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Original Article

Role of Panax Ginseng extract in ameliorating the effects of intermittent restraint stress on the hippocampus of adult male albino rats: Histological and Immunohistochemical study

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ABSTRACT

Introduction: Stress is a state of disrupted homeostasis and it is classified into physical and psychosocial. Hippocampus is a sensitive part of the brain to stress-induced damage. Panax ginseng neuroprotective effects on some neurodegenerative diseases have been proved.

Aim: This work aimed to study the role of Panax ginseng in ameliorating the effects of intermittent restraint stress on adult male rat hippocampus (Cornu Ammonis and Dentate).

Material and Methods: Thirty adult male albino rats were used in this study, five to seven months, 180-200 gm. Rats were divided into three groups: group I: rats were further subdivided into: subgroup IA: rats were kept as a negative control; subgroup IB: rats received single dose of Panax ginseng daily for three weeks. Group II: rats were housed as in group I except for the stress period, where each rat was kept in a reduced volume cage for 2 hrs daily for three weeks. Group III: rats were housed and exposed to stress as in group II and received a single dose of Panax ginseng daily for three weeks.

Results: The present work revealed that intermittent restraint stress induced marked histological changes in rat hippocampus of group II in the form of disorganized Cornu Ammonis and apparent decreased thickness of the granular layer of the Dentate gyrus. Group III showed nearly preserved histological structure.

Conclusion: Results obtained in this study suggested that Panax ginseng could provide an ameliorating effect on stress induced hippocampal histological changes.

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Key Words: Hippocampus, panax ginseng, restraint stress.

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INTRODUCTION

Stress is a state of disrupted homeostasis and it is classified into acute and chronic (intermittent or continuous), and further classified into physical (restraint) and psychosocial (anxiety, isolation, fear)[1]. Restraint stress is considered a modified type of immobilization stress, during which nonescape physical and mental stresses are done by imprisoning the animal in a closed tube. This type of experimental stress is involving both physical and psychological effects[2,3].

Chronic and acute stress have been proved to produce structural and functional changes in the brain[4]. The hippocampal formation is a brain structure that is formed of hippocampus proper (Cornu Ammonis), Dentate gyrus and Subiculum[5]. Its function includes episodic, declarative and spatial learning and memory[6]. Hippocampus is a sensitive part of the brain to stress-induced[7] and data suggested that hippocampus role in stress response has double action. The dorsal region was involved in adaptation to facilitate avoiding of the stressor and the ventral region involved in the emotional sides of the experience[8].

Panax ginseng is a popularly used Asian plant in herbal medicine, Panax roots major active compounds are ginsenosides (triterpenoid glycosides) which consist of a 4-ring steroid backbone structure. According to sugar type, quantity, and position, different types of ginsenosides could be recognized.

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Ginsenosides have been traditionally believed to restore the mental and physical vitality in Asia. Its use increased globally as a natural medicine. Its neuroprotective effects on some neurodegenerative diseases have been proved[9-11]. Therefore, the objective of this work was to study the role of Panax Ginseng in ameliorating the effect of intermittent restraint stress on adult male rat hippocampus (Cornu ammonis and Dentate).

MATERIAL AND METHODS

Chemicals:

Panax ginseng was purchased in the form of aqueous extract 120ml syrup (Pharco pharmaceuticals company, Egypt) each 5ml contained 47mg ginseng.

Animals:

Thirty adult male albino rats were used, aging five to seven months, weighting 180-200 gm, they were obtained and locally bred at the Medical Research Center (Faculty of Medicine, Ain-Shams University). Rats were kept three per medium sized metal cages in a room temperature with regular dark/light cycles with good ventilation. All rats were kept under the same circumstances throughout the experiment. The experiment followed the guidelines of Ain Shams University Ethics Committee.

Experimental Protocol:

Rats were divided equally into three groups:

• **Group I (Control):** composed of ten rats which were further subdivided into:

Subgroup IA (Negative Control): contained five rats that were housed at medium sized metal cages with free access to food and water for three consecutive weeks.

Subgroup IB (Panax Control): contained five rats, housed as group I and received single dose of 100 mg/kg/day Panax ginseng by gastric tube[13] for three consecutive weeks.

• **Group II (Stress):** composed of ten rats that were housed as in group I except for the stress period, where each rat was kept in a reduced volume cage inside its metal cage and that was done 2hrs daily (from 10am-12pm) for three consecutive weeks[12].

