Original Article	**Protective Role of L-arginine on the Suprarenal Gland of Adult Male Albino Rats Subjected to Recurrent Acute Restraint Stress: A Histological and Immunohistochemical Study**

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ABSTRACT

Background: The adrenal glands are small flattened glands closely applied to the upper pole of each kidney. The adrenal cortex consists of three layers that vary primarily in the arrangement of the secretory cells comprising each layer. The gland adapts to various forms of acute and chronic stress. Nitric oxide (NO) is an important endogenous biological modulator, produced by various cell types in different tissues and has diverse physiological actions, including the modulations of vascular resistance, tissue perfusion, blood pressure, and cell proliferation. L-arginine is one of the most metabolically versatile amino acids. In addition to its role in the synthesis of nitric oxide, L-arginine serves as a precursor for the synthesis of polyamines, proline, glutamate, creatine, agmatine and urea.

Aim of work: To study the microscopic changes that might occur in the suprarenal gland of adult male albino rats in response to recurrent episodes of acute restraint stress and the role of L-arginine in relieving or blocking these changes.

Material and Methods: Thirty adult male Albino rats, 200 - 250 gm body weight each, were used in the present study. The rats were divided into three groups (ten rats/group). Group I: Control group: were not exposed to stress and allowed to move freely. Group II: Rats subjected to recurrent episodes of acute restraint stress: rats were exposed to immobilization stress. The fore limbs and hind limbs of rats were tied separately and then together securing them with adhesive tape thereby immobilizing them for 2 hours daily for 7 days. Group III: Rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress: rats were administered L-arginine by gastric gavage, at a dose of 300 mg/kg/day.

At the end of the experiment, the rats were anaesthetized and their adrenals were extracted and processed for light microscopic examination, immunohistochemical studies and quantitative image analysis.

Results: Exposure of adult male rats to recurrent episodes of acute restraint stress resulted in diffuse vacuolar degeneration within the three zones of suprarenal cortex, with distortion of cellular cords, widened intercellular spaces and localized hemorrhage in suprarenal medulla. Acute restraint stress also caused statistically highly significant increase in the distribution of iNOS in both cortex and medulla within nerve cells and nerve fibers compared to the control group.

On the other hand, concomitant treatment with L-arginine caused an apparent improved cellular architecture with marked decrease in the extent of vacuolar degeneration within all the three zones of suprarenal cortex, but areas of hyperemia within the cortex and congested blood capillaries in the medulla could be observed. Moreover, treatment with L-arginine resulted in statistically highly significant decrease in the distribution of iNOS within suprarenal gland with shift of activity of iNOS from suprarenal medulla to suprarenal cortex compared to the stress group. Still, there were, statistically, highly significant increase in the distribution of iNOS in suprarenal cortex within nerve cells and nerve fibers compared to the control group.

Conclusion: The present study revealed that recurrent episodes of acute restraint stress caused microscopic degenerative changes in the suprarenal glands of adult male albino rats with decreased NOS activity within the suprarenal medulla. L-arginine improved such degenerative changes with increased NOS activity within the suprarenal medulla. However, a further study will be planned to correlate the dose and duration of L-arginine administration on the quality of such improvement.

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INTRODUCTION:

Stress is an aversive stimulus, which perturbs the physiological homeostasis, and its impact is reflected on a variety of biological systems. Complex mechanisms contribute to the adaptational processes resulting in various visceral, behavioral and endocrinological changes. It has been postulated that stress is involved in the pathogenesis of variety of diseases like depression and anxiety, immunosuppression, endocrine disorders, male impotency and cognitive dysfunction (Kulkarni and Juvekar, 2008). The response of an organism to stress is characterized by the activation of autonomic and neuroendocrine system responses. Stressful stimuli, whether physical, metabolic, endotoxic, or psychological, activate the hypothalamic–pituitary–adrenal (HPA) axis (Paca ´k and Palkovits 2001).

The adrenal gland adapts to various forms of acute and chronic stress *(Pignatelli et al. 1998)* by producing several molecules, such as cytokines, nitric oxide (NO), and prostaglandins (PGs) (John and Buckingham 2003; Ehrhart-Bornstein and Bornstein 2008). In the HPA axis , adrenocorticotropic hormone (ACTH), which is secreted from the anterior lobe of pituitary through corticotropin-releasing hormone (CRH), acts on the zona fasciculata of the adrenal cortex to produce the ‘‘anti-stress hormones’’, glucocorticoids, from intracellular cholesterol in the adrenal gland *(Hayashia et al., 2014)*. In addition to producing corticosteroids, stress induces free radical formation and oxidative tissue damages *(Bitgul et al., 2013)*.

In mammals, including humans, nitric oxide is an important cellular signaling molecule involved in many physiological and pathological processes *(Hou, et al., 1999)*. Nitric oxide is known as an endothelium-derived relaxing factor (EDRF). The endothelium of blood vessels uses nitric oxide to signal the surrounding smooth muscles to relax, thus resulting in vasodilation and increasing blood flow. Nitric oxide is highly reactive (having a lifetime of a few seconds), yet diffuses freely across membranes (Stryer, 1995).

Nitric oxide is formed from L-arginine through the action of numerous nitric oxide synthase (NOS) enzymes *(Kleinert et al. 2003)*. Similar to PGs, it can modulate the release of stress hormones such as CRH, vasopressin, ACTH, and corticosterone *(Bugajski et al. 2004; Rettori et al. 2009)*. NOS activity is increased during stress and infection *(Gadek-Michalska et al. 2005; Monau et al. 2009)*. It can also regulate cortical and medullary adrenal gland functions such as the secretion of aldosterone *(Sainz et al. 2004)* and corticosterone *(Cymeryng et al., 1998)*. Moreover, nitric oxide is involved in adrenaline secretion from the adrenal gland. All forms of stress, including restraint stress (RS) induce the production of cytokines and cause upregulation of cyclooxygenase and nitric oxide synthase (NOS) enzymes *(Mohn et al., 2011)*.

L-arginine, the physiological substrate of nitric oxide, has been demonstrated to improve peripheral circulation (Fossel, 2004), renal function (Klahr, 1999), and immune function *(Park et al, 1991)*. It also possesses antistress and adaptogenic capabilities *(Gupta et al, 2005)*. l-Arginine stimulates the release of growth hormone *(Collier et al., 2005)* as well as the release of pancreatic insulin, glucagon and pituitary prolactin (Boger and Bode-Boger, 2001). The antioxidant property of l-arginine has been well documented in several reports *(Boger et al., 1995; Lubec et al., 1997)*.

