

**HISTOMORPHOLOGICAL STUDIES OF THE EFFECTS
OF AMODIAQUINE ON THE OVARY IN SPRAGUE-
DAWLEY RATS**

BY

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**HISTOMORPHOLOGICAL STUDIES OF THE EFFECTS OF
AMODIAQUINE ON THE OVARY IN SPRAGUE-DAWLEY RATS**

**THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE
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BY

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SEPTEMBER, 2010

DECLARATION

We hereby declare that the thesis titled “HISTOMORPHOLOGICAL STUDIES OF THE EFFECTS OF AMODIAQUINE ON THE OVARY IN SPRAGUE-DAWLEY RATS” is a record of original research work carried out by GBOTOLORUN, Stella Chinwe in the Department of Anatomy, College of Medicine of the University Of Lagos, Nigeria.

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DEDICATION

This work is dedicated to my darling husband Olalekan, and my lovely children, Ayooluwa and Anuoluwapo who have made my life more meaningful and purposeful. God bless you all.

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What will it profit a man if he gains the whole world and loses his soul? That is the biggest question everyone must ask himself/herself one day and I want to thank God Almighty that he has helped me answer this question truthfully and also to make the right choice. I want to also thank God for life and inspiration because “in Him I live and move and have my being”.

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ABSTRACT

The prevalence of female infertility ranges from 7 to 28% depending on the age of the woman. Although the origin and frequency of infertility vary about 40 to 50% of the aetiology of infertility cases is as a result of female factors. Malaria and the treatment of malaria have being a major challenge for decades. A number of antimalarial drugs have been reported to have anti-fertility actions.

Amodiaquine (AQ) is an anti-malarial drug and belongs to a class of drugs known as the 4-aminoquinolines. It was removed from the essential drug list in 1990 as a result of hepatotoxicity and agranulocytosis that occurred with long- term use. AQ is now making a comeback and is in the spotlight as a partner drug with artemisinins in the World Health Organization (WHO) recommended artemisinin based combination therapies (ACTs). In malaria-endemic regions of the world, self medication with antimalarial drugs is common and it is possible for a person to be treated several times in a year. With the frequency of treatment the risk of adverse events associated with long-term use of AQ may arise.

A number of investigators have carried out studies on the effect of AQ on pregnancy and pregnancy outcomes, however, there remains a dearth of literature on the short-term or long-term effect of AQ on the structure and function of the ovary in the non-pregnant female.

This study was carried out to investigate the histomorphological changes and responses of the ovary of Sprague-Dawley rats to AQ administration. The study was divided into 4 experimental groups. AQ was given at a dose of 6.0 and 12 mg/kg bw via oral and intraperitoneal routes for 28 days. Vitamin C was administered at a dose of 0.1 mg/kg bw 3 days in a week while Vitamin E was given at a dose of 20 mg/kg bw 5 days in a week. In each of the experiments AQ was administered alone, co-administered with

Vitamin C and co-administered with Vitamin E (AQ alone, AQ + Vitamin C and AQ + Vitamin E) according to the treatment protocol. At the end of the experiment all the animals were sacrificed by cervical dislocation.

Oestrus cycle was determined using the vaginal smear method and ovulation was determined at 10:00hr on the day of estrus. The ovary was dissected and processed for histology, assayed for superoxide dismutase and catalase activities and also assayed for reproductive hormones (follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL)).

The result of the oestrous cycle showed that AQ prolonged the length of the oestrous cycle and was statistically significant in the group that received 12 mg/kg bw via intraperitoneal route. Co-administration of AQ + Vitamin C increased the cycle length even further and was statistically significant ($p < 0.05$) except in the group that received 6 mg/kg bw intraperitoneal route. The ovulation studies showed that AQ reduced the number of ova that was shed in the morning of estrus and was statistically significant ($p < 0.05$) in the group that received 6.0 and 12 mg/kg bw oral route.

Histology of the ovary showed that AQ increased the number of atretic follicles when compared with the control. There appeared to be more atretic follicles at 12 mg/kg bw than at 6.0 mg/kg bw AQ. AQ caused reduction in the activities of superoxide dismutase and catalase in the ovary and was statistically significant ($p < 0.05$) in the groups that received AQ + Vitamin C.

The expected surge in FSH, LH and PRL between 5 to 7 p.m. on proestrus that is expected for the follicular rupture that occurs at ovulation was experienced in this study. There was no statistically significant difference in the serum concentration of these hormones when compared with the control group. The results obtained in this study shows that AQ is deleterious to the ovary.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

1.1.1 Incidence of Malaria

Malaria has afflicted humans for thousands of years. The earliest references to the periodic fevers characteristic of the disease are recorded from every civilized society; from China in 2700 BC through the writings of Greek, Roman, Assyrian, Indian, Arabic, and European physicians up to the 19th century (Cox, 2002). The earliest detailed accounts are those of Hippocrates in the 5th century BC, and thereafter there were increasing numbers of references to the disease in Greece and Italy and throughout the Roman Empire as its occurrence became commonplace in Europe and elsewhere (Cox, 2002).

Today, it remains the most prevalent vector-borne disease in the world; ranking among the major health and development challenges of the world. The disease is prevalent in almost 102 countries; approximately 40% of people worldwide are at risk (WHO, 1991). It is estimated that 300-500 million people are at risk of contracting malaria, with 900,000 new cases diagnosed each year (Murray and Lopez, 1994; Olliaro *et al.*, 1996). Malaria kills one child every 30 seconds. In absolute numbers, malaria kills 3000 children younger than five years of age per day (WHO, 1998).

There are 1-2 million deaths reported annually due to severe cerebral malaria with the majority of these deaths being children in Africa (Zucker and Campbell, 1993). It was estimated by the use of epidemiological, demographic and geographical parameters, that there were 515 million clinical attacks of *P. falciparum* malaria in the

year 2002 (**Snow *et al.*, 2005**). About 70.9% of the clinical events occurred in Africa, 23.1% in South East Asia, 2.9% in western Pacific region, 2.3% in the East Mediterranean, 0.7% in America and 0.1% in Europe (**Murray and Lopez, 1997; Hay *et al.*, 2004; Snow *et al.*, 2005**). The present level of annual global incidence of malaria continues to be about 500 million: 350 million in the African region and 150 million in the South East Asian region irrespective of the global malaria eradication programmes, such as the Roll Back Malaria (RBM) initiative which aims to halve the burden of malaria by 2010 (**Nabarro and Taylor, 1998; WHO, 2001a**) and the Millennium Development Goal (MDG) that targets to halt the rising incidence of malaria by 2015 (**Attaran *et al.*, 2004**).

Sub-Saharan Africa with more than 80% of the world's malaria cases is the focus of most efforts in combating the disease due to the high morbidity and mortality rates in this part of the world. Southeast Asia and South America are of interest because of the early development of drug resistance among malaria parasites in these regions. Over time, the human immune system adjusts to combating the malaria parasite and adult mortality in endemic areas is fairly low (**WHO, 1991; Steketee *et al.*, 1996**).

The mortality is concentrated among children younger than five years, travelers, migrants from non-malarial into malarial regions, and among populations with repressed immune system, including pregnant women and individuals suffering from Human immunodeficiency virus (HIV) (**WHO, 1991; Steketee *et al.*, 1996**).

1.1.2 History of Antimalarial Compounds

The search for a drug that can prevent rather than cure an infestation with the malaria parasite has been long and complex. It began in 1917 with the testing of a range of compounds on deliberately infected patients with terminal paralytic syphilis, before it was shown in 1924 that canaries could be used instead.

With the observation that the *plasmodia* parasite takes up the dye methylene blue, work initially focused on quinoline/methylene blue hybrids. Within a year, clinical trials showed that one such drug pamaquin could cure naturally acquired *falciparum* malaria. However, it took a longer time to realize that it worked by killing the sporozoites lurking in the liver, and not the merozoites liberated by cyclical liver-cell rupture into the blood as compared to quinine.

A range of 4- and 8- aminoquinolones were then studied during the Second World War by the American Army's Malaria Research Programme and one of such studies gave birth to chloroquine and then amodiaquine in the late 1940s.

Resistance to chloroquine in *plasmodium flaciparum* malaria has become a major health concern of the developing world. This resistance has prompted a re-examination of the pharmacology of alternative anti-malarial compounds that may be effective against resistant strains in order to address this long standing problem (WHO, 2000).

1.1.3 Amodiaquine (AQ)

The synthesis of this 4-aminoquinoline compound was first reported at the meeting of the American Chemical Society in 1946 and has since been widely used mainly as an antimalarial drug (Kotecka *et al.*, 1997; Ngouesse *et al.*, 2001; Thomas *et al.*, 2004). Interestingly however, since its introduction its various therapeutic properties have also been explored. AQ is similar in structure and activity to chloroquine (CQ). It was generally used as a more palatable oral alternative to chloroquine (Olliaro *et al.*, 1996).

Most experts in malaria believe that AQ is still useful and effective in areas of low-grade CQ resistance (Nevill *et al.*, 1994; Muller *et al.*, 1996; Olliaro *et al.*, 1996). In Africa, AQ is significantly more effective than CQ in parasite clearance, with a faster clinical recovery (Schellenberg and Menendez, 2001; Olliaro and Taylor, 2004)

even in areas of high chloroquine resistance (**Olliaro et al.**, 1996; **Brasseur et al.**, 1999). However, this does not apply to Asia where *in-vivo* studies in India did not find any advantage in treating chloroquine resistant *Plasmodium falciparum* infections with AQ (**Barkakaty et al.**, 1980; **Misra et al.**, 1995). The use of AQ declined abruptly in 1986 and was eventually removed from the Essential Drug List in 1990 following reports of serious adverse events (**Anon**, 1990). When used for malaria chemoprophylaxis, AQ was associated with agranulocytosis, aplastic anemia and hepatotoxicity (**Neftel et al.**, 1986; **Markham et al.**, 2007). The frequency of agranulocytosis was estimated as 1 in 2,200 and that of serious hepatotoxicity as 1 in 15,650. The overall fatality rate was estimated as 1 in 15,650 (**Phillip-Howard and West**, 1990).

Subsequent re-evaluation led to appreciation that the risk of toxicity associated with short-term AQ treatment appeared to be lower than that associated with AQ chemoprophylaxis (**Olliaro et al.**, 1996). AQ is now recommended by the World Health Organization (WHO) for use in combination regimens to treat malaria (**WHO**, 2001b; 2006a).

1.1.4 Chemical Structure of Amodiaquine

AQ is chemically 4-[(7-chloro-4-quinolinyl) amino]-2-[(diethylamino) methyl] phenol dihydrochloride dehydrate. The salt is synthesized from 4, 7- dichloroquinoline and 4 – acetamido- diethylamino-o-cresol. It is also synthesized from 2- aminomethyl-p-aminophenol and 4, 7-dichloroquinoline (**The Merck Index**, 1983). The structural formula is $C_{20}H_{22}ClN_3O \cdot 2HCl \cdot 2H_2O$ with a molecular weight of 464.8g/mol.

AQ is known to exist in anhydrous, monohydrate and dihydrate forms. It is a yellow crystalline powder, odourless or almost odourless with a bitter taste often formulated as a dihydrochloride salt. It is soluble in water and ethanol but practically insoluble in

benzene, chloroform and ether. A 1% aqueous solution has a pH value of 4.0- 4.8 (**The Merck index**, 1983). The powder decomposes at temperatures between 150-160 °C.

1.1.5 Uses of Amodiaquine

AQ was formulated as a more palatable oral alternative to CQ. It has been in use for over 60 years. In addition to its antimalarial activity, AQ hydrochloride is also used in the treatment of teniasis, giardiasis, and amoebic liver abscess (**Mein**, 1951; **Gordon et al.**, 1955; **Wayne and Fernando**, 1993). It has also been used in the treatment of *Wuchereria bancrofti* and *microfilariae* (**McMahon**, 1979).

As a result of its anti-inflammatory and antipyretic properties, AQ is used in the treatment of lupus erythematosus, rheumatoid arthritis, and lepra reactions (**Bepler et al.**, 1959; **Pomeroy et al.**, 1959; **Ngouesse et al.**, 2001). AQ has also been used for mass administration as a medicated salt (**Thomas et al.**, 2004).

1.2 STATEMENT OF THE PROBLEM

The prevalence of female infertility ranges from 7 to 28%, depending on the age of the woman (**Yu and Yap**, 2003). Although the frequency and origin of different forms of infertility vary, however, 40 to 50% of the etiology of infertility cases is due to female factors (**Duckitt**, 2003; **Sule et al.**, 2008).

A number of authors have reported on the antifertility actions of some antimalarial drugs: CQ (**Okanlawon et al.**, 1990; **Adeeko et al.**, 1992; **Okanlawon and Ashiru**, 1992; **Oforah et al.**, 2004; **Asuquo et al.**, 2007; **Clewell et al.**, 2009), proguanil (**Singer et al.**, 2008), pyrimethamine (**Awoniyi et al.**, 1993) and quinine (**Osinubi et al.**, 2004; **Osinubi et al.**, 2007). AQ belongs to the class of 4-aminoquinolines. It is similar in structure and antimalarial activity with CQ. CQ has been reported to disrupt

the oestrous cycle, block ovulation and consequently reduce fertility in Sprague-Dawley rats (**Okanlawon and Ashiru, 1992**).

The alarming spread of parasite resistant strains to chloroquine has prompted the re-examination of antimalarial compounds in order to address this long-standing problem (**WHO, 2000**).

AQ is now making a come-back after its removal from the essential drug list as a result of agranulocytosis and hepatotoxicity (**Anon, 1990**). It is now in the spotlight as a partner drug in the WHO recommended Artemisinin Based Combination Therapies (ACTs) (**WHO , 2001b; 2006a**).

In malaria-endemic countries, self medication with antimalarial drugs is common (**Perret and Ngomo, 1993; Minzi et al., 2003**) and it is possible that a person be treated several times per year.

Although, when AQ is administered in the treatment of uncomplicated malaria, severe adverse reactions seem to be rare (**Olliaro and Taylor, 2004**). However, in countries where AQ is the first-line drug whether alone as a single regimen or in combinations such as with artesunate (AS-AQ), the issue of the toxicity of repeated treatments in malaria-endemic areas may arise.

AQ is also used in the treatment of chronic disorders such as lupus erythematosus, rheumatoid arthritis and lepra reactions (**Bepler et al., 1959; Pomeroy et al., 1959; Ngouesse et al., 2001**).

A few investigators have attempted to study the effect of AQ on pregnancy and pregnancy outcomes (**Steketee et al., 1987; Naing et al., 1988; Thet et al., 1988; Kone et al., 2002; Thomas et al., 2004**).

However, there remains a dearth of literature on the short-term or long-term effect of AQ administration on the structure and reproductive function of the ovary in the non-pregnant female.

1.3 OVERALL OBJECTIVE OF THE STUDY

The overall objective of this study is to determine the histomorphological changes and responses of the ovary of Sprague-Dawley rats to AQ administration.

1.4 SPECIFIC OBJECTIVES OF THE STUDY

The specific objectives of the study include the following: To

1. Investigate the effect of administration of AQ on the oestrous cycle and on ovulation in adult cyclic Sprague-Dawley rats.
2. Investigate the effect of AQ administration on the reproductive hormones: Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and Prolactin (PRL).
3. Determine the effect of AQ on enzymatic antioxidant status in the ovary and the co-administration of Vitamins, C and E.
4. Determine the histomorphological changes in the ovary following chronic administration of AQ in adult cyclic Sprague-Dawley rats.

1.5 SIGNIFICANCE OF THE STUDY

The result of this study will shed light on the morphological and physiological changes and responses of the ovary following AQ administration. This study will also provide information on the effect of the administration of AQ on fertility and fertility outcomes in females of reproductive age.

1.6 OPERATIONAL DEFINITIONS OF TERMS

ANTI-OXIDANTS – Substances that mop up or scavenge free radicals in the body. They minimize or prevent oxidation of vital components of the cells of the body by free radicals.

AMODIAQUINE – Belongs to the 4-aminoquinoline class of antimalarial drugs.

CATALASE – Catalase is an enzymatic anti-oxidant that detoxifies hydrogen peroxides generated by the action of superoxide dismutase.

FOLLICLE-STIMULATING HORMONE – Secreted by the gonadotropic cells of the anterior pituitary promotes ovarian follicle development and estrogen secretion in females.

LUTEINIZING HORMONE – Secreted by the gonadotropic cells of the anterior pituitary promotes ovarian follicle maturation, ovulation and progesterone secretion in females.

OXIDATIVE STRESS – Occurs as a result of imbalance between anti-oxidants and pro-oxidants.

PROLACTIN – Secreted by the mammatropic cells of the anterior pituitary, promotes milk secretion.

SUPEROXIDE DISMUTASE – Superoxide dismutase is an enzymatic anti-oxidant that catalyses the dismutation of superoxide radicals.

VITAMIN E – Is the major lipid-soluble anti-oxidant present in all cellular membranes.

VITAMIN C – Is regarded as the most important water-soluble antioxidant in human plasma and mammalian cells.

CHAPTER TWO

LITERATURE REVIEW

2.1 METABOLISM OF AMODIAQUINE

AQ has been used as both an antimalarial and an anti-inflammatory agent for more than 40 years. AQ exerts selective toxicity towards the erythrocytic stages of malaria parasites and often shows adequate clinical-parasitological efficacy in CQ-resistant infections. The usual route of administration for therapeutic use is by oral administration. However, AQ has been given by both constant rate intravenous injection and constant rate infusion in volunteers and patients (**White *et al.*, 1987**).

In humans after oral administration, AQ hydrochloride is rapidly absorbed and undergoes extensive metabolism into 4 metabolites which concentrate in blood cells. Two of these have been identified; one is desethylamodiaquine the principal biologically active metabolite and the other have been tentatively identified on the basis of the proton nuclear magnetic resonance spectroscopy as 2-hydroxydesethylamodiaquine. It has been suggested that desethylamodiaquine, and not AQ is responsible for most of the observed anti-malarial activity, and that the toxic effects of AQ after oral administration may in part be due to desethylamodiaquine (**Winstanley *et al.*, 1987**).

When given orally it is absorbed through the gastrointestinal tract where it undergoes a first order pharmacokinetics in such a way that very little orally administered AQ escapes untransformed into the systemic circulation and accumulates in the liver, kidneys, lung and spleen (**Ruscoe *et al.*, 1998**). In the liver, AQ is rapidly transformed into its active metabolite desethylamodiaquine which accumulates in blood cells, mainly in neutrophils (**Naisbitt *et al.*, 1997**; **Ruscoe *et al.*, 1998**).

In the rat, AQ is excreted in bile exclusively as the 5 thioether conjugate glutathione and cysteinyl (**Harrison *et al.***, 1992). In a study to determine the tissue distribution and excretion of AQ in rats, ¹⁴C-labelled AQ (14CAQ) was administered to male Wistar rats by oral and intravenous routes n = 6 for each route of administration. Excretion of total ¹⁴C-activity was predominantly in the faeces after both oral and intravenous administration. After oral administration 86± 8.3% (mean ± s.d) of the ¹⁴C administered had been excreted 77 ± 9% in the faeces, 7 ± 1% in the urine and 2 ± 2% in cage washings over 72h. Of the ¹⁴C administered, 4 ± 1% were recovered from the tissues, and this was widely distributed, with the main organs of accumulation being kidney, liver, red bone marrow and spleen. After intravenous administration, 102.6 ± 9.7% of the ¹⁴C had been excreted 90.9 ± 9.6% in faeces, 10.9 ± 0.8% in urine and 0.5 ± 0.2% in cage washings over 72h.

In healthy adults, the peak concentrations of AQ in whole blood and packed cells are 60 ± 10ng/ml at 0.5 ± 0.1h and 42 ± 6ng/ml at 0.5 ± 0.1h respectively. Thereafter it rapidly declines and is detectable for no more than 8h. The mean peak plasma concentration of desethylamodiaquine was 181 ± 26ng/ml for both whole blood and packed cells. **Winstanley *et al.***, (1987) recorded the times to peak for whole blood and packed cells as 2.2 ± 0.5h and 3.6 ± 1.1h respectively. **White *et al.***, (1987) reported that the distribution half times observed after intravenous injection of 3 mg base per kg over 10 minutes in healthy adult male volunteers was significantly faster than those observed after intravenous infusion of 10 mg base per kg over 4 hours in adults with *falciparum* malaria. The authors suggested that there was probably an additional distribution phase in the malarial patients obscured by the slower rate of infusion. High drug concentrations are found in the malaria parasite's digestive vacuoles (**AFHS**, 1988). The apparent terminal half-life of AQ was 5.2 ± 1.7 (range 0.4 to 5.5) minutes

and the geometric mean of the estimated elimination phase half-life were 2.1 (range 0.5 to 5.7) hours (**White et al.**, 1987).

AQ is closely related to CQ differing only by having a β -hydroxylanilino aromatic ring in its side chain. It is a more active inhibitor than CQ of the growth of *P. falciparum in vitro* (**Ekweozer et al.**, 1987). AQ competitively inhibits CQ accumulation and vice versa, suggesting that these compounds share a similar mechanism of accumulation (**Fitch**, 1973). Like CQ, AQ is a diprotic weak base, but the pKa values are lower (pKa₁ = 7.1, pKa₂ = 8.1).

AQ is accumulated more efficiently in the parasite's food vacuole. The parasite degrades hemoglobin in an acidic food vacuole, producing free heme and reactive oxygen species as toxic by-products. Within the vacuole AQ interferes with the polymerization of heme and the detoxification of the reactive oxygen species, effectively killing the parasite with its own metabolic waste (**Foley and Tilley**, 1998).

2.2 TOXICITY DATA ON AMODIAQUINE

Although AQ has been used in the treatment of malaria since 1946, concerns about safety of this drug still remain. AQ was thought to have a similar toxicity to CQ but it soon became evident that there were differences between the two compounds. Agranulocytosis and concomitant liver damage were reported as early as 1959 in rheumatoid patients with chronic exposure to AQ (**Bepler et al.**, 1959; **Pomeroy et al.**, 1959). All rheumatoid arthritis patients treated with daily AQ at a dose of 400 mg had mild toxic symptoms similar to those reported after CQ gastrointestinal manifestations such as anorexia, nausea, vomiting, abdominal cramps, and diarrhoea, and neurological manifestations such as tinnitus, depression, malaise, somnolence and tremors (**Bepler et al.**, 1959). A mild hepatic damage was reported in 10 of 29 patients towards the end

of a 7-month AQ treatment period and one patient died from malignant hepatoma after completion of treatment (**Bepler et al.**, 1959). In 1957, fatal agranulocytosis was reported in a patient with discoid lupus erythematosus treated with AQ for 8 weeks at 200 mg daily. Another patient developed jaundice, fever and agranulocytosis and died after 8 weeks of daily administration of 400 mg of AQ (**Glick**, 1957; **Kersley and Palin**, 1959).

Although symptomatic cardiac effects have not been reported after administration of 4-aminoquinolines, the electrocardiographic changes produced by CQ in healthy volunteers and malaria-infected patients are of potential concern in areas where AQ is extensively used because of the similarities in chemical structures and properties of CQ and AQ (**Bustos et al.**, 1994; **Sowunmi et al.**, 1999). In two studies to investigate the electrocardiographic effects of AQ in symptomatic, malaria-infected patients, and to correlate these changes with plasma concentrations of AQ and those of monodesethylamodiaquine, the authors reported that sinus bradycardia occurred frequently in these patients (**Huang et al.**, 1988; **Benjamin et al.**, 2001).

In two other studies the authors observed that sinus bradycardia and sinus arrhythmia occurred frequently up to 68% in the course of treatment with AQ (**Ekue et al.**, 1983; **Nosten et al.**, 1993). A more pronounced effect was seen in heart rate and QTc interval suggesting that the electrocardiographic effects of AQ may mimic to some extent those of quinidine and other IA antiarrhythmic agents such as disopyramide and procainamide which block the sodium channel (**Benjamin et al.**, 2001). These authors suggested that AQ should not be prescribed to patients with known congenital prolonged QTc syndrome.

A study in Kampala, Uganda compared the efficacy and safety of AS-AQ combination for the treatment of uncomplicated malaria in a cohort of HIV-infected children

receiving standard HIV care with the efficacy and safety of such treatment in a cohort of HIV-uninfected children. A total of 300 HIV-infected children were enrolled from a pediatric HIV clinic from October 2005 through August 2006. AS-AQ treatment was administered as follows: AQ; 10 mg/kg bodyweight on the first 2 days, and 5 mg/kg bodyweight on the third day and artesunate (AS); 4 mg/kg bodyweight for 3 days. The results showed that AS-AQ is efficacious for the treatment of uncomplicated malaria in both HIV-infected children and HIV-uninfected children (**Gasasira et al.**, 2008).

However, treatment with AS-AQ was associated with a remarkably higher risk of neutropenia in HIV-infected children compared with HIV-uninfected children. This neutropenia appeared to have clinical consequences, because HIV-infected study participants had an increased risk of pneumonia during neutropenic episodes, compared with matched control subjects. Neutropenia associated with AS-AQ treatment was most likely caused by AQ. This is because artemisinins have excellent safety profiles and the addition of AS to other drugs does not appear to adversely affect safety and tolerability (**Ribeiro and Olliaro**, 1998; **Price et al.**, 1999; **Adjuik et al.**, 2004). In contrast, neutropenia is a well documented, albeit uncommon, adverse effect associated with AQ (**Olliaro et al.**, 1996). Furthermore AQ and its metabolites exhibit cytotoxic effects on mononuclear leukocytes and inhibit granulocyte-monocyte colony formation (**Winstanley et al.**, 1990). Rate ratios of serious toxicity associated with AQ when used as chemoprophylaxis were reported as 1:2100 for blood dyscrasias and 1:31,000 for deaths due to blood dyscrasias (**Phillips-Howard and West**, 1990). A 6% risk of neutropenia that was observed in an HIV-uninfected cohort is consistent with prior studies of AQ safety (**Olliaro et al.**, 1996; **Adjuik et al.**, 2004).

2.3 AMODIAQUINE AND PREGNANCY

Few antimalarial drugs have been evaluated extensively in pregnancy because of fears over toxicity (**Tagbor et al.**, 2006). However, the most common complications of malaria are seen during pregnancy (**Goldsmith, 1998**).

Pregnant women are especially vulnerable to malarial infection. In endemic countries they have a higher risk for peripheral *parasitaemia* and placental malaria, with higher incidences in the first pregnancy (**Olliaro and Taylor, 2004**). Anaemia and low birthweight are adverse consequences associated with placental malaria infection and are more common in first or second pregnancies (**Brabin and Verhoeff, 2002**).

