ORIGINAL ARTICLE

Protective effects of zinc chloride on azathioprine-induced toxicity on germinal epithelium of albino rats: A morphometric study

Shahid Pervez Shaikh, Maria Mohiuddin, Amjad Ali, Zeeshan Qamar, Inayatullah, Hemant Kumar

Abstract

Objective: To determine the effects of azathioprine on germinal epithelium and evaluate the possible protection provided by co-administration of zinc chloride.

Design: A prospective experimental study.

Place of study: Anatomy Department, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Centre, Karachi from January 2016 till January 2017.

Material and Methods: For this study 60-male adult albino rats of 90-120 days of age were selected and distributed into three equal groups A, B and C. They were further divided into four sub-groups for 1st, 3rd, 6th and 8th weeks of the treatment. Group-A served as control, received only injection 0.9 % normal saline intraperitoneally daily, group-B received Inj. azathioprine 15mg/kg body weight intra-peritoneally daily and group-C received Inj. azathioprine 15mg/kg body weight plus Inj. zinc chloride 1mg/kg body weight intra-peritoneally daily. At the end of respective period of treatment animals were sacrificed. Both testes were excised, fixed in Bouin's solution for 24 hours. After processing in ascending grades of alcohol, cleared in xylene and fixed in paraffin, 5 µ thick sections were cut and stained with PAS-Iron Haematoxylin for study of seminiferous tubules morphology and micrometry.

Results: The tubules were compact, regularly arranged with intact basement membrane. The lumens of germinal epithelium contain mature spermatozoa in control group-A. The spaces between seminiferous tubules were increased with area of necrosis, the basement membrane of most of tubules were distorted and ruptured, the vacuoles between the germinal epithelium were appeared and the lumen contain slough without spermatozoa in group-B. There was widening of intertubular spaces, the tubules were wavy but basement membrane intact. Lumens were narrow and contained spermatozoa without slough in group-C, otherwise features similar to control.

Conclusion: Azathioprine produced germinal epithelium toxicity which is more pronounced with time period and provided protection by zinc chloride.

Keywords: Seminiferous tubules, azathioprine, zinc chloride, germinal epithelium, intraperitoneally, morphology, micrometry, spermatozoa.

Received
Date:13th December, 2018
Accepted
Date:7th October, 2019

Hamdard College of Medicine and Dentistry, Karachi
SP Shaikh
M Mohiuddin
H Kumar

CMH Multan Institute of Medical Sciences,
A Ali

Liaquat College of Medicine and Dentistry, Karachi
Z Qamar

Saidu Medical College, Swat
Inayatullah

Correspondence:
Dr. Shahid Pervez Shaikh,
Address: C-303, Yasir Terrace Phase -II,
Block-10, Gulistan-e-Johar, Karachi.
Cell No: +92 300-9212461
email: shahidpervez_dr@hotmail.com

Introduction:

The testis is sensitive following exposure to various toxicants. Therapeutic drugs, especially chemotherapeutics, can also adversely affect male fertility leading to testicular cells injury.[1]

Azathioprine is an immuno-modulatory and cytotoxic drug often used to treat inflammatory bowel disease, auto-immune disorders, organ transplant rejection, and cancer.[2,3] Despite of its effectiveness, it can cause drug-induced toxicity with increased risk of bone marrow suppression and vulnerability to infection, even when used in standard doses.[4,5] Some cases of testicular toxicity have been reported with azathioprine.[1] Azathioprine also increases oxidative stress; upon its administration, as it is rapidly metabolized into several toxic and non-toxic metabolic com-

pounds, including the active 6-mercaptopurine that is formed through a conjugation reaction with glutathione.[5,6] Its active metabolite 6-mercaptopurine damages rapidly dividing cells, such as those in the bone marrow, intestinal epithelium, and reproductive organs of adults.[7,8]

Azathioprine causes reductions in the organ weights of the thymus, spleen, liver, kidney, and testis in a dose-dependent manner. It severely affects spermatogenesis with lowered sperm count and reproductive failure in rats.[9-11]

The testicular atrophy and infertility, with genetic polymorphisms of the thiopurine methyltransferase enzyme, which is responsible for thiopurine metabolism, possibly contributing to its mechanism. Population studies have shown that patients with low enzymatic activity have a high risk for severe, potentially fatal toxicities.[10,12]

Zinc has been shown to have antioxidant and membrane stabilizing properties.

