Original Article

Ultrastructural Changes in Adrenocortical Zona Fasciculate Cells Upon Exposure to Chronic Immobilization Stress and the Ameliorating Effect of Panax Ginseng Extract

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ABSTRACT

Introduction: Panax ginseng extract (P. gin.) is a well-known adaptogen reducing blood cortisone particularly during chronic stress. Yet, its effect on the structure of the cells of the adrenal cortex during stress was not well studied. So, the present investigation aimed to demonstrate the ultrastructural changes of adrenocortical zona fasciculata (Z. fas.) cells after chronic stress and to appraise the influence of co-administration of P. gin. extract.

Materials and Methods: Four groups of adult male albino rats (10 animals each). Group I (-ve control). Group II (+ve control) received the extract of P. gin. (100mg/kg/ day) orally. Group III was exposed daily for 150 minutes to immobilization stress. Group IV was exposed daily to immobilization stress and received the extract of P. gin. The experimental period was 14 consecutive days. At the end, rats from all groups were sacrificed & suprarenal glands were excised & processed for ultrastructural study. Morphometric measurements of lipid droplets (lipid ds.) in zona fasciculate cells (Z. fas.) were done and the data were statistically analyzed.

Results: Compared to group I, group II showed no structural differences. In group III, Z. fas. cells had deeply stained nuclei, few (lipid ds.) and cytoplasmic vacuolation in semithin sections. The mitochondria appeared disrupted with hardly seen smooth endoplasmic reticulum by electron microscopic examination. Group IV showed most cells with vesicular nuclei and many lipid ds. in the cytoplasm in semithin sections. By electron microscope, the mitochondria appeared mostly with vesicular cristae with cisterns of smooth endoplasmic reticulum nearby. Morphometric study revealed a statistically significant reduction in the numbers and areas of lipid ds. /field in group III compared to group I. Moreover, the numbers and areas of lipid ds. /field were significantly increased in group IV compared to group III.

Conclusion: Chronic immobilization stress leads to structural degenerative changes in Z. fas. cells of adult male albino rats which could be ameliorated by P. gin. extract co-administration, suggesting a favorable effect of P. gin. on Z. fas. cells on exposure to chronic stress.

Received: 15 July 2017, **Accepted:** 06 September 2017

Key Words: Immobilization stress; p. ginseng; ultrastructure; zona fasciculata.

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The Egyptian Journal of Anatomy, ISSN: 0013-2446, Vol. 41 No. 2

INTRODUCTION

Stress has been suggested by many authors to be the first step in the pathogenesis of a diverse variety of diseases, ranging from mental disorders such as anxiety, depression, immunosuppression and endocrine disorders including diabetes mellitus, cognitive dysfunctions, male sexual disorders, peptic ulcer, ulcerative colitis and hypertension ***(Elliott & Eisdorfer, 1982)***. Immobilization is used widely as a physical method of stress induction in animals ***(Willich et al., 1994 & Wilbert-Lampen et al., 2006)***. Different models of acute or chronic stresses have been implemented with different time periods to elucidate the effects of stress experimentally

 DOI: 10.21608/EJANA.2021.171454

(Donadio et al., 2007; Kumari et al., 2007 & Crema et al., 2010). Immobilization resembles a stressful and inevitable life situation in which adaptation is inadequate to modify the physiological replies ***(Bhatia et al., 2011)***.

Several attempts were done to counter the undesirable consequences of stress, ranging from meditation and yoga to anti-stress drug therapy, chiefly benzodiazepines as anxiolytic. The benzodiazepine anxiolytics (BDZs), in spite of having considerable anti-stress activity against acute stress, have not proved to be effective against chronic stress induced unfavorable effects especially on immunity, cognition, behavior, hypertension and peptic ulcer ***(Bhattacharya & Muruganandam, 2003)***. Furthermore, BDZs have not only teratogenic consequences on the embryo but also hazardous outcomes on the neonate during lactation ***(Trevor & Way, 2001)***. The plant kingdom gave an answer to this intricate problem of countering stress prompted disorder on physiological homeostasis. A collection of plant-based drugs called the adaptogens seems to make a condition of nonspecific resistance, enabling the body to adapt and counteract different stressors that can unfavorably affect the physiological system ***(Wagner et al., 1994)***. Panax ginseng roots extract (P. gin.), one of the adaptogens, was established to be effective in diminishing stress-induced adverse effect in soldiers and spacemen ***(Brekhman & Dardymov, 1969)***. It is evident that P. gin. will be a better alternative for chronic stress treatment. It has a potent adaptogenic activity that is mediated by regulating adrenocorticotrophic hormone secretion from pituitary gland leading to reduced cortisone secretion from adrenal cortex ***(Rai et al., 2003)***.

