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كفاءات وطنية
معايير عالمية
دراسة
للاستشارات والدراسات والترجمة
UNIVERSITY
drasah1
www.drasah.com
00966555026526
00966560972772
info@drasah.com

خدماتنا

شركة دراسة
للاستشارات و الدراسات والترجمة

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الاستشارات الأكاديمية

جمع المادة العلمية

الترجمة المعتمدة

drasah1
info@drasah.com
00966555026526
00966560972772
drasah.com


دراسة
للاستشارات والدراسات والترجمة
تواصل معنا
00966555026526
00966560972772
متواجدون على مدار الســـاعة

Effect of Lipid Peroxidation and Mitochondrial Molecular Genetic Changes on Reproductive System in Male Rat Models of Interstitial Cystitis

By

Raghad Husam Al-Amro

Supervisor

Dr. Ezidin Kaddumi

Associate Professor

Co-supervisor

Dr. AbdulFattah Fararjeh

Assistant Professor

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Faulty of Graduate Studies

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Abstract

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Interstitial cystitis (IC) is a chronic pain syndrome which exact pathophysiology remains unknown. and complications of IC. Most male patients diagnosed with interstitial cystitis suffer from debilitating reproductive system complications. The integrity of male reproductive system is critical to maintain continuity of the human species. The structural integrity of the male reproductive system reflects majorly on the efficiency of spermatogenesis. Mitochondria are one of the few organelles reserved in mature spermatozoa, suggesting its importance throughout spermatogenesis. Reactive oxygen species (ROS) are generated as byproducts of mitochondrial oxidative phosphorylation are essential for maintaining the functionality of spermatozoa.

However, increased levels of ROS can lead to cellular oxidative stress, which involve in a wide range of pathological conditions, from chronic inflammatory diseases to male infertility. In this study, 36 Sprague-Dawley rats were used, animals were categorized into six groups: control, acute hydrochloric acid (HCl) group, chronic HCl group, cyclophosphamide (CYP) group, sham HCl group and sham CYP group. In HCl groups, IC was induced acutely and chronically through surgically instilling HCl into the bladder. In CYP group, IC was induced through intraperitoneally injecting each subject with CYP. Biochemical thiobarbituric acid assay was used for quantification of malondialdehyde (MDA) levels as an indicator of peroxidative damage in several structures including the urinary bladder, testicular tissue, and spermatozoa. Relative mRNA expression levels of mitochondrial respiratory chain genes including *mt-ND1*, *mt-ND5*, mt-*CYB*, mt-*Co1*, *mt-ATP6*, and *mt-ATP8* were assessed as indicators of mitochondrial respiratory chain dysfunction. Data obtained from this study showed a significant increase in MDA levels confirming peroxidative damage in the urinary bladder, testicular tissue, and spermatozoa. Levels of mt-*ND1* and mt-*ND5* from complex I were significantly upregulated in testicular tissue and spermatozoa of chronic HCl model. On the contrary these genes were significantly downregulated in spermatozoa of CYP model. Similarly, mRNA levels of mt-*CYB* and mt-*CoI* from complex III and complex IV respectively, were significantly upregulated in both testicular tissue and spermatozoa of chronic HCl model on the contrary these genes were significantly downregulated in spermatozoa of CYP model. From complex V, mRNA levels of *mt-ATP6* and mt-*ATP8* were significantly upregulated in testicular tissue and spermatozoa of chronic HCl model. On the contrary these genes were significantly downregulated in spermatozoa of CYP model. In this study we concluded the role of peroxidative damage and mitochondrial respiratory chain dysfunction as possible mechanisms in male reproductive system dysfunction in animal models of induced interstitial cystitis.

الملخص

تأثير فوق أكسدة الدهون والتغيرات الجزيئية للميتوكوندريا على الجهاز التناسلي الذكري في نماذج الفئران الذكور لالتهاب المثانة الخلالي

المشرف الرئيسي

د. عزالدين غالب القدومي

أستاذ مشارك

المشرف المشارك

د. عبد الفتاح فرارجة

أستاذ مساعد

التهاب المثانة الخلالي هو متلازمة ألم مزمن لا تزال الفيزيولوجيا المرضية الدقيقة للمرض غير معروفة. يعاني معظم المرضى الذكور الذين تم تشخيص إصابتهم بالتهاب المثانة الخلالي من مضاعفات منهكة في الجهاز التناسلي. سلامة الجهاز التناسلي الذكري أمر بالغ الأهمية للحفاظ على استمرارية الجنس البشري. تنعكس السلامة الهيكلية للجهاز التناسلي الذكري بشكل كبير على كفاءة تكوين الحيوانات المنوية. الميتوكوندريا هي واحدة من العضيات القليلة المحفوظة في الحيوانات المنوية الناضجة، مما يشير إلى أهميتها خلال عملية تكوين الحيوانات المنوية. يتم إنشاء أنواع الأكسجين التفاعلية كمنتجات ثانوية من الفسفرة المؤكسدة للميتوكوندريا ضرورية للحفاظ على وظائف الحيوانات المنوية. ومع ذلك، يمكن أن تؤدي المستويات المتزايدة من أنواع الأكسجين التفاعلية إلى الإجهاد التأكسدي الخلوي، والذي ينطوي على مجموعة واسعة من الحالات المرضية، من الأمراض الالتهابية المزمنة إلى العقم عند الذكور. في هذه الدراسة، تم استخدام 36 جرذًا من جرذان Sprague-Dawley، وتم تصنيف الحيوانات إلى ست مجموعات: مجموعة التحكم، مجموعة حمض الهيدروكلوريك الحاد، مجموعة حمض الهيدروكلوريك المزمن، مجموعة السيكلوفوسفاميد، مجموعة حمض

الهيدروكلوريك الزائف ومجموعة السيكلوفوسفاميد الزائف. في مجموعات حمض الهيدروكلوريك، تم إحداث التهاب المثانة الخلالي بشكل حاد ومزمن من خلال غرس حمض الهيدروكلوريك جراحياً في المثانة. في مجموعة السيكلوفوسفاميد، تم تحريض التهاب المثانة الخلالي من خلال الحقن داخل الصفاق لكل موضوع باستخدام السيكلوفوسفاميد. تم استخدام مقايسة حمض الثيوباربيتوريك البيوكيميائية لتقدير مستويات المالونداياالديهايد كمؤشر على تلف الأكسدة في العديد من الاجزاء بما في ذلك المثانة البولية وأنسجة الخصية والحيوانات المنوية. تم تقييم مستويات تعبير mRNA النسبية لجينات السلسلة التنفسية للميتوكوندريا بما في ذلك mt-ND1 وmt-ND5 وmt-CYB وmt-Co1 وmt-ATP6 وmt-ATP8 كمؤشرات لخلل في السلسلة التنفسية للميتوكوندريا. أظهرت البيانات التي تم الحصول عليها من هذه الدراسة زيادة معنوية في مستويات المالونداياالديهايد التي تؤكد الضرر التأكسدي في المثانة البولية وأنسجة الخصية والحيوانات المنوية. تم زيادة في مستويات mt-ND1 وmt-ND5 من المركب I بشكل كبير في أنسجة الخصية والحيوانات المنوية لنموذج حمض الهيدروكلوريك المزمن. على العكس من ذلك، تم انخفاض في هذه الجينات بشكل كبير في الحيوانات المنوية من نموذج السيكلوفوسفاميد. وبالمثل، تم ارتفاع مستويات mRNA من mt-CYB وmt-CoI من المركب III والمركب IV على التوالي، بشكل كبير في كل من أنسجة الخصية والحيوانات المنوية لنموذج حمض الهيدروكلوريك المزمن على عكس هذه الجينات تم انخفاضها بشكل كبير في الحيوانات المنوية من نموذج السيكلوفوسفاميد. من المركب V، تم ارتفاع في مستويات mRNA من mt-ATP6 وmt-ATP8 بشكل كبير في أنسجة الخصية والحيوانات المنوية لنموذج حمض الهيدروكلوريك المزمن. على العكس من ذلك، تم انخفاض هذه الجينات بشكل كبير في الحيوانات المنوية من نموذج السيكلوفوسفاميد. في هذه الدراسة، توصلنا إلى دور الضرر الناتج عن الأكسدة والخلل الوظيفي في سلسلة الميتوكوندريا التنفسية كآليات محتملة في ضعف الجهاز التناسلي الذكري في النماذج الحيوانية من التهاب المثانة الخلالي المستحث.

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Chapter One

Introduction

Interstitial cystitis (IC), often referred as a bladder pain syndrome (BPS), is defined as a syndrome of chronic pain with no apparent signs of infection and is often mistakenly diagnosed as a recurrent urinary tract infection. IC is characterized by pelvic and bladder pain associated with urinary frequency, sudden urge to void, and nocturia. The pain may vary among individuals from mild pressure to severe pain (Hanno et al., 2011). As IC advances pain usually worsens and becomes the most dominating and debilitating symptom, having a considerable influence on patients' personal and professional lives, the resulting pain may finally lead to cystectomy as a last resort. Up to date, the etiology and pathophysiology of this disease remain widely elusive. several theories regarding the pathogenesis of IC have been proposed including, epithelial and urothelium dysfunction, neurogenic inflammation, overactivation of mast cells, a possible role of autoimmunity, and the involvement of a crosstalk between pelvic viscera, in addition, Studies have shown alterations and anomalies in the gene expression of inflammatory proteins, proteins associated to oxidative and nitrosative stress, and proteins of tight junctions in epithelial cells of individuals with IC. (Keay et al., 2012; Nickel, 2004; Shie & Kuo, 2011).

Up to this day, the etiology of IC remains widely elusive. However, it is considered a complex disease with numerous components, it is thought to be a process that starts with unidentified bladder injury that triggers inflammatory responses, endocrine, and neural crosstalk, and sensitization events. In terms of pathogenesis, many pathophysiologic pathways are currently being explored. However, evidence suggesting that decreased blood flow, hypoxia, ischemia, and reperfusion resulting in the generation of free radicals and subsequent oxidative damage are the main cause of dysfunctions such as IC/BPS is steadily growing (Lovick, 2016; Malone et al., 2014).

Considering the complex nature of the pathophysiology of IC, several animal models have been proposed for a better understanding of the mechanisms responsible for IC/BPS. Bladder-centric models are frequently used to simulate situations of lower urinary tract inflammation (LUTI). Few methods were used widely over the years including, intravesical administration of acetic acid,

protamine sulfate, hydrochloric acid (HCl), acrolein, or injection of cyclophosphamide intraperitoneally (Bjorling et al., 2011).

Urogenital describes something that has both urinary and genital roots. Because males' urinary and reproductive systems merge, the term urogenital is employed. The testes, vas deferens, ejaculatory ducts, ureter, urethra, bladder, renal pelvis, and the penis, all are components of the male urogenital system. The systems are not entirely separated in males (semen travels through the urethra), but they are morphologically seperated in females. There is a strong correlation between inflammation of the male urogenital system and impairment of the reproductive system. (Gurung et al., 2020; Schuppe et al., 2017).

Infertility problems can be extremely difficult to overcome, given that spermatogenesis is dependent on the structural integrity of the testis, structural changes in the testis cause a major effect on sperm quality and therefore on fertility (Kaddumi et al., 2021).

Free radicals are normally produced at the end of metabolic processes. Detrimental oxidative reactions can occur during these activities, enzymatic and nonenzymatic antioxidant detoxification systems prevent these reactions from exacerbation. An oxidative state arises when the antioxidant systems fail to counteract the increased production and propagation of free radicals. The consequence of this imbalance “oxidative stress” develops which related to more than a hundred of diseases and considered a significant contributor and a key component of chronic pain diseases (Sánchez-Domínguez et al., 2015).

Reactive oxygen species (ROS) are produced endogenously during tissue injury and inflammation by macrophages and neutrophils infiltrating the area. Lipid peroxidation begins when free radicals covalently bind to membrane receptors, changing the unsaturated fatty acid/protein ratio. Lipid peroxides are unstable and readily decompose into a variety of compounds, including aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal (HNE). As a result, the amount of MDA and HNE measured represents the extent of lipid peroxidation in tissues (Bozkurt et al., 2018).

Mitochondria is a versatile organelle found almost in every eukaryote, commonly known as the powerhouse of the cell. In many cells, the mitochondrion creates a dynamically controlled network through several tightly linked mechanisms (Wu et al., 2019a). In addition to the production of adenosine triphosphate (ATP) as the main form of human energy, the mitochondria are involved in a vast array of fundamental and sophisticated cellular physiological functions from cellular homeostasis to apoptosis. ROS produced during oxidative phosphorylation play a critical role in

cellular communication and signaling by a variety of mechanisms in mitochondria. Excessive generation of ROS put the cell in a state of oxidative stress, and subsequent detrimental oxidation of vital cellular components (Herst et al., 2017).

Though all the cytoplasm is nearly removed during spermatogenesis, mitochondria are maintained in mature spermatozoa, suggesting the significant outstanding role they play in the integrity of reproductive system and male fertility (A. Agarwal, Durairajanayagam, Halabi, et al., 2014).

Although minimal amounts of ROS are essential to reassure the ongoing of spermatogenesis and spermatozoa functionality, structures with high content of lipid are more susceptible to undergo lipid peroxidation as a result of oxidative damage. The cell membranes of mature spermatozoa and testicular tissue contains high lipid content (mainly polyunsaturated fatty acid “PUFA”), excess ROS in the reproductive system with inability of the antioxidant defense system to counteract, PUFA-containing structures undergo lipid peroxidation (Agarwal, Durairajanayagam, & du Plessis, 2014; Agarwal, Durairajanayagam, Halabi, et al., 2014).

Oxidative stress (OS) plays a major role in the etiology of male infertility, with 30% to 80% of infertile men showing an elevation of ROS in seminal fluid. Male oxidative stress infertility (MOSI) is a novel term proposed in 2019 as a descriptor of subfertility and even infertility in men related to oxidative stress. The proposed term implies the need of incorporating ROS assessments and tests related to oxidative damage along with conventional semen analysis into the routine use and the diagnosis of infertility in men (Agarwal et al., 2019).

The overall health of the mitochondria is reflected on the mitochondrial respiratory chain (MRC). MRC, commonly known as the electron transport chain (ETC), is a four-protein complex found at the inner mitochondrial membrane. The mitochondrial respiratory chain in eukaryotes is where oxidative phosphorylation occurs, which results in energy generation. Coenzyme Q and Cytochrome C serve as intermediate substrates in the chain, which is made up of a sequence of enzyme complexes (I–IV). The movement of electrons from electron donors to electron acceptors via redox processes is combined with the transport of protons across the membrane, forming an electrochemical proton gradient that is utilized by the ATP synthase (complex V) to produce ATP. Reactive oxygen species are one of the byproducts of oxidative phosphorylation. Complex I and complex III mainly create superoxide, which is a frequent ROS in cells. Antioxidant enzymes that convert ROS into less harmful byproducts are produced by cells to protect them from oxidative damage. Many studies have connected mitochondrial respiratory chain malfunction to a wide range

of diseases, including diabetes, heart failure, neurodegenerative disorders, and the physiological process of aging (Bratic & Larsson, 2013; Cogliati et al., 2013).

There are both endogenous and exogenous sources of ROS in male reproductive system. Endogenous ROS are produced by the immature sperms and semen leukocytes. Exogenous sources are mainly due to infections and inflammatory reactions in the male reproductive ducts. According to studies, NADPH oxidase 5 (NOX5) and cytochrome c in spermatozoa play a key role in the formation of reactive oxygen species (ROS) during spermatogenesis (Agarwal et al., 2003; Sabeur & Ball, 2007; Tomlinson et al., 1992).

Numerous physiological elements of reproductive processes, including spermatogenesis and fertilization, are influenced by mitochondria. Sperm motility, acrosin activity, acrosome response, hyperactivation, capacitation, and DNA integrity are all dependent on mitochondrial general health, from mitochondrial functionality to an intact membrane potential. Therefore, energetic mitochondrial activity is necessary for the quality and functionality of human sperms. (Barbagallo et al., 2020).

Mitochondrial dysfunction has been related to a variety of neurodegenerative and neurological disorders, as well as the pathophysiology of chronic pain. While the underlying causes are complicated, research on fibromyalgia patients shows that inflammation, mitochondrial dysfunction, and oxidative stress all have a role in the duration and severity of chronic pain. (Cordero et al., 2010; Sánchez-Domínguez et al., 2015).

Cyclophosphamide (CYP) is a medication widely used as chemotherapy and an immuno-suppressor as a treatment for many autoimmune disorders including, systemic lupus erythematosus and rheumatoid arthritis (F. Liu et al., 2012). Despite its wide spectrum of beneficial effects and clinical use, CYP has numerous adverse effects, several studies have demonstrated the implication of CYP in both females and males' infertility. CYP is metabolized through cytochrome P450 into two toxic intermediates, acrolein and phophoramide. Acrolein is a reactive aldehyde that increases the propagation and therefore, the production of ROS, which in turn affect surrounding tissue. ROS generation affect reproductive system through the inhibition of several important enzymes, DNA damage, and lipid peroxidation (Qin et al., 2016).

The objective of this study was to investigate mitochondrial respiratory chain dysfunction and evaluate the detrimental effects of oxidative stress as a possible mechanism of male reproductive system dysfunction in animal models of interstitial cystitis.

1.1 Interstitial Cystitis

Interstitial cystitis (IC), also known as bladder pain syndrome (BPS) is collectively defined as a pelvic pain syndrome with no apparent signs of infection and is often misdiagnosed as a urinary tract infection (UTI). IC/BPS is characterized by a persistent sensation of pelvic and bladder pressure and/or pain associated with urinary frequency, urgency, and nocturia (Daniels et al., 2018; Marcu et al., 2018). The pain may vary among individuals, it ranges from mild pressure to severe pain (Akiyama et al., 2020). Pain usually worsens as IC/BPS advances and becomes the most dominating and debilitating symptom, having a significant impact on patients lives both personally and professionally (Gracely & Cameron, 2021).

According to the literature, IC was first reported in 1836 by physician Joseph Parrish, popularly known as the Philadelphia surgeon. Three different cases that mirrored the characteristics of IC/BPS were reported in his textbook "Practical Observations on Strangulated Hernia and Some Diseases of the Urinary Organs" under the term "tic doloureux of the bladder" (Gross, 1855). The three described cases showed severe recurrent lower urinary tract symptoms, such as nocturia, dysuria, frequency, and urgency. Bladder stones were formerly the primary source of these symptoms, despite the fact that all attempts to discover them failed, the three instances were grouped together under the umbrella name "tic doloureux of the bladder" because neither their pathology nor their cause could be determined. The physician Alexander Skene coined the term "interstitial cystitis" in 1887 to describe a variety of urinary tract symptoms (Moutzouris & Falagas, 2009). Later in 1915, Hunner published the most well-known study on interstitial cystitis later that year, documenting the erythematous lesions that patients had throughout the disease's various stages (Hünner, 1914). Up till now, the clinical description and symptomatology of this disease have fundamentally not changed for more than 170 years.

Women are more often diagnosed with IC than men and it was thought to be a female prepordance disease However, studies have shown that IC/BPS is underdiagnosed in men due to the fact that it is often misdiagnosed with prostatitis (Arora & Shoskes, 2015). Men and women of all cultures, regardless of socioeconomic class or age, are affected by IC/BPS. Recent studies revealed an increasing number of men and women being diagnosed in their twenties and younger, contrary to earlier theories that the disease was only associated with menopausal women (Rosenberg et al., 2007; Suskind et al., 2013). The scientific community's increased understanding of the condition

has contributed to more precise epidemiological data, disproving and undermining the concept that IC/BPS is a rare disease (Arora & Shoskes, 2015). According to recent studies, between 2.7 to 6.53 million women in the USA have IC symptoms, and up to 12% of women could be experiencing early IC/BPS symptoms. According to new research, there may be between 1.8 to 4.2 million more men than previously believed who suffer from interstitial cystitis symptoms (Anger et al., 2022). The etiology of this disease is still unkown. The exact pathophysiology for IC/BPS remains widely elusive. The disease itself is believed to be multifactorial, which makes it hard to comprehend the complex nature of the disease (Birder, 2019).