Zinc is an essential mineral for spermatogenesis and a hepatocellular metal lothionein inducer, and zinc co-treatment protects tissues against free radicals and oxidative stress.[13,14]

Evidence shows that during zinc deficiency, cellular oxidative stress is markedly induced by exposure to linoleic acid, TNF-α, or both and that this oxidative stress can be partially blocked by zinc supplementation. These facts indicate that the defense against oxidative stress plays critical roles in the maintenance of spermatogenesis and prevention of testicular atrophy.[15,16] Zinc is a trace mineral essential for normal functioning of the male reproductive system. In general, long-term deprivation of zinc renders an organism more susceptible to injury induced by a variety oxidative stress.[17]

The antioxidants protect germ cells against oxidative DNA damage and play important role in spermatogenesis. Zinc may stabilize lipid membrane and protects lipid per oxidation by free radicals, there by protecting tissues.[18]

The current study was undertaken to characterize the effects of azathioprine on testicular morphology in albino rats, and to examine if zinc can protect changes induced by azathioprine.

Material and methods:

This study was conducted in the department of Anatomy, Basic Medical Sciences Institute (BMSI), Jinnah Postgraduate Medical Centre (JPMC), Karachi from January 2016 till January 2017. For this study, 60-healthy male adult Albino rats of 90 -120 days of age were taken from the Animal House of Basic Medical Sciences Institute, Jinnah Postgraduate Medical Centre, Karachi. They were kept under observation for 10-days prior to the commencement of the study, for assessment of their health status, on the basis of weight gain or loss.

The animals were divided into three groups A, B and C and each comprising 20 rats:

Group-A served as control, group-B received azathioprine & group-C received azathioprine plus zinc chloride.

Each group further divided into four subgroups according to the period of treatment, 1st week, 3rd week, 6th week and 8th weeks. Each subgroup contains 5 animals.

Group-A: All subgroup (A_1, A_2, A_3 and A_4) received injection 0.9% normal saline intraperitoneally daily and sacrificed at the end of their respective period of treatment.

Group-B: All subgroups (B_1, B_2, B_3 and B_4) received Injection Azathioprine 15 mg/kg body weight intra-peritoneally as single daily dose and sacrificed at the end of their respective period of treatment.

Group-C: All subgroups (C_1, C_2, C_3 and C_4) given Injection Azathioprine 15mg/kg body weight intra-peritoneally daily plus Injection Zinc chloride 1mg/kg body weight intra-peritoneally daily and sacrificed at the end of their respective period of treatment.

The animals were kept in the cages of animal

Table 1: *Mean* Diameter (µm) of Seminiferous Tubules of Albino Rats in Different Groups at Variable Time Interval*

Groups	Treatment Received	Sub Groups	Duration of Treatment			
			1st Week	3rd Week	6th Week	8th Week
A (n=20)	Control	A1(n=5)	270.20±7.07	----	----	----
		A2(n=5)	----	282.16±1.48	----	----
		A3(n=5)	----	----	289.73±1.61	----
		A4(n=5)	----	----	----	300.91±4.15
B (n=20)	Azathioprine`	B1(n=5)	233.14±12.41	----	----	----
		B2(n=5)	----	221.75±2.24	----	----
		B3(n=5)	----	----	207.11±2.00	----
		B4(n=5)	----	----	----	194.14±1.65
C (n=20)	Azathioprine + Zinc Chloride	C1(n=5)	261.40±1.46	----	----	----
		C2(n=5)	----	281.71±1.71	----	----
		C3(n=5)	----	----	290.31±0.90	----
		C4(n=5)	----	----	----	303.20±3.12

**Mean±SEM*

Table 1: *Statistical analysis of mean diameter of seminiferous tubules between groups*

Statistical Comparison	P-value
B1 vs A1	P<0.5*
C1 vs A1	P<0.5*
C1 vs B1	P<0.5*
B2 vs A2	P<0.001****
C2 vs A2	P>0.5*
C2 vs B2	P<0.001****
B3 vs A3	P<0.001****
C3 vs A3	P>0.5*
C3 vs B3	P<0.001****
B4 vs A4	P<0.001****
C4 vs A4	P>0.5*
C4 vs B4	P<0.001****

**Insignificant, **Significant, ***Moderately Significant, ****Highly Significant*

House of BMSI, JPMC under natural environment, water and food supplied ad libitum.

