

Original article

## Blood-derived exosomes with anti-inflammatory properties as a new minimally invasive intratesticular therapy for aflatoxin B1-associated chronic testopathy

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**Abstract:** *Rationale* — Aflatoxin B1 (AB1) is a dangerous toxin causing severe poisoning and even death in human. Testopathy or even infertility is one of the adverse effects of AB1. Exosomes (Ex) with anti-inflammatory action are known as biological substances derived from body fluids. This experimental study aimed to investigate the possible therapeutic effects of blood-derived Ex on orchitis caused by chronic exposure to AB1.

*Methods* — Male rats (n=18) were distributed among three groups: control, AB1 and AB1+Ex. Orchitis was induced with AB1 (45 µL/kg of rat weight) for 40 days (3 times a week). A day later, 10 IU Ex were injected intratesticularly. A week later, testicular tissue and blood serum were sampled. To confirm the therapeutic effects of Ex, inflammatory genes (TNF-α, IL-6 and IL-10), nitric oxide (NO), malondialdehyde (MDA), testosterone (Tes) and sperm quality were fully evaluated. In addition, H&E staining was used to investigate tissue changes, and immunohistochemical (IHC) assessment was employed to detect the inflammatory protein, galectin-3. The results were analyzed using SPSS (v.19).

*Results and Discussion* — Our results confirmed that Ex therapy can significantly (p<0.05) increase normal morphology (25.01±2.23 in the AB1+Ex group vs. 10.1±0.9 in the AB1 group), number (76.12±6.7 in the AB1+Ex group vs. 27.8±3.2 in AB1 group), and vitality (53.4±6.12 in AB1+Ex vs. 41.9±4.6 in AB1) of sperm cells. Blood-derived Ex significantly (p<0.05) reduced the expression of inflammatory genes (6-fold change was observed in AB1+Ex vs. 17-fold in AB1 for TNF-α, 3-fold change was detected in AB1+Ex vs. 6-fold in AB1 for IL-6, and 2-fold change took place in AB1+Ex vs. 6-fold in AB1 for IL-10), NO (23.1±2.6 in AB1+Ex vs. 62.8±8 0.1 in AB1) and MDA (33.2±4.9 in AB1+Ex vs. 68.9±5.46 in AB1), and increased Tes level (7.1±0.5 in AB1+Ex vs. 1.14±0.3 in AB1) in AB1-affected rats. Histopathological evaluations revealed tissue regeneration after Ex injection. Also, the produced sperm cells were of high quality.

*Conclusion* — Intratesticular injection of Ex from the blood can be considered as a novel anti-inflammatory therapy after AB1-induced orchitis.

**Keywords:** reproductive, aflatoxin B, testopathy, orchitis, blood serum, exosome.

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### Introduction

Numerous scientific explorations are aimed at maintaining high-quality health status and reproductive capacity. Currently, reproduction and sexual health are considered important concepts in medical science. Any damage to the reproductive system can endanger human life or the quality of the resulting embryos. Meanwhile, the male reproductive system is given special attention. Disorders of male reproductive system can lead to poor sperm quality. Consequently, to improve sexual health, it is

necessary to identify factors that damage testicular tissue and develop a modern protocol for the treatment of comorbidities [1].

Aflatoxin B1 (AB1) is an inherently harmful poison produced by *Aspergillus flavus* and *Aspergillus parasiticus* [2]. This toxin, after intraperitoneal injection, is known as a carcinogen with an average toxicity index of 60 mg/kg of mice weight [3]. AB1 can contaminate corn, peanuts, and other grains [4], as well as animal feed, and plays an important role in causing liver cancer in humans [5]. Animal experiments demonstrated that AB1 is also a mutagenic [6], teratogenic [7] and immunosuppressing agent [8].

Testicular tissue is a sensitive organ in men exposed to various environmental factors, such as trauma, chemicals, drugs, and toxins [9]. Testicular inflammation is an acute or chronic process leading to orchitis. It can occur due to various reasons, mainly exposure to toxins. AB1, in addition to harmful effects on the liver and kidneys, can also cause inflammation of the testicles [10]. Due to the thick fibrous covering surrounding the testis, the tunica albuginea, any acute inflammation of the testes can cause tissue compression and reduced blood flow to the testicles. Following these pathological changes in the testicles, the level of oxidative stress increases and testicular dysfunction occurs. Testosterone (Tes) production may also be impaired and therefore sperm production may be reduced. These pathological conditions lead to azoospermia and male infertility [11].

