The behavior of adrenal progenitor stem cells in response to chronic stress and recovery

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SUMMARY

Stressors affect the differentiation of stem cells in the hypothalamic-pituitary-adrenal axis (HPA). This study was designed to ensure the presence of progenitor cells in the adult adrenal gland and to evaluate their behavior under chronic stress and after recovery. Also, to assess their ability to recruit new glial and chromaffin cells. Three groups of adult male rats (6 rats each): control, chronic-stress (rats were placed individually in stainless restrainers 2h/day for six days), recovered (rats were housed in an enriched circumference for seven days after the same stress modality). Both chronic-stress and recovered groups showed increased adrenal glands weight and cortisol levels with vacuolation, hemorrhage, and edema in the cortex and medulla. The chronic-stress group illustrated a significant increase in the chromaffin reaction, which was reduced in the recovered group. Evaluation of the immunohistochemical results revealed a significant decrease in the Nestin and GFAP (glial fibrillary acidic protein) reactions, but an increase in the chromogranin-A reaction in the chronic-stress group. The recovered group demonstrated a significant increase in the Nestin and GFAP and a reduction in the chromogranin-A immunohistochemical reactions. These results indicate the differentiation of the progenitor (Nestin expressing) stem cells into chromaffin (chromogranin-A expressing) cells under stress conditions for stress adaptation. Conversely, under normal conditions, the differentiation moved toward the glial cells.

Key words: Stem cell – Suprarenal gland – Nestin – GFAP – Chromogranin-A – Stress

ABBREVIATIONS

ACTH: Adrenocorticotropic hormone
ANOVA: analysis of variance
CN: control group
CRH: corticotropin-releasing hormone
CS: chronic stress
GFAP: glial fibrillary acidic protein
HPA: hypothalamic-pituitary-adrenal axis
Hx&E: Hematoxylin and eosin
IRP: Intuitional Research Board
MERC: Medical Experimental Research Center of Mansoura University
NIH: National Institutes of Health
REC: recovery after chronic stress
RIA: radioimmunoassay
SD: standard deviation
SPSS: Statistical package for social sciences
ZF: zona fasciculata
ZG: zona glomerulosa
ZR: zona reticularis

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INTRODUCTION

Generally, the term "stress" describes our difficulty in dealing with the challenges of life. It is linked to our perceptions of unexpected mental or physical efforts or any work burden we are exposed to (Stalder et al., 2017). Chronic stress results from repetitive, frequent, and intermittent stressors. It can initiate several responses: sensitization, desensitization, and habituation. Sustained activation of the HPA axis results in pulsatile and repetitive glucocorticoid secretion (Holinger et al., 2018).

In times of stress, the CRH (corticotropin-releasing hormone) is released from the hypothalamus and this motivates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH motivates the release of glucocorticoid from the inner adrenal cortex and the aldosterone from the outer cortex. CRH also impacts the, locus coeruleus, which triggers the sympathetic nervous system to secrete norepinephrine which stimulates the adrenal medulla to generate catecholamines (Berends et al., 2019). Despite the different origins and the nature of the hormones produced, the connection between the cortex and medulla is vital to the appropriate function of the adrenal gland and its adaptation to stress (Dutt et al., 2022).

From the first day of our life, internal and external stressors affect the process of stem and progenitor cell differentiation in the HPA to achieve a fully efficient endocrine stress system (Flak et al., 2012). Previously, the chromaffin stem cells were isolated and differentiated from the murine, bovine, and human adrenal medulla (Rizzoti et al., 2013; Vukicevic et al., 2015).

Various neural crest derivatives preserve in their tissues multipotent neural crest cells (Crane and Trainor, 2006), or even cells that have pluripotent characteristics (Sieber-Blum and Hu, 2008). Nestin is a class VI intermediate filament protein existent in proliferative cells in different fetal and embryonic tissues. Nestin expression is linked to the stem or progenitor cell with multipotent or regenerative properties (Yin et al., 2016). Regarding its distinctive expression pattern, Nestin is considered a marker of stem or progenitor cells (Obara et al., 2019; Bornstein et al., 2020).

A definite pool of Nestin-expressing glial-like progenitor cells was identified. These multipotent cells can differentiate into both neurons and chromaffin cells either in vitro or in vivo (Rubin de Celis et al., 2015). Moreover, Nestin-expressing progenitors are not only found in the adrenal medulla but also in the cortex. So, mature steroid-producing cells could be induced from this pool in response to any physical or metabolic stressors (Steenblock et al., 2017). However, it is uncertain if the multipotent neural stem cells still exist in the medulla of the adult adrenal gland and can participate in its adaptation to stress.

This study was designed to evaluate the adrenal histopathological changes induced by chronic stress and recovery. Also, to assess the existence of stem cells and their response to stress state and recovery by assessment of Nestin immunohistochemical expression. Lastly, to evaluate the capacity of those progenitor stem cells to recruit new glial and chromaffin cells by studying their specific markers (chromogranin-A and GFAP respectively).

MATERIALS AND METHODS

Animals used

Adult male Sprague Dawley rats (total number: 18, weight 220-250 g) were purchased from MERC (Mansoura University Medical Experimental Research Center). Two weeks before the experiment, the rats were housed six per cage under a comfortable environment (temperature: 22 °C, humidity: 55 ± 3%, reversed light/dark cycle: 12/12h) with *ad libitum* access to water and food for acclimatization. The current study was approved by the local ethical committee on animal experimentation, Intuitional Research Board (IRB) (code number: MD.18.03.17-2018/03/14), Faculty of Medicine, Mansoura University. It followed the NIH guidelines for animal care.

Experimental groups

The rats were divided into three experimental groups, six rats each. CN (Control**):** the rats were housed in ordinary stainless cages (60× 35×20 cm) under comfortable conditions during the experiment (El-Desouki et al., 2012). CS (Chron-

ic-stress): each rat in this group was retained individually in stainless-steel wire restrainers (12×7×5 cm to restrict the movement of the animals) (Soliman, 2006), daily for six days (2 h/day: 9 AM to 11 AM) without water or food (Rubin de Celis et al., 2015), then sacrificed on the 7th day. REC (Recovered): after exposure to the same stress design**,** the rats were returned to an enriched environment for seven days and then sacrificed on the 14th day.

Sacrifice of rats, specimen collection, and assessment of the adrenal gland weight

The body weight was determined at the beginning of the experiment and before sacrifice. After isoflurane anesthesia, the rats were decapitated, abdominal incisions were made, adrenal glands were carefully dissected, immediately weighed, and fixed in buffered formalin (10%) and used for paraffin sections. Other slices from the adrenal glands were fixed in a mixture of potassium chromate and potassium dichromate and then processed for the chromaffin reaction. Samples of blood were collected directly from the left ventricle and for plasma cortisone assessment.

Plasma glucocorticoid hormone

The blood was collected in an EDTA-treated glass tube and centrifuged (1800 rcf, 20 min, 4°C). The plasma was collected and stored at −80°C until used. The plasma glucocorticoid levels were determined by radioimmunoassay (RIA) using a Corticosterone Double Antibody RIA kit (#07–120102, MP Biomedicals, Santa Ana, California) (Bekhbat et al., 2018).

Histology and Immunohistochemistry

The fixed gland tissues were dehydrated using ascending graded concentrations of alcohol, cleared in xylene, and embedded in soft and then hard paraffin wax. Sections were cut (5 µm) and stained with Hematoxylin and eosin (Hx&E) (Bancroft and Gamble, 2002) for Histopathological evaluation. For immunohistochemical staining, sections of the adrenal gland were treated with hydrogen peroxide (3%) to block the activity of endogenous peroxidases, then were rinsed in phosphate buffered saline (pH 7.4). For antigen retrieval, the slides were incubated in sodium citrate buffer (0.01 M - pH 6.0) in a water bath (95°C – 30 minutes). After reaching room temperature, the slides were incubated (1 hr) with bovine serum albumin (1%), then with the primary antibodies (overnight - 4°C): Rabbit polyclonal anti-nestin antibody (N5413, Sigma Chemicals Co., St. Louis, MO, USA – dilution: 1/100) (Bellafiore et al., 2006; Klein et al., 2014), Rabbit polyclonal Anti-chromogranin-A antibody (A0430, Abcam chemicals, Kemet, Egypt – dilution: 1/1500) (Zhang et al., 2018), and Anti- GFAP antibody (ab7260, Abcam chemicals, Kemet, Egypt – dilution: 1/1000) (Nedzvetskii et al., 2016). The sections were treated with HRP-conjugated secondary antibodies (30 minutes - 37°C). The labeled streptavidin-biotin was then applied (30 minutes). The reactions were remarked with DAB (diaminobenzidine) and counterstained with Hx.

For the chromaffin reaction (which demonstrates catecholamine granules in chromaffin cells), the freshly excised tissues were immersed for 16 hours in a solution containing 10 volumes of 5% potassium dichromate and one volume of 5% potassium chromate. They were then washed in three changes of distilled water for a total of 60 minutes, trimmed properly, and embedded in gelatin at 37°C for one hour. Frozen sections were cut at 15 microns, and the slides dried for 2 hours at room temperature to ensure adherence. They were later mounted in glycerol jelly. Adrenaline secreting cells stain lightly (yellow or yellowish-brown), while noradrenaline secreting cells usually stain dark brown (Hillarp and Hokfelt, 1955).

