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RESEARCH

Effect of vitamin D in experimental varicocele model in rats

Sıçanlarda deneysel varikosel modelinde D vitamininin etkisi

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Abstract

Purpose: Varicocele is a pathological condition that affects approximately 20% of adult men, causing infertility and sperm deterioration. The aim of our study was to investigate the efficacy of vitamin D (Vit. D) in the pathophysiology of varicocele. We performed biochemical, gene expression analyses and histopathological to evaluate the efficacy of vitamin D in the experimental varicocele model.

Materials and Methods: In the study, 30 adult male Wistar Albino rats were used. The rats were divided into 3 groups equally as control group, experimental group (varicocele), treatment group (varicocele + D vit.). The treatment group received 500 IU/kg D vit. intramuscularly.

Results: Histopathological, TRPM2-8 gene expression and biochemical analyses were performed on testicular and blood samples collected at the end of the experiment. The experimental group showed a deterioration in tubular structure, a decrease in total antioxidant levels and an increase in total oxidant levels. The treatment group, on the other hand, showed an increase in TAS, a decrease in TOS and a beneficial improvement in tubular structure disorders. Analysis of gene expression levels showed that TRPM2-8 expression levels were significantly increased in the varicocele group and decreased in the treatment group.

Conclusion: In the varicocele model, the use of vitamin D had a significant effect on TRPM2-8 gene level, pathological seminiferous tubules and biochemical values. Further studies are needed to determine the clinical application of vitamin D in varicocele disease.

Keywords:. Varicocele, TRPM, Vitamin D, TAS, TOS

Öz

Amaç: Varikosel, yetişkin erkeklerin yaklaşık %20'sini etkileyen, infertilite ve sperm bozulmasına neden olan patolojik bir durumdur. Çalışmamızın amacı D vitamininin (Vit. D) varikosel patofizyolojisindeki etkinliğini araştırmaktır. Deneysel varikosel modelinde D vitamininin etkinliğini değerlendirmek için biyokimyasal, histopatolojik ve gen ekspresyon analizleri yaptık.

Gereç ve Yöntem: Çalışmada üç gruba ayrılmış 30 yetişkin erkek Wistar albino sıçan kullanıldı: kontrol grubu, deney grubu (varikosel) ve tedavi grubu (varikosel + D vitamini). Tedavi grubuna intramüsküler olarak 500 IU/kg D vitamini verildi.

Bulgular: Deney sonunda toplanan testis ve kan örneklerinde histopatolojik, TRPM2-8 gen ekspresyonu ve biyokimyasal analizler yapıldı. Deney grubunda tübüler yapıda bozulma, total antioksidan seviyelerinde azalma ve total oksidan seviyelerinde artış görüldü. Öte yandan, tedavi grubu TAS'da artış), TOS'da azalma ve tübüler yapı bozukluklarında faydalı bir iyileşme göstermiştir. Gen ekspresyon seviyelerinin analizi TRPM2-8 ekspresyon seviyelerinin varikosel grubunda anlamlı olarak arttığını ve tedavi grubunda azaldığını gösterdi.

Sonuç: Varikosel modelinde, D vitamini kullanımı TRPM2-8 gen seviyesi, patolojik seminifer tübüller ve biyokimyasal değerler üzerinde anlamlı bir etkiye sahipti. Varikosel hastalığında D vitamininin klinik uygulamasını belirlemek için daha ileri çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Varicocele, TRPM, Vitamin D, TAS, TOS