At present, much scientific attention is paid to the use of contemporary methods of treatment based on natural biological materials [12]. Exosomes (Ex), which are biological materials surrounded by bilayer phospholipids, naturally carry a variety of biological agents, including growth factors, anti-inflammatory factors and immunomodulators. An example of this biological material is blood-derived Ex. The anti-inflammatory properties of these biological substances have been stated in several published sources [13].

According to the above reports, the pathological condition of orchitis was developing with the introduction of AB1. In addition, to confirm the anti-inflammatory and therapeutic effects of blood-derived exosomes, they were injected into the scrotum of AB1-treated rats. Thus, the objective of our experimental study was evaluating the anti-inflammatory effect of blood-derived Ex on the alleviation of testopathy caused by the introduction of AB1 into the testes of rats.

## Material and Methods

### Animals

Experimental animals (rats) were provided from the Animal Breeding Center of the Pasteur Institute (Tehran, Iran). Their mean age and weight were  $8 \pm 1$  weeks and  $150 \pm 20$  g, respectively. The necessary living conditions were provided, including ambient temperature of  $37.5^\circ\text{C}$ , humidity of 30%, 12:12 (light – dark) photocycle, and free access to food and water. Manipulations with animals were carried out in accordance with the ethical principles of the 1975 Declaration of Helsinki (year 2000 revision) [14].

### Experimental modeling of orchitis with aflatoxin B1

According to Murad et al. (2015), AB1 (Fermentek, CAS NO:1162-65-8) with a maximum toxic dose of  $45 \mu\text{L}/\text{kg}$  of rat weight was administered 3 times a week for 40 days to finally induce testicular inflammation [15].

### Extraction of blood-derived exosomes

For high efficiency Ex extraction from blood serum, the protocol by Caradec et al. (2014) was employed via using an Ex-extraction kit (Exo-spin™, 8 columns, Cell Guidance System, Cat EX01) [16]. Separation of Ex was applied according to the physical characteristics of the droplets, including precipitation chromatography and size exclusion chromatography. Blood was aspirated from the right ventricle of adult rats (with the same gender, weight and age characteristics as the animals used in this study) and centrifuged twice ( $4,000 \text{ rpm}$ , 15 min) to isolate serum.

The resulting blood serum was again centrifuged five times ( $12,000 \text{ rpm}$ , 30 min) to remove cell debris and possible soluble erythrocytes. Finally, pure blood serum containing Ex was precipitated using Exo-spin™ Buffer. Phosphate-buffered saline (PBS) was used for rinsing the column matrix. The prepared solution contained the highest concentration of Ex with acceptable purity. Approximately 30 IU of a solution containing Ex was obtained after aspiration of 5 mL of blood according to the appropriate protocol. Finally, each animal affected by AB1 was injected with 10 IU of blood-derived Ex.

### Study groups and treatment protocol

Adult male rats ( $n=18$ ) were randomly distributed among three groups ( $n=6$  in each group): Normal (healthy control without experimental treatment), AB1 (disease control with a 40-day oral AB1 exposure), and AB1+Ex (treatment group; animals with AB1-induced orchitis were injected with a single intrascrotal dose of Ex). According to the protocol by Murad et al. (2015), AB1 was administered orally (3 times a week) at a maximum dose of  $45 \mu\text{L}/\text{kg}$  of rat body weight for 40 days. One day after the induction of AB1 testicular toxicity, the treatment with Ex was carried out at a dose of 10 IU administered intratesticularly as part of a minimally invasive procedure. After seven days, testicular tissue was dissected and analyzed in the laboratory settings [17].

### Animal dissection and tissue sampling

One week after treatment with Ex, rats were injected intraperitoneally with 70 IU of 40% ketamine/60% xylazine. After induction of deep anesthesia, the animals were sacrificed by inhalation of chloroform. A thoracotomy was quickly performed, blood was aspirated (10 mL) and centrifuged (15 min,  $4,000 \text{ rpm}$ ) for serological analyses. The right testicle was dissected and fixed in 10% formalin for histological examination, while the left testicle was frozen in liquid nitrogen for genetic evaluation. In addition, the epididymis was placed in a Petri dish (containing 10 mL of 5% DMEMF12/FBS culture medium) to collect the semen content [18].

