Original Article

Role of Adrenal Progenitor Cells in the Structural Response of Adrenal Gland to Various forms of Acute Stress and Subsequent Recovery in Adult Male Albino Rats

Doaa Ramadan Sadek, Seham Kamel Abunasef, Sahar Khalil[1]

Department of Histology, Faculty of Medicine, Ain Shams University, Cairo, [1]Department of Histology and Cell Biology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Abstract

Background: Stress is a response to stressogenic stimuli that interferes with an organism's homeostasis. The adrenal gland is crucial in the body's reaction to stress. **Objective:** This study compared the effects of immobility and cold as acute stressors and the subsequent recovery on the histological changes of the adrenal gland and the suspected role of the adrenal progenitor cells. **Materials and Methods:** Thirty-five adult male albino rats were divided equally into five groups. Group I: Control group, Group II: Rats subjected to the acute cold stress procedure, Group III: Rats subjected to the acute immobilization stress procedure, Group IV: The combined stress group, and Group V: Similar to the combined stress group and recovered for 6 days then sacrificed 1 day later. Serum cortisol level was determined, and the adrenal glands were processed for histological and immunohistochemical studies. **Results:** Serum cortisol concentration was higher in the acute-stress groups and decreased in the recovery group. The adrenal cortex had enlarged, vacuolated cells with pyknotic nuclei, sinusoidal dilatation, and congestion. Chromaffin cells were crowded, enlarged, and vacuolated. There was strong immunohistochemical reactivity for heat shock protein-70 and caspase-3. In addition, the combined group showed a significant increase in the optical density of chromogranin-A in the medullary cells as well as CD44+ve cells. These findings were decreased in the recovery group. **Conclusions:** The combined stress has more deleterious adrenal cortical changes than immobilization and cold stress alone. The progenitor and chromaffin cells apparently had an important regenerative role in recovery from both types of stress.

Keywords: Acute stress, adrenal gland, CD44, chromaffin cells, heat shock protein-70

Introduction

Stress is an organism's response to a variety of stressogenic stimuli, which tend to alter the organism's homeostasis. Stress has an impact on individual health, quality of life, and species propagation at the population level.[1,2] For homeostatic balance, the integrated hypothalamic-pituitary-adrenocortical (HPA) and sympatho-adreno-medullary systems, both of which have the adrenal glands as terminal effector organs, are required.[3] However, when the physiological response to stress becomes excessive and prolonged, homeostasis is disturbed.

The cortex and medulla make up the adrenal gland's histological structure. The release of steroid hormones that regulate body homeostasis is a concern for the adrenal cortex by its three concentric layers (zona glomerulosa [ZG], zona fasciculate [ZF], and zona reticularis [ZR]). Contrarily, the adrenal medulla produces and secretes catecholamines that mediate the body's reaction to acute stress. Both the morphology and the functionality of the adrenal gland can be directly impacted by stress exposure as mentioned in a previous study.[4]

Authors reported previously, on monitoring acute and chronic stress, that stress response increases blood circulating levels

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Address for correspondence: Dr. Doaa Ramadan Sadek, Department of Histology, Faculty of Medicine, Ain Shams University, Abbassia, Cairo-11566, Egypt.
E-mail: d.sadek@med.asu.edu.eg

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of corticotrophin (adrenocorticotropic hormone [ACTH]) due to the HPA axis activation. This activation would result in subsequent activation of the cortical ZF, to produce the stress hormone; cortisol that rebalances the body's functions and performances.[5]

Chronic stress, for example, increases the risk of developing adverse health consequences, such as dermatitis, cardiovascular disease, obesity, depression, and exacerbation of neurodegeneration. Acute stress has proven to be more mysterious.[2] The pathophysiological and behavioral changes brought on by stress in humans are partially replicated in the available animal models of stress. Acute or chronic stress models can be distinguished based on how long the stressor is applied. Acute stress refers to the administration of a stressor only once and for a brief length of time (between a few minutes and several hours). On the other hand, chronic stress refers to the application of stressors repeatedly over a longer period. Acute stress can be achieved by exposing animals to immobilization, exposure to extreme temperatures; hot or cold, loud noise, and forced swimming.[6]

Adult stem cell proliferation and differentiation help to maintain the body's organs and tissues. These cells are found in several organs, where they take part in cell regeneration.[7] The cortical and medullary cells of the adrenal gland include particular adult progenitor/stem cells, responsible for the renewal of cells specific to the adrenals. The adrenocortical stem cell niche plays a crucial role in stress adaption.[8] The cortical progenitor cells are dispersed through the cortex and can be differentiated into steroidogenic cells. The medullary progenitors can differentiate into three populations: glia, chromaffin, and neuronal cells, indicating their multipotent properties.[9]

In consideration of all of this, and up to our knowledge, there is a question regarding how the adrenal gland reacts to various acute stress situations and what is the suspected role of adrenal progenitor cells in these situations. Hence, this study aimed at detecting the histological changes of the adrenal gland and highlighting the suspected role of the adrenal progenitor cells under various forms of acute stress (like cold and immobility) and subsequent recovery in the adrenal gland using histological and immunohistochemical techniques.

