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Original Article

Potential Role of Human Chorionic Gonadotropin Supplementation in Spermatogenesis in Rats Subjected to Forced Swimming Exercise

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Abstract

Aim: The aim of this study was to evaluate the supportive effect of human chorionic gonadotropin (hCG) on the quality of spermatogenesis, including count, motility, morphology, viability and apoptosis of sperm following forced swimming exercise.

Materials and methods: Twenty-four adult male Sprague-Dawley rats were used in this study. All rats were divided into four groups: control group; swimming exercise group (S); hCG administration group and swimming (SG) with hCG administration group (G). The experimental group was trained to force swimming stress for 10 min for 6 days. Then the sperm quality parameters were measured after dissection and epididymis removal. Spermatogenesis and germ cell apoptosis were evaluated by using Miller & Johnsen's score and TUNEL staining respectively.

Results: Results showed the count (control: 113±3.1, S: 74±1.9, G: 111±3, and SG: 103±2.4), motility (control: 93±2, S: 67±2.8, G: 90±2.7, and SG: 78±1), morphology (control: 89±3%, S: 47±2.4%, G: 90±3.1%, and SG: 67±1.1%), and viability of sperm (control: 91±2.9, S: 50±2, G: 91±1.9, and SG: 70±1.3) in swimming exercised-hCG administered group, significantly enhanced by hCG treatment compared to the swimming exercise group ($p \leq 0.01$). Also the number of apoptotic germ cells significantly decreased in swimming exercised-hCG administered group compared to the swimming exercised group.

Conclusions: These results suggest that administration of hCG can protect the testes against the detrimental effect of forced swimming exercise in adult male rats.

Keywords

forced swimming exercise, human chorionic gonadotropin, quality of spermatogenesis

INTRODUCTION

Exercise has many health advantages including a reduced risk of cardiovascular disease, diabetes and cancer. Yet it is paradoxical that the contracting skeletal muscles produce free radicals and intense exercise can cause oxidative damage to cellular constituents. Also, intense exercise can lead to increased oxidative stress in athletes and non-athletes.[1] Cellular oxidative stress, by inducing reactive oxygen species (ROS) and reactive nitrogen species (RNS), causes many abnormalities in different tissues.[2] There are several studies in human and animal models which have reported the association of exercise with the production of ROS.[3-5] Several researchers have demonstrated that long-term exercise may cause dysfunctions in the male reproductive system.[6,7] Prolonged swimming has been shown to change the function of the male reproductive system such as decline of spermatogenesis, testosterone production and sperm fertilizing capacity.[8] Forced intensive swimming, as a strenuous exercise, leads to the oxidation of biological molecules (proteins, lipids, carbohydrates, etc.) with the ROS generation.[9] Like other tissues, the testes are affected by long physical exercises.[10] Indeed, the testes are more susceptible to peroxidation damage because of the lower anti-oxidative enzymes and higher amount of polyunsaturated lipids in the mitochondrial membrane.[11] By producing oxidative stress, exercise reduces spermatogenesis, spermiogenesis, and spermatozoa survival in the testes.[7] Also, intensive swimming exercise disrupts male fertility by declining the gonadosomatic index (GSI), the number of spermatocytes and spermatids and also the sperm count in epididymis.[12,13]

Human chorionic gonadotropin (hCG) is a glycoprotein hormone synthesized by syncytiotrophoblastic cells of the placenta during the pregnancy.[14] hCG plays important roles in the luteotropic function.[15] In males, hCG administration can irritate the Leydig cells to secrete testosterone.[16] This reaction to hCG is considered as a diagnostic test to evaluate the testis function.[17] In addition, previous studies have shown the protective effects of hCG against oxidative stress.[18] In order to protect men's infertility against exercise, antioxidants such as melatonin, vitamin A, and vitamin C have been evaluated.[3] Still, there is a necessity to find more effective methods for reducing the exercise-induced testicular oxidative stress.

AIM

The current study was conducted to examine the protective role of hCG on forced swimming exercise-induced abnormalities in adult male rats, including sperm parameters and spermatogenesis cell lines apoptosis.

