IMPACT OF ELEMENTAL IRON ON HUMAN SPERMATOZOA AND MOUSE EMBRYONIC DEVELOPMENT IN VITRO IN A DEFINED SYNTHETIC PROTEIN-FREE CULTURE SYSTEM

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ORIGINAL LITERARY WORK DECLARATION

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ABSTRACT

The element iron is essential for life and plays a number of key roles in biological processes and it is involved in cell development, some of which are intimately related to spermatogenesis and spermatozoa metabolism. Iron and its compounds within certain limits are not fundamentally toxic for the human organisms. However, the role of iron in infertility has not been widely investigated. Infertility, which known as a failure to achieve conception after one year of regular unprotected sexual intercourse, affects 10–15% of couples and approximately 80 million couples worldwide. The common causes of infertility include male factors, female factors, and unexplained causes. The rate of male factors is 30% to 40%, female factors 40%, and unexplained causes 10% of infertility cases.

This study aims to ascertain the optimal level of iron needed for optimal embryonic development (Spermatozoa and embryos) in protein free culture media, and toxic levels to enable its application in assisted reproductive technologies to help increase the pregnancy rates. Different levels of ferric iron concentrations were prepared using HEPES and protein- free culture media. The levels of ferric iron investigated closely resembled the normal range for human plasma iron (0.6-1.7 µg/ml). These levels were investigated to determine the optimal and tolerance levels of iron for human spermatozoa. Ferric chloride was used as source of iron in the HEPES-buffered synthetic protein-free medium. Normozoospermic semen samples (n=24) were incubated with different concentrations of iron (0.5, 1, 1.5, 2, 4, 8, 12, and 16 µg/ml) in HEPES buffered protein-free culture medium at room temperature for 1hr and 20hrs. Motility and vitality of spermatozoa were measured according to WHO Manual 2010. Spermatozoa activity and sperm DNA integrity were evaluated at room temperature at 1hr and 20hrs. 2.0 µg/ml (35.8 µM) of ferric iron was the optimal level of ferric iron

with range of tolerance levels (0.5- 2 µg/ml) which appeared beneficial for spermatozoa motility at 1hr and up to 20hrs. Levels above 4.0 µg/ml were toxic to human spermatozoa. Days 1-4 Quakenbush Special (Qs) mouse embryos were recovered (n=954) from stimulated females, pooled and randomly apportioned for individual treatments of different concentrations of ferric chloride (2, 5, 10, 20 and 50 µM for tolerance study and 100, 200, 300, 400 and 500 µM/L for toxicity study). The investigation on iron toxicity utilized 206 day 2 Qs mouse embryos. Medium supplemented with iron (2 to 50 µM/L of ferric chloride) had higher percentage of blastocysts than control in all treatments. In contrast, the percentages of blastocysts were lower than control in another experiment performed to investigate iron toxicity which contained 100 to 500 µM/L.

This study found that ferric iron at physiological levels of human plasma appears to enhance spermatozoa motility, preserve its DNA integrity and may increase percentage of blastocysts developed and embryo development because of its critical role in cell proliferation. However, increasing the concentrations of ferric iron above the physiological levels had harmful effects on spermatozoa motility, sperm DNA integrity and blastocysts development.

ABSTRAK

Zat besi merupakan elemen penting untuk hidup dan memainkan beberapa peranan penting dalam proses biologi dan ianya terlibat dalam perkembangan sel, berkait rapat dengan sintesis dan metabolisma sperma. Besi dan sebatiannya dalam batasan tertentu tidak bertoksik untuk organisma manusia. Walau bagaimanapun peranan zat besi dalam kemandulan tidak disiasat secara meluas. Kemandulan, yang dikenali sebagai kegagalan untuk mencapai konsepi selepas satu tahun hubungan seks tanpa perlindungan, memberi kesan kepada 10-15% daripada pasangan dan kira-kira 80 juta pasangan di seluruh dunia. Punca biasa ketidaksuburan termasuk faktor-faktor lelaki, faktor wanita, dan sebab-sebab yang tidak dapat dijelaskan. Kadar faktor lelaki adalah 30% kepada 40%, faktor perempuan 40%, dan sebab-sebab yang tidak dapat dijelaskan 10% daripada kes-kes kemandulan. Zat besi dan sebatiannya dalam had tertentu adalah pada asasnya tidak toksik untuk organisma manusia.

Kajian ini bertujuan untuk menentukan tahap optima zat besi yang diperlukan untuk perkembangan optima embrionik (spermatozoa dan embrio) dalam media kultur tanpa protin dan tahap toksik untuk membolehkan aplikasi nya dalam teknologi pembiakan dibantu untuk membantu meningkatkan kadar kehamilan. Tahap kepekatan besi ferik yang berbeza telah disediakan dengan menggunakan HEPES media kultur tanpa protin HEPES. Tahap besi ferik disiasat hampir menyerupai julat normal untuk zat besi plasma manusia (0.6- 1.7 μg/ml). Tahap-tahap ini telah disiasat untuk menentukan tahap optima dan toleransi zat besi untuk spermatozoa manusia. Ferik klorida digunakan sebagai sumber zat besi dalam medium HEPES sintetik tanpa protin. Sampel air mani normozoospermic (n=24) telah dieram dengan kepekatan zat besi yang berbeza dalam media kultur tanpa protin sintetik HEPES pada suhu bilik selama 1j dan 20 j. Motiliti dan kecergasan sperma telah diukur menurut manual WHO 2010. Aktiviti sperma dan

integriti DNA sperma telah dinilai pada suhu bilik selama 1 j dan 20 j. Kajian ini mendapati 2.0 μg / ml (35.8 μM) adalah tahap optima besi ferik dengan pelbagai tahap toleransi (0.5- 2 μg /ml) yang muncul bermanfaat untuk motiliti sperma pada 1 j dan 20 j. Tahap melebihi 4.0 μg/ml adalah toksik kepada sperma manusia. Embrio tikus "Quakenbush special" (Qs) pada hari 1-4 diperolehi (n = 954) dari tikus betina yang dirangsang, dikumpulkan dan dibahagikan secara rawak untuk rawatan individu. Sebanyak 206 embrio tikus perkembangan hari kedua digunakan untuk siasatan ke atas ketoksikan zat besi. Media ditambah dengan zat besi (2 hingga 50 μM/L ferik klorida) mempunyai peratusan blastosis yang lebih tinggi berbanding kawalan dalam semua kajian toleransi zat besi manakala peratusan blastosis adalah lebih rendah daripada kawalan dalam eksperimen lain yang dilakukan untuk menyiasat keracunan zat besi yang mengandungi 100 hingga 500 μM/L ferik klorida.

Kajian ini menunjukkan bahawa besi ferik pada tahap fisiologi yang dijumpai di dalam plasma manusia muncul untuk meningkatkan motiliti sperma, mengekalkan integriti DNA nya dan boleh meningkatkan peratusan perkembangan blastosis dan perkembangan embrio kerana peranan pentingnya dalam pertumbuhan sel. Walau bagaimanapun, peningkatan kepekatan besi ferik melebihi tahap fisiologi mempunyai kesan berbahaya pada motiliti sperma, integriti DNA sperma dan perkembangan blastosis.

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LIST OF SYMBOLS AND ABBREVIATIONS

AAS : Atomic Absorption Sperctrophotometer

ART : Assisted Reproductive Technology

ATP : Adenosine Triphosphate

^{o}C : Degree Celsius

CAT : Catalase

Cdk : Cyclin-dependent kinase

cECM : conventional Embryo Culture Media

CO_2 : Carbon dioxide

Cu_2 : Copper

Dcyt B : Duodenal cytochrome B :

dmfm : Drosophila melanogaster mitoferrin gene

DMT1 : Divalent Metal Transporter

DNA : Deoxyribonucleic Acid

DSP : Daily Spermatozoa Production

Fe^{+2} : Ferrous Iron

Fe^{+3} : Ferric Iron

$FeCl_3$: Ferric Chloride

$FeCL_3.6H_2O$: Ferric Chloride Hexahydrate

$FeSO_4.7H_2O$: Ferrous Sulfate Heptahydrate

GSH : Glutathione

hCG : Human Chorionic Gonadotropin

HCL : Hydrochloric Acid

HH : Hypogonadotropic Hypogonadism

•HO : Hydroxyl radicals

H_2O : Dihydrogen Monoxide

H_2O_2 : Hydrogen peroxide

HPG : Hypothalamic Pituitary Gonadal

HSA : Human Serum Albumin

ICP-MS : Inductively Coupled Plasma Mass Spectrometry

IRP1 : Iron Regulatory Protein1

IRP2 : Iron Regulatory Protein 2

mRNA : messenger Ribonucleic acid

MW : Molecular Weight

NADPH : Nicotinamide Adenine Dinucleotide Phosphate

O_2^- : Superoxide

OS : Oxidative Stress

PCOS : Polycystic Ovary Syndrome

PMSG : Pregnant Mare's Serum Gonadotropin

Ppb : Part per Billion

QS : Quakenbush Strain

ROO : peroxyl

ROS : Reactive Oxygen Species

SA : Serum Albumin

SCD : Sperm Chromatin Dispersion Assay

SDF : Sperm DNA Fragmentation

SDI : Spermatozoa DNA Integrity

SEM : Standard Error of Mean

SO : Swiss Outbred

SOD : Superoxide Dismutase

TfR1 : Transferrin Membrane Receptor 1

Tf : Transferrin

WHO : World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The element iron is essential for life. It plays a number of key roles in biological processes such as cellular respiration, electron transport, energy production, gas sensing, DNA synthesis, cell division and proliferation (Tverd et al., 2015b; Gozzelino & Arosio, 2016; Keller et al., 2016) However, for most living organisms, iron in excess is toxic, and iron deficiency is also a general problem in biology. As a result, the steady state level of the iron should be maintained within a range that prevents damage, but ensures sufficient iron supply for biosynthetic pathways in the cytoplasm and mitochondria (i.e. cell development). Although the importance of iron in cell development in general is well documented, the role of iron in infertility has not been widely investigated (Chavarroet al., 2006)

Infertility affects 10–15 % of couples and approximately 80 million couples worldwide (Gvozdjakova et al., 2015). In general, infertility is known as the failure to achieve conception after one year of regular sexual activity (Koroma & Stewart, 2012). The common causes of infertility include male factors, female factors, and unexplained causes. However, main causes were spermatozoa abnormalities, ovulation dysfunction, and fallopian tube obstruction (Hull et al., 1985). Male factor account for 30% to 40% of infertility (Marzec-Wróblewska et al., 2011; Gvozdjakova et al., 2015), female factor 40%, and unexplained causes 10% of infertility cases (Fritz & Speroff, 2011).

Lifestyle has an impact on fertility and pregnancy outcomes (Sharma et al., 2013). While, the most prevalent nutritional deficiency throughout the world is iron deficiency (Chavarro et al., 2006), and severe iron deficiency has a significant effect on fertility, and may be an important factor in unexplained infertility (Li et al., 2014). Moreover, iron supplements were associated with a lower rate of iron deficiency among women of reproductive age (Cogswell et al., 2003).

The role of iron in preimplantation development has not been fully elucidated. Also it is not clear whether the amounts of iron present in embryo culture media both the synthetic protein-free culture media (PFM) and commercial conventional embryo culture media (cECM) are sufficient for maintain normal embryo development in vitro. It is crucial to determine whether cECM/PFM needs to be supplemented with elemental iron to enhance the development and viability of the spermatozoa and embryo in vitro.

It was shown that iron depletion leaves harmful effects on cell proliferation in culture (Renton & Jeitner, 1996; Fu & Richardson, 2007; Nurtjahja-Tjendraputra et al., 2007). DNA synthesis was observed in the oligodendrocyte cell line [Cell line ref. N20.1] occurs after addition of ferric but not ferrous iron in the presence of apotransferrin (Silvestroff , 2013). It was also noted that the addition of ferrous iron in the deficiency of apotransferrin was harmful to cell proliferation. In relation to this, it was suggested that ferric iron has a beneficial effect on cellular processes in vitro. In addition, it is logical to say that iron is also needed for embryo development in vitro. According to Mojic (2014), cell culture medium supplemented with iron at physiological levels comparable to human plasma could prevent the accumulation of hydrogen peroxide (H_2O_2) which was one of the reactive oxygen species (ROS) involved in oxidative stress (OS) development.

The total iron concentration in human plasma is in the range of 0.6- 1.7 µg/ml (10–30 µM) (Ganz & Nemeth, 2011) and in order to mimic the physiological level of iron in plasma, this study was performed by using ferric iron (trivalent iron) because ferric iron is considered to be the physiological ion form for the organism. Tvrda et al. (2015a) suggested that ferric iron (Fe^{+3}) was less toxic than ferrous iron (Fe^{+2}). Furthermore, ferrous iron displays its deleterious effects in a shorter period of time and ferric iron takes more time to be reduced in vitro.

Ferric iron was used in the present study as a source of in vitro iron supplementation. Different concentrations of iron in Protein-Free Culture media (PFM) was prepared to investigate the effect of ferric iron on human spermatozoa activity and sperm DNA integrity and mice embryo development. Protein-Free Culture media (PFM) is devoid of Serum Albumin (SA). Protein-Free Culture media (PFM) has proven to be more efficient for human Assisted Reproduction Treatment (ART) with a pregnancy rate better than of conventional Embryo Culture Media (cECM). Being devoid of biological supplements it is thought to prevent disease transmission. The objectives of this were:

1- To assess the level of iron in PFM in order to know the exact amount of contaminated iron and thence to prepare the experimental concentrations of iron accordingly.

2- To determine the in vitro optimal concentration and tolerance concentrations of iron for spermatozoa function and embryo development.

3- To determine the in vitro toxic level of iron that affects spermatozoa viability and embryo development.

4- To investigate the effects of iron on the spermatozoa activity by measuring the motility, vitality and DNA integrity.

Although iron plays crucial roles in mammalian physiology, very little work has been conducted on iron supplementation in embryo culture media, and very little is known about the roles of or need for iron in spermatozoa physiology and its impact on fertilization and subsequent embryo development in vitro.

CHAPTER 2: LITERATURE REVIEW

Iron and its compounds are not fundamentally toxic to the human organism (Marzec-Wroblewska et al., 2011). Even so, prolonged intake of high doses of iron or in some pathological conditions regulative absorption mechanism disorders and a wide range of mechanism disturbances can occur (Defrere et al., 2008). In addition, iron deficiency minimizes the activity of iron-containing and iron-dependent enzymes (Mudron et al., 1996).

Elevated concentrations of iron during the cell development are harmful because iron can produce free radicals through Fenton reactions i.e. iron will become highly toxic. Consequently, it is natural that organisms developed complex routes to import, chaperone, sequester and export this metal ion. Iron is able to catalyze the propagation of reactive oxygen species which will then contribute to the generation of highly reactive radicals through Fenton reaction which leads to tissue damage.

Iron is a cofactor for several key enzymes in cellular respiration and metabolism, including enzymes of the citric acid cycle, and also ribonucleotide reductase. The latter enzyme catalyzes the reduction of ribonucleotides to deoxyribonucleotides, which is the rate-limiting step in DNA synthesis (Heath et al., 2013). Iron deficiency causes G1/S arrest and apoptosis (Yu et al., 2007). The mechanisms responsible for G1/S arrest and apoptosis after Fe‐depletion have been have been investigated. These studies revealed a multitude of cell cycle control molecules which are regulated by iron. These include p53, p27Kip1, cyclin D1 and cyclin‐dependent kinase 2 (cdk2). Additionally, iron‐depletion up‐regulates the mRNA levels of the cdk inhibitor, p21CIP1/WAF1, but paradoxically down‐regulates its protein expression. This effect can contribute to the apoptosis observed after iron‐depletion. Iron-depletion also leads to proteasomal

degradation of p21CIP1/WAF1 and cyclin D1 via an ubiquitin‐independent pathway. Under some conditions of iron deprivation, a G2/M arrest has also been identified (Renton & Jeitner, 1996). This means iron is required for macromolecule biosynthesis, necessary for cell growth and division (Fig. 2.1).

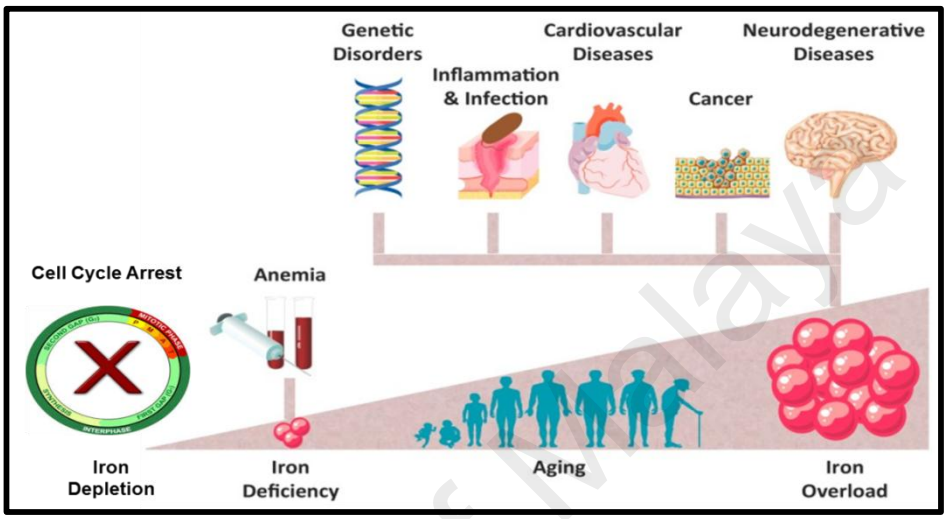

Figure 2.1: The importance of iron in pathophysiologic conditions. Iron is essential to ensure the survival. Many of pathophysiological conditions occur depending on the disruption of iron homeostasis, which include iron-overload related disorders, anemia, and cell cycle arrest in case of iron depletion. (Illustration modified from Gozzelino & Arosio, 2016).

2.1 Iron Metabolism

The amount of iron in the human body under normal conditions ranges around 3-4 g (Baynes & Dominiczak, 1999). While, the normal range of iron in human plasma is 0.6-1.7 µg/ml which is equivalent to 10–30 µM/L (Ganz & Nemeth, 2011). The daily loss of iron through exfoliation of epithelial cells in the gastrointestinal tract, urinary tract and on the integument is about 0.5 mg – 2.0 mg (Sharp & Srai, 2007). Thus, a dietary iron up-take is required. Iron can be divided into two groups: either bound to heme or non-heme. The main source of heme iron is meat products which contain hemoglobin. Heme in form of hemoproteins has many biological functions such as the catalysis of enzyme and electron transport (Paoli et al., 2002). Non-heme bound iron is obtained from vegetables, cereals and fruits rich in vitamin C (Zhou et al., 2005). Vitamin C

helps to stabilize optimal iron concentrations within the organism (Lahti-Koski et al., 2003). Heme iron is more easily absorbed than non-heme iron (Zhou et al., 2005).

