EFFECTS OF FOLIC ACID AND ZINC ON GONADAL SPERM RESERVE, GONADOSOMATIC INDICES AND SOME CYTOKINES IN EXPERIMENTALLY-INDUCED TESTICULAR TORSION IN WISTAR RATS

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN HUMAN PHYSIOLOGY

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DECLARATION

I declare that the work in this thesis entitled "EVALUATION OF EFFECTS OF FOLIC ACID AND ZINC ON GONADAL SPERM RESERVE, GONADOSOMATIC INDICES AND SOME CYTOKINES IN EXPERIMENTALLY-INDUCED TESTICULAR TORSION IN WISTAR RATS" has been carried out by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Prof. J. O. Ayo and Dr. Y. Tanko. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work was previously presented for another degree or diploma at this or any other institution.

Richard Jewo BEBEKAH _____ _____

Signature Date

CERTIFICATION

This thesis titled "EVALUATION OF EFFECTS OF FOLIC ACID AND ZINC ON GONADAL SPERM RESERVE, GONADOSOMATIC INDICES AND SOME CYTOKINES IN EXPERIMENTALLY-INDUCED TESTICULAR TORSION IN WISTAR RATS" by Richard Jewo BEBEKAH meets the regulations governing the award of the degree of Master of Human Physiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to Jesus Christ; my Saviour, the fountain of wisdom and knowledge and whom I owe my very being.

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ABSTRACT

Testicular torsion refers to the twisting of the spermatic cord structures and subsequent loss of the blood supply to the ipsilateral testicle. This is a urological emergency; early diagnosis and treatment are vital to saving the testicle and preserving future fertility. The aim of the study was to investigate the effects of folic acid and zinc on some cytokines, Gonadosomatic indices and gonadal sperm reserve in rats during experimentally induced testicular torsion. Thirty male Wistar rats were divided into six groups comprising five animals each. In Group 1 (control), rats were given (1 ml/kg) normal saline for 21 days. Group 2 (sham), rats were also given (1 ml/kg) normal saline for 21 days followed by operation. Group 3 (untreated torsion/detorsion), (torsion of the right testis was done by rotating the testis 720˚, twice around the longitudinal axis of the spermatic cord) in a clockwise direction for 2 hours, and thereafter , the right testis was detorted by rotating it (720˚) twice anti-clockwisely; and then fixing in the right position for 1 hour. Rats in Group 4 (folic acid), were treated with (2 mg/kg) folic acid for 21 days followed induction of torsion-detorsion of the testis. Group 5 (zinc), rats were treated with (50 mg/kg) zinc for 21 days followed by experimental torsion-detorsion .Group 6 (Aspirin), rats were treated with (200 mg/kg) aspirin for 21 days and induced with torsion-detorsion. At the end of 21 days, blood samples were collected through cardiac puncture and the serum harvested was used for tumour necrosis factor-alpha and prostaglandin assays. Gonado-somatic indices of the testes, epididymis and seminal vesicles and semen parameters were evaluated. The sperm count in all the treated groups was lower than in the control but sperm morphology did not differ between the groups. There was significant ($P<0.05$) difference in fast movement and non-motile cells of folic acid and zinc treated groups. There was no significant ($P>0.05$) difference in gonado-somatic indices of all treated groups, compared with the control. Treatment of the rats with either folic acid or zinc did not significantly alter their levels of prostaglandin E_2 and tumour necrosis factor-alpha but there is slight decrease in PGE_2 levels and TNF-α levels in folic acid and zinc treated groups,

respectively. In conclusion, testicular torsion results into generation of inflammatory cytokines and their adverse effects could be reduced by administration of folic acid and zinc.

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LIST OF ABBREVIATIONS

ANOVA	Analsis of Variance
CH_3-THF	Methyl tetrahydrofolate
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase -2
DHF	Dihydrofolate
DNA	Deoxyribonucleic Acid
dTMP	2'-deoxythymidine-5'-phosphate
dUMP	2'-deoxyuridine-5'-phosphate
ELISA	Enzyme linked immunosorbent assays
F	Folate
FH_4	Tetrahydrofolate
GSI	Gonadosomatic Index
H_2O_2	Hydrogen peroxide
HIV	Human immuno deficiency virus
IGF2	Insulin-like growth factor 2
IL-1α	Interleukine-1α
IL-1β	Interleukine-1β
IL-6	Interleukin-6
I-R	Ischaemic reperfusion
MAPKs	Mitogen activated protein kinases
MDA	Malondialdehyde
MPO	Myeloperoxidase
NADPH	Reduced Nicotinamide adenosine Dinucleotide Phosphate
NAP	National academy press
NF-kb	Nuclear factor kappa
NO	Nitric oxide
NSAIDs	Nonsteroid- anti-inflammatory drugs
NTDs	Neural Tube Defects
O_2	Oxygen
PGE_2	Prostaglandin E_2

RCTs	Ramdomised Control Trial
RDA	Recommended Daily Allowance
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
TGFβ3	Transfering Growth Factor-beta 3
TNF-a	Tissue Necrosis Factor-alpha
URI	Upper respiratory infection
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

Ischaemia-reperfusion injury is a complex phenomenon that induces cell damage through a bi-phasic process. Ischaemia initiates the injury by deprivation of the energy needed to maintain ionic gradients and homeostasis, which may ultimately lead to cellular dysfunction and death. Reperfusion exacerbates this damage triggering an inflammatory reaction, which precipitates oxygen free radicals, endothelial factors and leucocytes. The combination of the two processes called ischaemia-reperfusion, disrupts the delicate balance that maintains homeostasis in the microcirculation. Ischaemic-reperfusion induces attraction, activation, adhesion and migration of neutrophils, causing local tissue destruction by release of proteases and further oxygen free radicals. Mechanisms of ischaemic injury are common to all solid organs, but there are some specific characteristics for each one of them. Consequences of ischemia-reperfusion injury on organ function depend on duration of ischaemia, temperature and the nature of the organ (Clavien *et al.*, 1992; Grace, 1994; Zimmerman and Granger, 1994).

Testicular torsion is a urological emergency that usually results from rotation of testis around the axis of the spermatic cord, and it is frequently observed in the newborns, children and adolescents (Egur *et al.*, 2008). Testicular torsion can be divided into intravaginal torsion; torsion of the spermatic cord within the *tunica vaginalis*; and extravaginal torsion-torsion of the spermatic cord outside the *tunica vaginalis* (Cuckow and Frank, 2000; Frank and O'Brien, 2002; Visser and Heyns, 2003). Torsion of the testes initially results in obstruction of venous blood flow of the spermatic cord with secondary oedema and haemorrhage. Thereafter, oedema results in ischaemia and arterial obstruction leading to necrosis (Koseoglie *et al.*, 2009). The testicular torsion and detorsion process cause ischaemia-reperfusion damage to the testis. The pathophysiologic mechanisms are mainly due to the direct damage caused by ischaemia during torsion and the secondary effect of reperfusion occurring during the

untwisting of the spermatic cord. Although the restoration of testicular flow to the ischaemic testis is essential for tissue preservation, organ reperfusion could even have harmful effects (Greenstein *et al*., 2001; Minutoli *et al.,* 2005). This injury starts a pathophysiologic cascade, including activation of cytokines, which are the main mediators of inflammation, and the generation of nitric oxide and other reactive oxygen species (ROS) (Hashimoto *et al*., 2009). Thus, a possible mechanism underlying impairement of testicular functions during torsion-detorsion is ischaemia-reperfusion injury. Causes of testicular torsion include testicular tumours, increase in testicular volume, testicles with horizontal lie, history of cryptorchidism, spermatic cord with a long intrascrotal portion and even in the absence of any precipitating event (Noske *et al.,*1998; Arce, 2002).

A possible mechanism of torsion/detorsion is ischaemic reperfusion injury. Although the restoration of the blood flow to the testis is essential for tissue salvage, it may have detrimental effects (Minutoli *et al.*, 2005). Causes of testicular torsion include testicular tumours, increase in testicular volume, testicles with horizontal lie, history of cryptorchisism, spermatic cord with a long intrascrotal portion, absence of any precipitating event (Noske *et al.*, 1998; Arce, 2002). Several investigations carried on testicular torsion are mostly targeted at either preventive or ameliorative measures on testicular torsion. Results obtained in a recent investigation (Dokmeci, 2012) have shown that ischaemia-reperfusion injury in testes is associated with activation of neutrophils, inflammatory cytokines, and adhesion molecules with increased thrombogenicity and release of calcium. Another study carried out by Chiu *et al.* (2012), shows that season affects the rate of testicular torsion. It involved a total of 1782 patients of different ages hospitalized for testicular torsion between 2000 and 2009 in Taiwan. January had the highest rate, which decreased in April to a trough. The mechanism of ischaemia-reperfusion injury has been linked to increased generation of ROS in affected organs. Oxidative stress causes of ROS such as hydrogen peroxide, superoxide and hydroxyl ions and activation of myeloperoxide (MPO), which promote inflammation by activating the

inflammatory pathway. Activation of the pathway leads to expression of inflammatory cytokines in the tissues, including blood (Reuter *et al*., 2010). The onset of inflammation is characterised by release of pro-inflammatory mediators including tumour necrosis factor-alpha (TNF), Interlukin-1 (IL-1), adhesion molecules, vasoactive mediators and ROS (Pavlov *et al.,* 2003). Ischaemia-reperfusion of testis results in elevated production of ROS, activates mitogen- activated protein kinases, induce transcription factors and growth factors, including vascular endothelial growth factor, trigger apoptotic machinery and induce several inflammatory cytokines, including TNF-α and Interleukin-1β. This pathological cascade is responsible for the testicular atrophy, decreased blood flow and impaired spermatogenesis (Atavilla, 2012).

The early release of pro-inflammatory cytokines by activated macrophages has a pivotal role in triggering the local inflammatory response (Baumann and Gaudie, 1994). The mammalian testis is an immunologically-privileged site whereby tight junctions between Sertoli cells typically segregate germ cell antigens within the adluminal and luminal compartments of the seminiferous tubules (Head *et al*., 1983).

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can initiate chain reactions. When the chain reaction occurs in a cell, it can cause damage or death. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, zinc, folic acid or polyphenols (Sies, 1997; Dawson, 2000; Prasad, 2007).

Folic acid also known as vitamin B_9 or folacin (the naturally-occurring form) is a water-soluble vitamin (folic acid and folate inclusive) and it is essential to numerous bodily

functions. The human body needs folate to synthesise, repair and methylate DNA as well as co- factor in biological reactions, involving folates (Weisten, 2003). It is necessary for production and maintenance of new cells, DNA and RNA syntheses and for preventing changes to DNA (Kamen, 1997).

Zinc is an essential component of numerous proteins involved in the defence against oxidative stress and damage repairs. It has a role in maintaining DNA integrity, (Sung *et al*., 2009). According to Prasad *et al*. (2007), in humans, supplementation with zinc records reduced rate of infection and oxidative stress. It has both anti-inflammatory and antioxidant activities.

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used in the treatment of many diseases associated with inflammation. The major mechanism of these drugs is via the inhibition of cyclooxygenase (COX), the main enzyme of the inflammation process (Goldman *et al*., 1998). COX catalyzes the conversion of arachidonic acid to prostaglandins G_2 and H_2, the cytokines contributing to the inflammation process (Chen *et al*., 2008). The NSAIDs exert analgesic and antipyretic effects and in higher doses anti-inflammatory effects. The most prominent members of this group of drugs are aspirin, acetylsalicylic acid, ibuprofen, CoX- 1, CoX-2 and naproxen all of which are available over the counter in most countries. Aspirin differs from most other NSAIDs in its mechanism of action. It inhibits the enzyme COX (mostly the CoX-1 variant) in an irreversible manner thereby preventing or reducing the formation of prostaglandin (Burke *et al*., 2006).

1.1 STATEMENT OF RESEARCH PROBLEM

Each year, testicular torsion affects one in 4,000 males younger than 25 years in the United States. Early diagnosis and definitive management are the keys to avoid testicular loss (Ringidah *et al*., 2006). Testicular torsion is most common in males during adolescence. About 1 out of 160 males develops testicular torsion in the United States each year (Kadish *et*

al., 1998; Eaton *et al.,* 2005). A study was conducted at Jos University Teaching Hospital, Jos between August, 1993 and July, 2001 on cases of testicular torsion. A total 60 consecutive patients with acute scrotum (testicular torsion pain of onset) were explored during this period with a working diagnosis of testicular torsion (Ugwu *et al*., 2003).

Testicular torsion is a major contributor to male infertility in Zaria. From 1972 to 1984, scrotal exploration was performed in 192 patients who presented to Ahmadu Bello University Teaching Hospital, Zaria with pain of the scrotum. Testicular torsion was confirmed in 131 of the patients. Majority (80%) were aged 11- 25 years (Mabogunje, 1986). Late presentation or failure to diagnose testicular torsion may lead to decreased spermatogenesis, altered hormone production and sub-fertility (Fisch *et al*., 1988; Turner *et al*., 1997).

1.2 JUSTIFICATION OF THE STUDY

Testicular torsion can occur in the absence of any precipitating event (Noske *et al.*, 1998). Only 8% of cases are as a result of trauma (Seng *et al*., 2000). Therefore, many younger males may be at risk of infertility due to undiagnosed testicular torsion. With regard to normal development and function of the testis, both pro-inflammatory and an-inflammatory cytokines have been shown to play an important regulatory role that prevents damage to the testicular environment; but this does not preclude immune activation, resulting in inflammatory and potential damages. In men, infection and inflammation of the reproductive tract, including the testes, are widely accepted as important aetiological factors of infertility (Schuppe and Meinhardt, 2005).

These inflammatory molecules regulate mitosis and meiosis of the spermatogenic cells, organisation of the Sertoli cell cytoskeleton and intercellular junctions. They also regulates various Sertoli cell activities throughout the cycle of the seminiferous epithelium. Consequently, the presence of pathogenic molecules, cytokines and mediators of

inflammation produced within the testis, or entering from the circulation during systemic inflammation impinge upon the normal function of the Sertoli cells and spermatogenic cells, leading to disturbances in spermatogenesis (Hedger, 2011).

It is, therefore, conceivable that anti-inflammatory potentials of folic acid and zinc may be beneficial in ameliorating ischaemia-reperfusion injury in testes of Wistar rats. Many investigations have been carried on the protective effects of exogenous substances on testicular torsion in relation to oxidative stress biomarkers. They include the protective effect of selenium on ipsilateral and contralateral testes in testicular ischaemia-reperfusion injury (Alvan *et al*., 2005), protective effect of erythropoietin in pre-treatment in testicular ischaemia-reperfusion in rats (Egur *et al*., 2008). Peptide and non-peptide reactive oxygen scavengers, have also been been demonstrated to provide exogenous protection to testis after torsion (Lysia *et al*., 2002). However, there is thus need to investigate potential anti-inflammatory and antioxidant roles of folic acid and zinc in as an ameliorative measure in testicular torsion.

1.3 AIM OF THE STUDY

The aim of the study was to investigate the effects of administration of folic acid and zinc on ischaemia-reperfusion injury in the testes of Wistar rats.

1.4 THE SPECIFIC OBJECTIVES OF THE STUDY

1. Evaluate the effect of administration of folic acid and zinc on the inflammatory cytokines (tumour necrosis factor alpha and prostaglandin E_2) in ischaemia-reperfusion injury in testes induced by experimental torsion-detorsion in Wistar rats;
2. Investigate the effect of testicular torsion on GSI in Wistar rats;

3. Determine the effect of experimentally induced torsion-detorsion on sperm count, motility and morphology.
4. Compare the effects of aspirin (anti-inflammatory) and folic acid and zinc(anti-oxidants) in ischaema-reperfusion in testes of wistar rats. .

