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Amelioration effects of vitamin E on reproductive system and expression of zo-1 and vimentin in the epididymis of male rats exposed to busulfan

A thesis

Submitted to the Council of The College of Veterinary Medicine, University Of Karbala In Partial Fulfillment of the Requirements For the Master Degree of Science In Veterinary Medicine/ Physiology

BY

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Supervised by

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وَزَكَرِيَّا إِذْ نَادَى رَبَّهُ
رَبِّ لَا تَذَرْنِي فَرْداً
وَأَنْتَ خَيْرُ الْوَارِثِينَ
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صدق الله العلي العظيم

سورة الأنبياء

Supervisor Certification

I certify this thesis entitled (Amelioration effects of vitamin E on reproductive system and expression of zo-1 and Vimentin in the epididymis of male rats exposed to busulfan)
was prepared under my supervision at the College of Veterinary Medicine, University of Karbala in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Medicine/Physiology.

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The recommendation of the Department

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Linguistic Evaluator

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Dedication

I would like to thank my grandfather, Dr. Mohammed Ali Malallah, and my parents for their full support throughout my research , To My family is the beacon of light and guidance. I am forever grateful.

Mohammed Ali

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Smmary

Busulfan is a chemotherapy treatment for cancer, while Vitamin E is a chemical reactions as antioxidant against free radicals in the body and It helps in decrease the effect of treating by chemotherapy drugs like Busulfan .24 male rats ten weeks old, They weigh between 250-300 grams, were divided into four groups. First group as control, received an intraperitoneal injection of Dimethyl sulfoxide (DMSO) daily for four weeks (also use as a solvent for other experiment materials). Second group receive only single dose of Busulfan 40mg\kg BW\IP. Third group receive single dose of Busulfan as 40 mg\kg BW/IP and Vitamin E as 100 mg \kg BW/IP daily for 4 weeks and the fourth group receive only Vitamin E as 100 mg\kg BW\IP daily for 4 weeks. After the end of 4 weeks the Animals were sacrificed and the blood was collected for measurement of antioxidant enzymes (GSH-SOD-CAT) , gene expresstion (ZO-1 and Vimentin) hormonal measuring (FSH-LH-Testosteron) and epididymis was collected for sperm count and histological examination.

The results show that busulfan induces significant increase in serum FSH and LH level while testosterone level was significantly decreased. Moreover, busulfan caused significant decrease in sperm count, motility and viability and significant increase in sperm abnormality. On the other hand, - administration of vit-E with busulfan lead to ameliorate levels of hormone as well as sperm activity .The histological study showed a significant decrease in the diameter of the seminal tubes, as well as the occurrence of erosion of the testicle tissue in the animals injected with Busulfan (chemotherapy) compared to the control animals. But when animals are injected with Vitamin E that notice an improvement in damaged tissues as a result of chemotherapy toxicity.
The results of gene expression of Zo-1 and Vimentin genes that showed significant decrease in the second group of busulfan (when compare with other group). and showed significant increase in the fourth group of Vit E (when compare with other group)

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List of Abbreviations

Abbreviation term	Call name
A.O	antioxidant
Vit E	Vitamin E
ZO-1	Zona acculodenca
Vim	Vimentin gene
SOD	Superoxide dismutase
GSH	Glutathione
CAT	Catalase
b.w	body wight
BU	Busulfan
DMSO	Dimethyl sulfoxide
E-H.S	Eosin-Hematoxilin Stain
ELIZA	Enzyme linked immune sorbent assay
ROS	Reactive oxygen species
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin –releasing hormone
H&E	Hematoxylin and eosin
I.P	Intraperitoneal injection
L.S.D.	Least significant difference
LH	Luteinizing hormone
mg/kg	Milligram per kilogram
mIU/ml	Microliter international unit per milliliter
ROS	Reactive oxygen species
SCs	Sertoli cells
SD	Standard deviation
SEM	Standard error of mean
SPSS	Statistical Program for Social Sciences
T	Testosteron
HPG	Hypothalamic-pituitary-Gonadal
BMI	Body mass index
SFI	sperm fragmentation index
WHO	World Health Organization
µm	Micron meter
α-TOH	alpha- Tocopherol

Chapter One
Introduction

Introduction

Infertility is a condition of the reproductive system. It can be defined as the inability of an individual or a couple at reproductive age to achieve pregnancy after one year or more of regular unprotected sexual intercourse (Vander Borght and Wyns, 2018). Over the world, infertility challenges approximately 15% of couples who are trying to conceive (Jarow *et al*., 2002), wherein the fertility problem is equally distributed between the male and female partner (Oehninger, 2000 .,Coutton *et al*., 2016). However, that half of the infertility cases derive from the male's semen quality, maybe an underestimation, where inaccurate record-keeping, and inadequate health care and diagnosis antagonizes reliability of the statistical interpretations. Furthermore, clinical investigations adopt a different definition of infertility (Agarwal *et al*., 2015).

Male infertility can be hierarchically classed as either primary or secondary in etiological development, where primary infertility indicate a condition in which the male has never initiated a pregnancy, while secondary refers to the case where the male is unable to initiate pregnancy after twelve months following a previous success. (Mascarenhas,*et al* 2012)

Many drugs particularly alkylating agents have been shown to be gonadotoxic.(Howell, S. J. *et al* 2005) chemotherapies used in the treatment of lymphoma or in preparation for bone transplantation have been shown to cause azoospermia with feedback-raised follicle-stimulating hormone (FSH) levels in over 90% of men following cyclical chemotherapy (Elleuch, *et al* 2020).

Chemotherapy is the systematic treatment of cancer with anticancer agents. It works by stopping the growth of cancer cells, which abnormally grow and divide quickly. Unfortunately, it cannot selectively distinguish between healthy cells and cancer cells. Chemotherapy induced side effects are a result of hurt to

healthy cells(Supportive and . Board 2010) . chemotherapy is an effective way to treat many types of the cancer it also carries risk of number of side effects. Some chemotherapy side effects are mild, while other can cause serious complications including death. The side effects of the cancer chemotherapy come about because cancer cells aren't the only rapidly dividing cells; cells in the blood, mouth, intestine, trace, nose, nails, vagina, and hair are also undergoing constant division.(Chaitanya, *et al* 2017) Chemotherapy is a fundamental therapy this means it affect the whole body by going through the blood stream. Most chemotherapeutic drugs work by impairing mitosis (cell division), effectively targeting fast-dividing cells. They prevent mitosis by various mechanisms including damaging DNA and inhibition of the cellular machinery involved in cell division (Sahu, *et al* 2012) Normal cells that are most likely to be damaged by chemo are blood-forming cells inside the bone marrow, hair follicle, cells in the mouth, digestive tract, and reproduction system .(American Cancer Society, 2019).

Busulfan used in treating of chronic leukemia, cancer of ovary, and before transplantation of bone marrow in patients with cancer (Hassannejad *et al*, 2020). other chemicals that destroy differentiated spermatogonia, busulfan is a potent agent that preferentially kills spermatogonial stem cells (Moloody, *et al* 2018).

Vitamin E is a lipid soluble,chain-breaking antioxidant. It is called achain-breaking antioxidant because of its ability to terminate a free radical chain reaction (Bolle, *et al* 2002).

some evidence that suggests a relationship between daily antioxidant intake and better semen quality among healthy men (Nadjarzadeh, *et al* 2013) Higher levels of vitamin E intake were associated with higher levels of progressive sperm motility (Eskenazi, *et al* 2005) Vitamin E supplementation may also

play a role in reducing sperm DNA fragmentation. (Zini, *et al* 2009) The levels of ROS are normally limited by various antioxidant defense mechanisms ,such as alpha-tocopherol (vitamin E) that are present within the seminal plasma and plasma membrane (Shivhare, *et al* 2011) .

It is believed that a daily intake of 400 units of vitamin E would increase sexual desire. Getting higher amounts of vitamin E is associated with higher levels of testosterone in the body, which boosts sexual desire. (Mahmoud, *et al* 2013).

Aims of study

This study is conducted to achieve the following objectives

The present study investigated the toxic effects of single dose administration of busulfan on male rat reproductive system (testis). Evaluation the probable ameliorating effects of Vitamin E against the changes in the cells of testis caused by busulfan treatment.

At the end of experiment the following parameters will measured

-body weight, testis shape and weight.

- ZO-1 and Vimentin genetic expression in epididymis in the testis

-sperm analysis (sperm count , sperm viability , sperm abnormality).

- Hormone analyses FSH , LH and Testosterone

- Antioxidant Indices Assessment (SOD ,CAT and GSH)

- Histological changes in testis

Chapter Two

Literature Review

2. literature Review

2.1Infertility

Millions of individuals around the world are affected by infertility , The subjective quality of life is harmed in a significant percentage of men who suffer from sexual dysfunctions. (Taha;*et al*..2020) Approximately, 15% of couples of a reproductive age are unable to achieve the desired pregnancy (Farsimadan, M. *et al*2020) Various causes are attributed to the decline in sperm quality that may lead to infertility.(Babakhanzadeh, *et al* 2020)

2.2 Causes of Male infertility

Male fertility can be induced by a range of causes, one probable reason for the decreasing fertility is :-

2.2.1 lifestyle and environment lifestyle performance adds to the decline of semen features (Jurewicz *et al*., 2014). Lifestyle factors that influence infertility include age (when starting a family), weight management, nutrition, and exercise. The male reproductive system is highly susceptible to environmental factors (cosmetics, pesticides, herbicides, preservatives, cleaning materials, pharmaceuticals, and industrial by-products). These alien molecules enter our bodies in different forms that may lead to infertility (Sharma. A,2017) Existing data relating to the possible argumentative effects of the lifestyle problems on male fertility fluctuate in strength. Definite factors, for example, body mass index, cigarette smoking, alcohol consumption and physical activity are possible to apply an additive outcome, whereas other factors may posture a risk when exposed beside other environmental and occupational factors (Eisenberg *et al*., 2013; Gaskins *et al*., 2015; Povey *et al*., 2012).

2.2.2 Overweight and obesity: a prevalent medical condition connected with unnecessary fat accretion, which can be evaluated using the body mass index (BMI) (DuPles *et al*., 2010). BMI is linked with changes in sperm parameters in numerous reports. In a previous study examining factors related to semen quality among couples who visited an assisted reproduction hospital, the popularity of obesity among men with infertility was three times greater than among male partners of couples with a female factor or idiopathic infertility subgroup (Magnusdottir *et al*., 2005). It is assumed that with the growing rate of inactive lifestyles and dietary alterations, obesity is evolving as a significant source of argumentative health consequences, including male infertility. obesity has been proposed to affect male fertility by reducing semen quality and testosterone level (DuPles *et al*., 2010).) Physical issues can interfere with sperm development and obstruct the ejaculatory pathway. Varicocele is a condition characterized by sperm vessel enlargement, which is the most common cause of male infertility that affects around 40% of men (Sengupta,. *et al*2018) .

2.2.3 conventional medicine, post-testicular

infertility including infection, injury, or seminiferous tubules obstruction, sperm motility, sympathetic nervous system damage, maturation disorders, and problems associated with the penis and physical disability also cause infertility in men(Fode,. *Et al*2015) Moreover, chronic and acute genital tract infections can also be a common cause of infertility in men (Fallahi,. *Et al*2018) .

by defective spermatogenesis, ineffective transport, and ineffectual delivery of sperm. The presence of endocrine disorders such as hyperthyroidism and diabetes mellitus may cause azoospermia (failure to fertilize the ovum). Additionally, obstruction of the seminal vesicles and absence of the seminal ducts may affect the mobility of the sperm, causing infertility. Testicular

disorders such as undescended testis might affect fertility (Shehzad,. *etal* 2021) An estimated 40 to 90% of male infertility is due to deficient sperm production of indefinable origin (Sinclair,2000).

2.2.4 chemotherapy drugs

chemotherapy drugs affect the ability to firtilize ; reduce the number of sperm, affect the sperm's ability to fertilise an egg affect the production of the hormone testosterone (Muhammad, *et al* 2015),(Rostamzadeh,.*et al* 2020).

Drugs which may cause infertility in men and women include Alkylating agents-such as busulfan, cisplatin, cyclophosphamide, ifosfamide and melphalan.

Other categories of chemotherapy drugs, include Cytarabine, 5-flurouracil, methotrexate, vincristine, vinblastine, bleomycin, doxorubicin, and daunorubicin. (Weber,. 2015).

During spermatogenesis, testicles produce sperm, and problems (chromosomal defects, trauma, infection, interaction with chemicals/radiation, Varicocele, cryptorchidism) can cause infertility problems (Davidson,. *etal*2015).

One of the key factors related to interrupted sperm function in obese men is the OS produced by extra ROS, mostly including superoxide anion, hydrogen peroxide, and hydroxyl radicals. ROS can be formed regularly in cellular metabolism, however, in the unwarranted state, it can prompt OS which in turn damages sperm DNA and the plasma membrane integrity in sperm and elevate the stress on the testicular environment additionally (Rato *et al*., 2014). Exposure of spermatozoa to oxidative stress is a major causative agent of male infertility(Aitken,.*eta*2012).

2.3 Spermatogenesis

Spermatogenesis is a continuous highly regulated process of male germ cell proliferation and differentiation(Chen, et al 2021). This mechanism occurs in the testis' seminiferous tubules and allows for the creation of sperm throughout one's life. Differentiation of spermatogonia occurs through a linear process including mitotic expansions, meiotic reduction divisions, and morphological transformations(Khanehzad,et al 2021). The commitment of spermatogonia to differentiation pathway happens when undifferentiated spermatogonia undergo an irreversible transition (in mouse) or division (in primate) to produce differentiating spermatogonia(Khanehzad,.*et al* 2021)

2.4 The testes and epididymis

The epididymis plays an important role in post-testicular sperm maturation including the acquisition of forward motility and fertilizing ability (Caballero,.*et al* 2011).

When transiting through the lumen of the epididymis, sperm undergo maturation by interacting with proteins synthesized and secreted by the epididymal epithelium (Gervasi, *et al* 2017).

the epididymis also functions in sperm transport and concentration, immuno-protection of sperm, and serves as a sperm reservoir (Belleannee, *et al* 2012).

the epididymis is divided into four main anatomical regions: the initial segment (only present in rodents), the caput, the corpus and the cauda, with each epididymal region performing separate functions essential to the different steps of sperm maturation. In support of this view, early studies demonstrated that the caput and corpus provide microenvironments for sperm maturation, and that the cauda region primarily serves as a storage site for functional spermatozoa (Fang.,*et al* 2017).

The adult epididymal epithelium consists of different cell types, including principal, clear, basal, halo, narrow, and apical cells, which form a monolayer surrounding the lumen (Cornwall, 2009).

Although these cell types within the epididymal epithelium have individual functions, they also communicate with each other to maintain sperm maturation and storage via different cell junctions (Robaire, *et al* 2006) The principal cells form tight junctions between adjacent cells to create the blood epididymis barrier, which is necessary for the stable and specific microenvironment within the epididymal lumen. In the epididymis, epithelial cell–cell interactions are closely associated with cadherin-mediated cell adhesion. Occludin, cadherin, and tight junctional protein 1 (also known as zonulaoccludens-1 (ZO-1) are implicated in the formation of epididymal tight and adherens junctional complex (Cyr, *et al* 2007).

epithelial cell cross-talk by cell junctions in the epididymis is critical for the luminal microenvironment, which is responsible for sperm maturation (Zhou, *et al* 2018).

The epididymis is highly androgen-dependent and is responsible for maintaining epididymal structure and functions. In the epididymis, testosterone bound to androgen-binding protein (ABP) is taken up by the principal cells of the initial segment and caput epididymus through a receptor-mediated process (Robaire, and Hamzeh,. 2011).

2.4.1 Function of The testes

its responsible for spermatogenesis a process by which spermatozoa are formed, which is regulated hormonally by the hypothalamic-pituitary-gonadal (HPG) axis. Spermatozoon is the most specialized haploid cell, having a unique structure and function. To achieve natural fertilization sperms must have normal motility, morphology and intact intracellular contents and be present in adequate

numbers per ejaculation. Thus, conventional semen parameters measured macroscopically and microscopically in an andrology lab are inefficient to address the content or function of sperm at this level. Nevertheless, an andrology laboratory was traditionally used for diagnosis infertility(Dwyer, and Quinton,.2019).

The testes have a dual function: an exocrine function via the production of spermatozoa in the seminiferous tubules and an endocrine function via the secretion of testosterone by Leydig cells in the interstitial compartment. Thus, the testis is traditionally divided into two interdependent compartments: the seminiferous tubules—the site of spermatogenesis—and the interstitial tissue—containing Leydig cells and blood capillaries (Heinrich, *et al* 2019).

Spermatogenesis is a process of cell division and diterentiation leading to the production of spermatozoa from puberty to senescence. It takes place inside the seminiferous tubules in the testis, which are composed of Sertoli and germ cells. This process can be divided into three phases after puberty: (i) spermatogonial proliferation; (ii) spermatocyte meiosis leading to the production of haploid spermatids; and (iii) spermatogenesis, during which spermatids diterentiate into spermatozoa.

The duration of spermatogenesis according to species: 74 days are required to obtain spermatozoa in humans, 52 days in rats, and 35 days in mice(Marion D;.*et al* 2020).

Mammalian spermatogenesis involves cellular events, namely mitosis, meiosis, spermatogenesis and spermiation in the seminiferous tubules, where the passages of developing germ cells transverse from the basal to the luminal compartment of the seminiferous epithelium (Amann, 2008,. Tajaddini *et al*., 2014).

2.5 Chemotherapy and reproductive system

The chemotherapy and direct or indirect gonadal radiotherapy have substantial short-term and long-term harmful affects on spermatogenesis. Strategies to preserve fertility, including cryopreservation of ejaculated sperm or testis tissue, should be off erred to all post pubescent boys and men about to undergo such treatments. (Anderson *et al* 2015)(Tournaye *et al* 2014).

in cancer treatment increased have survival rates of both boys and men. However, cancer treatment itself can compromise fertility, especially exposure to alkylating agents and whole body irradiation, which cause substantial germ cell loss (Tournaye *et al* 2014).

2.5.1 Effects of Chemotherapy on Somatic Cells

Alkylating agents as chemotherapeutic drugs are often used to treat cancer and increase the survival rates of patients. Alkylating agents, affect cell division by adhesion to one strands of the DNA(Zhuang,. *et al* 2018).

Infertility observed after chemotherapy exposure can be the result of direct injury on germ cells or indirect damages on endocrine and paracrine control of somatic cells (Stukenborg,. *et al* 2018) reduced sperm counts found in male survivors of paediatric cancer were systemically associated with increased follicle stimulating hormone (FSH) levels, indirectly reflecting Sertoli cell alteration(vanCasteren,. *et al* 2009).

in contrast to germ cells, Sertoli cells seemed to develop a better resistance to chemotherapeutic agents in an animal model. In vitro studies using pre-pubertal rat testis showed no impact on the Sertoli cell number after 48 h exposure to doxorubicin, cisplatin, or cyclophosphamide (Smart, *et al* 2018).

Sertoli cell dysfunction was observed after in vivo exposure to chemotherapeutic molecules with a decreased production of androgen binding

protein (ABP) and transferrin, two proteins involved in the regulation of spermatogenesis (Stumpp,.*et a*l 2006).

Although most children treated for cancer develop secondary sexual characteristics and enter into puberty at a normal age, some patients might display sex hormone deficiency, delayed onset of puberty, and small testicular volume, suggesting a potential spermatogenesis impairment in addition to testicular endocrine dysfunction (Pietila, *et al* 2017).

2.5.2 Effects of Chemotherapy on Spermatogonia and Spermatocytes

Exposure to alkylating agents during childhood has been particularly associated with testicular damages characterized by Sertoli cell tubules, reduced tubular diameter, and interstitial fibrosis (Delessard, *et al* 2020).

In adult rats treated with low daily doses of etoposide during the prepubertal period (30- to 60-day-old rats), testicular weight is reduced and testicular tissues display severe alterations with Sertoli cell only tubules persisting in 113-day-old rats(Freitas, *et al* 2002).

2.5.3The effect of chemotherapy on spermatogenesis

Varies substantially depending on the combination of drugs used, and on the cumulative dose given. Nitrogen mustard derivatives, alkylating drugs, and cisplatin seem to have the most detrimental effect on germ cell proliferation . other agents are not likely to cause permanent sterility, and recovery to normal sperm counts can be expected. Alkylating agents are often used in combination with other chemotherapeutic drugs, substantially increasing their germ cell toxic effects (Meistrich *et al* 1992).

Chemotherapy, in combination with whole-body irradiation for leukemia is also detrimental for testicular function (Wallace *et al* 2005).

Chemotherapy and radiation therapy can result in DNA abnormalities of germ cells and potentially increase the risk of disturbed growth, childhood diseases, congenital abnormalities, and cancer in the children of individuals who have had cancer. Although only a few long-term follow-up studies have been done, initial data suggest no increase in abnormalities in children of men who have had chemotherapy or radiotherapy treatment for cancer (Chow ,.*et al* 2009).

Spermatogenesis is highly sensitive to the effects of chemotherapy and irradiation; therefore, the main strategy to minimize germ cell loss is to choose treatment and combinations that have a less severe eff ect on spermatogenesis. Gonadotropin-releasing hormone analogues or antagonists that temporarily suppress the production of gonadotropins have been used to preserve spermatogenesis, and although some promising data exist in non-human primates (Shetty ,. *et al* 2013).

Cigarette smoking and alcohol consumption are the main lifestyle factors with adverse impact on fertility. Generally, the distinct influences of either cigarette smoking or alcohol on sperm parameters have been widely examined, but outcomes are inconsistent (Boeri,. *et al.*, 2019).

Drugs with high risk of infertility include procarbazine, cyclophosphamide, ifosfamide, busulfan, melphalan, chlorambucil and chlormethine ; drugs like doxorubicin, cisplatin and carboplatin have medium risk while therapies with plant derivatives (such as vincristine and vinblastine), antibiotics (such as bleomycin and dactinomycin) and antimetabolites (such as methotrexate, mercaptopurine and 5-fluoruracil) have low risk of gonadotoxicity (Brydøy *etal*., 2007).

