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ORIGINAL ARTICLE

Investigation of the Protective Effects of Amlodipine on the Structure and Function of Testicular Tissue following Experimental Unilateral Cryptorchidism in Rats

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Abstract

Objective- Cryptorchidism, common birth defect of the male genital tract, is one of the causes of fertility problems. The elevation of tissue temperature following of cryptorchidism could induce oxidative stress which influences the cellular and tissue degeneration. Amlodipine is a third-generation of calcium channel blockers which has antioxidant activity. The aim of this study was to evaluate the protective effects of amlodipine on testicular tissue alterations in an animal model of cryptorchidism.
Design- Experimental study
Animals- Thirty adult male Sprague-Dawley rats weighing 200-220 g
Procedure- Experimental cryptorchidism was induced in adult rats. Amlodipine (10 mg/kg b.w.) was administrated orally for two and four consecutive weeks. The experimental groups consisted of non-treated cryptorchidism (n=10) and treated cryptorchidism (n=10) groups. Testicular tissue samples were collected on days 14 and 28 following of cryptorchidism form non-treated and treated groups. Histopathological and morphometrical studies with the evaluation of microscopic indices of spermatogenesis were prepared on tissue samples.
Results- Tubular atrophy with germinal epithelium disarrangement was observed in cryptorchidism groups. These changes were reduced dose-dependently in treated animals. The mean of Sertoli cells was reduced significantly (p=0.025) in four weeks non-treated and the mean of germ cells lineage was reduced significantly (p<0.0001) in four weeks non-treated and two weeks treated cryptorchidism groups compared to the control group. Similarly, all microscopic indices of spermatogenesis were reduced following the induction of cryptorchidism. These alterations were reduced time-dependently in amlodipine treated groups.
Conclusion and clinical relevance- The results of this study revealed that, the administration of amlodipine as an antioxidant agent, time-dependently could be effective on the reduction of cellular and tissue damages of testicular tissue induced by cryptorchidism. It seems some parts of these protective effects may be done through its activity as calcium blocker which declines apoptotic processes by reduction of cytoplasmic calcium levels.

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1. Introduction

Cryptorchidism is a defect in descending of testes into the scrotal sac. This condition is the most common birth defect of the male genital tract and is one of the causes of fertility problems in humans and animals.[1] Cryptorchidism is occurring in all domestic mammals but observed more in horses, some dogs' breeds and observed less commonly in bucks, rams and rarely in cats.[2, 3] In three to eight percent of infertile men and 20 percent of men with azoospermia the cryptorchidism has been reported.[4] Many studies demonstrated the effects of cryptorchidism on the structure of testicular tissue in laboratory animals.[5,6,7] It has been reported that, the elevation of tissue temperature could be induce an oxidative stress and production of reactive oxygen species (ROS) which influence the cellular and tissue degeneration.[8]

Amlodipine is a third-generation of calcium channel blockers with antioxidant and vascular activity.[9] Various effects such as the prevention of lipids peroxidation, the induction of nitric oxide (NO) production and the maintenance of superoxide dismutase (SOD) activity has been report for amlodipine.[10] Moreover, amlodipine could be effective in decrement of oxidative stress through increasing of the endothelial nitric oxide synthase 3 (eNOS) activity.[11]

According to the importance of oxidative stress in testicular tissue alterations related to cryptorchidism and the effects of amlodipine in decrement of oxidative stress in this study, the protective effects of the administration of amlodipine on testicular tissue were evaluated following the experimental induction of cryptorchidism in rat.

2. Materials and Methods

Animals' procedures and experimental design

All animals used for testing were housed under a 12 hour light-dark cycle with room temperature of 23-25° C and had access to food and water *ad libitum*. All animal procedures used in the present study were approved by the University of Tabriz standards for humane care and use of laboratory animals, in accordance with the Ethical Research Committee of the Ministry of Health and Medical Education of Iran (adopted in April 17, 2006) based on the Helsinki Protocol (Helsinki, Finland, 1975) with ethical number 9983/43/D. Thirty adult Wistar rat were randomly divided into a control group and two experimental groups (n = 10) as described below:

Group 2: experimental unilateral cryptorchidism. This group consisted of five animals which euthanized two weeks after induction of cryptorchidism (cryptorchidism 2W) and five animals which euthanized four weeks after induction of cryptorchidism (cryptorchidism 4W);

