Original Article

Effect of Chronic Stress on Suprarenal Cortex and Possible Therapeutic Potential of Ibuprofen versus Mesenchymal Stem Cells-Microvesicles in Adult Male Albino Rats: Histological and Immunohisochemical Study

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ABSTRACT

Introduction: Humans are constantly subjected to different stressors that endanger their ability to live a normal life. Since the adrenal gland is the primary organ implicated in the stress response, the purpose of this study was to assess the effects of stress on this organ and the contribution of ibuprofen and Mesenchymal stem cells microvesicles to ameliorate these effects.

Materials and Methods: Four groups of ten rats each: control group, stress group that subjected to various forms of stress for two weeks Ibuprofen group received an intraperitoneal injection of 60 mg/kg ibuprofen after the stress period. MSC-MVs group received a single intravenous injection of 15 ul of MSC-MVs in 0.5 ml PBS after the stress. The rats then were sacrificed and adrenal gland samples were prepared for immunohistochemical staining and light microscopy. Blood samples was taken for analysis of cortisol, aldosterone, malondialdehyde, and interleukin-6 levels.

Results: Sections stained with H&E and immunohistochemical stains showed a mild improvement in ibuprofen group. The biochemical results confirmed that MSC-MVs group results were equivalent to those of the control group at the same period. There was no observable difference between the control group and MSC-MVs group.

Conclusion: When given chronically and simultaneously under stressful settings to male albino rats, MSCs-MVs improves the histological alterations of the adrenal gland. Ibuprofen also helps these modifications, but to a lesser extent.

Key Words: Ibuprofen, microvesicles, stress.

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INTRODUCTION

Stress is characterized as a condition of disturbed equilibrium that enables living things to adapt to outside stimuli. Animals typically adjust to predictable environments. However, unanticipated life events are a form of stress that can affect a person's endocrine and metabolic health[1].

The main mechanisms by which stress is acted upon include the regulation of the steroid, catecholamine, peptide, and opioid systems. The hypothalamic-pituitary-adrenocortical axis (HPA) plays a role in a cascade of neurohumoral events that result in normalization (i.e., homeostasis) once the stress reaction has ended[2].

Through the glucocorticoid (GR) and mineralocorticoid (MR) receptors, corticosteroids control the stimulation and inhibition of the HPA axis[3]. The glucocorticoid receptors are associated with delayed genomic effects, whereas the mineralocorticoid receptors are associated with fast, non-genomic responses[4].

The sympathetic nervous system is activated by stress, which causes the adrenal medulla to release adrenaline quickly. Subsequently, the hypothalamus-pituitary-adrenal (HPA) system is activated, which causes the synthesis and release of corticosteroids, the primary adrenal stress hormone in humans. The central nervous system, the immunological system, the gastrointestinal tract, the cardiovascular system, and the endocrine system are just a few of the body systems that are impacted by the waves of adrenaline and corticosteroids[5].

Cyclooxygenase-2 (COX-2) upregulation is one underlying mechanism for stress-induced depression[6]. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) block the COX to reduce inflammation and relieve pain[7]. Although NSAIDs are widely known for their adjuvant efficacy in the management of inflammation and depression[8], nothing is known about their capacity to fend against long-term stress.

Microvesicles (MVs) are non-nucleated membrane fragments that have been liberated from a variety of cell types' surface membranes. Similar to the cells from which they are released, they include proteins, lipids, organelles, and mRNAs that they transport to the target cells. To avoid the negative effects of stem cells themselves, such as cancer, MVs can be used as an alternative therapeutic approach in regenerative medicine[9].

Stress defined as condition of disturbed homeostasis that permits the living organisms to adapt to environmental

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stimuli. Normally, animals get adapted to the predictable situations. However, the unpredictable situations of life represent a type of stress that include changes in the endocrine and metabolic status of them[1].

MATERIALS AND METHODS

• *Experimental animals*:

In this work, forty adult male albino rats weighing 200 –250 g (14-18 weeks old) were employed. They were acquired from Zagazig University's Faculty of Medicine's Animal House. The Zagazig University Research Ethics Committee gave the experimental protocol permission with the approval number (ZU-IACUC/3/F/263/2022). The rats were kept in a room-temperature environment with regular light and dark cycles for the length of the experiment, and they had unlimited access to food and water. They were separated into four groups, each of which has ten rats:

• **Group I (control group):** Ten rats were given two weeks of stress-free access to food and water without any other interruptions before being sacrificed.

• **Group II (Stress group):** For two weeks, ten rats were subjected to various forms of stress, including physical restraint, food deprivation, and tail pinching (1-min, 1 cm from the distal region of the tail) (The fore limbs and hind limbs of rats were tied separately and then together securing them with adhesive tape thereby immobilizing them for 2h). The rats were sacrificed after the stressors were given daily for two weeks in a random order, with no stressor being given on consecutive days[10].

• **Group III (Stress group with Ibuprofen):** Ten rats had two weeks of exposure to various forms of stress before receiving an intraperitoneal injection of ibuprofen at a dosage of 60 mg/kg for 21 days. Before the injection, ibuprofen was dissolved in distilled water. The rats were then sacrificed[11].

• **Group IV (Stress group+ MSC-MVs group):** Ten rats were exposed to different types of stress for 2 weeks then they received single IV injection of 15µl of MSCs-MVs dissolved in 0.5 ml PBS via tail vein. After 21 days, the rats were sacrificed[12].

II. Histological specimens

The rats were sacrificed, carefully dissected, and anaesthetized using ether inhalation. The adrenal gland samples were processed for light microscopic examination for the following:

- Hematoxylin & eosin (H&E) staining for demonstration of the histological structure.

- Mallory trichrome staining, for detection of collagen fibers deposition.

- Immunohistochemical staining for:

 PCNA, a marker for cellular proliferation

 Caspase-3, a marker for apoptosis

 CD44, as a marker for endogenous and exogenous mesenchymal stem cells and the microvesicles derived from them. Used for detection of homing of the injected microvesicles.

The specimens were then immersed in 10% neutral-buffered formalin, washed, dehydrated, cleared, and embedded in paraffin. H&E and Mallory stains were applied to sections that were 5 m thick[13]. In order to perform the immunostaining procedures for PCNA, Caspase3 & CD44, 3–4 µm thick paraffin was prepared. They underwent deparaffinization, rehydration, and a five-minute wash in PBS at a pH of 7.2. Utilizing peroxidase block (3% hydrogen peroxide in water), endogenous peroxidase activity was suppressed. Sections were stained using the antigen retrieval technique since numerous antigens were obscured by the tissues' formalin fixation, which created intermolecular cross-links. To prevent non-specific immunoglobulin binding, sections were coated with 2 drops of the blocking agent (normal rabbit serum) and placed in a humidified chamber for 30 minutes. Each portion received one to two drops of the monoclonal antibody[14].

III. Biochemical investigations

At the end of the experiment, blood samples were collected from the retro-orbital veins in non-heparinized tubes under light anesthesia with ether. Serum separation was done by centrifugation at 4000 g for 20 min and stored at −20°C.

- **Estimation of the levels of blood cortisol and aldosterone:** from all rats by using colorimetric kits (purchased from Bio-diagnostic Co., Giza, Egypt) according to the manufacturer's instructions.

- Estimation of malondialdehyde (MDA):

It is considered as lipid peroxidation index. MDA 586™ (R&D, Europe Ltd, Abingdon, Oxon, UK) was used. Adrenal glands samples were rinsed in ice cold Dulbecco's phosphate buffered saline (PBS) and frozen immediately at 70°C. Lipid peroxidation was estimated by adding 1 ml Dulbecco's PBS with 5 mM butylated-hydroxyltoluene to the samples and then homogenized. After homogenization the samples were centrifuged at 4000 × g for 10 minutes (min) at 4°C. An aliquot (200 µl) of the standard and/or the supernatant was added to a reaction mixture containing 640 µl of N-methyl-2-phelindole, 10 µl probucol and 150 µl of 12 M hydrochloric acid. The samples were then incubated in a water bath for 60 min at 45°C and centrifuged at 10000 × g for 10 min at 4°C. The absorbance of the standard and supernatant was measured by Spectrophotometry at 586 nm. The results were expressed as nmol MDA/g tissue[15].

