J Surg Med. 2024;8(2):22-30.
https://doi.org/10.28982/josam.7366

JOSAM

Research article
https://jsurgmed.com/

Journal of Surgery and Medicine

e-ISSN: 2602-2079

The investigation of retinoic acid on spermatogenetic cell types of rats

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Ethics Committee Approval
The animal experiments in this study were carried out in accordance with Decree No. 133 of the Ethics Committee of İstanbul University.

Conflict of Interest
No conflict of interest was declared by the authors.

Financial Disclosure
This study was granted by the Istanbul University Scientific Research Project Unit with project number 9515.

Published
2024 February 2

Abstract

Background/Aim: Vitamin A is crucial for male fertility and the progression of the spermatogenetic process. Retinoic acid (RA), a metabolite of vitamin A, binds to three nuclear receptors, functioning similarly to a hormone by activating them. Our study aimed to investigate the effects of exogenous RA on spermatogenetic cell types, assessing them histochemically, immunohistochemically, and ultrastructurally.
Methods: We used three groups: a control group and experimental groups treated with 40 mg/kg RA and 80 mg/kg RA. Each group contained eight adult Sprague-Dawley rats. RA, dissolved in corn oil, was administered to the experimental groups via gavage for 3 weeks. After 3 weeks, testes from the sacrificed animals were evaluated using light and electron microscopy. The sections were stained histochemically with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS). Seminiferous tubules in the rats were staged using PAS staining. The cellular localization of the RARα receptor in the seminiferous tubules was identified after immunohistochemical analysis.
Results: Immunoreactivity was qualitatively observed and graded from no staining to strong. In the immunohistochemical analysis, the experimental groups, particularly in stages VI, VII, VIII, and XIV, showed a significant difference in immunoreactivity compared to the control group. This difference was particularly evident in stage VI spermatogonia – the stage at which the first meiosis begins. A morphologically observed reduction in the seminiferous tubules, likely due to the loss of germ cells, was statistically significant in terms of the average diameter of the seminiferous tubules in the 80 mg/kg experimental group compared to both the control and the 40 mg/kg experimental group (P<0.001). Electron microscopic examination revealed an increase in intercellular distance, especially between basal compartment cells, in both experimental groups. Additionally, compared to the control group, both experimental groups showed an increase in the number of lipid-like granules on the membrane, particularly in the cytoplasm of spermatogonia and Sertoli cells.
Conclusion: Based on our observations, this study suggests that exogenous RA can impact the overall histology of the testis. Moreover, it may play a significant role in the meiosis process by influencing the internal dynamics of spermatogenetic cell types.

Keywords: 13-cis-retinoic acid, RARα, spermatogenesis, immunohistochemistry, electron microscopy

How to cite: Şentürk Ş, Sarıoğlu T, Kose T, Uyanıkgil Y. The investigation of retinoic acid on spermatogenetic cell types of rats. J Surg Med. 2024;8(2):22-30.

Introduction

Retinoic acid (RA) serves as the biologically active derivative of vitamin A. It is transported into the nucleus by two receptor families, namely retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which belong to the steroid/thyroid hormone receptor superfamily. Each of these receptor families is further divided into three isotypes: α, β, and γ, each with varying ligand binding affinities. RARs can bind to both all-trans and 9-cis retinoic acid, which are the two major naturally occurring derivatives of vitamin A. In contrast, RXRs exclusively bind to 9-cis RA. Ligands that specifically bind to RXRs are termed rexinoids [1].

In addition to its fundamental role in processes such as vision, growth, and cellular differentiation, RA is also essential for the proper functioning of reproductive organs. Numerous studies have demonstrated that both RA deficiency and excess have significant effects on the spermatogenic cells within the male reproductive organs, specifically the testes. However, the precise mechanisms underlying these effects have not yet been fully elucidated [2-4].

The presence of RARs in the development of male germ cells underscores the biological importance of RA in this process. In a study conducted on adult rats by Huang et al. [5] in 1994, it was observed that RARα was synthesized at varying rates not only in Sertoli cells but also in both spermatides and spermatocytes. The findings of this study suggest that RA may exert direct control over the differentiation of spermatogenic cells or do so indirectly through the actions of Sertoli cells. A deficiency or inactivity of RARα could potentially result in the selective deterioration of germ cells and trigger apoptosis [6]. Additionally, a connection has been proposed between RARs and germ cell apoptosis. In cases of RA deficiency or malfunction of the RARα receptor, male animals may become infertile due to testicle degeneration. Numerous studies have demonstrated that spermatogenesis can be reinstated by administering retinol, an RA precursor, to rats with vitamin A deficiency [7-10].

Our study aims to investigate the dose-dependent effects of RA on spermatogenic cells at histochemical, immunohistochemical, and electron microscopic levels. It is anticipated that our research will provide valuable insights into the role of RA in the mechanism of infertility.

Materials and methods

Experimental protocol

The animal experiments in this study were conducted in accordance with Decree No. 133 of the Local Ethics Committee of Istanbul University. Twenty-four adult male Sprague-Dawley rats, aged 6–8 weeks, were utilized for the study. Three groups, each comprising eight experimental animals, were formed in total. We employed 13-cis-RA (Isotretinoin, Roaccutane®, Hoffmann La Roche Ltd., Basel, Switzerland) in the form of 20 mg soft gelatin capsules for our experiment. To prepare the medication, 20 mg of 13-cis-RA was dissolved in a mixture of 4.5 ml of corn oil and 0.5 ml of ethanol. Each animal in the experimental groups received a daily dose of 1 ml of this medication via gavage feeding 5 days per week over a period of 3 weeks. The control group received normal saline treatment, whereas the experimental groups received 40 mg/kg and 80 mg/kg of 13-cis-RA.