MATERIALS AND METHODS

Animals

In this study, a total of 24 mature male Sprague-Dawley rats, weighing 180±30 g, were randomly allocated into four groups: control group (n=6); swimming exercise group (n=6); hCG administration group (n=6) and swimming with hCG administration group (n=6). In the hCG groups, the rats received 1000 IU of hCG (Sigma-Aldrich-CG10 MSDS), 3 times a week for 8 weeks by intramuscular injection **(Fig. 1)**.[19]

Exercise procedure

According to previous studies, in the swimming exercise and swimming with hCG administration groups, we performed an exercise protocol with 1 hour of swimming per day, 5 days a week for consecutive 8 weeks **(Fig. 1)**. The animals were trained for 6 days to adapt to swimming before the beginning of experiment. At first, the rats were trained to swim for 10 min in plastic tanks. Then the time of swimming was increased by 10 min daily until it reached 60 minutes. Rats swam in a plastic water tank with specified size (50×100×50 cm), and water temperature of 32-34°C.[20]

Sample collection

At the end of the study period, the rats were anaesthetized with a mixture of xylazine (10 mg/kg) and ketamine (80 mg/kg): (Alfasan Company, Woerden, the Netherlands), then the testes and epididymis were carefully removed and fixed in 10% buffered formaldehyde solution overnight for histopathological examinations. Sperm para-

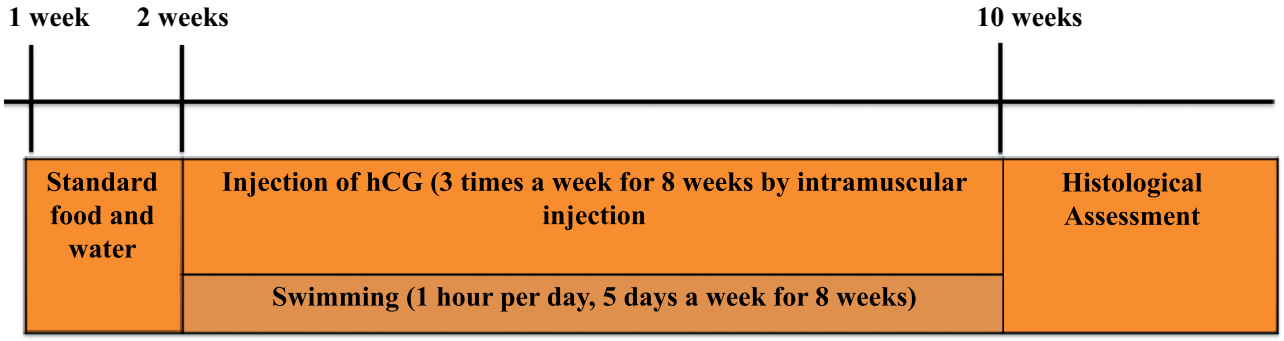

Figure 1. Time schedule of the experimental protocol.

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meters including count, morphology, viability, and motility were measured from the epididymal sperm samples.

Sperm analysis

The epididymis was minced with scissors in a petri dish containing 5 ml of Ham's F10 medium (Sigma Aldrich, N6635) and incubated at 37°C for 15 minutes to allow the spermatozoa to exit from the tissue. To analyze sperm motility, 10 µl of sperm suspension was placed on a slide and then covered with a coverslip. The percentage of motile sperms was calculated by selecting 10 microscopic fields at ×400 magnifications.[21] In order to determine the sperm viability, 10 µl of sperm suspension was blended with an equal volume of eosin-nigrosine dye (Sigma-Aldrich-198285 MSDS). The percentage of live sperms (colourless or light pink) and dead sperms (red or dark pink color) were calculated by counting 200 sperms in each slide with observation by light microscope at ×1000 magnification. The sperm count was calculated by mixing 50 µl of sperm suspension with 200 µl of distilled water. Ten microlitres of this diluted suspension was moved to each of the Neubauer haemocytometer and left for 5 minutes for cells sedimentation. Then sperm count was analyzed with a light microscope at ×400 magnification.[22] Sperm morphology was evaluated by using eosin Y (Sigma-Aldrich-CAS Number 15086-94-9) staining. One drop of sperm suspension was mixed with an equal amount of 1% eosin Y dye. After 30 minutes, smears were prepared and allowed to dry in the air, and were mounted and then covered with a coverslips. 200 sperm cells were inspected in each slide to investigate the morphological abnormalities at ×1000 magnification.[13] Unusual structure or morphology of head and tail of spermatozoa was considered as abnormal sperm.

