



Effect of Short and Long Term Restraint Stress on the Histology of Liver, Kidney and Suprarenal Gland in Albino Mice during Postweaning Period

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OHS and CJO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HTH and PBB managed the analyses of the study. Authors VSN, NFO and ESG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

On exposure to stress and for the sake of survival, cells make adjustments with the changes in their environment to the physiologic needs and non-lethal pathologic injury. When the environmental changes are greater than the capacity of the cell to maintain normal homeostasis the cell undergoes acute cell injury. If the injury or insult is removed on time, or the cell can adapt and withstand the injury, the term reversible injury is applied. The processes of adaptation include decreasing or increasing their size, increasing their number, or changing the pathway of phenotypic differentiation of cells.

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In the present study, albino mice of postweaning age of BALB C strain (21 days old) were exposed to short term (5 days) and long term (21 days) restraint stress to evaluate any histological changes in the kidney, liver, and suprarenal gland.

Mice subjected to long term stress showed in the kidney degeneration of the cells of the glomerulus and the convoluted tubules. In the liver, they showed congested sinusoids and the presence of some fatty change, whereas in the suprarenal gland mice subjected to 21 days of stress showed moderate hypertrophy and hyperplasia of the adrenal cortex with the presence of moderate lipid deposits when compared to controls. The overall effect on short term stress was mild when compared to exposure to 21 days stress

Long term stress causes degeneration in hepatic cells, infiltration in the liver, degeneration of glomerulus, Bowman's capsule, convoluted tubules in the kidney which could lead to nephrotoxicity. In the suprarenal gland, long term stress induces hypertrophy of the adrenal cortex. These morphological changes can explain the impaired immunity which develops in organisms that are exposed to chronic stress.

Keywords: Restraint stress; postweaned albino mice; liver; kidney; suprarenal gland.

1. INTRODUCTION

Stress is a response to biological and emotional changes that helps in the strengthening of the organism. Chronic stress induces a reduction in weight gain, raised corticosterone level, and changes in cognition, which affects both physiology and behavior and also leads to the development of psychological disorders [1]. Stressors that challenge homeostasis can be divided into three general categories: physical (restraint and exercise), psychosocial (isolation, anxiety, fear, or mental frustration), and metabolic (hypoglycemia and hemorrhage) [2]. Stress can involve single or intermittent exposure or prolonged intermittent or continuous exposure. Immobilization stress can be considered a mixture of physical and psychological stressors, restricting movement, and isolating the individual from his group [3]. Confusion still arises regarding what one believes defines and constitutes stress. Most scientists view stress as the situation when the hypothalamic-pituitary-adrenocortical (HPA) axis, represented mainly by elevated ACTH levels, is activated [4]. Others suggest that activation of other systems with or without an elevation in ACTH may reflect stress-induced disturbed homeostasis [5]. There is now evidence that specific stressors may elicit specific responses, and may activate different brain systems by using specific pathways within the central nervous system [3]. Equally observed is the fact that stressors are selective pressures from the physical and social environment that threaten or challenge an organism and elicit compensatory response patterns [6]. It can equally be a state of disharmony or threatened homeostasis, evoking physiologically and behaviorally adaptive responses that can be

specific to the stressor or generalized and nonspecific and that usually occur stereotypically, producing a "nonspecific" stress syndrome when the threat to homeostasis exceeds a threshold [7].

Chronic stress induces a reduction in weight gain, raised corticosterone level, and changes in cognition, which affects both physiology and behavior and leads to the development of psychological disorders [1]. Hypothalamus-pituitary adrenal and sympathetic adrenomedullary axes are the systems mainly involved in maintaining homeostasis during the stress response, and the adrenal gland is an essential organ common to both systems [8]. Stress triggers the activation of the hypothalamus-pituitary-adrenal (HPA) axis, culminating in the production of glucocorticoids by the adrenals. Receptors for these steroids are expressed throughout the brain; they can act as transcription factors and so regulate gene expression. Thus, glucocorticoids can have potentially long-lasting effects on the functioning of the brain regions that regulate their release. The suprarenal gland plays a major role when HPA and sympathetic adrenal medullary axis work to maintain homeostasis when the body is responding from the stress. This later gland is subject to dynamic structural changes which include cellular proliferation and apoptosis and these two processes must be balanced to ensure integrity and functionality of the adrenal gland [9]. The chief mediators of the stress axis are therefore corticotropin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus, adrenocorticotropin (ACTH) in the anterior pituitary gland, and glucocorticoids (corticosterone in rodents and cortisol in

humans) in the adrenal glands. To maintain its normal homeostasis the glucocorticoids secreted by the adrenal maintains a negative feedback mechanism through its specific receptors [10]. However in the event of repeated activation of the HPA axis or chronic stress and allostatic load is generated, which can alter its integrators (nervous neurotransmitters [11], endocrine hormones [12], and immune system secondary lymphoid organs [13]).

