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BASIC RESEARCH

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The effect of Vitamin D on testosterone and uncarboxylated osteocalcin levels in aged male rats

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ABSTRACT

There is a well-established complex interaction between vitamin D metabolism and bone and gonad functions. In this study, we aimed to investigate the potential effects of vitamin D therapy on testosterone and osteocalcin (OC) levels in aged male rats. Forty-five adult male rats were divided into three groups in this study. Unlike the control group, the two experimental groups received 50 IU/kg/day and 100 IU/kg/day of vitamin D_3 (cholecalciferol), respectively, for a 4-week period using the gavage method. Testicular tissue and blood samples from rats were collected under general anesthesia at the end of the 4-week period. Testicular tissue samples were examined using light and electron microscopy. Additionally, serum testosterone and OC levels were measured in blood samples. The 50 IU/kg dose of cholecalciferol increased testosterone and OC levels, which were lower than normal due to aging, and regulated the organization of the seminiferous tubule epithelium and interstitium more effectively than the 100 IU/kg dose of cholecalciferol. Male fertility functions and bone health, which degrade due to aging, were increased due to the use of exogenous vitamin D, although the higher dose was not associated with more effective results.

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Vitamin D; osteocalcin; testosterone; light microscopy; electron microscopy

Introduction

Vitamin D is an important prohormone that affects calcium and phosphorus homeostasis and bone mineralization in the body. There are two main forms: vitamin D_3 (cholecalciferol), which is produced in the skin as a result of exposure to ultraviolet light in sunlight, and vitamin D_2 (Ergocalciferol), which is obtained from plants. With sun exposure periods for approximately 90% of daily vitamin D needs being provided, while very little is compensated by diet. Therefore, it is recommended to provide oral vitamin D supplement, especially to children and the elderly in cases where there is not enough sun exposure. According to the Clinical Practice Guideline of the U.S. Endocrine Society, the daily vitamin D needs of children and young adults aged between 0–18 years ranges between 400–1000 IU, while in individuals aged ≥19 years increases up to 1500–2000 IU.[1–4] Relevant studies have demonstrated an association between vitamin D levels and bone mineral density, serum calcium level, and parathyroid hormone release.[5,6] Furthermore, it was found that the effects of vitamin D were not only limited with bone health.[7,8] It was reported that in cases of vitamin D deficiency the risk of cancer,[9] diabetes,[10] and autoimmune[11] and cardiovascular diseases[12] was increased. There is evidence that vitamin D modulates reproductive health in addition to sex steroid hormones.[13,14] Studies show that polycystic ovary syndrome (PCOS)[15] and endometriosis[16] in females and increased androgen levels and semen quality[17,18] in males are associated with vitamin D levels. However, its mechanism of action on the male reproductive system is not fully understood. Animal studies with rodents have reported vitamin D receptors (VDR) are found in the epithelial cells of epididymis, seminal vesicle, ductus deferens, and prostate, also Sertoli and Leydig cells, spermatids, spermatocytes, and spermatogonia.[19–21] Calcium is essential for spermatogenesis, sperm motility, hyperactivity, and the acrosome reaction in the male reproductive system. Vitamin D is also suggested to play a significant role in calcium metabolization. Study reported that vitamin D affected fertility by increasing intratesticular Ca^{+2} levels, sperm motility, and acrosin activity

