EFFECTS OF FERMENTED SOYABEAN (*Glycine max*) SUPPLEMENT ON LACTATION AND RELATED PHYSIOLOGICAL PARAMETERS INALBINO WISTAR RATS

BY

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DECLARATION

I, Ibrahim Malgwi Samaila declare that the work in this dissertation entitled: **Effects of Fermented Soya bean (*Glycine max)* Supplement on Lactation and Related Physiological Parameters in Albino Wistar Rats** was carried out by me in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, under the supervision of Prof. Aliyu Mohammed, Dr. Yusuf Tanko and Dr. M. U Kawu. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

_____ _____

Ibrahim Malgwi SAMAILA Date

CERTIFICATION

This thesis titled "**Effects of Fermented Soya bean (*Glycine max*) Supplement on Lactation and Related Physiological Parameters in Albino Wistar Rats**" by Ibrahim Malgwi Samaila, meets the requirement governing the award of Doctorate of Philosophy degree in Human Physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to God Almighty for His grace upon my life

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ABSTRACT

In mammals, lactation is the most energetically demanding period of a female's reproductive life. In the wild, investment in reproduction is associated with a decrease in survival in both large and small mammals. Energy intake typically increases by 66–188% but in some animals such as laboratory mice, intake can increase by up to 400 per cent. As a consequence, daily energy expenditure and basal metabolic rate can be increased in lactating females when compared with non-reproductives. This has led to the suggestion that oxidative stress may be elevated during lactation, unless antioxidant defences are sufficiently upregulated. The study was designed to evaluate the effect of fermented Soya bean and Vitamin C supplement in stimulating milk production in lactating albino rats. Thirty five (35) adult female rats were used for this study. At parturition, the animals were randomly divided into five groups of five (5) rats each. Except group four (4) that was subdivided into three (3) sub groups of five animals each (n=5). Treatment was carried out as follows: Group I: (Normal control) was given normal feed and distilled water, oraly (1 ml/kg), Group II: metoclopramide (5 mg/kg), Group III: 100 mg/kg of Vitamin C. The three (3) sub groups under group four (4) received 10%, 20% and 40% soya bean, respectively, Group V: was co-administered with 20% soya bean supplement and Vitamin C (100 mg/kg). Treatment was done for the period of ten (10) days at 06:00h daily. Milk yield was assessed daily at 18 h and 23 h after gavage for the period of ten (10) days. At the end of experiment, animals were euthanized using chloroform inhalation in a closed chamber and sacrificed afterwards. Blood samples were collected and the serum separated for determination of physiological parameters: prolactin, growth hormone, oxytocin, thyroxine (T3 and T4), insulin, progesterone oestrogen and biochemical parameters: SOD, catalase, GPx, MDA, ALP, ALT, AST, sodium, potassium, chloride, bicarbonate, calcium and magnesium. Mammary gland tissues excised were used for histological assessments. The result of this study showed no significant increase in milk yield 18 hours after gavage compared to the control. The result of 23 hours after gavage showed a significant increase in milk yield in SB 20% (2.36 ± 0.49), SB 40% (1.98 ± 0.42) and SB 20% + VIT C (1.90 ± 0.46) ($P< 0.05$) compared to the control (0.8 ± 0.26), with the highest milk yield in the SB 20% treated group. Although the level of milk yield in the METCL (1.16 ± 0.29 vs 0.8 ± 0.26), VIT C (1.24 ± 0.46 vs 0.8 ± 0.26) and SB 10% (1.70 ± 0.44 vs 0.8 ± 0.26) increased, it was however not statistically significant. The result for serum prolactin concentration (ng/ml) showed a significant increase in the group treated with SB 20% + VIT C (63.70 ± 2.27) when compared to the control (43.98 ± 5.57). The result of serum level of Growth hormone showed significant Increase in the groups treated with VIT C, SB 10%, SB 20% and SB 20% + VIT C. ($P< 0.05$) compared to the control. There was no statistically significant difference in serum oxytocin levels in all the treated groups, despite the relative increase in some of the treated groups when compared to the control ($P> 0.05$). The results of soya bean supplemention, metoclopramide and Vitamin C showed no significant difference ($P> 0.05$) on serum triiodothyronine (T3)

concentration compared to the control. There was however, significant increase in SB 40% (100.80 ± 9.95) in serum thyroxine (T4) compared to the control (68.00 ± 3.91) ($P< 0.05$). There was no significant difference ($P> 0.05$) in serum insulin level between treatment and control groups. However, serum progesterone level was significantly high in the groups treated with METCL (26.18 ± 1.87), VIT C (25.78 ± 1.53), SB 10% (26.52± 2.47) and SB 20% (28.70 ± 1.29) when compared to the control (16.66 ± 1.99) ($P< 0.05$). A significant difference in serum estrogen level was recorded in the groups treated with VIT C (669.60 ± 54.04), SB 10% (705.00 ± 27.53), and SB 20% + VIT C (725.40 ± 29.62) compared to the control (632.60 ± 42.96) ($P< 0.05$). The result on serum ALP level showed a significant increase in all the supplement treated groups; SB 10% (110.80 ± 1.63), SB 20% (127.60 ± 9.60), SB 40% (122.80 ± 2.60) and SB 20% + VIT C (129.40 ± 4.90) compared to the controls (86.60 ± 3.73) ($P< 0.05$). The co-administration of the soya bean supplement and Vitamin C showed a significant increase in serum ALP, ALT and AST levels suggesting a tendency of such combination to be more detrimental to the hepatocytes. Although there was an increase in serum MDA concentrations in all the treated groups compared to the control, lipid peroxidation was however significantly higher ($P< 0.05$) in the metoclopramide group relative to the soya bean supplemented groups. This study has shown that supplementation with soya bean induces a mild antioxidant effect by increasing serum level of superoxide dismutase. There was however a significant decrease in serum SOD in the 10% SB group compared to the control. There was a significant difference in serum catalase activity in the group treated with METCL (46.20± 1.53), SB 10% (44.00 ± 1.14) and SB 20% (45.20 ± 1.28) compared to the control (52.00 ± 0.71) ($P< 0.05$). Serum level of glutathione peroxidase GPx showed a significant difference in the group treated with VIT C, SB 10%and SB 20% compared to the control ($P< 0.05$). Soya bean supplementation showed no significant effect on serum electrolytes concentrations. There was a significant decrease in RBC count in the groups treated with SB 40% and SB 20% + VIT C, compared to the control ($P< 0.05$). Soya bean supplementation at SB 40% and SB 20% + VIT C boosted the immunity of the animal significantly by increasing WBC compared to the control. Although there was no significant difference in PCV and Hb concentration, there was however a significant decrease in platelet count in all the soya bean supplemented groups compared to the control ($P< 0.05$). All the treated groups showed an improvement in alveoli distention in the histology of the mammary gland, enhancing its lactogenic properties. In conclusion, soya bean supplement increased milk yield, especially at 20% supplementation compared to metoclopramide treated rats. However, supplementation with soya bean to improve milk yield should be done with caution due to the hepatoxicity in treated rats.

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LIST OF ABBREVIATIONS

AC	-	Adenylyl cyclase
ALP	-	Alkaline phosphotase
ALT	-	Alanine aminotransferase
ANOVA	-	Oneway analysis of variance
AO	-	Antioxidant
$Asc^{\cdot -}$	-	Ascorbyl radicals
AST	-	Aspartate aminotransferase
ATP	-	Adenosine triphospahte
AVP	-	Arginine vasopressin
BHA	-	Butylated hydroxyanisol
BHQ	-	Butylhydroquinone
BHT	-	Butalated hydroxytoluene
CAMP	-	Concentration of adenosine monophosphate
CAT	-	Catalase
COX-2	-	Cyclooxygenase-2
cPLA 2	-	Cytosolic phospholipase A2
D2R	-	Dopamine 2 receptor
DA	-	Dopamine
DAG	-	Diacylglycerol
DHAsc	-	Dyhydroascorbate
DNA	-	Deoxyribonucleic acid
EC	-	Extracellular
EDTA	-	Ethylenediaminetetraacetic acid
eEf2	-	Eukaryotic translation elongation factor 2

ERK	-	Extracellular signal-regulated kinase
FDA	-	Food and drug administration
GAS	-	g-interferon activated sequence
GBA	-	Y-aminobutaric acid
GH	-	Growth hormone
GHIH	-	Growth hormone inhibitory hormone
GHR	-	Growth hormone receptor
GPCR	-	Rhodopsin-type class I G-protein coupled receptor super family
GPx	-	Glutathione peroxidase
Grb2	-	Growth factor receptor bound protein
GTP	-	Guanosin triphosphate
H & E stain	-	Hematoxylin and Eosin Stain
H_2O_2	-	Hydrogen peroxide
HPNE	-	4- hydroperoxy-2(E) –nonenal
IC	-	Intracellular
IGF-II	-	Insulin like growth factor
JAK/STAT	-	Janus kinase/signal transducers
Jak2	-	Janus kinase 2
LDH	-	Lactate dehydrogenase
LPO	-	Lipid peroxidation
MAP/K	-	MAP kinase
MAPK	-	Mitogen- activated protein kinase
mg/dl	-	Milligram per deciliter
MMI	-	Mothers milk insufficiency
MPT	-	Mitochondrial permeability transition

MUFA	-	Monounsaturated fatty acid
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NO	-	Nitric oxide
NT	-	Neurotensin
OFR	-	Oxygen free radicals
OS	-	Oxydative stress
OTR	-	Oxytocin receptor
PCV	-	Packed Cell Volume
PI3K	-	Phosphatidylinositol 3-kinase
PIF	-	Prolactin inhibiting factor
PKC	-	Protein kinase type C
PL	-	Placenta lactogen
PLC	-	Phospholipase C
PMNs	-	Polymorphonuclear neutrophils
PPAR	-	Peroxisome proliferator activated receptors
PRF	-	Prolactin releasing factor
PRL	-	Prolactin
PTK	-	Protein tyrosine kinase
PVN	-	Paraventricular nucleus
RBC	-	Red cell count
rBST	-	recombinant bovine somatotropin
RNA	-	Ribonucleic acid
RNS	-	Reactive nitrogen species
ROS	-	Reactive oxygen species
ROS	-	Reactive oxygen species

RSS	-	Reactive sulfur species
SEM	-	Standard Error of Mean
SFA	-	Saturated fatty acid
SIB	-	SuboptImal infant breastfeeding
SOD	-	Superoxide dismutase
SON	-	Supraoptic nucleus
sos	-	Son of sevenless
SST	-	Somatostatin
TBA	-	Thiobarbituric acid
TocH	-	Tocopherol
TRH	-	Thyrotropin releasing hormone
WBC	-	White Blood Cell

CHAPTER ONE

1.0 INTRODUCTION

Milk production is essential for optimal feeding of infants and has a direct impact on growth, development, and health in neonatal period (Felipe *et al*., 2014). Breast feeding is influenced by nutritional and non-nutritional factors (associated with endocrinology, health, climate, and management) that affect synthesis and secretion. These factors modulate, physiological actions that regulate situations such as noninfectious agalactias and hypogalactias, the later being the main problem of breast feeding women (Zuppa *et al*., 2010). Galactogogues are synthetic or plant products used to induce, maintain, and increase milk production (Mortel and Mehta, 2013); by way of complex processes involving interaction between physical and physiological factors. Among the most important factors are hormones such as prolactin (PRL). However, somatotropin, thyroxine, insulin, leptin, estrogen, progesterone and medroxyprogesterone, oxytocin, recombinant bovine somatotropin (rBST), and thyrotropin releasing hormone (TRH) also play important role as galactogogues (Zuppa, *et al.*, 2010).

The most common synthetic galactogogues for human use are metoclopramide, domperidone, chlorpromazine, and sulpiride (Zuppa *et al.,* 2010). Their remarkable side effects in mothers are xerostomia (dry mouth syndrome or hyposalivation), gastrointestinal disorders, cardiac arrhythmia, lethargy, sedation, extra- pyramidal symptoms such as hypertension, tremor, tic facial seborrhea, hyperhidrosis, and even sudden death (Forinash *et al.,* 2012). In infants that ingest milk from treated mothers symptoms include intestinal discomfort, lethargy, and sedation (Zuppa *et al.*, 2010).

Plants with galactogogue properties include *Trigonella graecum* (fennel), *Foeniculum vulgare* (goats rue), *Galage officnalis* (asparagus), *Asparagus racemosus* (anise), *pimpenella*

anisum, and *Silybum marianum (milk thistle)* (Westfall, 2003). Nowadays, herbal preparations are known to increase significantly milk production in women, goats, cows, and other species. This work on fermented soyabean and vitamin C on milk production could be useful in human breastfeeding medicine and in Veterinary dairy industry.

Free radicals, also known simply as radicals, are organic molecules responsible for aging, tissue damage, and possibly a lot of other diseases. These molecules are very unstable therefore, they bond with other molecules, destroying their vigor and perpetuating the detrimental process. Reactive oxygen species (ROS) and oxygen free radicals (OFR) can change several intracellular process; as a result they induce mutagenesis or impairment of biological system that, in turn, can affect cell survival. The most familiar OFR are the singlet oxygen, the hydroxyl radical, and the superoxide anion, all of which are usually present in the cell because of metabolic oxidative processes which can increase in some conditions (Neifert *et al.,* 1990). Among these oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Gutteridge, 1984). It has been shown that deoxyribonucleic acid (DNA) is a major target of free radical induced damage. Under physiological conditions, the constant and endogenous rate of production of free radicals may lead to a minimal damage in DNA. However, if this production increases, oxygen radicals may attack DNA at either the sugar (deoxyrebose) or the base, giving rise to a large number of products. Free radicals are known to initiate lipid peroxidation that result in potential toxic compounds being produced and/or the destruction of cell membranes leading to cell death. The harmful action of free radicals can result in the death of milk secretary cells and thus, lower or stop milk production. (El-Deeb and Tharwat, 2015)

Mammalian cells have complex defence mechanisms for radical detoxification. Antioxidants are agents that scavenge free radicals and prevent the damage caused by them. A number of these compounds are of exogenous nature and are obtained from food. Examples include antioxidants like a-tocopherol, B- carotene, and ascorbic acid, and some micro nutrient elements such as zinc and selenium (Halliwell and Gutteridge, 1998). Synthetic antioxidants like butylated hydroxytolueone (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (BHQ) can be very expensive and toxic (Moure *et al.,* 2001).

Oxidative stress (OS) is considered a metabolic disturbance that affects organ systems and its presence affects not only the health status of animals but also the quality and quantity of final products, such as milk (Castilo, *et al.,* 2006). However, under proper nutritional and environmental conditions, it increases the antioxidant defense, maintaining a balance that favors lactation without compromising on maternal health. Recent research works are aimed at strengthening the antioxidant defense in the transition phase to counteract the effect of organic pathologies such as ketosis, hypocalcaemia, and mastitis, among others (Abuelo *et al.,* 2013). Feeds affect the nutrient composition of milk produced by animals, as well as metabolism and the availability of energy for the synthesis of milk (Mardalena, *et al.,* 2011).

People are exposed to many carcinogens and the most important seem to be reactive oxygen species (ROS) and reactive nitrogen species (RNS). Formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the human body can cause oxidative damage to biological macromolecules such as DNA, lipids and proteins that may contribute to many diseases (cancer, cardiovascular and neurological diseases, etc). To counteract the prooxidant actions in the human body, an intricate network of antioxidants (AO) is operative in biological system (Berg van den, 2001).

In a healthy body, there is a balance between ROS, RNS and AO. In normal pregnancies, the production of free radicals and lipoperoxidation increase towards the end of the pregnancy, as compared to non pregnant women. In the same vein, antioxidant capacity gradually increases during the pregnancy, leading to an oxidative balance that is maintained throughout the pregnancy (Hung, 2007). Lack of balance between free radicals and antioxidants leads to oxidative stress.

The human diet provides a range of different compounds that possess antioxidant activities or have been suggested to scavenge ROS/RNS based on their structural properties. The most prominent representatives of dietary antioxidants are ascorbate (vitamin C), tocopherols (vitamin E), carotenoids and flavonoids (Berg van den, 2001). Observational epidemiological studies clearly show a correlation between the increased consumption of food rich in antioxidants and a decreased risk of several oxidative stress related diseases. (Steinmetz and Potter, 1996) Protective effects of antioxidants have been found in animal studies as well as in epidemiological studies (Block and Patterson, 1992), and in some small–scale intervention studies (Porrini and Riso, 2000).

In mammals, reproduction, and particularly lactation, is the most energetically demanding period of a female's life. In the wild, investment in reproduction is associated with a decrease in survival in both large (Cluttonbrock *et al*., 1983) and small (Descamps *et al.,* 2006) mammals. Energy intake typically increases by 66–188% (Gittleman and Thompson, 1988), but in some animals such as laboratory mice, intake can increase by up to 400 per cent (Hammond, 1997). As a consequence, daily energy expenditure (Krol and Speakman, 2003), and basal metabolic rate (Speakman and McQueenie, 1996) can be increased in lactating when compared with non-reproductive females. This has led to the suggestion that oxidative

stress may be elevated during lactation, unless antioxidant defences are sufficiently upregulated (Speakman, 2008). There is evidence that oxidative damage increases during lactation in some domestic and laboratory animals (Upreti *et al.,* 2002). As yet, there has been very limited assessment of whether investment in reproduction elevates oxidative stress in mammals that have naturally selected levels of reproductive investment (Upreti *et al.,* 2002).

In this research, we stress that antioxidant supplementation not only will have a preventive effect on the health of the animals, but in turn can enhance the final product (milk), due to the increasing consumer's awareness of the relationship between diet, health, and disease prevention. We will revise how antioxidant supplementation given to lactating wistar rats for clinical purposes (disease prevention) can also affect the quality of the final product, adding value to production, with benefits not only for the health of animal.

Soybean is native to the Korean Peninsula and the Manchurian area (Ann and Jang, 2012), and soybean has been one of the major sources of protein in Korean food (Kim *et al.,* 2010). Numerous foods are made by soybeans, including doenjang (soybean paste), ganjang (soybean source), cheonggukjang (fast-fermented bean paste), bean curd, soybean milk, bean-curd dregs, etc (Yoo *et al.,* 2011). Soybean consumption is effective for the prevention of osteoporosis, arteriosclerosis, strokes and dementia, and can reduce the risk of cancer and obesity (Kim *et al.,* 2010). Furthermore, fermented soybean foods are effective in preventing and curing adult diseases (Yoo *et al.,* 2011).



Plate I: Image of fresh Soya bean plant (Yoo *et al.*, 2011)



Plate II: Soya bean seeds (Photograph by candidate, 2014)

1.1 Statement of Research Problem

Early and exclusive breastfeeding helps children survive, but it also supports healthy brain development, improves cognitive performance and is associated with better educational achievement at age 5. Breastfeeding is the foundation of good nutrition and protects children against disease. In this way, breastfeeding allows all children to thrive and develop to their full potential (UNICEF, 2015). Yet, less than half of the world's newborns benefit from

early breastfeeding and even fewer are exclusively breastfed for the first six months. Optimal breastfeeding of infants under two years of age has the greatest potential impact on child survival of all preventive interventions, with the potential to prevent over 800,000 deaths (13 per cent of all deaths) in children under five in the developing world (Mbori-Ngacha *et al.,* 2001). Problems of lactation are common in our environment, this may require the need to either induce or intervene to increase milk yield. Induction of lactation is a good option to overcome this problem, unfortunately not enough milk is produced to exclusively breastfeed the infant, but women find satisfaction in this rigorous process because of the maternal-infant bonding it promotes (Sharvan *et al.,* 2001). The Procedure commonly used to induce lactation includes both pharmacological and non-pharmacological methods, often in combination (Witting and Spatz, 2008). Agents that increase endogenous prolactin such as metoclopramide and domperidone have been used in a 7-10 day course to increase breast milk supply in mothers with lactational insufficiency (Gabay, 2002). However, one concern about these medications is their ability to penetrate the blood-brain barrier, which can result in central nervous system side effects such as depression and involuntary body movements (Dystonia), restlessness, drowsiness, fatigue especially with longer term use, (Anfision, 2002). For these reason alternative therapies are therefore needed.

Oxidative stress (OS) is considered a metabolic disturbance that affects functional systems and its presence affects not only the health status of the animal but also the quality and quantity of the final products, such as milk (Castilo, *et al.,* 2006). However, under proper nutritional and environmental conditions, it increases the antioxidant defense, maintaining a balance that favors lactation without compromising on maternal health (Abuelo *et al.,* 2013).

1.2 Significance of the Study

Although most mothers are capable of breastfeeding successfully, however approximately 25% of breastfeeding mothers will not produce enough milk to provide adequate nutrition for their infant, despite establishing a correct feeding pattern and feeding technique (Neifert *et al.,* 1990) This does not mean that they should stop breastfeeding their baby but that they will need additional supplementation throughout the breastfeeding period (Neifert, 2001). Concern for lactating women who are taking medications is the transfer of drugs into breast milk. Drugs circulating in the maternal blood stream can potentially be transferred into human breast milk, exposing breastfed infants to drugs that may potentially be harmful (Kimura *et al.,* 2006). Another concern is the effect of medication on the quantity and quality of breast milk produced, which may impact on the exclusivity, duration and success of breastfeeding (Hauck *et al.,* 2011). While orthodox treatments include conventional and complementary medicines, most studies to date have focused on evaluating the safety aspect of conventional medicines. Despite increasing popularity of herbal supplements, there are currently limited data available on the pattern of use and safety of these plants during breastfeeding (Tin *et al.,* 2013).

Breastfeeding provides numerous benefits for newborn infants and mothers. Breast milk provides tailored nourishment to the growing needs of infants, offering optimal nutrition, improved cognitive performance and neurological development (Foroushani *et al.,* 2010), and enhanced immunity (Abrahams and Labbok, 2011). It reduces the incidence of sudden infant death syndrome (SIDS), allergic/hypersensitivity diseases, and development of type 1 (insulin dependent) and type 2 (noninsulin dependent) diabetes mellitus, (Kimura *et al.,* 2006), relative to the use of infant formula. Women who have a history of breastfeeding

experience a reduced risk of osteoporosis and reduced incidence of breast and ovarian cancers (Jordan, *et al.,* 2010). Beside these health advantages, mothers and their babies are brought into closer emotional intimacy by nursing itself (Lawrence and Lawrence, 1999).

Several factors explain the tendency to use botanical galactogogues. These include adverse effects of synthetic drugs like hepatotoxicity, and an increased understanding of the chemistry, pharmacology and chemical use of botanical plants and their derivatives; the development of analytical methods that facilitate quality control, and the development of new ways of preparation and administration (Haya, 2007). With increased popularity of food supplements, there is need to further elucidate on the pattern of use and safety of these medicines during breastfeeding. This study aimed to identify the effect and pattern of use of fermented soybean and vitamin C as food supplement during breast feeding and to identify aspects which require further clinical research.

1.3 General Aim of the Study

This study was aimed at investigating the effects of supplementation with fermented soya bean and vitamin C on lactation process in female albino wistar rats

1.4 Objectives of the study

The objectives of this study were to investigate the following parameters in lactating female albino rats supplemented with fermented soya bean and vitamin C:

1. The galactopoietic effect of fermented Soya bean: Milk yield (18 and 23 hrs after gavage)
2. To determine the effect of administration of Soya bean supplement on Lactogenic hormones i.e.
   (i) Prolactin

(ii) Growth hormone

(iii) Oxytocin

(iv) Triidothyronine (T3)

(v) Thyroxine (T4)

(vi) Insulin

(vi) Progesterone

(vii) Oestrogen

3. To evaluate the effect of fermented soya bean and vitamin C supplement on serum levels of the following liver enzymes: Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST).

4. To evaluate the effect of the supplement on the serum levels of the following biomarkers of oxidative stress in lactating female albino rats:

i. Malondialdehyde (MDA)

ii. Superoxide dismutase (SOD)

iii. Catalase (CAT)

iv. Glutathione peroxidase (GPx)

5. To determine the effects of the Soya bean supplement on renal function, that is serum urea and serum level of the following electrolytes was determined:

(i) Calcium

(ii) Sodium

(iii) Potassim

(iv) Magnesium

(v) Chloride

(vi) Bicarbonate

6. To determine the effect of the supplement on the following hematological parameters

(i) Red blood cell count,

(ii) Hemoglobin (Hb) concentration

(iii) Packed cell volume (PCV)

(iv) White blood cell count

(v) Platelets count

7. To evaluate the effect of the supplement on histology of the mammary tissue

1.5 Research Hypothesis

H_0= Fermented soybean and Vitamin C supplementation does not increase milk production and serum levels of lactogenic hormones in lactating female albino rats.

H_1= Fermented soybean and Vitamin C supplementation increase milk production and serum levels of lactogenic hormones in lactating female albino rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

Mother's milk is undoubtedly a great and unique blessing of the God; our Almighty, Powerful, and Alert Creator, that has nourished the entire needs of a disabled infant at his/her birth and is regarded as the healthiest, the safest, the freshest, the most accessible and an ideal nourishment for a child during the first 2 years of his/her life. Mother's milk, an exceptional compound with unique characteristics, guarantees to nurture and maintain a child's physical and mental health. In addition, it has innumerable benefits for a nursing mother in terms of health and vitality. In other words, the matchless positive effect of this miraculous fluid on maternal and child health is undeniable. Human milk provides immunological, nutritional, and evolutionary benefits to an infant which cannot be provided by artificial (powder) milk. Extensive studies using advanced epidemiological methods and modern laboratory techniques have successfully proved various and significant advantages of breast-feeding for infants besides its positive influences on mothers, families, and communities (Baskabadi, 2012).

According to WHO, breast-feeding is the best way of providing young infants with the nutrients they need for healthy growth and development (WHO, 2009). Infant health benefits of breast-feeding include provision of nutritional, immunological and developmental benefits (Ramadhani *et al.,* 2011). Over the past several years, the health advantages of breast-feeding and recommendations for practice have been strengthened. The health benefits of breast-feeding are influenced by both the duration and intensity of breast-feeding and by age at which the child receives complementary foods (Horta *et al.,* 2007).

2.1 Physiologic Anatomy of the Breast

Milk is produced in the alveolus. The alveolus is made up of gland cells around a central duct. The milk is produced by the gland cells. Surrounding the gland cells are the myoepithelial cells which contract to cause milk ejection into the milk duct. The milk then travels down the lactiferous ducts (Pandya and Moore, 2011). An alveolus consists of single layer of cells attached to a basement membrane, a vascular system and myoepithelial cells (Figure 2.1). The alveolar cells are packed close together and are tightly attached to each other. The alveoli are kept together by a thin layer of connective tissue. It contains the nerves and blood vessels. Myoepithelial cells are of ectodermal origin. Myoepithehal cells lie on epithelial side of basement membrane. These cells are arranged in a criss-cross arrangement around the alveolus and smaller ducts (Bruckmaier and Blum, 1998). Milk is stored largely in the alveoli with little storage in the ducts between breast feedings. Mothers continue to make milk between feedings and they make more milk during feedings (Hale and Hartmann, 2007).

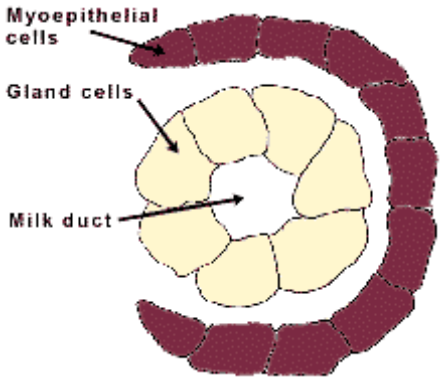


Figure 2.1: Histological presentation of the mammary gland (Hale and Hartmann, 2007)

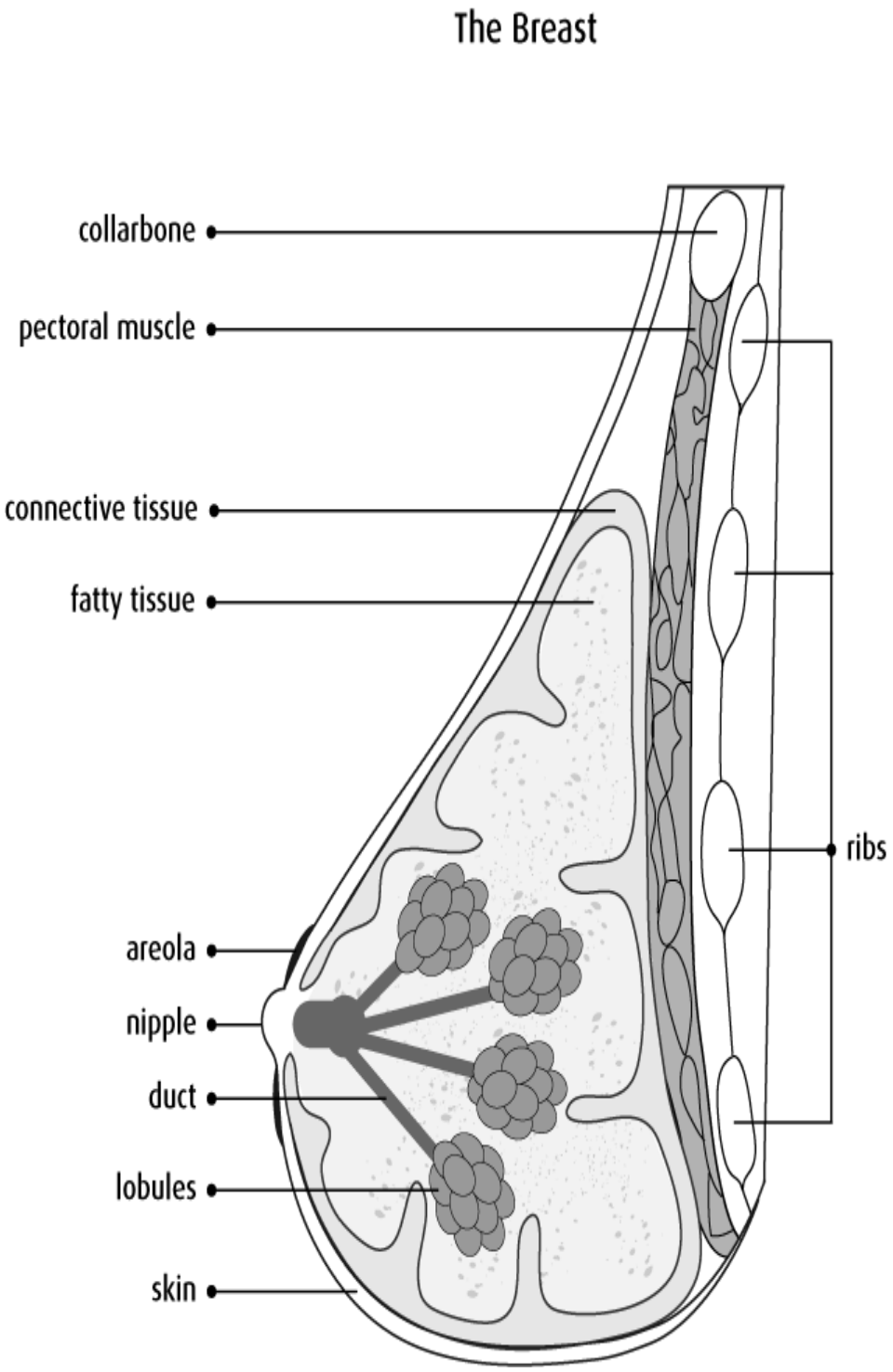


Figure 2.2: A cross-section view of the alveolus (Bruckmaier and Blum, 1998).

2.2 Mammogenesis

2.2.1 Mammary alveoli cells

Mammary alveoli are comprised of two distinct epithelial cell types—an inner layer of alveolar luminal cells, which selectively extract nutrients from the maternal circulation for secretion into milk, and a meshwork of myoepithelial cells on the basal surface that are responsible for generating the contractile force necessary for milk ejection (Watson and Khaled, 2008).

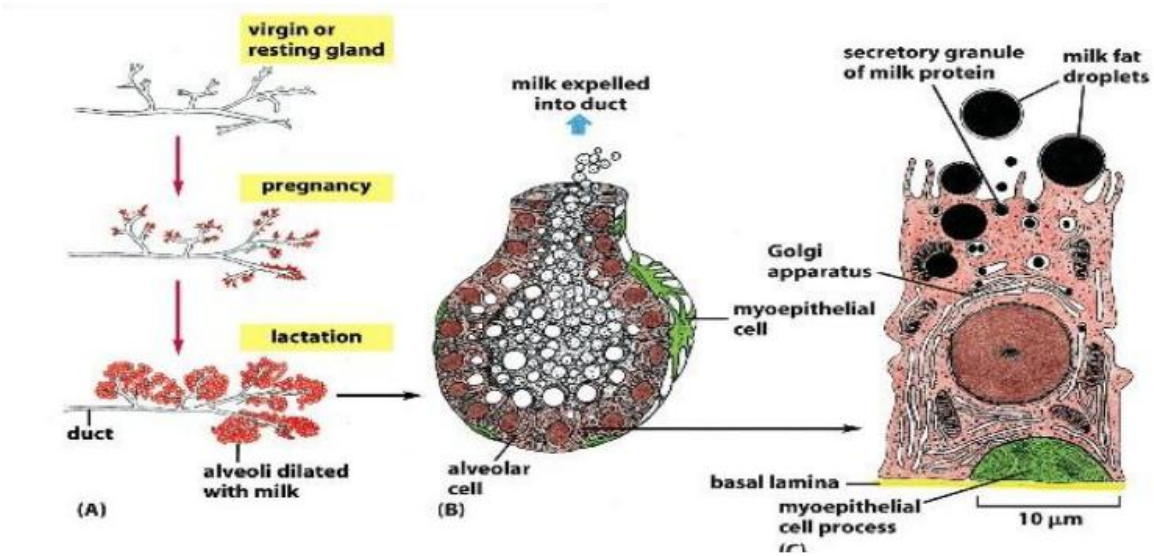


Figure 2.3: Mammary gland epithelium (Watkins *et al.*, 2008)

Mammary gland is a unique organ, which undergoes the majority of its development in the postnatal life of mammals, especially during puberty and pregnancy. It is a specialized epidermal appendage, whose development begins during embryogenesis but is not fully completed until the beginning of lactation (Cowen and Wysolmerski, 2010). It is a complex tissue comprised of many cell types. The actual glandular part is formed of two epithelial cell

subtypes: outer myoepithelial and inner luminal epithelia, which form a complicated net of ducts and lobules involved in milk synthesis and secretion during lactation.

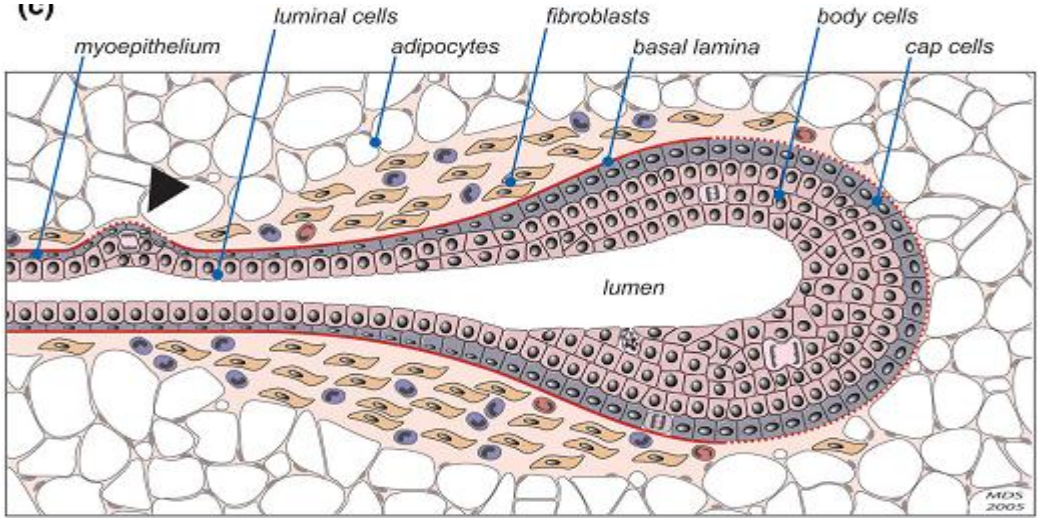


Figure 2.4: Mammary cells and layers (Sternlicht, 2005)

The glandular epithelium is embedded in the stroma composed of mesenchymal cells, such as fibroblasts, adipocytes, immune cells, and extracellular matrix (ECM). The stroma consists of fat cells and connective tissue, which is collectively known as the mammary fat pad. In women, the glandular tissue of the mammary gland is arranged in approximately 15-20 lobes. The lobes, lobules, and their ducts are surrounded and separated from each other by bundles of connective tissue or stroma. Each lobe consists of a branching structure made up of 20-40 lobules and finally terminates in clusters of acine (or alveoli). The alveoli are lined by a single layer of secretory epithelial cells, which are joined just below their luminal surface by tight junctional complexes and are surrounded by a diffuse layer of contractile basal-myoepithelial cells involved in milk ejection. The alveolus is then surrounded by a basement membrane. The lumen of the alveoli connects to collecting interlobular ducts, which empty

into the main 15 to 20 lobular collecting ducts. In turn, each lobe drains into the nipple. Within 24– 36 h of its formation, the mammary line resolves into one placode, which undergoes some changes. The general feature of the mammary gland development is universal for animals and humans; however, some differences in growth rate and hormonal control of the process can be distinguished between species. The mammary gland undergoes a series of well coordinated phases of development prior to lactation (Imagawa *et al*. 1990).

2.2.2 Mammary gland development

The mammary gland development during all stages is influenced by some factors, which certainly are the endocrine hormones that interact with the different growth factors and the epithelial and mesenchymal constituents (Hovey *et al*., 2002). The development and function of the mammary gland occurs through a cyclical process that changes during the physiological states of pregnancy, lactation and involution. During pregnancy, maternal hormones in circulation are responsible for the stimulation of the mammary gland development which ensures a sufficient number of mammary cells to produce milk during lactation (Capuco *et al*., 2003). Immediately prior to parturition, lactogenic hormones stimulate the differentiation of mammary cells, and they adopt a secretory phenotype (Boutinaud *et al*., 2004). In addition to the influence of the hormones, the mammary gland itself is thought to be involved in the regulation of mammary cell proliferation, differentiation and response to hormones. Ultimately, milk yield is dependent on the number and metabolic activity of secretory cells; however, both of these factors are tightly regulated by the endocrine system and physiological state, and also by local factors including the uptake of nutrients by the mammary gland, the connective tissue surrounding the epithelium, and the frequency and degree of milk removal (Akers, 2002). Therefore, improved lactation

performance can be achieved under conditions that enhance mammary cell proliferation (or decrease apoptosis), biochemical and structural differentiation of mammary epithelium, and synthesis and secretion of milk components. Because the deoxyribonucleic acid (DNA) content per mammary cell nucleus remains relatively constant during pregnancy and lactation, total DNA is considered an accurate indicator of mammary cell number (Tucker, 1987). Mammary cell secretory activity can be estimated by quantification of mammary ribonucleic acid (RNA), and the ratio of RNA to DNA (Paape and Tucker, 1969). Measurements of both DNA and RNA content have provided insight into the relationship between mammary cell number, secretory activity, and milk yield. The secretory activity of these cells is also an important factor involved in determining milk production potential. During lactogenesis, the mammary epithelium becomes highly differentiated. This period is associated with an overall increase in the size and metabolic activity of each cell, closure of tight junctions between cells, an increase in mitochondrial size, and development of the endoplasmic reticulum (Nickerson and Akers, 1984). During established lactation, any new cells that are formed are thought to become differentiated almost immediately (Tucker, 2000). The increase in milk yield during early lactation is associated with an increase in mammary DNA, followed by an increase in mammary cell secretory activity (Knight and Peaker, 1984). In response to the hormones of pregnancy, the epithelial compartment of the mammary gland undergoes proliferation and subsequent differentiation to generate alveoli that progressively fill the interductal spaces during late pregnancy, parturition, and lactation. Following weaning, removal of the suckling stimulus triggers collapse of the lobuloalveolar system through proteinase-mediated and apoptotic-mediated reductive remodeling processes, collectively termed involution as shown in figure 2.

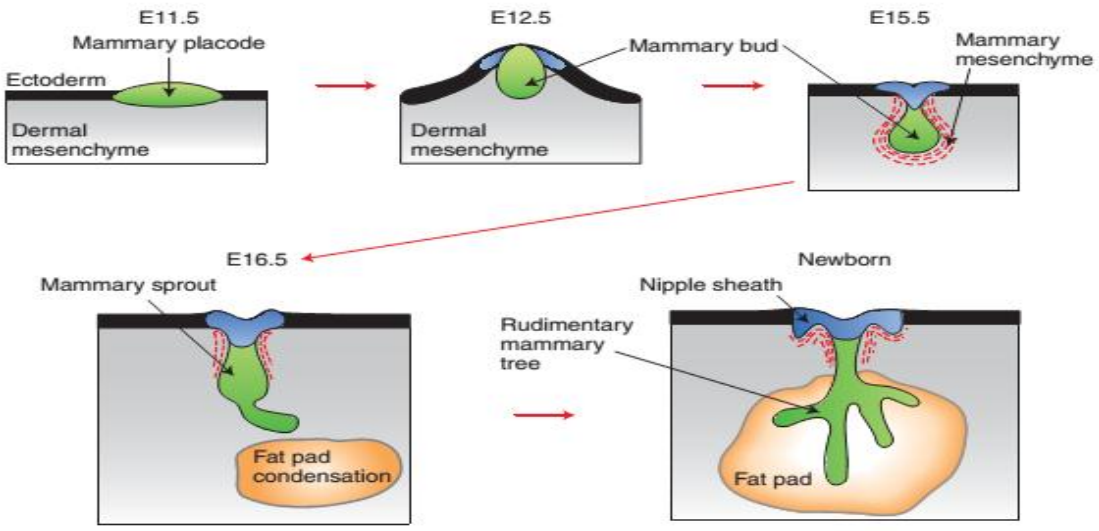


Figure 2.5: Formation of mammary gland (Cowen and Wysolmerski, 2010)

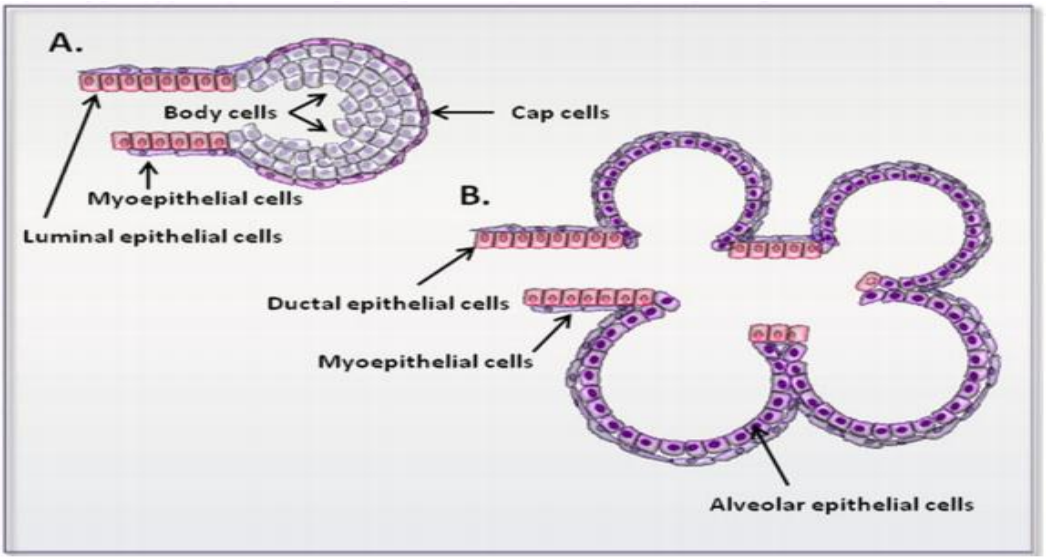


Figure 2.6: Mammary epithelium in different stages of differentiation. A: prepubertal and B: during pregnancy (Gajewska *et al*., 2013)

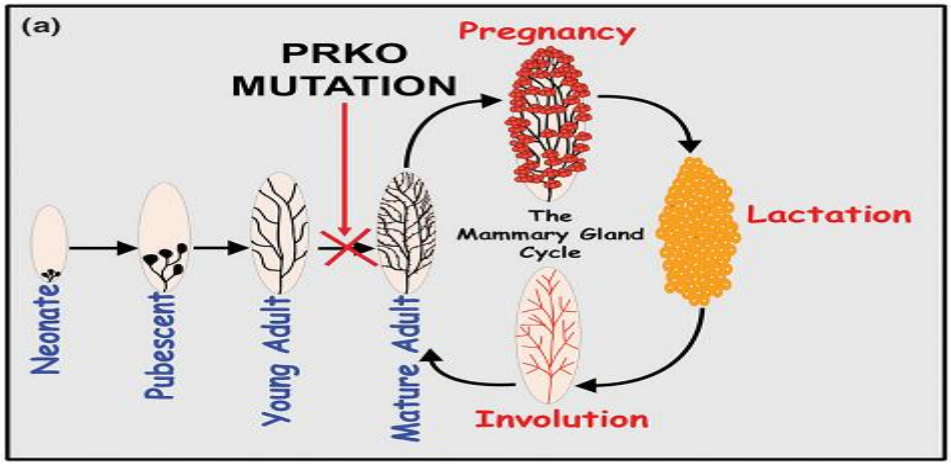


Figure 2.7: The mammary gland cycle (Lydon *et al*., 2000)

2.3 Lactogenesis

Lactogenesis or initiation of the capacity of the breast for milk secretion has been conceptually divided into 2 stages (Neville and Morton, 2001). Stage I begins as early as 12 weeks prior to birth. The high levels of prolactin during pregnancy do not usually increase large volumes of milk production due to the antagonism of progesterone hormone (Berens, 2004). Stage II typically occurs around day 2-3 postpartum, resulting in a copious increase in milk volume, often referred to by the mother as the "milk coming in", the first sudden sensation of breast fullness, engorgement and milk leakage after birth and may indicate a milk supply that is more than adequate for exclusively breastfeeding for 6 months (Chen *et al*., 1998). Milk synthesis starts in the epithelial cells of the mammary gland at the end of pregnancy to support the nutrition and promote the health of the offspring.