Several human and experimental animal studies have indicated that exogenous L-arginine intake has multiple beneficial pharmacological effects when taken in doses larger than normal dietary consumption. Such effects include reduction in the risk of vascular and heart diseases, reduction in erectile dysfunction, improvement in immune response and inhibition of gastric hyperacidity. The extracellular supply of L-arginine is essential

for proper endothelial nitric oxide synthase (eNOS) activity (Gad, 2009).

Thus, it became the aim of the present work to study the microscopic changes that might occur in the suprarenal gland of adult male albino rats in response to recurrent episodes of acute restraint stress and the role of L-arginine in relieving or blocking of these changes.

MATERIAL AND METHODS:

Thirty adult male Albino rats, aging 3-6 months and weighing 200 - 250 gm each, were used in the present study. The rats were divided into three groups (ten rats/group). Male rats were chosen to avoid sex differences. Animals were purchased from Research Unit and Bilharzial Research Center of Faculty of Medicine, Ain Shams University. Rats were maintained under routine conditions with free access to food and water, 12-hour light and 12-hour darkness. All experiments were carried out in accordance with the guide of the Committee of the Animal Research Ethics (CARE) - Faculty of Medicine-Ain Shams University.

Group I: Control group: rats were not exposed to stress and allowed to move freely (Bitgul, 2013).

Group II: Rats subjected to recurrent episodes of acute restraint stress: rats were exposed to immobilization stress. The fore limbs and hind limbs of rats were tied separately for 1 hour and then together for another hour securing them with adhesive tape thereby immobilizing them for 2 hours daily (from 8-10 every morning) for 7 days (Kulkarni & Juvekar, 2008).

Group III: Rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress: rats were administered L-arginine by gastric gavage, at a dose of 300 mg/kg/day *(Maia et al., 2013)*. L-arginine was purchased as powder from Eva pharma Egypt and dissolved in saline *(Wang et al., 2016)*.

At the end of the experiment, the rats were anaesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and fixed by perfusion through the heart with a mixture of 2.5% glutaraldehyde and 1% formaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) according to *Gundersen et al. (2001) and Cardoso et al. (2010)*. The rats were sacrificed applying the protocol of the animal care and use committee of Ain Shams University.

Light microscopy:

Rat's abdomen were opened through a midline incision. Suprarenal glands were dissected and then fixed in 10% neutral formalin for one week, dehydrated in ascending grades of ethanol and cleared in xylol. Paraffin blocks were prepared; serial 5µm sections were cut and stained with Hematoxylin and Eosin (Hx& E) (Bancroft & Gamble, 2002).

Immunohistochemical study iNOS:

The NOS immunohistochemistry was carried out, sections were rinsed in PBS containing 0.1% Triton X-100 and incubated with antiserum raised in rabbit against purified soluble NOS extracted from rat cerebellum 4~ at a dilution of 1:2500, at room temperature for 18 hours. Then they were washed in PBS (3 x 5 rain) and incubated with biotinylated donkey anti-rabbit antibody at a dilution of 1:250 for 1 hr. After washing with PBS (3 x 5 rain), sections were visualized by incubation with streptavidin fluorescein at a dilution of 1:100 for 1 hr, washed in PBS and mounted with citifluor mountant. *(Afework et al., 1996)*.

Quantitative Image Analysis:

The measurements were done by using the image analyzer (Image J program) in the Anatomy Department, Faculty of Medicine, Ain Shams University. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Each field was enclosed inside the standard measuring frame, and then the positively reacting iNOS immune-stained areas were masked by a blue binary color to be measured. The density (area percentage) of the immune-staining was measured in five fields from five serial sections from five animals from each group in accordance with *Gao et al. (2006)*.

Statistical analysis:

Analysis of variance (ANOVA) and Bonferroni post hoc t-test were used to compare the positive iNOS immune-stained area percentage/HPF in the three groups. The results were calculated as Mean + SD. *P-value* was calculated using the SPSS program. The significance of the data was determined by *P-value* ($P<0.05$ or equal to 0.05 was considered significant and $P<0.001$ or equal to 0.001 was considered highly significant) (Sawilowsky, 2005). *P-value* was corrected according to Bonferroni procedure by dividing the α value by the number of the compared groups (m) (α / m) so the significant *P-value* became

(P≤0.016666667) and highly significant *P-value* became *(P≤0.000333333)* (Frane, 2015).

RESULTS:

A] Hematoxylin & Eosin-stained sections results:

Group I (Control group):

Examination of Hx and E stained sections of the suprarenal gland of adult male control rat revealed that the parenchyma of suprarenal gland was formed of 2 main parts, the cortex and the medulla enclosed within a fibrous connective tissue capsule (Fig. 1). The adrenal cortex exhibited three distinct zones from outside inwards: zona glomerulosa just beneath the fibrous capsule, zona fasciculata and zona reticularis (Figs. 2 & 3). The zona glomerulosa appeared as clusters of columnar cells just beneath the capsule (Fig. 4). Cords of spongiocytes within zona fasciculata were observed. The cells exhibited partially vacuolated cytoplasm and rounded euchromatic nuclei. Some cells were binucleated (Fig. 4). In addition, anastomosing cords of cells in zona reticularis were detected (Fig. 4). Branching and anastomosing cords of polyhedral cells within the suprarenal medulla were arranged around the blood capillaries. Few cells were clumped together (Fig. 5).

Group II (Rats subjected to recurrent episodes of acute restraint stress):

Examination of suprarenal glands in rats subjected to recurrent episodes of acute restraint stress showed wide spread vacuolation (vacuolar degeneration) and distortion of cellular arrangement with pyknotic nuclei along the three zones within the cortex (Figs. 6 & 7). Widened intercellular spaces were seen. Cell boundaries were either partially or completely lost and numerous cells were amalgamated together. Detachment of the fibrous capsule was observed (Fig. 7). Localized hemorrhage in suprarenal medulla was also noticed (Fig. 6).