Materials and Methods

This experiment was done at (the Medical Ain Shams Research Institute, Faculty of Medicine, Ain Shams University) (FMASU). Rats were housed in groups at room temperature and in clean air. The rats were exposed to a 12:12-h light-dark cycle/day. They remained fed on balanced rat chow and water freely. The experiment was performed according to the guidelines of (the Ethical Committee, Faculty of Medicine, Ain Shams University) (No. FMASU R 149/2022) Approved date 16/10/2022.

Induction of stress procedure

The acute cold stress procedure

Rats were exposed to cold stress (CS) by keeping them at 4°C for 4 h in a refrigerated compartment in normal stainless cages (20 cm × 35 cm × 60 cm). CS was performed only once.[10]

The acute immobilization stress procedure

Rats were exposed to acute immobilization stress (AIS) only once for 4 h. The animals were individually placed in wire stainless steel mesh restrainers (5 cm × 7 cm × 12 cm in dimensions) without food or water, as described by Soliman.[11] This procedure restricted the movement of the animals effectively. The control group was housed in normal stainless-steel cages (20 cm × 35 cm × 60 cm).[12]

Animals and grouping

Thirty-five adult male albino rats (10–12 weeks old, weighing 200–260 g) were allocated randomly into five groups (7 rats each).

Group I (control group): Normal control.

Group II (acute cold stress [ACS]): Rats exposed to the ACS procedure.

Group III (AIS): Rats subjected to the AIS procedure.

Group IV (combined stress group): Rats were put in wire stainless steel mesh restrainers (5 cm × 7 cm × 12 cm in dimensions) at 4°C in a refrigerated compartment for 4 h for 1 day.

Group V (recovery group): Rats were subjected to a similar pattern of stress as in Group IV for only 1 day. Then, they were housed for 6 days and sacrificed on the 7th day.

The rats of Groups I, II, III, and IV were sacrificed on the 1st day after exposure to stress.

Serological study

At the termination of the experiment, collected blood from the epicanthus fold of the eyes of each rat was placed in tubes and stored at −20°C, and serum cortisol was determined using a radioimmunoassay kit (Biochemicals, Costa Mesa, CA, USA).[13]

Histological sample collection

After the collection of blood samples, each rat was anesthetized and cervically dislocated, and its abdomen was quickly opened. The kidneys were carefully extracted from the body with the surrounding tissue and then meticulously separated from the adrenal glands which located on the kidney's anterior tip. Suprarenal gland specimens were placed in paraffin blocks after being fixed in 10% buffered formalin, dehydrated, cleared, and mounted. For sectioning, the paraffin blocks are positioned in a horizontal plane. The embedded suprarenal glands were sectioned at 4 µm thick. Hematoxylin and eosin stain (H and E) was used to stain the first set for histological observation.

Immunohistochemical stains

The second sets of paraffin sections were cut on positively charged slides and subjected to immunohistochemical staining for anti-Caspase-3 antibody (rabbit polyclonal antibody, GTX110543, 1:1000, GeneTex, Irvine, CA,

USA); positive control was done on a section of tonsils; heat shock protein-70 (HSP-70) (mouse monoclonal, 1:5000 Sigma-Aldrich, Saint Louis, USA); positive control was done on a section of the brain, anti-chromogranin-A antibody (rabbit polyclonal Ab, 1:1000 Abcam, United Kingdom); rat adrenal gland was used as positive control; and finally anti-CD44 antibody (rabbit monoclonal Ab, 1:10,000, Abcam, United Kingdom) rat kidney was used as positive control.

The sections were left overnight at 4°C, and then, the samples were subjected to anti-rabbit secondary antibody (1:2000, Vector Lab., Burlingame, CA, USA) for 30 min at room temperature. In the end, sections were stained with diaminobenzidine chromogen and counterstained with Harris hematoxylin. The sections were passed through graded ethanol, sealed, and assessed microscopically. Recommended negative control was processed using the identical steps as the positive control, with the exception of using the primary antibody. The positive reaction appeared brown.

Morphometric study

The morphometric analysis of different parameters was done using the image analyzer Leica Q win V-3 program connected to a Leica DM2500 microscope (Wetzlar, Germany) in (the imaging unit of Histology and Cell Biology Department, Faculty of Medicine, Ain Shams University). Five different slides from each animal were used to acquire measurements (×40). For each slide, five randomly chosen nonoverlapping fields were examined to register the thickness of the adrenal cortex and each zone individually; the area percentage of Caspase-3-positive cells and HSP-70-positive cells; the number of CD44-positive cells and the optical density of chromogranin A.

Statistical analysis

All statistical analyses of biochemical blood tests and histomorphometric studies were performed using Statistical Package for the Social Sciences version 23 (SPSS Inc., Chicago, IL, USA). The registered data obtained from the experiment were expressed as mean values ± standard deviations. The significant differences among values were analyzed by using a one-way analysis of variance and the Tukey *post hoc* test. For all comparisons, $P < 0.05$ was considered statistically significant.

Results

Serum cortisol level

Assessment of the serum cortisol level revealed significant increases ($P < 0.05$) in the acute-stress groups compared to the control group. This elevation decreased in the recovered group. A significant elevation in serum cortisol level ($P < 0.05$) in the combined stress group was detected in comparison to the groups subjected to one type of stress and the recovery group [Figure 1].

