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Antisperm Antibodies and Hormonal Profiles Following Various Treatment Regimens of Broken Blood–Epididymal Barrier: A Rat Model Study

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ABSTRACT

The use of medications in the treatment of animals with antisperm antibodies (ASA) is questioned by conflicting information. The epididymal sperm extraction (ESE) rat model's broken blood epididymal barrier was the subject of investigation. Ten adult male Wister-Albino rats served as negative control, fifty rats were exposed to ESE and divided into five groups. The first group was untreated and considered as positive control. The remaining four groups were treated with dexamethasone (DEX), azathioprine (AZA), frankincense, and prepared secondary antibodies. Sperm counts, sperm viability %, ASA concentrations, testosterone assays, and histopathology of the testis and epididymis were performed. The results of the AZA and DEX-treated rats showed that at week 8, there was a significant decrease in ASA compared to the positive control group ($P<0.01$), whereas there was no significant difference between the testosterone concentrations, sperm count, or viability of the treated rats and the negative control group. Histologically, testis and epididymis displayed moderate degeneration and necrosis. There was a significantly lower ASA and index (%) than in the positive control group at weeks 6 and 8 following treatment with frankincense and secondary antibodies ($P<0.01$). The testosterone levels, however, recovered to normal levels since they were identical to those of the negative control and lower than those of the positive control ($P<0.05$). Histologically, the testis and epididymis clarified normal appearance. Conclusion, the findings support the idea that immune responses and acute inflammatory alterations in the testis are triggered by paracrine and immunological processes. Future research on comprehensive hormone profiling or analyses of the hypothalamo-hypophysial- gonadal axis following this operation would be of interest.

Key words: ASA, Epididymal Sperm Extraction, Testosterone, DEX, AZA, Frankincense,

INTRODUCTION

Away from the testis, sperms move into a long-tortuous tube, the epididymis. The epididymis is characterized by its four main types of pseudostratified epithelium, clear, principal, basal, and narrow cells, with specific morphologic and purposeful characteristics (Breton et al. 2019). Cells establish an elaborate interaction mediated by cell-to-cell connections that can form the blood-epididymis barrier (BEB). BEB protects the luminal microenvironment from attack (Breton et al. 2016). The BEB forms a distinctive physiological microenvironment that is critical for the protection, maturation, transport, and storing of spermatozoa in the epididymis (França et al. 2013). Anatomical, immunological, and physiological barriers make up the BEB. The first two barriers are composed of the tight junctions between cells, while the third barrier is made of certain transporters that are positioned along the cells and control the flow of molecules into and out of the lumen (Gregory and Cyr 2014). The BEB controls the permeability of the paracellular transport channel, the transfer of electrolytes, ions, water, nutrients, hormones, and chemicals (Hedger 2011). Enzymatic activity, the development of motility, the movement of the cytoplasmic droplet, and alterations to the sperm transcriptome and proteome are all examples of the amazing morphological, biochemical, and physiological changes that occur during epididymal transit (de Souza et al. 2017; Gervasi and Visconti 2017; Conine et al. 2018; James et al. 2020).

It is generally accepted that damage, and/or interruption in the blood-testis barrier (BTB) lead to the appearance of antisperm antibodies (ASA) due to the

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due to the exposure of immunogenic sperm antigens to the immune system (Xu et al. 2020). Because BEB function/integrity, which is assumed to be less robust than BTB, sperm cells are more likely to be exposed to the immune system and ASA can be created (Lotti et al. 2018).

Pre-testicular, testicular, or post-testicular causes are correlated with it. ASA is one of the post-testicular variables that contributes to immunological infertility (Jungwirth et al. 2019). Additionally, ASA can be created after the testis, when BEB safeguards the epididymis (Lotti and Maggi 2018). The epididymis is less immune-controlled and more susceptible to inflammation than the testis, despite the BEB physiological barrier being more advanced than the BTB's morphological barrier. Injury can result in the decrease of BEB function, exposure of the developing spermatozoa to the immune system and subsequently ASA production (Gregory and Cyr 2014).

According to results of the MAR test, epididymal abnormalities were more linked to the development of ASA than testicular irregularities (Lotti et al. 2018). Open testicular biopsy, percutaneous or microsurgical epididymal extraction, and testicular sperm extraction, with or without the use of a microscope, are the used sperm retrieval techniques. Epididymal sperm extraction (ESE) refers to the retrieval of sperm-containing fluid from animals with obstructive azoospermia.

In terms of pharmaceutical methods, immunosuppressive treatments utilizing dexamethasone (DEX) or cyclosporine and prednisolone have been prescribed to treat cases of ASA (Taiyeb et al. 2017). Contradictory ideas concerning this issue also be present. Another methodology has shown the efficiency of the seminal plasma therapy with *staphylococcus* protein A in the lessening of ASA titters in immune infertility disease (Al-Daghistani 2020). As for the outcome of the cyclosporine therapy, no firm conclusions can be made at this time. Shibahara et al. (2022) in their review article described that, the approach for removing ASA bound to sperm's is the effort to remove sperm- binding antibody from sperm. However, it has been discovered that it is challenging to dissociate firmly attached antibodies. There are many methods, including one that involves simply washing the sperm, splitting the ejaculate into medium that contains serum, immunological absorption of ASA, and enzymatic treatment. Simple sperm washing was unable to remove ASA from the surface of the sperm. The rate of oocyte fertilization may be increased by adding serum to sample collection pots. The last two techniques have not yet been used in clinical settings (Shibahara 2022).

