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Original Article

Effects of Exendin-4 on Male Reproductive Parameters of D-Galactose Induced Aging Mouse Model

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Purpose: The purpose of this study was to evaluate the role of exendin-4 on reproductive alteration in a D-galactose-induced aging mouse model.

Materials and Methods: In this experimental study, 72 male Naval Medical Research Institute mice (20～25 g) were randomly divided into six groups: control, exendin-4 (1 nmol/kg), exendin-4 (10 nmol/kg), D-galactose (500 mg/kg), D-galactose+exendin-4 (1 nmol/kg), and D-galactose+exendin-4 (10 nmol/kg). The aging model animals were gavaged with D-galactose for six weeks, and exendin-4 was injected intraperitoneally in the last 10 days. At the end of treatment serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone levels were evaluated and the cauda epididymis and testis were removed to analyze the sperm count and testis morphology.

Results: The testis weight and volume decreased in the D-galactose group ($p<0.01$ and $p<0.05$) respectively. Exendin-4 (1, 10 nmol/kg) increased these parameters in the normal and aging mouse models. Serum LH and FSH levels increased and the sperm count decreased in the D-galactose group ($p<0.05$). Further, exendin-4 (1 nmol/kg) decreased LH and FSH levels and increased the serum testosterone level and sperm count in both normal and aging animals.

Conclusions: D-galactose can induce aging alternations in the male reproductive system such as decreased sperm count and increased serum LH and FSH levels through reactive oxygen species over production and reduced antioxidant enzyme activity. Further, co-administration of exendin-4 reduced reproductive complications of D-galactose in an aging mouse model.

Key Words: Aging; Galactose; Exenatide; Testosterone; Spermatozoa

INTRODUCTION

Aging is a complex biological process involving molecular, cellular and organic changes. The result of age-related physiological disorders is a great deal of disarray such as homeostatic imbalance and pathology [1]. Aging males acquire hypogonadism characteristics because of relatively low androgen serum levels and certain morphological changes in the testis, which are partially coupled with the loss of gonadal endocrine function and fertility

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[2]. Further, the salient effect of aging on reproductive organs has been mainly analyzed in terms of testis morphology and epididymis. It has been shown that germ cell apoptosis enhanced senescence associated with the fall in serum levels of androgen and increase in tissue oxidative damage [3]. Aging testicular dysfunction is related to the diminished testosterone production reflected by the elevated serum levels [2]. Free and bound testosterone levels decline with senescence in men, as shown by lower than normal plasma levels of bioavailable testosterone. The causative mechanism of this phenomenon may be derived from the hypothalamic- pituitary- testicular axis. Further, it has been shown that luteinizing hormone (LH) levels have a reverse relationship with serum testosterone levels in the case of aging [2].

One of the various theories on the process of aging is free radical/oxidative stress; most scientists approve of this theory [4]. It states that the age-related accumulation of free radicals and superoxide leads to damage of macromolecular components such as carbohydrate, lipids, proteins and nucleic acids. Moreover, the pathological disorders that develop result in cell senescence and organism aging [5]. The most common reactive oxygen species (ROS) that have potential effects on the reproductive system are superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) [6]. Further, mammalian spermatozoa have a large amount of phospholipids, sterols, and saturated and polyunsaturated fatty acids, which makes them susceptible to ROS-mediated damage [7]. Oxidative damage induced by free radicals is caused by pituitary dysfunction that decreases the serum testosterone level and leads to undesirable changes in the LH and follicle-stimulating hormone (FSH) levels [8].

Under normal circumstances, there is equivalence between ROS generation and antioxidant enzyme activity in the male reproductive system [7]. However, excessive production of free radicals occurs due to imbalance between their formation and scavenging activity [9]. The scavenging potential in the testes and the seminal fluid is maintained by an adequate level of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) [9]. D-galactose is a reducing sugar that at higher levels is converted into aldose and H_2O_2 due to the catalysis of galactose oxidase and results in the generation of O_2^- and ROS [10]. These changes are similar to the normal aging process and natural senescence model which demonstrate the decrease in antioxidant enzyme activity [11]. D-galactose plays a primary role in the pathogenesis of aging. The most important hypothesis that clarifies the pathogenesis mechanism of aging is free radical injuries that occur due to increased ROS and decreased SOD, GPX, and CAT activity [12].

