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Investigating the Effects of Serratiopeptidase and N-acetyl cysteine against Methotrexate - induced damages in Male Rats testes

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

Submitted by

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بسمِ اللهِ الرحمنِ الرحيمِ

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I hereby declare that this dissertation is my original work, except for equations and citations which have been fully acknowledged. I also confirm that it has not been previously, Submitted , nor is it currently being submitted , for any other degree at the University Of Kerbala or any other institution .

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Dedication

To the Messenger of Mercy, the Prophet Muhammad, "May Allah,s peace be upon him and his progeny..."

To my homeland, Iraq, which is bleeding with martyrs.

To my father

And my beloved mother, who encouraged me to be faithful

To my dreams and aspirations..

To my brothers and sisters for their encouragement and love.

To everyone who benefits from this work especially my superviseres Prof. Dr. Wafaa Kadhim Jasim and Prof. Dr. Rana Fadhil Mousa

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Journey .

Summary

The study explored the Effects of Methotrexate induced damages on the male reproductive system of rats and the potential protective role of Serratiopeptidase and N-acetyl cysteine . Thirty-five adult male albino rats were divided into five groups : Negative control group: Received normal saline daily for 56 day, Methotrexate group: Received Methotrexate 5 mg/kg body weight, daily by intraperitoneal injection for 56 day. N-Acetylcysteine group : Received Methotrexate 5 mg/kg body weight, daily by intraperitoneal injection and N-Acetylcysteine 600mg/kg body weight orally , daily for 56 day. Serratiopeptidase group : : Received Methotrexate 5 mg/kg body weight, daily by intraperitoneal injection Serratiopeptidase 5mg/kg body weight orally , daily for 56 day. NAC+SER group: Received Methotrexate 5 mg/kg body weight, daily by intraperitoneal injection and N-Acetylcysteine 600mg/kg body weight orally , daily for 56 day + Serratiopeptidase 5mg/kg body weight orally , daily for 56 day. Rats were given a chloroform anesthetic to make them unconscious, and then blood samples were taken from the heart `to examine Oxidative stress biomarkers include the reduced glutathione(GSH), Malondialdehyde (MDA) and total antioxidant capacity (TAOC) , The reproductive hormone levels :Testosterone , follicle-stimulating hormone (FSH) and luteinizing hormone (LH) . and dissected animals to get the Testicular and epididymis tissues for histological investigation and sperm analysis and of Nuclear factor erythroid 2-related factor 2 (Nrf2). The results of experimental Shaw the Methotrexate toxicity significantly disrupted testicular function, causing a marked decline in testosterone and LH while increase in FSH levels. It also led to elevated levels of oxidative stress markers such as malondialdehyde (MDA), while the essential antioxidant enzymes including SOD show increase and decrease in glutathione (GSH) , also Sperm count, motility, morphology, and viability were all significantly impaired Histologically, MTX induced extensive damage in testicular tissue including necrosis, vascular congestion, and architectural disintegration of seminiferous tubules. The Efficacy of N-acetylcysteine and Serratiopeptidase treatment markedly improved hormonal profiles and antioxidant levels. Its function as a precursor to glutathione, N-acetylcysteine improve the sperm viability, motility, and histological structure by scavenging reactive oxygen species and enhancing cellular antioxidant capacity. And the Synergistic Effect of N-acetylcysteine with Serratiopeptidase demonstrated a synergistic protective effect, outperforming each agent used alone. It led to significant normalization of hormonal levels,

enhancement of antioxidant defense (including GSH and TAOC), and maintenance of normal testicular tissue.

Conclusions: The results provide strong evidence that NAC, especially when combined with SER, offers substantial protection against such damage, making this combination a promising approach for mitigating chemotherapy-related reproductive toxicity.

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List of Abbreviations

Abbreviations	Meaning
4-HNE	4 hydroxynonenal
ABP	androgen-binding protein
ANOVA	analysis of variance
AREs	antioxidant response elements
ATP	Adenosine triphosphate
BBB	blood-brain barrier
Cmax	maximal plasma concentration
DHFR	dihydrofolate reductase
ELISA	Enzyme-linked immunosorbent assay
ETC	electron transport chain

FDA	Food and drug Administration
FSH	Follicular Stimulating Hormone .
GI	gastrointestinal
GnRH	gonadotropin-releasing hormone
GSH	Glutathione
H2O2	hydrogen peroxide
HPG axis	hypothalamic-pituitary-gonadal
IPA	Institute for Public Accuracy
Keap1	Kelch-like ECH-associated protein
Kg	Kilogram
LH	Luteinizing Hormone .
LH-AB	Anti-luteinizig hormone antibody
MDA	Malondialdehyde .
Mg	Milligram
Mg/dl	Milligrams per deciliter
Mg/kg	Milligram per kilogram
MMP	Matrix metalloproteinase
MTX	Methotrexate
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
Nrf2	Nuclear factor erythroid 2-related factor 2
NSAID	nonsteroidal anti-inflammatory drugs
$O2^-$	superoxide anion
OS	Oxidative stress
PBS	phosphate buffered saline

PCR	Polymerase chain reaction
RA	rheumatoid arthritis
ROS	Reactive oxygen species
SER	Serratiopeptidase
SOD	Superoxide Dismutase.
T	Testosterone
TAC	total antioxidant capacity
TBA	Thiobarbituric Acid
Trolox	-tetramethyl-chroman-2-carboxylic acid
WHO	World Health Organization
XOD	xanthine oxidase

Chapter One
Introduction

Introduction

The creation of viable sperm is the result of the intricate interaction of several physiological processes in the male reproductive system. The highly controlled process of spermatogenesis, which takes place in the testes, turns spermatogonia into mature spermatozoa (**Santiago *et al*., 2020).**

This process can be hampered by a number of things, which could result in fewer or lower-quality sperm. These variables include lifestyle decisions, environmental exposures, hormone imbalances, and genetic abnormalities. Sperm production, motility, morphology, and eventually fertility can all be strongly impacted by each of these factors **(Kumar and Singh2022**).

Male factor infertility is on the rise and is acknowledged to be a major contributor to reproductive health and illness. Environmental exposures seem to be the main cause of the deterioration in seminal parameters and the increased male factor infertility **(Babakhanzadeh *et a*l., 2020**). This covers a wide range of toxins, endocrine disruptors, unhealthy eating habits, alcohol, tobacco, oxidation, and stress. Numerous environmental toxicants have been shown to directly lower sperm counts, induce testis illness, and cause male infertility in animal models (**Cano *et al*., 2021**).

A major contributing factor to male infertility is oxidative stress (OS), which upsets the equilibrium between antioxidants and reactive oxygen species (ROS**) (Barati *et al*., 2020)**.

Fertility is eventually hampered by this imbalance, which negatively impacts sperm viability and function. Further impairing sperm function and possible conception, OS also causes molecular abnormalities in sperm, such as DNA damage, lipid peroxidation, and changes in protein expression **(Barradas *et al*., 2021).** An overabundance of ROS damages spermatogenic, Leydig, and Sertoli cells and impairs a number of mature sperm activities. Additionally, polyunsaturated fatty acids' (PUFAs') double bonds can be broken by ROS **(Chakraborty and Roychoudhury, 2022)**.

Because testicular tissue has a very high rate of cell division and mitochondrial oxygen consumption, as well as relatively greater quantities of unsaturated fatty acids than other tissues, oxidative stress plays a significant role in the development of male infertility **(Ritchie and Ko, 2021).**

The maturation of spermatocytes, sperm motility, and sperm quality can all be hampered by oxidative stress. Increased rates of sperm apoptosis, lower sperm count, and poorer motility have all been associated with elevated ROS levels. Additionally, oxidative stress has been linked to a number of reproductive diseases, including infertility and testicular failure **(Chianese and Pierantoni, 2021).**

Methotrexate (MTX) is a commonly used chemotherapeutic and immunosuppressive drug, mainly prescribed for treating different types of cancer, autoimmune disorders, and inflammatory diseases. While MTX is very effective for treating these disorders, its application is frequently linked to various side effects affecting multiple organs, including the reproductive system. In clinical settings, one of the most important worries is its possible effects on male fertility, especially regarding how it affects testicular function and spermatogenesis **(Koźmiński *et al*., 2020)**.

The process of sperm production in the testes, known as spermatogenesis, is a biological process that is highly regulated and sensitive. It can be disrupted by various environmental and pharmacological factors. Recent research has underscored the harmful impact of MTX on male reproductive health, with multiple accounts suggesting that MTX can induce changes in the structure and function of the testes, disrupt spermatogenesis, and diminish sperm quality. These effects can result in infertility, which may have significant implications for those affected and their reproductive actions **(de Barros *et al*., 2025)**.

Antioxidants in testicular tissue boost general reproductive health, improve testosterone production, and preserve sperm quality. Antioxidants support healthy spermatogenesis, protect testicular cell integrity, and enhance fertility by lowering oxidative damage. Therefore, adding antioxidants to the diet or taking supplements may help people who are susceptible to testicular damage from oxidative stress **(Zhou *et al*., 2022).**

N-acetyl cysteine is one such antioxidant. Since NAC is a robust free radical scavenger, studies are being conducted to see whether it may be used orally to treat testicular damage. By modifying the expression of ROS, the N-acetyl derivative of the naturally occurring amino acid l-cysteine (**Tenório *et al*., 2021**).

NAC's capacity to function as a reduced glutathione (GSH) precursor accounts for its antioxidant activity; GSH is a well-known direct antioxidant and a substrate of multiple antioxidant enzymes **(Galicia *et al*., 2024).** Additionally, in some situations where endogenous Cys and GSH are significantly reduced, NAC can act as a direct antioxidant for oxidant species.NAC's antioxidant properties may potentially be influenced by its capacity to degrade thiolated proteins. Free thiols and reduced proteins are released during this process. which can have significant direct antioxidant activity in some situations (such as mercaptoalbumin**)(Altomare *et al*., 2020).**

Enzymes are essential for most metabolic processes and are either directly or indirectly required for the body to function normally. They are in charge of several physiological functions, such as respiration, immune response, digestion, metabolism, and reproduction (**Islam *et al*., 2023**) .

Enzymes, which can come from plant, animal, or microbial sources, are being used in clinical settings to manage and treat a variety of illnesses and disorders. Enzyme-based treatments have received increased attention lately due to their efficacy, safety record, and specificity **(Lombardero *et al*., 2023)**.

Serratiopeptidase, also known as serratiosin or serratia, is a proteolytic enzyme that is frequently utilized in therapeutic settings. It has demonstrated notable analgesic, anti-inflammatory, and anti-edema effects in addition to fibrinolytic qualities **(Jadhav *et al*., 2020).**

Serratiopeptidase contains zinc, It was first identified in silkworms as the non-pathogenic enterobacteria Serratia E15. The primary function of this enzyme is to reduce inflammation **(Mutzberg, 2021**), and its properties are more akin to those of an antioxidant. Because serratiopeptidase reduces oxidative damage, it may help maintain testicular health. A major cause of testicular dysfunction, which impairs spermatogenesis and causes problems with fertility, is oxidative stress **(Variyar and Premrajan, 2024) .**

Serratiopeptidase has fibrinolytic and anti-inflammatory properties that can improve testicular circulation and lessen edema. This enzyme may help shield testicular tissue from oxidative damage brought on by reactive oxygen species (ROS) by regulating inflammatory responses and encouraging tissue repair **(SESSION, 2021).**

Reduced sperm motility, sperm DNA damage, and consequent degradation of the embryonic genome are the outcomes of an imbalance between reactive oxygen species (ROS) levels and antioxidant defense ,numerous antioxidant genes are upregulated when oxidative stress occurs due to interactions between nuclear factor erythroid 2-related factor 2 (Nrf2) and the antioxidant response elements. **(Mottola *et al*., 2024).**

Erythroid 2-related factor 2 (Nrf2), a nuclear factor It is a master regulator of cellular responses to external stressors such oxidative damage, regulates the antioxidant response, and promotes cell division and proliferation **(He *et al*., 2020).**

Most eukaryotic cells expressed Nrf2 under physiological conditions, and it is responsible for inducing a wide spectrum of cellular defenses against both endogenous and foreign stressors, such as oxidants, Increased Nrf2 expression upregulates the genes encoding antioxidant enzymes to scavenge and control ROS levels **(Dia *et al*., 2020).**

Aims of the Study:

The current study attempted to investigate the antioxidant properties and protective effects of serratiopeptidase alone and when combined with N-acetyl cysteine against Methotrexate induced testicular damage in male rat

By measure the following parameters :

1- The sperm analysis sperm count and sperm motility).

2-The reproductive hormone levels :Testosterone , follicle-stimulating hormone (FSH) and luteinizing hormone (LH) .

3- Oxidative stress biomarkers : reduced glutathione(**GSH**), Malondialdehyde (**MDA**) and total antioxidant capacity **(TAOC)**

4-Expression levels of Nuclear factor erythroid 2-related factor 2 (**Nrf2).**

5-Histopathological changes in testes .

Chapter Two
Literature Review

2. Literature Review

2.1 Male Reproductive System

A complex structure of organs and processes, the male reproductive system is essential to human reproduction. In addition to producing, developing, and delivering sperm cells, it also synthesizes male hormones like testosterone, which have an impact on several physiological functions (**Yadav and Mali ,2024).** The primary function of the male reproductive system, a highly specialized and complex network of organs and structures, is the creation, maturation, and transport of sperm, which is necessary for human reproduction. This system, which is made up of both internal and exterior parts, such as the testes, epididymis, seminal vesicles, prostate gland, and external genitalia, functions in concert to guarantee that genetic material is successfully transferred during conception. The intricate interaction of hormones like testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which regulate spermatogenesis and sexual differentiation, is the main factor controlling the system's operation. **(Peate,2025).**

The reproductive organs on the exterior of the body are referred to as external genitalia , The male reproductive system is made up of numerous parts. These tissues are important for sexual reproduction and urine functions in both males and females (**Obukohwo *et al*.,2021).** The testes, epididymis, vas deferens, and prostate are the internal components of the male reproductive system, whereas the scrotum and penis are its outside components. With numerous glands and ducts, these structures are well-vascularized and facilitate the production of vital androgens for male development as well as the generation, storage, and ejaculation of sperm for conception **(Gurung *et al*.,2023**).

Penis is male copulatory organ made up of root of penis and body of penis. The male organ used for sexual activity is the penis. The portion of the penis that connects to your abdominal wall is called the root **(Ramirez-González, and**

Sansone, 2022). while The body or shaft it Shaped like a tube or cylinder, the body of the penis is made up of three internal chambers. Inside these chambers there's a particular, sponge-like erectile tissue that has millions of huge holes that fill with blood when you're sexually excitedd **(Кравець and Стойка ,2021) .**

Scrotum Testicles are a pouch of skin that surrounds and protects the testes. It aids in controlling testicular temperature, which is essential for sperm production. **(Samuel *et al* .,2025)** . The testicles and the lower portion of the spermatic cords are located in the scrotum, a suspended, two-chambered sac of muscle and skin. It is situated above the perineum and behind the penis. A little, vertical skin ridge that extends from the anus and passes through the center of the scrotum from front to back is called the perineal raphe. The testicular artery, testicular vein, and pampiniform plexus are among the abdominal tissues that are carried into the scrotum, which is also a distention of the perineum **(Carter *et al* .,2022)** .

The reproductive organs found inside the body are referred to as internal genitalia. Sexual reproduction, hormone synthesis, and the general operation of the reproductive system depend on these An essential component of the male reproductive system is the epididymis, a coiled tube found at the top of the testicle. It is in charge of the sperm cells made in the testes being stored, developed, and transported **(Peate,2025).** The testes make immature sperm cells, which are stored in the epididymis. It takes time for these sperm cells to develop inside the epididymis before they can fertilize an egg. Usually, this process takes two weeks. Sperm undergo modifications throughout this period that enable them to swim and fertilize an egg. The vas deferens, the tube that transports sperm to the urethra for ejaculation, receives the matured sperm from the epididymis **(Nordhoff and Wistuba,2023).**

Vas Deferens is A tube that transports mature sperm from the epididymis to the urethra during ejaculation. During ejaculation, sperm are transported from the epididymis to the urethra via the vas deferens, a pair of muscular tubes in the male reproductive system. Every testicle contains a vas deferens that travels to the prostate gland, where it unites with other tissues to form the ejaculatory duct, and the epididymis, where sperm develop **(Akhavizadegan *et al*.,2025).**

accessory gland refers to a type of gland that supports the primary function of the reproductive or digestive system by secreting fluids or other substances that aid in the physiological processes. In several organisms, accessory glands are present in both males and females **(Franjić ,2023) .** The seminal vesicles are one of these glands. A large amount of the seminal fluid, which is high in fructose and gives sperm cells energy, is produced by these glands **(Rathod *et al* .,2025).** Prostate Gland This gland secretes a fluid that makes up part of semen. Additionally, it aids in balancing the vagina's acidic environment, which promotes sperm survival **(Samuel *et al* .,2025).** and Bulbourethral Glands (Cowper's Glands) To prepare the urethra for sperm passage, these glands release a clear fluid that lubricates it and neutralizes any remnants of acidic urine **(GARCIA, 2023).**

2.2 Testes

The testes are male sex glands that have both an endocrine and exocrine function. The scrotal septum divides the oval-shaped testes, which are reproductive organs located in the scrotum. The testes have a bean-like form and are three centimeters long by five centimeters long by two to three centimeters wide. The testes are soft and smooth when felt via the scrotum. **(Hamed,2022).** The spermatic cord suspends the superior aspect of the testes. The scrotal ligament, the remains of the gubernaculum, connects the testes to the scrotum at

the inferior end. Generally speaking, the left testis is attached somewhat lower than the right **(Mulligan *et al*.,2023).**

The testes' lymphatic drainage and blood supply are essential to their healthy operation.The testicular arteries, which are branches of the abdominal aorta, provide blood to the testes. These arteries provide blood to the testes by passing via the inguinal canal **(Freeman and Parker,2022).** The Testicular Artery receives blood from two primary sources: This is the main blood supply that supplies the testes with oxygenated blood , Artery Cremasteric: The cremaster muscle and a portion of the scrotal sac are supplied by this branch of the inferior epigastric artery **(Kang *et al*.,2022).** The pampiniform plexus, a system of veins that encircles the testicular artery, controls the venous return from the testes. These veins combine to form the testicular vein, which empties into the left renal vein on the left side and the inferior vena cava on the right. **(Smith and Turek , 2024).**

The lymphatic drainage of the testes is important for removing waste and preventing infections. The lumbar lymph nodes, also known as para-aortic lymph nodes, are situated close to the aorta and receive the lymphatic veins from the testes. The scrotum, on the other hand, empties into the inguinal lymph nodes. These systems cooperate to maintain the healthy and normal operation of the testes because the lymphatic drainage from the testes follows the same route as the blood supply, and the drainage pattern is crucial clinically for comprehending the progression of testicular malignancies **(Mukenge et al.,2023).**

Both sperm and testosterone, which are essential for male fertility and the development and maintenance of several physiological processes, are produced by the testes. Endocrine hormones generated in the pituitary and brain, as well as locally inside the testis, control the synthesis of both products **(Das *et al*.,2023).**

Although follicle-stimulating hormone (FSH) and testosterone are both necessary for optimal testicular growth and maximum sperm production, testosterone is essential for sperm generation. **(Oduwole *et al*.,2021).**

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are released from the anterior pituitary gland and controlled by gonadotropin-releasing hormone (GnRH), which is generated by the brain, are significant hormones that influence these. These hormones work together to form the hypothalamic-pituitary-gonadal axis, which supports and sustains male sexual growth and function **(Victor et al.,2021).**

It is important that aromatase from adipose tissue can convert testosterone peripherally to estradiol. Peripherally, estradiol can be transformed into estrogen **(Bracht et al.,2020)**, In addition to having an inhibitory effect on the anterior pituitary and hypothalamus like testosterone, estradiol/estrogen can also have vascular effects, gynecomastia, bone resorption, and epiphyseal closure. Males can develop pathological changes such weak bones, breast growth, loss of libido, or infertility if their levels of estradiol rise **(Mandelli et al.,2022).**

Seminiferous tubules are crucial components of the male reproductive system, primarily located within the testes. Spermatogenesis, the process by which sperm cells are created, takes place in these structures. Understanding the architecture, cellular composition, and functional dynamics of seminiferous tubules is vital for grasping male fertility and associated diseases **(Houda *et al*.,2021).** At different stages of development, seminiferous tubules are densely coiled structures lined by a complex epithelium made up of leydig and Sertoli cells **(Tala'a and Hamdi,2023) .**Specialized endocrine cells called Leydig cells, often referred to as interstitial cells, are found in the testes between the seminiferous tubules. inhabit the testis' interstitial compartment, are the target of

luteinizing hormone, which promotes the generation of testosterone, and are essential for the early virilization and patterning of the male external genitalia **(Titi-Lartey,2020).**

An absolutely necessary component of male fertility, testosterone, which is produced by testicular Leydig cells, is necessary for the fetal differentiation of the male reproductive track, virilization of the male external genitalia, pubertal maturation of testicular Sertoli cells , followed by meiotic progression of male germ cells and spermiation, and regulation of sex drive libido **(Bhattacharya and Dey,2023)** Leydig cells influence spermatogenesis by promoting the growth of spermatogenic cells in the seminiferous tubules through the action of testosterone **(Ge *et al* .,2021).** Within the testes' seminiferous tubules are specialized somatic cells called sertoli cells, sometimes referred to as sustentacular cells. They are essential for spermatogenesis and the male reproductive system's general operation. **(Obukohwo *et al* .,2021)** Sertoli cells are the orchestrators of spermatogenesis and crucial for the development of germ cells, from the maintenance of the spermatogonial stem cell niche and spermatogonial populations through meiosis and spermiogeneis to the final discharge of mature spermatids during spermiation **(O'Donnell *et al* .,2021)** . Sertoli cells also have a major role in regulating androgen production within the testis, by specifying interstitial cells to a steroidogenic fate, contributing to androgen production in the fetal testis, and supporting fetal and adult Leydig cell development and function **(Xiao *et al* .,2025).**

2.3 Spermatogenesis

Is the process by which haploid spermatozoa develop from germ cells in the seminiferous tubules of the testicle. The process begins when the stem cells near the tubules' basement membrane divide mitotically**(Umoh, 2024)**. Is a highly proliferative and controlled developmental process that involves several germ cell

divisions to boost their quantity before they eventually differentiate into spermatozoa in the testes' seminiferous tubules. Humans generate 1000 spermatozoa with every heartbeat, and spermatogenesis begins at puberty. Spermatogonial stem cells in the basement of the seminiferous tubules sustain this massive spermatogenesis **(Nyaz*et al* .,2021)** .