Group III (Rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress):

In suprarenal glands of rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress, an apparent improved cellular architecture with marked decrease in the extent of vacuolar degeneration within all the three zones of suprarenal cortex was seen (Figs. 8&9). Occasional foci of hyperemia within the cortex were observed (Figs. 8 & 9). Congested blood capillaries within the suprarenal medulla were also detected (Figs. 8 & 10).

B] Immunohistochemical results:

Immunohistochemical staining of iNOS was performed to detect the distribution of iNOS in the suprarenal gland among the three groups. The immuno-positive reaction appeared as dark brown dots or star-shaped granules. The control group showed mildly positive brown staining with uniform distribution of iNOS activity within cortex and medulla in both intrinsic nerve cells and nerve fibers (Figs. 11 & 12). In suprarenal glands of rats subjected to recurrent episodes of acute restraint stress, the brownish coloration was apparently increased compared to the control, indicating increased activity of iNOS within both suprarenal cortex and medulla (Fig. 13) in both nerve cells and nerve fibers (Fig. 14). While, in suprarenal glands of rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress, the overall distribution of iNOS was apparently decreased compared to the stressed group. However, an apparently increased distribution of iNOS in both nerve cells and nerve fibers within suprarenal cortex and decreased iNOS within the suprarenal medulla was observed compared to the control group. Moreover, there was an apparent shift of activity of iNOS from suprarenal medulla to suprarenal cortex (Figs. 15 & 16).

C] Quantitative image analysis and statistical results:

Quantitative image analysis and ANOVA statistical analysis revealed that there was a highly significant difference in positive iNOS immunostaining area percentage/HPF *(P<0.001)* among the three groups (Table 1; Bar chart 1).

Bonferroni corrected post hoc test *(t-test)* for ANOVA revealed statistically highly significant increase in the mean of area fraction % (positive iNOS immunostaining area percentage / HPF) among group II (Rats subjected to recurrent episodes of acute restraint stress) *(P<0.000333)* compared to the control group (Table II). Also, there was a statistically highly significant increase in the mean of area fraction % (positive iNOS immunostaining area percentage / HPF) among group III (Rats treated with L-arginine and subjected to recurrent episodes of acute restraint

stress) *(P<0.000333)* compared to the control group (Table III).

On the other hand, there was statistically highly significant decrease in the mean of area fraction (positive iNOS immunostaining area percentage / HPF) among group III (Rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress) *(P<0.000333)* compared to group II (Rats subjected to recurrent episodes of acute restraint stress) (Table IV).

Overall results:

Exposure of adult male rats to recurrent episodes of acute restraint stress resulted in diffuse vacuolar degeneration within the three zones of suprarenal cortex, with distortion of cellular cords, widened intercellular spaces and localized hemorrhage in suprarenal medulla. Acute restraint stress also caused statistically highly significant increase in the distribution of iNOS in both cortex and medulla within nerve cells and nerve fibers compared to the control group.

On the other hand, concomitant treatment with L-arginine caused an apparent improved cellular architecture with marked decrease in the extent of vacuolar degeneration within all the three zones of suprarenal cortex, but areas of hyperemia within the cortex and congested blood capillaries in the medulla could be observed. Moreover, treatment with L-arginine resulted in statistically highly significant decrease in the distribution of iNOS within suprarenal gland with shift of activity of iNOS from suprarenal medulla to suprarenal cortex compared to the stress group. Still, there were, statistically, highly significant increase in the distribution of iNOS in suprarenal cortex within nerve cells and nerve fibers compared to the control group.

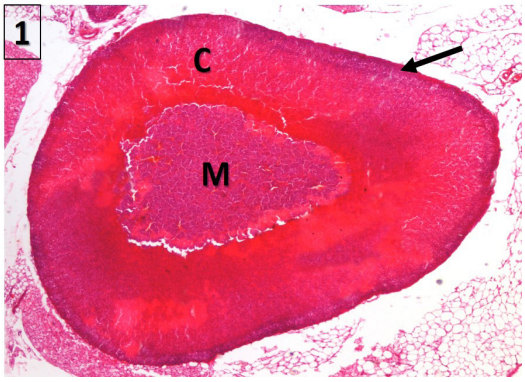


Fig.1: Photomicrograph of the suprarenal gland of a control adult male albino rat showing the suprarenal cortex (C) and suprarenal medulla (M) enclosed within a capsule (arrow).Hx & E x 40

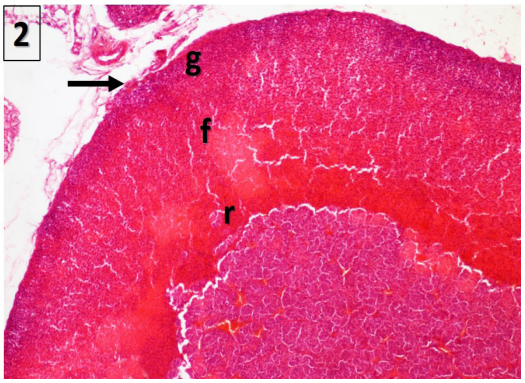


Fig.2: Photomicrograph of the suprarenal gland of a control adult male albino rat showing the three zones of suprarenal cortex, zona glomerulosa (g) just beneath the capsule (arrow), zona fasciculata (f) and zona reticularis (r)Hx & E x 100

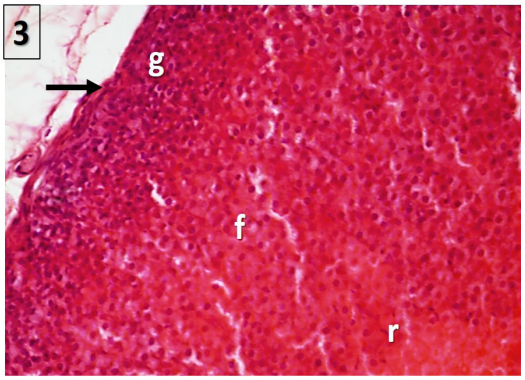


Fig.3: Photomicrograph of the suprarenal gland of a control adult male albino rat showing clusters of cells within zona glomerulosa (g) just beneath the capsule (arrow), cords of cells within zona fasciculata (f) and anastomosing cords of cells in zona reticularis (r). Hx & E x 400