A recent review indicates that the percentage of maternal deaths in Africa attributable to malaria ranged from 0.5– 23.0% in hospital studies and 2.9– 17.6% in community studies. Malaria-related maternal mortality rates during the past three decades have ranged from 1– 215 per 100 000 livebirths for hospital studies and 19 to 634 per 100 000 livebirths for community studies (**Brabin and Verhoeff, 2002**).

Intermittent preventive treatment (IPT) consists of at least two complete treatments of an effective antimalarial drug for all pregnant women at the beginning of the second and third trimester. IPT has a beneficial effect on the mother and child's health by preventing or eliminating current malaria infection and its consequences (**Thomas et al.**, 2004). However, information on the safety of and efficacy of drugs other than sulphadoxine-pyrimethamine to be given as IPT is very limited. This lack of data can be attributed to the systematic exclusion of pregnant women from drug trials for fear of toxicity to the fetus (**Nosten and McGready, 2003; Tagbor et al.**, 2006).

AQ can be effective where CQ resistance is high and it is a likely alternative, particularly when used in combination with another drug. However, its use in pregnancy raises several questions, the major one being about safety. A few studies

have investigated the safety of AQ during pregnancy (**Naing *et al.*, 1988; Thomas *et al.*, 2004; Nosten *et al.*, 2006**).

Indeed most of the publications did not collect any information on severe adverse events or at least they were not reported (**Thomas *et al.*, 2004**). No studies investigated AQ administration during gestation on the developing animal fetus; thus, no teratogenic data are available for organogenesis or structural abnormalities. Although no data is available for AQ, AQ like CQ may cross the placental barrier. Congenital abnormalities that have been noted in a few cases of CQ use during pregnancy are Tetralogy of Fallot, Congenital Hypothyroidism, Chorioretinitis and Deafness but no significant differences from the control group were seen (**Wolfe and Cordero, 1985**).

During pregnancy, chemoprophylaxis with AQ is contraindicated and there is no consensus for its use in treating clinical malaria. Some researchers believe AQ should not be used during pregnancy (**Kone *et al.*, 2002**). Other researchers believe its use should be restricted when no alternatives are available since there is not enough data on AQ safety (**Thomas *et al.*, 2004**). In a study in Kenya, 23 pregnant women who did not respond to CQ therapy were treated with AQ. At day 7 after treatment, 78% of the women were aparasitemic and all the treatment failures were reported in first pregnancies. However, no information was provided on adverse effects or on pregnancy outcomes (**Steketee *et al.*, 1987**).

AQ is now being implemented as first-line treatment alone in Cameroon or in combination with other drugs in Rwanda, Burundi, and Zanzibar in Tanzania (**Thomas *et al.*, 2004**). In these regions, pregnant women with clinical malaria are treated with AQ but there is little information on its safety and efficacy during pregnancy and none on its use as intermittent preventive treatment (**Thomas *et al.*, 2004**).

Two studies reported AQ treatment during pregnancy at a dose of 25 mg/kg over 3 days. Pregnant and non pregnant women attending Sao San Htun Hospital in Taunggyi Burma, with uncomplicated malaria were randomized either to AQ or quinine. No statistically significant difference was noted in fever and parasite clearance time between pregnant and non-pregnant women or between the two treatments. However, maternal and fetal outcomes were not reported in the treatment group and 50% of the patients were not followed-up. Among the 23 monitored pregnancies, there were 8 normal deliveries, 3 premature deliveries with low birth weight, one neonatal death, ten spontaneous abortions and one maternal death due to fulminant hepatic failure, apparently due to concurrent viral hepatitis, leading to a still-born baby (**Naing *et al.*, 1988; Thet *et al.*, 1988**).

AQ has also been used for mass administration as in medicated salt and it is probable that a certain number of pregnant women had been exposed to the drug although this information was not collected or not reported (**Thomas *et al.*, 2004**). WHO states that there is no evidence for stopping AQ use in pregnancy but also calls for additional safety data (**WHO, 2001a**). Nevertheless as countries change their first-line drug to AQ monotherapy or AQ-combinations, the collection of such data is an absolute priority.

2.4.0 THE OVARY

The ovary is the female gonad. Its function is the production of ova and the secretion of female sex hormones: estrogen and progesterone. Through these hormones, the ovary influences the cyclic maturation and discharge of the ova and the development and maintenance of the secondary female sex organs and secondary somatic sex characteristics of the female phenotype.

2.4.1 Gross Anatomy of the Ovary

Each ovary is a firm, almond-shaped organ, with an average dimension of 4 x 2 x 3 cm in reproductively mature women (**Standring, 2004**). Its surface, devoid of peritoneum is smooth until puberty; thereafter the scars left by the degenerating corpora lutea render it somewhat irregular. The ovary is suspended from the posterior lamina of the broad ligament by its own mesentery, the mesovarium, and is embraced anteriorly and laterally by the uterine tube. It is quite mobile and its position in the pelvic portion of the peritoneal cavity may vary. However, in the young, nulliparous woman, the ovary lies against the lateral pelvic wall, held there by its suspensory ligament (**Standring, 2004**). It has a lateral and medial surface, a mesovarium and a free border, and a tubal and uterine extremity or pole.

The lateral surface of the ovary contacts parietal peritoneum in the ovarian fossa. Behind the ovarian fossa, are retroperitoneal structures including the ureter, internal iliac vessels, obturator vessels and nerve, and the origin of the uterine artery (**Standring, 2004; Moore et al., 2009**). The medial surface faces towards the pararectal fossae and the rectouterine pouch and is in contact with loops of bowel. The tubal extremity or fimbriated lateral end of the uterine tube is tethered to the pole of the ovary that points laterally and upward. Hence, this pole called its tubal extremity is connected to the pelvic brim by the suspensory ligament of the ovary, a peritoneal fold draped over the ovarian vessels and nerves, and representing the lateral continuation of the broad ligament beyond the uterine tube (**Snell, 2003; Moore et al., 2009**).

The opposite pole of the ovary pointing towards the uterus and downwards is its uterine extremity. This is attached to the uterus at the inferior angle of the uterotubal junction by a fibromuscular band, the ligament of the ovary which is within the broad ligament and raises a ridge in its posterior lamina. The mesovarium, a reduplication of

the posterior lamina of the broad ligament, is attached along the anterior, or mesovarian border of the ovary. This mesovarium contains the vessels and nerves of the ovary which enter and leave the organ through its hilum to which the mesovarium is attached (**Moore et al., 2009**).

2.4.2 Arterial Supply of the Ovaries

The arterial blood supply of the ovaries is by the ovarian arteries branches of the abdominal aorta given off below the renal arteries. They descend along the posterior abdominal wall. At the pelvic brim, they cross over the external iliac vessels and enter the suspensory ligaments. The ovarian artery terminates by bifurcating into ovarian and tubal branches, which pass through the mesovarium to the ovary. Both branches anastomose with corresponding branches of the uterine artery (**Snell, 2003; Moore et al., 2009**).

2.4.3 Venous and Lymphatic Drainage of the Ovaries

The ovarian veins emerge from the ovary as a plexus (pampiniform plexus) of veins in the mesovarium and suspensory ligament. Two veins emerge from the plexus and ascend with the ovarian artery; they usually merge into a single vessel before entering either inferior vena cava on the right side or the renal vein on the left side (**Standring, 2004**). The lymphatic vessels follow the ovarian vein to para-aortic nodes situated near the origin of the renal artery. Drainage may also occur via pelvic nodes into lower para-aortic nodes (**Snell, 2003; Standring, 2004; Moore et al., 2009**)

2.4.4 Innervation of the Ovaries

The ovarian innervation is derived from autonomic plexuses. The upper part of the ovarian plexus is formed from branches of the renal and aortic plexuses, and the lower part is reinforced from the inferior and superior hypogastric plexuses. These plexuses

consist of postganglionic sympathetic, parasympathetic and visceral afferent fibres. The efferent sympathetic fibres are derived from the 10th and 11th thoracic spinal segments (Snell, 2003; Sandring, 2004; Moore et al., 2009).

2.5.0 HISTOLOGY OF THE OVARY

The ovary has an outer cortical and an inner medullary region. The medullary region contains a rich vascular bed within a cellular loose connective tissue. In the cortical region, ovarian follicles containing oocytes predominate. There are no sharp limits between the cortical and medullary zones. The stroma of the cortical region is composed of characteristic spindle-shaped fibroblasts that respond in a different way to hormonal stimuli than do fibroblasts of other organs. The surface of the ovary is covered by the germinal epithelium a simple squamous to cuboidal to pseudostratified columnar epithelium. (Auersperg et al., 2001).

Under the germinal epithelium, the stroma forms the tunica albuginea, a poorly delineated layer of dense connective tissue. The tunica albuginea is responsible for the whitish color of the ovary (Young et al., 2006). Cytoplasmic projections extend into the peritoneal cavity from the free surface of the cells and their lateral borders are connected by desmosomes. The nucleus is irregular and the dense cytoplasm contains many polysomes, free ribosomes and tonofilaments (Zuckerman and Weir, 1977). Intracellular vacuoles and extracellular channels contain materials of low to moderate density. Lipid droplets and compound aggregates are present and both coated and smooth vesicles occur in the peripheral cytoplasm. Focal degenerative changes may be seen which range from loss of density in the basal cytoplasm to complete desquamation (Papadaki and Beilby, 1971; Young et al., 2006).

2.5.1 Ovarian follicles

Ovarian follicles are embedded in the stroma of the cortex. A follicle consists of an oocyte surrounded by one or more layers of follicular cells, the granulosa cells. The total number of follicles in the 2 ovaries of a normal young adult woman is estimated to be 400,000, but most of them disappear through atresia during the reproductive years. This follicular regression begins before birth and continues over the entire span of reproductive life. After menopause, only a small number of follicles remain. Atresia can affect any type of follicle, from the primordial to those that are nearly mature. Since generally only one ovum is liberated by the ovaries in each reproductive cycle and the reproductive life of a woman lasts about 30-40 years, only about 450 ova are liberated. All the other follicles with their oocytes fail to mature; they become atretic and degenerate (**Young *et al.*, 2006**).

2.5.2 Primordial follicles

The primordial follicles are most numerous before birth. Each consists of a primary oocyte enveloped by a single layer of flattened follicular cells. The oocyte in the primordial follicle is a spherical cell about 25 μm in diameter. Its slightly eccentrically situated nucleus is large and has a large nucleolus. The chromosomes have become mostly uncoiled and do not stain intensely. The organelles in the cytoplasm tend to form a clump adjacent to the nucleus (**Peters and McNatty, 1980; Young *et al.*, 2006**). There are numerous mitochondria, several Golgi complexes, and cisternae of endoplasmic reticulum. The squamous follicular cells contain endoplasmic reticulum, mitochondria, and lipid droplets. They are joined to one another by desmosomes. A basal lamina underlies the follicular cells and marks the boundary between the avascular follicle and the surrounding stroma (**Peters and McNatty, 1980**).

2.5.3 Growing follicles

Follicular growth involves mainly the follicular cells but also the primary oocyte and the stroma surrounding the follicle. Oocyte growth is most rapid during the first part of follicular growth, with this cell reaching a maximum diameter of 125-150 μm . The nucleus enlarges and is now called a germinal vesicle. Mitochondria increase in number and become uniformly distributed throughout the cytoplasm; the endoplasmic reticulum hypertrophies, and the Golgi complex migrate to just beneath the cell surface. Follicular cells form a single layer of cuboidal cells and the follicle is now called a unilaminar primary follicle (Young *et al.*, 2006).

Follicular cells then proliferate by mitosis and form a stratified follicular epithelium or granulosa layer. The follicle is now called a multilaminar primary follicle, and gap junctions are found between follicular cells (Peters and McNatty, 1980; Young *et al.*, 2006). A thick coat, the zona pellucida, composed of at least 3 different glycoproteins surrounds the oocyte. It is thought that both the oocyte and follicular cells contribute to the synthesis of the zona pellucida (Peters and McNatty, 1980). Filopodia of follicular cells and microvilli of the oocyte penetrate the zona pellucida and make contact with one another via gap junctions (Young *et al.*, 2006).

While these modifications are taking place, the stroma immediately around the follicle differentiates to form the theca folliculi (Peters and McNatty, 1980). This layer subsequently differentiates into the theca interna and the theca externa. The cells of the theca interna, when completely differentiated have the same ultrastructural characteristics as cells that secrete oestrogen (Young *et al.*, 2006). These characteristics include abundant profiles of smooth endoplasmic reticulum, mitochondria with tubular cristae and numerous lipid droplets. As the follicle grows owing mainly to the increase in size and number of granulosa cells, accumulations of

follicular fluid- liquor folliculi appear between the cells. The cavities that contain this fluid coalesce and form a cavity, the antrum folliculi (**Guraya, 1977; Peters and McNatty, 1980; Young et al., 2006**). These follicles are termed secondary or vesicular follicles. Follicular fluid contains transudates of plasma and products secreted by follicular cells (**Papadaki and Beilby, 1971**). Most inorganic ions are present in concentrations similar to those found in plasma. Glycosaminoglycans, several proteins including steroid- binding proteins and high concentrations of steroids such as progesterone, androgens and estrogens are also present.

The cells of the granulosa layer are more numerous at a certain point on the follicular wall, forming a small hillock of cells, the cumulus oophorus, which contains the oocyte. The cumulus oophorus protrudes toward the interior of the antrum. The oocyte growth is thereafter arrested (**Young et al., 2006**).

2.5.4 Mature follicles

The mature Graafian follicle is about 2 cm in diameter and can be seen as a transparent vesicle that bulges from the surface of the ovary (**Standring, 2004; Young et al., 2006**). As a result of the accumulation of liquid, the follicular cavity increases in size, and the oocyte adheres to the wall of the follicle through a pedicle formed by granulosa cells. Since the granulosa cells do not multiply in proportion to the accumulation of liquid, the granulosa layer becomes thinner. The granulosa cells that form the first layer around the ovum are in close contact with the Zona pellucida. The oocyte and a surrounding ring of tightly adherent cells, the corona radiata breaks away from the follicle wall and floats freely in the follicular fluid. They accompany the ovum when it leaves the ovary. The corona radiata is still present when the spermatozoon fertilizes the ovum. It is retained for some time during the passage of the ovum through the oviduct (**Peters and McNatty, 1980; Young et al., 2006**)

2.5.5 Follicular atresia

Most ovarian follicles undergo follicular atresia, in which follicular cells and oocytes die and are disposed of by phagocytic cells. This process is characterized by cessation of mitosis in the granulosa cells, detachment of granulosa cells from the basal lamina and death of the oocyte (**Peters and McNatty, 1980**). Although follicular atresia takes place from before birth until a few years after menopause, there are times at which it is particularly intense. It is greatly accentuated just after birth, when the effect of maternal hormones ceases, and during puberty and pregnancy, when marked qualitative and quantitative hormonal modifications take place (**Young *et al.*, 2006**). The process of atresia can take place during any stage in the development of a follicle.

2.5.6 Corpus luteum

After ovulation, the granulosa cells and those of the theca interna that remain in the ovary form a temporary endocrine gland called the corpus luteum (**Young *et al.*, 2006**). The corpus luteum, which is localized in the cortical region of the ovary, secretes progesterone and estrogens. Progesterone prevents the development of new ovarian follicles and thus prevents ovulation. Release of the follicular fluid results in collapse of the follicular wall so that it becomes folded.

Some blood flows into the follicular cavity, where it coagulates and is later invaded by connective tissue. This connective tissue, with remnants of blood clots that are gradually removed, remains as the most central part of the corpus luteum (**Guraya, 1977; Young *et al.*, 2006**).

Although the granulosa cells do not divide after ovulation, they increase greatly in size to about 20 – 35 μm in diameter. They comprise about 80% of the parenchyma of the corpus luteum and are now called granulosa lutein cells, with the characteristics of steroid-secreting cells. This is in contrast to their structure in the preovulatory follicle,

where they appear to be protein-secreting cells (**Guraya, 1977; Peters and McNatty, 1980**).

Cells of the theca interna also contribute to the formation of the corpus luteum by giving rise to theca lutein cells. These cells are similar in structure to granulosa lutein cells but are smaller, about 15 µm in diameter and stain more intensely. They are located in the folds of the wall of the corpus luteum. The blood capillaries and lymphatics of the theca interna grow into the interior of the corpus luteum and form the rich vascular network of this structure (**Young *et al.*, 2006**).

The corpus luteum is formed as a result of the stimulus provided by luteinizing hormone (LH) synthesized by the pars distalis of the pituitary under hypothalamic control. Since the progesterone produced by the corpus luteum has an inhibitory effect on the production of LH, the corpus luteum soon degenerates unless it receives a stimulus from another source. This inhibitory effect of progesterone on LH production is indirect and is mediated through the hypothalamus (**Guraya, 1977; Young *et al.*, 2006**).

Hormone secretion in the corpus luteum ceases within 14 days after ovulation if the oocyte is not fertilised. In this case, the corpus luteum degenerates into a corpus albicans - whitish scar tissue within the ovaries (**Guraya, 1977; Peters and McNatty, 1980**).

2.6 THE OESTROUS CYCLE

The word “estrus” (oestrus or oestrum) is a Latin adaptation of the Greek word “oistros,” meaning gadfly, sting, or frenzy. This term was first used by **Heape** (1900) to describe the “special period of sexual desire of the female” and distinguish it from “rut” in the male.

Heape went further to describe the distinct stages of the cycles as applied to mammals during the breeding season. He used the term “anestrus” to describe the nonbreeding season or period of rest in the female mammal when the ovaries and accessory reproductive organs are relatively quiescent and attempts at mating by the male are resisted. Heape used the prefixes pro-, di-, and met-, along with the suffix estrus, to describe the stages of the cycle between the periods of estrus during the “sexual season”. The first part of the season he termed proestrus, characterized as the time when an animal is “coming on heat.” The next period, estrus, is described as time that the female is willing to receive the male, and fruitful coition may occur in most, if not all mammals. In the absence of conception, estrus is succeeded by a short recovery period called metestrus, during which the estrus changes in the reproductive tract subside. The following period, diestrus, is of variable duration in different species. During this time, the ovarian secretions prepare the reproductive tract for receipt of the ovum, which has become newly fertilized shortly after mating on estrus. If fertilization is unsuccessful, the mammal returns to proestrus, and the cycle begins anew.

The laboratory rat is a nonseasonal, spontaneously ovulating, polyestrus mammal. That is, the ovarian cycle continues throughout the year as opposed to ovarian cycles restricted to one season, as in the sheep. Ovulation is not dependent on overt nervous stimulation as opposed to the requirement of the mating stimulus in rabbits. Ovulation occurs every 4 to 5 days throughout the year.

The mean length of the 1,999 cycles in the rat, as studied by **Long and Evans** (1922) was 5.4 days, with a range of 3 to 38 days, which compared favourably with the 4.4 days as reported by **Blandau et al.**, (1941) and 4.5 days by **Astwood** (1939). In addition, the lengths of the various phases of the estrous cycle based on vaginal smear patterns have also been described. Proestrus lasts for 12 to 14 hr; estrus, 25 to 27 hr;

metestrus, 6 to 8 hr; and diestrus, 55 to 57 hr (**Astwood**, 1939; **Hartman**, 1944; **Mandl**, 1951).

2.7 PATTERN OF FSH SECRETION THROUGHOUT THE OESTROUS CYCLE

The pattern of FSH secretion throughout the estrous cycle is similar to that for LH (**Gay et al.**, 1970; **Daane and Parlow**, 1971; **Butcher et al.**, 1974). Specifically, basal levels of FSH are secreted from late on estrus through metestrus, diestrus, and midday on proestrus. From midafternoon onward, proestrus FSH secretion increases simultaneously with LH. That is, by 2 to 3 pm the circulating levels of FSH begin to increase rapidly and ultimately reach peak levels by 5 to 7 pm that same evening. Though both FSH and LH levels begin to decline to base line after this time, during the early morning of estrus a secondary rise of FSH begins and peaks shortly thereafter. Circulating levels of FSH then begin to decline and reach base line by the early evening of estrus. This initial phase of FSH secretion shares some controls with the LH surge.

2.8 PATTERN OF LH SECRETION THROUGHOUT THE OESTROUS CYCLE

The serum levels of LH are at the lowest from early on the morning of estrus, shortly after ovulation, through metestrus, diestrus and midday on proestrus (**Daane and Parlow**, 1971; **Butcher et al.**, 1974). Though most studies have reported basal unchanging levels of LH over this time (**Goldman et al.**, 1969; **Barraclough et al.**, 1971; **Smith et al.**, 1975; **Nequin et al.**, 1979), however one author reported a slight but significant diurnal variation throughout the cycle (**Gay et al.**, 1970). This circadian

pattern from estrus through diestrus consisted of a small elevation of LH each day at the midpoint of the light period, and the lowest levels by midnight of each day. On the afternoon of proestrus, about 2 to 3 p.m., the circulating levels of LH begin to increase rapidly and ultimately reach peak levels by 5 to 7 p.m. on the same evening. This rapid surge of LH induces follicular rupture and ovulation. Thereafter the LH blood levels begin to decline and reach basal levels early on the morning of estrus (**Gay et al.**, 1970; **Blake**, 1976; **Fox and Smith**, 1985). **Gallo** (1981) recorded infrequent pulses of LH by the late morning and throughout the day of estrus. However, **Fox and Smith** (1985) reported that these pulses were absent.

2.9 PATTERN OF PRL SECRETION THROUGHOUT THE OESTROUS CYCLE

The pattern of PRL secretion throughout the oestrous cycle is also similar to that for LH (**Gay et al.**, 1970; **Daane and Parlow**, 1971; **Butcher et al.**, 1974). Basal levels of PRL are secreted from the evening of estrus through the early morning of proestrus. During the afternoon of proestrus, a surge of PRL similar in timing to that of LH is observed (**Gay et al.**, 1970; **Smith et al.**, 1975). Although a number of authors have described a singular surge of PRL on proestrus (**Neill**, 1970; **Smith et al.**, 1975; **Gay et al.**, 1990) other authors have reported a secondary increase on estrus (**Butcher et al.**, 1974) or continuously elevated PRL levels on proestrus, estrus and metestrus (**Amenomori et al.**, 1970). These latter patterns may have been due to the method and frequency of blood collection. Therefore, it is generally accepted that the afternoon of proestrus is the only time that a major surge of PRL secretion occurs (**Freeman**, 1988).

2.10 FOLLICULOGENESIS

The major function of the female gonad is the differentiation and release of mature oocyte for fertilization and successful propagation of the species. Additionally, the ovary produces steroids that allow the development of female secondary sexual characteristics and support pregnancy. In mammalian ovaries the individual follicles consist of an innermost oocyte, surrounding granulosa cells, and outer layers of thecal cells. The fate of each follicle is controlled by endocrine as well as paracrine factors (**Richards *et al.*, 1995; Gougeon, 1996**).

The follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas a few of them, under the cyclic gonadotropin stimulation that occurs after puberty, reach the preovulatory stage (**Hirshfield, 1991; Gougeon, 1996**). These Graffian follicles are the major source of the cyclic secretion of ovarian estrogens in women of reproductive age.

In response to preovulatory gonadotropin surges during each reproductive cycle, the dominant Graffian follicle ovulates to release the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum (**Fauser and Van Heusden, 1997**).

The pool of oocytes in the mammalian ovary becomes fixed early in life; thus, ovarian senescence is linked to the dwindling supply and eventual exhaustion of the pool of primordial follicles. Studies have revealed that oocyte-secreting factors regulate the initiation of primordial follicle growth and moderate the known trophic actions of the gonadotrophins FSH and LH on preantral and antral follicle growth (**Picton, 2001; Knight and Glistler, 2006; McNatty *et al.*, 2007; Webb and Campbell, 2007**).

A plethora of putative regulators of folliculogenesis *in vivo* have been identified (Thomas and Vanderhyden, 2007). The tyrosine kinase receptor Kit and the two different isoforms of its ligand, kit ligand (KL), have been localised to oocytes and granulosa cells and have been shown to promote oocyte growth and maintenance of meiotic arrest in response to FSH receptor (FSHR) levels. While low concentrations of FSH promote oocyte growth by increasing KL-2 expression and by reducing the ratio of KL-1/KL-2, high concentrations of FSH enhance follicle development but impair oocyte growth (Thomas and Vanderhyden, 2007). Other regulators of follicle growth include: (1) epidermal growth factor (EGF) (Qu *et al.*, 2000) and its receptor activin (Hulshof *et al.*, 1997; Telfer *et al.*, 2008); (2) basic fibroblastic growth factor (bFGF) (Shikone *et al.*, 1992); (3) members of the insulin like growth factor (IGF) family and their binding proteins (Thomas *et al.*, 2007), transforming growth factor- β (TGFB) superfamily members (Knight and Glistler, 2006) including somatic derived anti-Müllerian hormone (AMH), oocyte derived growth differentiation factor-9 (GDF9) (McGrath *et al.*, 1995; Dong *et al.*, 1996); (4) the bone morphogenetic proteins (BMPs) especially BMP4, BMP7 and BMP15 (Shimasaki *et al.*, 1999; Otsuka *et al.*, 2001); and (5) retinoblastoma protein (RB1) (Bukovsky *et al.*, 1995). Changes in follicle morphology and cell number, together with the stage specific follicular responsiveness to the growth factors and the development of steroidogenic capacity can be used as functional markers to confirm the normality of follicle development *in vitro*.

Oocyte developmental competence is progressively acquired following a coordinated series of structural and functional changes in the gamete and surrounding cumulus cells. However, it is not until the final days of follicular development that oocytes acquire the capacity to undergo meiotic progression to metaphase II. The redistribution

of the cortical granules and changes in mitochondrial number, activity and distribution occur during the terminal stages of oocyte maturation *in vivo* (**Wilding *et al.*, 2001; Van Blerkom *et al.*, 2002**). Cytoplasmic and nuclear maturation of the oocyte is further characterised by a cascade of molecular events and check points which include changes in the transcription and translation of RNA, DNA replication and repair, chromosome condensation, spindle formation and development of the mechanisms for sperm head penetration (**Swain and Smith, 2007**).

Oocyte maturation *in vivo* is triggered by the preovulatory surge of gonadotrophins and particularly by high levels of LH. The LH driven follicular cascade results in a shift in steroid production by granulosa cells from predominately estrogen to a progesterogenic environment and a decrease in intracellular cAMP in the oocyte, which is induced at least in part by the loss of junctional contacts between the cumulus cells and oocyte (**Mehlmann, 2005; Sela-Abramovich *et al.*, 2006**).