### Assessment of sperm health factors

Sperm health factors were assessed based on four different indices according to Hosseinipour et al. protocol (2019): viability, number, morphology, and motility. Evaluation was conducted using a microscope ( $40\times$ ) and the eosin/nigrosin staining procedure was employed for sperm imaging [19].

### Evaluation of nitric oxide as a marker of oxidative stress

Elevated NO levels constitute the main factor in the pathological conditions of cells after their exposure to AB1. NO content was measured using Griess analysis according to the protocol used by Roshankhah et al. (2020). This technique used an ELISA kit (Griess reagent, Abcam, ab234044) with various dilutions of NO (wavelength  $540 \text{ nm}$ ) [20].

### Evaluation of malondialdehyde as a marker of oxidative stress

Malondialdehyde (MDA) is an organic reactive agent that indicates the level of lipid peroxidation in the blood serum. Accordingly, thiobarbituric acid reactive substances (TBARS) were measured using the MDA assay (MDA Assay Kit,

Colorimetric/Fluorometric, ab118970) according to the respective protocol [20].

#### Testosterone level as an indicator of Leydig cell damage

To assess possible damage to Leydig cells present in testicular interstitial tissue, an appropriate Tes ELISA kit (Abcam, CAT NO; 108666) was prepared for our study to measure Tes levels at the wavelength of 450 nm [20].

#### Hematoxylin and eosin staining for histopathological changes

After fixation of the right testis in 10% formalin, tissue samples were treated in various solutions of alcohol (for tissue dehydration), xylene (for tissue clearing), and paraffin (for tissue infiltration and embedding). A microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH; Germany) was used to prepare thin tissue sections (5 μm) followed by staining with hematoxylin and eosin (H&E) [21].

#### Protocol for immunohistochemical staining of the galectin-3 protein

We used an immunohistochemical (IHC) assessment of galectin-3 as an inflammatory testicular marker. Prepared histological sections of the testes were used for IHC staining of galectin-3. Hydrogen peroxide (0.3%) was used to inhibit endogenous peroxidase (10 min, in methanol). Then, non-target antibodies were blocked (in 5% bovine serum albumin, BSA, 30 min). Next, the section was incubated with rabbit anti-galectin-3 polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, USA) overnight at 4°C. This was followed by incubation with horseradish peroxidase (HRP) donkey IgG (H&L) (60 min). Peroxidase activity was detected by the 3,3'-diaminobenzidinetetrahydrochloride (DAB)-H<sub>2</sub>O<sub>2</sub> reaction. Slides were incubated with DAB (0.5 mg/mL PBS) containing 0.005% H<sub>2</sub>O<sub>2</sub> (10 min). Finally, the slides were counterstained with hematoxylin [22].

#### Evaluation of inflammatory gene expression by quantitative polymerase chain reaction

To assess the level of expression of inflammatory genes using quantitative polymerase chain reaction (qPCR), we measured three factors including TNF-α [23], IL-6 [24], and IL-10 [25]. The left testis was dissected and frozen with liquid nitrogen. The tissue was homogenized using the sonication procedure. The purity of tissue RNAs was then assessed using the NanoDrop spectrophotometer (260/280 nm). DNA synthesis was applied followed by real-time qPCR assay. Gene expression was measured and reported using the comparative Ct (2<sup>-ΔΔCt</sup>) method (Table 1).

#### Statistical data processing

After numerical data were collected, the normal distribution was evaluated using the Kolmogorov-Smirnov test performed with SPSS v.16 software. Data heterogeneity was assessed to ensure a normal distribution. Also, in some cases, laboratory experiments were repeated to obtain homogeneous data. One-way analysis of variance (one-way ANOVA) was used for statistical analysis, while Tukey's post hoc test was employed to determine differences between groups. The final plots were built using the GraphPad Prism software. The results were expressed as mean ± standard deviation (SD), and the level of significance was considered at p<0.05.

**Table 1. Inflammatory genes in testicular tissue and related primers. β-actin was considered an internal control**

Inflammatory Genes	Forward	Reverse	Primers
TNF-α	Forward	Reverse	CTTCTCCTTCTGATCGTGG GCTGGTTATCTCTCAGCTCCA
	Forward	Reverse	AAAGAGGCACCTGGCAGAAACAAC TTAAAGCTGCGCAGAATGAGATGA
IL-6	Forward	Reverse	GCCTAACATGCTTCGAGATC CTCATGGCTTTGTAGATGCC
	Forward	Reverse	GGCACCACACCTTCTACAATG GGGGTGTGAAGGTCTCAAC

TNF-α, tumor necrosis factor; IL-6, interleukin 6; IL-10, interleukin 10.

