Insulin improves spermatogenesis in a mouse model of aging

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SUMMARY

Aging can have adverse effects on the chance of fertility in men. In fact, men over 40 years old usually suffer from low sperm quality, sperm motility, and low level of serum testosterone that leads to infertility. In addition, the structure of DNA is more vulnerable in aged testes due to the high level of reactive oxygen species (ROS) and nitric oxide. However, it has been showed that insulin is able to decrease oxidative stress and apoptosis. Moreover, some studies showed that insulin can enhance both spermatogenesis and fertility. In this regard, we investigate the impacts of insulin on spermatogenesis, the number of sperm, and the level of gonadal hormones in aged testes of mice. Thirty-six adult male NMRI mice were used. They were kept under standard conditions. The animals were divided into two groups control and insulin. The insulin group received 100 µL insulin with a 72-hour interval by intraperitoneal (IP) injection for 33 weeks and the control group received water. At the 8, 12, and 33 weeks of treatment, the testes of mice were removed for histological, cellular (ROS and glutathione [GSH]) and molecular (Bcal2, caspase-3, and Bax) analysis. For measurement of the level of hormones (testosterone, LH, and FSH), the blood samples were collected from the heart. According to our findings, insulin could significantly increase the volume of the testes and the total number of spermatogonial stem cells, primary spermatocytes, round spermatids as well as leydig cells. In addition, activity of GPX was considerably high in the insulin group. Furthermore, in the insulin group, the level of ROS was reduced. In conclusion, insulin may be effective in enhancing the function of the testes in aged male mice.

Key words: Spermatogenesis – Insulin – Aging

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INTRODUCTION

Aging is a progressive process that is associated with impairment of physiological integrity, disability, and several diseases (López-Otín et al., 2013; Abdollahifar et al., 2019). During aging, free radicals attack cells and alter the cell membranes or inactive proteins which may lead to cell death (Salomon et al., 2013). Besides, aging affects fertility both in women and in men. Men who are over 40 years old have some infertility issues such as low sperm quality, reduction of testes volume, and low levels of serum testosterone (Bhanmeechao et al., 2018). However, in most developed societies, it is a trend amongst men to have children at older ages. Hence, they may face some problems with infertility (Almeida et al., 2017) and tend to try assisted reproductive techniques (ART) that are expensive and have many challenges in older persons (Abdollahifar et al., 2019; Almeida et al., 2017).

Aging has also adverse effects on spermatogenesis. Previous studies showed that ageing reduced semen quality, semen volume, the number of normal sperms, and sperm motility (Stone et al., 2013; Zhu et al., 2011). Salomon et al. revealed that the activity of antioxidant enzymes such as GPX and SOD in the testes of young rats (3-12 months) is significantly higher if compared to the old rats (24 months). Then, the testes of aged rats are more vulnerable to free radicals (Salomon et al., 2013). Moreover, a significant increase in sperm DNA fragmentation from the age of 30 to 35 years has been reported (Stone et al., 2013), because the production of nitric oxide increases in aged tissues that can damage the DNA structure (Salomon et al., 2013). On the other hand, it has shown that ageing in Leydig cells of testes can also reduce the production of serum testosterone (Barbutska et al., 2015). According to previous studies, mitochondrial dysfunction has a key role in Leydig-cell damages. The level of ROS in old cells are considerably higher than antioxidant enzymes, so exceeded ROS attacks mitochondria, which finally leads to cell apoptosis (Lacombe et al., 2006; Sastre et al., 2000).

Some studies showed that insulin can be considered a protective applicator, because it is able to decrease oxidative stress and apoptosis in neuron cells by activating SOD, CAT, and GSH-Px (Song et al., 2018). Moreover, it has been suggested that insulin can stimulate spermatogenesis (Nakayama et al., 2004) and enhance fertility through impact on hypothalamic-pituitary-gonadal axis, as well as normalize the levels of serum testosterone and LH (Schoeller et al., 2012). The aim of this study was to assess the effects of insulin on spermatogenesis, number of sperms, and level of gonadal hormones on testes aging according to stereological studies.

