Pathophysiological Changes of Hyperthermia Combined with Dehydration on Rat Submandibular Salivary Glands: Ultrastructural and Immunohistochemical Study

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Abstract: In warm climate areas, general dehydration is remarkable, most likely due to insufficient fluid intake and increased body temperature. Physiological and pathological factors generally influence salivary secretion rate. **Aim:** is to study the effects of elevated ambient temperature and dehydration on rat SMG histologically and ultrastructurally. **Material and Methods:** Rats were randomly divided into three groups: **Control group;** rats received dried food and water ad libitum at 21-23°C room temperature. **Hyperthermia group**; rats received dried food and water ad libitum for a period of 5 days at 39-41°C room temperature. **Hyperthermic dehydrated group**; rats were deprived of water with free access to dry food at 39-41°C room temperature. SMGs specimens were processed for histological, immunohistochemical and ultrastructural evaluation. **Results:** light microscopic examination of rat SMG of hyperthermia group, illustrated wider stromal spaces in between the parenchymal elements with obvious hyperemic vasodilation. Some acinar and ductal cells displayed obvious degenerative changes as vacuoles, pyknotic nuclei and apoptosis with striking ductal dilation. Wherease, in hyperthermic dehydratedgroup, the serous acini appeared pale-stained with marked cytoplasmic vaculation and very few secretory granules. Also, there were less ductal and vascular dilatation than those observed in hyperthermiagroup. Most of the dilated blood capillaries showed densely-packed disfigured (morphologically-deformed) RBCs and prominent swollen endothelial cells bulging into the capillary lumen. Extravasated RBCS were also observed within the stromal tissue. Immunohistochemically, SMG of hyperthermia group showed higher cytoplasmic Bax expression in some ducts and in their adjacent acinar cells in comparison to control and hyperthermic dehydrated groups. However, endothelial cells of blood capillaries of hyperthermic dehydrated group revealed marked Bax reaction. TEM examination of hyperthermia group demonstrated many acinar cells studded with numerous secretory granules. Degenerative changes in nuclear membrane, rough endoplasmic reticulum and mitochondria of the acinar as well as striated ductal cells were evident. Whereas, rat SMG of hyperthermic dehydrated group showed massive degenerative features in acinar and ductal cells as pyknotic hyperchromatic nuclei, destroyed Golgi apparatus, ruptured mitochondria, more vacuoles, few electronlucent secretory granules and loss of basal in foldings of striated ductal cells. **Conclusion:** Deficiency of water intake in high temperature environment was found to influence SMGs. It is important to emphasize on proper hydration to prevent serious damage to SMGs.

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1. Introduction:

Recent analytic studies predicted increases in temperature variability in tropical land regions(1). Garrabou et al. reported that summer heat waves are increasing in strength and frequency(2). The persistent extreme heat waves or droughts have severe impacts on many biological systems as a response to environmental changes(3). High temperature (Hyperthermia) severely affects activities and body organs and prompts physiological changes that adversely affects cerebrovascular, cardiac and respiratory systems(4,5). Vlad et al. reported various numerous vascular lesions in lung, heart, pancreas, liver, adrenal gland and kidneys after 30minutes of exposure to 40°C(6).

On the other hand, Water is essential for the maintenance of all body systems. Inadequate water drinking was established to be a high risk factor in a great proportion of children and adolescentsin cross-sectional surveys in 13 countries(7). Insufficient water intake reduces the extracellular fluid content that predispose to dehydration (8). Dehydration is defined as a decrease in the overall body water content owing to reduced fluid drinking, pathologic fluid loss or a combination of both (8). It contributes in the pathogenesis of many diseases of the urinary system, endocrine organs and gastrointestinal tract including oral cavity (9). Dehydration also increases body temperature while heating promotes significant shifts in water balance along with osmotic changes in

the peripheral blood vessels affecting hypothalamic thermosensitive neurons (10). Remarkably, water deficit concomitant with heat exposure promotes potentiation of hyperthermia and more enhanced loss of the extracellular fluid (11). Hyperthermia slows down the blood circulation and exposes organs to hypoxia and anoxia(12). Furthermore, a correlation between body hydration status and salivary gland function was established(13).