- Estimation of interleukin-6 (IL-6):

Adrenal samples were homogenized in 10 mM Tris buffer (pH 7.4) containing 2 M NaCl, 1 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride, and 0.01% Tween 80 followed by centrifugation at 8500 RPM for 30 min at 4°C. The resulting supernatant was collected and stored at−20°C for biochemical analysis[16].

IV. Morphometric study:

Digimizer 4.3.2 (MedCalc Software bvba, Belgium) was used to calculate the average area percentage of mallory stained sections from images taken at a magnification of X 400. Three different fields/rat from each group were randomly chosen. Using image J image analysis software (National Institute of Health; NIH, Bethesda, MD, USA), the mean colour intensity of positive immunohistochemistry reactions for PCNA Caspase3 & CD44 were performed within 6 fields for each rat at a total magnification of 400.[17].

STATISTICAL ANALYSIS

Statistical analysis was performed for the area percentage of mallory positive reaction sections and the area and the area % of positive immune reaction for PCNA Caspase3 & CD44. All values of the experiments were represented as mean ± Standard Deviation (SD). One-way analysis of variance (ANOVA) was used, followed by Post hoc least significant difference (LSD) test to evaluate the differences between the groups. For all comparisons $P<0.05$ were considered as significant difference. All analyses were performed using the IBM SPSS 19.0 software[17].

RESULTS

A) General observation:

All control subgroups revealed similar biochemical and histological results. Thus, they were collectively referred to as control group (group I).

B) Biochemical results and statistical analysis: (Table 1, Fig 1)

The mean values of serum aldosterone and serum cortisone were significantly increased in stress-group (group II) versus control group (I), and significantly decreased in both ibuprofen (III) and microvesicles treated (IV) groups. Moreover, the levels of tissue malondialdhyde (MDA) and interleukine 6 (IL-6) showed significant increase in the stress group (II) as compared to other groups. No significant difference was detected between microvesicle treated group (IV) and control (I) group.

C) Histological results:

- Light microscopic results:

1- H&E-stained sections:

Examination of sections of the suprarenal cortex of the control group revealed normal histological architecture. It was formed of cortex and medulla and surrounded by connective tissue capsule. The suprarenal cortex was composed of 3 zones: outer zona glomerulosa (ZG), middle zona fasciculata (ZF) and inner zona reticularis (ZR). The zona glomerulosa is consists of arched clusters of cells with blood sinusoids in between. The zona fasciculata is consisted of rows of large polyhedral cells with vesicular nuclei and vacuolated cytoplasm and separated by blood sinusoids (Fig 2A). Zona reticularis was composed of anastomosing cords of small dark stained cells with blood sinusoids in between (Fig 2B).

Examination of H&E-stained section of the suprarenal cortex of Stress group (group II) showed thickened capsule with subcapsular hyperplasia The cells of the zona glomerulosa shows marked vacuolations and numerous dark pyknotic nuclei (Fig 3A). Other section showed marked congestion of the capsular and subcapsular blood capillaries, subcapsular hyperplasia many vacuolated cells, while other cells show dark acidophilic cytoplasm and pyknotic nuclei (Fig 3B). Section in the Zona fasciculata (ZF) shows severe vacuolation of the cells, numerous darkly stained nuclei and massive congestion of the blood sinusoids (Fig 3C). Another section of the same group showing massive inflammatory cellular infiltrations (Fig 3D). The zona reticularis of the same group showed numerous vacuolated cells with pyknotic nuclei with massive congestion of the sinusoids. The medulla can be noticed (Fig 3E).

H&E-stained section of the suprarenal cortex from Ibuprufen-treated group showed irregular separated capsule, the zona glomerulosa (ZG) showing many vacuolated cells and residual pyknotic nuclei. The zona fasciculata (ZF) also has some vacuolated cells and residual pyknotic nuclei. Dilated blood sinusoids are still noticed (Fig 4A). The zona reticularis (ZR) shows residual congestion of the blood sinusoids. Some vacuolated cells and few residual dark pyknotic nuclei were still present (Fig 4B).

H&E-stained section of the suprarenal cortex from the microvesicles-treated group showed thin capsule, the zona glomerulosa (ZG) showing normal organization with apparently normal histological structure, cells have pale cytoplasm with vesicular nuclei. The cells of the zona fasciculata (ZF) normally organized with vesicular nuclei separated with blood sinusoids, few dark stained nuclei were still noticed (Fig 5A). most cells of the zona fasciculata are apparently normal with few residual vacuolated cells. Cells of the zona reticularis (ZR) appears normal with acidophilic cytoplasm and vesicular nuclei separated by blood sinusoids. Few residual dark pyknotic nuclei is present (Fig 5B).

2- Mallory trichrome-stained sections:

Mallory trichrome stained sections of the suprarenal cortex in the control group show minimal amount of bluish stained collagen fibers in the capsule with very thin reticular fibers in between cells (Fig. 6A). A section in the stress group shows excessive deposition of collagen fibers within the capsule with moderate deposition of reticular fibers in between cells (Fig. 6B). A section in the Ibuprofen-treated group showed moderate deposition of collagen fibers within the capsule with minimal deposition of reticular fibers in between cells (Fig. 6C). A section in the microvesicles-treated group shows minimal amount of

collagen fibers within the capsule with minimal deposition of reticular fibers in between cells (Fig. 6D).

• Immune-histochemical staining:

1- PCNA immune-stained sections:

Photomicrograph of PCNA stained sections of the suprarenal cortex in the control group showed some PCNA positive cells with moderate nuclear reaction (Fig 7A). Stress group section showed many PCNA positive cells (Fig 7B). Ibuprofen-treated group showed multiple positive cells for PCNA immune reaction (Fig 7C). microvesicles treated group shows numerous PCNA positive cells (Fig 7D).

2- Cleaved caspase-3 immune-stained sections

Photomicrograph of cleaved Caspase-3-stained sections of the suprarenal cortex in control group section shows few caspase-3 positive cells with weak nuclear reaction (Fig 8A). Stress group section shows numerous positive cells (Fig 8B). Ibuprofen-treated group shows many positive cells for caspase-3 immune reaction (Fig 8C). The microvesicles-treated group shows only few positive cells (Fig 8D).

3- CD-44 immune-stained sections

Photomicrograph of CD44 stained sections of the suprarenal cortex of the control group showed negative reaction (Fig 9A). The stress group section showed some positive cells (Fig 9B). While in the microvesicles-treated group extensive positive cytoplasmic reaction at most of the cells was detected (Fig 9C).

(D) Morphometrical results: (Table 2, Fig 10)

1- The mean area percentage of collagen fibers: It showed high significant increase in the stress group (II) as compared to the control (I) and high significant decrease in ibuprofen (III) and Microvesicles (IV) treated groups. No significant difference between group IV and group I (Fig 10A)

2- The mean number of PCNA immune-positive cells: It showed high significant increase in the Microvesicles treated group (IV) as compared to the other groups. Also, there is a significant increase in ibuprofen (III) treated group as compared to the control. No significant difference between group II and group I (Fig 10B)

3- The mean area% of cleaved caspase-3 positive reaction: It showed high significant increase in the stress group (II) as compared to the control (I) and high significant decrease in ibuprofen (III) and Microvesicles (IV) treated groups. No significant difference between group IV and group I (Fig 10C).

4- The mean area % of CD44 positive immune reaction: It showed high significant increase in the Microvesicles-treated group (IV) as compared to the control (I) and stress group (II) (Fig 10D).