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using the VDR.[22,23] Furthermore, infertility because of testicular dysfunction associated with decreased testicular weight, impaired testicular development and spermatogenesis, decreased sperm count, sperm quality, and testosterone levels was reported in cases of vitamin D deficiency.[24,25] Recent studies have investigated the physiological relationship between serum vitamin D level, VDR expression, and vitamin D metabolizing enzyme activities with serum testosterone levels.[26,27] Although a significant increase in total and free testosterone levels has been recorded in middle-aged men with sexual dysfunction undergoing vitamin D replacement therapy, the underlying mechanism has not yet been understood.[28–30] Testosterone is an important steroid hormone that plays a role in the functioning of various organs and tissues. A decrease in testosterone production in older men leads to increase in incidences of metabolic syndrome, as a result of which the use of exogenous testosterone for therapeutic purposes has increased. Recent studies reported azoospermia and kidney and cardiovascular dysfunctions parallely with an increase in the use of exogenous testosterone.[31,32] It has also been reported that, along with testosterone levels, serum vitamin D levels decrease as well due to age-related loss of function in bones and gonads.[33] The relevant evidence suggested a complex interaction between vitamin D metabolization and bone and gonad functions, glucose metabolism, and calcium absorption and release.[34–36] Furthermore, studies reported that osteocalcin (OC), a multifunctional hormone produced by osteoblasts responsible for bone formation, stimulated testosterone synthesis in Leydig cells and was regulated by dose-dependent vitamin D levels.[37,38] Nevertheless, there is no current study that describes this complex relationship between in vivo vitamin D application and OC and testosterone in aged male rats. Therefore, this study aimed to investigate the possible effects of vitamin D therapy on testosterone and OC levels in adult male rats. It was considered that vitamin D administration could be an alternative treatment protocol to protect both bone and male reproductive health against age-related androgen deficiency and the side effects of exogenous testosterone use.

Material and method

Experimental design

Twenty-one male rats aged 12 months that were provided by the Çukurova University Health Sciences Experimental Application and Research Center were divided into three groups: Group 1, Group 2, and Group 3. Group 1 was a control group in which rats only received a serum physiologic, while Group 2 and 3 rats received 50 IU/kg and 100 IU/kg cholecalciferol (C9756, Sigma-Aldrich, MO, USA), respectively, on a daily basis, for a 4-week period using the gavage method. Testicular tissue and blood samples from rats were collected under general anesthesia at the end of the 4-week period. Testicular tissue samples were investigated using light and electron microscopy and serum LH, testosterone, and OC levels were measured in the blood samples. The control and experimental group data were compared and interpreted using appropriate statistical methods. Approval of animal experiments from the ethics committee of the Çukurova University Health Sciences Experimental Medicine Research and Application Center was obtained before the onset of the study. (Date: 12.29.2014 No: 8)

Light microscopy methods

For the light microscopy examinations, the testicular tissue samples of rats from all the groups were fixed in Bouin solution and fixed at room temperature for 72 hours. The dehydrated tissue samples were then placed in a Leica TP 1020 Autotechnical Device (Germany). Following the blocking process, histological sections were taken using a microtome at a thickness of 5 μm and stained with hematoxylin-eosin. They were examined and photographed using an Olympus BX53 light microscope (Japan).

Electron microscopy methods

For the electron microscopy evaluations, the testicular tissue fragments were fixed in a 5% glutaraldehyde solution prepared with Millonig's phosphate buffer for approximately 4 hours. The tissues were then shaken twice in the Millonig's phosphate buffer for 10 minutes.

They were then fixed for a second time using a 1% Osmium tetroxide solution prepared with the Millonig's phosphate buffer and again rinsed twice for 10 minutes with the phosphate buffer. The tissue fragments, which were dehydrated by passing through an increasing alcohol series, were immersed in propylene oxide and mixed solution (propylene oxide + resin) and then transferred to tubes containing resin overnight in a rotator. The next day, the tissue fragments were buried in polyethylene capsules and polymerized for 48 hours at 60°C. 50 nm in thickness sections were taken from the blocks using a Reichert-Leica UltraCut S ultramicrotome. The sections were stained with uranyl acetate and lead citrate solutions. The stained sections were examined using the JEOL-JEM 1400 Transmission Electron Microscope (Japan) and their micrographs were obtained.