1.5 HYPOTHESIS

Folic acid and zinc do not have significant effects on ischaemia- reperfusion injury in the testes of Wistar rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALE REPRODUCTIVE ANATOMY

Male reproductive system is a network of external and internal organs that functions to produce, support, transport, and deliver viable sperm for reproduction. Prenatally, the male sex organs are formed under the influence of testosterone, secreted from the foetal testes; by puberty, the secondary sex organs further develop and become functional. Sperm is produced in the testes and is transported through the epididymis, *ductus deferens*, ejaculatory duct, and urethra. Concomitantly, the seminal vesicles, prostate gland, and bulbo-urethral gland produce seminal fluid that accompanies and nourishes the sperm as it is dischared from the penis during ejaculation and throughout the fertilisation process (Klaasen, 2011).

2.2 GROSS ANATOMY

2.2.1 The Scrotum

The scrotum is a fibro-muscular pouch divided by a median septum (raphe), forming two compartments; each of which contains a testis, epididymis and part of the spermatic cord. Layers of the scrotum consist of skin, dartos muscle, external spermatic fascia, cremasteric fascia and internal spermatic fascia, which are in close contact with the parietal layer of the *tunica vaginalis* (Klaasen, 2011).

2.2.2 The Testes

The testes are the primary male reproductive organ and are responsible for testosterone and sperm production. In humans, a testis is 4-5 cm long, 2-3 cm wide, weighs 10-14 g and

is suspended in the scrotum by the dartos muscle and spermatic cord (Klaasen, 2011).

The testis comprises compactly arrayed convoluted loops of seminiferous tubules separated by an interstitium, containing Leydig (interstitial) cells, vasculature, macrophages, a protein- and testosterone-rich ultrafiltrate, and supporting stroma. The seminiferous epithelium is formed by basally-located Sertoli cells supporting successive synchronised populations of maturing germ cells; that is, the spermatogonia, spermatocytes, round spermatids, and elongating spermatids. Seminiferous tubules are ensheathed by contractile myoid cells and converge, through *tubuli recti*, on the *rete testis*, which is continuous with the efferent ductules and epididymis (Boorman *et al*., 1990a).

Additionally, the testes receive blood from the cremasteric branch of the inferior epigastric artery and the artery to the *ductus deferens*. The pampiniform plexus drains both the testis and epididymis before coalescing to form the testicular vein, usually above the spermatic cord formation at the deep inguinal ring. Lymphatic drainage via the testicular vessels passes into the abdomen, ending in the lateral aortic and pre-aortic nodes. The tenth and eleventh thoracic spinal nerves supply the testes via the renal and aortic autonomic plexuses. Each testis has 600-1200 seminiferous tubules, with a total length of 280-400 m (Ovalle and Nahirney, 2007). At the mediastinum testis, on the posterior border of the testis, the seminiferous tubules empty spermatozoa into the tubuli recti and rete testis, eventually coalescing to form 6-8 efferent ductules..The seminiferous tubule epithelium consists of proliferating spermatogenic cells and the sustentacular Sertoli cells. Spermatogenic cells are at various stages of spermatogenesis and Sertoli cells are columnar cells that extend from the basement membrane to the lumen of the seminiferous tubule. Interstitial cells in the testis, including the Leydig cells, constitute 20-30% of the tissue in the gland and are found in between seminiferous tubules. The washed out cytoplasm of the Leydig cells is due to high lipid content in the form of cholesterol involved in the synthesis of testosterone (Klaasen, 2011).

2.2.3 The Epididymis

The epididymis is a highly-coiled duct linking the efferent ducts to the *vas deferens*. Sperm cells leave the testis, largely immotile and unable to fertilise the oocytes. By the time they reach the *cauda epididymis*, they are fully mature cells, with progressive forward motility and the ability to recognise and fertilise an oocyte. In addition to mediating this maturation process, the epididymis concentrates, protects, and stores the sperm prior to ejaculation. Protein secretion and endocytosis are major functions of the epididymal epithelium, and many of the proteins secreted and expressed are cell-specific and region-specific, underlining the importance of examination of the complete tissue. The luminal micro-environment of the entire epididymis is specialised and complex and varies along its length; it is maintained by secreted proteins, ions, and their transporters (Hermo and Robaire, 2002). The ducts of the *cauda epididymis* are surrounded by prominent layers of contractile smooth muscle, which aids in the expulsion of sperm during ejaculation. The epididymis develops from the Wolffian duct under the influence of testicular testosterone (Byskov and Hoyer 1998; Rodriguez *et al.,* 2002). Its position and dimensions vary with species. In rodents, the epididymis is relatively long and loosely attached to the testis compared to humans and domestic animals (Setchell *et al.*, 1988). It is generally subdivided into the initial segment and intermediate segment, into which the efferent ducts empty, followed by three main parts (caput, corpus, and cauda).It can also be subdivided further by various histologic and/or functional schemes into as many as 6 to 12 distinct zones, comprising a variety of cell types with a variety of functions. In general, epithelial height decreases and luminal diameter increases from the caput to the cauda, with some regional exceptions. Cell types that are present throughout the epididymis include principal cells, with apical stereocilia (the predominant cell type), basal cells, and halo cells (a subset of T lymphocytes and monocytes). In addition, the initial and intermediate segments contain apical and narrow cells, while clear cells are present in the caput, corpus, and cauda (Creasy *et al.,* 2012).

2.2.4 The *Ductus (vas) deferens*

The *ductus (vas) deferens* is the continuation of the epididymis; it is 30-45-cm long and conveys sperm to the ejaculatory ducts (Klaasen, 2011). *Ductus deferens* has relatively thick muscular walls and a minute lumen, giving a cord-like firmness. It begins in the tail of epididymis, at the inferior pole of the testis and ends by joining the duct of the seminal gland to form ejaculatory duct (Moore and Dalley, 2006).

2.2.5 The Spermatic cord

The spermatic cords are paired structures, extending between the abdomino-pelvic cavity and testes. Each spermatic cord consists of layers of fascia and muscle, enclosing the *ductus deferens* and the blood vessels, nerves, and lymphatic vessels that supply the testes. The blood vessels include deferential artery, testicular artery, cresmateric artery and pampiniform plexus of testicular vein. Branches of genito-femoral nerve, from lumbar plexus, provide innervations. Each spermatic cord begins at the enterance to the inguinal canal, a passage through the abdominal musculature. After passing through the inguinal canal, the spermatic cord descends into the scrotum (Martini and Nath, 2009).

The inguinal canals form during development as the testes descend into scrotum; at that time, the canals link the scrotal cavities with the peritoneal cavity. In normal adult males, the inguinal canals are closed, but the presence of the spermatic cords creates weak points in abdominal wall that remains throughout life. As a result, inguinal hernias - protrusions of visceral tissues or organs into the inguinal canal are relatively common in males (Martini and Nath, 2009).

2.2.6 The Ejaculatory ducts

The ejaculatory ducts are slender tubes that arise by the union of the ducts of a seminal glands with *ductus deferens*. They (approximately 2.5) arise near the neck of the bladder and run close together as they pass antero-inferiorly through the posterior part of the prostate and along the sides of prostatic utricle. The ejaculatory ducts converge to open on the seminal colliculus by tiny, slit-like apertures on, or just within, the opening of the prostatic utricle. Although the ejaculatory ducts traverse the glandular prostate, prostatic secretions join the seminal fluid in the prostatic urethra after the ejaculatory ducts have terminated (Moore and Dalley, 2006).

2.2.7 The Seminal vesicles

The two seminal vesicles are located between the bladder and the rectum and they measure approximately 5 cm in length. The anterior surface is in contact with the posterior wall of the bladder and the posterior surface is in contact with recto-vesical (Denonvilliers) fascia. The ampulla of the *ductus deferens* lies medial to the seminal vesicles and the prostatic venous plexus lies laterally. Arterial blood supply to the seminal vesicles includes branches from the inferior vesical and middle rectal arteries, while venous and lymphatic drainage accompany these arteries. The inferior division of the hypogastric plexus provides innervation to the seminal vesicles. The seminal vesicles are tubule-saccular glands consisting of connective tissue and secretory epithelium, projecting into the lumen of the gland (Klaasen, 2011).

2.2.8 The Bulbo-urethral glands

The bulbo-urethral or Cowper's gland is a small, paired organ that is difficult to dissect due to its location between the ischio-cavernosus and bulbocavernosus muscles. In rats, the pear-shaped, tubule-aveolar gland has acini, lined by a single layer of basophilic pyramidal cells with closely packed secretory granules (Boorman *et al.*, 1990b) that may have a filamentous

or reticular texture (Dünker and Aumüller, 2002). The secretion stains palely basophilic and is made up of acidic and neutral muco-substances. In mice, this gland is described as having a highly arborised arrangement of acini, lined by tall columnar epithelium that fills the acinar lumen (Dünker and Aumüller, 2002).

During ejaculation, secretions from the prostate, seminal vesicle, and coagulating gland are added to the sperm in the *vas deferens*. As the sperm enters the penile urethra, additional secretions from the bulbo-urethral gland are added (Creasy, 1998). Together, the secretions from these glands form the seminal fluid. Although the composition of the seminal fluid varies between species, its main role is to extend the viability of sperm during their transit through the female genital tract (Luke and Coffey, 1994). Species differences also exist in the coagulation of semen. Most muroid rodents form copulatory plugs (Hartung and Dewsbury, 1978), derived from the secretions of the seminal vesicle, coagulating gland, and bulbourethral gland. For example, in rats, the semen is composed of a fluid phase, which contains the sperm, followed by a solid phase, which forms the copulatory plug, involved in retention of the sperm in the vagina and enhances fertilisation. In the rat, the coagulation enzyme, vesiculase, is produced by the coagulating gland, converting procoagulase (secreted by the seminal vesicle) into coagulase. This process requires a co-factor produced by the bulbo-urethral gland (Creasy, 1998).

2.2.9 The Prostate Gland

The prostate gland is an ovoid structure encompassing the proximal portion of the urethra and is approximately 2.5-3.0-cm by 4.0-4.5-cm, normally weighing 20-25 g. The prostate in rodents consists of a paired ventral portion and a paired dorsolateral portion, which together encircle the urethra. Each of these portions has distinct physiologic functions and responds differently to hormonal perturbations and toxic injury. The acini of the ventral prostate are lined by a single layer of cuboidal to tall columnar epithelium, which varies in its height

depending on secretory activity. Additional cell types that are noted in low numbers in the prostatic epithelium include a non-secretory basal cell, a macrophage-type cell, and a lymphocyte-type cell. The microscopic features of the dorsal portion of the dorsolateral prostate are similar to the coagulating gland, while the lateral portion most closely resembles the ventral prostate, with the addition of a distinctive brush border. Acini are surrounded predominantly by stromal smooth muscle cells and occasional fibrocytes. Depending on the secretory activity, all lobes are filled with secretory material that may appear flocculent. In the ventral lobes, the secretion is palely eosinophilic, while in the lateral lobes it stains intensely eosinophilic. In the dorsal lobes, it stains intermediate between the other two lobes (Lee and Holland, 1987; Boorman *et al*., 1990b; Suwa *et al*., 2001, 2002).

2.2.10 The Urethra

The urethra stretches from the bladder to the tip of the *glans penis*, serving as a passage for urine and semen. The prostatic urethra extends vertically from the bladder neck, through the prostate before becoming the membranous urethra and before penetrating the perineal membrane. Of note, the prostatic urethra contains the orifice of the ejaculatory ducts. As the membranous urethra enters the deep perineal space, the urethra is surrounded by fibres of the external urethral sphincter, eventually entering the bulb of the *corpus spongiosum*, providing the orifice for the bulbo-urethral glands and, subsequently, becoming the penile urethra. When the urethra reaches the *glans penis* the diameter diminishes to that of the external ostium, the least dilatable portion of the urethral canal (Anson, 1966).

2.2.11 The Penis

The penis is the male copulatory organ and, by conveying the urethra, provides the common outlet for urine and semen. The penis consists of a root, body and glans. It is composed of three cylindrical bodies of erectile cavernous tissue: the paired *corpora cavernosa* dorsally and single *corpus spongiosum* ventrally. Distally, the *corpus spongiosum* expands to form the conical glans of the penis, or head of the penis. The margin of the glans projects beyond the ends of the *corpora cavernosa* to form the corona of the glans. The corona overhangs an obliquely grooved constriction, the neck of the glans, which separates the glans from the penis. The slit-like opening of the spongy urethra, the external urethral orifice (meatus), is near the tip of the glans (Moore and Dalley, 2006).

2.3 TESTICULAR TORSION

2.3.1 Background

Testicular torsion refers to the torsion of the spermatic cord structures and subsequent loss of the blood supply to the ipsilateral testicle. This is a urological emergency; early diagnosis and treatment are vital to saving the testicle and preserving future fertility.

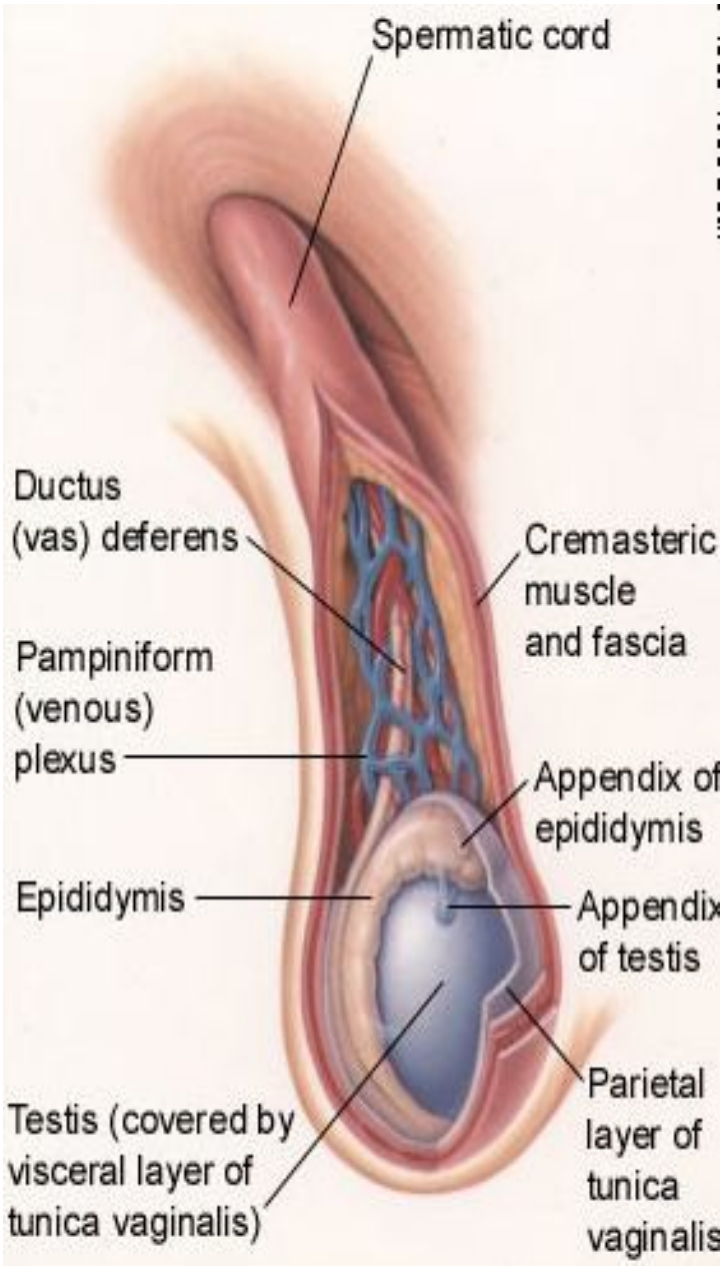


Plate 1: Spermatic cord and its contents (Adapted from Galejs and Kass, 1999)

