UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

EFFECT OF COCOA ON OVARIAN HISTOLOGY IN EXPERIMENTAL DIPSOMANIAC RABBITS

BY

EUGENIA ELIKEM ABENA KUMAGA

(10341393)

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN ANATOMY

DEPARTMENT OF ANATOMY

JULY, 2017

DECLARATION BY THE CANDIDATE

I hereby declare that except for references made to the work of other researchers; this project is the product of my own research carried out under supervision in accordance with regulations of the School of Research and Graduate Studies, University of Ghana. I further declare that this dissertation has neither in whole nor in part been presented for any degree elsewhere, and that I am entirely responsible for any residual flaws in this work.

Signature…… Date……..……

Eugenia Elikem Abena Kumaga

DECLARATION BY SUPERVISORS

We declare that the practical work and presentation of this thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

Principal supervisor:

Signature: …… Date: ……..……

Professor Frederick Kwaku Addai

Co-supervisors:

Signature: …… Date: ……..……

Dr. John Ahenkorah

DEDICATION

This work is dedicated to God, my parents and siblings.

ACKNOWLEDGEMENTS

All thanks and praise to God to whom I belong and owe my life.

I am grateful to the Department of Anatomy for the immense support and financial assistance in bearing the cost of the hormone analysis and for importing the anti-oxidant assay kit used.

My deepest gratitude goes to my Principal Supervisor, Prof. F. K. Addai, whose guidance saw me through to the completion of this work. I am thankful for your deep insights and mentorship.

To Dr. J. Ahenkorah, my co-supervisor, thank you for your guidance in all areas of this work. My appreciation also goes to Dr. (Mrs.) E. Dennis and Dr. B. A. Hottor, for your immense contribution to the success of this project.

I would like to thank Mr. S. Mensah and Mr. P. Atiah for offering your technical expertise. To Mr. Armah and staff of the Animal Experimentation Unit, thank you. I am grateful to Mr. Hope Azasu for helping me with the storage of samples.

My gratitude goes to Dr. B. Adu and Mr. E. Kyei-Baafour of NMIMR for their expertise and support in the running of the anti-oxidant assays.

Dr. R. Blay and Dr. B. Arko-Boham, thank you for your support in the drafting of my research proposal.

Mr. J. G. Akyeampong, Mr. B. G. Dzotefe, Mr and Mrs. W. Dzikunu, Mrs. G. A. Hammond, Mr. E. O. Fato, Ms. N. A. Baidoo, Mr. R. Abdulai, Mr. D. Odoom and Mr. E. Aidoo, I say thank you for your wonderful support.

I am greatly indebted to Dr. E. H. Owusu, Mr. and Mrs. E. Fiati (Ing) and Dr. D. Tormeti. For your prayers and support in diverse ways.

Also, I am thankful to Mawuli Fiati, Kafui D. Fiati, Nana Kofi Osei, H. Y. Akogo, B. Gapher, Maame Pokuaa, H. N. Anyomi, Mr. F. Antwi-Boasiako and S. R. Krause for your prayers and support.

I would like to thank my Mom and Dad, Aunty Yowome Dzah, my brothers; Mr. E. K. Adjogatse and Mr. I. K. Kumaga and my cousins; B. Ofori and E. K. Paul.

Finally, to all friends and loved ones whose encouragement and goodwill pulled me through, I say thank you.

I pray the Almighty God showers his blessings on you all.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADH: Alcohol Dehydrogenase

ALDH2: Aldehyde dehydrogenase 2

ATP: Adenosine Tri-Phosphate

BAC: Blood alcohol concentration

CI: Confidence Interval

CDC: Centres for Disease Control and Prevention

DNA: De-oxyribonucleic acid

FSH: Follicle stimulating Hormone

GAFCo: Ghana Agro Food Company

GF: Graafian follicle

GH: Growth Hormone

GnRH: Gonadotropin releasing Hormone

GRH: Growth releasing Hormone

GSH: Glutathione reductase

$GSHP_X$: Glutathione peroxidase

H_2O_2: Hydrogen peroxide

HPG: Hypothalamus- Pituitary- gonadal

LH: Luteinizing Hormone

LHRH: Luteinizing Hormone releasing Hormone

mRNA: Mitochondrial ribonucleic acid

NADH: Nicotinamide adenine dinucleotide

NMIMR: Noguchi Memorial Institute for Medical Research

NO: Nitric oxide

O^{2-}: Superoxide anion

OH^{-}: Hydroxyl radical

OS: Oxidative Stress

OTC: Over the counter

PCOS: Polycystic Ovary Syndrome

ROS: Reactive Oxygen Species

SBAHS: School of Biomedical and Allied Health Sciences

SOD: Superoxide dismutase

SRY: Sex determining region Y

WHO: World Health Organization

ABSTRACT

Background: Regular alcohol intake significantly increases reactive oxygen species (ROS) which magnify the levels of oxidative stress (OS) in the ovaries beyond physiological levels. Oxidative stress occurs due to an overabundance of ROS and the ineffectuality of scavengers. Alcohol is also known to perturb the feedback mechanisms of the hypothalamus-pituitary-gonadal (HPG) axis resulting in impairment of production and secretion of adequate quantity or potency of LH and FSH necessary for fertility. The usage of natural and synthetic antioxidants in treating and managing female infertility of OS etiology in patients is currently being investigated. Natural cocoa exhibits greater antioxidant capacity than many other flavanol-rich foods and food extracts. This suggests that cocoa as a nutraceutical may resolve OS related ovarian injury.

Aim: This study investigated the ameliorative effect of natural cocoa on alcohol induced ovarian injury in rabbits.

Methodology: Thirteen female rabbits of ages 5-7 months and weights 1.5-2.5 kg were put into 3 groups (Groups A, B and C) and given the following daily treatments for 7 weeks. Group A (n=5), Group B (n=5) and Group C (n=3). Group A, received cocoa from 6am to 6pm and 35% alcohol from 6pm to 6am *ad libitum*. Group B received water from 6am to 6pm and 35% alcohol *ad libitum* from 6pm to 6am. Group C received water for 24 hours. All rabbits had access to rabbit chow daily. Baseline serum levels of LH, FSH, GSH and SOD were measured and repeated at the end of the experiment (week 7). At termination, all rabbits were sacrificed and ovaries harvested. Volume density of follicles at different stages and stroma were estimated. Weight of rabbits and ovaries together with volume of ovaries were also determined.

Results: Group B had low levels of serum LH and FSH (ANOVA, $p<0.001$) as compared with Groups C. Post treatment concentrations of GSH and SOD in Group A were not significantly different from that of Group C (ANOVA, $p<0.05$). Stereologic assessment of volume density of primordial follicles, corpora atretica and stroma showed significant differences between groups (ANOVA, $p<0.05$). Functional elements of the ovary parenchyma were preserved in Group A rabbits compared to Group B and C (ANOVA, $p=0.05$). Ovary weights and volumes did not vary significantly between the groups (ANOVA, $p=0.75$).

Conclusion: Alcohol-induced structural and functional ovarian injury was significantly ameliorated in rabbits chronically fed alcohol but also given natural cocoa drink.

CHAPTER ONE

1 INTRODUCTION

1.1 Background of Study

The World Health Organization (WHO) Manual for standardized investigation and diagnosis as cited by Oremosu and Akang (2015) for infertile couple defines infertility as the inability of a couple to conceive after a year of regular unprotected sexual intercourse. The management and treatment of infertility is challenging and has become a global concern as the need to have children is of great priority in families. It is reported that about 15% of couples of reproductive age are infertile (Oremosu & Akang, 2015). In most developing countries, approximately 10% of all women's visits to doctors are due to childlessness-related problems and this could be due to environmental toxic agents (Moshfegh, Baharara, Namvar, Zafar-Balanezhad, & Amini, 2016).

The Centers for Disease Control and Prevention in 2011, reported that the effects of infertility on these couples can be devastating and this in turn leads to psychological stress, anxiety and depression (Akang et al., 2015). Infertility may not be considered as a disease in itself, nevertheless it is a social and public health issue as well as an individual problem (Akang et al., 2015).

Alcohol is widely used and it is the most abused chemical agent in the world (Kumar, Abbas, & Fausto, 2004). Alcoholic beverages come in beers, wine and spirits (Stiles, 2016). Consumption of alcohol in Ghana is pegged at 1.64 litres/person a year (Stiles, 2016). Also, a lot of herbal and traditional medications are made into bitters or tinctures which contribute to daily alcohol consumption either consciously or unconsciously. In

recent times, its intake among women has been taken up a notch due to the production of sweeter alcoholic beverages, cheaper pricing and free drinks for women at most pubs and clubs.

Alcohol is a psychoactive drug which affects diverse cellular and molecular processes in the liver and other organs of the body are no exception (Reddyvari et al., 2017). Up regulated OS due to the excessive liberation of ROS in ethanol metabolism affects the antioxidant defense system leading to organ and tissue injury and various disease conditions (Pyun, Mandal, Hong, & Lee, 2015). An increasing number of literatures suggest that chronic alcohol abuse may result in failure of female sexual and reproductive function. For example, an association between alcoholism and menstrual abnormalities, problems with reproduction, and changes in secondary sex characteristics has been established (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 1994). Moderate alcohol use may contribute to the risk of specific types of infertility in women (Grodstein, Goldman, & Cramer, 1994). Even in small amounts, it affects women differently from men (National Institutes of Health (NIH), 2015). As one of the key organs involved in the issues of infertility, the ovaries are not spared from the effects of alcohol intake by women. Alcohol significantly increases ROS which magnify the level of OS in the ovaries beyond physiological levels, leading to ovarian injury (Agarwal, Aponte-Mellado, Premkumar, Shaman, & Gupta, 2012)

Oxidative stress occurs when there is an imbalance between pro-oxidant molecules including ROS, reactive nitrogen species, and antioxidant defenses. This has been reported to play a key role in the pathogenesis of subfertility in both males and females. This imbalance also can lead to a number of gynecological diseases such as

endometriosis, polycystic ovary syndrome (PCOS), and unexplained infertility (Agarwal et al., 2012).

Alcoholic beverages such as beer and wine contain antioxidants, yet when abused, alcohol becomes a pro-oxidant instead of an antioxidant (Dasgupta & Klein, 2014). The consequences of alcohol metabolism include hypoxia in the liver and formation of ROS that can damage other cell and tissue components (Zakhari, 2017).

An understanding of alcohol metabolism provides the basis for expounding alcohol-induced ovarian injury. Alcohol is metabolized by several processes mainly in the liver. It can be metabolized by two distinct pathways: oxidative and non-oxidative alcohol metabolism, leading to the production of acetaldehyde, acetate, ROS, and fatty acid ethyl esters (Ghazali & Patel, 2016). The metabolism involves alcohol dehydrogenase (ADH) converting alcohol to acetaldehyde, a toxic chemical that causes DNA damage. This is further metabolized into acetic acid, a nontoxic metabolite in the body by aldehyde dehydrogenase 2 (ALDH2) (Hong, 2016). Acetic acid also removes other toxic aldehydes that can accumulate in the body. Acetic acid is further broken down into carbon dioxide and water (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2010). These processes make it possible for alcohol to be eliminated from the body. In as much as there are mechanisms to metabolize and excrete alcohol as a toxin from the body, the byproducts of these processes leave in their wake evidences of some degree of cell and tissue injury.

Consuming alcohol at the wrong time, even in insufficient amounts can cause a certain degree of tissue damage. This can also upset the sensitive balance crucial in maintaining

female reproductive hormonal cycles and result in some level of infertility. In a study conducted among social drinkers showed that even those who drank small amounts of alcohol ceased cycling normally and became at least temporarily infertile (Emanuele, Wezeman, & Emanuele, 2003; Gude, 2012). Some studies have reported that the ovaries of alcohol–exposed female rats were infantile and showed no indication of ovulation. Uteri also appeared totally deprived of estrogen (Gavaler, Van-Thiel, & Lester, 1980). Also, alcohol consumption temporarily increases testosterone levels. Testosterone is a well–known suppressor of the hypothalamic–pituitary unit. An increase in testosterone could therefore disturb normal female cycling (Sarkola, Adlercreutz, Heinonen, Von Der Pahlen, & Eriksson, 2001).

In the treatment and management of infertility, fertility supplements are employed. Ingestion of nutrients with antioxidant properties, including multivitamins, stifle the production of ROS and may play a helpful role in female fertility (Ruder, Hartman, & Goldman, 2009). Couples battling infertility resort to their usage either concurrently or as an adjunct to medical treatment to manage the problem (Polackwich & Sabanegh, 2015). Most of these supplements are obtained over the counter. Over-the-counter (OTC) medicines or non-prescription medicines are drugs one can buy without a prescription. They are considered safe and effective and requires the user to follow the instructions on the label and as directed by a health care professional (U.S Food and Drug Administration, 2016). The high costs associated with assisted reproductive techniques for male and female infertility and the side effects in the consumption of the OTC supplements have led consumers to find less expensive alternatives for potential

treatment. The use of Nutritional food supplements and nutraceuticals are some of such alternatives.

Any substance, considered as food or part of a food and provides medicinal or health benefits, encompassing, prevention and treatment of diseases is known as a 'nutraceutical' (Rajasekaran, Sivagnanam, & Xavier, 2008). The word 'nutraceutical', was made up by combining the terms 'nutrition' and 'pharmaceutical' in 1989 by Dr. Stephen DeFelice, chairman of the Foundation for Innovation in Medicine. Hippocrates correctly emphasized about 2000 years ago: "Let food be your medicine and medicine be your food". Nutraceuticals have received great attention for the reason that they are presumed to be safe and their possession of latent nutritional and therapeutic benefits (Rajasekaran *et al*., 2008). Different nutraceuticals, including herbs, fruits, vegetables, nutritional supplements, and vitamins, have been promoted to improve many aspects of male fertility. These include sperm function and semen analysis parameters, erectile function, and libido (Ko & Sabanegh, 2014).

Many supplements for fertility address the problem of ROS, suggesting that nutraceuticals used in treatment of infertility need to possess antioxidant properties to enable it to scavenge ROS (Polackwich & Sabanegh, 2015).

Historically, medicinal plants were considered as the only form of health care to which majority of populations could immediately access (Adaay & Mattar, 2012). Among edible plants known to contain antioxidants, it has been established that cocoa has the greatest variety and the most potent antioxidant. These properties can assist in mopping up free radicals resulting from alcohol metabolism and therefore avert their damaging

effect on ovaries (Lee, Kim, Lee, & Lee, 2003; Roy, Lundy, & Brantely, 2005). Cocoa possesses about 380 known chemicals, 10 of which are psychoactive compounds (Andújar, Recio, Giner, & Rios, 2012). The presence of phenolic compounds especially flavonoids in cocoa powder contribute to its antioxidant capacity. The flavonoids in cocoa were identified as catechin, epicatechin, dimers and trimers (Jalil & Ismail, 2008). Sokpor *et al* (2012) demonstrated that voluntary cocoa ingestion attenuated hepatic damage caused by experimental alcoholic toxicity in rats. Cocoa's effect is being assessed in this research to determine whether its consumption can demonstrably ameliorate ovarian tissue injury in rabbits induced by experimental dipsomania.

1.2 Problem Statement

Consumption of alcohol in Ghana is pegged at 1.64 litres/person a year according to *The Daily Viz* (Stiles, 2016). Even though this is among the low intake levels in Africa, many hospitals and clinics record high incidence of alcohol related diseases and tissue damage. Also, a lot of the herbal and traditional medications are made into bitters which influence daily alcohol consumption. Locally, alcohol is undoubtedly the most advertised beverage on many media platforms leading to greater awareness of its availability. In recent times, alcohol intake among women has risen due to the production of sweeter beverages, cheaper pricing and free drinks for women at some pubs and clubs (Personal Observation).

Fertility data gathered from the 2010 Ghana population census indicated that between 2000 and 2010, there had been a decline in all four fertility measures: specific fertility rate, total fertility rate (17.8% decline), general fertility rate (25.4% decline) and crude birth rate (19.4% decline) (Ghana Statistical Service, 2014a). In the 2015 Demographic and Health Survey Report by Ghana Statistical Service, total fertility rate has declined over a 20 year period (i.e. from 6.4 births per woman in 1988 to 4.0 births per woman in 2008, only increasing to 4.2 births per woman by 2014) (Ghana Statistical Service, 2014a). Primary infertility rate as reported by Larsen in 2000 stood at 2% with secondary infertility rate at 14% (Ghana Statistical Service, 2014b).

It has been shown that there is a direct association between alcohol intake and the risk of infertility in women (Eggert, Theobald, & Engfeldt, 2004). Few studies have shown the direct relationship between alcohol intake and ovarian health. The deleterious effects of alcohol on male sexual function are well documented and have been shown to be related

both to primary testicular failure and to suppression of hypothalamus-pituitary-gonadal (HPG) responsiveness. Also, most researches focus on alcohol as a reproductive toxin in males rather than in females. Alcohol significantly increases ROS which magnify the level of OS in the ovaries beyond physiological levels, leading to ovarian injury. However, the mechanism by which this occurs is not properly understood. The information available concerning the alcohol-ovarian injury relationship is inadequate and therefore the need for some further research in this area. The question is asked that would the antioxidant properties of natural cocoa be able to attenuate the OS related ovarian injury caused by experimental alcoholism?

1.3 Justification

Intake of alcohol is a causal factor of public health importance in the subject of infertility as consumption is wide spread and increasing in many countries. Consistent and unlimited use of alcohol leads to toxicity and alcohol-induced pathological problems and can institute a danger in the society. Thus, several studies are focusing on the mechanism of cell or tissue injury caused by alcohol-induced oxidative stress and protective methods.

ROS is significantly increased in regular alcohol intake. This magnifies the levels of OS in the ovaries, endometrium and fallopian tubes.

Natural cocoa is a readily available and affordable food substance, that presently is consumed widely and regularly in Ghana. Cocoa powder and cocoa extracts have been shown to exhibit greater antioxidant capacity than many other flavanol-rich foods. This can assist in mopping up free radicals resulting from alcohol metabolism and therefore avert their damaging effect on ovaries. This is anticipated to protect the ovarian architecture during regular alcohol consumption and also reverse the damaging effect alcohol has had on the ovaries.

It is of interest to establish the ameliorative effect of cocoa on impaired fertility caused by alcohol-induced OS. Natural cocoa, if found to give a positive outcome in this study may unveil a novel research area in cocoa and alcohol impaired fertility in humans. Natural cocoa as a possible antidote will definitely be a more affordable option.

In the Department of Anatomy, School of Biomedical and Allied Health Sciences (SBAHS), research that has been conducted have revealed the injurious effects of ROS and OS on organs and tissues in the body. This current research also seeks to aid in the

understanding of the mechanism ROS leading to OS achieve this injurious feat in the ovaries and how natural cocoa could help remedy the injury.

1.4 Hypothesis

Regular ingestion of natural cocoa has ameliorative effect on alcohol induced ovarian injury in rabbits.

1.5 Aim

To investigate the ameliorative effect of natural cocoa on alcohol induced ovarian injury in rabbits.

1.6 Specific Objectives

The specific objectives are as follows:

I. To produce alcohol-induced ovary tissue injury in rabbit model.
II. To determine the weight and volume of ovaries in the experimental and control groups.
III. To describe histological structure of ovarian follicles and stroma in the experimental and control groups.
IV. To determine the volume density of follicles and ovarian stroma using stereology in the experimental and control groups.
V. To determine the levels of serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the experimental and control groups.
VI. To determine the serum concentration of superoxide dismutase (SOD) and glutathione reductase (GSH) as surrogate for oxidative stress in the experimental and control groups.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Anatomy and Physiology of the Ovary

The ovary is the female gonad whose primary function is housing and releasing ova, which are necessary for reproduction. It is a distinctive organ that organizes menstruation, hormonal balance, bone metabolism and fertilization (Furuya, 2012). The human ovaries are paired oval structures, grayish in colour with an uneven surface and about the size of an almond. They are approximately 2 to 3 cm in length. The actual size of the ovary depends on a woman's age and hormonal status. They are about 3 to 5 cm in length during childbearing years and become much smaller and then become atrophic once menopause occurs (Katz, Gretchen, Lobo, & Gershenson, 2007; OpenStax-CNX, 2014). They are located in the pelvic cavity covered by modified peritoneum within the ovarian fossa, a space which has the external iliac vessels, obliterated umbilical artery, and the ureter as boundaries. Supported by an extension of the peritoneum; the mesovarium, they are connected to the broad ligament. An extension from the mesovarium is the suspensory ligament which carries the blood and lymph vessels (OpenStax-CNX, 2014). The ovaries are also attached to the uterus via the ovarian ligament. The ovarian surface is covered by cuboidal epithelium. This outer covering is superficial to the dense connective tissue covering known as tunica albuginea. Beneath the tunica albuginea is the cortex which is also made of tightly packed connective tissue. The cortex is composed of ovarian stroma which forms the bulk of the adult ovary. Furthermore the stroma is composed mainly of spindle-shaped fibroblasts arranged in whorls or storiform pattern that respond to hormonal stimulation differently from that of

other fibroblast in the body. Also present are luteinized stromal cells. These luteinized cells may also be found in the periphery of follicles and lutein cysts. They contain lipids, and look polygonal in shape. These active stromal cells are generally not distinguishable from non-reactive stromal cells shape-wise, but they may produce steroid converting enzymes (Furuya, 2012). Oocytes together with supporting cells which form the follicles develop within the outer layer of the ovarian stroma. Underneath the cortex is the inner ovarian medulla, the site of blood vessels, lymph vessels and nerves that supply and drain the ovary and is primarily made of loose stromal tissue (Chung, 2000; Gray, 1999; OpenStax-CNX, 2014). Blood supply to the ovary is from the ovarian artery and a branch of the uterine artery, which penetrate the ovarian hilus to reach the ovary in its distribution. Thus the ovarian hilus contains numerous blood vessels (Furuya, 2012).

The ovary is a multi-compartmental, constantly re-modeling organ with two main functions:

(i) Generation of a fertilizable oocyte with full capability for development.

(ii) reception and secretion of gonadotropins and steroid hormones essential for preparing the reproductive tract for fertilization and consequent establishment of pregnancy (Oktem & Oktay, 2008).

For the ovaries to be able to produce steroids and which affect ovulation, highly synchronized and intricate chain of events occur: folliculogenesis (Oktem & Oktay, 2008).

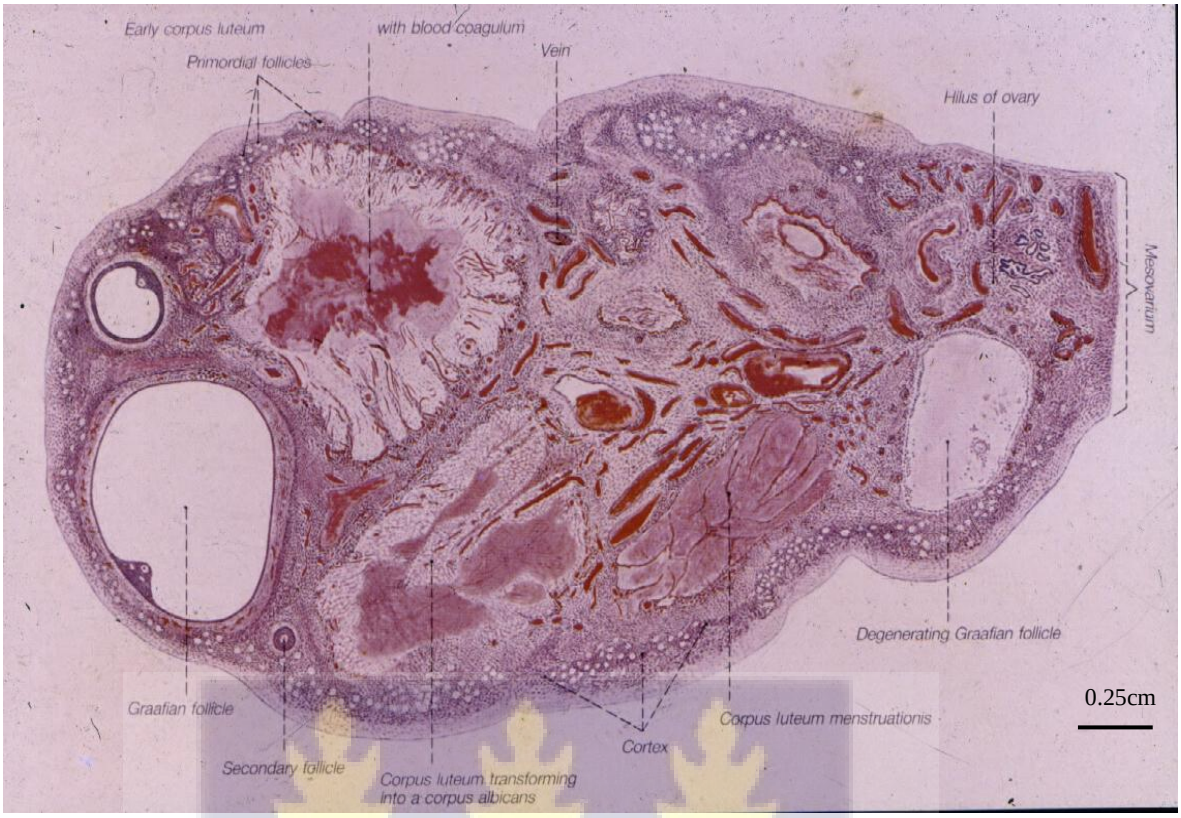

Figure 1: A picture showing a scanned slide of an ovary with labels. Source: (Sobotta & Hammersen, 1986).

2.1.1 Embryology of the Ovary

Development of the ovaries begins in the fifth fetal week (Healey, 2012). Initial gonad development in females and males is very similar, with germ cells migrating into undifferentiated gonads. In females with XX, the ovary then begins to develop and the subsequent structure and time course of germ cell then differs between males and females (Hill, 2017; Oktem & Oktay, 2008).

Since females do not have the Y chromosome, they have no SRY gene, only in cases when there is a translocation of the gene onto the X chromosome.

Two sections can be distinguished in the histology of the ovary:

- Cortex which contains all the elements of the parenchyma
- Medulla which shares stromal elements with the cortex (Celio *et al*., 2008).

The ovaries develop from primitive germ cells, the mesothelium of the posterior abdominal wall and next to the mesenchyme. Development begins in the fifth week of fetal life. The mesothelium medial to the mesonephros of the developing kidneys thickens, yielding the paired urogenital ridges (Healey, 2012). In studies using mice, many important genes that regulate early development and maintenance of the gonads in both sexes have been identified. Wnt4 and DAX1 are genes necessary for initiation of the pathway for ovary development. For instance, Wnt4 functions partially as an anti-testis gene by suppressing some developmental stages of differentiation in the course of the developing testes, since the gonadal ridges remain alike in both male and female fetuses until the seventh week (Healey, 2012; Hill, 2017).

In developing ovary, the bulk of the gonadal cords remain in close contact with the surface coelomic epithelium. The gonadal cords that are not in contact with the thickened coelomic epithelium lose contact and undergo atrophy (Celio *et al*., 2008).

Near the end of the embryonic period, the cortex with its gonadal cords and the medullary primordial germ cells in the ovary can be distinguished. During the 16th week the gonadal cords disband in the cortex. This results from sprouting blood vessels that are come from the medulla and isolated cell accumulations. These isolated cells surround the oogonia that are increasingly dividing mitotically (Celio *et al*., 2008). Germ cells migrate, undergo successive mitotic divisions, and colonize the prospective gonads, once specified (Richardson & Lehmann, 2010). Germ cells speedily increase from 600,000 at 8 weeks, to several millions of oogonia at 20 weeks of gestation (Healey, 2012; Oktem & Oktay, 2008). The singular cells are attached to each other by means of cellular bridges. As of this stage onwards, mitosis, meiosis, and atresia occur concurrently (Hill, 2017; Oktem & Oktay, 2008). The various zones in the cortex can now distinguished. The outermost layer where proliferating oogonia are found and the inner layer where oocytes that have naturally gone into the prophase of meiosis I. At this point, that the process is halted. All these occur prior to birth (Celio *et al*., 2008). After 20 weeks of fetal life, the rate of mitotical division of oogonia gradually drops and halts at about 28 weeks with nearly similar rate of increase in atresia, which heightens at 20 weeks of development (Oktem & Oktay, 2008).

Simultaneously, a third region which is towards the medulla in which the oocytes have already completed the prophase of meiosis I becomes visible. Oocytes are surrounded by a single layer of cells that have differentiated out of the gonadal cord cells. These cells

are known as follicle or granulosa cells. The primary oocytes, enveloped by follicle cells are now designated primordial follicles and stay in dictyotene stage of meiosis I (Celio *et al*., 2008).

The mitotically active oogonia, diploid (2n) germ cells represent the pool from which meiotic oocytes develop and differentiate. Mitotic activity of the oogonia is a key contributing factor of the size of the oocyte pool. The development of oocytes by meiosis from mitotic oogonia is termed oogenesis (Oktem & Oktay, 2008).

2.1.2 Oogenesis

Gametogenesis in females is called oogenesis. It is the process through which meiotic oocytes develop from mitotic oogonia (Oktem & Oktay, 2008). The process begins with the ovarian stem cells or oogonia with diploid chromosome number (2n). Oogonia are formed during fetal development and divide mitotically and form primary oocytes (2n) in the fetal ovary prior to birth. These primary oocytes begin a meiotic division but are then arrested at the dictyotene stage of prophase I of meiosis I. Shortly before birth, this meiotic resting phase then begins and lasts till puberty, during which each month and in the subsequent months until menopause, a pair of primary oocytes (2n) complete meiosis I. Just prior to ovulation, a surge of LH triggers the resumption to completion of meiosis I in a primary oocyte (Celio *et al*., 2008; OpenStax-CNX, 2014). This begins the transition from primary to secondary oocyte with a haploid chromosome number (n). However, this cell division does not result in two identical cells. The cytoplasm is divided unequally with one daughter cell much larger than the other. The daughter cell with relatively less cytoplasm is known as the first polar body with also haploid chromosome number (n) (Williams & Erickson, 2012). The secondary oocyte begins meiosis II. The secondary

oocyte together with the first polar body is ovulated. Once, the secondary oocyte is fertilized, meiosis II resumes. The oocyte splits into the ovum and the second polar body. The nuclei of the sperm cell and the ovum unite to form a diploid (2n) zygote (Celio *et al*., 2008).

Imperatively, folliculogenesis must occur somewhat simultaneously for successful ovulation to take place. In humans, a primordial follicle takes about 150 days to develop into a pre-antral or primary follicle and another 120 days to form an antral or secondary follicle. A number of antral follicles will then "vie" for 14 to 15 days to become the dominant follicle, which will undergo ovulation (Hill, 2017).