Evaluation of spermatogenesis

In the current study, Miller & Johnsen's score were used to evaluate the spermatogenesis. It was ranked by calculating Johnsen's score (from 1-10) and measuring the number of germinal cell layers in the testis. Ten seminiferous tubules were considered to count germinal epithelial layers according to the Miller's scores. The scores of spermatogenesis quality in seminiferous tubules were obtained according to the maturity of germ cells.[22]

Histopathological analysis

The left testis of each rat was used for histopathological analysis. The testis was fixed in fresh 4% paraformaldehyde (PFA) solution for 24 hours. After dehydration and clearing, the testis was embedded in paraffin wax and then 7-µm thick sections were obtained by using a rotary microtome. The deparaffinised sections in xylene were dehydrated in various grades of alcohol. After water wash, the sections were stained with hematoxylin stain for 7 min and then after washing in water for 10 min the differentiation was done in acid alcohol, the sections were incubated in lithium carbonate for 3 min and were stained with eosin for 15 sec and cleared in xylene and mounted using a suitable mountant. The slides were then observed using a simple light microscope.

Germ cell apoptosis by TUNEL assay

Germ cell apoptosis was evaluated by TUNEL staining according to the terminal deoxynucleotidyl transferase (TdT) enzyme-mediated dUTP nick end labeling [TUNEL] assay kit [HRP-DAB (ab206386)] protocol. 5-µm thick paraffin-embedded sections were deparaffinised and then rehydrated in graded alcohol. Sections were incubated in blocking solution (3% H_2O_2) to neutralize endogenous peroxidases for 10 minutes. Then, the sections were washed with PBS and were incubated with TdT for 60 minutes at 37°C. After washing the slides with PBS, they were incubated with anti-digoxigenin peroxidase antibodies. DAB substrate was applied for 10 minutes to stain positive apoptotic cell brown. At least, 10 seminiferous tubules were selected in each section for counting apoptotic cells by light microscope observation.

Statistical analysis

For all experiments, data were evaluated using SPSS16 (www.spss.com) using ANOVA with Tukey's multiple comparison. The data are expressed as mean ± standard error of the mean (SEM). The significance was set at $p<0.05$.

RESULTS

Sperm parameters

The effect of hCG administration on the sperm quality parameters, in swimming exercised and non-exercised groups is shown in **Fig. 2**. In the present study, forced swimming exercise caused significant reduction in all four sperm parameters compared to the control group ($p\leq0.0001$). Results showed the count (control: 113±3.1, S: 74±1.9, G: 111±3 and SG: 103±2.4), motility (control: 93±2, S: 67±2.8, G: 90±2.7 and SG: 78±1), morphology (control: 89±3%, S: 47±2.4%, G: 90±3.1%, and SG: 67±1.1%) and viability of sperm (control: 91±2.9, S: 50±2, G: 91±1.9, and SG: 70±1.3) in the swimming exercised hCG administered group, significantly enhanced by hCG treatment compared to the swimming exercise group ($p\leq0.01$). We found no significant differences in sperm parameters between control group and hCG treated group ($p\leq0.05$), although sperm parameters increased in control-hCG supplemented group compared to the control group.

