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Effect of bioactive peptides on heat stress-induced testiculopathies in mature rats: immunohistopathological evidence

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Abstract

Exposure to extreme temperature conditions such as occurs in certain occupations is known to induce male infertility. In humans and most of the mammals, it has been shown that whole-body heat stress (HS) decreases fertility and produces defective embryos. Hence, the present study aimed at gaining some insights into the mechanisms producing defects after HS. In the present study, 56 mature male Wistar rats were randomly categorized into eight groups (n = 7), including group 1: Control, groups 2: Bioactive peptides (BPs; 10.00 mg kg^{-1}), groups 3, 4, and 5: Heat-stressed (37.00, 39.00, and 43.00 ˚C for 20 min, respectively), and groups 6, 7, and 8: Heat-stressed along with BPs (10.00 mg kg^{-1}), respectively. All treatments were administered orally once *per* day. The HS was induced through the immersion of rat scrotums in a water bath. After 45 days, rats were sacrificed and left testes were removed, fixed, and used for histological and immunohistochemical studies. Harvested right testes were also used for oxidative stress assessments and molecular analyses. Heat stress increased testicular tissue damage, elevated oxidative stress and reactive oxygen species production, and increased germ cells apoptosis, *p53* and *caspase 3* expressions, and *Bax/Bcl-2* ratio. Treatment with BPs as a substance with anti-oxidant properties ameliorated the damage caused by HS. The results of this study highlight the protective role of BPs in the reproductive tract under HS. Bioactive peptides may have potential function against testicular tissue oxidative stress and apoptosis.

Introduction

It has been revealed that most of mammalian epididymides and testes temperature has to be 2.00 - 8.00 degrees below the core temperature to work efficiently.[1,2] In line with this issue, various epidemiological reports have illustrated that high environmental temperatures contact negatively affects spermatogenesis through scrotal temperature balance disruption.[3,4] Accordingly, it has been reported that in working men in high-temperature environments, including bakers, welders, and drivers, as well as in animals which are exposed to high temperatures, including boars, rams, rats, and mice, the testicular temperature has been increased dramatically.[5-7] Formerly conducted studies have shown the negative effects of increased testicular temperature on male fertility *via* oxidative stress induction and germ cells loss in testicular tissue.[8,9] Correspondingly, it has been revealed that the experimental hyperthermia induction in animal models leads to suppressed spermatogenesis, sperm abnormalities, and germ cells apoptosis, resulting in infertility.[4,10]

Furthermore, one of the most important adverse products of heat stress (HS) is reactive oxygen species (ROS) over-production.[2,11] It has been well documented that ROS induce oxidative stress,[12] leading to different pathological effects, including apoptosis,[13,14] disrupted spermatogenesis, reduced sperm production, and abnormal embryo development.[4] Moreover, HS impairs spermatogenesis indirectly due to its negative impacts on Sertoli and Leydig cells.[15] Additionally, it has been reported that ROS over-generation in HS condition enhances lipid peroxidation due to the testicular anti-oxidant defense system disturbance.[16]

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A recent study has shown that enhanced ROS level in testicular tissue results in mitochondria-dependent apoptosis.[17] It has also been well defined that up-regulated ROS in the testicular tissue lead to severe DNA damage.[4,18] Consistently, in ROS-induced DNA damage condition, several genes involving in apoptosis, such as *p53*, *p21*, *cyclin-D1*, *cyclin-dependent kinases*, *retinoblastoma tumor inhibitor*, *Bax*, and *Bcl-2*, have been reported to interact as double-edge swords to induce and/or ameliorate the apoptosis-induced DNA fragmentation in proliferating germ cells.[19,20] However, different genes have been reported to be over-expressed in progressive oxidative stress, and the *p53*, known as a DNA guardian gene, has got researchers attention.[21,22] Thus, formerly conducted studies have evinced the oxidative stress-induced DNA damage as a promoter of p53 in the testicular tissue.[22] However, *p53* as a potential DNA repair gene can cause DNA repair pathways activation, and it has been found that in the severe DNA damage condition, p53 plays a key role to trigger intrinsic apoptosis. Indeed, *via* binding to Bcl-xl, the p53 induces Bax and Bak oligomerization in the outer membrane of mitochondria and at the same time, suppresses *Bcl-2* and *Bcl-xl* expressions, leading to p53-mediated mitochondria-dependent apoptosis initiation. On the other hand, the p53 over-activation is able to transcriptionally stimulate the *p21* expression, being known as a p53/p21-initiated (p21-related) apoptosis.[22,23]

Several studies have explicated that HS initiates double-stranded DNA breaks directly by ionizing radiation;[24] however, HS-induced oxidative stress plays an efficient role in DNA damage process.[25] Accordingly, HS leads to different derangements in testicular tissue through oxidative stress induction. Considering former studies, it has been demonstrated that anti-oxidants play a critical role *versus* ROS in the testicular tissue, and anti-oxidant administration has been launched as an important therapeutic approach against testicular oxidative stress.[26]

It has been elucidated that anti-oxidants administration can positively improve the systemic and local anti-oxidant defenses against oxidative stress.[27] Regarding this issue, bioactive peptides (BPs) have gained researchers attention based on their low molecular weight, easy absorption, significant anti-oxidant activity, low sensitivity, and high stability under various conditions. The BPs are specific sequences of 10 - 20 amino acids, being basically inactive until being released by proteolytic enzymes.[28,29] Hence, BPs activation results in their various physiological functions, such as anti-oxidant,[28,30] anti-microbial,[31] anti-cancer,[32] immunomodulatory,[33] hypo-lipidemic,[34] and hypoglycemic effects.[35] Considering the large portion of marine organisms being wasted due to the low quality, low consumer preference, and small size each year, BPs extraction from the wasted source improves world population attention to gain a free resource of anti-oxidants with high quality.[36]

Based on this concept, in the current project, it was aimed to investigate BPs of sardine fish anti-oxidant effects on HS in a rat model. Regarding this issue, three different temperatures stimulating various HS conditions were induced as different groups. Then, BPs were administrated to evaluate the sardine-extracted BPs anti-oxidant effects on testicular tissue. Also, BPs ameliorating effects on HS condition using anti-oxidant and oxidative stress factors, as well as apoptosis-related proteins were evaluated.