Among the many consequences of chronic stress, including exacerbated stroke outcome in mice [14], with increased liver metastasis [15], increased susceptibility to endotoxic shock [16], and impaired antiviral immunity in wounded animals [17]. Social isolation has been shown to predict morbidity and mortality from a myriad of health conditions, including stroke and cerebrovascular disease [18]. In contrast, patients with high levels of social support or large social networks exhibit more rapid and extensive functional recovery after stroke than socially isolated individuals [19]. Social influences on experimental stroke outcome have been studied most often in the context of environmental enrichment studies. The environmental enrichment typically includes a social component, a means of voluntary exercise, and novel stimulus objects. Housing rats in an enriched environment beginning several days after induction of stroke does not significantly alter infarct volume, but it does improve functional recovery, [20] possibly through a mechanism that involves changes in dendritic structure in the contralateral hemisphere, [21] altered gene expression, [22] or increased neurogenesis [23]. However, normal glucocorticoid levels are important for normal brain maturation: they initiate terminal maturation, remodel axons and dendrites and affect cell survival; both suppressed and elevated glucocorticoid levels impair brain development and functioning. For example, administration of synthetic glucocorticoids to pregnant rats delays the maturation of neurons, myelination, glia, and vasculature in the offspring, significantly altering the neuronal structure and synapse formation and inhibiting neurogenesis [24]. Furthermore, juvenile and adult rats exposed to prenatal stress have decreased numbers of mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) in the hippocampus, possibly because of epigenetic effects on gene transcription [25]. Currently there is paucity of data on the direct evidence of stress and its dose on the liver,

kidney and supra renal glands. The study is therefore aimed at determining the effects of short term (5 days) and long term (21 days) restraint stress on histological changes in liver, kidney and supra renal glands in albino mice during their post weaning period

2. MATERIALS AND METHODS

2.1 Study Design and Site

This comparative study was conducted to see the effect of duration and nature of stress on the histological changes in liver, kidney, and suprarenal gland in post weaned mice exposed to short and long term restraint stress. In the present study, post-weaned albino mice (21 days old) of BALB/C strain of both sexes were used. The sample size was 30 mice; these animals were issued and maintained at the central animal house, Manipal University, Manipal. Animals of post-weaning age groups were used in these experiments. In particular, BALB/c mice are more stress-sensitive and have been proposed to be a model of pathological anxiety [26]. The mice were housed in polypropylene cages (3-4 mice/cage) and kept on steel racks. Paddy husk (sterilized) was used as bedding for animals and was changed thrice a week. The animal house was well ventilated with exhaust and ceiling fans. Room temperature was maintained at $27\pm 3^{\circ}\text{C}$. Throughout the study, 12 hours of dark and light cycle were maintained in the animal house [27].

2.2 Basic Methodology

2.2.1 Restrainers

In the present experiment, wire mesh restrainers of different dimensions were utilized for stressing the mice belonging to different age groups. These restrainers were locally fabricated with a wooden base on which wire mesh compartments of different dimensions were mounted on either side of a median partition. On each side, there are 6 compartments which were separated from each other by wooden partitions. The floor, sliding doors, median partition, and partition between the adjacent compartments were provided with many holes for ventilation.

2.2.2 Stress procedure

The mice were within the appropriate restrainer and the entrance was closed by sliding the door. These mice remained within the restrainer for

6hrs a day (9.00 A.M-3.00 PM) for 5 or 21 days based on the procedure. The restrainer with the mice was kept on a table in a room with sufficient ventilation. After 6hrs of stress, mice were released from the restrainer and were placed in their respective home cages. The restrainer was washed and dried after use.

2.2.3 Restraint stress group (RS)

Mice in this group were divided into 2 subgroups

5 days restraint stress: Mice were stressed 6h/day for 5 days in the restrainer (26 days old)

21 days restraint stress: Mice were stressed 6h/day, for 21 days in the restrainer (42 days old). The results obtained were compared with age-matched controls.

Control group (C):

5 days control: Mice were sacrificed on postnatal day 26.

21 days control: Mice were sacrificed on postnatal day 42.

After the last day of the stress, mice belonging to two groups (short and long term) were anaesthetized with anesthetic ether with age-matched controls and sacrificed. The chest cavity was opened and about 15ml of 0.9% heparinized saline was perfused at the rate of 1ml/min through the left ventricle. This was followed by injection with 10% formalin (about 250 ml) at the same rate. The liver, kidneys, and adrenal glands were dissected out and were put in 10% formalin for a week and processed further for paraffin block making and Hematoxylin and Eosin staining.