• **Group III (Stress + Panax):** composed of ten rats that were housed and exposed to stress as in group II and have received single dose of 100 mg/kg/day Panax ginseng by gastric tube[13] for three consecutive weeks.

The plastic cage used for stress was adapted to the rat size to prevent its mobility and contained five pores to allow aeration[14]. By the end of the experimental period, rats of each group were sacrificed by decapitation under anesthesia, the cerebral hemispheres were carefully dissected and processed for light microscopic examination.

Processing of samples:

Preparation of paraffin blocks and staining methods:

Each cerebral hemisphere was fixed in 10% buffered formalin, processed and embedded in paraffin blocks; parasagittal sections were obtained at 5μm from the medial surface and stained by Hematoxylin and Eosin (Hx. &E.)[15].

For Immunohistochemical study:

• Glial fibrillary acidic protein (GFAP) was used as an indicator for glial cells. Paraffin sections 5 μm thickness were deparaffinized and hydrated. The peroxidase activity was blocked with 10% hydrogen peroxide. Citrate buffer 0.01 mol/l (pH 6) was added to sections for 5 min. Thereafter, sections were incubated with the primary antibody (1: 500 monoclonal mouse anti-GFAP (Dako Carpenteria, Ca, USA.) at 4°C for 20 hrs. Sections then were incubated with biotinylated antibodies (ABC kit, 1: 200) and avidin-biotin complex followed by 0.05% diaminobenzidine. Finally, they were counterstained with Mayer's hematoxylin and mounted[16].

• Caspase 3 was used as apoptosis indicator, phosphate-buffered saline wash for 5 min was done for sections. Sections were incubated with antibody to cleave caspase 3 (Invitrogen, Sweden AB Stockholm Sweden (1:200)) at 4°C, then incubated with anti mouse antibody (Invitrogen, Molecular Probes, Eugene, Oregon, USA (1:500)) for 1h. Slides were then incubated in 3,3 Diaminobenzidene for 10 mins and

finally counterstained by Mayer's hematoxylin, dehydrated and mounted[17].

Image analysis:

Morphometric analysis was carried out on routine Hematoxylin and Eosin stained slides using image analyzer Leica Q win V.3 program on a computer in the Histology department, Faculty of Medicine, Ain Shams University. The computer was connected to a Leica DM2500 microscope (Wetzlar, Germany). Six randomly chosen fields in six sections obtained from six different rats from the same group were used for calculating the mean number of caspase 3 positive pyramidal cells of CA3 in all groups. Pixels were calibrated for actual measurements in micrometer. The magnification used was 400X using the objective lens of 40X.

Statistical Analysis

Data analysis was performed using PSPP freeware with one-way ANOVA and Bonferroni Post Hoc test to detect the significance between every two groups. Results were considered highly significant when $P\ value \leq 0.001$, significant when $P\ value \leq 0.05$ and nonsignificant when $P\ value > 0.05$.

RESULTS

Group I (Control):

Light microscopic examination of Hx. & E. stained sections of hippocampus of the control subgroups IA and IB showed regular structure of the pyramidal layer of Cornu Ammonis, CA1 and CA2 were formed of small pyramidal cells with vesicular nuclei arranged in three to four rows while CA3 and CA4 were formed of large pyramidal cells with vesicular nuclei. The molecular layer was formed of regular neuronal processes, scattered glial and nerve cells and the granular layer of the dentate gyrus was formed of small compact cells with granular cytoplasm (Figs. 1-4).

Examination of immunohistochemically stained sections for GFAP showed positive immunoreaction of scattered small astrocytes mainly in the molecular layer (Fig. 5), whereas, immunohistochemical staining for caspase-3 showed nearly negative immunoreaction of the pyramidal cells of Cornu Ammonis (Fig. 6).

Group II (Stress):

Light microscopic examination of Hx. & E. stained sections of the stress group hippocampus showed disorganized pyramidal layer of Cornu Ammonis with shrunken pyramidal cells, intracellular vacuolations and many ghost-like cells. Irregular neural processes were noticed in the molecular layer. The granular layer of the dentate gyrus showed apparent decrease in thickness with many flattened cells and marked pericellular spaces. (Figs. 7-9).

Examination of immunohistochemically stained sections for GFAP showed intensely positive stained astrocytes with increased cell body size, number and length of their processes (Fig. 10). Moreover, immunohistochemical staining for caspase-3 showed marked positive immunoreaction of most pyramidal cells of Cornu Ammonis (Figs. 11).