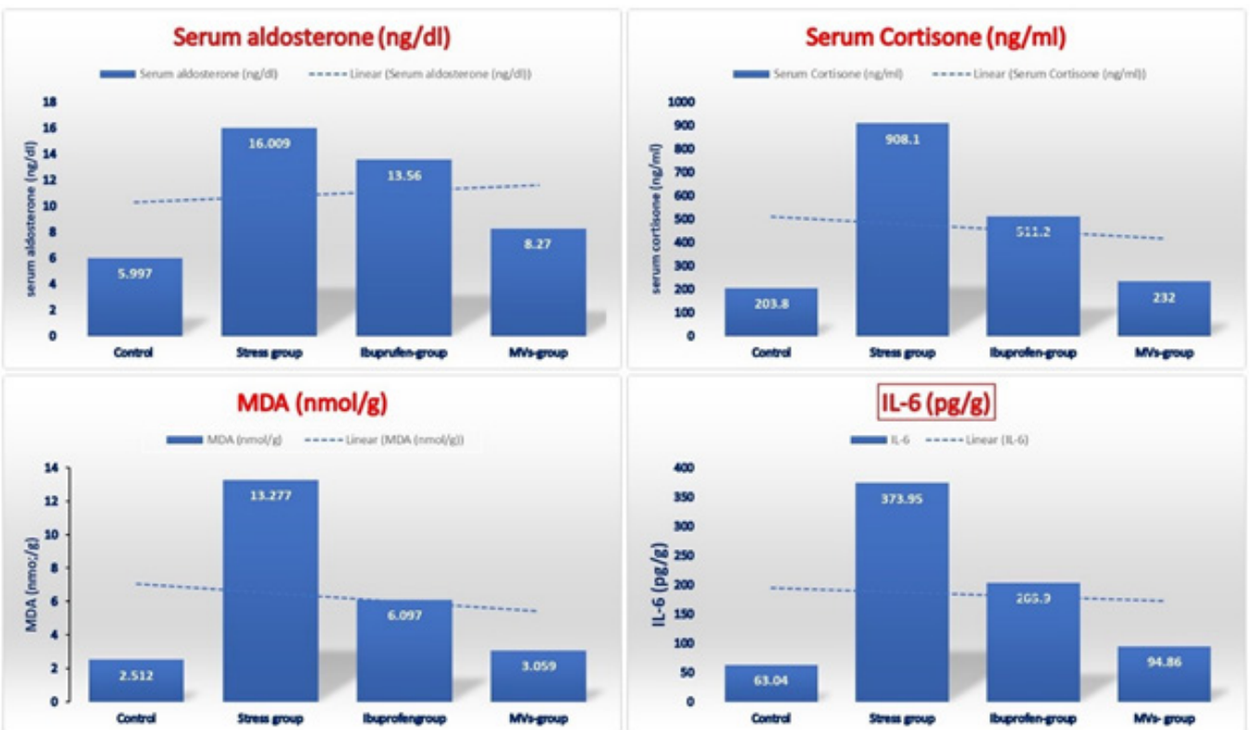

Fig 1: The mean values of serum aldosterone, serum cortisone, tissue MDA and tissue IL-6 in different studied groups

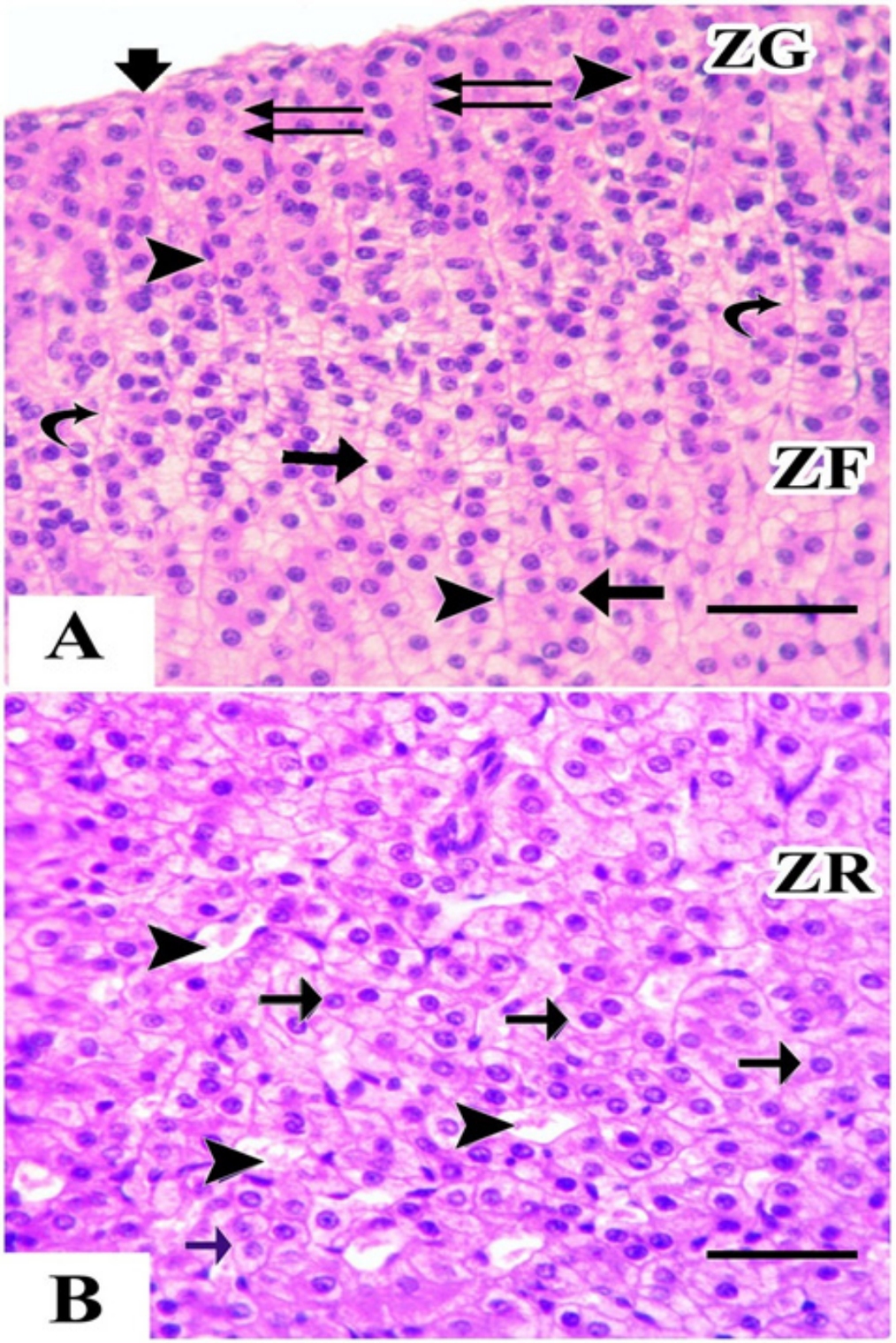

Fig 2: H&E-stained section of adrenal cortex of control group showing: A: connective tissue gland capsule (thick arrow). The zona glomerulosa (ZG) is formed of arched clusters of cells with acidophilic cytoplasm and rounded nuclei (double arrows). The zona fasciculata (ZF) consists of long straight parallel cords of polyhedral cells with vesicular rounded nuclei pale foamy cytoplasm (arrows). The cells are separated by blood sinusoids (arrowhead) and fine reticular fibers (curved arrow). B: the zona reticularis (ZR) is formed of interdigitating cords of closely packed polyhedral cells (arrows) which separated by sinusoidal capillaries (arrowhead). (H&E, X 400, Scale bar 40 um).