Azathioprine (AZA) is an immunomodulatory medication frequently used to treat cancer, autoimmune diseases, inflammatory bowel disease, and other conditions (Reggio et al. 2019; Bergasa 2022). When used therapeutically, 6-mercaptopurine (6-MP), an analog of AZA, prevents the DNA synthesis of highly proliferating cells like T and B lymphocytes and lowers the number of killer cells (Honkila et al. 2019). The botanical name for frankincense is *Boswellia sacra Fluck*, and it is a member of the Burseraceae family. Additionally, items derived from Boswellia oleo gum resin are sold all over the world for a variety of purposes, including mouth freshening, the treatment of digestive, skin, ear, and throat infections, the alleviation of menstruation pain, cardiovascular and neurological issues, etc. (Mojaverrostami et al. 2018). In Iran, frankincense is used with other herbs to treat changed male fertile potential, and it has long been used as an aphrodisiac and fertility enhancer in Jordan (Roy et al. 2019).

Scientists are in constant disagreement because of conflicting findings that support and refute the use of immune suppressant drugs in the treatment of ASA. To find potential treatments for ASA, we looked at treating ESE rat model with DEX, AZA, frankincense, and secondary antibodies.

MATERIALS AND METHODS

Ethical approval

All the study's methods have been given the go-ahead by the Animal Care and Use Committee of the Experimental Animal Centre at Qassim University No. 22-15-11. All the surgeries used diethyl ether as the anesthetic, and every attempt was made to decrease the pain.

Animals

Sixty adult male Wister-Albino rats were divided into six groups (n=10/cage) and kept in an environment-controlled room (22°C/40-60% RH) with a weight range of 180–200g on average.

Tap water and standard chow were available to rats *ad libitum*. Twelve-hour light-dark cycles were used for the whole trial as well as the week prior. Male rats were prepared for epididymal surgery at room temperature. Ten rats served as the negative control group and underwent scrotal sac surgery without having their ESE.

ESE Procedure and Treatment Groups

ESE has been applied previously in mice (Val and Robledano 2013). Each rat was put in a vacuum-sealed container and anesthetized by breathing diethyl ether. After receiving the anesthesia, the rat was placed on its back, and the scrotum was constricted to allow for testicular protrusion. The testicles were then held between the index and thumb and the scrotum was opened between them. The cauda epididymis was clamped before being punctured with a 32-gauge (0.202mm) hypodermic needle for extraction. The epididymal sperm drops were collected with capillary tubing (internal diameter, 0.58mm) attached to a mouth pipette. After that, a straightforward suture was created, and Sandoz Co.'s baneocin antibiotic powder was used to stop infection. ESE was carried out on both epididymis, with a two-week recovery time (Boersma et al. 2015).

Five groups of ESE rats (n=10) were separated into different cages. The ESE was performed on the first day of the trial in the positive control group, and the rats were not given any medication. Group receiving DEX treatment: This group received an intramuscular injection of DEX sodium phosphate 14 days after ESE. The dosage was 2.25mg/kg bwt twice a week for three weeks (Earp et al. 2008). DEX was used at ampule of 8mg/2mL/EPICO. AZA-treated group: Imuran 50mg tablets, the AZA medication, were purchased from a pharmacy

(Sitcopharma, Aspen Pharma Trading Limited Co. Germany). Following the dilution in 10mL of normal saline solution (NaCl 0.9% solution), the powder from one tablet was administered therapeutically orally through a stomach tube at a dosage of 1.0-1.5mg/kg bwt each day for two weeks (Simsek et al. 2018). Group that received frankincense treatment: Hojari, Salalah, Sultanate of Oman provided the frankincense male gum (Nusier et al. 2007). The raw material was crushed in a mortar and then dissolved in distal water to obtain a final concentration of 1gm/30mL DW. Each rat received 100mg daily for 30 days. Rats were orally administered frankincense male gum via a stomach tube. Group treated with secondary antibodies: 14 days following ESE, this group received an intraperitoneally injection of secondary antibodies with established potency. 50mg/kg bwt administered twice weekly for three weeks straight was the therapy dosage. Two weeks from the last injection, blood samples were taken from the rats' inner canthus of the eyes every two weeks till the end of experiment (ten weeks) for the purpose of obtaining serum.

Testosterone Levels

As directed by the manufacturer, a commercial ELISA kit was used to assess the testosterone levels. (Testosterone ELISA TEST SYSTEM Kits from Monocent, Inc. 9025 Eton Avenue, Ste. C, Canoga Park, California 91304, USA (REF. EL1-1263).

Histopathological Examination

The histology approach of Suvarna et al. (2018) was used. After postmortem examination, tissue samples from all rat groups' testis and epididymis were gathered and preserved in 10% neutral buffered formalin.

Preparation of ASA in Rabbits

Three mature male rats that appeared to be in good health were utilized to prepare the rat sperm protein as previously explained (Chiu et al. 2004). The corpus material was put into a microcentrifuge tube and left at room temperature for 30min to liquefy. The seminiferous tubules were removed and minced on a Petri dish with PBS (pH 7.4). Each aliquot was aspirated using a Pasteur pipette after the sperm had time to separate from the tubules, resuspended in 1mL of PBS (pH 7.4), and centrifuged at 1500g at 4°C. After washing process three times, sperm homogenization was done in a solution comprised 0.25 mol/L sucrose, 1 mmol/L EDTA, and 0.05 mol/L TRIS-HCL (pH 7.4) using a homogenizer (Staufen, Germany) at 0-4°C. The homogenate was then put on ice and sonicated for 30sec at 10 kHz (Takada et al. 1982). The sonicates were centrifuged for 90min at 8500g at 4°C, and the amount of total protein in the supernatants was determined using a colorimetric technique (Biuret reagent). Sperm proteins were given to three male rabbits to produce pooled hyperimmune serum. After an equivalent volume of Freund's complete adjuvant (Difico, USA) was applied, the mixture was emulsified. The homogenate, which included 400 mg of sperm total proteins, was administered to each animal by a 0.5mL intra-peritoneal injection. Boosters were given 2, 4, and 6 weeks after the initial injection. Ten days after the fourth injection, blood was taken through heart puncture. Aliquots of the separated, gathered sera were frozen at -20°C. This pooled hyper immune serum was used as a positive control in the ELISA experiment and to produce secondary antibodies as well as for the titration of their potency. Immunoglobulin precipitation was carried out using an ammonium sulfate solution, as described previously (Abd El Hafez et al. 2010). The produced immunoglobulins precipitate was dialyzed against 15 mM PBS using bag (Sigma-Aldrich). The dialyzed immunoglobulins were centrifuged for 20min at 4500rpm. The immunoglobulins were extracted from the supernatant, divided into the necessary amounts, and kept in tubes at -20°C until required.