At the end of respective period of treatment animals were sacrificed under ether anesthesia. A low midline abdominal incision extending up to the skin of scrotum, all coverings of scrotum were dissected and both testes were identified and removed and weighed on electro balance and results were calculated.

The testes were fixed in Bouin's fluid for 24-hours, before they were cut longitudinally into 2-equal halves and again post-fixed in fresh Bouin's fluid for next 24-hours. The tissues were dehydrated in the ascending strengths of alcohol, cleared in xylene. Infiltrated and embedded in paraffin wax, the tissue blocks were made, cut into 5 µm thick sections with the help of rotatory microtome. The sections were mounted on albumenized glass slides and stained with PAS-Iron Hematoxylin. Morphological study of testes was done, and morphometric study of germinal epithelium was measured with the help of ocular micrometer scale under light microscope.

The level of significance (p) was calculated by the help of student's t-distribution table. The significance level was considered as $p \leq 0.05$. All the calculations were done utilizing, SPSS 15.0.

Results:

The present study was planned to observe the germinal epithelium changes produced by azathioprine and possible protection provided by co-administration of zinc chloride.

Observations on control (group-A) under the light microscope shows seminiferous tubules compact with narrow interstitial space and intact basement membrane. The germinal epithelium was regularly arranged, and the lumens contain mature spermatozoa without sough as shown in Figure-1. Azathioprine-treated (group-B) shows the spaces between shrunken seminiferous tubules with area of necrosis, the basement membrane of most of tubules were detached, distorted and ruptured, the vacuoles between

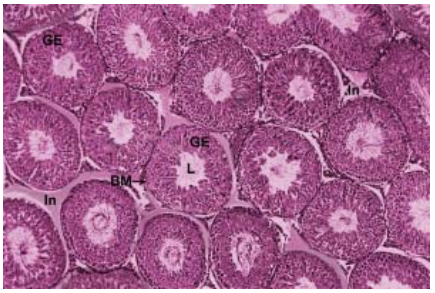

Figure-1: PAS-Iron haematoxylin stained, 4 µm thick section of testis of control albino rat, showing regular compact arrangement of seminiferous tubules, narrow interstitial space (In) with intact basement membrane (BM), germinal epithelium (GE), lumen of tubules (L) and spermatozoa in lumen. (Photomicrograph with low magnification after 8 weeks of treatment)

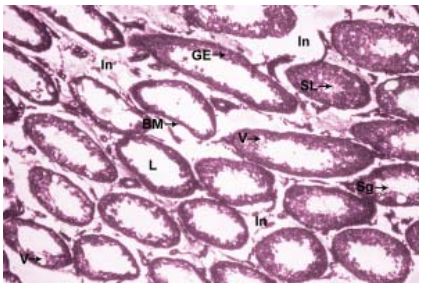

Figure-2: PAS-Iron haematoxylin stained, 4 µm thick section of testis after eight weeks of Azathioprine treatment in albino rat, showing shrunken seminiferous tubules with marked widening of interstitial spaces (In), distorted and ruptured basement membrane (BM), appearance of vacuoles (V) and lumen (L) of tubules contained slough (SL) without spermatozoa. (Photomicrograph with low magnification after 8 weeks of treatment)

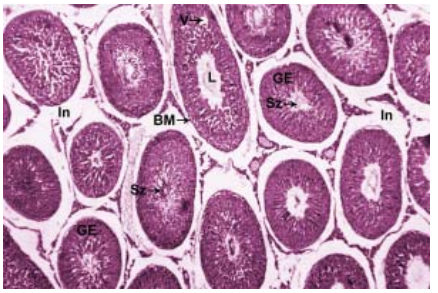

Figure-3: PAS-Iron haematoxylin stained, 4 µm thick section of testis with Azathioprine and zinc treatment in albino rat, showing regular seminiferous tubules with widening of interstitial space (In), germinal epithelium (GE) with wavy appearance without disruption of basement membrane (BM), appearance of few vacuoles (V) and lumen of tubules contained spermatozoa (Sz) without slough. (Photomicrograph with low magnification after 8 weeks of treatment)

Table 2: *Mean* Thickness of Germinal Epithelium (µm) of Seminiferous Tubules of Albino Rats in Different Groups at Variable Time Interval*

