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Correspondence to
BIOLOGICAL RESEARCH
Sociedad de Biología de Chile
Canadá 253, piso 3º, Dpto. F.
PO Box 16164, Santiago, Chile
Fax (56-2) 225 8427
Phone (56-2) 209 3503
E-mail socbiol@biologiachile.cl
Internet: www.biologiachile.cl

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Facultad de Medicina
Universidad de Chile
Fono: 56-2-9786044
E-mail: jprieto@med.uchile.cl

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Review Article

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Review Article

Contribution of environmental pollutants to male infertily: A working model of germ cell apoptosis induced by plasticizers

Raúl Lagos-Cabré and Ricardo D. Moreno[1]

[1] Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago Chile.

ABSTRACT

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA), 4-nonylphenol (NP) and di(2-ethylhexyl)phthalate (DEHP), and its metabolite mono-2-ethylhexyl phthalate (MEHP) are chemicals found in plastics, which act as endocrine disruptors (EDs) in animals, including human. EDs act like hormones in the endocrine system, and disrupt the physiologic function of endogenous hormones. Most people are exposed to different endocrine disruptors and concern has been raised about their true effect on reproductive organs. In the testis, they seem to preferentially attack developing testis during puberty rather than adult organs. However, the lack of information about the molecular mechanism, and the apparently controversial effect observed in different models has hampered the understanding of their effects on mammalian spermatogenesis. In this review, we critically discuss the available information regarding the effect of BPA, NP and DEHP/MEHP upon mammalian spermatogenesis, a major target of EDs. Germ cell sloughing, disruption of the blood-testis-barrier and germ cell apoptosis are the most common effects reported in the available literature. We propose a model at the molecular level to explain the effects at the cellular level, mainly focused on germ cell apoptosis.

Key words: Testis, spermatogenesis, Bisphenol A, nonylphenol, ADAM17

INTRODUCTION

Low sperm count (oligospermia), absence of spermatozoa in the semen (azoospermia) and morphological abnormalities are among the primary factors contributing to male infertility (WHO, 2010). The molecular and cellular bases of these pathologies are still not fully understood, but several studies suggest that increased germ cell death (apoptosis) during spermatogenesis may explain decreased sperm production in patients with oligo- and azoospermia. Spermatogenesis is highly influenced by external stimuli, such as drugs, radiation, reproductive and somatic pathologies, seasonal breeding, temperature and environmental pollutants, which increase the constitutive levels of apoptosis in germ cells (Tripathi et al., 2009).

Endocrine disruptors (EDs) involve a great number of molecules capable of inducing estrogenic or antiandrogenic responses in animals, including humans. Phenols and phthalates are among the EDs that can cause male infertility and other pathologies associated with developmental abnormalities. Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA), 4-nonylphenol (NP) and di(2-ethylhexyl)phthalate (DEHP) and its major metabolite mono(2-ethylhexyl)phthalate (MEHP) are found mainly in polycarbonate plastics, toys, dentist devices, food packaging, blood bags, cosmetics and currency paper (Guenther et al., 2002; ter Veld et al., 2006; EC-SCF, 2007; Liao and Kannan, 2011). Unfortunately they can leach from the lining of plastic packages, cans and baby bottles, and pipe walls. In this way, the human body is exposed to concentrations of 10µg/day of BPA, 7.5µg/day of NP and 30µg/day of DEHP, and they have been detected in human body samples, such as serum, urine, amniotic fluid of pregnant women, breast milk and even in semen (Guenther et al., 2002; Inoue et al., 2002; Calafat et al., 2005; Carlsen et al., 2005; Main et al., 2006; EC-SCF, 2007; Phillips and Tanphaichitr, 2008; Han et al., 2009; Huang et al., 2009; Zhang et al., 2009). Thus, experimental evidence clearly shows that humans are exposed to EDs, which may threaten normal physiology during development and adult life.

Even though these compounds are considered to mimic the effect of estrogen and other steroid hormones, deregulating the control of several hormone-dependent developmental processes (Phillips and Tanphaichitr, 2008; Roy et al., 2009), *in vitro* assays have shown that the potency of each ED (BPA, NP and DEHP, among others) is much lower than that of estrogen (~10,000 fold lower than estradiol, E2) (ter Veld et al., 2006). Therefore, it is plausible to propose that these molecules also act through a non-classical estrogenic pathway and probably bind and activate a wide range of proteins, activating different intracellular pathways. This would explain the multiple effects described at the cellular and physiological levels. Particularly relevant are the surviving and dead pathways that are disrupted and/or activated by these molecules, because by inducing germ cell apoptosis they could contribute to lower sperm production in the human testis.

First, we will briefly review the mechanism of apoptosis and the process of spermatogenesis. Then, we will critically discuss the available information linking plasticizers such as BPA, NP and DEHP/MEHP to alteration in normal spermatogenesis. Finally, we propose a molecular pathway in order to explain the deleterious effects of these molecules in spermatogenesis.

[1]Corresponding author: Dr. Ricardo D. Moreno, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. Alameda 340, Santiago, Chile. Fax: (562) 222 5515; E-mail: rmoreno@bio.puc.cl

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THE MECHANISM OF APOPTOSIS

Apoptosis is characterized by several hallmarks, such as: internucleosomal DNA fragmentation, caspase activation and externalization of phophatidyl serine (Degterev and Yuan, 2008; Fadeel et al., 2008; Youle and Strasser, 2008). Caspases are serine-proteases that are synthesized as inactive zymogens and become active upon death stimuli. The extrinsic pathway is initiated by activation of death receptors, such as Fas (CD95/Apo-1) or tumor necrosis factor receptor 1 (TNFR1). Trimerization of death receptors in response to ligand biding induces the formation of a multimeric complex termed death inducing signaling complex (DISC), which activates procaspase-8 in mice and capasase-10 in humans (Scaffidi et al., 1998; Scaffidi et al., 1999; Salvesen, 2002; Henkler et al., 2005). Caspase-8 and/or 10 proteolytically process caspase-3, -6 and -7. Among them, caspase-3 is the main executioner caspase involved in dismantling the Golgi apparatus, nuclear lamina fragmentation, DNA fragmentation (due in part to proteolysis of a DNAse inhibitor) and the decrease in mitochondrial membrane potential (Sato et al., 2004; Kuribayashi et al., 2006).

The intrinsic pathway of apoptosis could be activated by different stimuli, such as radiation, DNA fragmentation, starvation, oxidative stress and autophagy (Degterev and Yuan, 2008). This pathway is characterized by a decrease in mitochondria membrane potential and release of cytochrome C from the mitochondria, which along with dATP, the cytosolic protein Apaf-1 and procaspase-9 assemble a complex termed apoptosome. Within this complex, procaspase-9 becomes active and then activates caspase-3, connecting the intrinsic and extrinsic pathways (Shi, 2002; Shi, 2006). Mitochondria membrane stability is preserved by anti-apoptotic protein of the the B-cell lymphoma-2 (BCL-2) family. BCL-2, BCL-x and BCL-w are three anti-apoptotic proteins that interact with and repress the activity of pro-apoptotic proteins. Two general classes of pro-apoptotic family proteins exist: (1) those that share three homology regions (BH1, BH2 and BH3), and that are termed multidomain proteins; and (2) those that share little sequence homology, except for the conserved BH3 domain, also termed "BH3-only" proteins (Chen et al., 2005; Zhai et al., 2008; Ku et al., 2010; Young et al., 2010). Among the "BH3-only" group we find Bcl-2 antagonist of cell death (BAD) and p53 upstream modulator (PUMA) (Villunger et al., 2003). It appears that the multidomain pro-apoptotic proteins BAX and BAK are crucial for outer mitochondrial membrane (OMM) permeabilization and the subsequent release of apoptogenic molecules, such as cytochrome-c and DIABLO (also known as SMAC), which leads to caspase-9 activation (Riedl and Shi, 2004; Westphal et al., 2010). Thus, apoptosis is a complex process involving activation of several independent, but convergent pathways in order to induce cell death avoiding an inflammatory response.

MAMMALIAN SPERMATOGENESIS

The making of mammalian spermatozoa starts with engagement in a differentiation pathway of a diploid cell termed spermatogonium, which establishes itself at the basal lamina of seminiferous tubules (de Rooij and Russell, 2000; Oatley and Brinster, 2008). Through several mitotic divisions, type A spermatogonial stem cells either renew themselves or differentiate into later-stage spermatogonia to eventually initiate meiosis (Oatley and Brinster, 2008). Germ cells in meiosis, spermatocytes, will undergo two successive divisions, without a S phase, and will become haploid round spermatids, which eventually transform into mature spermatozoa (Hermo et al., 2010). Mingled among germ cells are the Sertoli cells, which are the somatic component of seminiferous epithelium that provide mechanical and nutritional support to germ cells (Figure 1). Germ cells in adult rat testes are grouped into 14 cell association or stages (numbered I-XIV) and six in humans (I-VI) (Moreno and Alvarado, 2006). Adjacent Sertoli cells bind to each other through tight junctions (TJs) constituting the blood–testis barrier (BTB) between 10 and 16 days of age in mice, and 20-25 days in rats, providing a protected environment for germ cell development termed the adluminal compartment (Dym and Fawcett, 1970; Sharpe et al., 2003; Yan et al., 2008). The TJs, which are the only known examples of occluding junctions, consist of three classes of integral membrane proteins, namely occludin, claudin and junctional adhesion molecules (Mruk and Cheng, 2004). In this way only spermatogonia and pre-leptotene spermatocytes are attached to the basal lamina and outside from the adluminal compartment (Fig 1).

Numerous studies indicate that Sertoli cells are involved in the progression of spermatogenesis through a variety of paracrine signals regulating gene expression and metabolism of germ cells (Skinner, 2005). Sertoli cells regulate survival of germ cells via paracrine secretion of trophic factors such as insulin growth factor (IGF), nerve growth factor (NGF), growth factor derived from glia (GDNF) and stem cell factor (SCF). Moreover, it has been shown that apoptosis of germ cells is somehow controlled by hormonal levels including testosterone, estrogen and FSH (Shetty et al., 1996; Yan et al., 2000b; Tesarik et al., 2002). Testosterone is essential for meiosis and subsequent differentiation of spermatids (De Gendt et al., 2004). Testosterone exerts its action through the Sertoli cell, which expresses the androgen receptor, and stimulates the synthesis of various proteins and trophic factors in specific periods of spermatogenesis (Wang et al., 2009). Therefore, germ cell development is a complex differentiation process controlled by juxta/paracine, and endocrine interactions.

APOPTOSIS DURING MAMMALIAN SPERMATOGENESIS

Sperm production relies on physiological and environmental factors, which may attenuate or even totally suppress testicular function. Germ cell apoptosis has been shown to play an important role in controlling sperm output in many species and has been linked to infertility in humans (Feng et al., 1999; Weikert et al., 2004; Ji et al., 2009). Germ cells undergoing meiosis (spermatocytes) are highly sensitive to heat shock, ionizing radiation, growth factor deprivation, and chemotherapeutic agents (Russell, 2004; Bieber et al., 2006; Lizama et al., 2009; Silva et al., 2011). Many studies have shown the relevance of apoptosis in regulating spermatozoa output and eliminating damaged germ cells (Knudson et al., 1995; Beumer et al., 1998; Yin et al., 1998; Allemand et al., 1999; Feng et al., 1999; Honarpour et al., 2000; Yan et al., 2000a; Russell et al., 2002; Moreno et al., 2006). To this end, it has been reported that massive germ cell death occurs under physiological conditions (constitutive apoptosis) during the first round of spermatogenesis (Oakberg, 1956; Rodriguez et al., 1997; Moreno et al., 2006). Different experimental

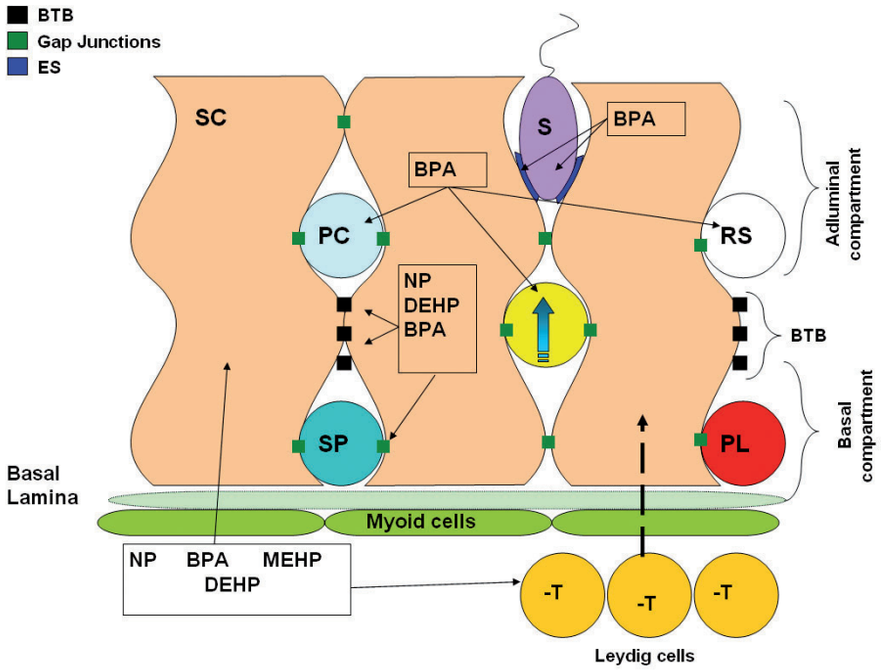

Figure 1: Major targets of BPA, NP and MEHP in a mammalian testis. Bisphenol A (BPA), 4-nonylphenol (NP) and di(2-ethylhexyl) phthalate (DEHP) disrupt spermatogenesis at different levels. They lower intratesticular and plasma testosterone (T) by affecting Leydig cells, resulting in decreased spermatogenesis. In addition, *in vitro* and *in vivo* studies show that Sertoli cells (SC) are primary targets of these compounds, affecting their metabolism, protein expression and morphology. BPA induces spermatogonium (SP), pachytene spermatocyte (PC) and preleptotene spermatocyte (PlC) apoptosis by affecting SC. The blood-testis barrier (BTB), which separates the adluminal compartment from the basal compartment within seminiferous tubules, ectoplasmic specializations (ES), which maintain attached elongated spermatids (S) to SC, and gap junctions (GJ) are disrupted by EDs, producing sloughing and apoptosis of germ cells.

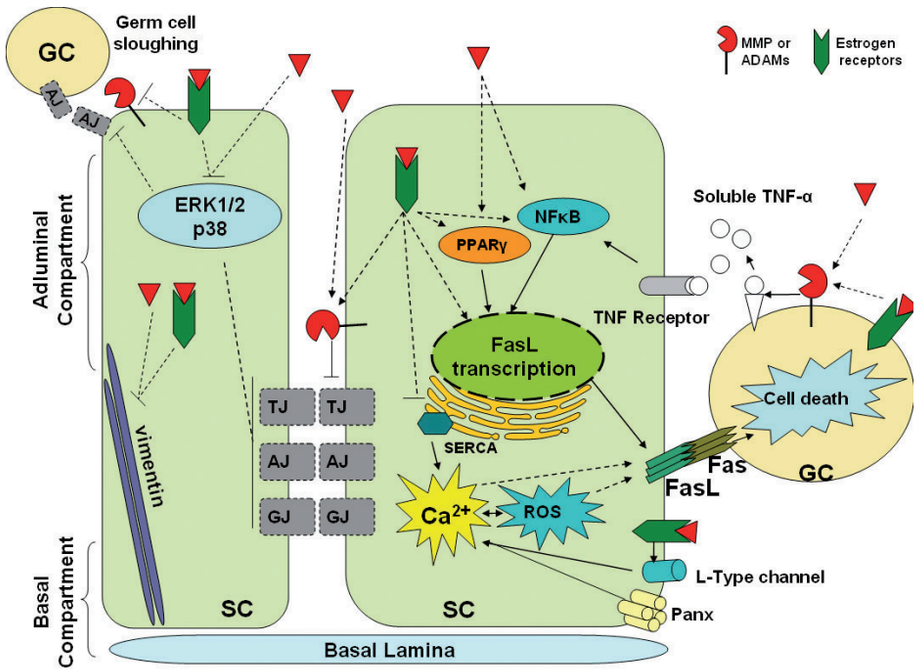

Figure 2: Model of the effects of EDs on Sertoli-germ cell interaction and germ cell apoptosis and sloughing. Depiction of a model of para/juxtacrine signaling events between germ cells (GC) and Sertoli cells during apoptosis induced by endocrine disruptors. Dotted lines indicate an unknown mechanism.
Triangles represent EDs molecules.

approaches have pointed out that spermatocytes are the main cell type undergoing apoptosis, with a smaller fraction of spermatogonia also undergoing the process (Jahnukainen et al., 2004; Moreno et al., 2006).

During spermatogenesis, about 75% of germ cell die in every round of spermatogenesis (Huckins, 1978). One possibility for this massive germ cell death is that the Sertoli cells provide an appropriate environment only to a certain amount of germ cells, so that apoptosis would serve as a mechanism to remove excess of germ cells that cannot be supported by Sertoli cells. Another hypothesis is that apoptosis is used to eliminate germ cells that do not pass the control points of the cell cycle. In several mammalian species, apoptosis occurs simultaneously with the mitotic divisions of the spermatogonia and with the beginning of meiosis of spermatocytes (Blanco-Rodriguez, 2002; Blanco-Rodriguez et al., 2003). This suggests that the checkpoints could be helping to correct the number of germ cells in relation to the number of Sertoli cells, acting specifically on cells with problems of chromosomal rearrangements during meiosis or damaged cells unable to repair the breaks in their DNA (Salazar et al., 2003; Salazar et al., 2005).

The importance of apoptosis in spermatogenesis is evident when pro-apoptotic genes are deleted (BAX, Bim or Bik) or anti-apoptotic genes are overexpressed (such as BCL-2). Both conditions are associated with infertility due to the arrest of spermatogenesis at the onset of meiosis (Knudson et al., 1995; Feng et al., 1999; Yamamoto et al., 2001; Russell et al., 2002; Yan et al., 2003; Coultas et al., 2005). Furthermore, inhibiting the engulfment of apoptotic bodies by Sertoli cells decreases sperm production and mice become sub-fertile (Maeda et al., 2002; Elliott et al., 2010). Results from our laboratory indicate an increase in the levels of the Fas receptor, the transcription factor p53 and the activation of caspases 8, 9, 3 2 and 6 in apoptotic germ cells (Lizama et al., 2007; Codelia et al., 2008). Interestingly, anti-cancer drugs, such as etoposide, which promotes DNA breaks by inhibiting topoisomerase II, induce apoptosis in spermatocytes (Ortiz et al., 2009; Codelia et al., 2010; Lizama et al., 2011; Lizama et al., 2012) by activating p73 and caspases. Thus, it seems that some elements of the mechanism involving constitutive (physiological) pathways are shared with externally induced apoptosis.

ADVERSE EFFECTS OF ENDOCRINE DISRUPTORS ON HUMAN FERTILITY

Clinical studies suggest that EDs could affect reproductive tract development because DEHP exposure during pregnancy correlates with low birth weight (Zhang et al., 2009) and a decrease in the anogenital distance (AGD) in females. In males, a reduced AGD accompanied by incomplete testicular descent has been observed in boys prenatally exposed to phthalates (Swan et al., 2005). Additionally, several abnormalities regarding secondary sexual characteristics have been observed in boys and girls when EDs are present, for example, girls exposed *in utero* to high doses of polybrominated biphenyls (PBB), >7 parts per billion (ppb) show an early menarche (at least one year earlier) than those who were not exposed to this ED (Blanck et al., 2000), while boys exposed *in utero* to pesticides show a significant decrease in penis length at the age of 3 months, along with a low testicular volume and decreased T levels (Andersen et al., 2008). Cultured human fetal testes treated with MEHP show an increase in apoptosis of germ cells and reduced expression of anti-Müllerian hormone mRNA, which may be linked to the feminization effect exerted *in utero* (Lambrot et al., 2009). In this regard, it is worth mentioning that millions of women in the USA between the 1950s and 1970s were treated with diethilbestrol (DES), an artificial estrogen that was prescribed to pregnant women to avoid miscarriages. 31.5% of the male children of these women showed abnormalities in their reproductive tracts, including epididymal cyst and hyposplastic testes in adulthood, compared to only 7.8% of males who presented these abnormalities when their mothers did not take DES (Bibbo et al., 1977; Gill et al., 1979; Jensen et al., 1995; Toppari et al., 1996). These males also showed decreased ejaculated volume and sperm abnormalities (Bibbo et al., 1977), suggesting that *in utero* exposure to estrogen is a major factor in male genital abnormalities observed in adulthood. The transgenerational and long-term negative effects on male testes were evidenced by the grandsons of women who received DES showing a high risk for hypospadias (Klip et al., 2002; Brouwers et al., 2006).

During a breastfeeding study, several phthalate monoesters were transferred to newborns from contaminated breast milk and these babies show low free T levels along with an increase in the luteinizing hormone (LH)/T ratio, indicating a possible adverse effect on Leydig cells or the gonadal-pituitary axis (Main et al., 2006). Similar effects have been observed in infertile men with high DEHP levels in semen, who also show an increase in serum estradiol (E2) and prolactin (PRL) (Li et al., 2011b). In fertile men, no significant associations were found among any semen parameters and urinary BPA concentrations (Mendiola et al., 2010). However, a significant inverse association was detected among urinary BPA concentrations free androgen index (FAI) levels and the FAI/LH ratio, as well as a significant positive association between BPA and sex hormone-binding globulin (SHBG). These results suggest that low BPA concentrations may be linked to subtle variations in sex hormones in fertile men.

The output and quality of sperm are useful tools to measure the effect of exogenous compounds on spermatogenesis. A high correlation has been observed between urine BPA levels and semen quality in Chinese men (including motility, viability, sperm count and sperm concentration), which also correlated with the educational level and longer employment history; men with better education and a long history of employment had lower levels of BPA since they were not in contact with EDs, unlike men who worked in factories (Li et al., 2011a). Urine BPA levels could also be associated with sperm abnormalities and sperm DNA fragmentation (suggesting apoptosis) in men from an infertility clinic (Meeker et al., 2010). However, direct application of BPA to human sperm samples does not produce any negative effects (Bennetts et al., 2008), suggesting that the observed negative effects could be induced during spermatogenesis and/or epididymal transit and not through a direct effect on spermatozoa. Regarding DEHP effects, it has been shown that infertile men in India who present DEHP levels of up to 0.77±1.2 µg/mL in semen have the following sperm abnormalities: reduced sperm count and motility, depolarized mitochondrial membrane, higher levels of reactive oxygen species (ROS) in semen and higher lipid peroxidation levels that correlate to the DEHP levels observed in these patients

(Pant et al., 2008). Similar effects have been observed in Chinese men where high urine BPA levels correlate with low sperm count, motility and concentration (Li et al., 2011a).

A recent study demonstrated that DEHP-contaminated air was associated with an increase in sperm DNA fragmentation and a decrease in sperm motility in polyvinyl chloride (PVC) factory workers (Huang et al., 2011). As in the case of DEHP-exposed workers, air BPA-exposed workers also show reduced sexual desire, accompanied by erectile dysfunction and ejaculation difficulties (Li et al., 2010). Diet and residential area (urban or rural) could be important factors related to the negative effects of EDs, since consumption of certain foods from contaminated areas could increase the levels of EDs in semen. In this regard, infertile men who live in urban areas and have a fish-based diet (probably from contaminated waters) have higher levels of polychlorinated biphenyls and phthalate esters than those of men who live in urban areas, but with a diet that does not include fish. These men showed reduced levels of progressive sperm motility, lower ejaculated volume and sperm vitality compared to fertile men (Rozati et al., 2002). It has been determined that high levels of organochlorine pesticides could be related to sperm abnormalities in greenhouse workers (Abell et al., 2000). These compounds have also been detected in young men (Carreno et al., 2007) who live near agricultural areas in southern Spain, suggesting possible risk factors of living and ingesting food from or near contaminated areas. The paucity of studies regarding the role of EDs in human reproductive functions limits the extent to which conclusions can be made. Despite that, the available data strongly suggest an adverse effect of BPA, NP and DEHP on sex hormone levels and semen parameters.

EDS AFFECT SPERMATOGENESIS AND INDUCE GERM CELL APOPTOSIS IN ANIMAL MODELS

A reduction in Leydig cell numbers and T plasma levels have been observed in pubertal mice orally receiving 160 to 960mg/kg of BPA for thirteen days (Li et al., 2009). However, treatment of adult mice and rats (Toyama et al., 2004) with 20 to 200µg/kg of BPA for six days produced abnormalities in ectoplasmic specializations (ES) in elonged spermatids, without major changes in Sertoli or Leydig cells. ES are testis-specific adherens junctions between elonged spermatids and Sertoli Cells, and their assembly and stability rely on T levels (Wong et al., 2005; Ruwanpura et al., 2010). E2 administration has also been shown to decrease ES stability by reducing T levels (Wong et al., 2005); therefore, it is possible that application of BPA mimics the effect of E2, thus affecting T levels and disrupting ES between elongated spermatids and Sertoli cells.

BPA increases Fas and FasL levels in germ and Sertoli cells, respectively, accompanied by activation of caspase-3 in germ and Leydig cells when administered by gavage to pubertal mice (Li et al., 2009). Administration of 1.2 to 10µg/day of BPA for five days to neonatal male rats lowers sperm count and motility in adulthood, accompanied by a low mating rate and sloughing of germ cells, hence demonstrating the long-term effects of these compounds and suggesting that EDs accumulate in the body and/or metabolic pathways are permanently disrupted (Salian et al., 2009). Male mice whose mothers received an implant containing varying BPA concentrations from before mating until four weeks postnatal (weaning) show increased T plasma levels with low doses of BPA (1.2µg/day), but decreased levels with higher doses of BPA (60µg/day), along with a sloughing of germ cells and a reduction of seminiferous tubules with elongated spermatids (Okada and Kai, 2008). Similarly, prepubertal mice administered 50µg/mL of BPA in drinking water have been shown to have decreased T levels and multinucleated germ cells (Takao et al., 2003).

DEHP and its active metabolite MEHP might be one of the most environmentally abundant phthalates and have been shown to deplete gonocytes (future germ cells) in fetal rat testes and decrease T levels (Chauvigne et al., 2009). These effects have also been observed in human fetal testes, but without a T level decrease (Lambrot et al., 2009). Both studies show that Sertoli cells are unaffected and that Leydig cells remain active, but only in human fetal testes. When administered during the gestational period, DEHP (10mg/kg) produces several negative effects on Leydig cells, such as a decrease in volume and number, and an increase in T plasma levels. On the contrary, higher doses of DEHP (750mg/kg or 1g/Kg) decrease T plasma levels (Lin et al., 2008) and increases germ cell apoptosis compared to wild type mice (Lin et al., 2010). A single dose of 2g/kg of MEHP by gavage to prepubertal rats has been observed to disrupt vimentin filaments in Sertoli cells and activates apoptosis only in germ cells, as evidenced only by TUNEL and DNA fragmentation assays These effects were observed as early as 12 hours after MEHP administration (Richburg and Boekelheide, 1996). Prepubertal mice treated with 1g/kg of MEHP show an upregulation of FasL and TNF-α 1.5 hours after exposure through activation of NFkB (Yao et al., 2007). The same study showed an increase in FasL (mRNA and protein levels) in primary Sertoli cell cultures and ASD17D cells (Sertoli cell line), which is akin to the effect of BPA on mice testes (Li et al., 2009). These results suggest that FasL increase in Sertoli cells could be a common pathway and a major player in BPA- and DEHP/MEHP-induced germ cell apoptosis (Figs 1, 2). Studies in *gld* mice harboring an inactivating mutation in FasL have shown decreased apoptosis of germ cells after MEHP exposure, demonstrating the role of this protein in EDs-induced apoptosis (Richburg et al., 2000). On the other hand, mice lacking FasL ($FasL^{-/-}$) show an increase in basal levels of germ cell apoptosis, and when exposed to 1g/kg of MEHP, they show a dramatic decrease in the high basal germ cell apoptosis (Lin et al., 2010). This could be because of a significant increase in c-FILP levels, and endogenous caspase-8 inhibitor, after MEHP treatment only in FasL $^{-/-}$ ice. Even though increases in c-FLIP levels in $FasL^{-/-}$ mice after MEHP may account for the observed decreases in germ cell apoptosis, the mechanism underlying c-FLIP protein levels regulation in these mice after MEHP exposure is not readily apparent (Lin et al., 2010).

Oral administration of NP (1, 10 and 100µg/kg/day) to male rats decreased epididymis and testis weight, as well as epididymal sperm count. Interestingly, NP-treated male rats show greater ROS production and decreased antioxidant enzyme levels compared to controls (Chitra et al., 2002). Administration of NP (125, 250 and 300mg/kg/day) for sixty days to 20-day old rats elicits Fas and FasL mRNA upregulation in testes and increases TUNEL-positive cells compared to controls (Han et al., 2004), which is in agreement

with the effects produced by BPA and DEHP/MEHP described above. Activation of the extrinsic pathway seems to have a major role in germ cell apoptosis induced by BPA, DEHP and NP, which is similar to the physiological conditions (Moreno et al., 2006).

During spermatogenesis, germ and Sertoli cells are in close physical and functional contact through gap junctions (GJ), tight junctions (TJ) and adheren junctions (AJ). GJs mediate communication by forming intercellular pores by the docking of two hemichannels of adjacent cells. These hemichannels are composed of connexins (Cx), which are a protein family composed of about 20 members of transmembrane proteins (Decrock et al., 2009). TJs and AJs, along with intermediate filament-based desmosome junctions, are located near the basement membrane of the seminiferous tubule forming the blood-testis barrier (BTB), which allows the existence of a basal and adluminal compartment (Fig 1) to generate a specific microenvironment for germ cell development (Cheng and Mruk, 2002; Lee and Cheng, 2004). *In vivo* and *in vitro* studies show that TJs, AJs and GJs are targets of EDs that affect Sertoli-Sertoli and Sertoli-germ cell interactions (Fig 1). In addition, disruption of vimentin filaments by MEHP promotes germ cell detachment due to Sertoli cells shrinking. NP administration to pregnant rats during gestation, lactancy and 10 weeks after weaning (corresponding to a complete lifespan exposed to NP) have been shown to decrease epithelial thickness, probably due to Sertoli cells shrinking and an increase in germ cell apoptosis (de Jager et al., 1999; McClusky et al., 2007).). In summary, all the evidence indicates that directly, or indirectly through the Sertoli cells, EDs sever the interaction between Sertoli and germ cells and thereby provoke detachment (sloughing) of germ cells.

5. A MOLECULAR MODEL OF EDS IN MAMMALIAN TESTES

The effect observed by EDs in testes may be mediated primarily by nuclear estrogen receptors (ER) alpha and beta (ERα and ERβ), which are expressed by Sertoli and germ cells (O'Donnell et al., 2001). ERs are activated by a large number of ligands (hormones, environmental pollutants and phytoestrogens, among others). In fact, BPA induces changes in the levels of ERα and ERβ in adult mice testes (Takao et al., 2003) and affects the recruitment of their coactivator (Routledge et al., 2000). These receptors can generate two possible responses: a genomic response, which leads to gene expression, and a non-genomic response, a faster response involving kinase phosphorylation and ion channel regulation (Marino et al., 2006; Fu and Simoncini, 2008). This non-classical membrane estrogen receptor (ncmER), which is a G-protein coupled receptor, produces a fast activation of voltage-gated channels, thus raising the intracellular Ca^{2+} (iCa^{2+}) concentration in the target cell (Carmeci et al., 1997; Marino et al., 2006).

It has been described that Sertoli cells *in vitro* exposed to a variety of EDs show an increase in iCa^{2+}, which could be due to an external influx or a depletion in intracellular stores, by inhibiting the SERCA pump at the endoplasmic reticulum (Hughes et al., 2000; Gong et al., 2008) (Fig 2). However, we cannot exclude the possibility of a genomic participation of ERs in germ cell apoptosis and sloughing, since there is an increase in FasL expression by a genomic pathway in TM4 (a Sertoli-like cell line) cells treated with E2 (Catalano et al., 2007). In addition to an increase in iCa^{2+} in Sertoli cells exposed to NP, increased ROS production has also been observed (Gong and Han, 2006), demonstrating another mode of action of these compounds. It is possible that elevated ROS production is associated with mitochondrial and/or endoplasmic reticulum stress. This hypothesis is supported by the findings that in primary Sertoli cell cultures, NP increases ROS levels and lipid peroxidation while decreasing mitochondrial membrane potential (Gong and Han, 2006), which are all characteristics of oxidative stress. In this regard, it has been shown that stress signals are related to an increase in ROS production, along with an activation of the p38MAPK pathway (Liu and Chang, 2009) that in turn upregulates the Fas/FasL system. However, EDs promote inactivation of p38MAPK and ERK1/2 in isolated Sertoli cells and TM4 cells (Aravindakshan and Cyr, 2005; Bhattacharya et al., 2005), which has been related to the downregulation of TJ, AJ and GJ proteins and in this way induces sloughing and apoptosis of germ cells, showing that the mechanisms of ED affects spermatogenesis are still a subject of controversy.

Several lines of evidence shown here suggest the participation of the Fas-FasL system in the apoptosis of germ cells elicited by EDs. It has been demonstrated in MCF7 cells that FasL expression depends on peroxisome proliferator-activated receptor-γ (PPARγ) through the binding of the SP1 transcription factor (Bonofiglio et al., 2009), both of which are expressed in mammalian testes (Fig 2). However, there are no data available supporting this proposal.

It has recently been shown that matrix metalloproteinase 2 (MMP2) is involved in the disruption of junction complexes between Sertoli-Sertoli and Sertoli-germ cells (Yao et al., 2010). They also observed an early increase in ADAM10 and ADAM17 protein levels, which can participate in the MEHP response. Following treatment with a single dose of MEHP, the levels of the inhibitor of MMP2, TIMP2, decrease significantly and as a consequence, the MMP2 activity increases (Yao et al., 2009). ADAM10 and ADAM17 belong to a family of extracellular proteases that are involved in the release of many protein ectodomains from the cell surface, including TNF-α, FasL, Notch, APP and TrkA, thus indicating a strong participation in autocrine, paracrine and juxta/paracrine signaling (Schlondorff and Blobel, 1999; White, 2003). They are widely distributed in the male reproductive tract, however the biological function of many of these proteases is still unknown (Moreno et al., 2011). It is possible that ADAM17 may participate in TNF-α shedding from germ cell plasma membrane and exert paracrine signaling on Sertoli cells. In addition, ADAM17 can also shed the extracellular domain of JAM-A (Koenen et al., 2009), a well known protein of TJs (see above), hence destabilizing the BTB and leading to germ cell sloughing (Fig. 2).

Our model puts forward ADAM metalloproteases as novel elements in germ cell apoptosis following ED treatment. Interestingly, we have recently shown that the ADAM17-mediated shedding of the c-kit extracellular domain is involved in germ cell apoptosis, suggesting that this could be a mechanism common to physiological and ED-induced germ cell apoptosis (Lizama et al., 2010).

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REFERENCES

ABELL A, ERNST E, BONDE JP (2000) Semen quality and sexual hormones in greenhouse workers. Scand J Work Environ Health 26: 492-500.

ALLEMAND I, ANGLO A, JEANTET AY, CERUTTI I, MAY E (1999) Testicular wild-type p53 expression in transgenic mice induces spermiogenesis alterations ranging from differentiation defects to apoptosis. Oncogene 18: 6521-6530.

ANDERSEN HR, SCHMIDT IM, GRANDJEAN P, JENSEN TK, BUDTZ-JORGENSEN E, KJAERSTAD MB, BAELUM J, NIELSEN JB, SKAKKEBAEK NE, MAIN KM (2008) Impaired reproductive development in sons of women occupationally exposed to pesticides during pregnancy. Environ Health Perspect 116: 566-572.

ARAVINDAKSHAN J, CYR DG (2005) Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-mitogen-activated protein kinase pathway. Biol Reprod 72: 1232-1240.

BENNETTS LE, DE IULIIS GN, NIXON B, KIME M, ZELSKI K, MCVICAR CM, LEWIS SE, AITKEN RJ (2008) Impact of estrogenic compounds on DNA integrity in human spermatozoa: evidence for cross-linking and redox cycling activities. Mutat Res 641: 1-11.

BEUMER TL, ROEPERS-GAJADIEN HL, GADEMAN IS, VAN BUUL PP, GIL-GOMEZ G, RUTGERS DH, DE ROOIJ DG (1998) The role of the tumor suppressor p53 in spermatogenesis. Cell Death Differ 5: 669-677.

BHATTACHARYA N, DUFOUR JM, VO MN, OKITA J, OKITA R, KIM KH (2005) Differential effects of phthalates on the testis and the liver. Biol Reprod 72: 745-754.

BIBBO M, GILL WB, AZIZI F, BLOUGH R, FANG VS, ROSENFIELD RL, SCHUMACHER GF, SLEEPER K, SONEK MG, WIED GL (1977) Follow-up study of male and female offspring of DES-exposed mothers. Obstet Gynecol 49: 1-8.

BIEBER AM, MARCON L, HALES BF, ROBAIRE B (2006) Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. J Androl 27: 189-200.

BLANCK HM, MARCUS M, TOLBERT PE, RUBIN C, HENDERSON AK, HERTZBERG VS, ZHANG RH, CAMERON L (2000) Age at menarche and tanner stage in girls exposed in utero and postnatally to polybrominated biphenyl. Epidemiology 11: 641-647.

BLANCO-RODRIGUEZ J (2002) Deoxyribonucleic acid replication and germ cell apoptosis during spermatogenesis in the rabbit. J Androl 23: 182-187.

BLANCO-RODRIGUEZ J, MARTINEZ-GARCIA C, PORRAS A (2003) Correlation between DNA synthesis in the second, third and fourth generations of spermatogonia and the occurrence of apoptosis in both spermatogonia and spermatocytes. Reproduction 126: 661-668.

BONOFIGLIO D, GABRIELE S, AQUILA S, QI H, BELMONTE M, CATALANO S, ANDO S (2009) Peroxisome proliferator-activated receptor gamma activates fas ligand gene promoter inducing apoptosis in human breast cancer cells. Breast Cancer Res Treat 113: 423-434.

BROUWERS MM, FEITZ WF, ROELOFS LA, KIEMENEY LA, DE GIER RP, ROELEVELD N (2006) Hypospadias: a transgenerational effect of diethylstilbestrol? Hum Reprod 21: 666-669.

CALAFAT AM, KUKLENYIK Z, REIDY JA, CAUDILL SP, EKONG J, NEEDHAM LL (2005) Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect 113: 391-395.

CARLSEN E, SWAN SH, PETERSEN JH, SKAKKEBAEK NE (2005) Longitudinal changes in semen parameters in young Danish men from the Copenhagen area. Hum Reprod 20: 942-949.

CARMECI C, THOMPSON DA, RING HZ, FRANCKE U, WEIGEL RJ (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45: 607-617.

CARRENO J, RIVAS A, GRANADA A, JOSE LOPEZ-ESPINOSA M, MARISCAL M, OLEA N, OLEA-SERRANO F (2007) Exposure of young men to organochlorine pesticides in Southern Spain. Environ Res 103: 55-61.

CATALANO S, RIZZA P, GU G, BARONE I, GIORDANO C, MARSICO S, CASABURI I, MIDDEA E, LANZINO M, PELLEGRINO M, ANDO S (2007) Fas ligand expression in TM4 Sertoli cells is enhanced by estradiol „in situ" production. J Cell Physiol 211: 448-456.

CODELIA VA, CISTERNA M, ALVAREZ AR, MORENO RD (2010) p73 participates in male germ cells apoptosis induced by etoposide. Mol Hum Reprod 16: 734-742.

CODELIA VA, CISTERNAS P, MORENO RD (2008) Relevance of caspase activity during apoptosis in pubertal rat spermatogenesis. Mol Reprod Dev 75: 881-889.

COULTAS L, BOUILLET P, LOVELAND KL, MEACHEM S, PERLMAN H, ADAMS JM, STRASSER A (2005) Concomitant loss of proapoptotic BH3-only Bcl-2 antagonists Bik and Bim arrests spermatogenesis. Embo J 24: 3963-3973.

CHAUVIGNE F, MENUET A, LESNE L, CHAGNON MC, CHEVRIER C, REGNIER JF, ANGERER J, JEGOU B (2009) Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis in vitro. Environ Health Perspect 117: 515-521.

CHEN L, WILLIS SN, WEI A, SMITH BJ, FLETCHER JI, HINDS MG, COLMAN PM, DAY CL, ADAMS JM, HUANG DC (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 17: 393-403.

CHENG CY, MRUK DD (2002) Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiol Rev 82: 825-874.

CHITRA KC, LATCHOUMYCANDANE C, MATHUR PP (2002) Effect of nonylphenol on the antioxidant system in epididymal sperm of rats. Arch Toxicol 76: 545-551.

DE GENDT K, SWINNEN JV, SAUNDERS PT, SCHOONJANS L, DEWERCHIN M, DEVOS A, TAN K, ATANASSOVA N, CLAESSENS F, LECUREUIL C, HEYNS W, CARMELIET P, GUILLOU F, SHARPE RM, VERHOEVEN G (2004) A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 101: 1327-1332.

DE JAGER C, BORNMAN MS, OOSTHUIZEN JM (1999) The effect of p-nonylphenol on the fertility potential of male rats after gestational, lactational and direct exposure. Andrologia 31: 107-113.

DE ROOIJ DG, RUSSELL LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21: 776-798.

DECROCK E, VINKEN M, DE VUYST E, KRYSKO DV, D'HERDE K, VANHAECKE T, VANDENABEELE P, ROGIERS V, LEYBAERT L (2009) Connexin-related signaling in cell death: to live or let die? Cell Death Differ 16: 524-536.

DEGTEREV A, YUAN J (2008) Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 9: 378-390.

DYM M, FAWCETT DW (1970) The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol Reprod 3: 308-326.

EC-SCF (2007) Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to 2,2-BIS(4-HYDROXYPHENYL) PROPANE (Bisphenol A). EFSA J: DOI:10.2903/j.efsa.2007.2428.

ELLIOTT MR, ZHENG S, PARK D, WOODSON RI, REARDON MA, JUNCADELLA IJ, KINCHEN JM, ZHANG J, LYSIAK JJ, RAVICHANDRAN KS (2010) Unexpected requirement for ELMO1 in clearance of apoptotic germ cells in vivo. Nature 467: 333-337.

FADEEL B, OTTOSSON A, PERVAIZ S (2008) Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. Cell Death Differ 15: 443-452.

FENG HL, SANDLOW JI, SPARKS AE, SANDRA A, ZHENG LJ (1999) Decreased expression of the c-kit receptor is associated with increased apoptosis in subfertile human testes. Fertil Steril 71: 85-89.

FU XD, SIMONCINI T (2008) Extra-nuclear signaling of estrogen receptors. IUBMB Life 60: 502-510.

GILL WB, SCHUMACHER GF, BIBBO M, STRAUS FH, 2ND, SCHOENBERG HW (1979) Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. J Urol 122: 36-39.

GONG Y, HAN XD (2006) Nonylphenol-induced oxidative stress and cytotoxicity in testicular Sertoli cells. Reprod Toxicol 22: 623-630.

GONG Y, PAN X, HUANG Y, GAO Z, YU H, HAN X (2008) NP-induced biophysical and biochemical alterations of rat testicular Sertoli cell membranes related to disturbed intracellular Ca(2+) homeostasis. Toxicol Lett 183: 10-20.

GUENTHER K, HEINKE V, THIELE B, KLEIST E, PRAST H, RAECKER T (2002) Endocrine disrupting nonylphenols are ubiquitous in food. Environ Sci Technol 36: 1676-1680.

HAN SW, LEE H, HAN SY, LIM DS, JUNG KK, KWACK SJ, KIM KB, LEE BM (2009) An exposure assessment of di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) in human semen. J Toxicol Environ Health A 72: 1463-1469.

HAN X, TU Z, WANG X, SHEN S, HOU Y (2004) Nonylphenol induced apoptosis in rat testis through the Fas/FasL pathway. Bull Environ Contam Toxicol 73: 620-627.

HENKLER F, BEHRLE E, DENNEHY KM, WICOVSKY A, PETERS N, WARNKE C, PFIZENMAIER K, WAJANT H (2005) The extracellular domains of FasL and Fas are sufficient for the formation of supramolecular FasL-Fas clusters of high stability. J Cell Biol 168: 1087-1098.

HERMO L, PELLETIER RM, CYR DG, SMITH CE (2010) Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. Microsc Res Tech 73: 241-278.

HONARPOUR N, DU C, RICHARDSON JA, HAMMER RE, WANG X, HERZ J (2000) Adult Apaf-1-deficient mice exhibit male infertility. Dev Biol 218: 248-258.

HUANG LP, LEE CC, HSU PC, SHIH TS (2011) The association between semen quality in workers and the concentration of di(2-ethylhexyl) phthalate in polyvinyl chloride pellet plant air. Fertil Steril 96: 90-94.

HUANG PC, KUO PL, CHOU YY, LIN SJ, LEE CC (2009) Association between prenatal exposure to phthalates and the health of newborns. Environ Int 35: 14-20.

HUCKINS C (1978) The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. Anat Rec 190: 905-926.

HUGHES PJ, MCLELLAN H, LOWES DA, KAHN SZ, BILMEN JG, TOVEY SC, GODFREY RE, MICHELL RH, KIRK CJ, MICHELANGELI F (2000) Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca(2+) pumps. Biochem Biophys Res Commun 277: 568-574.

INOUE K, WADA M, HIGUCHI T, OSHIO S, UMEDA T, YOSHIMURA Y, NAKAZAWA H (2002) Application of liquid chromatography-mass spectrometry to the quantification of bisphenol A in human semen. J Chromatogr B Analyt Technol Biomed Life Sci 773: 97-102.

JAHNUKAINEN K, CHRYSIS D, HOU M, PARVINEN M, EKSBORG S, SODER O (2004) Increased apoptosis occurring during the first wave of spermatogenesis is stage-specific and primarily affects midpachytene spermatocytes in the rat testis. Biol Reprod 70: 290-296.

JENSEN TK, TOPPARI J, KEIDING N, SKAKKEBAEK NE (1995) Do environmental estrogens contribute to the decline in male reproductive health? Clin Chem 41: 1896-1901.

JI G, GU A, HU F, WANG S, LIANG J, XIA Y, LU C, SONG L, FU G, WANG X (2009) Polymorphisms in cell death pathway genes are associated with altered sperm apoptosis and poor semen quality. Hum Reprod 24: 2439-2446.

KLIP H, VERLOOP J, VAN GOOL JD, KOSTER ME, BURGER CW, VAN LEEUWEN FE (2002) Hypospadias in sons of women exposed to diethylstilbestrol in utero: a cohort study. Lancet 359: 1102-1107.

KNUDSON CM, TUNG KS, TOURTELLOTTE WG, BROWN GA, KORSMEYER SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270: 96-99.

KOENEN RR, PRUESSMEYER J, SOEHNLEIN O, FRAEMOHS L, ZERNECKE A, SCHWARZ N, REISS K, SARABI A, LINDBOM L, HACKENG TM, WEBER C, LUDWIG A (2009) Regulated release and functional modulation of junctional adhesion molecule A by disintegrin metalloproteinases. Blood 113: 4799-4809.

KU B, LIANG C, JUNG JU, OH BH (2010) Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX. Cell Res 21: 627-641.

KURIBAYASHI K, MAYES PA, EL-DEIRY WS (2006) What are caspases 3 and 7 doing upstream of the mitochondria? Cancer Biol Ther 5: 763-765.

LAMBROT R, MUCZYNSKI V, LECUREUIL C, ANGENARD G, COFFIGNY H, PAIRAULT C, MOISON D, FRYDMAN R, HABERT R, ROUILLER-FABRE V (2009) Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. Environ Health Perspect 117: 32-37.

LEE NP, CHENG CY (2004) Adaptors, junction dynamics, and spermatogenesis. Biol Reprod 71: 392-404.

LI D, ZHOU Z, QING D, HE Y, WU T, MIAO M, WANG J, WENG X, FERBER JR, HERRINTON LJ, ZHU Q, GAO E, CHECKOWAY H, YUAN W (2010) Occupational exposure to bisphenol-A (BPA) and the risk of self-reported male sexual dysfunction. Hum Reprod 25: 519-527.

LI DK, ZHOU Z, MIAO M, HE Y, WANG J, FERBER J, HERRINTON LJ, GAO E, YUAN W (2011a) Urine bisphenol-A (BPA) level in relation to semen quality. Fertil Steril 95: 625-630 e621-624.

LI S, DAI J, ZHANG L, ZHANG J, ZHANG Z, CHEN B (2011b) An association of elevated serum prolactin with phthalate exposure in adult men. Biomed Environ Sci 24: 31-39.

LI YJ, SONG TB, CAI YY, ZHOU JS, SONG X, ZHAO X, WU XL (2009) Bisphenol A exposure induces apoptosis and upregulation of Fas/FasL and caspase-3 expression in the testes of mice. Toxicol Sci 108: 427-436.

LIAO C, KANNAN K (2011) High Levels of Bisphenol A in Paper Currencies from Several Countries, and Implications for Dermal Exposure. Environ Sci Technol.

LIN H, GE RS, CHEN GR, HU GX, DONG L, LIAN QQ, HARDY DO, SOTTAS CM, LI XK, HARDY MP (2008) Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. Proc Natl Acad Sci U S A 105: 7218-7222.

LIN YC, YAO PL, RICHBURG JH (2010) FasL Gene-Deficient Mice Display a Limited Disruption in Spermatogenesis and Inhibition of Mono-(2-ethylhexyl) Phthalate-Induced Germ Cell Apoptosis. Toxicol Sci 114: 335-345.

LIU WH, CHANG LS (2009) Arachidonic acid induces Fas and FasL upregulation in human leukemia U937 cells via Ca2+/ROS-mediated suppression of ERK/c-Fos pathway and activation of p38 MAPK/ATF-2 pathway. Toxicol Lett 191: 140-148.

LIZAMA C, ALFARO I, REYES JG, MORENO RD (2007) Up-regulation of CD95 (Apo-1/Fas) is associated with spermatocyte apoptosis during the first round of spermatogenesis in the rat. Apoptosis 12: 499-512.

LIZAMA C, LAGOS CF, LAGOS-CABRE R, CANTUARIAS L, RIVERA F, HUENCHUNIR P, PEREZ-ACLE T, CARRION F, MORENO RD (2009) Calpain inhibitors prevent p38 MAPK activation and germ cell apoptosis after heat stress in pubertal rat testes. J Cell Physiol 221: 296-305.

LIZAMA C, LUDWIG A, MORENO RD (2011) Etoposide induces apoptosis and upregulation of TACE/ADAM17 and ADAM10 in an in vitro male germ cell line model. Biochim Biophys Acta 1813: 120-128.

LIZAMA C, ROJAS-BENITEZ D, ANTONELLI M, LUDWIG A, BUSTAMANTE-MARIN X, BROUWER-VISSER J, MORENO RD (2010) TACE/ADAM17 is involved in germ cell apoptosis during rat spermatogenesis. Reproduction 140: 305-317.

LIZAMA C, ROJAS-BENITEZ D, ANTONELLI M, LUDWIG A, MORENO RD (2012) Involvement of TACE/ADAM17 and ADAM10 in etoposide-induced apoptosis of germ cells in rat spermatogenesis. J Cell Physiol 227: 829-838.

MAEDA Y, SHIRATSUCHI A, NAMIKI M, NAKANISHI Y (2002) Inhibition of sperm production in mice by annexin V microinjected into seminiferous tubules: possible etiology of phagocytic clearance of apoptotic spermatogenic cells and male infertility. Cell Death Differ 9: 742-749.

MAIN KM, MORTENSEN GK, KALEVA MM, BOISEN KA, DAMGAARD IN, CHELLAKOOTY M, SCHMIDT IM, SUOMI AM, VIRTANEN HE, PETERSEN DV, ANDERSSON AM, TOPPARI J, SKAKKEBAEK NE (2006) Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ Health Perspect 114: 270-276.

MARINO M, GALLUZZO P, ASCENZI P (2006) Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics 7: 497-508.

MCCLUSKY LM, DE JAGER C, BORNMAN MS (2007) Stage-related increase in the proportion of apoptotic germ cells and altered frequencies of stages in the spermatogenic cycle following gestational, lactational, and direct exposure of male rats to p-nonylphenol. Toxicol Sci 95: 249-256.

MEEKER JD, EHRLICH S, TOTH TL, WRIGHT DL, CALAFAT AM, TRISINI AT, YE X, HAUSER R (2010) Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. Reprod Toxicol 30: 532-539.

MENDIOLA J, JORGENSEN N, ANDERSSON AM, CALAFAT AM, YE X, REDMON JB, DROBNIS EZ, WANG C, SPARKS A, THURSTON SW, LIU F, SWAN SH (2010) Are environmental levels of bisphenol a associated with reproductive function in fertile men? Environ Health Perspect 118: 1286-1291.

MORENO RD, ALVARADO CP (2006) The mammalian acrosome as a secretory lysosome: new and old evidence. Mol Reprod Dev 73: 1430-1434.

MORENO RD, LIZAMA C, URZUA N, VERGARA SP, REYES JG (2006) Caspase activation throughout the first wave of spermatogenesis in the rat. Cell Tissue Res 325: 533-540.

MORENO RD, URRIOLA-MUÑOZ P, LAGOS-CABRÉ (2011) The emerging role of matrix metalloproteases of the ADAM family in male germ cell apoptosis. Spermatogenesis 1: 195-208.

MRUK DD, CHENG CY (2004) Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr Rev 25: 747-806.

O'DONNELL L, ROBERTSON KM, JONES ME, SIMPSON ER (2001) Estrogen and spermatogenesis. Endocr Rev 22: 289-318.

OAKBERG EF (1956) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 99: 391-413.

OATLEY JM, BRINSTER RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24: 263-286.

OKADA A, KAI O (2008) Effects of estradiol-17beta and bisphenol A administered chronically to mice throughout pregnancy and lactation on the male pups' reproductive system. Asian J Androl 10: 271-276.

ORTIZ RJ, LIZAMA C, CODELIA VA, MORENO RD (2009) A molecular evaluation of germ cell death induced by etoposide in pubertal rat testes. Mol Hum Reprod 15: 363-371.

PANT N, SHUKLA M, KUMAR PATEL D, SHUKLA Y, MATHUR N, KUMAR GUPTA Y, SAXENA DK (2008) Correlation of phthalate exposures with semen quality. Toxicol Appl Pharmacol 231: 112-116.

PHILLIPS KP, TANPHAICHITR N (2008) Human exposure to endocrine disrupters and semen quality. J Toxicol Environ Health B Crit Rev 11: 188-220.

RICHBURG JH, BOEKELHEIDE K (1996) Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicol Appl Pharmacol 137: 42-50.

RIEDL SJ, SHI Y (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 5: 897-907.

RODRIGUEZ I, ODY C, ARAKI K, GARCIA I, VASSALLI P (1997) An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. Embo J 16: 2262-2270.

ROUTLEDGE EJ, WHITE R, PARKER MG, SUMPTER JP (2000) Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 275: 35986-35993.

ROY JR, CHAKRABORTY S, CHAKRABORTY TR (2009) Estrogen-like endocrine disrupting chemicals affecting puberty in humans--a review. Med Sci Monit 15: RA137-145.

ROZATI R, REDDY PP, REDDANNA P, MUJTABA R (2002) Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 78: 1187-1194.

RUSSELL LB (2004) Effects of male germ-cell stage on the frequency, nature, and spectrum of induced specific-locus mutations in the mouse. Genetica 122: 25-36.

RUSSELL LD, CHIARINI-GARCIA H, KORSMEYER SJ, KNUDSON CM (2002) Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. Biol Reprod 66: 950-958.

RUWANPURA S, MCLACHLAN R, MEACHEM S (2010) Hormonal regulation of germ cell development. J Endocrinol 205: 117-131.

SALAZAR G, JOSHI A, LIU D, WEI H, PERSSON JL, WOLGEMUTH DJ (2005) Induction of apoptosis involving multiple pathways is a primary response to cyclin A1-deficiency in male meiosis. Dev Dyn 234: 114-123.

SALAZAR G, LIU D, LIAO C, BATKIEWICZ L, ARBING R, CHUNG SS, LELE K, WOLGEMUTH DJ (2003) Apoptosis in male germ cells in response to cyclin A1-deficiency and cell cycle arrest. Biochem Pharmacol 66: 1571-1579.

SALIAN S, DOSHI T, VANAGE G (2009) Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. Toxicology 265: 56-67.

SALVESEN GS (2002) Caspases: opening the boxes and interpreting the arrows. Cell Death Differ 9: 3-5.

SATO T, MACHIDA T, TAKAHASHI S, IYAMA S, SATO Y, KURIBAYASHI K, TAKADA K, OKU T, KAWANO Y, OKAMOTO T, TAKIMOTO R, MATSUNAGA T, TAKAYAMA T, TAKAHASHI M, KATO J, NIITSU Y (2004) Fas-mediated apoptosome formation is dependent on reactive oxygen species derived from mitochondrial permeability transition in Jurkat cells. J Immunol 173: 285-296.

SCAFFIDI C, FULDA S, SRINIVASAN A, FRIESEN C, LI F, TOMASELLI KJ, DEBATIN KM, KRAMMER PH, PETER ME (1998) Two CD95 (APO-1/Fas) signaling pathways. Embo J 17: 1675-1687.

SCAFFIDI C, SCHMITZ I, ZHA J, KORSMEYER SJ, KRAMMER PH, PETER ME (1999) Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. J Biol Chem 274: 22532-22538.