The primary hormones needed for spermatogenesis are testosterone, follicular stimulating hormone, and luteinizing hormone **(Waqas, 2021).** Spermatogenesis starts in the bottom part of seminiferous tubes and, progressively, cells go deeper into tubes and moving along it until mature spermatozoa reaches the lumen, where mature spermatozoa are deposited. The division occurs asynchronously, and several maturation phases may be seen if the tube is sliced transversally. A spermatogenic wave is a collection of cells at various stages of maturation that are being produced simultaneously **(Khalimova, 2024).** Spermatogenesis multi-stage process, which includes spermatogonia growth, meiosis, and spermiogenesis, is essential to male fertility. Numerous genetic, hormonal, and environmental variables carefully control each stage. According to recent studies, oxidative stress plays a part in many facets of spermatogenesis and can have a major effect on the reproductive health of men **(Obukohwo *et al* .,2021) .**High rates of mitochondrial oxygen consumption by the germinal epithelium are implied by the high rates of cell division that are inherent in this process **(Vertika *et al* .,2020).**

2.3.1. Stages of Spermatogenesis

The spermatogenic cycle is superimposed on the major divisions of spermatogenesis (spermatocytogenesis, Spermatidogenesis, and spermiogenesis). When the release of spermatozoa from the seminiferous epithelium is taken as a reference point, the cycle of the seminiferous epithelium is defined as a sequence of changes in a specific section of the epithelium between two appearances of the same developmental stages **(Zakariah *et al* .,2024).** Spermatogenesis occurs in the seminiferous tubules of the testes and can be divided into several distinct phases Its details are shown in the figure (2.1) and explanation below.

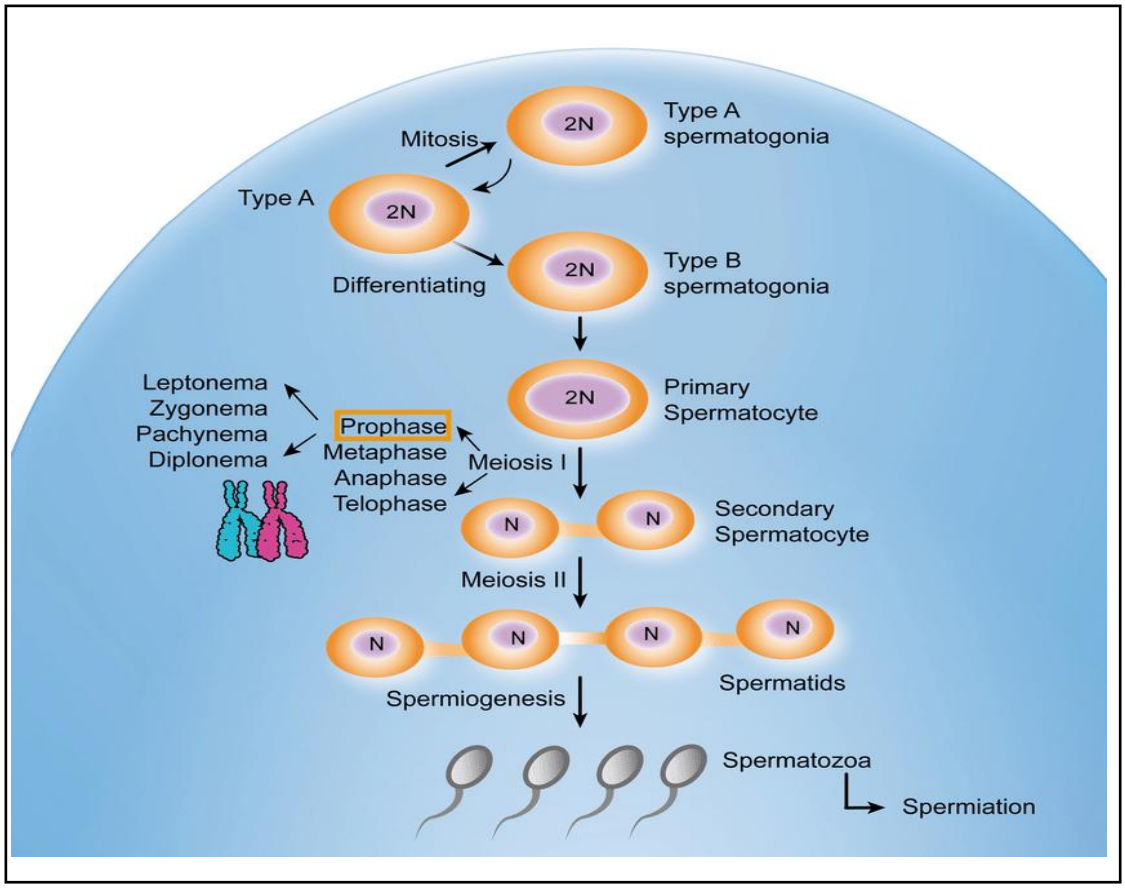


Figure (2.1) A schematic diagram of spermatogenesis(Gui, &Yuan,2021).

2.3.1.1. Spermatocytogenesis (Mitosis)

Spermatocytogenesis is the initial stage of spermatogenesis, which involves the division of spermatogonia through mitosis to produce primary and secondary spermatocytes. Maintaining a pool of stem cells and producing spermatogonia for subsequent proliferation and differentiation are its principal roles. entails stem cells proliferating to create a population of cells that will eventually develop into

sperm and to replace themselves **(Talapatra, 2021).** which occurs in the seminiferous tubules of the testes. Spermatogonial cells divide and differentiate into mature spermatozoa in this process. Spermatogonia, the stem cells that go through mitosis to become primary spermatocytes, are the first stage of the process. Following meiosis, these main spermatocytes divide into secondary spermatocytes, which in turn split into spermatids. Spermatids eventually develop into spermatozoa **(Umoh, 2024).**

2.3.1.2. Spermatidogenesis (Meiosis)

Is the creation of spermatids from secondary spermatocytes during spermatogenesis , process by which male gametes (sperm cells) develop during spermatogenesis, the process by which spermatids are created. After meiosis, which cuts the number of chromosomes in half, this process takes place in the testes' seminiferous tubules. Secondary spermatocytes initiate spermatidogenesis by undergoing meiosis II, which results in the production of haploid spermatids. Known as spermiogenesis, these non-motile spermatids go through additional development to become fully functioning sperm cells that can fertilize an egg. The development of a tail for movement, nucleus condensation, and the creation of the acrosome which facilitates penetration of the egg during fertilization are all examples of this metamorphosis **(Wang *et al* .,2022).**

2.3.1.3. Spermiogenesis

is the last phase of spermatogenesis, during which immature spermatids evolve structurally and morphologically to become mature, motile sperm cells. Round spermatids are changed into elongated spermatozoa during this process, which occurs in the testes' seminiferous tubules. Important alterations include the creation of the acrosome, a cap-like structure that aids sperm penetration of the egg, the condensation of the nucleus to create a streamlined head, and the production of a tail (flagellum) for movement. Furthermore, extra cytoplasm is

eliminated, and the mitochondria assemble around the tail to supply movement energy. When finished, the sperm are discharged into the seminiferous tubule lumen, where they will mature in the epididymis **(Khalimova,2024).**

From the first division of spermatogonia to the development of mature spermatozoa, spermatogenesis in rats normally takes 52–64 days. Hormones like testosterone and follicle-stimulating hormone (FSH) control this process, which takes place in the testes' seminiferous tubules. The epididymis stores spermatozoa for later maturation and ejaculation following spermatogenesis**(Wistuba *et al* .,2023).**

2.3.2. Role of Sertoli cells in Spermatogenesis

At all stages of differentiation Sertoli cells, which are believed to give the developing sperm cells structural and metabolic support, are in close contact with spermatogenic cells throughout the entire differentiation process. Although it is challenging to discern the cytoplasmic processes at the light microscopic level, a single Sertoli cell stretches from the basement membrane to the lumen of the seminiferous tubule **(Shah *et al* .,2021).** Sertoli cells play a crucial role in spermatogenesis, the process by which sperm are produced in the testes. These cells, also referred to as "nurse cells," nurture and govern the growth of sperm from spermatogonia to mature spermatozoa **(Rotimi *et al* .,2024).**Sertoli cells provide structural support to the developing germ cells by forming a physical scaffold in the seminiferous tubules, where spermatogenesis occurs. They surround and "nurse" the developing sperm cells through different stages of their maturation also form tight junctions between themselves to creating the blood-testis barrier. This barrier aids in maintaining a specific milieu for spermatogenesis by separating the growing germ cells from the blood supply **(Xiao *et al* .,2025)** . Additionally, it stops the blood's immune cells from attacking

the sperm, which supply nutrition, ions, and growth factors to the germ cells throughout development and include antigens that the immune system may perceive as alien. They contribute to the release of chemicals such as androgen-binding protein (ABP), which aids in the concentration of testosterone in the seminiferous tubules—a vital part of spermatogenesis **(Teves and Roldan ,2022).**

Hormones, especially FSH and testosterone, have an effect on sertoli cells and control their activity. While testosterone, which is generated by Leydig cells, collaborates with Sertoli cells to assist sperm synthesis, FSH stimulates Sertoli cells to boost spermatogenesis. Certain germ cells may experience apoptosis, or programmed cell death, during spermatogenesis. Sertoli cells engulf and remove these dying cells through phagocytosis, ensuring that only viable sperm are produced **(Osadchuk and Osadchuk,2023).**

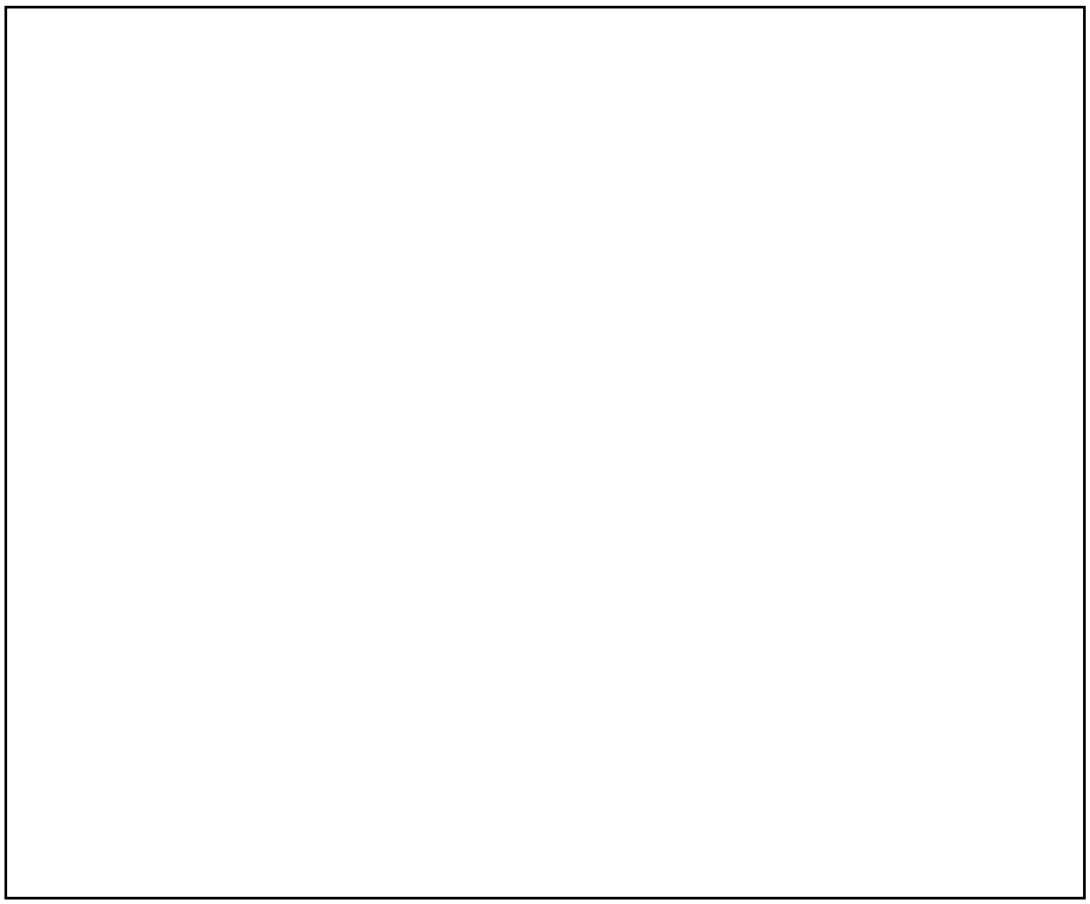
2.3.3. Influencing factors of Spermatogenesis

Many factor can Influence in Spermatogenesis especially the environment and genetic factor, Temperature and hormone changes in particular have a significant impact on the spermatogenesis process. Testosterone is required in large local concentrations to maintain the process, which is achieved via the binding of testosterone by androgen binding protein present in the seminiferous tubules **(Duan *et al* .,2024).** Interstitial cells, sometimes referred to as Leydig cells, are found next to the seminiferous tubules and manufacture testosterone. In humans and some other species, seminiferous epithelium is sensitive to high temperatures and will suffer from conditions as high as body temperature **(Batra *et al* .,2024).** The scrotum, where the testes are situated outside the body, keeps the temperature there two to three degrees Celsius below body temperature, which is necessary for healthy spermatogenesis. High temperatures (due to fever, tight clothing, or prolonged sitting) can impair sperm production **(Walker,2021).**

Deficits in minerals (zinc, selenium), vitamins (particularly C, E, and A), anabolic steroids, and metals. Alcohol, viral infections, dioxin, and x-ray exposure can all have an impact on spermatogenesis. Antioxidants are essential for preserving the health of sperm **(Assidi,2022).**

2.3.4. Hormonal control of spermatogenesis

Spermatogenesis is a highly regulated physiological process that occurs in the testes. The central nervous system, mainly through the hypothalamus and pituitary gland, as well as the testes themselves are involved in this complex hormonal interaction that ensures the healthy growth of sperm cells. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), gonadotropin-releasing hormone (GnRH), testosterone, and inhibin are the hormones that are involved in this process **(Li *et al* .,2024).Fig (2.2)**



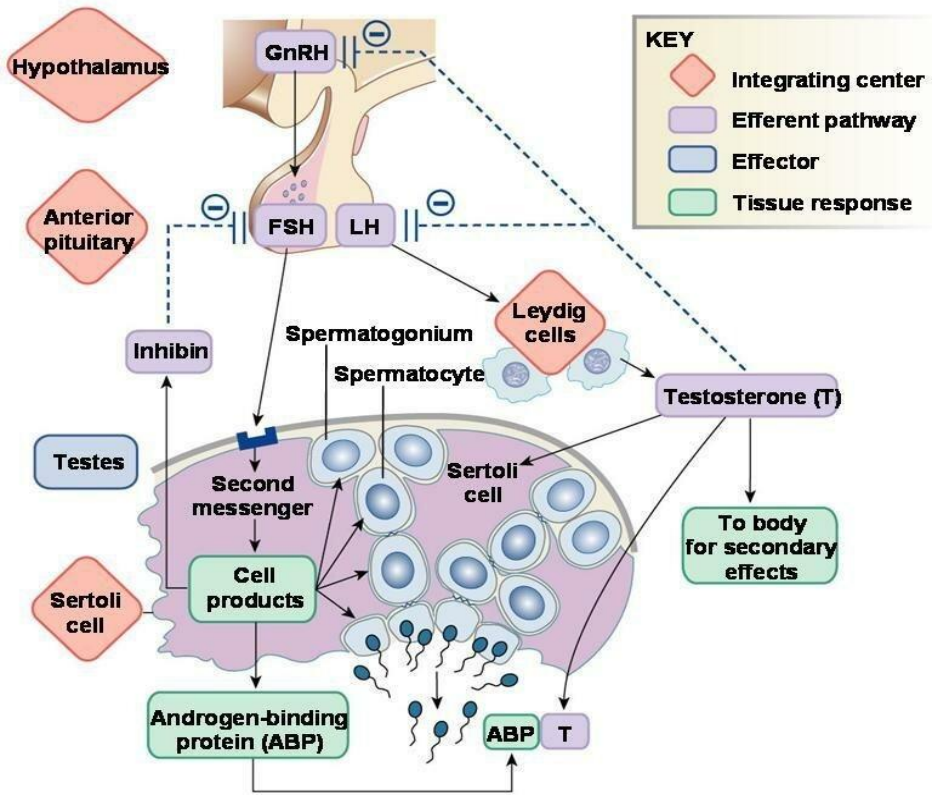


Fig.(2.2) Hormonal regulation of spermatogenesis (Plant *et al* .,2015).

The Hormonal control of spermatogenesis begins in the hypothalamus, which secretes Gonadotropin-Releasing Hormone (GnRH).

GnRH is released in a pulsatile manner, stimulating the anterior pituitary gland to secrete two important gonadotropins LH and FSH **(Marques *et al* .,2024).** LH contributes to spermatogenesis by inducing the production of testosterone by the testes' Leydig cells. The primary androgen hormone that propels the spermatogenesis process is testosterone. While FSH targets the Sertoli cells in the seminiferous tubules, it also aids in the maintenance of secondary sexual characteristics and helps control the actions of Sertoli cells **(Talebi *et al* .,2025).** The Sertoli cells "nurse" cells that provide the growing sperm cells with nutrition, structural support, and a blood-testis barrier are called sertoli cells. The function of Sertoli cells is improved by FSH, which aids in the maturation of spermatocytes into spermatozoa. Together with testosterone, FSH is essential for the development of spermatogenesis **(Al-Suhaimi and Khan,2022).** In the process of

spermatogenesis, testosterone have two functions. It is essential for sperm cell maturation and works together with FSH to control the activity of Sertoli cells. Moreover, testosterone has a negative feedback effect on the hypothalamus and pituitary gland, which helps regulate GnRH, LH, and FSH levels and keeps sperm production within normal limits **(Wistuba *et al* .,2023).**

Inhibin, a hormone that provides negative feedback specifically on FSH, is also secreted by Sertoli cells. When there is adequate sperm production, the levels of inhibin rise, which sends a signal to the pituitary gland to lower FSH secretion while If sperm production is low or insufficient, inhibin secretion decreases, which allows for higher FSH levels to stimulate the Sertoli cells and promote more spermatogenesis This feedback is useful in preventing the overstimulation of spermatogenesis **(Howard,2021).**

2.4. Methotrexate (MTX)

A folic acid antimetabolite(fig 2-3), is a chemotherapeutic agent widely used in broad spectrum treatment of malignant and non-malignant diseases, due to its antitumoural, anti-inflammatory, antimicrobial and immunosuppressive properties **(Guo *et al* .,2023).** MTX-induced cytotoxicity during the active stages of cell division. Therefore, in addition to cancer cells, it is toxic to bone marrow, gastrointestinal mucosa, hair follicles, and spermatogenic cells with a high potential for proliferation. **(Ingole *et al* .,2024).The following figure shows its chemical composition.**

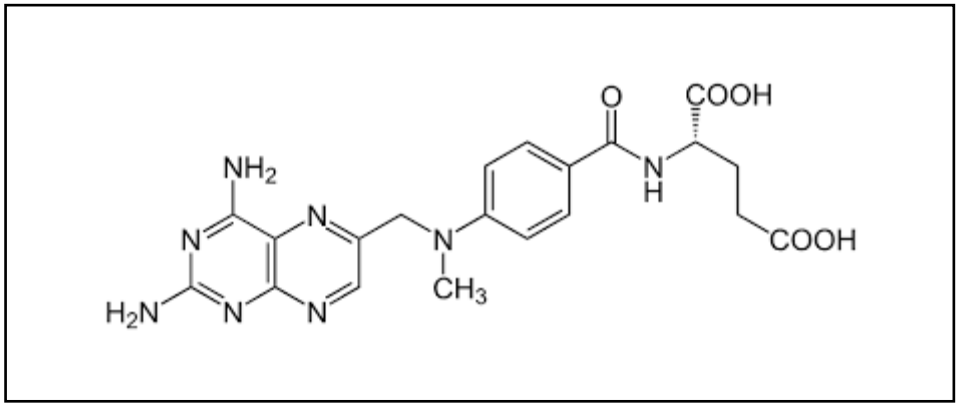


Figure (2.3) Methotrexate structure (Lotfi *et al*.,2016).