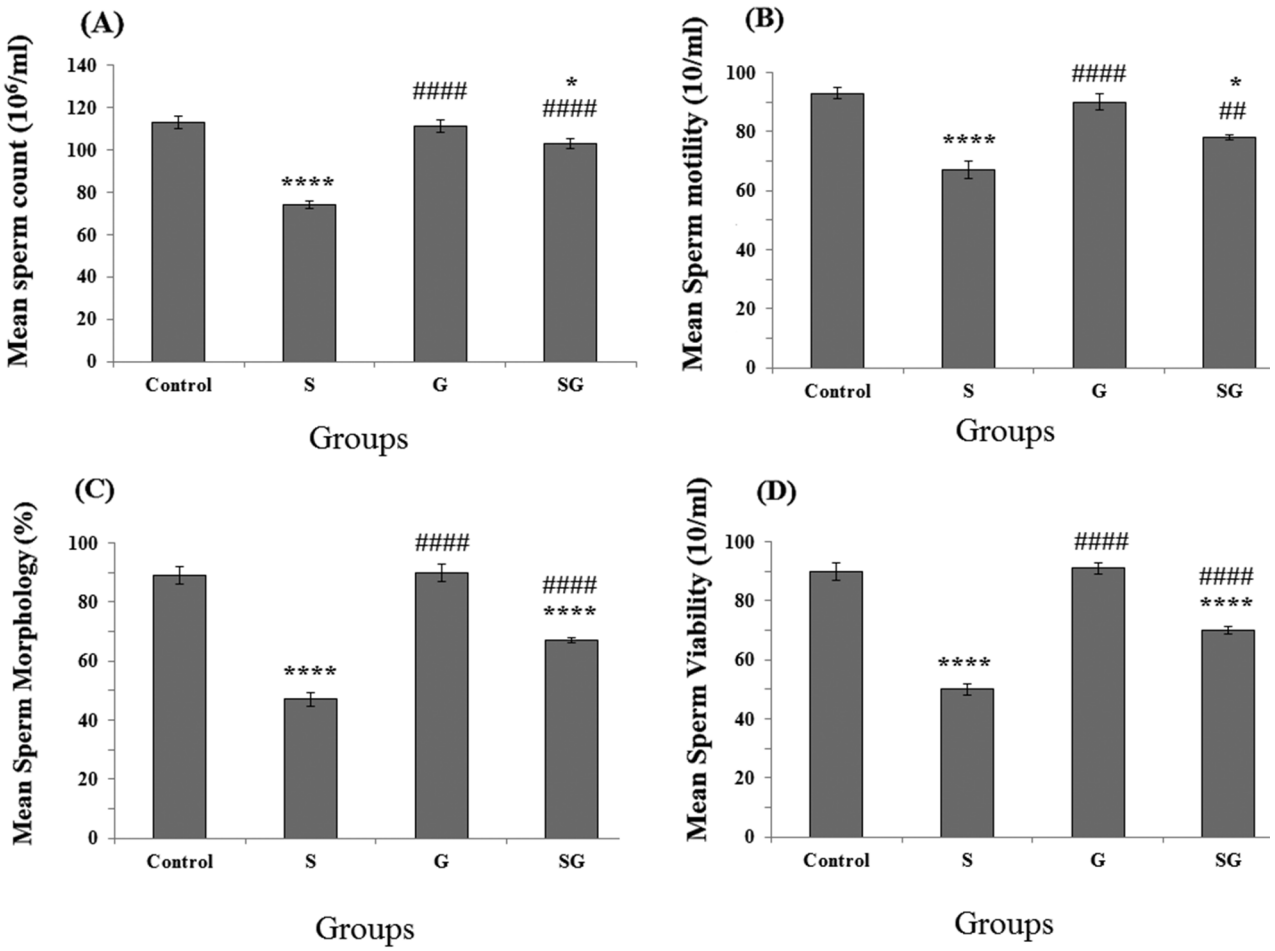

Figure 2. Changes in sperm parameters following hCG treatment in swimming exercise and control groups. (**A**) Sperm count, (**B**) Sperm motility, (**C**) sperm morphology, and (**D**) sperm viability.
S: swimming exercise group, G: gonadotropin group, SG: swimming exercise and gonadotropin group. * $p \leq 0.05$ vs. control group, ** $p \leq 0.01$ vs. control group, *** $p \leq 0.001$ vs. control group, **** $p \leq 0.0001$ vs. control group, # $p \leq 0.05$ vs. swimming exercise group, ## $p \leq 0.01$ vs. swimming exercise group, ### $p \leq 0.001$ vs. swimming exercise group, #### $p \leq 0.0001$ vs. swimming exercise group.

Apoptosis of germ cells

In the TUNEL assay, forced swimming exercise increased the apoptosis of germ cells **(Fig. 4)**. Histopathological examination of seminiferous tubules in different groups indicated the beneficial effect of hCG treatment during swimming exercise, by reducing the TUNEL positive cells (dark brown cells). The number of apoptotic germ cells, significantly decreased in swimming exercised-hCG administered group compared to the swimming exercised group. In the control and control-hCG administered groups, the number of apoptotic germ cells was negligible **(Fig. 3)**.

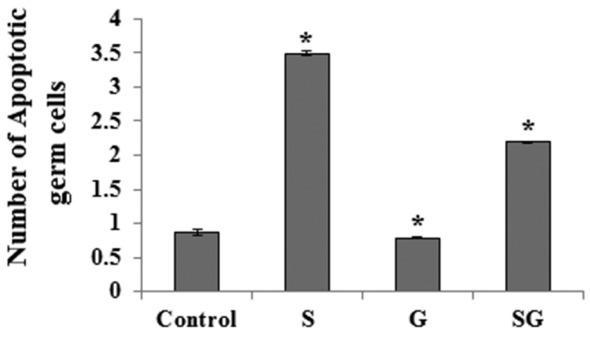

Figure 3. The number of apoptotic germ cells. * $p<0.05$, comparison of different groups with the control group. S: swimming exercised; G: gonadotropin administration; SG: swimming exercised and gonadotropin administration.