Materials and Methods

Chemicals and materials. The primary antibodies for p53 (Biocare Medical, Pacheco, USA), Bcl-2 (Biocare Medical), BAX (Merck, Darmstadt, Germany), and caspase 3 (Biocare) proteins were purchased from Life-Teb-Gen Company (Tehran, Iran). Primers for GAPDH (Sinaclon, Tehran, Iran), p53 (Sinaclon), caspase 3 (Sinaclon), BAX (Macrogen, Seoul, South Korea), and Bcl-2 (Macrogen) were also purchased from Life-Teb-Gen Company.

Peptide preparation. The BPs in this experiment were extracted from a traditional fermented fish sauce widely consumed in the southern part of Iran. The sauce is a mixture of Sardines (*Sardinella* sp.) or Anchovies (*Stelophorus* sp.), mustard (*Brassica juncea*), salt, and water. It is traditionally made from dried or fresh anchovy packed into glass jars with salt. The jars are kept in the sun for 25 - 30 days. Fermented sauce was dried by freeze dryer and then, mixed with distilled water (1:1). The mixture was kept in 30.00 ˚C water bath for 90 min and then, centrifuged in 10,000 *g* for 15 min at 4.00 ˚C. Finally, supernatant was dried by freeze dryer and extracted peptides were kept at –18.00 ˚C.[28]

Animals and grouping. Fifty-six mature male Wistar rats (200 ± 25.00 g) were provided from the Animal House of Faculty of Sciences, Urmia University, Urmia. Iran. All animals were kept in a standard condition, at 23.00 ± 2.00 ˚C and 12/12 light/dark photoperiod without food and water limitation, based on the Laboratory Animal Care Ethics of Urmia University, Urmia, Iran (Ethical Code: IR-UU-AEC 1383/3). Following 2 weeks' adaptation period, the animals were categorized into eight groups (seven rats in each group). Animals in group 1 (control group) received normal saline (0.50 mL) as a solvent for BPs; while, in group 2 animals received BPs (10.00 mg kg^{-1}). In the experimental groups 3, 4, and 5, animals were exposed to 37.00, 39.00, and 43.00 ˚C HS, and groups 6, 7, and 8 were heat-stressed along with BPs (10.00 mg kg^{-1}), respectively. All treatments were administered orally once *per* day. It should be noted that, in saline and BPs received groups, the water bath temperature was 23.00 ˚C and heat exposures (20 min) were performed according to the former reports. Following 45 days, all animals were euthanized following anesthesia induced by intra-peritoneal injection of 40.00 mg kg^{-1} ketamine (Alfasan,

Woerden, The Netherlands) and 5.00 mg kg^{-1} xylazine (Alfasan) and samples were collected.[4,14]

Assessment of malondialdehyde (MDA), total antioxidant capacity (TAC), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX). Testicular tissue (0.50 g) was homogenized in 0.10 M Tris-HCL buffer (Sigma-Aldrich, St. Louis, USA; pH: 7.40 at 4.00 ˚C) for each parameter. The homogenized tissue was transferred to the micro-tubes and centrifuged at 3,000 rpm for 10 min at 4.00 ˚C. The testicular solution was used to evaluate MDA and TAC levels, as well as activities of CAT, SOD, and GPX. These factors were measured using biochemical assay kits (Navand Salamat, Urmia, Iran) according to the manufacturer's instructions.

Tissue preparation and histological analyses. In order to perform histological analyses, the testes were dissected out and left testes were fixed in 10.00% formalin, while, the right testes were placed in – 80.00 ˚C for further biochemical and molecular measurements. The fixed testicles were undergone routine passage and embedded in paraffin. Then, using rotary microtome (Leitz Wetzlar GmbH, Wetzlar, Germany), the paraffin blocks were cut (6.00 µm sections), stained with Hematoxylin and Eosin and Periodic Acid-Schiff, and examined under a light microscope (Eclipse E200-LED; Nikon, Tokyo, Japan). In continue, Johnsen score, tubular differentiation index (TDI), spermiogenesis index (SPI), mitotic index (MI), and Sertoli cell index (SCI) were evaluated in 100 seminiferous tubules in each group. Moreover, the spermatogonia A (SpA) number *per* mm^2 was also determined at the same time.[37,38]

Immunohistochemical (IHC) staining. In order to perform IHC staining, the sections were prepared and placed at a hot air oven (Venticell; MMM Medcenter Einrichtungen GmbH, Munich, Germany; 60.00 ˚C for 30 min). Then, the sections were de-paraffinized in xylene and rehydrated using descending alcohol concentrations (96.00, 90.00, 80.00, and 70.00%). After that, antigen retrieval process was conducted by transferring the slides to a sodium citrate buffer (Sigma-Aldrich; 10.00 mM; pH: 7.20). Subsequently, the slides were undergone blocking process using a 0.03% hydrogen peroxide solution (Sigma-Aldrich). The sections were washed with phosphate-buffered saline, and primary antibodies (Bcl-2, Bax, p53, and caspase 3) were added to the section and placed at refrigerator for 18 hr. The next day, the slides were washed and transferred into the humidified chamber. Then, they were incubated with anti-polyvalent antibody (Sigma-Aldrich) and consequently with horseradish peroxidase (Sigma-Aldrich), each for 15 min. After washing the slides with phosphate-buffered saline, they were incubated with 3,3′-diaminobenzidine chromogen (Sigma-Aldrich) for 5 min, followed by washing and counterstaining with Hematoxylin for less than 5 sec. Finally, the slides were rinsed with distilled water and cover slipped. The percentage of cells with positive reaction for each antibody was analyzed in each group and compared between groups.