MATERIAL AND METHODS

Animals and treatments

Thirty-six male NMRI mice, weighing 25-30 g and aged 4 weeks, were purchased from the laboratory animal research center. The animal was kept in a standard situation. Healthy adult mice were divided into two groups, each group comprising sixteen mice. Control group: the animals were kept intact. Insulin group: the animals received insulin at the dose of 100 µL with a 72-hour interval by intraperitoneal (IP) injection for 8, 12, and 33 weeks. At the end of 8, 12, and 33 weeks the animals were euthanized, and their testes were collected and fixed in paraformaldehyde 4%. After that, all testes were embedded in paraffin blocks and cut into 5 µm and 25 µm thicknesses by using a systematic, uniformly random sampling (SURS) for the stereological studies. All tissue sections were stained with hematoxylin and eosin (H&E).

Stereological study

Volume of the testes and the length of seminiferous tubules

Using the Cavalieri principle, testis sections were examined under a projecting microscope. Using stereological software developed at Shahaid Beheshti University of Medical Sciences, a grid of points was superimposed on the images. Using a Nikon microscopy system (E-200: Japan) connected to a video camera, the volume of the testis was measured. The formula has been used for the estimation of the volume of testes (Raoofi et al., 2021):

$$V = \sum P \times \frac{a}{p} \times t$$

In this equation, Σp is the total points of the probe, (t) is the distance between the sections and a/p represents the area per point.

The length of the seminiferous tubules

To estimate the length density of seminiferous tubules using the optical dissector method, the following equation (described in the following section) was used (Hassani et al., 2020):

$$L_v = \frac{2\sum Q}{\sum P \times \frac{a}{f}}$$

In this equation, "ΣQ" is the number of seminiferous tubules, "a/f" is the area per frame, and "ΣP" is the number of filed views.

Total number of testicular cells

Optical dissector methods were used to in order to determine the number of testicular cells. We selected microscopic fields by systematic uniform random sampling, by moving the stage in equal distances in x- and y-directions. An unbiased bordered counting frame with inclusions and exclusions was superimposed on the images. To measure the z-axis travel, the microscope stage was fitted with a microcator. The upper and lower guard zones were used in order to avoid cutting artifacts that occurred at the upper and lower surfaces of the tissue sections. In each section, a guard zone defined the height of the dissector. Nuclei lying completely or partly within the counting frame and not touching the exclusion line were selected if they came into maximal focus within the next focal sampling plane. As a final step, the number of testicular cells was estimated using the following formula (Hasani et al., 2020):

$$\mathbf{N_v} = \frac{\sum \mathbf{Q}}{\sum \mathbf{P} \times \mathbf{h} \times \frac{\mathbf{a}}{\mathbf{f}}} \times \frac{\mathbf{t}}{\mathbf{BA}}$$

"ΣQ" represents the number of cells, "ΣP" represents the number of filed views, "h" represents the dissector height, "a/f" represents the frame area, "t" represents the real tissue section thickness, and "BA" represents the tissue section thickness. The total number of testicular cells was estimated by multiplying the numerical density by the total volume.

$$N_{total} = N_V \times V$$

Level of hormones

After deep anesthesia in mice, the blood samples were collected. Then, the blood samples were centrifuged at 6000 g for 5 minutes. Mice-specific ELISA kit has been utilized for measuring the levels of serum of testosterone, LH, and FSH in the blood (catalog No. CSB-E11162r).

Analysis of differential gene expression

All RNA samples were extracted. Then, for removing any contaminations caused by genomic DNA, DNase I (Roche; Basel: Switzerland) has been used. After that, for synthesizing cDNA a commercial kit (Fermentas: Lithuania) has been applied at 42 °C for sixty minutes in compliance with the directions defined in the Company's instructions. The next step, a real-time PCR (TaqMan) on the basis of the QuantiTect SYBR Green RT-PCR kit (Takara Bio Inc: Japan) has been utilized to quantify the genes' relative expression level. The PCR primers have been tested by the Primer-Blast tool that is available at the website, www.ncbi.nlm.nih.gov/tools/primer-blast.

Reactive oxygen species in testicular tissue

For isolating the testicular cells by adding trypsin EDTA and PBS, all tissue samples were centrifuged at 1200 RPM for 5 min at 4 ° C. After that, the DCFDA at a concentration of 20 µM in a 100 µl aliquot was added to the sample and stored in a 37° C incubator for 45 minutes in the dark. Finally, all samples were assessed by a flow cytometer with a wavelength of 495 nm (Hasani et al., 2020).