1.2 Animal Models of Interstitial Cystitis

Numerous animal models were developed to mimic bladder-related symptoms in order to better comprehend the pathophysiology of the disease because the exact mechanism of IC/BPS is still unknown (Song et al., 2017). These animal models are frequently produced by systemically or intravisecally administering harmful substances to healthy animals (Birder, 2019). These animal models are essential for understanding various mechanisms of the disease. Due to the multi-component complex nature of IC, chronic and subchronic animal models have been introduced to mimic the features of the disease. A single animal model cannot be totally represntative to the various components of the disease. Therefore, The following categories of animal models for IC were used: bladder-centric models, models with complicated processes, and models for physiological and psychological stressors/natural diseases. Studying these three categories can provide insight into a variety of diseases manifestations and etiopathogenesis (Bjorling et al., 2011; Kirimoto et al., 2007).

1.2.1 Bladder-centric Models

The models of this category are often created using rats or mice, to mimic the presence of a toxic substance in the urine. This model is based on the instillation of toxic irritant substances, several substances have been used in the creation of this model such as: acrolien, hydrochloric acid (HCl), acetic acid, cyclophoshphamide, lipopolysaccharide (LPS), etc (Sakata et al., 1989; Kato et al.,

1990; Jerde et al., 2000). Other models that fall under this category can also include an alteration in the expression of urothelial targets such as, claudins, and antiproliferative factors (Montalbetti et al., 2015). One of the most common used models as a bladder-centric model is the cyclophosphamide (CYP) IC model. A single intraperitoneal injection of CYP is frequently used to acutely induce the rodents CYP-induced cystitis model (Juszczak et al., 2010). Chronic CYP-induced cystitis is represntative model of the persistent bladder cystitis, this model is often induced by giving an injection of CYP intraperitoneally every third day of a ten days period (Boudes et al., 2011). CYP based bladder-centric models are represntative for the predominant features of the disease usually within 24 hours of the first injection, these features include activation of inflammation, bladder overreactivity, and pain-associated behaviours (Boudes et al., 2011). Multiple systemic injections of smaller dosages of CYP have been used to continually cause minor bladder pain in rats and mice, which results in chronic inflammation but no obvious behavioral effects (Golubeva et al., 2014). The pathogenic alterations in bladder neural pathways brought on by chronic inflammation have been investigated using the CYP models. Numerous researchers, including Vizzard and coworkers (Merrill et al., 2016) , have thoroughly studied the hallmark molecular pathways causing the pathogenic alterations of this type (Yoshimura & de Groat, 1999).

1.2.2 Models with Complex Mechanisms

On the premise that external variables, such as central nervous system (CNS) activity, are the primary drivers of the bladder alterations, models with complex mechanisms are based. These models mostly rely on injecting TNBS (2,4,6-trinitrobenzenesulfonic acid), pseudorabies virus (PRV) into the colon, autoimmune models, persistent pelvic pain, and vulvodynia into the tail, which results in bladder changes through activating CNS pathways (Yoshikawa et al., 2015).

1.2.3 Natural Disease Models (psychological and physiological stressors)

The feline IC model is included in this category, as are various stress models such as water avoidance stress (WAS), restraint stress, and models in which norepinephrine is injected or the temperature or light of the environment is modified. Even though animals are unable to articulate

pain, their behavior in reaction to noxious or other irritant stimuli can be observed and rated. Recent research has shown that additional factors, such as different types of psychological and physical stress, can cause analgesia or hyperalgesia in a number of circumstances. Pain studies can be influenced by anything, including changes in temperature and social conditions. Psychosocial stress can have a substantial impact on a variety of comorbid and other disorders, including fibromyalgia, skin diseases, asthma, and irritable bowel syndrome (IBS) (Lee et al., 2015). Numerous studies have demonstrated that stress has a significant impact on the communication of the (skin, bladder, and gut)-brain axis and that focusing on these signaling systems may have a good influence on health and disease (A. L. Smith et al., 2011). Furthermore, when IC/BPS patients' bladders are full, functional brain activation elevates in areas of the brain associated with pain and sensory perception. In this regard, there is evidence that chronic WAS in anxious-prone rats can result in a number of clinical and functional features similar to those seen in persons with IC/BPS. Long-term psychological stress has been shown in studies to cause suprapubic hyperalgesia, tactile hindpaw allodynia, and urinary frequency (Thompson & Montgomery, 2018). Furthermore, this model demonstrated that the components of the micturition circuit that respond to urgency are more actively involved. The WAS model has been observed to exhibit abnormalities in the gastrointestinal tract (GIT) as a model for IBS, which may be a comorbid illness associated to both lower urinary tract symptoms (LUTS) and IC/BPS (Matos et al., 2017). The activation of the sympathetic nervous system is thought to be a significant step in the chain of events that links psychological stress to functional pain disorders like IC/BPS, however the exact sequence of events is unknown. The involvement of the sympathetic system has been related to a number of chronic painful disorders, including complicated regional pain syndrome and fibromyalgia. According to literature, the sympatho-adrenal stress axis might contribute to the development and maintenance of mechanical hyperalgesia. Adrenergic stimulation has been shown to mediate the hypersensitivity of the pelvic viscera, influencing colorectal distension and bladder function in the long run. Chronic stress modifies sympathetic nerve activity by generating long-term changes in the neurological system, which can enhance the intensity and duration of pain episodes (Clemens et al., 2014).

1.3 Cyclophosphamide

Cyclophosphamide (CYP) is one of the oldest anticancer medications (Figure 1). This drug was discovered in 1958 and was initially introduced as an anti-cancer agent in 1959 (Mills et al., 2019). It continues to be a cornerstone in the treatment of certain epithelial tumors, such as breast, ovarian, and small-cell lung carcinomas, as well as of haematological malignancies like lymphoma and leukemia (Mills et al., 2019). In addition, CYP is utilized as an immunosuppressive agent in several conditioning regimens prior to bone marrow transplantation for aplastic anemia and haematological malignancies including, acute myeloid leukemia and myelodysplastic syndromes (AML, MDS). Others approved its indications as a chemotherapy in life-threatening complications of autoimmune and immune-mediated diseases such as Wegener's granulomatosis, lupus nephritis, and multiple sclerosis (Korkmaz et al., 2007).

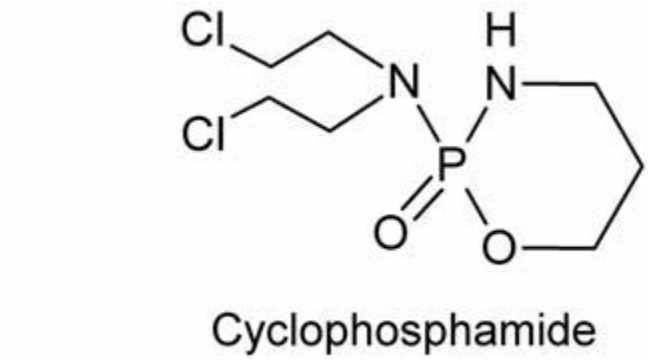

Figure 1. Chemical structural formula of cyclophosphamide (Korkmaz et al., 2007)**.**

The significant drawback of the drug including leuco- and thrombocytopenia, anemia, cardiotoxicity, and bladder toxicity. To prevent hemorrhagic cystitis, MESNA (sodium 2-sulfanylethanesulfonate) must be given before cyclophosphamide to neutralize the toxic metabolite acrolein in the urine. There is also nephrotoxicity, cardiotoxicity, and liver toxicity. All of these results are very dose dependent. Cardiotoxicity is dose-limited when using cyclophosphamide in high doses (Mills et al., 2019). CYP is regarded as a powerful alkylating agent. The active metabolite phoshoramide mustard generates a very reactive cyclic aziridinium

cation that can react with the N(7) of guanine and the cytidine from DNA (Korkmaz et al., 2007). The presence of these two reactive moieties makes it easier to create interstrand and intrastrand cross-links (Chatelanat et al., 2018).

The CYP induced bladder toxicity can cause potentially fatal hemorrhagic cystitis, which is a significant limiting factor in its use. The full efficacy of this medication and its clinical outcomes may not be fully realized due to its toxicity that restricts the dose and frequency of administration. Though water-soluble antioxidant MENSA was selected as a prophylaxis to minimize the high rate of bladder toxicity in uroprotective treatments, still reported that up to 10% of patients suffer from severe hematuria after receiving this drug for treatment of cancer (Dan et al., 2014). Additionally, patients may develop long-lasting adverse effects that impair their quality of life over time, such as pelvic pain, frequency, and pain when urinating (Atilla et al., 2020). Cyclophosphamide is also extensively used in experimental animals to cause cystitis due to these frequent urotoxic effects (Golubeva et al., 2014).

1.4 Pathophysiology of Interstitial Cystitis

The etiology of interstitial cystitis remains widely elusive. There is strong evidence that pain-related disorders are contributed by numerous physiological and psychological stressors, and these stressors facilitate the aggravation of symptoms (Fall et al., 2010). Previously, the disease was assumed to be mainly ulcerative in origin, and the diagnosis was based primarily on Hunner's lesions or hemorrhagic glomerulations in the bladder (Nickel, 2004). The malfunction in IC/BPS frequently arises in the bladder or a neighboring site (Akiyama et al., 2020). The urinary bladder walls are made up of four layers: mucosa, submucosa, muscularis, and serosa. These layers also contain several artery systems, nerve fibers, and immune cells (Alagiri et al., 1997). Multiple ruptures in the mucosa of the bladder wall, hemorrhages, and an abnormal number of immune cells are common pathological signs in patients with IC/BPS (Richter et al., 2010). In the literature, recent studies have noted that every subcellular layer of bladder tissue from IC/BPS patients exhibits some sort of alteration including, a reduction in urothelial layer (Graham & Chai, 2006), Low glycosaminoglycan content (Esko et al., 2009), decreased storage capacity (Lewis, 2000), abnormal smooth muscle cell pattern (HORN et al., 1998), high microvascular density , high mast

cell and nerve fiber density (Figure 2). Others proposed etiological factors including those that are neurologic, allergy, genetic, and stress-related (Pang, 1995).

Throughout history, numerous pathophysiological hypotheses about the pathogenesis of IC/PBS have been proposed, supported, and disproved. These pathophysiological hypotheses including, chronic stress theory, urothelial permeation theory, glycosaminoglycans theory, mast cell theory, infection theory, neuroendocrine theory, oxidative stress, and mitochondrial dysfunction are discussed in this section.

1.4.1 Chronic Stress Theory

Now there are many studies suggesting that chronic stress plays a part in the onset, ongoing, and even in the exacerbation of functional bladder disorders like IC/BPS (Gao & Rodríguez, 2022). In people prone to disorders like IC/BPS as well as in healthy people, chronic stress can both raise the likelihood of disease/pathology and itself cause hyperalgesia or pain. More than half of IC/BPS patients experience frequent urination and daily or chronic pain, both of which worsen by stressful situations (Bjorling et al., 2011). The activity of the sympathetic nervous system might alter as a consequence of the long-term changes in the neurological system caused by stress (Powell et al., 2017). Previous studies have demonstrated that a decrease in descending inhibition can lead to an increase of pain mediated by the sympathetic nervous system, which is referred to as stress-induced hyperalgesia. The increased sympathetic activity seen in IC/BPS patients could be a mechanism by which stress increases the intensity and length of pain episodes (Lee et al., 2015). Both clinical and preclinical (experimental) studies demonstrated these anomalies in sympathetic activity. For instance, it has been demonstrated that participants with IC/BPS displayed decreased vagal activity with a shift toward sympathetic nervous system dominance when utilizing heart rate variability as an indication of autonomic function (Leue et al., 2017).

1.4.2 Urothelial Permeation Theory

The urothelium, the epithelium that lines the inner surface of the urine bladder, is made up of three layers: a basal cell layer, an intermediate layer, and a top layer made up of "umbrella cells." This thin cellular line also has mucopolysaccharides, which form a protective barrier. One of the

fundamental functions of the urothelium is to provide a barrier to protect the underlying tissues from urine and to redirect certain molecules toward the bladder wall. Cell-to-cell tight junctions connect to certain membrane carrier proteins such as aquaporins, urea and ion transporters, and hormone receptors (androgen, mineralocorticoids, and estrogens) to perform this function (Acharya et al., 2004; Rubenwolf et al., 2012). The permeability of the urothelium serves as the foundation for this theory. The lamina propria connects the inner detrusor muscle layer and the mucosal layer. Understanding how a damaged or altered urothelium affects the function of the detrusor is dependent on the communication between these layers. Numerous neurotransmitter molecules, including ATP, acetylcholine (ACh), prostaglandins, and nitric oxide (NO), can modify bladder activity in response to a variety of chemical or physical stimuli supplied to the bladder wall (Fernandes & Hernández, 2016; Shyr et al., 2013). For example, ATP and ACh neuromodulators, which originate in the urothelium, have a function in blood flow regulation and detrusor contraction co-regulation, as well as transmitting physiological signals to the central nervous system as a pleasant sensation. These diseases can result in hyperactive bladder syndrome (IC/PBS), GAG layer or urothelium permeability loss (Nausch et al., 2010). NO has recently been recognized as a critical mediator in urinary tract neurotransmission, having a function in the modulation of ureteric smooth muscle activity. Overactive bladder syndrome, which is present in spinal cord injuries and other systemic illnesses, alters the ATP/NO ratio, with increased ATP generation and decreased NO urothelium production (Munoz et al., 2011). As a result, changes in urothelium permeability may influence how neuro-mediated communication between the mucosa, muscle layer, and afferent neural system works. Attempts to restore urothelium permeability could be performed with oral or intravenous GAG replacement therapy, albeit the results in people with IC/PBS have been mixed (Zhang et al., 2017).

1.4.3 Glycosaminoglycans Theory

This theory focuses on the extracellular and structural components of the bladder, particularly glycosaminoglycans (GAGs). GAGs, along with collagen, elastin, fibronectin, and laminin, are members of the polysaccharide (or mucopolysaccharide) family that makes up the extracellular

matrix (ECM) (Esko et al., 2009). GAG molecules are required by growth factors, protease inhibitors, cytokines, and growth factors. The most common GAG molecules found in the bladder are hyaluronate, keratan sulfate (KS), dermatan sulfate (DS), chondroitin sulfate (CS), heparin, and heparan sulfate (HS) (Hurst & Zebrowski, 1994). In terms of biomechanics, the GAG layer acts as a "hydrated gel" cushion that resists compressive pressures (Pang, 1995). A content analysis of bladder tissue revealed that the presence of GAG in the lumen was much lower than expected. This tissue was obtained from the bladder cell surface (luminal) as well as the ECM (the density of GAG is often higher in the ECM) (Hurst & Zebrowski, 1994). The basic function of bladder tissue is to retain urine and to allow for the selective exchange of electrolytes and non-electrolytes. This decreased constituent exchange and, as a result, the preservation of urine composition during storage is produced by a decrease in epithelial surface area for the volume of urine. This is made possible by a coating of GAGs covering the surfaces of these epithelial cells (Richter et al., 2010). Any biological (infection, inflammation, etc.) or physiological (damage, lesions, etc.) factors that affect this GAG layer can induce an imbalance in urine storage, resulting in frequent urination, decreased capacity, and pelvic pain (the signs/symptoms of IC/PBS). However, another theory claims that epithelial cells and tight junctions are involved in this penetration (Treutlein et al., 2012).

1.4.4 Mast Cells Activation Theory

Any abnormal change in the body's or cellular environment's normal homeostasis might result in a chemical release or a trigger signal as a response to that change; this signal activates a specific class of immune cells known as mast cells (MCs). Mast cells are derived from bone marrow and play a role in innate immunity, autoimmune immunity, and neurogenic immunity (Pang et al., 1995). MCs secrete biologically active molecules such as serotonin, heparin, histamine, kinins, proteases, phospholipases, chemotactic factors, cytokines, and vasoactive intestinal peptide; however, other molecules such as interleukin-6 (IL6), leukotrienes, platelet activating factor, prostaglandins, thromboxanes, NO, and tumor necrosis factor (Powell et al., 2017). MCs can be activated by anaphylatoxins, antigens, bradykinin, cytokines/lymphokines, hormones, IgE, neurotransmitters, neuropeptides, bacterial toxins, viruses, medicines, and stress (Yamada et al., 2000). Inflammation, discomfort, vasodilation, fibrosis, and smooth muscle contraction can all be caused by a damaged or malfunctioning urothelium, which is a powerful MC activator (Akiyama

et al., 2018). Histamine and its metabolites were detected in higher concentrations in the urine of IC/PBS patients as well as in animal sickness models, lending credence to these findings. According to Simmons and Bunce, MCs were responsible for the birth of IC/PBS. High MC density is a common histological finding in IC/PBS patients, particularly around the detrusor muscle (Yamada et al., 2000). According to this research, IC/PBS patients had increased PGE2 excretion and eosinophil leukocyte density, and high MC density is associated with lower epithelial number and higher urine protein levels. Numerous stimuli, including microorganisms (bacteria, viruses, etc.), neuropeptides (substance P), ACh, kinins, and others, may trigger MC to become active in IC/PBS patients. Surprisingly, Lundeberg et al. discovered a link between the neurological system and infammation between the MC numbers and the amount of nerve fibers detected in IC/PBS patients' sub-urothelium and detrusor muscle (Liu et al., 2012). Although they found no significant variations in mast cell expression in the detrusor muscle versus the mucosa layer in non-ulcerative IC/PBS patients, some experts believe the MC count is not a valid predictor (Liu et al., 2012).

1.4.5 Infection Theory

An infection in the bladder lumen can cause changes in the structure and composition of GAGs, resulting in permeation, autoimmune illness, and infection. Many doctors felt that IC/PBS was an infectious disease (of perhaps bacterial, viral, or even fungal origin) because of the presence of Helicobacter pylori (and other microorganisms) and its resemblance to chronic gastritis. A recent study suggests that a recently found pathogen known as nanobacteria (NB) may play a role in the development of IC/BPS (Atuğ et al., 2004). However, more research should be conducted to provide more insight on the pathogenic link between NB and the symptoms or development of IC/PBS. Further investigations (using polymerase chain reactions, electron microscopy, antibody detection, and so on) were carried out in a number of pathogen-related IC/PBS cases, and the presence of microorganisms (in the inflammatory process or disease etiology) was deemed to be a false positive response and not a contributing factor in the development of IC/PBS (M. Agarwal & Dixon, 2003). After merging the data of their global studies, experts concluded to rule out infection of the lower urinary tract as a symptom or diagnostic criterion of IC/PBS (Al-Hadithi et al., 2005).

1.4.6 Oxidative Stress and Mitochondrial Dysfunction

Multiple cellular activities depend on mitochondria, and there is strong evidence that mitochondrial malfunction contributes to a wide range of neurological illnesses as well as the etiology of chronic pain states. Despite the complexity of the underlying mechanisms, research from fibromyalgia patients suggests that inflammation, oxidative stress, and mitochondrial dysfunction may all contribute to persistent pain (Kullmann et al., 2019).

1.5 Male Urogenital system

The reproductive system and urinary tract constitute the male urogenital system; they both share an embryonic development and the urethra as an anatomical component. Regarding the reproductive system, this system varies with aging and environmental conditions. It has a very complex physiology that is closely connected to the circulatory and nervous systems and can adjust to environmental changes (Sanches et al., 2021).