Histological results

Examination of the H- and E-stained sections of the control group (Group I) adrenal gland revealed normal architecture

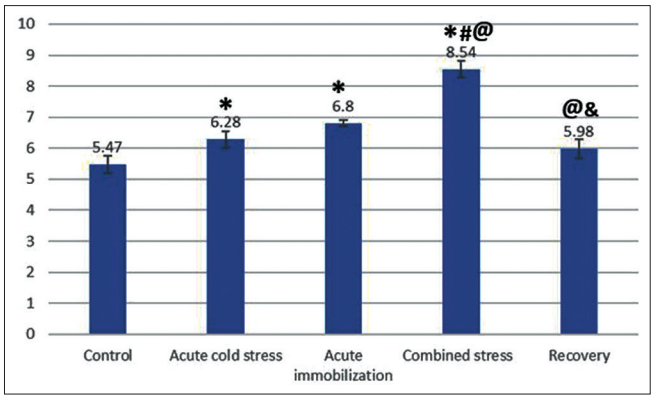

Figure 1: Effect of different types of acute stress and recovery on the mean serum cortisol level in different studied groups. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus ACS group, $^{@}P \leq 0.05$ versus acute immobilization group, $^{\&}P \leq 0.05$ versus combined stress group by One-way ANOVA with Tukey *post hoc* test. ACS: Acute cold stress

with a delicate capsule of fibrous connective tissue covering the cortex and a central medulla [Figure 2a]. The cortex was separated into three zones: outermost ZG, whose cells forming clusters of rounded or arched groups of columnar cells. The cells of the middle zone are named zona fasciculata (ZF) whose cells form parallel cords of one or two cell thickness and are separated by blood sinusoids between them. The cells were polyhedral vacuolated with acidophilic cytoplasm [Figure 2b]. The deepest layer or the ZR cells formed anastomosing cords as an erratic network intervening by blood sinusoids. Their cells were small, densely packed, and darkly pigmented polyhedral cells. The medulla was made up of chromaffin cells with vesicular nuclei [Figure 2c].

The microscopic examination of the adrenal gland specimens from the ACS group (Group II) revealed swollen edematous cells in ZG. Some cells had pyknotic nuclei with vacuolated cytoplasm. By examination of the ZF and ZR, there was sinusoidal dilatation and congestion. The medulla's chromaffin cells appeared packed, swollen, and highly vacuolated. There were signs of vascular congestion with extravasation of cells, in addition to follicular and interfollicular gap narrowing [Figure 3a and b].

The findings of the suprarenal gland of the acute immobilization (Group III) were similar to those of the CS group, but they were more prominent with eosinophilic pale stained areas in ZG, ZF, and ZR [Figure 3c and d].

Inspection of sections of the adrenal gland of the combined stress (Group IV) showed that most cells in ZG and ZF were vacuolated, swollen, and edematous with eosinophilic pale stained areas with karyorrhexis and karyolysis. Scattered cells with pyknotic nuclei were observed. The chromaffin cells in the medulla were crowded, swollen, and highly vacuolated. Furthermore, there were signs of vascular congestion, with the follicular and interfollicular gap narrowing [Figure 3e and f].

The core medullary architecture of the suprarenal gland was lost in some of the recovery group rats (Group V), although

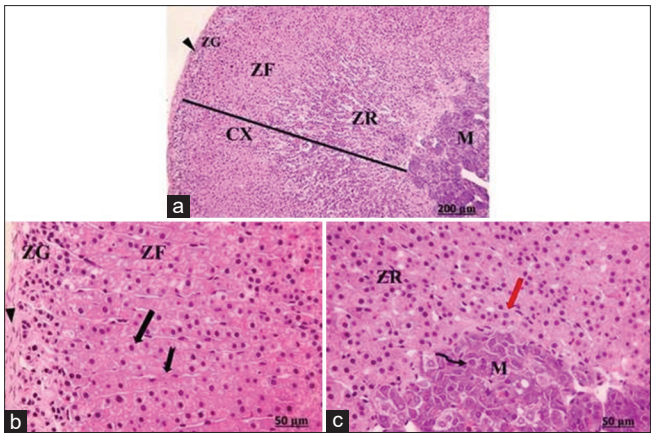

Figure 2: Photomicrographs of H and E-stained sections of the adrenal gland of the control group: (a-c) showing normal architecture with a thin capsule formed of fibrous connective tissue covering the gland (▲). Notice the outer CX and a central M. The cortex is formed of the outermost ZG; middle ZF cells form parallel cords that are separated by blood sinusoids (bifid arrow), cells are polyhedral vacuolated with acidophilic cytoplasm form radial cords one or two cells thick (black arrow); and the innermost ZR, whose cells form anastomosing cords. The small, closely packed and deeply stained polyhedral cells (red arrow) are separated by blood sinusoid. Notice the follicles of the chromaffin cells in the medulla with vesicular nuclei around blood sinusoids (curved arrow). (a, ×100, scale bar: 200 µm, b and c, ×400, scale bar: 50 µm). CX: Cortex, M: Medulla, ZG: Zona glomerulosa, ZF: Zona fasciculate, ZR: Zona reticularis

it was preserved in some areas of the gland in other rats. There were few vacuolations, hemorrhage, and damaged nuclei in the ZG, ZF, and ZR cells. Blood sinusoids were less congested [Figure 3g and h]. These findings were supported by histomorphometric data [Table 1].