SCHLONDORFF J, BLOBEL CP (1999) Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding. J Cell Sci 112: 3603-3617.

SHARPE RM, MCKINNELL C, KIVLIN C, FISHER JS (2003) Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125: 769-784.

SHETTY J, MARATHE GK, DIGHE RR (1996) Specific immunoneutralization of FSH leads to apoptotic cell death of the pachytene spermatocytes and spermatogonial cells in the rat. Endocrinology 137: 2179-2182.

SHI Y (2002) Apoptosome: the cellular engine for the activation of caspase-9. Structure 10: 285-288.

SHI Y (2006) Mechanical aspects of apoptosome assembly. Curr Opin Cell Biol 18: 677-684.

SILVA D, LIZAMA C, TAPIA V, R.D. M (2011) Propylthiouracil-induced hypothyroidism delays apoptosis during the first wave of spermatogenesis. Biol Res 44: 181-188.

SKINNER MK (2005). Sertoli cell secreted regulatori factors. Sertoli cell Biology. M. K. a. G. Skinner, M.D. New York, Elsevier: 107-121.

SOBARZO CM, LUSTIG L, PONZIO R, SUESCUN MO, DENDUCHIS B (2009) Effects of di(2-ethylhexyl) phthalate on gap and tight junction protein expression in the testis of prepubertal rats. Microsc Res Tech 72: 868-877.

SWAN SH, MAIN KM, LIU F, STEWART SL, KRUSE RL, CALAFAT AM, MAO CS, REDMON JB, TERNAND CL, SULLIVAN S, TEAGUE JL (2005) Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113: 1056-1061.

TAKAO T, NANAMIYA W, NAZARLOO HP, MATSUMOTO R, ASABA K, HASHIMOTO K (2003) Exposure to the environmental estrogen bisphenol A differentially modulated estrogen receptor-alpha and -beta immunoreactivity and mRNA in male mouse testis. Life Sci 72: 1159-1169.

TER VELD MG, SCHOUTEN B, LOUISSE J, VAN ES DS, VAN DER SAAG PT, RIETJENS IM, MURK AJ (2006) Estrogenic potency of food-packaging-associated plasticizers and antioxidants as detected in ERalpha and ERbeta reporter gene cell lines. J Agric Food Chem 54: 4407-4416.

TESARIK J, MARTINEZ F, RIENZI L, IACOBELLI M, UBALDI F, MENDOZA C, GRECO E (2002) In-vitro effects of FSH and testosterone withdrawal on caspase activation and DNA fragmentation in different cell types of human seminiferous epithelium. Hum Reprod 17: 1811-1819.

TOPPARI J, LARSEN JC, CHRISTIANSEN P, GIWERCMAN A, GRANDJEAN P, GUILLETTE LJ, JR., JEGOU B, JENSEN TK, JOUANNET P, KEIDING N, LEFFERS H, MCLACHLAN JA, MEYER O, MULLER J, RAJPERT-DE MEYTS E, SCHEIKE T, SHARPE R, SUMPTER J, SKAKKEBAEK NE (1996) Male reproductive health and environmental xenoestrogens. Environ Health Perspect 104 Suppl 4: 741-803.

TOYAMA Y, SUZUKI-TOYOTA F, MAEKAWA M, ITO C, TOSHIMORI K (2004) Adverse effects of bisphenol A to spermiogenesis in mice and rats. Arch Histol Cytol 67: 373-381.

TRIPATHI R, MISHRA DP, SHAHA C (2009) Male germ cell development: turning on the apoptotic pathways. J Reprod Immunol 83: 31-35.

VILLUNGER A, MICHALAK EM, COULTAS L, MULLAUER F, BOCK G, AUSSERLECHNER MJ, ADAMS JM, STRASSER A (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 302: 1036-1038.

WANG RS, YEH S, TZENG CR, CHANG C (2009) Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev 30: 119-132.

WEIKERT S, SCHRADER M, MULLER M, KRAUSE H, MILLER K (2004) Expression of the apoptosis inhibitor survivin in testicular tissue of infertile patients. Int J Androl 27: 161-165.

WESTPHAL D, DEWSON G, CZABOTAR PE, KLUCK RM (2010) Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta 1813: 521-531.

WHITE JM (2003) ADAMs: modulators of cell-cell and cell-matrix interactions. Curr Opin Cell Biol 15: 598-606.

WHO (2010). Examination and processing of human semen. Switzerland, World Health Organization.

WONG CH, XIA W, LEE NP, MRUK DD, LEE WM, CHENG CY (2005) Regulation of ectoplasmic specialization dynamics in the seminiferous epithelium by focal adhesion-associated proteins in testosterone-suppressed rat testes. Endocrinology 146: 1192-1204.

YAMAMOTO CM, HIKIM AP, LUE Y, PORTUGAL AM, GUO TB, HSU SY, SALAMEH WA, WANG C, HSUEH AJ, SWERDLOFF RS (2001) Impairment of spermatogenesis in transgenic mice with selective overexpression of Bcl-2 in the somatic cells of the testis. J Androl 22: 981-991.

YAN HH, MRUK DD, CHENG CY (2008) Junction restructuring and spermatogenesis: the biology, regulation, and implication in male contraceptive development. Curr Top Dev Biol 80: 57-92.

YAN W, HUANG JX, LAX AS, PELLINIEMI L, SALMINEN E, POUTANEN M, TOPPARI J (2003) Overexpression of Bcl-W in the testis disrupts spermatogenesis: revelation of a role of BCL-W in male germ cell cycle control. Mol Endocrinol 17: 1868-1879.

YAN W, SUOMINEN J, SAMSON M, JEGOU B, TOPPARI J (2000a) Involvement of Bcl-2 family proteins in germ cell apoptosis during testicular development in the rat and pro-survival effect of stem cell factor on germ cells in vitro. Mol Cell Endocrinol 165: 115-129.

YAN W, SUOMINEN J, TOPPARI J (2000b) Stem cell factor protects germ cells from apoptosis in vitro. J Cell Sci 113 (Pt 1): 161-168.

YAO PL, LIN YC, RICHBURG JH (2009) Mono-(2-Ethylhexyl) Phthalate-Induced Disruption of Junctional Complexes in the Seminiferous Epithelium of the Rodent Testis Is Mediated by MMP2. Biol Reprod.

YAO PL, LIN YC, RICHBURG JH (2010) Mono-(2-Ethylhexyl) Phthalate-Induced Disruption of Junctional Complexes in the Seminiferous Epithelium of the Rodent Testis Is Mediated by MMP2. Biol Reprod 82 516-527.

YAO PL, LIN YC, SAWHNEY P, RICHBURG JH (2007) Transcriptional regulation of FasL expression and participation of sTNF-alpha in response to sertoli cell injury. J Biol Chem 282: 5420-5431.

YIN Y, STAHL BC, DEWOLF WC, MORGENTALER A (1998) p53-mediated germ cell quality control in spermatogenesis. Dev Biol 204: 165-171.

YOULE RJ, STRASSER A (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9: 47-59.

YOUNG KW, PINON LG, DHIRAJ D, TWIDDY D, MACFARLANE M, HICKMAN J, NICOTERA P (2010) Mitochondrial fragmentation and neuronal cell death in response to the Bcl-2/Bcl-x(L)/Bcl-w antagonist ABT-737. Neuropharmacology 58: 1258-1267.

ZHAI D, JIN C, HUANG Z, SATTERTHWAIT AC, REED JC (2008) Differential regulation of Bax and Bak by anti-apoptotic Bcl-2 family proteins Bcl-B and Mcl-1. J Biol Chem 283: 9580-9586.

ZHANG Y, LIN L, CAO Y, CHEN B, ZHENG L, GE RS (2009) Phthalate levels and low birth weight: a nested case-control study of Chinese newborns. J Pediatr 155: 500-504.

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The effects of 4-nonylphenol contamination on livers of Tilapia fish *(Oreochromus spilurs)* in Jeddah

El-jawaher Abdulla Bin-Dohaish

Girls College of Science, University of King Abdul Aziz, Jeddah, Saudi Arabia.

ABSTRACT

Alkylphenol polyethoxylates is a group of estrogenic compounds. Natural or synthetic types of these compounds react with the endocrine system by binding hormone receptors, resulting in interference with their action, which is why they are called endocrine disrupting chemicals. Among their hydrolytic products are nonylphenols (NP), which are considered pollutants of aquatic environments. The objective of this study was to evaluate the pathological alterations on liver tissue of fish exposed to these compounds for long durations, starting from beginning of life and during the period of sexual maturity. Tilapia fish were obtained from Abhur fish farms, reared in the laboratory in special basins, and divided into two groups. The first maternal group was untreated and their larvae were divided into three sub-groups: control; exposed to 15µg/L; and exposed to 30 µg/L. The second maternal group was divided into 2 sub-groups: with larvae exposed to 15µg/L; and with their larvae exposed to 30 µg/L. Larvae and mother exposed to different concentrations of NP (15 and 30 µg/L) showed an increased accumulation of NP in both livers and muscles compared to the control group due to bioaccumulation. Tissue section examinations of the treated group (15 µg NP /L) showed disruption of liver architecture, with lyses, loss of nuclei, necrosis, and fatty infiltration. The changes were more marked in tissues exposed to (30 µg NP /L). Although this pollution was not lethal, its effect may be reflected in vital activities and in the economy.

Key words: nonylphenol, Tilapia fish ,liver, histology.

INTRODUCTION

Alkylphenol polyethoxylates are considered effective surface-acting agents (Nimord and Benson, 1996). Many studies have indicated that these compounds have the ability to change or stop the normal physiological functions of organisms exposed to them, due to their estrogenic nature. Natural or synthetic types of these compounds react with the endocrine system by binding hormone receptors, resulting in interference with their action and metabolism, which is why they are called endocrine disrupting chemicals (EDCs) (Tyler et al., 1998). They are considered a great threat to living organisms in water environments and consequently to human beings (Jessica et al., 2006). These estrogen analogues are originally derived from many sources, such as paper, dyes, paints, detergents, and agricultural activity. They are drained into sewage systems and finally seep into ground waters (Johnson et al., 2005). Among these synthetic estrogens is 17 alpha ethinyl estradiol, which is of special concern because of its use as an oral contraceptive and its widespread presence in treated sewage water (Ying et al.,2002). It is also considered a dangerous compound because of its effectiveness in nanogram concentrations (Ascenzo et al., 2003). It was synthesized specifically to resist lyses and metabolic degradation in vivo (Kime, 1998). In vitro alkylphenol polyethoxylate compounds are susceptible to hydrolysis to ethoxylates with short bonds and derivatives of carboxylic acid. Finally, they are degraded to alkylphenols, (Nimrod and Benson, 1996), lipophilic and persistent metabolites (Ahel and Giger, 1993). Among these hydrolytic products are nonylphenols (NP) that are considered pollutants found in practically all aquatic environments. They have not only been found in sewage water, but also in surface water, sediments with concentrations ranging from nano to milligram quantities per liter (Talmage, 1994). EDCs compounds disrupt the endocrine system that regulates most responses in living organism. They include synthetic products like biphenol A, polychlorinated biphenol, dioxins, phtalates, pesticides, heavy metals, alkyphenols, polycyclic aromatic hydrocarbons, ethinylestradiol and estradiol. All these compounds, among others, seep from sewage water to aquatic environments (Ying et al., 2002;Ma et al., 2005). It is likely that there is an association between exposure to these compounds and changes observed in secondary sex characteristics of male and female fish (Bindohaish, 2008).

Many studies have demonstrated that alkylphenols have estrogenic effects that inhibit testicular growth in rainbow fish (Oncorhynchus mykiss), carp (Platichthys flesus), and flounder (Platihthys flesus). They also stimulate production of egg forming cells in immature Japanese medaka fish (Oryzias Latipes). In addition, they stimulate synthesis of one protein present in yolk vitellogenin. The latter is considered a sensitive biomarker for estrogen exposure (Gimeno et al., 1998).

Mathiessen et al. (1998) found that the effect of estrogen on fish was much wider than previously thought. The presence of these compounds in water and their consequent ingestion by living organisms became a major concern.

The aim of this study is to evaluate the pathological alterations on fish livers due to exposure to different concentrations of 4-nonylphenol compounds for long durations, starting from the beginning of life and during the period of sexual maturity, as well as comparing levels of alterations between fish from contaminated and non-contaminated mothers.

*Corresponding author: El-jawaher Adbulla Bin-Dohaish; Girls College of Science, University of King Abduk Aziz, Jeddah, Saudi Arabia; E-mail: Awatefali@yahoo.com, AL_JOHARA_D@hotmail.com

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MATERIALS AND METHODS

Chemicals

4-nonylphenol was purchased from Luborchemikalien sigma-Aldrich, dissolved in ethanol, diluted to concentrations of 15 and 30 µg/L in basins prepared for rearing. Other basins with natural seawater were used for the control group.

Study site

The area of Abhur, close to the College for Marine Science, is considered an ideal region because it far from industrial areas. Specimens of water and sediments were collected in July 2008. Water (5 liters/region) was collected in polyethylene containers. Sediments (the top 15cm) were collected for analysis. The fish were handled in conformance with the Guiding Principles in the Care and Use of Laboratory Animals, endorsed by the American Physiological Society.

Fish

Tilapia fish (Oreochromus spilurs) were selected because they are easy to obtain and use in laboratory experiments. They are the most suitable species that can be reared in the Jeddah sea environment. In addition, they can live in a range of high levels of salt content, have a reproduction rate and are resistant to diseases and parasites.

The first group

Tilapia fish (weight: 16.25±0.43 g. and length 8.5±0.48 cm.) were obtained from fish farms in the Abhur area. They were reared in the laboratory in special basins from egg-laying, hatching and larvae formation, without treatment.

The larvae were collected and divided into three sub-groups. Each group contained 50 fish:

1. The first sub-group was reared without treatment until maturity.
2. The second sub-group was exposed to (15µg/L) nonylphenol.
3. The third was exposed to(30 µg/L) nonylphenol.

Specimens from young and mothers were obtained.

The second group

Tilapia fish (weight: 16.25±0.43 g. and length: 8.5±0.48 cm.) were obtained from fish farms in the Abhur area. They were reared in the laboratory in special basins, divided into two sub-groups, each group containing 50 fish:

1. The first sub-group was exposed to (15µg/L) nonylphenol.
2. The second was exposed to (30 µg/L) nonylphenol.

The experiment continued in the same conditions from egg-laying, hatching and larvae formation until maturity. Specimens from young and mother were obtained.

During the experiment, which extended from July until December 2008, fish were maintained under suitable light, temperature and feeding conditions. Basins were inspected daily to remove unconsumed food and excreta that could easily pollute the water. Waste pipelines and ventilation lines were routinely cleaned to remove dirt. The numbers of dead fish throughout the experiments were recorded.

Experimental studies

1. Histological examination

Fish were removed from the basins, dried on filter paper, dissected and livers were extracted. General morphological characteristics of the organ, such as color, size and texture, were registered. The organ was preserved in 10% formalin for 24 hrs. Livers were dehydrated, paraffinized and then cross-sectioned at 2-3 microns, followed by staining with hematoxylin and eosin for light microscopic examination.

2. Determination of bioaccumulation of nonylphenols in tissues

Concentrations of 4-nonylphenols were measured in livers and muscles. Specimens were dried, ground, and digested with nitric acid. The sediment (1-5 mm) was dissolved in one normal nitric acid, completed with deionized water, kept in clean bottles and the substance concentration was measured by atomic absorption spectroscopy.

RESULTS

Water and sediment analysis

Table (1) shows the mean concentrations of nonylphenol (Abhur area - Red Sea coast, Jeddah) in water (4.65±2.61 µg/L) and sediments (32.33±3.21 µg/g.) and this considered the natural concentration in the environment.

Bioaccumulation of 4-NP in selected organs

Table (2) demonstrates that:

1. concentrations of NP are higher in the livers (10.533± 1.528 µg./g.) of control mothers than in muscles (8.333 ±0.528 µg./g). Mothers exposed to different concentrations of NP (15, 30 µg/L) showed higher concentrations of NP in both livers and muscles than in those of the control groups with increasing exposure due to bioaccumulation.
2. concentrations of NP are higher in the livers (12.333±1.528 µg./g.) of control larvae than in muscles (7.333±2.517 µg./g). Larvae exposed to different concentrations of NP (15, 30 µg/L) showed higher concentrations of NP in both livers and muscles than in those of control groups with increasing exposure due to bioaccumulation. This is quite evident in the treated mother groups.

Morphological examination

The numbers of dead fish throughout the experiments were within a normal range and all of them were healthy.

TABLE 1
Concentration of 4-NPnonylephenol in water (µg/L) and sediments(µg/g) of Abhur site.

Site		Water	Sediments
Abhur site	Mean±S.D	4.65±2.61	32.33±3.21

TABLE 2
Bioaccumulation of 4-NP(µg/g.) in liver and muscles of Fish larvae From untreated and treated mothers with different concentration of 4-NP.

Groups	concentration of 4-NP in water Mg/L		liver	Muscles
Treated mother	15	Mean±S.D	37.00±9.786	27.600±5.598
	30	Mean±S.D	43.00±8.600	32.300±6.780
Larvae from treated mother	15	Mean±S.D	44.33±10.600	38.000±6.080
	30	Mean±S.D	51.667±9.127	52.333±6.506
Standard mother	4.65	Mean±S.D	10.533± 1.528	8.333 ±0.528
Larvae from untreated mothers	15	Mean±S.D	34.333±9.661	25.000±5.568
	30	Mean±S.D	36.667±10.786	27.333±4.933
Larvae reared at standard water	4.65	Mean±S.D	12.333±1.528	7.333±2.517

Histological examination of liver

Maternal control specimens

Histological examination of liver sections revealed a normal architecture according to sea fish (Roberts, 1978). Although this was a control group, some pathological changes were observed, such as light staining and loss of the hexagonal structure of liver cells. The rest of the cells were arranged in a normal hexagonal pattern with deeply stained central nuclei in acidophilic cytoplasm. The cells were separated by blood sinusoids from a central vein (Fig. 1).

1. First sub-group (control larvae)

Stained sections showed central vein with radiated hepatocytes, separated by sinusoid with Kupffer cells (Fig. 2).

2. Second sub-group (treated larvae from non-treated mothers)

Tissue section examinations of the group treated with low concentrations of NP (15 µg/L) showed disruption of liver architecture. In spite of the presence of normal cells, the majority showed lyses, loss of nuclei, the presence of vacuolar cells and stagnation of blood in sinusoids (Fig. 3).

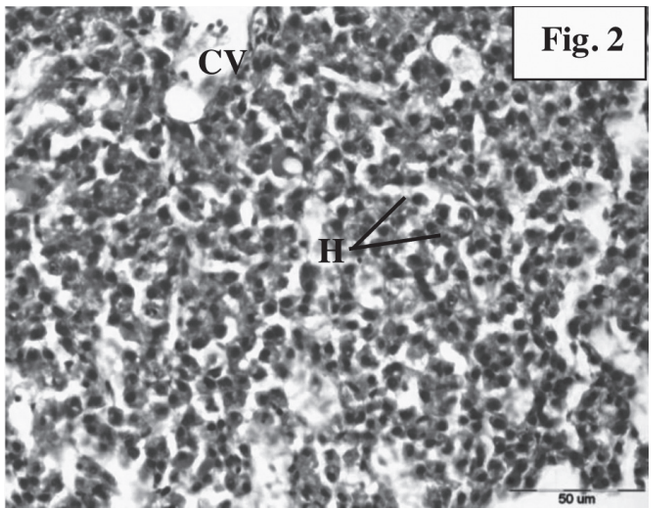

Fig. 2: Light micrograph for typical structure of standard liver of larva showing: central vein (CV) with radiated hepatocytes (H) separated by sinusoid with Kupffer cells(S).

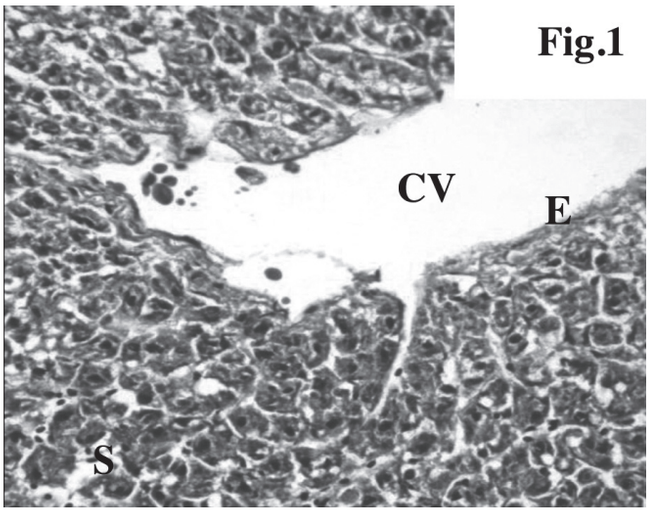

Fig. 1: Light micrograph for typical structure of maternal non-treated liver showing: central vein (CV) lined with endothelial cells (E) and sinusoid with Kupffer cells(S).

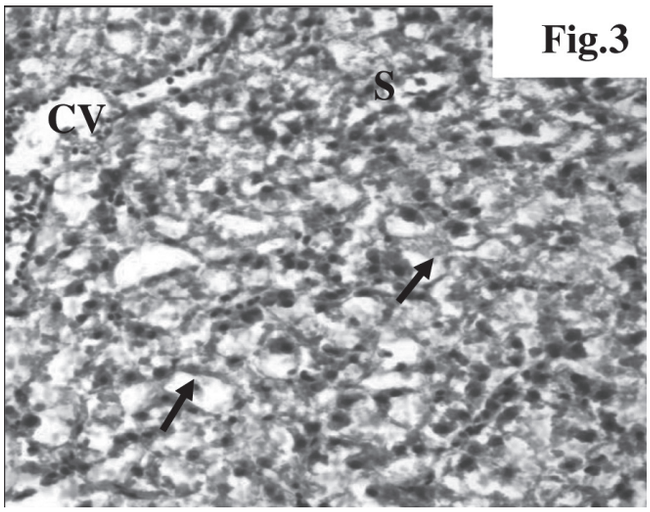

Fig. 3: Light micrograph of treated larvae liver from non-treated mother (15 mg NP/L) showing: congestion of both central vein (CV) and sinusoid(S) with vacuolar degeneration of hepatocytes (arrows).

3. Third sub-group (treated larvae from non-treated mothers)

The changes were more marked in tissues exposed to higher concentrations of NP (30 µg/L) where sections showed dilated blood vessels, loss of the hexagonal architecture of the liver, presence of fat globules, eccentric nuclei in cells and vacuolated cytoplasm. Other fields showed more damage represented by an increase in the number of cells, decreased affinity to eosin due to vacuolated cytoplasm, deep staining of pyknotic nuclei and the presence of clapped sinusoids full of stagnant blood (Fig. 4).

Treated maternal specimen

1. First sub-group (treated mothers and larvae)

Examination of the sections from the mothers of the group exposed to a lower concentration of NP revealed more loss of the ribbon-shaped structure, the presence of areas with lyses and some staining, but the rest maintained the normal hexagonal architecture, with deeply stained and degenerated nuclei in cytoplasm, and the central vein and sinusoids filled with stained fluid (Fig. 5). In the treated larvae from treated mothers, there are also some vessel abnormalities, such as a dilated central artery, ruptured vessel walls, dilated sinusoids, increased number of kuppfer cells, increased fat globules in cells and the presence of deeply stained degenerated nuclei (Fig. 6).

2. Second sub-group (treated mother and larvae from treated mothers)

The exposure of mothers to the higher concentration of NP resulted in changes that ranged from severe to moderate, represented by the presence of hemorrhaging in the central vein, increased wall thickness, lyses of cytoplasm in the majority of cells, loss of cell boundaries, dilated sinusoids, the vacuolated hepatic cord separated by dilated blood vessels that had ruptured endothelium, darkly stained cytoplasm and deeply stained nuclei (Fig. 7). The treated larvae from treated mothers showed loss of liver architecture, increased number of cells containing bare nuclei, increased fat content and the presence of short compressed unconnected sinusoids (Fig. 8).

DISCUSSION

Water and sediment analysis

The results showed that sediments contained high concentrations of NP compared to water in the selected zone

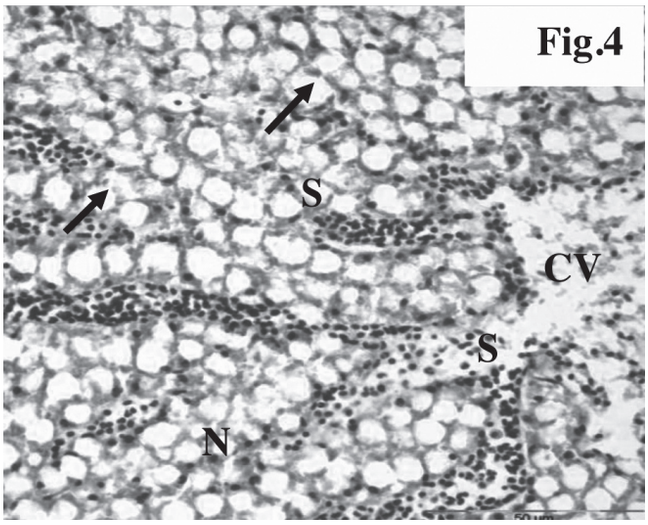

Fig. 4: Light micrograph of treated liver from non-treated mother (30 mg NP/L) showing: hepatocytes with lipid droplets (arrow) and marginal nuclei. Not, congestion and dilatation of both sinusoids (S) and central vein(CV).

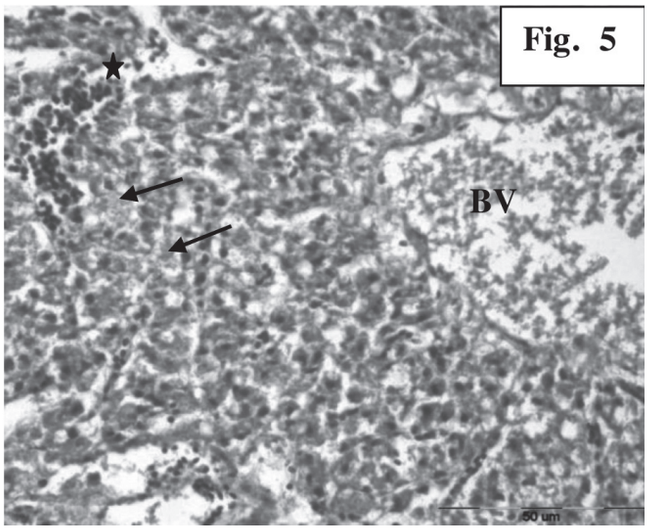

Fig. 5: Light micrograph of treated larvae liver from treated mother (15 mg NP/L) showing: Disturbance of hepatic architecture. Note, dilated and congested blood vessel (BV),stasis of red blood cells (stare) and necrotic area (arrows) (H&E x 40).

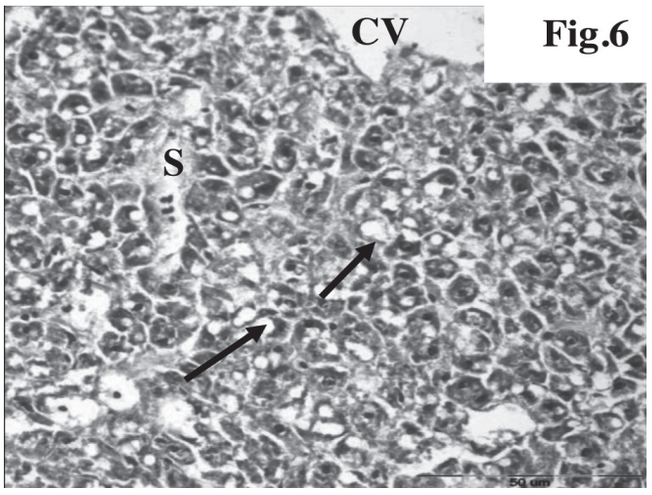

Fig. 6: Light micrograph of treated larvae liver from treated mother (15 mg NP/L) showing: dilated sinusoids (S) and central vein(CV). Note hepatocytes with variable sized of fat vacuoles (arrows).

and this was supported by previous studies that demonstrated the hydrophobic nature of NP compounds, their strong affinity to precipitate with non-soluble molecules, and that their final degradation with CO2 and water is very slow due to the slow breakdown of the phenol ring (Talmage, 1994; Nimord and Benson, 1996). Consequently fish living in this zone that are exposed to estrogenic alkylphenols, either directly from sediments or indirectly by feeding on the invertebrates living on these surfaces, will be seriously affected. In this work, the concentration of NP compounds in many specimens obtained from sediments in areas close to sewage outlets and areas close to industrial effluents reached (32.33±3.21 µg/g.), which contrasts with low concentrations that did not exceed 12.4 mg/kg (Hale et al., 2000), indicating that NP compounds exist among a mixture of other compounds, such as octyl and disulphonic, in spite of their low concentrations (Bennie, 1999).

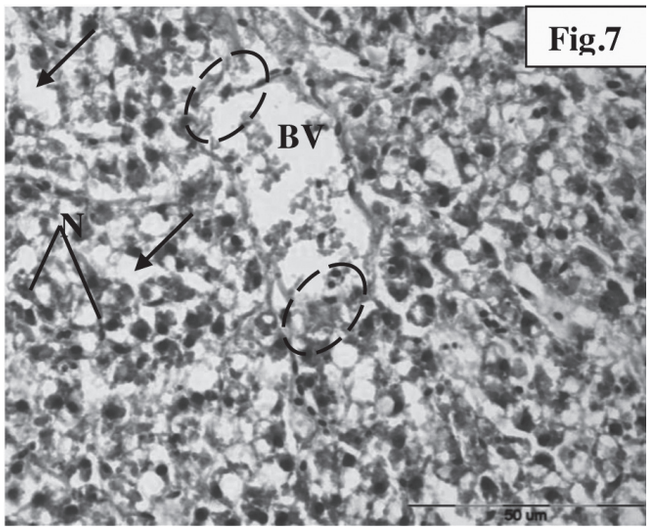

Fig. 7: Light micrograph of liver from treated mother (30 mg NP/L) showing: the vacuolated hepatic cord separated by dilated blood vessels (arrows) which have ruptured endothelium (dashed-line). Note, pyknotic nuclei (N). (H&E x40).

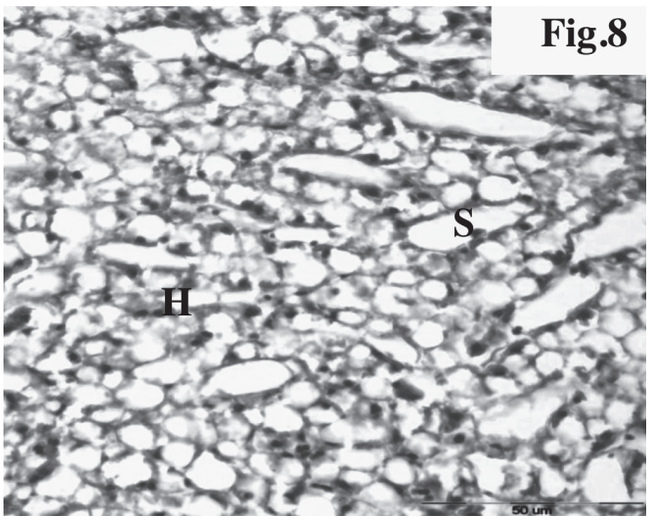

Fig. 8: Light micrograph of treated larvae liver from treated mother (30 mg NP/L) showing: marginal nuclei of atrophied hepatocytes (H) with fatty tissue appearance. Not, short and closed sinusoids (S) (H&E-X400).

Concentration of 4-NP in tissues

Low concentrations of NP were found in muscles (7.333±2.517 µg./g) and liver (12.333±1.528 µg./g.) of control larvae fish and in control mothers in muscle (8.333 ±0.528) and in liver (10.533± 1.528). There was a relative increase in concentrations in tissues among young fish from untreated mothers with the increase in exposure from 15 to 30 mg/L. There was also a marked increase in concentrations in tissues of fish from treated mothers, with both the lower and higher concentrations. This reflects the bioaccumulation of NP in fish tissues through the main sources, namely gills and the alimentary canal. Consequently these pollutants reach liver tissues through the blood (Roberts, 1978). It was clear from our results that the liver has more bioaccumulation than the muscles because of it role in storage, detoxification and regulation of all metabolic processes (Elrayis,1989).

This study showed that NP concentrations increased in the tissues of the young of mothers previously exposed, indicating long-term vertical transmission. Studies in the UK have demonstrated the presence in sewage systems of high concentrations of natural estrogenic compounds like estrone, 17-B-estradiol and synthetic estrogen from contraceptive pills made with ethinyl estradiol. The concentrations were high enough to stimulate vitellogenin. Many highly lipophilic and strongly adsorptive endocrine disrupting chemicals accumulate in sediments and tissue, increasing the probability that fish are exposed to the additive effects of other estrogenic compounds, which contributes to the overall contamination of the water environment (Virtanen, 1986).

Histological examination

Histological examination of tissues from fish exposed to different doses of NP compounds during pregnancy, development and maturity showed severe and variable affects, which increased with increased duration of exposure at the lower and higher concentrations. These results agree with previous studies that have demonstrated acute inflammatory edema and activation of kuppfer cells in cases of poisoning, especially with pesticides (Lutty et al., 2000). Burkitt et al. (1996) related these inflammatory changes to the immune reaction of the body to harm induced by chemicals and to infection by viruses and parasites that affected cells and tissues. The cellular edema of macrophages is considered a marker of chronic inflammation, where lymphocytes are responsible for the production of antitoxins and cell healing (Curran, 1996). Sileo et al. (2004) explained that the formation of cytoplasmic vacuoles in liver tissues of exposed birds living in water environment was due to acute intoxication by zinc and lead, which also appeared in the present tissue abnormalities. Mollendroff (1973) discovered that the formation of vacuoles is a defense mechanism by which the cell collects harmful and degraded substances to prevent interference with its viable activity. Burkitt et al. (1996) noted that interference with fatty acid metabolism leads to accumulation of neutral fat, resulting in equal-edged, and non-

membranous empty vacuoles. The neutral fat is dissolved by organic solvents during tissue preparation, leaving unstained empty vacuoles. Fatty infiltration can accompany hydropic degeneration, which appears as faintly stained vacuoles.

Examination of tissue sections treated with low doses showed that the liver cells maintain their ribbon-shaped structure, normal arrangement around the central vein, and normal content of cytoplasm and nuclei. Accumulation of fat droplets of different sizes in different cells was also observed. In addition, many dispersed cells with degenerated nuclei were observed, deeply stained with eosin. These cells separate from the rest due to dilation of intercellular spaces initiating cell necrosis. Studies have shown that the fatty infiltration and vacuolar degeneration are due to different contaminants. Khadre (1992) observed that exposure of T. Zilli fish to heavy metals resulted in swelling of liver cells due to accumulation of cytoplasmic vacuoles, nuclear atrophy and lyses of plasma membrane. Other studies have shown that exposure of Siganus rivulates fish to heavy metals results in abnormal liver architecture, fullness of cells with fat and their necrosis (Abdelaziz et al., 2006). Fabacher and Chambers (1971) determined that the amount of fat in Gambusia sp. fish is directly proportionate to their tolerance to insecticide DDT as fat gave them protection from toxins (Cheville, 1988). The effects on liver tissue increased with a higher doses and longer exposure, with the spread of necrotic cells in liver parenchyma. These cells can be distinguished from neighboring cells by their ribbon shape reflecting focal lysis. The cells degenerated and transformed to eosinic masses with no nuclei or atrophied and deeply stained nuclei. The other sections showed abnormalities, such as dilation of both sinusoids, central and portal veins and RBCs accumulation. There was a disturbance of liver architecture with many spaces that made the liver cells indistinguishable. This was probably due to loss of structural proteins (Burkitt et al., 1996). Kaptaner and Unal (2010) investigated that NP-dependent hepatotoxicity was causally related to the increase in apoptosis where NP (200 µg L^{-1}) caused significant increases in the extent of apoptosis in liver .

It is worth noting that the harmful effects on tissues, which varied in severity, were present in untreated fish reared in natural water and treated fish exposed to NP, indicating water pollution. Although this pollution was not lethal, its effect will be reflected in economic development and human reproduction.

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REFERENCES

ABDELAZIZ SH, ELGHAZALY N, BINDOHAISH GA (2006) Effect of pollutants in coastal water of Jeddah on2- the histological structure of liver of the fish Siganus rivulatus. Saudi Arabia. Egyption Journal of Aguatic research 32: 316-333.

AHEL M, GIGER W (1993) Partitioning of alkylphenols and alkylphenolethoxylates between water and organic solvents. Chemosphere 26:1471-1478.

ASENZO G, CORCIA A, GENTILI A, MANCINI R, MASTROPASQUA R, NAZ-ZARI M, SAMPERI R, (2003) Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Sci. Total Environ 302: 199-209.

BENNIE DT (1999) Review of the environmental occurrence of alkylphenols and alkylphenolethoxylates. Water Qual Res. J. Canada 34:79-122.

BINDOHAISH G (2008) Effects of environmental pollution with alkylphenol (4-Nonyl phenol) on reproduction of Tilapia, Oreochromus spilurs (Teleosts). Egyption Journal of Aquatic research 34:336-355.

BURKITT HG, STEVENS A, LOWE JS, YOUNG B (1996) Wheater's basic histopathology. International Student Edition. New York 299.pp: 88-93.

CHEVILLE NF (1988) Introduction to veterinary pathology. Iowa state University press. Ames, IA, pp: 537-540.

ELRAYIS OA (1989) Distribution of some heavy metals in sediments, water and different trophic levels from Jeddah coast, REDSEA. J. K. A. U.; Mar.sci 3:33-45.

FABACHER DL,CHAMBERS H (1971) A possible mechanism of insecticide resistance in mosquito-fish, Bull. Environ. Contam. Toxicol 6: 372-376.

GIMENO S, KOMEN H, JOBLING S, SUMPTER J, BOWMER T (1998) Demasculinisation of sexually mature male common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during spermatogenesis. Aquat Toxicol 43:93-109.

HALE RC, SMITH C L, DE FUR P O, HARVEY E , BUSH EO, LA GUARDIA M J,VADAS GG (2000) Nonylphenols in sediments and effluents associated with diverse waste water outfalls. Environ. Toxicol. Chem 19:946-952.

HARRIES JE, RUNNALLS T, HILL E, HARRIS CA, MADDIX S, SUMPTER JP, TYLER CR (2000) Development of a reproductive performance test for endocrine disrupting chemicals using Pair-Breeding fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 34:3003-3011.

JESSICA D, ROBERT M, FREDERIC S, ARNAUD B, DELPHINE L, JEAN-PIERRE T, PATERICK K (2006) Do sewage treatment plant discharges substantially impair fish reproduction in polluted rivers? Sci. Total Environ.

JOHNSON AC, AERNI HR , GERRITSEN A, GIBERT M, GIGER W, HYLLAND K, JURGENS M, NAKARI T, PICKERING A, SUTER M J F, SVENSON A, WETTSTEIN FE (2005) Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. Water Res 39: 47-58.

KAPTANER B, ÜNAL G (2010) Effects of 17-ethynylestradiol and nonylphenol on liver and gonadal apoptosis and histopathology in Chalcalburnus tarichi. Inc. Environ Toxicol 3:33-41.

KHADRE SE (1992) Cytological and cytochemical studies on the liver of *Tilapia zilli* surviving in lake maruit.Proceed of 2nd inter.Conf.Environ. Prot.Is a must 4:152-158.

KIME DE (1998) Endocrine Disruption in Fish.Kluwer Academic Publishers, Boston, MA.

LUTY S, LATUSZYNSKA J,OBUCHOWSKA P D,TOKAESKA M et.al. (2000) Subacute toxicity of orally applied alpha- cypermenthrin in swiss mice. Am. Agric. Environ. Med 7: 33-41.

MA T, WAN X, HUANG Q, WANG Z, LIU J (2005) Biomarker responses and reproductive toxicity of the effluent from a chinese large sewage treatment plant in Japanese medaka (Oryzias Latipes). Chemo-Sphere 59: 281-288.

MALTHIESSEN P, ALLEN Y T, ALLCHIN C R, FEIST S W, KIRBY M F, SCOTT A P, THAIN J E, THOMAS KV (1998) Estrogenic endocrine disruption in flounder (Platichthys Flesus) from United Kingdom estuarine and marine waters; Technical Report No. 107; The U. K. center for Environment Fisheries & Aquaculture science (CEFAS). Science Series; Lowest oft. Pp. 48.

MOLLENDROFF A (1973) cytology and cell physiology 3rd ed. New York. Academic press. pp: 50-55.

NIMROD AC, BENSON WH (1996) Environmental estrogenic effects of alkylphenol ethoxylates. Crit Rev Toxicol 26:335-364.

ROBERTS RJ (1978) Fish pathology. Bailliere tindall, London, pp. 489.

SILEO L, BEYER WN, MATEO R (2004) Pancreatitis in wild zinc-poisoned waterfowl. Avian pathology 32: 655-660.

TALMAGE SS (1994) Environmental and human safety of major surfactants; Lewis Publishers: Boca Raton. pp: 45-50.

TYLER CR, JOBLING S, SUMPTER JP (1998) Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev Toxicol 28: 319-361.

VIRTANEN MT (1986) Histological and Ultrastructural changes in the gills of *Poecilia reticulates* induced by an organochlorine pesticide. Jepto 7:73-86.

YING G, KOOKANA RS, RU Y (2002) Occurrence and fate of hormone steroids in the environment. Environ. Int 28: 545-551.

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The normal chain length distribution of the O antigen is required for the interaction of *Shigella flexneri* 2a with polarized Caco-2 cells

Anilei Hoare[1], Denisse Bravo[1], Mara Martinić[1], Miguel A. Valvano[2], Inés Contreras[1], Sergio A. Álvarez[1*]

[1] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile. PO Box 233, Correo 1, Santiago, Chile.
[2] Centre for Human Immunology, Department of Microbiology and Immunology, Siebens-Drake Research Institute, The University of Western Ontario, London, Ontario N6A 5C1, Canada.

ABSTRACT

Shigella flexneri causes bacillary dysentery in humans. Essential to the establishment of the disease is the invasion of the colonic epithelial cells. Here we investigated the role of the lipopolysaccharide (LPS) O antigen in the ability of *S. flexneri* to adhere to and invade polarized Caco-2 cells. The *S. flexneri* 2a O antigen has two preferred chain lengths: a short O antigen (S-OAg) regulated by the WzzB protein and a very long O antigen (VL-OAg) regulated by Wzz_{pHS2}. Mutants with defined deletions of the genes required for O-antigen assembly and polymerization were constructed and assayed for their abilities to adhere to and enter cultured epithelial cells. The results show that both VL- and S-OAg are required for invasion through the basolateral cell membrane. In contrast, the absence of O antigen does not impair adhesion. Purified LPS does not act as a competitor for the invasion of Caco-2 cells by the wild-type strain, suggesting that LPS is not directly involved in the internalization process by epithelial cells.

Key words: *Shigella*, epithelial cells, O antigen

INTRODUCTION

Species of the genus *Shigella* cause bacillary dysentery in humans, an acute intestinal infection characterized by bloody diarrhoea due to an intense inflammatory reaction accompanied by destruction of the colonic mucosa (Schroeder and Hilbi, 2008). An essential step to establish the disease is the invasion of the intestinal epithelial cells followed by bacterial multiplication and spread into adjacent cells. In the initial phase of infection, *Shigella* is transcytosed across M cells reaching the submucosa, and then free bacteria invade epithelial cells from the basolateral side (Schroeder and Hilbi, 2008). The invasive capacity of *Shigella* depends on proteins encoded in the large virulence plasmid (Parsot, 2009). This region contains more than 30 genes organized into two clusters: the *mxi-spa* locus that encodes the components of a type three secretion system (T3SS), and the *ipaBCDA* locus that encodes effector proteins that are translocated through the T3SS and cause cytoskeletal rearrangements that promote bacterial ingestion (Parsot, 2009).

The expression of *Shigella* virulence genes is controlled by environmental conditions prevalent in the large intestine (Dorman and Porter, 1998); however, full activation of the secretion system is triggered upon contact of *Shigella* with the host cells (Parsot, 2009). Thus, early interactions of bacteria with epithelial cells must take place before invasion can occur. Nonetheless, the nature of these interactions is not completely understood.

Lipopolysaccharide (LPS) has been implicated in the adherence and/or invasion of epithelial cells by several Gram-negative bacteria (Hoare et al., 2006; Kanipes et al., 2004; Klena et al., 2005; Zaidi et al., 1996). LPS consists of the inner lipid A portion, the core oligosaccharide, and the O polysaccharide or O antigen (OAg), which is exposed to the cell surface (Raetz and Whitfield, 2002). The LPS of *S. flexneri* 2a has O-antigen molecules with two preferred chain lengths, a short or S-OAg composed on average of 17 repeated units (RU) that is regulated by a chromosomally-encoded WzzB protein, and a very long or VL-OAg of about 90 RU that is regulated by Wzz_{pHS2}, which is encoded in plasmid pHS2.

The role of LPS in the invasion process of *Shigella* is unclear. Early reports using spontaneous or transposon-insertion mutants lacking O antigen or with truncated cores indicated that neither a complete core nor the O antigen is required for *S. flexneri* entry into HeLa cells (Okamura et al., 1983; Okamura and Nakaya, 1977; Rajakumar et al., 1994; Sandlin et al., 1995). More recently, a study conducted with mutants that were identified by signature-tagged mutagenesis indicated that a strain lacking O antigen was able to invade HeLa cells; however that work also showed that glycosylation of the O antigen enhances bacterial invasion, suggesting that a polymeric O antigen is necessary to this process (West et al., 2005). Only one study has addressed the role of LPS in the interaction of *S. flexneri* with polarized epithelial cells. Using mutants obtained by Tn*10* insertion, Köhler et al. (2002) found that adhesion to polarized human T84 colonic epithelial cells requires the presence of polymerized O antigen.

In this study, we have investigated the role of the O antigen in the ability of *S. flexneri* to adhere to and invade polarized Caco-2 cells, using a series of mutants with defined, non-polar deletions of the genes required for the assembly and polymerization of the O antigen. Our results indicate that polymerized O antigen is necessary for internalization and, moreover, a normal chain length distribution of the O antigen is required for the invasive process.

* Corresponding author: Sergio A. Álvarez, PO Box 233, Correo 1, Santiago, Chile, Phone: (56-2) 978 1681, Fax: (56-2) 2227900, E-mail: salvarez@uchile.cl

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MATERIAL AND METHODS

Bacterial strains, plasmids, media and growth conditions

Table I summarizes the properties of the bacterial strains and plasmids used in this study. Bacteria were grown in Luria-Bertani medium (LB) (10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl). Solid cultures were done in tryptic soy agar (TSA, Difco) containing 0.01% (w/v) Congo red (TSA-CR) at 30 ºC to ensure the presence of the virulence plasmid in the constructed strains (Schuch and Maurelli, 1997). Ampicillin (100 µg/mL), kanamycin (50 µg/mL), and tetracycline (10 µg/mL) were added when appropriate.

Mutagenesis of LPS biosynthesis genes

Mutagenesis was performed by the Red/Swap method to create chromosomal mutations by homologous recombination using PCR products (Datsenko and Wanner, 2000). *Shigella flexneri* 2a strain 2457T containing pKD46, which expresses the λ Red recombinase system, was transformed with PCR products that were generated using as template plasmid pKD4, which contains the FRT-flanked kanamycin-resistance gene (*aph*). Each primer pair also carried 30 bases that were homologous to the edge of the gene targeted for disruption. The sequences of the oligonucleotide primers used in this study are available on request. The kanamycin-resistant transformants were replica plated in the absence of antibiotic selection at 37 ºC and finally assayed for ampicillin sensitivity to confirm loss of pKD46. To obtain non-polar deletion mutants, the antibiotic resistance gene was removed by transforming the gene replacement mutants with pCP20, which encodes the FLP recombinase that mediates site specific recombination between the FRT sites (Cherepanov and Wackernagel, 1995). Correct insertional gene replacements and the deletion of the antibiotic gene cassettes were confirmed by PCR.

Cloning of the wzzB and wzz_{pHS2} genes

DNA fragments containing the *S. flexneri* 2457T wzz_{pHS2} (NC_002773) and *wzzB* (GeneID:1077593) genes and their promoter regions were amplified by PCR. The amplicons were cloned into pDA12 (Aubert et al., 2008). The sequences of the primers used for the cloning experiments are available upon request.

LPS purification

Large scale LPS preparations from *S. flexneri* wild type and the O-antigen mutants wzz_{pHS2}::*aph* and *wzzB* were obtained by the method of Darveau and Hancock (1983). The quality of the purified LPS samples was confirmed by Tricine-SDS-PAGE as described below. To rule out the presence of proteins, LPS samples were analyzed by SDS-PAGE and proteins were quantified using the Coomassie Plus protein assay kit (Pierce).

LPS analysis

Proteinase K-digested whole-cell lysates were prepared as described previously (Hitchcock and Brown, 1983), and LPS was separated on 12% (w/v) acrylamide gels using a Tricine-sodium dodecyl sulfate (SDS) buffer system (Lesse et al., 1990). Gel loadings were normalized so that each sample represented the same number of cells as previously described (Bravo et al., 2008). Gels were silver stained by a modification of the procedure of Tsai and Frasch (1982).

TABLE I
Strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Source or reference
***Shigella flexneri* 2a**		
2457T	wild type	ISP[b]
MSF1107	2457T wzz_{pHS2}::*aph*, Kan^R	This study
MSF1033	2457T *wzzB*	This study
MSF1749	2457T *wzy*	This study
MSF1210	2457T *waaL*	This study
MSF252	2457T *mxiH*::*aph*, Kan^R	This study
Plasmids		
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 *ori*TS	(Datsenko and Wanner, 2000)
pKD4	*bla* FRT *aph* FRT PS1 PS2 *ori*R6K	(Datsenko and Wanner, 2000)
pCP20	*bla cat c*I857 _PR *flp* pSC101 *ori*TS	(Cherepanov and Wackernagel, 1995)
pDA12	cloning vector, *ori*pBBR1, *mob*$^-$, *Pdhfr*, Tet^R	(Aubert et al., 2008)
pDAPF2	wzz_{pHS2} cloned into pDA12	This study
pDABF3	*wzzB* cloned into pDA12	This study

[a] Kan^R, kanamycin resistant.
[b] ISP, Institute of Public Health, Santiago, Chile.

Type three-secretion system induction

The secretion of Ipa proteins was induced by supplementing bacterial cultures with 20 µg/mL Congo red, as described by Bahrani et al. (1997). After the induction, supernatants were concentrated and the protein samples were analyzed by SDS-PAGE and silver stained.

Mammalian cell cultures

Caco-2 cells (American Type Culture Collection HTB-37), human colonic carcinoma cells, were maintained in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and non-essential amino acids (GIBCO BRL). Caco-2 polarized monolayers were obtained as follows: 3 µm pore size polycarbonate filter inserts (Millicell, Millipore) were coated with type A Gelatin (SIGMA) for 6 h before cell seeding. Then, 3 x 10^5 cells were seeded onto the inverted filter inserts and allowed to attach overnight. After that, inserts were placed upright in 24-well culture plates (basolateral side of monolayer upward) and maintained for 7 days in 5% CO_2 and 95% air, changing medium every 2 days. Electrical resistance was measured every day using a dual-voltage Ohmmeter clamp until a steady-state transepithelial cell resistance of approximately 400 to 800 Ω/cm was reached.

Adhesion and invasion assays

Bacteria were grown aerobically to an optical density at 600 nm of 0.3, washed twice with phosphate-buffered saline (PBS), suspended in DMEM supplemented with 10% (v/v) FBS and added to polarized Caco-2 cells monolayers at a multiplicity of infection of approximately 100. Adhesion assays were performed by co-incubating the monolayers with bacteria for 1 h at 4 ºC. Then, each well was rinsed three times with 100 µL of ice-cold PBS and cells were lysed by incubation with 100 µL of sodium deoxycholate (0.5% w/v in PBS) for 10 min. LB (900 µL) was added, and each sample was vigorously mixed to recover adherent bacteria, which were quantified by plating for colony-forming units (CFU) on TSA-CR plates. Adhesion was calculated as follows: percent adhesion = 100 x (number of cell-associated bacteria/initial number of bacteria added). For invasion assays, after addition of bacteria to the monolayers, the culture plates were centrifuged at 180 x *g* for 10 min and then incubated for 30 min at 37 ºC. Cells were washed three times with pre-warmed PBS and incubated for an additional 1 h in DMEM supplemented with 10% FBS and containing gentamicin (50 µg/mL) to kill extracellular bacteria. Cells were then washed three times with PBS and lysed with sodium deoxycholate (0.5% w/v in PBS). Intracellular bacteria were diluted and plated on TSA-CR plates. Invasion was calculated as the number of bacteria resistant to gentamicin/initial number of bacteria added. Invasion and adherence of the wild-type strain was arbitrarily defined as 100%.

For competition assays, purified LPS preparations (100 µL) at different final concentrations in PBS were added to polarized Caco-2 cells and incubated for 1 h at 37 ºC. Then, PBS was removed and invasion assays were performed as described above.

Data were obtained from at least three independent experiments performed in triplicate and were expressed as means ± standard errors. The statistical significance of differences in the data was determined using the one-way analysis of variance (ANOVA) test and the Dunnett's post-test.

RESULTS

Construction and LPS phenotypes of S. flexneri strains with defined mutations in O-antigen genes

Defined, non-polar deletions of genes responsible for assembly of the O antigen were constructed. The disrupted genes included: (i) wzz_{pHS2}, encoding the chain length regulator that determines polymerization of the VL-OAg (>90 RU), (ii) *wzzB*, encoding the chain regulator responsible for polymerization of the S-OAg (11-17 RU), (iii) *wzy*, encoding the O-antigen polymerase, and (iv) *waaL*, which encodes the O-antigen ligase; a protein catalyzing the link between O-antigen chain and the lipid A-core region (Schnaitman and Klena, 1993). Fig. 1A shows the LPS patterns of the parental and LPS mutant strains. As previously reported (Carter et al., 2007), strain wzz_{pHS2}::*aph* (MSF1107) produced O-antigen chains lacking VL-OAg (lane 2), whereas the *wzzB* mutant strain (MSF1033) did not exhibit S-OAg molecules (lane 3). Mutant *wzy* (MSF1749) showed a semi-rough phenotype with a very prominent band corresponding to core substituted with one O-antigen unit (lane 4). Finally, the *waaL* mutant (MSF1210) produced LPS with a complete core region and devoid of O antigen (lane 5).

To evaluate whether the LPS defects of the mutants affected the function of the T3SS, exponential cultures of each strain were incubated with Congo red to induce secretion of the effector proteins, as described in Materials and Methods. A mutant defective in type-three secretion (*mxiH*) was also analyzed as negative control. The parental strain and all the O antigen mutants showed the characteristic electrophoretic profile of Ipa proteins (Parsot et al., 1995), indicating a functional T3SS (Fig. 2).

Polymeric O antigen with normal chain-length distribution is required for invasion, but not for adhesion, to Caco-2 cells

To address the role of the O antigen in the initial interaction of *S. flexneri* with epithelial cells, we compared the abilities of mutants and the wild-type 2457T to adhere to and enter Caco-2 cells as a polarized colonic epithelial cell model.

We first investigated the role of O antigen in adhesion. The experiments were done under conditions in which invasion was inhibited by reduced temperature (4 ºC) as described in Material and Methods. As shown in Fig. 1B, all the mutants exhibited the same or higher levels of adhesion to Caco-2 cells compared to the parental strain. These results indicate that the presence of O antigen is not required for adhesion of *S. flexneri* to epithelial cells.

The ability of the O-antigen mutants to invade polarized epithelial cells was assayed. A *mxiH* mutant, which lacks one structural protein of the needle complex of the T3SS, was used as negative control. As shown in Fig. 1C, mutants in the wzz_{pHS2} or *wzzB* genes were significantly less invasive than the wild-type strain. This phenotype was also observed for the *wzy* and *waaL* mutants. We concluded from these data that entry of *S. flexneri* into epithelial cells from the basolateral surface requires not only polymerized O antigen but also, most importantly, a modal chain-length distribution of O-antigen molecules contributed by both WzzB and Wzz_{pHS2}.

To ensure that the defects in O-antigen chain length were responsible for the diminished invasive capacity of mutants

wzz_{pHS2} and *wzzB*, each mutant was complemented with the corresponding intact gene cloned into the low copy number plasmid pDA12 (Table I). The complemented strains showed wild-type LPS profiles (Fig. 3A). The invasion abilities of the complemented mutants were compared with the parental strain transformed with the vector pDA12. Both strains were able to enter Caco-2 cells as well as the wild type. The complemented *wzzB* showed higher invasion levels, but the differences with the wild type levels of invasion were not statistically significant (Fig. 3B).

LPS does not inhibit S. flexneri invasion of polarized Caco-2 epithelial cells

To elucidate whether O-antigen molecules are required for the first interaction with epithelial cells, LPS from the parental, wzz_{pHS2} and *wzzB* strains was purified and used as a competitor in the invasion assay. As shown in Fig. 4, pre-treatment of the monolayers with LPS purified (2 and 5 µg/mL) from any of the three strains did not affect the invasion ability of the parental strain. The same results were obtained when higher amounts of competitor LPS (100 and 1000 µg/mL) were added (data not shown). These results demonstrated that LPS does not compete for internalization of *S. flexneri* into epithelial cells by the basolateral membrane, supporting our finding that LPS is not required for adhesion, but rather it would be needed in a later step of the invasion process.