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INTRODUCTION

Varicocele is the dilation of the spermatic veins in the scrotum that form the pampiniform plexus. It can cause male infertility. Surgical intervention has been demonstrated to correct infertility related to varicocele[1]. Varicocele affects approximately 15-22% of adult males[2]. The formation of varicocele can be caused by various theories, including renal adrenal reflux, acrosome reaction, testicular blood flow, apoptosis, testicular-cellular fluid relationship, autoimmunity, hormonal dysfunction, venous pressure, oxidative stress and hyperthermia. While the physiopathology of varicocele has not been fully elucidated, numerous experimental studies have been conducted to understand vascular physiopathology. Varicocele-associated oxidative stress may cause testicular damage by increasing free radicals, according to some studies[3]. Vitamin D is a steroid molecule found in both animal and vegetable sources. It possesses anti-inflammatory and immune-regulating properties[4]. Vitamin D3, or cholecalciferol, and vitamin D2, or ergocalciferol, are two types of vitamin D. The D2 form is mostly produced by yeasts and is usually added to foods, while the D3 form is found in foods with high nutritional value. The structure of the D3 form of vitamin D is stronger and more stable than that of the D2 form[5]. The Vitamin D Receptor (VDR) is present in human tissues and cells and is responsible for some of the biological functions of Vitamin D[6]. The vitamin D receptor (VDR) is typically located in reproductive organs, including the placenta, skeletal system, calcium regulatory tissues, parathyroid glands, ovaries, intestines, uterus, and testes[7]. TRP (Transient Receptor Potential) cation channels are defined as voltage-dependent cation channels. TRP channels are ion channels that cause disruption of intracellular calcium homeostasis. Calcium plays a crucial role in ion exchange across the cell membrane[8]. It has been stated that the TRPM subgroup consists of eight members, each with distinct biophysical and physiological properties[9]. TRPM2 channels are found in various cells and tissues, including the brain, spleen, liver, and testes[10]. The TRPM2-8 channel has become particularly important due to its activation by oxidative stress products[11].

The treatment of varicocele in the clinic is surgical. Varicocele is a reversible cause of male factor infertility. However, due to its negative impact on the male reproductive system and the risk of recurrence, research into further treatment options is ongoing. Vitamin D is obtained both internally and externally through supplements. Our study was designed to provide preliminary clinical information with the aim of gaining a different perspective by investigating the activity of both biochemical and locally formed ion channels in varicocele.

MATERIALS AND METHODS

Animals

We carried out our study in the laboratory of Hatay Mustafa Kemal University, Medical School that approved with 2016/9-4 Ethics Committee number. We divided our study into three groups, 10 rats in each group. We used 30 male Wistar Albino rats weighing 350-350 gram. A power analysis was conducted to determine the number of group samples required for the experiment. The analysis was performed with an α error probability of 0.05, an effect size of $d = 1.2$, and a power of (1-β error probability) = 0.80. Based on the results, each group was planned to have $n = 10$. The rats in the experimental phase were kept in wire cages in rooms with an average ambient temperature of 21-23 °C, 12 hours during the day and 12 hours at night. Standard rat food and tap water were used to feed the rats. The experimental phase lasted for 30 days. After 30 days, operative laparotomy was performed on the subjects. Anesthesia was given to the patients before the procedure.

Experimental design

Animals were planned as 3 groups.

A. Sham Group (n:10): The abdomen was opened and the tissues around the spermatic vein were removed. It was dissected and closed without creating a varicocele model.

B. Experimental Group (n:10): An experimental varicocele model was created.

C. Treatment Group (n:10): After the experimental varicocele model was created, 500 IU/kg of D vit. was given intramuscularly every day for 4 weeks[12].

Experimental varicocele model

The surgical procedures were carried out under anaesthesia, with a maintenance dose administered and continued until the procedure was terminated. The abdominal wall was cleaned using a clipper, and

a surgical operation area was created. A midline incision was made, and the testicular vein was isolated in the area where the testicular and adrenal veins enter the left renal vein. The vessel was narrowed using a metal wire with a diameter of 0.85 mm. The vessel's outer diameter was reduced to 1 mm, A varicocele model was created after this pressure was transferred to the testicular vein[13]. The abdominal cavity was washed with isotonic saline fluid, and the abdominal wall and skin were sutured with 4.0 chromic catgut in the anatomical plane. After the study, orchiectomy was performed, and the testis tissues were weighed. The tissue was then divided into two equal parts for molecular and histopathological examinations.

