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Vitamin C Ameliorates the Malformed Tissue Integrity of Kidney and Liver in Swiss Albino Mice (*Mus musculus*) exposed to Restraint Stress and Dietary Salt

Sajeeb Khan A.[1]*, Manish K.[2], Praveen Kumar[1], Jaya Chithra S.K.[1] and Ayana Gayathri R.V.[3]
[1]Post graduate and Research Department of Zoology,
Government College for Women, Thiruvananthapuram (Kerala), India.
[2]Department of Zoology, VTM NSS College, Thiruvananthapuram (Kerala), India.
[3]Post graduate and Research Department of Zoology,
University College, Thiruvananthapuram (Kerala), India.

(Corresponding author: Sajeeb Khan A.)*
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ABSTRACT: Vitamin C, being an antioxidant, plays a crucial role in various physiological functions, and a critical regulator in cellular integrity. The effect of stressors like chronic restraining and salt-enriched diet in the kidney and liver histology and the role of Vitamin C in maintaining tissue integrity were studied in Swiss Albino mice *Mus musculus.* The mice were either exposed to restraint stress daily 1 hour alone or supplemented with ascorbic acid (330 mgL^{-1}) via drinking for three weeks. Similarly, groups of mice were either fed with 4% salt-rich diet alone or supplemented with ascorbic acid (330 mgL^{-1}) via drinking water for the same duration. Structural changes in kidney and liver were evident in mice exposed to chronic restraining and excess dietary salt. Volume expansion or necrosis of Bowman's capsule, shrunkenglomeruli, renal tissue gap formation, hyperplasia and fibrous tissue infiltration were observed in the kidney. Cellular hypertrophy, volume expansion, nuclear pyknosis and granulomatous lesions were observed in the liver tissue due to stress. Vitamin C supplementation under stressed conditions could effectively prevent these stress-induced changes by retaining the tissue integrity in mice and has thus proven its role in tissue protection during stress.

Keywords: Restraint stress, salt-rich diet, Vitamin C, Mice, kidney, liver.

INTRODUCTION

In animals, stress changes the structural and functional properties of organs, tissues, and cells in animals and reflects disturbed homeostasis**.** It is widely accepted that salt and water control by the kidney plays a vital role in the pathophysiology of salt-induced hypertension (Adaramoye *et al*., 2008; Bindhumol *et al*., 2003). High NaCl and urea concentrations are stressful, disrupting the function of cells and possibly causing death through apoptosis. Proteinuria and glomerular and tubule-interstitial damage are typical hallmarks of hypertensive renal damage. Additionally, a high salt diet is associated with damage to gastric mucosa and gastritis in humans and experimental animals and induces cancer when combined with proven gastric carcinogens. (Campese and Park 2006; Caso *et al*., 2007).

Anxiety and mood disorders are linked to exposure to confinement in animals and psychosocial stress in humans respectively (Chen and Herbert 1995). Many adverse effects of stress are linked with neurochemical and hormonal imbalances (Dallman *et al*., 2000). Moreover, restraint stress can also affect the cellular integrity of the tissues (Drake *et al*., 1996).

Vitamins and minerals are frequently used as dietary supplements to improve health, prevent chronic illnesses, and treat certain health issues (Fortmann *et al*., 2013; Gangadharan *et al*., 2001). Numerous studies have shown that ascorbic acid promotes collagen production and plays a crucial role in maintaining collagen (Kaplan, 2006). Vitamin C stimulates collagen synthesis and acts as a cofactor for lysyl hydroxylase and prolyl hydroxylase, enzymes vital for collagen synthesis (Kaplan, 2006). Therefore, a lack of ascorbic acid lowers the hydroxylation of proline and lysine, which in turn reduces collagen formation. Vitamin C acts as a cofactor of enzymes involved in the carnitine biosynthetic pathway (Kim *et al*., 2012). It is well known that the increase in reactive oxygen species (ROS) in the mitochondria leads to the breakdown of the mitochondrial membrane and the induction of apoptosis. The reduction of ROS in response to vitamin C treatment has a significant anti-apoptotic effect (Kovacs *et al*., 1996). Further, high dietary ascorbic acid intake has been shown to protect against gastric cancer and the ill effect of ionising radiations (McCormick *et al*., 1998; Mandl, 2009).