2.3.1 Some major lactational hormones

One of the major roles of the endocrine system is to coordinate mammary function with the reproductive state of the animal. This physiological synchronization is a very complex process that involves the action and interaction of multiple hormones, as well as the interplay

between hormones in circulation and local regulation of the mammary response to these hormones (Wall and Mcfadden, 2012). During lactation, several key hormones are involved in the regulation of mammary cell number, secretory activity, and consequent milk production potential.

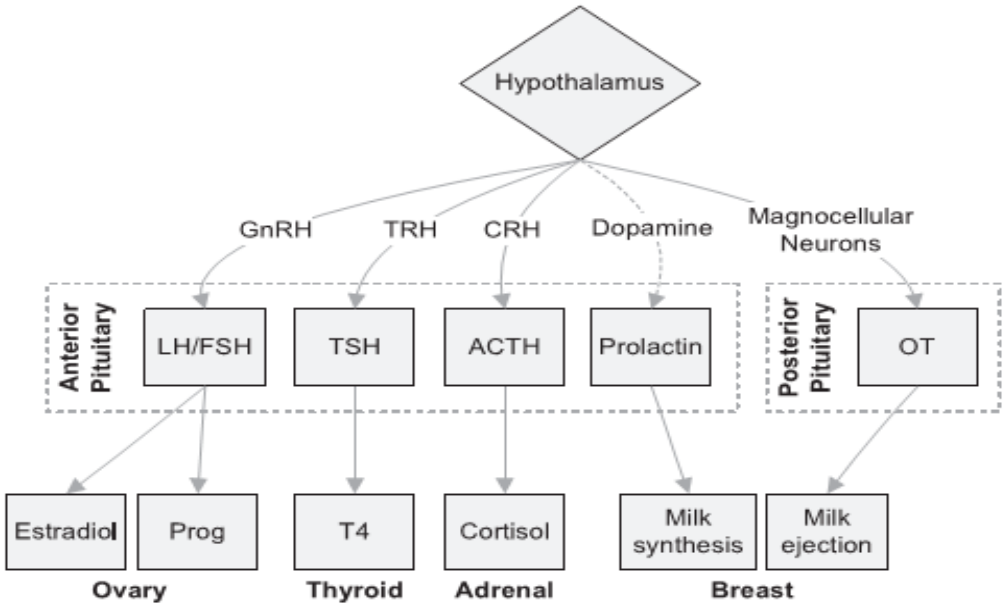


Figure 2.8: Neuroendocrine regulation of lactogenesis (Allison *et al.*, 2012)

2.3.1.1 Prolactin hormone

Prolactin (PRL) is mainly synthesized and secreted by the lactotrophic cells of the pituitary gland (Freeman *et al*., 2000), but also by extra pituitary sites such as mammary gland, placenta, uterus and T lymphocytes (Ben-Jonathan and Hnasko, 2001). Its amino acid sequence is similar to that of growth hormone (GH) and placental lactogen (PL), sharing genomic, structural and biological features and belonging to the same PRL/GH/PL protein family. It is now considered that PRL is a cytokine, based on both molecular and functional evidence (Horseman and Yu-Lee, 1994). As the name indicates, PRL is known as the

hormone of lactation. The varied effects of prolactin on the mammary gland include growth and development of the mammary gland (mammogenesis), synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis) (Freeman *et al*., 2002). Accordingly, it has received much attention from lactation biologists studying the hormonal regulation of mammary function. In ruminants, PRL and glucocorticoids provide the primary stimulus for lactogenesis (Akers, 1985). Prolactin has been shown to maintain both the structural integrity and the functional activity of the mammary epithelium during lactation. Prolactin, as well as PL and primate GH, binds the same PRL receptor (PRLR). Multiple isoforms of membrane-bound PRLR resulting from alternative splicing of the primary transcript have been identified in several species (Bole-Feysot *et al.*, 1998).

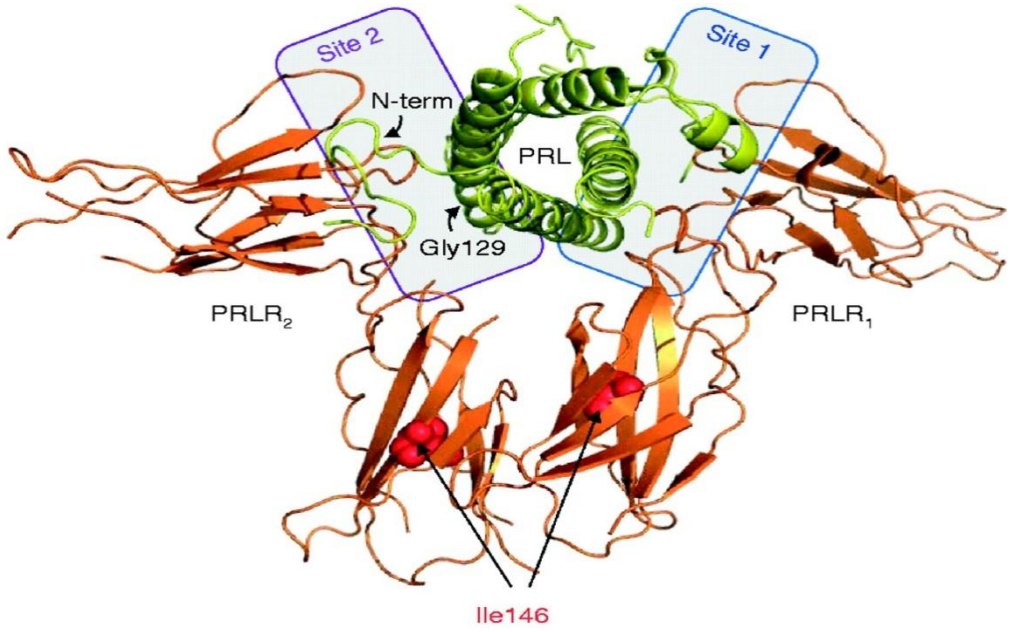


Figure 2.9: Chemical structure of prolactin (Hennighausen *et al*., 1997a)

Prolactin receptor activation

The action of PRL in the mammary gland is mediated through its receptor, which activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Hennighausen *et al*., 1997a). The understanding of how PRL transmits diverse signals to target cells is facilitated by the elucidation of the JAK/Stat pathway as the prototype signaling pathway used by all cytokine receptors. Prolactin is believed to activate sequentially the receptor by dimerizing two identical receptor subunits, leading to activation of Jak2 kinase associated with the cytoplasmic domain. Although the Jak2–Stat cascade is the major signaling pathway used by the PRL receptor, other transducing pathways are also involved in signal transduction by this receptor, the likes of signaling through MAP kinases (MAPK). Functional development of mammary epithelium during pregnancy depends mostly on PRL signaling. Proliferation and differentiation of secretory mammary epithelium is dependent on the presence of PRL receptor as well as its downstream Jak2–Stat5 pathway. Activation of prolactin-R involves ligand-induced sequential receptor dimerization driven by the prolactin molecule containing two binding sites (Boyle-Feysot *et al*., 1998). First, prolactin's binding site 1 interacts with a prolactin-R molecule. The extracellular (EC) domain of all prolactin-R isoforms consists of NH2-terminal (D1) and membrane-proximal (D2) sub-domains (Carstan *et al*., 1997), both of which show analogies with the fibronectin type III molecule driving the receptor-ligand interactions in cytokine receptors (Wells and Devos, 1996). The formation of the initial hormone-receptor complex induces the interaction of binding site 2 on the same prolactin molecule with a second prolactin-R (step 2) (Boyle-Feysot *et al*., 1998). Although the intracellular (IC) domains of prolactin-R isoforms differ in length and composition, there are two conserved regions, termed box 1 and box 2 (Murakami

et al., 1991). Both the presence of the proline-rich box 1 and strict homodimeric stoichiometry of prolactin-R dimers are necessary for the activation of the tyrosine kinase termed Janus kinase2 (Jak2), constitutively associated with the IC domain of the prolactin-R (1013). Jak2 kinases transphosphorylate each other (step 2) and phosphorylate (P) the Tyr residues (Y) of the prolactin-R itself (step 3) (Freeman *et al*., 2000). Although phosphorylation of Jak2 is a key event in the activation of all prolactin-R isoforms, Tyr phosphorylation of the receptor itself does not occur upon activation of the short form of the prolactin-R, despite the presence of four Tyr residues in its intracellular domain (Rui *et al*., 1992).

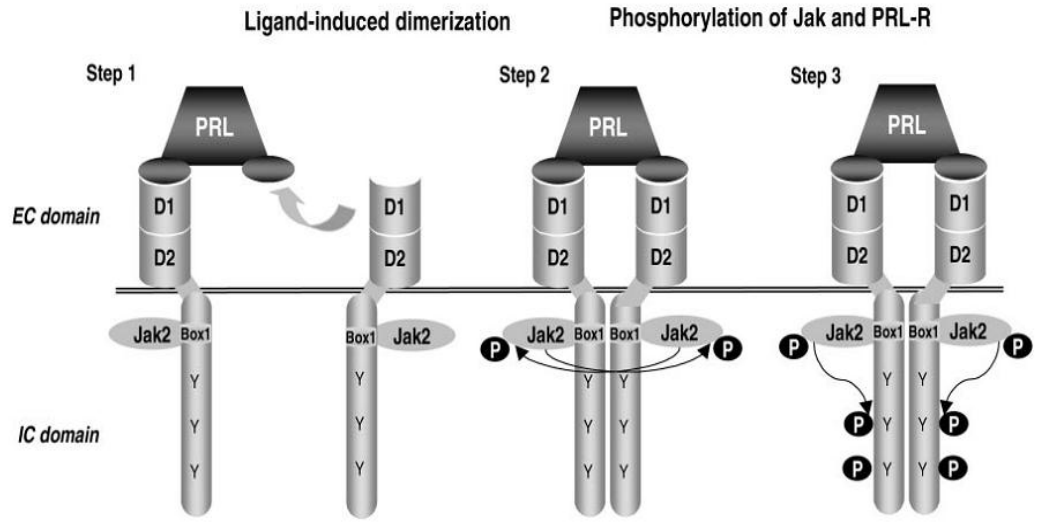


Figure 2.10: Mechanism of prolactin receptor activation (Freeman *et al*., 2000)

Prolactin signal transduction pathway

Signal transduction pathways are initiated by activation of the prolactin (PRL) receptor. Jak/STAT pathway: members of the signal transducer and activator of transcription (STAT) protein family (Finidor and Kelly, 1995), STAT1, STAT3, STAT5a, and STAT5b are the

central transducer molecules of the signal transduction pathways initiated by prolactin-R (PRL-R) activation (Jabbourhn *et al*., 1998). The STAT contains a DNA-binding domain, and SH3-like domain, an SH2 like domain, and an NH2- and a COOH-terminal trans-activating domain (Finidor and Kelly, 1995). A phosphorylated Tyr residue (Y) of the activated long prolactin-R isoform interacts with the SH2 domain of a STAT. The STAT, while docked at the receptor, is phosphorylated by the receptor-associated Jak kinase. Then, phosphorylated STAT dissociates from the receptor and hetero- or homodimerizes through its phosphotyrosine residues with the SH2 domain of another phosphorylated STAT molecule. Finally, the STAT dimer translocates to the nucleus and activates a STAT DNA-binding motif in the promoter of a target gene (Boyle-Feysot *et al*., 1998), termed GAS (g-interferon activated sequence) (Horseman and Yu-lee, 1994). The tyrosine residues of the short form of prolactin receptor are not phosphorylated by Jak2, but the phosphotyrosine of Jak2 can serve as docking site for Stat1 (Boyle-Feysot *et al*., 1998). The MAPK cascade: activation of the prolactin-R also activates the mitogen-activated protein kinase (MAPK) cascade (Pi and Grattan, 1998), which is involved in the activation of a wide range of transcription factors/immediate early genes by phosphorylation. Phosphotyrosine residues of the activated long prolactin-R isoform serve as docking sites for adapter proteins (Shc/Grb2/SOS) connecting the receptor to the Ras/Raf/MAPK cascade (Da and Vonderhaar, 1995). Novel data indicate communication between the Jak/STAT and MAPK pathways (Guera *et al*., 1998). Ion channels: box 1 of the intracellular domain of prolactin-R is also involved in the activation of a tyrosine kinase-dependent, calcium-sensitive K^+ channels through Jak2. The COOH terminal of prolactin-R's intracellular domain is involved in the production of the intracellular messengers (inositol 1,3,4,5-tetrakisphosphate (IP4) and inositol

hexakisphosphate (IP6)) that open voltage-independent Ca^{2+} channels. Src kinases: prolactin also induces the activation of members of the Src kinase family, c-src and Fyn, which are involved in the Tyr phosphorylation of phosphatidylinositol 3-kinase (PI3K). Down-regulation: Jak/STAT pathways can be inhibited by SOCS (suppressors of cytokine signaling) which inhibit Jak kinases or CIS (cytokine-inducible SH2-containing protein), which compete with STAT for docking sites on prolactin receptor.

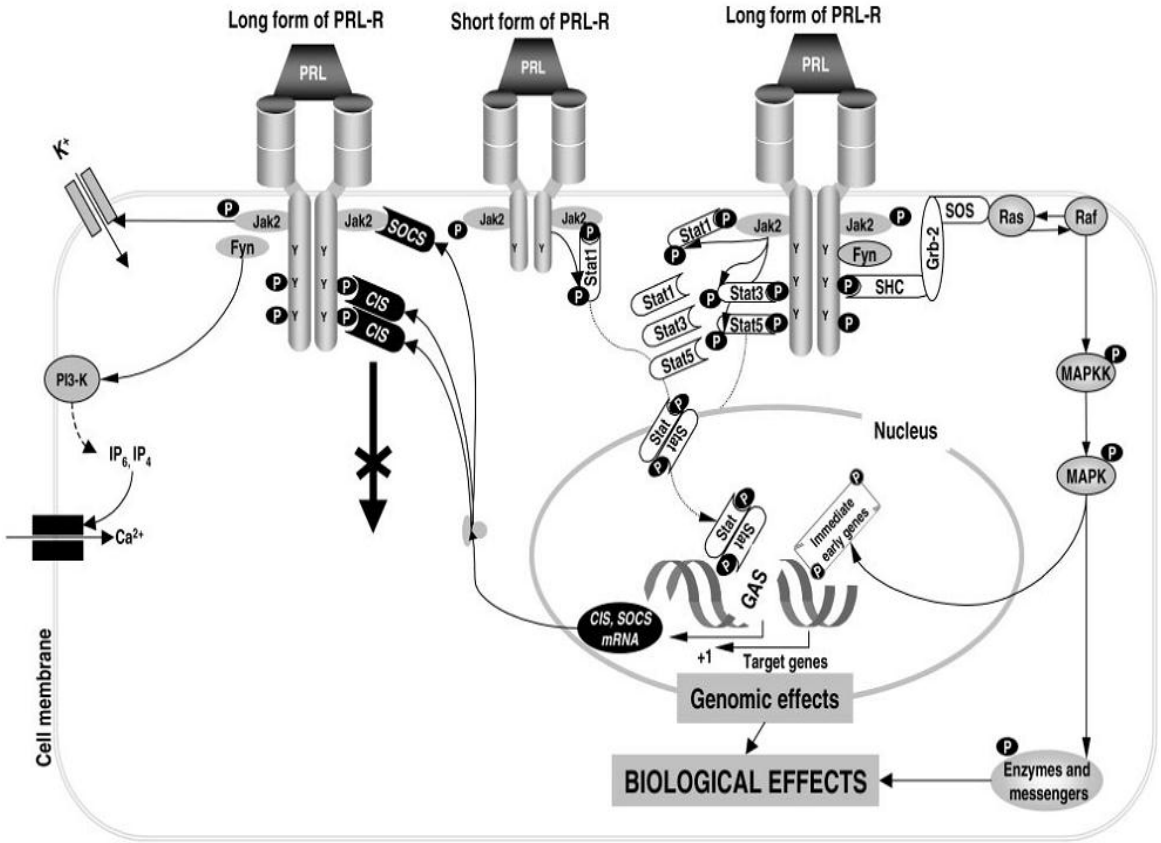


Figure 2.11: Signal transduction pathways of prolactin (Freeman *et al*., 2000)

Regulation of prolactin secretion

Prolactin secretion is affected by a large variety of stimuli provided by the environment and the internal milieu. The most important physiological stimuli that elevate pituitary prolactin secretion are suckling (Neill, 1974) stress and increased levels of ovarian steroids, primarily estrogen (Neill, 1974). Such stimuli are transduced by the hypothalamus which elaborates a host of prolactin releasing factors (PRF) and prolactin inhibiting factors (PIF). In mammals, the control exerted by the hypothalamus over pituitary prolactin secretion is largely inhibitory (Terkel *et al*., 1972). On the other hand, the hypothalamus is also involved in the acute stimulatory control of prolactin secretion by removal of the inhibition (disinhibition) and/or superimposition of brief stimulatory input. In addition, prolactin secretion is also influenced by numerous factors released by the lactotrophs themselves (autocrine regulation) or by other cells within the pituitary gland (paracrine regulation). Prolactin secretion is paced by a light-entrained circadian rhythm, which is modified by environmental input, with the internal milieu and reproductive stimuli affecting the inhibitory or stimulatory elements of the hypothalamic regulatory circuit. The final common pathways of the central stimulatory and inhibitory control of prolactin secretion are the neuroendocrine neurons producing prolactin inhibiting factors (PIF), such as dopamine (DA), somatostatin (SST), and γ-aminobutyric acid (GABA); or prolactin releasing factors (PRF), such as thyrotropin releasing hormone (TRH), oxytocin (OT), and neurotensin (NT). The PIF and PRF from the neuroendocrine neurons can be released either at the median eminence into the long portal veins or at the neuro-intermediate lobe, which is connected to the anterior lobe of the pituitary gland by the short portal vessels. Thus, lactotrophs are regulated by blood-borne agents of central nervous system or pituitary origin (a-melanocyte stimulating hormone) delivered to the anterior lobe

by the long or short portal veins. Lactotrophs are also influenced by PRF and PIF released from neighboring cells (paracrine regulation) or from the lactotrophs themselves (autocrine regulation) (Freeman *et al*., 2000).

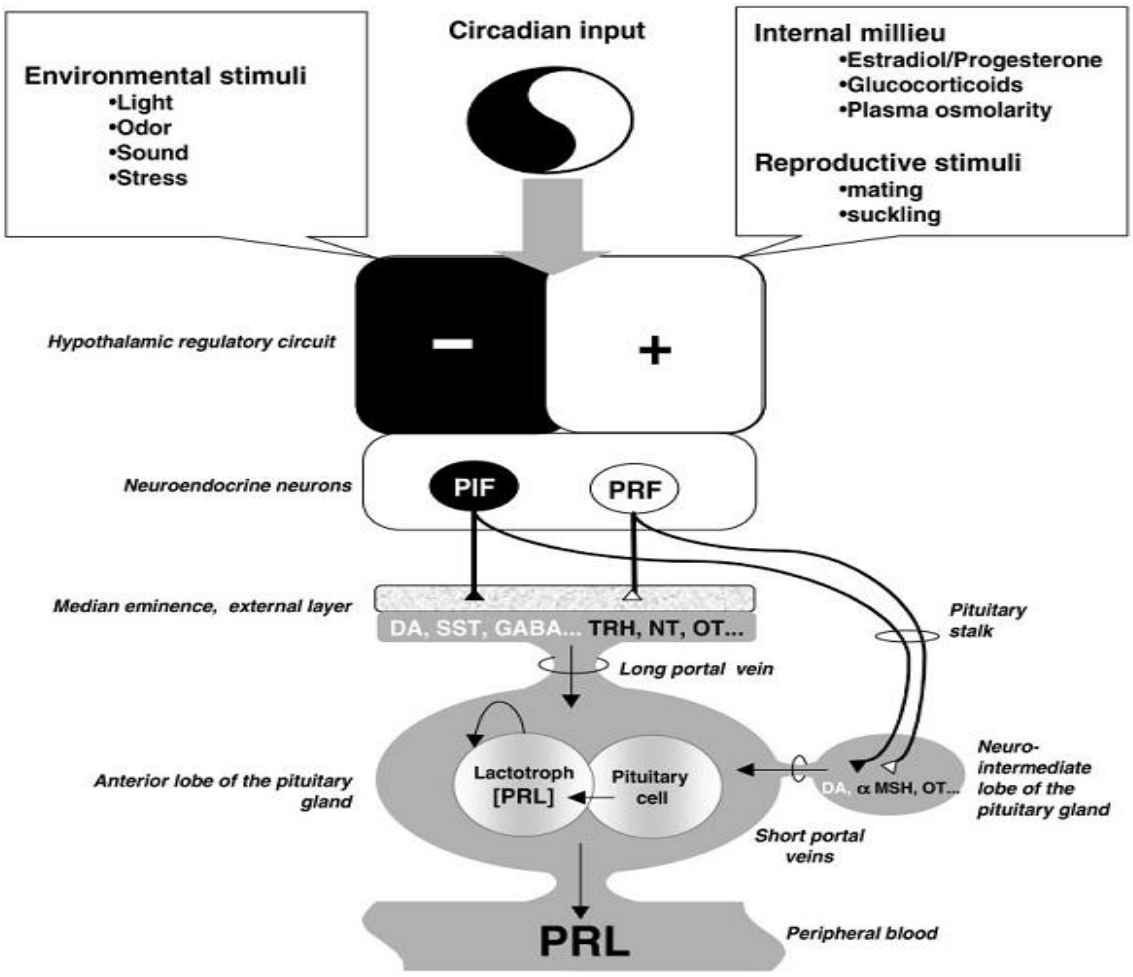


Figure 2.12: Regulation of prolactin secretion (Freeman *et al*., 2000)

2.3.1.2 Growth hormone

Growth hormone (GH), also called somatotropin, is a peptide hormone consisting of 190 or 191 amino acids. Four variants of Growth hormone have been described: either a leucine or valine at position 127, and either a 190- amino acid variant with a phenylalanine at the N-terminus or a 191-amino acid protein with an alanine at the N-terminus (Bauman and Vernon, 1993; Etherton and Bauman, 1998).



Figure 2.13: Chemical structure of growth hormone (Tuggle and Trenkle, 1996)

Growth hormone releasing hormone, secreted from the hypothalamus, stimulates the secretion of Growth hormone from the pituitary gland. Somatostatin also secreted from the hypothalamus, inhibits the release of Growth hormone (Tuggle and Trenkle, 1996). Growth hormone is secreted from the anterior pituitary gland in a pulsatile fashion (Gluckman *et al*., 1987), and acts on target tissues to promote growth. Secretion from the pituitary is the major

source of Growth hormone; however, several other tissues synthesize GH, including brain, placenta, mammary and pineal gland (Butler and Le Roith, 2001). The synergistic effects of estrogen and GH serve to lengthen and branch the rudimentary ductal system of the mammary gland (Kleinberg, 1997). It has been suggested that prolactin stimulates paracrine release of insulin-like growth factor II (IGF-II) within the mammary gland, which stimulates development of alveoli (Brisken *et al*., 2002). Growth hormone also promotes mammary epithelial proliferation, but to a much lesser extent than seen during gestation (Carstens *et al*., 1997; Sejrsen *et al*., 1986).

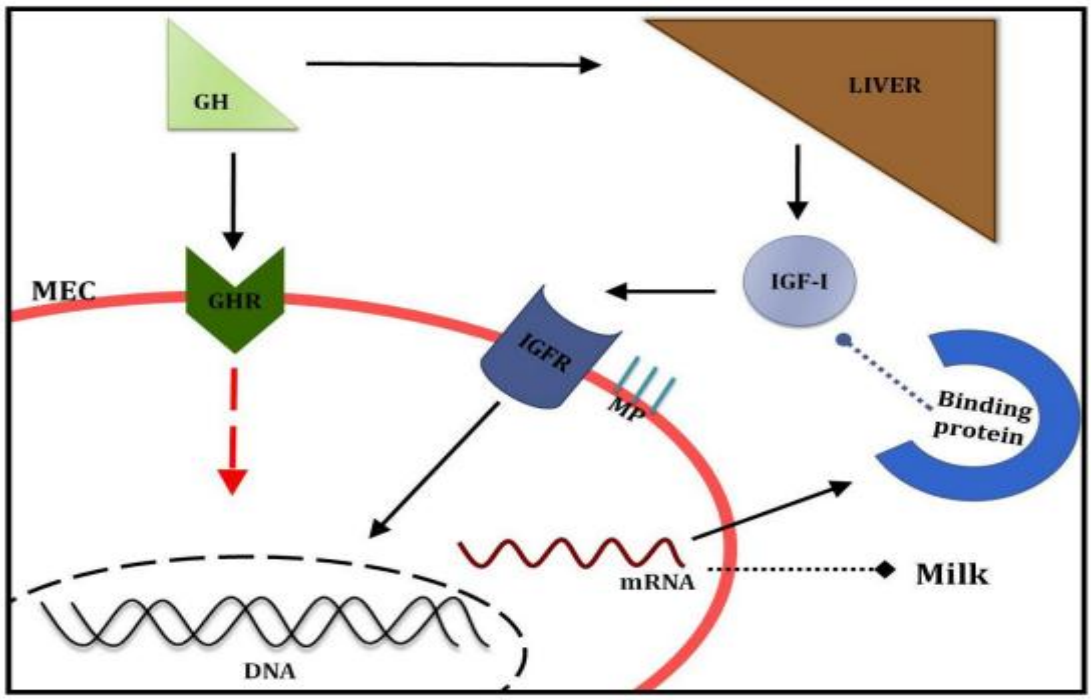


Figure 2.14: Activity of growth Hormone in lactation (Yamada and Lee, 2009)

Growth hormone receptor activation

The downstream signaling pathways mediated by the GHR are initiated upon the binding of GH to the extracellular domain of the GHR. Formation of this GH-GHR2 trimer complex was thought to be necessary and sufficient for GH responses (Wells, 1996). However, other studies indicated that dimerization of the GHR was insufficient for activation of GH mediated signaling (Rowlinson *et al*., 1998) and that preformed GHR dimers exist prior to GH binding (Gent *et al*., 2002).

Signal transduction

Growth hormone binding to Growth hormone receptor (GHR) activates the tyrosine kinase Janus kinase 2 (JAK2), thus initiating a multitude of signaling cascades that result in a variety of biological responses including cellular proliferation, differentiation and migration, prevention of apoptosis, cytoskeletal reorganization and regulation of metabolic pathways. A number of signaling proteins and pathways activated by GH have been identified, including JAKs, signal transducers and activators of transcription (Stats), the mitogen activated protein kinase (MAPK) pathway, and the phosphatidylinositol 3'-kinase (PI3K) pathway. Although these signal transduction pathways have been well characterized, the manner by which GH activates these pathways, the downstream signals induced by these pathways, and the cross-talk with other pathways are not completely understood. One of the more interesting recent developments in GH signaling is support for the hypothesis that not all GH signaling events lie downstream of JAK2 (Zhu *et al*., 2002). Despite the key role for JAK2 in GHR signaling, evidence continues to mount demonstrating a number of JAK2-independent signaling mechanisms, particularly using Src (sarcoma; a non receptor proto-oncogene encoding a tyrosine kinase) family kinases. Extracellular signal-regulated kinases (ERK1/2) activation

has been demonstrated to occur through an alternative JAK2-independent pathway (Zhu *et al*., 2002; Ling *et al*., 2003). The JAK2 also binds other adaptor proteins independently of the STATs as shown in the figure below. Binding of Shc to JAK2 leads to sequential activation of the growth factor receptor bound protein (Grb2), Son of sevenless (sos); so named because the sos protein it encoded was found to operate downstream of the sevenless gene in *drosophila melanogaster*, rat sarcoma (Ras), and rapidly accelerated fibrosarcoma (Raf); together referred to as Grb2-SOS-Ras/Raf- MAPK pathway in which activated ERK1/2 either directly, or via phosphorylation of the transcription factors Elk (Zhu *et al*., 2001).

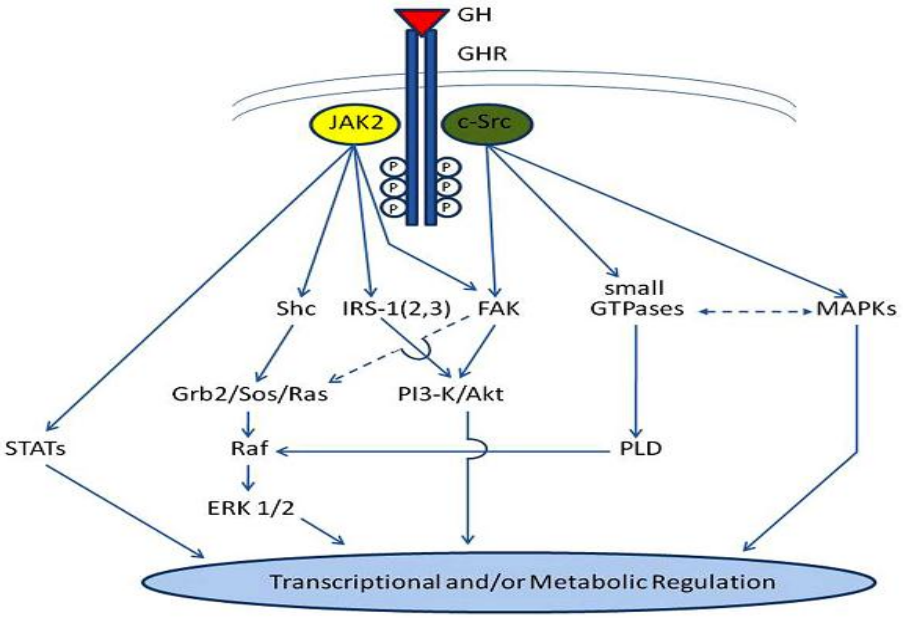


Figure 2.15: Growth hormone signal transduction pathway (Shafei *et al*, 2006**)**

Regulation of growth hormone

Secretion of growth hormone (GH) in the pituitary is regulated by the neurosecretory nuclei of the hypothalamus. These cells release the peptides Growth hormone releasing hormone (GHRH) or somatocrinin and Growth hormone-inhibiting hormone (GHIH) or somatostatin into the hypophyseal portal venous blood surrounding the pituitary gland. Growth hormone release in the pituitary is primarily determined by the balance of these two peptides, which in turn is affected by many physiological stimulators (e.g., exercise, nutrition, sleep) and inhibitors (e.g., free fatty acids) of GH secretion (Leung *et al*., 2002). Somatotropic cells in the adenohypophysis synthesize and secrete GH in a pulsatile manner, in response to these stimuli by the hypothalamus. The largest and most predictable of these GH peaks occurs about an hour after the onset of sleep with plasma levels of 13-17 ng/ML (Takahashi *et al*., 1968). Surges of secretion during the day occur at 3 to 5 hours intervals. The plasma concentrations of GH during these peaks may range from 5-45 ng/mL. A number of factors are known to affect GH secretion, such as age, sex, diet, exercise, stress and other hormones. Young adolescents secrete GH at the rate of about 700 µg per day, while healthy adults secrete GH at the rate of about 400 µg per day. Sleep deprivation generally suppresses GH release, particularly after early adulthood (Mullington *et al*., 1996).

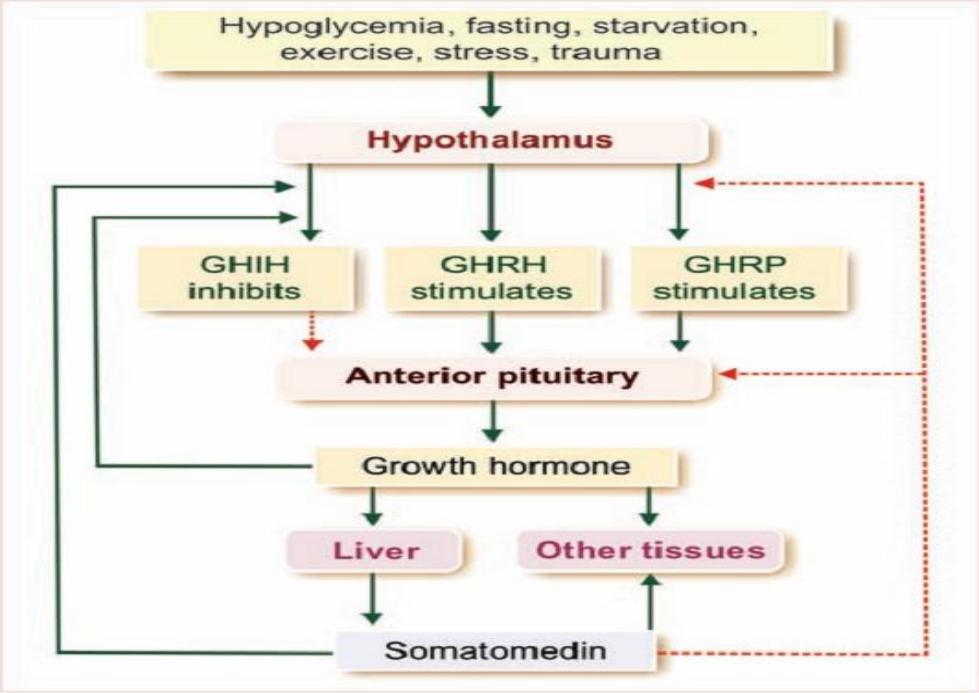


Figure 2.16: Regulation of growth hormone secretion
(Sembulingam and Sembulingam, 2012)

2.4 The Milk Ejection Reflex

In addition to the role of oxytocin in the stimulation of uterine contractions during labor and delivery, oxytocin continues to play an important role in lactation after birth. Stimulation of the nipple by suckling sends a signal via the spinal cord to the hypothalamus to induce the release of oxytocin from the pituitary into circulation (Neumann *et al*., 1996). The oxytocin then binds to oxytocin receptors (OTRs) on the smooth muscle cells of alveoli in the mammary glands, causing them to contract and eject milk into the ducts and cisterns in what is referred to as the milk ejection reflex. Just as OTRs in the uterus become more sensitive to oxytocin prior to birth, the sensitivity of OTRs in the mammary glands also increases leading up to delivery to make the mother more physiologically responsive to circulating oxytocin

(Soloff *et al*., 1979). Furthermore, by binding to OTRs on prolactin-releasing cells in the anterior pituitary gland, oxytocin also stimulates the release of prolactin, a hormone responsible for milk production (Jonas, 2009).

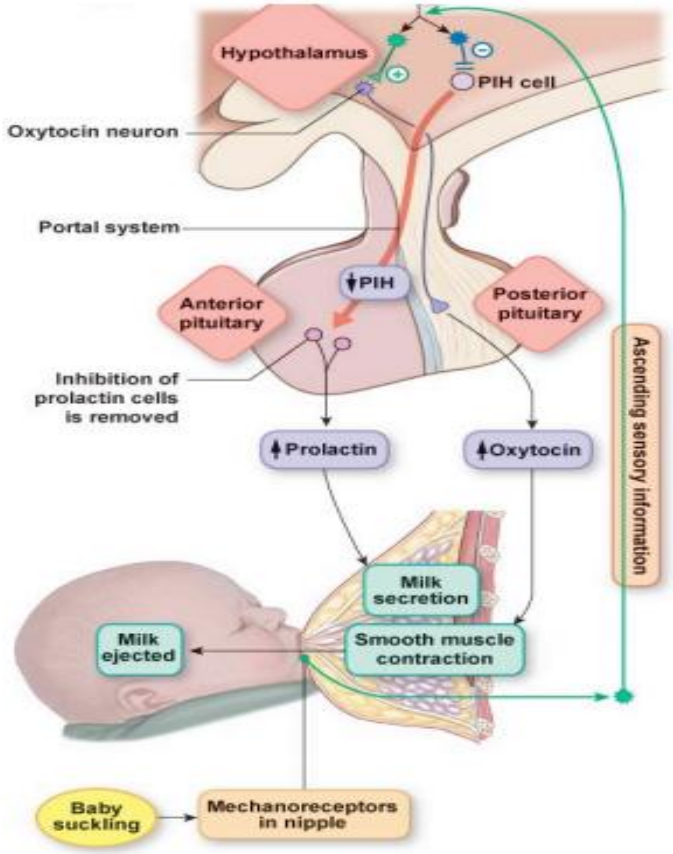


Figure 2.17: The milk ejection reflex (Silverthorn *et al*., 2013)

2.4.1 Oxytocin hormone

Oxytocin is synthesized in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) and axonally transported to the neural lobe where it is briefly stored until release from terminals. Axons of the oxytocin neurons passing through the median eminence already form synaptic boutons, which contact the capillary loops of the median eminence. Indeed, oxytocin release into the long portal vessels has been well-established (Gibbs, 1984; Nishinoh, 1993).

Moreover, short portal vessels connecting neural lobe and the inner zone of the anterior lobe also provide a potential route for delivering oxytocin to the adenohypophysis Oxytocin is secreted into the hypophyseal portal blood in 10 –15 times higher concentrations than found in the peripheral circulation (Gibbs, 1984), and high-affinity receptors resembling the uterine oxytocin receptors are present in the anterior lobe.

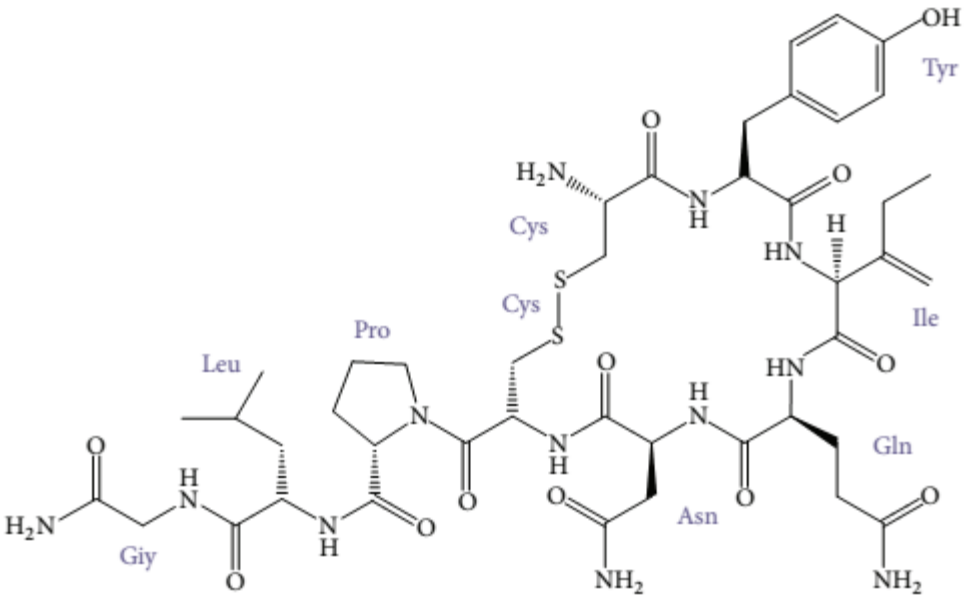


Figure 2.18: Chemical structure of oxytocin (Felipe *et al*., 2014)

In addition to acting as a hormone in the peripheral system, oxytocin is also released directly in the central nervous system from the cell bodies and dendrites of oxytocin-producing neurons to act as a neuromodulator in areas of the brain containing oxytocin receptors, such as the amygdala and hippocampus; (von Bohlen *et al*., 2002; Jonas, 2009). Oxytocin release is controlled by a positive feedback mechanism; for example, oxytocin release in the PVN stimulates further release of oxytocin by neurons in the same region (Kendrick, 2000).

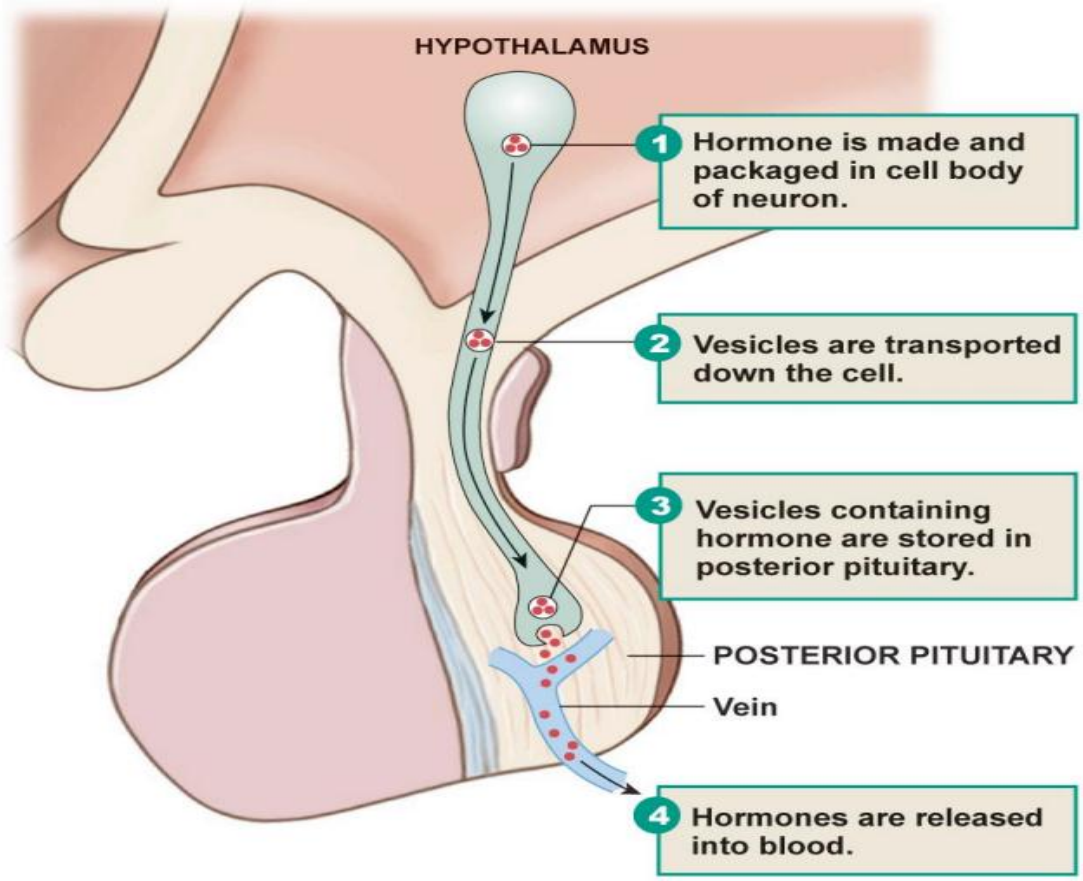


Figure 2.19: Oxytocin release from hypothalamus (Silverthorn *et al*., 2013)

2.4.1.1 Oxytocin receptor activation

Once oxytocin is released, it binds to oxytocin receptors (OTRs) located in neural tissue in the central nervous system and in the myoepithelial (smooth muscle) cells of the uterus and mammary glands in the peripheral system (Leng *et al*., 2008). The OTRs are G-coupled receptors, and the binding of oxytocin to OTRs on these myoepithelial cells causes a rise in intracellular calcium levels to facilitate contraction of the smooth muscle tissue (von Bohlen *et al*., 2002). Oxytocin has only one receptor, which belongs to the rhodopsin-type class I G-protein coupled receptor (GPCR) super family. The gene of the oxytocin receptor is present

in a single copy on chromosome 3p25 and contains 3 introns and 4 exons. Oxytocin and other molecules of similar structure, such as arginine vasopressin (AVP) and oxytocin agonists or antagonists, can bind to the receptor. The affinity for OT is about 10-fold higher than for AVP. The cell surface transmembrane OTR is activated after binding of OT molecule, and the receptor subsequently causes activation of the various intracellular signal pathways, thus resulting in the numerous effects of the hormone, including contraction. The OTR is coupled to the Gq/11 a- class guanosine triphosphate (GTP) binding proteins. Binding of OT activates, via Gαq/11, phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5- triphosphate (IP3) and diacylglycerol (DAG). The IP3 causes release of Ca^{2+} from intracellular stores, while DAG activates protein kinases type C (PKC), which further phosphorylates other proteins, thus bringing about a trophic effect on myometrial cells via the eukaryotic translation elongation factor 2 (eEF2). Release of Ca^{2+} initiates smooth muscle contractions as calcium ions binds to calmodulin and the Ca^{2+}-calmodulin system activates myosin light-chain kinase. This mechanism causes myometrial contractions, as well as contraction of mammary myoepithelial cells leading to milk ejection (Gimple and Fahrenholz, 2001). The major pathway that mediates the signal of OTR after binding of OT is the Gq/PLC/InsP3 pathway. The OTR is, however, also coupled with other G proteins, Gs and Gi, which give rise to various other cellular effects, e.g. inhibition of cellular growth (Viero *et al*., 2010).

The oxytocin receptor additionally acts on voltage-gated or receptor coupled channels; this activation, which leads to membrane depolarization and the entry of extracellular Ca^{2+} into the cells, eventually triggers various cellular responses and further promotes smooth muscle contractility. The OTR also activates the mitogen-activated protein kinase (MAPK) and the

Rho kinase pathways. The Rho associated protein kinases are involved in many cellular phenomena, among them cell migration, cell cycle control and cell contractility. Activation of OTR and MAPK results in both cases elevated cytosolic phospholipase A2 (cPLA2) activity. It hydrolyzes phospholipids while liberating arachidonic acid that results in increased production of prostaglandins via cyclooxygenase-2 (COX-2), an enzyme up-regulated by MAPK (Molnar and Hertelendy, 1995; Soloff *et al*., 2000). The RhoA kinase increases phosphorylated myosin light chains. The increase in intracellular Ca^{2+}, the activation of the Rho and MAP kinase pathways, and the increased production and secretion of prostaglandins all together result in the contractile effects of OT-OTR activation (Soloff *et al*., 2004).

