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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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Gastrodia elata Blume and its pure compounds protect bv-2 microglial-derived cell lines against β -amyloid: the involvement of GRP78 and CHOP

A light- and electron microscopic study of primordial germ cells in the zebra fish (*Danio rerio*)

Nazan Deniz Koç¹, Rikap Yüce²

¹ Sakarya University, Faculty of Science and Letters, Department of Biology, Sakarya-Turkey

² Marmara University, Faculty of Science and Letters, Department of Biology, Istanbul-Turkey

ABSTRACT

In sexually reproducing organisms, primordial germ cells (PGCs) give rise to the cells of the germ line, the gametes. In many animals, PGCs are set apart from somatic cells early during embryogenesis. This study explores the origin of primordial germ cells (PGCs) of the zebra fish and examines their morphology during early development (1st day-15th day). PGCs were selectively stained by the alkaline phosphatase histochemical reaction and viewed by light and electron microscopy from the time they are first detectable in the yolk sac endoderm. PGCs occurred in the subendodermal space on the syncytial periblast; differing from the surrounding endodermal cells. Later the PGCs moved to between the blastoderm and yolk sac and transferred to the dorsal mesentery where they formed gonadal anlage with mesoderm cells. PGCs were easily distinguished from somatic cells by their morphology and low electron density of their nuclei. Under light microscopy, PGCs were rounded with a distinct cytoplasmic membrane.

Key Words: Primordial germ cell, zebra fish, ultrastructure, morphology.

INTRODUCTION

In many different organisms, primordial germ cells (PGCs) are specified early in development in a position that is distinct from that of the gonad, the location where they differentiate into gametes (Nieuwkoop and Sutasurya 1979, Santos and Lehmann 2004, Raz 2004). Therefore, the cells have to reach the somatic part of the gonad in a process that, in addition to its crucial role in the propagation of the species, serves as a general model for long-range cell migration. PGC migration has been studied in diverse vertebrate and invertebrate species. These investigations highlighted the important role of somatic cells that act along the migratory route, offering a permissive environment for cell migration and directing the cells by providing attractive or repulsive cues.

Fish PGCs appear similar to those of mammals, reptiles and amphibians, in being large and having a large and spherical nucleus, and numerous lipid droplets in the cytoplasm (Fuyuta et al. 1974, Yasuda et al. 1992). There are numerous studies in the literature explaining the structural and histological features of primordial germ cells (Bendel-Stenzel et al. 1998, Braat et al. 1999b, Buehr 1997, Dixon 1994, Hamaguchi 2004c, Kobayashi 2003, Mackay et al. 1989, Raz 2004). Germ cell specification in fish was studied mainly in bony fish (teleosts) where the work relied on observations using light and electron microscopy. Early germ cells were studied by electron microscopy chiefly in freshwater species such as *Oryzias* (Hogan 1973), *Brachydanio* (Baumeister 1976), *Salmo* (Upadhyay 1977) and *Mugil* (Brusle 1978). The germ cells were recognized by their large size and by the typical electron dense nuage-like structures. Their peripheral cytoplasm shows high alkaline phosphatase activity; alkaline phosphatase is used as indicator enzyme. Alkaline phosphatase (an enzyme which is found in the cell or

in the cell membrane and active at pH 8.5-10 with the ability of detaching phosphate groups) activity was also observed in the peripheral cytoplasm of primordial germ cells.

It thus seemed of interest to use electron and light microscopy to define the histological characteristics of primordial germ cells in the embryo and larva of zebra fish.

MATERIAL & METHODS

Light Microscopy

The zebra fish embryos and larvae were fixed in Bouin's solution for 8 hours. Fixed embryos and larvae were dehydrated and embedded in paraffin wax and sectioned transversely at 5-7 µm thickness and stained with Hematoxylin&Eosin, Best Carmin, and Alkaline Phosphatase stain. Samples were evaluated by examining under light microscope.

Electron Microscopy

For transmission electron microscopy individual eggs were fixed by immersion in a solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 4 h. The chorion was subsequently removed and the eggs were fixed further overnight at 4° C using 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. After an additional fixation with 1% OsO₄ and pre-embedding staining with 1% uranyl acetate, eggs were dehydrated and embedded in Embed 812 resin. The sectioning was performed using a Leica Ultracut ultramicrotome. Thick sections were stained with toluidine blue and visualized in an Olympus light microscope to select the area of interest.

Corresponding Author: Nazan Deniz KOÇ. Address: Sakarya University, Faculty of Science and Letters, Department of Biology, Sakarya-Turkey. Fax Number:+902642955950. E-mail address: ndy31@hotmail.com

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Thereafter, thin sections were collected and counterstained with 1% uranyl acetate and lead citrate and examined using a transmission electron microscope.

RESULTS

In this study, histology and morphology of primordial germ cells in zebra fish embryos and larvae were examined following fertilization. Primordial germ cells in zebra fish were first identified in the segmentation phase after fertilization. Since primordial germ cells differ from somatic cells in their morphology and histology, these cells can be identified easily. In the development phase of embryo and larva, some changes were observed in the morphology and histology of cells in specific stages. It was observed that migrating cells were lengthened and spindle-shaped whereas static cells were rounded-elliptical shaped. These cells had more granulated structure than the somatic cells. "nuage material", which plays an important role in the identification of primordial germ cells in later phases of development, was first identified in the larval period.

Morphology: Primordial germ cells were identified according to their structural (morphological) properties; round shapes, large sizes, large nucleus and marked nuclear membrane. They are characterized by high electron cytoplasm density and have a very irregular outline.

Cytology:

Nuclear characteristics: The nucleus is voluminous (nucleus/cytoplasm) and eccentrically located. The outline of the nuclear envelope is irregular and the nucleoplasm contains widely dispersed chromatin. The single nucleolus situated eccentrically consists of center and a large.

Cytoplasmic characteristics: It was observed that these cells were morphologically larger and stained more densely than peripheral somatic cells. Furthermore, they were more evident in Best Carmin staining because glycogen density was greater. In the Best Carmin staining, dense red granular bodies were identified in the cytoplasm of these cells. Also, the structures with granules known as "nuage materials" were seen in the primordial germ cells studied with this staining method. In the identification of primordial germ cells during the light microscopy stage, "nuage material" played very important role due to its shape. Many ribosomes are freely dispersed throughout the cytoplasm, making it very electron-dense. Most mitochondria are round-shaped.

Light microscopy:

In the first day after fertilization, primordial germ cells in larger sizes than normal were observed under the endomesoderm in the area next to the vitellus and around the blastoderm. In the first 24 h after fertilization, the size of primordial germ cells was approximately 10-20 μm . When the cell morphology was examined in detail, it was observed that the cell structure was dense and granular. These dense granular bodies were considered as ribosomes or glycogen granules. It was observed that primordial germ cells were surrounded by somatic cells and they were positioned as groups of 2-3 cells near the vitellus in the dorsal side of

blastoderm. The cells were identified according to their large sizes, large nuclei and sometimes by their structure including two nucleoli. Primordial germ cell cytoplasm was more granular than the somatic cell cytoplasm. The size of primordial germ cell nucleus was approximately 6-8 μm and it covered the majority of the cytoplasm. It was observed that elliptical-shaped cell nucleus was modified by cytoplasmic alterations during the migration (Figure 1 a,b).

At day 5 post fertilization (5dpf), primordial germ cells were in the upper side of body cavity (in the area of gonadal anlage) and in the inferior boundaries of the vitellus. The measurements demonstrated that the cell sizes were between 10 and 20 μm . Primordial germ cells were considerably larger than the surrounding cells. In the semi-thin sections of pre-5 day old larvae (prelarvae) it was found that the elliptical shape of primordial germ cells was changed into a spindle form (This situation is related to alterations in cell shapes during migration). Developing primordial germ cells moved across the body close to the vitellus sac (Figure 1 c).

In the 7-day old zebra fish prelarvae, primordial germ cells were seen closer to the body cavity and in the boundaries of the vitellus. The cell sizes were not changed. The cells were nearly 10-20 μm and they were larger than somatic cells. The nuclei were large as in the other sections and they covered the majority of cytoplasm. Because granular structures called as nuage materials are specific to primordial germ cells, they could be seen obviously in the sections. These granules were observed as distributed in the cytoplasm around the nucleus (Figure 1 d).

We preferred this staining method because of high alkaline phosphatase activity in primordial germ cells. **In 13-day and 15-day old larva,** the cells with high alkaline phosphatase activity were stained as dark brown or black via Best Carmin staining. In this period, the cells were in the dorsal side of the vitellus sac and between the body cavity and somites, and were approximately 12 μm in size. The nuclei of primordial germ cells were large and 6-10 μm in size. It was determined that in these cells alkaline phosphatase activity in the nucleus and surroundings was quite high. The cells were monitored in the same sections stained by this method and the comparator staining method and it was confirmed that these cells were definitely primordial germ cells. The differences in size between primordial germ cells and somatic cells could be monitored easily in semi-thin sections of 1 μm stained by toluidine blue (Figure 1e,f and Figure 2a).

Electron microscopy: According to the results of examination by electron microscope, primordial germ cells from embryos and larvae of zebra fish were elliptical or flat due to the direction of section. The nuclei of primordial germ cells covered the majority of the cytoplasm and their boundaries were irregular. Chromatin was distributed homogeneously in the nucleus cytoplasm. The nucleolus was clear. The cytoplasm of primordial germ cells (germinal cytoplasm) was stained clearly. The cytoplasm was dense and granular. Although there were ribosomes, glycogen granules and many mitochondria in the cytoplasm, endoplasmic reticulum and the Golgi complex were insufficiently developed. In the comparison of primordial germ cells and

surrounding somatic cells, it was determined that there were differences in terms of size and structure. Primordial germ cells had more mitochondria compared to somatic cells. Also, electron-dense structures were striking in the cytoplasm. These structures were not surrounded by a membrane. The structure of cytoplasm was thin-fibrillary and reticular. Lipid droplets were seen sporadically in the germinal cytoplasm. It was noticed that nuage material was more dense around the mitochondria (Figure 2b,c,d).

DISCUSSION

Primordial germ cells in zebra fish embryos were examined as of the 10th-11th hour after fertilization (10 hpf) by light microscope and the structure of these cells was similar to that of members of Osteichthyes such as medaka (Hamaguchi

2004 a,b,c) and carp (van Winkoop et al. 1992, Winkoop et al. 2004). In hatched fish, primordial germ cells can be recognized histologically by their large size, low nucleus/cytoplasm ratio, clear nuclear borders, and granular nuclear chromatin (Dietrich and Krieger 2009). It was difficult to distinguish PGCs from other somatic cells before the bud stage using morphological features, especially their size and shape. In this study, primordial germ cells were detected in the later stages of the blastula phase (4hpf; late blastula phase). According to some of the literature it is not possible to detect the primordial germ cells as tissue before the segmentation phase. Primordial germ cells have different features morphologically and histologically than somatic cells. These cells are known to be more granular than somatic cells. The differences between the primordial germ cells and somatic cells were detected by PAS, Best Carmin and alkaline phosphatase staining methods.

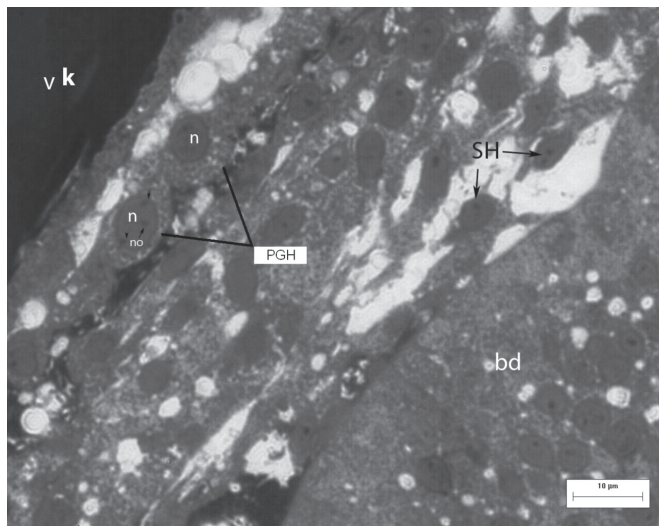


Figure 1. Primordial germ cells (PGH), blastoderm (bd), somatic cell (SH), vitellus sac (vk), nucleus (n), nucleolus (no) observed between blastoderm layer and vitellus sac in 1-day zebra fish embryo (x100).

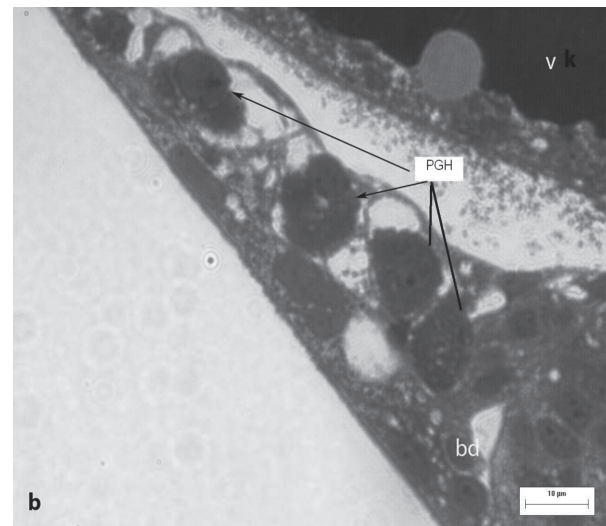


Figure 2. Primordial germ cells (PGH), blastoderm (bd), vitellus sac (vk) detected in the terminal area of blastoderm in 1-day zebra fish embryo. Toluidin blue. a-(x40),b- (x100).

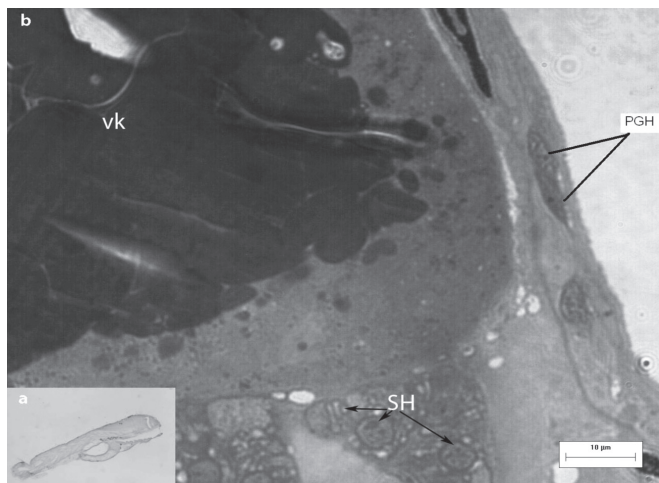


Figure 3. a- 5-day zebra fish prelarva, b-Primordial germ cells (PGH), vitellus sac (vk), somatic cell (SH) observed in 5-day zebra fish prelarva. Toluidin blue a-x4, b-x100.

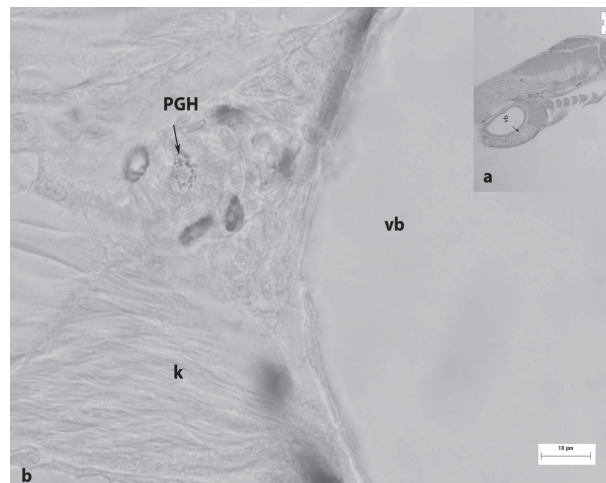


Figure 4. a- 7-day prelarva; b- Primordial germ cells (PGH), muscle layer (k), body cavity (vb) detected in 7-day prelarva. Best Carmin Staining x 100.

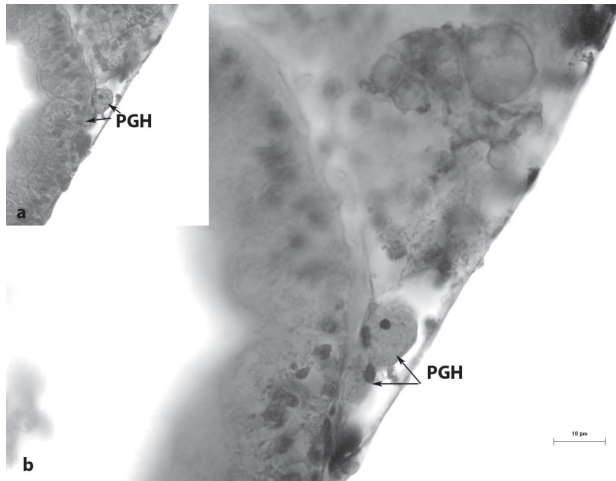


Figure 5. Primordial germ cell in 13-day larva.

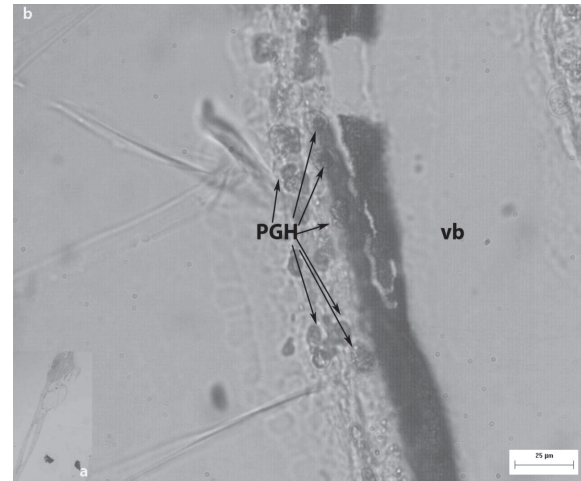


Figure 6. a-15-day zebra fish larva, b- Primordial germ cells (PGH), somatic cell (SH), body cavity (vb) chosen in 15-day zebra fish larva. Alkaline phosphatase stain. a-x4, b-x40

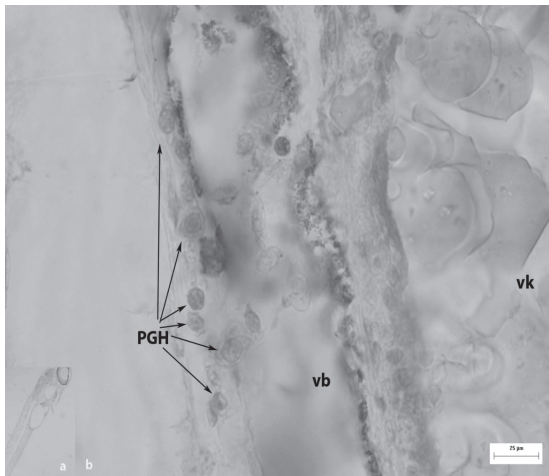


Figure 7. a-15-day zebra fish larva, b- Primordial germ cells (PGH), vitellus sac (vk), body cavity (vb) chosen in 15-day zebra fish larva. Toluidin blue. a-x4, b-x40. Best Carmin a-x4, b-x40.

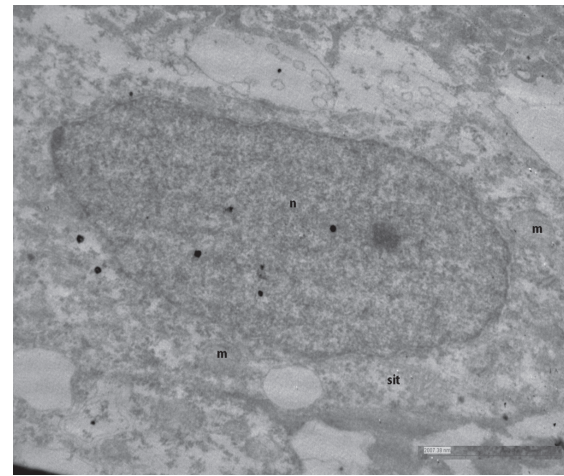


Figure 8. Thin structure of primordial germ cell (PGH), nucleus (n), cytoplasm (sit), mitochondria (m) in section area from blastoderm boundary in 7-day zebra fish embryo. X 15.000

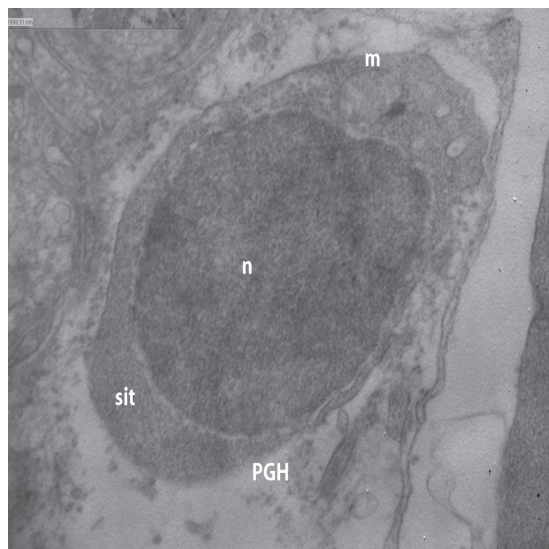


Figure 9. Primordial germ cell (PGH), nucleus (n), cytoplasm (sit), mitochondria (m) seen in boundaries of body cavity in 15-day zebra fish embryo. X30000.

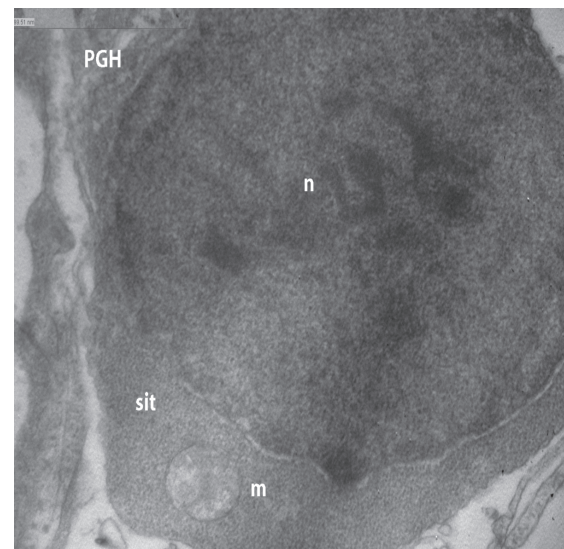


Figure 10. Primordial germ cell (PGH), nucleus (n), cytoplasm (sit), mitochondria (m) seen in boundaries of body cavity in 15-day zebra fish embryo. X30000.

Primordial germ cells in Osteichthyes were first identified in peripheral (surrounding) endoderm in 46-53 hour old *Fundulus heteroclitus* embryos by Richards and Thompson (1921). In this study, it was emphasized that there was a close relationship between the periblast layer and primordial germ cells. Johnston (1951) showed the presence of primordial germ cells in the exaggerated end of the periblast, bulging at the caudal end (tail area) of the blastodisc in *Micropterus salmoides*. It was detected that these cells moved from the caudal bulge of the yolk and in the later phases they migrated to the caudal periblast. Primordial germ cells in *Gambusia holbrooki* (Pala 1970) were observed in the early gastrula phase partially in the caudal portion of the blastodisc. Nedelea & Steopoe (1970) specified that primordial germ cell numbers were limited in the mesodermal layer of embryonic shield towards the end of vitellus growth in carp. Primordial germ cells were clearly distinguishable from somatic cells in the embryonic stage by the appearance of granules of melanin pigment in the eyes in medaka fish. The cells characteristically had a large size of the cell and nucleus (about 15 and 8/ μ m in diameter respectively), a distinct round outline, a less stained cytoplasm, and a single, clear nucleolus (Satoh and Egami 1972). Primordial germ cells (PGC) were oval unstained cells in *Epinephelus marginatus*. They were relatively large (12-24 μ m in diameter) and usually arranged in clusters of a few cells, both along the edge and the axis of the ovigerous folds. The diameter of the nucleus ranged between 9 and 15 μ m. The nucleus/cytoplasm ratio was about 0.7 (Mandich et al. 2002). The sizes of primordial germ cells cleaving from the somatic cells were examined in the early segmentation phase of *Micrometus aggregatus* (Eigenmann 1981). Primordial germ cells in carp were first observed in the second week of fertilization and then an increase was detected in the cell size in the 2nd-4th weeks. In previous studies, it was explained that there was a close relationship between the electron-dense structures and mitochondria and the presence of marked Golgi complex was shown (Van Winkoop et al. 1992). On the other hand, in primordial germ cells of zebra fish, the same close relationship was demonstrated for mitochondria and nuage material (deeply stained areas in the polyribosome structure) but the Golgi complex was insufficiently developed. In the 3rd month of fertilization in *Mugil cephalus* elliptical and large primordial germ cells with large nucleus were observed in gonads (Chang et al. 2005). In a study of *Gymnogobius* species, it was reported that primordial germ cells were elliptical-shaped with large nucleus and markedly surrounded by a nuclear membrane. In this study, primordial germ cells were found to be 10-20 μ m in the experimental species. Primordial germ cells in this species were monitored from the phase with 10 somites (Saito et al. 2004). On the other hand, in our study we detected primordial germ cells in 10th-11th hr approximately and observed that cells were in a close relationship with the periblast. The primordial germ cells and their nuclei were larger than somatic cells. Primordial germ cells were approximately 10-20 μ m. We observed that there were deeply stained granules around the nucleus in PAS and Best Carmin staining in zebra fish. Also a few eosinophilic granules were observed in the cytoplasm near the nuclei of primordial germ cells in medaka (*Oryzias latipes*) (Hamaguchi 2004a, b). Because the dense structures were stained especially around the nucleus in primordial germ cells in *Barbus conchoniensis*, they were considered as similar to "nuage material", and following staining with eosin and

gallocyenin, proteins and RNA could be seen easily (Gevers et al. 2004). Furthermore, it was shown that this nuage material observed around the nucleus membrane was in a close relationship with mitochondria. The nuage-mitochondria relationship was considered as an important parameter for germ cell development. The relation of nuage-mitochondria structure was examined in *Piaractus mesopotamicus* by Abdalla and Cruz-Landim (2004) and our results are consistent with their findings. Abdalla and Cruz-Landim reported that germ cell differentiation could not be occurring in the periods of defective mitochondria-nuage relation in the cytoplasm. Thin structure studies play an important role in understanding the structure of primordial germ cells. On the other hand, information about primordial germ cells in fish is limited in the literature. One of these studies was performed in *Cyprinus carpio* and gonadal development and primordial germ cell differentiation were identified in a six-week incubation period (Van Winkoop et al. 1992, Winkoop et al. 2004). It was noted that the cells differentiated rapidly following this period and due to the increase in the cell size, perinuclear dense structures became more pronounced. It was emphasized that a dense Golgi complex was detected in these cells by a thin structure study and in parallel to the size increase of cells, synthetic activity increased. In our study the Golgi complex was not detected rigorously whereas the cells were rich in ribosomes and mitochondria. vasa RNA embedded in electron-dense structures similar to the nuage material was found in *O. latipes* via a TEM study (Hamaguchi 2004c).

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Malnutrition affects the pressor response to microinjection of L-glutamate into the RVLM of awake rats

Fabiana Aparecida Rodrigues¹; Deoclecio Alves Chianca-Jr² and Luciano Gonçalves Fernandes^{3*}

¹ Nucleus of Biological Research (NUPEB), Federal University of Ouro Preto (UFOP). Ouro Preto, MG, Brazil – 35400-000.

² Department of Biological Sciences, Federal University of Ouro Preto (UFOP). Ouro Preto, MG, Brazil – 35400-000.

³ Department of Physiological Sciences, Federal Rural University of Rio de Janeiro (UFRRJ). Seropédica, RJ, Brazil – 23890-000.

ABSTRACT

Post-weaning protein malnutrition is often related to the development of cardiovascular and metabolic diseases in humans, as well to changed content of neurotransmitters in the central nervous system under experimental conditions. The rostral ventrolateral medulla (RVLM) is a bulbar region that contains sympathetic premotor neurons; the excitatory amino acid L-glutamate seems to be the main neurotransmitter at this level. The aim of the present study was to evaluate the possible change in the L-glutamate sensitivity of the RVLM neurons of malnourished animals. Male Fischer rats were divided into two groups: control (n = 15) and malnourished (n = 19). Four days before the experiments, guide cannulas were implanted bilaterally in direction of the RVLM for microinjection of L-glutamate. Twenty-four hours before the experiments, the femoral artery was cannulated for cardiovascular recordings. The results showed that the baseline heart rate increased in malnourished compared to control animals (412.18 ± 16.03 bpm *vs.* 370.74 ± 9.59 bpm, respectively). Malnourished animals presented a dissimilar concentration-dependent pressor response curve to L-glutamate and an attenuated baroreflex gain. Our results suggest that post-weaning protein restriction affects glutamatergic neurotransmission of the baroreflex at the RVLM level.

Key terms: baroreflex, blood pressure, L-glutamate receptors, heart rate, malnutrition, RVLM.

INTRODUCTION

Malnutrition denotes a nutritional state in which the diet does not provide adequate calories and protein for growth and organic maintenance (UNICEF, 2011). According to the World Health Organization, malnutrition is the biggest contributor to child mortality; hunger, underweight births and intra-uterine growth restrictions cause 2.2 million child deaths a year. (World Health Organization, 2009). Besides causing mortality, malnutrition often leads to morbidity. Studies from the literature (Cohen, 2004; Sawaya et al., 2003) suggest that a period of malnutrition during early childhood is directly related to development of hypertension and metabolic imbalance in adult life. These observations are in accordance with the “nutritional programming” hypothesis for cardiovascular diseases (Barker, 2007; Benabe and Martinez-Maldonado, 1993; Roseboom et al., 1999; Roseboom et al., 2000; Roseboom et al., 2000b).

Previous studies from our lab have shown that after-weaning malnutrition affects neural mechanisms involved in cardiovascular homeostasis (Bezerra et al., 2011a; Bezerra et al., 2011b; Loss et al., 2007; Martins et al., 2011; Oliveira et al., 2004; Penitente et al., 2007; Tropa et al., 2001). While the exact mechanism remains to be understood, there is evidence indicating that malnutrition can affect the release of neurotransmitters and receptor affinity in several areas of the central nervous system (El et al., 1999; Gotoh et al., 1996; Seidler et al., 1990). The rostral ventrolateral medulla (RVLM) is a bulbar site among the neural pathways of cardiovascular reflexes (e.g., baroreflex); it contains reticulo-spinal neurons that innervate the pre-ganglionic sympathetic neurons (Ross et al., 1984). According to anatomical (Aicher et al., 1995; Ross

et al., 1984) and experimental evidence (Lipski et al., 1996a; Lipski et al., 1996b; Sun et al., 1988a; Sun et al., 1988b; Sun et al., 1988c), it is well accepted that neurons located in the RVLM are involved in the maintenance of arterial pressure within levels compatible with life, through modulation of sympathetic activity. The RVLM has been associated with pathophysiological mechanisms that cause cardiovascular diseases (Colombari et al., 2001; Dampney et al., 2003; Guyenet, 2000).

Although there is evidence that malnutrition affects the cardiovascular system, there are few studies addressing the impact of malnutrition on the CNS areas involved in cardiovascular neural reflexes. Based on the assumption that the RVLM is a key region for sympathetic efferent activity and that L-glutamate is the major excitatory neurotransmitter in this area (Machado et al., 1997), the main objective of the present study was to evaluate the impact of malnutrition on the pressor response to microinjection of L-glutamate into the RVLM of freely moving malnourished rats.

METHODS

Animals

Male Fischer rats were used in this study. After birth, the offspring were randomly chosen and eight pups per dam were kept. The five dams used in the present study received normal rat chow (15% protein content) and water *ad libitum*. The pups were weaned after 28 days and then the males were divided into two groups according to the diet received; the control group received a diet containing 15% protein, and the experimental malnourished group received a diet containing

*Corresponding author: Prof. Luciano G. Fernandes, PhD. Universidade Federal Rural do Rio de Janeiro, Br 465 Km 47, Universitário Instituto de Biologia, Departamento de Ciências Fisiológicas Seropédica, RJ, 23890-000. Tel: +55 31 2682-3222. Fax: +55 31 2682-1763. e-mail: fernandeslg@gmail.com

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only 6% protein. Both groups received this diet for the next 35 days, after which the rats were weighted. During the following seven days, the rats underwent experimental protocols and continued receiving experimental rat chow (normal or low protein diet) according to the experimental group. The low protein diet was isocaloric with respect to the control diet. All the components of the diets were purchased from Rhostrer (São Paulo, Brazil). During all experimental protocols, the rats remained in controlled laboratory conditions (12/12 h light/darkness cycle, temperature: 23–25 °C). There were 15 control rats and 19 malnourished rats. All the experimental protocols followed the guidelines of the Brazilian Society for Laboratory Animal Science (SBCAL).

Stereotaxic surgery

Under tribromoethanol anesthesia through a stereotaxic apparatus (David-Kopf, Tujunga, CA, USA), the rats were submitted to stereotaxic surgery and guide cannulae were implanted in the direction of the RVLM bilaterally, according to stereotaxic coordinates adapted from (Paxinos and Watson, 2007): 3.9 mm caudal to the Lambda; 8.0 mm below the skull surface and 1.8 mm lateral to the midline for the control rats, and 2.9 mm caudal to the Lambda; 7.0 mm below the skull surface and 1.7 mm lateral to the midline for the malnourished rats. The 15 mm-long guide cannulae were made from hypodermic needles (25 x 0.7 mm). The needles used for microinjections were made from 30 gauge needles (Unoject, Rio de Janeiro, RJ, Brazil) and were 2.0-2.5 mm longer than the guide cannulae. In order to perform microinjections in the RVLM, the microinjector needle was connected to a 1 μ L syringe (Hamilton, Reno, NV, USA) through a PE-10 catheter (Clay Adams, Parsippany, NJ, USA). Each microinjection had a volume of 50 nL.

Cannulation of the femoral artery

Five days after the stereotaxic surgery, the animals were submitted to a second anesthetic protocol to insert a polyethylene catheter into the abdominal aorta through the femoral artery. Under tribromoethanol anesthesia (2.5%, 1 mL/100g, i.p.), a polyethylene catheter (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ, USA) filled with heparinized saline was inserted into the abdominal aorta, through the left femoral artery, for measurement of pulsatile arterial pressure (PAP). The free end of the catheter was tunneled subcutaneously and emerged through the back of the neck.

Cardiovascular measurements

The next day, the animals were placed in the experimental room between 7:00 and 8:00 AM to allow them to acclimatize to the experimental room. At the time of cardiovascular recordings (at 1:00 to 1:30 PM), the arterial catheter was connected to a pressure transducer (Model MLT0699; ADInstruments Pty Ltd., Castle Hill, NSW Australia). It is important to emphasize that during the cardiovascular recordings, all animals were fully recovered from surgery and anesthesia protocols. Data were obtained through an analog-to-digital data acquisition system (Model PowerLab 400; ADInstruments Pty Ltd., Castle Hill, NSW, Australia) and were sampled at 12 bits using a 200-Hz sampling rate. Heart

rate (HR) and mean arterial pressure (MAP) were derived off-line from PAP using the Chart for Windows software, version 4.1.2 (ADInstruments Pty Ltd., Castle Hill, NSW, Australia). The baseline of cardiovascular variables, MAP and HR were recorded during the 15 min before each experimental trial. All trials were performed on conscious and unrestrained rats.

Dose-response curve to L-glutamate microinjection into the RVLM

After stabilization of the cardiovascular parameters, solutions of L-glutamate (1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM) were microinjected in random order. Each animal received at most three microinjections of L-glutamate or aCSF (artificial cerebrospinal fluid) in the RVLM in order to avoid extensive damage to the neural tissue. According to previous studies (Gotoh et al., 1996a; Patel et al., 2008) the physiological concentration at the RVLM of rats is 67 nM. Therefore, the physiological concentration is in the range of the concentrations of L-glutamate used in the present study.

Drugs and microinjections

L-Glutamate was dissolved in artificial cerebrospinal fluid (aCSF) in order to obtain final solutions in the following concentrations: 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM and 10 nM. The aCSF contained (in mM) 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose (pH was adjusted to 7.4 with Tris-base). All microinjections were made in a volume of 50 nL. All drugs and salts were purchased from Sigma-Aldrich Chemical (St. Louis, MI, USA).

Histology

At the end of the experiments, the rats were sacrificed with an overdose of sodium thiopental (Abbot Laboratories, USA) and perfused with saline followed by 10% formalin. The brains were removed, stored in formalin for at least 48 hours and sliced in 40 μ m thick coronal sections in a cryostat (Leica). Sections were stained with neutral red stain for histological analysis and only the brains of rats with positive sites of microinjections in the RVLM were used. In the histological analysis we identified the path of the microinjector needle in direction to the RVLM as well the center of the microinjections.

Data analysis

After histological confirmation of the correct microinjection site, we analyzed the following cardiovascular parameters from each positive animal: a) baseline mean arterial pressure and heart rate before any microinjection, b) pressor response to microinjection of L-glutamate in the RVLM and c) Δ HR (bpm) / Δ MAP (mmHg) (first derivative of the baroreflex function) as a bradycardic index to estimate baroreflex gain activated by the pressor response to L-glutamate microinjection in the RVLM of awake rats.

Statistical analysis

The data were represented as mean $\pm$ SEM and were analyzed by the paired Student t-test followed by Bonferroni post-test or one-way for repeated measures, followed by the Tukey post-test as needed. In all analyses, the significance level was fixed at 5%.

RESULTS

Impact of malnutrition on body weight

In the present study we observed that the post-weaning protein restriction reduced the body weight of the malnourished rats by 70% in comparison to the control group (78.05 ± 3.6 g vs. 256.3 ± 7.72 g, respectively) on the 35th day of low protein feeding. This result has been observed in previous studies from our lab and has been recognized as a validation for our malnutrition protocol.

Effect of malnutrition on baseline heart rate and mean arterial pressure

For the analysis of the baseline mean arterial pressure and heart rate, we selected 15 minutes of cardiovascular recordings before any central manipulation. The mean basal HR levels in the malnourished group were significantly higher in comparison with those observed in the control group (412.18 ± 14.25 bpm vs. 370.74 ± 9.59 bpm, respectively). Regarding the mean arterial pressure, there was no difference in basal levels observed in the malnourished group compared to the control group (109.24 ± 4.74 mmHg vs. 108.58 ± 2.87 mmHg, respectively).

Cardiovascular changes subsequent to L-glutamate microinjection into the RVLM of awake rats

Figure 1 represents changes in MAP (mmHg) following microinjections of different concentrations of L-glutamate in the RVLM of malnourished and control rats. We observed the following pressor responses to each L-glutamate solution microinjected in the RVLM: 1 fmol: 8.35 ± 0.95 vs. 6.3 ± 1.4 mmHg, 10 fmol: 16.025 ± 2.19 vs. 6.13 ± 3.03 mmHg, 100 fmol:

20.66 ± 4.12 vs. 7.57 ± 1.64 mmHg, 1 pmol: 14.23 ± 3.07 vs. 16.23 ± 4.43 mmHg, 10 pmol: 12.32 ± 4.9 vs. 8.45 ± 2.05 mmHg, 100 pmol: 21.02 ± 5.39 vs. 8.7 ± 2.23 mmHg, 1 nmol: 17.78 ± 1.01 vs. 30.6 ± 1.25 mmHg, 10 nmol: 23.92 ± 3.11 vs. 32.24 ± 5.35 mmHg, 100 nmol: 24.8 ± 4.68 vs. 36.43 ± 7.15 mmHg in malnourished and control rats, respectively. As a volume control, aCSF was microinjected in the RVLM of awake malnourished or control rats and we observed that this vehicle produced negligible effects on the MAP of both groups.

Baroreflex gain to L-glutamate microinjection into the RVLM

From each animal we selected a period of cardiovascular recordings including a baseline MAP and HR as well as the observed changes in MAP and HR subsequent to L-glutamate microinjection into the RVLM. These data were plotted and fitted in the following sigmoid logistic equation:

$$HR = \frac{HR_{max} + (HR_{min} - HR_{max})}{1 + e^{\frac{MAP_{50} - MAP}{p}}}$$

Here HR_{max} stands for the upper plateau, HR_{min} represents the lower plateau, MAP₅₀ is the MAP value at the midpoint of the HR range and p is the curvature. As a result, we obtained a baroreflex sigmoid curve. These data are summarized in Figure 2, which shows that malnourished animals present altered baroreflex function in comparison to control rats. We also determined the continuous baroreflex gain function by calculating the first derivative of the baroreflex curve in the range of the baroreflex sigmoid function, applying the formula:

$$y' = \lim_{\Delta MAP \rightarrow 0} \frac{\Delta HR}{\Delta MAP}$$

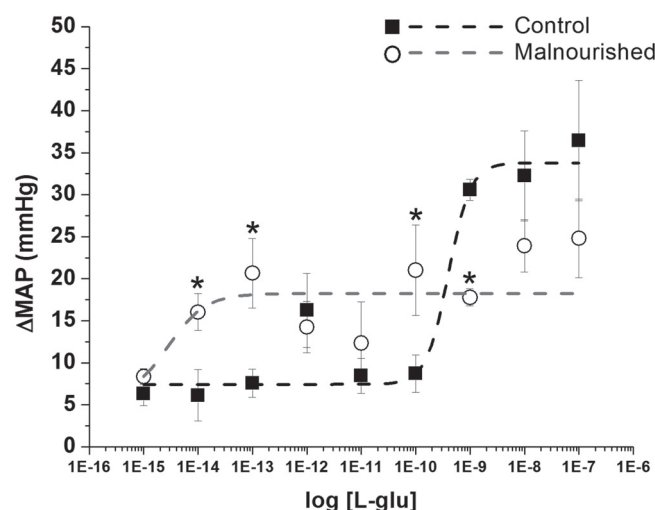


Figure 1: Concentration–pressor response curve (mmHg) obtained after the microinjection of different concentrations of L-glutamate (1fM, 10fM, 100fM, 1pM, 10pM, 100 pM, 1nM, 10 nM and 100 nM) into the RVLM of control (black squares, n=15) and malnourished (white circles, n=19) rats (* = different from the control, same concentration; $p < 0.05$). The dashed lines represent a sigmoidal regression of the data of the control (black line) and malnourished (gray line) groups.