Groups	Treatment Received	Sub Groups	Duration of Treatment			
			1st Week	3rd Week	6th Week	8th Week
A (n=20)	Control	A1(n=5)	76.55±1.11	----	----	----
		A2(n=5)	----	89.31±0.71	----	----
		A3(n=5)	----	----	90.72±0.14	----
		A4(n=5)	----	----	----	95.74±0.25
B (n=20)	Azathioprine`	B1(n=5)	69.43±0.51	----	----	----
		B2(n=5)	----	68.51±0.89	----	----
		B3(n=5)	----	----	56.25±0.35	----
		B4(n=5)	----	----	----	51.18±0.38
C (n=20)	Azathioprine + Zinc Chloride	C1(n=5)	79.44±0.47	----	----	----
		C2(n=5)	----	80.86±0.42	----	----
		C3(n=5)	----	----	87.95±0.43	----
		C4(n=5)	----	----	----	93.71±0.50

**Mean±SEM*

the germinal epithelium were appeared and the lumen contain slough without spermatozoa as shown in Figure-2. These features were more pronounced with time period. Azathioprine plus zinc treated rats (group-C) shows widening of intertubular spaces, the tubules were wavy but basement membrane intact. Lumens were narrow and contained spermatozoa without slough i.e. findings like control as shown in Figure-3.

The mean value of diameter of seminiferous tubules of A1, A2, A3 and A4 were 270.20±7.07 µm, 282.16±1.48 µm, 289.73±1.61µm and 300.91±4.15 µm respectively and of B1, B2, B3 and B4 were 233.14±12.41µm, 221.75±2.24µm, 207.11±2.00µm and 194.14±1.65µm respectively as shown in table-1. The mean value of thickness of germinal epithelium of seminiferous tubules was 76.55±1.11 µm, 89.31±0.71 µm, 90.72±0.14 µm and 95.74±0.25 µm respectively and of B1, B2, B3 and B4 were 69.43±0.51 µm,

Table 2: *Statistical analysis of mean thickness of germinal epithelium of seminiferous tubules-between groups*

Statistical Comparison	P-value
B1 vs A1	P<0.01***
C1 vs A1	P<0.001****
C1 vs B1	P<0.001****
B2 vs A2	P<0.001****
C2 vs A2	P<0.05**
C2 vs B2	P<0.001****
B3 vs A3	P<0.001****
C3 vs A3	P<0.01***
C3 vs B3	P<0.001****
B4 vs A4	P<0.001****
C4 vs A4	P<0.01***
C4 vs B4	P<0.001****

**Insignificant, **Significant, ***Moderately Significant, ****Highly Significant*

68.51±0.89 µm, 56.25±0.35 µm and 51.18±0.38 µm respectively as shown in table-2.

The mean value of diameter of seminiferous tubules of subgroup-CI was 261.40±1.46 µm. The insignificant decreased in diameter of seminiferous tubules observed (P<0.5), when compared with subgroup-A1, also insignificant decreased in diameter of seminiferous tubules observed (P<0.5), when compared with subgroup-B1 as shown in Table-1. The mean value of thickness of germinal epithelium of seminiferous tubules was 79.44±0.47 µm. The highly significant increased in thickness of germinal epithelium of seminiferous tubules observed (P<0.001), when compared with subgroup-A1. While comparing with B1, also highly significant increased thickness of germinal epithelium of seminiferous tubules observed (P<0.001), as shown in Table-2.

The mean value of diameter of seminiferous tubules of subgroup-C2 was 281.71±1.71 µm. The insignificant decreased in diameter of seminiferous tubules observed (P>0.5), when compared with subgroup-A2, while highly significant increased in diameter of seminiferous tubules observed (P<0.001), when compared with subgroup-B2, as shown in table-1. The mean value of thickness of germinal epithelium of seminiferous tubules was 80.86±0.42 µm. The significant decreased in thickness of germinal epithelium of seminiferous tubules observed (P<0.05), when compared with subgroup-A2, while comparing with B2 highly significant increased thickness of germinal epithelium of seminiferous tubules observed (P<0.001), as shown in table-2.

The mean value of diameter of seminiferous tubules of subgroup-C3 was 290.31±0.90 µm. The insignificant decreased in diameter of seminiferous tubules were observed (P>0.5), when compared with subgroup-A3, while highly significant increased in diameter of seminiferous tubules observed (P<0.001), when compared with subgroup-B3, as shown in table-1. The mean value of thickness of germinal epithelium of seminiferous tubules was 87.95±0.43 µm. The moderately significant decreased in thickness of germinal epithelium of seminiferous tubules observed (P<0.01), when compared with subgroup-A3, while comparing with B3 highly significant increased thickness of germinal epithelium of seminiferous tubules observed (P<0.001), as shown in table-2.