Although P. gin. was extensively investigated experimentally and clinically for its stress attenuating activity ***(Gaffney et al., 2001)***, its impact on the structure of suprarenal cortex during stress is not yet clear. Therefore, the present investigation aimed to demonstrate the ultrastructural changes of adrenocortical Z. fas. cells after chronic stress and to appraise the influence of co-administration of P. gin. extract.

MATERIAL AND METHODS

Animals

Forty male adult albino rats (weight; 200-250 gm.) were kept under standard circumstances (temperature 22 ± 2°C, lights on for 7:00 to 18:00 hours) with a free inlet to food and water. Clinical examination of the animals was evaluated before the experiment. All procedures of the experimental phase were accomplished according to the Ethics Council of Ain Shams University in Cairo, Egypt. After 7 days of adaptation to their surroundings, animals were placed individually in the experimental cage and allowed to move freely inside.

P. gin. extract preparation

Dried roots of P. gin. were obtained from a local market in Qurayyat, Al Jouf - KSA. The roots were grinded using an electrical blender into a fine powder. Twenty gm. of P. gin. powder were soaked in 100 ml of 20% ethanol for 3-5 days. Then filtering of the mixture was done and the dried extract was obtained by lyophilization ***(Mohan, 2004)***.

Experiment procedure

Rats were distributed into four equal groups. Group I was negative control. Group II (positive control) rats were receiving the extract of P. gin. (100 mg/kg/day) orally by gastric tube to ensure that all the animals received a constant supplementation of P. gin. extract. Rats of group III were exposed to immobilization stress by laying them in a tight transparent plastic tubes (6 cm in diameter × 15 cm long) with several 3 mm holes for breathing ***(Bitgul et al., 2013 & Shabir et al., 2013)***. This stress was applied for 150 minutes per day ***(Zareian et al., 2015)***. Rats of Group IV were exposed to immobilization stress (as mentioned in group III) and received the extract of P. gin. (100 mg/kg/day) orally by gastric tube. The extract of P. gin. was daily administered as aqueous suspensions using gum acacia (0.5%) as surfactant ***(Deepak et al., 2003)***. The experimental phase lasted for fourteen consecutive days ***(Das et al., 2000; Deepak et al., 2003 & Zareian et al., 2015)***. At the end of the experiment, rats from all groups were anaesthetized by chloroform and then sacrificed by decapitation. Both adrenal glands of each rat were extracted, freed of fatty tissues and prepared for light (semithin sections) and electron microscopic study.

Tissue preparation for ultrastructural study

The suprarenal glands of rats of all groups were cut to small pieces (3mm) and fixed in a solution formed of 2.5% paraformaldehyde and 2.5% glutaraldehyde for 24 hours at 4°C. Post-fixing of specimens was done in 1% osmium tetroxide and dehydration in ascending grades of alcohol. Then the specimens were immersed in two changes of

propylene oxide to be finally embedded in epon. Regional identification of adrenal sections was accomplished by staining semi-thin sections (1 um thickness) with 1% toluidine blue and examining them with the light microscope. Then ultra-thin sections (60 nm thickness) were cut, mounted on copper grids and stained with uranyl acetate and lead citrate. The grids were then examined with the transmission electron microscope (Seo- Russia) in Military Academy; Cairo ***(Oberley et al., 2008)***.

Morphometric study

Ten non overlapping high power fields of Z. fas. in semi-thin slides of each group were taken with an Olympus microscope connected with camera. The numbers of lipid ds. and areas occupied with lipid ds./field were calculated using digimizer software program version 4.6.1 ***(Maria Joao et al., 2009)***.

Statistical analysis

The recorded data were analyzed using SPSS software version 20 (SPSS Inc. Chicago, USA). One way ANOVA test was used to compare between numbers of lipid ds. and areas occupied with lipid ds. in control and other groups. Values in the text were expressed as means ±standard deviation (SD) and differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

Groups (I & II) (control group)

Light microscopic study

There were no ultrastructural differences found between positive control rats and negative control rats. The nuclei of cells of Z. fas. were rounded vesicular with prominent nucleoli. The cytoplasm was packed with numerous unstained lipid ds.. Multiple blood capillaries appeared separating the Z. fas. cells (Figure 1).