Group 3: experimental unilateral cryptorchidism + amlodipine (10 mg/kg BW oral gavage). In this group, after induction of cryptorchidism, the amlodipine besylate (5 mg tablets, NORVASC®, Pfizer) was administrated for two weeks (treatment 2W) or four weeks (treatment 4W) and the animals were euthanized following the end of administration of amlodipine.[12]

Induction of cryptorchidism

The animals were anaesthetized with xylazine hydrochloride (10 mg/kg IP) and ketamine hydrochloride (100 mg/kg IP). The skin of the scrotal region was shaved and prepared by povidone–iodine solution. The gubernaculum of the left testis was separated following of the incision of left inguinoscrotal region. The released testis was pushed back into the abdominal cavity through the internal inguinal ring. Subsequently, the external inguinal ring was closed by 4/0 nonabsorbable suture material (Nylon; Supa Co., Tehran, Iran).[13]

Tissue preparation and morphometric analysis

At the end of study, the animals were euthanized through sodium thiopental (100 mg/kg IP) and the left testis was immediately fixed in 10% buffered formaldehyde solution.

Paraffin embedded samples stained with hematoxylin and eosin (H&E) method. For morphometric assessment of seminiferous tubules, the slides were studied at 400× magnification. The analyses were performed from images obtained and digitalized using Dino-Eye Eyepiece Camera AM7023B (Dino-Lite Digital Microscope). The images were processed by Dino-Lite image analysis system software. To get extra precise results, only the seminiferous tubules (STs) that were sectioned transversely were studied and the shortest diameter of seminiferous tubules was considered for measurement.

Tubular differentiation index (TDI), repopulation index (RI) and spermiogenesis index (SPI) were used for estimation of spermatogenesis in testicular tissue. To determine the tubular differentiation index, the number of seminiferous tubules with more than three layers of germinal cells derived from type A spermatogonia was calculated. To find out the repopulation and spermiogenesis indices, the ratio of active spermatogonia to the inactive cells and the ratio of seminiferous tubules with spermatozoids to the empty tubules, were calculated, respectively.[14]

Statistical analysis

The results were analyzed using the GraphPad PRISM® software (version 5.04, GraphPad Software, Inc. USA). All data were reported as mean ± standard deviation (SD). The comparison of means between experimental groups was evaluated by using the one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Differences were considered to be statistically significant if $p< 0.05$.

3. Results

Testicular histomorphometry

Induction of cryptorchidism led to increase of capsular thickness. This increase was observed in more degrees four weeks after cryptorchidism. However, these changes were not significant ($p>0.05$). Accordingly, the mean of capsular thickness was reduced following the administration of amlodipine in comparison to cryptorchidism groups. The mean of tubular diameter was reduced in all experimental groups compared to control group. This reduction was significant in three experimental groups in comparison to control group ($p<0.05$). The most reduction of tubular diameter was observed four weeks after induction of cryptorchidism. In this regard, treatment with amlodipine for four weeks led to increase of tubular diameter. The mean of germinal epithelium height was reduced in all experimental groups in comparison to control group (Table 1). Accordingly, the lowest degree of this index was observed in (cryptorchidism 4W) and (treatment 2W) groups. Four weeks treatment of animals with amlodipine led to increase of the mean of germinal epithelium height.

Cellular population of seminiferous tubules

Table 2 shows the mean of cellular population of the seminiferous tubules. In this regard, the mean of Sertoli cells was decreased in experimental groups in comparison to control group. This decrement was significant after four weeks of cryptorchidism ($p<0.05$). The administration of amlodipine led to increase of Sertoli cells population time dependently. The mean of population of spermatogonia was reduced in experimental groups compared to control group. This reduction was significant in (cryptorchidism 4W) and (treatment 2W) groups in comparison to control group ($p<0.05$). Moreover, the administration of amlodipine led to increase of the mean of spermatogonia time dependently. The changes in the mean of the population of spermatocytes were similar to the changes mentioned for spermatogonia cells (Table 2).

Microscopic indices of spermatogenesis

Figure 1 shows the mean of three microscopic indices of spermatogenesis (TDI, SPI and RI) in all groups. Induction of cryptorchidism led to decrease of TDI in experimental groups in comparison to control group (Figure 1A). This

Table 1. Histomorphometry of testicular tissue.