The submandibular gland (SMG) is the largest of the three major salivary glands. Salivary glands secrete about 1.5 liters of saliva daily. Saliva is secreted by submandibular (65%), parotid (23%), sublingual (4%) glands and minor salivary glands (8%) (14). Dehydration was reported to affect the parotid gland causing its hypotension and eventually dysfunction(15).

BAX is a protein belongs to the BCL2 family members that are involved in a wide variety of cellular activities (16). This protein forms a heterodimer with BCL2, and functions as an apoptotic activator. BAX plays a role in the mitochondrial apoptotic process(17). Under normal conditions, BAX is largely cytosolic via constant retro-translocation from mitochondria to the cytosol, which avoids accumulation of toxic BAX levels at the mitochondrial outer membrane (18,19). Under stress conditions as hyperthermia or dehydration, it undergoes a conformation change that causes translocation to the mitochondrion membrane, leading to the release of cytochrome c that promotes activation of CASP3, and thereby apoptosis (20,21).

Even though the severe deleterious effects of hydropenia on cell physiology, little information is available about histological alterations due to dehydration. Moreover, few studies evaluated low water intake as a factor associated with hyperthermia and their influence on SMG but not from a histological point. Hence, in this study the SMG will be examined under the effects of elevated ambient temperature and dehydration histologically and ultrastructurally.

2. Material and Methods:

Animals

Fifteen male Albino rats, weighing approximately 200g, were obtained from the Pharmacology Department, Faculty of Pharmacy in Tanta University and were housed in individual wire cages under 12-hour alternating light-dark cycle in a room maintained at 21-23°C. Rats were given food and water ad libitum throughout the experimental period.

Experimental Protocol:

The protocol was first approved and conducted according to established animal welfare guidelines for the responsible use of animals in research as a part of scientific research ethics recommendation of Research Ethics Committee, Faculty of Dentistry, Tanta University. After of at least 1week acclimatization, rats were randomly divided into three equal groups. **Group I or *Control group*** received dried food and water ad libitum at 21-23°C room temperature. ***Group II or Hyperthermia group***, received dried food and water ad libitum for a period of 5 days at 39°C to 41°C room temperature. ***Group III or Hyperthermic dehydrated group,*** rats were deprived of water with free access to dry food at 39°C to 41°C room temperature. At the end of experimental period, rats were anesthetized with ketamine chloride (Ketalar, 40 mg/kg body weight), Ketalar (par pharmaceutical companies, Inc. suffern, NY, USA) and the glands were excised and then the rats were euthanized by cervical dislocation.

Histological and Immunohistochemical (IHC) studies

The specimens were immediately fixed in buffered formaldehyde solution (10%) for 48h and processed for routine technique of paraffin inclusion. Histological sections of 4 µm thickness were cut and stained with the Hematoxylin and Eosin (H & E) stain and examined under the Light Microscope (LM).

IHC staining was carried out to detect the expression of BAX as an apoptotic marker. The Avidin-Biotin Complex (ABC) technique was performed using the following reagents:

-Primary antibody against BAX Ab-1 (Clone: 2D2/BAX, Mouse monoclonal, Thermo Fisher Scientific Anatomical Pathology. Fremont, CA, USA). The ABC Universal Kit (Neomarkers, Fremont CA, USA) was used.

-The IHC procedure was done according to the manufacturers' instructions. The deparaffinized sections associated with IHC kit were processed acting as positive controls whereas negative controls consisting of tissue sections on which primary antibody was replaced with non-immune serum was also performed.

- The sections were evaluated for BAX expression by assessing the site of staining (nuclear and/ or cytoplasmic) and intensity of staining that was expressed as weak, moderate or strong staining.

Transmission Electron Microscopic (TEM) study

Specimens of SMG were fixed with a glutaraldehyde 2.5% then fixed with osmium tetroxide 2% buffered. Subsequently, the specimens were dehydrated in successive passes of ethanol at concentrations of 50%, 70%, 85%, 95% and 100%. Finally, they were placed in acetone. Then, they were embedded in a mixture of 1:1 epoxy propane and EPON for 2 hours and delivered to the capsule in a

40°C oven for 1 hour and a 60°C oven for 48 hours. The polymerized samples were used to obtain ultrathin sections of approximately 60 nm. The samples were stained for contrast with uranyl acetate and lead citrate. The sections were examined with JEOL transmission electron microscopy (JEOL JEM-100SX).