Activation of the molecular pathways leading to the production of hyaluronic acid by the cumulus cells occurs in response to the LH surge (**Schoenfelder and Einspanier, 2003**). The loss of junctional contacts between the oocyte and somatic cells in turn triggers the resumption of meiosis in mature oocytes as it further reduces cAMP concentrations (**Vaccari *et al.*, 2008**), leads to deactivation of cAMP dependent protein kinase A and reduces the inhibitory influences of purines on the maintenance of meiotic arrest (**Swain and Smith, 2007**).

2.10.1 EARLY FOLLICULOGENESIS IN HUMANS

In humans, primordial germ cells arrive in the gonadal ridge from the endoderm of the yolk sac by the seventh week of gestation to become oogonia, which proliferate by mitosis before differentiating into primary oocytes. Some oogonia begin transformation into primary oocytes and enter the first stages of meiosis at around 11–12 weeks of

gestation. The total germ cell number peaks at 20 weeks. After this time, the rate of oogonia division declines (**McGee and Hsueh**, 2000).

Primordial follicle formation begins around midgestation when a single layer of pregranulosa cells surround each oocyte and continues until just after birth (**Van Wagenen and Simpson**, 1965). After oocytes are within the primordial follicles, they remain arrested in the dictyate stage of meiosis I. From a peak of 6 to 7 million at 20 weeks of gestation, the oocyte number falls dramatically so that at birth, there are only 300,000 to 400,000 remaining (**Block**, 1953; **Forabosco et al.**, 1991). Oocytes not surrounded by granulosa cells to form primordial follicles are lost, probably via apoptosis (**Coucovanis et al.**, 1993; **Pesce and De Felici**, 1994; **De Pol et al.**, 1997).

Meanwhile, some primordial follicles leave the resting pool by initiating growth. Once entering the growing pool, most growing follicles progress to the antral stage, at which point they inevitably undergo atresia. After pubertal onset, a small number of the antral follicles can be rescued by gonadotropins to continue growth and normally one graffian follicle is formed each month in preparation for ovulation. Antral follicles 2–5 mm in diameter develop into graffian follicles in only 14 days during the follicular phase of the menstrual cycle. However, more than 85 days are needed for late secondary follicles to grow into preovulatory follicles.

In addition, it has been estimated that more than 120 days are needed for primary follicles to grow into the secondary stage and even longer for the development of primordial follicles into primary follicles (**Gougeon**, 1986; **Gougeon**, 1996). Thus, the entire growth phase of a follicle is much greater than 220 days or eight menstrual cycles. In the human ovary, primordial follicles are present by 20 weeks of fetal life, whereas primary follicles are found by 24 weeks. By 26 weeks, some follicles have

progressed to the secondary stage. Antral follicles develop in the third trimester and are also seen postnatally when FSH levels are elevated (**McGee** and **Hsueh**, 2000).

After puberty, cyclic increases in serum gonadotropins stimulate the antral follicles to become preovulatory follicles during each menstrual cycle. At the time of puberty there is an average of 200,000 follicles remaining in the ovary (**Block**, 1952).

During reproductive life, continuing growth of primordial and primary follicles into secondary and larger follicles leads to a gradual decrease in the original follicle pool. In addition, the primordial follicle pool could also be decreased due to apoptosis of resting follicles. More than 10 yrs before menopause, concomitant with subtle increases in serum FSH and decreases in circulating inhibins, increasing percentages of follicles are lost from the resting pool (**Richardson et al.**, 1987; **Faddy et al.**, 1992; **Gougeon et al.**, 1994; **Faddy and Gosden**, 1996). The diminishing follicle reserve serves as a ticking clock to time the onset of menopause. As the result of ovarian follicle exhaustion, menopause occurs (**Makinoda et al.**, 1988). With modern increases in longevity, a significant portion about one-third of a woman's life is now spent after menopause.

2.10.2 EARLY FOLLICULOGENESIS IN RODENTS

Important landmarks of ovarian development in rats are similar to those in the humans however, the timing is greatly compressed.

Primordial germ cells migrate to the gonadal ridge late in embryonic development to become oogonia. At birth, the rat ovary consists of cords and oogonia. Primordial follicles are formed by day 3 of age, and the first wave of follicles develops into antral follicles over the next 3 weeks (**Hirshfield**, 1989; **Rajah et al.**, 1992; **Gelety and Magoffin**, 1997; **McGee et al.**, 1997). Well developed secondary follicles are found by

day 7 of age. Minimal ovarian cell apoptosis can be found until day 18 when early antral follicles are apparent (McGee *et al.*, 1997; McGee *et al.*, 1998).

Puberty or first estrus occurs around day 34. The regular oestrous cycles continue until around 10–12 months of age when the cycles become prolonged and irregular (McPherson *et al.*, 1977; Wise, 1982). By age 12–15 months, animals enter persistent estrus, and this is followed by persistent diestrus and ultimately anestrus (Huang *et al.*, 1978; Dudley, 1982).

The timing of follicle growth has been meticulously evaluated in adult rats (Hirshfield, 1991). Primordial follicles grow from about 25 μm in diameter to 500–800 μm in diameter in preovulatory follicles, over a period of greater than 60 days or about 15 oestrous cycles. The time for primordial follicles to grow to the secondary follicle stage may be more than 30 days or comparable to the time (28 plus 2–3 days) to grow from the secondary stage to ovulation. Thus, as in the human, early follicle growth in rodents is very protracted. Of interest, the rate of development of the first wave of follicles in juvenile rats is more rapid than that in adult cycling animals (Hirshfield and DeSanti, 1995).

2.11 FOLLICLE-STIMULATING HORMONE RECEPTORS AND OOCYTES

The mechanism of maturation of germinal cells has always been the subject of great scientific interest. Studies on oocyte arrest and resumption of meiosis and also on the acquisition of the competence to become fertilized have been extensively carried out (Wassarman and Albertini, 1994; Gosden and Bownes, 1995; Fulka *et al.*, 1998).

In addition, scientific progress in assisted reproduction has increased interest in these mechanisms. Cloning by nuclear transfer objectifies the ability of the oocyte cytoplasm to set off a program of embryonic development even in differentiated nuclei

(**Campbell et al.**, 1996). The culture of oocytes to maturity is an emerging technology that may transform the practice of *in vitro* fertilization (**Eppig and O'Brien**, 1996; **Gosden et al.**, 1996).

Gonadotropins play a very important role and are supposed to act on somatic supportive cells and not directly on germ cells (**Cortvrindt et al.**, 1997; **Oktay et al.**, 1998). FSH is known to act on granulosa cells, whereas LH has mainly a steroidogenic activity in thecal cells. LH receptors (LHR) are present in granulosa cells only at later stages of development i.e. antral follicles. These conclusions were derived mainly from experiments involving stimulation by gonadotropins of isolated cells or from analysis of receptor distribution using radioactive gonadotropins and autoradiography (**Midgley**, 1973; **Roy et al.**, 1987).

Meduri et al. (2002) raised high affinity monoclonal antibodies against LHR and FSHR. They used the latter to study receptor distribution in human and pig ovaries, and observed the presence of FSH but not LH receptors in the oocytes. This observation, which was confirmed by the presence of receptor mRNA in denuded human preovulatory oocytes as well as autoradiography and Ca^{2+} mobilization studies, raises the possibility of direct control of oocyte development by FSH. The presence of FSHR and its mRNA in oocytes was confirmed by autoradiography, RT-PCR, and Ca^{2+} mobilization studies.

Ovarian follicular development is known to proceed to primordial and primary stages independent of the action of FSH. This has been observed in mice carrying invalidations of the FSH β and FSHR genes (**Kumar et al.**, 1997; **Dierich et al.**, 1998) and also in patients with mutations suppressing the function of the FSHR (**Aittomäki et al.**, 1995; **Beau et al.**, 1998; **Touraine et al.**, 1999). Increasing FSH concentrations seem, on the contrary, necessary for further stages of development (**Hillier**, 1994;

Fauser and Van Heusden, 1997). Although a minority of previous studies has given some hints of the existence of FSH-binding sites in the oocytes (**Oxberry and Greenwald, 1982; Wang and Greenwald, 1993**), a majority of studies showed FSH binding or FSHR mRNA only in the granulosa cells (**Uilenbroek and Richards, 1979; Xu et al., 1995a; Oktay et al., 1997; Simoni et al., 1997**). This has led to a consensus that this hormone would act only on granulosa cells (**Adashi, 1994**).

Oocyte development is considered secondary to these effects and also dependent on diffusible substances, possibly passing through gap junctions between granulosa cells and the oocyte. However, it is known that follicles enucleated from their oocytes fail to develop even in the presence of FSH (**Vanderhyden et al., 1992**). It is thus possible that a two-way exchange occurs and that a direct action of FSH on the oocyte produces compounds whose diffusion into the granulosa cells is necessary for their proliferation and maturation. In atretic follicles, **Meduri et al. (2002)** observed that FSHR are markedly decreased in the oocyte, the granulosa layers, or both. This may be a phenomenon secondary to atresia. Alternatively, it may be its primum movens, with the lack of activity of FSH in either the oocyte or the granulosa inducing atresia. It is thus possible that FSH must act in both cell types to promote follicular growth and development.

During development of the human ovarian follicle from the primordial to the preovulatory stage, the oocyte undergoes a 300-fold size increase, the number of organelles such as Mitochondria, Golgi, Ribosomes and Cortical granules are increased dramatically (**Wassarman, 1996**). A variety of RNA and protein molecules is synthesized and stored (**Gosden et al., 1997**). The oocyte is blocked in the diplotene stage of prophase I of the first meiotic division. The block will only be relieved at a later stage, secondary to the LH peak (**Eppig and O'Brien, 1996**). It is during this

growth period that oocytes acquire the ability to undergo meiosis i.e. meiotic competence. It is still largely unknown which of these events is FSH dependent and what are the underlying molecular mechanisms occurring in granulosa cells, oocytes, or both.

2.12 DOMINANT SELECTION OF OOCYTE

The FSH-initiated cyclic recruitment step is sometimes described interchangeably with the process of follicle selection (**Gougeon and Testart, 1990; Scheele and Schoemaker, 1996; Rombauts *et al.*, 1998**). The result is a negative selection of the remaining cohort, leading to its ultimate demise.

Concomitantly, increases in local growth factors and vasculature allow a positive selection of the dominant follicle, thus ensuring its final growth and eventual ovulation. After cyclic recruitment, it takes only 2 weeks for an antral follicle to become a dominant Graffian follicle in humans (**McGee and Hsueh, 2000**). The time required between the initial recruitment of a primordial follicle and its growth to the secondary stage is more than 30 days, whereas the time for a secondary follicle to reach the early antral stage is about 28 days. On reaching the early antral stage 0.2–0.4 mm in diameter, the follicles are subjected to cyclic recruitment, and only 2–3 days are needed for them to grow into preovulatory follicles (**McGee and Hsueh, 2000**).

Cyclic recruitment and selection of follicles represent a continuous process, eventually leading to the emergence of the preovulatory follicles. Cyclic recruitment and final follicle selection are most clearly illustrated during the human menstrual cycle. After increases in circulating FSH during the premenstrual period, a cohort of antral follicles escapes apoptosis due to the survival action of FSH. Among this group of about 10 antral follicles found in young adults, one of the leading follicles grows faster than the

rest of the cohort and produces higher levels of estrogens and inhibins (**Zeleznik** and **Benyo**, 1994).

Although the exact reasons why one follicle emerges as dominant are unclear, however, this follicle is likely to be more sensitive to FSH (**Fauser** and **Van Heusden**, 1997), perhaps because of enhanced FSH and or LH receptor expression or increases in local growth factors that augment FSH responsiveness as suggested by bovine studies (**Xu et al.**, 1995a; **Bao et al.**, 1997; **Evans** and **Fortune**, 1997).

Estrogens and inhibins produced by the largest follicle suppress pituitary FSH released during the midfollicular phase. As a result, the remaining growing antral follicles are deprived of adequate FSH stimulation required for survival (**diZerega** and **Hodgen**, 1981). In monkeys, it has been elegantly demonstrated that immunoneutralization of the actions of circulating estrogens during the midfollicular phase leads to sustained elevation of circulating FSH, thus allowing the development of multiple preovulatory follicles (**Zeleznik et al.**, 1987). Furthermore, administration of exogenous estrogens suppresses follicle development in women (**Tsai** and **Yen**, 1971; **Vaitukaitis et al.**, 1971), whereas treatment with high levels of exogenous gonadotropins during ovulation induction in women is widely known to stimulate the growth of multiple preovulatory follicles (**Fauser** and **Van Heusden**, 1997). Negative selection against subordinate follicles is therefore a result of estrogen and inhibin produced by the dominant follicle exerting negative feedback upon gonadotropin release.

Additionally, this rapidly growing follicle also produces higher levels of autocrine and paracrine growth factors that stimulate increases in vasculature and FSH responsiveness, thus constituting a local positive selection mechanism (**McGee** and **Hsueh**, 2000). Studies have demonstrated the importance of insulin-like growth factors (IGFs) and other local factors in the amplification of FSH action (**Giudice**, 1992;

Adashi, 1995). Although remaining to be characterized, atretogenic factors produced by the dominant follicle have been postulated to account for the lack of development of subordinate follicles after exogenous gonadotropin administration (**diZerega** and **Hodgen**, 1980; **Gougeon** and **Testart**, 1990). Other studies have proved that increased responsiveness of dominant follicles to FSH stimulates the expression of both FSH and LH receptors in the granulosa cells of this follicle (**Hsueh et al.**, 1984; **Harlow et al.**, 1988), thus providing a fail-safe mechanism to ensure the eventual ovulation of the selected follicle. Furthermore, computer modeling of ultrasound images in patients also suggested a suppressive effect of the dominant follicle on its neighboring follicles (**Gore et al.**, 1997).

Cyclic recruitment of early antral follicles and selection of dominant follicles in rodents is similar to that of the primates with the major exception that multiple follicles become dominant during each oestrous cycle. Monoovulatory and polyovulatory species likely differ in the threshold for negative feedback signals, presumably a genetically determined trait (**McNatty et al.**, 1986; **Spearow**, 1986). The law of follicular constancy proposed by **Lipschutz** (1928) emphasized that the ovulatory number remains constant in a given species even when a single ovary or a large portion of the remaining ovary is removed. Thus, findings of compensatory ovulation (**Baker et al.**, 1980) underline the importance of the putative central set point within a given species. In several high fecundity strains of sheep, the follicular negative feedback signals, estrogens and inhibins secreted by each individual follicle are decreased, thus allowing the selection of more preovulatory follicles (**Montgomery et al.**, 1992). In general, the preovulatory dominant follicles in these animals are of a smaller size than those found in the low fecundity strain (**Cahill et al.**, 1981; **Driancourt et al.**, 1996; **Baird** and **Campbell**, 1998). Furthermore, species and strain differences in follicular

responsiveness to FSH (**Abdennebi et al.**, 1999), or the available number of growing antral follicles, may also play a role in determining the number of preovulatory-size follicles.

2.13 OOCYTE RECRUITMENT

The term recruitment has been used frequently by different investigators to describe two important but distinct decision points during follicle development (**Gosden et al.**, 1983; **Gougeon and Testart**, 1990; **Meijs-Roelofs et al.**, 1990; **Rombauts et al.**, 1998). The dormant primordial follicles are recruited into the growing follicle pool in a continuous manner, whereas increases in circulating FSH during each reproductive cycle recruit a cohort of antral follicles.

During initial recruitment, intraovarian and/or other unknown factors stimulate some primordial follicles to initiate growth, whereas the rest of the follicles remain quiescent for months or years. Alternately, initial recruitment may be due to a release from inhibitory stimuli that maintain the resting follicles in stasis. Initial recruitment is believed to be a continuous process that starts just after follicle formation, long before pubertal onset.

Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles. After initial recruitment, oocyte growth is a prominent feature of the growing follicles, but these oocytes remain arrested in the prophase of meiosis 1. For those follicles not recruited, the default pathway is to remain dormant. Most investigators have monitored changes in the number of primordial and/or primary follicles that remain in the ovary at any given time, the supposition being that the decline of follicle numbers in this category is due to follicles leaving the resting pool to enter the growing pool. Because of the difficulties involved in distinguishing between nongrowing and

growing follicles, primordial and primary follicles have often been considered a contiguous group (**Pedersen and Peters, 1968; Edwards *et al.*, 1977; Gougeon, 1996; Van Wezel and Rodgers, 1996**).

By counting follicles of different categories in ovarian sections, rates of follicle progression and loss have been estimated (**Faddy and Gosden, 1996; Gougeon, 1996; Van Wezel and Rodgers, 1996**). Despite the limitations of this approach, several useful models have been formulated to describe the dynamics of initial recruitment of follicles. Although a radioactive decay model proposed a constant loss of a fixed number of resting follicles from the original pool over time (**Mandl and Zucherman, 1951; Edwards *et al.*, 1977**), some studies suggested a decreasing number of follicles initiate growth as the ovary ages, in proportion to the number of remaining follicles in the diminishing supply (**Jones and Krohn, 1961**). Morphometric studies further suggested that follicles initiate growth based upon the order in which they were formed (**Hirshfield, 1991**).

The decay model, proposed to account for the baseline rate of the initial recruitment of follicles, has been further modified based on observed variations in the rate of follicle loss from the resting pool over the reproductive life span. An accelerated loss of follicles from the resting pool has been found during the initial waves of follicle growth in infantile rodents (**Hage *et al.*, 1978**). In addition, morphometric studies have demonstrated that an increasing percentage of follicles are lost in the perimenopausal years in humans (**Richardson *et al.*, 1987; Faddy *et al.*, 1992**). In both of these situations, serum gonadotropins are elevated compared with those during the peak reproductive years. The accelerated rate of loss of follicles in infantile rodents has been attributed both to a lack of mature follicles that might exert a negative effect on initial recruitment and to qualitative differences in the first groups of growing follicles

(**Edwards *et al.*, 1977; Greenwald and Roy, 1994**). Likewise, the accelerated follicle loss in perimenopausal women may reflect intrinsic differences in their remaining follicles. Although the observed follicle depletion may be due to increasing serum gonadotropin levels, changes in gonadotropin secretion could also be the result of diminishing inhibitory influences from a lower number of growing follicles.

In contrast, cyclic recruitment starts after pubertal onset and is the result of the increase in circulating FSH during each reproductive cycle that rescues a cohort of antral follicles from atresia. In rodents, the recruitable early antral follicles are 0.2–0.4 mm in diameter, whereas human follicles at the comparable stage are larger (2–5 mm in diameter) and have acquired antrum for some time. During cyclic recruitment, only a limited number of follicles survive, and the default pathway is to undergo atresia. Oocytes in these follicles have already completed their growth, acquired a zona pellucida, and are competent to resume meiosis (**Tsafri, 1997; Trounson *et al.*, 1998**). Among this cohort, a leading follicle emerges as dominant by secreting high levels of estrogens and inhibins to suppress pituitary FSH release.

2.14 ROLE OF OOCYTE IN PRIMARY FOLLICULAR GROWTH

The role of the oocyte in the initial recruitment of follicles has also been evaluated. During development, granulosa cells of primordial follicles start to divide (**Hirshfield, 1989**), followed by morphological changes to the cuboidal shape characteristic of primary follicles. Because an increase in oocyte size is not evident until formation of the primary follicle, a passive role of the oocyte in initial recruitment has been suggested (**Tsafri, 1997**).

Based on the observation that the number of chiasmata, or crossing-over events, in ovulated oocytes decreases with the increasing age of an animal, it was proposed that

the order of follicle recruitment is related to the order in which the oocytes entered meiosis during development (**Edwards *et al.*, 1977**). This "production line" hypothesis predicts that the first oocytes entering meiosis are the first ones maturing and ovulating. In addition, **Hirshfield** (1991) demonstrated that rapidly progressing oocytes located near the corticomedullary junction of the ovary begin growth earlier and are enclosed into follicles that initiate growth during the neonatal and infantile period, a time of accelerated follicle loss. In contrast, oocytes that undergo slower meiotic progression are located closer to the cortex and are enclosed in follicles that grow later in life.

Meiotic competence of human follicles declines with age (**Volarcik *et al.*, 1998**) and this finding has been used in support of the "production line" hypothesis. However, the reduction of oocyte quality could be due to poorer conditions present during folliculogenesis after age 35. These issues have yet to be resolved (**McGee and Hsueh, 2000**). Factors involved in oocyte-granulosa cell communication in early follicles have also been proposed to have a role in initial recruitment. The Steel factor or kit ligand is expressed by granulosa cells of growing follicles whereas c-kit, a tyrosine kinase receptor of the platelet-derived growth factor receptor family, is located on oocytes and theca cells. Mutations in mice that prevent the production of the soluble form of the kit ligand lead to failure of follicular growth beyond the primary stage (**Kuroda *et al.*, 1988; Huang *et al.*, 1993; Bedell *et al.*, 1995**). Less severe mutations that result in reduced production of the soluble ligand allow a few follicles to grow to the antral stage. These animals ovulate sporadically and show limited fertility. Of interest, treatment of neonatal mice with a neutralizing antibody against the c-kit receptor caused apparent disturbances in initial follicle recruitment, primary follicle growth, and antrum formation in larger follicles (**Yoshida *et al.*, 1997**). **Ezoe *et al.*, (1995)** reported

that mutations affecting the function of c-kit in humans do not seem to affect female fertility.

Further evidence of the potential role of the oocyte in early follicle development is provided by studies of growth differentiation factor-9 (GDF-9), a homodimeric protein of the transforming growth factor- β (TGF β)/activin family that presumably signal via serine-threonine kinase receptors. GDF-9 is produced by growing mouse, rat, and human oocytes (**McGrath *et al.*, 1995; Aaltonen *et al.*, 1999; Hayashi *et al.*, 1999**) in primary and larger follicles but is absent in primordial follicles. However, in ovine and bovine ovaries, the GDF-9 message could be detected as early as the primordial follicle stage (**Bodensteiner *et al.*, 1999**).

In mutant mice, disruption of the GDF-9 gene prevents follicle development beyond the primary stage and also is associated with an absence of thecal cell markers and eventually, oocyte death (**Dong *et al.*, 1996; Elvin *et al.*, 1999**). These studies demonstrated the importance of oocyte-granulosa cell interactions during early stages of follicle development. Kit ligand and GDF-9 are highly expressed in secondary follicles. They also are likely to play important roles in preantral follicle development. In a study carried out by **Hayashi *et al.*, (1999)** these authors indicated that treatment with recombinant GDF-9 stimulates inhibin- α production by neonatal ovarian explants in rats as well as the growth and differentiation of cultured preantral rat follicles. It is possible that multiple paracrine factors are involved in the communication between oocyte and somatic cells during early follicle development. The mammalian oocyte and its surrounding cumulus cells are metabolically coupled through gap junctions that provide a unique means of entry into the ooplasm for several metabolites.

2.15 PREANTRAL FOLLICLE GROWTH AND DIFFERENTIATION

After initial recruitment, granulosa cells in primary follicles undergo profound changes, progressively acquiring the differentiated characteristics of epithelial cells found in secondary follicles. The oocyte continues to grow, the zona pellucida is formed, theca condenses around the preantral follicle, and the vascular supply develops.

In vitro studies have shown that granulosa-oocyte communication is essential for normal oocyte growth in early follicles. Immature oocytes separated from granulosa cells do not grow, but oocytes allowed to maintain gap junctions with granulosa cells grow at a near-normal rate (**Tsafiriri**, 1997). In mice, a gap junction protein, “Connexin 37”, is expressed at the oocyte-granulosa cell junction by the time follicles have developed to the secondary stage, whereas follicles of mice that lack Connexin 37 do not progress normally (**Simon et al.**, 1997). These defective follicles contain a normal zona pellucida and granulosa cell processes but lack oocyte-granulosa gap junctions and have impaired oocyte-granulosa communication. They progress normally to the late secondary stage and form a limited number of small antral follicles. The oocytes do not reach full size and are not competent to undergo meiosis (**McGee and Hsueh**, 2000).

Several studies have further demonstrated that oocytes secrete factors to regulate granulosa cell functions (**Eppig et al.**, 1997), including granulosa cell division (**Vanderhyden and Macdonald**, 1998), LH receptor formation (**Eppig et al.**, 1998), and steroidogenesis (**Nekola and Nalbandov**, 1971; **Vanderhyden and Macdonald**, 1998) as well as cumulus cell expansion (**Elvin et al.**, 1999). These studies underscore the concept that granulosa-oocyte communication is important for normal preantral follicle development.

Because preantral follicle development proceeds much slower than that of larger antral follicles, it is possible that ovarian growth and differentiation genes are suppressed during early follicle development. High levels of the Wilms' tumor gene, (WT1) are expressed in the granulosa cells of primary follicles in rats with lower levels in secondary follicles and negligible levels in antral and preovulatory follicles (**Hsu et al.**, 1995). WT1, a transcription factor with zinc finger domains (**Rauscher**, 1993), suppresses the expression of several growth factors and their receptors in different cell types. Furthermore, *in vitro* studies demonstrated that WT1 represses activities of the promoters for inhibin- α and FSH receptor, marker genes essential for follicle development.

In gel retardation assays, recombinant WT1 proteins interact directly with consensus DNA sequences in the inhibin- α gene promoter (**Chun et al.**, 1999). Thus, WT1 may act as a stasis factor on smaller follicles, and falling levels of WT1 allow the progression of early follicle development. Mutant mice with deletion of the WT1 gene die during embryonic development, thus preventing analysis of ovarian follicle development. In human males with inactivating mutations of WT1, gonadal dysgenesis is prominent (**Pelletier et al.**, 1991). However, detailed analysis of ovarian phenotypes in human females with WT1 mutations remains to be performed (**Henry et al.**, 1993). Further studies are needed to elucidate the regulation of WT1 expression in human follicles as well as the interaction of WT1 with other genes involved in follicle development.

Granulosa-theca cell interactions may also have a role in the development of early follicles (**Hillier et al.**, 1997; **Weil et al.**, 1998). Although the role of sex steroids in preantral follicle development is still unclear, studies have suggested that androgen treatment in intact monkeys increases the number of preantral and small antral follicles

up to 1 mm in diameter through androgen receptors (**Hillier *et al.*, 1997; Vendola *et al.*, 1998; Weil *et al.*, 1998**). In cultured mouse preantral follicles, androgen treatment also augments follicle growth (**Murray *et al.*, 1998**).