RNA isolation and cDNA synthesis. The testicles RNA was extracted based on TRIZOL method (Cinnagen, Tehran, Iran). In brief, TRIZOL reagent and chloroform were used in order to homogenize the frozen tissue (0.10 g). After 15 min incubation on ice, the solution was centrifuged in 13,000 rpm for 6 min and aqueous parts were removed into fresh micro-tubes. Then, isopropanol was added to the tubes; the mixture was incubated on ice for 10 min and centrifuged. Eventually, the supernatant was removed, washed with ethanol (70.00%), and air dried and then, 20.00 µL diethyl pyrocarbonate-treated water was added. The amount and quality of total RNA content were analyzed using NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA; 260 nm; A260/280 = 1.80 - 2.00). In order to cDNA synthesis, a mixture containing total RNA (10.00 ng ~ 5.00 µg), mRNA (1.00 ng ~ 0.50 µg), Oligo (dT) primer (20.00 : 50.00 µM), random hexamer (50.00 µM), sequence-specific primer (15.00 ~ 20.00 uM), 2.00 X RTPre-Mix (10.00 µL), and RNase-free water prepared and incubated for 5 min. Then, the mixture was heated for 30 min at 50.00 ˚C and 5 min at 95.00 ˚C in order to perform reverse transcriptase inactivation.

Quantitative reverse transcriptase polymerase chain reaction (qRT- PCR). The PCR reaction contained 0.50 µL (about 5.00 - 10.00 ng) of cDNA template, 10.00 µL 1.00 X SYBR GREEN master mix (Biofact Co., Ltd., Daejeon, South Korea), and 0.50 µL (600 nM) from each reverse and forward primers of the target genes. The PCR conditions were run as follows: General denaturation at 95.00 ˚C for 5 min, 1 cycle, followed by 45 cycles of 95.00 ˚C for 20 sec; annealing temperature: 61.00 ˚C for GAPDH, 59.00 ˚C for p53, 60.00 ˚C for caspase 3, 60.00 ˚C for Bax, and 61.00 ˚C for Bcl-2 for 10 sec; elongation: 72.00 ˚C for 1 min and 72.00 ˚C for 5 min. Values were normalized by subtracting the mean cycle threshold value of GAPDH. The primers pair's sequences for individual genes are presented in Table 1.

Statistical analyses and imaging. All results are presented as mean ± standard deviation. Differences between quantitative histological and biochemical data were analyzed with one-way analysis of variance, followed by Bonferroni test using SPSS Software (version 22.0; IBM Corp, Armonk, USA). A $p < 0.05$ was considered as statistically significant. The photomicrographs were taken by SONY onboard camera (Carl Zeiss Sony Cyber-shot, Tokyo, Japan) and presented using Adobe Photoshop CC 2018 (version 19.0; Adobe System Incorporated, San Jose, USA). ImageJ Software (National Institutes of Health, Bethesda, USA) was used for evaluating the pixel-based distribution of Bax, Bcl-2, p53, and caspase 3 positive cells *per* 253 × 10.00 µm.

Table 1. Sequences of the primer pairs used for quantitative reverse transcriptase polymerase chain reaction.

Primers	Sequence	Size (bp)
GAPDH	F: 5'- AAGAAGGTGGTGAAGCAGGCATC- 3' R: 5'- CGAAGGTGGAAGAGTGGGAGTTG- 3'	112
p53	F: 5'- GACTTCTTGTAGATGGCATGG- 3' R: 5'- ATGGAGGATTCACAGTCGGATA- 3'	250
Caspase 3	F: 5'- GTTAACACGAGTGAGGATGTG- 3' R: 5'- TACCCTGAAATGGGCTTGTGT- 3'	446
BAX	F: 5'- GACACCTGAGCTGACCTTGG- 3' R: 5'- GAGGAAGTCCAGTGTCCAGC- 3'	310
Bcl-2	F: 5'- ATCGCTCTGTGGATGACTGAGTAC- 3' R: 5'- AGAGACAGCCAGGAGAAATCAAAC- 3'	134

Results

General findings and biochemical analysis. Following 45 days, the total body and testicular weights were measured. Observation revealed that the total body weight did not change significantly ($p > 0.05$); meanwhile, the testicular weight reduced remarkably in 43.00 and 43.00 ˚C + BPs groups *versus* other groups (Fig. 1A).

To follow up testicular ROS content and anti-oxidant status, the MDA, TAC, CAT, SOD, and GPX levels were evaluated. The biochemical assessment showed a significant ($p < 0.05$) enhancement in the MDA level in HS-exposed groups compared to the control group; however, the BPs administrated groups showed a remarkable ($p < 0.05$) reduction in MDA concentration compared to the HS-treated animals (Fig. 1B). Moreover, the TAC, CAT, SOD, and GPX levels significantly diminished in HS-treated animals compared to the control ones; while, the BPs-received animals showed remarkable enhancement in their anti-oxidant enzymes levels *versus* HS-exposed animals. In contrast, there were no significant differences between 43 and 43.00 ˚C + BPs groups (Figs. 1C-1F).

Histological findings. The histological analysis illustrated that HS-exposed animals showed remarkable pathological defects, including cell dissociation, tubular depletion, tubular basal membrane disintegration, and arrested spermatogenesis in their testicular tissue. Meanwhile, the BPs administrated animals had less pathological defects, especially in 37.00 and 39.00 ˚C HS-treated groups (Fig. 2). In order to evaluate the testicular tissues changes more efficiently, Johnsen score (Fig. 3A), positive TDI percentage (Fig. 3B), positive SPI percentage (Fig. 3C), SCI (Fig. 3D), and MI (Fig. 3E), as well as SpA number *per* mm^2 (Fig. 3F) were analyzed, respectively.