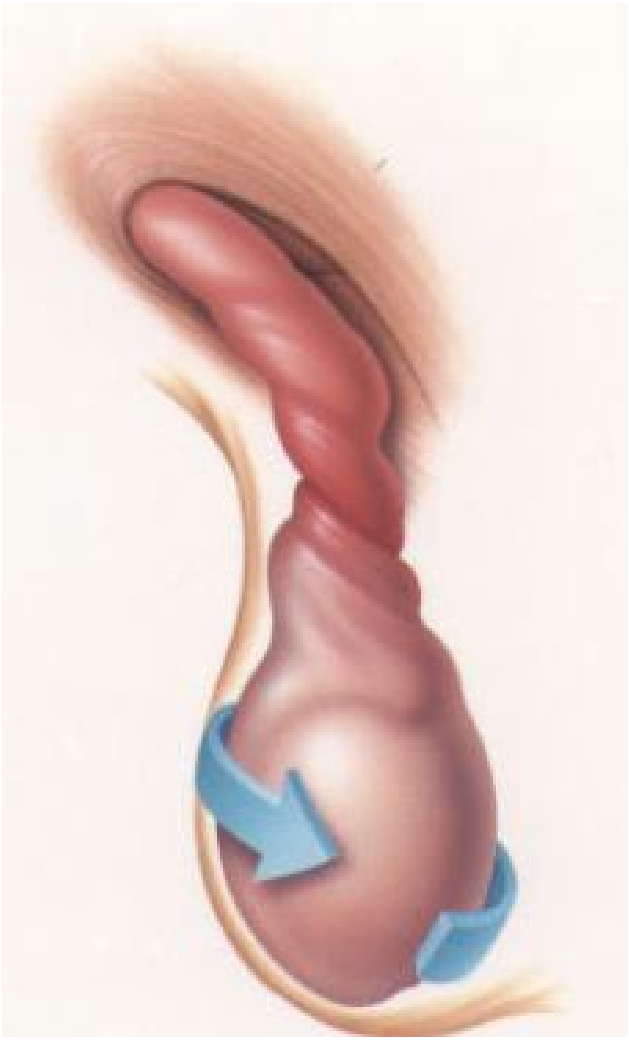
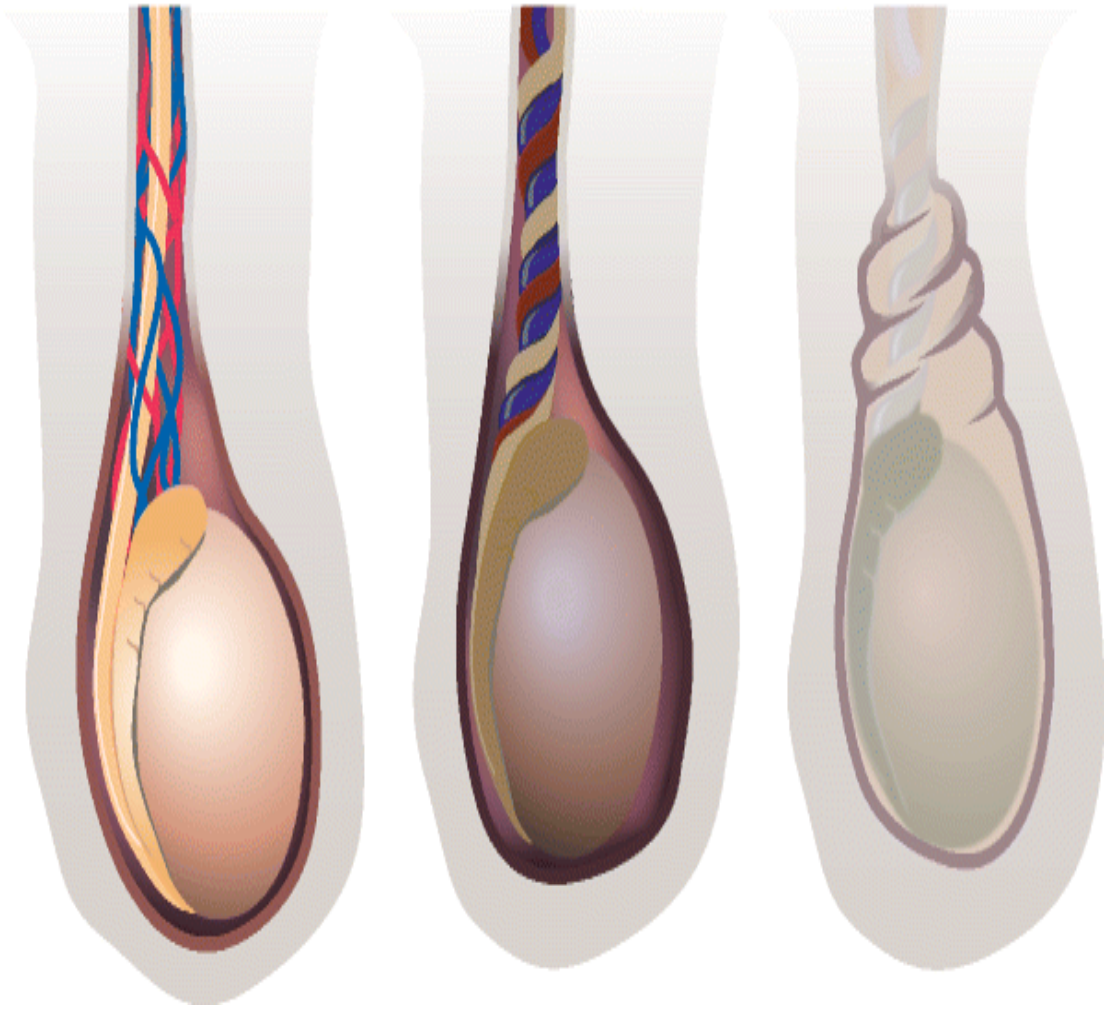


Plate 2: A twisted spermatic cord showing the direction of the tortion (Adapted from Galejs and Kass,1999)

Testicular torsion is a pathological condition, occurring in adolescents and neonates. It is the most common cause of testicular damage in these age groups. Testicular torsion is caused by twisting of the spermatic cord and the blood supply to the testicle. With matured, the *tunica vaginalis* is held securely to the posterior lateral aspect of the testicle, and, within it, the spermatic cord is not very mobile. If the attachment of the *tunica vaginalis* to the testicle is inappropriately high, the spermatic cord can rotate within it, which can lead to intravaginal torsion. This defect is referred to as the bell clapper deformity. This occurs in about 17 % of males (Schneck and Bellinger, 2007).

Intravaginal torsion most commonly occurs in adolescents. It is thought that the increased weight of the testicle after puberty, and sudden contraction of the cremasteric muscles (which inserts in a spiral fashion into the spermatic cord), is the impetus for acute torsion (Schneck and Bellinger, 2007). By contrast, neonates more often have extravaginal torsion. This occurs because the *tunica vaginalis* is not yet secured to the *gubernaculum* and, therefore, the spermatic cord, as well as the *tunica vaginalis*, undergoes torsion as a unit. Extravaginal torsion is not associated with bell clapper deformity. This can occur up to months prior to birth and, therefore, is managed differently depending on presentation. Surgical treatment may prevent further ischaemic damage to the testis. Rarely, observation is appropriate, depending on the pathology. Diagnosis of testicular torsion is clinical, and diagnostic testing should not delay treatment (Schneck and Bellinger, 2007).

Testicular torsion is associated with testicular malignancy, especially in adults; one study found a 64% association of testicular torsion with testicular malignancy. This is thought to be secondary to a relative increase in the broadness of the testicle compared with its blood supply (Schneck and Bellinger, 2007).



Normal (a)

Twisted Spermatic Cord (b)

Twisted Spermatic Cord and *Tunica Vaginalis* (c)

Plate 3: Normal situation (a) as opposed to intravaginal torsion of cord structures (b) and extravaginal testicular torsion where *tunica vaginalis* (and investing outer layers) are also involved (c) (Adapted from Callewaert and Kerrebroeck, 2009)

2.3.2 Testicular descent

For normal development and optimal sperm production, the testis must descend from its original position near the kidney into the scrotum. Around the 23rd week of gestation, the testis undergoes transabdominal migration to a location near the internal inguinal ring. The testis does not migrate transinguinally to its final position until after the 28th week of gestation, and this is usually complete between the 30th and 32nd week of gestation (Schneck and Bellinger, 2007).

2.3.3 Epidemiology

Extravaginal torsion constitutes approximately 5% of all torsions. Of these cases of testicular torsion, 70% occur prenatally and 30% occur post-natally. The condition is associated with high birth weight. Bilateral perinatal torsion is rare, although an increase in the number of case reports has been observed (Roth *et al*., 2011).

Intravaginal torsion constitutes approximately 16% of cases in patients presenting to an emergency department with acute scrotum. This form of testicular torsion is most often observed in males younger than 30 years, with most aged 12-18. Peak incidence occurs at age 13-14. The left testis is more frequently involved. Bilateral cases account for 2% of all torsions. The incidence of torsion in males younger than 25 years is approximately 1 in 4000 (Kar *et al*., 2010). In an Israeli study of paediatric patients presented for examination with scrotal/testicular pain of less than one week duration, only 17 (3.3%) had testicular torsion (Beni-Israel *et al*., 2010).

2.3.4 Prognosis

Success in the management of spermatic cord torsion is measured by immediate testicular salvage and the incidence of late testicular atrophy. A publication documented that approximately 32% of paediatric torsion cases resulted in the orchidectomy (Cost *et al*., 2011). The time elapsed between onset of pain and performance of detorsion, and the corresponding salvage rate, is as follows (Cattolica *et al*., 1982; Ringdahl and Teague, 2006):

1. < 6 hours – 90-100% salvage rate
2. 12-24 hours – 20-50% and
3. > 24 hours – 0-10%

Exocrine and endocrine function is substandard in men with a history of unilateral torsion. A correlation may exist between the duration of torsion and abnormal semen parameters. The following theories explain the contralateral disease noted in torsion patients:

1. Unrecognized or unreported repeated injury to both testes and
2. A preexisting pathologic condition, predisposing to both abnormal spermatogenesis and torsion of the spermatic cord (Sun *et al*., 2006).

In fact, clinical experience does not support either inherent bilateral testicular abnormalities or a humoral effect adversely affecting the contralateral testis in patients with unilateral torsion, since the fertility of adults with pre- pubertal testicular torsion does not appear to be reduced (Puri *et al*.,1985).

2.4 FOLIC ACID

Folic acid (also known as vitamin B_9) and folate (the naturally occurring form), as well as pteroyl-L-glutamic acid, pteroyl-L-glutamate, and pteroylmonoglutamic acid are forms of the water-soluble vitamin B_9. Folic acid is itself not biologically active, but its biological

importance is due to tetrahydrofolate and other derivatives after its conversion to dihydrofolic acid in the liver (Bailey and Ayling, 2009).Vitamin B_9 (folic acid and folate inclusive) is essential to numerous bodily functions. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a co-factor in biological reactions, involving folate (Weistein, 2003). A lack of dietary folic acid leads to folate deficiency which is uncommon in normal Western diets. Failure to replenish in the body folates might not manifest themselves as folate deficiency for four months. This is because a healthy individual has about 500–20,000 μg of folate in body stores. This deficiency can result in many health problems, the most notable one being neural tube defects in developing embryos. Common symptoms of folate deficiency include diarrhoea, macrocytic anaemia with weakness or shortness of breath, nerve damage with weakness and limb numbness (peripheral neuropathy), pregnancy complications and mental confusion. Other symptoms include forgetfulness or other cognitive declines, mental depression, sore or swollen tongue, peptic or mouth ulcers, headaches, heart palpitations, irritability, and behavioral disorders. Low levels of folate can also lead to homocysteine accumulation. DNA synthesis and repair are impaired and this could lead to cancer development (Weistein *et al*., 2003). Supplementation in patients with ischaemic heart disease may also cause increased rates of cancer (Ebbing *el al*., 2009)

All the biological functions of folic acid are performed by tetrahydrofolate and other derivatives. Their biological availability to the body depends upon dihydrofolate reductase action in the liver. This action is unusually slow in humans, being less than 2% of that in rats. Moreover, in contrast to rats, an almost-5-fold variation in the activity of this enzyme exists between humans. This low activity, limits the conversion of folic acid into its biologically-active forms, when folic acid is consumed at levels higher than the Tolerable Upper Intake Level (1 mg/d for adults) (Bailey and Ayling, 2009).

2.4.1 Deoxyribonucleic acid (DNA) and Cell Division

Folate is necessary for the production and maintenance of new cells, for DNA and RNA syntheses, and prevention of changes to DNA; and, thus, for preventing cancer (Kamen, 1997). It is especially important during periods of rapid cell division and growth, such as infancy and pregnancy. Folate is needed to carry one-carbon groups for methylation reactions and nucleic acid synthesis. The most notable one is thymine, but also purine bases may be carried (Figueiredo *et al*., 2009). Thus, folate deficiency hinders DNA synthesis and cell division, affecting haematopoietic cells and neoplasms. RNA transcription, and subsequent protein synthesis, is less affected by folate deficiency, as the mRNA can be recycled and used again. This unlike in DNA synthesis, where a new genomic copy must be created. Since folate deficiency limits cell division, erythropoiesis, production of red blood cells, is hindered and leads to megaloblastic anaemia, which is characterized by large immature red blood cells. This pathology results from persistently thwarted attempts at normal DNA replication, DNA repair, and cell division, and produces abnormally large red cells called megaloblasts (and hypersegmented neutrophils) with abundant cytoplasm capable of RNA and protein synthesis, but with clumping and fragmentation of nuclear chromatin. Some of these large cells (reticulocytes), although immature, are released early from the marrow in an attempt to compensate for the anemia. Both adults and children need folate to make normal red and white blood cells and prevent anaemia (Zittoun, 1993).

2.4.2 Biochemistry of DNA Base and Amino Acid Production

Metabolism of folic acid to produce methyl-vitamin B_{12}

In the form of a series of tetrahydrofolate compounds, folate derivatives are substrates in a number of single-carbon-transfer reactions, and also are involved in the synthesis of 2′-deoxythymidine-5′-phosphate (dTMP) from 2′-deoxyuridine-5′-phosphate (dUMP). It is a

substrate for an important reaction that involves vitamin $B_{12,}$ necessary for the synthesis of DNA, and so required for all dividing cells (Goh and Koren, 2009).

The pathway leading to the formation of tetrahydrofolate (FH_4) begins when folate (F) is reduced to dihydrofolate (DHF) (FH_2), which is then reduced to THF. Dihydrofolate reductase catalyses the last step. Vitamin B_3 in the form of NADPH is a necessary co-factor for both steps of the synthesis.

Methylene-THF (CH_2FH_4) is formed from THF by the addition of methylene groups from one of three carbon donors: formaldehyde, serine, or glycine. Methyl tetrahydrofolate (CH_3-THF) can be made from methylene-THF by reduction of the methylene group with NADPH. It is important to note that vitamin B_{12} is the only acceptor of methyl-THF. There is also only one acceptor for methyl-B_{12}, which is homocysteine in a reaction catalyzed by homocysteine methyltransferase. This is important because a defect in homocysteine methyltransferase or a deficiency of B_{12} can lead to a methyl-trap of THF and a subsequent deficiency (Hoffbrand and Weir, 2001).Thus, a deficiency in B_{12} can generate a large pool of methyl-THF that is unable to undergo reactions and will mimic folate deficiency. Another form of THF, formyl-THF or folinic acid results from oxidation of methylene-THF or is formed from formate donating formyl group to THF. Finally, histidine can donate a single carbon to THF to form methenyl-THF.