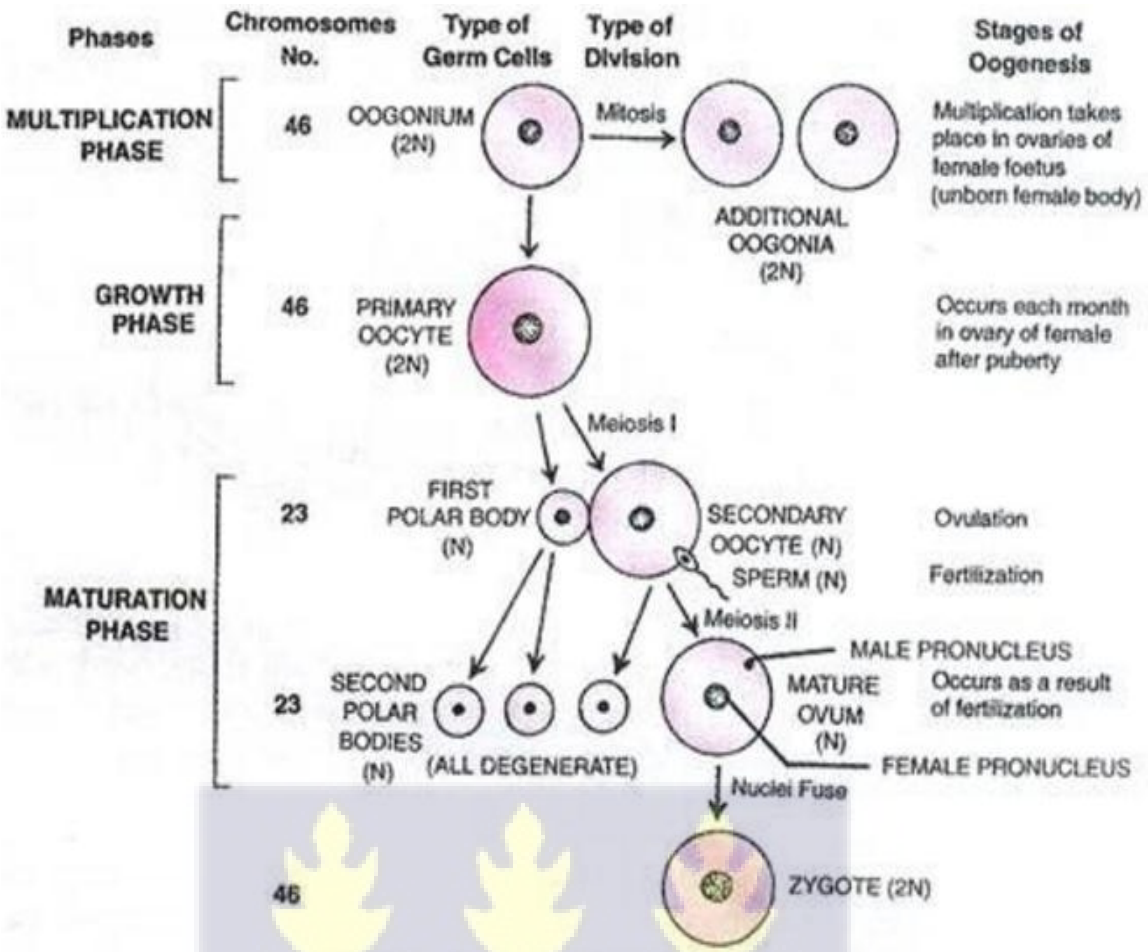

Figure 2: A flow diagram showing the process of oogenesis in the human ovary.

Source: (Mondal, 2016)

2.1.3 Folliculogenesis

Ovarian follicles are oocytes with their supporting cells. A follicle is made up of an oocyte surrounded by one or more layers of somatic cells referred to as granulosa cells. They are considered the functional units of the ovary. The oocyte and surrounding granulosa cells are distinguished from the stromal tissue by a membrane called the basal lamina (Oktem & Oktay, 2008). The process of follicular development is known as folliculogenesis. This process takes place within the cortex of the ovary. Folliculogenesis is also considered as a process of achieving sequentially advanced levels of organization by means of cell proliferation and cyto-differentiation. It comprises certain major developmental events; primordial follicle recruitment, pre-antral follicle development, selection and growth of the antral follicle, follicle atresia, steroid production, expression of gonadotropin receptor, maturation of oocyte, ovulation, luteinization, and corpora lutea formation (Oktem & Oktay, 2008; Williams & Erickson, 2012).

This multi-stage process leads to ovulation of one follicle approximately every 28 days. Mature follicles that do not make it to ovulation go through cell death. The death of ovarian follicles is known as atresia and this can occur at any point during follicular development (OpenStax-CNX, 2014).

Follicles progress from primordial, to primary, to secondary and tertiary stages prior to ovulation with the enclosed oocyte remaining as a primary oocyte until right before ovulation. Folliculogenesis begins with the follicles in a resting state. The admission of an arrested primordial follicle into the pool of growing follicles is termed primordial follicle activation or recruitment (OpenStax-CNX, 2014; Williams & Erickson, 2012). The activation of dormant primordial follicles into a maturing pool of primary follicles begins in fetal life and continues till the ovarian reserve is depleted, post-natally (Oktem

& Oktay, 2008). Primordial follicles are the abundant type of follicles in the adult ovary. They have only a flat single layer of follicular cells that surround the oocyte and can remain in this state for years; some until right before menopause (OpenStax-CNX, 2014). The activation for the recruitment of growth-arrested primordial follicles into their developing state as primary follicles needs to be expounded. This evolution gives the impression to be independent of gonadotropins due to the absence of mRNA receptor expression for FSH in the primordial follicles (Oktem & Oktay, 2008). After puberty, a few primordial follicles will respond to a recruitment signal and will join a pool of immature growing follicles called primary follicles. Primary follicles start with a single layer of follicular cells, but they become active and transition from squamous to cuboidal shape as they increase in size and proliferate and are now called granulosa cells. FSH receptors are thought to be initially expressed on granulosa cells of primary follicles. High levels of plasma FSH accelerate primary follicle development (Williams & Erickson, 2012). This is characterized by an increase in oocyte diameter during the primordial-to-primary transition (Oktem & Oktay, 2008; OpenStax-CNX, 2014). As granulosa cells divide, the follicles are now called secondary follicles. This stage of follicular development involves enlargement of oocyte i.e. an increase in diameter, multiplying of granulosa cells to form a multilayered structure of connective tissue. Also there is the formation of the basal lamina, blood vessels and the theca interna cell layer (Oktem & Oktay, 2008). Within the growing secondary follicle, the primary oocyte now secretes a thin cellular membrane called the zona pellucida. A thick fluid, known as follicular fluid formed between granulosa cells begin to collect into one large pool or antrum (OpenStax-CNX, 2014). Follicles with large and fully formed antrum are

considered tertiary or antral or Graafian follicles. Most follicles at the tertiary stage will undergo atresia. The one that does not die will expel its secondary oocyte surrounded by several layers of granulosa cells from the ovary (OpenStax-CNX, 2014).

In mammals, 99.9 % of the follicles become atretic. A fundamental property worthy of note in atresia is the stimulation of apoptosis in the oocyte and granulosa cells. Apoptosis is an intricate process involving signaling pathways tied to programmed cell death (Williams & Erickson, 2012). Atresia in follicles is moderated by a balance between pro-survival factors that encourage cell proliferation, follicle growth and differentiation and pro-apoptotic factors that promote cell death. Unlike the massive loss of oocytes during fetal development that occurs via apoptosis within the oocyte, in adults, follicle atresia and oocyte loss appear to be instigated by apoptosis in the granulosa cells (Hussein, 2005).

The concept of FSH as a survival factor for antral follicles is due to the role of FSH in supporting follicle growth after antrum formation and the prevention of apoptosis (Craig *et al*., 2007).

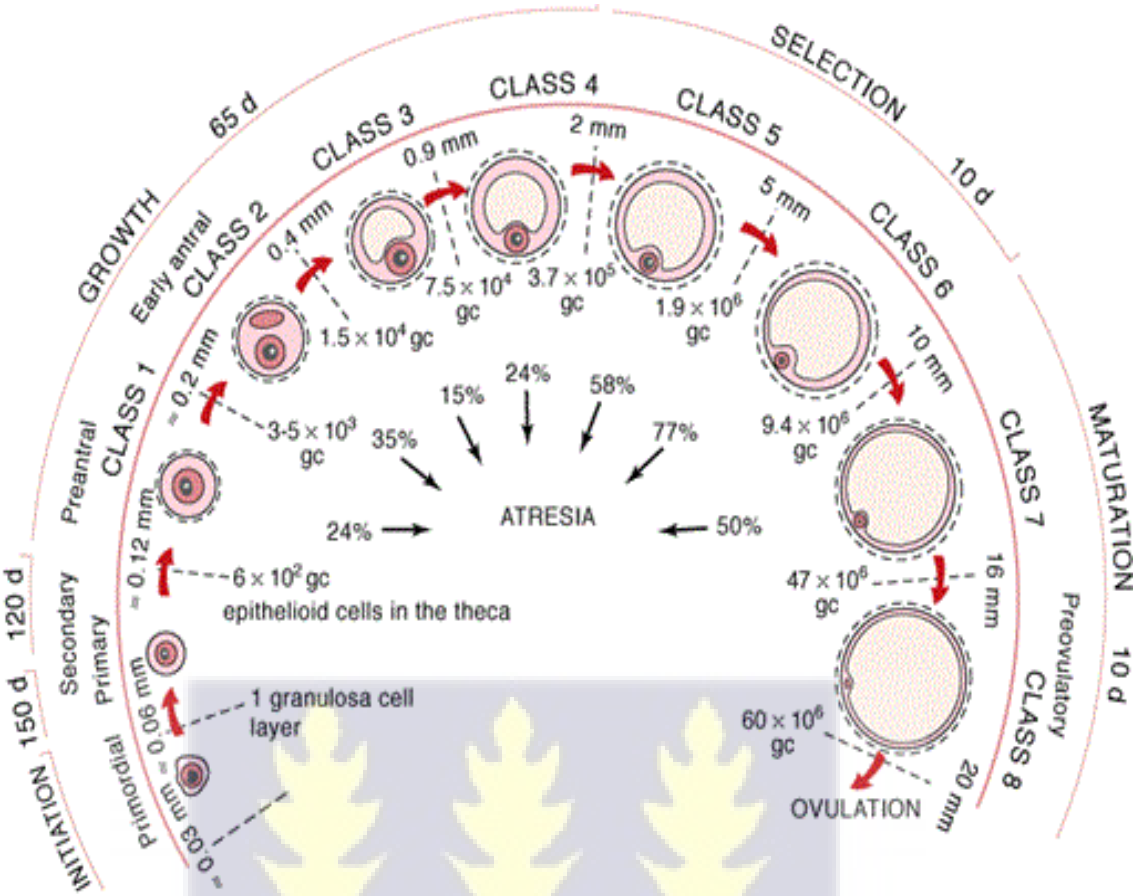

Figure 3: A flow diagram showing the chronology of folliculogenesis in the human ovary. Source: (Gougeon, 1996).

2.1.4 Hormonal control of the ovarian cycle

It is clear that the ovaries are not self-maintaining structures. Certain hormones of the anterior pituitary are essential to their maintenance and in turn the secretions of the ovaries influence the secretion of the gonadotropic hormones and probably other hormones of the hypophysis (Smith, 1940). It takes nearly two months for a primary oocyte to go from primordial follicle to pre-antral follicle. The final stages of development of a small group of tertiary follicles becoming a secondary oocyte ready to be released occur over a period of 28 days. These changes are regulated by gonadotropin releasing hormone (GnRH), LH and FSH (OpenStax-CNX, 2014). GnRH is produced by the hypothalamus; this hormone signals the anterior pituitary gland to produce the gonadotropins namely LH and FSH. This is because they stimulate the gonads. They are secreted from the cells known as gonadotrophs in the anterior pituitary gland. These gonadotropins leave the pituitary and travel through the bloodstream to the ovaries, where they bind to receptors on the granulosa and theca cells of the follicles. LH stimulates the granulosa and theca cells to secrete sex steroid hormone estradiol. Ovulation of mature follicles is induced by an upsurge of LH secretion which is known as the pre-ovulatory LH surge. It has been established that small amounts of LH are necessary to support follicular development. The action of LH on follicular development is not limited to providing androgen substrate for aromatization but also exerts a direct effect on the stimulation and modulation of folliculogenesis (Viudes-de-Castro, Pomares, Ribes, Marco-jiménez, & Vicente, 2015). Residual cells in ovulated follicles proliferate to form corpora lutea, which secrete the steroid hormones progesterone and estradiol. Progesterone is necessary for maintenance of pregnancy, and, in most mammals, LH is

required for continued development and function of corpora lutea. LH is derived from the effect of inducing luteinization of ovarian follicles. FSH stimulates the maturation of ovarian follicles (Bowen, 2004; OpenStax-CNX, 2014). After the release of estrogen and progesterone by the ovaries in response to LH, in a negative feedback loop, the sex steroids inhibit the secretion of GnRH and also affect the gonadotrophs negatively. This feedback loop leads to pulsatile secretion of LH and to a lesser extent, FSH (Bowen, 2004).

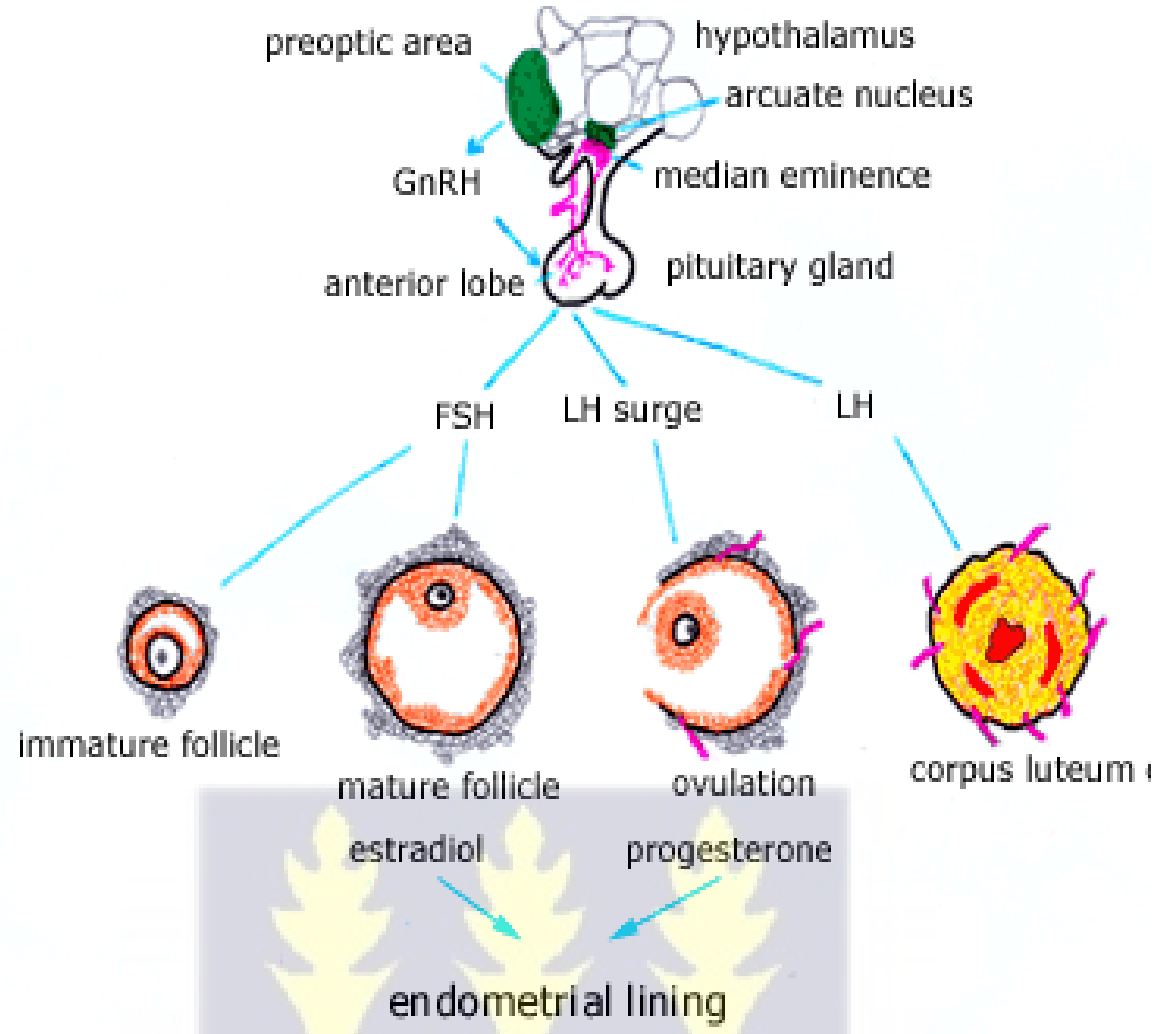

Figure 4: A diagram showing control of LH and FSH on the ovaries. Source: (Daiter, 2007)

2.2 Reproductive system of the rabbit

2.2.1 Taxonomy

Rabbits are considered small mammals and classified under the order Lagomorpha and family Leporidae. Eight different genera are classified as rabbits. These include Brachylagus, Bunolagus, Nesolagus, Oryctolagus, Pentalagus, Poelagus, Romerolagus, and Sylvilagus. New Zealand rabbit, commonly known as a European rabbit is known scientifically as *Oryctolagus cuniculus*. This species is the only tamed rabbit, and thus the only species from which unique breeds have been derived (Nowland, Brammer, Garcia, & Rush, 2015).

2.2.2 Sexual Maturity

The age of puberty varies with the breed of rabbit. Puberty generally occurs at 4–5 months of age in small breeds, 4–6 months in medium breeds, and 5–8 months in large breeds. Female New Zealand rabbits reach maturity at 5 months of age and males at 6–7 months. The breeding life of a doe lasts about 1–3 years, even though some remain fecund for up to 5 or 6 years. In later years, litter sizes usually reduce (Donnelly, 2004; Nowland *et al*., 2015).

2.2.3 Anatomy, Physiology and Estrus cycle of the Reproductive System of the Female Rabbit

The reproductive organ of the doe is considered as primitive. The split two-horned system is only observed in monotreme egg lying mammals and in lagomorphs (pika, hare and rabbit). The bicornuate organ is held in place by a broad ligament that is anchored at four points under the vertebral column (Praag, 2016). The ovaries are oval shaped and are about 1 to 1.5 cm in length. The oviduct is found beneath the ovaries and it consists of the

duct, the ampulla and the isthmus. Although outwardly the uterine horns are joined posteriorly into a single organ, there are in fact two separate uteri of about 7 cm, which open separately via two cervical ducts into the 6 to 10 cm vaginal tract. Bartholin's gland and the preputial glands can also be identified midway along the vagina (Lebas, Coudert, De Rochambeau, Thebault, & Rouvier, 1986).

Sexual differentiation occurs on the 16th following fertilization. Oogonial division starts on the 21st day of fetal life and continues till birth. The initial follicles appear on the 13th day after birth and the first antrum follicles, between the 65th to 70th days (Lebas *et al*., 1986). Follicular development occurs in waves, with 5 to 10 follicles on each ovary at a time. When the follicles reach maturity, they produce estrogen for about 12 to 14 days; if ovulation has not occurred during this period, the follicles degenerate with a corresponding reduction in estrogen level and sexual receptivity. Follicles not having evolved to the ovulatory stage because of lack of stimulation go into regression and are replaced by new follicles. These remain in pre-ovulatory state for a few days and may then regress (Lebas *et al*., 1986). After 4 to 7 days, a new wave of follicles develops and the doe becomes receptive again. This means the doe has a cycle of 16 to 18 days with about 12 to 14 days of receptivity followed by a period of non-receptivity for 2 to 4 days (Harcourt-Brown, 2017).

Does are induced or 'reflex' ovulators with no defined estrous cycle, although a cyclic rhythm in sexual receptivity exists (Harcourt-Brown, 2017). Receptivity is designated by periods (1–2 days every 4–17 days) of anestrus and recurrent disparities in reproductive performance (Hafez, 1970). During periods of receptivity, the vulva becomes swollen, moist, and dark pink. Does also assume lordotic posture in reaction to the bucks' attempts

to mount (Donnelly, 2004). When a doe accepts service she is considered to be in estrus, when she refuses she is in dioestrus (Lebas *et al*., 1986). Ovulation is induced and occurs approximately 10–13 hours after copulation. Ovulation can also be induced by administration of LH, human chorionic gonadotropin, or gonadotropic releasing hormone (Nowland *et al*., 2015).

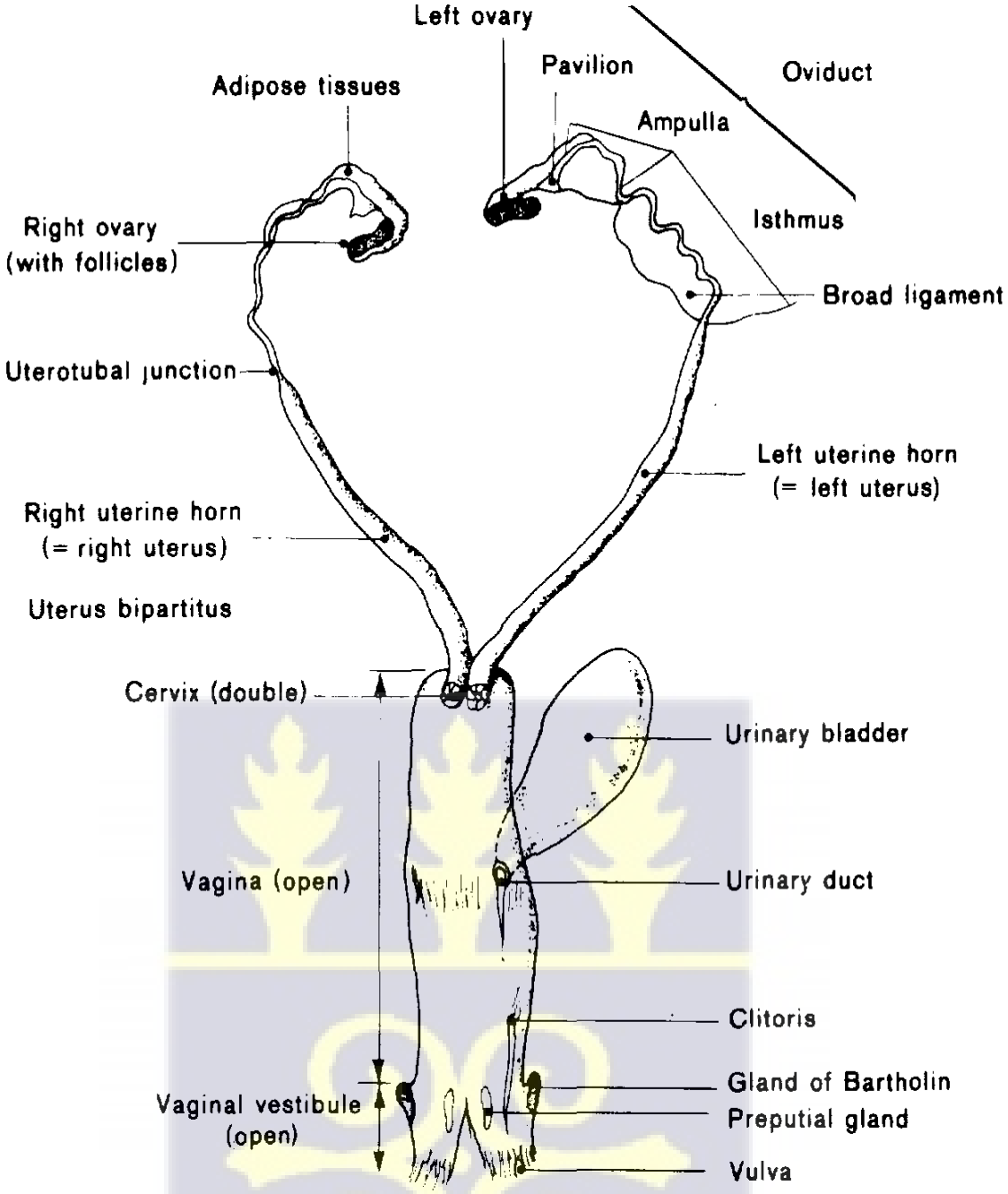

Figure 5 A picture showing the reproductive system of female rabbit. Source: ("Rabbit Reproduction," 2017)

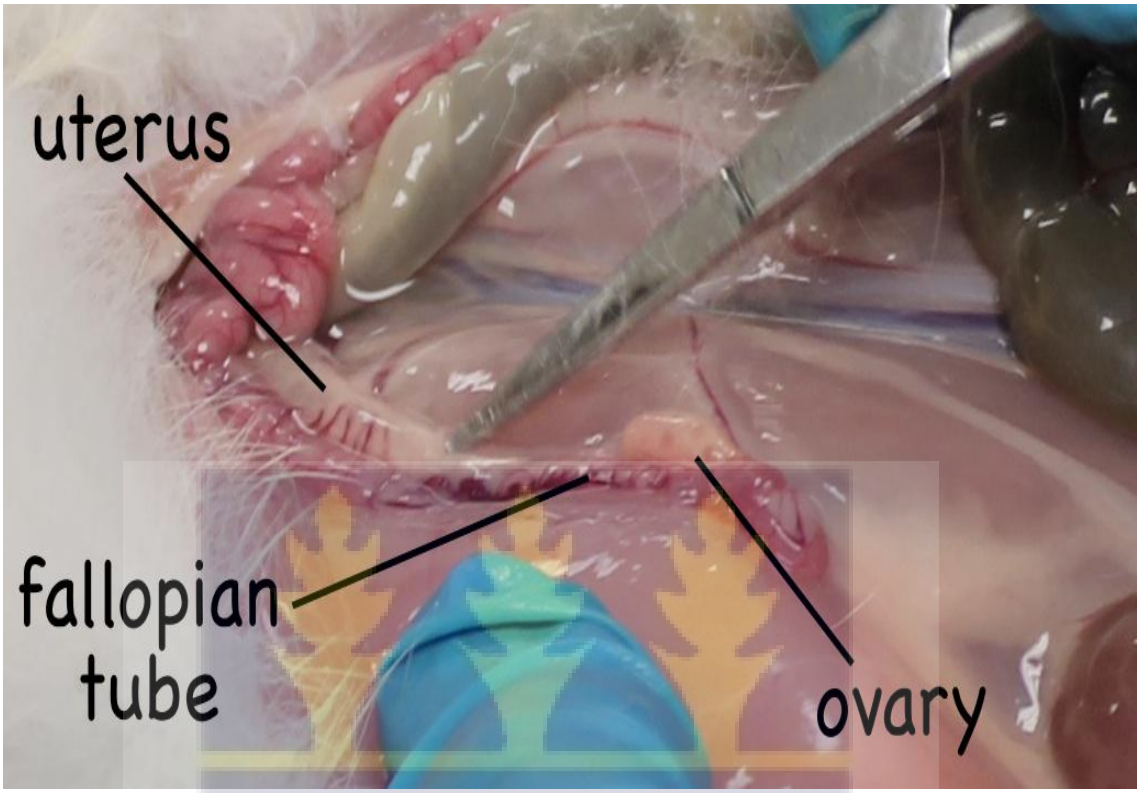

Figure 6: A picture showing rabbit ovary in situ. Source: (Urbano, 2013).

2.3 Alcohol Metabolism

Alcohol is a commonly abused psychoactive drug affecting diverse cellular and molecular processes in the liver and other organs of the body with no exception (Reddyvari *et al*., 2017). According to WHO in 2014, alcohol is the third leading cause of global deaths accounting for 6% of total deaths. Detrimental uses of alcohol is a significant cause of mortality and morbidity associated with a number of diseases with several pathologies including, malnutrition, gastritis, chronic pancreatitis, cardiomyopathy, alcoholic liver disease and cancers of all organs leading to death (Reddyvari *et al*., 2017). Up regulated OS due to the excessive liberation of ROS in alcohol metabolism affects the antioxidant defense system leading to organ and tissue injury and various disease conditions including cancer (Pyun *et al*., 2015). Mitochondria are highly dynamic and energy transducing cell organelles playing a key role in cellular ATP generation via oxidative phosphorylation (Yin & Cadenas, 2015). In addition, mitochondria involved in antioxidant defense system, fat oxidation, intermediary metabolic processes which includes alcohol metabolism and bioenergetics of the hepatocytes (Reddyvari *et al*., 2017).

The effects of alcohol on various tissues depend on its concentration in the blood (blood alcohol concentration [BAC]) over time. BAC is determined by how quickly alcohol is absorbed, distributed, metabolized, and excreted (Zakhari, 2017). Alcohol is primarily metabolized by the liver through ADH, cytochrome P450 2E1, and a catalase system (Amanvermez, Demir, Tuncel, Alvur, & Agar, 2005). Acetaldehyde, nicotinamide adenine dinucleotide (NADH), free radicals, and lipid and protein oxidation levels and the products of reactivity are increased during alcohol metabolism (Amanvermez *et al*., 2005).

Absorption of alcohol largely occurs the small intestine and then transported into the portal vein which leads to the liver and the veins that drain the stomach and intestines. It is then transported to the liver, where it is metabolized by being exposed to enzymes. The rate at which BAC rises is influenced by how rapidly alcohol is emptied from the stomach and the extent of metabolism during this first pass through the stomach and liver (Zakhari, 2017).

The kidneys are not left out in the process. Alcohol is also known to possess a diuretic effect. This is because of the direct action it has on the kidneys. Alcohol's effect on the renal epithelium is such that it allows water to pass but holds back potassium in large amounts while sodium, chloride and nitrogen are held back in smaller amounts. This also inhibits the reabsorption of water. Again, it is believed that alcohol can decrease the secretion of the anti-diuretic hormone or inactivate the circulation of it entirely (Strauss, Rosenbaum & Nelson, 1950).

Metabolism of alcohol occurs by several processes mainly in the liver. It can be metabolized by two distinct pathways: oxidative and non-oxidative alcohol metabolism, leading to the production of acetaldehyde, acetate, ROS, and fatty acid ethyl esters, to mention a few (Ghazali & Patel, 2016). ADH converts alcohol to acetaldehyde, a toxic chemical that causes DNA damage. This is further metabolized into acetic acid, a nontoxic metabolite in the body by ALDH2 (Hong, 2016). Acetic acid also removes other toxic aldehydes that can accumulate in the body. Acetic acid is further metabolized into carbon dioxide and water (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2010). Alcohol also is metabolized in non-liver (i.e. extra-hepatic) tissues that do not contain ADH, such as the brain, by the enzymes cytochrome P450 2E1 and

catalase. Overall, alcohol metabolism is realized by both oxidative pathways, which either add oxygen or remove hydrogen and non-oxidative pathways (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2010). These processes make it possible for alcohol to be eliminated from the human body.

2.3.1 Alcohol and the Female Reproductive System

Alcohol markedly disrupts normal menstrual cycling in female humans (Emanuele et al., 2003). Alcoholic women are known to have a variety of menstrual and reproductive disorders, from irregular menstrual cycles to complete cessation of menses, absence of ovulation (i.e., anovulation), and consequently, infertility. Alcohol abuse has also been associated with early menopause (Mello, Mendelson, & Teoh, 1993). Consuming alcohol at the wrong time, even in amounts inadequate to cause permanent tissue damage, as in the case of social drinkers, can disrupt the delicate balance critical to maintaining human female reproductive hormonal cycles and result in some degree of infertility (Emanuele et al., 2003; Gude, 2012). A study conducted among healthy nonalcoholic women reported that a considerable portion that were social drinkers ceased cycling normally and became at least temporarily infertile (Emanuele *et al*., 2003).

Acute and chronic alcohol exposure can alter the reproductive systems of rats and monkeys which are similar to that of humans. For instance, acute alcohol exposure in female rats has been found to upset cycling (Lapaglia, Steiner, & Kirsteins, 1997). Acute alcohol exposure given as a bolus to mimic binge drinking has also been shown to disturb the normal cycle at the time, but return to normal by the following cycle (Alfonso, Duran, & Marco, 1993).