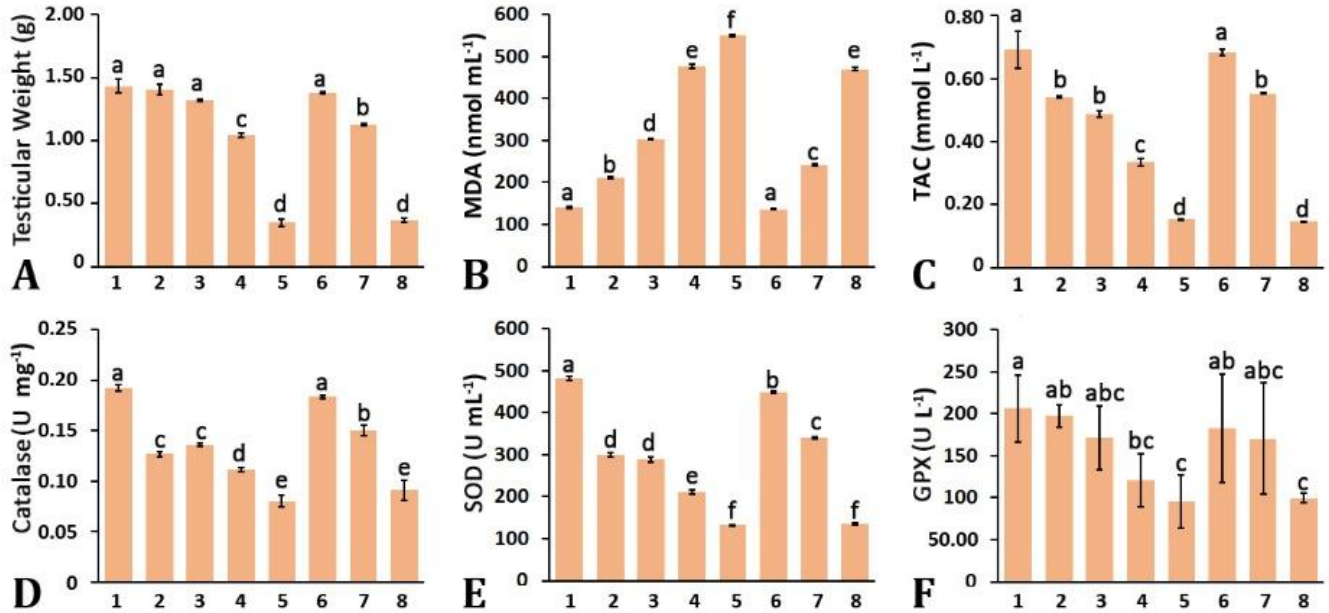

Fig. 1. A) Testicular weights changes are presented in different experimental groups. Moreover, biochemical findings, including **B)** Malondialdehyde (MDA), **C)** Total anti-oxidant capacity (TAC), **D)** Catalase, **E)** Superoxide dismutase (SOD), and **F)** Glutathione peroxidase (GPX) levels changes are shown, respectively. Group 1 (control group) received normal saline (0.50 mL), group 2 received bioactive peptides (10.00 mg kg^{-1}), groups 3, 4, and 5 were exposed to 37.00, 39.00, and 43.00 ˚C heat stress, and groups 6, 7, and 8 were heat-stressed along with bioactive peptides (10.00 mg kg^{-1}), respectively.
[a-f] Different symbols are presenting significant differences at $p < 0.05$.

Observation revealed that the histological parameters reduced significantly in HS-treated animals *versus* control group; however, BPs co-treatment could fairly ameliorate the negative impacts of HS and resulted in enhanced histological parameters in the BPs-received animals compared to the HS-treated groups. Moreover, our results indicated that BPs co-treatment in 43.00 ˚C exposed animals could not improve histological changes compared to the BPs-non-treated animals.

Bioactive peptide administration reduced *Bax* expression. Observation revealed a significant ($p < 0.05$) enhancement in Bax mRNA level in HS-treated groups *versus* control group. Meanwhile, BPs significantly ($p < 0.05$) down-regulated the Bax mRNA level (Fig. 4A). Moreover, the Bax protein IHC staining revealed a remarkable increment in Bax positive cells number percentage *per* mm^2 in HS-exposed animals compared to the control animals. In contrast, observation exhibited that BPs administration remarkably ($p < 0.05$) diminished Bax positive cells number percentage *per* mm^2 in BPs-co-treated groups *versus* HS-treated animals. In addition, the pixel based-frequency analysis using ImageJ Software was performed to approve the cell number percentage results and indicated that in BPs-received animals, the Bax protein content of cells diminished fairly compared to the HS-treated animals (Figs. 5 and 6).

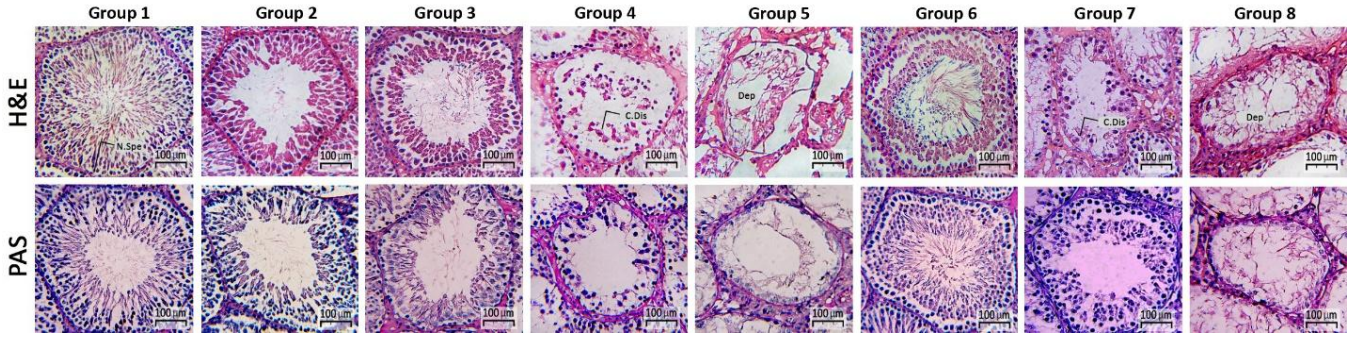

Fig. 2. Cross-sections of seminiferous tubules stained with Hematoxylin and Eosin (H & E) and Periodic Acid-Schiff (PAS) in all experimental groups. Germ cells dissociation (C.Dis) and tubular depletion (Dep) in heat stress groups, and normal spermatogenesis (N.Spe) in control group are evident. P: Bioactive peptide. Group 1 (control group) received normal saline (0.50 mL), group 2 received bioactive peptides (10.00 mg kg^{-1}), groups 3, 4, and 5 were respectively exposed to 37.00, 39.00, and 43.00 ˚C heat stress, and groups 6, 7, and 8 were heat-stressed along with bioactive peptides (10.00 mg kg^{-1}), respectively.