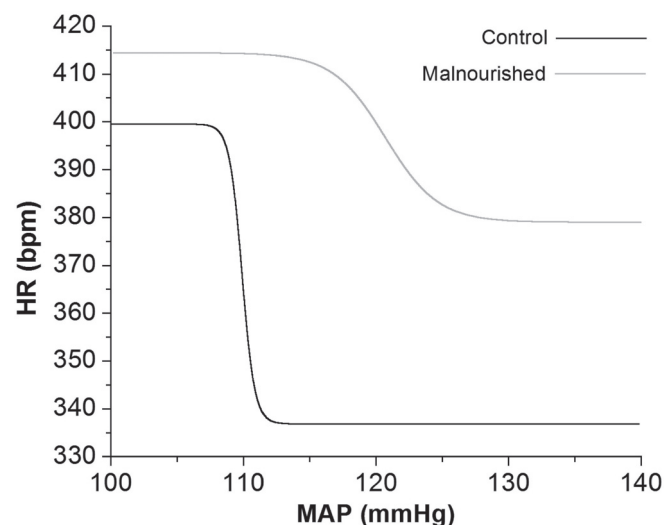


Figure 2: Baroreflex sigmoid curve of the control (black line, n=15) and malnourished rats (gray line, n=19).

Where y' represents the baroreflex gain (in bpm/mmHg), ΔHR the heart rate difference and ΔMAP the MAP difference. The highest baroreflex gain occurred at the MAP of 109.8 mmHg for the control animals at the rate of -32.9 bpm/mmHg, whereas the peak baroreflex gain occurred at 120.82 mmHg for the malnourished rats at the rate of -4.38 bpm/mmHg. The range of baroreflex gain can be assessed by this analysis; for the control group it was between 105.9 and 113.8 mmHg, while for the malnourished group it ranged from 108.8 to 130.16 mmHg. The data concerning this analysis are summarized in Figure 3.

DISCUSSION

The present study was undertaken to evaluate the sensitivity of the RVLM to L-glutamate of malnourished and control rats. We observed that rats submitted to malnutrition after weaning presented impaired sensitivity to L-glutamate microinjection in the RVLM. Moreover, the present study shows that the baroreflex gain is attenuated in rats submitted to protein restriction.

First, it is important to point out that our study was performed in awake and freely moving rats. This preparation is closer to the real physiological condition, since there is no influence of anesthetics on autonomic activity. It has been shown that the use of any anesthetic profoundly impacts the neural pathways of cardiovascular reflexes (Machado and Bonagamba, 1992). Also, it is important to note that the low protein diet used here had no calorie restriction, since it was isocaloric to the control diet.

The RVLM contains neurons whose activity seems to be related to the patterns observed in sympathetic vasomotor nerves. Therefore, those neurons are sensitive to changes in the main arterial pressure and show a pulse modulated activity at high levels of arterial pressure (Brown and Guyenet, 1985; Lipski et al., 1995; Lipski et al., 1996b; Morrison et al., 1988). The majority of these neurons project directly and/

or indirectly to the spinal cord, where they innervate the preganglionic sympathetic neurons (Milner et al., 1988; Ross et al., 1984). Under resting conditions, RVLM neurons are tonically inhibited by GABAergic afference from the caudal ventrolateral medulla – CVLM (Dampney, 1994; Schreihofer and Guyenet, 2002). Guertzenstein and Silver, (1974) depicted the RVLM as an important medullary region for the control and maintenance of baseline mean arterial pressure. It is therefore accepted that the excitatory premotor neurons of the RVLM are critical to maintain the activity of preganglionic sympathetic neurons (Dampney et al., 2003b; Dampney and Horiuchi, 2003). Most studies regarding the role of the RVLM in the maintenance of vasomotor sympathetic activity were conducted on anesthetized animals; stimulation of the RVLM increased the baseline mean arterial pressure (Bergamaschi et al., 1995; Campos and McAllen, 1999; McAllen, 1986), while bilateral lesions of the RVLM region reduced the baseline mean arterial pressure to levels similar to those observed after spinal cord transection (Dampney, 1994; Feldberg and Guertzenstein, 1976; Guertzenstein and Silver, 1974). On the other hand, a study (Cochrane and Nathan, 1989) observed that the cardiovascular responses to RVLM inhibition depended on the type of anesthetic protocol used, and the study of Bachelard et al. (1990), showing that chemical stimulation of the RVLM in conscious rats elicited a pressor response, raised the importance of investigating the role of RVLM in maintaining blood pressure in awake animals. Araujo et al. (1999) confirmed that the chemical stimulation of the RVLM with L-glutamate elicits a pressor response in awake rats. Therefore, the present study was conducted in awake and freely moving rats.

The first observation of the present study is that the microinjection of L-glutamate into the RVLM of malnourished rats provokes smaller pressor responses in comparison to those observed in control rats. In agreement with this observation, several studies in the literature report that malnutrition, irrespective of the model used, can cause changes in the neurotransmitter concentration in the CNS, in neurotransmitter/receptor affinity (Gotoh et al., 1996; Plagemann et al., 2000; Plagemann et al., 2006; Seidler et al., 1990; Zippel et al., 2003) and in the neuronal population and/or CNS nucleus morphology (Plagemann et al., 2000; Plagemann et al., 2006). These alterations may be responsible for deficits in memory and learning (Gray, 1989; Gray and Gray, 1989; Hall et al., 1983; Hall, 1983; Plagemann et al., 2000; Plagemann et al., 2006), motor activity (Gallo, 1981; Plagemann et al., 2000; Plagemann et al., 2006), affective behavior (Gallo, 1981; Hall et al., 1983; Hall, 1983), endocrine (Benabe and Martinez-Maldonado, 1993; Plagemann and Harder, 2009; Sawaya et al., 2003) and cardiovascular systems (Loss et al., 2007; Penitente et al., 2007; Tropia et al., 2001) observed in malnourished animals. Therefore, these changes in neurotransmitter release and/or receptor affinity may also be responsible for the impact of malnutrition on the cardiovascular system. Allied to this, rats submitted to protein malnutrition after weaning present increased pressor response to chemoreflex activation (Penitente et al., 2007), another important cardiovascular reflex. Loss et al. (2007) showed that the onset of the sympathoexcitation and parasympathoexcitation effects is changed in malnourished rats. These observations strengthen the hypothesis of an altered autonomic modulation in animals submitted to malnutrition.

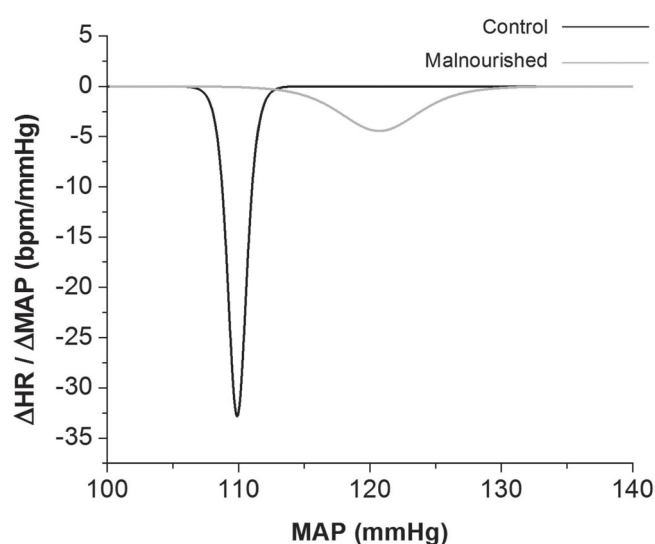


Figure 3: Baroreflex gain (expressed as bpm/mmHg²) associated with the pressor responses secondary to microinjection of different concentrations of L-glutamate into the RVLM of the control (black line, n=15) and malnourished (gray line, n=19) rats.

As depicted in Figure 2, the baroreflex curve is attenuated and shifted upward in malnourished animals, denoting malfunctioning of this important cardiovascular reflex in malnourished rats. We also analyzed the dynamic range of baroreflex gain and it was far greater in the malnourished than in the control rats. This is also reflected by the maximum baroreflex gain; while it occurred at normal levels in the control animals, the peak gain occurred at larger values in the malnourished animals. Moreover, although the baseline heart rate was greater in the malnourished animals in comparison to the control rats, the activation of baroreflex promoted by the pressor response to microinjection of L-glutamate into the RVLM and the subsequent sympathoinhibition directed to the heart (associated with parasympathoexcitation) produced only minor heart rate changes in the malnourished animals. This suggests that rats submitted to malnutrition present increased efferent sympathetic tonus. This is in accordance with previous study from our lab (Martins et al., 2011) which indicate that the low-frequency component of the heart rate variability is increased in malnourished rats. The increased sympathetic tonus was also suggested by Penitente et al (2007), so this may be responsible for the reduction in the baroreflex gain and range observed in these animals, and therefore for the increased baseline heart rate.

As limitations of the present study, we should consider that changes in the pressor response to L-glutamate microinjection into the RVLM could represent the impacts of malnutrition on the blood volume, cardiac output or structural changes of the peripheral vascular system. Moreover, the model of protein restriction used in the present study reflects a condition that very seldom is seen in humans (Sawaya et al., 2003). Our experimental model was formulated for the study of post-weaning protein restriction. Therefore, the rats were submitted to protein restriction beginning 28 days after birth, which is when weaning normally occurs. This model reflects a situation that occurs in many countries, where the newborn have adequate protein intake through breast feeding, but after weaning (which can be premature in some cases) the protein intake is greatly reduced because it is the most expensive diet component. There are few studies addressing post-weaning protein restriction, although the group of Dr. Sawaya has observed that teenagers from Brazilian slums are stunted and prone to develop metabolic and cardiovascular diseases (da Luz Santos et al., 2010; Franco et al., 2008). Moreover, this model seems to be a validated model to study the effects of protein restriction in a critical period of development on the occurrence of cardiovascular and metabolic diseases in adult life (Sawaya et al., 2003c). The changes in the pressor response to L-glutamate microinjection in the RVLM and in the baroreflex gain could be caused by changes in the neurotransmission and by changes in the neuronal morphology as well. In this regard, several studies have shown that malnutrition leads to changes in neurotransmitter systems (Benabe and Martinez-Maldonado, 1993; Seidler et al., 1990; Zippel et al., 2003). Moreover, Plagemann et al. (2000, 2006) showed that malnutrition causes malformations in the CNS nuclei involved in cardiovascular regulation. On the other hand, with our results we cannot assure whether this is the case in our experimental model of malnutrition. Further studies are needed to clarify this possibility. Also, there may be differences related to these two experimental models. Studies suggest that malnutrition during discrete periods of life (e.g., gestation, lactation, during weaning

and after weaning) can produce different levels of homeostasis imbalance; nevertheless, there is a consensus that protein deprivation in any period of life can lead to cardiovascular and metabolic imbalances (Barker, 2007; Cohen, 2004; El et al., 1999; Gallo, 1981; Oliveira et al., 2004; Roseboom et al., 1999). Unfortunately, there are few studies addressing post-weaning protein malnutrition and most of them are from our group. Another limitation may be related to the spread of L-glutamate to adjacent regions, affecting the cardiovascular responses to L-glutamate microinjection into the RVLM. For instance, the L-glutamate microinjected into the RVLM may diffuse and reach the CVLM, which contains GABAergic neurons that exert a tonic inhibition on the sympathetic premotor neurons of the RVLM (Dampney, 1994). Although CVLM is often regarded as a discrete region residing 1.0 – 1.5 mm caudal to the RVLM in the rat, barosensitive GABAergic neurons form a diffuse column of cells interspersed among other ventrolateral medullary neurons, including RVLM barosensitive neurons (Schreihofer and Guyenet, 2002). This region was first identified by Guertzeinstein (1973) in anesthetized cats by showing that the application of excitatory drugs in the CVLM region produces a marked decrease in the mean arterial pressure. Therefore, if L-glutamate has diffused from the RVLM to the CVLM, the cardiovascular responses may be a result of a summation of excitatory and inhibitory drives to sympathetic premotor neurons. If this is the case, it is more likely to occur in malnourished animals due to their smaller size.

The main finding of the present study is the lack of sympathetic modulation in malnourished rats. This is indicated by the highly linear blood pressure increase in response to L-glutamate microinjection into the RVLM. However, in previous studies our group showed that an autonomic imbalance does occur in post-weaning protein-deprived rats (Martins et al., 2011; Bezerra et al., 2011a; Bezerra et al., 2011b). This lack of modulation of sympathetic activity by the RVLM may explain, in part, the autonomic imbalance observed in previous studies of our group. This autonomic imbalance in turn may contribute to the changes observed in the cardiovascular responses to activation of the baroreflex (Loss et al., 2007), chemoreflex (Penitente et al., 2007) and von Bezold-Jarisch reflex (Bezerra et al., 2011a, 2011b). It is well established in the literature that autonomic imbalance and cardiovascular neural reflex impairment lead to hypertension and cardiovascular diseases. Whether or not such morbidities occur in our model is a matter for future long-term studies.

In conclusion, the present study suggests that malnutrition indeed affects the central mechanisms involved in cardiovascular regulation, especially regarding the glutamatergic pathways of the RVLM – the principal CNS area responsible for the modulation of sympathetic activity directed to the cardiovascular system. Further studies are needed to evaluate interaction with other neurotransmitters/neuromodulators (e.g., GABA, NO, angiotensins) at this level. The complete understanding of the central mechanisms involved in the autonomic imbalance found in malnourished individuals could lead to the development of therapeutic strategies and governmental policies to improve public health.

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Effects of simvastatin on bleomycin-induced pulmonary fibrosis in female rats

Baykal Tulek, MD^{1*}; Esen Kiyan, MD²; Aysel Kiyici, MD³; Hatice Toy, MD⁴; Hulagu Bariskaner, MD⁵; Mecit Suerdem, MD¹

¹ Department of Pulmonary Medicine, Selcuklu Faculty of Medicine, Selcuk University, Konya-Turkey

² Department of Pulmonary Medicine, Istanbul Faculty of Medicine, Istanbul University, Istanbul-Turkey

³ Department of Biochemistry, Selcuklu Faculty of Medicine, Selcuk University, Konya-Turkey

⁴ Department of Pathology, Meram Faculty of Medicine, Selcuk University, Konya-Turkey

⁵ Department of Pharmacology, Selcuklu Faculty of Medicine, Selcuk University, Konya-Turkey

ABSTRACT

Statins reduce cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase and have a major place in the treatment of atherosclerotic disease. Recent studies have shown anti-inflammatory properties of statins. The purpose of this study was to evaluate the anti-inflammatory effect of simvastatin on bleomycin (BLM)-induced pulmonary fibrosis in rats. A total of 31 female Sprague-Dawley rats were divided into four groups: (1) intratracheal (IT) phosphate-buffered saline (PBS) + intraperitoneal (IP) PBS (n=7); (2) IT BLM + IP PBS (n=8); (3) IT BLM + low dose (LD) simvastatin (1 mg/kg daily, n=8); (4) IT BLM + high dose (HD) simvastatin (5 mg/kg daily, n=8). Simvastatin was administered IP for 15 days, beginning 1 day prior to IT BLM. The effect of simvastatin on pulmonary fibrosis was studied by measurements of IL-13, PDGF, IFN- γ , TGF- β 1 levels in bronchoalveolar lavage (BAL) fluid and lung tissue hydroxyproline (HPL) content and by histopathological examination (Ashcroft score). BLM caused significant change in BAL fluid cytokine levels and increased both HPL content and histopathological score ($p < 0.001$ for all). While LD simvastatin had no effect on cytokine levels, HD significantly reduced IL-13 (15.12 ± 7.08 pg/ml vs. 4.43 ± 2.34 pg/mL; $p < 0.05$) and TGF- β 1 levels (269.25 ± 65.42 pg/mL vs. 131.75 ± 32.65 pg/mL; $p < 0.05$). Neither HD nor LD simvastatin attenuated HPL content or Ashcroft score. In conclusion, this study showed that LD simvastatin had no effect on a BLM-induced pulmonary fibrosis model, while the high dose caused partial improvement in profibrotic cytokine levels.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a fatal disease of unknown origin. Its incidence is estimated to be 20 per 10,000 for males and 13 per 100,000 for females. The survival rate varies from 2 to 4 years (Kim et al., 2006). Patients with IPF are usually treated with corticosteroids and/or cytotoxic agents such as azathioprine or cyclophosphamide; the poor efficiency and significant side effects of these medications create, however, an urgent need for novel drugs with better efficacy and tolerance.

Statins are widely prescribed drugs with a positive effect on morbidity and mortality in cardiovascular disease. In recent years several studies have shown these positive effects of statins to be due both to their lipid lowering and anti-inflammatory effect (Albert et al., 2001; Nissen et al., 2005; Ridker et al., 1998). Positive results in experimental trials have been obtained through the anti-inflammatory efficacy of statins in numerous respiratory disease models such as asthma, smoking-induced emphysema, pulmonary hypertension, IPF, acute lung injury and lung transplantation (Hothersall et al., 2006).

Bleomycin (BLM)-induced pulmonary fibrosis is the most common pulmonary experimental model in rodents (Chua et al., 2005). This model produces pathological findings similar to those in human pulmonary fibrosis; it is widely used in the assessment of potential antifibrotic drugs. A search of published literature shows that rodents used in studies carried out with BLM-induced pulmonary fibrosis model are male.

There is, on the other hand, a generally shared expectation that female animals may enhance fibrosis by increasing both inflammatory and immune responses through the estrogen hormones or a modified cytokine expression, although several studies indicate the opposite (Gharaee-Kermani et al., 2005; Voltz et al., 2008).

The aim of this study was to investigate the anti-inflammatory and antifibrotic efficiency of simvastatin, the effectiveness of which was already shown in the BLM-induced pulmonary fibrosis model in doses approaching those used in humans.

METHODS

Animals

The study was approved by Selcuk University Experimental Medical Research and Application Center Experimental Animal Ethics Committee. Thirty-one female Sprague Dawley rats weighing 200-230g were studied. The animals were maintained in a controlled environment and fed on rodent chow and tap water *ad libitum*, in 12-hour light, 12-hour dark cycles. They were free of respiratory and other diseases.

Preparation of simvastatin

Simvastatin (Merck, Sharp & Dohme, Middlesex, U.K.) was prepared as a 4 mg/mL stock solution. Briefly, 4 mg of simvastatin were dissolved in 100 μ L of ethanol and 150 μ L

*Corresponding author: Baykal TULEK, Yrd. Doc. Dr. Address: Selcuk Universitesi Selcuklu Tıp Fakultesi, Gogus Hastaliklari Anabilim Dalı 42075. Selcuklu-Konya/TURKEY. Tel: +90 532 7878327. E-mail: baykaltulek@yahoo.com

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of 0.1 N NaOH and incubated at 50 °C for 2 h, then the pH was adjusted to 7 and the total volume corrected to 1 mL. The stock solution was diluted to the appropriate concentration in sterile phosphate-buffered saline (PBS) immediately before use (McKay et al., 2004).

Experimental Model and Experimental Groups

All animals were anesthetized with a mixture of xylazine (10 mg/kg, Rompun; Bayer AG, Leverkusen, Germany) and ketamine (70 mg/kg, Ketalar®; Parke Davis, Eczacibasi, Istanbul, Turkey) administered by intramuscular (IM) injection before the surgical procedures. The trachea was exposed after a small cervical skin incision and separation of strap muscles, and punctured with a 26-gauge needle for the administration of bleomycin sulphate (BLM; Bleocin-S, Nippon Kayaku Co., Ltd, Tokyo, Japan) (Borzzone et al., 2001). A single dose of 2.5 U/kg BLM dissolved in 0.3 mL PBS was instilled by intratracheal (IT) injection. This dose of BLM was selected from previous experiments to cause consistent biochemical and histological damage without mortality (Serrano-Mollar et al., 2002). Control animals received 0.3 mL PBS IT.

Simvastatin solution was administered intraperitoneally (IP) in two different doses, low dose (LD, 1 mg/kg) or high dose (HD, 5 mg/kg) beginning 1 day before the start of BLM application. Control animals received PBS. The animals were randomly distributed into four weight-matched study groups: (1) IT PBS + IP PBS (n = 7); (2) IT BLM + IP PBS (n = 8); (3) IT BLM + IP LD simvastatin (n = 8); (4) IT BLM + IP HD simvastatin (n = 8).

The rats were sacrificed by transsection of the abdominal aorta while under an overdose of pentobarbital (80 mg/kg IP) 14 days after IT bleomycin or saline instillation.

Bronchoalveolar lavage fluid

After clamping the right main bronchus, bronchoalveolar lavage (BAL) fluid was obtained from the left lung. Lavage was performed four times with 3 mL NaCl 0.9%. Samples were stored in the refrigerator at -80 °C until analysis. Levels of interleukin (IL)-13, interferon (IFN)- γ , platelet derived growth factor (PDGF)-A and transforming growth factor (TGF)- β

were performed with the ELISA technique using commercially available kits. The Invitrogen Rat IL-13 (BioSource, Invitrogen, USA, Cat #KRC0132/KRC0131), Invitrogen Multispecies TGF- β (BioSource, Invitrogen, USA, Cat #KAC1688/KAC1688), Rat IFN- γ (BenderMedSystems, Austria, Cat #BMS621) and Quantikine Mouse/Rat PDGF-AB (R&D Systems, USA, Cat #MHD00) kits were used for determination of these parameters in BAL fluid.

Measurement of hydroxyproline concentration in the lung

The left lung was ligated and cut at the hilum, freed of extraneous tissue, blotted on filter paper and snap frozen in liquid nitrogen. The tissues were stored in Eppendorf tubes at -80 °C until the time of biochemical analysis. Frozen tissue samples were weighed before analysis and subjected to alkaline hydrolysis. Briefly, the tissue samples were placed in Eppendorf tubes with 1 mL of 2 N NaOH; the tubes were sealed with rubber stoppers and incubated for one hour in an autoclave at 120 °C. Derivatization followed hydrolysis and 50 μ L of the sample was injected into an HPLC system (Shimadzu Prominence /Japan). A commercially available HPLC hydroxyproline (HPL) analysis kit (Eureka, Italy) was used for this analysis with a UV/VIS detector at 495 nm. Lung tissue hydroxyproline contents were reported as mg/g frozen tissue.

Histological studies of the lungs

Histopathological evaluation was carried out on the right lungs that were not lavaged. The right lung was fixed by IT infusion of formalin under 25 cm hydrostatic pressure. Lung tissue was embedded in paraffin and sections stained with hematoxylin-eosin for examination by light microscopy. Ashcroft's scoring system (Ashcroft et al., 1988) was used to estimate the severity of pulmonary fibrosis on a numerical scale in a blind evaluation by the same pathologist (HT) (Table 1).

Statistical Analysis

We performed power analysis to determine the sample size. Effect size was based on results of the study of Ou et al. (2008), and sample size was set to 7 per group for a power of 80%

TABLE 1
Characterization of the Ashcroft Scale

Grade of fibrosis	Histological features
0	Normal lung
1	Minimal fibrous thickening of alveolar or bronchiolar vessels
2	
3	Moderate thickening of walls without obvious damage to lung architecture
4	
5	Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses
6	
7	Severe distortion of structure and large fibrous areas; "honeycomb lung" is placed in this category
8	Total fibrous obliteration of the field

and with a significance level of 0.05. Eight rats were included in groups receiving IT BLM considering probable animal loss during the study.

Descriptive results were expressed as mean $\pm$ 1 standard deviation. Inferential statistical analysis was carried out by the Kruskal-Wallis variance analysis, with Dunn's multiple comparison test as a *post hoc* test. A *p*-value <0.05 was considered as statistically significant.

RESULTS

Effect of simvastatin on IL-13, PDGF, IFN- γ and TGF- β 1 levels

BAL fluid recovery rate, ranging from 84 to 90%, was not significantly different among the four groups. IT instillation of BLM caused significant increases in IL-13, PDGF, and TGF- β 1 and a significant decrease in IFN- γ levels. HD simvastatin significantly decreased the levels of IL-13 and TGF- β 1 ($p<0.001$). There was, however, no significant change in the PDGF or IFN- γ levels. LD simvastatin administration did not correlate with any significant change in IL-13, PDGF, IFN- γ or TGF- β 1 levels. Details are shown in Table 2.

Effect of simvastatin on HPL content

IT BLM caused a significant increase in lung HPL concentration, which is a marker of collagen deposition, on day 14 ($p<0.001$). Neither LD nor HD administration of simvastatin

with IT BLM caused a significant decrease in HPL content (Table 3).

Histopathological assessment

Haematoxylin-eosin stained lung sections were examined by light microscopy on Day 14. The lungs of the control group animals showed normal, free alveoli and interalveolar spaces (Fig 1a). Those of animals given BLM presented inflammatory infiltration, alveolar wall thickening and increase in interstitial collagen deposition (Fig. 1b). The animals who had received pre-treatment with simvastatin before BLM showed moderate edema and inflammation of perialveolar tissues with increased fibrotic changes (Fig. 1c).

In the assessment of lung sections according to Ashcroft's scoring system, the BLM-PBS group had the highest Ashcroft-fibrosis score. Either LD or HD simvastatin before IT BLM did not result in significant decrease in histopathological scores (Table 3).

DISCUSSION

In this study of the effect of simvastatin on BLM-induced pulmonary fibrosis in rats, we could not determine a positive effect of either a high (5 mg/kg) or a low (1 mg/kg) dose of simvastatin on fibrosis formation. Only the levels of IL-13 and TGF- β 1 were found to be lower in the simvastatin group than in the BLM group, at the dose of 5 mg/kg.

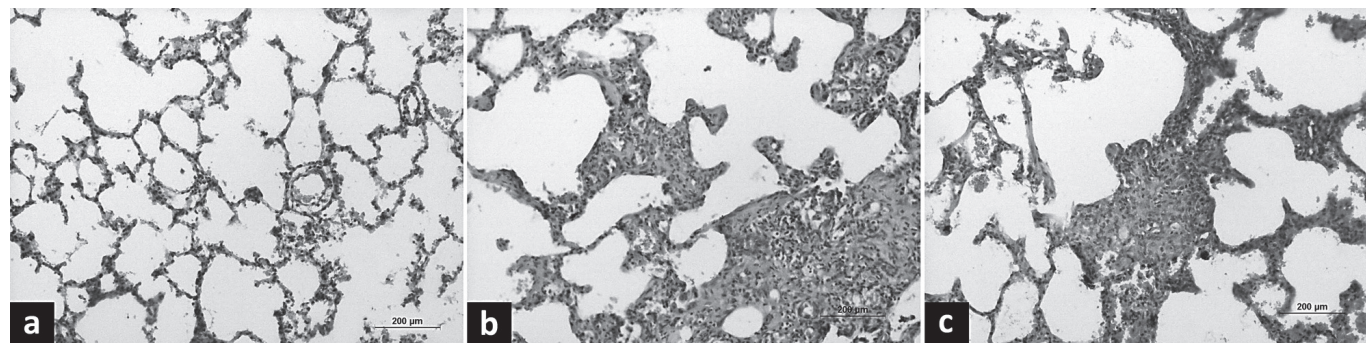


Figure 1: Histopathological examination of the anti-inflammatory and antifibrotic effects of simvastatin on bleomycin-instilled lungs. Lung sections stained with hematoxylin-eosin stain. **(a):** Normal lung tissues showing normal, open alveoli and interalveolar spaces with normal terminal bronchi **(b):** Bleomycin-treated lung sections showing severe congestion and edema of interalveolar space, inflammation, and thickened and collapsed alveoli with marked fibrosis. **(c):** High-dose simvastatin prior to bleomycin showing moderate inflammation, edema and fibrotic changes.

TABLE 2

Bronchoalveolar lavage (BAL) fluid levels of IL-13, PDGF-A, IFN- γ and TGF- β 1 in control rats, rats given bleomycin only, and rats given either low-dose or high-dose simvastatin

Groups	IL-13 (pg/ml)	PDGF-A (pg/ml)	IFN- γ (pg/ml)	TGF- β 1 (pg/ml)
Control (IT PBS + IP PBS, n: 7)	0.54 $\pm$ 0.23	15.48 $\pm$ 1.57	62.62 $\pm$ 16.01	24.42 $\pm$ 4.27
BLM+ IP PBS (n: 8)	15.12 $\pm$ 7.08*	58.70 $\pm$ 15.11*	18.95 $\pm$ 9.16*	269.25 $\pm$ 65.42*
BLM+1 mg/kg simvastatin (n: 8)	13.82 $\pm$ 5.73	54 $\pm$ 8.80	22.29 $\pm$ 6.16	231.75 $\pm$ 68.31
BLM+ 5 mg/kg simvastatin (n: 8)	4.43 $\pm$ 2.34**	24.11 $\pm$ 9.72	34.79 $\pm$ 8.79	131.75 $\pm$ 32.65**

IT: Intratracheal; IP: Intraperitoneal; PBS: Phosphate-buffered saline; BLM:Bleomycin; *: Compared to control group ($p<0.001$); **: Compared to BLM group ($p<0.05$).

TABLE 3

Hydroxyproline (HPL) contents and grade of pulmonary fibrosis in control rats, rats given bleomycin only, and rats given either low dose or high-dose simvastatin

Groups	HPL (mg/g)	Ashcroft score
Control (IT PBS + IP PBS; n: 7)	0.36 ± 0.06	0 ± 0
BLM+ IP PBS (n: 8)	0.80 ± 0.04*	3.12 ± 1.35*
BLM+1 mg/kg simvastatin (n: 8)	0.76 ± 0.05	3 ± 1.06
BLM+ 5 mg/kg simvastatin (n: 8)	0.48 ± 0.05	1.5 ± 0.53

IT: Intratracheal; IP: Intraperitoneal; PBS: Phosphate-buffered saline; BLM:Bleomycin;*: Compared to control group ($p < 0.001$). Ashcroft score; 0: Normal lung; 1: Minimal fibrous thickening of alveolar or bronchiolar vessels; 2-3: Moderate thickening of walls without obvious damage to lung architecture; 4-5: Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; 6-7: Severe distortion of structure and large fibrous areas; "honeycomb lung" is placed in this category; 8: Total fibrous obliteration of the field.

The anti-inflammatory effect of the statins is independent of their lipid reducing effect; different statins have different actions on inflammatory cells (Nagashima et al., 2006). Kiener et al. (2001) demonstrated that atorvastatin and simvastatin, the lipophilic statins, have a substantially greater anti-inflammatory effect than hydrophilic pravastatin. It is therefore important to realize that all the statins cannot have the same potential. Some statins may even show effects opposite to those of others. For example, in a study with 27 healthy volunteers comparing the 14-day effects of simvastatin, 40 mg and atorvastatin, 20 mg, simvastatin led to an increase in HLA-DR and MHC38 in T- cell expression, while the latter appeared to downregulate the same markers (Fehr et al., 2004). In addition, superantigen-mediated T-cell activation was inhibited by simvastatin, while atorvastatin, conversely, sensitized these cells. Simvastatin has been shown to be effective on the profibrogenic mechanisms in IPF and significantly inhibited the CTGF gene and protein expression, overriding induction by TGF β -1, a known potent inducer of CTGF (Watts et al., 2005). Several experimental trials showed an effect of simvastatin and pravastatin on BLM induced pulmonary fibrosis (Kim et al., 2010; Ou et al., 2008).

Experimental data suggest that the anti-inflammatory and antifibrotic effects of simvastatin could be utilized in clinical practice for pulmonary disease such as asthma and IPF. However, the positive experimental results could not be demonstrated in clinical trials. For example, in a study by Menzies et al. (2007) of the anti-inflammatory effect of simvastatin in asthma patients, simvastatin was not correlated with any significant changes in the inflammatory markers, lung volume or airway resistance. A retrospective review by Nadrous et al. (2004) of the contribution of ACE inhibitors and statins on IPF survival indicated that statins did not contribute to survival. Also, numerous case reports related to statin use in recent years have raised questions about the positive effects of statins in pulmonary fibrosis (Fernandez et al., 2008).

The effect of simvastatin on BLM-induced pulmonary fibrosis was previously discussed in one report only. In this study, Ou et al. (2008) evaluated the effect of simvastatin on histopathological scores, lung HPL content, inflammatory response, fibrogenic cytokines and profibrogenic markers. Administration of simvastatin for 28 days doses of 5 and 20 mg/kg showed statistically significant changes in all the parameters. The comparison of the antifibrotic activity of the high and low dose groups indicated a significant difference in

favor of the high-dose group. In our study, on the other hand, simvastatin was administered at lower doses and for a shorter time; no significant change was seen in the pulmonary fibrosis markers.

New drugs are usually tested for safety and efficacy in animal models before being used in clinical trials. It is well known that discrepancies exist between animal and human data and that a drug that is active in the animal may be useless in the human (Perel et al., 2007). Such an occurrence is often due to inappropriate translation of the drug dose from one animal species to another. Dose calculation based on body surface area (BSA) is accepted as the most appropriate method for translating doses among species (Reagan-Shaw et al., 2008). A BSA-based translation of the doses used in our study would result in doses of approximately 12 mg and 60 mg daily doses for a human weighing 60 kg. The dose range for simvastatin in human use is 20-80 mg/day for cardiovascular disease; statin-related side effects such as myopathy are known to be dosage-dependent (Escobar et al., 2008). Furthermore, in experimental studies high doses of simvastatin are reported to increase the risk of toxicity and mortality (Palmer et al., 2004). In the study by Palmer et al. (2004) of statin effect in collagen-induced arthritis, the anti-inflammatory activity of simvastatin was seen only after administration of 40 mg/kg; serious side effects were observed during treatment. Only 7 of 18 mice completed therapy.

Ou et al. (2008) sacrificed the rats after 28 days of IT BLM administration, while histological evaluation was performed after 14 days in our study. The time point of 14 days was considered as the most suitable for a comparative study in a BLM-induced PF model, as fibrosis is reported to decrease after 14 days (Izbicki et al., 2002). Also, in a study evaluating the immune modulating effects of statins (Fehr et al., 2004), a 14-day statin administration was shown to be sufficient for the immune system effects to occur. The selected 14-day evaluation period in our study appears to be optimal for judging both the simvastatin effect and the fibrosing effect of BLM.

TGF- β 1, PDGF-A, IL-13 and IFN- γ are biochemical endpoints used in many fibrosis evaluations (Scotton and Chambers, 2007). We found an increase of TGF- β 1, PDGF-A and IL-13 levels, and a decrease in the IFN- γ level, in line with the published literature. However, a significant decrease of IL-13 and TGF- β 1, compared to the BLM group, was only observed in the HD simvastatin group. TGF- β is one of the principal mediators in the pathophysiology of pulmonary

fibrosis. This cytokine affects extracellular matrix remodeling through multiple processes and is essential for several types of experimental fibrosis (Branton and Kopp, 1999). Therefore, inhibition of TGF- β signaling was intensively studied to evaluate the efficiency of antifibrotic therapies. Positive effects on fibrosis of therapies targeting TGF- β pathways were found (Bonniaud et al., 2005; de Gouville and Huet, 2006). Another profibrotic cytokine, IL-13, is a T-helper type 2 cytokine; it can stimulate the production of fibroblast collagen independently from TGF- β (Kolodsick et al., 2004). The important role of IL-13 in pulmonary fibrosis was clearly shown both in *in vivo* and *in vitro* studies (Gharaee-Kermani et al., 2001; Zhu et al., 1999). Jakubzick et al. (2003) assessed the efficiency of a chimeric IL-13 immunotoxin (IL-13-PE) in BLM-challenged mice. This study showed that IL-13-binding cells are essential for BLM-induced PF and the importance of antifibrotic therapies targeting IL-13 in pulmonary fibrosis. Examining experimentally the effects of simvastatin on allergic airway reaction and airway reactivity, Zeki et al. (2009) showed that simvastatin decreases airway inflammation, inhibits T-cell type 1 and 2, and improves lung physiology in the asthmatic mouse model. Simvastatin was administered in a much higher dose (40 mg/kg) than in our study; the IL-13 level in the lung lavage fluid decreased significantly.

Most experimental PF studies hitherto reported have been carried out in male rats, since the female gender is defined as a crucial factor for the development and prognosis of IPF. However, there are controversial indications from studies, suggesting that both the male and the female gender play a negative role in pulmonary fibrosis. Gharaee-Kermani et al. (2005) investigated the effects of gender and estradiol on pulmonary fibrosis in female and male rats in the BLM-induced pulmonary fibrosis model. They found more frequent and severe fibrotic lesions in the female rats. In this study, BLM was administered at a dose of 7.5 U/kg; all the male rats survived, while a high mortality rate of 80 $\pm$ 5% was calculated for the female animals. Whereas Voltz et al. (2008), reported that androgens, too, play a worsening role in the BLM-induced pulmonary fibrosis model. However, invasive pulmonary function tests were used in this study and no significant difference could be shown between animals of either sex in terms of the results of frequently used biochemical and histological indices of pulmonary fibrosis. Opposite results have, however, been reported in some clinical studies. In humans, even though the IPF incidence is higher in men, the incidence of the familial form of IPF is higher in women, who also have a comparatively poorer prognosis (Barzo, 1985; Wockel and Sultz, 1995). Several studies have, to the contrary, reported a poorer the prognosis in men with IPF compared to women (Gribbin et al., 2006; Olson et al., 2007). In our study, histologically confirmed fibrosis was achieved in female rats with 2.5 U/kg BLM, without mortality. This finding is one of the important results of the study.

In conclusion, simvastatin could not be shown to exert a favorable effect on BLM-induced PF in our study in female rats. Partial favorable effects in biochemical targets, in the group given HD treatment, suggest that favorable results could be possible at such high doses when interpreted in combination with results from the other studies. However, considering the relationship between high-dose administration and the risk of toxicity, we believe that further studies should be conducted on this subject.

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IGF2 expression in blood is not associated with its imprinting status in healthy pregnant Chinese women

Ru-Fei Gao¹, Xue-Qing Liu¹, Ying-Xiong Wang¹, Xue-Mei Chen¹, Yu-Bin Ding¹, Jun-Lin He¹

¹ Department of Reproductive Biology, Chongqing Medical University, Chongqing, China.

ABSTRACT

Loss of Imprinting (LOI) of IGF2 and over-expressed IGF2 are associated with tumorigenesis. Our previous epidemiological study found a relatively high frequency of IGF2 LOI in healthy mid-gestation pregnant women. The aim of this study is to determine whether the expression of IGF2 is associated with its imprinting status in healthy Chinese pregnant women. The IGF2 imprinting status of 300 pregnant women was analyzed. 20 cases of IGF2 LOI and 20 cases of IGF2 retention of imprinting (ROI) were selected randomly for IGF2 expression analysis. The expression pattern of IGF2 between the group with IGF2 ROI and group with IGF2 LOI in healthy Chinese pregnant women was evaluated by real time PCR and western blot. The result showed no significant differences between IGF2 ROI and LOI groups in mRNA and protein levels. These results imply that IGF2 imprinting status has no obvious impact on its expression. There may be some unknown important factors other than imprinting status driving IGF2 expression.

Key words: Insulin-like growth factor 2; loss of imprinting; retention of imprinting; pregnancy.

INTRODUCTION

Genomic imprinting is a common mechanism by which a gene is preferentially expressed based on the parental origin of the allele (Byun et al. 2007). Insulin-like growth factor 2 (IGF2), located at 11p15.5, has been known as a maternally imprinted and paternally expressed gene in most human tissues (Vu & Hoffman 1994). It plays a fundamental role in embryo growth, differentiation and placental development (Birnbacher et al. 1998; Constancia et al. 2002; Naeve et al. 2000; Wang et al. 1994). Studies have demonstrated that the IGF2 LOI gene is associated with the tumorigenesis of different types of cancers (Byun et al. 2007; Cui et al. 2002; Fu et al. 2008; Honda et al. 2008). 67-77% of IGF2 LOI was observed in sporadic Wilms' tumor cases (Ogawa et al. 1993), 47% in adult lung cancer (Suzuki et al. 1994), 86% in rhabdomyosarcoma (Zhan et al. 1994) and high incidence of LOI of IGF2 in other tumors including testicular germ cell tumors (van Gurp et al. 1994), choriocarcinoma (Hashimoto et al. 1995), bladder cancer (Byun et al. 2007) and colorectal tumors (Cui et al. 2003).