Iron is primarily found in erythrocyte hemoglobin and, small amounts of iron are also present in macrophages, muscle myoglobin, and blood plasma (Crichton & Charloteaux-Wauters, 1987; Mackenzie et al., 2008). For iron to be absorbed, ferric iron must be converted to ferrous iron (Fe^{+2}) by duodenal cytochrome b (Dcytb) enzyme, which possess ferric iron reducing characteristics, or by dietary components (McKie et al., 2001). Then ferrous iron (Fe^{+2}) is transported across the enterocyte membrane via the divalent metal transporter 1 (DMT1) (Gunshin et al., 1997). Ferrous iron is then transported across the basolateral membrane by ferroportin, oxidized to ferric iron by hephastin, and loaded into transferrin (Camaschella & Schrier, 2012 work is cited in Escobar-Morreale, 2012). Moreover, transferrin is the main transport protein for iron and represents an essential iron pool. Iron uptake is predominantly achieved via transferrin membrane receptor (TfR1) interaction in endocytosis. The micronutrient subsequently dissociates throughout the cytosol and is taken up by ferritin, the most effective iron storage protein (Wise et al., 2003), which is able to bind up to 4,500 atoms of iron in one molecule (Toebosch et al., 1987). As a transition metal, iron can easily donate an electron during oxidation to its active ferric form (Fe^{+3}) or remain in a stable reduced ferrous state (Fe^{+2}), which is more commonly found in the cytoplasm (Aitken et al., 1993; Lieu et al., 2001; Wise et al., 2003). Both ferritin and transferrin are regulated by the iron regulatory proteins (IRP-1/IRP-2) found in the cytoplasm (Mackenzie et al., 2008). Cellular Fe absorption, concentration, and accumulation are regulated via hepcidin. Hepcidin is a circulating protein secreted by the liver in response to increased body iron levels. It acts as the main negative regulator of iron absorption in villous enterocytes and to inhibit the release of iron from macrophages (Camaschella & Schrier, 2012. This work is cited in Escobar-Morreale, 2012) together with ferroportin,

an exporter protein, responsible for the excretion of iron from the cell (Nemeth et al., 2004). Ceruloplasmin, a Cu- dependent ferroxidase is associated with the oxidation of ferrous ion into ferric and is able to carry trivalent iron only (Roeser et al., 1970; Fig. 2.2).

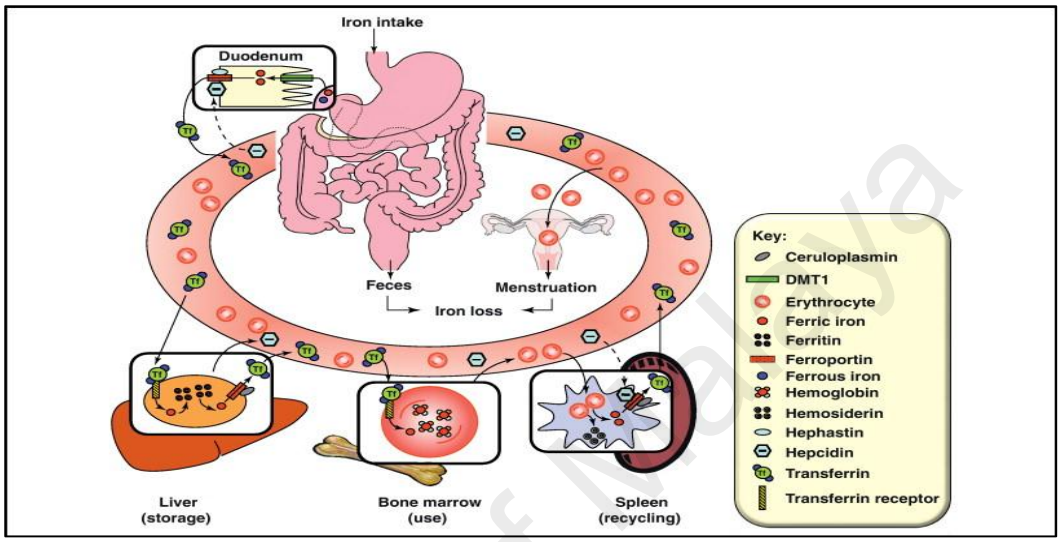

Figure 2.2: An overview of iron metabolism. Iron is absorbed in the duodenum. Non-heme iron enters into duodenum using the divalent metal transporter 1 (DMT1), ferrous iron is then transformed into ferric ion by hephastin and exported by ferroportin into the circulation bound to transferrin (Tf). Iron in tissues is bound to ferritin, most commonly in the bone marrow (haemoglobin synthesis), liver and spleen (iron storage in the liver as ferric ion into ferritin, and recycling by macrophages, especially in the spleen). Iron is excreted either via defecation (very small amount) or blood loss. Menstruation, pregnancy, and lactation may cause important iron loss in women (Escobar-Morreale, 2012).

2.2 Role of iron in Cellular Oxidative Balance

Reactive oxygen species (ROS) are different types such as superoxide (O_2^-), peroxyl (•ROO), hydroxyl radicals (•HO), and hydrogen peroxide (H_2O_2), which are implicated in oxidative stress (OS) development (Halliwell, 2007). In contrast, antioxidants including catalase (CAT), superoxide dismutase (SOD), vitamin E, selenium, or glutathione (GSH), overcome or neutralize ROS in order to reduce their ability to spread harm (Agarwal & Sekhon, 2010). A proper balance between ROS and antioxidants is essential and is maintained in all normal cells. This equilibrium may shift towards the pro-oxidant state when ROS production is increasing significantly or in case of

antioxidant depletion. The resulting state can subsequently lead to cellular damage or apoptosis (Halliwell, 2007; Agarwal & Sekhon, 2010).

Iron is one of the important components of superoxide dismutase (Peeker et al., 1997) and catalase (Beutler & Blaisdell, 1958). These antioxidants are the main antioxidant enzymes preventing variations in ROS and protecting the structure and function of cells against oxidative damage. Superoxide dismutase (SOD) automatically dismutates O_2^- to form oxygen (O_2) and H_2O_2, whereas CAT breaks H_2O_2 into O_2 and water (H_2O) (Maneesh & Jayalekshmi, 2006):

$2(O\text{-}_2) + 2H \rightarrow O_2 + H_2O_2$ (catalyzed by SOD)

$H_2O_2 \rightarrow H_2O + 1/2O_2$ (catalyzed by CAT)

Iron in the biological system is fundamental for electron transport (Arredondo & Nunez, 2005). At the same time, its transition and redox characteristics when free or unbound, determine its reactivity with oxygen via the Haber– Weiss and/ or Fenton reaction. ROS is overproduced as a consequence, being responsible for oxidative damage to biomolecules (Letelier et al., 2010). Toxic iron products may appear when hydrogen peroxide is formed by superoxide dismutase, which will react after that with free or poorly liganded iron through Fenton and Haber- Weiss reaction (Halliwell, 2006). Superoxide may also react with ferric iron through the Haber- Weiss reaction, producing ferrous iron, which affects again in the redox cycling (Kell, 2009, 2010; Khalil et al., 2011; Fig. 2.3). Ordinarily, both processes are slow, but in the presence of free or poorly liganded iron, they occur aggressively, leading to chain reactions with subsequent cellular damage (Kehrer, 2000; Thomas et al., 2009) Simultaneously, transferrin and ferritin can also support the ROS generation (Orino et al, 2001; Crane1& Low, 2012).

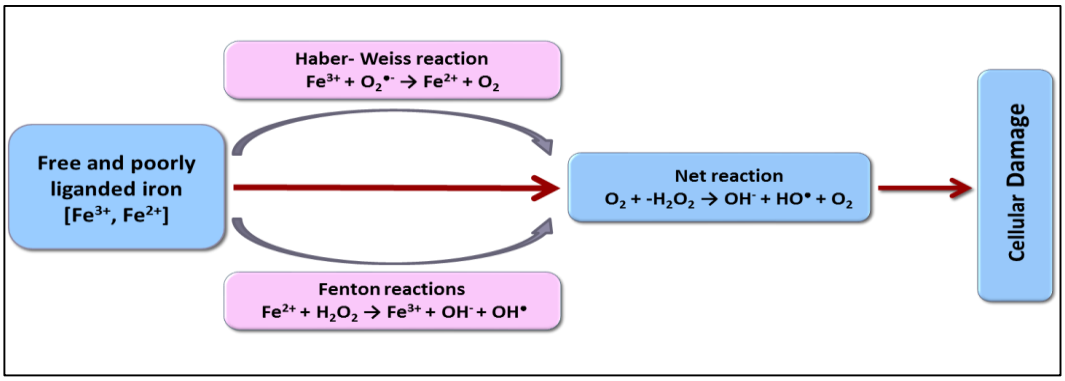

Figure 2.3: Generation of reactive and damaging hydroxyl radicals (OH•). Free Iron (Fe^{+2}) reacts through the Fenton reaction with hydrogen peroxide, leading to the generation of very reactive and damaging hydroxyl radicals (OH•). Superoxide can also react with ferric iron in the Haber- Weiss reaction leading to the production of Fe^{+2}, which then again affects redox cycling (Kehrer, 2000; Wimalasena et al., 2007; Thomas et al., 2009; Khalil et al., 2011).

2.3 Iron and Human Infertility

Ten to fifteen percent of couples are affected by infertility, which is approximately 80 million couple worldwide (Gvozdjakova et al., 2015) before undertaking the assisted reproductive treatment such as in vitro fertilization. The possibility of failing to achieve pregnancy for healthy couples in reproductive age with unprotected sexual intercourse and achieving pregnancy for each reproductive cycle is approximately 20 to 25%. Increasing probabilities of conception over time, for example in the first 6 months the rates of pregnancy may reach up to 60%, within the first 12 months may reach to 84% and in the first 48 months will be up to 92% (Kamel, 2010). Infertility or subfertility is diagnosed in a couple who have not achieved conception after 12 months of unprotected regular sexual activity where the female partner is under 35 years of age, or after 6 months if she is above 35 years of age (Kamel, 2010), or immediately where a clear cause is evident (Speroff et al., 1999). Explained infertility was shared equally between women and men (Evenson et al., 2002). There are two different types of infertility, Primary infertility which is determined when a couple never had any live birth and

secondary infertility when the same couple has had at least one live birth previously (Buy & Ghossain, 2013).

A number of different factors can cause infertility and some of which include: defects relating to genetics, urogenital and reproductive systems, gametogenesis, gamete function, fertilization and embryonic development (Matzuk & Lamb, 2008). Additionally, lifestyle factors have been shown to affect fertility in both men and women (Ferreira et al., 2010).

2.3.1 Iron and Male infertility

Infertility in men is one of the main stressful issues, involved in 30 to 40% of couples and generally dependent on the quality and quantity of spermatozoa (Marzec et al., 2011; Gvozdjakova et al., 2015). Trace elements have a significant influence on spermatozoa and whole semen quality in vivo (Marzec et al., 2011). Iron has a direct regulator role in three mammalian gene expressions (Lieu et al., 2001), two of which have an impact on male reproduction. Some of the protein kinase C family, such as the protein kinase C-beta, has been localized in human semen and associated with flagellar motility (Rotem et al., 1990). In addition, the enzymatic family in human spermatozoa has been detected by Kalina et al. (1995) who have proposed their involvement in several parts of spermatozoa physiology.

The liquefaction process of semen may be associated with presence of type 5-isozyme of acid phosphatase which is a Fe-containing molecule found in semen in large quantities and in prostatic origin as well (Upadhyaya et al., 1986). Besides, normal spermatogenesis, semen pH, viscosity and ejaculate thinning, are affected by iron and non-hemic ferroproteins (Nikolaev et al., 1997). Mitochondrial iron metabolism plays a role in spermatogenesis as a mitoferrin gene product and other proteins participate in iron metabolism that showed enriched expression in the testes (Hales, 2010; Metzendorf

& Lind, 2010). Mitochondria during spermatid development pass through several stages related to shaping and movement, including fusion and aggregation or elongation beside the growing axoneme (Hales, 2010). As a result disturbance of spermatozoa mitochondrial function usually affects male fertility.

Metzendorf and Lind, (2010) showed in *Drosophila Melanogaster* that the development of mature and motile spermatozoa need mitochondrial iron importer mitoferrin (dmfrn). Furthermore, mitoferrin could play a direct role in mitochondrial dynamics, comparable with dual roles in both ATP synthesis and the inner mitochondrial membrane shaping (Metzendorf & Lind, 2010). In addition, the role for the mitoferrin family members in Fe import from the cytosol into mitochondria was demonstrated in vertebrates and yeast (Zhang et al., 2006; Paradkar et al., 2009).

The relationship, between iron and the Krebs cycle is controlled by the mitochondrial aconitase enzyme (Tong & Rouault, 2007). Under ROS overproduction or in a state of iron deficiency, cellular respiration is inhibited by the nitrosylation of heme in mitochondrial enzymes aconitase and glyceraldehyde-3-phosphate dehydrogenase (Stamler, 1994) leading to a depletion of adenosine triphosphate (ATP) and a subsequent loss of spermatozoa motility (Loganathasamy, 2012).

The importance of iron in male fertility has been shown in a variety of in vivo and in vitro studies. According to Slivkova et al. (2009), total iron content of the human seminal plasma is 2.59 ± 0.21 mg/kg, this amount is important to preserve the motility and viability of spermatozoa after ejaculation, and to help the spermatozoa to maintain their functions in vivo. Iron was the only element evaluated in the seminal plasma of Niki-Rawi bulls, which was significantly and positively correlated with spermatozoa motility (Kanwal et al., 2000). Another study showed that iron quantified in bovine seminal plasma was positively associated with spermatozoa motility characteristics

(Tvrda et al., 2012). An in vitro study showed that iron (≤250 μM/L $FeSO_4.7H_2O$) sustained spermatozoa motility and energy metabolism, a main factor supporting spermatozoa function (Knazicka et al., 2012).

Diverse studies have reported on the role of CAT which is commonly found in the ejaculate (Kawakami et al., 2007) and SOD enzymes have a role in reproductive biology and act as antioxidants. Antioxidants including catalase (CAT) and SOD also eliminate superoxide radicals generated by NADPH oxidase in neutrophils and may play important roles in protecting spermatozoa during genitourinary inflammation (Comhaire et al., 1999). Catalase (CAT) and SOD activities have positive relationship with semen quality parameters in mammals including quick progressive motility, non-progressive motility, spermatozoa concentration and viability (Eghbali et al., 2008; Marzec et al., 2011; Khosrowbeygi et al., 2012). In contrast, decreased activities of CAT and SOD enzymes were detected in infertile men (Murawski et al., 2007; Abd-Elmoaty et al., 2010; Malkoc et al., 2013). Moreover, in vitro supplementation of both enzymes has significant improvement in human, dog and stallion semen (Rossi et al., 2001; Cocchia et al., 2011; Chaivechakarn et al., 2012).

2.3.1.1 Iron deficiency and Male infertility

Iron deficiency such as anemia (a decrease in the number of erythrocytes or hemoglobin leading to a lower capacity to transport oxygen), sickle cell disease (which is usually accompanied by low levels of ferritin and a general iron deficiency (Davies, 1983; Agbaraji, 1987) and beta thalassemia (a disease caused by a reduced production of beta chains of hemoglobin) have been shown to be highly associated with male infertility, sexual dysfunction, lack of effective pubertal growth, and inadequate sexual development (Chatterjee et al., 1993a; 1993b).

Therefore, males with iron deficiency-related anemia may present with poor semen parameters (Yassin et al., 2013). Common anemia may be manageable with iron supplementation or intravenous therapy, significantly enhancing the hormonal as well as semen profile of the patients (Alleyne et al., 2008). However, men with sickle cell disease generally have a smaller ejaculate volume, poorer spermatozoa motility, reduced spermatozoa density, and fewer spermatozoa with normal morphology (Agbaraji et al., 1987). While, β-thalassemia patients have hormonal complications due to iron overload from blood transfusions, administration of chelating agents, or splenectomy (Chatterjee et al., 1998; Chatterjee & Katz, 2000). Hypogonadism and abnormal spermatogenesis have been identified as primary causes of sexual dysfunction seen in β-thalassemia patients (De Sanctis et al., 1995; Chatterjee & Katz, 2000). The hypogonadism-like symptoms are related to either anemia (Merchant et al., 2011) or iron accumulation in the pituitary gland (Noetzli et al., 2012). Treating infertile male patients with β-thalassemia with growth hormone and gonadotropins improved semen parameters (Cisternino et al., 1997; Fig 2.4).

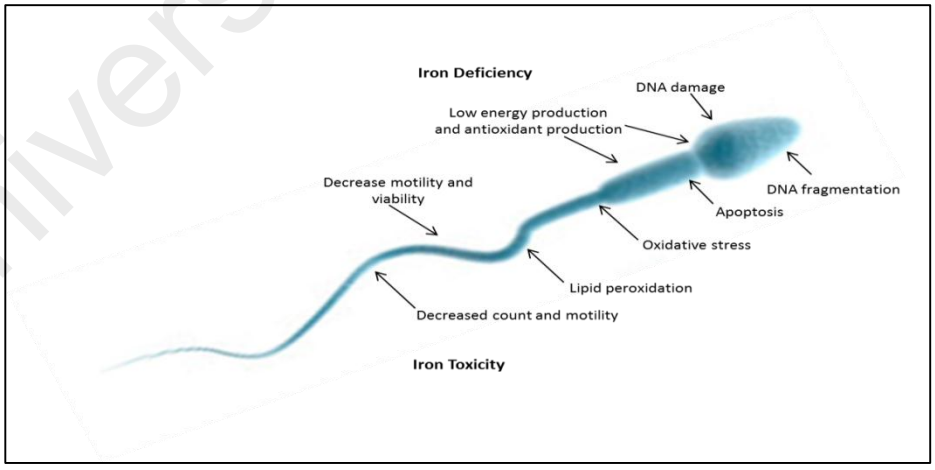

Figure 2.4: Iron effects on the spermatozoon. Based on the currently available data, it may be summarized that iron deficiency may lead to reduced spermatozoa vitality, DNA damage, and a high risk of oxidative damage. Inversely, iron overload may cause disturbances to spermatogenesis as well as to critical spermatozoa cell structures accompanied by oxidative stress and cell death (Tvrda et al., 2015b).