DISCUSSION

The role of LPS O antigen in the initial interaction of *S. flexneri* with epithelial cells is not yet well defined. Earlier studies carried out with HeLa cells indicated that O antigen is not required for *S. flexneri* invasion (Okamura et al., 1983; Okamura and Nakaya, 1977; Rajakumar et al., 1994; Sandlin et al., 1995). These studies were conducted using mutants that were not rigorously characterized genetically, obtained either spontaneously or by transposon insertions. In any case, secondary mutations and/or polar effects on genes located downstream the mutation site in the *wba* operon cannot be ruled out.

In this work, we dissected the contribution of O antigen to epithelial cell invasion by *S. flexneri* using mutants with defined deletions of genes for O antigen assembly and polymerization. We found that polymeric O antigen is required for invasion of polarized Caco-2 cells by the basolateral side. Our results are not in accordance to those of Köhler et al.

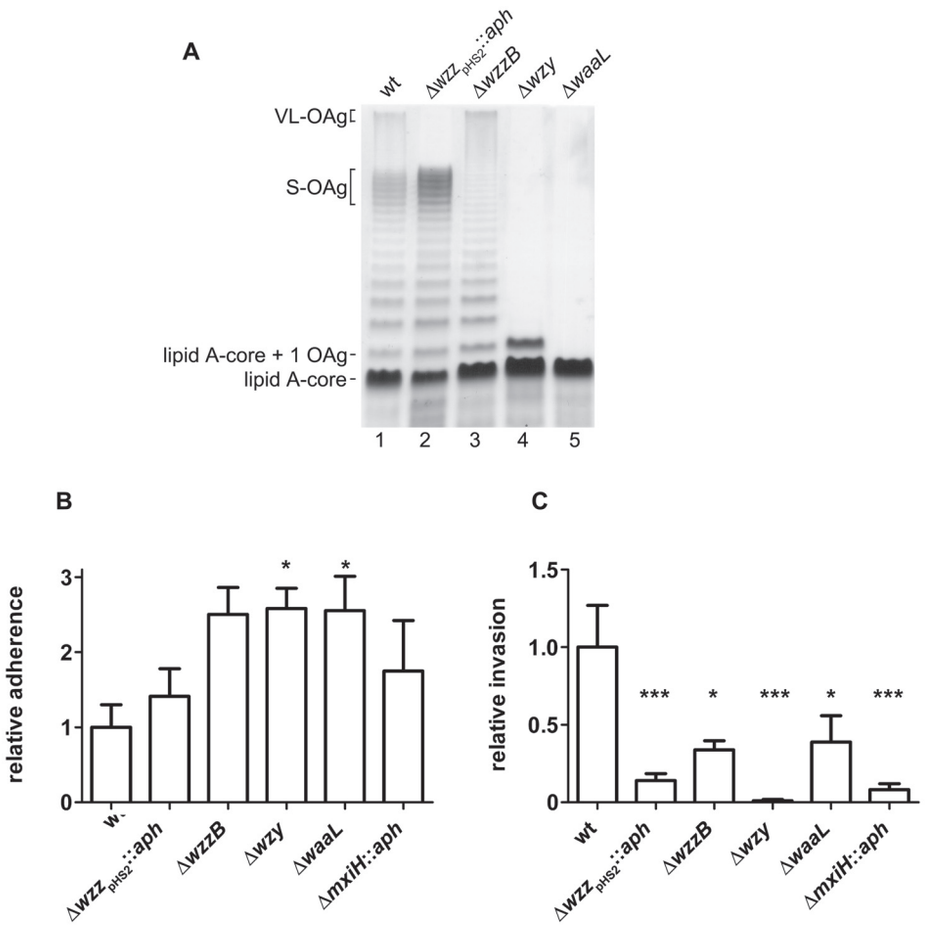

Figure 1: (A) LPS profiles from *S. flexneri* wild type and its isogenic O-antigen mutants. LPS samples from equal numbers of bacterial cells were loaded in each lane and were analyzed by Tricine/ SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel followed by silver staining. (B) Adhesion and (C) invasion of *S. flexneri* wild type and mutants to polarized Caco-2 cells. The assays were performed in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown. Values that are significantly different from those of the wild type are indicated by * ($p<0.05$) or *** ($p<0.001$). The strains are 2457T (wt), MSF1107 (Δwzz_{pHS2}::*aph*), MSF1033 (Δ*wzzB*), MSF1749 (Δ*wzy*), MSF1210 (Δ*waaL*), MSF252 (Δ*mxiH*::*aph*).

(2002), who reported that the presence of O antigen was not required for the invasion process itself, but it was essential for adhesion. In that study, invasion assays were performed by incubating the monolayer with bacteria at 37 ºC for 90 min, then gentamicin was added and incubation proceeded for additional 90 min. After lysis of the monolayer, CFU were counted. To estimate the number of surface-associated bacteria, the invasion values were subtracted from the CFU obtained after the initial 90 min of incubation without gentamicin. So, it is possible that during incubation with gentamicin, intracellular bacteria proliferated. Thus the invasion index was over estimated, lowering the adherence values. In consequence, these experimental conditions could explain why the authors attributed to an impaired adhesion a defect in invasion.

Interestingly, we found that polymerized O antigen with a normal chain length distribution, that is, containing S-OAg and VL-OAg, is required for polarized-cell invasion by *S. flexneri* (Fig. 1). Mutants with deletions of either *wzzB* or wzz_{pHS2} were significantly less invasive than the parental isolate. When these mutants were transformed with plasmids containing the corresponding intact gene, the invasive capacity was restored to wild-type levels (Fig. 3).

Our results suggest that no direct interaction occurs between O-antigen molecules and a possible ligand on the epithelial cell surface to promote invasion (Fig. 4). The invasion defects of the O-antigen mutants could be attributable to a general compromise of the outer membrane integrity. As the loss of bacterial cell envelope integrity often results in increased permeability and sensitivity to detergents, cationic peptides, antibiotics and other toxic compounds (Nikaido and Vaara, 1985), bacteria were tested for sensitivity to SDS, polymyxin B, EDTA, and novobiocin by the method described by Maloney and Valvano (2006). No differences were observed between any of the mutants and the wild-type strain (data not shown), suggesting that the cell envelope of the LPS mutants remains fully functional. Nonetheless, a normal LPS may be necessary for the assembly or exposure of other surface structure that mediates the entry process. In support of this notion, it has been reported that the O antigen contributes

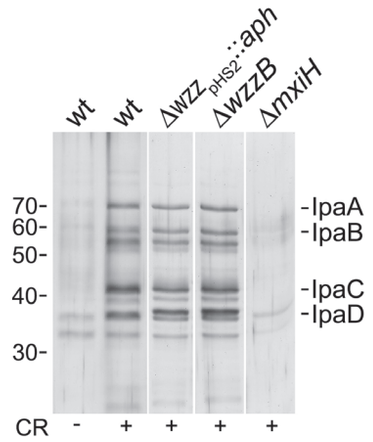

Figure 2: Secretion of Ipa proteins by *S. flexneri* wild type and mutants. Secretion was induced by incubating bacteria with Congo red (CR). Proteins in culture supernatants were electrophoresed and silver stained. The positions of Ipa proteins are indicated. Numbers indicate positions and sizes (in kDa) of standard proteins.

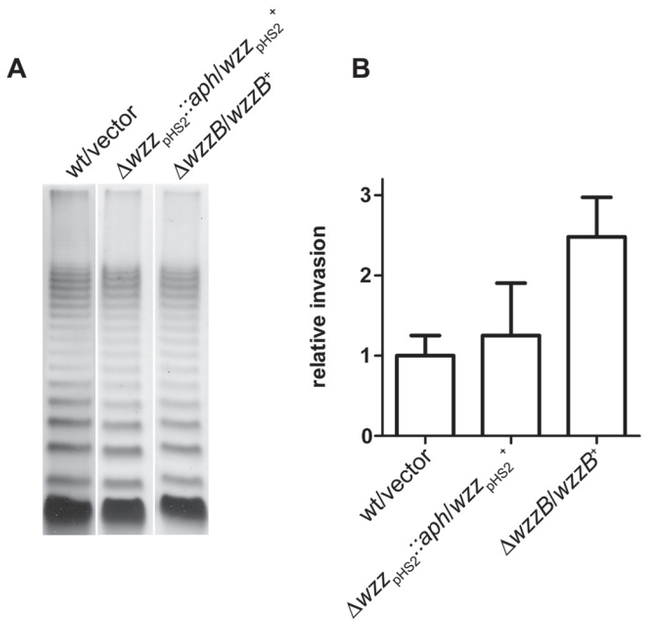

Figure 3: (A) LPS profiles from *S. flexneri* wild type and complemented O-antigen mutants (B) Invasion of polarized Caco-2 epithelial cells by *S. flexneri* wild type and complemented O-antigen mutants. The assays were performed in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown. Values were not significantly different from those of the wild-type *S. flexneri*. The strains are 2457T/pDA12 (wt/vector), MSF1107/pDAPF2 (Δwzz_{pHS2}::*aph*/wzz_{pHS2}^{+}) and MSF1033/pDABF3 ($\Delta wzzB/wzzB^{+}$).

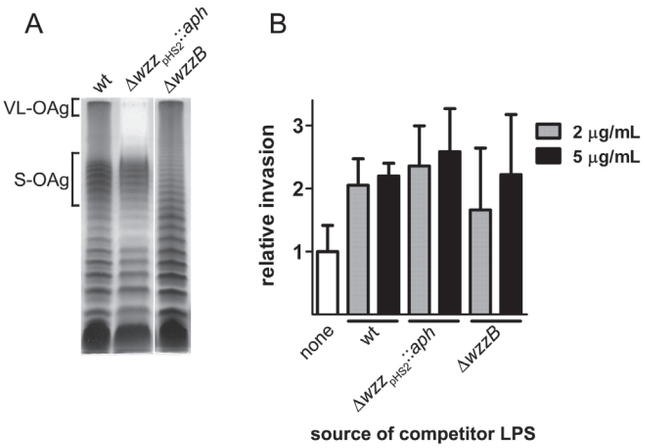

Figure 4: Effect of pre-treatment of Caco-2 cells with purified LPS on bacterial invasion. (A) LPS profiles of samples purified from *S. flexneri* wild type and its isogenic O-antigen mutants. (B) Competition assays. Caco-2 cells were pre-treated with 2 or 5 mg/mL of LPS obtained from each indicated strain before the addition of wild-type bacteria. Invasion assays were performed in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown. Values were not significantly different from that of non treated Caco-2 cells.

to stabilize the outer membrane by the interaction between adjacent O-antigen chains (Nikaido, 2003), and LPS also interacts with outer membrane proteins allowing their correct localization and function (Goldberg and Theriot, 1995).

In *S. flexneri*, mutants that are devoid of VL-OAg, but not of S-OAg, are highly sensitive to serum (Hong and Payne, 1997). These findings, together with the results of this study suggest that, while the presence of VL-OAg would be optimal for extracellular survival, a normal chain length distribution (S-OAg and VL-OAg) is required for internalization of bacteria into polarized epithelial cells.

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REFERENCES

AUBERT DF, FLANNAGAN RS, VALVANO MA (2008) A novel sensor kinase-response regulator hybrid controls biofilm formation and type VI secretion system activity in *Burkholderia cenocepacia*. Infect Immun 76: 1979-1991.

BAHRANI FK, SANSONETTI PJ, PARSOT C (1997) Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. Infect Immun 65: 4005-4010.

BRAVO D, SILVA C, CARTER JA, HOARE A, ÁLVAREZ SA, BLONDEL CJ, ZALDÍVAR M, VALVANO MA, CONTRERAS I (2008) Growth-phase regulation of lipopolysaccharide O-antigen chain length influences serum resistance in serovars of *Salmonella*. J Med Microbiol 57: 938-946.

CARTER JA, BLONDEL CJ, ZALDÍVAR M, ÁLVAREZ SA, MAROLDA CL, VALVANO MA, CONTRERAS I (2007) O-antigen modal chain length in *Shigella flexneri* 2a is growth-regulated through RfaH-mediated transcriptional control of the *wzy* gene. Microbiology 153: 3499-3507.

CHEREPANOV PP, WACKERNAGEL W (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158: 9-14.

DARVEAU RP, HANCOCK RE (1983) Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J Bacteriol 155: 831-838.

DATSENKO KA, WANNER BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97: 6640-6645.

DORMAN CJ, PORTER ME (1998) The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. Mol Microbiol 29: 677-684.

GOLDBERG MB, THERIOT JA (1995) *Shigella flexneri* surface protein IcsA is sufficient to direct actin-based motility. Proc Natl Acad Sci USA 92: 6572-6576.

HITCHCOCK PJ, BROWN TM (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 154: 269-277.

HOARE A, BITTNER M, CARTER J, ÁLVAREZ S, ZALDÍVAR M, BRAVO D, VALVANO MA, CONTRERAS I (2006) The outer core lipopolysaccharide of *Salmonella enterica* serovar Typhi is required for bacterial entry into epithelial cells. Infect Immun 74: 1555-1564.

HONG M, PAYNE SM (1997) Effect of mutations in *Shigella flexneri* chromosomal and plasmid-encoded lipopolysaccharide genes on invasion and serum resistance. Mol Microbiol 24: 779-791.

KANIPES MI, HOLDER LC, CORCORAN AT, MORAN AP, GUERRY P (2004) A deep-rough mutant of *Campylobacter jejuni* 81-176 is noninvasive for intestinal epithelial cells. Infect Immun 72: 2452-2455.

KLENA J, ZHANG P, SCHWARTZ O, HULL S, CHEN T (2005) The core lipopolysaccharide of *Escherichia coli* is a ligand for the dendritic-cell-specific intercellular adhesion molecule nonintegrin CD209 receptor. J Bacteriol 187: 1710-1715.

KÖHLER H, RODRIGUES SP, MCCORMICK BA (2002) *Shigella flexneri* interactions with the basolateral membrane domain of polarized model intestinal epithelium: Role of lipopolysaccharide in cell invasion and in activation of the mitogen-activated protein kinase ERK. Infect Immun 70: 1150-1158.

LESSE AJ, CAMPAGNARI AA, BITTNER WE, APICELLA MA (1990) Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J Immunol Methods 126: 109-117.

MALONEY K E, VALVANO M A (2006) The *mgtC* gene of *Burkholderia cenocepacia* is required for growth under magnesium limitation conditions and intracellular survival in macrophages. Infect Immun 74: 5477-5486.

NIKAIDO H, VAARA M (1985). Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49: 1-32.

NIKAIDO H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67: 593-656.

OKAMURA N, NAKAYA R (1977) Rough mutant of *Shigella flexneri* 2a that penetrates tissue culture cells but does not evoke keratoconjunctivitis in guinea pigs. Infect Immun 17: 4-8.

OKAMURA N, NAGAI T, NAKAYA R, KONDO S, MURAKAMI M, HISATSUNE K (1983) HeLa cell invasiveness and O antigen of *Shigella flexneri* as separate and prerequisite attributes of virulence to evoke keratoconjunctivitis in guinea pigs. Infect Immun 39: 505-513.

PARSOT C, MÉNARD R, GOUNON P, SANSONNETTI PJ (1995) Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. Mol Microbiol 16: 291-300.

PARSOT C (2009) *Shigella* type III secretion effectors: how, where, when, for what purposes? Curr Opin Microbiol 12: 110-116.

RAETZ CR, WHITFIELD C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71: 635-700.

RAJAKUMAR K, JOST BH, SASAKAWA C, OKADA N, YOSHIKAWA M, ADLER B (1994) Nucleotide sequence of the rhamnose biosynthetic operon of *Shigella flexneri* 2a and role of lipopolysaccharide in virulence. J Bacteriol 176: 2362-2373.

SANDLIN RC, LAMPEL KA, KEASLER SP, GOLDBERG MB, STOLZER AL, MAURELLI AT (1995) Avirulence of rough mutants of *Shigella flexneri*: requirement of O antigen for correct unipolar localization of IcsA in the bacterial outer membrane. Infect Immun 63: 229-237.

SCHNAITMAN CA, KLENA JD (1993) Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol Rev 57: 655-682.

SCHROEDER GN, HILBI H (2008) Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin Microbiol Rev 21: 134-156.

SCHUCH R, MAURELLI AT (1997) Virulence plasmid instability in *Shigella flexneri* 2a is induced by virulence gene expression. Infect Immun 65: 3686-3692.

TSAI CM, FRASCH CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119: 115-119.

WEST NP, SANSONETTI P, MOUNIER J, EXLEY RM, PARSOT C, GUADAGNINI S, PRÉVOST MC, PROCHNICKA-CHALUFOUR A, DELEPIERRE M, TANGUY M, TANG CM (2005) Optimization of virulence functions through glucosylation of *Shigella* LPS. Science 307: 1313-1317.

ZAIDI TS, FLEISZIG SM, PRESTON MJ, GOLDBERG JB, PIER GB (1996) Lipopolysaccharide outer core is a ligand for corneal cell binding and ingestion of *Pseudomonas aeruginosa*. Invest Ophthalmol Vis Sci 37: 976-986.

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Chronic ethanol consumption in mice does not induce DNA damage in somatic or germ cells, evaluated by the bone marrow micronucleous assay and the dominant lethal mutation assay

Manuel F Ellahueñe[1], L Patricia Pérez-Alzola[2] and M Isabel Olmedo[1]

[1] Unidad de Biodiversidad Acuática, Centro Nacional del Medio Ambiente (CENMA), Universidad de Chile, Santiago, Chile.
[2] Universidad Andrés Bello, Facultad de Ciencias Biológicas, República 252, Santiago, Chile.

ABSTRACT

Although alcohol is known to be a carcinogen for humans, ethanol-genotoxicity studies are incomplete. Ethanol seems not to be a bacterial mutagen, but the results are conflicting in rodent assays. We investigate the genotoxicity in the bone marrow micronucleus (MN) test and in the dominant lethal mutation (DLM) assay using two long-term ethanol exposure protocols. In the MN test, mice consumed three doses (5, 10 and 15% v/v) for 32 weeks. MN induction was compared to two control groups of 5- and 38-week-old mice (the ages of the treated mice when the treatment was initiated and when they were killed, respectively). For the three groups treated with ethanol there was no significant increase in MN induction as compared to the first control group, but observed MN frequencies were significantly lower than in the 38-week-old control group. This suggests a protective effect against genotoxic damage caused by aging, probably due to ethanol action as a hydroxyl radical scavenger.
In the DLM assay, male mice drank ethanol at 15% or 30% (v/v) for 20 weeks. In both groups the number of dead implants was similar to the control, but there was a significant reduction in total implants, indicating a pre-implantation loss.

Key words: dominant lethal mutation, ethanol, genotoxicity, micronucleus test

INTRODUCTION

Chronic alcoholism is a mayor public health issue around the world. Consumption of alcohol has been related to cardiovascular diseases (Friedman, 1998), hepatic effects (Lieber, 1985), brain toxicity (Harper, 1998), and increased incidence of esophagus, larynx and oral cavity cancers. The International Agency for Research on Cancer (IARC, 1998) has found that there is sufficient evidence for the carcinogenicity of alcoholic beverages in humans, and has classified alcoholic beverages as Group I carcinogens, although the mechanism of ethanol carcinogenicity is still not known (Kayani and Parry, 2010).

There are many studies about the genotoxic potential of ethanol, some of which have shown chromosomal effects in lymphocytes of alcoholics, like sister chromatid exchanges (reviewed in Obe and Anderson, 1987), induction of chromosomal aberrations (López et al., 2001) and increased incidence of aneuploidy (Kucheira et al., 1986), suggesting that alcoholism may cause chromosome damage in humans.

The genotoxicity testing of ethanol was first reviewed by an expert group of the International Commission for the Protection against Environmental Mutagens and Carcinogenesis (ICPEMC) (Obe and Anderson, 1987). No conclusion was reached about the effects of ethanol in relation to genetic damage. In 1995, the UK Department of Health's Committee on Mutagenesis of Chemicals in Food, Consumer Products and the Environment reviewed the evidence for the mutagenecity of ethanol, acetaldehyde and alcoholic beverages. The Committee agreed that the consumption of alcoholic beverages does not present any significant concern with respect to their mutagenic potential.

In 2001, Phillips and Jenkinson (Phillips and Jenkinson, 2001) made a review of the available information on the genotoxicity of ethanol, concluding that the data derived from studies using standard genotoxicity methods are incomplete. They reported that there is clear evidence that ethanol is not a bacterial or mammalian mutagen, but the results of some *in vivo* rodent assays are conflicting. The reported tests for chromosome aberrations *in vivo* are all negative, a minority of micronucleus tests have given positive results, dominant lethal assays are divided between positive and negative, and there is some limited evidence that high doses of ethanol can induce sister chromatid exchanges (SCE) and aneunogenic effects.

More recently, *in vitro* micronucleous (MN) tests with human TK6 cells showed non-MN induction with ethanol exposure up to 1.6% (v/v) (Bryce et al., 2007), while the cytokinesis blocked micronucleous assay (CBMN) revealed a dose dependent increase in the mean frequency of binucleated cells with MN with 0.8, 1.0 and 2.0% (v/v) of ethanol (Kayani and Parry, 2010)

The present study investigated the genotoxic potential of ethanol by using long-term exposure protocols to simulate chronic alcoholism in a mouse micronucleus bone marrow test and a mouse dominant lethal mutation assay.

MATHERIAL AND METHODS

Chemical Reagents

Ethanol (CAS Nº 6417-5) was obtained from Merck Chemical Co. (Germany).

Corresponding author: Manuel Ellahueñe, Centro Nacional del Medio Ambiente (CENMA), Av. Larraín 9975, La Reina, Santiago, Chile, Phone: (562)-2994151, FAX: (562) 2751688, E-mail: mellahuene@cenma.cl

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Animals and treatments

CF1 mice were obtained from the National Health Institute of Chile. They were kept with water and pellets (mouse chow Kimber, Chile) *ad libitum*, with a controlled temperature (24ºC) and humidity (40 - 50%) and a 12-hour light-dark cycle. During the study animals were kept under the same environmental conditions, except that ethanol treated groups received *ad libitum* an aqueous ethanol solution instead of water. Since mice do not like the taste of ethanol, we first began the ethanol treatment with a very low dose, which was daily increased to 5, 10, 15 or 30% (v/v)

Micronucleus Assay

For chronic ethanol treatment, three groups of male mice (n=6) drank an aqueous solution of ethanol with concentrations of 5, 10 or 15% (v/v) from 5 to 38 weeks of age. Control animals (n=6) received only water for the same period of time. The mouse bone marrow micronucleus assay was performed as previously reported (Ellahueñe et al., 1994). For each animal, 2000 polychromatic erythrocytes (PCE) were scored, and cytotoxicity was measured as the ratio of PCE/NCE (normochromatic erythrocytes) in 200 total erythrocytes. The results were analyzed statistically by the Mann-Whitney U test, with the level of significance set at a = 0.05.

Dominant Lethal Mutation Assay

Two groups of male mice (n=10) were exposed to 15% or 30% (v/v) of ethanol in the drinking water, as the only choice of liquid, from 10 to 30 weeks old. The control group (n=10) received only water for the same period of time. After the exposure interval, the ethanol solution was replaced by water in order to have minimal effects on mating performance. Each male was separately caged with two virgin females; each morning the females were examined for the presence of the vaginal plug, mated females were replaced and non-mated females were kept in cages. This mating procedure was performed for three consecutive days. The mated females were killed by cervical dislocation 12 days after the vaginal plug was observed and each uterus was removed and examined for the number and status of all implantation sites. The numbers of total live and dead implants were scored. The percentage of induced dominant lethal mutations (DLM) was calculated as:

$$\text{DLM (\%)} = 1 - \left(\frac{\text{Average of living embryos in the experimental group}}{\text{Average of living embryos in control group}} \right) \times 100$$

The results were analyzed statistically by the student's t test, with the level of significance set at a = 0.05.

RESULTS

Table I shows the micronucleus bone marrow test results. There were no significant increases in induced MNPCEs at any of the ethanol doses (5, 10, or 15% v/v) as compared to the 5-week-old control group. In addition, no differences were observed among the three doses. However, there were significant differences in induced MN between the two control groups, 2.7 ± 1.2 in 5-week-old mice and 6.5 ± 3.0 in 38-week-old mice. As well, the MN frequencies observed in the three ethanol treated groups were all significantly lower than the values observed in the 38-week-old control group.

The PCE/NCE ratio was within the normal range (>0.1) for both the treatment and control groups, showing no cytotoxic effect of ethanol ingestion on the cell population.

Dominant lethal mutation results are summarized in Table II. The number of total implants per pregnant female is similar for both ethanol doses, and significantly lower than the number observed in the control group. The mean numbers of living embryos per pregnant female also decreased in both ethanol treated groups, but not to statistically significant levels. No differences were observed for the mean number of dead implants, and the percentage of DLM was low and similar for both ethanol doses.

DISCUSSION

Alcohol abuse greatly increases the risk of different malignancies, including cancer, but the mechanisms by which ethanol could be a carcinogenic or co-carcinogenic agent remain unknown. The available data from studies on ethanol using standard genotoxicity methods are incomplete and inconclusive (Phillips and Jenkinson, 2001). Nevertheless, some studies have shown that chronic alcoholism may cause chromosome damage in humans, such as chromosome aberrations in peripheral blood lymphocytes (Burim et al., 2004), centromere positive MN in lymphocytes (Maffei et al., 2000), or micronuclei in tongue cells (de Almeida et al., 2002), suggesting a relationship between excessive consumption of ethanol-containing beverages and some degree of genotoxicity. On the other hand, the reports for chromosomal aberrations *in vivo*, designed to model the effects of alcoholism in animals, were all negative. Ethanol showed no effect on micronucleous incidence in rat bone marrow, when ethanol was administered

TABLE I

Mean frequency of micronucleous induction in 2000 polychromatic erythrocytes scored for each mouse, with 6 mice per group

Treatment	MN PCE ± sd	EPC/ENC ± sd
5-week control	2.7 ± 1.2 *	0.7 ± 0.3
38-week control	6.5 ± 3.0	0.5 ± 0.2
Ethanol at 5%	4.0 ± 2.7 *	0.7 ± 0.1
Ethanol at 10%	2.5 ± 0.5 *	0.5 ± 0.2
Ethanol at 15%	3.2 ± 1.6 *	0.6 ± 0.1

*: Significant difference at $p<0.05$ when compared to the 38-week control group. Abbreviations used: MN, micronucleus; PCE, polychromatic erythrocytes; EPC/ENC, the ratio of polychromatic/normochromatic erythrocytes in 200 total erythrocytes; sd, standard deviation.

in drinking water at 5% for 10-30 days (Balansky et al., 1993) or at 10% and 20% for 3 to 7 weeks (Tates et al., 1980). As well, a non-significant increase in chromosomal aberration frequency was observed at 20% of ethanol administered for 30 days (Tavares et al., 2001). These negative results could be the consequence of the short exposure time period (30 days), or the small number of animals (Tates et al., 1980). However, if ethanol *per se* is neither carcinogenic nor mutagenic, it could act as an enhancer for carcinogenicity. In this way it has been suggested that the ability of ethanol to induce CYP2E1 (Guegerich et al., 1994) could be the mechanistic basis of ethanol for enhancement of genotoxicity and carcinogenicity in mixtures containing carcinogens, such as alcoholic beverages (which contain urethane, and probably other known carcinogens). In support of this suggestion, alcoholic beverages, such as tequila and brandy, were demonstrated to be more genotoxic with the sister chromatid exchange test in mouse bone marrow cells than was ethanol itself (Pina Calva and Madrigal-Bujaidar, 1993).

In this work, we investigated the possible genotoxic effects of chronic ethanol exposure, so mice were drinking ethanol for a longer period of time than that used in the other cited *in vivo* ethanol studies. In the mouse bone marrow micronucleus assay (Table I), we observed that all ethanol doses tested did not increase the MNEPC frequency as compared to that in control animals. Because it has been reported that the MNEPC frequency increases in mice with age (Sato, 1995, Dass et al., 1997), we used two control groups, one 5 weeks old, the age at which the treated mice began drinking ethanol, and the other 38 weeks old, the age at which the treated mice were killed after drinking ethanol. Surprisingly, we observed that the MNEPC frequency was significantly lower in the three ethanol-treated groups compared to the 38-week-old control group, suggesting an ethanol protective effect against genotoxic damage caused by aging. This low MN frequency observed in our experiment could not be ascribed to an ethanol cytotoxicity effect, because the EPC/ENC ratio is within the normal range (≥ 0.1) in all of the treated and control animals (Table I).

Ethanol has previously been reported as a genotoxic protective agent. Different injected ethanol doses have been shown to reduce the induction of mouse MNEPC by urethane (Choy et al., 1995). The same authors also demonstrated that ethanol delays urethane genotoxicity for 12 hours (Choy et al., 1996).

Other authors have reported a radioprotective effect of ethanol. The addition of 10 mM ethanol reduced X-ray-induced chromosome aberrations in human lymphocytes *in vitro*, while ethanol was less effective in protection from carbon-induced chromosome aberrations. Since densely ionizing radiation produces lesions through direct action, while other ionizing radiation, like X-rays or g-rays, induces DNA lesions mostly by indirect action where free radicals play an important role, the authors concluded that ethanol protects DNA from X rays by scavenging hydroxyl (OH) radicals (Monobe and Ando, 2002). The same authors found that in mice that are given 1 ml of 5.5% ethanol orally 30 min before whole body irradiation, chromosome aberrations in spleen cells were significantly reduced by ethanol for g-ray irradiation, but not for carbon-ion irradiation (Monobe et al., 2003). These results may confirm the hypothesis that ethanol acts as a free radical scavenger. In human lymphocytes, ethanol showed a protective effect for hydrogen peroxide-induced DNA damage *in vitro* (Greenrod and Fenech, 2003), also apparently by acting as a free radical scavenger.

Paradoxically, oxidative metabolism of ethanol has been described as the mechanism by which ethanol can induce genotoxicity damage and some authors have demonstrated that antioxidants could markedly decrease the levels of ethanol induced DNA single-strand breaks in mouse brain cells (Guo et al., 2007)

The primary site of ethanol absorption is the gastrointestinal tract. Only 2% - 10% of the total ethanol ingested is eliminated by the kidney, and the rest is mainly oxidized in the liver (Lieber, 1997). Ethanol oxidation occurs in three places in hepatocytes, by different pathways: (a) in the cell cytoplasm by alcohol dehydrogenase; (b) in the endoplasmic reticulum by the microsomal ethanol oxidation system (MEOS); and (c) in the peroxisomes by catalase (Burim et al., 2004). Each of these three oxidation processes produces specific metabolites.

The first phase of ethanol biotransformation involves its oxidation to acetaldehyde, the main and primary metabolite of ethanol. Acetaldehyde is a highly reactive compound that can interact with DNA, forming DNA adducts of acetaldehyde like those observed in peripheral white blood cells of alcohol abusers (Fang and Vaca, 1995), or DNA strand breaks (Singh and Khan, 1995) and DNA cross-links in cultured human lymphocytes (Blasiak et al., 2000). Thus, the high levels of acetaldehyde accumulated during ethanol metabolism could be responsible for the positive genotoxic effects of ethanol reported in some papers. According to many authors, ethanol does not possess genotoxic potential and the observed ethanol genotoxicity is only due to acetaldehyde. Nevertheless, Kayani and Parry (2010) have shown that both ethanol and acetaldehyde can produce significant increases in MN induction, establishing that ethanol-MN induction is mainly through an aneugenic mechanism, while acetaldehyde does the same through a clastogenic effect. Different factors could

TABLE II
Uterine examination of females mated with control and chronic alcoholic mice

Treatment	Number of pregnant females	Mean of total implants ± sd	Mean of living embryos ± sd	Mean of dead embryos ± sd	DominantLethal Mutations (%)
Control	23	14.04 ± 1.89	13.30 ± 2.10	0.74 ± 1.32	
Ethanol at 15%	18	11.61 ± 1.86*	11.00 ± 1.85	0.61 ± 0.70	17.3
Ethanol at 30%	21	12.19 ± 1.86*	11.43 ± 2.16	0.76 ± 1.09	14.1

*: Significant difference at $p<0.05$ when compared to the control group.

regulate the rates of alcohol and acetaldehyde metabolism. One of these factors is alcohol deshydrogenase (ADH) and acetaldehyde deshydrogenase (ALDH) polymorphisms, both enzymes being primarily responsible for the amount of acetaldehyde generated. There could be a relationship between polymorphisms of ethanol-induced metabolism genes and alcoholism (Chen et al., 2009), and an effect has been observed of drinking alcohol and ADH/ALDH polymorphism on DNA damage, as measured by the alkaline comet assay (Weng et al., 2010). Thus, human polymorphisms of these enzymes could explain the different effects of the ethanol consumed by alcoholics.

Chronic ethanol consumption leads to its oxidation by MEOS, where P4502E1 is the main component of this system, generating an adaptive increase of ethanol metabolism (Lieber, 1997). This adaptation and/or tolerance to high concentrations of ethanol has been suggested to prevent ethanol that has entered the circulation from reaching excessive levels, so this progressively increasing rate of ethanol clearance from blood could explain the lack of genotoxic effect of chronic ethanol administration in rats (Tavares et al., 2001), as well as our negative results in mice. Furthermore, together with a lack of genotoxic effect, we also observed less genotoxic effects in older mice chronically exposed to ethanol. We suggest this could also be explained by ethanol metabolic pathways. Since oxidative damage of macromolecules plays a significant role in the aging of rodents (Martin et al., 1996) and enzymes involved in free radical detoxification, such as superoxide dismutase and catalase, are known to decrease in the liver and brain of mice with age (Dass et al., 1997), the genotoxic protection effect of ethanol could be due to ethanol acting as a hydroxyl radical scavenger by reducing free radical production. Chronic alcohol consumption could stimulate catalase activity, which is involved in ethanol oxidation, producing an adaptive increase similar to that observed for CYP2E1 activity. This hypothesis could also explain the absence of a synergistic or additive genotoxic effect of ethanol combined with cigarette smoke observed in alcoholic smokers (Burim et al., 2004), and the increase of cells with chromosomal aberrations in chronic alcoholics after 12 months of abstinence in comparison to the frequency at the beginning of an intensive treatment program (Huttner et al., 1999).

In germ cells, the dominant lethal mutation assay (Table II) gave no evidence of a significant increase in post-implantation lethality, while a moderate but significant reduction in mean total implants was observed, indicating pre-implantation loss. These results are similar to those reported by Rao et al, who found a significant reduction in mean total implants in a Swiss strain, but not in CBA mice after acute ethanol treatment (Rao et al., 1994). Thus, our results exclude the possibility that chronic ethanol exposure could induce germinal chromosome mutations in mice.

Finally, our results show that chronic treatment with ethanol does not induce genotoxic damage in somatic or germinal mouse cells evaluated by the micronucleus or the dominant lethal mutation assays. This suggests that ethanol could have a protective effect on age-related genotoxic damage, presumably due to free radical scavenging by ethanol, although further studies are required to confirm this effect and to elucidate the underlying mechanism.

REFERENCES

FRIEDMAN H (1998) Cardiovascular effects of alcohol in Recent Developments in Alcoholism, Volume 14: The Consequences of Alcoholism. Plenum Press: New York, New York.

LIEBER C (1985) Alcohol and the liver metabolism of ethanol, metabolic effects and pathogenesis of injury. Acta Med Scand Suppl 703: 1-55.

HARPER C (1998) The neuropathology of alcohol-specific brain damage, or does alcohol damage the brain? J Neuropathol Exp Neurol 57: 101-110.

IARC (1988) IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, no44, Alcohol Drinking, IARC, Lyon, 1-378.

KAYANI MA, PARRY JM (2010) The *in vitro* genotoxicity of ethanol and acetaldehyde. Toxicology in Vitro 24: 56-60.

OBE G, ANDERSON D (1987) Genetic effects of ethanol. Mutat Res 186: 177-200.

LÓPEZ MC, ROUBICEK M, ARZENO M (2001) Chronic alcohol ingestion and chromosomal aberrations. A popular study in Mar del Plata, Argentina. *J. Basic* Applied Genetics 14(1): 1-4.

KUCHEIRA K, TANEJA N, MOHAN D (1986) Chromosomal aberrations and sister chromatid exchanges in chronic male alcoholics. Indian J Med Res 83: 417-421.

PHILLIPS BJ, JENKINSON P (2001) Is ethanol genotoxic? A review of the published data. Mutagenesis 16(2): 91-101.

BRYCE SM, BEMIS JC, AVLASEVICH SL, DERTINGER SD (2007) In vitro micronucleous assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutat Res 630: 78-91.

ELLAHUEÑE MF, PÉREZ-ALZOLA LP, ORELLANA-VALDEBENITO M, MUÑOZ C, LAFUENTE-INDO N (1994) Genotoxic evaluation of eugenol using the bone marrow micronucleus assay. Mutat Res 320: 175-180.

BURIM RV, CANALLE R, TAKAHASHI CS, TAVARES DC, MARTINELLI AC, SAKAMOTO-HOJO ET (2004) Clastogenic effect of ethanol in chronic and abstinent alcoholics. Mutat Res 560: 187-198.

MAFFEI F, FIMOGNARI C, CASTELLI E, STEFANINI, GF, FORTI GC, HRELIA P (2000) Increased cytogenetic damage detected by FISH analysis on micronuclei in peripheral lymphocytes from alcoholics. Mutagenesis 15(6): 517-523.

DE ALMEIDA REIS SR, SADIGURSKY M, ANDRADE MGS, SOARES LP, DO ESPIRITO SANTO AR, VILAS BOAS DS (2002) Efeito genotoxico do etanol em células da mucosa bucal. Pesqui Odontol Bras 16(3): 221-225.

BALANSKY RM, BLAGOEVA PM, MIRCHEVA ZI, FLORA S (1993) Coclastogenicity of ethanol with cigarette smoke in rat erythroblasts and anticlastogenicity in alveolar macrophages. Cancer Lett 72: 183-189.

TATES AD, VOGEL N, NEUTEBOOM I (1980) Cytogenetic effects in hepatocytes, bone marrow cells and blood lymphocytes of rats exposed to ethanol in the drinking water. Mutat Res 70: 285-288.

TAVARES DC, CECCHI AO, JORDAO JR AA, VANNUCCHI H, TAKAHASHI CS (2001) Cytogenetic study of chronic ethanol consumption in rats. Teratogen Carcin Mut 21:361-368.

GUENGERICH FP, SHIMADA T, YUN CH, YAMAZAKI H, RANEY KD, THEIR R, COLES B, HARRIS TM (1994) Interactions of ingested food, beverage, and tobacco components involving human cytochrome P4501A2, 2A6, 2E1, and 3A4 enzymes. Environ Health Perspect 102 Suppl 9: 49-53.

PINA CALVA A, MADRIGAL-BUJAIDAR E (1993) SCE frequencies induced by ethanol, tequila, and brandy in mouse bone marrow cells in vivo. Toxicol Lett 66: 1-5.

SATO S (1995) Effect of aging on spontaneous micronucleus frequencies in peripheral blood of nine mouse strains: the results of the 7th collaborative study organized by CSGMT/JEMS.MMS. Mutat Res 388 (1-6): 51-57.

DASS SB, ALI, SF, HEFLICH RH, CASCIANO DA (1997) Frequency of spontaneous and induced micronuclei in the peripheral blood of aging mice. Mutat Res 381: 105-110.

CHOY WN, BLACK W, MANDAKAS G, MIRRO EJ, BLACK HE (1995) A pharmacokinetic study of ethanol inhibition of micronuclei induction by urethane in mouse bone marrow erythrocytes. Mutat Res 341(4): 255-263.

CHOY WN, MANDAKAS G, PARADISIN W (1996) Co-administration of ethanol transiently inhibits urethane genotoxicity as detected by a kinetic study of micronuclei induction in mice. Mutat Res 367(4): 237-244.

MONOBE M, ANDO K (2002) Drinking breer reduces radiation-induced chromosome aberrations in human lymphocytes. J Radiat Res 43: 237-345.

MONOBE M, KOIKE S, UZAWA, A, ANDO K (2003) Effects of beer administration in mice acute toxicities induced by X rays and carbon ions. J Radiat Res 44: 75-80.

GREENROD W, FENECH M (2003) The principal phenolics and alcoholic components of wine protect human lymphocytes against hydrogen peroxide – and ionizing radiation-induced DNA damage in vitro. Mutagenesis 18: 119-126.

GUO L, SUN B, JANG JY, ZHAO YQ, DONG YX, SPRANGER MI, WU CK (2007) Direct in vivo evidence of protective effects of grape seeds procyanidin fractions and other antioxidants against ethanol- induced oxidative DNA damage in mouse brain cells. J Agric Food Chem 55: 5881-5891.

LIEBER CS (1997)Ethanol metabolism, cirrosis and alcoholism.Clin Chim Acta 257: 59-84.

FANG JL, VACA CE (1997) Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers. Carcinogenesis 18(4): 627-632.

SINGH NP, KHAN A (1995) Acetaldehyde: genotoxicity and cytotoxicity in human lymphocites. Mutat Res 337(1): 9-17.

BLASIAK J, TRZECIAK A, MALECKA-PANAS E, DRZEWOSKI J, WOJEWÓDZKA M (2000) *In vitro* genotoxicity of ethanol and acetaldehyde in human lymphocytes and the gastrointestinal tract mucosa cells. Toxicol In Vitro 14: 287-295.

CHEN Y-C, PENG G-S, WANG M-F, TSA T-P, YIN S-J (2009) Polymorphism of ethanol-ethanol metabolism genes and alcoholism: correlation of allelic variation and pharmacokinetic and pharmacodinamic consequences. Chem Biol Interact 178: 2-7.

WENG H, WENG Z, LU Y, NAKAYAMA K, MORIMOTO K (2010) Effect of alcohol-drinking behavior and ADH1B and ALDH2 polymorphisms on basal DNA damage in human mononuclear cells as determined by the comet assay. Mutat Res 701: 132-136.

MARTIN GM, AUSTAD SN, JOHNSON TE (1996) Genetic anlysis of ageing: role of oxidative damage and environmental stresses. Nature Genet 13: 25-34.

HUTTNER E, MATTHIES U, NIKOLOVA T, EHRENREICH H (1999) A follow-up study on chromosomal aberrations in lymphocytes of alcoholics during early, medium and long-term abstinence. Alcoholism: Clin & Exp Res 23(2): 344.

RAO UN, ARAVINDAKSHAN M, CHAUHAN PS (1994) Studies on the effect of ethanol on dominant lethal mutations in Swiss, C57BL6 and CBA mice. Mutat Res 311(1): 69-76.

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Human dendritic cells sequentially matured with CD4+ T cells as a secondary signal favor CTL and long-term T memory cell responses

Thomas Simon*, Séverine Tanguy-Royer*, Pierre-Joseph Royer, Nicolas Boisgerault, Jihane Frikeche, Jean-François Fonteneau and Marc Grégoire

Institut de Recherche Thérapeutique de l'université de Nantes, INSERMU892, Nantes, France.

ABSTRACT

Dendritic cells (DCs) are professional antigen-presenting cells involved in the control and initiation of immune responses. *In vivo*, DCs exposed at the periphery to maturation stimuli migrate to lymph nodes, where they receive secondary signals from CD4+ T helper cells. These DCs become able to initiate CD8+ cytotoxic T lymphocyte (CTL) responses. However, *in vitro* investigations concerning human monocyte-derived DCs have never focused on their functional properties after such sequential maturation. Here, we studied human DC phenotypes and functions according to this sequential exposure to maturation stimuli. As first signals, we used TNF-α/polyI:C mimicking inflammatory and pathogen stimuli and, as second signals, we compared activated CD4+ T helper cells to a combination of CD40-L/IFN-γ. Our results show that a sequential activation with activated CD4+ T cells dramatically increased the maturation of DCs in terms of their phenotype and cytokine secretion compared to DCs activated with maturation stimuli delivered simultaneously. Furthermore, this sequential maturation led to the induction of CTL with a long-term effector and central memory phenotypes. Thus, sequential delivery of maturation stimuli, which includes $CD4^+$ T cells, should be considered in the future to improve the induction of long-term CTL memory in DC-based immunotherapy.

Key words: Cytotoxic T lymphocytes, dendritic cells, T memory cell, sequential maturation.

INTRODUCTION

The importance of DCs in adaptive immune responses has been extensively demonstrated in recent decades. DCs reside in skin, mucosa and peripheral tissues where they are exposed to foreign antigens. Through their capacity to sample their environment, to migrate to lymph nodes and to activate lymphocyte populations, these sentinel cells are at the origin of antigen-adapted immune responses, making a link between natural, innate and specific adaptive immunity (Banchereau and Steinman, 1998). In this context, DCs are at the heart of the immune surveillance mechanism, capturing and presenting antigens to specific T lymphocytes that can participate in response against pathogens or tumors.

Many studies in murine models have underlined the importance of CD4+ T cells in the induction of CD8+ T cell responses by DCs, and more recently in the establishment of a CD8+ T cell memory pool (Castellino and Germain, 2006). It is now well established in these animal models, that DCs must first be exposed to preliminary maturation signals in the periphery to induce their activation and migration to secondary lymphoid organs where they can interact and receive additional signals from CD4+ T helper cells. CD4+ T cell help is mediated by several factors, such as CD40-L. CD40-L expressed by activated CD4+ T cells is known to induce an increase in the expression of co-stimulatory molecules by DCs and in the secretion of bioactive IL-12p70, a cytokine required for Th1 differentiation and CTL activation (Bennett, et al., 1997, Mackey, et al., 1998, Ridge, et al., 1998, Schoenberger, et al., 1998). However, CD40-L independent signals, such as IFN-γ, can also act on DCs to increase their capacities to initiate CTL responses (Lu, et al., 2000, Schroder, et al., 2004). In addition, it was recently demonstrated in a murine model that these CD4+ T cell-derived signals induce secretion of the chemokines CCL3 and CCL4 by DC. These chemokines are reported to attract naïve CD8+ T cells, thus increasing the probability that an antigen-specific CD8+ precursor comes into contact with a mature antigen-presenting DC (Castellino and Germain, 2006, Castellino, et al., 2006, Arens and Schoenberger, 2010). Furthermore, these chemokines favored the differentiation of a long-term CD8+ T memory cell pool (Castellino and Germain, 2007, Obar and Lefrancois, 2010).

Thus, the murine models suggest that DC maturation require a two-step process in order to enable the efficient activation of CD8+ T cell responses and the generation of a T memory cell pool. Surprisingly, while numerous differences exist between murine and human DCs, no *in vitro* investigations concerning human DC maturation have taken into account this two-step maturation process. Indeed, they have focused on maturation stimuli, used alone or in combination, but delivered simultaneously, and not with $CD4^+$ T lymphocytes as a second signal. Thus, in the present study, we characterized the phenotype and function of human DCs sequentially matured with activated CD4+ T cells as a second signal in comparison to DCs activated with simultaneous delivery of maturation stimuli. We set up a DC maturation protocol composed of TNF-α/polyI:C as a first combined signal that mimics the peripheral stimuli, followed by exposure to allogenic CD4+ T cells activated with CD3 and CD28 specific mAb. We investigated the effect of this sequential maturation on the phenotype of DC and their ability to secrete cytokines and chemokines. We also compared their capacity to prime antigen-specific CD8+ T cell responses. We

*T. Simon and S. Tanguy-Royer contributed equally to this work.

Corresponding author: Dr. Marc Gregoire, INSERM U892, Institut de Recherche Thérapeutique de l'université de Nantes, 8 quai Moncousu BP70721, 44007 Nantes Cedex 1, France. Phone: +33-228-080-237; Fax: +33-240-084-082; E-mail: marc.gregoire@nantes.inserm.fr

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found that sequential maturation of human DCs, especially with activated CD4+ T cells as a second signal, lead to a better differentiation of anti-tumor CD8+ T cells with a long-term memory phenotype. Our work therefore reports a new approach using sequential maturation to optimize *in vitro* DC maturation and T cell activation for the development of DC-based immunotherapy.

METHODS

Cell culture

Human PBMCs were obtained at the Etablissement Français du Sang in Nantes from healthy donors (convention ATS 2000-12). After Ficoll-Paque density gradient centrifugation (Amersham, Uppsala, Sweden), monocytes were purified from PBMCs by elutriation using a Beckman Avanti J20 centrifuge equipped with a JE5.0 rotor and a 40 ml elutriation chamber. Monocyte purity (> 90%) was assessed by flow cytometry using scatter gating to define monocyte and lymphocyte populations.

Monocytes were cultured in flasks at $2x10^6$ cells/ml in RPMI 1% penicillin–streptomycin–glutamin supplemented with 2% human albumin (Laboratoire Français de Fractionnement et de Biotechnologies, Les Ulis, France), 500 U/ml recombinant human GM-CSF and 40 ng/ml recombinant human IL-4 (both from CellGenix, Paris, France). Immature DCs were harvested at day 5 and cultured in fresh medium with cytokines at $1x10^6$ cells/ml, in 24-well plates coated with poly(2-hydroxyethylmetacrylate) (Sigma, St Quentin Fallavier, France) to prevent cell adhesion. DC maturation was induced by the addition of TP: 20 ng/ml TNF-α (AbCys, Paris, France) and 50 µg/ml poly I:C (Sigma, St Quentin Fallavier, France), or CI: 0.5 µg/ml soluble CD40-L trimer (AbCys) and 1000 U/ml IFN-γ (AbCys), or T4+: activated CD4+ T cells at a DC/T4+ ratio of 1/2. In some conditions, T4+ or CI was added to DCs matured 10 hrs with TP. In order to activate CD4+ T cells (T4+), allogeneic CD4+ T lymphocytes were incubated for 6 hours in 24-well plates, coated with 0.5 µg/ml of anti-CD3 mAb (Orthoclone OKT3, Janssen-Cilag, France) and 0.5 µg/ml of anti-CD28 mAb (CD28.2, BD Biosciences, Paris, France), then washed and gamma-irradiated at 35 Gy to prevent proliferation.

The T2 cell line (Gift of T. Boon, Ludwig Institute for Cancer Research, Brussels) is a HLA-A*0201+ human T cell leukemia/B cell line hybrid defective for TAP1 and TAP2, thus expressing empty HLA class I molecules at its surface that can be loaded with exogenous peptides (Salter and Cresswell, 1986).

Immunofluorescence and flow cytometry

The surface phenotype of DCs was determined using the following PE-conjugated monoclonal antibodies (mAb): anti-CD80 (MAB104, Beckman-Coulter, Paris, France), anti-CD86 (HA5.2B7, Beckman-Coulter), anti-CD83 (HB15a Beckman-Coulter), anti-CD40 (MAB89, Beckman-Coulter), anti-HLA-DR (Immu-357, Beckman-Coulter), and FITC-conjugated anti-HLA-ABC mAb (B9.12.1, Beckman-Coulter). The fraction of responding CD8+ T cells was measured using APC-conjugated anti-CD8 mAb (RPA-T8, BD Biosciences) and PE-conjugated anti-IFN-γ mAb (4S.B3, BD Biosciences). The memory phenotype of CD8+ T cells was determined using PE-Cy5-conjugated anti-CD45RA mAb (HI100, BD Biosciences), PE-conjugated anti-CCR7 mAb(150503, RD system), FITC-conjugated anti-CD27 mAb (M-T271, BD Biosciences), APC-conjugated anti-CD28 mAb (CD28.2, BD Biosciences), FITC- or PE-conjugated anti-CD45RO mAb (UCHL1, BD Biosciences) and FITC-conjugated anti-CD127 mAb (hIL-7R-M21, BD Biosciences).

For cell surface staining, cells were incubated with mAb diluted in PBS containing 10% fetal calf serum (FCS, Sigma) for 30 min at 4°C in the dark, washed with PBS and then analyzed using a FACScalibur flow cytometer (Becton-Dickinson) and CellQuest Pro software. For IFN-γ intracellular staining, cells were fixed in PBS containing 4% paraformaldehyde (Electron Microscopy Science) for 10 min at room temperature, washed, permeabilized and stained with mAb in PBS containing 0.1% bovine serum albumin (BSA, Sigma) and 0.1% saponin (Sigma) at room temperature. Cells were washed and analyzed by flow cytometry. The percentage of positive cells was determined by comparison to the staining observed with isotype controls (FITC- or PE-conjugated mouse immunoglobulin G1 (IgG1) and IgG2b, BD Biosciences).

Cytokine production measurement

DCs were plated at $1x10^6$ cells/ml in RPMI +2% human albumin, supplemented with maturation stimuli. Supernatants were harvested after 48hrs of maturation. Cytokine production was determined using a CBA kit (BD Biosciences) in accordance with the manufacturer's protocol. The kit enables the simultaneous measurement of IL-10, IL-12p70, IL-6, CCL3 (Mip1-α) and CCL4 (Mip1-β) concentration by flow cytometry.

CD8+ stimulation by matured DCs

CD8+ T lymphocytes were negatively selected using Rosette Sep, according to the manufacturer's instructions (Stem Cell). Cells were then washed and cultured in RPMI supplemented with 8% pooled human serum (pHS) prepared locally.

For the induction of Melan-A-specific CTL, HLA-A*0201+ CD8+ T lymphocytes were cultured with autologous matured DC loaded for 2 hrs at 4°C with 10 µg/ml of Melan-$A_{(26-35)}$ A27L peptide (ELAGIGILTV, Eurogenetec) in U-bottom 96-well plates at a DC/T cell ratio of 1/10. Some 20 U/ml of IL-2 (AbCys) and 5ng/ml of IL-7 (AbCys) were added 5 and 7 days later, respectively. At day 6, the fraction of Melan-A-specific CTL was measured by CD8/IFN-γ co-staining after a 6 hrs re-stimulation with T2 cells loaded with 10 µg/ml of Melan-$A_{(26-35)}$ A27L peptide in the presence of brefeldin A. The cytotoxic activity of Melan-A specific CTL was determined by ^{51}Cr-release assay. At day 14, the profile of the CD8+ T memory cells was determined by immunofluorescence and flow cytometry.

^{51}Cr-release assay

Target cells (T2 cells) were pulsed for 1 hr with $Na_2{}^{51}CrO_4$ (NEN life science, Paris, France) and then washed. CD8+ T cells were co-cultured with $Na_2{}^{51}CrO_4$-pulsed T2 cells loaded with Melan-$A_{(26-35)}$ A27L peptides at several effector/target cell ratios. After 4 hrs of culture at 37°C, 25µL of supernatant was harvested and added to a 100 µL scintillation cocktail (optiphase supermix, Wallac, UK) before liquid scintillation counting. The percentage of specific lysis was calculated as

follows: (sample release - spontaneous release/maximum release - spontaneous release) x 100. The spontaneous release was calculated from targets incubated with culture medium, and the maximum release from targets incubated with 1% Triton X-100 (Sigma).

Statistical analysis

Statistical significance was assessed using the Kruskal-Wallis nonparametric test and Dunn's multiple comparison post-hoc test with GraphPad Prism 4 software.

RESULTS

Optimal DC maturation is induced by sequential exposure to maturation signals

In a first set of experiments, we measured the expression of DC maturation markers induced by the delivery of activated CD4+ T cells (T4+) after an initial exposure to TNF-α and poly I:C (TP) and determined the optimal sequence of the addition of these signals (Fig. 1). Immature DCs (iDC) exposed for 48 hrs to TP, to allogeneic T4+ or to both maturation stimuli delivered simultaneously, acquired a mature phenotype with an increased expression of surface CD80, CD86, CD83 and HLA class I molecules (Fig. 1A). Interestingly, the maturation level of these DCs was strongly improved when T4+ were added 10 hrs after the initial exposure to TP. The DC maturation level then decreased when the delay between exposure to TP and T4+ was extended to 24 hrs and 48 hrs. These results suggest that matured DCs are more sensitive to T4+-derived signals 10 hrs following initial exposure to TP and that this sensitivity then decreased because of their exhausted nature (Langenkamp, et al., 2000).

In addition to surface markers, we also measured cytokine productions by DC in culture supernatants (Fig. 1B). High levels of IL-6, IL-10 and IL-12 were observed in the culture of DC matured by the simultaneous addition of TP and T4+ (TP/T4+ sim). In comparison, high levels of IL-6 and IL-12 production were also measured for DCs matured sequentially by TP and, 10hrs later, activated CD4+ T cells, but the IL-10 production dropped dramatically. The addition of activated CD4+ T cells 24hrs or 48hrs after TP did not result in higher production of the three cytokines, confirming the observation shown in Figure 1A that DCs are less sensitive to CD4+ T cell signals 24 hrs after the exposition to TP. Therefore, for all subsequent experiments, exposure to second signals was performed ten hours after exposure to TP and is referred as TP+T4+.

Activated CD4+ T cells as second signals dramatically increases DC maturation

In a previous study, we reported that a combination of Poly I:C and TNF-α induces DC maturation and that IL-12p70 production by mature DC can be increased by addition, several hours later, of activated CD4+ T cell signals such as IFN-γ and CD40-L (Spisek, et al., 2003). In the present study, we used as first signals, TNF-α/polyI:C (TP) mimicking inflammatory and pathogen stimuli, and as second signals, we compared a combination of CD40-L/IFN-γ (TP+CI) to activated CD4+ T cells (TP+T4+). We then analyzed the expression of a large panel of surface maturation markers: CD80, CD86, CD83, CD40, HLA class I and class II (Fig. 2). The strongest expression of these phenotypic markers was observed on DCs exposed first to TP and then, ten hours later, to T4+ as a second signal, with significant differences for CD86, CD83, CD40 and HLA ABC ($P<0.05$). However, the expression of the HLA-DR molecule was not amplified by the sequential delivery of T4+. Comparatively, when we tried to mimic the effects of the activated CD4+ T cells by providing DCs with a combination of soluble CD40-L plus IFN-γ as a second signal (TP+CI), we also observed an increased maturation level, but not to the same extent as that observed when DCs were exposed to TP plus T4+. Our results suggest that the signal provided by activated CD4+ T cells to DCs cannot be mimicked by the simple addition of CD40-L and IFN-γ and that additional stimuli are required.