1.5.1 Ontogenesis and Phylogenesis

As components of the urogenital system, the reproductive system and urinary tract share a same embryonic development, and both partially derive from the intermediate mesoderm (Sanches et al., 2021). Throughout the evolution of our phylum, these systems have become increasingly separate while still utilizing certain similar elements. This connection is seen in how a human fetus develops. The mesonephron, the bottom section of the primitive kidney, canalizes when it connects to the cloaca near the conclusion of the fourth week of development. Later, it develops into the vas deferens, which connects the testes to the urethra, the epididymis, and seminal vesicles, which create the mesonephric duct, which grows to connect the mesonephros to the cloaca (Chaves et al., 2012). The ureteric bud, which later gives rise to the metanephric duct and metanephros, which eventually give rise to the ureters and definitive kidneys, respectively, first appears during the fifth gestational week. The ventral component of the cloaca produces the urogenital sinus, which forms the top region of the bladder and the lower portion of the urethra (Z. Chen et al., 2003). Finally,

the prostate, an auxiliary gland of the reproductive system, is formed by the interaction of the epithelium and mesenchyme of the urogenital sinus (Gunes et al., 2016).

1.5.2 Urogenital Infections and Inflammation in Male Sexual Dysfunction

Numerous research has reached the conclusion that urogenital infections and inflammation are the most common causes of male infertility (Tremellen & Tunc, 2010). Male infertility is primarily brought on by inflammation and urogenital infections. Silent genital tract inflammation, which has a negative effect on sperm quality, may be caused by chronic urethritis. In the chronic pelvic pain syndrome, there are changes to the seminal plasma and morphological abnormalities of spermatozoa. The majority of men with epididymitis experience a temporary reduction in the quality of their semen during the acute illness. Even after a full bacteriological healing, however, persistent unfavorable effects are not unusual. It is believed that chronic viral infections are underappreciated as an etiologic component in male infertility. The underlying illnesses seen here include chronic urethritis, several forms of prostatitis syndrome, epididymitis, and orchitis. Studies on chronic urethritis, which is typically brought on by persistent bacterial infections, have revealed a correlation between cases of epididymo-orchitis and testicular dysfunction, suggesting that it may be related to lower male fertility (Fall et al., 2010). Chronic prostatitis is another male urinary system disorder that has been associated with sexual dysfunction. People who have chronic prostatitis have abnormal ejaculate parameters and leukocytospermia (Chavarro et al., 2010). Other research on chronic prostatitis have demonstrated the presence of sexual dysfunction in 31 patients compared to the control group of healthy people (Rusz et al., 2012). Chronic prostatitis and chronic pelvic pain (CP/CPP) are the key characteristics of prostatitis syndrome, which is a spectrum of signs and symptoms (Weidner et al., 2013). The presence of the proinflammatory interlukin-8 (IL-8) in the semen of patients with prostatitis syndrome and lower urinary tract pain has been used by researchers to confirm the existence of an inflammatory component in the condition (Motrich et al., 2012). Studies have indicated that prostatitis syndrome has no discernible impact on sperm count or concentration. However, other sperm parameters, such morphology and motility, have changed, raising the possibility that CP/CPP has an impact on the quality of sperms and the male reproductive system (Fall et al., 2010; Schuppe et al., 2017b; Weidner et al., 2010, 2013). Throughout the literature, studies have shown the anatomical and physiological

interconnection between compartments of male urinary and reproductive systems (Table 1). Urinary tract infections and inflammation have a distinguishable effect on male reproductive system, semen quality, and sperm parameters.

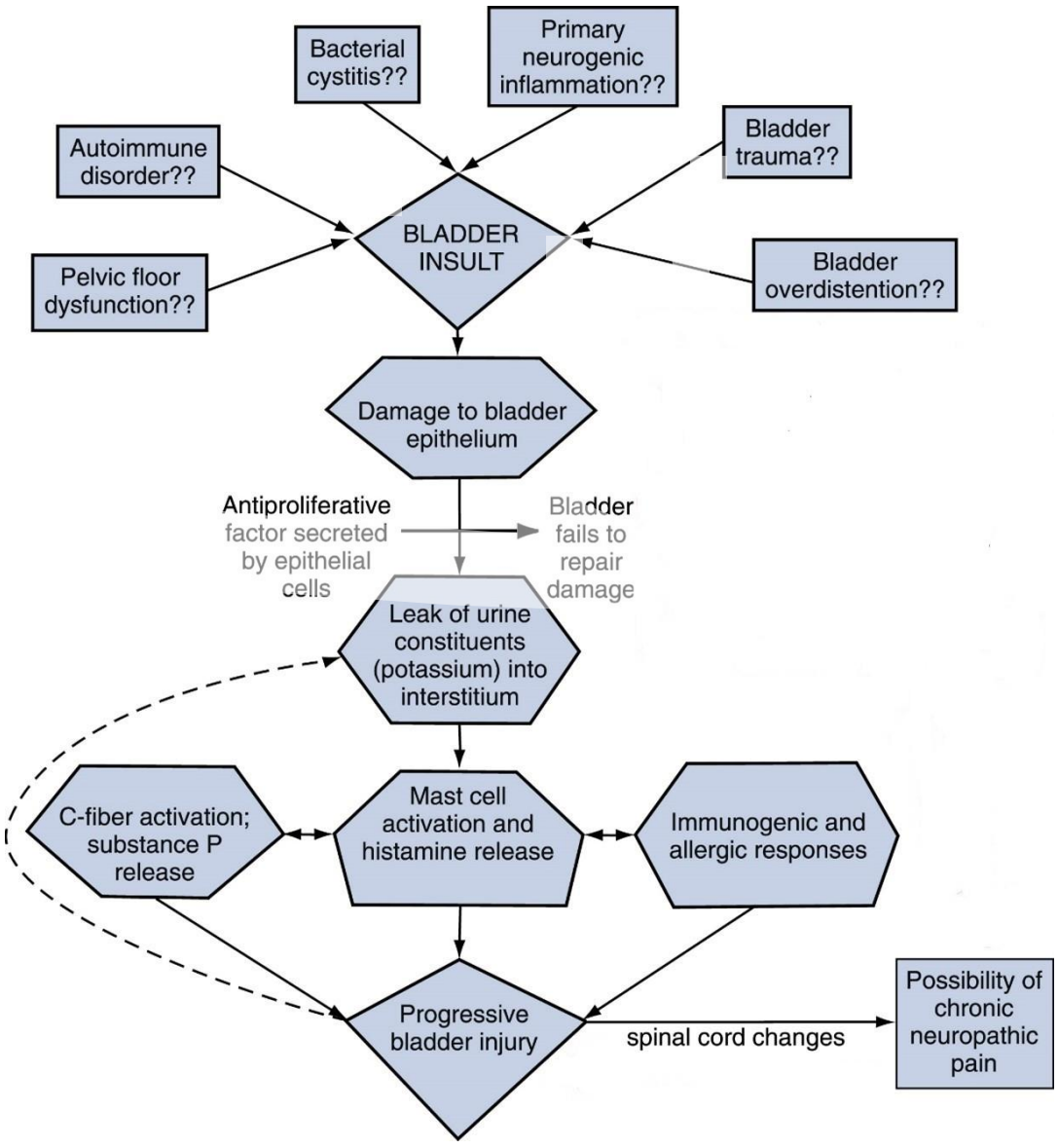

Figure 2. The Different Proposed Theories Regarding the Pathogenesis of Interstitial Cystitis (Chancellor & Yoshimura, 2004).

Table 1. A Summary of the Male Urogenital organs and structures.

Structure	Location	Function	Inter-connection
Kidney	Left and right in the retroperitoneal space.	fluid regulation and elimination of waste.	Indirect.
Renal pelvis	Within the medial concave surface (renal sinus) of the kidney.	Collection of urine, flowing to ureters and into the bladder.	Indirect.
Ureters	The upper part of the ureters is in the abdomen, while the lower part of the ureter is in the pelvic area.	Carry urine from the kidneys to the bladder.	Direct.
Bladder	In the retropubic space below the peritoneal cavity.	Stores urine.	Indirect.
Urethra	Originates at the bladder neck and terminates at the urethral meatus on the glans penis.	Empties urine from the bladder.	Direct.
Testis	Scrotum.	Spermatogenesis and production of hormone testosterone.	Direct.
Epididymis	Attached to each testis inside the scrotum.	Site for accumulation, storage, and further maturation of sperms.	Direct.
Prostate gland	Below the bladder in front of the rectum.	Secrete alkaline fluid to neutralize the acidity of the vagina and prostaglandins.	Direct.
Seminal Vesicle	On posterior surface of the bladder and anterior to the rectum.	Secrete fructose (main energy source for sperms motility) to energize the sperms and secrete seminal fluid.	Direct.

1.6 Free Radicals

Free radicals are the normal product of regular cellular metabolism. An atom or molecule that has one or more unpaired electrons in the valency shell or outer orbit and is capable of independent existence is referred to as a free radical. A free radical is unstable, short-lived, and extremely reactive due to its odd number of electrons. They can draw electrons from other compounds to become stable due to their high reactivity. Thus, the attacked molecule loses its electron and transforms into a free radical, starting a cascade of events that ultimately harms the live cell (Aitken et al., 2013). Free radicals and other non-radical reactive species are both comprised of reactive oxygen species (ROS) and reactive nitrogen species (RNS) collectively (Tripathi et al., 2021). The ROS and RNS have a dual purpose in the living system as both helpful and harmful molecules. At moderate or low levels, ROS/RNS have advantageous effects and are involved in several physiological processes, including immunological function (i.e., protection against harmful microorganisms), cellular signaling pathways, mitogenic response, and redox regulation (Akbar et al., 2016; C.-H. Wang et al., 2013). However, at larger concentrations, both ROS and RNS produce oxidative stress and nitrosative stress respectively, which may harm the biomolecules. When there is an excess of ROS/RNS generation on one side and a shortage of enzymatic and non-enzymatic antioxidants on the other, oxidative stress and nitrosative stress are created. The integrity of various biomolecules, including lipids, proteins, and DNA, can be damaged by excessive ROS, which is especially important because it increases oxidative stress in several human diseases, including diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory diseases, and the aging process (Federico et al., 2012; Singh et al., 2019).

1.6.1 Sources of ROS

The sources of ROS can either be endogenous or exogenous, different cellular organelles, such as mitochondria, peroxisomes, and endoplasmic reticulum, where oxygen consumption is substantial, are examples of endogenous producers of ROS. While exogenous sources include smoking, alcohol abuse, exposure to radiation and environmental sources (Phaniendra et al., 2015).

1.6.2 Mitochondria

The majority of intracellular ROS are produced by mitochondria. Two key sites in the electron transport chain (ETC) complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) produce superoxide radicals. The reduced form of coenzyme Q (QH2) is produced when electrons from complexes I or II are transferred to coenzyme Q or ubiquinone Q. Through the Q-unstable cycle's intermediate semiquinone anion ($\bullet Q^-$) the reduced form of QH2 regenerates coenzyme Q. Superoxide radical is immediately produced when the generated Q donates electrons to molecule oxygen. Superoxide cannot be produced by an enzyme, hence the higher the metabolic rate, the more ROS are produced (Ghezzi & Zeviani, 2012). Superoxide dismutase in the mitochondria converts the superoxide anion to hydrogen peroxide (MnSOD). Catalase (CAT) and glutathione peroxidase can detoxify H2O2 (GPx) (Wei et al., 2001). Monoamino oxidase, aketoglutarate dehydrogenase, glycerol phosphate dehydrogenase, and p66shc are some of the additional mitochondrial elements that aid in the production of ROS.

1.6.3 Peroxisomes

Unlike mitochondria, the respiratory pathway in peroxisomes involves the transfer of electrons from different metabolites to oxygen, which results in the creation of H2O2, but instead of producing ATP when paired with oxidative phosphorylation, free energy is released as heat (Phaniendra et al., 2015). The peroxisomes also generate H2O2, O2 •-, OH•, and NO• as free radicals. The main metabolic mechanism in the peroxisomes that produces H2O2 is the b-oxidation of fatty acids. It has been demonstrated that different ROS are produced by several peroxisomal enzymes, including acyl CoA oxidases, D-amino acid oxidases, L-a-hydroxy oxidases, urate oxidases, xanthine oxidases, and D-aspartate oxidases (Guerriero et al., 2014).

1.6.4 Endoplasmic Reticulum

The production of ROS is aided by endoplasmic reticulum enzymes such diamine oxidase, cytochrome p-450, and b5 enzymes (Aitken et al., 2014). In addition, Erop1p is a crucial thiol

oxidase enzyme that catalyzes the conversion of dithiols into molecular oxygen, which creates H2O2. Prostaglandin production, adrenalin autooxidation, phagocytic cells, decreased riboflavin, FMNH2, FADH2, cytochrome P450, immunological cell activation, inflammation, mental stress, excessive exercise, infection, cancer, aging, ischemia, etc. are some other endogenous causes of ROS (Höhn et al., 2017).

1.7 Exogenous Sources of ROS

The generation of free radicals is mainly carried out in intracellular compartments, however, the exposure to a variety of exogenous agents can lead to the generation of free radicals in human biological systems. Exogenous sources such as, alcohol abuse, tobacco smoke, pesticides, water and air pollution, exposure to high temperatures, ultraviolet (UV) light, industrial solvents, CCl_4, transition metals, heavy metals (Fe, Cu, Co, Cr, etc.), and drugs such as, Doxorubicin, Halothene, Bleomycine, Paracetamol, Metrenidazole, Ethanol, all can lead to detrimental effects to biological systems therefore to ROS generation (Lushchak, 2014).

1.8 Cellular Targets of Free Radicals

When free radicals (ROS/RNS) are generated in large concentrations it will lead to a state of oxidative stress and nitrosative stress, this is mainly due to an imbalance between their production and antioxidant defense systems. These highly reactive free radicals have the potential to harm all three major types of biological components, including lipids, proteins, and nucleic acids (Lushchak, 2014).

1.9 Mitochondria: Origin, Functionality and Dysfunction

A mitochondrion (plural form, mitochondria) is an organelle bound with a double membrane often found in most eukaryote cells (Osellame et al., 2012). The mitochondria are extensively known to be the powerhouse of eukaryote cells due to their ability to produce adenosine triphosphate (ATP),

the main source of energy for most eukaryotic cell's biochemical reactions (Durairajanayagam et al., 2021).

Structurally, mitochondria are bounded by a double membrane, thus the organelle is divided into four distinctive compartments: outer membrane, inner membrane, inter-membrane space and matrix, each serves a different function (Huang et al., 2019).

Besides energy conversion reactions that include, pyruvate and the citric acid cycle, NADPH and O_2 energy-releasing and heat production, mitochondria are a highly dynamic versatile organelles participating in numerous physiological processes due to their high adaptability to cellular requirements (Faja et al., 2019). These physiological processes include the transitory interactions with the membranes of other organelles such as, endoplasmic reticulum (ER) and lysosomes, this interaction is vital for glucose regulation and homeostasis, autophagy, the storage and influx of calcium ions (Ca^{2+}) and lipids, cellular regulation and apoptosis, in addition to the replication of mitochondrial DNA (mtDNA) (Ferree & Shirihai, 2012). Another essential function of the mitochondria is the generation of ROS promoted by the electron transport chain (ETC) located in the mitochondrial inner membrane. ETC is a series of proteins and other molecules located in the inner membrane of the mitochondria, these proteins participate in ATP synthesis process through oxidative phosphorylation. The imbalance in the signaling pathways of the ETC leads to the accumulation of free radicals, oxidative stress, and mitochondrial dysfunction (Fisher & Henkel, 2020).

1.9.1 Mitochondrial Genome

The endosymbiotic hypothesis suggests that the mitochondria originate from an aerobic prokaryotic ancestry. Recent studies have confirmed this mitochondrial endosymbiont ancestor is in fact related to α-proteobacteria, while it is still debated which lineage of this group is the direct ancestors (Gawryluk et al., 2014). In this regard, mitochondria typically contain their own genome. Unlike nuclear genome, the mitochondrial genome is built of double-stranded circular DNA, it is approximately 16.569 base pairs, and contains 37 genes that encodes 13 essential proteins, 2 rRNAs, and 22 tRNAs (Calvo & Mootha, 2010; Gawryluk et al., 2014). The 13 mitochondrial gene-encoded proteins all encode essential subunits of mitochondrial ETC system (Gawryluk et

al., 2014). Mitochondrial generation of ATP accounts for 80% of total cellular energy. Each gene encoded by mtDNA considered essential in ATP synthesis process through oxidative phosphorylation (Z. Wang & Wu, 2015). Unlike nuclear genome, each cell contains many mitochondria. In addition, each mitochondrion retains dozens of copies of its mtDNA. The mitochondrial genome is limited to the inability to produce all the proteins needed for its functionality independently; therefore, mitochondria is highly reliant on the importation of gene products from the nucleus (Roger et al., 2017).

Even in terminally differentiated cells like nerve cells and myocytes, mtDNA is constantly reproduced. As a result, mtDNA can accumulate mutations faster than nuclear DNA, especially in non-dividing cells (Holt & Reyes, 2012). Mutations in mitochondrial DNA frequently result in more apparent phenotypes in tissues with high energy demands, such as the brain, retina, skeletal muscle, cardiac muscle, and reproduction. (Holt & Reyes, 2012). Growing evidence implicate mitochondrial dysfunction in the pathophysiology of neurodegenerative disorders, chronic pain, and male infertility (Fischer & Maier, 2015; Mechta et al., 2017).

1.9.2 Mitochondrial Electron Transport Chain and Oxidative Phosphorylation

In introductory biology studies, we learned that aerobic respiration, a complex biochemical process takes place in the inner mitochondrial membrane by which pyruvate is converted to CO_2 and other reduced cofactors that drives the ETC chemiosmotically fueling the process of ATP generation, with oxygen being the final electron acceptor in this chain. Hence, most eukaryotic organisms require oxygen for survival (Chen et al., 2005).

Electron transport chain, also known as the respiratory chain, is located in the inner mitochondrial membrane and considered one of the most structural and functional parts of the mitochondrion (Ghezzi & Zeviani, 2012). It consists of a series of protein complexes, mostly membrane bound. This series of protein subunits consists of namely complex I, II, III, IV, and V that transfer electrons via redox reactions from electron donors to electron acceptors creating an electrochemical proton gradient that facilitates the phosphorylation of ADP to ATP (**Figure 3**) (Selivanov et al., 2011). Aerobic respiration that takes place in mitochondria and is responsible of the consumption of 90%

of total cellular oxygen content. It is estimated that 1-5% of this oxygen is converted into ROS under physiological conditions (Selivanov et al., 2011).

In this regard, Complex I (NADH ubiquinone oxidoreductase, mitochondrial complex I or NADH dehydrogenase I) is considered one of the primary sites of electron leakage to oxygen, which leads to the generation of superoxide anions (Voets et al., 2012). Complex I (NADH) and complex III (ubiquinone- cytochrome c oxidoreductuase) along the ETC are the primary sites of superoxide radicals, with complex III being considered the main site of ROS generation, under normal physiological conditions (Ghezzi & Zeviani, 2012).