3. Results

Light Microscopic results

Examination of H & Estained sections of SMG of control group showed the normal lobular architecture with thin intervening connective tissue septa. Each lobule contained closely packed serous acini and intralobular ducts. Acinar cells appeared pyramidal with darkly stained cytoplasm and rounded basophilic nuclei (Fig. 1A). The striated ducts were lined by columnar cells having central oval nuclei and characteristic basal infoldings (Fig. 1B).

In group II (hyperthermia group), It was observed that the normal salivary gland lobular architecture is preserved with obvious slightly wider stromal spaces in between the lobes, lobules and even between the serous acini (Fig. 2A). The acinar cells appeared smaller in size than normal and showed cytoplasmic vacuolation in the form of clear vacuoles at the apical part of the acinar cells in addition to dense cytoplasmic secretory eosinophilic granules. Marked ductal dilatation of intralobular and interlobular ducts was noted in addition to marked enlarged blood capillaries that were engorged with RBCs (Fig. 2B).

In group III (hyperthermic dehydrated group), the serous acini appeared swollen, pale-stained with numerous cytoplasmic vacuolation and very few secretory granules. There were also ductal and vascular dilatation but less obvious than those noted in (hyperthermia group) (Fig. 3A). Most of the dilated blood capillaries showed densely-packed disfigured (morphologically-deformed) RBCs and prominent endothelial cells bulging into the capillary lumen. Extravasated RBCS were also observed into the stromal tissue (Fig. 2B).

IHC results:

Inhyperthermia group, it was noted that BAX expression appeared as intense focal patchy brown mainly cytoplasmic granular stain in some ducts and in adjacent acinar cells, while it was absent or very weak at the remaining parts of salivary gland lobules. Moreover, prominent nuclear BAX immunostaining was also observed in some acinar and ductal cells (Fig. 1C & 2C).

In hyperthermic dehydrated group, negative or faint patchy granular BAX staining was demonstrated in few acinar and ductal cells as well as nuclear immunostaining. Endothelial cells also showed cytoplasmic BAX expression (Fig. 3C).

Transmission Electron Microscopic results

Ultrastructural examination of acinar cells of the control SMG showed normal architecture of seromucous acini as pyramidal cells having rounded euchromatic nuclei. In the basal part, they depicted parallel arrays of rough endoplasmic reticulum, mitochondria and Golgi saccules. Lateral and apical to the nucleus, there were variable sizes of membrane bounded secretory granules (Fig. 4A). Striated ducts showed tall columnar cells with rounded centrally placed nuclei surrounded by few rough endoplasmic reticulum and Golgi saccules. Basally, these cells depicted numerous mitochondria in between the deep infoldings of the plasma membrane (Fig. 4B).

Ultrastructural examination of acinar cells of hyperthermia group showed acinar cells studded with many secretory granules that many coalesce with each other. Also, dilated perinuclear space with folded nuclear membrane, slightly extended rough endoplasmic reticulum and multiple destructed mitochondria (Fig. 5A). Striated duct cells demonstrated loss of basal infoldings, ruptured mitochondria, few distended rough endoplasmic reticulum and many vacuoles in the apical cytoplasm (Fig. 5B).

Whereas, in hyperthermic dehydrated group, the degeneration was exaggerated. Acinar cells were smaller and showed pyknotic hyperchromatic nucleus, increased dilatation of perinuclear space and folding of the nuclear membrane. Degeneration of the cytoplasm was evident with destructed Golgi apparatus, ruptured mitochondria, more vacuoles, and few electronlucent secretory granules. The cisterns of rough endoplasmic reticulum were extended (Fig. 6A). Striated duct cells disclosed degeneration of the cytoplasm leaving few ruptured mitochondria, few distended rough endoplasmic reticulum and loss of basal infoldings (Fig. 6B).