Morphometric study

The thickness of the zona fasciculata (ZF) and medulla were measured in Hx&E-stained sections at 100X magnification. The value was the mean of three measurements of the maximum, medium, and minimum thickness (Gannouni et al., 2014). The optical density (OD) of chromaffin and immunohistochemical (Nestin, chromogranin-A**,** and GFAP) stained sections were evaluated in five non-overlapping randomized fields. The fields were photographed using a digital camera (Olympus® SC100) installed on a light microscope

(Olympus® CX41). The morphometric analysis was done using the NIH (National Institutes of Health, Bethesda, MD, USA) Image J program, observing the program instructions.

Statistical analysis

The data were analyzed by the statistical package for social science program (SPSS version 22) for descriptive statistics represented as mean±-standard deviation (SD) and analytical statistics for comparison between the different groups. The significance of difference was tested either by using analysis of variance (ANOVA) to compare between more than two groups of parametric data followed by post-hoc Tukey test, or Kruskal–Wallis test to compare between more than two groups of non-parametric data followed by Mann- Whitney test for multiple comparisons. *P*-value <0.05 was considered statistically significant.

RESULTS

Assessment of rat's body weight

Assessment of the body weight revealed a significant decrease (*P<0.011*) in CS group (184.2±10.034) as compared to CN group (200.8 ±10.848). Regards REC group (205.2±7.295), there was a significant increase (*P<0.002*) in body weight when compared to CS group and non-significant increases (*P<0.464*) in comparison to CN group (Fig. 1).

Assessment of the adrenal gland weight

The weight of the adrenal gland significantly increased (*P<0.0001*) in CS group (35.25±1.279) as compared to CN group (30.176±1.854). Although, the adrenal gland weight of REC group (35.11±1.279) demonstrated a non-significant (*P<0.867*) difference from CS group, it significantly increased (*P<0.0001*) when compared to CN group (Fig. 2).

Assessment of the plasma cortisol level

The plasma cortisol level significantly increased (*P<0.0001*) in CS group (10.156±0.769) as compared to CN group (5.554±0.252). The elevated cortisol level significantly reduced (*P<0.0001*) in REC group (8.482±0.82) as compared to CS group. However, it is still significantly elevated (*P<0.0001*) than CN group (Fig. 3).

Assessment of the histopathological results

Hematoxylin and eosin-stained sections

Sections of CN group revealed apparent normal histological structure. The adrenal gland was formed of an inner pale central medulla and outer dark cortex surrounded by a connective tis-

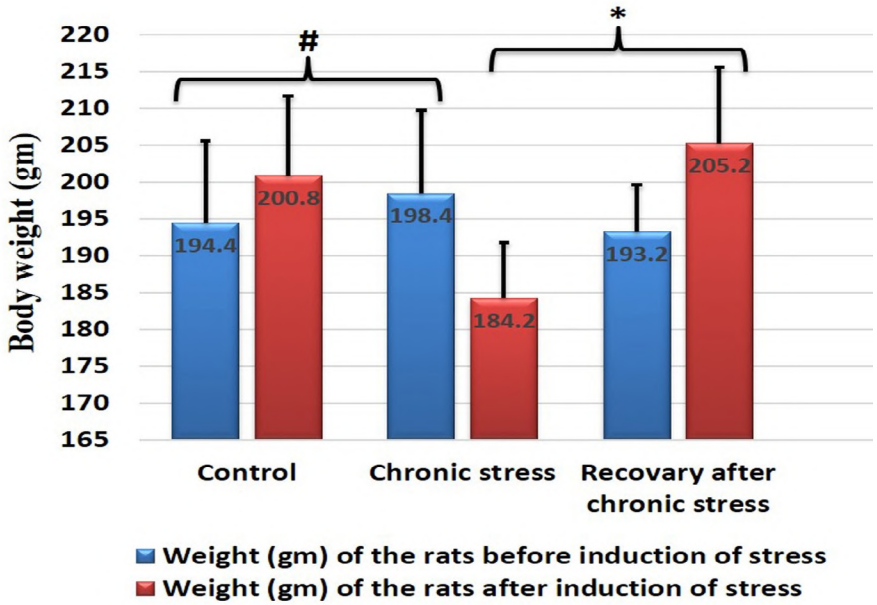

Fig. 1.- Histogram illustrating a significant decrease in the bodyweight of the CS group comparing to the CN group, and a significant increase in the REC group comparing to the CS group. #p<0.05, *p<0.01.

sue capsule**.** The cortex was formed of zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) (Fig. 4A). The cells of ZG were columnar with deeply stained, rounded nuclei, and arranged in small nests (Fig. 4B). The cells of ZF were large and polyhedral, containing central rounded vesicular nuclei; they were arranged in parallel cords, disjoined by blood sinusoids (Fig. 4C). The cells of ZR appeared small, rounded, and dark, with hyperchromatic nuclei, and characteristic brown wear and tear lipofuscin pigment; they were arranged in anastomosing cords separated by blood sinusoids (Fig. 4D). The adrenal medulla consisted of follicles of chromaffin cells that contain basophilic catecholamines granules and vesicular nuclei. Connective tissue trabeculae were seen extending between the medullary follicles. Blood sinusoids, medullary venules, and clusters of ganglion cells were seen in the interfollicular space (Fig. 4E).

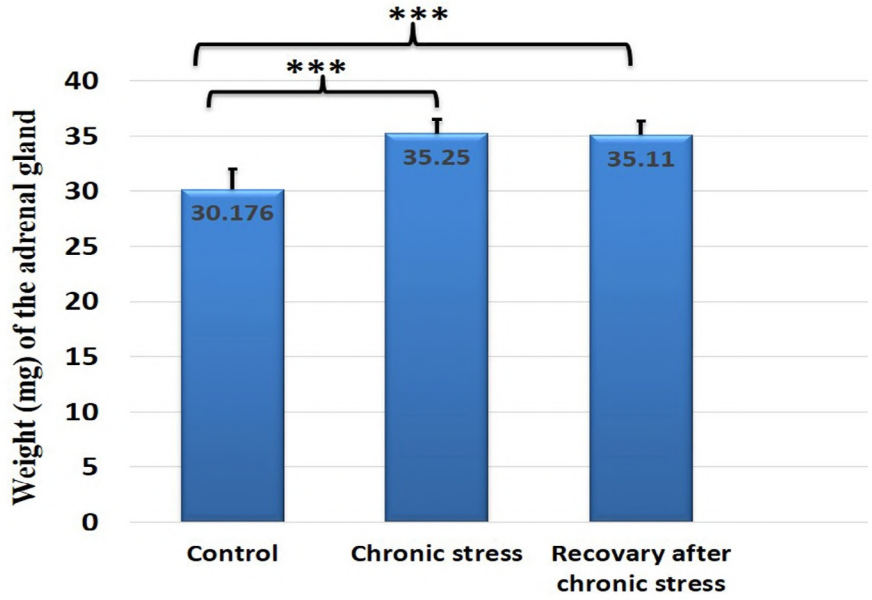

Fig. 2.- Histogram illustrating a significant increase in the adrenal gland weight of both CS and REC groups comparing to the CN group. ***p<0.0001.

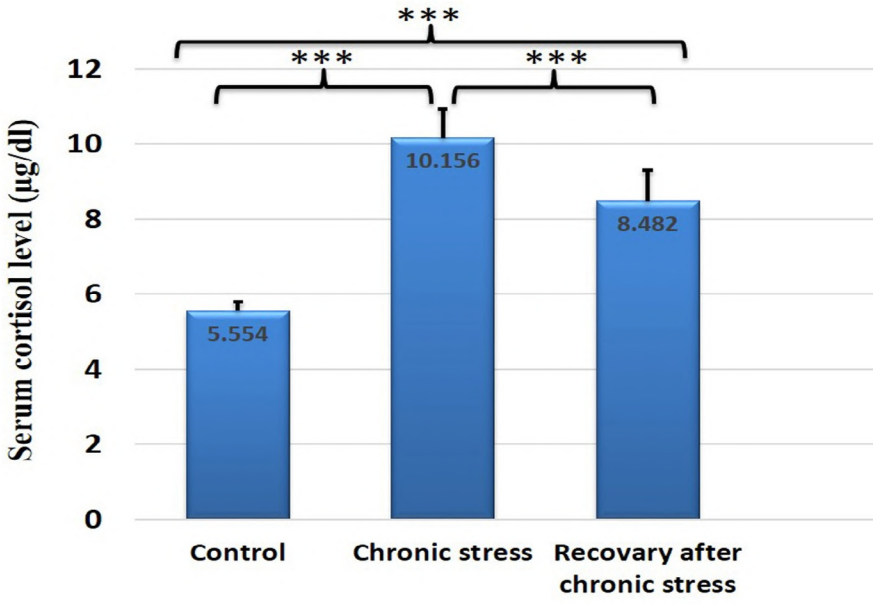

Fig. 3.- Histogram illustrating a significant increase in the serum cortisol level in both CS and REC groups comparing to the CN group. However, it shows a significant decrease in the REC group comparing to the CS group. ***p<0.0001.