Group III (Stress + Panax):

Light microscopic examination of Hx. & E. stained sections of group III hippocampus showed regular structure of the pyramidal layer of Cornu Ammonis with few shrunken cells, minimal intracellular vacuolations and few ghost-like cells. The molecular layer showed regular neuronal processes. Few flattened cells with minimal pericellular spaces could be detected in the granular layer of the Dentate gyrus. (Figs. 12-14).

Examination of immunohistochemically stained sections for GFAP showed positive immunoreaction of scattered small astrocytes (Fig. 15). Whereas, immunohistochemical staining for caspase-3 showed mainly negative immunoreaction of the pyramidal cells of Cornu Ammonis with only few cells retaining mild positive immunoreaction (Figs. 16).

Morphometrical results

Using morphometric studies, the mean number of caspase-3 immune positive pyramidal cells of CA3 in the three groups was counted and values were mentioned in table 1 and column chart 1. Statistical analysis revealed highly significant

decrease of caspase-3 immune positive pyramidal cells of the stress group (group II) as compared to the control group (group I) with a *P-value <0.001*. Similarly, a highly significant decrease of caspase-3 immune positive pyramidal cells of group II as compared to Panax received group (group III) has been found with a *P-value < 0.001*, On the other hand, the difference between group I and group III were statistically non-significant with a *P-value > 0.05* (Table 1). The comparisons between the morphometric results of the three groups were further illustrated in column chart 1.

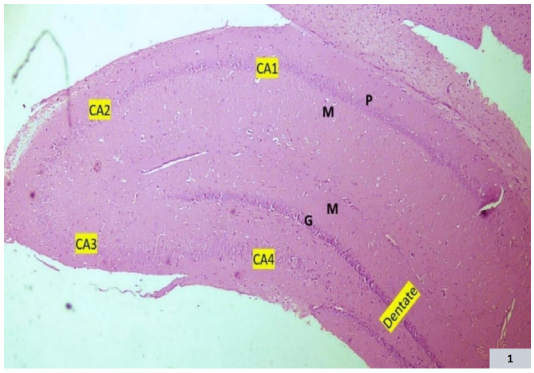


Fig. 1: A photomicrograph of a section of hippocampus of group I, showing Cornu Ammonis parts CA1, CA2, CA3, CA4 and dentate gyrus. Notice the pyramidal layer (P), the molecular layer (M) and the granular layer (G). (Hx. & E. X100).

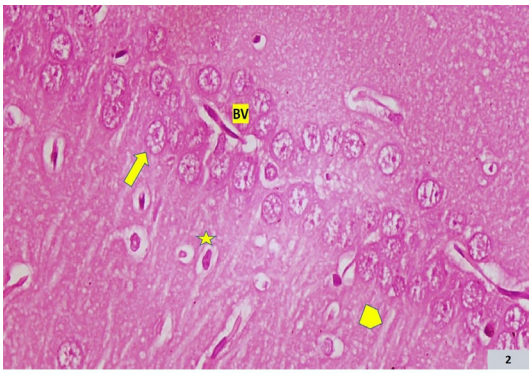


Fig. 2: A photomicrograph of a section of hippocampus of group I, showing CA1 region formed of small pyramidal cells with vesicular nuclei (arrow). Notice the small blood vessels (BV), the molecular layer with many glial cells (star) and regular neuronal processes (arrowhead). (Hx. & E. X400).

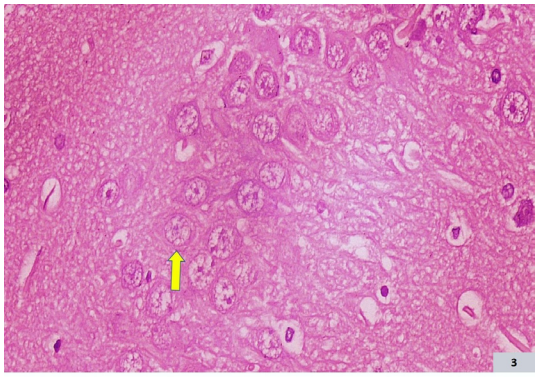


Fig. 3: A photomicrograph of a section of hippocampus of group I, showing CA3 formed of large pyramidal cells with vesicular nuclei (arrow). (Hx. & E. X400).