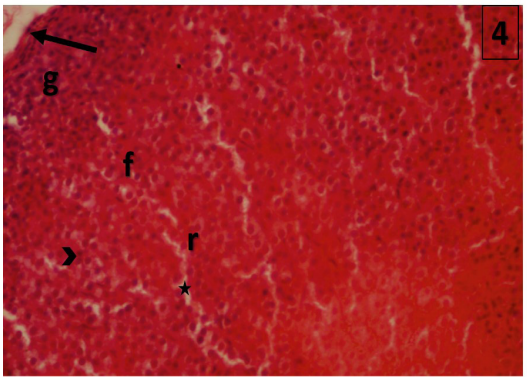


Fig.4: Photomicrograph of the suprarenal gland of a control adult male albino rat showing clusters of columnar cells within zona glomerulosa (g) just beneath the capsule (arrow), cords of spongiocytes within zona fasciculata (f) and anastomosing cords of cells in zona reticularis (r). Note the partial vacuolation (star) and the binucleated cells (arrow head). Hx & E x 400

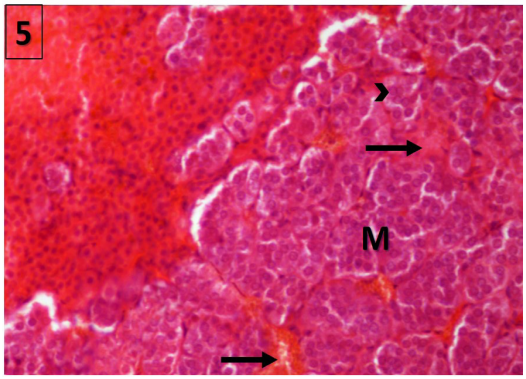


Fig.5: Photomicrograph of the suprarenal gland of a control adult male albino rat showing branching and anastomosing cords of polyhedral cells within the suprarenal medulla (M) arranged around the blood capillaries (arrows). Note the cellular clumps (arrow head). Hx & E x 400

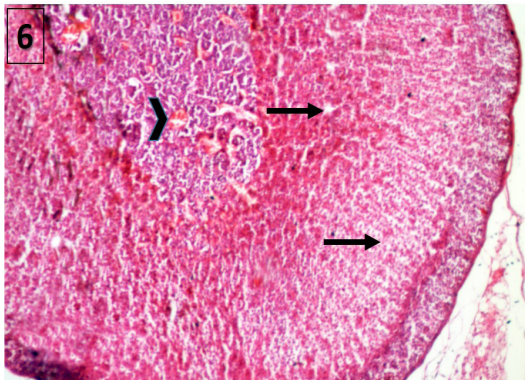


Fig.6: Photomicrograph of the suprarenal gland of an adult male albino rat subjected to recurrent acute restraint stress, showing wide spread vacuolation within the cortex (arrows) and localized hemorrhage in the medulla (arrow head). Hx & E x 100

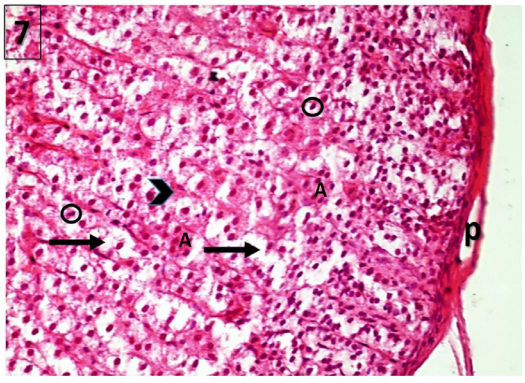


Fig.7: Photomicrograph of the suprarenal gland of an adult male albino rat subjected to recurrent acute restraint stress, showing wide spread vacuolation within the cortex (arrows), widened intercellular space (arrow head) and detachment of the fibrous capsule (p). Note the pyknotic nuclei (circles) and amalgamated cells (A). Hx & E x 400

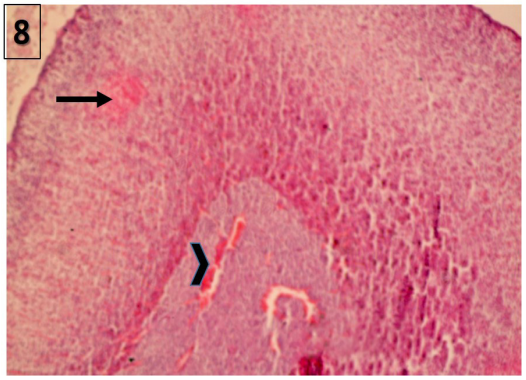


Fig.8: Photomicrograph of the suprarenal gland of an adult male albino rat treated with L-arginine and subjected to recurrent acute restraint stress, showing an apparent improved cellular architecture. Note the occasional foci of hyperemia within the cortex (arrow) and congested capillaries within the medulla (arrow head). Hx & E x 100

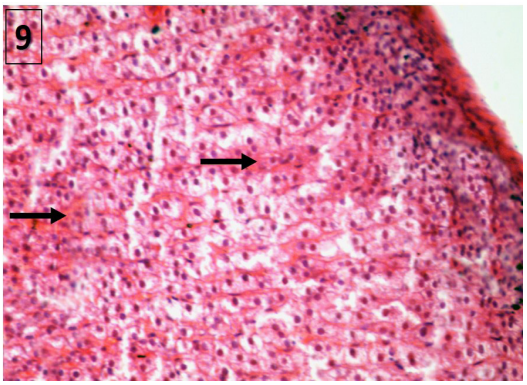


Fig.9: Photomicrograph of the suprarenal gland of an adult male albino rat treated with L-arginine and subjected to recurrent acute restraint stress, showing increased hyperemia within the suprarenal cortex (arrows). Hx & E x 400

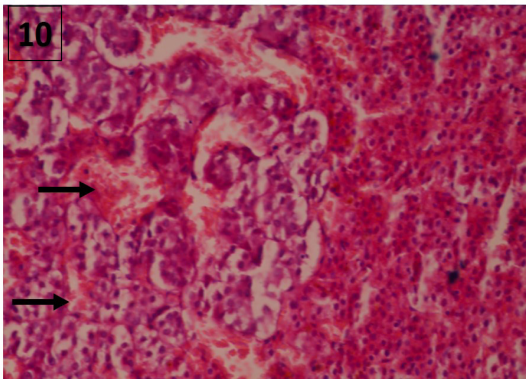


Fig.10: Photomicrograph of the suprarenal gland of an adult male albino rat treated with L-arginine and subjected to recurrent acute restraint stress, showing congested blood capillary within the suprarenal medulla (arrows). Hx & E x 400