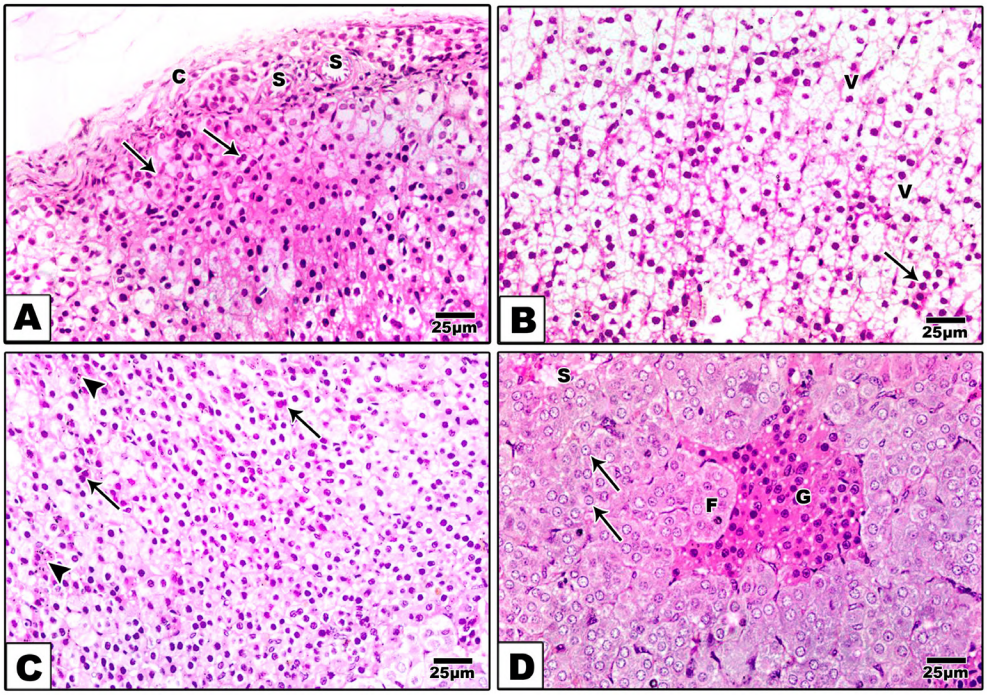

Fig. 4.- Photomicrographs of Hx&E-stained adrenal gland of a CN rat showing **A):** Capsular and subcapsular regions of rat suprarenal gland. The capsule (C) has blood sinusoids (S) between its cells and surrounds the peripheral subcapsular thin zona glomerulosa which consists of small nests of cells (arrows) with dark cytoplasm. **B):** The cells of the zona fasciculata are arranged in parallel cords of cells (arrow). The cells appear large and polyhedral containing large rounded nuclei. Their cytoplasm has tiny cytoplasmic vacuoles (V) and is stained faintly acidophilic. **C):** The zona reticularis cells are rounded dark cells arranged in anastomosing cords and have hyperchromatic nuclei and dense cytoplasm (arrows). A brown pigment (arrow heads) is seen between the cords. **D):** The medulla is formed of basophilic chromaffin cells with vesicular nuclei (arrows) which are arranged in follicles (F) surrounding blood sinusoids (S). A cluster of ganglion cells (G) is seen in the interfollicular space. Scale bars = 25 µm.

The adrenal gland of CS group revealed atrophy of ZG, congested blood sinusoids, and vacuolations (Fig. 5A). In ZF, many vacuoles were detected in between swollen cells, indicating edema. Some cells with pyknotic nuclei and hemorrhage were observed (Fig. 5B). The ZR showed blood engorgement, dilated sinusoids, and vacuolations (Fig. 5C). The adrenal medulla showed packed chromaffin cells with constriction of the follicular lumen and reduction of the space between the follicles. Extracellular eosinophilic material and degenerated chromaffin cells were detected. There was also an increasing number and congestion in medullary blood sinusoids as well as hemorrhage between the follicles (Figs. 5E, 5D).

The ZG of REC group showed hemorrhage, dilated congested blood sinusoids, and vacuolations (Fig. 6A). In ZF, swollen cells were still observed due to edema. Cellular mitotic figures, vacuoles, and hemorrhage are present between the cells (Fig. 6B). The ZR cells still showed vacuolations and hemorrhage (Fig. 6C). The medulla still showed vacuoles inside the follicular cells, as well as reduction in the follicular lumen and the interfollicular space. Extracellular eosinophilic material was detected. There was congestion in medullary blood sinusoids, as well as hemorrhage in between the follicles (Fig. 6D).

Zona fasciculata thickness: There was a significant increase (*P<0.025*) in ZF thickness in CS group (668±94.98) (Fig. 7B) compared to CN one (533.4±25.24) (Fig. 7A). The ZF thickness in REC group (579.8±37.16) (Fig. 7C) was little lower than CS group (*P<0.129*) and higher than CN group (*P< 0.415*) (Fig. 8).

Chromaffin-stained sections

The chromaffin reaction in CN group revealed a positively stained central brown medulla and a negatively stained peripheral yellowish cortex (Fig. 9A). The catecholamine granules in the adrenaline secreting cells are stained yellowish-brown, while those in the noradrenaline secreting cells are stained dark brown (Fig. 9B).

CS group (Figs. 9C, D) showed a non-significant increase (*P<0.663*) in OD of adrenaline cell

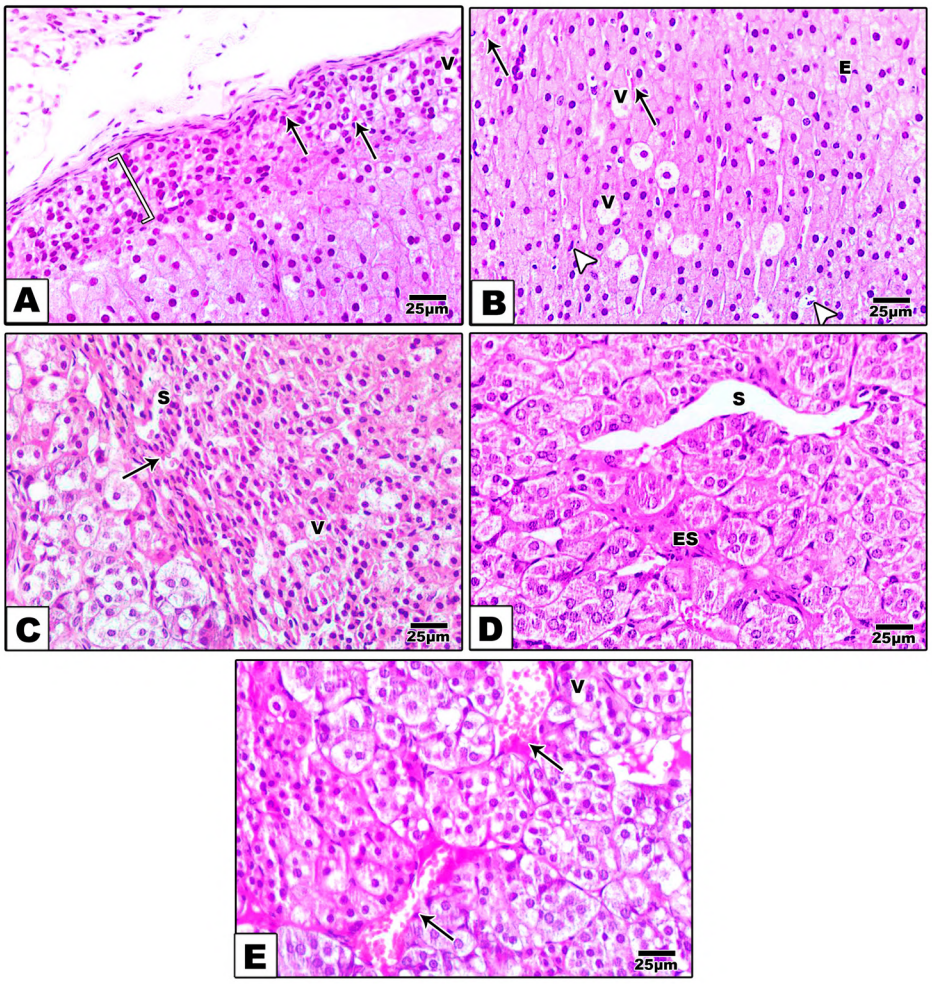

Fig. 5.- Photomicrographs of Hx&E-stained adrenal gland of a CS rat showing **A):** decreasing the zona glomerulosa thickness, vacuolation (V), and hemorrhage (arrows). **B):** The zona fasciculata represents, vacuolation (V), hemorrhage (arrows), and pyknotic nuclei (arrow heads). **C):** The zona reticularis represents dilated sinusoid (S), vacuolation (V), and hemorrhage (arrow). **D, E):** The medulla showing dilated blood sinusoids (S), hemorrhage in between chromaffin follicles (arrows), vacuolation (V)**,** and eosinophilic material (ES)**.** There is crowding and swelling of chromaffin cells with obliteration of many follicular lumens. Scale bars = 25 µm.