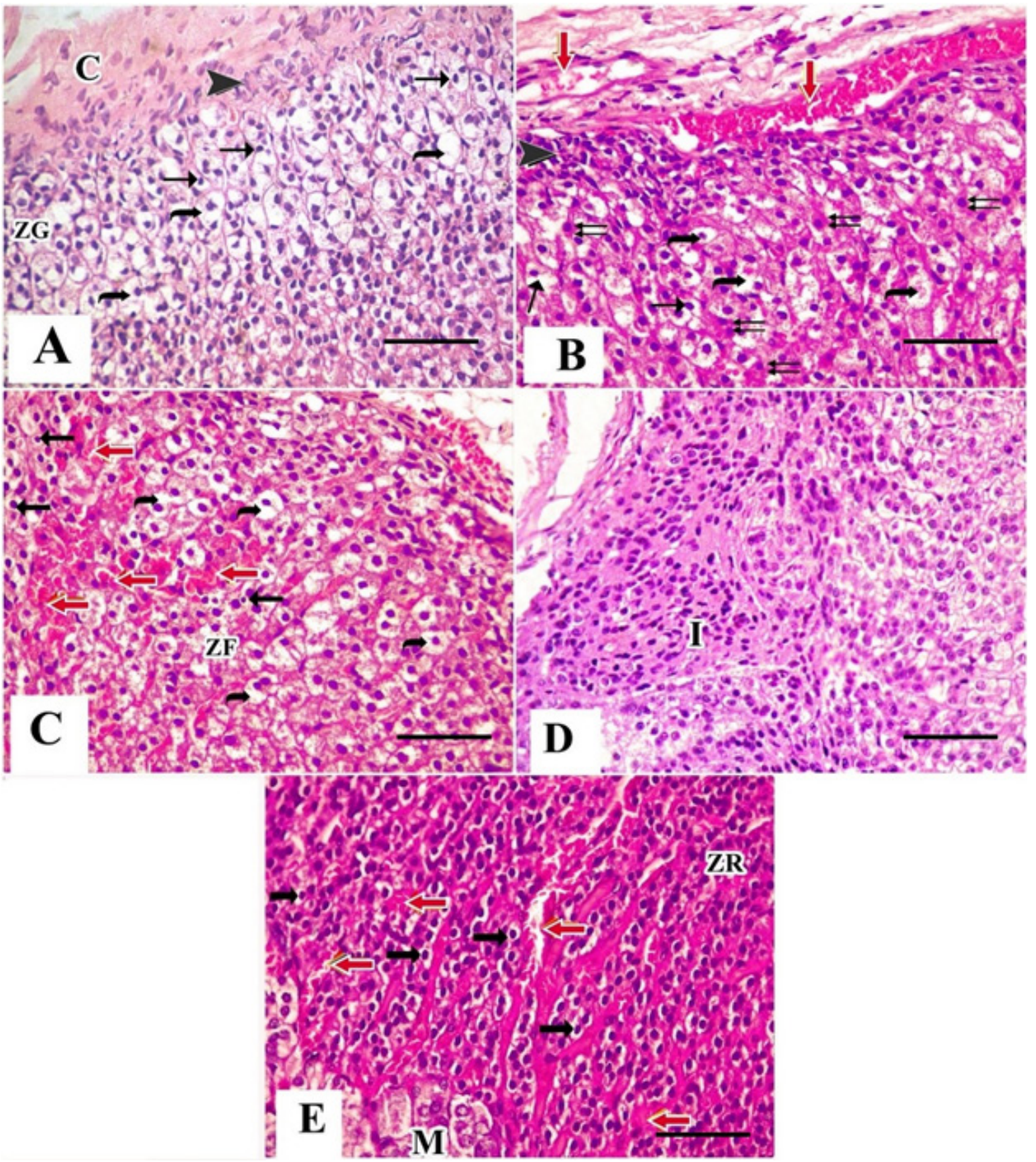

Fig 3: H&E-stained section of the suprarenal cortex of group II (stress group) showing: A: Thickened capsule (C) with area is of subcapsular hyperplasia (arrowhead), cells of the zona glomerulosa (ZG) shows marked vacuolations (curved arrow), numerous dark pyknotic nuclei (arrow). B: Marked congestion of the capsular and subcapsular blood capillaries (red arrows), subcapsular hyperplasia (arrowhead), many vacuolated cells (curved arrows) while other cells show dark acidophilic cytoplasm and pyknotic nuclei. C: section in the Zona fasciculata (ZF) shows severe vacuolation of the cells (curved arrows), numerous darkly stained nuclei (black arrow) and massive congestion of the blood sinusoids (red arrow). D: another section of the same group showing massive inflammatory cellular infiltrations (I). E: section of the zona reticularis of the same group showing numerous vacuolated cells with pyknotic nuclei (black arrow) with massive congestion of the sinusoids (red arrow). The medulla (M) can be noticed. (H&E X 400, Scale bar 40um).

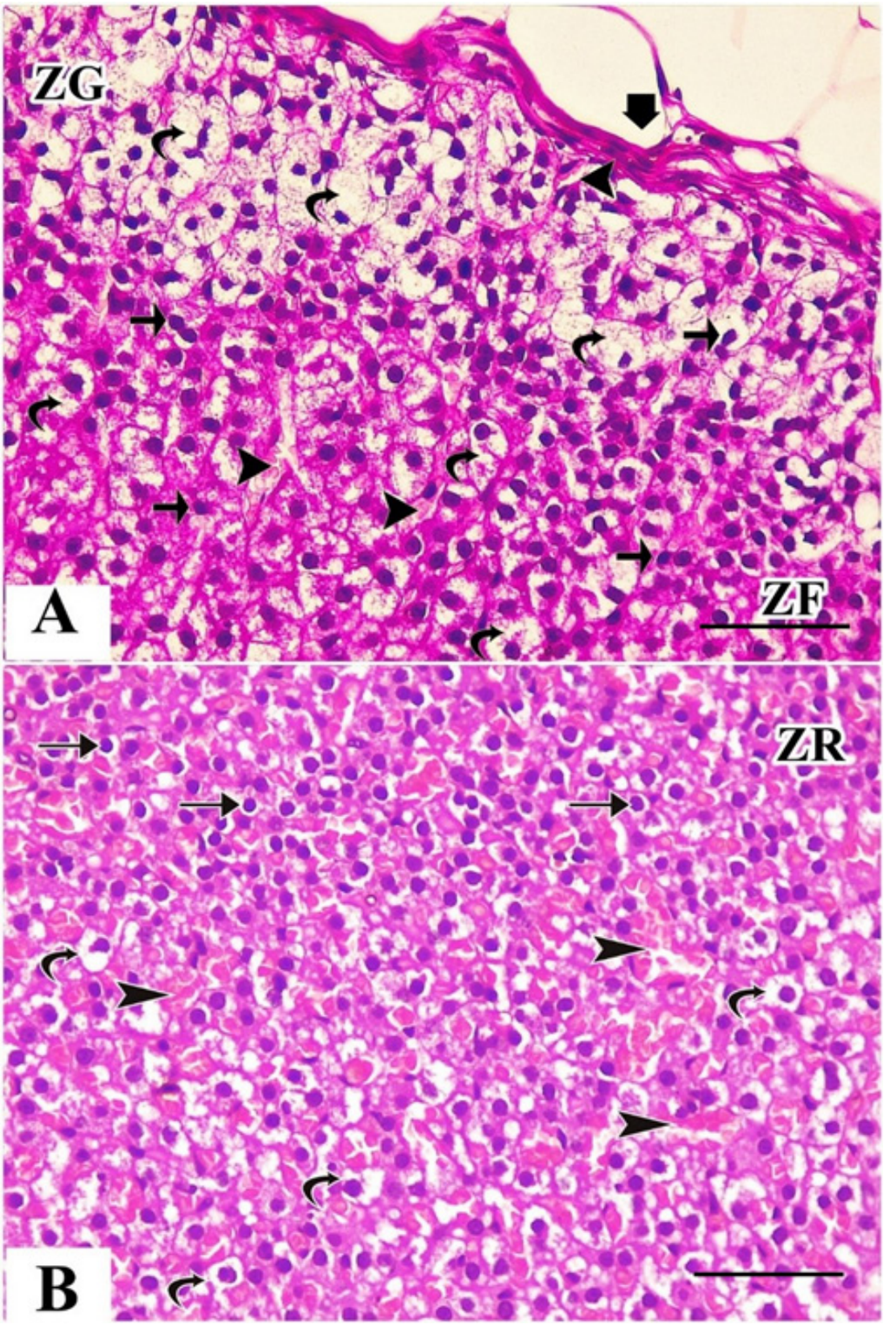

Fig 4: H&E-stained section of the suprarenal cortex from Ibuprufen-treated group showing: A: irregular separated capsule (thick arrow), the zona glomerulosa (ZG) showing many vacuolated cells (curved arrow). and residual pyknotic nuclei (arrow). the zona fasciculata (ZF) also has some vacuolated cells (curved arrow) and residual pyknotic nuclei (arrow). dilated blood sinusoids are still noticed (arrow head). B: The zona reticularis (ZR) shows residual congestion of the blood sinusoids (arrowheads). Some vacuolated cells (curved arrows) and few residual dark pyknotic nuclei(arrow) are still present (H&E, X 400, scale bar 40 um).