2.6 Buselfan

2.6.1 structure of Busulfan

(1,4-butanediol dimethanesulphonate) is an alkylatingagent that causes cytotoxicity due to its capacity to transfer alkyl groups to cellular constituents (Vasiliausha,. et al 2016).

Busulfan was approved by the US Food and Drug Administration (FDA) for the treatment of chronic myeloid leukemia (CML) in 1999. Busulfan was the mainstay of the chemotherapeutic treatment of chronic myeloid leukemia (CML) until it was displaced by the new gold standard, imatinib, though it is still in use to a degree as a result of the drug's relative low cost.(Joseph John,. (2006).

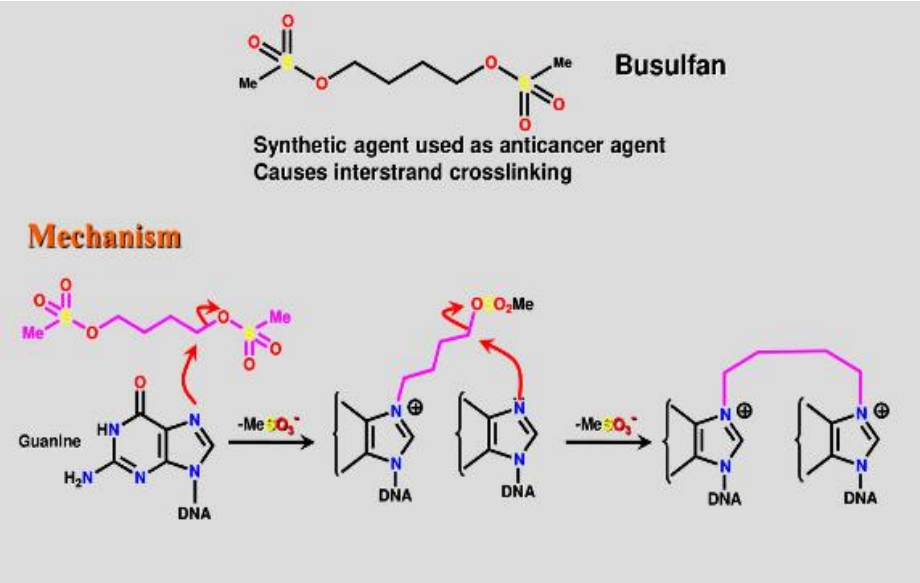

Fig.(2-1)Chemical structure of busulfan (butane-1,4-diyl dimethanesulfonate). (Moon, S. Y. *et al* 2014).

Busulfan toxicity occurs by several different mechanisms, including reactive oxygen species (ROS) formation and protein damage (oxidation) (Iwamoto,. *et al* 2004).

Busulfan is a chemotherapeutic and cytostatic agent that is widely used to remove endogenous germ cells from the testes of animal models (Galaup, and Paci, 2013) As a common chemotherapeutic agent for hematological diseases, busulfan has been reported to cause infertility in oncology patients (Vasiliausha, S.R et al 2016) and germinal epithelial damage is a recognized consequence of busulfan treatment in mice (Zohni, K.,et al 2012) Many drugs, particularly alkylating agents, have been shown to be gonadotoxic. Indeed, some chemotherapies used in the treatment of lymphoma or in preparation for bone transplantation have been shown to cause azoospermia, with feedback-raised follicle-stimulating hormone (FSH) levels in over 90% of men following cyclical chemotherapy (Qumel,*et al* 2019).

It is clear that certain chemo-therapeutic drugs specially alkylating agents influence spermatogenesis at least temporarily and in some cases permanently. Single doses of busulfan as an alkylating agent can permanently sterilize rats at non-lethal doses and cause long-term morphological damage to sperm produced by surviving spermatogonia (Panahi, *et al* 2015). Busulfan toxicity occurs by several different mechanisms, including reactive oxygen species (ROS) formation and protein damage (oxidation) (Iwamoto, *et al* 2004) . ROS are directly involved in precarious oxidative damage of cellular macromolecules such as proteins and nucleic acids in germ cells, which can lead to cell death(Aitken, *et al* 2016).

Busulfan can eliminate almost all endogenous germ cells in the recipient, creating an empty space in the spermatogonial stem cells niche; therefore, this drug has been used successfully to prepare recipients in rats (Jiang, ;& Short, (2019);Zhang and Renfree,2003) Busulfan blocks spermatogenesis in rats and other species by acting on early spermatogonia and germ cells to prevent mitosis; this may ultimately result in sterility ,When given prior to puberty, doses of 10 mg/kg or higher produce sterility, destruction of seminiferous

epithelium and abnormal gonadal development and maturation (Zhao *et al*.,2020).

2.6.2 Mechanism of action of busulfan

Busulfan is an alkylating chemotherapeutic agent. Alkylating agents are a broad category of antineoplastic agents, but all have a similar mechanism of action. Alkylating agents work specifically by substituting alkyl groups for hydrogen atoms on the cancer cell DNA molecule; this results in cross-linkage within the DNA chain, inhibiting the transcription of DNA into RNA. The inhibition of DNA into RNA causes inhibition of protein synthesis and further results in cytotoxic, mutagenic, and carcinogenic effects (Patel, R., & Tadi, P. (2021)).

Other inhibitory effects that busulfan has on DNA is binding to the cysteine molecules of histone proteins, which leads to DNA-protein binding. Busulfan also disrupts the cellular redox equilibrium by interacting with the sulfhydryl groups of glutathione, resulting in increased oxidative stress in cancer cells. (Myers . et al 2017).

2.6.3 Side Effect of busulfan

Increasing body of evidence have shown that busulfan causes negative effects on gonadal function and spermatogenesis through deregulation of follicular-stimulating hormone (FSH), luteinizing hormone and testosterone pathways (Tanaka,*et al* 2016)(Nasimi, *et al* 2015).

and contributes to the poor quality of lives in terms of slow cognitive function via mechanisms associated with increased reactive oxygen species (ROS) generation (Dehghani,.*et al* 2018) (Adaramoye,.*et al* 2014).

BUS is a DNA-destruction chemotherapy agent used in low doses for long-term treatment of chronic myelogenous leukemia and ovarian cancers, and in high doses for the treatment of bone marrow suppression in patients under bone marrow transplantation (JALILI, *et al* 2020)

One of the most common side effects of anticancer drugs is disruption of spermatogenesis process, leading to infertility in many cases (Solomon *et al* ,2019)

2.7 Vitamin E

2.7.1 structure of Vitamin E

Vitamin E is a fat-soluble vitamin with several forms, but alpha-tocopherol is the only one used by the human body.(Rizvi, *et al* 2014) Vitamin E is an essential fat-soluble micronutrient for higher mammals and functions as an antioxidant for lipids(Takada , Suzuki *et al* 2010).

is an important micronutrient in the human body. Vitamin E maintains various body functions. It plays a very important role in maternal health and child development (Bastani ;*et al* 2011).

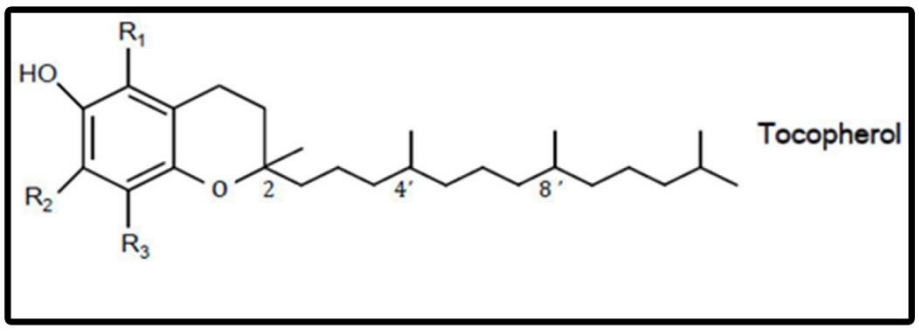

Fig. (2-2) structure of vitamin E (Tocopherol), (Lee., & Han, 2018)

2.7.2 History of vitamin E

American scientists Herbert McLean Evans and Katherine Scott Bishop discovered vitamin E in 1922. vitamin E was found as a cofactor responsible for fertility in rats. In the following years, vitamin E was described as a chain-breaking antioxidant preventing lipoxygenation. Next to its anti-oxidative capacity, Azzi and colleagues reported additional gene-regulatory properties of

vitamin E, more precisely α-tocopherol (α-TOH). In the following sections, that α-TOH has been announced as the only vitamin E form mediating the actual vitamin function (Younossi , Z.et al 2018).

Vitamin E is an essential lipid-soluble vitamin. that was necessary for reproduction. The vital role of vitamin E in reproduction was first investigated 80 years ago(Evans; Bishop. *et al* 1992).

It was named according to a consecutive alphabetical order preceded by the discovery of vitamins A to D. Later vitamin E was called alpha-tocopherol, according to the Greek term “tokos” childbirth, “phero” to bear, and -ol indicating alcohol. Vitamin E is also called the “protecting vitamin”(Obermuller Jevic, Lester Packer ;2004).

2.7.3 Food Sources of vitamin E

Vitamin E is found in plant-based oils, nuts, seeds, fruits, and vegetables.

- Wheat germ oil
- Sunflower, safflower, and soybean oil
- Sunflower seeds
- Almonds
- Peanuts, peanut butter
- Beet greens, collard greens, spinach
- Pumpkin
- Red bell pepper
- Asparagus
- Mango
- Avocado

(Rafeeq, *et al* 2019) , (Trela, & Szymańska (2020)

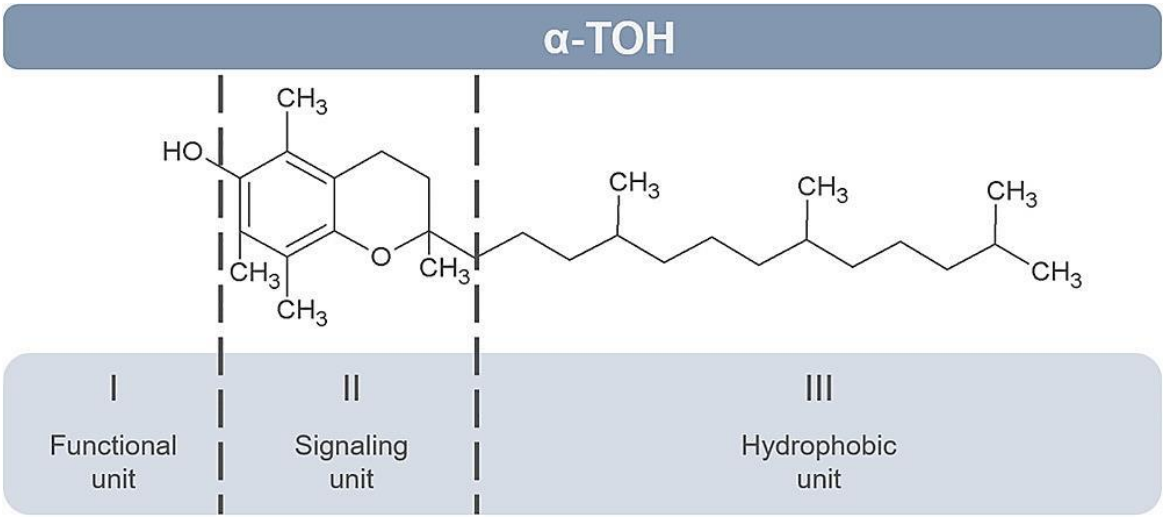

Figure (2-3): The properties of vitamin E depend on its molecular structure which can be divided in three functionally distinct units, as shown in Figure : the functional unit (I), the signaling unit (II), and the hydrophobic domain (III).

α-TOH, is the most potent form of vitamin E in terms of its properties as an antioxidant and ensuring fertility. Units I and II are responsible for the biological activity of α-TOH, and unit III, the side-chain, is considered as a passive domain that is responsible for the hydrophobicity of the molecule and the integration into lipoproteins and membranes.(Neuzil, J. et al 2002).

Vitamin E is also a major bioactive compound in human diet. Most research on vitamin E has focused primarily on α-tocopherol, because it is the predominant form of vitamin E in tissues. Numerous studies have shown the health benefits of tocopherols, including, among others, neuroprotective cardio protective , and anti-inflammatory activities (Mocchegiani et al., 2014).

Vit E improved the fertilization rate of fertile normospermic males with low fertilization rates after one month of treatment, possibly by reducing the lipid peroxidation potential, and with no change of the quantitative ultra morphologic analysis of subcellular organelles (Geva E, et al 1996).

2.7.4 Vitamin E and Gene Expression

Free radicals especially reactive oxygen species (ROS) are generated continuously in living cells, damaging cellular constituents if remaining uncontrolled.(Egea, *et al* 2020) . Differential transcriptional regulation by tocopherol isomers has demonstrated the low-density lipoproteins-scavenger receptor gene. ROS have been shown to induce lipid peroxidation and subsequent transactivation or repression of effector gene expression resulting in oxidative stress-induced cell damage. In the synovium of rheumatoid arthritis patients, transcriptional changes occur that reflect the genetic responses to a hypoxic/anoxic environment(Meydani, *et al* 2001).

Gene transcription in damaged renal tissue is upregulated for various transcription factors, cytokines, and extracellular matrix factors. Amyotrophic lateral sclerosis is a high-incidence motor neuron disease characterized by progressive neurodegeneration and cell death of lower motor neuron groups in the spinal cord and brain stem, and also of upper motor neurons in the motor cortex. Vitamin E regulation of gene regulation has implications for many biologic functions including those of vascular, neuronal, and immune systems as well as the diseases related to them.
(Mocchegiani, E et al 2014).

Improvement of basic sperm parameters by treatment with antioxidants has been stated in several studies (Alahmar, 2018; Terai *et al.*, 2020) . that 2-month supplementation of vitamin E and vitamin C (1 g/day) lead to a significant reduction in the percentage of DNA-fragmented spermatozoa Yet, there was no significant improvement in sperm parameters such as concentration, motility, and morphology after oral antioxidant treatment (Greco *et al.*, 2005). Pourmasumi and his colleagues(2018) also reported that 3 months of treatment with vitamin E and selenium can improve sperm parameters and

chromatin condensation in recurrent miscarriage male partner (Pourmasumi *et al*., 2018).

The major ROS in a sperm cell is (O_2.-), converts either spontaneously or by superoxide dismutase (SOD) to hydrogen peroxide. By catalase (CAT) enzymes the hydrogen peroxide is converted to the non-harmful products H_2O and O_2. Glutathione (GSH) acts as a substrate for the two enzymes, glutathione peroxidase (GP) and glutathione reductase (GR). These two enzymes carry out the CAT process leading to the elimination of H_2O_2 (Agarwal *et al*., 2014; Lopes *et al*., 1998). Thus, the amount of SOD, CAT, GSH, α-tocopherol in sperm cytoplasm and seminal plasma provides an indication of approximate ROS levels and the degree of SDF can be interpolated from that by calculating sperm fragmentation index (SFI). Finally, this index can be linked with patient infertility with OS and ROS.

2.8 Oxidative stress and cancer

Oxidative stress is tightly linked to initiation and progression of cancer. Moderate amounts of ROS promote stress signaling and contribute to mutation, thus favoring cancer development. Mitochondrial ROS production activates cell redox signaling linked to proliferative responses. The activation of transcription factors such as HIFs (hypoxia-inducible factor) promotes tumorigenesis. Mitochondrial DNA can be impaired by ROS and the resulted mutations were found to regulate the tumorigenic phenotype (N.M. Galigniana et al 2020).

Oxidative stress is the most common mechanism causing damage in the testicular tissue due to overproduction of reactive oxygen species and free radicals lead to a state of oxidative stress and resultant damages of lipids, protein, and DNA (Yahiya, Y. I. et al 2021) oxidative stress have been suggested as factors causing infertility in animal , sperm dysfunction is consequence of elevated testicular or seminal ROS . Additionally RSO lead to

DNA damage, bio membrane disruption and proteins denaturation of sperm. (Ommati, M.M. et al 2018).

2.9 antioxidant enzyme

There are several enzyme systems that catalyze reactions to neutralize free radicals and reactive oxygen species. These enzymes include:

- superoxide dismutase
- glutathione peroxidase
- glutathione reductase
- catalases (Ighodaro, O. M., & Akinloye, O. A. (2018). These form the body's endogenous defense mechanisms to help protect against free radical-induced cell damage. The antioxidant enzymes – glutathione peroxidase, catalase, and superoxide dismutase (SOD) – metabolize oxidative toxic intermediates.(Krishnamurthy, P., & Wadhwani, A. (2012).

2.9.1 Glutathione (GSH)

Is an antioxidant in plants, animals, fungiand some bacteria and archaea. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals (Sonei, A. *et al* 2020) It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and cysteine. The carboxyl group of the cysteine residue is attached by normal peptide linkage to glycine. (Silvagno, M. F *et al* 2020).

2.9.2 Glutathione biosynthesis involves two adenosine triphosphate-dependent steps:

- First, gamma-glutamylcysteine is synthesized from L-glutamate and cysteine. This conversion requires the enzyme glutamate–cysteine ligase (GCL, glutamate cysteine synthase).

  This reaction is the rate-limiting step in glutathione synthesis .Niknamian, S. (2019).

- Second, glycine is added to the C-terminal of *gamma*-glutamylcysteine. This condensation is catalyzed by glutathione synthetase.

While all animal cells are capable of synthesizing glutathione, glutathione synthesis in the liver has been shown to be essential. GCLC knockout mice die within a month of birth due to the absence of hepatic GSH synthesis.(Chen Y et al 2007).

The unusual gamma amide linkage in glutathione protects it from hydrolysis by peptidases.(Guoyao Wu et al 2004).

It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine, and the carboxyl group of cysteine is attached by normal peptide linkage to a glycine. Thiol groups are reducing agents, existing at a concentration around 5 mM in animal cells. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteine's by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG), also called L-glutathione (Pastore, A., et al 2001) (Couto, N., 2013).

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. The ratio of reduced glutathione to oxidized glutathione within cells is a measure of cellular oxidative stress (Couto, N., et al 2013) where increased GSSG-to-

GSH ratio is indicative of greater oxidative stress. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH), with the remainder in the disulfide form (GSSG).

Once a tumor has been established, elevated levels of glutathione may act to protect cancerous cells by conferring resistance to chemotherapeutic drugs. The antineoplastic mustard drug canfosfamide was modeled on the structure of glutathione. (Balendiran GK et al 2004).

2.9.3 Superoxide dismutase (SOD)

Irwin Fridovich and Joe McCord at Duke University discovered the enzymatic activity of superoxide dismutase in 1968.(Bilici, M. (2020). is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide ($O^{-}2$) radical into ordinary molecular oxygen (O2) and hydrogen peroxide (H 2O2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. (Hayyan M, et al 2016) SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. One exception is Lactobacillus plantarum and related lactobacilli, which use a different mechanism to prevent damage from reactive O2 (Hwang, Y. S. et al 2019).

SODs were previously known as a group of metalloproteinase with unknown function; for example, CuZnSOD was known as erythrocuprein (or hemocuprein, or cytocuprein) or as the veterinary anti-inflammatory drug "Orgotein" (Bilici, M. (2020).

SOD may reduce free radical damage to skin—for example, to reduce fibrosis following radiation for breast cancer. Studies of this kind must be regarded as tentative, however, as there were not adequate controls in the study including a lack of randomization, double-blinding (Kim, J. H. et al 2013).

Superoxide dismutase is known to reverse fibrosis, possibly through de-differentiation of myofibroblasts back to fibroblasts. (Vozenin-Brotons MC et al 2001).

2.9.4 Catalase (CAT)

is a common enzyme found in nearly all living organisms exposed to oxygen (such as bacteria, plants, and animals) which catalyzes the decomposition of hydrogen peroxide to water and oxygen.(Kim, H. K. (2019).

It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second. (Khyade, V. B., et al 2018) Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Wodu, E. et al 2019).

It contains four iron-containing heme groups that allow the enzyme to react with hydrogen peroxide. The optimum pH for human catalase is approximately 7 . (Pongsavee, M. (2021).

Cells have developed different antioxidant systems and various antioxidant enzymes to defend themselves against free radical attacks. SOD, the first line of defense against oxygen-derived free radicals, catalyzes the dis-mutation of the superoxide anion into hydrogen peroxide (H2O2). The H2O2 can be transformed into H2O and O2 by CAT (Nobari, H et al 2021).

2.10 Role of antioxidant enzyme on the reproductive system during oxidative stress

The primary defense against oxidative stress in the cell with antioxidants includes SOD, GPx, and catalase. SOD specifically converts superoxide radicals to hydrogen peroxide, and CAT and GPx detoxify hydrogen peroxide to water.Most problems that threaten the health of reproductive system, especially testicular function, are associated with free radical-induced oxidative stress. Life-threatening attacks of free radicals may cause arterial occlusion and serious damage to the reproductive system cells, and consequently defects in spermatogenesis. In other words, increased production of free radicals and per oxidants as well as weakened antioxidant defense system lead to oxidative stress (Biswas, A., et al 2016).

Therefore, it is necessary to create a balance between produced free radicals and its metabolism for appropriate function of testicular cells, because if the testicular biological system fails to detoxify or repair the adverse effects of free radicals, the cells and tissue are damaged seriously(Asadi, N. et al 2017). In this regard, antioxidants can avoid this damage by counteracting free radicals or preventing their formation in the testicular cells. It is noteworthy that a part of the body's antioxidant defense system, called preventive antioxidant system, is related to antioxidant enzymes such as Superoxide Dismutase (SOD), catalase, and Glutathione Peroxidase (GPX) (Eghbal, M., et al 2016).

These enzymes avoid oxidation by decreasing the rate of chain formation. These antioxidants can stop an oxidation chain forever by finding primer free radicals.(Shehabeldin, A. M. et al 2021).