2.3.2 The Effect of Alcohol on the Hypothalamic-Pituitary-Gonadal (HPG) Axis

The HPG and the hormones produced are necessary for the optimum functioning of the reproductive system. In people who consume alcohol, HPG dysfunction was shown to be associated with a decrease in infertility and gonadal atrophy. Chronic consumption of alcohol disrupts the communication between endocrine system and causes hormonal disturbances that lead to profound and serious consequences at behavioral and physiological levels (Rachdaoui & Sarkar, 2014). It has been established that alcohol has injurious effects on all three components of the HPG axis and also dampens the efficiency of the axis, modifying some reproduction factors such as hormone secretion, estrous cycle, ovulation, follicular maturation and fetal evolution (Alfonso *et al*., 1993; Rachdaoui & Sarkar, 2014). Additionally, it has been reported that alcohol exposure produces changes in neurotransmitter metabolism and there is clinical and experimental evidence indicating that alcohol can perturb pituitary or sex hormone secretion in humans and laboratory animals (Alfonso *et al*., 1993). Alcohol use in premenopausal women, in moderate amounts, has been related to a host of reproductive disorders such as irregular menstrual cycles, anovulation and early menopause (Rachdaoui & Sarkar, 2014). The human reproductive system is regulated by many hormones and the most important are androgens and estrogens. They are synthesized mainly by the testes and the ovaries and affect reproductive functions in various target tissues. Some other reproductive hormones are synthesized in the hypothalamus and pituitary. Although men and women produce many of the same hormones, their relative concentrations and their functions vary (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 1994). In women, hormones promote the development of secondary sexual characteristics, such as breast

development and distribution of body hair, regulate the menstrual cycle and are necessary to maintain pregnancy. Chronic heavy drinking can interfere with all these functions (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 1994). Moderate alcohol consumption for a long time has been shown to reduce ovarian reserve which is accompanied with increased FSH levels (Rachdaoui & Sarkar, 2014). In studies using female rats, Bo, Krueger and Rudeen (1982) reported that puberty, measured by vaginal opening, was markedly delayed in pre-pubertal female rats given alcohol. Furthermore, they stated that alcohol increased hypothalamic growth hormone releasing hormone (GRH) content which was associated with a decrease in circulating growth hormone (GH). In addition, alcohol decreased hypothalamic secretion of luteinizing hormone releasing hormone (LHRH). At the anterior pituitary, LHRH binds to specific receptors on gonadotroph cells and stimulates a cascade of events that lead to production and secretion of LH and FSH into the general circulation (Rachdaoui & Sarkar, 2014). In other works using female rats, they showed that alcohol administration blocked the pro-estrous surge of LH and ovulation (Ogilvie & Rivier, 1997; Rachdaoui & Sarkar, 2014). Alcohol-induced disturbances in HPG axis activity, during puberty, pre-menopause and post-menopause, could have far reaching consequences on growth which might persist through adult life, reproductive function and keeping balanced hormone levels respectively (Rachdaoui & Sarkar, 2014).

2.3.3 The Effect of Alcohol on the Ovary

Chronic and acute consumption of alcohol has been reported to cause fertility disturbances (Dosumu, Osinubi, & Duru, 2014). A considerable body of evidence establishes that alcohol is a metabolic poison not only for the traditional target organs, the liver and pancreas, but also for the heart, brain, kidney, ovary and other tissues (Van-Thiel, Gavaler, Lester, & Sherins, 1978). The major factor resulting in alcohol related tissue injury, chiefly through the production of ROS and OS in the tissues is the metabolism of alcohol (Zakhari, 2017). The production of ROS secondary to alcohol consumption causes harm to a variety of tissues not only by causing OS but also by encouraging apoptosis triggered by various stimuli (Bradford, Kono, & Isayama, 2005). The manifestation of several indicators of OS has been established in normal cycling ovaries (Sekhon, Gupta, Kim, & Agarwal, 2010). *In-situ* metabolism of alcohol to acetaldehyde increases the susceptibility of ovarian tissue of rats to OS and leads to cell destruction and ovarian dysfunction (Rachdaoui & Sarkar, 2014). It is suggested that the damaging action of alcohol on ovaries is partly facilitated through an elevated ovarian nitric oxide synthase and suppressed steroidogenic acute regulatory protein, two important intermediates in the production of steroid hormones (Srivastava, Dissen, & Ojeda, 2007).

2.3.4 Oxidative Stress

Aerobic metabolism comes with the production of pro-oxidant molecules called ROS and free radicals. These include the hydroxyl radicals (OH^-), nitric oxide (NO), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). They are characterized by having oxygen centers (Gupta, Sekhon, Aziz, & Agarwal, 2008; Krajcir, Chowdary, Gupta, & Agarwal, 2008). These radicals are usually very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. This starts a cascade of reactions of more free radicals which lead to a torrent of frenzied reactions (Krajcir, Chowdary, Gupta, & Agarwal, 2008). Each time the balance between pro-oxidants and antioxidants is affected, a state of OS is initiated. OS comes about when there is overabundance of ROS generation and antioxidants are not effective to neutralize the excessive loads of ROS (Gupta *et al*., 2008). ROS role is either oxidative or reducing in response to the changing molecular environments (Krajcir *et al*., 2008). According to Krajcir *et al* (2008), they also act differently when they occur in different tissue concentrations. For instance, ROS in tissues at optimum levels designates their function as normal cell signal responders. It is apparent that they act as triggers of DNA damage and turns up cell apoptosis, when in high concentrations. Reactive oxygen species are unstable and aggressive molecules which are incapable of diffusing across biological membranes. This by reason of their polarity when compared to other oxidants such as molecular oxygen (Krajcir *et al*., 2008).

OH^-, H_2O_2, and $O2^-$ are some ROS that are normally produced in the female reproductive tract (Krajcir *et al*., 2008).

The expression of numerous OS markers has been proven in normally cycling ovaries

(Suzuki, Sugino, & Fukaya, 1999; Tamate, Sengoku, & Ishikawa, 1995). Leukocytes, macrophages, and cytokines, are known sources of ROS and are found in the follicular fluid micro-environment. Within the follicular fluid environment, ROS play a role in moderating the maturation of oocytes, folliculogenesis, ovarian steroidogenesis, and luteolysis (Fujii, Iuchi, & Okada, 2005). Nitric oxide radical has been implicated in folliculogenesis and atresia (Sekhon *et al*., 2010). Optimal OS levels are essential for ovulation to occur. The rise and fall in levels of cytokines, prostaglandins, proteolytic enzymes, nitric oxide, and steroids that accompany the final stages of oocyte maturation are associated with increased the level of ROS, which affects blood flow to the ovaries and eventually facilitates rupturing of the follicle (Du *et al*., 2006). A degree of oxidative enzyme activity is exhibited by thecal cells, granulosa lutein cells, and hilus cells, illustrating the role of OS in ovarian steroidogenesis (Fujii *et al*., 2005). Reactive oxygen species is moderated and kept at optimal physiological levels within the ovary by various antioxidant systems. Some of them include catalase, vitamin E and glutathione (Attaran, Pasqualotto, & Falcone, 2000). Superoxide dismutase which is an enzymatic antioxidant that facilitates the decomposition of O_2- into H_2O_2 and oxygen has been described in the theca interna cells in the antral follicles. These cells may protect the oocyte from excess ROS during maturation of the follicle (Sugino *et al*., 2000). The overall scavenging ability of antioxidants within the follicular fluid micro-environment may reduce with reproductive aging. Carbone *et al* (2003) confirmed the decrease in concentrations of catalase and SOD localized in follicular fluid in older women. Their oocytes exhibited low fertilization rates and decreased blastocyst development in contrast with oocytes from younger women. The redox status of the follicle, for that reason is closely

associated to oocyte quality and fertilization capacity (Sekhon *et al*., 2010). Oxidative stress is associated in the etiopathologenesis of various causes of natural infertility (Krajcir *et al*., 2008).

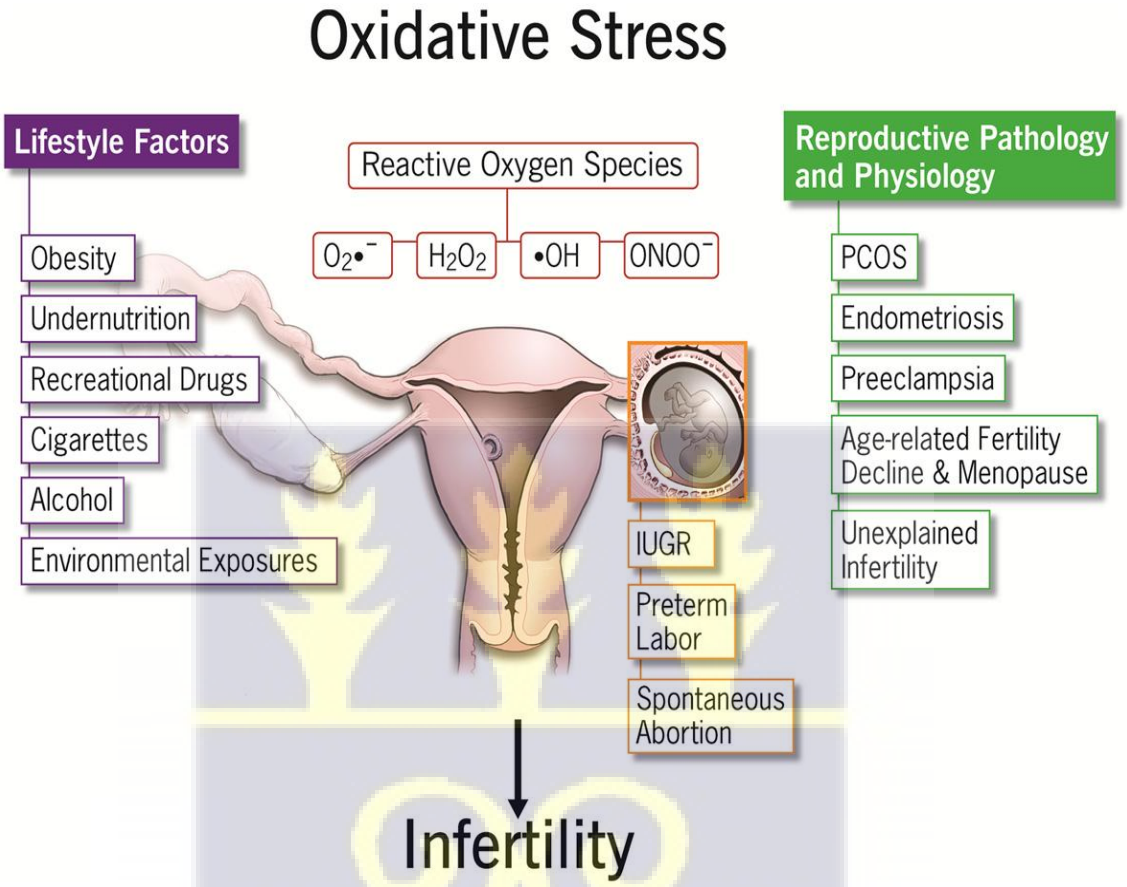

Figure 7: A picture illustrating the effects of OS on the female reproductive system. Source: (Agarwal *et al*., 2012).

2.3.5 Alcohol induced ovarian injury

Every so often, stroma in the adult ovary exhibits dynamic tissue remodeling during and after ovulation. Due to periodic ovulation of secondary oocytes by the ovary which is a form of mechanical stress, the surface of the ovary is lined by a single layer of epithelial cells (Furuya, 2012). Ovarian damage results in diminished fertility potential. Premature ovarian failure is a syndrome characterized by lack of folliculogenesis and ovarian estrogen production, associated with amenorrhea and infertility in women under the age of 40 years (Abd-allah *et al*., 2013). Chronic ingestion of high-dose alcohol is characterized by a number of biochemical and pathophysiologic changes in cells and in various organs including the ovaries (Amanvermez *et al*., 2005). It is probable that ovarian cells are exposed in the like manner as other cells to the devastating effects of oxidant agents (Amanvermez *et al*., 2005). OS reduces ovarian function due to oxidized protein and lipids as follicular fluid is very sensitive to slight changes in the ROS-antioxidant system in alcohol consuming subjects (Amanvermez *et al*., 2005). OS may lead to poor quality of oocytes, because it produces severe cell damage, including deterioration of membrane lipids, apoptosis, and inhibition of fertilization (Agarwal, Gupta, & Sharma, 2005; Chuffa *et al*., 2011). According to works done by Jensen *et al* (1998) it was understood that, alcohol given to experimental female rats and monkeys reduces ovarian weight and causes amenorrhea. Women noted for frequent alcohol consumption have also been found to have higher rates of menstrual disorders, including amenorrhea, dysmenorrhea, and irregular menstrual periods (Jensen, Hjollund, & Henriksen, 1998).

2.3.6 Alcohol and ROS/OS

Alcohol readily diffuses across membranes and distributes through all cells and tissues, and at these concentrations, it can acutely affect cell function by interacting with certain proteins and cell membranes (Zakhari, 2017). Alcohol is known to enhance ROS production and interferes with the body's antioxidant defense mechanism, particularly in the liver. Alcohol metabolism results in the generation of acetaldehyde, a highly reactive and toxic byproduct that may contribute to tissue damage, the formation of damaging molecules known as ROS, and a change in the reduction–oxidation state of liver cells (Zakhari, 2017). Reactive oxygen species "steal" hydrogen atoms from other molecules and then convert them into unstable and highly reactive free radicals. Again, ROS can combine with stable molecules to form free radicals. This results in molecular damage to proteins, lipids, and DNA (Agarwal, Virk, Ong, & Plessis, 2014; Zakhari, 2017). Chronic consumption of alcohol and its metabolism are strongly associated with tissue damage. Alcohol also is metabolized in non-liver (i.e. extra-hepatic) tissues that do not contain ADH, such as the brain, by the enzymes cytochrome P450 2E1 and catalase (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2010). The cytochrome P450 2E1, which is present largely in the vesicles of a network of membranes within the endoplasmic reticulum of cells, also contributes to the oxidation of alcohol during metabolism.

Cytochrome P450 2E1 is induced by chronic alcohol consumption and assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. In addition, cytochrome P450 2E1 dependent ethanol oxidation may occur in other tissues, such as the brain, where ADH activity is low. It also responsible for the production of ROS which increase the risk of tissue damage (Zakhari, 2017).

Rats that chronically consume alcohol by showed increased H_2O_2 production in pericentral regions of the liver and increased activity of catalase (Handler & Thurman, 1990; Misra, Bradford, Handler, & Thurman, 1992; Zakhari, 2017).

ROS are naturally occurring as a result of reactions in the cell. Through these mechanisms, ROS play an important role in cancer development, atherosclerosis, diabetes, inflammation, aging, and other harmful processes. To prevent the damage ROS can cause, numerous defense systems have evolved in the body involving compounds called antioxidants, which can interact with ROS and convert them into harmless molecules. Under normal conditions, a balance between ROS and antioxidants exists in the cells (Zakhari, 2017).

To make better the injurious effects following ROS, scientists are studying the effects and mechanism of antioxidant administration. Earlier works have shown that replacement of glutathione by the administration of the glutathione precursor S-adenosyl-l-methionine or the use of other antioxidants mitigated tissue damage secondary to alcohol metabolism (Wu & Cederbaum, 2003).

2.4 Antioxidants

Antioxidants are molecules that constrain or extinguish free radical reactions and delay or inhibit cellular damage (Balasaheb & Pal, 2015). They act to oppose ROS production by neutralizing oxidants, scavenge existing free radicals, and promote the repair of ROS-induced damage to cell structures (Agarwal & Allamaneni, 2004). Antioxidants can be characterized in multiple ways. Based on their mode of activity; non-enzymatic and enzymatic, solubility in water and lipids and their sizes; small-molecule antioxidants and large-molecule antioxidants (Shahidi & Zhong, 2010). Non-enzymatic antioxidants work

by interrupting free radical chain reactions (Balasaheb & Pal, 2015). Some of these include vitamin C, vitamin E, selenium, zinc, beta carotene, carotene, taurine, hypotaurine, cysteamine, and glutathione. Enzymatic antioxidants work by breaking down and removing free radicals (Balasaheb & Pal, 2015). The enzymatic antioxidants convert dangerous oxidative products to H_2O_2 and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese, and iron. These include SOD, glutathione peroxidase, catalase, glutaredoxin and glutathione reductase (Agarwal, Gupta, Sekhon, & Shah, 2008; Balasaheb & Pal, 2015; Krajcir *et al*., 2008). The water-soluble antioxidants (e.g. vitamin C) are present in the cellular fluids such as cytosol, or cytoplasmic matrix. The lipid-soluble antioxidants (e.g. vitamin E, carotenoids, and lipoic acid) are predominantly located in cell membranes (Balasaheb & Pal, 2015). The small-molecule antioxidants neutralize the ROS in a process called radical scavenging and carry them away. The main antioxidants in this category are vitamin C, vitamin E, carotenoids, and GSH. The large-molecule antioxidants are enzymes SOD, catalase, and glutathione peroxidase (GSHPx) and sacrificial proteins (e.g. albumin) that absorb ROS and prevent them from attacking other essential proteins (Balasaheb & Pal, 2015).

Antioxidants that protect cells against the damaging effects of alcohol include mainly GSH, vitamins C and E, and thiol-containing compounds such as cysteine, methionine, and others (Amanvermez *et al*., 2005). The occurrence of oxidant-antioxidant systems in reproductive tissues has aroused the curiosity of researchers in the role of OS in human reproduction (Krajcir *et al*., 2008).

Also, elevated OS and ROS levels and the decrease in antioxidant concentrations in peritoneal fluid and serum have been indicated in unexplained infertility. Increasing the

intake of natural antioxidants may help to maintain a tolerable antioxidant status, perhaps the normal physiological functioning (Balasaheb & Pal, 2015). Consequently, Sekhon *et al* (2010) recommended that antioxidant supplementation may seek to avert and ameliorate OS and its influence in the pathogenesis of obstetrical conditions such as pre-eclampsia, recurrent pregnancy loss, PCOS and endometriosis.

The degree of antioxidant defense present is often expressed as total antioxidant capacity (Agarwal *et al*., 2005).

2.4.1 Antioxidant Supplementation

Natural and synthetic antioxidants have been employed in the treatment and management of female infertility. Antioxidant derivative molecules are also in the developmental stages of research. They include phenolic, porphyrinic, and peptidsylic structures of zinc, copper and manganese. These complexes mimic SOD. Amongst the enzymatic antioxidants, SOD is the first enzyme to defend the cell from O_2- radicals and prevent the destruction of cellular molecules (Balasaheb & Pal, 2015). In addition, catalase, glutathione reductase, and glutathione peroxidase reduce H_2O_2 into water thereby, neutralizing possible ROS reactivity (Krajcir *et al*., 2008).

Dietary supplements also known as synthetic antioxidants fall under non-enzymatic antioxidants. These include Vitamins A, C, and E, zinc, glutathione, beta-carotene, and carotene. They supplement the female body's oxidant defense system. Glutathione is a non-enzymatic antioxidant which is localized in tubal fluid and the oocyte itself, promotes zygote development (Krajcir *et al*., 2008). Antioxidants may be advised when a specific etiology cannot be defined as in idiopathic infertility. Polyphenols represent a wide variety of compounds, which are divided into several classes. These are

hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005).

In the treatment of infertility, fertility supplements are employed. Intake of antioxidant nutrients, including use of multivitamins, impacts the generation of reactive oxygen species and may play a beneficial role in female fertility (Ruder *et al*., 2009). Couples battling infertility resort to their usage either concurrently or as an adjunct to medical treatment to manage the problem (Polackwich & Sabanegh, 2015). The high costs associated with assisted reproductive techniques (ARTs) for male and female infertility have led consumers to find less expensive alternatives for potential treatment. The use of NFS and nutraceuticals are some of such substitutes.

A 'nutraceutical' is any substance that may be considered as food or part of a food and provides medical or health benefits, encompassing, prevention and treatment of diseases (Rajasekaran *et al*., 2008). About 2000 years ago, Hippocrates correctly emphasized, "Let food be your medicine and medicine be your food". Nutraceuticals have received considerable interest because of their presumed safety and potential nutritional and therapeutic benefits (Rajasekaran *et al*., 2008). The term 'Nutraceutical', was coined by combining the terms 'Nutrition' and 'Pharmaceutical' in 1989 by Dr. Stephen DeFelice, Chairman of the Foundation for Innovation in Medicine. Different nutraceuticals, including herbs, fruits, vegetables, nutritional supplements, and vitamins, have been promoted to improve many aspects of male fertility. These include sperm function and semen analysis parameters, erectile function, and libido (Ko & Sabanegh, 2014).

Many supplements for fertility address the problem of ROS, suggesting that nutraceuticals used in treatment of infertility need to have antioxidant properties and hence the ability to scavenge oxidative species (Polackwich & Sabanegh, 2015).

Phenolic compounds present in medicinal plants have been reported to possess powerful antioxidants activity (Bhatia, Bhatia, & Grover, 2012). Amongst plants, cocoa and green tea are examples of rich sources of polyphenol antioxidants (Lee *et al*., 2003). Many plants and plant extracts as well as chemicals thought to contain high levels of antioxidants have been tested in both humans and experimental animals to discover the potential of tissue protection. Examples of such antioxidant-containing substances include wine, tea, grape fruits, selenium, sonchus asper, ginseng, taurine, thymoquinone and curcumin. Among these, cocoa beans have been found to contain the highest amount of antioxidants and their derivatives, such as cocoa powder and chocolate, are important sources of polyphenols. Also, it has been found out that natural cocoa powder contains the highest levels of total antioxidant capacity when compared to all other kinds of edible cocoa products (Lee *et al*., 2003; Sokpor *et al*., 2012).

2.4.2 Natural cocoa as an anti-oxidant supplement and a nutraceutical

The practical use of natural cocoa originated from Olmecs, Mayas, and Aztecs in South America. By the 16th and early 20th century in Europe and New Spain, over 100 medicinal uses of cocoa had been documented (Dillinger *et al*., 2000). Cocoa contains about 380 known chemicals, 10 of which are psychoactive compounds (Andújar et al., 2012). Studies indicate that the health promoting properties of cocoa powder were attributed mainly to their polyphenolic compounds and methylxanthines (Franco, Oñatibia-Astibia, & Martínez-Pinilla, 2013; Jalil & Ismail, 2008). These include mainly

procyanidins monomers, namely, catechin and epicatechin, dimer, trimer, tetramer, and up to tetradecamer (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Tomas-Barberan, F A Cienfuegos-Jovellanos, E Marin *et al*., 2007). In addition, methylxanthines, namely, caffeine, theobromine, and theophylline, had also been identified in cocoa (Kelm *et al*., 2006). Because of the significant amount of bioactive compounds, the study of their contribution toward health benefits is an area of interest (Jalil, Ismail, Pei, Hamid, & Kamaruddin, 2008). The antioxidant and antiradical properties *in vitro* of some of their polyphenolic constituents of natural cocoa, specially procyanidins and flavan-3-ols have increased its consumption worldwide (Andújar *et al*., 2012). Cocoa phenolics have been described as being bioactive compounds, especially prominent for their metabolic and cardiovascular effects. Cocoa has the property of being able to modulate the immune response. It possesses anti-inflammatory and anti-carcinogenic properties (Andújar *et al*., 2012). Cocoa powder and cocoa extracts have been shown to exhibit greater antioxidant capacity than many other flavanol-rich foods and food extracts such as green and black tea, red wine, blue berry, garlic and strawberry in vitro (Roy *et al*., 2005). The prevention of lipid peroxidation and the protection of low density lipoprotein-cholesterol against oxidation, and increase resistance to OS are indicators of the pharmacological properties (Andújar *et al*., 2012). Three groups of polyphenols can be identified in cocoa beans. These are catechins, which is about 37% of the polyphenol content in the beans, anthocyanidins, about 4%, and proanthocyanidins are about 58% (Andújar *et al*., 2012).

Moreover, the health-promoting properties of cocoa powder have chiefly been credited to their polyphenol compounds (Nehlig, 2012). Studies on the health benefits of cocoa and

cocoa products have been conducted over the past decade, with a major focus on degenerative diseases. These benefits could also be due to their significant amounts of flavonoid monomers (catechin and epicatechin) up to tetradecamers (Jalil & Ismail, 2008). Cocoa also contains mineral elements such as sodium, zinc, magnesium, boron, copper, calcium, phosphorus, potassium, nitrogen and manganese which help tissues in building their capacity to withstand injury (Oliveira & Genovese, 2013).

Addai (2010) stated that natural cocoa aids in precluding asymptomatic malaria and reducing age related cardiovascular conditions due its high antioxidant levels. There was a significant increase in white blood cells level suggesting that the ingestion of cocoa can boost the immune system in experimental rats fed on cocoa.

White blood cells are responsible for fighting infections, hence optimum levels are essential in guarding the body against infections (Abrokwa, Asamoah, & Esubonteng, 2009). Indications of the importance of natural cocoa on the heart, liver and kidney with alcohol toxicity, malaria and in other medical conditions of oxidative stress etiology have also been proven (Addai, 2010; Aidoo *et al*., 2012; Asah-Opoku, 2015 (M.Phil thesis); Asiamah, 2015 (M.Phil thesis); Hollenberg, 2006; Sokpor *et al*., 2012).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Experimental Protocol for Main Study

All experimentations were carried out with the approval of the ethical and protocol review committee of the University of Ghana Medical School (Korle-Bu, Ghana) (Protocol Identification number: CHS-Et/M.9-P 4.6/2016-2017). Practices involving animals and their care followed the institutional guidelines, in obedience with national and international laws and guidelines for the use of animals in biomedical research.

The following materials were acquired for the experiment: Ethanol-Analytical reagent grade. Code: E/0665DF/17 (Fisher chemical, Fisher Scientific UK) and Natural Cocoa Powder (Good Food Brand, Batch number: KK1604A, Lot number: CPC111413, Accra, Ghana).

This study was performed using thirteen (13) female New Zealand rabbits between 5 -7 months old and weighing between 1.5–2.5 kg. All rabbits were procured from the Animal Experimentation Department of Noguchi Memorial Institute for Medical Research (NMIMR) Legon, Ghana. They were housed in the Animal Experimentation Unit of the School of Biomedical and Allied Health Sciences, Korle – Bu for a week under the same laboratory conditions of ambient temperature (28 ± 2º C,) humidity (80 ± 2 %) and a 12 hour light/dark cycle which were necessary for the rabbit's circadian biorhythms before the start of the experiment. Rabbits received nutritionally standard rabbit chow (Ghana Agro Food Company Limited [GAFCO], Tema, Ghana) and clean drinking water. Rabbits were kept in cages (0.90 × 0.60 × 0.45 m) which hung at a height of 0.8 cm from

the ground so that excrement could fall out into collecting trays and be routinely observed for food consumption and fecal characteristics.

After one week of acclimatization, blood samples were taken from the rabbits via the lateral saphenous vein to be used as baseline hormonal markers of fertility (LH and FSH) and markers of antioxidative capacity (GSH and SOD). The rabbits were randomly put in three groups; Group A (n=5), Group B (n=5) and Group C (n=3). Group A received 35% alcohol, natural cocoa and rabbit chow. The cocoa drink was administered from 6 am to 6 pm *ad libitum* and the 35% alcohol was given from 6pm to 6am *ad libitum.* Group B received water from 6 am to 6 pm, and 35% alcohol *ad libitum* from 6 pm to 6. Group C received water in 24 hours. All three groups were given rabbit chow. Following the grouping, rabbits were weighed using the weighing balance and then subsequently every fortnight in order to monitor the growth rate of the animals.

At the end of the experiment (week 7), the rabbits were weighed and another blood samples were collected from each rabbit and placed into serum separation tubes for biochemical analyses. Blood samples were assayed for hormonal markers of fertility (LH and FSH) and markers of antioxidative capacity (GSH and SOD). Ovaries were harvested for histological studies.

The daily volumes of alcohol, natural cocoa drink and water consumed by rabbits were measured and recorded.

The above treatments for all the three groups were administered on daily basis for 7 weeks. The weight measurements of each rabbit were recorded against corresponding biochemical results.

At termination of the experiment, animals were sacrificed. Diethyl ether was used in sedating before decapitation. The ovaries were harvested and prepared for histological and stereological studies.

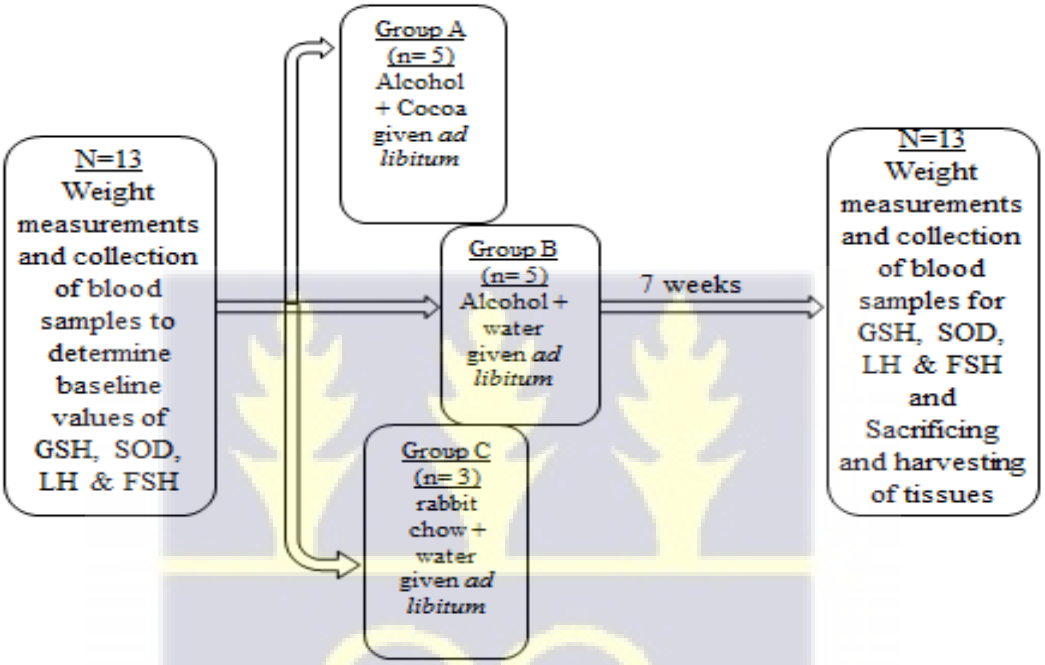

Figure 8: A flow diagram showing experimental schedule for 7 weeks.

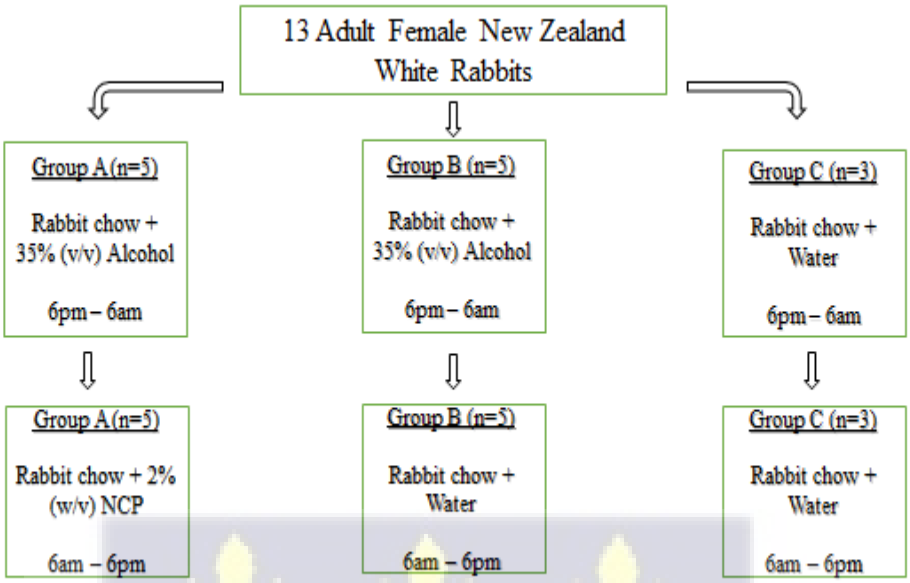

Figure 9: Flow diagram showing daily feeding treatments given to animals in the study.