Miller & Johnsen's scores

Miller's score indicated that the mean thickness of seminiferous layers in the S group (2.9±0.27) was significantly lower than that of the control group ($p \leq 0.01$). Also, SG group caused a significant increase than the S group

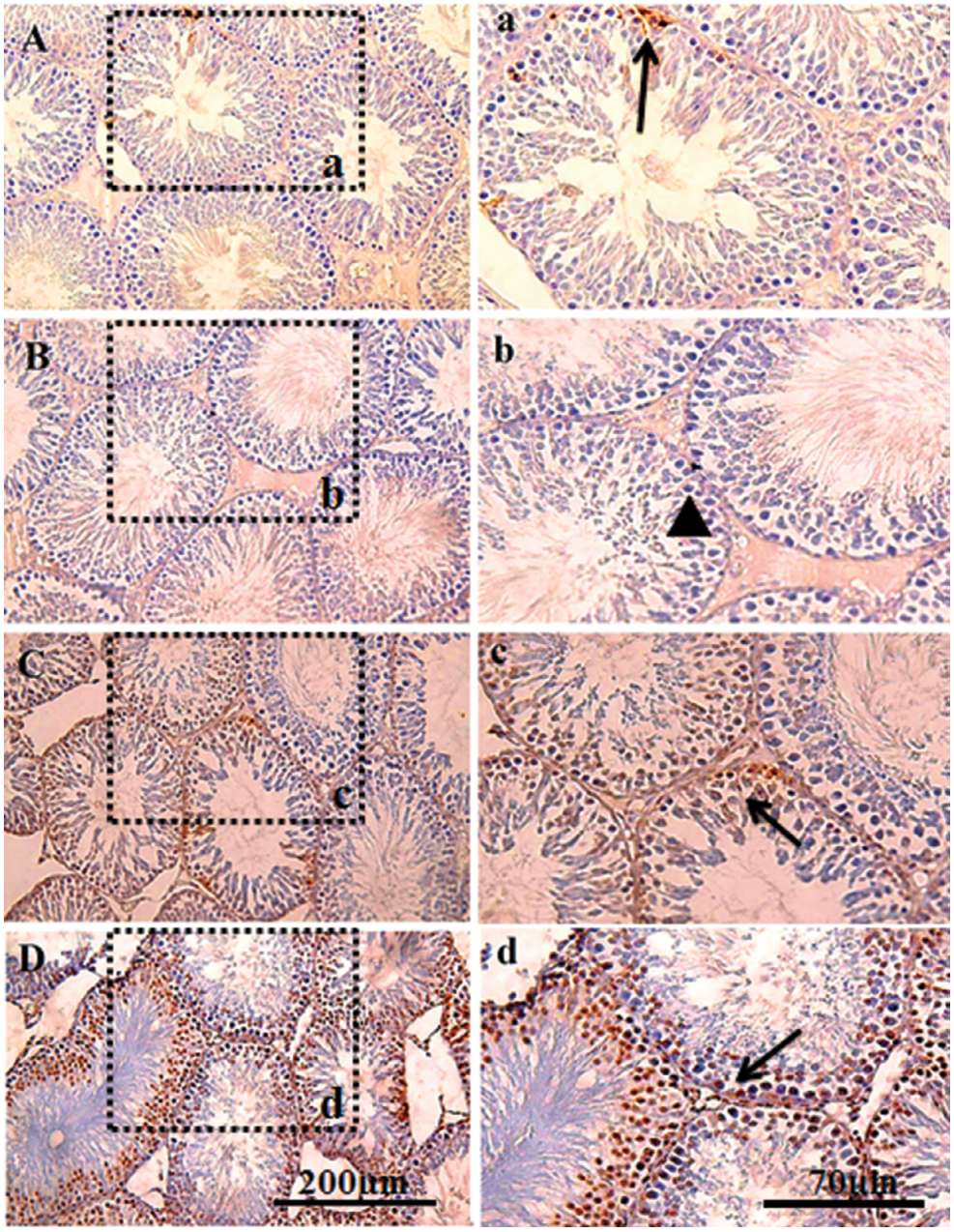

Figure 4. The germ cell apoptosis (TUNEL positive cells) in a cross-sectioned testis of different groups. Control group (**A**), gonadotropin administration group (**B**), swimming exercised group (**C**) and gonadotropin administration swimming exercised group (**D**); **a**, **b**, **c**, and **d** represent large insets of apoptotic germ cells showing details of morphological cells. The arrow shows apoptotic cells and the arrowhead indicates the normal cells.

(p<0.03). There were no significant differences in the G and SG groups as compared to the control group **(Fig. 5A)**.