2.3 Preparation of Tissue for Histological Examination

After one week in 10% formalin, the Liver, kidneys, and suprarenal gland were embedded in paraffin, sectioned serially (4 to 5 μ m slices), and further processed for Hematoxylin and Eosin staining.



Fig. 1. Photograph of Restrainer showing *Wooden restrainer of different dimensions used to induce stress to post-weaned mice*

3. RESULTS

3.1 Effect of Stress on Histology of Liver

Histological examination of the liver sections in the post-weaned age control groups (26 & 42 days old) showed a prominent central vein in the hepatic lobule surrounded by the rows of

hepatocytes with distinct nuclei and hepatic venous sinusoids between them (Fig. 2a & 2c). 5 days stressed group showed mild degeneration with the presence of hyperchromatic hepatic cells (26 days old) (Fig. 2b), whereas 21 days stressed group showed degeneration with congested Sinusoids and presence of some fatty change (42 days old) (Fig. 2d)

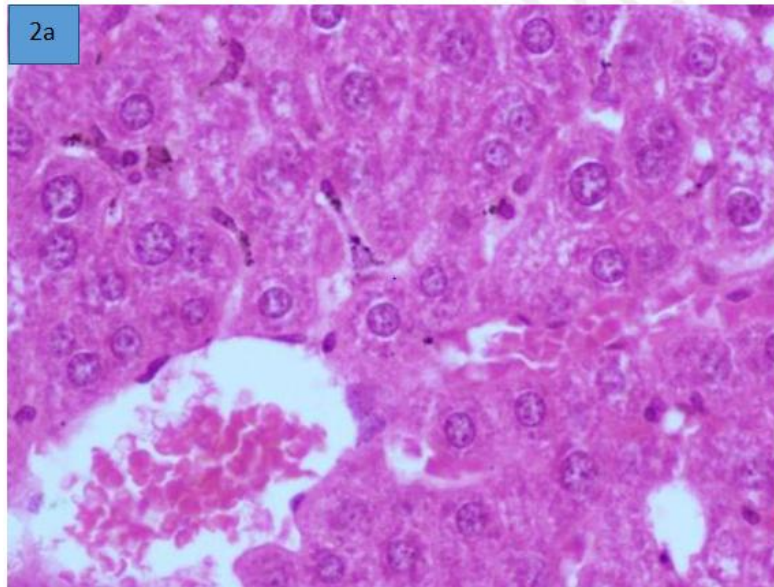


Fig. 2(a). Liver of post-weaned 5 days control showing normal hepatocytes (H & E 10X)

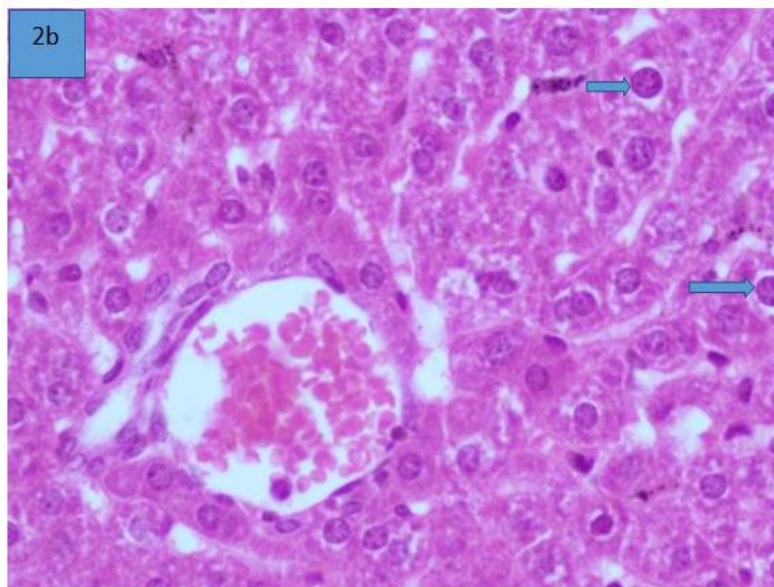


Fig. 2(b). Liver of 5 days post-weaned 5 days restraint stress showing mild degeneration with the presence of hyperchromatic hepatic cells (Blue filled arrow, H & E 10X)