Exendin-4 is a long-acting glucagon-like peptide-1; was approved by the Food and Drug Administration (USA) in 2005 [13,14]. This drug induces glucose-dependent insulin secretion and increases insulin-dependent glycogen synthesis and liver glucose uptake [15,16]. In some studies, exendin-4 has demonstrated a reducing effect on ROS and an enhancement effect on antioxidant defense in rats [17]. Therefore, given the knowledge of a D-galactose-induced aging model through excessive production of ROS and an imbalance between ROS formation and scavenging and the effects of exendin-4 on the reproductive system by improved antioxidant enzyme activity and reduced ROS generation, the present study was designed to evaluate the effects of this drug on gonadotropin hormones and the sperm count in D-galactose-induced aging mice.

MATERIALS AND METHODS

1. Animal preparation

In this experimental study, adult male Naval Medical Research Institute mice (age: 3 months) weighting 20 to 25 g, were obtained from the animal facility of Ahvaz Jundishapur University of Medical Sciences (AJUMS). Mice used in this study were treated in accordance with the AJUMS principles of guidelines on animal care and were housed in separate cages at 20°C to 24°C under a 12-hour darkness: 12-hour light cycle with free access to tap water and commercial chow.

2. Experimental design

After an initial acclimatization for one week, the animals were completely randomized into six groups (each group consisting of 12 animals): control (mice gavaged with drinking water for six weeks and injected intraperitoneally (IP) with phosphate buffered saline (Merck,

Darmstadt, Germany) in the last 10 days), exendin-4 (1 nmol/kg), exendin-4 (10 nmol/kg) (Sigma, St. Louis, MO, USA) (mice gavaged with drinking water for six weeks and injected IP with exendin-4 in the last 10 days) [18,19], D-galactose (500 mg/kg) (Merck) (aging mouse model: mice gavaged with D-galactose for six weeks) [20], D-galactose+exendin-4 (1 nmol/kg), D-galactose+exendin-4 (10 nmol/kg) (mice gavaged with D-galactose for six weeks and injected IP with exendin-4 in the last 10 days). Spermatogenesis in mice can be divided into 16 stages and is 229 hours (9.5 days) long. Therefore, to cover one complete spermatogenic cycle, exendin-4 was injected for 10 consecutive days [21].

The animals were sacrificed 24 hours after the last drug administration under deep anesthesia, and blood samples were collected by cardiac puncture and centrifuged at 3,000 rpm for 15 minutes. Then, serum samples were transferred to microtubes and maintained at -70^{o}C until the hormonal assays were performed.

3. Morphological analysis of the testis

After blood collection, the testes of all of the animals were immediately removed, and morphology factors such as weight, length, width and volume were analyzed. Further, testicular volume was calculated according to the following formula: volume $=(D^2/4\times\pi)\,L\times K$, where L denotes the length, and D, the width, Further, $K=0.9$ and $\pi=3.14$ [22].

4. Hormonal assessment

The serum sample concentrations of LH, FSH, and testosterone were measured by using enzyme-linked immunosorbent assay as described in the instructions provided with the assay kits (DRG Instruments GmbH, Marburg, Germany). The sensitivity of hormone detection per assay tube for testosterone, LH, and FSH was 0.083 ng/mL, 1.27 mIU/mL, 0.856 mIU/mL respectively.

5. Sperm count assessment

For the sperm count, the cauda epididymis of all animals was dissected and teased in 3 mL of normal saline 0.9% in the petri dish. Then one drop of petri dish solution containing the sperm in the normal saline solution was transferred into each chamber of a Neubauer hemocytometer (HBG, Hamburg, Germany) (Tiefe depth profondeur: 0.100 mm; area: 0.0025 mm^2), and the sperm number was monitored and manually counted by light microscopy (Olympus Light Microscope, Tokyo, Japan) in white blood cell grids. Ultimately, data were expressed as the number of sperm per milliliter [23].

6. Statistical analysis

The results were statistically analyzed using the SPSS software version 16 (SPSS Inc., Chicago, IL, USA) with one-way Analysis of variance and *post hoc* least significant difference tests. Further, data were represented as mean±standard error and differences were considered statistically significant at $p<0.05$.