3.1.1 Preparation of 2% (w/v) unsweetened natural cocoa drink

Natural cocoa powder (Good Food brand, Ghana) was used for the study. A concentration of 2% weight per volume (w/v) was administered as previously described in the works of Affram *et al.*, (2008) and Sokpor *et al.*, (2012). A suspension of 2% w/v unsweetened cocoa was freshly prepared daily by weighing out 18.0 g of the powder on a chemical weighing balance (Mettler Toledo P1200, Switzerland) and then thoroughly dissolving it in 1000 ml of pre-boiled tap water. The dissolution of cocoa particles was ensured by adequate stirring to form a uniform mixture. The freshly prepared and cooled 2 % (w/v) suspension was placed in the rabbits' cages in graduated feeding bottles to be taken *ad libitum*.

Animals in group A received 200 ml of cocoa drink for 12 hours during the day. The suspension was shaken intermittently (every 3 hours) as the cocoa particles had the tendency to settle and clog the teats of the bottles and hamper the flow of the drink when the rabbits sucked on them.

Before the administration of alcohol, the volumes of cocoa drink consumed by group A rabbits were determined by subtracting the final volume of drink on withdrawal, from the initial volume of the drink served. The mathematical expression below was used to determine the approximate amount of cocoa drink consumed by each rabbit in group A. Vc=Vi-Vf. Where Vc is the cocoa consumed by each rabbit, Vi is the initial volume of cocoa drink served and Vf is the final volume of cocoa left after 12 hours of feeding.

To ensure optimal hygiene among rabbits, the left over cocoa drink was always discarded and the bottles were properly cleaned for use the next morning. The bottle containing the

cocoa drink was placed in the cages such that the delivery of the cocoa drink was facilitated by gravity on suction by rabbits.

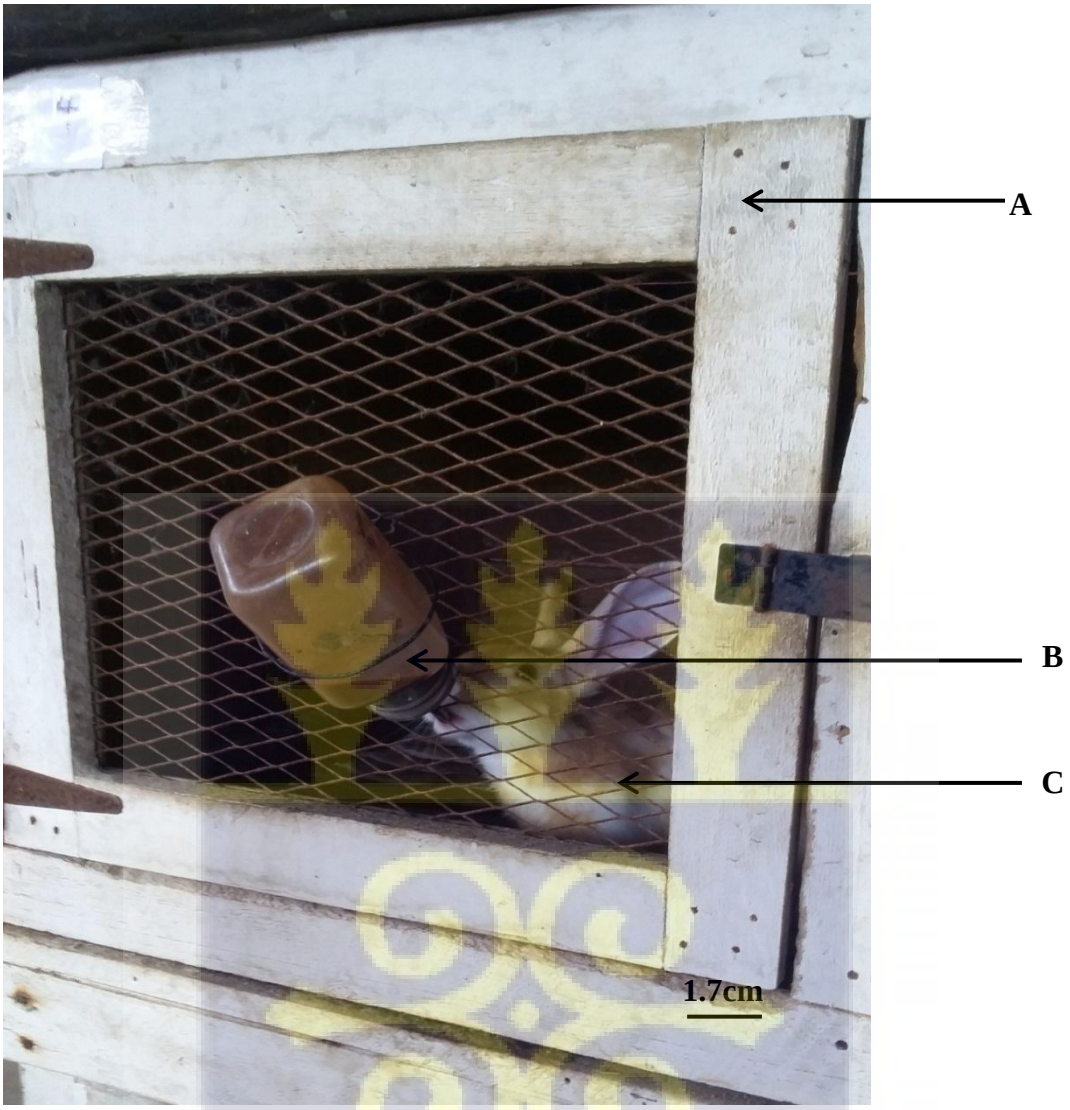

Figure 10: A picture showing experimental rabbit in a cage receiving unsweetened natural cocoa drink. A= Rabbit cage, B= Graduated bottle unsweetened natural cocoa drink, C= Female rabbit.

3.1.2 Preparation of 35% (v/v) alcoholic drink

The alcoholic drink was administered to the rabbits daily. It was prepared in such a way that the final ethanol concentration of the drink was 35% (v/v). This was achieved by diluting absolute ethanol (99.9% [v/v]) Fisher Scientific, UK to 35% concentration. Precisely, a total of 1000 ml of 35% (v/v) alcohol drink was prepared by measuring 350 ml of absolute ethanol into a 1000ml measuring cylinder and topped up with 650 ml with rabbit drinking water to the 1000 ml mark. This alcoholic drink was then shared amongst all the rabbits in Groups A and B. Each rabbit received 200 ml from 6pm to 6am. The volume of alcoholic drink consumed by each rabbit was determined using the same approach as that for the cocoa drink.

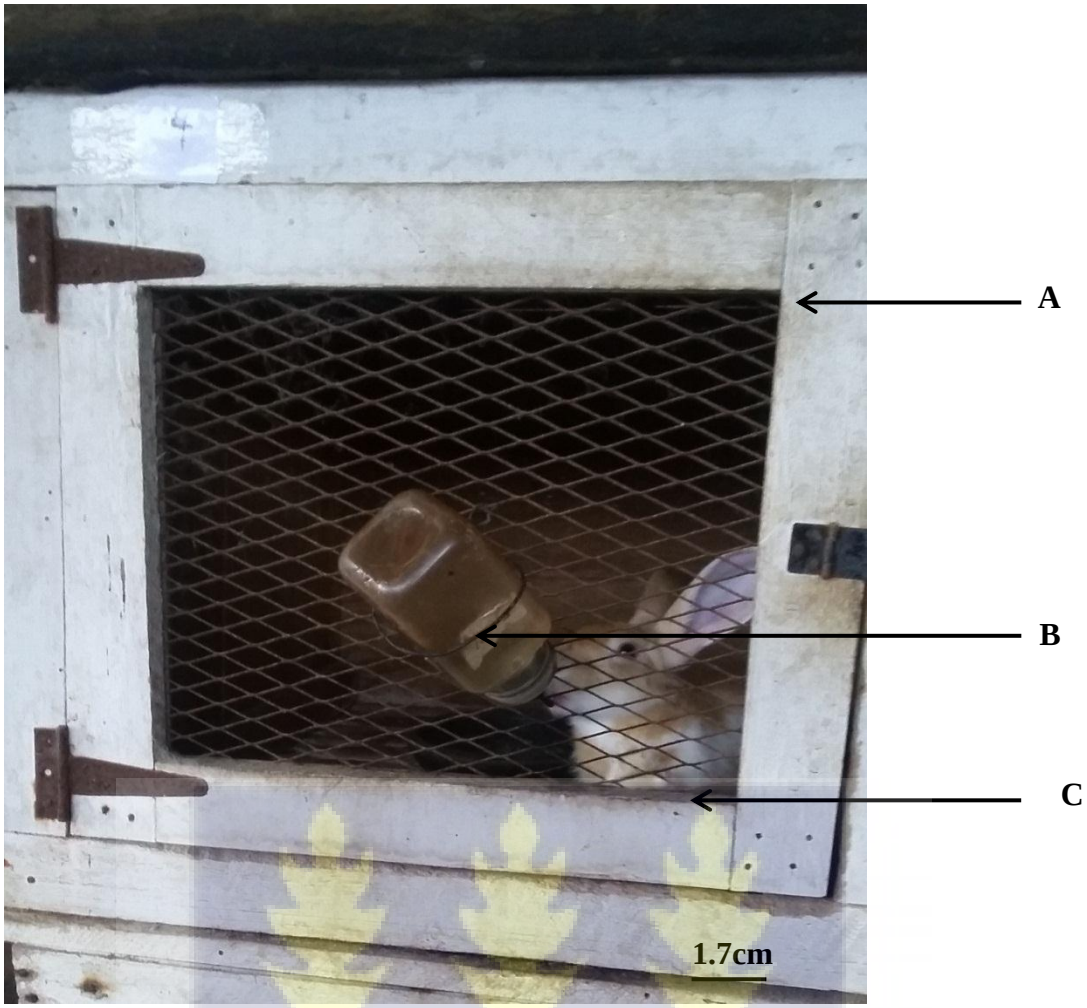

Figure 11: A picture showing experimental rabbit in a cage receiving 35 % alcohol. A= Rabbit cage, B= Graduated bottle containing alcohol, C= Female rabbit.

3.1.3 Blood Sampling to test for SOD, GSH, LH and FSH

At weeks 0 and 7, blood samples were collected from each rabbit to prepare serum for biochemical determination of LH and FSH levels and markers of antioxidant capacity (SOD and GSH). Each rabbit was restrained with a large towel which was wrapped around it leaving the head, ears and one hind leg uncovered. Blood was collected from the lateral saphenous vein. Fur was shaved from the lateral side of the thigh and a topical anaesthetic gel (Neon Lignocaine Hydrochloride I. P. 2% Jelly) was applied to the shaven site and allowed to sit for about 45 minutes. Using a 2 ml syringe (3 ml) 21G × 1 ½, 0.80 × 40 mm (Uni-Ject Quadact Limited, UK), about 3 ml of blood was collected and poured into serum separation tubes (All Pro Vacuum blood Collection tube [4 ml]) containing inert separating gel. The blood collected was allowed to stand at room temperature for 45 minutes to encourage clotting which facilitated serum preparation. The tubes with the blood samples were centrifuged at 5000 revolutions per minute (rpm) for 5 minutes to obtain serum. The serum was decanted by pouring into 1.0 ml Eppendorf tubes and kept on ice for biochemical and hormonal analysis.

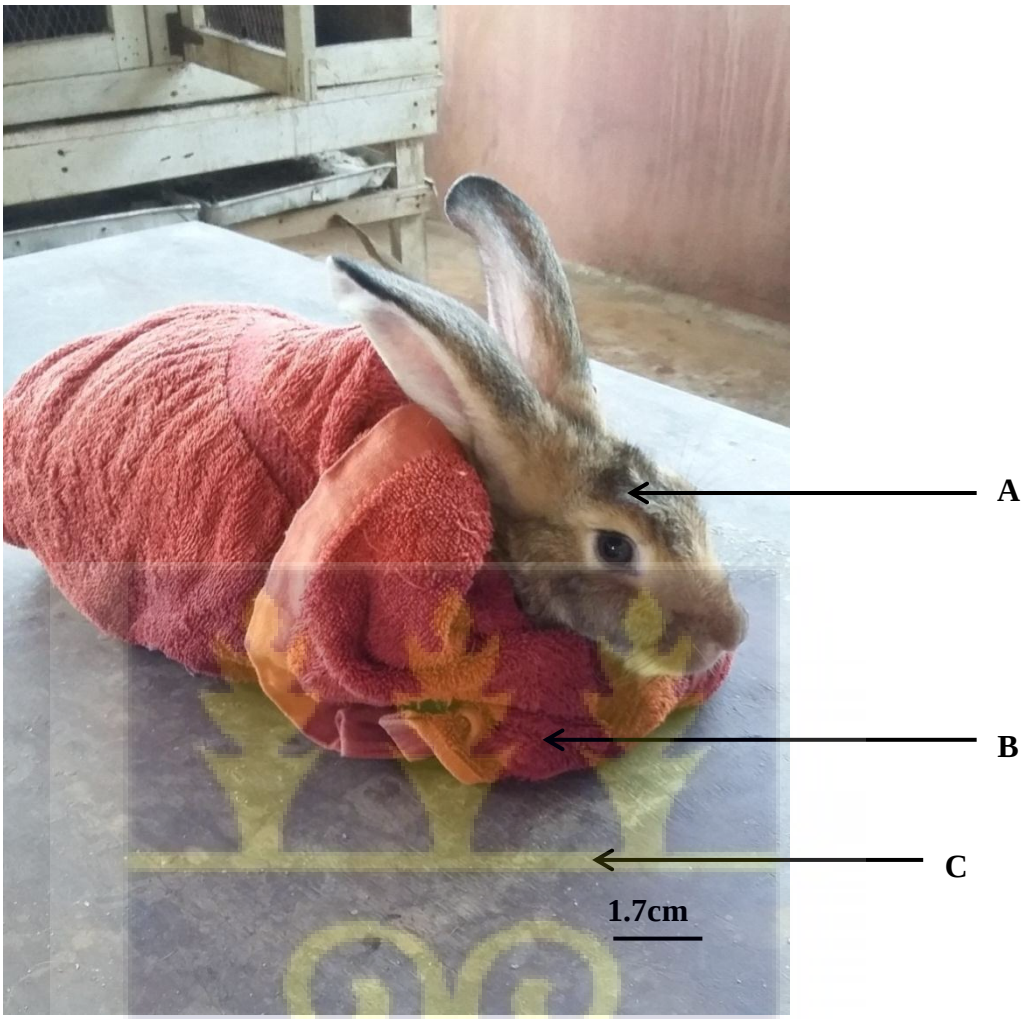

Figure 12: Picture showing female rabbit restrained using a large towel with head and ears exposed. A= Female rabbit, B= Large towel, C= Flat surface (table).

3.2 Tissue Processing

The animals were brought to the histology laboratory from the animal house in unit metal cages after they had been weighed. One animal was worked on at a time.

In the laboratory, each animal was sacrificed by decapitation. Animals were sedated using diethyl ether before decapitated. With this technique, about 6 ml of blood was obtained from each animal. The ovaries and fallopian tubes were removed, trimmed of all extraneous tissue, weighed, and representative sections were placed in 10% buffered formalin to fix them for histological study. Histological sections were prepared and studied with hematoxylin and eosin stain. Harvested tissues were taken through appropriate processing based on the tissue staining protocol being followed.

3.2.1 Rabbit ovary volume determination

Direct ovary volume was determined using the liquid displacement method. For determining the volume of each ovary, a measuring cylinder was filled with 40 ml water and recorded as the initial volume of water in measuring cylinder. The harvested ovary was gently and completely immersed in the measuring cylinder containing the water. The final volume of water after the immersion of ovary was recorded. The ovarian volume thus was obtained by deducting the initial volume of water from the final volume. The tissue was removed from the cylinder and placed in tissue container with 10 % phosphate buffered formalin for fixing and subsequent tissue processing.

3.2.2 Histological Preparations

The tissues were taken through normal histological processing of dehydration, clearing, embedding, sectioning, rehydration, staining and mounting. Tissues were dehydrated using a series of graded alcohols as detailed in appendix I. The alcohol in dehydrated tissues was cleared with two changes of xylene for two hours to allow embedding with paraffin wax. Embedding of tissues was done in two changes of molten paraffin wax at temperature of 60 ˚C for a total of 7 hours after which the tissues were blocked in 22 cm^3 metallic mould boxes. A small amount molten wax was poured into the mould box and the tissue was oriented in it with a forceps. More molten wax at the temperature of 60 ˚C was poured unto the tissue and labeled. As the wax solidified more wax was poured onto it. Care was taken not to create air bubbles in the block. The block was left overnight to harden and removed from the metal trays. The blocks were then put into a freezer to further harden them before sectioning. The blocks were sectioned at 5 microns using a Leica 2235 Rotary microtome.

Five sections were systematically selected. The first section was selected at random once full transverse profile of ovary was observable. Afterwards, every 50^{th} section was selected. Each section was collected on a small tray and placed in a water bath containing water at 40 ˚C. Alcohol solution (30%) in a wash bottle was used to unroll sections that rolled up by adding drops of it onto the tissue before transferring to warm water.

Labeled microscope glass slides were used to pick tissues from the water and dried overnight in an oven at a temperature of 30 ˚C. The sections were rehydrated by passing them through graded series of alcohol (from absolute to 70%) and then stained with haematoxylin and eosin (Appendix II) to assess the general ovarian structure.

3.2.3 Microscopy and Micrometry

Pictomicrographs of mounted slides were obtained using a digital Microscope Eyepiece (Lenovo Q350 USB PC Camera). The digital eyepiece was fitted to a microscope by removing one eyepiece of a Leica Galen III light microscope. The digital eyepiece was connected via a cable and used to transfer images of the tissues from the microscope to the computer HP Compaq dx2300 Microtower). The microscope stage was moved one stage unit interval on the x – axis and one stage unit interval on the y – axis, snapshots of ovarian tissue within the field of view were captured onto a computer. This was done till the whole area of tissue was covered. Using 13 animals in all, a total number of 10 micrographs each were randomly sampled per animal for stereological assessment to estimate the volume densities of ovarian follicles and stroma using the Cavalieri principle. In all 1,300 micrographs of the ovaries were used for the stereological study. Unenhanced images were opened and analyzed with Photoshop CS 6 (Adobe Systems, San Jose, CA, 2008).

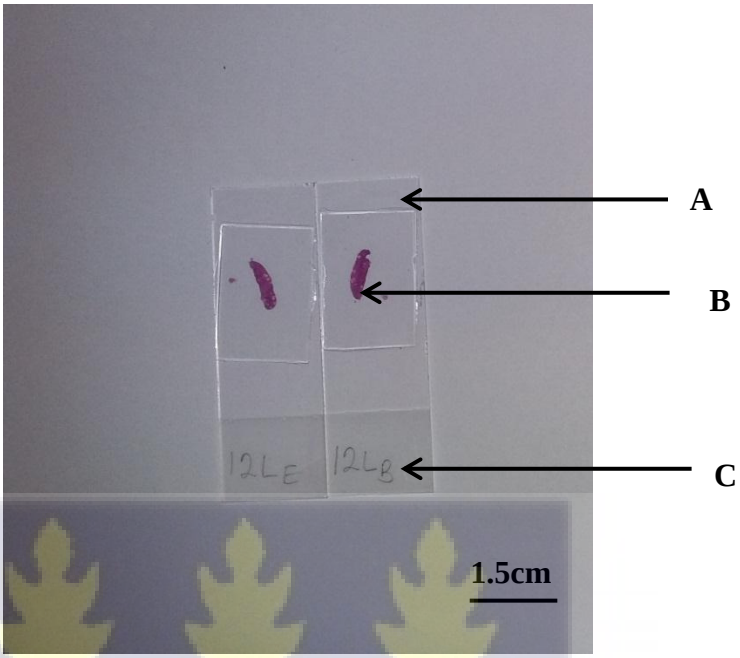

Figure 13: Picture showing ovary sections on glass slides. A= Glass slide, B= Transverse section of ovarian tissue, C= Slide label.

3.2.4 Stereological study of the ovary

The volumes of the follicles: primordial, primary, secondary, corpora atretica and stroma were determined using point counting with Cavalieri principle. Using Adobe Photoshop CS6 software, a grid of uniformly spaced points, 1cm × 1cm was superimposed over each micrograph of the ovary section. The number of test points, follicles and stroma which intersect with the grid points were counted for each section. The volume densities of the follicles and ovarian stroma were calculated using the equation (Heidari et al., 2003):

$$V = \frac{\Sigma P \times (\frac{a}{p}) \times t}{M^2}$$

Where 'V' is volume, 'ΣP' is the sum of all test points encountered, '(a/p)' is the area per point of the stereological grid, t is the thickness of the section and 'M' is the linear magnification.

For the Graafian follicles (GF), the average surface area was calculated for each micrograph. This was computed using the formula (Guven et al., 2013):

$$A\,(GF) = \frac{P \times b^2}{N}$$

Where 'A' is the surface area, P is the number of grid test points, overlying profiles of Graafian follicle within the counting frame, 'N' is the number of different profiles of Graafian follicles per micrograph and 'b' is the actual value of the distance between two points.

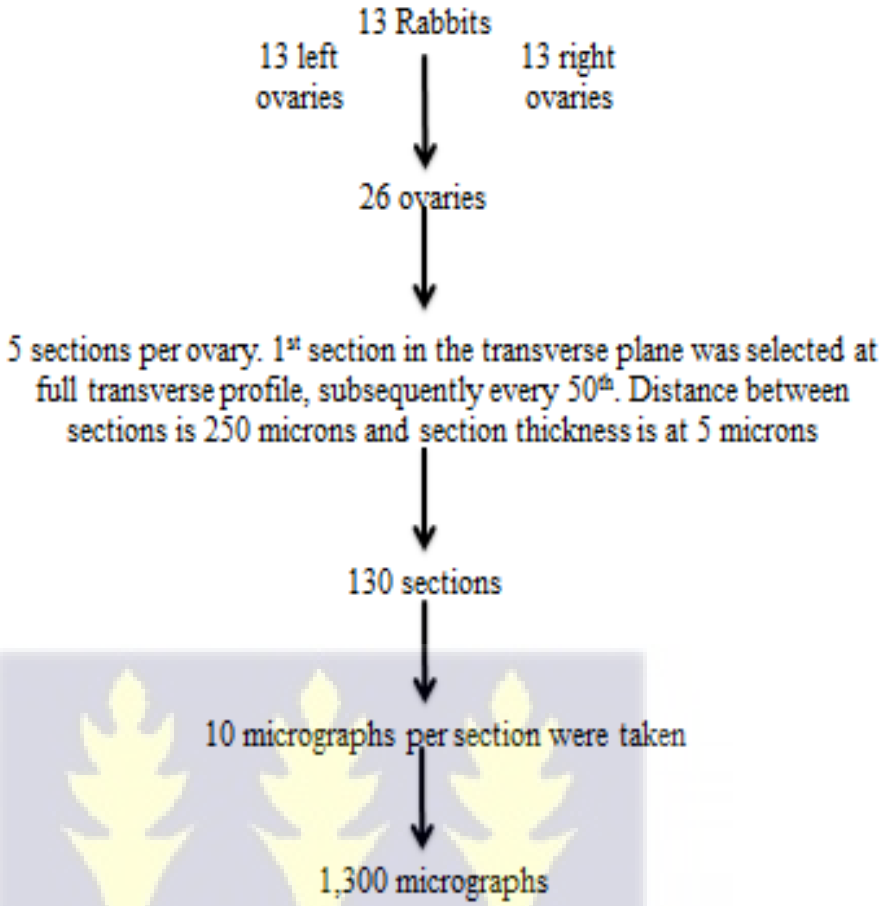

Figure 14: A flow diagram of multistage sampling adopted for stereological analysis.

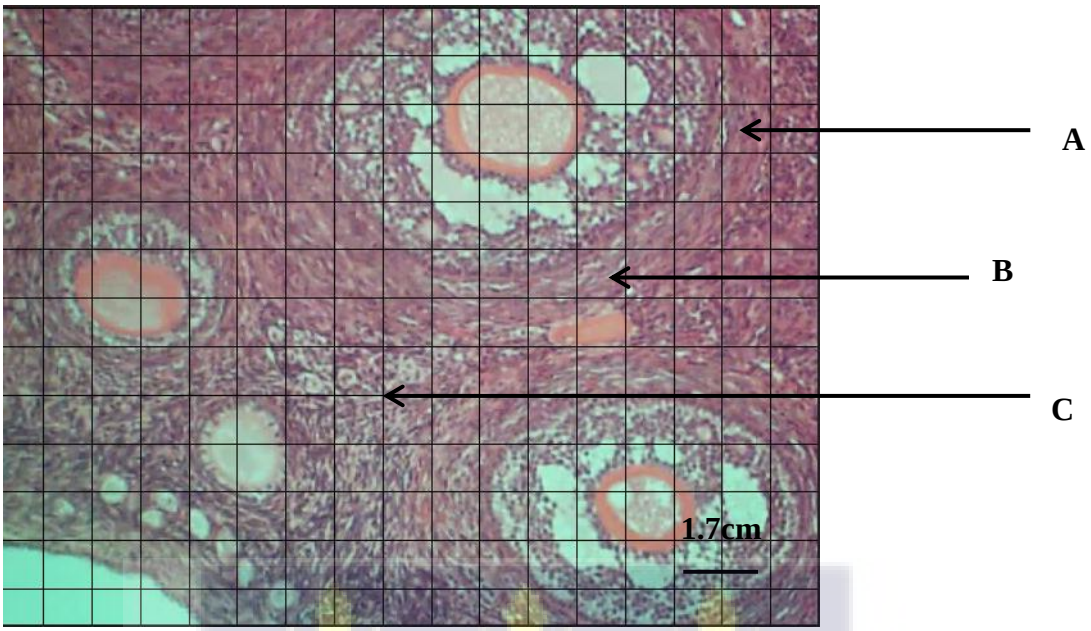

Figure 15: Picture showing test grid with square lattice superimposed on micrograph of ovary for stereological estimation volume densities of follicles and stroma. A= Pre-antral follicle, B= Theca cells secondary follicle, C= Test point hitting ovarian stroma.

3.3 STATISTICAL ANALYSIS

Graph pad Prism Version 5.0 was used for statistical analyses. All data sets were tested for normal distribution using Shapiro-Wilk normality test. Data sets with p-value less than 0.05 was concluded to be not normally distributed.

Data collected on ovary weights, body weights, volumes of fluids taken were normally distributed. Data were presented as mean ± S.E.M. Statistical significance of the difference between groups was performed by Paired T-Test and One way analysis of variance (ANOVA) for parametric data. For non-parametric data, Kruskal-Wallis Test and Mann-Whitney U Test were performed. Differences with $p < 0.05$ were considered to be statistically significant.

Data on volume fractions of follicles and stroma were represented as mean± S.E.M. One way (ANOVA) was used to test for significant differences of the above-mentioned variables among the three experimental groups. Paired T-test was used to test for significant difference of the variables for between any two experimental groups.

CHAPTER FOUR

4 RESULTS

4.1 MORTALITY

No animals were lost in the 7-week duration of the experiment.

4.2 AVERAGE DAILY FLUID INTAKE

4.2.1 Total fluid consumption

The mean and standard deviations of the volume of fluid consumed by each animal group per week are shown in Table 1 below. Comparing the mean volume of fluid consumed by each group per week by One-way ANOVA have shown that there were significant differences (95% confidence level) between the mean volumes of fluid for the groups during the entire course of the experiment. Tukey's post hoc analysis showed that the mean volume of fluids consumed were significantly greater in group B than group C during the 7 weeks while the difference was only statistically significant between group A and C only in week one (Table 1). Weekly assessment of within group mean total fluid consumption for groups A and B also showed significant differences of $p < 0.001$ and $p = 0.002$ respectively, during the experimental period at 95% confidence level. The weekly difference in the means of total fluid intake was not significant in group C ($p = 0.232$) (Table 1). Tukey's post hoc analysis however showed that within group B, only the mean in week 7 was significant (280.143±7.489 ml, $p < 0.05$). On the other hand, the means obtained from week 2 to week 7 were all significant for group A. The means obtained were: week 2 - 189.429±1.182 ml ($p < 0.01$), week 3 - 233.857±13.859 ml ($p < 0.001$), week 4 - 193.143±8.036 ml ($p < 0.01$), week 5 – 190.143±5.622 ml ($p < 0.01$), week 6 - 214.143±5.318 ml ($p < 0.001$) and week 7 - 213.000±6.916 ml ($p < 0.001$).

Table 1: Summary of statistics on mean amount of total fluid in milliliters (ml) consumed per week by Group A, B and C rabbits.

	Group A (alcohol-cocoa)	**Group B (alcohol-only)**	**Group C (Controls)**	***p*-value 1**
	n=5	n=5	n=3	
Week 1	160.857±10.634*	255.857±4.525**	198.571 ± 2.143	< 0.001
Week 2	189.429±1.182^{b}	243.143±4.395***	196.791 ± 0.630	< 0.001
Week 3	233.857±13.859^{c}	263.429±8.013**	198.571 ± 2.143	0.007
Week 4	193.143±8.036^{b}	255.517±5.148***	196.905 ± 0.238	< 0.001
Week 5	190. 143±5.622^{b}	260.857±4.330***	199.524 ± 1.260	< 0.001
Week 6	214.143±5.318^{c}	274.429±4.735***	195.952 ± 0.630	< 0.001
Week 7	213.000±6.916^{c}	280.143±7.489^{***a}	199.048 ± 1.717	< 0.001
***p*-value 2**	< 0.001	0.002	0.232	

Values are expressed as mean ± S.E.M. *p* - value 1 denotes significance level for One Way ANOVA (followed by Tukey's post hoc) for between-group comparison with *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$. *p* - value 2 indicates significance level for one way ANOVA (followed by Tukey's post hoc) for time course assessment within the groups with alphabets indicating significant difference as compared to week 1. c = $p < 0.001$, b = $p < 0.01$ and a = $p < 0.05$.

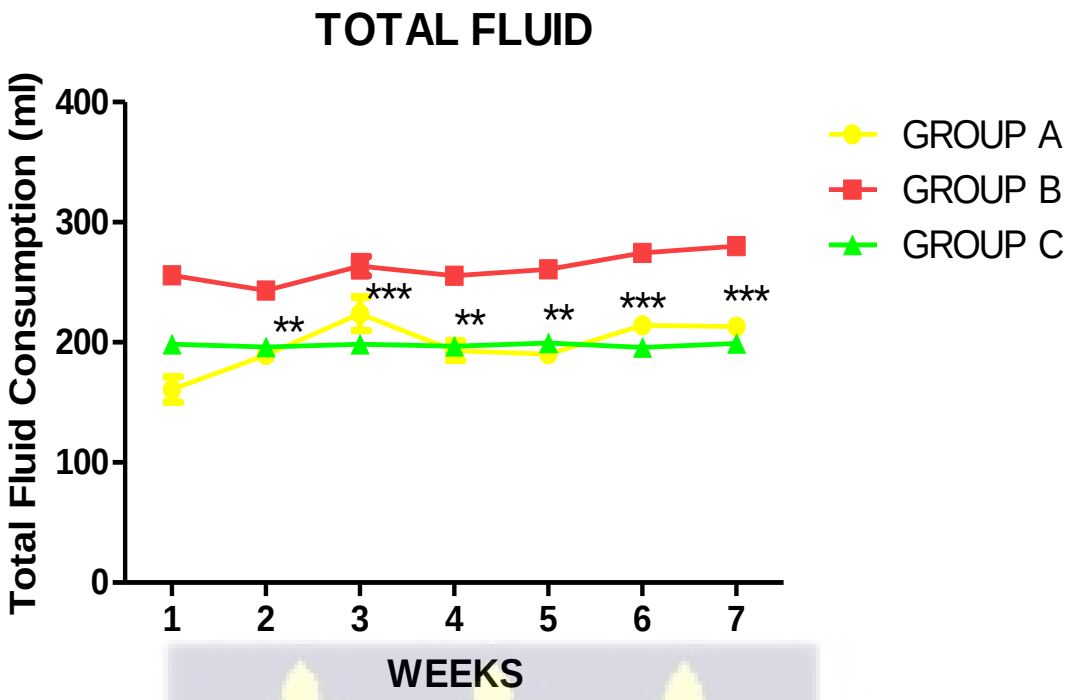

Figure 16: A line plot showing mean volume of total fluid consumption (ml) in the course of 7 weeks by rabbits in Groups A, B and C. Each point represents mean volume of fluid consumed by rabbits in the group for the specific week, error bars are S.E.M. (n = 13). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to respective controls (one-way ANOVA followed by Tukey's post hoc).

