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Gene and histomorphology alteration analysis in spermatogenesis arrest mouse model: a probable novel approach for infertility

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Introduction Approximately 15% of couples in the reproductive age are struggling with infertility which, in nearly half of them, is caused by male factors.
Material and methods The present study comprised of two groups of sixteen C57BL/6 mice; each mouse received either an intraperitoneal injection of 30 mg/kg of an alkylating agent or the same amount of distilled water. Testes were harvested 30 days following the injection. Morphometric analysis of hematoxylin and eosin (H&E) stained slides including mean tubular area, diameter and intratubular particles were performed. Spermatogenesis rate was assessed by spermatogonial markers including promyelocytic leukemia zinc finger protein (PLZF) and neurogenin-3 (NGN3). Moreover, the expression rate of Wilms Tumor-1 (WT-1), A-Kinase Anchoring Protein 4 (AKAP4) and adenosine deaminase domain containing 1 (ADAD1) genes were evaluated via real-time polymerase chain reaction (RT-PCR).
Results The body weight gradually increased in both groups after a period of 30 days, however, the increase was significantly (p-value = 0.023) lower in the chemically treated group. All the morphometric parameters were considerably decreased in the azoospermic mice. Also, promyelocytic leukemia zinc finger protein and neurogenin-3 expression dramatically declined (p-value <0.001 for both markers). In comparison with the negative control group, the expression rates of A-Kinase Anchoring Protein 4 and adenosine deaminase domain containing 1, two genes participating in the sperm structure, were remarkably reduced in the intervention group (p-value <0.001); however, our investigations demonstrated that the azoospermia model could induce a 5-fold upregulation in Wilms Tumor-1 gene expression.
Conclusions Development of an azoospermia model can upregulate Wilms Tumor-1 gene expression in a higher rate after 30 days; however, expression of the testis-specific genes, A-Kinase Anchoring Protein 4 and adenosine deaminase domain containing 1, decreased after the intervention. To the best of our knowledge, this upregulation could be related to spermatogenesis recovery after the follow-up period.

INTRODUCTION

Approximately 15% of couples in the reproductive age are struggling with infertility issues which are caused by male factors in nearly fifty percent of patients [1]. Different conditions are considered as the main triggers including varicocele, obesity, genetic factors, lifestyle-related factors and spinal cord injuries [2–5].

Busulfan (1,4-butanediol dimethanesulfonate) is an alkylating agent preventing the cell division by attaching to one of the DNA strands [6]. Organs, tissues

and cells with high division rates like germline cells are influenced by this agent. Applying the same properties of this alkylating agent for the treatment of chronic myeloid leukemia, it is employed as a potent factor to deplete mice testes stem cells [7, 8, 9]. According to the disparate impact of different doses on the body and the testes, detection of the optimal dose is indispensable to achieve the most efficient outcomes [10, 11].

Spermatogenesis is a rather complicated process, producing abundant spermatozoa from a paucity of stem cells [12]. In mature mice, spermatogonial stem cells are limited to undifferentiated type A spermatogonia underlying on the basement membrane of the testicular seminiferous tubules. This subcategory involves A_{single} (As) spermatogonia and their daughter cells, $A_{aligned}$ (Aal) and A_{paired} (Apr) spermatogonia [13, 14]. With the assistance of immunohistochemistry, different genes and markers including promyelocytic leukemia zinc finger protein (PLZF), neurogenin-3 (NGN3), SRY-box transcription factor 3 (SOX3), GDNF family receptor alpha-1 (GFRα1) and Notch homolog 1, translocation-associated (NOTCH1) have been specified for type A spermatogonia [15]. The promyelocytic leukemia zinc finger protein expression in A_{single}, $A_{aligned}$ and A_{paired} spermatogonia, has a prominent function in spermatogonial stem cells reproduction regulation [16, 17]. Neurogenin-3 is a transcription factor mainly functioning in the initial stages of the spermatogenesis; therefore, it could be an important factor to study the sperm production ratios [18].

Wilms Tumor-1 (WT-1) is a consequential gene in gonads formation with an imperative role in constitution of the urogenital system, and its mutation could result in gonadal agenesis and renal failure [19]. Wilms Tumor-1 is exclusively expressed by Sertoli cells in the epithelium of testes seminiferous tubules. Moreover, endothelial cells of small vessels and mesenchymal cells in embryonic and neonatal testes tissues possess the same feature [20, 21]. Wilms Tumor-1 gene suppression in Sertoli cells leads to decreased number of sperm, representing the remarkable role of this gene in the spermatogenesis [22]. Adenosine deaminase domain containing 1 (ADAD1) gene, detected in the spermatogenesis process, is testis-specific and is involved in different stages of meiosis [23]. Mutations in the aforementioned gene are understood to affect spermatogenesis and can cause infertility [24]. A-Kinase Anchoring Protein 4 (AKAP4) is the first known gene of its own kind participating in the structure of sperm flagellum and fibrous sheath [25]. The level of this gene's expression has been substantiated to be lower in infertile men with non-obstructive azoospermia compared to a normal fertile group [26]. As in the Baccetti's study, the model of A-Kinase Anchoring Protein 4 gene knockout demonstrated that the absence of it could result in structural disturbances and amorphous sperm; however, no decrease in the sperm count was reported [27].