According to the **Fig. 5B**, Johnsen's scores significantly decreased in the swimming exercised group compared with the control group (p<0.05). hCG administration caused an increase in Johnsen's scores in the swimming exercised-hCG and control-hCG groups compared to the non-hCG groups. Also, the results of this study demonstrated that the testicular tissue in the control group was supported with an albugineous layer and the seminiferous tubule cell series was discernible **(Fig. 6A)**. The histological structure in the gonadotropin administration group (**G**) corresponded to that of the control group **(Fig. 6D)**. The finding showed serious injury at the seminiferous tubules, loss of spermatogenic cells and haemorragia at the interstitial area in swimming exercised group **(Fig. 6B)**. The number of spermatogenic cells in the in swimming exercised and gonadotropin administration group (SG) was increased compared to the swimming exercised group, and amelioration in the seminiferous tubule structure was seen **(Figs 6A, 6C)**.

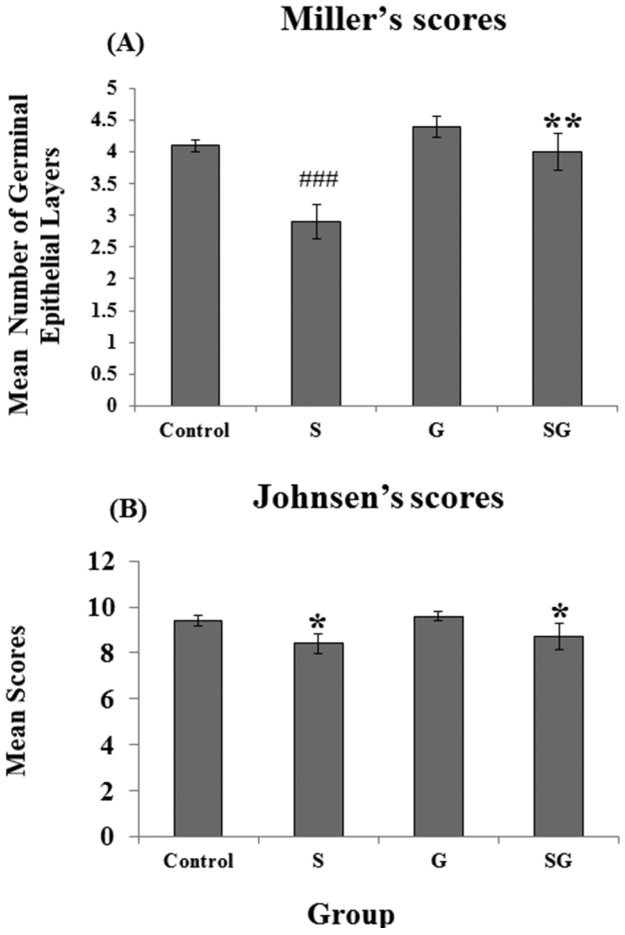

Figure 5. Miller & Johnsen's scores. (**A**) the mean number of germinal epithelial layers was counted. ** indicates the significance of the mean number of germinal epithelial layers in SG than the S group (p<0.03). ### showed the significance of S than the other groups (p<0.001). (**B**) Comparison of different groups with the control group. S: swimming exercised, G: gonadotropin administration, SG: swimming exercised and gonadotropin administration (*p<0.05).

DISCUSSION

In this study, we evaluated the protective effect of hCG treatment against detrimental changes of forced swimming exercise on spermatozoa. The results of our study showed that forced swimming exercise led to harmful effects on the testes and sperm quality parameters. The swimming exercise in male rats significantly reduced sperm count, motility, and viability and enhanced abnormal morphology. Also, exercise decreased Johnsen's scores as an indicator of spermatogenesis quality and increased apoptosis of germ cells.

Several studies compatible with our data have confirmed the detrimental effect of exercise on testis and sperm parameters.[20,23] Moreover, other studies have shown that swimming exercise promoted germ cell apoptosis and decreased spermatogenesis quality.[23,24] Literature data confirm that intensive exercise such as intensive swimming exercise leads to dysfunction of the male reproductive system by two main mechanisms: 1. by reducing testosterone secretion and 2. by inducing increased oxidative stress.[3,25] Intensive exercise decreases the testosterone level along with other reproductive hormone, which inhibits the spermatogenesis in male athletes.[26] Decline of testosterone secretion is due to the reduction in testicular blood flow during exercise. In addition to the decrease of blood flow, exercise promotes the temperature of testicle which can decrement testosterone secretion and spermatogenesis.[27]