To further define the effects of sequential exposure to TP and T4+ on DC maturation level, we measured the secretion of major cytokines (IL-6, IL-10 and IL-12p70) and chemokines (CCL3 (Mip-1α) and CCL4 (Mip-1β)) by DC. Supernatants of the different DC cultures were analyzed by cytometric bead array (Fig. 3). We observed that secretion of IL-6, IL-10 and IL-12p70 by DC was induced by the mixture of TNF-α and Poly I:C as we previously reported (Spisek, et al., 2003). These secretions were significantly increased ($P<0.05$) by the sequential addition of activated CD4+ T cells, which, alone, did not produce any of these cytokines (data not shown).

In comparison, the addition of soluble CD40-L and IFN-γ (TP+CI) after the first signal did not affect the production of IL-6, IL-10 and IL-12p70. Our results suggest that additional unknown signals delivered by activated CD4+ T cells participate with IFN-γ and CD40-L to increase IL-12p70 secretion. In addition, we observed that secretion of CCL3 and CCL4 chemokines, which are implicated in the attraction of naïve T cells (Castellino, Huang, Altan-Bonnet, Stoll, Scheinecker and Germain, 2006), is also greatly increased by the sequential addition of T4+ ten hours after the exposure of DC to TP. Thus, the sequential combination of TNF-α / Poly I:C and activated CD4+ T cells conditions the DC to produce the maximum levels of the cytokines IL-6 and IL-12p70 and the chemokines CCL3 and CCL4, associated with a highly mature surface phenotype. The large amount of IL-12p70 induced by the sequential maturation of DC by TP plus T4+ would probably provide a microenvironment favorable to the initiation and development of Th1 and CTL responses. In contrast, the amount of IL-10 production by TP plus T4+-matured DCs has a negative effect on these T cell responses (O'Garra and Vieira, 2007).

DCs matured sequentially with CD4+ T cells as a second signal induce cytotoxic CD8+ T cell responses against the tumor-associated antigen Melan-A

Since we observed a strong maturation profile and a high production of IL-12p70 by DCs sequentially matured by TP and T4+ (Fig. 2 and 3), we hypothesized that this sequential maturation would favor induction of CTL specific for a tumor-associated antigen. Thus, autologous HLA-A*0201+ CD8+ T lymphocytes from healthy donors were stimulated once with iDC or differentially matured DCs pulsed with the Melan-A_{26-35} A27L peptides derived from the melanocytic differentiation antigen Melan-A/MART1 (Fig. 4). After six

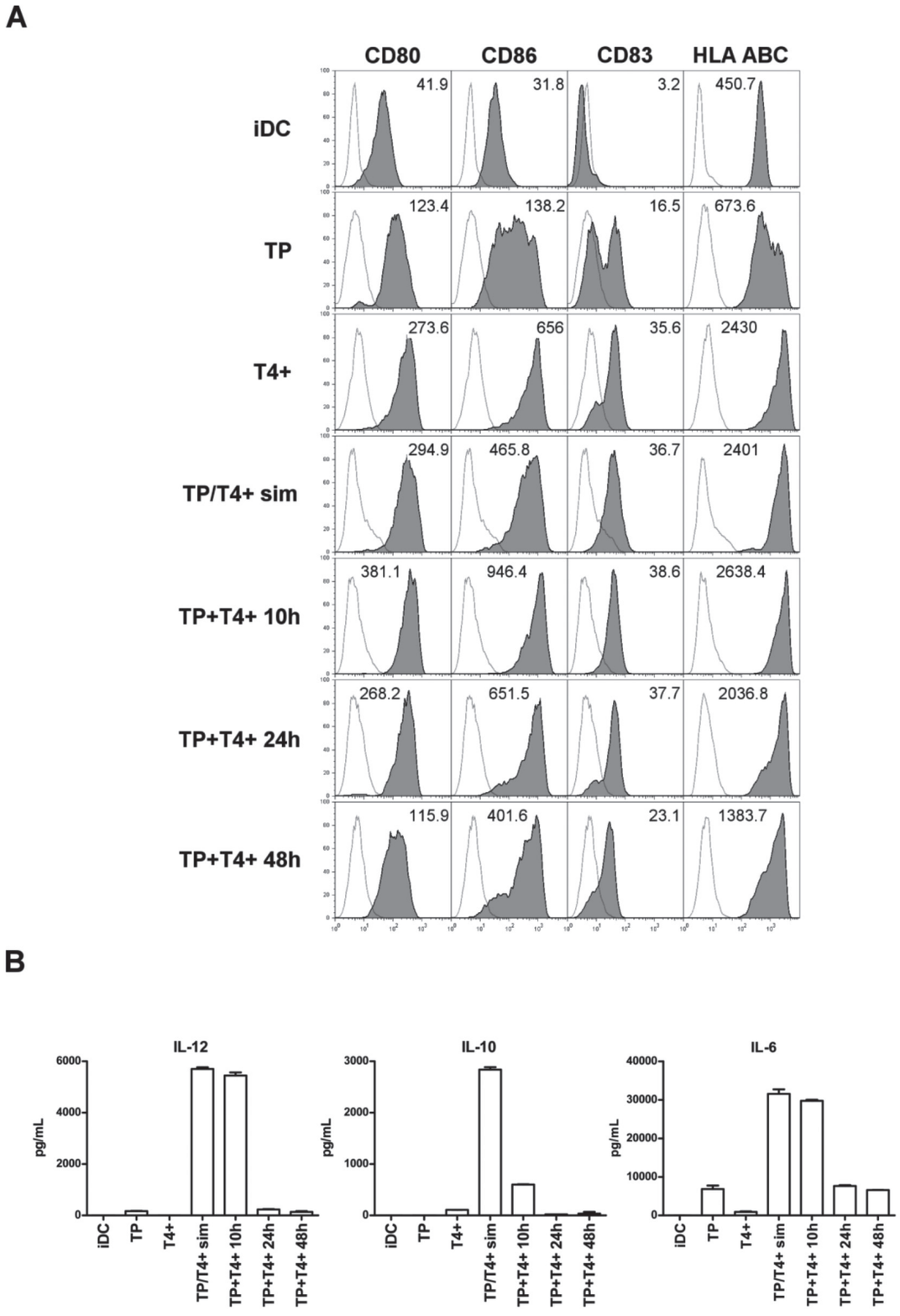

Fig. 1 Optimal DC maturation is induced by addition of activated CD4+ T cells as a second signal, ten hours after the first maturation signal. (A) phenotypic analysis of immature dendritic cells (iDC) and mature DC exposed for 48 hrs to different maturation stimuli: TNF-α and poly I:C (TP), irradiated activated CD4+ T cells (T4+), both stimuli added simultaneously (TP/T4+ sim) or TP followed by T4+, 10 hrs, 24 hrs or 48 hrs later (TP+T4+ 10h, TP+T4+ 24h or TP+T4+ 48h). Median fluorescence intensity (MFI) is indicated on the corresponding histogram plot. (B) Analysis of IL-6, IL-10 and IL-12 production in the supernatant of DC culture exposed 48hrs to the different maturation stimuli. Measurement of cytokine production was performed by commercially available BD Cytometric Bead Array kits, according to the manufacturer's protocol.

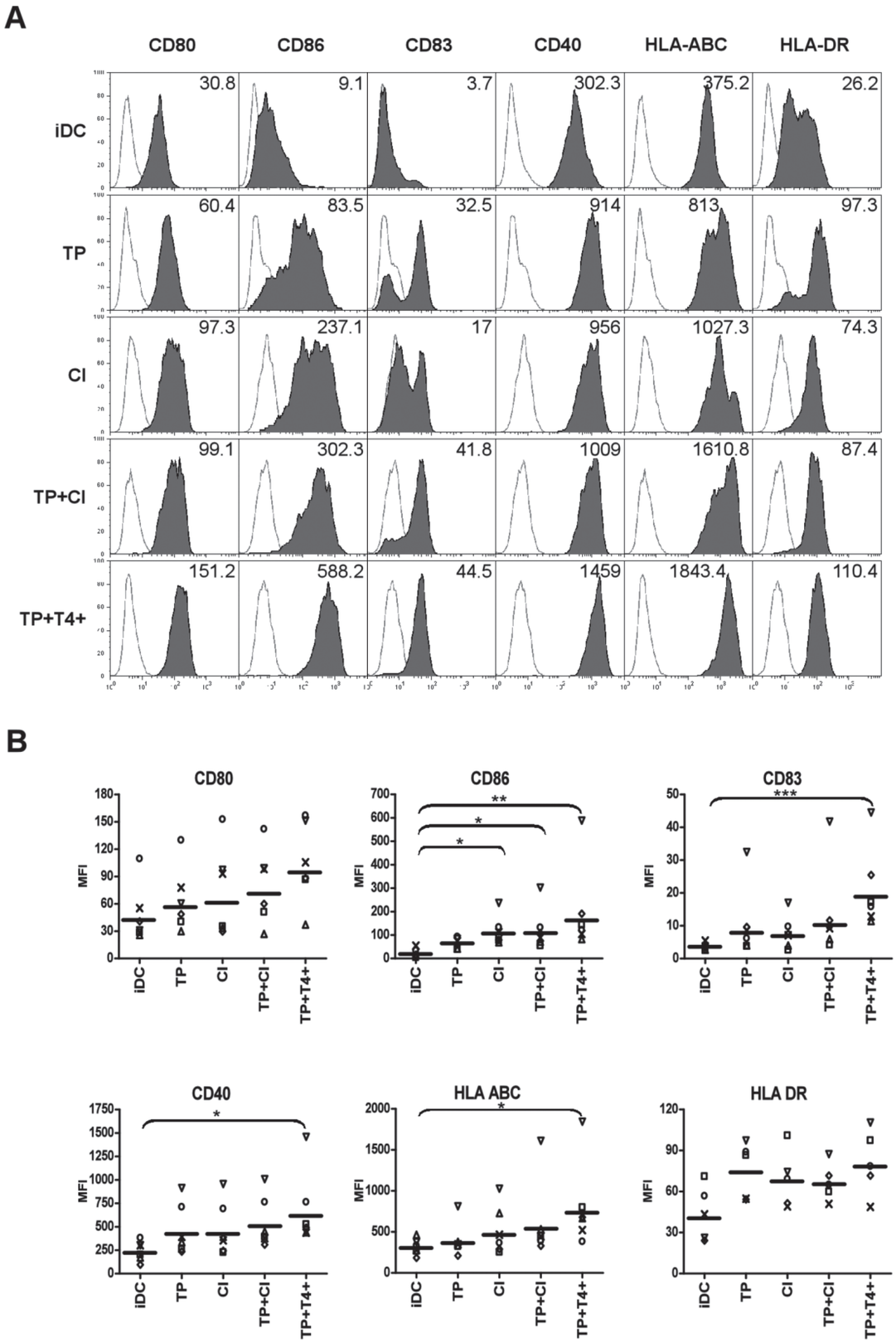

Fig. 2 DCs treated with activated CD4+ T cells as a secondary maturation signal exhibit a more mature phenotype. (A) Comparative phenotypic analysis of iDCs and mature DCs exposed for 48 hrs to different maturation stimuli: TNF-α and poly I:C (TP), CD40-L and IFN-γ (CI), TP followed 10 hrs later by CI (TP+CI) and TP followed 10 hrs later by T4+ (TP+T4+). (B) Analysis from six healthy donors of iDC and mature DC phenotypes, exposed for 48 hrs to different maturation stimuli. Results are expressed as MFI and each symbol represents one donor.

days of culture, part of the T cell population was stimulated by peptide-pulsed T2 cells in the presence of brefeldin A. The proportion of Melan-A-specific CTL was assessed by measurement of intracellular IFN-γ and surface CD8 staining by flow cytometry (Fig. 4A and 4B). The greatest expansion of Melan-A-specific CTL was observed in the culture where CD8+ T cells were stimulated by DCs that had been sequentially matured by TP and T4+. Indeed, we observed two-fold more CD8+ T cells able to produce IFN-γ in response to the Melan-A peptides in culture stimulated with peptide-pulsed DC sequentially matured by TP and T4+ compared to TP-matured DC. This difference was significant ($P<0.01$). The addition of CD40-L and IFN-γ to DC matured with TP did not allow reaching the amplification level of Melan-A-specific CTL obtained with DCs matured with TP plus T4+. These results suggest that signals other than CD40-L and IFN-γ are probably involved in the induction of Melan-A-specific CTL response.

The cytotoxic activity of the expanded Melan-A-specific CTL was also analyzed six days after the stimulation with DC, using Melan-A-pulsed T2 cells (Fig. 4C). Cytotoxic activities were well correlated to the Melan-A-specific CTL frequency determined by IFN-γ/CD8 staining. The highest Melan-A/MART-1 specific lysis was observed in the culture where CD8+ T cells were stimulated by DC matured with TP plus T4+, suggesting that sequentially matured DC prime functional cytotoxic T lymphocytes .

Anti-tumor cytotoxic CD8+ T cells primed by sequentially matured DCs with CD4+ T cells as a second signal exhibit a long-term memory phenotype

It has been established in mouse models that CD4+ plays a role in the differentiation of long-term CD8+ T cell memory responses (Castellino and Germain, 2006). Thus, fourteen days after the stimulation we characterized the effector/memory phenotype of T cells surviving after the contraction phase in Melan-A-specific CTL cultures with markers currently used in the literature: CD27, CD28, CD45RO, CD45RA, CD127 and CCR7 (Powell, et al., 2005, Miller, et al., 2008). Cells were stained with an anti-CD45RO+ mAb to identify CD8+ T memory cells, and their expression of CD27, CD28 and CD45RA was analyzed (Fig. 5). Indeed, these three markers are expressed in naïve T cells, but tend to disappear upon the acquisition of effector function and are strongly re-expressed (CD27 and CD28) or moderately (CD45RA) when T cells acquire the memory phenotype. We found that a population of intermediate CD45RO+ CD27+ CD28+ CD45RA cells among the CD8+ T cells, persisted in the culture and were more frequent in the condition where DCs had received secondary signals from activated helper cells (TP+T4+). The combination of CD40-L, plus IFN-γ as a second signal (TP+CI), had a weaker effect. We then studied CD127 (IL-7Rα) expression on this population. Indeed, it is now acknowledged that selective

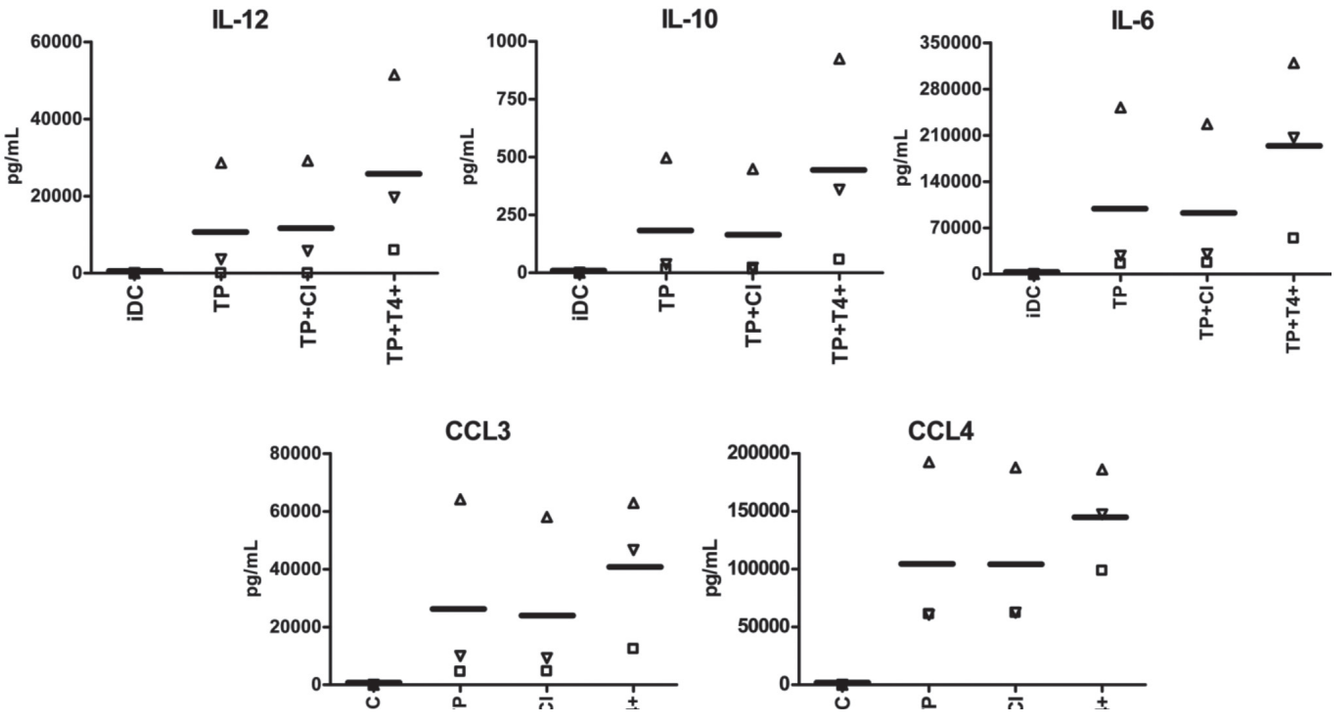

Fig. 3 DCs matured sequentially with CD4+ T cells as a secondary signal produce high levels of inflammatory cytokines and chemokines. Supernatants were collected from iDC and mature DCs exposed for 48 hrs to different maturation stimuli: TNF-α and poly I:C (TP), TP followed 10 hrs later by CD40-L and IFN-γ (TP+CI) or T4+ (TP+T4+). Cytokines (IL-10, IL-12p70, IL-6) and chemokines (CCL3, CCL4,) were measured in supernatants using commercially available BD Cytometric Bead Array kits, according to the manufacturer's protocol. Each symbol represents one donor.

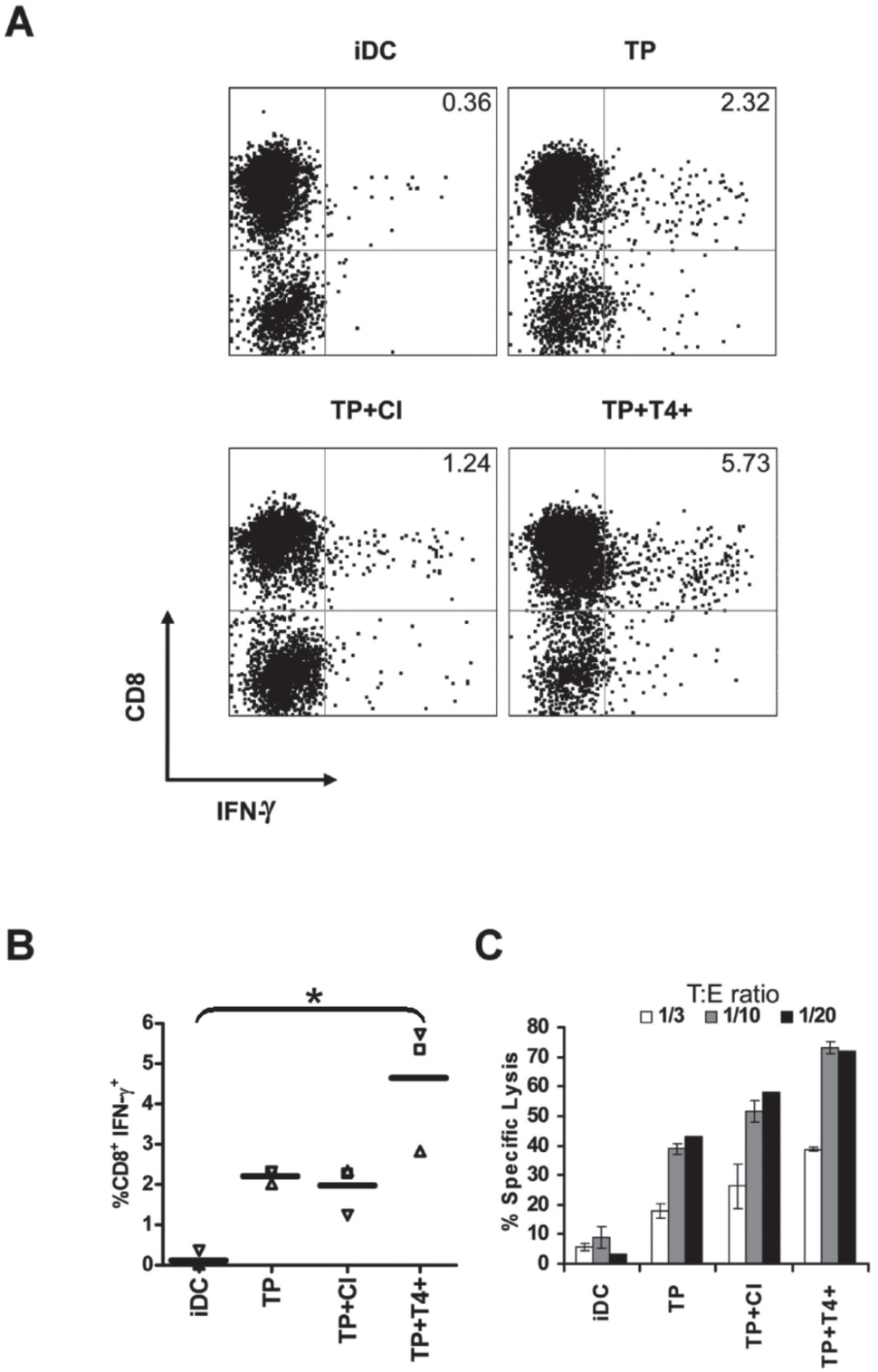

Fig. 4 DCs matured sequentially with CD4+ T cells as a secondary signal generate cytotoxic T cells. DCs activated by the indicated maturation agents were loaded with Melan-$A_{(26-35)}$ A27L peptide, and used to stimulate autologous CD8+ T lymphocytes from healthy donors at a 1:10 DC/T cell ratio. (A) After 6 days, T cells were re-stimulated with unloaded or Melan-A peptide-loaded T2 cells for 6 hrs and the production of IFN-γ by CD8+ T cells was measured by intracellular staining and flow cytometry. The percentages of IFN-γ+ CD8+ T cells are indicated on the dot plot. A representative experiment for one donor out of three is shown. (B) The percentage of IFN-γ+ CD8+ T cells responding to Melan-A peptide was determined from three independent experiments. Each symbol represents one donor. (C) CD8+ T cells were incubated for 4 hrs with Melan-A peptide and ^{51}Cr loaded T2 cells at Effector:Target ratios of 3:1 (white), 10:1 (grey), 20:1 (black). The percentage of lysis induced by DC-stimuladed CD8+ T cells was then measured by Cr^{51} release compared to spontaneous and maximal release. A representative experiment for one donor out of three is shown.

expression of CD127 identifies memory cells (Kaech, et al., 2003). We observed that CD45RO+CD28+CD45RAint cells expressed CD127, with much higher intensity in the "TP+T4+" condition (MFI=107 compared to MFI<65 for all other conditions). These cells identified as CD45RO+ CD45RAint CD27+CD28+CD127hi display a phenotype typical of long-lived T memory cells.

To complete the characterization of these T memory cells, we analyzed their expression of CCR7. Indeed, two mains populations of CD8+ T memory cells have been described depending on their expression of CCR7: CCR7- effector T memory cells (Tem) and CCR7+ central T memory cells (Tcm) (Sallusto, et al., 1999). We observed that the T memory cells obtained from stimulation with DCs sequentially matured with T4+ as second signals, exhibit both memory profiles, Tem (37.6%) and Tcm (55.7%), whereas the majority of T memory cells obtained from other mature DCs were mainly CCR7- Tem. Furthermore, CCR7 expression was stronger on the Tcm obtained from sequentially matured DCs compared to the Tcm obtained from other conditions (MFI=21 compare to MFI<10 for all other conditions). Thus, our results demonstrate that sequential maturation of DCs in the presence of helper cells contributes to the generation of a long-term T cell pool composed of both Tem and Tcm memory components.

DISCUSSION

Description of DCs maturation in mouse model showed that this process is a multi-step phenomenon, with a first maturation signal received in the periphery (inflammatory

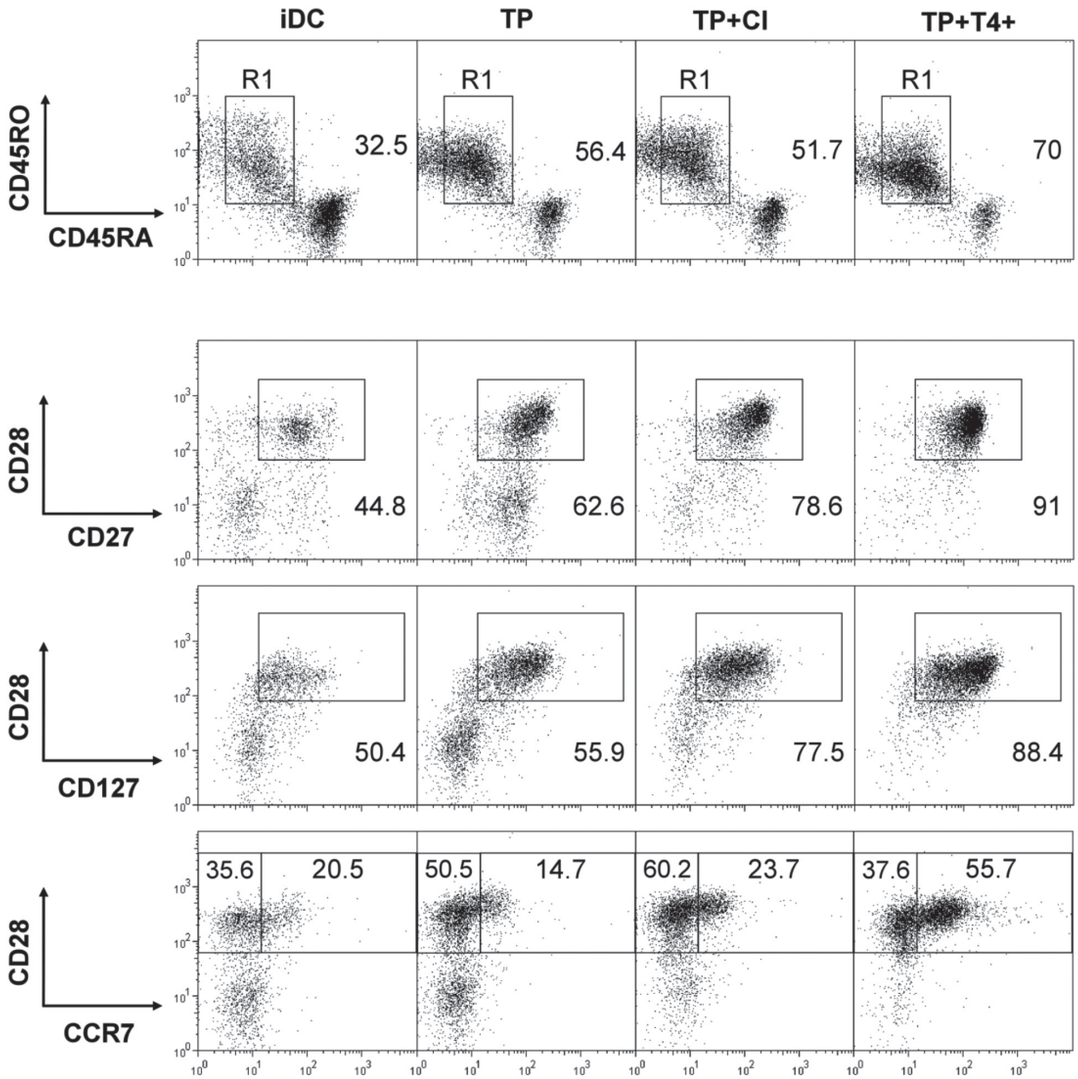

Fig. 5 DCs matured sequentially with CD4+ T cells as a secondary signal favor the generation of CD8+ T cells exhibiting a memory phenotype. DCs were activated by the indicated maturation agents, loaded with Melan-$A_{(26-35)}$ A27L peptide, and used to stimulate autologous CD8+ T lymphocytes at a DC/T cell ratio of 1:10. After 15 days, the CD8+ T cell memory profile was determined by immunofluorescence and flow cytometry. T cells were gated according to their morphological properties and CD28, CD27, CD127 and CCR7 expression was studied on the CD45RO+ CD45RAint T cell population (R1 gate). The percentage of gated cells is indicated on the dot plot. A representative experiment for one donor out of three is shown.

and/or pathogenic signals), which at least induces DC activation and their migration to lymph nodes, where they receive additional maturation signals from CD4+ T cells (Banchereau and Steinman, 1998). In our *in vitro* study, we characterized, for the first time, this sequential maturation of human DCs with activated CD4+ T cells as a second signal. We used TNF-α and poly I:C as first maturation signals, since these two signals are relevant as inflammation and pathogenic stimuli, respectively, that DCs encounter in periphery. In a previous study we demonstrate that the combination of TNF-α and poly I:C is the most efficient in inducing the complete activation of human DCs compared to a large variety of maturation molecules or cytokine cocktails (Spisek, 2001; Gregoire, 2003). These two molecules are also relevant for clinical applications of this maturation process (Mc IlRoy et al., *2003;* Royer et al., *2006*). In this study, we show that DC maturation requires second signals delivered by activated CD4+ T cells a few hours after exposure to peripheral maturation stimuli to fully initiate CD8+ T cell responses. DCs activated by this sequential procedure exhibit a more profoundly mature phenotype, which improves their ability to induce differentiation of tumor antigen-specific CTL with a long-term memory phenotype.

It was previously demonstrated in murine models that in secondary lymphoid organs, activation of CD4+ T helper cells by mature DC results in stimulation and conditioning of DCs by CD4+ T cells that "license" DCs to induce CTL responses. It has been well established that CD40-L is one of the main activated CD4+ T cell signals provided to DCs and that CD40-L/CD40 interactions initiate differentiation of naïve CD8+ T cells to effector CTL by DCs (Bennett, et al., 1998, Ridge, et al., 1998, Schoenberger, et al., 1998, Johnson, et al., 2009). In our study, we show that activated allogeneic CD4+ T cells used as a second signal are more efficient at maturing monocyte-derived human DCs than the combination of CD40-L and IFN-γ, suggesting a more complex exchange of signals between these two types of cells. In addition to CD40-L and IFN-γ, CD4+ T cells produce other factors such as molecules of the TNF family (TNF-α; TRANCE…) and cytokines such as IL-2, which may explain the difference observed in DC between the CD40-L/IFN-γ combination and activated CD4+ T cells used as second signals.

Furthermore, these CD4+ T cell/DC interactions not only affect the maturation phenotype of DCs, but also induce the secretion of chemokines by DCs, such as CCL3 and CCL4 (Mip1-α and -β). Chemokines are reported in murine models to have a chemotactic effect on naïve CD8+ T cells (Castellino, et al., 2006) and to favor the encounter of rare antigen-specific naïve CD8+ precursors with mature DCs. In our study, we show that DCs exposed to activated CD4+ T cells secrete high quantities of these two chemokines, which in humans may have the same chemotactic effect on naïve CD8+ T cells as observed in mice. Furthermore, it has been shown that CCL3 and CCL4 produced by T helper-conditioned DCs play a role in the generation of pre-memory CD8+ T cells characterized by a high expression of IL-6Rα and IL-7Rα (Castellino and Germain, 2007). Survival of these pre-memory CD8+ T cells is dependent on IL-6, even in the presence of IL-7 (used under our experimental conditions). In addition, it has been shown that IL-6 produced upon TLR signaling can block CD4+CD25+ T cell-mediated suppression (Pasare and Medzhitov, 2003). These effects of IL-6, CCL3 and CCL4 on the immune response fit well with our present observations. Indeed, activated CD4+ T cells exposed to mature DCs, which produced the highest amount of IL-6, CCL3 and CCL4, induced Melan-A-specific CTL, which exhibited the strongest long-term memory phenotype (CD27+ CD28+ CD45RAint CD45RO+ CD127+ CD8+) (Powell, et al., 2005).We also observed a dramatic effect of activated CD4+ T cells on cytokine production, notably IL-12, by mature DCs. IL-12 secretion by DCs skews the immune response towards a Th1 profile (Macatonia, et al., 1995, Hilkens, et al., 1997), whereas IL-10 inhibits it (O'Garra and Vieira, 2007). In our study, activated CD4+ T cell-exposed mature DCs, which produced high amounts of IL-12, were the most efficient to induce a CTL response against a tumor-associated antigen such as Melan-A from HLA-A*0201+ healthy blood donors. Therefore, the balance of effect between IL-10 and IL-12 is in favor of IL-12, skewing the immune response towards a Th1 profile.

Several anti-tumor immunotherapeutic strategies have been developed during the last decade, such as injection of anti-tumor-specific T cells or tumor antigen-loaded DCs, notably to treat melanoma patients. Despite induction of anti-tumor T cell responses and encouraging clinical results, one common problem encountered during these protocols is the failure to induce a long-lasting anti-tumor T cell response (Dudley and Rosenberg, 2007, Palucka, et al., 2007). Indeed, in adoptive immunotherapy clinical trials, anti-tumor-specific T cells are detected for only a few days following injection in the patients (Dudley, et al., 2001, Yee, et al., 2002). In DC-based clinical trials, progression of the disease after a period of stabilization was described when the anti-tumor-specific CTL response ceases (Andersen, et al., 2001), and a re-stimulation of anti-tumor-specific T cells is required for these cells to be detected several weeks after the DC injection (Palucka, et al., 2005, Simon, et al., 2009). Interestingly, Rosenberg's group designed a modified clinical trial based on immunotherapy to increase the survival of tumor-antigen-specific CTLs injected to patients (Dudley, et al., 2002). They treated the patients with a lympho-depleting chemotherapeutic regimen prior to the injection of T cells to limit the negative effect of CD4+CD25+ regulatory T cells and to disrupt homeostatic T cell regulation. The authors succeeded in inducing a long-lasting anti-tumor-specific CTL response that was still detectable in the blood of responding patients two months after the injection. They were also able to define the phenotype of these tumor-reactive cells as effector T memory cells (Tem) characterized by a CD27+CD28+CD45RAintCD62L-CCR7-CD127+ phenotype (Powell, et al., 2005). However, in a mouse model, transfer of anti-tumor central T memory cells (Tcm) was shown to be more efficient at inducing tumor regression than transfer of anti-tumor Tem (Klebanoff, et al., 2005). This suggests that both memory subsets play a complementary role in T cell memory responses. In the periphery, Tem are able to quickly lyse cells harboring the antigen. This cytotoxic response is reinforced by a new pool of effector T cells differentiated from Tcm exposed to the antigen in secondary lymphoid organs. Thus, it is likely that the presence of both subsets is required for optimal T memory cell responses. In our study, we demonstrate that DC sequentially matured with poly I:C plus TNF-α followed by activated CD4+ T cells, allow the priming of tumor-associated antigen-specific CTL, which differentiate *in vitro* into both Tem and Tcm memory subsets. The Tem

exhibited a CD27+CD28+CD45RAintCD127+ phenotype similar to the persistent anti-tumor T cells described by Powell et al (Powell, et al., 2005).

Altogether, the results of our study demonstrate for the first time that sequential maturation of human DCs using activated CD4+ T cells as a second signal greatly increases the capacity of DCs to prime a complete CTL response, with the establishment of a long-lasting anti-tumor T memory cell pool containing both effector and central components. This work has important implications for the development of DC-based immunotherapy and highlights the requirement of T helper-derived signals in the DC maturation process.

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The authors declare no conflict of interest.

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REFERENCES

ANDERSEN MH, KEIKAVOUSSI P, BROCKER EB, SCHULER-THURNER B, JONASSEN M, SONDERGAARD I, STRATEN PT, BECKER JC, KAMPGEN E (2001) Induction of systemic CTL responses in melanoma patients by dendritic cell vaccination: cessation of CTL responses is associated with disease progression. Int J Cancer 94:820-824.

ARENS R, SCHOENBERGER SP (2010) Plasticity in programming of effector and memory CD8 T-cell formation. Immunol Rev 235:190-205.

BANCHEREAU J, STEINMAN RM (1998) Dendritic cells and the control of immunity. Nature 392:245-252.

BENNETT SR, CARBONE FR, KARAMALIS F, MILLER JF, HEATH WR (1997) Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med 186:65-70.

BENNETT SR, CARBONE FR, KARAMALIS F, FLAVELL RA, MILLER JF, HEATH WR (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 393:478-480.

CASTELLINO F, GERMAIN RN (2006) Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol 24:519-540.

CASTELLINO F, HUANG AY, ALTAN-BONNET G, STOLL S, SCHEINECKER C, GERMAIN RN (2006) Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature 440:890-895.

CASTELLINO F, GERMAIN RN (2007) Chemokine-guided CD4+ T cell help enhances generation of IL-6RalphahighIL-7Ralpha high prememory CD8+ T cells. J Immunol 178:778-787.

DUDLEY ME, WUNDERLICH J, NISHIMURA MI, YU D, YANG JC, TOPALIAN SL, SCHWARTZENTRUBER DJ, HWU P, MARINCOLA FM, SHERRY R, LEITMAN SF, ROSENBERG SA (2001) Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. J Immunother 24:363-373.

DUDLEY ME, WUNDERLICH JR, ROBBINS PF, YANG JC, HWU P, SCHWARTZENTRUBER DJ, TOPALIAN SL, SHERRY R, RESTIFO NP, HUBICKI AM, ROBINSON MR, RAFFELD M, DURAY P, SEIPP CA, ROGERS-FREEZER L, MORTON KE, MAVROUKAKIS SA, WHITE DE, ROSENBERG SA (2002) Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298: 850-854.

DUDLEY ME, ROSENBERG SA (2007) Adoptive cell transfer therapy. Semin Oncol 34:524-531.

GREGOIRE M, LIGEZA-POISSON C, JUGE-MORINEAU N, SPISEK R. Anti-cancer therapy using dendritic cells and apoptotic tumour cells: pre-clinical data in human mesothelioma and acute myeloid leukaemia. (2003) 21(7-8):791-4.

HILKENS CM, KALINSKI P, DE BOER M, KAPSENBERG ML (1997) Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood 90:1920-1926.

JOHNSON S, ZHAN Y, SUTHERLAND RM, MOUNT AM, BEDOUI S, BRADY JL, CARRINGTON EM, BROWN LE, BELZ GT, HEATH WR, LEW AM (2009) Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells. Immunity 30:218-227.

KAECH SM, TAN JT, WHERRY EJ, KONIECZNY BT, SURH CD, AHMED R (2003) Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol 4:1191-1198.

KLEBANOFF CA, GATTINONI L, TORABI-PARIZI P, KERSTANN K, CARDONES AR, FINKELSTEIN SE, PALMER DC, ANTONY PA, HWANG ST, ROSENBERG SA, WALDMANN TA, RESTIFO NP (2005) Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector t memory cells. Proc Natl Acad Sci U S A 102:9571-9576.

LANGENKAMP A, MESSI M, LANZAVECCHIA A, SALLUSTO F (2000) Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol 1:311-316.

LU Z, YUAN L, ZHOU X, SOTOMAYOR E, LEVITSKY HI, PARDOLL DM (2000) CD40-independent pathways of T cell help for priming of CD8(+) cytotoxic T lymphocytes. J Exp Med 191:541-550.

MACATONIA SE, HOSKEN NA, LITTON M, VIEIRA P, HSIEH CS, CULPEPPER JA, WYSOCKA M, TRINCHIERI G, MURPHY KM, O'GARRA A (1995) Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol 154: 5071-5079.

MACKEY MF, GUNN JR, MALISZEWSKY C, KIKUTANI H, NOELLE RJ, BARTH RJ, JR. (1998) Dendritic cells require maturation via CD40 to generate protective antitumor immunity. J Immunol 161:2094-2098.

MCILROY D, GREGOIRE M. (2003) Optimizing dendritic cell-based anticancer immunotherapy: maturation state does have clinical impact. Cancer Immunol Immunother 52(10):583-91.

MILLER JD, VAN DER MOST RG, AKONDY RS, GLIDEWELL JT, ALBOTT S, MASOPUST D, MURALI-KRISHNA K, MAHAR PL, EDUPUGANTI S, LALOR S, GERMON S, DEL RIO C, MULLIGAN MJ, STAPRANS SI, ALTMAN JD, FEINBERG MB, AHMED R (2008) Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. Immunity 28:710-722.

O'GARRA A, VIEIRA P (2007) T(H)1 cells control themselves by producing interleukin-10. Nat Rev Immunol 7:425-428.

OBAR JJ, LEFRANCOIS L (2010) Memory CD8+ T cell differentiation. Ann N Y Acad Sci 1183:251-266.

PALUCKA AK, DHODAPKAR MV, PACZESNY S, UENO H, FAY J, BANCHEREAU J (2005) Boosting vaccinations with peptide-pulsed CD34+ progenitor-derived dendritic cells can expand long-lived melanoma peptide-specific CD8+ T cells in patients with metastatic melanoma. J Immunother 28:158-168.

PALUCKA AK, UENO H, FAY JW, BANCHEREAU J (2007) Taming cancer by inducing immunity via dendritic cells. Immunol Rev 220:129-150.

PASARE C, MEDZHITOV R (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299:1033-1036.

POWELL DJ, JR., DUDLEY ME, ROBBINS PF, ROSENBERG SA (2005) Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector t memory cells in humans after adoptive cell transfer therapy. Blood 105:241-250.

RIDGE JP, DI ROSA F, MATZINGER P (1998) A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 393:474-478.

ROYERr PJ, TANGUY-ROYER S, EBSTEIN F, SAPEDE C, SIMON T, BARBIEUX I, OGER R, GREGOIRE M. (2006) Culture medium and protein supplementation in the generation and maturation of dendritic cells. Scand J Immunol. 63(6):401-9.

SALLUSTO F, LENIG D, FORSTER R, LIPP M, LANZAVECCHIA A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401:708-712.

SALTER RD, CRESSWELL P (1986) Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. EMBO J 5:943-949.

SCHOENBERGER SP, TOES RE, VAN DER VOORT EI, OFFRINGA R, MELIEF CJ (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 393:480-483.

SCHRODER K, HERTZOG PJ, RAVASI T, HUME DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75:163-189.

SIMON T, FONTENEAU JF, GREGOIRE M (2009) Dendritic cell preparation for immunotherapeutic interventions. Immunotherapy 1:289-302.

SPISEK R, BRETAUDEAU L, BARBIEUX I, MEFLAH K, GREGOIRE M. (2001) Standardized generation of fully mature p70 IL-12 secreting monocyte-derived dendritic cells for clinical use. Cancer Immunol Immunother 8 :417-27.

SPISEK R, BOUGRAS G, EBSTEIN F, MASSE D, MEFLAH K, MCILROY D, GREGOIRE M (2003) Transient exposure of dendritic cells to maturation stimuli is sufficient to induce complete phenotypic maturation while preserving their capacity to respond to subsequent restimulation. Cancer Immunol Immunother 52:445-454.

YEE C, THOMPSON JA, BYRD D, RIDDELL SR, ROCHE P, CELIS E, GREENBERG PD (2002) Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A 99:16168-16173.

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Effects of silk sericin on the proliferation and apoptosis of colon cancer cells

Waraporn Kaewkorn[a], Nanteetip Limpeanchob[a], Waree Tiyaboonchai[b], Sutatip Pongcharoen[c*], Manote Sutheerawattananonda[d]

[a]Departments of Pharmacy Practice and [b]Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, [c]Department of Medicine, Faculty of Medicine, Naresuan University, Phitsanulok, and [d]School of Food Technology, Institute of agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

ABSTRACT

Sericin is a silk protein woven from silkworm cocoons *(Bombyx mori)*. In animal model, sericin has been reported to have anti-tumoral action against colon cancer. The mechanisms underlying the activity of sericin against cancer cells are not fully understood. The present study investigated the effects of sericin on human colorectal cancer SW480 cells compared to normal colonic mucosal FHC cells. Since the size of the sericin protein may be important for its activity, two ranges of molecular weight were tested. Sericin was found to decrease SW480 and FHC cell viability. The small sericin had higher anti-proliferative effects than that of the large sericin in both cell types. Increased apoptosis of SW480 cells is associated with increased caspase-3 activity and decreased Bcl-2 expression. The anti-proliferative effect of sericin was accompanied by cell cycle arrest at the S phase. Thus, sericin reduced SW480 cell viability by inducing cell apoptosis via caspase-3 activation and down-regulation of Bcl-2 expression. The present study provides scientific data that support the protective effect of silk sericin against cancer cells of the colon and suggests that this protein may have significant health benefits and could potentially be developed as a dietary supplement for colon cancer prevention.

Key words: Silk sericin, colon cancer, apoptosis, Bcl-2, caspase-3.

INTRODUCTION

Colon cancer is one of the most common that causes cancer related deaths worldwide. The incidence is high, especially in developed countries, and it is now rising in developing countries like Thailand (Labianca et al., 2004; Khuhaprema and Srivatanakul, 2008). Genetics, environment, lifestyle, and diet are considered important factors that contribute to the development of colon cancer. Epidemiological studies have indicated that diet significantly influences the risk of colon cancer. Western dietary habits, especially high consumption of fat and red meat, increase risk of colon cancer, whereas high consumption of fruits, vegetables, and dietary fibers probably protect against it (van Breda et al., 2008). Because diet is an important factor for identifying risk of colon cancer, it is believed that 90% of colon cancer cases could be prevented by dietary intervention or by supplementary diets that protect against colon cancer (Johnson, 2004).

Numerous studies in different experimental systems have shown that fruits and vegetables exert beneficial effects through various mechanisms. The protective effects of fruits and vegetables to reduce colon cancer risk might be the result of their high content of anti-oxidant vitamins and fibers (Campos et al., 2005; Levi et al., 2001; Wogan, 1985). Apart from fruits and vegetables, dietary proteins such as soy, whey, and buckwheat proteins have also been found to reduce the development of colon cancer in animals (Belobrajdic et al., 2003; Hakkak et al., 2001; Liu et al., 2001). Interestingly, a non-dietary protein called sericin has been reported to suppress colon tumorigenesis in both mice and rats (Zhaorigetu et al., 2001; Zhaorigetu et al., 2007).

Sericin, a silk protein, is one of the main constituents of silk cocoons, comprising 20-30% of total cocoon weight (Sasaki et al., 2000). Sericin is insoluble in cold water although it is easily dispersed or solubilised in hot water. Sericin is specifically synthesized in the middle gland of the silk worm, *Bombyx mori*. Sericin was found to suppress lipid peroxidation and tyrosinase inhibitory activity in an *in vitro* study (Kato et al., 1998). Interestingly, it is resistant to several proteases, which might make sericin beneficial for colon health (Sasaki et al., 2000; Kato and Iwami, 2002). Moreover, because of its proteinous nature, sericin is a biocompatible and biodegradable material. In addition to its anti-oxidant activity (Fan et al., 2009), sericin exhibits chemopreventive effects by suppressing 7,12-dimethylbenze(*a*)anthracene/12-*O*-tetradecanoyl-phorbol-13-acetate(DMBA-TPA)-induced mouse skin tumorigenesis (Zhaorigetu et al., 2003) and colon tumorigenesis in 1,2-dimethylhydrazine (DMH)-induced colon cancer in animal models (Zhaorigetu et al., 2001; Zhaorigetu et al., 2007). The mechanisms of its chemoprevention are associated with its ability to reduce colonic oxidative stress and colonic cell proliferation, and also to suppress aberrant crypt foci in animals (Zhaorigetu et al., 2001; Zhaorigetu et al., 2007).

It should be noted that sericin preparation from silk cocoons is generally heterogeneous, with various sizes of polypeptides. Since sericin is a polymeric protein, different extraction techniques can provide different sizes or molecular weights of polypeptides consequently, exhibiting different effects (Terada et al., 2005). Thus, the size of sericin appears to be important for its activity. For this reason, the present study investigated the effect of two differently sized sericin preparations on the viability of colonic cell lines, including

*Corresponding author: Dr. Sutatip Pongcharoen Department of Medicine, Faculty of Medicine, Naresuan University, Phitsanulok 65000, Thailand Tel.: +66 55 965053 Fax: +66 55 965021 E-mail: sutatipp@nu.ac.th

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cancerous cells SW480 and normal cells FHC. The effect of sericin on the cell cycle and apoptosis of these cells were investigated. Certain apoptotic cellular pathways involving caspase-3 activity as well as Bcl-2 and Bax protein expression were also investigated.

METHODS

Preparation of Sericin

Silk sericin was supplied by the Institution of Agricultural Technology, Suranaree University of Technology, Nakorn Ratchasrima, Thailand. The sericin was classified into 2 types according to isolation techniques; large-size sericin (MW 191-339 kDa) and small-size sericin (MW 61-132 kDa). Briefly, silk sericin was extracted with deionized water from raw silk yarns of the silkworm *Bombyx mori* under high pressure and high temperature. The specific extraction condition was under a pending Thai patent (application number 080595). The extract was later dried at 130 °C, and then ground and sieved through a 0.75 mm screen. The resulting sericin powder was sealed in sterile plastic bags and kept at room temperature until used. The sericin was used by reconstituting it in phosphate-buffered saline (PBS) and was sterilized by autoclaving at 121°C for 15 minutes.

Cell culture

The human colorectal cancer cells (SW480: ATCC number CCL-228) and normal human fetal colonic mucosal cells (FHC: ATCC number CRL-1831) were purchased from the American Type Culture Collection (ATCC: Manassas, VA, USA). The SW480 cells were cultured in Dulbecco's modified Eagle's medium with HamF-12 (DMEM/F-12), supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 mg/ml streptomycin, all procured from Gibco, NY, USA. FHC cells were cultured in the above medium, supplemented with 25mM N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid (HEPES) (JRH Biosciences, KS, USA), 5 mg/ml insulin (Sigma, MO, USA), and 100 ng/ml hydrocortisone (Sigma). Both SW480 and FHC cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Cell viability assay

The cells were plated in 96-well plates at a concentration of 1×10^4 cells per well with complete culture medium. After an overnight incubation, the cells were exposed to various concentrations (25-1,600 mg/ml) of each type of sericin for 24, 48 and 72 h. MTT solution (0.5 mg/ml) was added to the culture medium 2 h before the end of the treatment period. Formazan crystals were then lysed with DMSO:ethanol (1:1), and the absorbance was read at 595 nm using a micro-plate reader (Beckman Coulter, NSW, Australia).

Cell apoptosis assay

Apoptosis was detected with an annexin V-FITC kit (Becton Dickinson, NJ, USA) according to the manufacturer's instructions. Initially, the cells were plated in a 60-mm culture dish at a concentration of 1×10^6 cells per dish with complete culture medium. After an overnight incubation, the cells were treated with sericin at a concentration of 1600 mg/ml for 72 h. After treatment, the cells were collected, washed with ice-cold phosphate-buffered saline (PBS) pH 7.4, centrifuged, and resuspended in 1X binding buffer. Cells were then stained with annexin V-FITC and propidium iodide (PI) solution, incubated for 15 min in the dark, and analyzed by FACSCalibur using CellQuestPro software (Becton Dickinson).

Caspase-3 activity assay

The cells were plated in 60-mm culture dishes at a concentration of 1×10^6 cells per dish with DMEM/F-12 with 10% FBS. After overnight incubation, the culture medium was replaced with a new complete medium and then treated with sericin at a concentration of 1600 mg/ml for 72 h. Then, the cells were harvested by trypsinization before caspase-3 activity was detected by using Caspase-3 fluorimetric kit (Sigma). According to the manufacturer's instructions, caspase-3 activity was detected by using acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) as a substrate. The fluorescence of 7-amido-4-methylcoumarin (AMC) was measured at Ex 360 nm and Em 460 nm.

Western blot analysis

The cells were plated in 60-mm culture dishes at a concentration of 1×10^6 cells per dish with DMEM/F-12 with 10% FBS. After overnight incubation, the culture medium was replaced with new complete medium and then treated with sericin at a concentration of 1600 mg/ml for 72 h. After treatment, the cells were lysed and the protein concentrations in cell lysate were determined by using a BCA protein assay kit (Thermo scientific, Rockford, USA). Twenty micrograms of each cell lysate sample were loaded and separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting electrophoresis products were transferred onto a PVDF membrane and were then incubated with specific antibodies against Bcl-2 or Bax and then a secondary antibody conjugated with alkaline phosphatase. The protein bands were detected by using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a substrate.

Cell cycle analysis

The cells were plated in 60-mm culture dishes at a concentration of 1×10^6 cells per dish with DMEM/F-12 with 1% FBS. After overnight incubation, the culture medium was replaced with new complete medium and then treated with sericin at a concentration of 1600 mg/ml for 72 h. After treatment, the cells were harvested by trypsinization, washed with PBS pH 7.4, and fixed in 70% ethanol at 4 °C for 1 h. The cells were then treated with 100 mg/ml of RNase A at 37 °C for 30 min and then stained with 20 mg/ml of PI (Molecular Probes, Invitrogen, NY, USA) for 30 min at room temperature in the dark. The cell analysis was performed by FACSCalibur using CellQuestPro software (Becton Dickinson).

Statistical analysis

All data was expressed as mean ± standard deviation (SD). The statistical analysis was performed by using a one-way analysis

of variance (ANOVA) and a two-tailed Student's t-test. The p values < 0.05 were considered statistically significant.

RESULTS

Effect of sericin on SW480 and FHC cell viability

SW480 colon cancer cells and FHC normal colon cells were treated with small or large sericin at various concentrations (25-1,600 mg/ml) for 24, 48 and 72 h. Both sizes of sericin at all tested concentrations did not affect the viability of either type of cell at treatment periods of 24 and 48 h (data not shown). At 72 h of sericin treatment, the viability of SW480 and FHC cells gradually decreased with an increasing concentration of sericin (Figure 1). These findings suggest that sericin has considerably low cytotoxicity to both colon cancer cells and normal colon cells. To compare between small and large sericin, the small sericin has a slightly higher anti-proliferative effect than that of the large sericin (Table 1). Large sericin seemed to reduce the viability of colonic adenocarcinoma SW480 better than normal FHC cells (Figure 1A), whereas small sericin did not show such differences (Figure 1B). The results suggest that sericin possesses low to moderate effect on the cellular viability for colon cells.

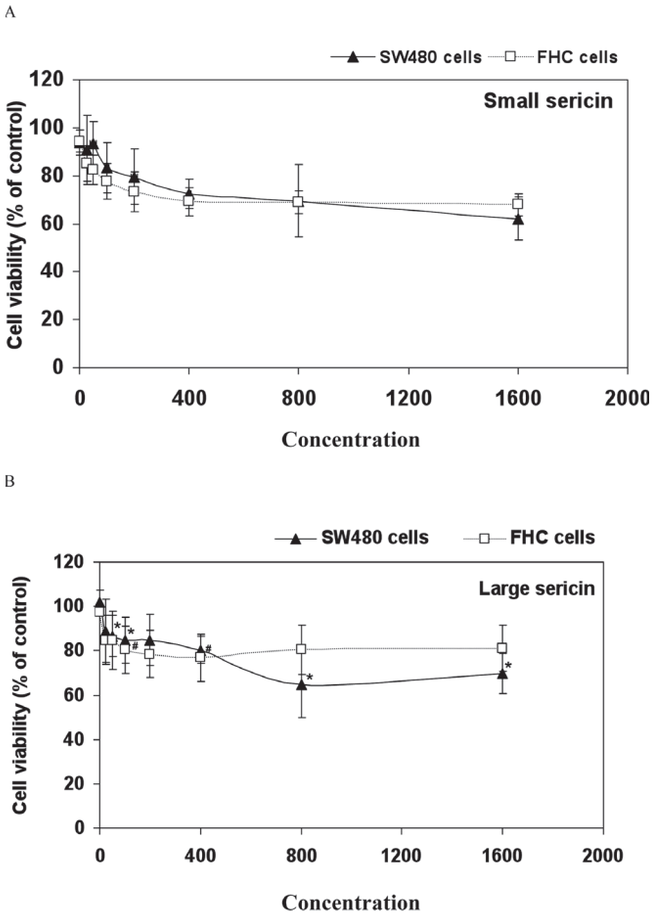

Figure 1: Effect of sericin on SW480 and FHC cell viability. SW480 and FHC cells were incubated with small (A) and large (B) sericin at various concentrations (25 – 1,600 mg/ml) for 72 h. The cell viability was determined by MTT assay. Results are expressed as mean±SEM and represent the average values from five experiments. * two-tailed Student's *t*-test indicates significant difference compared to control SW480 cells with *p* values of less than 0.05. # two-tailed Student's t-test indicates significant difference compared to control FHC cells with *p* values of less than 0.05.

Effect of sericin on SW480 and FHC cell apoptosis

At 72 h of treatment, sericin at 1,600 mg/ml showed a significant reduction of SW480 and FHC cell viability. These cells were then tested as to whether they underwent cell apoptosis or necrosis. The result showed that small but not large sericin significantly increased the percentage of late apoptotic cells in adenocarcinoma SW480 cells (Figure 2A). Neither size of sericin changed the percentage of necrotic cells when compared to the control cells and PBS-treated cells (Figure 2A). In contrast, the colonic normal FHC cells did not undergo cell death either via apoptotic or necrotic processes following sericin treatment (Figure 2B).