RESULTS

1. Effect of exendin-4 on testis morphology of normal and aging mouse models

As the results show, there was a significant decline in the testis weight ($p<0.01$), width ($p<0.01$) and volume ($p<0.05$) in the aging mouse model followed by D-galactose administration. Exendin-4 (1 nmol/kg) injection in normal mice showed a significant increase in testis weight, length, width and volume ($p<0.001$) when compared with the D-galactose group. A similar observation was made for this dose of the drug on testis length ($p<0.001$), width ($p<0.01$) and volume ($p<0.001$) in comparison with the control group. Administration of exendin-4 (10 nmol/kg) in normal mice demonstrated a significant increase in testis weight ($p<0.05$), length ($p<0.01$), width ($p<0.001$) and volume ($p<0.001$) as compared to the D-galactose group. Moreover, this increasing effect was observed for testis length, width, and volume ($p<0.05$) when compared with the control group. The morphologic factors of testis such as weight ($p<0.05$), length, width and volume ($p<0.001$) increased significantly in the D-galactose+exendin-4 (10 nmol/kg) group compared with the D-galactose group. Further, the results of the D-galactose+exendin-4 (10 nmol/kg) group showed this increasing effect in testis width ($p<0.01$), length ($p<0.001$) and volume ($p<0.001$) in comparison with the control group. Testis weight in the D-galactose+exendin-4 (1 nmol/kg) group decreased as compared to the

Table 1. Effect of exendin-4 on testis morphology of normal and aging mouse models (n=12)

Groups	Testis weight (mg)	Testis length (mm)	Testis width (mm)	Testis volume (mm^3)
Control	58.25±2.88	6.75±0.25	4.37±0.18	92.99±9.71
Exendin-4 (1 nmol/kg)	62.64±2.07***	8.55±0.17$^{\#\#\#}$***	5.33±0.16$^{\#\#}$***	174.42±13.83$^{\#\#\#}$***
Exendin-4 (10 nmol/kg)	52.78±3.12*	7.80±0.37$^{\#}$**	5.10±0.15$^{\#}$***	137.76±6.60$^{\#}$***
D-galactose	42.97±2.37$^{\#\#}$	6.40±0.33	3.55±0.28$^{\#\#}$	63.60±8.21$^{\#}$
D-galactose+exendin-4 (1 nmol/kg)	44.02±3.4$^{\#}$	7.50±0.32$^{\#}$**	5.00±0.18$^{\#}$***	136.44±15.71$^{\#}$***
D-galactose+exendin-4 (10 nmol/kg)	52.15±2.65*	8.75±0.45$^{\#\#\#}$***	5.37±0.26$^{\#\#}$***	186.69±18.23$^{\#\#\#}$***

Values are presented as mean±standard error.
*p<0.05, **p<0.01, ***p<0.001 compared with the D-galactose group. $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared with the control group.

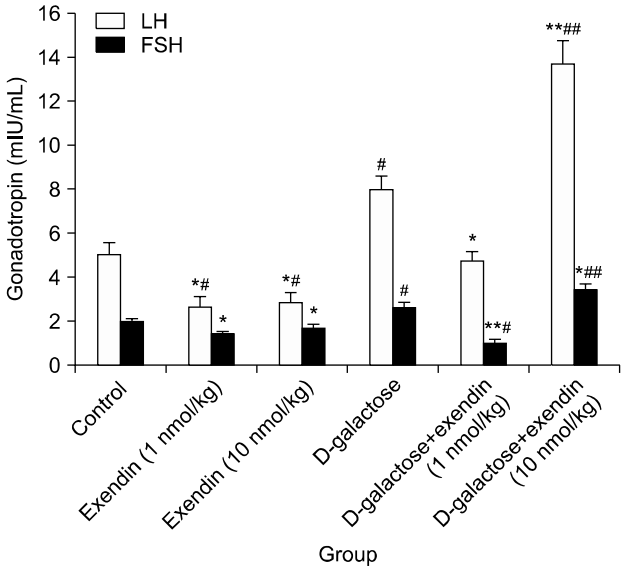

Fig. 1. Effect of exendin-4 on serum LH and FSH levels of normal and aging mouse models. Values are presented as mean± standard error; n=12. LH: luteinizing hormone, FSH: follicle-stimulating hormone. *p<0.05, **p<0.01 compared with the D-galactose group, $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared with the control group.

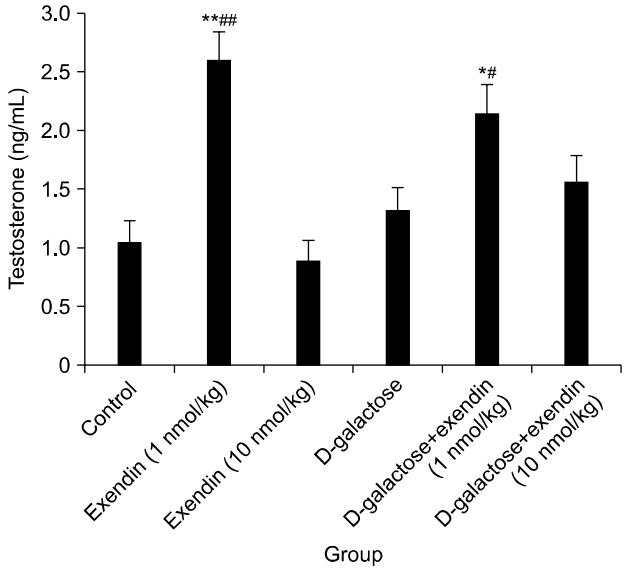

Fig. 2. Effect of exendin-4 on serum testosterone levels of normal and aging mouse models. Values are presented as mean± standard error; n=12. *p<0.05, **p<0.01 compared with the D-galactose group, $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared with the control group.