Glutathione disulfide content assessments

For determining the level of GSH in testis tissue samples, GPX assay kit (Zelbio GmbH) was used with 5U/ml sensitivity (5KU/L) according to the previous study (Hasani et al., 2020). All samples were assessed using a Shimadzu RF5000U fluorescence spectrophotometer set for 495 nm excitation and 530 nm emission wavelengths.

Statistical analysis

Our findings were analyzed by two-way repeated measures ANOVA test, using the SPSS software version 20 (IBM Corp., Armonk, NY, USA). P≤0.05 were considered significant.

RESULTS

Stereological Evaluations

Statistical analysis of stereological assay of histological study demonstrated precise results (Fig. 1).

Volume of testes and Length of seminiferous tubules

The total volume of testes at 33 weeks and the length of seminiferous tubules at 8, 12 and 33 weeks in the insulin-treated group were considerably higher compared to the control group (Fig. 2A and B). There was no significant different in the volume of testis at 8 and 12 weeks between study groups (Fig. 2B)

Total number of testicular cells

A significant increase was observed in the total number of spermatogonia, primary spermatocyte round spermatid, and Leydig cells in the testes of the insulin group at 33 weeks of treatment compared to the control group. However, the mean total number of Sertoli cells did not show a considerable change between groups (Fig. 3A-E).

DCF absorption and GPX activity

We also demonstrated that the absorption of DFC has been reduced in the insulin group after 33 weeks of treatment (P< 0.05) (Fig. 4). Besides, the activity of GPX in the insulin group was higher

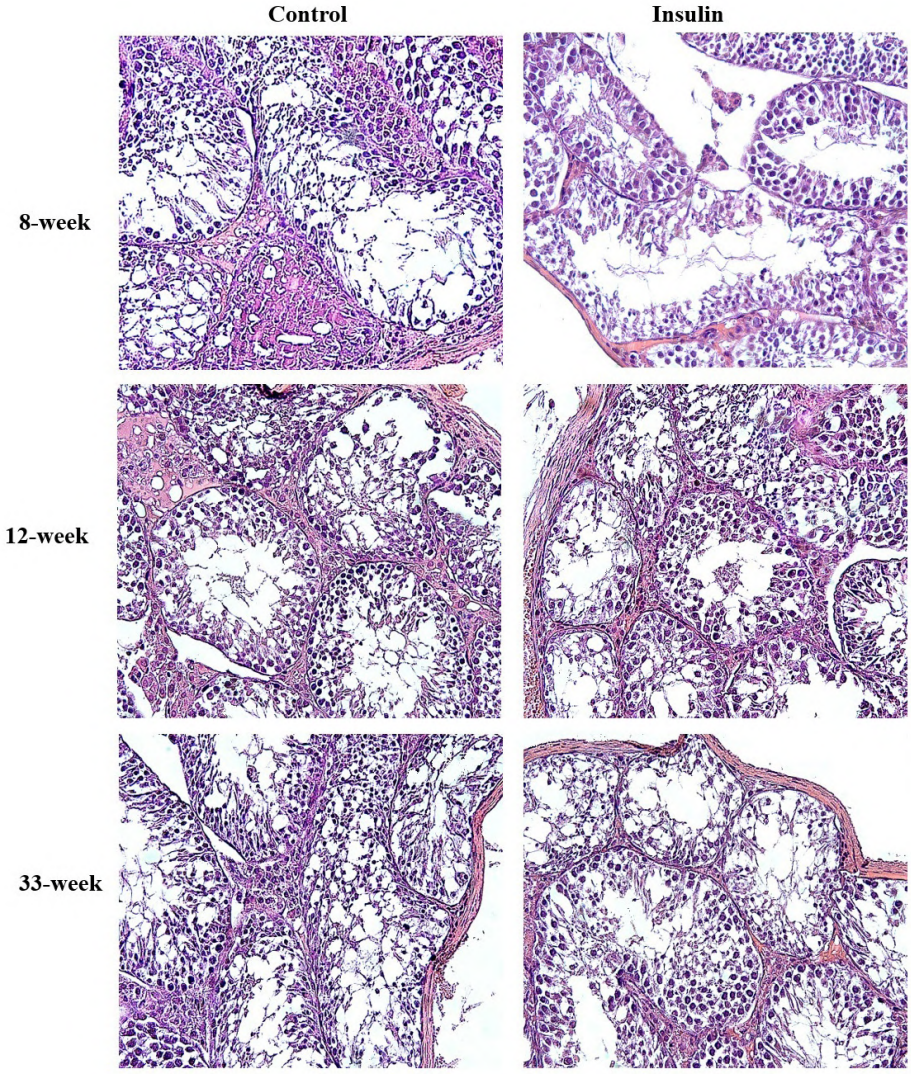

Fig. 1.- Photomicrograph of the testis stained with H&E (40x).