2.3.1.2 Iron overload and male infertility

In contrast, iron overload has a toxic effects on spermatozoa production (Wang & Pantopoulos, 2011), may be due to genetics, lifestyle, and environmental factors transferrin is unable to effectively regulate the amount of iron in the body this will lead to iron accumulation (Fig.2.4). Wise et al. (2003) showed that iron and ferritin levels were negatively correlated with testicular weight in boars. Furthermore, fewer spermatozoa were produced in boars with high iron levels. As a result, when testicular iron concentration was increased, the daily spermatozoa production (DSP) would decline. Additionally, this study concluded that hypogonadism was associated with high levels of ferritin.

Excessive dietary doses of iron resulted in morphological changes in the testes, testicular atrophy, epididymal lesions, impaired spermatogenesis, and impaired reproductive performance (Lucesoli & Frage, 1995; Merker et al., 1996; Pereira & Costa, 2003). In vitro experimental administration of ferrous sulfate at doses ≥ 125 μM/L significantly inhibited a variety of motion parameters in bulls (Knazicka et al., 2012). Iron accumulation ultimately reaches a critical point when transferrin is unable to effectively manage the large iron amounts in the organism (Gottschalk et al., 2000).

Iron toxicity in association with hypogonadism results in atrophied testes with morphological changes and lesions in the seminiferous tubules, epididymes and Sertoli cells (Gunel-Ozcanet al., 2009). Spermatozoa DNA damage was also observed in iron toxicity, which has a high risk of inheritance to offspring (Anderson et al., 2014). Males with hemochromatosis tend to have symptoms related to impotence and lack of sexual desire due to iron toxicity in the pituitary gland. This reduces the regular flow of gonadotropins leading to decrease testosterone levels (Uitz et al., 2013).

Generally, iron overload increases ROS in the testes and epididymis causing a depletion of lipid soluble antioxidants such as alpha-tocopherol, ubiquinol-9, and ubiquinol-10, accompanied by damage to the lipids, proteins and DNA, impaired spermatogenesis and a subsequent infertility (Lucesol & Frage, 1995; Lucesoli et al., 1999; Huang et al., 2001). Moreover, spermatozoa lack cytoplasm – an important component containing antioxidants to counteract the damaging effect of ROS, and large amounts of polyunsaturated fatty acids in the spermatozoa membrane can be easily degraded by ROS derived lipid peroxidation (Aitken et al., 1993; Tvrda et al., 2011). Furthermore, iron intoxication on epididymal and testicular cells in vivo as well as in vitro may cause oxidative damage in rat spermatozoa DNA (Wellejus et al., 2000). Male patients with diseases related to iron overload present with substantial oxidative damage to the spermatozoa, worsened by reduced antioxidant concentrations.

2.3.2 Iron and Female Infertility

The most common identifiable conditions in female infertility are as follows: ovulatory disorders, endometriosis, pelvic adhesions, tubal blockage and others. Each of these conditions results in dysfunction of reproductive physiology (Harris-Glocker & Mclaren, 2013). However, one of the most prevalent causes of female infertility is ovulatory dysfunction (Grigorescu et al., 2014) with 30%–40% of cases of female infertility (Hornstein & Schust, 1996).

The role of iron has been highlighted by some studies especially the iron-transporting proteins in key ovarian cells. Moreover, it has been documented that transferrin and its receptor are present in granulosa cells and oocytes in several studies (Balboni et al., 1987; Aleshire et al., 1989; Briggs et al., 1999). More recently, it has been reported that granulosa cells can synthesize transferrin; which may be translocated to the oocytes (Briggs, 1999). Although it is possible that transferrin and the transferrin receptor are

redundant in the ovary or do not have an important role in local iron metabolism, it has been proposed that these proteins are essential for ovum development and are required to support the increased iron demand of the developing follicle (Aleshire et al., 1989).

In addition, a higher risk of infertility among women with subclinical celiac disease, which characterized by malabsorption and associated with iron deficiency (Smukalla et al., 2014), has been detected in several studies. Undiagnosed celiac disease is more common among women with unexplained infertility than among fertile controls (Collin et al., 1996; Meloni et al., 1999).

Elemental iron plays a vital role in fertility and is essential for normal growth and development of the foetus (Dorea, 2000; Knazicka, 2012). Iron also plays an important role in the success of culture of 8-cell embryos, morula, and blastocysts, and long-term lack of iron increase the number of apoptotic blastomeres (Gao et al., 2007). However, the role of iron in preimplantation embryos is not fully elucidated.

2.3.2.1 Iron Deficiency and Female Infertility

A substantial number of infertile women have signs of iron deficiency including iron deficiency anemia (Collin et al., 1996) and low ferritin levels without evidence of other nutrient deficiencies (Meloni et al., 1999). Intake of non heme iron was found to be related to a lower risk of infertility due to anovulation in a large prospective cohort study (Chavarro et al., 2006). Women in the highest quartile of non heme iron intake (median intake 76 mg/day) had 40% lower risk of infertility due to anovulation than women in the lowest quintile of intake (median intake 9.7 mg/day). Heme iron intake was unrelated to fertility (Chavarro et al., 2006).

Significant evidence supports a role for iron and other supplements such as folate, and vitamin D in ovulation. The high non heme iron intake has been shown to reduce

the chances of an ovulatory infertility. Also there is significant evidence that supports a role for iron in ovulation (Tremellen & Pearce, 2015). According to Chavarro et al. (2006) women who consumed iron supplements had a significantly lower risk of ovulatory infertility than women who did not consume iron supplements.

Anemia during pregnancy is associated with multiple adverse outcomes for both mother and infant, including an increased risk of hemorrhage, sepsis, maternal mortality, perinatal mortality, and low birth weight (Nacher et al., 2002; Yazdani et al., 2004).

2.3.2.2 Iron Overload and Female Infertility

Female patients with beta-thalassemia major usually suffer from hypogonadotropic hypogonadism (HH) associated with amenorrhea, anovulation, and infertility, attributed to the iron effect on the pituitary gland as well as on the female reproductive system. Beta-thalassemia is characterized by iron overload because the multiple blood transfusions and inappropriately increased iron absorption associated with ineffective erythropoiesis (Hershko et al., 2010). To overcome this problem regular chelation therapy is very important (Toumba et al., 2007).

On the basis of all the presented data, it can be concluded that OS plays a major role in the pathophysiology of infertility in females with thalassemia. This OS is mainly caused by tissue injury due to overproduction of free radicals by secondary iron overload, alterations in serum trace elements, and alterations in the level of antioxidant enzymes. Consequently, there is a rationale for iron chelation to eliminate the free iron species which, in this respect, act like antioxidants (Roussou et al., 2013). Polycystic ovary syndrome (PCOS) patients had iron excess and higher hepcidin levels, which were associated with metabolic disruptions (Kim et. al., 2014). Polycystic ovary syndrome (PCOS) is the most common endocrine-metabolic disorder, affecting 10%–

15% of women of reproductive age (Ehrmann, 2005; Azziz et al., 2006). Patients with PCOS have elevated serum ferritin levels, indicating increased body iron stores (Escobar-Morreale et al., 2005; Martínez-García et al., 2009). The polycystic ovary syndrome (PCOS) is associated with abnormal glucose tolerance and insulin resistance. Iron overload may also lead to insulin resistance and diabetes (Escobar-Morreale., 2012; Rossi et al., 2016). Serum ferritin levels are increased in PCOS, especially when glucose tolerance is abnormal, proposing slight iron overload. Factors contributing to potential iron overload in PCOS include the iron sparing effect of chronic menstrual dysfunction, insulin resistance, and a decrease in hepcidin leading to increased iron absorption. Enhancement of erythropoiesis by androgen excess is unlikely, because soluble transferrin receptor levels are not increased in PCOS. The presence of hyperferritinemia in PCOS patients, in the absence of confounding factors such as inflammation or infection, suggests increased body iron storage in these women (Escobar-Morreale., 2012). Rossi et al. (2016) demonstrated that iron accumulation in the HPG axis lead to disruptions of ovarian follicular development and steroidogenesis. In addition, there was evidence that morphology impairment in the pituitary, ovary and uterus could be attributable to an imbalance in pro-oxidant and antioxidant pathways (Rossi et al., 2016).

CHAPTER 3: METHODOLOGY

3.1 Experimental Design

The experimental design for the present study involved preparing different concentrations of ferric iron which closely resemble the normal range for human plasma iron in a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) protein-free culture medium (PFM) and a protein-free culture medium developed by Professor Jaffar Ali. Protein-free culture medium (PFM) has the ability to prevent any disease transmission because it is devoid of Human Serum Albumin (HSA), which is the main source of iron in the media as well. Protein-free culture medium (PFM) may have a little amount of iron as a contaminant. Accordingly, the first investigation of this study was to determine the amount of iron present in HEPES PFM, as well as to calculate the exact amount of iron needed for optimal spermatozoa activity, and eventually for mice embryo development.

Total iron concentration in human plasma is in the range of 0.6 - 1.7 µg/ml; equal to 10 – 30 µM (Ganz & Nemeth, 2011). Mojic et al. (2014) found that cell culture medium with iron at physiological levels of human plasma prevents the accumulation of hydrogen peroxide (H_2O_2), which is one of the reactive oxygen species (ROS) involved in oxidative stress (OS) development. In order to mimic the physiological level of iron in plasma, firstly there is a need to investigate the optimal tolerance levels of iron on spermatozoa activity and sperm DNA integrity (tolerance investigation). The concentrations of ferric iron ($FeCl_3.6H_2O$; Merck, KGaA, Germany) were in the range of human plasma iron in the HEPES protein free culture medium at different lengths of culture times 1 hour (hr) and 20 hours (hrs). However, to determine the toxic level of ferric iron on spermatozoa (toxicity investigation), the concentrations of iron supplementation ($FeCl_3.6H_2O$) were multiplied by the highest level of the normal range (almost 2 µg/ml) for many times in the HEPES protein free culture medium, at different

culture times (1 hr and 20 hrs). Sperm DNA integrity (SDI) tests were carried out after determining the optimal tolerance level and the toxic level of ferric chloride on HEPES PFM, in order to find out in which ferric iron level resulted in the highest and lowest percentages of spermatozoa motility and vitality.

Secondly, to investigate optimal tolerance levels (tolerance investigation) of iron on mice embryos development, concentrations of ferric iron were increased from 0.11 µg/ml, to 2.8 µg/ml in a PFM culture medium for 6 days of observation after in vitro culture. To investigate the iron toxic level of day two embryos of Quakenbush Special (QS) mouse embryos, the levels of ferric chloride used for toxicity investigation were increased from 5.6 µg/ml to 28 µg/ml in a protein-free culture medium. The embryos were observed until day 6 of fertilization (Figure 3.1).

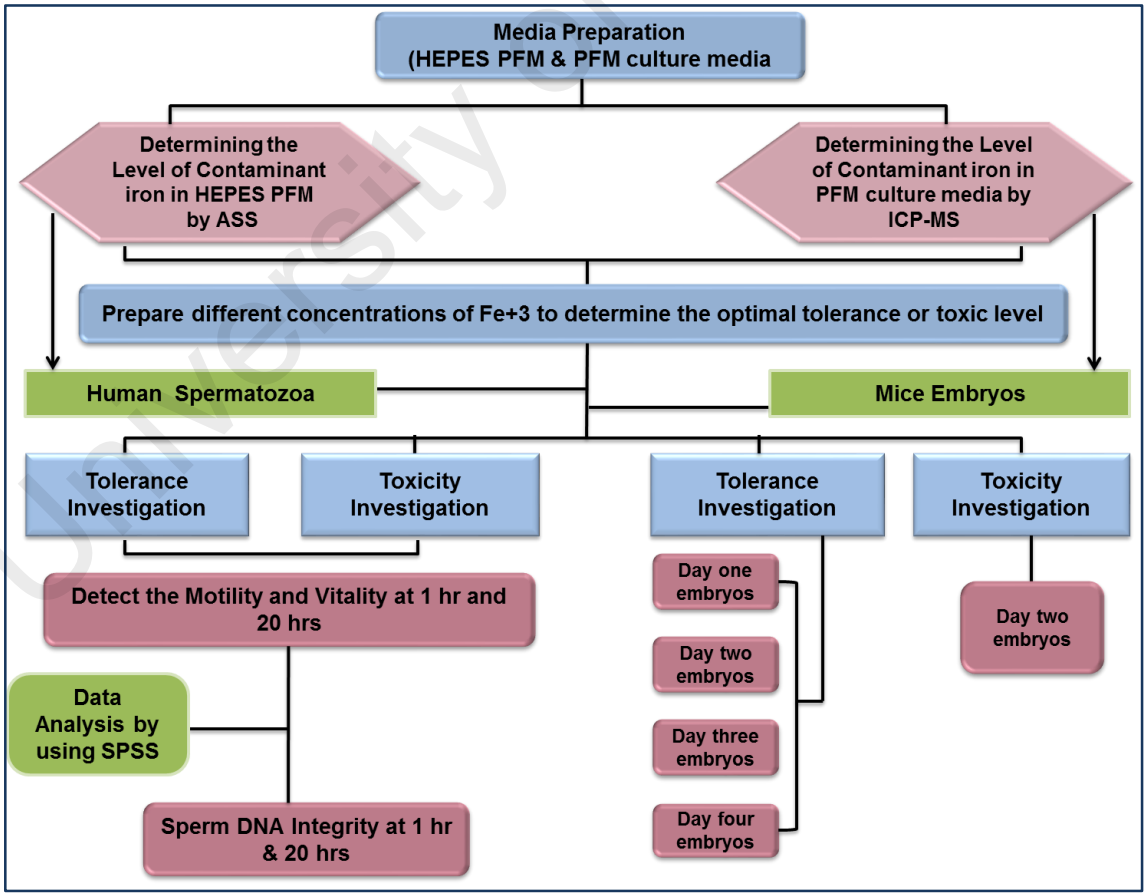

Figure 3.1: Flow Chart of the Experimental Design

3.2 Determining the iron concentration of HEPES PFM and the PFM culture medium

The iron level was determined by flame atomic absorption spectrophotometer (AAS) (Perkin Elmer, Analyst 400) in the HEPES PFM which was used to culture the human spermatozoa. A sample preparation was performed according to procedures outlined by Altekin et al. (2005) with slight modifications. The sample was diluted in deionized water with 0.1 M of hydrochloric acid (HCL; Sigma Chemical Co, USA) with a dilution factor 1:10. Calibration was achieved by diluting the stock solution of Iron (III) Nitrate AAS Standard [1 mg/mL] (Ajax Finechem Pty Ltd, Australia) at 1, 2, and 3 mg/L. Three readings for each replicate were done. The result of AAS showed that the concentration of iron in HEPES PFM is 0.004µg/mL (0.07 µM/L). This amount of iron was considered for each treatment of tolerance and toxic investigations for spermatozoa activity and sperm DNA integrity evaluation.

On the other hand, for the protein-free culture medium used to culture mice embryos, iron level was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent, 7500 Single Turbo System). Inductively coupled plasma mass spectrometry (ICP-MS) multi-element standard solution (Merck, Millipore) was diluted in nitric acid at 100 mg/L. The result of the ICP-MS showed that the concentration of iron in PFM culture medium was 0.59 ppb (0.00059 µg/ml). This amount of iron was considered for each treatment of tolerance and toxic investigations for the evaluation of mice embryo development.

3.3 Media preparation

The PFM is made from highly purified components according to European/US pharmacopeias. The PFM medium (HEPES and culture media) used in this study was developed and prepared by Professor Dr. Jaffar Ali. (Ali, 2000).

3.3.1 Detecting spermatozoa activity using HEPES medium with different concentrations of ferric iron

As mentioned before, the different concentrations of iron levels in this study, were planned to closely resemble the normal range for human plasma iron; 0.6 - 1.7µg/ml, equal to 10 - 30 µM (Ganz & Nemeth, 2011). In order to mimic the physiological level of iron in plasma, 4 treatments in addition to one control group were prepared for both tolerance and toxic investigations of spermatozoa activity. The control group was HEPES medium without iron supplementation (Table 3.1 & table 3.2).

The calculations of the amount of ferric iron in HEPES PFM were done using the formula below:

- Note: $FeCl_3.6H_2O$ (MW = 270.33 g/ mol), Fe (III) (MW = 55.84 g/mol)

The amount of Fe (III) in 100ml of the medium =

$$\frac{Amount\ of\ Fe\ (III)\ in\ gram}{Molar\ mass\ (M)\ of\ Fe(III)}\ x\ Molecular\ weight\ (MW) of\ FeCl3.6\ H2O$$

Table 3.1: Levels of iron in control media and different treatments of HEPES PFM after adding different amounts of $FeCl_3.6H_2O$ (Tolerance investigations for spermatozoa activity)

Specification	Level of iron before adding $FeCl_3.6H_2O$ detected by AAS (µg/ml)	Level of Fe^{+3} after adding $FeCl_3.6H_2O$ (µg/ml)	Final concentration of iron	
			µg/ml	µM/L
HEPES PFM (Control)	0.004 ± 0.0002	0	0.004	0.07
Treatment 1 (T1)	0.004 ± 0.0002	0.5	0.504	8.9
Treatment 2 (T2)	0.004 ± 0.0002	1	1.004	17.9
Treatment 3 (T3)	0.004 ± 0.0002	1.5	1.504	26.8
Treatment 4 (T4)	0.004 ± 0.0002	2	2.004	35.8

Table 3.2: Levels of iron in control media and different treatments of HEPES PFM after adding different amounts of $FeCl_3.6H_2O$ (Toxicity investigation for spermatozoa activity)

Specification	Level of iron before adding $FeCl_3.6H_2O$ detected by AAS (µg/ml)	Level of Fe^{+3} after adding $FeCl_3.6H_2O$ (µg/ml)	Final concentration of iron	
			µg/ml	µM/L
HEPES PFM (Control)	0.004 ± 0.0002	0	0.004	0.07
Treatment 1 (Tt1)	0.004 ± 0.0002	4	4.004	71.7
Treatment 2 (Tt2)	0.004 ± 0.0002	8	8.004	143.4
Treatment 3 (Tt3)	0.004 ± 0.0002	12	12.004	214.9
Treatment 4 (Tt4)	0.004 ± 0.0002	16	16.004	286.6

3.3.2 Detecting embryos development using PFM culture media with different concentrations of ferric iron

To study the embryos' development, five treatments of PFM culture media with different iron concentrations and one control of PFM culture medium without iron supplementation were prepared for both tolerance and toxicity investigation (Table 3.3 & Table 3.4).