**Table 2. Parameters of sperm health in the control group, and groups AB1 and AB1+Ex**

Groups	Normal morphology (%)	Count (10 <sup>6</sup> )	Motility (%)	Vitality (%)
Control	32.4±2.13	76.12±8.16	58.13±7.1	71.01±5.01
AB1	10.1±0.9 *	27.8±3.2 *	23±1.03 *	41.9±4.6 *
AB1+Ex	25.01±2.23 **	67.12±6.7 **	49.3±4.2 **	53.4±6.12 **

Data were presented as mean±SD, \* indicates p<0.05 in AB1 group vs. the control; \*\* indicates p<0.05 in AB1+Ex group vs. AB1. N=18 rats in each group. AB1, aflatoxin B<sub>1</sub>; Ex, exosome.

**Table 3. Impact of AB1 and Ex on serological indices**

Groups	NO (OD)	Tes (ng/m)	MDA (nm/gKW)
Control	12.2±6.3	10.1±1.2	12.32±0.2
AB1	62.8±8.1 *	1.14±0.3 *	68.9±5.46 *
AB1+Ex	23.1±2.6 **	7.1±0.5 **	33.2±4.9 **

Data were reported as mean±SD, \* indicates p<0.05 in AB1 group vs. the control; \*\* indicates p<0.05 in AB1+Ex group vs. AB1. N=18 rats in each group. NO, nitric oxide; OD, optical density; Tes, testosterone; MDA, malondialdehyde.

## Results

### Change in sperm quality after injection of exosomes

The results in Table 2 regarding the quality of sperm after AB1 administration imply that all sperm parameters including morphology (p=0.01), number (p=0.001), motility (p=0.02) and vitality (p=0.03) were affected and statistically significantly reduced vs. the control group. This finding points to the destructive effect of AB1 on sperm parameters. Our results also demonstrated that after a single injection of Ex in rats treated with AB1 (AB1+Ex group), all sperm quality parameters had significant (p=0.01 for normal morphology, p=0.001 for quantity, p=0.04 for motility and p=0.04 for vitality) incremental changes vs. AB1 group animals, indicating a positive effect of Ex on sperm quality. According to these data, even though significant positive changes were observed, they differed from those in normal control animals (Table 2).

### Change in serological parameters after administration of exosomes

Based on the results presented in Table 3, all serological factors including Tes, NO, and MDA represented Leydig cell activity levels and oxidative stress levels, respectively. A significant reduction in Tes levels (p<0.001) in the AB1 group vs. control animals implied detrimental damage to Leydig cells, and a significant increasing incremental change in oxidative stress level (p=0.003 for NO and p=0.002 for MDA values) designated a destructive effect of AB1 on testicular tissue in AB1 animals vs. the

control animals. The results also showed that after administration of Ex in rats treated with AB1 (AB1+Ex group), Tes production significantly increased ( $p=0.03$ ), compared with the AB1 group, implying positive effect of Ex on restoration of both Leydig cell function and Tes secretion. Serum oxidative stress levels ( $p=0.02$  for NO and  $p=0.01$  for MDA) were also significantly reduced in AB1+Ex animals vs. the AB1 group indicating an inhibitory effect of treatment with Ex on testicular tissue damage after exposure to AB1 ([Table 3](#)).