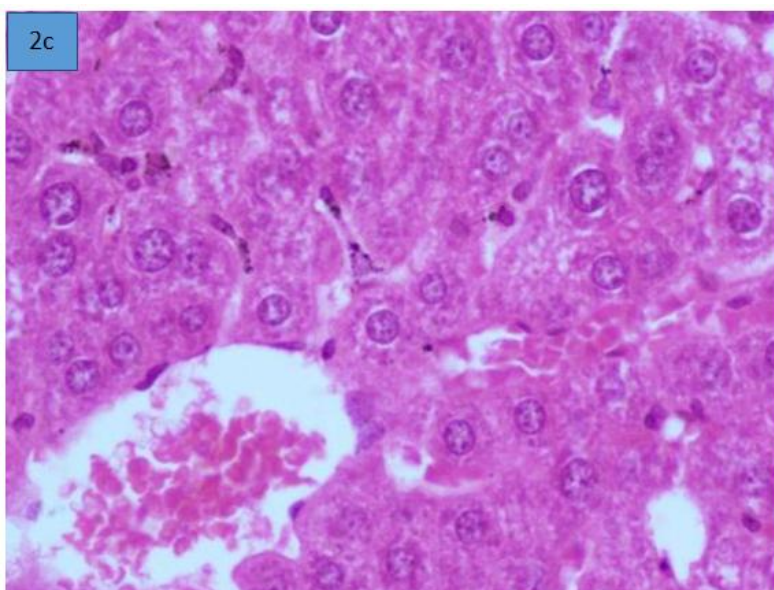


Fig. 2(c). Liver of post-weaned age 21 days control showing normal hepatocytes (H & E 10X)

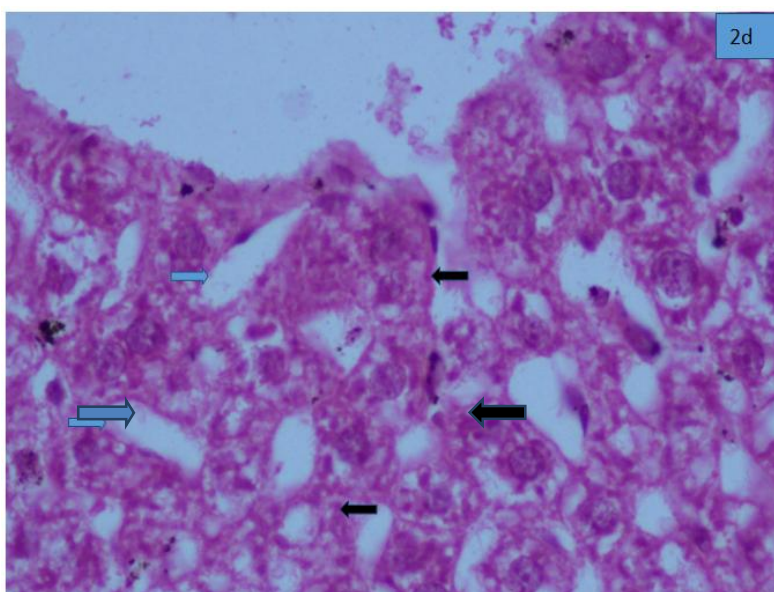


Fig. 2(d). Liver of post-weaned age 21 days restraint stress showing congested sinusoids (black filled arrow) and the presence of some fatty change (Blue filled arrow) (H & E 10X)

3.2 Effect of Stress on Histology of Kidney

Histological examination of the kidney in control groups (26 and 42 days old) showed normal histological features of the tissue, in the cortical part, for example, the Bowman's capsule with glomerulus displayed normal features and the convoluted tubules on the other hand conserved

their general architecture. In mice subjected to 5 days of stress, the histological slides revealed mild degeneration with hypercellularity of glomerulus and convoluted tubules. More degeneration of the glomerulus and the tubules were found in the histological sections of the post-weaned mice undergone 21 days of restraint stress.

3.3 Effect of Stress on Histology of Suprarenal Gland

Histological examination of haematoxylin and eosin sections from the control group (post-weaning 26 and 42 days old) revealed the normal histological architecture of the adrenal cortex. The cells of zona glomerulosa were separated by blood sinusoids and arranged in

the form of rounded or arched clusters beneath the adrenal gland capsule (Fig. 4a). The cells of zona fasciculata were arranged in long straight cords separated by blood sinusoids. These cells were large and polyhedral with pale vacuolated cytoplasm and vesicular rounded nuclei (Figs. 4a & 4c). Zona reticularis cells were disposed of in the form of cords anastomosing with one another.