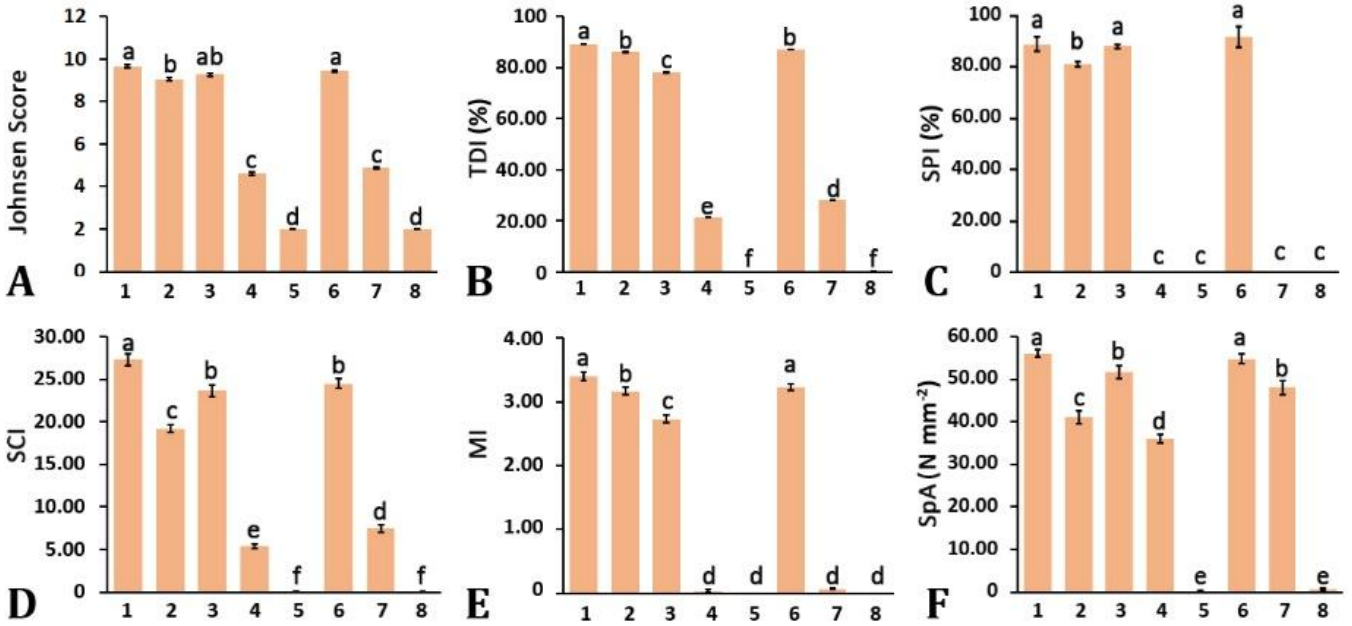

Fig. 3. Histological findings in different experimental groups. **A)** Johnsen score is presented in different groups. Moreover, mean percentages of seminiferous tubules with positive tubular differentiation index (TDI; **B**) and spermiogenesis index (SPI; **C**) are reported. Additionally, the Sertoli cell index (SCI; **D**), mitotic index (MI; **E**), and spermatogonia A (SpA) number *per* mm^2 **(F)** are presented. Group 1 (control group) received normal saline (0.50 mL), group 2 received bioactive peptides (10.00 mg kg^{-1}), groups 3, 4, and 5 were respectively exposed to 37.00, 39.00, and 43.00 ˚C heat stress, and groups 6, 7, and 8 were heat-stressed along with bioactive peptides (10.00 mg kg^{-1}), respectively.

$^{a\text{-}f}$ Different symbols are presenting significant differences at $p < 0.05$.

Bioactive peptide administration enhanced *Bcl-2* expression. Although the mRNA expression of *Bcl-2* analysis revealed a significant ($p < 0.05$) reduction in HS-treated groups *versus* control group, the BPs-treated animals exhibited a remarkable ($p < 0.05$) enhancement in the Bcl-2 mRNA level *versus* HS-treated animals (Fig. 4B). Additionally, the Bcl-2 protein examination showed that HS resulted in significantly diminished Bcl-2 positive cells number percentage *per* mm^2 *versus* control animals; while, BPs administration enhanced Bcl-2 positive cells number percentage *per* mm^2 *versus* HS-only-treated animals. Moreover, software analysis demonstrated the same results and showed BPs protective effects against HS (Figs. 5 and 6).

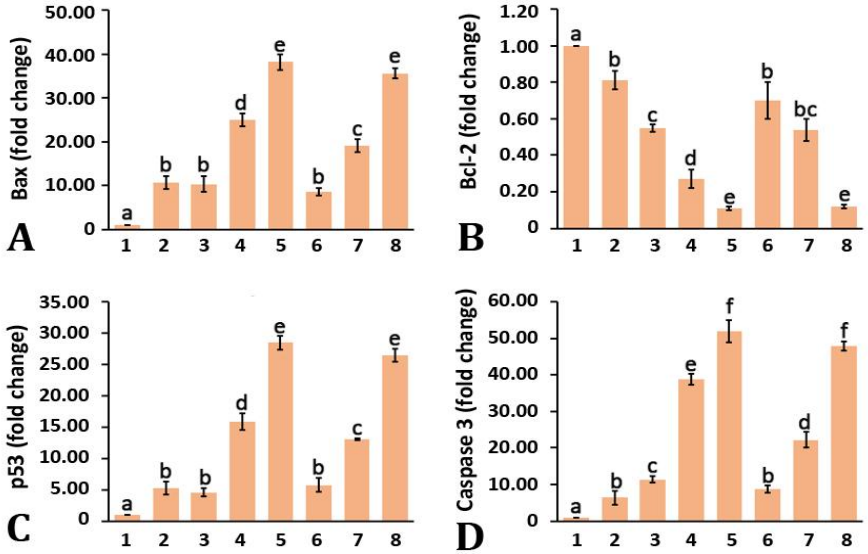

Fig. 4. The mRNA expressions of **A)** Bax, **B)** Bcl-2, **C)** p53, and **D)** caspase 3 in different experimental groups. Group 1 (control group) received normal saline (0.50 mL), group 2 received bioactive peptides (10.00 mg kg^{-1}), groups 3, 4, and 5 were respectively exposed to 37.00, 39.00, and 43.00 ˚C heat stress, and groups 6, 7, and 8 were heat-stressed along with bioactive peptides (10.00 mg kg^{-1}), respectively.
$^{a\text{-}f}$ Different symbols are presenting significant differences at $p < 0.05$.