#### ***Change in histopathological features after injection of exosomes***

Histopathological analysis was performed using two different H&E and IHC staining procedures. In the H&E protocol, structural changes in the density of interstitial cells (containing Leydig cells), spermatogenic lineages, and the density of spermatozoa in the seminiferous tubules were assessed using a microscope. Inflamed spermatogenic cell lineages containing galectin-3 were detected by the IHC protocol. After comparison of the interstitial tissue ([Figure 1A-C](#), dashed lines), a reduction in the area affected by AB1-induced inflammation was observed. Several gaps were also observed in most microscopic sections ([Figure 1B](#), dashed line). In addition, the introduction of a single dose of blood-derived Ex caused a substantial improvement in the state of this connective tissue and a decrease in the number of pathological gaps. These findings imply that Ex has a restorative effect on the interstitial tissue of testes. The spermatogenic lineage in normal sections was regular and compact ([Figure 1A](#), black arrow), while after the onset of tissue inflammation, spermatogenic cell density decreased and irregularities appeared ([Figure 1B](#), black arrow). Finally, in the AB1+Ex group, the natural arrangement of these cell lineages was shifted from the pathological state to the physiological type ([Figure 1C](#), black arrow). All these tissue changes led to changes in sperm production: the lumen of the seminiferous tubules was saturated with spermatozoa ([Figure 1A](#), blue arrow), while the production of sperm was significantly reduced, which was indicated by an empty lumen ([Figure 1B](#), blue arrow). Thus, generated sperm cells filled in the seminiferous tubules of the testes in the AB1+Ex group significantly more than in AB1 animals ([Figure 1C](#), blue arrow). Regarding IHC images of inflamed cells with or without the galectin-3 protein, none were observed in the control group ([Figure 1D](#), black arrow) stained with hematoxylin, whereas the spermatogenic lineages were rich in inflamed cells ([Figure 1E](#), black arrow). After treatment of AB1-affected rats (in the AB1+Ex group), the inflamed cell population decreased significantly ([Figure 1F](#), black arrow).

#### ***Changes in the expression of inflammatory genes after the introduction of exosomes***

As shown in [Figure 2](#), all inflammatory genes (TNF- $\alpha$ , IL-6, and IL-10) were highly expressed in the AB1 group contrary to the control ( $p<0.001$  for TNF- $\alpha$ ,  $p=0.01$  for IL-6, and  $p=0.01$  for IL-10), thereby confirming the inflammatory effects of AB1 on the testes ([Figure 2](#)). Besides, our results showed lower expression of inflammatory genes ( $p=0.002$  for TNF- $\alpha$ ,  $p=0.02$  for IL-6, and  $p=0.03$  for IL-10) in the AB1+Ex group, compared with AB1 animals, after administration of blood-derived Ex implying an inhibitory effect of Ex on the expression of inflammatory genes ([Figure 2](#)).

#### **Discussion**

Efforts undertaken by medical science to save life or preserve health are of utmost importance, even more so if they are aimed at human offspring. In this regard, the testicles in the male reproductive system are among major organs involved in the preservation of future offspring. Although the testes constantly produce sperm, they are vulnerable to environmental toxins that may cause poor sperm quality, azoospermia, and permanent infertility [26]. Among the toxins, AB1 has long-term adverse effects on the liver, kidneys, brain, and especially the testes. This toxin can cause testicular inflammation resulting in impaired sperm production [27]. Various remedies have been proposed for the treatment of orchitis, but preference has been given to the use of biological constituents rather than chemical synthetic materials.

Ex are spherical structures surrounded by bilayer phospholipid membranes containing active biological molecules, such as growth, anti-inflammatory and immunomodulatory factors. They are present in body fluids (cerebrospinal fluid, extracellular fluid, blood, and peritoneal secretions) [28]. These biological materials can be used when administered homologously with few side effects and tissue immunological reactions. With these interpretations in mind, we aimed to investigate the effect of blood-derived Ex with anti-inflammatory properties on AB1-induced inflammation in the testes.

The goal of our study was to develop a treatment plan for orchitis by administering blood-derived Ex. We think that the proposed treatment based on homologous transplantation could not only slow down the complications of immunological response, but also cure orchitis quicker. In our study, we examined the anti-inflammatory effects of Ex by histological, genetic, and serological analyses. Our results implied that blood-derived Ex have an anti-inflammatory effect on orchitis caused by AB1. Therefore, by injecting a single dose of Ex, a significant improvement can be achieved. Blood-derived Ex can reduce the expression of inflammatory genes and suppress the production of inflammatory interleukin. Consequently, damaged testicular tissue is repaired and sperm production resumes.