During physical exercise, ROS generation significantly increases because of the higher oxygen consumption.[5] The testis is one of the most susceptible organs to oxidative stress which highly affects it during intensive exercise. In animal model studies, drastic exercise reported to decrease sperm parameters and quality of spermatogenesis, and these findings are consistent with our studies.[7,23] In a study by Naraghi et al., swimming exercise caused several ultrastructural changes in the testes of adult male rats.[20] Reduction in the number of Leydig cells, irregularity of basement membrane, several lipid droplets and several apoptotic germ cells were found in the transmission electron microscope (TEM) study in swimming-exercised rats.[20] Moayeri et al. have shown that forced swimming exercise decreased sperm parameters, antioxidant enzymes and increment apoptosis of germ cells in the testis.[23] In the mentioned study, treatment with melatonin considerably improved sperm motility and reduced apoptosis of germ cells along with increased antioxidant enzymes in rats performed the forced swimming practice.

In another study, strenuous swimming exercise was shown to decrease the epididymal sperm count and testosterone level in testicle and increase the ROS generation, lipid peroxidation and abnormal morphology of sperm.[28] Co-administration of α-lipoic acid and N-acetylcysteine showed a protective effect against these harmful changes.

Several studies have documented the beneficial effects of hCG treatment in different male and female reproductive system disorders such as cryptorchidism, non-obstructive azoospermia, idiopathic hypogonadotropic hypogonadism and idiopathic male infertility.[29-36] The effect of hCG administration is similar to the LH activity, because they have the same receptor.[37] Many studies have reported that hCG therapy stimulates testosterone secretion, which improves spermatogenesis and fertility rates.[17] hCG injection for at least 6 to 12 months is considered as an initial treatment protocol for male infertility.[38] Recent studies showed that normal fertile men need fewer doses of hCG to initiate spermatogenesis than do infertile men, because fertile men are more susceptible to hCG and have mature Leydig cells.[39] Rulli et al. have reported that hCG hyper stimulation improved testicular steroidogenesis and proliferated the Leydig cell.[40] In one study, hCG treatment in patients with idiopathic male infertility was demonstrated to increase the sperm parameters, a finding which is similar to our present results.[41] In our previous study, hCG treat-

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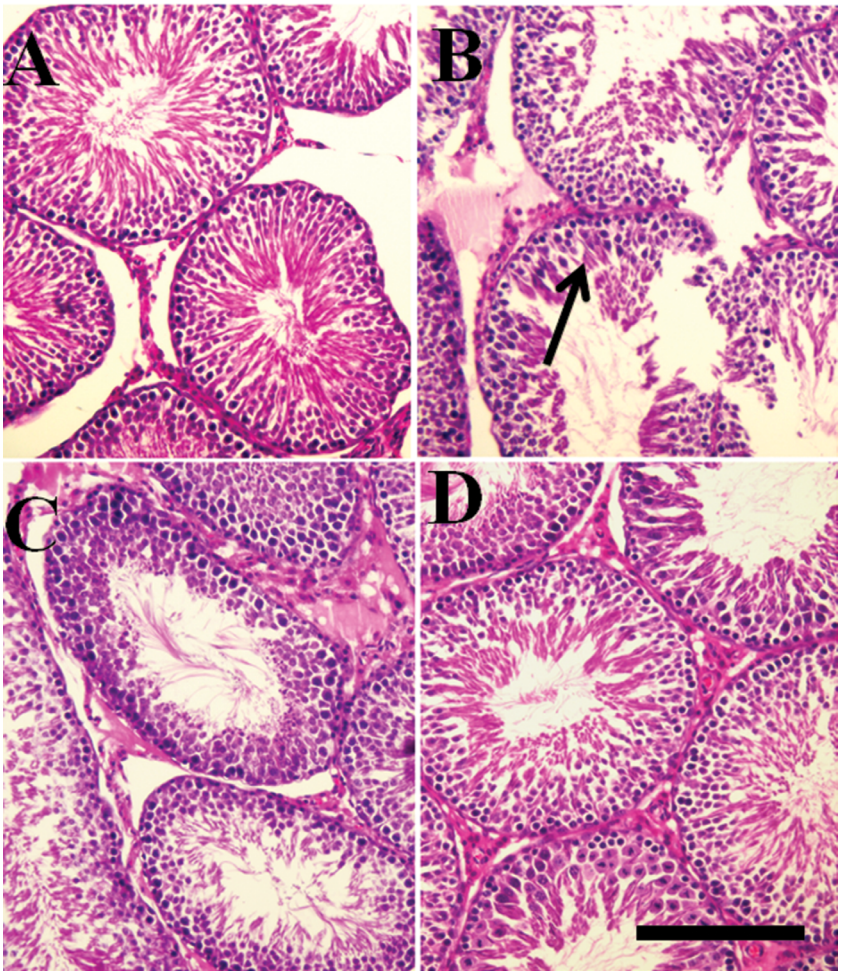