The present study was designed to assess the effect of vitamin C supplements in retaining tissue integrity, mainly at the kidney and liver tissue, in mice exposed to chronic restraint stress and excessive dietary salt.

MATERIALS AND METHODS

Healthy adult male and female Swiss albino mice (*Mus*

musculus) were used for the experiments in this study. Animals were kept in the remote building and shielded from dust, smoke, noise, wild rodents, insects, birds, and other environmental irritants. Animals weighing 32±3 g were kept in groups of five in polypropylene cages for mice (Size: 29 × 22 × 14cm) with a stainless steel-wire mesh top. All animals were maintained under a 12-hour light/12-hour dark cycle at a temperature of 24 ± 4ºC and relative humidity of 70 ± 10. Animals were allowed *ad libitum* access to a standard pelleted maintenance diet (Sri Sai Durga Feeds and Products, Bangalore) and purified tap water. Cage bedding was changed once every two days. The animals were brought to the laboratory in home cages for conducting the experiments, and care was taken to provide similar conditions. The experimental animals were managed following the institutional ethics committee protocols of the University of Kerala (No.IAEC-KU-31/2011-12-ZOO-MCSP (2)).

The Vitamin C supplement was prepared as described by Massip *et al*. (2010). To supplement vitamin C, 330 mg/L L-ascorbic acid was prepared and subsequently, 20 µl 0.01mM EDTA was added to increase the stability of the ascorbic acid.

The standard pelleted feed was powdered and made into small round pellets by soaking the powder with drops of water. The round pellets were sun-dried to remove moisture. This feed was considered as the normal diet. Similarly, the salt-enriched diet (4 %) was prepared by adding 40 g of NaCl (Sisco Research laboratories India Ltd) into 960 gm of standard feed powder. The mixture was again soaked with water drops to make small round pellets and sun-dried.

Animals were immobilised individually using hollow tubes made of polyvinyl chloride with length and diameter of 12cm and 5cm, respectively. One end of the tube was closed, and the opposite end was perforated with a few holes for ventilation. The tubes with animals were placed vertically. The animals were immobilised due to this procedure, generating a reasonably powerful and stressful stimulus.

Twenty adult male Swiss albino mice of 4 weeks old were randomly selected and divided into four groups, each comprising five animals. The first group, designated as **A**, served as a control that received the normal diet and drinking water (200ml/cage) daily without any disturbance except for handling for bedding change during the experiment. The second group B was provided with a normal diet and drinking water supplemented with Vitamin C. The third group **C** was subjected to restraint stress for 60 minutes daily (11.00am-12 pm). The fourth group D received both restraint stress and drinking water supplemented with Vitamin C. The treatment continued for twenty-one days. The feed and water were supplied *ad libitum*.

Twenty adult female Swiss albino mice of4 weeks old were randomly divided into four groups, comprising five animals. The first group A served as control, which received a normal diet and water (200ml/cage) daily without any disturbance except for handling for bedding change during the experiment. The second group B was provided with a normal diet and drinking water supplemented with Vitamin C. The third group **C** was subjected to salt-induced hyperosmotic stress by providing 4% salt-enriched diet and drinking water (200ml/cage) daily. The fourth group D, received both salt-induced hyperosmotic stress through the 4% salt enriched diet and drinking water supplemented with Vitamin C. The treatment continued for 21 days. All animals were provided with food and water *ad libitum*.