Spermatogenic cells' toxicity to the testis, where they are formed, is significant since it may result in gonadal dysfunction and infertility. As a result, it has been shown that MTX-induced testicular toxicity rapidly causes sperm count to drop and abnormal sperm shape to rise. It has also been shown to cause decreased levels of follicle-stimulating hormone FSH, testosterone, luteinizing hormone (LH), and testicular weight. Additionally, it causes testicular degeneration **(Sun *et al* .,2023).**

There are several ways to deliver methotrexate, and the dosage might vary greatly. It may be given subcutaneously, intravenously, intramuscularly, or orally. **(Rubio-Romero *et al* .,2024).** Its bioavailability ranges from 64 to 90% however, at oral dosages beyond 25 mg, this falls because the carrier-mediated transport of methotrexate becomes saturated **(Ali *et al* .,2024).**

Although to a limited degree, methotrexate can pass the blood-brain barrier (BBB) and enter several tissues, such as the liver, kidneys, and lungs **(Koźmiński *et al* .,2020).** Additionally, in the liver and tissues, folylpolyglutamate synthase converts it to methotrexate polyglutamate.1, 7 The glutamyl chains of methotrexate polyglutamates are hydrolyzed by gamma-glutamyl hydrolase, which then transforms them back into methotrexate.1, 7 Additionally, a little quantity of methotrexate is changed into 7-hydroxymethotrexate **(WOO *et al* .,1999).**Methotrexate is excreted predominantly by the kidneys. Methotrexate is >80% excreted as the unchanged drug and approximately 3% as the 7-hydroxylated metabolite. elimination half-life varies significantly (around 3 to 10 hours for low doses; can be longer for high-dose regimens) **(Zhang *et al* .,2022).**

2.4.1. Mechanism Action of Methotrexate

Methotrexate (MTX) is an antimetabolite and folate antagonist that functions by blocking a number of enzymes necessary for cell division and DNA creation. It works by preventing the conversion of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR). Purines and pyrimidines are vital building blocks for the synthesis of DNA and RNA, and tetrahydrofolate is a crucial component needed for their creation **(Wu *et al* .,2021).** and by Methotrexate decreases tetrahydrofolate availability by blocking DHFR, which impairs DNA and RNA synthesis. Because this impact is more noticeable in cells that divide quickly, such immune or cancer cells, methotrexate is used to treat autoimmune illnesses and some types of cancer **(Riaz *et al* .,2020).**

further suppresses cell proliferation by influencing the synthesis of purines and pyrimidines, which are the building blocks of nucleic acids. Because of this, methotrexate works well for diseases like cancer and rheumatoid arthritis that involve excessive cell proliferation **(Katturajan *et al* .,2021)** and Methotrexate's effect on cell division also inhibits the immune system in autoimmune illnesses (such as psoriasis and rheumatoid arthritis), especially by preventing T cells from functioning and reducing cytokine production. When the body's tissues are attacked by the immune system, this immunosuppressive effect can be helpful. **(Efferth and Oesch ,2021).**Methotrexate inhibits the metabolism of folate, resulting in a folate shortage that can be hazardous, particularly in healthy, normal tissues. Folate supplements are frequently administered to individuals on long-term methotrexate therapy in order to lessen this **(de Rouw *et al* .,2021).**

2.4.2. Medical Uses Of Methotrexate

Methotrexate (MTX) is widely used in cancer chemotherapy and to treat a number of autoimmune conditions, including psoriasis and rheumatoid arthritis **(Hoque *et al* .,2023).**Methotrexate is useful in cancer and autoimmune diseases

when inflammation or high cell turnover are present because it prevents the metabolism of folate, which is essential for cell division **(Marin *et al* .,2022).**

2.4.3. Side Effects Of Methotrexate

Although MTX is the first choice for RA treatment, the side effects of MTX must also be widely considered. The primary adverse effects of MTX include gastrointestinal issues, hepatotoxicity, lung toxicity, haematological toxicity, and renal toxicity. Anorexia, nausea, vomiting, diarrhea, and stomach pain are among the dose-dependent GI adverse effects of MTX. Changes in plasma homocysteine are linked to the incidence of GI side effects. In RA patients, MTX's hepatotoxicity primarily shows **(Ezhilarasan ,2021).**The liver, kidney, and testis are the organs of the body that methotrexate affects the most. MTX reduces spermatogenesis, testicular cellular redox state, and reproductive failure in the testis, which reduces fertility **(Marin *et al* .,2022).**

Significant testicular damage is caused by methotrexate administration in male rats, as evidenced by increased oxidative stress markers, decreased spermatogenic activity, and seminiferous tubule degeneration. Reduced testosterone levels and impaired fertility may be the outcome of these changes. The oxidative stress generated by MTX is principally due to the formation of reactive oxygen species (ROS), which overwhelms the antioxidant defense systems in testicular tissue **(Koc *et al* .,2018).**Testicular damage is regarded to be a significant adverse effect of MTX that causes male infertility . Variations in spermatogenetic control or irregular germ cell growth might be driven by anomalies in genomic status, all of which could be factors in lower fertility **(Sarman *et al* .,2023).**

2.5 Oxidative Stress

Oxidative stress is the result of an accumulation of Reactive Oxygen Species (ROS) that exceeds the capacity of the body’s antioxidant defenses. ROS

are molecules that are created during regular cellular metabolism, especially in the mitochondria, and comprise free radicals (like superoxide anion and hydroxyl radical) and non-radical species (like hydrogen peroxide) **(Sikder *et al* .,2025).** Under typical circumstances, ROS are kept within acceptable bounds by the body's antioxidant systems, which include enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (SOD). But when these defenses are overpowered by pathological circumstances or outside stresses, ROS may cause oxidative damage to proteins, lipids, and DNA, which exacerbates cellular dysfunction **(Chaudhary *et al* .,2023).**The primary sources of ROS generation, primarily during cellular respiration and Adenosine triphosphate (ATP) creation, are the mitochondria, the cell's engine. ROS production and NADPH Oxidases are markedly elevated by mitochondrial dysfunction, including decreased electron transport chain (ETC) activity These enzymes are essential for producing ROS, especially in immune cells such as endothelial cells, neutrophils, and macrophages **(Adhab *et al* .,2025).** Many illnesses, including as atherosclerosis and hypertension, have been linked to their overactivation **(Kuznetsov *et al* .,2022).**

The other sources of ROS generation is Environmental Factors: Tobacco smoking, UV radiation, environmental contaminants, and chemical poisons can all cause ROS to be produced, which in turn causes oxidative stress in tissues and cells. And the Inflammation: As immune cells, such as neutrophils and macrophages, produce ROS to fight infections, chronic inflammation sets off the creation of ROS. On the other hand, tissue damage and excessive oxidative stress can result from chronic inflammation. **(Albano et al .,2022).**

2.5.1. Oxidative Stress and Testicular Health

Oxidative stress (OS) refers to the imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms in the body.

Because oxidative stress affects spermatogenesis, hormone synthesis, and overall male fertility, it is especially problematic in the testes. Developing treatment techniques to lessen the consequences of oxidative stress in the testes requires an understanding of the processes behind this stress and its implications for male reproductive health **(Dutta *et al* .,2021).**The Oxidative stress effect on testicular function. As shown in the figure below (Fig.2.4) oxidative changes to DNA brought on by elevated ROS levels can result in fragmentation and decreased fertility **(Takeshima *et al* .,2020)** Increased ROS levels can harm sperm cells' plasma membranes, which lowers sperm motility and viability. Oxidative stress can also decrease sperm motility and viability **(Serrano *et al*., 2020)** and impact the synthesis of hormones ,Leydig cell malfunction brought on by oxidative stress (OS) affects spermatogenesis, steroidogenesis, and eventually male infertility. Preventing oxidative damages to Leydig cells that also affect testosterone levels

Hormonal abnormalities that further impair reproductive function might arise from oxidative damage to Leydig cells, which are responsible of producing testosterone. **(Monageng *et al* .,2023).**

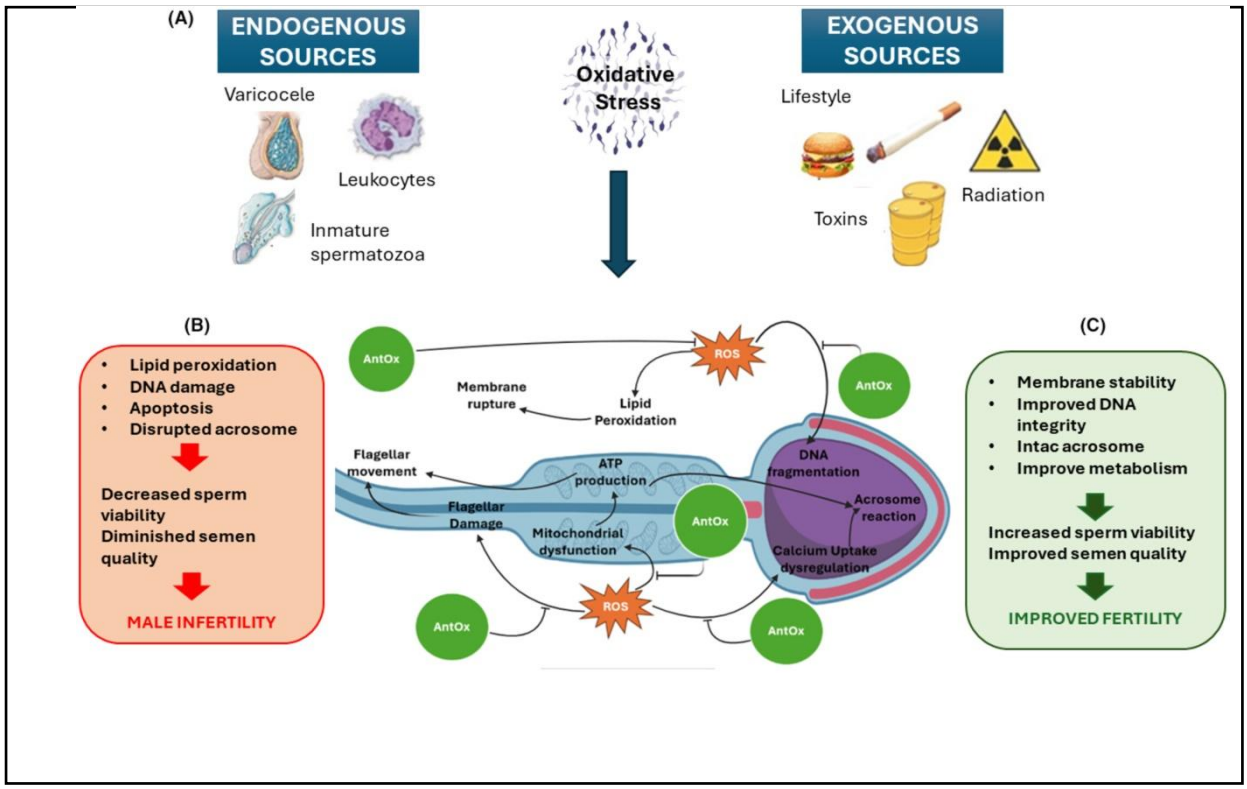


Figure (2.4) Impact of oxidative stress on male fertility (Sengupta *et al*.,2024)

Sperm damage morphological , Free radicals can lower sperm fertility because they can greatly affect a number of sperm parameters, including count, motility, and morphology. **(Gualtieri *et al* .,2021)** The free radical-induced oxidative stress contributes significantly in producing and increasing abnormal sperm and decreasing sperm count and transformation and fragmenting sperm DNA **(Younus *et al* .,2024)** Infertility is caused by these alterations in sperm DNA. Infertile men's sperm function is fatally affected by Reactive Oxygen Species (ROS) generated by leukocytes or spermatozoid **(Sengupta *et al* .,2022).**

2.6 Role Of Antioxidant In Testes Health

Antioxidant enzymes shield germ and somatic cells from harm caused by free radicals, and spermatogenesis occurs in a hypoxic environment **(Sengupta *et al* ., 2024)** An essential defensive mechanism, the testicular antioxidant system shields germ and somatic cells from harm caused by free radicals.

As a consequence of aerobic metabolism, reactive oxygen species (ROS) are often generated in the testis during spermatogenesis and steroidogenesis. ROS support a number of regulatory mechanisms during proper spermatogenesis and steroid hormone synthesis, as well as spermatozoa's acquisition of fertilizing capacity, at physiological concentrations **(Baskaran *et al* ., 2021).**

The antioxidant defense system uses enzymatic processes to shield cells from oxidative stress caused by elevated ROS levels **(Kıran *et al* ., 2023)** Free radicals are unstable chemicals that can lead to oxidative stress, and these compounds aid in their neutralization. An imbalance between antioxidants and free radicals causes oxidative stress, which damages cells. Oxidative stress in the testes can have a detrimental effect on hormone balance, sperm production, and general reproductive health **(Unsal *et al* ., 2021).**

The testes are highly susceptible to oxidative stress due to their high metabolic activity and exposure to environmental toxins such as pollutants and chemicals. As byproducts of biological functions, free radicals can build up excessively and harm testicular cells, such as Leydig and Sertoli cells, which are essential for the generation of testosterone and the maturation of sperm. These free radicals are neutralized by antioxidants including glutathione, selenium, and vitamins C and E, which lower oxidative damage and support normal testicular function **(Monageng *et al* ., 2023).**

2.7. N-acetyl cysteine (NAC)

Is a medication that is frequently used as a mucolytic and to treat paracetamol overdose as show in (fig.2.5). It is a supplement and pharmaceutical with a well-established safety profile and rare toxicity. It may be used as a mucolytic agent and antioxidant, among other things **(Schwalfenberg,2021) .**

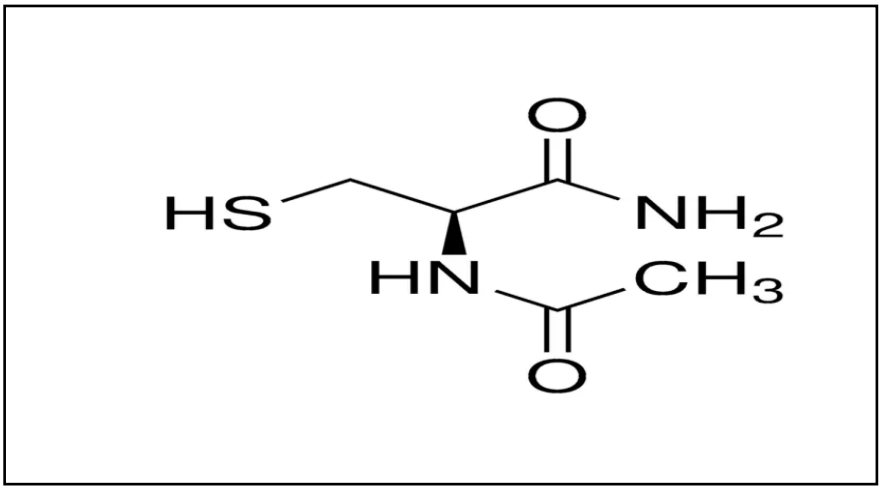


Figure (2.5) structure of N-acetyl cysteine (Rodrigo *et al*., 2021).

Is a powerful scavenger of free radicals; studies on the possible use of NAC as an oral therapy for testicular damage By changing how ROS is expressed **(Adel *et al* ., 2024)** It is well-known for its ability to cure acetaminophen (Tylenol) overdose and is frequently used to thin mucus in order to aid with respiratory disorders. designated as an essential medicine by the World Health Organization (WHO) and authorized by the Food and medicine Administration (FDA). is frequently offered as an over-the-counter dietary supplement that has anti-inflammatory and antioxidant qualities **(Tenório *et al.*, 2021).**

N-acetylcysteine (NAC) is derived from cysteine, an amino acid. The amine functional group (-NH2), which is a feature of amino acids, is part of its chemical structure. Additionally, the amino acid's acidic portion is known as the carboxyl group (-COOH). Moreover, the Thiol Group (-SH): This group that contains sulfur is in charge of many of the biological characteristics of NAC. and Group Acetyl (-COCH3): This is the source of the "acetyl" in N-acetylcysteine, which improves its bioavailability and stability. **(Noreen and Bernkop-Schnürch ,2024).**The molecular formula for NAC is C5H9NO3S, and its IUPAC name is (2S)-2-acetamido-3-sulfanylpropanoic acid **(GeTenório *et al*., 2021)**

2.7.1. Pharmacokinetics and Bioavailability of N-acetyl cysteine (NAC)

It Can be administered orally, intravenously, or by inhalation, being commonly safe and well tolerated, even in high doses. When taken orally, it is quickly absorbed by the intestines and metabolized by the liver, which uses the majority of the released cysteine to produce GSH. Its maximal plasma concentration (Cmax) happens around one to two hours after oral dosing. **(Tieu *et al*., 2023).** The elimination half-life of NAC is about 5 to 6 hours.It is primarily excreted in the urine as metabolites **(ASSESSMENT and HANDBOOK ,2022).**Oral Bioavailability As mentioned, it ranges from 6% to 10% due to first-pass metabolism. This is a consideration for dosing, especially in therapeutic contexts **(Galicia-Moreno *et al*., 2024).**On other hand Intravenous Use IV administration provides 100% bioavailability, making it effective for acute conditions like acetaminophen overdose Additionally, Inhalation: Depending on the formulation and administration mechanism, bioavailability may vary, and this route permits localized effects in respiratory disorders. **(Mokra *et al*., 2023).**

2.7.2. Effect of N-acetyl cysteine in testicular damage

N-acetyl cysteine seems to provide protective advantages in testicular damage situations. Because of its anti-inflammatory and antioxidant properties It has been suggested that NAC is a great option for treating male reproductive toxicity. NAC's high anti-inflammatory and antioxidant properties make them possible preventive medicines for diseases linked to Reactive oxygen species **(Gad *et al*., 2024)**. Infertile males may benefit from oral N-acetyl cysteine supplementation in terms of sperm parameters and oxidative/antioxidant state. may increase the quantity and quality of sperm following testicular damage **(Dimitriadis *et al*., 2023)**. In contrast to aberrant morphology and DNA fragmentation, patients' sperm count and motility rose dramatically.

Improvements in hormonal profiles were linked to higher testosterone levels and decreased levels of FSH and LH **(Benjamongkolchai *et al*., 2024).**

Malondialdehyde MDA decreased and total antioxidant capacity (TAC) increased dramatically; there was a strong negative relationship between TAC and MDA. **(Esalatmanesh *et al*., 2022).** NAC's capability to function as a reduced glutathione (GSH) precursor—a well-known direct antioxidant and substrate of many antioxidant enzymes—is what gives it its antioxidant activity. Additionally, in some situations where endogenous Cys and GSH are significantly reduced, NAC can act as a direct antioxidant for oxidant species.NAC's antioxidant properties may potentially be influenced by its capacity to degrade thiolated proteins. In certain circumstances, the reduced proteins and free thiols released by this process (such mercaptoalbumin) can exhibit strong direct antioxidant action **(Aldini *et al*., 2018) .**

2.8. Serratiopeptidase

(Serratia E-15 protease also known as serral-ysin/serratia-protease/serrapeptase) Serratopeptidase enzymes, are biological molecules which are usually proteins, serve as catalysts to quicken chemical reactions in living things. Numerous bodily functions, including digestion, metabolism, DNA replication, and cellular respiration, depend on them **(SAFDAR and ÖZASLAN ,2023)**, Enzymes act as Catalytic Function to speed up reactions by lowering the activation energy required for the reaction to occur, Enzymes are highly specific to the substrates (reactants) they act upon. This is because of their active site's distinct structure, which precisely binds to the substrate and prevents enzymes from being consumed in the activities they catalyze. Enzymes may be controlled by a number of variables, such as temperature, pH, and the quantity of substrates or inhibitors **(Patadiya *et al*., 2021).as Figure (2.6).**

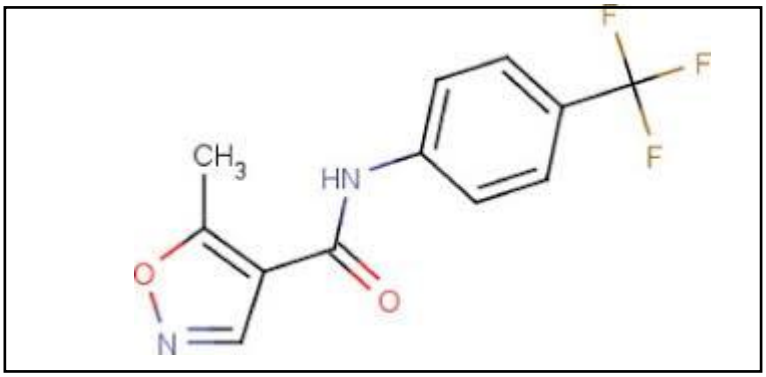


Figure (2.6) structure of Serratiopeptidase (LuthraU *et al.,2022)*

It has shown significant anti-inflammatory, anti-edemic, and analgesic effects and fibrinolytic properties **(Jadhav *et al*., 2020)** .Numerous illnesses have been treated with microbial products. One of the most effective bacteria made from serratiopeptidase is Serratia sp. Serratia E15, a non-pathogenic enterobacteria present in silkworms, is where it was first isolated. Serratiopeptidase, an enzyme that primarily fights inflammation, is a zinc that has characteristics more akin to those of an antioxidant **(Patil and Wagdarika ,2024).** Because of its analgesic, anti-inflammatory, and anti-edema properties, it is prescribed in a number of specialties, including dentistry, gynecology, orthopedics, otorhinolaryngology, and surgery. Because of its fibrinolytic and caseinolytic qualities, some anecdotal accounts indicate that it also has anti-atherosclerotic benefits. is utilized in several therapeutic specialties due to its analgesic, anti-inflammatory, and anti-edema properties. It is even being advertised as a health supplement to reduce cardiovascular morbidity **(Jadhav *et al*., 2020).** Serratiopeptidase has been used in therapeutic settings more frequently lately, either by itself or in conjunction with other medications. as an anti-inflammatory substance to protect the body from harm and illness. may be administered in conjunction with nonsteroidal anti-inflammatory medicines (NSAIDs) or in addition to other medications to treat acute inflammation., **(Hashmi *et al*., 2023).**