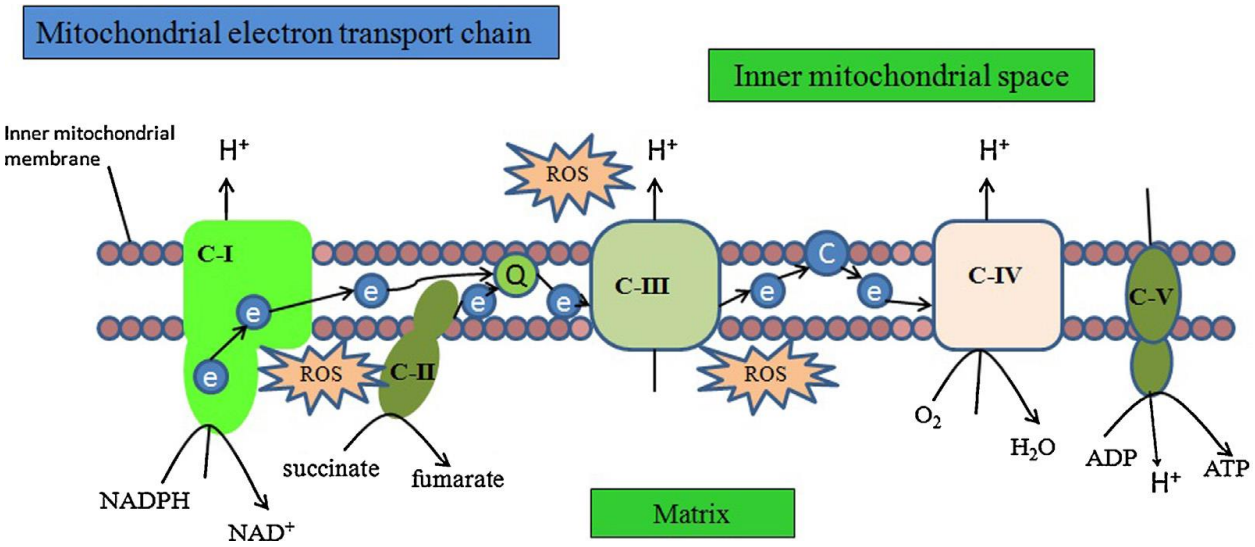

Figure 3. The Mitochondrial Electron Transport Chain (Bhat et al., 2015).

1.10 Mitochondrial Functionality and Male Reproductive System

Besides being the cellular powerhouse, mitochondria are key organelles playing an essential role in several metabolic and physiologic processes. The mitochondria play a central role in reassuring the quality of male reproductive system throughout spermatogenesis and fertilization (Hill et al., 2012). Mitochondria undergo substantial changes during spermatogenesis, the morphology and size of mitochondria in spermatocytes and early spermatogonia is typical, while in late

spermatocytes and spermatids the featured mitochondria tend to be more condensed and with an increased metabolic efficiency (Amaral et al., 2013). During the maturation of spermatids into spermatozoa, the mitochondria are conserved mainly in the midpiece, while most of the cytoplasm is lost suggesting the crucial role of the remaining mitochondria (Ramalho-Santos & Amaral, 2013). Sperm motility is dependent on the ability of mitochondria to generate ATP. In fact, any alteration in the ultrastructure of mitochondria can lead to drastic changes in the motility of sperms and even asthenozoospermia (Pelliccione et al., 2011). However, the motility of sperms is sustained either by glycolysis or oxidative phosphorylation. Metabolic pathway can be used as a dual energy system based on the availability of substrates, demonstrating high versatility in spermatozoa (du Plessis et al., 2015). The maintenance of a positively charged membrane potential is a key requirement to produce ATP. The use of an oxidative uncoupler on human sperm decreases mitochondrial membrane potential, decreasing sperm motility and fertility (du Plessis et al., 2015; Barbagallo et al., 2020). As a result, spermatozoa from infertile patients have been found to have low mitochondrial membrane potential and significant ROS production (da Silva et al., 2014).

Under physiological conditions, ROS are generated as a result of several metabolic pathways, mitochondria are considered the main site of ROS generation in cells (**section 5.1**) (X. Li et al., 2013). ROS are obligatory metabolic products that serve as signaling molecules in various processes of antioxidant systems, such as CAT, glutathione peroxidase (GPXs), thioredoxins, superoxide dismustase (SOD), and so on, are responsible for sustaining the levels of ROS under physiologic control (Ishii et al., 2005; X. Li et al., 2013; Ramalho-Santos & Amaral, 2013).

A set of complex antioxidant mechanisms and systems have evolved in the testis (X. Li et al., 2013), it also relies on several non-enzymatic agents that serves as free radical scavengers, such as, vitamin c that mainly produced by sertoli cells and spermocytes. Deficiency in vitamin c can lead to oxidative stress and further detrimental oxidative damage in the testis (Ishii et al., 2005). Another agent that considered a core component of SOD is zinc, the mechanism of action of zinc is capable to counteract lipid peroxidation in the testis (Khan et al., 1991). In addition, melatonin is considered a key protective agent against oxidative damage in germinal epithelium, due to the ability of this pineal hormone to cross the blood-barrier of the testes (Meccariello et al., 2014).

The process of spermatogenesis includes the occurrence of a series of dynamic and complex events, where immature sperm cells undergo a successive mitotic and meiotic divisions, also known as

spermocytogenesis, and other morphological changes that aim in the cellular differentiation of immature sperm cell to the production of mature spermatozoa (spermiogenesis) (Guerriero et al., 2014).

Physiologically, ROS are generated as normal by-products during the metabolism and differentiation of germ line cells. However, minimal amounts of ROS generated under physiological control is essential for regular functions of spermatozoa such as, controlling internal homeostasis, serving as cellular signaling molecules, participating in sperm capacitation process, and mediating sperm-egg interactions after fertilization (Ray et al., 2012). In regard of sperm capacitation, ROS are involved by mediating the generation of cAMP, the inhibition of tyrosine phosphatase, and importantly the efflux of cholesterol throughout plasma membrane from areas containing cholesterol, including both the head and flagellum (Aitken & Drevet, 2020; Migliaccio et al., 2019).

In contrast, the over-production of ROS and the consequent accumulation of ROS in male reproductive system can lead to serious detrimental effects on its structures (Aitken & Baker, 2006). In sperm cell, the accumulation of ROS lead to drastic morphological alteration, genomic and epigenomic changes, protein modifications, and lipid peroxidation (Bisht et al., 2017). In the testis, several morphological alterations in the seminiferous epithelium are observed, vacuolization of the cytoplasm of both Sertoli cells and germ-line cells, and apoptosis (Kocer et al., 2015; Noblanc et al., 2013).

1.10.1 Oxidative Stress and Male Reproductive System

When ROS production exceeds the physiological levels and antioxidant mechanisms are unable to prevent further damage, oxidative stress occurs (Kocer et al., 2015). The overexcitation of NADPH and mitochondrial ETC result in the excessive creation of ROS, which is why mitochondria are thought to be the main location of cellular ROS generation (Figure 4). Overproduction of ROS can result in oxidative stress and additional oxidative cellular damage even though physiologically controlled levels are necessary for several metabolic pathways, including cellular signaling, defense against pathogenic pathogens, and apoptosis (Aitken, 2018). In numerous neurological diseases, the problems associated with chronic pain, and male infertility have all been linked to oxidative stress as a contributing factor in their development (Bauer et al., 2015). Spermatogenesis

is primarily responsible for male fertility and the overall health of the male reproductive system (Smith et al., 2013). In the seminiferous tubules of the testis, spermatogenesis is the process by which mature haploid spermatozoa differentiate from germ line cells (Sharma et al., 2019). Abnormally large heads, double heads and/or tails, malformed head and tails, and crooked tails are all spermatozoa deformities that have been related to excessive ROS production which compromise the activity of these cells (Ménézo et al., 2014). Additionally, spermatozoa with such defects might cause sexual dysfunction and reproductive complications due to their increased production of ROS in semen (Q. Chen et al., 2016). These cells are particularly vulnerable to ROS-mediated damage because most of the cytoplasmic contents, including a significant number of antioxidant enzymes, are lost during spermatogenesis (Chu et al., 2020). Lacking an adequate integrated antioxidant defense system in chromatin condensation phase, high concentration of polyunsaturated fatty acids, and deficiency in DNA repair pathways, make the spermatozoa more vulnerable to oxidative stress and ROS-mediated damage (Figure 4). Additionally, spermatozoa naturally produce ROS throughout various stages of spermatogenesis, and the prolonged isolation of spermatozoa in the male and female genital tracts also contribute to oxidative damage (Aitken et al., 2014).

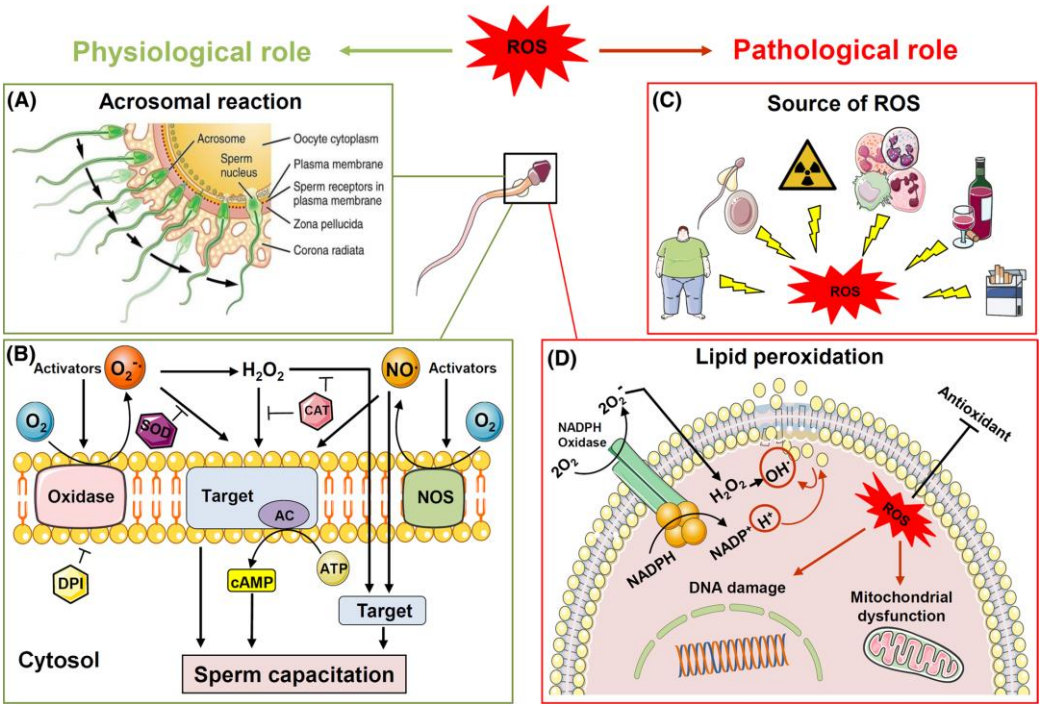

Figure 4. Reactive Oxygen Species and Spermatocytes (Barati et al., 2020).

1.10.2 Lipid Peroxidation

Lipid peroxidation is a series of oxidative lipid breakdown processes. Through which free radicals "steal" electrons from the lipids in cell membranes, harming the cells. This activity is driven by a free radical chain reaction mechanism. Due to the presence of methylene bridges (-CH2-) with extremely reactive hydrogen atoms between multiple double bonds in polyunsaturated fatty acids, these acids are the most frequently damaged. The reaction has three main stages: initiation, propagation, and termination, just like any other radical reaction (Figure 5). The chemical byproducts of this oxidation are lipid peroxides or lipid oxidation products (LOPs) (Ayala et al., 2014).

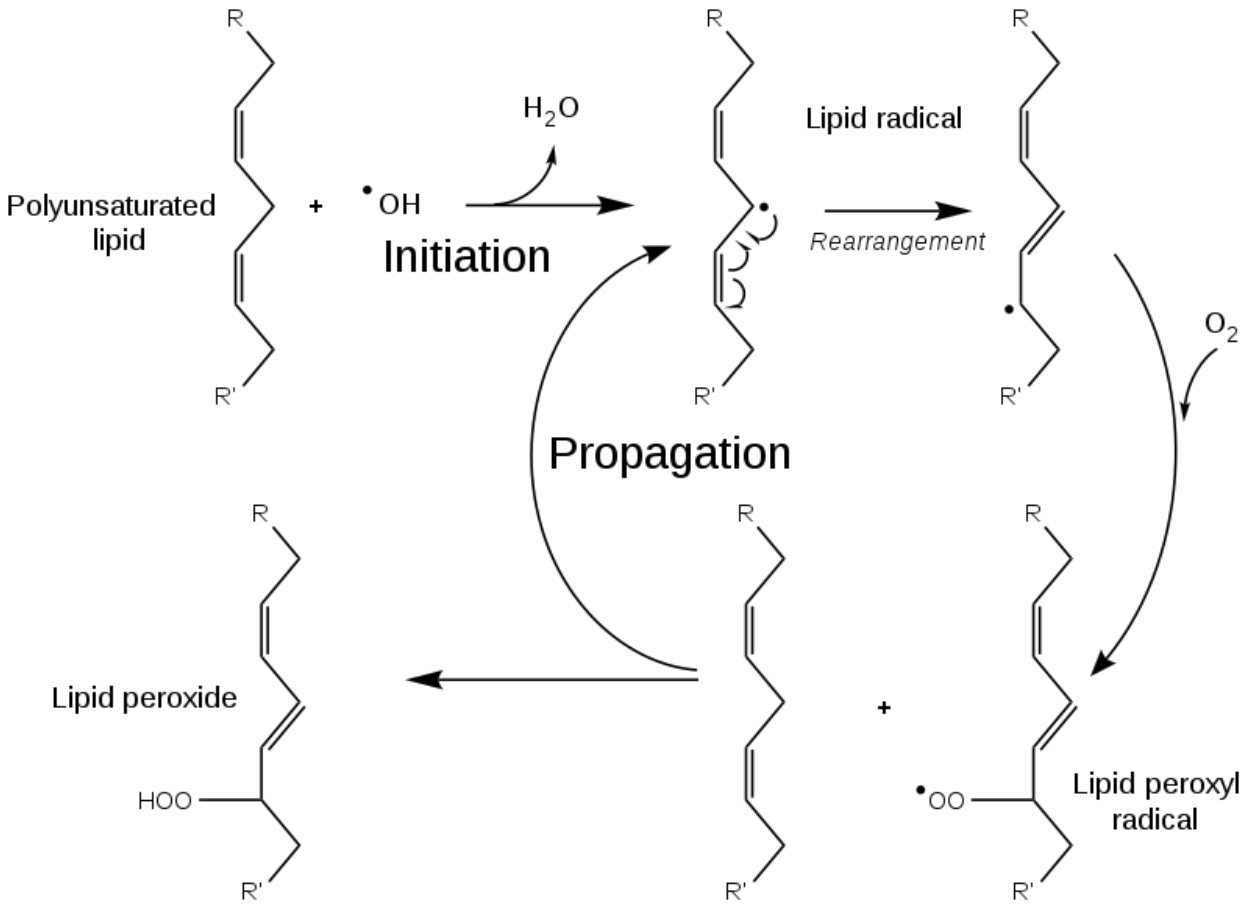

Figure 5. The Mechanism of Lipid Peroxidation (González-Minero et al., 2020).

Sperms are particularly prone to ROS damage because of the high concentration of polyunsaturated fatty acids (PUFA) in their plasma membranes and the extremely low levels of antioxidant enzymes in their cytoplasm. An overabundance of ROS in stressed sperms leads to the development of lipid peroxidation cascades, which lowers sperm function (Wathes et al., 2007).

Excessive lipid peroxidation alters the accumulation, structure, and metabolic dynamics of lipid membranes depending on the degree of lipid peroxidation, and because these lipids are so reactive, it can produce a sizable amount of ROS (Moazamian et al., 2015). Lipid peroxidation is effectively activated by the hydroxyl radical (OH). Most of the unsaturated fatty acids in the sperm membrane are non-conjugated double bonds that are divided by methylene groups. The likelihood of hydrogen separation increases when double bonds are present adjacent to the methylene group, which weakens the methylene H-C bonds. Hydrogen splitting results in the formation of free radicals and a shift in the position of double bonds, which allows the newly produced radical to be stabilized. This radical is made up of a single bond bridging two double bonds. As a result, lipids close to methylene groups that have a lot of double bonds are particularly susceptible to O2 peroxidation and radical production. Lipid peroxyl radicals (ROO), which swiftly combine with conjugated radicals to generate lipid hydroperoxide (ROOH), remove hydrogen atoms from other lipid molecules (Aitken et al., 2014). Increased ROS levels decrease axonal protein phosphorylation and sperm motility by oxidizing sulfhydryl groups (SH-), one reaction that is induced by elevated ROS levels. One type of ROS, hydrogen peroxide, can pass through the sperm membrane and enter the cytoplasm. Several enzymes, notably G6PD, are inhibited by hydrogen peroxide in the cytoplasm of sperm (John Aitken et al., 1989).

Furthermore, by removing electrons from the lipids in plasmid membranes, ROS can cause lipid peroxidation. This leads to a series of reduction-oxidation reactions that result in the production of electrophilic aldehydes that are highly mutagenic and genotoxic, including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and acrolein. Lipid hydroperoxides (LOOHs) of fatty acids-6, such as linoleic acid and arachidonic acid, are used to create 4-HNE (Ayala et al., 2014). Malondialdehyde (MDA) is a result of lipid peroxidation that is employed in several biochemical methods to evaluate spermatozoa's peroxidation damage (Kumar, 2007). MDA is highly mutagenic, but 4-HNE is a byproduct that is extremely genotoxic. Acrolein and 4-HNE significantly speed up apoptosis, mitochondrial ROS production, DNA damage, and lipid

peroxidation (Aboua et al., 2012; Kumar, 2007). High levels of ROS can damage mitochondrial membranes, activating caspases and ultimately leading to apoptosis. The generation of cytochrome c during apoptosis boosts ROS levels, causing DNA damage and fragmentation to increase and perhaps accelerating the apoptotic cycle (Aboulmaouahib et al., 2018; Aitken et al., 2013). As a result, ROS have a major effect on the sperm plasma membrane and can alter the genetic makeup of these cells by triggering cascade signaling. In a recent study, lipid peroxidation, NO, ascorbic acid, and GSH were evaluated in both infertile and indeterminate reproductive potential groups by comparing sperm parameters using MDA levels as a factor. Variable comparison reveals that as NO, GSH, and ascorbic acid levels rise in the infertile group, sperm quality decreases. According to the results, GSH and ascorbic acid levels presumably increased to counteract ROS's harmful effects and stop lipid peroxidation (Gaschler & Stockwell, 2017).

Chapter Two

Materials and Methods

2.1 Experimental Animals

36 adult male Sprague-Dawley rats (~250 g) were used. All the animals were obtained from Jordan University of Science and Technology's animal house. The animals of this study were maintained under the same conditions, each animal was housed individually in special cages, kept away from any noise pollution, and was maintained in an environment of 12-hour dark cycle and 12-hour light cycle at approximately 25°C. No special diet was required for the subjects, food and water were provided ad libitum. All procedures regarding the experimental animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals applied by the National Institutes of Health (NIH) (Council, 2010).

2.2 Experimental Design and Categorization

The animals were randomly categorized into six groups (six animals in each group) as follows:

1. Cyclophosphamide group: each subject in this group was treated using cyclophosphamide injected intraperitoneally.
2. HCl acute group: each animal in this group was treated at the day of experiment using 0.1M HCl administered into the urinary bladder.
3. HCl chronic group: each animal in this group was treated one week before the experiment using 0.1M HCl administered into the urinary bladder.
4. Cyclophosphamide sham group: instead of cyclophosphamide, this group was treated using 0.9% normal saline injected intraperitoneally.
5. HCL sham group: instead of HCl, this group was treated using 0.9% normal saline administered in the urinary bladder.
6. Control group: no treatment.

2.2.1 Cyclophosphamide-induced Cystitis Model

According to (Dattilio & Vizzard, 2005), this model was induced by giving each subject 75 mg/kg of cyclophosphamide intraperitoneally. Each animal in this group was shaved prior to the procedure and each injection was three days apart from another on a total duration of ten days.