	Capsular thickness (µm)	Tubular diameter (µm)	Germinal ept. height (µm)
Control	45.22±5.06	260.2±15.46	79.33±16.88
Cryptorchidism 2W	48.39±9.49	213.2±25.87$^{\alpha**}$	64.11±8.91
Cryptorchidism 4W	52.95±9.14	197.6±17.30$^{\alpha***}$	58.74±15.94$^{\alpha*}$
Cryptorchidism 2W + amlodipine	44.58±8.78	214.1±34.65$^{\alpha**}$	57.81±8.59$^{\alpha**}$
Cryptorchidism 4W + amlodipine	46.33±8.33	229.2±27.74	73.98±15.85
***p*-value**	0.272	<0.0001	0.002

$^{\alpha}$ Significant different in comparison to control group. The number of stars indicates the degree of significant difference.

Table 2. Cellular population of seminiferous tubules

	Sertoli cells (mean/20 tubules)	Spermatogonia (mean/20 tubules)	Spermatocyte (mean/20 tubules)
Control	17.84±1.85	61.80±7.64	92.95±10.99
Cryptorchidism 2W	15.48±2.66	55.80±11.90 $^{\beta***}$	77.07±10.61
Cryptorchidism 4W	13.97±2.34$^{\alpha**}$	32.10±9.58$^{\alpha***}$	64.22±15.32$^{\alpha***}$
Cryptorchidism 2W + amlodipine	15.63±2.57	43.60±10.92$^{\alpha**}$	73.27±20.46$^{\alpha*}$
Cryptorchidism 4W + amlodipine	16.00±2.91	47.70±14.60$^{£*}$	83.66±13.91$^{\beta*}$
***p*-value**	0.025	<0.0001	<0.001

Sertoli cells: $^{\alpha}$ Significant different in comparison to control group. **Spermatogonia:** $^{\alpha}$ Significant different in comparison to control group; $^{\beta}$ Significant different in comparison to (Cryptorchidism 4W.) group; $^{£}$ Significant different in comparison to (Cryptorchidism 4W.) group. **Spermatocyte:** $^{\alpha}$ Significant different in comparison to control group; $^{\beta}$ Significant different in comparison to (Cryptorchidism 4W.) group. The number of stars indicates the degree of significant difference.

reduction was observed significantly following of cryptorchidism and also two weeks administration of amlodipine ($p<0.05$). The administration of amlodipine time dependently led to increase of this index compared to cryptorchidism groups.

A significant reduction of spermiogenesis index was observed in all experimental groups compared to control group ($p<0.05$). Moreover, this index was increased time dependently following of the administration of amlodipine in comparison to cryptorchidism groups (Figure 1B).

The mean of repopulation index was decreased in experimental groups in comparison to control group (Figure 1C). Accordingly, the induction of cryptorchidism led to decrease of this index in time dependent manner. Moreover, the administration of amlodipine led to increase of this index compared to non-treated cryptorchidism groups.

Histopathology of testicular tissue

The histopathological evaluation of testicular tissue showed a various changes in testicular tissue of experimental groups compared to control group. Accordingly, induction of experimental cryptorchidism led to disarrangement of cellular architecture in seminiferous tubules and the population of germ cells was reduced. In this regard, tubular atrophy, nuclear shrinkage and tubular vacuolation were the notable changes observed in the seminiferous tubules. These structural alterations were observed in more degrees four weeks after induction of cryptorchidism. The above mentioned histologic changes were reduced following the administration of amlodipine in time dependent manner (Figure 2).

4. Discussion

Cryptorchidism is a condition of alteration in normal descending of testes into scrotum.[15] It is the most common abnormality of the male reproductive system and present in more than 5% of newborn males.[16,17] Experimental induction of cryptorchidism leads to alteration and disruption of spermatogenesis due to higher temperature of