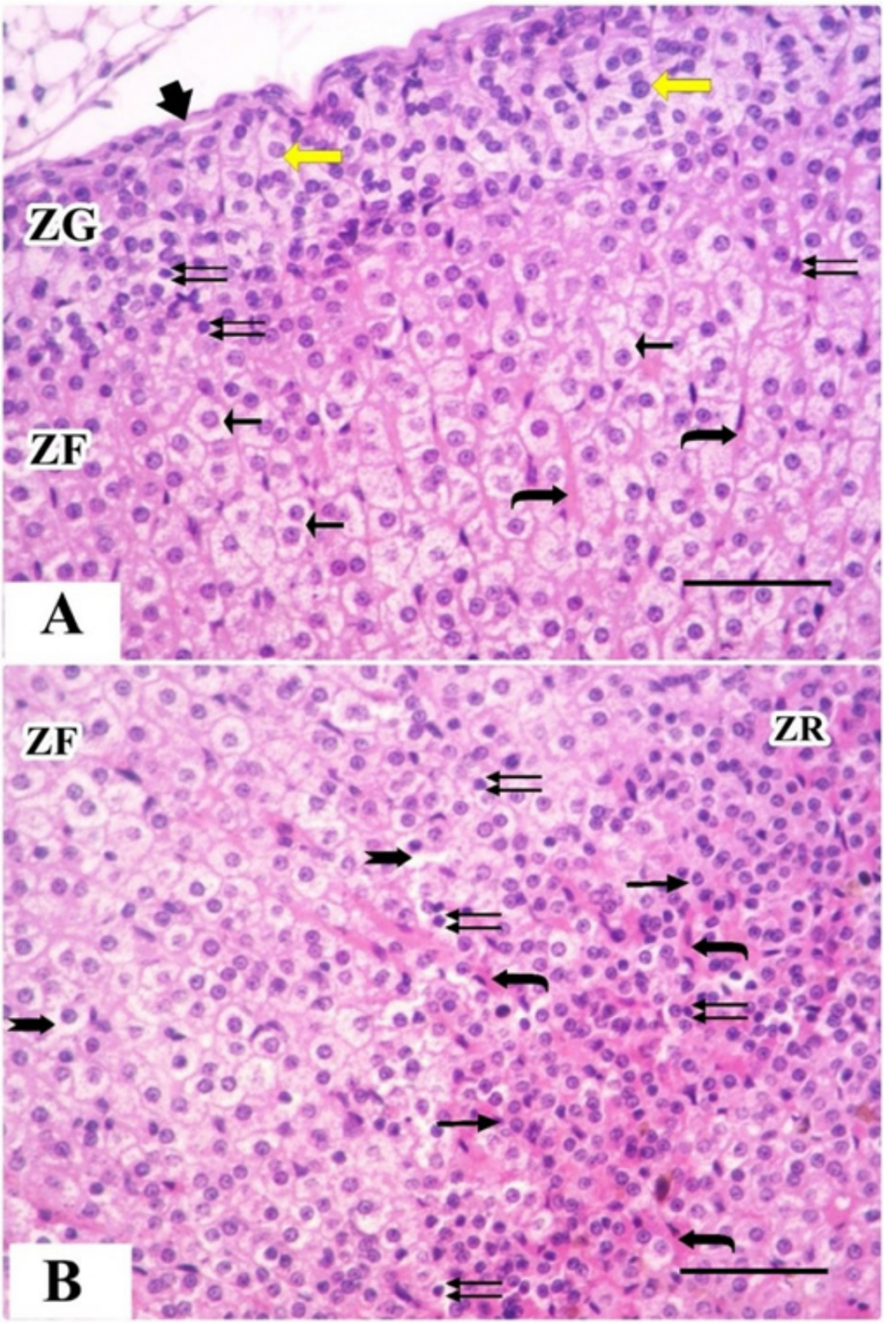

Fig 5: H&E-stained section of the suprarenal cortex from microvesicles-treated group showing: A: thin capsule (thick arrow), the zona glomerulosa (ZG) showing normal organization with apparently normal histological structure, cells have pale cytoplasm with vesicular nuclei (yellow arrow), cells of the zona fasciculata (ZF) normally organized with vesicular nuclei (arrows) separated with blood sinusoids (curved arrow), few dark stained nuclei can be still notice (double arrow). B: ZF is apparently normal with few residual vacuolated cells (notched arrow). Cells of the zona reticularis (ZR) appears normal with acidophilic cytoplasm and vesicular nuclei(arrow) separated by blood sinusoids (curved arrow). Few residual dark pyknotic nuclei is present (double arrow) (H&E, x 400, scale bar 40 um).

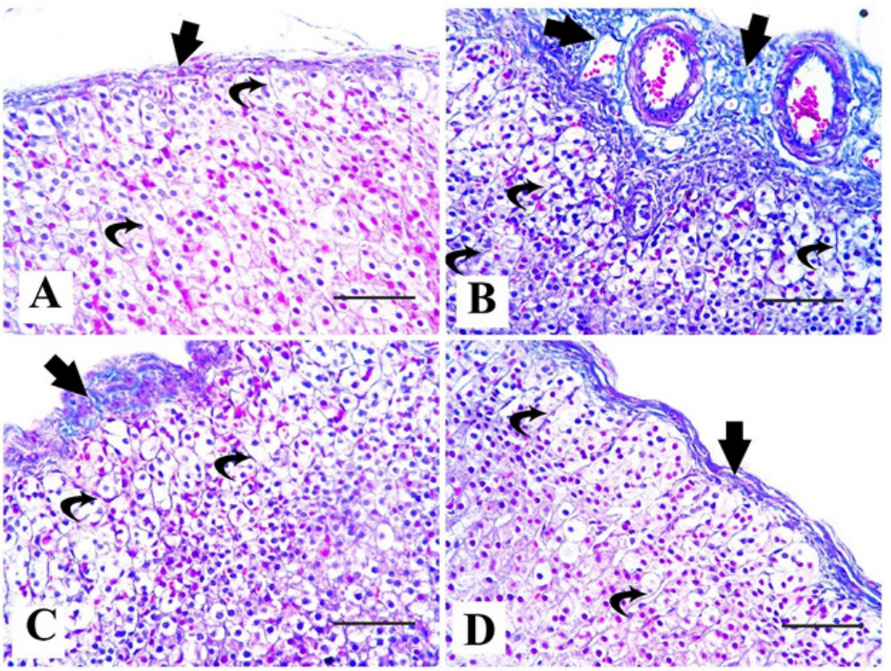

Fig. 6: Mallory trichrome stained sections of the adrenal cortex: (A) a section in the control group shows minimal amount of bluish stained collagen fibers in the capsule (thick arrow) with very thin reticular fibers in between cells (curved arrow). (B) A section in the stress group shows excessive deposition of collagen fibers within the capsule (thick arrow) with moderate deposition of reticular fibers in between cells (curved arrow). (C) A section in the ibuprofen treated group shows moderate deposition of collagen fibers within the capsule (thick arrow) with minimal deposition of reticular fibers in between cells (curved arrow). (D) A section in the microvesicles treated group shows minimal amount of collagen fibers within the capsule (thick arrow) with minimal deposition of reticular fibers in between cells (curved arrow) (Mallory trichrome stain X400, scale bar 40 um)