A literature review regarding spermatogenesis arrest was performed and, as far as we know, no study has been carried out to investigate the regulatory genes expression: Wilms Tumor-1, A-Kinase Anchoring Protein 4 and adenosine deaminase domain containing 1 in the azoospermic murine model testis. In this study, we intended to evaluate the effects of a chemotherapy agent on the content of type A spermatogonial cells and primary genes expression rates in the mouse testis. In addition, we introduced an efficient model to pave the path for future studies and therapeutic strategies requiring spermatogenesis cessation and clearance of testes from germ cells.

MATERIAL AND METHODS

Animals

A total of 32 male C57BL/6 mice were maintained in the animal facility at the Children's Medical Center Hospital of Tehran University School of Medicine under an approved institutional animal care and use committee protocol. Mice in age ranging from 8 to 12 weeks' years of old and 26 to 36 grams in weight, were enrolled in the present study, and divided randomly into two groups of 16. The average temperature was 24°C, with a 12-h light-dark cycle.

Azoospermia development

The mice were randomly allotted to two groups of control and injection group. Initially, busulfan (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and then the same amount of distilled water was added to reach a final aqueous solution of 30 mg/kg. All of the 15 mice in the injection group received an intraperitoneal injection of the aforementioned compound. In the control group, 10 ml of distilled water was inoculated in the same site. Thirty days after injection, mice were initially weighed and thereafter sacrificed after which both testes were excised in a sterile setting and their other organs were collected. Subsequently, other organs of the mice were collected for use in other studies at the facility.

Hematoxylin and eosin staining

The extracted testes were preserved in formaldehyde and then a paraffin embedding process was performed

on the tissues. Sections of 5 microns were prepared and stained with hematoxylin and eosin (Abcam, Germany) for light microscopic examinations.

Immunohistochemistry staining

From the provided blocks, sections were obtained, deparaffinized by heating to 60°C for 1 hour, followed by three passages in xylenes for 10 minutes and rehydrated by the aid of absolute, 90% and 80% alcohol for 3 minutes, respectively. For further rehydration, slides were placed in phosphate-buffered saline (PBS) wash bath for 30 minutes at room temperature. For antigen retrieval, sections were incubated in sodium citrate in a microwave oven for 10 minutes at 100°C. Endogenous peroxidase activity and non-specific binding sites were blocked by application of 3% hydrogen peroxide and 5% goat serum on slides, separately. After performing the later steps, the slides were treated with antibodies against promyelocytic leukemia zinc finger protein (Abcam, Germany) and neurogenin-3 (Abcam, Germany) overnight at 4°C. The following day, 3 phosphate-buffered saline washes were done by incubation for 1 hour at room temperature in biotinylated horse-anti-rabbit and rabbit-anti-goat IgG (Abcam, Germany), afterwards colorimetric detection was performed using ABC Vectastain Elite reagents with 3,3′-diaminobenzidine (DAB) (Abcam, Germany). Sections were counterstained with hematoxylin (Abcam, Germany) for 2 minutes, then were rinsed gently with distilled water and running tap water for 5 minutes. The slides were mounted using glycerol gelatin. Images of stained sections were captured using an Olympus BX-51 microscope, afterwards expression rates of mentioned antibodies in each group were determined.

4′,6-diamidino-2-phenylindole staining

Considering the penetration of 4′,6-diamidino-2-phenylindole (DAPI, Abcam, Germany) through an intact cell membrane and its extreme binding nature to DNA, this staining of the prepared tissues was carried out to support the immunohistochemistry (IHC) and hematoxylin and eosin staining results. New slices were obtained from the embedded tissues and were deparaffinized at 56°C for 15 minutes. The slides were transferred to xylene, alcohol series of 100%, 96%, 80% and 50% and finally deionized water (DW) respectively. A phosphate-buffered saline bath was considered prior to introduction of 4′,6-diamidino-2-phenylindole. Slides were incubated in 4′,6-diamidino-2-phenylindole solution for 10 minutes followed by three times of deionized water wash for 10 minutes each. Finally, the tissue

sections were covered with mounting solution and coverslips and were stored in a dark place at 4°C. Images of established sections were captured using a fluorescence microscope.

Morphometric analysis

Hematoxylin and eosin stained slides' images were imported to the ImageJ software (IMAGEJ Software, United States) for further analysis regarding the tubular area, diameter and intratubular particles. For entire measurements, tubules with a circular or relatively semi-circular shape from all over the slide were selected. The selected tubules margins were delineated and the area of them were determined. Subsequently the diameter of each tubule was calculated using the area formula:

$$A = \pi \times D^2 / 4$$

The exact number of cells within the tubules including spermatogonial stem cells, spermatogonia, spermatocytes, spermatids and sperm cells were counted and the acquired data were analyzed for cell depletion ascertainment.