Table 1. Primer gene sequences used.

β-Actin Left 5'	CCC GCG AGT ACA ACC TTC T-3' 58.8 19
β-Actin Right 5'	CGT CAT CCA TGG CGA ACT-3' 56.0 18
TRPM2 Left 5'	AAT TTG CTC ATC GCC ATG TT-3' 53.2 20
TRPM2 Right 5'	GAT CTG GTC TGT GTG CTC CTG- 61.8 21
TRPM8 Left 5'	GCC CAG TGA TGT GGA CAG TA- 59.4 20
TRPM8 Right 5'	GGA CTC ATT TCC CGA GAA GG-' 59.4 20

Biochemical analysis

Cardiac blood samples were obtained at room temperature and serum was extracted by centrifugation at 5000 rpm for 10 minutes at +4 oC. TAS and TOS analyses were conducted on the Olympus AU 400 biochemistry autoanalyzer using commercial kits (Rel Assay Diagnostics; Catalog no: RL0017 for TAS and RL0024 for TOS) following the protocol described by Erel [14]. Serum samples were obtained and used to measure Total Antioxidant Levels (TAS) and determine Oxidative Stress Index (OSI) levels by measuring Total Oxidant Levels (TOS).

Histopathological analysis

Testicular tissue samples were evaluated for histopathological examinations, and the Johnsen Score (JS) was used for evaluation.

Gene analysis

Testicular tissue samples underwent RT-PCR (quantitative real-time) analysis to examine TRPM2-8 channels. The transcription levels of TRPM2-8 and the housekeeping gene β-Actin (EllaBiotech, Deutschland) were measured using a QIAGEN Rotor-Gene Q device. Quantitative values were determined using the normalization coefficient shown in Table 1. 5 µl of each cDNA sample was added to PCR-strip tubes, followed by 20 µl of qRT-PCR mix to each sample. The qRT-PCR reaction was carried out on a QIAGEN Rotor-Gene Q instrument using a 25 µl volume. The ΔCt values and results were subsequently analysed.

Statistical analysis

Data were collected using the 'Graphpad Prism 7' computer package program. Normality was determined using the D'Agustino & Pearson test. The ANOVA (one-way analysis of variance) test was used for data that showed normal distribution between groups, while Tukey's multiple comparison test was used to compare between groups. Data that did not show normal distribution were analyzed using the Kruskal-Wallis test. Dunn's multiple comparison test was used to compare data between groups. A statistically significant level of $p<0.05$ was accepted.

RESULTS

Figure 1 shows a comparison of TAS values between groups, indicating differences in TAS assessments. The varicocele group showed an increase in TOS evaluation between groups ($p<0.05$), while the treatment group showed a decrease in TOS level (Figure 2) ($p<0.01$). The percentage of measured TOS values to TAS values was used to obtain the OSI assessment. The varicocele group had higher OSI values, while the other two groups had lower values ($p<0.01$). A significant decrease was observed when comparing the treatment group with the varicocele group ($p<0.05$) (Figure 3).

Evaluation of histopathological analysis

At the end of the study, testicular tissue sections were stained with H&E and scored between groups according to JS. Scoring revealed a significant increase in the deterioration of seminiferous tubule elements in the varicocele group ($p<0.01$). Similarly, a significant difference was observed when comparing the varicocele group with the treatment group ($p<0.05$). Figure 4 shows the testicular

morphologies (A: control, B: varicocele, C: varicocele + D vit).

Evaluation of gene analysis

Figures 5 and 6 show the digital measurement results of each gene obtained by qRT-PCR. Analysis of the TRPM2 group data showed a significant increase in TRPM2 levels in the varicocele group ($p<0.05$). Vitamin D only caused a numerical decrease in the TRPM2 channel (Figure 5). Examination of the TRPM8 channel showed a significant increase in channel levels ($p<0.05$). Vitamin D was found to affect only the TRPM8 channel, resulting in a numerical decrease ($p<0.01$) (Figure 6).