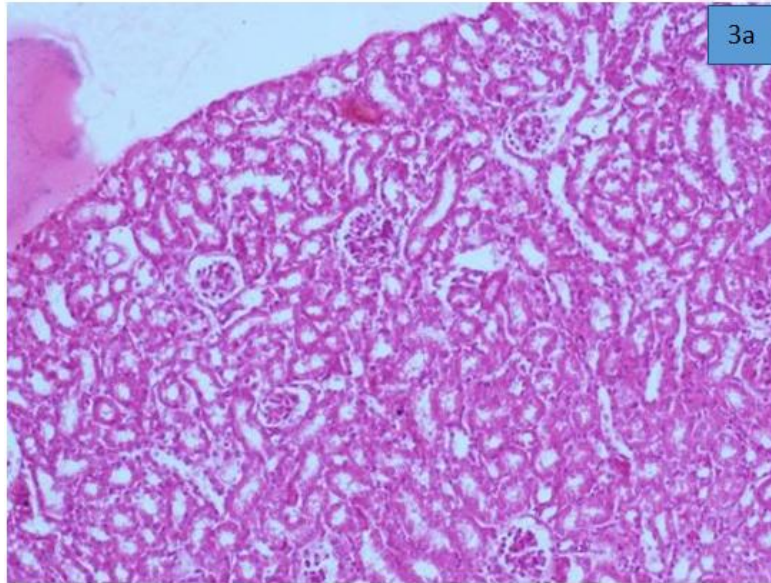


Fig. 3(a). Kidney of post-weaned age 5 days control (26 days old) showing normal Glomerulus (H & E 4X)

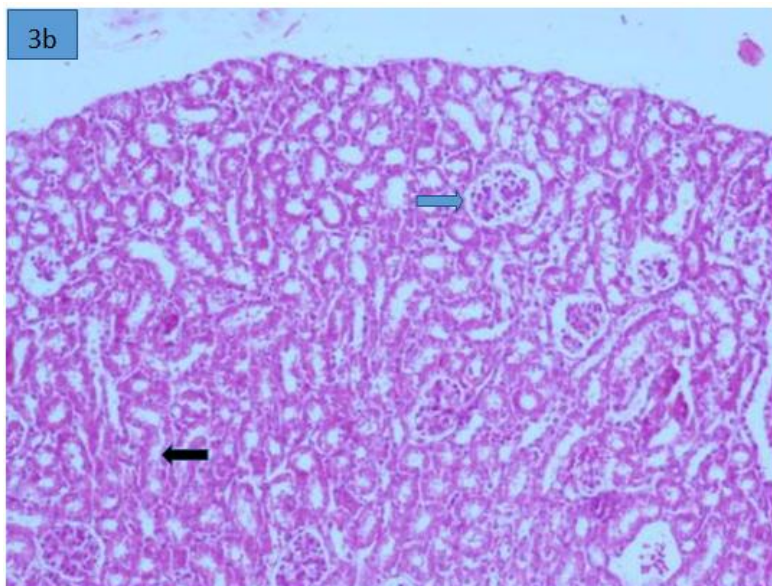


Fig. 3(b). Kidney of post-weaned 5 days restraint stressed showing mild hypercellularity of the glomerulus (blue filled arrow) and convoluted tubules (black filled arrow) (H & E 4X)

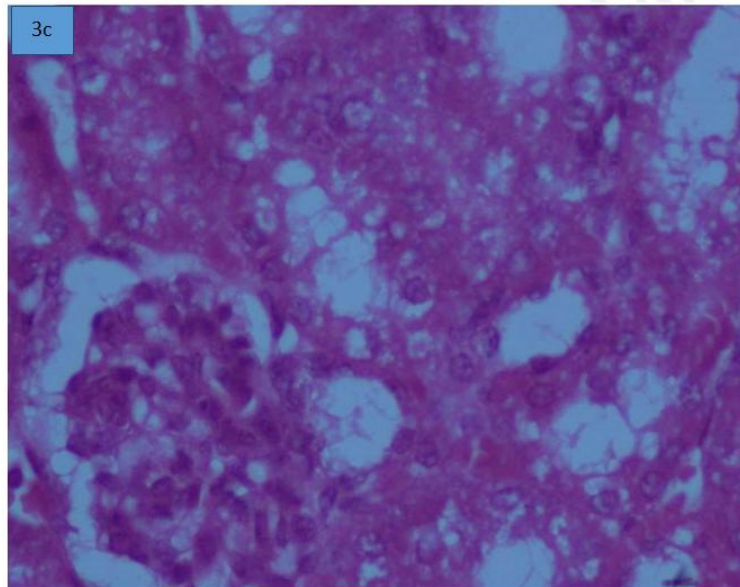


Fig. 3(c). Kidney of post-weaned mice control (42 days old) showing normal glomerulus and convoluted tubules (H & E 10X)