Electron microscopic study

The ultrastructure in positive and negative control rats was the same. The cells of Z. fas. from control rats were consisting of euchromatic nuclei with regular outlines in most of them (Figures 2&3). The cytoplasm contained numerous rounded or oval lipid ds. of varying sizes with distinguishable outlines (Figure 2).The mitochondria were rounded or ovoid in shape with double layered membrane (Figure 4) and closely packed vesicular cristae in moderate electron dense matrix (Figures 2, 3&4) and smooth endoplasmic reticulum appeared in the form of a network of small tubules which were in strict topographic relation with mitochondria (Figures 3&4).

Group (III)

Light microscopic study

The nuclei of Z. fas. cells had different sizes, shapes and some of them appeared deeply stained. The cytoplasm showed apparently few lipid ds. (Figures 5&6). Vacuolation in the cytoplasm and indented small nuclei could be also seen in some Z. fas. cells (Figure 6).

Electron microscopic study

In Z. fas. of rats subjected to chronic immobilization stress, the nuclei of many cells appeared irregular euchromatic with dense heterochromatin on the inner aspect of the nuclear membrane (Figure 7). Moreover, the nuclei of some cells showed irregular outline and condensed chromatin (Figure 8). The cytoplasm had few lipid ds. The outlines of the lipid ds. were not discernible. Additionally, confluence of lipid ds. could be seen formed of fusion of the lipid ds. with each other (Figures 7&9). Some mitochondria showed disrupted cristae. Other mitochondria were swollen with no outer membrane. Abnormal empty space could be also seen in some of them. The tubules of smooth endoplasmic reticulum could be hardly seen close to the mitochondria (Figure 10).

Group (IV)

Light microscopic study

Most of cells of Z. fas. had vesicular nuclei and their cytoplasm showed many lipid ds. The nuclei of some cells had varying sizes and shapes. The cytoplasm of them revealed few lipid ds. (Figure 11).

Electron microscopic study

Rats subjected to chronic immobilization stress and receiving P. gin. extract showed euchromatic nuclei with a nearly regular outline in most of the cells. Numerous lipid ds. with discernible outlines were detected in the cytoplasm (Figures 12&13). Most of the mitochondria contained vesicular cristae in a moderate electron dense matrix. Disruption of cristae was seen in very few mitochondria. The cisterns of the smooth endoplasmic reticulum still could be observed near mitochondria (Figure 13).

Morphometric study

The morphometric analysis revealed a nonsignificant difference in the numbers and area of lipid ds./field between the negative and positive control groups. The number and the area of lipid ds./field of group III (the group of rats exposed to stress only) were significantly reduced as compared to the negative control group. Meanwhile, the number and area of lipid ds./field of group IV (the group of rats exposed to stress and treated with ginseng) were significantly increased as compared to group III (Table1, Histogram 1,2).

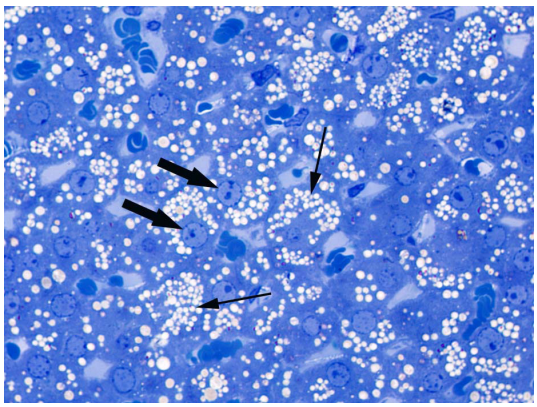


Fig. 1: A photomicrograph of a semithin section showing vesicular nuclei (thick arrow) and numerous unstained lipid ds. (thin arrow) in Z. fas. cells of control group rats. (Toluidine blue; X 1000)

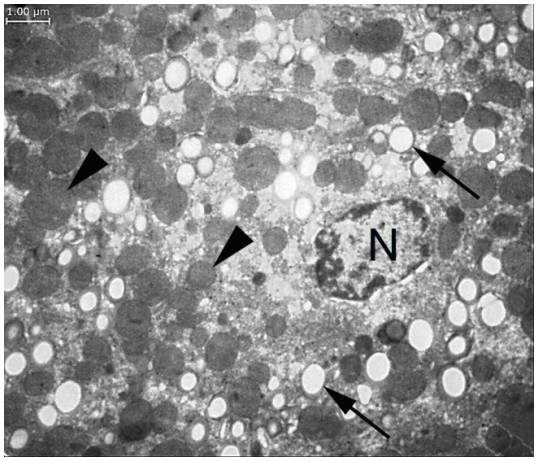


Fig. 2: An electron micrograph showing euchromatic nucleus (N), numerous lipid ds. with distinguishable outline (arrow) and mitochondria (arrow head) in Z. fas. cells of control group rats. (Uranyl acetate & lead citrate; X6000)