However, recent studies revealed that IGF2 LOI was not a phenomenon specific to cancer, it may occur in normal tissues such as normal cervical tissues adjacent to cervical cancers (Douc-Rasy et al. 1996) and noncancerous liver specimens around hepatocellular carcinomas (Takeda et al. 1996). In the human fetal liver IGF2 appeared to be imprinted, while relaxing its imprinting in the second half of the 1st year of postnatal life (Davies 1994); LOI remained in the adult liver (Kalscheuer et al. 1993). An epidemiological study also showed that IGF2 LOI is found in the cord blood of newborns (Dai et al. 2007).

In theory, IGF2 LOI would lead to a doubling of IGF2 mRNA abundance and a 2-fold increase in IGF2 production (Vu et al.). However, mixed results were shown on the relationship between IGF2 expression and its imprinting status. Wu et al. (1997) found that LOI had strong association with IGF2 overexpression and

played an important role in carcinogenesis of cancers. IGF2 LOI also resulted in overexpression of IGF2 in childhood tumors (Muller et al. 2000; Ogawa et al. 1993; Roy et al. 2000). Micha et al. (1999) observed that none of the pancreatic tissues with IGF2 LOI showed concomitant overexpression of IGF2 transcripts. The LOI of IGF2 may induce both increased or decrease expression, which could initiate the onset of Wilms' tumor (Yun 1998). Wang et al. (1996) reported that increased expression of the IGF2 gene in Wilms' tumor did not depend on loss of genomic imprinting or loss of heterozygosity. In prostate cancer, IGF2 imprinting correlated with IGF2 expression in tissues associated with the tumor, but not within the tumor foci (Bhusari et al.). In addition, no association of IGF2 LOI with increased expression of IGF2 mRNA was observed in Ewing's sarcoma (Zhan et al. 1995). Waterland et al. (2006) revealed that a folate-deficient diet caused significant IGF2 LOI in mice offspring and IGF2 expression was significantly reduced in the treatment group. And LOI patterns have no direct influence on altered expression levels of imprinted genes on growth in adult mouse hybrids (Shi et al. 2005). These studies indicated that LOI may not be always involved in the regulation of IGF2 expression and some other unknown factors may be driving IGF2 expression.

To obtain a better understanding of the relationship between IGF2 expression and the imprinting status of IGF2, peripheral blood of healthy pregnant women was evaluated by screening the expression and imprinting status of IGF2. Here we examine the correlation of LOI and IGF2 expression in pregnant women.

MATERIALS AND METHODS

Sample Collection

All the peripheral blood samples (n=300) chosen for our study were obtained from healthy pregnant women aged 20 to 30

Corresponding author: Yu-Bin Ding and Jun-Lin He, Chongqing Medical University, No.1 Yixueyuan Road, Yuzhong Street, Chongqing 400016, China. Tel: +86-23-68485001. Fax: +86-23-68485001. E-mail: dingyb@gmail.com and hejunlin_11@yahoo.com.cn.

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years who visited the First and the Second Affiliated Hospital of Chongqing Medical University in the period from February 2008 to August 2009. The research was approved by Local Ethics Committee and informed written consent was obtained from all participants. With clinical examination and ultrasound examination, the gestation (20.6 ± 1.2 weeks) was calculated. The subjects who had a history of gastrointestinal disease or major pregnancy complications were excluded. The pregnancy process and pregnancy outcome were followed up till the children were born.

With standard procedures, fasting blood samples were obtained into EDTA-containing vacuum tubes and 0.5 ml peripheral blood was frozen at -20°C for DNA and protein extraction. The separated lymphocytes were used for RNA extraction.

DNA extraction

Genomic DNA was extracted using a TIANamp Blood DNA Kit (TIANGEN Biotechnology Co. Ltd, Beijing, China) according to the manufacturer's instructions and the extracted DNA was stored at -20°C for genotyping analysis.

cDNA synthesis

Total RNA extraction was performed using Trizol reagent (Invitrogen Corporation, USA). Quantification and purity were assessed by optical density (OD) measurement at 260nm and 280nm. Integrity of the total RNA was examined by agarose gel electrophoresis and stored at -20°C . The reverse transcription of cDNA was performed with 0.5 μg of total RNA in a 10 μl reaction using the First Strand synthesis for RT-PCR kit (TAKARA Biotechnology Co. Ltd, Dalian, China). cDNA was stored at -80°C for analysis of IGF2 LOI and ROI and Real-time PCR.

Analysis of IGF2 LOI and ROI

To identify heterozygous cases for imprinting analysis, polymorphic pattern of genomic DNA was examined by *Apal* polymorphism of axon 9 for IGF2. Cases heterozygous for IGF2 were further analyzed with PCR products of the first strand cDNA as described and for the analysis of imprinting status. The 236bp RT-PCR products (Fig. 1 A) of the IGF2 gene were digested with *Apal* endonuclease, followed by electrophoresis on 3% Biowest agarose gel. The digestion products showing both 236bp and 173bp fragments are indicative of LOI and retention of IGF2 imprinting (ROI) (Fig. 1 B). All the protocols were described in our previous study (Gao et al. 2011).

Real-time PCR of IGF2 mRNA expression between LOI and ROI

All Real-time PCR were performed using SYBR Premix Ex Taq (TAKARA Biotechnology Co. Ltd, Dalian, China) and a Light Cycler (Bio-Rad iQTM5 Multicolor Real-time PCR detection system). Briefly, 2 μl of aliquots containing cDNA were amplified in a total volume of 20 μl containing 4 μl of a 5 $\times$ SYBR PreMix Ex Taq and 0.2 μM of each primer. For internal controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified. The following specific primers for IGF2 and GAPDH were used: IGF2 (forward: 5'-CACGCCAAACACTGAAT-3', reverse,

5'-GCAAGCGTTACAAGGTTA-3', product size: 142bp), GAPDH (forward: 5'-CTCTCTGCTCCTCCTGTTTCGACAG-3', reverse, 5'-GTGGAATCATATTGGAACATGT-3', product size: 230bp) (Sangon Biological Engineering Technology & Services Co. Ltd, Shanghai, China). All samples were run in triplicate. For appropriate negative controls, the RNA template was replaced with nuclease-free water in each run. Melting curves of the products were obtained after cycling with a stepwise increase of temperature from 55°C to 95°C . At the end of 40 cycles, reaction products were separated electrophoretically on an agarose gel and stained with ethidium bromide for visual confirmation of the PCR products. Relative gene expression levels were calculated with the $2^{-\Delta\Delta\text{CT}}$ method.

Western blotting analysis

The total protein concentration of plasma samples was evaluated using the Bradford assay. Equal amounts (30 μg per lane) of diluted plasma samples were incubated in SDS-PAGE sample buffer, subjected to SDS-PAGE analysis with 12% acrylamide gel, and then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The membrane was subsequently blocked in 5% skim milk powder in 0.1% Tris-buffered saline/Tween-20 and then incubated with rabbit polyclonal to IGF2 primary antibody (Abcam, Cambridge, MA, USA) followed by incubation with the appropriate secondary antibody of peroxidase-conjugated goat anti-rabbit IgG (Zhongshan Goldenbridge Biotechnology Co., Ltd, Beijing, China). Immuno-reactive proteins were visualized by a chemiluminescence reaction using the ECL Western blocking detection system (Amersham).

Bisulfite genomic sequencing for methylation analysis

The IGF2 DMR0 region (GenBank nucleotides 631–859, accession No. Y13633) has been previously reported to be hypomethylated in colorectal cancers with LOI at IGF2 (Cui et al. 2002). In this study, the methylation status of this region was quantified in normal pregnancy via BSP. Genomic DNA extracted from peripheral blood was treated with sodium bisulfite using EZ DNA Methylation-Gold kit according to the manufacturer's instructions. Each PCR

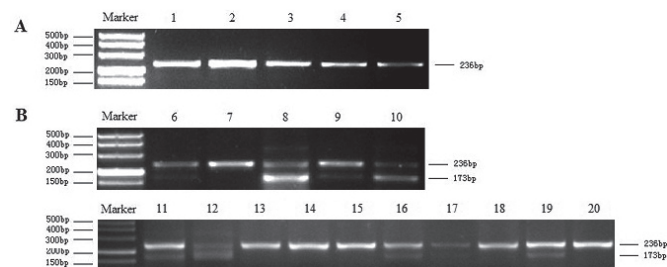


Figure 1. Analysis of IGF2 LOI and ROI in pregnancy. A. 2 μl cDNA of the heterozygote informative cases was amplified by RT-PCR. B. In each case of IGF2, the RT-PCR product was digested with 20 units of *Apal* endonuclease. The presence of a 236bp fragment indicates an IGF2 ROI case and the presence of both the 236bp and 173bp fragments indicates an IGF2 LOI case. Lanes 7, 13-15, 18, 20 are the cases of IGF2 ROI. Lanes 6, 8-12, 16, 17, 19 are the representative cases of IGF2 LOI.

mix reaction contained 10 pmol of the forward primer (5'-TAGGGTGGTGTGTTGGGA-3'), 10 pmol of the reverse primer (5'-AAAACCTCCCRATACTAATAAAAAAACC-3'), 3 μ l of bisulfite-modified DNA, 1 μ l of 10 pmol/ μ l primers, 1 μ l of 10 mmol/L dNTP, 5 μ l of 10 $\times$ PCR buffer, 0.8 μ l of HotStart Taq DNA polymerase (Takara). PCR amplification conditions were as follows: initial denaturation at 98 $^{\circ}$ C for 5 min; 40 cycles of 45 sec at 94 $^{\circ}$ C, 45 sec at 56 $^{\circ}$ C, and 1 min at 72 $^{\circ}$ C; followed by a final extension at 72 $^{\circ}$ C for 10 min. Products purified with the Universal DNA Purification kit (Tiangen, Beijing, China) were cloned into pUC18-T and transformed into chemically induced competent *E. coli*. Five insert-positive clones of each sample were chosen for sequencing by the Sangon company.

Statistical analysis

All statistical analyses were carried out using SAS for WINDOWS software (version 8.2; SAS Institute Inc, Cary, NC). Data are presented as the means $\pm$ S.D. Differences in IGF2 mRNA and protein levels between IGF2 LOI and ROI were compared using Student's *t* test. The relationship between the

expression and the imprinting status of IGF2 was examined with logistic regression models. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

For the *Apal* polymorphism site in exon 9 of IGF2, the unrestricted PCR product (AA genotype) has a size of 236bp, and complete restriction (GG genotype) generates bands of 173bp and 63bp. Samples with 236bp and 173bp fragments were heterozygotes (AG genotype), which made them informative for further analysis of imprinting status (Figure.1). Of the 300 women analyzed, 164 (54.67%) cases were heterozygous with the AG genotype and informative for imprinting status analysis of IGF2. Of the 164 cases analyzed for imprinting status, 44 (26.83%) were IGF2 LOI cases while 120 (73.17%) were IGF2 ROI. The IGF2 DMR0 region has been previously reported to be hypomethylated in colorectal cancers with LOI at IGF2 (Cui et al. 2002). Our BSP results showed that, except for CpG 2, all the CpG sites in this region were also hypomethylated in blood lymphocytes in normal pregnancy with IGF2 LOI (Figure. 2).

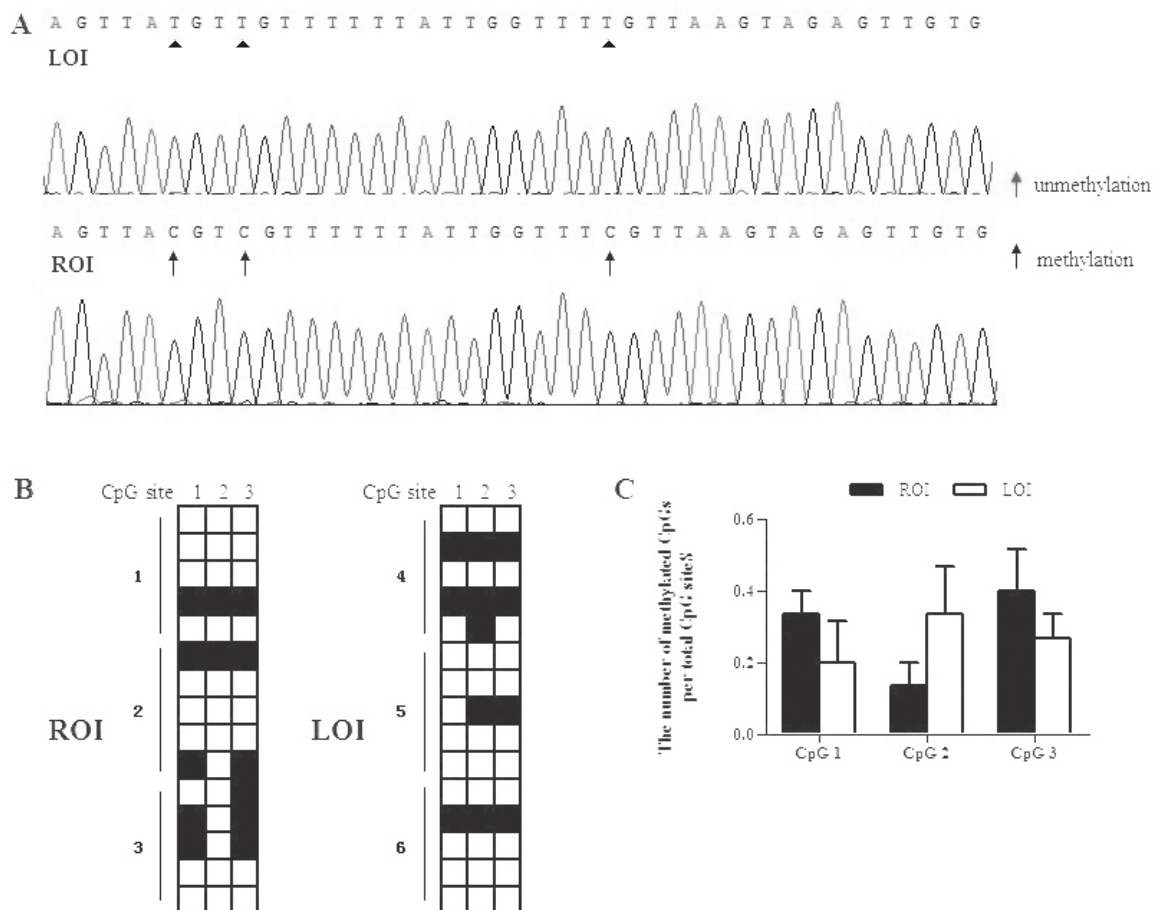


Figure 2. Bisulfite sequencing of IGF2 DMR0 region. Representative sequences of the IGF2 DMR0 region (GenBank nucleotides 631–859, accession No. Y13633) in the ROI group and LOI group. Methylated CpG sites are indicated by *black arrows* and *non-methylated* CpG sites by *arrow heads*. B. The methylation status at each of 3 CpGs. The white squares represent non-methylated CpGs, and the black squares represent methylated CpGs. Samples are marked by numbers. Each line represents one clone. C. The bar chart shows the average number of methylated CpGs per CpG site according to the BSP results. Except for CpG 2, all the CpG sites in this region were hypomethylated in blood lymphocytes with IGF2 LOI ($P < 0.05$).

20 cases of IGF2 LOI and 20 cases of IGF2 ROI were selected randomly for further analysis. The general characteristics of subjects with IGF2 LOI and IGF2 ROI are presented in Table 1. The mean age was 25.3 ± 4.2 years in the group with ROI and 24.2 ± 2.8 years in the group with LOI. The mean gestational age at enrollment was 20.9 ± 1.5 weeks in the group with ROI and 20.6 ± 1.7 weeks in the group with LOI. 5.7% of the group with ROI and 6.1% of the group with LOI took antibiotic medicine for a cold during pregnancy. 3.9% of the group with ROI and 4.0% of the group with LOI had bad habits such as smoking and drinking alcohol which could be harmful to embryo development. None of the subjects had complications related to pregnancy. No obvious difference was found in the general characteristics between LOI and ROI.

Expression of IGF2 mRNA was detected by Real-time PCR. The amplification curve and melt curve for Real-time PCR are shown in Fig. 3A and Fig. 3B, respectively. The relative amounts of IGF2 mRNA (IGF2/GAPDH mRNA) in blood lymphocytes in normal pregnancy are shown in Fig. 3C. The IGF2 mRNA level in the group with LOI was a little lower than in the group with ROI, but the difference between the group with ROI and LOI was not significant ($P=0.281$).

The protein level of IGF2 in different groups with ROI and LOI in plasma was detected by western blot (Figure 4). The IGF2 protein expression was associated with mRNA expression. Densitometric analysis revealed that there was no difference of IGF2 protein expression between the two groups ($P=0.275$). This showed that there was not a close correlation between the mRNA ($OR=1.401$, $P=0.290$) and protein ($OR=1.418$, $P=0.284$) expression and the imprinting status of IGF2. Pregnancy outcome was followed up, and no birth defects were found in the two groups.

DISCUSSION

The frequency of IGF2 LOI, which ranges from 12% to 100% in various tumors, suggested that aberrant imprinting is an important epigenetic cause of human carcinogenesis. However,

a relatively high frequency of IGF2 LOI was also found in normal human tissue including human fetal liver in the second half of the 1st year of postnatal life (Davies 1994) and the cord blood of newborns (Dai et al. 2007). In our previous epidemiological study, 14.67% (44/300) of pregnant women showed IGF2 LOI. These results indicated that IGF2 LOI is not tumor specific and may be required in some normal biological processes. The association of IGF2 LOI and IGF2 expression in

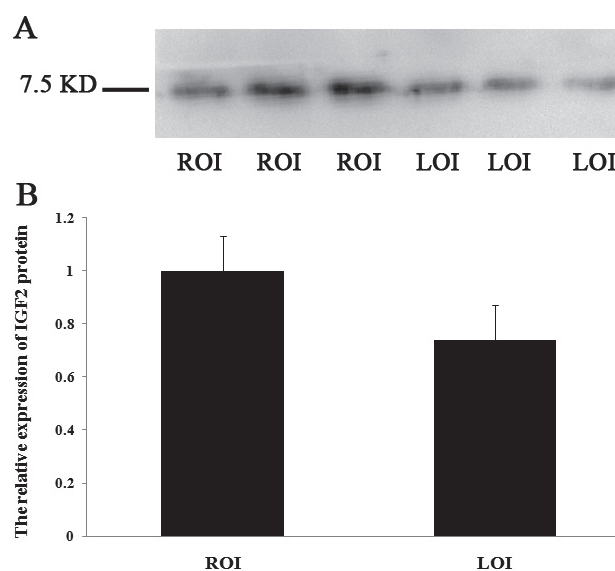


Figure 4. Western blot analysis of IGF2 protein expression in groups with LOI and ROI. Total protein was extracted from plasma samples of the subjects with IGF2 ROI and LOI. Protein level of IGF2 in the two groups was analyzed by western blot. As control for equal protein loading in western blot analysis, the membrane was re-stained with Ponceau. A, IGF2 protein expression in representative cases. B, Densitometric analysis of the western blot.

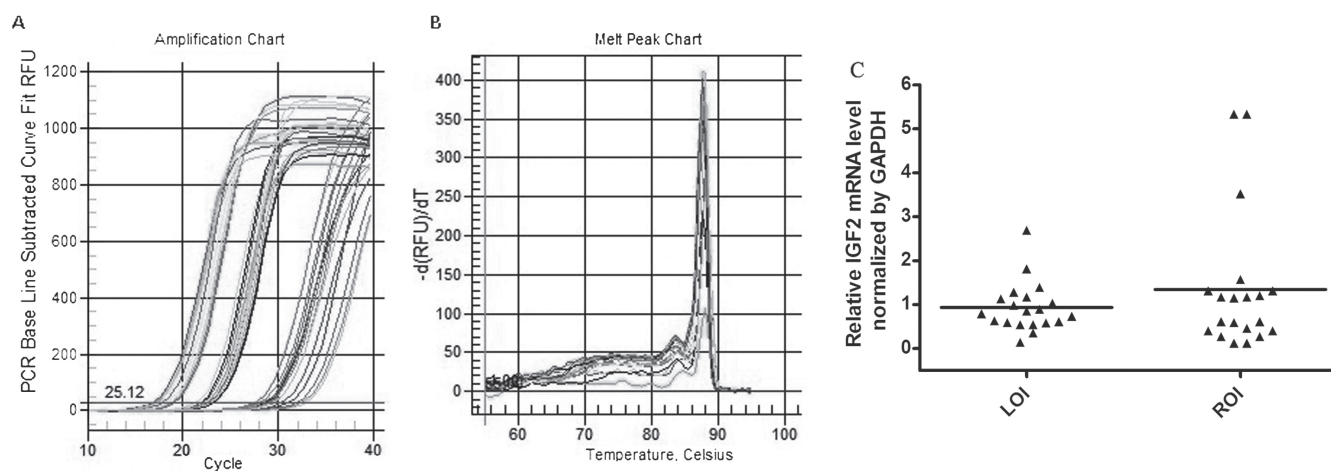


Figure 3. Real time PCR analysis of IGF2 mRNA expression in groups with LOI and ROI. Total RNA isolated from heterozygous with AG genotype was subjected to real-time PCR. A, Amplification chart. B, melt peak chart. C, Relative mRNA expression of IGF2 was calculated (normalized by GAPDH) and plotted in the graph. The ROI IGF2 mRNA level was set as control. Data are presented as mean $\pm$ S.D. The difference of IGF2 mRNA levels between IGF2 LOI and ROI groups was compared using Student's *t* test ($P=0.281$). The horizontal line represents the mean value of each group.

TABLE 1
General characteristics of the subjects

Variables	ROI(n=20)	LOI(n=20)	P value
Age of gestation	25.3±4.2	24.2±2.8	0.693
Weeks of gestation at survey	20.9±1.5	20.6±1.7	0.782
Times of gestation	1-4	1-4	0.887
Times of birth	0-3	0-3	0.856
Smoking	1.9%	2.2%	0.643
Drinking	2%	1.8%	0.748
Medicine	5.7%	6.1%	0.720

tumor tissues has been demonstrated in several clinical and animal studies, although the results were mixed. Some studies showed that IGF2 overexpression in mRNA was associated with IGF2 LOI in various tumors (Hajdu et al.; Martin-Trujillo et al.; Xu et al. 2006). However, some other studies also showed that IGF2 overexpression was not dependent on loss of genomic imprinting or loss of heterozygosity (Bhusari et al. ; Wang et al. 1996; Zhan et al. 1995). Some researchers demonstrated that IGF2 LOI was associated with decreased expression of IGF2 (Shi et al. 2005; Waterland et al. 2006). In our study, the IGF2 expression was evaluated in order to find out whether LOI had effects on IGF2 expression in mid-gestation pregnant women. We found that the IGF2 mRNA level in the group with LOI was a little lower than in the group with ROI in peripheral blood lymphocyte of normal pregnancy. But there was no significant difference between the group with ROI and LOI. These results suggest that some unknown factors other than LOI might drive the expression of IGF2.

Although the precise effect of IGF2 LOI on biological processes is not clear, LOI has been demonstrated to be associated with increased cellular proliferation and therefore plays a role in tumorigenesis (Byun et al. 2007; Cui et al. 2003; Hashimoto et al. 1995; Kaneda et al. 2007; Suzuki et al. 1994; van Gurp et al. 1994; Zhan et al. 1994). Little is known of the importance of IGF2 in adults, except the brain and liver, where there is biallelic expression of IGF2. In this study, the follow-up analysis of pregnant outcome showed no obvious harmful effects on offspring in the IFG2 LOI group (data not shown). The imprinting status of IGF2 in fetus and placenta would be more important to produce an effect on pregnancy and offspring. It may indicate that IGF2 LOI might be a common phenomenon in mid-gestation pregnant women, or it might be a salvage pathway for some unknown bad effects of pregnancy. This may also give us a clue to explain why frequent IGF2 LOI is found in some persons who do not suffer from a disease such as cancer.

ACKNOWLEDGEMENTS

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Effect of neuroserpin in a neonatal hypoxic-ischemic injury model *ex vivo*

Jiao Ma^{1, 3}, Dan Yu^{1, 2, 3}, Yu Tong^{1, 3}, Meng Mao^{1, 2, 3}

¹ Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, PR China

² Key Laboratory of Obstetric & Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, Chengdu, PR China

³ Laboratory of Early Developmental and Injuries, West China Institutes for Woman and Children's Health, West China Second University Hospital, Sichuan University, Chengdu, PR China

ABSTRACT

Hypoxia-ischemia (HI) occurring in immature brains stimulates the expression of tissue-type plasminogen activator (tPA). Neuroserpin is a selected inhibitor of tPA in the central nervous system. However, the role that neuroserpin plays and the possible mechanisms involved during neonatal HI are poorly defined. In this study, an oxygen-glucose deprivation and reoxygenation (OGD/R) model was generated with cultured rat cortical neurons mimicking neonatal HI injury *ex vivo*, and an acute neuronal excitatory injury was induced by exposure to a high concentration of N-methyl-D-aspartic acid (NMDA). Cells received either neuroserpin or MK-801, an antagonist of the NMDA receptor, during OGD/R, and were incubated with or without neuroserpin after NMDA exposure. Cell viability and morphology were detected by a Cell Counting Kit-8 and immunohistochemical staining, respectively. tPA expression and activity were also assessed. We found that MK-801 alleviated injuries induced by OGD/R, suggesting an excitatory damage involvement. Neuroserpin provided a dose-dependent neuroprotective effect in both OGD/R and acute excitatory injuries by inhibiting the activity of tPA, without affecting neuronal tPA expression. Neuroserpin protected neurons against OGD/R even after a delayed administration of 3h. Collectively, our data indicate that neuroserpin protects neurons against OGD/R, mainly by inhibiting tPA-mediated acute neuronal excitotoxicity.

Key terms: excitotoxicity, hypoxia-ischemia, neuroserpin, N-methyl-D-aspartic, tissue-type plasminogen activator.

INTRODUCTION

Tissue-type plasminogen activator (tPA), a serine proteinase cleaving plasminogen into plasmin, is well known as a thrombolytic enzyme in the intravascular space (Collen, 2001). However, tPA is also expressed in the central nervous system (CNS) and displays both physiological and pathological activity (Benarroch, 2007). Neuroserpin (NSP), an axonally secreted serine protease inhibitor (Osterwalder et al., 1996), is a selective inhibitor of tPA in the CNS. Both of these proteins are associated with neuronal activity and stimulated to release by neuronal depolarization (Qian et al., 1993; Berger et al., 1999).

Studies propose that neuroserpin functions as a neuroprotective factor by eliminating the deleterious effects of tPA in some pathological conditions. The genetic knockout of tPA or administration of neuroserpin has shown better results in ischemic stroke models (Wang et al., 1998; Zhang et al., 2002; Yepes et al., 2000). During the course of cerebral ischemia, endogenous neuroserpin increases and then blocks microglial activation, decreases the number of apoptotic cells, preserves the integrity of blood brain barrier (BBB) and attenuates tPA-mediated inflammation (Yepes and Lawrence, 2004; Rodríguez-González et al., 2011). Furthermore, tPA has been indicated to enhance N-methyl-D-aspartic acid (NMDA)

receptor-mediated excitatory neuronal death, and tPA-deficient mice show a dramatic resistance to excitotoxicity (Nicole et al., 2001; Tsirka et al., 1995). Additionally, neuroserpin protects neurons against low concentration NMDA-induced chronic excitotoxicity both *in vitro* and *in vivo* (Lebeurrier et al., 2005). A negative correlation between a decrease of serum neuroserpin level and an increase of glutamate has been found in adult ischemic stroke patients recently (Rodríguez-González et al., 2011).

Although the role of neuroserpin in the adult ischemic stroke model has already been investigated, its effect in neonatal hypoxia-ischemia (HI) injury as well as in acute excitotoxicity has not been revealed. Unlike adult brains, HI only transiently impairs blood perfusion in newborn (Mujscs et al., 1990; Adhami et al., 2008). Reperfusion is accompanied by increased glucose levels, which has been shown to be detrimental (Sheldon et al., 1992), and excessive oxygen and free radicals, producing further brain injury (Harukuni and Bhardwaj, 2006). Based on this evidence, we used an oxygen-glucose deprivation and reoxygenation (OGD/R) model to mimic neonatal HI injury *ex vivo*. An acute neuronal excitatory injury model was generated by exposure to high NMDA concentration. We tested the hypothesis that neuroserpin would display neuroprotective effects by restraining tPA-mediated acute neuroexcitotoxicity in neonatal HI.

Abbreviations: BBB: blood brain barrier; BSA: bovine serum albumin; CCK-8: Cell Counting Kit-8; CNS: central nervous system; DMEM: Dulbecco's modified Eagle medium; EAAs: excitatory amino acids; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; HI: hypoxia-ischemia; HIE: hypoxic-ischemic encephalopathy; LTP: long-term potentiation; MAP2: microtubule-associated protein 2; NB: neurobasal medium; NMDA: N-methyl-D-aspartic; NSP: neuroserpin; OGD/R: oxygen-glucose deprivation and reoxygenation; PAI1: plasminogen activator inhibitor-1; QRT-PCR: Quantitative real-time reverse-transcriptase Polymerase Chain Reaction; tPA: tissue-type plasminogen activator.

Corresponding authors: Meng Mao, West China Second University Hospital, Sichuan University, Chengdu 610041, PR China, Tel: 13608088613, Fax: +86 28 87461751, E-mail address: dffmmao@126.com, Dan Yu, West China Second University Hospital, Sichuan University, Chengdu 610041, PR China, Tel: 15928623580, Fax: +86 28 85501319, E-mail address: yd540@126.com

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METHODS

Material

NMDA and poly-D-lysine were from Sigma-Aldrich ((St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), neurobasal medium (NB), B27, fetal calf serum (FCS), glutaMax and penicillin/streptomycin were obtained from Gibco (Grand Island, NY, USA). Human recombinant neuroserpin was bought from PeproTech (Rocky Hill, NJ, USA). The Cell Counting Kit-8 (CCK-8) was from Dojindo (Dojindo, Kumamoto, Japan). Primary antibody, mouse MAP-2 antibody, was from Abcam (Abcom, Cambridge, UK) and a FITC-conjugated rabbit anti-mouse IgG is a product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the reagents for QRT-PCR were from Bio-Rad (Biorad Laboratories, Hercules, CA).

Animals and primary culture of rat cortical neurons

All animal procedures were approved by the Sichuan University Committee on Animal Research. Pregnant Sprague-Dawley rats were supplied by the Experimental Animal Center of Sichuan University. Each test was repeated 3 times.

Cortical neurons were prepared from the brains of rat embryos (E15-16) as described previously (Zhang et al., 2009). Cells were planted on plates coated with 0.1mg/ml poly-D-lysine in a DMEM supplement containing 10% FCS, 2mM glutaMax, 100U/mL penicillin and 100mg/mL streptomycin. The cultures were incubated in a humidified atmosphere of 5% CO₂, maintained 37 °C for 4 hours, then the DMEM supplement was replaced with neurobasal medium with 2% B27 and 2mM glutaMax, 100 U/mL penicillin and 100 mg/mL streptomycin. Half medium replacement was performed every 3 days, and the following experiments were implemented on *in vitro* days 7-10.

Oxygen-glucose deprivation/ reoxygenation (OGD/R) and treatment

To initiate the HI condition *in vitro*, oxygen-glucose deprivation was performed as previously described (Zhang et al., 2009). Briefly, the cells were washed three times with glucose-free medium (125 mM NaCl, 2.8 mM KCl, 1.5 mM MgCl₂, 0.05 mM MgSO₄, 2 mM CaCl₂, 0.83 mM NaH₂PO₄, 24 mM NaHCO₃, 2 mM HEPES) prior to oxygen removal and placed in an anaerobic chamber containing 95% nitrogen and 5% CO₂ at 37 °C for 2h in the same glucose-free medium. To generate a reoxygenation injury, cells were then placed in the original neurobasal medium and returned to the incubator under normoxic conditions.

To study the effect of neuroserpin on OGD/R neurons, progressive concentrations of neuroserpin (0 to 1.2μmol/L) were added to normal neurobasal medium immediately after OGD (this time point is referred to as reoxygenation 0h, R0h). To explore the window of neuroserpin treatment, 900nmol/L neuroserpin was administrated at different time points (R0h, R1h, R2h, R3h, R4h).

To explore the excitatory effect induced by OGD/R, a subset of neurons was incubated with MK-801 10μmol/L at R0h. All samples were collected at R24h.

Effects of neuroserpin and MK-801 on normal neurons

To test the effects of neuroserpin and MK-801 on neurons, 900nmol/L neuroserpin and 10μmol/L MK-801 were added to

neurons without OGD/R injury. Application of normal saline (NS) provided controls. Samples were harvested for the CCK-8 test at 0h, 4h, 8h, 12h and 24h after treatment.

Excitotoxic neuronal injury and neuroserpin treatment

A quickly-triggered excitotoxic insult was induced in the cultured cortical neurons by 2h exposure to 0 to 800μmol/L NMDA; then neurons were cultured in original neurobasal medium. Neuroserpin 900nmol/L was added to neurobasal medium after 2h exposure to 600μmol/L NMDA. Samples were harvested 24h later.

Neuronal viability assay

For quantitative analysis of cell viability, the CCK-8 test was used, critically following the manufacturer's instructions. Briefly, at harvest time 10μL CCK-8 solution was added to each well; the total volume of culture medium was 100μL per well. After incubation at 37 °C for 1 h in a humidified CO₂ incubator, absorbance at 540 nm was monitored with the Model 680 microplate reader (Bio-Rad). The values were used to calculate cell viability by setting the normoxic control as 100%.

Immunocytochemistry

Immunocytochemistry was performed 24 h after OGD/R and NMDA exposure (Zhang et al., 2009). Neuronal cultures were fixed with 4% paraformaldehyde for 10min at 4 °C, washed with PBS and incubated for 30min at 4 °C in the presence of PBS containing 0.3% Triton-X100 and 1% bovine serum albumin (BSA). Primary antibody, mouse MAP-2 antibody (1:250), was incubated overnight at 4 °C and then washed 3 times with PBS containing 0.1% Tween-20 (PBST). Cells were incubated for 1 h with a FITC-conjugated rabbit anti-mouse IgG (1:200). Cultures incubated without primary antibody were used as negative controls. Images were observed using a light microscope (Leica). Eight images were randomly selected from one slice and each group was observed on 3 independent slices. MAP2 expression was analyzed by NIS-Elements AR 3.00, SP7 (Build 547), Imaging Software, setting normal MAP2 expression as 1.

Quantitative real-time reverse-transcriptase PCR

Total RNA strands were extracted from cultured cells using the iScript™ RT-qPCR Sample Preparation Reagent. RNA (1 μg) was reverse transcribed using an iScript™ cDNA Synthesis Kit. The following primer sequences were used: (a) tPA: sense primer 5'-GGCCAAATGCCATCAAGCT-3', antisense primer 5'-CGTGGTATACTTCCCTGCCTTAAA -3'; (b) GAPDH : sense primer 5'-CAAGTTCAACGGCACAGTCAA -3', antisense primer 5'-TGGTGAAGACGCCAGTAGACTC -3'. Two negative controls were performed: samples without reverse transcription, and RNase-free water instead of cDNA. Assays were run using SsoFast™ EvaGreen Supermix and the iCycler iQ real-time PCR detection system. Amplification conditions were: 95 °C for 2 minutes followed by 40 cycles at 95 °C for 1 second and at 62.5 °C for 7 seconds. The levels of expression of the gene of interest were computed as follows: relative mRNA expression = 2^{-(Ct of gene of interest)}, where Ct is the threshold-cycle value. They were also computed with respect to the mRNA

expression level of the reference gene transcript using the formula:

$$\text{Relative mRNA expression} = 2^{-(Ct \text{ of gene of interest} - Ct \text{ of gene of reference})}$$

tPA activity assay

tPA activity in the cultured medium was detected by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (NovoNordisk, Bagsvaerd, Denmark).

Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Student's *t* test was used to compare between 2 groups. ANOVA with Fisher post hoc test was used for comparing more than 2 groups. A value of $P < 0.05$ was defined as statistically significant.

RESULTS

Neuroserpin protects neurons against OGD/R injury

Figure 1B shows that exposure to OGD/R conditions decreased neuronal viability to $24.32 \pm 3.96\%$. The treatment with neuroserpin produced a concentration-dependent increase in neuronal viability, $36.82 \pm 3.61\%$ when administered at 900nmol/L, $p < 0.05$. Normal saline (NS) showed no protective effect on OGD/R neurons when neuroserpin was not present. This indicated that the neuroprotective effect of neuroserpin in OGD/R condition was independent of NS.

The administration of 900nmol/L neuroserpin to normoxic neurons showed no effect on cell viability compared to the normal saline-treated group. Cell viability was $93.67 \pm 1.22\%$ (neuroserpin treated group) and $91.67 \pm 2.02\%$ (normal saline-treated group), respectively, after 24 hours exposure, $p = 0.27$ (Fig. 1A). This indicated that neuroserpin had no proliferous or detrimental effect under normal conditions.

We also found that treatment with 900nmol/L neuroserpin within 3h after OGD/R provided efficient neuroprotection (Fig. 1C). Cell morphological changes were observed by MAP2

immunofluorescence (Fig. 2A). OGD/R induced a breakdown of neuritic networks with a decreased MAP2 expression. However, treatment with 900nmol/L neuroserpin clearly preserved neurons with a lesser extent of neurite fragmentation (Fig. 2).

Neuroserpin protects neurons against NMDA-induced acute excitotoxicity

The 2h-exposure to NMDA caused a dose-dependent decrease in neuronal viability (Fig. 3A). In this acute neuroexcitotoxic state induced by 600 μ mol/L NMDA, 900nmol/mL neuroserpin promoted neuronal viability from $56.67 \pm 8.7\%$ to $89 \pm 7.5\%$ (Fig. 3B). Cell morphological changes were also detected by MAP2 immunofluorescence (Fig. 2A). We found that NMDA exposure led to a retraction of neurites, and that administration of neuroserpin protected neurons efficiently.

NMDAR-mediated neuronal excitotoxicity in OGD/R

The incubation of 10 μ mol/L MK-801 with normoxic neurons showed no proliferous or damage effects during a 24h time-course compared to the saline treated group. Cell viability was $91.2 \pm 2.33\%$ (MK-801 treated group) and $91.67 \pm 2.02\%$ (normal saline-treated group), respectively, after 24 hours exposure, $p = 0.49$ (Fig. 1A). However, MK-801 raised cell viability from $25.23 \pm 3.95\%$ to $33.57 \pm 3.86\%$ during OGD/R (Fig. 1C). These results indicate that neuronal excitatory damage constitutes the total neuronal injury caused by 2h OGD and 24h reoxygenation.

Effects of neuroserpin on tPA expression and activity in OGD/R injury and NMDA-induced acute excitotoxicity

tPA mRNA expression and enzyme activity were detected by quantitative Real-time PCR (QRT-PCR) and ELISA, respectively. We found that tPA mRNA and enzyme activity was dramatically increased within 4h after OGD/R and maintained a high level at R24h. Although neuroserpin had no effect on mRNA expression of tPA (Fig. 4A), it inhibited tPA activity efficiently (Fig. 4B).

Similarly, acute induction of excitotoxicity raised the mRNA expression of tPA and elevated tPA activity. Neuroserpin

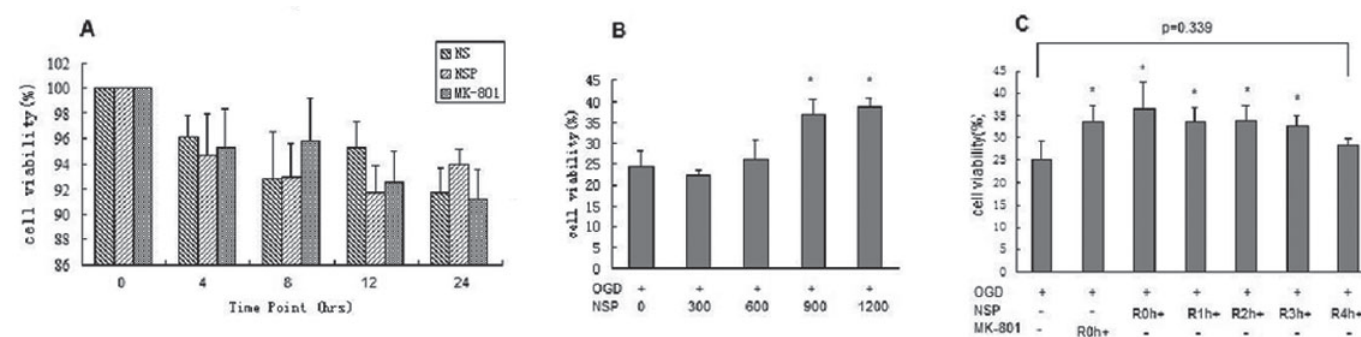


Figure 1. The neuroprotective effects of neuroserpin and MK-801 against OGD/R insult detected by CCK-8. (A) Administration of neuroserpin and MK-801 to normoxic neurons showed no increase in cell viability compared with the normal saline (NS) treated group. (B) Neuroserpin increased the cell viability in a concentration-dependent way, 900nmol/L neuroserpin protected neurons efficiently. (C) 900nmol/L neuroserpin protected neurons with a delayed administration of 3 hours after OGD/R and 10 μ mol/L MK-801 also increased cell viability. Normoxic cells were defined as control group. Mean cell viability was calculated by setting the control group as 100%, and represented as mean $\pm$ SD. * $P < 0.05$ compared to the control group. Each result was repeated 3 times.