Tissues were collected and preserved in Bouin's fluid from the sacrificed animals at the end of the experiment. Histological preparations of the tissues (Renal and hepatic tissues) were carried out as per the procedures of McCormick *et al*. (1998) with slight modifications. After infiltration, the tissues were embedded in wax and blocks were prepared. Sections were cut at a thickness of 5 µ using a rotatory microtome (Leica, Germany) and they were subsequently mounted on glass slides using Mayer's albumin as an adhesive. The slides were kept in staining racks and cleared the paraffin in three changes of xylene for 3 minutes per change. Following the clearing procedure the slides were transferred through ethanol series of 100%, 95% and 70% concentrations respectively for three changes of 2 minutes duration per change. Lastly the slides were rinsed in running tap water at room temperature for 2 minutes. The slides were then dipped in hamatoxylin solution for 3 minutes and placed under running tap water at room temperature for 5 minutes. This is followed by staining in working eosin Y solution for 2 minutes. Dehydration was done by passing the slides in 90% ethanol for 20 minutes and further through 95% and 100% ethanol for 10 minutes per change. The slides were then cleared in three changes of xylene for 2 minutes per change. Mounting procedures were undertaken by placing a drop of Permount over the tissue on each slide and put a cover slip. After completing the entire procedure, the tissues were observed under the microscope (Leica, Germany) and images were captured.

RESULTS AND DISCUSSION

Histological analysis of stressed mice revealed his to pathological changes in the renal and hepatic tissues. Our results showed that control and vitamin C supplemented mice maintained the normal histological structures in the kidney and liver. Restraint stress, which causes immobilisation, is an unpleasant stimulus that disrupts physiological homeostasis and impacts many biological systems in the body (Kumar *et al*., 2010). The restrain causes hormonal imbalance; for instance, the restraint results in elevated levels of plasma corticosterone and adrenocorticotropic hormone (Hirotaka and Tsuyoshi 2020).

The histological analysis of the control and vitamin C supplemented mice groups revealed a normal his to architecture with standard morphological features in the renal tissues (Fig. 1). However, deformed kidney architecture was observed in mice exposed to restraint stress. In nephrons, increased thickness of Bowman's capsule due to the proliferation of fibrous connective tissue with mononuclear infiltration was observed. Additionally, gap formation in kidney cortex with

shrunken glomeruli was seen in mice subjected to chronic restraint stress. Hyperplasia of tubular epithelium resulted in increased tubular diameter (Figures 1&2). The deformation in renal architecture was very meagre in the mice group, which were vitamin C supplemented but exposed to chronic restraint stress. The fibrous tissue infiltration was less and almost normal capsular thickness was observed. The glomerular shrinkage and renal tissue gap formation were also not evident in this group (Fig. 1&2).

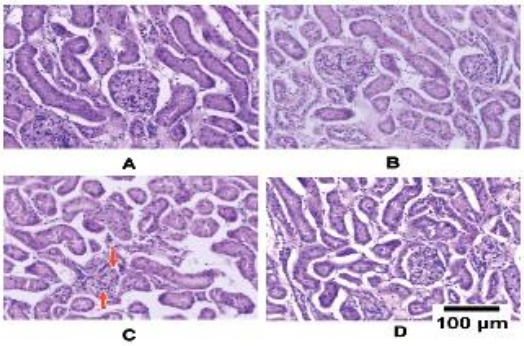

Fig. 1. Histological sections of mouse kidney stained with Hematoxylin-eosin (A) Control, (B) Vitamin C, (C) Restraint and (D) Vitamin C+Restraint. Red arrows indicate capsular hypertrophy and shrunken glomeruli due to fibroid tissue infiltration

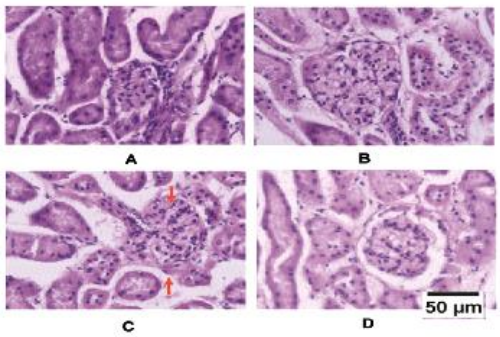

Fig. 2. Histological sections of mouse kidney stained with Hematoxylin-eosin. (A) Control, (B) Vitamin C, (C) Restraint and (D) Vitamin C+Restraint. Red arrows indicate capsular hypertrophy due to fibroid tissue infiltration

In the current investigation, restraint stress-induced alterations were noted, including an increase in Bowman's capsule thickness and a growth of fibrous connective tissue with mononuclear infiltration. Renal tissue gap formation and reduced glomeruli were also detectable. These alterations were all indications of renal tissue necrosis. Additionally, it had already been shown that restraint stress raises blood pressure and heart rate, primarily due to the activation of the sympatho- adrenal system (Pinnell, 1985). The kidneys from the restraint group in our study likewise displayed glomerulonephritis caused on by hypertension. Neurochemical and hormonal abnormalities, which are frequently associated to oxidative stress, enhance the adverse effects of stress (Dallmann *et al*., 2000). Moreover, it is well known that sub-acute immobilisation stress affects neurologic outcomes by activating excite toxic and inflammatory brain pathways, which results in a rise in infarct volume (Radimer *et al*., 2012).