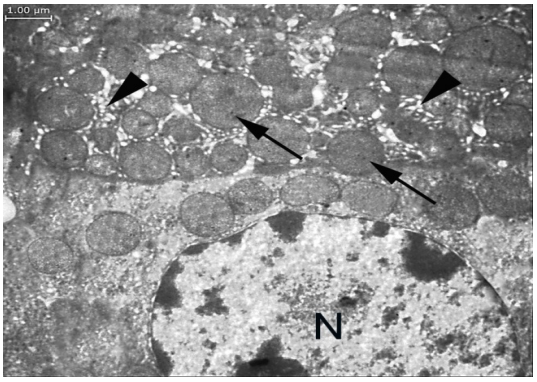


Fig. 3: An electron micrograph showing euchromatic nucleus (N) with regular outline, numerous mitochondria (arrow) and smooth endoplasmic reticulum (arrow head) in Z. fas. cells of control group rats. (Uranyl acetate & lead citrate; X10000)

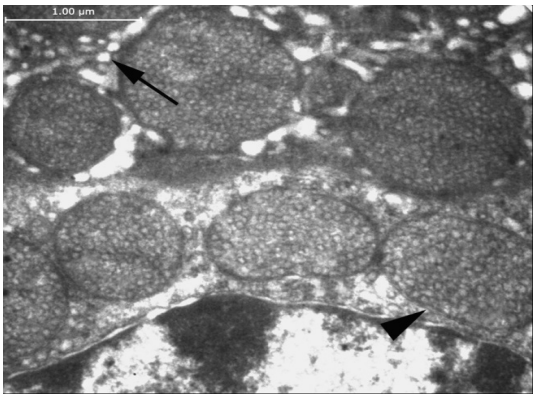


Fig. 4: An electron micrograph showing Z. fas. cells of control group rats with rounded and oval mitochondria, closely packed vesicular cristae in a moderate electron dense matrix and the tubules of the smooth endoplasmic reticulum (arrow). The double layered membrane of mitochondria could be seen (arrow head). (Uranyl acetate & lead citrate; X30000)

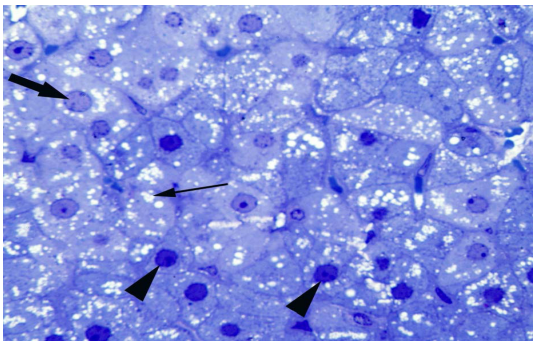


Fig. 5: A photomicrograph of a semithin section in Z. fas. of group (III) showing vesicular nuclei (thick arrow), deeply stained nuclei (arrow head) & lipid ds. (thin arrow). (Toluidine blue; X 1000)

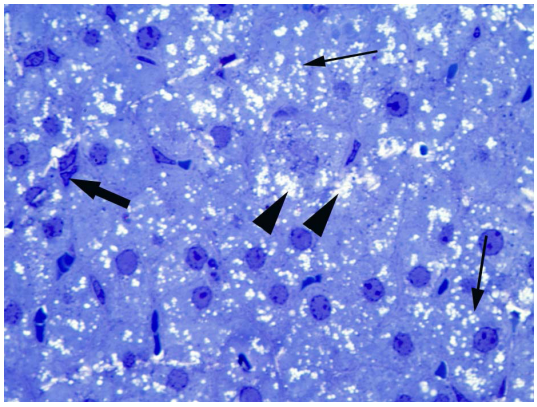


Fig. 6: A photomicrograph of a semithin section in Z. fas. of group (III) showing indented small nuclei (thick arrow), lipid ds. (thin arrow). The vacuolation in the cytoplasm of some cells could be seen (arrow head). (Toluidine blue; X 1000)