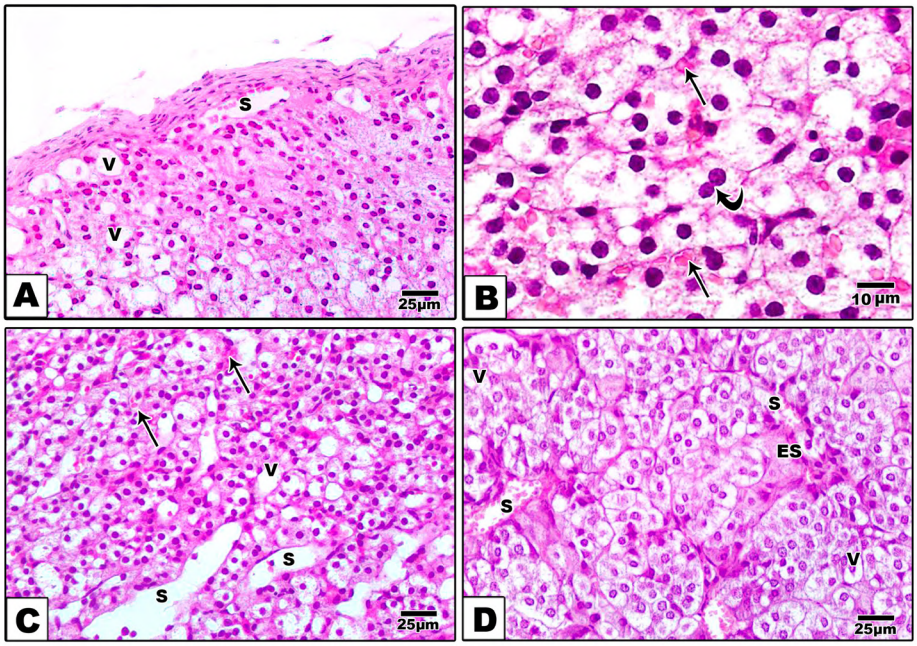

Fig. 6.- Photomicrographs of Hx&E-stained adrenal gland of a REC rat after chronic stress showing **A):** vacuolation (V) and dilatation in blood sinusoids (S) of zona glomerulosa. **B).** The zona fasciculata illustrates hemorrhage (arrows) between its cells. The curved arrow points to the mitotic nucleus**. C):** The zona reticularis represents vacuolation (V), hemorrhage (arrows), and dilated blood sinusoids (S). **D)**: The medulla showing dilated blood sinusoids (S), vacuolation (V)**,** and extracellular eosinophilic material (ES)**.** Scale bars = 25 µm.

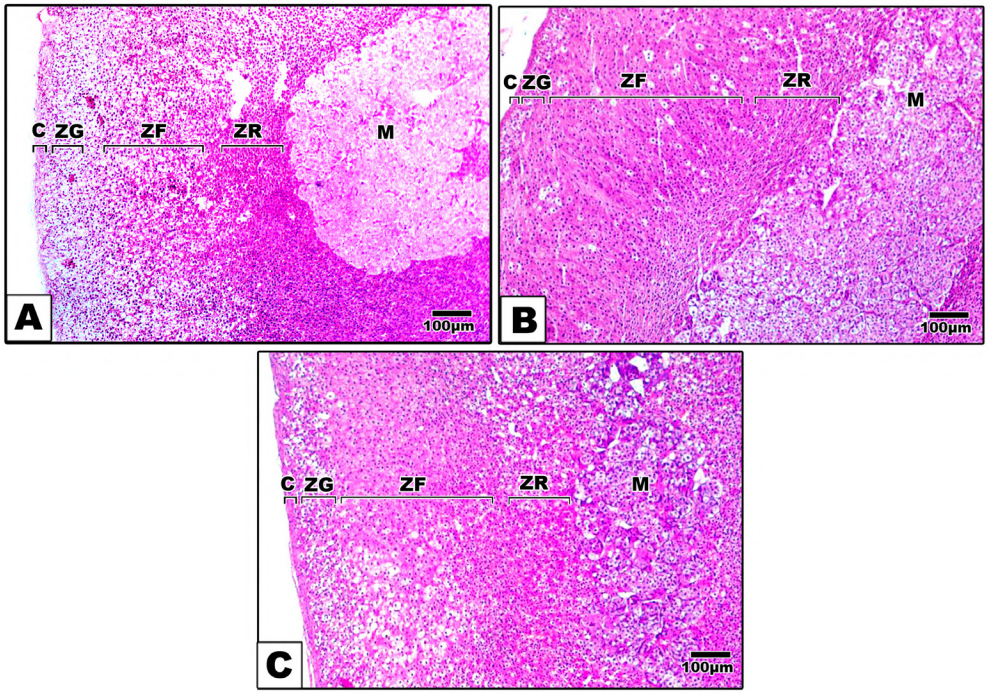

Fig. 7.- Panoramic of rat adrenal glands of **A):** CN, **B):** CS, and **D):** REC rats showing the capsule (C), the three cortical layers; zona glomerulosa (ZG), zona fasciculate (ZF), zona reticularis (ZR), and medulla (M). Scale bars = 100 µm.

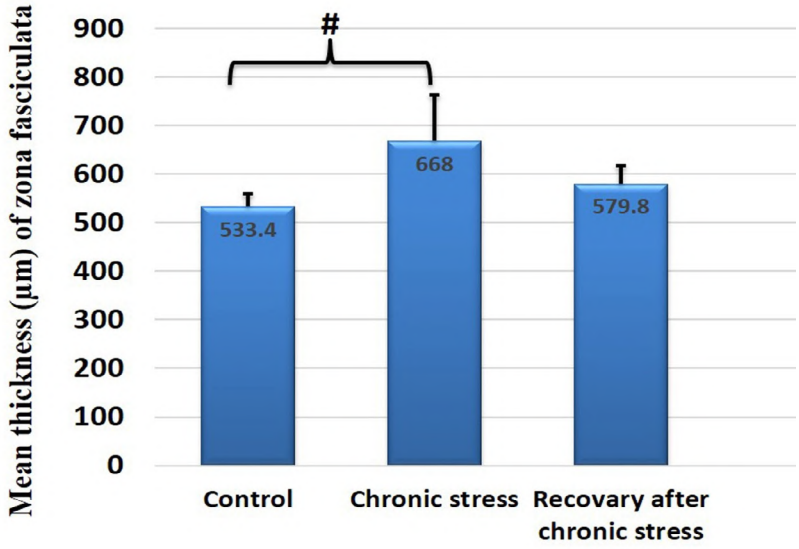

Fig. 8.- Histogram showing significant increase in the ZF thickness in the CS group. #p<0.05.

(0.171±0.023), and a significant increase (*P<0.01*) in the nor-adrenaline cells (0.277±0.066). Regarding REC group (Figs. 9E, F), although it illustrated a non-significant difference (*P<0.992, P< 0.655*) in the OD of adrenaline cell (0.171±0.036) compared to CS and CN groups, and in OD of nor-adrenaline cells (0.21±0.034) compared to CN group (*P<0.102*), it had a significant decrease effect (*P<0.034*) on OD of nor-adrenaline cells compared to CS group (Fig. 10).

Assessment of the immunohistochemical results

Immunohistochemistry for Nestin

The Nestin immunohistochemical reaction in CN group was represented as diffuse and dotted in the cytoplasm of ZG cells (Fig. 11A). The immune stain was scattered in cells of ZF (Fig. 11B) and ZR (Fig. 11C). The medulla showed a strong positive reaction (Fig. 11D).

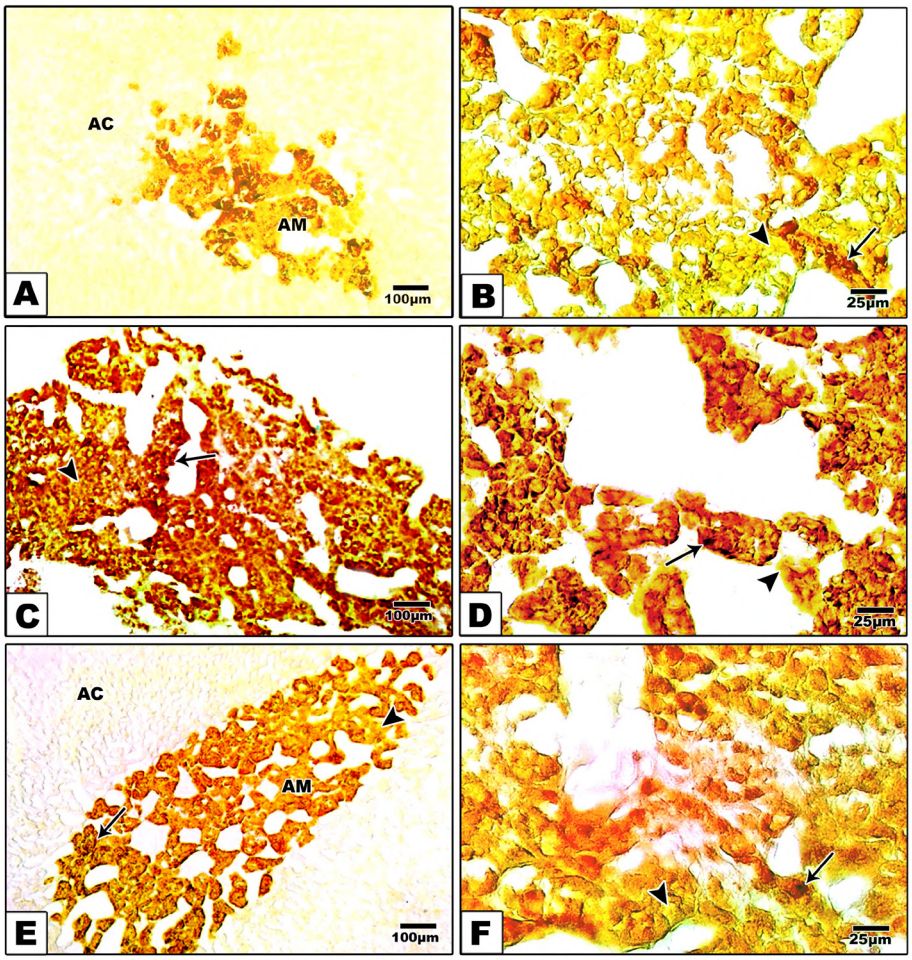

Fig. 9.- Chromaffin reaction in the adrenal glands of **A, B):** CN rat showing positively stained central brown medulla (M) and a negatively stained peripheral yellowish cortex (C). The catecholamine granules in adrenaline secreting cells (blue arrow) are stained yellowish-brown while those in noradrenaline secreting cells are stained dark brown (black arrow). **C, D):** CS rat. Showing the catecholamine granules in adrenaline secreting cells (blue arrow) were moderately stained yellowish-brown while those in noradrenaline secreting cells are strongly stained dark brown (black arrow). **E, F):** The REC rat after chronic stress showing the catecholamine granules in adrenaline secreting cells (blue arrow) were moderately stained yellowish-brown and those of noradrenaline secreting cells were moderately stained dark brown (black arrow). Scale bars A, C,E = 100 µm; B,D,F = 25 µm.