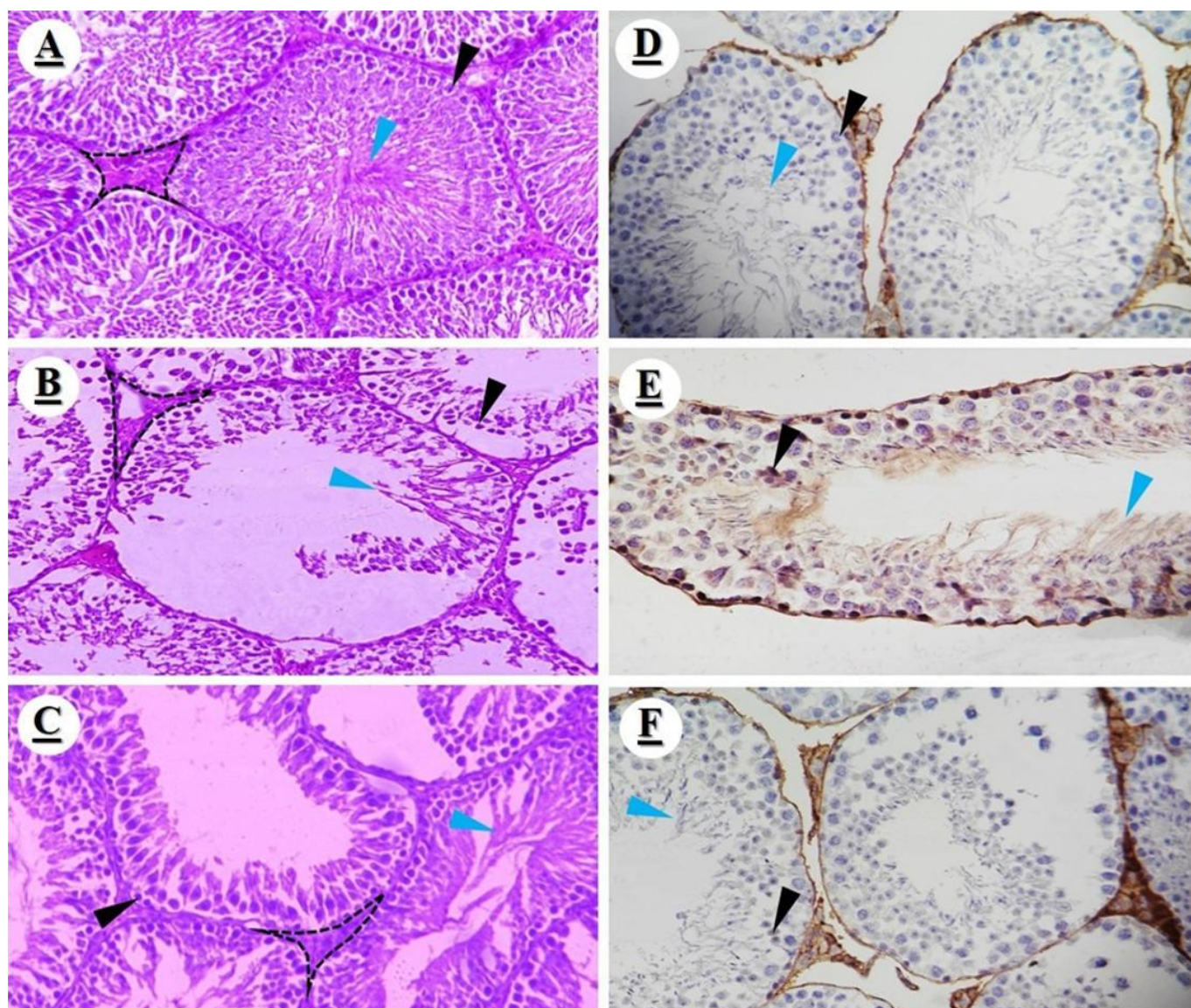
The results of numerous studies suggested that toxins can slightly increase the expression of inflammatory genes, such as TNF- $\alpha$ , and interleukins 6 and 10, which leads to the onset of inflammation in tissues [29]. After the intracellular presence of TNF- $\alpha$  in high concentration, a temporary cessation of cellular activity occurs. In this pathological situation, the cell cannot perform its physiological function properly [30]. Increased expression of this gene may eventually lead to the induction of premature cell death and apoptosis [31]. IL-6 [32] and IL-10 [33] also suppress the physiological function of cells by activating intracellular cascades and inhibiting cell growth, along with all secretory products of cells. In our study, we discovered that with the development of cellular inflammation in the testes, the density of cells in spermatogenic lineages decreased, as well as the density of interstitial connective tissue cells, which was probably associated with cell death in conditions of an increase in TNF- $\alpha$  gene expression.