Effect of sericin on caspase-3 activity

Caspase-3 is an executioner caspase, whose activity is increased when cells are about to undergo apoptosis. Therefore, we determined whether or not caspase-3 activity was directly involved in cell apoptosis induced by sericin. As shown in Figure 3, SW480 cells treated with sericin at 1,600 mg/ml for 72 h exhibited increased caspase-3 activity. Only the small sericin significantly enhanced caspase-3 activity in SW480 cells. This suggests that sericin, particularly the small-sized, induced adenocarcinoma colonic cells to undergo apoptosis through a caspase-3 dependent pathway.

Effect of sericin on Bcl-2 and Bax expression

The expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax were also expected to correlate with the degree of apoptosis of SW480 cells in the previous experiment. The expression of these proteins was examined by using Western blotting. After treating the cells with both sizes of sericin at 1,600 mg/ml for 72 h, there was a slight reduction of Bcl-2 protein levels, but only the small sericin showed a significant effect (Figure 4). The expression of the Bax protein in sericin-treated cells did not differ from that in the control cells (Figure 4). This suggests that sericin down-regulated anti-apoptotic protein Bcl-2, which may then

TABLE 1
The effect of sericin (1,600 mg/ml, 72 h) on SW480 and FHC cell viability

Sericin	Cell viability (% of control)		p-value
	SW480 (mean±SD)	FHC (mean±SD)	
Small	62.16 ± 8.27	67.90 ± 4.52	0.17
Large	69.78 ± 8.60	81.16 ± 10.53	0.22
p-value	0.24	0.03	

Statistical calculation was performed using Student's t-test.

enhance adenocarcinoma SW480 cells to undergo an apoptotic process.

Effect of sericin on the cell cycle

To further investigate whether or not the inhibitory effect of sericin on SW480 cell viability might partially be due to an arrest of cell proliferation, a cell cycle analysis was also conducted by using flow cytometry. After treating the SW480 cells with sericin at 1,600 mg/ml for 72 h, they were harvested and stained with PI. As shown in Table 2, the colon adenocarcinoma SW480 cells had a higher percentage of cells in the S phase (19.22±6.33%) than FHC normal colonic cells (7.64±1.01). After treatment with either small or large sericin, the percentage of SW480 cells in the S phase fell below the control level, although the reductions were not statistically significant. Interestingly, both sizes of sericin caused a significant increase in the percentage of the S-phase of FHC cells as compared to the control cells. These results suggest that the cell cycle of adenocarcinoma and normal colonic cell lines are differently regulated by sericin treatment.

DISCUSSION

Sericin has previously been reported to suppress colon tumorigenesis in animal models (Zhaorigetu et al., 2001; Zhaorigetu et al., 2007). However, there is still a limited number of studies that explain sericin's mechanisms of action for this process. Thus, this present study investigated whether sericin has any effects on colon cancer cells and normal colonic cells. The results showed that sericin exerted a greater inhibitory effect on SW480 cells than on FHC cells. Sericin also exerted a moderate inhibitory effect on the cell viability of SW480 and FHC cells. Small-size sericin (61-132 kDa) reduced the viability of SW480 cells via the induction of cell apoptosis as well as by the reduction of the S-phase cell population. Large-size sericin (191-339 kDa) seems to decrease SW480 cell viability through cell cycle regulation. The sericin-induced apoptsis of SW480 cells was associated with an increase in caspase-3 activity and a down-regulation of Bcl-2 anti-apoptotic proteins. Normal FHC colonic cells did not undergo apoptosis, and the cell cycle of these cells changed only slightly after the sericin treatment.

The observed chemopreventative effect may be attributed to several mechanisms, such as suppression of cancer cell proliferation, induction of cell apoptosis, inhibition of angiogenesis, and regulation of the cancer cell cycle. Among these mechanisms, cell apoptosis is the major target for the treatment and prevention of colon cancer (Lifshitz et al., 2001; Sun et al., 2004; Huerta et al., 2006). In the present study, sericin induced late apoptosis in SW480 colon cancer cells but not in FHC normal colonic cells. Early apoptosis is characterized by the changes in the cellular membrane. Phosphatidyl serine, normally located inside the cell membrane, moves to the outer surface, and mitochondrial membrane potential drops. Late apoptosis involves fragmentation of nuclear chromatin. The stages of apoptosis depend on differential apoptotic stimuli, as well as functions of anti-apoptotic and pro-apoptotic proteins (Indran et al., 2011; Kelly et al., 2011; Ascenzi et al., 2011). Malignant transformation of epithelial cells, including those of the colon, also involves various proteins that promote or inhibit apoptosis at different stages (Royer and Lu, 2011). Thus, it is possible that sericin might have an influence on some as yet unidentified proteins associated with the late apoptotic stage in colon cancer cells.

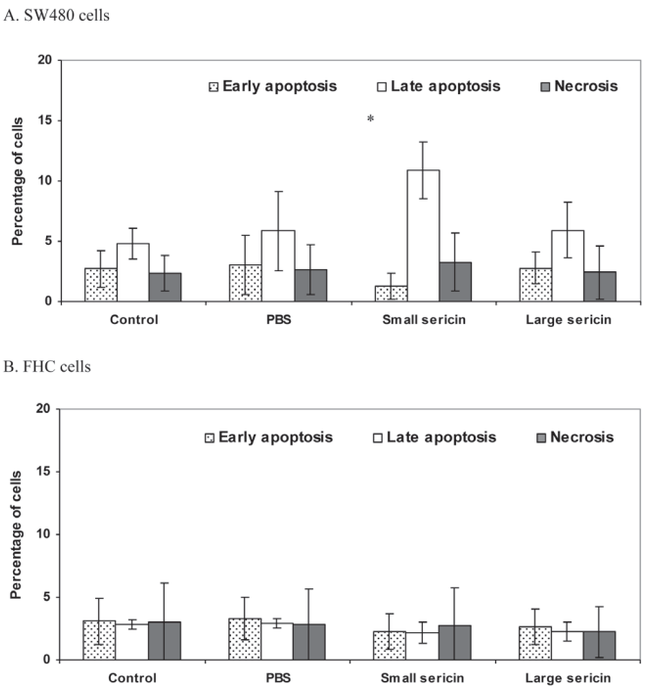

Figure 2: Effect of sericin on SW480 and FHC cell apoptosis. Flow cytometry analysis of cell apoptosis was measured after SW480 (A) and FHC (B) cells were treated with 1,600 mg/ml of small and large-sizes of sericin for 72 h. Results are expressed as mean±SEM and represent the average values from five experiments. * two-tailed Student's t-test indicates significant difference compared to control cells with *p* values of less than 0.05.

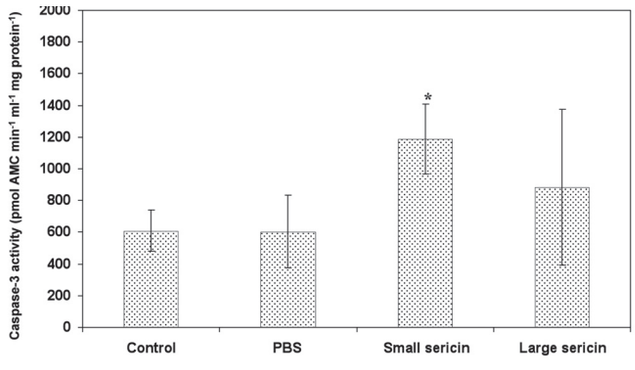

Figure 3: Effect of sericin on caspase-3 activity of SW480 cells. Cells were treated with 1,600 mg/ml of small and large sizes of sericin for 72 h. The caspase-3 activity was determined by using Ac-DEVD-AMC, a specific fluorometric substrate for caspase-3. Results are expressed as mean±SEM and represent the average values from three experiments. * two-tailed Student's t-test indicates significant difference compared to control cells with *p* values of less than 0.05.

TABLE 2
The effect of sericin (1,600 mg/ml, 72 h) on cell cycle of FHC and SW480 cells

Treatment	Stage of cell cycle					
	G0/G1		S		G2/M	
	FHC	SW480	FHC	SW480	FHC	SW480
Control	69.79 ± 4.04	60.46 ± 6.17	7.64 ± 1.01	19.22 ± 6.33	17.08 ± 3.02	16.49 ± 8.09
PBS	68.56 ± 5.12	66.20 ± 2.67	7.90 ± 2.60	13.54 ± 3.08	17.06 ± 3.72	20.11 ± 5.90
Small sericin	69.97 ± 6.43	70.51 ± 3.54	9.93 ± 1.31*	10.46 ± 3.01	13.11 ± 2.78	17.98 ± 2.07
Large sericin	67.23 ± 8.01	67.91 ± 1.41	10.02 ± 1.57*	11.00 ± 2.13	15.26 ± 2.89	20.21 ± 0.66

Results are expressed as mean±SD of three experiments. * p value of less than 0.05 compared to control cells.

The mechanisms of apoptosis mainly involve two signaling pathways, namely the intrinsic pathway, involving the activation of the mitochondria and several caspases, and the extrinsic pathway, involving the activation of death receptors (Elmore, 2007). The key element in a mitochondrial pathway is the release of cytochrome *c* from mitochondria to cytosol to bind to Apaf-1 and caspase-9, and finally to activate caspase-3 (Shi, 2002). Caspase-3 activity is increased when the cells undergo apoptosis (Stennicke and Salvesen, 1998; Wang et al., 2005). In addition to caspase-3, apoptosis regulatory proteins Bcl-2 and Bax associated with the mitochondrial pathway were also examined. In sericin treated SW480 cells, Bcl-2 was down-regulated, but Bax remained unchanged. These findings suggest that sericin may promote cell apoptosis via the mitochondrial pathway. However, the reduced viability of FHC normal cells induced by sericin was not associated with cell apoptosis. However, the decrease in cell viability after the sericin treatment might have been a result of mechanisms other than apoptosis such as cell cycle regulation.

Cell cycle is a complex process involved in cellular proliferation. A defect in the normal pattern of cell cycle may lead to excessive proliferation and finally to the formation of cancerous cells (Schafer, 1998). Although the present findings showed that sericin had no significant effect on the cell cycle of SW480 cells, the percentage of cells in the G0/G1 phase tended to increase. Interestingly, the cell cycle of FHC cells was arrested at the S phase, indicating that sericin, at least in part, may accelerate cell proliferation in normal colonic cells.

Since sericin is a polymeric protein, different extraction techniques can produce different sizes of sericin. Biological activities of sericin are associated with its molecular weight. Small-size sericin (5–100 kDa) rather than large-size sericin (50-200 kDa) can accelerate the proliferation of hybridoma cells (Terada, 2005). The present results showed that small-size sericin (61-132 kDa), but not large-size sericin (191-339 kDa), significantly reduced cell viability and induced cell apoptosis in SW480 cell. In conclusion, the present study demonstrates that a small sericin of 61-132 kDa has anti-proliferative effects for SW480 human colon carcinoma cells. Sericin could induce cell apoptosis associated with an increase in caspase-3 activity and a decrease in Bcl-2 anti-apoptotic protein expression. Sericin had no cytotoxicity, and indeed it might accelerate cell proliferation of FHC normal colonic cells. Thus, sericin appeared to exert a chemopreventive effect against colon cancer by inducing apoptosis in cancerous cells, whereas this protein had no effect on normal colonic cells. Therefore, sericin may have significant health benefits and it could potentially be developed as a dietary supplement for colon cancer prevention.

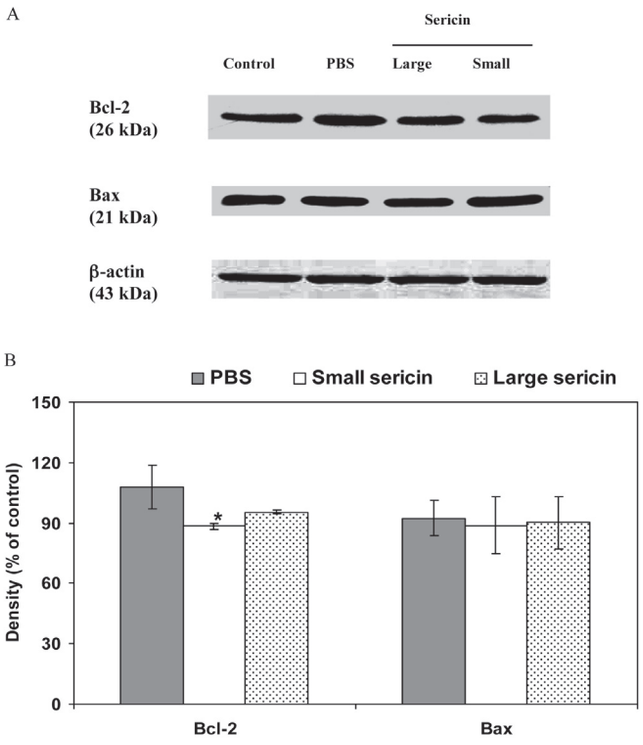

Figure 4: Effect of sericin on the expression of Bcl-2 and Bax proteins in SW480 cells. Cells were treated with 1,600 mg/ml of small and large sizes of serica in for 72 h. Twenty micrograms of cell lysates were subjected to SDS-PAGE and immunoblotting was conducted with a specific antibody against Bcl-2, Bax or β-actin (A). The relative abundance of each protein band to its control β-actin band was estimated by densitometric scanning and calculated to a percentage of the control (B). Results are expressed as mean±SD and represent the average values from three experiments. * two-tailed Student's t-test indicates significant difference compared to control cells with *p* values of less than 0.05.

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REFERENCES

ASCENZI P, POLTICELLI F, MARINO M, SANTUCCI R, COLETTAM (2011) Cardiolipin drives cytochrom c proapoptotic and anti-aoptotic actions. IUBMB Life 63:160-165.

BELOBRAJDIC DP, MCINTOSH GH, OWENS JA (2003) Whey proteins protect more than red meat against azoxymethane induced ACF in Wistar rats. Cancer Lett 198:43-51.

CAMPOS FG, LOGULLO WAITZBERG AG, KISS DR, WAITZBERG DL, HABR-GAMA A, GAMA-RODRIGUES J (2005) Diet and colorectal cancer: current evidence for etiology and prevention. Nutr Hosp 20:18-25.

ELMORE S (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495-516.

FAN JB, WU LP, CHEN LS, MAO XY, REN FZ (2009) Antioxidant activities of silk sericin from silkworm *Bombyx Mori*. J Food Biochem 33:74-88.

HAKKAK R, KOROURIAN S, RONIS MJ, JOHNSTON JM, BADGER TM (2001) Soy protein isolate consumption protects against azoxymethane-induced colon tumors in male rats. Cancer Lett 166:27-32.

HUERTA S, GOULET EJ, LIVINGSTON EH (2006) Colon cancer and apoptosis. Am J Surg 191:517-526.

INDRAN IR, TUFO G, PERVIZ S, BRENNER C (2011) Recent advances in apoptosis, mitochondria and durg resistance in cancer cells. Biochim Biophy Acta 1807:735-745.

JOHNSON IT (2004) New approaches to the role of diet in the prevention of cancers of the alimentary tract. Mutat Res 551:9-28.

KATO N, IWAMI K (2002) Resistant protein: its existence and function beneficial to health. J Nutr Sci Vitaminol 48:1-5.

KATO N, SATO S, YAMANAKA A, YAMADA H, FUWA N, NOMURA M (1998) Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. Biosci Biotechnol Biochem 62:145-147.

KELLY RJ, LOPEZ-CHAVEZ A, CITRIN D, JANIK JE, MORRIS JC (2011) Impacting tumor cell-fate by targeting the inhibitor of apoptosis protein surviving. Mol Cancer 10:35.

KHUHAPREMA T, SRIVATANAKUL P (2008) Colon and rectum cancer in Thailand: an overview. Jpn J Clin Oncol 38:243-273.

LABIANCA R, BERETTA G, GATTA G, DE BRAUD F, WILS J (2004) Colon cancer. Crit Rev Oncol Hematol 51:145-170.

LEVI F, PASCHE C, LUCCHINI F, LA VECCHIA C (2001) Dietary fibre and the risk of colorectal cancer. Eur J Cancer 37:2091-2096.

LIFSHITZ S, LAMPRECHT SA, BENHARROCH D, PRINSLOO I, POLAK-CHARCON S, SCHWARTZ B (2001) Apoptosis (programmed cell death) in colonic cells: from normal to transformed stage. Cancer Letters 163:229-238.

LIU Z, ISHIKAWA W, HUANG X, TOMOTAKE H, KAYASHITA J, WATANABE H, KATO N (2001) A Buckwheat Protein Product Suppresses 1,2-Dimethylhydrazine-Induced Colon Carcinogenesis in Rats by Reducing Cell Proliferation. J Nutr 131:1850-1853.

ROYER C, LU X (2011) Epithelial cell polarity: a major gatekeeper against cancer? Cell Death Different 18:1470-1477.

SASAKI M, YAMADA H, KATO N. (2000) Consumption of silk protein, sericin elevates intestinal absorption of zinc, iron, magnesium and calcium in rats. Nutr Res 20:1505-1511.

SCHAFER KA (1998) The cell cycle: a review. Vet Pathol 35:461-478.

SHI Y (2002) Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 9:459-470.

STENNICKE HR, SALVESEN GS (1998) Properties of the caspases. Biochim Biophys Acta 1387:17-31.

SUN SY, HAIL N, LOTAN R (2004) Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 96:662-672.

TERADA S, SASAKI M, YANAGIHARA K, YAMADA H (2005) Preparation of silk protein sericin as mitogenic factor for better mammalian cell culture. J Biosci Bioeng 100:667-671.

VAN BREDA SG, DE KOK TM, VAN DELFT JH (2008) Mechanisms of colorectal and lung cancer prevention by vegetables: a genomic approach. J Nutr Biochem 19:139-157.

WANG ZB, LIU YQ, CUI YF (2005) Pathways to caspase activation. Cell Biol Int 29:489-496.

WOGAN GN (1985) Diet and nutrition as risk factors for cancer. Princess Takamatsu Symp 16:3-10.

ZHAORIGETU S, SASAKI M, WATANABE H, KATO N (2001) Supplemental silk protein, sericin, suppress colon tumorigenesis in 1,2-dimethylhydrazine-treated mice by reducing oxidative stress and cell proliferation. Biosci Biotechnol Biochem 65:2181-2186.

ZHAORIGETU S, YANAKA N, SASAKI M, WATANABE H, KATO N (2003) Silk protein, sericin, suppresses DMBA-TPA-induced mouse skin tumorigenesis by reducing oxidative stress, inflammatory responses and endogenous tumor promoter TNF-alpha. Oncol Rep 10:537-543.

ZHAORIGETU S, SASAKI M, KATO N (2007) Consumption of sericin suppress colon oxidative stress and aberrant crypt foci in 1,2-dimethylhydrazine-treated rats by colon undigested sericin. J Nutr Sci Vitaminol 53:297-300.

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Cyclooxygenase-2 and hypoxia-regulated proteins are modulated by basic fibroblast growth factor in acute renal failure

Sandra Villanueva[1], Pía Escobar[1], Ioram Jacubovsky[1], Carlos Irarrázabal[1], Juan E Carreño[1], José M. Erpel[2], Carlos Céspedes[2], Alexis A. González[2], Carlos P. Vio[2] And Victoria Velarde[2]

[1] Laboratory of Integrative and Molecular Physiology, Universidad de los Andes, Santiago, Chile. [2] Department of Physiology, Center for Aging and Regeneration, Pontificia Universidad Católica de Chile, Santiago, Chile.

ABSTRACT

Acute renal failure (ARF) can be caused by injuries that induce tissue hypoxia, which in turn can trigger adaptive or inflammatory responses. We previously showed the participation of basic fibroblast growth factor (FGF-2) in renal repair. Based on this, the aim of this study was to analyze the effect of FGF-2 signaling pathway manipulation at hypoxia-induced protein levels, as well as in key proteins from the vasoactive systems of the kidney. We injected rat kidneys with FGF-2 recombinant protein (r-FGF) or FGF-2 receptor antisense oligonucleotide (FGFR2-ASO) after bilateral ischemia, and evaluated the presence of iNOS, EPO and HO-1, in representation of hypoxia-induced proteins, as well as COX-2, renin, kallikrein, and B2KR, in representation of the vasoactive systems of the kidney. A reduction in iNOS, HO-1, EPO, renin, kallikrein, B2KR, and in renal damage was observed in animals treated with r-FGF. The opposite effect was found with FGF-2 receptor down-regulation. In contrast, COX-2 protein levels were higher in kidneys treated with r-FGF and lower in those that received FGFR2-ASO, as compared to saline treated kidneys. These results suggest that the protective role of FGF-2 in the pathogenesis of ARF induced by I/R is a complex process, through which a differential regulation of metabolic pathways takes place.

Key words: ARF, COX-2, FGF-2, kidney regeneration.

INTRODUCTION

Acute renal failure (ARF) is known to be associated with high mortality and morbidity (Schrier et al., 2004). ARF has an initiating phase characterized by organ dysfunction (Sutton et al., 2002), an extension phase marked by inflammatory responses (Molitoris and Sutton, 2004), and a resolution phase in which cellular repair typically occurs (Sutton et al., 2002).

Acute tubular necrosis (ATN) is a common cause of ARF (Esson and Schrier, 2002), and is observed in hypoxic conditions such as hemorrhagic shock or sepsis (Mehta, 2003). The transcriptional response to hypoxia can be adaptive or inflammatory (Taylor and Colgan, 1999). Adaptive responses are controlled through the nuclear accumulation of hypoxia-inducible factor 1 alpha (HIF-1α), and by other proteins that support tissue survival that are controlled by HIF-1α (Bunn and Poyton, 1996), such as erythropoietin (EPO), heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) (Ashley et al., 2002; Lee et al., 1997; Melillo et al., 1997; Noiri et al., 2001). Accordingly, the increased expression of these proteins may be linked to the resolution phase of ARF (Kirkby and Adin, 2006). We previously reported changes in the expression of EPO and HO-1, using an experimental approach of renal ischemia/reperfusion (I/R) as a model of ATN (Villanueva et al., 2007).

A healthy kidney produces vasoactive substances through the activation of the renin-angiotensin system (RAS), and the kallikrein-kinin system (KKS). These vasoactive systems not only participate in maintaining renal blood flow and adequate glomerular filtration rates, but are also involved in renal development (Shen and El-Dahr, 2006). In addition, eicosanoids, the products of cyclooxygenases (COX type 1 and 2), exert physiological activity in the kidney and other organs, thus playing an important role in the regeneration of damaged tissue, as reported for endothelial (Eligini et al., 2009), gastric mucosal (Brzozowski et al., 2000; Mizuno et al., 1997), skin (Hamamoto et al., 2009), skeletal muscle (Bondesen et al., 2004), corneal endothelial (Jumblatt and Willer, 1996), lung (Fukunaga et al., 2005), colon (Reuter et al., 1996) and glomerular mesangial cells (Ishaque et al., 2003). Although COX-2 has been postulated to cause adverse events in the kidney, including ARF (Braden et al., 2004, Seibert et al., 1994), this enzyme has a physiological role in the normal adult kidney (Vio et al., 1997; Vio et al., 2001; Leon et al., 2001) and is necessary for normal kidney development (Vio et al., 1999). In experimental I/R we have observed alterations in the KKS and in the RAS. The observed increase in renin and decrease in kallikrein and COX-2 protein levels suggest a differential regulation of these proteins during ischemic damage (Villanueva et al., 2007). Considering that these enzymes are important in renal development, they could also be part of the regenerative phase described in ARF.

Fibroblast growth factor (FGF) has been suggested to be a renal protector in I/R (Cuevas et al., 1999). Furthermore, basic-FGF (FGF-2) has been postulated to be involved in epithelial-mesenchymal transition and early tubulogenesis (Perantoni et al., 1995). Using a recombinant protein (r-FGF), we have observed a correlation between the increase of FGF-2 and the increase of some proteins during ATN recovery, which are known to be implicated in kidney development (Villanueva et al., 2006a; Villanueva et al., 2006b, Villanueva et al., 2008). Nevertheless, the complete regenerative responses induced by FGF-2 in experimental I/R cannot be understood based solely on the induction of these proteins. Several publications have

Corresponding author: Sandra Villanueva, Ph.D. Laboratory of Integrative and Molecular Physiology, Universidad de los Andes, San Carlos de Apoquindo 2200, Santiago, Chile, Phone: 56-2-4129344, Fax: 56-2-2141756, Email: svillanueva@uandes.cl

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also shown an FGF effect on proteins regulated by hypoxia in different tissues (Akiba et al., 1997; Neuvians et al., 2004; Schmerer et al., 2006).

In light of this evidence, we hypothesized that FGF modulation of the protein levels induced by hypoxia and key proteins from the vasoactive systems of the kidney could contribute to the resolution phase of ARF. To this purpose, we manipulated FGF-2 levels in an experimental model of I/R, and studied the relative abundance of iNOS, EPO and HO-1 in representation of hypoxia-induced proteins, as well as levels of COX-2, renin, kallikrein, and the B2 kinin receptor (B2KR) in representation of the vasoactive systems of the kidney.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (220 to 250 g, n=7 for each I/R group: 24h, 48h, 72h and 96h) were housed in a 12h light/dark cycle. Animals were weighed at the time of bilateral ischemic injury initiation and after completion of experiments. Animals had free access to food and water, and were kept at the animal care facilities of the Universidad Católica de Chile. All experimental procedures were in accordance with institutional and international standards for the humane care and use of laboratory animals (Animal Welfare Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, National Institutes of Health).

Renal ischemia/reperfusion injury

An established model was performed of renal I/R injury that resembles structural and functional consequences of renal ischemia, including apoptotic tubular epithelial cells (Perantoni et al., 1995). Animals were anesthetized with ketamine: xylazine (25:2.5 mg/kg, ip), maintaining body temperature at 37ºC. Both kidneys were exposed by a flank incision and both renal arteries were occluded with a non-traumatic vascular clamp for 30 minutes. Then, while clamps were in position, the left kidney was injected in the parenchymal medullary area with 200 µl of r-FGF (30 mg/kg) (Villanueva et al., 2008; Unger et al., 2000) or FGFR2-ASO (112 µg/kg) (Villanueva et al., 2006b; Carstens et al., 2000) (preliminary experiments using methylene blue dye or Bouin's solution demonstrated that the volume used allowed extensive diffusion over the tissue); the right kidney was injected with the same volume of saline NaCl, 0.9 % (S), used as a vehicle for the oligonucleotides, and also used for control purposes. After injection clamps were removed, renal blood flow was reestablished and both incisions were sutured. Rats were allowed to recover in a room at warm temperature levels. 24, 48, 72 and 96 hours after reperfusion, both kidneys were removed and the animals were sacrificed by exsanguination under heavy anesthesia (ketamine: xylazine). The kidneys were then processed for immunohistochemistry and Western blotting.

Tissue processing and immunohistochemical analysis

Tissue processing for immunohistochemical studies in paraplast-embedded sections was carried out according to methods previously described (Vio et al., 1999; Vio et al., 2001). Immunolocalization studies were performed using an indirect immunoperoxidase technique (Villanueva et al., 2006a). Briefly, tissue sections were dewaxed, rehydrated, rinsed in 0.05 M tris-phosphate-saline (TPS) buffer (pH 7.6) and incubated with the primary antibody overnight at 22°C. Sections were subsequently washed, followed by 30 minutes of incubation with the corresponding secondary antibody and with the peroxidase-antiperoxidase (PAP) complex. Immunoreactive sites were revealed using 0.1% of 3,3'-diaminobenzidine (wt/vol) and 0.03% (vol/vol) of hydrogen peroxide solution.

Antibodies and chemicals

The following primary antibodies were used: monoclonal antibodies against macrophages (clone ED-1, Biosource, Camarillo, CA), monoclonal antibodies against a-smooth muscle actin (α-SMA: clone 1A4, Sigma Aldrich Co., St. Louis, MO), monoclonal antibodies against iNOS (Transduction Labs., Lexington, KI), goat polyclonal antibodies against COX-2 (Cayman Chemical Company, Ann Arbor, MI), goat polyclonal antibodies against EPO (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat polyclonal antibodies against HO-1 (StressGen Biotechnologies Corp., Victoria, Canada), and rabbit monoclonal against FGFR2 (clone EPR5180, Epitomics, Inc. Burlingame, CA). Rabbit polyclonal antibodies against renin, B2KR and kallikrein were prepared as described previously (Velarde et al., 1995).

Secondary antibodies and the corresponding PAP complexes were purchased from MP Biomedicals, Inc. (Aurora, OH). Triton X-100, 3,3'-diaminobenzidine, carrageenan, tris-HCl, hydrogen peroxide, phosphate salts, and other chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO).

Western-blotting

For Western-blotting experiments, longitudinal sections from kidneys including the inner and outer medulla (approximately 1 mm thick) were homogenized with an Ultra-Turrax homogenizer in PBS 1X buffer containing 0.05 M of EDTA and a protease inhibitor cocktail (Pierce, Rockford, IL). The protein concentration was determined by the method of Bradford (Bio-Rad, Richmond, CA). Western blotting was performed as previously described (Villanueva et al., 2006a). Briefly, sixty micrograms of protein were mixed with an equal volume of SDS-PAGE sample buffer (100 mM tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Proteins were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blocking was carried out by incubation in blocking solution (8% nonfat dry milk in tris-buffered saline-0.1% tween) for 2 hours at room temperature. After blocking, membranes were probed with the corresponding primary antibody for 18 hours at 4ºC, washed with tris-buffered saline-tween, and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Proteins were detected by using enhanced chemiluminescence techniques (Pierce, Thermo Scientific, Rockford, IL).

Subsequently, blots were scanned and densitometric analysis was performed by using the public domain NIH Image program v1.61 (US National Institutes of Health, http://

rsb.info.nih.gov/nih-image). The expression of α-tubulin was used to correct variation in sample loading.

Determination of tissue damage and immunohistochemical quantification

Tissue damage was evaluated through periodic acid-Schiff (PAS) staining. Immunolocalization of ED-1 and interstitial α-SMA were used as tissue damage markers. The immunoreactive area in each field (whole kidney sections) was determined by image analysis using Simple PCI software (Compix). Total immunostained (brown) cells were averaged and expressed as the mean absolute values or the mean percentage of stained cell area per field, as previously described (Villanueva et al., 2006a) with minor modifications.

Statistical Analysis

The differences were assessed with the Mann-Whitney nonparametric test for pair-wise comparisons, when overall significance was detected. The significance level was defined at $P<0.05$. Protein densitometry values are presented as mean ± SD. All values are represented by Arbitrary Units (AU).

RESULTS

Determination of tissue damage

According to our hypothesis, if FGF-2 modulates the levels of proteins involved in the different phases of ARF, the reduction of FGF-2, or its signaling should therefore alter the levels of such proteins. With this idea in mind, we injected the kidneys with FGF receptor type 2 (FGFR2) antisense oligonucleotide (ASO). To confirm that the antisense oligonucleotide reduced the expression of the receptor, kidneys injected with ASO (Fig. 1A) and control kidneys injected with a scrambled oligonucleotide (Fig. 1B) were stained against this receptor. As shown in Fig. 1, immunostaining for the FGF receptor was not observed when kidneys were injected with the ASO, thus confirming the effectiveness of the antisense oligonucleotide on the expression of the receptor.

Renal functional damage produced by I/R was assessed by serum creatinine levels. Rats subjected to I/R, and injected with saline had creatinine levels of 0.6±0.1 mg/dL two days after injection. Creatinine levels were 0.40±0.02 mg/dL in animals subjected to I/R and injected with r-FGF, whereas such levels were 0.9±0.2 mg/dL ($P<0.05$) in rats subjected to I/R and injected with FGFR2-ASO.

PAS staining of sections from kidneys injected with saline 48 hours after I/R showed alterations in morphology, which is consistent with ATN (Fig. 2B) as compared to sham operated animals (Fig. 2A). These alterations were characterized by flattening of the brush border epithelia and a high percentage of proximal tubule cells undergoing mitosis. However, detection of such alterations did not occur 96 hours after I/R (data not shown), in accordance with previous results (Villanueva et al., 2006b). Kidneys treated with r-FGF showed a morphology closer to normal at 48 hours after I/R (Fig. 2C), compared to those injected with saline (Fig. 2B). Nevertheless, an important level of mitosis was maintained. Kidneys treated with FGFR2-ASO showed altered morphology, with an increase in the damaged area as mentioned above. These alterations in renal structure were observed 24 hours after I/R and were maintained for 96 hours (Fig. 2D).

Regarding renal damage markers, an increased number of interstitial macrophages (ED-1) and interstitial myofibroblasts (α-SMA) were observed 48 hours after I/R in kidneys injected with saline (ED-1: 238 μm^2 and α-SMA: 22,103 μm^2) (Fig. 2F, J), when compared to sham operated animals (Fig. 2E, I), similar to what was reported previously (Villanueva et al., 2006b). In contrast, r-FGF treated rats showed lower numbers of both markers (ED-1: 180 μm^2 and α-SMA: 4,392 μm^2) at the same period of time, without apparent morphological alterations (Fig. 2G, K). Kidneys injected with FGFR2-ASO showed an increased number of macrophages (ED-1: 650 μm^2) and interstitial α-SMA (47,369 μm^2), which was maintained up to 96 hours after I/R (Fig. 2H, L). This histological data was well correlated with the creatinine levels mentioned above.

iNOS, HO-1 and EPO levels in I/R kidneys treated with r-FGF or FGFR2-ASO

To understand the mechanism associated with experimental I/R and the putative effect of FGF-2, we analyzed three stress proteins induced by hypoxia, namely iNOS, HO-1

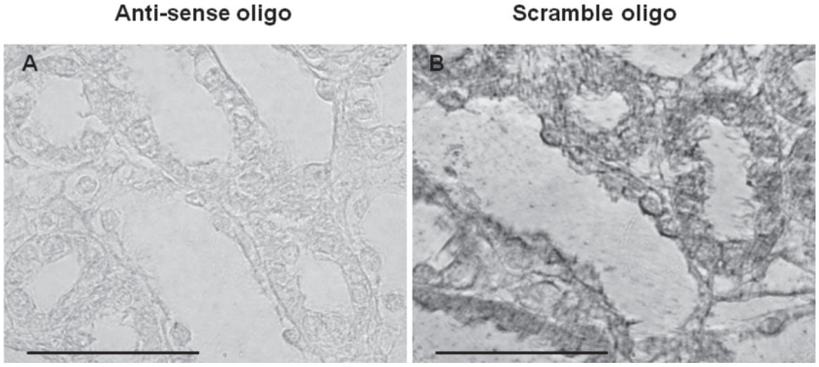

Figure 1: ASO effect on FGFR2 expression in medullary renal tubules. IHQ was performed on kidneys samples obtained 48 hours after injection with (**A**) an FGF receptor type 2 antisense oligonucleotide (**FGFR2-ASO**), or (**B**) a scrambled oligonucleotide (**SO**), (n=5 for each group). An important reduction in bFGFR2 staining was observed in kidneys treated with ASO, compared to kidneys injected with scrambled oligonucleotides. Staining was observed in the entire cell area. Scale bar= 50 μm.

and EPO, and compared the relative abundance of these proteins when kidneys were injected with r-FGF or FGFR2-ASO. When kidneys were injected with saline, the levels of the three proteins were higher when observed 24 hours after I/R. However, as can be seen in Fig. 3, the levels of the three proteins decreased at 48 hours compared to their levels at 24 hours (iNOS: 185 ± 16AU at 24h v/s 82 ± 15 AU at 48h; HO-1: 126 ± 34AU at 24h v/s 15 ± 8 AU at 48h; and EPO: 125 ± 26AU at 24h v/s 77 ± 9 AU at 48h; $P<0.05$) (Fig. 3, circles A,B & C). Nevertheless, an important reduction in the levels of these markers was observed at 24 hours in kidneys treated with r-FGF compared to saline injected kidneys (iNOS: 37 ± 2 AU; HO-1: 15 ± 4 AU and EPO: 88 ± 5 AU; $P<0.05$) (Fig. 3, squares). Furthermore, a significant increase in these proteins was maintained in kidneys treated with FGFR2-ASO for at least 96 hours after experimental I/R (iNOS: 214 ± 18 AU; HO-1: 126± 28 AU and EPO 165 ± 24 AU at 96h; $P<0.05$) (Fig. 3, triangles). Since two bands were observed for HO-1 and EPO, only the band closer to the molecular weight described for each of these proteins was used for densitometry.

The protein distribution of iNOS, HO-1 and EPO was analyzed by immunohistochemistry. At 48 h after I/R, iNOS was observed in collecting ducts localized in the outer medulla (Fig. 4, A, B & C), and HO-1 was localized in the cortical area, mainly in interstitial cells. iNOS was still observed in the outer medulla in r-FGF treated kidneys, but was limited to certain tubules, while HO-1 was mainly localized in some tubular cells, possibly due to a treatment effect (Fig. 4, D, E & F). In the case of FGFR2-ASO treated rats, immunostaining for iNOS was still observed in all tubular segments, while HO-1 was again observed in the interstitium. Accordingly, when the immunostaining was quantified, kidneys from animals treated with r-FGF showed a lower staining for iNOS and HO-1, 48 hours after experimental I/R (iNOS: 6,232 μm^2 and HO-1: 433 μm^2) (Fig. 4, B & E), when compared to the group treated with saline (iNOS: 13,645 μm^2 and HO-1: 966 μm^2) (Fig. 4, A & D). Moreover, this reduction was still observed 96 hours after I/R (data not shown). In contrast, kidneys treated with FGFR2-ASO showed an increase in the staining for both proteins 48 hours after I/R (iNOS: 20,469 μm^2 and HO-1: 948 μm^2) (Fig 4, C & F), which was maintained for 96 hours after I/R, compared to the group treated with saline (Fig. 4, A & D). Immunostaining for EPO was localized mainly in a few cells of the kidney cortex in animals submitted to I/R and injected with saline after 48 hours (Fig. 4, G). The staining was higher in animals treated with FGFR2-ASO (2,124 μm^2) (Fig. 4, I), compared to animals treated with r-FGF (535 μm^2) (Fig. 4, H). The distribution was mainly peritubular, close to proximal tubules and loops of Henle. However, in the r-FGF group it was also localized in tubular cells, probably due to a treatment effect.

Renal levels of vasoactive system components are regulated by the FGF-2 pathway

Normal kidney function is due to the proper balance between the KKS and the RAS (Schmaier, 2003). In addition, these

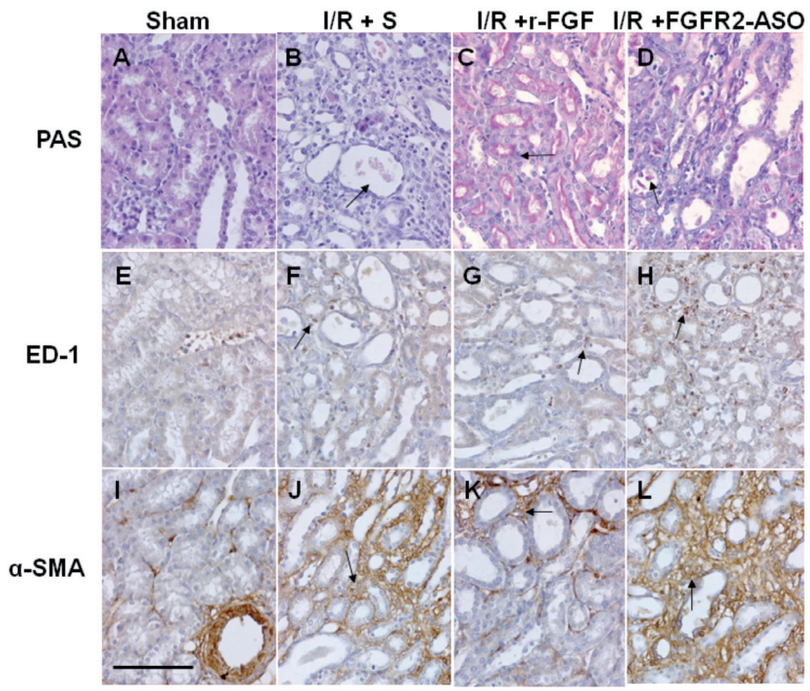

Figure 2: Evidence of histological renal damage in hypoxic kidney induced by I/R and treated with r-FGF or FGFR2-ASO. Immunohistochemistry was performed on kidney samples from sham operated animals (**A,E & I**), or obtained 48 hours after I/R and saline (S) injection (**B,F & J**) or FGF-2 recombinant protein (r-FGF) (**C,G & K**), and at 96 hours after I/R in kidneys injected with FGF-2 receptor 2 antisense oligonucleotides (FGFR2-ASO) (**D,H & L**) (n=7 for each group). Histological damage was evaluated by PAS staining (**A,B,C & D**). Brush border, epithelial flattening and mitosis are shown. Induction of renal damage markers such as ED-1 (**E,F,G & H**) as a measurement of macrophages, or α-SMA (**I,J,K & L**) as a measurement of myofibroblasts, are shown. Scale bar=100μm. The arrows in F,G,H,J,K,and L show one area where the markers are localized.

systems are involved in renal development and maturation (Shen and El-Dahr, 2006). We evaluated the effect of FGF in the distribution and levels of certain components of these systems in kidneys after I/R (Fig. 5 and 6). First, we analyzed renin expression in the three conditions described above: saline, r-FGF and FGFR2-ASO. Kidneys treated with r-FGF showed an important reduction in renin levels at 24 hours compared to the saline group. This low level was maintained at 96 hours after I/R, which was when the renin levels in the saline group also decreased to a similar level (24 ± 3 AU at 24 hours v/s 23 ± 3 AU at 96 hours; $P<0.05$) (Fig. 5, A). Kidneys treated with FGFR2-ASO showed an increase in renin levels at all times analyzed after I/R (182 ± 16 AU at 24 hours and 170 ± 36 AU at 96 hours; $P<0.05$) (Fig. 5, A). We previously demonstrated that kallikrein levels can be modulated after I/R (Villanueva et al., 2007). In the present study, we found that kallikrein levels were low at all analyzed times in kidneys treated with r-FGF, compared to the saline condition (46 ± 7 AU v/s 76 ± 5 AU at 24 hours) (Fig. 5, B). In contrast, kidneys treated with FGFR2-ASO showed an increase in kallikrein levels 24 hours after I/R, which was maintained at all analyzed times (115 ± 13 AU at 24 hours; $P<0.05$) (Fig. 5, B).

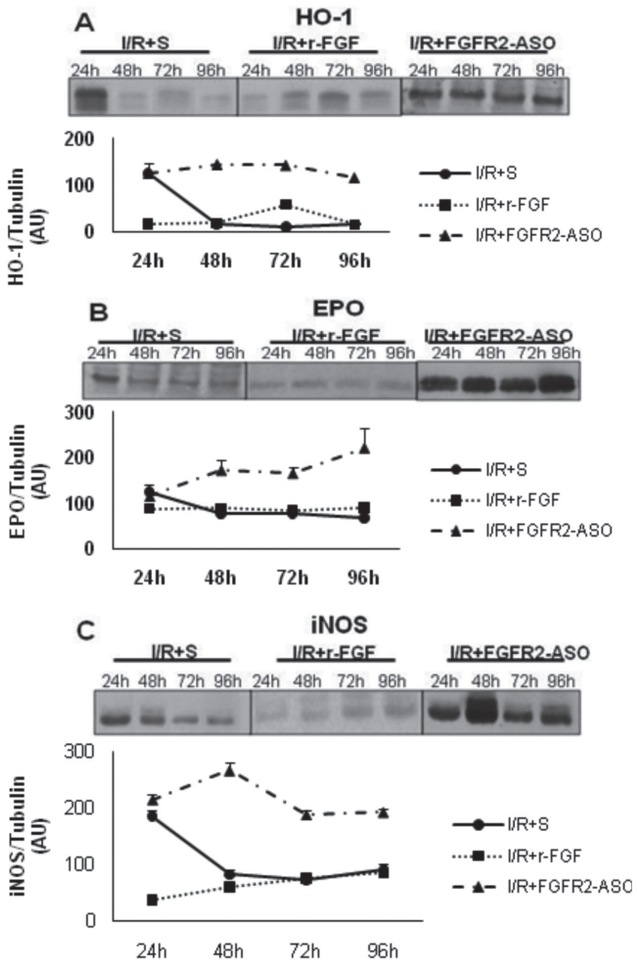

Figure 3: FGFR2-ASO and r-FGF effects on HO-1, EPO and i-NOS response in I/R-induced ARF. Proteins induced by hypoxia (**A**)HO-1, (**B**)EPO and (**C**)i-NOS were analyzed at 24, 48, 72 and 96 hours after a 30 min ischemia in kidneys injected with saline (**circles**), r-FGF (**squares**) or FGFR2-ASO (**triangles**). n=7 for each group. *=$P<0.05$ compared to saline at the same point in time.

Another important KKS protein is the B2 kinin receptor (B2KR). In the case of kidneys treated with r-FGF, B2KR levels decreased at all evaluated times when compared to the saline group (27 ± 5 AU v/s 149 ± 5 AU at 24 hours; $P<0.05$) (Fig. 5, C). In contrast, kidneys treated with FGFR2-ASO showed higher B2KR levels at all the analyzed times in contrast to the saline group (172 ± 10 AU at 24 hours $P<0.05$) (Fig. 5, C).

Immunostaining for renin, kallikrein and B2KR observed at 48 hours after treatment showed similar results to those observed by Western blot, that is, immunoreactivity in the r-FGF group decreased in cell number and in intensity (renin: 43 μm^2, kallikrein: 136 μm^2 and B2KR: 117 μm^2) (Fig. 6, E-G), compared to saline group (renin: 50 μm^2, kallikrein: 345 μm^2 and B2KR: 345 μm^2) (Fig. 6, A-C). In contrast, the kidneys treated with FGFR2-ASO showed increased immunoreactivity for renin, kallikrein and B2KR compared to the saline group (renin: 151 μm^2, kallikrein: 371 μm^2 and B2KR: 177 μm^2) (Fig. 6, I-K).

Kidneys submitted to I/R and injected with saline solution had almost undetectable COX-2 protein levels when measured by Western blot at 24, 48 and 96 hours after I/R, with an increase at 72 hours after reperfusion (Villanueva et al., 2007). In contrast, kidneys treated with r-FGF showed an increased expression of COX-2 24 hours after I/R, which was maintained at all evaluation times (126 ± 35 AU at 24 hours; $P<0.05$) (Fig. 4, D). Conversely, COX-2 was not detectable by Western blot at any evaluation time in kidneys treated with FGFR2-ASO, (Fig. 5, D). In addition, IHC revealed that kidneys injected with r-FGF had an increased COX-2 immunoreactivity 48 hours after I/R (118 μm^2) (Fig. 6, H), whereas kidneys treated with FGFR2-ASO showed a decreased COX-2 staining (14 μm^2) (Fig. 6, L) when compared to control saline kidneys (54 μm^2) (Fig. 6, D).

As expected, COX-2 and B2KR were mainly observed in thick ascending limbs (Vio et al., 1999; Vio et al., 2001), renin was present in the afferent arteriole, and kallikrein was located in connecting tubule cells in the kidney cortex. The treatments with FGFR2-ASO or r-FGF did not modify the localization of these proteins.

DISCUSSION

ARF induced by ischemia is a reversible pathological condition, whose recovery is characterized by the restoration of the renal tubular system (Villanueva et al., 2006a). However, there are persistent post-ischemic alterations in renal function that can lead to permanent failures in urinary concentrating ability, associated with enduring vascular damage. One particular protein that has been observed to be altered, even several weeks after I/R, is Kallikrein (Basile et al., 2005).

We recently reported that FGF-2 prevents renal damage and stimulates kidney regeneration in an adult kidney I/R model, through the induction of proteins involved in embryonic kidney development (Villanueva et al., 2008). In addition, recent studies performed in different tissues (esophagus, gastric mucosa, corpus luteum and red blood cells), have shown an important interaction between FGF-2 and the vasoactive renal systems, or FGF-2 and stress proteins

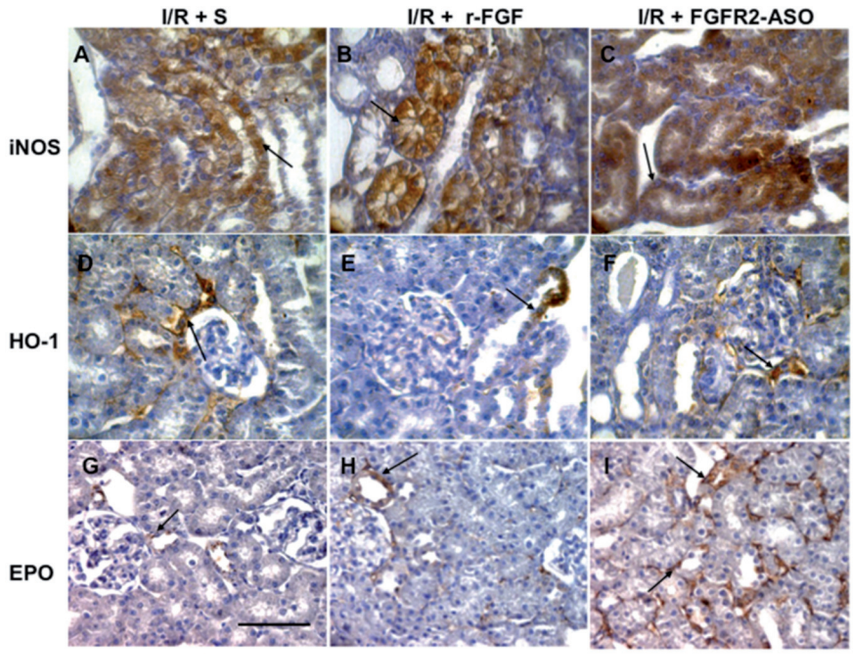

Figure 4: Immunolocalization of proteins that respond to hypoxia in I/R-induced ARF. Staining for iNOS (**A,B & C**), HO-1 (**D,E & F**) and EPO (**G,H & I**) was observed 48 hours after I/R in kidneys injected with saline (**A,D & G**), r-FGF **(B,E & H**) or FGFR2-ASO (**C,F & I**) (n=7 for each group). Scale bar=100μm. The arrows indicate representative immunostained (brown) areas.

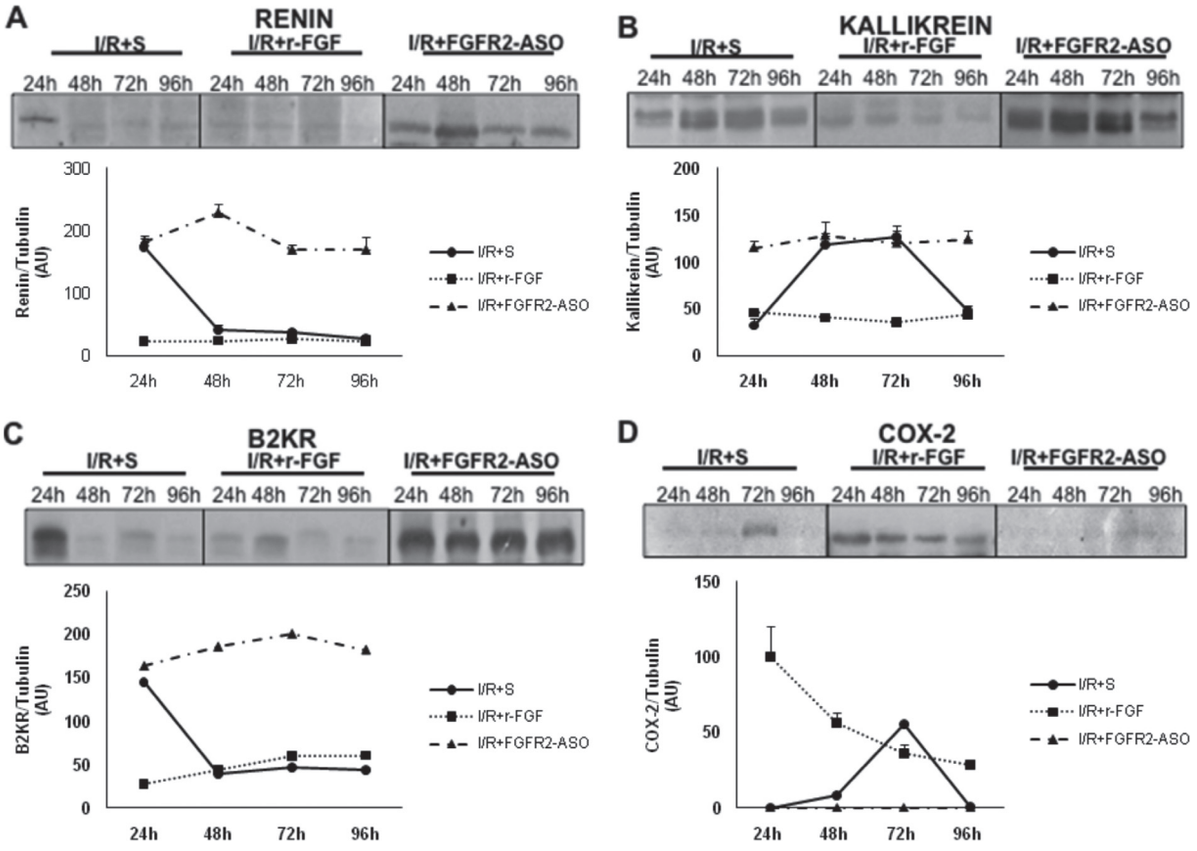

Figure 5: Immunoblot for vasoactive proteins in by I/R-induced ARF. Vasoactive proteins: renin (**A**), kallikrein (**B**), B2KR (**C**) and COX-2 (**D**), were analyzed at 24, 48, 72 and 96 hours after 30 min of ischemia in kidneys injected with saline (**circles**), r-FGF (**squares**) or FGFR2-ASO (**triangles**). (n=7 for each group). *=$P<0.05$ compared to saline at each time point.

induced by hypoxia (Schmerer et al., 2006; Unger et al., 2000; Carstens et al., 2000; Baguma-Nibasheka et al., 2007;Martins et al., 2008). This raises the possibility that FGF-2 may have a double role in kidney repair, by increasing the expression of repairing proteins, and by modulating the vasoactive systems of the kidney as well as the proteins related to the hypoxic stress response. In this study we performed complementary experiments to evaluate the direct effects of FGF-2 on the regulation of the components of these vasoactive systems and stress-related proteins induced by hypoxia, as well as their participation in renal damage and their involvement in persistent alterations.

FGFs mediate their biological effects by binding to, and activating receptors with tyrosine kinase activity. This interaction results in a series of molecular events that culminate in biological responses such as mitosis, differentiation or migration (Friesel and Maciag, 1999). In this regard, FGFR2 is localized throughout the whole cell and, as observed in some cases, staining up to the apical membrane, mainly in tubular segments of the inner stripe of the outer medulla, which is similar to what has been observed by other researchers (Cancilla et al., 2001). It can be speculated that the growth factor reaches its receptor from the blood vessels in normal conditions, but it can also reach the receptor through the tubular lumen when the filtration barrier is damaged.

We analyzed three stress proteins regulated by hypoxia (HO-1, EPO, and iNOS), and also proteins that are not only involved in urine concentration and blood pressure regulation, but in kidney development as well (B2KR, kallikrein, renin and COX-2). The major findings of this study were the significant decreases of most of these proteins in kidneys treated with r-FGF. To explain these results, we could speculate that FGF-2 is directly inhibiting the overexpression of these proteins, or that the effect of FGF-2 is indirect, probably mediated by HIF-1α or other transcription factors that are regulated by FGF-2. This pattern was the opposite for COX-2 proteins, since they increased in the r-FGF group. This result suggests that COX-2 might be regulated by a different mechanism, as discussed later.

Although three HO isoforms have been reported, only the inducible isoform HO-1 is expressed in renal tissue (Yang et al., 2004). HO-1 induction occurs as an adaptive and beneficial response to several injury processes, including oxidative injuries (Morimoto et al., 2001), rhabdomyolysis (Carstens et al., 2000), cisplatin nephrotoxicity and ARF (Shimizu et al., 2000). This may be explained by the generation of products from heme degradation, iron, carbon monoxide, biliverdin and bilirubin, which exert important antioxidant, anti-inflammatory, and cytoprotective functions (Kirkby and Adin, 2006). We found increased levels of HO-1 in kidneys with high damage induced by FGFR2-ASO. This could be explained by a compensatory mechanism against maladaptive responses stimulated by hypoxia. In addition, the low levels of HO-1 observed even at 24 hours after I/R, when r-FGF was injected, could be explained by the anticipation of HO-1 level reduction, as compared to the effect under the saline injection condition.