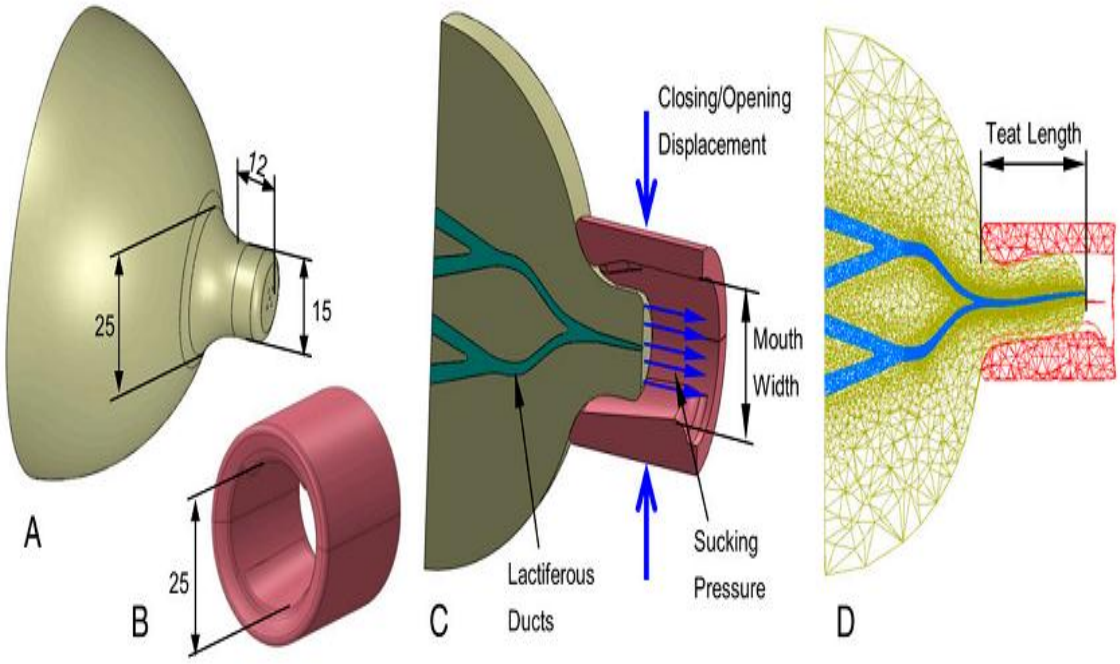


Figure 2.20: Biomechanics of milk ejection (David *et al*., 2014)

2.4.2 Role of Oxytocin in irregular Ca^{2+} oscillations

Using a novel, 3D imaging strategy, live oxytocin-induced alveolar unit contractions in the mammary gland was visualized, and demonstrated that milk is ejected by way of pulsatile contractions of these alveolar units. In mammary glands of ORAI1 knockout mice, these contractions are infrequent and poorly coordinated. Oxytocin was shown to also induce a large transient release of stored Ca^{2+}in mammary myoepithelial cells followed by slow, irregular Ca^{2+} oscillations. These oscillations, and not the initial Ca^{2+} transient, are mediated exclusively by ORAI1 and are absolutely required for milk ejection and pup survival, an observation that redefines the signaling processes responsible for milk ejection. These findings clearly demonstrate that Ca^{2+} is not just a substrate for nutritional enrichment in mammals but is also a master regulator of the spatiotemporal signaling events underpinning mammary alveolar unit contraction (Davisa *et al*., 2015).

2.5 Hypogalactia

Lactation failure or hypogalactia is defined as the need to start top feeds for the baby within 3 months of delivery because of inadequate breast milk supply (Joshi *et al*., 1993). Total lactation failure was defined as either a total absence of milk flow or secretion of just a few drops of breast milk following suckling for at least 7 days (Banapurmath *et al*., 2003). Partial lactation failure was defined as either inadequate milk output or the need for supplemental feedings to sustain growth (Banapurmath *et al*., 2003). The age, parity, education, socioeconomic status, religion, family structure, and urban versus rural status of mothers all had a bearing on the occurrence of lactation failure (Mathur *et al*., 1992). More so, the perception of not having enough milk often leads to infrequent suckling, leading to a true reduction in production of breast milk (Mathur *et al*., 2009). Low milk supply is the most

commonly reported reason for early discontinuation of breastfeeding (Odom *et al*., 2013). This is despite the fact most mothers are able to produce sufficient quantities of milk for the baby, if given the correct support and information (Amir, 2006). Risk factors for women experiencing hypogalactia include maternal illness, caesarean birth, maternal smoking, previous breast surgery, mother-infant separation and indirect lactation (i.e., breast pump or manual milk expression). It was also reported that mothers of infants who are unwell and require supportive care after birth, such as preterm infants, are particularly vulnerable to experiencing low milk supply (Zuppa *et al*., 2010). Some of the synonyms of lactation insufficiency include lactational inadequacy (Sharma *et al*., 1996), breast milk insufficiency (Oberlin and Wilkinson, 2008), lactation failure (Joshi *et al*., 1993; De *et* al., 2002), mothers milk insufficiency (MMI) (Oberlin and Wilkinson, 2008), perceived insufficient milk (PIM) (Millan *et al*., 1994; Kent *et al*., 2012), insufficient breast milk, partial lactation failure (Seema *et al*., 1997; Banapurmath *et al*., 2003), neonatal insufficient milk syndrome (IMS), hypogalactia or lactation inadequacy, breast-feeding failure (Osman *et al*., 2009), and suboptimal infant breastfeeding (SIB) (Miller *et al*., 2009).

2.5.1 Causes of hypogalactia

The most common cause of hypogalactia is insufficient secretion of prolactin from the pituitary gland (Prabhakar and Shalet, 2006). D_2 receptor antagonists are used to correct this insufficiency by blocking D_2 receptors in the anterior pituitary, which in turn disinhibits prolactin release (Bennett and Jensen, 1996). Other causes of low pituitary prolactin secretion include:

i. Lymphocytic hypophysitis: inflammation of the pituitary gland due to autoimmunity

ii. Sheehan's syndrome: postpartum hypopituitarism due to ischemic necrosis from blood loss and hypovolemia during and after child birth (Prabhakar and Shalet, 2006).

iii. Adult growth hormone deficiency: hypopituitarism in general can impair secretory activities of the pituitary gland such as prolactin and growth hormone, which in turn results in hypogalactia (Robert *et al.*, 2009).

iv. Other causes include insufficient glandular tissue of the breasts or breast hypoplasia, caused by lack of normal mammary gland growth and maturation during puberty or pregnancy (Karen, 2014). Overweightness/obesity can also result in hypogalactia by inhibiting the amount of prolactin secreted in response to suckling in the first week after childbirth (Peckenpaugh, 2013). Also certain drugs such as D_2 receptor antagonists like bromocriptine and pergolide as well as indirect D_2 receptors activators like amphetamines can suppress prolactin secretion from the pituitary gland and thereby cause hypogalactia (Peckenpaugh, 2013).

2.5.2 Assessment of milk adequacy

When milk is not sufficient, the infant does not feel satisfied after feedings, cries a lot, wants to nurse frequently, takes very long feedings, and does not gain weight properly (<20 gm/day). The number of wet diapers a day (less than six–eight) and infrequent bowel movements, with a small amount of stools, which are dry and hard, indirectly indicate low intake of milk (Powers, 2001). The following signs indicate that an infant is not receiving enough milk in the first weeks of life: weight loss greater than 10% of the birth weight, not regaining birth weight up to 2 weeks of life, no urinary output for 24 hours, and absence of

yellow stools in the first week and clinical signs of dehydration (Powers, 2001). An infant's milk ingestion is accurately measured by test weighing, which entails weighing the infant before and after breastfeeding without changing clothes or accessories between the two weighing (Kent *et al*., 2012).

2.5.3 Strategies for management of hypogalactia

Although the problem of hypogalactia looks simple, it is rather a more difficult situation (Gupte, 2004). In addition to motivation, encouragement and moral support, the use the following is also recommended:

i. Nipple stimulation exercises by nipple stroking, massaging the breast, and rolling the nipple between thumb and the index finger.
ii. Frequent suckling, at least 8–10 times a day, each session lasting 10–15 minutes for each breast.
iii. Drop and drip method may be employed if the infant fails to suckle for 8–10 minutes. The method involves expressing some breast milk or top milk in a cup and gradually pouring it over as drops over the breast. As the drops slide over the nipple down into infant's mouth, he is stimulated to suckle at the breast (Gupte, 2004).

2.6 Galactogogues

Galactogogues are synthetic or plant compounds used to induce, maintain and increase milk production (Mortel and Mehta, 2013), through complex mechanisms involving interaction between physical and physiological factors. They are medications that aid in initiating, maintaining, and augmenting of adequate milk production. The term galactogogue refers to substances that augment established lactation, whereas the term 'galactopoietics' is used

independently to describe the hormone preparations which enhance milk production in an animal already in lactation (Asimov and Krouze, 1991). Among the most important factors are hormones such as prolactin (PRL). However, somatotropine, cortisol, insulin, leptin, estrogen, progesterone and oxytocin also play important role as galactogogues (Zuppa *et al*., 2010). Galactogogues may be synthetic, plant-derived or endogenous products. They act through exerting an influence on adreno-hypothalamo-hypophyseal-gonadal axis by blocking hypothalamic dopaminergic receptors or by inhibiting dopamine producing neurons. These medications increase prolactin secretion by antagonizing dopamine receptors (Gabay, 2002).

2.6.1 Synthetic galactogogues

Among synthetic molecules used to increase lactation, the dopamine antagonists, such as antiemetics, metoclopramide and domperidone; and antipsychotics, sulpiride and chlorpromazine; hormone synthetic analogs such as oxytocin, thyroxine and medroxyprogesterone are also included as synthetic galactogogues (Zuppa *et al*., 2010).

2.6.2 Metoclopramide

This drug was originally commercialized in Europe as an antipsychotic and later in the US as a gastro-kinetic agent that increases gastrointestinal motility. Its first reported use as a galactogogue was in 1975 (Guzman *et al*., 1979). It has also been evaluated in many clinical trials (Anderson and Valdes, 2007). It is reported to have adverse side effects in humans including; anxiety, insomnia, several gastrointestinal disorders, severe depression and seizure. Infants that consumed milk from treated mother had intestinal discomfort (Zuppa *et al*., 2010). It has a half life of about 156.7 minutes in humans (Bateman *et al*., 1980), and when administered at, 10mg/kg by oral route three times daily for 10 days it increases milk production (Ingram *et al*., 2012).

2.6.3 Domperidone

Its first use as a galactogogue was first reported in 1983 (Barone, 1999). It was used to increase milk production in mothers of premature infants (Wan *et al*., 2008), but was not approved by the Food and Drug Administration (FDA) in the United States; although its use in human clinical trials was not associated with adverse effects in infants. However, it was associated with xerostomia, gastrointestinal disorders, cardiac arrhythmias, and sudden death (Osadchy *et al*., 2012). Its half life is reported to be about 7.5 hours in humans (Heykants *et al*., 1981), and a dosage of 10 mg (PO) 3 times daily enhances lactation in women (Da-silva and Knoppert, 2004).

2.6.4 Chlorpromazine

As is the case with other neuroleptics, little is known about pharmacokinetics of chlorpromazine in mothers or infants during breast feeding (Maguire, 2002). Chlorpromazine administered in doses of 15 mg/kg of body weight in rats during 5 days was effective in inducing lobuloalveolar growth and initiation of milk secretion (Talwalker *et al*., 1960); also it increased milk production and weight gain in women with hypogalactia at doses of 25 mg, 3 times daily for a week (Gabay, 2002). It has a half life of about 16-30 hours (Wampler, 1983), with documented side effects of extrapyramidal symptoms in mothers and lethargy in infants that consumed the milk (Gabay, 2002).

2.6.5 Herbal galactogogues

Some of the plants with galactogogues components include fenugreek (*Trigonella graecum foecum*), fennel (*Foeniculum vulgare*), goat's rue (*Galega officinalis*), asparagus (*Asparagus racemosus*), anise (*Pimpinella anisum*), and milk thistle (*Silybum marianum*) (Agrawala *et*

al., 1968; Westfall, 2003). *Hibiscus sabdariffa linn* has also been reported as a safe, affordable and tolerable galactogogue (Bako *et al.,* 2013).

2.7 Nutritional Components of Breast milk

Milk contain vital nutrients such as proteins, carbohydrates, lipids, minerals and vitamins, together with bioactive substances including immunoglobulins, peptides, antimicrobial factors, hormones and growth factors (Grosvenor, *et* al., 1993; Clare and Swaisgood, 2000). The mean macronutrient composition of mature, term milk is estimated to be approximately 0.9 to 1.2 g/dL for protein, 3.2 to 3.6 g/dL for fat, and 6.7 to 7.8 g/dL for lactose. Energy estimates range from 65 to 70 kcal/dL, and are highly correlated with the fat content of human milk (Nommsen *et al*., 1991). The average content of human milk is around 86-87% water and 13-14% dry matter. Dry matter is composed of the following substances: organic matter which is mostly formed of proteins (about 2.5%) in colostrum and up to 1.5% in mature milk. The proteins for the most part include casein, lactalbumin and lactoglobulin. Casein is the major milk protein, present in at least four forms; it represents a complete protein, rich in all essential amino acids. Because of its complex composition, lactogenesis requires the concerted action of many transport processes and the presence of many milk constituents may have biological effects on both the mother and child (Silanikove *et al*., 2000).

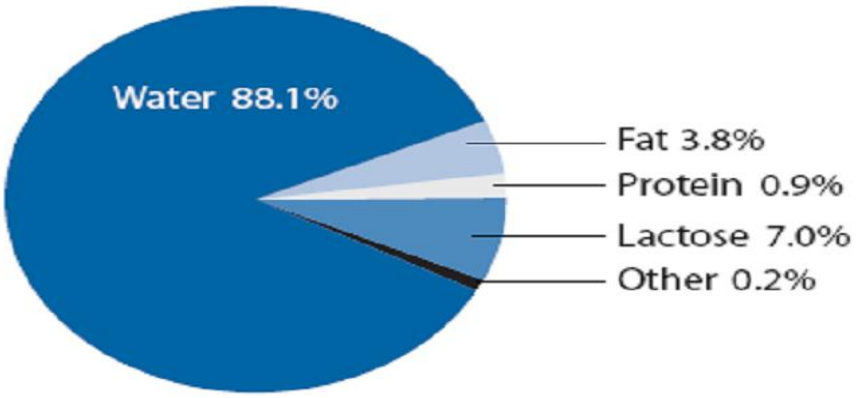


Figure 2.21: Milk composition (Silanikove *et al*., 2000)

2.7.1 Physical nature of breast milk

Though its structure appears to be continual and homogeneous, milk is composed of at least five physically and functionally discrete components:

(i) Whey

Milk serum, commonly known as whey, is the medium in which all compartments are homogeneously dispersed. Whey is composed of water in which minerals, various organic molecules, proteins and peptides are dissolved (Heid and Keenan, 2005).

(ii) Fat globules

The fat is dispersed in milk as small droplets that are enveloped by a plasma membrane rich in phospholipids and commonly known as the milk fat globule membrane (MFGM) (Heid and Keenan, 2005).

(iii)Casein micelles

The main protein in milk is casein which is in form of colloidal particles known as micelles. High resolution field-emission scanning electron microscope micrographs of casein micelles

revealed spherical particles in the range of 200 nm; the surface of the micelle is not smooth but contains gaps between the tubular substructures (Dalgleish *et* al., 2004).

(iv) Membrane vesicles

The secretion of various membrane vesicles into the extracellular space is a frequent phenomenon described in normal and tumor cells (Hugel *et al*., 2005). Two types of vesicles are secreted by cells. Exosomes, typically 40–100 nm in diameter, originate from endocytic multivesicular bodies, and are released in an exocytic manner. Although the functions of exosomes remain largely unresolved, they are thought to play immunoregulatory and antitumoral roles (Fevrier and Raposo, 2004). Microvesicles, with a diameter in the range 100–1000 nm, originate from the cell surface membrane and are shed directly into the extracellular space, a process that seems to be important for membrane turnover, tumor ganglioside metabolism and vascular regulation (Hugel *et al*., 2005). The processes of exosome secretion and membrane shedding are poorly understood.

(v) Milk cells

The milk of various mammals contains a heterogeneous population of cells, commonly referred to as somatic cells (SC). In most species, the predominant cells are leukocytes, composed of lymphocytes, polymorphonuclear neutrophils (PMNs) and macrophages, which serve as important components in the mammary defense against potential pathogens, mostly bacteria (Paape *et al*., 2003). The area between the milk particles represents the milk serum (whey), the phase in which all other phases are homogenously dispersed as shown in the image below:

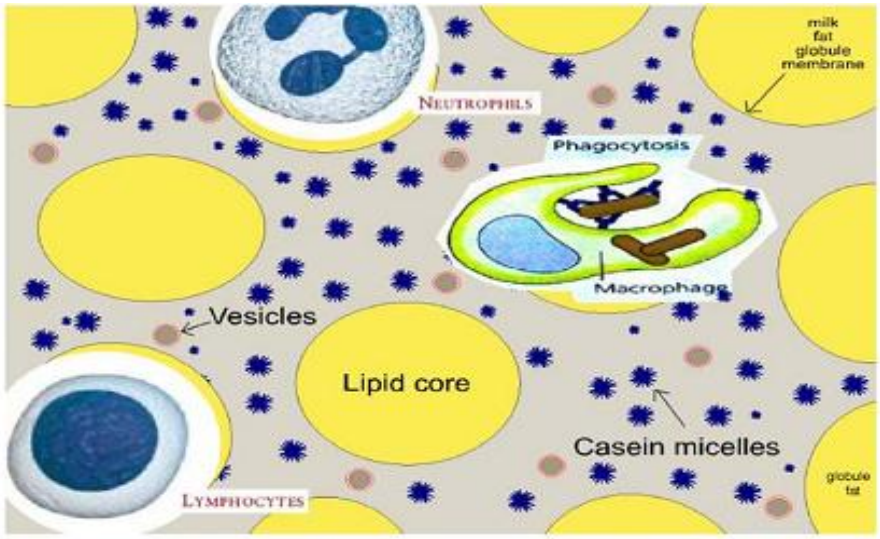


Figure 2.22: Schematic representation of the physical phases of milk (Silanikov *et al*., 2000)

2.7.2 Milk enzyme distribution

Milk is not a homogenous solution of enzymes; rather, specific enzymes that are specifically associated with one or more of the above described five distinct phases. Over 60 indigenous enzymes have been identified so far in the milk of various mammalian species (Fox and McSweeney, 2003). Milk contains numerous complex biomolecules such as proteins, oligosaccharides, and lipids. In addition to these components, milk contains plasmin, immunereactive anionic trypsin, most likely present in complex with IgA, and cathepsin. Since the amount of proteins in colostrum is much, a small amount of milk should satisfy all the needs of a newborn before suckling strength is attained (Kelly *et al*., 2006).

2.7.2.1 Lactate-dehydrogenase

Lactate dehydrogenase (LDH) is a nicotinamide adenine dinucleotide (NAD-dependent) enzyme included in the metabolism of glucose in aerobic and anaerobic conditions. Therefore, the distribution of isoenzyme forms changes depending on the supply of oxygen to

tissues and metabolic activity of tissues. The major source of enzymes in milk is the epithelium of the mammary gland (Fox and Kelly, 2006).

2.7.2.2 Amylase

Amylase is an enzyme that belongs to the class of hydrolytic enzymes included in the digestion of starch. It was the first enzyme isolated in milk at the end of the 19th century. It was shown later that the main component is α-amylase and it is interesting that amylase activity in human milk is more than 20-40 times higher than in cow's milk. This enzyme is homologous to the salivary amylase and is 15 to 140 times more active than in plasma. Considering that milk does not contain starch, one of the assumptions is that amylase is necessary for breaking down the oligosaccharide units which are present in milk (Heyndrickx, 1963; Kunz *et al*., 2002). One of the hypotheses is that amylase is also produced as a compensatory component in the undeveloped digestive enzymatic system of a newborn child who possesses only 0.2-0.5% of the total amylase activity that takes place in adults. This enzyme is capable of breaking down the polysaccharide bacteria capsules, so its antimicrobial effect is undeniable. The decrease in activity during lactation after a period of one month is in accordance with the decrease in proteins as well as with the development of the digestive system of infants who do not need this enzyme anymore. The richness of mother's milk in amylase explains why children feeding on human milk are able to digest carbohydrate meals earlier and much better than children taking artificial food (Lindberg and Skude, 1982; Heitlinger *et al*., 1983).

2.7.2.3 Alkaline phosphatase

Alkaline phosphatase in human milk was discovered at the beginning of the 20th century. It is a membranous enzyme particularly present in lipoprotein structures of the Golgi complex.

Therefore, it is not surprising that it is secreted in milk in high concentrations. Alkaline phosphatase from milk is homologous to the enzyme in liver and placenta. It also has a similar bond with phosphatidylinositol in the lipoprotein complex of the membrane. These kinds of membranous fat globules are actively secreted in milk. More complex researches of its structure and origin have shown that milk contains two forms of enzymes, one from myoepithelial cells and the other from cytoplasm (Fox and Kelly, 2006).

2.8 Liver Enzymes

The liver is the primary site of drug metabolism; other tissues, such as kidney, brain, skin, blood, lung and gastrointestinal mucosa, also contribute (Krishna and Klotz, 1994). The liver is a large, complex organ that is well adapted for its central role in carbohydrate, protein and fat metabolism. It is the site where waste products of metabolism are detoxified through processes such as amino acid deamination, which produces urea. In conjunction with the spleen it is involved in the destruction of spent red blood cells and the reclamation of their constituents. It is responsible for synthesizing and secreting bile and synthesizing lipoproteins and plasma proteins, including clotting factors (Burkitt *et* al., 1993). It maintains a stable blood glucose level by taking up and storing glucose as glycogen (glycogenesis), breaking this down to glucose when needed (glycogenolysis) and forming glucose from non-carbohydrate sources such as amino acids (gluconeogenesis) (Figueiredo *et al.,* 2009).

System or function	Marker	Site or significance	Function
Hepatocyte integrity	Aspartate aminotransferase	Liver, heart skeletal muscle, kidney, brain, red blood cell	Catabolizes amino acids, permitting them to enter the citric acid cycle.
	Alanine aminotransferase	Liver	
Cholestasis	Alkaline phosphatase	Bone, intestine, liver, placenta	Canicular enzyme that plays a role in bile production.
	γ-Glutamyl-transpeptidase	Correlated levels with alkaline phosphatase indicate hepatobiliary origin	Catalyzes transfer of γ-glutamyl group from peptides to other amino acids.
	Bilirubin	Elevations may indicate hepatic or extrahepatic disorder	Breakdown product of hemolysis taken up by liver cells and conjugated to water-soluble product excreted in bile.
Liver function mass	Serum albumin	Diet or liver	Liver synthesizes albumin
	Prothrombin time	Liver synthesizes vitamin K-dependent clotting factors	Bile salts are synthesized in the liver and necessary for vitamin K absorption

Figure 2.23: Hepatic biochemical markers (Edoardo *et al*., 2005).

Most medications are transformed in phase I or II reactions. Phase I reactions result in conversion of the parent drug, usually by oxidation, reduction or hydrolysis, to a more hydrophilic metabolite. Phase II reactions result in inactivation of the medication through chemically coupling to an endogenous substance, such as glucuronic acid, glycine, glutathione or glutamine, or conjugation with acetate, sulfate or methyl groups, thus facilitating renal excretion. Oxidative reactions are among the most important of the phase I reactions. They are mediated by the family of enzymes that constitute the CYP system. At least 17 mammalian CYP gene families have been defined, and more than 30 human gene products have been identified (Nelson *et al*., 1996; Nelson, 1999). Abnormal liver enzyme levels may signal liver damage or alteration in bile flow. Liver enzyme alteration may be either the accompanying biochemical picture in a patient with symptoms or signs suggestive of liver disease or an isolated, unexpected finding in a patient who has undergone a wide range of laboratory tests for a non-hepatic disease or for minor, vague complaints.

Measurement of liver enzymes remains the most practical tool to diagnose liver damage, and includes mainly alanine aminotransferase (ALT), an enzyme present in hepatocytes, and alkaline phosphatase (ALP), an enzyme present in cells lining the biliary ducts of the liver. Based on elevation of, and ratio between elevations of, these enzymes, hepatocellular, cholestatic or mixed liver injury is diagnosed (Benichou, 1990; Danan and Benichou, 1993).

2.8.1 Alkaline phosphatase (ALP)

Alkaline phosphatase is an enzyme that transports metabolites across cell membranes. Liver and bone diseases are the most common causes of pathological elevation of ALP levels, although ALP may originate from other tissues, such as the placenta, kidneys or intestines, or from leukocytes (Fishman, 1990). The ALP is present in mucosal epithelia of small intestine, proximal convoluted tubule of kidney, bone, liver and placenta. It performs lipid transportation in the intestine and calcification in bone. The serum ALP activity is mainly from the liver with 50% contributed by bone (Mauro *et al*., 2006). Normal serum ALP is 41 – 133 U/L. The half-life of ALP in circulation is about 1 week (Dufour *et al*., 2000), and drug-induced liver injury may present with a cholestatic pattern (preferential increase in ALP or ALT/ALP ratio < 2), although the degree of ALP alteration is variable and may be accompanied by hyperbilirubinaemia (Velayudham and Farrell, 2003).

2.8.2 Alanine amino transferase (ALT)

Alanine amino transferase is found in kidneys, heart, muscle and greater concentration in liver compared with other tissues of the body. The ALT is purely cytoplasmic catalyzing the transamination reaction (Mauro *et al*., 2006). Normal serum ALT is 7-56 U/ L. Any type of liver cell injury can reasonably increases ALT levels. Elevated values up to 300 µ/L are considered nonspecific. Marked elevations of ALT levels greater than 500 µ /L is observed

most often in persons with diseases that affect primarily hepatocytes such as viral hepatitis, ischemic liver injury (shock liver) and toxin-induced liver damage. Despite the association between greatly elevated ALT levels and its specificity to hepatocellular diseases, the absolute peak of the ALT elevation does not correlate with the extent of liver cell damage (Kallei *et al*., 1964). Furthermore, increased ALT level was associated with reduced insulin sensitivity, adiponectin and glucose tolerance as well as increased free fatty acids and triglycerides (James *et al*., 2006).

2.8.3 Aspartate amino transferase (AST)

Aspartate amino transferase catalyze transamination reactions. It exists in two different isoenzyme forms which are genetically distinct, the mitochondrial and cytoplasmic form. The AST is found in highest concentration in heart compared with other tissues of the body such as liver, skeletal muscle and kidney (Mauro *et al*., 2006). Normal serum AST is 0 to 35 U/L (Diana, 2007), and elevated mitochondrial AST is seen in extensive tissue necrosis during myocardial infarction and also in chronic liver diseases like liver tissue degeneration and necrosis. About 80% of AST activity of the liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme. However, the ratio of mitochondrial AST to total AST activity has diagnostic importance in identifying the liver cell necrotic type condition and alcoholic hepatitis (Panteghini *et al*., 1983).

2.8.4 Main types of drug-induced liver injury

Drugs can cause liver injury in several ways, but three main types usually described; referred to as dose-dependent (or intrinsic) toxicity, dose-independent (or idiosyncratic metabolic) toxicity and drug allergy (or idiosyncratic immunological). Overdose of most drugs can

represent a threat to the liver, and psychotropic drugs are often involved (Watkins *et al*., 2006). Intrinsic toxicity is predictable and often caused by high concentrations of parent drug and/or a metabolite when the liver`s defense systems against toxic damage are overwhelmed. Most people ingesting large amounts of a drug are affected, and liver injury is apparent within a few days. Injury is usually reversible within days to weeks if liver failure does not develop (Adams *et al*., 2010).

2.8.5 Main mechanisms of drug-induced liver injury

The liver has a central role as a detoxifying organ towards xenobiotics and chemicals. However, biotransformation to less toxic substances can actually involve production of molecules that can induce liver injury through various pathways. Important mechanisms involved in non-allergic drug-induced hepatic injury can be divided into:

a. Drug metabolism and reactive metabolite formation
b. Covalent binding
c. Reactive oxygen species generation
d. Activation of signal transduction pathways that modulate cell death or survival
e. Mitochondrial damage.

2.8.5.1 Drug metabolism and reactive metabolite formation

Biotransformation of lipophilic xenobiotics to more hydrophilic substances before excretion in urine or bile is an important function in the liver. Phase I enzyme reactions involve among others cytochrome-P450 (CYP-450) reactions which converts xenobiotics to reactive metabolites. The metabolites can via Phase II enzymes be conjugated to hydrophilic molecules (glucuronide conjugation-most important reaction among several) before excretion. The reason why reactive metabolites can induce damage through idiosyncratic

liver injury is based on a complex interplay between several factors. Age, sex, liver function and disease, environmental (enzyme induction and inhibition) and genetic factors (polymorphism) can influence the level or function of CYP-450, and thereby production of reactive metabolites. Inherent toxicity of these, their distribution and accumulation within the organ, cells and sub-cellular organelles together with the status of defense systems are likely to be of additional importance (Grattagliano *et al*., 2009).

2.8.5.2 Covalent bonding

Bio-activation of drugs through Phase I reactions can produce electrophilic metabolites with intrinsic chemical reactivity toward several cellular macromolecules. Covalent bonding of short-lived reactive metabolites to cellular components includes proteins, peptides, lipids and nuclear acids, and this modification is thought of as essential in hepatotoxicity of drugs (Kemper, 2008). An important pathway for bio-activation of many acidic NSAIDs involves formation of reactive acylglucuronides (Bailey and Dickinson, 2003). Thus, Phase II reactions of bioactivation, usually thought of as a detoxifying process, can also produce reactive metabolites associated with hepatotoxicity. Furthermore, covalent modification of cellular components can initiate both toxic and/or mechanisms of liver injury (Bailey and Dickinson, 2003).

2.8.5.3 Reactive oxygen species generation

Metabolic activation of drugs can involve production of reactive oxygen species through chemical modification of cellular macromolecules that can induce hepatocellular injury. Reactive oxygen species can initiate protein and lipid peroxidation, and deplete antioxidant defences like reduced glutathione (GSH), and modify sulphydryl (SH) groups on cellular components. The subsequent increased cellular stress and uncontrolled increase in cytosolic

Ca^{2+} concentration stimulate Ca^{2+} -activated degradative enzymes with further cellular injury. Disruption of cellular membranes and sub-cellular organelles including mitochondria, can affect energy production and further disrupt ionic homeostasis, and initiate cell death pathways.

2.8.5.4 Activation of signal transduction pathways that mediates cell death or survival

The stress in hepatocytes induced by reactive metabolites, including reactive oxygen species, can activate several signal transduction pathways. Several endogenous signaling substances modify signal transduction including various cytokines. The pathways activate pro- death or survival proteins that determine the fate of the cell (Han *et al*., 2010). Important for liver injury is c-Jun N-terminal protein kinases (JNK). JNK can be activated with many stresses including reactive oxygen species, and represents a common mechanism of cellular injury associated with several diseases (Han *et al*., 2010).

2.8.5.5 Mitochondrial damage

Mitochondria have a central role in liver injury. Cytotoxic drugs and/or metabolites can attack the organelle directly or through pathways described above. Important pathological mechanisms are mitochondrial permeability transition (MPT) through opening of MPT pore in the membranes of the organelle, and activation of signals, death receptors and pro-apototic pathways. As a result acute necrosis, apoptosis and autophagic cell death can occur (Kass, 2006). Mitochondrial dysfunction is involved in most forms of drug-induced liver injury, and through dysfunction of the organelle more sub-acute or chronic injury can develop. Valproic acid can inhibit mitochondrial fatty oxidation, uncouple mitochondrial respiration and induce MTP (Pessayre *et al*., 2010).

2.8.5.6 Immunological mechanisms

Various types of immune cells, including lymphocytes, reside in the liver, and other leukocytes are distributed to this organ during inflammation (Adams *et al.*, 2010). Furthermore, antidrug antibodies and auto- antibodies can be detected during liver injury (Liu and Kaplowitz, 2002). A drug-modified protein formed due to reactions described previously (reactive metabolite formation and covalent bonding) can initiate either classic immune responses against the drug or an autoimmune response against a modified protein. The classic immune response is characterized with short time period to onset of symptoms and a memory component, while the autoimmune response develops after a long time period with no memory. The different courses are based on interaction with different T helper cells, but involvement of B cells and other immune cells could be of importance (Adams *et al*., 2010).

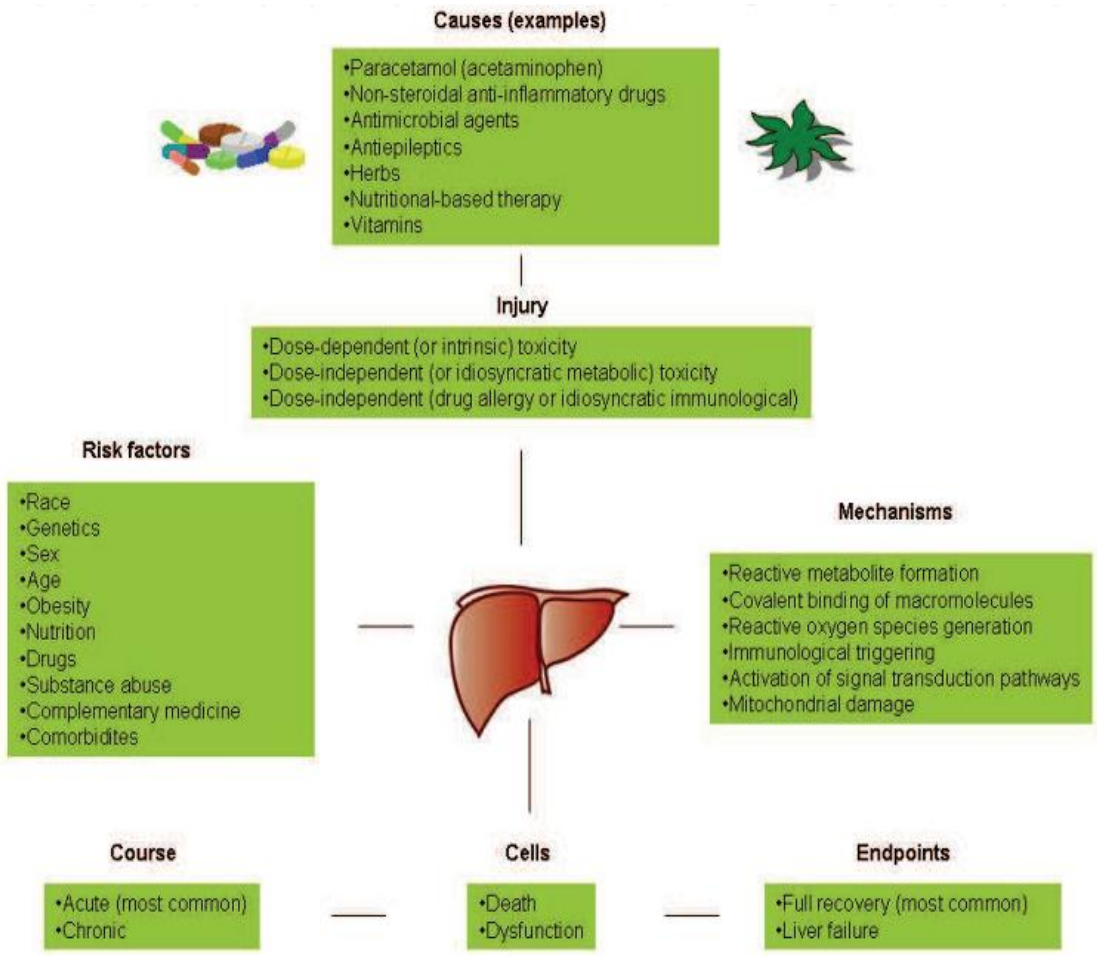


Figure 2.24: Pathophysiology of drug-induced liver damage (Han *et al*., 2010)

2.9 Free Radicals and Oxidative Stress

Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and active towards chemical reactions with other molecules. They are derived from three elements: oxygen, nitrogen and sulfur, thus creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS). The ROS include free radicals like the superoxide anion ($O_2^{-}\cdot$), hydroperoxyl radical ($HO_2\cdot$), hydroxyl radical ($OH\cdot$), nitric oxide (NO), and other species like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). The RNS derive from NO by reacting with $O_2^{-}\cdot$,

and forming ONOO. The RSS are easily formed by the reaction of ROS with thiols (Lü *et al*., 2010). The balance between the production and neutralization of ROS by antioxidants is very delicate, and if this balance tends to the overproduction of ROS, the cells start to suffer the consequences of oxidative stress (Wiernsperger, 2003).

2.9.1 Effects of oxidative stress

Internally, free radicals are produced as a normal part of metabolism within the mitochondria, through xanthine oxidase, peroxisomes, inflammation processes, phagocytosis, arachidonate pathways, ischemia, and physical exercise. External factors that help to promote the production of free radicals are smoking, environmental pollutants, radiation, drugs, pesticides, industrial solvents and ozone. It is ironic that these elements, essential to life (especially oxygen) have deleterious effects on the human body through these reactive species (Lobo *et al.*, 2010). The main targets of ROS, RNS and RSS are proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) molecules, sugars and lipids (Lü *et al*., 2010; Craft *et al*., 2012).

There are three distinct ways they can be oxidatively modified: (1) oxidative modification of a specific amino acid, (2) free radical-mediated peptide cleavage and (3) formation of protein cross-linkage due to reaction with lipid peroxidation products (Lobo *et al*., 2010).

The damage induced by free radicals to DNA can be described both chemically and structurally having a characteristic pattern of modification: production of base-free sites, deletions, modification of all bases, frame shifts, strand breaks, DNA–protein cross-links and chromosomal arrangements. An important reaction involved with DNA damage is the production of the hydroxyl radical through the Fenton reaction. This radical is known to react

with all the components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone. The peroxyl and OH-radicals also intervene in DNA oxidation (Dizdaroglu *et al.*, 2002; Valko *et al.*, 2004).

The formation of oxygen free radicals during early glycation could contribute to glycoxidative damage. During the initial stages of non-enzymatic glycosylation, sugar fragmentation produces short chain species like glycoaldehyde whose chain is too short to cyclize and is therefore, prone to autoxidation, forming the superoxide radical. The resulting chain reaction propagated by this radical can form a and b-dicarbonyls, which are well known mutagens (Benov and Beema, 2003). Lipid peroxidation is initiated by an attack towards a fatty acid's side chain by a radical in order to abstract a hydrogen atom from a methylene carbon. The more double bonds present in the fatty acid, the easier it is to remove hydrogen atoms and consequently form a radical, making monounsaturated (MUFA) and saturated fatty acids (SFA) more resistant to radicals than polyunsaturated fatty acids (PUFA). After the removal, the carbon centered lipid radical can undergo molecular rearrangement and react with oxygen forming a peroxyl radical. These highly reactive molecules can then abstract hydrogen atoms from surrounding molecules and propagate a chain reaction of lipid peroxidation. The hydroxyl radical is one of the main radicals in lipid peroxidation, it is formed in biological systems, as stated above, by the Fenton reaction as a result of interaction between hydrogen peroxide and metal ions (Carocho and Ferreira, 2013).

2.9.2 Antioxidants

Antioxidant is defined as any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell, 2007). It can also be defined as "any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defences or inhibit ROS

production" (Khlebnikov *et al*. 2007). Antioxidants neutralize free radicals or inhibit the oxidation they cause in target cells by donating one of their own electrons, ending the electron-gain reaction (Asshokkumar *et al*., 2012). The antioxidant nutrients themselves don't become free radicals by donating an electron because they are stable in either form. They act as scavengers, helping to prevent cell and tissue damage that could lead to disease (Reiter, 2003; Reiter *et al*., 2004).

The antioxidant activity can be effective through various ways: as inhibitors of free radical oxidation reactions (preventive oxidants) by inhibiting formation of free lipid radicals; by interrupting the propagation of the autoxidation chain reaction (chain breaking antioxidants); as singlet oxygen quenchers; through synergism with other antioxidants; as reducing agents which convert hydroperoxides into stable compounds; as metal chelators that convert metal pro-oxidants (iron and copper derivatives) into stable products; and finally as inhibitors of pro-oxidative enzymes (lipooxigenases) (Min and Boff, 2002; Pokorny´, 2007; Kancheva, 2009).

2.9.3 Classification of antioxidants

The human antioxidant system is divided into two major groups;

A. Enzymatic antioxidants

i. Primary (e.g. superoxide dismutase, glutathione peroxidase and catalase)
ii. Secondary enzymatic defences (e.g. glutathione reductase and glucose-6-phosphate dehydrogenase; Rahman, 2007).

Superoxide dismutases (SOD): These are enzymes that catalyze the dismutation of superoxide anion into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defence in nearly all cells exposed to oxygen. Superoxide dismutase exists in

cytosolic, mitochondrial, and extracellular forms, all of which catalyze the dismutation of superoxide anion by successive oxidation-reduction of the transition metal at the enzyme's active site (Hsieh *et al.*, 1998).

Catalase (CAT): Is is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in reproductive reactions. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. Catalase exists in only 1 form and is a highly efficient, intracellular enzyme converting hydrogen peroxide to hydrogen and water.

Glutathione peroxidase (GPx) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect tissue from oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Glutathione peroxidase exists in 5 different forms, with the predominant form depending on the tissue. Glutathione peroxidase IV, also known as phospholipid hydroperoxide GPX, is the predominant form in the mouse testis, whereas GPXs III and V are predominant in the mouse epididymis. In whatever form, this glutamate-based system is a major defence against oxidative stress (Roveri *et al*, 1992).

Glutathione reductase reduces glutathione (antioxidant) from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH (nicotinamide adenine dinucleotide phosphate – coenzyme used in anabolic reactions) creating a reducing environment. These two enzymes do not neutralize free

radicals directly, but have supporting roles to the other endogenous antioxidants (Ratnam *et al.,* 2006).

B. Non-enzymatic antioxidants

There are a number of non-enzymatic endogenous antioxidants e.g, vitamins, enzyme cofactors (Q10), nitrogen compounds (uric acid), and peptides (glutathione).

Vitamin A or retinol is a carotenoid produced in the liver and results from the breakdown of β-carotene. There are about a dozen forms of vitamin A that can be isolated. It is known to have beneficial impact on the skin, eyes and internal organs. What confers antioxidant activity to vitamin A is its ability to combine with peroxyl radicals before they propagate peroxidation to lipids (Palace *et al.,* 1999; Jee *et al.,* 2006).

Coenzyme Q10 is present in all cells and membranes; it plays an important role in the respiratory chain and in other cellular metabolic reactions. Coenzyme Q10 acts by preventing the formation of lipid peroxyl radicals, although it has been reported that this coenzyme can neutralize these radicals even after their formation. Another important function is the ability to regenerate vitamin E; some authors describe this process to be more likely than regeneration of vitamin E through ascorbate (vitamin C) (Turunen *et al*., 2004). Uric acid is the end product of purine nucleotide metabolism in humans and during evolution its concentrations have been rising. After undergoing kidney filtration, 90% of uric acid is reabsorbed by the body, showing that it has important functions within the body. In fact, uric acid is known to prevent the overproduction of oxo-hem oxidants that result from the reaction of hemoglobin with peroxides. On the other hand it also prevents lysis of erythrocytes by

peroxidation and is a potent scavenger of singlet oxygen and hydroxyl radicals (Kand'ár *et al.*, 2006).

Glutathione is an endogenous tripeptide which protects the cells against free radicals either by donating a hydrogen atom or an electron. It is also very important in the regeneration of other antioxidants like ascorbate (Steenvoorden and Henegouwen, 1997).

C. Other antioxidants

Despite its remarkable efficiency, the endogenous antioxidant system does not suffice, and humans depend on various types of antioxidants that are present in the diet to maintain free radical concentrations at low levels (Pietta, 2000).

Vitamins C and E are generic names for ascorbic acid and tocopherols. Ascorbic acid includes two compounds with antioxidant activity: L-ascorbic acid and L-dehydroascorbic acid which are both absorbed through the gastrointestinal tract and can be interchanged enzymatically *in vivo*. Ascorbic acid is effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitrogen oxide (Barros *et al.*, 2011).

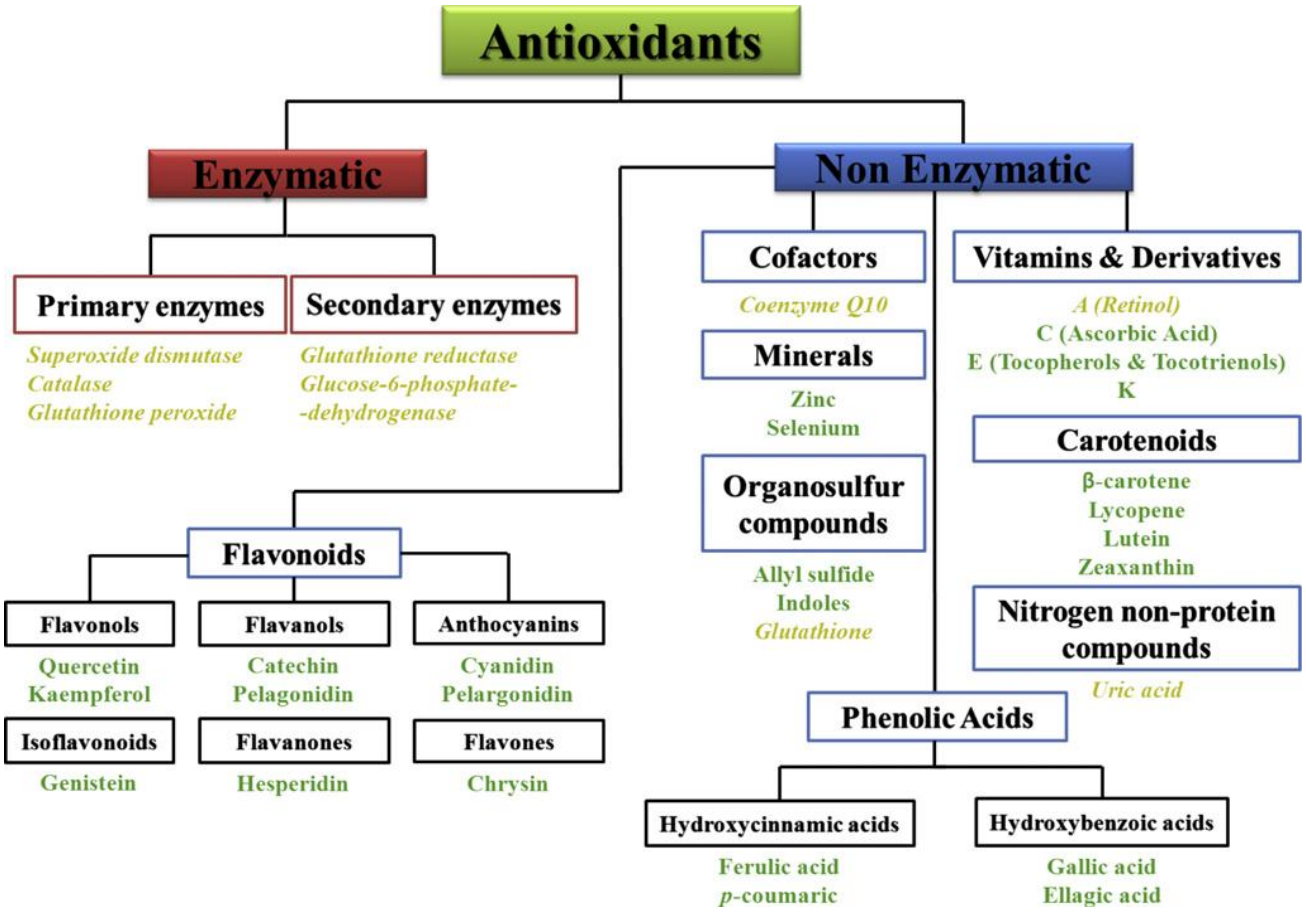


Figure 2.25: Classes of natural antioxidants. (Carocho and Ferreira, 2013)

Vitamin E is composed of eight isoforms, with four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol) and four tocotrienols (α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol); with α-tocopherol being the most potent and abundant isoform in biological systems. The chroman head group confers the antioxidant activity to tocopherols, but the phytyl tail has no influence. Vitamin E halts lipid peroxidation by donating its phenolic hydrogen to the peroxyl radicals forming tocopheroxyl radicals that, despite also being radicals, are unreactive and unable to continue the oxidative chain reaction.