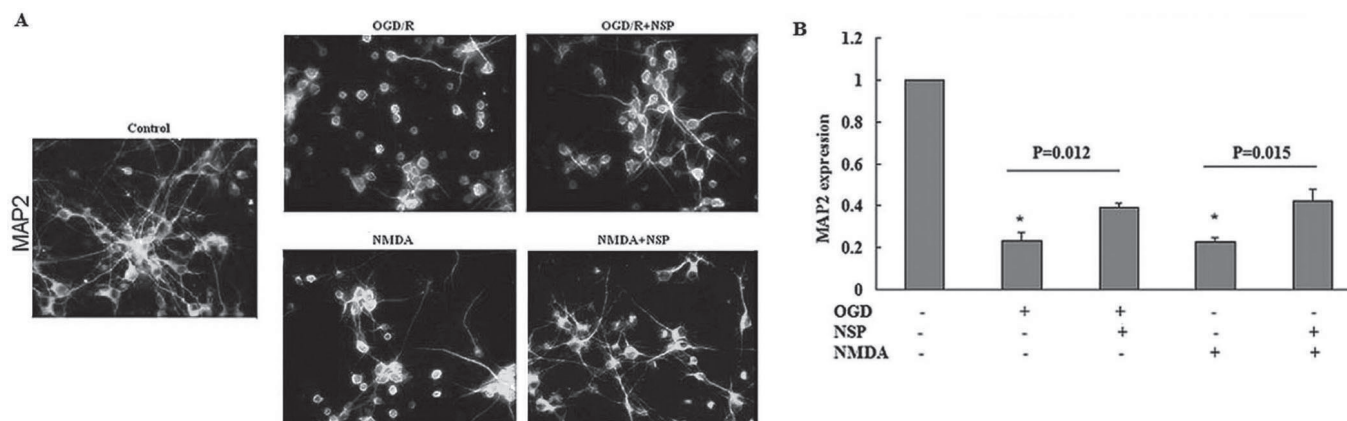


Figure 2. MAP-2 detection by immunofluorescence. (A) Cell morphological change was observed by MAP2 immunostaining. Normoxic cells were defined as the control group. Bar=40 μm. (B) MAP2 expression was analyzed by NIS-Elements AR 3.00, SP7 (Build 547), Imaging Software, setting the control group MAP2 expression as 1. Eight images were randomly selected on one slice and each group was observed on 3 independent slices.

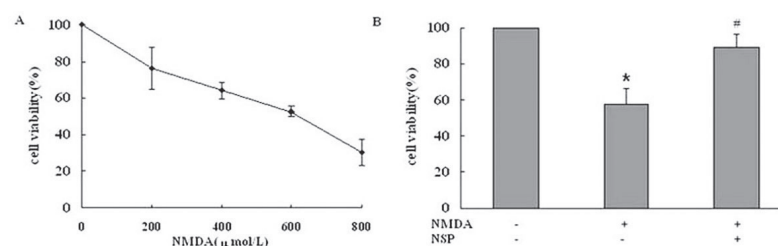


Figure 3. Protective effects of neuroserpin against NMDA-induced acute excitatory injury detected by CCK-8 test. (A) NMDA induced a dose-related decrease in cell viability. (B) 900nmol/L neuroserpin protected neurons against acute excitatory injury induced by 2h-exposure to 600 μmol/L NMDA. Untreated cells were taken as the control group. Mean cell viability was calculated by setting the control group as 100%, and represented as mean ± SD. *P<0.05 compared to the control group, #P<0.05 compared to the NMDA-treated group.

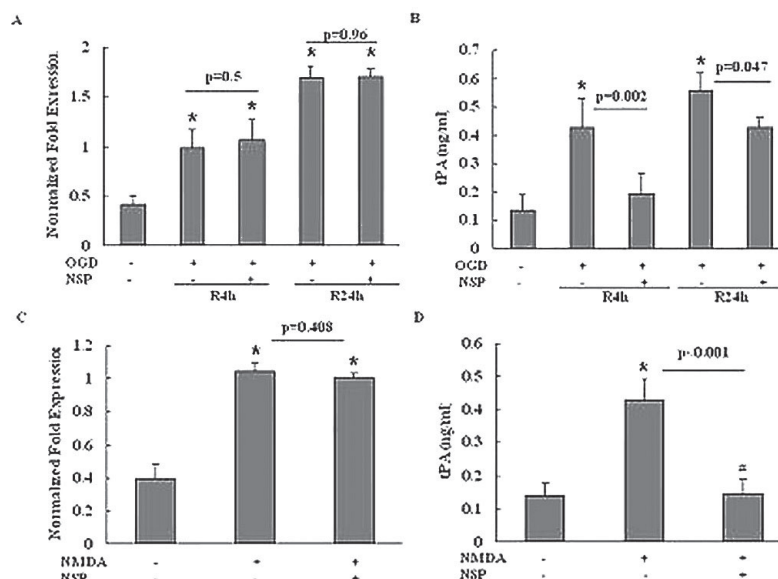


Figure 4. The regulation of tPA mRNA expression and enzyme activity. QRT-PCR showed tPA mRNA expression in the OGD/R model (A) and NMDA-induced acute excitatory injury models (C). ELISA revealed the enzyme activity of tPA in the culture medium in OGD/R model (B) and NMDA-induced acute excitatory injury models (D). Neurons in normal condition were taken as the control group. *P<0.05 compared to the control group. #P<0.001 compared with neurons treated with NMDA alone. Each observation was repeated 3 times.

dramatically inhibited the enzyme activity of tPA but failed to affect the neuronal expression of tPA (Fig. 4C and 4D).

DISCUSSION

In the present study, we demonstrate that neuroserpin protects neurons against HI, mainly depending on the regulation of tPA-mediated acute excitotoxicity, even with a delayed administration of 3h after the HI insult.

Perinatal HI brain damage is a leading cause of early mortality and permanent neurological morbidity in infants (Ferriero, 2005). A growing body of evidence suggests that the PA system, including tPA, plays a detrimental role in HI events (Wang et al., 1998; Adhami et al., 2008; Yang et al., 2009). Recently, the involvement of tPA has been demonstrated in a rat neonatal hypoxic-ischemic encephalopathy (HIE) model ((Adhami et al., 2008; Yang et al., 2009). The use of plasminogen activator inhibitor-1 (PAI1), another member of the serpin superfamily and the major inhibitor of tPA in the circulatory system, prevents HI-induced brain injury (Yang et al., 2009). However, the ubiquitously expressed character of PAI-1 limits its use in the CNS. In contrast, the brain tissue-specific expression characteristic of neuroserpin provides an optimum candidate for the treatment of newborn HI injury. Consistent with these findings, our experiments show an increase in tPA expression and activity. The therapeutic administration of neuroserpin dramatically inhibits tPA activity and preserves cell viability. Unexpectedly, neuroserpin had no effect on neuronal tPA mRNA expression. This result goes against previous *in vivo* findings in which the inhibitor of the PA system decreased both tPA expression and activity (Adhami et al., 2008). Neuroserpin has been shown to reduce the tPA expression produced by microglial cells (Cinelli et al., 2001). Integrating this data with our results, we propose that neuroserpin has a cell-type specific effect on tPA expression.

The cerebral vascular developmental differences between preterm and term infants produce a variation of the vulnerable region caused by HI (Distefano and Pratico, 2010). Glucose is an obligate energy fuel for the brain, but the immature brain has a low capacity for glucose transport. The glucose transport limit initiates the cerebral energy failure during HI (Vannucci and Hagberg, 2004). Energy failure leads to depolarization of neurons and glia, intracellular calcium accumulation and increased levels of excitatory amino acids (EAAs), combined with excessive activation of NMDA receptors (Towfighi et al., 1997; Dirnagl et al., 1999). In the immature brain, NMDA receptors play important roles in long term potentiation (LTP), neuronal migration and synaptic pruning, containing subunits that allow the channel to be opened more easily for a longer period than in the adult. These developmental features make immature brains more susceptible to glutamate excitotoxic insults, which could be induced by an HI state (Johnston, 1995; Vannucci and Hagberg, 2004).

tPA has been shown to aggravate chronic NMDA-induced neuronal excitotoxicity with 24h exposure to low concentration of NMDA (Nicole et al., 2001; Tsirka et al., 1995), and neuroserpin shows the ability to limit the exacerbation of the glutamatergic signaling stimulated by long-term exposure to NMDA (Lebeurrier et al., 2005). However, 2h exposure to an OGD condition also induces an acute excitotoxic injury, where MK-801 protects neurons efficiently. To mimic this kind of acute excitotoxicity, we exposed neurons to 600 μ mol/L NMDA for 2h, which decreased cell viability to approximately 50%. However,

it is important to indicate that the exact extent of excitotoxicity during neonatal HI is still undefined, and we are undecided whether the acute excitatory model used in our studies reflects that seen *in vivo*. Previous research proves that acute NMDA toxicity is accompanied by autophagy induction and subsequent neuronal death (Sadasivan et al., 2010). In conjunction with this observation, our experiment also initiated a decrease of cell viability, a loss of neurons and an alteration of the neuronal morphology, which are detected by MAP2 expression. Although MAP2 marks the neuronal bodies and axons and its expression reflects the breakdown of neurites indirectly, the exact change of neurite length could not be measured. The administration of neuroserpin results in a more intact neuritic network and protects neurons against acute NMDA-induced excitotoxicity by restraining tPA activity *ex vivo*.

However, tPA has been shown to rescue neurons from apoptosis and to be a neuroprotective agent in the mouse hippocampus (Liot et al., 2006; Echeverry et al., 2010); neuroserpin is also capable of protecting neurons from ischemia-induced cell death independently of tPA inhibition (Wu et al., 2010). This differs from our result mainly because of the different types of experimental procedures, and it must be noted that neuroserpin actually exhibits tPA-independent roles in HI. Besides tPA, neuroserpin also reacts with uPA and plasmin. UPA is upregulated following ischemia (Adhami et al., 2008), while plasmin participates in the excitotoxic process (Tsirka et al., 1997). Neuroserpin has already been shown to abrogate the excitotoxic neuronal death induced by plasmin (Wu et al., 2010). Moreover, it is known that methionine residues constitute an important antioxidant defense mechanism (Levine et al., 1996). There are 20 methionine residues contained in the neuroserpin molecular structure, which partly explains the oxidative tolerance produced by neuroserpin, and constitutes neuroserpin's neuroprotective effects against the oxidative stress condition during HI (Mohsenifar et al., 2007). In addition, systemic inflammatory stimulus is detrimental to HI and neuroserpin is reported to possess anti-inflammatory activity by modifying Th cell responses and reducing plaque growth (Munuswamy-Ramanujam et al., 2010).

In conclusion, the currently available collaborative data support the idea that during HI, neuroserpin can display an age-related neuroprotective effect by restraining tPA-mediated neuroexcitotoxicity. A neuroserpin-based intervention seems to be an extremely promising future pathway for the successful treatment of neonatal hypoxic-ischemic encephalopathy. To develop a clinically feasible drug for neonatal hypoxic-ischemic encephalopathy, further *in vivo* studies are necessary.

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Assessment of allelopathic properties of *Aloe ferox* Mill. on turnip, beetroot and carrot

Sunday Arowosegbe and Anthony J. Afolayan

Phytomedicine Research Centre, Department of Botany, University of Fort Hare, Alice, 5700, South Africa.

ABSTRACT

Turnip (*Brassica rapa* var. *rapa* L.), beetroot (*Beta vulgaris* L.) and carrot (*Daucus carota* L.) are common vegetables in South Africa. The allelopathic potential of aqueous leaf and root extracts of *Aloe ferox* Mill.- a highly valued medicinal plant- was evaluated against seed germination and seedling growth of the three vegetables in Petri dish experiments. The extracts were tested at concentrations of 2, 4, 6, 8, and 10 mg/mL. Leaf extract concentrations above 4 mg/mL inhibited the germination of all the crops, while the root extract had no significant effect on germination irrespective of concentration. Interestingly, the lowest concentration of leaf extract stimulated root length elongation of beetroot by 31.71%. Other concentrations significantly inhibited both root and shoot growth of the vegetable crops except the turnip shoot. The most sensitive crop was carrot, with percentage inhibition ranging from 29.15 to 100% for root and shoot lengths. Lower percentage inhibition was observed for the root extract than the leaf extract against shoot growth of beetroot and carrot. The results from this study suggested the presence of allelochemicals mostly in the leaves of *A. ferox* that could inhibit the growth of the turnip, beetroot and carrot.

Key Terms: allelopathy, *Aloe ferox*, aqueous extracts, intercrops, vegetable crops.

INTRODUCTION

Aloe ferox Mill., commonly called bitter or red aloe, is highly valued in South Africa for its various medicinal uses. The plant is a popular ingredient in cosmetics, herbal remedies and food supplements both locally and internationally. The processed part of *A. ferox* is usually taken orally as laxative or purgative and to improve digestion. It could also be applied to treat skin infections. This plant is also an income earner for South Africa through huge exportation of its products to other countries, particularly the European countries (Knapp, 2006). *A. ferox* (Fig. 1) is a single-stemmed seed plant that grows up to 2 or 3 metres in height. The stem, when old, is usually covered in dried leaves. The leaves are succulent and arranged in a rosette, each up to 60 – 100 cm long at maturity. The leaves have spines along the margins and sometimes on the surfaces. The flowers are carried in a large candelabra-like flower head, with about five to eight branches. Each branch carries a spike-like head of many flowers. The flower colour varies from yellow-orange to bright red, which attracts pollinating birds and insects. In South Africa, the plant grows both in the open and on mountainsides.

At present, *A. ferox* is readily available throughout South Africa and it is not currently listed in the Red List of Threatened Species (IUCN, 2010). However, since most of the *A. ferox* materials used locally and as international commercial products are harvested in the wild, the increase in demand for this plant will likely threaten the sustainability of these products. According to Gopalan (1992), overexploitation of a plant from the natural habitat could cause habitat degradation and reduction in its population. The highest priority is to conserve species in their natural habitats (*in situ*). To complement the *in situ* efforts of conserving plant biodiversity, there is a need for *ex situ* conservation of *A. ferox* to keep it

as a sustainable crop, by bringing the plant into cultivation. For this to be successful, there is a need for the evaluation of its chemical relationships with other cultivated plants (Nandakumar, 2009), most especially as intercrops.

This chemical relationship is usually the ability of the plant to inhibit, retard or promote the germination of seeds



Fig.1 *Aloe ferox*, African Flowering Plants Database". South African National Biodiversity Institute. Retrieved 14 May 2009.

Corresponding author: Anthony Jide Afolayan. Phytomedicine Research Centre, Department of Botany, University of Fort Hare, Alice, 5700, South Africa. E- mail: aafolayan@ufh.ac.za. Telephone: +27 82 202 2167. Fax: +27 866 282 295

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or seedling growth of other plants through the production of allelochemicals. This phenomenon is known as allelopathy (Inderjirt, 1996; Rice, 1984). Allelochemicals may be present in any part of the plant, from where they are released to the soil in many ways (Rice, 1984; Chou 1990).

The allelopathic property of some medicinal species is of special interest in recent years. For example, Fujii *et al* (2003) screened 239 medicinal plants for allelopathic activity and reported that 223 species were inhibitory, while 17 species enhanced radicle growth in lettuce. Again, 11 Thai medicinal plants that were evaluated against five plant species by Piyatida and Kato–Noguchi (2010) were reported to have inhibitory effects on all the test species. Nazir *et al.* (2007) investigated the allelopathic potential of three medicinal species against some crops. Germination of all the crops was reduced by 2 of the 3 medicinal species. The present study was therefore carried out to investigate the allelopathic potential of aqueous leaf and root extracts of *A. ferox* on turnip (*Brassica rapa* var. *rapa* L.), beetroot (*Beta vulgaris* L.) and carrot (*Daucus carota* L.). The seeds were chosen as test crops because they are good representatives of crops planted as homestead crops in South Africa, supplying the community with the required vitamins, fibres and some other essential nutrients.

MATERIALS AND METHODS

Extract preparation

Fresh leaves and roots of *A. ferox* were collected from the field, taken to the laboratory and washed several times with tap water. Both materials were cut into small pieces, oven-dried at 50 °C for 48 h, and pulverized using a grinder (Polymax PX-MFC 90 D, Switzerland). 100g each of the materials were soaked in 2 litres of distilled water and allowed to shake in an orbital shaker for 24 h at room temperature (23 - 25 °C) for extraction. The extracts of the leaf and root samples were filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrates were quickly frozen at - 40 °C and dried for 48 h using a freeze dryer (Savant Refrigerated Vapour Trap, RV T41404, USA) to obtain the leaf and root extract samples.

Germination experiment

Appropriate portions of the leaf and root samples were each reconstituted in 100 mL distilled water to give five concentration levels of 2, 4, 6, 8 and 10 mg/mL, respectively. Certified seeds of turnip (*Brassica rapa* var. *rapa*), beetroot (*Beta vulgaris* L.) and carrot (*Daucus carota* L.) were purchased from a seed store in Alice, South Africa. Ten uniform and surface sterilized (2% sodium hypochlorite for 15 min) seeds each of the three crops were placed in 9 cm sterilized Petri dishes lined with two Whatman No.1 filter papers. The papers were moistened with 6 mL of different concentrations of the aqueous extracts. Treatment in the same manner, but with distilled water, served as the control. Each treatment had three replicates maintained in a complete randomized design. The Petri dishes were kept on the germination table under laboratory conditions (room temperature 23 - 25 °C at mid day, and diffuse light during the day). After 7 days, seed germination counts and measurements of the root and shoot growth (cm) of seedlings were made using the method of Abugre and Sam (2010).

Statistical analysis

The data were subjected to one way analysis of variance (ANOVA) and the mean values were separated at $P < 0.05$ using Duncan's Multiple Range Test. Correlations between the extract concentrations and germination, root and shoot lengths of the crops were estimated. The percentage contribution of extract concentrations to the variation observed in the studied parameters was also analysed using the coefficient of determination (R^2). All statistical analyses were done using SAS software, 1999 version.

RESULTS

Effect of extracts on turnip

The aqueous leaf extract of *A. ferox* at the lowest concentration (2 mg/mL) had no significant effect on the germination of turnip (Table 1). However, concentrations above 2 mg/mL significantly inhibited seed germination of the crop in a concentration dependent manner. The root extract had no significant effect on the germination of turnip irrespective of concentration.

The root length elongation of this crop at all concentrations of both the leaf and the root extracts was significantly reduced compared to the control. The percentage inhibition also increased as the concentration of the extracts increased.

The reduction in shoot length at 10 mg/mL of the extracts was not significantly different from the control. However, concentrations below this seemed to have produced a stimulatory effect on the shoot elongation of turnip, with the percentage stimulation increasing as the concentration of the extracts decreased. The correlation between the leaf extract concentrations and germination percentage and root elongation were strong and negative (Table 4). The coefficient of determination (R^2) of the leaf extract concentrations on turnip was about 0.932 and 0.740 for germination and root length, respectively. Meanwhile, significant correlation of root extract concentrations was found only with root length, with an R^2 value of 0.740.

Effect of extracts on beetroot

Effects of *A. ferox* aqueous extracts on the germination of the seeds of beetroot were found to vary with the part used for the extract preparation and the concentration of the extract (Table 2). All the applied concentrations of the root extract had no significant effect on the germination of the crop. By contrast, leaf extract at 8 and 10 mg/mL caused significant inhibition in germination, by 35.01% and 50.01%, respectively. However, 2 mg/mL of the leaf extract appeared to stimulate germination of this crop, although it was not significantly different from the control.

Leaf extract at 2 mg/mL had a significant stimulatory effect on the root elongation of beetroot by 31.71%. Concentrations above this however, caused a reduction in root elongation, with percentage inhibition ranging from 23.98 to 63.41%. The root extract of *A. ferox* was comparatively less active than the leaf extract, with only the 10 mg/mL having significant inhibitory effect on root length elongation.

The shoot length of beetroot was significantly inhibited by the leaf extract at all concentrations; the percentage inhibition

was between 14.63% and 45.07%. This was also the same for the root extract except at 2 mg/mL with no significant difference from the control. There was significant and negative correlation between the leaf extract concentrations and all the parameters studied in beetroot (Table 4). The coefficient of determination (R^2) of the leaf extract concentrations on beetroot was 0.686, 0.678 and 0.855 for germination, root and shoot lengths, respectively. However, root extract concentrations correlated significantly only with the shoot length of the crop, with an R^2 value of 0.841.

Effect of extracts on carrot

Carrot seemed to be the most sensitive of all the crops to *A. ferox* leaf extract (Table 3). All the concentrations significantly inhibited seed germination of carrot, with 10 mg/mL completely preventing any seed germination. The root extract had no significant effect on the germination of carrot at all concentrations. While all concentrations of the leaf extract imposed significant reduction in the root elongation of carrot, with 100% inhibition at 10 mg/mL, the root extract

TABLE 1

Effect of aqueous leaf and root extracts of *Aloe ferox* on the germination (%), root and shoot lengths (cm) of turnip ($\pm$ SE).

Treatments (mg / mL)	Leaf extract			Root extract		
	Germination	Root length	Shoot length	Germination	Root length	Shoot length
Control	100.00 $\pm$ 0.00 ^a	6.17 $\pm$ 0.108 ^a	1.16 $\pm$ 0.026 ^{cd}	100.00 $\pm$ 0.00 ^a	6.17 $\pm$ 0.108 ^a	1.16 $\pm$ 0.026 ^c
2	86.67 $\pm$ 3.333 ^a *(-13.33)	2.45 $\pm$ 0.044 ^b (-60.29)	2.82 $\pm$ 0.240 ^a (+143.10)	100.00 $\pm$ 0.00 ^a (0)	2.47 $\pm$ 0.260 ^b (-59.97)	2.27 $\pm$ 0.020 ^a (+95.69)
4	63.33 $\pm$ 8.82 ^b (-36.67)	1.12 $\pm$ 0.079 ^c (-81.85)	2.44 $\pm$ 0.162 ^{ab} (+110.34)	96.67 $\pm$ 3.333 ^a (-3.33)	1.39 $\pm$ 0.110 ^c (-77.47)	2.26 $\pm$ 0.144 ^a (+94.83)
6	53.33 $\pm$ 6.667 ^b (-43.67)	0.33 $\pm$ 0.022 ^d (-94.65)	1.99 $\pm$ 0.087 ^b (+71.55)	100.00 $\pm$ 0.00 ^a (0)	0.94 $\pm$ 0.060 ^d (-84.76)	2.16 $\pm$ 0.111 ^a (+86.21)
8	36.67 $\pm$ 3.333 ^c (-63.33)	0.23 $\pm$ 0.021 ^{de} (-96.27)	1.44 $\pm$ 0.239 ^c (+24.14)	93.33 $\pm$ 3.333 ^a (-6.67)	0.50 $\pm$ 0.015 ^e (-91.90)	1.85 $\pm$ 0.079 ^b (+59.48)
10	16.67 $\pm$ 3.333 ^d (-83.33)	0.11 $\pm$ 0.013 ^e (-98.22)	0.71 ^d (-38.79)	93.33 $\pm$ 3.333 ^a (-6.67)	0.42 $\pm$ 0.42 ^e (-93.19)	1.13 $\pm$ 0.120 ^c (-2.59)
C.V.(%)	12.91	6.31	14.10	4.47	10.06	8.45

Values with the same letter within a column are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test (DMRT). C.V: Coefficient of Variation. *Data in brackets represent the % inhibition (-) or stimulation (+) over control

TABLE 2

Effect of aqueous leaf and root extracts of *Aloe ferox* on the germination (%), root and shoot lengths (cm) of beetroot ($\pm$ SE)

Treatments (mg / mL)	Leaf extract			Root extract		
	Germination	Root length	Shoot length	Germination	Root length	Shoot length
Control	66.67 $\pm$ 3.333 ^{ab}	2.46 $\pm$ 0.177 ^b	3.35 $\pm$ 0.093 ^a	66.67 $\pm$ 3.333 ^a	2.46 $\pm$ 0.177 ^a	3.35 $\pm$ 0.093 ^a
2	73.33 $\pm$ 3.333 ^a *(+9.99)	3.24 $\pm$ 0.145 ^a (+31.71)	2.86 $\pm$ 0.108 ^b (-14.63)	66.67 $\pm$ 3.333 ^a (0)	2.49 $\pm$ 0.173 ^a (+1.22)	3.21 $\pm$ 0.126 ^a (-4.18)
4	63.33 $\pm$ 3.333 ^{ab} (-5.00)	1.87 $\pm$ 0.224 ^c (-23.98)	2.38 $\pm$ 0.195 ^c (-28.96)	63.33 $\pm$ 3.333 ^a (-5.01)	2.49 $\pm$ 0.220 ^a (+1.22)	2.70 $\pm$ 0.128 ^b (-19.40)
6	53.33 $\pm$ 3.333 ^{bc} (-20.00)	1.26 $\pm$ 0.164 ^d (-48.78)	2.26 $\pm$ 0.080 ^{cd} (-32.54)	66.37 $\pm$ 8.819 ^a (-0.45)	2.43 $\pm$ 0.066 ^a (-1.22)	2.62 $\pm$ 0.132 ^b (-21.79)
8	43.33 $\pm$ 6.667 ^{cd} (-35.01)	1.11 $\pm$ 0.144 ^d (-54.88)	2.04 $\pm$ 0.037 ^{de} (-39.10)	63.33 $\pm$ 6.667 ^a (-5.01)	2.41 $\pm$ 0.134 ^a (-2.03)	2.40 $\pm$ 0.048 ^{bc} (-28.36)
10	33.33 $\pm$ 8.819 ^d (-50.01)	0.90 $\pm$ 0.153 ^d (-63.41)	1.84 $\pm$ 0.117 ^e (-45.07)	70.00 $\pm$ 0.000 ^a (+4.99)	1.26 $\pm$ 0.058 ^b (-48.78)	2.2 $\pm$ 0.038 ^c (-32.84)
C.V.(%)	14.57	16.65	6.54	14.26	10.66	5.91

Values with the same letter within a column are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test (DMRT). C.V: Coefficient of Variation. *Data in brackets represent the % inhibition (-) or stimulation (+) over control

only at 8 and 10 mg/mL had significant inhibitory effect. The same trend was observed in the response of shoot length to the leaf extract (Table 3). All root extract concentrations except 2 mg/mL also inhibited shoot elongation of this crop. However, the percentage inhibition on both the root and shoot elongation were small compared to the effects caused by the leaf extract. Negative significant correlations were observed between the leaf and the root extract concentrations and all the studied parameters in carrot except germination (Table 4). The coefficients of determination (R^2) of the leaf extract concentrations on carrot were 0.926, 0.815 and 0.950 for germination, root and shoot lengths, respectively, while the R^2

value of the root extract concentrations was 0.715 and 0.829 for root and shoot lengths, respectively.

DISCUSSION

The results of this study demonstrated that extracts from *Aloe ferox* plant contain indeed some phytochemicals capable of affecting germination and seedling growth of the tested vegetable crops. The extent of the effect of the phytochemicals seemed to be dependent on the part of the plant used for the extract preparation, the concentration of the extracts as well as the target vegetable crop involved. Leaf extracts

TABLE 3
Effect of aqueous leaf and root extracts of *Aloe ferox* on the germination (%), root and shoot lengths (cm) of carrot ($\pm$ SE)

Treatments (mg/mL)	Leaf extract			Root extract		
	Germination	Root length	Shoot length	Germination	Root length	Shoot length
Control	96.67 $\pm$ 3.333 ^a	2.56 $\pm$ 0.096 ^a	2.47 $\pm$ 0.104 ^a	96.67 $\pm$ 3.333 ^a	2.56 $\pm$ 0.096 ^a	2.47 $\pm$ 0.104 ^a
2	80.00 $\pm$ 0.000 ^b *(-17.24)	1.56 $\pm$ 0.047 ^b (-39.06)	1.75 $\pm$ 0.135 ^b (-29.15)	90.00 $\pm$ 5.773 ^a (-6.90)	2.54 $\pm$ 0.083 ^a (-0.78)	2.39 $\pm$ 0.112 ^{ab} (-3.24)
4	66.67 $\pm$ 6.667 ^b (-31.03)	0.76 $\pm$ 0.123 ^c (-70.31)	1.09 $\pm$ 0.107 ^c (-55.87)	86.67 $\pm$ 6.667 ^a (-10.34)	2.44 $\pm$ 0.116 ^a (-4.69)	2.18 $\pm$ 0.046 ^{bc} (-11.74)
6	23.33 $\pm$ 6.667 ^c (-75.87)	0.92 $\pm$ 0.039 ^c (-64.06)	0.80 $\pm$ 0.087 ^c (-67.61)	93.33 $\pm$ 3.333 ^a (-3.46)	2.32 $\pm$ 0.032 ^{ab} (-9.38)	2.03 $\pm$ 0.074 ^c (-17.81)
8	20.00 $\pm$ 5.773 ^c (-79.31)	0.85 $\pm$ 0.047 ^c (-66.80)	0.42 $\pm$ 0.027 ^d (-83.00)	90.00 $\pm$ 5.773 ^a (-6.90)	2.05 $\pm$ 0.110 ^b (-19.92)	1.93 $\pm$ 0.056 ^{cd} (-21.86)
10	0.00 $\pm$ 0.000 ^d (-100.00)	0.00 $\pm$ 0.000 ^d (-100.00)	0.00 $\pm$ 0.000 ^e (-100.00)	86.67 $\pm$ 6.667 ^a (-10.34)	1.65 $\pm$ 0.159 ^c (-35.55)	1.74 $\pm$ 0.059 ^d (-29.55)
C.V.(%)	16.06	7.99	15.64	11.35	8.49	7.00

Values with the same letter within a column are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test (DMRT). C.V: Coefficient of Variation. *Data in brackets represent the % inhibition (-) or stimulation (+) over control

TABLE 4
Correlation coefficient and Coefficient of determination (R^2) between extract concentrations and studied parameters of the test vegetable crops

Test crops	Parameters	Plant extracts			
		Correlation Coefficient		R^2	
		Leaf	Root	Leaf	Root
Turnip	germination	- 0.965**	- 0.545NS	0.932	0.297
	root length	- 0.860**	- 0.856**	0.740	0.740
	shoot length	- 0.436NS	- 0.144NS	0.190	0.021
Beetroot	germination	- 0.828**	0.065NS	0.686	0.004
	root length	- 0.824**	- 0.623NS	0.678	0.388
	shoot length	- 0.925**	- 0.917**	0.855	0.841
Carrot	germination	- 0.962**	- 0.249NS	0.926	0.062
	root length	- 0.903**	- 0.845**	0.815	0.715
	shoot length	- 0.975**	- 0.910**	0.950	0.829

NS: No significant difference at $P < 0.05$; ** significant difference at $P < 0.01$ level of probability.

were found to have more inhibitory property than the root extracts. For instance, leaf extract of *A. ferox* above 4 mg/mL exhibited significant inhibitory effects on seed germination of all the vegetable crops studied, whereas no significant inhibitory effect was recorded for root extract on germination of the crops. Similarly, stronger inhibitory allelopathic effects of leaf extract than root extract of *A. ferox* was observed in the elongation of root and shoot lengths of beetroot and carrot.

The observed stronger allelopathic impact of leaf extracts than root extracts on the tested vegetable crops might be connected with the presence of higher concentrations of allelochemicals such as tannins, alkaloids and flavonoids in the leaves than in the roots (Kanchan and Jayachandra, 1980). Different tissues of plants (leaves, stems and roots) are capable of releasing different amounts of allelochemicals into the surrounding environment (Scrivanti, 2010), thereby inflicting different effects on other plants in that vicinity. Stronger inhibitory potential of leaf extracts than other vegetative parts was also reported for some other plants like *Parthenium hysterophorus* (Tefera, 2002; Maharjan *et al.*, 2007) and *Croton bonplandianum* (Sisodia and Siddiqui, 2010).

The results of this study also indicated that the inhibitory effects of the extracts on germination, root and shoot growth of the three crops, in most cases increased as the extract concentration increased, and hence were concentration dependent. Maharjan *et al.* (2007) reported that high concentrations of aqueous leaf extract of *Parthenium hysterophorus* also had strong inhibitory effect on the germination of some cultivated and wild herbaceous plant species. In all extracts of sunflower, allelopathicity was also found to increase with increase in concentrations (Ashrafi *et al.*, 2008). The negative but significant correlation observed between the extract concentrations and most of the parameters of the target crops in this study further confirmed that the effect of *A. ferox* extracts on the germination and seedling growth of these crops is concentration dependent. Singh *et al.* (2005) also found a strong positive correlation between extract concentrations of residues of *Parthenium hysterophorus* and reduction in seedling length of *Brassica* species. (i.e. extract concentration negatively correlated with increase in seedling length). About 74.03% of the variation observed in the root length of turnip seedlings as a result of application of *A. ferox* leaf extract could be attributed to differences in the concentration of the extract, as indicated by the R^2 value of 0.740. Moreover, differences in the extract concentrations contributed between 67.80 - 85.20% (R^2 : 0.678 - 0.852) and 81.50 - 95.00 (R^2 : 0.0815 - 0.950) to the effects of the leaf extract on the germination, root and shoot lengths of beetroot and carrot, respectively.

The 100% inhibition of seed germination, root and shoot elongation of carrot by the 10 mg/mL leaf extract of *A. ferox* could occur only when some allelochemicals present in the leaves prevented growth of the embryo or caused complete death of the cells. The allelochemicals might have also prevented water uptake (Tawaha and Turk, 2003), or caused an alteration in the synthesis or activation of gibberelic acid (Olofsdotter, 2001), a growth promoting hormone.

It was also observed in this study that there were stronger inhibitory effects of extracts on seedling roots than the shoots of the tested crops in most cases. The difference in the sensitivity might be due to the fact that roots were in direct contact with the extract and subsequently with inhibitory

chemicals, as described in earlier studies on some other plant species (Bhowmik and Doll, 1984; Quasem, 1995; Tefera,

2002). According to Chon *et al.* (2000), root length was the best indicator of allelopathic effects of plant extracts due to its higher sensitivity to phytotoxic compounds than the hypocotyls. Root tissue was also reported to be more permeable to allelochemicals than shoot tissue (Nishida *et al.*, 2005). The difference in the genetic makeup of the target vegetable crops is likely to be responsible for the differential responses to the extract treatments, with carrot being the most sensitive crop.

Aqueous extracts of *A. ferox* exhibited allelopathic activity on the three vegetables, with the greatest effect on carrot. The leaf extract of the plant had more inhibitory effect than the root extract. Whereas the lowest concentration of leaf extract (2 mg/mL) stimulated root growth of beetroot, leaf extract concentrations above 4 mg/mL generally inhibited seedling growth of the vegetable crops investigated in a concentration dependent manner. At present, *A. ferox* is not vulnerable in the wild; however, there is concern that continuous harvesting from the wild could lead to local extinction (Van Wyk and Smith, 1996). The results of this study could be a way forward to the assertion of Pfab and Scholes (2004) that sustainability of plants harvested from the wild can never be achieved without *ex situ* cultivation. Even though the effects obtained under laboratory conditions may not always be what are obtained in the field, it can be concluded that *A. ferox* is better planted as a sole crop since it could affect crops like carrot, beetroot and turnip as intercrops.

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Interleukin-8 gene polymorphism and susceptibility to gastric cancer in a brazilian population

Aledson V. Felipe¹, Tiago D. Silva¹, Célia A. Pimenta¹, Paulo Kassab², Nora M. Forones¹

¹ Grupo de Oncologia, Disciplina de Gastroenterologia, Universidade Federal de São Paulo, SP, Brasil

² Departamento de Cirurgia, Santa Casa de Misericórdia de São Paulo, SP, Brasil

ABSTRACT

BACKGROUND: Studies have demonstrated that some polymorphisms in different interleukin genes may increase the risk of cancer. The aim of this study was to investigate the association between the *IL-8* (rs4073) -251A/T gene polymorphism and the risk of gastric cancer (GC). **PATIENTS AND METHODS:** A case-control study was conducted on patients with noncardia gastric cancer. DNA was extracted from leukocytes and the *IL-8* (rs4073) -251A/T polymorphism was analyzed by PCR-RFLP. Infection with *Helicobacter pylori* was investigated in the serum by ELISA. **RESULTS:** The sample consisted of 104 patients with GC and 196 controls. Cigarette smoking ($P=0.007$) and high fat intake ($P=0.01$) were more frequent in patients with GC. The proportion of patients infected with *H. pylori* was similar in the two groups ($P=0.101$). The frequency of the genotype A/T was higher in the cancer group ($P=0.008$). An increased risk of GC was found in subjects carrying the genotype A/T (OR=2.50, CI: 1.27-4.90), subjects with high fat intake (OR=1.92, CI: 1.17-3.15), and smokers (OR=2.00, CI: 1.20-3.31). **CONCLUSIONS:** Subjects with the heterozygous A/T genotype, high fat intake and smokers or ex-smokers presented an increased risk of GC. Individuals with A/A genotype may have protective effect for GC.

Key words: IL-8, polymorphism, gastric cancer.

INTRODUCTION

Gastric cancer (GC) is the fourth most common type of cancer in the world and the second leading cause of death due to cancer. In general, the incidence of GC is two to three times greater in developing countries and the frequency of this cancer is higher among men than among women (Matysiak-Budnika and Me'graub, 2006; Ferlay et al., 2007; Wen and Moss 2009; INCA, 2010).

IL-8 is a chemokine produced by macrophages and other cells such as epithelial and endothelial cells (Wolff et al., 1998). This cytokine is involved in the inflammatory response to infection with *Helicobacter pylori*, recruits phagocytes and causes damage to the gastric mucosa by inducing the release of reactive oxygen radicals (Torok et al., 2005; Ohyauchi et al., 2005). Furthermore, studies have shown that IL-8 induces cell proliferation and angiogenesis (Karashima et al., 2003; Taguchi et al., 2005).

In the early 1990s, Clore et al., (1990) described the three-dimensional structure of IL-8. The locus in the human genome was identified on chromosome 4q21.1. In 2002, IL-8 was renamed CXCL8 by the Chemokine Nomenclature Subcommittee of the International Union of Immunological Societies, although the symbol IL-8 has remained (Bacon et al., 2002).

Several risk factors associated with GC have been identified, including *H. pylori* infection, dietary factors such as salt-preserved foods and dietary nitrite, as well as smoking and alcohol drinking habits (Ladeiras-Lopes et al., 2008; Joossens et al., 1996).

More than 50% of the world population is infected with this bacterium (Danesh, 1999). Most case-control and cohort studies

have shown that the risk of patients with *H. pylori* infection for developing GC is increased from two to six fold (Esllick et al., 1999). Moreover, some of the trials on *H. pylori* eradication revealed that cure of its infection reduces the development of GC in high risk populations (Wong et al., 2004; Fukase et al., 2008). Accumulated evidence indicates that there are three steps in gastric carcinogenesis: *H. pylori* infection, development of gastric precancerous conditions and carcinogenesis (Hamajima et al., 2006). Severe gastric atrophy and corpus-predominant gastritis, intestinal metaplasia and dysplasia are well recognized as predominant predispositions to GC (Correa, 1988; Uemura et al., 2001).

Studies have indicated that activation of the transcription factor NF- κ B has a dominant role in *H. pylori*-induced IL-8 production in gastric epithelial cells. NF- κ B can be activated by phosphorylation via different signaling pathways leading to subsequent proteolytic degradation of I κ B. Activated NF- κ B translocates to the nucleus where it up-regulates *IL-8* gene transcription (Crabtree et al., 1995; Yamaoka et al., 2000).

Many investigators have reported associations between single nucleotide polymorphisms (SNPs) in genes that regulate the host's inflammatory response and GC, with sometimes conflicting results. The inflammatory response-related genes that have been most frequently studied in relation to GC are interleukin (IL) genes *IL1B*, *IL1RN*, *IL-8*, and *IL10*, coding for the proteins IL-1 β , IL-1ra, IL-8, and IL-10, respectively. These cytokines are important mediators in gastric physiology and pathophysiology and could play important roles in the etiology of GC (Sharma et al., 1998). There is growing interest in identifying the association between IL-8 (rs4073) genotypes and the risk of GC (Ohyauchi et al., 2005; Taguchi et al., 2005; Sarvestani et al., 2006; Shirai et al., 2006 Persson et al., 2011).

Corresponding author: Aledson Vitor Felipe. Laboratório de Gastro-Oncologia Molecular da Disciplina de Gastroenterologia da Universidade Federal de São Paulo. R: Pedro de Toledo, 781, 10º andar. CEP 04039-032 - São Paulo - Brasil. Phone/Fax: 55-11-5084-1743. E-mail: aledson.felipe@gmail.com.