In mice treated with a high salt diet, renal cells were shrunken, particularly in the cortical region (Fig. 3-5). A high salt diet caused a significant infiltration of lymphocytes and macrophages in the interstitial areas of the kidney cortex. Inflammatory tissue proliferation, macrophage infiltration and necrosis of Bowman's capsule were seen in this group (Fig. 4). Additionally, renal tubule constriction, vascular degradation, epithelial shedding, and an accumulation of inflammatory cells in the interstitial region were seen. (Fig. 3, 4). Higher magnification showed shrinkage of glomeruli, cuboidal cells of nephron tubules and gap formation in mice treated with a high salt diet (Fig. 5). In the mice group, which were vitamin C supplemented but fed with a high salt diet; the renal tissue showed the integrity of cortical and medullary regions with less inflammatory and macrophage cells (Fig. 3, 4). Capsular necrosis and tubular constrictions were also inconspicuous. Glomeruli were intact with very little gap formation (Fig. 3, 4).

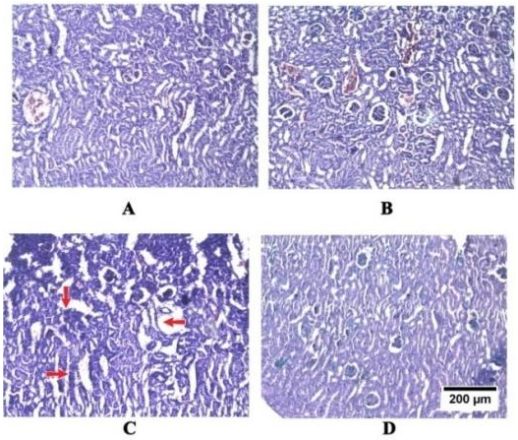

Fig. 3. Histological sections of mouse kidney stained with Hematoxiline-eosin. (A) Control, (B) Vitamin C, (C) 4% salt-rich diet and (D) Vitamin C+ 4% salt-rich diet. Red arrows indicate tubular hyperplasia and necrotic lesions in the cortical regions.

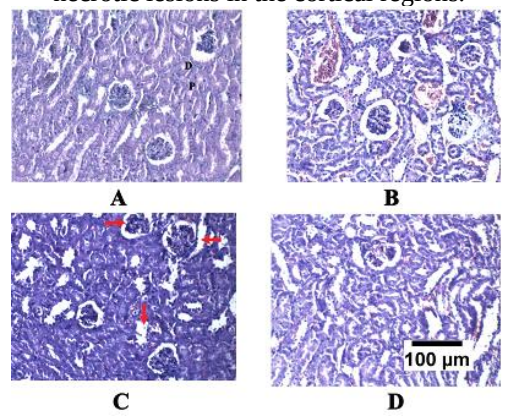

Fig. 4. Histological sections of mouse kidney stained with Hematoxylin-eosin. (A) Control, (B) Vitamin C, (C) 4% salt-rich diet and (D) Vitamin C+ 4% salt-rich diet. Red arrows indicate necrosis of Bowman's capsule due to the proliferation of inflammatory tissue with macrophage infiltration.