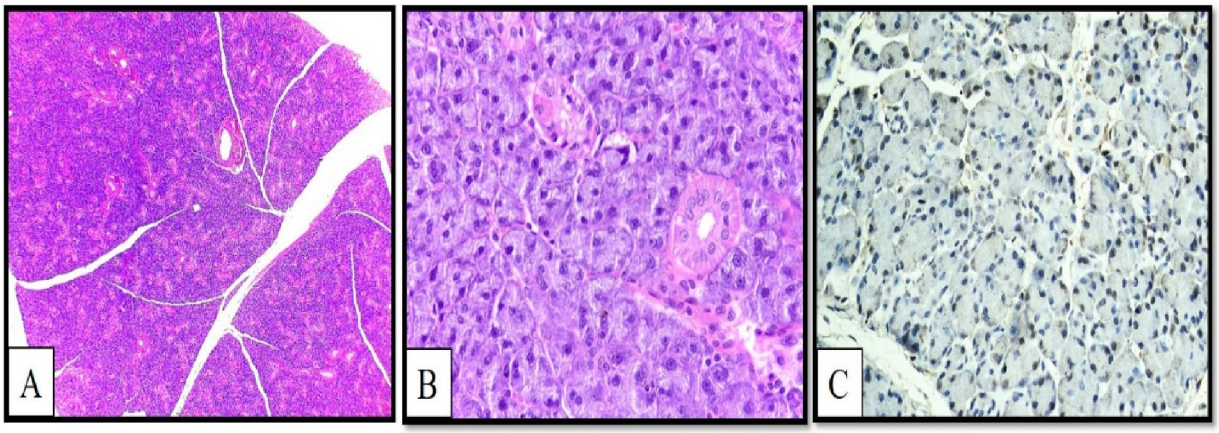

Figure (1): Photomicrograph of group I (control group) displays: (A)Normal lobular architecture of SMG with intervening connective tissue stroma (H & E, x100). (B) Serous acini with granular acinar cells and normal-sized intralobular ducts (H & E, x400). (C) Negative BAX expression in acinar and ductal cells. (ABC, x400)

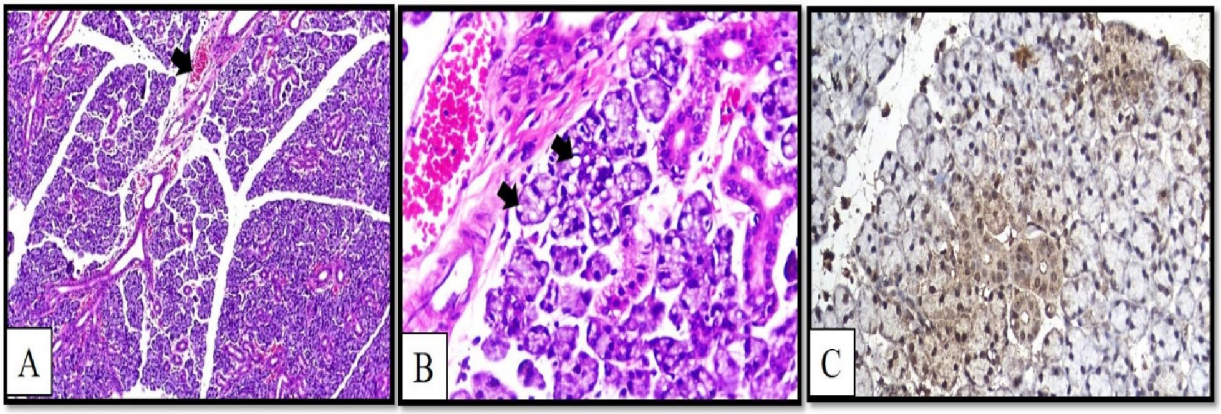

Figure (2): Photomicrograph of group II (hyperthermia group) illustrates: (A) Lobules of SMG tissue that demonstrate abnormally small-sized serous acini with wider inter-acinar stromal spaces and marked dilatation of blood capillaries (arrow) (H & E, x100). (B) Cytoplasmic vacuolar degeneration of acinar cells is observed (arrows) with marked capillary and ductal dialation (H & E, x400). (C) Focal patchy moderate to strong cytoplasmic granular as well as nuclear BAX immunostaining of some aggregates of serous acini while the remaining acini and ductal cells show negative stain. (ABC, x400)