control group, but there were no significant differences between the D-galactose group and this group. As the data show, testis length (p<0.01), width (p<0.001) and volume (p<0.001) increased in the D-galactose+exendin-4 (1 nmol/kg) group as compared to the D-galactose and control (p<0.05) groups (Table 1).

2. Effect of exendin-4 on serum luteinizing hormone, follicle-stimulating hormone, and testosterone levels of normal and aging mouse model

The LH levels obtained in this study showed a significant increase in the D-galactose group compare with the control group (p<0.05), and the administration of exendin-4 (1, 10 nmol/kg) in normal mice demonstrated a significant decrease when compared to the control and D-galactose groups (p<0.05). The same effect was observed in the D-galactose+exendin-4 (1 nmol/kg) group as compared to the D-galactose group (p<0.05) but this hormone was increased in the D-galactose+exendin-4 (10 nmol/kg) (p<0.01) group compared with the normal and aging animals models (Fig. 1).

As the results of FSH show, this hormone increased in the D-galactose group compared with the control group (p

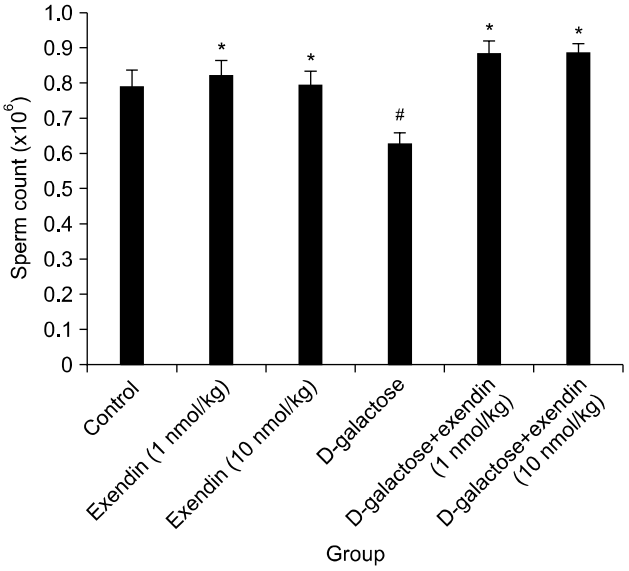

Fig. 3. Effect of exendin-4 on the sperm count of normal and aging mouse models. Values are presented as mean±standard error; n=12. *$p<0.05$ compared with the D-galactose group, #$p<0.05$ compared with the control group.

<0.05). The serum level of FSH in the exendin-4 (1, 10 nmol/kg) group decreased significantly in comparison with the D-galactose group ($p<0.05$). Further, a similar effect was observed in the D-galactose+exendin-4 (1 nmol/kg) group as compared to the D-galactose ($p<0.01$) and control ($p<0.05$) groups. The data of the aging model animals' treatment with exendin-4 (10 nmol/kg) illustrated a significant increase in FSH as compared to the D-galactose ($p<0.05$) and control ($p<0.01$) groups (Fig. 1).

After injection of exendin-4 (1 nmol/kg) in normal and aging mouse models, serum level of testosterone increased significantly in comparison with the D-galactose ($p<0.01$, $p<0.05$) and control ($p<0.01$, $p<0.05$) groups respectively (Fig. 2).

3. Effect of exendin-4 on sperm count of normal and aging mouse models

The results of this study demonstrated a significant decline in sperm count upon D-galactose administration. Moreover, exendin-4 injection increased the sperm count ($p<0.05$) in normal and aging mouse models (Fig. 3).