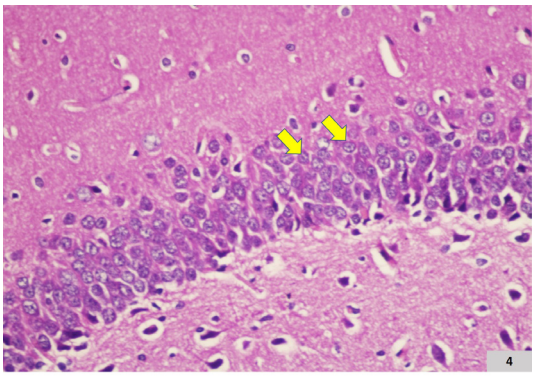


Fig. 4: A photomicrograph of a section of hippocampus of group I, showing Dentate gyrus formed of small dark granular cells (arrows). (Hx. & E. X400).

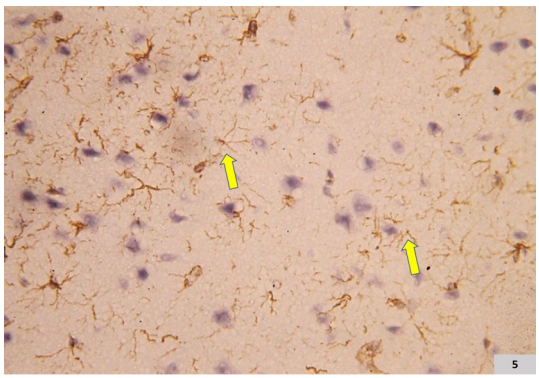


Fig. 5: A photomicrograph of a section of hippocampus of group I, showing positive immunoreaction for GFAP of scattered small astrocytes (arrows). (GFAP X400).

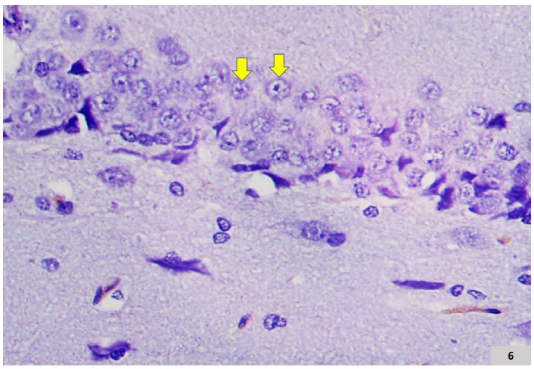


Fig. 6: A photomicrograph of a section of hippocampus of group I, showing negative immunoreaction for caspase-3 of CA3 pyramidal cells (arrows). (Caspase-3, X400).

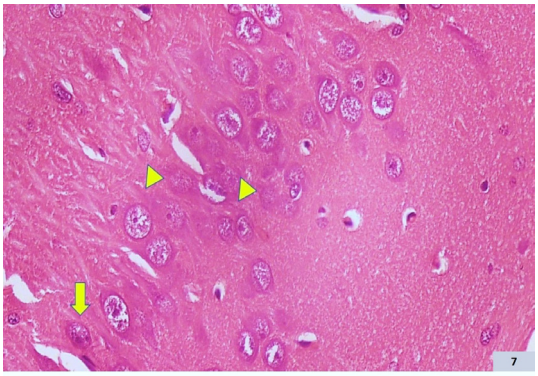


Fig. 7: A photomicrograph of a section of hippocampus of group II, showing the pyramidal layer of CA1 with flattened cells (arrow) and many ghost-like cells (arrow heads). (Hx. & E. X400).

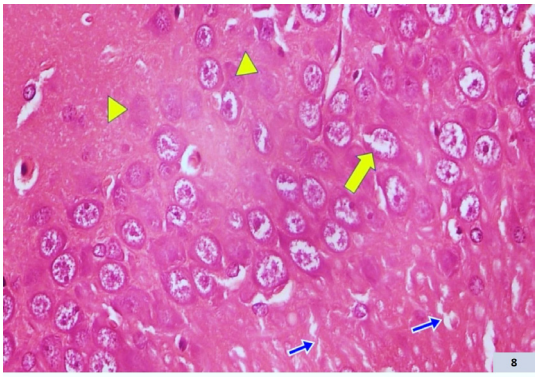


Fig. 8: A photomicrograph of a section of hippocampus of group II, showing disorganized pyramidal layer of CA3 with intracellular vacuolations of pyramidal cells (arrow) and ghost-like cells (arrow heads). Notice the irregular neural processes (blue arrows). (Hx. & E. X400).