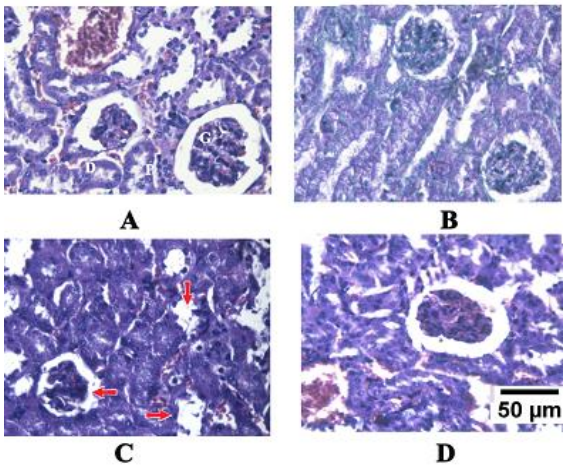

Fig. 5. Histological sections of mouse kidney stained with Hematoxylin-eosin (A) Control (B) Vitamin C (C) 4% salt rich diet and (D) Vitamin C+ 4% salt-rich diet. Red arrows indicate necrosis of glomerulus and tubular hypertrophy with fibroid infiltration.

Hepatocytes with centrally positioned nuclei and radially organised hepatic cords around the centralve in could be seen in the liver histology of the control (Fig. 6&7) and vitamin C-supplemented mice (Fig. 6, 7). The restraint stress altered the hepatic tissues (Fig. 6&7). The liver showed a disorganised his to architecture with changes represented by multiple granulomatous lesions active from an aggregation of macrophages and lymphocytes, scattered through the liver parenchyma (Fig. 6&7). Stress-induced hyperplasia of hepatocytes could also be observed (Fig. 6, 7).

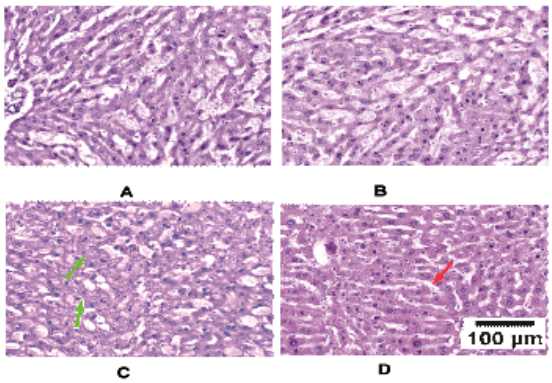

Fig. 6. Histological sections of mouse liver stained with Hematoxylin-eosin. (A) Control (B) Vitamin C (C) Restraint and (D) Vitamin C+Restraint, Green arrows indicate reduced or disappeared sinusoids. The red arrow indicates normal sinusoids due to the radial arrangement of hepatocytes.

Further, areas of coagulative necrosis characterised by pyknotic or disappeared nuclei could be observed (Fig. 6, 7). Mice exposed to chronic restraint stress and vitamin C supplementation showed no granulomatous lesions but random macrophages in the parenchyma (Fig. 6&7). Hepatocytes were normal with prominent nuclei. Radial arrangement of hepatic cords was also visible (Fig. 6&7). In mice, fed with a salty diet, the enlargement of hepatic cells resulted in volume expansion and cellular hypertrophy (Fig. 8-10). This group showed dilated central vein with hypertrophy of hepatocytes with pyknotic nuclei, vacuoles and hyalinisation. The radial arrangement of hepatocytes was lost due to vacuolisation and hyalinisation (Fig 8-10). The liver histology of the mice group supplemented with vitamin C but fed with as salty diet seemed more or less normal (Fig. 8-10). Cellular hypertrophy was significantly less, and nuclei were intact in the hepatocytes. The hepatocytes were seen radially arranged around the central vein (Fig. 8-10).

The salt increases the sodium ion concentration in the blood and impairs homeostasis, lowering the ability of the kidneys to excrete water. Thus, it works on the kidneys to make the mice hold on to more water. The excess fluid and strain on the sensitive blood arteries cause a rise in blood pressure, damaging the kidneys. The anatomical features of mice on a high-sodium diet underwent significant changes, including glomerular and tubular necrosis, glomerular fibrillation, cell shrinkage in distal and convoluted tubules, and gap formation in renal tissue.