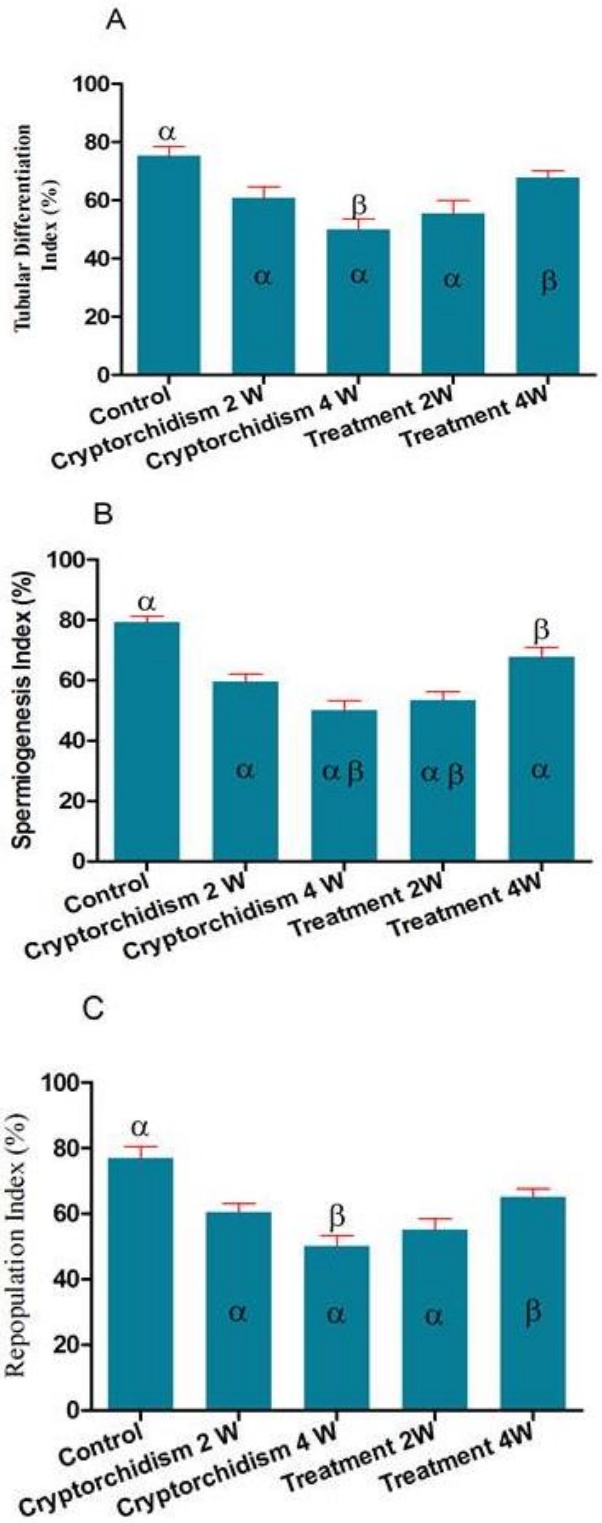

Figure 1. The mean of microscopic indices of spermatogenesis. Similar symbol letters (α, β) indicates significant difference between experimental groups. **TDI:** ᵅ Significant difference compared to control group; ᵝ Significant difference compared to Cryptorchidism 4W. **SPI:** ᵅ Significant difference compared to control group; ᵝ Significant difference compared to Treatment 4W. RI: ᵅ Significant difference compared to control group; ᵝ Significant difference compared to Cryptorchidism 4W.