2.4.3 Human Reproduction

Adequate folate intake during the peri-conception period, the time right before and just after a woman becomes pregnant, helps protect against a number of congenital malformations, including NTDs, which are the most notable birth defects that occur from folate deficiency (Shaw *et al*., 1995). NTDs produce malformations of the spine, skull, and brain including spina bifida and anencephaly. The risk of NTDs is significantly reduced when supplemental folic acid is consumed in addition to a healthy diet prior to and during the first month

following conception (Mulinare *et al*., 1988; Milunsky *et al*.,1989). Supplementation with folic acid has also been shown to reduce the risk of congenital heart defects, cleft lips (Wilcox *et al*., 2007), limb defects, and urinary tract anomalies (Goh and Koren, 2008). Folate deficiency during pregnancy may increase the risk of preterm delivery, infant low- birth weight and foetal growth retardation. It may also increase homocysteine level in the blood, which may lead to spontaneous abortion and pregnancy complications, such as placental abruption and pre-eclampsia (Scholl and Johnson, 2000). Women who could become pregnant are advised to eat foods fortified with folic acid or take supplements in addition to eating folate-rich foods to reduce the risk of serious birth defects (Wilton and Foureur, 2010).

Taking 400 micrograms of synthetic folic acid daily from fortified foods and/or supplements has been suggested. The recommended daily allowance (RDA) for folate equivalents for pregnant women is 600–800 µg; twice the normal RDA of 400 µg for women who are not pregnant. The mechanisms and reasons why folic acid prevents birth defects is unknown. It is hypothesized that the insulin-like growth factor-2 gene is differentially methylated and these changes in IGF2 result in improved intrauterine growth and development (Steegers *et al*., 2009). Approximately 85% of women in an urban Irish study reported using folic acid supplements before they become pregnant, but only 18% used enough folic acid supplements to meet the current folic acid requirements due, reportedly, to socio-economic challenges (McGuire and Cleary, 2010). Folic acid supplements may also protect the foetus against disease, when the mother is sick taking medications or smoking during pregnancy (Jia *et al*., 2010). Folic acid may also reduce chromosomal defects in sperm. A benefit is indicated even for more than 700 µg folate per day, which, though below the tolerable upper intake levels of 1,000 µg/day, was 1.8 times the recommended dietary allowance (Young, 2008).

2.5 ZINC

Zinc is the second most abundantly distributed trace element in the body after iron (King, 2006). Zinc is an essential micronutrient for human metabolism that catalyses more than 100 enzymes. It facilitates protein folding and regulates gene expression. Patients with malnutrition, alcoholism, inflammatory bowel disease, and malabsorption syndromes are at an increased risk of zinc deficiency. Symptoms of zinc deficiency are non-specific, including growth retardation, diarrhoea, alopecia, glossitis, nail dystrophy, decreased immunity, and hypogonadism in males. In developing countries, zinc supplementation may be effective for the prevention of upper respiratory infection and diarrhoea, and as an adjunct treatment for diarrhoea in malnourished children. Zinc in combination with antioxidants may be modestly effective in slowing the progression of intermediate and advanced age-related macular degeneration. Zinc is an effective treatment for Wilson disease. It is well tolerated at recommended dosages. Adverse effects of long-term high-dose zinc use include suppressed immunity, decreased high-density lipoprotein cholesterol levels, anaemia, copper deficiency, and possible genitor-urinary complications (Saper and Rash, 2009)

It is found in a variety of foods, such as beef, poultry, seafood, and grains (Groff and Gropper, 2001; NAP, 2001). Commercial zinc supplements contain 7 to 80 mg of elemental zinc, and are commonly formulated as zinc oxide or salts with acetate, gluconate, and sulphate. Zinc supplements are commonly used to alleviate a number of conditions, including zinc-deficient states, diarrhea, age-related macular degeneration, upper respiratory infection, wound healing, and human immunodeficiency virus.

2.5.1 Pharmacology

Zinc is absorbed in the small intestine. Severe decreases or increases in zinc intake are necessary to substantially affect zinc stores (Cousins, 1989). Zinc is a co-factor for

polymerases and proteases involved in many cellular functions (for example wound repair (Iwata, 1999), intestinal epithelial cell regeneration) (Saper and Rash, 2009). Zinc is also a cofactor for thymulin, a thymic hormone essential for T-cell maturation (Mocchegiani, 1995). Zinc has antioxidant properties and may protect against macular degeneration from oxidative stress (Grahn, 2001).

2.5.2 Zinc Deficiency

Zinc deficiency caused by malnutrition is the 11th major risk factor in the global distribution of disease burden and is associated with 1.8 million deaths annually (WHO, 2002). Serum zinc levels are not a reliable measure of zinc stores and, therefore, are not recommended for routine screening. A presumptive diagnosis of zinc deficiency can be made in the context of zinc-deficiency symptoms, signs of malnutrition (for example, underweight, hypoalbuminaemia), or conditions commonly associated with zinc deficiency (NAP, 2001; King, 2006). A meta-analysis of 33 randomised controlled trials enrolling prepubertal children from North and South America, Europe, Africa and Asia, who were at risk of zinc deficiency showed that zinc supplementation modestly enhanced linear growth and weight gain (effect sizes of 0.35 and 0.31, respectively) compared with the control group (Brown *et al*., 2002).

2.5.3 Characteristics of Zinc Deficiency

2.5.3.1 Diarrhoea

A meta-analysis of 22 of randomised controlled trials zinc supplements versus placebo for the treatment of diarrhoea in children from developing countries found an 18 percent reduction of diarrhoea symptoms, compared with placebo (Lukacik *et al*., 2008). A meta-analysis of 15 prevention studies demonstrated that zinc supplementation conferred a 14 percent reduced risk in the incidence of diarrhoeal episodes compared with placebo (Aggarwal *et al*., 2007).

2.5.3.2 Upper respiratory infection

A meta-analysis including 12 randomised controlled trials with a total of 5,512 children in developing countries found a reduction in upper respiratory infection incidence in those using zinc supplements, compared with placebo (8 percent; 95% CI, 1 to 15 percent) (Aggarwal *et al*., 2007). Regarding treatment, a meta-analysis of eight randomised controlled trials with 890 predominantly adult participants with upper respiratory infection in industrialized countries who were treated with zinc lozenges found no evidence of a statistically significant reduction in duration (Jackson *et al*., 2001). Methodologic problems included poor blinding, small sample size, high drop-out rates, and variability in zinc dosage and formulation. Subsequent studies in adults (Turner *et al*., 2000; Mossad, 2003; Prasad *et al*., 2007) and children (Macknin *et al*., 1998; Kurugol *et al*., 2006) showed similarly mixed results. Overall, robust data are lacking for the effectiveness of zinc lozenges in reducing the duration or severity of upper respiratory infection.

2.5.3.3 Wound healing

Zinc deficiency is associated with impaired wound healing (Rojas and Phillips, 1999). Although zinc is a common ingredient in topical products used to treat skin conditions such as ulcers, diaper rash and haemorrhoids, relatively few studies support its use for accelerating wound healing. A randomised controlled trial of 46 infants with diaper dermatitis found no significant difference in resolution between zinc oxide ointment and ointment base alone (Wananukul *et al*., 2006). A meta-analysis of 181 participants from six randomised controlled trials of oral zinc sulphate versus placebo for venous or arterial leg ulcers found no significant difference in time to ulcer resolution (Wilkinson and Hawke, 1998). A randomised controlled trial comparing surgical mesh impregnated with zinc oxide ointment versus mesh with ointment base alone found no statistically significant difference in time to secondary closure of pilonidal surgery wounds (Agren *et al*., 2006).

2.5.3.4 Other uses

Zinc deficiency is associated with the rapid progression of HIV (Jones *et al*., 2006). However, most of zinc randomised controlled trials in persons who are HIV positive have shown no increase in CD4 cell counts or decrease in viral load (Bobat *et al*., 2005; Villamor *et al*., 2006). Wilson disease can be successfully treated with zinc because of its ability to compete with copper for binding sites (Czlonkowska *et al*., 1996). Zinc acetate has been shown to be effective in the long-term treatment of Wilson disease and is approved by the U.S. Food and Drug Administration for maintenance therapy (Brewer *et al*., 1998). Although oral zinc has been used for acne, minocycline (Dynacin) is twice as effective (Dreno *et al*., 2001).

2.5.4 Zinc Contra-indications, Adverse Effects and Interactions with other agents

Chronic ingestion of zinc supplements up to the tolerable upper intake level (40 mg elemental zinc per day in adults) is generally considered safe. Use of zinc supplements above the tolerable upper intake level in well-nourished pregnant and lactating women is contra-indicated (NAP, 2001). Common adverse effects of excessive zinc intake include metallic taste, nausea, vomiting, abdominal cramping, and diarrhoea (NAP, 2001). Prolonged exposure to amounts greater than the tolerable upper intake level may suppress immunity, decrease high-density lipoprotein cholesterol levels, and cause hypochromic microcytic anaemia and copper deficiency (Fosmire, 1990). Furthermore, the Health Professionals Follow-up Study of 46,974 adult men found a 2.3 increased relative risk of advanced prostate cancer in men using elemental zinc in amounts of 100 mg per day or more (Leitzmann *et al*., 2003).

2.5.5 Zinc supplements

2.4.5.1 Dosage

Mild zinc deficiency should be treated with zinc supplementation at two to three times the recommended daily allowance (RDA), whereas moderate to severe deficiency can be treated at four to five times the RDA (Walravens *et al*., 1989; Beers, 2006). Treatment should last for six months. For acute diarrhoea in malnourished children six to 36 months of age, 20 mg per day of elemental zinc has been used (Lukacik *et al*., 2008).

2.4.5.2 Common Oral Zinc Preparations

Despite the many essential roles of zinc in human body, no robust data support zinc supplementation alone for persons with a normal zinc status. However, zinc in combination with antioxidants may be modestly effective in slowing the progression of age-related macular degeneration. In children living in developing areas of the world, zinc supplementation may be an effective preventive measure for diarrhoea and upper respiratory infection, and an adjunct treatment for diarrhoea. Zinc has been shown to be an effective treatment for Wilson disease. No consistent benefit of zinc has been found for treatment of upper respiratory infection, wound healing, or HIV. On-going zinc supplementation up to the tolerable upper intake level is generally safe. Higher doses should be limited to short-term use because of an increased risk of gastro-intestinal adverse effects, copper deficiency, anaemia, and genitourinary complications (Saper and Rash, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Cages, normal saline, scissors, emmersion oil, Gimsa stain, buffer distilled water, water bath, cover slip containers, syringes (1 ml, 2 ml and 5 ml), soap, making tape, oral intragastric tube, watch, cotton wool, dissection kit, filter paper, red blood cell pipette, chromic suture, Petri dish, electronic balance, improved Neubauer counting chamber (Hawksley, England) and HM-Lux microscope (Germany) were obtained in Zaria City, Nigeria and Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria.

3.1.1 Animals

Thirty male Wistar rats weighing 104 - 295 g were bought from the Animal House of the Department of Human Physiology, Ahmadu Bello University Zaria, Nigeria. They were kept in well aerated laboratory cages and given access to growers' mesh Vital feeds, Grand Cereals Ltd, Jos, Nigeria and water *ad libitum*.

3.1.2 Chemicals

Folic acid, 653-100 (Masons Vitamins, INC. Miami Lakes, Florida), zinc gluconate, B16875 (Good 'N Natural, Bohemia, New York) and aspirin tablets were obtained from pharmaceutical stores in Zaria, Nigeria. Enzyme-linked immunosorbent assay (ELISA) kits for tumour necrosis factor-alpha (TNF-α1) and prostaglandin E_2 (PGE_2) were purchased from Ausmausco Pharmaceutical Company, Shanghai, China.

3.2 METHODS

3.2.1 Experimental Design

Thirty male Wistar rats were weighed and then divided randomly into seven groups, with each group containing five rats. The treated groups were administered folic acid, zinc and aspirin via oral route for twenty one days.

GROUP 1 (CONTROL): In this group, the rats were sacrificed without treatment after giving them normal saline 1 ml/kg for 21 days.

GROUP 2 (SHAM): In this group, the testes were brought through the scrotal incision and then replaced to the scrotal sac after giving them normal saline 1ml/kg for 21 days.

GROUP 3 (IRI): Rats in this group were experimentally-induced with torsion for two hours followed by detorsion for one hour after giving them normal saline 1ml/kg for 21 days

GROUP 4 (IRI + FA): In this group, the rats were treated with 2 m/kg of folic acid for 21 days. Thereafter, they were experimentally induced with torsion-detorsion as in group 3.

GROUP 5 (IRI + ZINC): Rats in this group were treated with 50 mg/kg of zinc gluconate for 21 days and then induced experimentally with torsion-detorsion as in group 3.

GROUP 6 (IRI+ ASPIRIN): Rats in this group were treated with 200 mg/kg of aspirin for 21 days and then induced experimentally with torsion-detorsion as in group 3.

3.2.2 Surgical Induction of torsion and detorsion

The rats were anaesthetised by chloroform inhalation in a closed chamber and thereafter sacrificed. The testes were exposed through identically-opened and closed right-sided ilio-inguiral incision. The testicles were exposed by incising the *tunica vaginalis*. The spermatic cords were exposed and torsions were created by rotating the testes 720^0 clockwise. The rotated testes were maintained for two hours by fixing them medially and laterally to the scrotum using a surgical silk suture. The detorsions were performed for one hour during which reperfusion occurred. The testicles were surgically removed through a lower abdominal incision according to the method described by Akusu *et al.* (1985) and Oyeyemi and Ubiogoro (2005).

3.2.3 Semen Analysis

The epididymis was crushed using a blunt forceps in a Petri dish. Normal saline(1 ml) was added and mixed thoroughly. The semen mixture was then sucked into a red blood cell pipette to the 0.5 mark, then normal saline was sucked up to the 101 mark. The normal saline in the stem of the pipette was discarded and the content of the bulb of the pipette was mixed thoroughly. A drop of the mixture was then placed on the counting chamber, which then spread under the cover slip under capillary action. The counting chamber was then mounted on the microscope and viewed under x 40 magnification. A grid system divided the counting chamber into five major squares each containing 16 smaller boxes. The count included all the sperm cell within all the five major squares using the Top-Right or Bottom-Left system of counting (Oyeyemi et *al.*, 2006).

For the sperm morphology, the epididymis was crushed in a Petri dish and 1ml of normal saline was added. A smear of it was made using microscope slides and allowed to dry. Gimsa stain was added on the smear and allowed to remain for twelve minutes. Buffer distil water was also added on the smear and allowed to remain for another eight minutes. The slide was then washed and allowed to dry. After drying, a drop of immersion was placed on the slide and viewed under the microscope using x 100 objective. A total of 100 cells were counted and classified as normal and abnormal.

To assess the motility, the epipidymis was crushed in 2 ml of pre-warmed normal saline (36˚C). A drop of the crushed epipidymis was put on a microscope slide and covered with a cover slip and viewed under the microscope using x 10 objective. Hundred cells were viewed and classified as fast, slow and non-motile cells.

3.2.4 Gonado-Somatic Index

The testes, epididymes and seminal vesicles were dissected and immediately separated out from the adherent tissue and weighed to the nearest mg on an electronic balance according to the method described by Franca *et al*. (2006). The GSI calculated using the formula:

$$\text{GSI} = \frac{\text{Gonad weight}}{\text{Total body weight}} \times 100$$

Where gonad weight = (weight of right testis + weight of left testis)/2

3.2.5 Assay of Pro-inflammatory Cytokines

Tumour necrosis factor alpha (TNF-α) levels in the serum was measured by using TNF-α ELISA kit (assay range: 10 ng/L – 360 ng/L). The kit was removed from the refrigerator and brought to room temperature before the commencement of the experiment. Each (150 µl) standard was added to appropriate wells in the TNF-α micro plate coat. The wells were covered with closure plate membrane and incubated for 55 minutes at 28˚C. The plate was brought out from the incubator, the closure membrane removed and the liquid inside each well was discarded. The plate was dried by swinging, then wash solution was added to each well and allowed to remain for 30 seconds and drained. The washing was repeated five times and the wells were dried by pad. HPR-Conjugate reagent (50 µl) was added to each well and incubated for 53 min at 30˚C. Washing and drying repeated, 50 µl of Chromogen solution A and Chromogen solution B were added to each well and incubated for 20 min at 31˚C. At the end of incubation, 50 µl of stop solution was added to each well to stop the reaction and the absorbance was taken at 450nm from the reader.