Vitamin E is the only major lipid-soluble, chain breaking antioxidant found in plasma, red cells and tissues, allowing it to protect the integrity of lipid structures, mainly membranes (Burton and Traber, 1990).

Vitamin K is a group of fat-soluble compounds, essential for posttranslational conversion of protein-bound glutamates into carboxy-glutamates in various target proteins. The 1,4-naphthoquinone structure of these vitamins confers the antioxidant protective effect. The two natural isoforms of this vitamin are K1 and K2 (Vervoort *et al.*, 1997).

Flavonoids are an antioxidant group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones. Flavanones and flavones are usually found in the same fruits and are connected by specific enzymes, while flavones and flavonols do not share this phenomenon and are rarely found together. Anthocyanins are also absent in flavanone-rich plants. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups attached to ring structures and they can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators. They also activate antioxidant enzymes, reduce a-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid and low molecular weight molecules. Some of the most important flavonoids are catechin, catechin-gallate, quercetin and kaempferol (Rice-Evans *et al.,* 1996; Procházková *et al.*, 2011).

Phenolic acids are composed of hydroxycinnamic and hydroxybenzoic acids. They are ubiquitous to plant material and sometimes present as esters and glycosides. They have antioxidant activity as chelators and free radical scavengers with special impact over

hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites. One of the most studied and promising compounds in the hydroxybenzoic group is gallic acid which is also the precursor of many tannins, while cinnamic acid is the precursor of all the hydroxycinnamic acids (Krimmel *et al.,* 2010; Terpinc *et al*., 2011).

Carotenoids are a group of natural pigments that are synthesized by plants and microorganisms but not by animals. They can be separated into two vast groups: the carotenoid hydrocarbons known as the carotenes which contain specific end groups like lycopene and β-carotene; and the oxygenated carotenoids known as xanthophyls, like zeaxanthin and lutein. The main antioxidant property of carotenoids is due to singlet oxygen quenching which results in excited carotenoids that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. This can occur while the carotenoids have conjugated double bonds within. The only free radicals that completely destroy these pigments are peroxyl radicals. Carotenoids are relatively unreactive but may also decay and form non-radical compounds that may terminate free radical attacks by binding to these radicals (Paiva and Russell, 1999).

Minerals are only found in trace quantities in animals and are a small proportion of dietary antioxidants, but play important roles in their metabolism. Regarding antioxidant activity, the most important minerals are selenium and zinc. Selenium can be found in both organic (selenocysteine and selenomethionine) and inorganic (selenite) forms in the human body. It does not act directly on free radicals but is an indispensable part of most antioxidant enzymes (metalloenzymes, glutathione peroxidase, thioredoxin reductase) that would have no effect without it (Tabassum *et al*., 2010).

Zinc is a mineral that is essential for various pathways in metabolism. Just like selenium, it does not directly attack free radicals but is quite important in the prevention of their formation. Zinc is also an inhibitor of NADPH oxidases which catalyze the production of the singlet oxygen radical from oxygen by using NADPH as an electron donor. It is present in superoxide dismutase, an important antioxidant enzyme that converts the singlet oxygen radical into hydrogen peroxide. Zinc induces the production of metallothionein that is a scavenger of the hydroxyl radical. Finally zinc also competes with copper for binding to the cell wall, thus decreasing once again the production of hydroxyl radicals (Prasad *et al.,* 2004).

2.9.4 Ascorbic acid (Vitamin C)

Vitamin C is a powerful antioxidant found in many fruits. A strong positive relationship between the amount of vitamin C, and total antioxidant activity has been reported (Kim *et al.,* 2002). Vitamin C is known as an antioxidant (Kondakova *et al.,* 2009) because of its ability to donate electrons (Padayatty *et al*., 2003) which can prevent cell damage from oxidation of fat (Evans, 2000). As an antioxidant, vitamin C works as an electron donor by moving one electron to the free compound (Padayatty *et al.,* 2003). Vitamin C is an essential phytonutrients for the metabolism of living cells that occurs in different concentrations in natural foods, especially fruits and their products. Gardner *et al*. (2000) have revealed that Vitamin C accounted for 65-100% of antioxidant capacity of citrus juices.

Vitamin C (ascorbic acid) is an important requirement for a variety of biological functions. Humans and other primates have lost the ability to synthesize ascorbic acid due to a defect in L-gulono-1,4-lactone oxidase, an enzyme that catalyzes the conversion of L-gulonolactone into ascorbic acid. Humans, primates and a few other animals (e.g., guinea pigs) depend on

the diet as a source of vitamin C to prevent the vitamin C deficiency disease, scurvy, and to maintain general health (Bowman *et al.,* 2009). The health-promoting effects of vitamin C can be attributed to its biological function as a co-factor for a number of enzymes, most notably hydroxylases involved in collagen synthesis, and as a water-soluble antioxidant (Wilson, 2009).

2.9.4.1 Vitamin C as an anti-oxidant

Role of vitamin C in lipid peroxidation (LPO) can be considered as an example of a radical chain reaction (Figure: 2.24). Reactive oxygen species (ROS) produced by a variety of sources, such as the electron transport chain, xanthine oxidase, myeloperoxidase and NADPH oxidase, initiate the radical reaction through abstraction of hydrogen atoms from bisallylic C–H bonds thereby forming lipid radicals (Halliwell and Gutteridge, 1999). Lipids are often prime targets of oxygen radicals because many of the enzymes producing ROS are embedded in lipid bilayers and because the bisallylic C–H bond in polyunsaturated fatty acids (PUFAs) is relatively weak compared to other C–H bonds. Carbon-centered lipid radicals react with molecular oxygen to form peroxyl radicals that, if not neutralized by α-tocopherol in membranes, may participate in the radical propagation reaction. Lipid hydroperoxides are chemically unstable and, when not reduced by glutathione-dependent reductases to hydroxy-fatty acids, constitute a source of a variety of LPO products, including 2-alkenals, epoxides, and malondialdehyde. Vitamin C has the ability to protect against LPO by acting as a scavenger of ROS and by one-electron reduction of lipid hydroperoxyl radicals via the vitamin E redox cycle (Halliwell and Gutteridge, 1999). Furthermore, studies support a role for vitamin C in protection against cellular damage from LPO-derived 2-alkenals. Vitamin C-adequate cultured human THP-1 cells exposed to the LPO product, 4-hydroxy-2(*E*)-nonenal

(HNE) showed a significant reduction in protein carbonylation compared to THP-1 cells that were not pre-incubated with vitamin C (Chavez *et al.,* 2010). The protective effects of ascorbate were associated with an increase in the formation of GSH-HNE conjugate and its phase I metabolites, measured by LC-MS/MS, and with increased transport of GSH conjugates from the cells into the medium

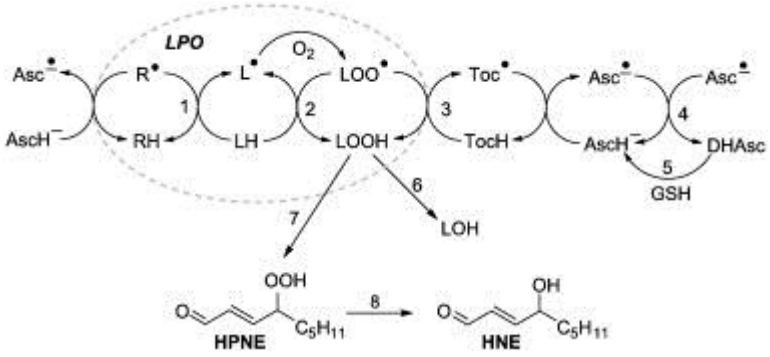


Fig 2.26: Antioxidant effects of vitamins C on lipid peroxidation (LPO)
(Miranda *et al.,* 2009)

The LPO chain reaction can be initiated by many radical species (indicated by R•) and converts LH into LOO•, which attacks another LH generating L• (paths 1 and 2, dotted oval). Ascorbic acid may scavenge the initiating radical species R• and reduce the tocopheroxyl radical, generating the ascorbyl radical, which can be reduced by glutathione-dependent enzymes. Key to reaction steps: 1, initiating event; 2, radical propagation reaction; 3, termination of the radical reaction by tocopherol (TocH); 4, dismutation of ascorbyl radicals (Asc•⁻); 5, reduction of dehydroascorbate (DHAsc) by GSH-dependent dehydroascorbate reductase; 6, GSH peroxidase (GPx); 7, further oxygenation and nonenzymatic cleavage of carbon–carbon bonds yields 4-hydroperoxy-2(*E*)-nonenal (HPNE); 8, reduction yields 4-hydroxy-2(*E*)-nonenal (HNE).

Some authors have reported that vitamin C promotes the formation of LPO products from lipid hydroperoxides in vitro, even in the absence of catalytic iron (Lee *et al.*, 2001). The in vivo implications of a vitamin C-dependent conversion of lipid hydroperoxides into reactive 2-alkenals have been disputed by others who have argued that redox-active Fe^{2+} ions, considered necessary for homolytic cleavage of hydroperoxides via Fenton chemistry, are not freely available under physiological conditions (Suh *et al.*, 2003).

Lipid peroxidation contributes to the development and progression of chronic diseases with an inflammatory component. Biomarkers of oxidative stress that are derived from LPO, such as F_2-isoprostanes, HNE, and malondialdehyde, are often elevated in plasma, urine, or tissues obtained from patients diagnosed with cardiovascular diseases (Mattson, 2009) neurodegenerative disease (Mattson, 2009), and diabetes (Traverso *et al.*, 1998). These observations raise the question whether there is a causal relationship between oxidative stress and these diseases. If so, antioxidant therapy should prove beneficial in chronic inflammatory disease. Indeed, many clinical studies have been conducted using vitamins C to improve biomarker levels and clinical outcomes (Carr and Frei, 1999). Disappointing results of antioxidant therapy in a variety of trials (Lykkesfeldt and Poulsen, 2010) led some critics to hypothesize that antioxidant therapy may not be effective to provide secondary prevention in advanced stages of cardiovascular disease (Duffy *et al.*, 1999). In a 2002 review of prospective studies on vitamin C status and cardiovascular disease, Loria (2002) noted that outcomes were more likely to be positive when serum vitamin C concentrations were used to assess vitamin C status as opposed to vitamin C intake and when a wider definition of cardiovascular disease was used with inclusion of endpoints related and unrelated to oxidative stress. Others have argued that ‘overdosing’ with antioxidants proves ineffective in

reducing oxidative stress due to suppression of normal physiological response systems following an oxidative stress insult, for example, physical exercise (Ristow, 2009). The effect of vitamin C supplementation (500 mg twice daily for 17 days) on urinary levels of metabolites of 4-hydroperoxy-2(*E*)-nonenal, as an indicator of oxidative stress was investigated in a double-blind, placebo-controlled, randomized crossover study in 22 young adults (Kuiper, 2010). Vitamin C supplementation was found to decrease the urinary concentrations of these LPO product metabolites by 20-30% (Kuiper, 2011), which supports the notion that vitamin C supplementation exerts antioxidant effects and reduces oxidative stress *in vivo*. Harrison and May, (2009) have recently reviewed the cellular uptake, recycling, and neuroprotective functions of vitamin C in the brain. Tveden-Nyborg and Lykkesfeldt (2009), suggested that the neonatal brain is particularly susceptible to low vitamin C concentrations and that vitamin C deficiency may adversely affect early brain development. A similar conclusion was reached by Harrison *et al.,* (2010) who studied the relationship between vitamin C deficiency and oxidative stress during development in $Gulo^{-/-}$ mice. The authors found that $Gulo^{-/-}$ mice pups had low ascorbic acid levels with accompanying elevations in liver malondialdehyde and brain F_2-isoprostanes, both biomarkers of oxidative stress. The authors emphasized the critical role of ascorbic acid in preventing oxidative stress in the developing brain in animals that, like humans, cannot synthesize their own ascorbic acid.

2.10 Soybean

Soybean is a type of legume (plant that belongs to the pea family) that originates from China. Cultivation of soybean started 3500 years BC. Soybean is mostly cultivated in temperate areas with hot summers. Increased demand for products made of soybean accelerates

deforestation of Amazon rainforests (deforested areas are converted into agricultural lands used for cultivation of soybean). People cultivate soybean as a source of food and oil that has application in numerous industries. (Gottstein *et al.,* 2003)

Size of soybean depends on the variety. It can reach from 0.66 to 6.56 feet in height. Soybean has compound leaves that consist of 3 to 4 green leaflets. Leaves are covered with miniature grey hairs. Soybean produces white, pink or purple flowers. They grow in the axils of leaves. Flowers contain both types of reproductive organs (pistil and stamens) and they are able to perform self-fertilization. (Sasamura *et al.,* 2004)

Soybean produces hairy pods (fruit) with 2 to 4 seeds. Fruit grows gathered in clusters composed of 3 to 5 pods. Color of the hull (outer coat of seed) depends on the variety of soybean. It can be black, blue, green, yellow or mottled. Soybean is propagated via seed. It has short vegetative season. Soybean is usually harvested 80 to 120 days after planting.

Soybean is the only plant that contains "complete protein" (all essential amino acids that can be found in meat). Soybean is also rich source of vitamin K and vitamins of the B group, dietary fibers and minerals such as iron, magnesium, manganese and phosphorus. (Sasamura *et al.,* 2004).

2.10.1 Phytochemicals in Soybeans and their health effect

Consumption of soy foods has been associated, at least in part, with lower incidences of a number of chronic diseases indicated by epidemiological studies (Boyapati *et al.,* 2005). Soy-based foods have been consumed in Asian countries such as China, Japan and Korea for many centuries. The lower rates of several chronic diseases in Asia, including cardiovascular

diseases and certain types of cancer, have been partly attributed to consumption of large quantities of soy foods (Wu *et al.,* 1998).

In the last few decades, extensive efforts have been made towards identifying bioactive components in soy foods that are responsible for the health benefits. Among them, isoflavones and soy proteins, are the two major groups of components that have received the most attention (Xiao, 2008). Isoflavones belong to a broad group of plant-derived compounds that have structural and functional similarities to estrogens, which has led to the term phytoestrogens (Cabot, 2003). Consumption of isoflavones has been suggested to have multiple beneficial effects in a number of chronic diseases and medical conditions (Zhou, 2004). However, accumulating evidence has also suggested that isoflavones only reflected certain aspects of the health effects associated with soy consumption. Other components in soy, such as soya saponins, phytic acid or plant sterols, display a wide range of bioactivities, including anti-cancer, anti-oxidative, anti-viral, cardiovascular protective effects, and hepatoprotective actions (Rochfort and Panozzo, 2007).

Composition and contents of phytochemicals in soya bean vary dramatically depending on the variety and growing environment. In general, the contents of major phytochemicals from high to low in soybean are: phytic acid (1.0-2.2%) (Schlemmer *et al.,* 2009), sterols (0.23-0.46%) (Piironen *et al.,* 2000), saponins (0.17-6.16%) (Lin and Wang, 2004), isoflavones (0.1-0.3%) (Messina, 1999), and lignans (0.02%) (Penalvo, 2004). Secondly, the potential health effects of the major phytochemicals are outlined. For many years, the studies of health effects of soybean phytochemicals primarily focused on isoflavones. However, other phytochemicals in soy, such as soya saponins, phytosterols, lignans, phytic acid, and

oligosaccharides, have also been found to exert biological activities. These may contribute to overall health effects observed with soy consumption.

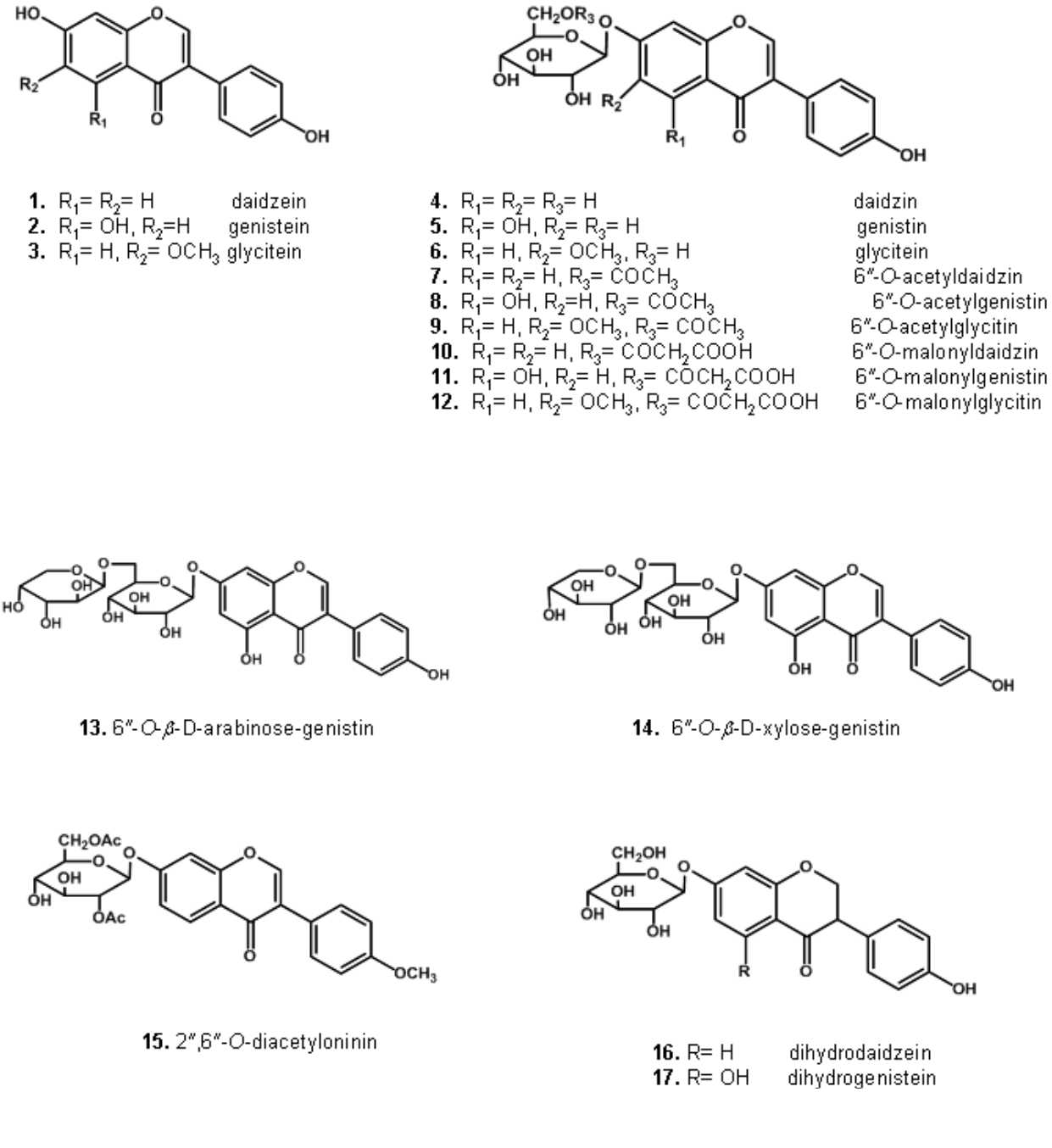


Fig. 2.27 Chemical structures of isoflavones (Wang and Murphy, 1994)

2.10.2 Health effects of isoflavones

Health effects of isoflavones were initially thought to be related to their estrogenic activity (Messina, 1999). The molecular structures of isoflavones, especially genistein (Boyapati, *et al.,* 2005), is similar to that of 17B- estradiol (Figure 2). Isoflavones can bind to both isoforms of estrogen receptor (ER), but their binding affinity to ER is about 20 times higher than that to ER (11). However, compared to physiological estrogen such as 17-estradiol, isoflavones have approximately 100 times weaker affinities (Barnes, 2010). Estrogen-like effects have been proposed as one of the major mechanism of action of isoflavone related to their health effects (Barnes *et al.,* 2000). A second mechanism of action of isoflavones, particularly of genistein, was discovered that genistein is a protein tyrosine kinase (PTK) inhibitor (Barnes *et al.,* 2000). Since then, isoflavones have been shown to affect a diverse array of intracellular signaling pathways (Messina, 2010). For instance, genistein and other isoflavones were found to interact with the peroxisome proliferator activated receptors, PPAR. These nuclear receptors are activated by fatty acids (PPAR) and prostaglandins (PPAR) and serve as transcription factors (Barnes, 2010). As polyphenols, isoflavones also have antioxidant activities, which were proposed as another important mechanism of action of isoflavones. However, isoflavones are not strong antioxidants and may not be able to scavenge oxidants directly. They therefore are considered as antioxidants because of their effects on gene expression of enzymes that enhance antioxidant defenses (Barnes, 2010). In addition to what has been discussed above, several other mechanisms have been proposed for the activities of isoflavones, including stimulation/inhibition of enzyme activities involved in steroid synthesis and metabolism, targeting thyroid peroxidase, and inhibiting cancer metastasis (Barnes, 2010). Consumption of isoflavones has been suggested to have multiple

beneficial effects in a number of chronic diseases and medical conditions, including certain types of cancer (Sarkar, and Li, 2003), heart disease (Clair and Anthony, 2005), bone functions (Zhang *et al.,* 2008) and most recently, prevention of obesity (Orgaard, and Jensen, 2008).

2.10.3 Soya saponins and soya sapogenols

Saponins are sterol or triterpene glycosides that occur in a wide variety of plants. Soy-based foods are primary dietary sources of saponins (Oakenfull, 1981). Chemical studies of saponins in soy dates back to the 1930's (Sumiki, 1930).

Plant saponins as a whole have been reported to have a wide range of biological activities, which were summarized with a long list in a recent review (Guclu-Ustundag, and Mazza, 2007). Soya saponins and soya sapogenols have been reported to display diverse health effects (Lee, 2005), including anticancer, cardiovascular protective effects, anti-virus, hepatoprotective actions and antioxidant activities (Rochfort, and Panozzo, 2007). Their health effects largely depend on their chemical structures (Gurfinkel, and Rao, 2003). Since this class of soy compounds are so poorly absorbed, their bioactivity maybe caused by indirect actions within the GI tract (Shuichi *et al.,* 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The materials that were used include:

(i) Microplate reader, Rayto (RT 6000)

(ii) Bench Centrifuge (Hawksley RegNo: 891481 Ser. No. 07. 4. 26)

(iii) Automated Chemistry Analyzer (Rayto Chemistry 120)

(iv) Haematology analyzer (Systmex model k21N, USA)

(v) Automated electrolyte Analyzer, Audicom USA (AC 9900 model)

(vi) Electronic weighing machine, (Salter, HoMedics Group Ltd. Production no. IB-1066-1011-03) and Microplate Reader

(vii) EDTA and plain tubes sample bottles, cotton wools, dissecting kits

(viii) Cages and feeders

(ix) Cannula, syringes

(x) Rats Prolactin, Oxytocin, Growth hormone and insulin (Wkea med Supplies) Thyroid hormones (T3 and T4), 17-β estradiol and Progesterone (Accubind) Elisa kits.

3.2 Experimental Animals and Care

Adult 40 females and 8 males Wistar rats weighing 190 to 230 g were obtained from the Animal House of the Department of Human Physiology, Ahmadu Bello University, Zaria, Kaduna State. The animals were kept and maintained under routine laboratory condition of temperature, humidity and light. The animals were housed five animals per cage. They were fed on standard commercial feeds with water *ad libitum.*

3.3 Drugs and Soy beans

The drugs and soya bean variety used were as follows:

(i) Metoclopramide hydrochloride tablets 10 mg; Jiangsu Pengyao Pharmaceutical Inc. China, Manufacturing Licence No.: B131205, NAFDAC Reg. No.: 04-6476.

(ii) Vitamin C (L Ascobic acid A5960 (Sigma-Aldrich) Germany)

(iii) Soy beans (TGX 1448-2E)- sourced from IAR

3.4 Experimental Design

In the experiment, a total of 35 mature female rats (190g -230g b.w) were used. The animals were divided in to five groups as follows:

Group 1 (n=5): Normal control = Normal feed + Water

Group 2 (n=5): Received metoclopramide (5mg/kg) (Simelane *et al.*, 2012)

Group 3 (n=5): Received 100 mg/kg of Vitamin C (Eteng *et al.*, 2006)

Group 4 (a) (n=5): Received 10% soybean

(b) (n=5): Received 20% soybean

(c) (n=5): Received 40% soybean. (Fatahnia *et al.*, 2010)

Group 5 (n=5): Received 20% supplement plus 100 mg/kg of Vitamin C. (Maghsoud *et al*., 2013)

The Vitamin C and metoclopramide were given orally. The supplements and drugs were administered for 10 days, starting from day 4 to day 13 of lactation.

3.5 Evaluation of Milk Production

Milk production was estimated 18hr and 23hr after gavage. Milk yield and body weight of dams and weight gain of pups was measured each day (Sampson and Jansen, 1984). Every

day during the study period, the pups was weighed at 07hr (W1), and subsequently isolated from the dams for 4hr. At 11hr, the pups were weighed (W2), and were returned to their mother and allowed to feed for 1hr. At 12hr, the pups were weighed (W3) again. Milk yield at 18hr after the gavage was estimated as W3-W2. Daily milk yield was corrected for weight loss due to metabolic processes in the pup (respiration, urination and defecation) during suckling. The value used was be (w2-w1)/4 (Morag, 1970). This value was then multiplied by the number of suckling hours per day and was added to the daily sucking again. Daily weight gain of pups was calculated from the pup weight at W2 (Reddy and Donker, 1965). The same procedure was repeated for 23hr. Daily milk yield was corrected for weight loss due to metabolic processes in the pup (respiration, urination and defecation) during suckling. The value to be used for correction of weight lost will be [(w2-w1) + (w4-w3)]/8. This value was multiplied by the number of suckling hours per day and added to the daily suckling gain (Ouedraogo et al., 2004). Daily weight gain of pups was again calculated from the pup weight at w2.

3.6 Blood Sample Collection and Serum Preparation

Following end of treatment all animals from each group were sacrificed using light chloroform euthanesia after 24 hours; and blood (4ml) was collected through cardiac puncture into heparinized sample bottles for haematological analysis, osmotic fragility test and erythrocytes malondialdehyde concentration. The other portion of blood sample was collected into specimen bottles and was allowed to clot and separated by centrifugation at 2000 revolutions per minute for 10 minutes using Centrifuge Hettich (Universal 32, Made in Germany) and the supernant obtained was used for the determination of the physiological and biochemical parameters, respectively.

3.7 Tissue Sample Collection

On the day 14 of lactation, at the termination of the experiment, thirty five (35) lactating Wistar rats consisting of groups I to V were anaesthetized by chloroform euthanesia in a closed chamber and thereafter sacrificed. Inguinal mammary gland tissues, two from each group were harvested using a pair of scissors and a forceps, and thereafter deposited in a labeled test tube containing a physiological solution; 10 % formal saline.

3.8 Determination of Serum Lactogenic Hormones Levels

3.8.1 Serum Prolactin Assay

Prolactin analysis was carried out using the Prolactin rat ELISA kit according to the manufacturer's manual.

3.8.1.1 Principle

The Rat Prolactin ELISA kit is a solid phase enzyme immunometric assay (ELISA) in the microplate format, designed for the quantitative measurement of rat prolactin. The microplate is coated with a first monoclonal antibody specific for rat prolactin. Calibrators and samples are pipetted into the antibody coated microplate. During 2 hours incubation, endogenous rat prolactin in the sample bind to the antibodies fixed on the inner surface of the wells. Non-reactive sample components are removed by a washing step. Afterwards, a second polyclonal horseradish peroxidase-labeled antibody, directed against another epitope of the prolactin molecule, is added. During 1 hour incubation, a sandwich complex consisting of the two antibodies and the rat prolactin is formed. An excess of enzyme conjugate is washed out. A chromogenic substrate, TMB (3,3',5,5'-Tetra-Methyl-Benzidine), is added to all wells. During 30 minutes incubation, the substrate is converted to a colored end product (blue) by the fixed enzyme. Enzyme reaction is stopped by dispensing of hydrochloric acid as stop solution

(change from blue to yellow). The color intensity is direct proportional to the concentration of rat prolactin present in the sample. The optical density of the color solution is measured with a microplate reader at 450 nm. Bi-chromatic measurement with a 600 - 690 nm reference filter is recommended.

3.8.1.2 Procedure

0.1 mL of the reconstituted Master Calibrator was pipetted into tube E (40 ng/mL), and mix thoroughly. This process was repeated successively to complete the 2-fold dilution series. The reconstituted Calibrator served as the highest calibrator F (80 ng/mL). The calibrator/sample diluent was used as the zero calibrator A (0 ng/mL). 25 µL of each calibrator, control and sample was introduced into the wells prepared. 50 µL of sample buffer was added to every well. It was then shaken for 2 hours at room temperature (18 - 28 °C). The plate shaker was set at 600-900 rpm. The content of the wells were discarded and wash 4 times with 300 µL wash buffer which was then removed as much as possible by tapping the microplate against an a paper towel carefully. 200 µL of enzyme conjugate was added to all the wells, and shaken again for an hour at 600-900 rpm. The content of the wells were then discarded and washed 4 times with 300 µL buffered wash buffer, which was also removed as much as possible by beating the microplate carefully. 200 µL of liquid TMB/Substrate solution was added to all wells, and incubated without shaking for 30 minutes in the dark. 50 µL of stop solution was added to each well and mixed carefully. The optical density at 450 nm was measured.

3.8.2 Serum Growth Hormone Assay

The growth hormone analysis was carried out according to the manufacturer's manual, using the growth hormone ELISA kit.

3.8.2.1 Principle

The MICRO-ELISA growth hormone test is based on the principle of a solid phase enzyme linked immunosorbent assay. The sample sera were allowed to react simultaneously with the coated and conjugated antibodies which resulted in the rat growth hormone molecule being sandwiched between the solid phase and enzyme linked antibodies. It was the incubated for 45 minutes at room temperature and the unbound enzyme washed out. Hydrogen peroxide and tetra-methylbenzidine (TMB) were added to the well and incubated for 30 minutes at room temperature which resulted in the formation of a blue color. 1.0 N of H_2S0_4 was added to stop the reaction which converted the colour to yellow. The intensity of the yellow color is directly proportional to the concentration of rat growth hormone in the sera.

3.8.2.2 Procedure

100 µl dilution buffer was added into the first two wells which served as blanks. Subsequently 100 µl standard diluted control sera were poured into wells designated for standards (A-G). The wells were covered with sealing tape and incubated for 1 hour at room temperature with a shake at 350 rpm. After incubation, the content of the wells was aspirated and the wells washed 3 times with 300 µl wash buffer. The plate was tapped firmly against several layers of folded paper towels to remove excess wash buffer. 100 µl of the antibody conjugate was added into each well and covered with sealing tape and the plate incubated for an hour at room temperature with a shake at 350 rpm. The wells were then washed after incubation as described above after which 100 µl of the enzyme conjugate was put into each well and covered with sealing tape and incubated for 30 minutes at room temperature (shake 350 rpm). The wells were washed 3 times with wash buffer as described above, then 100 µl of the TMB substrate solution was pipetted into each well and incubated for 30 minutes in the

dark at room temperature (20 – 25°C). The reaction was brought to an end by adding 100 µl of Stop Solution. The color reaction was measured within 30 minutes at 450 nm.

3.8.3 Serum Oxytocin Assay

Oxytocin analysis was carried out using the Oxytocin rat ELISA kit according to the manufacturer's manual.

3.8.3.1 Principle

Oxytocin analysis was carried out using the Oxytocin rat ELISA kit according to the manufacturer's manual. This oxytocin species specific enzyme-linked immunosorbent assay (ELISA) kit in microplate was designed for the quantitative evaluation of rat oxytocin. The sera sample was pipetted into an antibody coated microplate, and allowed for two (2) hours during which the endogenous oxytocin in the sample bound to the antibodies fixed on the inner surface of the wells. After the addition of a second polyclonal horseradish peroxidase-labeled antibody, a sandwich complex which consisted of two antibodies and the rat prolactin was formed. The excess enzyme conjugate was washed out and a chromogenic substrate, tetra-methyl benzidine (TMB) was added to the wells and allowed for 30 minutes incubation, during which the substrate was converted to a colored end product (blue) by the fixed enzyme. The enzyme reaction was then stopped by dispensing hydrochloric acid as the stop solution. The optical density of the colour solution was read using a microplate reader at 450 nm. A standard curve was obtained by plotting the concentration of standard versus the absorbance. The oxytocin concentration of the specimens and controls run concurrently with standards and were calculated from standard curve.

3.8.3.2 Procedure

150 µl ELISA buffer was pipetted into non specific binding wells whereas a 100 µl of ELISA buffer was added to maximum binding wells. 50 µl of oxytocin tracer was added to all the wells except the total activity wells (TA) and the blank wells (Blk). Plates were then covered with plastic covers and incubated overnight 12-18 hours at 4^{o}C. 50 µl of oxytocin polyclonal antiserum was then added to the wells after incubation except TA, non-specific binding wells (NSB). The wells were then emptied and washed 5 times with wash buffer solution. Afterward, 200 µl of Ellman's reagent was added to all the wells, and 5 µl of the tracer to the TA wells. This preparation was covered with plastic covers and incubated for 90 minutes.

3.8.4 Serum Thyroid Hormones Assay (T4 and T3)

The thyroid hormone (T4) and (T3) analysis was carried out according to the manufacturer's manual, using the thyroid hormones ELISA kit.

3.8.4.1 Principle (T4)

This assay employs the competitive inhibition enzyme immunoassay technique. An antibody specific to T4 has been pre-coated onto a microplate. Standards or samples are added to the appropriate micro titer plate wells with biotin-conjugated T4 and incubated. A competitive inhibition reaction is launched between T4 (Standards or samples) and Biotin-conjugated T4 with the pre-coated antibody specific for T4. The more amount of T4 in samples, the less antibody bound by Biotin-conjugated T4. Then Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The substrate solutions are added to the wells, respectively. And the color develops in opposite to the amount of T4 in the sample. The color development is stopped and the intensity of the color is measured.

3.8.4.2 Procedure (T4)

A Blank well without any solution was set and 50µl of standard was added per well. 50µl of Biotin-conjugate was also added to each well, excluding the blanks and mixed well and then incubate for 1 hour at 37°C. Each well with Wash Buffer (about 200µl), stayed for 10 seconds and Spinning. This process was repeated for a total of three washes. The remaining wash buffer was removed by aspiration. The plate was inverted and blotted against clean paper towels. 50µl of HRP-avidin was added to each well and Incubated for 30 min at 37°C. The aspiration and washing of wash buffer and wells respectively was repeated and addition was made of 50µl of substrate A and Substrate B to each well and Incubated for 15 minutes at 37°C. 50µl of stop Solution was added to each well to stop the reaction. The optical density of each well was measured within 10 minutes using a microplate reader set to 450 nm.

The Triiodothyronine (T3) analysis was carried out according to the manufacturer's manual, using the thyroid hormones ELISA kit.

3.7.4.3 Principle (T3)

This assay employs the competitive inhibition enzyme immunoassay technique. An antibody specific to T3 has been pre-coated onto a microplate. Standards or samples are added to the appropriate microtiter plate wells with biotin-conjugated T3 and incubated. A competitive inhibition reaction is launched between T3 (Standards or samples) and Biotin-conjugated T3 with the pre-coated antibody specific for T3. The more amount of T3 in samples, the less antibody bound by Biotin-conjugated T3. Then Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The substrate solutions are added to

the wells, respectively. And the color develops in opposite to the amount of T3 in the sample. The color development is stopped and the intensity of the color is measured.

3.8.4.4 Procedure (T4)

25 mL of the appropriate was put into the assigned well. 100 mL of working T3-Enzyme conjugate solution to was added to all wells. The plate cover the plate was incubated for 60 minutes at room temperature with shaking. Liquids from all wells were removed and washed using absorbent paper towels. 100 mL of TMB of substrate solution was added to all wells. It was covered and incubated at room temperature for 15 minutes. 50 mL of stop solution was added to each well and gently mixed for 15–20 seconds. The absorbance on ELISA Reader of each well at 450 nm within 15 minutes was taken.

3.8.5 Serum Insulin Assay

Serum Insulin analysis was carried out according to the manufacturer's manual, using the thyroid hormones ELISA kit.

3.8.5.1 Principle

This ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with anti-insulin antibodies bound to the micro titration well and with peroxidase-conjugated anti-insulin antibodies. A washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB, a frequently used chromogenic in ELISAs). The reaction is stopped by adding acid to

give a colorimetric endpoint that is read spectrophotometrically at a waive length of 450 nm using a microplate reader.

3.8.5.2 Procedure

25 µL each of calibrators was pipetted into appropriate wells, with 25 µL each of the samples. 100 µL of enzyme conjugate 1X solution was also pipetted into each well. It was then covered with a plastic cover and then incubated on a plate shaker (700-900 rpm) for 2 hours at room temperature (18-25°C). Each well was washed 6 times with was buffer IX solution and the solution discarded by inverting the microplate over a sink. 350 µL wash solution was then added to each well after which it was discarded by tapping firmly against absorbent paper and the excess liquid removed. This procedure was repeated 5 times after which 200 µL substrate TMB was put into each well and incubated for 15 minutes at room temperature (18 -25°C). 50 µL stop solution was added to each well and the plate placed on the shaker for approximately 5 seconds and mixing ensured. Optical density was read at 450 nm within 30 minutes.

3.8.6 Serum Progesterone Assay

The serum progesterone analysis was carried out according to the manufacturer's manual, using the thyroid hormones ELISA kit.

3.8.6.1 Principle

Progesterone ELISA is used for the quantitative measurement of progesterone in rat serum or plasma. It is a solid phase competitive ELISA. The samples and Progesterone enzyme conjugate are added to the wells coated with anti-Progesterone monoclonal antibody. Progesterone in the sample competes with a Progesterone Enzyme Conjugate for binding

sites. Unbound progesterone and progesterone enzyme conjugate are washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of progesterone in the samples. A standard curve is prepared relating color intensity to the concentration of the progesterone.

3.7.6.2 Procedure

20 mL of Progesterone standards, control, and serum samples was added into the wells after which 100 mL of Progesterone enzyme conjugate was added also and incubated for 60 minutes at room temperature (18–26 °C) with shaking. Liquid from all wells was removed and the wells washed 3 times with 300 mL of 1x wash buffer and Blotted on an absorbent paper towels. 100 mL of TMB substrate was added to all wells and incubated for 15 minutes at room temperature. 50 mL of stop solution was added to all wells, and the plate was gently agitated to mix the solution. Absorbance on ELISA Reader at 450 nm was measured within 15 minutes after adding the Stop Solution.

3.8.7 Determination of Serum 17-β Estradiol Level

The 17-β estradiol analysis was carried out according to the manufacturer's manual, using the estrogen ELISA kit.

3.8.7.1 Principle

The Rat Estradiol ELISA is intended for the quantitative determination of Estradiol (E2) concentration in mouse/rat serum and plasma. It is based on the principle of competitive binding between E2 in the test specimen and E2 Enzyme Conjugate for a constant amount of anti-Estradiol polyclonal antibody. In the incubation, anti-E2 antibody coated wells are incubated with E2 standards, controls, samples, and E2 enzyme conjugate at room

temperature for 120 minutes. During the incubation, a fixed amount of HRP-labeled E2 competes with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. E2 Peroxidase Conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 Peroxidase Conjugate is then removed and the wells are washed. Next, a solution of TMB Reagent is added and incubated at room temperature for 15 minutes, resulting in the development of blue color. The color development is stopped with the addition of Stop Solution, and the absorbance is measured spectrophotometrically at 450 nm. A standard curve is obtained by plotting the concentration of the standard versus the absorbance.

3.8.7.2 Procedure

All reagents were brought to room temperature (18–26 °C) before use. 25 mL of standards, specimens, and controls was dispensed into appropriate wells, after which 100 mL of working reagent of Estradiol Enzyme Conjugate was also dispensed into each well of the wells and mixed by placing on shaker for 20 seconds and incubated at room temperature (18–25 °C) for 120 minutes. All liquid from all wells was poured out in a sink and the wells washed 3 times with 300 mL of 1x Wash buffer, and blotted out on an absorbance paper or paper towel. 100 mL of TMB Reagent was dispensed into each of the wells and gently mixed for 10 seconds, and incubated at room temperature (18–25 °C) for 30 minutes. The reaction was stopped by adding 50 mL of stop solution to each well and gently mixed for 30 seconds until all the blue color changed to yellow completely. The absorbance was measured at 450 nm with a microplate reader within 15 minutes.

3.9 Determination of Serum Antioxidant Enzyme Activities

3.9.1 Serum Malondialdehyde Assay

Malondialdehyde (MDA) is one of many low molecular weight end-products of lipid hydroperoxide decomposition and is the most often measured as an index of lipid peroxidation. Malondialdehyde (MDA) activity was estimated using The NWLSS™ Malondialdehyde assay kits (Northwest Life Sciences Specialies, Product NWK-MDA01, Vancouver WA, Specificity: Malondialdehyde, Sensitivity: 0.08µ) based on the method adapted by Janero (1990).

3.9.1.1 Principle

The microtiter plate provided in this kit has been pre-coated with an antibody specific to MDA. Standards or samples are then added tothe appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for MDA and Avidin conjugated to Horseradish Peroxidase (HRP) is addedto each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain MDA, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of MDA in the samples is then determined by comparing the O.D. of the samples to the standard curve. The test principle is based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm.

3.9.1.2 Procedure

100 µl of Standard, Blank and sample was added per well and covered with the Plate sealer. It was then Incubate for 2 hours at 37℃, after which the liquid in the wells was dispensed

into a sink. Without washing, 100 µl of detection reagent A working solution was dispensed into each well and covered with the plate sealer and incubated for 1 hour at 37℃ and mixed gently until the solution appeared uniform. Each well was aspirated and washed and the process repeated 3 times using the wash buffer solution (approximately 400 µl). After the last wash, the plate was inverted and blotted against clean paper towels. 100 µl of detection reagent B working solution to was dispensed into each well and covered with a new plate sealer, and incubated for 1 hour at 37℃. Aspiration was repeated and a described earlier as well as the washing. 90 µl of Substrate Solution was added to each well which was then covered with a new cover plate and incubated within 30 minutes. 50 µl of Stop Solution was dispensed into each well after which, the optical density of each well was measured at once, using a microplate reader set to 450 nm.

3.9.2 Serum Superoxide Dismutase Assay

Superoxide Dismutase (SOD) activity was assayed using the North West Life Science Specialties (NWLSS™) SOD assays kit (Product NWK-SOD02, Specificity: Cu/Zn, Mn and Fe Superoxide Dismutase, Sensitivity: 5 U/mL).

3.9.2.1 Principle

This assay employs the quantitative sandwich enzyme immunoassaytechnique. A monoclonal antibody specific for SOD has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any SOD present is bound by the immobilized antibody. An enzyme-linked monoclonal antibody specific for SOD is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of SOD bound in the initial step. The color development is stopped and the intensity of the color is measured.

3.9.2.2 Procedure

100 µl of Standard, Blank and sample was added per well and covered with the adhesive strip. It was then Incubate for 2 hours at 37℃, after which the liquid in the wells was dispensed into a sink. Without washing, 100 µl of detection reagent A working solution was dispensed into each well and covered with the plate sealer and incubated for 1 hour at 37℃ and mixed gently until the solution appeared uniform. Each well was aspirated and washed and the process repeated 3 times using the wash buffer solution (approximately 400 µl). After the last wash, the plate was inverted and blotted against clean paper towels. 100 µl of detection reagent B working solution to was dispensed into each well and covered with a new plate sealer, and incubated for 1 hour at 37℃. Aspiration was repeated and a described earlier as well as the washing. 90 µl of substrate solution was added to each well which was then covered with a new cover plate and incubated within 30 minutes. 50 µl of Stop Solution was dispensed into each well after which, the optical density of each well was measured at once, using a microplate reader set to 450 nm.

3.9.3 Serum Catalase Assay

Catalase (CAT) activity was estimated using North West Life Science Specialties (NWLSS™) NWLSS™ Catalase assay kits (Product NWK-CATO1, Specificity: 6.0 U Catalase/mL) based on the method of (Beers and Sizer, 1952) with modifications to increase robustness and convenience.

3.9.3.1 Principle

This assay is based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for CAT has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CAT present is bound by the immobilized

antibody. An enzyme-linked monoclonal antibody specific for CAT is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CAT bound in the initial step. The color development is stopped and the intensity of the color is measured.

3.9.3.2 Procedure

100 µL of sample dilution buffer was added and placed in the reference cuvette holder and the wavelength of spectrophotometer was set to 240 nm. To a clean semi-micro UV cuvette, 950 µL of working assay buffer was added. Then 50 µL of diluted standard or sample was pipetted to the cuvette and mixed as quickly as possible by repeated pipetting (About 10 times) with the same pipette tip. The absorbance of the mixture was then read immediately at 240 nm.

3.9.4 Serum Glutathione Peroxidase

Glutathione peroxidase (GPx) activity was assessed using North West Life Science Specialties (NWLSS™) cGPx (GPX1) ELISA kit (Product NWK-GPX02, Specificity: Gluthathione peroxidase, Sensitivity: 12.5pg/ml).