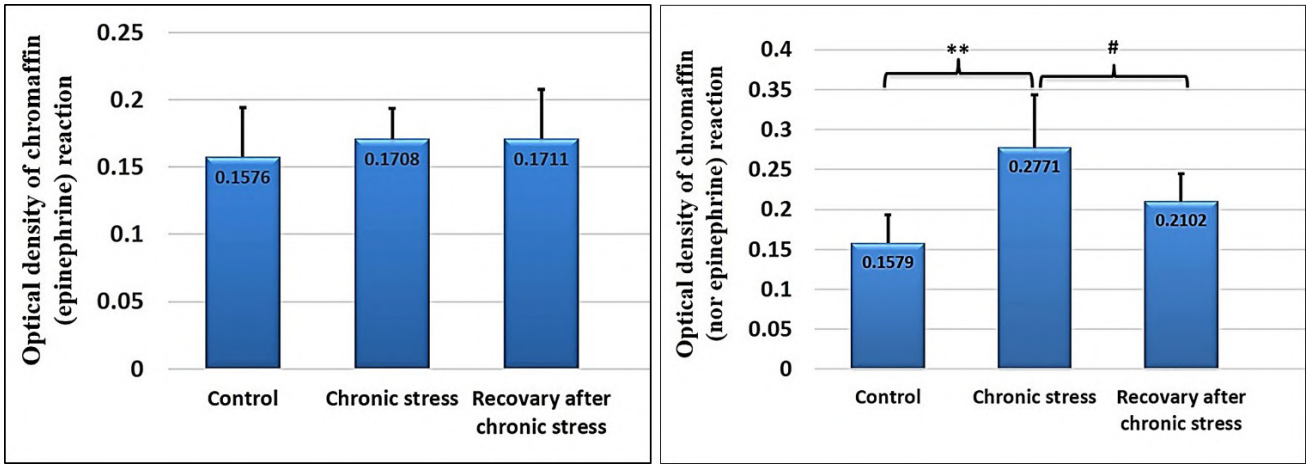

Fig. 10.- Histograms illustrate the statistical differences in the OD of the chromaffin reaction (epinephrine and nor-epinephrine respectively) between the studied groups. The epinephrine reaction represents a non-significant difference between the studied groups. The nor-epinephrine reaction represents a significant increase in the CS group comparing both CN and REC groups. #p<0.05, **p<0.001.

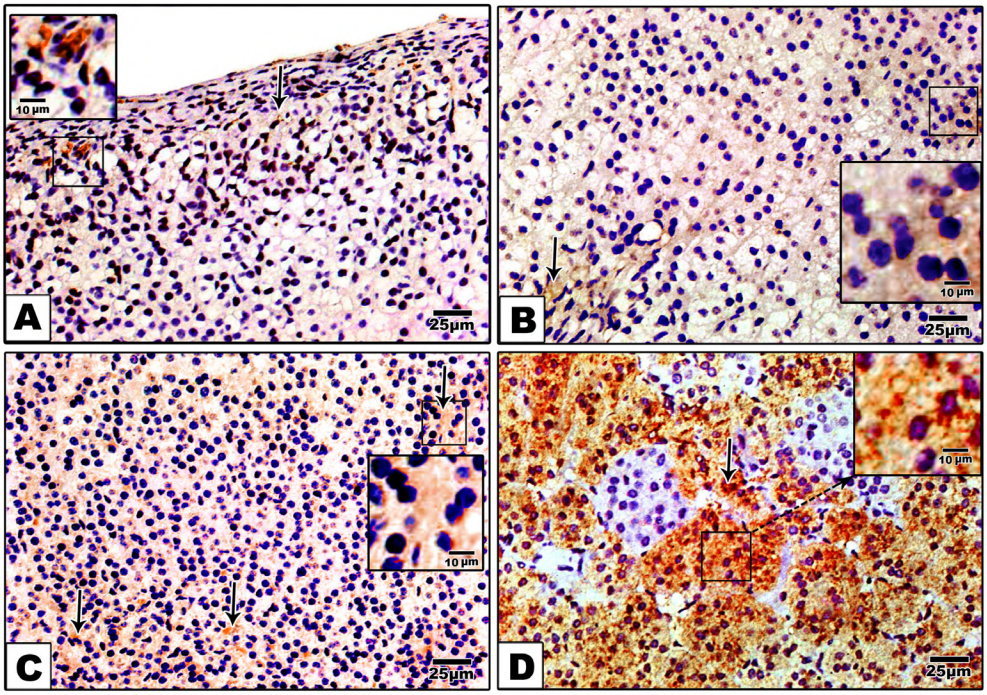

Fig. 11.- Nestin immunostained suprarenal gland sections of the CN rat showing **A):** diffuse and dotted (magnified square) faint immunohistochemical reaction (arrow) in the cytoplasm of the capsular and subcapsular region (zona glomerulosa)**. B):** scattered immunostaining (arrow) is in the cells of zona fasciculate (magnified square)**. C):** dispersed immunoreaction (arrows) in the zona reticularis. **D):** There is a strong positive immune reaction (arrow) in the medulla**.** (Nestin immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

The CS group showed weak Nestin immunostaining in the cytoplasm of capsular and subcapsular (ZG) regions (Fig. 12A), ZF (Fig. 12B), ZR (Fig. 12C), and medulla (Fig. 12D), with a highly significant decrease (*P<0.0001*) in OD of the total adrenal Nestin-stained cells (0.097±0.023) as compared to CN group (0.160±0.029). The REC group showed a mild Nestin immunohistochemical reaction in ZG

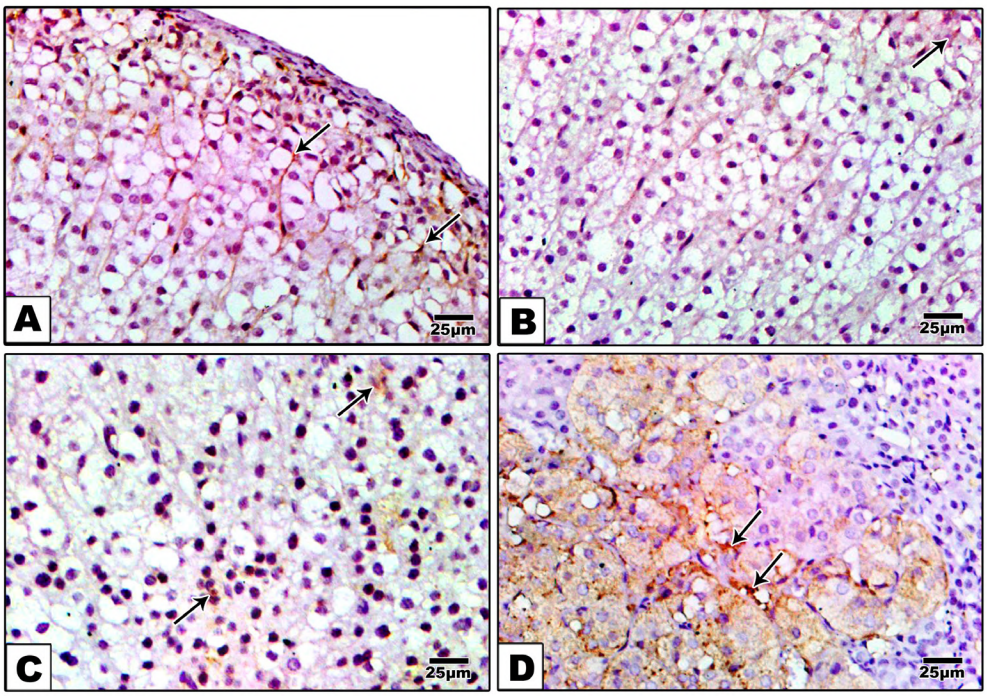

Fig. 12: Nestin immunostained suprarenal gland sections of the CS rat showing **A):** moderate weak positive immunoreaction (arrows) in the cytoplasm of capsular and subcapsular region (zona glomerulosa)**. B):** faint immunoreaction (arrows) in zona fasciculata**. C):** Cells of the zona reticularis weakly express the immune stain (arrows). **D):** There is decreased positive immune reaction in the medulla (arrow)**.** (Nestin immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