Immunological study

Immunohistochemical examination for heat shock protein-70

Control group (Group I) adrenal gland examination showed HSP-70 negative immunohistochemical reaction. Scattered medullary cells had positive cytoplasmic immunohistochemical reactions [Figure 4a and b]. In ACS (Group II) [Figure 4c and d], acute immobilization groups (Group III) [Figure 4e and f], and the combined stress group (Group IV) [Figure 4g and h], there was strong positive HSP-70 immunohistochemical reactions in the cortical and some medullary cells. There was mild-to-moderate reaction in the cortex and medulla of the recovery group (Group V) [Figure 4i and j].

Statistically, a significant increase ($P < 0.05$) in the mean HSP-70 area percent in Group III compared to the control group and in Group IV compared to Group II. On the other hand, a significant decrease ($P > 0.05$) was detected in the recovery (Group V) compared to Group II and Group IV [Table 2].

Immunohistochemical examination for Caspase-3

A negative immunohistochemical reaction to Caspase-3 was shown in the ZG and ZF of the suprarenal gland in the control group, even though few positive cells in the ZR and

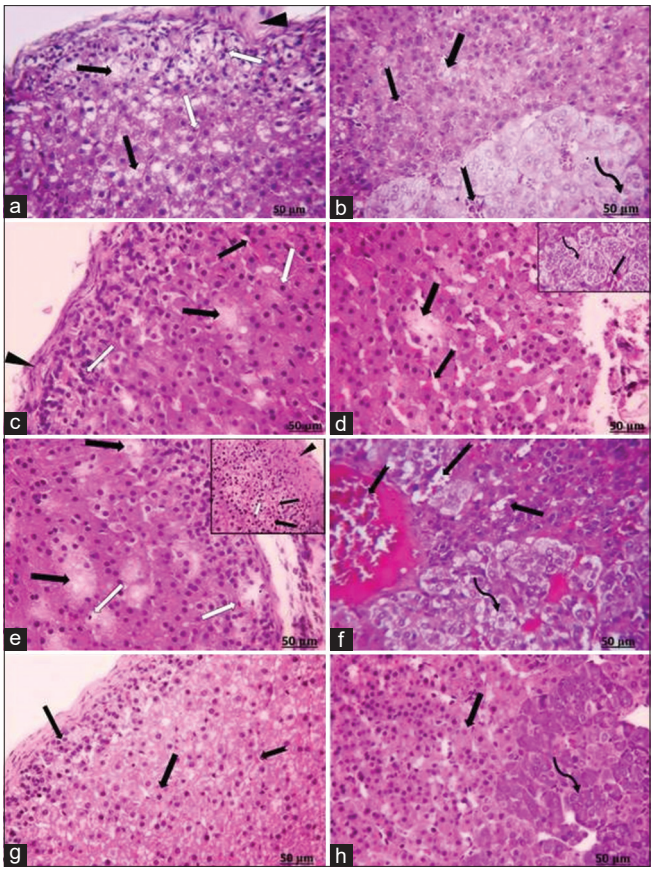

Figure 3: Photomicrographs of H and E-stained sections of the adrenal gland: (a and b) ACS group; (c and d, inset) acute immobilization group showing the capsule (▲). The adrenal cortex has edematous cells with cytoplasmic vacuolations (black arrow). Some cells have pyknotic nuclei (white arrow), blood congestion and sinusoidal dilatation are visible (bifid arrow). Chromaffin cells in the medulla are crowded, swollen, and displayed a lot of vacuoles (curved arrows) with vascular congestion, and narrowing of the follicular lumen and interfollicular gaps (bifid arrow). In the acute immobilization group, the findings are more prominent with eosinophilic pale stained areas in the adrenal cortex. (e and f, inset) combined stress group, showing the capsule (▲), vacuolated, swallowed, and edematous cells with eosinophilic pale stained areas in ZG and ZF (black arrow) and some cells with pyknotic nuclei (white arrow). The medulla's chromaffin cells are packed, enlarged, and showed a lot of vacuoles. Signs of hemorrhage and vascular congestion are seen (bifid arrow). (g and h) recovery group showing loss of the adrenal core medullary architecture in some rats, whereas, in others, it is preserved in parts of the adrenal gland. Few vacuolations and less congested blood sinusoids are seen in the ZG, ZF, ZR (black arrow) and medullary cells (curved arrow) (×400, scale bar: 50 µm). ZG: Zona glomerulosa, ZF: Zona fasciculate, ZR: Zona reticularis, ACS: Acute cold stress

the medulla were detected [Figure 5a and b]. The cortex of the adrenal gland and some medullary cells showed a moderate increase in caspase-positive cells in the ACS group [Figure 5c and d]. A strong positive immunohistochemical reaction was seen in both Group II [Figure 5e and f] and Group IV [Figure 5g and h]. There were some positive cells in the recovery group (Group V) [Figure 5i and j]. The histomorphometric parameters of the caspase area percent are summarized in Table 2.