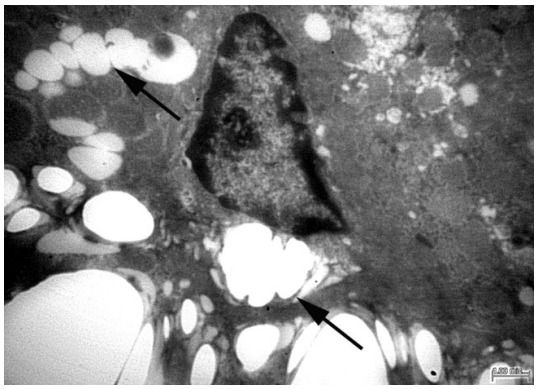


Fig. 7: An electron micrograph of Z. fas. cells of (Group III) showing irregular euchromatic nucleus with dense heterochromatin on the inner surface of the nuclear membrane and the confluence of lipid ds. (arrow). (Uranyl acetate & lead citrate; X3000)

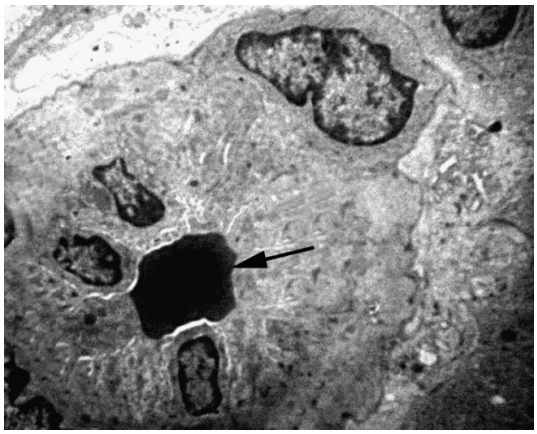


Fig. 8: An electron micrograph of Z. fas. cells of (Group III) showing irregular nuclei of different sizes. One of them has condensed chromatin (arrow). No lipid ds. are seen. (Uranyl acetate & lead citrate; X3000)

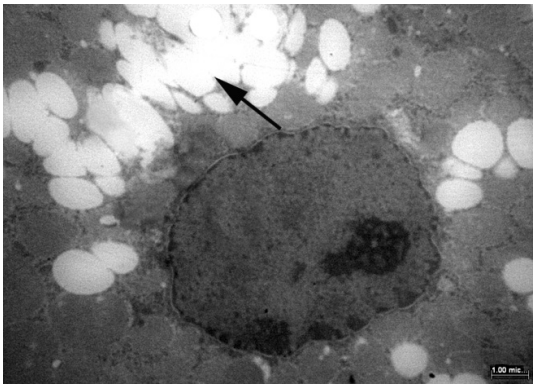


Fig. 9: An electron micrograph of Z. fas. cells of (Group III) showing marked confluence of the lipid ds. (arrow). Most of the lipid ds. were without discernible outline. (Uranyl acetate & lead citrate; X3000)

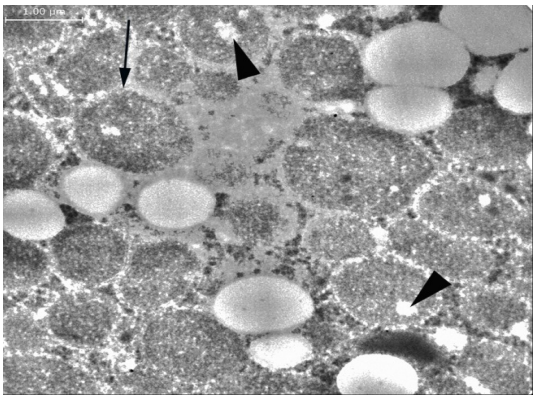


Fig. 10: An electron micrograph of Z. fas. cells of (Group III) showing mitochondria with disrupted cristae. Some swollen mitochondria with no outer membrane (arrow) and with abnormal empty space (arrow head). The tubules of smooth endoplasmic reticulum could be hardly seen close to the mitochondria. (Uranyl acetate & lead citrate; X15000)

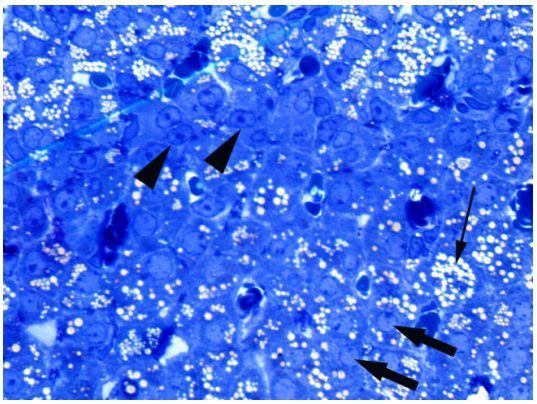


Fig. 11: A photomicrograph of a semithin section of group (IV) showing vesicular nuclei (thick arrow) and numerous lipid ds. (thin arrow) in Z. fas. cells. Some cells have few lipid ds. and irregular nuclei (arrow head). (Toluidine blue; X 1000)