Studies on the reproductive effects of 13-cis-RA have indicated that doses up to 30 mg/kg did not have significant effects on spermatogenesis [11]. However, a dose of 50 mg/kg resulted in testicular weight reduction and decreased spermatogenesis [12]. Hixson et al. [13] administered 40 mg/kg of 13-cis-RA to Sprague-Dawley rats and did not report any signs of toxicity.

At the conclusion of the experiment, the animals were anesthetized using 50 mg/kg of ketamine, and their abdominal cavities were opened. Subsequently, the testes were carefully extracted and immersed in appropriate solutions for examination under both light and electron microscopes.

Histochemical investigation

For light microscope analysis, the extracted testis tissues from the rats were initially placed in Bouin solution for 24 h. Subsequently, routine tissue processing methods were employed, and the tissues were embedded in paraffin. Sections were prepared and then evaluated using hematoxylin-eosin (H&E) and periodic acid Schiff (PAS) staining. The seminiferous tubules of the rats were staged using PAS staining.

RARα immunoreactivity

After dehydration, the 2 µm-thick sections were immersed in a 3% hydrogen methanol solution in the dark for 20 min to quench endogenous peroxidase activity. Subsequently, they were rinsed with phosphate-buffered saline, subjected to antigen retrieval, and then treated with a Kitin Blocking solution. After this blocking step, the sections were incubated with a 1/50-diluted primary antibody, RARα (Santa Cruz), overnight in a humidified container at +4°C.

On the following day, the sections were once again incubated, this time with a Biotinylated Goat Anti-Rabbit Secondary Antibody, in a humidified container for 20 min. Later, Streptavidin Peroxidase was applied for 20 min. Following these procedures, chromogen was applied to the sections in a dark container for 7–10 min. To assess background staining, the sections were counterstained with Ehrlich's Hematoxylin.

Transmission electron microscopy

For ultrastructural analysis, the testis tissues were initially immersed in a cacodylate-buffered solution containing 2.5% glutaraldehyde for fixation, and they were left in this solution for one day. Subsequently, routine tissue processing methods for transmission electron microscopy were carried out.

Statistical analysis

The data obtained in this study are presented as mean (standard deviation). To assess inter-group differences in seminiferous tubule diameters, a one-way analysis of variance (ANOVA) was employed. Subsequently, paired comparisons were analyzed using the Dunnett T3 test. The significance threshold was set at *P*-value <0.05, and the analyses were conducted using IBM SPSS Statistics 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

Results

Histochemical findings

All PAS-stained testis tissues from both the control and experimental groups were staged, and their spermatogenic serial cells were examined. In the control group, we observed spermatogonia and Sertoli cells in the basal compartments of seminiferous tubules, along with primary and secondary spermatocytes, as well as round, elongating, and elongated spermatids in the adluminal compartments (Figure 1a). The basal membrane structure of the seminiferous tubules and the acrosome structures of spermatids were positively stained with PAS (+).

Upon comparing the experimental groups with the control group, we observed a narrowing in the seminiferous tubule epithelium in both the 40 mg/kg-RA and 80 mg/kg-RA groups. Additionally, apart from the narrowing, which could be attributed to germ cell loss, the spermatogenic cells in most seminiferous tubules were found to be deformed (Figure 1b; Figure 1c).

RARα immunoreactivity

The results of immunohistochemical staining, conducted on both the control and experimental groups using the Rabbit Polyclonal Santa Cruz© brand RARα (c-20) antibody, were categorized based on seminiferous tubule sections. Each stage's spermatogenic serial cells and Sertoli cells were assessed individually, and you can find the corresponding findings in Table 1.

In stage VI, it was noted that both Sertoli cells and spermatogonia in the experimental groups exhibited higher RARα immunoreactivity in comparison to the control group. However, the spermatocytes and spermatids in the adluminal compartment exhibited lower immunoreactivity (Figure 2a, b, c).

In stages VII and VIII, both the control and experimental groups exhibited similar levels of RARα immunoreactivity in Sertoli cells, spermatogonia, and spermatocytes, with no significant differences. However, there was an increase in RARα immunoreactivity in spermatids and spermatozoa. Spermatozoa were only observed in stages VII and VIII, and initially, these cells showed no staining. Nevertheless, in stage VIII, a faint staining was observed.

Conversely, the experimental groups that received 40 mg/kg RA and 80 mg/kg RA displayed moderate and strong staining, respectively. In other words, RARα immunoreactivity in spermatozoa was significantly heightened in the RA-administered groups (Figure 3a, b).

In stage XIV, the RARα immunoreactivity in the Sertoli cells and spermatogonia within the basal compartment exhibited moderate staining in the control group. In contrast, both experimental groups displayed strong staining in these cell types. However, the degree of staining in spermatocytes and spermatids remained consistent between the control and experimental groups (Figure 4a, b).