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The aim of this study was to determine the frequency of the IL-8 (rs4073) promoter polymorphism (-251A/T) genotypes A/A, A/T and T/T in patients with GC and to correlate this polymorphism with *H. pylori* infection, consumption of red meat, fat and alcohol and smoking.

PATIENTS AND METHODS

A case-control study was conducted on patients of both genders with adenocarcinoma of the stomach aged > 18 years and treated at the Gastroenterology Division of Universidade Federal de Sao Paulo and Santa Casa de Misericórdia de Sao Paulo, Brazil. Patients with cancer of the cardia or diffuse-type adenocarcinoma were excluded. The control group consisted of healthy subjects who attended the Central Laboratory of the hospital for blood collection. Patients with cancer were excluded from the control group. The study was approved by the Ethics Committee of the two hospitals and all patients agreed to participate in the study by signing an informed consent form.

All patients filled out a questionnaire on the frequency of food intake, including meat, fat, vegetables, and fruits. Habits such as smoking and alcohol consumption were also recorded and classified as 'never used' and 'current user'.

Serum anti-*H. pylori* IgG levels were measured by a commercially available kit (R-Biopharm GmbH, Germany) based on the enzyme-linked immunosorbent assay (ELISA) method.

Genotyping procedure: DNA was extracted from peripheral venous blood leukocytes collected with EDTA as anticoagulant using the Set Invisorb™ (Invisorb, Blood Spin Mini kit, Germany). The samples were submitted to the polymerase chain reaction (PCR) and subsequently genotyped by restriction fragment length polymorphism (RFLP) analysis. The following primers were used: forward primer: 5' - TTCTAACACCTGCCACTCTAG - 3' and reverse: 5' - CTGAAGCTCCACAATTTGGTG - 3'. PCR was performed in a final volume of 10 µL containing 40 ng DNA, 1x buffer reagent, 0.125 mmol/L of each deoxynucleotide triphosphate (dNTP), 1.5 mmol/L MgCl₂, 0.75 mmol/L of each primer, and 0.5 units Platinum Taq DNA polymerase. The PCR conditions were denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. After amplification, the PCR products were digested with 5 units of *Mfe*I for 12h at 37 °C. The digestion products were separated by electrophoresis on 5% agarose gels stained with ethidium bromide (Taguchi et al., 2005). Genome sequencing was used to confirm the PCR and RFLP techniques using random samples of the two groups. The amplicons were purified by with the Big Dye X Terminator Kit (Applied Biosystems) and sequenced in an ABI Prism 3100 sequencer (Applied Biosystems). The reverse primer was used for sequencing. The electropherogram was analyzed with the Sequence Scanner v1.0 program.

Statistical analysis:

The data were analyzed using Minitab (Version 15) (Minitab, State College, PA, USA). The Student t-test and χ^2 test were used for comparison and odds ratios (OR) and confidence intervals (CI) (95%) were calculated using the Cox regression model to examine the impact of the polymorphism on GC risk.

Multivariate logistic regression was applied to identify risk factors. A *P* value < 0.05 was considered statistically significant.

RESULTS

All samples were submitted to genotyping by digestion of the amplicons by RFLP. Subjects carrying the homozygous T/T genotype showed a single band of 108 bp, A/A homozygotes had two bands of 76 and 32 bp, and subjects carrying the heterozygous A/T genotype presented three bands of 108, 76, and 32 bp as shown in Figure 1.

The case group consisted of 104 patients with GC, including 47 (45.2%) women. The control group consisted of 196 subjects, including 107 (54.6%) women. The patients of the case and control groups were admitted during the same period. There was no difference in gender (*P*=0.121) or age (*P*=0.117) between the groups. Lifestyle analysis showed no significant differences between the control and case groups in terms of the consumption of alcohol (*P*=0.391), fruits (*P*=0.809), vegetables (*P*=0.340), cereals (*P*=0.167), or red meat (*P*=0.499). There was a relationship between smoking and GC; nearly 61% of GC patients were current smokers plus former smokers (those who had smoked in some period during the past year), statistically different (*P*=0.007) than the control group (57.6%) who never smoked. The GC patients consumed significantly (*P*=0.005) more fat foods (48.1%) compared to the control group (31.6%). There was no statistically significant difference between GC and persons with *H. pylori* positivity IgG antibody, which was detected in 45.2% of the GC patients vs. 54.6% of the controls (*P*=0.101). The frequencies of the A/A, A/T and T/T genotypes were 26.5%, 43.4% and 30.1%, respectively, in the control group, and 14.4%, 55.8% and 29.8% in the case group. The genotypic distribution is in Hardy-Weinberg equilibrium in the control group (*p*>0.05). The frequency of the genotypes differed between the two groups ($\chi^2=6.654$, *P*=0.036), and on the other hand, there was no statistical difference in allele frequency

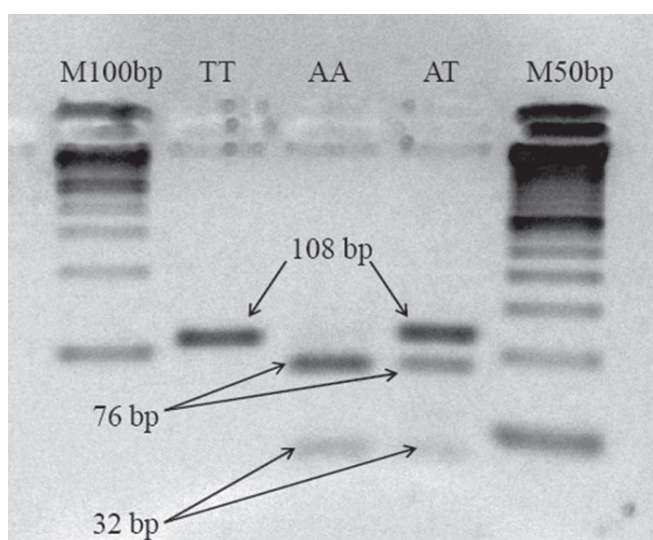


Figure 1. Subjects carrying the homozygous TT genotype showed a single band of 108 base pairs (bp), AA homozygotes had two bands of 76 and 32 bp, and subjects carrying the heterozygous AT genotype presented three bands of 108, 76, and 32 bp. DNA Molecular Weight Marker (100 bp ladder).

between groups ($\chi^2=1.677$, $P=0.631$). Comparing GC patients with T/T genotype to the subgroup with A/T+A/A genotypes did not show a significant difference from the control group ($P=0.866$). However, comparing the sub group with A/A genotypes *vs.* A/T+T/T genotypes, there was a significant difference compared to the controls ($P=0.003$). The proportion of GC patients was significantly greater in the T carriers (A/T+T/T) than in the non-T carriers (A/A) ($P=0.015$). Although a positive association was observed among individuals for the A/T genotype and GC (OR = 2.50, CI 1.27-4.90, $P=0.008$), and among T/T genotype *vs.* A/A genotype and GC, the results were not statistically significant ($P=0.106$). Individuals with

A/A genotype had a significantly lower risk of GC (OR = 0.45, CI 0.24-0.86, $P=0.003$) compared to (A/T+T/T) genotypes. The T allele was not statistically significantly associated with CG risk ($P=0.631$); however, T carriers was associated with an increased risk of GC (OR = 2.21, CI 1.17-4.20, $P=0.015$). These results remained consistent for IL-8 (rs4073) genotypes associated with GC in unadjusted and age-sex adjusted models, which are presented in Table 1.

In multivariate logistic regression analysis adjusted for age and sex, the following factors were associated with high risk of GC in Current + Ex-smokers ($P=0.030$; OR:1.79, 95% CI = 1.06-3.01), high-fat diet ($P=0.047$; OR:1.66, 95% CI = 1.02-2.95), and

TABLE 1

Characteristics of the study population. Odds ratios (OR) for gastric cancer according to Cigarette smoking, Fat intake, *H. pylori* infection, Genotype and Alleles frequencies, T Carriers using case-control data

Variable	Cases N = 104		Controls N = 196	P	OR* (95% CI)	P	OR** (95% CI)
	N (%)	N (%)	N (%)				
Gender							
Male	57 (54.8)		89 (45.4)	0.121	-		-
Female	47 (45.2)		107 (54.6)				
Age							
Male	59.9 ±14.4		64.0 ±10.3	0.117 [#]	-		-
Female	60.4 ±11.6		62.0 ±14.0				
Total	60.7 ±11.8		63.0 ±12.4				
Smoker							
Never	40 (38.5)		113 (57.6)		1.00 Reference		1.00 Reference
Current +Ex-Smokers	64 (61.5)		83 (42.4)	0.007	2.00 (1.20-3.31)	0.002	2.18 (1.34-3.54)
Fat Intake							
Low ¹	54 (51.9)		134 (68.4)		1.00 Reference		1.00 Reference
High ²	50 (48.1)		62 (31.6)	0.010	1.92 (1.17-3.15)	0.005	2.00 (1.23-3.26)
<i>H. pylori</i> infection							
Negative	57 (54.8)		89 (45.4)		1.00 Reference		1.00 Reference
Positive	47 (45.2)		107 (54.6)	0.101	0.71 (0.44-1.15)	0.121	0.69 (0.43-1.11)
Genotypes							
A/A	15 (14.4)		52 (26.5)	0.036 ⁺	1.00 Reference		1.00 Reference
A/T	58 (55.8)		85 (43.4)	0.008	2.50 (1.27-4.90)	0.005	2.37 (1.22-4.60)
T/T	31 (29.8)		59 (30.1)	0.106	1.83 (0.88-3.79)	0.143	1.82 (0.89-3.74)
A Allele	88 (43.3)		189 (48.2)		1.00 Reference		1.00 Reference
T Allele	120 (57.7)		203 (51.8)	0.631	1.09 (0.76-1.56)	0.195	1.27 (0.90-1.78)
A/T + A/A	73 (70.2)		137 (69.9)		1.00 Reference		1.00 Reference
T/T	31 (29.8)		59 (30.1)	0.866	0.96 (0.56-1.62)	0.958	0.99 (0.59-1.66)
A/T + T/T	89 (85.6)		144 (73.5)		1.00 Reference		1.00 Reference
A/A	15 (14.4)		52 (26.5)	0.003	0.45 (0.24-0.86)	0.014	0.47 (0.25-0.88)
No T Carriers	15 (14.4)		52 (26.5)				
T Carriers	89 (85.6)		144 (73.5)	0.015	2.21 (1.17-4.20)	0.017	2.14 (1.14-4.03)

Values are means ± standard deviation for continuous variables and n (%) for categorical variables. Low¹: almost never, 3-4 times/mo; High²: 3-4 times/wk, more than once/d. *P* for trend Pearson Chi-Square (χ^2) test; [#]Two-Sample T-Test. *OR (Odds Ratios) and CI (Confidence interval) adjusted by age and sex. **Unadjusted; ⁺*P* for Genotypes trend.

A/T genotype individuals ($P=0.014$; OR: 2.36, 95% CI = 1.19-4.67) as shown in Table 2.

Figure 2 shows the survival estimated by Kaplan-Meier analysis and comparison of curves by log-rank test; our study showed no difference between the probability of survival of GC patients and IL-8 (rs4073) polymorphism ($P=0.038$). GC patients with advanced stage were significantly associated ($P=0.003$) with decreased survival.

DISCUSSION

Gastric cancer is the fourth most common cancer and the second cause of death from cancer worldwide (Crew et al., 2006; Jemal et al., 2008). Data from the Brazilian National Cancer Institute (INCA) show that GC is more frequent among men (64.3%), with a higher incidence in patients over the age of

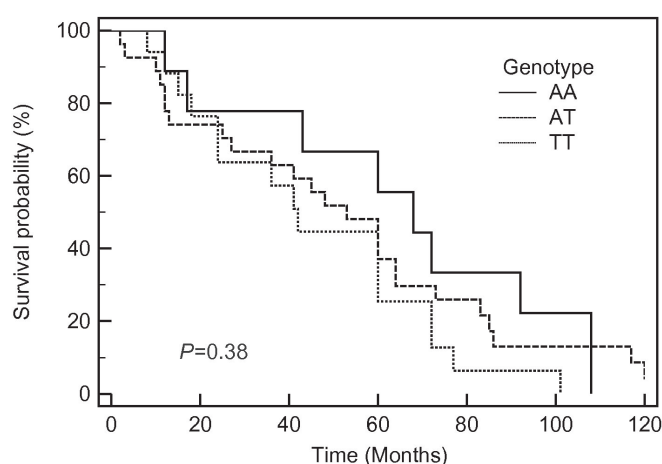


Figure 2. Kaplan-Meier survival plot for gastric cancer patients by interleukin-8 genotype. Survival was estimated Kaplan-Meier analysis and comparison of curves by long-rank test, our study showed no difference between the probability of survival of GC patients and IL-8 polymorphism.

50 in both genders (INCA, 2010). A higher prevalence among men (54.8%) was also observed in the present study, with the mean age being 60.7 years in the two genders.

Studying patients from Nagoya, Japan, Shirai et al. (2006) demonstrated an association between GC and infection with *H. pylori*, with a higher rate of infection being observed among patients with GC (91.1%) compared to those without cancer (58.8%). In the present study, no significant difference in the proportion of patients infected *H. pylori* was diagnosed ($P=0.101$). Similarly, Lu et al. found no increased frequency of patients infected with *H. pylori* in the cancer group ($P=0.18$) (Lu et al., 2005).

The occurrence of GC has also been associated with exposure to intrinsic (genetic) and extrinsic factors, such as the consumption of diets containing high concentrations of sodium chloride, nitrates and nitrites found in smoked foods, sausages and potato chips (Teixeira and Nogueira, 2003). In the present study, no significant differences in dietary habits (consumption of fruits, cereals, red meat, and vegetables) were observed for patients with GC. However, the consumption of fried foods and fats was significantly greater among cancer patients compared to subjects without cancer.

Epidemiological studies have demonstrated a relationship between nutrition and GC, with the observation of a protective effect of diets containing fresh vegetables and fruits, probably due to the presence of vitamin C and carotene which reduce the risk of cancer (Kono and Hirohata, 1996; Latorre, 1997). However, this relationship was not observed in the present study, possibly because of the difficulty in measuring the intake of these foods throughout life. A food frequency questionnaire on the consumption of certain foods and beverages was applied to the subjects to evaluate the relationship between diet and disease (Salvo and Gimeno, 2002). In a multicenter study involving 10 European countries, Buckland et al. (2010) found a significant reduction in the risk of GC in countries where adherence to the Mediterranean diet was greater.

The -251A/T polymorphism in the promoter region of the IL-8 (rs4073) gene has been the target of studies investigating which genetic profile is associated with a higher risk of or

TABLE 2
Multivariate logistic regression analysis stratified by the selected variables

Variable	Cases N = 104	Controls N = 196	P	OR* (95% CI)
<i>Smoker</i>				
Never	40 (38.5)	113 (57.6)		1.00 Reference
Smokers**	64 (61.5)	83 (42.4)	0.030	1.79 (1.06-3.01)
<i>Fat Intake</i>				
Low ¹	54 (51.9)	134 (68.4)		1.00 Reference
High ²	50 (48.1)	62 (31.6)	0.047	1.66 (1.02-2.95)
<i>Genotypes</i>				
A/A	15 (14.4)	52 (26.5)		1.00 Reference
A/T	58 (55.8)	85 (43.4)	0.014	2.36 (1.19-4.67)
T/T	31 (29.8)	59 (30.1)	0.130	1.78 (0.84-3.74)

Low¹: almost never, 3-4 times/mo; ²High: 3-4 times/wk, more than once/d; P for trend; *OR (Odds Ratios) and CI (Confidence interval) adjusted by age and sex; **Current Smokers + Ex-Smokers.

protection against gastrointestinal disease. In a recent study, Song et al. (2010) found a significant association between the IL-8 (rs4073) polymorphism and cachexia in GC patients (OR=1.765, 95% CI: 1.192-2.615; $P=0.004$). However, the number of cases and controls was relatively small in that study. A Polish study reported no association between GC and the -251A/T polymorphism (Savage et al., 2006). However in another case-control study, Japanese investigators found no significant difference in genotype ($P=0.95$) or allele ($P=0.93$) frequencies of the IL-8 (rs4073) polymorphism (Shirai et al., 2006). Similar results have been also reported by Kamangar et al. (2006) in Finland ($P=0.82$) who also studied the IL-8 (rs4073) polymorphism (OR=0.92, 95% CI: 0.42-2.00). Sarvestani et al. (2006) studied Iranian patients with GC, and observed a higher frequency of genotype T/T ($P=0.04$) and a marginal difference ($P=0.07$) in the frequency of allele T between patients (62%) and controls (56%). Similarly, Lee et al. (2005) found a higher frequency of genotype T/T in Chinese patients with GC ($P=0.002$), with 90% of these patients showing an increased risk of this malignancy (OR=1.93, 95% CI: 1.26-2.95). These authors also reported a significant difference ($P=0.004$) in T allele proportion between the cancer group (64.8%) and the control group (57.5%). No association between allele frequency and the risk of GC was observed in our study.

In contrast, a study conducted in Mexico showed that the IL-8 (rs4073) -251A allele is a risk factor for the development of noncardia GC (Garza-Gonzalez et al., 2007). In China, Lu et al. (2005) observed a greater risk of GC in subjects with genotype A/A and *H. pylori* infection (OR=2.54, 95% CI: 1.38-4.72; $P=0.012$). In the study of Taguchi et al. (2005), the A/A genotype was found to be strongly associated with gastric carcinogenesis compared to genotype T/T in Japanese patients (OR=2.22, 95% CI: 1.08-4.56; $P=0.03$).

In the present study, the individuals with homozygous A/A genotype may have a protective effect on CG (OR=0.45, 95% CI: 0.24-0.86; $P=0.003$). The heterozygous A/T genotype increased more than twice the risk of GC (OR=2.50, 95% CI: 1.27-4.90; $P=0.008$). Similar results have been reported by Ohyauchi et al., (2005) who showed that this genotype was more frequent in patients with GC (OR=2.02, 95% CI: 1.37-2.97; $P=0.0005$).

Sarvestani et al. (2006) also compared the frequency of IL-8 (rs4073) genotypes between patients with gastritis, peptic ulcer and GC and found a higher prevalence of the A/A genotype in patients with GC compared to those with benign diseases ($P=0.013$). These differences in genotypes may be due to differences in ethnic groups and disease etiology and/or sample size limitations.

We compared the IL-8 (rs4073) polymorphism with survival time in 104 patients with GC. The median survival time was 68 months for patients with the IL-8 (rs4073) -251A/A genotype, 53 months for patients with the A/T genotype, and 42 months for patients with the T/T genotype. The IL-8 (rs4073) -251 genotypes were not associated with the prognosis of GC ($P=0.38$, log-rank test). By contrast, Lurje et al. (2010) found a significant result ($P<0.001$) between the probability of survival of GC patients and IL-8 (rs4073) polymorphism, and they found the A/A genotype associated with increased survival. The limitation of the study was the relatively small sample size, which limits the generality of the results.

In conclusion, the presence of the A/T genotype was associated with an increased risk of GC in the population

studied. Individuals with A/A genotype may have protective effect on GC. No association was observed between allele frequency and the risk of GC. Subjects consuming large amounts of fried foods and ex-smokers/smokers are at higher risk of GC.

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FOOTNOTES

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Biological screening of selected flora of Pakistan

M. Zia-Ul-Haq^{1*}, M. Raza Shah², Mughal Qayum³, Sezai Ercisli⁴

¹ Department of Pharmacognosy, University of Karachi, Karachi-75270, Pakistan

² H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

³ Department of Pharmacy, Abdul Wali Khan University, Mardan-23200, Pakistan

⁴ Department of Horticulture, Ataturk University, Erzurum-25240, Turkey

ABSTRACT

Methanolic extracts of different parts of five medicinal plants, *Ferula assafoetida* L. resin, *Grewia asiatica* L. leaves, *Ipomoea hederacea* Jacq. seeds, *Lepidium sativum* L. seeds and *Terminalia chebula* Retz. fruits were tested *in vitro* for their cytotoxic, phytotoxic, insecticidal, nematocidal and anthelmintic activities. *Ipomoea hederacea* showed very significant phytotoxic and cytotoxic activity, with 100% inhibition of *Lemna minor* growth and 100% death of *Artemia salina* at concentrations of 1000 and 100 µg mL⁻¹. *Grewia asiatica* exhibited very weak activities while *Lepidium sativum* and *Ferula assafoetida* showed moderate to good potential in all three bioassays. The results suggest screening of *Ipomoea hederacea* seeds further for isolation of bioactive compounds that may be responsible for its toxic potential.

INTRODUCTION

Pakistan has varied geographic, climatic and vegetation features which are essential to the formation of one of the most diverse flora, which varies through latitudinal and longitudinal gradients. The country is characterized by a wide range of macrohabitats across elevation and climatic gradients, generating high environmental heterogeneity. Some important thematic areas of indigenous flora have been recently covered (Zia-Ul-Haq et al., 2009; 2010; 2011 a, b, c, d, e; 2012 a, b, c, d, e, f; Nisar et al., 2010 a, b, c; 2011), however most of medicinal flora of Pakistan remains unexplored. All plants may not be as useful as claimed, or may have more therapeutic properties than are known traditionally. Claimed therapeutic efficacies of medicinal plants are often criticized due to dearth of research, critical evaluation and *in vivo* studies, questioning the safety of their use. Therefore, proper scientific investigations are required to explore the exact medicinal potential of plants.

Ferula assafoetida L. (Umbelliferae), *Grewia asiatica* L. (Malvaceae), *Ipomoea hederacea* Jacq. (Convolvulaceae), *Lepidium sativum* L. (Brassicaceae), and *Terminalia chebula* Retz. (Combretaceae) are multi-purpose medicinal plants used in various parts of Pakistan. *Ferula assafoetida* resin is believed to possess sedative, expectorant, analgesic, carminative, stimulant, antiperiodic, antidiabetic, antispasmodic, emmenagogue, vermifuge, laxative, anti-inflammatory, contraceptive and antiepileptic properties (Zia-Ul-Haq et al., 2012d). *Grewia asiatica* is believed to possess antipyretic, antidiabetic, analgesic, antifertility, antibiotic and antimicrobial properties (Zia-Ul-Haq et al., 2012a). *Ipomoea hederacea* seeds are diuretic, anthelmintic, a blood purifier, and are used for treatment of constipation, inflammations, abdominal diseases, fevers, headaches, bronchitis and dropsy (Nasir and Ali, 1979; Chopra et al. 1986; Joshi, 2000). *Lepidium sativum* seeds are used in chronic enlargement of liver and spleen. The bruised seeds mixed with lime juice are used as local application for the relief of inflammatory and rheumatic pains. The seeds are bitter, themogenic, depurative, rubefacient,

galactagogue, emmenagogue, tonic, aphrodisiac and diuretic (Manohar et al., 2012). *Terminalia chebula* fruit is a mild laxative, stomachic, tonic and antispasmodic. It is useful in ophthalmia, hemorrhoids, dental caries, bleeding gums and oral cavity ulcers. Its paste with water is found to be anti-inflammatory, analgesic and to have purifying and healing capacity for wounds (Nadkarni, 1976). Besides their diverse and broad spectrum ethnopharmacological uses, all these plants have some common medicinal uses, since they all possess antimicrobial, antioxidant and anti-inflammatory potential.

Biological screening including cytotoxic, phytotoxic, insecticidal, nematocidal and anthelmintic activities is an important part of the development of new drugs from medicinal plants. These are low cost, quick, and easy benchtop general bioassays used for screening and monitoring of crude extracts of plants. To the best of our knowledge, no systematic investigation has been made on biological screening of the medicinal plants that we have selected. The current study has been designed to screen the methanolic extracts of selected plants for phytotoxicity, cytotoxicity and nematocidal activities. The present research will provide the needed preliminary observations necessary to select among crude extracts, those with potentially useful properties for further biochemical investigations.

MATERIALS AND METHODS

Preparation of crude extract

The plant materials, *Ferula assafoetida* L. (resin), *Grewia asiatica* L. (leaves), *Ipomoea hederacea* Jacq. (seeds), *Lepidium sativum* L. (seeds), and *Terminalia chebula* Retz. (fruits) were purchased from a local market. These were identified by Dr. Shakeel Ahmad, Bahuddin Zakariya University Multan and a voucher specimen was deposited in the Botany Department of this university. The plant material was air-dried in shade and crushed to a coarse powder separately using mortar and pestle. Plant material (0.5 kg each) was macerated with aqueous methanolic mixture (80:20; v/v), at room temperature for

*Corresponding author: Sezai Ercisli. Department of Horticulture, Ataturk University, Erzurum-25240, Turkey. E-mail: sercisli@gmail.com

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fifteen days with occasional shaking. The process was repeated three times with the same quantity of solvent mixture. The extracts so obtained were combined, filtered through filter paper under vacuum and concentrated under reduced pressure in a rotary evaporator (model Q-344B – Quimis, Brazil) using a warm water bath (model Q-214M2 – Quimis, Brazil) to obtain a thick gummy mass, which was further dried in a desiccator and stored in an air-tight vial until further use.

Phytotoxic activity

Phytotoxic activity was determined by using the modified protocol of *Lemna minor* (Ali et al., 2009; Nisar et al., 2010 b, c). The medium was prepared by mixing various constituents in 100 ml distilled water and the pH was adjusted (5.5-6.5) by adding KOH solution. The medium was then autoclaved at 121 °C for 15 minutes. The extracts dissolved in ethanol (20 mg/ml) served as stock solution. Nine sterilized flasks, three for each concentration, were inoculated with 1000 µl, 100 µl and 10 µl of the stock solution for 500, 50 and 5 ppm respectively. The solvent was allowed to evaporate overnight under sterile conditions. To each flask, medium (20 ml) and plants (10), each containing a rosette of three fronds of *Lemna minor* L., were added. All flasks were plugged with cotton and kept in the growth cabinet for 7 days. The number of fronds per flask were counted and recorded on day seven and their growth regulation in percentage was calculated by the following formula:

Growth regulation (%) =

$$\text{Mortality}(\%) = \left(\frac{100 - \text{Number of fronds in test sample}}{\text{Number of fronds in negative control}} \right) \times 100$$

The result was calculated with reference to the positive and negative control (Table 1). Paraquat was used as a standard drug, while paraquat and volatile solvent were used as positive and negative controls (Nisar et al., 2010 b, c).

Cytotoxic activity

This activity is an excellent and simple preliminary method to determine the cytotoxicity of crude plant extract and pure natural compounds (Nisar et al., 2010 b, c). In this method, artificial “sea water” was prepared by dissolving 3.8 g sea salt per liter of double distilled water and filtered (Meyer et al., 1982). “Sea water” was placed in a small tank; brine-shrimp eggs were added (1mg) (*Artemia salina*) and the tank was darkened by covering with aluminum foil. It was allowed to stand for 24 hours at 25 °C, which provided a large number of larvae. Twenty milligrams of the concentrated sample was dissolved in 2 ml CHCl₃ (20 mg/2 ml) and transferred to 500, 50 and 5 µl vials corresponding to 1000, 100 and 10 µg per ml, respectively. Three replicates were prepared for each concentration, making a total of nine vials. The vials containing material were concentrated, dissolved in DMSO (50 µl) and 5ml “sea water” added to each. Then 10 shrimp were added per vial and allowed to stand for 24 hours; then shrimp were counted and the number of surviving shrimp recorded. Etoposide was used as positive control. The data were analyzed with a Finney computer program to determine the LD₅₀ values (Table 2).

Insecticidal activity

Crude extract and all fractions were evaluated against different insects, viz. *Tribolium castaneum*, *Callosobruchus analis*, and *Rhyzopertha dominica*. The test sample was prepared by dissolving 200 mg of crude fractions in 3 ml acetone and loading in Petri dishes covered with filter paper. After 24 hours, 10 test insects were placed in each plate and incubated at 27 °C for 24 hours with 50% relative humidity in a growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls (Table 3). Permethrin was used as a standard drug, while permethrin, acetone and test insects were used as positive and negative controls (Ali et al., 2009; Rashid et al., 2009).

The percentage mortality was calculated by the formula:

$$\text{Growth regulation}(\%) = \left(\frac{\text{Number of insects alive in test}}{\text{Number of insects alive in control}} \right) \times 100$$

TABLE 1
Phytotoxic activity of extracts by *Lemna minor* bioassay

Samples	Conc.	% Growth regulation	Conc. of std. drug (µg/ml)
<i>F. assafoetida</i>		60	
<i>G. asiatica</i>		30	
<i>I. hederacea</i>	1000	100	0.015
<i>L. sativum</i>		64	
<i>T. chebula</i>		37	
<i>F. assafoetida</i>		43	
<i>G. asiatica</i>		21	
<i>I. hederacea</i>	100	100	0.015
<i>L. sativum</i>		42	
<i>T. chebula</i>		30	
<i>F. assafoetida</i>		30	
<i>G. asiatica</i>		17	
<i>I. hederacea</i>	10	80	0.015
<i>L. sativum</i>		30	
<i>T. chebula</i>		22	

TABLE 2
Cytotoxic activity of extracts by brine shrimp lethality bioassay

Sample	% deaths at doses			LD ₅₀
	1000 µg/ml	100 µg/ml	10 µg/ml	
<i>F. assafoetida</i>	50	40	25	310.3220
<i>G. asiatica</i>	26	25	22	41389.2
<i>I. hederacea</i>	100	100	70	0.0332
<i>L. sativum</i>	60	48	28	262.4612
<i>T. chebula</i>	26	24	22	346568.9
Etoposide (standard)				7.4625

Nematicidal activity

For isolation of *Helicotylenchus indicus* nematodes, 500 mg of soil samples (depth 15-25 cm) were collected from rice paddy (*Oryza sativa* L.) fields. Soil samples were processed by Cobb sieving (Cobb, 1918) and a modified Baermann funnel method (Baermann, 1917). The soil sample was put into a large bucket containing water and the mixture was vigorously stirred into a suspension, which was allowed to settle for about 2 minutes. The heavy soil particles sank to the bottom but nematodes remained suspended in the water. The remaining suspension was slowly poured over a coarse sieve (60 mesh aperture), which was continuously tapped by hand to avoid blocking. The deposit on the sieve was washed with a gentle jet of water into a beaker. This water suspension, containing eel-shaped nematodes, was passed through 200 and 300 mesh sieves. The nematodes thus recovered were mixed and water was decanted after allowing sufficient time for the nematodes to settle down. Then the nematode suspension was poured over a piece of tissue paper attached to a perforated plastic sheet placed in a funnel fitted with a rubber tube and clamped at the lower end. The water contained in the funnel barely touched the bottom of the tissue paper. Care was taken not to allow the debris to float off the edges of the tissue paper. After 24 hours the nematodes wriggled out into the clear water in the funnel and settled at the bottom, and then 100 ml of water containing the nematodes was drawn into a beaker. The nematode suspension was allowed to settle for 2 hours or more, the excess supernatant water was poured off, and the remaining concentrated content was transferred into a cavity block for examination under a stereomicroscope and selecting nematodes (Naqvi et al., 1992). Crude extracts were dissolved in water (passed through Whatman filter paper No.1) to make dilutions of 2%, 1%, 0.5%, and 0.25 %. Experiments were performed under laboratory conditions at 28±2 °C. Glass tubes 15 cm long and 8 cm were used for bioassay. Three ml were taken from all dilutions in each tube. The required amount of nematode suspension (100 freshly hatched second stage juveniles/3ml suspension) was poured into tubes to each of which an equal amount of plant extract had already been poured). Distilled water with nematode larvae was taken as control. The dead nematodes were observed under a stereoscopic binocular microscope after 24 48 and 72 hours and percentage mortality was calculated (Table 4). Nematodes were considered dead if they did not move when probed with a fine needle (Cayrol et al., 1989).

Larvicidal activity

The infective L3 larvae of *Haemonchus contortus* were obtained by culturing fecal samples directly from the rumen of the sheep naturally infected by mixed gastrointestinal nematodes. L3 Larvae were incubated with the plant extracts used at concentrations of 50, 25, 12.5, 6.25 and 3.12 mg/ml of saline phosphate buffer (Ph 7.2). Ivermectin was used as standard. Mortality of the larvae subjected to the above treatments was used as the criterion for anthelmintic activity. Motility was recorded at 0, 1, 2, 3, 4, and 8 hour intervals (Iqbal et al., 2004). LC₅₀ was determined using regression analysis and solving the regression equation (Table 5).

RESULTS AND DISCUSSION

With a rich endemic flora, Pakistan has many plant species which are not yet explored; detailed compendia of medicinal species native to Pakistan are being published. Strategies like high-throughput screening, phytochemical profiling, quality controls and standardization of raw materials and finished products, clinical trials and herbal therapeutics will lead to maximum benefits from these plants. The compiled data will be helpful for chemotaxonomy and breeding, as well as authenticity testing of phytopharmaceuticals containing these extracts.

Phytotoxic results of crude extracts of selected plants are shown in Table 1. The criteria used for phytotoxicity were as follows: 0-39% inhibition (low activity), 40-59% inhibition (moderate activity), 60-69% inhibition (good activity),

TABLE 3
Insecticidal activity of of extracts against various insects

Samples	% Mortality		
	C. analis	T.castaneum	R. dominica
F. assafoetida	30	30	25
G. asiatica	20	20	20
I. hederacea	80	70	75
L. sativum	70	65	70
T. chebula	50	50	45

TABLE 4
Nematicidal activity of extracts against *Helicotylenchus indicus*

Samples	% Mortality observed against concentration						Control
	24 hr			48 hr			
	2	1	0.5	2	1	0.5	
<i>F. assafoetida</i>	49	31	25	57	45	37	1
<i>G. asiatica</i>	22	17	10	39	23	20	1
<i>I. hederacea</i>	90	65	55	96	82	58	2
<i>L. sativum</i>	63	48	28	69	55	33	2
<i>T. chebula</i>	40	29	15	52	39	24	2

TABLE 5
Larvicidal activity (LC₅₀ mg/ml) of extracts against *Haemonchus contortus*

Samples	Larvicidal Activity(LC ₅₀)
F. assafoetida	9.11
G. asiatica	17.21
I. hederacea	2.07
L. sativum	6.52
T. chebula	4.82
Ivermectin (standard)	3.19

above 70% inhibition (significant activity). *Ipomoea hederacea* appeared to have very significant phytotoxic activity, with 100% inhibition of *Lemna minor* growth at high concentrations (1000 and 100 µg/mL). *Ferula assafoetida* and *Lepidium sativum* showed a good response (60-64%) at a concentration of 1000 µg/mL. *Grewia asiatica* and *Terminalia chebula* showed low weedcidal activity (30-37%) at a concentration of 1000 µg/mL. The data suggests the potential of *Ipomoea hederacea* for further phytochemical analysis as a potent herbicide. Interference of weeds obviously reduces the quality and quantity of agricultural crops and is responsible for huge economic losses all over the world. Synthetic herbicides are extensively used for the control of weeds in agricultural sectors. However, various factors that restrict the use of synthetic herbicides include water and soil pollution, herbicide-resistant weed populations, herbicide residues and detrimental effects on non-target species. In recent times, more emphasis has been placed on natural allelochemicals from plants for weed control in crop production especially to cope with the problem of weed resistance (Li et al., 2003; Saeed et al., 2010).

Cytotoxicity of crude extracts of selected plants is shown in Table 2. *Ipomoea hederacea* exhibited significant cytotoxic effect at high concentrations of 1000 and 100 µg/mL. *G. asiatica* and *T. chebula* exhibited weak lethality, with LD₅₀ values of 41389.2 and 346568.9 µg/mL, respectively. *Ferula assafoetida* and *Lepidium sativum* showed a moderate response, with 50-60% deaths at a concentration of 1000 µg/mL. This test is used as litmus test for presence of anticancer compounds. Our results suggest the presence of natural anticancer products in *Ipomoea hederacea* seed extract. Our results are supported by recent findings of Parekh (Parekh et al., 2012) which proved anticancerous activity of *Ipomoea hederacea* seed extract.

Insects control relies heavily on the use of synthetic insecticides; however, their widespread use has led to various problems such as the development of insect strains resistant to insecticides (Zettler and Cuperus, 1990; White, 1995; Ribeiro et al., 2003), toxic residues on stored grain, toxicity to consumers and increasing costs of application. There is an urgent need to develop safe alternatives that are of low cost, convenient to use and environmentally friendly. Insecticidal results of investigated plant extracts are shown in Table 3. The results indicated that *Ipomoea hederacea* and *Lepidium sativum* seed extracts showed the highest insecticidal activity against all three insects, while the remaining extracts had moderate to average activity against all insects.

Nematodes are pathogens which cause serious damage to agricultural crops and plants. These nematodes may be controlled by synthetic nematicides. However, environmental and human health concerns are resulting in increased restrictions on use of synthetic nematicides. Some safe procedures for nematode control have been developed based on biological control agents and organic amendments (Noling and Becker, 1994); one of them is use of plant extracts against these nematodes. This method has drawn great interest from researchers in the prevention of nematodes due to the environmental pollution problems induced by synthetic nematicides. Three different concentrations were used; 2%, 1% and 0.5% with examination after 24 and 48 hours. The results (Table 4) indicated that *Ipomoea hederacea* seed extract exhibited very strong nematocidal capacity against *H. indicus*. *Ferula assafoetida* has shown activity against *Pheretima posthuma* (Naikwadi and Bhalodia, 2009).

The LC₅₀ value for methanolic extracts against *Haemonchus contortus* are presented in Table 5. The results indicate that *L. sativum* and *T. chebula* and *I. hederacea* have significant anthelmintic activity. The greatest anthelmintic activity was shown by *I. hederacea*, whose LC₅₀ was 2.07 mg/ml and the lowest was observed for *G. asiatica*, 17.21 mg/ml. *Haemonchus contortus* is responsible for severe economic losses in sheep and goat breeding globally. The results confirm the traditional use of *L. sativum* and *T. chebula* for anthelmintic purposes (Abbas et al., 2002; Kamaraj and Rahuman, 2011).

The results clearly indicated that all activities are dose dependent, i.e. high activity at high concentrations and *vice versa*. The possible action of plant extracts is attributed to the bioactive compounds present as secondary metabolites in the form of condensed tannins, saponins, polyphenols, and flavonoids. Chemical analyses and biological and pharmacological assays provide objective evidence to validate traditional uses by indigenous communities. Further, to export herbs it is necessary to put them into value-added products. The current study will open avenues for future in depth research on these plants.

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Age-related changes in quality and fertility of porcine semen

Ioannis A Tsakmakidis^{1*}, Tarek AA Khalifa², Costas M Boscos¹

¹ Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, St. Voutyra 11, 54 627, Thessaloniki, Greece,

² EquiBiotech - Research Services in Animal Breeding, Thessaloniki, Greece

ABSTRACT

The aim of this study was to investigate the effect of boar age on quality traits and fertility of liquid-stored semen. Boars were allocated into 3 age groups: 7-10 months (young), 18-33 months (mature), 51-61 months (old). Ejaculates of $\geq 200 \times 10^6$ sperm/ml and 85% total motile sperm were extended to 30×10^6 sperm/ml, stored at 17-18 °C and used within 12-24 h for artificial insemination (AI) of 2062 multiparous sows. After 24 h of storage, aliquots of diluted semen were assessed for sperm progressive motility (SPM), incidence of sperm chromatin instability (SCI), proportion of live morphologically normal sperm (LMNS) and head morphometry of LMNS. The results showed that young boars had higher percentages of SCI and lower proportions of LMNS than those of the mature ($p < 0.05$) and old ($p < 0.001$) boars, respectively. Sperm head dimensions of young and old boars were greater ($p < 0.03$ - 0.001) than those of mature boars. The farrowing rate of young boars (65%) was significantly lower ($p < 0.001$; $\chi^2 = 30$ -61) than those of the mature (87.2%) and old (84.7%) boars. The relationship between sperm head dimensions and boar fertility was non-significant. In conclusion, boar age is an important physiological factor contributing to the success of swine AI.

Key terms: boar age; boar semen; morphology; morphometry; sperm chromatin.

1. INTRODUCTION

Artificial insemination (AI) performance in pig farms requires evaluation of semen characteristics, aiming to ensure its quality before service. Male aging is accompanied with hormonal and cellular changes affecting sperm quality as well as its fertilizing capacity. Kennedy and Wilkins (1984) found that maximum semen volume and sperm concentration could be obtained from boars of 24–29 months old, while according to Smital (2009) sperm output tended to increase up to boar age of 3.5 years. For basic semen characteristics, an age-related decrease in testosterone has been reported (Araujo and Wittert, 2011). The age-related reduction of testosterone is complex, involving intrinsic and extrinsic factors to the Leydig cells (Midzak et al., 2009). It is known that boar age and semen quality are taken into account as factors for boar culling. Replacement rates are variable, but generally boars are replaced about their third birthday unless they are truly exceptional, in which case they are kept up to 5 years of age. Huang et al. (2010) demonstrated that Duroc breed boars could be used as semen donors at least until the age of 4 years, but their reproductive longevity could be extended up to 6 years old if they were kept strictly in a temperature lower than 22 °C.