Morphometric methods

The epithelial height and diameter of the seminiferous tubules were quantitatively measured by light microscopy at a magnification of 20X and averages were calculated by the CellSens Standard Program 1.6 (Olympus, 2011). Additionally, Johnsen's Tubular Biopsy Score (JTBS) was used to semiquantitatively evaluate the spermatogenesis in 20 seminiferous tubules from each testicular section (Johnsen, 1970). JTBS was calculated by dividing the sum of all scores in each group by the total number of seminiferous tubules. A decrease in the average value was evaluated as an impairment in spermatogenesis. A 10-point scoring grade system was established.

Score 10: complete spermatogenesis with regular tubules;
Score 9: many sperms, irregular germinal epithelium;
Score 8: few sperms;
Score 7: no sperms, many spermatids;
Score 6: few spermatids;
Score 5: no sperm or spermatids;
Score 4: few spermatocytes;
Score 3: presence of spermatogonia;
Score 2: presence of Sertoli cells;
Score 1: no cells.

All morphometric parameters were analyzed statistically.

Biochemical methods

Intracardial blood samples were centrifuged at 3600 rpm, testosterone (CSB-E05100r, Cusabio) and undercarboxylated OC (Glu-OC) (E-EL-R2477, Elabscience) levels were measured using the E170 immunoassay (Roche) in the serum samples, and biochemical data were evaluated statistically.

Statistical analysis

The GraphPad Prism 5 (GraphPad Software Inc., USA) software was used for statistical analysis of study data. The D'Agostino & Pearson omnibus normality test was used to test the normal distribution hypothesis for the study data. The intergroup differences were analyzed using the Mann-Whitney U test, where $p < .05$ was considered statistically significant.

Results

Light microscopic results

Regressive changes were observed in both seminiferous tubules and the interstitial area in the testicular tissue samples of Group 1 rats. Vacuolization, a relatively reduced number of spermatogenic cells, and Sertoli cells in the seminiferous tubular epithelium were observed. Due to the arrest of spermatogenesis in many tubules, there was an accumulation of immature and multinucleated giant cells in the lumen. The interstitium in the structure of loose connective tissue was expanded, where the number of Leydig cells was decreased (Figure 1a). In the examination of the testicular tissue samples from Group 2 rats, which received daily 50 IU/kg cholecalciferol (Vitamin D3), tubule epithelial cells showed a regular organization and there was no vacuolated appearance in tubule epithelium, indicating continued spermatogenesis in most seminiferous tubules. The interstitium also had a relatively normal appearance (Figure 1b). However, examination of the testicular tissue samples from Group 3 rats, which received daily 100 IU/kg cholecalciferol

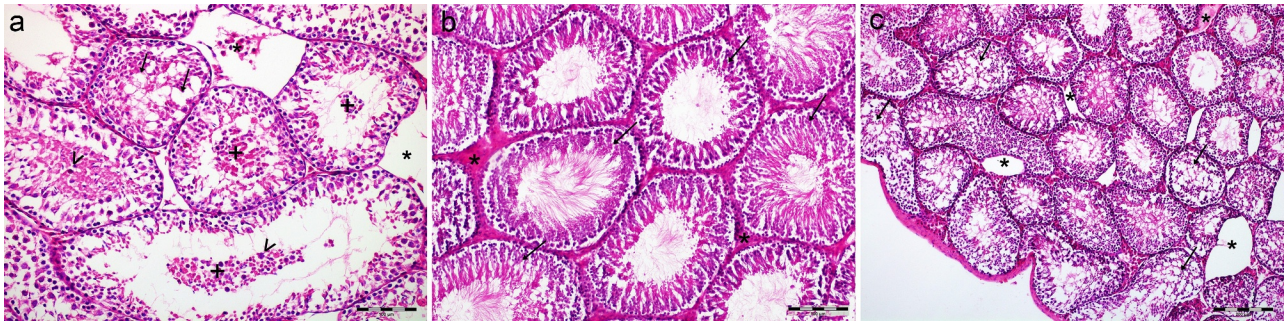


Figure 1. Light microscopic examination of the testicular tissue samples of aged rats. **A)** There were vacuolization (arrow) in the seminiferous tubule epithelium, immature cell accumulation (+), and multinucleated giant cells (arrowhead) in the tubule lumen of the testicular tissue samples belonging to the control group. There were expansion (*) in the interstitium. **B)** Seminiferous tubules and interstitium were relatively normal in testicular tissue samples of rats that received 50 IU/kg Cholecalciferol. **C)** Vacuolization (arrow) and expansion (*) continued in some seminiferous tubular epithelium in the testicular tissue samples of rats that received 100 IU/kg Cholecalciferol.