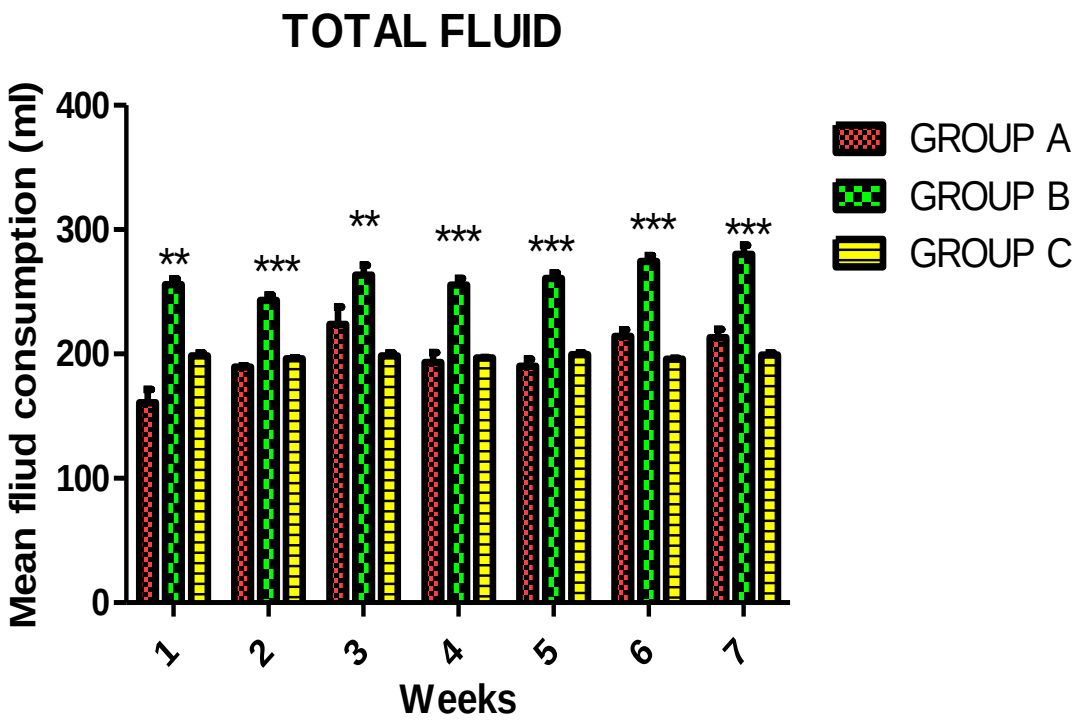

Figure 17: A bar chart showing mean volume of total fluid consumption (ml) in the course of 7 weeks by rabbits in Groups A, B and C. Error bars represent standard error of mean (S.E.M) (n = 13). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to respective controls (one-way ANOVA followed by Tukey's post hoc).

4.2.2 Alcohol consumption

The lowest mean volume of alcohol consumption by rabbits in group A (cocoa-alcohol) was 41.286±1.054 ml (week 2) while the highest was 82.000±3.645 ml (week 6). In group B (alcohol-only), the lowest mean volume of alcohol consumption was 45.143±3.680 ml (week 2) while the highest was 82.857±7.002 ml (week 7). The results indicate that consumption of alcohol in the two experimental groups was highest in the 6^{th} and 7^{th} weeks of the experimental period. Also the difference between means of group A and group B for each week was not significant ($p > 0.05$) (Table 2).

Weekly assessment of within group mean volume of alcohol consumption for groups A (cocoa-alcohol) and B (alcohol-only) however showed significant differences of $p < 0.001$ and $p < 0.001$ respectively, during the experimental period at 95% confidence level. The means of the first week were 55.143±3.617 ml for group A (cocoa-alcohol) and 56.143±2.365 ml for group B (alcohol-only). The results showed that in group A, the mean alcohol intake for week 6 (82.000±3.645 ml, $p < 0.05$) and week 7 (80.000±4.646 ml, $p < 0.05$) were significantly different from the mean. In group B (alcohol-only) however, only the mean for week 7 (82.857±7.002 ml, $p < 0.01$) was significant.

Table 2: The mean volume of alcohol drink in milliliters (ml) consumed per week by Group A and B rabbits.

	Group A (alcohol-cocoa)	**Group B (alcohol-only)**	**t-value**	***p*- value 1**	**95% CI**
	n=5	n=5			
Week 1	55.143±3.617	56.143±2.365	0.134	0.448	-22.732 to 24.733
Week 2	41.286±1.054	45.143±3.680	0.517	0.348	-19.371 to 27.594
Week 3	75.571±9.571	66.000±7.541	1.284	0.210	-33.301 to 14.162
Week 4	55.143±4.282	57.000±5.818	0.249	0.422	-21.874 to 25.593
Week 5	57.286±6.142	62.429±4.235	0.690	0.307	18.590 to 28.871
Week 6	82.000±3.645[a]	74.714±3.955	0.977	0.253	31.022 to 16.451
Week 7	80.000±4.646[a]	82.857±7.002[a]	0.383	0.387	-20.872 to 26.591
***p* - value 2**	< 0.001	< 0.001			

Values are expressed as mean ± S.E.M. *p*-value 1 denotes significance level of t-test for between-group comparison with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. *p*-value 2 indicates significance level for one way ANOVA (followed by Tukey's post hoc) for time course assessment within the groups. Alphabets indicate significant difference from week 1. c = $p < 0.001$, b = $p < 0.01$ and a = $p < 0.05$.

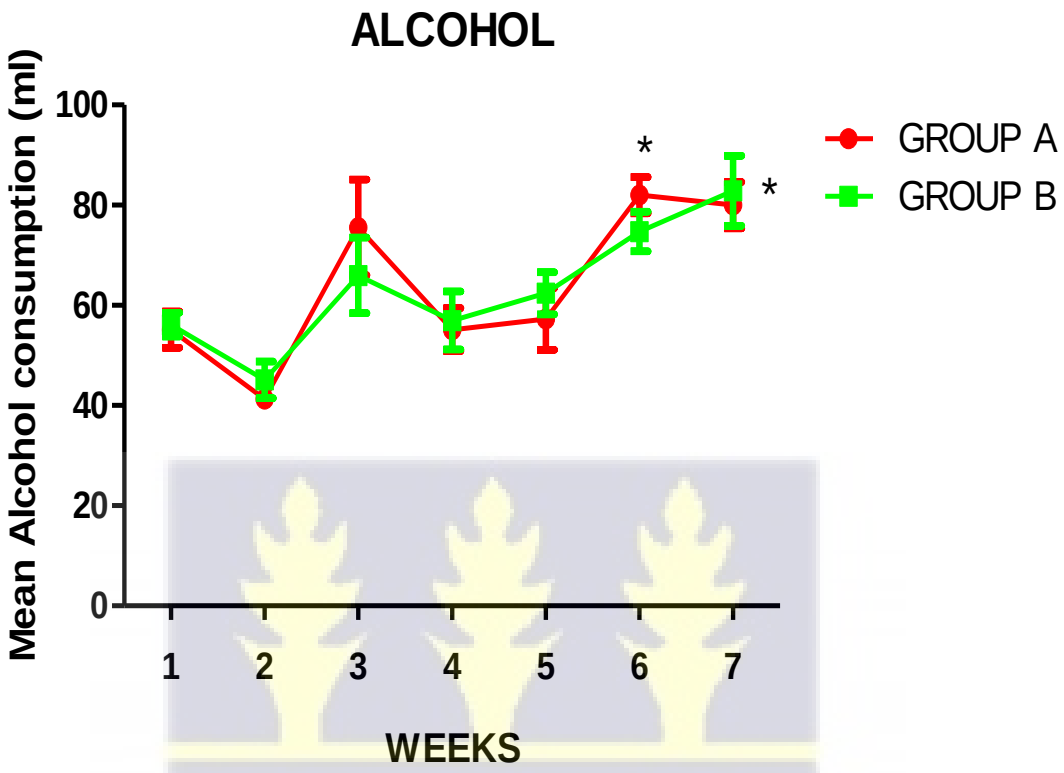

Figure 18: A line plot showing mean volume of alcohol consumption (ml) in the course of 7 weeks by rabbits in Groups A and B. Each point represents mean volume of alcohol consumed by rabbits for the specific week, error bars are S.E.M. (n = 10). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (one-way ANOVA followed by Tukey's post hoc).

4.2.3 Cocoa consumption

The results show that the difference between the means of volume of cocoa consumed was significant ($p < 0.001$). At week one, the mean volume of cocoa was 105.714±7.246 ml which also represented the least amount of cocoa consumed. All the mean volumes of cocoa consumed from week two through to seven were significantly different from that of week one. The means were 148.143±2.102 ml ($p < 0.001$) for week two, 148.286±6.839 ml ($p < 0.001$) for week three and 138.000±4.056 ml ($p < 0.001$) for week four. Weeks five, six and seven recorded 132.857±2.485 ml ($p < 0.05$), 132.143±2.893 ml ($p < 0.05$) and 133.000±3.517 ml ($p < 0.05$) as the mean volumes of cocoa consumed respectively.

The mean volume of cocoa consumption was highest in week three and lowest in the first week. The results showed a decline in cocoa consumption after the third weak (see Figure 19).

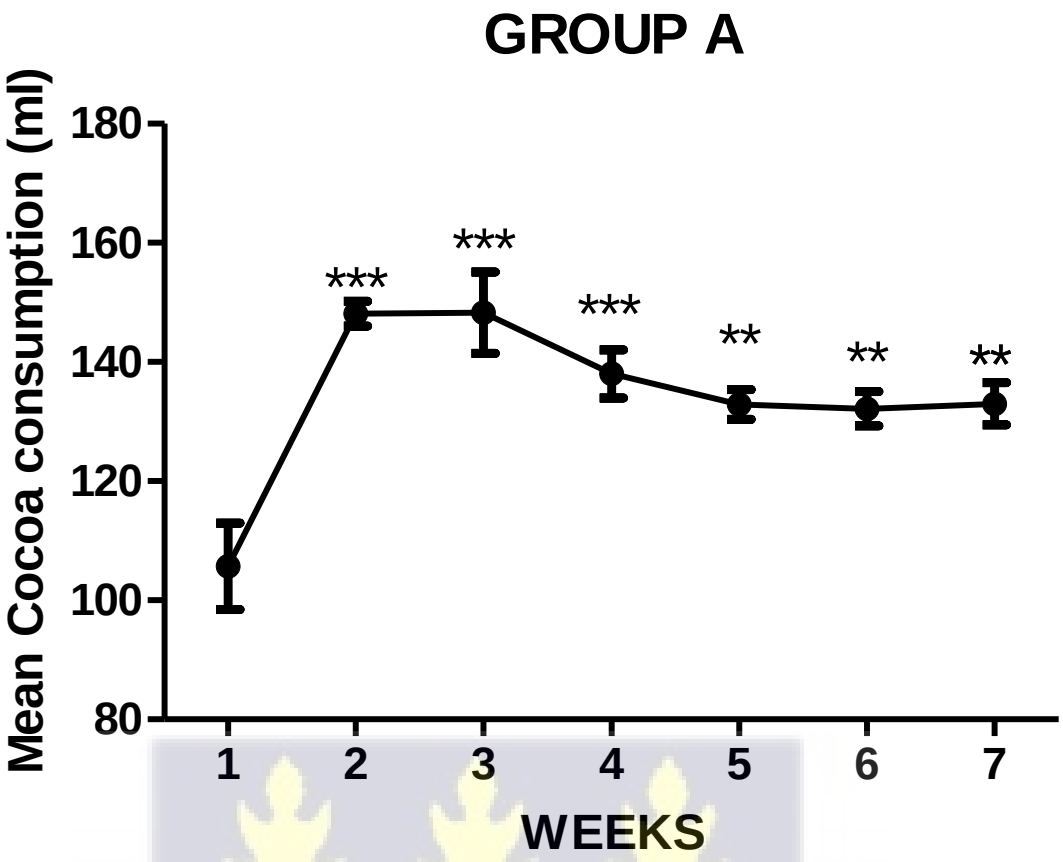

Figure 19: A line plot showing mean volume of cocoa consumption (ml) in the course of 7 weeks by rabbits in Groups A only. Each point represents mean volume of cocoa consumed by rabbits for the specific week, error bars are S.E.M. (n = 5). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (one-way ANOVA followed by Tukey's post hoc).

4.3 Weight Assessment

The mean bi-weekly weights of the rabbits in all groups are shown in table 3. Comparing the mean weights within group B showed that there were no significant differences over the seven-week experimental period ($p < 0.111$). The difference in the mean weights was however significant within group A ($p = 0.0058$) and group C ($p = 0.001$). Within group A, the results revealed that the mean weight increased significantly during week 5 (2.070±0.044 kg, $p < 0.01$) and during week 7 (2.024±0.044 kg, $p < 0.05$). Similarly, the increase in mean weight within group C was also significant during week 5 (2.217±0.262 kg, $p < 0.05$) and week 7 (2.417±0.252 kg, $p < 0.001$) (Figure 21).

A comparison of mean weights between groups A, B and C showed no significant differences in the first five weeks of the experiment ($p > 0.05$). The difference in mean weight was however significant in the seventh week ($p = 0.01$) (Table 3). Further analysis showed that it was the mean weight of rabbits in group B (1.782±0.064 kg, $p < 0.01$) that was significantly lower than the mean in group C (2.417±0.252 kg) (Figure 20). The mean weight of 2.024±0.044 kg ($p > 0.05$) in group A was not significant at this time.

Table 3: Biweekly assessment of mean weights (kg) for Groups A, B and C.

	Group A (alcohol-cocoa)	Group B (alcohol-only)	Group C (controls)	P- Value 1
	n=5	n=5	n=3	
Week 0	1.660±0.103	1.600±0.082	1.667±0.176	0.894
Week 1	1.860±0.103	1.790±0.078	1.917±0.208	0.843
Week 3	1.964±0.039	1.824±0.035	2.110±0.219	0.152
Week 5	2.070±0.044^{b}	1.840±0.058	2.217±0.262^{a}	0.108
Week 7	2.024±0.044^{a}	1.782±0.064**	2.417±0.252^{c}	0.010
P - value 2	0.006	0.111	0.001	

Values are expressed as mean ± S.E.M. *p* - value 1 denotes significance level for One Way ANOVA (followed by Tukey's post hoc) for between-group comparison with *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$. *p* - value 2 indicates significance level for one way ANOVA (followed by Tukey's post hoc) for time course assessment within the groups. Alphabets indicate significant difference from week 0. c = $p < 0.001$, b = $p < 0.01$ and a = $p < 0.05$.

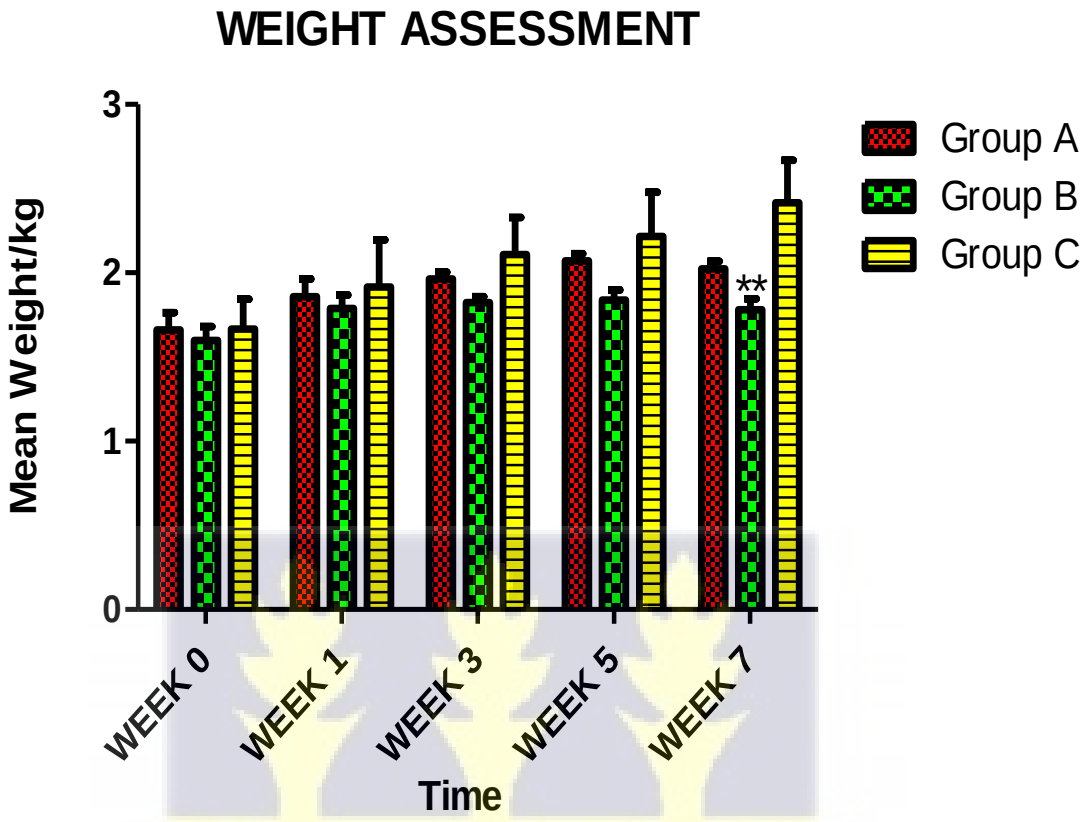

Figure 20: A bar chart showing the weight (kg) of rabbits measured biweekly and averaged over time in experimental groups of rabbits. Error bars represent standard error of mean (S.E.M.) * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to respective controls.

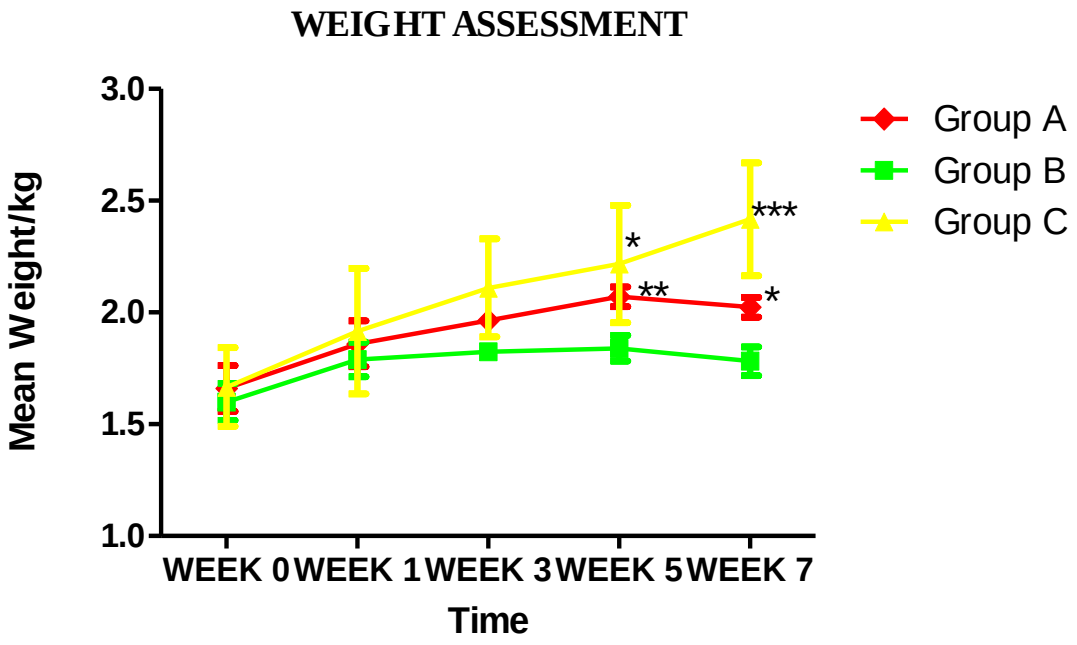

Figure 21: A line plot showing the effect of alcohol and natural cocoa over time (7weeks) on weight (kg) in experimental groups of rabbits. Each point represents the mean weight of rabbits in the group for specific week, error bars are S.E.M. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ compared to control (One-way ANOVA followed by Tukey's post hoc).

4.4 Serum FSH concentration (IU/L)

The mean pre-test and post-test serum FSH concentrations for rabbits that consumed alcohol and cocoa were 0.102±0.033 IU/L and 0.006±0.005 IU/L respectively. Analysis showed that even though there was a drastic drop in mean serum FSH concentration after the experiment, this difference was not significant (t = 2.410, p = 0.06). The mean concentrations for rabbits that consumed alcohol and water were 0.136±0.030 IU/L and 0.003±0.02 IU/L for pre-test and post-test respectively. The difference between the pre-test mean and post-test mean in group B (alcohol-only) was significant (t = 3.702, p = 0.013). In group C (controls), the mean serum FSH concentration from the pre-test was 0.137±0.007 IU/L and 0.085±0.007 IU/L from the post-test. The difference between the two means was also not significant (t = 1.109, p = 0.317) (Table 4).

The mean pretest FSH serum concentrations of the three groups were not significantly different from each other (p = 0.661). Group C had the highest mean (0.137±0.007 IU/L) while Group A (cocoa-alcohol) had the lowest mean (0.102±0.033 IU/L). The results showed that mean serum concentrations were significantly different (p < 0.001). The analysis revealed that the mean post-test FSH concentrations of both group A (0.006±0.005, p < 0.001) and group B (alcohol-only) (0.003±0.02, p < 0.001) were significantly different from the group C (0.085±0.007) mean concentration (Table 4).

Table 4: Summary of statistics on mean total FSH concentration (IU/L) in serum of Groups A, B and C rabbits.

	FSH		t-value	*p*- value 1	95% CI
	Pre-Test	**Post-Test**			
Group A (alcohol-cocoa)	0.102±0.033	0.006±0.005^{c}	2.410	0.061	-0.225 to 0.032
Group B (alcohol-only)	0.136±0.030	0.003±0.020^{**c}	3.702	0.013	-0.248 to -0.018
Group C (controls)	0.137±0.007	0.085±0.007	1.109	0.317	-0.284 to 0.013
***p* - value 2**	0.661	< 0.001			

Values are expressed as mean ± S.E.M. P-value 1 represents significance level for paired t-test between pre-test and post-test with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. P-value 2 indicates significance level for one way ANOVA (followed by Tukey's post-hoc) for between group pre-test and between group post-test comparisons. Alphabets indicate significant difference from group C. c = $p < 0.001$, b = $p < 0.01$ and a = $p < 0.05$.

4.5 Serum LH concentration (IU/L)

The mean pre-test and post-test serum concentrations for LH in rabbits that consumed alcohol and cocoa (Group A) were 0.126±0.024 IU/L and 0.03±0.003 IU/L respectively. Analysis revealed that even though there was a sharp decline in mean serum LH concentration after the experiment, this difference was significant (t = 3.440, p = 0.006). The mean concentrations for rabbits that consumed alcohol and water were 0.220±0.076 IU/L and 0.004±0.001 IU/L for pretest and post-test respectively. The difference between the pre-test mean and post-test mean in group B was significant (t = 5.388, p < 0.001). In group C, the mean serum LH concentration from the pre-test was 0.160±0.012 IU/L and 0.074±0.005 IU/L from the post-test. The difference between the two means was also not significant (t = 2.977, p = 0.012) (Table 5).

The mean pre-test LH serum concentrations of the three groups were not significantly different from each other (p = 0.376). Also, the mean post-test LH serum concentrations of the three groups were not significantly different from each other (p = 0.237) (Table 5).

Table 5: Summary of statistics on mean total LH concentration (IU/L) in serum of Groups A, B and C rabbits.

	LH		t-value	*p*- Value 1	95% CI
	Pre-Test	**Post-Test**			
Group A (alcohol-cocoa)	0.126±0.024	0.03±0.0003**	3.440	0.006	-0.344 to -0.087
Group B (alcohol-only)	0.220±0.076	0.004±0.001***	5.388	< 0.001	-0.238 to -0.082
Group C (controls)	0.160±0.012	0.074±0.005*	2.977	0.012	-0.286 to -0.011
P - value 2	0.3756	0.2376			

Values are expressed as mean ± S.E.M. *p* - value 1 represents significance level for paired t-test between pre-test and post-test with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. P-value 2 indicates significance level for one way ANOVA (followed by Tukey's post-hoc) for between-group pre-test and between group post-test comparisons.

4.6 GSH

One way analysis of variance test showed that the activity of GSH was not significantly different in the various experimental groups. Paired t-test analysis showed that GSH activities post-test in Group C (8.920±0.181 mmol/g, t=0.231) rabbit serum were not significantly greater than those in Group A (6.788±0.939 mmol/g, t=1.076). However, there was significant decrease when the value for Group B (2.886±0.370 mmol/g, t=3.931) post-test was compared to Group C post-test concentration (Table 6).

Table 6: Summary of statistics on mean total GSH concentration (mmol/g) in serum of Groups A, B and C rabbits.

	GSH		t-value	*p*- value 1	95% CI
	Pre-Test	**Post-Test**			
Group A (alcohol-cocoa)	7.888±0.805	6.788±0.939	1.076	0.197	-4.461 to 2.262
Group B (alcohol-only)	6.886±0.866	2.866±0.370^{*a}	3.931	0.029	-7.382 to -0.658
Group C (controls)	9.156±0.830	8.920±0.181	0.231	0.419	-3.598 to 3.125
P - value 2	0.298	0.012			

Values are expressed as mean ± S.E.M. *p* - value 2 represents significance level for One Way ANOVA (followed by Tukey's post hoc) for between group comparison with a = $p < 0.05$, b = $p < 0.01$ and c = $p < 0.001$. *p* - value 1 indicates significance level for one way ANOVA (followed by Tukey's post hoc) for before and after assessment within the groups *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$ with alphabets indicating significant difference from Pre-test.

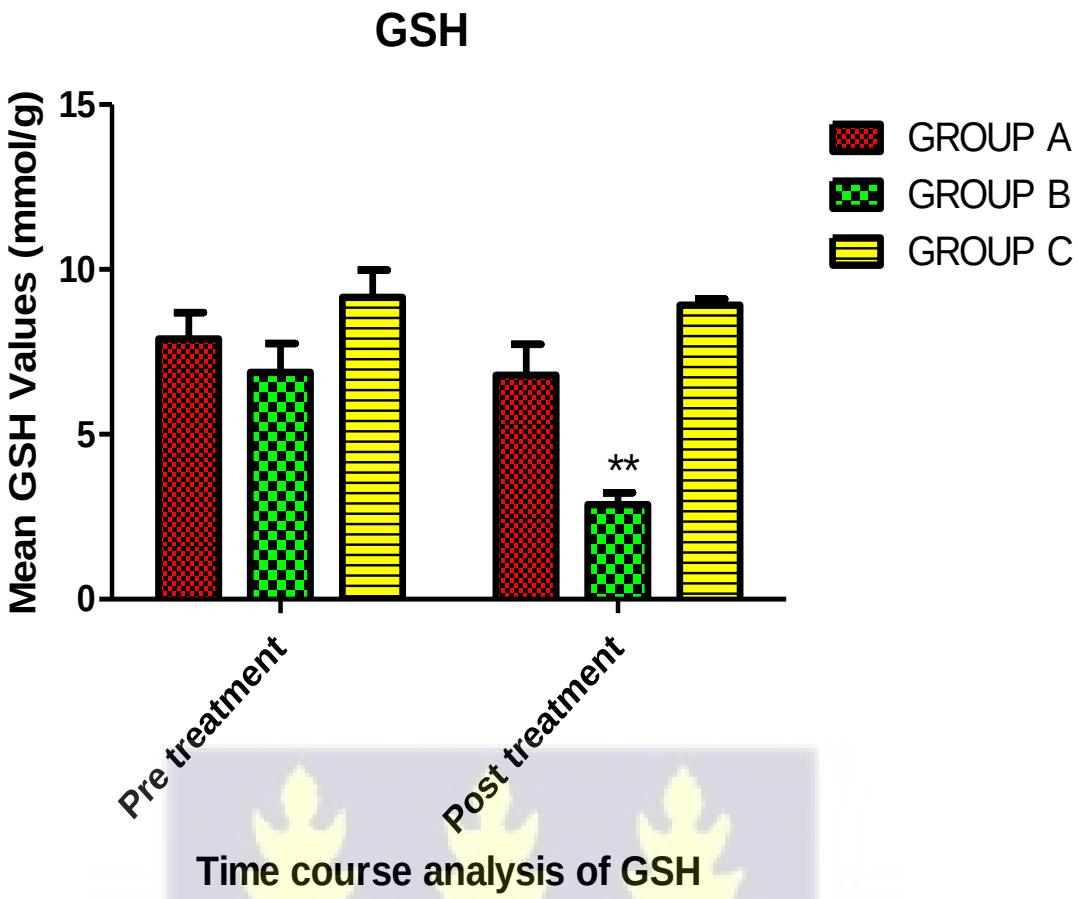

Figure 22: A bar chart showing mean concentrations of GSH for before and after treatment in Groups A, B and C rabbits. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to respective controls.

4.7 SOD

One way analysis of variance test showed that the activity of SOD was significantly different in the various experimental groups. Paired t-test analysis showed that SOD activities post-test in Group C (3.301±0.013 U/mg/protein, t=0.092) rabbit serum were not significantly greater than those in Group A (3.295±0.099 U/mg/protein, t=1.027). However, there was significant decrease when the value for Group B (1.055±0.007 U/mg/protein, t=8.194) post-test was compared to Group C post-test value (Table 7).

Table 7: Summary of statistics on mean total SOD concentration (U/mg/protein) in serum of Groups A, B and C rabbits.

	SOD		t-value	*p*- Value 1	95% CI
	Pre-Test	**Post-Test**			
Group A (alcohol-cocoa)	4.154±0.008	3.295±0.099	1.027	0.170	-1.509 to 0.791
Group B (alcohol-only)	3.920±0.047	1.055±0.007^{***C}	8.194	0.001	-4.015 to -1.716
Group C (controls)	3.349±0.134	3.301±0.013	0.092	0.196	-1.117 to 1.182
P - value 2	0.567	0.0002			

Values are expressed as mean ± S.E.M. *p* - value 2 represents significance level for One Way ANOVA (followed by Tukey's post hoc) for between group comparison with a = $p < 0.05$, b = $p < 0.01$ and c = $p < 0.001$. *p* - value 1 indicates significance level for one way ANOVA (followed by Tukey's post hoc) for before and after assessment within the groups with *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$ indicating significant difference from Pre-test.