The mean value of diameter of seminiferous tubules of subgroup-C4 was 303.20±3.12 µm. The insignificant increased in diameter of seminiferous tubules observed (P>0.5), when compared with subgroup-A4, while highly significant increased in diameter of seminiferous tubules observed (P<0.001), when compared with subgroup-B4, as shown in table-1. The mean value of thickness of germinal epithelium of seminiferous tubules was 93.71±0.50 µm. The moderately significant decreased in thickness of germinal epithelium of seminiferous tubules observed (P<0.01), when compared with subgroup-A4, while comparing with B4 highly significant increase thickness of germjinal epithelium of seminiferous tubules observed (P<0.001), as shown in Table-2

Discussion:

The present study was designed to observe the effects of azathioprine and the protection offered by zinc on germinal epithelium in different groups at different time intervals.

The observation on germinal epithelium of

testes of azathioprine-treated animals showed significant reduction in the thickness and diameter of germinal epithelium. The number of primary spermatocytes and spermatogonia were decreased. These findings showed that azathioprine induced morphometric changes. These morphometric changes could be protected by zinc chloride.[19]

Spermatogenesis is highly susceptible to testicular inflammation, which causes damage to the seminiferous epithelium and increases apoptosis of spermatogenic cells. The effect of inflammation on spermatogenesis is evidenced by the histological results in this study, suggesting a direct association between azathioprine treatment and testicular inflammation. This association is supported by the study of Ramonda et al. 2014.[20]

Azathioprine is an immunosuppressive agent that acts as an antagonist of purine metabolism, resulting in the inhibition of DNA, RNA, and protein synthesis.[3] Iwasaki et al proved that azathioprine induced impairment of spermatogenesis by direct inhibition of germinal epithelium or indirect by influencing the axis between hypothalamus-pituitary and gonads leads to testicular atrophy and azoospermia.[21]

Zinc is necessary for immune function, DNA replication, RNA polymerases and metabolic processes.[17] Results of present study showed in group-C very little dis-arrangement of germinal epithelium, so zinc provides significant protection of testicular tissues, like control. This agrees with a previous study observed that; zinc supplementation ameliorates lead-induced rat testicular damage.[22]

Zinc exists in spermatozoa within the seminiferous tubules and helps spermatogenesis. It has also been suggested that Zinc acts as an anti inflammatory factor and is involved in sperm's oxidative metabolism.[14] According to findings of study there was no reversibility in reduction of germinal epithelium width and number of spermatogonia and primary spermatocytes in azathioprine treated animals as compare to zinc treated animals. Fereshte torabi et al, reported protective role of Zinc supplementation against cyclophosphamide induced germinal epithelium damage in wistar rats.[23]

Conclusion:
The current study concluded the germinal epithelium toxicity by azathioprine, which is more pronounced with increase time period and can be protected by the anti-oxidant zinc chloride.

Conflict of interest: None

Funding source: None

Role and contribution of authors:
Dr. Shahid Pervez Shaikh, idea and conception, data collection and initial drafting.

Dr. Maria Mohiuddin, did final drafting and reference collection.

Dr. Amjad Ali, did data collection & data analysis.

Dr. Zeeshan Qamar, did statistics and final drafting.

Dr. Inayatullah, did data collection.

Dr. Hemant Kumar, collected the data, references, critically went through the article and made final chnags.

References:
1. Schaalan MF, Ramadan BK, Abd Elwahab AH. Ameliorative effect of taurine- chloramine in azathioprine-induced testicular damage; a deeper insight into the mechanism of protection. BioMed Central Complement Altern Med. 2018; 18: 255.
2. La Duke KE, Ehling S, Cullen JM, Bäumer W. Effects of azathioprine, 6- mercaptopurine, and 6-thioguanine on canine primary hepatocytes. Am J Vet Res. 2015; 76(7): 649–655.
3. Ponticelli C, Glassock RJ. Prevention of complications from use of conventional immunosuppressants: a critical review. Journal of nephrology. 2019 Mar 29:1-20.
4. Ramadan BK, Schaalan MF, Mahmoud ES. Protective Effect of Taurine on Thiopurine-Induced Testicular Atrophy in Male Albino Rats. J Steroids Horm Sci. 2017;9(1):192.
5. van Asseldonk DP, Sanderson J, de Boer NK, Sparrow MP, Lémann M, Ansari A. Difficulties and possibilities with thiopurine therapy in inflammatory bowel disease–proceedings of the first Thiopurine Task Force meeting. Dig Liver Dis. 2011; 43(4): 270–276.
6. da Silva Coelho MC, Botelho ACP, Bruno PB. Effectiveness of Azathioprine in Maintaining Remission in Crohn's Disease: A Systematic Review. Gastroint Hepatol Dig Dis. 2019;2(1):1-