RNA extraction

The RNA was extracted from control and treated tissues using Hybrid-R™ Mini kit GeneAll, Republic of Korea) according to the manufacturer's guidelines. Quality identification of extracted RNA was implemented by electrophoresis on agarose gel containing GelRed™ (Biotium, USA) and was measured its absorption on A260/280 nm by NanoDrop® ND1000 spectrophotometer (Isogen Life Science, The Netherlands).

cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA extracted from the samples was reverse transcribed using HyperScript™ First-strand Synthesis Kit (GeneAll, Republic of Korea) under the guide of manufacturer's instructions. 10 μl of total RNA, 1 μl deoxyribose nucleoside triphosphate (d-NTP), 1 μl Random hexamer, 2 μl real-time reaction buffer (10X), 2μl Nuclease-free water, 2 μl MDTT (0.1), 2 μl HyperScript Reverse Transcriptase (200 U/μl) and 1 μl ZymAll™ RNase Inhibitor were included in the cDNA synthesis reactions. The total mentioned agents (20 μl) were incubated for 5 min at 65°C, 1 minute on ice, 40 minutes at 50°C, 5 minutes at 85°C, and were kept at 4°C. Subsequently real-time polymerase chain reaction was fulfilled employing SYBR®

Premix Ex Taq™ II (Dalian, Takara Co., Ltd), produced cDNA and eligible primers (Table 1). Real-time PCR reactions were included a total of twenty μl containing 10 μl SYBR® Premix Ex Taq™ II, 1 μl genomic template DNA, 7.6 μl nuclease-free water, 0.4 μl Rox, 0.5 μl forward primer, 0.5 μl reverse primers. Ensued process was driven on the ABI StepOne Plus real-time polymerase chain reaction system (Applied Biosystems Company, USA): one cycle of 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds and finally 70°C for 15 seconds. Real-time polymerase chain reaction was conducted in optical 8-cap strips (MicroAmp® Applied Biosystems, Singapore). As a housekeeping gene, the β-actin was administered for normalizing the 8 amplification. $2^{-\Delta\Delta Ct}$ method regarding the calculation of the relative amounts of polymerase chain reaction product and StepOne Software Version 2.2.2 (Applied Biosystems, USA) in terms of the quality of graphs, melting curves and quantitative analyses of the data, assisted us.

Statistical analysis

All the data were analyzed by SPSS software version 20.0 for Windows (SPSS Inc., Chicago, IL, USA).
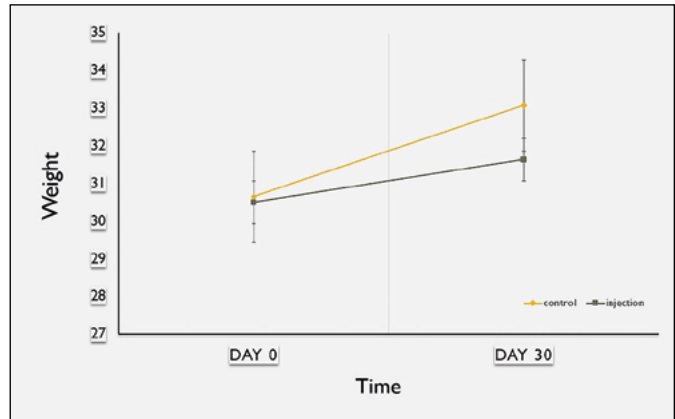

Figure 1. *Changes in body weight of C57BL/6 mice over time after injection.*

Table 1. *Primers used in real time-polymerase chain reaction*

No.	Gene name	Accession number	Primer Sequences 5' → 3'
1	Actin Beta	NM_007393.5	5'- GTGACGTTGACATCCGTAAAGA-3' 5'- GCCGGACTCATCGTACTCC-3'
2	Wilms Tumor-1	NM_144783.2	5'- AATGACCTCCCAGCTTGAATG-3' 5'- CCGTGGGTGTGTATTCTGTACT-3'
3	Adenosine Deaminase Domain Containing 1	NM_009350.3	5'- CCAGTATGCCAAGATTTCAC-3' 5'- GTAGTTGTATTCGCCTGTGC-3'
4	A-Kinase Anchoring Protein 4	NM_001042542.2	5'- GACAACAAGATCAGGACCGA-3' 5'- TGAAAAGGCACACAGATCCC-3'

Table 2. *The changes in area, diameter and intratubular particles of seminiferous tubules after a treatment period of 30 days. As shown in the column of p-value, all the parameters in the intervention group compared to the control group were significantly decreased*

	Control group	Azoospermia group	P-value
Area (μm²)	26390 ±7172.47393	16569 ±3120.45778	0.035
Diameter (μm)	182.076 ±23.45817	143.0559 ±13.17943	0.016
Intratubular particles (number)	284 ±53.24941	25 ±3.83116	<0.001

Table 3. *Comparison of spermatogenesis related genes expression rate in two groups of C57BL/6 mice. The expression rates of the 3 genes were significantly changed after the 30-day interval. As demonstrated in the table, Wilms Tumor-1 expression had a 5-fold upregulation compared to the control group; though, two other testis-specific genes were meaningfully downregulated*

Genes	Control group (n = 16)	Azoospermia group (n = 16)	p-value
Wilms Tumor-1	1.0223 ±0.10292	5.2504 ±3.23865	<0.001
A-Kinase Anchoring Protein 4	1.0230 ±0.04571	0.0293 ±0.10396	<0.001
Adenosine Deaminase Domain Containing 1	1.0029 ±0.09934	0.0020 ±0.00125	<0.001

n – number

A p-value <0.05 was considered statistically significant.