Transmission electron microscopic evaluation

Upon detailed examination of thin sections, it became evident that the basal compartment contained both A and B-type spermatogonia along with Sertoli cells. In the adluminal compartments, primary and secondary spermatocytes were observed, while the region near the lumen contained round, elongating, and elongated spermatids, as well as spermatozoa.

Primary spermatocytes, characterized by type B spermatogonia with circular nuclei, were observed to have nuclei that appeared large and vesicle-like (Figure 5a).

As the largest cells within the seminiferous epithelium, primary spermatocytes were prominent in stages VII to XII (Figure 5b). In stage XIV, various phases of spermatocyte meiosis were observed (Figure 5c). The acrosome caps of round spermatids had expanded to cover approximately one-third of the spermatid head in stage VI and half of the head in stage VII. The Golgi bodies of round spermatids appeared normal (Figure 5d). In stage VIII, the circular nucleus of the spermatid was in contact with the plasma membrane (Figure 5e).

Spermatozoa were only visible in stages VII and VIII, characterized by their distinctive head, neck, and tail structures (Figure 5f). Examination of thin sections during these stages revealed the presence of tail sections, head structures, and residual material related to the spermatozoa within the lumen.

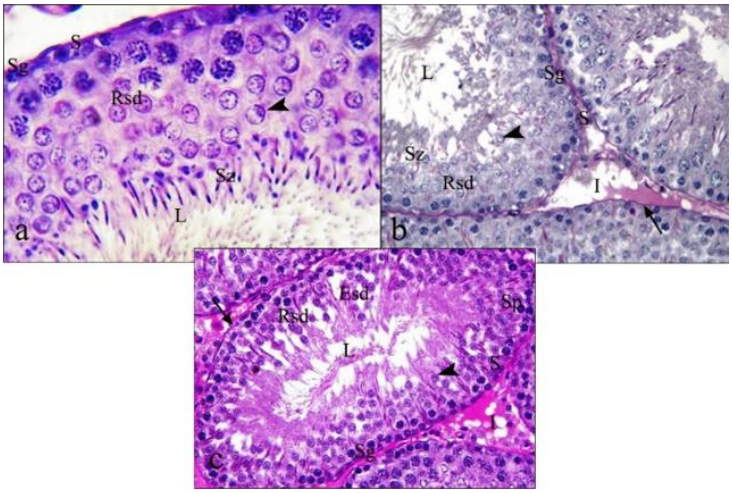
When comparing the semi-thin sections taken from the testes of rats in the 40 mg/kg RA-group with those from the control group, several notable differences were observed. In many stages, it was evident that the intercellular distance between basal compartment cells had increased. Additionally, there was an augmentation in the number of dark-stained, granule-like structures within the cell cytoplasm, particularly in both the basal compartment cells and the cells of the adluminal compartment. This increased intercellular distance among basal compartment cells manifested as cell separation in the thin sections (Figure 6a).

The dark-stained granule-like structures in the semi-thin sections were found to possess membranes when examined under an electron microscope. These sections appeared black in electron microscopy and were hence termed "electron-intense." These structures varied in size, with some being small and numerous while others were larger and less numerous in different locations. In thin sections from stage VIII, the structures within the basal compartments ranged from small to large. Notably, thin sections from stages XI and XIV exhibited a greater number of these structures, and they were larger compared to the control group (Figure 6b).

Primary spermatocytes' nucleus and cytoplasm structures appeared normal, particularly during stages VII-XII. Spermatocytes in various stages of meiosis were also observed to be normal in stage XIV (Figure 6c). Deformation in the cistern appearance of the Golgi body was observed in thin sections from stage VI, which belonged to circular spermatid cells undergoing spermiogenesis. Similarly, in thin sections from stages VII and VIII, deformities in the Golgi bodies of round spermatids were evident, consistent with stage VI (Figure 6d).

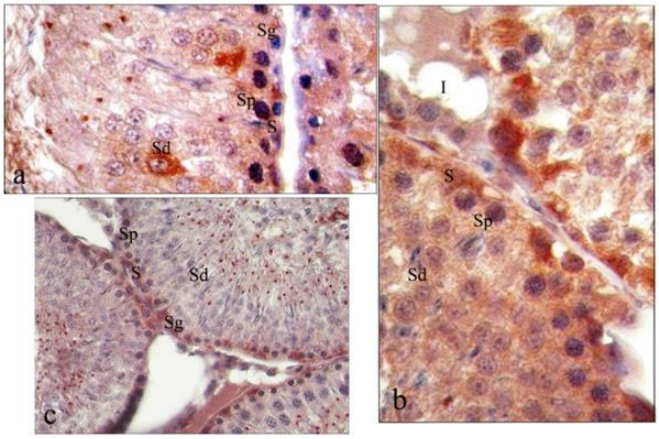
Elongating spermatid cells with oval-shaped heads in stages IX-XI exhibited normal morphology. In thin sections from stages VI and XIV, numerous membrane-enveloped granule-like structures were observed, with these structures being fewer and smaller in volume within the adluminal compartment of the tubule (Figure 6e). In comparison to the control group, there was an increase in the residual material left behind by mature spermatozoa in stage VIII (Figure 6f).

Figure 1: a) Control Group Stage VII Stage; b) 40 mg/kg retinoic acid administrated group VIIth stage; c) 80 mg/kg retinoic acid administrated group VIth stage.