Five species of Serratia, which produce extracellular protease, have been discovered from the numerous bacterial strains that have been isolated from the silk worm's digestive canal. S. indica, S. marcescens, S. plymuthica, S. piscatorum, and S. species E-15 are among them.37 Of them, strain E-15 of Serratia generates more than three times as many proteases than Serratia marcescens. **(Vélez-Gómez *et al*., 2019).**The zinc atom in the Serratia protease enzyme, a metalloprotease with a molecular weight of 45,000–60,000, is crucial to its proteolytic action. 470 amino acids make up the amino acid sequence inferred from the nucleotide sequence of the gene encoding the enzyme. **(Adaki *et al*., 2023).**

2.8.1. Effect of Serratiopeptidase in testicular damage

Serratiopeptidase's impact on testicular injury and enhanced spermatogenesis: In diseases that cause testicular discomfort or swelling, it may offer symptomatic relief by lowering inflammation and oxidative stress, improving sperm production and quality. has an impact on male infertility since it can address the root cause, but it can also improve sperm parameters by enhancing the conditions that allow spermatozoa to develop and mature **(Mafruchati,2024).**

Also it shows potential in treating testicular injury through its anti-inflammatory and tissue healing qualities By lowering inflammation and facilitating healing in the testicular tissue, it may help minimize damage caused by diseases including trauma, infection, or oxidative stress **(Shahbaz *et al*., 2021).**

2.9. Nuclear factor erythroid 2-related factor 2 (Nrf2)

It is a crucial transcription factor that regulates the defense mechanisms of cells against oxidative stress, inflammation, and other types of cellular damage. It does this by expressing genes related to drug detoxification and the oxidative stress response. Activation of NRF2 makes cells resistant to inflammatory

challenges and chemical carcinogens. Beyond its first thought-out roles, NRF2 is engaged in several other cellular processes, such as metabolism and inflammation, in addition to antioxidant responses **(Sadiq ,2023)**Through internal metabolism and exposure to toxins in the environment, organisms come into contact with reactive oxidants. Because they generate oxidative stress, reactive oxygen and nitrogen species (ROS, RNS) are typically regarded as harmful. Nuclear factor erythroid 2-related factor 2 (Nrf2), a putative regulator of cellular defense, and its endogenous inhibitor Kelch-like ECH-associated protein 1 (Keap1) function as a shared, genetically maintained intrinsic defensive mechanism to fight oxidative stress. **(Tripathi *et al*., 2024) .** primarily functions as a transcriptional activator, controlling the expression of genes involved in antioxidant defense, detoxification, and cellular repair. When Nrf2 and Keap1 are exposed to toxic insults or oxidative stress, their relationship is broken down. As a result, Nrf2 can build up and go into the nucleus, where it attaches itself to target gene promoters' antioxidant response elements (AREs) **(Mukherjee and Gopalakrishnan,2023).**

Chapter Three

Methodology

3. Materials and methods

3.1. Materials

3.1.1 Chemicals and Kits:

All chemical materials, purified reagents and standard kits that used in this study with their origin are listed in tables (3-1) and (3-2) respectively below.

Table (3-1): Chemical, Manufacture and Source Country.

NO.	Chemicals	Manufacture	Country
1	Serratiopeptidase	BioActiveT	Poland
2	**N-acetyl-L-cysteine**	Neutec	Turkey
3	**Methotrexate**	Medwise	Britain

Table (3-2): Standard Kits with their Suppliers

NO.	Kit	Supplied company	Country
1	Follicle Stimulating Hormone (FSH) ELISA Kit	sunlong	China
2	Anti-luteinizig hormone antibody (LH-AB) elisa kit	sunlong	China
3	Testesterone elisa kit	sunlong	China
4	Glutathione	solarbio	China
5	Malondialdehyde(MDA) Spectrophotometer kit	solarbio	China
6	RNA extraction kit	Genaid	Korea
7	cDNA synthesis kit	ADDBio	Korea
8	qPCR master mix kit (Syber master)	ADDBio	Korea

3.1.2. Instruments and Equipments:

The study's equipment and instruments that were utilized in the investigations of the current study are listed and described with their full details (company, city, and country) in the table 3-3, below.

Table (3-3): Instruments, Manufacture and Country Sources.

NO.	Instrument	Manufacture	Country
1	Anatomical set (Scissors, Forceps, Scalpe	Chemo lab	China
2	Beakers (100, 250, 500, 1000)	Chemo lab	India
3	Camera Digital	Sony	Japan
4	Centrifuge	Hettich Roto fix11	Japan
5	Microplate Reader "Asys Expert 96"	Biochrom	US
6	Embedding center	Leed	Chine
7	Incubator	Nuve	Japan
8	Micropipette	Dragon	Chine
9	UV-visible spectrophotometer	Shimadzu	Japan
10	Microscopic	Olympus	Japan
11	Water Bath	WNB	Belgium
12	Microplate Elisa Reader	Biotelk	USA
13	Microplate ElisaWashe	Biotelk	USA
14	Electronic Balance	Metter company	Switzerland
15	Electronic microscope	Hitachi	Japan
16	Graduated Tube	Chemo lab	India
17	Real time qPCR machine	BioRad	USA
18	Ethanol (molecular grade)	Sigma	Germany
19	Quantus	Promega	USA
20	Spectrophotometer	Labomed	UK

3.2 Methods:

3.2.1 Animal Management:

The present study was conducted in the college of Veterinary medicine –at University of Kerbala, in the animal house of the department of physiology. A total number of 35 male adult albino rats (Rattus), with an average weight between (200±20g) and the ages of animals were ranged (from 8-to 10) weeks, were used in the current study. They were housed for two weeks for an adaptation before the experiment. Every seven animals were housed in an individual plastic cage measured as 15x35x50cm. They were fed ad libitum with the meal of standard pellet of diet supplied from IPA (Institute for Public Accuracy), counter for agriculture research. They had free access to water to drink, and they were kept

under the exact condition of temperature (22-25) C° and light, the regime of 14hours of light, and 10 hours, of darkness. At the beginning of the experiment, the initial body weight of adult rats was recorded and then obtained until the end of the investigation; body weight gain was also recorded.

3.2.2 The Experiments Design

Animals in the study were divided into five groups. Each group consists of 7 male rats used for the design experiments as the following in Figure **(3-1):**

1.First group: received only normal saline.
2. Second group: received IP injection 5 mg/kg **MTX** daily
3.Third group: received IP injection of 5 mg/kg **MTX** daily and treated by **NAC** (600mg/kg/d orally).
4.Fourth group: received IP administered of 5 mg/kg **MTX** daily and treated by **serrati peptidase** (5 mg/kg orally).
5.Fifth group: received IP administered of 5 mg/kg MTX daily and treated by**(NAC+SER)**.

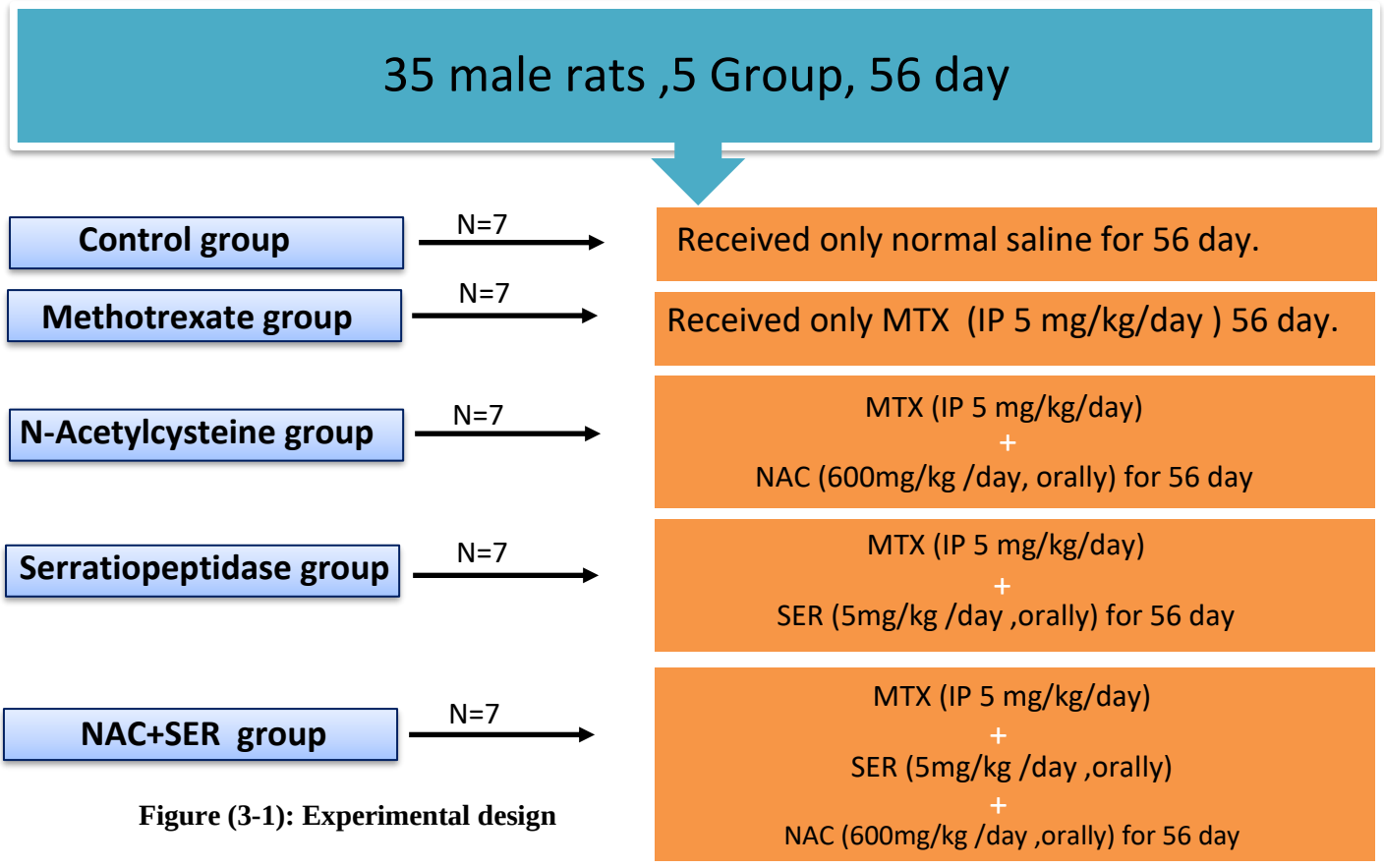


Figure (3-1): Experimental design

The Blood sample were obtained via heart puncture, immediately prior to organ collection and the serum was stored, the testes was fixed in bouin solution to analyze histopathological parameters ,while the other was snap- frozen in liquid nitrogen and stored at -80C.

3.3 Ethical approve

Under the reference number UOK.VET.PH.2024.110, this research was carried out in the anatomical laboratory of the College of Veterinary Medicine at the University of Kerbala - Iraq

3.4. Chemicals preparations

3.4.1 Methotrexate (MTX)

In this experiment, each rat was administered 5mg/kg of Methotrexate (prepared as Take 1.25mg of MTX so, each 1ml of MTX has 25mg so 1.25mg÷25mg =0.05 ml, It is very small so need to dilution for safe the accurate IP injection by use 0.95ml from NS +0.05 MTX =1ml) intraperitoneal on a daily basis for 56 day **(Mohamed and Nor-Eldin , 2018) (Figure 3-2).**

Figure (3-2):The Methotrexate

3.4.2 N-acetyl-L-cysteine (NAC)

Each rat was administered 600mg/kg/d orally grams of NAC (prepared as Take on NAC Effervescent tab 600mg in 2ml to reach (300mg/ml) ,If the animal BW 250 so the animal needs 150mg) approximately all rats was (250g) so token 0.5ml (150mg) orally per day basis for 56 day (**Abdelkader *et al*., 2021**) Figure (3-3).

Figure (3-3): N-acetyl-L-cysteine

3.4.3 Serratiopeptidase (SER)

Each rat was administered 5 mg/kg orally of SER (prepared as Take SER CAP 40000 IU cap: 40mg in 8ml to reach (5mg/ml) If the animal BW 250 so the animal needs 1.25mg . approximately all rats was (250g) so token 0.25ml (150mg) orally per day basis for 56 day (**Abdelkader**, **2019**) **Figure (3-4).**

Figure (3-4): Serratiopeptidase

3.5 Blood & Samples Collection:

In all of the studies, rats were sacrificed at the end of the treatment period during the two months of the experiment. The controlled and treated animals, before sacrificing, were initially put to anaesthetised with diethyl ether an anesthesia using cotton swabs in a covered container. The chest and the abdominal cavities were widely opened to give a clear view of the reproductive organs (testis, epididymis) needed in the studies. The blood was drawn using the Hoff and Ralatg technique of cardiac puncture. A 5ml disposable syringe was used to draw blood from the heart using the non-heparinized plane tube, which was then centrifuged for 15 minutes at (3000 rpm) to extract the serum, which was

then transferred to epndroffe tubes and kept at (-20C) until all tests were completed.

3.6 Hormones Assay

Assay for Hormones (ELISA) Testosterone, Follicular Stimulating Hormone (FSH), and Luteinizing Hormone (LH). The premise behind enzyme-linked immunosorbents is that an enzyme is used to detect antigen-antibody binding. Using a colorless substrate, the enzyme creates a brightly colored end product, showing the incidence of Ag: Ab binding (Ma, and Shieh 2006).

3.6.1Estimation of Testosterone Hormone (T) Concentration (ng/ml).

Serum testosterone hormone(T) concentration in the current study was measured by ELISA technique using commercial test kit as listed in (Table 3-2). Uses a biotin double antibody sandwich technology-based enzyme-linked immunosorbent assay (ELISA) to measure Testosterones levels in samples (**Mujika *et al*., 1996**). testing procedure was according the manufacturer's instruction as illustrated in appendix I.

3.6.2 Estimation Follicular Stimulating Hormone (FSH) Concentration (mlU/ml).

Serum follicle stimulating hormone concentration in the current study was measured by ELISA technique using commercial test kit as listed in (Table 3-2). Uses a biotin double antibody sandwich technology based enzyme-linked immunosorbent assay (ELISA) to measure FSH levels in samples (**Di-Simoni *et al*., 1997)** . testing procedure was according the manufacturer's instruction as illustrated in appendix II.

3.6.3 Estimation of Luteinizing Hormone (LH) Concentration (ng/ml).

Serum Luteinizing hormone concentration in the current study was measured by ELISA technique using commercial test kit as listed in (Table 3-2). Uses a biotin double antibody sandwich technology-based enzyme-linked immunosorbent assay (ELISA) to measure LH levels in samples (Uotila et al., 1981), testing procedure was according the manufacturer's instruction as illustrated in appendix III.

3.7 The Oxidant and Anti-oxidant Parameter:

3.7.1 Serum Malondialdehyde Measurement (MDA)

The ability to accurately measure lipid peroxidation in disease states necessitates this method of assessing oxidative stress. MDA and 4 hydroxynonenal (4-HNE) are the natural bi-products of lipid peroxidation. One of the most commonly acknowledged methods for assessing oxidative damage is to measure the lipid peroxidation products. It is easy to use the MDA Microplate Assay Kit to detect MDA in a range of samples. Thiobarbituric Acid (TBA) reacts with MDA in the sample to form the MDA-TBA adduct. You may readily measure the MDA-TBA adduct using a colorimeter (λ= 532 nm). According to **(Kavsak, 2017)** ,as illustrated in the appendix IV.

3.7.2 Superoxide Dismutase (SOD) Activity.

Dismutation of superoxide radicals (O2-) into hydrogen peroxide (H2O2) and elemental oxygen (O2) is catalysed by superoxide dismutases (SODs), which act as a crucial defence against superoxide radical toxicity. Tumor cells are protected from apoptosis in mice lacking SOD1 and SOD2, however these animals spontaneously acquire liver cancer. NBT is converted to NBT-diformazan by xanthine oxidase (XOD) and hydrogen peroxide in the Superoxide

Dismutase Microplate Assay Kit. Light with a wavelength of 560 nm or longer is absorbed by NBT-diformazan. SODs diminish the levels of superoxide ions, which in turn reduces the rate at which NBT-diformazan is formed. NBT diformazan decrease is a good indicator of SOD activity in experimental samples. According to (Kavsak, 2017) .In Appendix V

3.7.3 Estimation of Serum reduced glutathione concentration (GSH)

The assay involves carefully optimized enzymatic recycling method using glutathione reductase and Ellman's reagent according to **(Abdelrazek et al ., 2022).**

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 **,** as described in **Appendices IV.**

3.7.4 Estimation of Serum Total antioxidant capacity (TAOC)

Estimation of serum total antioxidant capacity with the ABTS decolourization method, Serum AOC levels were tested using 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolourization method, expressed as serum6-Hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (**Kambayashi *et al*.,2009**). Radical cations were generated by the oxidation of ABTS with potassium persulfate (K2S2O8). The freshly prepared ABTS stock solution was diluted in 40- fold with 5 mM phosphate buffered saline (PBS) to prepare the ABTS working

solution. Regent blank was prepared by mixing equal volumes of distilled water and K2S2O8 (10 µL in each) were mixed with 800 µL of 5 mM PBS. Test serum from each sample was mixed with ABTS working solution in 1: 9 rations and kept exactly for 1 min to complete the scavenging process in dark. Samples were analyzed in duplicates and absorbance were measured at 734 nm against the reagent blank using spectrophotometer (UV Spectrophotometer, Shimadsu, Japan) A two-fold dilution series of 6-Hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox) (12.5–400 µM) was mixed at the same ration with ABTS working solution and the standard curve was plotted using absorbance values. TAOC was calculated using the Trolox standard curve as described in Appendix VII

3.8 Gene Expression

3.8.1 Expression levels of Nuclear factor erythroid 2-related factor 2 (Nrf2):

This approach was carried out according to the comparative Ct approach (ΔΔCt) with normalization to the level of the control group in the presence of the transcript levels to those of GAPDH. This was achieved according to the recommendation of **(Schmittgen and Livak, 2008).**

3.8.1.1 Quantitative Reverse Transcription Real-Time PCR (RT-qPCR):

A-RNA extraction (Genaid Korea):

1. 200 µg of each tissue sample was lysed by adding 600 µl of Triazol in a 1.5 ml micro-centrifuge tube and homogenised by inserting pestle several times.

2. This followed by centrifugation at 13000 rpm for 2 minutes meanwhile the supernatant was transferred into new tube and 600 µl of ethanol was added and vortexed.
3. The lysate was carefully transferred into the upper reservoir of the spin column for RNA binding which then centrifuged at 13,000 rpm for 1 minute meanwhile the flow-through was discarded.
4. The spin column was washed with washing buffer then exposed to DNase by adding 45 µl of DNase buffer and 5 µl of DNase which then left for 15 minutes.
5. The spin column was then washed three times with washing buffer by adding 500 µl of washing buffer and centrifugation at 13000 rpm for 1 minute.
6. For RNA elution, the column was centrifuged empty to dry out the silica gel followed by adding 50 µl of elution buffer and centrifugation at 13000 rpm for 2 minutes.

B. RNA concentration measurement by Quantus™ Fluorometer (Promega, USA):

1. 1X of TE buffer was prepared by diluting 20X TE Buffer (pH 7.5) with nuclease-free water.
2. The QuantiFluor® Dye working solution was prepared with 1X TE buffer to make a 1:400 dilution by combining 10µl of QuantiFluor® Dye with 3,990µl of 1X TE buffer with mixing.
3. The blank sample was prepared for the QuantiFluor® ONE RNA System by adding 200 µl of QuantiFluor® RNA Dye to a 0.5ml PCR tube.
4. The nucleic acid standard (RNA) was added in a 0.5ml PCR tube as recommended by the manufacturer (2 µl of standard to 200 µl of working solution).

5. Set up standard measurement by the Quantus Fluorometer reading then measure the RNA samples according to the recommended procedures by the Quantus machine.

C. cDNA synthesis

A- A total of RNA was reversed transcribed to cDNA using the kit from ADDBio (Korea) as following:

Table (3-4): Protocol for cDNA Synthesis Using ADDRio Kit".

Substance	Amount
H2O	6 µl
Reverse transcriptase (RT) 2X add script cDNA	20 µl
dNTPs	4 µl
random oligos hexamer	2 µl
RNA	8 µl
Total volume	40 µl

B- The thermal conditions were as following:

Table (3-5): Temperature Conditions for cDNA Synthesis"

Temperature	Time	Purpose
25 C	10 min	Priming
50 C	60 min	Reverse transcriptase (RT)
80 C	5 min	RT inactivation
4 C	On hold	Store cDNA

3.8.1.2 Quantitative Reverse transcriptase PCR (RT-qPCR): Preparation

Initially, the amplification was achieved using AddScript RT-qPCR Syber master (AddBio, Korea) and the reaction was including:

Table (3-6): RT-qPCR Preparation Details (AddBio, Korea)"

Substance	Amount
H2O	3 µl
AddScript RT-qPCR	10 µl
Forward primer (0.05 pmol/20 µl)	2 µl
Reverse primer (0.05 pmol/20 µl)	2 µl
cDNA	3 µl
Total	20 µl

3.9 Histological Studies

The testis were collected from all studied groups to prepare slides for a histological examination according (**meschar method *et al*.,2010**) with the used of the light microscope as the shown in Appendix VIII.