RESULTS

Body weight

The animals were weighed followed by randomized selection and prior to sacrificing. As illustrated in Figure 1, it seemed that after a 30-day interval, a slight increase in weight was observed in both groups of mice; although, this enhancement was remarkably lower in azoospermic mice (p-value = 0.023).

Histopathologic evaluations

The light microscopy images of hematoxylin and eosin staining demonstrated the germ cell depleting effect of the agent on murine testes. Fully spermatogenic tubules including all generations of spermatogonial stem cells turned into hollow and vacant lumens, thus, as displayed in Figure 2, almost no germ line cells were detected within the seminiferous tubules of azoospermia induced mice. In order to proclaim this prominent effect on the germ cells, of the primary blocks, additional slides were stained with 4′,6-diamidino-2-phenylindole. Owing to the DNA-binding nature of 4′,6-diamidino-2-phenylindole and the illustrated fact of empty tubules derived from hematoxylin and eosin images, depleted tubules were scintillated much less under the fluorescent microscope (Figure 3).

Morphometric analysis

The mean area, derived diameter and intratubular particles of each group were calculated. As shown in Table 2, all of the aforementioned parameters in the intervention group compared to control group were significantly decreased, thus with the seal of approval
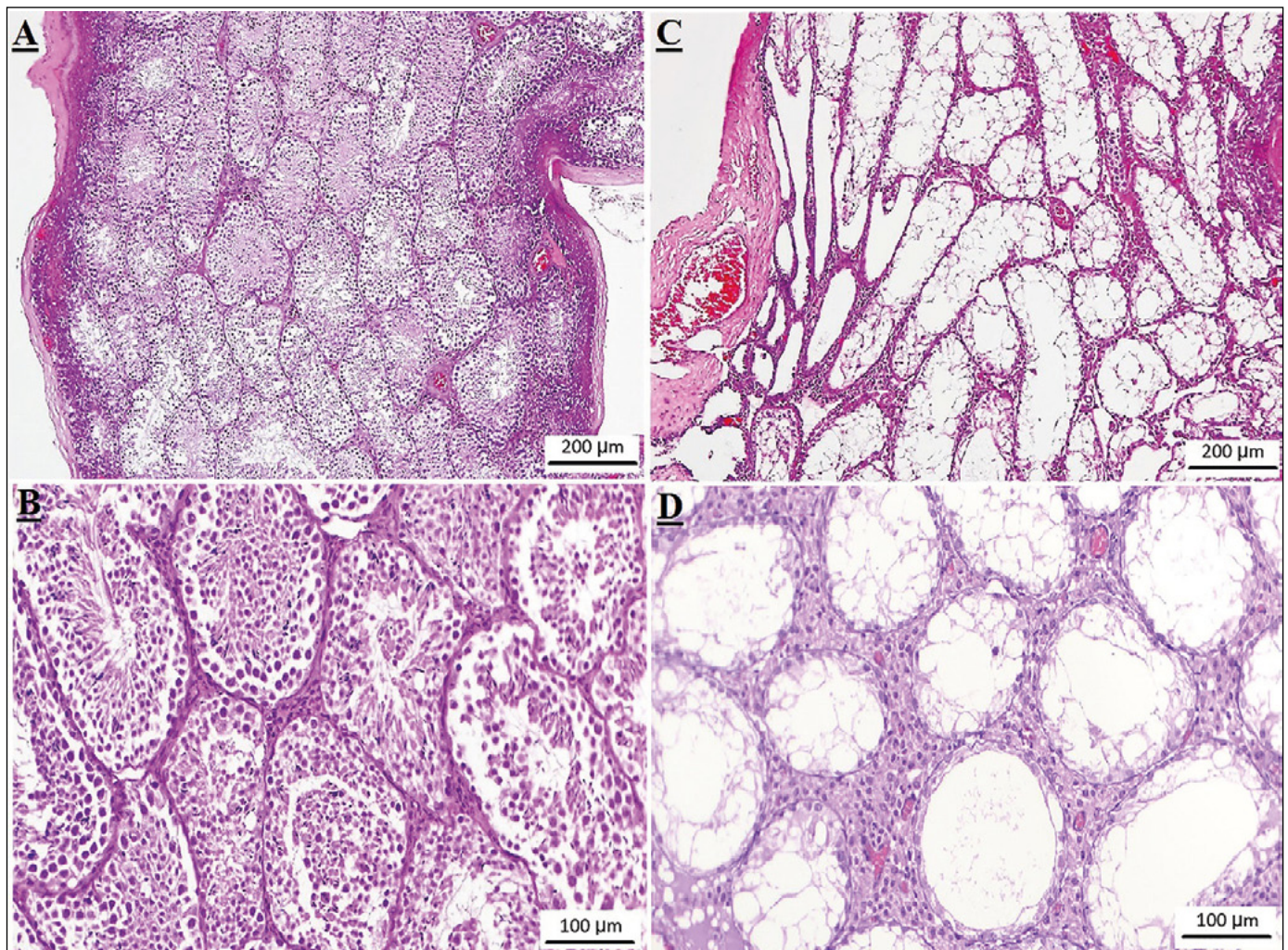