3.9.4.1 Principle

This assay is based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for GPx has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any GPx present is bound by the immobilized antibody. An enzyme-linked monoclonal antibody specific for GPx is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is

added to the wells and color develops in proportion to the amount of GPx bound in the initial step. The color development is stopped and the intensity of the color is measured.

3.9.4.2 Procedure

100 µl of Standard, Blank and sample was added per well and covered with the adhesive strip. It was then Incubate for 2 hours at 37℃, after which the liquid in the wells was dispensed into a sink. Without washing, 100 µl of detection reagent A working solution was dispensed into each well and covered with the plate sealer and incubated for 1 hour at 37℃ and mixed gently until the solution appeared uniform. Each well was aspirated and washed and the process repeated 3 times using the wash buffer solution (approximately 400 µl). After the last wash, the plate was inverted and blotted against clean paper towels. 100 µl of detection reagent B working solution to was dispensed into each well and covered with a new plate sealer, and incubated for 1 hour at 37℃. Aspiration was repeated and a described earlier as well as the washing. 90 µl of substrate solution was added to each well which was then covered with a new cover plate and incubated within 30 minutes. 50 µl of Stop Solution was dispensed into each well after which, the optical density of each well was measured at once, using a microplate reader set to 450 nm.

3.10 Determination of Liver Enzymes Concentrations

3.10.1 Serum alkaline phosphatase Assay

3.10.1.1 Principle

Alkaline Phosphatase in a sample hydrolyses paranitrophenyl phosphate into paranitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405 nm due to liberation of paranitrophenol is proportional to the alkaline phosphatase activity.

3.10.1.2 Procedure

100µL of detection reagent A working solution was dispensed into each well and incubated Incubate for 1 hour at 37^0C after covering it with the Plate sealer. The solution was afterwards aspirated and the wells washed with 350µL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette and dispenser and allowed sit for 1~2 minutes. Remove the remaining. Washing was carried out 3 times after this and the remaining liquid completely removed from the wells by blotting with an absorbent paper towels. 100µL of Detection Reagent B working solution was dispensed to each well and incubated for 30 minutes at 37^0C after covering with plate sealer. Aspiration and washing was conducted afterwards as described above and 90µL of Substrate Solution was added into each well. It was then covered with a new plate sealer and incubated for 10 - 20 minutes at 37^0C. 50µL of Stop Solution was later dispensed in to each well and the plate tapped gently to ensure thorough mixing. Measurement at 450nm was conducted using a microplate reader immediately.

3.10.2 Serum aspartate aminotransferase Assay

3.10.2.1 Principle

In this reaction L-Aspartate and á-Ketoglutarate react in the presence of GOT in the sample to yield oxaloacetate and L-glutamate. The oxaloacetate is reduced by malate dehydrogenase (MDH) to yield L-malate with the oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340nm. The rate of deduction in absorbance is proportional to GOT activity in the sample.

3.10.2.2 Procedure

100µL of detection reagent A working solution was dispensed into each well and incubated Incubate for 1 hour at 37^0C after covering it with the Plate sealer. The solution was afterwards aspirated and the wells washed with 350µL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette and dispenser and allowed sit for 1~2 minutes. Remove the remaining. Washing was carried out 3 times after this and the remaining liquid completely removed from the wells by blotting with an absorbent paper towels. 100µL of Detection Reagent B working solution was dispensed to each well and incubated for 30 minutes at 37^0C after covering with plate sealer. Aspiration and washing was conducted afterwards as described above and 90µL of Substrate Solution was added into each well. It was then covered with a new plate sealer and incubated for 10 - 20 minutes at 37^0C. 50µL of Stop Solution was later dispensed in to each well and the plate tapped gently to ensure thorough mixing. Measurement at 450nm was conducted using a microplate reader immediately.

3.10.3 Serum alanine aminotransferase Assay

3.10.3.1 Principle

In this reaction, L-Alanine and á-ketoglutarate react in the presence of GPT in the sample to yield pyruvate and L-glutamate. Pyruvate is reduced by lactate dehydrogenase to yield lactate with oxidation of NADH to NAD. The reaction is monitored by measurement of the in absorbance of NADH at 340nm. The rate of reduction in absorbance is proportional to GPT activity in the sample.

3.10.3.2 Procedure

100µL of detection reagent A working solution was dispensed into each well and incubated Incubate for 1 hour at 37^0C after covering it with the Plate sealer. The solution was afterwards aspirated and the wells washed with 350µL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette and dispenser and allowed sit for 1~2 minutes. Remove the remaining. Washing was carried out 3 times after this and the remaining liquid completely removed from the wells by blotting with an absorbent paper towels. 100µL of Detection Reagent B working solution was dispensed to each well and incubated for 30 minutes at 37^0C after covering with plate sealer. Aspiration and washing was conducted afterwards as described above and 90µL of Substrate Solution was added into each well. It was then covered with a new plate sealer and incubated for 10 - 20 minutes at 37^0C. 50µL of Stop Solution was later dispensed in to each well and the plate tapped gently to ensure thorough mixing. Measurement at 450nm was conducted using a microplate reader immediately.

3.11 Determination of Serum Electrolytes Levels

3.11.1 Serum Urea assay

3.11.1.1 Principle

Urea Assay Kit is designed to measure urea directly in biological samples without any pretreatment. The improved Jung method utilizes a chromogenic reagent that forms a colored complex specifically with urea. The intensity of the color, measured at 520nm, is directly proportional to the urea concentration in the sample. The optimized formulation substantially reduces interference by substances in the raw samples.

3.11.1.2 Procedure

Twenty (20) µL water, standard (50 mg/dL) and samples were dispensed into appropriately labeled tubes. 5 mg/dL standard and 200 µL instead of 20 µL was used for low urea samples 1000 µL working reagent was added afterward and tapped lightly to mix. It was then incubate 20 min and absorbance measured at (430nm).

3.11.2 Serum Sodium and Potassium assay

Serum sodium and potassium ions were measured by the Audicom Electrolyte Analyser.

3.11.3 Serum Bicarbonate assay

Bicarbonate ion was determined using the Audicom Electrolyte Analyser.

3.11.4 Serum Chloride assay

Chloride ion was analyzed using the Audicom Electrolyte Analyser.

3.12 Determination of Haematological Parameters

Hematological parameters (Red blood cell count, Hemoglobin concentrations, packed cell volume, white blood cell count, differential white blood cell countamd platelet count) was analyzed using Automated hematology analyzer (Systmex model k21N, USA), at Department of Hematology and Blood transfussion, Ahmadu Bello University Teaching Hospital, Zaria.

3.13 Histology of the Mammary gland

The method of H and E staining technique was used for the histological preparation. The mammary gland tissue sections were hydrated in descending grades of alcohol from 100%, 95%, 90% and finally 70% (Laporta *et al*., 2013). Each of the steps lasted for three (3) minutes and the tissues were washed in a running tap water and stained afterward with haematoxylin for twenty five (25) minutes, washed with water and then differentiated in acid

alcohol. The tissues were then counter stained with eosin and blued in Scott water. Afterward, the tissues were hydrated in ascending grades of alcohol and cleared in xylene for three (3) changes in five (5) minutes each. The tissues were then mounted with cover slips using a mounting media. The tissues were then viewed under a light microscope and the photomicrographs taken.

3.14 Statistical Analysis

Data obtained were expressed as mean ± SEM. The data was statistically analyzed using ANOVA with Tukey's *Post hoc test* to compare the levels of significant between the control and experimental groups. All statistical analysis was evaluated using SPSS version 20.0 software and Microsoft Excel (2007). The values of $p \leq 0.05$ were considered as significant.

CHAPTER FOUR

RESULTS

4.1: Effect of administration of Soya bean supplement and Vitamin C for 10 days on milk yield; 18 and 23 hours after gavage in female lactating Wistar rats.

Figure 4.1 shows the result of female lactating wistar rats 18 and 23 hours after gavage. Although there was increased milk yield 18 hours after gavage in all the treated groups; METCL (1.68 ± 0.37 g/pup vs 1.28 ± 0.47), VIT C (1.54 ± 0.47 vs 1.28 ± 0.47), SB 10% (1.64 ± 0.47 vs 1.28 ± 0.47), SB 20% (1.90 ± 0.45 vs 1.28 ± 0.47), SB 40% (2.14 ± 0.38 vs 1.28 ± 0.47) and SB 20% + VIT C (2.30 ± 0.411 vs 1.28 ± 0.47); the increase was however not significant ($P > 0.05$) compared to the control. There was however a significant increase in milk yield 23 hours after gavage in SB 20% (2.36 ± 0.49 vs 0.8 ± 0.26), SB 40% (1.98 ± 0.42 vs 0.8 ± 0.26) and SB 20% + VIT C (1.90 ± 0.46 vs 0.8 ± 0.26) ($P < 0.05$) compared to the control, with the highest milk yield in the SB 20% treated group. Although the level of milk yield in the METCL (1.16 ± 0.29 vs 0.8 ± 0.26), VIT C (1.24 ± 0.46 vs 0.8 ± 0.26) and SB 10% (1.70 ± 0.44 vs 0.8 ± 0.26) was also increased compared to the control, the increase was however not statistically significant ($P > 0.05$).

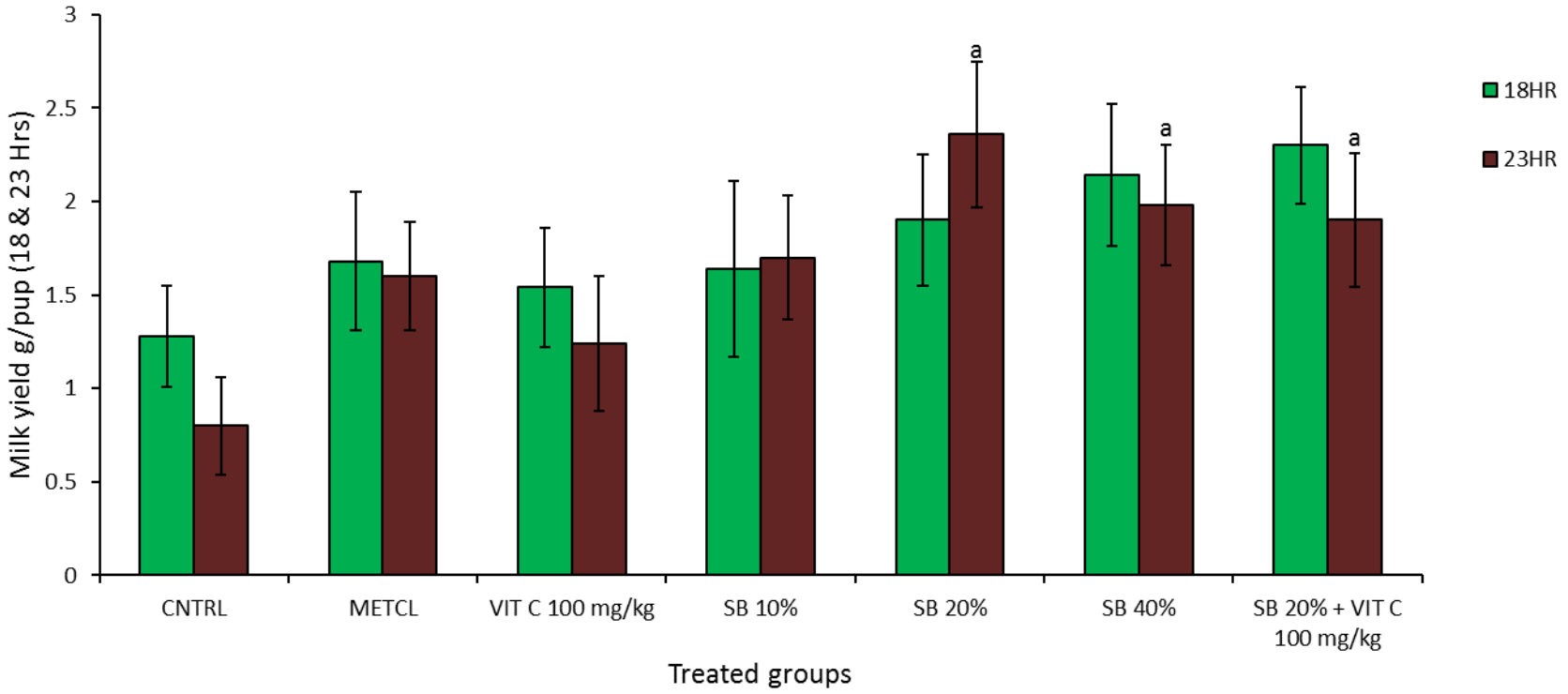


Figure 4.1: Effect of ten (10) days administration of Soya bean supplement and Vitamin C on milk yield; 18 and 23 hours after gavage in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 40% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg). 18HR = 18 hours after gavage, 23HR = 23 hours after gavage, superscript letter (a) represents significance when compared to control ($P< 0.05$).

4.2: Effect of administration of Soya bean supplement and Vitamin C for 10 on daily milk yield 18 hours after gavage in female lactating Wistar rats.

Figure 4.2 shows the distribution of the daily milk yield 18 hours after gavage in all the treated groups compared to the control, during 10 days treatment duration. The highest daily milk yield were as follows; METCL (Day 13; 2.80 ± 0.37 g/pup), VIT C (Day 13; 2.60 ± 0.75g/pup), SB 10% (Day 13; 3.00 ± 0.55 g/pup), SB 20% (Day 11; 3.20 ± 0.74 g/pup), SB 40% (Day 13; 4.60 ± 0.40) and SB 20% + VIT C (Day 13; 3.2 ± 0.37 g/pup). Although an increase in milk yield was observed in the treated groups, the increase was however not significant.

4.3: Effect administration of Soya bean supplement and Vitamin C for 10 days on daily milk yield 23 hours after gavage in female lactating Wistar rats.

Figure 4.3 shows the distribution of the daily milk yield 23 hours after gavage in all the treated groups compared to the control, during the 10 days treatment duration. The highest daily milk yield were as follows; METCL (Day 8 & 10; 1.80 ± 0.37 g/pup and 1.80 ± 0.20 g/pup), VIT C (Day 11 & 13; 1.8 ± 0.58 g/pup and 1.80 ± 0.58 g/pup), SB 10% (Day 13; 3.4 ± 0.25 g/pup), SB 20% (Day 7 & 13; 3.4 ± 0.4 g/pup and 3.4 ± 0.93) and SB 40% (Day 13; 3.2 ± 0.20 g/pup), SB 20% + VIT C (Day 13; 2.8 ± 0.97). There was a significant increase in milk yield in SB 20%, SB 40%, and SB 20% + VIT C when compared to the control ($P < 0.05$).

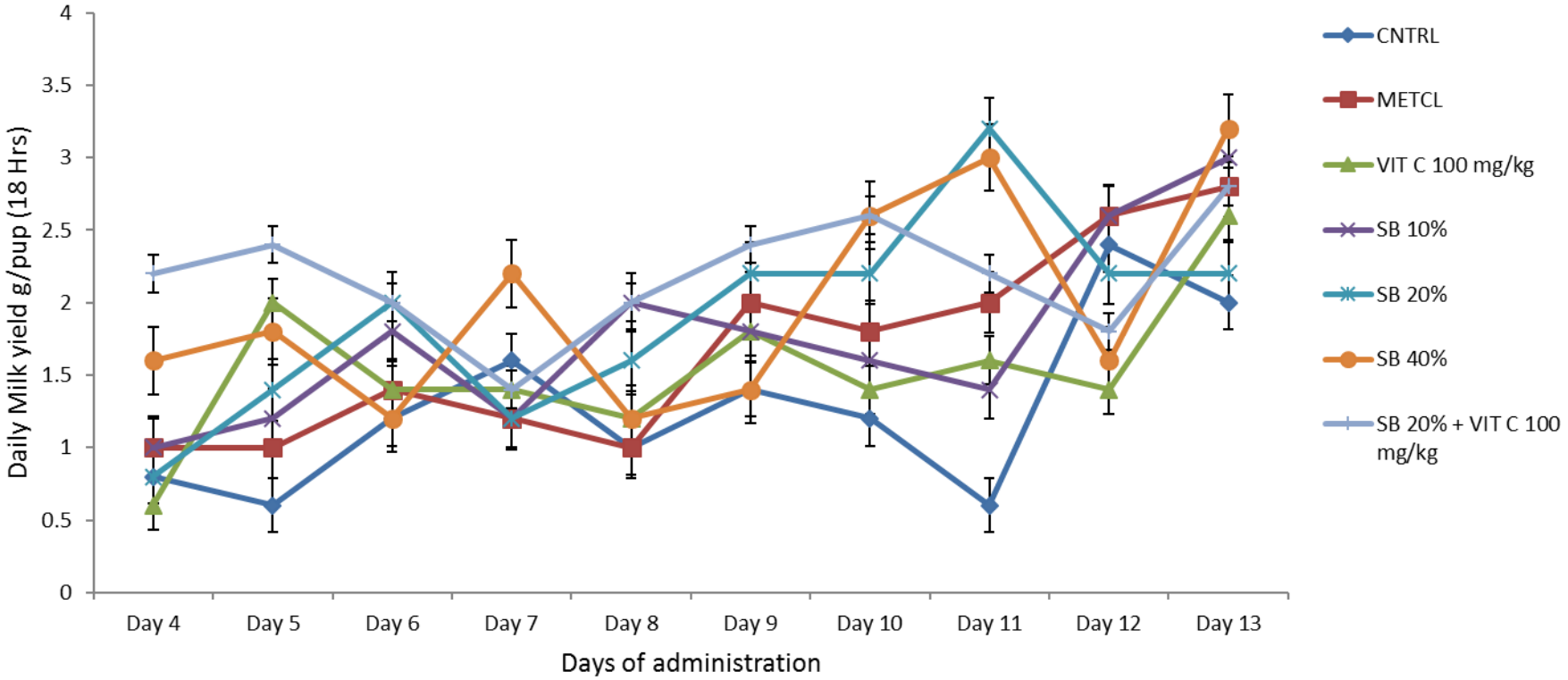


Figure 4.2: Effect of days administration of Soya bean supplement and Vitamin C for 10 days on daily milk yield 18 hours after gavage in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

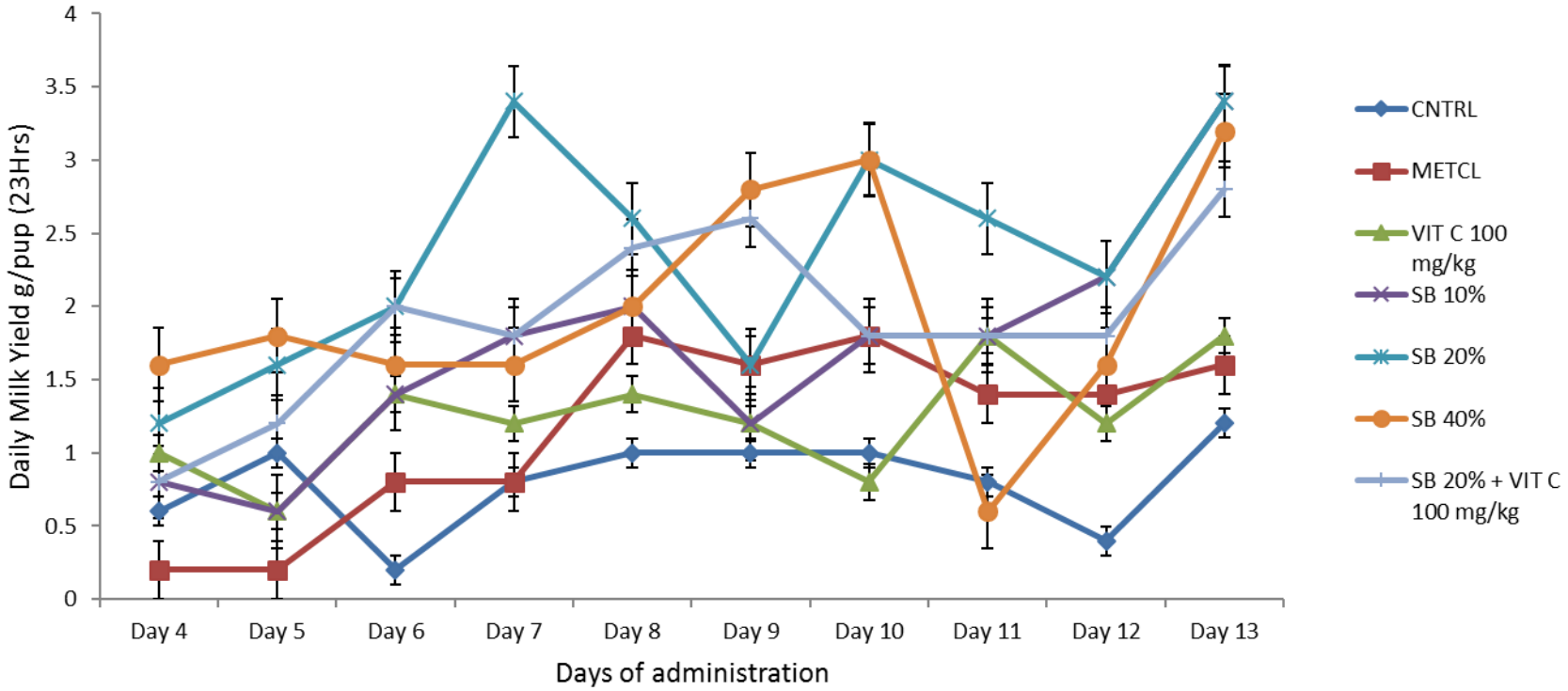


Figure 4.3: Effect of administration of Soya bean supplement and Vitamin C for 10 days on milk yield 23 hours after gavage in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.4: Effect of administration of Soya bean supplement and Vitamin C for 10 days Serum Prolactin level in female lactating Wistar rats.

Figure 4.4 shows serum prolactin level (ng/ml) compared in all the treated and control groups after ten (10) days of admininistration. There was a non significant increase ($P > 0.05$) in the serum prolactin level in treated as compared to control groups viz; METCL (52.24 ± 6.73 vs 43.98 ± 5.57), VIT C (59.06 ± 3.93 vs 43.98 v 5.57), SB 10% (50.86 ± 3.90 vs 43.98 ± 5.57), SB 20% (52.84 ± 2.93 vs 43.98 ± 5.57) and SB 40% (47.80 ± 1.06 vs 43.98 ± 5.57). However, the increase in the group treated with SB 20% + VIT C (63.70 ± 2.27 vs 43.98 ± 5.57) was significant ($P < 0.05$) when compared to the control.

4.5: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Growth hormone level in female lactating Wistar rats.

The effect of oral administration of Metoclopramide, Vitamin C and Soya bean is shown in figure 4.5. The serum level of Growth hormone (ng/ml) compared to the control were as follows: METCL (6.86 ± 1.42 vs 4.90 ± 0.37), VIT C (10.64 ± 0.79 vs 4.90 ± 0.37), SB 10% (10.34 ± 1.26 vs 4.90 ± 0.37), SB 20% (12.48 ± 0.84 vs 4.90 ± 0.37), SB 40% (8.76 ± 0.95 vs 4.90 ± 0.37) and SB 20% + VIT C (9.48 ± 0.85 vs 4.90 ± 0.37). There was a significant increase in serum GH in the groups treated with VIT C, SB 10%, SB 20% and SB 20% + VIT C. ($P < 0.05$) compared to the control rats.

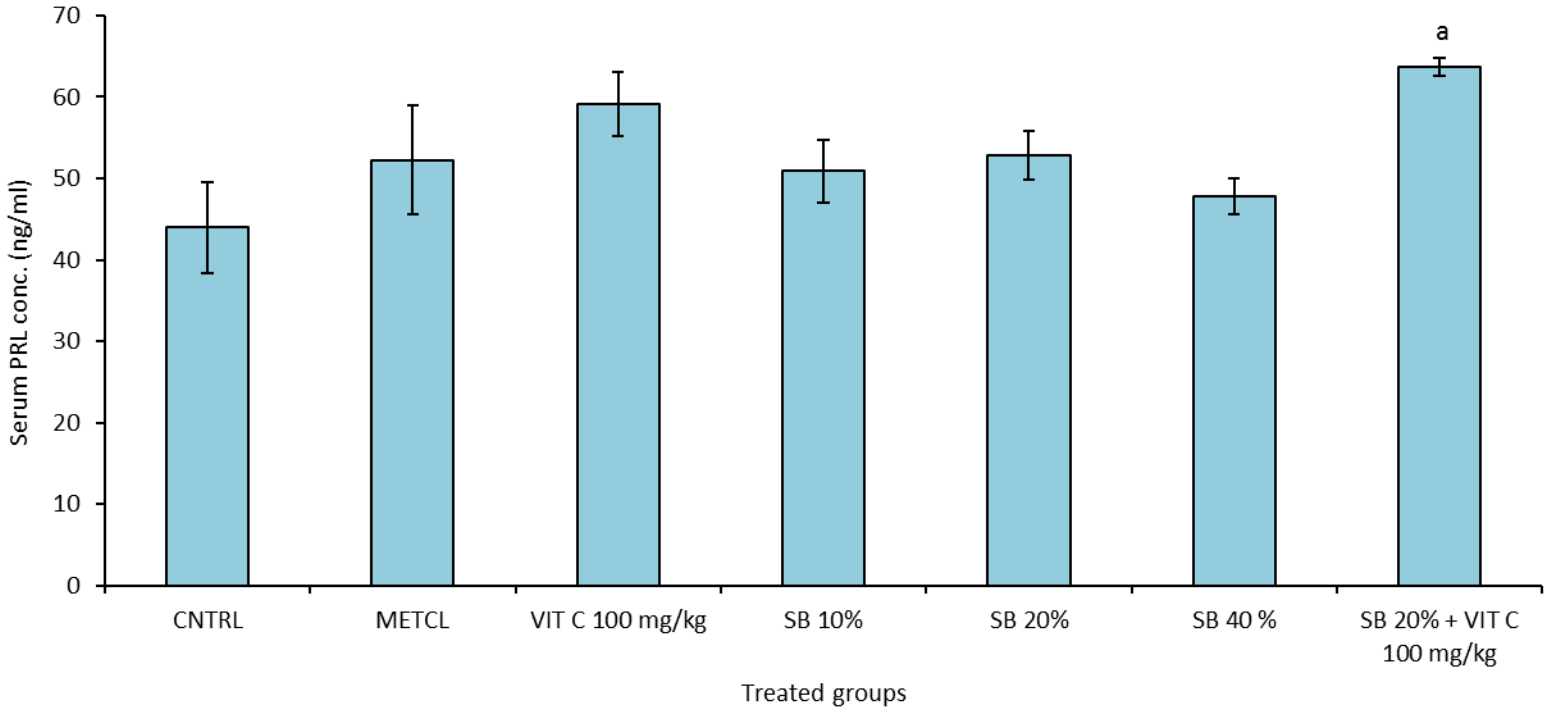


Figure 4.4: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum prolactin level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

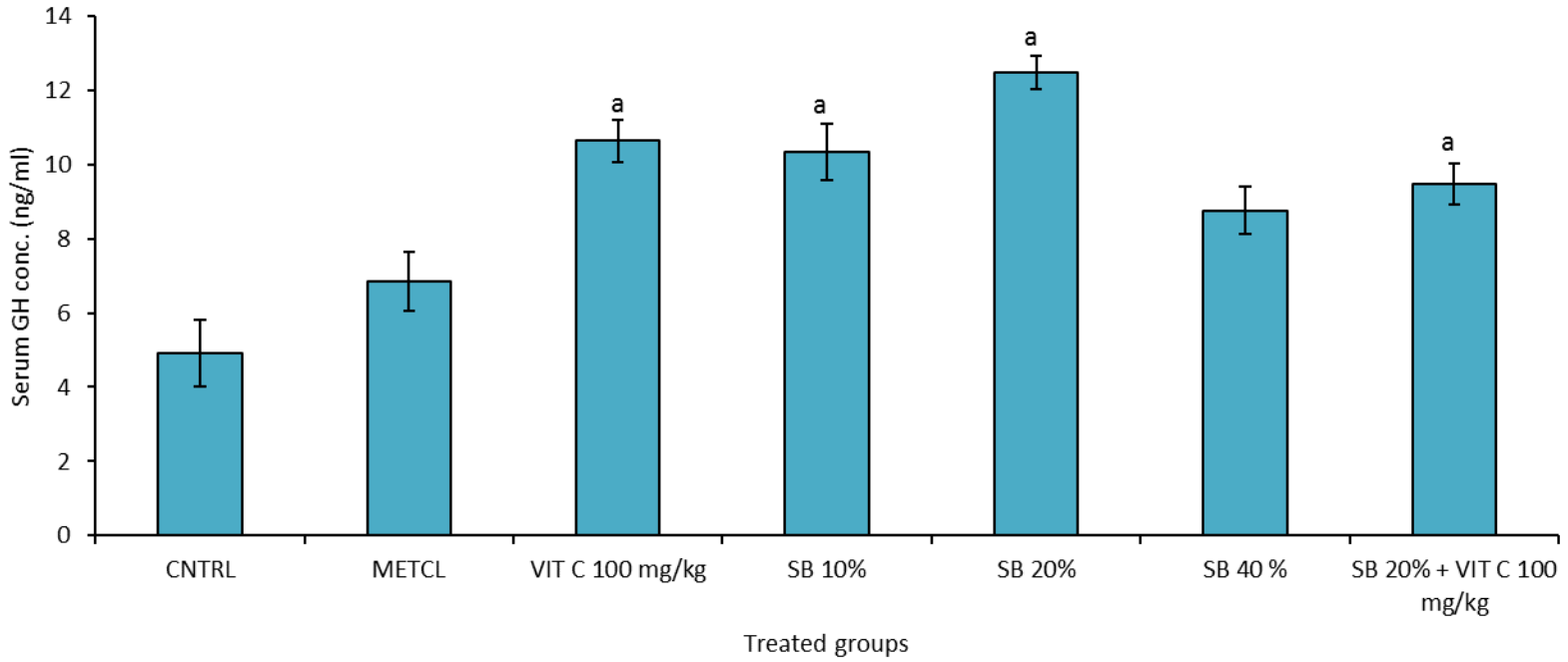


Figure 4.5: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Growth hormone level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance compared to control ($P < 0.05$).

4.6: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Oxytocin level in female lactating Wistar rats.

The effect of oral administration of Metoclopramide, Vitamin C and Soya bean on serum oxytocin is shown in figure 4.6. The serum level of Oxytocin (ng/ml) in treated compared to the control rats were as follows: METCL (404.20 ± 23.42 vs 342.80 ± 22.1), VIT C (298.60 ± 20.84 vs 342.80 ± 22.1), SB 10% (335.20 ± 42.72 vs 342.80 ± 22.1), SB 20% (395.20 ± 26.82 vs 342.80 ± 22.1), SB 40% (343.40 ± 15.17 vs 342.80 ± 22.1) and SB 20% + VIT C (338.40 ± 26.48 vs 342.80 ± 22.1). The differences in serum oxytocin levels between treated and control groups were not significant (P> 0.05). However, METCL and SB 20% groups had remarkably higher serum oxytocin levels compared to the control rats.

4.7: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Triiodothyronine (T3) level in female lactating Wistar rats.

The effect of oral administration of Metoclopramide, Vitamin C and Soya bean on T3 serum level (ng/ml) is shown in figure 4.7. The serum level of Triiodothyronine (T3) compared to the control were as follows: METCL (1.38 ± 0.12 vs 1.22 ± 0.15), VIT C (1.24 ± 0.10 vs 1.22 ± 0.15), SB 10% (1.42 ± 0.12 vs 1.22 ± 0.15), SB 20% (1.22 ± 0.09 vs 1.22 ± 0.15), SB 40% (1.32 ± 0.10 vs 1.22 ± 0.15) and SB 20% + VIT C (1.28 ± 0.12 vs 1.22 ± 0.15). There was no significant difference in serum T3 concentrations between treated and control groups (P> 0.05).

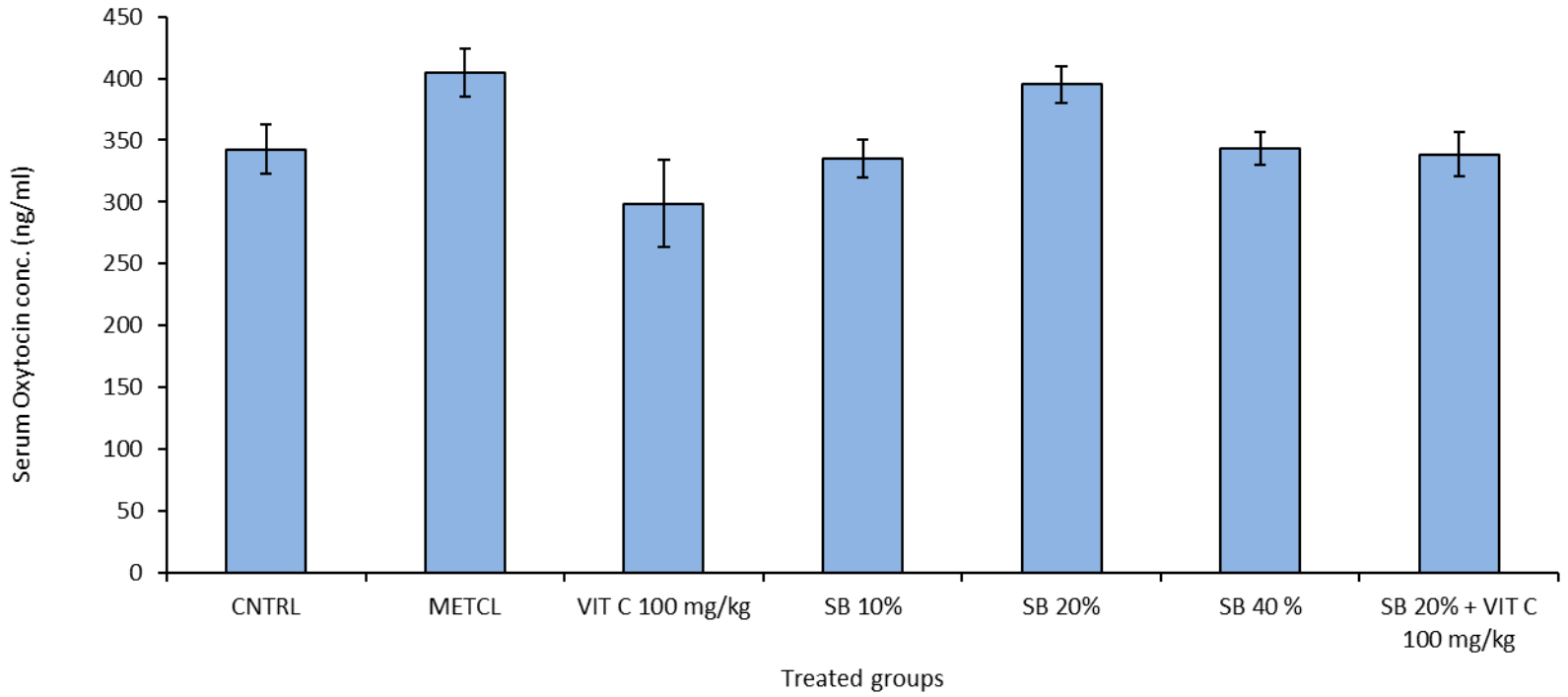


Figure 4.6: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Oxytocin level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

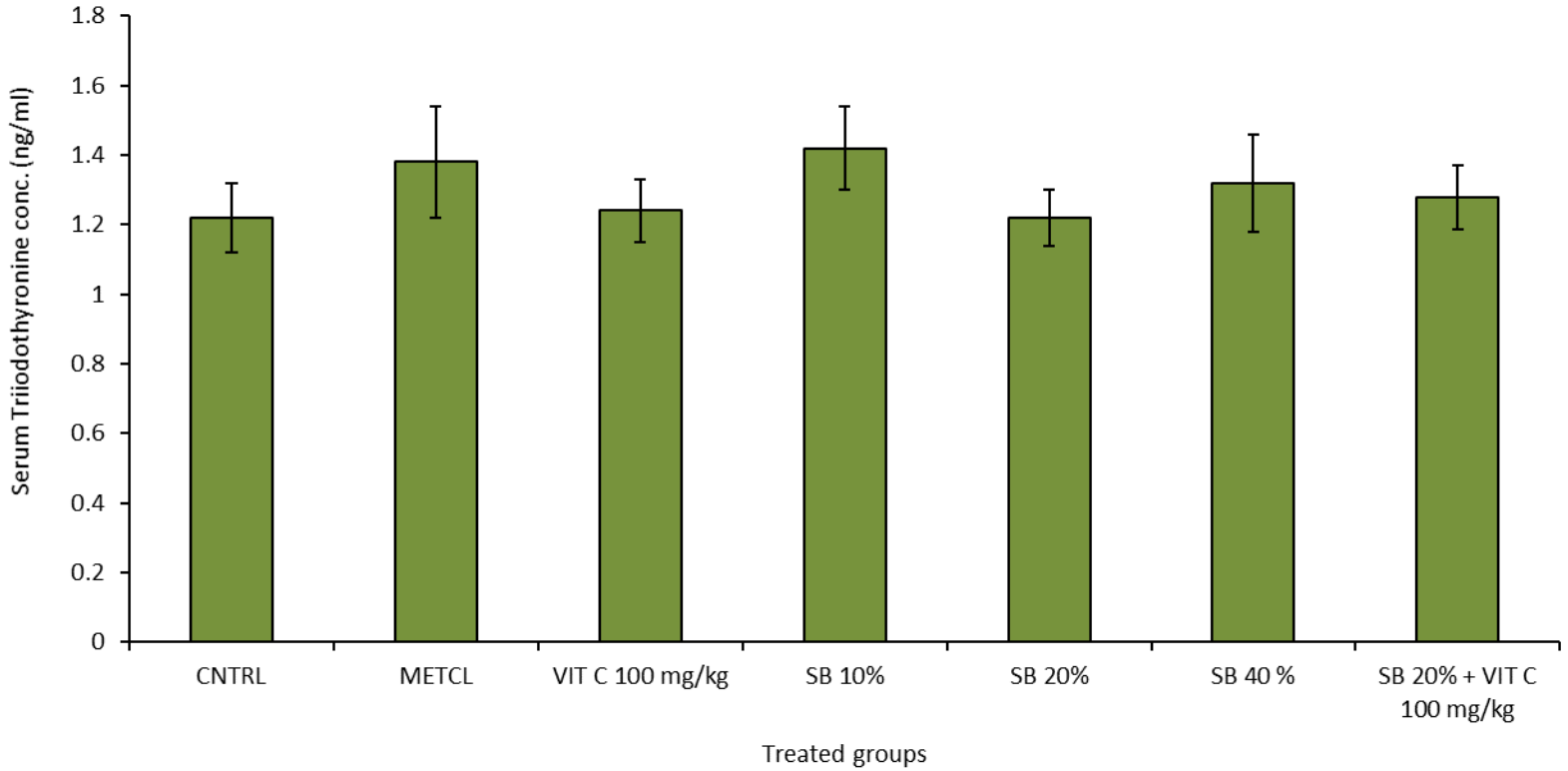


Figure 4.7: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Triiodothyronine (T3) level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.8: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Thyroxine (T4) level in female lactating Wistar rats.

Figure 4.8 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean on serum T4 levels (ng/ml) in female lactating Wistar rats. The serum levels of Thyroxine (T4) compared to the control were as follows: METCL (70.40 ± 2.98 vs 68.00 ± 3.91), VIT C (68.40 ± 4.43 vs 68.00 ± 3.91), SB 10% (56.74 ± 12.29 vs 68.00 ± 3.91), SB 20% (65.40 ± 3.31 vs 68.00 ± 3.91), and SB 20% + VIT C (73.60 ± 4.13 vs 68.00 ± 3.91). The differences were significant between treated and control groups ($P > 0.05$). However, there was a significant increase in serum T4 level in the SB 40% (100.80 ± 9.95 vs 68.00 ± 3.91) compared to the control ($P < 0.05$).

4.9: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Insulin level in female lactating Wistar rats.

Figure 4.9 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean on serum insulin level (ng/ml) in female lactating Wistar rats. The serum levels of insulin compared to the control were as follows: METCL (1.50 ± 0.13 vs 1.50 ± 0.13), VIT C (1.54 ± 0.11 ± vs 1.50 ± 0.13), SB 10% (1.66 ± 0.16 vs 1.50 ± 0.13), SB 20% (1.22 ± 3.82 vs 1.50 ± 0.13), SB 40% (1.54 ± 0.12 vs 1.50 ± 0.13) and SB 20% + VIT C (1.90 ± 0.14 vs 1.50 ± 0.13). There was no significant difference between treated and control groups in serum insulin levels ($P > 0.05$); though some treated groups showed remarably higher insulin levels when compared to control rats.

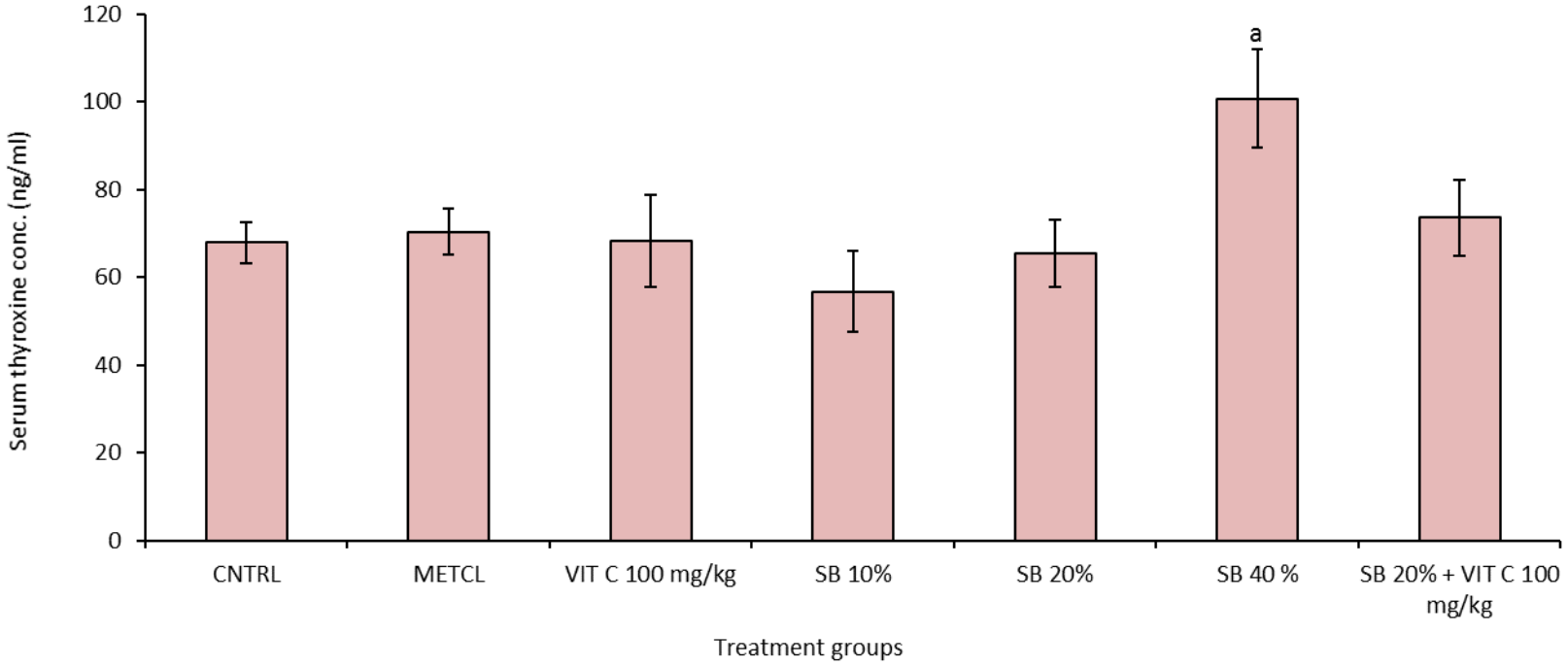


Figure 4.8: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Thyroxine (T4) hormone level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

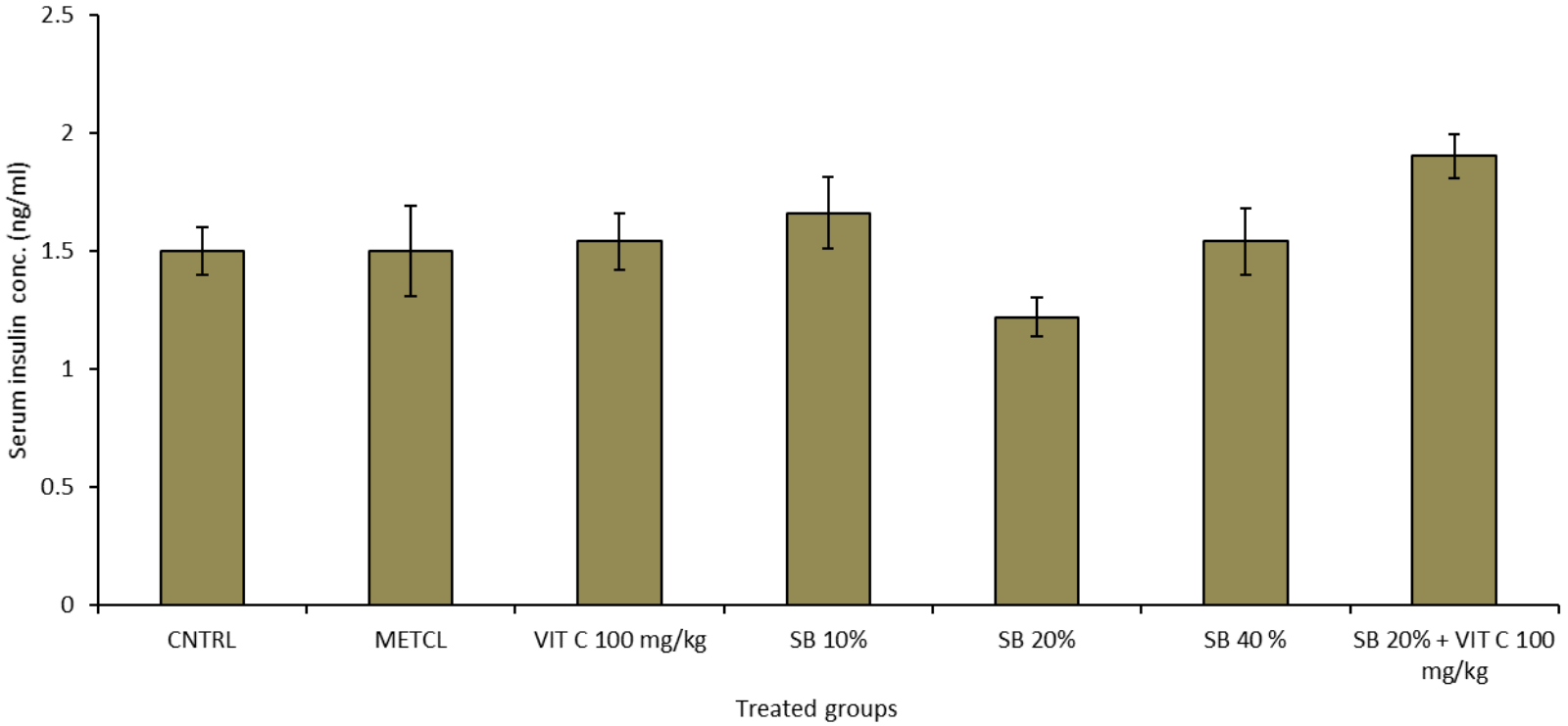


Figure 4.9: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Insulin level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.10: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Progesterone level in female lactating Wistar rats.