6.
7. Panettieri RA, Schaafsma D, Amrani Y, Koziol-White C, Ostrom R, Tliba O. Non-genomic effects of glucocorticoids: an updated view. Trends in pharmacological sciences. 2018 Nov 26.
8. Bendre SV, Shaddock JG, Patton RE, Dobrovolsky VN, Albertini RJ, Heflich RH. Effect of chronic Azathioprine treatment on germ–line transmission of Hprt in mice. Environ Mol Mutagen. 2007; 48(9): 744–753.
9. Nawab A, Zhao Y, Ibtisham F, Li G, Xiao M, Wu J, Liu W, Tang S, An L. The Potential Effect of Zinc Deficiency on Reproductive Profile of Male Rat and its Possible Consequences. Animal Review. 2018;5(2):12-21.
10. Zeyneloglu HB, Oktem M, Durak T. Male infertility after renal transplantation: achievement of pregnancy after intracytoplasmic sperm injection. Transplant Proc.2005; 37(7): 3081-4.
11. Shenuka S, Thiyagarajan T, Kumar SR. A Review on the Effect of Immunosuppressants oon Fertility. Research Journal of Pharmacy and Technology. 2019 Mar 1;12(3):1441-7.
12. Lee MN, Kang B, Choi SY, Kim MJ, Woo SY, Kim JW, Choe YH, et al. Impact of genetic polymorphisms on 6-thioguanine nucleotide levels and toxicity in pediatric patients with IBD treated with azathioprine. Inflamm Bowel Dis.2015; 21(12): 2897-2908.
13. Afonne OJ, Orisakwe OE, Ekanem IA, Akumka DD. Zinc protects chromium- induced testicular injury in mice. Indian J Pharmacol 2002; 34: 26-31.
14. 14. Adedara IA, Abiola MA, Adegbosin AN, Odunewu AA, Farombi EO. Impact of binary waterborne mixtures of nickel and zinc on hypothalamic-pituitary-testicular axis in rats. Chemosphere. 2019 Dec 1;237:124501.
15. Yang Y, Cheng JZ, Singhal SS, et al. Role of glutathione S-transferases in protection against lipid peroxidation. J. Biol. Chem. 2001; 276: 19220-19230.
16. Kabel AM. Zinc/alogliptin combination attenuates testicular toxicity induced by doxorubicin in rats: Role of oxidative stress, apoptosis and TGF-β1/NF-κB signaling. Biomedicine & Pharmacotherapy. 2018 Jan 1;97:439-49.
17. Powell SR, The Antioxidant Properties of Zinc. Journal of Nutrition. 2000; 130: 1447S-1454S.
18. Rostan EF, DeBuys HV, Madey DL, Pinnell SR. Evidence supporting zinc as an important antioxidant for skin. Int J Dermatol 2002; 41:606-11.
19. Batra N, Nehru B, Bansal MP. Reproductive potential of male portan rats exposed to various levels of lead with regard to zinc status. Br J Nutr 2004; 91: 387-91.
20. Ramonda R, Foresta C, Ortolan A, Bertoldo A, Oliviero F, Lorenzin M. Influence of tumor necrosis factor α inhibitors on testicular function and semen in spondyloarthritis patients. Fertil Steril. 2014;101(2):359–365.
21. Iwasaki M, Fuse H, Katayama T. The effects of cyclosporine azathioprine and mizoribine on male reproduction in rats. Nippon Hinyokika Gakkai Zasshi. 1996; 87: 42-49.
22. Rafique M, Naheed K, Khalida P, Anjum N. The effects of lead and zinc on the quality of semen of albino rats. J Coll Physicians Surg Pak 2009; 19: 510-3.
23. Torabi F, Shafaroudi MM, Rezaei N. Combined protective effect of zinc oxide nanoparticles and melatonin on cyclophosphamide-induced toxicity in testicular histology and sperm parameters in adult Wistar rats. International Journal of Reproductive BioMedicine. 2017 Jul;15(7):403.