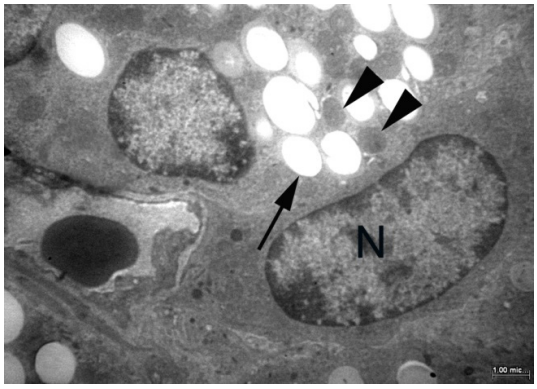


Fig. 12: An electron micrograph of Z. fas. cells of (Group IV) showing euchromatic nuclei with a nearly regular outline (N), lipid ds. with discernible outline (arrow) and the mitochondria (arrow head). (Uranyl acetate & lead citrate; X3000)

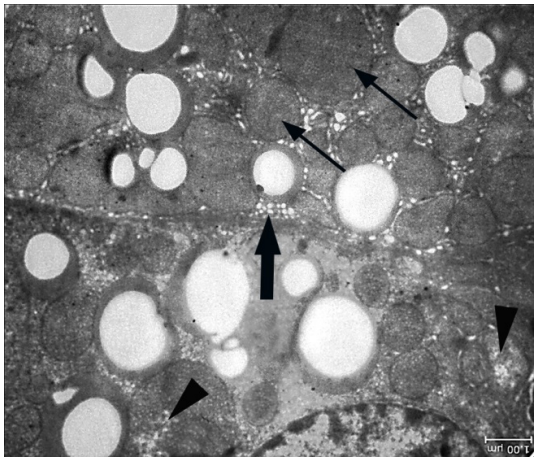


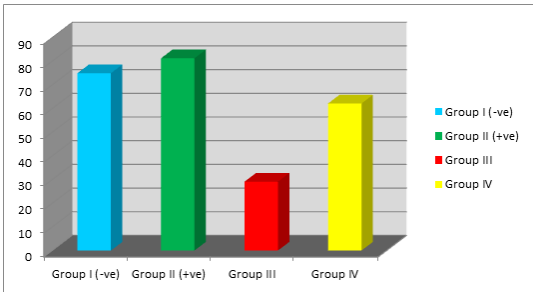
Fig. 13: An electron micrograph of Z. fas. cells of (Group IV) showing many lipid ds. with discernible outline, normal mitochondria (arrow) and few mitochondria showed disruption of cristae (arrow head). Note the cisterns of the smooth endoplasmic reticulum near mitochondria (thick arrow). (Uranyl acetate & lead citrate; X10000)

Table 1: The number of lipid ds./field and area of lipid ds. (um2)/field in the different groups

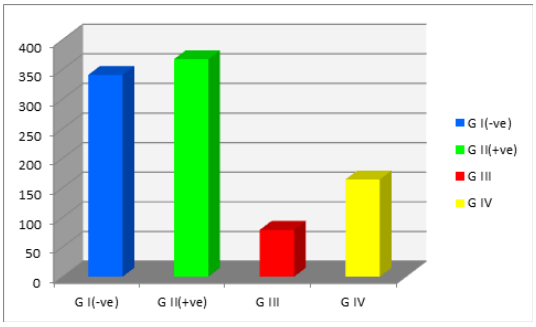
Groups	No. of lipid ds.	area of lipid ds. (um2)/field
Group I (-ve control)	74.90+4.48	340.67±82.97
Group II (+ve control)	81.20+5.31	367.28±74.02
Group III	29.10+6.72*	78.97±20.75*
Group IV	62.10+13.49**	164.13±23.56**

Data is expressed as mean ± standard deviation, *P. value* = probability of chance, $P < 0.05$ is significant;

* indicates a significant difference (*$P<0.005$ vs negative control group. ** $P<0.005$ vs group III, ANOVA test).



Histogram 1: Showing the relation between the numbers of lipid ds./field in the different groups studied.



Histogram 2: Showing the relation between the mean areas of lipid ds. (um2)/field in the different groups studied.

