Assiut University Journal of Multidisciplinary Scientific Research (AUNJMSR)
Faculty of Science, Assiut University, Assiut, Egypt.
Printed ISSN 2812-5029
Online ISSN 2812-5037
Vol. 51(1): 57–70 (2022)
https://aunj.journals.ekb.eg/

Busulfan induced azoospermia in rat via oxidative stress, hormonal change, and down-regulation of MND1 gene expression in testicular tissue

Mai Emad [1*], Sohair MM Rajb [1], Abdel-Razik Khalifa[2], Emad A. Ahmed[1], Emad AbdelRhim Taha[3] and Hossam El-Din M.Omar[1]

[1] Laboratory of Physiology, Zoology Department, Faculty of Science, Assiut Univerisity, Assiut, Egypt;

[2] Laboratory of Animal Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Assiut Univerisity, Assiut, Egypt;

[3] Department of Dermatology, Venereology and Andrology, Faculty of Medicine, Assiut University, Egypt;

[*]Corresponding Author: emai21837@gmail.com

ARTICLE INFO

Article History:
Received: 6/12/2021
Accepted: 22/12/2021
Online: 14/2/2022

Keywords:
Azoospermia, Gene expression, Testicular dysfunction, Oxidative stress, Sex hormones

ABSTRACT

This prospective study compares the diagnostic and predictive value of MND1 gene expression in testicular dysfunction in rats. Thirteen rats given a single intraperitoneal injection of 10 mg/kg of body weight busulfan to induce azoospermia, and 15 untreated rats remained as the control group. After 35 days, blood and testis were collected for analysis. MND1 gene expression was done by PCR. Follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were measured in blood by ELISA. Oxidative stress markers and lipid profile were determined colimetrically. Histopathological and immunohistochemical studies were used H&E and YAP stain. Body weight, MND1 gene expression, TAC and testosterone significantly decreased in busulfan-treated rats compared to control ($p<0.0001$). Serum FSH, LH, NO, LPO, and HDL were significantly increased in busulfan treated group compared to control. Testis of busulfan-treated rats showed a significant decrease in YAP immuno-expression compared to control. In conclusion, significant decrease in MND1 expression in testicular tissue with increase in serum level of FSH and LH are promising predictor for testicular function.

INTRODUCTION

Azoospermia means that there is no sperm in the ejaculate and 15% of males are infertile . There are two types of azoospermia: obstructive azoospermia (OA), which accounts for 40%, and non-obstructive azoospermia (NOA), which accounts for 60%. OA is caused by a physical blockage of the male excurrent ductal system, which results in normal endocrine function and spermatogenesis in the testis. NOA is caused by either primary or secondary testicular failure (when LH and FSH are increased) [1]. Animal models of azoospermia are extremely useful for research. Busulfan decrease cell division by adhering to one of the DNA strands [2]. Busulfan treatment resulted in an increase in cell apoptosis and a decrease the weight of testis [3]. Furthermore, a single dose of busulfan can permanently infertile mice at non-lethal doses and cause long-term morphological damage to sperm produced by surviving spermatogonia [4]. The cells of spermiogenesis were reduced, and meiosis was delayed. However, some cells remained unchanged [2]., and dyslipidemia has been linked to low testosterone levels [5]. Furthermore, busulfan injection increased apoptotic sperm and induced oxidative stress [6].

In mice, 47 genes involved in DNA repair, spermatogenesis were found to have differential testicular gene expression and were linked to male infertility [7]. Changes in the spermatogenesis process caused by genetics or environmental factors can result in male sterility [8]. Meiotic nuclear division 1 (MND1) is gene connected with human and animal spermatogenesis [9]. MND1 appear prior to meiotic division, and its corresponding proteins are involved in meiotic progression during spermatogenesis [9]. As a result, studying the expression patterns of MND1 and its housekeeping gene, GAPDH, in rats provides a way to diagnose and predict testicular function. This study aimed to see if MND1/GAPDH is a candidate predictor of testicular function in animals' model of azoospermia.