(Vitamin D3) for a 4-week period, showed that there was vacuolization and partial spillage in the epithelium of some seminiferous tubules. The interstitial expansion was also more pronounced compared to the testicular tissue samples of Group 2 rats (Figure 1c).

Electron microscopic results

Electron microscopic examination of testicular tissue samples from aged rats of Group 1 showed expansion of the agranular endoplasmic reticulum cisternas in the Sertoli cells and disruption in the tight junctions between Sertoli cells. Blebbing in the nuclear membranes and large spaces between spermatogenic cells were observed (Figure 2a). There were degenerative mitochondria and decreased lipid droplets in the Leydig cell cytoplasm, which became electron-dense (Figure 2b). Whereas, the expansion of the agranular endoplasmic reticulum cisternas in Sertoli cell cytoplasm decreased and the tight junctions between the neighboring Sertoli cells maintained their integrity in testicular tissue samples of Group 2 rats. The blebbing in the nuclear membranes and the large spaces between the spermatogenic cells were relatively reduced. Late spermatids with abnormal orientation were also found (Figure 2c). While Leydig cell electron density was normal and mitochondria in the cytoplasm were intact, it was noted that the number of lipid droplets increased slightly compared to Group 1 rats (Figure 2d). In the testicular tissue samples of Group 3 rats, Sertoli cells were normal, similar to that of Group 2 rats, whereas blebbing in the nuclear membrane of spermatogenic cells and junctions between spermatogenic cells were slightly observed to increase (Figure 2e). Mitochondrial degeneration of Group 3 rats was higher compared to that of Group 2 rats, where the number of lipid droplets in Leydig cell cytoplasm was normal, similar to Group 2 rats (Figure 2f).

Morphometric results

Thickness and diameter of seminiferous tubules and Johnsen's score values for each group are shown in Table 1. The morphometric analysis indicated that Group 2 had significantly higher seminiferous epithelium thickness, diameter, and Johnsen's scores compared to the control group (Group 1) ($p < .05$). However, there was no significant difference in all morphometric parameters of Group 3 compared to the control group (Group 1) ($p > .05$).

Biochemical results

The serum testosterone level of the Group 2 rats was significantly higher compared to the control group (Group 1), while there was no significant difference between the Group 3 rats and the control group (Group 1) (Table 2). Similarly, serum (Glu-OC) levels in Group 2 rats were significantly higher compared to the control group, while there was no significant difference between the Group 3 rats and the control group (Table 3).