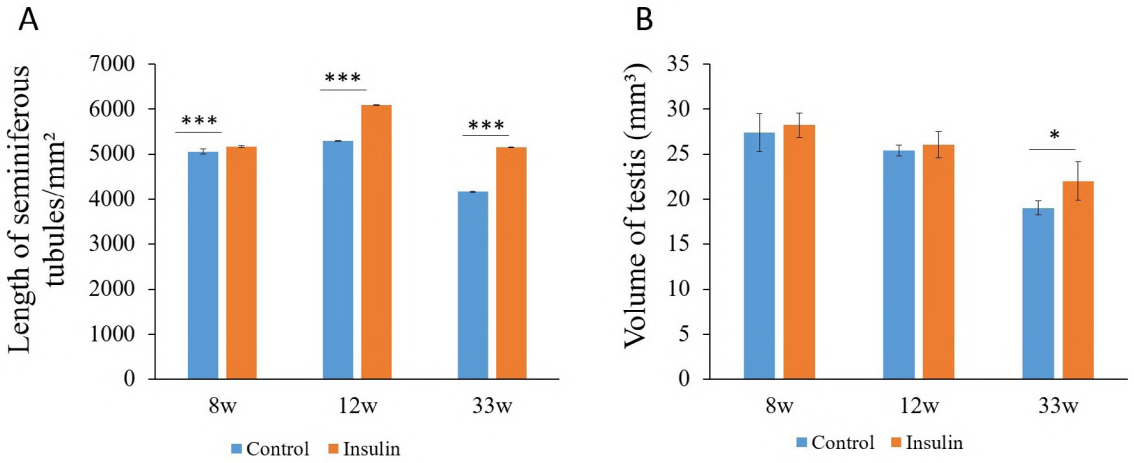

Fig. 2.- A: The effect of insulin on testis volume and seminiferous tubules length in mouse models of ageing. **B:** Mean±SD of the testis volume, seminiferous tubules length of testis in the study groups as compared by the ANOVA; (*P<0.05, **P<0.01, ***P<0.001).