EPO is a protein involved in renal erythropoiesis, and as such, it is regulated by hypoxia. EPO has protective effects on retina and renal tubular cells; in the specific context of ARF (Sharples et al., 2005), EPO exerts its action through the activation of Janus kinase 2, Akt, and multiple targets with antiapoptotic effects (Sharples et al., 2004). In addition, FGF has been reported to inhibit EPO-induced erythropoiesis (Schmerer et al., 2006; Kreja et al., 1993). It is possible that the observed EPO induction in ischemic kidneys treated with FGFR2-ASO could be related to the molecular machinery

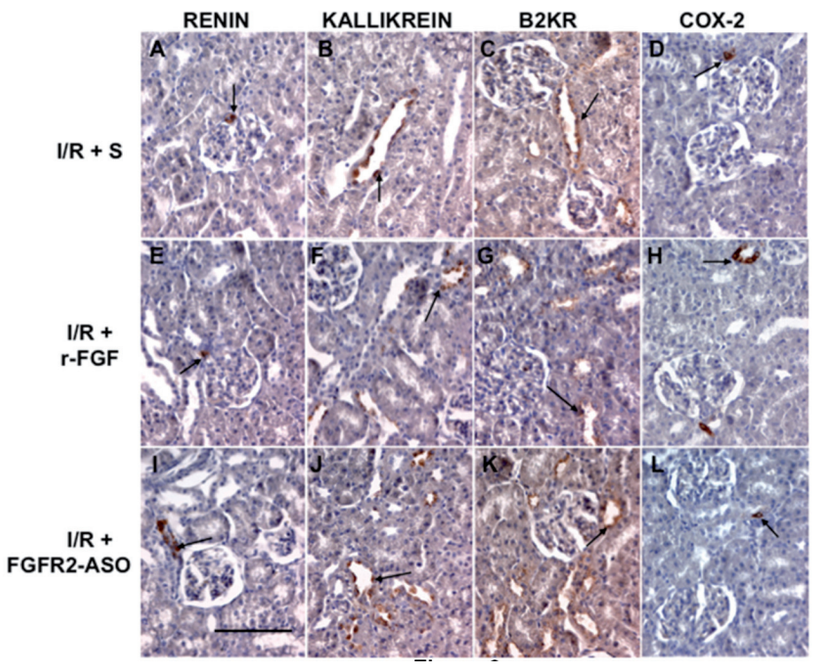

Figure 6: Immunolocalization of vasoactive proteins in I/R-induced ARF . Staining for renin (**A,E & I**), kallikrein (**B,F & J**), B2KR (**C,G & K**) and COX-2 (**D,H & L**) was evaluated 48 hours after ischemia in kidneys injected with S (**A-D**), r-FGF (**E-H**) and FGFR2-ASO (**I-L**) (n=7 for each group). Scale bar=100μm. The arrows indicate representative immunostained (brown) areas.

elicited in the protective response to I/R damage, and that this response is not necessary or is anticipated in the presence of FGF-2.

Recent studies have shown that nitric oxide (NO) generated by iNOS is involved in many pathological states (including renal I/R), contributing to oxidative damage of critical cellular macromolecules (Chatterjee et al., 2002; Viñas et al., 2006). In addition, the increase in HO-1, together with an increase in iNOS, the former producing CO and the latter producing NO, could contribute to the production of NO in a regulated and beneficial form (Lee and Yen, 2009). In this study, we have shown that FGF-2 is involved in the suppression of iNOS and HO-1 induction, suggesting that additional production of NO and CO is not needed to preserve renal function in the presence of this growth factor or, as previously speculated for HO-1 and EPO, the effects on iNOS regulation were anticipated at least 24 hours when r-FGF was administered.

We have also analyzed the FGF effect on some proteins from renal vasoactive systems that have been involved in renal development, and that could participate in the recovery of renal damage. As mentioned previously, the activity of the KKS is important for normal kidney development (Shen and El-Dahr, 2006). Kallikrein cleaves kininogen to produce bradykinin (BK), which binds to its receptor B2KR, activating intracellular signaling (Velarde et al., 1995). B2KR is constitutively expressed in renal tissues, and has been associated with the differentiation of the distal nephron and epithelial cells derived from embryonic stem cells (El-Dahr, 2004). When BK concentration increases, the receptor is down-regulated by internalization, followed by degradation (Velarde et al., 1995). The up-regulation of B2KR observed in saline treated kidneys after 24 hours of I/R could be explained by a decrease in bradykinin, caused by the reduction of kallikrein, observed at that same time period. In addition, it has been observed that the B2KR is induced by hypoxia in endothelial cells (Liesmaa et al., 2009), which is also consistent with the high levels of B2KR observed 24h after I/R. The negative feedback of BK toward its receptor could explain the increase in kallikrein and the reduction of the receptor observed 48 hours after I/R. Recent reports have shown that B2KR expression and BK secretion decrease during *in vitro* differentiation (Martins et al., 2008), suggesting an additional regulatory mechanism. The participation of FGF in tissue differentiation and the observations of Martins et al., could explain the reduction of kallikrein and the B2KR in the presence of r-FGF at 96 hours after I/R, as well as the opposite effect with FGFR2-ASO observed in the present study.

The effect of COX-2 on renal damage is controversial; while Matsuyama *et al*. (2005) proposed that COX-2 might induce renal tissue damage triggered by I/R, other authors have suggested a role for COX-2 in regeneration (Feitoza et al., 2005). Several studies have shown COX-2 participation in nephron development (Vio et al., 1999) and tissue repair (Eligini et al., 2009; Brzozowski et al., 2000; Mizuno et al., 1997; Hamamoto et al., 2009; Bondesen et al., 2004; Reuter et al., 1996; Leahy et al., 2002). In addition, a beneficial effect of COX-2-derived mediators has been reported; examples are lipoxin in acute lung injury (Fukunaga et al., 2005) and prostaglandin E2 in corneal endothelial injury (Jumblatt and Willer, 1996). Furthermore, an inhibitory effect on apoptosis has been described in renal glomerular mesangial cells (Ishaque et al., 2003).

The results obtained in the present study might be explained by the effects of COX-2 derivatives on proteins induced by renal damage. In this sense, the regulation of COX-2 expression acquires great importance. Our observations indicate that COX-2 has an opposite regulation from those of the other studied proteins. We postulate that COX-2 has a similar effect to the one reported previously in kidney tubular cells, in terms of its involvement in repairing tissue damage. However, this COX-2 effect might only be observed when renal tubules are preserved and COX-2 can be induced as occurs in the presence of r-FGF. In contrast, when tissue damage is high, as observed in I/R+S, the induction of COX-2 would not be possible. Additionally, recent publications have shown a direct association between COX-2 and FGF-2 (Baguma-Nibasheka et al., 2007), and COX-2 mRNA has been shown to increase in response to FGF-2 administration in retinal epithelial cells, (Ershov and Basan, 1999). A decrease in COX-2 and an increase in HO-1 activity have also been observed in endothelial cells (Morimoto et al., 2001; Li et al., 2003), suggesting that the heme-hemoxygenase system could be a negative regulator for COX-2 expression.

In summary, it is obvious that this study has some limitations due to two facts, namely that it is an *in vivo study* and, as such, it is hard to isolate one single effect, and that most of the experiments are not quantitative, so they only reflect a relationship between the compared proteins. However, despite such limitations, it is possible to postulate that FGF could be acting as a master regulator in I/R. In this sense, we suggest a model in which iNOS, HO-1, EPO, Renin, Kallikrein, B2KR are initially stimulated by FGF to induce cell proliferation and tissue regeneration. The continuous presence of FGF-2 could act as negative feedback to the abovementioned proteins to end a phase, and as a positive stimulus to COX-2 that, after the reduction of HO-1, could increase and act on the following phase of differentiation. Consequently, further studies are needed to evaluate this new model. Nevertheless, this study opens a new perspective for the use of FGF-2 in future research for kidney damage and regeneration.

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REFERENCES

AKIBA Y, NAKAMURA M, ODA M, KIMURA H, MIURA S, TSUCHIYA M, ISHII H (1997) Basic fibroblast growth factor increases constitutive nitric oxide synthase during healing of rat gastric ulcers. *J Clin Gastroenterol* 25:S122-S128.

ASHLEY RA, DUBUQUE SH, DVORAK B, WOODWARD SS, WILLIAMS SK, KLING PJ (2002) Erythropoietin stimulates vasculogenesis in neonatal rat mesenteric microvascular endothelial cells. *Pediatr Res* 51:472-478.

BAGUMA-NIBASHEKA M, BARCLAY C, LI AW, GELDENHUYS L, PORTER GA, BLAY J, CASSON AG, MURPHY PR (2007) Selective cyclooxygenase-2 inhibition suppresses basic fibroblast growth factor expression in human esophageal adenocarcinoma. *Mol Carcinog* 46:971-980.

BASILE DP, FREDRICH K, ALAUSA M, VIO CP, LIANG M, RIEDER MR, GREENE AS, COWLEY AW JR (2005) Identification of persistently

altered gene expression in the kidney after functional recovery from ischemic acute renal failure. Am J Physiol Renal Physiol 288:F953-F963.

BONDESEN BA, MILLS ST, KEGLEY KM, PAVLATH GK (2004) The COX-2 pathway is essential during early stages of skeletal muscle regeneration. Am J Physiol Cell Physiol 287:C475-C483.

BRADEN GL, O'SHEA MH, MULHERN JG, AND GERMAIN MJ (2004) Acute renal failure and hyperkalaemia associated with cyclooxygenase-2 inhibitors. *Nephrol Dial Transplant* 19:1149-1153.

BRZOZOWSKI T, KONTUREK PC, KONTUREK SJ, PAJDO R, SCHUPPAN D, DROZDOWICZ D, PTAK A, PAWLIK M, NAKAMURA T, HAHN EG (2000) Involvement of cyclooxygenase (COX)-2 products in acceleration of ulcer healing by gastrin and hepatocyte growth factor. *J Physiol Pharmacol* 51:751-773.

BUNN HF, AND POYTON RO (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* 76:839-885.

CANCILLA B, DAVIES A, CAUCHI JA, RISBRIDGER GP, BERTRAM JF (2001). Fibroblast growth factor receptors and their ligands in the adult rat kidney. *Kidney Int* 60:147-155.

CARSTENS RP, WAGNER EJ, GARCÍA-BLANCO MA (2000) An intronic splicing silencer causes skipping of the IIIb exon of fibroblast growth factor receptor 2 through involvement of polypyrimidine tract binding protein. *Mol Cell Biol* 20:7388-7400.

CHATTERJEE PK, PATEL NS, KVALE EO, CUZZOCREA S, BROWN PA, STEWART KN, MOTA-FILIPE H, THIEMERMANN C (2002) Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury. *Kidney Int* 61:862-871.

CUEVAS P, MARTÍNEZ-COSO V, FU X, ORTE L, REIMERS D, GIMÉNEZ-GALLEGO G, FORSSMANN WG, SÁENZ DE TEJADA I (1999) Fibroblast growth factor protects the kidney against ischemia-reperfusion injury. *Eur J Med Res* 4:403-410.

EL-DAHR SS (2004) Spatial expression of the kallikrein-kinin system during nephrogenesis. *Histol Histopathol* 19:1301-1310.

ELIGINI S, ARENAZ I, BARBIERI SS, FALERI ML, CRISCI M, TREMOLI E, COLLI S (2009) Cyclooxygenase-2 mediates hydrogen peroxide-induced wound repair in human endothelial cells. *Free Radic Biol Med* 46:1428-1436.

ERSHOV AV, BAZAN NG (1999) Induction of cyclooxygenase-2 gene expression in retinal pigment epithelium cells by photoreceptor rod outer segment phagocytosis and growth factors. *J Neurosci Res* 58:254-261.

ESSON ML, SCHRIER RW (2002) Diagnosis and treatment of acute tubular necrosis. *Ann Intern Med* 137:744-752.

FEITOZA CQ, CÂMARA NO, PINHEIRO HS, GONÇALVES GM, CENEDEZE MA, PACHECO-SILVA A, SANTOS OF (2005) Cyclooxygenase 1 and/or 2 blockade ameliorates the renal tissue damage triggered by ischemia and reperfusion injury. *Int Immunopharmacol* 5:79-84.

FRIESEL R, MACIAG T (1999) Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. Thromb Haemost. 82:748-754.

FUKUNAGA K, KOHLI P, BONNANS C, FREDENBURGH LE, LEVY BD (2005) Cyclooxygenase 2 plays a pivotal role in the resolution of acute lung injury. J Immunol 174:5033-5039.

HAMAMOTO T, YABUKI A, YAMATO O, FUJIKI M, MISUMI K, MATSUMOTO M (2009) Immunohistochemical analysis of cyclooxygenase-2 induction during wound healing in dog skin. *Res Vet Sci* 87:349-354.

ISHAQUE A, DUNN MJ, SOROKIN A (2003) Cyclooxygenase-2 inhibits tumor necrosis factor alpha-mediated apoptosis in renal glomerular mesangial cells. *J Biol Chem* 278:10629-10640.

JUMBLATT MM, WILLER SS (1996) Corneal endothelial repair. Regulation of prostaglandin E2 synthesis. *Invest Ophthalmol Vis Sci* 37:1294-1301.

KIRKBY KA, ADIN CA (2006) Products of heme oxygenase and their potential therapeutic applications. *Am J Physiol Renal Physiol* 290:F563-F571.

KREJA L, THOMA S, SELIG C, LAMPING C, ZIEGLER BL, NOTHDURFT W (1993) The effect of recombinant human stem cell factor and basic fibroblast growth factor on the in vitro radiosensitivity of CD34+ hematopoietic progenitors from human umbilical cord blood. *Exp Hematol* 21:1436-1441.

LEAHY KM, ORNBERG RL, WANG Y, ZWEIFEL BS, KOKI AT, MASFERRER JL (2002) Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* 62: 625-631.

LEE PJ, JIANG BH, CHIN BY, IYER NV, ALAM J, SEMENZA GL, CHOI AM (1997) Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem* 272: 5375-5381.

LEE CY, YEN MH (2009) Nitric oxide and carbon monoxide, collaborative and competitive regulators of hypertension. Chang Gung Med J. 32: 12-21.

LEÓN CG, MARCHETTI J, VIO CP (2001) Renal cyclooxygenase-2: evidence for recruitment of thick ascending limb of henle cells in microdissected nephron segments. *Hipertension* 38:630-634.

LI VOLTI G, SETA F, SCHWARTZMAN ML, NASJLETTI A, ABRAHAM NG (2003) Heme oxygenase attenuates angiotensin II-mediated increase in cyclooxygenase-2 activity in human femoral endothelial cells. *Hypertension* 41:715-719.

LIESMAA I, LESKINEN HK, KOKKONEN JO, RUSKOAHO H, KOVANEN PT, LINDSTEDT KA (2009) Hypoxia-induced expression of bradykinin type-2 receptors in endothelial cells triggers NO production, cell migration, and angiogenesis. *J Cell Physiol* 221:359-366.MARTINS AH, ALVES JM, TRUJILLO CA, SCHWINDT TT, BARNABÉ GF, MOTTA FL, GUIMARAES AO, CASARINI DE, MELLO LE, PESQUERO JB, ULRICH H (2008) Kinin-B2 receptor expression and activity during differentiation of embryonic rat neurospheres. *Cytometry A* 73:361-368.

MATSUYAMA M, YOSHIMURA R, HASE T, KAWAHITO Y, SANO H, NAKATANI T (2005) Study of cyclooxygenase-2 in renal ischemia-reperfusion injury. *Transplant Proc* 37:370-372.

MEHTA R (2003) Outcomes research in acute renal failure. *Semin Nephrol* 23:283-294.

MELILLO G, TAYLOR LS, BROOKS A, MUSSO T, COX GW, VARESIO L (1997) Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. *J Biol Chem*; 272:12236-12243.

MIZUNO H, SAKAMOTO C, MATSUDA K, WADA K, UCHIDA T, NOGUCHI H, AKAMATSU T, KASUGA M (1997) Induction of cyclooxygenase 2 in gastric mucosal lesions and its inhibition by the specific antagonist delays healing in mice. *Gastroenterology* 112:387-397.

MOLITORIS BA, SUTTON TA (2004) Endothelial injury and dysfunction: role in the extension phase of acute renal failure. *Kidney Int* 66:496-499.

MORIMOTO K, OHTA K, YACHIE A, YANG Y, SHIMIZU M, GOTO C, TOMA T, KASAHARA Y, YOKOYAMA H, MIYATA T, SEKI H, KOIZUMI S (2001) Cytoprotective role of heme oxygenase (HO)-1 in human kidney with various renal diseases. *Kidney Int* 60:1858-1860.

NEUVIANS TP, SCHAMS D, BERISHA B, PFAFFL MW (2004) Involvement of pro-inflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin F2alpha-induced luteolysis in bovine corpus luteum. *Biol Reprod* 70:473-480.

NOIRI E, NAKAO A, UCHIDA K, TSUKAHARA H, OHNO M, FUJITA T, BRODSKY S, GOLIGORSKY MS (2001) Oxidative and nitrosative stress in acute renal ischemia. *Am J Physiol Renal Physiol* 281:F948-F957.

PERANTONI AO, DOVE LF, KARAVANOVA I (1995) Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc Natl Acad Sci USA* 92:4696-4700.

REUTER BK, ASFAHA S, BURET A, SHARKEY KA, WALLACE JL (1996) Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J Clin Invest 98:2076-2085.

SCHMAIER AH (2003) The kallikrein-kinin and the renin-angiotensin systems have a multilayered interaction. *Am J Physiol Regul Integr Comp Physiol* 285:R1-R13.

SCHMERER M, TORREGROZA I, PASCAL A, UMBHAUER M, EVANS T (2006) STAT5 ACTS AS A REPRESSOR TO REGULATE EARLY EMBRYONIC ERYTHROPOIESIS. *BLOOD* 108:2989-2997.

SCHRIER RW, WANG W, POOLE B, MITRA A (2004) Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. *J Clin Invest* 114:5-14.

SEIBERT K, ZHANG Y, LEAHY K, HAUSER S, MASFERRER J, PERKINS W, LEE L, ISAKSON P (1994) PHARMACOLOGICAL AND BIOCHEMICAL DEMONSTRATION OF THE ROLE OF CYCLOOXYGENASE 2 IN INFLAMMATION AND PAIN. *PROC NATL ACAD SCI USA* 91:12013-12017.

SHARPLES EJ, PATEL N, BROWN P, STEWART K, MOTA-PHILIPE H, SHEAFF M, KIESWICH J, ALLEN D, HARWOOD S, RAFTERY M, THIEMERMANN C, YAQOOB MM (2004) Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. *J Am Soc Nephrol* 15:2240-2241.

SHARPLES EJ, THIEMERMANN C, YAQOOB MM (2005) Mechanism of disease: Cell death in acute renal failure and emerging evidence for a protective role of erythropoietin. *Nat Clin Pract Nephrol* 1:87-97.

SHEN B, EL-DAHR SS (2006) Cross-talk of the renin-angiotensin and kallikrein-kinin systems. *Biol Chem* 387:145-150.

SHIMIZU H, TAKAHASHI T, SUZUKI T, YAMASAKI A, FUJIWARA T, ODAKA Y, HIRAKAWA M, FUJITA H, AKAGI R (2000) Protective

effect of heme oxygenase induction in ischemic acute renal failure. *Crit Care Med* 28:809-817.

SUTTON TA, FISHER CJ, MOLITORIS BA (2002) Microvascular endothelial injury and dysfunction during ischemic acute renal failure. *Kidney Int* 62:1539-1549.

TAYLOR CT, COLGAN SP (1999) Therapeutic targets for hypoxia-elicited pathways. *Pharm Res* 16:1498-1505.

UNGER EF, GONCALVES L, EPSTEIN SE, CHEW EY, TRAPNELL CB, CANNON RO 3RD, QUYYUMI AA (2000) Effects of a single intracoronary injection of basic fibroblast growth factor in stable angina pectoris. *Am J Cardiol* 85:1414-1419.

VELARDE V, HUMPHREYS J, FIGUEROA CD, VIO CP (1995) Postnatal maturation of tissue kallikrein-producing cells (connecting tubule cells) in the rat kidney: a morphometric and immunohistochemical study. *Anat Embryol* 192:407-414.

VILLANUEVA S, CÉSPEDES C, GONZÁLEZ A, VIO CP (2006a) bFGF induces an earlier expression of nephrogenic proteins after ischemic acute renal failure. *Am J Physiol Regul Integr Comp Physiol* 291:R1677-R1687.

VILLANUEVA S, CÉSPEDES C, VIO CP (2006b) Ischemic acute renal failure induces the expression of a wide range of nephrogenic proteins. *Am J Physiol Regul Integr Comp Physiol* 290:R861-R870.

VILLANUEVA S, CÉSPEDES C, GONZÁLEZ AA, VIO CP, VELARDE V (2007) Effect of ischemic acute renal damage on the expression of COX-2 and oxidative stress-related elements in rat kidney. *Am J Physiol Renal Physiol* 292:F1364-F1371.

VILLANUEVA S, CÉSPEDES C, GONZÁLEZ AA, ROESSLER E, VIO CP (2008) Inhibition of bFGF-receptor type 2 increases kidney damage and suppresses nephrogenic protein after ischemic acute renal failure. *Am J Physiol Regul Integr Comp Physiol* 294:R819-R828.

VIÑAS JL, SOLA A, GENESCA M, ALFARO V, PI F, HOTTER G (2006) NO and NOS isoforms in the development of apoptosis in renal ischemia/reperfusion. Free *Radic Biol Med* 40:992-1003.

VIO CP, CÉSPEDES C, GALLARDO P, MASFERRER JL (1997) RENAL IDENTIFICATION OF CYCLOOXYGENASE-2 IN A SUBSET OF THICK ASCENDING LIMB CELLS. *HYPERTENSION* 30: 687-692.

VIO CP, BALESTRINI C, RECABARREN M, CÉSPEDES C (1999) Postnatal development of cyclooxygenase-2 in the rat kidney. *Immunopharmacology* 44:205-210.

VIO CP, AN SH, CÉSPEDES C, MCGIFF J, FERRERI N (2001) Induction of cyclooxygenase-2 in thick ascending limb cells by adrenalectomy. *J Am Soc Nephrol* 12:649-658.

YANG L, QUAN S, NASJLETTI A, LANIADO-SCHWARTZMAN M, ABRAHAM NG (2004) Heme oxygenase-1 gene expression modulates angiotensin II-induced increase in blood pressure. *Hypertension* 43:1221-1226.

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Reinstatement of short-latency responses after asymptotic Pavlovian conditioning training by the presentation of an extraneous stimulus

Edgar H. Vogel

Universidad de Talca, Talca, Chile and Yale University, New Haven, CT, USA.

ABSTRACT

The purpose of this study was to examine whether the progressive disappearance of short-latency conditioned responses, or inhibition of delay, observed in Pavlovian conditioning with long inter-stimulus intervals, could be reverted by the presentation of a novel stimulus. In one experiment, two groups of rabbits received extensive training with a short (250 ms) or a long (1500 ms) tone that overlapped and terminated with a periorbital shock unconditioned stimulus. After training, the presentation of an extraneous stimulus prior to tone onset produced a reinstatement of short latency CRs in the group trained with the long CS, but did not affect CR latency in the group trained with the short CS.
This finding is consistent with Pavlov's (1927) view that conditioning with long conditioned stimuli involves the acquisition of response tendencies in the early portion of the stimulus that are subsequently suppressed by the development of an inhibitory process.

Key words: Inhibition of delay, conditioning, temporal discrimination, eyeblink conditioning, timing

INTRODUCTION

Inhibition of delay is an empirical fact of Pavlovian conditioning that consists of an increase in the latency of the conditioned response (CR) over training. The first systematic observations of this phenomenon date back to 1927 in Pavlov's studies of salivary conditioning in dogs. He noticed that over the course of training with a long conditioned stimulus (CS) that precedes the unconditioned stimulus (US, typically food) there was a gradual reduction in the amount of saliva elicited during the early portion of the CS, and an increase in the amount of saliva elicited during the latter portion of the CS. Since the responses that originally occurred in the early portion of the CS seemed to be "inhibited" as training progressed, he proposed the term "inhibition of delay" (Pavlov, 1927).

During the 1960s, there was marked interest among theorists in the nature of the inhibition of delay reported by Pavlov. Consequently, Pavlov's findings were frequently analyzed and scattered evidence was reported in several theoretical papers (e.g., Kimmel, 1965; Prokasy, 1965; Sheffield, 1965). For instance, Sheffield (1965) reported the lengthening of the latency of conditioned salivation in a dog trained with a 4-sec CS and Kimmel (1965) observed a virtually linear increase in the latency of human Galvanic Skin Response with a light CS followed by a fi nger shock US.

For early theoreticians, inhibition of delay is closely related to other temporal phenomena of Pavlovian conditioning, such as the fact that the latency of onset and peak of the CR increases with the CS-US interval, and that the prominent time of occurrence of the CR is at the US locus (Bitterman, 1964; Davis et al., 1989; Gallistel and Gibbon, 2000; Mauk and Ruiz, 1992). Nevertheless, although these observations are not incompatible with the notion of inhibition of delay, and probably are regulated by partially common mechanisms of temporal discrimination, they should not be taken as indicative that an excitatory tendency has been inhibited during the early portion of the CS. Vogel et al. (2003) pointed out that a genuine demonstration of inhibition of delay should include both, evidence of conditioned responses in the early portion of the CS at initial stages of training, presumably due to the development of the so-called "excitatory CS-US associations", and a subsequent suppression of these responses at the end of conditioning, presumably due to the progressive development of the so- called "inhibitory CS-US associations" in the early portion of the CS.

As a theoretical interpretation of inhibition of delay, Pavlov (1927) proposed that training with a single CS can be seen as a situation where animals learn to discriminate among a complex combination of hypothetical elements present over the duration of the CS. This implies that the CS is made up of a number of components that establish separable associations with the US. A critical aspect of this CS representation is the assumption that the pattern of activity of each element varies in time following CS initiation, such that they are differentially eligible for reinforcement and non-reinforcement depending on their temporal location with respect to the US.

Figure 1 presents an example of how the temporal discrimination hypothesis can be implemented in modern theories of Pavlovian conditioning (e.g., Vogel et al., 2003). It is assumed that some CS-elements have a more positive correlation with reinforcement across trials than other elements do, in such a way that they may develop comparatively more associative strength. In Figure 1, processing of the CS is represented by a set of binary elements, which can be in an "on" state of activity (represented as a black circle) or in an "off" state of activity (represented as a white circle), at any time during the CS. The figure depicts the pattern of activity of six elements in a training situation in which the US is presented in the last temporal segment of the CS. Although the elements differ in their loci of activity, they can be classified roughly into three categories. The "A-elements" that are solely activated at the US locus, the "X-elements" that are

Correspondence to: Edgar Vogel, Universidad de Talca, Escuela de Psicología, Casilla N° 747, Talca/Chile, Phone: (5671) 201566, Fax: (5671) 201510, E-mail: evogel@utalca.cl

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activated in both the US locus and at some other moments, and the "B-elements" that are activated only when the US is not present. Thus, temporal discrimination would be an instance of an AX+BXdiscrimination in which the elements A, B and X develop differential associations with the US, according to their differential temporal contiguity with the US.

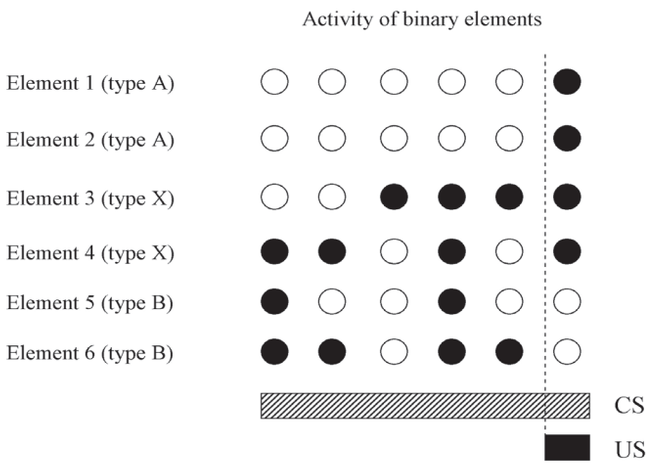

Figure 1. Schematic representation of the temporal discrimination hypothesis of inhibition of delay: It is assumed that the CS activates a sequence of binary elements whose state of activity varies between off (white circles) and on (black circles) states over the duration of the CS. The black rectangles at the bottom represent the duration of the CS and the US.

According to the theoretical analysis presented above, inhibition of delay is the result of conditioned inhibition to these CS elements that are consistently activated in absence of the US (the "B" elements) in position of overlap with those elements that are partially associated with the US (the "X" elements). This notion implies that excitatory and inhibitory CS-US associations coexist in the early part of the CS. Very few studies, however, have evaluated the nature of the association existing in the early portion of the CS.

Evidence of inhibition in the early portion of the CS has been provided by means of a summation test by Rodnick (1937). He demonstrated that the human eyelid conditioned response to a brief vibratory CS was significantly diminished in amplitude when presented 5-sec after the onset of a 21-sec light that had been paired with a wrist shock US, as compared to trials without the light CS. Although the light CS produced some decrement in the conditioned eyelid response before it was paired with the US, this decrement was reliably greater after it had been paired with the US.

Pavlov (1927) suggested another method to detect excitatory and inhibitory tendencies in the early portion of the CS. He reported that animals that had developed inhibition of delay would show a reappearance of short latency CRs in the presence of an extraneous, "disinhibiting" stimulus. The disinhibition procedure was further explored by Rescorla (1967, Experiment 2) in dogs trained to avoid a CS paired with footshock. Inhibition of delay was demonstrated by a progressive reduction in the rate of avoidance responses to the early portion of the CS. In the last session of training, a novel stimulus presented during the early portion of the CS produced a uniform increase in the response rate, whereas it did no affect responding when it was presented in the later portion of the CS.

Apart from the early reports described above, the evidence on the existence of inhibition of delay in other preparations is controversial. That is, although a progressive increase in the latency of CR has been demonstrated in the conditioned suppression of instrumental behavior in rats (Hammond and Maser, 1970; Hendry and Van Toller, 1965; Millenson and Hendry, 1967; Smith et al., 1969; Zielinsky, 1966) and fear conditioning in dogs (Rescorla, 1967), in human and rabbit eyelid conditioning, the literature is ambiguous. For instance, while some studies of human eyelid conditioning reported increases in the latency of CR (Runquist and Muir, 1965) others showed decreases (Ebel and Prokasy, 1963; Hilgard and Campbell, 1936; Subosky, 1967). Likewise, most research conducted with rabbit eyelid conditioning reports that the CR latency decreases with training towards an asymptote at about half of the CS duration (e.g., Coleman and Gormezano, 1971; Gormezano, 1972; Salafia et al., 1974; Salafia et al., 1975; Schneiderman, 1966; Schneiderman and Gormezano, 1964; Smith et al., 1969), which is the opposite of an inhibition of delay effect.

On the face of these controversial results, Vogel et al. (2003) suggested that the predominant absence of inhibition of delay seen in rabbit eyeblink conditioning could be due primarily to the fact that the reported data were based on moderate amounts of training, typically between eight and ten sessions, and with relatively short CSs, which might not be sufficient to observe the development of inhibition of delay. In agreement with this reasoning, Vogel et al. reported two experiments that demonstrated inhibition of delay when rabbits were trained with relatively long but not with short CSs. Specifically, in Experiment 2, two groups of rabbits received 20 daily sessions of eyelid conditioning training, in which a tone CS overlapped and terminated with a shock US. Each session consisted of 56 reinforced trials and 6 nonreinforced trials. For the animals in the group designated as "short CS group" (n=8), the CS was 250 ms duration, while in the group designated as "long CS group" (n=7), the CS was 1500 ms duration. Inhibition of delay was apparent in animals trained with the long 1500 ms CS but not in animals trained with the shorter-250 ms CS, whose CR latency decrease, rather than increased over training.

The present experiment was designed to extend the findings of Vogel et al. (2003) by investigating a disinhibition procedure in the same 15 animals that were trained with short and long intervals. The rationale of the experiment is that if the animals of the two groups of Vogel et al. (Experiment 2) had developed differential inhibition of delay, and the difference was due to differential conditioned inhibition, then the presentation of an extraneous stimulus prior to the initiation of the CS (the disinhibition procedure) may have a differential effect on the two groups. Specifically, it was expected that there would be a reinstatement of short latency CRs in the 1500 ms animals, which had presumably developed inhibition of delay, but there would be no effect on the animals trained with the 250 ms CS, which did not develop inhibition of delay.

METHOD

Animals

Male New Zealand white rabbits (Oryctolagus cuniculus; n=16) weighing between 2 and 3 kg, were individually housed and

maintained with ad libitum food and water, except during experimental sessions

Apparatus

The experiment was conducted in 66 cm × 48 cm × 48 cm isolation chambers. Each chamber was completely lined with aluminium foil to provide a homogeneous visual surrounding and illuminated by a 1W neon bulb. Ventilation fans provided a constant background masking noise that raised the ambient sound pressure level to approximately 70 dB (re 20 uN/m2) in each chamber. During the experimental sessions, the rabbit was loosely restrained within a 51 cm ×18 cm ×14 cm Plexiglas box, from which its head protruded. The conditioned stimulus was a 3000-Hz 90-dB tone that coterminated with a 50-ms 3-mA shock US delivered to the right paraorbital region. The "extraneous stimulus," (see procedure below) was a 1000 ms sequence of alternating presentations of a 250 ms light and a 250 ms vibrotactile stimulus applied to the animal's chest. The light stimulus was a 12/sec flashing light generated by a strobe lamp located behind the animal so as to reflect diffusely from the walls of the chamber. The vibrotactile stimulus was a 30-Hz oscillation provided by a hand massager mounted on the floor of the restraining box so as to maintain firm contact with the animal's chest. For half of the animals in each group, the extraneous stimulus began with the light and for the other half it began with the vibrotactile stimulus.

Closure of the rabbit's eye was monitored by an adaptation of the photoresistive transducer described by Gormezano and Gibbs (1988). The resulting signal was displayed on a polygraph, adjusted so that a 1 mm eyelid closure produced a 1 mm deflection of a recording pen.

Procedure

The experiment is a continuation of the study reported by Vogel et al (2003, Experiment 2). In this previous study, two groups of rabbits received 20 daily sessions of eyelid conditioning training, in which a tone CS overlapped and terminated with a shock US. Each session consisted of 56 reinforced trials and 6 nonreinforced trials. For the animals in the group designated as "short CS group" (n=8), the CS was 250 ms duration, while in the group designated as "long CS group" (n=8), the CS was 1500 ms duration.

The experiment reported here began the day following the last session of this training. Here a "disinhibition test" was conducted in two successive sessions. Each test session consisted of 56 regular training trials and 7 test trials in which the extraneous stimulus preceded the CS. This stimulus was delivered 1000 ms prior to the onset of the CS on designated test trials. The test trials were distributed such that one test trial occurred after approximately each 6 training trials.

Scoring

Movements of the rabbit's eyelid were recorded automatically by a computer program with a frequency of one sample every 10 ms. An eyelid CR was scored when the record indicted an eyelid closure of 0.5 mm or more, relative to the pre-stimulus baseline, occurring from 50 ms after the CS onset to 30 ms after the US onset. The variable of interest was the latency of the CR, computed as the delay between the onset of the CS and the onset of the CR. To avoid distortions due to the different durations of the CS, the latency was expressed as "relative latency" by dividing the latency by the duration of the CS in each group.

RESULTS AND DISCUSSION

Figure 2 depicts the mean relative latency of the CR for the trials that immediately preceded each test trial with the extraneous stimulus, for the trials that contained the extraneous stimulus, and for the trials that immediately followed the test trials with the extraneous stimulus. Although both groups showed shorter latency of CRs in those trials in which the novel stimulus was presented as compared to the immediately preceding and subsequent trials, the decrease of the Short CS group was negligible, within the range of within session variability, whereas the decrease in the Long CS group was very striking and substantial.

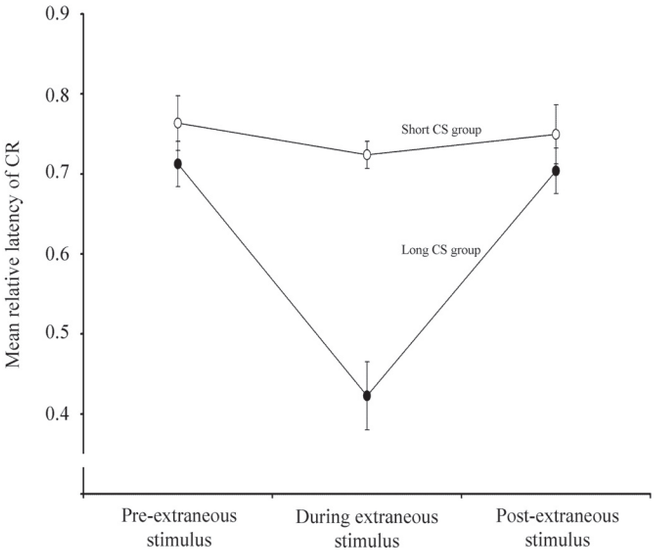

Figure 2. Mean relative latency of CR for the trials that immediately preceded each test trial with the extraneous stimulus, for the trials that contained the extraneous stimulus and for the trials that immediately followed the trials with the extraneous stimulus. The error bars represent the standard error of the means.

Statistical analyses confirmed the reliability of these observations. A 2 (group: 250 ms, 1500 ms) x 3 (trial type: Pre-extraneous stimulus, during extraneous stimulus, Post-extraneous stimulus) ANOVA revealed reliable main effects of group ($F_{1,11}$ =9.003, p =0.009) and trial type ($F_{2, 22}$ =33.271, p <0.001) and reliable group x trial type interaction ($F_{2, 22}$ =20.93; p <0.001).

The main effect of the trial type was due to shorter relative latency of the CR during the extraneous stimulus trials than in the pre-extraneous stimulus and post-extraneous stimulus trials in both groups. Likewise, the main effect of group was due to shorter relative latency of the CR in the long-CS group than in the short-CS one.

The most interesting effect is the trial type x group interaction, which was examined by LSD pairwise comparisons

of the three trial types in each group. This analysis indicated a reliable decrement in relative latency of CR from the pre and post-extraneous stimulus trials to the extraneous stimulus trials (ps <0.01) in the 1500 ms group, but no reliable effects in the 250 ms group (ps >0.239).

Note that the two groups were responding with similar relative latency in regular trials (see the pre-extraneous and post-extraneous stimulus trials in Figure 2). However, just as the groups approached these similar relative latencies from different directions (i.e. one group increased and the other decreased its latency, as reported by Vogel et al., 2003), they also exhibited differential effects of the extraneous stimulus. This is consistent with the notion that an inhibitory process may underlie inhibition of delay and that excitatory and inhibitory associations coexist in the early portion of the CS in those cases in which inhibition of delay occurred.

There are some alternative explanations for the differential effect of the extraneous stimulus in the two groups that should be noted. For example, it can be hypothesized that the decrease in the latency of CR during test trials was due to a CR "potentiation" or "sensitization" caused by the introduction of the extraneous stimulus. According to this interpretation, the reinstatements of short latency responses would not be the result of disinhibition caused by the extraneous stimulus, but rather of the manifestation of an unconditioned response controlled by the latter stimulus. That this effect was evident in the long but not in the short CS group might be attributed to a delay in the potentiation effect, so that it would be more evident when the CR was delayed (in the 1500 ms group) than when the CR was more immediate (in the 250 ms group). The possibility of CR potentiation could be investigated by replicating the basic design of this experiment and including test trials with several different extraneous stimulus-CS intervals. The hypothetical potentiation effect might be more likely to be seen in the short CS if the extraneous stimulus were lengthened. Likewise, if the extraneous stimulus is a source of response potentiation, it would be expected that a similar increase in responding in the early portion of the CS (or decrease in latency of CR) would be observed in the early stages of training as well as at asymptote. Conversely, if the effect of the extraneous stimulus is to remove inhibition, no effect of the extraneous stimulus should be expected in early stages of training, when inhibition of delay has presumably no yet developed.

It should be pointed out that even if the decrease in the latency of CRs in the 1500 ms group were due to a removal of inhibition by the extraneous stimulus, this experiment does not comment on why the "disinhibition" effect is obtained. One interpretation is that a novel stimulus specially disrupts inhibition, leaving the excitatory tendency relatively intact, by virtue of inhibition being more labile than excitation, as has been suggested by Pavlov (1927). One could equally argue, however, that the CS elements that developed excitation might be more context-independent than those CS elements that developed inhibition, such that the latter elements would be more readily changed in its perceptual properties by the extraneous stimulus. This view has the advantage that it does no appeal to differential properties of inhibition and excitation, but nevertheless it clearly requires further theoretical and empirical examinations in order to specify the conditions under which these differential effects may occur (see Wagner, 2003; Wagner and Vogel, 2008).

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REFERENCES

BITTERMAN ME (1964) Classical conditioning of the goldfish as a function of the CS-US interval. J. Comp Physiol Psychol 58: 359-366.

BONEAU CA (1958) The interstimulus interval and the latency of the conditioned eyelid response. J Exp Psychol 56: 464-471.

COLEMAN SR, GORMEZANO I (1971) Classical conditioning of the rabbit's *Oryctolagus cuniculus* nictitating membrane response under symmetrical CS-US interval shifts. J Comp Physiol Psychol 77, 447-455.

DAVIS M, SCHLESINGER LS, SORENSON CA (1989) Temporal specificity of fear conditioning: effects of different conditioned stimulus-unconditioned stimulus interval on the fear-potentiated startle effect. J Exp Psychol Anim Behav Process 15: 295-310.

EBEL HC, PROKASY WF (1963) Classical eyelid conditioning as a function of sustained and shifted interstimulus interval. J Exp Psychol 65, 52-58.

GALLISTEL R, GIBBON J, (2000) Time, rate and conditioning. Psychol Rev 107: 289-344.

GORMEZANO I (1972) Investigations of defense and reward conditioning in the rabbit In: BLACK AH, PROKASY WF (Eds) Classical conditioning II. New York: Appleton-Century-Crofts. pp: 151-181.

GORMEZANO I, GIBBS CM (1988) Transduction of the rabbit's nictitating membrane response. Behav Res Methods Instrum Comput 20: 18-22.

HAMMOND LJ, MASER J (1970) Forgetting and conditioned suppression: role of a temporal discrimination. J Exp Anal Behav 13: 333-338.

HENDRY DP, VAN TOLLER C (1965) Alleviation of conditioned suppression. J Comp Physiol Psychol 49: 458-460.

HILGARD ER, CAMPBELL AA (1936) The course of acquisition and retention of conditioned eyelid response in man. J Exp Psychol 19: 227-247.

KIMMEL HD (1965) Instrumental inhibitory factors in classical conditioning In: PROKASY WF (Ed) Classical Conditioning: A symposium. New York: Appleton-Century-Crofts. pp: 148-171.

MAUK MD, RUIZ BP (1992) Learning-dependent timing of Pavlovian eyelid responses: differential conditioned using multiple interstimulus. Behav Neurosci 106: 666-681.

MILLENSON JR, HENDRY DP (1967) Quantification of response suppression in conditioned anxiety training. Can J Psychol 21: 242-252.

PAVLOV IP (1927) Conditioned Reflexes. London: Oxford University Press.

PROKASY WF (1965) Classical eyelid conditioning: experimenter operations, task demands, and response shaping In: PROJASY WF (Ed) Classical conditioning: A symposium. New York: Appleton-Century-Crofts. pp: 208-225.

RESCORLA RA (1967) Inhibition of delay in Pavlovian fear conditioning. J Comp Physiol Psychol 64: 114-120.

RODNICK EH (1937) Does de interval of delay of conditioned responses possess inhibitory properties? J Exp Psychol 20: 507-527.

RUNQUIST WN, MUIR WR (1965) Intrasession decrements in the performance of the classically conditioned eyelid reflex. J Exp Psychol 70: 520-525.

SALAFIA WR, DATSON AP, BARTOSIAK RS, HURLEY J, MARTINO LJ (1974) Classical nictitating membrane conditioning in the rabbit *Oryctolagus cuniculus* as a function of unconditioned stimulus locus. J Comp Physiol Psychol 86: 608-636.

SALAFIA WR, TERRY SW, DATSON AP (1975) Conditioning of the rabbit *Oryctolagus cuniculus* nictitating membrane response as a function of trials per session, ISI, and ITI. Psychon Soc 6: 505-508.

SCHNEIDERMAN N (1966) Interstimulus interval function in the nictitating membrane response under delay versus trace conditioning. J Comp Physiol Psychol 62: 397-402.

SCHNEIDERMAN N, GROMEZANO I (1964) Conditioning of the nictitating membrane of the rabbit as a function of CS-US interval. J Comp Physiol Psychol 57: 188-195.

SHEFFIELD FD (1965) Relation between classical conditioning and instrumental learning, In: PROKASY WF (Ed) Classical conditioning: A symposium. New York: Appleton-Century-Crofts. pp: 302-322.

SMITH MC, COLEMAN SR, GORMEZANO I (1969) Classical conditioning of the rabbit's nictitating membrane response at backward, simultaneous, and forward CS-US intervals. J Comp Physiol Psychol 69: 226-231.

SUBOSKY MD (1967) UCS intensity and the latency of the classically conditioned eyelid response. J Exp Psychol 74: 31-35.

VOGEL EH, BRANDON SE, WAGNER AR (2003) Stimulus representation in SOP: II An application to inhibition of delay. Behav Processes 62: 27-48.

WAGNER AR (2003) Context-sensitive elemental theory Q J Exp Psychol, 56B, 7-29

WAGNER AR, VOGEL EH (2008). Configural and elemental processing in associative learning: Commentary on Melchers, Schanks and Lachnit. Behav Processes 77: 446-450.

ZIELINSKY K (1966) Inhibition of "delay" as a mechanism of the gradual weakning of the conditioned emotional response. Acta Neurobiol Exp 37: 299-309.

Biol Res 45: 67-73, 2012

Relaxant effects of different fractions from *Tymus vulgaris* on guinea-pig tracheal chains

Rana Keyhanmanesh[1,2,3] and Mohammad H. Boskabady[4,*]

[1] Department of Physiology, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Tuberculosis and lung research center, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Drug applied research center, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Department of Physiology, Medical School and Pharmacological Research Centre of Medical Plants, Mashhad University of Medical Sciences, Mashhad, Iran

ABSTRACT

In previous studies, the relaxant effect of *Tymus vulgaris* has been demonstrated on guinea pig tracheal chains. Therefore, in the present study, the relaxant effects of n-hexane, dichloromethane, methanol and aqueous fractions of *Tymus vulgaris* on tracheal chains of guinea pigs were examined. The relaxant effects of four cumulative concentrations of each fraction (0.4, 0.8, 1.2 and 1.6 g%) in comparison to saline as negative control and four cumulative concentrations of theophylline (0.2, 0.4, 0.6 and 0.8 mM) were examined for their relaxant effects on precontracted tracheal chains of guinea pig by 60 mM KCl (group 1) and 10 μM methacholine (group 2, n = 7 for each group). In group 1, all concentrations of the n-hexane fraction and theophylline and three last concentrations (0.8, 1.2 and 1.6 g%) of dichloromethane and two higher concentrations (1.2 and 1.6 g%) of methanol fractions showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$). In group 2, all concentrations of theophylline, n-hexane and dichloromethane fractions and three concentrations (0.8, 1.2 and 1.6 g%) of methanol and two higher concentrations (1.2 and 1.6 g%) of aqueous fractions showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$). In addition, with group 1, the relaxant effect of all concentrations of all fractions except the n-hexane fraction, were significantly less than those of theophylline ($p<0.05$ to $p<0.001$). The n-hexane fraction showed higher relaxant effect than theophylline. The relaxant effect of all concentrations of the n-hexane fraction and the three last concentrations (0.8, 1.2 and 1.6 g%) of dichloromethane and aqueous fractions were significantly greater in group 2 than in group 1 ($p<0.05$ to $p<0.001$). There were significant positive correlations between the relaxant effects and concentrations for theophylline and all fractions (except aqueous fraction in group 1) in both groups, but a negative correlation for the aqueous fraction in group 1 ($p<0.05$ to $p<0.001$). These results showed a potent relaxant effect for n-hexane and weaker relaxant effect for other fractions from *Tymus vulgaris* on tracheal chains of guinea pigs.

Key words: *Tymus vulgaris*; Fractions; Relaxant effects; Guinea pig; Trachea

INTRODUCTION

The main active constituents of *Tymus vulgaris* L include: terpenes, phenols, thymol, carvacrol, terpenoids, glycosides of phenolic monoterpenoids, eugenol and aliphatic alcohols, flavonoids such as thymonin, cirsilineol and 8-methoxycirsilineol, biphenyl compounds of monoterpenoid origin, caffeic and rosmarinic acids and saponins (ESCOP, 1997). Other constituents include tannins, labiatic acid, ursolic acid, and oleanolic acid. Thyme also contains apigenin, luteolin, and 6-hydroxyluteolin glycosides, as well as di-, tri-, and tetramethoxylated flavones (Mossa, 1987).

Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, pharmaceuticals, and cosmetics (Simon et al., 1999; Javanmardi et al., 2002; Senatore, 1996). Traditionally, thyme possesses various beneficial effects, e.g., antiseptic, carminative, antimicrobial, and antioxidative properties (Baranauskiene et al., 2003). Thyme extract has been used orally to treat dyspepsia and other gastrointestinal disturbances; coughs due to colds, bronchitis and pertussis, laryngitis and tonsillitis (as a gargle). Topical applications of thyme extract have been used in the treatment of minor wounds, the common cold, disorders of the oral cavity and as an antibacterial agent in oral hygiene. Both essential oil and thymol are ingredients of a number of proprietary drugs including antiseptic and healing ointments, syrups for the treatment of respiratory disorders and preparations for inhalation (Mossa, 1987). It has also been used to improve digestion (ESCOP, 1997) and to treat pertussis, stomatitis, and halitosis (Stecher, 1968).

Previous studies have shown different pharmacological effect for this plant. In vitro studies have shown that both thyme essential oil and thymol have antifungal activity against a number of fungi including Cryptococcus neoformans, Aspergillus, Saprolegnia, and Zygorhynchus species (Vollon and Chaumont, 1994; Perrucci et al., 1995; Pasteur N et al., 1995; Tantaouielaraki and Errifi, 1994). Both essential oil and thymol have antibacterial activity against Salmonella typhimurium, Staphylococcus aureus, Escherichia coli and a number of other bacterial species (Janssen et al., 1987; Juven et al., 1994). As an antibiotic, thymol is 25 times more effective than phenol, but less toxic (Czygan, 1989). The relaxant effect of this plant on tracheal and ileal smooth muscle has been shown (Meister et al., 1999; Boskabady et al., 2006; Babaei et al., 2008). The relaxant effect of other plants of this family on tracheal and ileal smooth muscle of guinea pigs has also been reported previously (Reiter and Brandt, 1985).

Therefore, in the present study, the relaxant effects of n-hexane, dichloromethane, methanol and aqueous fractions

*Corresponding author: M.H. Boskabady, M.D., Ph.D., Dept. of Physiology, Medical School, Mashhad, Post Code 9177948564, Iran, Fax: 0098 511 8828564, Phone: 0098 511 8828565. E-mail: boskabadymh@mums.ac.ir, mhboskabady@hotmail.com

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of *T. vulgaris* on tracheal chains of guinea pigs were examined. These fractions are different ingredients of the plant that have not been characterized in terms of composition.

MATERIALS AND METHODS

Plant and fractions

T. vulgaris was collected from the Tabas mountains (centre east region of Iran) and identified by botanists at Ferdowsi University in the city of Mashhad. A voucher specimen was preserved in the Herbarium of the School of Agriculture, Ferdowsi University (Herbarium No: 153-2613-2). Four fractions were prepared as follows (Fig. 1): 300 ml n-hexane was added to two hundred grams of chopped, dried seeds and the solution was kept at room temperature for 48 hours. The solution was then separated by maceration and dried by a rotary evaporator at 50ºC under reduced pressure. The same as above, dichloromethane, methanol and distilled water were respectively added to the remaining powder and extractions were carried out by maceration at room temperature for 48 hours. Finally, four different fractions including n-hexane, dichloromethane, methanol and water extracts were obtained. The 40 g% solutions were prepared for all fractions by adding distilled water to dried fractions.

Tissue preparation

Guinea pigs (400-700 g, both sexes) were killed by a blow on the neck and tracheas were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain (Holroyde, 1986). Tissue was then suspended in a 20 ml organ bath (Schuler organ bath type 809, March- Hugstetten, Germany) containing Krebs-Henseliet solution of the following composition (mM): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11.

The Krebs solution was maintained at 37ºC and gassed with 95% O_2 and 5% CO_2. Tissue was suspended under an isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with Krebs solution every 15 min.

Protocols

We examined the relaxant effects of four cumulative concentrations (0.4, 0.8, 1.2 and 1.6 g%) of n-hexane, dichloromethane, methanol and aqueous fractions of *T. vulgaris* and theophylline anhydrous (Sigma Chemical Ltd, UK) (0.2, 0.4, 0.6 and 0.8 mM) as positive control, and saline (1 ml) as negative control. To produce the first concentration of each fraction, 0.2 ml of 40 g% was added to a 20 ml organ bath and for other three concentrations; 0.2 ml of 40 g% was added to the organ bath respectively three times. For theophylline, 0.2 ml of 20 mM concentrated solution was added to the organ bath 4 times. The consecutive volumes were added to the organ bath at five minutes intervals.

In each experiment, the effect of four cumulative concentrations from each fraction, theophylline or saline on contracted tracheal smooth muscle was measured after exposing tissue to each concentration of the solution for 5 min. An increase in the length of tracheal chain was considered to be a relaxant (bronchodilatory) effect and expressed as a positive percentage change in proportion to the maximum contraction. A decrease in length of tracheal chain was considered as a contractile (bronchoconstrictory) effect, which was expressed as a negative percentage change (Holroyde, 1986).

The relaxant effect of different solutions was tested with two different experimental designs, (n= 7 for each group) as follows:

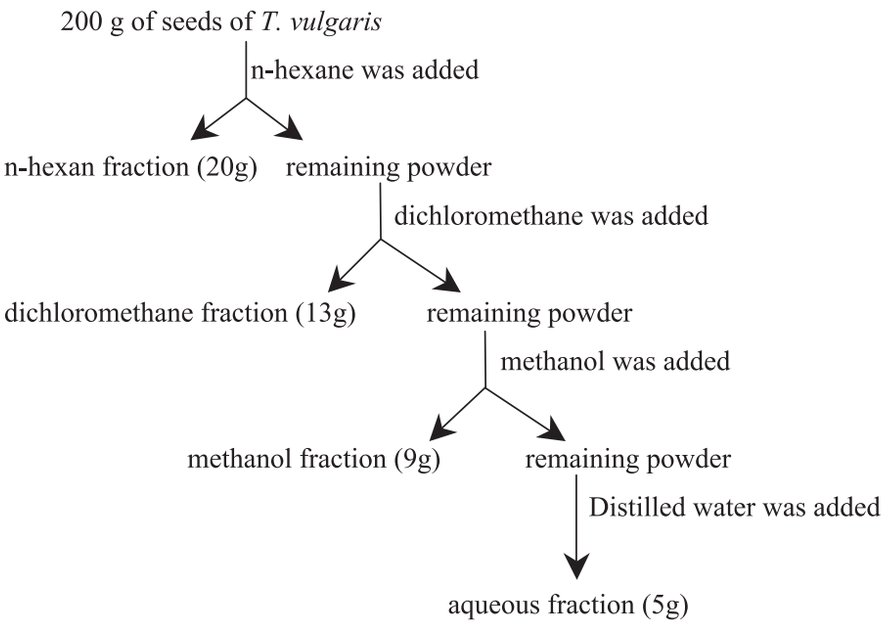

Figure 1: Separation of different fractions of the seeds of Tymus vulgaris. The numbers in grams refer to dried yield fraction.

1. On tracheal chains contracted by 60 mM KCl (group 1 experiment).
2. On tracheal chains contracted by 10 µM methacholine hydrochloride (Sigma Chemical Ltd, UK), (group 2 experiment).

The relaxant effects in two groups of experiments were examined in two different series of tracheal chains. All of the experiments were performed randomly with a 1 h resting period for tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution. In all experiments, responses were amplified with an amplifier (ML/118 quadribridge amp, March- Hugstetten, Germany) and recorded on power lab (ML-750, 4 channel recorder, March-Hugstetten, Germany).

Statistical analysis

All data were expressed as mean±SEM. Data of relaxant effects of different concentrations of each fraction were compared to the results of negative and positive controls using paired t-test. The data of relaxant effects obtained in two groups of experiments were compared using the unpaired t-test. The relaxant effects of different concentrations of four different fractions were compared to each other using ANOVA. The relaxant effect of different concentrations of four fractions and theophylline were related to their concentrations using least square regression. Significance was accepted at $p<0.05$.

RESULTS

Relaxant (bronchodilatory) effect

In group 1 experiments, all concentrations of theophylline and n-hexane fraction, three last concentrations (0.8, 1.2 and 1.6 g%) of dichloromethane and two higher concentrations (1.2 and 1.6 g%) of methanol fractions showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$). However, all concentrations of aqueous fraction showed contractile effects compared to that of saline in this group which was statistically significant for three last concentrations (0.8, 1.2 and 1.6 g%), ($p<0.05$), (Fig. 2).

In group 2 experiments, all concentrations of theophylline and n-hexane and dichloromethane fractions, three last concentrations (0.8, 1.2 and 1.6 g%) of methanol fraction and two higher concentrations of aqueous fraction (1.2 and 1.6 g%) showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$), (Fig. 3).

Comparison of the relaxant effect of theophylline with different fractions

In both groups 1 and 2, the relaxant effect of all concentrations of dichloromethane, methanol and aqueous fractions were significantly less than those of theophylline ($p<0.05$ to $p<0.001$). The relaxant effect of n-hexane fraction was significantly higher than that of theophylline in group 2 ($p<0.01$ to $p<0.05$), (Fig. 2 and 3).