Prostaglandin E_2 level in the serum was measured by using PGE_2 ELISA kit (assay range: 15 ng/L – 480 ng/L). The kit was removed from the refrigerator and brought to room temperature before the commencement of the experiment. Each standard (150 µl) was added to appropriate wells in the PGE_2 micro plate coat. The wells were covered with closure plate

membrane and incubated for 60 min at 30˚C. The plate was brought out from the incubator, the closure membrane removed and the liquid inside each well was discarded. The plate was dried by swinging, then wash solution was added to each well and allowed to remain for 30 seconds and drained. The washing was repeated five times and the wells were dried by pad. HPR-Conjugate reagent (50 µl) was added to each well and incubated for 60 min at 31˚C. Washing and drying repeated, 50 µl of chromogen solution A and chromogen solution B were added to each well and incubated for 20 min at 31˚C. At the end of incubation, 50 µl of stop solution was added to each well to stop the reaction and the absorbance reading is taken at 450 nm from the reader.

3.2.5 Statistical Analysis

All values were expressed as mean ± standard error of the mean (mean ± SEM). The significance of the data obtained was evaluated by using one-way analysis of variance (ANOVA). Differences between means were analysed using Turkey's post hoc test. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1.1 SPERM COUNT

Table 4.1.1 shows the effect of folic acid and zinc on sperm count in Wistar rats induced with testicular torsion. There was a significant decrease in treated groups compared with that of the controls. The folic acid-treated group had the minimum sperm count ($0.75 \pm 0.06 \times 10^{12}$ cells/L) while the torsion/detorsion group has the maximum sperm count ($0.90 \pm 0.03 \times 10^{12}$ cells/L)

Table 4.1.1: Effect of folic acid and zinc on sperm count in experimentally induced testicular torsion in Wistar rats.

Group	Mean ± SEM (x 10^{12} cells/L)	Minimum (x 10^{12} cells/L)	Maximum (x 10^{12} cells/L)
Control	1.14 ± 0.06^{a}	0.99	1.33
Sham	0.79 ± 0.02^{b}	0.75	0.83
Torsion/Detorsion	0.90 ± 0.03^{b}	0.86	0.99
Aspirin	$0.83 \pm 0.`07^{b}$	0.71	0.96
Folic acid	0.75 ± 0.06^{b}	0.67	0.86
Zinc	0.82 ± 0.02^{b}	0.78	0.85

[ab] Means along the same column with different superscripts are significantly($P < 0.05$) different

4.1.2 SPERM MORPHOLOGY OF NORMAL CELLS

Table 4.1.2 shows the effect of folic acid and zinc on morphology of normal cells in Wistar rats induced with testicular torsion. There was no significant ($P > 0.05$) difference in the treated groups compared to that of the controls.

Table 4.1.2: Effect of folic acid and zinc on morphology of normal sperm cells in Wistar rats induced with testicular torsion

Group	Mean ± SEM (x 10^{12} cells/L)	Minimum (x 10^{12} cells/L)	Maximum (x 10^{12} cells/L)
Control	70.00 ± 8.94[a]	35.00	85.00
Sham	70.00 ± 7.64[a]	55.00	80.00
Torsion/Detortion	68.75 ± 7.47[a]	50.00	85.00
Aspirin	67.50 ± 5.95[a]	50.00	75.00
Folic acid	75.00 ± 10.41[a]	55.00	90.00
Zinc	68.75 ± 4.27[a]	60.00	80.00

[a] Means along the same column with the same superscript are not significantly (P> 0.05) different

4.1.3 SPERM MORPHOLOGY OF ABNORMAL CELLS

Table 4.1.3 shows the effect of folic acid and zinc on the morphology of abnormal sperm cells in testicular torsion induced experimentally in Wistar rats. There was no significant difference in the treated groups compared to that of the control.

Table 4.1.3: Effect of folic acid and zinc on morphology of abnormal sperm cells in testicular torsion induce experimentally in Wistar rats

Group	Mean ± SEM (x 10^{12} cells/L)	Minimum (x 10^{12} cells/L)	Maximum (x 10^{12} cells/L)
Control	30.00 ± 8.94[a]	15.00	65.00
Sham	30.00 ± 7.64[a]	25.00	45.00
Torsion/Detortion	31.25 ± 7.47[a]	15.00	50.00
Aspirin	32.50 ± 5.95[a]	25.00	50.00
Folic acid	25.00 ± 10.4[a]	10.00	45.00
Zinc	31.25 ± 4.27[a]	20.00	40.00

[a] Means along the same column with the same superscript are not significantly (P> 0.05) different

4.1.4 SPERM MOTILITY OF FAST MOVING CELLS

Table 4.1.4 shows the effect of folic acid and zinc on sperm motility of fast movement cells in testicular torsion induced in Wistar rats. The result obtained shows a significant ($P < 0.05$) decrease in folic acid and zinc-treated groups compared with that of the controls. The zinc-treated group has a minimum value of 26.32±00 x 10^{12} cells/L while the aspirin treated group has the maximum value of 71.67±8.82 x 10^{12} cells/L

Table 4.1.4: Effect of folic acid and zinc on motility of fast movement cells in Wistar rats induced with testicular torsion

Group	Mean ± SEM (x 10^{12} cells/L)	Minimum (x 10^{12} cells/L)	Maximum (x 10^{12} cells/L)
Control	62.00 ± 5.39[a]	50.00	75.00
Sham	53.00 ± 10.03[a]	35.00	75.00
Torsion/Detorsion	43.29 ± 8.44[a]	22.92	59.14
Aspirin	71.67 ± 8.82[a]	55.00	85.00
Folic acid	26.35 ± 4.36[b]	21.05	35.00
Zinc	17.83 ± 4.53[b]	5.00	26.32

[ab] Means with different superscripts along the same column are significantly($p < 0.05$) different

4.1.5 SPERM MOTILITY OF SLOW MOVING CELLS

Table 4.1.5 shows the effect of folic acid and zinc on slow moving cells in experimentally-induced testicular torsion in Wistar rats. There was no significant ($p>0.05$) difference in the number of slow moving cells in treated groups compared with that of the controls.

Table 4.1.5: Effect of folic acid and zinc on motility of slow movement cells in experimentally-induced testicular torsion

Group	Mean ± SEM (x 10^{12} cells/L)	Minimum (x 10^{12} cells/L)	Maximum (x 10^{12} cells/L)
Control	29.80 ± 5.21[a]	15.00	45.00
Sham	41.75 ± 9.47[a]	20.00	60.00
Torsion/Detorsion	37.29 ± 9.11[a]	21.51	56.70
Aspirin	21.67 ± 3.33[a]	15.00	25.00
Folic acid	48.21 ± 2.28[a]	45.00	52.63
Zinc	55.59 ± 8.20[a]	45.00	80.00

[a] Means along the same column with the same superscript are not significantly (P> 0.05) different

4.1.6 SPERM MOTILITY OF NON-MOTILE CELLS

Table 4.1.6 shows the effect of folic acid and zinc on non-motile cells in experimentally induced testicular torsion in Wistar rats. There was significant ($P < 0.05$) decrease in folic acid and zinc-treated groups, compared with that of the controls. The sham operated group had a minimum value ($5.25 \pm 1.03 \times 10^{12}$ cells/L) while the zinc treated group recorded the maximum value ($26.58 \pm 4.25 \times 10^{12}$ cells/L).

Table 4.1.6: Effect of folic acid and zinc on sperm motility (non-motile cells) in experimentally-induced testicular torsion in Wistar rat

Group	Mean ± SEM (x 10^{12} cells/L)	Minimum (x 10^{12} cells/L)	Maximum (x 10^{12} cells/L)
Control	7.20 ± 3.15^{a}	0.00	18.00
Sham	5.25 ± 1.03^{a}	3.00	8.00
Torsion/Detorsion	19.43 ± 4.45^{a}	7.22	56.70
Aspirin	21.67 ± 3.33^{a}	15.00	28.13
Folic acid	25.45 ± 2.92^{b}	20.00	35.00
Zinc	26.58 ± 4.25^{b}	15.00	35.00

[ab] Means along the same column with different superscripts are significantly($p < 0.05$) different

4.2.1 TESTICULAR BODY RATIO

Table 4.2.1 shows the effect of folic acid and zinc on testicular body ratio in Wistar rats experimentally induced with testicular torsion. There was no significant ($P > 0.05$) difference in the testicular body ratio in the treated groups compared to that of the controls.

Table 4.2.1: Effect of folic acid and zinc on testicular body ratio in testis of Wistar rats experimentally-induced with testicular torsion

Group	Mean ± SEM (kg)	Minimum (kg)	Maximum (kg)
Control	0.61 ± 0.08[a]	0.31	0.82
Sham	0.63 ± 0.09[a]	0.45	0.86
Torsion/Detorsion	0.60 ± 0.05[a]	0.48	0.71
Aspirin	0.66 ± 0.03[a]	0.62	0.72
Folic acid	0.58 ± 0.04[a]	0.52	0.65
Zinc	0.56 ± 0.04[a]	0.48	0.68

[a] Means along the same column with the same superscript are not significantly (P> 0.05) different

4.2.2 EPIDIDYMAL BODY RATIO

Table 4.2.2 shows the effect of folic acid and zinc on the epididymal body ratio in Wistar rats experimentally-induced with testicular torsion. There was no significant ($P > 0.05$) difference in the treated groups compared to that of the controls.

Table 4.2.2: Effect of folic acid and zinc on epididymal body ratio in Wistar rats experimentally-induced with testicular torsion

Group	Mean ± SEM (kg)	Minimum	Maximum
Control	0.12 ± 0.01[a]	0.08	0.15
Sham	0.13 ± 0.02[a]	0.08	0.17
Torsion/Detorsion	0.12 ± 0.01[a]	0.10	0.14
Aspirin	0.14 ± 0.01[a]	0.13	0.16
Folic acid	0.13 ± 0.01[a]	0.12	0.15
Zinc	0.13 ± 0.01[a]	0.10	0.16

[a] Means along the same column with the same superscript are not significantly ($P > 0.05$) different

4.2.3 SEMINAL BODY RATIO

Table 4.2.3 shows the effect of folic acid and zinc on seminal body ratio in experimentally-induced testicular torsion in Wistar rats. There was no significant ($P > 0.05$) difference in the treated groups compared to that of the controls.

Table 4.2.3: Effect of folic acid and zinc on seminal body ratio in experimentally-induced testicular torsion in Wistar rats

Group	Mean ± SEM (kg)	Minimum (kg)	Maximum (kg)
Control	0.58 ± 0.05[a]	0.45	0.73
Sham	0.70 ± 0.13[a]	0.50	1.03
Torsion/Detorsion	0.47 ± 0.10[a]	0.31	0.75
Aspirin	0.68 ± 0.07[a]	0.55	0.75
Folic acid	0.93 ± 0.04[a]	0.85	0.98
Zinc	0.59 ± 0.09[a]	0.33	0.74

[a] Means along the same column with the same superscript are not significantly ($P > 0.05$) different

4.3.1 PROSTAGLANDIN E_2 LEVELS

Table 4.3.1 shows the effect of folic acid and zinc on PGE_2 levels in to experimentally-induced testicular torsion in Wistar rats. There was no significant ($P > 0.05$) difference in PGE_2 levels in the treated groups, compared to that of the controls.

Table 4.3.1: The effect of folic acid and zinc on PGE_2 levels in experimentally-induced testicular torsion in Wistar rats

Group	Mean ± SEM (ng/l)	Minimum	Maximum
Control	393.40 ± 132.79[a]	126.20	395.80
Sham	449.60 ± 176.60[a]	135.40	401.40
Torsion/Detorsion	464.67 ± 187.07[a]	138.00	414.40
Aspirin	537.28 ± 277.12[a]	130.08	407.20
Folic acid	265.00 ± 5.00[a]	130.00	135.00
Zinc	394.50 ± 133.46[a]	125.60	397.40

[a] Means along the same column with the same superscript are not significantly ($P > 0.05$) different

4.3.2 TUMOUR NECROSIS FACTOR-ALPHA LEVELS

Table 4.3.2 shows the effect of folic acid and zinc on TNF-α levels in experimentally-induced testicular torsion in Wistar rats. There was no significant ($p>0.05$) difference in the treated groups, compared to that of the controls.

Table 4.3.2: The effect of folic acid and zinc on TNF-α levels in experimentally-induced testicular torsion in Wistar rats

Group	Mean ± SEM (ng/l)	Minimum	Maximum
Control	252.95 ± 6.12[a]	121.80	135.60
Sham	254.27 ± 9.24[a]	119.70	135.60
Torsion/Detorsion	252.33 ± 7.20[a]	120.60	132.90
Aspirin	241.10 ± 7.95[a]	112.60	128.50
Folic acid	256.00 ± 5.30[a]	125.50	130.80
Zinc	245.45 ± 4.50[a]	118.30	127.90

[a] Means along the same column with the same superscript are not significantly (P> 0.05) different

CHAPTER FIVE

5.0 DISCUSSION, SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The results of the present study demonstrated the effects of folic acid and zinc in Wistar rats experimentally-induced with testicular torsion. The obtained decrease ($P < 0.05$) in the sperm count in the treated groups compared to that of the controls showed that ischaemia-reperfusion injury in testis resulted in disruption of spermatogenesis that may be permanent with short term suppression of testosterone secretion (Turner *et al.,* 2005). This finding is in agreement with that of Shiva *et al.* (2014) who showed that during testicular torsion, haemorrhagic necrosis and cell apoptosis lead to the accumulation of testicular neutrophils (inflammatory response) and increased testicular stress. According to experimental studies (Turner and Brown, 1993), germ cells are more vulnerable than Sertoli cells and spermatogenesis may be impaired despite the reflow of blood to the tissues and the preserved Sertoli cells (Turner and Brown, 1993). The decrease in the sperm cells observed in this study is due to increased generation of ROS, reactive nitrogen species and inflammatory cytokines generated during torsion through apoptosis (Garcia *et al.,* 2014). The disruption experienced during Ischaemic reperfusion injury was not restored by treatment with antioxidants. Cytokines are paracrine regulators of spermatogenesis and steroidogenesis in the normal testis. There is complex and multiple interactions among immune and germ cells generally resulting in the alteration of spermatogenesis. The complexity of these cell interactions is multiplied because sertoli and legdig cells are also producers of pro and anti-inflammatory cytokines and chemokines. This bidirectional immunoregulatory function of cytokines in acute testicular inflammation shows up regulation of IL-1β, IL-1α, IL-6 and TNF-α, which induce adverse effects on germ cells (Guazzone *et al.,* 2009). The folic acid and zinc could not significantly improve the sperm count, compared with the torsion/detorsion group.

Folic acid is considered to play a role in the maintenance of normal spermatogenesis and sperm maturation and DNA metabolism, synthesis, repair and transcription (Young *et al*., 2008). Zinc is an essential trace element for maintenance of germ cells, the progression of spermatogenesis and the regulation of sperm motility (Yamagushi, 2009). The significant reduction in the sperm cells obtained in this study in the folic acid-treated group disagree with the result of study done by Azizollahi *et al*. (2013) who recorded increase in the sperm count in human subjects treated with folic acid. The decrease in the sperm count in zinc- treated group recorded in the present study agrees with the findings of the author.