The boar age factor has been investigated concerning its effect on ejaculate volume, sperm concentration, motility and abnormalities (Wolf and Smital, 2009). Moreover, morphology of spermatozoa has been used in fertility evaluation of the male (Waberski et al., 1990; Abu Hasan Abu et al., 2011) and it is recommended as part of the spermiogram for domestic animals (Rodriguez-Martinez, 2003). In addition, sperm head morphometry has been shown to correlate with fertility in various species including pigs (Pena et al., 2005). Gil et al. (2009) showed that sperm motion characteristics of porcine spermatozoa are affected by head morphometry. However, to our knowledge, there is no report of a study on the influence of boar age on sperm head dimensions.

Sperm chromatin integrity is an independent quality parameter that may have a better diagnostic value than the standard semen analysis (Sun et al., 1997). Previous studies demonstrated that chromatin integrity reflected the possible disorders in spermatogenesis and epididymal sperm maturation (Hingst et al., 1995). It is directly related to boar fertility (Evenson et al., 1994) and has a very important contribution to the normal development of the early embryo (Ahmadi and Ng, 1999). In humans, paternal age is positively correlated with increased DNA damage in men of infertile couples (Singh et al., 2003). On the other hand, boar spermatozoa have a highly condensed chromatin where protamines pack tightly and protect the haploid DNA (Rodriguez-Martinez et al., 1990). However, in vitro handling of spermatozoa including semen storage has been discussed as a factor that may compromise chromatin-DNA integrity (Boe-Hansen et al., 2005). Other studies analyzed the relationship between chromatin integrity and sperm morphology. Although the relationship between sperm head dimensions and chromatin status remains controversial, many of the studies reported no relationship between them (Saravia et al., 2007; Gassuto et al., 2011). Aiming at improving the predictive value of semen analysis, earlier investigations supported a relation between field fertility and chromatin integrity after the combination of sperm traits such as live normal spermatozoa and stability of the deoxyribonucleic acid complex (Boe-Hansen et al., 2008; Tsakmakidis et al., 2010).

The objective of this study was to investigate the influence of boar age on some sperm quality traits (motility, viability, normal morphology, morphometry and chromatin integrity) and the fertility of liquid-stored semen. An additional objective of this study was to determine the possible differences in sperm head dimensions according to boar age (young, mature and old) and to relate them to field fertility.

*Corresponding author: Ioannis A. Tsakmakidis. DVM, PhD. Faculty of Veterinary Medicine, Aristotle University of Thessaloniki. 11, St. Voutyra str., 546 27, Thessaloniki, Greece. tel./fax: +302310994467. e-mail: iat@vet.auth.gr
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2. MATERIALS AND METHODS

2.1. Chemical reagents

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich Co., Greece. Buffers and staining solutions were sterilized by filtration through 0.22- μ m filters.

2.2. Animals

The study was conducted over a 5-month period (November, 2009 to March, 2010) in a private enterprise (Thessaloniki, Greece) and involved seventeen crossbred boars and 2062 sows. Boars of similar sire were imported from a European breeding company. Boars were selected according to the normal semen quality in routine tests (sperm concentration, morphology and motility). They were individually penned and fed on a ration formulated according to NRC standards. All boars were sexually active and under a weekly semen collection regimen throughout the study period.

2.3. Experimental design

Boars were allocated into three groups. Each group consisted of 5-7 boars aged 7-10 months (young, $n=5$), 18-33 months (mature, $n=7$) or 51-61 months (old, $n=5$). Semen samples were collected (1-2 ejaculates/boar/week) by the 'gloved hand' technique. Gel-free ejaculates ($n=300$, 11-25/boar) of $\geq 200 \times 10^6$ sperm/ml and 85% total motile spermatozoa (TMS) were diluted in one step with a commercial semen extender (BTS, Minitub-Germany) to 30×10^6 sperm/ml, packaged in 100 ml insemination doses and stored at 17-18 °C for 24 h. Stored semen with at least 75% TSM was then used for AI of multiparous sows in post-weaning standing estrus. Briefly, each sow was checked for heat twice a day. Once detected in standing heat, sows were further stimulated by back pressure and inseminated twice, 12 h and 24 h after the standing heat. Furthermore, after 24 h of semen storage doses of some ejaculates ($n=90$, 4-6/boar) were transported at 17-18 °C to the AI laboratory and assessed for sperm progressive motility (SPM), sperm chromatin instability (SCI), live morphologically normal spermatozoa (LMNS) and head morphometry of LMNS. It is worthy to note that we employed assays of SCI and LMNS in the current study in view of their correlation with fertility and ease of use together with the potential of integration of these assays into the routine work of AI centers with limited equipment (Tsakmakidis et al. 2010).

2.4. Evaluation of seminal parameters

2.4.1. Sperm concentration and motility

Sperm concentration of fresh semen was measured by a pre-calibrated photometer (SpermaCue, Minitub-Germany). In preserved samples, 1 μ l of semen was diluted with 99 μ l of 3% NaCl solution containing 0.02% eosin and sperm cell concentration was estimated using an improved Neubauer hemocytometer chamber. The percentage of TMS in fresh semen was subjectively examined ($\times 1000$, phase-contrast microscope, Leica, Germany) after dilution 10 μ l of semen with 100 μ l of BTS extender at 37 °C. In stored semen, the percentage of SPM was assessed using a computer-assisted

cell motion analyzer (Strömberg Mika, Germany). Sperm cells with both VAP>MVV and STR value >70% were included in the percentage of SPM (VAP=average pathway velocity, μ m/s; MVV=medium VAT cut-off value; STR=straightness, VSL divided by VAP; VSL=velocity straight line, μ m/s). Semen samples were diluted to 20×10^6 sperm/ml with BTS extender, incubated under aerobic conditions at 37 °C for 30 min and placed (2 μ l) onto a pre-warmed disposable 4-chamber slide (Leja Products B.V., The Netherlands). The image was digitalized through a camera mounted on a phase-contrast microscope ($\times 100$) and at least 200 spermatozoa of a minimum of eight sequential fields were tracked and analyzed using the following settings: temperature 37 °C; depth 20 μ m; velocity limit immotile 9 μ m/s; velocity limit local motile 15 μ m/s; linearity limit 90%; maximum radius for circles 30 μ m.

2.4.2. Sperm chromatin structure

The incidence of SCI was evaluated by the acridine orange test (AOT), as described by Tejada et al., (1984). AOT measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of AO fluorescence from green (native/double-stranded DNA) to red (denatured/single-stranded DNA). Semen (5 ml) was transferred to a 15-ml conical plastic tube and centrifuged at $2400 \times g$ for 3 min at 25 °C. The sperm pellet was resuspended in phosphate buffer saline (PBS, pH 7.2) to 100×10^6 cells/ml and 20 μ l of sperm suspension was spread onto a pre-cleaned glass microscope slide and allowed to dry for 20 min at 37 °C. The slides ($n=2$ /ejaculate) were fixed in Carnoy's solution (300 ml methanol and 100 ml glacial acetic acid) for 12 h at room temperature (RT). The slides were then removed from the fixative, allowed to dry for 10 min at 37 °C and stained in the dark at RT for 5 min in a jar containing AO staining solution (prepared by mixing 40 ml of 0.1% AO stock solution with 160 ml of 2.1014% citric acid monohydrate solution and 10 ml of 4.26% Na_2HPO_4 solution, pH 2.5). The slides were thoroughly rinsed by dipping in three changes of water bath, allowed to dry in the dark, mounted with anti-fading solution (0.2468 g Dabco™ (1, 4-Diazabicyclo [2.2.2] octane) dissolved in 9 ml of glycerol and 1ml PBS) and overlaid with a 24x50-mm cover slip. The cover slip was then sealed with nail polish and all slides were examined (500 spermatozoa/slide) by a single observer under Leica fluorescence microscopy (I3 filter cube, blue excitation range, BP 450/490 excitation filter, dichromatic mirror 510 and LP 515 suppression filter, $\times 1000$). The slides were randomized and coded before examination and percentage of sperm heads; a spectrum of colors ranging from yellowish green to partially or entirely red was scored as cells susceptible to chromatin instability (Khalifa et al., 2008).

2.4.3. Sperm plasma membrane integrity, morphology and head morphometry

The proportions of live (intact plasma membrane) and morphologically normal spermatozoa (LMNS) were simultaneously assessed in smears ($n=2$ /ejaculate) stained with eosin-nigrosin stain as described by Barth and Oko (1989). Five hundred spermatozoa from each preserved ejaculate were examined under a bright-field microscope ($\times 1000$). The deviations of sperm head, midpiece and principal piece, as well as the proximal and distal cytoplasmic droplets were

scored as cells with morphological abnormalities. Furthermore, head dimensions of LMNS were manually examined in the same stained smears under phase-contrast microscopy equipped with a preset eye-piece ocular micrometer ($\times 1000$). The length and width (μm) of fifty live normal sperm heads from each preserved ejaculate (300 spermatozoa/boar; 1800 spermatozoa/age group) were measured.

2.5. Fertility data analysis

The farrowing rate (number of farrowed sows/number of inseminated ones) and the litter size (total number of piglets born alive/farrowed sow) of the pig farm were recorded and analyzed.

2.6. Statistical analysis

Data were analyzed according to the statistical procedures described by Petrie and Watson (2006), using the SPSS statistical package (SPSS® 11.01.1 Statistical Software Inc., Chicago, 2001). The assumption of data normality was verified by the Kolmogorov-Smirnov test. All data were nearly normally distributed. The inter- and intra-assay coefficients of variation (CV) were estimated for sperm head dimensions. Hypothesis testing was performed by parametric tests which included analysis of variance (ANOVA), chi-squared (χ^2) analysis and Pearson's correlation coefficient (PCC). Multifactorial ANOVA models were used to test the effects of age group, boar, semen ejaculate and their possible interactions on SPM, SCI, LMNS, sperm head dimensions and litter size. If ANOVA revealed significant differences among means (main effects), a planned multiple comparison of the means were performed using Duncan's multiple range test. The variations among farrowing rates due to age groups or boars were examined by χ^2 analysis. PCC was used to measure degrees of association (r) between sperm head morphometry and fertility traits. Differences were considered significant at $p < 0.05$. Unless otherwise stated, results were expressed in percentages and are presented as means $\pm$ SEM.

3. RESULTS

The percentage of SPM varied between 64.20 and 83.60% among semen ejaculates and between $62.94 \pm 2.72\%$ and $71.55 \pm 2.48\%$ among boars ($p < 0.05$), with an overall mean value of $67.83 \pm 0.98\%$. The effect of age (Table 1) and its

interaction with boar or semen ejaculate on SPM were non-significant.

The incidence of SCI ranged between 0 and 10% among semen ejaculates and between $0.16 \pm 0.05\%$ and $5.20 \pm 1.32\%$ among boars, with overall mean values of $2.16 \pm 0.46\%$. The effect of boar ($p < 0.001$) and age ($p < 0.05$) on SCI was significant. Mature boars had a lower value ($1.62 \pm 0.30\%$) of SCI than those of the young ($3.51 \pm 0.76\%$) and old ($3.33 \pm 0.70\%$) boars (Table 1). No significant interactions were found between age group, boar and ejaculate on SCI.

The proportion of LMNS varied between 41 and 93.60% among semen ejaculates and between $45.81 \pm 1.32\%$ and $80.26 \pm 1.76\%$ among boars, with overall mean values of $65.40 \pm 1.25\%$. Both boar and age significantly influenced the percentage of LMNS ($p < 0.001$). Semen samples of young ($59.29 \pm 2.28\%$) and mature ($61.48 \pm 3.03\%$) boars had lower values of LMNS than that ($72.67 \pm 2.89\%$) found in semen samples of the old boars (Table 1). The interactions between boar, age group and semen ejaculate on LMNS were statistically non-significant.

The overall mean value of sperm head length was $9.00 \pm 0.02 \mu\text{m}$ (SD = 0.53; CV = 5.89%). The intra-assay CV ranged between 4.57% and 7.22%. Head length varied between 7.50 and $10.5 \mu\text{m}$ among semen ejaculates and between 8.77 ± 0.06 and $9.24 \pm 0.08 \mu\text{m}$ among boars. Significant effects were found for boar ($p < 0.001$) and age ($p < 0.03$) on head length. Mature boars had lower mean values of sperm head length ($8.95 \pm 0.03 \mu\text{m}$) than those of the young ($9.04 \pm 0.03 \mu\text{m}$) and old ($9.06 \pm 0.03 \mu\text{m}$) boars (Table 2). No significant interactions were found between boar, age group and ejaculate on sperm head length.

The overall mean value of sperm head width was $4.95 \pm 0.01 \mu\text{m}$ (SD = 0.24; CV = 4.85%). The intra-assay CV ranged between 3.33% and 6.85%. Head width fluctuated between 4 and $6 \mu\text{m}$ among semen ejaculates and between 4.80 ± 0.05 and $4.99 \pm 0.04 \mu\text{m}$ among boars. The effect of boar ($p < 0.01$) and age ($p < 0.001$) on head width was significant. As shown in Table 3, mature boars had lower mean values of sperm head width ($4.93 \pm 0.01 \mu\text{m}$) than that ($4.98 \pm 0.01 \mu\text{m}$) of the old boars. Nevertheless, sperm head length of young boars ($4.95 \pm 0.02 \mu\text{m}$) did not vary significantly from those of the mature and old boars. Furthermore, boar and ejaculate by age interactions on head width were non-significant.

The overall values of farrowing rate and live litter size were 84.77% and 10.87 ± 0.23 . Farrowing rate varied between 59.30% and 87.67% among boars ($P < 0.005$; $\chi^2 = 9.87$). As indicated in Table 4, young boars had a significantly ($p < 0.001$) lower

TABLE 1

Effect of boar age on incidence (mean $\pm$ SEM) of sperm progressive motility (SPM), chromatin stability (SCI) and live morphologically normal spermatozoa (LMNS) in 24-h liquid-stored semen

Age group	N1	N2	SPM (%)	SCI (%)*	LMNS (%)**
Young boars (7-10 months, n= 5)	6	30	68.81 ± 1.63 a	3.51 ± 0.76 a	59.29 ± 2.28 a
Mature boars (18-33 months, n= 7)	4-5	30	69.33 ± 1.75 a	1.62 ± 0.30 b	61.48 ± 3.03 a
Old boars (51-61 months, n= 5)	6	30	66.92 ± 2.01 a	3.33 ± 0.70 a	72.67 ± 2.89 b
Overall	4-6	90	67.83 ± 0.98	2.16 ± 0.46	65.40 ± 1.25

Values with dissimilar letters within columns are significantly different ($P < 0.05^*$ and $< 0.001^{**}$). The effect of boar on SPM, SCI and LMNS is significant ($P < 0.05$ -0.001). N1 and N2 denote number of ejaculates used per boar and per group, respectively.

TABLE 2
Effect of boar age on head length (μm) of live morphologically normal spermatozoa in 24-h liquid-stored semen

Age group	N1	N2	Mean	SD	SEM	CV (%)
Young boars (7-10 months, n= 5)	6	30	9.04 a	0.46	0.03	5.09
Mature boars (18-33 months, n= 7)	4-5	30	8.95 b	0.38	0.03	4.25
Old boars (51-61 months, n= 5)	6	30	9.06 a	0.35	0.03	3.86
Overall	4-6	90	9.00	0.53	0.02	5.89

Values with dissimilar letters are significantly different ($P < 0.03$). The effect of boar on sperm head length is significant ($P < 0.001$). N1 and N2 denote number of ejaculates used per boar and per group, respectively.

TABLE 3
Effect of boar age on head width (μm) of live morphologically normal spermatozoa in 24-h liquid-stored semen

Age group	N1	N2	Mean	SD	SEM	CV (%)
Young boars (7-10 months, n= 5)	6	30	4.95 ab	0.27	0.02	5.45
Mature boars (18-33 months, n= 7)	4-5	30	4.93 b	0.25	0.01	5.07
Old boars (51-61 months, n= 5)	6	30	4.98 a	0.16	0.01	3.21
Overall	4-6	90	4.95	0.24	0.01	4.85

Values with dissimilar letters are significantly different ($P < 0.001$). The effect of boar on sperm head length is significant ($P < 0.01$). N1 and N2 denote number of ejaculates used per boar and per group, respectively.

TABLE 4
Effect of boar age on fertility of liquid-stored semen

Age group	N1	N2	Farrowing rate (%)	Live litter size (mean $\pm$ SEM)
Young boars (7-10 months, n= 5)	11	55	65.00 (117/180) a	11.62 $\pm$ 0.51 a
Mature boars (18-33 months, n= 7)	25	175	87.24 (1259/1443) b	10.53 $\pm$ 0.25 a
Old boars (51-61 months, n= 5)	14	70	84.74 (372/439) b	11.13 $\pm$ 0.23 a
Overall	11-25	300	84.77 (1748/2062)	10.87 $\pm$ 0.23

Values with dissimilar letters within columns are significantly different ($P < 0.001$; $\chi^2 = 29.99$ -61.42). The effect of boar on farrowing rate is significant ($P < 0.005$). N1 and N2 denote number of ejaculates used per boar and per group, respectively.

farrowing rate (65%) compared to those of mature (87.24%; $\chi^2 = 61.42$) and old (84.74%; $\chi^2 = 29.99$) boars. There was no significant difference between farrowing rates of mature and old boars, and the age of the boar had no significant effect on litter size (Table 4). Furthermore, boar and ejaculate by age interactions on litter size were non-significant.

Pearson correlation analysis did not show significant relationships between head length of LMNS and farrowing rate ($r = -0.04$) or litter size ($r = -0.24$). Likewise, there were no relationships ($p > 0.05$) between head width of LMNS and farrowing rate ($r = 0.20$) or litter size ($r = 0.02$).

4. DISCUSSION

Many factors including age could affect boar semen quality. The present study investigated effect of boar age on semen parameters and fertility. Significant differences were detected among boar age groups. Meanwhile, the non-significant age by boar and age by ejaculate interactions indicate that influences of boar and ejaculate were homogeneously distributed among

studied age groups. To our knowledge, there are no previous studies that have investigated boar age in relation to SCI.

In dairy bulls, Hallap et al. (2005) reported that animal age did not significantly influence SCI and total sperm morphological abnormalities. However, Karabinus et al. (1990) recorded a high incidence of chromatin instability and a low proportion of intact acrosomes in spermatozoa of young bulls. Furthermore, in a recent study on dairy sheep in our lab, Lymberopoulos et al. (2010) observed that mature rams had significantly lower values of sperm hyperactive motility and peroxidized sperm, greater percentages of sperm with intact plasma membrane, functional mitochondria and condensed chromatin, as well as greater lambing rate and ewe prolificacy. These results are in accordance with the results of our investigation, in which young boars demonstrated higher values of sperm morphological abnormalities, chromatin instability and sperm head length than mature boars after 24 h of semen storage. All these characteristics reflected in a significantly lower farrowing rate compared to the other groups.

In addition, high SCI without reduction of farrowing rate was noticed in old boars. Considering that this phenomenon has been extensively studied in humans, these results are in agreement with previous studies that reported a higher DNA fragmentation in aged men (Wyrobek et al., 2006; Rybar et al., 2011). It is known that aging induces apoptosis in the epididymis, which seems to be caused by the fall of testosterone levels and is probably related to an increase of oxidative stress in tissues (Jara et al., 2004). It has been stated by Sailer et al. (1996) that abnormal chromatin structure may lead to problems in sperm nuclear packaging material, which can be related to morphologically abnormal spermatozoa. This was noticed in our study with respect to young boars, but not in old boars; SCI was also at a high level in this group. Therefore, more uninvestigated age-related factors are involved in spermatozoa production and function.

From the literature it is clear that a DNA fragmentation index (percentage of abnormal *vs.* normal cells for DNA fragmentation) beyond 6% may lead to reduced farrowing rate and/or decreased average total number of pigs born per litter (Didion et al., 2009). In the present study the percentage of SCI was less than 6% (3.51% and 3.33% for young and old boars, respectively). Moreover, Boe-Hansen et al. (2008) demonstrated that differences in litter size can be observed with thresholds as low as 2.1% or 3.0% for the DNA fragmentation index in liquid-stored boar semen. In our study no differences in litter size were found, but we tested crossbred boars of different ages, while in the previous study Hampshire, Danish Large White and Landrace boars were used. Furthermore, Waberski et al. (2011) reported no effect of DNA fragmentation index greater than 5% compared to that of less than 5% on farrowing rate and litter size, using ejaculates from Pietrain boars of mean age 36 months. This is consistent with our results of boars with similar age concerning the fertility data (mature boar group). In the present study, the SCI of the mature boars was less than 5% (1.62%), while the fertility measures remained at acceptable levels (farrowing rate, 87.24% and live litter size, 10.53).

In general, our study revealed that the susceptibility of boar spermatozoa to SCI was dependent on boar age. Young and old boars were more susceptible to SCI compared to mature boars and this property had a negative effect on the farrowing rate but not on the number of piglets born live. However, the increase of chromatin instability of old boars was not associated with a decrease in farrowing rate as observed in young boars. An explanation could be that the higher percentage of LMNS found in semen of old boars compared to young ones counterbalanced the detrimental effect of SCI. More studies are needed to rationalize the impact, or to associate the above-mentioned parameter with lower farrowing rates observed after AI with semen of young boars.

Spermatozoa differ in shape and dimensions among species and even between and within individuals (Thurston et al., 2001). Gil et al. (2009) suggested that boar spermatozoa head morphometry influenced the motility characteristics. According to Smital (2009) the highest incidence of sperm with pear-shaped heads occurred in boars up to 12 months of age and the highest incidence of sperm with simple bent tails occurred in boars older than 30 months. The results obtained in the present study demonstrated differences among boar age groups in LMNS and sperm head dimensions. The sperm head of mature boars was found to be significantly shorter than those of the other groups, and the sperm head of old boars was

significantly wider than those of the mature boars. However, no significant relationship was found between sperm head length or width and farrowing rate. These results indicate that this level of differences in sperm head dimensions is not a sensitive marker to explain the variation in farrowing rates observed among boar age groups. To our knowledge, this is the first study which analyzed the effect of boar age on sperm head dimensions. Nevertheless, similar studies have been conducted in other animal species. Marti et al. (2011), aiming at using morphometry as an indicator of ram sexual maturity, pointed out a direct relationship between sexual maturity and sperm head dimensions and shape. They observed significant differences for all variables (area, perimeter, length and width) of sperm head dimensions between young and middle-aged or old rams, while except for length, all morphometric dimension values decreased with increasing ram age.

In conclusion, the results of this study revealed significant differences among boars of different ages in terms of chromatin stability, morphology and sperm head morphometry. Young and old boars are more susceptible to SCI compared to mature boars, while the risk of obtaining unacceptable farrowing rates is higher after AI with semen from young than from mature and old boars. Therefore, boar age is an important physiological factor that contributes to the success of AI with preserved semen and should be taken into account.

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Antioxidant, Antibacterial activity and Brine shrimp toxicity test of some Mountainous Lichens from Nepal

Babita Paudel¹, Hari Datta Bhattarai¹, Durga Prasad Pandey², Jae Seoun Hur³, Soon Gyu Hong¹, Il-Chan Kim¹, Joung Han Yim^{1*}

¹ Division of LifeSciences, Korea Polar Research Institute, KOPRI, Songdo Technopark, Songdo-dong 7-50, Yeonsu-gu, Incheon 406-840, South Korea, Tel. +82-32-260-6340, Fax +82-32-260-6301

² Research Center for Applied Science and Biotechnology, Tribhuvan University, Kirtipur, P.O.Box. 5026. Kathmandu, Nepal. Tel. 977-1-4330348

³ Korean Lichen Research Institute, Suncheon National University, Suncheon 540-742, Korea

ABSTRACT

A total of twenty four lichen species belonging to six families were collected from mountainous region of Nepal. The methanol extracts of each species were tested for antimicrobial and antioxidant activities *in vitro*. It was found that extracts of twenty one lichen species were active against *B. subtilis* and seven species were active against *S. aureus*. Similarly, in DPPH assay, three species *Peltigera* sp., *Cladonia* sp., and *Canoparmelia* sp. showed comparable activity with commercial standard, BHA. In ABTS⁺ assay, extracts of *Parmotermia* sp., *Ramalina* sp., *Peltigera* sp. and *Cladonia* sp. showed stronger activity than ascorbic acid. The observed data after comparison with previously published reports indicated that the high altitude lichens contain stronger antioxidant and antibacterial constituents. Similarly, the methanol extracts of *Heterodermia* sp. and *Ramalina* sp. showed comparable toxicity effect with commercial standard berberine chloride indicating a potent source of anticancer drugs.

Key words: Antibacterial; Antioxidant; *Nepal lichens*; Free radicals

INTRODUCTION

Lichens are cosmopolitan in distribution from arctic to tropical regions and from plains to the highest mountains and some survive in the extreme environment of deserts. From the ancient time, lichens have been used for various purposes such as food, dyes, perfumes, remedies in folk medicines and decoration. Various lichen metabolites from several classes as- aliphatic acids, pulvinic acid derivatives, depsides and depsidones, dibenzofurans, diterpenes, anthraquinones, naphthoquinones, xanthenes as well as epidithiopiperazinediones have been described. These compounds have shown a wide ranges of biological activities such as antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, cytotoxic effects and antioxidant properties¹⁻².

To overcome the pace of drug resistance phenomenon in several pathogenic bacteria, several novel antibacterial compounds need to be developed. After the discovery of penicillin a number of lichens were screened for antibacterial activity³ between 1940 and 1950. Several lichen compounds were found active against gram-positive bacteria and mycobacteria⁴.

Reactive oxygen species (ROS) are harmful or lethal for living organism⁵. ROS are ions or very small molecules which include free radicals, oxygen ions and peroxides. ROS are formed inside the living organisms as natural byproducts of the normal metabolism of oxygen and help in cell signaling. Thus, living organisms accumulate various ROS through both normal metabolic processes and exogenous sources like environmental stresses such as UV light, cigarette smoke, environmental pollutants, γ -radiation. Various types of ROS are

produced inside the living organisms⁶ such as hypochlorous acid (HClO), hydrogen peroxide (H₂O₂) and free radicals such as the hydroxyl radical ($\cdot$ OH), superoxide anion (O₂⁻), alkoxy and peroxy radicals (RO $\cdot$ and ROO $\cdot$), peroxyne (ONOO $\cdot$). These ROS are highly reactive and unstable due to the presence of unpaired electron in their outer shell. They have been associated with several diseases such as inflammation, cardiovascular diseases, cancer, aging-related disorders, metabolic disorders, and atherosclerosis⁷. ROS react with various cellular components including DNA, proteins, lipids, fatty acids. Thus, the reaction between ROS and cellular components lead to damage DNA, attack unsaturated fatty acids and cause membrane lipid peroxidation, decreases in membrane fluidity, loss of enzyme receptor activities, and damage to membrane proteins, ultimately leading to cell inactivation⁸. Most of the known antioxidant activities of lichens and their chemical investigations are derived from the species of tropical and sub-tropical origin. Fewer studies have been conducted on the antioxidant activities of lichens from the extreme environment such as the Antarctic regions⁹⁻¹⁰.

Nepal is a mountainous country which contains a diverse geographic locations ranging from 60 m to 8848 m above sea level. In Nepal, 465 species of lichens (2.3% of the world species) from 79 genera (19.7% of the world genera) have been recorded, of which 48 species are endemic¹¹. Because of its unique bio-geographic location, altitudinal variation and diverse climatic and topographic conditions, several lichen species can be obtained within a small geographical area. Traditionally, a variety of lichen species are used in medication for several diseases such as inflammation, dysentery, vomiting by various indigenous community people. There are very few reports of chemical investigation of lichens

*Address for correspondence. Joung Han Yim. Polar BioCenter, Korea Polar Research Institute, KOPRI, Songdo Technopark, Songdo-dong 7-50, Yeonsu-gu, Incheon 406-840, South Korea. Tel. +82-32-260-6340, Fax. +82-32-260-6301. E-mail: jhyim@kopri.re.kr

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species from Nepal². In this report, we describe the biological activity of some mountainous lichen species which are yet to be phytochemically explored.

MATERIAL AND METHODS

Collection and identification of lichen species

Twenty-four species (Table 1) were collected from three different geographical locations of Nepal in late September and in early October, 2010. Six lichen species: NL-1 to NL-6

were collected from Nargarjun mountain, Nepal (E85°21'-85°22', N27°19'-27°23'). Similarly, nine lichen species: NL-7 to NL-15 were collected from Sarankot (E83°57'-83°58', N28°14'-28°15'), Pokhara, Nepal and remaining nine lichen species: NL-16 to NL-24 were collected from Srinagar, Palpa, Nepal (E85°17'-85°34', N27°34'-27°44'). All the lichen specimens were identified by phylogenetic analysis of ITS rDNA sequences with sequences available from GeneBank sequence database. The voucher specimens were kept in lichen herbarium, Korea Polar Research Institute, KOPRI, Incheon, South Korea.

TABLE 1
List of test lichen species and their methanol extracts' antibacterial, antioxidant and toxicity activities

Symbol	Lichen species	Family	Inhibition zone (mm)		MIC (µg/mL)		50% inhibition conc. (IC ₅₀) (µg/mL)		LC ₅₀ conc. (µg/mL)	
			B. subtilis	S. aureus	B. subtilis	S. aureus	DPPH	ABTS	Reducing power ^a	Artimia sp.
NL-1	<i>Parmotermia</i> sp.	Parmeliaceae	9	na	85±8.5	na	40.5±4.1	35.5±7.1	2.6±0.1	400
NL-2	<i>Cladonia</i> sp.	Cladoniaceae	17	9	11.7±0.6	83±6.7	38.5±3.8	51.6±6.5	1.7±0.1	200
NL-3	<i>Parmotermia</i> sp.	Parmeliaceae	10	na	81.2±7.5	na	34.4±4.4	45.6±6.7	4.6±0.4	nt
NL-4	<i>Heterodermia</i> sp.	Physciaceae	11	na	65.2±5.2	na	41.6±2.5	60.3±5.1	3.4±0.3	100
NL-5	<i>Heterodermia</i> sp.	Physciaceae	10	na	77.7±5.1	na	51.8±7	84.2±6.8	2.9±0.4	nt
NL-6	<i>Usnea</i> sp.	Parmeliaceae	25	18	35.9±2.4	89.2±8.2	45.2±5.2	86.6±10.1	4.5±0.3	nt
NL-7	<i>Parmotrema</i> sp.	Parmeliaceae	20	na	21.7±1.3	na	45.7±0.9	50.3±5.9	3.4±0.6	200
NL-8	<i>Coccocarpia</i> sp.	Coccocarpiaceae	14	na	79.4±6.4	na	72.1±5.4	48.2±3.9	2.8±0.4	nt
NL-9	<i>Heterodermia</i> sp.	Physciaceae	11	na	76.1±8.5	na	49.9±3.8	65.2±10.5	3.8±0.2	200
NL-10	<i>Parmotrema</i> sp.	Parmeliaceae	11	10	11.1±0.2	18.2±0.8	61.8±7.4	42.3±3.4	3.5±0.6	200
NL-11	<i>Parmotrema</i> sp.	Parmeliaceae	16	11	23.2±2.6	35.6±2.7	98.6±10.7	60.3±14.4	6.2±0.8	nt
NL-12	<i>Peltigera</i> sp.	Peltigeraceae	na	na	na	na	5.6±0.2	6.9±0.3	1.3±0.1	nt
NL-13	<i>Heterodermia</i> sp.	Physciaceae	na	na	na	na	48.8±5.7	95.7±9.3	6.7±1.3	200
NL-14	<i>Bulbothrix</i> sp.	Parmeliaceae	19	na	41.1±4.2	na	41.1±3.8	71.8±5.6	4.9±1.7	400
NL-15	<i>Heterodermia</i> sp.	Physciaceae	9	na	89.8±8.2	na	43.4±4.1	99.9±8.7	3.7±0.6	100
NL-16	<i>Cladonia</i> sp.	Cladoniaceae	na	na	na	na	10.1±2.2	7.0±0.3	1.8±0.2	200
NL-17	<i>Ramalina</i> sp.	Ramalinaceae	26	na	15.1±1.2	na	32.9±3.5	52.0±5.5	2±0.1	nt
NL-18	<i>Ramalina</i> sp.	Ramalinaceae	24	11	8.5±0.1	65.3±5.7	8.7±2.7	33.2±2.8	1.2±0.1	100
NL-19	<i>Canoparmelia</i> sp.	Parmeliaceae	17	10	4.2±0.02	32.6±2.4	80.7±8.4	68±7	3.7±0.5	nt
NL-20	<i>Parmotrema</i> sp.	Parmeliaceae	31	16	2.1±0.03	21.4±2.2	45.7±6	32.4±3.1	2.3±0.1	nt
NL-21	<i>Everniastium</i> sp.	Parmeliaceae	11	11	91.9±11	71.7±5.9	70.1±8.5	70.5±6.9	4.1±0.1	nt
NL-22	<i>Parmotrema</i> sp.	Parmeliaceae	9	na	85.3±8.2	na	58.4±10.1	42.8±6.1	2.4±0	na
NL-23	<i>Parmotrema</i> sp.	Parmeliaceae	14	na	99.9±10	na	61.2±5.9	31.4±5.3	2.6±0.2	nt
NL-24	<i>Parmotrema</i> sp.	Parmeliaceae	12	na	87.6±4.7	na	41.7±5.9	73.7±6.2	2.7±0.1	na
BHA	-	-	-	-	-	-	3.5±0.2	-	-	
Trolox	-	-	-	-	-	-	-	46.4±0.1	-	
Ampicillin	-	-	-	-	0.4±0.1	0.35±0.01	-	-	-	
Berberine chloride										8.4

^a 1 µg of BHT equivalent; na-no activity; nt, not tested

Extraction

A completely freeze-dried and grinded lichen samples (Table 1) were extracted separately in a mixture of methanol and water (80:20 v/v). Then, the extract was filtered and solvent was evaporated at 45 °C in vacuum. This extraction procedure was repeated three times to ensure the complete extraction of extractable compounds. Extracts were washed with hexanes three times to remove pigments and fats. The extracts were then lyophilized and stored at -20 °C until further use.

Chemical reagents

Antioxidant assay kit (Product code CS0790), chemicals and reagents used for *in vitro* antioxidant assays were purchased from Sigma-Aldrich, USA. All used chemicals and reagents were of analytical grade. The solvents used during extraction were HPLC grade and were purchased from DUKSAN pure chemicals Co. Limited, Korea. The media, NA (Nutrient agar), NB (Nutrient broth) and YM (Yeast mannitol), used for antimicrobial assays were purchased from Difco USA.

Antimicrobial Assay

Target microorganisms and culture condition

Five clinical microorganisms, including two Gram-positive (*Bacillus subtilis* KCTC1022 and *Staphylococcus aureus* KCTC3881) and two Gram-negative (*Escherichia coli* KCTC1039 and *Pseudomonas aeruginosa* KCTC1636) bacteria and a fungus, *Candida albicans* KCTC 7965, were purchased from Korean Collection of Type Culture (KCTC). Bacterial strains were grown on nutrient agar (NA) at 30-37 °C and *C. albicans* was grown on yeast mannitol (YM) agar at 25 °C.

Disk diffusion assay

Antimicrobial testing was carried out by using a previously described paper disk assay¹². Sterile paper disks (Adventic, Japan) of 8 mm size were loaded with lichen extract at a concentration of 500 µg/disk in triplicate and allowed to dry at room temperature under sterile conditions. The disks were kept on the surface of NA (Nutrient agar) or YM (Yeast mannitol) agar plates, which had been freshly swabbed with the overnight grown broth culture of the target microbial strains. Then, the plates were incubated at optimum growth temperature of each strain for 24 - 48 h. The zones of inhibition around the lichen extract loaded paper disks were reflective of the antimicrobial effectiveness of the extract. Paper disks loaded with methanol, a solvent used to dissolve crude extract, were used as negative controls and the paper disks loaded with ampicillin were used as positive controls.

Determination of minimum inhibitory concentration (MIC)

MIC was determined by a broth dilution method¹³. Serial dilutions of lichen extract ranging from 0 to 1000 µg/mL in 5 mL of nutrient broth in triplicate were prepared in 15 mL sterile falcon tubes. The range of the test concentration for the extracts was determined after completion of an initial range finding experiment. An overnight grown broth culture of the target strains seed, having a final cell density 10⁵ CFU/mL,

was used to inoculate in fresh nutrient broth media containing a range of test extract concentrations. The inoculated tubes were incubated at optimum temperature of each target strain (Table 1) and shaken at 250 rpm for 24 h. Microbe growth was measured spectrophotometrically (SCINCO-AMERICA) by absorbance at 600 nm. The MICs were analyzed after the linear regression analysis of the experimental data. Ampicillin was used as a positive control.

Antioxidant assay

Various *in vitro* antioxidant activities such as DPPH and ABTS^{•+} free radical scavenging capacity¹⁴⁻¹⁵ and Fe³⁺ reducing power¹⁶ of the lichen extracts were determined by comparing with available commercial standards (Table 1). These experiments were modified at various degrees as described previously¹⁷. **The experiments were conducted in triplicate and data were analyzed by using linear regression analysis.**

Brine shrimp lethality test

Brine shrimp lethality test (BST) was used to evaluate the toxicity of various lichen's crude extracts¹⁸ with slight modification. The eggs of *A. salina* were hatched in aerated seawater in light at 25 °C. The hatched active larvae will be attracted towards the direction of light. The active larvae (about 100) are selected and treated with various concentrations of test samples (0-2000 µg/ml). The effects of test samples were monitored after 24 h of treatment by observing the live larvae. The mortality rate of the larvae indicated the toxicity of the test samples. Berberine chloride a standard anticancer drug was taken as positive control and brine shrimp larvae in only sea water were taken as negative control.

RESULT AND DISCUSSION

Antibacterial activity

In the present study the antimicrobial activities of lichens extracts were determined qualitatively and quantitatively by measuring inhibition zone and MIC values. The experimental data of antimicrobial activities of twenty four lichen species are presented in Table 1. In the first step, antibacterial strength against the Gram-positive bacteria, *B. subtilis* and *S. aureus*, Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* and the yeast, *Candida albicans* was measured in terms of zone of inhibition produced by extracts of various lichen species. Eight lichen species NL-2, NL-6, NL-10, NL-11, NL-18, NL-19, NL-20 and NL-21 showed the antibacterial activity against *B. subtilis* and *S. aureus*. The antibacterial activity, as indicated by zone of inhibition size were various among the above eight lichens extract. However, *B. subtilis* was more susceptible than *S. aureus* to lichens extract. The zone of inhibition for active species extracts (500 µg/disk) was in the range of 9-31mm against *B. subtilis* and 9-18 mm against *S. aureus*. NL-20 produced highest inhibition zone against *B. subtilis* but NL-6 showed the highest inhibition zone against *S. aureus*. Similarly, seven lichen species NL-5, NL-7, NL-8, NL-14, NL-17, NL-23 and NL-24 produced remarkable inhibition zone against *B. subtilis* only. In addition, five lichens species NL-1, NL-3, NL-9, NL-15 and NL-22 showed very weak antibacterial activity against *B. subtilis*. Four lichen NL-4, NL-

12, NL-13, NL-16 did not show inhibition zone upto 500 µg/disk. The obtained result showed that none of the lichen extract was active against the Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* and the pathogenic fungus, *Candida albicans*.

The MIC (100% of growth inhibition) showing by test lichen extracts against two gram positive strains *B. subtilis* and *S. aureus* are various (Table 1). Among the tested lichen's extract, NL-18, NL-19 and NL-20 showed comparatively strong antibacterial activity against *B. subtilis*. Among them, NL-20 showed strongest activity against *B. subtilis* while NL-10 showed similar result with *S. aureus*.

Antioxidant activity

In this experiment, three *in-vitro* antioxidant assays based on electron transfer (ET) or hydrogen atom transfer (HAT) system- (DPPH free radical, ABTS^{•+} scavenging capacities and Fe⁺³ reducing power) were used to investigate the antioxidant activities of methanol-water (80:20) extract of twenty-four lichen species. ET-based assays measured the capacity of antioxidants which reduce an oxidant by changing the color¹⁹. Thus, the degree of color change was correlated with antioxidant potential. Antioxidant capacities of methanol extract of all twenty four lichen species are presented in table 1.

DPPH is commercially available stable free radical in aqueous or methanol solution and becomes a stable molecule by accepting an electron or hydrogen radical from antioxidant compounds¹⁴. All the tested lichen extracts and the commercial standard (BHA) exhibited DPPH free radical scavenging capacity in the concentration dependent manner that could easily be read by a spectrophotometer by obtaining a decreased absorbance at 517 nm. BHA is a strong commercial antioxidant agent and the IC₅₀ (50% inhibition) of this compound was 4.98 µg/mL in the present experiment. In the present experiment, the test lichens showed the various strengths to scavenge DPPH free radical. Overall, the IC₅₀ of DPPH free radical scavenging capacity of twenty-four lichens extract was found between 5.6± 0.2 and 98.6± 10.7 µg/mL. Three species NL-12, NL-16 and NL-18 showed comparatively strong DPPH free radical scavenging capacity. Similarly, NL-1, NL-2, NL-3 and NL-17 showed moderate activity.