MATERIALS AND METHODS

Study design

This prospective study is conducted in the Zoology Department, Faculty of Science, Assiut University. Thirty male rats were divided into two groups of 15 rats. The intervention group given a single intraperitoneal injection of busulfan (10 mg/kg body weight) dissolved in distilled water [2], whereas the control group received only distilled water.

Collection of samples

At the end of experiment rats were sacrificed. Blood and testes were collected for biochemical histological investigation. Blood was centrifuged at 3000 rpm for separation of serum. One testis from each rat were fixed in formal alcohol for histological and immunohistochemical studies, small part of the other testis was examined under a light microscope for sperm detection and the other part of the same testis was preserved in liquid nitrogen for MND1 gene expression and biochemical analysis.

Testicular gene expression analysis

For the isolation of RNA, each part of testis samples was treated with QIAzol lysis reagent containing RNase inhibitor (Qiagen, Germany). Before processing, the redissolved RNA in RNase-free water was cleaned with a miRNeasy Mini Kit (Qiagen, Germany) to remove all contaminants, inhibitors, and residuals. Following this, cDNA was synthesized for each extracted sample using a high-capacity cDNA Reverse Transcription with RNase Inhibitor Kit (Applied Biosystems, UK). The expression level of the MND1 target gene was measured using SYBR Green-based real-time PCR and subsequent relative quantification analysis on the Step One Real-Time PCR system using GAPDH as a housekeeping gene (Applied Biosystems, UK). For each sample, the 20 L PCR reaction mixture included 15 L master mix with 9 L PCR-grade water, 1 L forward primer for each parameter and the housekeeping gene (20 pmol/L), 1 L reverse primer for each parameter and the housekeeping gene (20 pmol/L), 4 L ready-to-use SYBR Green Universal Master Mix (Applied Biosystems, UK), and 50 ng cDNA. The thermal profile was as follows: initial denaturation at 95°C for 10 minutes, then 45 cycles of amplification, beginning with denaturation at 95°C for 10 seconds, annealing at 66°C for 20 seconds, and extension at

72°C for 25 seconds. Following amplification, an additional melting curve analysis cycle was performed for product characterization by heating the reaction mixture at a rate of 0.2 °C/s from 65 °C to 95 °C. The gene expression values were calculated automatically by step one of the real-time PCR system software using relative quantitative analysis [10].

Hormonal and biochemical measurements

VIDAS® (BioMérieux Corporate) was used to measure follicle-stimulating hormone (FSH) levels, luteinizing hormone (LH) levels, and testosterone levels in serum. Total antioxidant capacity (TAC) was determined by the method of Koracevic [11], lipid peroxidation (LPO) was measured by the method of Ohkawa et al. [12], and nitric oxide (NO) through the method of Ding [13]. Triglycerides were measured with liquizyme CHOD-PAP (single reagent) [14]. Cholesterol was measured with liquizyme CHOD-PAP (single reagent) [15].

Histological examination

Following fixation, ethanol and xylene were used to dehydrate the testes, which were then embedded in paraffin wax and sectioned at thicknesses of 5–7 m (16). Using tissue for immunocytochemistry and staining with YES1 was used to identify primary and secondary spermatocytes. A microscope was used to capture approximately 15–20 randomly selected fields in each of three nonadjacent sections per testis. The total number of primary and secondary spermatocytes was determined using the ImageJ programme [17].

Statistical analysis

Where applicable, baseline characteristics were summarized as mean and standard deviations (SDs), median and interquartile range, and numbers with percentages. All analyses were carried out using Prism version 8 and the R Statistical Package**.**

RESULTS

The gene expression of MND1 in the tissue of testicular showed highly significant decrease (P 0.0001) in busulfan group compared to the normal group. **Figure 1.**