DISCUSSION

Immobilization was used widely to investigate stress-related physiological, biological and biochemical outcomes. The evident advantage of the usage of immobilization as a stressor is being in the fact that it produces both an inescapable psychological as well as physical stress ***(Marty et al., 1997 & Kasuga et al., 1999)***.

Stress activates the hypothalamic – pituitary – adrenal axis leading to a release of ACTH (adrenocortictrophic hormone) that acts on the Z. fas. cells of adrenal cortex to increase corticosterone levels in blood ***(Gesi et al., 2001)***, so the present study aimed to demonstrate the ultrastructural changes of adrenocortical Z. fas. cells after chronic stress and to appraise the influence of co-administration of P. gin. extract.

In the current study, the light microscopic picture and the ultrastructure of Z. fas. cells in control rats was the same as that reported in other references ***(Kadioglu & Harrison, 1975 & Nussdorfer et al., 1981)***. The exposure to chronic immobilization stress resulted in several changes in Z. fas. cells. Semithin sections revealed deeply

stained nuclei, cytoplasmic vacuolation and the lipid ds. became few. The reduction in the number and area of lipid ds./field was confirmed morphometrically where the mean numbers and areas of lipid ds. were 29.10 ± 6.72/field and 78.97 ± 20.75 um2/field in group (III) compared to 74.90+4.48/field and 340.67±82.97 um2/field in group (I) respectively. Ultrastructurally, lipid ds. showed no discernible outline and some of them were confluent. Different studies reported that stress exposure resulted in a substantial decrease in the number of lipid ds. in the cells of all adrenocortical zones, predominantly in the Z. fas. cells ***(Pellegrini et al. 1998; Koko et al., 2004 & Koldysheva & Lushnikova, 2008)***.

Moreover, the examination by electron microscope revealed that ,the nuclei of some cells in the group of rats subjected to immobilization stress had condensed chromatin and the mitochondria were swollen and showed no outer membrane and disrupted cristae. The same results were reported in Z. fas. cells in rats exposed to noise stress ***(Soldani et al., 1999)***. In the present study, the observed decrease in smooth endoplasmic reticulum tubules in some Z. fas. cells in group (III) rats could be interpreted as a sign of cell exhaustion ***(Penny & Brown, 1971)***.

In group (IV), the perception of P. gin. favorably improved the degenerative changes induced by chronic immobilization stress as compared to group (III) rats. In semi-thin sections, Z. fas. cells revealed vesicular nuclei and contained many lipid ds. Ultrastructurally, lipid ds. were surrounded with distinct outlines and most of the mitochondria were the same as in control groups. However, few mitochondria were still showing disrupted cristae. The morphometric study confirmed that the mean numbers and areas of lipid ds./field (62.10+13.49 and 164.13±23.56 um2/field respectively) were increased significantly in group (IV) as compared to those of group (III) (29.10±6.72 and 78.97±20.75 um2/field respectively). The ameliorating effect of P. gin.in the current study was in accordance with the preceding reports of some investigators whose results suggested that P. gin. improved the microscopic picture and ultrastructure of adrenal gland of rats exposed to Di-(2-ethylhexyl) phthalate ***(Ezzat et al., 2009)***. P. gin. could protect the Z. fas. cells and prevents their exhaustion in chronic stress due to its reducing effect on cortisol production by them ***(Rai et al., 2003)***.

It was reported that immobilization stress resulted in oxidative stress ***(Liu et al., 1994)***. Immobilization stress leads to oxidative damage to brain proteins, lipids, and DNA. Mitochondria exhibited a considerably greater increase in oxidation of proteins and peroxidation of lipids than cytosol. These suggestions supported the idea that stress induces oxidants, and that the oxidative damage in stress could accelerate the occurrence of degenerative diseases of elderly, including brain dysfunction ***(Liu et al., 1996)***. Immobilization decreases the activities of glutathione-S-transferase, superoxide dismutase and catalase enzymes leading to oxidative stress in brains of rats ***(Zaidi & Banu, 2004)***. Theoretically, exogenous antioxidant supplementation could support endogenous antioxidant system, thus decreasing oxidative damage ***(Selman et al., 2006)***. It was postulated that ginseng could reduce oxidative stress. The active compounds of ginseng are the ginsenosides. They are accountable for the anti-inflammatory, anti-oxidative and anticancer properties of ginseng ***(Lü et al., 2009)***. Ginsenosides also have additional neuroprotective effects ***(Chen et al., 2005; Kim et al., 2013 & Shin et al., 2013)***.