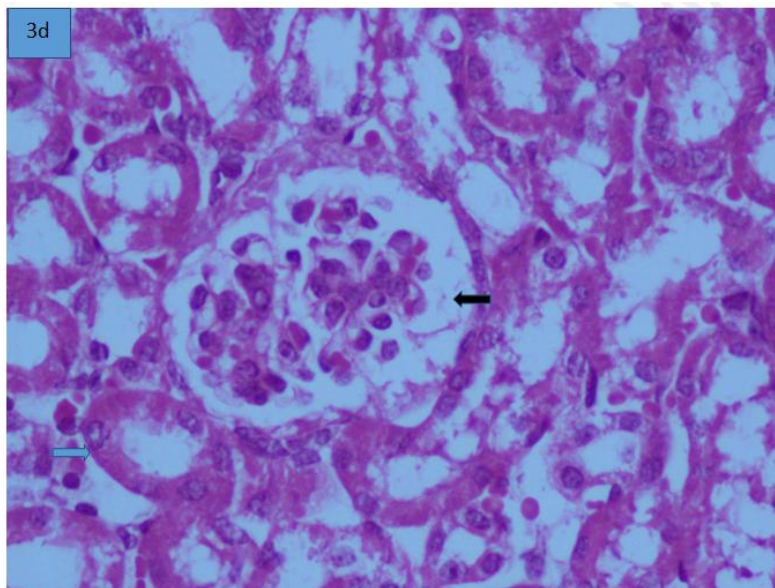


Fig. 3(d). Kidney of post-weaned age (42 days old) subjected to 21 days restraint stress showing degeneration in the form of swellings of both glomerulus (black filled arrow), and convoluted Tubules (blue filled arrow) (H & E 10X)

In 5 days stressed group slight irregular orientation of zona glomerulosa and less increased vacuolation of zona fasciculata were observed in mild hypertrophy whereas in 21 days restraint stressed group, the entire cortex showed irregular orientation of the cells with swelling and hyperplasia. The presence of

moderate lipid deposits was observed (Fig. 4d).

4. DISCUSSION

Stress is a process that originates when environmental demands exceed the adaptive

capacity of a human being or an animal. This can lead to biological and psychological changes, which in turn can cause disease. Stressors are many ranging from physical to psychological and all are likely to cause histological changes in the liver and other viscera. Experimental research in

animals is an effective method of investigating stress since animal models are much easier to control environmentally, and that these models are genetically identical. Animal immobilization or restraint is known to be an applicable, easy, and convenient model to induce both psychological

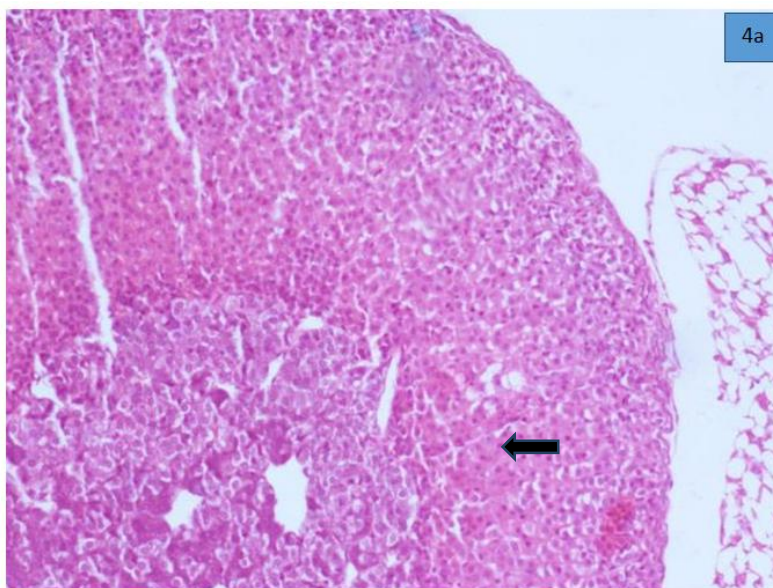


Fig. 4(a). Suprenal gland of post-weaned age 5 days control (26 days old) showing normal adrenal cortex (H & E 4X)

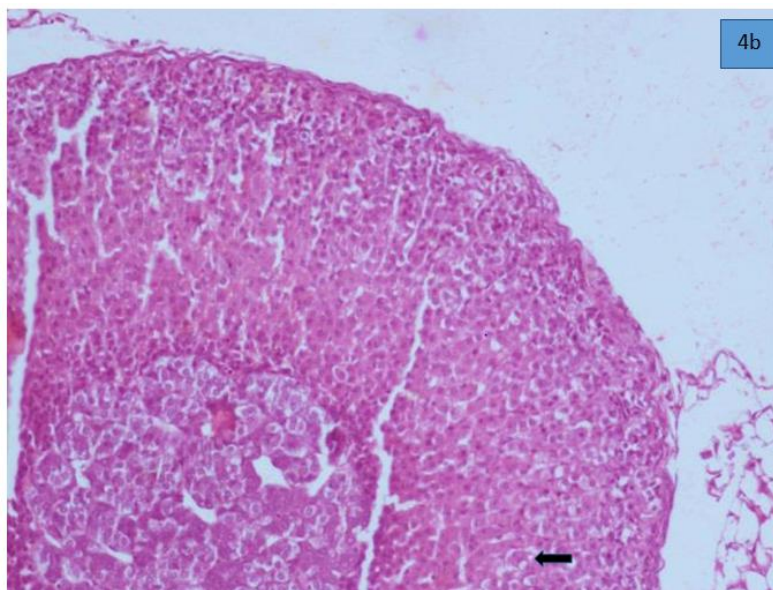


Fig. 4(b). Suprenal gland of post-weaned 5 restraint stress (26 days old) showing mild hypertrophy of adrenal cortex (black filled arrow) (H & E 4X)