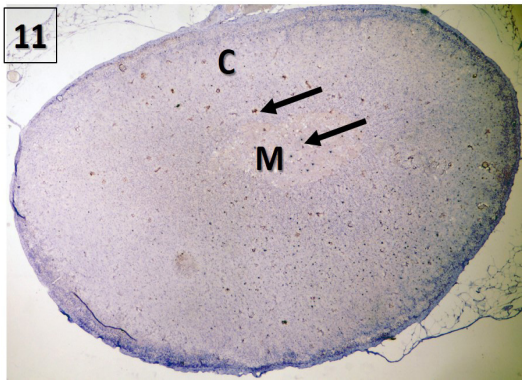


Fig.11: Photomicrograph of the suprarenal gland of a control adult male albino rat showing the distribution of iNOS within suprarenal cortex (C) and suprarenal medulla (M) apparent as dark brown dots (arrows). iNOS immuno staining x 40

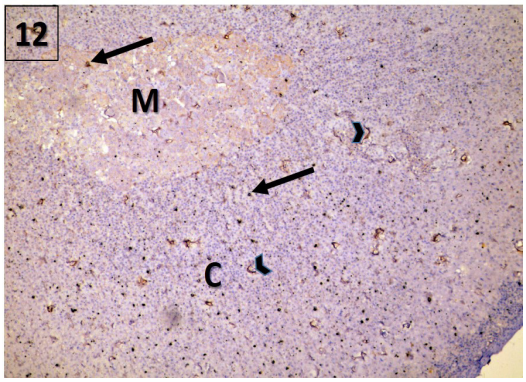


Fig.12: Photomicrograph of the suprarenal gland of a control adult male albino rat showing the distribution of iNOS within suprarenal cortex (C) and suprarenal medulla (M) in nerve cells (arrows) and nerve fibers (arrow heads). iNOS immuno staining x 100

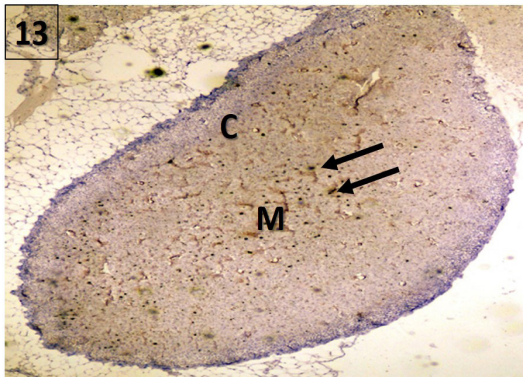


Fig.13: Photomicrograph of the suprarenal gland of adult male albino rat subjected to recurrent acute restraint stress showing an apparently increased distribution of iNOS within suprarenal cortex (C) and suprarenal medulla (M) apparent as dark brown dots (arrows). iNOS immuno staining x 40

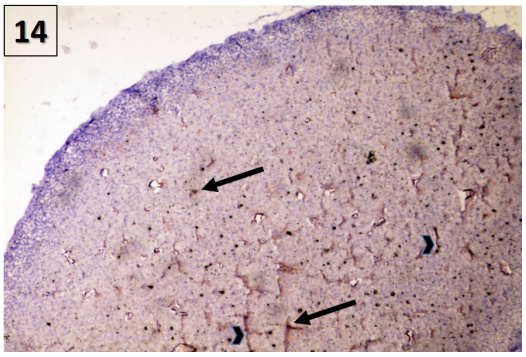


Fig.14: Photomicrograph of the suprarenal gland of adult male albino rat subjected to recurrent acute restraint stress showing an apparently increased distribution of iNOS within nerve cells (arrows) and nerve fibers (arrow heads). iNOS immuno staining x 100

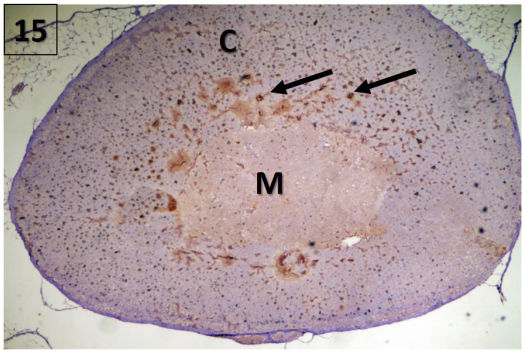


Fig.15: Photomicrograph of the suprarenal gland of adult male albino rat treated with L-arginine and subjected to recurrent acute restraint stress showing an apparently increased distribution of iNOS within suprarenal cortex (C) and decreased iNOS within the suprarenal medulla (M) in the form

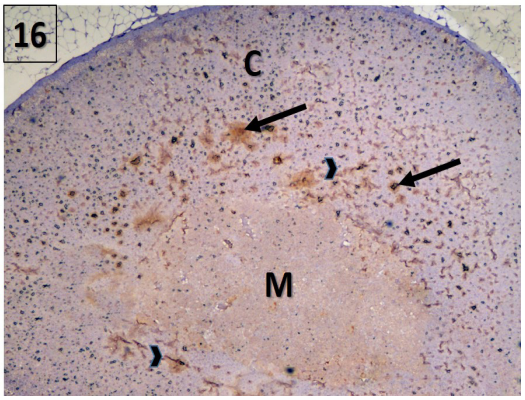
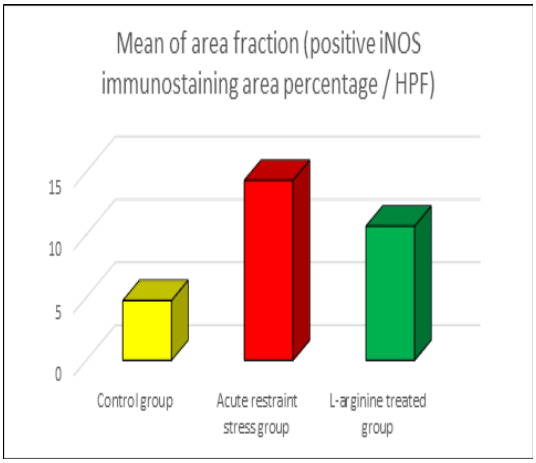