S: Sertoli cell, Sg: spermatogonium, Sp: spermatocyte, Rsd: round spermatid, Esd: elongated spermatid, Sz: spermatozoa, L: lumen, I: interstisium, arrowhead: acrosome, arrow: basal membrane, PAS x100.

Figure 2: a) Control group stage VI; b) 40 mg/kg RA administrated group stage VI; c) 80 mg/kg administrated group stage VI.



S: Sertoli cell, Sg: spermatogonium, Sp: spermatocyte, Sd: spermatid, I: interstisium RARα Immunostaining, ×100.

Figure 3: a) Control group stage VIII; b) 80 mg/kg RA administrated group stage VII. Sz: spermatozoon, RARα İmmunostaining, ×40, ×100 respectively.

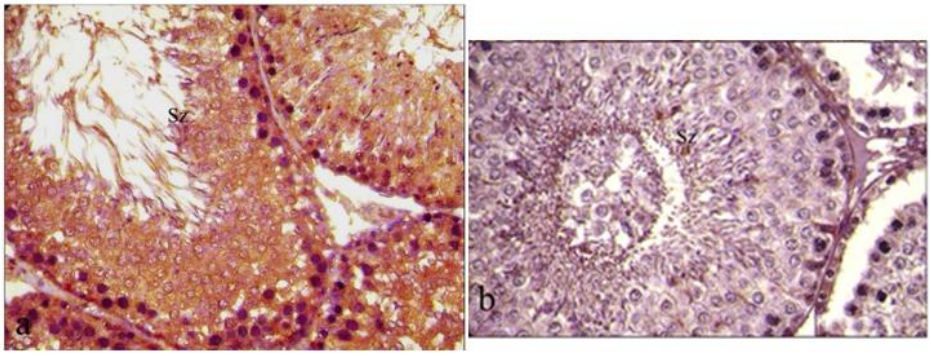


Figure 4: a) Control group stage XIV, b) 80 mg/kg administrated group stage XIV. Sg: spermatogonium, Sp: spermatocyte, Sd: spermatid, L: lumen. RARα İmmunostaining, ×100.

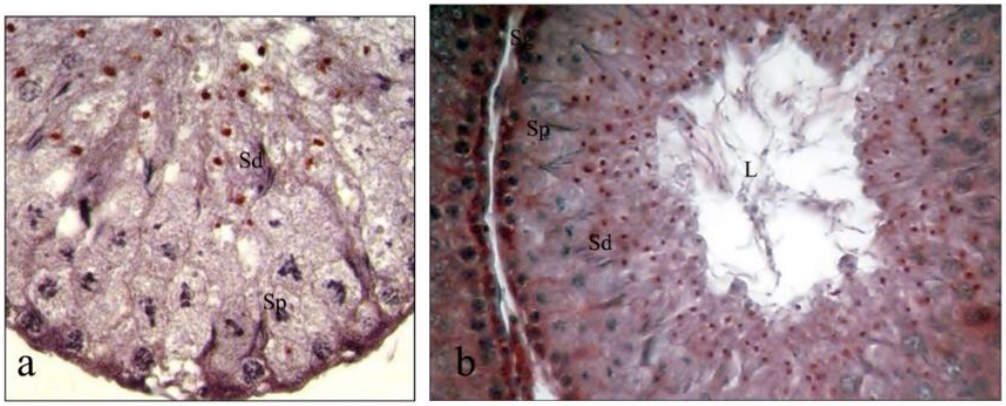
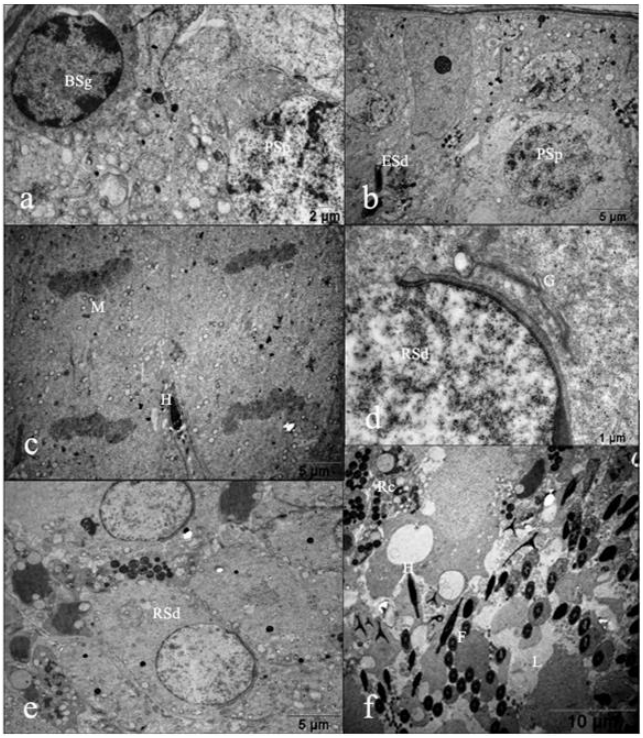
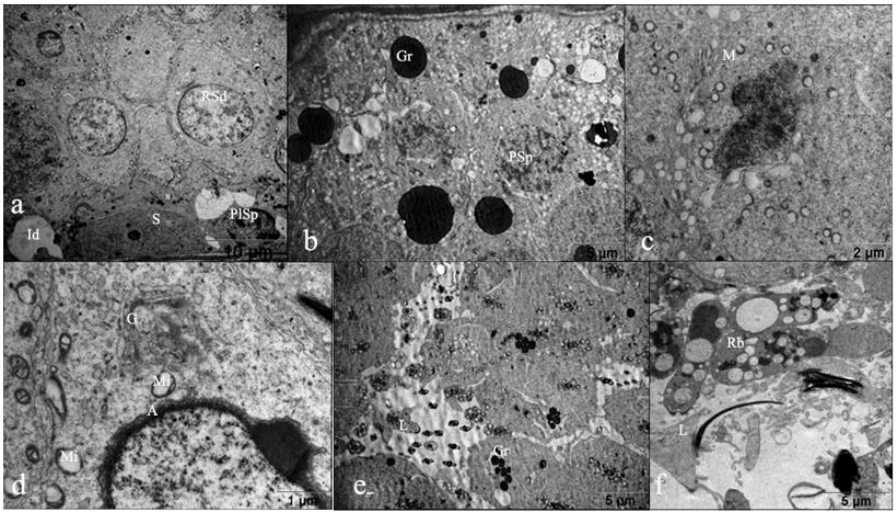