Table 1: The mean thickness of the different zones of adrenal cortex (zona glomerulosa, zona fasciculate, zona reticularis) and adrenal medulla (μm) in the studied groups

Variable	Mean±SD				
	Control	ACS	Acute immobilization	Combined stress	Recovery
Thickness of the adrenal CX	727.06±35.41	817.12±6.91*	965.86±37.55*,#	1162.82±37.36*,#,@	856.21±6.26*,@,&
Thickness of ZG	51.69±3.90	53.39±4.73	54.92±1.59	60.28±5.70*	53.08±3.12
Thickness of ZF	442.19±23.75	486.07±56.12	576.75±24.04*	768.59±85.61*,#,@	451.92±80.81@,&
Thickness of ZR	216.53±10.93	258.46±76.19	281.61±20.42	305.6±63.91*	265.96±52.16

*P<0.05, versus control group, #P<0.05, versus ACS group, @P<0.05, versus acute immobilization group, &P<0.05, versus combined stress group by one-way ANOVA with Tukey *post hoc* test. ANOVA: Analysis of variance, SD: Standard deviation, ZG: Zona glomerulosa, ZF: Zona fasciculate, ZR: Zona reticularis, ACS: Acute cold stress, CX: Cortex

Table 2: The mean area percent of caspase-3 and heat shock protein-70 positive cells, number of CD44 positive cells and the optical density of chromogranin A in the studied groups

Variables	Mean±SD				
	Control	ACS	Acute immobilization	Combined stress	Recovery
Area percent of HSP-70 positive cells	8.18±2.48	12.99±5.18	18.76±2.05*	29.04±2.45*,#,@	10.47±5.18@,&
Area percent of caspase-3	6.03±2.62	14.12±2.29	19.72±3.75*	27.86±4.27*,#	9.83±2.07&
Number of CD44 positive cells	0.8±0.82	2.8±0.83	3.6±1.8*	10±1.52*,#,@	7±1.58*,#,@,&
The optical density of chromogranin A	58.08±1.40	68.64±0.41*	70.4±4.19*	76.41±0.14*,#,@	63.17±0.17*,#,@,&

*P<0.05, versus control group, #P<0.05, versus ACS group, @P<0.05, versus acute immobilization group, &P<0.05, versus combined stress group by one-way ANOVA with Tukey *post hoc* test. ANOVA: Analysis of variance, SD: Standard deviation, HSP-70: Heat shock protein-70, ACS: Acute cold stress

Immunohistochemical examination for CD44

Despite a few positive cells in the adipose tissue surrounding the adrenal gland, the immunohistochemical reaction for the control group showed negative immunoexpression of CD44 in the adrenal gland [Figure 6a]. However, there were some spindle-shaped CD44+ve cells in the subcapsular area, among the gland's cortex, and in the nearby adipose tissue in the ACS (Group II) and acute immobilization groups (Group III) [Figure 6b and c], respectively. There was a noticeable increase in CD44+ve cells in both the combined group and the recovery group [Figures 6d and e], respectively. These results were supported by histomorphometric data in Table 2.

Immunohistochemical examination for chromogranin-A

The control group medullary cells displayed scattered cytoplasmic responses [Figure 6f]. A considerable increase in the optical density of chromogranin-A was seen in the medullary cells of the ACS group [Figure 6g], acute immobilization group [Figure 6h], and combined stress group [Figure 6i] compared to the control group. The optical density of GpV was noticeably lower than that of the combined Gp and significantly higher than that of the control group [Figure 6j], indicating a moderately positive immunoreaction [Table 2].

Discussion

Indeed, stress is prevalent in the current culture. Daily issues, significant life events, abuse, trauma, maintaining a work-life balance, and many other things can be stressful. Financial difficulties, when combined with other stressors, might increase the risk of developing so-called stressor-related disorders.[14] Therefore, this study aimed to compare the effects of immobility and cold as an example of acute stressors and the recovery from these stressors on the histological changes of the adrenal gland and the subsequent role of the adrenal progenitor cells. Only male rats were used in this study because hormone receptors differ across the sexes, making the stress response sex specific.[15]

Serum cortisol levels significantly increased in ACS, AIS, and the combined stress groups. When the two acute stressor models were compared, no statistical-significant difference was detected between the ACS and acute immobilization. Compared to the acute cold and acute immobility groups separately, the combined stress group had a statistically significant increase. In the recovery group, this rise was suppressed and nearly brought back to the control level.

Similar results were observed by Jameel *et al*.[16] and Tsukada *et al*.,[17] as they subjected the animals to a single stress model, including an increase in corticotropin-releasing factor (CRF) and blood cortisol levels. The CRF and mRNA estimations are used to explain their results. They concluded that 6 h after acute (single) restraint stress, the expression of the CRF mRNAs and arginine vasopressin (AVP) in the paraventricular nucleus varied. The tests indicate that a cascade of neurohumoral events mostly takes place at the level of the HPA axis, which results in an elevated level of blood cortisol. When exposed to a single stressor, a spike in cortisol levels terminates the stress response and returns the body to equilibrium more quickly. Meanwhile, multiple stressors take a longer period for the levels to drop and fail to return the body to homeostasis.