Rat preantral follicles have been shown to secrete proteins that enhance the growth and differentiation of theca cells before their expression of LH receptors (**Gelety and Magoffin, 1997**). Likewise, co-cultures of theca and granulosa cells enhance proliferation and steroidogenesis of both cell types (**Kotsuji and Tominaga, 1994**). The observed interactions between granulosa and theca cells are probably mediated by paracrine growth factors. Studies have indicated that keratinocyte growth factor, or fibroblast growth factor-7, a paracrine hormone secreted by theca cells, enhances the growth of preantral rat follicles in culture (**Parrot and Skinner, 1998; McGee *et al.*, 1999**).

Treatment of dissociated ovarian cells from juvenile rats with activin and FSH enhances formation and growth of follicular structure (**Li *et al.*, 1995**). Activin treatment also enhances FSH-stimulated inhibin production in dispersed ovarian cells from neonatal rats (**Drummond *et al.*, 1996**).

In mice, cultured preantral follicles secrete activin, and treatment with recombinant activin enhances FSH-stimulated inhibin and estrogen production (**Smitz *et al.*, 1998**). Furthermore, studies using co-cultures of mouse follicles at different stages of development suggested that activin secreted from secondary follicles causes small preantral follicles to remain dormant (**Liu *et al.*, 1998; Mizunuma *et al.*, 1999**). The exact stage-dependent effects of activin in early follicle development remain to be elucidated.

High levels of IGF and IGF-I receptors have been found in postnatal rats during preantral follicle development (**Levy *et al.*, 1992**). However, follicles seem to develop

normally to the early antral stage in mutant mice lacking IGF-I, although numerical morphometrics were not performed (**Baker et al.**, 1996). Studies using these mutant mice further suggested that ovarian IGF-I expression serves to enhance granulosa cell FSH responsiveness by augmenting FSH receptor expression (**Zhou et al.**, 1997). A large body of data exists on the effects of growth factors on monolayer cultures of granulosa and theca-interstitial cells as well as on cultures of antral and preovulatory follicles (**Hsueh et al.**, 1984; **Greenwald and Roy**, 1994; **Kol and Adashi**, 1995; **Findlay et al.**, 1996). It is clear that paracrine growth factors are also involved in preantral follicle development.

2.16 GONADOTROPIN REGULATION OF PREANTRAL FOLLICLES

Several studies investigated the role of FSH on follicle development based on mutant mice with a defective FSH- β or FSH receptor gene (**Kumar et al.**, 1997; **Dierich et al.**, 1998) and in patients with loss-of-function FSH receptors (**Aittomaki et al.**, 1995). Although the growth of preantral follicles has been considered to be gonadotropin independent because follicles can develop to the antral stage in animals or humans with minimal circulating FSH or defective FSH receptors (**Halpin et al.**, 1986; **Hillier**, 1994; **Aittomaki et al.**, 1995; **Kumar et al.**, 1997), studies in rodents have suggested that the development of early follicles is under the influence of gonadotropins.

Treatment with dihydrotestosterone propionate during the first week of life decreases serum gonadotropins and leads to a delay in ovarian FSH receptor acquisition (**Smith and Ojeda**, 1986). Conversely, treatment of infantile rats with PMSG increases ovarian weight (**Goldenberg et al.**, 1973), whereas treatment of neonatal rats with a GnRH antagonist reduces the number of growing ovarian follicles found at puberty (**Meijs-Roelofs et al.**, 1990).

Taking advantage of the relatively uniform development of the first wave of follicles in the postnatal rat ovary, the role of endogenous and exogenous gonadotropins on preantral follicle development was evaluated (McGee *et al.*, 1997). Reduction of the high levels of gonadotropins present in juvenile rats by either hypophysectomy or GnRH antagonist treatment decreases ovarian weight at day 19 of age and reduces the number of developing follicles together with increasing atresia of the remaining ones. In contrast, treatment with FSH in intact, hypophysectomized, or GnRH antagonist-treated juvenile rats increases ovarian weight and preantral follicle development (McGee *et al.*, 1997).

In vitro studies on the role of FSH in preantral follicle development have been conflicting. In one model, the authors reported that FSH treatment promotes the progression of cultured rat follicles to the antral stage (Cain *et al.*, 1995). In another study, Li *et al.*, (1995) reported that treatment with FSH alone does not enhance granulosa cell division or steroidogenesis. In mice, FSH treatment enhances antral formation without increasing granulosa cell numbers in cultured follicles (Boland *et al.*, 1993) whereas, in cultured hamster follicles, FSH treatment increases granulosa cell division (Roy and Greenwald, 1989). Using a cGMP analog to suppress apoptosis in preantral rat follicles in serum-free cultures, it was demonstrated that FSH treatment increases both follicle size and cell number (McGee *et al.*, 1997).

FSH has been shown to stimulate the expression of cyclin D2, a cell cycle protein important in the G₁ phase of cell division. Mice lacking cyclin D2 are infertile, and granulosa cell replication is impaired as early as the secondary follicle stage (Sicinski *et al.*, 1996). The paucity of granulosa cells results in the formation of small atypical antral follicles that cannot ovulate properly.

Thus, these results demonstrate that preantral follicles respond to gonadotropins with cell division and differentiation. However, follicles can progress to the antral stage in the absence of gonadotropins. In hypogonadal mice (**Halpin et al.**, 1986) or hypophysectomized rats (**Hirshfield**, 1985), ovarian follicles can develop to the secondary and early antral stages, but more slowly and in fewer numbers. In individuals with hypogonadotropic hypogonadism, treatment with exogenous gonadotropins leads to the development of preovulatory follicles within 2 weeks, suggesting that antral follicles are present and available for cyclic recruitment (**Santen and Paulsen**, 1973). **Scaramuzzi et al.** (1993) argued that continued development of antral and larger follicles is dependent on the presence of FSH, but gonadotropin responsiveness may occur earlier in follicle development than is widely believed. This concept is further supported by studies on human early follicles in ovarian xenografts transplanted into the kidney capsule of immunodeficient and hypogonadal mice (**Oktay et al.**, 1998). In this model, FSH was shown to be required for the growth of follicles beyond the two-layer granulosa cell stage. Therefore, gonadotropin fluctuations during the oestrous cycle in the rodent may not only advance the development of antral follicles but may also affect smaller growing follicles that are several cycles away from becoming the leading cohort.

In humans, exogenous gonadotropins could have an effect on follicle development for several months after a controlled ovarian hyperstimulation cycle, although the exact role of gonadotropins remains to be elucidated.

2.17 FSH RESCUE OF ANTRAL FOLLICLES

Before the onset of puberty, the normal fate of growing follicles is atretic demise. After puberty, stimulation by cyclic gonadotropins allows the survival and continued growth

of only a limited number of antral follicles that will reach the preovulatory stage. Morphological and biochemical studies have demonstrated that the demise of both somatic and germ cells in the ovary is mediated by apoptosis (**Hsueh *et al.*, 1994; Pesce and De Felici, 1994; Morita and Tilly, 1999**). Although apoptosis can occur at all stages of follicle development, in rodents, the preantral to early antral transition is most susceptible to atresia (**Hirshfield, 1991**).

Most studies on follicular cell apoptosis have been performed in rats, but there are a few studies on human tissues (**Fukaya *et al.*, 1997; Yuan and Giudice, 1997; Shikone *et al.*, 1997**).

FSH and LH are important trophic factors for the proliferation and survival of follicular somatic cells and the cyclic recruitment of antral follicles. Suppression of serum gonadotropins after hypophysectomy leads to atresia and apoptosis of developing follicles (**Nahum *et al.*, 1996**), whereas FSH treatment of cultured early antral follicles prevents the spontaneous onset of follicular apoptosis (**Chun *et al.*, 1996**). However, LH/hCG treatment alone is ineffective, suggesting that FSH is the predominant survival factor at this stage of follicle development (**Chun *et al.*, 1996**). Although the role of estrogen on human follicles is still unclear, in rats, estrogens are potent antiapoptotic hormones in early antral follicles (**Billig *et al.*, 1993**). Follicular estrogen production is dependent upon both FSH stimulation of aromatase in the granulosa cells and LH stimulation of androstenedione production by the theca (**Hsueh *et al.*, 1984**). Therefore, both gonadotropins play a role in the continued survival of growing follicles, but the cellular mechanism by which FSH or estrogen ensures the survival of early antral follicles is unknown.

2.18 CYCLIC CHANGES OF THE OVARIAN SURFACE EPITHELIUM (OSE) IN THE RAT

The OSE is a single cell layer of squamous and cuboidal cells that express both epithelial and mesenchymal characteristics (**Auersperg *et al.*, 2001**). These cells do not contain discrete markers when compared with other tissues derived from coelomic epithelia, suggesting that they are less differentiated and more pluripotent (**Auersperg *et al.*, 2001**). The OSE is important to the integrity of the ovary and serves as the regulated barrier at the time of ovulation. The OSE was originally studied to investigate its contribution to ovarian follicular rupture and the subsequent repair (**Bjersing and Cajander 1975**). The OSE plays pivotal roles during ovulation and postovulatory wound repair. The cycle of the OSE consists of a proliferative phase that lasts for two consecutive oestrous cycles and a quiescent phase of variable duration. Cyclic changes in the OSE were related to the underlying ovarian structure.

OSE areas covering growing follicles enter into the proliferative phase during the transition from proestrus to estrus, with the appearance of fast-growing class 1 follicles, destined to ovulate at the end of the current oestrous cycle (**Gaytan *et al.*, 2005**; **Burdette *et al.*, 2006**). As follicle rupture occurs at the apex, that is at the follicle side facing the ovarian surface local disruption and subsequent repair of the OSE has to occur during ovulation. However, the role of the OSE in ovulation is still controversial. Although an early study proposed an active role for the OSE in the ovulatory process (**Bjersing and Cajander, 1975**), this notion was discounted thereafter by the observation that ovulation still occurred in some rabbit follicles after OSE scraping (**Rawson and Espey, 1977**). However, more recent studies strongly suggest that the OSE plays an active role in ovulation, participating in the proteolytic breakdown of the basement membrane and the ovarian tunica albuginea (**Murdoch and McDonnel,**

2002), thus contributing to the spatial targeting of follicle rupture at the apex (**Gaytán et al.**, 2003).

Reparative mitogenic activity of OSE cells is currently considered as a main factor favoring accumulation of mutagenic events leading to OSE cell transformation and ovarian cancer (**Murdoch et al.**, 2001; **Murdoch and McDonnel**, 2002). In this context, knowledge of the mechanisms regulating OSE cell proliferation is essential to the understanding of OSE biology either in physiological or pathophysiological conditions. In spite of its morphological simplicity, **Auersperg et al.**, (2001) have pointed out the complexity of OSE cell biology and functional regulation. As the ovary is under the control of gonadotropins and steroids, these hormones are strong candidates to regulate the OSE.

The potential for gonadotropins and steroids to regulate OSE cell proliferation is suggested by the demonstration of receptors for these hormones in the OSE of several species (**Zheng et al.**, 1996; **Pelletier et al.**, 2000; **Kuroda et al.**, 2001; **Okada et al.**, 2002). However interspecies variations have been reported (**Hess et al.**, 1999; **Pelletier et al.**, 2000). In general, *in vivo* studies have reported that gonadotropins (**Davies et al.**, 1999; **Hess et al.**, 1999; **Stewart et al.**, 2004) and estrogens (**Adams and Auersperg**, 1983; **Bai et al.**, 2000) stimulate OSE cell proliferation. However, *in vitro* studies have provided variable results (**Gaytán et al.**, 2005). Whereas some studies have reported that gonadotropins and estrogens stimulate the proliferative activity of OSE cells in culture (**Bai et al.**, 2000; **Murdoch and van Kirk**, 2002), other studies having found a lack of mitogenic effects of gonadotropins and steroids in isolated OSE cells (**Karlan et al.**, 1995; **Syed et al.**, 2001; **Wright et al.**, 2002), suggesting that the effects of gonadotropin and estrogens could be mediated by the local release of growth-promoting factors.

At the site of ovulation, OSE cells suffer DNA oxidative damage and express the tumor suppressor p53, showing the potential of ovulation as a stress factor on OSE cells to give rise to a transformed progenitor cell. The expression of FSH and LH receptors in the OSE reflects on their ability to directly respond to the gonadotropins FSH and LH independent of an ovulatory event (**Zheng *et al.*, 1996; Parrot *et al.*, 2001**).

Steroid hormones released by the follicle and/or the corpus luteum are strong candidates for factors which regulate the proliferative activity of OSE cells. In general, like in many other tissues, several studies have reported that estradiol stimulates OSE cell proliferation (**Murdoch and van Kirk, 2002; Ho, 2003**), whereas progesterone has been reported to inhibit estrogen-mediated OSE cell proliferation (**Murdoch and van Kirk, 2002**). **Gaytan *et al.*, (2005)** demonstrated that the proposed inhibitory effects of progesterone are in line with the low proliferative activity of OSE cells found in their study during pregnancy. However, due to the existence of OSE areas showing different proliferative activity at the same phase of the oestrous cycle, a clear correlation between serum hormone concentrations and OSE cell proliferation could not be established. It is unclear whether estradiol and progesterone effects on the OSE are direct or mediated by local factors. Interestingly, the proliferative response of OSE cells to estrogen is enhanced when epithelial cells are grown together with ovarian stromal cells (**Bai *et al.*, 2000**), suggesting the existence of stromal-epithelial interactions. Moreover, it has been reported that estrogens do not have mitogenic effects on isolated OSE cells (**Karlan *et al.*, 1995; Wright *et al.*, 2002**).

In another study, **Gaytan *et al.*, (2005)** reported that the immunostaining of OSE cells for ER α was not regionalized, and the expression of ER α was equivalent at least qualitatively in both proliferating and quiescent areas. This suggests that the proposed effects of estrogens on OSE cell proliferation are mediated by local

microenvironmental factors (**Gaytan *et al.*, 2005**). Similarly, the absence of progesterone receptor (PR) expression in the rat OSE strongly suggests that the proposed progesterone effects on OSE cell proliferation are indirect.

However, the possibility of non-genomic responses to steroid hormones, independent of classic genomic steroid receptors cannot be discarded (**Bramley, 2003**).

Some previous studies have analysed the proliferative activity of the OSE in immature gonadotropin-primed rats and mice (**Beller *et al.*, 1995; Hess *et al.*, 1999**). It is worthy to note that in immature animals, the development of a large cohort of follicles about 50 and subsequent corpora lutea is stimulated by equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) treatment. In addition, the ovary considerably enlarges as a consequence of superovulation. Accordingly, large areas of the OSE should enter into the proliferative phase in immature gonadotropin-primed rats. This would likely lead to overestimation of the proliferative response of the OSE to gonadotropin treatment when compared with adult cycling rats, and could mask the existence of quiescent, non-proliferating areas.

In adult animals, the increasing volume of growing follicles and newly formed corpora lutea is compensated by the loss of volume of several generations of regressing corpora lutea of previous cycles, this occurs at each transition from proestrus to estrus, triggered by the preovulatory PRL surge. In this way, the ovarian volume, and hence the ovarian surface does not undergo significant changes throughout the oestrous cycle. Prostaglandins are essential factors during the ovulatory process (**Tsafiriri *et al.*, 1993**) and some of the changes that happen in the OSE during ovulation have been reported to be mediated by prostaglandins (**Ackerman and Murdoch 1993; Gaytán *et al.*, 2002**).

Gaytan *et al.*, (2005) analysed the proliferative response of the OSE after ovulation in rats treated with indomethacin. The equivalent proliferative activity of OSE cells in vehicle or indomethacin-treated rats indicates that post-ovulatory proliferative activity of OSE cells was neither mediated by prostaglandins, nor affected by indomethacin treatment through prostaglandin-independent routes. Data from indomethacin-treated rats also provided information on the mechanism of OSE cell proliferation at the site of ovulation. These investigators argued that although rupture of the integrity of the OSE has been considered as the main factor determining OSE cell proliferation after ovulation however ovulation-triggered OSE cell proliferation was not directly due to the occurrence of rupture of the OSE but to ovulation-related events, as indicated by the similar proliferative activity in newly-formed corpora lutea, in which rupture at the apex did not occur.

2.19 OOCYTE PROGRAMMED CELL DEATH

In mammals, oogenesis begins with the formation of primordial germ cells and encompasses a series of cellular differentiation events, from primordial germ cells to oogonia, from oogonia to oocytes, and from oocytes to eggs. Extensive degeneration of germ cells has been described during embryonic, fetal, and early postnatal stages of oogenesis before follicle formation (**Labascio *et al.***, 2007)

In the mouse embryo, early morphological studies have shown that cell death may occur in primordial germ cells or oogonia 12–13 days *post coitum*, (dpc), but mainly in oocytes at the zygotene/pachytene stage of meiotic prophase I (MPI); from 16.5 dpc through birth (**Bakken and McClanahan**, 1978). Following such episodes, the number of germ cells decreases from ~20, 000 at 13.5 dpc to about 6000–10 000 after 6 days, at birth (**Burgoyne and Baker**, 1981; **Tam and Snow**, 1981).

McClellan et al., (2003) performed a careful study of the number of oocytes throughout MPI in the embryonic mouse ovaries and reached the conclusion that the decrease of oocyte population during this period (about 65% loss) is a continuous process without apparent peaks of degeneration. If or not oocyte degeneration is a continuous or a stage-dependent process, it shows characteristics of programmed cell death (PCD). That is cell death which occurs in predictable places (fetal ovary) and at predictable times (mid-late gestation) and results in rapid elimination of considerable numbers of cells (oocytes) without any inflammatory reaction.

Three types of PCD have been described. Type I is apoptotic cell death, which includes the morphological changes of cell shrinkage, membrane blebbing, and extensive chromatin condensation followed by nuclei fragmentation. Type II is autophagic cell death, characterized by the formation of autophagic vacuoles in the cytoplasm of dying cells. Type III is necrotic cell death, characterized by a rapid loss of plasma membrane integrity and spillage of the intracellular contents (**Labascio et al.**, 2007).

According to several reports, fetal oocyte PCD takes place in the form of apoptosis (**De Felici et al.**, 2005). The process of oocyte death remains, however, incompletely characterized and alternative forms of PCD for embryonic germ cells remain possible (**Wartenberg et al.**, 2001).

Furthermore, the causes and the molecular mechanisms underlying oocyte PCD are little understood (**Morita and Tilly**, 1999; **Tilly**, 2001; **De Felici et al.**, 2005). Apoptosis may occur via a death receptor-dependent (extrinsic) or receptor-independent (intrinsic) pathway. The extrinsic pathway is activated by the binding of ligands (Fas ligand and tumour necrosis factor) to death receptors on the cell membrane. **De Felici et al.**, (2005) postulated that there is no evidence that such a

pathway can operate in fetal oocytes. The intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth factor withdrawal, hypoxia, and DNA damage. On these bases, two main hypotheses have been advanced to explain germ cell death in the embryo. Germ cells like most other cell types could depend on growth factors for survival, and a limited supply of growth factors might determine oocyte apoptosis. In fact, the primordial germ cells, the precursors of both oocytes and spermatogonia, appear to undergo apoptosis in the absence of certain growth factors such as kit ligand (KL) and leukemia inhibitory factor (LIF) (**De Felici**, 2000). Evidence is increasing that fetal oocytes also need specific growth factors to avoid apoptosis. Growth factors reported to exert direct and/or indirect anti-apoptotic effect on the fetal oocytes include besides KL and LIF also insulin growth factor-I (IGF-I), interleukin α/β , neurotrophin 4/5, and brain-derived neurotrophic factors (**Morita and Tilly**, 1999; **Tilly**, 2001; **De Felici et al.**, 2005). In the fetal ovary, growth factor dependence for survival could represent a way to limit the number of oocytes supported by somatic cells during the formation of follicles. In addition or alternatively, oocytes with defects in chromosome crossover, a process requiring critical events such as chromosome pairing, recombination, DNA repair, and synapses, might be eliminated by apoptosis to assure oocyte quality control. In the mouse, genetic mutations resulting in crossover defects during prophase I, which cause fetal oocyte depletion, have been identified: *Atm*, **Barlow et al.**, (1998); *Msh4* and *Msh5*, **de Vries et al.**, (1999); **Edelmann et al.**, 1999; **Kneitz et al.**, (2000); *Dmc1*, **Yoshida et al.**, (1998). Moreover, female mice that lack a second X chromosome (X0) show oogenesis failure presumably due to failed chromosome pairing, which leads to increased oocyte degeneration during the development of the fetal ovaries (**Burgoyne and Baker**, 1985).

A similar situation occurs in the Turner (X0) syndrome in humans, which is associated with massive oocyte apoptosis in the fetal ovary (**Modi et al.**, 2003).

Though inadequate supply of growth factors and defects in the crossover are likely causes of fetal oocyte apoptosis, only little is known about the molecular pathways which in oocytes link such deficiencies to PCD.

A diverse spectrum of pro- and anti-apoptotic susceptibility genes including members of the *Bcl-2* (*Bax*, *Bcl-x*) and *casp* (caspase-2) gene families have been reported to be expressed in germ cells of the ovary and seem to be involved in controlling apoptotic pathways in oocytes (**Tilly**, 2001). While mice lacking *Bax* did not show alteration in the number of oocytes at birth (**Perez et al.**, 1999), *Bcl-x*- or *Bcl-2*-deficient mice show a marked reduction of the postnatal oocyte pool (**Ratts et al.**, 1995; **Rucker et al.**, 2000). Moreover, evidence exists that increased levels of ceramide (**Bergeron et al.**, 1998) and activation of the aromatic hydrocarbon receptor (**Matikainen et al.**, 2001) may trigger apoptosis in fetal mouse oocyte.

2.20 GONADOTROPIN SURGE-ATTENUATING FACTOR

The putative ovarian hormone, gonadotrophin surge-attenuating factor (GnSAF) has the specific biological action of reducing pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in response to gonadotrophin-releasing hormone (GnRH). However, the factor remains to be conclusively identified (**Fowler et al.**, 2003). GnSAF is secreted by granulosa cells (**Fowler et al.**, 2002) and there is a large body of data demonstrating that FSH administration stimulates GnSAF production both *in vivo* (**Messinis et al.**, 1993; 1994; **Fowler and Price**, 1997) and *in vitro* (**Fowler and Mason**, 2000). However, there are a number of concerns about both kinds of data. FSH treatment *in vivo* stimulates a considerable number of protein and

gene changes within the ovary. Such changes in expression levels *e.g.* in estradiol and inhibin-B can, in turn, exert effects at the level of the hypothalamus and pituitary, making it difficult to determine whether any effect of FSH on GnSAF is direct or indirect. On the other hand, FSH administration to cultured granulosa cells, while excluding the possibility of indirect effects, stimulates protein synthesis in the absence of normal structural relationships between granulosa cells and between granulosa, oocyte and thecal cells. There is the potential, therefore, for the response of cultured granulosa cells to deviate from the normal response to such stimulation *in vivo*.

Two publications have questioned a direct link between FSH and GnSAF. In the first, **Tio *et al.***, (1998) immunoneutralised FSH in female rats and concluded that there was no difference in circulating GnSAF. In the second, **Schuiling *et al.*** (1999) found that FSH treatment in female rats suppressed estradiol-augmented LH responses to GnRH without affecting GnRH self-priming, a definite aspect of GnSAF from *in vitro* pituitary models (**Fowler and Templeton**, 1996). In both of these studies the interpretation of the data is complex and debatable, due to multiple factors operating *in vivo*. What is required is an analysis of FSH actions on the production of GnSAF from a model system demonstrating normal function *in vitro*. The intact rodent follicle model (**Spears *et al.***, 1994; **McGee *et al.***, 2001; **Murray *et al.***, 2001; **Spears *et al.***, 2002) retains the cell–cell interactions and structural framework within which the granulosa cell normally functions.

2.21 OXIDATIVE STRESS (OS)

A paradox in metabolism is that while the large majority of complex life on earth requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species (**Davies**, 1995). Free radical

species are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease (**Attaran et al.**, 2000; **Van Landendonck et al.**, 2002; **Szczepanska et al.**, 2003; **Pierce et al.**, 2004). There are two major types of free radical species (1) reactive oxygen species (ROS) (2) reactive nitrogen species (RNS) (**Agarwal et al.**, 2005). The three major types of ROS are: superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl (OH^{\bullet}). The superoxide radical is formed when electrons leak from the electron transport chain (**Halliwell et al.**, 1992). The dismutation of superoxide results in the formation of hydrogen peroxide. The hydroxyl ion is highly reactive and can modify purines and pyrimidines and cause strand breaks resulting in DNA damage (**Mello et al.**, 1984). Some oxidase enzymes can directly generate the hydrogen peroxide radical.

ROS have been implicated in more than 100 diseases (**Aitken and Baker**, 2004; **Gibson and Huang**, 2004; **Madamanchi et al.**, 2005). They have a physiological and pathological role in the female reproductive tract. Numerous animal and human studies have demonstrated the presence of ROS in the female reproductive tract: ovaries, (**Sabatini et al.**, 1999; **Suzuki et al.**, 1999; **Behrman et al.**, 2001), fallopian tubes (**El Mouatassim et al.**, 1999) and embryos (**Guerin et al.**, 2001). ROS is involved in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, corpus luteal function and luteolysis (**Ishikawa**, 1993; **Sabatini et al.**, 1999; **Behrman et al.**, 2001).

ROS-related female fertility disorders may have common etiopathogenic mechanisms. ROS may also originate from embryo metabolism and from its surroundings. Nitric oxide (NO) is synthesized during the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) (**Rosselli et al.**, 1998; **Dong et al.**, 2001).

With an unpaired electron, NO, a highly reactive free radical, damages proteins, carbohydrates, nucleotides and lipids and, together with other inflammatory mediators, results in cell and tissue damage, low-grade, sterile inflammation and adhesions (**Dong et al.**, 2001). NO potently relaxes arterial and venous smooth muscles and, less strongly, inhibits platelet aggregation and adhesion. NO donors, acting as vasodilating agents, are therefore a possible therapeutic approach (**Ohl et al.**, 2002). NO acts in a variety of tissues to regulate a diverse range of physiological processes, but excess of NO can be toxic (**Rosselli et al.**, 1998; **Dong et al.**, 2001; **Osborn et al.**, 2002).