In her review (2012), M.S. Theas, stated that inflammation was observed in 15% of patients after testicular biopsy. She concluded that although the presence of immunomodulatory ILs is necessary for proper testicular tissue activity, elevated IL levels may eventually lead to severe inflammatory conditions [34]. In our experiment, we also revealed high expression levels of

proinflammatory genes after administration of AB1. The decrease in the level of Tes could be associated with apoptosis of Leydig cells caused by increased expression of TNF- $\alpha$ . Lahijani et al. (2012) have also reported a similar pattern. They discovered that quinazolinone, due to its anticancer properties, inhibited the high expression of TNF- $\alpha$ , which led to the preservation of spermatogenic cell lineages [35]. It was also observed that with a decrease in density and an increase in the rate of cell apoptosis, the number of spermatozoa also declined.

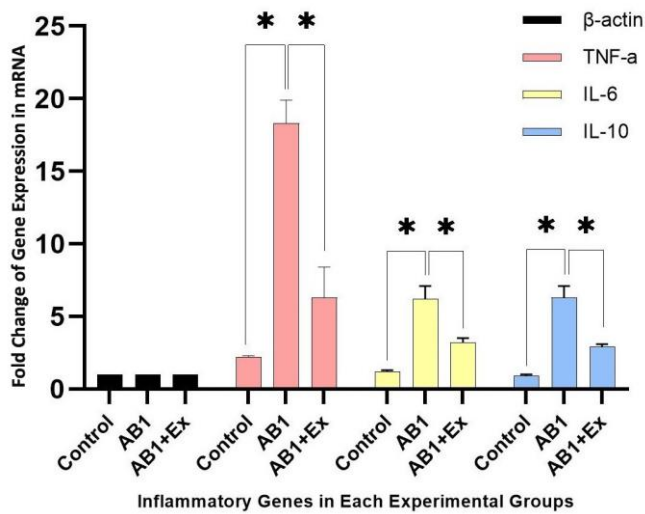
Inflammation appears to not only reduce sperm production but also reduce sperm quality [36]. The study demonstrated that in groups with pathology (AB1 animals), sperm quality expressed via its morphology, vitality, and motility, was drastically reduced. Several published studies suggested that the cause of destructive changes during inflammation was a decrease in the morphological and motile qualities of spermatozoa in the event of oxidative

stress. Shojaeepour et al. (2022) concluded that any exogenous toxin with acute inflammatory properties, such as cadmium, has the potential to reduce sperm quality [37]. We also discovered a decrease in sperm quality levels in our experimental study. The chronic presence of toxins in tissue cells causes severe damage to the cellular antioxidant system resulting in the cell inability to withstand environmental toxins. Najafpour et al. (2020) established that a high concentration of environmental toxins can seriously change the protein and lipid composition of cell membranes, as well as membranes of intracellular organs, and make them more susceptible to damage [1]. Hence, in the presence of a toxin in the environment, besides the possibility of membrane destruction in mitochondria with a subsequent cell expiry, the cell membrane becomes fragile resulting in cell death as well. This process can exacerbate inflammation and alter tissue functioning.



**Figure 1.** Normal (A, D), pathological (B, E), and treatment (C, F) images stained by H&E (A-C) and IHC (D-F) protocols in different groups of control (A, D), AB1 (B, E), and AB1+Ex (C, F).

Blue arrows indicate sperm in the lumen of seminiferous tubules, black arrows show spermatogenic lineages, and black dashed lines represent interstitial connective tissue containing Leydig cells. Magnification 100 $\times$ . N=6 rats per group. H&E, hematoxyline and eosin; IHC, immunohistochemistry.



**Figure 2.** Inflammatory gene expression of TNF- $\alpha$ , IL-6, and IL-10 in Control, AB1, and AB1+Ex groups.

N=6 rats in each group. \*Indicates  $P < 0.05$  in comparisons between the groups. TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; IL-6, interleukin-6; IL-10, interleukin-10; AB1, aflatoxin B<sub>1</sub>.

One of our findings suggested that the use of AB1 toxin caused extensive inflammation and increased levels of NO and MDA. Blood-derived Ex with their extensive anti-inflammatory properties, as well as a tissue mitogen, reduced inflammation and promoted cell proliferation. Some studies demonstrated that these Ex-derived tissue mitogens, when released, bound to the surface tissue receptor stimulating cell growth [38]. Consequently, the tissue can restore its function. As we established in our study, after tissue damage and cell death following the application of AB1, a minimum number of cells of spermatogenic origin with high mitotic properties remained, which were probably affected by these mitogens. This is how tissue repair begins.