2.2.2 Hydrochloric acid-induced Cystitis Model

This model was induced surgically under sterile conditions by the direct instillation of hydrochloric acid (HCl). First, each subject was anesthetized using a mixture of ketamine (80mg/kg) and xylazine (10 mg/kg), using a surgical blade an incision was made in the lower anterior wall of the abdomen to gain access into the urinary bladder, a retractor was used to maintain the incision open while operating. A 24-gauge catheter was directly administered into the urinary bladder and fixed during the treatment process. One ml of 0.1M HCl was administered into the urinary bladder for 10 minutes, followed by the neutralization and washing with normal saline for three times. After that the incision was sutured. In the chronic HCl group, each animal was additionally treated with 2.5 mg/kg Ketoprofen once a day for two days as analgesic to eliminate any anticipated pain. Additionally, 5 mg/kg of Gentamicin was given once a day for five days to avoid any post-operative urogenital or wound infections.

2.3 Tissue collection

Tissue samples were collected as the final step of the experiment. Animals were euthanized by an anesthetic overdose of urethane. The epididymis was exposed by a scrotal incision and was collected by cutting both vas deferens and corpus epididymis with a scissor. Next, cauda epididymis was transferred to pre warmed 0.9% normal saline, obtained sperms was used in further biochemical and gene expression studies. Similarly, after the extraction of cauda epididymis, each animal was perfused by 0.9% phosphate buffer saline (PBS), bladder and testes were kept on ice until biochemical and gene expression studies.

2.4 Quantification of Lipid Peroxidation (LPO)

The peroxidation of cell membranes, lipoproteins, and other lipid-containing structures is a well-known form of oxidative damage. Malondialdehyde (MDA) was measured spectrophotometrically in the urinary bladder, sperms and testicular tissue samples using the thiobarbituric acid reactive substance (TBARS) as described by (Guidet & Shah, 1989). Reagents: 17.5% trichloroacetic acid (TCA), 70% TCA, 0.6% thiobarbituric acid (TBA).

1. Tissue samples and spermatozoa were removed from each animal rapidly and homogenized in 0.02 M phosphate buffered saline (PBS, pH 7.4).

2. 1 mL of 17.5% trichloroacetic acid (TCA) was added to 1 mL of the homogenate and placed on ice.

3. 1 mL of 0.6% thiobarbituric acid (TBA) was added to the homogenate.

4. The tubes were placed in a boiling water bath (80°C) for 20 min, all tubes were allowed to cool at room temperature.

5. 1 mL of 70% of TCA was added. then incubated for about 20 minutes, centrifuged at 2000 rpm for 15 minutes. The supernatant was collected, and absorbance was read against a reagent blank at 532 nm.

2.5 Gene Expression Analysis

The expression of mRNA levels of vital mitochondrial respiratory chain genes was assessed using quantitative polymerase chain reaction (qPCR) analysis, these genes include NADPH dehydrogenase 1 (*mt-ND1*), NADPH dehydrogenase 5 (*mt-ND5*), cytochrome B (*mt-CYB*), cytochrome C (*mt-Co1*), ATP synthase 6 (*mt-ATP6*), and ATP synthase 8 (*mt-ATP8*).

2.5.1 Primer Preparation

The primers in this study were designed using Perl Primer software (Table 2). The housekeeping gene β-actin was used as the reference gene. The National center for Biotechnology Information (NCBI) Primer-BLAST was used to confirm the primers specificity to each of the templates.

Table 2. List of Primers used in Real time qPCR.

Gene	Primer sequence	Product size (Kb)
mt-ND1	F: ATGGCCTTCCTCACCCTAGT R: GCTCGTAGGGCTCCGAATAG	356
mt-ND5	F: ACTCCCGTCTCTGCCTTACT R: GGCCTAGTTGGCTGGATGTT	214
mt -CYB	F: TGCCGAGACGTAAACTACGG R: GTGGAATGCGAAGAAGCGTG	311
mt -CoI	F: ATCGCAATTCCTACAGGCGT R: GCAAAGTGGGCTTTTGCTCA	339
mt-ATP6	F: AACGCCTAATCAGCAACCGA R: TGCTCATAGGGGGATGGCTA	227
mt-ATP8	F: TGACATGCCACAACTAGACACA R: TGGGGGTAATGAAAGAGGCAA	197
Actb	F: CGAGTACAACCTTCTTGCAGC R: GTCAGGATGCCTCTCTTGCT	255

mt-ND1 – *mt-ND5*, NADPH dehydrogenase subunits 1, 5; *mt-CYB*, mitochondrially encoded cytochrome b; *mt-CoI*, cytochrome c oxidase subunits 1; *mt-ATP6*, mitochondrially encoded ATP synthase 6; *mt-ATP8*, mitochondrially encoded ATP synthase 8.

2.5.2 RNA Extraction

Total RNA was extracted from both testicular tissue and fresh sperm using TRIzol isolation method, slightly modified (Rio et al., 2010). Each sample was homogenized in 1 ml of TRI reagent (Zymo Research, CA, USA) per 50 to 100 mg of tissue and 0.1 to 0.2 ml of fresh sperms and were incubated at room temperature for 20 minutes to ensure complete cellular dissociation. For phase separation 0.2 ml of chloroform was added to each sample, capped securely and vortexed vigorously and incubated at room temperature. Each sample was centrifuged at 10,000xg for 15 minutes, the upper aqueous phase was transferred carefully to a fresh nuclease free eppendrof tube. For RNA precipitation 0.5 ml of isopropanol was added, gently mixed, and left to set on room temperature for at least 20 minutes, samples were centrifuged at 10,000xg for 10 minutes, isopropanol was completely discarded leaving a pellet of RNA at the bottom of the tube. RNA pellet was washed twice using 1 ml of 75% ethanol each time and centrifuged at no more than 7,500xg for 5 minutes, ethanol was discarded, and the RNA was air-dried. For RNA re-dissolving, nuclease free water was used and set on ice for 15 – 20 minutes for complete dissolving.

The quality and concentration of RNA was assessed using Nabi UV/vis nanodrop spectrophotometer (Nabi, GyungGi-Do, Korea), each sample had a concentration of ~ 1 µg. The ratio absorbance of the RNA samples at 260/230 were 2.0 – 2.2 and for 260/280 ratio the absorbance was generally ~ 1.8 – 2.0 which is consider "pure" for both ratios.

2.5.3 Reverse Transcription and cDNA Synthesis

The previously extracted RNA was used as template for complementary DNA synthesis (cDNA). PrimeScript™ RT Master Mix (Takara, Japan) was used for the generation of cDNA following manufacturer's protocol. 500 ng/ul of total RNA was used as a template for cDNA synthesis, 2 ul of 5x PrimeScript RT master mix, 1 ul of total RNA and 7 ul nuclease free water to make a total volume of 10 ul (volumes were adjusted based on the concentration of the RNA sample).

The mixtures were incubated at 37°C for 1 hour in a dry heat block, then placed at 85°C for 5 seconds for the inactivation of the reverse transcriptase enzyme. Samples were set on ice at room temperature and then stored at -20°C.

2.5.4 Real-Time qPCR

Quantitative real-time PCR (qPCR) analysis was carried on using the respective cDNA template for each sample and *TB Green Premix Ex Taq II* (Takara, Japan) with a total volume of 20 ul in each reaction well following manufacturer's protocol, qPCR then was performed using real-time thermal cycler qTOWER^3 (Analytik Jena, Germany). The step by step followed protocol for the thermal cycler is illustrated in Figure 6. The annealing temperatures for each set of primers are listed in (**Table 3)**.

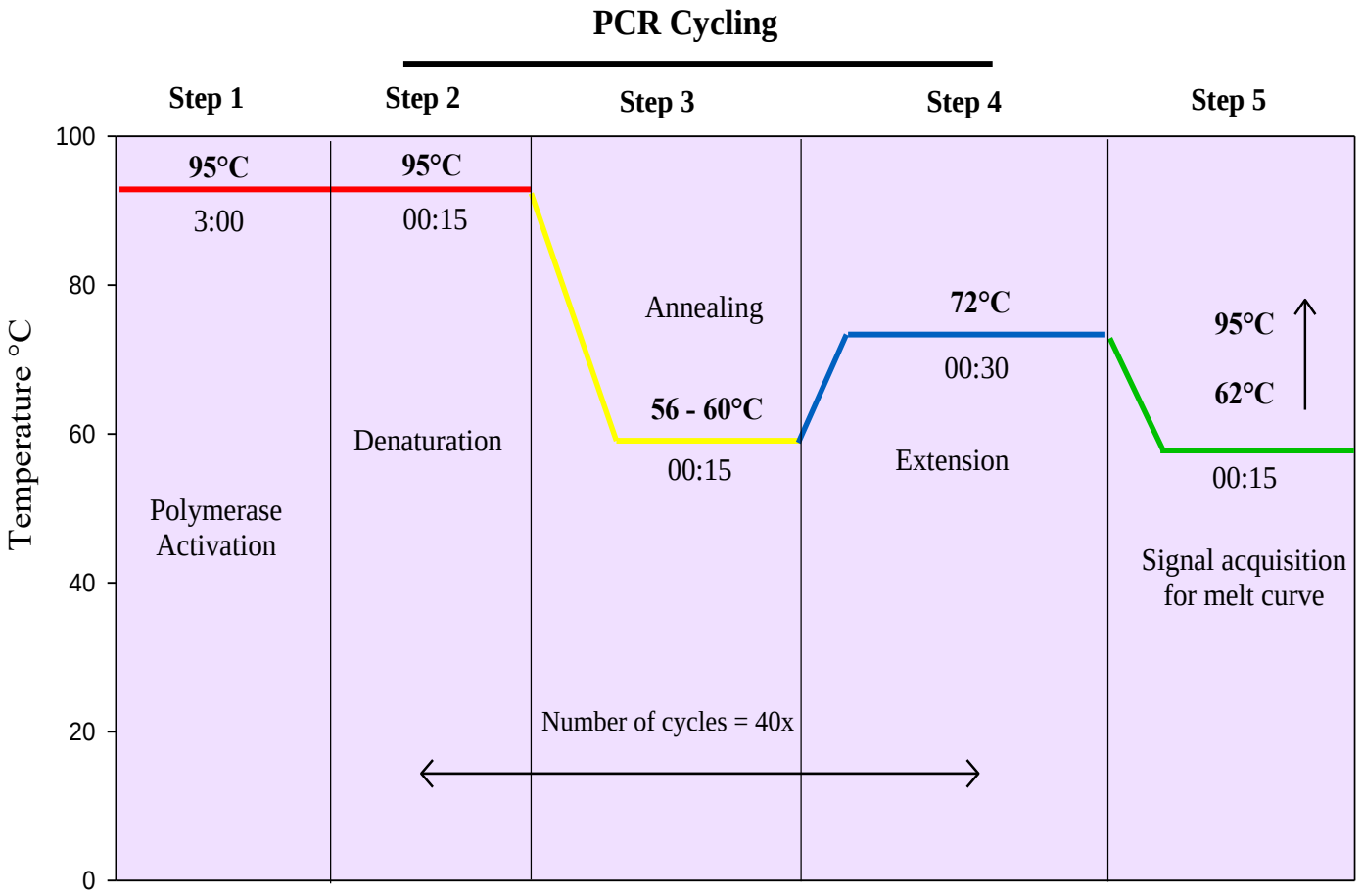

Figure 6. Real-time PCR Step-by-step Thermal Cycler Followed Protocol

The first step represents DNA polymerase activation by the pre-denaturation heating of the thermal cycler (95°C for 3 minutes). Step 2 represents the denaturation step in which the double stranded DNA starts unwinding (95°C for 15 seconds). Step 3, the annealing temperature for each pair of primer differ in which each primer would anneal to its specific site on each strand (56 - 60°C for 15 seconds). Step 4, extension (72°C for 30 seconds). Step 5 was optional for the acquisition of melting curve (the temperature lowers to 62°C and gradually rise to 95°C, 15 seconds).

Table 3. List of Annealing Temperature for Each Set of Primers

Gene	Annealing Temperature (Tm)
mt-ND1	58.5°C
mt-ND5	58.5°C
mt-CYB	57.6°C
mt-CoI	60°C
mt-ATP6	59.4°C
mt-ATP8	59.4°C
Actb	56°C

After performing all qPCR experiments, the Livak method, also known as the delta delta C_T method (C_T = cycle threshold) was used for measuring relative mRNA levels of expression for each gene (Livak & Schmittgen, 2001). The levels of gene expression were standardized against the gene β-actin which was used as an endogenous reference gene. Regarding relative gene expression, first each sample was normalized against the endogenous reference gene (C_T of the gene of interest (GOI) - C_T of the reference gene), the resulting represents the first delta C_T (ΔC_T). All the obtained ΔC_T values will be furthermore normalized against the average of ΔC_T values for the control group ($\Delta\Delta C_T$). Finally, the following formula: $2^{-\Delta\Delta CT}$ was applied to all the values.

2.6 Statistical Analysis

One-way ANOVA and Tukey's tests were used to assess significance of differences among treatment groups. The results were represented as mean ± SEM. All statistical analysis was performed using IBM SPSS statistics 20 software (USA, 2012). Levels of statistical significance were set at $P<0.05$. All of figures were generated using GraphPad Prism 9.0 software (GraphPad Software, USA).

Chapter Three

Results

3.1 Histopathological Evaluation of Inflammation in the Urinary Bladder

3.1.1 Hematoxylin and Eosin (H & E) of the Bladder in Control Group

The presence of an inflammatory response in the urinary bladder was assessed in each animal using hematoxylin and eosin stain. The degree of inflammation in the urinary bladder tissue for each group was measured using microscopic qualitative tissue characterization. In the control group a normal characteristic appearance of the bladder tissue was observed, the lining urothelium, appear to be intact with no apparent signs of inflammation (**Figure 7**).

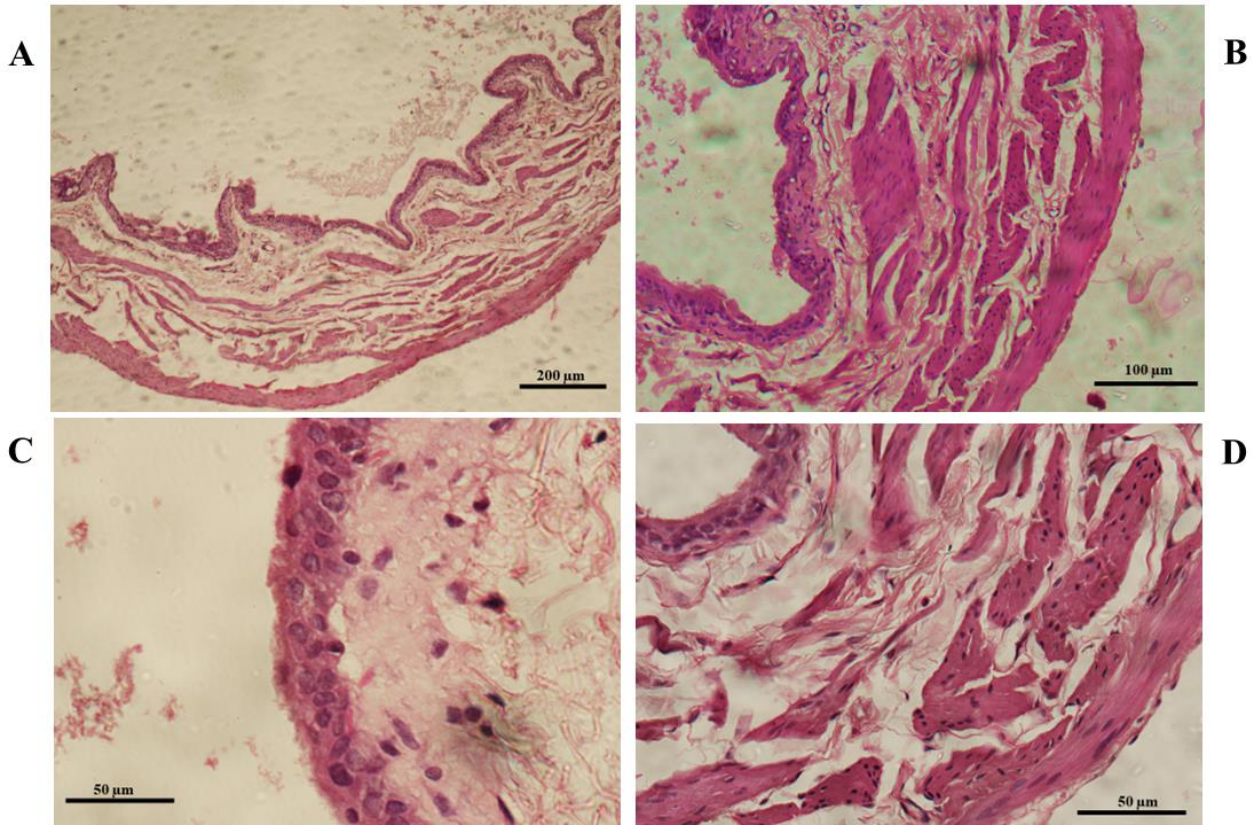

Figure 7. Hematoxylin and Eosin (H & E) stained sections of the urinary bladder tissue of the control group demonstrated a normal appearance of the urothelium with no signs of inflammation.

3.1.2 Hematoxylin and eosin (H & E) of the Bladder in Acute HCl Group

An acute inflammatory response was observed in the acute HCl group, histopathological features included urothelial sloughing, vascular congestion, edema, and infiltration of inflammatory cells (**Figure 8**).

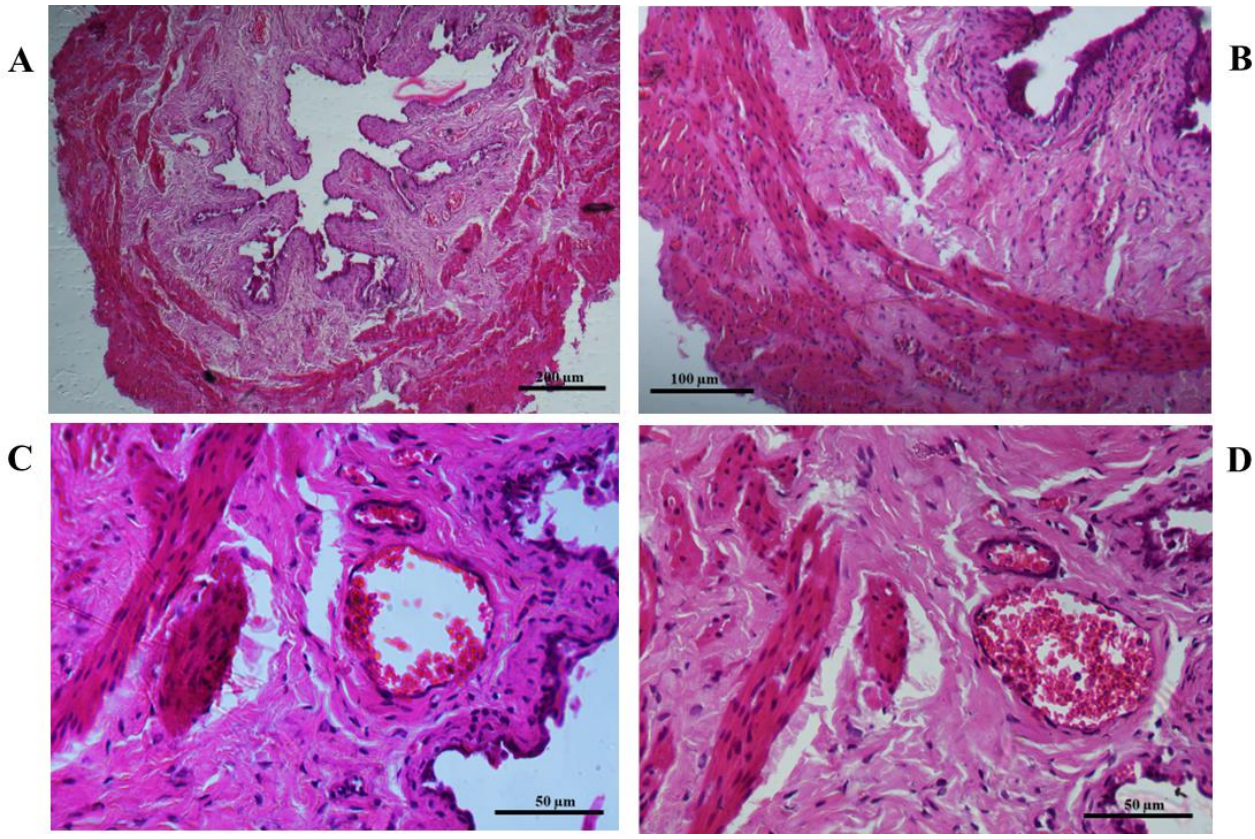

Figure 8. The urinary bladder tissue in acute HCl-induced cystitis. Hematoxylin and eosin (H & E) stained sections of the urinary bladder tissue (A) demonstrated a significant urothelial sloughing, (B) infiltration of inflammatory cells, (C, D) vascular congestion and edema.