Figure 2. *Hematoxylin and eosin stained sections of testis. (A and B): Control group, (C and D): intervention group.*
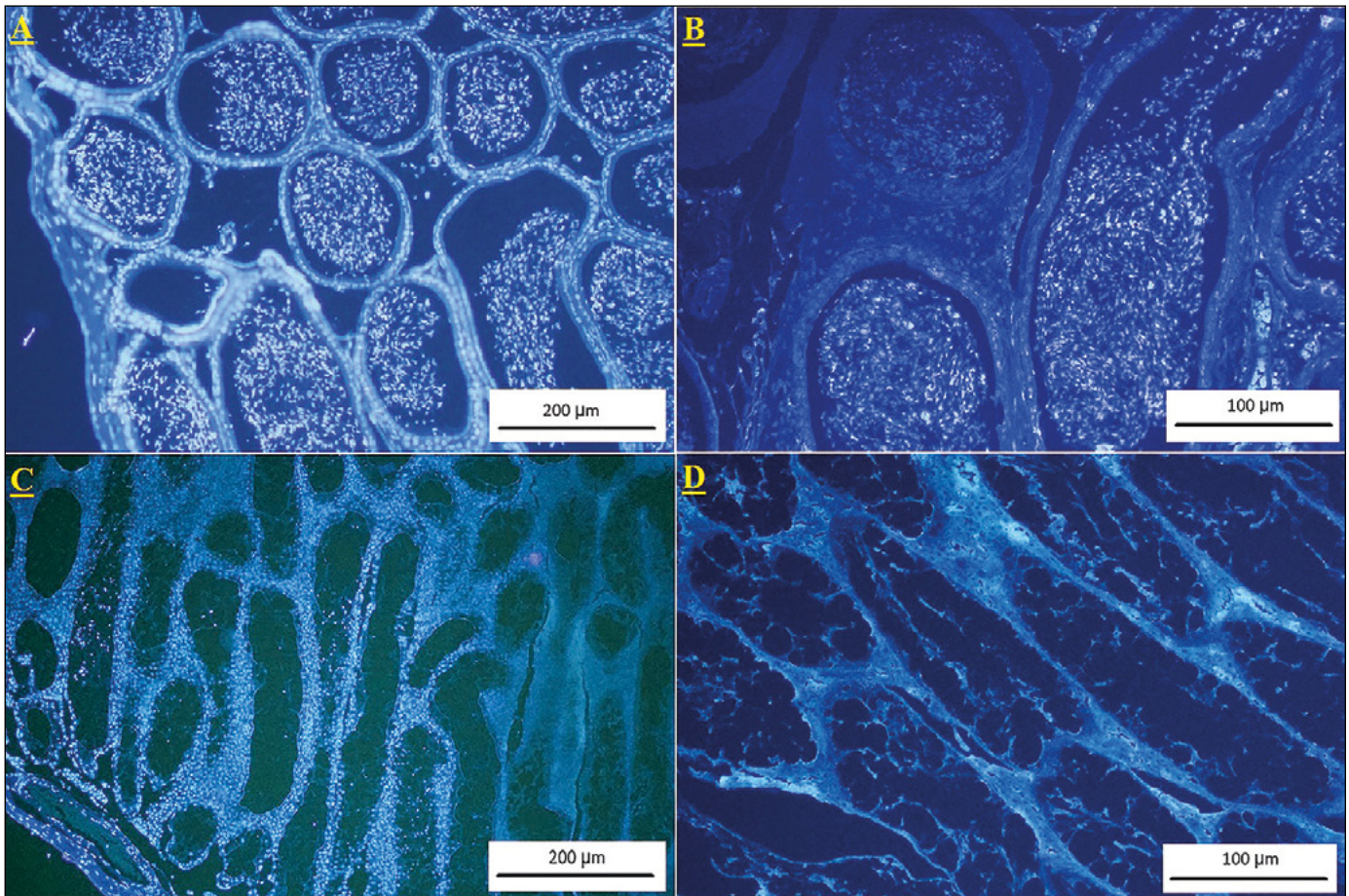
Figure 3. *4',6-diamidino-2-phenylindole stained sections of testis. (A and B): Control group, (C and D): intervention group.*

of later analysis, administration of an alkylating agent made the tubules smaller in area and diameter; moreover, the evaluations demonstrated its destructive effect on germ line cells.