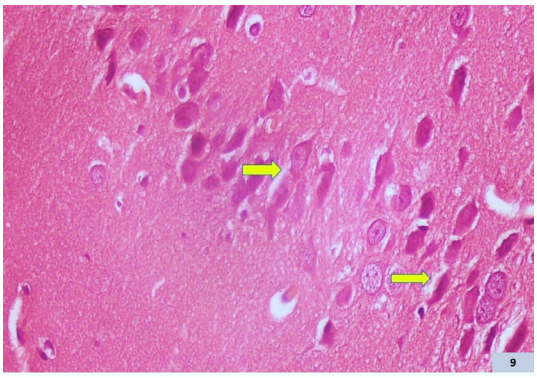


Fig. 9: A photomicrograph of a section of hippocampus of group II, showing apparent decrease in thickness of the granular layer of Dentate gyrus, flattened cells with marked pericellular spaces (arrows). (Hx. & E. X400).

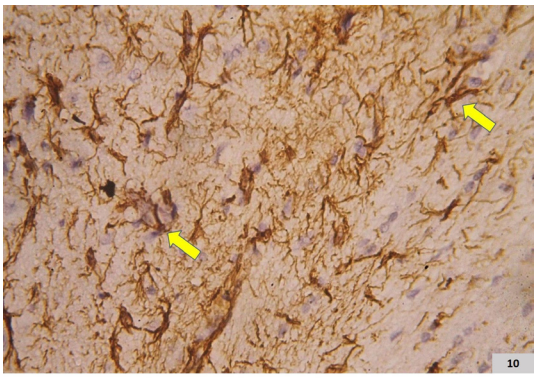


Fig. 10: A photomicrograph of a section of hippocampus of group II, showing intensely positive immunoreaction for GFAP of astrocytes with increased cell body size, number and length of their processes (arrows). (GFAP, X400).

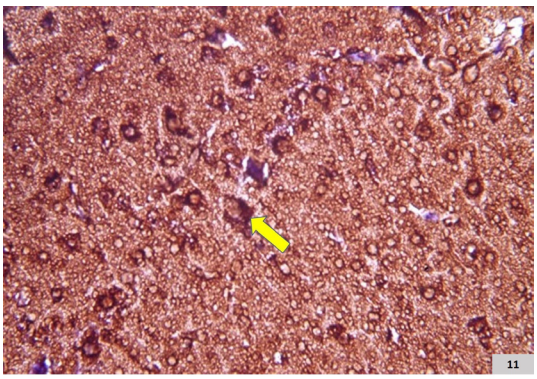


Fig. 11: A photomicrograph of a section of hippocampus of group II, showing intense positive immunoreaction for caspase-3 of CA3 pyramidal cells (arrow). (Caspase-3, X400).

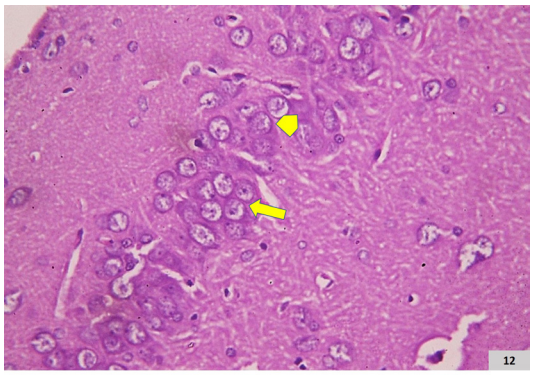


Fig. 12: A photomicrograph of a section of hippocampus of group III, showing CA1 with nearly regular pyramidal cells (arrow) with few ghost-like cells (arrowhead). (Hx. & E. X400).

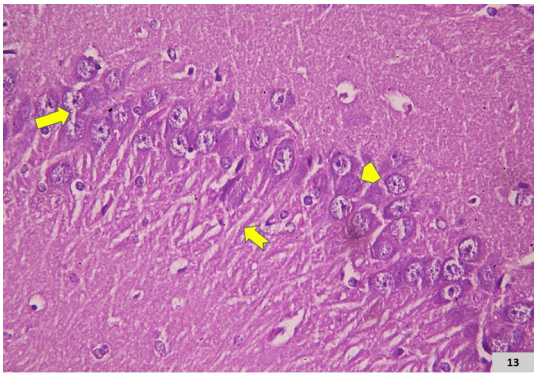


Fig. 13: A photomicrograph of a section of hippocampus of group III, showing regular pyramidal layer of CA3 with minimal intracellular vacuolations of some pyramidal cells (arrow) and few ghost-like cells (arrowhead). Notice the regular neural processes (notched arrow). (Hx. & E. X400).