Furthermore, ginseng pre-treatment reduced the expression of the pro-apoptotic gene caspase-3, while it augmented expression of the anti-apoptotic gene Bcl-2. Consistent with this, immunoblot investigations exhibited that pre-treatment of cultured neuroblastoma cells with ginseng prevented expression of the pro-inflammatory gene COX-2. Accordingly, ginseng can protect against oxidative stress provoked cultured cell death by repression of the genes mediating apoptosis and inflammation ***(Eun-Hye et al., 2010)***. The previously mentioned studies could explain the favorable role of P. gin. on the degenerative changes that occurred in Z. fas. cells upon exposure to chronic stress.

CONCLUSION

Chronic immobilization stress leads to structural degenerative changes in Z. fas. cells of adult male albino rats which could be ameliorated by P. gin. extract co-administration, suggesting a favorable effect of P. gin. on Z. fas. cells on exposure to chronic stress.

CONFLICT OF INTERESTS

There are no Conflicts of Interest.

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التغيرات التركيبية الدقيقة فى خلايا المنطقة الحزمية بالقشرة الكظرية عند التعرض لضغط التجميد الحركى المزمن والآثر المحسن لمستخلص الباناكس جينسينغ

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ملخص البحث

مقدمة: إن مستخلص الباناكس جينسينغ مكيف طبيعى معروف يقلل مستوى الكورتيزون بالدم خاصة عند التعرض للضغط المستمر، وإلى الآن لم يتم جيداً دراسة تأثيره على تركيب خلايا قشرة الغدة الكظرية، ولذلك هدفت الدراسة الحالية لتوضيح التغيرات التركيبية الدقيقة لخلايا منطقة فاسيكولاتا بالقشرة الكظرية بعد التعرض للضغط العصبى المزمن، ولتقييم تأثير تناول مستخلص الباناكس جينسينغ مصاحبا له.

أدوات وطرق البحث: تم تحديد أربعة مجموعات من ذكور الفئران البيضاء البالغة (10 فئران بكل مجموعة)، المجموعة الأولى (المراقبة السالبة) والمجموعة الثانية (المراقبة الموجبة) تم اعطائها مستخلص الباناكس جينسينغ عن طريق الفم (100مج/كج/يوميا)، والمجموعة الثالثة تم تعرضها يوميا لضغط التجميد الحركى لمدة 150 دقيقة، والمجموعة الرابعة تم تعرضها يوميا لضغط التجميد الحركى مع تناول مستخلص الباناكس جينسينغ وكانت مدة التجربة 14 يوم متتالية. فى النهاية تم ذبح الفئران من كل المجموعات واستئصال الغدد الكظرية ومعالجتها للدراسة التركيبية الدقيقة، وتم عمل قياسات شكلية لقطرات الدهون فى منطقة فاسيكولاتا وتم تحليل البيانات إحصائياً.

النتائج: لم تظهر المجموعة الثانية تغيرات تركيبية مقارنة بالمجموعة الأولى، وفى المجموعة الثالثة كان لخلايا منطقة فاسيكولاتا نوى مصبوغة بعمق وقليل من قطرات الدهون مع فجوات عصارية فى المقاطع النصف رفيعة، كما ظهرت الميتوكوندريا ممزقة وظهرت الشبكة الإندوبلازمية الناعمة بصعوبة عند الفحص بالميكروسكوب الإلكترونى. وفى المجموعة الرابعة ظهرت معظم الخلايا بنوى حويصلية وقطرات دهنية متعددة فى العصارة بفحص مقاطع نصف رفيعة، وبالميكروسكوب الإلكترونى ظهرت معظم الميتوكوندريا بأعراف حويصلية مع أوعية الشبكة الإندوبلازمية الناعمة بالقرب منها. وأوضحت الدراسة القياسية الشكلية إنخفاض إحصائى هام فى أعداد ومساحات القطرات الدهنية فى المجال فى المجموعة الثالثة مقارنة بالمجموعة الأولى، علاوة على ذلك كانت أعداد ومساحات القطرات الدهنية زائدة بشكل كبير فى المجموعة الرابعة مقارنة بالمجموعة الثالثة.

الإستنتاج: إن ضغط التجميد الحركى يؤدى إلى تغيرات انحلالية تركيبية فى خلايا منطقة فاسيكولاتا لذكور الفئران البيضاء البالغة والذى أمكن تحسينه بالتناول المصاحب لمستخلص الباناكس جينسينج مما يوحى بتأثير ملائم للباناكس جينسينج على خلايا منطقة فاسيكولاتا عند التعرض للضغط المزمن.