3.10 Statistical Analysis

The result of our treatments parameters was viewed as mean ± SE. One way analysis of variance (ANOVA) was used in the comparison between the experimental groups by using computerized (SPSS) program version 13 , with $P<0.05$ lest limit of significance **(SPSS ,2001).**

Chapter Four
Results and Discussion

4. Results And Discussion

4.1 Semen analysis.

4.1.1 Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on total sperm motility of Male Rats as show in Figure (4.1).

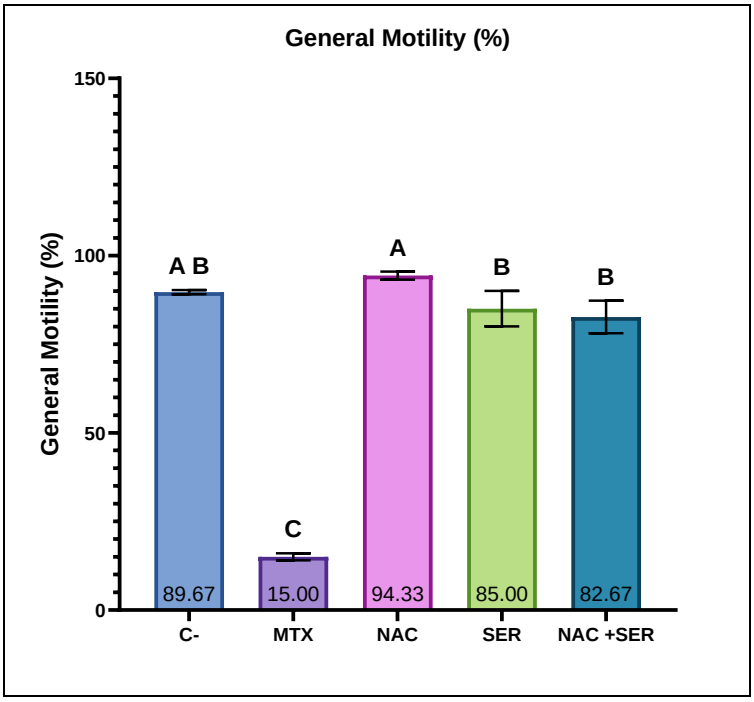


Figure (4.1) Comparison of total sperm motility in the Control, MTX, NAC, SER, and NAC+SER groups.

A statically significant (P<0.05) decrease in total sperm motility was recorded in MTX group as compared to Control; however, the motility was significantly improved in NAC, SER, and NAC+SER.

Sperm concentration was show statistically different between the groups, although the MTX group was numerically lower than control, while NAC had better values.

This data highlights the significant changes seen in sperm parameters as a result of oxidative stress caused by Methotrexate (MTX) and the protective

effects of N-Acetylcysteine (NAC), Serratiopeptidase (SER) alone and in combination.

MTX impedes mitochondrial operation and inhibits stay of ATP and dawdles axonemal stability, which bends approach motility **(Al-Khatib,2024).** Additionally, oxidative injury to the plasma membrane hinders the cellular flexibility required for motility (**Ofosu *et al*.,2023).** NAC facilitated rates of motility (94.33%) due to its stimulating glutathione properties, in agreement with (**Adel *et al*.,2024**). who showed enhanced motility using antioxidant therapy.

4.1.2 Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on progressive sperm motility of Male Rats.

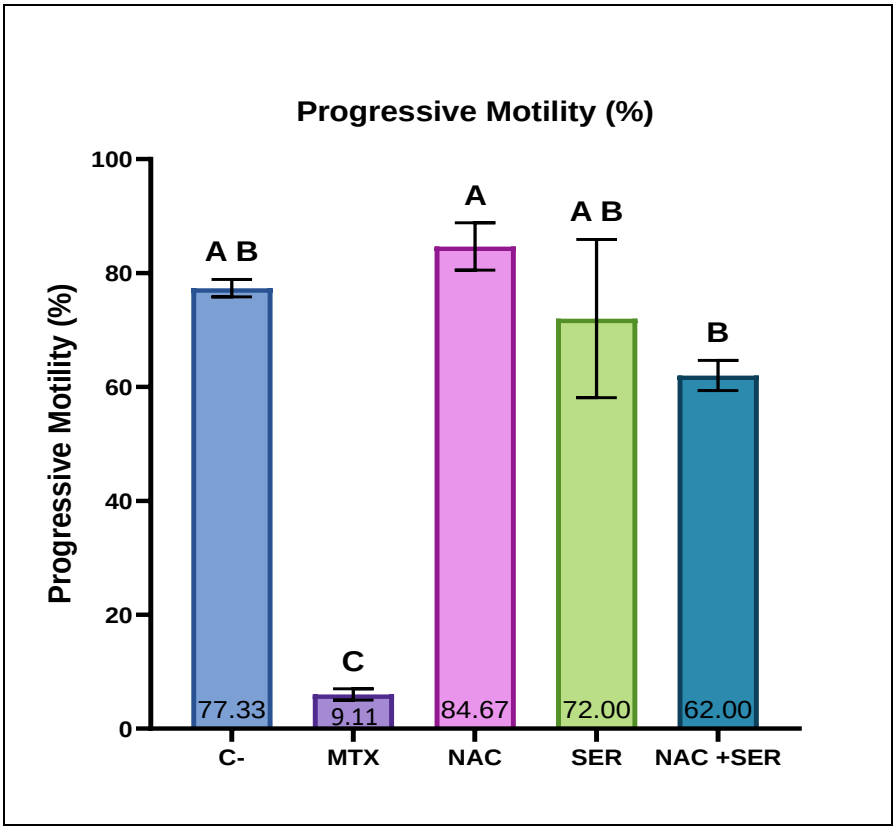


Figure (4.2) Comparison of progressive sperm motility in the Control, MTX, NAC, SER, and NAC+SER groups.

Progressive sperm motility significantly (P<0.05) decreased in MTX group as compared to Control while NAC and SER showed significant recovery as compared to MTX.

AS seen in Fig. (4.2) Vital for fertilization, progressive motility in the MTX group was severely impaired (6.00%). It is commonly caused by mitochondrial membrane depolarization and decreased flagellar beat frequency (**Giaretta *et al.*, 2022**). Similar extreme recovery is demonstrated by NAC (84.67%), probably due to Matrix metalloproteinase (MMP) preservation and peroxynitrite radical scavenging as shown in the study done by (**Sönmez *et al.*, 2015**). While SER treatment showed patchy improvement (72.00%), recovery was slightly reduced in the NAC+SER group (62.00%), likely because of pharmacodynamic interactions.

4.1.3 Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on sperm viability of Male Rats

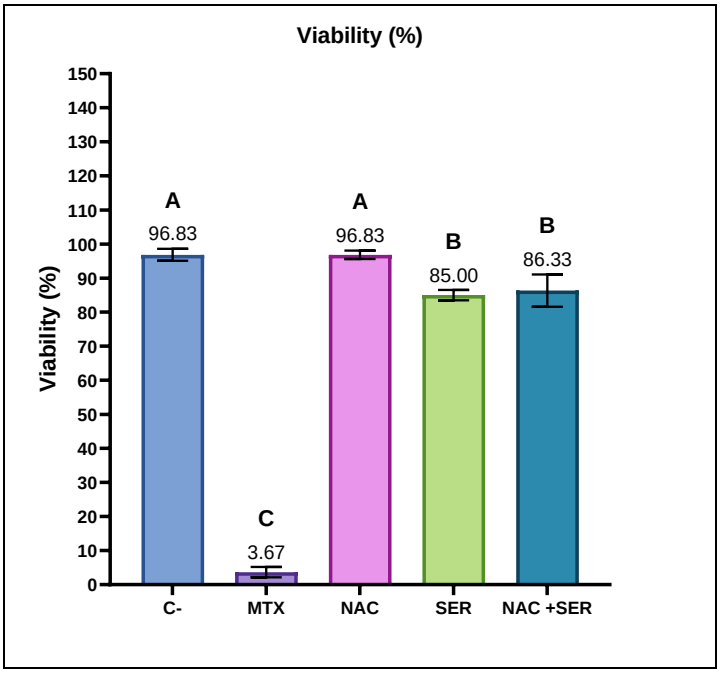


Figure (4.3) Comparison of sperm viability in the Control, MTX, NAC, SER, and NAC+SER groups.

Sperm viability was significantly ($P < 0.05$) reduced in MTX group and NAC and NAC+SER groups showed a significant improvement ($P < 0.05$) approaching control values.

In the MTX group, sperm viability dropped dramatically to 3.66% Fig.(4.3), reflecting extensive membrane lipid peroxidation and necrosis. It is in agreement with results from (**Nasr *et al*. 2018**), which observed similar decreases in viability as a result of MTX-mediated lipid damage. Both the NAC, SER and NAC+SER-treated groups nearly reached their normal function (96.83 and 86.33% restoration, respectively), suggesting that by its ability to donate thiols, NAC is able to guarantee the preservation of phospholipid bilayer integrity (**Guerini *et al*., 2021**). This up to 85% restored viability is significant in light of the anti-inflammatory and microcirculatory potential of SER (**Calogero *et al*., 2017**).

4.1.4 Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on percentage of morphologically normal sperm cells of Male Rats.

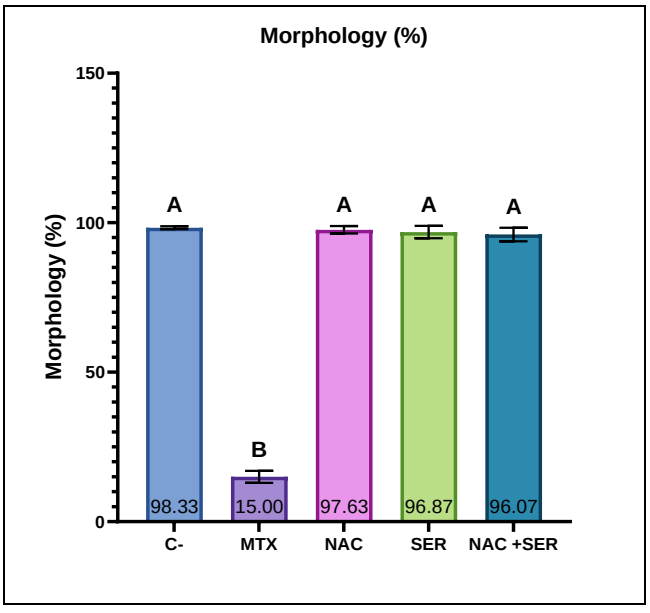


Figure (4.4)Comparison of the percentage of morphologically normal sperm cells in the Control, MTX, NAC, SER, and NAC+SER groups.

The significant (P<0.05) decrease in percentage of morphologically normal sperm Cells was observed in MTX group. The NAC , SER and NAC+SER groups in each row reestablished similar morphology as in the healthy state.

In the Fig. (4.4),the MTX group also showed abnormal morphology (15.00% normal forms),suggesting aberrant spermatogenesis and chromatin remodeling. (**Zini & Libman 2006**) reported that high levels of ROS lead to fragmentation of chromatin and abnormal acrosome development. In contrast, morphology was preserved above 96% in the NAC, SER, and NAC+SER groups, and these agents effectively maintained spermatogenic microenvironment .

4.5.5 Effect of Methotrexate, N-Acetyl cysteine, Serratipeptidase, and Their Combination on sperm cells Concentration of of Male Rats.

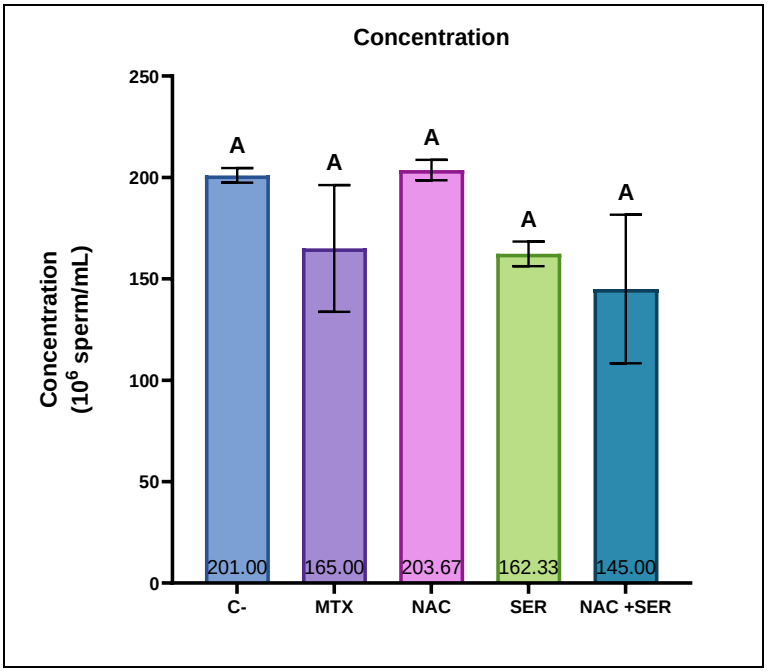


Figure (4.5) Comparison of the percentage of Concentration of sperm cells in the Control, MTX, NAC, SER, and NAC+SER groups.

As illustrated in Fig(4.5), Definitely not significant differences were detected in sperm concentration levels among the groups. In contrast, MTX was reduced (165 million/mL vs. 201 in control), consistent with earlier work by (**Zribi *et al*. 2016),** who found late-stage spermatogenic arrest to be a dose-dependent phenomenon of MTX. The preserved concentration of NAC (203.66) continued to add data to the existing literature as NAC is known for maintaining functions of Sertoli cell and attenuating apoptotic signaling (**Kanter *et al*., 2013).**

4.2. Hormonal Study

4.2.1. Effect of Methotrexate (MTX), N-Acetylcysteine (NAC), Serratiopeptidase (SER), and Their Combination on Serum Testosterone Levels in Male Rats.

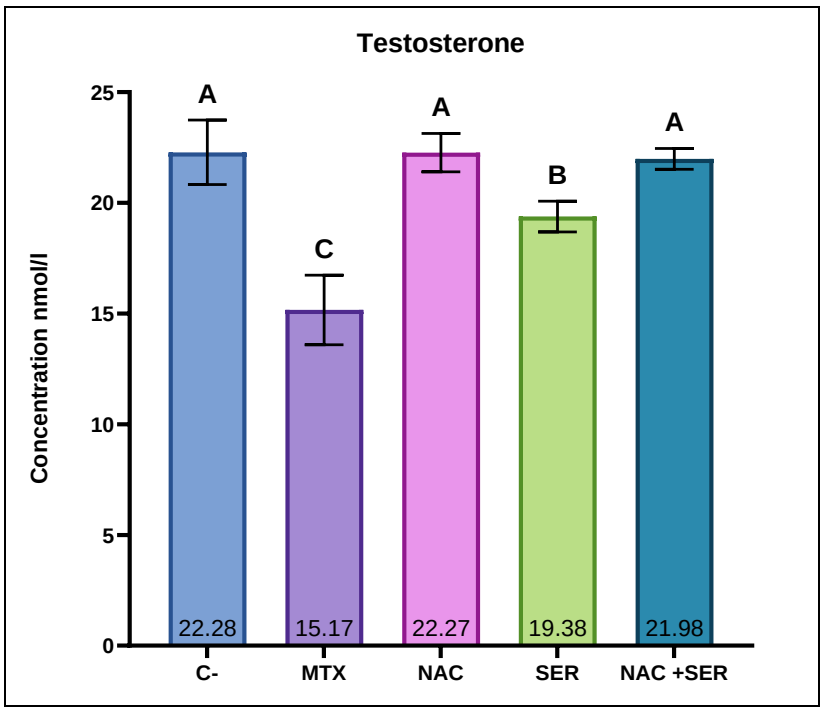


Figure (4.6) Comparison of serum testosterone levels in the Control, MTX, NAC, SER, and NAC+SER groups

The mean ± SD serum testosterone level (nmol/l) were significantly (P<0.05) lower in MTX groups compared to Controlling groups and significantly (P<0.05) higher in NAC and NAC+SER groups compared to MTX. The SER group had also a moderate significant (P<0.05) increase.

Table (4.1) Comparison of the serum testosterone levels (mean ± SD), (p<0.05) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Gropes	Mean	Std. Deviation
Testosterone (nmol/l)	C-	22.28 A	1.45797
	MTX	15.17 C	1.57056
	NAC	22.27 A	.86872
	SER	19.38 B	.69690
	NAC +SER	21.98 A	.47081
LSD	1.308		

The results of this study that appears in table (4.1) revealed that testosterone levels in the Methotrexate (MTX) group were significantly lower than the healthy control group (C-). Meanwhile, the testosterone levels were substantially higher in groups treated with the antioxidant N-Acetylcysteine (NAC), Serratiopeptidase (SER) or a combination of NAC and SER, being NAC and NAC+SER groups resemble those obtained in the control group.

Testosterone is the major male hormone secreted from the Leydig cells in testes, triggered by luteinizing hormone (LH) , (**Oduwole *et al.*, 2021**). Low levels cause dysfunction in the male reproductive system, and this decline is often

correlated with toxic, inflammatory, or oxidative factors affecting testicular tissue , This fact was reached by (**Yadav & Mali, 2024)** as we are.

As previously reported by (**Atas *et al,* 2024)** were found methotrexate is toxic to testicular tissue since it directly mediates oxidative damage to Leydig cells, leading to a decrease in steroidogenic capacity and, consequently, reduced testosterone production, this supports the results of the current research. In addition to sex hormones. The researcher's (**Hassanein *et al.,*2023)** agreement with the our results which found that Methotrexate decreases the activity of enzymes involved in testosterone synthesis, including 17β-hydroxysteroid dehydrogenase (**Hassanein *et al*., 2023**).

In contrast, N-Acetylcysteine (NAC) treatment, whose powerful scavenging properties of free radicals may have helped maintain testosterone levels. N-Acetylcysteine has shown to be a precursor to glutathione, the most critical intracellular antioxidant that reduces oxidative damage to testicular tissue, thus enforcing hormonal function. In this regard, one study demonstrated that N-Acetylcysteine reduces oxidative stress in rats and improves their testicular structure and function when subjected to toxic agents **(Li *et al*., 2024).**

Serratiopeptidase (SER) (a proteolytic enzyme) having the role of a very powerful anti-inflammatory agent, also helps to increase the availability of the drug within the tissue, leads to the slight rise in the testosterone levels, but of the least extent unlike (NAC) (**Kumar,Verma,& Abbot, 2023**). This indicates that the anti-inflammatory effect alone is not enough to protect the testicles from the toxic effect of methotrexate . As a result, protective effect becomes more powerful and effective when Serratiopeptidase is used in combination with NAC (**Demirci *et al*., 2019**).

The group NAC results were the best and the (NAC + SER) was near to the NAC group so helpful in preserving testosterone levels, indicating a synergistic

effect of the antioxidant NAC and anti-inflammatory SER. This could be attributed to the simultaneous reduction of oxidative stress and inflammation resulting in a more stable cellular environment for Leydig cell function. This was also shown by (**Soliman *et al*., 2024)**, where they also demonstrated amelioration of the toxic effects in the testes post-MTX treatment when a combination of an antioxidant and an anti-inflammatory drug was used.

4.2.2. Effect of Methotrexate (MET), N-Acetylcysteine (NAC), Serratiopeptidase (SER), and Their Combination on Serum of luteinizing hormone (LH) Levels in Male Rats.

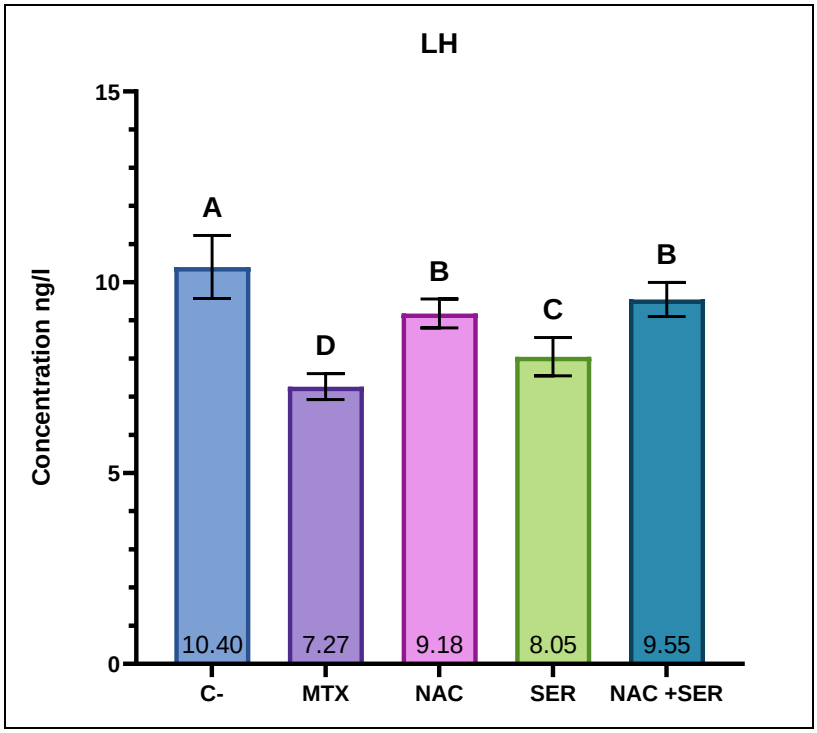


Figure (4.7) Comparison of luteinizing hormone (LH) levels in the Control, MTX, NAC, SER, and NAC+SER groups.