Fig.16: Photomicrograph of the suprarenal gland of adult male albino rat treated with L-arginine and subjected to recurrent acute restraint stress showing an apparently increased distribution of iNOS in suprarenal cortex (C) within both nerve cells (arrows) and nerve fibers (arrow heads) with an apparent decrease in iNOS within the suprarenal medulla (M). iNOS immuno staining x 100

Table I: Means of area fraction (positive iNOS immunostaining area percentage / HPF) in the three groups (ANOVA single factor)

Groups	Count	Sum	Average	Standard deviation	Variance		
Control group	6	28.4964	4.7494	0.2637	0.055643		
Acute restraint stress group	6	85.8984	14.3164	0.5924	0.280785		
L-arginine treated group	6	64.0128	10.6688	0.4621	0.170822		
Source of Variation	SS	df	MS		F**	P-value*	F crit**
Between Groups	279.743542	2	139.8718		827.234	4.48E-16	3.6823
Within Groups	2.5362552	15	0.169084				
Total	282.2797974	17					

*P-value among all groups **F value was greatly higher than F critical value



Bar chart 1: Means of area fraction (%) (Positive iNOS immunostaining area percentage / HPF)

Table II: Means of area fraction (positive iNOS immunostaining area percentage / HPF) in the control & acute restraint stress group (Bonferroni post hoc test)

	Control group	Acute restraint stress group
Mean	4.7494	14.3164
Variance	0.0695543	0.3509818
Observations	5	5
Pooled Variance	0.21026805	
Hypothesized Mean Difference	0	
df	8	
t Stat	-32.98823918	
P(T<=t) one-tail	3.88922E-10	
t Critical one-tail	1.859548038	
P(T<=t) two-tail*	7.77844E-10	
t Critical two-tail	2.306004135	

**P-value< 0.000333* (i.e. Highly significant)

Table III: Means of area fraction (positive iNOS immunostaining area percentage / HPF) in the control & L-arginine treated group (Bonferroni post hoc test)

	Control group	L-arginine treated group
Mean	4.7494	10.6688
Variance	0.0695543	0.2135277
Observations	5	5
Pooled Variance	0.141541	
Hypothesized Mean Difference	0	
df	8	
t Stat	-24.87749013	
P(T<=t) one-tail	3.64463E-09	
t Critical one-tail	1.859548038	
P(T<=t) two-tail*	7.28925E-09	
t Critical two-tail	2.306004135	

**P-value< 0.000333* (i.e. Highly significant)

Table IV: Means of area fraction (positive iNOS immunostaining area percentage / HPF) in acute restraint stress group & L-arginine treated group (Bonferroni post hoc test)

	Acute restraint stress group	L-arginine treated group
Mean	14.3164	10.6688
Variance	0.3509818	0.2135277
Observations	5	5
Pooled Variance	0.28225475	
Hypothesized Mean Difference	0	
df	8	
t Stat	10.85566872	
P(T<=t) one-tail	2.29187E-06	
t Critical one-tail	1.859548038	
P(T<=t) two-tail*	4.58374E-06	
t Critical two-tail	2.306004135	

**P-value< 0.000333* (i.e. Highly significant)

DISCUSSION:

The present study aimed to clarify the microscopic changes that might occur in the suprarenal gland of adult male albino rats in response to recurrent episodes of acute restraint stress and the role of L-arginine in relieving or blocking these changes.

The adrenal gland is a complex, polyfunctional organ whose secretions are required for the maintenance of life. These glands are commonly susceptible to toxins and stress factors *(Soliman et al., 2015)*. The cells of the adrenal cortex (adrenocorticocytes) secrete more than 30 different hormones called corticosteroids; these hormones include mineralocorticoids (aldosterone) and glucocorticoids (corticosterone), which play roles in homeostasis and stress response *(Randall et al., 2002)*. Adrenomedullary chromaffin cells secrete catecholamines, epinephrine and norepinephrine. In addition, numerous transmitters, neuropeptides, and proteins, are released together with the catecholamines (Ehrhart-Bornstein and Bornstein, 2008). The two endocrine tissues interact, thus, chromaffin cells regulate steroid-hormone release by the adrenal cortex, and steroids induce catecholamine production in the medulla *(Merke et al., 2005)*.

On the other hand, prolonged psychological stress potentially exerts harmful influence on various internal organs. Appropriate biomechanical responses to stress must be driven in order to maintain an internal homeostasis of the organs. The hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic–adrenomedullary (SA) axis are the common systems that are responsible for playing these roles during a stress state, the adrenal gland being an essential organ common to both systems, with hypersecretion of adrenal hormones, particularly glucocorticoids from the adrenal cortex and epinephrine from the adrenal medulla *(Hayashia et al., 2014)*

The effects of stress that have formed on people are examined on animals by applying a variety of methods. The animal model of stress used in the present study, was the acute restraint stress model which was adopted by Kulkarni & Juvekar, 2008. Immobilization (IMO) stress method causes both psychological and physiological stress by the restriction of movement, aggression, feeling of distress, and burnout. Consequently, the immobilization stress model is considered as an easy and convenient method *(Yazawa et al., 1999)*. Immobilization is a severe stressor that triggers both physiological and behavioral responses. An inflammatory process is developed by imbalanced release of pro- and anti-inflammatory cytokines in response to IMO *(Lee et al., 2016)*.

Additionally, the immobilization stress has been proved to increase the reactive oxygen species that caused damage in cells which, in turn, might inhibit mitochondrial energy metabolization *(Gameiro et al., 2006)*. Oxidative stress may also induce many damaging processes in stress disorders such as disruption of energy pathways, mitochondrial dysfunction and dysregulation of calcium homeostasis *(Sevgi et al., 2006)*. Accumulating evidence suggests that a single exposure of stress leads to a significant increase in circulating cortisol level and nuclear intensity and activity *(Lee et al., 2014)* as a result of HPA axis activation *(Garcia et al., 2000)*. It has been reported, however, that chronic exposure to stress induces hypocorticism *(Adzic et al., 2009)*.