Preparation of Secondary Antibodies

First blood was drawn before the immunization to provide a negative control for further testing. According to Muro et al. (1997), three rabbits of the same breed were immunized. After being well combined with an equal amount of complete Freund's adjuvant, rabbit IgG was administered subcutaneously at a dosage of 40mg/kg bwt. The same injection schedule was followed again.

Checkerboard Titration

Checkerboard titration was used to determine the ideal antigen dilution and to test the potency of the rabbit-made sperm protein antisera. Different dilutions of the tested anti sera were evaluated with serial dilutions of the tested antigen that was used to coat the ELISA plate and a negative control serum. Under the known dilution of conjugate and substrate, the serum dilution with the lowest concentration of antigen/well that produced a distinct signal was deemed the ideal situation and used in further testing. The antisera's ideal dilution was 1:32 when the potency of the rabbit antisera against the antigen (300ng of sperm protein) was tested. The volume of rat sperm protein used is determined by the standard log-dose response curve of rat sperm protein.

Standard Log - Dose Response Curve

Following the negative control antigen, serial sperm protein dilutions of 250, 125, 62.5, 31.25, and 15.62ng were added to the plate (dilution/row). The plate was washed three times with the washing buffer, loaded with 100µL/well of the rabbit antisera (1:32 in PBS), and then incubated for an hour at 37°C while being shaken. A volume of 100µL/well of HRP-conjugated sheep anti-rabbit IgG (product no. A-5279, Sigma co., St. Louis, U.S.A) (1:4000 dilution in PBS, PH7.2) was added to the plate after incubation, and it was incubated for a further hour at 37°C in a shaking water bath. A volume of 100µL/well of HRP-conjugated sheep anti-rabbit IgG (1:4000 dilution in PBS, PH 7.2) was added to the plate after incubation, and it was incubated for a further hour at 37°C in a shaking water bath. Following incubation, the plate was rinsed three times before each well received 100µL of the substrate solution. After allowing the plate to sit at room temperature for 7min in the dark, the response coloring was visible. The reaction was then stopped by adding 50 µL of the stopping solution per well. Using a microplate ELISA reader, the enzyme-mediated signal was detected at a wavelength of 492nm. The optical density was then plotted against the log dose

of antigen to construct a standard curve. Standard log - dose response curve of secondary antibodies as antigen was done using a similar procedure. Secondary antibodies were serially diluted in the plate (one dilution per row) in the following amounts: 5.61, 2.81, 1.40, and 0.70µg, followed by the negative control.

Evaluation of ASA Levels in Serum of Rats

The levels of immunoreactive ASA were determined using modified technique of the standard indirect solid phase ELISA. The sensitivity% and specificity% were also calculated (Esmailnejad et al. 2020). To evenly distribute the protein in the wells, the microplate wells were coated with 100µL/well of rat sperm proteins (300ng), diluted in carbonate bicarbonate coating buffer (PH 9.6), and then incubated for two hours at 37°C in a shaking water bath. The plates were covered with an adhesive tape to prevent evaporation and incubated at 4°C overnight to allow the solid phase to completely adsorb. Any extra unattached sperm proteins were removed after three washings with the washing buffer (PBS PH7.2 containing 0.05% Tween-20). Plates were shaken for 2 hours at 37°C while the remaining binding sites were blocked with 200 µL/well of blocking buffer composed of bovine serum albumin (BSA2%: PH7.2). Each well received 100µL of serially diluted rat serum samples after being rinsed three times with the washing buffer. Each well was then covered with an adhesive tape and incubated for an hour at 37°C with shaking. After repeating the washing process, 100µL of the1:2000 dilutions of the HRP-conjugated sheep anti-rat IgG (product no. A-5287, Sigma co., St. Louis, U.S.A) was added to each well. The wells were then incubated for an additional hour at 37°C in a shaking water bath. The substrate solution (TMB Innovative Diagnostics) was applied to each well after the washing process, 100µL/well at a time. After the plates had been incubated for 7min at room temperature in the dark, the reaction was subsequently terminated using the stopping solution (50µL/well). The enzyme-mediated signal was identified at 492nm using a microplate ELISA reader.

Evaluation of sperm parameters

The caudal regions of both epididymis were utilized for the study of sperm parameters (Owagboriaye et al. 2017). Using a scalpel, the tissue was chopped into small pieces and placed in a Petri dish containing 2mL of normal saline that had been pre-warmed to 37°C for 30min. Two drops of the mixed sperm suspension were placed on a microscope slide, covered with a coverslip, and pipetted numerous times to measure motility. At least five microscopic fields at a magnification of 400 were examined on each slide, and the proportion of motile sperm was noted. 1mL of sperm suspension was diluted with 4mL of formaldehyde solution for sperm counts (1:5 dilutions). The number of sperm heads was transferred into a haemocytometer then counted in five fields and expressed as 10^6 sperm/mL. By using eosin Y staining (5% in saline), viability was evaluated. On a glass slide, 40 microliter samples of the freshly formed sperm solution were applied, mixed with 10mL eosin, and then examined under a light microscope (X400 magnification). After staining, live sperms remained unmarked, whereas those that displayed any pink or red coloring were dead. Each sample had at least 200 sperm counted in 10 randomly chosen fields of vision, and the proportion of viable sperm was noted.