Figure 5: a) Thin section of basal compartment of control group, stage V, b) Thin section of control group, stage V, c) Thin section of Stage XIV meiotic spermatocyte of control group, d) Thin section of stage VII of control group, e) Thin section of stage VIII of control group, f) Thin section of stage VIII of control group.



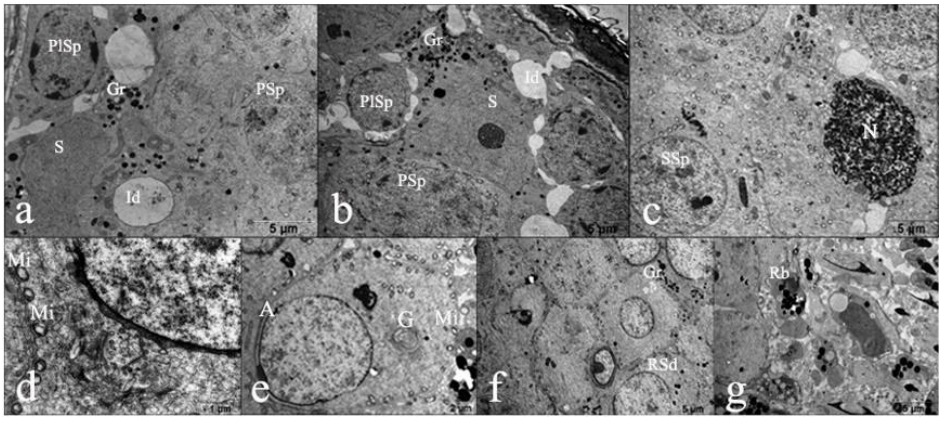
BSg: B-type spermatogonium, PSp: pakiten spermatocyte, ESd: elongated spermatid, H: Head of elongated spermatid (5c), M: Meiotic spermatocyte, Rsd: round spermatid, G: Golgi body, F: Cross section of flagellum of spermatozoon, H: Head of spermatozoon (5f), Rc: residual body, L: lumen.

Figure 6: a) Thin section of stage VII basal compartment of 40 mg/kg RA administrated group, b) Thin section of stage XI basal compartment of 40 mg/kg RA administrated group, c) Thin section of stage XIV meiotic spermatocyte of 40 mg/kg RA administrated group, d) Thin section of stage VII round spermatid of 40 mg/kg RA administrated group, e) Thin section of stage XIV lumen of 40 mg/kg RA administrated group, f) Thin section of stage VIII lumen of 40 mg/kg RA administrated group.



S: Sertoli cell, PlSp: preleptoten spermatocyte, PSp: pachytene spermatocyte, RSd: round spermatid, Id: intercellular distance, Gr: granul, M: Meiotic spermatocyte, G: Golgi body, Mi: mitochondrion, A: acrosome, L: lumen, Rb: residual body.

Figure 7: a) Thin section of stage VI of 80 mg/kg RA administrated group, b) Thin section of stage VIII basal compartment of 80 mg/kg RA administrated group. c)Thin section of stage XIV of 80 mg/kg RA administrated group. d)Thin section of stage VII round spermatid of 80 mg/kg RA administrated group. e) Thin section of stage VIII round spermatid of 80 mg/kg RA administrated group, f) Thin section of stage VIII of 80 mg/kg RA administrated group, g) Thin section of stage VIII lumen of 80 mg/kg RA administrated group



S: Sertoli cell, PlSp: preleptoten spermatocyte, PSp: pachytene spermatocyte, N: necrotic spermatocyte, SSp: seconder spermatocyte, RSd: round spermatid, Id: intercellular distance, Gr: granule, G: Golgi body, Mi: mitochondrion, A: acrosome, Rb: residual body.

Table 1: Qualitative immunoreactivity evaluation of spermatogenetic cell types belonging to control and experimental groups.