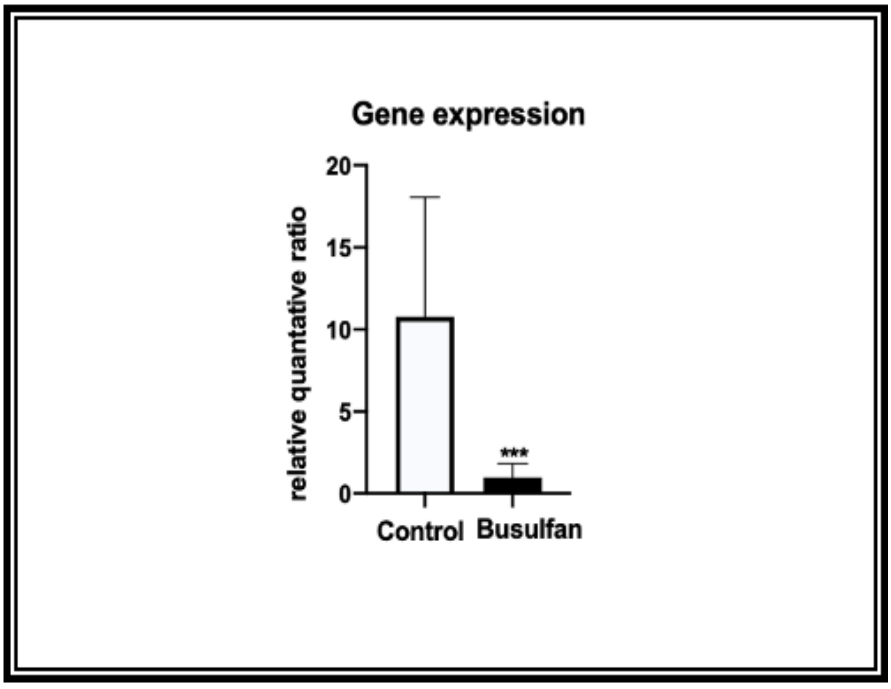

Figure 1. MND1 gene expression in rats after treatment with busulfan to induce azoospermia versus control rats.

Histological and immunohistochemical observation

Figure 2. Showed the testes of control rats (A&B) which revealed normal testicular tissue with the normal pattern of seminiferous tubules, a cluster of Leydig cells in the interstitial space between the seminiferous tubules lined by spermatogenic cells and Sertoli cells, (SCs) and that the tissue was full of spermatozoa (A). Seminiferous tubule lined by different stages of spermatogenic cells (B). Testes of rats treated with busulfan as seen in C&D showed testicular tissues damage, congested blood vessels, an absence of sperms in seminiferous tubules, a wide interstitial cell, oedema in the interstitial tissue, and macro vacuolation within the tubules in between degenerative spermatogenic cells (C). Moreover, magnification of the germ layer reveals degeneration with some germ cells losing contact with the surrounding Sertoli cell cytoplasmic processes and being shed into the tubular lumen ("germ cell exfoliation") and presence of spermatid giant cells, hypo spermatogenesis, and seminiferous epithelial vacuolation (D).

Immuno-expression of YAP in the testes appeared as brown dots in the primary and secondary spermatocytes. This expression was significantly decreased (P 0.0001) in busulfan treated group

rats testis (Figure 3A&B) in comparison to normal rats. Changes in YAP immuno-expression calculated by the ImageJ programme showed 72.4 % decrease (P 0.0001) in busulfan treated group rats testis compared to the normal group **(Figure 3C).**