In addition, through stabilizing metal radicals such as copper and iron, they can inhibit their oxidation, preventing various diseases (Pham-Huy, L. A. et al 2008). Another part of the body's antioxidant defense system is called

scavenging antioxidant system. These antioxidants delete Reactive Oxygen Species (ROS) produced in the body's different tissues to prevent peroxidation of plasma membrane lipids (Rafieian-Kopaie M, Baradaran A.et al 2013).

Vitamins such as E and C are some examples of these antioxidants. These antioxidants neutralize free radicals and prevent them from damaging the cell and tissues.(Pham-Huy, L. A. et al 2008).

Given that the antioxidants produced by the body are not able to neutralize all free radicals, then use of antioxidant supplements can play an important role in increasing the body's capacity to fight free radicals (Pryor WA et al 2006).

Human spermatozoa represent a growing list of cell types that exhibit a capacity to generate reactive oxygen species (ROS). ROS are important mediators of sperm function.

Mammalian spermatozoa membranes are rich in unsaturated fatty acid, which are sensitive to oxygen-induced damage mediated by lipid peroxidation (LPO) (Bansal, A. K., & Bilaspuri, G. S. (2011)).

However, antioxidants are known as general compounds which dispose, scavenge, and suppress the formation of ROS or oppose their actions of the antioxidants where the enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) play significant roles (Stoleru, E., et al 2020).

2.11 Effective Antioxidants in Decreasing Testicular Oxidative Stress

Vitamins C and E: Vitamin E (α-tocopherol) is a potent, lipophilic antioxidant, which is vital to protect and maintain mammalian sperm. Besides

that, this element contributes greatly to the activity of Sertoli cell lines and spermatocytes (Dare, B. J., et al 2014).

Similarly, vitamin C (ascorbic acid) plays an important part in the process of spermatogenesis. As a result, vitamins C or E deficiency leads to induction of testicular oxidative stress and hence disturbs spermatogenesis and production of testosterone . In contrast, a study demonstrated that feeding with ascorbate caused stimulation of spermatogenesis and secretion of testosterone in healthy animals(Asadi, N., et al 2014).

In addition, use of vitamins C and E is highly effective to fight testicular oxidative stress due to exposure to oxidants such as arsenic, cadmium, endosulfan and alcohol and can considerably decrease the complications due to these substances(Ukwenya, V., et al 2013).

A study demonstrated that vitamin E was effective on testicular function through suppressing lipid peroxidation in testicular and mitochondrial microsomes and fighting adverse effects of oxidative stress due to exposure to certain agents such as ozone gas, iron overload, intense exercise, aflatoxin, cyclophosplamide and formaldehyde (Poljsak, B., & Fink, R. (2014)) ,(Asadi, N., et al 2014).

2.12 The Effect of Oxidative Stress on Sperm Morphological Characteristics:

2.12.1 Damage to sperm morphological characteristics

Various parameters of sperm such as count, motility, and morphology are significantly susceptible to free radicals and hence free radicals can reduce sperm fertility (Shiva, M., et al 2011). The free radical-induced oxidative stress contributes significantly in producing and increasing abnormal sperm and

decreasing sperm count and transformation and fragmenting sperm DNA. These changes in sperm DNA result in infertility(Lanzafame, F. M. et al 2009) .

In this regard, incubation of spermatozoa under high oxygen pressure reduces the rate and motility of sperm; however, adding catalase to the culture medium prevents this effect (Martin-Hidalgo, D. et al 2019).

Increased production of Hydrogen Peroxide (H_2O_2) by spermatozoa under high oxygen pressure may be a factor for reducing sperm motility (Choudhary, R., et al 2010).

Researchers believe that the sperm is more susceptible to oxidative stress than other cells due to the limited amount of cytoplasm in a mature sperm and the concentration of ROS-suppressing antioxidants in the sperm as well as high levels of unsaturated fatty acids in the sperm structure (Zhaku, V. et al 2020).

In addition, according to the particular morphology of sperm, antioxidant enzymes in the sperm fail to protect plasma membrane surrounding acrosome and tail. Therefore, the health and fertility of sperm are greatly dependent on the availability of the antioxidants,(Chłopik, A., & Wysokińska, A. (2020). Which mostly is related to the antioxidant systems in the seminal plasma. If the antioxidants are separated from the semen for any reason such as washing, sperm becomes susceptible to oxidative damage. Researchers believe that sperms can fight oxidative stress conditions mainly due to the antioxidant properties of semen (Sikka, S. C. (2004).)

Such that ROS generated in the semen is constantly deactivated by seminal antioxidants under normal conditions. Therefore, one of the reasons for establishment of oxidative stress conditions is the imbalance between the production of ROS and its inactivation by antioxidants of semen.(Ghareeb, D. A., & Sarhan, E. M. E. (2014).)

2.12.2 Lipid peroxidation of sperm plasma membrane:

Lipid peroxidation in the cell membrane can disrupt fluidity and permeability of cell membranes and damage all cells. In other words, when the cell membranes are damaged by free radicals, their protective cell is lost and thus the total cell is exposed to risk.(Suwalsky, M., et al 2007) In this regard, increased production of ROS induces lipid peroxidation in spermatozoa, which has two important effects: 1) Reducing sperm combination with oocyte; 2) Increasing spermatozoa ability to bind to the transparent area (zona placida) (Moubashera, A. E et al 2020) .

As well, lipid peroxidation caused abnormality in the middle section of sperm and loss of acrosome capacity of fertilization Malondialdehyde (MDA) molecules cause asymmetric distribution of lipid membrane components by penetrating into the cell membrane structure(ANGHEL, *et al* 2013) . Notably, the rate of lipids peroxidation is determined according to the resulting product of secondary failure of primary lipid hydro peroxides (Oleszko, *et al* 2015) . MDA is produced due to the degradation of the peroxides of unsaturated fatty acids. It is used as a marker (biomarker) to determine the rate of oxidative damage to lipids that differs depending on biotic and abiotic stress.(Anjum, *et al* 2015) This issue has been one of the used indicators in the studies on lipids peroxidation in humans and animals. It is noteworthy that currently, the damage caused by lipid peroxidation is the most important factor for testicular dysfunction (Lone, *et al* 2015).

2.12.3Damage to DNA sperm

It is important to protect integrity and accuracy of DNA in the sperm nucleus to transfer genetic material completely from one generation to another, because genetic material disorder causes defective transmission of genetic

information to embryo. Oxidative stress causes increase in DNA breakdown (Cocuzza, *et al* 2007).

Besides that, evidence indicates that fragmentation of DNA is, commonly seen in infertile people's spermatozoa, is due to the ROS high concentration in the sperm (Esteves, *et al* 2018).

In a study on DNA samples in people with teratozoospermia, the DNA damage rate was higher in the samples of people with spermatozoospermia than in those of healthy people; moreover, this damage was demonstrated to be mainly due to the amount of ROS produced by these sperms which can be the cause of infertility in people with spermatozoospermia. Therefore, a main reason for infertility is believed to be excessive production of ROS or decreased antioxidant capacity in semen that causes oxidative stress conditions and ultimately decrease in sperm motility, increase in sperm death, and fragmentation of DNA (Bansal, & Bilaspuri, 2011).

A study reported that the oxidative damage to DNA is 100 times higher in infertile men than in fertile men. Relevantly, ROS concentration is very high in the sperm of the men whose wives have previous abortion; therefore, increased oxidative stress in these people's testicles leads to destruction of sperm membrane and hence damage to DNA. This can be associated with abortion among such people's wives (Wright, *et al* 2014).

In this regard, semen has been reported to contribute significantly to maintaining sperm DNA's health such that gonads are thought to be one of the main sources of sperm-protective antioxidants (Araujo, *et al* 2021). In a study, the effect of Hamster gonads removal was investigated on sperm health. The findings demonstrated that removing the gonads caused increase in damage to hamster DNA (Di Santo, *et al* 2011). Therefore, gonads can be considered the main source of antioxidants in the semen.(Nazmara, *et al* 2021)

2.13 Gonadotropin-releasing hormone (GnRH)

drives pituitary secretion of luteinizing hormone and follicle-stimulating hormone, which in turn regulate gonadal functions including steroidogenesis. (Veldhuis, *et al* 2009).

The pattern's of GnRH release and thus fertility depend on gonadal steroid feedback. Under homeostatic (negative) feedback conditions, removal of the gonads from either females or males increases the amplitude and frequency of GnRH release and alters the long-term firing pattern of these neurons in brain slices. (Oyola, & Handa, 2017).

The episodic release of gonadotropin-releasing hormone (GnRH) from the brain is key to successful reproduction in both sexes. GnRH regulates pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone, which activate gonadal functions including the production of sex steroids. These steroids feed back to regulate the pattern of GnRH release and pituitary response to GnRH. Aspects of this feedback system are well established as sexually differentiated; for example, estradiol-positive feedback is exclusive to females under normal physiologic conditions and induces the pre-ovulatory surge of GnRH (DeFazio, & Moenter, 2021).

2.13.1 Follicle Stimulating Hormone (FSH)

Follicle-stimulating hormone (FSH)is a gonadotropin a glycoprotein polypeptide hormone.

FSH is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland, and regulates the development, growth, pubertal maturation, and

reproductive processes of the body. FSH and luteinizing hormone (LH) work together in the reproductive system (Yaron, & Levavi Sivan, 2011).

FSH regulates the development, growth, pubertal maturation and reproductive processes of the human body. (Diao, & Lin, 2012).

2.13.2 Effects in males

FSH stimulates primary spermatocytes to undergo the first division of meiosis, to form secondary spermatocytes. (Praveen, *et al* 2020) FSH enhances the production of androgen-binding protein by the Sertoli cells of the testes by binding to FSH receptors on their basolateral membranes, and is critical for the initiation of spermatogenesis. (Grover, *et al* 2004).

2.13.3 Luteinizing Hormone (LH)

Luteinizing hormone (LH, also known as lutropin and sometimes lutrophin) is a hormone produced by gonadotropic cells in the anterior pituitary gland. (Kaur, Prabha, 2014) .

The production of LH is regulated by Gonadotropin Releasing Hormone (GnRH) from the hypothalamus. In females, an acute rise of LH ("LH surge") triggers ovulation and development of the corpus luteum. In males, where LH had also been called interstitial cell stimulating hormone (ICSH), it stimulates Leydig cell production of testosterone. It acts 2

synergistically with follicle-stimulating hormone(FSH) (Sengupta, Arafa, & Elbardisi, 2019).

Effects of LH on the body

In both males and females, LH works upon endocrine cells in the gonads to produce androgens.

2.13.4 Effects in males

LH acts upon the Leydig cells of the testis and is regulated by gonadotropin-releasing hormone (GnRH) (Yao & Kawaminami. 2008).

The Leydig cells produce testosterone under the control of LH. LH binds to LH receptors on the membrane surface of Leydig cells. Binding to this receptor causes an increase in cyclic adenosine monophosphate (cAMP), a secondary messenger, which allows cholesterol to translocate into the mitochondria. Within the mitochondria, cholesterol is converted to pregnenolone by CYP11A1(Zirkin,2018).

LH is released from the pituitary gland, and is controlled by pulses of gonadotropin-releasing hormone (DeFazio & Moenter, 2021)

As the levels of testosterone increase, it will act on the pituitary through a negative feedback loop and inhibit the release of GnRH and LH consequently (Ajayi & Akhigbe 2020) .

Androgens (including testosterone and dihydrotestosterone) inhibit monoamine oxidase (MAO) in the pineal gland, leading to increased melatonin and reduced LH and FSH by melatonin-induced increase of Gonadotropin-Inhibitory Hormone (GnIH)(Ubuka *et al* ,. 2014).

2.13.6 Testosterone

the primary sex hormone and anabolic steroid in males. In male humans, testosterone plays a key role in the development of male reproductive tissues such as testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair, in addition, testosterone is involved in health and well being (Bassil *et al*,.

2009) testosterone in both sexes is involved in health and well-being, including moods, behavior, and in the prevention of osteoporosis (Serpell *et al* 2020).

In humans and most other vertebrates, testosterone is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females. On average, in adult males, levels of testosterone are about seven to eight times as great as in adult females (Torjesen Sandnes ,.2004) As the metabolism of testosterone in males is more pronounced, the daily production is about 20 times greater in men (Morssinkhof, *et al* 2020).

In addition to its role as a natural hormone, testosterone is used as a medication in the treatment of hypogonadism in men and breast cancer in women (American Society of Health System Pharmacists,2016).

In males, testosterone is synthesized primarily in Leydig cells. The number of Leydig cells in turn is regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In addition, the amount of testosterone produced by existing Leydig cells is under the control of LH, which regulates the expression of 17β-hydroxysteroids dehydrogenase. (Chen, *et al* 2014).

testosterone levels decrease as men age, testosterone is sometimes used in older men to counteract this deficiency. It is also used illicitly to enhance physique and performance, for instance in athletes(Institute of Medicine (US) ,2004).

Testosterone is responsible for the growth and development of the Wolffian duct to the esophagus in fetal, vas deferens, seminal vesicle and Prostate, as well as its responsibility to show secondary sexual characteristics such as coarse sound and hair appearance (Zurita *et al*.,2003). Testosterone also plays a role in the regulation of leydig and myoid cells where it affects steroidogenic functions resulting in a spermatogenic arrest at the round spermatid stage (Wang *etal.*,2009).

2.14 Zonula occludens-1 (ZO-1)

Tight junction protein-1, also known as TJP1, is a 220-kD peripheral membrane protein encoded by the TJP1 gene in humans.(Lehman, D. M et al 2006) Tight junctions (TJs) create the epithelial barrier and are considered to control epithelial polarity by forming a membrane fence. although the roles of TJs in epithelial polarity remain controversial. Claudins, together with the cytoplasmic scaffolds ZO-1 and ZO-2, create TJ strands, which are essential for epithelial barrier development.

It belongs to the family of *zonula occludens proteins* (ZO-1, ZO-2, and ZO-3), which are tight junction-associated proteins and of which, ZO-1 is the first to be cloned (Otani, T. et al 2019) .

Stevenson and Goodenough were the first to identify it in 1986, using a monoclonal antibody produced in rat liver to recognize a 225-kD polypeptide in whole liver homogenates and tight junction-enriched membrane fractions. (Stevenson BR et al 1986).

Zonula occludens (ZO)-1 was the first tight junction protein to be cloned and has been implicated as an important scaffold protein. . It contains multiple domains that bind a diverse set of junction proteins (McNeil, E. et al 2006).

Some of these proteins, such as the claudins and occludin are transmembrane proteins that form intercellular homophilic and heterophilic adhesions, whereas others such as ZO-1, ZO-2, ZO-3, cingulin, MAGI-1, Pals1, and PATJ are intracellular plaque proteins that form a scaffold between the transmembrane proteins and the actin cytoskeleton A knockout of ZO-1 in mouse epithelial cells recently demonstrated that ZO-1 is not essential for tight junction formation but that loss of ZO-1 causes a pronounced delay in junction

assembly, as measured after withdrawal and replacement of calcium in the medium (Umeda, K et al 2004).

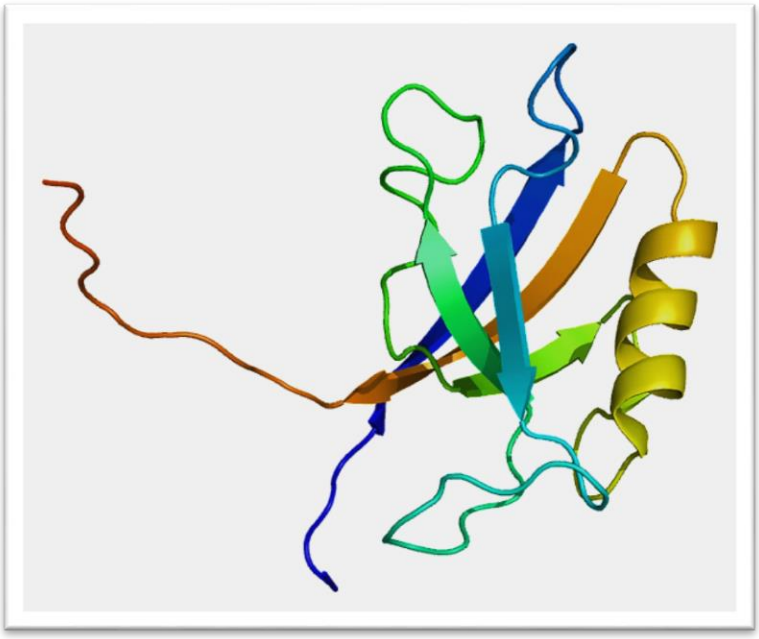

Figure (2-4)Structure of the TJP1 protein.(Ebnet, K., *et al* 2003)

It functions as a scaffold protein that cross-links and attaches Tight Junction (TJ) strand proteins to the actin cytoskeleton. TJ strand proteins are fibril-like structures inside the lipid bilayer. (Huang, W et al 2019).

Tight junctions are cell-cell adhesion complexes that control solute transport para-cellular and restrict pathogen entrance across epithelial and endothelial cell layers, including the blood-brain barrier. (S. Citi 2018).

2.14.1 Function of zo-1 gene

The protein encoded by this gene is found on the cytoplasmic membrane surface of intercellular tight junctions. The encoded protein might have a role in signaling at cell–cell junctions.. Two transcript variants encoding distinct isoforms have been identified for this gene ("Gene: TJP1 tight junction protein 1 (zona occludens 1) .

2.14.2 Gene Summary for TJP1 Gene :

This gene produces a tight junction adaptor protein that also controls adherens junctions. It belongs to the membrane-associated guanylate kinase (MAGUK) family of proteins. (Heinemann, U., & Schuetz, A. (2019) . Tight junctions regulate the movement of ions and macromolecules between endothelial and epithelial cells(Gupta, S. et al 2019).

TJP1 (Tight Junction Protein 1) is a gene that codes for a protein. Neovascular Glaucoma and Brain Edema are two diseases linked to TJP1. (Munoz-Erazo, L. E. (2016).)

TJP1, TJP2, and TJP3 are closely related scaffolding proteins that link tight junction (TJ) transmembrane proteins such as claudins, junctional adhesion molecules, and occludin to the actin cytoskeleton (Bauer, H., & Traweger, A. (2016).) The tight junction serves to limit material across the adjacent cell and as a boundary between apical and basal plasma membrane domains distinct to form epithelial and endothelial cells. (Haskins, J. et al 1998).

2.15 Vimentin gene

VIM is a structural protein that is encoded by the VIM gene in humans. Its name is derived from the Latin vimentum, which means a collection of flexible rods.(Battaglia, R. A et al 2018).

Vimentin is a mesenchymal cell-expressed type III intermediate filament (IF) protein. All animal cells have IF proteins. (Zhou, T. et al 2019) In general, Vimentin is the cytoskeletal component important for preserving cell integrity, and cells lacking Vimentin were shown to be very fragile when disrupted with a micro puncture. (Goldman, R. D., et al 1996).

A Vimentin monomer, like all other intermediate filaments, has a central α-helical domain, capped on each end by non-helical amino (head) and carboxyl (tail) domains (D'Amore, C. et al 2019).

the Vimentin protein was found to be expressed in the majority of the 44 tissues analyzed, 14 of which showed high levels of Vimentin expression. These tissues included skin, lung, kidney bone marrow and lymph node .

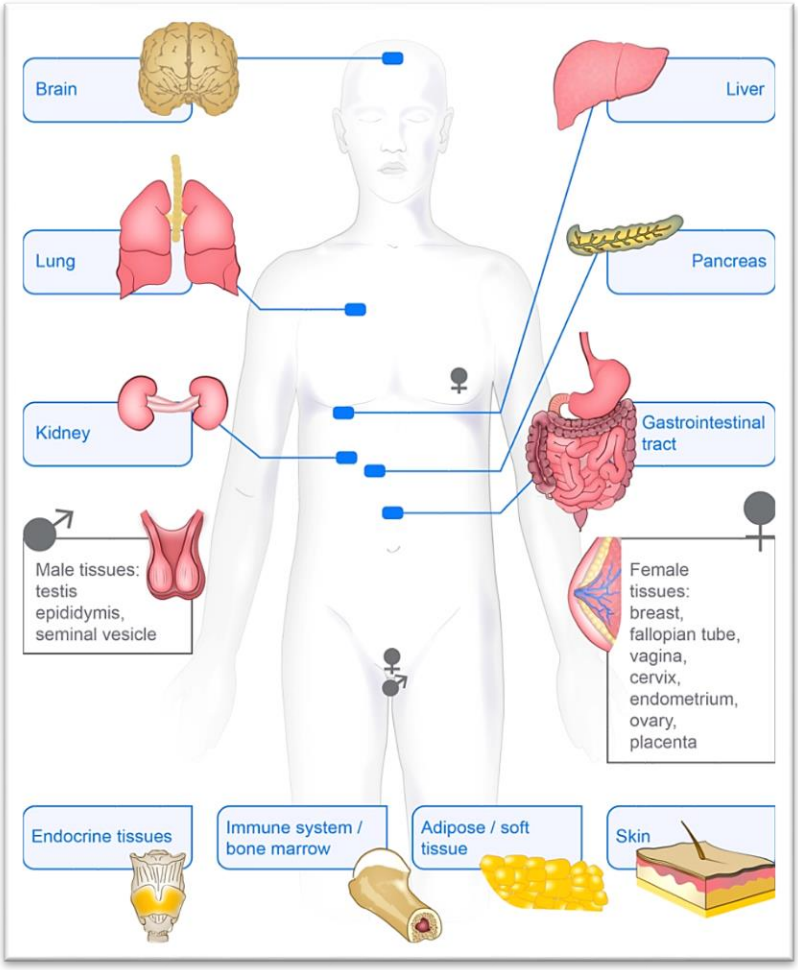

Figure (2-6) . Example of tissue groups in which Vimentin protein has been identified in low, medium or high levels. Gender-neutral and gender-specific tissue groups are indicated by blue or white squares, respectively. (Danielsson, F. et al 2018).

In cells, Vimentin forms a network that surrounds the nucleus. From there, it extends throughout the entire cytoplasm, with shorter soluble forms more abundant in the cell periphery (Lowery, J. et al 2015).