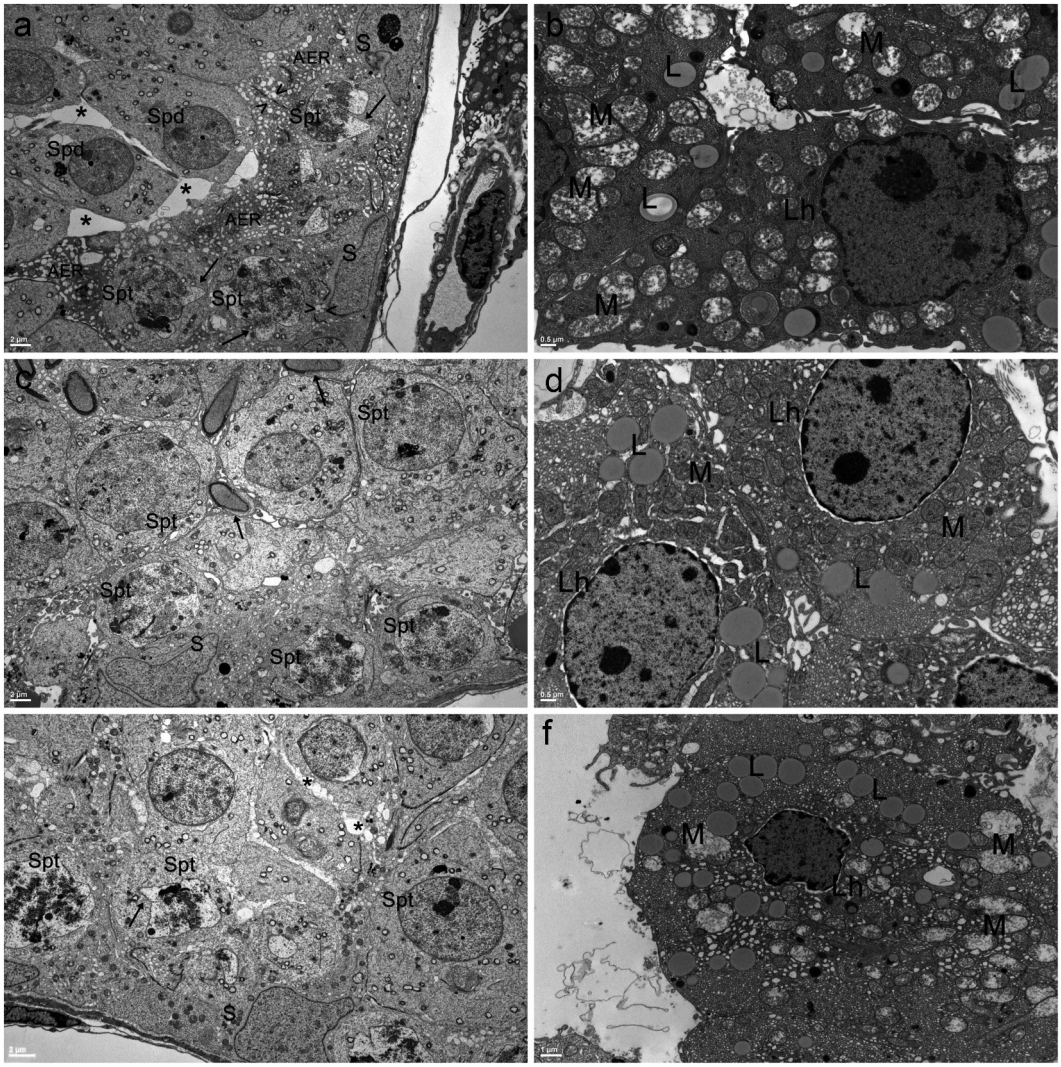


Figure 2. Electron microscopic examination of testicular tissue samples of aged rats. **A)** In the testicular tissue samples of the control group, there were expansion in agranular endoplasmic reticulum (AER) cisternas and impairment in the tight junctions (arrowhead) between Sertoli cells (S). Blebbing (arrow) between the nuclear membrane of some spermatocytes (Spt) and large intercellular spaces (*) between spermatids (Spd) were observed. **B)** There was an increase in electron density in the Leydig cells (Lh) and degenerative mitochondria (M) and decreased lipid droplets (L) in their cytoplasm. **C)** Sertoli cells (S) were intact in the testicular tissue samples of Group 2 rats. There was decreased blebbing in the nuclear membrane of the spermatocyte (Spt). However, there were late spermatids (arrow) with abnormal orientation. **D)** Mitochondria (M) were in normal structure and the number of lipid droplets (L) in the Leydig cell (Lh) cytoplasm were normal **E)** While the Sertoli cells (S) were normal in structure, blebbing (arrow) continued in the nuclear membrane of some spermatocytes (Spt) upon examination of the testicular tissue samples of Group 3 rats. **F)** The number of lipid droplets (L) in the cytoplasm of Leydig cells (Lh) was relatively normal, where degenerative changes continued in the mitochondria (M) of testicular tissue samples of Group 3 rats.