Figure 4.10 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean on serum progesterone level (ng/ml) in female lactating Wistar rats. The serum levels of progesterone compared to the control were as follows: METCL (26.18 ± 1.87 vs 16.66 ± 1.99), VIT C (25.78 ± 1.53 vs 16.66 ± 1.99), SB 10% (26.52 ± 2.47 vs 16.66 ± 1.99), SB 20% (28.78 ± 1.29 vs 16.66 ± 1.99), SB 40% (23.58 ± 1.48 vs 16.66 ± 1.99) and SB 20% + VIT C (25.30 ± 2.94 vs 16.66±16.66). There was a significant difference in serum progesterone concentrations between the groups treated with METCL (5 mg/kg), VIT C (100 mg/kg), SB 10% and SB 20% when compared to the control ($P< 0.05$).

4.11: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Estrogen level in female lactating Wistar rats.

Figure 4.11 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean on serum estrogen level in female lactating Wistar rats on serum Estrogen level. The serum levels of estrogen (ng/ml) compared to the control were as follows: METCL (660.00 ± 46.62 vs 632.60 ± 42.96), VIT C (669.60 ± 54.04 vs 632.60 ± 42.96), SB 10% (705.00 ± 27.53 vs 632.60 ± 42.96), SB 20% (633.00 ± 22.27 vs 632.60 ± 42.96), SB 40% (645.80 ± 45.68 vs 632.60 ± 42.96) and SB 20% + VIT C (725.40 ± 29.62 vs 632.60 ± 42.96). There was significant difference in the groups treated with VIT C (100 mg/kg), SB 10%, SB 20% and SB 20% + VIT C compared to the control ($P< 0.05$).

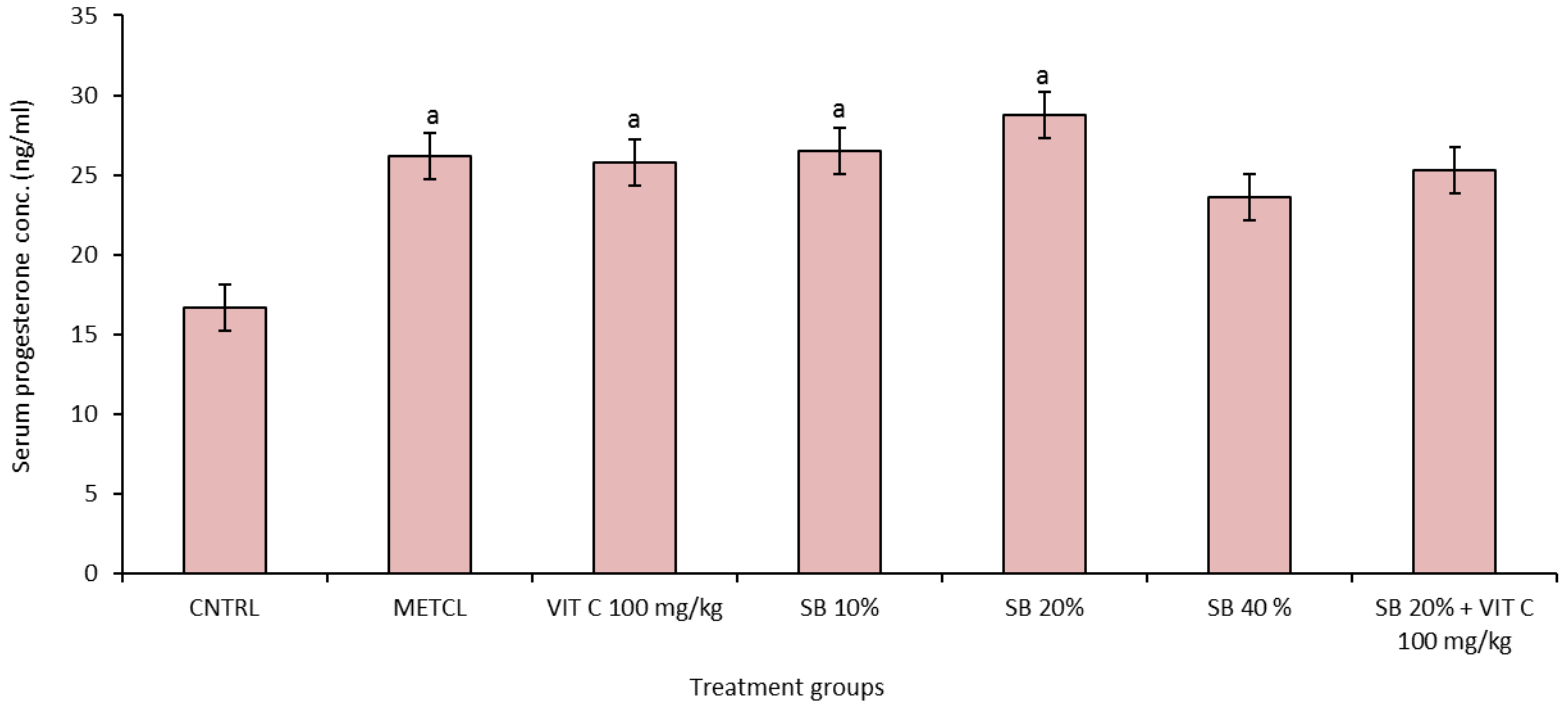


Figure 4.10: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum progesterone hormone level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

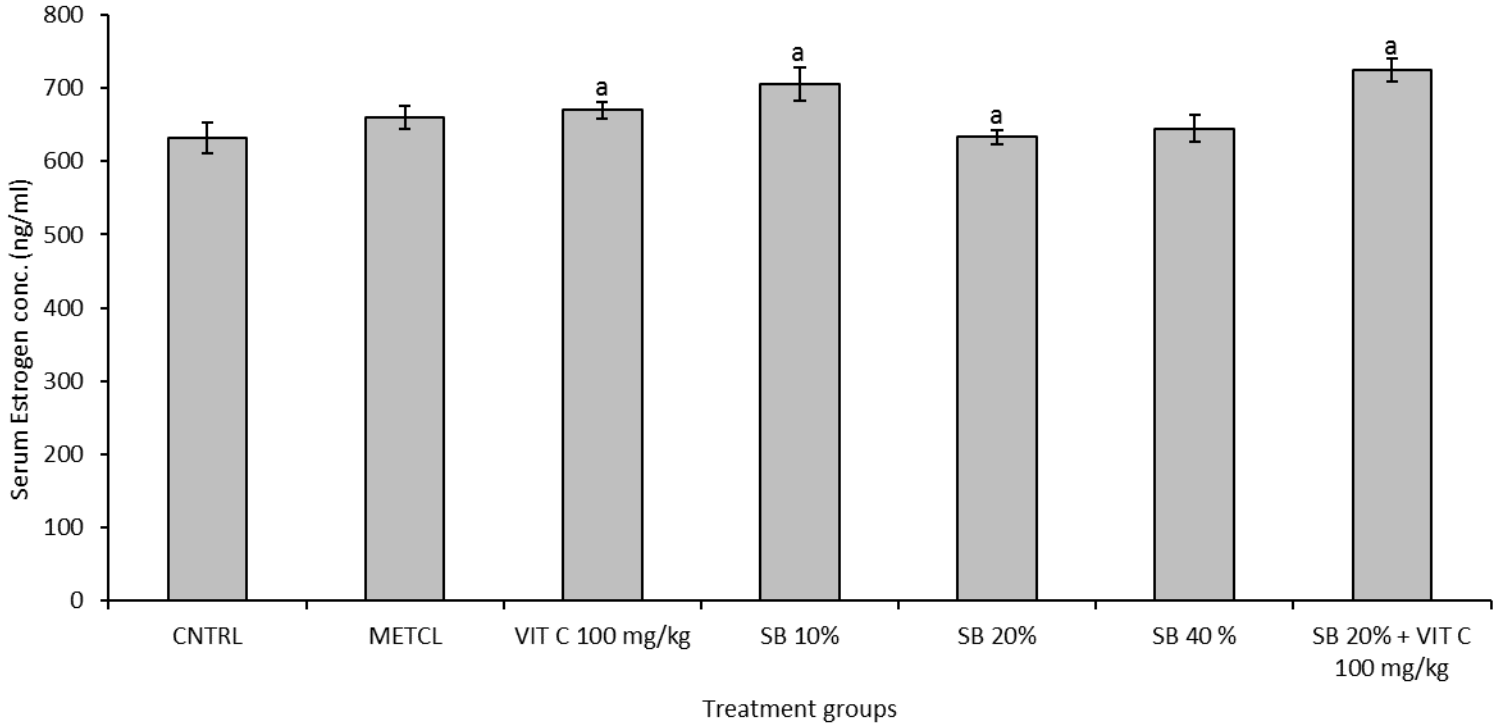


Figure 4.11: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Estrogen hormone level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.12: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Alkaline phosphatase (ALP) level in female lactating Wistar rats.

Figure 4.12 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean in female lactating Wistar rats on serum alkaline phosphatase (ALP) level (Iu/L). The serum levels of ALP compared to the control were as follows: METCL (115.60 ± 3.61 vs 86.60 ± 3.73), VIT C (99.80 ± 4.54 vs 86.60 ± 3.73), SB 10% (110.80 ± 1.63 vs 86.60 ± 3.73), SB 20% (127.60 ± 9.60 vs 86.60 ± 3.73), SB 40% (122.80 ± 2.60 vs 86.60 ± 3.37) and SB 20% + VIT C (129.40 ± 4.90 vs 86.60 ± 3.73). There was a significant difference in the groups treated with METCL (5 mg/kg), SB 10%, SB 20%, SB 40% and SB 20% + VIT C, compared to the control ($P< 0.05$).

4.13: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Alanine amino transferase (ALT) level in female lactating Wistar rats.

Figure 4.13 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean in female lactating Wistar rats on serum alanine amino transferase (ALT) level (Iu/l). The serum levels of ALT compared to the control were as follows: METCL (52.40 ± 2.40 vs 38.20 ± 1.67), VIT C (44.20 ± 1.24 vs 38.20 ± 1.67), SB 10% (44.60 ± 1.21 vs 38.20 ± 1.67), SB 20% (42.20 ± 1.66 vs 38.20 ± 1.67), SB 40% (43.40 ± 1.21 vs 38.20 ± 1.67) and SB 20% + VIT C (47.20 ± 1.74 vs 38.20 ± 1.67). There was a significant difference between groups treated with METCL (5 mg/kg) and SB 20% + VIT C, compared to the control ($P< 0.05$).

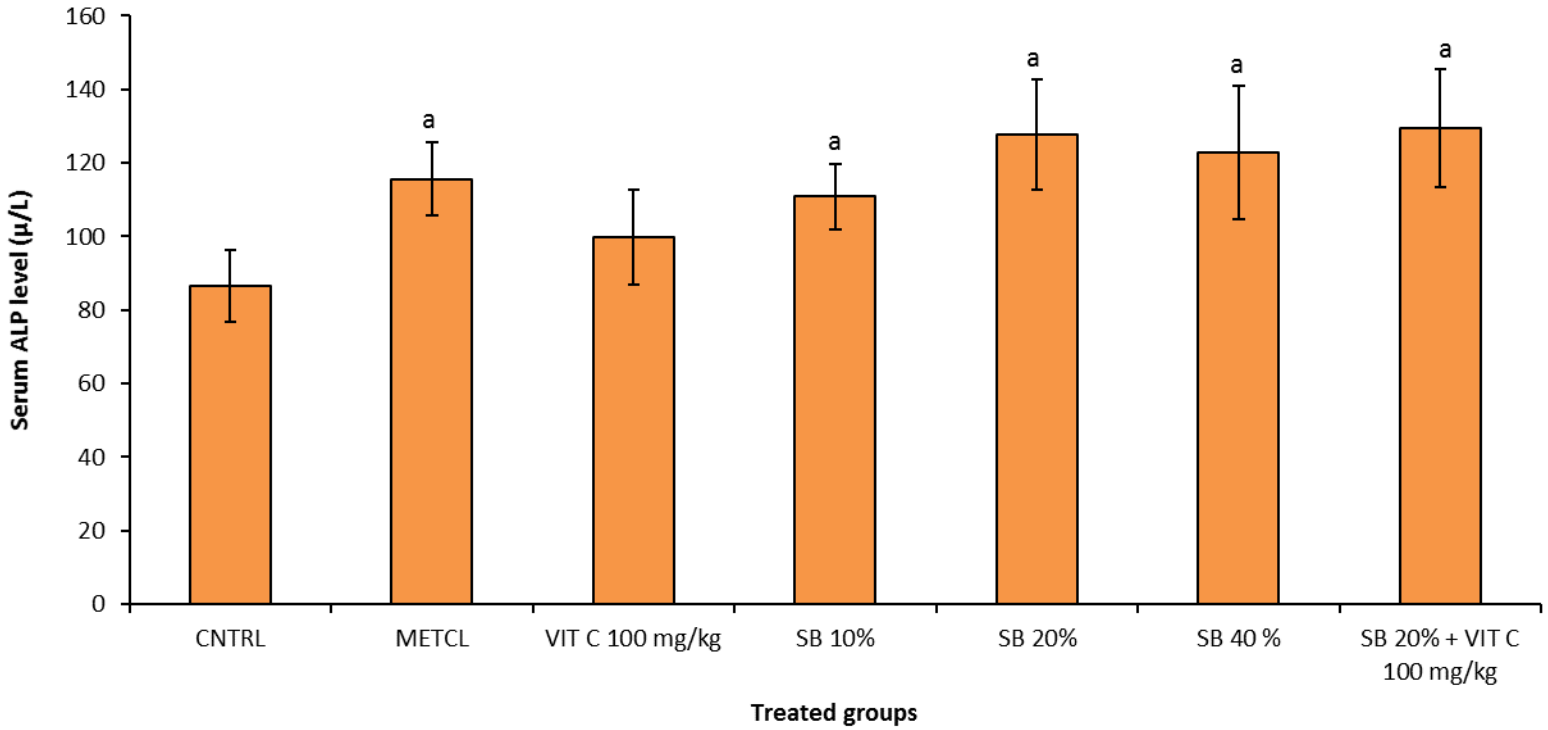


Figure 4.12: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Alkaline phosphatase (ALP) level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

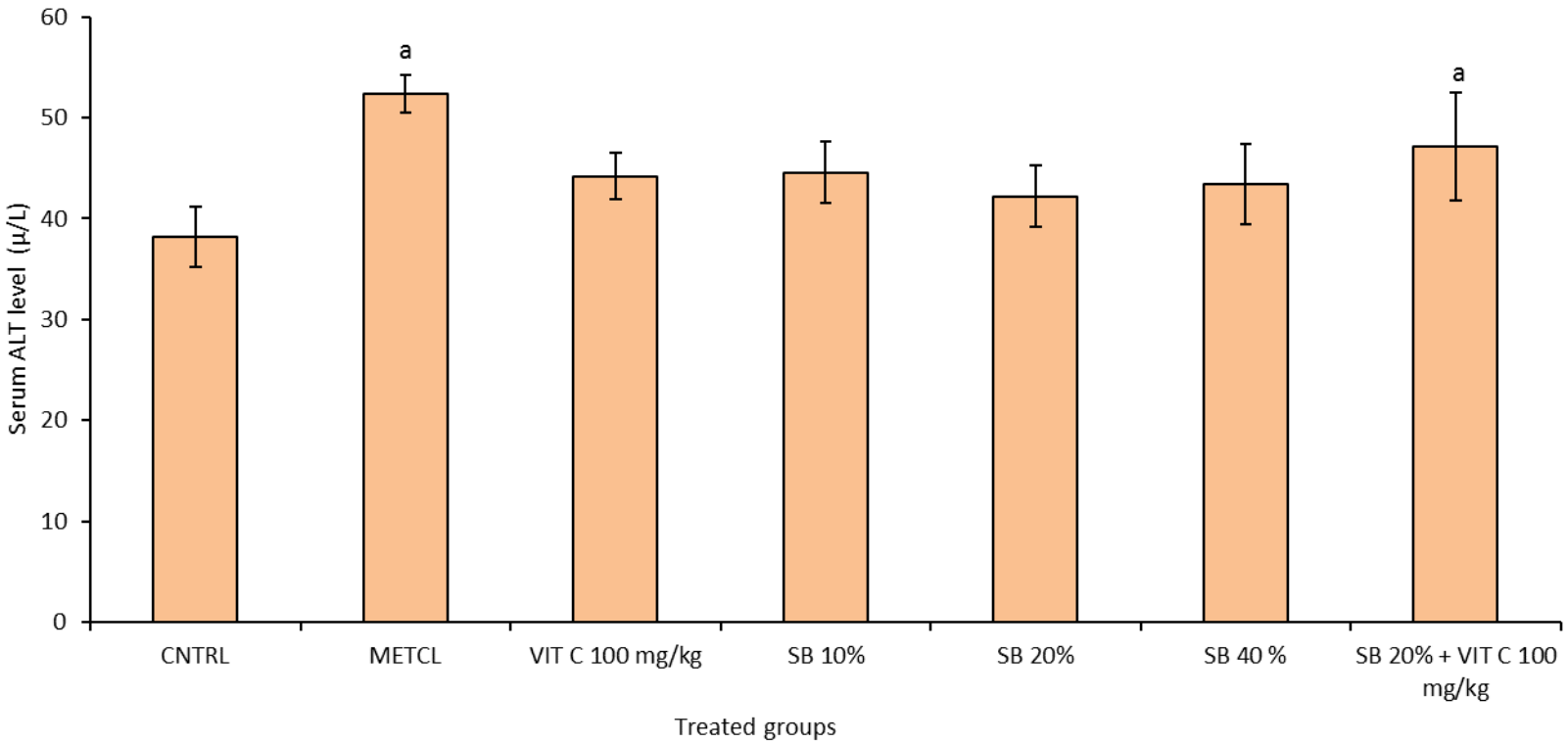


Figure 4.13: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Alanine amino transferase level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

4.14: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Aspartate amino transferase level in female lactating Wistar rats.

Figure 4.14 shows the effect of oral administration of Metoclopramide, Vitamin C and Soya bean in female lactating Wistar rats on serum aspartate amino transferase (AST) level (Iu/L) The serum levels of AST compared to the control were as follows: METCL (48.20 ± 2.08 vs 34.20 ± 1.59), VIT C (40.20 ± 1.12 vs 34.20 ± 1.59), SB 10% (42.20 ± 1.07 vs 34.20 ± 1.59), SB 20% (41.00 ± 2.00 vs 34.20 ± 1.59), SB 40% (45.00 ± 2.10 vs 34.20 ± 1.59) and SB 20% + VIT C (50.80 ± 2.33 vs 34.20 ± 1.59). There was a significant difference in the groups treated with METCL (5 mg/kg), SB 40% and SB 20% + VIT C, compared to the control ($P < 0.05$).

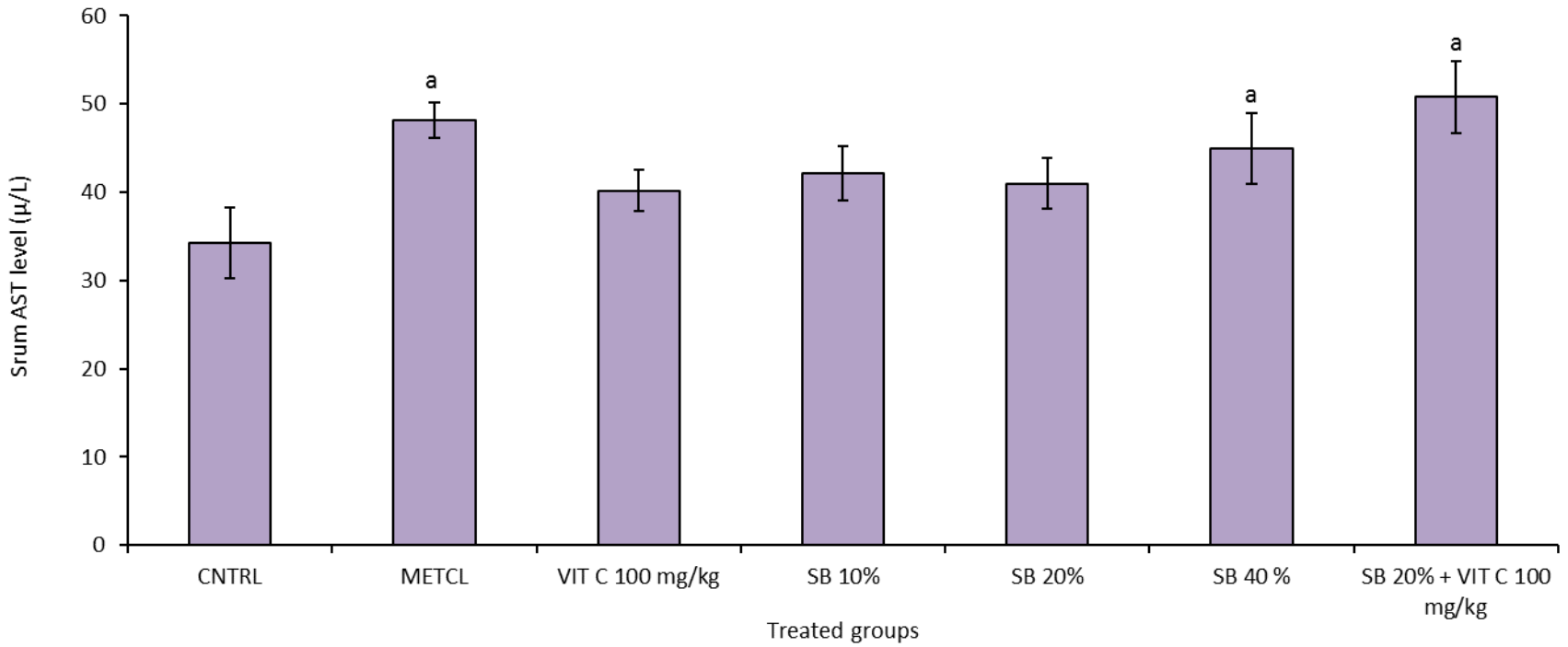


Figure 4.14: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Aspartate amino transferase level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

4.15: Effect of administration of Soya bean supplement and Vitamin C for 10 days on biomarker of Lipid peroxidation;

Malondialdehyde (MDA) level in female lactating Wistar rats

In figure 4.15, the levels of Serum MDA (umol/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C were as follows: METCL (1.78 ± 0.15 vs 1.32 ± 0.11), VIT C (1.52 ± 0.09 vs 1.32 ± 0.11), SB 10% (1.42 ± 0.11 vs 1.32 ± 0.11), SB 20% (1.60 ± 0.07 vs 1.32 ± 0.11), SB 40% (1.40 ± 0.05 vs 1.32 ± 0.11) and SB 20% + VIT C (1.40 ± 0.07 ± 1.32 ± 0.11). There was a significant difference in the group treated with METCL (5 mg/kg) compared to the control ($P< 0.05$).

4.16: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Superoxide dismutase (SOD) level in female lactating Wistar rats.

In figure 4.16, the levels of Serum SOD (Iu/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C were as follows: METCL (1.88 ± 0.07 vs 2.24 ± 0.09), VIT C (2.08 ± 0.10 vs 2.24 ± 0.09), SB 10% (1.74 ± 0.10 vs 2.24 ± 0.09), SB 20% (1.84 ± 0.05 vs 2.24 ± 0.09), SB 40% (2.30 ± 0.13 vs 2.24 ± 0.09) and SB 20% + VIT C (1.96 ± 0.12 vs 2.24 ± 0.09). There was a significant difference in the group treated with SB 10% compared to the control ($P< 0.05$).

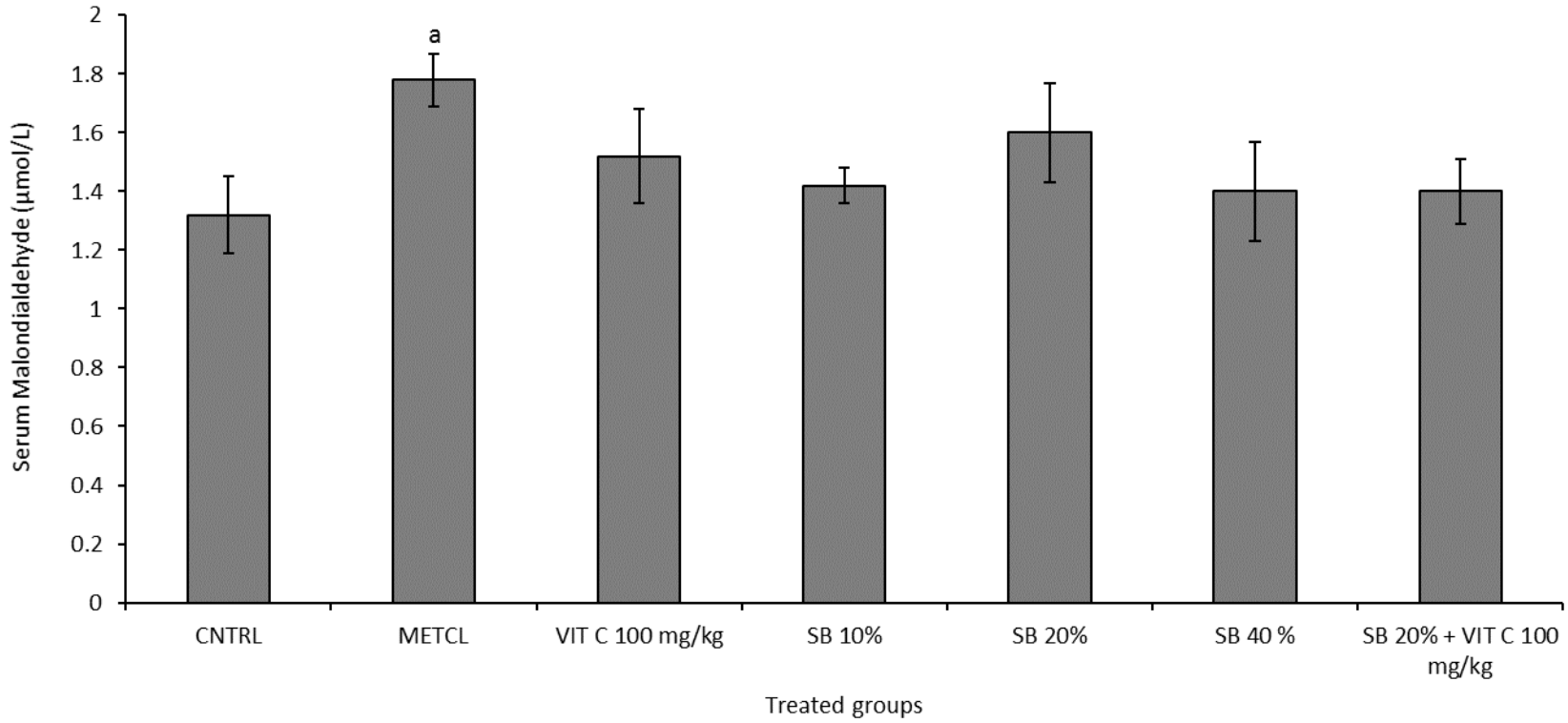


Figure 4.15: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Malondialdehyde level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

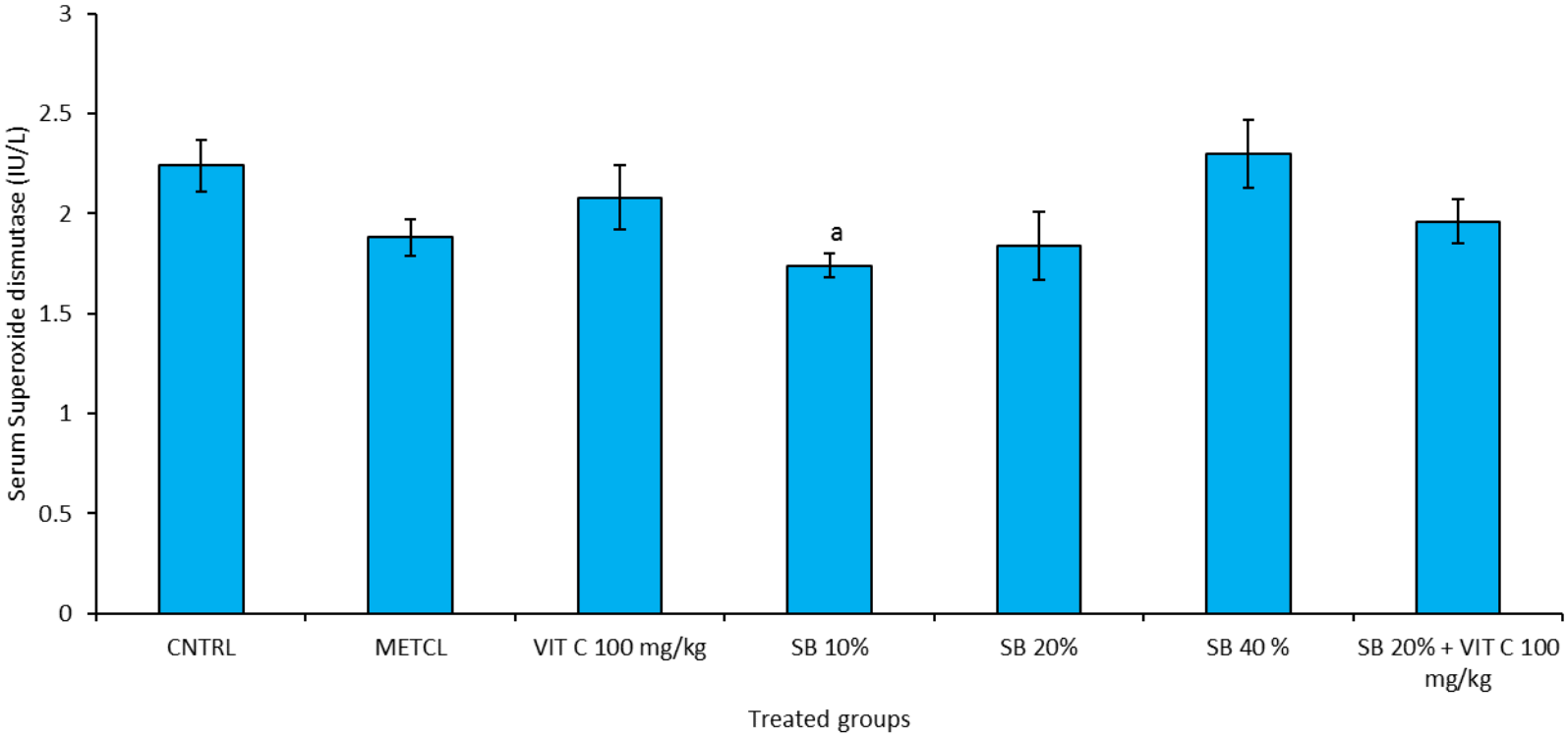


Figure 4.16: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Superoxide dismutase level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

4.17: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Catalase level in female lactating Wistar rats.

In figure 4.16, the levels of Serum catalase (CAT) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C were as follows: METCL (46.20 ± 1.53 vs 52.00 ± 0.71), VIT C (46.80 ± 1.59 vs 52.00 ± 0.71), SB 10% (44.00 ± 1.14 vs 52.00 ± 0.71), SB 20% (45.20 ± 1.28 vs 52.00 ± 0.71), SB 40% (50.40 ± 1.17 vs 52.00 ± 0.71) and SB 20% + VIT C (48.20 ± 1.02 vs 52.00 ± 0.71). There was a significant difference between the groups treated with METCL (5 mg/kg), SB 10% and SB 20% compared to the control ($P< 0.05$).

4.18: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Glutathione peroxidase level in female lactating Wistar rats.

In figure 4.16, the levels of Serum glutathione peroxidase (GPx; Iu/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C were as follows: METCL (44.20 ± 1.56 vs 48.40 ± 0.93), VIT C (42.60 ± 0.93 vs 48.40 ± 0.93), SB 10% (41.60 ± 1.50 vs 48.40 ± 0.93), SB 20% (42.60 ± 1.50 vs 48.40 ± 0.93), SB 40% (48.00 ± 0.94 vs 48.40 ± 0.93) and SB 20% + VIT C (45.80 ± 0.86 vs 48.40 ± 0.93). There was a significant difference between the groups treated with VIT C, SB 10%and SB 20% compared to the control ($P< 0.05$).

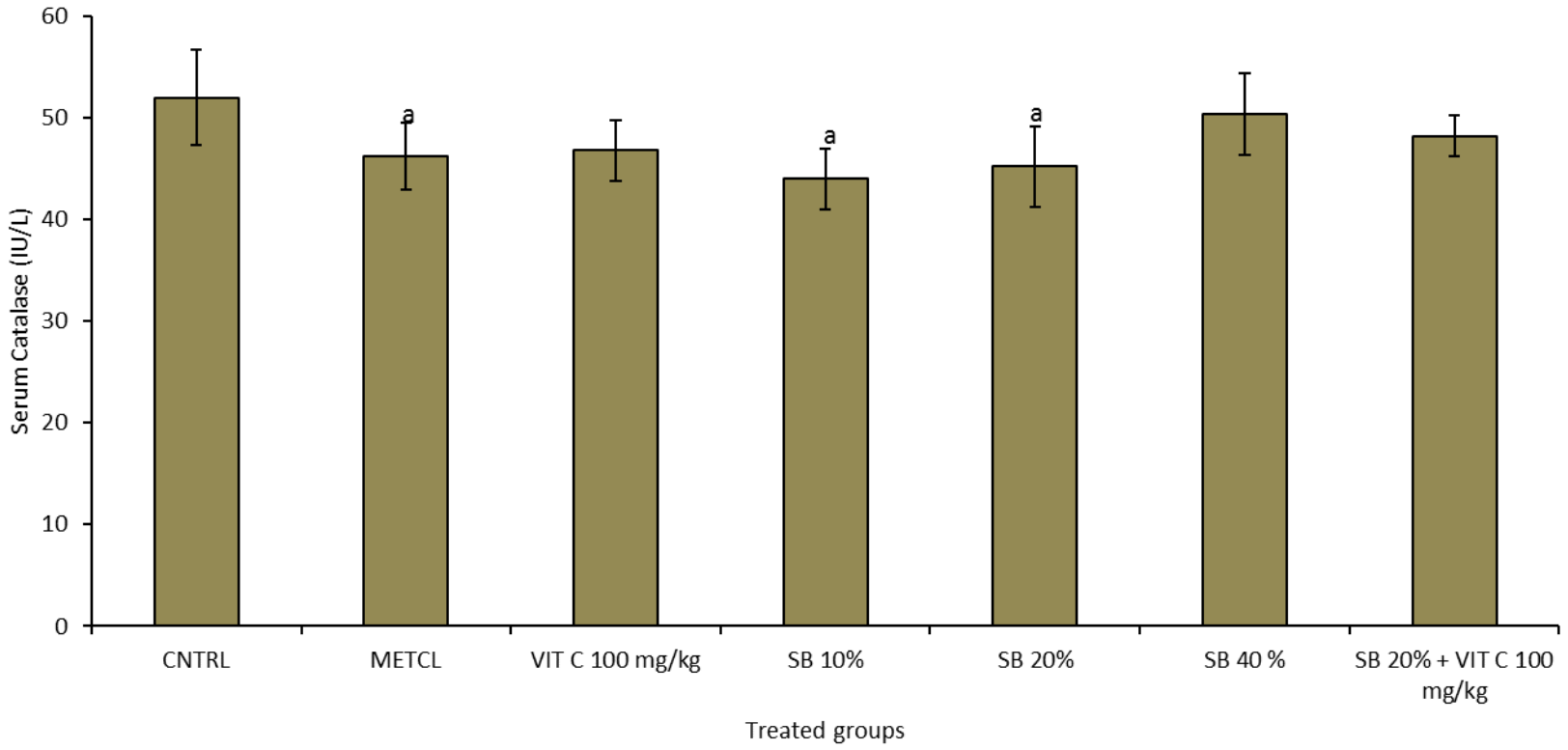


Figure 4.17: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Catalase level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

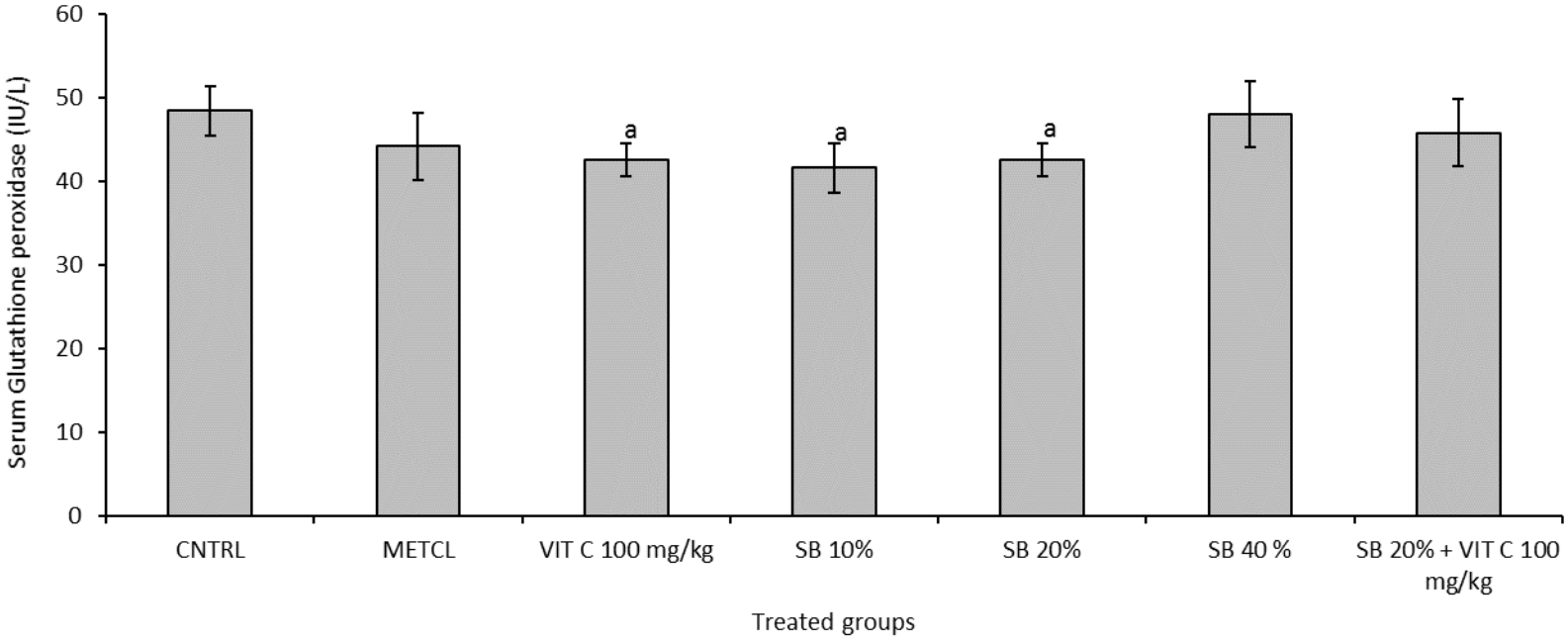


Figure 4.18: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Glutathione peroxidase level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg), superscript letter (a) represents significance when compared to control ($P < 0.05$).

4.19: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Calcium level in female lactating Wistar rats.

In figure 4.19; the levels of serum calcium (meq/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C were as follows: METCL (2.28 ± 0.43 vs 2.26 ± 0.40), VIT C (2.38 ± 0.42 vs 2.26 ± 0.40), SB 10% (2.28 v 0.04 vs 2.26 ± 0.40), SB 20% (2.28 ± 0.04 vs 2.26 ± 0.40), SB 40% (2.28 ± 0.08 vs 2.26 ± 0.40) and SB 20% + VIT C (2.33 ± 0.07 vs 2.26 ± 0.40). There was no significant difference in serum calcium concentration between treated and control groups (P> 0.05).

4.20: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Sodium level in female lactating Wistar rats.

In figure 4.20; the levels of Serum sodium (meq/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C were as follows: METCL (141.00 ± 1.00 vs 139.80 ± 1.39), VIT C (142.00 ± 1.14 vs 139.80 ± 1.39), SB 10% (142.00 ± 1.70 vs 139.80 ± 1.39), SB 20% (140.00 ± 0.71 vs 139.80 ± 1.39), SB 40% (138.6 ± 0.81 vs 139.80 ± 1.39) and SB 20% + VIT C (139.00 ± 1.09 vs 139.80 ± 1.39). There was no statistical significant difference in serum sodium concentration between treated groups when compared to the control (P> 0.05).

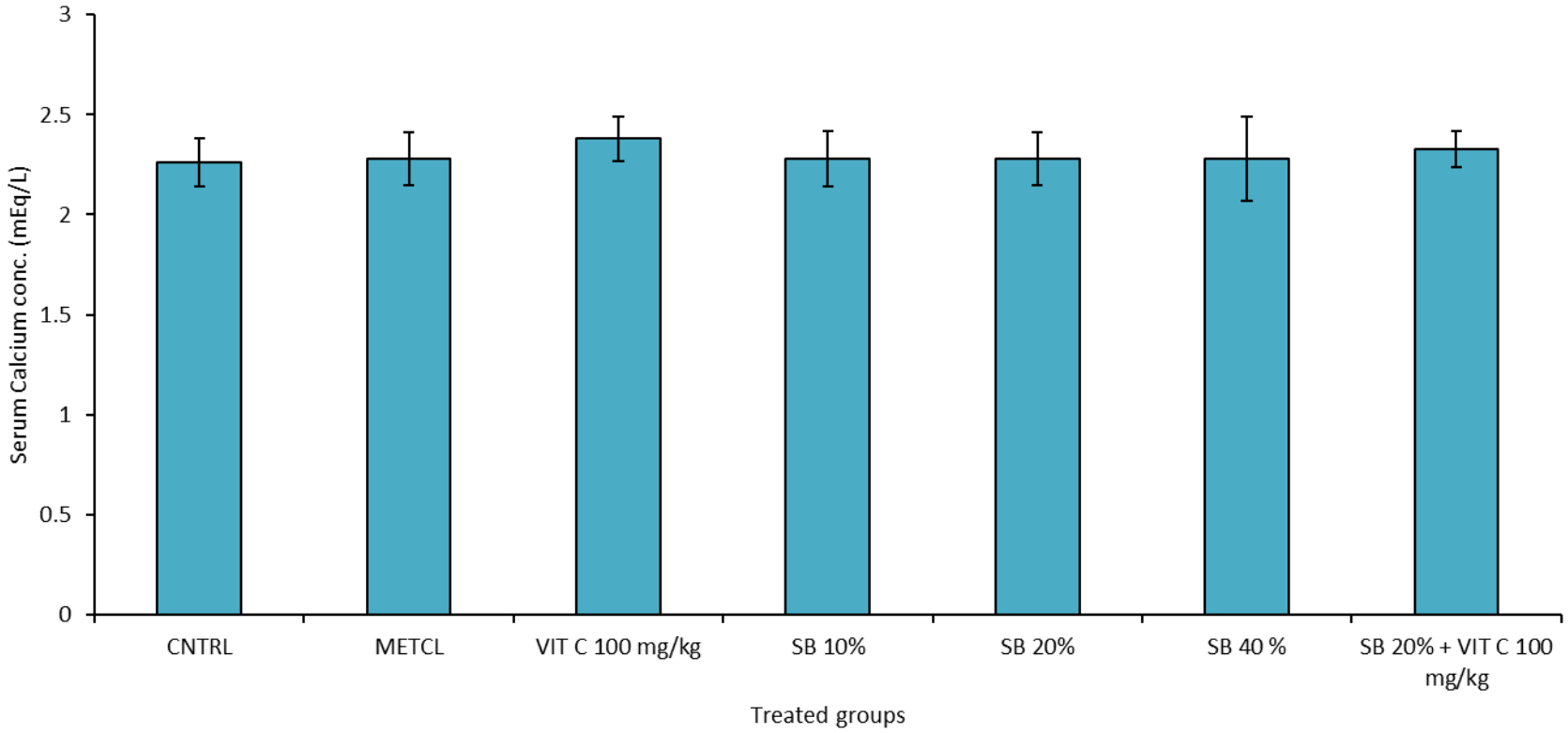


Figure 4.19: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Calcium level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

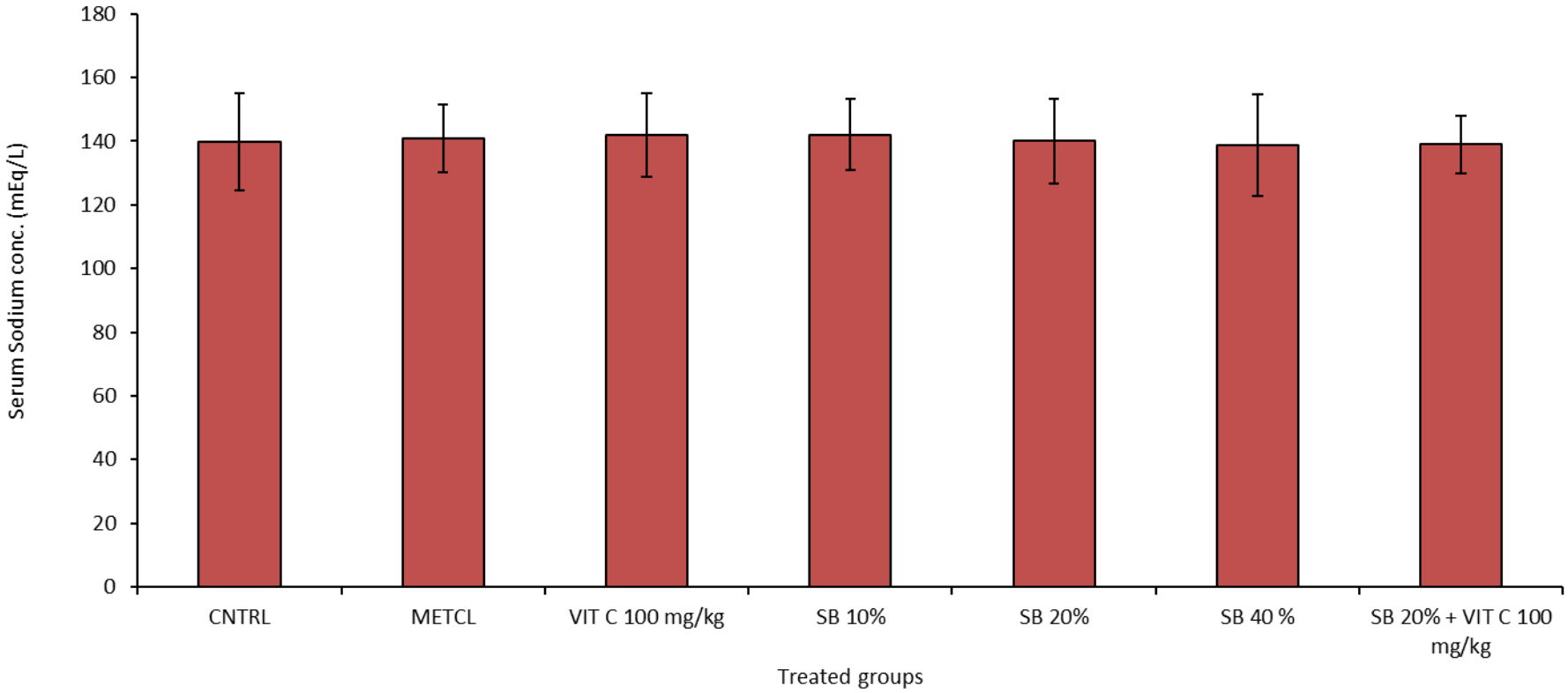


Figure 4.20: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Sodium level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.21: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Potassium level in female lactating Wistar rats.