There was a significant ($P<0.05$) decrease in the mean LH level in the MTX group, when compared to Control, while NAC and NAC+SER groups had significantly raised LH level in comparison to MTX (Table 2). The increase in SER group was intermediary significant ($P<0.05$).

Table (4.2) Comparison of the serum LH hormone levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation
LH (ng/l)	C-	10.4 A	.82462
	MTX	7.26 D	.33862
	NAC	9.18 B	.37639
	SER	8.05 C	.50100
	NAC +SER	9.55 B	.44609
LSD	0.626		

As noted in the table (4.2), results reported the highest levels of LH for control and then progressively less for NAC+SER, while MTX group Record the lowest score as compare with other groups. It is similar to what was found in the research of (**Erdogan *et al*.,2015**).The marked drop of LH in MTX group reinforces the notion of the involvement of methotrexate on hypothalamic-pituitary-gonadal (HPG axis) axis inhibiting secretion of this hormone (**Ghosh, *et al.*, 2022**).

LH is released from the anterior pituitary and acts on Leydig cells in the testes to promote testosterone synthesis. Thus any fluctuation in its levels creates an imbalance in the male hormones (**Sengupta *et al*., 2019; Oduwole, 2021; Li, *et al*., 2024).**

The dramatic drop in LH in the MTX group might have been because of direct inhibition of the pituitary or possibly due to the increase in oxidative stress disrupting feedback signals via a decrease in testosterone itself. This was reinforced by a study conducted by **Roy *et al*.. in 2021**, that concluded the breakdown of LH and FSH secretion is a direct consequence of methotrexate-induced oxidative damage to the pituitary gland, ultimately impairing its reproductive functions.

By contrast, NAC and (NAC+SER) treatment significantly alleviated reduced LH levels (**Fang *et al.*,2024**). As discussed above, NAC helps improve the antioxidant balance in the body and protects pituitary tissue from damage. Serratiopeptidase also helps reduce mild cerebral inflammation accompanying oxidative stress (**Zhao *et al.*, 2023**). The significant elevation of LH levels post-exposure to methotrexate in (NAC+SER) group support the rationale of combination of antioxidants and anti-inflammatoires acts synergistically towards resetting of HPG axis. Such a consistency of results was recently reported by (**Sahoo *et al.*, 2024**) where antioxidant treatments could improve LH secretion by protecting neural tissue from oxidative damage.

4.2.3. Effect of Methotrexate (MTX), N-Acetylcysteine (NAC), Serratiopeptidase (SER), and Their Combination on Serum follicle-stimulating hormone (FSH) Levels in Male Rats.

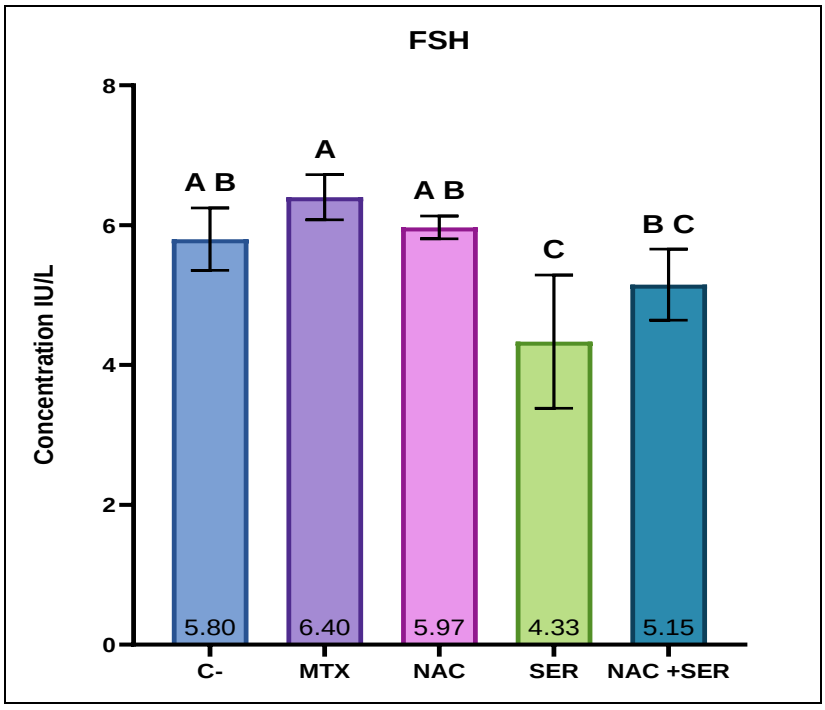


Figure (4.8) Comparison of follicle-stimulating hormone (FSH) levels in the Control, MTX, NAC, SER, and NAC+SER groups.

There was a significant (P<0.05) increase in mean FSH in MTX group compared to Control and moderate reductions in NAC and NAC+SER groups. FSH level was significantly (P<0.05) lower in SER group in comparison with MTX.

Table (4.3) Comparison of the serum FSH hormone levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation
FSH (IU/L)	C-	5.80 AB	.44721
	MTX	6.40 A	.32249
	NAC	5.97 AB	.16330
	SER	4.33 C	.95219
	NAC +SER	5.15 BC	.50892
LSD	0.65		

As shown in the table above, the average levels of follicle-stimulating hormone (FSH) were elevated in the Methotrexate (MTX) group compared to the control group, while there was a stepwise decline in the levels of FSH in the NAC, NAC+SER and SER groups with statistically significant differences.

FSH is secreted by the anterior pituitary gland and is important for stimulating the seminiferous tubules and promoting spermatogenesis via activation of the Sertoli cells. Under physiological conditions FSH levels are regulated by negative feedback, predominantly through Inhibin B secreted from Sertoli cells (**Stamatiades and Kaiser,2018**).

The increased serum FSH levels noted in the MTX group may be due to the damage exerted on the Sertoli cells by the oxidative stress or even due to the inflammation process, resulting in reduced secretion of Inhibin B and a potentially

impaired negative feedback mechanism. This is in agreement with the findings of (**Yan *et al*. 2024**) further reported that MTX toxicity leads to Sertoli cell necrosis and subsequent increase in FSH.

A previous study conducted by (**Jannatifar *et al*.,2019**), agreed with these results, as it showed that the enhanced FSH levels seen due to the antioxidant and anti-inflammatory properties of N-Acetylcysteine (NAC), where he works a precursor to glutathione that helps with cellular redox balance and integrity of Sertoli cells. Serratiopeptidase (SER) that down-regulates inflammatory cytokine expression and improves microvascular perfusion. These findings are in agreement with (**Ma *et al*. 2025**) who demonstrated that such combined antioxidant and anti-inflammatory therapies effectively restore hypothalamic-pituitary-gonadal axis homeostasis.

4.3 Oxidative stress study

4.3.1. Effect of Methotrexate (MET), N-Acetylcysteine (NAC), Serratiopeptidase (SER), and Their Combination on Serum GSH Levels in Male Rats.

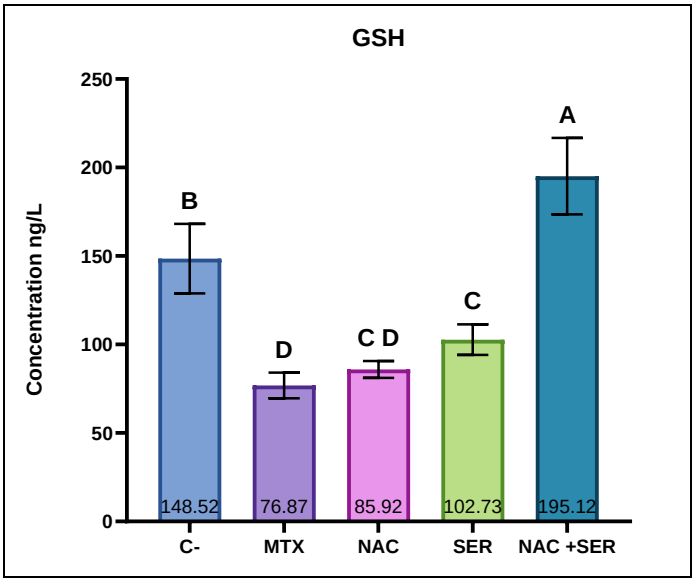


Figure (4.9) Comparison of glutathione (GSH) concentrations in the Control, MTX, NAC, SER, and NAC+SER groups.

GSH concentration was significantly (P<0.05) decreased in MTX group as compared to Control, on the other hand NAC+SER group had shown significant (P<0.05) increase in GSH concentration . NAC and SER alone also increased GSH levels compared with MTX.

Table (4.4) Comparison of the serum GSH levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation
GSH (ng/l)	C-	148.52 B	19.69156
	MTX	76.87 D	7.23012
	NAC	85.92 CD	4.73262
	SER	102.73 C	8.64353
	NAC +SER	195.12 A	21.62595
LSD	16.85		

Statistical of Table (4.4) showed that levels of GSH were highest in the NAC+SER group, followed by healthy control, SER, NAC while showed a striking drop of GSH levels that was significant.

GSH is a tripeptide and one of the most crucial endogenous antioxidants, being the major cellular antioxidant against oxidative stress. Its drastic depletion in the MTX group indicates how much oxidative imbalance was taking place this induced a damage of letal oxidative stress in DNA, proteins and lipids membranes (**Canevarolo *et al*., 2022**).

(Yüce *et al*., 2019). support this result in (2019) when showed that MTX dramatically decreases GSH because of excessive ROS generation, which depletes intracellular antioxidant stores.

Conversely, administration of N-Acetylcysteine (NAC) significantly recovered GSH levels owing to its direct precursor in glutathione synthesis (**Sahasrabudhe *et al*., 2023**).Reduce glutathione (GSH) consumption has seen an evident decrease due to the contribution of serratiopeptidase as well, through the limitation of the inflammatory responses and oxidative damage. These effects are in line with the findings by (**El-Sheikh *et al.*,2022**), which suggested that NAC-based therapies have the potential to improve tissue oxidative markers under toxic assault.

4.3.2. Effect of Methotrexate (MET), N-Acetylcysteine (NAC), Serratiopeptidase (SER), and Their Combination on Serum Superoxide dismutase (SOD) Levels in Male Rats.

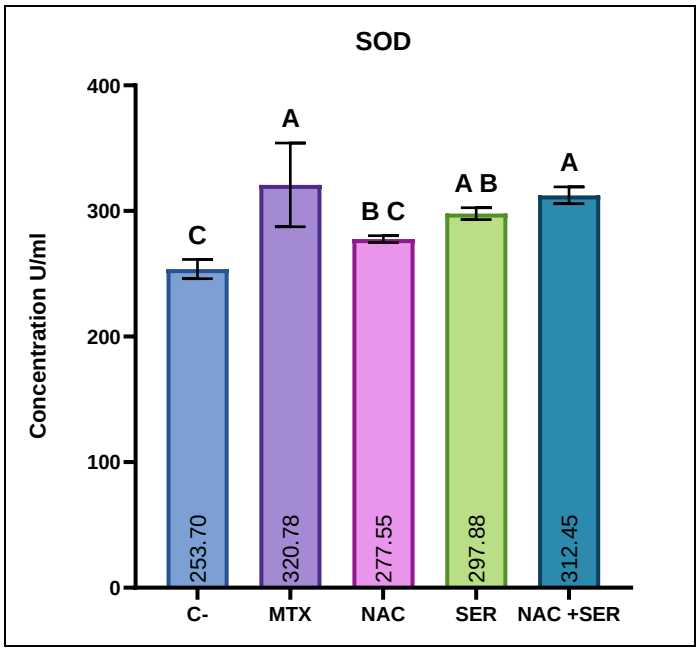


Figure (4.10) Comparison of superoxide dismutase (SOD) activity in the Control, MTX, NAC, SER, and NAC+SER groups.

Superoxide activity was significantly ($P<0.05$) higher in MTX group as compared to Control. SOD activity was decreased with Sufficient treatment of NAC, SER and (NAC+SER) while approaches normal values in the group NAC.

Table (4.5) Comparison of the serum (SOD) levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation
SOD (U/ml)	C-	253.70 C	7.70013
	MTX	320.78 A	33.27530
	NAC	277.55 BC	2.75445
	SER	297.88 AB	4.72754
	NAC +SER	312.45 A	6.67675
LSD	18.733		

SOD activity was significantly higher in the MTX group relative to NAC+SER, SER and the lowest amount was observed in the NAC group as seen in (Table 4.5).

Superoxide dismutase is an important antioxidant enzyme that causes the dismutation of superoxide radicals to hydrogen peroxide, even if upregulation of SOD occurred in the MTX group, it does not mean that antioxidant status was improved (**Zheng *et al*.,2023**).

Rather, it is a compensatory feedback for excessive MTX-induced ROS levels. Yet, if the elevation of SOD is not balanced with adequate concentrations of other antioxidants like GSH or TAOC, it indicates ongoing oxidative stress and possible cytotoxic effect (**Demirci-Çekiç *et al*., 2022**)

Chatterjee *et al* .,2017 also observed this paradoxical increase in SOD in toxic environment by noted that high SOD may be a marker of oxidative burden instead of a measure of effective protection.On the contrary, the modest amounts of SOD showed by NAC, SER and NAC+SER groups may indicate a more equilibrated redox environment, leading to coordinated antioxidant enzymes and cellular resistance (**Mushtaq *et al*., 2021**).

4.3.3. Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on Serum malodialdehyde (MDA) Levels in Male Rats.

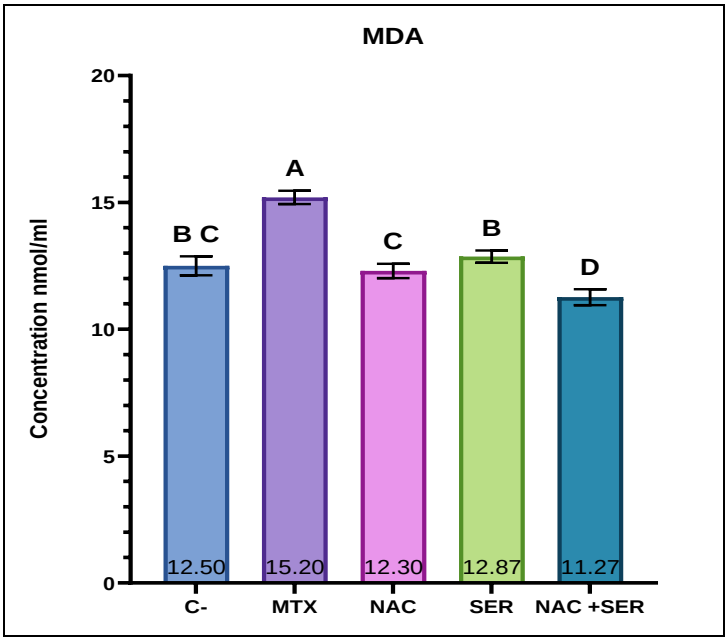


Figure (4.11) Comparison of malondialdehyde (MDA) levels in the Control, MTX, NAC, SER, and NAC+SER groups.

Data is mean ± SD of MDA levels is represented in table 4.6, ($P<0.05$) (compared between Control and MTX group). MDA levels were significantly reduced by NAC, SER and NAC+SER treatment and NAC+SER produced the lowest values.

Table (4.6) Comparison of the serum (MDA) levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation
MDA	C-	12.50 BC	.37417
	MTX	15.20 A	.26077
	NAC	12.30 C	.28284
	SER	12.86 B	.24221
	NAC +SER	11.26 D	.31411
LSD	0.354		

The level of MDA, a well-known indicator of lipid peroxidation, were significantly highest in MTX group. NAC dropped with SER and negative control(C-)and NAC+SER group .

The elevation of the MDA levels in the MTX group suggests a considerable oxidation of the membrane lipid indicating the unregulated activity of ROS released as a side effect of MTX application. This finding agreed with that of (**Türkez *et al*. 2016**), for increased MDA in response to MTX exposure correlating with histological evidence of cellular degeneration.

Combined, these results highlight the protective synergism between the antioxidative capacity of NAC and the anti-inflammatory action of SER, as shown by the notably low MDA levels in the NAC+SER group. By minimizing lipid peroxidation and maintaining membrane stability, this combination regimen probably prevents cascades that lead to oxidative lesion formation. Similar results were observed by (**Küçük *et al*.,2021**), demonstrating that comorbidity treatments are more effective than monotherapy in models of oxidative stress.

4.3.4 Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on Serum total antioxidant capacity (TAOC) Levels in Male Rats.

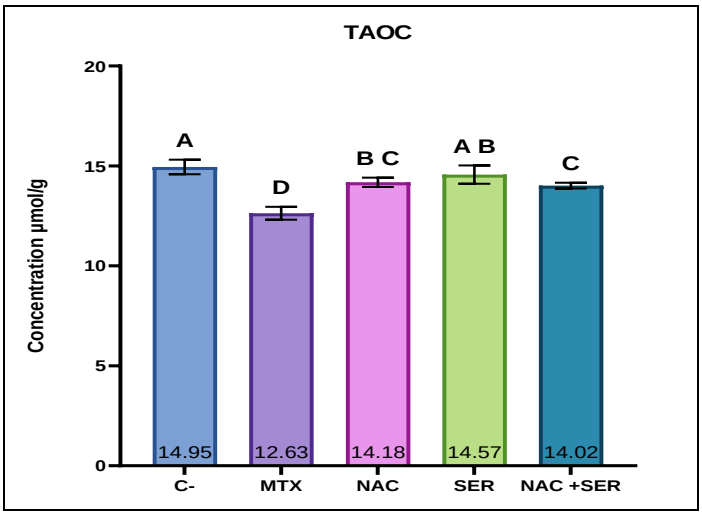


Figure (4.12) Comparison of total antioxidant capacity (TAOC) in the Control, MTX, NAC, SER, and NAC+SER groups.

total antioxidant capacity (TAOC) levels decreased significantly ($P<0.05$) in the MTX group compared to the Control, whereas NAC, SER and NAC+SER restored to different levels, NAC+SER and SER being closer to control.

Table (4.7) Comparison of the serum (TAOC) levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation
TAOC (umol/g)	C-	14.95 A	.36194
	MTX	12.63 D	.32042
	NAC	14.18 BC	.23166
	SER	14.57 AB	.45461
	NAC +SER	14.02 C	.14720
LSD	0.381		

However, the control group showed the highest level of TAOC, and followed by SER, NAC, NAC + SER, and MTX at the lowest level (Table 4.7).

Total antioxidant capacity is a comprehensive index that reflects the sum of the effects of all antioxidants in the plasma (**Silvestrini *et al*.,2023**). The lower TAOC in the MTX group confirms depletion of systemic antioxidant defenses and may indicate vulnerability to ROS-induced damage (**Sies *et al*., 2022**). **Arafa et al.in 2020** provide additional support for these data,who observed similar TAOC reductions from experimental models subjected to chemotherapeutic oxidative agents.

While both NAC and SER increased TAOC alone, the NAC+SER group did not exceed the individual phases in this parameter. It perhaps indicates a complex inter-play of the antioxidant systems or increased consumption of

antioxidant in a system rehabilitating after a marked oxidative stress, this is what (**Senol *et al*., 2014**) agreed with us on this research.

Nevertheless, the impressive partial recovery of TAOC in both subsets of treated animals emphasizes the beneficial potential of these agents in restoring the redox homeostasis and lowering the global oxidative burden (**Salman *et al*., 2022**).

4.4.5 Effect of Methotrexate, N-Acetylcysteine, Serratiopeptidase, and Their Combination on Serum Nuclear factor erythroid 2-related factor 2 (Nrf2) Levels in Male Rats.

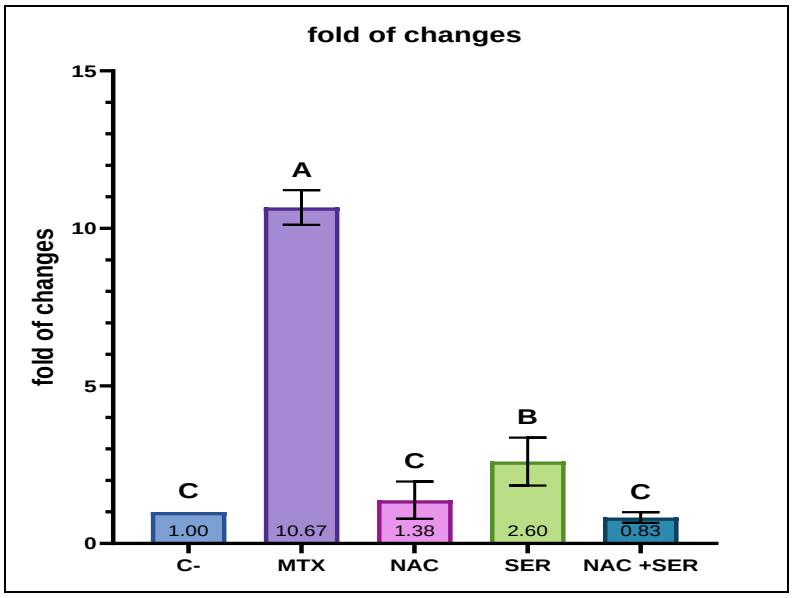


Figure (4.13) Comparison of Nuclear factor erythroid 2-related factor 2 (Nrf2) level in the Control, MTX, NAC, SER, and NAC+SER groups.

Methotrexate group had significantly ($P<0.05$) elevated Nrf2 as compare to Control. Nrf2 levels were significantly decreased by treatment in the NAC, SER, whereas NAC+SER treatment returned levels back to near control.