3.1.3 Hematoxylin and eosin (H & E) of the Bladder in Chronic HCl Group

In chronic HCl-induced cystitis model, the intravesical instillation of HCl two weeks prior to the sacrifice of animals had led to a state of chronic inflammation. Unlike acute HCl-induced cystitis models, an ameliorated form of inflammation is observed in H&E-stained sections of chronic HCl-induced cystitis models. Observed histopathological features included a regeneration of the urothelium, thickening in some areas of the urothelium, edema and fibrosis. Additionally,

hyperplastic changes in some areas were observed. A significant tissue repair and renewal was observed in chronic HCl-induced cystitis compared to both acute HCl model and the control group (**Figure 9**).

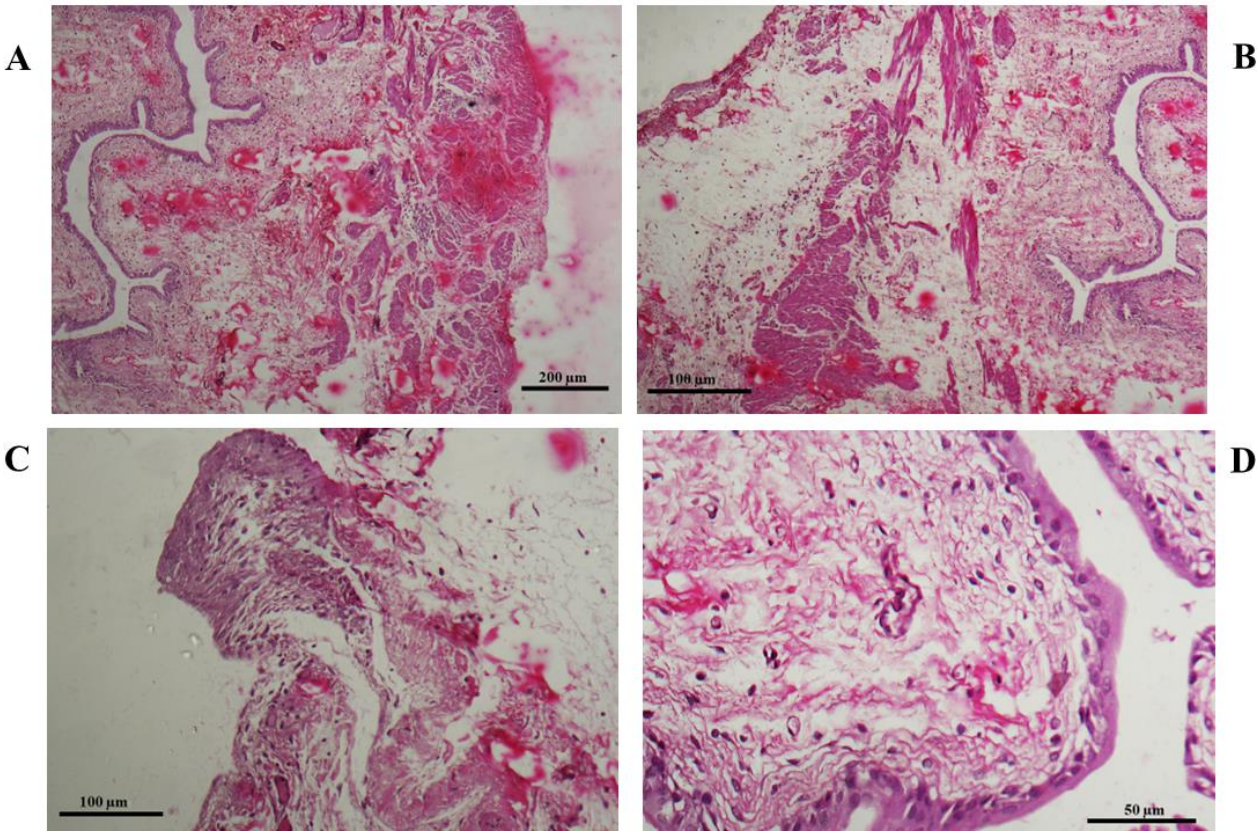

Figure 9. Histology of the urinary bladder in chronic HCl-induced cystitis. Hematoxylin and eosin (H & E) stained sections of the urinary bladder tissue (A) demonstrated a significant tissue repair, (B) edema, (C) thickening in some areas of the urothelium. (B - D) Hyperplastic changes were observed.

3.1.4 Hematoxylin and eosin (H & E) of the Bladder in CYP Group

In CYP-induced cystitis model, several histopathological features of inflammation were observed including urothelium atypia, abnormal thickening of the urothelium, areas of hyperplasia, vascular congestion, a significant increase in the content of smooth muscle, in addition to infiltration of inflammatory cells (**Figure 10**).

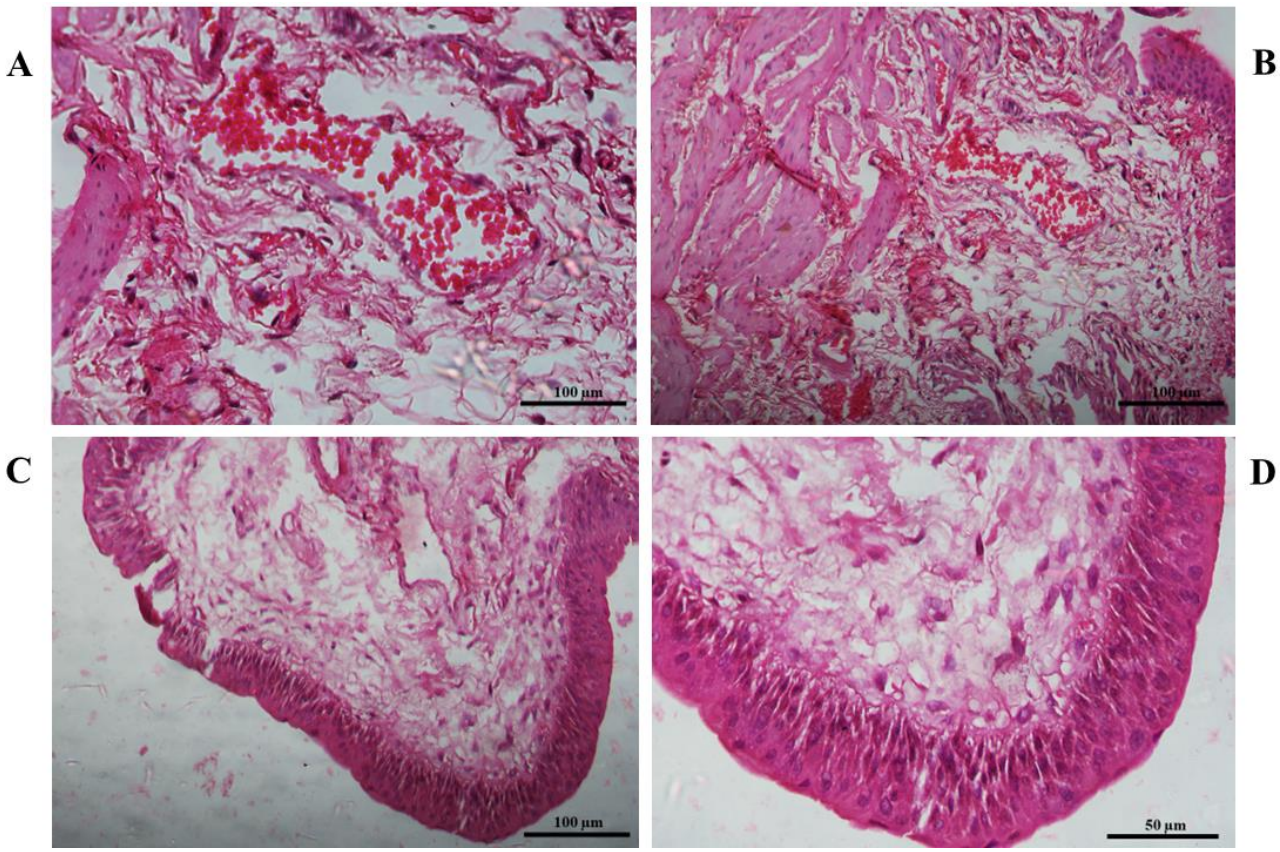

Figure 10. Histology of the urinary bladder in CYP-induced cystitis. Hematoxylin and eosin (H & E) (A) stained sections demonstrated a significant increase in the content of smooth muscle, (B) vascular congestion, (C) hyperplastic changes including urothelium atypia and edema, (D) a significant thickening of the bladder urothelium.

3.2 Quantification of Lipid Peroxidation Through the Thiobarbituric Acid Reactive Substances (TBARS) Assay

The TBARS assay for the quantification of MDA levels was used in this study as an indicator of peroxidative damage in different structures. MDA levels were measured in testicular tissue, spermatozoa, and the urinary bladder of the control group, acute HCl group, chronic HCl group, CYP group, sham HCl group and sham CYP group.

3.2.1 MDA Levels in Testicular Tissue

The levels of MDA as an indicator of peroxidative damage in testicular tissue were assessed. A significant increase of MDA levels ($P < 0.001$) in chronic HCl model was observed compared to the control group, acute HCl group, sham HCl group and sham CYP group. Similarly, a significant increase ($P < 0.001$) was also observed in CYP model compared to the other groups. No significant change was observed in acute HCl, sham HCl and sham CYP groups (**Table 4, Figure 11A**).

Table 4. Levels of MDA in Testicular Tissue.

Group	N	MDA (nmol/mg)
Control	n=6	0.040 ± 0.008
Acute HCl	n=6	0.051 ± 0.006
Chronic HCl	n=6	0.19 ± 0.025 *
CYP	n=6	0.37 ± 0.025 *
Sham HCl	n=6	0.034 ± 0.002
Sham CYP	n=6	0.035 ± 0.005

Values are represented as mean ± SEM, a significance of ($P < 0.001$) between groups was observed in testicular tissue.

* ($P < 0.001$) compared to the other groups.

3.2.2 MDA Levels in spermatozoa

The levels of MDA in spermatozoa were assessed as an indicator of peroxidative damage. A significant increase in the levels of MDA were observed in chronic HCl group ($P < 0.007$) compared to the control group, a significance of ($P < 0.024$) compared to acute HCl group, and a significance of ($P < 0.05$) compared to the sham HCl group. In CYP group the levels of MDA were significantly increased ($P < 0.001$) compared to the control group and the acute HCl group, a significance of ($P < 0.001$) compared to the sham HCl group and a significance of ($P < 0.002$) compared to the sham CYP group. No significant change in the control, acute HCl, sham HCl and sham CYP was observed (**Table 5, Figure 11B**).

Table 5. Levels of MDA in Spermatozoa.

Group	N	MDA (nmol/mg)
Control	n=6	0.013 ± 0.006
Acute HCl	n=6	0.019 ± 0.005
Chronic HCl	n=6	0.036 ± 0.006 *
CYP	n=6	0.061 ± 0.009 †
Sham HCl	n=6	0.019 ± 0.002
Sham CYP	n=6	0.025 ± 0.003

Values are represented as mean ± SEM, a significance of ($P < 0.001$) between groups was observed in spermatozoa.

* The levels of MDA were significantly increased in chronic HCl group ($P < 0.007$) compared to the control group, a significance of ($P < 0.024$) compared to acute HCl group, and a significance of ($P < 0.05$) compared to the sham HCl group.

† The levels of MDA were significantly increased in CYP group ($P < 0.001$) compared to the control group and the acute HCl group, a significance of ($P < 0.001$) compared to the sham HCl group and a significance of ($P < 0.002$) compared to the sham CYP group.

3.2.3 MDA Levels Evaluated in the Urinary Bladder

The levels of MDA in the urinary bladder tissue were assessed as an indicator of peroxidative damage. A significant increase in chronic HCl group ($P < 0.003$) was observed compared to the control group, a significance of ($P < 0.012$) compared to acute HCl group, and a significance of ($P < 0.008$) compared to the sham HCl group. In CYP group a significant increase was observed in MDA levels ($P < 0.001$) compared to the control group and the acute HCl group, a significance of ($P < 0.001$) compared to the sham HCl group and a significance of ($P < 0.001$) compared to the sham CYP group. Additionally, the MDA levels in the CYP showed a significant increase of ($P < 0.006$) compared to the chronic HCl group. No significant change in the control, acute HCl, sham HCl and sham CYP was observed (**Table 6, Figure 11C**).

Table 6. MDA Levels in the Urinary Bladder Tissue.

Group	N	MDA (nmol/mg)
Control	n=6	0.012 ± 0.005
Acute HCl	n=6	0.018 ± 0.003
Chronic HCl	n=6	0.044 ± 0.006 *****
CYP	n=6	0.071 ± 0.008 †
Sham HCl	n=6	0.015 ± 0.002
Sham CYP	n=6	0.023 ±0.004

Values are represented as mean ± SEM, In the urinary bladder tissue, a significance in the MDA levels ($P < 0.001$) was observed between groups.

* The levels of MDA were significantly increased in chronic HCl group ($P < 0.003$) compared to the control group, a significance of ($P < 0.012$) compared to acute HCl group, and a significance of ($P < 0.008$) compared to the sham HCl group.

† The levels of MDA were significantly increased in CYP group ($P < 0.001$) compared to the control group and the acute HCl group, a significance of ($P < 0.001$) compared to the sham HCL group and a significance of ($P < 0.001$) compared to the sham CYP group. Additionally, the MDA levels in the CYP showed a significant increase of ($P < 0.006$) compared to the chronic HCl group.

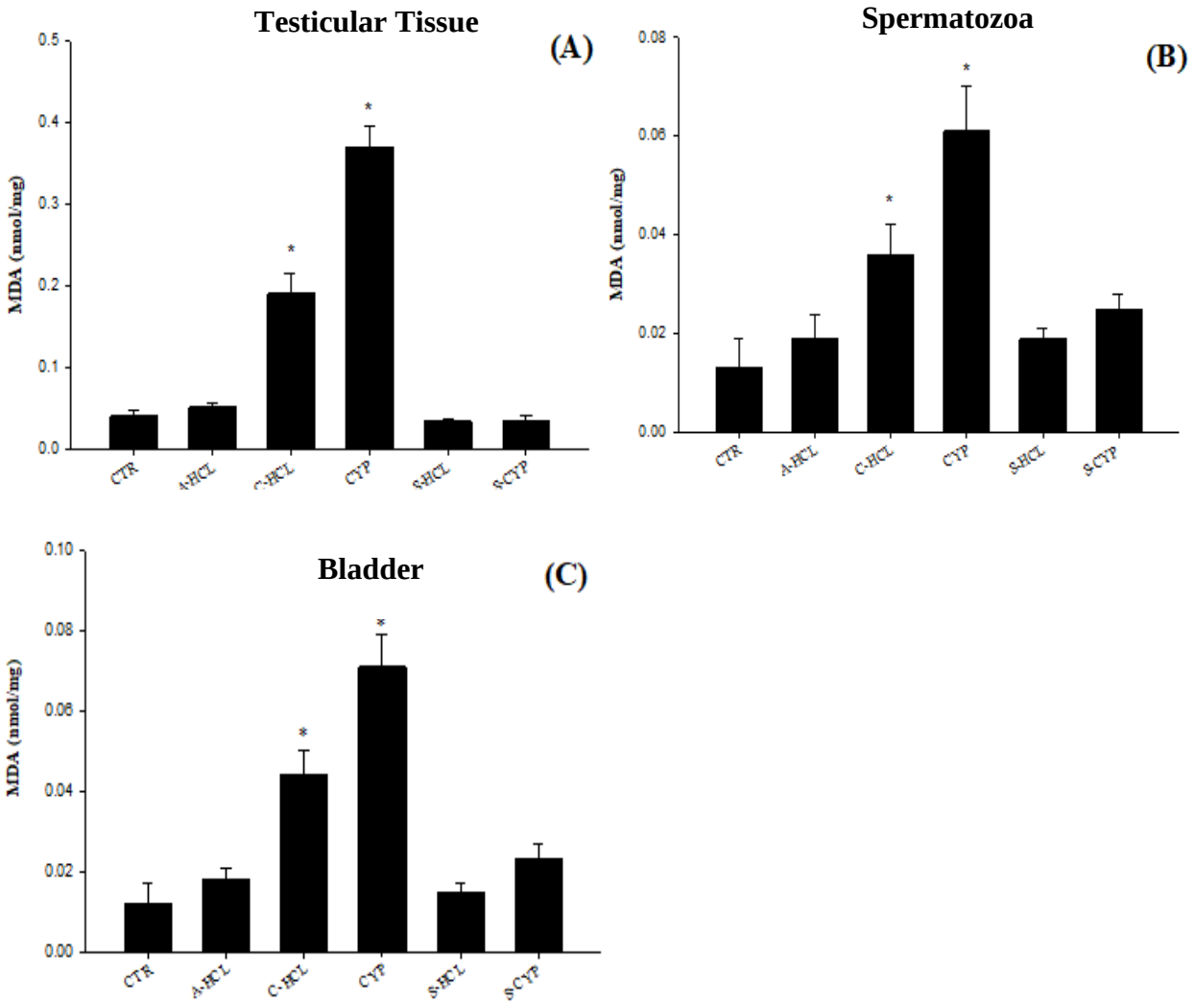

Figure 11. The Level of MDA Evaluated in Various Structures in Models of Induced-Interstitial Cystitis

Levels of MDA obtained from the thiobarbituric acid assay. **(A)** in testicular tissue, the levels of MDA had significantly increased in both chronic HCl and CYP models. **(B)** in spermatozoa, the levels of MDA were significantly higher in both chronic HCl and CYP compared to the other groups. **(C)** in the bladder the content of MDA was also significantly higher in both chronic HCl and CYP compared to the other groups.

3.3 Relative Expression of Mitochondrial Respiratory Chain Genes

Quantitative real-time PCR was used for the evaluation of relative gene expression levels of several mitochondrial ETC genes including, NADPH dehydrogenase subunit 1 (*mt-ND1)*, NADPH dehydrogenase subunit 5 (*mt-ND5)*, cytochrome b (mt-*CYB)*, cytochrome c oxidase subunit I (mt-*CoI)*, adenine triphosphate synthase subunit 6 (mt-*ATP6)* and adenine triphosphate synthase subunit 8 (mt-*ATP8)*.