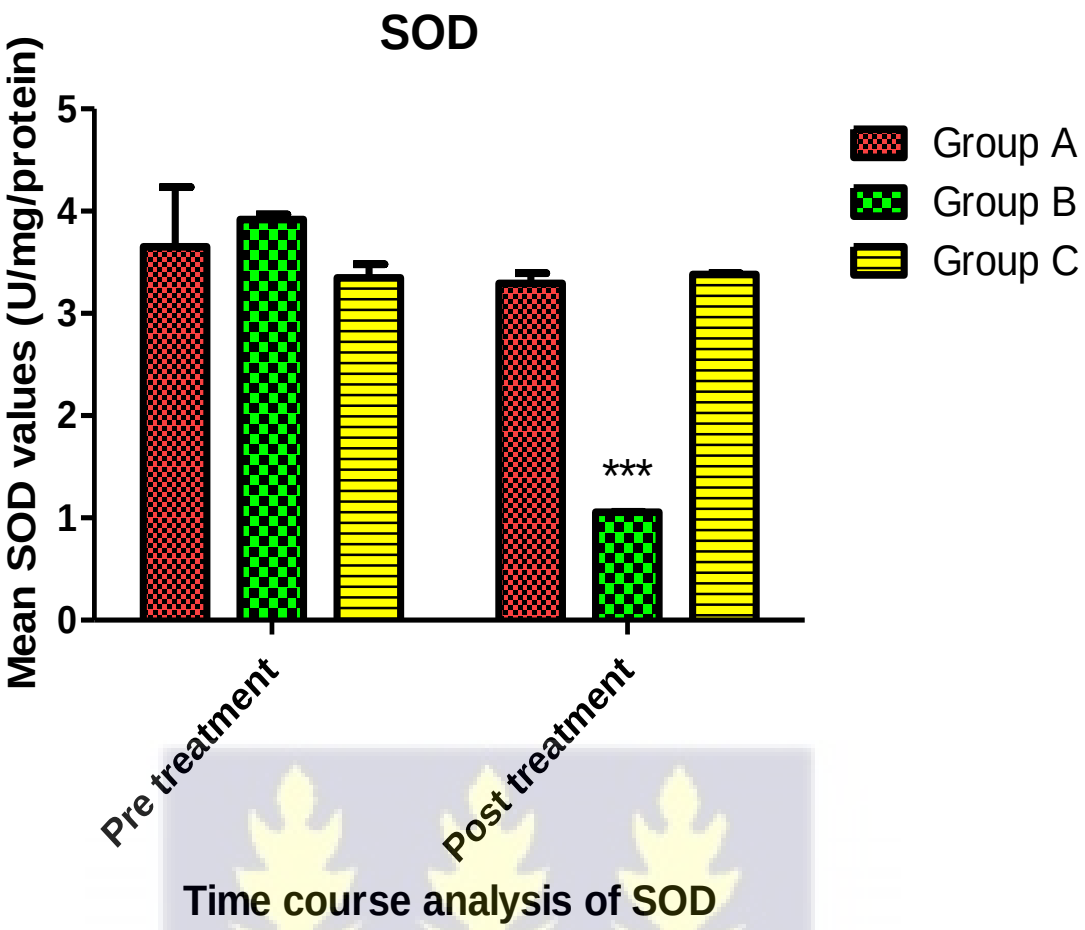

Figure 23: A bar chart showing mean concentrations of SOD for before and after treatment in Groups A, B and C rabbits. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to respective controls.

4.8 Mean ovary weights and volume in Group A, B and C.

Mean ovary weights between the three groups was shown not to be significant in the One-Way ANOVA test (p = 0.754). The mean ovarian weight in group A was 0.082±0.007 g. Group C had a mean ovary weight of 0.083±0.013 g. However, group B recorded an insignificant decrease in ovarian weight following alcohol exposure (0.074±0.007 g) as compared to group C.

For the mean ovary volumes, there was no significant difference between the three groups in the One-Way ANOVA test (p = 0.761). The mean ovary volume in group A was 0.039±0.004 ml. Group B with mean ovary volume of 0.036±0.004 ml saw an insignificant decrease when compared to the control group, C (0.040±0.006 ml).

Table 8: Summary of statistics on mean ovary weights and volume of Groups A, B and C rabbits.

	Group A (alcohol-cocoa)	**Group B (alcohol-only)**	**Group C (controls)**	***p – value***
	n=5	n=5	n=3	
Ovary weights (g)	0.082±0.007	0.074±0.007	0.083±0.013	0.754
Ovary volume (ml)	0.039±0.004	0.036±0.004	0.040±0.006	0.761

Values are expressed as mean ± S.E.M. *p* - value denotes significance level for One Way ANOVA (followed by Tukey's post hoc) for between-group comparison with *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$.

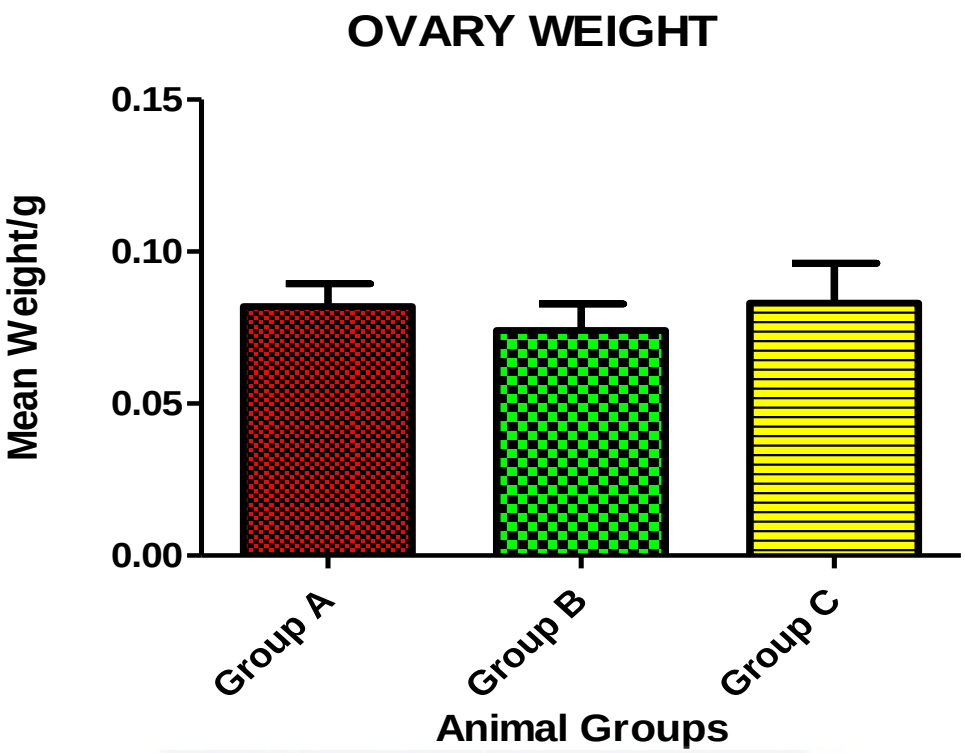

Figure 24: A bar chart showing means of ovary weights for rabbits in Groups A, B and C. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to control group.

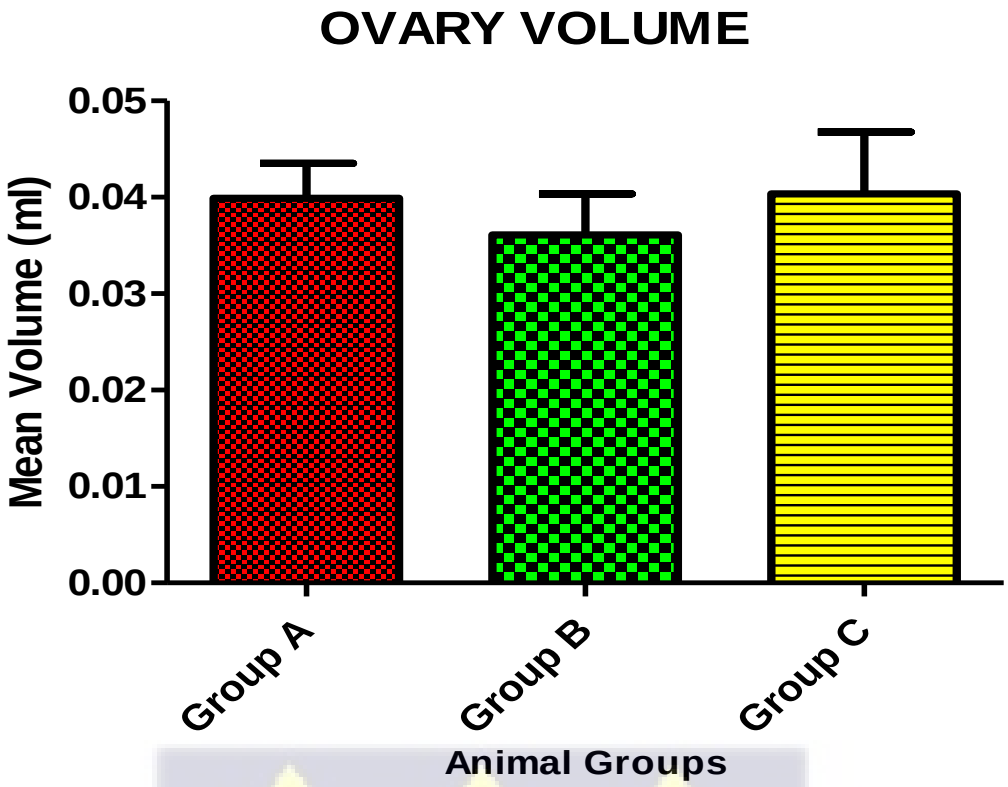

Figure 25: A bar chart showing means of ovarian volume for rabbits in Groups A, B and C. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to control group.

Table 9: Summary of statistics on mean volume densities of follicles and stroma and surface area of Graafian follicles of Groups A, B and C rabbits.

	Group A (alcohol-cocoa)	Group B (alcohol-only)	Group C (controls)	*p – value*
Primordial follicle (μm^3)	10.17±1.13	17.33±0.92**	11.38±1.32	0.002
Primary follicle (μm^3)	1.84±0.59	2.53±0.91	1.50±0.41	0.639
Secondary follicle (μm^3)	2.15±0.28	1.79±0.41	2.55±0.11	0.696
Ovarian stroma (μm^3)	275.50±43.25	442.00±26.83**	238.60±5.81	0.004
Corpora Atretica (μm^3)	1.38±0.23	2.34±0.10*	1.24±0.28	0.006
Graafian follicle (μm^2)	21.55±4.14	10.77±2.10	19.93±1.95	0.068

Values are expressed as mean ± S.E.M. *p* - value denotes significance level for One Way ANOVA (followed by Tukey's post hoc) for between-group comparison with *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$. n=1,300 micrographs.

4.9 Primordial follicles

The mean volume density of primordial follicles for the various groups of rabbits is shown in table 9 and figure 26. The difference between mean volume densities of primordial follicles was significant when the three groups were compared (p = 0.002). Further comparison of the means showed that it was the mean volume density in group B (17.33±0.92 µm^3) that was significantly larger than that of the control group (p < 0.01). The difference between the means of group A (10.17±1.13 µm^3) and group C (11.38±1.32 µm^3) was not significant (Figure 26).

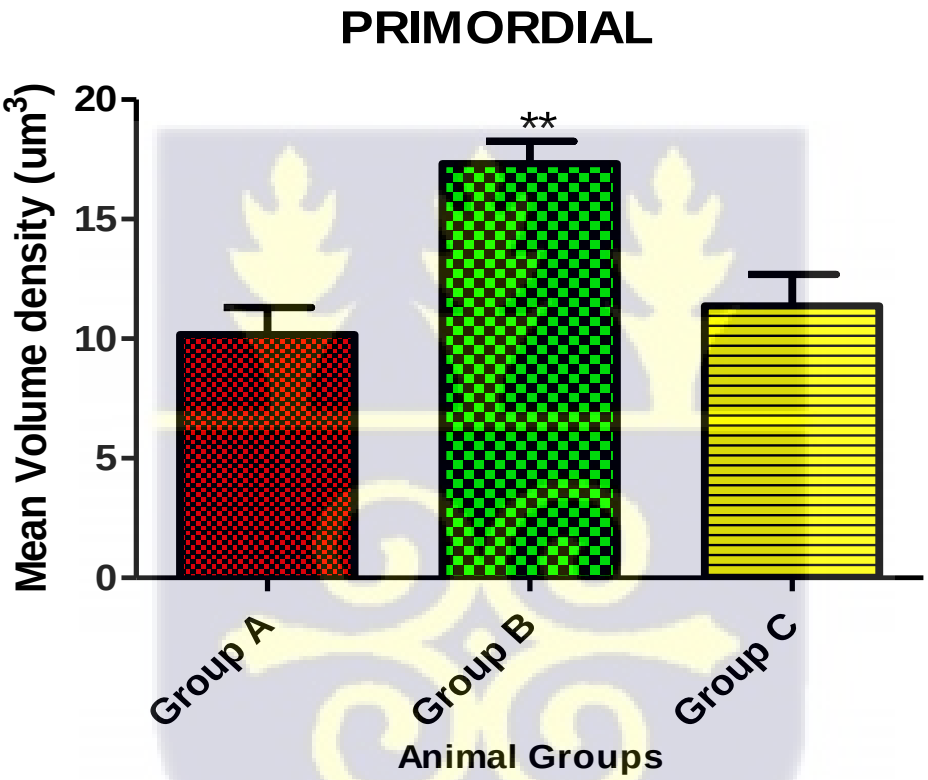

Figure 26: A bar chart showing mean volume densities of primordial follicles for rabbits in Groups A, B and C. Error bars represent S.E.M. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to control group.

4.10 Primary follicles

The mean volume densities for primary follicles did not significantly differ from each other when the three groups were compared (p = 0.638). The means were 1.84±0.59 µm^3 for group A, 2.53±0.91 µm^3 for group B and 1.50±0.41 µm^3 for group C (Figure 27).

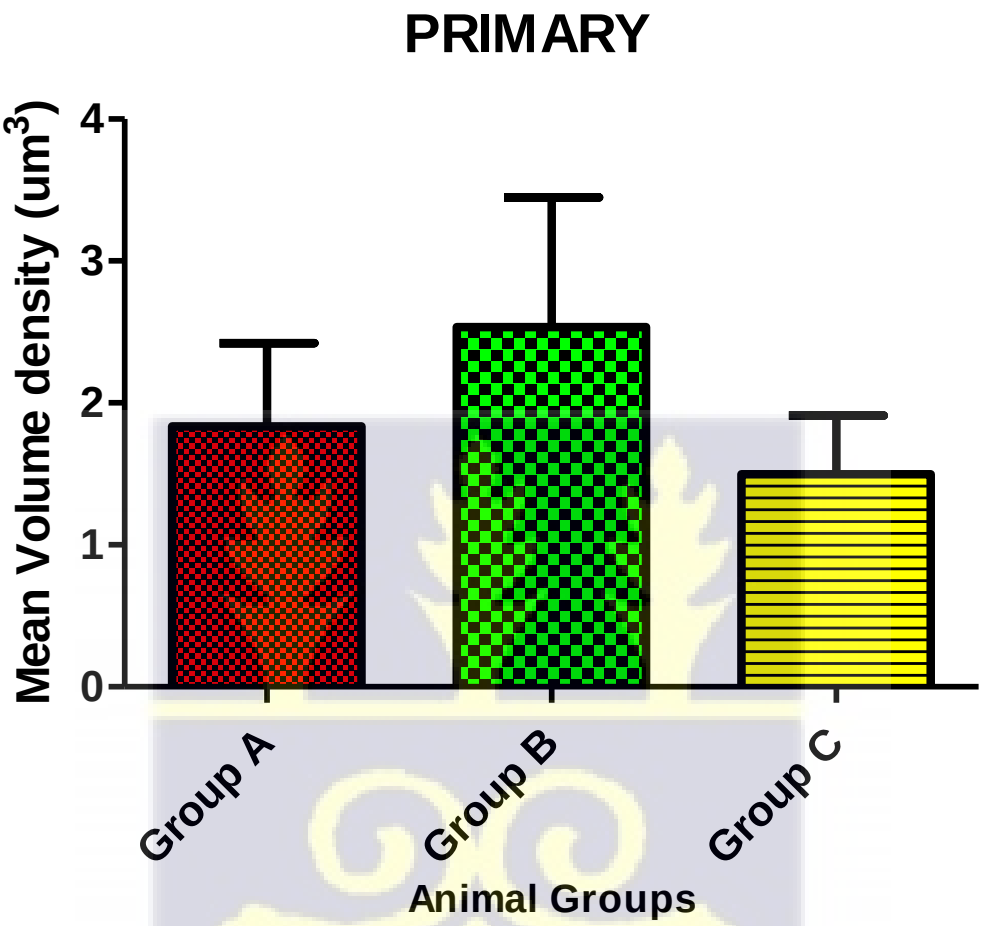

Figure 27 A bar chart showing mean volume densities of primary follicles for rabbits in Groups A, B and C. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to control group.

4.11 Secondary follicles

The results for the mean volume densities of the secondary follicles showed no statistically significant differences (p = 0.639). The means were 2.15±0.28 µm^3 for group A, 1.79±0.41 µm^3 for group B and 2.55±0.11 µm^3 for group C (Figure 28).

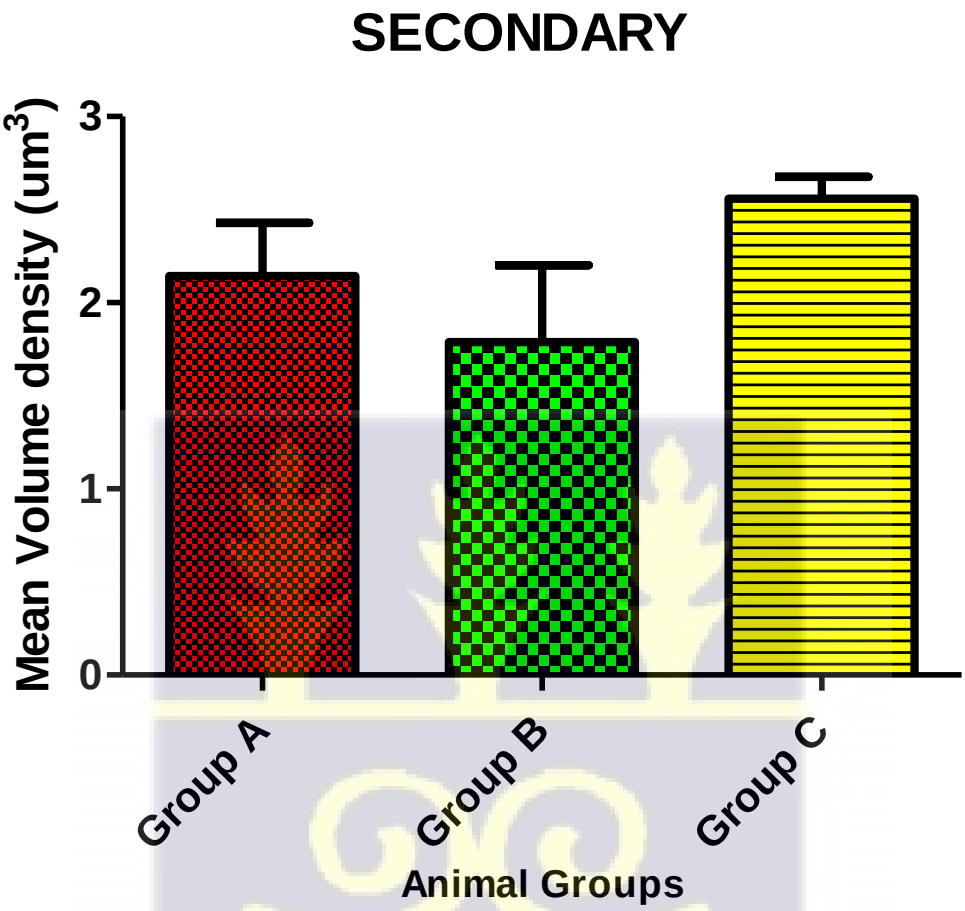

Figure 28 A bar chart showing mean volume densities of secondary follicles for rabbits in Groups A, B and C. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to control group.

4.12 Ovarian Stroma

The difference between mean volume densities of ovarian stroma was significantly different when the 3 groups were compared ($p = 0.004$). Further comparison of the means showed that it was the mean volume density in group B (442.0±26.83 μm^3) that was significantly larger than that of the control group (238.6±5.81 μm^3) ($p < 0.01$). The difference between the means of group A (275.5±43.25 μm^3) and group C was not significant (Figure 29).

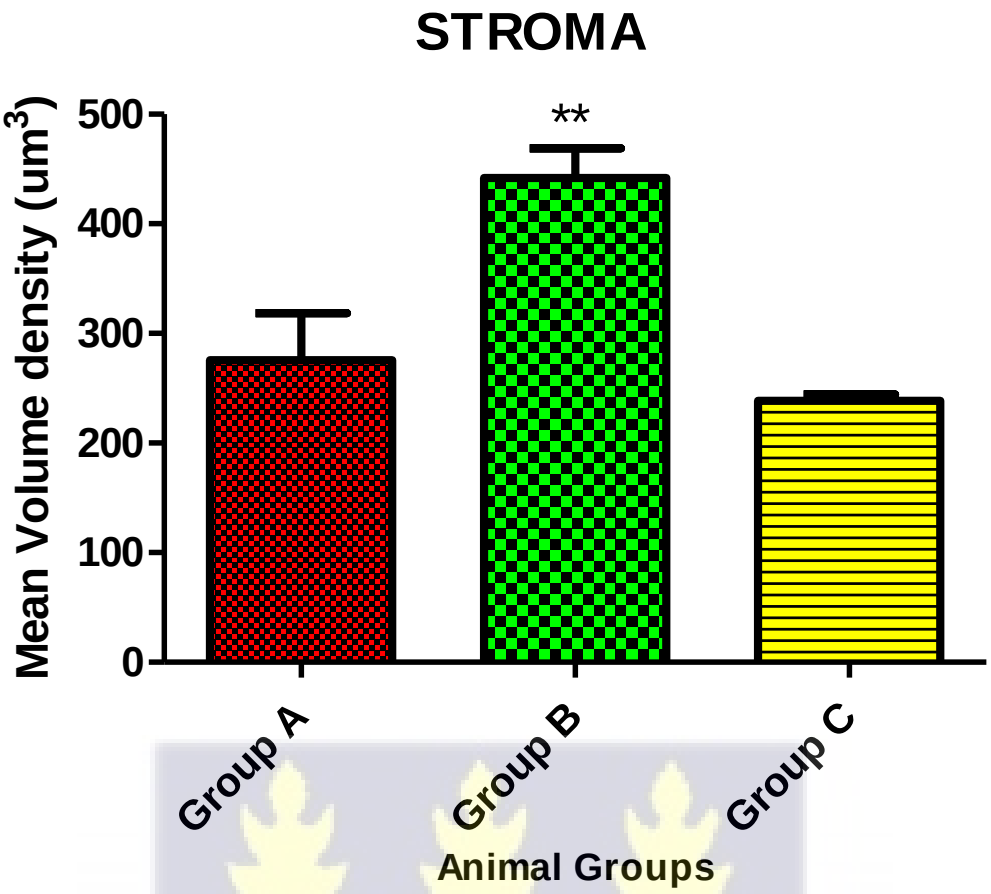

Figure 29 A bar chart showing mean volume density of ovarian stroma for rabbits in Groups A, B and C. Error bars represent S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to control group.

4.13 Corpora Atretica

The difference between mean volume densities of corpora atretica was significant when the three groups were compared (p = 0.006). Supplementary comparison of the means revealed that the mean volume density in group B (2.33±0.10 µm^3) was significantly larger than that of the control group, C (p < 0.01). The difference between the means of group A (1.375±0.23 µm^3) and group C (12.42±0.28 µm^3) was not significant (Figure 30).

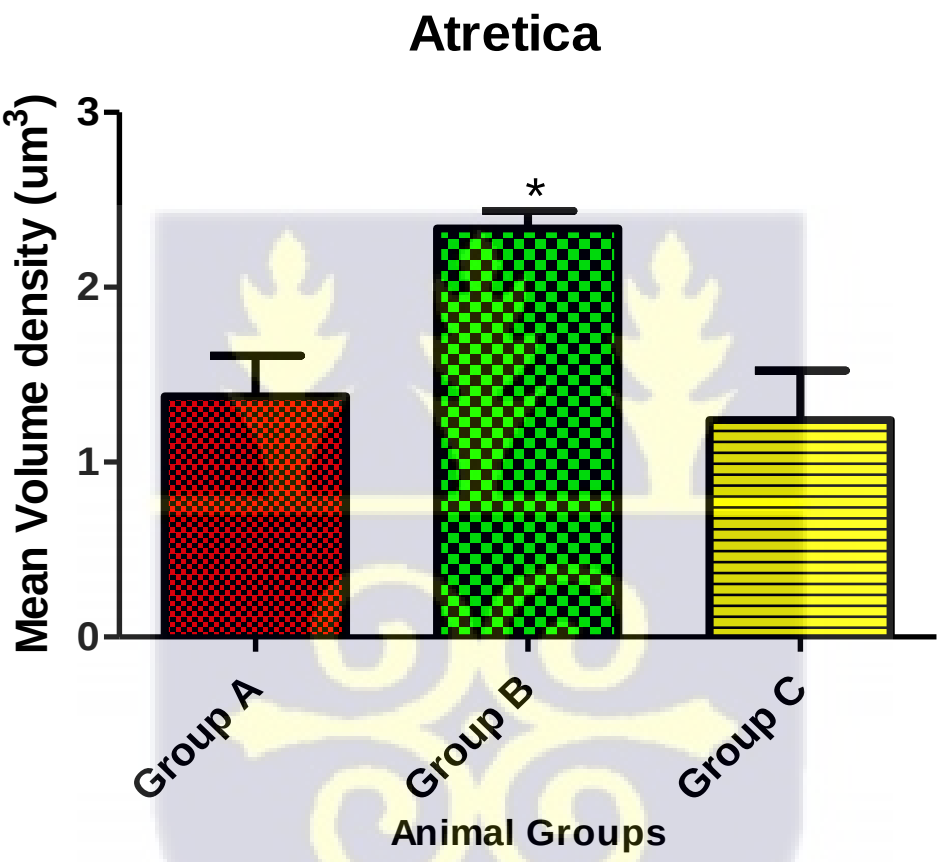

Figure 30 A bar chart showing mean volume densities of corpora atretica for rabbits in Groups A, B and C. Error bars represent S.E.M. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to control group.

4.14 Graafian follicles

The results for the mean surface areas of Graafian follicles showed no statistically significant differences (p = 0.0678). The means were 21.55±4.14 µm^2 for group A, 10.77±2.10 µm^2 for group B and 19.93±1.93 µm^2 for group C (Figure 31).

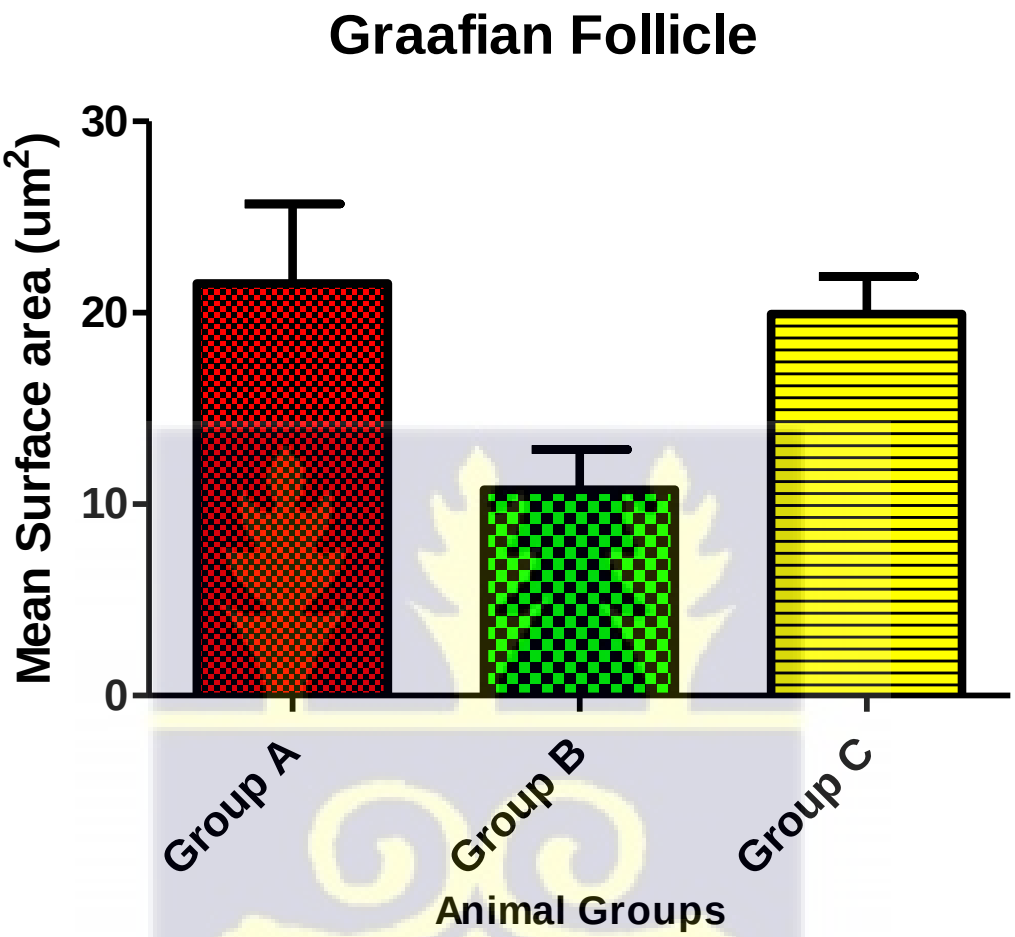

Figure 31 A bar chart showing mean surface areas of Graafian follicles for rabbits in Groups A, B and C. Error bars represent S.E.M. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to control group.

4.15 Morphology of ovarian tissue

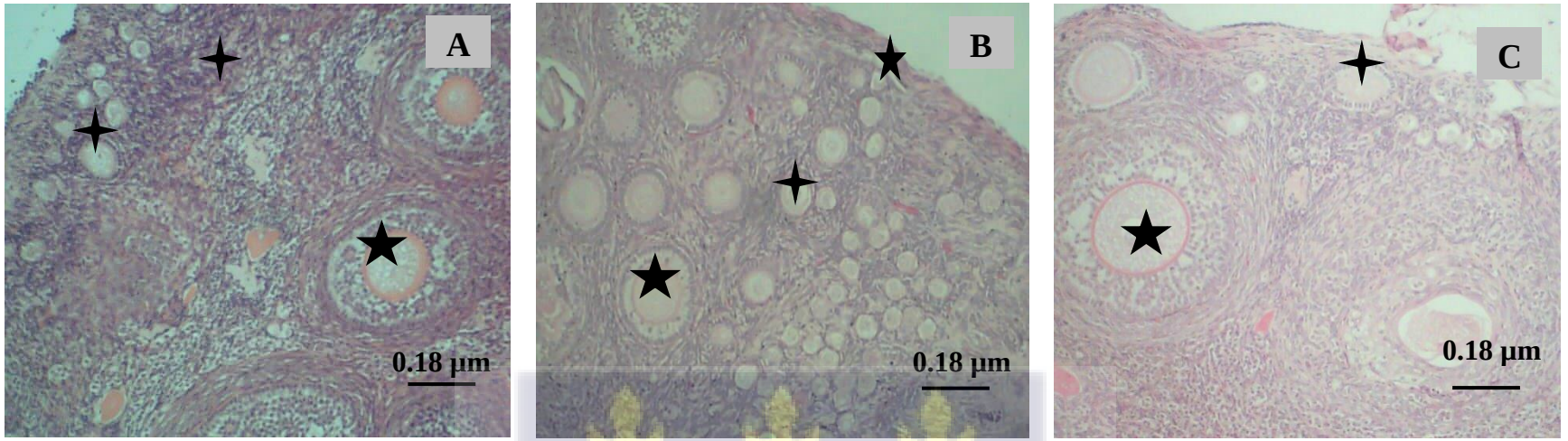

Figure 32 Histomorphology of the ovary showing primary and secondary follicles groups A, B and C. Primary follicle is indicated by (four-point star) ✦ and secondary follicle is indicated by (five-point star) ★ .