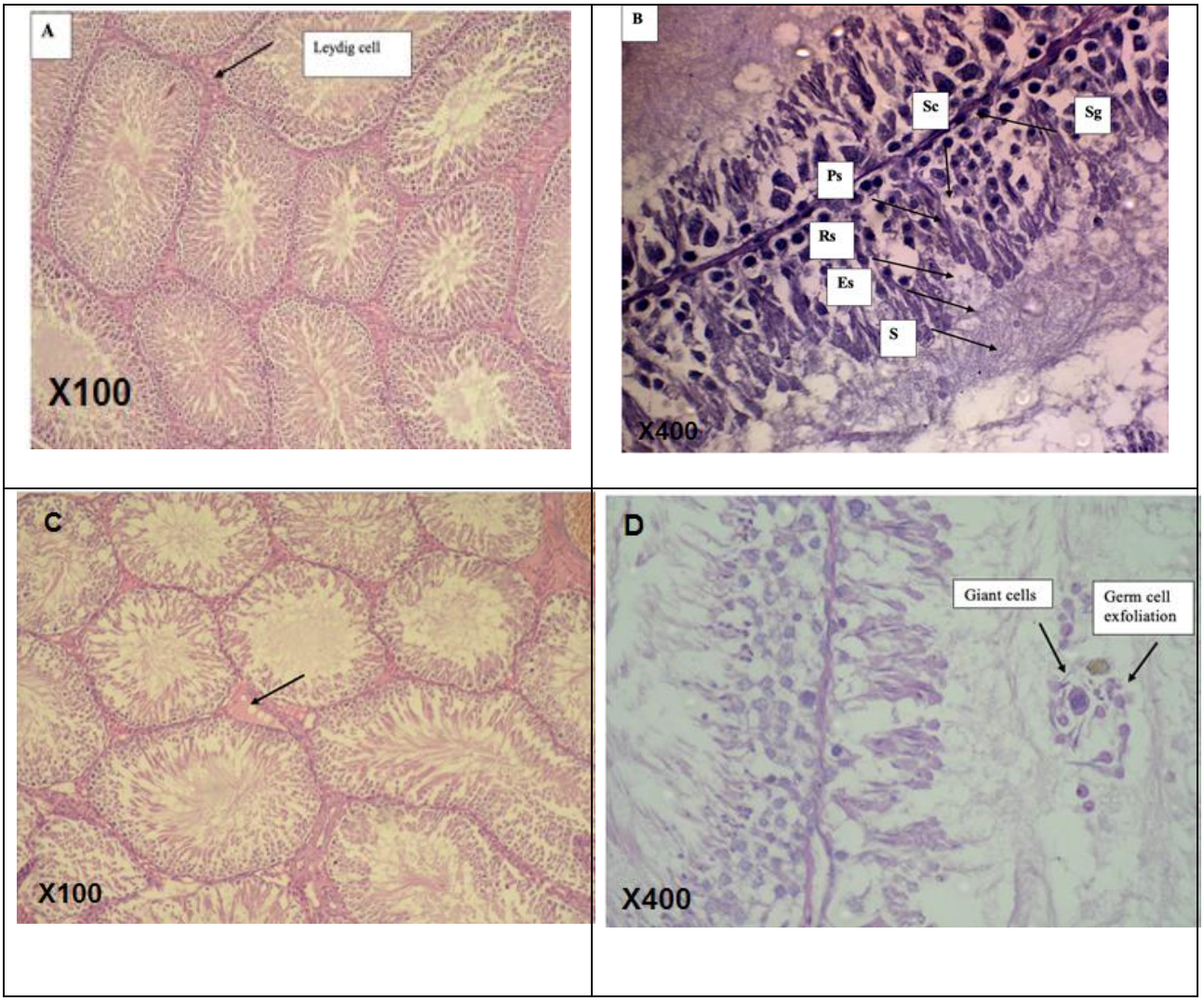

Figure 2. Photomicrographs of testis from the control (A&B) and busulfan treated rats (C&D) showing normal pattern of seminiferous tubules with cluster of Leydig cells are present in the interstitial space between the tubules (A), Seminiferous tubule lined by spermatogenic cells; spermatogonia (Ps), primary spermatocyte, rounded spermatids (Rs), elongated spermatids (Es), spermatozoa (S) and Sertoli cells (Sc) (B), damaged testicular tissue showing congested blood vessels, tubule with no sperm and edema in interstitial tubules (arrow) (C), and macro vacuolation within the tubules with presence of spermatid giant cells (D). Hematoxylin and Eosin stained.