Efficient transport of vimentin is required for the maintenance of the intracellular network. Vimentin transport has been shown to occur along microtubules, and to depend upon actin filaments, microtubules, the microtubule-associated motor protein kinesin-1 and the cytoskeletal regulators PAK and ROCK (Leduc, C.; et al 2017).

The soluble shorter forms of Vimentin are found inside the cell, on the cell surface and in the extracellular environment. The organization of the filamentous intracellular network of Vimentin varies in different cells. For example, it can form relatively homogenous distributions within the cytoplasm of primary, senescent or non-dividing mesenchymal cells, and is rapidly reorganized towards the nucleus upon exposure to PDGF, oncogenes or viruses (Rathje, L.-S.Z. et al 2014) The subcellular spatial organization of Vimentin fibers is regulated by the post-translational modifications and by the assembly state and solubility of Vimentin (Robert, A.; et al 2015).

Function

Vimentin is important for maintaining and fixing the location of organelles in the cytoplasm. Vimentin is either laterally or terminally linked to the nucleus, endoplasmic reticulum, and mitochondria. (Jarullah, H. A. et al 2014) Vimentin is responsible for maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeletal interactions. In asymmetric division of mammalian cell lines, Vimentin has been demonstrated to remove harmful proteins from JUNQ and IPOD inclusion bodies. The transport of low-density lipoprotein (LDL)-derived cholesterol from a lysosome to the esterification site is regulated by vimentin. (Sarria AJ et al 1992).

Vimentin is involved in the development of aggresomes, where it creates a cage around a core of aggregated protein. (Matsumoto, G., et al 2018).

Chapter Three Materials and Methods

3.Materials and Methods:

3.1 Materials:

3.2 Equipment's and Instruments:

Table (3.1): The equipments and instruments which were used in this study.

NO.	Equipment & Instrument	Company	Country
1	Analytical sensitive balance	Sartorius	Germany
2	Balance for animals	Shimadu company	China
3	Centrifuge	Hettich Roto fix11	Japan
4	Digital camera	Toup Cam	China
5	Electronic Balance	Metter company	Switzerland
6	ELISA reader	Biotek	USA
7	ELISA washer	Biotek	USA
8	Filter paper		China
9	Freezer	Hitachi	Japan
10	Incubator	BINDER	Germany
11	Jell tube	AFMA-Dispo	Japan
12	Latex gloves	Great glove	Malaysia
13	Light microscope	Leica	China
14	Micropipette 100-1000 µl	CYAN	Germany
15	Micropipette 1-100 µl	CYAN	Germany
16	Optical microscope with camera	OPTICA	Italy
17	Rotor-gene q	Qiagen	Germany
18	Scan drop	Analytik jena	India
19	Sterile syringes 5, 10 ml	PROTON	Malaysia
20	Vortex	Vortex Genie Pulse	USA
21	Water bath	K.F.T.LAB.Equipment	Italy

3.3 Chemicals :

Table (3.2): The chemicals were used in this study and their sources:

No.	Chemicals	company	Country
1	Architect Testosterone ELISA Kit	Abbott 1000sR	USA
2	Architect (FSH) ELISA Kit	Abbott 1000sR	USA
3	Architect Luteinizing Hormone (LH) ELISA Kit	Abbott 1000Sr	USA
4	Busulfandrug (chemotherapy)	Xi'an Horlen Bio industries Inc	China
5	Chloroform	Noorbrok	England
6	DMSO	LOBA	China
7	Eosin-Hematoxylin Stain	Merck	Germany
8	Ethanol	Merck	Germany
9	Formalin 10 %	TEDIA Company.	USA
10	Normal saline	Labort	India
11	Paraffin Wax	Merck	Germany
12	Sodium Citrate	Sigma-aldrich	Germany
13	Trizol	Thermofisher	Girmany
14	Vitamin E	Merck	India
15	Xylole	Scharlau	Spain

3.4 Primers

Two primers were used in this study, first primer used for **zo-1** gene as House keeping gene and second primer used for **Vimentin** gene as target gene. These primers were designed by using NCBI- Gene Bank data base and Primer 3 design online, the primers used in quantification of gene expression using q-PCR techniques based SYBER Green DNA binding dye, and supported from Trans company.

Table (3-3): The Primers and their sequences, gene bank accession number, and references.

Primer	sequences	
ZO-1	F	5 GAGTGGACTATCAAGTGAGCCTAA 3
	R	5 ATCCAAGTTGCTCGTCAATCTAA 3
Vimentin	F	5 AGAGCACCCTGCAGTCATTCAGA 3
	R	5 CACTTTACGTTCAAGGTCAAGAC 3
References		(Fang, F., et al 2017)

3.5 Prepare Drug Busulfan (Chemotherapy)

Busulfan is a bi-functional alkylating agent is known chemically as 1,4-butanediol, dimethane-sulfonate. BUSULFEX® (busulfan) were intraperitoneally Injection (Benkessou, F. (2019).

The activist ingredient, a white crystalline powder with a molecular formula of CH3SO2O(CH2)4OSO2CH3 and a molecular weight of 250 g/mole .

Busulfan 40mg/kg was initially dissolved in10ml dimethyl sulfoxide(DMSO; Wako, Japan) and further diluted with 10 ml an equal volume of sterile distilled water, the busulfan solution was prepared immediately before use (Fang, F., et al (2017).

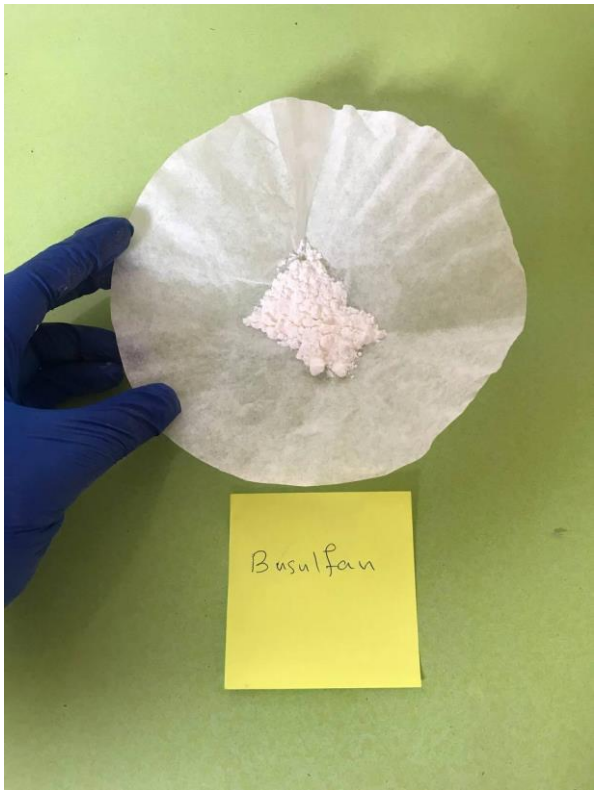

Fig. (3-1):The Dose Of Busulfan

3.6 Prepare Drug vitamin E (alpha-tocopherol)

Vitamin E is a group of eight fat soluble compounds that include four tocopherols and four tocotrienols .

Vitamin E is fat-soluble antioxidant protecting cellmembranes from reactive oxygen species .Traber MG, Bruno RS (2020).

Fig. (3-2) α-tocopherol. (vitamin E) *merke* 400 IU (268 mg.) was dissolved in DMSO to prepare 100 mg/kg, daily, b.w/IP

3.7 Experimental animals:

the experimental animal model selected for the present study is albino rat *Rattus norvegicus* rats were used in this experiment and they were obtained from the laboratory animal unit, faculty of Pharmacy , university of Karbala , Iraq. the age ranges between (2.5–3month) old with an average body weight (350-390gm) for male. the animals were apparently healthy, kept under hygienic conditions and air-conditioned room. the light system was 14/10 hrs. light/dark cycle;22 ±2 c° with a relative humidity of 30 to 60 % from (10/1/2021 to 8/2/2021). The animals were housed in plastic cages , tap water was provided via glassy bottles. Food and water were offered daily. the animals were

accommodated to the laboratory conditions for one week before beginning the experiment.

3.8 Experimental Design:

Twenty-four adult male rats weighing (350–390 gm) were randomly divided into Four groups comprising sex animals for each group as the following:-

*** first group: (Group A)** : six male Rats were given **DMSO** (0.1 mL, daily, intraperitoneal injection) and served as the control.

*** second group: (Group B)** : six male Rats were given single dose of **busulfan**(40 mg/kgIP.) intraperitoneal injection (Fang, f ., *et al*., 2019).

*** third group: (Group C)** : six male Rats were given single dose of **busulfan** (40mg/kgIP.)**plus vitamin E** (100 mg/kg, daily b.w/IP) intraperitoneal injection.

*** fourth group: (Group D)** : six male Rats were given a dose Of the **vitamin E** (100 mg/kg, daily, b.w/IP) intraperitoneal injection (Salehinezhad, F. *et al* 2019).

The drugs were administered intraperitoneally injection for 28 days, animals from each group will be scarified ,

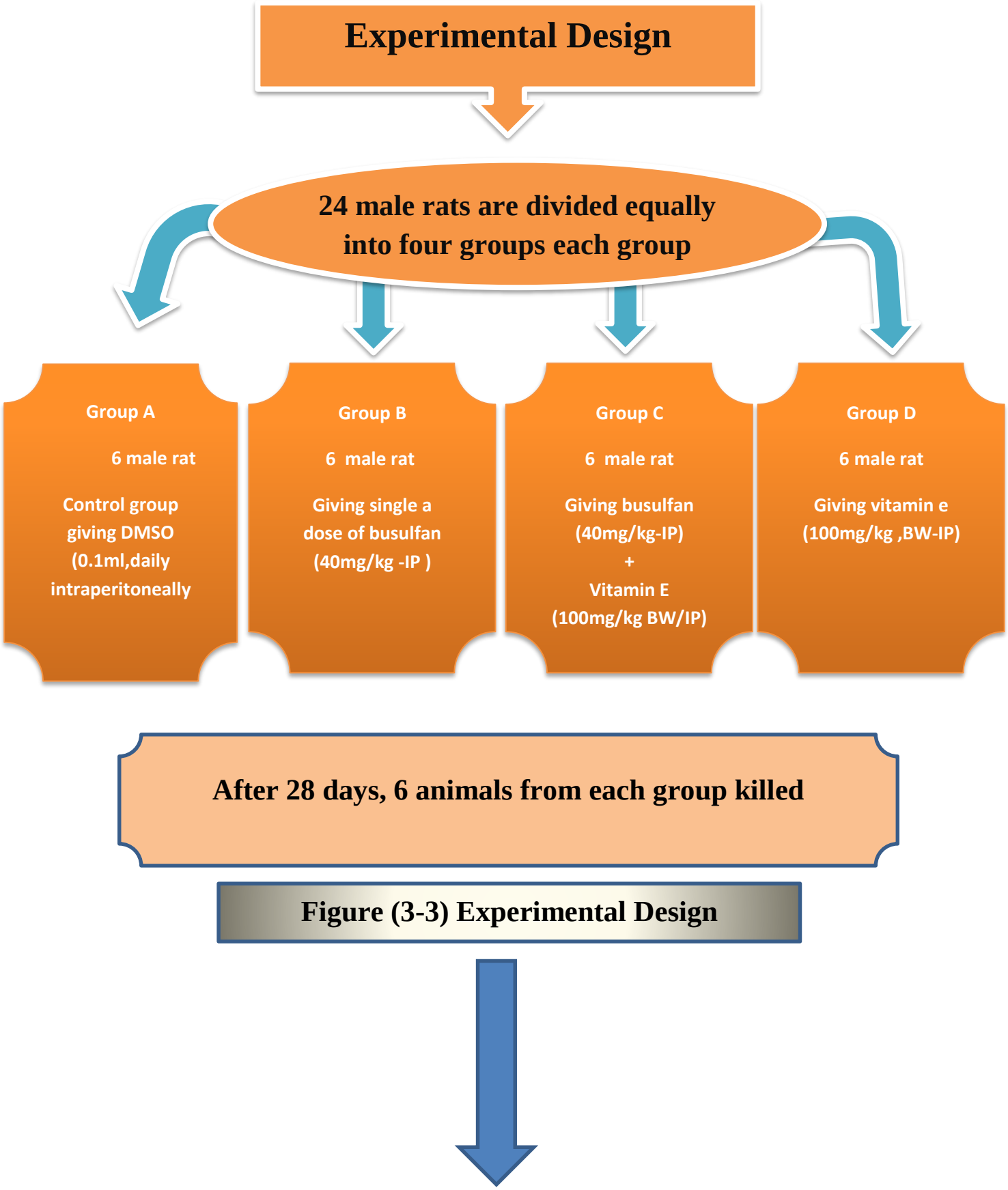

Figure (3-3) Experimental Design

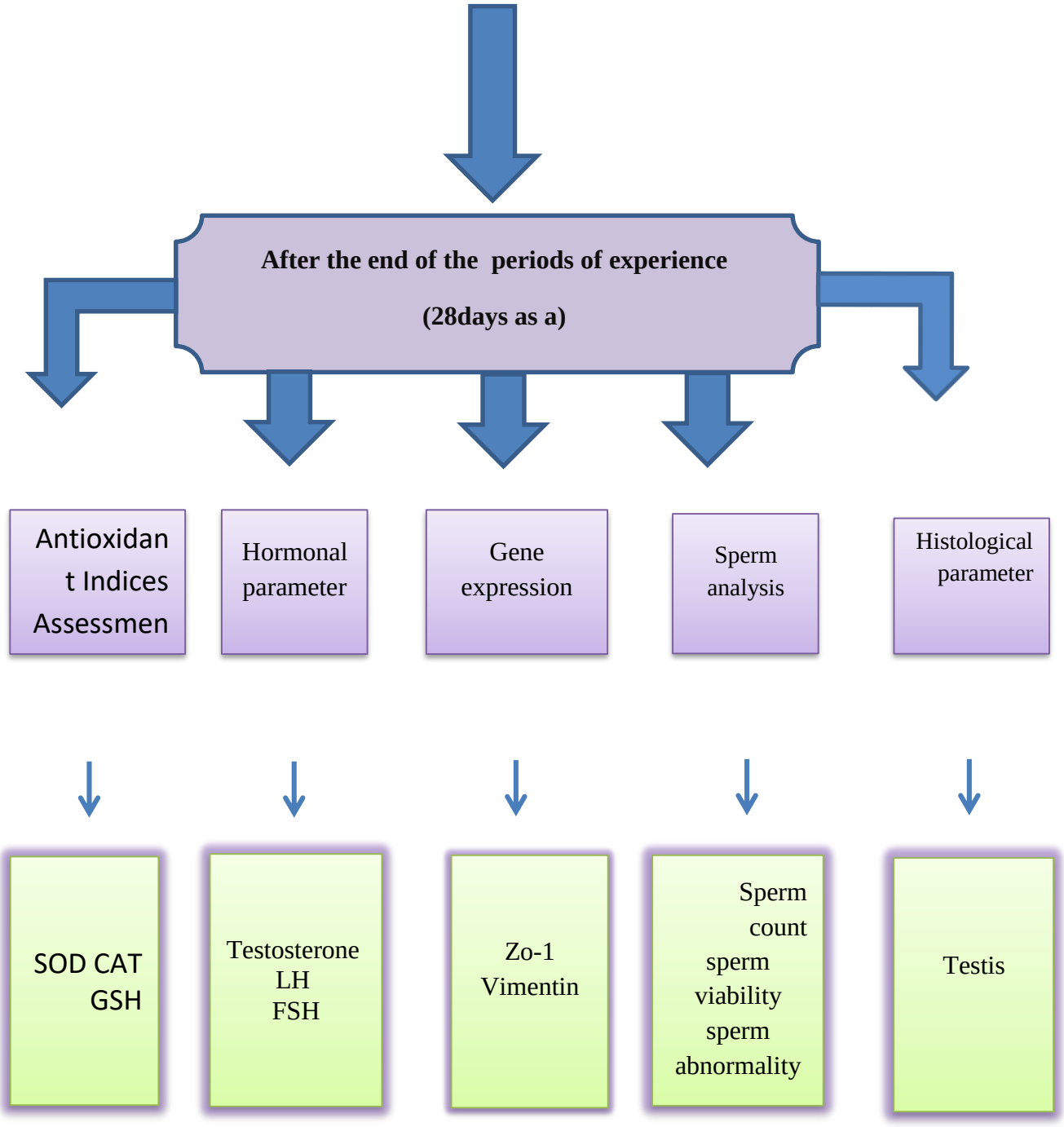

Figure (3-4) study design.

3.9 Preparation and collection of the samples:

At the end of the experiment, the animals that were (anesthetized by chloramphenicol inhalation). The chest and abdomen were open by thoracotomy and laparotomy. followed by collection of blood to perform biochemical and hormonal testing then after sacrificed collection of caudal sperm for caudal sperm analysis and testes isolated to histopathological studies and take epididymis and put it in Trizol for gene expression .were done laborotary of research and study in company of Al Fadhil in Babilon City.

3.9.1 Blood sample :

Blood sample were collected via cardiac puncture. from each male rat. The blood samples were placed in serum tube and left for 30 minutes. Then the blood sample were dropped directly from the heart by using 5 ml disposable syringe the blood putting in the gel tube and then to be centrifuged (3000 rpm for 10 minutes) and kept frozen at -20 °C to obtain .the serum which then were transferred to the Eppendorf tubes. for assessment of reproductive hormones concentrations in serum (FSH, LH, testosterone) . All these of tubes were stored at (-4c) until analyzed. All sample were collected at the morning (9:00 AM – 12:30 PM) in order to minimize the diurnal variation of the hormone levels.

3.9.2 Organs

testes were to be removed and the organs were fixed into 10 % of formalin for histological examination. Epididymis storage in Trizol

3.10 Methods:

3.10.1Estimation of Follicle Stimulating Hormone (FSH) Concentration (µlU/ml) by using a special kit (mono bind USA Follicle Stimulating Hormone (FSH) Test System Product Code: 425-300

Estimation of Follicle Stimulating Hormone (FSH) Concentration in the serum, the level of the hormone FSH was measured using a Kit Specific to measure the hormone level depending on the method of using Kit according to the instructions of the producing company , as shown in appendix I

3.10.2 Estimation of Hormone (LH) Concentration (ng/ml) by using a special kit (mono bimd USA Luteinizing Hormone (LH) Test System ProductCode:625-300

In the serum, the level of the hormone LH was measured using a Kit Specific to measure the hormone level depending on the method of using Kit according to the instructions of the producing company , by using a special kit (mono bimd USA Luteinizing Hormone (LH) Test System Product Code: 625-300 as shown appendix II.

3.10.3 Estimation of Testosterone (T) Concentration (ng/ml) by using a special kit

Testosterone ELISA Kit (Catalog No: E-EL-0155 96T): Measurement of the serum follicular stimulating hormone concentration was generally regarded as valuable tool in the diagnosis of homeostasis of fertility regulation via the hypothalamic –pituitary –gonad axis; the kit

were used(Architect abbott,park,IL60064 USA).Clinical and laboratory standards institute(CLSI),2014. appendix III.

3.10.4 Determination of serum Reduced Glutathione concentration

Determination of serum Reduced Glutathione concentration (GSH) Reduced glutathione was measured following the method of (Sedlak and Lindsay ,1968) , As Show appendix IV .

3.10.5 Determination of serum concentration of (cat)

M. H.Hadwan and H.N. Abed . (2016). Data supporting the spectrophotometric method for the estimation of catalase activity. Data in Brief 6 :194-199. As Show appendix V.

3.10.6 Determination of serum concentration of (sod)

SOD activity determination According to Marklund and Marklund (1974) As Show appendix VI.

3.10.7 Determination of (zo-1) (Vimentin) gene expression

take part of epididymis and put in the Trizol for storage and use special kit by using special technology (real time pcr) (Trans**)** appendix VII , VIII

3.11 Total RNA extraction:

Total RNA were extracted from rat epididymis by using (Trans® reagent kit) and done according to company instructions as following steps in the appendix VII

3.12 DNase inactivation (DNase I) Treatment:

The extracted total RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to method described by Trans company, USA instructions as in the appendix VII.