Statistical analysis

Data values were represented as means standard errors. For each measured parameter, a straightforward one-way analysis of variances (ANOVA) test was conducted. The Mann-Whitney test was used in post hoc analysis to compare the positive control to other experimental groups and the negative control to the positive control at $P<0.05$ that indicated a statistical difference. A general formula for a cut-off value is as follows: Cut-off = a*X + f*SD. Where a and f are two multipliers, X is the mean, SD is the standard deviation of independent negative control values.

RESULTS

On each sample, there were six readings taken (Table 1). The samples' ODs at first dilution (1:1) and their calibrated ASA concentration were the first two readings. The next two points represented the mean ODs of the ASA concentration and rat sera cutoff values. The last two calibrated readings, however, were antibody indexes (%) and the dilution point at which the cutoff value was reached. The concentration of ASA in the rat serum was 13.73±0.3280 ng/100uL, and the mean OD of the cutoff values was 0.0392±0.0018. However, the cutoff value's dilution point (antibody titer) and antibody indices (%) varied depending on the treatment. According to the findings, the concentrations of ASA associated to antigen sperm protein at weeks 2, 4, 6, and 8 were 18.4, 19.25, 18.2, and 18.4ng/100µL, respectively. The dilution point antibody titer at cut-off values was, however, 1/4 at all weeks. The findings showed that the positive control ESE rats' ASA titers were 1/128 at weeks 2 and 4 and 1/64 at weeks 6 and 8, while the total mean antibody index (%) was substantially higher at ($P<0.001$).

The study showed that at week 2, the OD at the first dilution (1/1) was 1.0815±0.009 and the ASA concentration was 116.5±14.5ng/100µL in the positive control ESE rats. The OD at the first dilution (1/1) was 1.0515±0.289 at week 4, whereas the ASA concentration was 112.4±29.16 ng/100µL. Additionally, the data revealed that at week 6, the OD at first dilution (1/1) was 0.9545±0.083 and the ASA concentration was 103.9±27.016ng/100µL. The OD at the first dilution (1/1) was also 0.8865±0.050 at week 8, and the ASA concentration was 97.5±15.5ng/100µL. At every one of the studied weeks, the positive control's ASA concentration levels were all considerably ($P<0.001$) greater than those of the negative control.

We measured the cut-off values in AZA-treated ESE rats. The findings revealed that during weeks 2, 4, and 6, the cut-off values of the group were observed at the 1/128, 1/32, and 1/32 serial dilutions respectively. Additionally, it was noticed at a serial dilution of 1/16 at week 8. The findings also revealed that there was no significant difference between the positive control group and the ASA concentrations at first dilution (1/1) of 122.3±12.9 in the second week, 88.5±14.5 in the fourth week, and 110.3±17.06ng/100µL in the sixth week. Additionally, the

Table 1: Estimated ELISA assay parameters of rat epididymal sperm extraction (ESE) treated with various therapeutic immune effectors.

Animal grouping	Sampling week	OD of samples at first dilution (1:1)	Concentration at first dilution (1:1) ng/100µL	Antibody titer at cut-off values	Antibodies Index (%)
Control negative	Wk2	0.135±0.003	18.4±0.2	1/4	11.774±3.11
	Wk4	0.1375±0.001	19.25±0.25	1/4	15.043±2.09
	Wk6	0.1325±0.001	18.2±0.033	1/4	16.119±3.17
	Wk8	0.1315±0.001	18.4±0.220	1/4	17.417±4.02
Control positive	Wk2	1.0815±0.009	116.5±14.5*	1/128	111.775±11.43*
	Wk4	1.0515±0.289	112.4±29.16*	1/128	115.043±16.41*
	Wk6	0.9545±0.083	103.9±27.016*	1/64	116.119±19.66*
	Wk8	0.8865±0.050	97.5±15.5*	1/64	107.417±5.09*
AZA treated ESE	Wk2	1.010±0.010	122.3±12.9	1/128	81.552±11.42
	Wk4	0.799±0.043	88.5±14.5	1/32	72.374±12.54
	Wk6	1.043±0.00	110.3±17.06	1/32	99.766±22.30
	Wk8	0.485±0.025	52.5±2.5[a]	1/16	46.821±10.42a
DEX treated ESE	Wk2	0.961±0.119	100.2±14.55	1/128	72.045±9.51
	Wk4	1.0385±0.002	112.5±2.5	1/32	98.577±8.33
	Wk6	1.054±0.008	117.6±19.83	1/32	102.104±11.00
	Wk8	0.7615±0.119	69.54±7.73[a]	1/16	63.933±9.46[a]
Frankincense	Wk2	0.815±0.055	105.2±13.25	1/16	69.3109±6.81
treated ESE	Wk4	0.86±0.010	108.7±21.03	1/16	79.0481±12.45
	Wk6	0.57±0.110	72.8±22.7	1/32	53.2238±4.66[a]
	Wk8	0.2235±0.136	26.65±9.65[a]	1/16	12.1854±3.80[a]
Secondary	Wk2	0.795±0.142	108.6±17.4	1/32	57.566±12.25[c]
antibody treated	Wk4	0.5795±0.043	63.5±15.5[b]	1/8	51.181±5.71[c]
ESE	Wk6	0.2985±0.055	31.5±4.0[a]	1/4	20.194±3.60[a]
	Wk8	0.225±0.045	27.1±3.1[a]	1/8	12.384±2.32[a]

Values (Means±SE) bearing asterisk are significantly (P<0.001) different than control negative group. Values bearing a, b, c are significantly different than control positive group at P<0.001, P<0.01 and P<0.05, respectively. AZA: azathioprine; DEX: dexamethasone; epididymal sperm extraction: ESE.

Table 2: Evaluated serum testosterone (pg/mL) of ESE rat treated with various therapeutics.