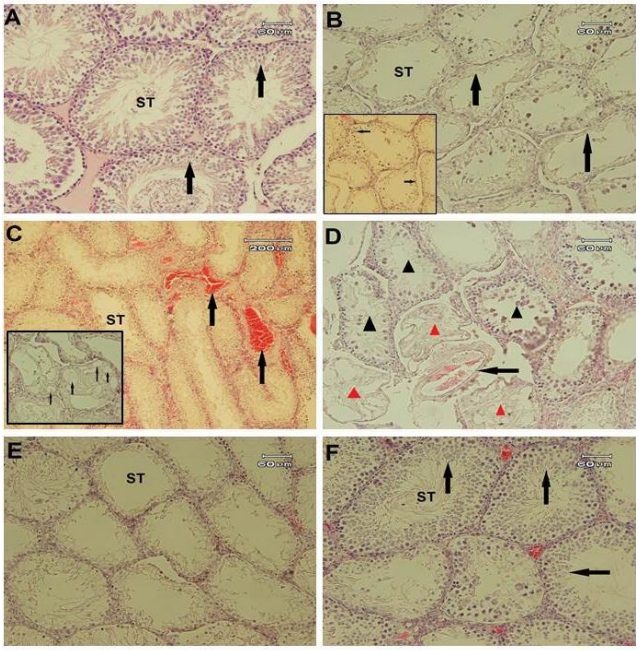

Figure 2. Cross section of testicular tissue in experimental groups. **A:** Seminiferous tubules (ST) with normal cellular architecture. All types of germ cells with spermatozoa are observed within the lumen of seminiferous tubules (black arrows); **B:** Testicular tissue two weeks following induction of cryptorchidism. Tubular atrophy with reduction of cellular population and disarrangement of germinal epithelium (black arrows) is observed. Nuclear shrinkage (small square) also observed in tubules; **C:** Vascular hyperemia (black arrows) in testicular tissue after four weeks of cryptorchidism. Tubular vacuolation (small square) was observed; **D:** Testicular tissue following four weeks of cryptorchidism. Hyperemia (black arrow) and tubular atrophy with increase of interstitial connective tissue are observed. Reduction of germinal epithelium population (black and red arrow heads) and the absence of germ cells (red arrow heads) are prominent changes of seminiferous tubules; **E:** Testicular tissue in amlodipine treated group following two weeks of cryptorchidism. No significant changes are observed in seminiferous tubules; **F:** Testicular tissue four weeks after cryptorchidism and treatment with amlodipine. Tubular atrophy is reduced and most of seminiferous tubules are seen with normal structure (black arrows), (H&E staining. Magnification: C: ×10; A, B, D, E & F: ×20).

abdominal cavity compare to scrotum.[8,14] Increase of testicular temperature above normal levels may influence the normal spermatogenesis through the induction of temperature elevation which can initiate the oxidative stress in seminiferous tubules.[18,19] Heat induced production of reactive oxygen species (ROS) in testicular tissue could stimulate the lipid peroxidation which affecting the spermatogonia.[20,21] Consequently, the elimination of ROS

production reported as an important factor in treatment of cryptorchidism.[19] In this regard, it seems that the administration of antioxidants could have a protective effect on testicular tissue alterations following the heat induced oxidative stress of testicular tissue in cryptorchidism.

Amlodipine is a third-generation of dihydropyridine-type calcium channels blockers which has an antioxidant activity.[22] Oxidative stress and ROS generation are important factors in abnormal fertility.[23] In this study, the results of testicular histomorphometry revealed numerous tissue alterations such as decrease in cellular population and structural changes in seminiferous tubules following the induction of cryptorchidism. In this regard, decrement in diameter of STs was accompanied with depletion in the height of germinal epithelium which causes the atrophy of seminiferous tubules. These histological observations, illustrate the depressed cellular activity of spermatogenic cells in cryptorchidism conditions. Programmed cell death (apoptosis) is a normal regulatory process in testicular tissue and occurs in various phases in germinal epithelium.[24] Cellular stress such as sever DNA damage induced by exogenous factors can activate the initiation of apoptosis or repair pathways in cell.[25] Through an oxidative stress which leads to production of high levels of reactive oxygen species, the normal cellular physiological pathways disrupted which cause cell death. Oxidative stress causes influx of Ca^{2+} from extracellular environment into the cytoplasm. The increase of cytoplasmic calcium concentration related to oxidative stress; influence the influx of calcium into mitochondria. This factor disrupts the normal cell metabolism mediated by mitochondria and leading to cell death.[26,27]

In this study, the population of germ cells was reduced in all experimental groups. Moreover, the results showed that the most alteration in cell population was occur in spermatocytes. According to the results of this study, it seems that, the reduction of spermatocytes has occurred following of the reduction of spermatogonia cells. Moreover, our data showed that, the administration of amlodipine has not prominent effect on cellular population of spermatogonia cells consequently; the increment of spermatocytes after the administration of amlodipine could be occurred due to the improvement of cell division process of spermatogonia lineage. The results obtained from the repopulation index and spermiogenesis index, confirm this effect of amlodipine on testicular tissue cellular activity. It has been reported that, the spermatocytes are the most sensitive cells which susceptible to cryptorchidism and heat induced oxidative stress.[28,29] The results of this study showed that the long term induction of cryptorchidism could induce significant reduction of the population of Sertoli cells. In this regard, some studies revealed that the elevation of oxidative stress related to cryptorchidism could induce irreversible changes in Sertoli cells which consequently may influence the activities of spermatogenic cells lineage.[30] It has been reported that the elevation of intracellular Ca^{2+} levels, can influence the production of xanthine oxidase from dehydrogenases which has an important role in the production of reactive oxygen radicals.[31] Increase of the permeability of inner mitochondrial membrane leads to elevation of cytoplasmic calcium levels.[32] Base on the results of this study and the previous reports, we can propose at least two possible mechanisms which may be related to heat-induced testicular tissue alteration following the over production of reactive oxygen species and consequently, the elevation of cytoplasmic calcium levels: 1) the influx of calcium into mitochondria and disruption of the normal cell metabolism which may lead to cell death and, 2) production of xanthine oxidase and subsequently the production of intracellular reactive oxygen species. These changes could initiate the various apoptotic pathways which induce the reduction of cellular population of seminiferous tubules. According to our histopathological data of testicular tissue which revealed a various cellular alterations and the activity of amlodipine as calcium channel blocker, we can suggest that, the