RNS have been associated with asthma, ischemic/reperfusion injury, septic shock and atherosclerosis (**Schrier and Wang**, 2004; **Reyneart et al.**, 2005; **Schulman et al.**, 2005). The two common examples of RNS are nitric oxide (NO) and nitrogen dioxide (**Van Langendonck et al.**, 2002; **Szczepanska et al.**, 2003; **Pierce et al.**, 2004). NO is produced by the enzyme NO synthase. There are 3 types of nitric oxide synthase (NOS) isoenzymes in mammals: Neuronal NO synthase (NO synthase 1), Inducible NO synthase (NO synthase 2) and Endothelial NO synthase (NO synthase 3). Neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS) are constitutive NO synthases, and responsible for the continuous basal release of NO. Inducible NO synthase (iNOS) is present in mononuclear phagocytes (monocytes and macrophages) and produces large amounts of NO. This is expressed in response to proinflammatory cytokines and lipopolysaccharides (**Ota et al.**, 1998; **Osborn et al.**, 2002). Inducible NO synthase is activated by cytokines such as, interleukin-1, and TNF- α and lipopolysaccharides. Endothelial NO synthase is expressed in thecal cells, granulosa cells, and the surface of oocyte during the follicular development (**Agarwal et al.**, 2005). In pathological conditions, inducible NO synthase might play a major role in

NO production. In most organs, inducible NO synthase is expressed only in response to immunological stimuli (**Lee *et al.*, 2000**).

2.21.1 OXIDATIVE STRESS IN FEMALE REPRODUCTION

Cells have developed a wide range of antioxidant systems to limit production of ROS, inactivate them and repair cell damage (**Van Langendonck *et al.*, 2002**; **Agarwal and Allamaneni, 2004**; **Pierce *et al.*, 2004**). OS influences the entire reproductive span of women's life and even thereafter i.e. menopause. **de Bruin *et al.*, (2002)** suggest that the age-related decline in fertility is modulated by OS. OS plays a role during pregnancy (**Myatt and Cui, 2004**) and normal parturition (**Fainaru *et al.*, 2002**; **Mocatta *et al.*, 2004**) and in initiation of preterm labor (**Wall *et al.*, 2002**; **Pressman *et al.*, 2003**). The pathological effects are exerted by various mechanisms including lipid damage, inhibition of protein synthesis, and depletion of ATP (**Ray *et al.*, 2004**).

The control of ovarian stromal cells and germ cell function is a diverse paradigm and oxidative stress may be one of the modulators of ovarian germ cell and stromal cell physiology. A number of autocrine and paracrine factors affect the modulation of various ovarian functions and steroidogenesis. Cytokines are polypeptides or glycoproteins secreted into the extra cellular compartment by the leukocytes (**Bedaiwy *et al.*, 2002**). Mammalian ovulation or follicular rupture was proposed to result from the vascular changes and the proteolytic cascade (**Tsafri and Reich, 1999**).

The cross talk between these two cascades is mediated by cytokines, vascular endothelial growth factor (VEGF), and ROS both reactive nitrogen and oxygen radicals. Interleukin-1 β causes nitrite to accumulate in rat ovarian dispersates, demonstrating the close interaction between cytokines and NOS (**Ben-Shlomo *et al.*, 1994**). OS and cytokines are proposed to be interlinked and act as intercellular and

intracellular messengers in the ovary. A number of investigators have investigated the synthesis of NOS and ROS in the ovaries (**Van Voorhis *et al.*, 1994; Sugino *et al.*, 1996; Roselli *et al.*, 1998**).

Markers of oxidative stress such as superoxide dismutase (SOD), Cu-Zn SOD, Mn SOD, glutathione peroxidase, γ glutamyl synthetase and lipid peroxides have been investigated by immunohistochemical localization, m-RNA expression and thiobarbituric acid method (**Suzuki *et al.*, 1999; Attaran *et al.*, 2000; Sugino *et al.*, 2000**). The expression of various biomarkers of OS has been demonstrated in normal cycling human ovaries (**Shiotani *et al.*, 1991; Suzuki *et al.*, 1999**). All the various follicular stages have been examined for SOD expression including primordial, primary, preantral and nondominant antral follicles in follicular phase, dominant follicles and atretic follicles (**Suzuki *et al.*, 1999**). ROS may have a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis and luteolysis. There is a delicate balance between ROS and antioxidant enzymes in the ovarian tissues. The antioxidant enzymes neutralize ROS production and protect the oocyte and embryo.

The presence of superoxide dismutase in the ovary, revealed intense staining by immunohistochemistry in the theca interna cells in the antral follicles (**Shiotani *et al.*, 1991**). Antibody to Ad4-binding protein (Ad4BP) was utilized to localize Ad4BP in the nuclei of theca and granulosa cells. Ad4BP is a steroidogenic transcription factor that induces transcription of the steroidogenic P450 enzyme. Thus, it controls steroidogenesis in the ovaries. The correlation between Ad4BP and superoxide dismutase expression suggested an association between OS and ovarian steroidogenesis (**Suzuki *et al.*, 1999**).

Behrman *et al.*, (2001) postulated that both human granulosa and luteal cells respond to hydrogen peroxide with an extirpation of gonadotropin action and inhibition of

progesterone secretion. The production of both progesterone and estradiol hormones is reduced when hydrogen peroxide is added to a culture of human chorionic gonadotropin-stimulated luteal cells. Hydrogen peroxide lowers both cAMP dependent and non-cAMP dependent steroidogenesis (**Vega *et al.*, 1995**). The role of human chorionic gonadotropin (hCG) in the expression of the antioxidant enzyme SOD has been investigated. **Suzuki *et al.*, (1999)** carried out a study on the corpora lutea collected from patients at hysterectomy and surgeries for ectopic pregnancy. These authors observed that the Cu-Zn SOD expression in the corpora lutea paralleled levels of progesterone and these levels rose from early to mid luteal phase and decreased during the regression of the corpus luteum. However, in the corpus luteum from pregnant patients, the mRNA expression for Cu-Zn superoxide dismutase was significantly higher than that in midcycle corpora lutea. The authors suggested that this enhanced expression of luteal Cu-Zn SOD may be due to hCG. Furthermore they concluded that hCG may have an important role in maintenance of corpus luteal function in pregnancy.

Levels of three oxidative stress biomarkers, conjugated dienes, lipid hydroperoxide and thiobarbituric acid were determined in preovulatory follicles. Concentration gradient was found to exist as levels of all three markers were significantly lower in the follicular fluid compared with serum levels (**Jozwik *et al.*, 1999**). The preovulatory follicle has a potent antioxidant defense, which is depleted by the intense peroxidation (**Jozwik *et al.*, 1999**). Glutathione peroxidase may also maintain low levels of hydroperoxides inside the follicle and thus may play an important role in gametogenesis and fertilization (**Paszkowski *et al.*, 1995**).

2.22 ANTIOXIDANTS

The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines (**Matill**, 1947).

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (**German**, 1999). Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms (**Jacob**, 1996; **Knight**, 1998).

The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized (**Moreau** and **Dufraisse**, 1922). Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevents oxidative reactions, often by scavenging reactive oxygen species before they can damage cells (**Wolf**, 2005).

An antioxidant is therefore a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves (**Wolf**, 2005). As a result, antioxidants are often reducing agents

such as thiols or polyphenols. Although oxidation reactions are crucial for life, they can also be damaging. Hence, plants and animals maintain complex systems of multiple types of antioxidants, such as Glutathione, Vitamin C and Vitamin E as well as enzymes such as Catalase (CAT), SOD and various Peroxidases (**Jacob, 1996; Knight, 1998**).

Low levels of antioxidants, or inhibition of the antioxidant enzymes causes oxidative stress and may damage or kill cells (**Wolf, 2005**). As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatment for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful (**Bjelakovic *et al.*, 2007**). In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses such as preservatives in food and cosmetics. There are two types of antioxidants in the human body: enzymatic antioxidants and non-enzymatic antioxidants (**Van Langendonck *et al.*, 2002; Pierce *et al.*, 2004**).

Enzymatic antioxidants are also known as natural antioxidants; they neutralize excessive ROS and prevent it from damaging the cellular structure. Enzymatic antioxidants are composed of SOD, CAT, glutathione peroxidase and glutathione reductase, which also causes reduction of hydrogen peroxide to water and alcohol (**Agarwal *et al.*, 2005**).

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is influenced by dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, beta carotene, and carotene (**Van Langendonck et al.**, 2002; **Szczepanska et al.**, 2003; **Pierce et al.**, 2004).. Taurine, hypotaurine and transferrin are mainly found in the tubal and follicular fluid where they protect the embryo from OS (**Guerin et al.**, 2001). Glutathione is present in the oocyte and tubal fluid and has a role in improving the development of the zygote beyond the 2-cell stage to the morula or the blastocyst stage (**de Matos and Fumus**, 2000).

The relative importance and interactions between these different antioxidants is a very complex question, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another (**Sies**, 1993; **Chaudiere and Ferrari-Iliou**, 1999). The action of one antioxidant may therefore depend on the proper function of other members of the antioxidant system (**Vertuani et al.**, 2004). The amount of protection provided by any one antioxidant will also depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts (**Vertuani et al.**, 2004).

2.23 VITAMIN C

Vitamin C or L-ascorbic is an essential nutrient for humans, in which it functions as a vitamin. Ascorbate, an ion of ascorbic acid is required for a range of essential metabolic reactions in all animals and plants. Deficiency in this vitamin causes the disease scurvy in humans (**Higdon**, 2006). It is also widely used as a food additive (**McCluskey**, 1985).

Scurvy has been known since ancient times. People in many parts of the world assumed it was caused by a lack of fresh plant foods. The British Navy started giving sailors lime juice to prevent scurvy in 1795 (**Wilson**, 1975). Ascorbic acid was finally isolated in 1933 and synthesized in 1934.

The pharmacophore of Vitamin C is the ascorbate ion. In living organisms, ascorbate is an anti-oxidant, since it protects the body against oxidative stress and is a co-factor in several vital enzymatic reactions (**Padayatty**, 2003).

Vitamin C is purely the L-enantiomer of ascorbate; the opposite D-enantiomer has no physiological significance. Both forms are mirror images of the same molecular structure. When L-ascorbate, which is a strong reducing agent, carries out its reducing function, it is converted to its oxidized form L-dehydroascorbate (**Meister**, 1994). The L-dehydroascorbate can then be reduced back to the active L-ascorbate form in the body by enzymes and glutathione (**Meister**, 1994). During this process semidehydroascorbic acid radical is formed. Ascorbate free radical reacts poorly with oxygen, and thus, will not create a superoxide. Instead two semidehydroascorbate radicals will react and form one ascorbate and one dehydroascorbate. In the presence of glutathione, dehydroascorbate is converted back to ascorbate (**Nualart et al.**, 2003). Glutathione is crucial since it spares ascorbate and improves the anti-oxidant capacity of blood (**Gropper et al.**, 2004).

The vast majority of animals and plants are able to synthesize their own Vitamin C, through a sequence of four enzyme-driven steps, which convert glucose to Vitamin C (**Banhegyi and Mandl**, 2001). The glucose needed to produce ascorbate in mammals and perching birds is extracted from glycogen in the liver while in reptiles and birds; glycogen is extracted from the kidneys. Ascorbate synthesis is a glycogenolysis-dependent process (**Banhegyi and Mandl**, 2001).

Ascorbic acid is absorbed in the body by both active transport and simple diffusion. Sodium Dependent Active Transport- Sodium-Ascorbate Co-Transporters (SVCTs) and Hexose transporters (GLUTs) are the two transporters required for absorption. SVCT1 and SVCT2 imported the reduced form of ascorbate across plasma membrane (**Savini et al.**, 2008). GLUT1 and GLUT3 are the two glucose transporters and only transfer dehydroascorbic acid form of Vitamin C (**Rumsey et al.**, 1997). Although dehydroascorbic acid is absorbed in higher rate than ascorbate, the amount of dehydroascorbic acid found in plasma and tissues under normal conditions is low as cells rapidly reduce dehydroascorbic acid to ascorbate (**Packer**, 1997; **May et al.**, 2003). Thus, SVCTs appear to be the predominant system for Vitamin C transport in the body. SVCT2 is involved in Vitamin C transport in almost every tissue (**Savini et al.**, 2008), the notable exception being red blood cells which lose SVCT proteins during maturation (**James et al.**, 2007). Knockout animals for SVCT2 die shortly after birth suggesting that SVCT2-mediated Vitamin C transport is necessary for life (**Sotiriou et al.**, 2002).

With regular intake the absorption rate varies between 70 to 95%. However, the degree of absorption decreases as intake increases. At high intake (12 g), the human body can absorb ascorbic acid as low as 16% while at low intake (<20 mg) the absorption rate could reach up to 98% (**Levine et al.**, 1996).

2.23.1 IS VITAMIN C AN ANTI-OXIDANT OR A PRO-OXIDANT

Paradoxically, vitamin C is also known to act as a pro-oxidant *in vitro*. Mixtures of Vitamin C and copper or iron have been used for decades to induce oxidative modifications of lipids, proteins and DNA (**Halliwell and Gutteridge** 1999).

Vitamin C may contribute to oxidative damage formation by reducing ferric Fe^{3+} to ferrous Fe^{2+} ions and Cu^{2+} to Cu^{1+} (**Duarte and Lunec**, 2005). This in turn can reduce

hydrogen peroxide to hydroxyl radicals. However, although this Vitamin C-mediated Fenton reaction occurs *in vitro*, some authors have argued that because the human body is able to control these reactions, the pro-oxidant effect of Vitamin C may not be relevant *in vivo* (**Carr and Frei**, 1999; **Halliwell** 1999). Nevertheless, vitamin C supplements have not been recommended in people with high iron levels or in pathological conditions associated with iron overload such as thalassaemia or haemochromatosis (**Herbert et al.**, 1996).

Moreover, **Lee and Blair** (2001) argued that it is also possible that not all the undesired effects of vitamin C depend on the presence of transition metals. They reported that a mechanism has been provided by which vitamin C induces the decomposition of lipid hydroperoxides to genotoxic bifunctional electrophiles *in vitro* without the need for free transition metal ions. However, it is still unknown whether this mechanism is relevant *in vivo* (**Lee and Blair**, 2001).

Other authors have argued that in the more physiological context of human plasma incubated *in vitro*, vitamin C prevents lipid peroxidation even in the presence of added redox-active transition metals and Hydrogen peroxides (H₂O₂) (**Suh et al.**, 2003).

2.23.2 Human intervention studies

The controversy around the *in vivo* anti- or pro-oxidant nature of vitamin C has been the subject of several human intervention studies in recent years (**Duarte and Lunec**, 2005). The ability of vitamin C to modulate oxidative DNA damage *in vivo* is of particular interest because some oxidative DNA lesions are thought to be pre-mutagenic (**Wang et al.**, 1998).

In a review, **Duarte and Lunec** (2005) reported that most of the intervention studies on humans have looked at the effects of vitamin C supplementation on oxidative damage to DNA of blood cells. The most popular base lesion has been 8-oxo-7,8-dihydro-2'-

deoxyguanosine (8-oxo-dG). The detection of 8-oxo-dG is relevant because not only is it one of the most abundant DNA lesions formed during oxidative stress, it is also mutagenic and is implicated in carcinogenesis (**Kasai**, 1997). In addition, it can be quantitatively measured as either the base product (8-oxo-G) or as the deoxynucleoside (8-oxo-dG) in tissues, serum and urine following exposure to oxidative stress (**Duarte and Lunec**, 2005).

Duarte and Lunec (2005) went further to state that some studies have also measured oxidative stress by measuring DNA single-strand breaks as an indirect indicator of antioxidant status. To achieve this purpose, researchers challenge white blood cells *ex vivo* with a DNA strand breaking oxidant usually H₂O₂ or ionising radiation to assess the donor's antioxidant status, i.e. assuming that the intracellular antioxidants would prevent DNA breakage (**Duarte and Lunec**, 2005).

The following studies report the effects of vitamin C administered to healthy or diseased individuals as a dietary supplement or intravenously, either alone or in combination with other antioxidants, and either as a single dose or for several weeks or months.

As regarding the measurement of DNA base lesions, an earlier study showed that vitamin C content in the semen was inversely related to the level of 8-oxo-dG in sperm DNA (**Fraga *et al.***, 1991). This report was in agreement with the report of **Lenton *et al.***, (1999) who reported that 8-oxo-dG in lymphocyte DNA from human healthy volunteers was negatively correlated with the intracellular vitamin C levels. In spite of these findings, the question still remains – Can supplementation with Vitamin C contribute to the reduction of the levels of the DNA base lesions *in vivo*?

Healthy individuals were administered supplements of 500 mg vitamin C for 6 weeks (**Podmore *et al.***, 1998). The authors observed a decrease in 8-oxo-G in lymphocyte

DNA relative to both placebo and baseline levels. This result suggests that vitamin C was acting directly as an anti-oxidant *in vivo*. However, these workers found a simultaneous increase in the level of another base oxidation product, 8-oxoadenine (8-oxo-A), suggesting a concomitant pro-oxidant effect. 8-oxo-A is, however, much less mutagenic than 8-oxo-G, so the authors argued that the study has shown an overall protective effect of vitamin C (**Podmore *et al.*, 1998**). In a later publication, but as part of the same study, **Cooke *et al.*, (1998)** found significant decreases in DNA levels of 8-oxo-dG that were strongly correlated with increase in plasma vitamin C concentration *in vivo*, and reported significant subsequent increases in serum and urinary 8-oxo-dG levels, which could be products of DNA repair. The authors suggested that vitamin C did not inhibit 8-oxo-dG formation but rather promoted its removal. It was hypothesised that vitamin C would initially cause oxidative DNA damage via a pro-oxidant activity and consequently cause the up-regulation of DNA repair processes that promote removal of highly mutagenic lesions.

In another study, **Cooke *et al.*, (2003)** reported that vitamin C supplementation increased the levels of deoxycytidine glyoxal (gdC), a putative product of lipid peroxidation and autoxidation of vitamin C and glucose, which was also suggestive of a pro-oxidant effect *in vivo*. Deoxycytidine glyoxal levels were, however, significantly reduced upon continued vitamin C supplementation, suggesting once again that vitamin C may promote lesion removal by up-regulating repair processes.

Further evidence for a pro-oxidant effect of vitamin C *in vivo* came from **Rehman *et al.* (1998)**. The authors observed a significant rise in several oxidative DNA base damage products (5-OH methylhydantoin, 5-OH hydantoin and FAPY guanine) in the white blood cells of healthy human volunteers with a high initial plasma vitamin C concentration after 6 weeks of co-supplementation with iron and vitamin C. On the

other hand, levels of 8-oxo-G decreased following 12 weeks of supplementation. In individuals with lower initial levels of plasma vitamin C, pre-supplemental levels of oxidative DNA damage were higher and decreased on supplementation.

In two other placebo-controlled supplementation trials with healthy individuals, **Welch *et al.*** (1999) claimed that vitamin C supplementation alone or in combination with vitamin E for 4 weeks did not have an effect on oxidative damage, while **Vojdani *et al.*** (2000) observed a reduction in lymphocyte 8-oxo-dG following vitamin C supplementation with 1000 mg per day for 2 weeks.

In addition, four placebo-controlled supplementation trials were performed with smokers, a condition that is known to be associated with increased oxidative damage to DNA. **Lee *et al.*** (1998) supplemented smokers with 500 mg vitamin C per day for 4 weeks and observed a decrease in 8-oxo-dG levels in white blood cells, even though this effect was not statistically significant. On the other hand, **Jacobson *et al.*** (2000), reported on the effect of supplementation with vitamin C in combination with vitamin E and β -carotene on smokers. The authors reported a decrease in 8-oxo-dG levels both in treated and in placebo groups. But a combination of the same antioxidants was able to decrease the endogenous levels of oxidised pyrimidines assessed as endonuclease III sensitive sites with the use of the comet assay in lymphocyte DNA of smokers and non-smokers.

Moller *et al.* (2004) have also reported that oral supplementation with 500 mg vitamin C in combination with 182 mg vitamin E per day protected blood mononuclear cells of smokers against oxidative DNA damage by decreasing the amount of endonuclease III and FPG sensitive sites. Interestingly, the protective effect was only evident for a few hours after ingestion when the vitamin C was supplied as plain release tablets;

however, supplementation with slow release tablets afforded a longer-term protection that was still evident at the end of the 4 weeks trial.

In a different study, whole blood DNA from individuals exposed to environmental tobacco smoke contained increased levels of 8-oxo-dG, which decreased to levels below those in the control group on supplementation with an antioxidant cocktail containing vitamin C (**Howard *et al.*, 1998**).

Furthermore three trials have assessed the effect of vitamin C supplementation on DNA base oxidation in other conditions that have been associated with oxidative stress. **Evans *et al.* (2000)** reported no effect of vitamin C supplementation on lymphocyte DNA 8-oxo-dG of systemic lupus erythematosus (SLE) patients. **Jaruga *et al.* (2002)** reported that HIV-infected patients had significantly higher levels of two oxidised DNA bases (8-oxo-G and 5-OH-Ura) that were decreased upon vitamin C supplementation in combination with vitamins A and E. A randomised, placebo-controlled study with chronic haemodialysis patients showed additional evidence for a role of vitamin C in promoting removal of 8-oxo-dG. In these subjects, vitamin C intravenous supplementation for 8 weeks was able to reduce the lymphocyte 8-oxo-dG levels (**Tarng *et al.*, 2004**).

Results from studies in which white blood cells were collected from patients and subsequently challenged *ex vivo* with an oxidant insult are more promising but still conflicting. Three studies investigated the effect of a single-dose vitamin C supplementation. **Green *et al.* (1994)** reported a protection against *ex vivo* exposure to ionising radiation that started as early as 1 h after a single vitamin C ingestion (500 mg) and peaked at 4 h. **Panayiotidis *et al.* (1997)** reported that a single high dose (1 g) vitamin C supplement afforded protection against oxidative DNA damage caused by *ex vivo* exposure to H₂O₂. In agreement with the study of **Green *et al.* (1994)**, this

protection peaked at 2-4 h after the ingestion. However, another study failed to show any effect of a single dose of vitamin C alone, or in combination with vitamin E, on resistance to an *ex vivo* oxidative challenge, despite the clear increase in plasma ascorbate (**Choi *et al.***, 2004).

Other trials have investigated the effects of long-term vitamin C supplementation. Antioxidant supplementation with a combination of vitamin C, vitamin E and β -carotene for 20 weeks has decreased the damage induced when lymphocytes were challenged *ex vivo* with H₂O₂ (**Duthie *et al.***, 1996). **Brennan *et al.*** (2000) supplemented individuals with vitamin C and observed a similar protective effect, which correlated with increases in patient plasma and lymphocyte intracellular vitamin C levels. However, other studies have failed to show a protective effect (**Anderson *et al.***, 1997; **Welch *et al.***, 1999; **Astley *et al.***, 2004).

Duarte and Lunec (2005) suggested that a possible reason for the discrepancies encountered between different studies might be the vitamin C intracellular saturation. It is known that blood cells saturate at lower vitamin C concentrations than human plasma and that this intracellular saturation occurs at plasma concentrations that can easily be obtained from the diet (**Levine *et al.***, 1996). However, most studies only report increase in plasma levels. It is likely that if tissue saturation is achieved, then the additional beneficial effects are small and difficult to detect, leading to non-significant or null effects. Consistent with this notion, most of the studies that have shown a protective effect were carried out with smokers or patients with pathological conditions associated with oxidative stress and low plasma vitamin C levels (**Duarte and Lunec**, 2005). Although these studies have focused on oxidative modifications to DNA but other biomolecules like proteins and lipids are also susceptible to oxidation by ROS and naturally vitamin C protection to these biomolecules would also appear beneficial.

In this respect, vitamin C supplementation was able to reduce *in vivo* levels of protein carbonyls, a biomarker of protein oxidation even though this effect only occurred in subjects with low baseline ascorbate levels (**Carty et al.**, 2000). It is thus possible that the initial level of vitamin C in the cell predetermines whether supplementation trials may have a positive or null response. In this case, the lower the vitamin C level the more positive the response would be (**Duarte and Lunec**, 2005).

2.24 VITAMIN E

The term Vitamin E was introduced to describe a factor in the diet that is important for reproduction in animals and was given the name tocopherol, meaning child birth in Greek (**Evans and Bishop**, 1922). Vitamin E deficiency produces degeneration of the seminiferous epithelium in male and fetal resorption in female rats (**Evans and Bishop**, 1922; **Horwit**, 1980). Different forms of tocopherols and tocotrienols have been identified and of these, alpha-tocopherol is the most biologically active member of the vitamin E family (**Ingold et al.**, 1987; **Sokol**, 1989; **Lieber**, 1993). It is found in polyunsaturated vegetable oils, major mammalian cell types, and cell membranes (**Bauernfeind et al.**, 1970; **Gruger and Tappel**, 1971; **Chow**, 1985). It passively reaches the blood stream and liver after emulsifying together with the fat soluble components of the food and shows tissue-specific distribution (**Arita et al.**, 1995; **Hosomi et al.**, 1997). A selective transfer of α -tocopherol into lipoproteins was mediated by the specific α -tocopherol transfer protein (α -TTP) in the hepatocyte (**Boscoboinik et al.**, 1991). In addition, a protein capable of specifically binding tocopherol, a tocopherol-associated protein, was found in a large number of tissues, such as liver, prostate, and neural tissues (**Stocker et al.**, 1999).

The mechanism of the physiological action of vitamin E was not very clear but some of the biological activities are attributed to its antioxidant activity (**Burton and Ingold, 1981; Burton et al., 1983; Packer et al., 2001**). It plays a major role in the prevention of lipid peroxidation in biological membranes and is an important intramembrane antioxidant, membrane stabilizer, and lipid-soluble antioxidant (**Burton et al., 1983; Burton and Ingold, 1986; Herrera and Barbas, 2001; Traber and Atkinson, 2007**). Numerous *in vitro* experiments have demonstrated antioxidant synergism between alpha-tocopherol and ascorbate, reduced glutathione, NADPH, and cellular electron transport proteins (**Lambelet et al., 1994; Stahl and Sies, 1997**). Studies of vitamin E regeneration in a protein-denaturing system revealed that ascorbate regenerates vitamin E by a nonenzymatic mechanism (**Chan, 1993**). This is in line with findings showing that α -tocopherol, but not water-soluble antioxidants, efficiently protects glutathione peroxidase 4 (GPx4)-deficient cells from cell death (**Seiler et al., 2008**). These studies suggest that significant interaction occurs between water- and lipid-soluble vitamins at the membrane cytosol interface and that ascorbic acid (AA) may function *in vivo* to repair membrane-bound oxidized vitamin E (**Sokol, 1989; Beyer, 1994**).