	Control					40 mg/kg RA administrated group					80 mg/kg RA administrated group				
Stages	Sertoli cells	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa	Sertoli cells	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa	Sertoli cells	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa
I.	+	+	-	+		+	+	-	+		+	+	-	+	
II-III.	+	+	-	+		+	+	+	+		+	+	+	+	
IV-V.	++	±	-	++		++	+	±	++		++	+	±	++	
VI.	+	+	++	++		++	++	+	+		++	++	+	+	
VII.	++	++	+	±	-	++	++	+	+	+	++	++	++	+	++
VIII.	++	++	++	±	±	++	++	++	+	+	++	++	++	+	++
IX.	±	±	+	-		±	±	±	-		-	-	±	-	
X.	-	-	+	+		-	-	+	+		-	-	+	+	
XI.	-	-	-	+		-	-	-	+		-	-	-	+	
XII.	-	-	-	+		-	-	-	+		-	-	-	+	
XIII.	-	+	-	+		-	+	-	+		-	+	-	+	
XIV.	+	+	±	++		++	++	±	++		++	++	+	++	

(-): no staining, (±): weak immunoreactivity, (+): moderate immunoreactivity, (++): strong immunoreactivity

When comparing the thin sections obtained from rats in the 80 mg/kg-RA group with those from the control group, a significant increase in the intercellular distance between basal compartment cells, as observed in the other experimental group, was noted. Furthermore, the dark-stained granule-like structures seen in adluminal compartment cells were also evident in this group, particularly within basal compartment cells. In thin sections from stage VI of the 80 mg/kg-RA group, the membrane-enveloped granular structures appeared small in size and were numerous, especially in basal compartment cells, coinciding with the extension of the intercellular distance (Figure 7a). Similar deformations were observed in stages VII and VIII, mirroring the observations made in stage VI (Figure 7b).

In contrast, the membrane-enveloped granular structures within the basal compartment appeared small and rare in thin sections from stage XIV when compared to the other experimental group. Both primary spermatocytes, the largest cells within the seminiferous epithelia, and secondary spermatocytes, which can only be observed in stage XIV during various stages of meiosis, displayed a normal morphology. In close proximity to these cells, a necrotic meiotic cell was observed (Figure 7c). Deformation of the Golgi body in round spermatids during stages VI, VII, and VIII was also pronounced in this experimental group, as in the other group (Figure 7d, e). Particularly notable in the basal compartment, membrane-enveloped granule-like structures were small in size and numerous in the cytoplasm of round spermatids during stage VIII (Figure 7f).

Elongating spermatid cells, characterized by their rounding spermatid heads, as well as elongated spermatids, displayed a normal morphological appearance. In comparison to the control group, there was an increase in the number of residual structures left behind by spermatozoa in stages VII and VIII (Figure 7g).

Statistical analysis

The diameters of seminiferous tubules were measured in microns using the Image-Pro Express 4.5 program (Media Cybernetics, Inc., USA). We measured the diameters of ten seminiferous tubules randomly selected from prepared samples of each animal. Four diameter measurements were conducted for each seminiferous tubule, and the arithmetic mean of these measurements was calculated. Subsequently, the obtained values underwent statistical analysis (Table 2a).

Based on the one-way ANOVA, there was a statistically significant difference in the average diameters of seminiferous tubules (P<0.001). Subsequent paired comparisons were conducted using the Dunnett T3 test following the unilateral variance analysis. The results of this test revealed a statistically significant difference when comparing the 80 mg/kg-group with the other groups (P<0.001). However, no statistically significant difference was observed when comparing the 40 mg/kg-RA group with the control group (P=0.147) (Table 2b).

Table 2A: Seminiferous tubules diameter measurement analysis of control and experimental groups.

Group	Mean	SD	n
Control	277.46	35.49	113
40 mg/kg	266.95	45.31	116
80 mg/kg	204.32	36.73	117
Total	249.20	50.96	346

SD: Standard Deviation, One-Way ANOVA, P<0.001

Table 2B: Pairwise comparison of seminiferous tubules diameter means between control and experimental groups with Dunnett T3 test.

Group	Control	40 mg/kg
40 mg/kg	0.147	
80 mg/kg	<0.001	<0.001

Dunnett T3 test, The Dependent Variable: Tubulus Diameter

Discussion

RA, the active metabolite of Vitamin A, plays a crucial role in various essential physiological processes within the body. It serves as a vital signal molecule for normal fetal development, cell proliferation, and differentiation [14]. Vitamin A metabolism is also pivotal for proper spermatogenesis, requiring precise regulation of RA availability. This regulation is essential for spermatogonial differentiation, maintaining the blood-testis barrier function, initiating meiosis, and facilitating proper spermiation [15]. Research has indicated that the highest expression of RARα occurs in round spermatids during stage VIII of the spermatogenetic cycle in adult rats. During stages IX to XI, RARα is predominantly found in the nuclei of elongating spermatids rather than elongated ones, and it is also present in

germ cells undergoing prophase of meiosis, with lower expression in Sertoli cells [16]. The same study emphasizes the role of RARα in Sertoli cells during testis development and its impact on the transformation of round spermatids to elongating spermatids during the meiosis stage of spermatogenesis.

In our study, where we examined RARα immunoreactivity, we observed that Sertoli cells and spermatogonia displayed either moderate or strong staining intensity in stages I-VIII. However, in stage IX, they exhibited weak staining. By stage XIV, all cells displayed staining. Spermatocytes in stages VI-X and spermatids, except for stage IX, exhibited either moderate or strong staining, while spermatozoa in stage VIII showed weak staining.