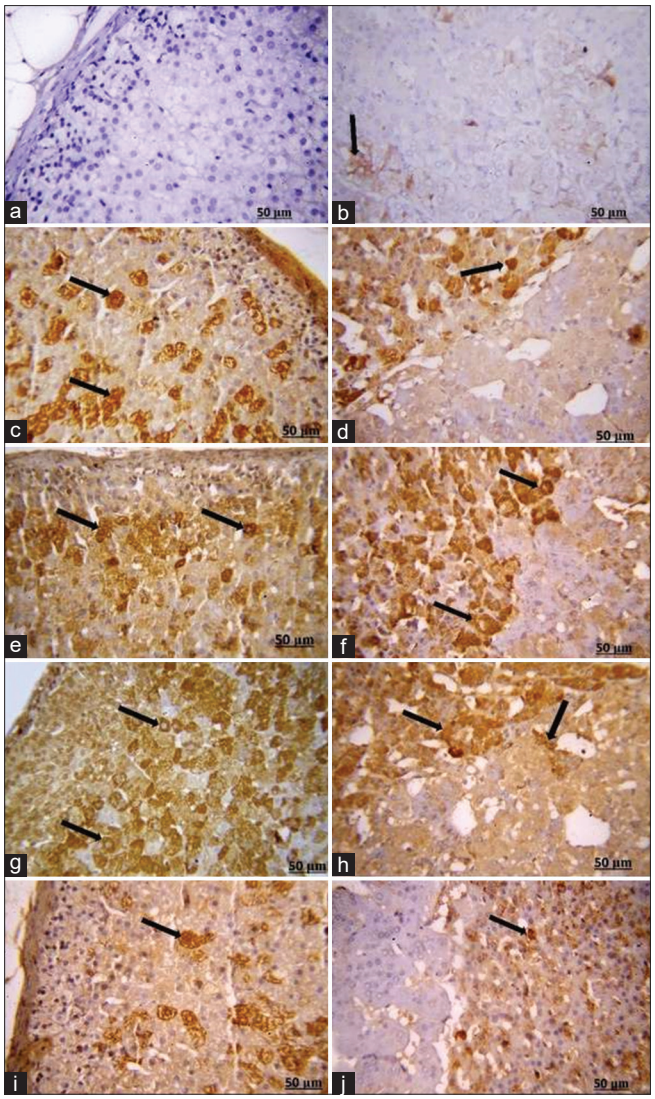

Figure 4: Photomicrographs of HSP-70 immunohistochemical sections of the adrenal gland: (a and b) control group, showing the negative immunohistochemical reaction of the cells forming the adrenal cortex. Notice some positively stained cells in the medulla. (c and d) ACS, (e and f) acute immobilization groups, (g and h) combined stress group, showing strong HSP-70 positive immunohistochemical reaction of the cytoplasm of cortical and medullary cells (arrows). (i and j) recovery group; mild to moderate positive immunohistochemical reaction in the cortex and medulla (↑) (×400, scale bar: 50 µm). HSP-70: Heat shock protein-70, ACS: Acute cold stress

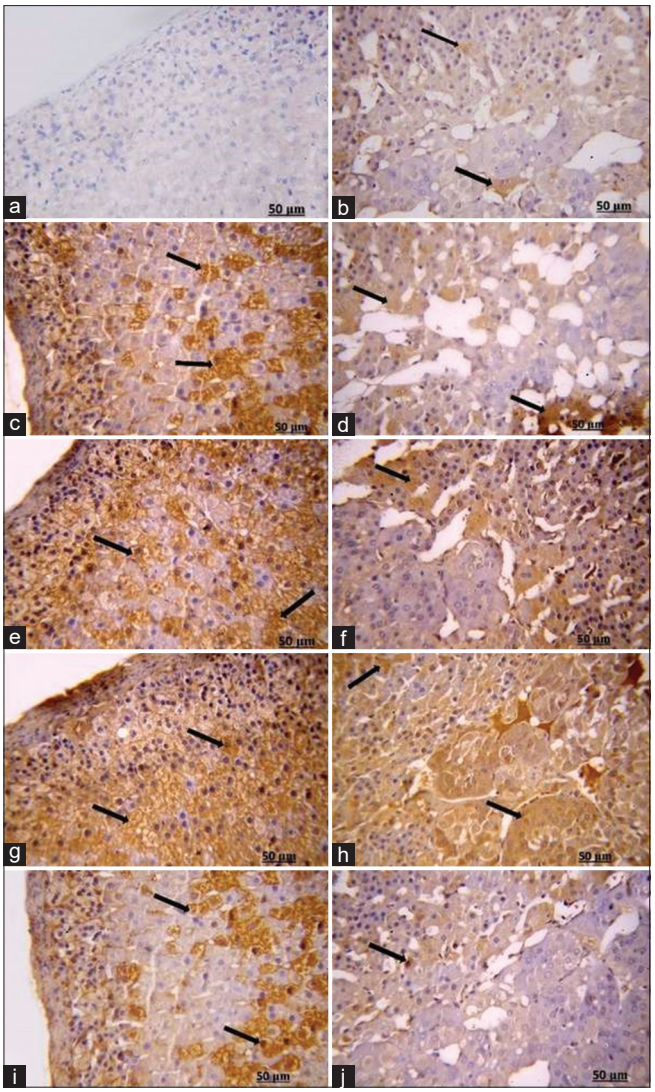

Figure 5: Photomicrographs of Caspase-3 immunohistochemical stained sections of the suprarenal gland: (a and b) control group, there is a negative immunohistochemical reaction of caspase-3 in the ZG and ZF. Notice scattered positively reacted cells in the ZR and the medulla. The ACS (c and d), acute immobilization (e and f), and combined stress groups (g and h) have strong positive immunohistochemical reactions in both cortex and medulla of the adrenal gland (arrows). In the recovery group (i and j), there are persistence of some positive cells (↑) in the cortex compared to few cells in the medulla (× 400, scale bar: 50 µm). ACS: Acute cold stress, ZG: Zona glomerulosa, ZF: Zona fasciculate, ZR: Zona reticularis

In the current study, rats that were exposed to either ACS or acute immobilization displayed numerous histopathological characteristics in the adrenal cortex (more prominent in the acute immobilization group), including a disorganized ZG with swollen cells. Both ZF and ZR had obvious cells with cytoplasmic vacuolations and pyknotic nuclei, denoting degenerative changes and subsequent necrosis. Vascular congestion as well as hemorrhage in the medulla and adrenal cortex, were both evident. The chromaffin cells of the adrenal medulla were also swollen and vacuolated.