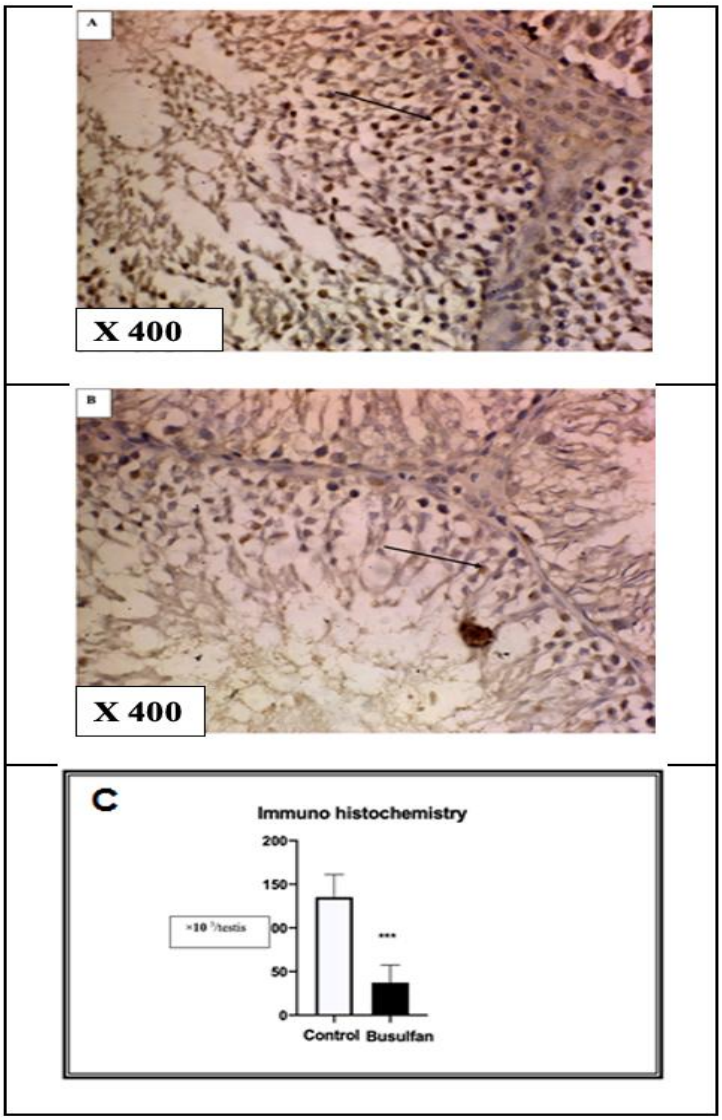

Figure 3. Changes in YAP immuno-expression calculated by the ImageJ programme, which revealed a 72.4 per cent decrease (P 0.0001) in busulfan treated group rats testis compared to the normal group.

Table 1. showed the change in body weight and measured biochemical parameters in busulfan treated rats in comparison with control rats. Body weight and the level of testosterone and TAC were significantly decreased. The level of FSH, LH, NO, LPO, and HDL were significantly increased, however, the level of cholesterol and triglycerides not changed in comparison with the control groups.

Table 1. Showed the effect of busulfan treatment on body weight gain, sexual hormones, oxidative stress markers and lipid profile

Parameters	**Control group**	**Busulfan group**	**p-value**
Body weight (gram)	301±19.70	248.7±31.60	0.0001
FSH (IU/L)	3.31±0.50	8.48±1.02	<0.0001
LH (IU/L)	3.49±0.60	7.74±1.30	<0.0001
Testosterone (nmol/L)	5.42±0.30	2.18±0.80	<0.0001
TAC (nmol/L)	1.53±0.20	1.20±0.20	0.0009
NO (nmol/L)	34.77±1.30	46.45±3.60	<0.0001
LPO (nmol/L)	9.21±0.40	11.87±0.80	<0.0001
HDL Cholesterol (mg/dl)	50.32±4.60	61.61±12.90	0.0416
Cholesterol (mg/dl)	135.2±11.4	137.3±13.90	0.7406
Triglycerides (mg/dl)	221.4±24.19	207.5±52.10	0.5182

Results are expressed as mean ± standard deviation, n=15

DISCUSSION

The lack of a reliable method for predicting the prognosis of azoospermia is a significant issue. MND1 is germ cell-specific gene, its expression may be altered in the testes of azoospermia. The current study showed that a combination of high FSH and a low level of gene expression in rats with NOA indicate a poor prognosis for sperm recovery and testicular histological appearance. Moreover, the current study found that busulfan-treated rats had lower serum levels of testosterone and higher levels of LH, which agreed with previous findings by Ghosh [18] and Nasimi [19]. In this regard, it was reported that busulfan can affect the exocrine and endocrine compartments of the testis, resulting in a persistent impairment of Leydig cell

function [20]. Because the hypothalamic-pituitary axis controls Leydig cell functions via LH to produce testosterone, the obvious increase in LH and FSH levels denotes the escape of the hypothalamic-pituitary circuit from the negative feedback effect of testosterone [20,21]. A significant positive correlation between LH and FSH indicate a strong link between Leydig cell dysfunction and germinal epithelial damage [20].