Table 3.3: Levels of iron in control media and different treatments of PFM culture media after adding different amounts of $FeCl_3.6H_2O$ (tolerance investigation for embryos developments)

Specification	Level of iron before adding $FeCl_3.6H_2O$ detected by ICP-MS (µg/ml)	Level of Fe^{+3} after adding $FeCl_3.6H_2O$ (µg/ml)	Final concentration of iron	
			µg/ml	µM/L
PFM culture Medium (Control)	0.00059	0	0.00059	0.01
Treatment 1 (T1)	0.00059	0.11	0.11	2.0
Treatment 2 (T2)	0.00059	0.28	0.28	5.0
Treatment 3 (T3)	0.00059	0.56	0.56	10.0
Treatment 4 (T4)	0.00059	1.12	1.12	20.1
Treatment 5 (T5)	0.00059	2.79	2.80	50.0

Table 3.4: Levels of iron in control media and different treatments of PFM culture media after adding different amounts of $FeCl_3.6H_2O$ (toxicity investigation for embryos developments)

Specification	Level of iron before adding $FeCl_3.6H_2O$ detected by ICP-MS (µg/ml)	Level of Fe^{+3} after adding $FeCl_3.6H_2O$ (µg/ml)	Final concentration of iron	
			µg/ml	µM/L
PFM culture Medium (Control 1)	0.00059	0	0.00059	0.011
Treatment 1 (Tt1)	0.00059	5.58	5.6	100
Treatment 2 (Tt2)	0.00059	11.17	11.2	200
Treatment 3 (Tt3)	0.00059	16.75	16.8	300
Treatment 4 (Tt4)	0.00059	22.34	22.3	400
Treatment 5 (Tt5)	0.00059	27.92	28.0	500

3.4 Ethics Approval

This study received its ethics approval from the University of Malaya Medical Centre Ethics Committee (Ref. No. 1198.52) for work with human semen samples. Furthermore, this study was approved by the Institutional Animal Experimentation Ethics Committee with Ref. No. 2014-07-01/ONG/R/"JAMA, for animal samples.

3.5 Ferric iron effects on spermatozoa activity

3.5.1 Semen Samples Collection and in vitro culture

Semen samples of normozoospermic characteristics were collected from 24 male patients undergoing semen assessment at National Population and Family Development Board (LPPKN). Semen samples were obtained by masturbation after 2– 3 days of sexual abstinence. Each sample was collected in a clean and wide mouthed, 60ml container, and the patient's name, date, and time of collections were labelled. All of the semen samples used in this study were used within 2 to 4 hours of collection. Semen analysis was carried out by using the procedures to measure characteristics as outlined by the World Health (2010) including; semen volume, pH, sperm concentration, percentage motility and vitality. In summary, semen volume was measured by aspirating the whole sample into a 10 ml graduated pipette. To measure the pH level of sample, a drop (50 µL) of semen was placed equally onto pH test paper, and after 30 seconds, the colour of the impregnated zone of pH test paper was compared to the calibration strip. For semen concentration assessment, a wet mount of semen sample was examined with a phase contrast microscope and sperm numbers for each ejaculate were determined using a Neubauer Haemocytometer chamber with a single count of 200 spermatozoa per replicate. The motility of all spermatozoa within the field of a defined area were assessed and scored using the following descriptors; progressive, non-progressive or non-motile. Progressive motility is when the examined spermatozoa move fast either in a linear or largely circular pattern, whereas all other patterns of

motility are considered to be non-progressive according to WHO (2010). Spermatozoa that are absent of any movement are considered to be non-motile. Sperm vitality was measured by placing a drop (50 µL) of semen onto a labelled microscope slide, and one drop (50 µL) of spermatozoa VitalStain™ (Nidacon International AB, Sweden) was then mixed well using a pipette tip and smeared by dragging another microscope slide over the drops. This was then allowed to dry and 200 consecutive spermatozoa were counted under a bright field microscope using (40x) objective lens.

Finally, well-mixed sibling spermatozoa were cultured in HEPES PFM supplemented with various concentrations of ferric iron (e.g. for iron tolerance investigation: C; 0.0 µg/ml, T1; 0.5 µg/ml, T2; 1.0 µg/ml, T3; 1.5 µg/ml, and T4; 2.0 µg/ml and for iron toxicity investigation: C; 0.0 µg/ml, Tt1; 4.0 µg/ml, Tt2; 8.0 µg/ml, Tt3; 12.0 µg/ml, and Tt4; 16.0 µg/ml) depending on the original spermatozoa concentration, with a dilution ratio of 1:20, in 96-well plates (MTP, Greiner, Germany) There were many treatments for each sample (1 control and 4 treatments for iron tolerance investigation and another control and 4 treatments for iron toxicity investigations, which in total resulted in 10 treatments just for one sample) for 24 hours at room temperature (22-25 ℃). The experiment was performed with ease.in spite of the large number of treatments Spermatozoa activity was evaluated after 1 hr and 20 hrs.

3.5.2 Evaluation of spermatozoa motility at 1 hour and 20 hours for tolerance and toxicity investigations

The motility analysis of all treatments of tolerance and toxicity investigations were performed using a modified Neubauer Chamber (Hawksley, Lancing, England) according to WHO semen analysis standards (2010) using a phase contrast microscope (Olympus- CH2, Japan) at 20x magnification. Spermatozoa were scored as progressive,

non-progressive, and non-motile. The Neubauer chamber was incubated for 10 minutes at 37°C before a 10µml wet amount of each treatment was placed and examined under a phase contrast microscope after 1 hr and 20 hrs of culturing.

3.5.3 Evaluation of spermatozoa vitality at 1 hour and 20 hours for tolerance and toxicity investigations

Spermatozoa vitality for each treatment was examined by using spermatozoa VitalStain™ solution (Nidacon International AB, Sweden). This was to differentiate and quantitate life and dead spermatozoa. Spermatozoa VitalStain contains eosin and nigrosin stains. The by mixing a drop (50 µL) of the specimen with one drop (50 µL) of VitalStain, which were then mixed thoroughly. Then, one drop (50 µL) of the mixture was placed onto a labelled microscope slide. 200 spermatozoa were observed under a bright field microscope using (40x) objective lens at 1 hr and 20 hrs. The percentage of spermatozoa vitality was calculated by dividing live sperms count by total sperm count x100.

3.5.4 Sperm DNA Integrity (SDI) test

Sperm DNA analysis was carried out using a Halosperm® G2 kit (Halotech DNA S.L., Spain). The procedures were conducted according to manufacturer instructions. After adjusting the concentration of sperms to 20 million per milliliter, for each of the following samples: Control (C); 0.0 µg/ml, T4; 2.0 µg/ml, Tt1; 4.0 µg/ml, and Tt4; 16.0 µg/ml at 1 hr and 20 hrs. Analysis was carried out using the reagents supplied in the Halosperm® G2 kit plus additional reagents which included ethanol (95%), microscope slides and cover slips, distilled water and a water bath (37°C). The low-melting-point agarose gel supplied with the kit was placed in a water bath at 90°C - 100°C for 5 minutes to melt, before 50µL was transferred into an Eppendorf tube at 37 °C for 5 minutes to permit temperature equilibration. The semen sample (25 µL), of each

treatment was then added to the 50µL of liquefied and temperature equalized agarose gel and mixed until fully incorporated. The semen-agarose mix 8 µL was pipetted onto a Halosperm® pretreated slide, covered with a 22 mm by 22 mm coverslip and placed on a pre-cooled metal tray inside a refrigerator at 4°C for 5 minutes to enable the spermatozoa to become embedded in the agarose gel. The slide was then placed in a horizontal position on top of a petri dish inside a glass container before the coverslip was gently removed and an acid denaturation solution (Solution 1) was applied for 7 minutes and then drained. For negative control, this step was omitted. This was followed by 20 minutes of incubation in a lytic solution (Solution 2) before draining. After that, the slide was washed in distilled water and drained. This was followed by dehydration in 70% and 100% ethanol for 2 minutes each, with draining between each step. Subsequently, slide was air-dried and stained with Eosin dye (Solution 3) for 7 minutes and then drained. Thereafter, an Azure B solution (Solution 4) was applied for another 7 minutes before final draining.

Sperm DNA integrity of each treatment was detected comparison to a negative control. Spermatozoa with a halo of chromatin around the sperm head was considered to have preserved its DNA integrity, whilst spermatozoa without halo indicated DNA fragmentation. A total of 200 treated spermatozoa were observed under bright field light microscope (Olympus- CH2, Japan) at 40x magnification. Spermatozoa DNA Integrity Level was calculated by dividing the number of spermatozoa (with Halo) by total number of spermatozoa x 100 (Figure 3.2).

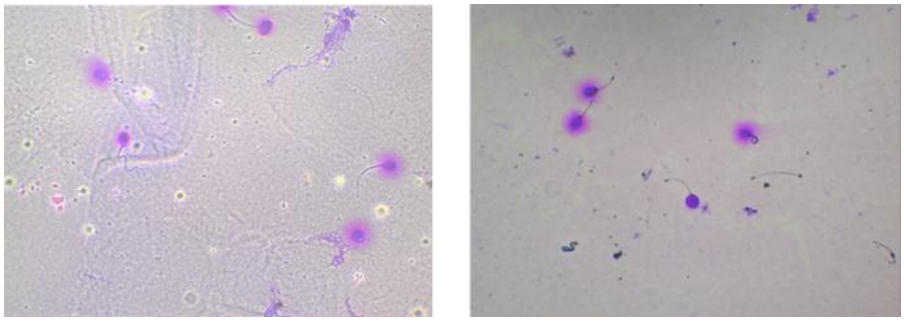

Figure 3.2: On the left, negative (-ve) control of sperm DNA integrity test, spermatozoa with a halo of chromatin around the sperm head. On the right, the picture was shown an example of the results of this experiment (sperm with and without halo).

3.6 Ferric iron effects on mice embryos development

3.6.1 Mouse embryos collections and in vitro culture

Swiss outbred (SO) mice, also known as Quakenbush strain (QS), are often used in many laboratories as oocyte/ embryo donors as they are good breeders, produce more oocytes/ embryos than most strains and are easy to handle. The methodology employed in the generation and recovery of mouse embryos is described by Ali et al. (1993; 2000) and Ali and Shelton (1995). Thick leather gloves were used for protection against mouse bites during mouse handling. Superovulation by an intraperitoneal injection of 10 IU of PMSG, then by 10 IU of hCG after 44-48 hours were done for all female mice (Figure 3.3). Then, the females were mated with stud SO males of proven virility and fertility (Figure 3.4). The females were examined for copulatory plugs early next morning. Presence of copulatory plug is considered proof of copulation (Figure 3.5). For recovery of embryos, the mice were euthanized by either CO_2 asphyxiation or cervical dislocation (Figure 3.6). The euthanized mice were placed in a row over layers of tissue paper, ventral side uppermost. The skin of the abdomen was grasped by forceps and small incision was made using the scissors (Figure 3.7). Then the thumb and forefinger of both hands and with a single twist pulling motion the skin was broken and separated to reveal the ventral abdominal muscles (Figure 3.8). With a pair of sterile scissors the abdominal muscles were cut and the contents exposed. After that, the

oviducts were excised and placed on a sterile dish containing HEPES - embryo culture medium (Figure 3.9). The embryos were recovered from the oviducts by flushing with Hepes medium. Flushing was performed by using a syringe fitted with a 0.5 inch 30-gauge filled with medium. Generally, most SO mice produced over 20 embryos per oviduct. Recovered embryos were pooled and subsequently randomly apportioned for individual treatment and cultured at 37°C and 5% CO_2 (Figure 3.10).

The embryos were cultured in PFM embryo culture media with different concentrations of ferric iron as previously described. Observations of embryo development were carried out on daily at 9 to 10 am. It is well documented in vitro cultured embryos develop a little slower than embryos in vivo. It thus follows that 2 & 4 cell embryos were considered as day 2 embryos; 4 to 8 cell embryos as day 3 embryos; 16-cell morula, compacting and compacted morula as day 4 embryos; early blastocysts and blastocysts as day 5 embryos; and expanded, hatching and hatched blastocysts as day 6 embryos. The end point was the development of day 6 embryos.

Figure 3.3: Superovulation by an intraperitoneal injection of PMSG, then by hCG after 44-48 hours for female Qs mouse.

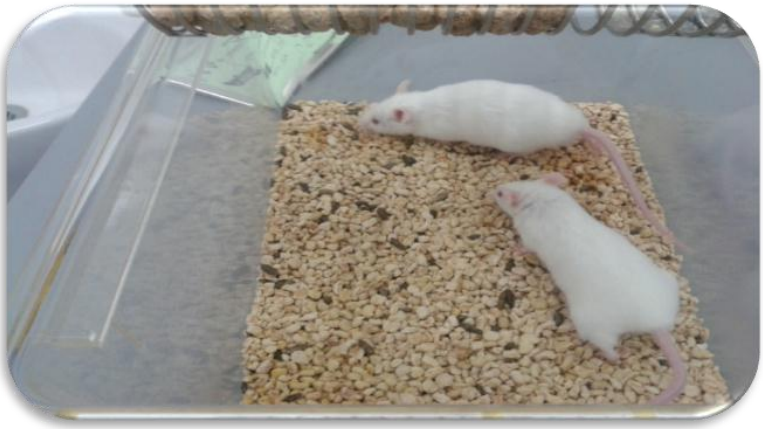

Figure 3.4: The female was mated with stud Qs male.

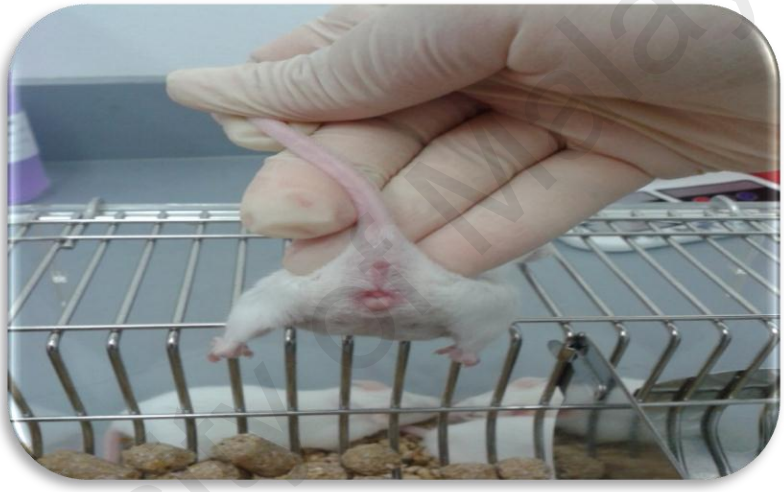

Figure 3.5: Presence of copulatory plugs is indicative of mating.

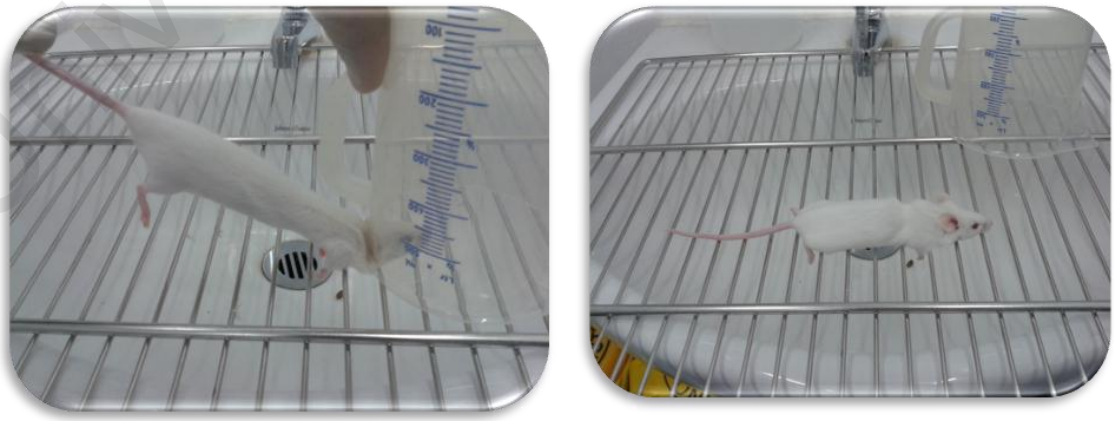

Figure 3.6: Left: Cervical dislocation, Right: After cervical dislocation.

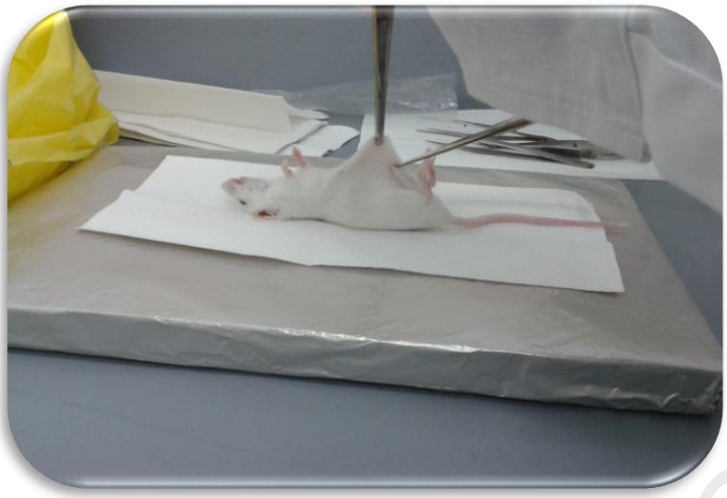

Figure 3.7: Small incision was made on the abdomen skin by using forceps and scissors.