Previous researches have similar findings to the current study,[9,18] and according to Liu *et al*.[19] oxidative stress alters the apoptosis-related genes expression levels and encourages cell apoptosis and degeneration through BCL-2, bax, and caspase-3 signaling pathways. These findings can explain the degenerative changes in the cortical cells as a result of the elevated stress-induced ACTH release, which induces the ZF and ZR to release cortisol. Due to their critical involvement in the production of glucocorticoids, stress could have a damaging effect on the mitochondria and smooth endoplasmic reticulum (sER). It was shown to be followed by decreased steroidogenesis, which might be used to explain cytoplasmic vacuolations.[20] The deposition of lipid globules containing cholesterol could function as a reserve material. This

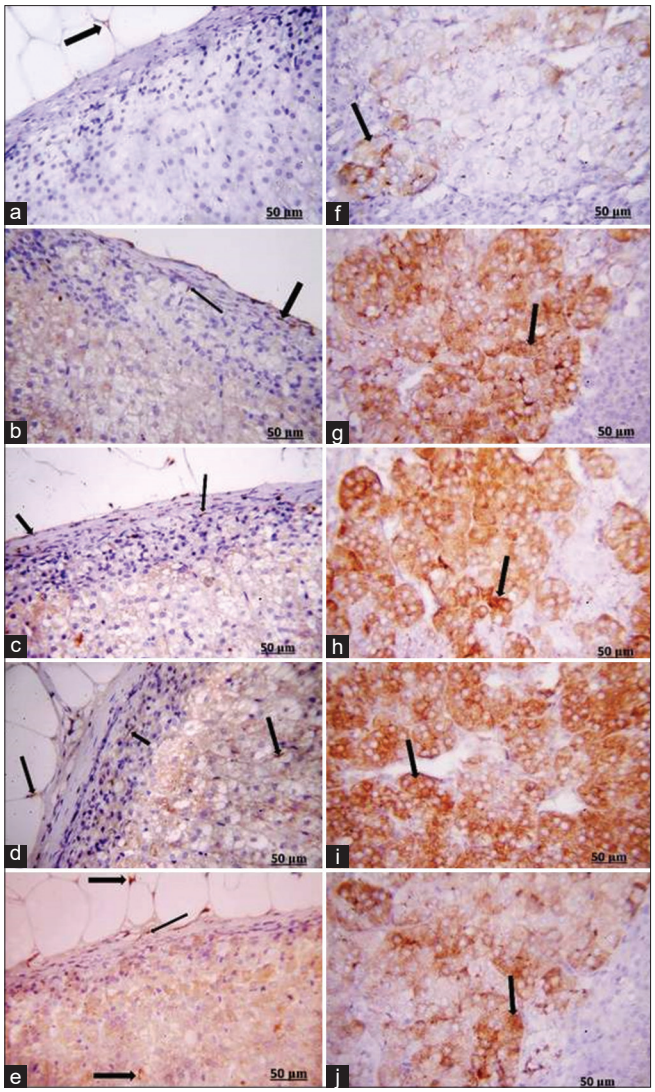

Figure 6: Photomicrographs of immunohistochemically stained sections (a-e) against CD44 of the suprarenal glands: (a) control group, only a few positive cells are seen in the adipose tissue surrounding the gland. However, in ACS (b), acute immobilization groups, (c), and the combined stress group (d), there are some CD44+ve cells in the subcapsular region, among the cortex of the gland, and in the adjacent adipose tissue (e) recovery group, showing an apparent increase in CD44-positive cells (↑) (f-j) immunohistochemically stained sections by anti-Chromogranin-A in the medulla of the suprarenal gland, (f) control group, showing scattered cytoplasmic responses. In ACS (g), acute immobilization groups (h), and the combined stress group (i), there is a strong positive immunoreaction in the medullary cells (j) recovery group shows moderate positive immunoreaction (↑) (×400, scale bar: 50 µm). ACS: Acute cold stress

cholesterol is the main precursor in the manufacture of steroid hormones caused by stress. This explanation is considered a cause of the observed cytoplasmic vacuolization in cortical cells.

Acute stress, however, led to an increase in ZG cell volume. Aldosterone is produced by these cells and its physiological effects correspond to the hypothalamic hormone, vasopressin (AVP). In addition, this hormone can increase cortisol release. It was demonstrated that stress enhanced the secretion of both ACTH and AVP. In stressful situations, ACTH is secreted in large amounts, which can stimulate the secretion of aldosterone. Hence, under conditions of acute stress, both ACTH and AVP cause an increase in the volume of ZG cells, the location of aldosterone accumulation.[21] Adrenal congestion and hemorrhage may be explained by a high level of ACTH, which stimulates catecholamine release and increases blood flow to the adrenal gland. A surge in ACTH also promotes the production of prostaglandins, which causes congestion.[22] Hemorrhage is more likely to happen because endotoxins produced by increased gland activity cause endothelial cell damage, and hypoxia brought on by congested glands causes endothelial cell damage also.[9]

Rats of the combined group have a thick adrenal cortex. The cortical cells, specifically ZG, ZF, and medullary cells, are enlarged and vacuolated, as well as having vascular congestion and hemorrhage. Due to the combination of two different types of stress, the burden on the adrenal gland increases. All these pathological abnormalities became more obvious than in other groups subjected to acute cold or immobility.