A water-soluble form of Vitamin E, tocopheryl succinate polyethylene glycol 1000 (TPGS), is used as a vitamin supplement in children with cholestatic liver disease who are incapable of absorbing alpha-tocopherol or alpha-tocopheryl acetate (**Sokol et al., 1983; Traber et al., 1986; Sokol et al., 1987a**). TPGS does not depend on fat absorption for uptake into intestinal cells because TPGS forms micellar solutions at low concentrations, thereby eliminating the need for bile acids for Vitamin E absorption (**Gallo-Torres, 1970**). Supplementation with TPGS improves neurologic function in Vitamin E-deficient children with cholestatic liver disease (**Sokol et al., 1987b**).

Rats given vitamin E-deficient diet were found to develop pathological abnormalities. **Machlin *et al.*** (1977) showed that weaning rats fed with Vitamin E deficient diet chronically exhibited growth retardation and necrotizing myopathy when they were older. Vitamin E has also been shown to have some effects on bone growth. Vitamin E was able to stimulate the growth of trabecular bone (**Xu *et al.***, 1995b). Alpha-tocopherol and Vitamin E derived from palm oil was able to reverse the negative effects of estrogen deficiency on bone mineral density (**Norazlina *et al.***, 2000). **Norazlina *et al.*** (2001) in another study reported that optimum Vitamin E levels are needed to maintain bone calcification and bone mineral density.

In terms of bodily growth, vitamin E has been shown to improve growth retardation in glucocorticoid treated rats (**Ohtsuka *et al.***, 1998). Another study did not find any significant difference in body weight of growing male rats supplemented with two different doses of palm vitamin E-rich extract (**Yee and Ima-Nirwana**, 1998). **Ima-Nirwana *et al.*** (2000) showed that intact and ovariectomized rats given long-term Vitamin E deficient diet failed to increase their body weight as compared to rats given adequate dietary Vitamin E. However, in a study, to determine the effect of Vitamin E-deficiency and supplementation on body weight and body composition in intact and ovariectomized growing female rats the authors reported that Vitamin E played an important role in the weight gain of female rats and the gain was primarily due to the increase in fat mass, irrespective of the effect of ovariectomy (**Azman *et al.***, 2001).

In a study to determine the effect of selenium-vitamin E injections of ewes on reproduction and growth of their lambs, **Koyuncu and Yerlikaya** (2007) reported that The injections of Selenium plus Vitamin E significantly increased the incidence of oestrus, fertility and prolificacy in ewes, lamb body weight at day 60 and daily weight gain of ewes for 60 days postpartum compared with the control group.

A study on the protective effect of Vitamin E on ischaemia-reperfusion injury in ovarian grafts was determined (**Nugent *et al.*, 1998**). Also, the effects of administering an antioxidant, Vitamin E, on total lipid peroxides and malondialdehyde concentrations were also tested by these researchers. Results showed that products of lipid peroxidation were higher in non-supplemented murine autografts compared with control ovaries ($p < 0.05$), and were significantly reduced on day 3 by Vitamin E administration ($p < 0.05$). Similarly, in human xenografts, there was a significant reduction in lipid peroxidation with Vitamin E administration. These results correspond to a significantly greater total follicle survival in the murine grafts of the supplemented group (45 versus 72%; $p < 0.05$). The authors concluded that antioxidant treatment improved the survival of follicles in ovarian grafts by reducing ischaemia-reperfusion injury (**Nugent *et al.*, 1998**).

In another study the *in vivo* effects of nickel chloride and/or potassium dichromate in the ovary of adult mice was determined (**Rao *et al.*, 2009**). The protective role of Vitamin E (2 mg/kg body weight) along with their combination was also studied. Nickel and/or chromium to mice enhanced the levels of lipid peroxides in the ovary, which was accompanied by a significant decline in the levels of protein, glutathione, total ascorbic acid and activities of superoxide dismutase and catalase. Supplementation of Vitamin E along with nickel chloride and potassium dichromate significantly lowered the levels of lipid peroxidation and enhanced the antioxidant status. In conclusion, these authors suggested that Vitamin E exerts its protective effect against nickel and/or chromium induced toxicity by preventing lipid peroxidation and protecting antioxidant system in the mouse ovary (**Rao *et al.*, 2009**).

2.25 ANTIMALARIAL DRUGS AND THE REPRODUCTIVE SYSTEM

It has been shown that the gonads are affected by a series of factors like exposure to physical agents, irradiation, hypoxia and certain types of drugs (**Heywood and Wardsworth**, 1980). Information on the safety of drugs during pregnancy and lactation is derived from experimental and preclinical animal studies. Human experience accumulates in most cases from inadvertent drug exposure during pregnancy and lactation.

To explore the effect of *in utero* CQ exposure on fetal male sexual development, pregnant Sprague-Dawley rats were given a daily dose of either water or CQ diphosphate from gestational day (GD) 16–18 by oral gavage. CQ was administered as 200 mg/kg CQ base on GD 16, followed by two maintenance doses of 100 mg/kg CQ base on GD 16 and 18. Three days of CQ treatment resulted in a significant reduction in fetal testosterone levels in the testes. Examination of the fetal testes revealed significant alterations in vascularization and seminiferous tubule development after short-term CQ treatment; however Anogenital distance was not altered. Microarray and reverse transcription polymerase chain reaction (RT-PCR) showed down-regulation of several genes associated with cholesterol transport and steroid synthesis in the fetal testes. This indicates that CQ inhibits testosterone synthesis and normal testis development in the rat fetus at human relevant doses (**Clewell *et al.***, 2009).

In another study to investigate the effect of CQ on the fertility of adult male rats, CQ was administered at a daily intra-peritoneal dose of 10 mg/kg body weight and 40 mg/kg body weight per rat, five days a week for 16 weeks. Females mated with treated males showed a dose dependent decrease in the number of litter per female rat. In the *in-vitro* studies, more than 80% of spermatozoa population was immotile in all

concentrations of chloroquine tested. These results suggest that CQ brings about its antifertility effect by decreasing sperm motility (**Okanlawon *et al.*, 1990**).

Ebong *et al.* (1999) reported on the effect of chronic administration of CQ on leydig cells and plasma testosterone level. Twenty-five albino Wistar rats divided into five groups (A, B, C, D and E) were used for the study. Group A animals received a normal dose of 0.57 mg/kg body weight of chloroquine for 3 days. Groups B, C and D received chronic doses of 0.57 mg/kg body weight of CQ for 4, 5 and 6 days respectively. Group E animals, which served as control, were administered normal saline. Histological examination of the processed sections of groups B, C and D indicated numerical reduction of the Leydig cells when compared with the control group. Group A appeared normal. The basement membrane of the seminiferous epithelium in groups B, C and D were disrupted, leading to the detachment of many spermatocytes. Groups B, C and D recorded reduced level of plasma testosterone when compared with the control group. However, the concentration of plasma testosterone in group A (2.15 ± 1.63g/ml) and control (2.40 ± 1.48g/ml) were similar. Chronic administration of CQ reduced the number of leydig cells with concomitant reduction of testosterone production. It also disrupted seminiferous epithelium, leading to the detachment of spermatocytes.

Okanlawon and Ashiru (1992) conducted an experiment to determine the effects of CQ on the oestrous cycle and ovulation in 4-day cyclic rats. There were two treatment groups. Group 1 animals had CQ phosphate (40 mg/kg body weight) administered intraperitoneally (i.p.) starting from diestrus day 1 once a day, 5 days a week for 4 weeks. Oestrous smear was monitored by daily saline vaginal lavage. Control rats received an equal volume of physiological saline. All animals were sacrificed at the end of the 4th week of treatment. Group 11 animals also received CQ phosphate (40

mg/kg body weight) administered in a single dose at either 0900 h or 1800 h proestrus, and on the morning of estrus the rats were killed. Trunk blood was collected in each group at the time of sacrifice, centrifuged and serum stored for subsequent radioimmunoassay of LH, FSH and estrogen. The fallopian tube was dissected out and a search made for the ova. Results showed that administration of chloroquine altered the oestrus cycle (i.e. the rats showed a persistent diestrus smear), lowered serum estrogen and LH levels while serum FSH was unaltered, prevented the expected ovulation when injected at 0900 h proestrus and did not affect ovulation in rats injected at 1800 h proestrus. This study supports that CQ has an adverse effect on hypothalamo-pituitary ovarian systems.

Pyrimethamine (PYR) is an inhibitor of dihydrofolate reductase and is used in the treatment of malaria and toxoplasmosis. **Kalla *et al.*, (1997)** carried out studies to determine the antifertility and reversibility effect of PYR in adult male mice. The parameters mainly included sperm count and motility, fertility, histoarchitecture of testis and testicular cell kinetics quantitatively following oral administration of PYR (50 mg/kg body weight) daily for 30 days. The same parameters were also studied in PYR-treated animals which were allowed to recover for 45 days (recovery group). These authors reported that sperm motility as well as counts was significantly decreased in PYR-treated animals, and the fertility rate fell to zero. Testicular histology as well as germ cell kinetics was altered. However, in the animals of the recovery group, the values of all the parameters studied were almost the same as control values demonstrating the antifertility as well as reversibility effect of PYR. PYR's antifertility effects in the male mouse suggest that this agent has potential as a male contraceptive.

This dihydrofolate reductase inhibitor was administered to 72 adult male Swiss-Webster mice over a 50-day period at dosages ranging from 10-200 mg/kg/day (**Cosentino et al.**, 1990). During the last 10 days of drug administration, the study mice were exposed to 3 female mice of proven fertility. The female mice were examined for gravidity 19 days after the onset of the breeding cycle. Male infertility was dose-dependent, with no pregnancies occurring among the partners of mice who received the maximum dosage of PYR. Also inversely proportional to dosage were the number and motility of epididymal sperm in the treated mice and mean seminiferous tubule diameter and testicular and epididymal weights. Time course analysis revealed that the drug begins to exert its antifertility effect 33 days after administration and nearly complete infertility is achieved with 50 days, suggesting that PYR acts on early-midspermatogenesis. All mice returned to normal fertility status 44 days after treatment ended, and epididymal sperm reserves, sperm motility, and testicular and epididymal weights also returned to baseline values within this time period. Of particular interest was the finding that when PYR was administered to another group of mice for 80 days, infertility was significantly reduced beyond that achieved in 50 days, yet there were no further effects on testicular epididymal function. It would appear that PYR's mechanism of action is its antifolate action, with the main effect occurring on the testes rather than the epididymis (**Cosentino et al.**, 1990)

In another study designed by **Aydemir** and **Bilaloğlu** (1996), the authors examined the cytogenetic effects of PYR. Adult male mice were given doses of 20, 40, 80, and 120 mg/kg PYR intraperitoneally. Animals were killed by cervical dislocation on the 3rd, 6th, 9th, and 12th day after treatment, and the primary spermatocytes were harvested from their testes. These cells were analyzed for gaps, breaks, acentric fragments, and exchanges, as well as for numerical aberrations such as univalency. A dose-related

increase in chromosomal aberrations was found in the PYR group compared with the control group. The authors suspected that PYR is a possible clastogen that may affect human germ cells.

Quinine (QU) an alkaloid derived from the cinchona bark has been in use for the treatment of malaria for the past 350 years. A single injection of quinine in a maximum tolerable dose to BALB/c mice suppressed spermatogenesis and caused morphological changes in the testes. Gonocytes in all layers of the spermatogenic epithelium, interstitial endocrinocytes, and sustentocytes proved to be sensitive to the toxic effect of the drug. The majority of detected morphological changes were reversible (**Borovskaya *et al.*, 2000**).

In a study to determine the long-term morphological response of the testis to long-term administration of QU using stereological parameters (**Osinubi *et al.*, 2004**) 64 adult male Sprague-Dawley rats weighing 180-200g were used. The animals were randomly divided into 8 groups of 8 rats each. Every experimental animal had intramuscular QU at a dose of 10 mg/kg body weight per day (5 times in a week, with the exception of group 1 animals). Group 1 rats had QU for 1 week (7 days consecutively) and were sacrificed on the last day of injection. Groups 2 and 3 rats had QU for 4 and 6 weeks and were sacrificed at the end of the 4th and 6th week respectively. Group 4, 5, 6 and 7 rats had QU for 8 weeks and were sacrificed at the end of week 8, 12, 16 and 20 respectively. Group 8 animals constituted the controls and had equal volume of distilled water intramuscularly for 8 weeks. All sacrifices were by decapitation. The testes were carefully dissected out, their volumes measured, weighed and histological sections prepared. Morphometric assessment was carried out using the diameter, cross-sectional area, number of profiles per unit area, numerical density and volume density of the seminiferous tubules and the relative and absolute volume of the seminiferous

epithelium, stroma and lumen of tubules. The results showed that there was a general destruction of cells of the seminiferous tubules and the testicular interstitium that persisted even after the discontinuation of QU suggesting that QU has deleterious effect on the seminiferous tubules of Sprague-Dawley rats (**Osinubi *et al.*, 2004**).

Some anti-malarial drugs are much more abused and some have been found to be teratogenic. For example, PYR has been implicated in several forms of skeletal anomalies when administered to pregnant rats in doses higher than the therapeutic dose (**Akpaffiong *et al.*, 1986**). Studies on reproductive and developmental toxicity carried out on male rats using Halofantrine hydrochloride showed that there was no drug related effects on male fertility or reproductive performance in low or mid dose groups (**Reno, 1982**). The epididymus and seminal vesicles were organs of toxicity in the high dose group.

Drugs constitute a substantial environmental factor, which could affect ovarian hormones. Hormones play very important roles in the body of animals, which includes the maintenance of hormonal body homeostasis as well as in the reproductive life of animals. Female sex hormones have particular roles in the development of follicles and ovulation. Estradiol is needed for the maintenance of the normal menstrual cycle.

A study carried out by **Adjene and Agoreyo (2003)** shows that halofantrine hydrochloride caused reduction in follicle size and cytoplasmic vacuolation in the ovary of the rats. Studies also carried out with some laboratory animals revealed the extent to which various substances such as hormonal defect affect the reproductive system. The results showed that following 5 and 10 days treatment with 0.5ml/kg and 1ml/kg body weight of halofantrine hydrochloride, there was significant increase ($p < 0.05$) in estradiol levels in both groups. The results therefore appear to suggest that

halofantrine hydrochloride may be inducing the steroidogenic enzymes thereby causing high levels of estradiol.

Espay (1980) also found that pre-ovulatory gonadotropic surge may induce mammalian ovulation of initiating inflammatory processes in the wall of mature ovarian follicle. This would influence both endocrine balance and reproductive activities, thereby affecting both anatomical and physiological functions of the ovary (**Adjene and Agoreyo, 2003**). Evidence from female users of this drug has shown that the drug administered at a normal human therapeutic dose induces menstrual flow, which could probably be attributed to increase in estradiol levels. However more studies are recommended to confirm this proposition (**Ugochukwu *et al.*, 2008**)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental animals

A total of 220 female Sprague-Dawley rats weighing 120 – 180 g were used for this study. Sixty rats were used for the pilot study and 160 for the main study. The animals were allowed unrestricted access to water and rat chow (Agric Farms, Lagos Nigeria). The animals were maintained under standard natural photoperiodic condition of twelve hours of light alternating with twelve hours of darkness (*i.e.* L: D; 12: 12). The weights of the animals were determined at procurement and once every week throughout the duration of the experiment using a weighing balance.

3.2 EXPERIMENTAL PROTOCOLS

3.3 Pilot study

3.3.1 A study of the effect of amodiaquine on the histology of the ovary

Thirty rats were randomly divided into six groups:

Group 1– Control – oral (p.o.) route

Group 2– Control – intraperitoneal (i.p.) route

Group 3– 6 mg/kg bw AQ (low dose) – p.o. route

Group 4– 6 mg/kg bw AQ (low dose) – i.p. route

Group 5– 12 mg/kg bw AQ (high dose) – p.o. route

Group 6– 12 mg/kg bw AQ (high dose) – i.p. route

3.3.2 A study of the effect of amodiaquine on ovulation

Thirty rats were randomly divided into six groups

Group 1– Control – p.o. route

Group 2– Control – i.p. route

Group 3– 6 mg/kg bw AQ (low dose) – p.o. route

Group 4– 6 mg/kg bw AQ (low dose) – i.p. route

Group 5– 12 mg/kg bw AQ (high dose) – o.p. route

Group 6– 12 mg/kg bw AQ (high dose) – i.p.route

3.4 Main study

There were four experimental groups. The same group of rats used for experiment 1 was used for experiment 3; similarly, the same sets of rats used for experiment 2 were used for experiment 4. Each group was further divided into subgroups.

3.4.1 Experiment 1: Determination of the effects of chronic administration of amodiaquine for 28 days on the oestrous cycle and on the histomorphology of the ovary

Seventy mature female Sprague-Dawley rats weighing 120-180 g were used for this study. They were randomly divided into 14 groups of 5 rats each:

Control groups: Group 1A received distilled water orally; Group 1B received distilled water intraperitoneally.

AQ alone groups: Group 1C received low oral dose of AQ; Group 1D received high oral dose of AQ; Group 1E received low intraperitoneal dose of AQ; Group 1F received high intraperitoneal dose of AQ.

AQ + Vitamin C groups: Group 1G received low oral dose of AQ; Group 1H received high oral dose of AQ; Group 1I received low intraperitoneal dose of AQ; Group 1J received high intraperitoneal dose of AQ. Each group received vitamin C intramuscularly once every other day for 28 days.

AQ + Vitamin E groups: Group 1K received low oral dose of AQ; Group 1L received high oral dose of AQ; Group 1M received low intraperitoneal dose of AQ; Group 1N

received high intraperitoneal dose of AQ. They all received vitamin E intramuscularly once a day, 5 days in a week (Monday to Friday) for 4 weeks.

AQ was administered for 28 days during which the oestrous cycle was monitored every morning between 8 a.m. to 10 a.m. AQ was administered alone or co-administered with vitamin C or vitamin E according to the treatment group. At the end of the experiment the animals were sacrificed by cervical dislocation and the ovaries were processed for light microscopic study.

3.4.2 Experiment 2: Determination of the effect of amodiaquine on ovulation

A total of 90 mature female Sprague-Dawley rats weighing between 120-180 g were randomly divided into 18 groups of 5 rats each.

Negative control groups: Group 2A received distilled water orally; Group 2B received distilled water intraperitoneal.

Positive control groups: Group 2C received distilled water orally + Vitamin C; Group 2D received distilled water intraperitoneally + Vitamin C; Group 2E received distilled water orally + Vitamin E; Group 2F received distilled water intraperitoneally + Vitamin E.

AQ alone groups: Group 2G received low oral dose of AQ; Group 2H received high oral dose of AQ; Group 2I received low intraperitoneal dose of AQ; Group 2J received high intraperitoneal dose of AQ.

AQ + Vitamin C groups: Group 2K received low oral dose of AQ; Group 2L received high oral dose of AQ; Group 2M received low intraperitoneal dose of AQ; Group 2N received high intraperitoneal dose of AQ. Each group also received a single intramuscular dose of vitamin C.

AQ + Vitamin E groups: Group 2O received low oral dose of AQ; Group 2P received high oral dose of AQ; Group 2Q received low intraperitoneal low dose of AQ; Group

2R received high intraperitoneal dose of AQ. All the groups received a single dose of vitamin E intramuscularly.

Animals in this experiment were given a single dose of AQ in the morning on proestrus between 8 a.m. and 10 a.m. A single dose of Vitamin E or Vitamin C was given concurrently with AQ according to the treatment groups. The animals were sacrificed the next day in the morning on estrus by cervical dislocation. The oviducts were dissected placed on slides and viewed under the light microscope for ova count.

3.4.3 Experiment 3: Determination of the effect of AQ on enzymatic antioxidant status in the ovary and co-administration of Vitamin C and Vitamin E

For this experiment a total of 70 mature female Sprague-Dawley rats weighing between 120-180 g were randomly divided into 14 groups of 5 rats each.

Control groups: Group 3A received distilled water orally; Group 3B received distilled water intraperitoneally.

AQ alone groups: Group 3C received low oral dose of AQ; Group 3D received high oral dose of AQ; Group 3E received low intraperitoneal dose of AQ; Group 3F received high intraperitoneal dose of AQ.

AQ + Vitamin C groups: Group 3G received low oral dose of AQ; Group 3H received high oral dose of AQ; Group 3I received low intraperitoneal dose of AQ; Group 3J received high intraperitoneal dose of AQ. Each group received vitamin C intramuscularly once every other day for 28 days.

AQ + Vitamin E groups: Group 3K received low oral dose of AQ; Group 3L received high oral dose of AQ; Group 3M received low intraperitoneal dose of AQ; Group 3N received high intraperitoneal dose of AQ. They all received vitamin E intramuscularly once a day, 5 days in a week (Monday to Friday) for 4 weeks.

Animals in this experiment received AQ for 28 days in addition with Vitamin C and Vitamin E were given intramuscularly according to the treatment group. At the end of the treatment regimen, the animals were sacrificed by cervical dislocation and the ovaries were dissected out and assayed for superoxide dismutase and catalase activities.

3.4.4 Experiment 4: Determination of the effect of AQ on reproductive hormone

In this experiment a total of 90 mature female Sprague-Dawley rats weighing between 120-180 g were randomly divided into 18 groups of 5 rats each.

Negative control groups: Group 4A received distilled water orally; Group 4B received distilled water intraperitoneal.

Positive control groups: Group 4C received distilled water orally + Vitamin C; Group 4D received distilled water intraperitoneally + Vitamin C; Group 4E received distilled water orally + Vitamin E; Group 4F received distilled water intraperitoneally + Vitamin E.

AQ alone groups: Group 4G received low oral dose of AQ; Group 4H received high oral dose of AQ; Group 4I received low intraperitoneal dose of AQ; Group 4J received high intraperitoneal dose of AQ.

AQ + Vitamin C groups: Group 4K received low oral dose of AQ; Group 4L received high oral dose of AQ; Group 4M received low intraperitoneal dose of AQ; Group 4N received high intraperitoneal dose of AQ. Each group also received a single intramuscular dose of vitamin C.

AQ + Vitamin E groups: Group 4O received low oral dose of AQ; Group 4P received high oral dose of AQ; Group 4Q received low intraperitoneal low dose of AQ; Group 4R received high intraperitoneal dose of AQ. All the groups received a single dose of vitamin E intramuscularly.

Animals in this experiment were given AQ in the morning on proestrus between 8 a.m. and 10 a.m. A single intramuscular dose of Vitamin E or Vitamin C was given concurrently with AQ according to the treatment groups. Blood was collected into heparinized bottles from the angular vein of the eye at 6 p.m. on proestrus. The blood sample was spun at 2500 revolutions per minute for 10 minutes in an angle head desktop centrifuge at 25°C. Serum samples were stored at -80°C in a refrigerator for immunoassay.

3.5 AMODIAQUINE ADMINISTRATION

Pure substance of AQ with a potency of 99.1% a product of Rhone Poulenc Pharmaceutical Company, France: Expiry date; December, 2014 was used as the drug source. AQ was revalidated using Ultraviolet Spectrophotometer and high-performance liquid chromatography (HPLC) at the Faculty of Pharmacy, University of Lagos, Nigeria. AQ was constituted into a solution by the addition of distilled water. AQ was administered based on the body weight of the rats by extrapolating from the recommended therapeutic human dose of 400 mg/70kg (**Kersley and Palin, 1959**). Two different doses were given: 6 mg/kg bw (low dose) and 12 mg/kg bw (high dose) and by two different routes: oral and intraperitoneal routes.

3.6 VITAMIN C ADMINISTRATION

Vitamin C manufactured by Linling Pharmaceuticals China, was administered intramuscularly at a dose of 0.1 mg/kg body weight per day 3 days in a week - Mondays, Wednesdays and Fridays (**Mishra and Acharya, 2004**) concurrently with AQ for 28 days. The animals were sacrificed at the end of 28 days by cervical dislocation.

3.7 VITAMIN E ADMINISTRATION

Vitamin E manufactured by Pharco Pharmaceuticals, Egypt was administered intramuscularly at the dose of 20 mg/kg bodyweight per day 5 days in a week- Mondays to Fridays (**Mishra and Acharya, 2004**) concurrently with AQ for a period of 28 days. The animals were sacrificed at the end of 28 days by cervical dislocation.

3.8 DETERMINATION OF OESTROUS CYCLE

Oestrous cyclicity was determined between 8 a.m. and 10 a.m. using the vaginal smear method. Vaginal secretion was collected with a plastic pipette filled with 10 µL of normal saline (NaCl 0.9%). The vagina was flushed two or three times with the pipette and the vaginal fluid was placed on a glass slide. A different slide was used for each animal. The unstained secretion was observed under a light microscope.

A proestrus smear consists of a predominance of small nucleated epithelial cells; an estrus smear primarily consists of anucleated cornified cells; a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells; and a diestrus smear primarily consists of a predominance of leukocytes (**Long and Evans, 1922; Mandl, 1951; Nobunaga and Nakamura, 1968**).

After confirming regular 4-day cyclicity for 2 weeks, the animals were selected for this study. The effect of AQ on the oestrous cycle was monitored for 28days (**Gasco et al., 2008**) after which the animals were sacrificed by cervical dislocation. AQ was administered at two doses- 6 mg/kg body weight and 12 mg/kg bw and via two routes- oral and intraperitoneal. Control groups received equivalent volume of distilled water.

3.9 DETERMINATION OF OVULATION

Ovulation was examined as follows: briefly in the morning on proestrus, the animals received AQ between 08:00 and 10:00 hr at a dose of 6 mg/kg bw and 12 mg/kg bw via oral and intraperitoneal routes. The rats were sacrificed by cervical dislocation at 10:00 hr in the morning of estrus. The lower abdominal wall was dissected and both ovaries were removed and trimmed of fat. The oviducts were removed and placed on glass slides with a drop of saline. The oviducts were covered with cover slips and squeezed with both sides being gently rocked (**Kim et al.**, 1994). Each ovum found in the distended ampulla was counted under a light microscope. The animals in this experiment were divided into 18 sub groups. Ovulation was examined at 10:00hr for all the groups with various treatments including the control.

3.10 TISSUE PROCESSING FOR LIGHT MICROSCOPY

The animals were sacrificed on the 28th day by cervical dislocation. The lower abdominal wall was dissected and the ovaries were removed, trimmed of fat and fixed in 10% formol saline. The fixed tissues were dehydrated in increasing concentrations of ethanol and thereafter embedded in paraffin wax. Serial sections of 5 µm thick were made with a rotatory microtome. These were stained with haematoxylin and eosin after being mounted on plain glass slides in the routine H&E preparation.