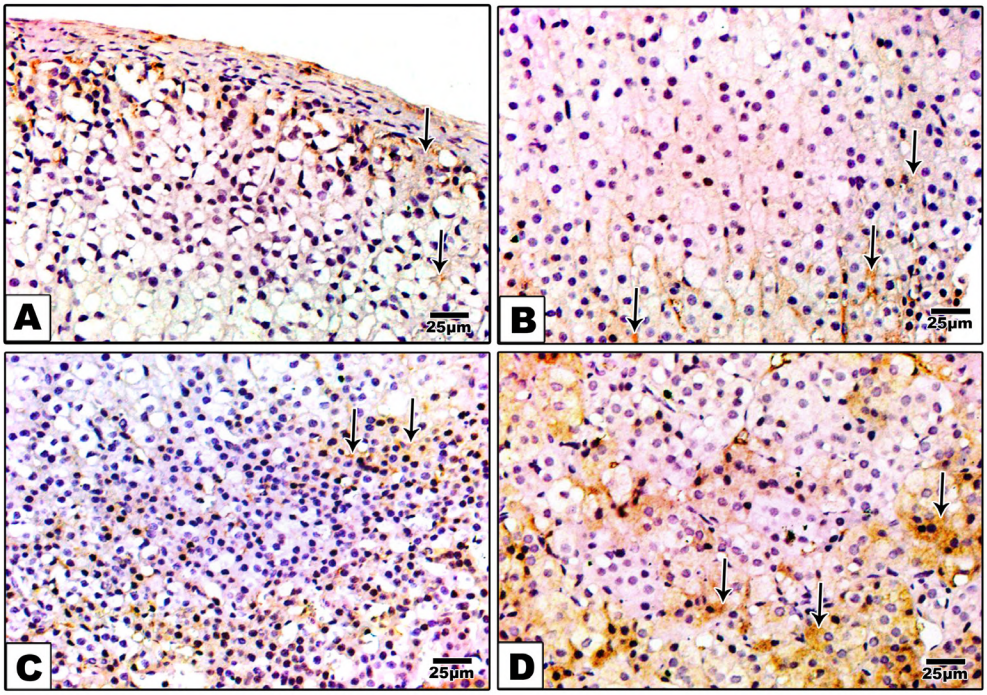

Fig. 13.- Nestin immunostained suprarenal gland sections of the REC rat after chronic-stress showing **A):** weak positive immunoreaction (arrow) in the cytoplasm of the capsular and subcapsular region (zona glomerulosa layer)**. B):** weak immunoreaction (arrows) in zona fasciculata**. C):** faint immunoreaction (arrows) in the zona reticularis. **D):** There is moderate positive immunoreaction (arrow) in the medulla**.** (Nestin immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

(Fig. 13A). Cells of ZF (Fig. 13B) and ZR (Fig. 13C) showed weak expression of the immune stain. There is a moderate immunohistochemical reaction in the medulla (Fig. 13D). The REC group showed a significant increase (*P<0.012*) in OD of total adrenal Nestin-stained cells (0.137±0.033) as compared to CS group, and a non-significant decrease (*P<0.155*) with CN group (Fig. 14).

Immunohistochemistry for Chromogranin-A

The chromogranin-A immunohistochemical reaction in CN group represented as diffuse and dotted cytoplasmic reaction in the cells of adrenal medulla (Fig. 15A). The CS group revealed a strong cytoplasmic reaction (Fig. 15B) with a significant increase (*P1=0.0001*) in OD (0.130±0.002)

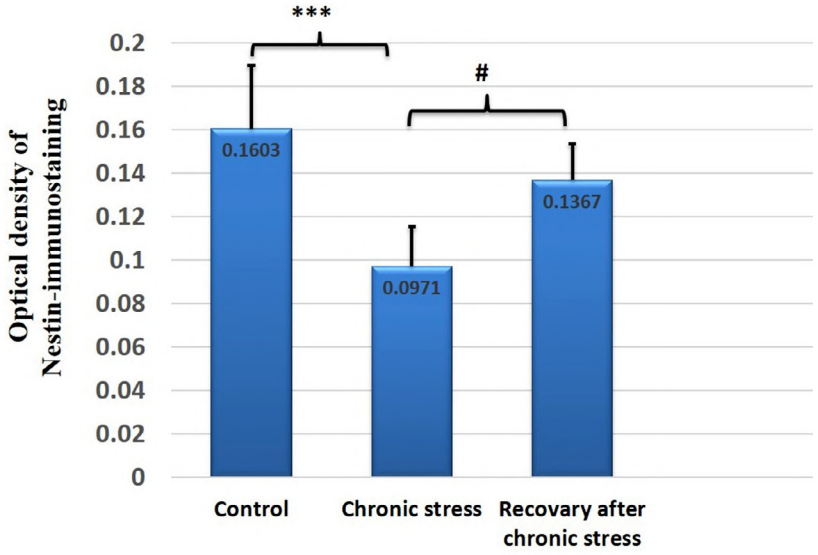

Fig. 14.- Histogram illustrating the statistical difference in the OD of Nestin immunohistochemical staining between the studied groups. It represents a significant decrease in the CS group comparing to both CN and REC groups. #p<0.05, ***p<0.0001.

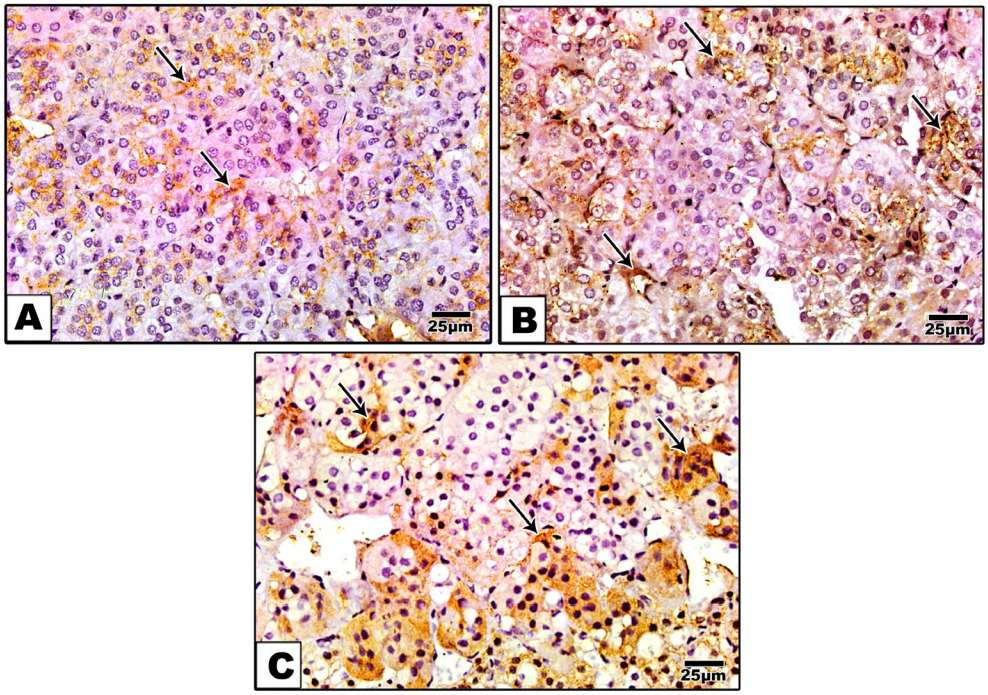

Fig. 15.- Chromogranine-A immunostained suprarenal gland sections of A) CN rat showing strong diffuse and dotted immunoreaction (arrow) in the adrenal medulla. **B):** CS rat showing stronger medullary immunohistochemical reaction (arrows). **C):** REC rat after chronic stress showing moderate medullary immunohistochemical reaction (arrows). (Chromogranine-A immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

as compared to CN group (0.130±0.002). The REC group showed a moderate immunohistochemical reaction (Fig. 15C) with a significant reduction (*P<0.004*) in OD of chromogranin-A positive cells (0.276±0.013) as compared to CS group, and significant elevation (*P< 0.0001*) as compared to CN group (Fig. 16).

Immunohistochemistry for GFAP

The positive GFAP immunoreaction in the CN group appeared diffuse cytoplasm staining in the cortical and medullary cells. The capsule showed strong positive immunoreaction (Fig. 17A). The ZG (Fig. 17A), ZF (Fig. 17B), and ZR (Fig. 17C) showed moderate positive reactions. The medulla showed a strong positive reaction (Fig. 17D).

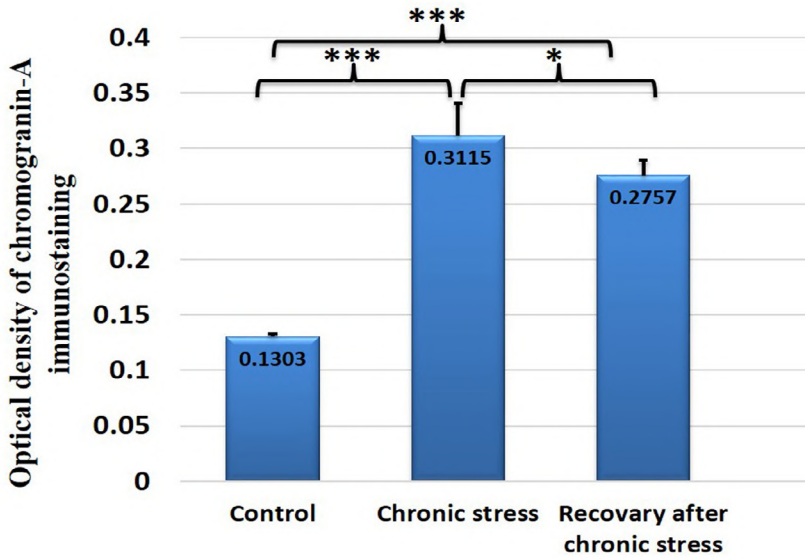

Fig. 16.- Histogram illustrates the statistical difference in the OD of the chromogranin-A immunohistochemical staining between the studied groups. It represents a significant increase in both CS and REC groups comparing to the CN group. *p<0.01, ***p<0.0001.