Figure 6. Representation of H&E staining in different groups. The arrow shows degenerating cells. (**A**) control group; (**B**) swimming exercised group; (**C**) swimming exercised and gonadotropin administration group; (**D**) gonadotropin administration group.

ment ameliorated the detrimental effect of nandrolone decanoate on sperm parameters of male rats.[19,42]

In the present study, injection of 1000 IU hCG ameliorated the sperm quality and further promoted the spermatogenesis (In this study, two different doses of hCG were used, and since the findings of 1000 IU and 500 IU were not statistically significant and, on the other hand, numerical data from a dose of 1000 IU hCG showed further improvement in the testis and sperm quality parameters because we reported the dose of 1000 IU in this study) which is consistent with previous investigations.[43,44]

Anti-oxidative effect of hCG, plays an important role against oxidative-induced disorders.[18] In a cell culture, hCG showed a protective effect against oxidative stress in human umbilical vein endothelial cells (HUVEC), also hCG indicated antiapoptotic and cell survival actions.[18] Recent studies have shown that in addition to the increase in ROS generation, intensive swimming exercise decreases antioxidant enzymes in the testis, which leads to more severe damage to the this tissue.[23,45] In this study, swimming exercise significantly increased germ cell apoptosis, which resembles other previous studies.[13,46] Increase in apoptosis of germ cells is described as a result of oxidative stress in the testes.

CONCLUSIONS

Our investigations lead to the conclusion that apoptosis is a normal, hormonally controlled phenomenon in the testis after force exercises. The present study indicates that administration of hCG can protect the testis against the detrimental effect of forced swimming exercise in adult male rats. Forced swimming exercise decreased the Johnsen's scores, sperm count, motility, and viability. Also this drastic exercise increased abnormal morphology and apoptotic germ cells in this study, treatment with hCG ameliorated the harmful effect of forced swimming exercise, including enhancing the sperm parameters, spermatogenesis quality and reducing apoptosis compared to the non-treatment group. These beneficial effects of hCG administration are due to the induction of testosterone secretion and anti-oxidative properties.

Ethics approval

This research was approved by the Ethics Committee of Ilam University of Medical Sciences (ethical code: ir.medilam.rec.1394.46). All animal experiments we performed were in strict compliance with the laws of working with animals.

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Возможная роль добавок хорионического гонадотропина человека на качество сперматогенеза у крыс, подвергшихся принудительному плаванию

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Резюме

Цель: Целью этого исследования было оценить поддерживающий эффект хорионического гонадотропина человека (ХГЧ) на качество сперматогенеза, включая количество сперматозоидов, подвижность, морфологию, жизнеспособность и апоптоз после упражнений принудительного плавания.

Материалы и методы: В этом исследовании использовали 24 крысы Sprague-Dawley. Все крысы были разделены на четыре группы: группу с плаванием с упражнениями (S), группу, получавшую ХГЧ и плавание (SG), и группу, получавшую ХГЧ (G). Опытная группа подвергалась стрессу принудительного плавания в течение 10 минут в течение 6 дней. Затем измеряли параметры качества спермы после рассечения и удаления придатка яичка. Сперматогенез и апоптоз зародышевых клеток оценивали с использованием шкалы Миллера и Йонсена и окрашивания TUNEL соответственно.

Результаты: Результаты показали количество (контроль: 113±3.1, S: 74±1.9, G: 111±3 и SG: 103±2.4), подвижность (контроль: 93±2, S: 67±2.8, G: 90±2.7 и SG: 78±1), морфология (контроль: 89±3%, S: 47±2.4%, G: 90±3.1% и SG: 67±1.1%) и жизнеспособность сперматозоидов (контроль: 91±2.9, S: 50±2, G: 91±1.9 и SG: 70±1.3) в группе плавания и ХГЧ, значительно увеличилось при лечении ХГЧ по сравнению с группой плавания ($p \leq 0.01$). Кроме того, количество апоптотических половых клеток значительно снизилось в группе плавания и ХГЧ по сравнению с группой плавания.

Заключение: Эти результаты предполагают, что введение ХГЧ может защитить яички от вредного воздействия упражнений по плаванию у взрослых самцов крыс.

Ключевые слова

принудительное плавание, хорионический гонадотропин человека, качество сперматогенеза