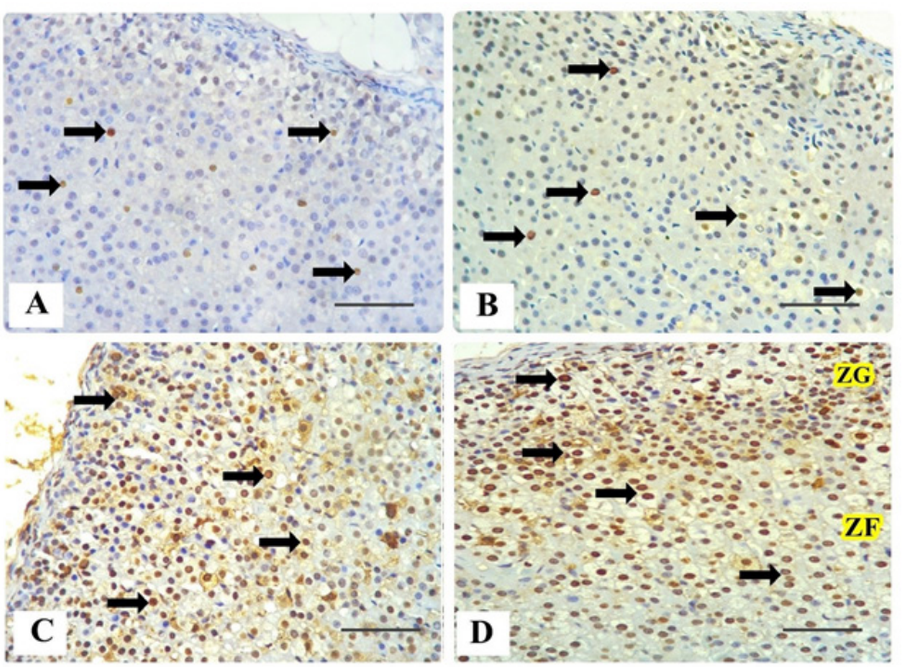

Fig. 7: Photomicrograph of PCNA stained sections of the adrenal cortex in different groups: (A) control group section shows some PCNA positive cells with moderate nuclear reaction (arrow). (B) stress group section shows many PCNA positive cells (arrows). (C) ibuprofen treated group shows multiple positive cells (arrows) for PCNA immune reaction. (D) microvesicles treated group shows numerous PCNA positive cells (PCNA X 400, scale bar 40 um).

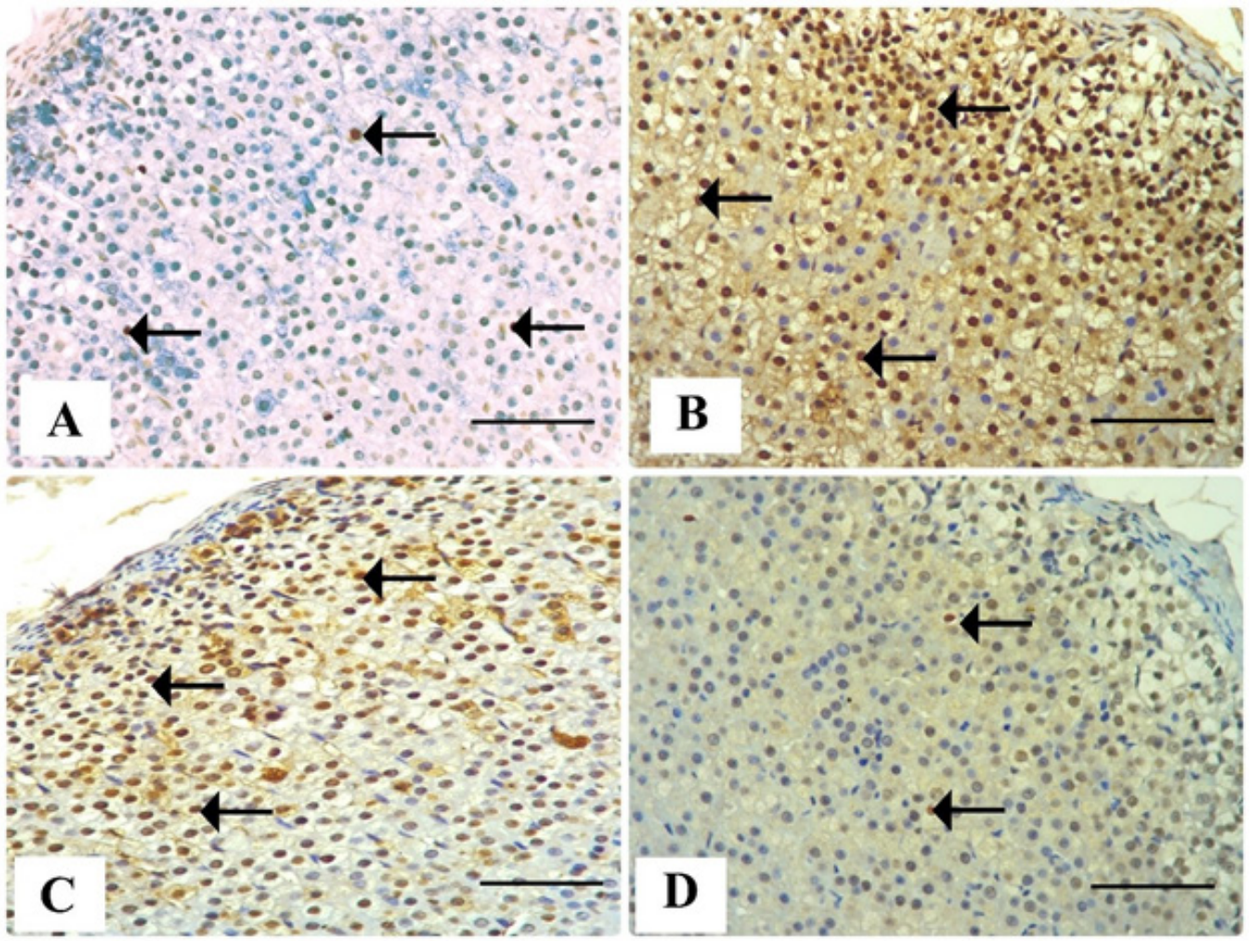

Fig 8: Photomicrograph of cleaved CASPASE-3-stained sections of the adrenal cortex in different groups: (A) control group section shows few caspase-3 positive cells with weak nuclear reaction (arrow). (B) stress group section shows numerous positive cells (arrows). (C) ibuprofen treated group shows many positive cells (arrows) for caspase-3 immune reaction. (D) microvesicles treated group shows only few positive cells (Caspase-3 X 400, scale bar 40 um).