Immunohistochemistry analysis

Promyelocytic leukemia zinc finger protein and neurogenin-3 were applied as specific markers to locate the spermatogonia underlying the basement membrane of seminiferous tubules which were traced by immunohistochemistry. Expression of Promyelocytic leukemia zinc finger protein and neurogenin-3 are displayed in Figure 4. According to Figure 5, expression rates of two markers in the seminiferous tubules of intervention group in contrast to control group were considerably diminished (p-value <0.001 for both).

Genes expression

Since the β-actin extensively has an equivalent expression rate in the community and different samples and is not affected under the pathologic and

therapeutic conditions, regarding the molecular tests in this study β-actin was chosen as the house keeping gene; additionally, the target genes expression rates were corrected and normalized through this gene. After preparation of the samples by MasterMix, the optimal temperature, acquisition of distinct peak and products melting curve for all primers were determined in order to minimize the unspecific products. Following completion of molecular tests, obtained melting and amplification curves were evaluated; consequently, expression intensity, product concentration and amplified product degree of purity were acquired.

All genes' melting point was 84–87°C and just a single peak was seen in every curve, demonstrating the target gene specific proliferation and no production of unspecific output. Eventually gene expression results were represented as mean±standard error of means and were analyzed by SPSS software version 24. The final outcome is provided in Table 3.

According to Table 1, Wilms Tumor-1 gene expression in the testes of azoospermic mice interestingly had an approximately 5-fold upregulation compared to mice
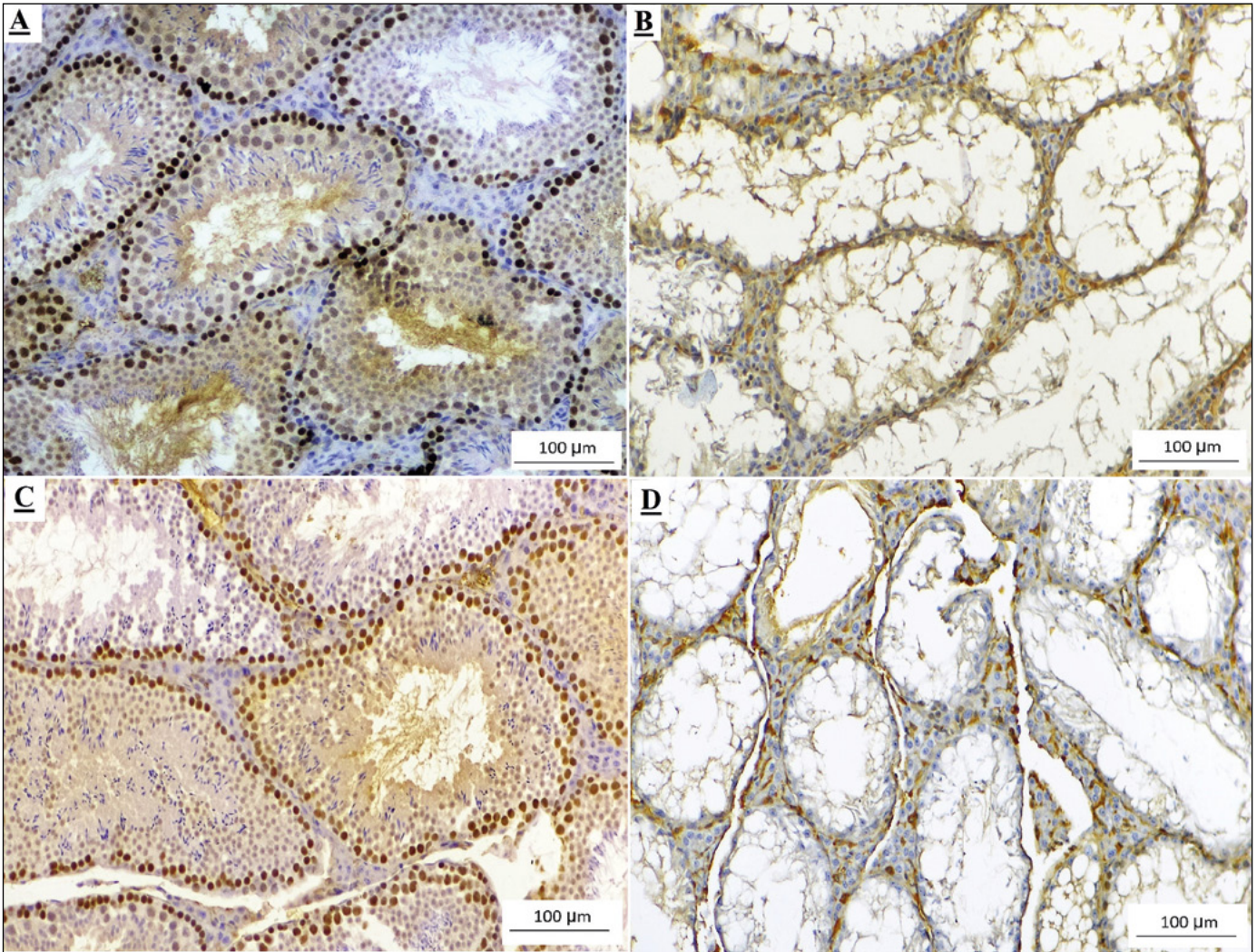
Figure 4. *Immunohistochemistry staining of promyelocytic leukemia zinc finger protein and neurogenin-3 in testis of C57BL/6 mice.* **(A):** *expression of promyelocytic leukemia zinc finger protein in testis of control group,* **(B)** *expression of promyelocytic leukemia zinc finger protein in testis of azoospermic mice,* **(C)** *expression of neurogenin-3 in testis of control group,* **(D)** *expression of neurogenin-3 in testis of azoospermia induced mice.*
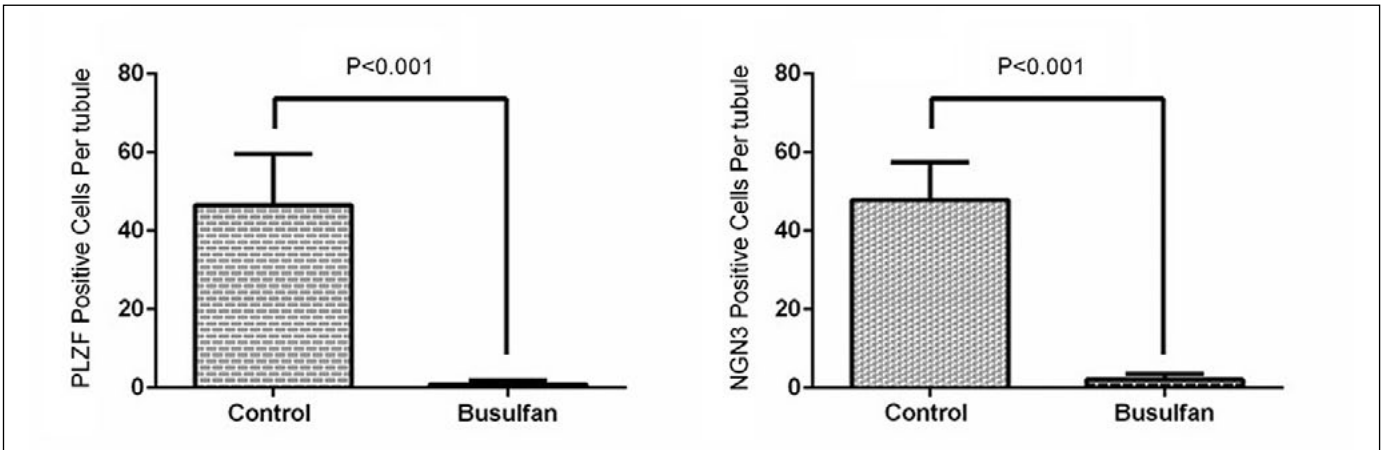