Table (4.8) Comparison of the serum (Nrf2) levels (mean ± SD) among the Control, Methotrexate (MTX), Serratiopeptidase (SER), N-acetylcysteine (NAC), and NAC+SER groups in male rats.

Parameter	Groups	Mean	Std. Deviation	Std. Error
	C-	1.00 C	.00000	.00000
Nrf2	MTX	10.67 A	4.85421	2.42710
	NAC	1.37 C	.59090	.29545
	SER	2.60 B	.76158	.38079
	NAC +SER	0.82 C	.17078	.08539
LSD	3.337			

The Expression levels of Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates the expression of antioxidant response elements (ARE)-driven genes, including glutathione S-transferases (GST), heme oxygenase-1 (HO-1), and NAD(P)H quinone oxidoreductase 1 (NQO1). These enzymes are critical for maintaining redox homeostasis and protecting tissues from oxidative damage (**Zheng *et al*., 2024**).

Nuclear factor erythroid 2-related factor 2 (Nrf_2) stretches an idea about the number of cells undergoing oxidative damage and its levels were highest in the MTX group and were gradually decreasing from SER→NAC→C−→NAC+SER which had the least count.

Methotrexate Group showed a significant increase in (Nrf2) level, which is also an indicative of excessive oxidative stress leading to cellular injury (**Al-kraaled *et al*., 2022**). MTX is known to cause apoptosis and necrosis via ROS overproduction, leading to impaired mitochondrial and nuclear functions (**Ezhilarasan, 2021**). As seen from (Nrf2) in the NAC+SER group, the cellular oxidative injury were effectively alleviated using NAC+SER combination.

This combination, will be potentially beneficial in cytoprotection by simultaneously reducing ROS generation, augmenting antioxidant responses, and preserving cellular integrity (**Chaudhary *et al*., 2023**).

(Abd El-Aziz *et al*., 2021) proved the same result when found More importantly, the multi-target therapies using antioxidants and anti-inflammatory agents simultaneously were shown to produce better results in preventing histological damage and markers of oxidative cells than the single agents .

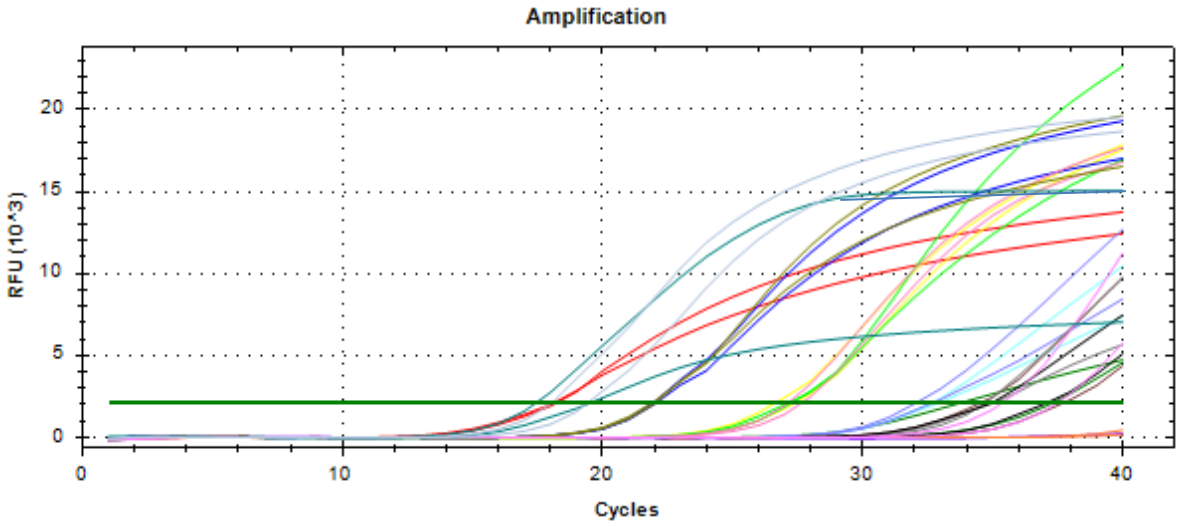


Figure (4.14) Graph of Nuclear factor erythroid 2-related factor 2 (Nrf2) level in the Control, MTX, NAC, SER, and NAC+SER groups.

This graph shows an amplification plot typical of a qPCR experiment, tracking fluorescence (RFU, relative fluorescence units) over cycles. The x-axis represents the number of PCR cycles, and the y-axis shows fluorescence, indicating the amount of DNA amplified.

This curved of the expression level of Nrf2 across different sample groups, including a MTX (methotrexate)-treated group and control or comparison groups show the amplification plot demonstrates an increased expression of Nrf2 in the MTX-treated group, as indicated by the earlier and steeper rise in fluorescence compared to other groups (**Ebrahimi *et al*., 2019**). The MTX

group curves cross the threshold earlier (lower Ct values), reflecting higher initial levels of Nrf2 mRNA. In contrast, the control or untreated groups show delayed or minimal amplification, suggesting normal or baseline expression levels of Nrf2. This upregulation of Nrf2 in response to MTX may indicate a cellular stress response, consistent with the role of Nrf2 in oxidative stress regulation , These effects are in line with the findings by (**Al-khawalde *et al*., 2022**).

4.4 Histological study

Histopathological examination of the testicular tissue was conducted for **five** experimental groups using hematoxylin and eosin (H&E) staining under a light microscope at 10x magnification, aiming to assess the structural alterations following various treatments. The control group exhibited normal testicular architecture, with well-organized seminiferous tubules, clearly distinguished germ cells, Sertoli cells, and interstitial Leydig cells. The interstitial connective tissue appeared intact and well preserved.

Sections from the N-acetylcysteine (NAC)-treated group showed generally preserved testicular histology, with mild changes in the interstitial connective tissue, ranging from slight disruption to minimal hyperplastic features.

The Serratiopeptidase-treated group also demonstrated mild hyperplastic changes in the interstitial area, while maintaining the overall integrity of the seminiferous epithelium. In the group treated with a combination of NAC and Serratiopeptidase, the tissue appeared mostly normal, with only mild structural alterations observed in the interstitial regions.

In contrast, the Methotrexate-treated group revealed marked pathological changes, including extensive damage to the seminiferous tubules, degeneration of germ cells and Sertoli cells, disruption of interstitial architecture, and pronounced

vascular congestion. These histological findings reflect varying degrees of testicular tissue alterations across groups depending on the type of intervention applied.

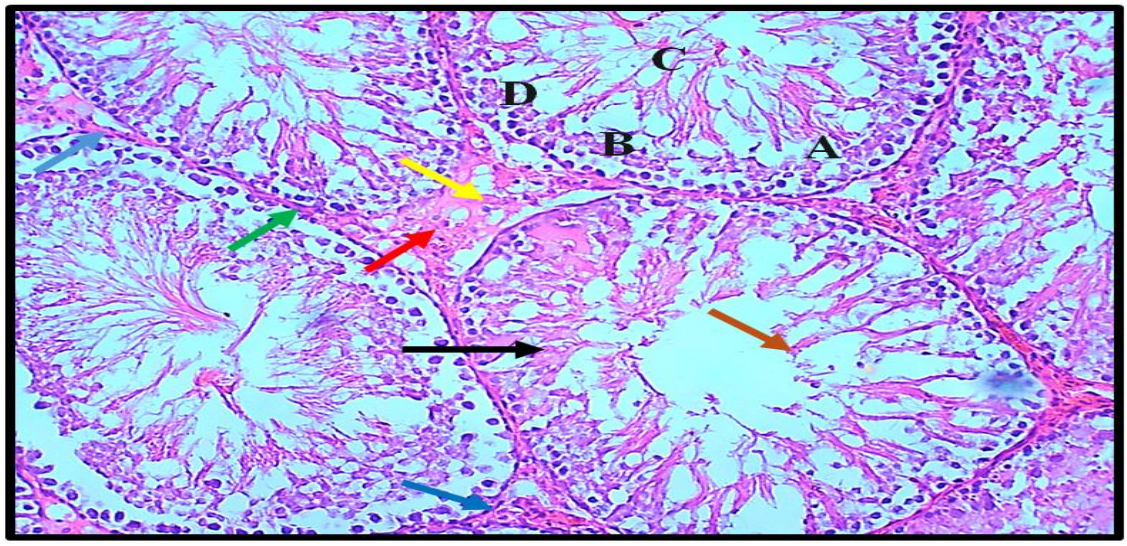


Figure (4.15) The histological section of the testis in rats in the control group. The section shows the normal texture of testicular tissue as appear as : seminiferous tubules (Black arrows), interstitial cells of Leydig (red arrow), interstitial connective tissue (yellow arrow), fibroblast (blue arrow), base rent membrane (green arrow), spermatide (brown arrow) , primary spermatocytes **(A),** Sertoli cells **(B),** Spermatid **(C)** and Spermatogonia **(D)**. The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnification scale.

The Figure above shows the histological section of the testis obtained from the control group. Photomicrographs of testis sections from display intact architecture characterized by normal seminiferous tubules with well-defined basement membrane and clear differentiation of the germinal epithelium including spermatogonia, primary spermatocytes, spermatids and Sertoli cells in addition to normal appearance of interstitial Leydig cells. The well-developed histology of the testicular tissue in control healthy rats confirms the structural and functional integrity, serving as a baseline reference for experimental groups. An intact basement membrane, coupled with orderly germ cell arrangement, indicates normal spermatogenesis while, (**D. S. Johnston *et al*. 2018**) stated that both testes

morphology and Sertoli-germ cell adjacent relationship are required for normal spermatogenic cycle.

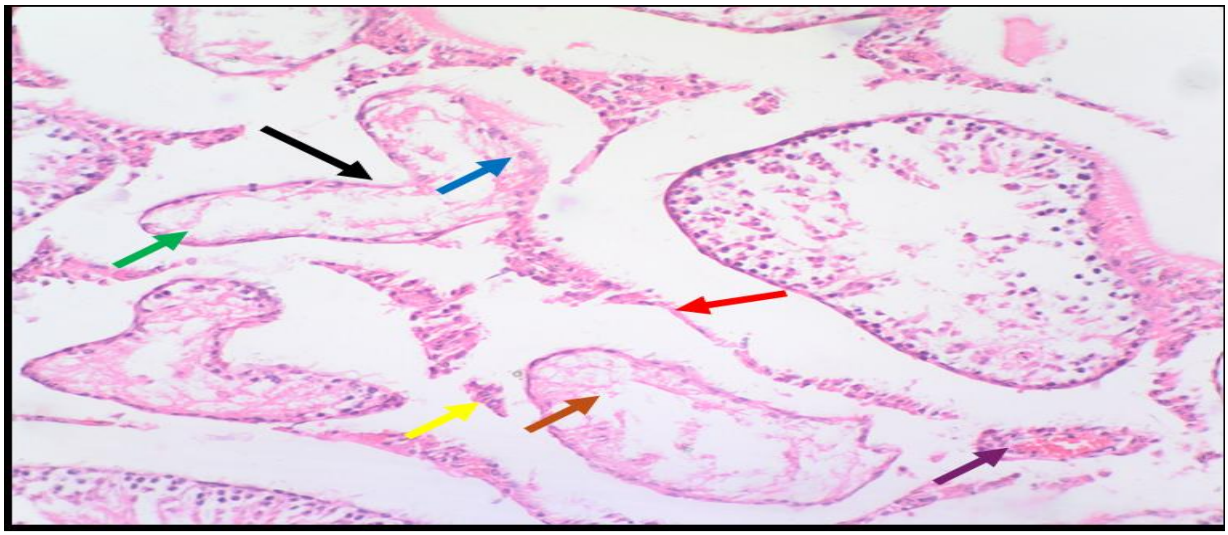


Figure (4.16) The histopathological section in testes of rats that treated with Methotrexate (IP administered 5 mg/kg daily). The section shows sever damage of seminiferous tubules of testicular tissue (Black arrow) with severe damage of interstitial connective tissue (Red arrow). The section shows sever necrotic lesion in the spermatocytes (Green arrow), Sertoli cells (Blue arrow) , spermatids (Brown arrow) and interstitial cells of Leydig (Yellow arrow). The section shows sever blood vessels congestion (Purple arrow).The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnification scale.

Severe damage of seminiferous tubules, extensive necrosis of germ cells (spermatocytes, spermatids, Sertoli cells and Leydig cells), congestion of blood vessels with destruction of interstitial connective tissue is shown in this image.

This trend developed as expected for MTX-induced testis toxicity, which is mediated by excessive oxidative stress, mitochondrial dysfunction, and DNA breakage. According to (**Yang *et al.*, 2021**), MTX causes arrest of the spermatogonia cell cycle and apoptosis of Sertoli and Leydigcells which leads to the collapse of endocrine and spermatogenic function of the testis (**Rajizadeh *et al.*,2024).**

Vascular congestion and cellular necrosis resemble what was described in (**Adlan *et al*.,2024**), and suggest that MTX-induced organ damage is oxidative but also inflammatory and ischemic. This outcome is consistent with the marked decline noted in hormonal and semen analysis, providing further support for the importance of oxidative injury in MTX toxicity(**Borovskaya *et al*., 2021**).

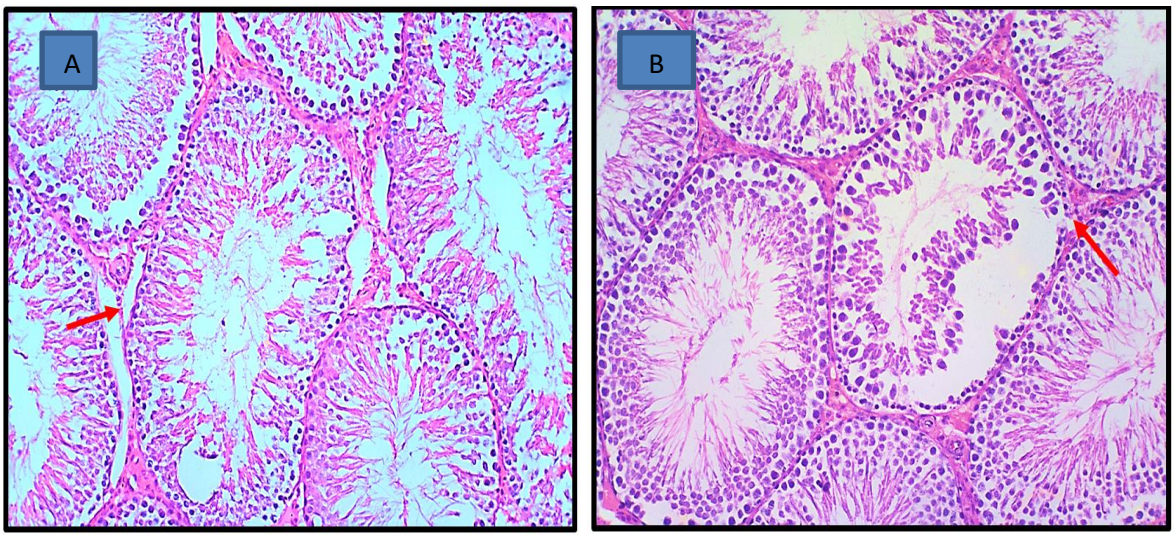


Figure (4.17) The histopathological section of the testis in rats that treated with (Nacetylcysteine, 600mg/kg/day, orally). Section A shows the normal texture of testicular tissue with mild damage of testicular interstitial connective tissue (Red arrows). Section B shows the normal texture of testicular tissue with mild hyperplastic changes of testicular interstitial connective tissue (Red arrows).The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnification scale.

This section demonstrates generally preserved testicular architecture with minor damage to interstitial connective tissue. There are no large areas of necrosis or germ cell degeneration; seminiferous tubules appear intact. This one minor modification is consistent with the cytoprotective and antioxidant function of NAC, including free radical scavenging, membrane stabilization, and restoration of glutathione levels. Experimental studies like **(Mohammadi-Sardoo *et al*.,2018; Abedini Bajgiran *et al*.,2023)** confirm that in chemically induced

oxidative stress, NAC protects testicular tissue, especially Sertoli cell function and apoptosis.

The testicular tissue in this section still looks generally normal except mild hyperplastic changes of the interstitial connective tissue are seen. These changes might be considered a somewhat oxidative stress response regulated by the N-acetylcysteine protective effect.

These hyperplastic changes are most likely a low-grade regenerative response and not pathological fibrosis, which reflects an ongoing balance of damage and healing. How support recent search (**Heinrich, & DeFalco,2020**).

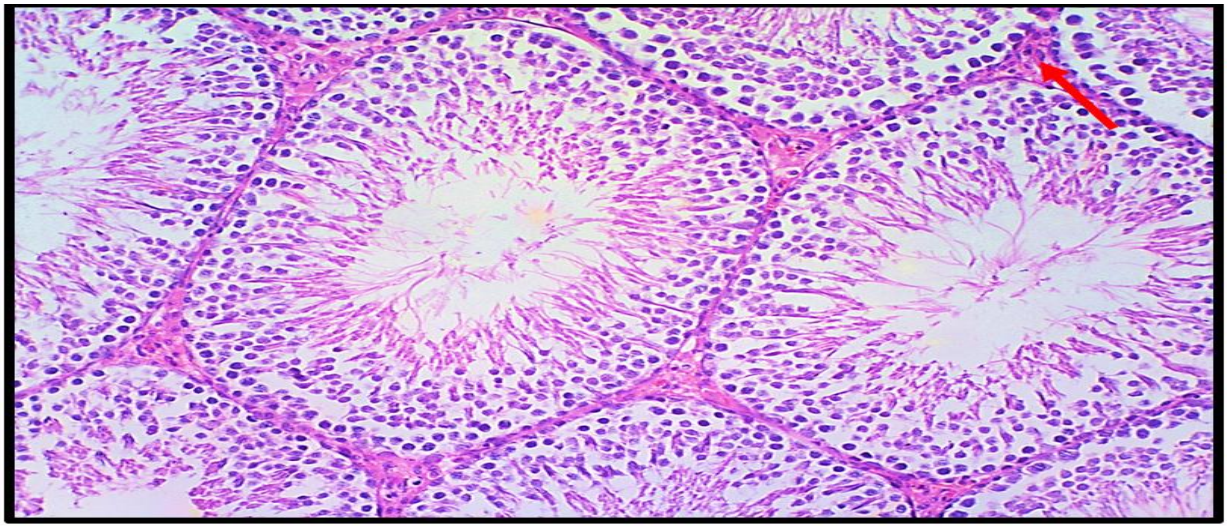


Figure (4.18) The histopathological section of the testis in rats that treated with (Serratiopeptidase 5 mg/kg/day, orally). The section shows the normal texture of testicular tissue with mild hyperplastic changes of testicular interstitial connective tissue (Red arrows). The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnification scale.

Mild hyperplastic changes in the interstitial tissue are also demonstrated in this section, but overall seminiferous architecture is preserved. Serratiopeptidase, a proteolytic enzyme with anti-inflammatory abilities, may have helped minimize edema and improve microvascular blood flow in the testis.

Studies such as Jamal et al. (2025) have demonstrated that Serratiopeptidase enhances blood flow and lymphatic drainage, thus promoting resolution of local inflammation and prevention of residual fibrosis. Whilst hyperplasia is apparent, its subtle degrees allow SER to ameliorate inflammation and tissue remodelling (**Che Man *et al.*, 2020**).

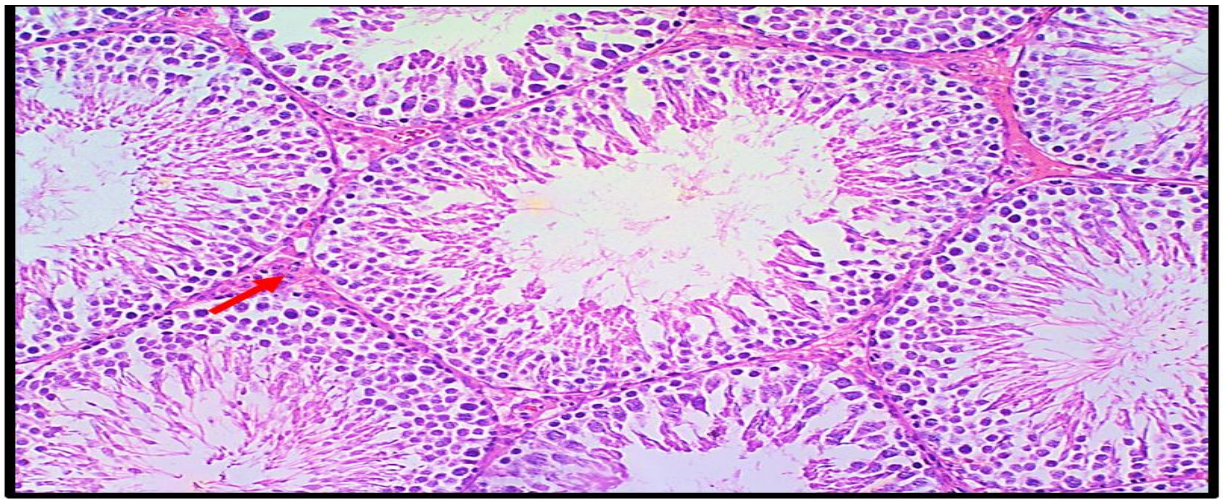


Figure (4.19) The histopathological section of the testis in rats that treated with Serratiopeptidase (5 mg/kg/day, orally) + N acetylcysteine (600mg/kg/day, orally). The section shows the normal texture of testicular tissue with mild hyperplastic changes of testicular interstitial connective tissue (Red arrows). The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnification scale.

The combination therapy group shows testicular architecture very similar to control and only mild hyperplastic changes in the interstitial space.