Figure 4.21 shows the levels of serum potassium (meq/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C was follows: METCL (4.88 ± 0.23 vs 4.20 ± 0.15), VIT C (4.24 ± 0.18 vs 4.20 ± 0.15), SB 10% (4.28 ± 0.19 vs 4.20 ± 0.15), SB 20% (4.24 ± 0.14 vs 4.20 ± 0.15), SB 40% (4.40 ± 0.27 vs 4.20 ± 0.15) and SB 20% + VIT C (4.26 ± 0.15 vs 4.20 ± 0.15). There was no statistical significant difference in serum potassium concentration between the treated groups compared to the control (P> 0.05).

4.22: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Magnesium level in female lactating Wistar rats.

Figure 4.22 shows the levels of Serum magnesium in female lactating Wistar rats treated with Soya bean supplement and Vitamin C as follows: METCL (1.10 ± 0.07 vs 1.02 ± 0.12), VIT C (0.90 ± 0.13 vs 1.02 ± 0.12), SB 10% (1.06 ± 0.10 vs 1.20 ± 0.12), SB 20% (1.12 ± 0.08 vs 1.02 ± 0.12), SB 40% (1.06 ± 0.07 vs 1.02 ± 0.12) and SB 20% + VIT C (0.98 ± 0.37 vs 1.02 ± 0.12). There was no significant difference in serum magnesium concentration between the treated groups compared to the control (P> 0.05).

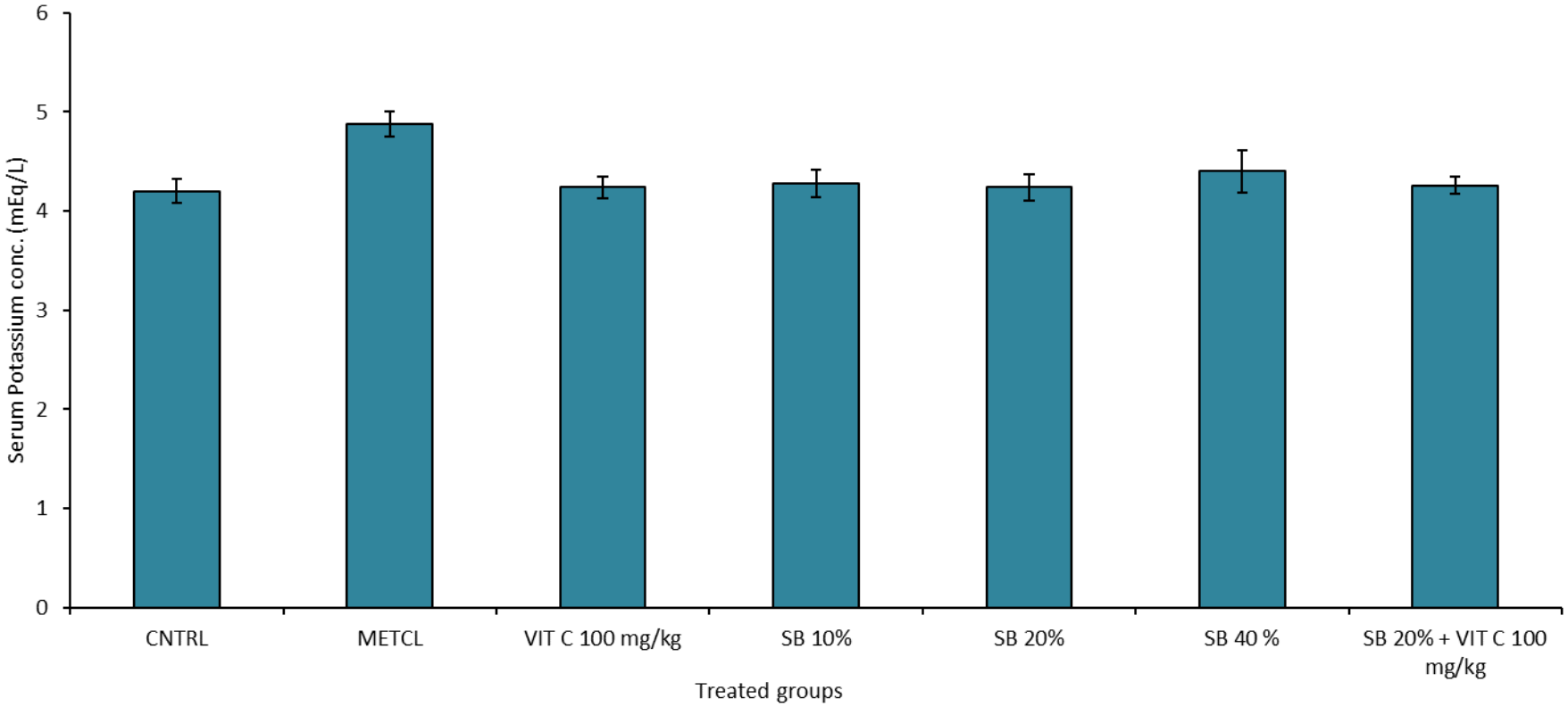


Figure 4.21: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Potassium level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

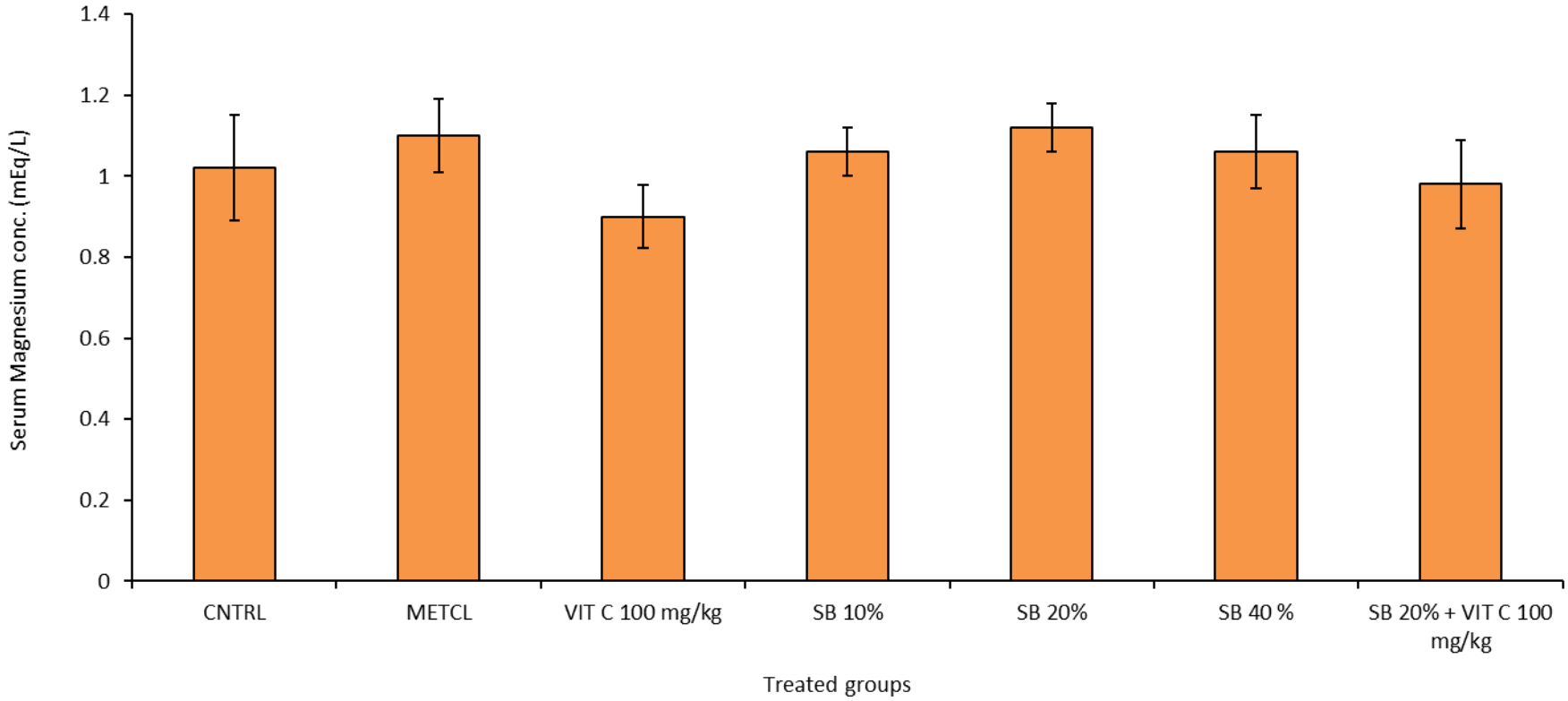


Figure 4.22: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Magnesium level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.23: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Chloride level in female lactating Wistar rats.

Figure 4.23 shows the levels of Serum chloride (meq/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C as follows: METCL (102.80 ± 0.86 vs 101.80 ± 0.86), VIT C (103.00 ± 1.30 vs 101.80 ± 0.86), SB 10% (103.60 ± 1.36 vs 101.80 ± 1.39), SB 20% (102.00 ± 0.71 vs 101.80 ± 1.39), SB 40% (100.60 ± 1.33 vs 101.80 ± 1.39) and SB 20% + VIT C (99.40 ± 0.93 vs 101.80 ± 1.39). There was no significant difference in serum chloride concentration between the treated and control groups ($P > 0.05$).

4.24: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Bicarbonate level in female lactating Wistar rats.

Figure 4.24 shows the levels of Serum bicarbonate (meq/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C as follows: METCL (21.60 ± 1.17 vs 23.00 ± 1.00), VIT C (24.20 ± 0.66 vs 23.00 ± 1.00), SB 10% (23.20 ± 1.02 vs 23.00 ± 1.00), SB 20% (23.60 ± 0.81 vs 23.00 ± 1.00), SB 40% (23.00 ± 1.00 vs 23.00 ± 1.00) and SB 20% + VIT C (22.00 ± 0.95 vs 23.00 v 1.00). There was no statistical significant difference in serum chloride concentration between the treated and control groups ($P > 0.05$).

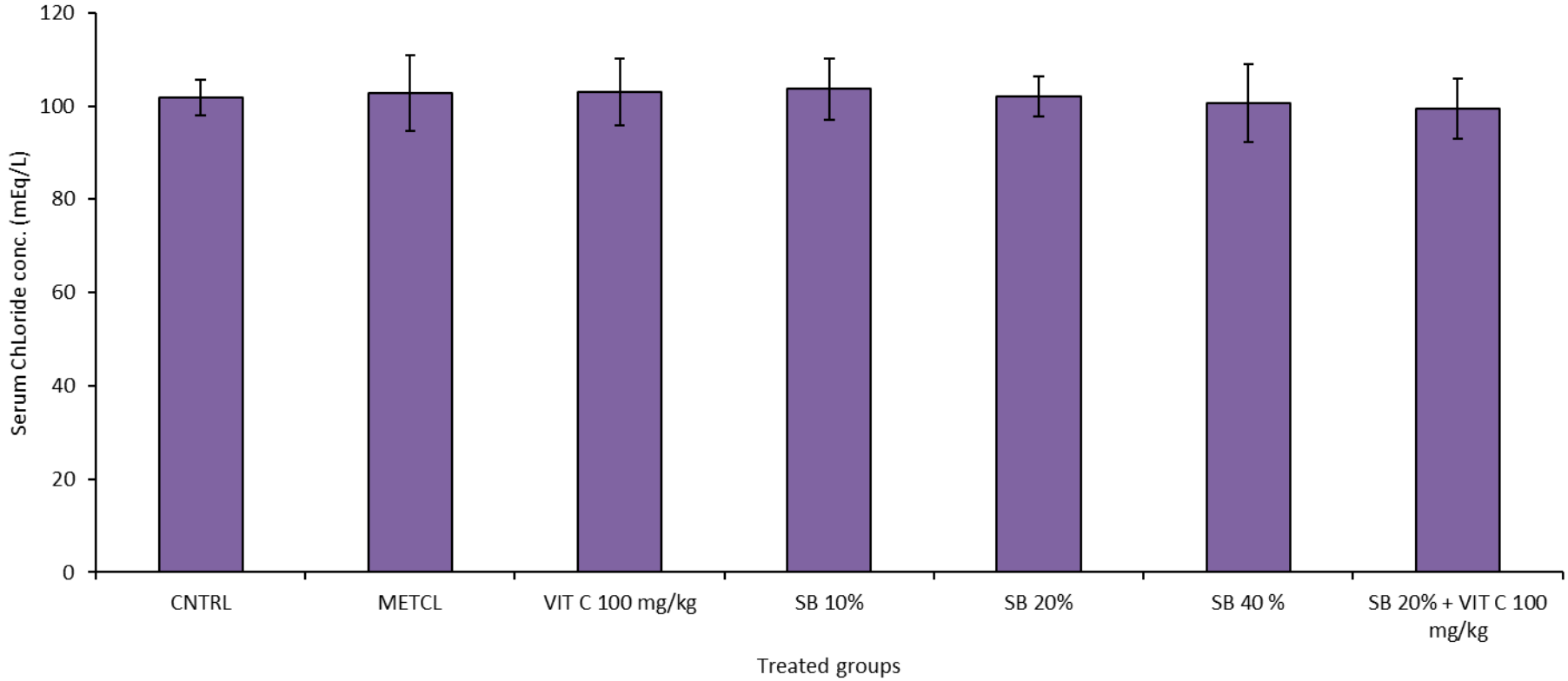


Figure 4.23: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Chloride level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

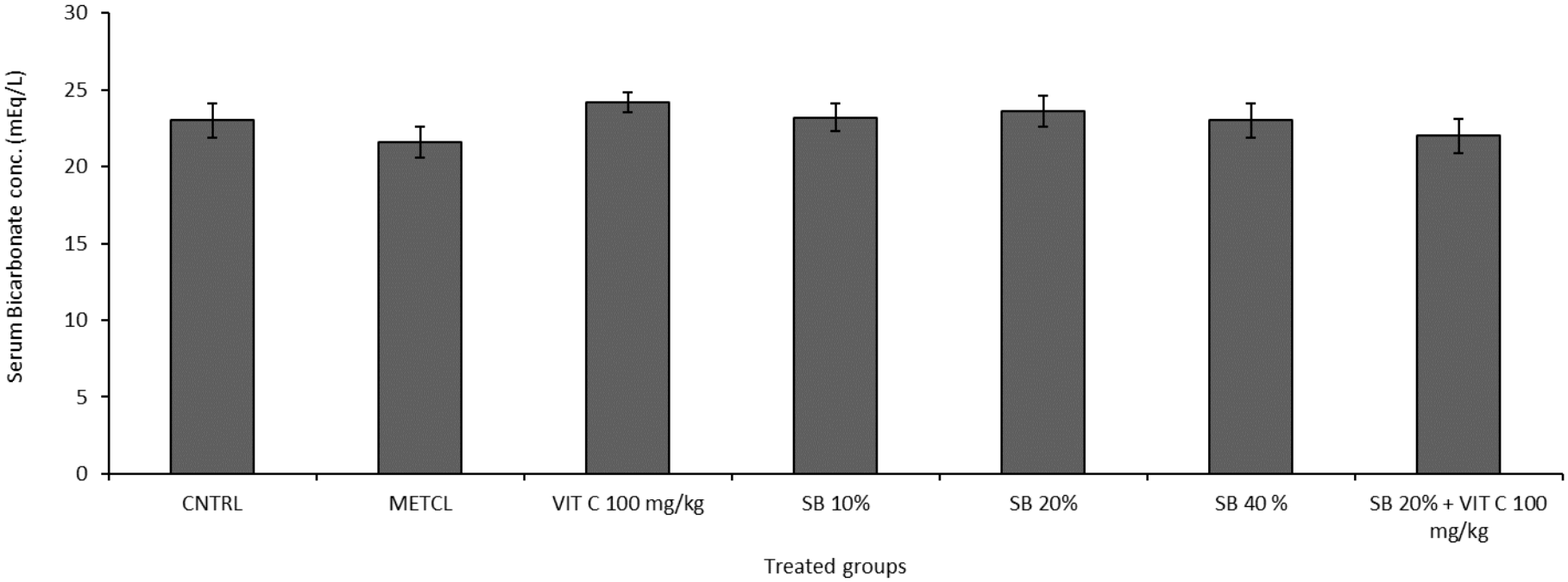


Figure 4.24: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Bicarbonate level in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.25: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Urea level in female lactating Wistar rats

Figure 4.25 shows the levels of serum urea (meq/L) in female lactating Wistar rats treated with Soya bean supplement and Vitamin C as follows: METCL (4.62 ± 0.29 vs 3.60 ± 0.27), VIT C (3.90 ± 0.25 vs 3.60 ± 0.27), SB 10% (4.00 ± 0.25 vs 3.60 ± 0.27), SB 20% (4.08 ± 0.16 vs 3.60 ± 0.27), SB 40% (3.28 ± 0.34 vs 3.60 ± 0.27) and SB 20% + VIT C (3.90 ± 0.28 vs 3.60 ± 0.27). There was no statistical significant difference in serum urea concentration between treated control groups ($P > 0.05$).

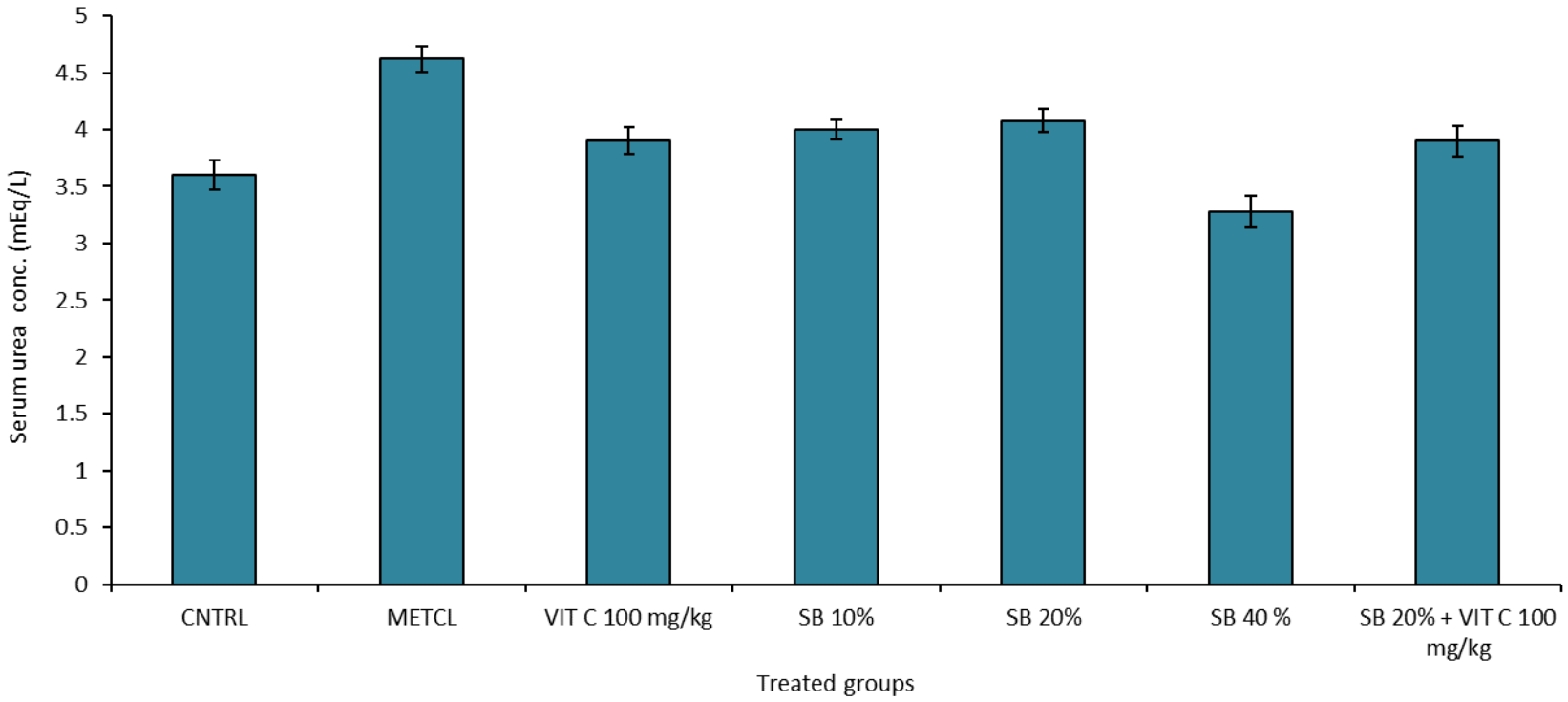


Figure 4.25: Effect administration of Soya bean supplement and Vitamin C for 10 days on Serum urea concentration in female lactating Wistar rats

CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 20% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (20%) and Vitamin C (100 mg/kg).

4.26: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Red blood cell counts, White blood cell count, Packed cell volume, Hemoglobin concentration and Platelet count in female lactating Wistar rats.

Table 4.26 shows RBC count in treated and control rats as follows; METCL (6.69 ± 0.16 vs 7.23 ± 0.11), VIT C (6.58 ± 0.11 vs 7.23 ± 0.11), SB 10% (6.02 ± 0.30 vs 7.23 ± 0.11), SB 20% (5.57 v 0.61 vs 7.23 v 0.11), SB 40% (4.24 ± 0.65 vs 7.23 ± 0.11) and SB 20% + VIT C (5.42 ± 0.43 vs 7.23 ± 0.11). There was a significant decrease in red blood cell count between the groups treated with SB 40% and SB 20% + VIT C, compared to the control ($P< 0.05$). The WBC count were as follows; METCL (8.42 ± 0.45 vs 8.24 ± 1.81), VIT C (6.38 ± 0.89 vs 8.24 1.81), SB 10% (9.74 ± 0.69 vs 8.24 ± 1.81), SB 20% (12.20 ± 1.73 vs 8.24 ± 1.81), SB 40% (13.62 ± 4.45 vs 8.24 ± 1.81) and SB 20% + VIT C (11.88 ± 1.29 vs 8.24 ± 1.81). There was a significant increase in white blood cell count between the groups treated with SB 40% and SB 20% + VIT C, compared to the control ($P< 0.05$). The values of PCV were as follows; METCL (34.38 ± 1.88 vs 34.86 ± 0.49), VIT C (32.20 ± 1.04 vs 34.86 ± 0.49), SB 10% (28.76 ± 1.44 vs 34.86 ± 0.49), SB 20% (31.74 ± 2.82 vs 34.86 ± 0.49), SB 40% (31.64 ± 3.23 vs 34.86 ± 0.49) and SB 20% + VIT C (29.78 ± 1.14 vs 34.86 ± 0.49). There was no statistically significant difference in packed cell volume between the treated groups compared to the control ($P> 0.05$). The values of Hb concentration were as follows; METCL (12.72 ± 0.31 vs 13.18 ± 0.40), VIT C (12.54 ± 0.26 vs 13.18 ± 0.40), SB 10% (11.06 ± 0.48 vs 13.18 ± 0.40), SB 20% (11.64 ± 0.12 vs 13.18 ± 0.40), SB 40% (12.16 ± 1.16 vs 13.18 ± 0.40) and SB 20% + VIT C (11.88 ± 0.33 vs 13.18 ± 0.40). There was no statistically significant difference in Hb concentration between the treated groups compared to the control ($P> 0.05$). The Platelet counts were as follows; METCL (658.00 ± 55.52 vs 681.60 ± 44.81), VIT C (623.20 ± 86.87 vs 681.60 ± 44.81), SB 10% (327.80 ± 81.76 vs 681.60 ± 44.81), SB

20% (356.00 ± 81.76 vs 681.60 ± 44.81), SB 40% (338.00 ± 70.31 vs 681.60 ± 44.81) and SB 20% + VIT C (344.00 ± 42.26 vs 681.60 ± 44.81). There was a statistically significant decrease in platelet count in all the soybean supplemented treated groups compared to the control ($P < 0.05$).

Table 4: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Red blood cell counts, White blood cell count, Packed cell volume, Hemoglobin concentration and Platelet count in female lactating Wistar rats.

Groups	Red Blood Cell (10^{12}/L)	White Blood Cell (10^{9}/L)	Packed Cell Volume (%)	Hemoglobin conc. g/dl	Platelet Count (10^{9}/L)
CON.	7.23 ± 0.11	8.24 ± 1.81	34.86 ± 0.49	13.18 ± 0.40	681.60 ± 44.81
METC	6.69 ± 0.16	8.42 ± 0.45	34.38 ± 1.88	12.72 ± 0.31	658.00 ± 55.52
VIT C	6.58 ± 0.11	6.38 ± 0.89	32.20 ± 1.04	12.54 ± 0.26	623.20 ± 86.87
SB10	6.02 ± 0.30	9.74 ± 0.69	28.76 ± 1.44	11.06 ± 0.48	327.80 ± 81.76*
SB20	5.57 ± 0.61	12.20 ± 1.73	31.74 ± 2.82	11.64 ± 0.12	356.00 ± 81.76*
SB40	4.24 ± 0.65*	13.62 ± 4.45*	31.64 ± 3.23	12.16 ± 1.16	338.00 ± 70.31*
20V	5.42 ± 0.43*	11.88 ± 1.29	29.78 ± 1.14	11.88 ± 0.33	344.00 ± 42.26*

* -Statistically Significant at ($p < 0.05$) when compared with Control

Group 1: Control Rats; **Group 2:** Metoclopramide treated Rats (5 mg/kg); **Group 3:** Vitamin C treated Rats (100 mg/kg); **Group 4a:** Rats Treated with 10% of Soya bean Supplement; **Group 4b:** Rats Treated with 20% of Soya bean Supplement; **Group 4c:** Rats Treated with 40% of Soya bean Supplement; **Group 5:** Rats Treated with 20% of Soya bean Supplement + Vitamin C (100 mg/kg).

* -Statistically Significant at ($p < 0.05$) when compared with Control

4.27: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Histology of the mammary gland.

Photomicrograph sections of the mammary gland following 10 days of oral administration were presented as follows; CNTRL = Rats treated with normal saline (1 ml/kg), METCL = Rats treated with Metoclopramide (5 mg/kg), VIT C 100 mg/kg = Rats treated with Vitamin C (100 mg/kg), SB 10% = Rats treated with Soya bean supplement (10%), SB 20% = Rats treated with Soya bean supplement (20%), SB 40% = Rats treated with Soya bean supplement (40%), SB 40% + VIT C 100 mg/kg = Rats treated with co-administration of Soya bean Supplement (40%) and Vitamin C (100 mg/kg).

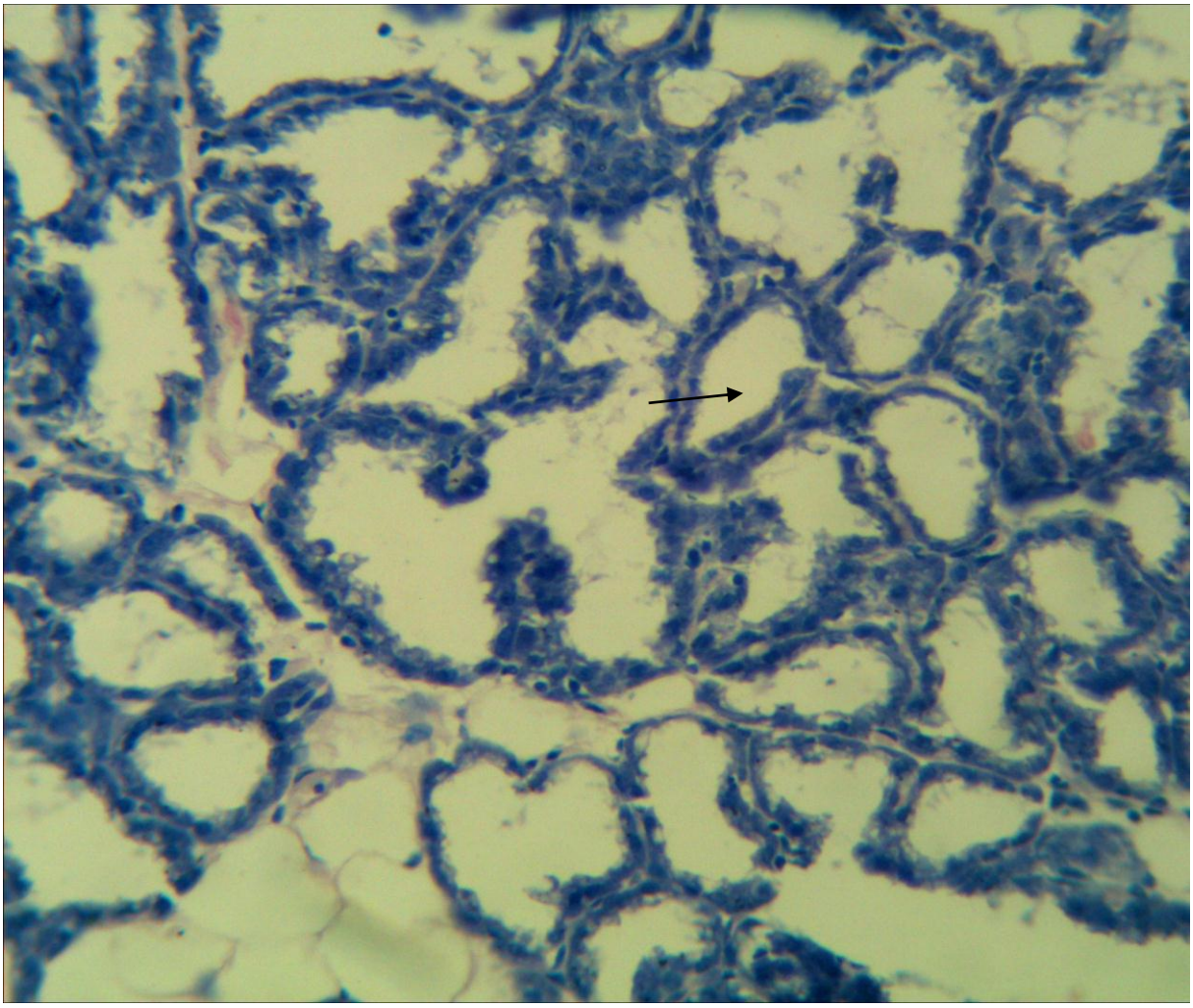


Plate III: (Control) Photomicrograph section of mammary gland tissue showing the normal histological appearance of alveoli with milky secretions in the lumen. (H & E stained x250).

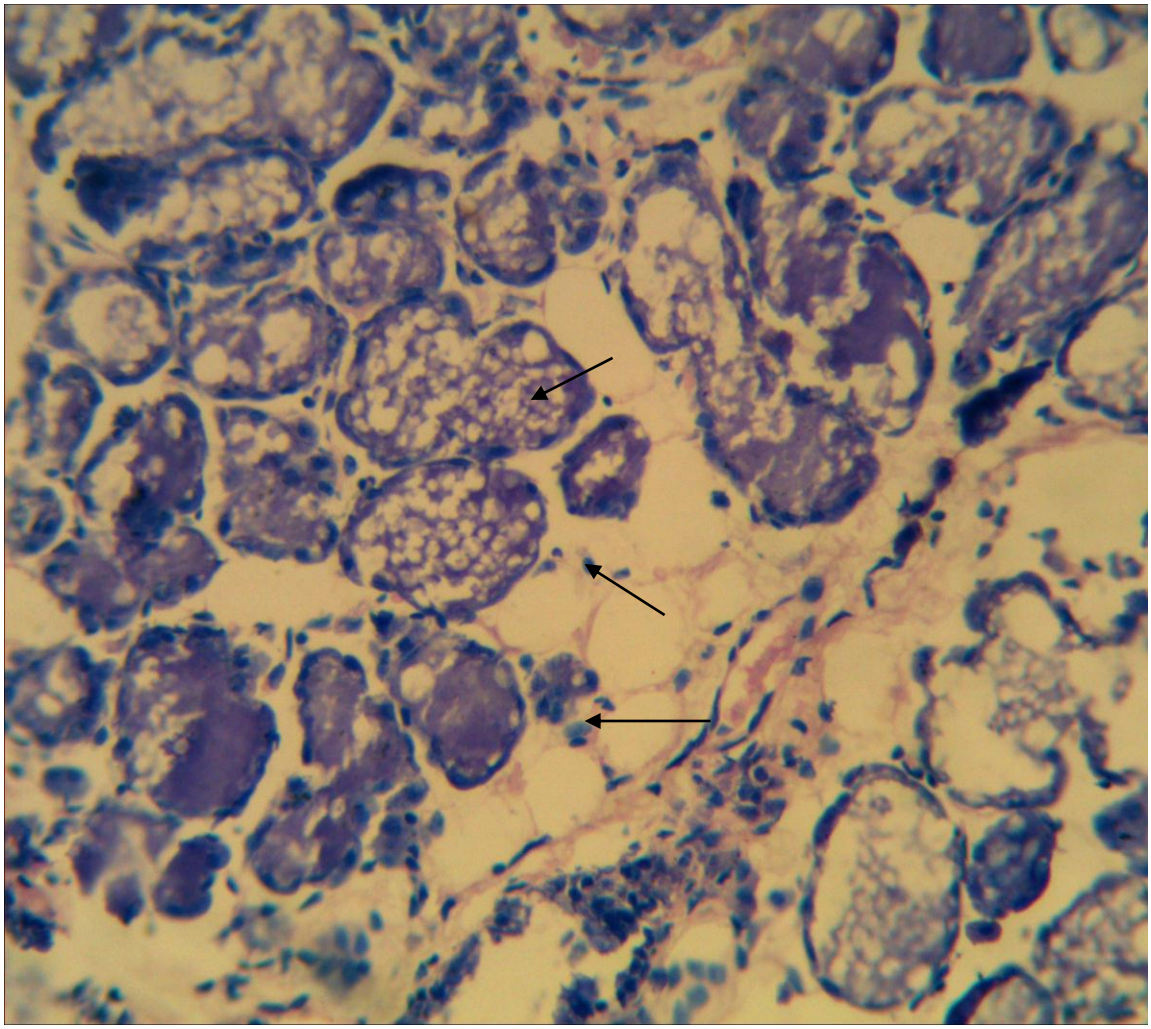


Plate IV: (Metoclopramide) Photomicrograph section of tissue showing an active mammary gland with milky secretions in the lumen of the secretory alveoli (black arrows). Pockets of lymphocytes were also seen (black arrow heads). (H & E stained x250).

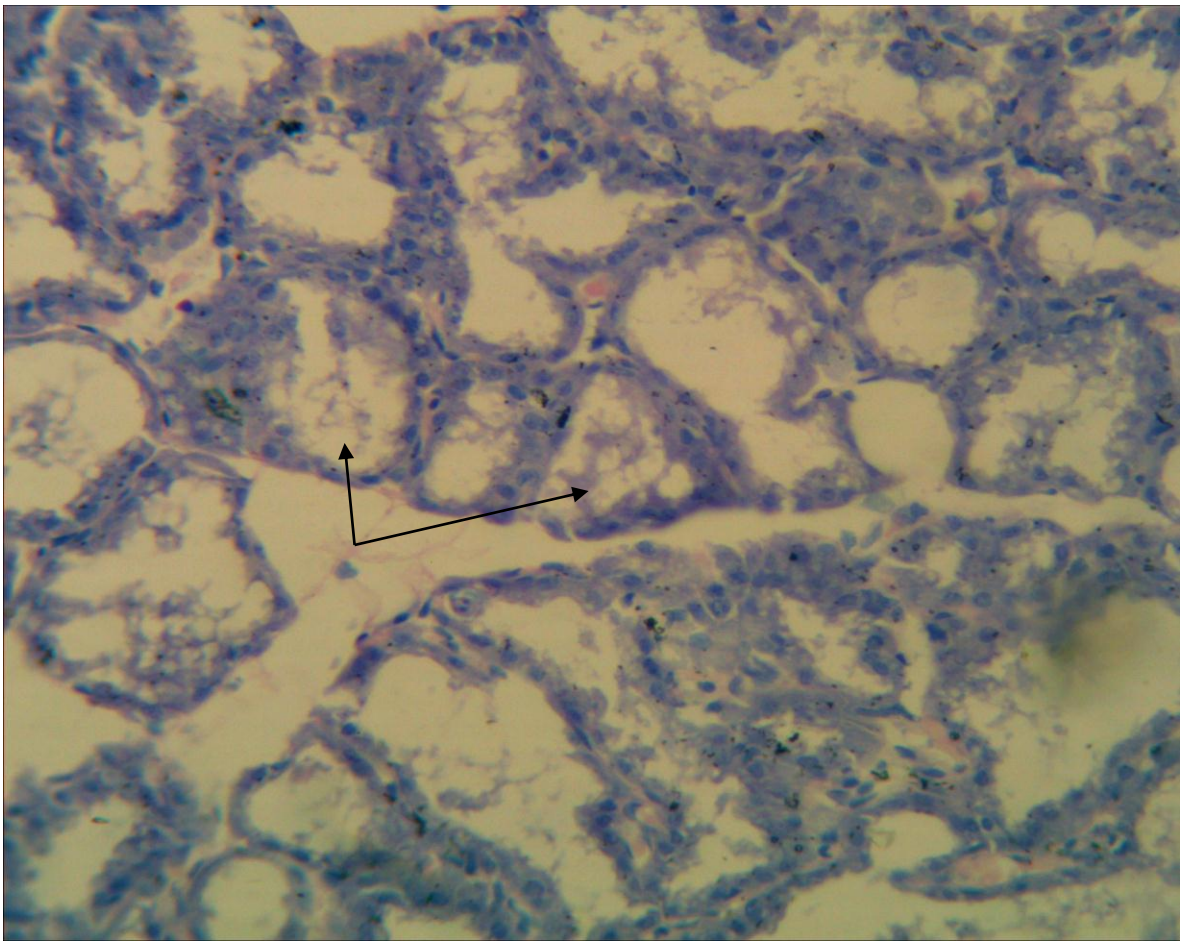


Plate V: (VIT C) Photomicrograph section of mammary gland tissue showing reduced activity with reduced milky secretions in the lumen of the alveoli. (H & E stained x250).

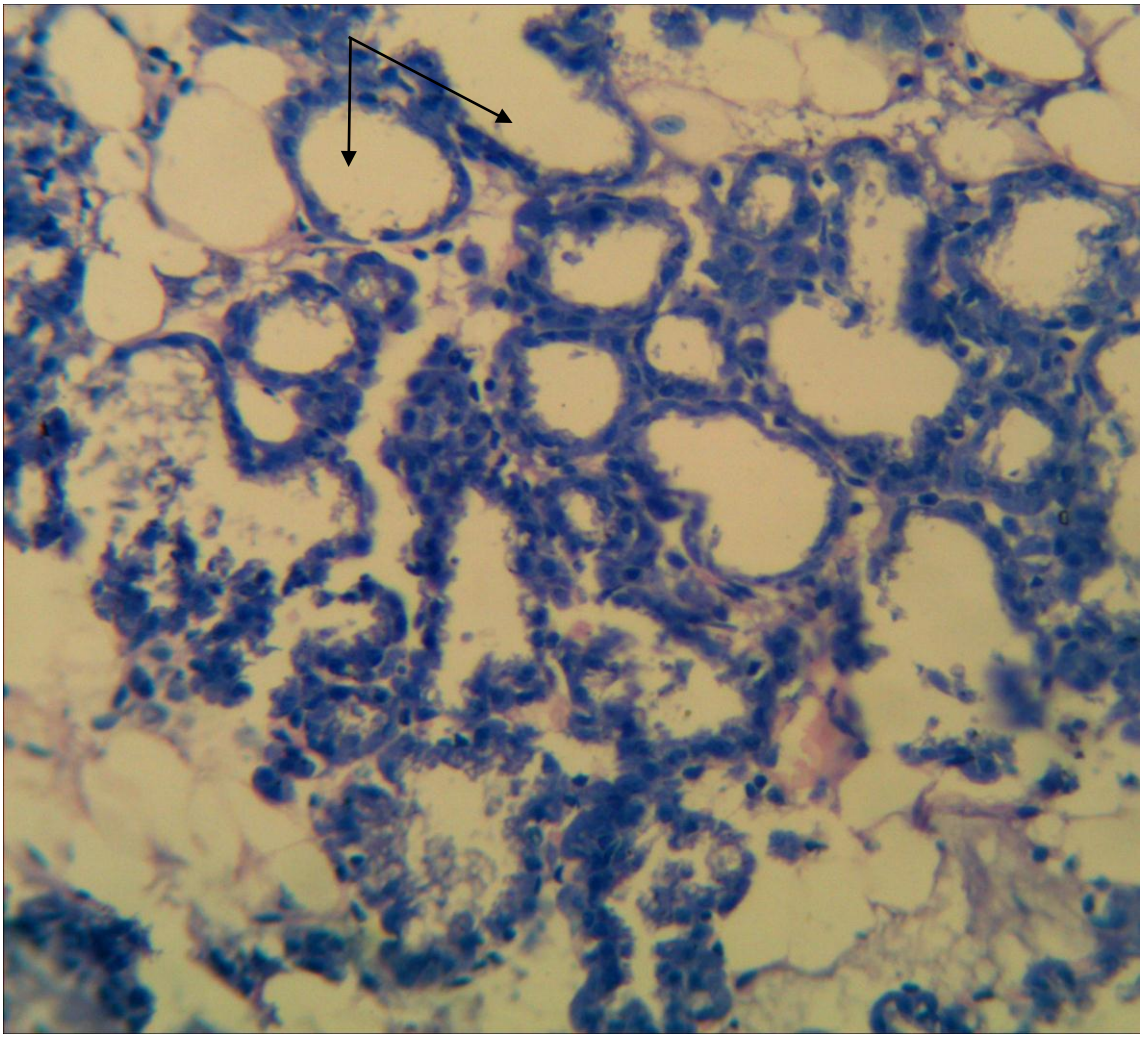


Plate VI: (SB 10%) Photomicrograph section of mammary gland tissue showing decrease of milky secretions in the lumen of the alveoli of the gland. (H & E stained x250).