3.13 qRT-PCR based SYBER Green I Dye Detection

qRT-PCR was performed using AccuPower® Greenstar™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). According to method described by Cheon *et al.* (1999). The Syber Green based q PCR PreMix reagent kit is designed for PCR amplification of cDNA for (zo-1 and Vimentin) target gene by using, zo-1 and Vimentin primer and Housekeeping gene. The Syber Green dye that used in this kit is DNA binding dye which reacted with new copies of amplification specific segment in target and housekeeping gene, then the fluorescent signals recorded in Real Time PCR Thermocycler. VIII

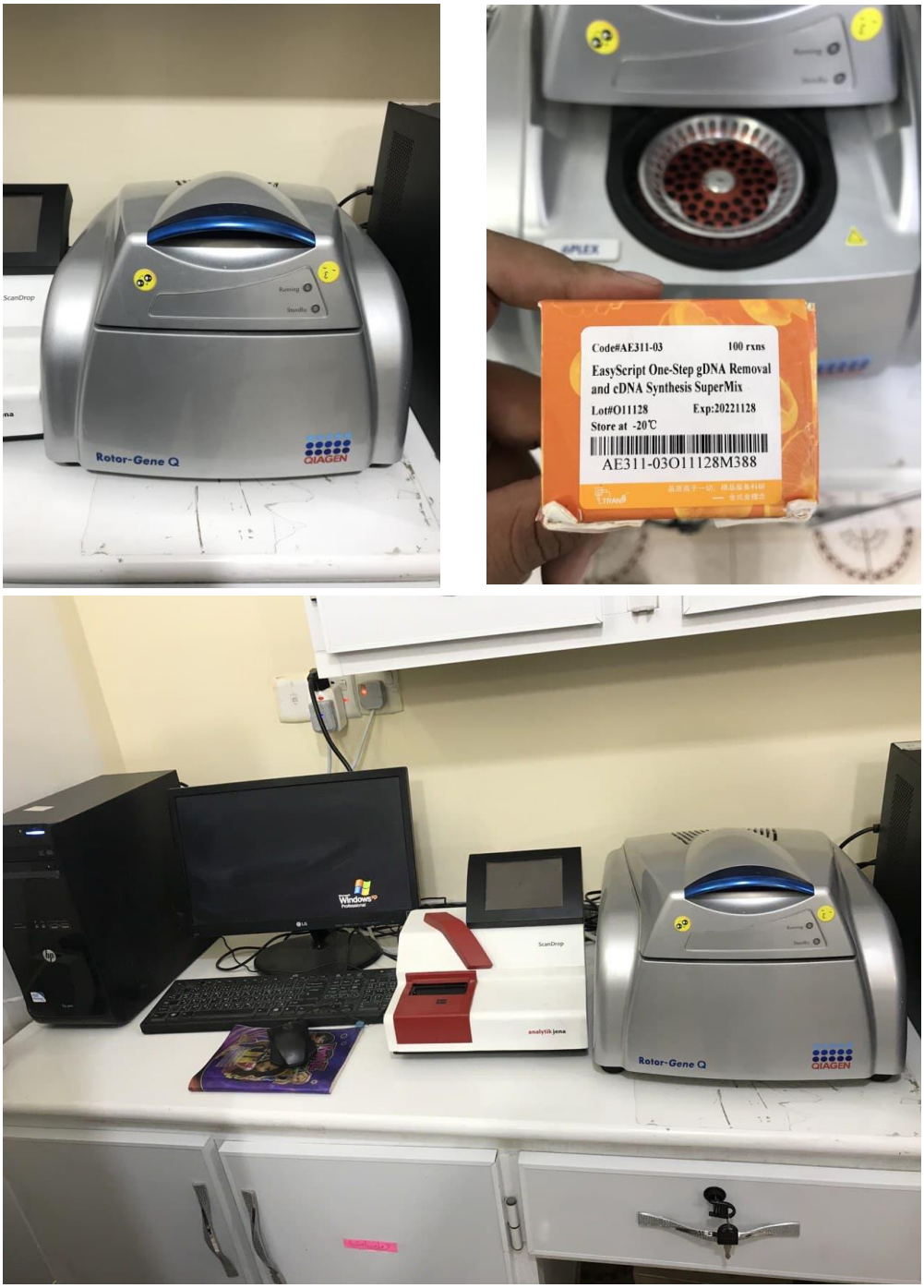

Fig. (3-6) qRT-PCR based SYBER Green I Dye Detection Machines

3.14 Sperm Count Solutions :

Eosin –Nigrosin stain: It was prepared from :

1. (1gm) of Eosin stain soluble in (100 ml) of 3% sodium citrate.

2. (5gm) of Nigrosin stain soluble in (100 ml) of 3% sodium citrate (Hancook, 1951).

3.15 Seminal Analysis:

3.15.1 Sperm Concentration:

The sperms were counted according to the method of (Fernandes et al., (1978) by using Neubauer hemocytometer chamber which used for RBC and WBC count.

3.15.2 Procedure

1. The epididymis wasput in a Petri dish contained 2ml of 0.9% Normal saline .
2. The epididymis was cut into 6-10 pieces by using sharp scalpel .
3. The suspension resulted from the previous step was filtered by Clean of piece by gauze into a test tube .
4. One drop from the filtrate was dropped on the neubauerb chamber which was covered previously with cover slide.
5. The sperms found on the five squares that use for counting the RBCS by using the objective lens (40x) .
6. The sperms were calculated in one mm3 as follwing:

Sperms/cmm=nx10000

N=number of sperms in 5 squires .

3.15.3 Sperm Motility Percentage:

Number of motile sperms were calculated according to (Lio, et al, 1986). Collection of spermatozoa for evaluation of sperm motility. Epididymal spermatozoa were collected by cutting the caudal region of the epididymis into small pieces in 2ml of normal saline pre warmed to 37°C. Sperm was forced out of the caudal epididymis with fine forceps by putting pressure on lower region of caudal epididymis, not forcing out excess material i.e. immature cells. Number of motile spermatozoa were calculated per unit area and expressed as percentage sperm motility. Sperm counts were done using hemocytometer and results were expressed as millions/ml of suspension.

3.16 Percentage of Spermatozoa Viability:

The viability of sperms was counted according to(Graaf and Beilby.,2009).

3.17 Procedure.

1. Diluted semen was dropped on a warm clean slide.

2. warm eosin-negrosin stain was dropped on the semen and mixed together carefully by the use of aglass rod .Asmear was done by using clean slide which was put angularly on the semen slide and was dragged horizontally.

3. The slide was left to dry, and the slide was assessed under the light of microscope of using 40x power; Live sperms appeared non stained and the dead sperms appeared stained.

3.18 Percentage of the Abnormal Spermatozoa:

The abnormal spermatozoa were counted in the same slide that was used to measure the dead and live spermatozoa using account 200 sperms under the light microscope using 100 X power. The number of the result sperm divided on 200 and multiple by 100 according to Filler (1993).

3.19 Preparation of Histological Solutions:

3.19.1 Eosin stain: it was prepared by mixing :

1. Eosin stain (1 gm.)

2. Distilled water (30ml)

3. Ethyl alcohol (70 ml). (AL-Karawi,et al., 2019)

3.19.2 Histological study:

The animals anatomy in the laboratory and the organs of rats were dissected (testes) rapidly excised for histological study and rinsed in normal saline then fixed by immersing deeply in a large volume of 10% formalin at least ten times the volume of the tissue as rapidly as was feasible there by keeping postmortem changes at a minimum. The samples were put in a labeled container contain 10% formalin, and shaking of the container gently several times to make certain that the fluid reached all surface and that the pieces were not sticking to the bottom or sides (A shank of glass wool placed in the container will aid in keeping the tissue free of the bottom) according to (Al-khalissi., 2014).

3.19.3 Histopathological Technique:

The testes of each animal were quickly removed and rapidly weighed then prepared for histological study according to (Al-khalissi., 2014). with the aid of the light microscope as the following steps:

1.Fixation

The specimen was fixated in the natural buffered formalin 10 % for 24 – 48 hours.

2.Washing and dehydration

After fixation the specimens were washed with water to remove the fixative in order to avoid the interaction between the fixative and staining materials used later. By dehydration the water had been completely extracted from fragments by bathing them successively in a graded series of ethanol and water (70 %, 80 %, 90 %, and 100 % ethanol).

3.Clearing

Bathing the dehydrated fragments in solvent (xylene) for 30 – 60 minutes; this step was repeated 3 times .As the tissues were cleared ,they generally became transparent .

4.Infiltration and embedding

Once the tissue fragments were impregnated with the solvent, they were placed in melted paraffin in an oven, typically at 52 – 60 oC . The heat causes the solvent to evaporate, and the space within the tissues becomes filled with paraffin.

5.Sectioning

After removing the specimens from the oven and allowing them to cool to room temperature before sectioning, they were placed in the rotary microtome and cut into 5 micrometer thick slices using the microtome's steel blade. The sections were floated on water bath (50 – 55 o C) , then transferred into glass slides coated with Mayer's albumin as adhesive substance and left to dry.

6.Staining

The histological sections of the studied organs were stained with Hematoxylin - Eosin

3.20 Statistical Analysis:

Statistical analysis of the results was conducted according to SPSS (2016) version 24.00 where one way (ANOVA) was used to assess the significance of changes between the groups' results. The data were expressed as Mean, ± Standard Errors (SE) and P-value ≤ 0.05 and ≤ 0.01 was considered as statistically significant, LSD test was carried out to test the significant levels among means of treatments (Green & Salkind, 2016).

Chapter four

Results

4. Results

4. 1. Effects of Buselfan and Vitamin E on the Body weight and Testes weight

The results in table (4-1) are for the group of male rats treated with Busulfan, Busulfan +Vit E and Vit E alone for 28 days, they showed asignificant ($p<0.05$) decrease in body weight and testes weight in Buselfan group and Buselfan + Vit E group compared with the control group and Vit E alone group.

Table (4-1) show Effects of Buselfan and Vitamin E on the Body weight and Testes weight

Parameters / Groups	Body weight (gm)	Testes weight (gm)
Control	338.83±8.681A	1.5383±0.12906A
Buselfan	289.50±18.218B	1.2283±0.06494C
Buselfan+ Vitamin E	304.83±11.788B	1.4000±0.04000B
Vitamin E	327.50±19.705A	1.5900±0.07925A

- **Values Means ± SD, n = 6**
- **The different letters refer to the significant change between groups ($(p \leq 0.05)$)**

The results also show the different in size of the testes between group as show in figure (4-1)

Fig. (4-1)show difference in size of the testes between control and Vit E group when compared with buselfan and buselfan + Vit E group

4.2 Effects of Buselfan and Vitamin E on Sperm parameters

The results in table (4-2) are for the group of male rats treated with Busulfan, Busulfan +Vit E and Vit E alone for 28 days, they showed a significant ($p<0.05$) decrease in the sperm count, sperm motility, sperm viability in Busulfan group when compared with control group, busulfan +Vit E group and Vit E alone group and there is a significant ($p<0.05$) increase in sperm abnormality in Busulfan group when compared with control group, busulfan +Vit E group and Vit E alone group.

Also, there is a significant increase in the sperm count, sperm motility, sperm viability in busulfan +Vit E group when compared with busulfan alone group.

Table (4-2) show Effects of Buselfan and Vitamin E on sperm parameters

Parameter / Group	Sperm count (10^6/ml)	Sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)
Control	70.67±5.854 B	69.17±8.280 A	72.83±3.764 B	6.00±1.414C
Buselfan	11.83±2.639 D	15.83±3.920 C	17.00±3.162 D	43.67±4.719 A
Buselfan+Vitamin E	25.33±3.777 C	28.33±5.164 B	29.33±2.733 C	16.83±3.430 B
Vitamin E	77.83±5.492 A	74.33±7.033 A	77.17±4.708 A	3.83±1.472C

•Values Means ± SD, n = 6

The different letters refer to the significant change between groups ((p ≤ 0.05)

4.3 Effects of Buselfan and Vitamin E on Reproductive Hormones

The results presented in Table (4-3) showed a significant ($p < 0.05$) decrease in testosterone level and a significant ($p< 0.05$) increase in LH & FSH in the Busulfan group compared to the control group, busulfan + vitamin E, and vitamin E group alone.

Table (4-3) show Effects of Buselfan and Vitamin E on reproducytive hormones

Parameters / Groups	Testosterone (ng/mL)	FSH (ng/mL)	LH (ng/mL)
Control	1.721 ± .254 B	1.856 ± .224 C	1.728 ± .072 BC
Buselfan	.810 ± .118 D	3.545 ± .885 A	2.768 ± .717 A
Buselfan+ Vitamin E	1.263 ±.355 C	2.993 ± .985 AB	2.053 ± .389 B
Vitamin E	2.480 ± .531 A	2.343 ± .735 BC	1.862 ± .247 BC

•Values Means ± SD, n = 6

- **The different litters refer to the significant change between groups ((p ≤ 0.05)**

4.4 Effects of Buselfan and Vitamin E on enzymatic antioxidants

The results presented in Table (4-4) showed a significant ($p < 0.05$) decrease in antioxidant enzyme in the Busulfan group compared to the control group, busulfan + vitamin E, and vitamin E group alone. While there is a significant increase in the same enzyme in buselfan + vit E group when compared with buselfan group alone .

Table (4-4) show Effects of Buselfan and Vitamin E on enzymatic antioxidants

Parameters / Groups	GSH	CAT	SOD
Control	30.90717±5.708335 B	70.24850±10.799293 A	95.5783±2.26974 AB
Buselfan	16.29933±0.865983 D	32.95417±1.943827 C	90.4067±2.44874 C
Buselfan+ Vitamin E	22.61183±1.667184 C	48.12600±9.198610 B	93.3200±5.89853 B
Vitamin E	36.10433±7.591289 A	73.75333±8.834798 A	98.5750±3.07569 A

•Values Means ± SD, n = 6

- **The different letters refer to the significant change between groups ((p ≤ 0.05)**

4.5 Histological Picture and Changes of the Testes

The histological section was read under a magnification of x10 and x40 Examination of the tissues of the normal testes of rats shows that the seminiferous tubules are spherical or oval in shape, lined with germinal epithelium consisting mainly of spermogenic cells that are in the gradual stages of spermatogenesis in the lumen of the seminiferous tubules, between the seminiferous tubules there are interstitial spaces with the group that produce interstitial leydig cells.

Histological examination in this study showed a decrease in the diameter of the seminiferous tubules and the thickness of the seminal epithelium. The seminiferous tubules also suffer from enlargement with some absence in the process of spermatogenesis in groups of male rats treated in the second group.

Normal seminiferous tubules appear with increased spermatogenesis and spermatogenesis in groups of male rats treated with vitamin E in the fourth group.

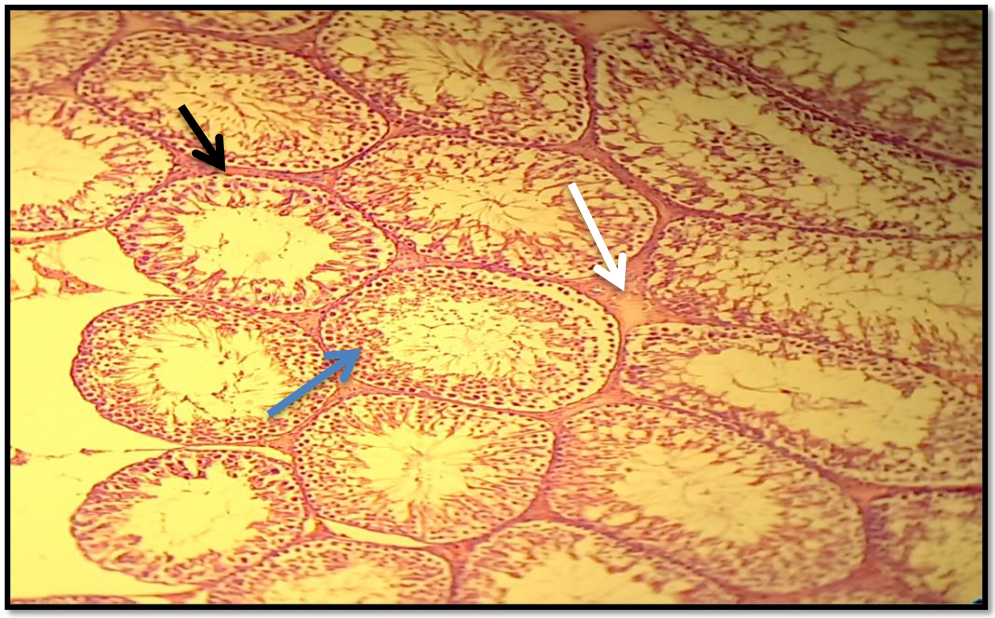

Figure (4-2) Photomicrograph of rat testis shows normal histological features in control group , normal histological structures of seminiferous tubules (black arrow) and interstitial tissues (white arrow) and normal appearing spermatogenesis activity in seminiferous tubules (blue arrow) . (Hematoxylin & Eosin, X 10)

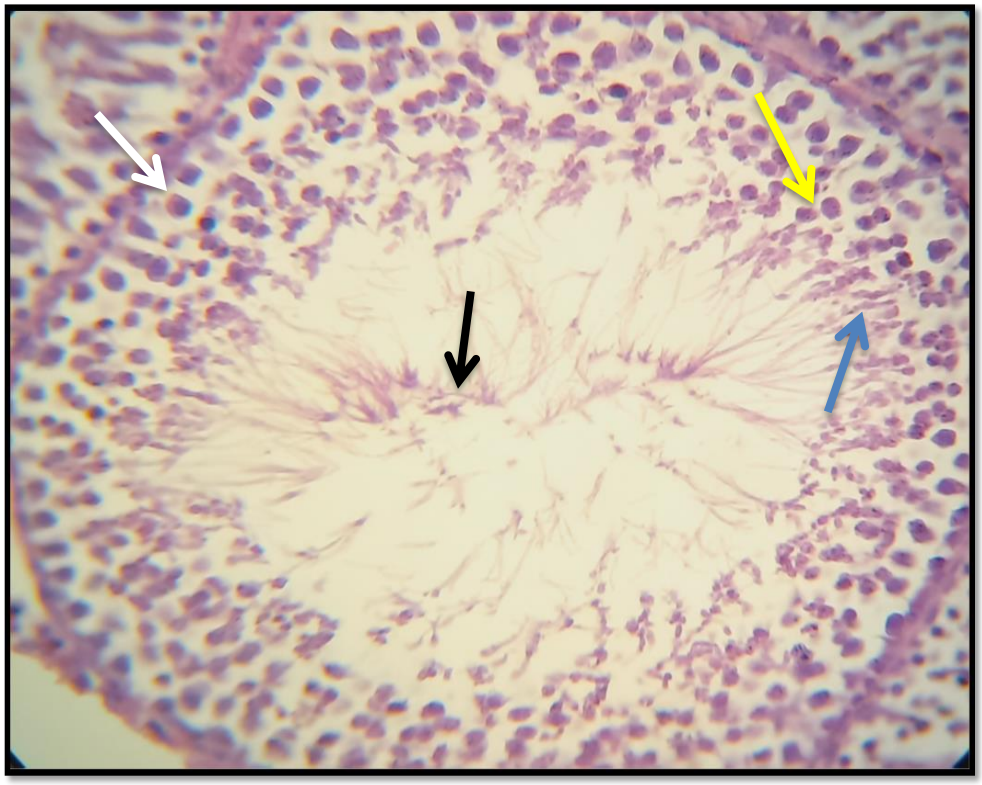

Figure (4-3) Photomicrograph of seminiferous tubule of control group , reveals normal oval to rounded seminiferous tubule are seen. Normal histological structure of the germinal epithelium . Spermatozoa (black arrow) are filling the lumina of the tubule . There are different types of spermatogenic cells lining the tubule including spermatogonia (white arrow), primary spermatocytes (yellow arrow), spermatids (blue arrow), and spermatozoa . (Hematoxylin and Eosin , X40)

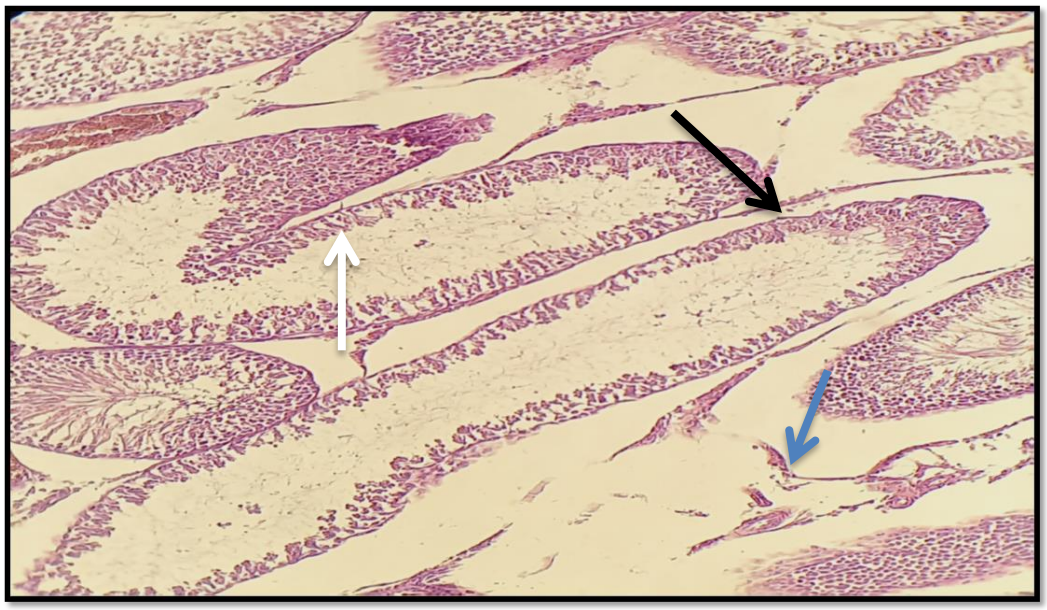

Figure (4-4) Photomicrograph of busulfan treated group showing damage in testicular tissues . The seminiferous tubular atrophy and germinal epitheliums aplasia represent no spermatogenetic activity. The most of spermatogonia (black arrow) and primary spermatocytes cells (white arrow) are destroyed in busulfan treated group, the interstitial tissue were atrophied (blue arrow) with few Leydig cells .(H&E stain X10)

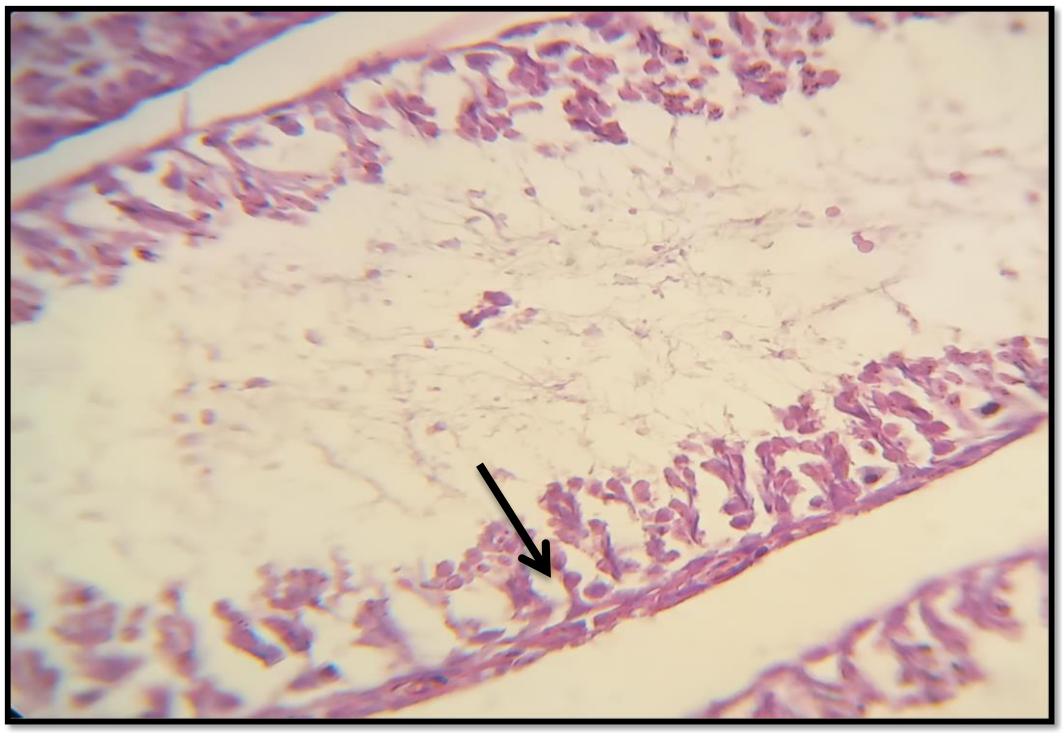

Figure (4-5) Photomicrograph of busulfan treated group showing the seminiferous tubular atropy , decreased thickness of germinal epithelium and vacuolar space on the basement membrane of the seminiferous tubules were observed (black arrow). The spermatogonia and primary spermatocytes cells are destroyed , no spermatozoa in the lumen of the seminiferous tubule.(H and E , X40)