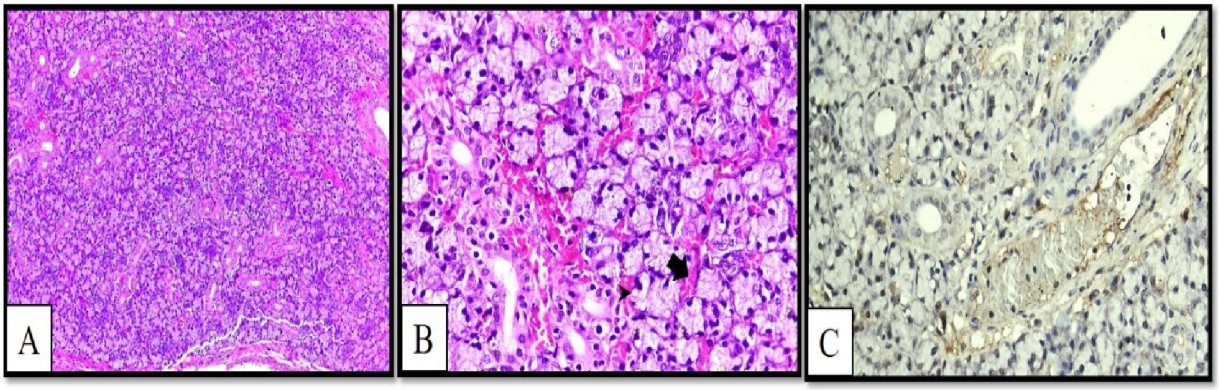

Figure (3): Photomicrograph of group III (hyperthermic dehydrated group) depicts: (A) Densely packed serous acini with ductal dialation (H & E, x100). (B) Marked cytoplasmic vacuolar degeneration of acinar cells (arrowheads) as well as slightly dilated blood capillaries that are engorged with compressed disfigured RBCS, in addition to extravasated RBCs (arrows) (H & E, x400). (C) Cytoplasmic BAX staining of dilated ductal as well as endothelial cells. Nuclear staining of some adjacent acinar cells is seen while other acini shows negative BAX staining. (ABC, x400)

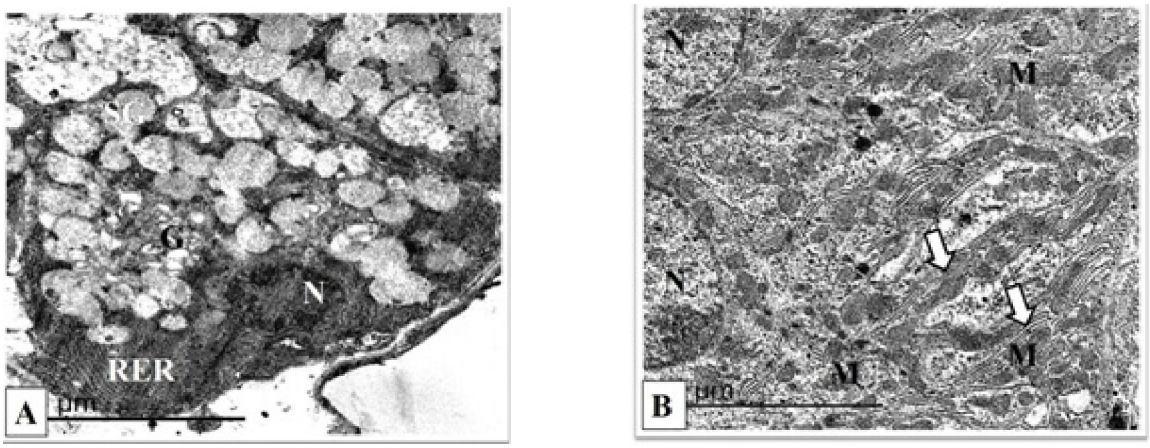

Figure (4): Electron micrograph of control group reveals: (A) Normal pyramidal shaped acinar cell that has a basal nucleus, basal parallel arrays of RER cisternae and electronlucent seromucous granules (SG). (B) Normal striated duct columnar cells that have euchromatic centrally located nucleus with basally situated profiles of elongated mitochondria (M) interspersed between the folded basal membrane (arrows). (Mic. Mag. A & B: x1500).