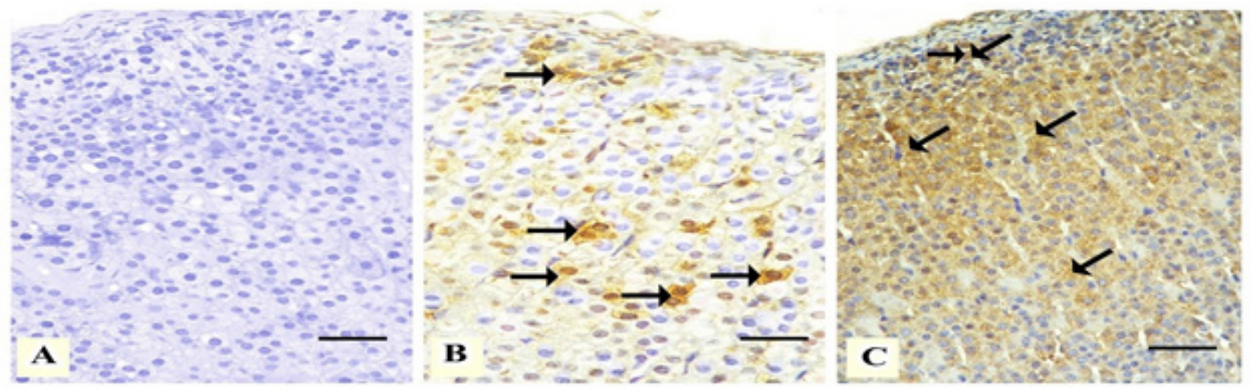

Fig (9): Photomicrograph of CD44 stained sections of the adrenal cortex in different groups: (A) control group section shows negative reaction. (B) stress group section shows some positive cells (arrows). (C) microvesicles treated group shows extensive positive cytoplasmic reaction at most of the cells (CD44 X 400, scale bar 40 um)

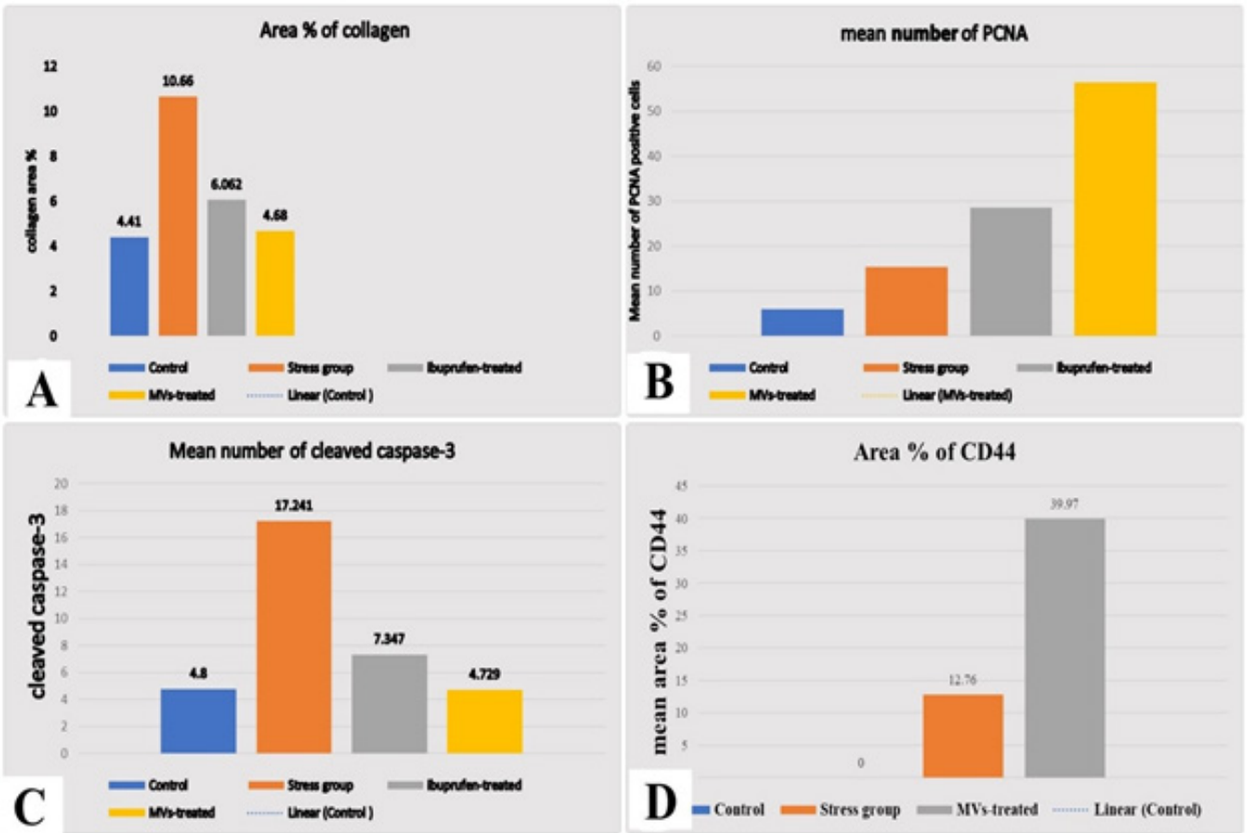

Fig 10: comparison between mean area% of collagen fibers, mean number of PCNA, cleaved casase-3 & mean area % of CD44 positive reaction in different studied groups.

Table 1: Comparison between different studied groups regarding the mean values (± SD) of serum aldosterone, serum cortisone, tisuue MDA and tissue IL-6 using ONE WAY ANOVA:

	Control	Stress group	Stress + Ibuprofen	Stress + MVs	*F value*	*P value*
Serum aldosterone (ng/dl)	5.997 ±1.79	16.009±3.2 *	13.65 ±1.14	8.27 ±1.35	50.879	0.00001<***
Serum cortisone (ng/ml)	203.8 ±29.66**	908.1 ±74.25*	511.2 ±59.64	232 ±48.2**	348.63	0.00001 <***
MDA (nmol/g)	2.512 ± 0.962	13.277 ± 2.75*	6.097 ± 1.297	3.059 ± 1.132**	85.3178	0.00001 <***
IL-6 (pg/g)	63.04 ±8.91	373.95 ±95.66*	203.9 ±37.9	94.86 ±15.59**	72.1518	0.00001 <***

*** high significant difference
*High statistical increase as compared with other groups
** No significant difference between group I & IV

Table 2: Comparison between different studied groups regarding the mean area % of collagen fibers, number of PCNA, area % of cleaved caspase-3 and area % of CD44 using ONE WAY ANOVA:

	Control	Stress group	Stress + Ibuprufen	Stress + MVs	*F value*	*P value*
Area % of collagen fibers	4.41 ±0.89	10.66 ±1.519*	6.062 ±1.196	4.68 ±0.703	59.9727	0.00001<***
Number of PCNA positive cells	5.9 ±2.28	15.3 ±3.3	28.6 ±6.7	56.4 ±15.18	65.982	0.00001<***
Number of cleaved caspase-3 positive cells	4.8±0.99	17.241±1.07*	7.347 ±0.926	4.729 ±1.164	324.164	0.00001<***
Area % of CD44 positive reaction	Not detected	12.76 ± 2.22	-	39.97 ± 9.62	128.175	0.0001<***

*** high significant difference
*High statistical increase as compared with other groups

DISCUSSION

Human individuals are exposed to various kinds of stressors all the time which threat the physiological and the psychological normal life of them[18]. We studied the effect of stress on the adrenal gland as it is the main organ involved in stress response. It is an essential stress-responsive organ that is part of both the hypothalamic-pituitary-adrenal axis and the sympatho-adrenomedullary system[19].

The best model for this experiment was a male in order to minimize the impact of sex hormones throughout the estrous cycle on the adrenal cortex, which is more sensitive in female than male[20]. The animal model of stress employed in the current study was subjected to a number of different techniques, including a 45°cage tilt (for 24 hours), physical restraint (for 2 hours), tail pinching (for 1 minute, 1 cm from the distal region of the tail), and food deprivation (48 h). To avoid the animals developing an expectation of stressors, the stressors were presented every day for two weeks in a random order, avoiding the same stressor on two consecutive days[17].

Stress is brought on by physical constraint because it releases pro- and anti-inflammatory cytokines[21]. Additionally, it worsens calcium homeostasis and raises reactive oxygen species, two factors that injure cells with dysfunctional mitochondria[22].

According to Beck and Luine (1999), hunger operates as a stress by raising arousal and attention to the things. It raises blood corticosteroid levels as well as basal serotonin activity in the prefrontal cortex and hippocampus[23].