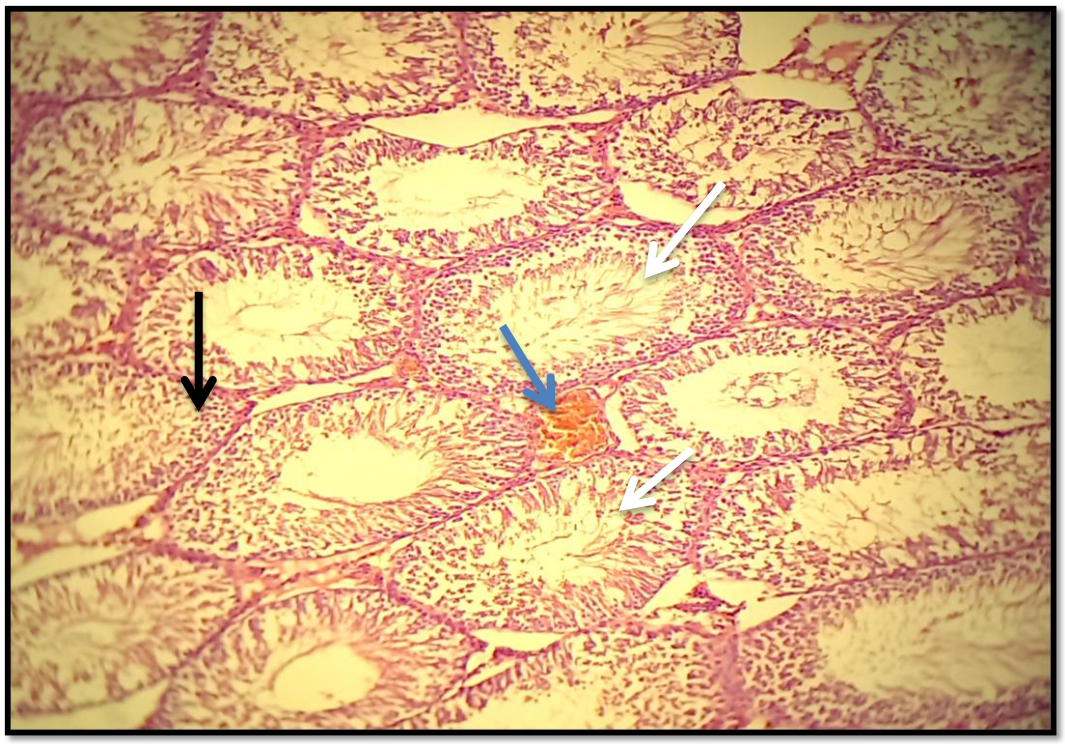

Figure (4-6) Photomicrograph of seminiferous tubules of group treated with busulfan and Vitamin E , showed almost normal arrangement of germinal epithelium (white arrows) and represent reestablishment of spermatogenesis (black arrow) , mild congestion of interstitial blood vessel (blue arrow) .(H and E , X10)

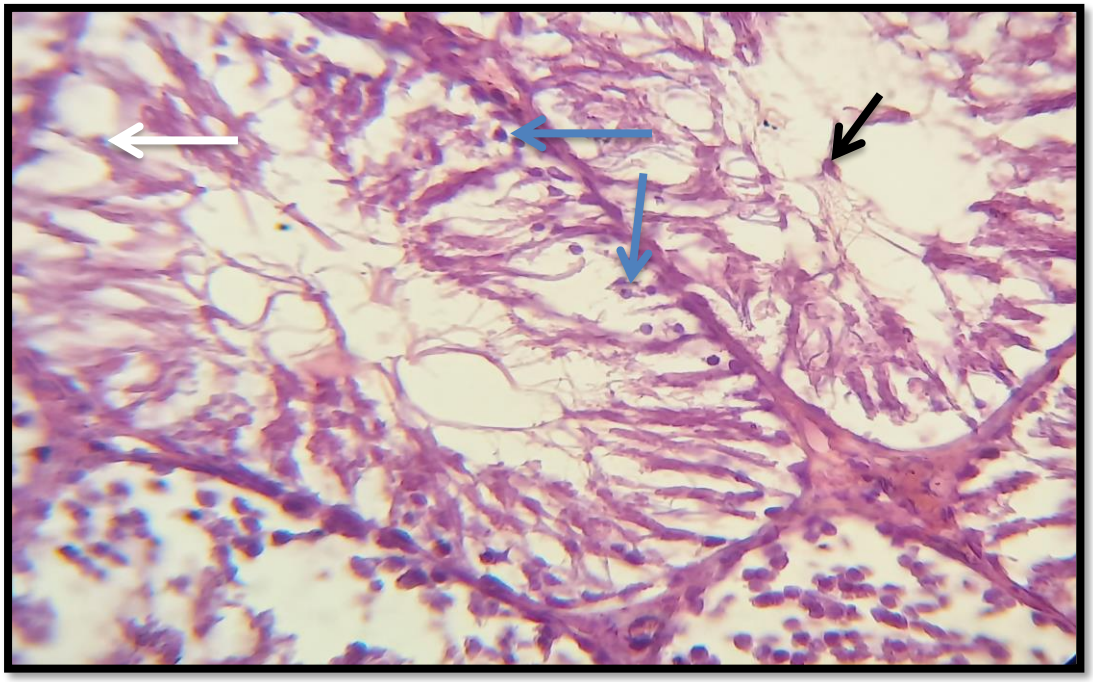

Figure (4-7) Photomicrograph of seminiferous tubules of group treated with busulfan and Vitamin E , showed mild reduction of germinal lining represented by the view of moderate number of germinal cells (blue arrows) with a number of spermatids in the lumen of the recovered seminiferous tubules (black arrow) , congested interstitium with average Leydig cells (white arrow) and prescence of vacuolation of the seminiferous tubules (white arrow) .(H and E , X40)

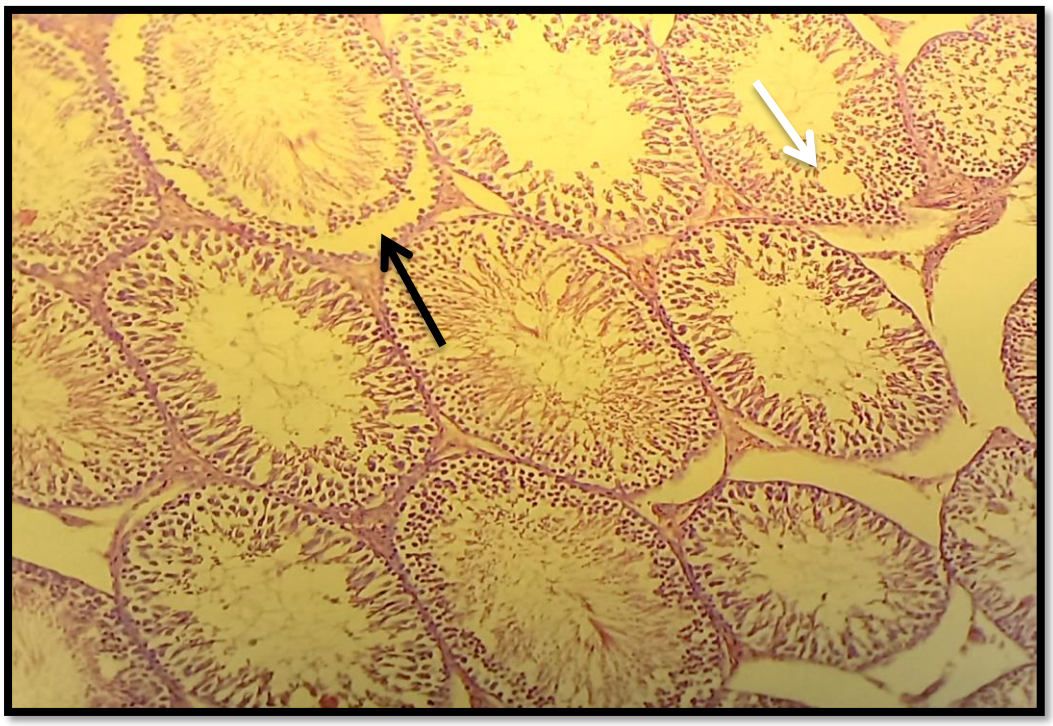

Figure (4-8) Photomicrographs of group treated with vitamin E , showing most of seminiferous tubules with normal appearance , with restoration of active spermatogenesis in most of seminiferous tubules and with focal areas of vacuolation (white arrow) and sloughing of germinal epithelium (black arrow) . (H & E, X 10) .

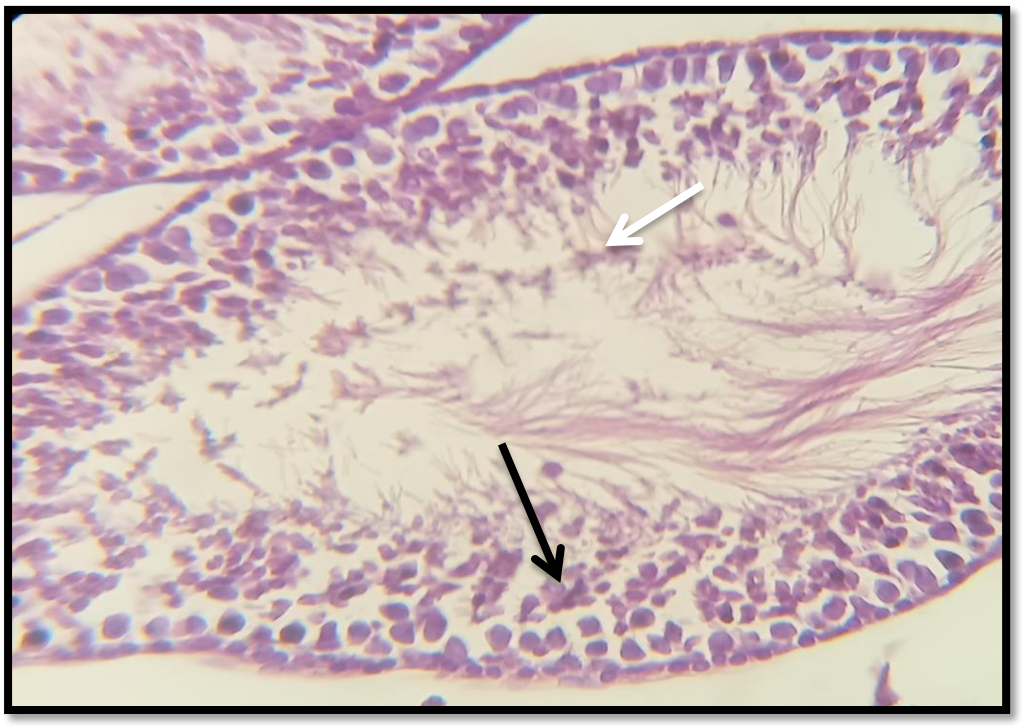

Figure (4-9) Photomicrographs of rat testis from group treated with vitamin E , showed the normal arrangement of germinal epithelium and normal histology in all successive stages of spermatogenesis (black arrow)with lumen filled with sperm (white arrow) (H & E, X 40)

4.6 Effects of Buselfan and Vitamin E on gene expression of zo-1 :

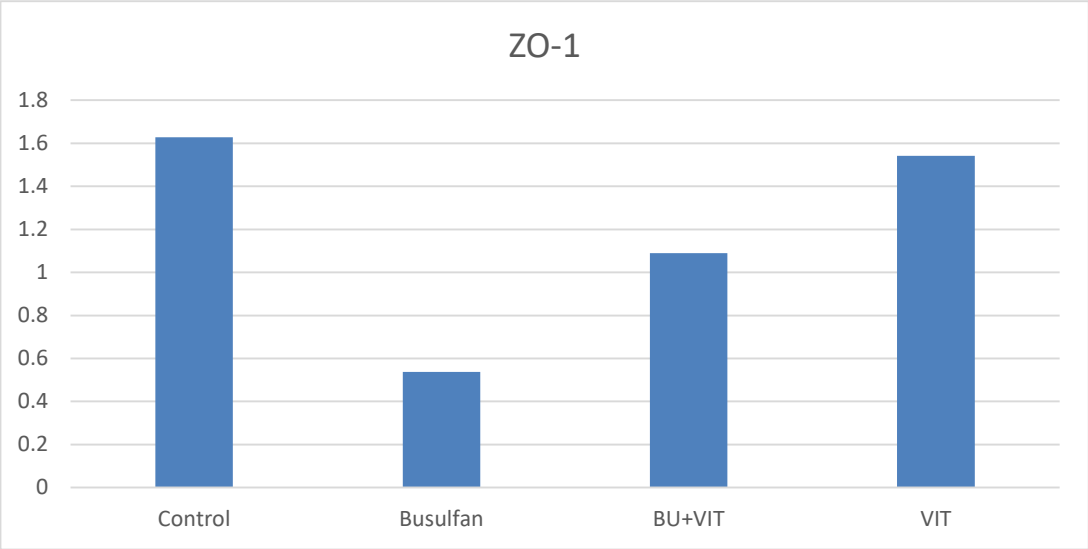

Table (4-5) Effects of Buselfan and Vitamin E on gene expression of zo-1 :

ZO-1 gene expression	groups
1.627161	Control
0.537052	Busulfan
1.089094	BU+VIT
1.541172	VIT

first group: (Group A) : six male Rats were given **DMSO** (1 mL, daily, intraperitoneally injection) observe the gene expression of zo-1 is (1.627161)

second group: (Group B) : six male Rats were given single dose of **busulfan** (40mg/kg IP.) intraperitoneal injection . observe the gene expression of zo-1 is decrease (0.537052)

third group: (Group C) : six male Rats were given single dose of **busulfan** (40mg/kg IP.)**plus vitamin E** (100 mg/kg, daily b.w/IP) intraperitoneal injection. . observe the gene expression of zo-1 is increase compare it with second group due to that effect of vit (E) is decrease the effect of BU (1.089094)

fourth group: (Group D) : six male Rats were given dose Of the **vitamin E** (100mg/kg, daily, b.w/IP) intraperitoneally injection) observe the gene expression of zo-1 is (1.541172)

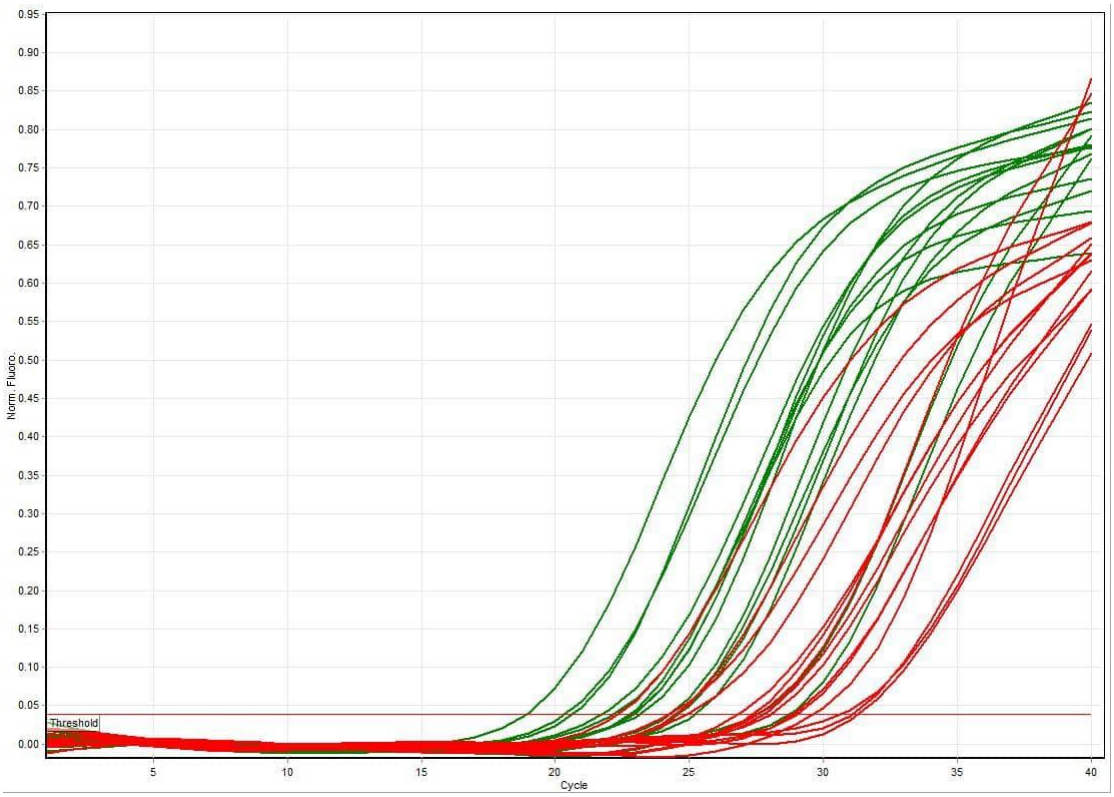

Fig. (4-10) Peak analysis of ZO-1 gene showing immersion of SYBER Green overtime

4.7 Effects of Buselfan and Vitamin E on gene expression of Vimentin gene :

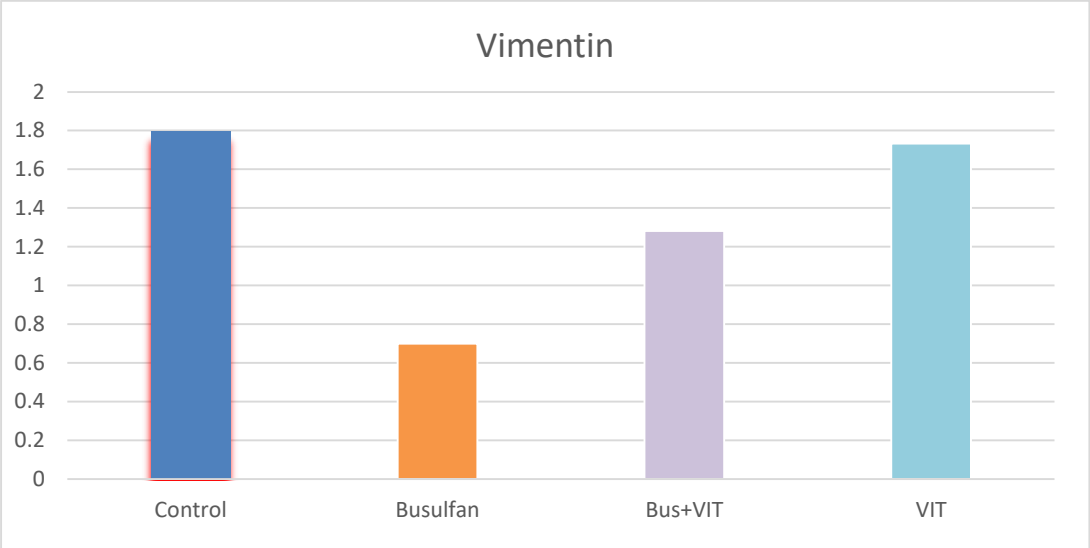

Table (4-6) Effects of Buselfan and Vitamin E on gene expression of Vimentin gene

Vimentin gene expression	groups
1.796736	Control
0.699031	Busulfan
1.281176	BU+VIT
1.732872	VIT

first group: (Group A) : six male Rats were given **DMSO** (1 mL, daily, intraperitoneal injection) observe the gene expression of Vimentin is (1.796736)

second group: (Group B) : six male Rats were given single dose of **busulfan**(40mg/kg IP.) intraperitoneal injection . observe the gene expression of Vimentin decrease (0.699031)

third group: (Group C) : six male Rats were given single dose of **busulfan** (40mg/kg IP.)**plus vitamin E** (100 mg/kg, daily b.w/IP) intraperitoneally injection. . observe the gene expression of Vimentin is increase compare it with second group due to that effect of vit (E) is(1.732872)

fourth group: (Group D) : six male Rats were given dose Of the **vitamin E** (100mg/kg, daily, b.w/IP) intraperitoneally injection) observe the gene expression of Vimentin is (1.28117)

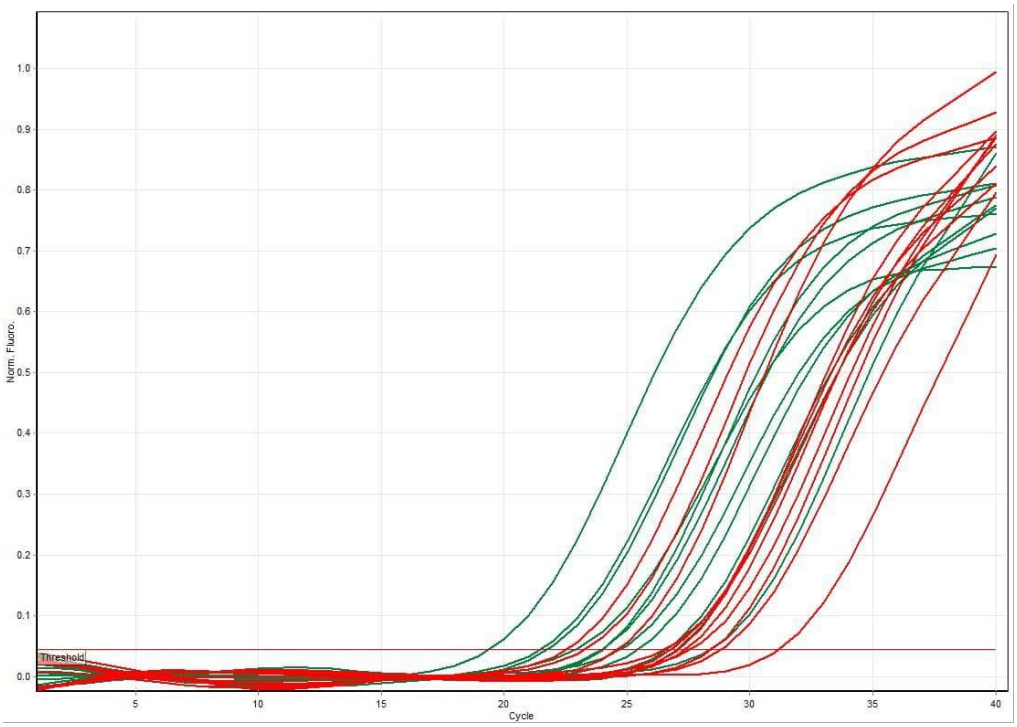

Figure (4-11) Peak analysis of Vimentin gene showing immersion of SYBER Green overtime

as show Appendix VIII.