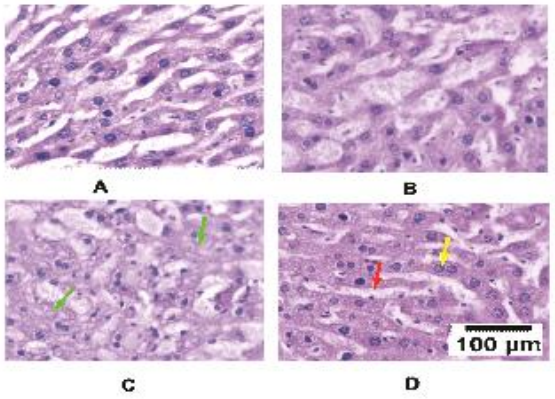

Fig. 7. Histological sections of mouse liver stained with Hematoxylin-eosin. A) Control, (B) Vitamin C, (C) Restraint and (D) Vitamin C+Restraint, Green arrows indicate apoptotic hepatocytes with heteropyknotic nuclei. The red arrow indicates normal sinusoids due to the radial arrangement of hepatocytes. Yellow arrow indicates normal hepatocyte with the nucleus.

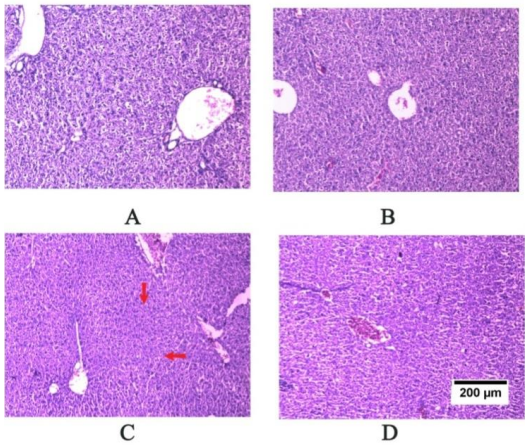

Fig. 8. Histological sections of mouse liver stained with Hematoxylin-eosin. (A) Control, (B) Vitamin C, (C) 4% salt-rich diet and (D) Vitamin C+ 4% salt-rich diet. The red arrows indicate shrunken sinusoids due to hepatocyte hypertrophy.

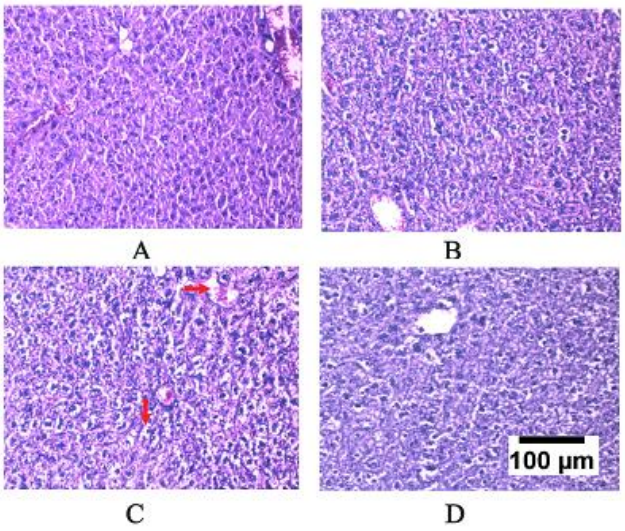

Fig. 9. Histological sections of mouse liver stained with Hematoxylin-eosin. (A) Control, (B) Vitamin C (C) 4% salt-rich diet (D) Vitamin C+ 4% salt-rich diet. The red arrow indicates dilated central vein and hypertrophy of hepatocytes with pyknotic nuclei.

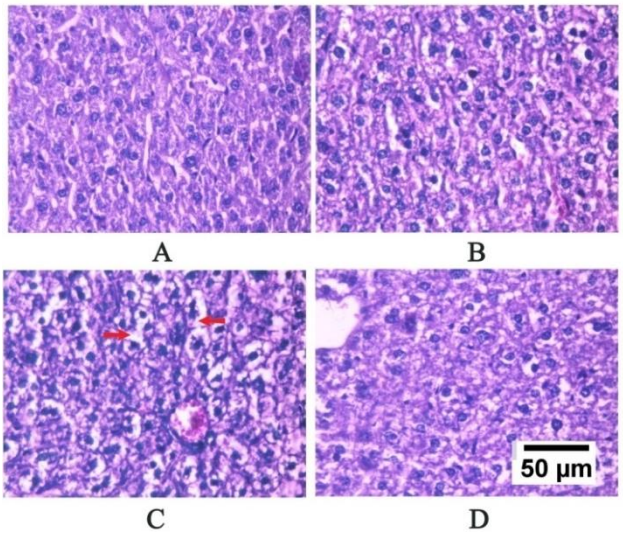

Fig. 10. Histological sections of mouse liver stained with Hematoxylin-eosin. (A) Control, (B) Vitamin C (C) 4% salt rich diet (D) Vitamin C+ 4% salt-rich diet. The red arrows indicate hypertrophy, vacuolization and hyalinization of tissue.