Stress-group adrenal gland sections stained with H&E revealed enlarged capsules. Studies in morphometry and statistics support this conclusion. Comparing the stress group to the other groups, the mean area % of collagen fibers in the capsule and intracellular areas in Mallory staining slides. According to Said *et al*. 2021's explanation, psychological or physical stress causes the adrenal medulla to secrete the catecholamines adrenaline (epinephrine) and noradrenaline (norepinephrine), which cause cytotoxicity and tissue injury that results in fibrosis[24]. In line with Zaki *et al*. 2018's assertion that the same outcome was observed in the stress-group[25].

The migration theory, which contends that adrenal cell regeneration starts in the outer region of the cortex and subsequently migrates from ZG to ZF to ultimately reach ZR, can explain why the stress group exhibited subcapsular hyperplasia[26].

When compared to the MSC-MVs treated group and the ibuprofen treated group, the mean values of (cortisol and aldosterone) in the adrenal tissue in our study showed a highly statistically significant increase in the stress group; however, the improvement was greater in the MVs treated group, which was explained by failure of the negative feedback of cortisol hormone on the hypothalamic corticotropin releasing hormone[27]. Additionally, according to Angelina *et al*. 2020, stress raises serum aldosterone levels while stimulating the renin-angiotensin system[27].

ZF revealed vacuolated cells. Some nuclei had cytoplasmic vacuolation, which gave them a pyknotic appearance. Stress's role in cell apoptosis is the primary cause of these alterations. It raises the levels of reactive oxygen species and antioxidant enzymes, which activate cysteine protease enzymes and cause a rise in lipid peroxidation[28].

In compared to the other groups, the stress group had statistically significant higher mean values of MDA in the adrenal tissue. According to Sahu *et al*. (2015), Malondialdehyde (MDA), an oxidative marker that is the byproduct of lipid peroxidation, promotes cell death by disrupting enzymatic activity and ionic transport, which leads to a failure of permeability and fluidity in the cell membrane. In the end, it results in cell vacuolation and breakdown of the intracytoplasmic organelle material[29].

Studying the mean area percent of Caspase-3 immune-positive cells, which revealed a highly statistically significant increase in the stress group compared to the other groups, confirms the effect of stress as a primary cause of apoptosis.

Additionally, a very statistically significant rise in the stress group was seen when the mean values of (IL 6) in adrenal tissue were analyzed statistically for our study. IL6 levels had dramatically decreased in comparison to the stress group in both the MSC-MVs treated group and the ibuprofen treated group, however the improvement was greater in the MVs-treated group.

According to Lee *et al*. (2016), stress causes the release of pro- and anti-inflammatory cytokines, such as IL6[22]. Additionally, stress-induced lipid peroxidation causes apoptotic cells to release pro-inflammatory mediators like interleukin-6, which attracts more macrophages and other inflammatory cells, explaining the rise in IL6 in the stress-group[30].

In the stress-group, extravasation with dilated, congested blood sinusoids was found. It can be explained by increased blood flow in order to deal with the elevated level of ZF stimulation caused by prolonged stress[31].

Extravasation with dilated congested blood vessels were discovered in the group that was under stress. Increased blood flow to deal with the elevated degree of ZF stimulation brought on by continuous stress may explain it[31].

Reduced inflammatory mediator release during stress is one of the defensive mechanisms against its negative effects. Ibuprofen also reduces lipid peroxidation and adrenal cell death by reducing NO through a non-cyclooxygenase-dependent route[32].

Recently, there have been serious trials using mesenchymal stem cell derived microvesicles (MSC-MVs) to avoid the negative effects of stem cells, such as mal-differentiation and tumor formation[33].

According to Xin *et al*. (2013), who demonstrated improvement in the lung tissue after being insulted by Escherichia coli and treated by MSC-MVs, H&E-stained sections of the MSC-MVs treated group in the current study appeared virtually normal[34].

The main contribution of MSC-MVs to therapy comes from the release of proteins, lipids, organelles, and mRNAs that are identical to those of the stem cells from whence they originate. It was established that stem cells lost their therapeutic properties when their MVs were removed, and that their MVs are primarily responsible for the paracrine effects of stem cells[35].

According to Raposo *et al*. (2013), stem cells have CD44 receptors on their surface that interact with L-selectin and osteopontin ligands on damaged cells to enhance the uptake of MVs. Following their ingestion by the target cells, the MVs are then incorporated into the damaged cells through endocytosis or membrane fusion to begin acting therapeutically[36]. In contrast to other synthetic drug nano-carriers, which can have their diffusion through tissues disrupted by various biological barriers, microvesicles' nanoscale size increases their permeability and retention impact[37].

MSC-MVS reduces the inflammatory mediators to combat stress. According to Tricarico *et al*. (2017), MVs have anti-inflammatory properties by suppressing T lymphocyte functions and altering the cytokine production of dendritic cells, naive T cells, and natural killer cells. This inhibits the release of inflammatory mediators during stress and counteracts the effects of stress[38].

In contrast to the other groups, the MVs treated group demonstrated a significantly higher number of PCNA positive cells. Additionally, there was a considerable reduction in caspase-3 reaction level. This outcome can be explained by the ability of MVs to transfer proliferative genes and proteins to recipient cells, increasing the proliferation and apoptotic resistance of the remaining mature cells. In vivo investigations by Xin *et al*. (1995) showed that MSC encourage cell proliferation and survival by lowering apoptosis in models of brain or kidney damage[39]. Other investigations that revealed that incubating cultured renal cells with MSCs dramatically decreased the tubular apoptosis brought on by cisplatin in vitro also corroborated this finding[40].

In our study, the mean number of cells with a positive CD44 immunological reaction in the MVs-group was significantly higher than that of the control group and the stress-group. In comparison to the control group, the stress-group displayed some positive cells that had significantly increased. One theory was that CD44 expression is necessary for the initial steps in the inflammatory response to stress that include circulating neutrophils penetrating adrenal tissue[33].

The finding of CD44 receptors is solid proof that MVs homing actually took place. They have L-selectin and osteopontin ligands on their surface, which they bind to cause the MVs to endocytose into the wounded cells to begin their therapeutic action[36].

CONCLUSION

MSCs-MVs improve the histological changes of the adrenal gland of the male albino rats when it is administered chronically and concurrently under stressful situations. Also, Ibuprofen improve these changes but with less degree.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

تأثيرالإجهاد المزمن على قشرة الغدة الكظرية والإمكانات العلاجية المحتملة للإيبوبروفين مقابل الحويصلات الدقيقة للخلايا الجذعية فى ذكور الجرذان المهق البالغة (دراسة هستولوجية و هستوكيميائية مناعية)

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الهدف من البحث: دراسة تأثير الإجهاد المزمن على التركيب النسيجى لقشرة الغدة الكظرية ومقارنة الدور العلاجى لكلا من الإيبوبروفين والحويصلات الدقيقة المستخلصة من الخلايا الجذعية

المواد وطرق البحث: تم تقسيم الفئران الى أربعة مجموعات تحتوى كل منها على عشرة فئران : مجموعة ضابطة , مجموعة الإجهاد تعرضت لأشال مختلفة من الإجهادات لمدة إسبوعين ,مجموعة الإيبوبروفين ومجموعة الحويصلات الدقيقة. تمت معالجة عينات قشرة الغدة الكظرية لإجراء الدراسات الهستولوجية والهستوكيميائية. و كذلك عينات الدم لإجراء الدراسات الكيميائية.

النتائج: لقد أظهر فحص الشرائح تحسن بسيط فى مجموعة الإيبوبروفين فى نفس الوقت الذى تحسنت مجموعة الحويصلات الدقيقة لتصبح مشابهة للمجموعة الضابطة.

الإستنتاج: إن إعطاء الحويصلات الدقيقة للخلايا الجذعية بالتزامن مع حدوث الإجهاد المزمن فى ذكور الجرذان المهق قد أدى الى تحسين التركيب النسيجى للغدة الكظرية. وكذلك قد ساعد الإيبوبروفين فى التغيير ولكن بصورة أقل.