Table 1. Values of morphological parameters compared among the groups according to sacrificed time.

Analyzed parameters (mean ± SD)	Group 1	Group 2	Group 3
Thickness of seminiferous epithelium (μm)	72,87 ± 1,2	81,12 ± 1,8*	80,62 ± 1,22
Diameter of seminiferous tubules (mm)	238.53 ± 1,5	254,7 ± 1,7*	252,46 ± 1,08
Johnsen's score	7,4 ± 0,2	11,62 ± 0,22*	10,21 ± 1,28

Discussion

Vitamin D, which is produced in the skin as a result of ultraviolet radiation from sunlight exposure, regulates calcium and phosphorus metabolism by physiologically effecting bones, intestines, kidneys, and parathyroid glands in the body.[1] It has recently

Table 2. Statistical evaluation of serum testosterone levels. *p < 0.01

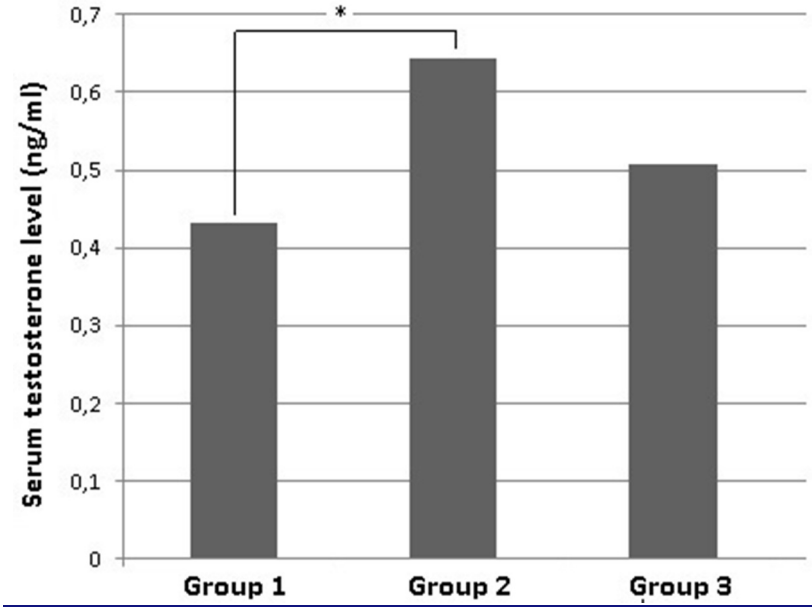
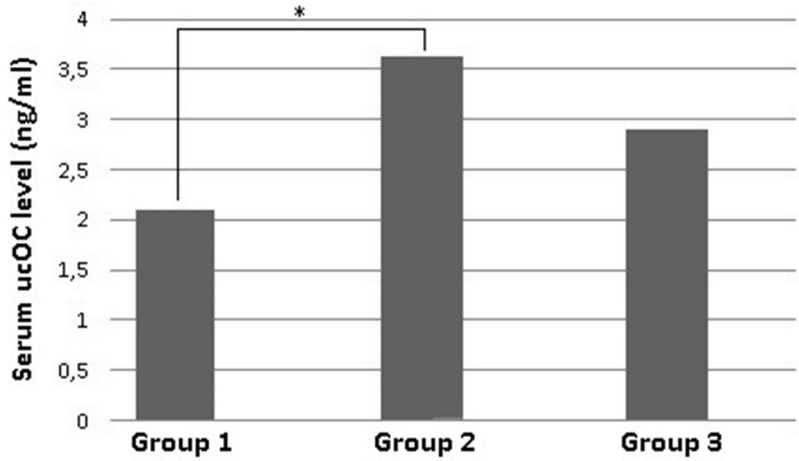