In the current study, excessive salt loading may lead to a state comparable to glomerulonephritis in which the membrane is disrupted and the glomeruli become scarred. Glomerulonephritis impairs the kidney's capacity to filter blood adequately, causing waste to accumulate up in the bloodstream and possibly resulting in renal failure. Previous works has demonstrated that high salt treatment increased kidney weight in rats, which may have been caused by hypertrophy of existing cells or elevated interstitial tissue, water, or fat (Tatematsu *et al.*, 1975) Salt-induced oxidative stress might produce ROS and other free radicals, eventually leading to lipid peroxidation of biomembranes. As previously observed, the kidney was more susceptible to oxidative damage than the liver (Eatemad and Awadalla, 2010).

Animals supplemented with Vitamin C and a diet high in salt or those subjected to restraint stress revealed that the membrane deformation brought on by stress was either recovered from or resisted. According to several findings, vitamin C possesses antioxidant properties that can prevent oxidative stress induced by free radicals (Rebouche 1991; Lee *et al.*, 2007). Animals that are provided with Vitamin C maintained their tissue structures similar to that of the controlled group, indicating stress recovery. The liver is a vital organ that plays a crucial part in regulating animal homeostasis. Evidences support that immobilisation increases lipid peroxidation in the acute stress model, suggesting stress-induced liver tissue damage (Weyers *et al.*, 2001; Sanchez *et al.*, 2002). The liver tissue of the restraint mice in the current study indicated granulation, hypertrophy, apoptotic hepatocytes, and necrotic sinusoids. The consequences of hepatotoxic substance-induced liver tissue damage are severe. Ionic and fluid balance alterations could result from the high sodium doses. Thus, too much sodium may result in the accumulation of intracellular water, resulting in localised hepatic oedema. The critical barrier, the plasma membrane, is frequently disrupted by the enlargement of the cell, allowing cell contents to leak and eventually killing the affected cell. As salt loading and the frequency of cell wounding in tissues are associated, hyperosmotic stress is suggested as the disrupting factor. The body's xenobiotic metabolism and excretion are mostly carried out by the liver and kidneys. Our findings show that by restoring membrane integrity, vitamin C supplementation can inhibit disruption-induced cellular death. It has been documented that Vitamin C prevents free radical damage in membrane lipids (Olga *et al.*, 2003). Thus vitamin C supplementation might enable the hepatocytes to resist the salt-induced cellular hypertrophic state.

CONCLUSIONS

Taking all our results together, we conclude that emotional or physical stress damages cellular integrity in tissues. Stressors like chronic restraining and intake of foods with high salt levels negatively affect the integrity of tissues, as demonstrated by the histological examination results. Restraint stress had induced such structural changes in the kidney like increased thickness of Bowman's capsule, shrunken glomeruli and increased tubular diameter, whereas, in the liver, it caused a disorganised his to architecture like multiple granulomatous lesions, hyperplasia of hepatocytes with coagulative necrosis characterised by pyknoticnuclei. The hypertrophy, hyperplasia, nuclear enlargement, and gap development in the kidney and liver are indications of structural deformity driven on by the high salt diet. However, Vitamin C supplementation has countered these ill effects by either slowing down tissue damage or by maintaining the tissue integrity with reduced inflammation in mice exposed to either restraint stress or dietary salt excess. Overall, the findings of this study showed that vitamin C supplementation had significant effects in restoring the distorted his to architecture of

kidney and liver in mice exposed to high dietary salt or restraining stressors. These findings indicate the protective role of vitamin C against stressors.

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Conflict of interest. None.

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