In the current study, the stressed groups (ACS, AIS, and combined stress group) have HSP-70 and caspase-3 immunopositivity. The adrenal cortex has most of the HSP-70-positive cells. The mean HSP-70 area percent is significantly increased, reaching its highest in the combined stress group, followed by the immobilization group. A similar result was obtained by Udelsman *et al*.[23] who stated that rats exposed to restraint, which is a mild physiologically relevant stress, induced HSP70 mRNA expression in the adrenal cortex. Consistent with our findings, Li *et al*.[24] declared a higher level of HSP70 by western blotting analysis after acute heat stress. This can be explained by the fact that heat stress causes oxidative stress with reactive oxygen species (ROS) generation. Mitochondrial heat-induced alterations due to increased ATP production thus promotes cellular oxidative stress due to the release of ROS from the mitochondria. Mitochondria have their own antioxidant system allowing the scavenging and neutralization of the radicals generated. Induction of the HSP70 as a chaperone protein that binds to hydrophobic protein sequences liberated by denaturation so that they prevent their irreversible interaction with other cellular proteins, thus preventing the loss of the protein function causing cytoprotection. HSP synthesis is triggered by the activation of so-called 'heat shock transcription factors' (HSFs). ROS increases the nuclear translocation and DNA binding activity site of HSF1, which makes a redox mechanism in heat-induced signal transduction pathways during apoptosis very likely.[25,26]

For assessment of the degree of apoptosis in the adrenal cells, the immunoreactivity for caspase-3 displays an increase in caspase-3 area percent, indicating cellular apoptosis, mainly in the cells of the adrenal cortex of all acute stress groups. The combined stress group has the highest levels of these changes. Similar findings are evident by Altayeb and Salem,[18] as they investigated how immobility stress affected rats'

adrenal glands. According to Liu *et al*.,[19] excessive oxidative stress causes cell apoptosis and degeneration by altering the expression levels of "apoptosis-related genes" after being exposed to various types of acute stress.

To elucidate the role of the progenitor cells, the current study demonstrated that both the ACS group and the acute immobilization group had a small number of CD44+ve spindle cells at the subcapsular region, within the cortex, and in the surrounding adipose tissue. Morphometry showed that the combined stress and recovery group have a considerable increase in CD44+ve cells.

The proliferation and differentiation of adult stem cells contribute to the preservation of bodily organs and tissues. These cells are present in various organs and contribute to cell regeneration.[7] Adrenal glands possess specific adult progenitor/stem cells in the cortex and medulla. They are in charge of the adrenal gland-specific cell renewal, which is essential for stress adaptation.[8] In addition, adipose tissue and bone marrow contain mesenchymal stem cells, which are sources of endogenous stem cells that circulate through the blood to the injured organs. Mesenchymal stem cells have the typical immunostaining for CD44. This study confirmed the existence of stem cells, which is consistent with findings made by previous researchers who found stem/progenitor cell pools in the juxtamedullary, subcapsular, and adrenal capsular regions that can differentiate to reinhabit zones trying to overcome the pathological changes resulting from the stress conditions affecting the adrenal gland.[18,27] The current increase in CD44+ve cells in the combined stress group and recovery group, the number of these cells has a positive association with the weight/amount of the stress, the subpopulation of newly formed adrenocortical cells, and the time for recovery.

The primary protein for the adrenomedullary chromaffin cells, chromogranin A, is found in the cytoplasm of the medullary cells in the current study. When compared to the control group, all acute stress groups and recovery groups have significantly higher expression and optical densities. Similar findings are published by Lashine *et al*.,[9] who claimed that under conditions of acute stress, the adrenal glands Nestin-positive progenitor cells could differentiate into chromaffin cells that express chromogranin-A. This may be explained by the fact that mesenchymal stem cells, which normally exhibit the characteristic immunostaining for CD44 positivity, can develop into a wide variety of cell types, including glial, neuronal, and chromaffin cells. They do, however, favorably develop into chromaffin cells under stress, which causes the adrenal to exhibit an excessive adaptive response. In addition, it has been demonstrated that chromaffin progenitor cells can self-renew in culture and develop into chromaffin cells that produce hormones.[28,29]

Thus, the end organ of the endocrine and neuroendocrine stress system contains a complex network of neuronal and cellular connections. Most probably, the cortical and chromaffin cells within the gland actively interact on cellular and functional levels. Catecholamines control the release of steroids and the cellular activity of the adrenal cortex. Meanwhile, the adrenocortical glucocorticoids are essential for the manufacture of adrenomedullary epinephrine.

Conclusion

In summary, different acute stressors have varying degrees of impact on the histological structure of the adrenal gland. The combination of CS and immobility has more harmful consequences than immobilization and CS alone. The adrenal gland attempted to reverse the pathological abnormalities in the recovery group, although it may take more time to get back to normal, denoting the importance of both progenitor and chromaffin cells in the regenerative process.

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Conflicts of interest

There are no conflicts of interest.

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