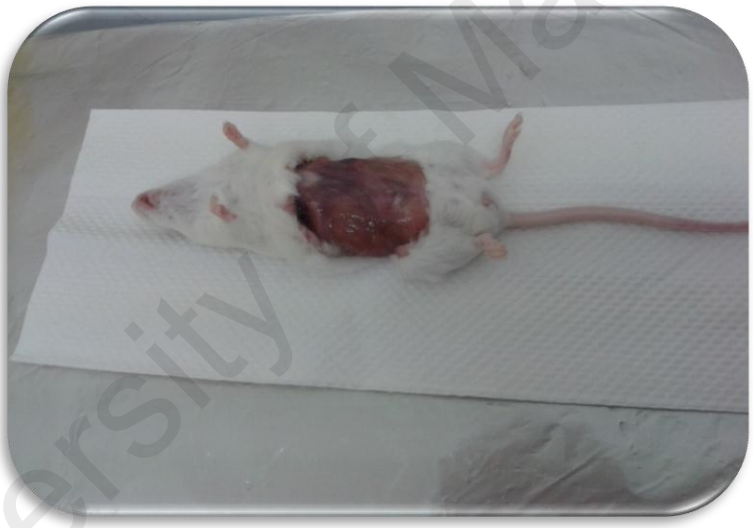

Figure 3.8: The skin was torn back to reveal the ventral abdominal muscles.

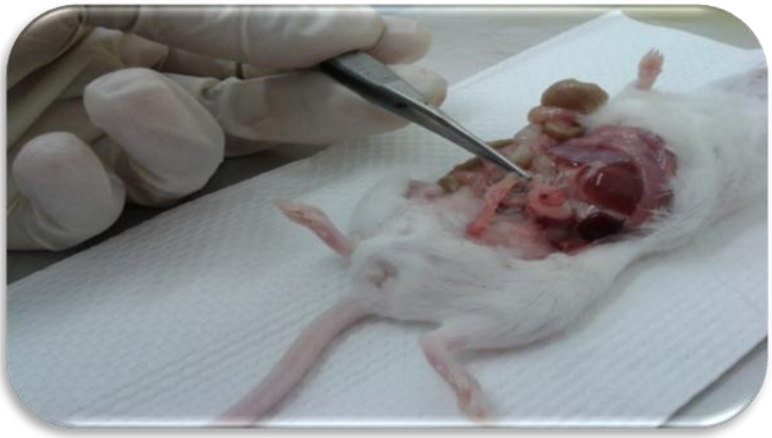

Figure 3.9: The oviducts were excised on day one or day two and placed on sterile dish containing HEPES - Embryo culture medium.

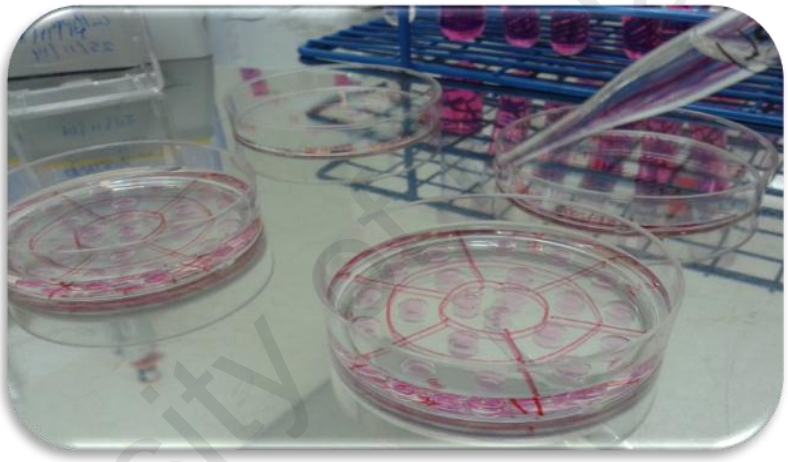

Figure 3.10: Recovered embryos were pooled and subsequently randomly apportioned for individual treatments.

3.6.2 Tolerance investigation: determination of tolerance and optimal levels of elemental iron on mouse embryo development and viability in PFM

In the tolerance investigation, mouse zygotes were cultured in PFM containing a series of different levels of ferric iron, C; 0.0 µM, T1; 2 µM, T2; 5 µM, T3; 10 µM;, T4; 20 µM and T5; 50 µM) at 37ºC and 5% of CO_2. The observation of mice embryos developments were done until day 6 of fertilization for all treatments. The groups of embryos generated in the best treatment was compared with the control and the worst

treatment. Besides that, this experiment was done in 4 stages; day 1, day 2, day 3 and day 4 embryos, with 6 replicates for day 1 and day 2 embryos and 4 replicates for both day 3 and day 4 embryos. For day 3 and 4 embryos culture, the day 2 embryos were kept in the control medium until the required date of cultivation. Then, the embryos were placed in treatments of different levels of ferric iron in PFM culture medium (Table 3.5).

Table 3.5: Numbers of mouse embryos of different developmental stages used to determine the optimal and tolerance levels of iron in mouse embryo development

Iron concentration	Treatments						Total embryos for all replicates
	C	T1	T2	T3	T4	T5	
µM/L ($FeCl_3.6H_2O$)	0	2	5	10	20	50	
µg/ml (Fe^{+3})	0	0.11	0.28	0.56	1.1	2.8	
	Expt. On day 1 embryos						
No. of replicates	6	6	6	6	6	6	
No. of embryos in individual treatments	62	58	60	58	60	59	357
	Expt. On day 2 embryos						
No. of replicates	6	6	6	6	6	6	
No. of embryos in individual treatments	43	43	43	42	43	45	259
	Expt. On day 3 embryos						
No. of replicates	4	4	4	4	4	4	
No. of embryos in individual treatments	31	34	33	32	31	32	193
	Expt. On day 4 embryos						
No. of replicates	4	4	4	4	4	4	
No. of embryos in individual treatments	25	24	24	23	25	24	145
Total embryos for all replicates							954

3.6.3 Toxicity investigation: determination of toxic levels of elemental iron on mouse embryo development and viability in PFM

In contrast, for the toxicity investigation, mouse zygotes of day 2 embryos were cultured in PFM containing different levels of ferric iron namely: C; 0.0 µM, Tt1; 100 µM, Tt2; 200 µM, Tt3; 300 µM. Tt4; 400 µM and Tt5; 500 µM at 37ºC and 5% of CO_2.

The observation of mice embryos developments were done until day 6 of fertilization for all treatments (Table 3.6).

Table 3.6: Numbers of mouse embryos of different developmental stages used to determine the toxic levels of iron on mouse embryos development

Iron concentration	**Treatments**						**Total embryos for all replicates**
	C	**Tt1**	**Tt2**	**Tt3**	**Tt4**	**Tt5**	
µM/L ($FeCl_3.6H_2O$)	0	100	200	300	400	500	
µg/ml (Fe^{+3})	0	5.6	11.2	16.8	22.3	28	
	Expt. On day 1 embryos						
No. of replicates	5	5	5	5	5	5	
No. of embryos in individual treatments	34	34	34	35	34	35	
Total embryos for all replicates							**206**

3.7 Statistical Analysis

Microsoft Excel 2010 (Microsoft, Redmond, USA) software was mainly used for organizing data from trial protocols to calculate the means and standard error of mean (SEM), as well as producing statistical illustrations. In addition, IBM's SPSS Statistical Software Version 22 (International Business Machines Corp, New York 10504) was also used to analyze semen samples results, and Statistix 9 analytical software version 9.1 was used to analyze the mice embryos results.

The samples were examined for normal distribution. This was done by an evaluation of histograms, QQ plots and performing the Shapiro–Wilk test for normality. The data showed normal distribution. Statistical evaluations of spermatozoa parameters were performed by one-way ANOVA, followed by Dunnett post hoc test to statistically determine significant difference between the control group and the test groups. Tukey's test was also performed to determine the significant differences between treatments. Correlation analyses were performed using Pearson's correlation. Values lower $P<0.05$ are considered significantly different.

To examine the relationship between the effects of different concentrations of iron and mice embryos development, Pearson's Chi-square was performed using the Statistix^{TM} software, and $P < 0.05$ was set as the minimum level of significance.

CHAPTER 4: RESULTS

4.1 Spermatozoa Parameters

Each sample in this study (n = 24) was examined for a number parameters according to WHO (2010) guidelines. Ejaculate volume, pH, sperm concentration, motility, vitality and DNA integrity were measured (Table 4.1).

Table 4.1: Characteristics of human semen samples

Parameters : (n = 24)	Mean ± SEM
Ejaculate volume (ml)	3.1 ± 0.26
pH	7.8 ± 0.16
Sperm concentration (million/ml)	55.5 ± 11.68
Total motility (%)	68.1 ± 2.46
Vitality (%)	70.6 ± 2.41
DNA integrity (%)	79.9 ± 4.66

4.2 Spermatozoa motility

4.2.1 Tolerance investigation at 1 hour

The percentage of total motility and progressive motility in all treatments group of tolerance investigation (0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, and 2 µg/ml of Fe^{+3}) significantly increased, ($p = 0.001$ for total motility and $p = 0.008$ for progressive motility). The progressive motility of spermatozoa when compared individually between treatments and the control group found that the 2 µg/ml (T4) of ferric iron was significantly higher than control group after 1 hr of ferric exposure. Total motility was significantly increased for 2 µg/ml (T4) compared to the control group. The percentage of immotile spermatozoa decreased significantly at 2 µg/ml of Fe^{+3} compared to the control group (Table 4.2).

Table 4.2: Comparison between the control group and the test groups for progressive and total motility of spermatozoa after 1 hr of (Fe^{+3}) exposure at physiological levels of iron in HEPES buffered protein-free culture medium

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation (Means ± SE)			
	C	T1	T2	T3	T4
µM/L	0	8.9	17.9	26.8	35.8
µg/ml	0	0.5	1.0	1.5	2
% Progressive Motility	44.2±2.23	44. 5±1.98	46.0±1.82	46.9±1.87	53.4±1.86
p. value		ns	ns	ns	0.005
% Total Motility	57.3± 2.23	58.3±1.62	59.9±1.61	60.4±1.72	67.6±1.88
p. value		ns	ns	ns	0.001
% Immotile	42.7 ± 2.23	41.7 ± 1.62	40.2± 1.61	39.6± 1.72	32.4±1.88
p. value		ns	ns	ns	0.001

ns= not significant

The progressive motility of spermatozoa was significantly increased in the 2 µg/ml (T4) treatment group of ferric iron compared to the T1 group (0.5 µg/ml Fe^{+3}) after 1 hr of exposure. Total motility was likewise significantly increased for 2µg/ml (T4) compared to T1, T2, and T3 groups; 0.5 µg/ml, 1 µg/ml, and 1.5 µg/ml of Fe^{+3} treatment groups, respectively. The percentage of immotile spermatozoa decreased significantly at 2 µg/ml of Fe^{+3} compared to all other treatments 0.5 µg/ml, 1 µg/ml, and 1.5 µg/ml (Table 4.3).

Table 4.3: Comparison of progressive and total motility of spermatozoa after 1 hr exposure of (Fe^{+3}) in HEPES buffered protein-free culture medium for control and all groups

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation (Means ± SE)			
	C	T1	T2	T3	T4
µM/L	0	8.9	17.9	26.8	35.8
µg/ml	0	0.5	1.0	1.5	2
% Progressive Motility	44.2±2.23	44. 5±1.98	46.0±1.82	46.9±1.87	53.4±1.86
p. value		0.014	ns	ns	
% Total Motility	57.3± 2.23	58.3±1.62	59.9±1.61	60.4±1.72	67.6±1.88
p. value		0.004	0.028	0.049	
% Immotile	42.7 ± 2.23	41.7 ± 1.62	40.2± 1.61	39.6± 1.72	32.4±1.88
p. value		0.004	0.028	0.049	

ns= not significant

Figure 4.1 shows both progressive and total motility investigated after 1 hr of ferric exposure with 2.0 µg/ ml of Fe^{+3} supplementation.

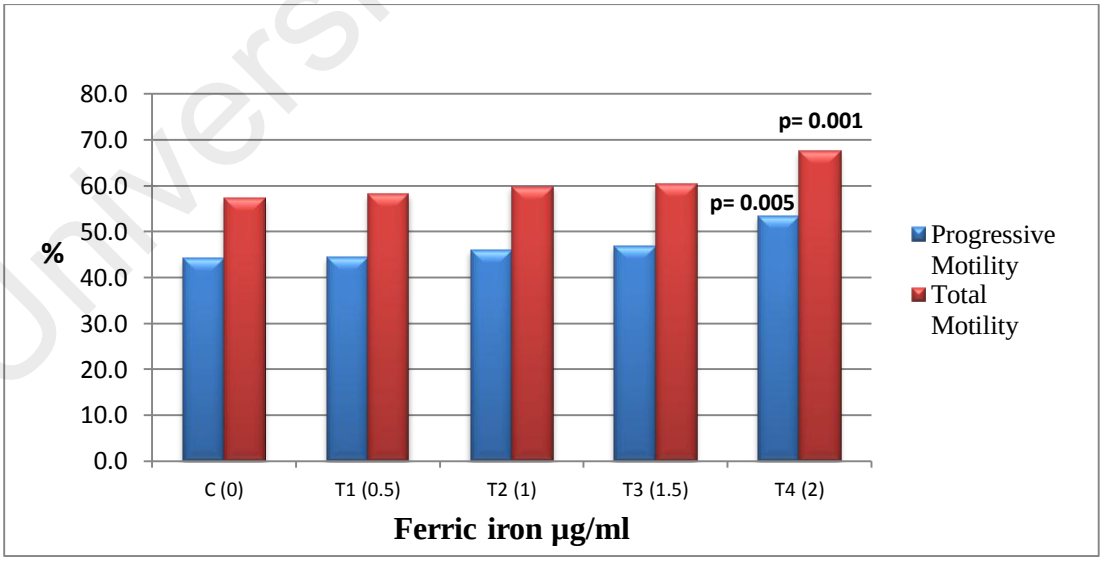

Figure 4.1: Progressive and total motility percentage of spermatozoa at 1 hr of exposure.

Figure 4.2 shows a weak positive correlation of r = 0.367 between different concentrations of Fe^{+3} and total spermatozoa motility ($p < 0.001$).

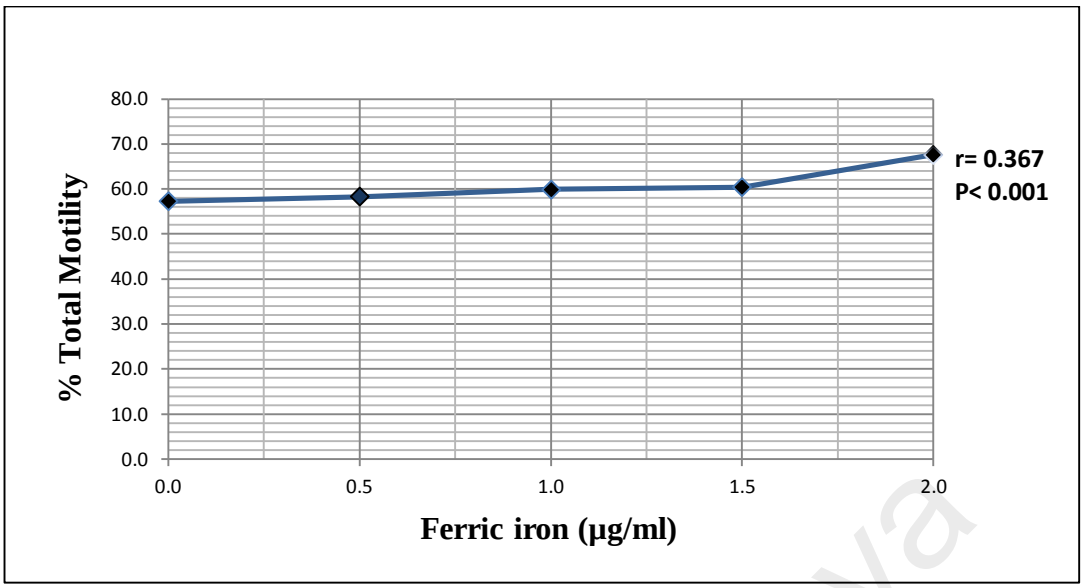

Figure 4.2: Correlation between the different concentrations of ferric iron and total motility of the spermatozoa at 1 hr of exposure.

There is a weak positive correlation of r = 0.322 also between increasing ferric iron concentration within the normal range of human plasma iron and progressive spermatozoa motility (Figure 4.3).

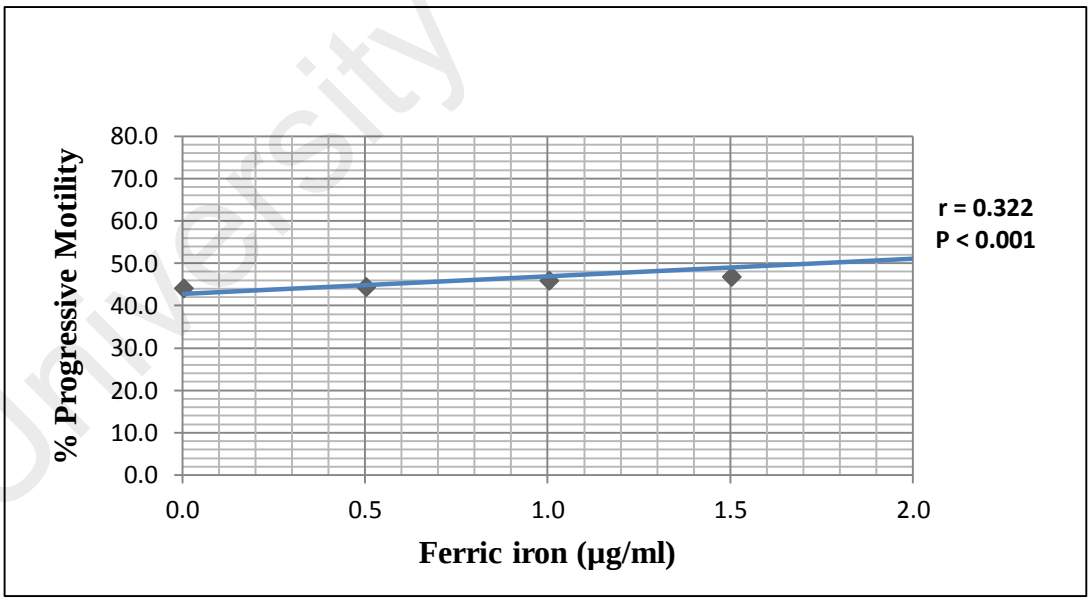

Figure 4.3: Weak positive correlation between different concentrations of ferric iron (Fe^{+3}) and progressive motility at 1 hr of exposure.