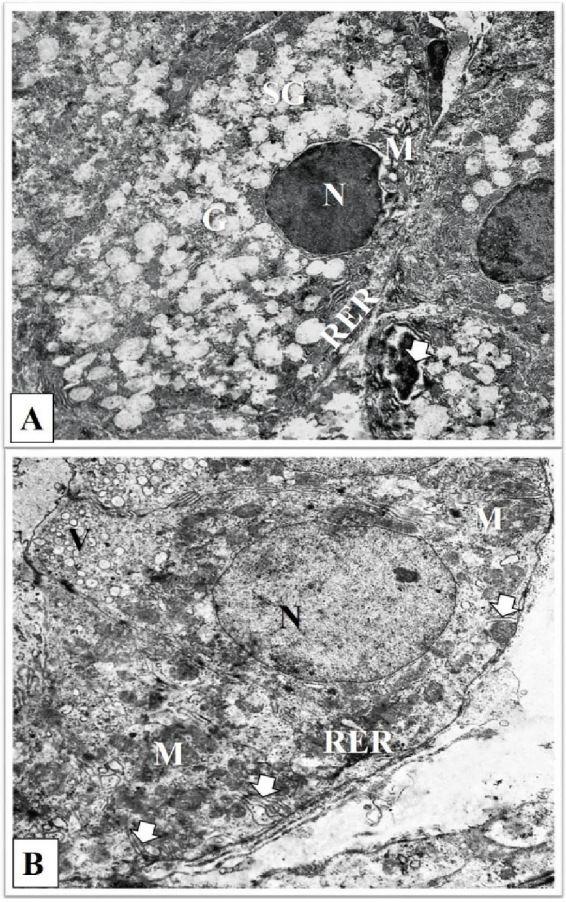

Fig. (5): Electron micrograph of group II (hyperthermia group) shows: (A) Acinar cell appears loaded with many fused secretory granules. Also, dilated perinuclear space, folded nuclear membrane and deformed mitochondria (M) are detected. Cisternae of RER are slightly dilated. Moreover, acinar cell with pyknotic nucleus is noticed (arrow). (B) Striated duct cells shows degenerative changes manifested as loss of basal infoldings (arrows), degenerated mitochondria (M) distended RER and many vacuoles (V). (Mic. Mag. A & B: x1000).