Groups	Control negative	Control positive ESE	AZA treated ESE	DEX treated ESE	Frankincense treated ESE	Secondary antibody treated ESE
Testosterone	97.844±6.54	70.43±3.52*	66.43±4.70	55.03±5.11	84.63±3.01a	91.42±5.11a

Means ± SE. Values with * is significantly different than control negative values at P<0.05. Values with letter a are significantly different than control positive values at P<0.05. AZA: Azathioprine: DEX: dexamethasone; epididymal sperm extraction: ESE.

results showed that at weeks 8, there was a statistically significant reduction in ASA concentrations of 52.5±2.5ng/100µL compared to the positive control group (P<0.001). The first three collections' mean ASA index (%) values did not substantially differ from those of the positive control group. The ASA indices, however, seem to be lower in the week 8 (P<0.001).

The results showed that the cutoff value was detected at serial dilution 1/128 in the serum samples of DEX-treated ESE rats at week 2. The cutoff value for sera samples was seen at serial dilutions of 1/32 for the fourth and sixth weeks of therapy and 1/16 for the eighth week. The data demonstrated that at first dilution (1/1), the ASA concentration was 100.2±14.55ng/100µL at week 2 in the DEX-treated ESE rats. The ASA concentration was 112.5±2.5ng/100µL at week 4, nevertheless. Additionally, the data showed that the ASA concentration was 117.6±19.83ng/100µL at week 6. None of the three values deviated significantly from the positive control. Furthermore, the ASA concentration at week 8 was 69.54±7.73ng/100µL, which differed substantially from the positive control group (P<0.05). Except for week 8, where the antibody index% mean values were 63.933±9.46 compared to the positive control values (P<0.05), none of the sample collection weeks' antibody index% mean values were significantly different.

Serum samples from ESE rats treated with frankincense were measured for their cut-off values. The data demonstrated that for blood samples collected at the sixth week, the cut-off value in the 2, 4, and 8 weeks was found at the 1/16 serial dilution and the 1/32 serial dilution, respectively. The findings showed that at week 2, the ASA concentration was 105.2±13.25ng/100µL. The ASA content at first dilution (1/1) was, however, 108.7±21.03ng/100µL at week 4. Additionally, the findings revealed that in week 6, the ASA concentration at first dilution (1/1) was 72.8±22.7ng/100µL. Furthermore, the ASA concentration in week 8 was 26.65±9.65ng/100µL, which was significantly lower than the positive control group (P<001). The mean antibody index (%) only significantly decreased (P<001) at weeks 6 and 8 of treatment.

The results showed that the cut-off value for the ESE rats treated with secondary antibodies was discovered at the 1/32 serial dilution for the second week, at the 1/8 serial dilution for the fourth and eighth weeks, and at the 1/4 serial dilution for the sixth week. The results demonstrated that at week 2, the ASA concentration at first dilution (1/1) was 108.6±17.4ng/100µL. When compared to the positive control group, the ASA concentration at the first dilution (1/1) at week 4 was 63.5±15.5ng/100µL (P<0.01). Additionally, the outcomes showed that at weeks 6 and 8, respectively, the ASA

concentration was considerably lower than the positive control group at 31.5±4.0 and 27.1±3.1ng/100µL (P<0.001). In samples taken at weeks 2 and 4, as well as weeks 6 and 8, the mean values of the antibody indexes (%) were considerably lower at (P<0.05) and (P<0.001), respectively.

The sperm count (10^6/mL), sperm viability (%) and testosterone concentrations for each treatment group, after the end of experimental period, were determined. The ESE rats treated with either AZA or DEX were not significantly different from the positive control rats (Table 3). The testosterone levels in ESE rats treated with frankincense or secondary antibodies, however, recovered to normal levels since they were identical to those of the negative control and significantly lower than those of the positive control (P<0.05).

Histopathology

Seminiferous tubules in the testes of negative control rats were normal, demonstrating the existence of several spermatogenic cell stages occluding most of the lumen of seminiferous tubules (Fig. 1A). The epididymis showed normal epididymal tubules that were bordered with healthy, undamaged pseudo-stratified columnar epithelium and totally filled with seminal fluid (Fig. 2A). In the positive control group, some of the seminiferous tubules exhibited hyaline degeneration, while others exhibited full destruction had extensive peri-tubular edema together with focal inflammatory cell infiltrations (Fig. 1B). Infiltrations of multifocal inter-tubular inflammatory cells could be seen in the epididymis of the control positive rats. (Figs. 2B). Testis of the DEX-treated group showed hyaline degeneration in few of the seminiferous tubules. Some other tubules displayed severe desquamation in the spermatogenic cells to the tubular lumen. Also, atrophy and shrinkage were noticed in few of tubules (Fig. 1C). The epididymal tubules displayed dilatation and engorgement with the epididymal sperms. Hyperplasia takes the finger like projection was noticed in the pseudo-stratified epithelial lining the epididymal some tubules associated with detachment for some of the hyperplastic cells toward the lumen. Other tubules showed rupture in the lining epithelium of tubule (Figs. 2C). Concerning AZA -treated group, severe hyaline degeneration in some seminiferous tubules, sever desquamation of the spermatogenic cells associated with edematous fluid infiltration among some tubules was noticed (Figs. 1D). The epididymal tubules displayed multifocal hyperplasia in the pseudo-stratified columnar lining epithelium. Atrophy of some other epididymal tubules was noticed associated with rupture in the wall of some other epididymal tubules (Fig. 2D). The testicular tissue of the frankincense -treated group exhibited good spermatogenic activity with presence of the different stages of spermatogenic cells occludes most of the lumen of the seminiferous tubules associated with the presence of normal intact active Sertoli cells (Figs. 1E). The sperm cells filled the epididymis' tubular lumen (Fig. 2E). The spermatogenic cells in various stages that occlude most of the tubular lumen were visible in the testicular tissue of the secondary antibody-treated group, and the epididymal tubules were only moderately loaded with stored sperm cells (Figs. 1F and 2F).