4.2.2 Tolerance investigation at 20 hours

The motility of spermatozoa in all treatments was similar to the control group with no significant difference at 20 hrs. The total motility of spermatozoa when compared individually between the treatments and the control group were increased significantly ($p< 0.05$) at 2.0 µg/ml of Fe^{+3} (Table 4.4 & Figure 4.4).

Furthermore, immotile spermatozoa percentage was decreased significantly at 2 µg/ml (T4) of Fe^{+3} when compared with the control group ($p <0.05$).

Table 4.4: Comparison of the effect of different concentrations of physiological levels of ferric iron against progressive and total motility at 20 hrs of exposure.

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation (Means ± SE)			
	C	T1	T2	T3	T4
µM/L	0	8.9	17.9	26.8	35.8
µg/ml	0	0.5	1.0	1.5	2
% Progressive Motility	24.5± 1.87	24.0± 2.04	25.2± 2.02	25.4± 1.96	30.5± 2.33
p. value		ns	ns	ns	ns
% Total Motility	43.0± 2.34	44.2± 2.91	46.1± 2.43	46.6± 2.23	52.0± 2.57
p. value		ns	ns	ns	0.046
% Immotile	57.0± 2.35	55.9± 2.91	53.9± 2.43	53.4± 2.23	48.0± 2.56
p. value		ns	ns	ns	0.046

ns= not significant

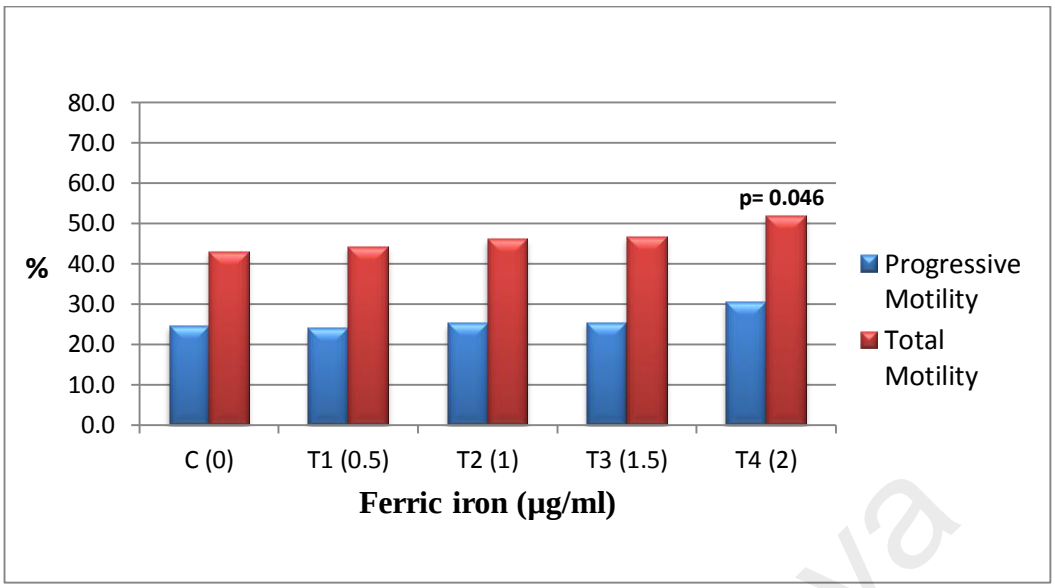

Figure 4.4: Proportion of spermatozoa exhibiting progressive and total motility at 20 hrs of exposure.

The correlation between increasing ferric iron concentration (within normal range of human plasma iron) and total motility was increased significantly when compared with the control group ($p < 0.05$) (Figure 4.5).

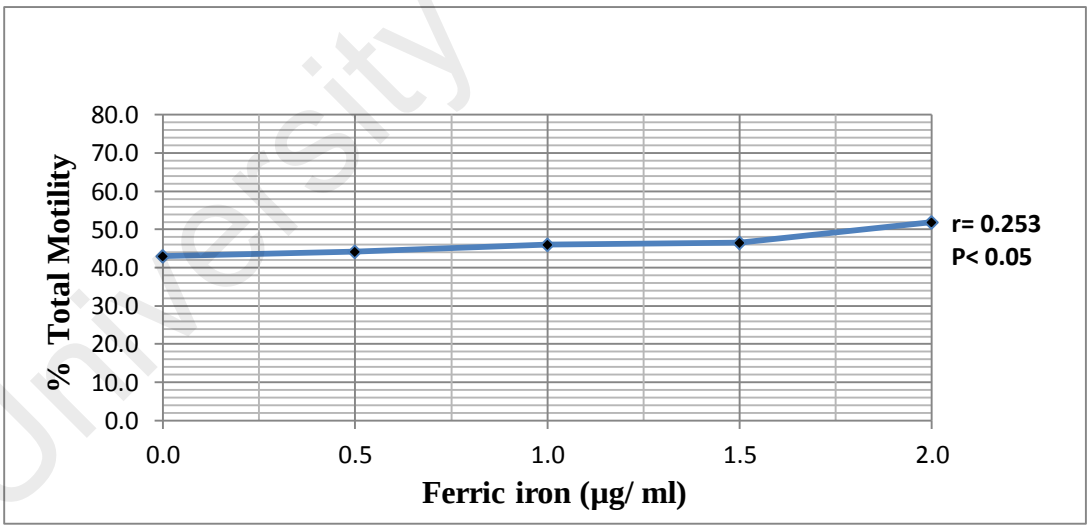

Figure 4.5: Correlation between different concentrations of ferric iron (Fe^{+3}) and total spermatozoa motility at 20 hrs of exposure.

There was a weak positive correlation ($r = 0.204$) between different iron concentrations (within normal range of human plasma iron) and control group at 20 hrs for progressive motility (Figure 4.6).

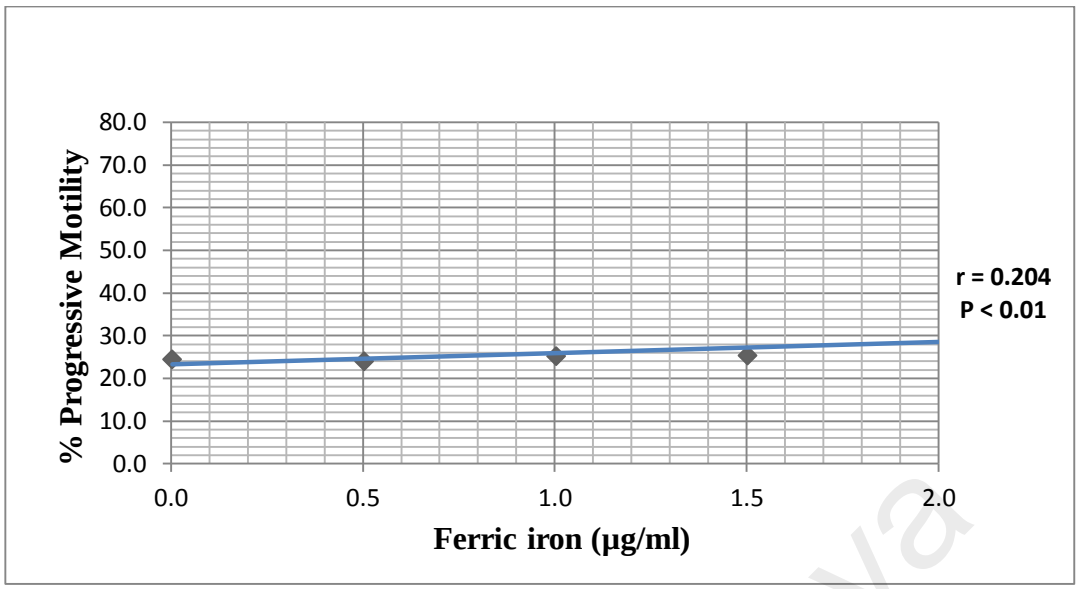

Figure 4.6: Weak positive correlation of different concentrations of ferric iron (Fe^{+3}) and progressive motility at 20 hrs of exposure.

4.2.3 Toxicity investigation at 1 hour

The percentage of total motility and progressive motility in all treatments of the toxicity investigation (4 µg/ml, 8 µg/ml, 12 µg/ml, and 16 µg/ml of Fe^{+3}) and control group expressed significant difference ($p < 0.05$). The progressive motility of spermatozoa for 12 µg/ml and 16 µg/ml (Tt3 & Tt4) of ferric iron was significantly lower than the control group at 1 hr of exposure. Furthermore, total motility had significantly decreased even further at 16 µg/ml (Tt4). Proportion of immotile spermatozoa increased in Tt2, Tt3 & Tt4 but only Tt4 was statistically different (Table 4.5).

Table 4.5: Comparison between the control group and the test groups for progressive and total motility of spermatozoa after 1 hr of (Fe^{+3}) exposure above the physiological levels of iron in HEPES buffered protein-free culture medium.

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation Means ± SE			
	C	Tt1	Tt2	Tt3	Tt4
µM/L	0	71.7	143.4	214.9	286.6
µg/ml	0	4	8	12	16
% Progressive Motility	44.2 ± 2.23	47.5± 2.21	38.8 ± 2.15	35.9 ± 2.42	29.7 ± 2.59
p. value		ns	ns	0.046	0.0001
% Total Motility	57.3 ± 2.23	58 ± 2.15	52.3 ± 2.19	50.4 ± 2.27	45.0 ± 2.36
p. value		ns	ns	ns	0.001
% Immotile	42.7 ± 2.23	41.3± 2.15	47.7 ± 2.20	49.6 ± 2.27	55.0 ± 2.36
p. value		ns	ns	ns	0.001

ns= not significant

Statistically, when the control group was considered as a treatment, the progressive motility of spermatozoa exposed for 1 hr to Tt3 and Tt4 of ferric iron was significantly lower than that of Tt1. Also the progressive motility in Tt1 was significantly higher than the control. Total motility decreased significantly after exposure to Tt4 (16 µg/ml Fe^{+3}) for 1hr compared to the control whereas it was significantly higher for treatment Tt1 compared than Tt4. Immotility of spermatozoa was significantly increased in Tt2, Tt3 and Tt4 compared to Tt1 and the control (Table 4.6)

Table 4.6: The effect of ferric iron concentrations above physiological levels of iron on progressive and total motility after 1 hr of ferric iron exposure in synthetic HEPES protein-free culture medium.

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation (Means ± SE)			
	C	Tt1	Tt2	Tt3	Tt4
µM/L	0	71.7	143.4	214.9	286.6
µg/ml	0	4	8	12	16
% Progressive Motility	44.2 ± 2.23	47.5± 2.21	38.8 ± 2.15	35.9 ± 2.42	29.7 ± 2.59
p. value		ns	ns	0.006 (Tt1)	0.0001 (C,Tt1)
% Total Motility	57.3 ± 2.23	58 ± 2.15	52.3 ± 2.19	50.4 ± 2.27	45.0 ± 2.36
p. value		ns	ns	ns	0.002 (C) 0.0001(Tt1)
% Immotile	42.7 ± 2.23	41.3± 2.15	47.7 ± 2.20	49.6 ± 2.27	55.0 ± 2.36
p. value		ns	ns	ns	0.002 (C) 0.0001(Tt1)

ns= not significant

Figure 4.7 shows progressive and total motility percentages for all treatments and control group of toxicity investigation at 1 hr.

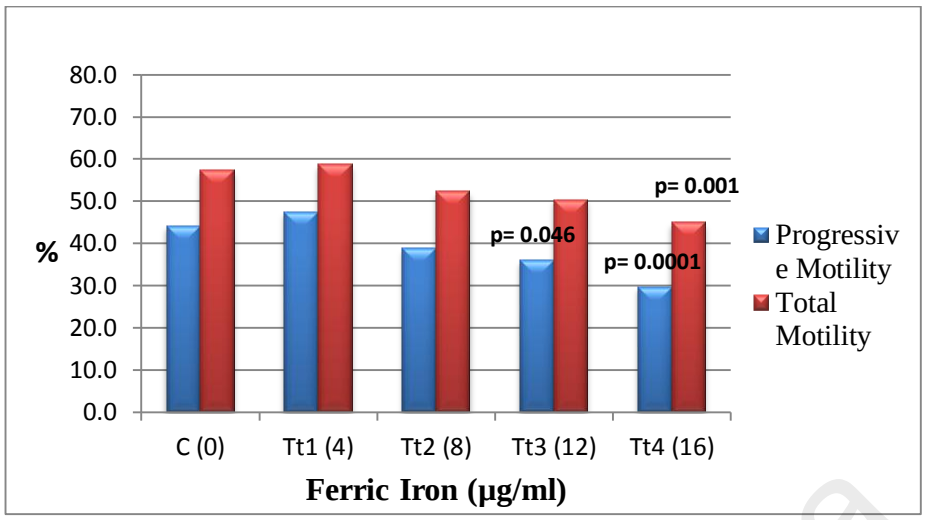

Figure 4.7: Progressive and total motility percentage of spermatozoa at 1 hr of toxicity investigation.

The correlation between increasing ferric iron (Fe^{+3}) concentration (more than the physiological level of human plasma iron) and total motility increased slightly (4.0 µg/ml Fe^{+3}) as compared to the control group. On the other hand, total motility decreased significantly when compared with the control group ($p < 0.05$) (Figure 4.8).

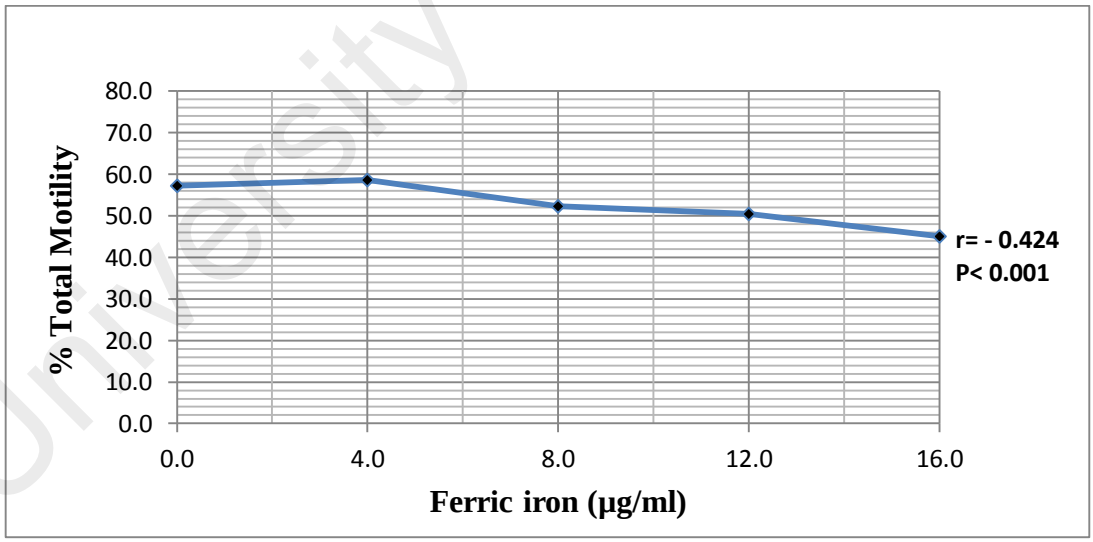

Figure 4.8: The relation of various concentrations of ferric iron (Fe^{+3}) with spermatozoa motility at 1 hr of toxicity investigation.

There is a moderate negative correlation ($r = -0.482$) between increasing iron concentration (more than physiological level in human plasma iron) with progressive motility at 1 hr of exposure (Figure 4.9).

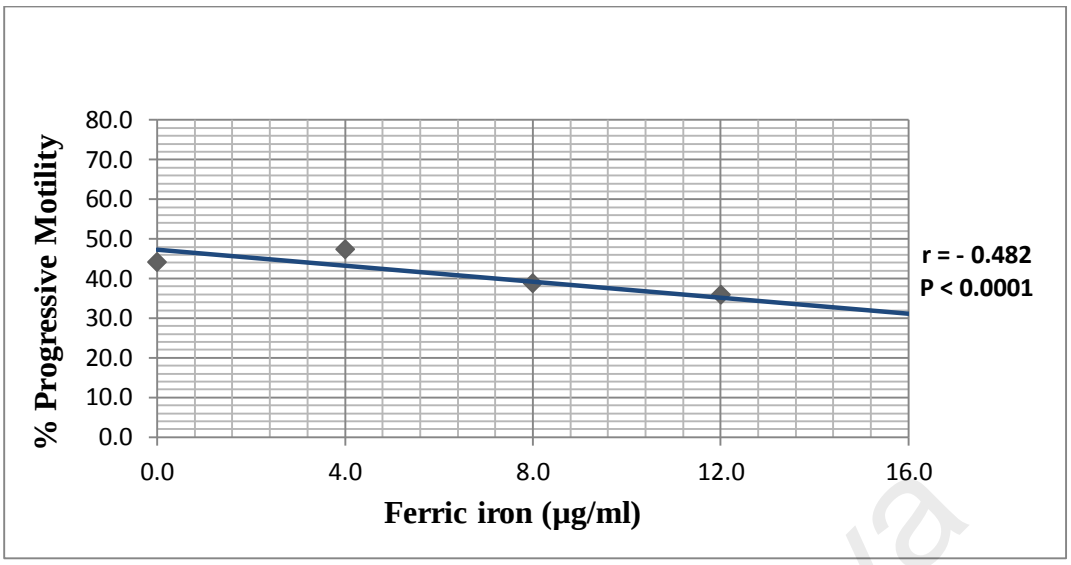

Figure 4.9: Moderate negative correlation between different concentrations of ferric iron (Fe^{+3}) and progressive motility at 1 hr of toxicity investigation.

4.2.4 Toxicity investigation at 20 hours

The one-way ANOVA test showed a significant difference in the percentage of total motility and progressive motility in all treatments of the toxicity investigations for ferric iron concentrations above the physiological level (4 µg/ml, 8 µg/ml, 12 µg/ml, and 16 µg/ml of Fe^{+3}) compared to the control group ($p < 0.05$). When compared individually between treatments and the Tt4 treatment group (16 µg/ml) showed significantly lower progressive motility than the control group. Besides that, progressive and total motility at Tt1 (4 µg/ml Fe^{+3}) was significantly increased compared to the control group. Conversely, immotile spermatozoa at Tt1 (4 µg/ml Fe^{+3}) was significantly lower than control group (Table 4.7).

Table 4.7: The effect of high concentrations of ferric iron on progressive and total motility of spermatozoa after 20 hrs of exposure in synthetic HEPES protein-free culture medium.