In the present work, recurrent episodes of acute restraint stress caused wide spread vacuolation (vacuolar degeneration) and distortion of cellular arrangement with pyknotic nuclei and widened intercellular spaces along the three zones within the cortex. Similar changes were detected by *El-Shenawany et al. (2007)* in their study on N-butyl benzene sulphonamide-induced toxicity in adrenal cortex of albino rats. Physiologic vacuolation of the adrenal cortex is normally present in a diffuse pattern and may be an indirect response of some stressors upon the animal. In the normal rat, the zona fasciculata contains the most prominent vacuoles; which consist of small or large clear round intracytoplasmic structures of neutral lipids and cholesterol *(Frith et al., 2000)*. Lipid droplets in adrenocorticocytes usually exhibit a uniform appearance. The staining of these lipid droplets depends on the degree of free fatty acid saturation, which reflects the impairment of steroidogenesis. Enlargement of these droplets with its subsequent dissolution, is caused by accumulation of cholesterol needed for steroidogenesis *(Cole et al, 2000*; Gadek-Michalska and Bugajski, 2004).

In addition, the present work revealed that the cell boundaries were either partially or completely

lost and numerous cells were amalgamated together with detachment of the fibrous capsule. The loss of cell boundaries might affect the integrity of the cell membranes, which are essential for steroidogenesis *(Soliman et al., 2015). Freedman et al. (2013)* revealed that adrenal maintenance involves cellular conversion from zona glomerulosa into zona fasciculata cells. The adrenal capsule is a signaling center controlling cell renewal and zonation through the capsular enzymes that induce ß- catenin signaling and imprint glomerulosa cell fate *(Vidal et al., 2016)*.

Areas of localized hemorrhage in suprarenal medulla were also observed in the present study. *El-Desouki et al. (2014)* observed that skeletal muscle cells of immobilization stressed rats showed large areas of leucocytic infiltration and widely dilated and engorged blood vessels. Hemorrhage was the most frequent adrenal gland pathology observed in fatal bacterial infections. Adrenal hemorrhage usually occurs in life-threatening conditions where there are low cortisol levels and consequently increased adrenocorticotropic hormone *(Gaurner et al., 2008)*.

Immunohistochemical study in the current work showed that the brownish coloration was apparently increased compared to the control, indicating increased activity of iNOS within both suprarenal cortex and medulla in both nerve cells and nerve fibers. Nitric oxide (NO) was proved to have an important role in the regulation of adrenal blood flow and adrenal corticomedullary functions during normoxaemia and hypoxaemia functions in the late gestation llama fetus. It is an important regulator of fetal plasma cortisol and catecholamine concentrations during acute hypoxaemia. *(Riquelme et al, 2002)*. NO could also regulate cortical and medullary adrenal gland functions such as the secretion of aldosterone *(Sainz et al. 2004)* and corticosterone *(Cymeryng et al. 1998).*

Furthermore, statistical analysis in the present study revealed a highly significant increase in positive iNOS immunostaining area percentage/ HPF *(P<0.001)* in suprarenal glands of rats subjected to recurrent episodes of acute restraint stress as compared to the control group reflecting increased NOS activity. The increase of both NO & PGs caused by stress in many tissues has been described before by *Gadek-Michalska et al. (2005) and Grion et al. (2007)*. PGs and NO are important signal transducers involved in neurotransmitter and neurohormone secretion during basal and stress conditions *(Bugajski et al., 2004; Rettori et al., 2009)*.

Nitric oxide is regarded as a major modulator of a variety of physiological reactions (Stern 2004). It is formed from L-arginine through the action of nitric oxide synthase (NOS). NO has several isoforms including, calcium dependent neuronal NOS, endothelial NOS, and calcium-independent inducible NOS (iNOS) *(Kleinert et al. 2003)*. Each isoform has a specific distribution in the body including the adrenal gland *(Kishimoto et al. 1996; Cymeryng et al. 2002; Lai et al. 2005)*. NOS activity had been reported to increase during stress and infection *(Gadek-Michalska et al. 2005; Monau et al. 2009)*. Moreover, nitric oxide (NO), as a free radical gas, is a well known intracellular messenger in a wide range of physiological processes such as neurotransmission, vasodilation, and immune response (Bohlen, 2015). It also behaves as a regulator of cell death and survival. It can turn on or shut off apoptotic pathways depending largely on its concentration and exposure time *(Leong et al., 2002)*.

Though, its physiological function is not yet fully understood. However, current interest in L-arginine is focused mainly on its close relationship with the important signal molecule nitric oxide. L-Arginine is the only substrate in the biosynthesis of NO, which plays critical roles in diverse physiological processes in the human body (Gad, 2009).

L-arginine, the physiological substrate for nitric oxide (NO) synthesis, improves endothelium-dependent vasodilation in hypercholesterolemic humans and, in animal models, has anti-atherogenic actions, reducing oxidative stress, platelet aggregation, monocyte adhesion and the formation of intimal lesions. Decreased platelet aggregation has also been observed in humans. Such actions suggest a potential therapeutic role for L-arginine *(Walker et al., 2001)*. These attributes are similar to the modern concept of adaptogenic agents, which are known to afford protection of the human physiological system against diverse stressors (Juvekar & Nachankar, 2005).

The current study revealed that, treatment with L-arginine caused evident improvement in the cellular architecture of suprarenal glands and marked decrease in the extent of vacuolar

degeneration within all the three zones of suprarenal cortex of adult male albino rats, despite their exposure to recurrent episodes of acute restraint stress. Although ACTH has been largely recognized as the primary regulator of adrenal cortex development and function, locally produced factors may synergize or antagonize its biological effects. Several lines of evidence support that NO among the regulators of adrenal function. The endogenous production of NO could depend on extracellular L-arginine levels, and therefore, the activity of the L-arginine transport system could modulate NO production in adrenal cells *(Repetto et al., 2006).)*. In addition, the oral bioavailability of L-arginine was about 70% of the administered dose which proposed the dose dependent effect of L-arginine. The maximum plasma concentration after oral ingestion was considerably lower than intravenous infusion due to the delay of absorption from the intestinal tract *(Bode-Boger et al., 1998)*.

However, occasional foci of hyperemia within the cortex and congested blood capillaries within the suprarenal medulla were observed in the current work. Hyperemia of the adrenal cortex has been described in response to administration of ACTH or following exposure to stress *(Silva et al., 2004)*. van Duijnie (2012) observed adrenalitis, congestion, hemorrhage, necrosis, apoptosis, hypertrophy, edema, cysts, extracellular eosinophilic material and vacuolar degeneration in postmortem examination of the adrenal glands of harbor porpoises, in an attempt to correlate to stress exposure.