3.11 DETERMINATION OF SUPEROXIDE DISMUTASE

Superoxide dismutase was assayed utilizing the technique of **Kakkar et al.**, (1984). A single unit of enzyme was expressed as 50% inhibition of Nitroblue tetrazolium (NBT) reduction/min/mg/protein.

3.12 DETERMINATION OF CATALASE

Catalase was assayed colorimetrically at 620 nm and expressed as $\mu\text{moles of H}_2\text{O}_2$ Consumed/min/mg/protein as described by **Sinha** (1972). The reaction mixture 1.5 ml contained 1.0 ml of 0.01 M pH 7.0 phosphate buffer, 0.1 ml of ovary homogenate and 0.4 ml of 2 M H_2O_2 . The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent 5% potassium dichromate and glacial acetic acid was mixed in 1:3 ratios.

3.13 DETERMINATION OF REPRODUCTIVE HORMONES

Blood was obtained from the angular vein of the eye and collected into a heparinised bottle. Each blood sample was spun at 2500 revolution per minute for 10 minutes in an angle-head desktop centrifuge at 25°C. Serum samples were assayed in batches with control sera at both physiological and pathological levels by Standard Quantitative Enzyme Linked Immunosorbent Assay (ELISA) technique with Microwel kit from Syntrobioresearch Inc. California, U.S.A.

3.13.1 Determination of FSH

Assay procedure: The desired numbers of antibody-coated wells were secured in the holder. Exactly 50 μl of standard, specimens and controls were dispensed into appropriate wells. Enzyme conjugate reagent measuring 100 μl was dispensed into each well. The contents of the well were thoroughly mixed for 30 seconds and incubated at room temperature (23 - 25°C) for 60 minutes. The incubation mixture was removed by flicking the content of the plate into a waste container. The microtiter wells were flicked and rinsed 5 times with washing buffer. Thereafter the wells were struck sharply onto absorbent paper to remove all residual water droplets. Later, 100 μl of TMB solution was dispensed into each well, mixed gently for 5 seconds and then

incubated at room temperature in the dark for 20 minutes. The reaction was stopped by the addition of 100 µl of 1 N HCL acid to each well. The solution was mixed gently for another 30 seconds and care taken to ensure that all the blue color changed completely to a yellow colour. Optical density was read at 450 nm with a microtiter reader within 30 minutes.

3.13.2 Determination of LH

Assay procedure: The desired numbers of antibody-coated wells were secured in the holder. Exactly 50 µl of standard, specimens and controls were dispensed into appropriate wells. Thereafter, 100 µl of enzyme conjugate reagent was dispensed into each well. The contents of the well were thoroughly mixed for 30 seconds and incubated at room temperature (22 - 25°C) for 60 minutes. The incubation mixture was removed by flicking the content of the plate into a waste container. The microtiter wells were flicked and rinsed 5 times with washing buffer (IX). Thereafter the wells were struck sharply onto absorbent paper to remove all residual water droplets. Later, 100 µl of TMB solution was dispensed into each well, mixed gently for 5 seconds and then incubated at room temperature for 20 minutes. The reaction was stopped by the addition of 100 µl of 1 N HCL acid to each well. The solution was mixed gently for another 30 seconds and care taken to ensure that all the blue color changed completely to a yellow colour. Optical density was read at 450 nm with a microtiter reader within 30 minutes.

3.13.3 Determination of PRL

Assay procedure: The desired numbers of antibody-coated wells were secured in the holder. Exactly 50 µl of standard, specimens and controls were dispensed into appropriate wells. Thereafter 100 µl of enzyme conjugate reagent was dispensed into

each well. The contents of the well were gently mixed for 10 seconds and incubated at room temperature for 45 minutes. The incubation mixture was removed by flicking the content of the plate into a waste container. The microtiter wells were flicked and rinsed 5 times with deionized water. Thereafter the wells were struck sharply onto absorbent paper to remove all residual water droplets. Later, 100 μ l of TMB solution was dispensed into each well, mixed gently for 10 seconds and then incubated at room temperature in the dark for 20 minutes. The reaction was stopped by the addition of 100 μ l of 1 N HCL acid to each well. The solution was mixed gently for another 30 seconds and care taken to ensure that all the blue color changed completely to a yellow colour. Optical density was read at 450 nm with a microtiter reader within 15 minutes.

3.14 STATISTICAL ANALYSIS

Results were expressed as mean \pm standard deviation (SD) and subjected to statistical analysis using analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Statistical significance was considered at $p < 0.05$ or 5%.

CHAPTER FOUR

RESULTS

4.1 EFFECT OF AMODIAQUINE ON BODY WEIGHT OF SPRAGUE-DAWLEY RATS

The results displayed in tables 1 and 2 show that there was an increase in the mean bodyweight of rats in all the control and treatment groups. This increase in mean body weight was statistically significant ($p < 0.05$) in the groups that received 6 mg/kg bw AQ alone and AQ + Vitamin C and also in the groups that received 12 mg/kg bw AQ + Vitamin C.

4.2 EFFECT OF AMODIAQUINE ON OESTROUS CYCLE

The result displayed in table 3 showed that the mean period of the estrous cycle was prolonged in all the groups that received AQ alone when compared with the control. This increase in mean cycle length was statistically significant ($p < 0.05$) in the group that received 12 mg/kg via the intraperitoneal route (5.49 ± 1.09). When compared with the control, co-administration with Vitamin C prolonged the mean cycle length even further. This increase in mean cycle length was statistically significant ($p < 0.05$) in the groups that received 6 mg/kg (p.o.), 12 mg/kg (p.o.) and 12 mg/kg (i.p.); 6.86 ± 2.01 , 7.58 ± 3.50 and 5.32 ± 0.82 respectively. However, Co-administration with Vitamin E reduced the mean cycle length but was not able to return it to the control values. There was no statistically significant difference observed when dose and route of administration were compared in all the treatment groups. Table 4 and 5 shows that there was a disruption in the pattern of the phases of the oestrus cycle in the AQ alone, AQ + Vitamin C and AQ + Vitamin E treated animals when compared with the control. This disruption in cycle pattern occurred as a result of the animals spending

increased number days ($p < 0.05$) in the diestrus phase as compared to the metestrus, proestrus and estrus phases of the cycle.

4.3 EFFECT OF AMODIAQUINE ON OVULATION

Table 6 shows the effect of AQ at 6 mg/kg bw and 12 mg/kg bw administered orally between 8.00 a.m. and 10.00 a.m. on proestrus on the number of ova shed in the oviduct on the morning of estrus. From table 6, it was observed that the number of ova shed in the oviduct in the morning of estrus was reduced in the groups treated with AQ alone, AQ + Vitamin C and distilled water + Vitamin C when compared to the control. This reduction in ova count was statistically significant ($p < 0.05$) at 6 mg/kg bw AQ alone (5.00 ± 0.71), 6 mg/kg bw AQ + Vitamin C (5.80 ± 1.30), 12 mg/kg bw AQ + Vitamin C (3.60 ± 2.51) and distilled water + Vitamin C (6.00 ± 2.24). Co-administration of AQ with vitamin E brought about an increase in the number of ova shed and was comparable to control values in all the treatment groups that received vitamin E: 6 mg/kg bw AQ + Vitamin E (8.50 ± 0.58); 12 mg/kg bw AQ + Vitamin E (7.00 ± 2.16); Distilled water + Vitamin E (6.00 ± 2.24).

Table 1: The effect of oral administration of AQ alone for 28 days, AQ +Vitamin C and AQ + Vitamin E on bodyweight of Sprague-Dawley rats.

Treatment (6 mg/kg bw)	Before (g) Mean ± SD	After (g) Mean ± SD	Treatment (12 mg/kg bw)	Before (g) Mean ± SD	After (g) Mean ± SD
Control	117±28.90	142 ±28.90	Control	117 ±28.90	142 ± 28.9
AQ Alone	132 ±6.02	149 ± 7.69*	AQ Alone	176 ± 2.30	178 ± 7.01
AQ + Vit C	125 ± 0.00	198 ± 2.89*	AQ + Vit C	125 ± 0.00	225 ± 0.00*
AQ + Vit E	145 ± 27.40	160 ± 22.40	AQ + Vit E	175 ±39.50	180 ± 27.40

*** = $p < 0.05$**

bw = body weight

Vit C = Vitamin C

Vit E = Vitamin E

Table 2: The effect of intraperitoneal administration of AQ alone for 28 days, AQ + Vitamin C and AQ + Vitamin E on bodyweight of Sprague-Dawley rats.

Treatment	Before (g)	After (g)	Treatment	Before (g)	After (g)
(6 mg/kg	Mean± SD	Mean ±SD	(12 mg/kg	Mean± SD	Mean± SD
bw)			bw)		
Control	108 ±14.40	133±14.40	Control	108± 14.40	133 ±14.40
AQ Alone	146 ± 5.44	158 ±9.02*	AQ Alone	163± 4.12	165 ± 11.50
AQ + Vit C	152 ± 2.89	225± 0.00*	AQ + Vit C	162 ± 12.60	217 ± 28.9*
AQ + Vit E	170 ± 37.1	175 ± 39.50	AQ + Vit E	170 ± 11.20	180 ± 27.40

*** = $p < 0.05$**

bw = body weight

Vit C = Vitamin C

Vit E = Vitamin E

Table 3: The effect of the administration of AQ alone for 28 days, AQ + Vit C and AQ + Vit E on the length of the oestrous cycle in Sprague-Dawley rats.

Treatment groups	6 mg/kg	12 mg/kg	6 mg/kg	12 mg/kg
	bw (p.o.)	bw (p.o.)	bw (i.p.)	bw (i.p.)
Mean \pm SD				
Control	4.00 \pm 0.00	4.00 \pm 0.00	4.00 \pm 0.00	4.00 \pm 0.00
AQ Alone	5.04 \pm 0.43	6.06 \pm 1.63	5.25 \pm 0.98	5.49 \pm 1.09*
AQ + Vit C	6.86 \pm 2.01*	7.58 \pm 3.50*	5.52 \pm 2.16	5.32 \pm 0.82*
AQ + Vit E	5.04 \pm 0.92	4.70 \pm 0.58	4.56 \pm 0.46	5.50 \pm 0.59*

* = $p < 0.05$

bw = body weight

p.o. –Per oral

i.p. = Intraperitoneal route

Table 4: The effect of the oral administration of AQ alone for 28 days, AQ + Vitamin C and AQ + Vitamin E on the mean number of days spent in the phases of the oestrus cycle in Sprague-Dawley rats.

Treatment groups	Metestrus	Diestrus	Proestrus	Estrus
	(Mean \pm SD)			
Control	7.00 \pm 0.00	7.00 \pm 0.00	7.00 \pm 0.00	7.00 \pm 0.00
AQ (6 mg/kg)	6.00 \pm 1.00	11.80 \pm 3.70*	5.80 \pm 1.64	6.60 \pm 1.34
AQ (12 mg/kg)	5.00 \pm 2.16*	14.80 \pm 5.85*	5.25 \pm 2.36*	5.00 \pm 1.83*
AQ (0.6 mg/kg) + Vit C	3.67 \pm 1.15*	16.70 \pm 5.86*	3.67 \pm 2.08*	4.67 \pm 2.08*
AQ (1.2 mg/kg) + Vit C	3.67 \pm 0.58*	18.30 \pm 2.31*	3.33 \pm 0.58*	3.67 \pm 1.15*
AQ (0.6 mg/kg) + Vit E	6.00 \pm 1.00	9.80 \pm 2.59*	5.40 \pm 0.89	6.40 \pm 1.14
AQ (1.2 mg/kg) + Vit E	6.00 \pm 1.22*	9.40 \pm 4.34*	5.80 \pm 1.10*	6.40 \pm 2.51*

*= $p < 0.05$

Vit C = Vitamin C

Vit E = Vitamin E

Table 5: The effect of the intraperitoneal administration of AQ alone for 28 days, AQ + Vitamin C and AQ + Vitamin E on the mean number of days spent in the phases of the oestrus cycle in Sprague-Dawley rats.

Treatment groups	Metestrus	Diestrus	Proestrus	Estrus
	(Mean ± SD)			
Control	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
AQ (6 mg/kg)	5.80 ± 1.30	12.80 ± 4.44*	5.40 ± 1.52*	5.60 ± 1.52
AQ (12 mg/kg)	5.60 ± 0.89	11.80 ± 2.59*	5.80 ± 1.30	6.80 ± 1.10
AQ (0.6 mg/kg) + Vit C	5.33 ± 1.53*	11.70 ± 4.73*	5.33 ± 2.08*	6.00 ± 2.65
AQ (1.2 mg/kg) + Vit C	4.67 ± 1.53*	12.70 ± 4.73*	5.33 ± 2.08*	5.33 ± 1.15*
AQ (0.6 mg/kg) + Vit E	5.00 ± 1.41*	12.20 ± 4.99*	5.25 ± 1.71*	5.25 ± 1.71*
AQ (1.2 mg/kg) + Vit E	4.80 ± 1.10*	11.60 ± 3.78*	5.00 ± 1.22*	6.60 ± 1.67

*= $p < 0.05$

Vit C = Vitamin C

Vit E = Vitamin E

Table 7 shows the effect of AQ administered intraperitoneally at 6 mg/kg bw and 12 mg/kg bw on the number of ova shed in the oviduct in the morning of estrus. The result showed a reduction in ova count in the groups treated with AQ alone (6 mg/kg bw, 6.80 ± 1.48 ; 12 mg/kg bw, 5.80 ± 3.42), AQ + Vitamin C (6 mg/kg bw + Vitamin C, 7.00 ± 1.41 ; 12 mg/kg bw + Vitamin C, 4.80 ± 2.77) and distilled water + Vitamin C (DW + Vitamin C, 6.00 ± 2.24) when compared to the control. However, this reduction in number of ova shed was not statistically significant.

Co-administration of AQ with vitamin E brought about an increase in the number of ova shed and was comparable to control values (6 mg/kg bw AQ + Vitamin E, 8.75 ± 0.50 ; 12 mg/kg bw AQ + Vitamin E, 8.25 ± 0.50 ; DW + Vitamin E, 8.25 ± 0.50). No statistically significant difference was observed when dose and route of administration were compared.

4.4 EFFECT OF AMODIAQUINE ON SERUM CONCENTRATION OF PRL

There was no statistically significant difference in the level of the concentration of prolactin in the evening of proestrus among the treatment groups when compared with the control. When dose and route of administration were compared, no statistically significant difference was observed (Table 8).

4.5 EFFECT OF AMODIAQUINE ON SERUM CONCENTRATION OF FSH

There was no statistically significant difference in the level of the concentration of follicle-stimulating hormone in the evening on proestrus among the treatment groups when compared with the control group. A comparison of the dose and route of administration did not show any significant difference (Table 9).

4.6 EFFECT OF AMODIAQUINE ON SERUM CONCENTRATION OF LH

There was no statistically significant difference in the level of the concentration of luteinizing hormone in the evening of proestrus among the treatment groups when compared with the control. When dose and route of administration were compared, no statistically significant difference was observed (Table 10).

Table 6: The effect of the oral administration of AQ alone, AQ + Vitamin C and AQ + Vitamin E on the number of ova shed (Mean \pm SD) in the oviduct in the morning of estrus in Sprague-Dawley rats

Treatment groups	Number of ova shed in oviduct (Mean \pm SD)	
	6 mg/kg bw	12 mg/kg bw
Control	8.40 \pm 0.89	8.40 \pm 0.89
AQ Alone	5.00 \pm 0.71*	4.60 \pm 3.51*
AQ + Vit C	5.80 \pm 1.30*	3.60 \pm 2.51*
AQ + Vit E	8.50 \pm 0.58	7.00 \pm 2.16
DW + Vit C	6.00 \pm 2.24*	6.00 \pm 2.24
DW + Vit E	8.25 \pm 0.50	8.25 \pm 0.50

* = $p < 0.05$

bw = body weight

DW = Distilled water

Vit C = Vitamin C

Vit E = Vitamin E

Table 7: The effect of the intraperitoneal administration of AQ alone, AQ + Vitamin C and AQ + Vitamin E on the number of ova shed (Mean \pm SD) in the oviduct in the morning of estrus in Sprague-Dawley rats.

Treatment groups	Number of ova shed in the oviduct	
	(Mean \pm SD)	
	6 mg/kg bw	12 mg/kg bw
Control	8.25 \pm 0.96	8.25 \pm 0.96
AQ Alone	6.80 \pm 1.48	5.80 \pm 3.42
AQ +Vit C	7.00 \pm 1.41	4.80 \pm 2.77
AQ +Vit E	8.75 \pm 0.50	8.25 \pm 0.50
DW + Vit C	6.00 \pm 2.24	6.00 \pm 2.24
DW + Vit E	8.25 \pm 0.50	8.25 \pm 0.50

bw = body weight

DW = Distilled water

Vit C = Vitamin C

Vit E = Vitamin E

Table 8: The effect of the oral and intraperitoneal administration of AQ alone, AQ + Vitamin C and AQ + Vitamin E on the concentration of Prolactin (PRL) at 6 p.m. on proestrus in Sprague-Dawley rats.

Treatment	Concentration of PRL in the blood at 6p.m. on proestrus			
Groups	(Mean \pm SD)			
	6 mg/kg (p.o.)	12 mg/kg (p.o.)	6 mg/kg (i.p.)	12 mg/kg (i.p.)
Control	0.295 \pm 0.02	0.295 \pm 0.02	0.292 \pm 0.02	0.292 \pm 0.02
AQ Alone	0.300 \pm 0.02	0.312 \pm 0.01	0.295 \pm 0.02	0.289 \pm 0.06
AQ + Vit C	0.297 \pm 0.02	0.290 \pm 0.02	0.325 \pm 0.03	0.289 \pm 0.02
AQ + Vit E	0.314 \pm 0.02	0.378 \pm 0.08	0.328 \pm 0.04	0.303 \pm 0.02
DW + Vit C	0.326 \pm 0.02	0.326 \pm 0.02	0.319 \pm 0.02	0.319 \pm 0.02
DW + Vit E	0.323 \pm 0.04	0.323 \pm 0.04	0.328 \pm 0.03	0.328 \pm 0.03

bw = body weight

DW = Distilled water

p.o. = Per Oral

i.p. = Intraperitoneal route

Table 9: The effect of the oral and intraperitoneal administration of AQ alone, AQ + Vit C and AQ + Vit E on the concentration of Follicle stimulating Hormone (FSH) at 6 p.m. on proestrous in Sprague-Dawley rats.

Treatment Groups	Concentration of FSH in the blood at 6p.m. on proestrus			
	(Mean \pm SD)			
	6 mg/kg (p.o.)	12 mg/kg (p.o.)	6 mg/kg (i.p.)	12 mg/kg (i.p.)
Control	0.339 \pm 0.02	0.339 \pm 0.02	0.348 \pm 0.03	0.348 \pm 0.03
AQ Alone	0.330 \pm 0.01	0.337 \pm 0.02	0.451 \pm 0.02	0.289 \pm 0.06
AQ + Vit C	0.351 \pm 0.02	0.347 \pm 0.03	0.338 \pm 0.01	0.289 \pm 0.02
AQ + Vit E	0.331 \pm 0.05	0.408 \pm 0.13	0.404 \pm 0.11	0.303 \pm 0.02
DW + Vit C	0.363 \pm 0.02	0.363 \pm 0.02	0.336 \pm 0.01	0.336 \pm 0.01
DW + Vit E	0.413 \pm 0.06	0.413 \pm 0.06	0.339 \pm 0.01	0.339 \pm 0.01

bw = body weight

DW = Distilled water

p.o. = Per Oral

i.p. = Intraperitoneal route

Vit C = Vitamin C

Vit E = Vitamin E

Table 10: The effect of the oral and intraperitoneal administration of AQ alone, AQ + Vit C and AQ + Vit E on the concentration of Luteinizing Hormone (LH) at 6 p.m. on proestrous in Sprague-Dawley rats.

Treatment	Concentration of LH in the blood at 6p.m. on proestrus			
Groups	(Mean \pm SD)			
	6 mg/kg (p.o.)	12 mg/kg (p.o.)	6 mg/kg (i.p.)	12 mg/kg (i.p.)
Control	0.142 \pm 0.04	0.142 \pm 0.04	0.196 \pm 0.14	0.196 \pm 0.14
AQ Alone	0.134 \pm 0.07	0.111 \pm 0.02	0.302 \pm 0.02	0.191 \pm 0.01
AQ + Vit C	0.177 \pm 0.10	0.256 \pm 0.11	0.184 \pm 0.10	0.149 \pm 0.09
AQ + Vit E	0.264 \pm 0.15	0.378 \pm 0.49	0.364 \pm 0.05	0.180 \pm 0.09
DW + Vit C	0.119 \pm 0.04	0.119 \pm 0.04	0.157 \pm 0.05	0.157 \pm 0.05
DW + Vit E	0.147 \pm 0.04	0.147 \pm 0.04	0.145 \pm 0.02	0.145 \pm 0.02

bw = body weight

DW = Distilled water

p.o. = Per Oral

i.p = Intraperitoneal route

Vit C = Vitamin C

Vit E = Vitamin E

4.7 EFFECT OF AMODIAQUINE ON SUPEROXIDE DISMUTASE ACTIVITY IN THE OVARY OF SPRAGUE-DAWLEY RATS

The result obtained from the enzymatic antioxidant status of superoxide dismutase in the ovary revealed that the administration of AQ alone at both doses (6 mg/kg bw and 12 mg/kg bw) and by both routes (oral and intraperitoneal) reduced the activities of superoxide dismutase in the ovary when compared to the control (Table 11). The reduction in the activity of superoxide dismutase in the ovary was further reduced by co-administration with Vitamin C however, was not statistically significant in all the treatment groups (6 mg/kg bw AQ (p.o.) + Vitamin C, 0.15 ± 0.00 ; 6 mg/kg bw AQ (i.p.) + Vitamin C, 0.15 ± 7.50 ; 12 mg/kg bw AQ (p.o.) + Vitamin C, 0.14 ± 2.30 ; 12 mg/kg bw AQ (i.p.) + Vitamin C, 0.17 ± 3.39). Co-administration of AQ with Vitamin E resulted in an increase in the activity of superoxide dismutase in the ovary when compared to the AQ alone and the AQ + Vitamin C groups. This increase was comparable to control values in the groups that received intraperitoneal doses of 6 mg/kg bw AQ + vitamin E (1.43 ± 0.63) and 12 mg/kg bw AQ + vitamin E (1.38 ± 0.88) demonstrating the ameliorating effect of vitamin E. When dose and route of administration were compared, no statistically significant difference was observed.

4.8 EFFECT OF AMODIAQUINE ON CATALASE ACTIVITY IN THE OVARY OF SPRAGUE-DAWLEY RATS

The administration of AQ alone resulted in a reduction in the activities of catalase in the ovary when compared to the control: 6 mg/kg bw (p.o.), 55.5 ± 26.50 ; 6 mg/kg bw (i.p.), 52.1 ± 27.20 ; 12 mg/kg bw (p.o.), 46.5 ± 26.70 and 12 mg/kg bw (i.p.), 54.7 ± 26.60 . The activity of catalase was further reduced by the co-administration of AQ with Vitamin C. This reduction was statistically significant ($p < 0.05$) in all the groups

that received Vitamin C: 6 mg/kg bw AQ (p.o.) + Vitamin C, 26.8 ± 3.24 ; 6 mg/kg bw AQ (i.p.) + Vitamin C, 29.2 ± 1.98 ; 12 mg/kg bw AQ (p.o.) + Vitamin C, 27.3 ± 2.90 ; 12 mg/kg bw AQ (i.p.) + Vitamin C, 24.4 ± 0.75 . However, co-administration of AQ with Vitamin E increased the activity of catalase in the ovary but was not able to restore to control values (Table 12). When dose and route of administration of AQ were compared, there was no statistically significant difference observed.

Table 11: The effect of the administration of AQ alone for 28 days, AQ + Vitamin C and AQ + Vitamin E on enzymatic anti-oxidant activities of superoxide dismutase (SOD) in the ovary of Sprague-Dawley rats.

Treatment groups	Anti-oxidant level (SOD/min/mg protein)			
	(Mean \pm SD)			
	6 mg/kg (p.o.)	6 mg/kg (i.p.)	12 mg/kg (p.o.)	12 mg/kg (i.p.)
Control	1.40 \pm 0.85	1.45 \pm 1.06	1.40 \pm 0.85	1.45 \pm 1.06
AQ Alone	0.96 \pm 0.71	0.98 \pm 0.76	1.09 \pm 0.63	1.11 \pm 0.65
AQ + Vit C	0.15 \pm 0.00	0.15 \pm 7.51	0.14 \pm 2.30	0.17 \pm 3.39
AQ + Vit E	0.99 \pm 0.81	1.43 \pm 0.63	1.11 \pm 0.75	1.38 \pm 0.88

p.o. = Per Oral

i.p. = Intraperitoneal route

Vit C = Vitamin C

Vit E = Vitamin E

Table 12: The effect of the administration of AQ alone for 28 days, AQ + Vitamin C and AQ + Vitamin E on enzymatic anti-oxidant activities of catalase (CAT) in the ovary of Sprague-Dawley rats.

Treatment groups	Anti-oxidant level (CAT/Mmol/min/mg protein)			
	(Mean ± SD)			
	6 mg/kg (p.o.)	12 mg/kg (p.o.)	6 mg/kg (i.p.)	12 mg/kg (i.p.)
Control	84.8 ± 49.10	84.8 ± 49.10	85.7 ± 47.30	85.7 ± 47.30
AQ Alone	55.5 ± 26.50	46.5 ± 26.70	52.1 ± 27.20	54.7 ± 26.60
AQ + Vit C	26.8 ± 3.24*	27.3 ± 2.90*	29.2 ± 1.98*	24.4 ± 0.75*
AQ + Vit E	74.7 ± 31.30	72.3 ± 31.70	76.1 ± 29.60	65.7 ± 24.80

* = $p < 0.05$

bw = body weight

p.o. = Per Oral

i.p. = Intraperitoneal route

Vit C = Vitamin C

Vit E = Vitamin E

4.9 EFFECT OF AMODIAQUINE ON THE HISTOMORPHOLOGY OF THE OVARY IN SPRAGUE-DAWLEY RATS

Microscopic examination of the ovaries did not show any difference between the control rats that received distilled water via oral or intraperitoneal routes. Histological sections of the ovaries in the control group were covered by a single, focal layer of pseudostratified modified peritoneal cells. The cells varied from flat to cuboidal and columnar types. Cortical and medullary stroma was continuous, and the boundary between these two zones was ill-defined and arbitrary. Ovarian follicles of various sizes with well defined theca interna and theca externa cells were seen. Each follicle contains a single oocyte in the stroma of the cortex. The oocyte within the graffian follicle is enveloped by the zona pellucida (ZP) and guarded by the granulosa cells. Few corpora lutea were observed in the cortex. Stromal cells immediately surrounding the follicle formed a sheath of connective tissue. Vessels penetrated into the cortex through the stroma. A mature graffian follicle in the control rat ovary appeared oval in shape exhibiting a spacious antrum, cumulus oophorus, corona radiata, zona pellucida and an oocyte (Plates 1 and 2).