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation (Means ± SE)			
	C	Tt1	Tt2	Tt3	Tt4
µM/L	0	71.7	143.4	214.9	286.6
µg/ml	0	4	8	12	16
% Progressive Motility	24.5± 1.87	35.1± 2.31	21.2 ± 2.03	20.4 ± 1.78	15.9 ± 1.46
p. value		0.001	ns	ns	0.007
% Total Motility	43.0± 2.34	55.0± 2.45	40.2 ± 2.62	39.1 ± 2.16	35.0 ± 2.11
p. value		0.002	ns	ns	ns
% Immotile	57.0± 2.34	45.0± 2.45	59.8 ± 2.62	61.1 ± 2.16	65.1 ± 2.11
p. value		0.002	ns	ns	ns

ns= not significant

The progressive motility for Tt2, Tt3 and Tt4 (8 µg/ml, 12 µg/ml and 16 µg/ml, respectively) at 20 hrs was significantly lower compared to Tt1 (4 µg/ml Fe^{+3}). Moreover, total motility was significant decreased for Tt2, Tt3 and Tt4 (8 µg/ml, 12 µg/ml and 16 µg/ml, respectively) compared to control; while for Tt1 (4 µg/ml Fe^{+3}) it was significantly higher than the control. The proportion of immotile spermatozoa at Tt2, Tt3 and Tt4 (8 µg/ml, 12 µg/ ml and 16 µg/ml, respectively) was significantly higher than Tt1 (4 µg/ml Fe^{+3}) (Table 4.8).

Table 4.8: The effect of exposure to high concentrations ferric iron (Fe^{+3}) for 20 hrs on the progressive and total motility of spermatozoa cultured in synthetic HEPES protein-free culture medium.

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation (Means ± SE)			
	C	Tt1	Tt2	Tt3	Tt4
µM/L	0	71.7	143.4	214.9	286.6
µg/ml	0	4	8	12	16
% Progressive Motility	24.5± 1.87	35.1± 2.31	21.2 ± 2.03	20.4 ± 1.78	15.9 ± 1.46
p. value		0.001(C)	0.0001 (Tt1)	0.0001 (Tt1)	0.017 (C) 0.0001 (Tt1)
% Total Motility	43.0± 2.34	55.0± 2.45	40.2 ± 2.62	39.1 ± 2.16	35.0 ± 2.11
p. value		0.004 (C)	0.0001 (Tt1)	0.0001 (Tt1)	0.0001 (Tt1)
% Immotile	57.0± 2.34	45.0± 2.45	59.8 ± 2.62	61.1 ± 2.16	65.1 ± 2.11
p. value		0.004 (C)	0.0001 (Tt1)	0.0001 (Tt1)	0.0001 (Tt1)

ns= not significant

Figure 4.10 shows progressive and total motility percentage for all treatments and control group for the toxicity investigation at 20 hrs.

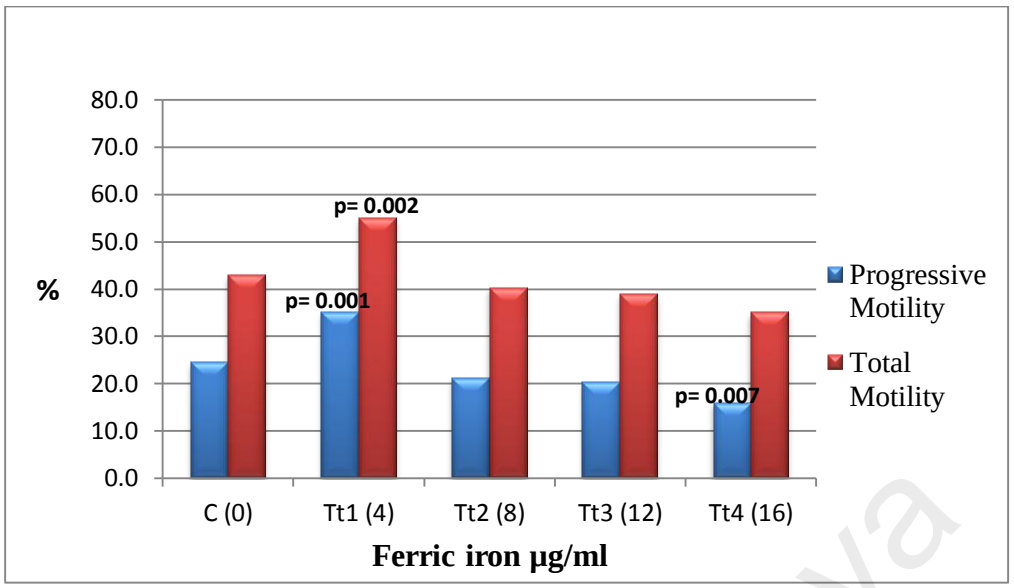

Figure 4.10: Progressive and total motility percentage of spermatozoa at 20 hrs of toxicity investigation.

The correlation between increasing ferric iron concentration (more than physiological level in human plasma iron) and total motility significantly increased at 4.0 µg/ml of Fe^{+3} as compared with the control group ($p < 0.05$) (Figure 4.11).

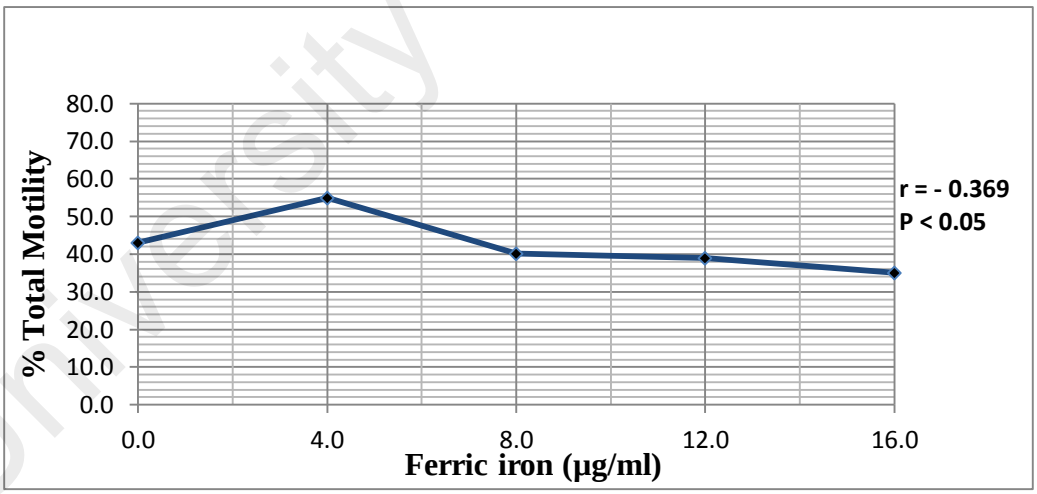

Figure 4.11: The relation of various concentrations of ferric iron (Fe^{+3}) with spermatozoa motility at 20 hrs.

A moderate negative correlation (r = -0.429) was shown between increasing iron concentration (more than physiological level in human plasma iron) with progressive motility at 20 hrs (Figure 4.12).

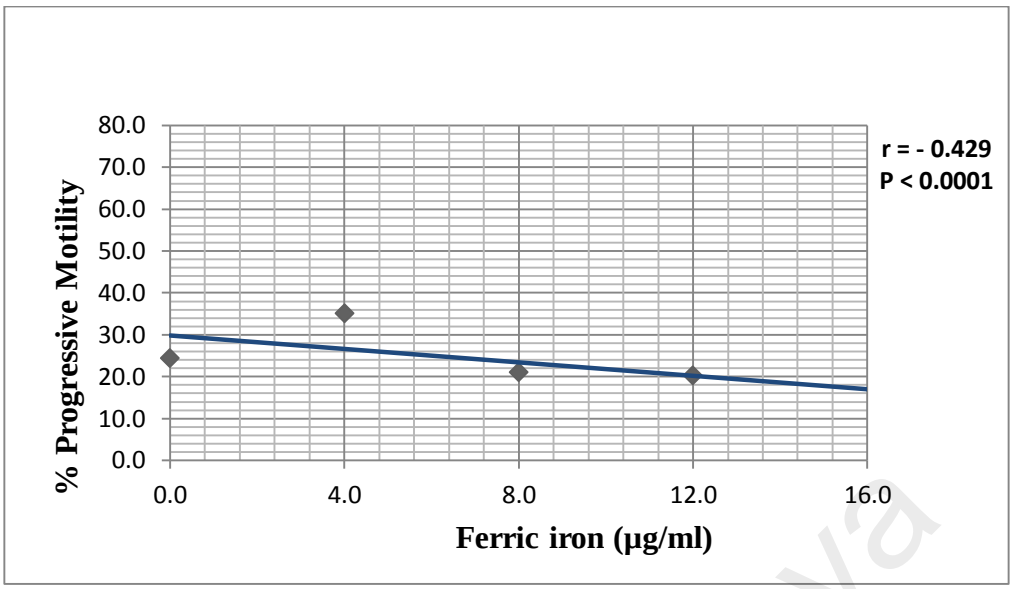

Figure 4.12: Moderate negative correlation between different concentrations of ferric iron (Fe^{+3}) and progressive motility at 20 hrs of toxicity investigation.

4.3 Spermatozoa vitality

4.3.1 Tolerance investigation

The vitality of spermatozoa at 1h was significantly higher in 2.0 µg/ml of Fe^{+3} supplementation media (within normal range of human iron plasma) than the control group ($p < 0.05$) (Table 4.9). However, there is no significant difference at 20 hrs in treatments found in the tolerance investigation (0.5 µg/ml, 1.0 µg/ml, 1.5 µg/ml, and 2.0 µg/ml) compared to the control media (Table 4.9).

Table 4.9: Spermatozoa vitality after supplementation with physiological levels of iron (Fe^{+3}) at 1 hr & 20 hrs of exposure.

Parameters	Control media	HEPES PFM media with Fe^{+3} supplementation Means ± SE			
	C	T1	T2	T3	T4
µM/L	0	8.9	17.9	26.8	35.8
µg/ml	0	0.5	1.0	1.5	2
% Vitality At 1 h	59.7± 2.21	60.4± 1.57	62.3± 1.50	62.6 ± 1.50	70.4 ± 1.70
p. value		0.001 (T4)	0.010 (T4)	0.016 (T4)	0.0001 (C)
% Vitality At 20 hrs	45.7± 2.21	46.1 ± 2.67	47.1 ± 2.62	47. 8 ± 2.57	54.1± 2.56
p. value		ns	ns	ns	ns

ns= not significant

4.3.2 Toxicity Investigation

Spermatozoa treated with high concentrations of ferric iron (namely 4 µg/ml, 8 µg/ml, 12 µg/ml, and 16 µg/ml (more than the physiological range of human plasma iron) for 1 hr, demonstrated significant drop in vitality compared to the control. Whereas after 20 hrs of exposure there was no significant difference in vitality levels between Tt1 or Tt2 and the control group. However, the vitality level for Tt3 was significantly lower compared to Tt1 and Tt4 was significantly lower compared to the control and Tt1. (Table 4.10).

Table 4.10: Spermatozoa vitality after ferric supplementation above the physiological levels of iron (Fe^{+3}) at 1 hr & 20 hrs of ferric exposure.

Parameters	Control media (C)	HEPES PFM media with Fe^{+3} supplementation Mean ± Se			
	HEPES	Tt1	Tt2	Tt3	Tt4
µM/l	0	71.7	143.4	214.9	286.6
µg/ml	0	4	8	12	16
% Vitality at 1h	45.7 ± 2.21	56.2 ± 2.02	41.2± 2.22	40.5± 1.74	36.3 ± 1.71
p. value		0.003 (C)	0.0001 (Tt1)	0.0001 (Tt1)	0.01 (C) 0.0001(Tt1)
% Vitality at 20hrs	59.7 ± 2.21	61.3 ± 1.94	55.0± 1.98	52.6± 2.11	47.1± 2.20
p. value		ns	ns	0.032 (Tt1)	0.0001 (C) 0.0001 (Tt1)

ns= not significant

4.4 Spermatozoa DNA Integrity test (SDI)

The comparison between control media (HEPES; 0µg/ml) and different concentrations of ferric (T4; 2.0, Tt1; 4.0, and Tt4; 16.0 µg/ml) showed that average SDI value was the highest in 2.0 µg/ml (78.0 ± 1.82 at 1 hr and 70.1 ± 2.86 at 20 hrs) than control and other treatments, but not significantly different ($p > 0.05$) at 1 hr and 20 hrs, respectively. While the lowest average SDI value was in 16.0 µg/ml compared to the control and the other treatments (69.9 ±1.56 at 1hr and 63.9 ± 1.75 at 20 hrs) without significant difference at 1 hr and 20 hrs as well ($p > 0.05$) (Table 4.11).

Table 4.11: Spermatozoa DNA integrity in presence of various levels of ferric iron.

Exposure Time	Parameter	% Spermatozoa DNA Integrity Means ± SE			
		C	T4	Tt1	Tt4
	µM/L of Fe^{+3}	0	35.8	71.7	286.6
	µg/ml of Fe^{+3}	0	2	4	16
1h	% SDI	72.6 ± 2.98	78.0 ± 1.82	74.7 ± 1.21	69.9 ± 1.56
	P. value		ns	ns	ns
20hrs	% SDI	65.4 ± 4.19	70.1 ± 2.86	65.4 ± 3.79	63.9 ± 1.75
	P. value		ns	ns	ns

ns= not significant

4.5 Mouse embryos development

4.5.1 Tolerance Investigation

Four stages of embryo development were performed to detect the effect of different concentrations of ferric iron (within normal human physiological level of plasma iron). Nine hundred and fifty four (954) of day 1, 2, 3 and 4 mice embryo with 4 to 6 replicates of sibling zygotes were used to perform this experiment (Table 4.12).

Table 4.12: Effect of different concentration of ferric iron levels (within physiological human level) on embryo development of different stages.

Days	Categories	Treatments					
		C	T1	T2	T3	T4	T5
		Iron Concentration ($FeCl_3.6H_2O$) µM/L					
		0	2	5	10	20	50
		Iron Concentration (Fe^{+3}) µg/ml					
		0	0.11	0.28	0.56	1.1	2.8
1	Alive	13(21%)	17(29.3%)	15(25%)	7(12.1%)	9(15%)	14(23.7%)
	Dead	49 (79%)	41(70.7%)	45(75%)	51(87.9%)	51(85%)	45(76.3%)
	p. value		ns	ns	ns	ns	ns
2	Alive	20(46.5%)	31(72.1%)	24(55.8%)	29(69%)	23(53.5%)	30(66.7%)
	Dead	23(53.5%)	12(27.9%)	19(44.2%)	13(31%)	20(46.5%)	15(33.3%)
	p. value		0.0158	ns	0.0355	ns	ns
3	Alive	24(77.4%)	29 (85.3%)	24(72.7%)	25(78.1%)	26(83.9%)	28(87.5%)
	Dead	7 (22.6%)	5 (14.7%)	9 (27.3%)	7(21.9%)	5 (16.1%)	4 (12.5%)
	p. value		ns	ns	ns	ns	ns
4	Alive	22 (88%)	18 (75%)	17(70.8%)	19(82.6%)	23 (92%)	20(83.3%)
	Dead	3 (12%)	6 (25%)	7 (29.2%)	4 (17.4%)	2 (8%)	4 (16.7%)
	p. value		ns	ns	ns	ns	ns

ns= not significant

4.5.1.1 Day one embryos

A total of 357 sibling zygotes was used in this experiment with 6 replicates for each treatment group. The development of embryos in all treatments from day 1 to day 6 of fertilization was not significantly different to the control group of sibling embryos (p

>0.05). A comparison of embryos development in control group in the daily development of embryos and supplemented iron media treatments showed no significant difference ($p > 0.05$) from lower to higher concentrations (0.11 to 2.8 µg/ml) of ferric iron (Table 4.12 & Figure 4.13).

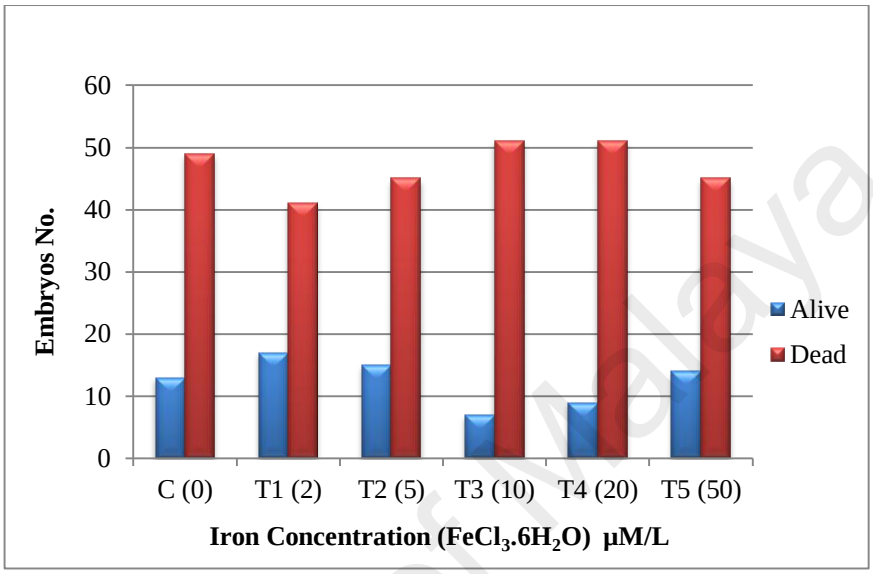

Figure 4.13: Live and dead of day one embryos in tolerance investigation.

4.5.1.2 Day two embryos

A total of 259 sibling zygotes was used in this experiment with 6 replicates for each treatment group. Embryos development in all treatments from day 1 to day 6 of fertilization was not significantly different to the control group of sibling embryos ($p >0.05$). A comparison of embryo development in the control group and the group with supplemented iron treatments showed significant increases at 2 µM/L = 0.11 µg/ml and 0.56 µg/ml ($p< 0.05$). The other treatments were not significantly different than the control group (Figure 4.14).