administration of amlodipine could be effective in decrease of cellular and tissue alterations of testicular tissue through the reduction of apoptotic process which mediated by heat-induced oxidative stress in cryptorchidism conditions. Consequently, the approval of these beneficial effects of amlodipine requires some additional studies.

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Conflict of Interests

None.

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چکیده

بررسی اثرات محافظتی آملودیپین بر ساختار و عملکرد بافت بیضه به دنبال ایجاد کریپتورکیدیسم تجربی یک‌طرفه در موش صحرایی

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هدف- کریپتورکیدیسم، نقص مادرزادی شایع دستگاه تناسلی نر، یکی از علل بروز ناباروری است. افزایش دمای بافت بیضه ناشی از کریپتورکیدیسم با ایجاد استرس اکسیداتیو همراه بوده که می‌تواند موجب آسیب سلولی و بافتی شود. آملودیپین یک داروی بلوک‌کننده کانال کلسیمی نسل سوم است که دارای ویژگی‌های آنتی‌اکسیدانی است. هدف از این مطالعه بررسی اثرات محافظتی آملودیپین بر ساختار بافت بیضه در مدل حیوانی کریپتورکیدیسم بود.

طرح مطالعه- مطالعه تجربی

حیوانات- ۳۰ سر موش صحرایی نر نژاد اسپاراگ-داولی با وزن ۲۲۰-۲۰۰ گرم

روش کار- کریپتورکیدیسم تجربی در موش‌های صحرایی ایجاد شد. آملودیپین (۱۰ میلی‌گرم/کیلوگرم) به مدت دو هفته یا چهار هفته به‌صورت روزانه به روش گاواژ دهانی مصرف گردید. گروه‌های تجربی شامل گروه کریپتورکیدیسم (تعداد=۱۰) و گروه کریپتورکیدیسم درمان شده (تعداد=۱۰) بود. نمونه‌های بافت بیضه در روزهای ۱۴ و ۲۸ پس از کریپتورکیدیسم از گروه‌های تحت مطالعه اخذ شد. مطالعات هیستوپاتولوژی و مورفومتری به همراه ارزیابی شاخص‌های میکروسکوپی اسپرماتوژنز صورت گرفت.

نتایج- آتروفی لوله‌های اسپرم‌ساز به‌همراه بی‌نظمی اپیتلیوم دیواره لوله‌ها در گروه‌های کریپتورکیدیسم مشاهده شد. این تغییرات در موش‌های درمان شده به‌صورت وابسته به زمان کاهش یافت. میانگین تعداد سلول‌های سرتولی به‌صورت معنادار (p=0.025) در گروه کریپتورکیدیسم چهار هفته و میانگین تعداد سلول‌های زایا در گروه‌های کریپتورکیدیسم چهار هفته و درمان شده دو هفته به‌صورت معنادار (p<0.0001) در مقایسه با گروه کنترل کاهش یافت. تمام شاخص‌های میکروسکوپی اسپرماتوژنز به‌دنبال ایجاد کریپتورکیدیسم کاهش نشان داد. تغییرات مذکور به‌صورت وابسته به زمان در گروه‌های تحت درمان کاهش یافت.

نتیجه‌گیری و کاربرد بالینی- نتایج نشان داد که استفاده از آملودیپین به‌عنوان یک ترکیب آنتی‌اکسیدان، می‌تواند به‌صورت وابسته به زمان در کاهش تغییرات سلولی و بافتی بیضه متعاقب کریپتورکیدیسم مؤثر باشد. بخشی از اثرات محافظتی مذکور ممکن است از طریق مهار کلسیم و کاهش روند آپوپتوز به‌دنبال کاهش سطح کلسیم سیتوپلاسمی رخ دهد.

واژه‌های کلیدی- آملودیپین، کریپتورکیدیسم، موش صحرایی، بافت بیضه