3.3.1 Relative mRNA Expression of *mt-ND1* in Testicular Tissue and Spermatozoa

The relative mRNA levels of *mt-ND1* in both testicular tissue and spermatozoa were evaluated. C_T values of qPCR analysis of *mt-ND1* were normalized to that of β-actin and expressed as mRNA levels against the control group. In testicular tissue, mRNA expression levels of *mt-ND1* were significantly up-regulated in chronic HCl group compared to the other groups (**Figure 12A**). In spermatozoa, mRNA expression levels of *mt-ND1* were significantly up-regulated in chronic HCl group compared to the other groups, on the contrary the levels of *mt-ND1* were significantly down-regulated in CYP group compared to the other groups (**Figure 12B**).

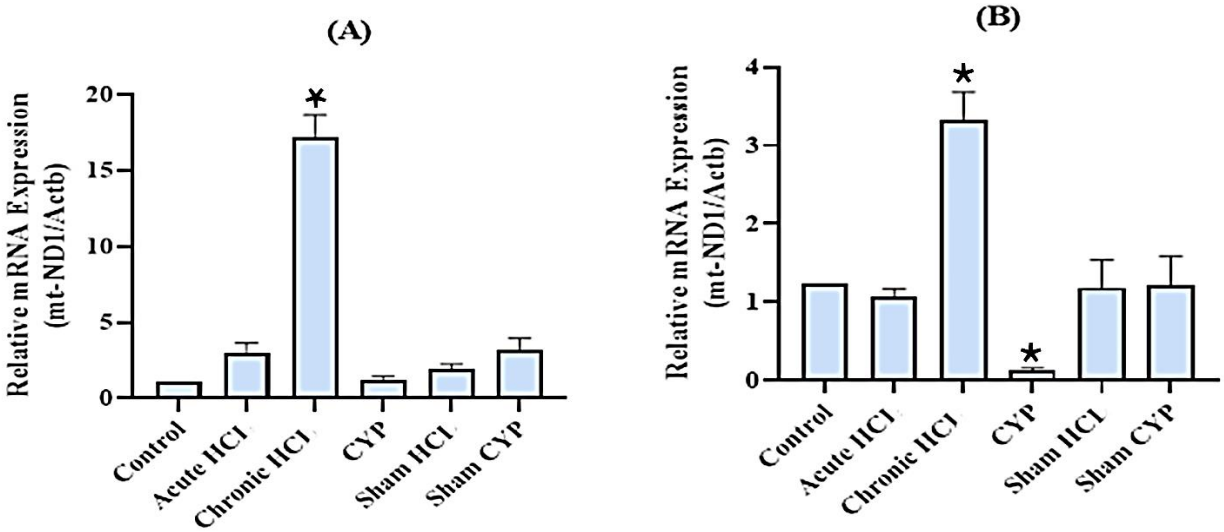

Figure 12. Relative mRNA Expression Levels of mt-ND1 in Testicular Tissue and Spermatozoa

Expression levels of mt-ND1 was evaluated in both testicular tissue and spermatozoa. **(A)** In **testicular tissue**, mRNA levels of *mt-ND1* were significantly upregulated in chronic HCl group ($P < 0.001$) compared to other groups. **(B)** In **spermatozoa**, mRNA levels of *mt-ND1* in chronic HCl model was up-regulated ($P < 0.001$) while in CYP group was significantly down-regulated group ($P < 0.001$) compared to other groups. Mean values are represented by vertical bars. * $P < 0.001$

3.3.2 Relative mRNA Expression of *mt-ND5* in Testicular Tissue and Spermatozoa

The relative mRNA levels of *mt-ND5* in both testicular tissue and spermatozoa were evaluated using quantitative real-time PCR. C_T values of qPCR analysis of *mt-ND5* were normalized to that of β-actin and expressed as mRNA levels against the control group. In testicular tissue, mRNA levels of *mt-ND5* were significantly up-regulated in chronic HCl group compared to the other groups (**Figure 13A**). In spermatozoa, mRNA expression levels of *mt-ND5* were significantly up-regulated in chronic HCl group compared to the other groups, on the contrary the levels of *mt-nd5* mRNA expression were significantly down-regulated in CYP group compared the other groups (**Figure 13B)**.

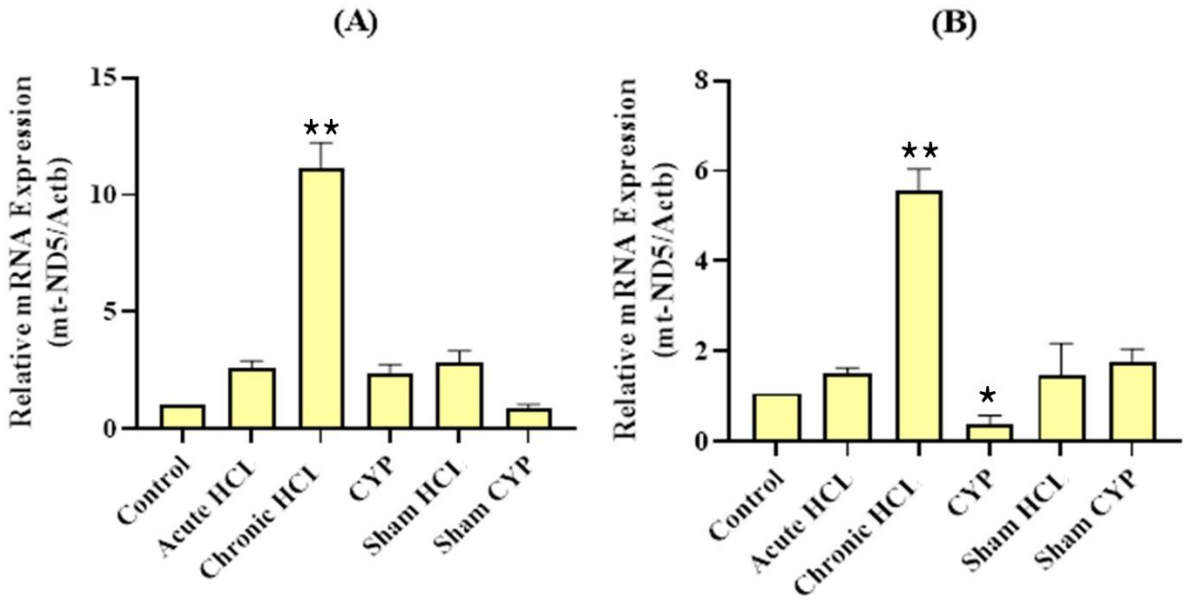

Figure 13. Relative mRNA Expression Levels of *mt-ND5* in Testicular Tissue and Sperms

Relative mRNA expression levels of *mt-ND5* were evaluated using qPCR analysis. **(A)** In **testicular tissue**, mRNA levels of *mt-ND5* were significantly upregulated in chronic HCl group ($P < 0.001$) compared to the other groups. **(B)** In **spermatozoa**, mRNA levels of *mt-ND5* in chronic HCl model was up-regulated ($P < 0.001$) compared to other groups, while in CYP model was significantly down-regulated ($P < 0.005$) compared to other groups. Mean values are represented by vertical bars.

** $P < 0.001$

* $P < 0.005$

3.3.3 Relative mRNA Expression of *mt-CYB* in Testicular Tissue and Spermatozoa

The expression levels of *mt-CYB* were evaluated in both testicular tissue and spermatozoa using qPCR analysis. In testicular tissue, levels of *mt-CYB* were significantly upregulated in chronic HCl models compared to the other groups. No significant change was observed in the expression levels of *mt-CYB* in acute HCl, CYP, sham HCl and sham CYP (**Figure 14A**). In spermatozoa, the expression levels of *mt-CYB* in chronic HCl models were significantly upregulated compared to the other groups. On the contrary, in CYP models the levels of expression were significantly downregulated compared to the other groups (**Figure 14B**).

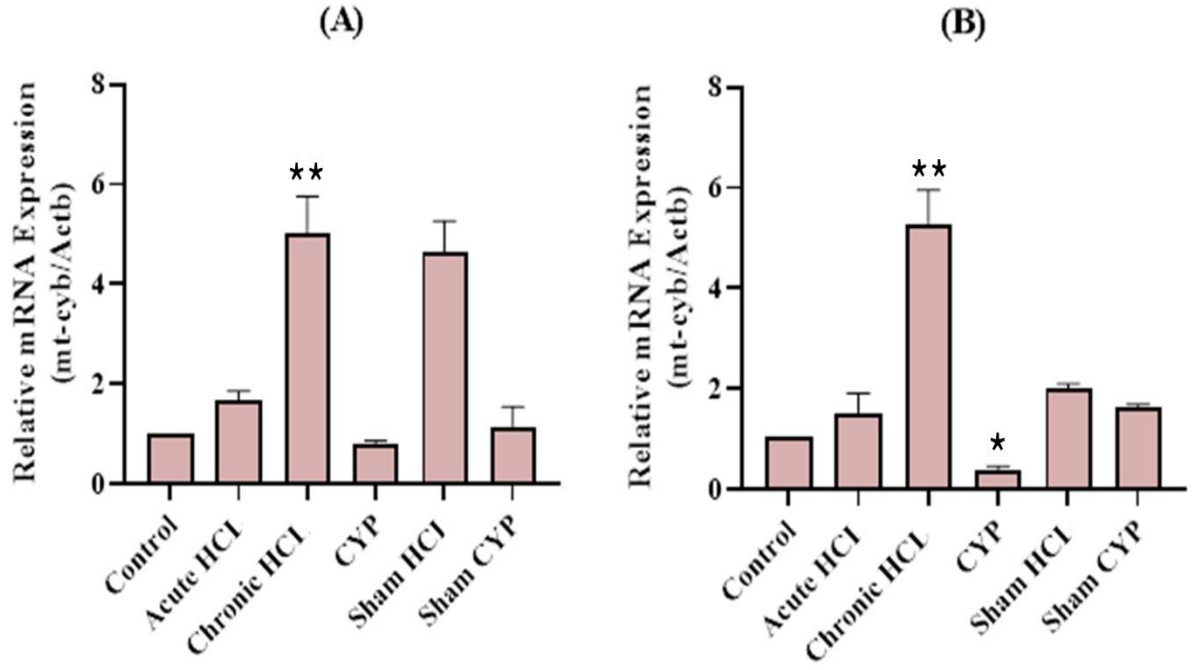

Figure 14. Relative mRNA Expression Levels of *mt-CYB* in Testicular Tissue and Spermatozoa

C_T values of qPCR analysis of *mt-CYB* were normalized to that of β-actin and expressed as mRNA levels against the control group. **(A)** In **testicular tissue**, mRNA levels of *mt-CYB* were significantly upregulated in chronic HCl group ($P < 0.001$) compared to other groups. **(B)** In **spermatozoa**, mRNA levels of *mt-CYB* in chronic HCl model was up-regulated ($P < 0.001$) while in CYP model was slightly down-regulated ($P < 0.005$) compared to other groups. Mean values are represented by vertical bars.

** $P < 0.001$

* $P < 0.005$

3.3.4 Relative mRNA Expression of *mt-CoI* in Testicular Tissue and Spermatozoa

The relative mRNA levels of *mt-CoI* in both testicular tissue and spermatozoa were evaluated. In testicular tissue, mRNA expression levels of *mt- CoI* was significantly up-regulated in chronic HCl model of interstitial cystitis compared to the other groups (**Figure 15A**). In spermatozoa, mRNA expression levels of *mt- CoI* were significantly up-regulated in chronic HCl group compared to other groups, on the contrary the levels of *mt-CoI* mRNA levels were significantly down-regulated in CYP group compared to the other groups (**Figure 15B**).

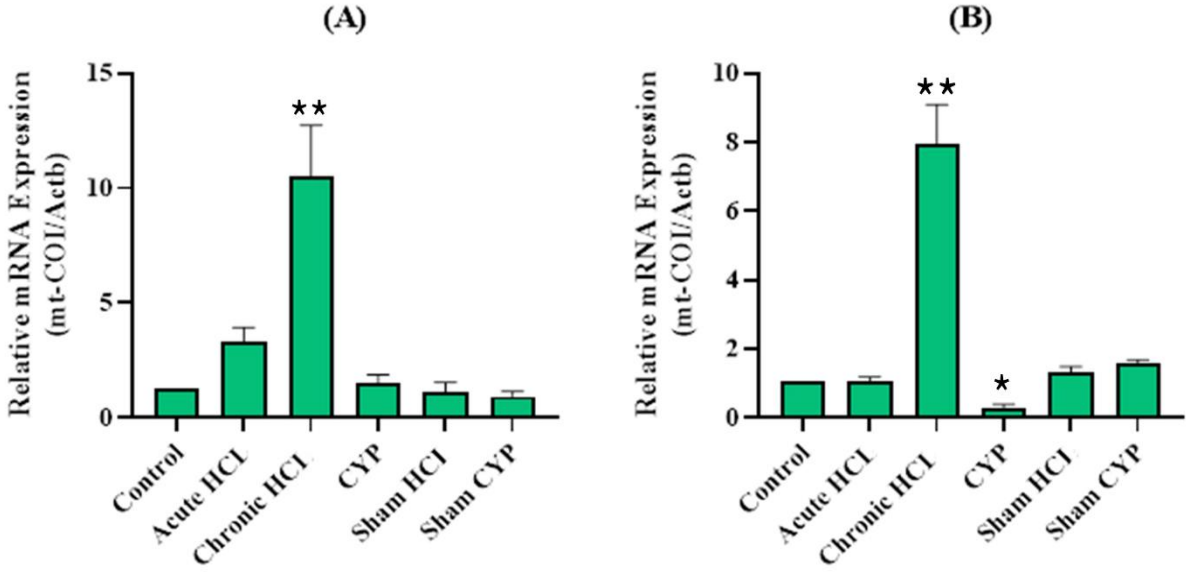

Figure 15. Relative mRNA Expression Levels of *mt-CoI* in Testicular Tissue and Spermatozoa

mRNA levels *mt-CoI* of both testicular tissue and spermatozoa were evaluated using qPCR analysis, results were normalized to that of β-actin and expressed as mRNA levels against the control group. (A) In **testicular tissue**, mRNA levels of *mt-CoI* were significantly upregulated in chronic HCl group ($P < 0.001$). (B) In **spermatozoa**, mRNA levels of *mt-CoI* in chronic HCl model was up-regulated ($P < 0.001$) while in CYP model was significantly down-regulated ($P < 0.01$). Mean values are represented by vertical bars.

** $P < 0.001$

* $P < 0.01$

3.3.5 Relative mRNA Expression of *mt-ATP6* in Testicular Tissue and Spermatozoa

From complex V, mRNA expression levels of *mt-ATP6* in testicular tissue was significantly upregulated in chronic HCl models compared to the other groups. In contrast, no significant change was observed in the levels of expression in acute HCl, CYP, sham HCl and sham CYP (**Figure 16A**). In spermatozoa, expression levels of *mt-ATP6* in chronic HCl models were significantly upregulated compared to the other groups. On the contrary, in CYP models the levels of expression were significantly downregulated. No significant change was observed in the expression levels of *mt-ATP6* in acute HCl, sham HCl and sham CYP group (**Figure 16B**).

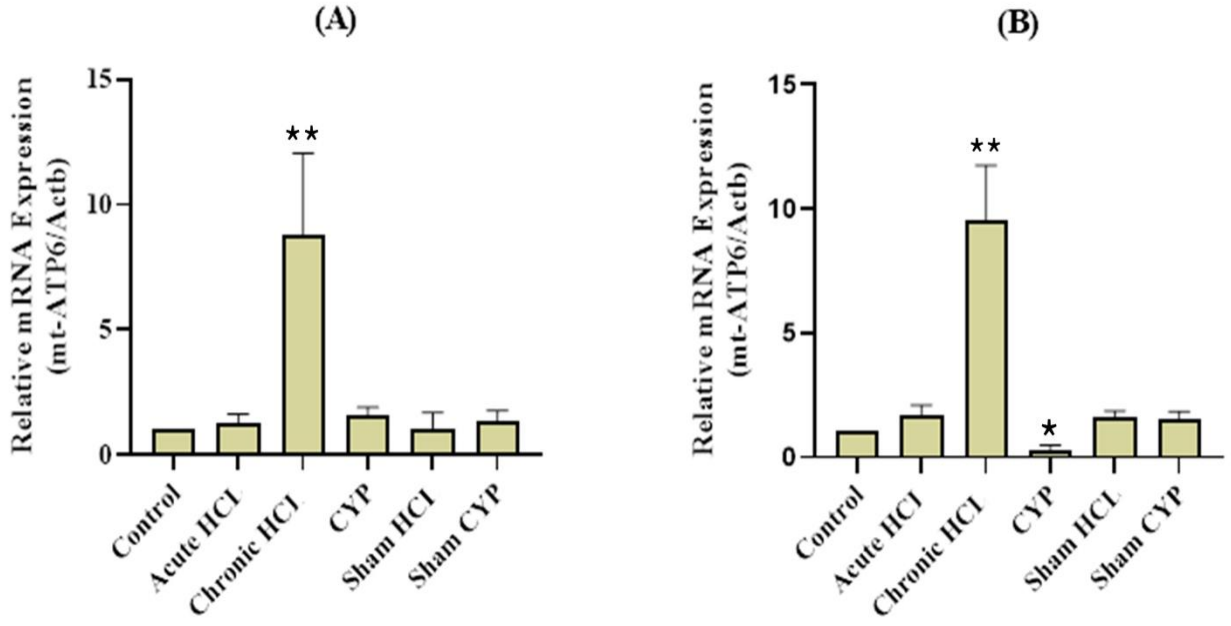

Figure 16. Relative mRNA Expression Levels of *mt-ATP6* in Testicular Tissue and Spermatozoa

mRNA levels *mt-ATP6* of both testicular tissue and spermatozoa were evaluated using qPCR analysis, results were normalized to that of β-actin and expressed as mRNA levels against the control group. (A) In **testicular tissue**, mRNA levels of *mt-ATP6* were significantly upregulated in chronic HCl group ($P < 0.001$). (B) In **spermatozoa**, mRNA levels of *mt-ATP6* in chronic HCl model was up-regulated ($P < 0.001$) while in CYP model was significantly down-regulated ($P < 0.01$). Mean values are represented by vertical bars.

** $P < 0.001$

* $P < 0.01$

3.3.6 Relative mRNA Expression of *mt-ATP8* in Testicular Tissue and Spermatozoa

The levels of mRNA gene expression of *mt-ATP8* from complex V in testicular tissue was significantly upregulated in chronic HCl models compared to the other groups. In contrast, no significant change was observed in the levels of expression in acute HCl, CYP, sham HCl and sham CYP group (**Figure 17A**). In spermatozoa, the expression levels of *mt-ATP8* in chronic HCl models were significantly upregulated compared to the other groups. On the contrary, in CYP models the levels of expression were significantly downregulated. No significant change in the expression levels of *mt-ATP8* in acute HCl, sham HCl and sham CYP (**Figure 17B**).