There is no statistically significant difference in the normal cells count in the treated groups compared with the control. However there is slight decrease in the group treated with folic acid and this agrees with several researches which confirm the repairing and maintenance role of folic acid in the cells of the body during oxidative stress (Weistein, 2003; Young, 2008). The result obtained also agrees with the findings of Shiva *et al.* (2014) which shows decreased in abnormal cells in melatonin treated rats during inequality-induced damages on testicular tissue. According Raigani *et al.* (2013) zinc sulphate and folic acid supplementation did not ameliorate sperm quality in infertile men with severely compromised sperm parameters which agrees with lack of significance recorded in the result obtained.

The groups treated with folic acid and zinc recorded significant decrease in motile cells compared with that of the controls. The result corroborates the findings of Collard and Gelman (2001), which explains that ischaemia-reperfusion disrupt protective physiologic responses or immune suppression thereby affecting the motility of the sperm cells. The number of the non-motile cells decreased significantly in the folic acid and zinc treated groups. The decrease in zinc treated group could be due to the anti-inflammatory properties of zinc (Prasad *et al*., 2007) and the folate pathway involved in spermatogenesis via DNA synthesis, repair and methylation (Mfady *et al.*, 2014).

The Gonadosomatic index which is either the testicular volume per body weight or testicular weight per body weight is species-specific and varies with age and breeding season, even within the same species (Pochron *et al.,* 2002). It was reported that this index predicts the rates of sperm function in a given species (Adebayo, 2009). In this study, there was no significant difference between the treated groups and the controls. The result obtained collaborates with the findinds of Ekoja (2011). The lack of significance may be due to relatively short duration of ischaemia before the reperfusion or the short duration of the experiment after the induction.

In the epididymal body ratio, when the results of the treated groups were compared with the control, there was no statistically significant difference between the treated group and the control. The result obtained could also be due to relatively short duration of ischaemia during the testicular torsion. This result agrees with the findings reported by Ekoja (2011). However, there was slight increase in epididymal body ratio in the aspirin treated group compared with the other treated groups and also the control. Asprin, a non-steroidal anti-inflammatory drug (NSAIDs), in higher doses reduces inflammation by inhibiting the enzyme cyclooxygenase (mostly the COX-1 variant) in an irreversible manner, thereby preventing or reducing the formation prostaglandin (Burke *et al*., 2006).

The result obtained in seminal body ratio shows no statistically significant difference between the treated groups and the control. Arterial blood supply to the seminal vesicles includes branches from the inferior vesical and middle rectal arteries, while venous and lymphatic drainage accompanies these arteries. The testes and epididymis share the same vasculature and their vessels all pass through the twisted spermatic cord during the experiment but the vessels of the seminal vesicles do not pass through it (Boorman *et al.,* 1990; Ovalle and Nahirney, 2007). The result obtained agrees with the findings of Ekoja (2011).

Prostaglandin E_2 (PGE_2) is a bioactive lipid that elicits a wide range of biological effects associated with inflammation and cancer. PGE_2 exerts diverse effects on cell proliferation, apoptosis, angiogenesis, inflammation and immune surveillance (Nakanishi, 2013). PGE_2 can be produced by all cell types of the body, with epithelial, fibroblasts and infiltrating inflammatory cells representing the major sources of PGE_2 in the course of an immune response (Lambeau, 1999). Arachidonic acid metabolites are since known to have significant roles in initiating and or terminating inflammatory processes. PGE_2 is one of the most abundant metabolites of arachidonic acid, generated through an enzymatic-cascade controlled by cyclooxygenase (COX) enzymes. PGE_2 has a role in increasing vascular permeability, fever generation and hyperalgesia (Chizzolin and Brembilla, 2009). The results obtained in this study shows no significant difference between treated groups compared with the controls. This finding disagrees with the study of Solini *et al*. (2006) which states that folic acid and magnesium have anti-inflammatory properties in human subjects that had folic acid supplementation. This study also disagrees with a study carried out by Mangoni *et al*. (2003) on human subjects that short-term folic acid supplementation has no significant effects on inflammatory markers but it induced a significant reduction in homocystein level.

Tumour necrosis factor-alpha (TNF-α) is another important inflammatory cytokine that is secreted mainly from activated macrophages and induced by ROS production in many body cells (Moore *et al.,*1999). During testicular inflammation as obtained in testicular torsion, there is upregulation of interleukin-1β (IL-1β), interleukin (IL-1α), interleukin (IL-6), and TNF-α (Guazzone *et al.*, 2009). TNF-α is one of the most important pro-inflammatory cytokines, it participates in vasodilatation and edema formation, and leucocyte adhesion to epithelium molecules, it regulates blood coagulation, contributes to oxidative stress in sites of inflammation (Zelova and Hosek, 2013). Increased concentrations of TNF-α are found in acute and chronic inflammatory conditions for example trauma, sepsis, infection, rheumatoid arthritis. In this study, there was no significant difference between the treated and control

groups but there is slight decrease in TNF-α in the zinc treated group. The slight decrease in TNF-α in the zinc treated group agrees with the findings of Prasad (2007) that slight decrease in increased plasma oxidative markers and increased generation of inflammatory cytokines in the human elderly subjects were corrected by zinc inducing A20, which inhibits nuclear factor-kappa (NF-kB) activation and resulting in decreased generation of inflammatory cytokines.

5.2 SUMMARY

In the study carried out, it was found out that two-hour Ischaemia followed by one-hour reperfusion significantly reduced the number of sperm cells that even treatment with antioxidant could not improve it. There was slight decrease in the number of abnormal cells in the folic acid treated group. The findings also show significant decrease in the number of motile and non-motile cells in folic acid and zinc treated groups. GSI were not affected by acute testicular torsion however the PGE_2 levels in the folic acid treated group was slightly reduced and this has shown that folic acid has potential anti-inflammatory properties that can be used to alleviate the adverse effect of testicular torsion.

5.3 CONCLUSION

The results obtained in this study demonstrate that testicular torsion significantly reduce sperm count and motility. It also leads to generation of TNF-α and PGE_2 levels which were slightly reduced following treatment with folic acid and zinc. It can be concluded that antioxidant therapy may present a new option within a broader therapeutic strategy in testicular torsion with oxidative stress-mediated infertility.

5.4 RECOMMENDATIONS

The following are our recommendations for future studies:

1. More than two inflammatory cytokines should be studied at a time for purpose of comparison.

2. The duration of ischaemia and reperfusion should be increased
3. The cytokines assay should also be conducted in the tissues so as to compare the results with the serum levels.
4. The experiments should be conducted in two different seasons for comparative studies.

REFERENCES

Abou-Haila, A. and Fain-Maurel, M. (1984). Regional differences of the proximal part of mouse epididymis: Morphological and histochemical characterization. *Anatamical Record,* 209: 197–208.

Adebayo, A. O., Oke, B., and Akinloye, A. K. (2009). Characterizing the Gonado-somatic index and its relationship with age in greater cane Rat (*Thryonomys swinderianus,Temminck).Veterinary Anatomy,* 2 (2): 53-59.

Aggarwal, R., Sentz, J. and Miller, M. A. (2007). Role of zinc administration in prevention of childhood diarrhea and respiratory illnesses: a meta-analysis. *Paediatrics,* 119(6): 1120–1130.

Agren, M. S., Ostenfeld, U. and Kallehave, F. (2006). A randomized, double-blind, placebo-controlled multicenter trial evaluating topical zinc oxide for acute open wounds following pilonidal disease excision. *Wound Repair and Regeneration,* 14(5): 526–535.

Akusu, M. O., Akpokodje, J. U., Ogwnegbu, S. O. and Oke, B. O. (1985). Differences in morphology of bull spermatozoa from normal and pathological testis during epididymal transit. *Nigerian Veterinary Journal,* 14 (2): 30-33.

Alvan, D., Erdouan, K. and Cimen, B. (2005). The protective effect of selenium on ipsilateral and contralateral testes in testicular reperfusion injury. *Paediatric Surgery International,* 21: 274-278.

Arce, J. D., Cortes, M. and Vargas, J. C. (2002). Sonographic diagnosis of acute spermatic cord torsion. Rotation of the cord: a key to the diagnosis. *Paediatric Radiology,* 32: 485-491.

Atavilla, D., Romeo C., Squadrito, F., Marini, H., Morqia, G., Antonuccio, P. and Minutol, L. (2012). Molecular pathways involved in the early and late damage induced bytestis ishaemia: evidence for rational pharmacological modulation *Current Medicinal Chemistry*, 10(8): 1219-1224.

Azizollahi, G., Azizollahi, S., Babaei, H., Kianinejad, M., Baneshi, M. R. and Nematollahi-mahani, S. N. (2013). Effects of supplement therapy on sperm parameters, protamine content and acrosomal integrity of varicocelectomised subjects. *Journal of Assisted Reproduction and Genetics,* 30(4): 593-599.

Bailey, S. W. and Ayling, J. E. (2009). The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. *Proceedings of the National Academy of Sciences of the United States of America,*106 (36): 15424–1529.

Barada, J. H., Weingarten, J. L. and Cromie, W. J. (1998). Testicular salvage and age-related delay in presentation of testicular torsion. *Journal of Urology,*142: 746-748

Beers, M. H. (2006). The Merck Manual of Diagnosis and Therapy. 18. Whitehouse Station, N.J: Merck Research Laboratories. *Nutritional Disorders,* P. 55.

Beni-Israel, T., Goldman, M., Bar Chaim, S. and Kozer E. (2010). Clinical predictors for testicular torsion as seen in the paediatric ED. *American Journal of Emergency Medicine*. 28(7): 786-789.

Bobat, R., Coovadia, H. and Stephen, C. (2005). Safety and efficacy of zinc supplementation for children with HIV-1 infection in South Africa: a randomised double-blind placebo-controlled trial. *Lancet*, 366(9500): 1862–1867.

Boorman, G. A., Chapin R. E. and Mitsumori, K. (1990). Testis and epididymis. In: *Pathology of the Fischer Rat, Reference and Atlas*. Boorman, G. A., Eustis, S. L., Elwell, M. R., Montgomery, C. A. Jr and MacKenzie W. F. (editors). Academic Press, San Diego, California, Pp. 405–418.

Boorman, G. A., Elwell, M. R. and Mitsumori K. (1990). Male accessory sex gland, penis and scrotum. In: *Pathology of the Fischer Rat, Reference and Atlas*. Boorman, G. A., Eustis, S. L., Elwell, M. R., Montgomery, C. A. Jr and MacKenzie, W. F. (editors). Academic Press, San Diego, California, Pp 419–428.

Boorman, G. A., Eustis, S. L. and Elwell, M. R. (1990). Neoplasms of the testis. In: *Atlas of Tumor Pathology of the Fischer Rat.* Stinson, S. F., Schuller, H. M. and Reznik, G. K. (editors). CRC Press, Boca Raton, Florida, Pp. 409–414.

Bozlu, M., Co kun, B. and Cayan, S. (2004). Inhibition of poly (adenosine diphosphate-ribose) polymerase decreases long-term histologic damage in testicular ischemia repersusion injury. *Urology,*63: 791-795.

Bradley, J. R. (2008). TNF-mediated inflammatory disease. *Journal of Pathology Disease*, 414: 149-160.

Brewer, G. J., Dick, R. D., Johnson, V. D., Brunberg, J. A., Kluin, K. J. and Fink, J. K. (1998). Treatment of Wilson's disease with zinc: XV long-term follow-up studies. *Journal of Laboratory Clinical Medicine,* 132(4): 264–278.

Brown, K. H., Peerson, J. M., Rivera, J. and Allen, L. H. (2002). Effect of supplemental zinc on the growth and serum zinc concentrations of prepubertal children: a meta-analysis of randomized controlled trials. *American Journal of Clinical Nutrition,* 75(6): 1062–1071.

Brzezinska-Slebodzinska, E. (2003). Species differences in the susceptibllity of erythrocytes exposed to free radicals *in vivo*. *Veterinary Research Communications*, 27: 211 – 217.

Burke, A., Smyth, E. and FitzGerald, G. A. (2006). "26: Analgesic Antipyretic and Antiinflammatory Agents". *Goodman and Gilman's the pharmacological basis of therapeutics* (11th ed.). New York: McGraw-Hill, Pp. 671–716.

Buege, J. A. and Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods Enzymology,* 52: 302-310.

Byskov, A. G. and Hoyer, P. E. (1988). Embryology of the mammalian gonads and ducts. In: *The Physiology of Reproduction*. Knobil, E. and Neill, J. D. (editors), 2nd edition., Volume 1, Raven Press, New York, Pp487–540.

Callewaert, P. R. H. and Kerrebroeck, P. V. (2009). New insights into perinatal testicular torsion. *European Journal of Paediatrics,* 10.1007/s00431-009-1096-8.

Candocia, F. J. and Sack-Solomon, K. (2003). An infant with testicular torsion in the inguinal canal. *Paediatric Radiology,* 33: 722-724.

Carden, D.L., Granger, D N. (2000). Pathophysiology of ischaemia-reperfusion injury. *Journal of Pathology,*190: 2255-2266.

Cattolica, E. V., Karol, J. B., Rankin, K. N. and Klein R. S. (1982). High testicular salvage rate in torsion of the spermatic cord. *Journal of Urology*, 128(1): 66-68.

Chen, Y. F., Jobanputra, P. and Barton, P. (2008). Cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: a systemic review and economic evaluation. *Health Technology Assesement,* 12: 1-278.

Chiu, B., Chen, C., Keller, J J., Lin, C and Lin H. (2012). Seasonality of testicular torsion: A 10-year nationwide population based study. *Journal of Urology,* 187(5): 1781-1785.

Chizzolini, C. and Brembilla, N. C. (2009). Inflammation Prostaglandin E_2: Igniting the fire. *Immunology and Cell Biology,* 87: 510-511.

Chong, E. W., Wong, T. Y., Kreis, A. J., Simpson, J. A. and Guymer, R. H. (2007). Dietary anti-oxidants and primary prevention of age related macular degeneration: systematic review and meta-analysis. *British Medical Journal,* 335(7623): 755.

Clavien, P. A., Harvey, P. R. C. and Strasberg, S. M. (1992). Preservation and reperfusion injuries in liver allografts. *Transplantation,* 53: 957-958.

Coley, B. D (2006). The Acute Paediatric scrotum. *Ultrasound Clinics*, 1: 485-496.

Collard, C. D. and Gelman, S. (2001). Pathophysiology, clinical manifestations and prevention of Ischaemia-reperfusion injury. *Anaesthesiology,* 94: 1133-1138.

Cost, N. G., Bush, N. C., Barber, T. D., Huang, R and Baker L. A. (2011). Pediatric testicular torsion: demographics of national orchiopexy versus orchiectomy rates. *Journal of Urology*, 185(6): 2459-2463.

Cousins, R. J. (1989). Systemic transport of zinc. In: Mills, C. F (editor). *Zinc in Human Biology*. New York, NY: Springer-Verlag; pp. 79–93.

Creasy, D., Bube, A., de Rijk., Kandori, H., Kuwahara, M., Masson, R., Nolte, T., Reams, R., Regan, K., Rehm, S., Rogerson, P. and Whitney, K. (2012). Proliferative and non-proliferative lesions of the rat and mouse male reproductive system. *Toxicological Pathology,* doi: 10.1177/0192623312454337. 40(6): 40s-121s.

Creasy, D. M. (1998). The male reproductive system. In: *Target Organ Pathology*. Turton, J and Hooson, J. (editors). Taylor and Francis Ltd., London, pp. 371–406.

Cuckow, P. M and Frank, J D. (2000). Torsion of the testis. In: *British Journal of Urology International,* 86 (3,5): 349-353.

Czlonkowska, A., Gajda, J. and Rodo, M. (1996). Effects of long-term treatment in Wilson's disease with D-penicillamine and zinc sulphate. *Journal of Neurology,* 243(3): 269–273.

Dawson, R. M. C. (1989). *Data for Biochemical Research,* 3rd Edition, Oxford University Press, Oxford, pp. 134.