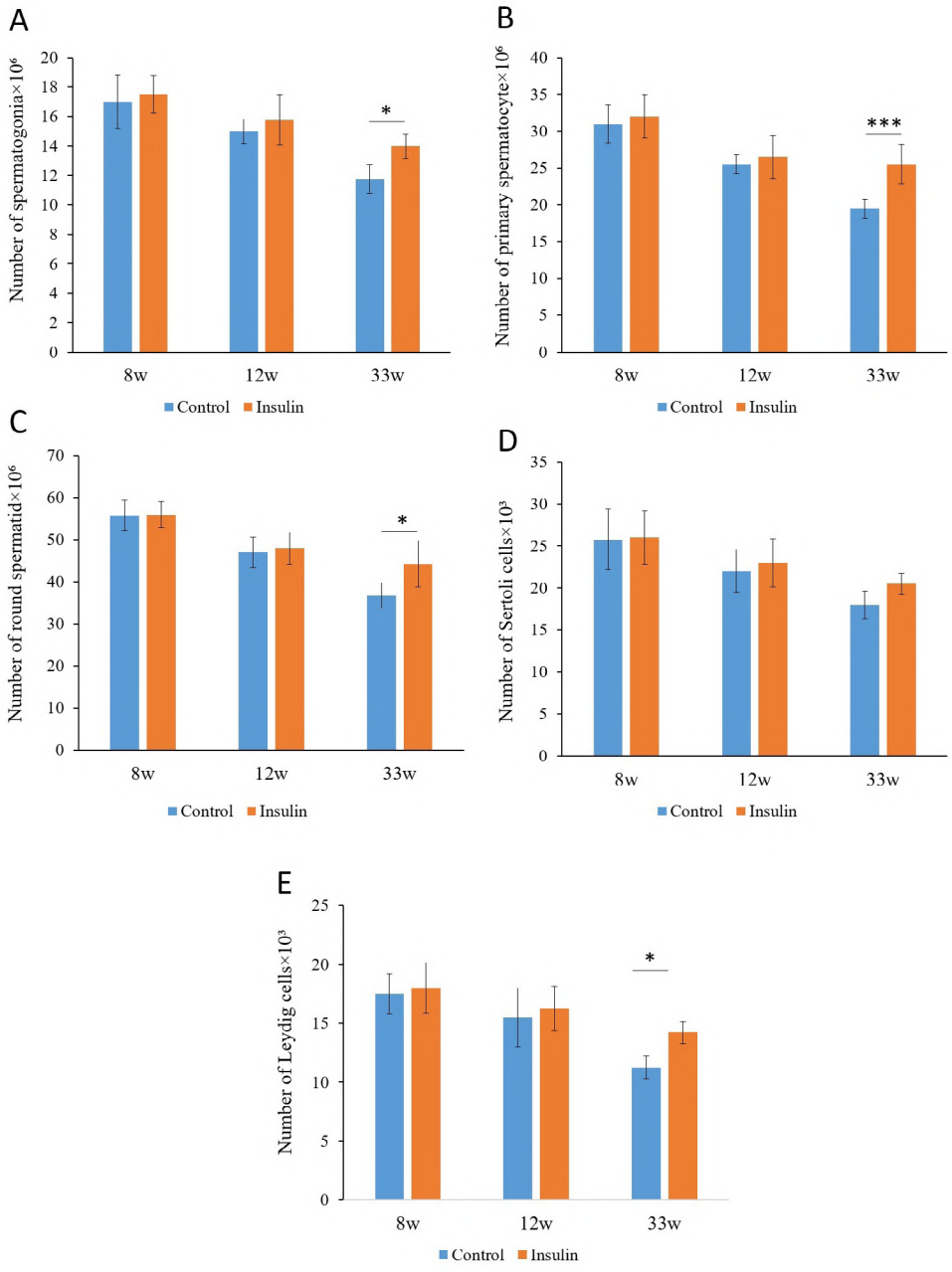

Fig. 3.- The effect of insulin on spermatogenic cells in mouse models of ageing. (*P<0.05, **P<0.01, ***P<0.001).

than the control group at 33 weeks of treatment (P<0.05) (Fig. 4A and B).

Gene expression

The levels of expression of caspase3 and Bax as apoptotic genes and Bcl2 as an inhibitor of apoptosis were assessed in the testes of mice in both insulin and control groups at 8, 12, and 33 weeks by using real-time qPCR. According to our findings, the level of gene expression of caspase3, Bax, and Bcl2 did not show any significant change between groups (Fig. 5A-C).

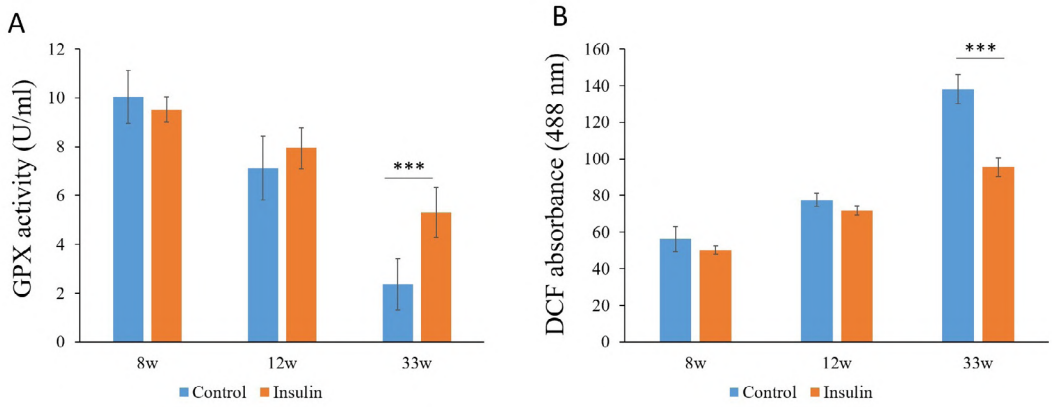

Fig. 4.- The effect of insulin on Reactive Oxygen Species (ROS) production and the level of glutathione (GSH) in mouse models of ageing. Mean±SD of the ROS production, GPX activity of testis in the study groups as compared by the ANOVA and LSD; (*P<0.05, **P<0.01, ***P<0.001).