In addition, Maya et al. reported that many cell-to-cell communications were carried out using Ex. They concluded that, based on the nature of these biological substances, the spread of the disease and metastases of the tumor took place [39]. In contrast, we detected that blood-derived Ex acted as an anti-inflammatory agent against orchitis caused by AB1. Although the exact mechanism of damaged tissue restoration after injections of these Ex is still unclear, it is likely that the vital activity of two important cell lines (interstitial connective tissue and spermatogenic lineage) restored the physiological status of damaged cells. When cells in the interstitial tissue begin to regenerate, Leydig cells proliferate resulting in the production of Tes. Another finding in our study was that after administration of a single dose of Ex, blood level of Tes increased to its normal physiological state. The same result was also reported by Shabanizadeh et al. (2022). In that study, low Tes levels accompanying a pathological condition caused by morphine were improved by administration of Sumach extract. The authors suggested that Tes levels could possibly be restored by Leydig cell regeneration [40].

One of the important effects of high Tes levels is a positive impact on the rate of mitosis in cells of spermatogenic origin. The positive effect of Tes, along with the activity of mitogens that are derived from blood Ex, can fully stimulate the proliferation of

spermatogenic lineage cells. As we discovered in our study, after the injection of Ex, tissue was repaired, and production of Tes resumed. Accordingly, tissue proliferated, and sperm production began. As demonstrated by tissue sections, the lumen of the seminiferous tubules was filled with sperm after the healing of tissue damage.

The role of galectin-3 in the control and regulation of inflammation after Ex therapy should be specifically addressed. Some studies suggested that galectin-3, as a protein involved in inflammation, can direct the inflammatory phase of tissue damage from the acute stage to the chronic stage and ultimately leads to tissue fibrosis [41]. Recent research showed that inhibition of galectin-3 expression or activity in damaged tissue is a way to treat tissue inflammation [42]. There are approximately 15 varieties of galectin in mammals, of which type 3 is the most common and effective in many biological processes occurring in tissues [43]. The expression of galectin occurs in different cell lines. This molecule is generated and secreted into the extracellular environment in macrophage cells. Here it acts as a chemotactic factor and introduces many inflammatory cells and leukocytes into the tissue [44]. Some published evidence suggested that this process in pneumonia exacerbated the inflammation and aggravated chronic form of pneumonia. Many other articles discussed the role of galectin-3 in the control of apoptosis [45]. Intracellular galectin inhibits apoptosis, while extracellular galectin, mainly secreted by macrophages, can induce apoptosis. The results of our study showed high expression of galectin-3 in spermatogenic cell lineage after the exposure to AB1, while tissue sections demonstrated that galectin-3 was inhibited after Ex administration; hence, this factor can inhibit inflammation and eliminate the pathology caused by AB1.

### Conclusion

Ex therapy was proposed as an innovative method of treating inflammatory diseases. The results of our study, based on the anti-inflammatory properties of blood-derived Ex, demonstrated that these biological substances can heal inflammation caused by AB1. This anti-inflammatory property is likely due to the inhibition of galectin-3 in testicular tissue, which suppresses inflammation and restores the physiological activity of testes. Since these biological substances are derived from the blood of homologous animals, they can inhibit the pathogenesis of AB1 by suppressing inflammation without immunological side effects. Future comprehensive studies should investigate the use of blood-derived Ex in controlling post-AB1 inflammation as a new treatment procedure for testicular pathology.

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### Authors' contributions

Fatemeh Mansouri Torghabeh performed primary manipulations with animals. Mona Keivan conducted reproductive assays. Mandana Fakoor, as leading toxicologist, assisted the research team in preparing the exact dose of the toxin and modeling the disease. Reza Dadfar and Mahsa Nazarzadeh participated in animal manipulation and data analysis. The concept of this study was proposed by Amir Abdolmaleki, who also served as the corresponding author. The accuracy of the manuscript was approved by all authors and was signed by Amir Abdolmaleki.

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**Availability of data and research materials**

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

**Conflict of interest**

The authors declare no conflicts of interest.

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