Figure 32 represents haematoxylin and eosin staining of ovarian tissue at the end of the experiment. The alcohol-cocoa group (B) showed numerous secondary and primary follicles. For groups A and C primary and secondary follicles were few in number.

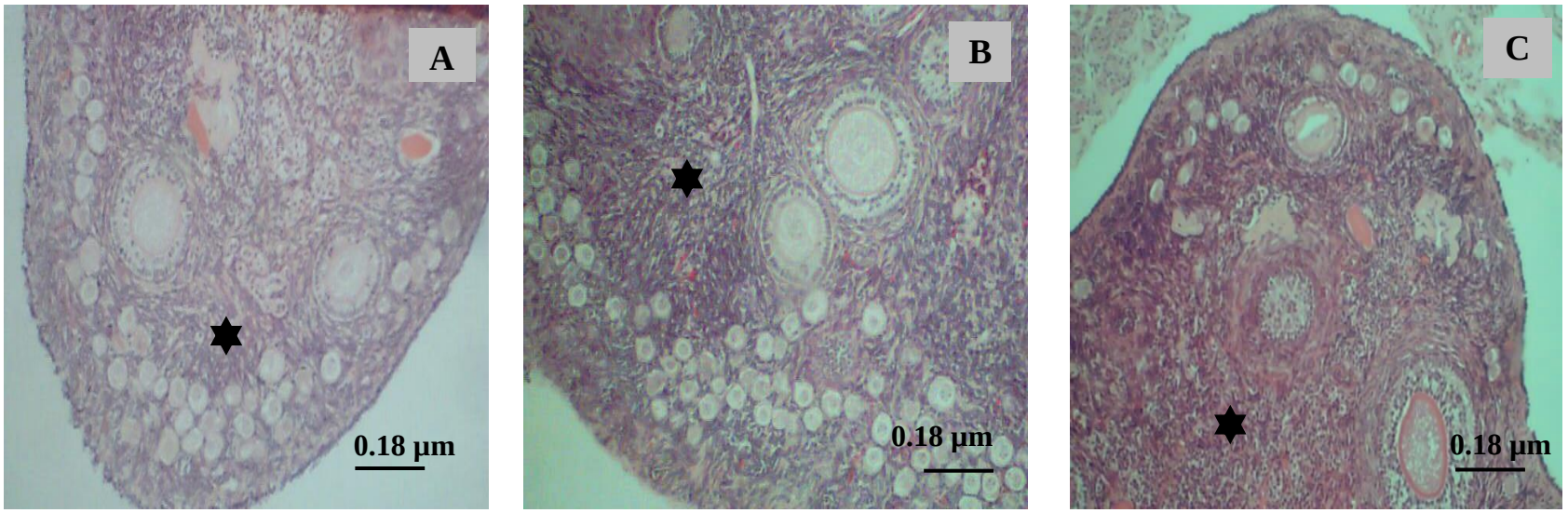

Figure 33 Histomorphology of the ovary showing primordial follicles and stroma in the groups A, B and C. Stroma is indicated by (six-point star) ✸.

Figure 33 denotes haematoxylin and eosin staining of ovarian tissue at the end of the experiment. The alcohol-cocoa group showed numerous primordial follicles located within the inner layer of the ovarian cortex with little ovarian stroma. For groups A and C, primordial follicles were located at the outer layer of ovarian cortex with ample ovarian stroma.

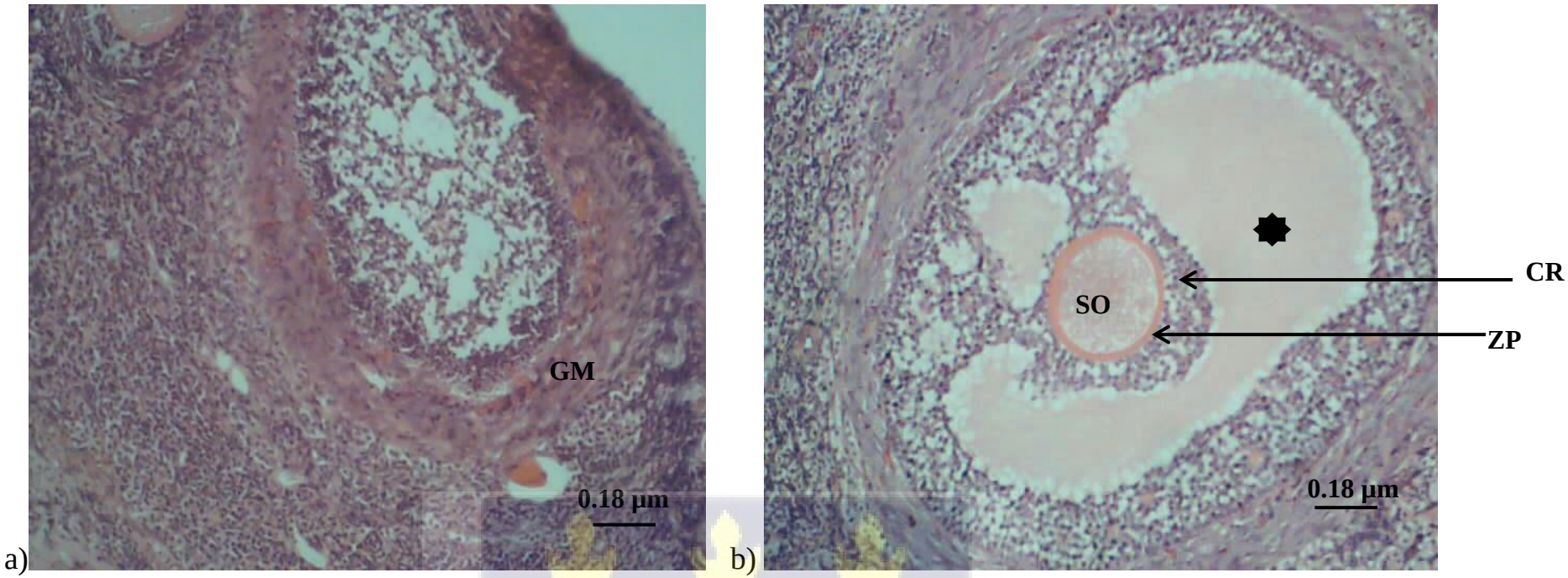

Figure 34 Histomorphology of the ovary showing: a) corpus atreticum b) Graafian follicle.

Figure 34 represents haematoxylin and eosin staining of ovarian tissue showing an atretic follicle and Graafian follicle. The corpus atreticum is characterized by the basement membrane that separates the granulosa cells from the vascularized theca interna. This structure persists in the atretic follicles and appears as a collapsed structure known as the "glassy membrane" (GM). The Graafian follicle in (b) is characterized by a large antrum ✹, which contains the liquor folliculi, the secondary oocyte (SO), surrounded by the zona pellucida (ZP) and a layer of several cells known as the corona radiata (CR).

CHAPTER FIVE

5 DISCUSSION

5.1 General

There is a convincing body of evidence showing that ROS leading to OS is often implicated in ovary related infertility conditions which eventually lead to the diagnosis, usually termed as unexplained infertility (Agarwal et al., 2012; Balasaheb & Pal, 2015). Balasaheb & Pal (2015) stated in their work that by increasing the intake of natural antioxidants, tolerable antioxidant status may be maintained and perhaps normal physiological function could be achieved. As it was expected, the antioxidant property of natural cocoa ameliorated the effect alcohol had on the ovarian tissue. The antioxidant properties of natural cocoa may also be accountable for many of their pharmaceutical effects, which include the prevention of lipid peroxidation and the protection of low density lipoprotein-cholesterol against oxidation, and increase resistance to OS (Andújar et al., 2012). This was manifested by the close resemblance in histology of ovarian tissue in rabbits of Group A (cocoa-alcohol fed) to that of rabbits in Group C (controls). Also, biochemical tests conducted before and after treatment reinforced the results exhibited by rabbits in Group A (cocoa-alcohol fed).

5.2 Total fluid consumption

Total fluid in this research consist of alcohol and natural cocoa, alcohol and water and water only consumed by the experimental and control groups (Groups A, B and C) respectively. Percentage of alcohol and the frequency at which it is consumed are some factors considered in assessing the damaging effects of alcohol on major tissues in the body including the ovaries. The total fluid intake by rabbits of the various groups

corresponded to the findings associated with the results obtained. From the results, it was evident that feeding rabbits 35% alcohol for 12 hours created changes in the histology of the ovary (see figure 32 and 33).

Rabbits in Group B (alcohol-only) recorded the highest total fluid intake throughout the 7 weeks of the experiment as compared to the other two groups. It is apparent that alcohol only fed animals consumed more water as they were likely to be dehydrated. This could have contributed to the high volumes of water consumed by group B (alcohol-only) rabbits which added up to the total volumes of fluid consumed prospectively. It has been shown that once alcohol has been consumed and absorbed, it is circulated to all tissues and fluids of the body in direct quantity to the water content (Wilkinson, Sedman, & Sakmar, 1977). The equilibrium concentration of alcohol in a tissue is dependent on the relative water content which may lead to dehydration, and could explain why the rabbits consumed more water than they normally would. The diuretic effect of alcohol has been attributed also to the direct action it has on the kidneys. Alcohol's effect on the renal epithelium allows water to pass but holds back potassium in large quantities while sodium, chloride and nitrogen are held back in smaller quantities. This inhibits the reabsorption of water, hence the passing out of copious volume of urine. Again, it is believed that alcohol can decrease the secretion of the anti-diuretic hormone or inactivate the circulation of it entirely (Strauss et al., 1950).

It is likely that, rabbits in groups A and B (alcohol-cocoa and alcohol only) had to consume more cocoa and water respectively to compensate for the dehydration they suffered due to alcohol intoxication.

The experimental model used in replicating alcohol-induced ovarian injury in rabbits in this study was partially successful. However, the duration of experiment can be extended to about 14 weeks to actually establish the visible histological changes observed in the study performed by Gavaler et al., (1980).

5.3 Effect of natural cocoa on LH and FSH in alcohol induced ovarian injury

It has been proven that alcohol has injurious effects on all three components of the hypothalamus, pituitary, and gonads and also dampens the efficiency of the axis, modifying some reproduction factors such as hormone secretion, estrous cycle, ovulation, follicular maturation and fetal evolution (Alfonso et al., 1993; Rachdaoui & Sarkar, 2014). Chronic consumption of alcohol upsets the communication between endocrine system and causes hormonal imbalance that lead to serious consequences at physiological levels (Rachdaoui & Sarkar, 2014).

It was noted in this current study that rabbits that consumed alcohol only (group B) had significantly reduced levels of FSH post-treatment (see table 4). A similar pattern is also noticed with respect to LH levels. Rabbits in group B recorded a significant decrease post-treatment in LH levels (paired t-test, $p < 0.001$) (table 5). These results are in support of earlier study reports.

Natural cocoa consumption seemed to have ameliorated the effect alcohol had on these hormones. For rabbits in group A (alcohol-cocoa), LH and FSH levels post-treatment were not significantly different even though there were obvious reductions, looking at the pre-treatment values of LH and FSH levels.

A study conducted by Van-Thiel *et al* (1978), in rats which were alcohol-fed, pair-fed

isocalorically and intact ad-libitum control groups, it was reported that plasma levels of FSH within the groups of rats weren't statistically different. In the case of LH, plasma levels of the alcohol-fed rats were increased. This contradicts what was reported by Alfonso *et al*., (1993) that pre-ovulatory surge is inhibited by alcohol. These differences could be attributed to the fact that rats have regular estrus cycles whereas, whereas, rabbits don't. It takes coitus for ovulation to occur in rabbits and that usually happens 10 to 12 hours post-coitus. This is the time pre-ovulatory surge of LH occurs to initiate ovulation. Ogilvie and Rivier (1997) reported that alcohol administration blocked the pro-estrous surge of LH and ovulation in rats. These findings were supported by Rachdaoui and Sarkar (2014).

With the above reports, it is clear that alcohol interrupts the usual sequence of events during ovulation, whether it increases or decreases plasma or serum LH levels. Pre-mature initiation of the pre-ovulatory surge could lead the release of incompetent secondary oocytes and drastically reducing the LH levels in plasma could also lead to anovulation which sends the mature follicles into regression.

Low serum levels of FSH could be responsible for the high volume densities of primordial follicles in rabbits in group B (alcohol-only). This is because the recruiting of follicles to mature into antral follicles via the process of folliculogenesis could not occur. FSH is not only responsible for the stimulation of follicle growth in the ovary; it is also indicated in assisting follicle growth after antrum formation and in stopping apoptosis. This has led to the theory that FSH is a survival factor for antral follicles (Craig *et al*., 2007).

5.4 Effect of alcohol and natural cocoa on GSH and SOD activities

Alcoholic beverages such as beer and wine contain antioxidants, yet when abused, alcohol becomes a pro-oxidant instead of an antioxidant (Dasgupta & Klein, 2014). The consequences of alcohol metabolism include hypoxia in the liver and formation of ROS that can damage other cell and tissue components (Zakhari, 2017). Alcohol metabolism is followed by the generation of acetaldehyde, that may be implicated in tissue damage, the formation of ROS, and a change in the redox state of liver tissue (Zakhari, 2017).

Alcohol also is metabolized in non-liver tissues that do not contain ADH, such as the brain, by the enzymes cytochrome P450 2E1 and catalase (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2010). Cytochrome P450 2E1 is induced by chronic alcohol consumption and assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. It also implicated in the production of ROS, including OH^-, $O2^-$, H_2O_2 which increase the risk of tissue damage (Zakhari, 2017).

Catalase oxidizes alcohol in vitro in the presence of a H_2O_2 generating system, such as the enzyme complex NADPH oxidase or the enzyme xanthine oxidase. Increased catalase activity has been indicated in rats that chronically consume alcohol. This has been shown to result in increased H_2O_2 production in pericentral regions of the liver (Handler & Thurman, 1990; Misra et al., 1992; Zakhari, 2017).

In this study, experimental rabbits that were exposed to only alcohol saw a significant reduction in the activities of GSH and SOD after seven weeks of treatment. This is in line with studies conducted by Dosumu *et al* (2014). They submitted that alcohol depletes GSH levels via the generation of oxidants as well as by inhibiting the mitochondrial glutathione transporter. Inhibition of the transport of GSH from the cytosol into the

mitochondria leads to depletion in the mitochondrial pool of GSH after alcohol intake. Glutathione, however, is a non-enzymatic antioxidant which works by interrupting free radical chain reactions (Balasaheb & Pal, 2015). GSH and SOD are enzymatic antioxidants. These antioxidants convert dangerous oxidative products to H_2O_2 and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese, and iron (Agarwal et al., 2008; Balasaheb & Pal, 2015; Krajcir et al., 2008).

High OS levels together with antioxidant concentrations in peritoneal fluid and serum have also been validated in infertility of unknown etiology. Increasing intake of natural antioxidants has been said to help to maintain a tolerable antioxidant status, perhaps restore and sustain normal physiological function (Balasaheb & Pal, 2015). Consequently, Sekhon *et al*., (2010) suggested that oral antioxidant supplementation may ameliorate OS and its contribution to the pathogenesis of obstetrical conditions.

Consumption of natural cocoa drink by group A (alcohol-cocoa) rabbits showed higher concentrations of GSH and SOD. Post-test values of group A (cocoa-alcohol) were not significantly different when compared to group C (control group) (see tables 6 and 7).
The closeness of the means of the post-test to pre-test GSH and SOD values of rabbits in group A ((alcohol-cocoa) suggested that natural cocoa antioxidant polyphenols like catechin and epicatechin may have contributed significantly in maintaining the levels of these enzymatic antioxidants even in the presence of excessive ROS production caused by the consumption and metabolism of alcohol.

5.5 Stereological evaluation of ovarian injury

Design-based stereology, in recent years has been a useful tool employed by researchers to quantify damage or injury to tissues. Stereology is a method that utilizes random and systematic sampling to provide unbiased and quantitative data. The volume fraction of a component within a reference volume is a simple and very widely used parameter in biomedical sciences. In this study, stereological and histomorphometric methods were used for analysis. The Cavalieri principle, one of the stereological methods, was employed to estimate the volume densities of the primordial, primary and secondary follicles, stroma and corpora atretica. Surface area was used to quantify the area fraction of the Graafian follicles (Guven *et al*., 2013). The systematic, random approach used in sampling the ovary tissue made it possible to stereologically study and quantify damage in all the ovaries (left and right) of rabbits in the experimental groups. Because of the number of micrographs (1,300) used in the stereology, results obtained, stand to be descriptive with significant statistical precision.

The high volume density of primordial follicles in the ovaries of alcohol-only fed rabbits makes certain organ injury. High volume densities of primordial follicles could be as a result of low or irregularities in levels of FSH in the blood making it difficult for follicles to be recruited and initiate follicular maturation. Ovarian injury is characterized by lack of folliculogenesis and ovarian oestrogen production (Abd-allah *et al*., 2013; Altunkaynak *et al*., 2016). Abd-allah *et al* (2013), however, reported that a few primordial follicles and degenerated growing and antral follicles were noticed. This could explain the high volume fractions of primordial follicles which ended up in degeneration.

As shown by the stereological study of volume density (table 9), cocoa intake

significantly prevented the effect of alcohol toxicity had on rabbits in group A (cocoa-alcohol) when compared to the controls, group C.

For other functional elements of the ovarian parenchyma such as primary, secondary and Graafian follicles, there were no significant differences in the volume fractions between the experimental groups. Abd-allah *et al* (2013), reported, reduction of total number of primary, secondary and tertiary follicles. Also, mean volume of medulla, cortex, and corpora lutea was decreased. In another study, thickening of tunica albuginea, irregular follicle and oocyte borders and hydropic degeneration in follicular granulosa and increase of fibril in connective tissue were detected (Altunkaynak *et al*., 2016). The observation of irregular follicle and oocyte borders were not made in this study. However, in this study, many maturing follicles were degenerated and this was characterized by the smooth appearance the basement membrane exhibits while being detached from the theca cells.

Van-Thiel *et al* (1978), showed in his study that ovarian tissue in alcohol-fed animals consisted of dense stroma, a few immature follicles but no or little large well developed follicles were seen. They also purported corpora lutea were absent in most of the alcohol-fed ovaries. The absence of corpora lutea indicates anovulation. Dense stroma, hypercellularity of stromal cells usually contains numerous blood vessels, hypervascularity. Blood vessels had prominent wall thickening and smooth muscle proliferation. Some of these findings corroborate what was obtained in this study.

Volume density of stroma in alcohol-only fed ovaries recorded up to 95% increase when compared to group C (controls). Group A (cocoa-alcohol) rabbits had their ovaries record similar values as those of group C (controls), indicating that cocoa ameliorated the toxic effects of alcohol on ovarian tissue.

The differences in the means of the volume density of corpora atretica between the experimental groups corresponded to Van-Thiel *et al* (1978) study. They recorded increased volumes of atretic follicles. Ovaries of Group B (alcohol-only fed) rabbits recorded a mean volume density of 2.335±0.10 μm^3 which was significantly different when compared to groups A (cocoa-alcohol) (1.375±0.23 μm^3) and C (controls). It is probable that Group B (alcohol-only) recorded relatively high volume densities of corpora atretica because of the degeneration of large numbers of primordial follicles and also mature Graafian follicles that could not ovulate their secondary oocytes. Group A (cocoa-alcohol) had close to the volume density of atretic follicle group C (controls) (1.242±0.28 μm^3) ovaries had. This points towards the protective effect natural cocoa has on the follicles, whether maturing or matured.

5.6 Effect of alcohol on ovary weight and volume of rabbits

Some studies have shown that ovaries of alcohol-fed rats weighed significantly less ($p < 0.05$) than their isocaloric controls (Gavaler et al., 1980). However, in this study there were no significant differences between the mean weights and volume of the ovaries. Group B (alcohol-only) rabbits recorded relative reductions in ovary weight and volume with respect to rabbits in group C (controls) but these were statistically not significant. Gavaler *et al* (1980) however, observed a striking histological appearance of the ovaries from alcohol-fed rats. The histology revealed loose stroma, a few immature follicles, and a complete absence of corpora lutea and corpora hemorrhagica. The absence of corpora lutea and corpora hemorrhagica in Gavaler *et al* (1980) study indicates ovulation failure. Their study lasted for four months.

The histology of ovaries of rabbits fed alcohol-only in this study revealed dense stroma

and an increase in the volume density of primordial follicles. These structural changes did not translate into the gross level such that they did not significantly affect the weights and volume of the ovaries of rabbits in group B. This is likely due to the duration of the experiment; seven weeks. Also, these variations were not observed in this study because rabbits are considered to be 'reflex ovulators' and only ovulate post-coitus. In an earlier study by Van-Thiel, Gavaler, Lester and Sherins (1978), ovaries of alcohol-fed animals weighed two-fifths as much as those from the pair-fed isocaloric controls and one-third as much as the intact *ad libitum* controls. In this particular study, the experiment ran for 49 days. They reported that the greater part of the ovarian tissue of the alcohol-fed animals consisted of dense stroma. A few immature follicles could be identified but no large, well-developed follicles were seen. Corpora lutea and corpora heamorrhagica were absent in 90% of the ovaries obtained from alcohol-fed animals. Thus, the reduction in ovarian mass in the alcohol-fed animals was due primarily to an absence of developing follicles, corpora lutea, and corpora hemorrhagica. These findings support what was obtained in this particular study, which were low volume fractions of maturing follicles; primary secondary, Graafian follicles and high volume fractions of dense stroma.

In comparing the mean ovary weights and volume, it is clear that the values of ovary weights and volume of rabbits of group A (cocoa-alcohol) are as close to those in group C (controls). Since there was no significant reduction in the ovary weight and volume of rabbits that were fed with cocoa and alcohol as compared to the controls, it is likely that cocoa antioxidants reduced ROS activities; which is involved in alcohol induced ovarian injury.

5.7 Body weight assessment of rabbits

One of the known indicators of chronic alcohol consumption is malnutrition which may lead to weight loss. As part of evaluating the effect of natural cocoa on the ovaries of dipsomaniac rabbits, body weights were determined as an index of growth during the 7 weeks of the experiment. In the first five weeks of the experiment, no significant differences in weight between groups were recorded ($p > 0.05$). However, in the seventh week the differences between the mean weights were significant ($p = 0.01$) (Table 3). Rabbits in group B (alcohol-only) suffered a significant decrease in weight (1.782±0.064 kg, $p < 0.01$) when compared to group C (2.417±0.252 kg) (see figure 20).

It is known that alcohol interferes with the absorption of essential nutrients and leads to avitaminosis (Agarwal *et al*., 2012; Reddyvari *et al*., 2017). Dipsomaniacs experience OS for two reasons: poor nutritional status which as a result of consumption of alcohol in excess lowers the concentration of antioxidants in the blood. Again, dipsomaniacs are malnourished because they ingest fewer nutrients such as carbohydrate, protein and vitamins than non-dipsomaniacs do. It could also be that alcohol and its metabolism interfere with appropriate digestion and absorption of these nutrients. It has been established that dipsomaniacs suffer from protein, mineral, and vitamin deficiencies, especially deficiency of vitamin A (Dasgupta & Klein, 2014). This could explain the 74% decrease in weight of rabbits that took only alcohol when compared to rabbits in the control group in the seventh week of treatment.

5.8 Summary of key findings

Post-treatment levels of LH and FSH levels were strikingly decreased in Group B (alcohol-only) rabbits after 7 weeks of 12 hours daily alcohol feeding. Regular consumption of natural cocoa significantly moderated the effect alcohol has on the HPG axis which was evident in the LH and FSH levels of Group A (alcohol-cocoa) as compared to Group C (controls).

Concentrations of GSH and SOD were also decreased in serum of rabbits that consumed alcohol only (Group B). This suggests high anti-oxidant activity in the presence of OS induced tissue injury. Natural cocoa mitigated and somewhat restored antioxidants levels optimally in rabbits that took cocoa and alcohol (Group A).

Natural cocoa appears to positively affect weight and prevent drastic weight loss secondary to alcohol consumption. However, due to time constraints and the feeding protocol used in this research, alcohol could not impact significant weight loss hence cocoa's effect remains dicey in that regard.

Ovarian weight and volume were not significantly affected by alcohol toxicity. This could be as a result of time used, percentage of alcohol and procedure of administration. For this reason, the direct effect of consuming of natural cocoa regularly on organ weight and volume cannot be precisely quantified.

Feeding rabbits with 35% (v/v) alcohol caused changes in ovarian microstructure after 7 weeks of treatment. The pathology achieved by this model of alcohol toxicity was largely hyper-cellularity of ovarian stroma and abundance of primordial follicles located in the

outer and inner cortex of ovary parenchyma. Histologically, this was indicated by hyper-vascularity in the stromal tissue and numerous degenerated and atretic follicles.

Quantification of the volume densities of functional components of ovary parenchyma by means of design-based random and systematic stereology suggested the protective effect of natural cocoa ingestion on the ovary structure and function after 7 weeks of daily alcohol consumption.

5.9 Conclusion

The present study has proven ovarian injury by chronic and voluntary ingestion of 35% alcohol. It has also revealed the probable effect of natural cocoa consumption in decreasing damage to the ovary secondary to chronic alcohol consumption. This however needs further investigation to quantitatively ascertain the fact. It is suggested that cocoa consumption ameliorated and eventually preserved normal ovary morphology significantly. This research further reinforces the knowledge that the pathogenesis of ovarian injury is basically facilitated by OS and anti-oxidant supplementation; the use of nutraceuticals may potentially ameliorate the damage.

5.10 Limitations of the study

1. Further stereological studies, such as estimation of total follicle number and Graafian follicle diameter which could quantify more of the morphological changes, were not performed due to time constraints.
2. Due to time constraints, the maximum effect of alcohol and cocoa were not fully observed.

3. For lack of research funds and other resources, the study did not incorporate the determination of some of the reproductive hormones which could have emphasized the implication of OS in the injury caused by alcohol consumption.

5.11 Recommendations

1. The accurate mechanism by which ovary deals with early onset of injury secondary to alcohol consumption needs to be further explored.
2. The mechanism by which the potential therapeutic use anti-oxidant properties of natural cocoa in ameliorating the ovary from alcohol induced OS needs to be expounded in further research.
3. Percentage of alcohol used, mode of administration and duration of experiment need to be reviewed in further research to clearly ascertain ovarian injury.
4. Hormones such as oestrogen and progesterone should be tested for as proxy markers for ovarian function.
5. Level of alcohol intoxication should be evaluated by determining blood alcohol concentrations to establish a relationship between the exact volumes of alcohol consumed and the degree of injury achieved.
6. Reliable and modern stereological software like *stereo investigator* should be used for stereological studies to avoid going through arduous and time consuming traditional methods.

REFERENCES

Abd-allah, S. H., Shalaby, S. M., Pasha, H. F., El-shal, A. S., Raafat, N., Shabrawy, S. M., … El-kelawy, H. M. (2013). Mechanistic action of mesenchymal stem cell injection in the treatment of chemically induced ovarian failure in rabbits. *Journal of Cytotherapy*, *15*(1), 64–75. https://doi.org/10.1016/j.jcyt.2012.08.001

Abrokwa, F. K., Asamoah, K. A., & Esubonteng, P. K. A. (2009). Effect of intake of natural cocoa powder on some biochemical and haematological indices in the rat. *Ghana Medical Journal*, *43*(4), 164 – 168.

Adaay, M. H., & Mattar, A. G. (2012). Effect of Aqueous and Ethanolic Extracts of Tribulus terrestris , Phoenix dactylifera and Nasturtium officinale Mixture on Some Reproductive Parameters in Male Mice. *Baghdad Science Journal*, *9*(4), 640–650.

Addai, F. K. (2010). Natural cocoa as diet-mediated anti-malarial prophylaxis. *Medical Hypotheses*, *74*, 825–830.

Agarwal, A., & Allamaneni, S. (2004). Role of free radicals in female reproductive diseases and assisted reproduction. *Reproductive BioMedicine Online*, *9*(3), 338–347.

Agarwal, A., Aponte-Mellado, A., Premkumar, B. J., Shaman, A., & Gupta, S. (2012). The effects of oxidative stress on female reproduction : A review. *Reproductive Biology and Endocrinology*, *10*(49), 1–31.

Agarwal, A., Gupta, S., & Sharma, R. K. (2005). Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology*, *3*(28), 1–46. https://doi.org/10.1186/1477

Agarwal, A., Virk, G., Ong, C., & Plessis, S. S. (2014). Effect of Oxidative Stress on Male Reproduction. *The World Journal of Men's Health*, *32*(1), 1–17. https://doi.org/10.5534/wjmh.2014.32.1.1

Agarwal, Gupta, S., Sekhon, L., & Shah, R. (2008). Redox considerations in female reproductive function and assisted reproduction. *Molecular Mechanisms to Health Implications. Antioxidant Redox Signal*, *10*, 1376–1388.

Aidoo, E., Addai, F. K., Ahenkorah, J., Hottor, B., Bugyei, K. A., & Gyan, B. A. (2012). Natural cocoa ingestion reduced liver damage in mice infected with Plasmodium berghei (NK65). *Research Reports in Tropical Medicine*, *3*, 107–116.

Akang, E. N., Oremosu, A. A., Osinubi, A. A., Dosumu, O. O., Kusemiju, T. O., Adelakun, S. A., & Umaru, M. L. (2015). Histomorphometric studies of the effects of Telfairia occidentalis on alcohol-induced gonado-toxicity in male rats. *Toxicology Reports*, *2*, 968–975.

Alfonso, M., Duran, R., & Marco, J. (1993). Ethanol-induced alterations in gonadotrophins secretion during the estrous cycle of rats. *Alcohol and Alcoholism*, *28*(6), 667–674.

Altunkaynak, B. Z., Akgül, N., Yahyazadeh, A., Altunkaynak, M. E., Turkmen, A. P., Akgül, H. M., & Ünal, B. (2016). Effect of mercury vapor inhalation on rat ovary:

Stereology and histopathology. *Journal of Obstetrics and Gynaecology Research*, *42*(4), 410–416. https://doi.org/10.1111/jog.12911

Amanvermez, R., Demir, S., Tuncel, O. K., Alvur, M., & Agar, E. (2005). Alcohol-Induced Oxidative Stress and Reduction in Oxidation by Ascorbate/L-Cys/L-Met in the Testis, Ovary, Kidney, and Lung of Rat. *Advances in Therapy*, *22*(6), 548–558.

Andújar, I., Recio, M. C., Giner, R. M., & Rios, J. L. (2012). Cocoa Polyphenols and Their Potential Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, 1–23. https://doi.org/10.1155/2012/906252

Asah-Opoku, M. N. A. (2015). *Renal histology and function in gentamicin-induced acute kidney injury (AKI) in rats given natural cocoa* (Unpublished M.Phil Thesis). University of Ghana, Legon.

Asiamah, E. (2015). *Kidney histology in experimentally-induced type 2 diabetic rats given natural cocoa or standard drug treatment* (Unpublished M.Phil Thesis). University of Ghana, Legon.

Attaran, M., Pasqualotto, E., & Falcone, T. (2000). The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertiliza- tion. *International Journal of Fertility and Women's Medicine*, *45*, 314–320.