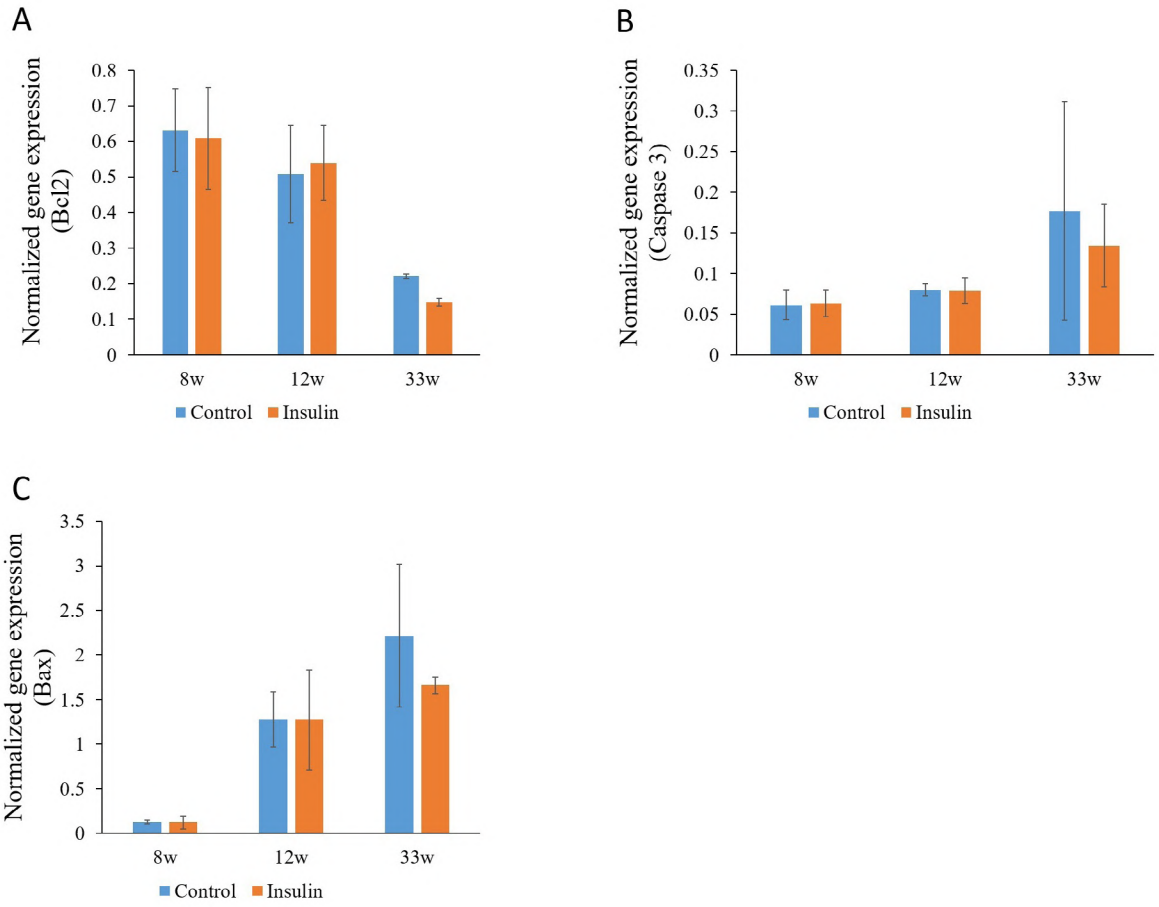

Fig. 5.- The effect of insulin on mRNA expression levels of Caspase3, Bax and Bcl2 in mouse models of ageing. **(A-C)** Mean±SD of the mRNA expression levels of Caspase3, Bax and Bcl2 of testis in the study groups as compared by the ANOVA; (**P<0.01 and ***P<0.001).

Hormone level of testosterone, LH, and FSH

According to the data, there were no any significant differences in the level of LH, FSH, and Testosterone hormones between groups (Fig. 6 A-C).