DISCUSSION

The effect of aging on the reproductive system is associated with regressed reproductive organs such as the testis [9]. The findings of the present study showed that D-galactose decreased testis weight; this was accompanied with a decrease in testis volume after induced aging in mice. Further, after administration of exendin-4, the testis weight and volume increased in normal and aging animal models. Reproductive failure in males is characterized by various changes in the testes that lead to a decrease in spermatogenesis and steroidogenesis. Moreover, reproductive aging is related to an increase in oxidative stress and overproduction of ROS [24]. In some studies, D-galactose has been used to induce oxidative stress and aging in mice by the generation of oxygen-derived free radicals and a decrease in antioxidant enzyme activity [25]. Patil et al [7,26] reported that D-galactose administration decreased the testis weight via induced oxidative stress and overproduction of ROS in an aging mouse model. Therefore, it can be suggested that; D-galactose decrease testis weight and volume through the above mentioned mechanism. The study by Peltola et al [27] on antioxidant enzyme activity in the maturing rat testis demonstrated that this activity is significantly greater in young animals than in aged animals. Moreover, animals treated with antioxidants experienced an increase in testicular weight [28]. Gezginci-Oktayoglu et al [29] indicated that the administration of exendin-4 increases antioxidant enzyme activity; Vaghasiya et al [17] showed similar effects of this drug on the generation of ROS and antioxidant defense in rats. Therefore, it can be suggested that the exendin-4 injection in this study increased testicular weight and volume by decreasing free radicals and increasing antioxidant enzyme activity.

The prevalence of serum testosterone deficiency increases with age. Some studies have shown that there is a slow decline in serum testosterone levels in normal aging animals and the absence of disease [30,31]. Further, serum LH and FSH levels rise modestly or do not change and serum testosterone level decrease in this situation [32]. The mechanism of the age-related decrease in testosterone has been a subject of investigation. However, some researchers have reported that one of the important intercurrent mechanisms in this process is the free radical/antioxidant mechanism. Further, there are age-related increases in ROS production in geriatric patients [33]. Some

findings in Leydig cells suggest that an imbalance between oxidative stress and antioxidant enzyme activity may play an important role in age-related deficits in steroid hormone formation [34]. Thus, the steroidogenic capacity of Leydig cells declined by about 50% with aging. There is some evidence that ROS; derived from the mitochondrial electron transport chain, affects on cyclic adenosine monophosphate production and cholesterol transport into the mitochondria by altering Leydig cell aging. These modifications result in relative resistance to LH signaling and reduced testosterone levels characterized by aging Leydig cells [35]. Therefore, the results of present study demonstrated that D-galactose, followed by induced aging, could increase serum LH and FSH levels due to the stimulation of the pituitary gland as a compensatory response. Further, exendin-4 (1 nmol/kg) increased the testosterone level and decreased the LH and FSH levels in normal and aging animal models, but a high (10 nmol/kg) dose of this drug did not show any treatment effect on them. Hence, it can be concluded that exendin-4 (10 nmol/kg) has no anti-infertility effects through testicular functioning while exendin-4 (1 nmol/kg) leads to testicular treatment alterations in aging mice. Further, D-galactose can affect Leydig cells in an aging mouse model by increasing ROS generation and decreasing antioxidant enzyme activity, ultimately leading to relative insensitivity to LH signaling and no increase in testosterone levels. On the other hand, according to the results of exendin-4 (1 nmol/kg), we can conclude, that dosagee of drug the recovered the effects of D-galactose on gonadal hormones due to improved antioxidant defense and excessive free radical production in Leydig cells.

Aging causes a number of alternations in the male reproductive system, such as testicular damage, decrease in sperm count, and increase in serum FSH levels on the basis of the free radical theory and because of excessive production of ROS [36]. Suzuki and Sofikitis [37] proved that the administration of antioxidants enhanced testicular functions and epididymis sperm in rats. On the basis of the results of the present study, it can be concluded that D-galactose decreases sperm count and enhances FSH levels via excessive production of free radicals in an aging mouse model. It can be concluded that co-administration of exendin-4 improves these effects by increasing the antioxidant enzyme activity. In another study Patil et al [38] revealed that D-galactose administration causes structural deformities in the testis and decreases the sperm count. Further, utilization of a plant with antioxidant properties can increase sperm count by protecting male reproductive organs from ROS overproduction. Therefore, we can conclude that the results of D-galactose administration obtained in and the antioxidant properties of exendin-4 observed in this study agree with the finding of previous studies, but further research is necessary to explore the main mechanisms of exendin-4 in the reproductive system.

CONCLUSIONS

In conclusion, this study revealed that D-galactose could induce aging alterations in the male reproductive system, such as decline in sperm count, increase in serum LH and FSH levels, and no change in the testosterone level, by the overproduction of free radicals and a decrease in the antioxidant enzyme activity. Further, the co-administration of exendin-4 (1 nmol/kg) in the aging mouse model demonstrated an improvement in the D-galactose-induced reproductive complications. Therefore, it seems that a low dose of exendin-4 may be useful in treating age-induced infertility problems through the hypothalamic- pituitary- testicular axis.

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