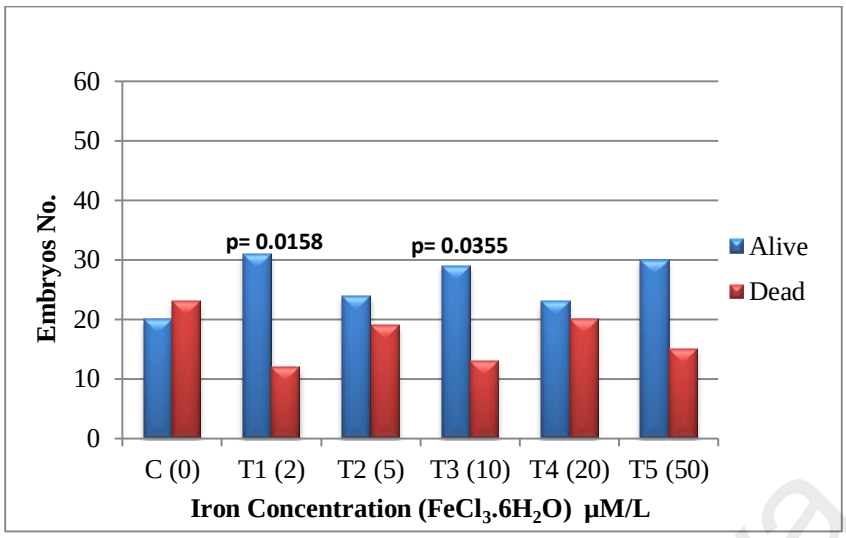

Figure 4.14: Live and dead of day two embryos in tolerance investigation.

4.5.1.3 Day three embryos

A total of 193 sibling zygotes was used in this experiment with 4 replicates for each treatment group. The development of embryos in all treatments from day 1 to day 6 of fertilization was not significantly different to the control group of sibling embryos ($p > 0.05$). A comparison of embryos development in the control group and in the treatments showed no significant difference between of them (Figure 4.15).

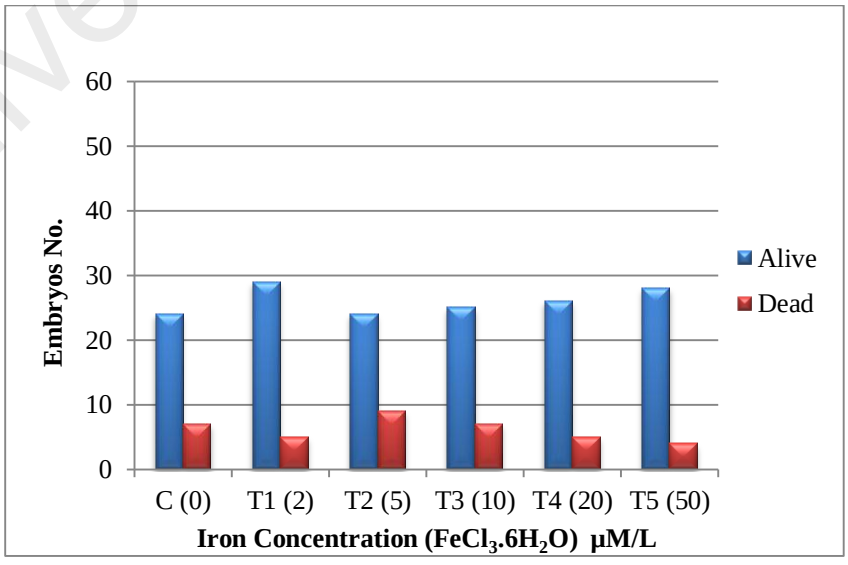

Figure 4.15: Live and dead of day three embryos in tolerance investigation.

4.5.1.4 Day four embryos

A total of 145 sibling zygotes was used in this experiment with 4replicates for each treatment group. Embryos development in all treatments from day 1 to day 6 of fertilization was not significantly different to the control group of sibling embryos ($p > 0.05$). The development of day 6 embryos (expanded, hatching and hatched blastocysts) was not significantly different for all treatments when compared to the control group ($p > 0.05$) (Figure 4.16).

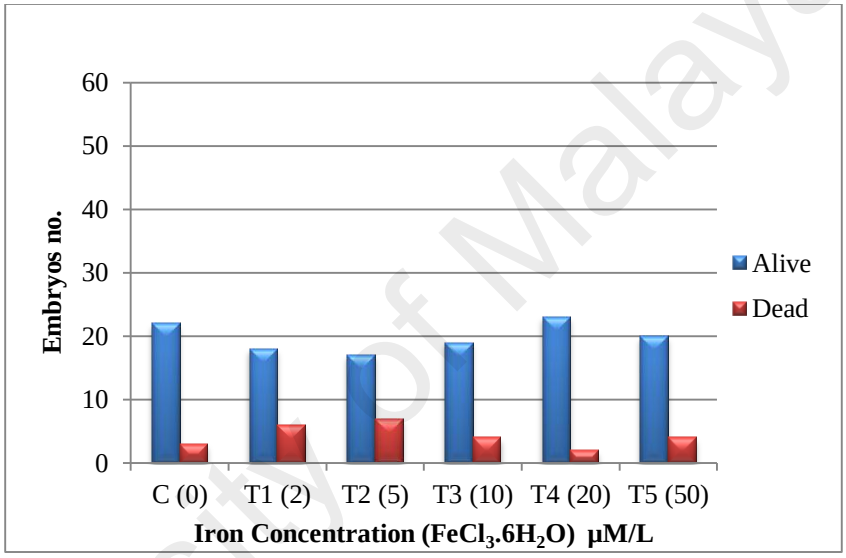

Figure 4.16: Live and dead of day four embryos in tolerance investigation.

4.5.2 Toxicity investigation

For the toxicity investigation, the effect of high concentrations of ferric iron (above the human physiological level of plasma iron) on day 2 embryos was detected. A total of 206 sibling zygotes of day 2 was used in this experiment with 5 replicates for each treatment group. Embryos development in all treatments from day 1 to day 6 of fertilization was not significantly different to the control group of sibling embryos ($p > 0.05$). However, the development of embryos was significantly lower for Tt3, Tt4 and

Tt5 treatments (16.8 µg/ml, 22.34 µg/ml and 27.9 µg/ml, respectively) when compared to the control group ($p < 0.05$) (Table 4.13 & Figure 4.17).

Table 4.13: Effect of added various ferric iron levels (above the physiological human level) on embryo development of day 2 embryos.

Categories	Treatments					
	C	Tt1	Tt2	Tt3	Tt4	Tt5
	Iron Concentration ($FeCl_3.6H_2O$) µM/L					
	0	100	200	300	400	500
	Iron Concentration (Fe^{+3}) µg/ml					
	0	5.6	11.2	16.8	22.3	28
Alive	18 (52.9%)	13 (38.2%)	15 (44.1%)	10 (28.6%)	10 (29.4%)	10 (28.6%)
Dead	16 (47.1%)	21 (61.8%)	19 (55.9%)	25 (71.4%)	24 (70.6%)	25 (71.4%)
p. value		ns	ns	0.0393	0.0487	0.0393

ns= not significant

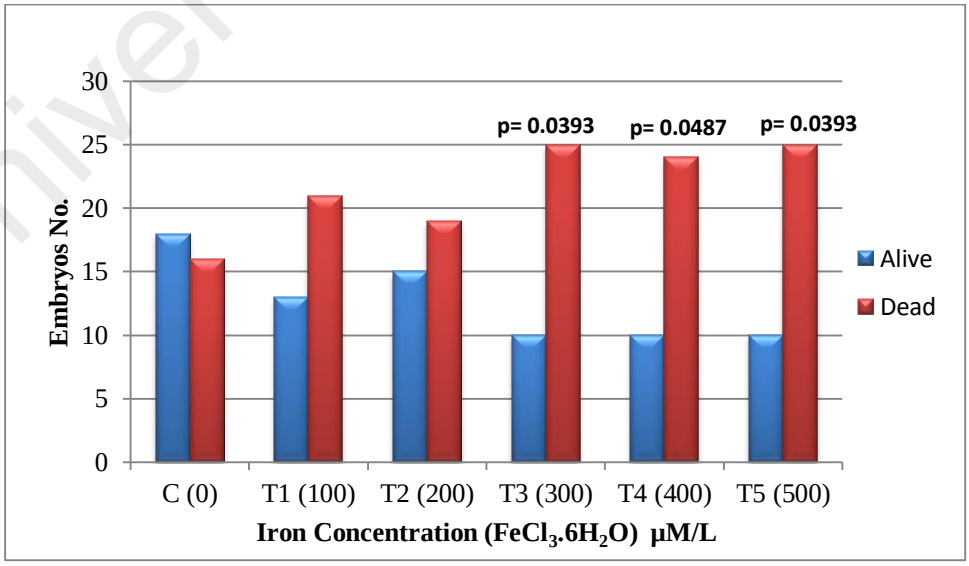

Figure 4.17: Live and dead of day two embryos in toxicity investigation.

CHAPTER 5: DISCUSSION

This is the first study on the human spermatozoa and mice embryonic development that has taken into account physiological concentrations of iron. Generally, iron is a main micronutrient which has the ability to preserve cellular homeostasis. However, it can disturb this accurate balance due to its role as an important catalyst for the reproductive dysfunction (Tvrda et al., 2015b). Testicular atrophy, epididymal lesions, morphological changes in testes, and defect in spermatogenesis and reproductive performance, are problems resulting from iron disproportionate levels (Merker et al., 1996; Lucesoli et al., 1999; Whittaker et al., 1997) Therefore, the main aim of this study was to determine the optimal or toxic levels of ferric iron on spermatozoa activity, sperm DNA integrity and mice embryos development in vitro to increase the knowledge about the role of iron in embryonic development.

5.1 Ferric iron effects on spermatozoa activity

The amount of iron present in synthetic protein-free medium as a contaminant is very low (0.004 µg/ml = 0.07 µM) but has proven sufficient for eliciting excellent in vitro fertilization (Ali, 2000; 2004). Present studies have shown that in vitro culture medium with ferric iron in physiological level of iron plasma of human (tolerance investigation) has significant effect on human spermatozoa motility and vitality with high percentage of DNA integrity for short and long term culture (1 hr and 20 hrs). It has been shown through in vitro studies that iron effects on spermatozoa activity dependent on culturing period in relation to the doses applied to culture media (Ali, 2000: 2004; Tvrda et al., 2012). Moreover, in vivo studies have shown that iron in mammalian seminal plasma was positively correlated to spermatozoa motility characteristics (Eghbali, 2012; Tvrda,

2015a). According to Eghbali et al. (2012) iron content in seminal plasma would assist spermatozoa motility and vitality after ejaculation.

The present study demonstrated that culture media with ferric iron supplementation (≤ 2 µg/ml (35.8 µM) to 4 µg/ml (71.6 µM)) has positive effect on spermatozoa motility and vitality at 1h and 20hrs of exposure. These findings were compatible with previous in vitro studies which was performed on animal semen. Knazicka et al. (2012) reported that the spermatozoa motility increased with in vitro concentration of ferrous iron $FeSO_4.7H_2O$ (≤ 62.50 µM) and iron in low concentrations (≤ 250 µmol/ L $FeSO_4.7H_2O$) sustained the motility of spermatozoa and energy of metabolism.

Tvrdá et al (2012, 2015a) found that low levels of iron (≤ 10 µmol/ L $FeCl_2$ and ≤ 50 µmol/L $FeCl_3$) were able to activate spermatozoa motility, in addition to stabilization of the oxidative balance. The authors suggested that concentrations of ferrous (50 µmol/L of $FeCl_2$) and ferric (100 µmol/L of $FeCl_3$; equivalent to 5.59 µg/ml of ferric iron) in vitro are critical. It will become toxic and a pro-oxidant substance (Tvrda, 2012, 2015a) at higher concentrations. It has also been reported that in vitro fertilization rates in mice was significantly improved when the spermatozoa was incubated with low concentrations of iron (Kodama et al., 1996; Tvrda, 2012, 2015a).

In spite of the previous conflicting reports, present study provide an insight into the optimal level of ferric iron needed to obtain the optimal activity for in vitro spermatozoa culture with high percentage of sperm DNA integrity compared to control medium (without iron supplementation). From the present study it could be inferred that human spermatozoa may not survive concentrations of iron above the 2.0 µg/ml for long periods of time. The optimal level of ferric iron was 2 µg/ml (35.8 µM) with tolerance levels range of 0.5- 2 µg/ml were beneficial for spermatozoa motility for short and long time of exposure (1 hr and 20 hrs). While 4 µg/ml had significantly positive effect on

motility at 20 hrs but the percentage of sperm DNA Integrity was lower than that in 2 µg/ml at 20 hrs.

Short and long duration of ferric exposure resulted in higher toxicity being expressed with increasing levels of iron. This study demonstrated that the toxic level of ferric iron was ≥ 16 µg/ml because of its negative effect on spermatozoa total motility and vitality beside its toxic effect on sperm DNA integrity at 1 hr and 20 hrs. In progressive motility studies, toxicity began at 12 µg/ml of ferric iron. The correlation of ferric iron increasing levels (more than physiological level of human iron serum level) and progressive motility was a moderate negative correlation at 1 hr and 20 hrs of ferric exposure.

5.2 Ferric iron effects on mice embryos development

Free iron present in synthetic protein-free culture medium as a contaminant is in very low concentration, 0.59 ppb (0.00059 µg/ ml = 0.0105µM) but has proven sufficient for clinical pregnancy and live birth rates in the human (Ali, 2000; Ali, 2013). The first aim in this part of this study was to determine at which stage of mice embryo development iron supplementation is needed in PFM culture medium and what is the optimal level of ferric iron. To achieve this objective, present experiments were performed at different stages of mouse embryo development with iron supplementation at the physiological level of iron based on the normal range in human plasma (tolerance investigation). There is no significant difference between the control and the treatments groups of all stages of day one, three and four of fertilization. The only significant different was on the second stage of the second day of fertilization (2- 4 cell embryos) at 2 µM/L (0.11 µg/ml) of ferric iron. It was significantly higher than the control group. In general, media supplemented with iron at 2 to 50 µM/L of ferric chloride (equivalent to 0.11 to

2.8 µg/ml) had the highest percentage of blastocysts than control in all treatments in the iron tolerance investigation.

Present study demonstrated that culture media supplemented with ferric iron (2 to 50 µM/L; equivalent to 0.11 to 2.8 µg/ml) has positive effect on mice embryos development in all stages of mice embryos. This finding was compatible with the work of Gao et al. (2007). The authors found that media containing iron (0.45 to 3.26 µg/ml) had higher rates of 8-cell embryos, morula, and blastocysts of bovine embryos than the control media (without iron). It was reported that the presence of iron in the culture media would accelerate the formation of 8-cell embryos, morula, and blastocysts (Gao et al., 2007).

The present investigation demonstrated that 2 µM/ L (0.11 µg/ ml) of iron was the optimal level and provide sufficient iron for the mice embryos at 2-cell stage in PFM media without serum proteins. This finding confirmed the work in a previous study (Ali, personal communication, 2016) on the effect of iron supplementation on mouse embryo development in vitro. The author noted that iron at even 2 µM/L concentration was sufficient to elicit an improved blastulation rate and embryo development in vitro in the presence of added donor serum proteins. Very minute concentration of iron may be sufficient for assisted reproduction procedures probably because the embryos have intracellular iron storage of maternal origin which provide sufficient iron to meet most physiological needs as previously suggested by Ali (2000, 2008, 2013).

This finding further proves the need for iron supplementation in embryo culture media. The consumption of trace elements below the standard requirement can cause deficiency (Goldhaber et al., 2003). According to Gao et al. (2007), the rate of blastocysts apoptosis in bovine embryos was decreased when it was cultured in trace amounts of iron supplemented media. In vitro culture of bovine embryos lacking of iron

had higher rates of apoptotic blastomeres. In addition, iron at physiological concentrations provided protection irrespective of metastatic potential of the cell lines (Mojic et al., 2014). As a result, a long-term lack of trace amounts of iron in culture medium would produce low quality of blastocysts (Gao et al., 2007).

The second aim of this study was to determine the toxic level of ferric iron on mice embryo development in vitro. The present investigation demonstrated that supplemented media with high level of iron i.e. more than the physiological level of human serum iron (toxicity investigation) has negative effects on mouse embryo development.

There is no significant difference between the control and the treatments groups of day two of fertilization at 5.6 µg/ml and 11.2 µg/ml with low rate of blastocysts comparing with the control group. In contrast, blastocysts in groups supplemented with 16.8, 22.3 and 28 µg/ml of ferric iron were significantly lower than the control group. Present study established that culture media with more than 300 µM/L (16.8 µg/ ml) of ferric iron had toxic effects on mice embryos development. Present study provides insight into the toxic level of ferric iron in the culture medium for both human spermatozoa and mice embryos which is surprisingly identical for both human spermatozoa and mouse embryos at the levels of 16 µg/ ml and above.

In so far, as human assisted reproductive technology is concerned, it is recommended that the 0.11 µg/ ml and 2 µg/ ml (2 µM/L and 35.8 µM/L, respectively) of ferric iron are recommended to be supplemented in the culture medium thus improve the quality of embryonic development. In contrast, it appears frank iron toxicity is expressed at the 16 µg/ ml and above for both human spermatozoa and mice embryos.

CHAPTER 6: CONCLUSION

In conclusion, the present study confirmed that iron present in protein-containing medium as a contaminant. Iron appears essential for embryo development but the amount of this element present in culture medium is just barely sufficient (and likely vary by batches) to meet the metabolic needs of the embryonic development. The amount of unbound free iron present in synthetic protein-free medium as a contaminant is very low but has proven sufficient for eliciting excellent in vitro fertilization, clinical pregnancy and live birth rates in the human.

The present study found that ferric iron at physiological levels similar to that in human plasma, especially at the 2.0 µg/ ml level (35.8 µM/ L) enhances the culture milieu by improving spermatozoa motility and spermatozoa DNA integrity. The rate of blastocyst formation in mice embryos was improved when the embryo culture medium was supplemented with 0.11 µg/ ml (2 µM/ L) of ferric iron.

On the other hand, our study provides insight about the toxic level of ferric iron in the culture medium for both human spermatozoa and mice embryos. Increasing the concentrations of ferric iron above physiological levels may cause hazardous for the embryonic development and possible to the in vitro fertilization procedure. It appears that iron has frank toxicity observed at 16 µg/ ml and above for both human spermatozoa and mice embryos.

Future studies using iron supplementation medium for washing mice spermatozoa and mice oocytes and in vitro fertilization. Subsequently return the recovered cultured embryos to female mouse as recipient are now required. This will be further improved by a purposely constructed study to measure the pregnancy rate, embryos safety, and long term effects of iron on the embryos. However due to the harmful nature of iron

prudence must be practiced in ensuring the amount of iron utilized in culture media are of the minimal level needed to obtain a positive response.

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