Dawson, V. L. and Dawson, T. M. (2000). Neuronal ischemic preconditioning. *Trends in Pharmacological Science,* 21: 423-424.

Dogra, V. and Bhatt, S. (2004). Acute painful scrotum. *Radiologic Clinics of North America*. 42(2): 349-363.

Dokmeci, D. (2012). Oxidative stress and testicular torsion. *Oxidative Stress in Applied Basic Research and Clinical Practice,* pp. 355-389.

Dreno, B., Moyse, D. and Alirezai, M. (2001). Multicenter randomized comparative double-blind controlled clinical trial of the safety and efficacy of zinc gluconate versus minocycline hydrochloride in the treatment of inflammatory acne vulgaris. *Dermatology,* 203(2): 135–140.

Dünker, N. and Aumüller, G. (2002). Transforming growth factor-beta 2 heterozygous mutant mice exhibit Cowper's gland hyperplasia and cystic dilations of the gland ducts (Cowper's syringoceles). *Journal of Anatomy,* 201: 173–183.

Eaton, S. H., Cendron, M. A., Estrada, C. R., Bauer, S. B., Borer, J. G., Cilento, B. G., Diamond, D. A., Retik, A. B. and Peters, C. A. (2005). Intermittent testicular torsion: diagnostic features and management outcomes. *Journal of Urology,* 174(4 Pt 2): 1532-1535.

Ebbing, M., Bønaa, K. H. and Nygård O. (2009). Cancer incidence and mortality after treatment with folic acid and vitamin B_{12}. *Journal of American Medical Association,* 302 (19): 2119–2126.

Egur, B. U., Kiray, M., Pekcetin, C., Bagriyanik, H. A. and Erbil, G. (2008). Protective effect of erythropoietin pretreatment in testicular ischaemic-reperfusion in rats. *Journal of Paediatric Surgery,* 43: 722-728.

Ekoja, B. (2011). Modulatory role of folic acid and vitamin C in ischaemic reperfusion injury in Testes of Wistar rats. *A Project submitted in Partial Fulfillment* of *the Requirements for the Bachelor's Degree in Human Physiology*. Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria, pp. 36-41.

Evans, J. R. (2006). Antioxidant vitamin and mineral supplements for slowing the progression of age-related macular degeneration. *Cochrane Database System Review*, 2: CD000254.

Evans, J. R. and Henshaw, K. (2008). Antioxidant vitamin and mineral supplements for preventing age-related macular degeneration. *Cochrane Database System Review*, 1: CD000253.

Farrer, J. H., Walker, A. H. and Rajfer, J. (1985). Management of the postpubertal cryptorchid testis: a statistical review. *Journal of Urology,*134(6): 1071-1076.

Ferrero-Miliani, L., Nielsen, O. H., Andersen, P. S and Girardin, S. E. (2007). Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clinical Experimental Immunology,*147 (2): 061127015327006.

Figueiredo, J. C., Grau, M. V., Haile, R. W., Sandler, R. S., Summers, R. W., Bresalier, R. S., Burke, C. A., McKeown-Eyssen, G. E. and Baron, J. A. (2009). Folic acid and risk of prostate cancer: results from a randomized clinical trial. *Journal of the National Cancer Institute,*101 (6): 432–435.

Filho, D. W., Torres., M.A., and Bordin, A. L. (2004). Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. *Molecular Aspects of Medicine,* 25: 199-210.

Fisch, H., Laor, E. and Reid, R. E. (1998). Gonadal dysfunction after testicular torsion: luteinizing hormone and follicle-stimulating hormone response to gonadotropin releasing hormone. *Journal of Urology,* 139: 961-964.

Flaherty, J. Y. (1991). Ischaemia/ reperfusion injury. *American Journal of Medicine,* 91: 79S

Fosmire, G. J. (1990). Zinc toxicity. *American Journal of Clinical Nutrition,* 51(2): 225–227.

Franca, L. R., Suescun, M. O., Miranda, J. R., Giovambattista, A., Perello, M., Spinedi E. and Calandra, R. S. (2006). Testis structure and function in anongenetic hyperadipose rat model at prepubertal and adultages. *Endocrinology,* 147(3): 1556–1563.

Frank, J. D. and O'Brien, M. (2002). Fixation of the testis. *British Journal of Urology International,* 89(4, 5): 331-333.

Galejs L. E. and Kass. E . J. (1999). Diagnosis and treatment of the acute scrotum. *American Family Physician,* 15: 59(4): 817-824.

Garcia, J. J., Lopez-Pingarron, L., Almeida-Souza, P., Tres, A., Escudero, P., Garcia-Gil, F. A., Tan, D., Reiter, R. J., Ramirez, J. M. and Bernal-Perez, M. (2014). Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: a review. *Journal of Pineal Research,* 56(3): 225-237.

Goh, Y. I. and Koren, G (2008). Folic acid in pregnancy and foetal outcomes. *Journal of Obstetrics and Gynaecology,* 28 (1): 3–13.

Goldman, A. P., Williams, C. S., Sheng, H. Lamps, L. W., Williams, V. P., Pairet, M., Morrow, J. D. and DuBois, R. N. (1998). Meloxicam inhibits the growth of colorectal cancer cells, *Carcinogenesis,* Pp 2195–2199.

Gomendio, M., Martin-Coello, J., Crespo, C., Magana, C and Rolden, E. R. S. (2006). Sperm competition enhances functional capacity of mammalian spermatozoa. *Proceedings of National Academy of Sciences of the United States of America,* 103: 15113-15117.

Grace, P. A. (1994): Ischemia-reperfusion injury. *Brtish Journal of Surgery,* 81: 637-647.

Grahn, B. H., Paterson, P. G., Gottschall-Pass, K. T. and Zhang, Z. (2001). Zinc and the eye. *Journal of American College of Nutrition,* 20(2): 106–118.

Greenstein, A., Schreiber, L. And Matzkin, H. (2001). The effect of methylene blue on histological damage after spermatic cord torsion in a rat model. *British Journal of Urology International,* 88: 90–92.

Groff, J. L. and Gropper, S. A. (editors). *Advanced Nutrition and Human Metabolism.* 3. Belmont, Calif: West/Wadsworth; 2000. *Microminerals,* Pp. 401–470.

Guazzone,V. A., Jacobo, P., Theas, M. S. and Lustig, L. (2009). Cytokines and chemokines in testicular inflammation: a brief review. *Microscopy and Research Technique,* 72620–72628.

Hartung, T. G. and Dewsbury, D. A. (1978). A comparative analysis of copulatory plugs in muroid rodents and their relationship to copulatory behavior. *Journal of Mammals,* 59: 717–723.

Hashimoto, H., Ishikawa, T., Yamaguchi, K., Shiotani, M., Fujisawa, M., and Fujisawa, M. (2009). Experimental ischaemia-reperfusion injury induces vascular endothelial growth factor expression in the rat testis. *Andrologia,* 41: 216–221.

Head , J. R., Neaves, W. B. and Billingham, R. E. (1983). Immune privilege in the testis. *Basic Parameters of Allograft Survival,* 36: 423-429.

Hedger, M. P. (2011a). Immunophysiology and pathology of inflammation in the testis and epididymis. *Journal of Andrology,* 32: 6.

Hedger, M. P. (2011b). Toll-like receptors and signalling in spermatogenesis and testicular responses to inflammation—a perspective. *Journal of Reproduction Immunology*, 88: 130 –141.

Heindel, R. M., Pakyz, R. E., Reinking, L. N. and Cosentino, M. J. (1990). The effect of various degrees of unilateral spermatic cord torsion on fertility in the rat. *Journal of Urology,* 144: 366-369.

Hermo, L., and Robaire, B. (2002). Epididymal cell types and their functions. In: Robaire, B and Hinton B. T.(editors). *The Epididymis: From Molecules to Clinical Practice.* Kluwer Academic/Plenum Publishers, New York, Pp. 81–102.

Hoffbrand, A. V. and Weir, D. G. (2001). The history of folic acid. *British Journal of Haematology,* 113(3): 579–589.

Holma, R., Salmenpera, P. (2001). Acute effects of the cys-leukotriene-1 receptor antagonist,montelukast, on experimental colitis in rats. *European Journal of Pharmacology,* 429: 3009-30018.

Iwata, M., Takebayashi, T., Ohta, H., Alcalde, R. E., Itano, Y and Matsumura, T. (1999). Zinc accumulation and metallothionein gene expression in the proliferating epidermis during wound healing in mouse skin. *Histochemistry Cell Biolology,*112(4): 283–290.

Jackson, J. L., Lesho, E. and Peterson, C. (2000). Zinc and the common cold: a meta-analysis revisited. *Journal of Nutrition,* 130(5S): 1512S–1515S.

Jia, ZL., Li, Y., Chen, C., Li, S., Wang, Y., Zheng, Q. and Shi, B. (2010). Association among polymorphisms at MYH9, environmental factors, and nonsyndromic orofacial cleftsin western China. *DNA and Cell Biology,* 29 (1): 25–32.

Johnson, A. R., Munoz, A., Gottlieb, J. L and Jarrard, D. F. (2007). High dose zinc increases hospital admissions due to genitourinary complications. *Journal of Urology,* 177(2): 639–643.

Jones, C. Y., Tang, A. M., Forrester J. E., Castaneda-Sceppa, C., Bermudez, O. I. and Wanke, C. (2006). Micronutrient levels and HIV disease status in HIV-infected patients on highly active antiretroviral therapy in the Nutrition for Healthy Living cohort. *Journal of Acquired Immune Deficiency Syndrome,* 43(4): 475–482.

Jorge, G. M., Michael, L., Arnold. G. C., Of Native, E. and Sukhotnik (2005). Effect of diclofenac on germ cell apoptosis following testicular ischaemic-reperfusion injury in rats. *Paediatric Surgery International,* 22: 99-105.

Kadish, H. A. and Bolte, R. G. (1998). A retrospective review of pediatric patients with epididymitis, testicular torsion, and torsion of testicular appendages. *Paediatrics,* (1 Pt 1): 73-76.

Kamen, B. (1997). Folate and antifolate pharmacology. *Seminars in Oncology,* 24(5): 18.

Kar, A., Ozden, E., Yakupoglu, Y. K., Kefeli, M., Sarikaya, S. and Yilmaz, A. F. (2010) Experimental unilateral spermatic cord torsion: the effect of polypolymerase enzyme inhibitor on histopathological and biochemical changes in the early and late periods in the ipsilateral and contralateral testicles. *Urology,* 76(2): 507.e1-5.

Kehinde, E. O., Anim, J. T., Mojiminiyi, O. A., Al-awadi, F., Omu, A. E. and Vanghese, R. (2005). Significance of determining the point of reperfusion failure in experimental torsion of testis. *International Journal of Urology,* 12: 81-89.

Kelly, K. J., Williams, W. W. Jr., Colvin, R. B., Meehan, S. M., Springer, T. A., Gutierrez-Ramos, J. C. and Bonventre, J. V. (1996). Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *Journal of Clinical Investigation,* 97: 1056-1063.

King, J. C. Zinc. In: Shils, M. E and Shike, M. (editors). *Modern Nutrition in Health and Disease.* 10. Philadelphia, Pa: Lippincott Williams & Wilkins; 2006; Pp. 271–285.

Klaasen, Z. W. A. (2011). In: *Male Reproductive organs Anatomy.* Htt:// emedicine.medscape.com/article/1899075-Overview Retrieved on July 13, 2011.

Koseoglie, B. Yilmaz, E., Ceylan, K., Uzun, E., Bayram, I. and Hizh, F. (2009). The protective effect of erythropoietin on testicular torsion/detorsion: an experimental study. *International Journal of Urology,* 41: 85-91.

Kurugöl, Z., Akilli, M., Bayram, N. and Koturoglu, G. (2006). The prophylactic and therapeutic effectiveness of zinc sulphate on common cold in children. *Acta Paediatrica,* 95(10): 1175–1181.

Lambeau, G. and M. Lazdunski. (1999). Receptors for a growing family of secreted phospholipases A2. *Trends in Pharmacological Science,* 20: 162–170.

Leitzmann, M. F., Stampfer, M. J., Wu, K., Colditz, G. A., Willet, W.C., and Giovannucci E. L. (2003). Zinc supplement use and risk of prostate cancer. *Journal of National Cancer Institute*, 95(13): 1004–1007.

Lee, C. and Holland, J. M. (1987). Anatomy, histology and ultrastructure correlation with function, prostate, rat. In: Jones, T. C., Mohr, U. and Hunt R. D. (editors). *Monographs on Pathology of Laboratory Animals, Genital system*pp. Springer, Berlin, pp 239–251.

Lin, W. W. and Karin, M.A. (2007). Cytokine-mediated link between innate immunity, inflammation, and cancer. *Journal of Clinical Investigation,* 117: 1175–1183.

Lukacik, M., Thomas, R. L and Aranda, J. V. (2008). A meta-analysis of the effects of oral zinc in the treatment of acute and persistent diarrhea. *Paediatrics,* 121(2): 326–336.

Luke, M. A. and Coffey, D. S. (1994). The male accessory sex tissues. In: *The Physiology of Reproduction*. Knobil, E and Neill, G. D. (editors). Vol. 1, Raven Press, New York, Pp 1435–1488.

Lysia, J. J., Nguyen, Q. A. and Turner, T.T. (2002). Peptide and nonpeptide reactive oxygen scavengers provide artificial rescue of the testis after torsion. *Journal of Andrology,* 23: 400-409.

Mabogunje, O. A (1986). Testicular torsion and low relative humidity in a tropical country. *British medical journal (Clinical Research Edition),* 292(6517): 363.

Macknin, M. L., Piedmonte, M., Calendine, C., Janosky, J. and Wald, E. (1998). Zinc gluconate lozenges for treating the common cold in children: a randomized controlled trial. *Journal of medical association of America,* 279(24): 1962–1967.

Malinow, M. R. (1995). Plasma homocyst(e)ine and arterial occlusive diseases: A mini-review. *Clinical Chemistry,* 41(1): 173–176.

Mangoni, A. A., Arya, R., Ford, E., Asonganyi, B., Sherwood, R. A, Ouldred, E., Swift, C. G. and Jackson S. H. (2003). Effects of folic acid supplementation on inflammatory and thrombogenic markers in chronic smokers. A randomised controlled trial. *Thrombosis Research,* 110(1): 13-17.

Martini, F. H. and Nath, J. L . (2009). The reproductive system. In: Fundamentals of Anatomy and Physiology. Pearson,United States of America, 8th edition, pp 1045.

McGuire, M., Cleary, B., Sahm, L. and Murphy, D. J. (2010). Prevalence and predictors of periconceptional folic acid uptake—prospective cohort study in an Irish urban obstetric population. *Human Reproduction,* 25 (2): 535–453.

Mfady, D. S., Sadiq, M. F., Khabour, O. F., Fararjeh, A. S., Abu-Awad, A. and Khader, Yousef. (2014). Associations of variants in *MTHFR and MTRR* with male infertility in the Jordanian population. *Gene,* 536: 40-44.

Milei, J., Ferreira, R. and Llesuy, S. (1992). Reduction of reperfusion injury with preoperative rapid intravenous infusion of taurine during myocardial revascularization. *American Heart Journal,* 123: 339-345.

Milunsky, A., Jick, H., Jick, S. S., Bruell, C. L., MacLaughlin, D. S., Rothman, K. J. and Willett, W. (1989). Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. *Journal of the American Medical Association,* 262 (20): 2847–2852.

Minutoli, L., antonyccio, P., Romeo, C., Nicotina, P.A., Bitto, A., Arena, S., Polito, F., Altavilla, D., Turiaco, N., Cutrupi, A., Zuccarello, B. and Squadrito, F. (2005). Evidence for a role of nitrogen activated kinase3/nitrogen activated kinase in the development of testicular ischaemic kinase in the development of testicular ischaemic reperfusion injury. *Biology and Reproduction,* 73(4): 730-736.