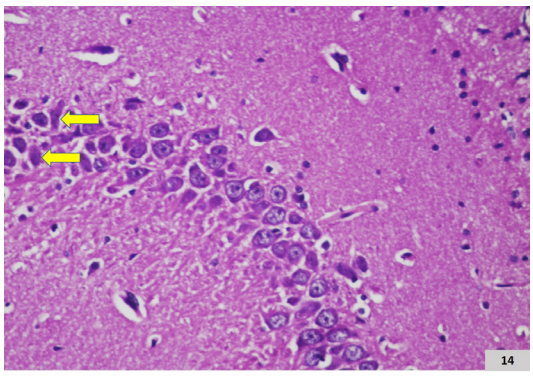


Fig. 14: A photomicrograph of a section of hippocampus of group III, showing almost regular structure of the granular cells of dentate gyrus with few flattened cells (arrows). (Hx. & E. X400).

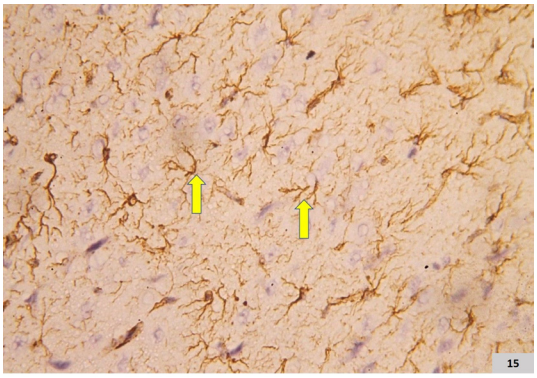


Fig. 15: A photomicrograph of a section of hippocampus of group III, showing positive immunoreaction for GFAP of small astrocytes (arrows). (GFAP, X400).

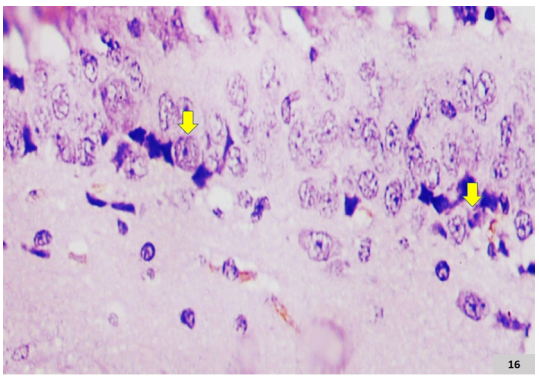
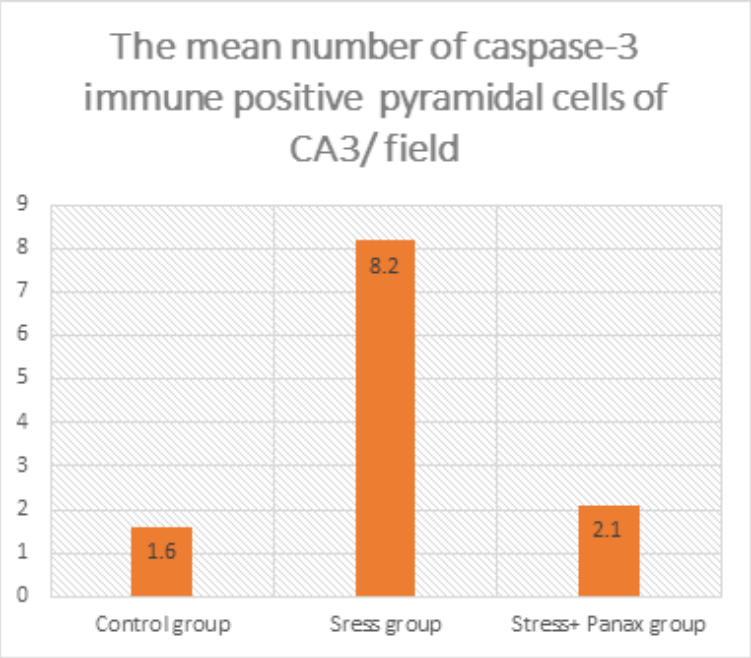


Fig. 16: A photomicrograph of a section of hippocampus of group III, showing mild positive immunoreaction for caspase-3 for some CA3 pyramidal cells (arrows). (Caspase-3, X400).