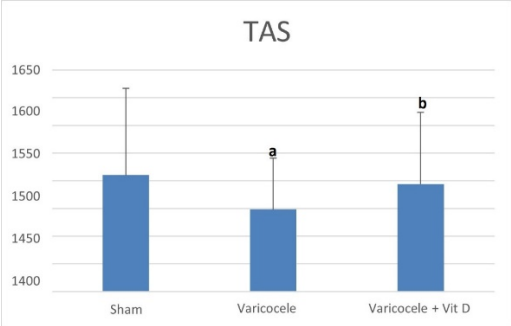

Figure 1. Comparison of TAS values between groups (a: Sham vs Varicocele, $p<0.05$; b: Varicocele+Vit. D vs Varicocele, $p<0.01$).

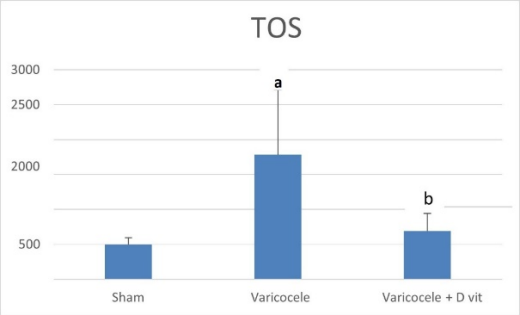

Figure 2. Comparison of TOS values between groups (a: Sham vs Varicocele, $p<0.05$; b: Varicocele+Vit. D vs Varicocele, $p<0.01$).

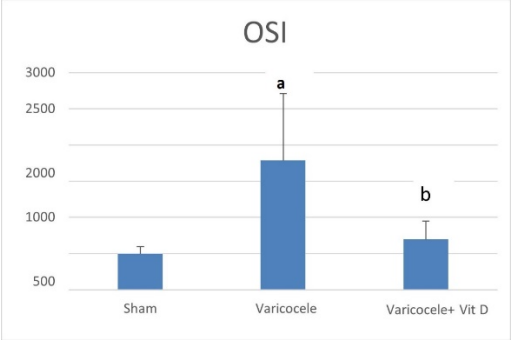

Figure 3. Comparison of OSI values between groups. (a: Sham vs Varicocele, $p<0.05$; b: Varicocele+vit D vs Varicocele, $p<0.01$).

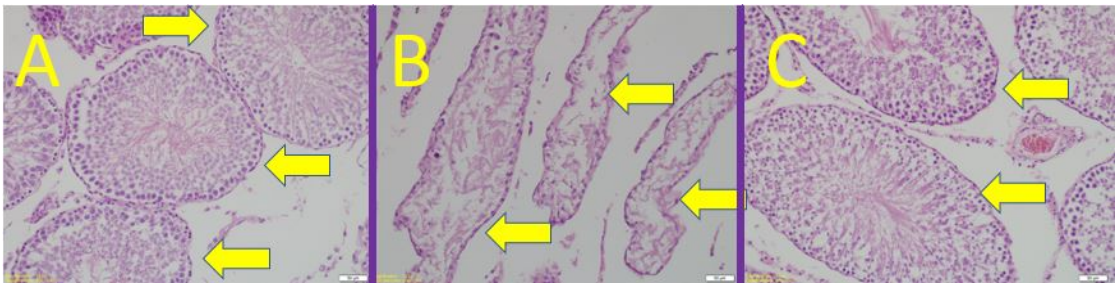

Figure 4. Histopathological image of rat testis tissue. (The seminiferous tubules are marked with a yellow arrow.) A). Preserved testicular morphology visible in the control group (H&Ex200).