Free radicals' ability to react with all cellular components, including polyunsaturated fatty acids in the cell membrane, resulted in cell lysis [22]. In the current study, lipid peroxidation as MDA was significantly higher in the challenged group than in the control group which matched with previous report by Moghadam [23]. This result could be attributed to a steady increase in free radical production via NADPH, which is induced by busulfan [24]. Previous research has shown that oxidative stress markers are induced in several testosterone-depleted animal models via increased gene expression, depletion of enzymatic and non-enzymatic antioxidants, and overproduction of reactive oxidants [25]. Under testosterone deprivation, the excessive generation of reactive oxygen and nitrogenous species overcomes the antioxidant protective network, which initiates and propagates chain reactions. This was confirmed by a significant increase in NO levels in the busulfan group, which is consistent with its ability to up-regulate gene expression of endothelial nitric oxide synthase [26]. Moreover, busulfan caused significant decrease in the TAC in male rat sera due to suppression of enzymatic antioxidants and depletion of non-enzymatic antioxidants [23,27].

The histopathological changes in testicular histoarchitecture observed after busulfan exposure are consistent with those found previously by Aboul Fotouh [28]. The alkylating nature of busulfan may be responsible for spermatogenesis disorders [29] and Sertoli cell apoptosis [29]. Because the germ cells rely heavily on the function of the Sertoli cells, supporting cells apoptosis can also endanger the germ cells' viability. Furthermore, busulfan increases oxidative stress, which may be a cause of histopathological changes such as germ cell death [31]. in conjunction with a decrease in testosterone levels, which is required to maintain the normal structure and function of the testis [32]. Immunohistochemical staining revealed the presence of YAP in the cytoplasm and membrane of primary and secondary spermatocytes. The stage-specific increase in YAP expression corresponded well with the development of primary spermatocytes. These findings imply that under physiological conditions, this kinase may not be abundantly expressed.

MND1 was found to be appeared prior to meiotic division, and the protein corresponding to them play a role in meiotic progression during spermatogenesis [9]. The current study found a reduction in the MND1 expression, which is like earlier findings by Qian [33], who found a significant decrease in mRNA expression of meiosis genes in mice suffering from busulfan-induced testicular toxicity. Changes in YAP levels in the cellular nucleus, as a transcriptional coactivator, play important role for YAP activity and the transcriptional state of target genes [34]. In the current study, busulfan increased HDL but did not affect cholesterol or triglyceride levels compared to the control. Male reproductive function depends on cholesterol homeostasis because cholesterol is a precursor of steroid synthesis, a requirement of normal sperm production [35].

CONCLUSION

These findings have the potential to predict the future of testicular function by using hormonal profile and MND1 gene expression. In addition, our study confirms the need of patient with cancer and undergoing chemotherapy to have their sperm cryopreserved to protect their future fertility.

REFERENCES

[1] M. Wosnitzer, M. Goldstein, , & M.P. Hardy: Review of Azoospermia. Spermatogenesis, 4(1), e28218 (2014).

[2] M. Panahi, S. Keshavarz , F. Rahmanifa , A. Tamadon , D. Mehrabani , N. Karimaghai , M. Sepehrimanesh, M, & H. Aqababa: Busulfan induced azoospermia: Stereological evaluation of testes in rat. Veterinary Research Forum. ; 6(4):273-8 (2015).

[3] Y.J. Choi, , D.W. Ok , D.N. Kwon, , J.I. Chung, , H.C. Kim, S.M. Yeo, T. Kim, H.G. Seo, & J.H.Kim : Murine male germ cell apoptosis induced by busulfan treatment correlates with loss of c-kit-expression in a Fas/FasL- and p53-independent manner. FEBS Letters, 575(1–3), 41–51(2004).

[4] L.R. Bucci & M.L. Meistrich: Effects of busulfan on murine spermatogenesis: cytotoxicity, sterility, sperm abnormalities, and dominant lethal mutations. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 176(2), 259–268 (1987).