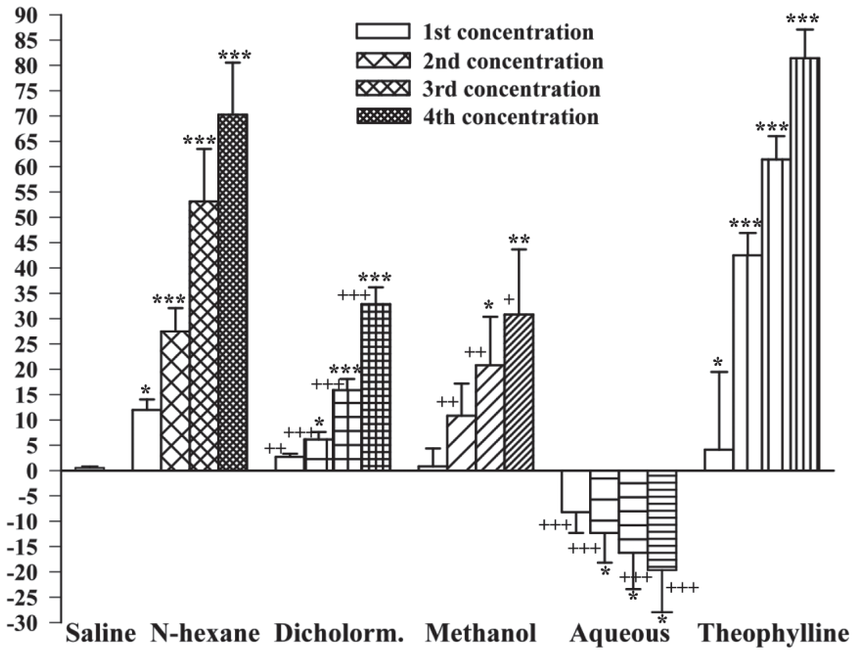

Figure 2: Relaxant effect (mean±SEM) of four different fractions (n-hexane, dichloromethane, methanol and aqueous) from *T. vulgaris* in comparison with negative control (saline) and positive control (theophylline) in Group 1 experiments (contracted tracheal chains by 60 mM KCl, n=7)**.** The four different concentrations for fractions were 0.4, 0.8, 1.2 and 1.6 g%, and for theophylline, 0.2, 0.4. 0.6 and 0.8 mM. Dichlorom.: dichloromethane. Statistical differences between the effect of fractions and theophylline with that of saline; *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$. Statistical differences between the effect of fractions and theophylline; +: $p<0.05$, ++: $p<0.01$ and +++: $p<0.001$.

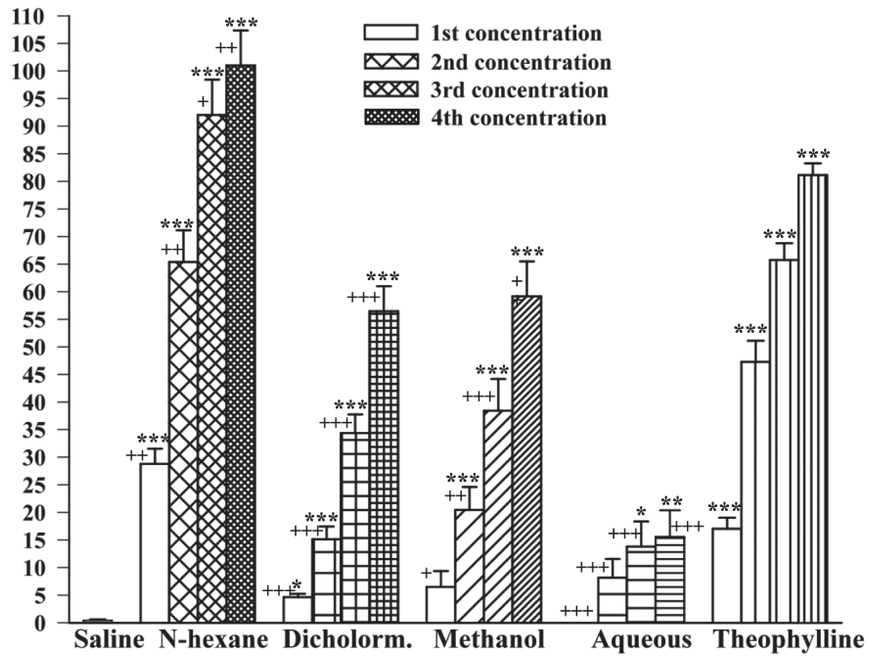

Figure 3: Relaxant effect (mean±SEM) of four different fractions (n-hexane, dichloromethane, methanol and aqueous) from *T. vulgaris* in comparison to negative control (saline) and positive control (theophylline) in Group 2 experiments (contracted tracheal chains by 10 mM methacholie, n=7)**.** The four different concentrations for fractions were 0.4, 0.8, 1.2 and 1.6 g%, and for theophylline, 0.2, 0.4. 0.6 and 0.8 mM. Dichlorom.: dichloromethane. Statistical differences between the effect of fractions and theophylline with that of saline; *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$. Statistical differences between the effect of fractions and theophylline; +: $p<0.05$, ++: $p<0.01$ and +++: $p<0.001$.

Comparison of the relaxant effect of different fractions

In both groups, the relaxant effects of all concentrations of the n-hexan fraction were significantly greater than those of others ($p<0.01$ to $p<0.001$). In addition, the relaxant effects of all concentrations of aqueous fractions were significantly lower than those of other fractions in both groups ($p<0.05$ to $p<0.001$).

Comparison of the relaxant effect between two groups of experiments

The relaxant effect of all concentrations of the n-hexane fraction and the three last concentrations (0.8, 1.2 and 1.6 g%) of dichloromethane and aqueous fractions were significantly greater in group 2 than in group 1 experiments ($p<0.01$ to $p<0.001$). However, there was no significant difference in the relaxant effect of different concentrations of the theophylline and methanol fraction between two groups (Fig. 4).

Correlation between concentrations of solutions and their relaxant effect

There were significant positive correlations between the relaxant effects and concentrations for theophylline and all fractions (except aqueous fraction in group 1) in both groups ($p<0.05$ to $p<0.001$). However, the correlation between the relaxant effects and concentrations for aqueous fraction in group 1 was not significant (Table 1).

DISCUSSION

In this study, the relaxant effects of four different fractions of *T. vulgaris* in comparison to saline as negative control and theophylline as positive control were studied. In group 1 experiment (contracted tracheal chains by KCl), all fractions (except aqueous fraction) and theophylline showed relaxant effect on tracheal smooth muscle. The relaxant effects of dichloromethane and methanol fractions were significantly less than that of theophylline. However almost, all fractions of *T. vulgaris* showed relatively potent relaxant effects compared with the effect of saline. In group 2 experiments, the relaxant effects of dichloromethane, methanol and aqueous fractions were significantly lower than that of theophylline but n-hexane fraction showed a relaxant effect higher than that of theophylline. Theophylline is a non-specific relaxant drug for airways with different possible mechanisms including inhibition of phosphodiesterase to increase intracellular cAMP levels (Rabe et al. 1995) and nonselective adenosine receptor antagonist effect (Daly et al. 1987). Therefore, it was used as a positive control in the present study. There were positive correlations between concentrations and the relaxant effects of all fractions in both groups of experiments. The contractions obtained by 10 µM MC and 60 mM KCl were 70% of maximum contraction by the corresponding substance. Therefore, the comparison of the relaxant effects of different solutions in pre-contracted tracheal muscle by two substances is valid.

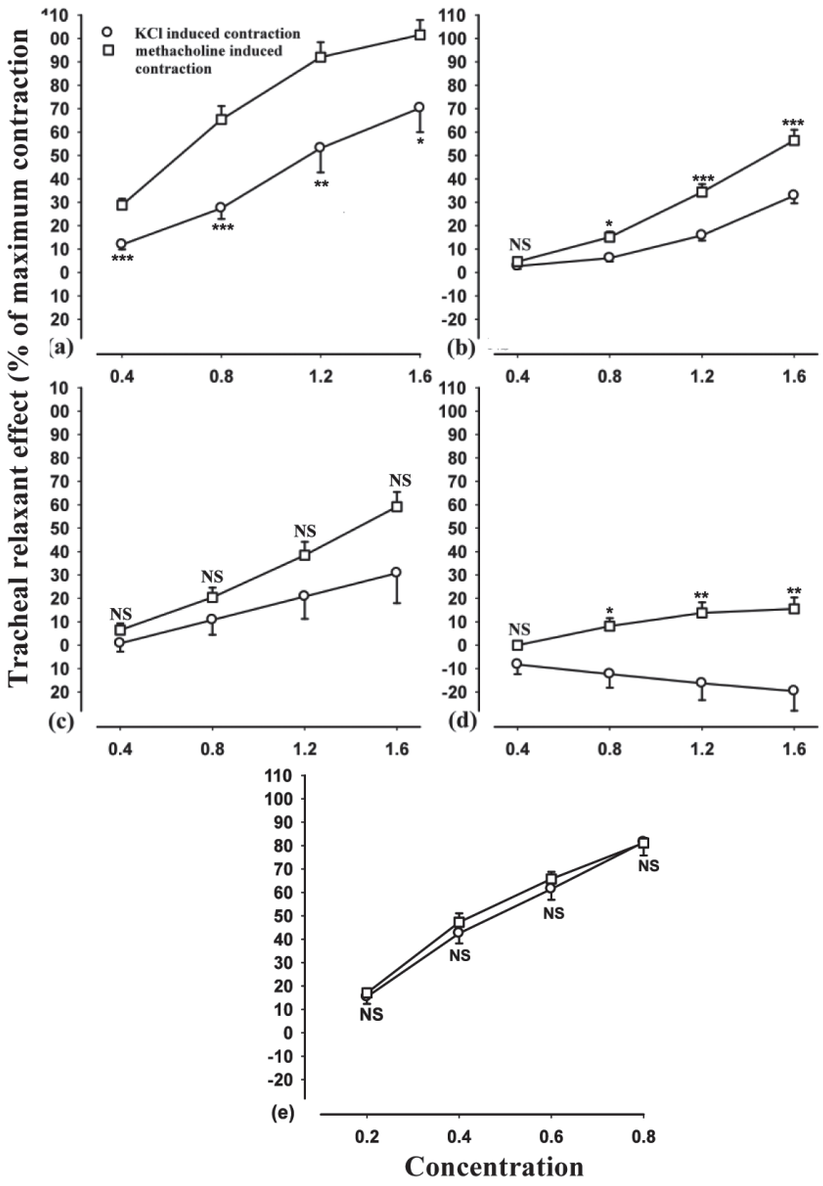

Figure 4: Concentration response curves of the relaxant effect of n-hexane (a), dichloromethane (b), methanol (c) and aqueous fractions (d) from *T. vulgaris* and theophylline (e) in two groups of experiments. Group 1: KCl induced contraction of tracheal chains (o) and Group 2: methacholine induced contraction of tracheal chains, (n=7 for each groups). Concentrations for fractions were 0.4, 0.8, 1.2 and 1.6 g%, and for theophylline, 0.2, 0.4. 0.6 and 0.8 mM. Statistical differences in the relaxant effect of different concentrations of each fraction of Group 1 and those of Group 2; NS: non-significant difference, *; p<0.05, **; p<0.01, ***; p<0.001. The concentration unit for fractions was g%, and for theophylline, mM.

TABLE 1
Correlation (r) among the relaxant effects of four different fractions (n-hexane, dichloromethane, methanol and aqueous) from *T. vulgaris* and theophylline with their concentrations in two groups of experiments

Different solutions	n-hexane fraction		Dichloromethane fraction		Methanol fraction		Aqueous fraction		Theophylline	
	R	P value	R	P value	r	P value	R	P value	r	P value
Group 1	0.762	p<0.001	0.869	p<0.001	0.460	p<0.05	-0.257	NS	0.908	p<0.001
Group 2	0.876	p<0.001	0.920	p<0.001	0.849	p<0.001	0.534	p<0.01	0.947	p<0.001

The results of this study confirmed those of Meister et al. (1999), Boskabady et al. (2006), Babaei et al. (2008) and Reiter and Brandt (1985) indicating relaxant effect of this plant on tracheal and ileal smooth muscles. In our previous study, the relaxant effect of macerated and aqueous (Soxhlet extracted) extracts of the plant was studied (Boskabady et al. 2006). However, the present work studied the effect of four different fractions of the plant that were different in composition. The results of the present study showed that n-hexane is the main fraction of the plant responsible for the relaxant effect of the plant and aqueous fraction showed the least relaxant effect. Therefore, the results showed that mainly lipid soluble ingredients of the plant are responsible for its relaxant effect.

The spasmolytic and antitussive activity of thyme has been most often attributed to its constituents: thymol and carvacrol, which make up a large percentage of the volatile oil (Meister et al., 1999). Although these compounds have been shown to prevent contractions induced in the ileum and the trachea of the guinea pig, by histamine, acetylcholine and other reagents, the concentration of phenolics in aqueous preparations of the drug is insufficient to account for this activity (Van Den Broucke, 1980; Van Den Broucke and Lemli, 1981). Our previous study (Boskabady and Jandaghi, 2003) showed a potent effect for carvacol, which is one of the constituents of *T. vulgaris.* Therefore, the carvacol content of the plant may be responsible for its relaxant effect on tracheal chains. Experimental evidence also suggests that the in vitro spasmolytic activity of thyme preparations is due to the presence of polymethoxy flavones (Van Den Broucke and Lemli, 1983). In vitro studies have shown that flavones and thyme extracts inhibit responses to agonists of specific receptors such as acetylcholine, histamine and L-norepinephrine, as well as agents whose actions do not require specific receptors, such as barium chloride. The flavones of thyme were found to act as noncompetitive and non-specific antagonists. They were also shown to be Ca_2-antagonists and musculotropic agents that act directly on smooth muscle (Van Den Broucke and Lemli, 1983).

The relaxant effect of the plant is not due to its main constituent, thymol because another study we conducted failed to show any relaxant effect for thymol on tracheal chains of guinea pig (Boskabady et al., 1998).

Methacholine caused contraction by stimulating muscarinic receptors. However, KCl causes depolarisation of the muscle fibbers leading to increase of sarcopelasmic calcium concentration and contraction. Therefore, the results of the present study may indicate that the relaxant effect of different fractions from *T. vulgaris* on tracheal chains of guinea pigs might be produced due to their inhibitory effect on muscarinic receptors because, the relaxant effect on methacholine induced contraction was greater than KCl induced contraction. In addition, the relaxant effect of muscarinic receptors has been documented previously (Leonards et al., 1992). However, due to the existence of relaxant effect of relaxant effect of n-hexane, dichloromethane and methanol fraction on KCl induced contraction of tracheal muscle (group 1); these fractions may have inhibitory effect on calcium channels and/or opening effect on potassium channels. In fact, the relaxant effect of potassium channels openers (Buckle et al., 1993; Perez-Guerrero et al., 1997) and calcium channel blockers (Miyahara et al., 1993; McCaig and DeJonckeere, 1993) has been shown. In addition, it is well known that KCl can affect calcium channels. However, with regard to the potent relaxant effect of β-adrenergic receptors (Lronards et al., 1992; Buckle et al., 1993), stimulator effect of fractions of the plant on β_2-adrenoceptors could not be excluded by the results of this study. Therefore, more studies are required to reveal the different therapeutic effects, effective substance(s), and extract mechanism(s) of relaxant effect of *T. vulgaris.*

A secretomotoric activity has also been suggested for thyme oil, which has been associated with a saponin extract from *T. vulgaris* (Vollmer, 1932). Stimulation of ciliary movements in the pharynx mucosa of frogs treated with diluted solutions of thyme oil, thymol or carvacrol has also been reported (Freytag, 1933). Furthermore, an increase in mucus secretion of the bronchi after treatment with thyme extracts has been observed (Schilf, 1932). Muller-Limmroth and Frohlich (1980) have suggested a protective effect on mucous layers in the hypopharynx and spasmolytic, secretolytic and bactericide effects. In contrast, Lemiere et al (1996) have reported the occupational asthma due to the exposure of subjects to thyme. The antioxidant effects of this plant have been also shown. Therefore, this plant may have theraputic effect on respiatory diseases (Haraguchi et al., 1996). It was also shown that the thyme extract may help in the treatment of diseases related to endothelin hyper-reagibility of the bronchus system, such as asthma and COPD (chronic obstructive pulmonary disease), but thymol is not involved in this effect (Engelbertz et al., 2008).

In the present study, 4 concentrations of each fractions and positive control (theophylline) were used to evaluate the concentration dependency of the relaxant effect of each solution. A complete dose-response curve, achievement of Emax and measurement of EC_{50} values were only valid for a pure substance that affects a certain receptor. For an unknown solution such as fractions used in the present study the examination of the effect of few concentrations is valid. In fact, in further studies the pure effective substance of each fraction needs to be identified and its complete dose-response curve, Emax and EC_{50} should be measured. The aim of the present study was to evaluate the relaxant effect of different fractions of *T. vulgaris*. However, the exact mechanism(s) of the relaxant effects of different fractions and their effective substances such as the effect of different fractions on calcium channels should be examined in further studies.

As is made clear in the Method section (Plant and fractions), all fractions were dried (the solvents were removed). Therefore, the effects of the vehicles n-hexane, dichloromethane and methanol were not contributed in the observed relaxant effects.

Nonploar substances, lipids and essential oil are extracted in n-hexane fraction, polar terfenadines is extracted in dichlormethan fraction, glycosides and saponins are extracted in methanol fraction and carbohydrates and solutes are extracted in aqueous fraction. Considering the best effect of volatile oil-containing n-hexane fraction, the observed relaxant activity may be attributed to mainly the non-polar constituents of the plant such as carvacrol and thymol. In fact, the relatively potent relaxant effect of carvacrol on tracheal chains was observed in our previous study, which supports the findings of the present study (Boskabady and Jandaghi, 2003). However, the relaxant effects of other fractions, especially dichlormethan and methanol fractions indicated that other ingredients of the plant also contributed to its relaxant effect on tracheal smooth muscle.

In conclusion, the results of this study indicate a potent relaxant effect for n-hexane and weaker relaxant effect for other fractions from *T. vulgaris* on tracheal chains of guinea pigs. The weakest relaxant effect was seen for aquous fractions.

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REFERENCES

BABAEI M, ABARGHOEI ME, ANSARI R, VAFAEI AA, TAHERIAN AA, AKHAVAN MM, TOUSSY G, MOUSAVI S (2008) Antispasmodic effect of hydroalcoholic extract of Thymus vulgaris on the guinea-pig ileum. Nat Prod Res 22: 1143-1150.

BARANAUSKIENE R, VENSKUTONIS PR,VISKELIS P, DAMBRAUSKIENE E (2003). Influence of nitrogen fertilizers on the yield and composition of thyme (Thymus vulgaris). J Agri Food Chem 51: 7751–7758.

BOSKABADY MH, ASLANI MR, KIANI S (2006) Relaxant effects of *Tymus volgari* on guinea pig tracheal chains and its possible mechanism(s). Phytother Res 20: 28-33.

BOSKABADY MH, JANDAGHI P (2003) Relaxant effect of carvacrol on guinea pig tracheal chainsand its possible mechanisms. Pharmazie 58: 661-663.

BOSKABADY MH, RAKHSHANDAH H, MOETAMEDSHARIATI V (1998) Bronchodilatory and anticholinergic effects of *Carum copticum* on isolated guinea pig tracheal chains. Med J I R Iran 11: 329-334.

BUCKLE DR, ARCH JRS, BOERING NE, FOSTER KA, TAYLOR JF, TAYLOR SG, SHAW DJ (1993) Relaxation effect of potassium channel activators BRL 38227 and Pinacidil on guinea pig and human airway smooth muscle, and blockade of their effects by libenclamide and BRL 31660. Pulmon Pharmacol 6: 77-86.

CZYGAN C-F (1989) Thymian, Thymi Herba. In: Wichtl M. ed. Teedrogen, 2nd ed. Stuttgart, Wissenschaftliche Verlagsgesellschaft: 498–500.

DALY JW, JACOBSON KA, UKENA D. (1987). Adenosine receptors: development of selective agonists and antagonists. Prog Clin Biol Res. 230: 41-63.

ENGELBERTZ J, SCHWENK T, KINZINGER U, SCHIERSTEDT D, VERSPOHL EJ (2008) Thyme extract, but not thymol, inhibits endothelin-induced contractions of isolated rat trachea. Planta Med 74: 1436-1440.

ESCOP (1997) "Thymi herba." Monographs on the Medicinal Uses of Plant Drugs. Exeter, U.K.: European Scientific Cooperative on Phytotherapy.

FREYTAG A. ber den Einflu von (1933) Thymianl, Thymol und Carvacrol auf die Flimmerbewegung. Pflügers Archiv, Eur J Physiol 232: 346– 350.

HARAGUCHI H., SAITO T., ISHIKAWA H., DATE H., KATAOKA S., TAMURA Y, MIZUTANI K (1996) Antiperoxidative components in Thymus vulgaris. Planta Med 62: 217-221.

HOLROYDE MC (1986) The influence of epithelium on the responsiveness of guinea pig isolated trachea. Br J Pharmacol 87: 501-507.

JANSSEN AM, SCHEFFER JJC, BAERHEIM-SVENDSEN A (1987) Antimicrobial activity of essential oils: A1976–1986 literature review. Aspects of the test methods. Planta Med 53: 395–398.

JAVANMARDI J, KHALIGHI A, KASHI A, BAIS HP, VIVANCO JM (2002) Chemical characterization of basil (Ocimum basilicum L.) found in local accessions and used in traditional medicines in Iran. J Agri Food Chem 50: 5878–5883.

JUVEN BJ, KANNER J, SCHVED F, WEISSSLOWICZ H (1994) Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl Bacteriol 76:626–631.

LEMIERE C, CARTIER A, LEHRER SB, MALO JL (1996) Occupational asthma caused by aromatic herbs. Allergy 51: 647-649.

LEONARDS B, RAMPART M, HERMAN AG (1992) Selective M_3 muscarinic receptors inhibit smooth muscle contraction in rabit trachea without increasing the release of acetylcholine. J Pharmacol Exp Ther 263: 773-770.

MCCAIG D, DEJONCKEERE S (1993) Effect of two Ca^{2+} modulator in normal and albumin sensitised guinea pig trachea. Eur J Pharmacol 249: 53-63.

MEISTER A, BERNHARDT G, CHRISTOFFEL V, BUSCHAUER A (1999) Antispasmodic activity of Thymus vulgaris extract on the isolated guinea-pig trachea: discrimination between drug and ethanol effects. Planta Med 65: 512-516.

MIYAHARA Y, KIZAWA Y, SANO M, MURAKAMI H (1993) Effect of organic and inorganic Ca^{2+} antagonists on acetylcholine induced contraction in molluscan (Mytilus edulis) smooth muscle. Gen Pharmacol 24: 1419-1423.

MOSSA JS, AL-YAHYA MA, AL-MESHAL IA (1987) Medicinal plants of Saudi Arabia. Riyadh, Saudi Arabia, King Saud University Libraries 1.

MULLER-LIMMROTH W, FROHLICH HH (1980) Effect of various phytotherapeutic expectorants on mucociliary transport. Fortschr Med 98: 95-101.

PASTEUR N, MENASHEROV M, RAVID U, JUVEN B (1995) Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. J food protection 58: 81– 85.

PEREZ-GUERRERO C, SUAREZ J, HERRERA MD, MARHUNNDA E (1997) Spasmolytic effect of tetrazepam on rat duodenum and guinea pig ileum. Pharmacol Res 35: 493-494.

PERRUCCI S CECCHINI S, PRETTI C, VARRIALE COGNETTI AM, MACCHIONI G, FLAMINI G, CIONI PL (1995) In vitro antimycotic activity of some natural products against Saprolegnia ferax. Phytother Res 9: 147–149.

RABE, KF, MAGNUSSEN, H, DENT, G (1995) Theophylline and selective PDE inhibitors as bronchodilators and smooth muscle relaxants. Eur Respir J 8: 637–642.

REITER M, BRANDT W (1985) Relaxant effects on tracheal and ileal smooth muscles of the guinea pig. Arzneimittelforschung 35: 408-414.

SCHIIF F, EINFLUSS VON (1932) Azetylcholin, Adrenalin, Histamin und Thymianextrakt auf die. Bronchialschleimhautsekretion; zugleich ein Beitrag zur Messung der Bronchialschleimhautsekretion. Naunyn-Schmiedebergs Archiv für Pharmakologie 166: 22-25.

SENATORE F (1996) Influence of harvesting time on yield and composition of the essential oil of a thyme (Thymus pulegioides L.) growing wild in Campania (southern Italy). J Agri Food Chem 44: 1327–1332.

SIMON JE, MORALES MR, PHIPPEN WB,VIEIRA RF, HAO Z (1999) A source of aroma compounds and a popular culinary and ornamental herb. In J Janick (Ed.), Perspectives on new crops and new uses (pp.499–505). Alexandria,VA: ASHS Press.

STECHER PG (1968) The Merck Index: An Encyclopedia of Chemicals and Drugs, 8th ed.

Rahway, N.J.: Merck & Co., Inc.

TANTAOUIELARAKI A, ERRIFI A (1994) Antifungal activity of essential oils when associated with sodium chloride or fatty acids. Grasas-y-aceites 45: 363–369.

VAN DEN BROUCKE CO, LEMLI JA (1983) Spasmolytic activity of the flavonoids from Thymus vulgaris. Pharm Weekbl 5: 9-14.

VAN DEN BROUCKE CO, LEMLI JA (1981) Pharmalogical and chemical investigation of thyme liquid extracts. Planta Medica 41: 129-135.

VAN DEN BROUCKE CO (1980) Chemical and pharmacological investigation on Thymi herba and its liquid extracts. Planta Med 39: 253-254.

VOLLMER H (1932) Untersuchungen über Expektorantien und den Mechanismus ihrer Wirkung. 21Klinische Wochenschrift 11: 590–595.

VOLLON C, CHAUMONT JP (1994) Antifungal properties of essential oils and their main components upon Cryptococcus neoformans. Mycopathol 128: 151-153.

Biol Res 45: 75-80, 2012

Proteomic Investigation of Changes in Rat Skeletal Muscle after Exercise-Induced Fatigue

Liping-zhao[1#], Wenhui-Yan[2#], Heng-Xiang[1], Xiaoyang-wang[3*], Haixuan-qiao[2*]

[1] Department of Rehabilitation and sports medicine, Tianjin Medical University, Tianjin, China
[2] School of biomedical engineering, Tianjin Medical University, Tianjin 300070, China (E-mail: qiaohaixuan@yahoo.com.cn)
[3] Department of Physical Education, Shanxi University, Shanxi, Taiyuan 030006, (E-mail:wangxy1212@163.com)

ABSTRACT

The mechanisms of exercise-induced fatigue have not been investigated using proteomic techniques, an approach that could improve our understanding and generate novel information regarding the effects of exercise. In this study, the proteom alterations of rat skeletal muscle were investigated during exercise-induced fatigue. The proteins were extracted from the skeletal muscle of SD rat thigh, and then analyzed by two-dimensional electrophoresis and PDQuest software. Compared to control samples, 10 significantly altered proteins were found in exercise samples, two of them were upregulated and eight of them were downregulated. These proteins were identified by MALDI TOF-MS. The two upregulated proteins were identified as MLC1 and myosin L2 (DTNB) regulatory light-chain precursors. The eight decreased proteins are Glyceraldehyde-3-phosphate Dehydrogenas (GAPDH); Beta enolase; Creatine kinase M chain (M-CK); ATP-AMP Transphosphorylase (AK1); myosin heavy chain (MHC); actin; Troponin I, fast-skeletal muscle (Troponin I fast-twitch isoform), fsTnI; Troponin T, fast-skeletal muscle isoforms (TnTF). In these proteins, four of the eight decreased proteins are related directly or indirectly to exercise induced fatigue. The other proteins represent diverse sets of proteins including enzymyes related to energy metabolism, skeletal muscle fabric protein and protein with unknown functions. They did not exhibit evident relationship with exercise-induced fatigue. Whereas the two identified increased proteins exhibit evident relationship with fatigue. These findings will help in understanding the mechanisms involved in exercise-induced fatigue.

Key words: Proteomics, exercise-induced fatigue, skeletal muscle, rat

INTRODUCTION

Exercise-induced fatigue is a reduction in maximal voluntary muscle force that results from intense physical activity (Gandevia. S. C. 2001). Exercise-induced fatigue is a complex process. In the course of exercise the physiological environment changes, which could result in the alteration of gene and protein expression levels and the alteration of posttranslational modifications. Importantly, intense and prolonged exercise can result in changes in cell signaling pathways in eukaryotic cells (Reid MB. 2008; Powers SK et al. 2008). Thus, the proteome profile of skeletal muscles during exercise-induced fatigue is distinct from that of the normal physiological state. Although many proteins associated with exercise have been identified (Adam J et al. 2009; Borja Guerra et al, 2010; Craig A. Goodman et al. 2009), the molecular mechanisms involved in exercise-induced fatigue have not been well elucidated. Investigation into the alterations of proteins in exercise-induced fatigue will help to discover mechanisms, pathways and new players involved in the induction and regulation of fatigue.

Proteomics is the study of protein properties (expression level, posttranslational modification, interactions, etc.) on a large scale to obtain a more global view of physiological processes. The application of proteomics to investigate exercise is in its infancy (D.S. Hittel et al. 2007), and few studies have been published in this area (Jatin G. Burniston 2008; Yamaguchi W et al. 2010). in order to understand the mechanisms of exercise-induced fatigue and search for new proteins involved in it. In the present study, an exercise-induced fatigue model of rat SD was built using swimming. Biochemical and behavioral indices were used to verify the credibility of the model. After the modeling, the skeletal muscle of rat SD thigh was separated at the low temperature condition and the proteins were extracted.

Proteins of skeletal muscle were separated by two-dimensional (2D) gel electrophoresis. The protein patterns of scanned 2D images were analyzed with PDQuest software and 10 proteins were found to be upregulated or downregulated. These altered proteins were then identified by MALDI TOF-MS/MS and searched against the SWISS-PROT and NCBInr database. Our findings reveal novel changes in the expression of well-known metabolic enzymes and myofibrillar proteins. Some of these are related to fatigue, while others have not been confirmed as involved in the process of fatigue. Therefore, further investigation of their roles during fatigue will help to elucidate the mechanism of exercise induced fatigue.

MATERIALS AND METHODS

The model of exercise-induced fatigue

The model of exercise-induced fatigue was established as described by (Shin Terada. 2001, HOU L. 2005). Nine- to

Note: Liping-zhao and Wenhui-Yan contributed equally to this paper.

*Corresponding author: Dr. Haixuan-Qiao· School of biomedical engineering, Tianjin Medical University, Tianjin 300070, China (E-mail: qiaohaixuan@yahoo.com.cn) or Xiao Yang-Wang Departament of Physical Education, Shanxi University, Shanxi, Taiyuan 030006, China (E-mail: Wangxy122@163.com)

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ten-week-old male Sprague-Dawley rats with initial body weights of 200±20g were used for this study. Sixteen male SD rats were randomly divided into exercise and rest groups according to weight. In the exercise group, the rats repeated 30-minute swimming bouts daily for the first 2 days. This was increased by 30 min each day for the next 3 days, reaching 120 minutes of swimming by the fifth day. This was increased to 180 minutes for the sixth and seventh days. For the final three days the exercise was doubled by introduced 180-minute swimming bouts interspaced with six hours for rest. (Table 1). All rats swam in a barrel filled to a depth of 80cm and an average surface area of 190 cm2/rat. Water temperature was maintained at 35°C during swimming training. Changes in body weight and the biochemical index blood urea nitrogen (BUN) were used to verify the credibility of the model.

Preparation of skeletal muscle proteins

The rats from the exercise and control groups were killed by decollation, proteins were extracted from the skeletal muscle of SD rat thigh at a low temperature and dissolved in 9 mol/L urea, 2% CHAPS (Sigma), Pharmalyte pH 3-10 (Amersham Pharmacia), 1% DTT, and then separated from insoluble material by centrifugation. The total protein concentration was determined by the Bradford assay (Kruger N J et al. 1996).

2-Dimensional gel electrophoresis and image analysis

Two-dimensional gel electrophoresis (2DE) was carried out in the IPGphor system (BioRad) as described (Gorg et al. 1998). Isoelectric focusing (IEF) of 500μg samples was performed in pre-cast immobilized pH gradient (IPG) strips (pH3–10, L=18cm; Pharmacia) using the following rehydration and running conditions: rehydration 14hours, step 1: 250V, 30min, step 2: 1 000V, 1 hour, step3: 1 000–10 000V, 5–6hours , step 4: 10 000V, 60 000Vh. After IEF separation, the extruded strips were incubated in buffer 1 (50 mmol Tris-HCl, pH 8.18, 6 mol urea, 30 % glycerol, 2 % SDS, 015 %DTT) and buffer 2 (50 mmol/L Tris-HCl, pH 8.18, 6mol/L urea, 30% glycerol, 2% SDS, 4.5% iodoacetamide) for 15 min, respectively. The equilibrated gels were immediately applied to 12 % polyacrylamide slab gels for second separation. Gel electrophoresis was carried out at a constant current (30mA/gel) and a set temperature of 20ºC. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue. Analysis of the gels, including background subtraction, spot detection, volume normalization and differences in protein expression levels among samples were analyzed by using PDQuest software (version 6.0, BioRad), To determine the variation, 3 gels were prepared for each sample. The protein spots that varied >2 fold changes and were specific for the test groups and the control group were manually labeled and considered for MS analysis.

In-gel digestion and mass spectrometric identification

Gel spots were excised from the stained gel, and cut to 1–2mm^2 slices. The excised pieces were destained with 25 mmol ammonium bicarbonate/50 % acetonitrile (Fisher), and were washed with 100% acetonitrile, and then dried with a SpeedVac Plus SC110A vacuum concentrator (Savant, Holbook, NY, USA). Trypsin in digestion buffer (25 mmol ammonium bicarbonate P5 mmolPL calcium chloride) was added to the dry gel pieces and incubated for 15 hours for rehydration at 37ºC.The resulting peptide mixture was extracted with 5% TFA for 1 hour at 40ºC and followed by 2.5% TFA/50% acetonitrile for 1 hour at 30ºC. The supernatants were pooled and the peptides were dried by a vacuum concentrator. Peptide sequences were identified using matrix assisted laser desorption Pionization time of flight mass spectrometry (MALDI-TOF MS). Prior to mass spectrometry, the peptide samples were reconstituted with 0.5% TFA. Peptide mass mapping of tryptic digests was carried out with a Micromass Tof Spec MALDI-TOF mass spectrometer. The datasets of the MS spectra, including peptide sequence information, were searched against the SWISS-PROT (GeneBio, Geneva, Switzerland) and NCBInr database using Mascot Daemon (Matrix Science, London, UK) as a client attached to the Mascot search protocol.

Immunoblot analysis

The proteins were extracted from the skeletal muscle of SD rat thigh and lysed in a buffer (0.5 M Tris HCl, 0.4% SDS, 20% glycerol, 1.5% BromphenolBlue, 1.0%beta-mercap toethanol) by boiling for 5min. Equal amounts of protein were

TABLE 1
Sswimming periods per day over the ten day experiment

	SWIMMING PERIODS
First day	30min
Second day	30min
Third day	60min
Fourth day	90min
Fifth day	120min
Sixth day	180min
Seventh day	180min
Eighth day	180min, two times, The interval between the two times measures 6 hours
Ninth day	180min, two times, The interval between the two times measures 6 hours
Tenth day	180min, two times, The interval between the two times measures 6 hours,

subjected to 12% SDS gel electrophoresis and transferred to polyvinylidenediuoride membranes (AmershamBiosciences, Piscataway, NJ, USA). Membranes were probed with antibodies as indicated below and detected using ECL Advance or Plus (Amer-sham Biosciences). The blots were probed with the following antibodies, Rabbit anti-Actin (1:1000, cell signal), Rabbit anti-alpha Tubulin (1:1000), anti-rabbit HRP-conjugated secondary antibody (1:5000), and polyclonal anti-GAPDH antibody (1:500) (Santa Cruz, CA, USA).

Statistic analysis

Protein expression data for control and treated groups are expressed as the mean±SD of 3 replicate gels for fold changes of normalized spot volumes. For the statistic analysis of data, the Student-t-test was used, with $p<0.05$ considered as significant.

RESULT

Effect of swimming on body weight and BUN of rats

After ten days of loading-increasing swimming exercise, the body weight of the exercise group had decreased significantly and the BUN had increased significantly compared to the control groups (Fig 1, Fig 2). These changes of behavioral and biochemical index showed that the rats were in the condition of fatigue. The model was suitable for the use of the experiments about exercise-induced fatigue.

Protein expression profile

To investigate the potential proteomic alterations of rat skeletal muscle during exercise-induced fatigue, the proteins were extracted from the skeletal muscle of SD rat thigh and separated by two dimensional gel electrophoresis, The muscles were stained with Coomassie Brilliant Blue and the data was analyzed by PDQuest software (Fig 3).

Figure 3 shows a pair of Coomassie Brilliant Blue stained gels obtained from rat skeletal muscle proteins during exercise-induced fatigue. The distributing modes of overall skeletal muscle proteins were very similar to each other, the range of pI was 4.0–9.0, and the Mr range was 20–100 kD. The overall protein profiles in the control and exercise groups were compared by image analysis. Using pH 3–10 stripes, computer assisted analysis of the gels detected about 400 spots. Compared to control samples, ten proteins were apparently upregulated or downregulated in treated rats, determined by two dimensional gel electrophoresis and PDQuest software analysis. These proteins exhibited >2 fold changes between the exercise and control group. Two of them were upregulated and eight of them were down regulated.

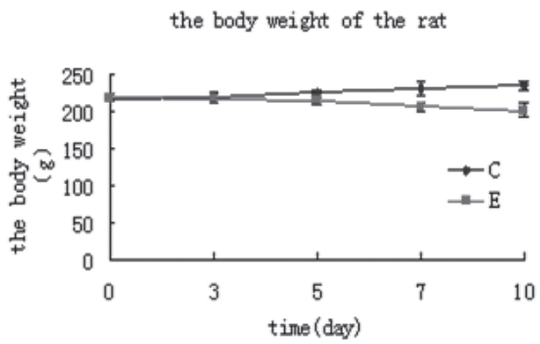

Fig. 1: The changes of the rat body weight in the course of exercise (C: contral group, E exercise group)

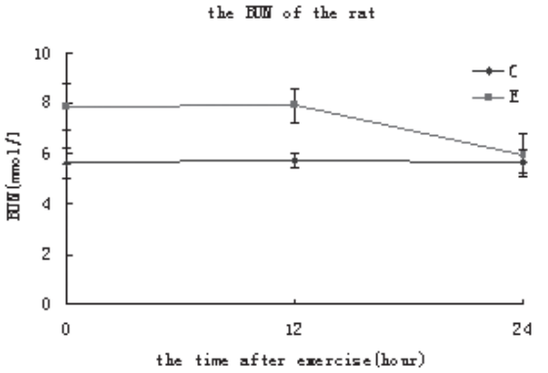

Fig. 2: The changes of the BUN concentration of rat after exercise (C: contral group, E exercise group)

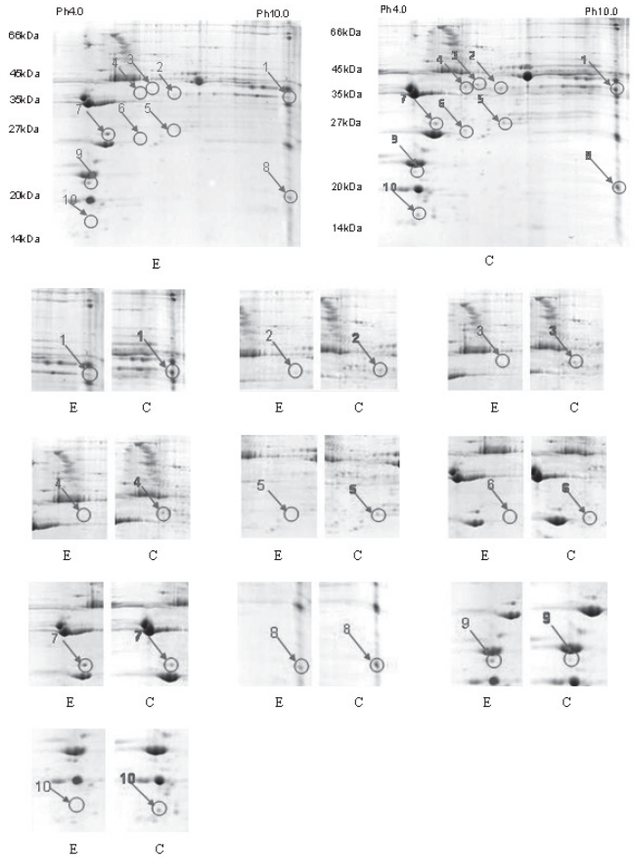

Fig. 3: 2-D map of SD rat thigh (E: exercise group, C: control groups), number1 to number 10 indicate the up-regulated or down-regulated proteins in exercise group.

These proteins were identified by MALDI-TOF MS and the Mascot search program. Two of the upregulated proteins were identified as myosin L2 (DTNB) regulatory light chain precursor (pI 4.82, Mr 19kD), and Myosin light chain 1 (MLC1, pI 5.03, Mr 22kD).

The eight downregulated proteins were identified as Beta enolase (pI 7.74, Mr 46.8 kD); Glyceraldehyde-3-phosphate Dehydrogenas (GAPDH, pI 8.44, Mr 35.8kD); Creatine kinase M chain (M-CK, pI 6.58, Mr 43kD); Adenylate kinase isoenzyme 1, ATP-AMP Transphosphorylase (AK1, pI 7.71, Mr 21.6 kD); Myokinase; myosin heavy chain (MHC, pI 5.31, Mr 24 kD); actin (pI 5.23, Mr 42 kD); Troponin I, fast skeletal muscle (fsTnI, Troponin I, fast-twitch isoform, pI 8.86, Mr 21.2 kD); Troponin T, fast skeletal muscle isoforms (TnTF, pI 6.18, Mr 30.6kD).

The four downregulated proteins (GAPDH; Beta enolase; M-CK; ATP-AMP Transphosphorylase) are enzymes related to energy metabolism; the other four downregulated proteins, MHC; actin; fsTnI; TnTF) are muscle fabric proteins (Table 2, Table 3).

Immunoblot verification of Actin and GAPDH

Downregulation of actin and GAPDH expression was found in the muscles of the exercise group by 2-DE. Western blot analysis was then performed for these two proteins in an independent set of skeletal muscle samples. Western blot showed the consistent downregulation of actin and GAPDH in the exercise group compared to the control group, Levels of these two proteins were significantly lower in the muscle of the exercise sample compared to controls (Fig 4).

DISCUSSION

During intense activation of skeletal muscle, contractile function becomes impaired. This is known as fatigue. The cause of fatigue is probably multifactorial, but a central role is changes in high-energy phosphates (i.e. ATP and ADP). The accumulation of by-products of rapid energy metabolism has also been postulated (Westerblad H et al. 1998). Therefore, the proteome profile of skeletal muscle during exercise-induced fatigue is distinct from that of the normal physiological state. In this study, the alterations of skeletal muscle proteins during exercise-induced fatigue were analyzed by two-dimensional electrophoresis. The results showed that 10 proteins were apparently upregulated or downregulated after exercise-induced fatigue. Two of them were upregulated and eight were downregulated.

In these proteins, four of the eight decreased proteins may relate to exercise induced fatigue directly or indirectly. The

TABLE 2
Identification of differentially expressed protein spots in treated rats by mass spectrometry and database searches

Spots number	SWISS-PROT ID	Theoretical pI	Theoretical Mr(kDa)	Sequence covered	MASquot Score	Protein name
1	XP217111.1	8.44	35.8	14%	275	glyceraldehyde-3-phosphate dehydrogenase
2	XP229872.1	7.62	46.2	8%	199	beta enolase
3	P00564	6.58	43.0	8%	80	creatine kinase, M chain
4	ATRT	5.23	42.0	10%	154	Actin
5	P39069	7.71	21.6	17%	133	ATP-AMP transphosphorylase
6	P09739	6.18	30.6	6%	33	troponinT, fast skeletal muscle isoforms
7	P16409	5.03	22.0	22%	203	myosin light chain 1
8	P27768	8.86	21.2	16%	161	troponin I, fast-twitch isoform
9	MORTL2	4.82	19.0	5%	85	myosin L2 (DTNB) regulatory light chain precursor
10	AAA41656.1	5.31	24.0	5%	45	myosin heavy chain

TABLE 3
Fold changes of differentially expressed proteins in treated group compared to untreated group ($p<0.05$)

Spot no	Protein name	Fold changes
1	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	-4.87
2	beta enolase	-2.05
3	creatine kinase, M chain (M-CK)	-2.14
4	Actin	-2.27
5	ATP-AMP transphosphorylase	-2.16
6	troponinT, fast skeletal muscle isoforms (TnTF)	-1.93
7	myosin light chain 1(MLC1)	+2.73
8	troponin I, fast-twitch isoform (fsTnI)	-2.68
9	myosin L2 (DTNB) regulatory light chain precursor	+2.58
10	myosin heavy chain (MHC)	-2.47

Data represents mean±SD of 3 experiments. (+) indicates upregulation, whereas (–) indicates downregulation

other proteins represent diverse sets of proteins including enzyme related to energy metabolism, skeletal muscle protein that do not exhibit evident relationship with exercise.induced fatigue. Whereas, the two increased proteins that were identified exhibit evident relationships with fatigue.

GAPDH is a classical glycolytic protein in energy production (Anders J et al, 2000). However, recent evidence has demonstrated that mammalian GAPGH displays a number of diverse activities unrelated to its glycolytic function. These include its role in membrane fusion, RNA transport, microtubule assembly, DNA replication, DNA repair, and apoptosis (Chuang DM et al. 2005). Exercise promotes consumption of energy sources such as glycogen by mobilizing internal energy metabolism to the maximum and using and depleting the energy source. The role of GAPDH in fatigue may correlate with its energy supply for movement and transport.

Creatine kinase (CK) is a key enzyme for maintaining a constant ATP/ADP ratio during rapid energy turnover. It catalyzes phosphate exchange between the high free energy phosphates ATP and phosphocreatine (PCr) via the reaction: PCr + ADP + H+ ⇋ creatine (Cr) + ATP. In skeletal muscle there are two major forms of CK: one is found in the cytosol (M-CK) and the other is associated with mitochondria (ScCKmit). M-CK dominates in fast-twitch muscles and is considered important for energy utilization at sites of high energy turnover (LaBella J J et al. 1998), Several studies have demonstrated that the complete absence of CK activity is associated with an immediate marked decline in skeletal muscle force generation and power output during repetitive activation (Steeghs K A et al.1997; Anders J et al. 2000; Watchko J F et al.1997; Momken I et al.2005), Our result is consistent with these previous findings.

Beta enolase is a glycolytic enzyme of particular interest because it has muscle specific isoforms (Chosa E et al. 2003). In adults, subunits of the muscle isoform of enolase (b-enolase) accumulate preferentially in fast-twitch muscle fibers in which the b-isoenzyme accounts for more than 90% of total enolase. In this study, Beta enolase was found to have decreased in skeletal muscle. However, its role in fatigue remain unclear and deserves further investigation.

Actin is a globular, roughly 42-kDa protein found in all eukaryotic cells. Actin participates in many important cellular processes including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape.

Myosin, a large family of motor proteins found in eukaryotic tissues, is a thick filament-based molecule that harnesses the free energy furnished by ATP hydrolysis to perform mechanical work against actin proteins of the thin filament. The cyclic attachment and detachment of myosin with actin that generates muscle force and shortening is Ca^{2+} regulated and is responsible for actin-based motility (Vandenboom R. 2004).

MHC is a structurally bound contractile protein of the thick filaments, which has a ATPase function that could involve binding with actin. In this work, MHC and actin were found to have decreased in skeletal muscle. It is probable that during intense activation of skeletal muscle, contractile function becomes impaired, and attachment of myosin with actin, which generates muscle force, was no longer needed.

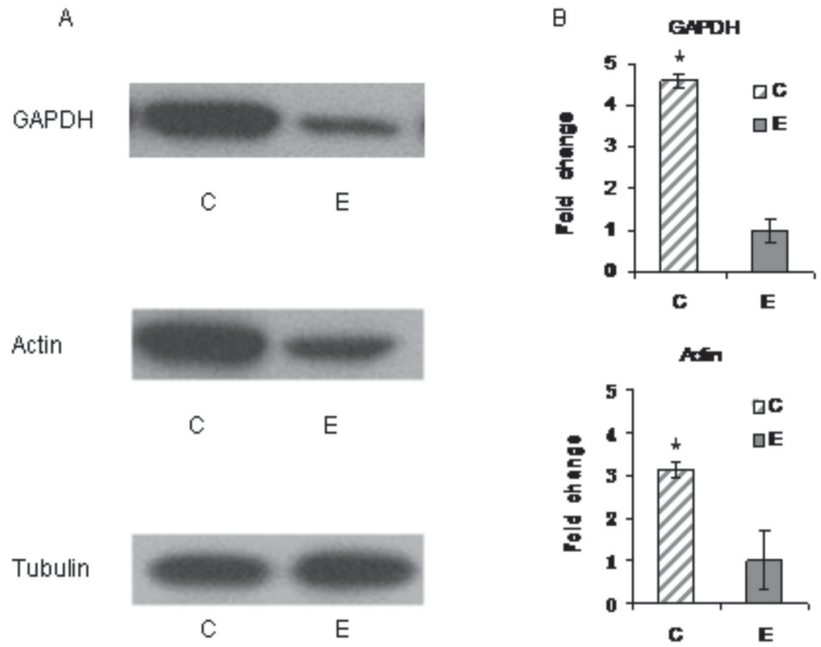

Fig. 4: (A) Westem blots showing expression of GAPDH an actin among control and treated groups. Equal loading of the samples was evaluated by reprobingmthe menbranes with tubuin antiboby. (B)Quantitative fold change. *indicates significant difference, p<0,05.

In contrast to MHC, there are only three TnI isoforms, one for slow-twitch skeletal muscle, one for fast-twitch skeletal muscle, and one for myocardium (North KN et al. 2008). Our results provide evidence that the high muscle force associated with eccentric contraction causes rapid removal of fsTnI. These findings support the concept that eccentric exercise initiates a series of events that result in rapid disruption of the cytoskeletal network and contractile apparatus, which could be the mechanism for deterioration of the contractile response and loss in force generation.

Skeletal muscle is the most important effector of exercise-induced fatigue, although several proteins have been found to be associated. However, their role in exercise-induced fatigue is not well understood. Therefore, further investigation of their role will help to elucidate the mechanism of exercise induced fatigue.

REFERENCES

ADAM J ROSE and ERIC A (2009) Richter Regulatory mechanisms of skeletal muscle protein turnover during exercisen J Appl Physiol, 106: 1702 - 1711.

ALAN H and MICHAEL J MCKENNA (2009) Dissociation between force and maximal Na+, K+-ATPase activity in rat fast-twitch skeletal muscle with fatiguing in vitro stimulation Craig A Goodman, , 105 (4), 575-583.

ANDERS J, DAHL STEDT, ABRAM KATZ, HÅKAN WESTERBLAD (2000) Is creatine kinase responsible for fatigue? Studies of isolated skeletal muscle deficient in creatine kinase. The FASEB Journa14:982-990.

BORJA G, AMELIA GG, TERESA F, et al. (2010) SIRT 1, AMP-activated protein kinase phosphorylation and downstream kinases in response to a single bout of sprint exercise: influence of glucose ingestion, 109, 4, 731-743.

BURNISTON JG (2008) Changes in the rat skeletal muscle proteome induced by moderate-intensity endurance exercise. Biochimica Biophysica Acta 1784:1077-1086.

CHOSA E, SEKIMOTO T, SONODA N, YAMAMOTO K, MATSUDA H, TAKAHAMA K, TAJIMA N (2003) Evaluation of human beta-enolase as a serum marker for exercise-induced muscle damage.Clin J Sport Med. 13(4):209-12.

CHUANG DM, HOUGH C, SENATOROV VV (2005) Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol. 45:269–90.

GORG A, POSTEL V, GUNTHER S (1988) The current state of two-dimensional electrophoresis with immobilized pH gradients Electrophoresis 9:531-546.

GANDEVIA SC (2001) Spinal and Supraspinal Factors in Human Muscle Fatigue. Physiol. Rev. 81: 1725-1789.

HITTEL DS, HATHOUT Y, HOFFMAN EP (2007) Proteomics and systems biology in exercise and sport sciences research, Exerc. Sport Sci. Rev. 35,5–11.

HOU L, LIU X, QIAO D (2005) The Establishment of Exercise-induced Fatigue Model in Rats. Laborarory Animal Science and Management. 22(1):1-3

KRUGER NJ, WALKER JM (1996) The Protein Protocols Handbook. Totowa NJ: Human Press:15-20.

KELLER A, OTT MO, LAMANDE N, LUCAS M, GROS F, BUCKINGHAM M and LAZAR M (1992) Mech Dev 38:41-54.

LABELLA JJ, DAOOD MJ, KORETSKY AP, ROMAN BB, SIECK GC (1998) Absence of myofibrillar creatine kinase and diaphragm isometric function during repetitive activation. J Appl Physiol 84:1166–1173.

MOMKEN I, LECHENE P, KOULMANN N, FORTIN D, MATEO P, DOAN BT, HOERTER J, BIGARD X, VEKSLER V, VENTURA-CLAPIER R (2005) Impaired voluntary running capacity of creatine kinase-deficient mice. J Physiol. 15;565 (Pt 3):951-64.

MICHAEL A, SIROVER (1999) New insights into an old protein: the functional diversity of mammalian glyceraldehyde 3 phosphate dehydrogenase. Biochim Biophys Acta , 1432: 159–184.

NORTH KN, LAING Skeletal muscle alpha-actin diseases. Adv Exp Med Biol. (2008); 642:15-27.

POWERS SK, JACKSON MJ (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev. 88(4):1243-76.

REID MB (2008) Free radicals and muscle fatigue: Of ROS, canaries, and the IOC. Free Radic Biol Med. 15;44(2):169-79.

STEEGHS KA, BENDERS A, OERLEMANS F, HAAN A, HEERSCHAP A, RUITENBEEK W, JOST C, VAN DJ, PERRYMAN B, PETTE D, BRUCKWILDER M, KOUDIJS J, JAP P, and WIERINGA B (1997) Altered Ca21 responses in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. Cell 89: 93–103.

TERADA S, YOKOZEKI T, KAWANAKA K, OGAWA K, HIGUCHI M, EZAKI O, and TABATA I. Effects of high-intensity swimming training on GLUT-4 and glucose transport activity in rat skeletal muscle. J Appl Physiol 90: 2019-2024, (2001).

VANDENBOOM R (2004)The myofibrillar complex and fatigue:a review.Can Applied Physiology, 29(3):330-56.

WESTERBLAD H, ALLEN DG, BRUTON JD, ANDRADE FH, LANNERGREN J (1998). Mechanisms underlying the reduction of isometric force in skeletal muscle fatigue. Acta Physio Scand 162:253-260.

WATCHKO JF, DAOOD MJ, SIECK GC, LABELLA JJ, AMEREDES BT, KORETSKY AP, and WIERINGA B (1997) Combined myofibrillar and mitochondrial creatine kinase deficiency impairs mouse diaphragm isotonic function. J Appl Physiol 82: 1416–1423.

YAMAGUCHI W, FUJIMOTO E, HIGUCHI M, TABATA I (2010) A DIGE proteomic analysis for high-intensity exercise-trained rat skeletal muscle. J Biochem. 148 (3):327-33.

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Melatonin Protects the Heart, Lungs and Kidneys from Oxidative Stress under Intermittent Hypobaric Hypoxia in Rats

Jorge G. Farías[1], Andrea B. Zepeda[1] And Gloria M. Calaf[2, 3]

[1] Facultad de Ingeniería, Departamento de Ingeniería Química, Ciencias y Administración, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.
[2] Instituto de Alta Investigación, Universidad de Tarapacá, Arica,-Chile.
[3] Center for Radiological Research, Columbia University Medical Center, New York, NY, USA.

ABSTRACT

Melatonin (N-acetyl-5-methoxytryptamine) is the main secretory product of the pineal gland in all mammals including humans, but it is also produced in other organs. It has been previously demonstrated to be a powerful organ-protective substance under oxidative stress conditions. The aim of this study was to evaluate the protective effect of melatonin in several organs such as heart, lung, kidney, and of the reproductive system, such as testis and epididymis in animals exposed to intermittent hypobaric hypoxia and therefore exposed to oxidative stress and analyzed by lipid peroxidation. Ten-week-old male Wistar rats were divided into 6 groups for 96 hours during 32 days under: 1) Normobaric conditions, 2) plus physiologic solution, 3) plus melatonin, 4) intermittent hypobaric hypoxia, 5 plus physiologic solution and 6) plus melatonin. The animals were injected with melatonin (10 mg/kg body weight) at an interval of 96 hours during 32 days. Results indicated that melatonin decreased lipid peroxidation in heart, kidneys and lung under intermittent hypobaric hypoxia conditions. However, it did not exhibit any protective effect in liver, testis, epididymis and sperm count.

Key words: intermittent hypobaric hypoxia, lipid peroxidation, melatonin, oxidative stress

INTRODUCTION

Oxidative stress can be triggered by a series of endogenous and exogenous factors, including exposure to high altitude. Exposure to high altitudes has been associated with an increase in the production of reactive oxygen species that are generated during the re-oxygenation phase of intermittent continuous hypobaric hypoxia and contribute to physiological responses (Nanduri et al., 2008: Farias et al., 2005a). The highly reactive property and oxidative capability that characterize the reactive oxygen species and the free radicals result in overall damage that affects the main cell components and tissues (Blokhina et al., 2003; Radak et al., 1997; Nakanishi et al. 1995; Radak et al., 1994). The use of antioxidants in pharmacological concentrations to reduce the effects of intermittent hypobaric hypoxia by oxidative stress has been reported (Vagas et al., 2011, Farias et al., 2010). Oxidative stress *per se* may be associated with complications such as myocardial injury, pulmonary edema, kidney and liver failure, and increased mortality (Kücükakin et al., 2007).