DISCUSSION

On each sample, there were six readings taken. The samples' ODs at first dilution (1:1) and their calibrated ASA concentration were the first two readings. The next two points represented the mean ODs of the ASA concentration and rat sera cutoff values. The last two calibrated readings, however, were antibody indexes (%) and the dilution point at which the cutoff value was reached.

The findings showed that the positive control rats' ASA titers were 1/128 at weeks 2 and 4 and 1/64 at weeks 6 and 8, while the total mean antibody index (%) was substantially higher than negative control values. At every one of the studied weeks, the positive control's ASA concentration levels at first dilution (1/1) were all considerably greater than those of the negative control. Testis of the control positive rats showed multifocal peri-tubular edematous fluid infiltrations among the seminiferous tubules. Also, severe detachment for the spermatogenic cells from the basement membrane into the lumen of seminiferous tubule was observed. Epididymis showed moderate inter-tubular inflammatory cell infiltrations mainly lymphocytes among the epididymal tubules. The epididymis is more motionless to inflammation than the testis, as is common knowledge. Inflammation may lead to immune system exposure of maturing spermatozoa, loss of BEB function and ASA synthesis (Hedger 2011; Gregory and Cyr 2014). According to earlier reports (Lotti et al. 2018), ESE led to the creation of ASA because the loss of BEB function or integrity can expose sperm cells to the immune system more easily than BTB. According to WHO recommendations, Lotti and colleagues' findings are strongly suggestive of chronic epididymal inflammation in ASA-positive cases (Lotti et al. 2018). Notably, the concentration of sperm autoantigens in the epididymis is equal to or higher than that in the testis, making it a preferred site for an ASA response (Pöllänen and Cooper 1994). Additionally, unlike seminiferous tubules, immune cells are widespread in the epididymal epithelium and interstitium, and their quantity and activity rise during inflammation (Hedger 2011). According to Wang and Duan (2016), the development of ASA is not solely dependent on a testicular injury; rather, it is caused by an inflammation of the epididymis that subsequently spreads

Table 3: Evaluation of sperm parameters of ESE rat treated with various therapeutics.

Groups	Control negative	Control positive	AZA treated ESE	DEX treated ESE	Frankincense treated ESE	Secondary antibody treated ESE
Sperm count (10^6 / ml)	48.54±3.81	33.71±3.77*	39.66±2.61	35.61±3.40	46.02±2.77a	44.59±4.19a
Viability%	92.62±2.82	73.79±3.91*	78.18±2.36	81.55±1.65	90.72±3.24a	82.18±2.11

Means ± SE. Values with * is significantly different than control negative values at P<0.05. Values with letter a are significantly different than control positive values at P<0.05. AZA: Azathioprine: DEX: dexamethasone; epididymal sperm extraction: ESE.

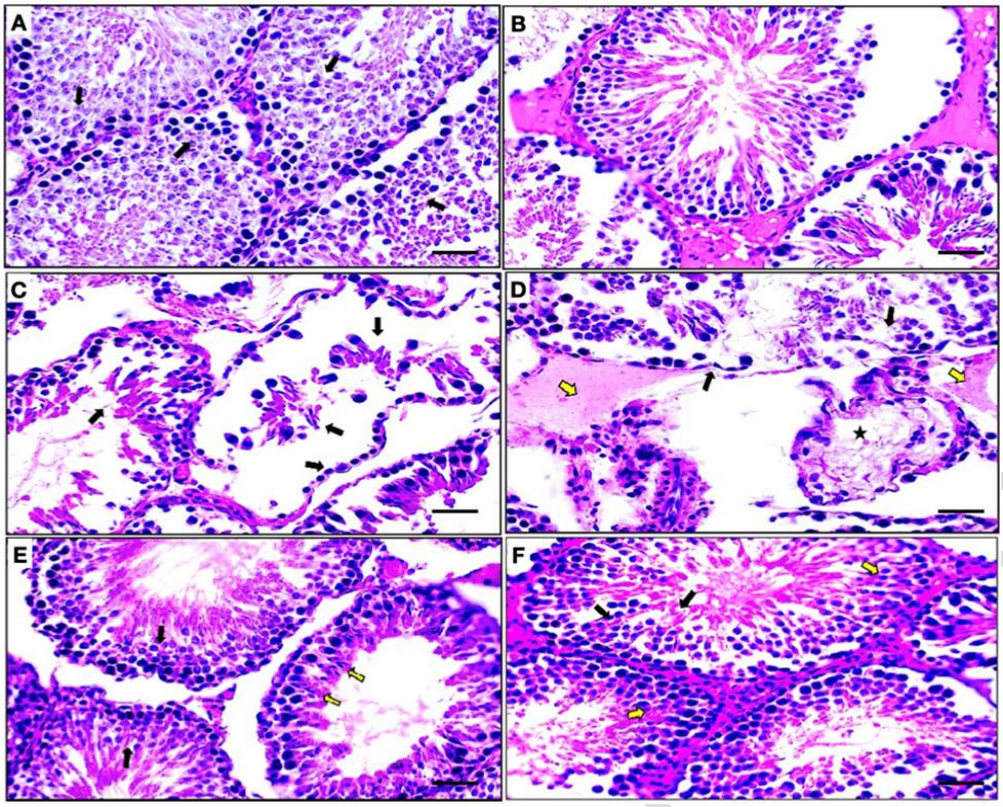

Fig. 1: 1A) Testis of the negative control group showed good spermatogenic activity with presence of the different stages of spermatogenic cells that completely occludes the lumen of the seminiferous tubules (arrows).1B) Testis of the control positive ESE treated rats showed multifocal peri-tubular edematous fluid infiltrations among the seminiferous tubules (yellow arrows). Also, severe detachment for the spermatogenic cells from the basement membrane into the lumen of seminiferous tubule was observed (black arrows). 1C) Testis of the AZA- ESE treated rat illustrated severe diffuse exfoliations of the spermatogenic cells from the basement membrane except the spermatogonial cells (black arrows). 1D) Testis of the ESE -DEX treated group displayed moderate degeneration and necrosis in the spermatogenic cells of seminiferous tubules (black arrows). Atrophy of some of tubules was observed (star). Moderate inter-tubular edematous fluid infiltrations (yellow arrows) were also noticed. 1E) High magnification for the testis of the frankincense ESE treated group clarified high spermatogenic activity with presence of the different stages of spermatogenic cells in the seminiferous tubules (black arrows). Normal intact active Sertoli cells were also noticed. 1F) Testis of the secondary antibody ESE treated rat illustrated the presence of the active and proliferative different stages of the spermatogenic cells (yellow arrows). Also, the healthy intact Sertoli cells were found (black arrows). H and E; X 400.