When a testis becomes completely deficient in RA, the only germ cells found within the seminiferous tubules are undifferentiated spermatogonia. However, when RA is reintroduced into this environment, spermatogenesis resumes normally, albeit in a synchronized manner [17-18]. A recent study has demonstrated that a reduced RA environment within the testes compromises the integrity of the blood-testis barrier and leads to an increased number of meiotic defects, both of which negatively impact fertility [19]. Targeted mutagenesis of the RARα gene has revealed its significant role in spermatogenesis. While cells in all stages of spermatogenesis were still present in RAR-/- testes, there was an elevated occurrence of degenerating pachytene spermatocytes and a temporary developmental arrest in step 8–9 spermatids during the first wave of spermatogenesis.

Additionally, a delay in the onset of the second wave and a temporary arrest in preleptotene to leptotene spermatocytes were observed in the first, second, and third waves. On another note, issues were reported regarding the alignment of spermatozoa in the lumen during stage VIII of the mutant phenotype. In vivo BrdU labeling indicates a significant decrease in germ cell proliferation in both juvenile and adult RARα-/- testes, confirming a halt in spermatids at steps 8 and 9. Consequently, retinoid signal transduction through RARα is deemed crucial for the synchronous progression of spermatogenesis and the formation of the cellular body [20].

In a study conducted by Boulogne et al. [21], the cellular distribution of RAR and RXRs in rats was examined through immunohistochemistry from fetal day 13.5 to postnatal day 8, with comparisons made to the findings in adult rat testes. RARα exhibited pronounced staining in the interstitial connective tissue on fetal day 14.5 and in gonocytes from fetal day 20.5 until postnatal day 8. By the eighth postnatal day, nuclei of all cell types displayed a faint RARα staining. The immunostaining of these receptors predominantly appeared in the cytoplasmic regions of fetal and neonatal testicles, whereas it was notably localized within the nuclei of adult testicles. In our study, however, RARα immunoreactivity was observed as nuclear staining in some spermatogenetic cells, although it was generally observed as cytoplasmic staining.

In a study, it was noted that the rate of spermatogenesis and the diameter of seminiferous tubules exhibited a significant decrease compared to the control group when rats were intraperitoneally administered 6 mg of 13-cis-RA three times a week for 6 weeks [2]. In contrast, the study conducted by Livera et al. [22] introduced selective agonists and antagonists of RARs or RXRs into an organotypical culture system to discern which receptors of RA influenced Leydig, Sertoli, and germ cell development. It was determined that, aside from RAR β predominantly affecting Sertoli cell proliferation, all the effects of RA on fetal and neonatal testicle development were mediated by RAR α. Similar to its RA agonist, RARα disrupted the arrangement of testis cords that had been established on the 14.5th postnatal day and reduced the diameter of testis cords cultivated on the third postnatal day. When comparing the average seminiferous tubule diameters of testis sections between our control and experimental groups, the decrease observed in the 80 mg/kg-RA group was statistically significant compared to both the control group and the 40 mg/kg-RA group.

It has been reported that oral administration of a pan-RAR antagonist inhibited spermatogenesis in mice even at low doses (2.5 mg/kg for 4 weeks) with no discernible side effects other than abnormal testicular histology. Importantly, the impaired spermatogenesis and induced sterility were reversible. Such an impact on fertility suggests that testes are highly sensitive to disruptions in retinoid signaling, and these receptors could be potential targets for pharmacological interventions in male contraception [23]. In a related study [24] by the same research team [23], the effects of daily doses as low as 1.0 mg/kg were examined over dosing periods of 4, 8, and 16 weeks. In all regimens, 100% sterility was observed, with fertility being restored upon discontinuation of the drug treatment, even after 16 weeks. There were no changes in testosterone levels in these males, and the offspring examined from two of the recovered males were healthy and fertile, with normal testicular weight and histology.

Snyder et al. [4] reported that exogenous RA leads to germ cell apoptosis, particularly in type A spermatogonia, and the loss of round spermatids in stages VII and VIII. In another study conducted by Gençoğlan and Tosun [3], which aimed to examine the dose- and time-dependent effects of 13-cis-RA on spermatogenesis in rats, three groups were formed. The experimental groups were daily administered 1 mg/mL and 2 mg/mL of isotretinoin for 21 days, and they exhibited an increase in p53-positive cells compared to the control group. According to the conclusions of this study, it was suggested that long-term treatment with high doses of retinoids could be employed as a method of birth control for men. On the contrary, a study conducted by Ismail et al. [25] reported that rats with vitamin A deficiency experienced testicular deterioration, spermatogenetic cycle arrest at stage VIII, cell apoptosis, and the disappearance of many germ cells at various stages of differentiation. In our study, the observed narrowing in the seminiferous tubule epithelium, possibly attributed to germ cell loss, aligns with the findings of the study mentioned above.

Numerous studies have underscored the pivotal role of RA in germ cell differentiation [26-27]. In a study conducted by Anderson et al. [26] involving juvenile rats of inbred C57BL/6 genetic origin, it was highlighted that the gene Stra8 was essential for initiating meiosis in testis germ cells. Stra8 serves as a distinct cytoplasmic factor in vertebrates, expressed by germ cells in response to RA. The expression of Stra8, a crucial factor for successful meiosis in spermatogenesis, is directly linked to

the presence of RA. Abdelghani et al. [28] identified Stra8 transcripts on the basal surface of seminiferous tubules through their in situ hybridization (ISH) analyses. In the same study, the distribution of this protein in the cryostat sections of adult rat testis was demonstrated at the immunoelectron microscopic level using anti-Stra8 antibodies. Intensive immunoperoxidase staining was also observed in the cytoplasm of germ cells in contact with the basal lamina.