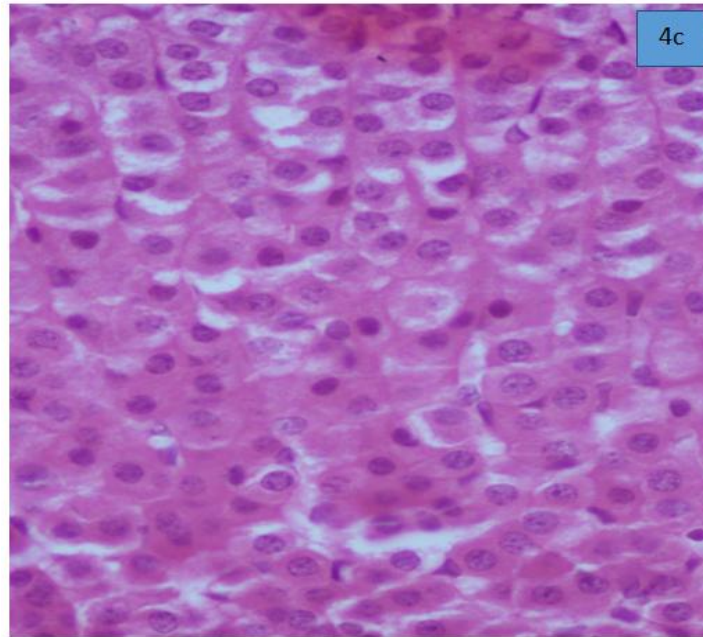


Fig. 4(c). Suprarenal gland of post-weaned mice control (42 days old) showing normal adrenal cortex (H & E 10X)

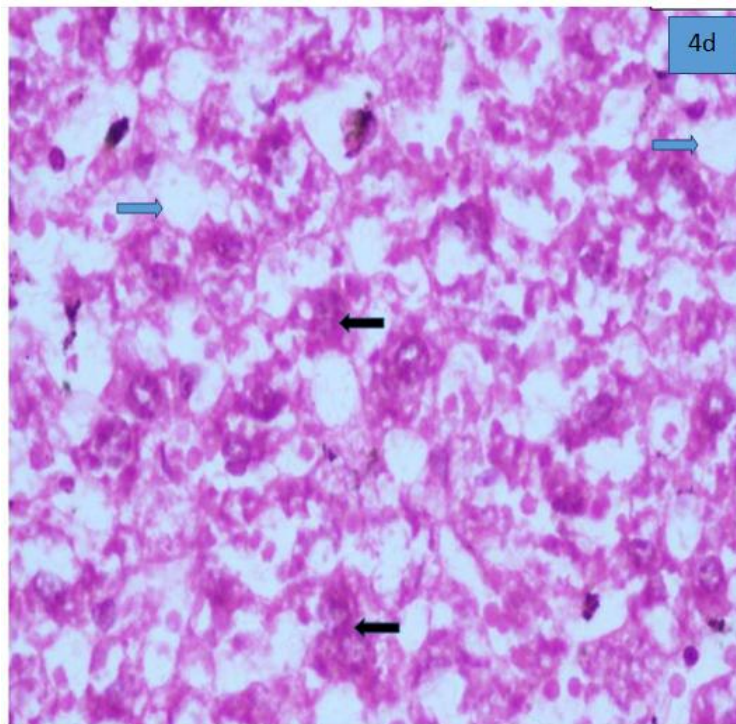


Fig. 4(d). Suprarenal gland (42 days old age) subjected to 21 days restraint stress showing hypertrophy and hyperplasia (black filled arrow) of the adrenal cortex with the presence of lipid deposits blue filled arrow) (H & E 10X)