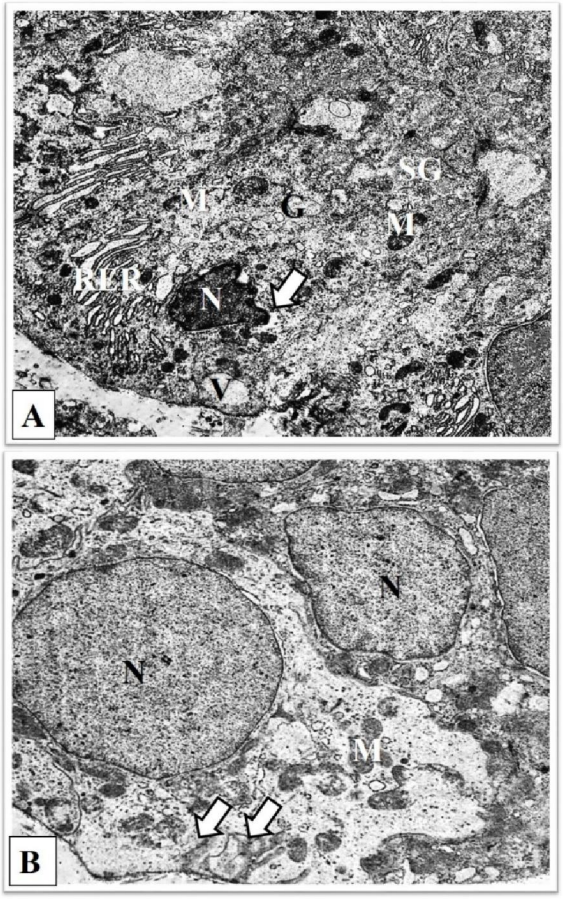

Fig. (6): Electron micrograph of group III (hyperthermic dehydrated group) shows: (A) Pyknotic hyperchromatic nucleus (N), increased dilatation of perinuclear space (arrow) and folding of the nuclear membrane. Degeneration of the cytoplasm is evident with destructed Golgi apparatus (G), ruptured mitochondria (M), vacuoles (V), few electronlucent secretory granules (SG), extended rough endoplasmic reticulum (RER). (B) Striated duct cells are showing degeneration of the cytoplasm leaving few ruptured mitochondria and distended RER with loss of basal infoldings (arrows). (Mic. Mag. A: x1000, B: x2000.).

4. Discussion

Elevated ambient temperature leads to extreme heat gain which exposes body to severe life threatening condition. In humans, the high body temperature is suppressed by enhancing heat dissipation via sweat evaporation along with cutaneous vasodilation (22). At ambient temperatures of more than 35°C, rats do not sweat nor pant but they dissipate their heat by dispersion of saliva on their skin (23). The initial phase of heat acclimation (exposure to 40°C for 3-4 h) is manifested by over discharge of saliva that results in a great water loss. Vaporization cooling of saliva from SMG resolves more than 60% of the heat dissipation. Parasympathetic nervous system controls this process via a cholinergic pathway. Throughout the acclimation period, rats modulate central thermoregulatory mechanisms to achieve water balance and regulate body temperature (24)through reducing the response of muscarinic receptors in SMGs (25). Sugimoto et al., found that heat acclimation process at 32°C for 5 days includes enhanced expression of aquaporin water channel molecules AQP5 and AQP1, production of vascular endothelial growth factor (VEGF), a major angiogeneic promotor (26) and induction of heat shock proteins which break down abnormal proteins or correct folding of proteins or, alternatively, collaborate with protective molec-ular signaling pathways (22).

In the current study, light microscopic examination of SMG of hyperthermia group, illustrated wider stromal spaces in between the parenchymal elements with obvious hyperemic vasodilation. These were in agreement with (23) who reported a large swelling developed in the surrounding soft tissues of SMG at an ambient temperature of 40°C. Saliva has an important role in the heat balance of rodents at 40°C and SMGs are the main effectors of the thermolytic processes. Hence, these changes could be related to the increased functional demand of salivation to dissipate the heat. Horowitz et al., explained this increase in size by hyperplasia and hypertrophy of the acini(24). Damas also linked the vasodilation to the cholinergic autonomic nerve stimulation and release of bradykinin, nitric oxide and prostanoids(23)which are potent vasodilators that increase vascular permeability (27). Moreover, heat stimulates kallikrein-kinin system facilitating the secretion of saliva by enhancing the exchange of water into the salivary glands (28). Furthermore, Bouchama et al. and Roberts et al. recognized the endothelial cells as the primary target of heat stress (29,30) that may lead to their apoptosis (31).

Also, in hyperthermia group, acinar and ductal cells showed obvious degenerative changes as vacuoles, pyknotic nuclei and apoptosis in some acinar cells that may be explained by the heat stress induced cytotoxicity via intracellular Ca^{+2} overload, oxidative stress and free radical damaging effect (32).

Striking ductal dilation in SMG was eminent in this group. Vanmuylder et al. highlighted the presence of Heat shock proteins (HSPs) in the cytoplasm of striated duct cells and in myoepithelial cells(33). This suggested the pathological effect of heat on embracing myoepithelial cells that impairs flow of secretion into the oral cavity resulting in glandular dys-function.

Furthermore, hyperthermia group showed higher Bax expression in comparison to control and hyperthermic dehydrated groups. This was in agreement with Gu et al. who confirmed that in response to heat stress, p53 accumulated in mitochondria and stimulated Bax mitochondrial translocation that consequently resulted in an obvious increase in the amount of Bax (32), which induced mitochondrial apoptotic pathways (34).