On the basis of histological examination, the ovarian sections of the rats administered 6 mg/kg bw AQ alone orally for 28 days when compared to the control showed increased number of atretic follicles characterized by degenerated oocytes and zona pellucida, disintegrated granulosa cell layers and also a distortion/destruction of the basement membrane separating the theca cells from the granulosa cell layers (Plates 3 and 4). Co-administration with vitamin C showed more atretic follicles when compared to the control (Plates 5 and 6), however, this appeared consistent with the AQ alone treated group. The atretic follicles demonstrated degenerated oocytes and zona pellucida however, the basement membrane was intact. When AQ was co-

administered with vitamin E, the number of atretic follicles observed were fewer compared to the AQ alone group and the AQ + vitamin C treated groups, about 2 per profile (Plates 7 and 8). The atretic follicles were characterized by degenerated oocytes and zona pellucida and disintegrating granulosa cells.

In the rats that received 6 mg/kg bw AQ intraperitoneally, the ovarian histology appeared to show more atretic follicles, about 3 per profile characterized by degenerated oocytes and zona pellucida and disintegrated granulosa cell layers in comparison to that of the control. Also observed was a destruction of the basement membrane separating the theca cells from the granulosa cells in some areas (Plates 9 and 10). The ovarian histology of the rats that received AQ + Vitamin C and AQ + Vitamin E showed more atretic follicles when compared to the control, however, no significant difference was observed in comparison to the AQ alone group (Plates 11 to 14). The atretic follicles were characterized by degenerated oocytes, degenerated zona pellucida and disintegrating granulosa cells. However, a well defined theca cell layer and intact basement membrane was observed in these treatment groups.

At 12 mg/kg bw of AQ oral route, histological sections showed numerous atretic follicles, about 6 per profile characterized by degenerated oocytes, degenerated zona pellucida, disintegrating granulosa cell layers and also a distortion/destruction of the basement membrane separating the theca cells from the granulosa cell layers when compared with the control rat ovary (15 and 16).

When this treatment group was compared with the 6 mg/kg bw of AQ oral route, there appeared to more atretic follicles at the high dose of treatment.

Co-administration of AQ with vitamin C showed more atretic follicles when compared with the control (Plates 17 and 18). The atretic follicles were characterized by degenerated oocytes, degenerated zona pellucida and disorganization of the granulosa

cell layer. There appeared to be more atretic follicles in the AQ alone group than in the AQ + Vitamin C treatment groups. Fewer atretic follicles about 2 per profile were observed in the AQ + vitamin E treatment groups when compared to the AQ alone and the AQ + vitamin C groups (Plates 19 and 20).

When AQ was administered intraperitoneally at 12 mg/kg bw, ovarian sections showed atretic follicles about 6 per profile characterized by degenerated oocytes, degenerated zona pellucida and disintegrating granulosa cell layers compared to the control rat ovary (21 and 22).

The AQ + vitamin C group revealed atretic follicles characterized by degenerated oocytes, degenerated zona pellucida and disintegrating granulosa cells (Plates 23 and 24). Also observed was a distortion/destruction of the basement membrane separating the theca cells from the granulosa cell layers. The ovarian histology of the rats that received AQ + vitamin E displayed atretic follicles demonstrating degenerated oocytes, degenerated zona pellucida and disorganization of the granulosa cell layers (Plates 25 and 26).

Plate 1: Micrograph of cross-section of ovary of rat in control group showing a normal ovary demonstrating blood vessel (BV) in the interstitium. A mature graffian follicle is seen showing oocyte (O), corona radiata (CR), granulosa cells (GCs) antrum (A) and well defined theca cells (TCs) (H & E) x100.

Plate 2: Micrograph of cross-section of ovary of rat in control group showing a mature graffian follicle demonstrating oocyte (O), zona pellucida (ZP), cumulus oophorus (CO) and corona radiata (CR) (H & E) x400.

Plate 3: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ oral route for 28 days, showing atretic follicles (AF). (H & E) x100.

Plate 4: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ oral route for 28 days, showing atretic follicle characterized by degenerated oocytes and zona pellucida, disintegrating granulosa cells (GCs) and a destruction of the basement membrane (BM) separating the theca cells (TCs) from the granulosa cell layer. (H & E) x400

Plate 5: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ oral route and co-administration with 0.1 mg/kg bw Vitamin C for 28 days showing atretic follicles (AF). (H & E) x100.

Plate 6: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ oral route and co-administration 0.1 mg/kg bw Vitamin C for 28 days showing atretic follicle characterized by degenerated oocytes and zona pellucida and disorganization of the granulosa cell (GCs) layer (H & E) x400.

Plate 7: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ oral route and co-administration with 20 mg/kg bw Vitamin E for 28 days, showing atretic follicle (AF). (H & E) x100.

Plate 8: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ oral route and co-administration with 20 mg/kg bw Vitamin E for 28 days, showing atretic follicle characterized by degenerated oocyte and zona pellucida and disorganization of the granulosa cells (GCs). (H & E) x400.

Plate 9: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ intraperitoneal route for 28 days, showing atretic follicles (AF) (H & E) x100

Plate 10: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ intraperitoneal route for 28 days showing, atretic follicles demonstrating degenerated oocytes and zona pellucida, disintegrating granulosa cells (GCs) and a destruction of the basement membrane (BM) separating granulosa cell layer from theca cells (TCs) (H & E) x400.

Plate 11: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 0.1 mg/kg bw Vitamin C showing atretic follicle (AF) (H & E) x100.

Plate 12: Micrograph of cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 0.1 mg/kg bw Vitamin C showing, atretic follicle demonstrating degenerated oocyte and zona pellucida, disintegrating granulosa cells (GCs) and destruction of the basement membrane (BM) separating the theca cells (TCs) from the granulosa cell layer (H & E) x400.

Plate 13: Micrograph oh cross-section of ovary of rat in treatment group 6 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 20 mg/kg bw Vitamin E showing atretic follicles (H & E) x100.

Plate 14: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 20 mg/kg bw Vitamin E showing atretic follicle characterized by degenerated oocytes and zona pellucida (H & E) x400.

Plate 15: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ oral route for 28 days showing atretic follicles (AF) (H & E) x100.

Plate 16: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ oral route for 28 days showing atretic follicle characterized by degenerated oocyte and zona pellucida, disintegrating granulosa cells (GCs) and destruction of the basement membrane (BM) separating the theca cells (TCs) from the granulosa cell layer (H & E) x400.

Plate 17: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ oral route for 28 days and co-administration with 0.1 mg/kg bw of Vitamin C showing atretic follicles (H & E) x100.

Plate 18: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ oral route for 28 days and co-administration with 0.1 mg/kg bw of Vitamin C showing atretic follicle demonstrating degenerated oocyte and zona pellucida, disintegrating granulosa cells (GCs) and destruction of the basement membrane (BM) separating the theca cells (TCs) from the granulosa cell layer (H & E) x400.

Plate 19: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ oral route for 28 days and co-administration with 20 mg/kg bw of Vitamin E showing atretic follicles (H & E) x100.

Plate 20: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ oral route for 28 days and co-administration with 20 mg/kg bw of Vitamin E showing atretic follicles characterized by degenerated oocytes and zona pellucida and disintegrating granulosa cells (GCs) (H & E) x400.

Plate 21: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days showing atretic follicles (AF) (H & E) x100.

Plate 22: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days showing atretic follicle characterized by degenerated oocytes and zona pellucida, disintegrating granulosa cells (GCs) and disruption of the basement membrane (H & E) x400.

Plate 23: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 0.1 mg/kg bw of Vitamin C showing atretic follicle (AF) (H & E) x100.

Plate 24: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration of 0.1 mg/kg bw of Vitamin C showing atretic follicle characterized by degenerated oocytes and zona pellucida and disintegrating granulosa cells (GCs) (H & E) x400.

Plate 25: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 20 mg/kg bw Vitamin E showing atretic follicles (AF) (H & E) x100.

Plate 26: Micrograph of cross-section of ovary of rat in treatment group 12 mg/kg bw of AQ intraperitoneal route for 28 days and co-administration with 20 mg/kg bw Vitamin E showing atretic follicles characterized by degenerated oocyte and zona pellucida (H & E) x400.

CHAPTER FIVE

DISCUSSION

5.1 BODYWEIGHT

The result from this study showed that there was a significant increase in weight in the treatment groups relative to the control. This significant weight increase could be as a result of chronic stress produced by AQ. Stress can affect virtually any part of the body and produce physical, mental and emotional symptoms including allergies, dizziness, headache, heart palpitations, environmental sensitivity, impaired coordination, impaired immunity and weight-gain (**James**, 2001).

Under stress, the body excretes corticotrophin-releasing hormone and adrenalin. This reaction stimulates the release of cortisol from the adrenal cortex. In turn, cortisol, a glucocorticoid, stimulates glucose release into the bloodstream, which, during periods of chronic stress, creates an excessive release of insulin. Insulin, which is part of the endocrine system, is a fat-storage hormone that overrides the stress signal from adrenalin to burn fat. The excess release of insulin gives the body the message to store fat in the abdomen (**Facchini et al.**, 2000).

The study showed that there was a relatively higher body weight increase in the groups administered by intraperitoneal route compared to those that received AQ via the oral route however, this increase in body weight was not statistically significant.

5.2 OESTROUS CYCLICITY STUDIES

Rats typically have rapid cycle times of 4 to 5 days and the events of the cycle are strongly influenced by photo-periodicity (**Freeman**, 1994).

The results from this study show that the administration of AQ caused a disruption in the oestrus cycle. Compared to the control, the length of the estrous cycle was

prolonged in the AQ alone treatment groups. Nevertheless, the mean cycle length of 5.04 and 5.25 days recorded in the groups that received 6 mg/kg bw of AQ via oral and intraperitoneal routes, respectively is in concert with the study of **Long and Evans** (1922), **Astwood** (1939) and **Blandau *et al.***, (1941) who recorded a mean cycle length of 5.4, 4.5 and 4.4 days respectively. The groups that received the high dose (12 mg/kg bw of AQ) via oral and intraperitoneal routes recorded mean cycle lengths of 6.06 and 5.49 days, respectively demonstrating that the animals had a longer mean cycle length at 12 mg/kg bw than at 6 mg/kg bw in both routes of administration. However, this difference in mean cycle length was not statistically significant. The increase in mean cycle length observed in this study occurred as a result of a disruption in cycle pattern. The reason for this disruption was because the animals had prolonged diestrus with resultant increase in the cycle length. The result of this study is in concert with the findings of **Okanlawon and Ashiru** (1992). In their study to determine the effects of CQ on the oestrus cycle in 4-day cyclic Sprague-Dawley rats, the authors administered 40 mg/kg bw chloroquine phosphate intraperitoneally once a day five days in a week. Result showed that the administration of CQ altered the oestrus cycle through a persistent diestrus smear. The similarity in our findings may possibly be due to the similarity in the structure of chloroquine and AQ.

Co-administration of AQ with Vitamin C significantly ($p < 0.05$) increased the mean cycle length in all the treatment groups that were administered AQ + Vitamin C. However, co-administration of AQ with Vitamin E reduced the cycle length but was not able to return the cycle to control values.

It has been reported previously that progesterone secretion remains higher during diestrus in the 5-day cyclic rat than in the 4-day (**Nequin *et al.***, 1979). These researchers suggested that the 5-day cycle is due to the prolonged progesterone

secretion from a new crop of corpora lutea during diestrus. The LH-independent luteal secretion of progesterone during diestrus phase begins to rise in the afternoon of metestrus, reaches peak values by early morning of diestrus, and falls to basal levels shortly thereafter (**Smith *et al.*, 1975**).

It is well established that the progesterone secreted during diestrus intensifies the negative feedback of estradiol on basal LH secretion and that when these levels of progesterone fall at luteal regression, the secretion of estradiol and LH rises and ovulation occurs within the following 6- 8 h (**Beattie and Corbin, 1975; Smith *et al.*, 1975; Taya *et al.*, 1981; Kaneko *et al.*, 1986**). Therefore the action of gonadotropins on estrogen secretion is not a process triggered immediately in an all-or-none fashion. Instead, gonadotropins must continue to act for some time for ovarian estrogen secretion to increase. The prolongation of luteal progesterone secretion in an estrous cycle suppresses pituitary gonadotropin secretion. Rather than blocking the estrogen triggering of ovulatory LH surge directly, it delays the estrogen secretion itself, which decreases the threshold of the neural and/or hypophyseal structures for ovulatory LH release.

In this study progesterone level was not determined so it can not be substantiated at this stage whether the persistent diestrus observed in this study was as a result of prolonged progesterone levels.

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5.3 OVULATION AND HORMONAL STUDIES

The ovary is the primary organ of female reproduction. Successful growth and differentiation of the ovarian follicle is known to be under the control of the FSH and LH produced by the pituitary.

Studies have shown that the process of folliculogenesis is mediated by cAMP and is modulated by many local paracrine and autocrine factors in addition to the gonadotrophins themselves (**Winter *et al.*, 1977; Williams *et al.*, 1982**). In this study, no statistically significant difference was observed in the serum concentrations of LH, FSH and PRL when compared to the control. The expected surge in serum LH concentration between 5 – 7 p.m. on proestrus that is responsible for follicular rupture at ovulation was observed in all the animals both in the treated and control groups. It has been established that serum levels of FSH, LH and PRL are at the lowest from early on the morning of estrus shortly after ovulation (**Gay *et al.*, 1970; Daane and Parlow, 1971**). This circadian pattern from estrus through diestrus consisted of a small elevation of FSH, LH and PRL each day at the midpoint of the light period, and the lowest levels were reached midnight of each day. On the afternoon of proestrus, about 2 to 3 p.m., the circulating levels of LH begins to increase rapidly and ultimately reached peak levels by 5 to 7 p.m. on that same evening. This proestrus surge of LH is responsible for the process of and events following ovulation. This rapid surge of LH induces follicular rupture and ovulation (**Freeman, 1988**). Since the expected surge in FSH, LH and PRL was observed in the study, the result of this study suggests that AQ does not exert its effect on ovulation via a direct effect on pituitary gonadotropin secretion.

In this study, a partial block in ovulation was observed. This is in agreement with the result of previous investigators. **van der Schoot *et al.* (1982)** and **Okanlawon and Ashiru (1992)** reported that the administration of CQ and sodium pentobarbital at 9 a.m. on proestrus blocked ovulation completely. **Gbotolorun *et al.* (2004)** and **Gbotolorun *et al.* (2008)** reported a partial block in ovulation at 9 a.m. with the

administration of *Neem* seeds and flowers respectively. **Akpantah et al.** (2005) also reported a partial block in ovulation with the administration of *Garcinia Kola*.

AQ, is a potent inhibitor of histamine N-methyltransferase (**Yokoyama et al.**, 2007), it acts by accumulating in the lysosomes of the parasites bringing loss of its function, and also binds to their nucleoproteins inhibiting the DNA and RNA polymerase which end up generating free radicals (**Maggs et al.**, 1988; **O'Neill et al.**, 1998).

AQ generates free radicals in the forms of AQ quinone immine and semi quinone immine and these have been implicated in lipid peroxidation in the membranes of hepatocyte (**Maggs et al.**, 1988). AQ circulating in the blood therefore reaches the ovary with its free radicals.

In addition, AQ has been reported to have anti-inflammatory properties (**Bepler et al.**, 1959; **Pomeroy**, 1959; **Ngouesse et al.**, 2001). Studies have revealed that the process of ovulation is comparable to an inflammatory process (**Epsey**, 1980). Anti-inflammatory drugs have been employed in blocking ovulation (**Gaytan et al.**, 2002). Anti-inflammatory drugs exert their action through inhibition of cyclo-oxygenase enzyme (**Liang et al.**, 1999). This implies that the mechanism of the anti-ovulatory property may be similar to that induced by indomethacin, a potent cyclo-oxygenase enzyme inhibitor (**Epsey**, 1983) suggesting that AQ may not inhibit ovum maturation but causes abnormal follicle rupture (**Gaytan et al.**, 2002). Cyclo-oxygenase, which converts arachidonic acid derived from cell membranes to prostaglandins (PG), has two isomers, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (**Osau et al.**, 2001). COX-1 is the endogenous form of the enzyme necessary for production of PG while COX-2 is thought of as being an inducible enzyme associated with inflammation. The latter is thought to be essential for the ovulatory mechanism. It was revealed that all traditional non-steroidal anti-inflammatory drugs affect the action of

both COX-1 and COX-2 but produce most of their effects by blocking COX-2 (**Staud, 2000**). COX-2 deficient mice suffer from defect in reproductive functions such as ovulation and fertilization (**Lim *et al.*, 1997**), implying that COX- 2 is important in ovulation. These reports (**Katori and Majima, 2000; Lim *et al.*, 1997**) indicate that COX-2 is an essential enzyme for follicular rupture through metabolites of arachidonic acids, which play important roles in follicular rupture through the activation of proteases, neovascularization, leukocyte migration and smooth muscle contraction (**Katori and Majima, 2000**).

The partial block in ovulation observed in this study may be as a result of increased circulation of free radicals due to overwhelmed scavenging mechanisms and also via inhibition of cyclooxygenase activity (perhaps COX-2) and PG Synthesis.

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5.4 ANTI-OXIDANT STUDIES

All forms of aerobic life face the threat of oxidation from molecular oxygen (O₂). Conceivably to cope with various reactive oxygen species (ROS), different classes of enzymatic and nonenzymatic antioxidants have evolved. Antioxidants function by preventing the accumulation of toxic levels of oxygen-derived free radicals, which can damage the cells by modifying proteins, lipids, and DNA. An imbalance between free radical pro-oxidants and anti-oxidants has important implications for both physiological and pathological processes in the reproductive tract (**Agarwal *et al.*, 2005**).

The enzyme superoxide dismutase catalyses dismutation of superoxide radical, leading to formation of hydrogen peroxide, which in turn is detoxified by the enzyme catalase (**Rzeuski *et al.*, 1998**).

In this study decreased activities of superoxide dismutase and catalase in all the treatment groups that received AQ alone was observed. AQ, is a potent inhibitor of histamine N-methyltransferase (**Yokoyamaa et al.**, 2007), it acts by accumulating in the lysosomes of the parasites bringing loss of its function, and also binds to their nucleoproteins inhibiting the DNA and RNA polymerase which end up generating free radicals (**Maggs et al.**, 1988; **O'Neill et al.**, 1998). AQ generates free radicals in the forms of AQ quinone imine and semi quinone imine and these have been implicated in lipid peroxidation in the membranes of hepatocytes (**Maggs et al.**, 1988). AQ circulating in the blood therefore reaches the ovary with its free radicals.

The result of this study is in concert with the report of **Rao et al.** (2009) in which the authors reported a reduction in the activities of superoxide dismutase and catalase in the mouse ovary. A reduction in cellular enzymatic antioxidant activities of superoxide dismutase and catalase is clearly indicative of oxidative stress.

Vitamin C is a potent reducing agent and scavenger of free radicals in biological systems (**Rose and Bode**, 1993; **Buettner and Jurkiewicz**, 1996; **May**, 1999).

In this present study, co-administration of AQ + Vitamin C produced a further reduction in the enzymatic antioxidant activities of superoxide dismutase and catalase. The reduction in the activities of catalase was statistically significant ($p < 0.05$) in the entire treatment groups. The controversy around the *in vivo* anti- or pro-oxidant nature of Vitamin C has been the subject of several human intervention studies in recent years. It is known that blood cells saturate at lower vitamin C concentrations than human plasma and that this intracellular saturation occurs at plasma concentrations that can easily be obtained from the diet (**Levine et al.**, 1996).

However, most studies only report increase in plasma levels. It is likely that if tissue saturation is achieved, then the additional beneficial effects are small and difficult to

detect, leading to non-significant or null effects. Consistent with this notion, most of the studies that have shown a protective effect were carried out with smokers or patients with pathological conditions associated with oxidative stress and low plasma vitamin C levels. It is thus possible that the initial level of vitamin C in the cell predetermines whether supplementation trials may have a positive or null response. Therefore, the lower the vitamin C level the more positive the response would be (**Duarte and Lunec, 2005**).

From this study, it can be deduced that Vitamin C had a pro-oxidant effect on the ovary by exacerbating the oxidative stress produced by AQ administration.

Co-administration of AQ with Vitamin E on the other hand, increased the activities of superoxide dismutase and catalase. The result of this study is in concert with the studies of **Nugent *et al.* (1998)** and **Rao *et al.* (2009)**. These authors reported that Vitamin E administration improved the survival rate of follicles in ovarian grafts by reducing ischaemia-reperfusion injury, and also exerts a protective effect against nickel and/or chromium induced toxicity by preventing lipid peroxidation and protecting antioxidant system in the mouse ovary respectively.

This study therefore suggest that co-administration of AQ with Vitamin E exerts a protective effect against AQ induced oxidative stress in the ovary of Sprague-Dawley rats.

5.5 HISTOLOGICAL STUDIES

Ovarian sections from control rats consistently displayed good histological preservation indicating that the fixation method and tissue processing was optimal. The sections demonstrated ovarian follicles in various stages of development with normal appearance in the ovarian cortex. A follicle is considered to be undergoing

atresia/regression whenever the following structural changes occur: two or more pyknotic granulosa cells are found in a single section, hypertrophy of theca cells, denuded oocyte and zona pellucida or thinning of cumulus oophorus and destruction of basal lamina (**Osman**, 1985; **Azarnia et al.**, 2004; **Koc et al.**, 2009). The histopathological examination of ovaries of animals treated with AQ for 28 days revealed that the most remarkable change was widespread ovarian follicle atresia in comparison to the control group. The atretic follicles were characterized by degenerated oocytes and zona pellucida, disintegrated granulosa cells and a distortion/destruction of the basement membrane separating the theca cells from the granulosa cell layer (**Osman**, 1985; **Azarnia et al.**, 2004; **Koc et al.**, 2009). The result of this study is in concert with previous investigators who reported increase in the number of atretic follicles when Lead and *Artemisia annua* were administered to mouse and rat ovaries respectively (**Azarnia et al.**, 2004; **Ajah and Etang** 2010). This study suggests that AQ may have inhibited the maturation process of the follicles.

The ovarian histology of the groups that received AQ + Vitamin C showed more atretic follicles compared to the control group. When these groups were compared with the AQ alone treated groups there appeared to be no difference in the number of atretic follicles observed at both dosages and routes of administration however, the atretic follicles showed some structural differences. Unlike those in the AQ alone treated groups these atretic follicles still had intact basement membrane suggesting that Vitamin C may have conferred some protection or controlled the process of atresia.

In the groups that received AQ + Vitamin E for 28 days, compared to the control, the ovaries displayed fewer numbers of atretic follicles suggesting that Vitamin E may attenuate the inhibitory effects of AQ on maturation of the follicles.

Granulosa cells are essential in the normal follicular maturation process since they produce steroidal hormones and growth factors and also play a crucial role in follicular atresia. Apoptosis of granulosa cells seems to have a negative effect on follicular maturation. A higher incidence of apoptotic granulosa cells has been associated with fewer oocytes retrieved and poorer quality of oocytes and embryos (**Jancar *et al.*, 2007**).

Various pathological stimuli such as OS can initiate apoptosis in mammalian oocytes (**Roth and Hansen, 2004**). Intra-cellular accumulation of ROS, can damage cells by causing nucleic acid strand breaks, lipid peroxidation, protein degradation and ultimately, cell death (**Yu, 1994**). It has been suggested that steroidogenically active cells such as granulosa cells of antral follicles, require high levels of energy production and thus generate large amounts of ROS (**Rapoport *et al.*, 1999**). Therefore it is possible that OS is involved in the mechanisms that trigger apoptosis in healthy steroidogenic antral follicles (**Jancar *et al.*, 2007**).

Some evidence based histopathological changes of oxidative stress have been observed and characterized in the ovary. They include vascular congestion, atretic follicles, haemorrhage, edema and inflammatory cells infiltration (**Coskun, *et al.*, 2007**; **Güven *et al.*, 2008**; **Halici *et al.*, 2008**). In this study, although not all the classical features of oxidative-stress induced histopathological changes were observed, it can not be completely excluded that the ovary may be under some form of oxidative stress. Therefore, it can be deduced from this study that chronic administration of AQ produces deleterious effect in the ovary by increasing follicular atresia.

CHAPTER SIX

SUMMARY OF FINDINGS AND CONCLUSION

6.1 SUMMARY OF FINDINGS

1. AQ increases the number of atretic follicles in the ovary of the treated rats.
2. AQ disrupts the estrous cycle by prolonging the length of the cycle in all the groups that were treated with AQ alone. The increase in cycle length was caused by a persistent diestrus phase. AQ also causes a partial block in ovulation.
3. AQ produces oxidative stress in the ovary by reducing the activities of superoxide dismutase and catalase. Co-administration of AQ with Vitamin C causes a further reduction in the activities of superoxide dismutase and catalase in the ovary. This reduction was statistically significant in the activities of catalase. However, Co-administration with Vitamin E was able to ameliorate the effect of AQ on the activities of superoxide dismutase and catalase in the ovary of Sprague-Dawley rats.
4. AQ has no direct effect on serum concentrations of FSH, LH and PRL.

6.2 CONTRIBUTIONS TO KNOWLEDGE

1. The study demonstrates that AQ has deleterious effect on the histomorphology of the ovary with accompanying prolongation of the diestrus phase of the oestrus cycle and partial block in ovulation.
2. The effect of AQ on ovulation is possibly through a free radical and cyclo-oxygenase inhibitory mechanisms rather than via a direct effect on FSH, LH and PRL concentrations.
3. AQ produces oxidative stress in the ovary and this is ameliorated by co-administration with Vitamin E.

6.3 CONCLUSIONS

This study demonstrates that AQ causes increased ROS production, oxidative damage, and decreased antioxidant defense in the rat ovary, which result in an oxidized state in the cells. This AQ-induced oxidative stress leads to increased atresia which in turn leads to reduced follicle numbers, and reduced oocyte numbers at ovulation. Consequently, this will lead to a reduction in the fertility of female Sprague-Dawley rats.

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