Furthermore, the present study elucidated a statistically highly significant increase in the mean of area fraction (positive iNOS immunostaining area percentage / HPF) among rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress compared to the control group. Whereas, there was statistically highly significant decrease in the mean of area fraction (positive iNOS immunostaining area percentage / HPF) among rats treated with L-arginine and subjected to recurrent episodes of acute restraint stress compared to stressed rats which were not treated with L-arginine (group II).

The adrenal gland is involved in the complex set of reactions evoked by tissue trauma, chemical irritants or infection. Corticosteroids produced in the zona fasiculata act as a major feedback control preventing the immune system from overreacting or causing autoimmunity and in this sense they are essential for survival in critical conditions. The existence of modulatory mechanisms controlling hormone production, such as the NOS system, is very important as they enable the gland to provide appropriate response in stressful situations *(Grion et al., 2007)*. However, *Wang et al. (2015)* proved that the overproduction of nitric oxide contributes to mitochondrial oxidative stress in adrenocortical cells and subsequently leads to adrenal insufficiency.

In conclusion, the present study revealed that recurrent episodes of acute restraint stress caused microscopic diffuse degenerative changes in the suprarenal glands of adult male albino rats with increased overall NOS activity within the suprarenal gland. Whereas, L-arginine improved such degenerative changes with decreased overall NOS activity and shift of activity of iNOS from suprarenal medulla to suprarenal cortex. However, a further study will be planned to correlate the dose and duration of L-arginine administration on the quality of such improvement.

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الدور الوقائي لعقار L-arginine على الغدة الكظرية للجرذان البيضاء البالغة المعرضة للتوتر القيدي الحاد المتكرر: دراسة نسيجية وكيميائية نسيجية مناعية

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ملخص البحث

المقدمة: الغدد الكظرية هي غدد صغيرة تطبق بشكل وثيق إلى القطب العلوي من كل كلية. قشرة الغدة الكظرية تتألف من ثلاث طبقات التي تختلف في المقام الأول في ترتيب الخلايا الإفرازيةالتى تضم كل طبقة. الغدة تتكيف مع أشكال مختلفة من التوتر الحاد والمزمن. أكسيد النيتريك (NO) هو المغير البيولوجي الذاتى، الذي تنتجه مختلف أنواع الخلايا في الأنسجة المختلفة، وله إجراءات فسيولوجية مختلفة، بما في ذلك التحويرات من مقاومة الأوعية الدموية، نضح الأنسجة، وضغط الدم، وتكاثر الخلايا. يعتبر ال L-arginine واحد من الأحماض الأمينية الأكثر تنوعا في عملية الأيض، بالإضافة إلى دوره في تخليق أكسيد النتريك، يخدم L-arginine تمهيدا لتركيب الأمينات، البرولين، الجلوتامات، الكرياتين، أجماتين واليوريا.

الهدف من البحث: لدراسة التغيرات المجهرية التي قد تحدث في الغدة الكظرية في ذكور الجرذان البيضاء البالغين في الاستجابة لنوبات متكررة من التوتر الحاد ودور L-arginine في تخفيف أو منع هذه التغييرات.

المواد والطرق المستخدمه: استخدم في هذه الدراسة ثلاثون ذكرا بالغا من الجرذان البيضاء، يزن كل منها -200 250 جرام. تم تقسيم الجرذان إلى ثلاث مجموعات (عشرة جرذان / مجموعة). المجموعه الاولى: المجموعة الضابطة لم يتعرض للإجهاد وسمح لهم بالتنقل بحرية، المجموعة الثانية: يتعرض الجرذان لنوبات متكررة من التوتر القيدى الحاد عن طريق تجميد وتعادل أطرافه الصدارة وأطرافه الخلفية على حدة مع تأمينها بشريط لاصق وبالتالي شل حركة منهم لمدة 2 ساعة يوميا لمدة 7 أيام. المجموعة الثالثة: اعطيت الجرذان L-arginine بواسطة أنبوب تغذية في المعدة، بجرعة 300 ملغ / كغ / يوم. مع التعرض لنوبات متكررة من التوتر القيدي الحاد.

في نهاية التجربة، تم تخدير الجرذان وانتزعت الغدة الكظرية وتم تجهيزها للفحص بالمجهر الضوئى والدراسة الكيميائية النسيجية المناعية وتم تحليل البيانات الكيميائية النسيجية المناعية كميا وإحصائيا.

النتائج: أظهر فحص مقاطع الهيماتوكسلين والايوسين ان تعرض الجرذان الذكور البالغين لنوبات متكررة من التوتر القيدي الحاد في تحلل فجوي منتشر في ثلاث مناطق القشرة الكظرية، مع تشويه الحبال الخلوية، اتسعت المساحات بين الخلايا مع نزيف منتشر في النخاع الكظري. تسبب التوتر القيدي الحاد أيضا الى زيادة إحصائية هامة للغاية في توزيع iNOS في كل من القشرة والنخاع داخل الخلايا العصبية والألياف العصبية مقارنة مع المجموعة الضابطة. من ناحية أخرى، العلاج المصاحب ب L - arginineتسبب في انخفاض ملحوظ في مدى الانحطاط الفجوي في جميع المناطق الثلاث من القشرة الكظرية، ولكن يمكن ملاحظة انتشار بؤر واسعة من النزيف داخل القشرة والكبسولة. أيضا، كانت هناك شعيرات دموية مزدحمه داخل النخاع. أدى العلاج بعقار L-arginine الي انخفاض في توزيع iNOS داخل الغدة الكظرية مع تحول النشاط من النخاع الكظري إلى القشرة الكظرية مقارنة مع مجموعة التوتر. ومع ذلك، كانت هناك، من الناحية الإحصائية، زيادة كبيرة للغاية في توزيع iNOS في القشرة الكظرية داخل الخلايا العصبية والألياف العصبية مقارنة مع المجموعة الضابطة.

الخاتمه: كشفت هذه الدراسة أن نوبات متكررة من التوتر القيدى الحاد تسبب تغيرات مجهرية في الغدد الكظرية لذكور الجرذان البيضاء البالغة مع انخفاض نشاط NOS داخل النخاع الكظري. ومع ال L-arginineتحسنت هذه التغيرات مع زيادة نشاط NOS داخل النخاع الكظري. ومع ذلك، سيتم التخطيط لمزيد من الدراسة لربط الجرعة ومدة اعطاء ال L-arginine على نوعية هذا التحسن.