Melatonin (N-acetyl-5-methoxytryptamine) is the main secretory product of the pineal gland in all mammals including humans, but it is also produced in other organs (Okutan et al., 2004). It is a highly effective antioxidant, scavenging hydroxyl radicals and inhibiting the production of nitric oxide (Gitto et al., 2011; Serel et al., 2004) and other antioxidants like vitamins E and C (Kücükakin et al., 2009). The ability of melatonin to counteract ROS formation is due to the special characteristic of this substance to cross morpho and physiological barriers distributed in tissues, cells and sub-cellular compartments due to its distinct physical and chemical properties (Costa et al., 1995; Tomás-Zapico et al., 2005). In addition, the "hormone of darkness" and messenger of the photoperiod is also well known to exhibit strong direct and indirect antioxidant properties, one of which is capturing ROS directly and another is stimulating gene expression and the activity of some enzymes that can activate enzymatic antioxidants (Kücükakin et al., 2009). This demonstrates that the surgical removal of the pineal gland, one source of melatonin, exacerbates the tissue damage caused by free radicals (Reiter et al., 2001). Melatonin has previously been demonstrated to be a powerful protective substance in numerous injury models as ischemia/reperfusion, where it is cardio-protective in both physiological and pharmacological concentrations. These beneficial effects have been attributed to the capacity of this hormone to act as a radical scavenger (Kücükakin et al., 2009; Mathes, 2010).

During the last 20 years, intensive mining activity has been developed in Chile, particularly in the Andean mountain range, where the altitude is over 4,000 meters above sea level. It is estimated that there is a working population of over 55,000 who are exposed to hypobaric hypoxia (high altitude sickness). Miners work under these conditions for 4 to 20 days, followed by days off at sea level, and this is repeated over several years (Jalil et al., 1994, Germack et al., 2002; Richalet et al., 2002). Intermittent exposure to high altitudes is virtually unknown to date; however it is an unusual condition for workers involving a series of changes at the physiological and cellular levels aimed to compensate the decrease in partial oxygen pressure (PO_2). On the other hand, exposure at 7,576 m.a.s.l. for 5 days caused an increase in lipid peroxidation in the plasma of rats (Kumar et al., 1999). It has been previously shown that melatonin can also protect tissues from oxidative damage (e.g: testicles) under intermittent hypobaric hypoxia conditions (Bustos-Obregón et al., 2010; Hartley et al., 2009). The aim of this study was to evaluate the protective effect of melatonin in several organs, such as heart, lung, kidney, and reproductive system as testis and epididymis in animals exposed to IHH and therefore exposed to oxidative stress.

Corresponding Author: Jorge G. Farias. Facultad de Ingeniería, Departamento de Ingeniería Química, Ciencias y Administración, Universidad de La Frontera. e-mail: jfarias@ufro.cl Phone: +56-45-325472/592189 Fax: +56-45-325053

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MATERIAL AND METHODS

Experimental design

Ten-week-old male Wistar rats (246 ± 11g; n= 36) were obtained from the Animal Facility at the University of Valparaiso. Rats were housed under a 12L: 12D cycle, with water and rat chow being provided ad libitum. They were killed by cervical dislocation. All procedures were performed in agreement with the Principles of Laboratory Animal Care, advocated by the National Society of Medical Research, and the Guide for the Care and Use of Laboratory Animals (Institute of Animal Laboratory Resources, 1996). Rats were divided into 6 groups (6 rats per group): 1) normobaric conditions (Nx), 2) Nx + physiologic NaCl solution (Nx + PS), 3) Nx + Melatonin (Nx+Me), 4) intermittent hypobaric hypoxia (IHH), 5) IHH+ PS, and 6) IHH + Me. A 3x2-factorial experimental design was used to consider 3 injection treatments (no injection, PS, and Me) and 2 environmental treatments (Nx and IHH). The animals were intraperitoneally injected melatonin (10 mg /kg body weight) or vehicle (0.1 mL of PS) at intervals of 96 hours. The IHH group of animals were exposed to hypobaric hypoxia for 96 hours (428 torr; PO2 89.6 mm Hg) followed by the Nx condition for 96 hours (96 hours of hypoxia/96 hours of normoxia) for a total period of 32 days. Pressure changes in the hypobaric chamber were achieved by steps of 150 mm Hg per minute, which simulated altitude changes. The Nx animals were housed in the same room next to the IHH animals.

Determination of Thiobarbituric Acid-Reactive Substances (TBARS)

Body weight was determined 32 days after initiation of the protocol. Rats were then killed by cervical dislocation, and the different organs were collected in phosphate-buffered saline (PBS; pH 7.2). Determination of thiobarbituric acid–reactive substances (TBARS) was undertaken as an index of lipid peroxidation. TBARS were estimated at 532 nm, and their concentrations were calculated using a molar extinction coefficient of 1.56 $X10^5$ M^{-1} cm^{-1} obtained utilizing malondialdehyde (MDA; Sigma-Aldrich, St Louis, Missouri) as a standard (Farias et al., 2010). The results were expressed as nmol of MDA equivalents/mg tissue.

Isolation and sperm count

Sperm count was performed as previously described (Farías et al., 2010). Briefly, epididymal spermatozoa were separated by cutting the caudal epididymis into segments of approximately 1 mm^3 with a sharp razor blade in 1 mL of PBS (pH 7.2). Spermatozoa from caudal regions were completely removed by vortexing gently in PBS, and the tissue debris was allowed to settle for 5 minutes. Spermatozoa released in the buffer were aspirated, centrifuged at 800 xg for 15 minutes, and used for biochemical determinations. All of these procedures were performed at 4°C. The number of sperm in the suspension was counted using a Neubauer chamber.

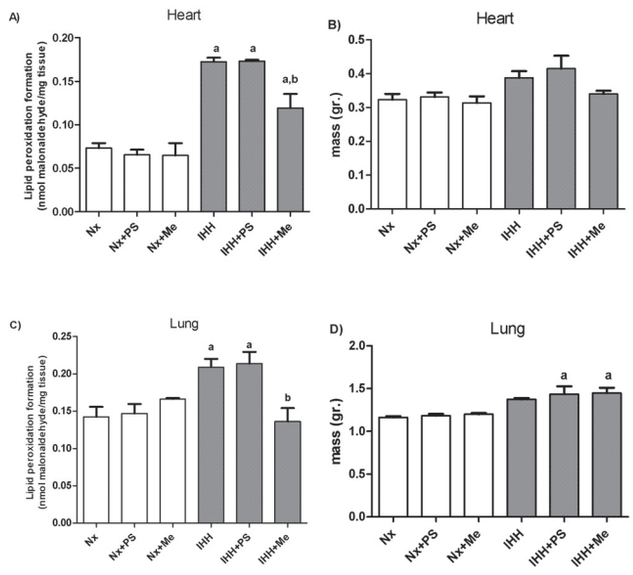

Figure 1. Levels of lipid peroxidation and organ size in heart and lung after IHH: Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) condition, with or without treatment of melatonin (Me). PS: rats treated with physiological NaCl solution. At the end of treatment (32 days), lipid peroxidation and organ size were determined in heart (A, B) and lung (C, D). Bars indicate the mean SD (n=6). a $p<0.05$ (Hx versus Nx); b $p<0.05$.

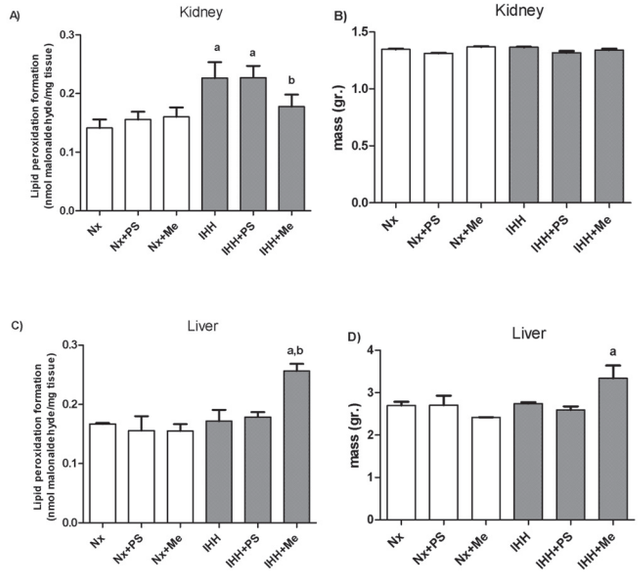

Figure 2. Levels of lipid peroxidation and organ size in kidney and liver after IHH: Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) condition, with or without treatment of melatonin (Me). PS: rats treated with physiological NaCl solution. At the end of treatment (32 days), lipid peroxidation and organ size were determined in kidney (A, B) and liver (C, D). Bars indicate the mean SD (n=6). a $p<0.05$ (Hx versus Nx); b $p<0.05$.

Statistical analysis

The results were analyzed using a 2-way variance analysis (ANOVA) followed by a Bonferroni analysis. The level of statistical significance was set at p <0.05. The data were analyzed using the GraphPad Prism software version 4.0 (GraphPad Software, San Diego, California). The results are presented as means ± standard deviation (SD).

RESULTS

Determination of thiobarbituric acid–reactive substances (TBARS) was undertaken as an index of lipid peroxidation. The present results show that intermittent hypobaric hypoxia induced lipid peroxidation in heart in comparison to normoxic (Nx) conditions as indicated by the levels of TBARS (P<0.05) and shown in Figure 1A. However, melatonin diminished such effect that was corroborated by a decrease in mass of the organ (Figure 1B). Similar results were observed in lung (P<0.05) as shown in Figure 1C: However, but there was a significant (P<0.05) increase in the mass of such organ (Figure 1D).

Lipid peroxidation in kidneys has been found to under IHH conditions more than under Nx conditions (P<0.05) as shown in Figure 2A. It is interesting to note that there was a decrease induced by melatonin (P<0.05). However, there was no significant difference in organ mass (P>0.05), as can be observed in Figure 2B. Figure 2C shows that there was no significant difference in lipid peroxidation in the liver under IHH and Nx conditions (P>0.05). However, there was an increase in lipid peroxidation in melatonin-treated animals, compared to the Nx and IHH treated groups (P<0.05). As well, the lipid peroxidation in the melaton-treated group was accompanied by an increase in organ mass (P<0.05), as shown in Figure 2D.

Figure 3A shows that IHH induced a significant (P<0.05) increase in lipid peroxidation in testis in comparison to Nx conditions. Melatonin did not have any effect, but there was a decrease in organ mass (P<0.05) compared to IHH conditions (Fig3 B). Similar results were observed in epididymis (P<0.05) in comparison to Nx (Fig 3C), although there was no significant difference (Fig3D) in organ mass (P>0.05). There was a non-significant difference in sperm counts under Nx conditions. However, there was a decrease in sperm number under IHH conditions (P<0.05), as observed in Figure 4. Melatonin in the doses used in this study did not have any effect.

DISCUSSION

The protective role of melatonin against oxidative stress in rats exposed to intermittent hypobaric hypoxia was analyzed in this study. Oxidative stress can be triggered by a series of endogenous and exogenous factors, exposure to intermittent hypobaric hypoxia being one of them (Farias et al., 2010; Vargas et al., 2011). Exposure to high altitudes is associated with an increase in the production of reactive oxygen species, which are generated during the phase of re-oxygenation of intermittent hypobaric hypoxia and contributes to the physiological responses (Nanduri et al., 2008). The main cause of oxidative stress is lower availability of oxygen as it converts to H_2O by cytochrome oxidase. Hypoxia appears to affect enzyme activities such as superoxide dismutase (SOD), glutathione reductase (GSR) and glutathione peroxidase (GPX), which are usually reduced (Maiti et al., 2006). Recently, studies have demonstrated melatonin interactions with many

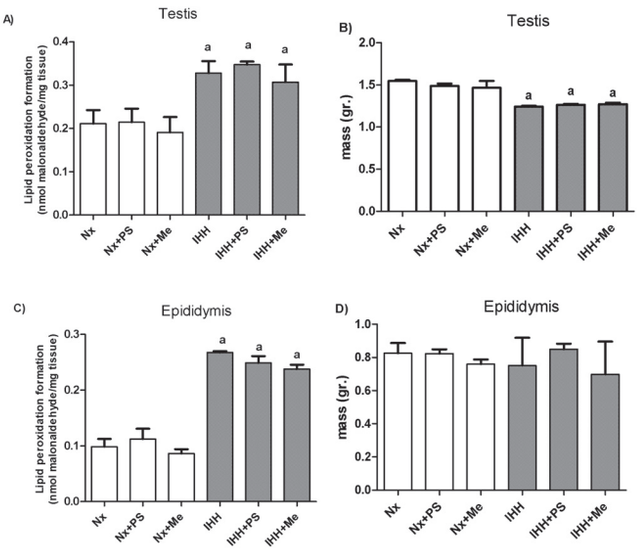

Figure 3. Levels of lipid peroxidation and organ size in testis and epididymis after IHH: Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) condition, with or without treatment of melatonin (Me). PS: rats treated with physiological NaCl solution. At the end of treatment (32 days), lipid peroxidation and organ size were determined in testis (A,B) and epididymis (C,D). Bars indicate the mean SD (n=6). a p<0.05 (Hx versus Nx); b p<0.05.

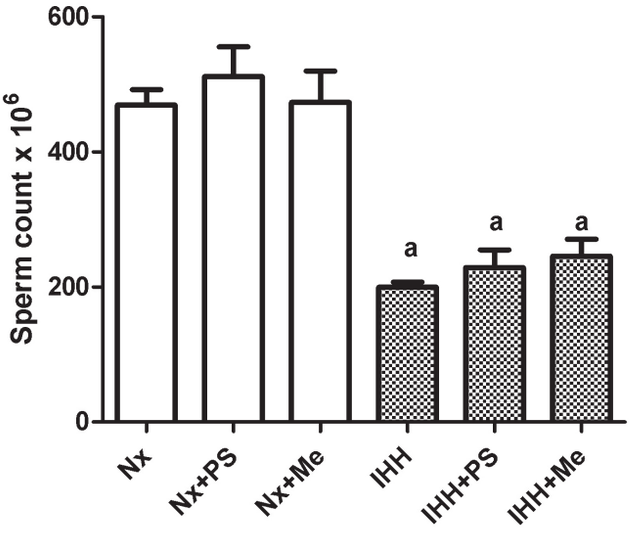

Figure 4. Effect of intermittent hypobaric hypoxia in sperm number: Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) condition, with or without treatment of melatonin (Me). PS: rats treated with physiological NaCl solution. At the end of treatment (32 days), epidydimal cauda spermatozoa were counted. Hx induced a significant decrease in sperm count, which was not prevented by melatonin treatment. Bars indicate the mean SD (n=6). a p<0.05.

physiological processes and diseases (Peliciari-Garcia et al., 2011). Therefore, the effects of melatonin could depend on physiological processes of the organs when these are subjected to environmental stress like IHH.

Little is known about the level of oxidative damage suffered by cell structures in several organs and tissues exposed to high altitude. The study of lipid oxidative damage by estimating malonydialdehyde (MDA) content as a product of lipid peroxidation has been performed on humans, as well as animal models (Moller et al., 2001; Joanny et al., 2001; Radak et al., 1994; Kurmar et al., 1999).

These studies have demonstrated that melatonin plays a protective role in heart, lung and kidney in animals exposed to intermittent hypobaric hypoxia since intermittent lipid peroxidation results in lower malonydialdehyde content in such tissues. The effect of melatonin was corroborated by changes in the organ mass. It is interesting to note that melatonin membrane receptors like MT1 and MT2 have been described in heart, lung, liver and kidney (Sanchez-Hidalgo et al., 2009; Ishii et al., 2009). Authors (Nakanishi et al. (1995) have reported that exposure to hypobaric hypoxia induced at 5,000 meters above see level results in increased oxidative stress in heart, lungs, kidneys and liver. However, these authors did not consider the melatonin effect.

Our results showed that the administration of melatonin to rats induced lipid peroxidation and an increase in liver mass that was not affected by melatonin exposure when injected into the experimental animals. Despite the enormous amount of data supporting the idea of melatonin as a liver protective agent, it should be noted that there are also other reports that have indicated no hepato-protective effect of melatonin to the effect of 2-nitropropane and ethanol in rat liver, which supports our results (Mathes, 2010). On the other hand, lower rates of ROS generation in the liver and decreases in mitochondrial energy metabolism has been reported in rats subjected to hypobaric hypoxia (Costa et al., 1993; Nouette-Gaulain et al., 2011 and Farías et al., 2005a).

The present study demonstrated that intermittent hypobaric hypoxia induces lipid peroxidation in testis and epididymis, as previously reported (Farias et al., 2010; Vargas et al, 2011). However, in the aforementioned studies melatonin did not have any effect with the doses used in contrary to other reports where melatonin had a protective role in testis and spermatogenesis of rats subjected to intermittent hypobaric hypoxia (Hartley et al., 2009; Bustos-Obregón et al., 2010; Vargas et al., 2011); Ahmad & Haldar (2010) as well as hamsters and rats, where melatonin seems to act directly on Leydig cells to suppress testosterone release *in vitro*. When administered orally at 10 mg/kg bodyweight, melatonin has been described as a counteracting substance for testis and spermatogenesis damage in rats subjected to intermittent hypobaric hypoxia (Vargas et al., 2011). The present results showed that melatonin, with the doses given via intra-peritoneal, had no protective effect in testis and epididymis. Furthermore, it has been reported that injecting hamsters with melatonin induced damage in testis by decreasing levels of LH and FSH (Tamarkin et al., 1976).

Some authors have reported that melatonin treatment induced a marked reduction in sperm quality and did not prevent the reduction in sperm concentration under an ischemia/reperfusion condition (Oosthuizen et al. 1986; Kurcer et al., 2010), which concurs with the results of the present work. Luboshitzky et al. (2002) reported that melatonin administration (3mg) is associated with decreased semen quality in healthy men, probably through the inhibition of aromatase at the testicular level. Aromatase is the terminal enzyme responsible for forming estrogens from androgens and it plays a physiological role in the maintenance of male gonadal functions (Carreau et al., 2001). This enzyme is expressed in Leydig cells, Sertoli cells, germ cells and ejaculated spermatozoa (Carreau and Hess, 2010) in animals and humans. In conclusion, melatonin plays a protective role in heart, lung and kidney under intermittent hypobaric hypoxia conditions.

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REFERENCES

AHMAD R, HALDAR C. (2010). Effect of intra-testicular melatonin injection on testicular functions, local and general immunity of a tropical rodent Funambulus pennant. Endocr. 37:479–488.

BLOKHINA O, VIROLAINEN E, FAGERSTEDT KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot. 91; 179-94.

BUSTOS-OBREGÓN E, CASTRO-SÁNCHEZ R, RAMOS GONZÁLEZ B. AND TORRES-DÍAZ L (2010) Rat spermatogenesis damage in intermittent hypobaric hypoxia and the protective role of melatonin. II: testicular parameters. Int. J. Morphology. 28(2): 537-547.

CARREAU S AND HESS RA (2010) Oestrogens and spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 365(1546):1517-35.

CARREAU S, BOURGUIBA S, LAMBARD S, GALERAUD-DENIS I, GENISSEL C, BILINSKA B, BENAHMED M AND LEVALLET J (2001) Aromatase expression in male germ cells. J Steroid Biochem Mol Biol. 79(1-5):203-8.

COSTA EJ, LOPES RH, LAMY-FREUND MT (1995) Permeability of pure lipid bilayers to melatonin. J Pineal Res 1995. 19:123–126.

COSTA LE, LLESUY S, BOVERIS A (1993) Active oxygen species in the liver of rats submitted to chronic hypobaric hypoxia. Am J Physiol. 264 (6 Pt 1): C1395-400.

FARÍAS JG, BUSTOS-OBREGÓN E, ORELLANA R, BUCAREY, JL, QUIROZ E, REYES JG (2005a) Effects of chronic hypobaric hypoxia on testis histology and round spermatid oxidative metabolism. Andrologia. 37:47–52.

FARÍAS JG, PUEBLA M, ACEVEDO A, TAPIA P, GUTIÉRREZ E, ZEPEDA A, JUANTOK C, CALAF G, REYES, J (2010) Oxidative Stress in Testis and Epididymis Under Intermittent Hypobaric Hypoxia in Rats: Protective Role of Antioxidant Supplementation. Journal of Andrology. 31(3):314-321.

GERMACK R, LEÓN-VELARDE F, VALDÉS R, FARÍAS J, SOTO G, RICHALET JP (2002) Effect of intermittent hypoxia on cardiovascular function, adrenoceptors and muscarinic receptors in Wistar rats. Experimental Physiology 84: 4-12.

GITTO E, AVERSA S, REITER RJ, BARBERI1 I, PELLEGRIN S (2011) Update on the use of melatonin in pediatrics. J. Pineal Res. 50:21–28.

HARTLEY R, CASTRO-SÁNCHEZ R, RAMOS-GONZÁLEZ B AND BUSTOS-OBREGÓN E (2009) Rat spermatogenesis damage in intermittent hypobaric hypoxia and the protective role of melatonin. I: Caudal Epididymal spermatozoa. Int. J. Morphology. 27(4): 1275-1284.

ISHII H, TANAKA N, KOBAYASHI M, KATO M, SAKUMA Y (2009) Gene structures, biochemical characterization and distribution of rat melatonin receptors. J Physiol Sci. 59; 37–47.

JALIL J, BRAUN S, CHAMORRO G, CASANEGRA P, SALDÍAS F, BEROÍZA T, FORADORI A, RODRÍGUEZ R, MORALES M (1994) Cardiovascular response to exercise at high altitude in workers chronically exposed to intermittent hypobaric hypoxia. 122(10):1120-1125.

JOANNY P, STEINBERG J, ROBACH P, RICHALET JP, GORTAN C, GARDETTE B, JAMMES Y (2001) Operation Everest III (Comex'97): the effect of simulated sever hypobaric hypoxia on lipid peroxidation and antioxidant defence systems in human blood at rest and after maximal exercise. Resuscitation. 49(3); 307-14.

KÜCÜKAKIN B, GÖGENUR I, REITER RJ, ROSENBERG J (2009) Oxidative Stress in Relation to Surgery: Is There a Role for the Antioxidant Melatonin?. Journal of Surgical Research. 152: 338–347.

KÜCÜKAKIN B, GÖGENUR I, ROSENBERG J (2007) Melatonin against surgical stress. Ugeskr Laeger. 169(14); 1306-8.

KUMAR D, BANSAL A, THOMAS P, SAIRAM M, SHARMA SK, MONGIA SS, SINGH R, SELVAMURTHY W (1999) Biochemical and immunological changes on oral glutamate feeding in male albino rats. Int J Biometeorol. 42(4); 201-4.

KURCER Z, HEKIMOGLU A, ARAL F, BABA F AND SAHNA E (2010) Effect of melatonin on epididymal sperm quality after testicular ischemia/reperfusion in rats. Fertil Steril. 93(5):1545-9.

LUBOSHITZKY R, SHEN-ORR Z, NAVE R, LAVI S AND LAVIE P (2002) Melatonin administration alters semen quality in healthy men. J Androl. 23(4):572-8.

MAITI P, SINGH SB, SHARMA AK, MUTHURAJU S, BANERJEE PK, ILAVAZHAGAN G (2006) Hypobaric hypoxia induces oxidative stress in rat brain. Neurochem Int. 49(8); 709-16.

MATHES AM (2010) Hepatoprotective actions of melatonin: Possible mediation by melatonin receptors. World J Gastroenterol.16(48): 6087-6097.

MOLLER P, LOFT S, LUNDBY C, OLSEN NV (2001) Acute hypoxia and hypoxic exercise induce DNA strand breaks and oxidative DNA damage in humans. FASEB J. 15(7); 1181-6.

NAKANISHI K, TAJIMA F, NAKAMURA A, YAGURA S, OOKAWARA T, YAMASHITA H, SUZUKI K, TANIGUCHI N, OHNO H (1995) Effects of hypobaric hypoxia on antioxidant enzymes in rats. J Physiol. 489(3); 869-76.

NANDURI J, YUAN G, KUMAR GK, SEMENZA GL, PRABHAKAR NR (2008) Transcriptional responses to intermittent hipoxia. Respiratory Physiology & Neurobiology 164: 277–281.

NOUETTE-GAULAIN K, BIAIS M, SAVINEAU JP, MARTHAN R, MAZAT JP, LETELLIER T, SZTARK F (2011) Chronic hypoxia-induced alterations in mitochondrial energy metabolism are not reversible in rat heart ventricles. Can J Physiol Pharmacol. 89(1); 58-66.

OKUTAN H, SAVAS C, DELIBAS N (2004) The antioxidant effect of melatonin in lung injury after aortic occlusion–reperfusion. Interact CardioVasc Thorac Surg. 3:519-522.

OOSTHUIZEN JMC, BORNMAN MS AND SCHULENBURG GW (1986) Melatonin impairs sperm motility-a novel finding. S. Afr. Med. 70:566.

PELICIARI-GARCÍA RA, ZANQUETTA MM, ANDRADE-SILVA J, GOMES DA, BARRETO-CHAVES ML, CIPOLLA-NETO J (2011) Expression of circadian clock and melatonin receptors within cultured rat cardiomyocytes. Chronobiol Int. 28(1):21-30.

RADAK Z, ASANO K, LEE KC, OHNO H, NAKAMURA A, NAKAMOTO H, GOTO S (1997) High altitude training increases reactive carbonyl derivatives but not lipid peroxidation in skeletal muscle of rats. Free Radic Biol Med. 22(6); 1109-14.

RADAK Z, LEE K, CHOI W, SUNOO S, KIZAKI T, OH-ISHI S, SUZUKI K, TANIGUCHI N, OHNO H, ASANO K (1994) Oxidative stress induced by intermittent exposure at a simulated altitude of 4000 m decreases mitochondrial superoxide dismutase content in soleus muscle of rats. Eur J Appl Physiol Occup Physiol.69(5); 392-5.

REITER RJ, TAN D, MANCHESTER LC, QI W (2001) Biochemical Reactivity of Melatonin with Reactive Oxygen and Nitrogen Species. Cell Biochemistry and Biophysics. 34: 237–256.

RICHALET JP, VARGAS M, JIMÉNEZ D, ANTEZANA AM, HUDSON C, CORTÉS G, OSORIO J, LEÓN G (2002) Chilean miners commuting from sea level to 4500 m: A prospective study. High. Alt. Med. Biol. 3:159–166.

SÁNCHEZ-HIDALGO M, GUERRERO JM, CARRASCOSA-SALMORAL M-P, NARANJO M-C, LARDONE PJ, ALARCÓN C (2009) Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. J. Pineal Res. 46; 29–35.

SEREL TA, ÖZGUNNER F. AND SOYUPEK S (2004) Prevention of shock wave-induced renal oxidative stress by melatonin: an experimental study. Urol. Res 32:69-71.

TAMARKIN L, WESTROM WK, HAMILL AI, GOLDMAN BD (1976) Effect of melatonin on the reproductive systems of male and female Syrian hamsters: a diurnal rhythm in sensitivity to melatonin. Endocrinology. 99(6):1534-41

TOMÁS-ZAPICO C, COTO-MONTES A (2005) A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J. Pineal Res. 39:99–104.

VARGAS A, BUSTOS-OBREGÓN E, HARTLEY R (2011) Effects of hypoxia on epididymal sperm parameters and protective role of ibuprofen and melatonin. Biol Res. 44: 161-167.

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Anatomy of corpus callosum in prenatally malnourished rats

Ricardo Olivares[1*], Carlos Morgan[2], Hernán Pérez[2], Alejandro Hernández[3], Francisco Aboitiz[4], Rubén Soto-Moyano[2], Julio Gil[5], Alexis Ortiz[6], Osvaldo Flores[2], Miguel Gimeno[5] and Jesús Laborda[5]

[1] Departamento de Ciencias Biológicas Animales, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile.
[2] Laboratorio de Nutrición y Regulación Metabólica, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile.
[3] Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago Chile.
[4] Departamento de Psiquiatría, Escuela de Medicina y Centro Interdisciplinario de Neurociencia, Pontificia Universidad Católica de Chile
[5] Departamento de Anatomía, Embriología y Genética Animal, Facultad de Veterinaria, Universidad de Zaragoza, España.
[6] Universidad Andrés Bello, Facultad de Ecología y Recursos Naturales, Av. República 252, Santiago.

ABSTRACT

The effect of prenatal malnutrition on the anatomy of the corpus callosum was assessed in adult rats (45-52 days old). In the prenatally malnourished animals we observed a significant reduction of the corpus callosum total area, partial areas, and perimeter, as compared with normal animals. In addition, the splenium of corpus callosum (posterior fifth) showed a significant decrease of fiber diameters in the myelinated fibers without changing density. There was also a significant decrease in diameter and a significant increase in density of unmyelinated fibers. Measurements of perimeter's fractal dimensions from sagittal sections of the brain and corpus callosum did not show significant differences between malnourished and control animals. These findings indicate that cortico-cortical connections are vulnerable to the prenatal malnutrition, and suggest this may affect interhemispheric conduction velocity, particulary in visual connections (splenium).

Key Words: corpus callosum, prenatal malnutrition, rat, splenium

INTRODUCTION

A commonly used protocol for inducing prenatal malnutrition in rats consists of a restriction of food intake in pregnant females at a 40% of daily requirements since the eighth day of pregnancy up to birth (Soto-Moyano et al., 1993). This and other types of caloric-protein malnutrition of pregnant rats correlate with lower body weight of the offspring as well as with a high mortality rate during early postnatal life (Morgane et al., 1993).

In humans, fetal undernutrition has been found to be associated to disease in a variety of physiological systems, such as the cardiovascular system (hypertension, coronary heart disease, stroke, atherosclerosis, coagulation disorders, pre-eclampsia), reproductive system (polycystic ovary syndrome, early adrenarche/menarche, early menopause), respiratory system (chronic obstructive pulmonary disease, asthma), endocrine system (hypercortisolism, hypothyroidism), skeletal system (osteoporosis), and the nervous system (neurological disorders, schizophrenia, dementia) (for review see Fowden et al. 2006). In animal models of experimental malnutrition (rat, guinea pig and sheep), changes in the fetal nutritional environment lead to permanent alterations of the developmental pattern of cellular proliferation and differentiation in key tissue and organ systems (hearth, endothelium, vascular smooth muscle, skeletal muscle, kidney, rennin-angiotensin system, pancreatic growth, adipose tissue and adipocyte secretion, hypothalamo-pituitary-adrenal axis, and peripheral and central nervous system) that results in pathological consequences in adult life (for review see McMillen and Robinson, 2006).

Concerning the rat central nervous system, prenatal malnutrition has been shown to alter brain development and growth, thus desynchronizing cell migration, retarding or blocking cell differentiation and increasing cell death associated to a diminished number of neurons (Morgane et al., 1993). Those impairments are frequently associated to behavioral alterations (Morgane et al., 1993; Levitsky and Strupp, 1995). In addition, inadequate prenatal nutrition increases central noradrenaline levels and release (Soto-Moyano et al., 1994; Soto-Moyano et al., 1998a; Soto-Moyano et al., 1998b), which is most relevant because noradrenaline is a critical factor for regulating regressive events of neuronal projections during normal brain development (Soto-Moyano et al., 1994).

The corpus callosum (CC) is the major commissure in the brain of placental mammals, being formed by numerous transversal fibers connecting mostly homologous but also heterologous cortical areas in both hemispheres (Aboitiz et al., 1993). Its function is to integrate sensory and cognitive experience of the two sides of the brain and is a likely target of developmental injuries. In fact, the CC and total brain size are affected by prenatal insults like ethanol ingestion and overt protein malnutrition (Morgane et al., 1978; Zimmerberg and Mickus 1990; Gressens et al., 1997). Overt prenatal protein malnutrition also reduces spontaneous forebrain neuronal activity in adulthood (Stern et al., 1983). Moreover, projecting fields of transcallosal evoked responses are affected in overt and hidden forms of prenatal protein malnutrition (Soto-Moyano et al., 1993; Soto-Moyano et al., 1998a; Forbes et al., 1975). Although reduction in CC size and functionality has been reported in malnourished animals, it has not yet been studied at the fine anatomical and ultrastructural levels.

On these bases, it seems likely that the CC may be an important target in the pathophysiology of prenatal

* Corresponding author: Ricardo Olivares. Depto. de Ciencias Biológicas Animales, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile. Postal Address: Av. Santa Rosa 11735, La Pintana, Santiago, Chile. Fono: 9785528 Fax: 9785593, E-mail: rolivare@uchile.cl

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malnutrition. In order to assess this hypothesis, the effect of prenatal malnutrition on CC anatomy was determined in adult rats (45-52-day old). The perimeter, total area and partial areas of CC sagittal cuts, as well as the fractal dimension of CC shape were determined. In addition, we considered of interest to study the possible effects of malnutrition on the histology of the callosal splenium, which may affect the velocity of cortical-cortical occipital conduction, and particularly the visual information.

METHODS

Laboratory Animals and Experimental Treatment

The experimental protocols and animal management were carried out in accordance to NIH guidelines (NIH, 1996) and approved by the Committee of Bioethics at INTA, University of Chile. The experiments were conducted on male and female Sprague-Dawley rats (INTA, Santiago, Chile) born from dams subjected during pregnancy to one of the following nutritional conditions (8 pregnant rats per groups): 1) well-nourished pregnant rats, with free access to a 21% protein non purified diet (Champion®, Santiago, Chile: 21.4% protein, 6.9% fat, 45.9% carbohydrate, 4.6% minerals, 0.5% vitamins, 10% water, 10.7% non-nutritive filler and 13.3KJ/g.) (Soto-Moyano et al., 1998b), and 2) malnourished pregnant rats, with free access to food until 7 days post-conception; after this date the nonpurified diet was restricted to 10 g/day until parturition. This amount of food is about 40% of that consumed by normal pregnant rats during weeks 2 and 3 of gestation (Soto-Moyano et al., 1993; Soto-Moyano et al., 1998b), and was given two times daily (5 g at 09:00 h and 5 g at 19:00h) in order to minimize anxiety for feeding in food restricted pregnant dams. At birth, to ensure adequate nutrition during lactation, prenatally malnourished pups were fostered to well-nourished dams giving birth on that day, according to rearing procedures already described (Morgane et al., 1993); pups born from well-nourished mothers were also fostered to well-nourished dams, in order to equalize among groups other factors that may depend on the rearing conditions (i.e. stress due to cross-fostering) (Navarrete et al., 2007).

During the suckling period, all litters were adjusted to 8 pups per dam (4 males and 4 females), and all dams continued to receive the standard laboratory diet *ad libitum*. After weaning at 21 days of age, the offspring were given free access to the nonpurified diet up to sacrifice (45-52 days of age). Body weight of pregnant rats and offspring were measured daily. All these procedures were performed as previously reported (Soto-Moyano et al., 1993; Soto-Moyano et al., 1998b).

Macroscopic study

After i.p. injection of 40 mg/kg sodium thiopental and intracardial perfusion of 10% formalin in saline solution, at body temperature and at equivalent volume

of 20% to body weight, animals (malnourished = 8 and control = 8) were sacrificed by decapitation. Encephala were extracted and kept in 10% formalin until sectioned and photographed. Digital photographs of mid-sagittal sections were analyzed using the software *Scion Image for Windows* (Merlo et al., 2002), under 100X total magnification, to determine total and partial areas of CC as well as perimeters, according to Witelson (1989), who divided arbitrarily the corpus callosum into three regions in relation to its maximum length: the anterior third (genu), the middle third (body), and the posterior third which is often divided into fifth posterior (splenium) (Fig. 1). Brain weight measurements were performed excluding cerebellum, brainstem and olfactory bulbs.

We calculated the fractal dimension of the perimeters of CC areas with the FDC (*Fractal Dimension Calculator*) software, which uses the box-counting technique. The math formula used for calculations is log N(s) = D log (1/s), where: **D** is fractal dimension, **N** is the number of boxes containing image dots, and **s** is the box side iterated as expressed in pixels (Guzmán et al., 1993). Mathematicians have demonstrated that fractal dimension could be used to measure the space complexity of a structure, the greatest values of fractal dimensions being associated to high geometric complexity of any structure. We made the calculations over the perimeter of the problem zone from original images filtered by *Photoshop* software. From these images we calculated the fractal dimensions. Thereafter, we compare the fractal dimension of sagittal cuts from brain and corpus callosum from eutrophic and malnourished groups (Fig. 1).

Microscopy study

Eight animals and their respective controls were perfused transcardially with Karnosky's solution (1.5% glutaraldehyde; 0.8% paraformaldehyde) in sodium phosphate buffer, at body temperature and at equivalent volumen of 20% to body weight. Thereafter the brain was extracted and the splenium of CC (posterior fifth) dissected, which contains visual fibers from the occipital cortex and other regions (Witelson, 1989). Tissues were fixed in 2.5% glutaraldehyde for 8h at 4 ºC and afterward washed in 0.1 M sodium phosphate buffer, pH 7.3. Tissue samples were post-fixed in 1% osmium tetroxide for 1 h at room temperature, and finally included in EPON. Semi-thin 1 µm slices were obtained and stained with toluidine blue in 1% sodium borate to show the splenium (posterior region), allowing axons to be counted (expressed as number of axons per mm^2) and diameters of both myelinated and non-myelinated callosal fibers measured (expressed in µm). Thereafter, 60 nm thin slices were mounted on 200 mesh grids, and left in uranyl acetate and Reynolds stain for 10 min, before observing them at the transmission electron microscope

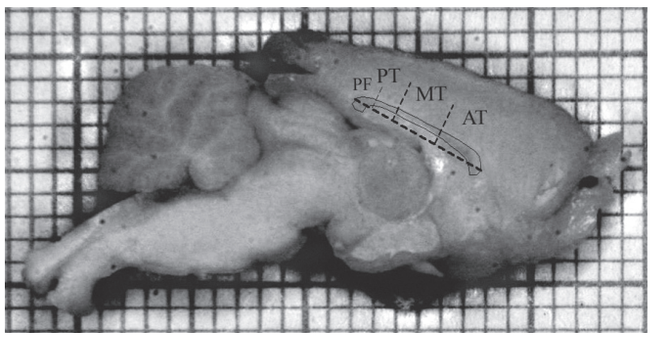

Figure 1: Cross section of corpus callosum indicating the three regions and posterior fifth in relation to its maximum length. AT = anterior third, MT = middle third, PT = posterior third, and PF = posterior fifth. Each square in the background is of 1 mm side

(Zeiss EM 109). Observations were made at an adequate magnification to get a clear visualization of myelinated and unmyelinated axons. Five of ten fields (27,000 x 23,000 µm) were photographed per every splenium respective to each animal, and analyzed with the software *Scion Image for Windows* (Merlo et al., 2002; Olivares et al., 2007) using total magnification of 28,606X. All fibers were counted excepting those touching the left and inferior adjacent sides and the lower right of the images (Gundersen, 1977). An average number of 109 myelinated and 593 unmyelinated fibers were counted per field. Regarding the myelinated fibers, their internal diameters were considered excluding the myelin sheat, and considering the minimal diameter of every fiber, in order to minimize factors such as elongation of the tissue and oblique cutting of fibers. Glial processes cannot not distinguished from unmyelinated fibers with the panoramic magnification used for fiber counting; however, these processes are more frequent between adjacent myelinated fibers, regions that were not considered in the counting process.

Statistical analysis

Resulting values from malnourished (n=8) and control animals (n=8), were corrected at their real magnification, and data are reported as means ± SD.

We used the Program SPSS (Statistical Package for the Social Sciences) from IBM® SPSS® Statistics. The statistic Mann-Whitney U test (Ferrán, 2001) is a nonparametric test that we used to determine the effect of diet on the performed measurements. We used it because it is a distribution free test or nonparametric one, then does not require assumptions about the shape of the underlying compared distributions. It tests the hypothesis that two independent samples come from populations having the same distribution, statistical differences were considered significant at a probability level equal or less than 0.05.

RESULTS

The macroscopic study showed that the offspring of malnourished dams presented lower body weight than eutrophic animals since birth up to weaning ($p < 0.001$), but no differences were observed when sacrificed at adulthood (45-52 days of age). Nevertheless, malnourished animals exhibited lower brain weight at the moment of sacrifice ($p < 0.01$) as compared to control animals. Additionally, we calculated the percentage of weight gain of malnourished animals respective to eutrophic controls, from birth to weaning and from weaning to sacrifice. In the first case there was a decrease in weight gain ($p < 0.01$) and in the latter case an increase ($p < 0.01$). Finally, the difference between brain weight/body weight ratios between the two groups was not significant (Table I).

Regarding the total and partial areas of CC, a reduction was observed in the malnourished group ($p < 0.05$) as well as in total perimeter ($p < 0.01$; Table II) as compared to controls. No significant statistical differences were detected when comparing fractal dimension of brain and CC from malnourished and control animals (Table III) using Mann-Whitney U test statistics.

TABLE I
Average body weight, brain weight, gain weight and brain:body weight relation

WEIGHTS	**Malnourished** (n=8)	**Control** (n=8)	**M-W U Test**
Body weight at birth (g)	6.06 ± 0.14	7.55 ± 0.24	$p < 0.001$
Body weight at weaning (g)	42.69 ± 1.28	59.35 ± 3.19	$p < 0.001$
Body weight 45-52-day-old (g)	154.50 ± 18.88	170.64 ± 22.00	NS
Weight gain from birth to weaning (%)	604.58 ± 30.12	686.67 ± 46.34	$p < 0.01$
Weight gain from weaning to sacrifice (%)	261.63 ± 39.62	187.38 ± 31.95	$p < 0.01$
Brain weight (g)	1.10 ± 0.07	1.23 ± 0.06	$p < 0.01$
Brain: Body weight relation	0.73 ± 0.07	0.73 ± 0.09	NS

TABLE II
Average callosal measurements

CALLOSAL MEASUREMENTS	**Malnourished** (n=8)	**Control** (n=8)	**M-W U Test**
Total area (mm^2)	2.65 ± 0.61	3.93 ± 0.31	$p < 0.01$
Anterior third (mm^2)	0.95 ± 0.22	1.49 ± 0.3	$p < 0.01$
Middle third (mm^2)	0.74 ± 0.26	1.08 ± 0.16	$p < 0.05$
Posterior third (mm^2)	0.97 ± 0.17	1.36 ± 0.24	$p < 0.01$
Posterior fifth (mm^2)	0.62 ± 0.13	0.87 ± 0.14	$p < 0.01$
Total perimeter (mm)	14.54 ± 1.37	17.13 ± 1.29	$p < 0.01$

TABLE III
Average fractal dimensions

FRACTAL DIMENSION	**Malnourished** (n=8)	**Control** (n=8)	**M-W U Test**
Brain	1.08 ± 0.03	1.09 ± 0.02	NS
Corpus callosum	1.35 ± 0.09	1.29 ± 0.07	NS

The CC of malnourished rats exhibited thinner myelinated fiber diameters than the control group ($p < 0.05$). In addition, the malnourished group also presented lower values in the diameter of unmyelinated fibers than the control group ($p < 0.001$; Table IV, Fig. 2 and 3). Density of myelinated fibers was not different between groups. In contrast, malnourished rats presented higher density of unmyelinated fibers than controls ($p < 0.05$; Table V, Fig. 2 and 3).

DISCUSSION

Macroscopic study

Malnutrition during gestation resulted in a significant body weight deficit of pups at birth and at weaning age, and in significantly less weight gain from birth to weaning, indicating that reduction of food intake by the dams during pregnancy caused fetal growth retardation. Although the weight gain from weaning to sacrifice was significant higher in the malnourished group, no differences were observed in body weight of young rats at sacrifice and in the brain/body weight relation, which is in agreement with previous works by our group (Soto-Moyano et al., 1998a; Soto-Moyano et al., 1998b; Navarrete et al., 2007). Malnutrition during gestation also resulted in a significant brain weight deficit in newborns (Table I). The postnatal recuperation of body weight, but not of brain weight, complies with the concept of a prenatal correlated growth between body and brain that becomes unlocked postnatally, body weight then increasing much faster than brain weight (Aboitiz, 1996).

Our findings regarding the CC are in agreement with Zimmerberg and Mickus (1990), who investigated the size of CC in rats prenatally exposed to alcohol. Those animals exhibited smaller total CC areas than control animals and the callosal size was negatively correlated to open field activity, suggesting a possible role in normal exploratory behavior. Furthermore, in a previous work from our group, rats malnourished prenatally and during lactation with a low-protein, isocaloric diet were shown to have smaller CC than controls, which was consistent with differences in brain weight. In contrast, prenatally malnourished rats rehabilitated with high-protein diet during lactation showed normal brain weight and development of the mid-third and posterior third of the callosum, but reduced size of the anterior third at 45-52 days of age (Olivares et al., 2002). This observation may imply that those rehabilitated animals are specifically damaged in certain frontal functions (Kolb, 1984). In our current study the experimental conditions were more dramatic, since all partial areas of CC were significantly smaller ($p < 0.05$) in malnourished than in control animals (Table II). These results suggest that prenatal malnutrition affects interhemispheric connections in different regions of the cerebral cortex.

We used the same morphometric software utilized by Merlo et al., (2002) who found changing values of callosal areas and perimeters with age in humans with no significant differences between genders. We used the Box counting technique to calculate fractal dimensions, as previously used in works on kidney (Gil et al., 2006) and the bronchial tree (Canals et al.,

TABLE IV
Average diameter fibers

µm	**Malnourished** (n=8)	**Control** (n=8)	**M-W U Test**
Myelinated Fibers	0.42 ± 0.03	0.49 ± 0.07	$p < 0.05$
Unmyelinated Fibers	0.12 ± 0.01	0.18 ± 0.02	$p < 0.001$

TABLE V
Average density fibers

axons / mm^2	**Malnourished** (n=8)	**Control** (n=8)	M-W U Test
Myelinated Fibers	0.19 ± 0.04	0.16 ± 0.02	NS
Unmyelinated Fibers	1.18 ± 0.29	0.73 ± 0.24	$p < 0.05$

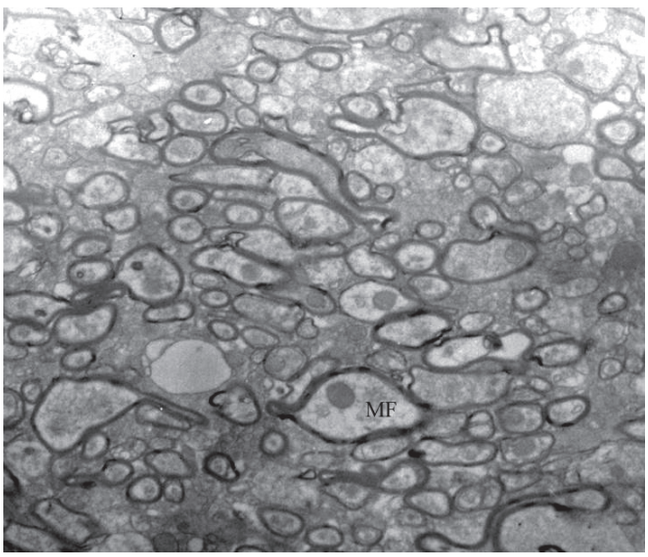

Figure 2: Electron micrograph of the splenium from a representative control animal. MF: myelinated fiber, Bar: 1µm.

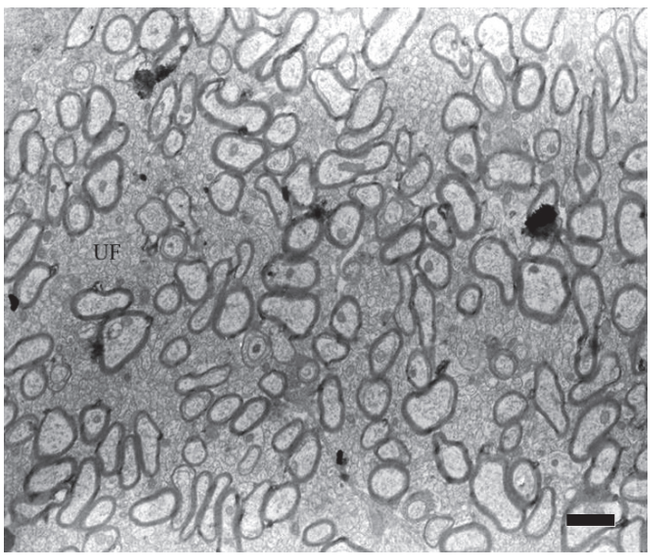

Figure 3: Electron micrograph of the splenium from a representative malnourished animal. UF: unmyelinated fiber, Bar: 1µm.

2003). We have not found previous works showing calculations of CC fractal dimension. Kiselev et al., (2003) determined fractal dimension of the whole cerebral cortex by a volumetric method from data obtained by magnetic resonance imaging, a different methodology than that used by us.

Microscopic Study

Diverse factors can affect the normal histology of CC. For example, the effects of alcohol consumption as well as thiamin deficiency on CC size have been studied in rats (He et al., 2007). When examined at the electron microscopy, the CC from those animals showed thinner diameters, higher fiber density, a high proportion of small diameter fibers and thinner myelin sheaths than groups ingesting either alcohol-thiamin or water-thiamin (He et al., 2007).

Concerning the reported inverse relationship between axon diameter and density (Aboitiz, 1992), our data showed that the callosal splenium evidenced a significant decrease in diameters in both myelinated and unmyelinated fibers ($p < 0.05$; $p < 0.001$, respectively) and an increase of density of unmyelinated fibers in malnourished animals ($p < 0.05$), but not of myelinated fibers (Fig. 2 and 3). These observations are in line with previous studies in rats subjected to prenatal protein malnutrition. These animals, though calorically compensated, exhibited significant smaller axon diameters in the splenium while densities of both types of fibers were unchanged (Olivares et al., 2007). Differences observed in the current work are stronger than those detected by Olivares et al. (2007), probably due to less severity of the malnutrition model used in that study. In addition, we have presently increased the number of animals per group. Whether there was postnatal axon growth, which may compensate during lactancy the effects of prenatal malnutrition, remains unanswered but possible mechanisms involving development of myelin and axon glial sheaths are expected.

CONCLUSIONS

We conclude that malnourished animals show significant reductions in both total and partial callosal areas as well as in callosal perimeters. However, prenatal malnutrition did not modify the geometry associated to morphologic complexity of brain and CC, according to our calculations of fractal dimensions, finding an isometric reduction of callosal measurements. In fact, we found isometric reductions in any measures of brain parts, thus meaning that there are changes in the absolute values of the measurements obtained but not in the relationship between these measures. Therefore, these isometric variations do not affect inferences about relationships, and the causes of these geometric variations are changes in the size of the whole body (Bonner and Horn, 2000; Brown et al., 2000).

Finally, both myelinated and unmyelinated types of fibers decreased significantly in diameter, and a concomitant increase of unmyelinated fiber density, but not of myelinated axons, was observed. These findings suggest that cortico-cortical (interhemispheric) connections are vulnerable to prenatal malnutrition and this may affect interhemispheric conduction velocity, particularly in relation to visual connections (splenium).

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REFERENCES

ABOITIZ F (1992) Brain connections: interhemispheric fiber systems and anatomical brain asymmetries in humans. Biol Res 25: 51-61.

ABOITIZ F (1996) Does bigger mean better? Evolutionary determinants of brain size and structure. Brain Behav Evol 47: 225-245.

ABOITIZ F, IDE A, OLIVARES R (2003) The Cognitive Neuroscience of the Corpus Callosum. Corpus Callosum Morphology in Relation to Cerebral Asymmetries in the Postmortem Human. In: Zaidel E, Iacoboni M, editors. A Bradford Book The Mit Press Cambridge, Massachussets; pp: 33-49.

BONNER JT, HORN HS (2000) Allometry and Natural Selection in Scaling in Biology a volume in the Santa Fe Institute Studies in the Sciences of Complexity. Oxford University Press; pp: 60-73.

BROWN JH, WEST GB, ENQUIST BJ (2000) Patterns and Processes, Causes and Consequences**.** in Scaling in Biology a volume in the Santa Fe Institute Studies in the Sciences of Complexity. Oxford University Press; pp: 1-50.

CANALS M, OLIVARES R, LABRA F, CAPUTO L, RIVERA A, NOVOA FF (1998) Measuring the fractal geometry of the bronquial tree in mammals. Rev Chil Anat 16:237-244.

FERRÁN M (2001) Análisis estadístico SPSS para Windows. Osborne McGraw-Hill; pp: 1-165.

FORBES WB, RESNICK O, STERN WC, BRONZINO JD, MORGANE PJ (1975) The effect of chronic protein malnutrition on trans-callosal evoked responses in the rat. Devl Psychobiol 8: 503-509.

FOWDEN AL, GIUSSANI DA, FORHEAD AJ (2006) Intrauterine programming of physiological systems: causes and consequences. Physiology (Bethesda) 21: 29-37.

GIL J, GIMENO M, LABORDA J, NUVIALA J (2006) Fractal dimension of dog kidney proximal convoluted tubuli sections by mean box-counting algorithm. Int J Morphol 24: 549-554.

GRESSENS P, MUAKU SM, BESSE L, NSEGBE E, GALLEGO J, DELPECH B, GAULTIER C, EVRARD P, KETELSLEGERS JM, MAITER D (1997) Maternal protein restriction early in rat pregnancy alters brain development in the progeny. Dev Brain Res 103: 21-35

GUNDERSEN HJ (1977) Notes on the estimation of the numerical density of arbitrary profiles. The edge effect. J Microsc 111: 219-223.

GUZMÁN M, MARTÍN MA, MORÁN M, REYES M (1993) Estructuras Fractales y sus Aplicaciones. Labor S.A. Barcelona; pp: 1-230.

HE X, SULLIVAN EV, STANKOVIC RK, HARPER CG, PFEFFERBAUM A (2007) Interaction of thiamine deficiency and voluntary alcohol consumption disrupts rat corpus callosum ultrastructure. Neuropsychopharmacol 32: 2207–2216.

KISELEV VG, HAHN KR, AUER DP (2003) Is the brain cortex a fractal? Neuroimage 20: 1765-1774.

KOLB B (1984) Functions of the frontal cortex of the rat: a comparative review. Brain Res Rev 8: 65-98.

LEVITSKY DA, STRUPP BJ (1995) Malnutrition and the brain: changing concepts, changing concerns. J Nutr 125: Suppl 2212-2220.

MCMILLEN IC, ROBINSON JS (2005) Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 85: 571-633.

MERLO A, ALBANECE AM, MIÑO J, GÓMEZ E, SAUBIDET A, MASCITTI T, INGRATA A, ALBANECE E (2002) Corpus callosum in the midsagital planum of magnetic resonance image: perimeter, surface and shape in relation with age and sex. Rev Chil Anat 20: 131-138.

MORGANE PJ, MILLER M, KEMPER T, STERN W, FORBES R, HALL J, BRONZINO J, KISSANE J, HAWRYLEWICZ J, RESNICK O (1978) The effects of protein malnutrition of the developing central nervous system in the rat. Neurosci Biobehav Rev 2: 137-230.

MORGANE PJ, AUSTIN-LA FRANCE R, BRONZINO J, TONKISS J, DIAZ-CINTRA S, CINTRA L, KEMPER T, GALLER JR (1993) Prenatal malnutrition and development of the brain. Neurosci Biobehav Rev 17: 91-128.

NATIONAL RESEARCH COUNCIL, GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS (1996) National Institutes of Health, Bethesda; Publications 80-23 rev.

NAVARRETE M, NÚÑEZ H, RUIZ S, SOTO-MOYANO R, VALLADARES L, WHITE A, PÉREZ H (2007) Prenatal undernutrition decreases the sensitivity of the hypothalamo-pituitary-adrenal axis in rat, as revealed by subcutaneous and intra-paraventricular dexamethasone challenges. Neurosc Letters 419: 99-103.

OLIVARES R, SOTO-MOYANO R, HERNÁNDEZ A. ABOITIZ F (2002) Mild protein prenatal malnutrition specifically affects development of the anterior corpus callosum. Rev Chil Anat 20: 159-163.

OLIVARES R, SOTO-MOYANO R, HERNÁNDEZ A, GIL J, GIMENO M, LABORDA J, ABOITIZ F (2007) Effect of mild protein prenatal malnutrition in the callosal splenium histology. Int J Morphol 25: 717-721.

SOTO-MOYANO R, HERNÁNDEZ A, PÉREZ H, RUIZ S, CARREÑO P, BELMAR J (1993) Functional alterations induced by prenatal malnutrition in callosal connections and interhemispheric asymmetry as revealed by transcallosal and visual evoked responses in the rat. Exp Neurol 119: 107-112.

SOTO-MOYANO R, HERNÁNDEZ A, PÉREZ H, RUIZ S, CARREÑO P, ALARCÓN S, BELMAR J (1994) Clonidine treatment during gestation prevents functional deficits induced by prenatal malnutrition in the rat visual cortex. Int J Neurosci 76 : 237-246.

SOTO-MOYANO R, ALARCÓN S, BELMAR J, KUSCH P, PÉREZ H, RUIZ S, HERNÁNDEZ A (1998a) Prenatal protein restriction alters synaptic mechanisms of callosal connections in the rat visual cortex. Int J Dev Neurosc 16: 75-84.

SOTO-MOYANO R, ALARCÓN S, HERNÁNDEZ A, PÉREZ H, RUIZ S, CARREÑO P, KUSCH C, BELMAR J (1998b) Prenatal malnutrition-induced functional alterations in callosal connections and in interhemispheric asymmetry in rats are prevented by reduction of noradrenaline synthesis during gestation. J Nutr. 128: 1224-1231.

STERN WC, PUGH WW, JOHNSON A, MORGANE PJ (1983) Spontaneous forebrain neuronal activity in developmentally protein malnourished rats. Brain Res 285: 95-98.

WITELSON SF (1989) Handedness and sex differences in the isthmus and genu the human corpus callosum: a postmortem morphological study. Brain 112: 799-835.

ZIMMERBERG B, MICKUS LA (1990) Sex differences in corpus callosum: influence of prenatal alcohol exposure and maternal undernutrition. Brain Res 537: 115-122.