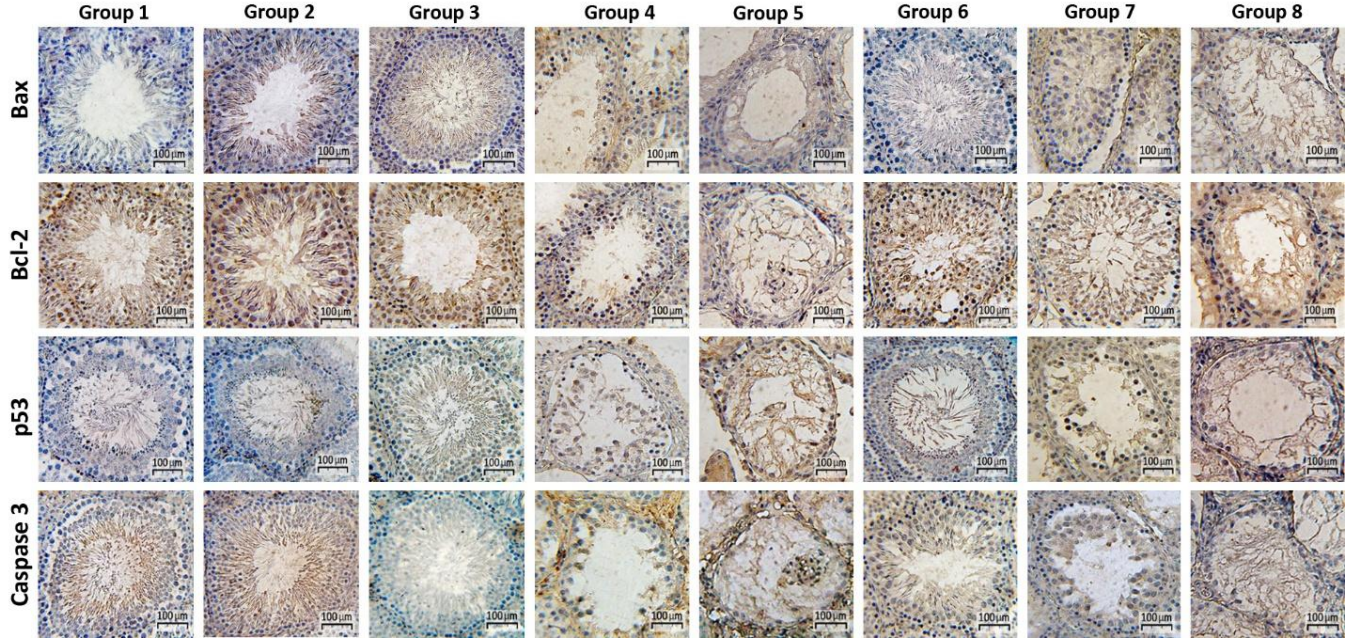

Fig. 5. Cross-sections of seminiferous tubules with immunohistochemical staining for Bax, Bcl-2, p53, and caspase 3 proteins. Accordingly, the immunopositive cells were marked by brown reaction; while, negative cells did not represent any brown color. Group 1 (control group) received normal saline (0.50 mL), group 2 received bioactive peptides (10.00 mg kg^{-1}), groups 3, 4, and 5 were respectively exposed to 37.00, 39.00, and 43.00 ˚C heat stress, and groups 6, 7, and 8 were heat-stressed along with bioactive peptides (10.00 mg kg^{-1}), respectively.