Figure 5. *Comparative expression rate of promyelocytic leukemia zinc finger protein and neurogenin-3 in two groups. Both of the markers were significantly downregulated (p-value <0.001).*
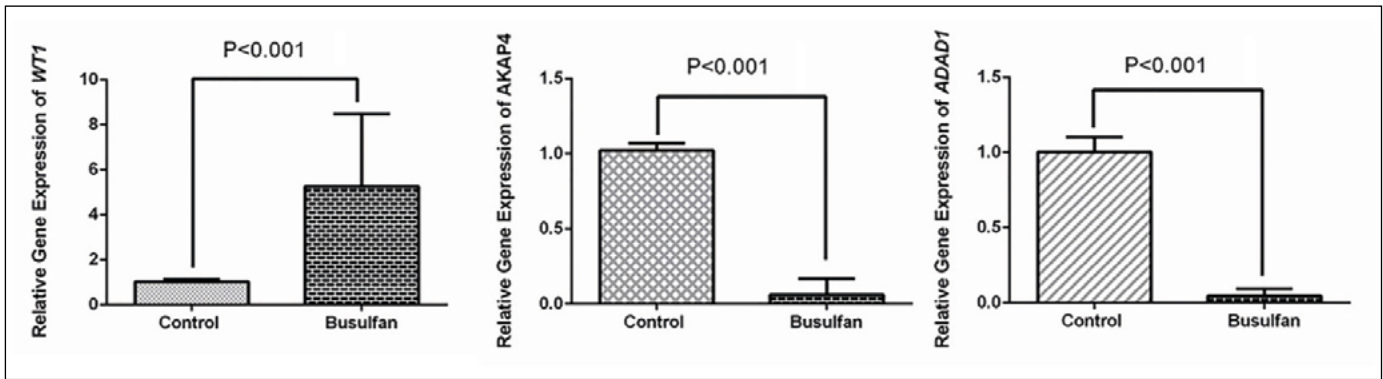
Figure 6. *Relative expression of Wilms Tumor-1, A-Kinase Anchoring Protein 4 and Adenosine deaminase domain containing 1 genes in control and intervention groups. Wilms Tumor-1 expression demonstrated a significant rise; while, A-Kinase Anchoring Protein 4 and adenosine deaminase domain containing 1 remarkably downregulated in the azoospermia group.*