DISCUSSION

This study aimed to evaluate the beneficial effects of insulin on the testes of aging male rats according to a stereological study. We observed that insulin could significantly increase the spermatogenesis, the length of seminiferous tubules, and the activity of GPX.

In aging testes, various changes occur at the level of molecules and genes, which eventually lead to a reduction in steroidogenesis and spermatogenesis simultaneously (Matzkin et al., 2016). ROS play a key role during the aging of testes. In fact, ROS impair the normal function of cell organelles and stimulate lipid peroxidation (Matzkin et al., 2016; Amaral et al., 2013; Amaral et al., 2013). One of the important sources of the production of ROS is the energy transduction in the mitochondria (Valko et al., 2007). Mitochondria convert about 1 to 5 percent of oxygen consumption into the ROS. It has been proved that the integrity of mitochondrial function decreases during the process of ageing. Therefore, the production of ROS dramatically increases while mitochondria convert to the main source of free radicals (Abdollahifar et al., 2019; Valko et al., 2007). As a result, the balance between the generation of ROS and the antioxidant defense system is impaired (Amaral et al., 2013).

The testicles are prone to oxidative damage and lipid peroxidation. Because, besides other cells, Leydig cells and P450 enzymes involved in steroidogenesis produce ROS by the mitochondrial electron transport chain. However, the level of enzymatic and non-enzymatic antioxidants is enough in the normal and healthy male gonad to suppress the adverse effects of ROS (Brown-Borg et al., 2012; Chen et al., 2009). In aged testis, the level of oxidative stress and the production of free