ABTS^{•+} scavenging capacities of lichens extracts are presented in table (1). In this reaction system, the chromogen cations of ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) are produced by oxidizing ABTS with ferryl myoglobin radicals. The ferryl myoglobin radicals are produced by the reaction of metmyoglobin with hydrogen peroxide. The ABTS^{•+} free radical produces a green color and can be read spectrophotometrically at 405 nm¹⁵. Like trolox, a commercially available vitamin E analogue, the water soluble crude extracts reduced the production of ABTS^{•+} radicals in the reaction mixture in a concentration-dependent manner. The rate of scavenging capacities were various among different lichen species. Among the twenty-four lichens species tested, nine lichens NL-1, NL-3, NL-10, NL-12, NL-16, NL-18, NL-20, NL-22 and NL-23 showed stronger ABTS^{•+} scavenging capacity than commercial standard trolox. Similarly, the other four species NL-2, NL-7, NL-8 and NL-17 showed comparable activity with commercial standard.

It was observed that the reducing power of compound was directly correlated with its potential antioxidant activity²⁰⁻²¹.

The reducing activities of compounds are based on the presence of reductones, which exert antioxidant effects by donating a hydrogen atom to break the free radical chain²². The ferric ion reducing antioxidant (or reducing power) assay measured the electron transferring capacity of the test samples to convert Fe⁺³ to Fe⁺² inside a complex molecule. In the present experiment, the reducing power of the crude extracts of several tested lichen species was comparable with that of the commercial antioxidant, BHT (Table 1).

Toxicity against *Artemia salina*

A total of hundred percent of *Artemia* larvae were alive after 24 h of experiment in the negative control. LC₅₀ of positive control sample was obtained as 8.4 µg/mL. The test extract showed LC₅₀ in the range of 100-400 µg/mL even at crude level. The activity of purified compounds can be expected much stronger than that of the berberine chloride. Among the tested lichen extracts, NL-4, NL-15 and NL-18 showed comparatively strong activity.

CONCLUSION

In conclusion lichens from mountains of Nepal showed comparable antibacterial and antioxidant activities even at crude extract level with commercial standards. In previous studies²³ antioxidant activity (in term of IC₅₀) of lichens extracts from tropical and other mountainous regions were reported in mg/mL concentration level. But, in this study we found the activity were in µg/mL level which means at least thousands times stronger activity. In addition, no or very few bioactive natural products researches of mountainous lichens of Nepalese origin were published so far. Therefore, there is a high probability of getting novel and active antioxidant and antibacterial natural products from these lichens species. Such metabolites could be better candidates for future therapeutic applications. In addition, the *A. salina* test revealed that lichen extracts even contains anticancer compounds probably stronger than the positive control when tested in pure form.

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Sporogenesis and development of gametophytes in an endangered plant, *Tetracentron sinense* Oliv

Xiaohong Gan, Dan Xie, Lingling Cao

College of Life Science, China West Normal University, Nanchong, Sichuan Province 637009, China

ABSTRACT

The sporogenesis and development of gametophytes in *Tetracentron sinense* Oliv. were studied with light microscopy. The anther has four microsporangia; its primary anther wall consists of an epidermis, an endothecium, one or two middle layers and one glandular tapetum. Simultaneous cytokinesis follows meiosis, forming a tetrahedral tetrad. Mature pollen grains are two-celled at the time of anther dehiscence. Its ovule is anatropous, bitegmic and crassinucellate; the development of the female gametophyte is of the monosporic 8-nucleate Polygonum type. Significantly, some striking features were first found in *T. sinense*: (1) anther dehiscence occurs soon after the endothecium fibrously thickens and the intersporangial septum degenerates; (2) tapetum degeneration is retarded, persisting up to the stage of two-celled pollen grain; (3) a few cellular events such as the vacuolization and the contraction and deformation of the pollen mother cell (PMC) and microspore are not normal at the PMC, dyad and tetrad stages. The abnormalities during male reproduction might be one of important factors resulting in the poor natural regeneration of *T. sinense*.

Key words: *Tetracentron sinense* Oliv.; Sporogenesis; Development of gametophytes; Natural regeneration.

INTRODUCTION

Tetracentron sinense Oliv. is a deciduous tree with catkin-like inflorescences, placed either in its own family, Tetracentraceae (Fu and Bartholomew, 2001), or more commonly in the Trochodendraceae together with the monotypic Trochodendron (Martyn and Peter, 2007). This species is today restricted to central and southern China, northern Vietnam, northern Burma, and south of the Himalayas to eastern Nepal, Bhutan and northeastern India (Fu and Bartholomew, 2001), and mainly scattered within a region 24°-34.5°N, 98°-111.5°E and 900-3500 m above sea level, where the annual average temperature is about 11°C, the annual rainfall is about 1200 mm, and the relative humidity about 85% (Zhang, 1999). Flowering occurs from April to July and fruiting from July to October (Fu and Bartholomew, 2001). Due to its rarity and poor ability to regenerate naturally, it has been listed as a national second-grade protected plant species in China (Fu, 1992).

T. sinense has been used for furniture by local people for many centuries, and also used as an ornamental plant for forestation as well as a medicinal plant (Luo, 1998; Wang et al., 2006; Lai et al., 2010). As its vessel-free wood is quite rare in angiosperms, suggesting primitiveness, the species has received much taxonomic attention (Ren et al. 2007). So far, there are comprehensive studies on its morphology, anatomy, floral organogenesis (Chen et al., 2007; Ren et al. 2007), palynology (Zhang et al., 1999), phytochemistry (Wu et al., 2000; Zheng et al., 2000; Yi et al., 2000; Lai et al., 2010) and systematics (APG, 2009). Previous studies have mainly focused on discussing its systematic status. Recently, a few investigations on seed germination and seedling initial growth have been reported (Xu et al., 2006; Zhou, 2007; Gan et al., 2008; Luo et al., 2010).

Plant natural regeneration is correlated with the sexual reproduction process, through which a plant can maintain its

genetic diversity for adapting to the external environment. Knowledge of reproductive biology is necessary for effective protection of endangered plants. It has been reported that abnormalities during plant sexual reproduction, such as abnormal development of sporogenesis and loss of the pollination vector (Pan et al., 2001, 2003; Xue et al., 2005; Xiao and Xu, 2006; Zhao et al., 2008) can influence the effectiveness of reproduction, resulting in poor natural regeneration. To date, little is known about sporogenesis and gametogenesis in *T. sinense* or the impact of abnormalities during these processes on natural regeneration.

In this study, sporogenesis and development of gametophytes in *Tetracentron sinense* were studied with light microscopy. The objective of this study was to deepen the understanding of the reproductive biology of *T. sinense* and to discuss its influence on natural regeneration, as well as to provide important information for the conservation of this endangered species.

MATERIALS AND METHODS

Plant materials

Plant material of *Tetracentron sinense* was collected for the reproductive biology study from a natural population in the Emei Mountain Natural Reserve (Sichuan, China). The vouchers (Gan Xiaohong 200700A) were deposited in the Herbarium of China West Normal University (CWNU).

Cytological studies

The inflorescences of a range of developmental stages were collected from 2007 to 2009. For light microscopy, the developing inflorescences were dissected and fixed for 1 h in Carnoy's fixative solution (100% ethanol and acetic

Corresponding author: Xiaohong Gan. Address: College of Life Science, China West Normal University, Nanchong, 637009, P.R.China. E-mail: bhgan@163.com. Tel: 86-817-2568352. Fax: 86-817-2568352

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acid, 1:1 v/v), and then stored in 70% ethanol at 4 °C. After being stained with Ehrlich's hematoxylin; specimens were embedded in paraffin and the blocks were sectioned at 6-8 μ m with a Leica 2126 microtome (Germany). Observations and photography were carried out using a Motic BA300 microscope.

RESULTS

Microsporogenesis and male gametophyte formation

The anther has four sporangia (Fig.1, A), and each has a row of archesporia differentiated just beneath the epidermis. The archesporial stage of microsporogenesis was first observed in buds collected in mid March. Microsporogenesis and male gametophyte development take place from mid March to early August.

Formation of anther wall

In mid March, the primary anther wall was observed to be composed of four to five cell-layers: from the outer to the inner layer, one single layer of epidermis, one row of endothecium,

one to two rows of middle layers and one row of mononucleate tapetal cells (Fig.1, B). At the beginning of pollen mother cell (PMC) meiosis the tapetal cells are binucleate (Fig.1, C), and then become vacuolated (Fig.1, D) and degenerate in situ gradually (Fig.1, A, E-F). Thus the tapetum is of glandular type. With the development of the tapetal cells, the endothelial cells expand gradually and become vacuolated (Fig.1, D). After undergoing U-shaped thickening (Fig.1, F), the endothecium develops into a fibrous layer (Fig.1, G). All middle layers are ephemeral (Fig.1, D) and disintegrate (Fig.1, E) quickly, while the endothecium develops fibrous thickening. Thus the mature anther wall contains two cell layers; the epidermis and the fibrous layer (Fig.1, G-H).

At the time of middle layer disintegration, some sub-epidermal cells between the two locules of each theca become specialized to form the intersporangial septum, and a small set of specialized epidermal cells develop into the stomium (Fig.1, E). Afterward, degeneration of the intersporangial septum occurs first (Fig.1, G) uniting the two locules of each theca into a confluent chamber (Fig.1, H). Following this stage, the stomium splits. As a result of the process, the anther wall dehisces by two longitudinal slits and the pollen grains are released from the anther (Fig.1, I).

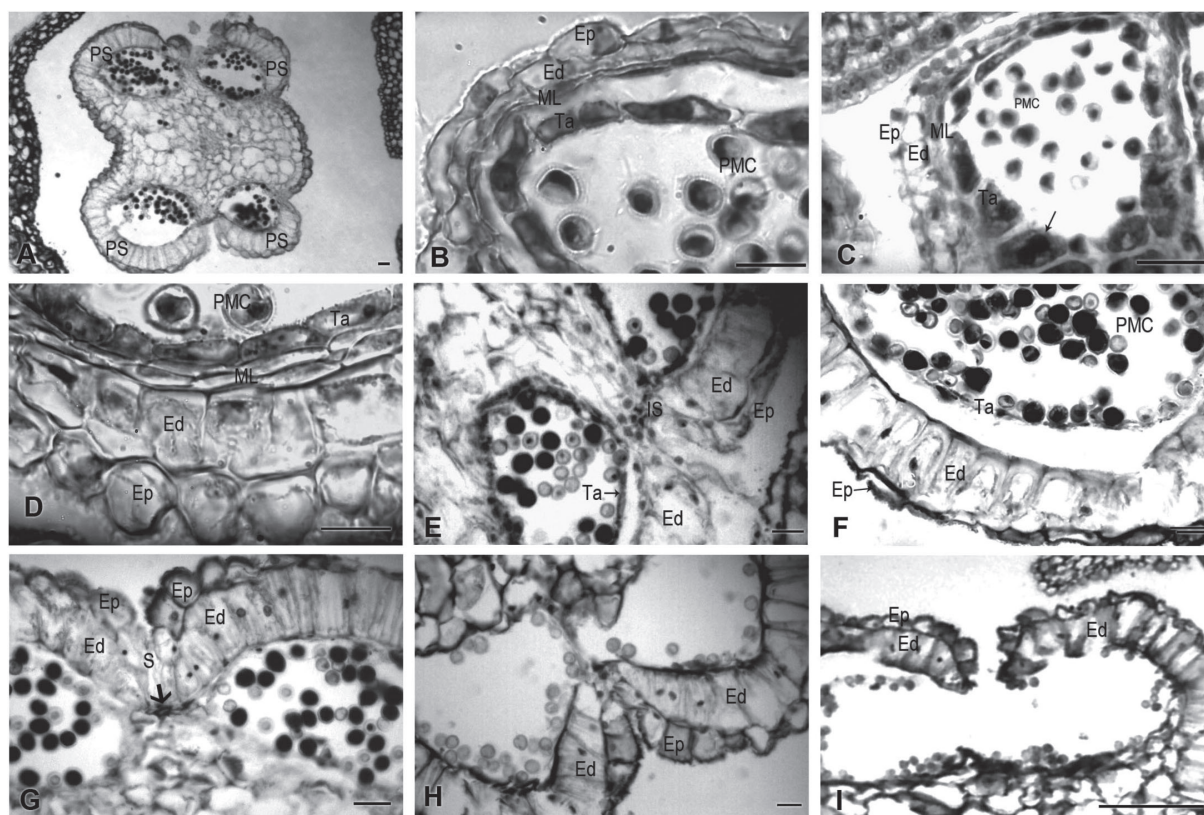


Fig. 1 The development of anther wall in *Tetracentron sinense*.

A. The anther of each stamen has four sporangia; B. At the PMC stage, the primary anther wall is composed of an epidermis, an endothecium, one or two middle layers and a tapetum; C. The tapetal cells are binucleate; D. The endothecium expands its volume and is vacuolated, and two middle layers are flattened; E. The formation of intersporangial septum and stomium, and the degeneration of middle layers; F. The U-shape thickening of endothecium and the degeneration of tapetal cells; G. The fibrous layer and the degeneration of intersporangial septum (arrow); H. The two locules of each theca were united to a confluent chamber after the degeneration of the intersporangial septum; I. Anther dehiscence after the splitting of the stomium.

Scale bar = 10 μ m. Ed: endothecium; Ep: epidermis; IS: intersporangial septum; ML: middle layer; PS: pollen sac; PMC: pollen mother cell; S: stomium; Ta: tapetum.

Microsporogenesis and male gametophyte formation

In early April, a row of sporogenous cells deriving from archesporial cells give rise to a mass of PMCs (Fig.2, A). The PMC undergoes one meiotic division, contributing to form a tetrahedral tetrad (Fig.2, B-E). The cytokinesis of microspores follows the simultaneous type. Cell plate is not laid down after meiosis I (Fig.2, B-C), but simultaneously comes into being after meiosis II (Fig.2, E). In early July, the microspores separate from each other and individually develop into pollen grains with dense cytoplasm and a central nucleus (Fig.2, F). Afterward, the microspore cytoplasm gradually becomes

sparse and vacuolated, and the nucleus takes a peripheral position when its tricolpate apertures form (Fig.2, G). In mid July the microspore nucleus proceeds into mitosis, resulting in the formation of two unequal cells, a large vegetative one and a smaller generative one. The generative cell becomes spindle-shaped and is enclosed in the vegetative cell (Fig.2, H). As development proceeds into early August, the generative cell is integrated into the vegetative one, indicating the formation of mature pollen. At the time of anther dehiscence, the pollen grains are 2-celled.

Significantly, a few abnormalities were investigated during microsporogenesis and male gametophyte development. At the

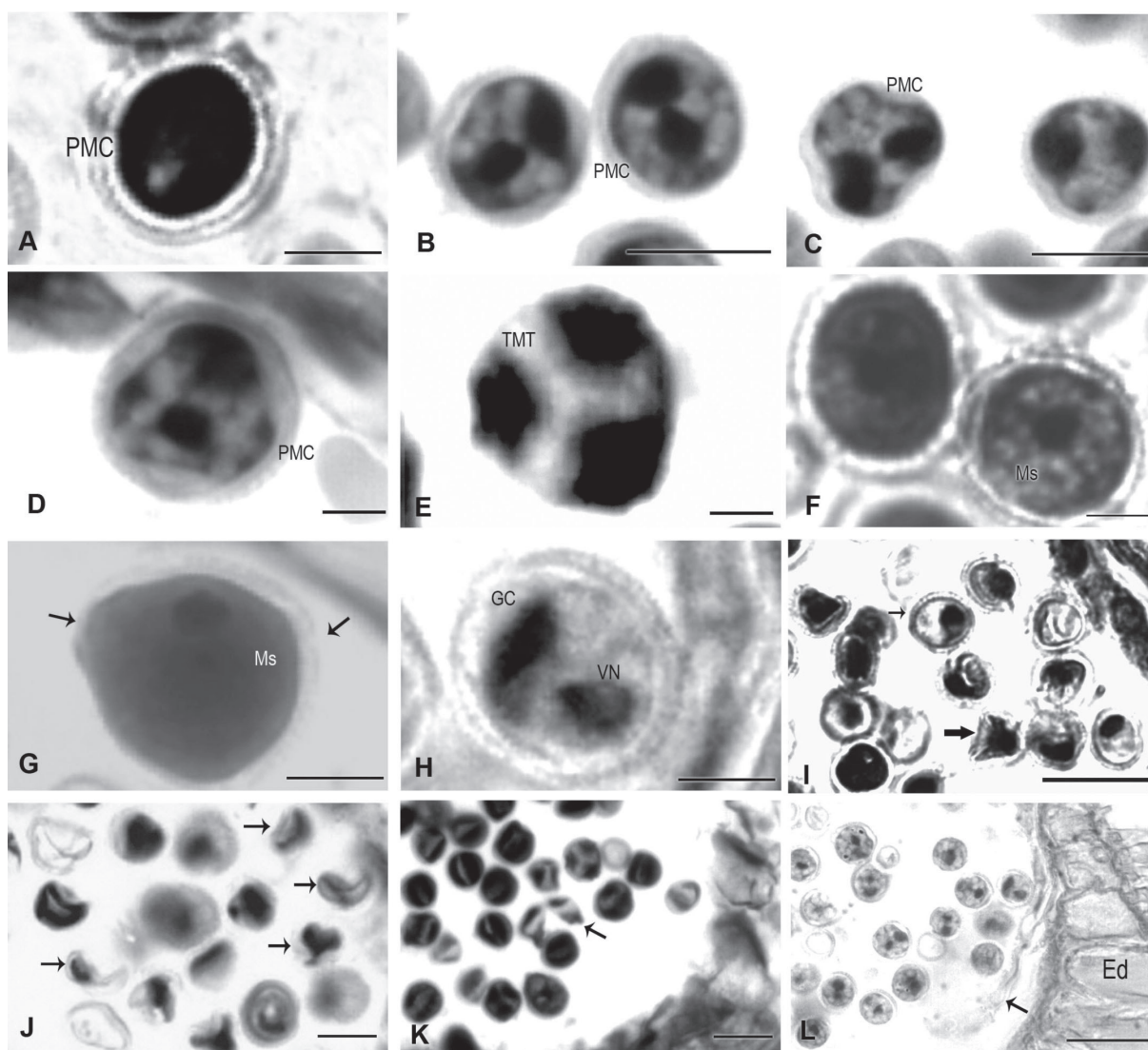


Fig. 2 Microsporogenesis and abnormal development during the process.

A. The microspore mother cell; B. Two daughter nuclei may be observed in a PMC, showing telophase I of PMC meiosis; C. The PMC was tetrahedral and only two daughter nuclei in a PMC, showing prophase II of PMC meiosis; D. Four daughter nuclei can be observed in a PMC, but the cell plate was not investigated, showing telophase II of PMC meiosis; E. The tetrahedral tetrad of microspores; F. Newly released uninucleate microspores; G. The uninucleate microspore with apertures (arrow); H. Two-celled pollen with generative cell and vegetative cell; I. The PMC stage, showing the vacuolated PMC with adhesion of nucleus and cytoplasm (small arrow), and the shrinkage PMC (large arrow); J. The meiosis I stage of PMC, showing the shrinkage PMC (arrow); K. The tetrad stage, showing the PMC contracted and bony (arrow); L. The two-celled pollen stage, showing the persistent tapetum (arrow).

Scale bars = 50µm in figures A, E-H; 10µm in figures B-D, I-L. Ed: endothecium; GC: generative cell; Ms: microspore; PMC: pollen mother cell; TMT: tetrahedral tetrad; VN: vegetative cell nucleus.

PMC stage, PMCs partly become vacuolated, and their nuclei are adhesive with their cytoplasm (Fig.2, A). The contraction and deformation of PMCs was observed at the stage of PMC (Fig.2, I), dyad (Fig.2, J) and tetrad (Fig.2, K). Especially, over 50% PMCs contract seriously to become bony at the tetrahedral tetrad stage (Fig.2, L). The tapetum of the anther wall degenerates in situ slowly, and is persistent at the stage of two-celled pollen grain (Figure 21).

Megasporogenesis and female gametophyte formation

In early April, a parenchyma cell beneath the epidermis of the ovule in the micropyle end develops into an archesporial cell, characterized by dense cytoplasm and large nucleus (Fig.3, A). Afterwards, the archesporial cell divides periclinally into

two cells, the parietal cell and the sporogenous cell (Fig.3, B). In mid June, the parietal cell undergoes several periclinal and anticlinal divisions to form nucellus cells, and the sporogenous cell develops directly into the megaspore mother cell (MMC), recognized by dense cytoplasm and large nucleus (Fig.3, C). The functional MMC becomes deeply embedded in the tissue of the nucellus, hence the type of ovule is crassinucellate. At the same time, inner and outer integument with 2-layer cells are well defined (Figure 24), and then the ovule becomes gradually anatropous (Fig.3, D).

After undergoing successive changes during meiosis, the MMC divides into two equal dyad cells (Fig.3, E) and then the linear tetrad forms. After this the chalazal cell functions as a megaspore, but the other three cells gradually degenerate (Fig.3, F). Finally, the functioning megaspore

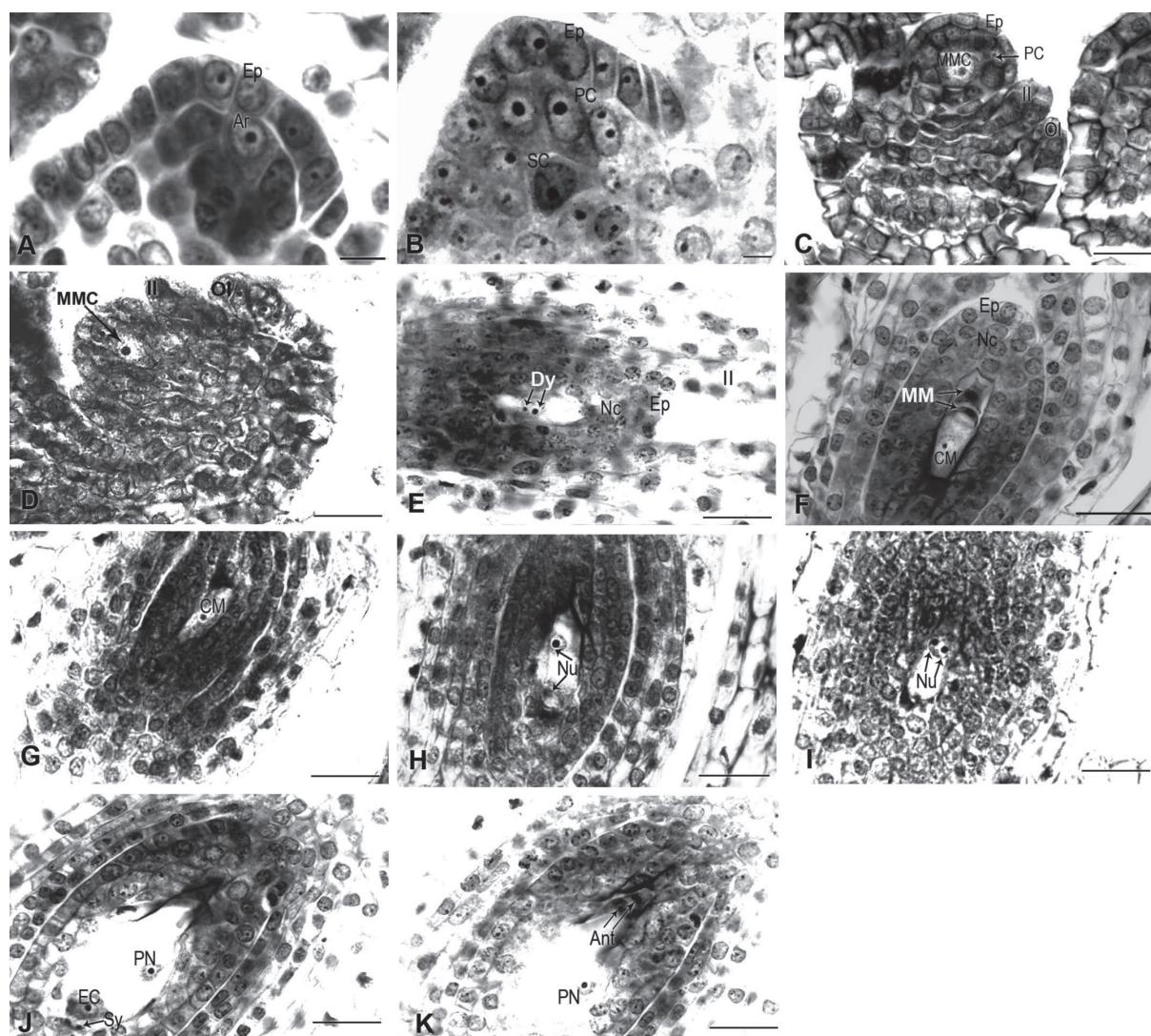


Fig. 3 Megasporogenesis and the development of the female gametophyte.

A. The archeposporium of the female gametophyte; B. The parietal cell and sporophyte cell; C. Megaspore mother cell and the formation of inner and outer integument; D. The anatropous ovule; E. Dyad; F. The functional megaspore with the degenerating megaspores; G. Uninucleate embryo sac; H. Binucleate embryo sac; I. Two nuclei from the four nucleate gametophyte are visible in a transverse plane; J-K. Consecutive sections, showing the mature 8-nucleate embryo sac.

Scale bars = 10µm. ANT: antipodal cell; Ar: archesporium; CM: chalazal megaspore; Dy: dyad; EC: egg cell; Ep: epidermis; II: inner integument; MM: micropylar megaspore; MMC: megaspore mother cell; Nc: nucellus; Nu: nucleus; OI: outer integument; PC: parietal cell; PN: polar nucleus SC: sporophyte cell; Sy: synergid.

becomes incrassate and vacuolated, and the nucleus is suspended in the center (Fig.3, G), indicating the completion of megasporogenesis.

In early July, the uni-nucleate female gametophyte coming directly from the functional megaspore increases its volume, then produces two daughter nuclei which move apart to the two poles of the female gametophyte (Fig.3, H). After three mitotic divisions, the megaspore develops into an eight-nucleate female gametophyte (Fig.3, I-K). The development of the female gametophyte thus conforms to the monosporic 8-nucleate Polygonum type. As nuclear division proceeds the female gametophyte enlarges its volume prominently, and three nuclei at the micropylar end constitute the egg apparatus, consisting of an egg cell and two synergids, and the three cells at the chalazal end become the antipodals (Fig.3, J-K). Simultaneously, one nucleus migrates to the middle of the female gametophyte from the micropylar or chalazal pole, leading to the formation of polar nuclei (Fig.3, J-K).

DISCUSSION

In this study, the sporogenesis and development of gametophytes in *T. sinense* were analyzed in order to reveal the factors affecting natural regeneration. The reproductive biology features of *T. sinense* may be summarized as follows. The anther of each stamen is of four sporangia. The anther wall prior to maturation consists of an epidermis, an endothecium, one or two middle layers and one glandular tapetum. Simultaneous cytokinesis follows meiosis to produce tetrahedral tetrads. The mature pollens are two-celled at the time of anther dehiscence. The ovule is anatropous, bitegmic and crassinucellate, and the development of the female gametophyte is of the monosporic 8-nucleate Polygonum type. The results presented are in agreement with the previous report. Significantly, some striking features were first found in *T. sinense*: (1) anther dehiscence occurs soon after the endothecium fibrously thickens and the intersporangial septum degenerates; (2) tapetum degeneration is retarded, persisting up to the stage of two-celled pollen grain; (3) a few cellular events such as the vacuolization and the contraction and deformation of PMC and microspore are not normal at the stage of PMC, dyad and tetrad.

Anther dehiscence is the terminal step in anther development that causes the release of pollen grains from the anther (Scott et al., 2004). As poor dehiscence of anthers causes a decreased yield of seed and influences the reproduction of plants (Matsui et al., 1999), the anther dehiscence mechanism has received considerable attention. Hitherto, the anther dehiscence mechanisms in angiosperms are of four basic types. (1) In most angiosperms, the expansion and fibrous thickening of the endothecium and the splitting of the stomium are required for anther dehiscence. (2) In tobacco and other solanaceous plants, two specialized cell types, the stomium and the circular cell, cluster within the notch region and are associated with anther dehiscence (Beals and Goldberg, 1997). (3) In most non-solanaceous plants (D'Arcy, 1996; Sanders et al., 2005), specialized septum cells in the notch region of these anthers function analogously in dehiscence. (4) In rice, the U-shaped thickening in the endothecium wall and the rupture of the septum and splitting of the stomium contribute to anther dehiscence (Matsui et al., 1999). Interestingly in *T. sinense*, the endothelial layer fibrously thickens after the

U-shaped thickening, and then the intersporangial septum degenerates and the stomium splits, leading to anther dehiscence. The result suggests that the anther dehiscence of *T. sinense* should be attributed to a synergetic effect between the fibrous thickening of endothecium and the degeneration of the intersporangial septum and stomium, which is in disagreement with previous reports (Beals and Goldberg, 1997; Matsui et al., 1999; Sanders et al., 2005). Further studies on the cytological and cytochemical dynamics of the endothecium and intersporangial septum during development in this species will probably reveal the mechanism of anther dehiscence.

There are a few reports about abnormal development of the PMC during microsporogenesis, such as the vacuolization and contraction of PMC, and the adhesive phenomenon of PMC and pollen (Pan et al., 2001, 2003; Wang et al., 2005; Zhao et al., 2008). In general, the abnormalities during PMC development usually correlated with pollen abortion, affecting the efficiency of reproduction (Zhao et al., 2008). Additionally, a persistent tapetum was usually present in some male sterile plants (Gorman and McCormick, 1997; Chaubal et al., 2000), and the retarding of its degeneration can block the nutrient transportation which is necessary for the microspores to develop into viable pollen grains (Buyukkartal et al., 2005). In the present study, the abnormal development of the microspore and tapetum during male reproduction of *T. sinense* would result in pollen abortion and affect the efficiency of sexual reproduction and thus affect seed set, which might be one of the important factors resulting in the poor natural regeneration of this species. To date, little is known about the cause of the abnormalities during sporogametes of *T. sinense*. To conserve effectively this medicinal plant and accelerate the development of medicine in China, further research should be carried out to reveal the reason for the abnormal development of the microspore and tapetum during male reproduction in *T. sinense*.

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Leishmanicidal activity of carvacrol-rich essential oil from *Lippia sidoides* Cham

Paulo A Farias-Junior¹, Marcos C Rios¹, Tauanny A Moura², Roque P Almeida², Pericles B Alves³, Arie F Blank⁴, Roberta P M Fernandes¹, Ricardo Scher^{5*}

¹ Departamento de Fisiologia, Universidade Federal de Sergipe, Av. Marechal Rondon, São Cristóvão, Sergipe, Brasil

² Departamento de Medicina, Universidade Federal de Sergipe, Rua Claudio Batista, Aracaju, Sergipe, Brasil

³ Departamento de Química, Universidade Federal de Sergipe, Av. Marechal Rondon, São Cristóvão, Sergipe, Brasil

⁴ Departamento de Agronomia, Universidade Federal de Sergipe, Av. Marechal Rondon, São Cristóvão, Sergipe, Brasil

⁵ Departamento de Morfologia, Universidade Federal de Sergipe, Av. Marechal Rondon, São Cristóvão, Sergipe, Brasil

ABSTRACT

Leishmaniasis is a disease that affects more than 2 million people worldwide, whose causative agent is *Leishmania* spp. The current therapy for leishmaniasis is far from satisfactory. All available drugs, including pentavalent antimony, require parenteral administration and are potentially toxic. Moreover, an increase in clinical resistance to these drugs has been reported. In this scenario, plant essential oils used traditionally in folk medicine are emerging as alternative sources for chemotherapeutic compounds. In this study, *in vitro* leishmanicidal effects of a thymol- and a carvacrol-rich essential oil from leaves of *Lippia sidoides* Cham. were investigated. The essential oils were extracted and their constituents were characterized by gas chromatography coupled to mass spectrometry (GC/MS). Both essential oils showed significant activity against promastigote forms of *Leishmania chagasi*. However, we found that carvacrol-rich essential oil was more effective, with IC₅₀/72 h of 54.8 µg/mL compared to 74.1 µg/mL for thymol-rich oil. Carvacrol also showed lower IC₅₀ than thymol. Our data suggest that *L. sidoides* essential oils are indeed promising sources of leishmanicidal compounds.

Key words: carvacrol, p-cymene, essential oil composition, *Leishmania chagasi*, *Lippia* spp., thymol, Verbenaceae.

INTRODUCTION

Natural products obtained from a wide range of plant species, such as essential oils, have been traditionally used to treat a number of diseases, including leishmaniasis (Hammer and Johns, 1993; Franca et al., 1996; Bezerra et al., 2006).

The causative agent of leishmaniasis is a parasitic protozoan of the genus *Leishmania* (Trypanosomatidae) which is transmitted to humans by sand flies, either of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World). According to the World Health Organization, leishmaniasis affects 350 million people in 88 countries. It is estimated that 2 million new cases occur each year, with at least 12 million people presently infected worldwide (WHO, 2010). Depending on both the infecting vector species and the host immunological response to the pathogenic agent, leishmaniasis can be classified as tegumentary or visceral. The latter presents more severe symptoms and, in the New World, is caused by *Leishmania chagasi*.

The most common measures to control leishmaniasis rely on chemotherapy and vector control to reduce transmission (Handman, 2001). The first-line drug currently used in treatments is pentavalent antimony Sb(V), which was introduced at the beginning of 1940's (Herwaldt, 1999) and presents high toxicity. Moreover, an increase in clinical resistance to this drug has been reported (Lira et al., 1999; Mittal et al., 2007; Sen and Chatterjee, 2011). Second-line choice treatments include pentamidine and amphotericin B, which are also toxic and too expensive for routine use in developing countries (Murray, 2001). Therefore, the necessity

of new studies to find safe, less expensive and more effective treatments against leishmaniasis becomes evident.

Verbenaceae is a family consisting of more than 150 genera and about 2300 species widely distributed in tropical and subtropical regions (Cavalcanti et al., 2010). The species *Lippia sidoides* Cham. is a perennial bushy plant native to the Caatinga, which is rich in aromatic essential oils. The medicinal properties of *L. sidoides* essential oil include antifungal (Fontenelle et al., 2007), antibacterial (Oliveira et al., 2006; Lobo et al., 2011), anti-helminthic (Camurça-Vasconcelos et al., 2008), larvicidal, and acaricidal activities (Carvalho et al., 2003; Cavalcanti et al., 2010). Some of these biological properties are attributed to the presence of thymol and carvacrol in its essential oil (Carvalho et al., 2003; Botelho et al., 2007). The leishmanicidal activity of *L. sidoides* essential oil was recently demonstrated in promastigote forms of *L. chagasi* (Oliveira et al., 2009) and *L. amazonensis* (Medeiros et al., 2011). Since in both studies thymol was the major component, further studies are needed to evaluate the leishmanicidal activity of essential oils with different compositions. Therefore, in the present study, we evaluated the inhibitory effect of a carvacrol-rich essential oil obtained from leaves of *L. sidoides* on promastigotes of *L. chagasi*.

MATERIAL AND METHODS

Plant material

Two accessions of *L. sidoides* (LSD102 and LSD104) were collected at Poço Redondo, Sergipe, Brazil (9° 58' 07,6" S; 37°

* Corresponding author: Ricardo Scher, Universidade Federal de Sergipe, Departamento de Morfologia, Av. Marechal Rondon, sem número, São Cristóvão, Sergipe, Brasil. CEP: 49100-000; Phone: 55+79-2105-6629; Fax: 55+79-2105-6494, e-mail: scherr@ufs.br

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51' 49,2" W) and were cultivated in the Research Farm of the Universidade Federal de Sergipe, Department of Agronomical Engineering, São Cristóvão, Brazil. The leaves were harvested for essential oil extraction at the flowering stage. The voucher specimens of accessions LSD102 and LSD104, under numbers 8224 and 8226, respectively, are deposited at the Herbarium of Universidade Federal de Sergipe.

Dried and powdered leaves (75 g) were submitted to hydrodistillation in a Clevenger-type apparatus for 2 hours. At the end of each distillation the oils were collected and kept at a temperature of -18°C for further analysis.

Analysis of the essential oils

Quantitative and qualitative analysis of the chemical composition of the essential oils were carried out using a gas chromatograph (Shimadzu, QP 5050A model) coupled to a mass spectrometer (MS) that was equipped with an auto-injector AOC-20i (Shimadzu) and a J&W Scientific fused silica capillary column (30 m by 0.25 mm; film thickness, 0.25 μm) with helium used as the gas carrier (1.2 ml/min). The MS was carried out with an ion capture detector operating in electronic impact mode, with an impact energy of 70 eV. The temperature was programmed to remain at 50°C for 2 min, followed by an increase at a rate of $4^{\circ}\text{C}/\text{min}$ up to 200°C , then another increase of $15^{\circ}\text{C}/\text{min}$ until 300°C was reached, and maintained at this temperature for 15 min. The injector and detector (or interface) temperatures were 250°C and 280°C , respectively. Identification of the oil constituents was based on direct comparison of their spectra with spectra from the equipment database (NIST21 and NIST107) and from the literature (Adams, 2007). Additionally, the retention rates were compared to n-alkanes (C8-C18) injected under the same chromatographic conditions as the samples (Vandendool and Kratz, 1963).

Parasite cultures

Promastigote forms were isolated from a patient of the University Hospital of Universidade Federal de Sergipe and coded as HU-UFS14 (Ethics Committee number CAAE -0151.0.107.000-07). The isolate was characterized as *Leishmania chagasi* using monoclonal antibody and isoenzyme approaches. The culture was maintained by weekly transfer in Schneider's *Drosophila* medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA).

Leishmanicidal Activity Assay

The essential oils and major compounds (carvacrol, thymol and p-cymene) were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 4%. This solution was dissolved in culture medium to obtain a stock solution at 0.064%. In a microplate (96 wells) this solution was serially diluted to concentrations ranging from 160 to 2.5 nL/mL for essential oil, 64.0 to 0.5 for carvacrol and p-cymene and 200.0 to 1.6 $\mu\text{g}/\text{mL}$ for thymol. Promastigotes (1×10^5 cells) in the log phase of growth were seeded in each well and incubated for $24^{\circ}\text{C}/72$ h. Wells without oil and with amphotericin B were used as controls. In all cases the final concentration of DMSO never exceeded 0.4%, a concentration which is not toxic for the protozoan (Oliveira et al., 2009).

The effect of essential oils on promastigote viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium

bromide (MTT) assay. After 72 h of treatment with essential oils, MTT ($1\mu\text{g}/\text{mL}$, pH 7.4) was added in each well and the plate was incubated overnight in the dark at 24°C . After this, isopropanol 50% and Sodium Dodecyl Sulphate (SDS) 10% were added and the plate was incubated at $37^{\circ}\text{C}/4$ h, until the formazan crystals had been completely dissolved. Finally, the plate was read at 540 nm in a microplate reader (Labsystems apparatus multiskar MS).

All experiments were performed in triplicate. The concentration that inhibited culture growth by 50% (IC_{50}) was determined by regression analysis. IC_{50} values were obtained in nL/mL and were converted to $\mu\text{g}/\text{mL}$ based on oil and molecule densities.

RESULTS

The essential oils from two different accessions of *L. sidoides* (LSD102 and LSD104) were obtained with 6.7% and 6.8% w/v yield, respectively. Their chemical compositions were analyzed by GC/MS and the chromatograms are presented in Figure 1.

Fourteen (LSD102) and 15 (LSD104) compounds were identified and their retention indices and relative amounts, listed in order of elution, are shown on Table I. The oxygenated

TABLE I
Essential oil composition from accessions of *L. sidoides* characterized by GC/MS.

RI	Compound	LSLSEO 102 %(*)	LSEO 104 %(*)
924	α -Thujene	1.1 (1)	1.7 (1)
931	α -Pinene	0.3 (2)	0.5 (2)
988	Myrcene	3.3 (3)	3.5 (3)
1006	α -Phellandrene	0.0	0.2 (4)
1007	δ (3)Carene	0.1 (4)	0.1 (5)
1016	α -Terpinene	1.9 (5)	3.1 (6)
1024	p-Cymene	34.1 (6)	17.8 (7)
1028	Limonene	0.5 (7)	0.4 (8)
1035	(Z)- β -Ocimene	0.0	0.3 (9)
1057	γ -Terpinene	6.8 (8)	16.6 (10)
1180	Terpinen-4-ol	0.7 (9)	0.9 (11)
1228	Methyl thymol	9.4 (10)	4.1 (12)
1292	Thymol	38.7 (11)	6.0 (13)
1298	Carvacrol	0.6 (12)	43.7 (14)
1344	Acetate thymol	1.8 (13)	0.0
1418	β -Caryophyllene	0.6 (14)	0.0
1477	NI	0.0	0.8 (15)
1506	β -bisabolene	0.0	0.2 (16)
	Monoterpenes	99.4	99.0
	Sesquiterpenes	0.6	0.2
	Total	100.0	99.2

RI: Relative retention index calculated against n-alkanes applying the Van den Dool equation

%: Compound percentage.

* Numbers inside parenthesis correspond to respective peaks in the chromatograms reproduced in Figure 1

monoterpene thymol was the main component of the essential oil from accession LSD102 (38.7%) while carvacrol was more abundant in LSD104 (43.7%). Henceforth we will name the essential oil from LSD102 as LSEO102 and that from LSD104 as LSEO104.