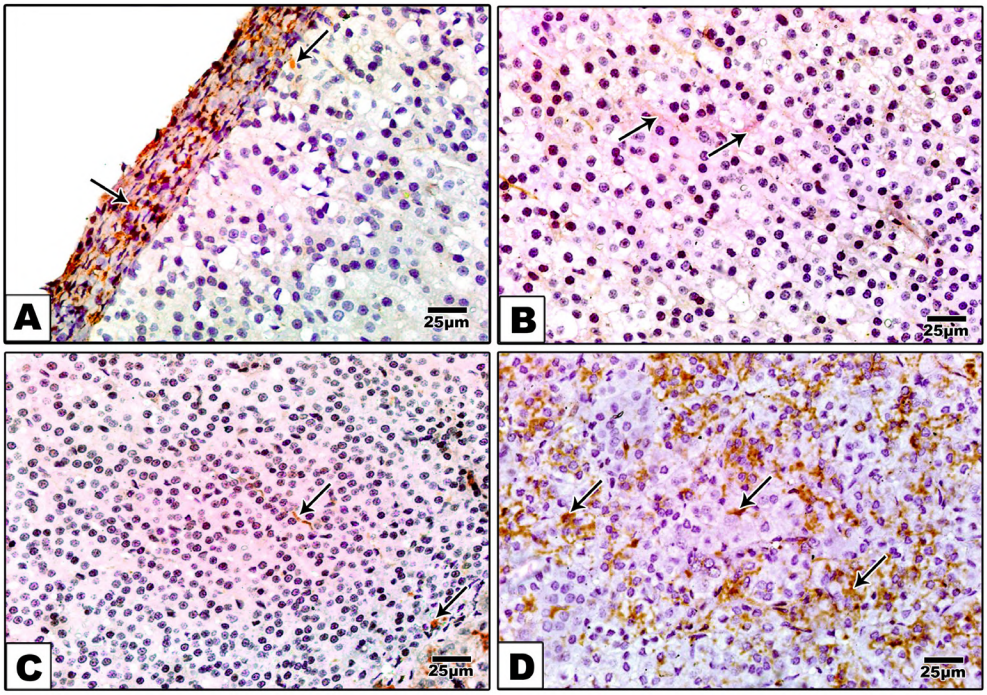

Fig. 17.- GFAP immunohistochemical stained suprarenal gland sections of CN rat showing **A):** diffuse strong immunoreaction (arrows) in the cytoplasm of the capsular and subcapsular region (zona glomerulosa)**. B):** moderate immunostaining (arrows) is in the cells of zona fasciculate**. C):** moderate immunoreaction (arrows) in the zona reticularis. **D):** There is a strong immunoreaction (arrow) in the medulla (magnified square)**.** (GFAP immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

The CS group revealed a strong cytoplasmic reaction in the capsular and ZG cells (Fig. 18A). Cells of ZF (Fig. 18B) and ZR (Fig. 18C) showed weak GFAP immune expression. There was a weak positive immune reaction in the medulla (Fig. 18D). This CS group illustrated a significant decrease (*P<0.01*) in OD of total adrenal GFAP positive cells (0.086±0.020) as compared to the CN group

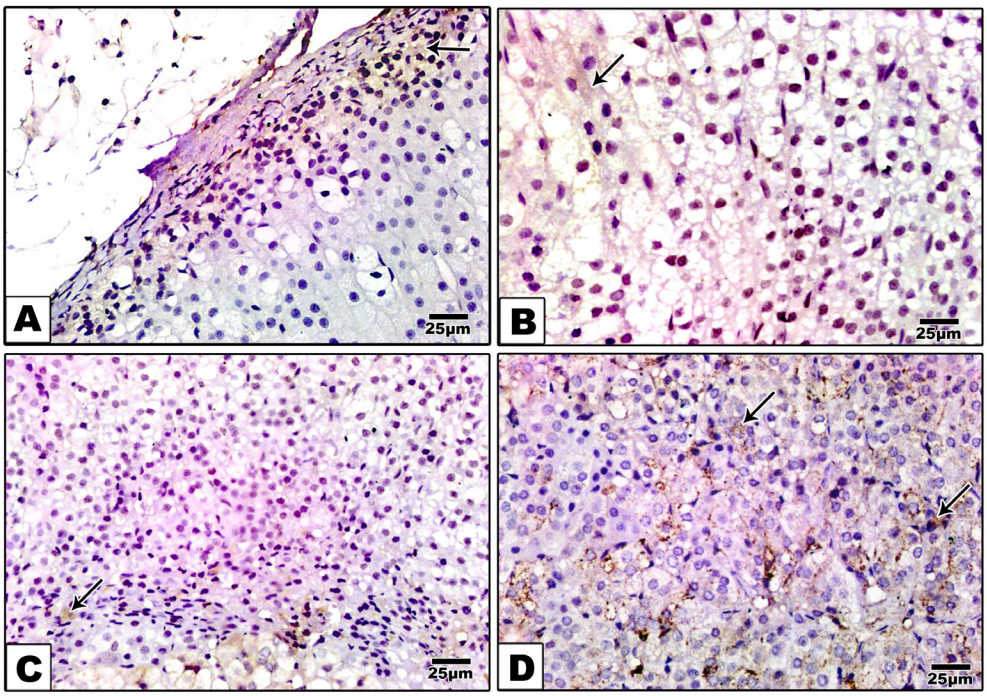

Fig. 18.- GFAP immunohistochemical stained suprarenal gland sections of CS rat showing **A):** strong cytoplasmic reaction (arrows) in the capsular and subcapsular region (zona glomerulosa)**. B):** weak immunoreaction (arrow) in zona fasciculata**. C):** Cells of the zona reticularis faintly express the immune stain (arrows). **D):** There is a weak positive immune reaction in the medulla (arrows)**.** (GFAP immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

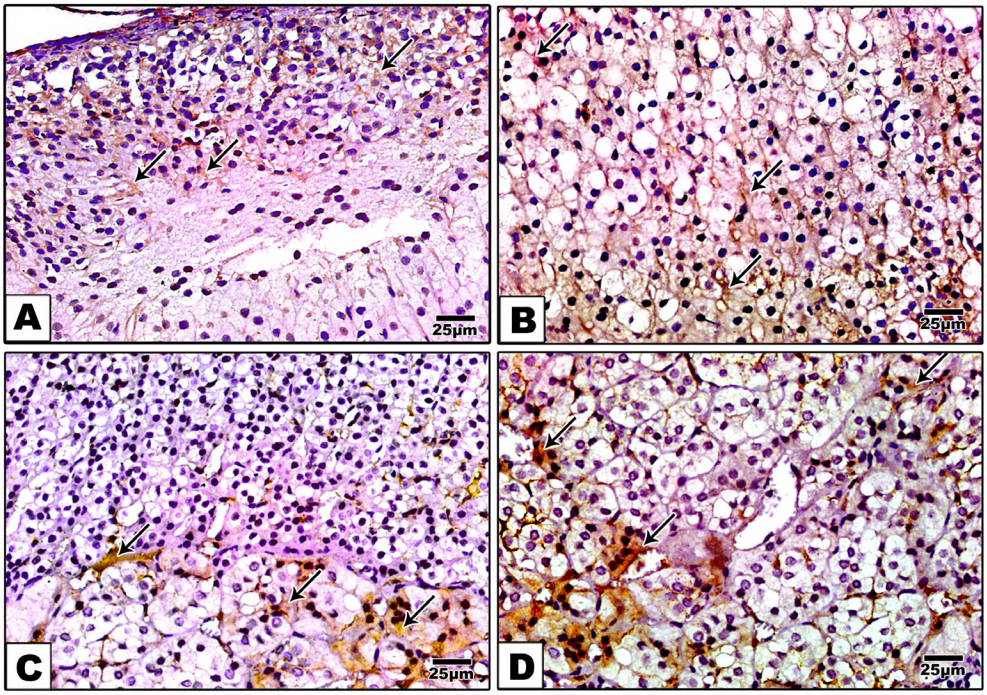

Fig. 19.- GFAP immunostained suprarenal gland sections of the REC rat after chronic stress showing **A):** moderate positive immunoreaction (arrow) in the cytoplasm of capsular and subcapsular region (zona glomerulosa layer)**. B):** weak immunoreaction (arrows) in zona fasciculata**. C):** weak immunoreaction (arrows) in the zona reticularis. **D):** There is a strong positive immunoreaction (arrow) in the medulla**.** (GFAP immunohistochemical stain with Hx&E counterstain). Scale bars = 25 µm.