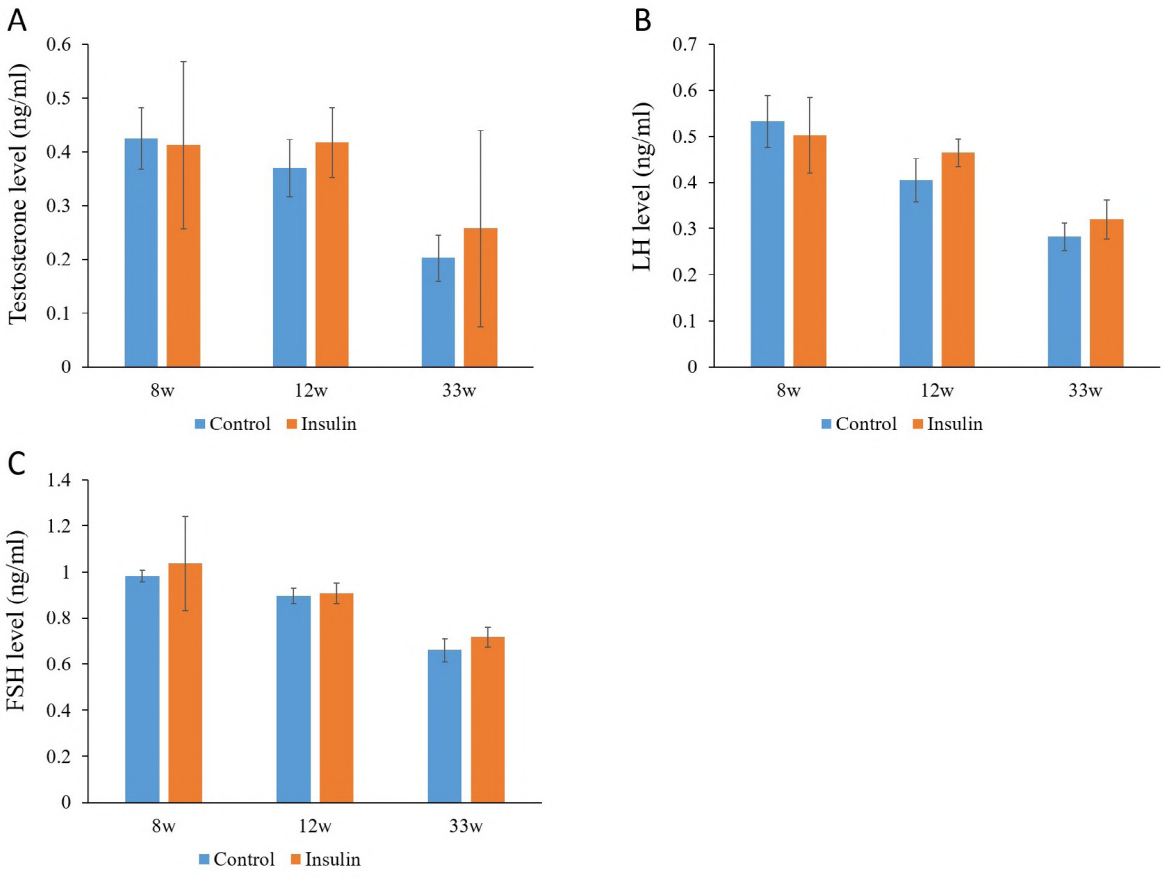

Fig. 6.- The effect of insulin on serum testosterone **(A)**, LH **(B)** and FSH **(C)** levels in mouse models of ageing. Mean±SD of the testosterone, LH and FSH level in the study groups as compared by the ANOVA (*P<0.05, **P<0.01, ***P<0.001).

radicals increase because of the alterations in the antioxidant enzymes such as glutathione-s-transferase (GST), an important enzyme that protects testes cells from oxidative stress, superoxide dismutase (SOD), and GPX (Salomon et al., 2013; Mueller et al., 1998; Cao et al., 2004). Overall, ROS and following oxidative stress significantly stimulate apoptosis in the testes of old men (Matzkin et al., 2016). In addition, with increasing age, the expression of pro-inflammatory cytokines including IL1β, IL6, and TNFα dramatically increase in the testicular mesenchymal stem cells (Matzkin et al., 2016).

We observed some histomorphological alterations in aged testes such as reduction of length of seminiferous tubules, reduction of Leydig cells, and a reduction in the volume and weight of them. These results were in line with previous studies (Johnson et al., 1984, 1986, 2015). Other studies also showed that aging has adverse effects on spermatogenesis, semen quality, and the thickness of the basal membrane of seminiferous tubules; besides, vacuolization, and multinucleation of spermatids are reported (Santiago et al., 2018; Miranda et al., 2018; Plas et al., 2000).

Insulin is a peptide hormone that has a fundamental role in maintaining blood glucose. This peptide is produced by the β cells of pancreatic islets of Langerhans (Wilcox et al., 2005). In addition to maintaining blood glucose, insulin has other vital cellular functions, such as transportation of amino acid, ion regulation, lipid metabolism, synthesis of glycogen, degradation and synthesis of protein, and DNA synthesis. Besides, insulin is effective in the cellular growth and differentiation (Cheatham et al., 1995).

Along with the effects of insulin, which are mentioned above, it is very important during spermatogenesis. It has been suggested that human sperms are able to release insulin. Besides, insulin receptors have also been found in sperms (Schoeller et al., 2012). According to our results, insulin can stimulate spermatogenesis and increase the number of testicular cells in the aged rats. Besides, insulin revitalize seminiferous tubules and the volume of the testis. It is suggested that insulin can regenerate the function of seminiferous tubules in culture medium by stimulating spermatogonial DNA synthesis in rats (Söder et al., 1992). In a study, Schoeller and et al. investigated the effect of insulin on the spermatogenesis of Akita mice with type-1 diabetes. They reported that these mice are infertile because of the absence of insulin due to *ins2* gene mutation in both testis and pancreas. The size and weight of the testes, and the level of serum testosterone were low in Akita mice. They showed that treatment with insulin in the Akita mice for 8 weeks led to regeneration of the seminiferous tubules and increase of volume and weight of testes. In addition, the total number of Sertoli cells, spermatogonia, and spermatid cells significantly increase (Schoeller et al., 2012).

Oxidative stress and apoptosis are common at aged testis. Mitochondria are well known as the main source of ROS damage. During aging of testis, the mitochondria of Leydig cells, as an endocrine cell, dramatically release free radicals (Barbutska et al., 2015). As a result, the proapoptotic genes such as Bax and caspase-3 significantly express (Dias et al., 2013). According to our results, insulin also has protective effects by increasing the activity of GPX as an antioxidant enzyme. It has been shown that a low level of insulin in diabetic patients causes inflammation and oxidative stress, while insulin therapy after 24 and 48 weeks enhanced the activity of enzymatic antioxidants such as GPX in them (Goyal et al., 2011).

We concluded that insulin improves spermatogenesis and restores morphological features of aged testes. In other words, insulin can alleviate the negative effects of aging on testicular function and increase the likelihood of fertility in older men.

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