to the testis. In up to 50% of situations, epididymitis causes inflammation of the testis (Lotti and Maggi 2015). Furthermore, Prithiviraj (2013) demonstrated that ASA mean values and histological changes in the ESE group were higher than those determined for the control and sham-control animals using Wistar albino rats.

The findings in AZA and DEX-treated rats revealed that during weeks 2, 4, and 6, the cut-off values of the AZA-treated group were observed at higher dilutions indicating high ASA levels which are the 1/128, 1/32, and 1/32 serial dilutions respectively. Additionally, it was also noticed at a relatively low serial dilution of 1/16 at week 8. The findings also revealed that there was no significant difference between the positive control group and the ASA concentrations at first dilution (1/1) in the second, fourth and the sixth weeks. Additionally, the results showed that at weeks 8, there was a statistically significant reduction in ASA concentrations of compared to the positive control group. The first three collections' mean ASA index (%) values did not substantially differ from those of the positive control group. The ASA indices, however, seem to be lower in the eighth week. The ESE rats treated with either AZA or DEX were not significantly different from the positive control rats in terms of their testosterone concentrations, sperm count and sperm viability% compared to the negative control group. The ESE rats treated with either AZA or DEX were not significantly different from the positive control rats in terms of their testosterone concentrations. Histologically, testis of the AZA treated rat illustrated severe diffuse exfoliations of the spermatogenic cells from the basement membrane except the spermatogonial cells. Testis of the DEX treated group displayed moderate degeneration and necrosis in the spermatogenic cells of seminiferous tubules. Epididymis of the AZA or DEX treated rat showed focal desquamation in the pseudo-stratified columnar lining epithelium of the epididymal tubules. Mild inter-tubular infiltration of the inflammatory cells among some of epididymal tubules was found. Simsek et al. (2018) shown that AZA is used to reduce immunity and has previously been utilized to treat autoimmune disorders and leukemia. It increased the anti-inflammatory and immunomodulatory actions of T cells at a lower dose (azathioprine 2.0–2.5mg/kg). If the concentrations are higher than the therapeutic dose, it is likely that AZA interferes with cell division and, in the