in the control group. Meanwhile, on the contrary, the two other purposed genes, A-Kinase Anchoring Protein 4 and adenosine deaminase domain containing 1 expression exhibited a notable downregulation in the same mice and all the genes' expression changes were statistically meaningful. The genes expression rate diagrams are presented in Figure 6.

DISCUSSION

Our findings cast a new light on testis-specific genes expression ratio in an azoospermic mice model. The outcomes of this experiment illustrated an efficient method of chemically induced azoospermia for infertility simulation in small animal models.
In the study designed by Jiang et al., 10 mg/kg of a chemotherapeutic agent was administered intraperitoneally to Long-Evans male rats with weights ranging from 250–300 grams. Our results tie well with a later study wherein there was a mutual weight gain in both groups of animals but the increment in the chemical exposed rats was significantly lesser than the control group [28]. In line with the previous study and ours, Choi et al. reached consistent results towards weight changes by utilizing 30–40 grams Institute of Cancer Research (ICR) mice [29]. Partly in contrary to our results in this theme, study of Wang et al. referring to azoospermia model development using BALB/c mice by different single doses of 10, 20, 30, 40, 50 and a double dose of 10 mg/kg of the same agent showed the subjacent weight increment of the intervention group; though, no statistical significance had established (11).
When it comes to morphometric and stereological changes of the testis structure, the study of Panahi et al. emerges. Three groups of Sprague-Dawley rats were enrolled in the study, the first group was injected by single dose of busulfan (10 mg/kg), the

second group received double doses of busulfan with 21 days' interval and the third group experienced no treatment. At 35 days after the final injection, the testes were extracted and stereological analyses were performed. Tubular, cellular and luminal area and the diameter of seminiferous tubules were calculated. All of these parameters in the one injection group, as well as the luminal area and the diameter of the two injection group, were meaningfully increased compared with the control group. Nevertheless, only the cellular area and diameter of the two injection group had a considerable decrease; besides, no statistical significance was reported regarding the total area and diameter of the two injection group compared to the control group. When comparing our morphometric results to those of the former study, it must be pointed out that there is an unexpressive connection among these two. Panahi et al. concluded that this index was gradually decreased due to the increased dose of the chemical agent and the amount of this decrement was notable among the three groups; this finding is in accordance with our results regarding the intratubular particles [30].
To the best of our knowledge, this is the first investigation evaluating the spermatogenesis regulating genes, Wilms Tumor-1, A-Kinase Anchoring Protein 4 and adenosine deaminase domain containing 1 expression in an azoospermia model; nevertheless, studies related to the suppression of the aforementioned genes or knockout model and their effects on spermatogenesis have been previously conducted. Wang's group, by detecting 6 major mutations in Wilms Tumor-1 gene, generated an inactivation model of this gene resulting in a massive germ cell loss and testicular atrophy; while only the Sertoli cells were remained. Their investigations indicated that Wilms Tumor-1 is essential for spermatogenesis and Sertoli cells polarity [31]. In another study con-

ducted by Fang et al. indicating a murine A-Kinase Anchoring Protein 4 knockout model, testes and sperms motility were examined. In their study, spermatogenesis did not cease to the extent of chemically induced azoospermia; nonetheless, the sperm viability was totally diminished and all of the sperm motility parameters showed a considerable decrease [25]. A major limitation to our investigation is lack of administration of a regenerative agent following the testicular germ cell depletion. We believe that future studies could be focused on regeneration of the provided disease model.

CONCLUSIONS

In this study, we established an azoospermia murine model with a 30 mg/kg intraperitoneal dose of a chemical agent. According to our evaluations, this was confirmed as an efficient technique, and an environment fairly devoid of host germ cells was provided. In the developed animal model, the expression rate of two spermatogonia-specific proteins and two genes contributed in sperm structure were thoroughly abated; even though, the expression rate of Wilms Tumor-1 showed an approximate 5-fold upregulation. According to the previously noted studies and the reversible nature of this kind of azoospermia, we believe that Wilms Tumor-1 gene was engaged in the first stages of this regenerative process and maybe had a pivotal role in the recommencement of spermatogenesis. From this standpoint, suppression of Wilms Tumor-1 gene can be considered as an approach to maintain the spermatogenesis arrest models; moreover, its expression stimulation probably could provide a novel approaching strategy for the burdensome problem of infertility; although, until now, no study has been performed to properly confirm this hypothesis. Hence, further investigations are needed to vividly elucidate the underlying mechanism of this phenomenon and its plausible assistance to medical research.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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