In a study by Zhou et al. [27] aimed at examining the effects of exogenous RA, 8-day-old rats were injected with 350 µg of all-trans RA, while normal adult rats received 750 µg of it. The peak expression of Stra8 mRNA correlated with the onset of meiosis in postnatal testicles. In this study, the earliest detection of Stra8 protein in gonocytes was on postnatal day 5. The expression of Stra8 protein in neonatal testes was not consistent among spermatogonia, potentially indicating the precursor stage of asynchronous spermatogenesis. The highest levels of Stra8 mRNA and protein in seminiferous epithelia were found between stages VI-VIII. The Stra8 protein was identified in certain type A and B spermatogonia, preleptonen spermatocytes, and early leptotene spermatocytes. In vitamin A-sufficient adult rats, RA induced stra8 mRNA expression rather than retinol. In adult spermatogonia, RA stimulated the expression of the Stra8 protein, confirming its role in spermatogonial differentiation. Additionally, RA increased the number of preleptotene spermatocytes, indicating heightened synchronized premeiotic DNA replication. All the studies mentioned above investigating Stra8 transcript and protein induction by retinoic acid and their role in meiosis initiation yielded findings parallel to our study, which also concluded that RA is necessary for the initiation of meiosis, as evidenced by the increase in basal compartment cells in stage VI when the first meiosis begins in rats, based on the immunohistochemical analysis of the RARα protein—one of the nuclear receptors for RA.

Chung et al. [29] conducted a study examining the effects of defective retinoid signal transduction on cell-cell interactions in rats. They demonstrated that hypertonic fixation treatment of RARα-deficient testis tubules disrupted the integrity of Sertoli cell barriers, leading to abnormal intercellular connections during the transition from the basal to adluminal compartments in stain-transfer experiments. This study raises the possibility that the defective spermiogenesis observed in RARα-deficient testicles may result from the deficient cyclic expression of structural RA components, inadequate regulation of connexin-40 within cells, and delayed involvement of zona occludens-1 in Sertoli cell junction complexes.

In a study by Russell et al. [30], cytochalasin D was used to disrupt the actin filaments, which are essential cytoskeletal components of Sertoli cells, affecting spermatids. It was reported that this treatment led to an 88% reduction in the ectoplasmic specialization of the head section of round spermatids in stage VIII compared to the control group. The study also suggested that cytochalasin D treatment caused Sertoli cells to detach from round spermatids, particularly in areas where ectoplasmic specialization was lost on the Sertoli cell surface. Actin in the ectoplasmic specialization area played a significant role in cell-cell interactions. Following cytochalasin D treatment, there was a 5.8-fold increase in the Sertoli cell basal surface orientation of spermatid acrosomes in stage VIII, with the ectoplasmic specialization area playing a crucial role in this orientation.

Taking the findings of these studies into account collectively, it is evident that the RA administered to rats through gavage disrupted Sertoli cell junction complexes, resulting in an increased intercellular distance. This, in turn, accelerated spermiation and led to the accumulation of residual material in the lumen during stages VII-VIII.

In a study conducted by Wu et al. [31], they observed a noteworthy change in the Golgi body of a human glioma cell culture that had been exposed to all-trans RA. Specifically, the Golgi body disappeared, and perinuclear vacuolization occurred. This led to the recognition of the possibility that retinoids could directly influence intracellular traffic by affecting the Golgi body.

In light of this study and the electron microscopic findings in our own research, it is worth noting that the Golgi body in round spermatids at stages VI, VII, and VIII in the experimental groups exhibited a distinct cysterna-like deformation, particularly in the cytoplasmic region near the nucleus.

In a study conducted by Kastner et al. [32] on RXRβ mutant rats, electron microscopy revealed abnormalities in epididymal spermatozoa. Specifically, mutants exhibited an increased occurrence of acrosomes that had detached from the nuclear membrane compared to the wild type, and some spermatozoa lacked acrosomes altogether. It was proposed that these abnormalities, more prevalent in mutant spermatozoa, hindered the proper binding of the acrosomal membrane to the nucleus. Additionally, the large and round vacuoles located in the periphery of the seminiferous tubules in the testes were found to be highly osmophilic in semi-thin sections, indicating their lipid content.

In contrast, electron microscopy revealed that these lipid droplets were devoid of membranes within the Sertoli cell cytoplasm. In our study, we observed granular structures resembling lipid droplets in the basal compartments of the seminiferous tubules in semi-thin sections. However, in electron microscopy examination, these structures were observed to be enveloped by a membrane. Notably, these structures were widespread in basal compartment cells and showed an increased prevalence in the experimental groups.

Conclusion

In conclusion, when we integrate these observations with the results of our study, several key points emerge. Firstly, the impact of exogenous RA on the adult rat testis appears to be dose-dependent. This dosing effect potentially leads to a reduction in the diameter of seminiferous tubules due to germ cell loss. Secondly, there is an indication that the cellular distribution of RARα protein may undergo alterations, influencing its immunoreactivity strength and potentially playing a pivotal role in the initiation of meiosis. Lastly, it is plausible that this mechanism also affects intracellular traffic, possibly through its influence on the Golgi body.

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