In order to investigate the leishmanicidal activity, promastigotes of *L. chagasi* were incubated in the presence of increasing concentrations of LSEO102 and LSEO104 and cell viability was determined after 72 hours. As shown in Figure 2a, both essential oils inhibited parasite growth. The IC_{50} values obtained were 74.1 and 54.8 $\mu\text{g/mL}$ for LSEO102 and LSEO104, respectively. In addition, we also evaluated the leishmanicidal potential of thymol and carvacrol, the main compounds of LSEO102 and LSEO104, respectively. Thymol exhibited an IC_{50} of 9.8 $\mu\text{g/mL}$ and carvacrol, 2.3 $\mu\text{g/mL}$.

DISCUSSION

The leishmanicidal and trypanocidal activity of essential oils from *Lippia* spp have been already demonstrated in some studies (Sulsen et al., 2006; Escobar et al., 2010). With regards to *L. sidoides*, leishmanicidal activity was demonstrated for the first time in promastigotes of *L. chagasi* with an IC_{50} of 89 $\mu\text{g/mL}$ (Oliveira et al., 2009). More recently, Medeiros et al.

(2011) reported a more efficient activity of *L. sidoides* essential oil against promastigotes of *L. amazonensis*, since they found a lower IC_{50} (44.38 $\mu\text{g/mL}$). In both studies, remarkable morphological changes in the parasite were observed and thymol was reported as the main constituent of the essential oils. It is important to note that the IC_{50} value obtained in our study for LSEO102 was similar to those obtained in the previous works (Oliveira et al., 2009; Medeiros et al., 2011).

Although a previous study has demonstrated the leishmanicidal activity of *Lippia origanoides* essential oil, with a relative amount of carvacrol greater than 35% (Escobar et al., 2010), this is the first report on the leishmanicidal properties of essential oil from a new *L. sidoides* accession in which carvacrol, rather than thymol, is the main constituent. The composition of the carvacrol-rich essential oil herein evaluated (LSEO104) was similar to the one characterized by Cavalcanti et al. (2010). In that study, the authors verified that both the essential oil and pure monoterpene carvacrol showed acaricidal activity.

Interestingly, in the present study, the IC_{50} obtained for LSEO104 was lower than that found for LSEO102. Accordingly, carvacrol (LSEO104 major compound) presented IC_{50} lower than thymol (LSEO102 major compound). These results suggest that the greater efficiency of the carvacrol-rich essential oil against *L. chagasi* promastigotes probably is due to its major component. Although the relative amounts of thymol in LSEO102 and of carvacrol in LSEO104 are about 40%, LSEO104 has 6% thymol in addition to the carvacrol content. On the other hand LSEO102 had no detectable amount of carvacrol in its composition. Therefore, we cannot rule out the contribution of a synergistic effect between thymol and carvacrol in LSEO104 for the greater leishmanicidal effect observed for this oil. It is important to note that, although relevant amounts of p cymene were detected in LSEO102 and LSEO104 (34.1% and 17.8%, respectively), this molecule did not show leishmanicidal effect in concentrations up to 64 nL/mL.

CONCLUSIONS

Our data show that *L. sidoides* essential oil is a natural product with leishmanicidal activity, suggesting its potential for the development of drugs against leishmania in the future. However, further studies are necessary to verify its effects on amastigote forms and its potential toxicity *in vivo* before clinical evaluation is performed.

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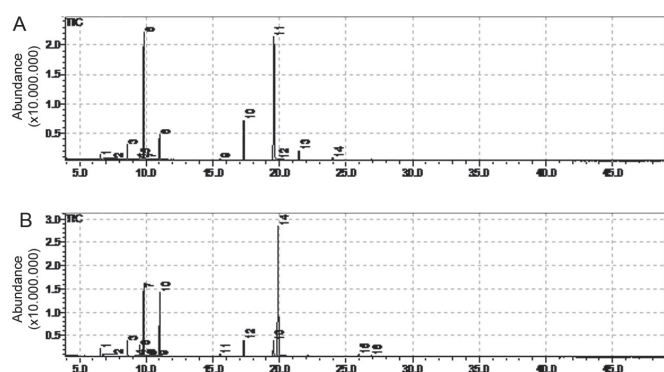


Figure 1: Total ion chromatogram (T) GC/MS chromatogram of essential oil from *L. sidoides*. A) LSEO102. B) LSEO104

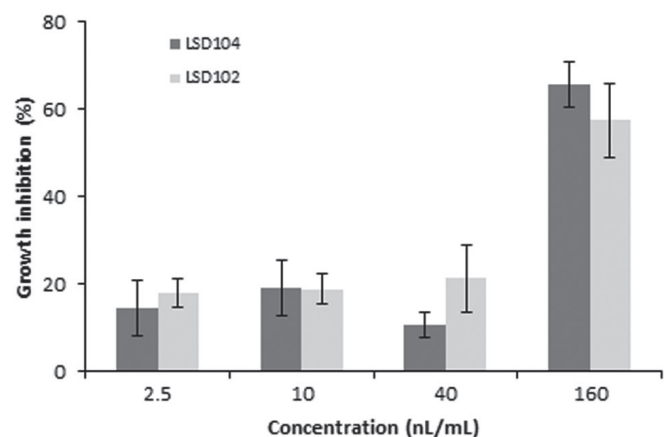


Figure 2: Effects of essential oil from accessions of *L. sidoides* on growth of *L. chagasi* promastigote forms. Each bar represents the mean $\pm$ standard deviation of three independent experiments.

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Gastrodia elata Blume and its pure compounds protect BV-2 microglial-derived cell lines against β -amyloid: The involvement of GRP78 and CHOP

Geum-Hwa Lee¹, Hyung-Ryong Kim¹, Sang-Yong Han¹, Bidur Bhandary², Do-Sung Kim², Min-Gul Kim³, Byung-Ok So³, Sun-Young Kim³, Kyu-Sik Jo⁴, Bo-Hee Lee⁴, Hee-Nam Seo⁵, Soo-Wan Chae^{2, 3}, and Han-Jung Chae²

¹ Department of Dental Pharmacology and Wonkwang Dental Research Institute, School of Dentistry, Wonkwang University, Iksan, Chonbuk, South Korea

² Department of Pharmacology and Cardiovascular Research Institute, Medical School, Chonbuk National University, Jeonju, Chonbuk, South Korea

³ Clinical Trial Center for Functional Foods, Chonbuk National University Hospital, Jeonju, South Korea

⁴ Muju Chunma Native Local Industrial Center, Muju, Chonbuk, South Korea

⁵ Department of Pharmacy, Kangbuk Samsung Hospital, Seoul, South Korea.

Objectives: *Gastrodia elata* (GE) Blume (Orchidaceae) has been previously known for its therapeutic benefits against neurodegenerative diseases. Microglial activation and death have been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer's disease. In this study, GE and its pure components, gastrodin and 4-hydroxybenzyl alcohol (4HBA), were applied to β -amyloid-induced BV2 mouse microglial cells.

Materials and Methods Cell viability was assessed by the MTT assay and Western blotting was also performed.

Results: β -amyloid-induced cell death was shown to be induced time- and dose-dependently. To examine the cell death mechanism, we confirmed the involvement of ER stress signaling. C/EBP homologous protein (CHOP), a pro-apoptotic ER stress protein, was expressed at high levels but glucose-regulated protein 78 (GRP78), an anti-apoptotic ER stress protein with chaperone activity, was only slightly affected by treatment with β -amyloid. However, pretreatment with GE and its components inhibited the expression of CHOP but increased that of GRP78 in β -amyloid-treated cells. This study also showed that a single treatment with GE extracts, gastrodin, or 4HBA induced the expression of GRP78, a marker for enhanced protein folding machinery, suggesting a protective mechanism for GE against β -amyloid.

Conclusions: This study reveals the protective effects of GE against β -amyloid-induced cell death, possibly through the enhancement of protein folding machinery of a representative protein, GRP78, and the regulation of CHOP in BV2 mouse microglial cells.

Key words: *Gastrodia elata*, 4HBA, GRP78, CHOP, β -amyloid.

INTRODUCTION

Alzheimer's disease, the most common form of dementia, is a chronic neurodegenerative disease that causes the progressive impairment of memory and other cognitive functions. Neuritic plaques, neurofibrillary tangles and neuronal loss represent the primary pathological characteristics in the brains of Alzheimer's patients. Amyloid β -protein (β -amyloid), the central component of senile plaques, is produced from sequential proteolytic cleavages of type I transmembrane β -amyloid precursor protein by β - and γ -secretases [Glenner and Wong, 1984, Hardy and Selkoe, 2002]. The integrity of adult mouse brain neurogenesis in Alzheimer's disease-associated pathological models has also been examined [Siman et al., 2000]. The mouse brains studied exhibited amyloid deposition and microgliosis. It has also been reported that β -amyloid accelerates neurodegeneration by activating microglia and that the activated microglia exert cytotoxic effects on neurons [Ryu et al., 2009, Folden et al., 2005]. In addition to neuronal cells, microglial CNS-resident professional sensor cells also respond to many pathological events. Localized activation of microglia has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer's disease. Aggregated β -amyloid has been shown to interfere with several cellular processes and can result in endoplasmic reticulum (ER) stress. As neurons are highly susceptible to the toxic effects of the aggregated

β -amyloid of Alzheimer's disease, ER-stress-mediated cell death might have an important role in the pathogenesis of this disease [Wiley et al., 2010, Chafekar et al., 2008].

Under normal conditions a chaperone protein, glucose-regulated protein 78 (GRP78), sequesters three key signal transducers on the ER membrane by forming an inactive complex that includes a double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF-6), and inositol-requiring transmembrane kinase and endonuclease, IRE-1 [Bertolotti et al., 2000, Xu et al., 2005]. Dissociation from GRP78 is required for the activation of the three key signal transducers. The activation results in a decrease in translation, increased protein degradation and increased levels of ER chaperones. When there is prolonged ER stress the cells can no longer maintain homeostasis, and thus the cell death signaling pathway is triggered [Kim et al., 2008, Gu et al., 2010]. In contrast, an adaptive ER stress response known as the unfolded protein response (UPR), including XBP-1 and GRP78, has been suggested to have a protective role against ER stress through enhancement of the protein-folding capacity, a protective mechanism against ER stress-associated neuronal degenerative diseases including Alzheimer's disease [Casas-Tinto et al., 2011, Kogel et al., 2003].

There have been continuous efforts to develop drugs or nutraceuticals to treat or prevent Alzheimer's disease. Numerous candidate treatment agents have been examined, including various kinds of herbal and natural products.

Address correspondence to: Han-Jung Chae, PhD, Department of Pharmacology and Institute of Cardiovascular Research, Medical School, Chonbuk University, Jeonju, Chonbuk, South Korea. Tel: 82-63-270-3092. Fax: 82-63-275-2855. E-mail: hjchae@jbnu.ac.kr.

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Among these natural compounds, *Gastrodia elata* (GE) has demonstrated its potential, considering that the material was traditionally and frequently used for cerebral ischemia or symptoms similar to those of Alzheimer's disease. In addition, gastrodin, the primary component of the functional extracts of GE, can reduce the seizure score in seizure-prone gerbils and facilitate learning and memory [Hsieh et al., 1997]. In addition, some studies found that gastrodin had protective effects in rat cortical neurons and astrocytes against damage induced by *in vitro* simulated cerebral ischemia, and gastrodin has been associated with a decreased mortality rate resulting from ischemia in mice [Zeng et al., 2007]. 4-Hydroxybenzyl alcohol (4HBA), an aglycone of gastrodin and an active ingredient of GE, attenuates learning deficits in an inhibitory avoidance task, suggesting that 4HBA may act by suppressing dopaminergic and serotonergic activities, thereby improving learning abilities [Wu et al., 1996]. Although microglial cells are also important for the pathological conditions of neurodegenerative diseases, fewer studies have been conducted with microglial cells than with neuronal cells. Since the ways in which GE and its pure components facilitate the learning and memory processes and protect against neuron damage have not yet been clarified, GE and its components were applied to mouse microglial cells, BV2 cells.

In this study, the functional roles and mechanisms of GE and its pure components in the protection against β -amyloid-induced microglial cell death were investigated.

MATERIALS AND METHODS

Materials

Dried *Gastrodia elata* was acquired from the MooJoo Hangto Research Group (MooJoo, Korea). 26.52 g of *G. elata* powder was prepared from 1800 g of dried *G. elata*; three replicates were cut into small pieces and extracted with 1500 ml of 100% methanol at 60 °C for 3 h. The extract was filtered and evaporated in a rotary vacuum evaporator at 50 °C before being freeze-dried. Lyophilized, HPLC-purified β -amyloid (1-42 peptides) was purchased from American Peptide (Sunnyvale, CA) and β -amyloid reconstituted in sterile water at a concentration of 400 μ M and incubated at 37 °C for 3 d or 24 hr to form aggregated β -amyloid, or maintained at 4 °C in soluble or nonaggregated form. Oligomeric and fibrillar β -amyloid were prepared using the method of Karie et al [2002]. The antibodies against actin, GRP78, PERK, IRE-1, spliced XBP-1 and CHOP were acquired from Santa Cruz Biotechnology (SantaCruz, CA). The antibodies against eIF-2 α and phospho-eIF-2 α were purchased from Cell Signaling Technologies (Beverly, Mass). All other chemicals were of at least analytical grade and were purchased from Sigma Chemical Company (St. Louis, MO).

Cell culture

The BV2 mouse microglial cell line [Byun et al., 2012] was cultured in DMEM supplemented with 10% fetal bovine serum and a 1% penicillin-streptomycin mixture (Invitrogen, Carlsbad, CA) at 37 °C in a humidified incubator (5% CO₂ and 95% air). Cells were plated one day before treatment at 80% density according to each experimental scale.

Determination of cell viability

Cell viability was measured and calculated using the MTT reduction assay, as described previously [Yu et al., 2008]. After the incubation of BV2 cells (4 X 10⁴ cells/300 μ L in 24-well plates) with oligomeric and fibrillar β -amyloid (1-42 amino acid residues) for various time periods in the presence or absence of GE or one of its pure components, gastrodin or 4HBA, MTT solution (final concentration 1 mg/ml) was added. The dark blue formazan crystals, which formed in viable cells with normal mitochondrial function, were dissolved with dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured with a microplate reader (EMax, Molecular Device, Sunnyvale, CA, USA). Cell viability was expressed as percentage (%) MTT reduction, assuming that the optical density of formazan formed in the vehicle-treated control cells was 100%.

Caspase-3 activity assay

Cytosolic cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl, pH 7.4, 10 Ag/ml leupeptin, aprotinin (0.27 trypsin inhibitor/U/ml) and 100 AM PMSF. The caspase protease activity was determined by incubating the cell lysates (25 Ag of total protein) with 50 AM of a fluorogenic substrate, AC-DEVD-AMC (caspase-3) in a cell-free buffer system (10 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol). The release of fluorescent 7-amino-4-methylcoumarin was measured at 2-min intervals for 1 h using a spectrofluorometer at an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

Hoechst staining

Cell nuclei were stained with chromatin dye (Hoechst 33258). Briefly, cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed twice with PBS and incubated with 10 μ M Hoechst 33258 in PBS at room temperature for 30 min. After three washes, cells were observed under a fluorescence microscope (MPS 60, Leica).

Western blotting

After drug treatment for the indicated times, treated cells were harvested by washing twice with ice-cold PBS and the cell pellet was obtained by centrifuging at 3,000 rpm for 5 min. After removing the supernatant, cells were lysed on ice through the addition of RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP40, 1 mM EDTA, 0.1% SDS), protease inhibitor cocktail set III, and phosphatase inhibitor cocktail set II (EMD Biosciences, La Jolla, CA, USA). Cell lysates were transferred to microtubes and incubated for 30 min on ice, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. Supernatants were collected and used as protein extracts. Protein extracts were added to sample buffer, boiled in a water bath for 5 min, and stored at -20 °C until use. Samples of 10 μ g of protein extract were analyzed on a polyacrylamide gel and transferred onto a nitrocellulose membrane, which was blocked for 30 min at

room temperature with TBS containing 0.05% Tween-20. Blots were probed overnight at 4 °C with appropriate antibodies, washed, and probed again with species-specific secondary antibodies coupled to horseradish peroxidase (Santa Cruz, CA, USA). Chemiluminescent reagents (GE Healthcare, Piscataway, NJ, USA) were added for blot detection, and immunoreactive bands were visualized using an LAS-3000 Imager (Fuji Film, Japan).

Statistical analysis

Results are presented as mean $\pm$ SEM of n experiments; one-way ANOVA was used for comparison and post-hoc tests to compare different groups. OriginPro7.5 software [Northampton, MA, USA] was used for the statistical calculations.

RESULTS

β -Amyloid induces cell death as an ER stress response in BV2 cells

MTT reduction assays were performed to assess β -amyloid-induced toxicity. Oxidoreductase, a mitochondrial enzyme, converts MTT to a crystalline blue formazan in viable cells. The β -amyloid-induced toxicity was assessed in BV2 cells after incubation with different concentrations of β -amyloid (5, 10, 15, or 20 μ M) for 48 hours. A dose-response curve was created for percentage cell viability against the concentration of β -amyloid (Fig. 1A). Caspase-3 activity was also examined in the treated cells. Caspase-3 activity was also increased by treatment with β -amyloid (right). Similarly, we assessed the time-dependent response of β -amyloid-induced cytotoxicity. For this experiment, we incubated BV2 cells with β -amyloid (10 and 20 μ M) and assessed cell viability at different time intervals (6, 12, 24, 36, and 48 hours). Cell viability and caspase-3 activity clearly increased in a time-dependent manner (Fig. 1B). In addition, nuclei staining clearly showed apoptosis in 10 or 20 μ M β -amyloid-treated cells (Fig. 1B, right).

To determine the activation status of major ER stress sensors including GRP 78, CHOP, IRE-1 α , spliced XBP-1, PERK and p-eIF2 α , we performed Western blot analysis with specific antibodies in neuronal cells treated with increasing concentrations of β -amyloid (5, 10, 15, and 20 μ M) for 24 hours. Interestingly, the levels of CHOP, IRE-1 α , spliced XBP-1, PERK, and p-eIF2 α were markedly increased at all concentrations of β -amyloid, although the expression of GRP78 was only slightly affected, showing a slightly increased pattern in the presence of low concentrations of β -amyloid (Fig. 1C). Similarly, we observed increased ER stress protein expression when cells were treated for various durations of time (0, 3, 6, 9, 12, 18, or 24 hours) with 15 μ M β -amyloid (Fig. 1D). The expression of GRP78, a well-known chaperone protein, was not affected by the presence of β -amyloid. The quantitative analysis of each protein is shown in the lower panel.

GE, gastrodin, and 4HBA regulate β -amyloid-induced cell death and have a regulatory effect on ER stress proteins in BV2 cells

The MTT assay relies primarily on the mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state. After the incubation of BV2 cells pretreated with β -amyloid alone or together with GE, gastrodin, or 4HBA, the cells were

treated with MTT solution. The experimental results showed that the cytotoxicity induced by β -amyloid was inhibited in the presence of GE, gastrodin, or 4HBA (Fig. 2A, left). The time-response cell viability percentage was also measured in BV2 cells with the same pretreatments (i.e., β -amyloid alone or together with GE, gastrodin, or 4HBA for 6, 12, 24, 36, and 48 hours). We observed the inhibition of β -amyloid-induced toxicity in cells pretreated with GE, gastrodin, or 4HBA up to 48 hours (Fig. 2A, right). Fig. 2B shows that caspase-3 activity was also correlated with the MTT assay. With the treatment of GE, gastrodin or 4HBA, the caspase-3 activity was significantly inhibited in β -amyloid-treated cells. Consistently, Hoechst staining data confirmed the protective effect of GE and its components against β -amyloid-induced apoptosis (Fig. 2C). To test whether GE-, gastrodin-, or 4HBA-induced protection is related to ER stress, we analyzed the levels of ER stress proteins; GRP78, a chaperone protein with protective roles, and CHOP, an executive transcription factor for pro-apoptotic genes, using Western blot analysis of the lysates of cells treated with GE, gastrodin, or 4HBA. The results showed that CHOP expression induced by β -amyloid was inhibited in the presence of GE, gastrodin, or 4HBA (Fig. 2D). The GRP78 signal, which was not affected by β -amyloid treatment, was somewhat increased by GE and its components. Figure 2E also shows the concentration-dependent regulation of GE and its components on GRP78 and CHOP expressions in the BV cells. The quantitative data of the protein expressions are shown in the lower panel.

GE, gastrodin, or 4HBA alone induce the expression of the chaperone protein, GRP78

Since GRP78 is a chaperone protein which enhances protein folding and rehabilitation of the unfolded protein, we tested the effects of GE, gastrodin, and 4HBA on the expression of GRP78 in the absence of β -amyloid. The expression of GRP78 was significantly increased by treatment with GE, gastrodin, or 4HBA (Fig. 3A). The quantitative results of GRP78 expression are shown in the lower panel of Fig. 3A. At 10 μ g/ml of GE, gastrodin, or 4HBA, a time-dependent increase in the expression of GRP78 in BV2 cells was observed (Fig. 3B). However, there was no expression of CHOP, another ER stress protein, in the presence of GE, gastrodin, or 4HBA (data not shown). This study suggests that a chaperone protein expression and its linked chaperone function may be related to the ER stress response and its related cell death regulation in β -amyloid-treated BV cells.

DISCUSSION

In this study, *Gastrodia elata* (GE) protected microglial cells against β -amyloid-induced cell death. Two major components of GE, gastrodin and 4HBA, also regulated β -amyloid-induced cell death. β -amyloid-induced ER stress response was also regulated by GE and its components, gastrodin and 4HBA, through the expression of a chaperone protein, GRP78. GE protected cells against β -amyloid-induced cell death in an *in vitro* model of Alzheimer's disease. GE shows promise as a potential agent for use against Alzheimer's disease. The natural compound has been traditionally used for cerebral ischemia or symptoms similar to those of Alzheimer's disease [Yu et al., 2005, Wu et al., 2010, Park et al., 2009]. Furthermore,

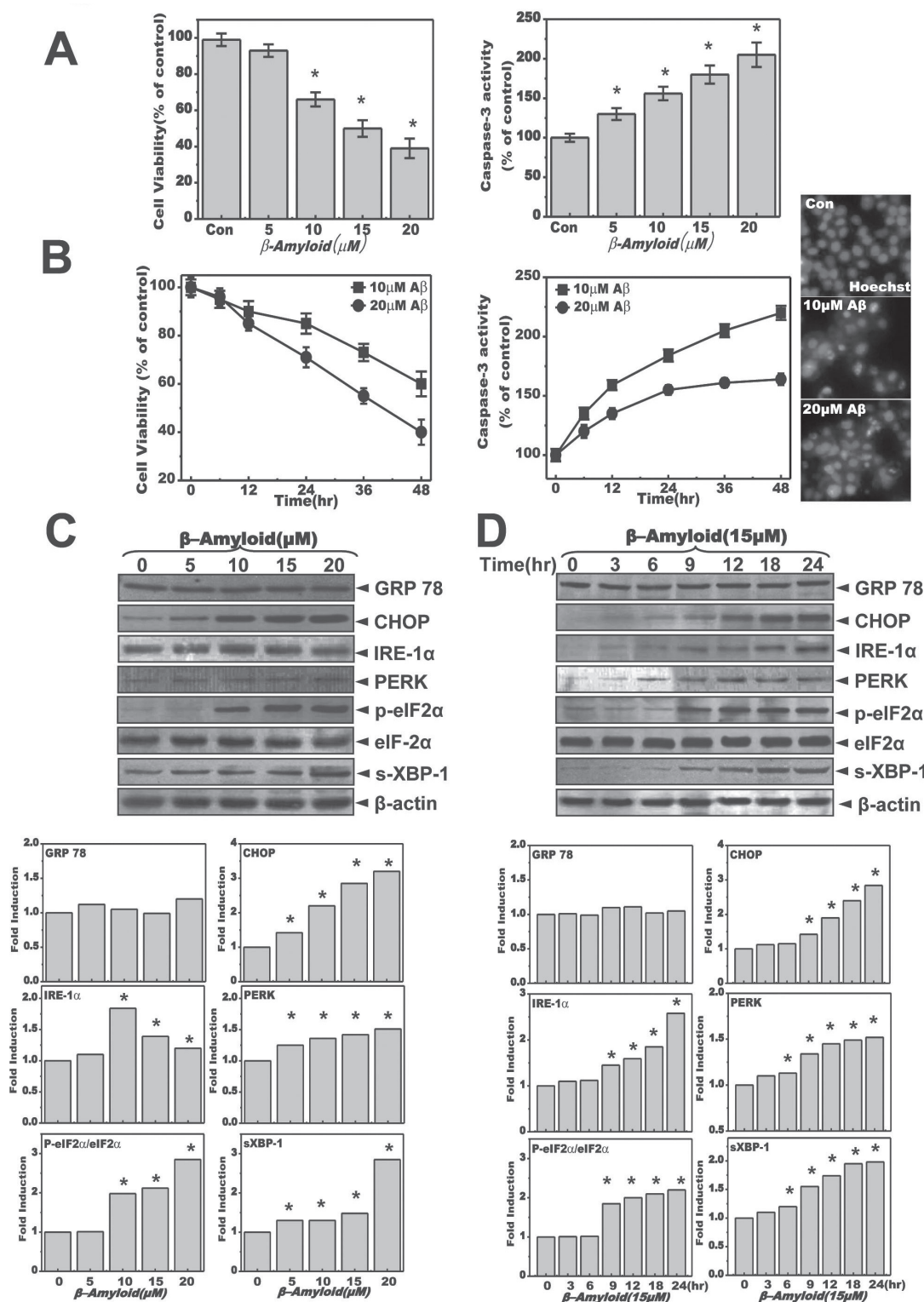


Figure 1: β -Amyloid induces cell death and causes ER stress response in BV2 cells.

BV2 cells were treated with the indicated concentrations of β -amyloid for a 48-hour period (A). Separately, BV2 cells were treated with 10 or 20 μ M β -amyloid for the indicated time periods (B) and MTT and caspase-3 activity assays were conducted. BV2 cells were treated with 10 or 20 μ M β -amyloid for 48 hours and Hoechst staining was performed as described in Materials and Methods (right). BV2 cells were treated with the indicated concentrations of β -amyloid for 48 hours (C). Separately, BV2 cells were treated with 15 μ M β -amyloid for the indicated time periods (D) and immunoblotting was performed with antibodies to GRP78, CHOP, IRE-1 α , spliced XBP-1, PERK, p-eIF2 α , eIF2 α , or β -actin. Representative data is shown from three independent experiments. Quantification of the protein density is shown in the lower panel. * $p < 0.05$, significantly different from the control.

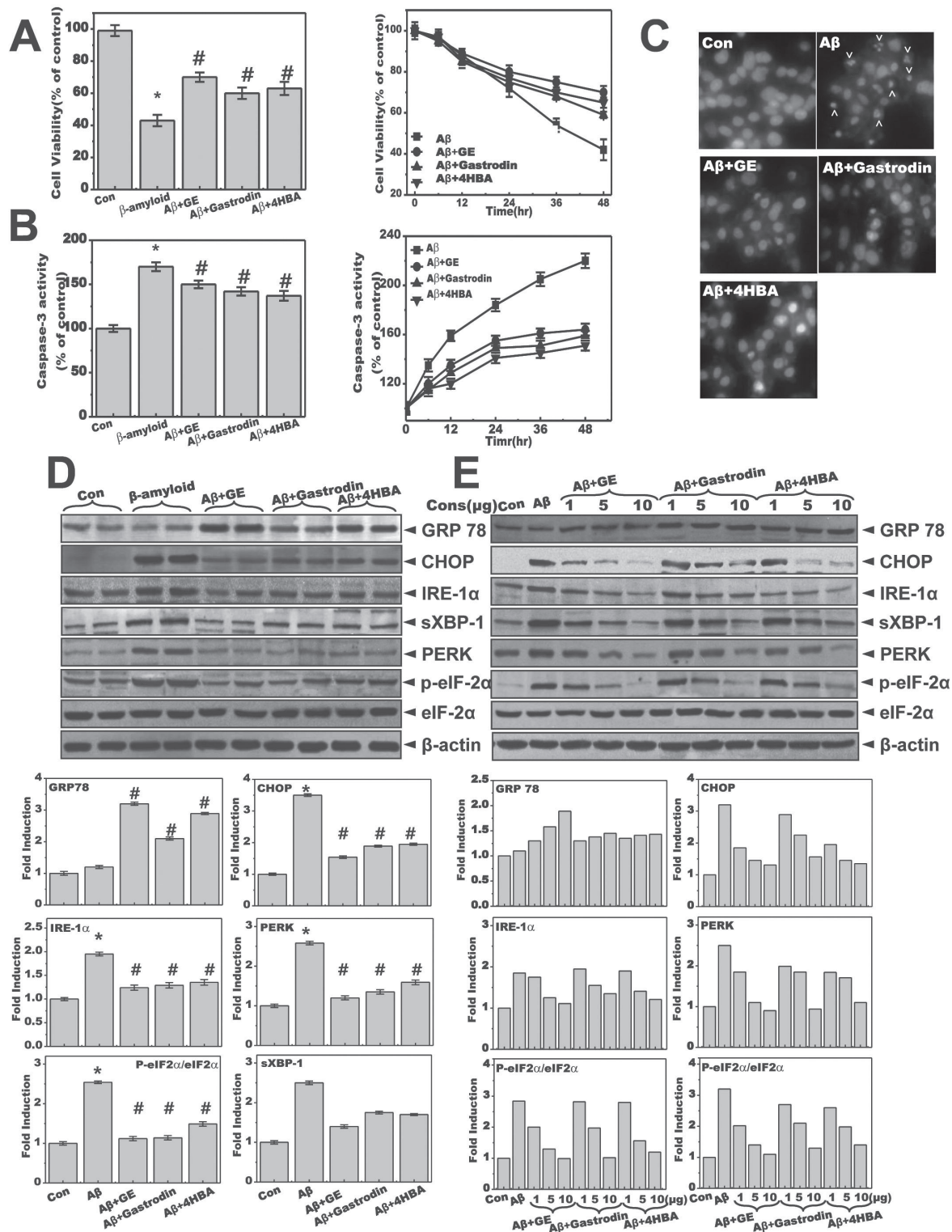


Figure 2: GE, gastrodin, and 4HBA regulate β -amyloid-induced cell death and show a regulatory effect on ER stress proteins in BV2 cells. BV2 cells were treated with 20 μ M β -amyloid with or without 10 μ g/ml GE, gastrodin, or 4HBA for 48 hours (A, B, left). Separately, BV2 cells were treated with 10 μ g/ml GE, gastrodin, or 4HBA with 20 μ M β -amyloid for the indicated times (A, B, right) and MTT and caspase-3 activity assays and Hoechst staining (C) were performed. BV2 cells were treated with 10 μ g/ml GE, gastrodin, or 4HBA with 20 μ M β -amyloid for 24 hours and immunoblotting was performed with antibodies to GRP78, CHOP, IRE-1 α , spliced XBP-1, PERK, p-eIF-2 α , eIF-2 α or β -actin (C). BV2 cells were treated with the indicated concentrations of GE, gastrodin, or 4HBA with 20 μ M β -amyloid for 24 hours, and immunoblotting was performed with antibodies to GRP78, CHOP, IRE-1 α , spliced XBP-1, PERK, p-eIF-2 α , eIF-2 α or β -actin (D). Representative data is shown from three independent experiments. Quantification of the protein density is shown in the lower panel. * p < 0.05, significantly different from the control; # p < 0.05, significantly different from β -amyloid-treated cells.

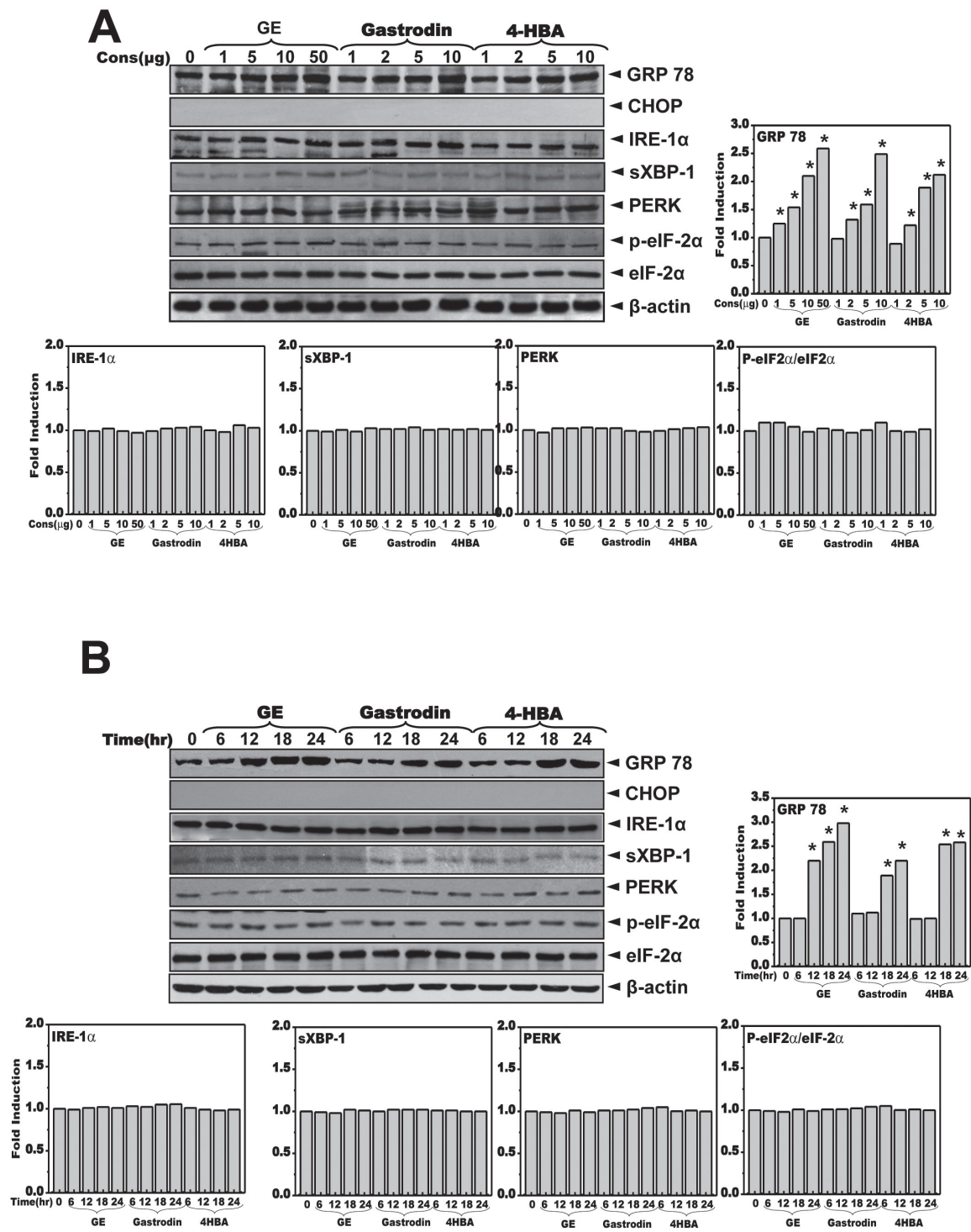


Figure 3: GE, gastrodin, and 4HBA induce the expression of the chaperone protein, GRP78. BV2 cells were treated with the indicated concentrations of GE, gastrodin or 4HBA for 24 hours (A). Separately, BV2 cells were treated with 10 μg/ml of GE, gastrodin, or 4HBA during the indicated time periods (B) and immunoblotting was performed with anti-GRP78, CHOP, IRE-1α, spliced XBP-1, PERK, p-eIF-2α, eIF-2α or β-actin antibody. Representative data is shown from three independent experiments. Quantification analysis was performed using densitometry (lower). **p* < 0.05, significantly different from the control.

gastrodin and 4HBA showed effects similar to those of GE. The role of gastrodin in facilitating learning and memory was previously investigated [Wu et al., 1996, Hsieh et al., 1997]. It has also been reported that gastrodin has protective effects against cerebral ischemia-induced neuronal cell death [Yu et al., 2005, Xu et al., 2007]. Furthermore, a gastrodin-mediated decrease in mouse mortality rate was also correlated with the *in vitro* results [Zeng et al., 2007]. In addition to GE, several potential drug or natural product candidates to treat or prevent Alzheimer's disease have been discovered, including curcuminoids from *Curcuma longa* (turmeric) [Ahmed and Gilani, 2009], shogaols from *Zingiber officinale* (ginger) [Ghayur et al., 2008], huperzine from herbal moss *Huperzia serrata* (toothed clubmoss) [Zhang et al., 2008], and galanthamine from *Ungernia* sp. (daffodil bulbs) [Keller et al., 2011]. In another study of the protective effects of herbs and natural compounds against neurotoxicity, *Uncariae Ramulus et Uncus*, *Gastrodia elata*, *Evodia officinalis* and *Panax ginseng* were the most successful [Kim et al., 2006]. These findings suggest that these plants are also potential candidates with neuroprotective effects that could be useful in the treatment of neurodegenerative diseases such as Alzheimer's disease. However, these plant extracts represent only a small fraction of the herbs used worldwide. Thus, it is of interest to identify additional herbs that could be used as sources for new potential drug candidates to treat Alzheimer's disease. Additionally, the known candidates for the treatment of Alzheimer's disease require further study, especially with respect to the mechanisms responsible for their potential effects against Alzheimer's disease models *in vitro* and *in vivo*.

This study suggests a mechanism that involves the regulation of β -amyloid-induced cell death through GRP78 expression. Stress conditions such as glucose starvation, energy deficiency, chemical toxicity, acidosis and hypoxia, oxidative stress, ER Ca^{2+} depletion and inhibitors of glycosylation also cause increased cell damage and the induction of the glucose-regulated protein family members [Casas-Tinto et al., 2011, Kogel et al., 2003]. GRP78 was identified as a prototypic ER stress marker and master regulator of unfolded protein response (UPR). The accumulation of misfolded proteins in the ER due to environmental and/or metabolic stress conditions triggers GRP78 liberation from transmembrane ER signaling proteins to initiate GRP78 mRNA re-synthesis. Under normal or protective conditions, the GRP78 protein prevents the release of ATF6 from the ER membrane and the expression of CHOP, a transcription factor of pro-apoptotic genes, which acts as its own negative regulator [Casas-Tinto et al., 2011, Kim et al., 2008]. Throughout this study, the expression of GRP78 is suggested as a protective mechanism. Similarly, several studies have revealed the role of GRP78 as a protective chaperone protein against pathological conditions. It was reported that GRP78 has an essential role against neuronal apoptosis [Wang et al., 2010, Yuet et al., 1999]. In other cell types such as cardiomyocytes and renal epithelial cells, GRP78 expression has been suggested as a main protective mechanism against programmed cell death [Xiao-Hong et al., 2006, Liu et al., 1997].

Our finding regarding the involvement of GRP78 and the ER stress response in β -amyloid-induced cell death regulation with GE is novel, given that previous reports showing the beneficial effects of GE/pure components only linked the benefits to the antioxidant properties of the substances or to a mitochondrial cell death mechanism [An et al., 2010, Yu

et al., 2005]. In this study, the observed increase in GRP78 levels due to GE application may reflect an adaptation by the cell for increased protein synthesis in order to avoid ER overload. Upon β -amyloid treatment we observed an increase in cell death, in which the expression of GRP78 changed slightly at a relatively low concentration of β -amyloid, 5 μM , but not at other concentrations (10, 15, or 20 μM). However, the expression of a pro-apoptotic protein, CHOP, was strongly increased in a β -amyloid concentration-dependent manner (Fig. 1C). The elevated expression of CHOP could be interpreted as β -amyloid-associated cell death, just as the GE-mediated reduction in CHOP expression might be explained as one of the protective mechanisms of GE against β -amyloid stimuli. In Fig. 2D, GE and its components inhibited the expression of CHOP, the pro-apoptotic ER stress protein, while increasing that of GRP78, the anti-apoptotic chaperone protein. Consistent with this, concentration-dependent regulation of GRP78 and CHOP expression in BV cells was shown in Fig. 2E. Although the GE-induced expression of GRP78 provides a protective mechanism against β -amyloid, prolonged treatment with GE will not overcome the deleterious effects of chronic β -amyloid exposure, generally longer than four days, nor will it counteract the effects of an excessively high concentration of β -amyloid. These findings indicate the possibility of multiple mechanisms that facilitate cognition and memory without providing a cell-protective function. However, our results highlight a novel mechanism, whereby GE may inhibit the progression to Alzheimer's disease by allowing for a temporary adaptation to cell stress, a cellular protection phenotype that lasted for at least a few days in an *in vitro* study model.

In conclusion, GE and its pure components, gastrodin and 4HBA, protect against β -amyloid-induced cell death. The protective mechanism involved is suggested to be the induction of the chaperone ER stress protein, GRP78. However, further studies are needed to clarify the *in vivo* neuroprotective effects of GE, gastrodin, and 4HBA.

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