Table 1: Comparing the number of caspase-3 immune positive pyramidal cells of CA3/ field of hippocampus between the three groups showing, P-value either; non-significant (*) or highly significant (**).

		Group I (Control)	Group II (Stress)	Group III (Stress+Panax)
Number of caspase-3 immune positive pyramidal cells pyramidal cells of CA3/field (mean ± standard deviation)		1.6± 0.5	8.2 ± 0.6	2.1 ± 0.8
T test	Between Group I&II		*P= 0.0005***	
	Between Group II&III		*P = 0.0001***	
	Between Group I&III		*P= 0.1**	



Column chart 1: Demonstrating the morphometric comparison between the three groups as regards; the mean number of caspase-3 immune positive pyramidal cells of CA3/ field.

DISCUSSION

Stress is a common experience of daily life and it is essential for survival and enhancing performance. Maladaptive responses to chronic stress lead to both neurological and psychiatric illnesses[18].

The results of the present study revealed that intermittent restraint stress for three weeks caused marked structural changes of both Cornu Ammonis and Dentate parts of the hippocampus. Hx. & E. stained sections showed disorganized pyramidal layer of Cornu Ammonis with flattened cells, intracellular vacuolations and many ghost-like cells. Ghost cells are defined as dead cells with lost nuclei, but the cellular outline remnants are still visible[19]. Irregular neural processes were also detected in the molecular layer. The granular layer of the Dentate gyrus showed apparent decrease in thickness with flattened cells and marked pericellular spaces. Magarinos *et al*.[20] and Huang *et al*.[21] reported that rats exposed to repeated restraint stress revealed, atrophy and dendrites remodeling of pyramidal neurons. The authors explained that these changes were mostly due to increased intracellular calcium with subsequent depolymerization of the dendritic cytoskeleton and atrophy. Cotella *et al*.[22] and Kathleen[23] also explained that changes of hippocampus with restraint stress could be due to activation of excitatory amino acids release from

mossy fibers and stress hormones (cortisol and epinephrine) from the adrenal gland. Complex interaction between the nervous system and the endocrine system synergize to cause the stress effects on hippocampus. The hippocampus is known to be one of the adrenal steroids target. Its neurons express receptors for adrenal steroids, type I receptors for mineralocorticoids and type II receptors for glucocorticoids, that affect neurons excitability with subsequent neurological consequences during stress[24, 25].

Chronic stress was reported to spoil synaptic plasticity, inhibit genesis of neurons and change the actin expression (the cytoskeleton of the dendritic spines). In addition to change of the amyloid precursor protein, that is known to be associated with Alzheimer's disease[26].

Additionally, GFAP immunohistochemical stain revealed that astrocytes were intensely stained with increased cell body size, number and length of their processes. Glial cells play important roles in regulating the activity of neurons and synaptic plasticity. Chronic stress in animal models proved to increase astrocyte size and density and it was found that astrocyte gene expression reacted to stress by changing the astrocyte specific proteins leading to the cognitive impairments that occur with stress and mood disorders[27]. Baydas *et al*.[28] attributed the increase in GFAP expression (reactive gliosis) in diabetic rat hippocampus to the effect of increased reactive oxygen species. This was in contradiction to Zhao *et al*.[29] who reported that chronic stress reduced hippocampal astrocytic size and they explained that astrocytes are the only cells in the brain that store energy in the form of glycogen and chronic stress greatly affects glycogen synthesis and glycogenolysis in brain, which was suggestive of metabolic dysfunction and atrophy of astrocyte with chronic stress.

The stress group also, showed marked expression of the caspase-3 in almost all pyramidal cells, which was further confirmed by the morphometric studies that revealed a highly significant increase in the mean number of caspase-3 immune positive pyramidal cells of CA3 when compared to those of the control group. Previous studies have shown that repeated stress elevate the circulating glucocorticoids which have destructive effects on pyramidal neurons of hippocampus especially in CA3 region[30]. Chronic stress altered hippocampal neurochemistry, neuronal morphology, excitability and increase in the apoptotic cells suggesting increased turnover of neurons in rats with subsequent cognitive deficits[31-34]. Repeated stress has been proved to impair antioxidant defenses and increase the free radicals particularly lipid peroxidation leading to oxidative damage in the hippocampus[35].