Balasaheb, S., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Advances*, *5*, 27986–28006. https://doi.org/10.1039/C4RA13315C

Bautista, A. P. (1997). Chronic alcohol intoxication induces hepatic injury through enhanced macrophage inflammatory protein-2 production and intercellular adhesion molecule-1 expression in the liver. *Hepatology*, *25*, 335–342.

Bhatia, L., Bhatia, V., & Grover, M. (2012). Nephroprotective effect of fresh leaves extracts of Sida Cordifolia Linn in gentamicin induced nephrotoxicity in rats. *International Journal of Research in Pharmacy and Science*, *2*(2), 151–158.

Bo, W. J., Krueger, W. A., & Rudeen, P. K. (1982). Ethanol-induced alterations in the morphology and function of the rat ovary. *The Anatomical Record*, *202*, 255–260.

Bowen, R. (2004). Gonadotropins: Luteinizing and Follicle Stimulating Hormones. Retrieved April 30, 2017, from http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/hypopit/lhfsh.html

Bradford, B. U., Kono, H., & Isayama, F. (2005). Cytochrome P450 CYP2E1, but not nicotinamide adenine dinucleotide phosphate oxidase, is required for ethanol induced oxidative DNA damage in rodent liver. *Hepatology*, *41*, 336–344.

Carbone, M. C., Tatone, C., Delle, M. S., Marci, R., Caserta, D., Colonna, R., & Amicarelli, F. (2003). Antioxidant enzymatic defenses in hu- man follicular fluid: characterization and age-dependent changes. *Molecular Human Reproduction*, *9*, 639–643.

Celio, M. R., Hornung, J. P., Burri, P., Adé-Damilano, M., Kasas, S., & Müller, O. (2008). Ovaries : differentiation Development of the stroma. Retrieved from http://www.embryology.ch/anglais/ugenital/diffmorpho03.htm

Chuffa, L. G. A., Amorim, J. P. A., Teixeira, G. R., Mendes, L. O., Fioruci, B. A., Pinheiro, P. F. F., … Martinez, F. E. (2011). Long-Term Exogenous Melatonin Treatment Modulates Overall Feed Efficiency and Protects Ovarian Tissue Against Injuries Caused by Ethanol-Induced Oxidative Stress in Adult UChB Rats. *Alcoholism: Clinical and Experimental Research*, *35*(8), 1498–1508. https://doi.org/10.1111/j.1530-0277.2011.01486.x

Chung, K. W. (2000). *Gross Anatomy*. Philadelphia: Lippincott Williams & Wilkins.

Cook, R. T., Schlueter, A. J., Coleman, R. A., Tygrett, L., Ballas, Z. K., Jerrells, T. R., … Waldschmidt, T. J. (2007). Thymocytes, pre-B cells, and organ changes in a mouse model of chronic ethanol ingestion--absence of subset-specific glucocorticoid-induced immune cell loss. *Alcohol Clinical Experimental Research*, *31*, 1746–1758.

Craig, J., Orisaka, M., Wang, H., Orisaka, S., Thompson, W., Zhu, C., … Tsang, B. K. (2007). Gonadotropin and intra-ovarian signals regulating follicle development and atresia: the delicate balance between life and death. *Frontiers BioScience*, *12*, 3628–3639.

D'Souza El-Guindy, N. B., Kovacs, E. J., De-Witte, P., Spies, C., Littleton, J. M., De-Villiers, W. J. S., … Meadows, G. G. (2011). Laboratory models available to study alcohol-induced organ damage and immune variations; choosing the appropriate model. *Alcohol Clinical Experimental Research*, *34*(9), 1489–1511. https://doi.org/10.1111/j.1530-0277.2010.01234.x.Laboratory

Daiter, E. (2007). Are there any good (safe, effective & affordable) alternatives to IVF for the treatment of infertility? Retrieved June 7, 2017, from http://www.infertilitytutorials.com/infertility_landing.php

Dasgupta, A., & Klein, K. (2014). Oxidative Stress Caused by Cigarette Smoking , Alcohol Abuse, and Drug Abuse: Treatment and Prevention of Disease. In *Antioxidants in Food, Vitamins and Supplements*. (pp. 59–75). San Diego: Elsevier Inc. https://doi.org/10.1016/B978-0-12-405872-9.00004-5

Dillinger, T. L., Barriga, P., Escarcega, S., Jimenez, M., Lowe, D. S., & Grivetti, L. E. (2000). Food of the Gods: Cure for humanity? A cultural history of the medicinal and ritual use of chocolate. *Journal of Nutrition*, *130*, 2057S–2072S.

Donnelly, T. M. (2004). *Ferrets, Rabbits, and Rodents, Clinical Medicine and Surgery*. (J. W. Quesenberry, K E, Carpenter, Ed.) (2nd ed.). Philadelphia, PA: Saunders, W B.

Dosumu, O. O., Osinubi, A. A. A., & Duru, F. I. O. (2014). Alcohol induced testicular damage : Can abstinence equal recovery ? *Middle East Fertility Society Journal*, *19*(3), 221–228. https://doi.org/10.1016/j.mefs.2014.01.003

Du, B., Takahashi, K., Ishida, G. M., Nakahara, K., Saito, H., & Kurachi, H. (2006). Usefulness of intraovarian artery pulsatility and resistance indices measurement on the day of follicle aspiration for the assessment of oocyte quality. *Fertility and Sterility*, *85*(2), 366–370.

Eggert, J., Theobald, H., & Engfeldt, P. (2004). Effects of alcohol consumption on female fertility during an 18-year period. *Sterility And Fertility*, *81*(2), 379–383. https://doi.org/10.1016/j.fertnstert.2003.06.018

Emanuele, M. A., Wezeman, F., & Emanuele, N. V. (2003). Alcohol's Effects on Female Reproductive Function. *National Institute on Alcohol Abuse and Alcoholism*, *26*(4), 274–281. Retrieved from https://pubs.niaaa.nih.gov/publications/arh26?4/274?281.htm

Franco, R., Oñatibia-Astibia, A., & Martínez-Pinilla, E. (2013). Health Benefits of Methylxanthines in Cacao and Chocolate. *Nutrients*, *5*, 4159–4173. https://doi.org/10.3390/nu5104159

Fujii, J., Iuchi, Y., & Okada, F. (2005). Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reproductive Biology and Endocrinology*, *3*(43).

Furuya, M. (2012). Ovarian cancer stroma: Pathophysiology and the roles in cancer development. *Cancers*, *4*(3), 701–724. https://doi.org/10.3390/cancers4030701

Gavaler, J. S., Van-Thiel, D. H., & Lester, R. (1980). Ethanol: A Gonadal Toxin in the Mature Rat of Both Sexes. *Alcoholism: Clinical and Experimental Research*, *4*(3), 271–276.

Ghana Statistical Service. (2014a). *Ghana 2014 Demographic and Health Survey: Key Findings*.

Ghana Statistical Service. (2014b). *Ghana Demographic and Health Survey: Key Indicators*.

Ghazali, R., & Patel, V. B. (2016). Chapter 2 - Alcohol Metabolism: General Aspects BT - Molecular Aspects of Alcohol and Nutrition. In *General Aspects BT- Molecular Aspects of Alcohol and Nutrition* (pp. 17–21). San Diego: Academic Press. https://doi.org/http://dx.doi.org/10.1016/B978-0-12-800773-0.00002-1

Gougeon, A. (1996). Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocrine Reviews*, *17*, 121.

Gray, H. (1999). *Anatomy, Descriptive and Surgical, The Unabridged Gray's Anatomy*. Philadelphia: Running Press.

Grodstein, F., Goldman, M. B., & Cramer, D. W. (1994). Infertility in Women and Moderate Alcohol Use. *American Journal of Public Health*, *84*(9), 1429–1432.

Gude, D. (2012). Alcohol and fertility. *Journal of Human Reproductive Sciences*, *5*(2), 226–228. https://doi.org/10.4103/0974

Gupta, S., Sekhon, L., Aziz, N., & Agarwal, A. (2008). The Impact of Oxidative Stress on Female Reproduction and ART : An Evidence- Based Review. *Infertility and Assisted Reproduction*, 178–186.

Guven, D., Altunkaynak, B. Z., Ayranci, E., Kaplan, S., Bildircin, F. D., Kesim, Y., & Ragbetli, M. C. (2013). Stereological and histopathological evaluation of ovary and uterine horns of female rats prenatally exposed to diclofenac sodium. *Journal of Obstetrics and Gynaecology*, *33*(3), 258–263. https://doi.org/10.3109/01443615.2012.761185

Hafez, E. S. E. (1970). Rabbits. In E. S. E. Hafez (Ed.), *Reproduction and Breeding Techniques for Laboratory Animals.* Philadelphia, PA: Lea & Febiger.

Handler, J. A., & Thurman, R. G. (1990). Redox interactions between catalase and alcohol dehydrogenase pathways of ethanol metabolism in the perfused rat liver. *Journal of Biological Chemistry*, *265*, 1510–1515.

Harcourt-Brown, F. M. (2017). Disorders of the Reproductive Tract of Rabbits. *Veterinary Clinics of North America: Exotic Animal Practice*, *20*(2), 555–587. https://doi.org/10.1016/j.cvex.2016.11.010

Healey, A. (2012). Embryology of the Female Reproductive Tract. *Imaging of Gynecological Disorders in Infants and Children*. Berlin Heidelberg: Springer-Verlag. https://doi.org/10.1007/174

Heidari, Z., Sagheb, H. R. M., Rafighdoost, H., Moein, A., Mugahi, M. H. N., & Minaii, B. (2003). A Stereological study of Diabetic kidney following administration of different doses of Streptozotocin. *DARU*, *11*(3).

Hill, M. A. (2017). Embryology Ovary Development. Retrieved May 29, 2017, from https://embryology.med.unsw.edu.au/embryology/index.php/Ovary_Development (https://embryology.med.unsw.edu.au/embryology/index.php/Ovary_Development)

Hollenberg, N. K. (2006). Vascular action of cocoa flavanols in humans: The root of the story. *Journal of Cardiovascular Pharmacology*, *47*(Suppl 2), S99–S102.

Hong, Y. H. (2016). Effects of the herb mixture , DTS20 , on oxidative stress and plasma alcoholic metabolites after alcohol consumption in healthy young men. *Integrative Medicine Research*, *5*(4), 309–316. https://doi.org/10.1016/j.imr.2015.10.001

Hussein, M. R. (2005). Apoptosis in the ovary: molecular mechanisms. *Human Reproduction Update*, *11*, 162–177.

Jalil, A. M. M., & Ismail, A. (2008). Polyphenols in Cocoa and Cocoa Products: Is There a Link between Antioxidant Properties and Health?, 2190–2219. https://doi.org/10.3390/molecules13092190

Jalil, A. M. M., Ismail, A., Pei, C. P., Hamid, M., & Kamaruddin, S. H. S. (2008). Effects of Cocoa Extract on Glucometabolism , Oxidative Stress , and Antioxidant Enzymes in Obese-Diabetic (Ob-db) Rats. *Journal of Agricultural and Food Chemistry*, *56*(17), 7877–7884.

Jensen, T. K., Hjollund, N. H., & Henriksen, T. B. et al. (1998). Does moderate alcohol consumption affect fertility? Follow up study among couples planning first pregnancy. *BMJ*, *317*, 505–510.

Katz, V. L., Gretchen, M. L., Lobo, R. A., & Gershenson, D. M. (2007). *Comprehensive Gynecology*. (Droegemueller, Stenchever, Mishell, & Herbst, Eds.) (Fifth). Philadelphia: Mosby Elsevier.

Kelm, M. A., Johnson, J. C., Robbins, R. J., Hammerstone, J. F., & Schmitz, H. H. (2006). High-performance liquid chromatography separation and purification of cacao (Theobroma cacao L.) procyanidins according to degree of polymerization using a diol stationary phase. *Journal of Agricultural and Food Chemistry*, *54*, 1571–1576.

Ko, E. Y., & Sabanegh, E. S. (2014). The Role of Nutraceuticals in Male Fertility. *Urologics Clinics of North America*, *41*, 181–193. https://doi.org/10.1016/j.ucl.2013.08.003

Krajcir, N., Chowdary, H., Gupta, S., & Agarwal, A. (2008). Female Infertility and Assisted Reproduction: Impact of Oxidative Stress. *Current Women's Health Reviews*, *4*(1), 9–17.

Kumar, V., Abbas, A. K., & Fausto, N. (2004). *Robbins and Contrar Pathologic Basis of Diseases* (7th ed.). India: Thompson Press Ltd.

Lapaglia, N., Steiner, J., & Kirsteins, L. (1997). The impact of acute ethanol on reproductive hormone synthesis, processing and secretion in female rats at proestrous. *Alcoholism: Clinical and Experimental Research*, *21*(9), 1567–1572.

Lebas, F., Coudert, P., De Rochambeau, H., Thebault, R. G., & Rouvier, R. (1986). *The rabbit: husbandry, health and production. The rabbit.* (Vol. 121). Retrieved from http://www.fao.org/docrep/t1690E/t1690e04.htm#anatomy and physiology

Lee, K. W., Kim, J. Y., Lee, J. H., & Lee, Y. C. (2003). Cocoa Has More Phenolic Phytochemicals and a Higher Antioxidant Capacity than Teas and Red Wine. *Journal of Agricultural and Food Chemistry*, *51*, 7292–7295.

Lieber, C. S., & DeCarli, L. M. (1970). Quanititative relationship between amiount of dietary fat and severity of alcoholic fatty liver. *American Journal of Clinical Nutrition*, *23*, 474–478.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. *American Journal of Clinical Nutrition (Suppl)*, *81*, 230S– 242S.

Mello, N., Mendelson, J., & Teoh, S. (1993). Overview of the effects of alcohol on the neuroendocrine function in women. *Alcohol and Endocrine System*, 139–169.

Misra, U. K., Bradford, B. U., Handler, J. A., & Thurman, R. G. (1992). Chronic ethanol treatment induces H2O2 production selectively in pericentral regions of the liver lobule. *Alcoholism: Clinical and Experimental Research*, *16*, 839–842.

Mondal, P. (2016). Gametogenesis Process in Human: Spermatogenesis and Oogenesis. Retrieved June 7, 2017, from http://www.yourarticlelibrary.com/biology/gametogenesis-process-in-human-spermatogenesis-and-oogenesis/11843

Moshfegh, F., Baharara, J., Namvar, F., Zafar-Balanezhad, S., & Amini, E. (2016). Effects of date palm pollen on fertility and development of reproductive system in female Balb / C mice. *Journal of HerbMed Pharmacology*, *5*(1), 23–28.

National Institute on Alcohol Abuse and Alcoholism (NIAAA). (1994). Alcohol and Hormones. Retrieved May 8, 2017, from https://pubs.niaaa.nih.gov/publications/aa26.htm

National Institute on Alcohol Abuse and Alcoholism (NIAAA). (2010). *Molecule Repairs Alcohol Metabolism Enzyme*. Bethesda. Retrieved from https://www.niaaa.nih.gov/news-events/news-releases/molecule-repairs-alcohol-metabolism-enzyme

National Institutes of Health (NIH). (2015). *Alcohol; A Women's Health Issue* (2015th ed.). NIH.

Nehlig, A. (2012). The neuroprotective effects of cocoa flavanol and its influence on cognitive performance. *British Journal of Clinical Pharmacology*, *10*, 1365–1370.

Nowland, M. H., Brammer, D. W., Garcia, A., & Rush, H. G. (2015). Biology and Diseases of Rabbits. In *Laboratory Animal Medcine* (3rd ed., pp. 411–461). Elsevier Inc. https://doi.org/10.1016/B978-0-12-409527-4.00010-9

Ogilvie, K. M., & Rivier, C. (1997). Effect of alcohol on the proestrous surge of luteinizing hormone (LH) and the activation of LH-releasing hormone (LHRH) neurons in the female rat. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(7), 2595–2604.

Oktem, O., & Oktay, K. (2008). The Ovary: Anatomy and Function throughout Human Life. *Annals of the New York Academy of Sciences*, *1127*, 1–9. https://doi.org/10.1196/annals.1434.009

Oliveira, T. B., & Genovese, M. I. (2013). Chemical composition of cupuassu (Theobroma grandi florum) and cocoa (Theobroma cacao) liquors and their effects on streptozotocin-induced diabetic rats. *Food Research International*, *51*(2), 929–935.

OpenStax-CNX. (2014). Anatomy and Physiology of the Female Reproductive System. OpenStax College. Retrieved from https://cnx.org/exports/9cccba49-6490-4e5b-a366-9991b7dbc56c@5.pdf/anatomy-and-physiology-of-the-female-reproductive-system-5.pdf

Oremosu, A. A., & Akang, E. N. (2015). Impact of alcohol on male reproductive hormones , oxidative stress and semen parameters in Sprague – Dawley rats. *Middle East Fertility Society Journal*, *20*, 114–118. https://doi.org/10.1016/j.mefs.2014.07.001

Polackwich, S. A., & Sabanegh, E. S. (2015). The Role of Over-the-Counter Supplements in Male Infertility. In *Handbook of Fertility: Nutrition, Diet, Lifestyle and Reproductive Health* (pp. 369–381). Elsevier Inc. https://doi.org/10.1016/B978-0-12-800872-0/00033-0

Praag, E. (2016). Reproductive tract of the female rabbit. Retrieved January 1, 2016, from http://www.medirabbit.com/EN/Uro_gen_diseases/Fem_rabbit/Fem_en.html

Pyun, C., Mandal, P. K., Hong, G., & Lee, C. (2015). Effect of Chronic Alcohol Consumption on Phosphatidylcholine Hydroperoxide Content of Rat Liver and Brain. *Tropical Journal of Pharmaceutical Research*, *14*(7), 1225–1230.

Rabbit Reproduction. (2017). Retrieved June 12, 2017, from http://www.ansci.wisc.edu/jjp1/ansci_repro/misc/project_websites_07/tue07/rabbit_ai/Reproduction.html

Rachdaoui, N., & Sarkar, D. K. (2014). Effects of Alcohol on the Endrocrine System. *National Institute of Health*, *42*(3), 593–615. https://doi.org/10.1016/j.ecl.2013.05.008.Effects

Rajasekaran, A., Sivagnanam, G., & Xavier, R. (2008). Nutraceuticals as therapeutic agents : A Review. *Research Journal of Phamacy and Technology*, *1*(4), 328–340.

Ramaiah, S. K., Rivera, C., & Arteel, G. E. (2004). Early-Phase Alcoholic Liver Disease: An Update on Animal Models, Pathology and Pathogenesis. *International Journal of Toxicology*, *23*, 217–231.

Reddyvari, H., Govatati, S., Kumar, S. M., Swapna, V. K., Sravanthi, M., Sreenivasa, R. P., … Varadacharyulu, N. (2017). Therapeutic effect of green tea extract on alcohol induced hepatic mitochondrial DNA damage in albino wistar rats. *Journal of Advanced Research*, *8*(3), 289–295. https://doi.org/10.1016/j.jare.2017.02.002

Richardson, B. E., & Lehmann, R. (2010). Mechanisms guiding primordial germ cell migration: strategies from different organisms. *Nature Reviews Molecular Cell Biology*, *11*(1), 37–49. https://doi.org/10.1038/nrm2815.Mechanisms

Roy, H., Lundy, S., & Brantely, P. (2005). Cocoa Polyphenols. *Pennington Nutrition Series*, *1*.

Ruder, E. H., Hartman, T. J., & Goldman, M. B. (2009). Impact of oxidative stress on female fertility. *Current Opinion Obstetrics Gynecology*, *21*(3), 1–10.

Sarkola, T., Adlercreutz, H., Heinonen, S., Von Der Pahlen, B., & Eriksson, C. J. P. (2001). The role of the liver in the acute effect of alcohol on androgens in women. *Journal of Clinical Endocrinology and Metabolism*, *86*(5), 1981–1985. https://doi.org/10.1210/jc.86.5.1981

Sekhon, L. H., Gupta, S., Kim, Y., & Agarwal, A. (2010). Female Infertility and Antioxidants. *Current Women's Reviews*, *6*, 84–95. https://doi.org/1573-4048/10

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the Oxidative stability. *European Journal of Lipid Science and Technology*, *112*, 930–940.

Smith, P. E. (1940). The Physiology of the Ovaries. *The Bulletin*, (March), 153–170.

Sobotta, & Hammersen. (1986). *Slides in Human Histology*. (F. Hammersen, Ed.) (3rd ed.). Baltimore-Munich: Urban & Schwarzenberg.

Sokpor, G., Addai, F. K., Gyasi, R. K., Bugyei, K. A., Ahenkorah, J., & Hottor, B. (2012). Voluntary Ingestion of Natural Cocoa Extenuated Hepatic Damage in Rats with Experimentally Induced Chronic Alcoholic Toxicity. *Functional Foods in Health and Disease*, *2*(5), 166–185.

Srivastava, V. K., Dissen, G. A., & Ojeda, S. R. (2007). Effects of alcohol on intraovarian nitric oxide synthase and steroidogenic acute regulatory protein in the prepubertal female rhesus monkey. *Journal of Studies on Alcohol and Drugs.*, *68*, 182–191.

Stiles, M. (2016). *Visualizing World Alcohol Consumption : How Much Does Each Country Drink? The Daily Viz*. Retrieved from http://thedailyviz.com/2016/08/25/charting-world-alcohol-consumption-how-much-does-each-country-drink/

Strauss, M., Rosenbaum, J. D., & Nelson, W. P. (1950). The effect of alcohol on the renal excretion of water and electrolyte.

Sugino, N., Takiguchi, S., Kashida, S., Karube, A., Nakamura, Y., & Kato, H. (2000). Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. *Molecular Human Reproduction*, *6*(1), 19–25.

Suzuki, T., Sugino, N., & Fukaya, T. (1999). Superoxide dismutase in normal cycling human ovaries: immunohistochemical localization and characterization. *Fertility and Sterility*, *72*(4), 720–726.

Tamate, K., Sengoku, K., & Ishikawa, M. (1995). The role of superoxide dismutase in the human ovary and fallopian tube. *Journal of Obstetrics and Gynaecology*, *21*(4), 401–409.

Tomas-Barberan, F A Cienfuegos-Jovellanos, E Marin, A., Muguerza, B., A, G. I., Cerdaa, B., Zafrilla, P., Morillas, J., … Espin, J. C. (2007). A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *Journal of Agricultural and Food Chemistry*, *55*, 3926–3935.

Tsukamoto, H., Towner, S. J., Ciofalo, M. L., & French, S. (1986). Ethanol-induced liver fibrosis in rats fed high fat diet. Hepatology. *Hepatology*, *6*, 814–22.

U.S Food and Drug Administration. (2017). Drug Applications for Over - the - Counter (OTC) Drugs. Retrieved from https://www.fda.gov/drugs/developmentapprovalprocess/howdrugsaredevelopedandapproved/approvalapplications/over?the?counterdrugs/default.htm

Urbano, L. (2013). Dissecting Rabbits, from Montessori Muddle. Retrieved June 12, 2017, from http://montessorimuddle.org/

Van-Thiel, D. H., Gavaler, J. S., Lester, R., & Sherins, R. J. (1978). Alcohol-Induced Ovarian Failure in the Rat. *The Journal of Clinical Investigation*, *10*, 624–632.

Viudes-de-Castro, M. P., Pomares, A., Ribes, J. M. D. S., Marco-jiménez, F., & Vicente, J. S. (2015). Effect of luteinizing hormone on rabbit ovarian superstimulation and embryo developmental potential. *Theriogenology*, *84*(3), 446–451. https://doi.org/10.1016/j.theriogenology.2015.04.001

Wilkinson, P. K., Sedman, A. J., & Sakmar, E. (1977). Pharmacokinetics of ethanol after oral administration in the fasting state. *Journal of Pharmacokinetics Biopharmaceutics*, *5*, 207–224.

Williams, C. J., & Erickson, F. G. (2012). *Morphology and Physiology of the Ovary*. (D. K. De Groot L J, Chrousos G, Ed.). South Dartmouth (MA): MDText.com, Inc. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK278951/

Wu, D., & Cederbaum, A. I. (2003). Role of p38 MAPK in CYP2E1-dependent arachidonic acid toxicity. *The Journal of Biological Chemistry*, *278*(2), 1115–1124. https://doi.org/10.1074/jbc.M207856200

Yin, F., & Cadenas, E. (2015). Mitochondria : The Cellular Hub. *Antioxidant & Redox Signaling*, *22*(12), 961–964. https://doi.org/10.1089/ars.2015.6313

Zakhari, S. (2017). Overview : How Is Alcohol Metabolized by the Body ? Retrieved from https://pubs.niaaa.nih.gov/publications/arh294/245?255.html

APPENDIX I

Protocol for Tissue processing

- Place tissue in tissue cassettes and into 50% alcohol overnight
- Remove tissue and place into 70% alcohol for 45 minutes
- Transfer tissue into 90% alcohol for 30 minutes
- Remove tissue and place in absolute alcohol (100%) I, II and III for 45 minutes each.
- Transfer tissue into xylene I and II for 30 minutes each
- Remove and place tissue into xylene III for 45 minutes.

Infiltration

- Place tissue in tissue cassettes in molten wax I for 1 hour in an oven
- Remove and place into molten wax II and III for 30minutes each in an oven
- Tissues in cassettes are embedded in wax outside of oven and allowed to harden on ice.

APPENDIX II

Protocol for Haematoxylin and Eosin staining

With the aid of Leica Auto Stainer XL with the following programmed methods, the paraffin embedded liver section with haematoxylin and eosin for histomorphometry

Technique

1. De-wax sections in xylene for 1 minute.

2. Rehydrate tissues by passing them through graded series of alcohol in the order 100%, 95% and 70%

3. Stain in Haematoxylin (see below for preparation) for 15 minutes.

4. Wash in water for 2-3minutes

5. Differentiate in 1% hydrochloric acid in 70% alcohol for 1 minute

6. Wash in water for 10 minutes

7. Stain in 1% aqueous eosin (see below for preparation) for 5 minutes

8. Rinse gently in a bowl under running tap water to wash off surplus stain.

9. Dehydrate in graded series of alcohol (75%, 95%, 100% and 100%) and keep in xylene for subsequent mounting with Dysterene Plasticised Xylene (DPX)

Preparation of Solution

Haematoxylin Solution		**Aqueous Eosin Solution**	
Haematoxylin (Sigma)	1.0g	Eosin	1.0g
Distilled Water	100ml	Distilled Water	100ml

APPENDIX III

Protocol for preparation of 10% buffered Formaldehyde PH 7.3

10% buffered formaldehyde pH 7.3 (IL)

Formalin (37 - 40% w/v - BDH, England)……..……100 mL

Distilled water…….. 900 mL

Sodium hydrogen orthophosphate (NaH_2PO_4)…..…….4g

Disodium hydrogen orthophosphate (Na_2HPO_4)…….6.5g

Apparatus and equipment

- ✓ Electronic balance (Mettler CH – 8606)
- ✓ 1000 mL flask
- ✓ Magnetic stirrer
- ✓ pH meter (Philips, PW9418)
- ✓ Conical flasks and beakers
- ✓ Plastic weighing container
- ✓ Measuring cylinder

APPENDIX IV

Types of animal models for demonstrating alcohol induced ovarian injury

Oral liquid diets

For the fact that animals show an aversion towards drinking alcohol readily, the incorporation of alcohol accounting for 36% of total calories in a specialized liquid diet ensures animals such as rats to consume high amounts of alcohol in an experimental study (Lieber & DeCarli, 1970). This approached was used by Van-Thiel *et al* (1978 and 1980) in inducing ovarian injury in rats and also in using alcohol as a gonadal toxin in both sexes of rats. This oral liquid diet is known as Lieber-DeCarli formula. In Van-Thiel's experiment to investigate alcohol-induced ovarian injury, alcohol-fed animals received a liquid diet with alcohol accounting for 36% of total calories (Group 1), rats in pair-fed isocaloric controls were fed a similar diet in which dextri-maltose was isocalorically substituted for alcohol (Group 2), while *ad libitum*-intact controls (Group 3) and oophorectomized *ad libitum* controls (Group 4) were given Wayne Lab Blox F4 diet obtained from Best Feeds, Oakdale, Pa. The experiment ran 49 days from age 28 through age 77 days (Van-Thiel *et al*., 1978). The ovaries of the alcohol-fed animals weighed two-fifths as much (30.6±2.2 mg) as those obtained from the pair-fed isocaloric controls (75.5±3.9 mg) and one-third as much as those of the intact *ad-libitum* controls (91.4±0.2 mg; both $P < 0.01$). One merit that this model comes with is that the diet is affordable and the contents can be manipulated with ease. It also allows for the gradual increase of alcohol (D'Souza El-Guindy *et al*., 2011).

Even though this is a notable improvement over alcohol exposure in drinking water, the Lieber-DeCarli formula of *ad-libitum* feeding of alcohol containing liquid diet still have some limitations. The model does not simulate human drinking accurately as animals are

obligated to ingest alcohol each time they are hungry for food or thirsty (D'Souza El-Guindy *et al*., 2011).

The Intragastric Alcohol Infusion Model

This model was established on the hypothesis that rats have a higher rate of alcohol breakdown than humans do and may require sustained higher blood alcohol levels than humans to induce liver and any other organ damage (Tsukamoto, Towner, Ciofalo, & French, 1986). This model employs the use of a catheter which is implanted into the stomach under aseptic conditions. Alcohol, a Lieber-DeCarli formulation is added to the liquid diet and then infused by means of the catheter directly into the stomach using an infusion pump. The experimental animals can be kept on the diet for quite a good length of time without any complications. The blood alcohol levels achieved and sustained when using this method are between 250–500 mg/dl (D'Souza El-Guindy *et al*., 2011). The model however, is not physiological. It is expensive, labor intensive, requires constant monitoring of animals, and highly trained technical personnel to perform the catheter implantation surgery (D'Souza El-Guindy *et al*., 2011).

Alcohol Agar Block Model

This model of chronic alcohol abuse was developed by Bautista (1997). It involves maintaining rodents on rodent chow, agar block containing 40% (v/v) of alcohol with 0.5 g/kg of peanut butter and 10% (v/v) of alcohol supplemented in water. The agar blocks are given to the animals in Petri dishes. The concentration of alcohol in the agar block is gradually increased throughout the experiment (Bautista, 1997). The pair-fed animals receive isocaloric chow, similar amount of agar without alcohol, and alcohol-free water. By using this model, Bautista (1997; 2002) and Bautista and Spitzer (1999) described the

presence of significant amounts of endotoxin in plasma, a 6-fold increase in serum aspartate aminotransferase, polymorphonuclear neutrophils infiltration, and a mild fat accumulation in the livers of Sprague-Dawley rats fed alcohol for 16 weeks (D'Souza El-Guindy *et al*., 2011). The model is easy to handle, affordable and has leeway for dietary and cofactors manipulation. However, it is not be ideal to attain and sustain very high blood alcohol levels. This is due to significant loss of alcohol from the agar blocks by evaporation (D'Souza El-Guindy *et al*., 2011).

Alcohol in Drinking Water Model

This is a more practical solution for long- term alcohol exposure. This model has been used in several species, including mice, rats and guinea pigs. Subject to the research question, it can be modified from single bottle – no choice, to two bottles – choice between water and alcohol, multiple bottles – choice between water and alcohol of varying concentrations and drinking in the dark (D'Souza El-Guindy *et al*., 2011). In this model, serum corticosterone levels are unaffected and animals do not become dehydrated (Cook *et al*., 2007). The model is physiological, inexpensive, and can be manipulated to mimic human drinking. The animal husbandry is not as cumbersome as is with some of the other models. Unlike some of the other models, it is easy to maintain sterility of the food and water provided to the animals. The model allows for manipulation of the diet and to superimpose other cofactors into the experimental design. It may however be difficult to control for and maximize alcohol intake using any of three available variations of the model (D'Souza El-Guindy *et al*., 2011).