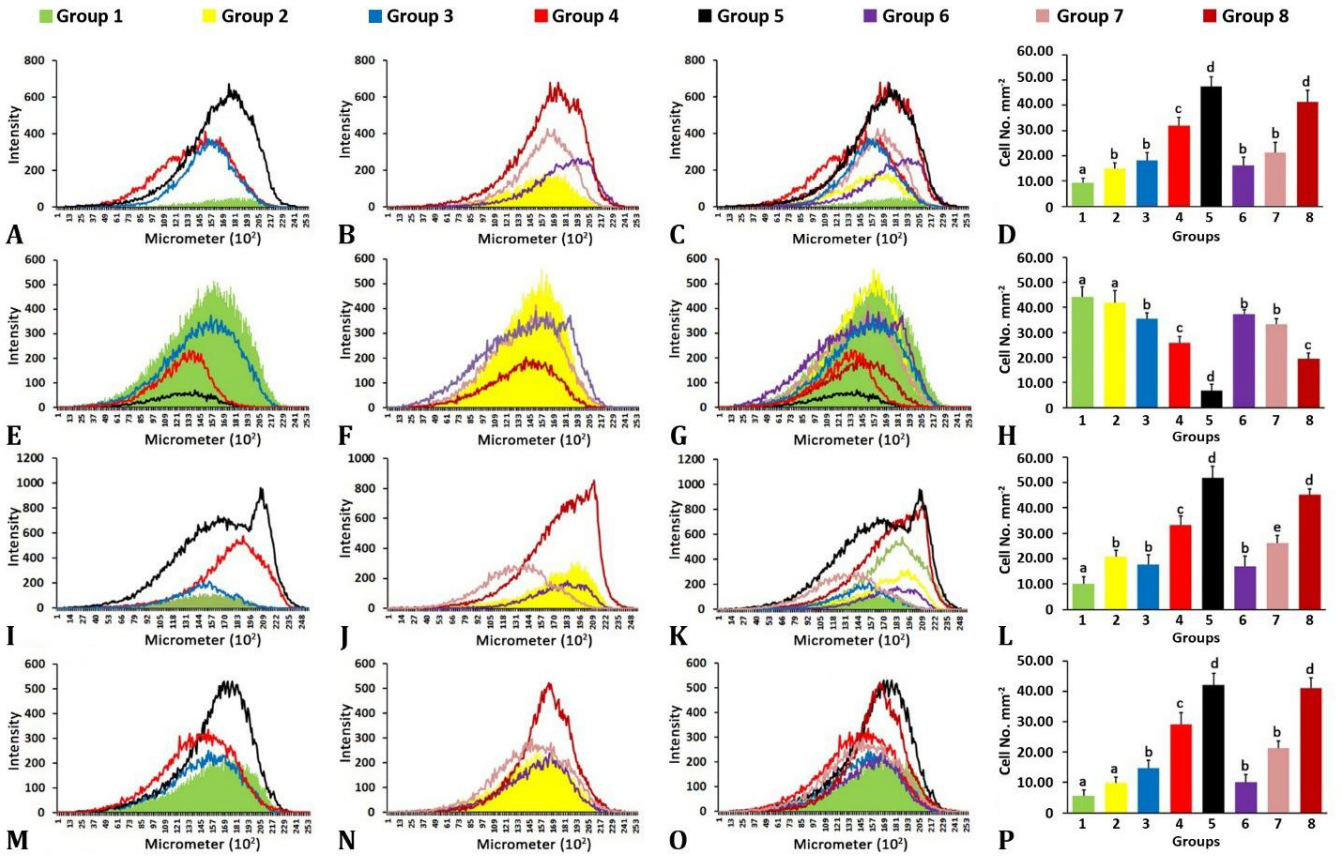

Fig. 6. Quantitative immunohistochemical analyses in different experimental groups. Pixel-based intensity for brown reaction represented Bax (**A**, **B**, and **C**), Bcl-2 (**E**, **F**, and **G**), p53 (**I**, **J**, and **K**), and caspase 3 (**M**, **N**, and **O**) stained cells in various experimental groups. Moreover, Bax **(D)**, Bcl-2 (**H**), p53 (**L**), and caspase 3 (**P**) positive cells numbers *per* mm² of tissue in different experimental groups are reported. Group 1 (control group) received normal saline (0.50 mL), group 2 received bioactive peptides (10.00 mg kg^{-1}), groups 3, 4, and 5 were respectively exposed to 37.00, 39.00, and 43.00 °C heat stress, and groups 6, 7, and 8 were heat-stressed along with bioactive peptides (10.00 mg kg^{-1}), respectively.
[a-e] Different symbols are presenting significant differences at $p < 0.05$.

Bioactive peptide administration down-regulated *p53* expression. The animals in HS-exposed groups displayed increased mRNA level and p53^{+} cells number percentage *versus* control and BPs-received groups; however, the administration of BPs significantly down-regulated the mRNA and protein levels of p53 (Fig. 4C). Moreover, the IHC staining for p53 was conducted and the p53^{+} cells number percentage *per* mm² of tissue was compared between groups. Observation revealed a significant enhancement in p53 protein level in HS-exposed groups *versus* control group. In contrast, the animals in BPs-received groups exhibited a remarkable reduction *versus* HS-exposed animals. Similar to p53^{+} cells number percentage results, the pixel-based intensity analysis represented significantly higher p53 intensity in HS-treated groups *versus* other groups. No significant changes were found between control and BPs-received animals (Figs. 5 and 6).

Bioactive peptide administration reduced *caspase 3* expression. The qRT-PCR analysis revealed a remarkable increase in mRNA level of *caspase 3* expression in HS-treated groups compared to the control group. In line with that, BPs-treated animals showed a significant reduction in *caspase 3* mRNA expression *versus* HS-exposed animals (Fig. 4D). Additionally, IHC staining was performed in order to assess the caspase 3 protein level. Observations demonstrated that HS exposure resulted in enhanced caspase 3^{+} cells number percentage *per* mm² compared to the control and BPs-received groups. Meanwhile, the BPs administration could fairly ameliorate the condition and led to reduction in caspase 3^{+} cells number percentage *per* mm² in BPs-treated animals *versus* HS-received animals. The quantitative results were confirmed by software analyses and the same pattern was observed in pixel-based frequency analysis (Figs. 5 and 6).

Discussion

Our results illustrated that BPs administration could significantly protect testicular tissue against HS-induced spermatogenesis arrest, germ cell loss, and cellular apoptosis. Our preliminary findings revealed that BPs

co-treatment could remarkably enhance testicular tissue weight, up-regulate anti-oxidant enzymes, diminish MDA level, and improve histological parameters, such as TDI, SPI, SCI, and MI. In order to clarify that how BPs administration could exert the aforementioned effects, the mRNA and protein contents of *Bax*, *Bcl-2*, *caspase 3*, and *p53*, important genes involving in intrinsic apoptosis, were assessed. Observations showed that BPs treatment significantly enhanced *Bcl-2* expression and diminished *p53*, *Bax*, and *caspase 3* expressions compared to the HS-only-treated animals.

It has been reported that HS results in various dysfunctions in reproductive system, including germ cells apoptosis, sperm motility reduction, sperm chromatin abnormalities, and reduced embryo developments.[4,10] Moreover, HS is considered as an important environmental factor involving in human and animals infertility.[4,15] In line with that, it has been indicated that HS exposure is correspondent for approximately 10.00% of male infertility around the world.[39] Due to the high rate of HS-induced infertility around the world and based on various adverse effects of HS exposure on testicular tissue, different researchers were attracted to this subject and this phenomenon was led to different conducted studies focus.[4,40] Accordingly, it is well-documented that HS adversely impacts testicular tissue through germ cell apoptosis initiation[41] and autophagy induction,[42] as well as ROS over-generation.[40] Among the above-said factors, ROS have been considered as a key factor in HS-inducing negative effects on testicular tissue.[40] In normal testicular tissue, ROS play a pronounced role in maintaining various cellular activities in an acceptable level; however, HS-induced ROS have been shown to up-regulate this amount, resulting in anti-oxidants down-regulation.[43] Consistently, testicular heat promotes ROS generation from mitochondria and mainly impacts germ cells by lipid peroxidation.[44] In coordination with earlier studies, our results showed that HS-exposed animals exhibited a significant higher amount of MDA level *versus* control group. Moreover, testicular anti-oxidant enzymes level decreased in HS-exposed animals compared to the control group. Further, the anti-oxidant administration has been established as an important approach to use against the HS-induced testicular tissue oxidative stress.[4,45] Being in conformity with this approach, it was observed in the current study that using BPs as an anti-oxidant resulted in reduced MDA level and enhanced the enzymatic anti-oxidant defense of testicular tissue. Furthermore, evaluating the histopathological effects of HS exposure revealed that HS adversely impacts testicular tissue, resulting in reduced TDI, SPI, SCI, MI, and SpA number *per* mm^2 *versus* control group. In contrast, the BPs-co-treated animals delineated that using BPs could remarkably alleviate the histopathological injuries in testicular tissue compared to the HS-treated animals.