Chapter Five Discussion

5.Discussion

5.1 Body and testis weight

The results of this study show a significant ($p<0.05$) decrease in the body weight and Testes weight compared with the control group. This result comes from that one of the side effect of busulfan administration is diarrhea and weight loos as a result of injection and this result is agreement with YuSheng Qin et al 2016 and Mao Zhang et al 2021 while, disagreement with Ahar et al 2014 who found no significant difference in body weight but there is a significant difference in testis weight between busulfan group and control group.

This result could be due to cytotoxic effects of Busulfan with Transferring the alkyl group(s) to various body organ.

This result may be due to the cytotoxic effects of busulfan with transfer of the alkyl group(s) to a different cellular. However, DNA alkylation events may constitute major inducers that lead to cell death (Molenaar et al 1985). Busulfan partially eliminates stem cells due to its alkylating nature (Ray, 2012. It kills cells by producing free radicals (Ray, 2011). Therefore, busulfan appears to inhibit spermatogenesis, particularly via oxidative damage. Another mechanism suggested that busulfan increased the level of ck18, a Sertoli cell surface marker. Elevation of this mark causes impaired spermatogenesis and infertility (Ray, 2011). In this study, busulfan reduces body weight and testes weight. Zheng Wei et al showed that there is a direct relationship between testicular weight and the number of germ cells in primates (Zheng et al 1997). Bucci et al. showed that busulfan caused chromosomal abnormalities and dominant killer mutations in sperm (Bucci et al 1987).

5.2 Sperm parameters

Infertility Is One of the most important problems in the life of a married couple. Many factors can affect sperm production and the risk of infertility, and among these factors is the use of chemotherapy drugs for cancer. Busulfan is one of those drugs that significantly affect the testicles. (Aboul Fotouh *et al* .,2018). These agents can reduce sperm concentration while generating free radicals and oxidation of germ cells in the testes (Amin and Hamza,2006). In this study, we examined the protective effect of vitamin E against busulfan-mediated sperm damage in rats, and a four-week period was chosen to monitor the possible recovery of spermatogenesis in busulfan-treated animals. According to the study prepared by (Nagano et al., 1999), it took about 4 weeks after busulfan treatment for the effect of busulfan to differentiate between cells secreted from the seminiferous tubular lumen.

Our results showed that administration of busulfan in a single dose and daily administration of vitamin E for 4 weeks can significantly reduce testicular destruction caused by busulfan injection. The results showed a significant decrease in the busulfan group compared to the other groups ($P < 0.05$), (Table 4-2) in sperm count, motility and vitality as well as a significant increase ($P < 0.05$) in sperm abnormalities. Our findings were in agreement with (Moloody et al .,2018and Jalili et al ., 2020). It was confirmed by Choi et al., 2004 that busulfan chemotherapy can induce apoptosis in spermatozoa. Busulfan also increases the production of reactive oxygen species and leads to the death of sperm(Dehghani et al ., 2013).

The presence of sperm with abnormal morphology in the semen is another possible consequence of busulfan treatment in mice and this was

found by (Panahi et al., 2015). These studies have shown that busulfan is involved in stopping spermatogenesis, although some changes are amenable to Reversible and dose-dependent, Busulfan inhibits spermatogenesis, particularly due to oxidative damage that increases the level of ck-18, a superficial marker of Sertoli cells. Elevation of this marker caused impaired spermatogenesis, infertility, and reduced sperm motility by reducing flagellum length (Maymon et al., 2004).

We chose vitamin E as an antioxidant based on previous data in the literature on male fertility (Showell et al 2014, Matorras et al 2020). Supplementation of infertile males with the antioxidant vitamin E has been suggested as a potential treatment for idiopathic male infertility (wen,2006). In our results we found there is a significant increase in sperm count in vit E plus Busulfan group when compare with Buselfan alone group. This result comes from that This vitamin has the strong antioxidant properties and inhibits the lipid peroxidation created by the free hydroxyl and superoxide radicals. This vitamin protects the cell membrane of sperm cell from damages of ROS. (Zubair, 2017)

Oral supplementation of this vitamin has significant beneficial effects on sperm motility by reducing malondealdehyde (MDA), which is known as the end product of lipid peroxidation. (Zubair, 2017)

Vitamin E deficiency can damage the reproductive organs Such as damage to spermatogenesis, testicular weakness The contraction of the seminiferous tubules. Use this vitamin Enhances the functions of the testicles in the form of weight gain From the testicles and epididymis(wang et al 2007).

An interesting finding in the sperm viability and count test was that vitamin E alone increased these parameters compared to the control group. This effective result of vitamin E may also be due to its role as an antioxidant. This vitamin plays an important protective role in preventing the production of lipid peroxides by scavenging free radicals that are toxic to biological membranes and this result is agreement with Yue et al 2010, Momeni and Eskandari, 2012)

5.3 Hormonal parameters

As shown in (Table 4-3) we found that LH and FSH levels were significantly increased ($P \leq 0.05$) while testosterone levels were decreased in busulfan-treated rats compared to the other groups. Busulfan is a drug that greatly affects the testicles. It causes a pronounced increase in apoptosis and affects spermatogenesis. Busulfan has toxic effects on the spermatic epithelium of rat and causes infertility (Abul-Fotouh et al.,2018) mainly by killing sperm stem cells. A primary gonadal defect is caused by a testicle when damage has a detrimental effect on spermatogenesis and/or defect in Leydig cells (Kiserud et al., 2009).

Exposure to chemotherapy and cytotoxic radiotherapy results in an associated elevation of FSH levels with decreased sperm count and, consequently, a suppressive effect of testosterone analogues and gonadotropins on spermatozoa. Also , chemotherapy is often used with increased testicular weakness and damage to the epithelial microbiota. To enable treatment of male infertility after cancer treatment (Qu et al., 2019). On the other hand, accumulation of free radicals and thus oxidative injury in Leydig cells in the testis by specific chemical oxidants (eg busulfan) may degrade their response and performance of testosterone synthesis. (Banihani,2018).

According to the above, the elevated FSH level in this study may be due to impaired spermatogenesis, while the low testosterone levels associated with elevated LH may occur as a result of Leydig cell dysfunction. (Hassan & Jasim, 2020).

Our results in table 4-3 also show a significant increase in testosterone level in busulfan +Vit E group when compared with busulfan group this result comes from that Vitamin E is another antioxidant found in Cell membranes protecting the cell hydrophobic membranes peroxides. Some reports indicated that The main role of vitamin E in reducing if Oxidative stress in the testicle (Kutlubay et al 2007, Momeni et al 2012).

The assumption is vitamin E due to enhancing steroid hormones levels to Sertoli cells, can have a positive effect on nutrition of germ cells (Abbas, S. M., & Khalil, L. W. (2016) Therefore, vitamin E have a direct effect on sexual hormones production or decreased level of FSH and LH may be as a compensatory mechanism to elevate testosterone level(Ayinde et al 2012).

Furthermore, the present study demonstrates that vitamin E administration enhances testosterone production in rat. It was found that a specific binding material for vitamin E is present in cytosol and nuclei from liver. Recently, It can be presumed that this specific receptor may also be present in the pituitary and testis (Abbas and Khalil, 2017).

5.4 antioxidant enzyme parameters

According to the serum's antioxidant status, the Changes in serum levels of some enzymatic antioxidants are shown in Table 4-4. A significant decrease in activity of SOD, CAT, and GSH levels were

observed in the treated rats with busulfan compared to the other groups. Co-administration of vitamin E with medicated busulfan Rats cause elevated serum SOD, CAT activity, and GSH compared to the busulfan group alone. The significant decrease in of antioxidant enzyme busulfan group is in agreement with ROHNAVAZ et al 2016 and de Paula Eduardo et al 2020.

Many factors can affect sperm production and infertility risks. Among these factors are chemotherapy drugs for cancer. These drugs can reduce sperm concentration with generation of free radicals and oxidation of germ cells in the testes (Amin and Hamza 2006).

Busulfan is highly cytotoxic and genotoxic factor (Panahi et al 2015) that can cause various damages Effects, both acute and chronic, such as DNA damage hence it activates apoptosis or aging in a Cell type dependent method possibly due to oxidation Stress in many biological organs one of them is reproductive organs. (Iwamoto et al 2004). it has been Confirmed that chemotherapy with busulfan can increased generation of ROS and Resulting in the death of sperm (Dehghani et al 2013).

Antioxidants neutralize free radicals thus The oxidative reactions it causes. food antioxidants May be helpful in reducing lipid peroxidation and DNA damage in sperm during busulfan treatment (Khaki et al 2009). Vitamin E (A-tocopherol) is a fat-soluble organic compound They are generally located in cell membranes. This dominant antioxidant quenches superoxide anions and free hydroxyl The radicals thus reduce lipid peroxidation caused by reactive oxygen species in plasma membranes (Lü et al 2010) and thus protect the cell Membrane damage caused by ROS. This is an antioxidant Reduces testicular tissue damage caused by

cytotoxic agents By increasing the expression of related antioxidants genes. In the male reproductive system, antioxidants The property of this vitamin in inhibiting the destructive effects The presence of free radicals has been confirmed in testes (Mendiola et al 2010) and sperm [Lu et al 2018]. In addition, some studies have shown that vitamin E is effective in Protect the testicles from damage caused by oxidative stress Reducing this damage can be achieved through vitamin E treatment (Aydin et al 2015).

Besides the antioxidant properties of this Vitamin, and antioxidant enzymes such as superoxide dismutase (SOD) and Glutathione peroxidase is enhanced due to the use of this vitamin.(wang et al 2007)

5.5 Histopathological Effect of Busulfan and vit E on Testes Tissue:

The results of the current study showed that busulfan reduces germinal epithelium elevation in rats. There was desquamation and irregularity in the sperm cells with exfoliation in the lumen of the seminiferous tubules. Thus, the decrease in the thickness of the gothic tubules of the epithelium could be a consequence of the degenerative effects of busulfan(Babazadeh and Najafi,2017).

The histopathological examination of testicular tissue in our study showed degenerating in seminiferous convoluted tubules with decrease in the diameter of seminiferous tubules and necrosis of spermatids, spermatocytes, epithelial sloughing, absence and degeneration of the spermatogenesis, this result agreed with (Demir et al., 2007) Histo-morphometric effect show the thickness of testicular capsule was

increased in busulfan groups. Compared to the control group, germinal epithelium height was decreased in busulfan and busulfan & vit E group.

5.6 Gene expression of ZO-1 and Vimentin gene

The epididymis is where sperm maturation occurs, it is important to recognize the effects of busulfan treatment on this organ which is an area often overlooked. The present study determined the effect of busulfan treatment on the epididymis. Approximately 4 weeks after busulfan treatment, germ cells in the process of spermatogenesis were cleared from the seminiferous tubules; therefore, we used different time points from week 1 to 4 to observe the effects of busulfan on the epididymis (Zohni, K., et al 2012).

In the epididymis, epithelial cell–cell interactions are mediated by adhering junctions, gap junctions, and tight junctions, which are necessary for cell adhesion and the formation of the blood–epididymal barrier, respectively (Cyr, D.G. et al 2007).

Previous studies reported that Vimentin was associated with the desmosome-like junctions in the testis special in the epididymis, the collapse of Vimentin caused by busulfan treatment might result in an impaired anchoring function of these junctions between adjacent principal cells, leading to the loss of structural integrity of the epididymal epithelium; however, this speculation requires further confirmation. As shown by immunostaining, Vimentin was also located in spermatozoa in the lumen, indicating the decreased expression of Vimentin might be related to the reduced number of sperms that enter the epididymis. in our results showed significant decrees in the gene expression of ZO-1and

Vimentin in the busulfan group compared with control group .this result agreement with (Ruan, Y.C., et al 2014) (Mendez, M.G., et al 2010) (Fang, F., et al 2017).

in other result showed significantly increased in the gene expression of ZO-1 and Vimentin in the fourth group (Vit E) compared with third group (Busulfan with Vit E) that result agreement with (Salehinezhad, F., et al 2019)

Chapter Six

Conclusions& Recommendations

6.1 Conclusion:

Administration of Vit E in busulfan-treated animals improved histological and sperm quality. The curreat study were concluded that :

1. Busulfan has a sperm-toxic effect.
2. Chemotherapy (busulfan) has direct effect on body wight and testes wight.
3. Busulfan that reduce the gene expression of Zo-1 and vimenten in rats treated of busulfan .
4. Vit E have effects of antioxidant action and protection effects of against oxidative stress, according to the results of this study, Vit E has positive therapeutic and protective effects on testicular tissue and increases sperm production in the rat treated by busulfan
5. Vit E have positive effect on reproductive system . The best treatment for men who suffer from a lack of sperm (oligospermia)

6.2 Recommendations:

1. Using a technique Tunnel assay to measure cell damage in the rat's epididymis instead of Q PSR .

2**.** . Re design the experiment to include implantation of cancerous tumors (cancer cell line) in rats (Xenograph technique) and then follow up to the protective effect of vitamin E on tumor growth.
3.Use other antioxidant such as Selenium ,olive oil as an effective and powerful antioxidant .

4. Recommend to use Vit E in cases that need to be treated with

busulfan for long time and Investigate the mechanism behind the adverse effect of busulfan on the testicles.

5.We recommend use of vitamin E as a treatment for people with oligospermia and izospremia .

6.Study side effects of busulfan drug on other organs such prostate gland and ovary in female.

7.study of effects of busulfan on the other adrinal gland and thyroid gland .

8. We Recommend study use for long period for example (6 mounth).

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Appendix

Appendix I

Estimation of Follicle Stimulating Hormone (FSH) Concentration

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at 2- 30°C for up to 60 days.

2. Working Substrate Solution – Stable for one year Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note1 : Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C). Test Procedure should be performed by a skilled individual or trained professional

1. Format the microplate wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.050 ml (50µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Add 0.100 ml (100µl) of FSH-Enzyme Reagent solution to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 60 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 350µl of wash buffer (see Reagent Preparation Section) decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.100 ml (100µl) of working substrate solution to all wells

(see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells

Do not shake the plate after substrate addition

9. Incubate at room temperature for fifteen (15) minutes.

10. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds). Always add reagents in the same order to minimize reaction time differences between wells

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Calculation of results

A dose response curve is used to ascertain the concentration of follicle stimulating hormone in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding FSH concentration in mIU/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of FSH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in mIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.214) intersects the dose response curve at 43.2mIU/ml FSH concentration (See Figure 1).

Appendix II

Estimation of Hormone (LH) Concentration

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at 2-30°C for up to 60 days.

2. Working Substrate Solution – Stable for one year Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

Test procedure

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

Test Procedure should be performed by a skilled individual or trained professional

1. Format the microplate wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C

2. Pipette 0.050 ml (50µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Add 0.100 ml (100µl) of LH-Enzyme Reagent to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 60 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section) decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells

Do not shake the plate after substrate addition

9. Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds). Always add reagents in the same order to minimize reaction time differences between wells

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Calculation of results

A dose response curve is used to ascertain the concentration of luteinizing hormone (LH) in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding LH concentration in mIU/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of LH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in mIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.005) intersects the dose response curve at 42.7 mIU/ml LH concentration

Appendix III

T(Testosterone) ELISA Kit

Catalog No: E-EL-0155

96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)

Email: techsupport@elabscience.com

Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service

Intended use

This ELISA kit applies to the in vitro quantitative determination of T concentrations in serum, plasma,

urine and saliva. Please contact tech-support for other sample type detection.

Specification

Sensitivity: 0.17ng/mL.

Detection Range: 0.31-20ng/mL.

Specificity: This kit recognizes T in samples. No significant cross-reactivity or interference between T and analogues was observed.

Repeatability: Coefficient of variation is <10%.

Background

Testosterone is a hormone found in humans, as well as in other animals. The testicles primarily make testosterone in men. Women's ovaries also make testosterone, though in much smaller amounts. Testosterone production starts to increase significantly during puberty, and begins to dip after age 30 or so [1]. Testosterone is most often associated with sex drive, and plays a vital role in sperm production. But it also affects bone and muscle mass, the way men store fat in the body, and even red blood cell production. A man's testosterone levels can also affect his mood. Testosterone is used as a medication for the treatment of males with too little or no natural testosterone production, certain forms of breast cancer, and gender dysphoria in transgender men. This is known as hormone replacement therapy(HRT) or testosterone replacement therapy(TRT), which maintains serum testosterone levels in the normal range. Decline of testosterone production with age has led to interest in androgen replacement therapy [2].

Mooradian, A. D., Morley, J. E., & Korenman, S. G. (1987). Biological actions of androgens. Endocrine reviews, 8(1), 1-28.

Myers, J. B., & Meacham, R. B. (2003). Androgen replacement therapy in the aging male. Reviews in urology, 5(4), 216.

Test principle

This ELISA kit uses the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with T. During the reaction, T in the sample or standard competes with a fixed amount of T on the solid phase supporter for sites on the Biotinylated Detection Ab specific to T. Excess conjugate and unbound sample or standard are

washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of T in the samples is then determined by comparing the OD of the samples to the standard curve.

Kit components & Storage

An unopened kit can be stored at 4C for 1 month. If the kit is not used within 1 month, store the items separately according to the following conditions since the kit is received.

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	8 wells ×12 strips	-20C, 6 months
Reference Standard	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 120 uL	
Concentrated HRP Conjugate (100×)	1 vial, 120 μL	-20C(shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	4C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	4C(shading light)
Stop Solution	1 vial, 10 mL	4C

Plate Sealer 5 pieces

Product Description 1 copy

Certificate of Analysis 1 copy

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.

The volume of reagents in partial shipments is a little more than the volume marked on the label,

please use accurate measuring equipment instead of directly pouring.

Other supplies required

Microplate reader with 450 nm wavelength filter

High-precision transfer pipette, EP tubes and disposable pipette tips

37℃ Incubator

Deionized or distilled water

Absorbent paper

Loading slot for Wash Buffer

Note

Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.

A freshly opened ELISA Plate may appear to have a water-like substance, which is normal and will not have any impact on the experimental results.

Do not reuse the diluted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.

The microplate reader should be able to be installed with a filter that can detect the wave length at 450 ±10 nm. The optical density should be within 0~3.5.

Do not mix or use components from other lots.

Change pipette tips in between adding of each standard level, between sample adding and between reagent adding. Also, use separate reservoirs for each reagent.

Sample collection

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4CC before centrifugation for 20 min at 1000×g at 2-8CC. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin.

Plasma: Collect plasma using EDTA-Na2 as anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8CC within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for ELISA assay!

Urine: Use a sterile container to collect urine samples. Remove particulates by centrifugation for 15 minutes at 1000×g at 2-8°C. Collect the supernatant to carry out the assay.

Saliva: Remove particulates by centrifugation for 10 minutes at 4000×g at 2-8°C. Collect the supernatant to carry out the assay. Recommend to use fresh saliva samples.

Note for sample:

Samples should be assayed within 7 days when stored at 4CC, otherwise samples must be divided up and stored at -20CC (≤1 month) or -80CC (≤3 months). Avoid repeated freeze-thaw cycles.

Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

It is recommended to do the experiment with undiluted human serum, plasma and saliva samples, urine samples diluted at about 10 fold.

It is recommended to do the experiment with undiluted serum and plasma samples from mouse, rat, chicken, porcine, bovine and sheep.

Reagent preparation

Bring all reagents to room temperature (18~25 C) before use. Follow the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.

Wash Buffer: Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40 Cwater bath and mix it gently until the crystals have completely dissolved.

Standard working solution: Centrifuge the standard at 10,000×g for 1min. Add 1.0mL of Reference Standard &Sample Diluent, let it stand for

10min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 20 ng/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 20, 10, 5, 2.5, 1.25, 0.62, 0.31, 0 ng/mL.

Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 20 ng/mL working solution to the first tube and mix up to produce a 10 ng/mL working solution. Pipette 500uL of the solution from the former tube to the latter one according to this step. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.

Biotinylated Detection Ab working solution: Calculate the required amount before the experiment (50μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.

Concentrated HRP Conjugate working solution: Calculate the required amount before the experiment (100μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100×Concentrated HRP Conjugate to 1×working solution with Concentrated HRP Conjugate Diluent.

Assay procedure (A brief assay procedure is on the 11th page)

Add the Standard working solution to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side(50 uL for each well). Add the samples to the other wells(50 uL

for each well). Immediately add 50 μL of Biotinylated Detection Ab working solution to each well. Cover the plate with the sealer provided in the kit. Incubate for 45 min at 37℃.Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

Aspirate or decant the solution from each well,add 350 uL of wash buffer to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.

Add 100 μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.

Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 2.