Mocchegiani, E., Santarelli, L., Muzzioli, M. and Fabris, N. (1995). Reversibility of the thymic involution and of age-related peripheral immune dysfunctions by zinc supplementation in old mice. *Internatinal Journal of Immunopharmacology,* 17(9): 703–718.

Moon, C., Kim, J. S and Jang, H. (2008). Activation of Akt/protein kinase B and extracellular signal-regulated kinase in rats with acute experimental testicular torsion. *Journal of Veterinary Medical Science,* 70: 337-341.

More, K. L. and Dally, A. T (1999). Clinical oriented Anatomy (4th Edition). Lippincut Milliams and Williams; a Woller Klumner corporation, Philadelphia, pp 263 – 271

More, K. L. and Dally, A. T. (2006). Clinical oriented Anatomy (4th Edition). Lippincut Milliams and Williams; a Woller Klumner corporation, Philadelphia pp 405 –406, 453-456

Mossad, S. B. (2003). Effect of zincum gluconicum nasal gel on the duration and symptom severity of the common cold in otherwise healthy adults. *Quarterly Journal of Medicine,* 96(1): 35–43

Mulinare, J., Cordero J. F., Erickson, J. D. and Berry, R. J. (1988). Periconceptional use of multivitamins and the occurrence of neural tube defects. *Journal of the American Medical Association,* 260 (21): 3141–3145.

Milunsky, A., Jick, H., Jick, S.S., Bruell, C. L., MacLaughlin, D. S., Rothman, K. J. and Willett. W. (1989). Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. *Journal of the American Medical Association,* 262(20): 2847–2852.

Nakanishi, M. and Rosenberg, D. W. (2013). Multifaceted roles of PGE_2 inflammation and cancer. *Seminars in immunopathy,* 35(2): 123-137.

National Academy Press (2001). Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. Washington, DC

Noske, H. D., Krau, S. W., Altinkilic, B. M. and Weidner, W. (1998).Historical milestones regarding torsion of the scrotal organs. *Journal of Urology*,159: 13-16.

Oyeyemi, M. O., Towobola, B. A. and Ola-Davies, O. E. (2006).The Reproductive implication of Clomiphene citrate on sperm cells during the epididymal transit of spermatozoa in male Wistar rats. *Folia Veterinaria,* 50(3): 131-133.

Oyeyemi, M. O. and Ubiogoro, O. (2005). Spermiogram and morphological characteristics in testicular and epididymal spermatozoa of Large White boar in Nigeria. *International Journal of Morphology,* 23 (3): 235-239.

Pavlov,V. A., Wang, H., Czura., Friedman, S. G. and Tracey, K. J. (2003). The cholinergic anti-inflammatory pathway: A missing link in neuroimmunomodulation. *Molecular Medicine,* 9(5-8): 125-134.

Popa, C., Netea, M. G.,van Riel, P. L. M., van der Meer, J. W. M. and Stalenhoef, A. F. H. (2007). The role of TNF-α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *The Journal of Lipid Research,* 48: 751-762.

Pochon, S. T., Wright, P. C., Schaentzter, E., Ippolito, M., Rakonirina, G. and Rakotosoa, R. (2002). Effect of season and age on Gonado-somatic index of Milne Ewards' silfakas (*Propithecus diadema Edwards*) in Ranomafana National Park, Madagascar. *International Journal of Primatology,*53(2): 355-364.

Prasad A. S., Fitzgerald J. T., Bao, B. and Beck, F. W (2000). Chandrasekar PH. Duration of symptoms and plasma cytokine levels in patients with the common cold treated with zinc acetate. A randomized, double-blind, placebo-controlled trial. *Annals of International Medicine,* 133(4): 245–252.

Prasad, A. S., Beck, F. W., Bao, B., Fitzgerald, J. T., Snell, D. C., Steinberg, J. D. and Cardozo, L. J. (2007). Zinc supplementation decreases incidence of infections in the elderly: effect of zinc on generation of cytokines and oxidative stress. *American Journal Clinical Nutrition,* 85(3): 837–844.

Prasad, A. S., Bao, B., Beck, F.W.J. and Sarker, F. H. (2011). Zinc-suppressed inflammatory Cytokines by induction of A20-mediated inhibition of nuclear factor-Kb. *Nutrition,* 27(7-8): 816-823.

Prasad, A. S., Beck, F. W. J., Bao, B., Fitzgerald, J. T., Snell, D.C., Steinberg, J. D. and Cardozo, L.J. (2007). Zinc supplementation decrease incidence of injections in the elderly: effect of zinc on generation of cytokines and oxidative stress. *American Journal of Clinical Nutrition,* 85(3): 837-844.

Puri, P., Barton, D and O'Donnell, B. (1985). Prepubertal testicular torsion: subsequent fertility. *Journal of Paediatric Surgery*, 20(6): 598-601.

Raghuvanshi, P., Mathur, P., Sethi, P. and Bhatnagar, P. (2012). Evaluation of the changes in sperm morphology,sperm count and Gonadotrophic ratio of Swiss Albino male mice fed continuously with micro wave exposed food. *Advances Bioresearch,* 3(4): 95-99.

Raigani, M., Yaghmaei, B., Amirjannti, N., Lakpour, W., Akhondi, M. M., Zeraati, H., Hajihosseinal, M. and Sadeghi, M. R. (2013). The micronutrient supplements, Zinc sulphate and folic acid did not ameliorate sperm functional parameters in oligoasthenoteratozoospermic men. *Andrologia,* DOI: 10.1111/and.12180.

Reuter, S., Gupta, S. C., Chaturvedi, M. M. and Aggarwal B. B. (2010). Oxidative stress,inflammation and cancer: how are they linked? *Free Radical biology and medicine,* 49(11): 1603-1616.

Ringdahl, E. and Teague, L. (2006). Testicular Torsion. *American Family Physician,* 15; 74(10): 1739-1744.

Rodriguez, C. M., Kirby, J. L. and Hinton, B. T. (2002). The development of the epididymis. In: Robaire, B and Hinton, B. T. (editors). *The Epididymis: From Molecules to Clinical Practice.* Kluwer Academic/Plenum Publishers, New York, pp 251–268

Rojas, A. I. and Phillips, T. J. (1999). Patients with chronic leg ulcers show diminished levels of vitamins A and E, carotenes, and zinc. *Dermatological Surgery,* 25(8): 601–604.

Romeo, C., Antonuccio, P., Esposito, M., Marini, H., Impellizzeri, P., Turiaco, N., Altavilla, D., Bitto, A., Zuccarello, B. and Squadrito, F. (2004). Raxofelast, a hydrophilic vitamin E-like antioxidant, reduces testicular ischemia-reperfusion injury. *Urological research*, 32(5): 367-71.

Roth, C. C., Mingin, G. C. and Ortenberg, J. (2011). Salvage of bilateral asynchronous perinatal testicular torsion. *Journal of Urology*, 185(6): 2464-2468.

Saper, R.B. and Rash, B. (2009). Zinc: An essential micronutrient. *American Family Physician,* 1;79(9): 768.

Schneck, F. X and Bellinger, M. E. (2007). Abnormalities of the testis and scrotum and their surgical management. In: Wein, A. J., Kavoussi, L. R., Novick, A. C, Partin, A. W and Peters, C. A. (editors). *Campbell- Walsh Urology*. 9th edition. Chapter 127. Philadelphia, Pa: WB Saunders.

Scholl, T. O. and Johnson, W. G. (2000).Folic acid: influence on the outcome of pregnancy. *The American journal of clinical nutrition,* 71(5): 1295S–1303S.

Seng, Y. J., and Moissinac, K. (2000). Trauma induced testicular torsion. A reminder for the unwary. *Journal of Accident and Emergency Medicine.* 17: 381-382.

Setchell, B. P., Maddocks, S and Brooks, D. E. (1988). Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: knobil, E and Neill, J. D. (editors). *The Physiology of Reproduction.* Vol. 1, 2nd edition. Raven Press, New York, pp. 1063–1176.

Shaw, G. M., Schaffer, D., Velie, E. M., Morland, K. and Harris, J. A. (1995). Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. *Epidemiology,* 6 (3): 219–226.

Shiva, N., Kazem, P., Albolhasan, A., Mansoureh, M., Mahdavi, V. and Reza, M. (2014). Protective effect of melatonin against inequality-induced damages on testicular tissue and sperm parameters. *International journal of fertility and sterility,*7(4): 313-322.

Shuppe, H. Meinhardt, A. and Markert, U. R. (2005). Immunology of Gametes and Embryo Implantation. *Chemical Immunology and Allergy,* Basel, Karger, Volume 88, pp 1-14.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology,* 82(2): 291–295.

Solini, A., Santini, E. and Ferrannini, E. (2006). Effect of short-term folic acid supplementation on insulin sensitivity and inflammatory markers in overweight subjects. *International Journal of Obesity,* 30(8): 1197-1202.

Steegers-Theunissen, R. P., Obermann-Borst, S. A., Kremer, D., Lindemans, J., Siebel, C., Steegers, E. A., Slagboom, P. E. and Heijmans, B. T. (2009). Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. PloS one 4 (11): e7845. doi:10.1371/journal.pone.0007845.

Sun, J., Liu, G. H., Zhao, H. T. and Shi, C. R. (2006).Long-term influence of prepubertal testicular torsion on spermatogenesis. *Urology International*, 77(3): 275-278.

Sung, Y., Scott, W., Leonard, Maret., G. Traber and Emily, I. (2009). Zinc deficiency affects DNA damage oxidative stress, antioxidant defense and DNA repairs. *Journal of Nutrition*, 139: 9125-1631.

Suwa, T., Nyska, A., Peckham, J. C., Hailey, J. R., Mahler, J. F. and Haseman, J. K. (2001). A retrospective analysis of background lesions and tissue accountability for male accessory sex organs in Fischer-344 rats. *Toxicological Pathology,* 29: 467–478.

Suwa, T., Nyska, A., Haseman, J. K., Mahler, J. F. and Maronpot, R. R. (2002). Spontaneous lesions in control B6C3F1 mice and recommended sectioning of male accessory sex organs. *Toxicological Pathology,* 30: 228–234.

Szewczyk, B., Kubera, M., and Nowak, G. (2011). The role of Zinc in neurogenerative inflammatory pathways in depression. *Progress in neuro-psychopharmacology and Biological psychiatary,* 3: 693-701.

Tan, J. S., Wang, J. J., Flood, V., Rochtchina, E., Smith, W. and Mitchell, P. (2008). Dietary antioxidants and the long-term incidence of age-related macular degeneration: the Blue Mountains Eye Study. *Ophthalmology,*115(2): 334–341.

The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The alpha-Tocopherol beta carotene cancer Prevention Study Group (1994). *New England Journal of Medicine*, 330(15): 1029–1035.

Timbo, B. B., Ross, M. P., McCarthy, P. V and Lin, C. T. (2006). Dietary supplements in a national survey: prevalence of use and reports of adverse events. *Journal of American Diet Association,* 106(12): 1966–1974.

Tong, L., Li, J. and Qiao, H. (2006) .Taurine protects agains ischemia-reperfusion injury in rabbit livers. *Transplant Proceedings,* 38: 1575-1579.

Turner, R. B. and Cetnarowski, W. E. (2000). Effect of treatment with zinc gluconate or zinc acetate on experimental and natural colds. *Clinical Infectious Diseases*, 31(5): 1202–1208.

Turner, T. T. and Brown, K. J. (1993). Spermatic cord torsion: loss of spermatogenesis despite return of blood flow. *Biology and Reproduction,* 49: 401-407.

Turner, T. T., Bang, H. J. and Lysiak, J. J. (2005) Experimental testicular torsion: reperfusion blood flow and subsequent testicular venous plasma testosterone concentrations. *Urology,* 65(2): 390–394.

Turner, T. T., Lysiak, J. J., Shannon, J. D., Nguyen, Q. A. and Bazemore-Walker, C. R. (2006). Testicular torsion alters the presence of specific proteins in the mouse testis as

well as the phosphorylation status of specific proteins. *Journal of Andrology*, 27(2): 285–293.

Turner, T.T., Tung, K.S and Tomomasa, H. (1997). Acute testicular ischemia results in germ cell-specific apoptosis in the rat. *Biology and Reproduction,* 57: 1267-1274.

Ugwu, B. T., Dakura, N. K., Yiltok, S. J., Mbah, N., and Legbo, J. N . (2003). Testicular torsion in the Jos, Plateau.*West African Journal of Medicine,* 22(4-6): 2.

Villamor, E., Aboud, S. and Koulinska, I. N. (2006) . Zinc supplementation to HIV-1-infected pregnant women: effects on maternal anthropometry, viral load, and early mother-to-child transmission. *European Jornal Clinical Nutrition,* 60(7): 862–869.

Visser, A. J. and Heyns, C. F. (2003) . Testicular function after torsion of the spermatic cord. *British Journal International,* 92(3): 200-203.

Walravens, P. A., Hambidge, K. M. and Koepfer, D. M. (1989). Zinc supplementation in infants with a nutritional pattern of failure to thrive: a double-blind, controlled study. *Paediatrics,* 83(4): 532–538.

Wananukul, S., Limpongsanuruk, W., Singalavanija, S. and Wisuthsarewong, W. (2006). Comparison of dexpanthenol and zinc oxide ointment with ointment base in the treatment of irritant diaper dermatitis from diarrhea: a multicenter study. *Journal of Medical Association of Thailand,* 89(10): 1654–1658.

Weinstein, S. J. (2003). Null association between prostate cancer and serum folate, Vitamin B6, Vitamin B12, and Homocysteine. *Cancer Epidemiology, Biomarkers & Prevention,* 12: 1271–1272.

Wilcox, A. J., Lie, R. T., Solvoll K., Taylor J., McConnaughey, D. R., Abyholm, F., Vindenes, H. and Vollset, S. E (2007). Folic acid supplements and risk of facial clefts: national population based case-control study. *British Medical Journal),* 334 (7591): 464.

Wilkinson, E. A. and Hawke, C. (1998). Does oral zinc aid the healing of chronic leg ulcers? A systematic literature review. *Archives of Dermatology*, 134(12): 1556–1560.

Wilton, D. C. and Foureur, M. J. (2010). A survey of folic acid use in primigravid women. Women and birth. *Journal of the Australian College of Midwives,* 23 (2): 67–73.

Wolter, J. M., Rodwell, R. L., Bowler, S. D. and McCormack, J. G. Cytokines and mediators do not indicate acute infection in cystic fibrosis. *Clinical and Diagnostic Laboratory immunology,* 6(2): 260-265.

World Health Organisation (2010) laboratory manual for the examination and processing of human semen - 5th edition, WHO Press, Geneva.

World Health Organization (2002). The World Health Report, 2002: Reducing Risks, Promoting Healthy Life. Geneva, Switzerland: World Health Organization; 2002.

Yamagachi. S., Miura, C. , Kikuchi, K., Celino. F. T., Agusa. T., Tanade, T. Miura. and T. (2009). Zinc is essential trace element for spermatogenesis. *Proceedings of National Academy of Science of the United States of America,*106: 10859.

Young, S. S., Eskenazi, B., Marchetti, F. M., Block, G. and Wyrobek, A. J. (2008). The association of folate, zinc and antioxidant intake with sperm aneuploidy in healthy non-smoking men. *Human Reproduction,* 23 (5): 1–9.

Zelova, H. and Hosek, J. (2013). TNF-α signalling and inflammation: interactions between old acquaintances. *Inflammation Research,* 62(7): 641-651.

Zimmerman, B. J. and Granger, D.N. (1994). Mechanisms of reperfusion injury. *American Journal of Medical Science,* 307: 284-292.

Zittoun, J. (1993). Anemias due to disorder of folate, vitamin B12 and transcobalamin metabolism. *La Revue du praticien,* 43 (11): 1358–1363.