B). Significant damage to the seminiferous tubules after varicocele model (H&Ex200).
C). Vitamin D structural improvement in seminiferous tubules after application. (H&Ex200).

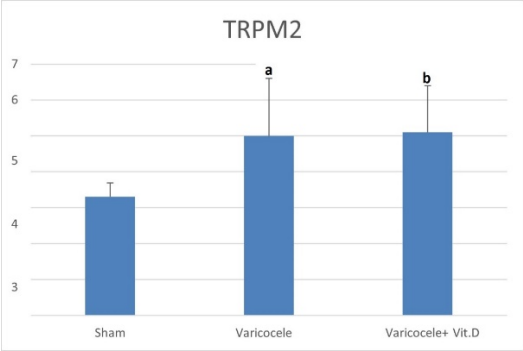

Figure 5. Comparison of TRPM2 gene expression levels between groups. (a: Sham vs Varicocele, p<0.05; b: Varicocele+vit D vs Varicocele, p<0.01).

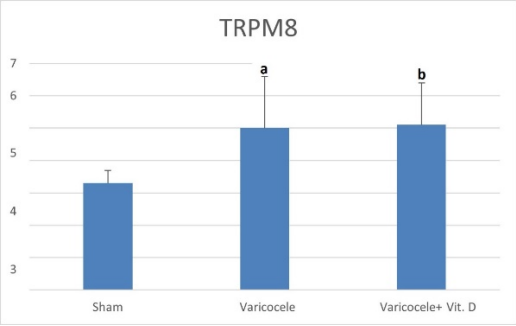

Figure 6. Comparison of TRPM8 gene expression levels between groups. (a: Sham vs Varicocele, p<0.05; b: Varicocele+vit D vs Varicocele, p<0.01).

Comparison of testicular weights

At the end of the 4-week varicocele model period, the testicular tissues of all experimental animals were weighed using a precision balance (Pioneer, OHAUS). Comparison of testicular weights between groups is shown in Table 2. A decrease in testicular weight was observed in the varicocele group compared to the control group ($p<0.05$) for the left testis. The weight of the left testis in the treatment group was similar to that in the sham group. Although there was a numerical difference in the weight of the right testis between the groups, no significant difference was observed (Table 2).

Table 2. Comparison of right and left testicular weights (Mean±SD

	Sham	Varicocele	Varicocele+ Vit D
Right Testis	2.1 ±0.15 ^{b***}	1.6 ±0.27^{b**}	1.8±0.22 ^{c***}
Left Testis	2+0.16	1.2 ±0.23 ^{a***}	1.6±0.23 ^{c**}

[a]: vs Sham; [b]: vs Varikosel; [c]: vs Varikosel+ D vit. *:p<0,05; **p<0,01; ***:p<0,001.

DISCUSSION

There is evidence that vitamin D is crucial for male reproduction, along with key hormones in the reproductive system. VDR and metabolic enzymes have been detected in human ejaculatory ducts, germ cells and mature sperm. It has also been found to be associated with the calcium balance of sperm function[15]. The study observed deterioration of the tubules in the testicular tissue of the varicocele group. The structure of the seminiferous tubules observed in the treatment group was similar to that of the control group.

It has been reported that there is an association between ROS and varicocele in infertile population including people with varicocele. The study conducted TAS and TOS measurements to evaluate the oxidant and antioxidant balances in total. The serum was obtained from blood in accordance with the studies. The study obtained OSI values, which are an important indicator of oxidative stress. The OSI value is calculated by dividing the ratio of TOS values to TAS values as a percentage. The study found that the varicocele group had a significantly higher OSI value ($p<0.001$) compared to the sham and treatment groups ($p>0.01$).