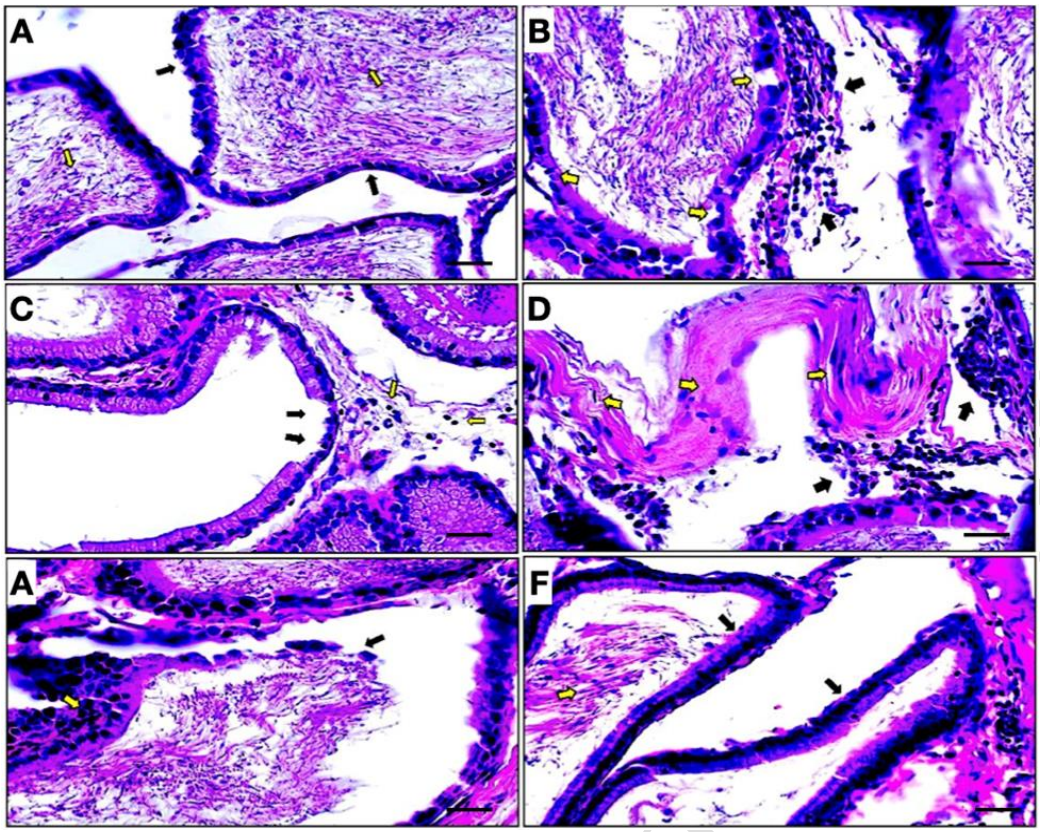

Fig. 2: 2A) High magnification for the epididymis of the control negative rat clarified normal epididymal tubules lined with healthy intact pseudo-stratified columnar epithelium (black arrows). The epididymal tubules completely filled with the sperm cells (yellow arrows). 2B) Epididymis of the control positive ESE treated rats showed moderate inter-tubular inflammatory cell infiltrations mainly lymphocytes among the epididymal tubules (black arrows). Mild desquamation in the lining epithelium of the epididymal tubules was found (yellow arrows). 2C) Epididymis of the AZA-ESE treated rat showed focal desquamation in the pseudo-stratified columnar lining epithelium of the epididymal tubules (black arrows). Mild inter-tubular infiltration of the inflammatory cells among some of epididymal tubules was found (yellow arrows). 2D) Epididymis of the DEX- ESE treated rats exhibited moderate multifocal inter-tubular inflammatory cell infiltrations mainly lymphocytes among the epididymal tubules (black arrows). Also folding and annulation in the tunica albuginea were observed around the epididymal tubules (yellow arrows). 2E) Epididymis of the frankensence-ESE treated group showed hyperplasia in the pseudo-stratified epithelial lining the epididymal tubules (yellow arrow) associated with detachment for some of the hyperplastic cells toward the lumen (black arrow). 2F) Epididymis of the secondary antibody ESE treated rat showed completely normal epididymal tubules lined with normal intact pseudo-stratified columnar epithelium (black arrows). Also, the epididymal tubules moderately filled with stored sperm cells (yellow arrow). H and E; X 400.

treatment of immunological disorders, negatively affects the germ cells and their proliferation. This medication is both cytotoxic and immunosuppressive (Dobrange et al. 2019). In terms of sperm density, motility, morphology, ejaculate volume, or total sperm count, AZA has no negative effects on the quality of semen (Dejaco et al. 2002). Lower doses of AZA (azathioprine 2.0–2.5mg/kg) have anti-inflammatory effects and modulate the immune system by causing T-lymphocyte apoptosis (Chavez-Alvarez et al. 2020). AZA treatment for male infertility is still debatable (Vermeire et al. 2012). According to Barz (2020), a member of the Center for Infertility Treatment and Embryo Research, there have recently been contradictory findings regarding the DEX treatment that support and oppose treating ASAs with corticosteroids. The outcomes revealed no evidence of a therapeutic benefit (Taiyeb et al. 2017). Due to these conflicting DEX readings, specialists are constantly at odds. Kadam et al. (2016) discovered a positive effect of autoimmune illness treatment in mice. However, Bubanovic et al. (2005) discovered that corticosteroids had a favorable effect on the immune system and a direct effect on immunoglobulin responses, making DEX a safe treatment for male ASA infertility.

Serum samples from ESE rats treated with frankincense were measured for their cut-off values, mean antibody index (%) and ASA concentration. The mean antibody index (%) only significantly decreased (P<001) at weeks 6 and 8 of treatment. The testosterone levels in rats treated with frankincense, however, recovered to normal levels since they were identical to those of the negative control and significantly lower than those of the positive control. High magnification for the testis of the frankincense treated group clarified high spermatogenic activity with presence of the different stages of spermatogenic cells in the seminiferous tubules. Normal intact active Sertoli cells were also noticed. Epididymis of the frankincense treated group showed hyperplasia in the pseudo-stratified epithelial lining of the epididymal tubules associated with detachment for some of the hyperplastic cells toward the lumen. This finding confirms the previous results of Alharbi et al. (2022), who discovered that frankincense enhanced the semen profile and boosted testosterone by protecting the testicles from

cyclophosphamide-induced damage. They mentioned that antioxidant effect may shield the testicles from oxidative injury by lowering the quantity of reactive oxidative species in the testis.

The cut-off value for the ESE rats treated with secondary antibodies was discovered at the 1/32 serial dilution for the second week, at the 1/8 serial dilution for the fourth and eighth weeks, and at the 1/4 serial dilution for the sixth week. In samples taken at weeks 2 and 4, as well as weeks 6 and 8, the mean values of the antibody indexes (%) were considerably lower than positive control group. The testis of the secondary antibody treated rat illustrated the presence of the active and proliferative different stages of the spermatogenic cells with healthy intact Sertoli cells. Epididymis showed completely normal epididymal tubules moderately filled with stored sperm cells. The sperm count and viability and the testosterone levels in rats treated with frankincense or secondary antibodies, however, recovered to normal levels since they were identical to those of the negative control and significantly lower than those of the positive control. A second rabbit of the same strain received an injection of a purified ASA produced in rabbits. According to Großerichter-Wagener et al. (2020), the second antibody isolated from the rabbits may contain anti-idiotypic antibodies. They are known as surrogate antigens because these anti-idiotypic antibodies mimic the original antigen. The likelihood of obtaining anti-isotypic and anti-allotypic antibodies depended on which strain or species the first antibody was administered to. Anti-idiotypic antibodies have been developed as an alternative to immunotherapy, according to Pan et al. (2020), since they can target specific antibodies, have fewer toxicity and side effects, and may offer long-lasting protection. In their review, Stanova et al. (2020) presented the hypothetical statement that anti-idiotype antibodies have the capacity to simulate an antigen's biologic activity in addition to competing with it for antibody binding. The antibodies produced by rabbits immunized with biopharmaceutical drugs are thought to be drug-specific antibodies that can be used as positive controls for anti-drug antibody measurements and assay reagents to determine the pharmacokinetics of original drugs (Boysen et al. 2016; Jiskoot et al. 2016). Therefore, in the current investigation, secondary antibodies have been tried as a treatment for ASA. Naz et al. (1993)'s research showed that secondary antibodies can counteract anti-fertilization antibodies' ability to prevent fertilization, provides evidence in support of this theory. Additionally, it may inhibit sperm antibodies from adhering to the sperm surface.

Conclusion

The findings support the idea that immune responses and acute inflammatory alterations in the testis are triggered by paracrine and immunological processes, which is how epididymal aspiration affects testis function. Future research on comprehensive hormone profiling or analyses of the hypothalamo-hypophysial-gonadal axis following these operations would be of interest.

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