Add 90 μL of Substrate Reagent to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.

Add 50 μL of Stop Solution to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.

Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

Calculation of results

Average the duplicate readings for each standard and samples. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample is under the lowest limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

Concentration(ng/mL)	20	10	5	2.5	1.25	0.62	0.31	0
OD	0.269	0.545	0.740	1.006	1.299	1.608	1.919	2.426

Reference values

Samples from different species were evaluated for the presence of T in this assay.

Sample type	Reference range of T in different species(ng/mL)				
	Human	Female			
Rat	Female				
Mouse	Chicken	Porcine	Sheep	Cattle	

Serum(n=10)	1.43-9.69	4.97-6.58	0.36-0.62	4.61-8.45	0.75-1.99	0.94-2.72
0.85-1.00						
Plasma(EDTA)(n=10)	0.69-8.05	1.01-1.08	ND	1.51-2.53	0.30-0.97	0.77-3.73
0.83-1.44						
Urine(n=5)	3.88-21.05	-	-	-	-	-
Saliva(n=5)	0.84-1.67	-	-	-	-	-

The above values were all from normal healthy samples.

Precision

Intra-assay Precision (Precision within an assay): 3 human samples with low, mid range and high level

T were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 human samples with low, mid range and high level

T were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	
Mean(ng/mL)	1.33	5.22	10.88	1.54	5.73	10.02
Standard deviation	0.11	0.35	0.46	0.14	0.35	0.68
CV(%)	8.13	6.72	4.27	9.23	6.18	6.75

Recovery

The recovery of T spiked at three different levels in human samples throughout the range of the assay

was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum(n=5)	80-90	84
EDTA plasma(n=5)	82-94	87
Urine(n=5)	85-102	95

Linearity

Human Samples were spiked with high concentrations of T and diluted with Reference Standard &

Sample Diluent to produce samples with values within the range of the assay.

		Serum(n=5)	Plasma (EDTA)(n=5)	Urine (n=5)
1:2	Range (%)	83-90	88-108	85-101
	Average (%)	85	96	93
1:4	Range (%)	80-95	87-95	80-102
	Average (%)	88	89	92
1:8	Range (%)	89-100	82-89	83-96
	Average (%)	93	86	88
1:16	Range (%)	87-115	87-105	84-99
	Average (%)	97	98	87

Troubleshooting

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by gentle mixing.
	Wells are not completely aspirated	Completely aspirate wells in between steps.
Low signal	Insufficient incubation time	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Checkpipettes and ensure correct **preparation**.
	Improper dilution	
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB,rapid coloring.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting onthe Microplate reader.
	Open the Microplate Reader ahead to pre-heat.	
Large CV	Inaccurate pipetting	Check pipettes.
High		

background	Concentration of target protein is too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution is not added	Stop solution should be added to each well before measurement.

SUMMARY

1. Add 50 μL standard or sample to each well. Immediately add 50 μL Biotinylated Detection Ab to each well. Incubate for 45 min at 37℃

Aspirate and wash 3 times

Add 100 μL HRP Conjugate to each well. Incubate for 30 min at 37℃

Aspirate and wash 5 times

Add 90 μL Substrate Reagent. Incubate 15 min at 37℃

Add 50 μL Stop Solution. Read at 450nm immediately.

Calculation of results.

Declaration

Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.

The final experimental results will be closely related to the validity of products, operational skills of the operators and the experimental environments. Please make sure that sufficient samples are available.

To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions!

Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.

Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.

Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from the above reasons, too.

Appendix IV

Determination of serum Reduced Glutathione concentration

Reduced glutathione was measured following the method of Sedlak and Lindsay (1968). First, 3.0 mL precipitating solution containing metaphosphoric acid, Na2EDTA and NaCl was added to 2.0 mL of the sample. The mixture was centrifuged at 4500 × g for 10 min. 1.0 mL of supernatant was added to 4.0 mL of 0.3 M Na2HPO4 solution and 0.5 mM DTNB (5,5 -dithiobis-2-nitrobenzoic acid) was then added to this

solution. Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was calculated as nmol GSH/mg protein in the tissues and mmol GSH/g Hb in whole blood using the reduced glutathione as a reference (hemoglobin levels were estimated in whole blood using the Drabkins' solution)

Appendix V

Determination of serum concentration of (cat)

Procedure

Catalase activity was assessed by incubating the enzymes ample in 1.0 ml substrate (65 mmol/ml hydrogen peroxide in 60 mmol/l sodium–potassium phosphatebuffer,pH7.4)at37 °C for three minutes. There action was stopped with ammonium molybdate. Absorbance of the yellow

complex of molybdate and hydrogen peroxide is measured at374nm against the blank.

Reagents

1. Sodium, potassium phosphate buffer (50mM,pH7.4): this buffer isprepared by dissolving 1.1g of Na2HPO4 and 0.27g of KH2PO4 in 100ml distilled water.

2. H2O2 (20 mM) in 50mmol/l sodium, potassium phosphate buffer: this solution is freshly diluted and standardized daily using a molar extinction coefficient of 43.6M_1 cm_1 at 240nm.

3. Ammonium molybdate (32.4mmol/l).

3. Calculation The rate constant of a first-order reaction (k) equation is used to determine catalase activity:

t: time.

S°: absorbance of standard tube

S: absorbance of test tube.

M: absorbance of control test (correction factor).

Vt: total volume of reagents in test tube. Vs: volume of serum.

Appendix VI

Determination of serum concentration of (sod)

SOD activity determination

Preparation

1. Tris buffer (pH 8.0): was prepared by dissolving 0.258 gm of tris and 0.111 gm of Ethylenediaminetetraacetic acid (EDTA) in dH2O and completing the volume to 100 ml.

2. Pyragallol solution (0.2 mM): was prepared by dissolving 0.0252 gm of pyragallol with 10 ml of HCl and completing the volume to 100 ml with dH2O.

Procedure

According to Marklund and Marklund (1974), reaction mix is consisting of 50 µl crude enzyme extract with 2 ml of tris buffer and 0.5 ml of pyragallol (0.2 mM) which absorbs light at 420 nm. Control solution contains the same materials except for the enzyme extract that was replaced by dH2O. As a blank, dH2O was used. Single unit of enzyme is defined as the amount of enzyme that is capable of inhibiting 50% of pyragallol oxidation. SOD activity was calculated using the following equation (Ma et al., 2009) :

SOD activity (u/ml)= (Vp-Vs)/(Vp*0.5)*(Vt/Vs)*n

Vp=Auto oxidation rate of pyrogallol rate of pyrogallol (control)

Vs= Auto oxidation rate of sample (with enzyme)

Vt=Total reaction volume (ml)

Vs= volume of enzyme used for the assay (ml)

n= dilution fold of the SOD sample

0.5= factor for 50% inhibition

3.5. Determination of Serum Lipid profile:

Appendix VII

EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix

Cat. No. AE311

Storage: at -20°C for two years

Description

Unique genomic DNA remover is combined with *EasyScript*® First- Strand cDNA Synthesis SuperMix to achieve simultaneous

genomic DNA removal and cDNA synthesis. After cDNA synthesis, gDNA remover and reverse transcriptase are inactivated by heating at 85°C for 5 seconds.

Hightlights

- Simultaneous genomic DNA removal and cDNA synthesis in one tube to minimize RNA contamination.
- The product obtained from 15 minutes reaction is used for qPCR; the product obtained from 30 minutes reaction is used for PCR.
- cDNA up to 8 kb.

Applications

Component	AE3l1-02 (50 rxns)	AE311-03 (100 rxns)	AE311-04 (500 rxns)
EasyScript® RT/RI Enzyme Mix	50 µI	100 µI	5x100 µI
gDNA Remover	50 µl	100 µl	5x100 µl
2xES Reaction Mix	500 µl	!ml	5x) ml
Random Primer(N9) (0.1 µg/µl)	50 µl	100 µl	5xl00µl
Anchored Oligo(dT)$_{18}$ Primer (0.5 µg/µl)	50 µI	100 µI	5x100 µI
RNase-free Water	500 µI	1 ml	5ml

Multiple
copy
gene
detection
Kit
Contents

First-Strand cDNA synthesis

1. Reaction Components

Component	Volume
Total RNA/mRNA	0.1 ng-5 µg/10 pg-500 ng
Anchored Oligo(d T)$_{18}$ Primer (0.5 µg/µI)	I µl
or Random Primer (0.1 µg/µl)	l µI
orGSP	2pmol
2xES Reaction Mix	10 µI
EasyScript® RT/RI Enzyme Mix	1 µI
gDNA Remover	I µl
RNase-free Water	to 20 µl

Optional: for higher efficiency, suggest to mix RNA, primer and water first. Incubate the mixture at 65°C for 5 minutes, on ice for 2 minutes. Then add other components.

2. Incubation

- For anchored oligo(dT)$_{18}$ primer or GSP, incubate at 42°C for 15 minutes (for qPCR) or incubate at 42°C for 30 minutes (for PCR).
- For random primer, incubate at 25°C for 10 minutes. After that, incubate at 42°C for 15 minutes (for qPCR) or incubate at 42°C for 30 minutes (for PCR).

3. Incubate at 85°C for 5 seconds to inactivate enzymes.

RT-PCR

Reaction Components

Component	Volume	Final Concentration
Template	Variable	as required
Forward Primer (10 μM)	1 μl	0.2 μM
Reverse Primer (10 μM)	1 μl	0.2 μM
10×*TransTaq*® HiFi Buffer II	5 μl	1×
2.5 mM dNTPs	4 μl	0.2 mM
TransTaq® HiFi DNA Polymerase	0.5-1 μl	2.5-5 units
Nuclease-free Water	Variable	-
Total volume	50 μl	-

Thermal cycling conditions

94°C	2-5 min	
94°C	30 sec	30-35 cycles
50-60°C	30 sec	
72°C	1-2 kb/min	
72°C	5-10 min	

FOR RESEARCH USE ONLY

Appendix VIII

The BEST for Life Science

TransStart® Green qPCR SuperMix

Cat. No. AQ101
Storage: at -20°C in dark for two years

Description

TransStart® Green qPCR SuperMix is a ready-to-use qPCR cocktail containing all components, except primer and template. It contains *TransStart*® *Taq* DNA Polymerase, SYBR Green I, dNTPs, PCR enhancer and stabilizer. qPCR SuperMix is provided at 2× concentration and can be used at 1× concentration by adding template, primer, passive reference dye (optional) and Nuclease-free Water.

Highlights

- *TransStart*® *Taq* DNA Polymerase, hot start with double blocking technique, improves sensitivity, enhances specificity and generates more accurate data.
- Double cation (K^+, NH_4^+) buffer enhances specificity and reduces primer-dimer formation.
- Passive reference dyes are provided for different qPCR instruments.

Passive Reference Dye

- Passive Reference Dye I (50×)
  ABI Prism® 7000/7300/7700/7900, ABI Step One®, ABI Step One Plus®
- Passive Reference Dye II (50×)
  ABI Prism® 7500, ABI Prism® 7500 Fast, ABI Q6, ABI QuantStudio® 6/7 Flex, ABI ViiA® 7, Stratagene Mx3000® /Mx3005P®, Qiagen Corbett Rotor-Gene® 3000
- No Passive Reference Dye
  Roche LightCycler® 480, Roche Light Cycler® 96, MJ Research Chromo4®, MJ Research Opticon® 2, Takara TP-800®, Bio-Rad iCycler iQ®, Bio-Rad iCycler iQ5®, Bio-Rad CFX96®, Bio-Rad C1000® Thermal Cycler, Thermo Scientific Pikoreal® 96, Qiagen Corbett Rotor- Gene® 6000, Qiagen Corbett Rotor-Gene® G, Qiagen Corbett Rotor-Gene® Q

Kit Contents

Component	AQ101-01	AQ101-02	AQ101-03
TransStart® Green qPCR SuperMix (2×)	1 ml	5×1 ml	15×1 ml
Passive Reference Dye (50×)	40 µl	200 µl	600 µl
Nuclease-free Water	1 ml	5 ml	3×5 ml

Reaction Components (20 µl)

Component	Volume	Final Concentration
Template	Variable	as required
Forward Primer (10 µM)	0.4 µl	0.2 µM
Reverse Primer (10 µM)	0.4 µl	0.2 µM
2×*TransStart*® Green qPCR SuperMix	10 µl	1×
Passive Reference Dye (50×) (optional)	0.4 µl	1×
Nuclease-free Water	Variable	-
Total Volume	20 µl	-

For genomic DNA, we suggest using 1 pg-1 µg template; for plasmid DNA, we suggest using 10-10^7 copies.

The BEST for Life Science

Thermal cycling conditions (three-step)

94°C 30 sec
94°C 5 sec
50-60°C 15 sec* } 40-45 cycles
72°C 10 sec*
Dissociation Stage

Thermal cycling conditions (two-step)

94°C 30 sec
94°C 5 sec
60°C 30 sec* } 40-45 cycles
Dissociation Stage

Fluorescent signals can be collected during the annealing or extension stage. For ABI qPCR instrument, we suggest using the following signal collecting time:

* For ABI Prism® 7700/7900, the time to 30 seconds.
* For ABI Prism® 7000/7300, the time to 31 seconds.
* For ABI Prism® 7500, the time to 34 seconds.
* For ABI ViiA® 7, the time is at least 19 seconds.

Two-step qPCR is more suitable for higher specificity assay.
Three-step qPCR is more suitable for higher sensitivity assay.

Note

Completely thaw the contents in the tube and mix well before each use.

FOR RESEARCH USE ONLY

Website www.transgenbiotech.com
E-mail info@transgenbiotech.com
Customer Service +86-400-898-0321
Phone +86-10-57815027

Appendix IX

		house1 ct	gene1 ct		house2 ct	gene2 ct
	zo-1		Avg.	vimentin		
control	1	16.21	28.18	1	31.04	32.64
control	2	16.52	29.62	2	24.64	27.6
control	3	16.71	27.41	3	24.65	29.71
control	4	17.35	30.94	4	22.93	28.91
busulfan	5	31.01	26.87	5	22.25	29.07
busulfan	6	29.32	26.53	6	28.81	31.12
busulfan	7	30.15	31.2	7	27.56	30.7
busulfan	8	29.91	24.66	8	20.72	24.57
bus+vite	9	23.73	27.08	9	24.34	27.81
bus+vite	10	24.28	28.46	10	25.29	31.35
bus+vite	11	25.34	23.31	11	20.43	24.32
bus+vit e	12	25.08	22.57	12	18.95	22.39
vit e	13	19.33	27.36	13	22.83	26.88
vit e	14	18.21	26.8	14	22.99	27.42
vit e	15	20.53	28.84	15	23.19	28.07
vit e	16	20.34	27.57	16	21.78	24.94

Vimetine gene expresion

	target	avarage	DELTA ct	DD ct	negative	folding	
control	32.64	32.64	-1.6	2.3	-2.3	0.203063	1.796736
control	27.6	27.6	-2.96	0.94	-0.94	0.521233	
control	29.71	29.71	-5.06	-1.16	1.16	2.234574	
control	28.91	28.91	-5.98	-2.08	2.08	4.228072	
busulfan	29.07	29.07	-3.76	0.14	-0.14	0.907519	0.699031

Appendix……..……...…..

busulfan	31.12	31.12	-2.31	1.59	-1.59	0.332171	
busulfan	30.7	30.7	-3.14	0.76	-0.76	0.590496	
busulfan	24.57	24.57	-3.85	0.05	-0.05	0.965936	
bus+vite	27.81	27.81	-3.47	0.43	-0.43	0.742262	1.732872
bus+vite	31.35	31.35	-6.06	-2.16	2.16	4.469149	
bus+vite	24.32	24.32	-3.89	0.01	-0.01	0.993092	
bus+vit e	22.39	22.39	-3.44	0.46	-0.46	0.726986	
vit e	26.88	26.88	-4.05	-0.15	0.15	1.109569	1.281176
vit e	27.42	27.42	-4.43	-0.53	0.53	1.443929	
vit e	28.07	28.07	-4.88	-0.98	0.98	1.972465	
vit e	24.94	24.94	-3.16	0.74	-0.74	0.598739	
avarage deelta ct control	-3.9						

Appendix……..……...…….

Zo-1 gene expresion

		house1 ct			gene1 ct						
	zo-1										
			Avg.		target	Avg.	ḊCT	DDCT	negative	folding	
control	1	25.67	25.67	control	28.18	28.18	-2.51	0.4075	-0.4075	0.753929	1.627161
control	2	25.65	25.65	control	29.62	29.62	-3.97	-1.0525	1.0525	2.074121	
control	3	25.45	25.45	control	27.41	27.41	-1.96	0.9575	-0.9575	0.514948	
control	4	26.36	26.36	control	30.94	30.94	-4.58	-1.6625	1.6625	3.165646	
busulfan	5	24.56	24.56	busulfan	26.87	26.87	-2.31	0.6075	-0.6075	0.656333	1.089094
busulfan	6	22.92	22.92	busulfan	26.53	26.53	-3.61	-0.6925	0.6925	1.616082	
busulfan	7	28.42	28.42	busulfan	31.2	31.2	-2.78	0.1375	-0.1375	0.909093	
busulfan	8	21.51	21.51	busulfan	24.66	24.66	-3.15	-0.2325	0.2325	1.174869	
bus+vite	9	27.04	27.04	bus+vite	27.08	27.08	-0.04	2.8775	-2.8775	0.136077	0.537052
bus+vite	10	26.62	26.62	bus+vite	28.46	28.46	-1.84	1.0775	-1.0775	0.473849	
bus+vite	11	21.79	21.79	bus+vite	23.31	23.31	-1.52	1.3975	-1.3975	0.379586	
bus+vit e	12	19.44	19.44	bus+vit e	22.57	22.57	-3.13	-0.2125	0.2125	1.158694	
vit e	13	23.45	23.45	vit e	27.36	27.36	-3.91	-0.9925	0.9925	1.98963	1.541172
vit e	14	23.58	23.58	vit e	26.8	26.8	-3.22	-0.3025	0.3025	1.23328	
vit e	15	25.48	25.48	vit e	28.84	28.84	-3.36	-0.4425	0.4425	1.358957	
vit e	16	23.99	23.99	vit e	27.57	27.57	-3.58	-0.6625	0.6625	1.582823	
				avg.Control	-3.255						

Appendix……..……...…….

الملخص

بوسولفان هو علاج كيماوي للسرطان ، بينما فيتامين هـ عبارة عن تفاعلات كيميائية كمضاد للأكسدة ضد الجذور الحرة في الجسم ويساعد في تقليل تأثير العلاج بأدوية العلاج الكيميائي مثل بوسلفان. 24 فأر ذكر اعمارهم 10 اسابيع . تتراوح اوزانهم بين 250 – 300 جرام تم تقسيمها إلى أربع مجموعات. المجموعة الأولى كمجموعة سيطره ، تلقت حقنة داخل الصفاق من ثنائي ميثيل سلفوكسيد (DMSO) يوميًا لمدة أربعة أسابيع (استخدم أيضًا كمذيب لمواد التجربة الأخرى) . تلقت المجموعة الثانية جرعة واحدة فقط من بوسلفان 40 ملغم / كغم من وزن الجسم \ داخل الصفاق . وتلقت المجموعة الثالثة جرعة واحدة فقط من بوسولفان 40 ملغم / كجم من وزن الجسم \داخل الصفاق . وفيتامين هـ بمقدار 100 ملغم / كجم من وزن الجسم \داخل الصفاق يوميًا لمدة 4 أسابيع. والمجموعة الرابعة تلقت فقط فيتامين هـ بنسبة 100 ملغم / كجم من وزن الجسم \داخل الصفاق يوميًا لمدة 4 أسابيع. بعد النهاية فترة 4 أسابيع تم التضحية بالحيوانات وتم جمع الدم للقياس الهرموني (LH-FSH-Testosteron) وقياس قيم انزيمات مضادات الاكسدة (GSH-SOD-CAT) وتم جمع البربخ من أجل تعداد الحيوانات المنوية وقياس التعبير الجيني لجيني (ZO-1\ vimentin) . واجراء الفحص النسيجي للخصية .

أظهرت النتائج أن بوسولفان يؤدي إلى زيادة معنوية في مستوى FSH و LH في الدم بينما انخفض مستوى التستوستيرون بشكل كبير. علاوة على ذلك ، تسبب بوسولفان في انخفاض كبير في عدد الحيوانات المنوية وحركتها وحيويتها وزيادة كبيرة في تشوه الحيوانات المنوية. من ناحية أخرى ، يؤدي تناول فيتامين هـ مع بوسولفان إلى تحسين مستويات الهرمون وكذلك نشاط الحيوانات المنوية.

أظهرت الدراسة النسيجية انخفاضاً معنوياً في قطر الأنابيب المنوية ، وكذلك حدوث تآكل في أنسجة الخصية في الحيوانات المحقونة بالبوسلفان (العلاج الكيميائي) مقارنة بالحيوانات في المجموعة الاولى .

ولكن عندما يتم حقن الحيوانات بفيتامين هـ ، نلاحظ تحسنًا في الأنسجة التالفة نتيجة تسمم العلاج الكيميائي.

نتيجة Vimentinو ZO-1كما اضهرت نتائج التعبير الجيني انخفاضا ملحوظا لجيني العلاج الكيميائي في المجموعة الثانيه مقارنتا مع المجموعة الاولى . وايضا نلاحظ تحسن ملحوظ في التعبير الجيني في المجموعة الثالثة نتيجة اعطاء فيتامين هـ.

جمهوريه العراقيه

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء /كلية الطب البيطري

فرع الفسلجة والكيمياء الحياتية والادوية

التأثير المحسن لفيتامين هـ على الجهاز التناسلي والتعبير الجيني لجيني zo-1 والفيمنتين في البربخ لذكور الجرذان المعرضة للبوسولفان

رسالة مقدمة الى

مجلس كلية الطب البيطري جامعة كربلاء وهي جزء من متطلبات نيل-

درجة الماجستير في علوم الطب البيطري / الفسلجة

من قبل

محمد علي ناظم خداداد محمد

بكالوريوس طب وجراحة بيطرية

2018-2017

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