[5] S.M. Haffner , L. Mykkäinen ,R.A. Valdez, &M.S. Katz : Relationship of sex hormones to lipids and lipoproteins in nondiabetic men. Journal of Clinical Endocrinology and Metabolism, 77(6), 1610–1615 (1993).

[6] A. Vahdati, A.R. Fathi , P. Nasimi, & G. Saki: Busulfan induces apoptotic and cytotoxic effects on testis and epididymal sperm of adult male mouse following low dose treatment International Journal of Biosciences | IJB |. Int. J. Biosci, 6(5), 70–78 (2015).

[7] J.C. Rockett, P. Patrizio, J.E. Schmid, N.B. Hecht, & D.J. Dix: Gene expression patterns associated with infertility in humans and rodent models. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. 549:225–40 (2004).

[8] V. Gatta, F. Raicu, A. Ferlin, I. Antonucci, A.P. Scioletti,A. Garolla,G. Palka, C. Foresta,L. Stuppia,P.J.I. Ellis,R.A. Furlong, S.J. Conner,J. Kirkman-Brown, M. Afnan, C. Barratt,D.K. Griffin, , N.A. Affara, J.C. Rockett,P. Patrizio: A language and environment for statistical computing. Human Reproduction, 11(1), 1034–1038(2019).

[9] A. Dorosh, O. Tepla, E. Zatecka, L. Ded,K. Koci, & J. Peknicova: Expression analysis of MND1/GAJ, SPATA22, GAPDHS and ACR genes in testicular biopsies from non-obstructive azoospermia (NOA) patients. Reproductive Biology and Endocrinology, 11(1), 42(2013).

[10] A.B. Peter, Daniel Penrice : A Vision for Venture Capital: Realizing the Promise of Global Venture Capital and Private Equity (Winthrop Group). ISBN 978-1584657996(2009).

[11] D. Koracevic, G. Koracevic, V. Djordjevic, S. Andrejevic, V. Cosic: Method for the measurement of antioxidant activity in human fluids. J Clin Pathol 2001; 54:356–361(2001).

[12] H. Ohkawa, N. Ohishi, K. Yagi : Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem.1979Jun;95(2): 351-8.doi: 10.1016/0003-2697(79)90738-3(1979).

[13] A. H. Ding, C. F. Nathan, D. J. stuehr: Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritonial macrophages: comparison of activating cytokines and evidence for independent production. J. immunol. 141;2407-2412 (1988).

[14] N.W. Tietz, T. Borden and J.D. Stepleton: An improved method for the determination of

lipase in serum. American Journal 0/ Clinical Pathology, 31, 148-154(1959).

[15] Richmondw: Preparation and properties of bacterial cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. Clinical Chemistry 19, 1350-1 356(1973).

[16] A.A. Dury and E.A.Wallington : Carletons histological technique.5th Ed.,Oxford Univerisity press, NewYork, Toronto (1980).

[17] Y. Liang, Y. Dong, J. Zhao, and W. Li: YES1 Activation Elicited by Heat Stress Is Anti-Apoptotic in Mouse Pachytene Spermatocytes. BIOLOGY OF REPRODUCTION (2013) 89(6):131, 1–11. DOI 10.1095/biolreprod.113.112235 (2013).

[18] D. Ghosh, U.B. Das, S. Ghosh, M. Mallick, & J. Debnath: Testicular gametogenic and steroidogenic activities in cyclophosphamide treated rat: A correlative study with testicular oxidative stress. Drug and Chemical Toxicology, 25(3), 281–292(2002).

[19] P. Nasimi, M.R. Tabandeh, & S. Roohi : Busulfan-mediated oxidative stress and genotoxicity decrease in sperm of Satureja Khuzestanica essential oil-administered mice. Systems Biology in Reproductive Medicine, 64(5), 348–357(2018).

[20] A. Gerl, D. Mühlbayer,G. Hansmann,W. Mraz, & W. Hiddemann: The impact of chemotherapy on leydig cell function in long term survivors of germ cell tumors. Cancer.; 91(7): 1297-1303(2001).