On the other hand, examination of Hx. & E. stained sections of hippocampus of the Panax group showed apparent regular pyramidal layer of Cornu Ammonis with few flattened cells, minimal intracellular vacuolations and few ghost-like cells. The granular layer of Dentate gyrus showed few flattened cells with minimal pericellular spaces. Panax ginseng is called "adaptogen" that means, it helps the body to adapt to physical and biochemical stressors and enhance the mental capacity[36]. Panax ginseng anti-stress mechanisms are still not well understood but experiments have proved its action on both the adrenal glands and the hypothalamic-pituitary-adrenal axis[23]. It has been reported that it has an improving effect on neurite growth and in protecting the hippocampal neuron from ischemic damage and disorders associated with ageing[37]. Marked improvement in learning and memory in rats with brain damage were also noticed after ginseng administration[38].

In addition, Panax group showed scattered small astrocytes by GFAP immunohistochemical stain, nearly similar to that of the control group. Baydas *et al*.[28] reported that antioxidants could prevent the stress reactive gliosis by reducing the damaging effects of the reactive oxygen species in the hippocampus. Vitamins E and C administration as an antioxidant were able to decrease stress induced memory impairment[39]. Panax was reported to have hepatoprotective effect by enhancing the antioxidant defense mechanism[40]. Moreover, The Panax group showed, mild expression of the caspase-3 of pyramidal cells. This was further confirmed by the morphometric studies that revealed highly statistically significant decrease in the mean number of caspase-3 immune positive pyramidal cells of CA3 when compared to that of group II and showed non-significant difference compared to group I. Hou *et al*.[41] reported that prolonged administration of ginsenoside was able to enhance cell survival of Dentate gyrus and improved spatial recognition memory of mouse. Panax ginseng has been proved to increase the body resistance to

many harmful factors and can protect tissues from damage by toxins and stress[42].

CONCLUSIONS

Results obtained in this study suggested that Panax ginseng could provide an ameliorating effect on stress induced hippocampal histological changes in rats.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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دور مستخلص نبات باناكس جينسنغ في تخفيف آثار اجهاد التقييد المتقطع على الحصين لذكور الجرذان البيضاء البالغة: دراسة هستولوجية وهستو كيميائية مناعية

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ملخص البحث

مقدمة: الإجهاد هو حالة من خلل توازن الجسم ويصنف إلى جسدى ونفسي اجتماعي. الحصين هو جزء من المخ حساس للضرر الناتج عن الإجهاد. وقد أثبتت قدرة الباناكس جينسنغ على حماية الاعصاب من بعض الأمراض التنكسية العصبية.

الهدف: هدف هذا العمل إلى دراسة دور الباناكس جينسنغ في تخفيف آثار إجهاد التقييد المتقطع على حصين ذكور الجرذان البالغين .

المواد والأساليب: تم استخدام ثلاثين من ذكور الجرذان البيضاء البالغة في هذه الدراسة ، تتراوح أعمارهم بين خمسة إلى سبعة أشهر ، وتزن 180-200 جم. تم تقسيمهم بالتساوي إلى ثلاث مجموعات ، **المجموعة الأولى:** تم تقسيمها بالتساوي إلى:

المجموعة الفرعية IA: تم الاحتفاظ بالجرذان كمجموعه ضابطه سلبية

المجموعة الفرعية IB: تلقت الجرذان فيها جرعة واحدة من الباناكس جينسنغ يوميًا لمدة ثلاثة أسابيع. **المجموعة المجموعة الثانية:** تم إيواء الجرذان كما في المجموعة الأولى باستثناء فترة التعرض للإجهاد ، حيث تم وضع كل فأر في قفص اصغر حجما ,ساعتين يوميًا لمدة ثلاثة أسابيع.

المجموعة الثالثة: تم إيواء الجرذان وتعريضها للإجهاد كما في المجموعة الثانية مع تلقى جرعة واحدة من الباناكس جينسنغ يوميا لمدة ثلاثة أسابيع.

النتائج: كشف العمل الحالي أن الإجهاد المتقطع تسبب في حدوث تغييرات نسيجية ملحوظة في حصين الجرذان في شكل قرن أمون غير المنظم وانخفاض ظاهرى فى سماكة الطبقة الحبيبية في التلفيف المسنن كما أظهرت المجموعة الثالثة حماية ملحوظة من هذه التغييرات.

الخلاصة: أشارت النتائج التي تم الحصول عليها في هذه الدراسة إلى أن الباناكس جينسنغ يمكن أن يوفر تأثيرًا مُحسّنًا للتغيرات النسيجية للحصين الناجمة عن الإجهاد.