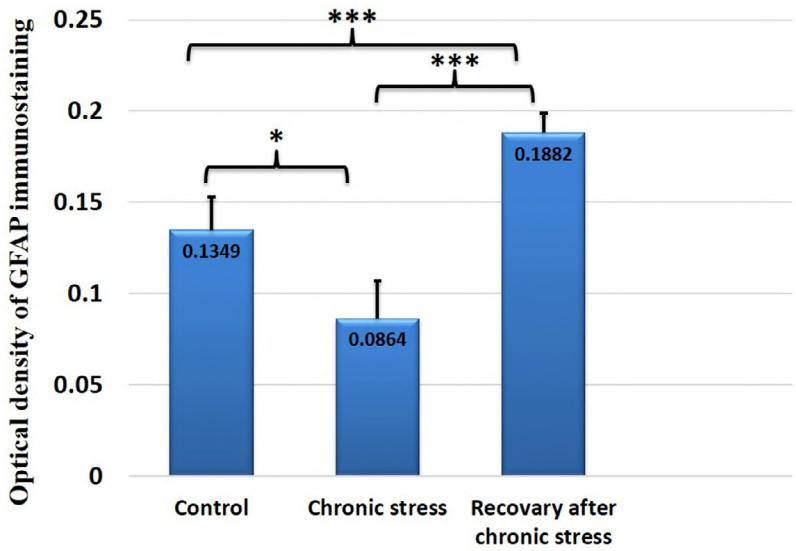

Fig. 20.- Histogram showing the statistical differences in the OD of GFAP immunohistochemical staining between the studied groups. It represents a significant decrease in the CS group comparing both CN and REC groups. The REC group illustrates a significant increase comparing to the CN group. *p<0.01, ***p<0.0001.

(0.135±0.018). The REC group showed a moderate immunoreaction in the cytoplasm of the capsular and ZG cells (Fig. 19A). The ZF (Fig. 19B) and ZR (Fig. 19C) faintly express the immune stain. There is a strong immunoreaction in the medulla (Fig. 19D). The REC group represented a highly significant elevation (*P<0.0001*) in OD of total adrenal GFAP positive cells (0.188±0.0108) as compared to CS and CN groups (Fig. 20).

DISCUSSION

The outcomes of this experiment are of interest from two points of view: first, they extend our knowledge about the impact of the chronic stressor on the function and structure of the adrenal gland; and second, they assess the existence of adrenal gland progenitor cells, with their possibility of transformation into new chromaffin and glial cells. The present study showed that there were a decreased number of progenitor cells after exposure to stress due to its transformation into chromaffin and glial cells.

In the present work, chronic stress was achieved by restricting the movement of each rat in a mesh restrainer two hours per day for six days. Restraint stress is the most widely used stressor in rodents. The effects of restraint stress are evidenced with impairments in the immune, neuroendocrine, and cognitive functions (Mo et al., 2019).

Our study showed a decrease in the body weight of CS group compared with CN group, while, when the rats were allowed to recover for seven days, they regained their body weight. These findings were in agreement with previous studies (Bhatnagar et al., 2006; Rubin de Celli et al., 2015). The reduction in body weight of CS rats can be explained by the affection of appetite. Stress decreases body weight by reduction of food and water intake (Ranjbaran et al., 2013) and anorexia induction (Halataei et al., 2011) due to the central release of CRH during stress that suppresses food ingestion (Grill et al., 2000; Heinrichs et al., 2001). Also, this effect is linked to the involvement of either the amygdala or the ventral tegmental area (Nasihatkon et al., 2014; Sadeghi et al., 2015).

The present study showed that CS had a significant increase in the adrenal gland weight versus CN. The REC group showed a non-significant effect on the adrenal gland weight versus the CS group, and a significant increase versus the CN group. Our findings were in agreement with Rostamkhani et al. (2012), who stated that chronic stress causes a significant increase in the weight of adrenal glands. Also, Rubin de Celli et al. (2015) found that the adrenals of chronically strained rats are increased in size by approximately 22%. The increase in the adrenal weight could be explained by the increased corticosterone demand and the capacity of the gland to produce higher amounts of corticosterone.

The present study showed a marked elevation in the cortisol level of CS group. The REC group showed a highly significant reduction versus CS group, but still significantly higher than CN group. Similar results were documented by several studies (Kiank et al., 2010; Cho et al., 2011; Zhang et al., 2011; Jameel et al., 2014). The endocrine system responds to stress by the interaction between the HPA and the adrenomedullary neuroendocrine system, accompanied by the release of adrenocortical glucocorticoids and adrenomedullary epinephrine (Demirci and Sahin, 2017).

In this study, the CS group showed several pathological changes in the adrenal cortices such as cellular edema, vacuolations, pyknosis, vascular congestion, and atrophy (decrease in its thickness) of ZG. Inconsistent with these results, Howard (2018) documented that vacuolar degeneration in ZG and ZR is a nonspecific pathology related to trauma, stress, or disease. In the medulla, the present study reported extracellular eosinophilic material, dilated congested blood sinusoid, and degenerated chromaffin cells. The extracellular eosinophilic material noted in the adrenal medulla is probably leaked serum from the arteries and veins.

Adrenal hemorrhage, congestion, and dilatation of blood sinusoids were associated with chronic stress in the present study. These changes were due to an increased ACTH level, which motivates the catecholamines release, thus increasing the adrenal blood flow and inducing vasoconstriction. This results in congestion with increased pressure in the capillaries (Simon and Palese, 2009). Moreover, Zidan and Elnegris (2013) reported that ACTH elevation enhances the prostaglandins formation, which is responsible for tissue congestion.

In the current study, the chromaffin reaction in the CS group illustrated a marked increase in the nor-epinephrine secreting cells which was significantly decreased to CN level after recovery. Sabban et al. (2012) found that the adrenal medulla increased the neurosecretory capacity

with more efficient catecholamine storage in response to repeated exposure to stress. Tank and Lee Wong (2015) demonstrated that the body responds to stress by activating catecholaminergic neurons in the brain stem; then the spinal cord efferent stimulates the sympathetic nervous system and adrenal medulla to secrete epinephrine and nor-epinephrine.

In the current study, the evaluation of immunohistochemical data revealed that the positive immunoreaction reaction of Nestin is diffuse and dotted in the cytoplasm of ZG cells, and dispersed in ZF and ZR. The medulla showed a strong positive reaction. Inconsistent with our findings, Steenblock et al. (2018) demonstrated the highest prevalence of Nestin-stained cells is in the medulla and ZG directly beneath the capsule, while a few positive cells were scattered in ZF and ZG. Chang et al. (2013) and Finco et al. (2018) reported that distinct populations of adrenocortical progenitors located in the subcapsular region were responsible for the regeneration of the adrenal gland. Langton et al. (2018) have demonstrated the presence of Nestin-immunostained cells in the medulla adult adrenal gland.

Under basal conditions, these progenitors are displaced centripetally through the different zones of the adrenal cortex until they reach the corticomedullary boundary, where they become apoptotic (Chang et al., 2013; Steenblock et al., 2018). Nestin-positive cells traverse the whole adrenal, so that they connect the medulla with the capsule and make direct signaling. Moreover, the sub-capsular Nestin-positive cells are interconnected all around the capsule (Steenblock et al., 2018).

It was reported that Nestin levels reduced on cell differentiation, but elevated transiently after neuronal injury (Bott et al., 2019). The present study demonstrated a highly significant decrease in the total adrenal Nestin-immunoreaction in the CS group as compared to the CN group. However, the REC group showed a significant increase as compared to the CS group and a non-significant decrease with the CN group. In line with our results, Rubin de Celli et al. (2015) documented low proliferation rate of Nestin-positive cells at basal conditions, and increased proliferation after acute stress (one-day stress). When stress became chronic, the number of Nestin cells in the medullae was significantly decreased by 50%, indicating their participation in the gland adaptation. After the resting period, the number of Nestin-stained cells had returned to their basal levels.

Under basal conditions, Nestin-positive cells can differentiate into neuronal, glial, and chromaffin cells. However, under stress, they preferentially differentiate into chromaffin cells (Rubin de Celli et al., 2015). The chromogranin-A (Co-A) is the chief protein for the medullary chromaffin cells (Machado et al., 2010). There was no detected cellular co- staining for Nestin and chromogranin-A in the previous studies on the adult adrenal gland, suggesting that mature chromaffin cells never express Nestin protein (Taupenot et al., 2003).

This study detected positive chromogranin-A cytoplasmic immunoreaction in the medullary cells. This reaction increased significantly in CS group versus CN group. The recovery for seven days resulted in a significant decrease as compared to CS group but still significantly higher than CN group.

It has been shown that repeated immobilization stress resulted in increased the level of catecholamine. This was related to augmented catecholamines synthesis due to the increased expression of enzyme that converts norepinephrine to epinephrine (Kvetnansky et al., 2013). In addition to this molecular adaptation, the results in Rubin de Celli et al. (2015) illustrated cellular adaptation by recruiting new chromaffin cells from the stem cell pool located in the medulla of the adult adrenal gland.

Unlike the chromogranin-A, Rubin de Celli et al. (2015) showed that nearly 62% of the cells in the adrenal medulla which are stained with Nestin were co-stained for GFAP, specifying parallel features of with Nestin stem cells.

In this study, the adrenal GFAP immunohistochemical reaction illustrated a significant decrease in the CS group versus the CN group. The REC group had a highly significant increase versus the CS and CN groups. Our results were in agreement with Rubin de Celli et al. (2015),as who

found that GFAP/Nestin-positive immune stained cells were significantly reduced in the stressed groups compared with the CN group. These results suggested that the Nestin-positive medullary stem cells behave as activated glial cells and they proliferate to increase this glia population.

In conclusion, under normal conditions, the differentiation of adrenal progenitor cells was towards the glial cell lineage. Conversely, with chronic stress, the differentiation significantly proceeded towards the chromaffin lineage resulting in overproduction of chromaffin cells for stress adaptation.

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