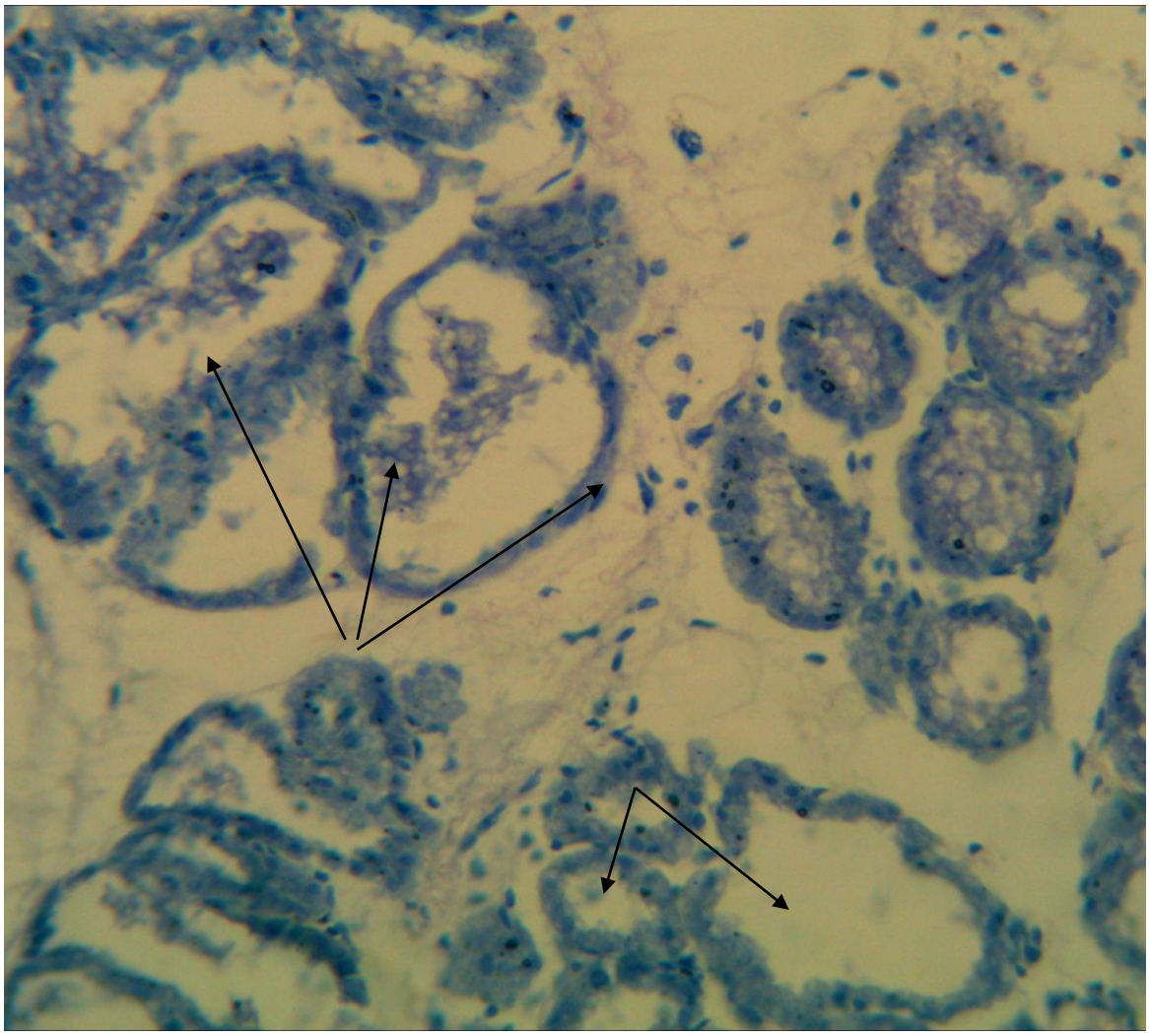


Plate VII: (SB 20%) Photomicrograph section of mammary gland tissue showing increased alveoli distention and increased accumulation of milky secretions in the lumen of the alveoli of the gland. (H & E stained x250).

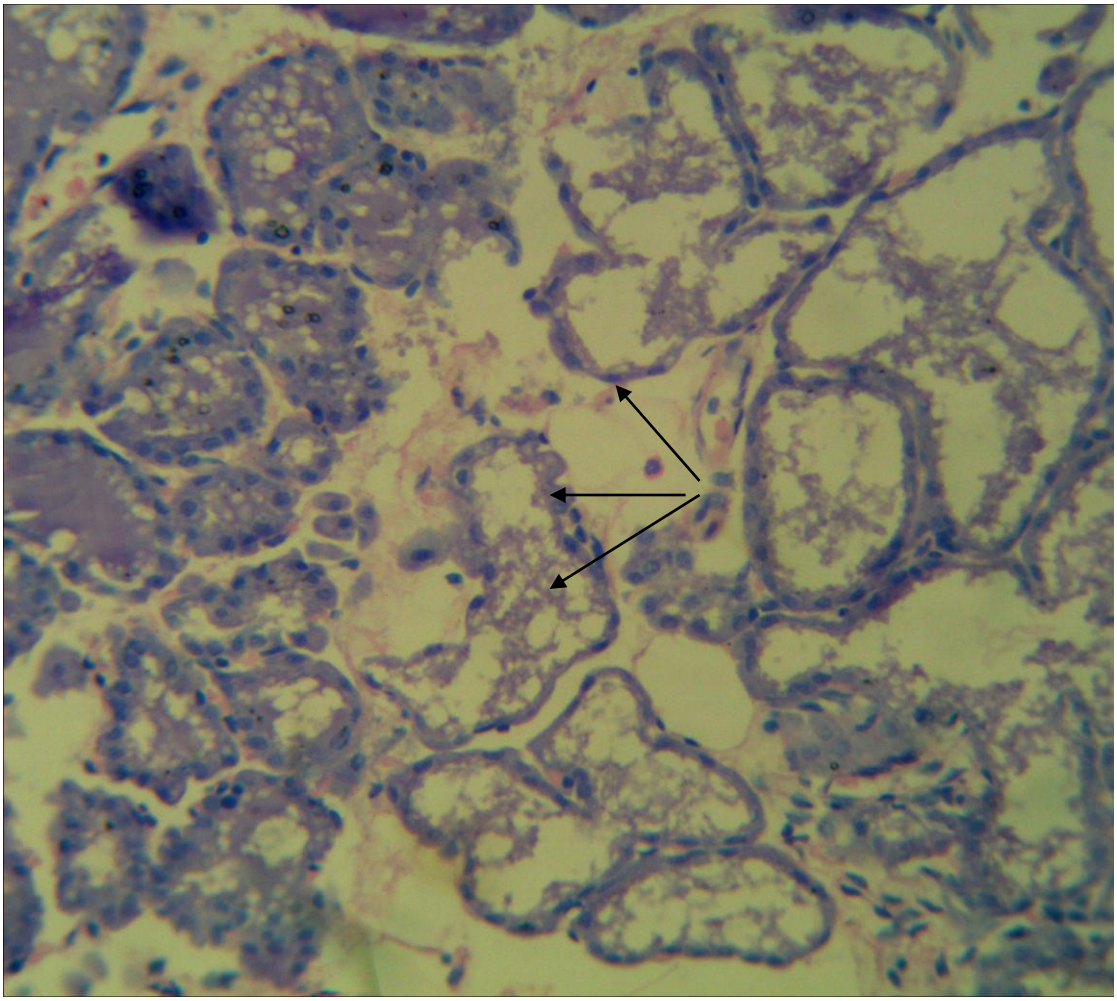


Plate VIII: (SB 40%) Photomicrograph section of mammary gland tissue showing increased accumulation of milky secretions in the lumen of the alveoli of the gland. (H & E stained x250).

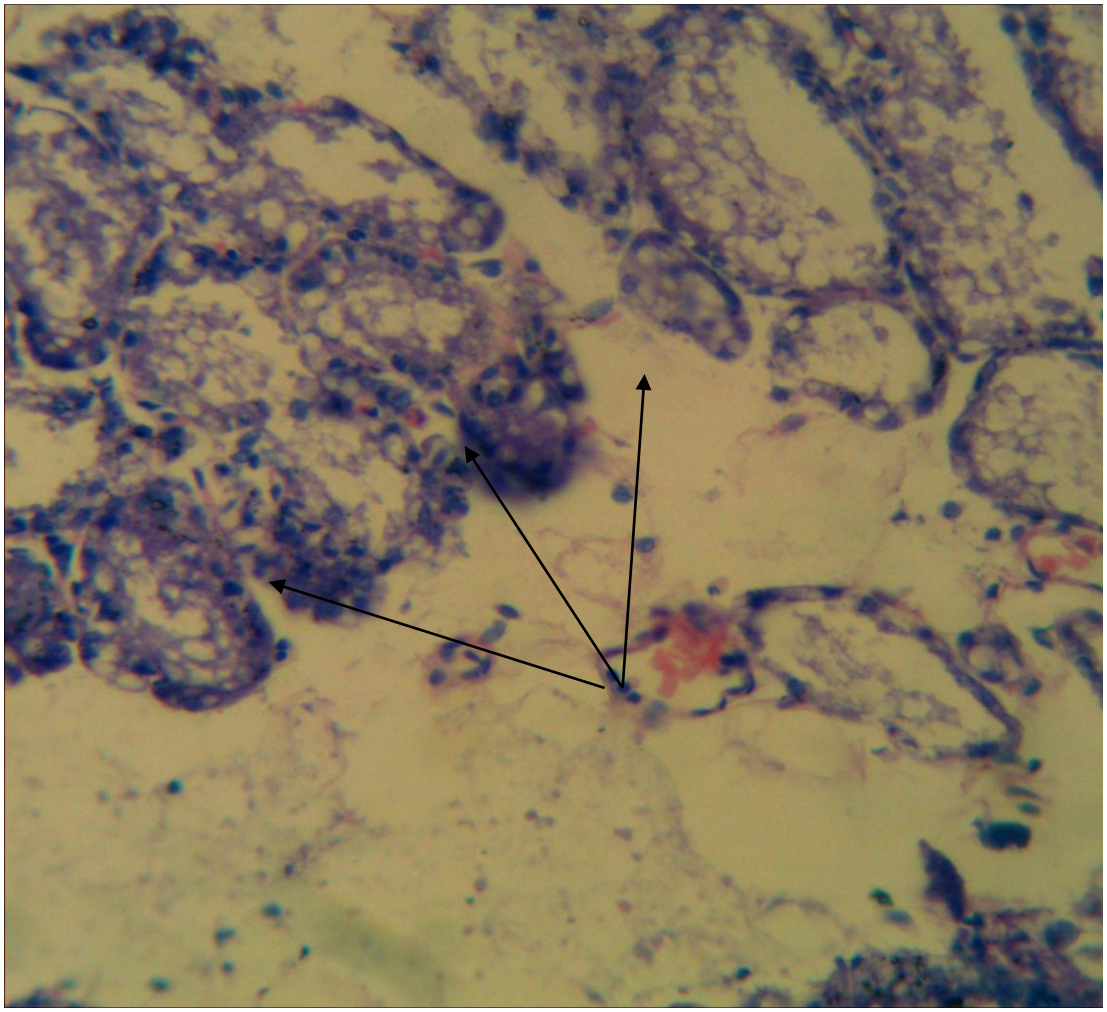


Plate IX: (SB 20% + VIT C) Photomicrograph section of mammary gland tissue showing increase of milky secretions in the lumen of the alveoli of the gland. (H & E stained x250).

CHAPTER FIVE

5.0 DISCUSSION

This study clearly indicated that soy bean gavage when administered in lactating female albino Wistar rats increase milk yield. Although there was increased milk yield 18 hours after gavage in all the treated groups compared to the control, the increase was however not significant (P> 0.05). The highest milk yield was observed in the group treated with SB 20% + VIT C. This result could have been due to the effect of both supplements on serum prolactin level which was significantly increased in this particular group compared to the other treated groups and the controls. This result is in concert with the work of Bako *et al*., (2013), which showed that increase in serum prolactin level could cause a subsequent increase in milk yield in lactating female wistar rats. The action of SB 20% + VIT C on serum prolactin could have been due to its direct excitatory stimulation of these supplements on the cells of the adenohypophysis; responsible for synthesis and secretion of prolactin hormone. This effect could also have been due to a possible antagonistic activity of these supplements on doperminergic cells along the tubero-infundibular axis resulting in a possible decrease of dopamine (prolactin inhibiting hormone). This activity is typical of antipsychotics through which they raise prolactin levels. The result of the milk yield 18 hours after gavage in the supplement treated groups showed a dose dependent increase. The increased milk yield in the SB 20% + VIT C group compared to the SB 40 % treated could have been due to a sort of augmentative effect of the two substances at 18 hours after gavage. Vitamin C is a known antioxidant because of its ability to donate electrons (Padayatty *et al*., 2003) which can prevent cell damage from oxidation of fat (Evans, 2000). The increase in milk yield could have also have been due to the activity of growth hormone

which is known to facilitate galactopoiesis. Although the increase in serum growth hormone was more in the SB 20% than SB 20 % + VIT C, the significant increase in the milk yield of the latter relative to the former as opposed to what one would have expected would be complemented by the increased serum prolactin level in the latter more than the former. Inspite of the higher in serum oxytocin level in the SB 20% treated group as compared to the SB 20% + VIT C, the increase in milk yield 18 hours after gavage in the latter more than in the former could be attributed to the difference in serum prolactin level, suggesting more lactogenesis in the SB 20% + VIT C than the SB 20% treated group. The increase in milk yield 18 hours after gavage in the Metoclopramide treated group could have been due to the antagonistic activity of this anti-emetic drug on D_2 receptors. The result observed in the VIT C treated group could have been due to its antioxidant activity, hence decreasing lipid peroxidation and scavenging free radicals which are generated during lactation.

Milk yield 23 hour after gavage was significantly increased ($P < 0.05$) in the SB 20%, SB 40% and SB 20% + VITC treated groups compared to the control. The highest milk yield 23 hours after gavage was however observed in the SB 20% treated group as opposed to the SB 20% + VIT C seen at 18 hours after gavage. This result could have been due to the increased level of serum growth hormone from either direct stimulation of the growth hormone receptors in the adenohypophysis or from release of growth hormone releasing hormone from the hypothalamus in the SB 20% treated group compared to the other groups. Allan *et al*. (2002) suggested that GH is involved in maintaining mammary cell number during lactation which in effect affects milk production and subsequently milk yield. Therefore, improved lactation performance can be achieved under conditions that enhance mammary cell proliferation (or decrease apoptosis), biochemical and structural differentiation of

mammary epithelium, and synthesis and secretion of milk components. Moreover, any factor involved in the regulation of these processes can directly impact mammary function and milk yield (Akers, 2002). The result obtained could have also been due to the direct activity of soya bean at 20% supplementation on the histology of the mammary gland. The SB 20% treated group showed more alveoli distension, resulting in the increased capacity of the mammary glands to accommodate more milk than the other treated groups. Subcutaneous administration of TRH was also effective to increase plasma PRL levels in lactating cows (Johke, 1978). Women treated with synthetic TRH 20 mg PO three times a day had high blood concentrations of PRL (Zárate *et al*., 2012). The current study also showed that with increasing dosage, the pro lactogenic activity of soya bean supplement on milk yield 23 hours after gavage decreases.

In the present study, the increase in serum level of growth hormone in the metoclopramide treated group was not significant ($P > 0.05$). The VIT C treated group showed a significant increase in serum growth hormone. This result is due to the anti-oxidant activity of VIT C, hence, ameliorating the oxidative stress resulting in optimal function of the pituitary gland cells. There was a significant increase ($P < 0.05$) in serum growth hormone level in the VIT C, SB 10%, 20% and the SB 20% + VIT C. There was however a non-significant decrease in the SB 40% treated groups ($P < 0.05$) compared to the other SB treated groups. This result suggests that SB at higher doses has an antagonistic activity on serum growth hormone level by possibly stimulating the release of growth hormone inhibiting hormone from the hypothalamus.

In the present study, there was no statistical difference ($P > 0.05$) in the serum level of oxytocin in all the treated groups when compared to the control. The metoclopramide

treated group however showed the highest increase in serum oxytocin level compared to the control and the other groups. This was due to the action of metoclopramide on the oxytocin producing neurons in the hypothalamus. Co-administration of SB 20% + VIT C however showed the least increase in serum oxytocin when compared to the remaining SB treated groups. This result suggests that co-administration of SB 20% + VIT C could exert an inhibitory effect on the serum oxytocin which could be due to the effect of VIT C, which showed the least effect on serum oxytocin compared to all the treated groups.

From this study there was no significant difference (P> 0.05) in the level of serum T3 in all the treated groups when compared to the control. The SB 10% treated group showed the highest activity on serum T3 level when compared to the control and the other treated groups. This result suggests that SB 10% could be more potent than the doses 20% and 40%. This result contradicts the findings of Ishizuki *et al*., (1991) which showed that Soya bean might contain goitrogens - substances that depress thyroid function. The metoclopramide treated group also showed a non-significant increase in serum T3 level compared to the control.

Similarly, there was a non- significant difference in the serum level of T4 in all the treated groups when compared to the control except in the SB 40% treated group, which showed a significant increase in serum T4 level. This result could be attributed to the stimulating activity of SB 40% on thyroid hormone releasing hormone resulting in subsequent release of serum T4. However, the level of T4 in SB 10% and SB 20% showed a non-significant decrease (P> 0.05) when compared to the control. This result suggests that Soya bean supplement at lower dose could have an inhibitory activity on thyroid function. This is in concert with the work of Ishizuki *et al*., (1991). More so, soy isoflavones may bind with

some of the iodine that the body would normally use to make thyroid hormones resulting in decreased synthesis of T4 hormone and hence decreased secretion (Divi *et al*., 1997). The results of metoclopramide and vitamin c treated groups showed no significant changes suggesting that both substances have no obvious effect on serum T4 levels in female lactating Wistar rats.

The result of the current study also showed no significant difference (P> 0.05) in serum level of insulin in all the treated groups when compared to the control. Metoclopramide treated group showed no difference in serum insulin when compared to the control suggesting the absence of any activity on the beta cells of the islets. There was an non-significant increase in serum insulin level in the SB 10% treated group when compared to the control. Isoflavones in soybeans which has been shown to enhance insulin secretion and insulin sensitivity in experimental animal models of diabetes could have caused this result (Noriega-López *et al*., 2007; Lu *et al*., 2008; Cederroth *et al*., 2008;). Soy proteins are rich in amino acid arginine and glycine, which play major role in insulin and glucagon secretion from the pancreas. There was however a non- significant increase in the group treated with the co-administration of SB 20% + VIT C which suggest that this might have a pro activity on serum insulin level. This could also have resulted from the antioxidant activity of vitamin C. Fermentation of soybeans leads to structural changes in proteins and phytoestrogen, which may contribute to more beneficial effects on glucose metabolism (Lu *et al*., 2008).

The result of this current study showed significant increase (P< 0.05) in the serum level of progesterone hormone in the treated groups except SB 40% and SB 20% + VIT C which showed a non- significant increase (P> 0.05) compared to the control. Soya bean is the richest dietary source of bioactive phytoestrogen (plant estrogen) called isoflavones. The

result of this present study is in agreement to that of Duncan *et al.* (2000) who showed that consumption of soya bean increased progesterone hormone level in female Wistar rats. This is however in contrast to the work of Serag *et al*. (2011) who showed that administration of soya bean decreased serum progesterone level in female rats. A possible stimulatory activity of soya bean supplement on the Gonadotrophic releasing hormone secreting cells of the hypothalamus which stimulates the secretion of follicle stimulating hormone (FSH) and LH resulting in an increased progesterone secretion from the ovaries could have caused this result.

From the present study, the result showed a significant increase ($P< 0.05$) in the serum level of estrogen in all treated groups except SB 40% which showed a non- significant increase ($P> 0.05$) compared to the control. Isoflavones bind to and activate ER-dependent gene transcription through both ER isoforms, generally with higher binding affinity estrogen receptors. The result of co-administration showed increased serum estrogen when compared to the treated groups. This result suggests an augmentative effect of the co-administration of SB 20% + VIT C on serum estrogen level. This study shows a significant increase in serum alkaline phosphatase (ALP) level in all the treated groups except the vitamin C treated group. The ameliorative effect of Vitamin C is in agreement to the work of Mallo *et al.* (2013), which shows that administration of Vitamin C causes a decrease in serum liver enzymes level. However, the co-administration of Soya bean and Vitamin C showed a significant increase in serum ALP level which could suggest that the co-administration has rather a counteractive relationship as opposed to augmentative effect.

The result of metoclopramide treatment is suggestive of a possible detrimental effect that metoclopramide and the Soya bean supplement could exert on the hepatocytes. The possible

hepatotoxicity of Soya bean supplement appears to be dose dependent as suggested by the effects of the groups treated with 10% and 20% respectively. However, the trend is not observed between the 20% and the 40% treated groups; as a sudden decrease is observed in the 40% treated relative to the 20%. This pattern could suggest that at lower dosages of 10% and 20%, the supplement exerts more deleterious effect on the hepatocytes than at a higher dose of 40%. The action of the supplement at 10% and 20% dosages could be through an increase lipid peroxidation at these doses than at 40%. This is evident in the results of serum levels of malondialdehyde, with that of 20% showing higher concentrations than that of 10% and 40%. The co-administration of the supplement (20%) with vitamin C didn't seem to alleviate the hepatotoxicity of the supplement as one would have expected, taking into cognizance the antioxidant activity of vitamin C. However, the result of the co-administration of the supplement with vitamin C, seem to have exacerbated the hepatotoxicity of the supplement, with the result showing the highest level of ALP.

Although all the treated groups showed an increase in the level of serum ALT, the increase was however not significant except in the metoclopramide treated group and SB 20% + VIT C treated. This result suggests that the co-administration of the supplement with vitamin C has a more hepatotoxic effect than the administration of each substance apart, which could have be attributed to the formation of a possible complex which exerts damage to the hepatocytes during metabolism of the supposedly formed complex in the liver, as part of a normal metabolism of substances to form active metabolites or to deactivate them for excretory purposes. In this present study, the serum level of AST was significantly increased in the groups treated with metoclopramide, SB 40% and SB 20% + VIT C. This results further supports the assertion that the co-administration of the supplement with vitamin C

tends to have more detrimental effect on the hepatocytes compared to when each substance is given separately.

The result on the level of serum MDA shows a significant increase in the group treated with metoclopramide compared to control. Metoclopramide acting on the poly unsaturated fatty acids along the membrane of the hepatocytes resulted in increased lipid peroxidation. The group treated with 20% SB showed an increase in the level of serum MDA compared to the other treated groups. This could also be due to a possible pro activity of the supplement on oxidants resulting in increased lipid peroxidation of cells and a subsequent oxidative stress. The result could also indicate the null anti oxidative activity of the supplement at 20% dosage hence, the prevalence of lipid peroxidation as opposed to the other groups treated with a different dosage. Lipid peroroxides, which are free radicals, originate from the oxidation of lipids presumably from plasma membrane. Membrane lipids succumb easily to deleterious actions of reactive oxygen species. The measurement of lipid per oxidation is a convenient method to monitor oxidative damage (Wallin *et al*., 1993).

The result of SOD in the metoclopramide treated group shows a decrease compared to the control, although not significant. This result could be due to the depletion of the endogenous antioxidant as a result of its utilization in combating the existing oxidative stress as suggested by the level of MDA. The result also could have been due to the inability of metoclopramide to stimulate the release of endogenous antioxidants release as well as its tendency to cause increase in reactive oxygen species within the system. There was however, a remarkable increase in serum SOD in the Vitamin C treated group which could have been be due to its antioxidant activity. The increase was however, rivaled by that of the SB 40%, which showed the highest increase in serum SOD level. This result is suggestive of

SB 40% as having an antioxidant activity more than the standard anti-oxidant. The anti-oxidant activity of the supplement could be due to the presence of tannins and saponins. The result of the co-administration of SB 20% and vitamin C increased the level of SOD more than when the supplement was given singly in the 10% and 20% treated groups. This suggests that the co-administration of the supplement and vitamin C could be more potent than the supplement alone at lower doses of 10% and 20%. However, Vitamin C administered solely and the supplement solely administered at 40% seems to have a more antioxidant activity than the co-administration. The result of the supplement at 40% dosage seems to follow through in the results of both Catalase (CAT) and Glutathione peroxidase (GPx). The higher serum level of both CAT and GPx in the 40% treated group relative to the other groups, strongly consolidates the inference made earlier that the supplement at 40% could have more antioxidant activity than at 10% and 20% doses, the co-administration and the standard antioxidant drug.

In this study, there was an insignificant increase in serum calcium ion concentrations in all the treated groups compared to the control. Metoclopramide, vitamin C, and soya bean supplement could have stimulated the parathyroid hormone (PTH) or estrogen which serves to increase serum calcium concentrations, or due to an inhibitory activity on calcitonin which opposes the effect of parathyroid hormone. There was also a non-significant increase in sodium ion concentration in all the treated groups except in SB 40% and SB 20% + VIT C treated, which showed an insignificant decrease relative to the control. There was an insignificant increase in serum potassium ion concentrations in all the treated groups compared to the control. This result could have been due to the action of these substances on serum aldosterone resulting in reabsorption of this electrolyte. There was an insignificant

increase in serum magnesium ion concentrations in METCL, SB10%, SB 20% and SB 40% treated groups. There was no statistical significant difference in the serum chloride ion concentration all the treated groups when compared to the control. Similarly, there was no significant difference in serum bicarbonate concentrations in all the treated groups when compared to the control. Urea was non-significantly increased in the metoclopramide treated than the other treated groups. An elevation in serum urea level signifies a decline in renal function (Sapna *et al*., 2009), or increased N metabolism and urea synthesis beyond the renal tubular load for urea.

In this present study there was a significant decrease in RBC count in the groups treated SB 40% and SB 20% + VIT C. The decrease observed is attributed to increased lipid peroxidation caused by the actions of free radicals on the membrane of the RBCs. There was a significant increase in WBC in the group treated with SB 40% when compared to the control. This however suggests the role of soya bean supplemention in boosting immunity. There was also an insignificant decrease in PCV and Hb concentration in all the treated groups compared to the control. Thus, soya bean supplementation at 10%, 20%, 40% and 20% + vitamin C could result in decreased oxygen carrying capacity of the blood with respect to decrease hemoglobin concentration. There was also a significant decrease in platelet count in all the supplement treated groups when compared to the control. Such decrease could have been due to a possible activity of the supplement on liver and kidneys which could have affected the production and secretion of thrombopoietin. This supposition is based on the level of damage that could be inferred from the raised levels of liver enzymes in the supplement treated groups.

In this present study, the mammary tissue of the control showed normal histological appearance of alveoli with milky secretions in the lumen. There was however no much alveoli distension as shown in some of the treated groups which is one of the indicators of milk accumulation. With slightly more alveoli distension in the metoclopramide group compared to the control, there were also pockets of lymphocytic infiltration into the mammary tissue. The 20% supplement treated group showed increased alveoli distension and increased accumulation of milky secretions in the alveoli when compared with the control group. This result could be due to the activity of the supplement on serum growth hormone levels, which is known to cause tissue growth and development. The presence of numerous mitotic figures within the epithelial cells in the SB 20% treated groups is indicative of higher cellular division and proliferation, thus resulting in increased milk synthesis and secretion. This result agrees with the findings of Neville and Morton, (2001), who reported the distension of alveoli and mitotic presence as a pro-factor for increased secretory activity and milk synthesis in mammary epithelial cells.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Soya bean supplement has been found to increase milk yield significantly at SB 20% (2.36 ± 0.49 vs 0.8 ± 0.26), SB 40% (1.98 ± 0.42 vs 0.8 ± 0.26) and SB 20% + VIT C (1.90 ± 0.46 vs 0.8 ± 0.26) at 23 hours after gavage. Serum prolactin concentration showed a significant increase in the group treated with SB 20% + VIT C (63.70 ± 2.27 vs 43.98 ± 5.57). Serum level of growth hormone showed significant increase in the groups treated with VIT C, SB 10%, SB 20% and SB 20% + VIT C. The supplement also caused a significant increase in serum thyroxine in the SB 40% (100.80 ± 9.95 vs 68.00 ± 3.91). Serum progesterone level was significantly higher in the groups treated with METCL (5 mg/kg), VIT C (100 mg/kg), SB 10% and SB 20%. A significant increase in serum estrogen level was observed in the groups treated with VIT C (100 mg/kg), SB 10%, SB 20% and SB 20% + VIT C. Increased activity of liver enzymes was also observed in this study as follows: serum ALP level; SB 10% (110.80 ± 1.63 vs 86.60 ± 3.73), SB 20% (127.60 ± 9.60 vs 86.60 ± 3.73), SB 40% (122.80 ± 2.60 vs 86.60 ± 3.37) and SB 20% + VIT C (129.40 ± 4.90 vs 86.60 ± 3.73). Although there was an increase in serum MDA concentrations in all the treated groups compared to the control, lipid peroxidation was however significant in the metoclopramide treated group (1.78 ± 0.15 vs 1.32 ± 0.11). Soya bean supplement from this study has been shown to possess a mild antioxidant property by increasing serum Superoxide dismutase level, although not significantly. Similarly, serum level of catalase showed a significant difference in the group treated with METCL (5 mg/kg) (46.20 ± 1.53 vs 52.00 ± 0.71), SB 10% (44.00 ± 1.14 vs 52.00 ± 0.71), and SB 20% (45.20 ± 1.28 vs 52.00 ± 0.71). Whereas that of glutathione peroxidase showed a significant difference in the group treated with VIT

C (42.60 ± 0.93 vs 48.40 ± 0.93), SB 10% (41.60 ± 1.50 vs 48.40 ± 0.93) and SB 20% (42.60 ± 1.50 vs 48.40 ± 0.93). The results of the supplementation on serum electrolytes levels was not significant. There was a significant decrease in RBC count in the groups treated with SB 40% (4.24 ± 0.65 vs 7.23 ± 0.11) and SB 20% + VIT C (5.42 ± 0.43 vs 7.23 ± 0.11). Soya bean supplementation at SB 20% (12.20 ± 1.73 vs 8.24± 1.81), SB 40% (13.62 ± 4.45 vs 8.24 ± 1.81) and SB 40% (13.62 ± 4.45 vs 8.24 ± 1.81) boosted the immunity of the animal significantly by increasing WBC compared to the control. Although there was no significant difference in PCV and Hb concentration, there was however a significant decrease ($P< 0.05$) in platelet count in all the supplement treated groups when compared to the control SB 10% (327.80 ± 81.76 vs 681.60 ± 44.81), SB 20% (356.00 ± 81.76 vs 681.60 ± 44.81) and SB 20% + VIT C (344.00 ± 42.26 vs 681.60±44.81). There was evidence of enhanced mammary alveoli development and secretory activity in the mammary gland of the treated groups, (influencing milk production) as compared to the control group.

6.2 Conclusion

The study confirmed the lactogenic activity of Soya bean supplement in female lactating wistar rats by increasing the milk yield at 23 hours after administration more than the standard drug metoclopramide. This study has also confirmed the anti-oxidative properties of soya bean. The study further demonstrated evidence of enhanced mammary alveoli development and secretory activity in the mammary gland of the treated groups.

6.3 Recommendations

i. The use of soya bean supplement should be considered as a possible galactagogue in lactation deficit situation.

ii. Soya bean supplement could be considered as an anti-oxidant hence could be consumed to ameliorate lactation stress in mothers.

iii. Further studies should be carried out on the effect of soya bean supplementation on Gene expressions as well as the possible effect it might have on pups fed with milk from supplemented dams.

6.4 Contributions to Knowledge

1. Soya bean supplement increases significantly milk yield 23 hours after gavage; SB 20% (2.36 ± 0.49), SB 40% (1.98 ± 0.42) and SB 20% + VIT C (1.90 ± 0.46) in female lactating wistar rats in female lactating wistar rats.

2. Soya bean supplement at 20% dosage increased milk yield significantly (2.36 ± 0.49) more than the standard drug metoclopramide (1.16 ± 0.29) 23 hours after gavage in female lactating wistar rats.

3. Soya bean at doses 10%, 20% and co-administration of 20% + Vitamin C significantly increased serum growth hormone concentration; 10.34 ± 1.26, 12.48 ± 0.84 and 9.48 ± 0.85, respectively in female lactating wistar rats.

4. Co-administration of soya bean supplement and vitamin C significantly increased serum prolactin concentration (63.70 ± 2.27 ng/ml) in female lactating wistar rats.

5. Soya bean supplement at 40% significantly increased serum thyroxine (T4) concentrations in female lactating wistar rats.

6. Soya bean supplement significantly increased serum progesterone concentrations at 10 % and 20 % dose; 26.52 ± 2.47 vs 16.66 ± 1.99 and 28.78 ± 1.29 vs 16.66 ± 1.99 respectively in female lactating wistar rats.

7. Soya bean supplement significantly increased serum estrogen concentrations at 10%, 20% and 20% + Vitamin C; 705.00 ± 27.53 vs 632.60 ± 42.96, 633.00 ± 22.27 vs 632.60 ± 42.96 and 725.40 ± 29.62 vs 632.60 ± 42.96 respectively in female lactating wistar rats.

8. Soya bean at all doses significantly increased the serum level of ALP in female lactating wistar rats; SB 10% (110.80 ± 1.63 vs 86.60 ± 3.73), SB 20% (127.60 ± 9.60 vs 86.60 ± 3.73), SB 40% (122.80 ± 2.60 vs 86.60 ± 3.73) and SB 20% + VIT C (129.40 ± 4.90 vs 86.60 ± 3.73).

9. The supplement significantly decreased RBC count in the groups treated with the supplement at 40% (4.24 ± 0.65 vs 7.23 ± 0.11) and SB 20% + VIT C (5.42 ± 0.43 vs7.23 ± 0.11).

10. There was no significant difference in PCV and Hb concentration.

12. There was however a significant decrease in platelet count in all the Supplement treated groups SB 10% (327.80 ± 81.76 vs 681.60 ± 44.81), SB 20% (356.00 ± 81.76 vs 681.60 ± 44.81) and SB 20% + VIT C (344.00 ± 42.26 vs 681.60 ± 44.81).

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APPENDICES

APPENDIX I: Effect of administration of Soya bean supplement and vitamin C for 10 days on total milk yield (18 and 23 hours) in female lactating Wistar rats

Treatment Groups	Milk yield 18h (g/pup)	Milk yield 23h (g/pup)
Control (1 mg/kg Normal saline)	1.28±0.47	0.8±0.26
Metoclopramide (5 mg/kg)	1.68±0.37	1.16±0.29
Vitamin C (100 mg/kg)	1.54±0.47	1.24±0.46
Soya Bean supplement (10%)	1.64±0.47	1.70±0.44
Soya Bean supplement (20%)	1.90±0.45	2.36±0.49*
Soya Bean supplement (40%)	2.14±0.38	1.98±0.42*
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	2.30±0.41	1.90±0.46*

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$).

APPENDIX II: Effect of administration of Soya bean supplement and vitamin C for 10 days on serum prolactin level in female lactating Wistar rats

Treatment Groups	Prolactin hormone ng/ml
Control (1 mg/kg Normal saline)	43.98 ± 5.57
Metoclopramide (5 mg/kg)	52.24 ± 6.73
Vitamin C (100 mg/kg)	59.06 ± 2.93
Soya Bean supplement (10%)	50.86 ± 3.90
Soya Bean supplement (20%)	52.84 ± 2.93
Soya Bean supplement (40%)	47.80 ± 1.06
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	63.70 ± 2.27*

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$).

APPENDIX III: Effect of administration of Soya bean supplement and vitamin C for 10 days on serum growth hormone level in female lactating Wistar rats

Treatment Groups	Growth hormone ng/ml
Control (1 mg/kg Normal saline)	4.90 ± 0.37
Metoclopramide (5 mg/kg)	6.86 ± 1.42
Vitamin C (100 mg/kg)	10.64 ± 0.79*
Soya Bean supplement (10%)	10.34 ± 1.26*
Soya Bean supplement (20%)	12.48 ± 0.84*
Soya Bean supplement (40%)	8.76 ± 0.95
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	9.48 ± 0.85*

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$).

APPENDIX IV: Effect of ten (10) days administration of soya bean supplement and vitamin C on serum oxytocin level in female lactating Wistar rats

Treatment Groups	**Oxytocin (ng/ml)**
Control (1 mg/kg Normal saline)	342.80 ± 22.10
Metoclopramide (5 mg/kg)	404.20 ± 23.42
Vitamin C (100 mg/kg)	298.60 ± 20.84
Soya Bean supplement (10%)	335.20 ± 42.72
Soya Bean supplement (20%)	395.20 ± 26.82
Soya Bean supplement (40%)	343.40 ± 15.17
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	338.40 ± 26.48

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$).

APPENDIX V: Effect of administration of Soya bean supplement and vitamin C for 10 days on Serum Triiodothyronine (T3) level in female lactating Wistar rats

Treatment Groups	T3 (ng/ml)	T4 (ng/ml)
Control (1 mg/kg Normal saline)	1.22 ± 0.15	68.00 ± 3.91
Metoclopramide (5 mg/kg)	1.38 ± 0.12	70.40 ± 2.98
Vitamin C (100 mg/kg)	1.24 ± 0.10	68.40 ± 4.43
Soya Bean supplement (10%)	1.42 ± 0.12	56.74 ± 12.29
Soya Bean supplement (20%)	1.22 ± 0.09	65.40 ± 3.31
Soya Bean supplement (40%)	1.32 ± 0.10	100.80 ± 9.95*
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	1.28 ± 0.12	73.60 ± 4.13

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$).

APPENDIX VI: Effect of administration of Soya bean supplement and vitamin C for 10 days on Serum thyroxine (T4) level in female lactating Wistar rats

Treatment Groups	**Tyroxine (ng/ml)**
Control (1 mg/kg Normal saline)	68.00 ± 3.91
Metoclopramide (5 mg/kg)	70.40 ± 2.98
Vitamin C (100 mg/kg)	68.40 ± 4.43
Soya Bean supplement (10%)	56.74 ± 12.29
Soya Bean supplement (20%)	65.40 ± 3.31
Soya Bean supplement (40%)	100.80 ± 9.95*
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	73.60 ± 4.13

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$).

APPENDIX VII: Effect of administration of Soya bean supplement and vitamin C for 10 days on Serum insulin level in female lactating Wistar rats

Treatment Groups	**Insulin (ng/ml)**
Control (1 mg/kg Normal saline)	1.50 ± 0.13
Metoclopramide (5 mg/kg)	1.50 ± 0.13
Vitamin C (100 mg/kg)	1.54 ± 0.11
Soya Bean supplement (10%)	1.66 ± 0.16
Soya Bean supplement (20%)	1.22 ± 3.82
Soya Bean supplement (40%)	1.54 ± 0.12
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	1.90 ± 0.14

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$) compared to the control

APPENDIX VIII: Effect of administration of Soya bean supplement and vitamin C for 10 days on Serum Progesterone and Estrogen level in female lactating Wistar rats

Treatment Groups	Progesterone (ng/ml)	Estrogen (ng/ml)
Control (1 mg/kg Normal saline)	16.66 ± 1.99	632.60 ± 42.96
Metoclopramide (5 mg/kg)	26.18 ± 1.87*	660.00 ± 46.62
Vitamin C (100 mg/kg)	25.78 ± 1.53*	669.60 ± 54.04*
Soya Bean supplement (10%)	26.52 ± 2.47*	705.00 ± 27.53*
Soya Bean supplement (20%)	28.70 ± 1.29*	633.00 ± 22.27*
Soya Bean supplement (40%)	23.58 ± 1.48	645.80 ± 45.68
Soya Bean supplement (20%) + Vitamin C	25.30 ± 2.94	725.40 ± 29.62*

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$) compared to the control.

APPENDIX IX: Effect of administration of Soya bean supplement and vitamin C for 10 days on Serum Liver enzymes level in female lactating Wistar rats

Treatment Groups	ALP (µ/L)	ALT (µ/L)	AST (µ/L)
Control (1 mg/kg Normal saline)	86.60 ± 3.73	38.20 ± 1.67	34.20 ± 1.59
Metoclopramide (5 mg/kg)	115.60 ± 3.61*	52.40 ± 2.40*	48.20 ± 2.08*
Vitamin C (100 mg/kg)	99.80 ± 4.54	44.20 ± 1.24	40.20 ± 1.12
Soya Bean supplement (10%)	110.80 ± 1.63*	44.60 ± 1.21	42.20 ± 1.07
Soya Bean supplement (20%)	127.60 ± 9.60*	42.20 ± 1.66	41.00 ± 2.00
Soya Bean supplement (40%)	122.80 ± 2.60*	43.40 ± 1.21	45.00 ± 2.10*
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	129.40 ± 4.90*	47.20 ± 1.74*	50.80 ± 2.33*

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$) compared to the control

APPENDIX X: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum MDA level in female lactating Wistar rats

Treatment Groups	**MDA (µmol/L)**
Control (1 mg/kg Normal saline)	1.32 ± 0.11
Metoclopramide (5 mg/kg)	1.78 ± 0.15*
Vitamin C (100 mg/kg)	1.52 ± 0.09
Soya Bean supplement (10%)	1.42 ± 0.11
Soya Bean supplement (20%)	1.60 ± 0.07
Soya Bean supplement (40%)	1.40 ± 0.05
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	1.40 ± 0.07

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$) compared to the control

APPENDIX XI: Effect of administration of Soya bean supplement and vitamin C for 10 days on Serum Antioxidant enzymes level in female lactating Wistar rats

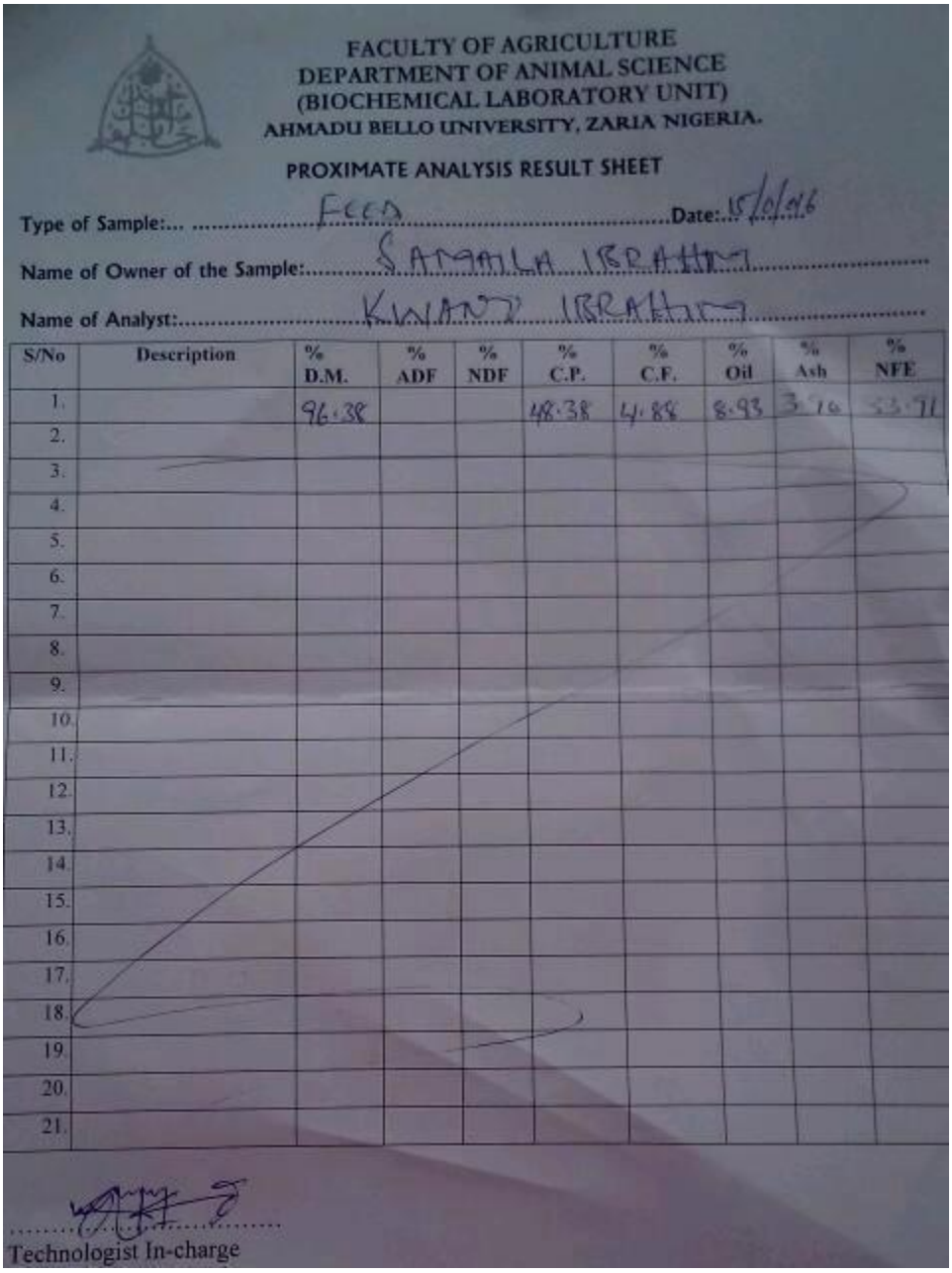
Treatment Groups	**SOD (IU/L)**	**CAT (IU/L)**	**GPx (IU/L)**
Control (1 mg/kg Normal saline)	2.24±0.0.09	52.00±0.71	48.40±0.93
Metoclopramide (5 mg/kg)	1.88±0.07	46.20±1.53*	44.20±1.46
Vitamin C (100 mg/kg)	2.08±0.10	46.80±1.59	42.60±0.93*
Soya Bean supplement (10%)	1.74±0.10*	44.00±1.14*	41.60±1.50*
Soya Bean supplement (20%)	1.84±0.05	45.20±1.28*	42.60±1.50*
Soya Bean supplement (40%)	2.30±0.13	50.40±1.17	48.00±0.94
Soya Bean supplement (20%) + Vitamin C (100 mg/kg)	1.96±0.12	48.20±1.02	45.80±0.86

± = SEM; Means with superscripts (*) within columns are statistically significant ($P < 0.05$) compared to the control.

APPENDIX XII: Effect of administration of Soya bean supplement and Vitamin C for 10 days on Serum Electrolytes level in female lactating Wistar rats

Groups	Calcium (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Magnesium (mmol/L)	Chloride (mmol/L)	Bicarbonate (mmol/L)	Urea (mmol/L)
Control	2.26±0.04	139.80±1.39	4.20±0.15	1.02±0.12	101.80±0.86	23.00±1.00	3.60±0.27
Metcl	2.28±0.43	141.00±1.00	4.88±0.23	1.10±0.07	102.80±0.86	21.60±1.17	4.62±0.29
Vit C	2.38±0.42	142.00±1.14	4.24±0.18	0.90±0.13	103.00±1.30	24.20±0.66	3.90±0.25
SB 10%	2.28±0.04	142.00±1.70	4.28±0.19	1.06±0.10	103.60±1.36	23.20±1.02	4.00±0.25
SB 20%	2.28±0.04	140.00±0.71	4.24±0.14	1.12±0.08	102.00±0.71	23.60±0.81	4.08±0.16
SB 40%	2.28±0.08	138.60±0.81	4.40±0.27	1.06±0.07	100.60±1.33	23.00±1.00	3.28±0.28
SB20%+Vit C	2.33±0.07	139.00±1.09	4.26±0.15	0.98±0.37	99.40±0.93	22.00±0.95	3.90±0.37

APPENDIX XIII: Proximate Analysis Result for Soya Bean



FACULTY OF AGRICULTURE
DEPARTMENT OF ANIMAL SCIENCE
(BIOCHEMICAL LABORATORY UNIT)
AHMADU BELLO UNIVERSITY, ZARIA NIGERIA.

PROXIMATE ANALYSIS RESULT SHEET

Type of Sample:... Feed ..... Date: 15/6/16

Name of Owner of the Sample: SAMAILA IBRAHIM

Name of Analyst: KWANU IBRAHIM

S/No	Description	% D.M.	% ADF	% NDF	% C.P.	% C.F.	% Oil	% Ash	% NFE
1.		96.38			48.38	4.88	8.93	3.10	33.71
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Technologist In-charge