[21] T. Tanaka, M. Kanatsu-Shinohara, Z. Lei, C.V. Rao & T. Shinohara: The Luteinizing Hormone-Testosterone Pathway Regulates Mouse Spermatogonial Stem Cell Self-Renewal by Suppressing WNT5A Expression in Sertoli Cells. Stem Cell Reports, 7(2), 279–291(2016).

[22] R. Radi : Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. Proceedings of the National Academy of Sciences of the United States of America, 115(23), 5839–5848 (2018).

[23] M.T. Moghadam , R. Dadfar, & L. Khorsandi : The effects of ozone and melatonin on busulfan-induced testicular damage in mice. Jornal Brasileiro de Reproducao Assistida, 25(2), 176–184 (2021).

[24] V. Probin, Y. Wang, & D. Zhou: Busulfan-induced senescence is dependent on ROS production upstream of the MAPK pathway. Free Radical Biology and Medicine, 42(12), 1858–1865 (2007).

[25] T. Kataoka, Y. Hotta, Y. Maeda & K. Kimura : Testosterone Deficiency Causes Endothelial Dysfunction via Elevation of Asymmetric Dimethylarginine and Oxidative Stress in Castrated Rats. Journal of Sexual Medicine, 14(12), 1540–1548 (2017).

[26] S. Al-Hashmi, P.J.M. Boels, F. Zadjali,B. Sadeghi,J. Sällström, K. Hultenby, Z. Hassan,A. Arner & M. Hassan: Busulphan-cyclophosphamide cause endothelial injury, remodeling of resistance arteries and enhanced expression of endothelial nitric oxide synthase. PLoS ONE, 7(1) (2012).

[27] D. Ma, P. Han, M. Song, H. Zhang,W. Shen, G. Huang, M. Zhao, Q. Sun,Y. Zhao & L. Min: β-carotene Rescues Busulfan Disrupted Spermatogenesis Through Elevation in Testicular Antioxidant Capability. Frontiers in Pharmacology, 12, 74. (2021).

[28] G.I. Aboul Fotouh, M.M. Abdel-Dayem, D.I. Ismail &H.H. Mohamed: Histological Study on the Protective Effect of Endogenous Stem Cell Mobilization in Busulfan-Induced Testicular Injury in Albino Rats. Journal of Microscopy and Ultrastructure, 6(4), 197–204 (2018).

[29] S.G. Hakemi, F. Sharififar, T. Haghpanah, A. Babaee &S.H. Eftekhar-Vaghefi : The effects of olive leaf extract on the testis, sperm quality and testicular germ cell apoptosis in male rats exposed to busulfan. International Journal of Fertility and Sterility, 13(1), 57–65 (2019).

[30] C.J. Murphy & J.H. Richburg (2014): Implications of Sertoli cell induced germ cell apoptosis to testicular pathology. Spermatogenesis, 4(2), e979110 (2014).

[31] N.H. Ahar, A. Khaki, G. Akbari & M.G. Novin: The effect of Busulfan on body weight, Testis weight and MDA enzymes in male rats. International Journal of Women's Health and Reproduction Sciences, 2(5), 316–319 (2014).

[32] M.F. Abd-Ellah, H.A.A. Aly, H.A.M. Mokhlis & A.H. Abdel-Aziz: Quercetin attenuates di-(2-ethylhexyl) phthalate-induced testicular toxicity in adult rats. Human and Experimental Toxicology, 35(3), 232–243 (2016).

[33] C. Qian, Q. Meng , J. Lu , L. Zhang , H. Li & B. Huang: Human amnion mesenchymal stem cells restore spermatogenesis in mice with busulfan-induced testis toxicity by inhibiting apoptosis and oxidative stress. Stem Cell Research and Therapy, 11(1). (2020).

[34] F. Haderk, V. Olivas & T.G. Bivona: Immunohistochemistry to Study YAP in Human Tissue Samples. Methods in Molecular Biology, 1893, 89–95 (2019).

[35] F. Vignon, M.H. Koll-Back,A. Clavert & C. Cranz (1989): Lipid composition of human seminal plasma. Systems Biology in Reproductive Medicine, 22(1), 49–53 (1989).