NAC had a dual protective role; it scavenged free radicals, which were released from inflammatory cells, while SER inhibited inflammatory cascades, the synergistic role of NAC and SER when co-administered likely protected against SER-associated inflammatory damage. This concurs with what was presented by (**Raghu *et al.*,2021**), which showed that combination therapies provide better histological and functional recovery in oxidative tissue injury models. This finding with minimal pathological finding among this group, indicate effective cellular protection, mirroring the results observed on hormonal

and semen parameters confirmatory to protective synergy as in search down as (**Ezzat et al.,2023**).

Chapter Five

Conclusions and Recommendations

5. Conclusions & Recommendations

5.1 Conclusions

This study investigated the protective effects of N-acetylcysteine (NAC) and Serratiopeptidase (SER), individually and in combination, against methotrexate (MTX)-induced testicular damage in male rats. The experimental results demonstrated the following conclusions:

1. Methotrexate Toxicity: MTX significantly disrupted testicular function, causing a marked decline in testosterone and LH while increase in FSH levels. It also led to elevated levels of oxidative stress markers such as malondialdehyde (MDA), while the essential antioxidant enzymes including SOD show increase and decrease in glutathione (GSH) , also Sperm count, motility, morphology, and viability were all significantly impaired Histologically, MTX induced extensive damage in testicular tissue including necrosis, vascular congestion, and architectural disintegration of seminiferous tubules.
2. Efficacy of N-acetylcysteine (NAC) and Serratiopeptidase (SER): NAC and SER treatment markedly improved hormonal profiles and antioxidant levels. Its function as a precursor to GSH ,NAC improve the sperm viability, motility, and histological structure by scavenging reactive oxygen species and enhancing cellular antioxidant capacity.
3. Synergistic Effect of NAC + SER: The combination of NAC and SER demonstrated a synergistic protective effect, outperforming each agent used alone. It led to significant normalization of hormonal levels, enhancement of antioxidant defense (including GSH and TAOC), and maintenance of normal testicular tissue. This group showed the closest resemblance to the control group in terms of both biochemical and histological parameters
4. The results provide strong evidence that NAC, especially when combined with SER, offers substantial protection against such damage, making this combination a promising approach for mitigating chemotherapy-related reproductive toxicity.

5.2 Recommendations

1. further molecular and ultrastructural studies are needed to gain a better understanding of how methotrexate works.

2. further studies are required to investigate the therapeutic potential of serratiopeptidase, clarify its mechanism(s) of action, and assess the safety of various doses and formulations. Thoroughly designed in vitro, in vivo, and clinical studies will aid in establishing the application of serratiopeptidase across different therapeutic domains.

Employing a differential dose or period of gavage could potentially strengthen the effects of NAC because of its capacity to enhance other antioxidant states.

Chapter Six

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APPENDIXES

Appendix(I): Estimation of Testosterone Hormone (T) Concentration (ng/ml).

Principle:

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to DHT. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for DHT is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain DHT and HRP conjugated DHT antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of DHT. You can calculate the concentration of DHT in the samples by comparing the OD of the samples to the standard curve.

Sample preparation .

Serum preparation.

After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2000 - 3000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifugated again.

The test procedure was conducted in accordance with the following steps:

1. Dilution of Standards Ten wells are set for standards in a Microelisa stripplate. In Well 1 and Well 2, 100µl Standard solution and 50µl Standard Dilution buffer are added and mixed well. In Well 3 and Well 4, 100µl solution from Well 1 and Well 2 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is

discarded from Well 3 and Well 4. In Well 5 and Well 6, 50µl solution from Well 3 and Well 4 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 7 and ell 8, 50µl solution from Well 5 and Well 6 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 9 and Well 10, 50µl solution from Well 8 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 9 and Well 10. After dilution, the total volume in all the wells are 50µl and the concentrations are 480 pg/ml, 320 pg/ml,160 pg/ml, 80 pg/ml and 40 pg/ml, respectively

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.
3. Incubation: incubate 30 min at 37℃ after sealed with Closure plate membrane.
4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).
5. ashing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.
6. Add 50 µl HRP-Conjugate reagent to each well except the blank control well.
7. Incubation as described in Step 3.
8. ashing as described in Step 5.
9. Coloring: Add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubate at 37℃ for 15 minutes. Please avoid light during coloring.

10. Termination: add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.
11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Precision .

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level Rat DHT were tested 20 times on one plate, respectively. Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level Rat DHT were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%.

appendix (II): Estimation Follicular Stimulating Hormone (FSH) Concentration (mlU/ml).

Principle.

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to FSH Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for FSH is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain FSH and HRP conjugated FSH antibody will appear blue in color and then turn

yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of FSH You can calculate the concentration of FSH in the samples by comparing the OD of the samples to the standard curve.

Sample preparation .

Serum preparation.

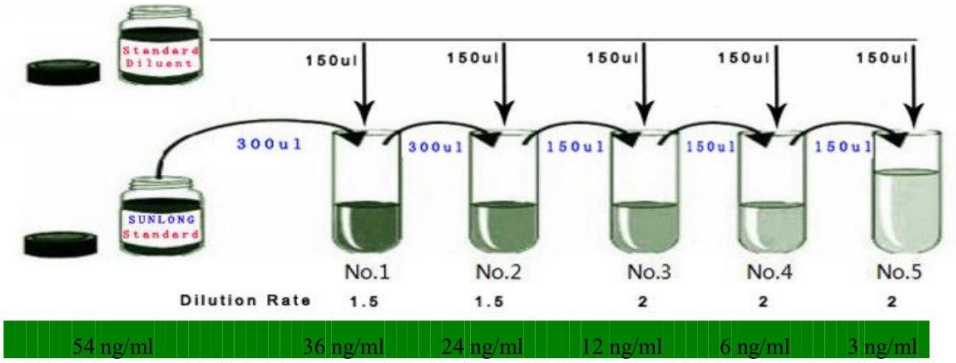
After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2000 – 3000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifugated again.

Procedure.

1. Dilution of Standards

   Dilute the standard by small tubes first, then pipette the volume of 50ul from each tube to microplate well,each tube use two wells, total ten wells.

36 ng/ml	Standard No.1	300µl Original Standard + 150µl Standard diluents
24 ng/ml	Standard No.2	300µl Standard No.1 + 150µl Standard diluents
12 ng/ml	Standard No.3	150µl Standard No.2 + 150µl Standard diluent
6 ng/ml	Standard No.4	150µl Standard No.3 + 150µl Standard diluent
3 ng/ml	Standard No.5	150µl Standard No.4 + 150µl Standard diluent



2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution

factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: incubate 30 min at 37℃ after sealed with Closure plate membrane.
4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and20 times for 48T).
5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.
6. Add 50 μl HRP-Conjugate reagent to each well except the blank control well.
7. Incubation as described in Step 3.
8. Washing as described in Step 5.
9. Coloring: Add 50 μl Chromogen Solution A and 50 μl Chromogen Solution B to each well, mix with gently shaking and incubate at 37 ℃ for 15 minutes. Please avoid light during coloring.
10. Termination: add 50 μl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.
11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes afteradding stop solution.

Precision .

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level Rat DHT were tested 20 times on one plate, respectively. Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level Rat DHT were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%.

appendix (III): Estimation of Luteinizing Hormone (LH) Concentration (ng/ml).

Principle.

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antigen specific to LH-Ab. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antigen. Then a Horseradish Peroxidase (HRP)-conjugated antigen specific for LH-Ab is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain LH-Ab and HRP conjugated LH antigen will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of LH-Ab. You can calculate the concentration of LH-Ab in the samples by comparing the OD of the samples to the standard curve.

Sample preparation .

Serum preparation.

After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2000 - 3000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifugated again.

Procedure.

1. Dilution of Standards

Dilute the standard by small tubes first,then pipette the volume of 50ul from each tube to microplate well,each tube use two wells ,total ten wells.

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40μl Sample dilution buffer and 10μl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.
3. Incubation: incubate 30 min at 37℃ after sealed with Closure plate membrane.
4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and
   20 times for 48T).
5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash
   solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.
6. Add 50 μl HRP-Conjugate reagent to each well except the blank control well.
7. Incubation as described in Step 3.
8. Washing as described in Step 5.
9. Coloring: Add 50 μl Chromogen Solution A and 50 μl Chromogen Solution B to each well, mix with gently shaking and incubate at 37 ℃ for 15 minutes. Please avoid light during coloring.
10. Termination: add 50 μl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.
11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Precision .

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level Rat DHT were tested 20 times on one plate, respectively. Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level Rat DHT were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%.

appendix (IV): Serum Malondialdehyde Measurement (MDA).

Malondialdehyde was estimated by Thiobarbituric acid (TBA) assay method of Buege & Aust, (1978) on spectrophotometer

Principle:

This method quantifies lipid peroxides by measuring aldehyde breakdown products of lipid peroxidation. Basic principle of the method is the reaction of one molecule of malondialdehyde and two molecules of Thiobarbituric acid to form a red MDA-TBA complex which can be measure at 535 nm.

Stock TCA – TBA – HCl

Reagent:

It was prepared by dissolving 15% W/V trichloroacetic acid and 0.375% W/V Thiobarbituric acid and 0.25N HCl to make 100 ml (2.1 ml of concentrated HCl in 100 ml). This solution was mildly heated to assist in the dissolution of TBA. Dissolved 15 gm TCA and 0.375 mg Thiobarbituric acid in 0.25 N HCl and volume was made up to 100 ml with 0.25 N HCl.

Procedure:

To 0.4 ml of serum, 0.6 ml TCA-TBA-HCl reagents were added. It was mixed well and kept in boiling water bath for 10 minutes. After cooling 1.0 ml freshly prepared 1N NaOH solution was added to eliminate centrifugation. This absorbance of pink colour was measured at 535 nm against blank which contained distilled water in place of serum. In blank 0.4 ml distilled water and 0.6 ml TCA-TBA-HCl reagent was mixed and boiled. Blank was always taken.

Calculation:

Extinction coefficient of MDA at 535 nm is = 1.56 × 105

MDA concentration = χ / 0.0624 nmol / ml.

Appendix (V) : Superoxide Dismutase (SOD) Activity.

Preparation

1. Tris buffer (pH 8.0): was prepared by dissolving 0.258 gm of tris and 0.111 gm of Ethylene diamine tetra acetic 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

Appendix (VI): Serum reduced glutathione concentration (GSH)

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).

Table 1

Reagents	Test	Control-test*	Standard	Blank
Serum	100 µl	100 µl	–	–
D.W.	–	1000 µl	100 µl	1100 µl
Hydrogen peroxide	1000 µl	–	1000 µl	–
Mix with vortex and incubate at 37 °C for 3 min, after that, add:				
Ammonium molybdate	4000 µl	4000 µl	4000 µl	4000 µl
After that, the tubes were kept at room temperature. Changes in absorbance were recorded at 374 nm against the reagent blank.				

4. 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 (VII): Serum Total antioxidant capacity (TAOC)

Determine the total antioxidant level composed of various antioxidant substances and antioxidant enzymes in the object. In biological, medical, and pharmaceutical research, the total antioxidant capacity of various body fluids such as plasma, serum, saliva, urine, cell or tissue lysates, plant or herbal extracts, and various antioxidant solutions are often tested.

The ability to reduce Fe3+- triphenyltriazine (Fe3+- TPTZ) to produce blue Fe2+- TPTZ under acidic conditions reflects the total antioxidant capacity.

Reagents and Equipment Required:

Spectrophotometer, 1 mL glass cuvette, water bath/constant temperature incubator, low temperature. centrifuge, mortar/homogenizer/cell ultrasonic crusher, sulfuric acid (>95%, AR), ice and distilled water.

Procedure:

Ⅰ. Sample preparation:

1. Serum, plasma, saliva or urine samples Plasma (anticoagulation with heparin or sodium citrate, avoid using EDTA), centrifuge at 5000 rpm/min for 10 min, take supernatant for test. Take serum, saliva or urine samples for direct determination, or they can be frozen at -80℃ (not exceeding 30 days) before measurement.

2. Cells or bacteria samples Collect cells or bacteria in centrifuge tubes. According to the ratio of cell or bacterial count (104): extract volume (mL) of 500-1000:1, add 1.0mL of pre cooled extract solution (it is recommended to take 5 million cells and add 1mL of pre cooled extract solution), sonicate the cells (power 200W, ultrasound on for 3 seconds, off for 9 seconds, total time for 3 minutes), then centrifuge at 10000rpm, 4℃ for 10 minutes, take the supernatant and place it on ice for testing.

3. Tissue sample: According to the ratio of tissue mass (g) to extract solution volume (mL) of 1:5-10 (it is recommended to weigh about 0.1g of tissue and add 1mL of pre cooled extract solution), perform ice bath homogenization, then centrifuge at 10000rpm and 4℃ for 10 minutes. Take the supernatant and place it on ice for testing.

Ⅱ. Determination procedure:

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1. Preheat the spectrophotometer for more than 30 min, adjust wavelength to 593 nm and set zero with distilled water.

2. Preparation of standard solution: Dilute 40 μmol/mL standard solution with distilled water to 0.1, 0.05, 0.025,0.0125, 0.00625, 0.003125 μmol/mL standard solution for later use.

3. The standard solution dilution can refer to the following table:

Number	Pre dilution concentration (μmol/mL)	Standard solution volume (μL)	Distilled water volume (μL)	Post dilution concentration (μmol/mL)
1	40	50	950	2
2	2	100	1900	0.1
3	0.1	1000	1000	0.05
4	0.05	1000	1000	0.025
5	0.025	1000	1000	0.0125
6	0.0125	1000	1000	0.00625
7	0.00625	1000	1000	0.003125

4. Take 500 μL of standard solution (distilled water for blank control), add to 500 μL of Reagent II. Mix thoroughly for 10 min, detect the absorbance in 593 nm, calculate ΔA=AS-AB. (AS: standard solution tube, AB: blank control tube.) The final concentration of Fe2+ is 0.05、0.025、0.0125、0.00625、0.003125 0.00156 μ mol/mL. The standard curve only needs to be done 1-2 times.

5. Operation table:

Reagent Name	Blank tube (A_B)	Test tube (A_T)
Solution mixture (μL)	900	900
Sample (μL)	-	30
Double distilled water (μL)	120	90
Mix thoroughly and react accurately at room temperature for 10 minutes. Take 1mL and measure the absorbance at 593nm in a 1mL glass cuvette. Calculate $\Delta A_T=A_T - A_B$. The blank tube only needs to be measured 1-2 times.		

Ⅲ. Calculation:

1. Create standard curve

Establish a standard curve based on the final concentration of Fe2+ (x, μmol/mL) and absorbance ΔA standard(y, ΔAS). According to the standard curve, calculate the sample concentration (x, μmol/mL) by substituting the Δ AT (y, ΔAT) into the formula**.**

2. Formula

Unit definition: the sample antioxidant capacity is indicated by the standard liquid ion concentration required for the same absorbance change (ΔA).

A. Protein concentration:

Total antioxidant capacity (μmol/mg prot) = x × Vrv÷ (Vs× Cpr) = 34× x ÷ Cpr

B. Sample weight:

Total antioxidant capacity (μmol/g weight) = x × Vrv÷ (Vs ÷ Ve ×W) =34× x÷ W

C. Cell amount:

Total antioxidant capacity (μmol/104cell) = x × Vrv÷ (Vs ÷Ve × N) = 34× x÷ N

D. Solution volume:

Total antioxidant capacity (μmol/mL) =x× Vrv÷ Vs =34×x

Ve: extract solution volume, 1 mL;

Vrv: total reaction volume, 1.02 mL;

Vs: sample volume, 0.03 mL;

W: sample weight, g;

Cpr: sample protein concentration, mg/mL;

N: cell amount, unit based on 104 (ten thousand).

Appendix (VIII): Procedure of Histological study

with aid of the light microscope as the following steps:

1) Fixation: - The specimen fixated in the formalin 10 % for 24 – 48 hours.

2) Washing and dehydration:- After fixation the specimens 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 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 clearing, 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 ℃. The heat causes the solvent to evaporate and the space within the tissues becomes filled with paraffin.

5) Sectioning :- After holds from the oven, the specimen let at room temperature to be solid and removed from their containers in order to sectioning they were put in the rotary microtome and were sliced by the microtome, a steel blade into sections 5 micrometers thick. The sections were floated on water bath (50–55oC), then transferred into glass slides coated with Mayers albumin as adhesive substance and left to dry.

6) Staining :- The histological sections of the studied organs were stained with Hematoxylin - Eosin stain.

الخلاصة

استكشفت الدراسة آثار الأضرار التي يسببها الميثوتريكسات على الجهاز التناسلي الذكري للجرذان والدور الوقائي المحتمل لسيراتيوببتيداز ون-أسيتيل سيستين. تم تقسيم خمسة وثلاثين من ذكور الجرذان البيضاء البالغة إلى خمس مجموعات: مجموعة التحكم السلبية: تلقت محلول ملحي طبيعي يوميًا لمدة 56 يومًا، مجموعة الميثوتريكسات: تلقت الميثوتريكسات 5 ملغم/كغم من وزن الجسم يوميًا عن طريق الحقن داخل الصفاق لمدة 56 يومًا. مجموعة ن-أسيتيل سيستين: تلقت الميثوتريكسات 5 ملغم/كغم من وزن الجسم يوميًا عن طريق الحقن داخل الصفاق و ن-أسيتيل سيستين 600 ملغم/كغم من وزن الجسم عن طريق الفم، يوميًا لمدة 56 يومًا. مجموعة سيراتيوببتيداز: تلقت ميثوتريكسات ٥ ملغ/كغ من وزن الجسم يوميًا عن طريق الحقن داخل الصفاق، وسيراتيوببتيداز ٥ ملغ/كغ من وزن الجسم عن طريق الفم، يوميًا لمدة ٥٦ يومًا. مجموعة :NAC+SER تلقت ميثوتريكسات ٥ ملغ/كغ من وزن الجسم يوميًا عن طريق الحقن داخل الصفاق، و٦٠٠ ملغ/كغ من إن-أسيتيل سيستئين عن طريق الفم، يوميًا لمدة ٥٦ يومًا، بالإضافة إلى سيراتيوببتيداز ٥ ملغ/كغ من وزن الجسم عن طريق الفم، يوميًا لمدة ٥٦ يومًا. أُعطيت الفئران مخدرًا بالكلوروفورم لإفقادها الوعي، ثم أُخذت عينات دم من القلب لفحصها. تشمل المؤشرات الحيوية للإجهاد التأكسدي انخفاض الجلوتاثيون(GSH) ، والمالونديالدهيد(MDA) ، والقدرة الكلية المضادة للأكسدة .(TAOC)مستويات الهرمونات التناسلية: التستوستيرون، هرمون منبه الجريبات (FSH) وهرمون ملوتن .(LH) وتم تشريح الحيوانات للحصول على أنسجة الخصية والبربخ للتحقيق النسيجي وتحليل الحيوانات المنوية وعامل نووي كريات الدم الحمراء المرتبط بالعامل 2 .(Nrf2) أظهرت نتائج التجارب أن سمية الميثوتريكسات عطلت وظيفة الخصية بشكل كبير، مما تسبب في انخفاض ملحوظ في هرمون التستوستيرون وهرمون الملوتن مع زيادة في مستويات .FSH كما أدى ذلك إلى ارتفاع مستويات علامات الإجهاد التأكسدي مثل مالونديالدهيد(MDA) ، في حين أظهرت إنزيمات مضادات الأكسدة الأساسية بما في ذلك SOD زيادة وانخفاض في الجلوتاثيون(GSH) ، كما تضرر عدد الحيوانات المنوية وحركتها وشكلها وقابليتها للحياة بشكل كبير. من الناحية النسيجية، تسبب الميثوتريكسات في تلف واسع النطاق في أنسجة الخصية بما في ذلك النخر واحتقان الأوعية الدموية والتفكك المعماري للأنابيب المنوية. أدت فعالية علاج-N أسيتيل سيستين وسيراتيوبيبتيديز إلى تحسين ملحوظ في مستويات الهرمونات ومضادات الأكسدة. يعمل-N أسيتيل سيستين، باعتباره مادة أولية للجلوتاثيون، على تحسين حيوية الحيوانات المنوية وحركتها وبنيتها النسيجية من خلال التخلص من أنواع الأكسجين التفاعلية وتعزيز قدرة الخلايا المضادة للأكسدة.وقد أظهر التأثير التآزري لـ N-acetylcysteine مع Serratiopeptidase تأثيرًا وقائيًا تآزريًا، متفوقًا على

كل عامل يُستخدم بمفرده. وقد أدى ذلك إلى تطبيع ملحوظ لمستويات الهرمونات، وتعزيز دفاعات مضادات الأكسدة (بما في ذلك GSH و(TAOC، والحفاظ على أنسجة الخصية الطبيعية.

الخلاصة: تُقدم النتائج دليلًا قويًا على أن NAC، وخاصةً عند دمجه مع SER، يوفر حماية كبيرة من هذا الضرر، مما يجعل هذا المزيج نهجًا واعدًا لتخفيف السمية التناسلية المرتبطة بالعلاج الكيميائي.

جمهورية العراق

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دراسة تأثير السيراتوبيبتيداز و ن-أسيتيل سيستين ضد الأضرار الناجمة عن الميثوتريكسات في خصيتي ذكور الفئران

رسالة مقدمة إلى

مجلس كلية الطب البيطري جامعة كربلاء لاستيفاء جزء من متطلبات

درجة ماجستير في الطب البيطري / الفسلجة

الطالب

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1446هـ **2025م**