Based on previous findings, it has been declared that HS-induced ROS significantly suppress spermatogenesis process through intrinsic apoptosis pathway induction.[46] Likewise, it has been detailed that HS enhances testicular content of pro-apoptotic proteins, such as p53 and Bax,[47] and diminishes anti-apoptotic proteins, including Bcl-2; while, caspases play a fundamental role in HS-induced apoptosis.[46,47] Herein, it was focused on the HS-induced impact on mitochondrial-dependent apoptosis in order to unearth the BPs protective effects on this pathway inhibition.

Further, ROS have been well recognized as DNA damage inducers. Analogously, it was found that HS-induced ROS cause DNA damage in various cells in testicular tissue.[4] Indeed, the DNA fragmentation in testicular tissue is known as a main originator of apoptosis, triggering DNA guardian gene recognized as *p53* among several genes.[48] In fact, *p53* is involved in cellular DNA damage control in G1/S and G2/M cell cycle machinery. Therefore, excessive DNA damage initiates *p53* over-expression, resulting in *p53*-mediated DNA repair or *p53*-dependent apoptosis. Evidently, the *p53* directly up-regulates the *Bax* and *Bak* expressions, leading to the mitochondria-dependent apoptosis initiation.[49] Considering our results, HS-exposed animals showed a remarkable enhanced *p53* expression at mRNA and protein levels; while, the BPs-co-treated animals exhibited a significant reduction in *p53* expression *versus* HS-treated animals. In order to clarify whether p53 initiates DNA repair or mitochondria-dependent apoptosis, the *Bax*, *Bcl-2*, and *caspase 3* genes mRNA and protein expressions were determined. Thus, our observation revealed that in HS-induced animals *Bax* and *caspase 3* mRNA and protein expressions have significantly increased compared to the control animals; while, the *Bcl-2* expression reduced in HS condition. Moreover, our findings revealed that BPs administration could remarkably diminish pro-apoptotic genes expression and enhance *Bcl-2* level *versus* HS-induced animals. On the other hand, our results depicted that BPs administration could not improve 43.00 ˚C -induced damages/apoptosis in testicular tissue.

Based on the aforementioned findings, it should be noted that the Bcl-2 and Bax are the hallmarks of intrinsic apoptosis and/or cell survival, and *Bcl-2* serves as a member of anti-apoptotic genes; while, *Bax* is recognized as a pro-apoptotic gene.[49] Correspondingly, it has been rendered that reduced *Bcl-2* expression results in cytochrome C release from mitochondria to cytoplasm, triggering caspase-dependent pathways. Thence, any reduction in *Bcl-2* expression initiates Bax protein oligomerization, resulting in mitochondria permeability transition, leading to mitochondria inner trans-membrane potential disruption, leakage of cytochrome C to the cytoplasm, and subsequently a cascade of caspases activation.[50] Minding reduced *p53*, *Bax*, and *caspase 3*

expressions in BPs-received groups, as well as increased *Bcl-2* expression, it can be hypothesized that BPs could fairly inhibit HS-induced mitochondria-dependent pathway. In addition, it should be considered that the positive effects of BPs co-treatment were observed on 37.00 and 39.00 ˚C HS inductions, and in order to inhibit 43.00 ˚C HS, a higher dose of BPs administration may be needed.

Regarding increased anti-oxidant enzymes and *Bcl-2* expression, and reduced *p53*, *Bax*, and *caspase 3* expressions in BPs-received groups, it can be suggested that BPs co-treatment protects the testicular tissue against HS-induced mitochondria-dependent apoptosis not only by improving enzymatic anti-oxidant defense but also *via* provoking the *Bcl-2* over-expression in germ cell lineage in HS condition (Fig. 7). Although in this study BPs administration at the selected dose could not improve the HS-induced side effects at 43.00 ˚C, it would be more logical to conclude that BPs as a potential source of anti-oxidant can fairly repress HS-induced damages; however, higher dose(s) possible salutary effects are needed to be meticulously unveiled.

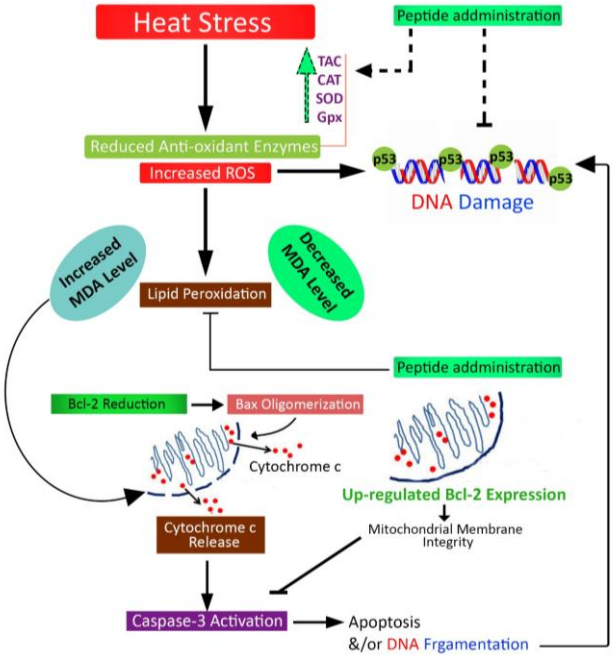

Fig. 7. Heat stress (HS) induces reactive oxygen species (ROS) overload and anti-oxidant enzymes reduction; while, these phenomena result in lipid peroxidation and DNA damage. Moreover, these two effects of HS lead to reduced Bcl-2 protein concentration and enhanced Bax oligomerization, resulting in mitochondria-dependent apoptosis. Moreover, HS induces *p53* over-expression through DNA damage elevation. In contrast, it was upheld that bioactive peptides (BPs) administration could improve enzymatic anti-oxidant defense as well as Bcl-2 up-regulation in mitochondria membrane. Thus, BPs protected the testicular tissue against HS-induced apoptosis, not only by enhancing testicular enzymatic anti-oxidant defense but also *via* *Bcl-2* expression up-regulation.

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Conflict of interest

All authors declare no competing interests.

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