Excessive accumulation of ROS and oxidative stress has been identified as one of the underlying pathological causes of male reproductive issues, which has been the focus of research in this area. TAS as an antioxidant marker was found to be close to the control group in the treatment group. The TOS level, which shows oxidant levels, was found to be increased in the varicocele group compared to the control and treatment groups. Hirai et al. (2009) reported that vitamin D treatment improved impaired testicular histology in mice with cryptorchidism[16]. A study was conducted to examine the function of vitamin D in mice with a suppressed VDR gene. The study found that the testes showed histological abnormalities, which resulted in a

decrease in sperm count and motility[17]. Thomas et al. (2002) reported that there is a relative atrophy of the testis on the same side in varicocele in their studies[18]. Lin et al. (2005) study on rats, cerebral oxidative stress was induced by zinc and compared with the effects of vitamin D, melatonin, beta estradiol, and vitamin E, all of which have antioxidant properties. The study reported that vitamin D has an inducing effect on lipid peroxidation and autoxidation, and shares similar properties with the other antioxidants that were compared[19]. Our study is similar to other studies of vitamin D seminiferous tubules.

In testicular anatomy, the left testicle is slightly lower than the right. Clinically, the majority of varicocele cases are seen in the left testicle. Our study found a statistically significant decrease in left testicular volume in the varicocele group compared to the sham and treatment groups. We compared the weight of the left testicle between the groups and found a statistically significant decrease in the varicocele group. As a result, Vitamin D reported that the testis plays an important role in genomic effects that can be triggered by protein kinase A and rapid responses involving Ca^{+2}/K^{+} channels in the plasma membrane[20]. A numerical increase in the expression level of the TRPM2-8 gene, one of the indicators of calcium levels, was found in the varicocele group. There was also a numerical decrease in expression levels in the treatment group. Previous studies have reported that TRPM ion channels can be activated by oxidative stress. Studies have shown that the TRPM2 channel increases with intracellular Ca^{+2} due to oxidative stress. Our results indicate an increase in the TRPM2 channel in the varicocele group under oxidative stress. In contrast, vitamin D only caused numerical changes in the channel. Recently, TRPM 8 has been shown to cause sensory inhibition in painful animal experiments[21]. It has been suggested that TRPM8 plays a significant role in the pathophysiology of prostate cancer[22]. In our study, we found that TRPM8 levels were increased in the varicocele group ($p<0.05$). Additionally, vitamin D caused only numerical changes on the channel ($p<0.01$). Histopathological examinations revealed a decrease in the elements in the seminiferous tubules in the varicocele group ($p<0.01$), according to the data obtained from JS. It is important to note that all evaluations are objective and free from bias. Significant differences were observed when comparing the varicocele group with the treatment group ($p<0.05$). It can be concluded that the treatment had a positive and regulating effect on the testicular tissue formed as a result of vitamin D varicocele, with a value similar to that of the patient group.

The mechanism of vitamin D action on human testes and sperm is not fully understood. In the absence of sufficient clinical data, it is important to determine the preferred dose and efficacy of vitamin D in daily use. We believe that vitamin D should be supported by in vitro studies. As this was a preliminary study, some parameters sperm hareketliliği sperm sayısı ve akrozom reaksiyonu gibi could not be evaluated.

In conclusion, our study showed that vitamin D reduced biochemical, genetic and histopathological parameters of oxidative stress in the testis. We believe that the results of our study should be supported and confirmed hem in vitro clinically.

Author Contributions: Concept/Design : GA, CT, OT; Data acquisition: GA, HDG, TO, OO, GGD, MT; Data analysis and interpretation: GA, CT, OT, OO, HDG, GGD, MT; Drafting manuscript: GA, MT; Critical revision of manuscript: -; Final approval and accountability: GA, CT, OT, HDG, TÖ, OÖ, GGD, MT; Technical or material support: GA, CT, OT, HDG, TÖ, OÖ, GGD, MT; Supervision: -; Securing funding (if available): n/a.
Ethical Approval: This study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee. MKÜ (No 2016/9-4).
Peer-review: Externally peer-reviewed.
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