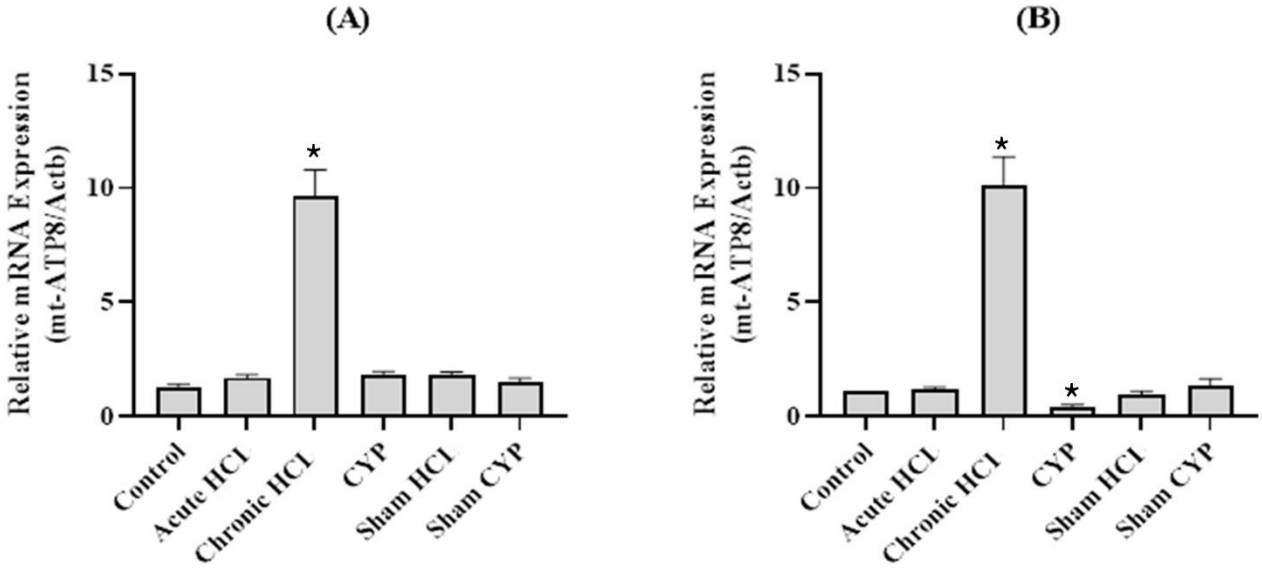

Figure 17. Relative mRNA Expression Levels of *mt-ATP8* in Testicular Tissue and Spermatozoa

mRNA levels *mt-ATP8* of both testicular tissue and spermatozoa were evaluated using qPCR analysis, results were normalized to that of β-actin and expressed as mRNA levels against the control group. (A) In **testicular tissue**, mRNA levels of *mt- ATP8* were significantly upregulated in chronic HCl group ($P < 0.001$). (B) In **spermatozoa**, mRNA levels of *mt- ATP8* in chronic HCl model was up-regulated ($P < 0.001$) while in CYP model was significantly down-regulated ($P < 0.001$). Mean values are represented by vertical bars. * $P < 0.001$

Chapter Four

Discussion

In the present study, we aimed to investigate the detrimental effects of lipid peroxidation and mitochondrial respiratory chain dysfunction on male reproductive system in different animal models of induced-interstitial cystitis.

The inflammatory response induced in different animal models of interstitial cystitis was confirmed by histopathological assessment of H & E sections of the urinary bladder in the six groups. In acute HCl model, an acute inflammatory response was observed characterized by a significant sloughing of the urothelium, inflammatory cells infiltration, edema, and vascular congestions, all are histopathological features of an acute inflammatory response. In contrast, the chronic HCl model showed a more ameliorated form of inflammation. Regeneration of the urothelium was the highlight histopathological feature in this model, in addition to the presence of scarring and fibrosis in some areas of the urothelium consistent with the persistence of the inflammatory process in the urinary bladder of chronic HCl model. All obtained features from both HCl models were representative and reflective of the inflammatory status of the urinary bladder in interstitial cystitis.

Due to the systemic effect of CYP on several organs including the urinary bladder, it has been widely used throughout the literature as a chemically induced model of interstitial cystitis. Data obtained from CYP-induced cystitis model in this study confirm the presence of several histopathological features of inflammation including urothelium atypia, abnormal thickening of the urothelium, areas of hyperplasia, vascular congestion, a significant increase in the content of smooth muscle, in addition to infiltration of inflammatory cell, which validates the use of this as a model of interstitial cystitis in this study. To eliminate the possible direct effect of CYP on male reproductive system (specifically the testis), two animal models of interstitial cystitis were represented in this study.

For the quantification of peroxidative damage as a possible mechanism that directly affect the male reproductive system in interstitial cystitis models, the MDA levels were evaluated in testicular tissue, spermatozoa, and urinary bladder tissue of the six groups. The significant increase in MDA levels is an indicator of peroxidative damage in both chronic HCl and CYP groups, this may be attributed to the high content of polyunsaturated fatty acids in both the testes and sperm cells, which

make these structures more vulnerable to peroxidative damage. Physiologically during the process of spermatogenesis, the total content of fatty acids in maturing spermatozoa is dramatically reduced, with a notable increase in the levels of docosahexaneioc acid. While in defective spermatozoa, this morphogenetic physiological transformation does not take place rendering spermatozoa with a high content of both free and total fatty acids, and a decreased content of docosahexaneioc acid. Besides the association of high fatty acids content with decreased membrane permeability and fluidity, this increase in unsaturated fatty acids content can enhance the generation of free radicals from the mitochondria of defective spermatozoa, which in turn triggers the lipid peroxidation cascade that ultimately drive these cells into a state of oxidative stress and further oxidative damage (Koppers et al., 2008).

On the contrary, the urinary bladder is structurally different with relatively lower lipid content. Throughout the literature, the levels of MDA have been used as a reliable biomarker for estimating peroxidative damage in the urinary bladder. This increase in MDA levels in both chronic HCL and CYP groups is an indicator of the presence of peroxidative damage as a consequence of the inflammatory status due to the direct instillation of HCL into the bladder, or systemically through the effect of CYP on the bladder.

Evidence that the reproductive system in animal models of interstitial cystitis was affected by peroxidative damage was obtained through the quantification of MDA levels in testicular tissue and spermatozoa. In Chronic HCL models the levels of MDA in testicular tissue was significantly higher than both the control and acute HCL groups. Similarly, the levels of MDA were significantly higher in CYP group compared to the other groups. Peroxidative damage in CYP group was reported higher among the other groups, this may be due to the toxic direct effect CYP has on multiple organs including the testes. No significance was observed in sham HCL and sham CYP groups, the levels of MDA in both groups were similar to that of the control group.

The significant increase in MDA levels in both IC models is attributed to the chemically induced inflammatory status of the bladder. In the chronic HCL model, the direct intravesical instillation of HCL into the urinary bladder had induced membranous degradation and altered the lipid composition in the membranes of bladder urothelium. The exposure of bladder tissue to HCL resembles ischemic reperfusion injury that increase the levels of ROS at the location of insult, which in turn contributes to the occurrence of peroxidative damage (Koppers et al., 2008). The

increase of MDA levels in CYP can be explained by the systemic toxicity of CYP on multiple organs, it has been reported that the use of CYP predisposes patients to bladder related complications including, chronic cystitis, hemorrhagic cystitis and even bladder carcinoma (Tremellen, 2008).

Another possible explanation for the observed reproductive system toxicity in CYP models is the potential effect of CYP metabolic products on DNA damage. The presence of cytoplasmic morphological anomalies including, cytoplasmic droplets have been associated with the excessive generation of ROS, these anomalies are considered a sign of sperm immaturity, which has been positively correlated with signs of DNA damage and mitochondrial gene expression alterations (Dan et al., 2014; Mills et al., 2019).

Additionally, MDA and 4-hydroxynonenal, two byproducts of lipid peroxidation, can lead to the formation of adducts with mitochondrial proteins in the ETC, which in turn promotes the formation of mitochondrial ROS (Koppers et al., 2008). Along with the structure and signaling mechanisms of the sperm tail, it also dysregulates sperm bioenergetic pathways directly affecting the motility and viability of these cells (Aitken et al., 2012a; Aitken & Drevet, 2020).

In epididymal spermatozoa, levels of MDA were significantly elevated in CYP compared to the other groups, this may be due to the potential toxicity that CYP exhibits on multiple systems including the male reproductive system. Throughout the literature, exposure to CYP has been reported to be associated with abnormalities in sperm production and function (Korkmaz et al., 2007; F. Liu et al., 2012). MDA levels in spermatozoa of chronic HCL models were significantly higher than the control group and acute HCL group. The levels of MDA in both sham HCL and sham CYP models were lower than that of chronic HCL group and CYP group, with no significance compared to the control and acute HCL group.

Throughout the literature, ROS are a normal by-product of the oxidative phosphorylation pathway, and it has been associated with oxidative damage of mitochondria in many pathological cases (Piomboni et al., 2012). Additionally, ROS changes the electrical charge of proteins by oxidizing and cross-linking certain amino acids (cysteine and methionine residues), which makes proteins more vulnerable to proteolysis or destruction by proteases (Birben et al., 2012).

In spermatozoa, several studies have reported the detrimental consequences of excessive ROS generation and oxidative stress on the overall function and structure of sperm cells (Aitken et al., 2012a; Koppers et al., 2010; Zhu et al., 2015; Zhu, Fan, et al., 2017; Zhu, Ren, et al., 2017).

Mitochondria are one of the few organelles retained during spermatogenesis, even though most of the cytoplasm and its content is lost which suggests the importance of these organelles for male fertility. Human sperm functionality, both in terms of quality and capacity to fertilize, is fundamentally dependent on mitochondrial activity (Wu et al., 2019b).

The relative expression levels of mitochondrial genes (*mt-ND1*, *mt-ND5*, *mt-CYB*, *mt-COI*, *mt-ATP6* and *mt-ATP8*) was analyzed using quantitative real-time PCR as an indicator of mitochondrial dysfunction as a possible mechanism of male reproductive system dysfunction in animal models of interstitial cystitis.

Each gene was normalized to that of β-actin and were compared with the control group. Data obtained in this study revealed that the expression levels of mitochondrial genes were significantly affected in models of interstitial cystitis relative to the other groups. When compared to the other groups, the relative expression levels of mt-ND1 and mt-ND5 from complex I were considerably raised in testicular tissue in chronic HCl animals. In contrast, no significant change in the expression levels of mt-ND1 or mt-ND5 was seen in the other groups.

Similarly, in epididymal spermatozoa the expression levels of both *mt-ND1* and *mt-ND5* were significantly upregulated in chronic HCl models compared to other groups. While in CYP models the levels of expression were significantly downregulated.

In recent studies, the vital role of *mt-ND5* in the production of physiological levels of ROS important to ensure the ongoing of the spermatogenesis process was determined (Sabeur & Ball, 2007). The observed dysfunctions in the expression of *mt-ND5* in both chronic HCl and CYP groups are indicators to the impairments of the spermatogenesis process and the male reproductive system. A study has reported that the overexpression of *mt-ND5* have been positively correlated with the elevation of ROS generation in asthenozoospermic males compared to non-asthenozoospermic males (Vignini et al., 2006).

From complex III, the expression levels of *mt-CYB* in testicular tissue were significantly upregulated in chronic HCl models. The expression levels of *mt-CYB* gene in acute HCL, CYP,

sham HCL and sham CYP mirrored that of the control group with no significant change. In epididymal spermatozoa, the expression levels of *mt-CYB* in chronic HCl models were significantly upregulated compared to the other groups. On the contrary, in CYP models the levels of expression were significantly downregulated. The changes in mitochondrial respiratory chain expression are an indicator of the increased ROS produced from the mitochondria. Mature spermatozoa exhibit very low rates of spontaneous ROS production, although in some cases dysfunctional spermatozoa tend to generate large amounts of ROS. Studies have shown that disruption of the electron transport chain doubles the amount of oxygen consumed by cells normally, which in turn significantly increases the production of ROS in mitochondria.

As previously mentioned, physiological levels of unsaturated fatty acids should dramatically decrease throughout spermatogenesis. High content of PUFA not can only trigger the lipid peroxidation cascade but can furthermore lead to an increase in ROS generation which in turn lead to the inhibition of complex I of the respiratory chain, with a minor inhibition of complex III (Amaral et al., 2013; Federico et al., 2012), which was consistent with the data obtained from the CYP group.

Consistent with the literature, studies have shown a direct relationship between the production of ROS in mature spermatozoa and poor motility, the latter has been explained by the effect of peroxidative damage in spermatozoa undergoing oxidative stress.

The main site of energy conversion in the mitochondria is the cristae. In addition to the presence of complexes I – V in the mitochondrial cristae, large amounts of small soluble electron carrier proteins namely cytochrome c (Ghezzi & Zeviani, 2012). Cytochrome c is the mean of transporting electrons from complex III into complex IV, the leakage of electrons into the cytoplasm can trigger apoptosis (Li et al., 1997). From complex IV, the expression levels of COI in testicular tissue were significantly upregulated in chronic HCl models compared to the other groups. The expression levels of COI gene in acute HCl, CYP, sham HCl and sham CYP mirrored that of the control group with no significant change. Similarly, in epididymal spermatozoa, the expression levels of COI in chronic HCL models were significantly upregulated compared to the other groups. On the contrary, in CYP models the levels of expression were significantly downregulated. The expression levels of COI gene in acute HCl, sham HCl and sham CYP mirrored that of the control group with no significant change. Studies have shown that in mice lacking the cytochrome c gene in the testis,

the production of abnormally immotile spermatozoa was significantly increased, furthermore the ability of these spermatozoa to successfully fertilize oocytes was significantly lower (Hessle et al., 2002).

As we previously demonstrated, mitochondria are considered the main source of ROS generation in spermatozoa, and often is the target of ROS-mediated damage (Shamsi et al., 2008). Moderate levels of ROS are essential for maintenance of spermatozoan physiological functions including, the phosphorylation of tyrosine, which mediate the capacitation of spermatozoa and aid in the maintenance of telomere length (Mishra et al., 2016). Another contributor to the inflammatory status that was induced in both HCL and CYP models of interstitial cystitis are the leucocytes present in the seminal fluid, infiltrating leukocytes are reported to generate 1000 times free radicals than spermatozoa (Tremellen, 2008). This generation of free radicals is considered an immune response that takes place due to the presence of infection-related and/or inflammation-related stimuli in male genital tract, a process referred to as “the respiratory burst” is responsible for the generation of high amounts of ROS and/or RNS by activated seminal leukocytes in case of stimulation by an inflammation related agent. The presence of an immunogenic stimuli along the male urogenital tract may trigger this phenomenon to take place (Aitken et al., 2012b; Pasqualotto et al., 2000).

The levels of MDA and peroxidative damage in CYP model have been negatively correlated with the downregulation of vital mitochondrial genes of the ETC, the downregulation of these genes can be attributed to the elimination of spermatozoa at various stages of development, the atrophy of Leydig cells, and the significantly decreased rates of spermatogenesis. The extent of lipid peroxidation in spermatozoan cells and the excessive generation of ROS can lead to the corruption of the structure of lipid matrix in the spermatozoan cellular membranes.

On the contrary, peroxidative damage in chronic HCl model have been positively correlated with the upregulation of ETC genes.

Besides its involvement in ATP synthesis, the mitochondrial ETC promotes the production of reactive oxygen species (ROS), which can both function in signaling pathways and cause oxidative damage, if produced in an unchecked manner. Remarkably, the mobile ETC carrier cytochrome c moonlights as an active participant in the mitochondria-mediated intrinsic apoptotic pathway. In fact, one of the hallmark triggers of this process is cytochrome c release into the cytoplasm.

From complex V, the levels of gene expression for both *mt-ATP6* and *mt-ATP8* in testicular tissue were significantly upregulated in chronic HCL models. No significant change was observed in the expression levels of both *mt-ATP6* and *mt-ATP8* in acute HCl, CYP, sham HCl and sham CYP groups.

In epididymal spermatozoa, the expression levels of both *mt-ATP6* and *mt-ATP8* in chronic HCl models were significantly upregulated compared to the other groups. On the contrary, in CYP models the levels of expression of both genes were significantly downregulated. The expression levels of both *mt-ATP6* and *mt-ATP8* genes in acute HCl, sham HCl and sham CYP mirrored that of the control group with no significant change.

The primary function of the mitochondria is the generation of ATP from ADP and phosphate ions during cellular respiration (citric acid cycle) by the mitochondrial ATP synthase (Ghezzi & Zeviani, 2012b). The production of ATP is directly associated with the oxidative phosphorylation that takes place in the mitochondria and result in the generation of ATP molecules that aid in the functional linear motility of mature spermatozoa (Zhu, Fan, et al., 2017). The excessive generation of ROS has been shown to mainly target the production of ATP, which results in dysfunctional movement patterns and even immobility of spermatozoa (Piomboni et al., 2012). In CYP models the production of ATP, and mitochondrial activity were decreased with increasing ROS levels due to the exposure of cyclophosphamide. Additionally, several studies have shown the effect of cyclophosphamide on sperm viability and motility patterns, as expected the linear motility of spermatozoa was significantly decreased in correlation with peroxidative damage in CYP models. The decreased expression of both ATP6 and ATP8 from complex V in addition to the increased peroxidative damage is presumably the main cause of dysfunctional sperm motility.

In chronic HCL models, the expression levels of both ATP6 and ATP8 from mitochondrial complex V were interestingly upregulated. Data from our lab regarding sperm parameters have shown that in chronic HCL models the motility of spermatozoa was not completely lost as the case in CYP models and most of spermatozoa were still viable. The flagellar movement of spermatozoa is completely dependent on ATP production and an intact internal structure, in mammalian cells including spermatozoa the generation of ATP is fulfilled either through mitochondrial oxidative phosphorylation or throughout glycolysis, these two metabolic pathways are switched based on the presence or absence of the required substrates and the availability of oxygen for each pathway (du

Plessis et al., 2015). In the case of chronic HCL models it is presumed that the overexpression of ATP6 and ATP8 was due to the increased demand of ATP on these two subunits but the fact that the extent of peroxidative damage on testicular tissue and spermatozoa were irreversible, had led to the disruption of the physiological overflow of electrons throughout the ETC, rendering spermatozoa dysfunctional but still viable.

It has been reported throughout the literature that the production of ATP is powered by a tight control of the mitochondrial microenvironment pH (Ghezzi & Zeviani, 2012a). It is presumed that the fluctuations in pH are due to the instillation of HCL (a highly acidic substance), had led to the dysfunction of the electrochemical gradient across the mitochondrial inner membrane and subsequently had led to dysfunctional ATP production.

The downregulation of *mt-ATP6* and *mt-ATP8* subunits of complex V in CYP models can lead to the depletion of intracellular levels of ATP in sperm cells leading to axonemal and flagellar damage which directly affect the motility of spermatozoa. Additionally, studies have shown that the motility parameter in sperm cells of CYP models was extensively affected rendering spermatozoa immotile with little to even no viability at all.

Reactive oxygen species are involved in all cells physiological processes. In testis, they may be beneficial or even indispensable in the complex process of male germ cells' proliferation and maturation, from diploid spermatogonia through meiosis to mature haploid spermatozoa (Guerriero et al., 2014). Conversely high doses, and/or inadequate removal of ROS caused by several mechanisms, i.e., ionizing radiation, bioactivation of xenobiotics, inflammatory processes, increased cellular metabolism, activation of oxidases, and oxygenases, can be very dangerous, modifying susceptible molecules including DNA, lipids, and proteins.

Conclusions

In this study we demonstrated the potential effect of ROS and mitochondrial dysfunction on male reproductive system through the assessment of lipid peroxidation and the expression of mitochondrial respiratory chain genes. Induction of IC in the urinary bladder had led to a significant peroxidative damage in both testicular tissue and spermatozoa, the upregulation of mitochondrial respiratory genes in the chronic HCl model, and a downregulation in these genes in the CYP model, both an indicator of mitochondrial dysfunction. Thus, the results obtained in this study showed that a problem in one viscera could possibly affect other organs. In our case, the inflammatory status of the urinary bladder in animal models of interstitial cystitis had a significant effect on male reproductive system. The results of this study can be utilized for further studies to fully understand the mechanistic insights of interstitial cystitis and its complications. It also could be applied for diagnosis, and management purposes of interstitial cystitis.

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