Table 3. Statistical evaluation of serum ucOC levels. *p < 0.01



been reported that, along with the steroid hormones, vitamin D is among the main regulators of the reproductive system. VDR and metabolizing enzymes have been shown in the prostate, seminal vesicle, epididymis, and germ and Sertoli cells.[24,39] Furthermore, decreased sperm count and motility and abnormal testicular histology have been reported in VDR deficient mutant male mice.[40] In mice fed on vitamin D-deficient diet, testicular weight and number of spermatozoa were decreased and germ cell proliferation was suppressed.[24] Studies on vitamin D deficiency have reported an association with decreased testicular and epididymal sperm counts, Sertoli cell dysfunction, decreased number of Leydig cells, and degenerative changes in germinal epithelium.[25] Another study further suggested that fertility is decreased in male rats fed vitamin D-deficient diet compared to rats fed vitamin D-rich diet. In particular, male rats with vitamin D deficiency had reduced mating rate of 45% and 73% reduction in fertilization.[40] Although there are several studies that report an association between serum vitamin D levels and total and free testosterone levels,[26,27,33,41] certain

studies report contradictions in this association, especially in elderly subjects. While certain studies did not report any association between circulating vitamin D levels and total and free testosterone levels,[42] others have suggested a positive relationship between them.[43] It has been reported that increased cell proliferation, decreased apoptosis, and decreased oxidative stress in aged rat testis are the the causes of antiaging effects of vitamin D. Treatment with vitamin D3 resulted in a significant reduction in the serum and testicular advanced glycation end products (AGE) levels in the aged rat testis. It can be thought that vitamin D3-mediated AGE/RAGE system in aged testis regulates oxidative stress and controls proliferation and apoptosis in aged testis.[44]

In addition to the risk of hypogonadism and infertility as a result of vitamin D deficiency, musculoskeletal diseases, such as osteoporosis and muscle weakness, have also been reported.[2] In this respect, OC is another hormone that acts as a mediator for the regulatory effect of vitamin D on the male reproductive system. Released from osteoblasts and involved in bone metabolism, OC has been shown to stimulate testosterone production in Leydig cells in in vivo, ex vivo, and in vitro studies.[45] It has also been reported that OC stimulates the synthesis of vitamin D in Leydig cells.[46]

Given the complex relationship between vitamin D, testosterone, and Glu-OC, this study found that 50 IU/kg cholecalciferol increased the age-related decrease in testosterone and Glu-OC levels; repaired degenerative changes in seminiferous tubules at morphological, morphometric, and ultrastructural levels; and maintained normal morphology, especially at the ultrastructural level of Leydig cells. However, it was noted that in higher doses (100 IU/kg) cholecalciferol was not as effective as the lower dose, and degenerative changes partially continued in seminiferous tubule epithelium and Leydig cells. Although the testosterone and OC levels of the Group 3 rats that were given 100 IU/kg cholecalciferol were higher compared to the control group, they were not as high as Group 2 that received the 50 IU/kg cholecalciferol dose. These results show that vitamin D may have some cellular effects in high doses and is associated with hypercalcemia in the cytoplasm, which leads to impairment of steroidogenesis, especially in Leydig cells, due to mitochondrial damage. In addition to hypercalcemia, another side effect of high-dose vitamin D is known to be hyperphosphatemia. Remarkably, similar to our study, hypertrophy protection in osteoarthritis rat model was reported at low doses of vitamin D, whereas the same was not observed at high doses. This lack of dose response may be thought to be related to the different effects of vitamin D on different cells. Also, more studies are needed to examine various molecules associated with the degenerative process of high-dose vitamin D.[47]

Reproductive potential declines with age, and this decline in reproductive capacity with age may be due to a combination of morphological and molecular changes in the reproductive organs together with bone health problems. The role of vitamin D3 treatment at different doses in aging men on testicular activity has not been investigated yet, in parallel with the OC level. Therefore, based on the results of the present study, it was concluded that vitamin D replacement therapy may be dose-dependently effective in the treatment of testicular dysfunctions related to aging. It was observed that while it protects male reproductive and bone health at low doses, it may cause different systematic disorders at high doses.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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