and physical stress. Immobilization or restraint can induce psychological escape reaction and physical muscle work, which result in restricted mobility and aggression [28]. Restraint stress has continued to dominate stress-induced methodologies, especially for experiments that employ rodents as subjects [29]. Our present study which showed hepatic cells dilatation and congestion of sinusoids, coupled with the frequent fatty change, in liver hepatic cells of post-weaned age mice subjected to 21 days of restraint stress compared to mild degeneration of hepatic cells among the 5 days restraint stress group is a clear indication that duration of exposure could harm the cellular integrity of the hepatic cells. Other workers had equally observed that restraint stress affects cellular integrity in many tissues including the heart, stomach, and brain, and especially the liver [29], [30]. All these alterations may be caused by catecholamine. Psychological or physical stress stimulates the adrenal medulla to secrete the two catecholamines, adrenaline (epinephrine) and noradrenaline (norepinephrine), which initiate the "fight or flight" response. Many researchers have found that catecholamine and adrenoceptors are implicated in the modulation of cytotoxicity and tissue injury including the liver. It has been reported that adrenaline can promote hydroxyl radical generation in isolated rat hepatocytes [31]. The liver injury, caused by stress, may also be the consequence of enhanced free radical generation and altered antioxidant enzyme activities [32,33]. Oxidative damage to the DNA as well as lipid peroxidation and protein oxidation are among the few alterations of restraint stress and catecholamine is postulated to be responsible for all aforesaid changes [34]. Some workers have observed that impaired liver function and hepatocyte injury may be caused by continuous administration of norepinephrine hormone in the peritoneal cavity employing implantable mini-osmotic pump [35].

The effect of duration was equally shown on the histology of the kidney as mice subjected to 5 days stress showed mild degeneration of glomerulus and convoluted tubules compared to a higher level of degeneration of glomerulus and convoluted tubules from 21 days stress. The effect was normal on the controls used. Several studies confirmed that restraint stress triggers the generation of free radicals particularly in the mitochondria, peroxisomes, and cell membrane, and significantly lowers the antioxidant enzyme activities. The so formed free radicals from cell metabolism have been attributed to kidney

histological changes and cellular toxicity in general. The observed changes may be due to an imbalance between reactive oxygen species generation and its suppression by the antioxidant defense system [36,37,38]. The kidney is one of the vital sources of antioxidant enzymes, including glutathione peroxidases, and therefore, the impairment of this organ in the course of kidney malfunction could be associated with decreased levels of these enzymes as well as increased levels of pro-oxidants [39]. Another stress model attributed to kidney damage is heat stress and is said to interfere with animal performance. In animal fields, heat stress can be noted by the rise in body temperature and an increase in respiration rate. This rise in body temperature affects the blood flow, reduces food intake, and causes hormonal imbalance, which can consequently affect the productive and reproductive functions of the animals, [40]. Epinephrine induced stress has also been reported to cause histological changes in the kidney of mice [41]. In their study, one group of Swiss Albino mice was treated with epinephrine as a stress model. Frequent vacuolization, dilated bowmen's capsules, clusters in the glomerulus, and degenerated endothelium of PCT are a few of the many histological changes observed in the cortical region of the kidney.

The suprarenal gland under 21 days restraint stress in our study showed irregular orientation with moderate hypertrophy and hyperplasia of the adrenal cortex and also the presence of moderate lipid deposits. Chronic stress activates the HPA axis which leaves a cumulative effect such as adrenal gland hypertrophy and thymic atrophy due to glucocorticoid-mediated apoptosis. These effects are directly connected to the effects of the stressors on CRH neurons. Considering the dynamics underlying the progression of chronic stress exposure is of great importance. The first few days of stress exposure are characterized by significant increases in glucocorticoid secretion with the largest somatic and HPA axis effects. A good example is that of profound initial weight loss that is maintained throughout the stress exposure period [42]. Activated HPA axis during stress response causes rapid liberation of ACTH which in turn influences the synthesis of glucocorticoid in the zona fasciculata of the adrenal cortex. Continuous exposure of the stress irrespective of its strength will gently return the normal levels of pituitary-adrenal hormones particularly that of ACTH. However, the higher levels of Glucocorticoids (GC) will be maintained

[43]. Chronic immobilization stress in rats has been recently documented to be associated with adrenal gland hypertrophy, increased serum corticosterone levels, and anxiety-like behavior [44].

5. CONCLUSION

Stress causes hepatic cell degeneration with congested sinusoids which could lead to improper function of the liver. It may equally cause degeneration of glomerulus and convoluted tubules as well as irregular orientation, swelling, and a marked increased number of cells of the adrenal cortex. All these effects are capable of showing duration-response characteristics with stress and a more deteriorating effect as the level of stress increases. These morphological changes can explain the impaired immunity which develops in organisms that are exposed to chronic stress.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Prior permission for this undertaking was obtained from the institutional animal ethical committee (IAEC) at Manipal University, (No: IAEC/KMC/08/2017). All the experimental procedures were appropriately followed as per the guidelines of the IAEC.

DISCLAIMER

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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