TEM examination of hyperthermia group demonstratedmany acinar cells studded with numerous secretory granules that was explained by the continuous stimulation of salivary secretion. This may also suppose accumulation of secretory material that could be elucidated by adaptation of the gland to reduce secretion that controls water loss and allows surviving under high temperature (24). Moreover, in this study, it was observed that nuclear membrane, rough endoplasmic reticulum and mitochondria of the acinar as well as striated ductal cells showed degenerative changes that were in agreement with increased Bax expression with its apoptotic changes.

It is noteworthy to know that reduction of water intake predisposes the body to hypovolemia (35). The physiological compensatory mecha-nisms start as fluid moves from the intracellular compartment into the extracellular space to maintain water and mineral balance leading to cellular dehydration which was coincided with our results of decreased gland size in the DH group. As the hypovolemia is increased due to failure of rehydration, the plasma concentration of sodium and chloride ions or the plasma osmolality increased (36) that are involved in epithelial transport, and might affect salivary secretion in addition to hyperosmotic effects (37). Barney & Folkerts demonstrated that fluid deficit associated with heat exposure potentiate hyperthermia (11) and this may explain the more destructive findings in this group.

Obviously, hyperthermia with dehyd-ration induced sever degenerative changes in the SMG. At

the light microscopic level, hyper-thermic dehydrated group revealed many packed serous acini that had smaller, pale-stained acinar cells with numerous cytoplasmic vacuoles and very few secretory granules. These changes were more severe than the study previously described in the parotid salivary gland under dehydration only (38). They interpreted these degenerative changes with inability of the compensatory mechanisms to overcome these stresses due to underdevelopment in young ages (39). Otherwise, this may be explained as the intensity of these stresses together exceeded beyond the capacity of these mechanisms to try to overcome this condition. There were also dilated blood capillaries showing densely-packed disfigured extravasated RBCs and swollen endothelial cells. These could be clarified by that hyperthermia release bradykinin, nitric oxide and prostanoids which are potent vasodilators that activate various cellular signaling pathways that lead to activation of pro-inflammatory and pro-fibrotic cytokines (23).

In hyperthermic dehydrated group, Bax expression of acinar cells and ductal cells was weak, whereas endothelial cells of blood capillaries showed marked Bax reaction. These observations were in agreement with Niu et al.(40) who found increased Bax expression of thoracic blood capillaries in the dehydration-heat exposure group than only in heat exposure group. The lesser Bax expression of acinar cells and ducts in this study may be explained by the role of Bax as a pro-apoptotic gene detecting early degenerative changes, however in the hyperthermic dehydrated group it showed severe degenerative changes.

Ultrastructurally, rat SMG showed exaggerated degeneration in acinar and ductal cells as pyknotic hyperchromatic nuclei. Degeneration of the cytoplasmic constituents was evident as destroyed Golgi apparatus, ruptured mitochondria, more vacuoles, and few electron-lucent secretory granules. The cisterns of RER were extended. Striated ductal cells also revealed degeneration of their cytoplasmic components in addition to loss of basal infoldings. These degenerative changes may be explained by lipid peroxidation, denaturation of proteins and nucleic acid damage by reactive oxygen species (ROS) like superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) that was formed by dehydration which may affect cell metabolism and consequently lead to DNA damage (41).

5. Conclusion:

Deficiency of water intake in high temperature environment was found to influence many metabolic activities of the body that are reflected on the structure of many organs including salivary glands. Possibly, the effect of heat on SMG is similar to its effect on other organs. In humans, Kavanagh et al. (42) established that the flow rate of saliva was inversely associated with ambient temperature even in minor temperature variations. Horowitz et al. (43) reported hypoplastic changes following an exposure to heat as low as 34 °C in rat parotid glands. Unfortunately, Ship and Fischer., reported that after rehydration, parotid flow rate decreased during the 24-h dehydration period, yet did not completely return to baseline values (44). Thus, it is important to emphasize on proper hydration to prevent serious damage to SMGs. Also, additional studies are needed to elucidate whether the same results are detected in humans. Further studies with longer experimental periods may be required to exclude this reactive hyper salivation in rats. These results suggest that it may be useful to apply local heat on the gland to increase the salivary secretion to be used in treating certain cases of dry mouth.

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