Effect of different sources of Selenium supplementation on performance of bucks

THESIS SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

OF

MASTER OF VETERINARY SCIENCE

IN

ANIMAL NUTRITION

BY

Sunil Kumar

Enrolment No.: V-1283/14

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura-281 001 (UP)

(2021)

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This is to certify that thesis entitled, **"Effect of different sources of Selenium supplementation on performance of bucks"** submitted by **Dr. Sunil Kumar, Enrollment No. V-1283/14** in partial fulfillment of the requirements for the award of the **Master of Veterinary Science** in **Animal Nutrition** of the **Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go- Anusandhan Sansthan, Mathura (UP), India,** is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated 23.10.2021

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%	:	Percent
@	:	At the rate of
<	:	Less than
>	:	More than
µM	:	Micro mole
^{0}C	:	Degree centigrade
100X	:	Magnification hundred times
10X	:	Magnification ten times
400X	:	Magnification four hundred times
40X	:	Magnification forty times
45X	:	Magnification forty five times
ADF	:	Acid detergent fibre
ADG	:	Average daily gain
ADL	:	Acid detergent lignin
AI	:	Artificial Insemination
AIA	:	Acid insoluble ash
ALP	:	Alkaline phosphatase
ALT	:	Alanine aminotransferase
AOAC	:	Association of official analytical chemists
AR	:	Analytical Grade
ARC	:	Agricultural Research Council
AST	:	Aspartate aminotransferase
ATP	:	Adenosine Triphosphate
AV	:	Artificial Vagina
BW	:	Body weight
Ca	:	Calcium
CAT	:	Catalase
CF	:	Crude fibre
Conc.	:	Concentrate

CP	:	Crude protein
Cu	:	Copper
CuSO4	:	Copper sulphate
DCP	:	Digestible crude protein
DM	:	Dry matter
DMI	:	Dry matter intake
DMSO	:	Dimethyl sulfoxide
DUVASU	:	Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan
DW	:	Dry weight/ Distilled water
e.g.	:	For example
EE	:	Ether extract
ELISA	:	Enzyme linked immunosorbant assay
et al.	:	*Etalli / alia*
etc	:	And so forth
FAO	:	Food and Agriculture Organization
FCR	:	Feed conversion ratio
Fe	:	Iron
Fig.	:	Figure
g	:	Gram
g/dl	:	Gram per deciliter
g/ml	:	Gram per milliliter
GSH	:	Glutathione
GSH-Px	:	Glutathione Peroxidase
H_2SO_4	:	Sulphuric acid
Hb	:	Haemoglobin
HCl	:	Hydrochloric acid
$HClO_4$	:	Perchloric acid
HNO_3	:	Nitric acid
HOST	:	Hypo Osmotic Swelling Test
hr (s)	:	Hours
i.e.	:	id est (that is)

ICAR	:	Indian Council of Agricultural Research
ICP-OES	:	Inductively coupled plasma-optical emission spectrometry
IU	:	International Unit
IU/ L	:	International Unit per liter
kg	:	Kilogram
$kgW^{0.75}$	:	Metabolic body weight
KH_2HPO_4	:	Potassium di hydrogen phosphate
l	:	Litre
LH	:	Leutinizing Hormone
LN_2	:	Liquid Nitrogen
LPO	:	Lipid peroxide
MDA	:	Malondialdehyde
meq/l	:	Miliequivalent per litre
mg	:	Milligram
mg/dl	:	Milligram per deciliter
mg/l	:	Milligram per litre
mg/ml	:	Milligram per milliliter
min	:	Minutes
ml	:	Milliliter
Mm	:	Mill mole
mOsm	:	Milliosmole
MSIS	:	Multimode sample introduction system
NDF	:	Neutral detergent fibre
NDS	:	Neutral detergent solution
NFE	:	Nitrogen Free Extract
ng / ml	:	Nanogram per milliliter
NH_3-N	:	Ammonia nitrogen
nm	:	Nanometer
Nmol	:	Nano mole
NS	:	Non Significant
OD	:	Optical density

OM	:	Organic matter
OS	:	Oxidative stress
P	:	Phosphorous
P≤ 0.01	:	1% level of significance
P≤0.05	:	5 % level of significance
PBS	:	Phosphate buffer saline.
PCV	:	Packed cell volume
pH	:	Negative logarithm of Hydrogen ion
ppm	:	Parts per million
RBCs	:	Red blood cells
ROS	:	Reactive oxygen Species
rpm	:	Revolution per minute.
SE	:	Standard Error
Se	:	Selenium
sec (s)	:	Seconds
Selenop	:	Seleno protein P
SEM	:	Standard error of mean
SOD	:	Superoxide dismutase
TA	:	Total ash
TBA	:	Thio barbituric acid
TCA	:	Tri-chloral acetic acid
TMR	:	Total mixed ration
TOAC	:	Total antioxidant capacity
U	:	Unit
U.P.	:	Uttar Pradesh
v/v	:	volume by volume
WBC	:	White blood cell

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Sunil

(Sunil Kumar)

Date: 23/10/21

Place: Mathura (U.P.)

CONTENTS

ABSTRACT

The present study was designed to evaluate the effect of dietary supplementation of different sources of Se on growth performance, nutrient utilization, seminal attributes, haemato-biochemicals, antioxidant, mineral and hormonal profile of bucks. For this study, 24 experimental bucks were selected and equally divided into four groups (n=6) on body weight basis. The animals in control group were fed on basal diet i.e., concentrate mixture, gram straw and corn silage as per ICAR (2013) feeding standard whereas, the groups T1, T2 and T3 were fed basal diet along with supplementation of inorganic Se (Sodium selenite), organic Se (Se-Yeast) and Se nano particles at level of 0.3mg/kg DM offered respectively. The experimental feeding was done for 90 days and a digestion trial of seven days was conducted to appraise the nutrient utilization. Blood sample were collected at day 0 followed by monthly interval. Semen from each buck in a group was collected and pooled, a total of six pooled ejaculates from each group were evaluated at start and end of experimental feeding. The results revealed dietary supplementation of Se from either source have no significant ($P<0.05$) effect on BW, ADG, FCR, feed intake and digestibility of nutrients. Plasma globulins and total protein concentration were significantly ($P<0.05$) higher in Se supplemented groups. However, all other haemato-biochemical parameters monitored were under normal physiological range. Significant ($P<0.05$) increase in plasma SOD and GSH-Px activity with concomitant decrease in ROS and LPO levels was found in treatment groups. However, on comparing the source of Se supplementation, nano Se seems to be more effective in enhancing antioxidant status. Plasma Se concentration significantly ($P<0.05$) increased with Se supplementation irrespective of source, however, nano Se exhibited highest plasma Se concentration. The assay of IgG, Testosterone and LH significantly ($P<0.05$) increased in treatment groups with highest concentration in nano Se group. Semen analysis showed significant ($P<0.05$) improvement in progressive motility, viability, Acrosomal integrity, ROS, membrane fluidity, intracellular Ca levels, and HOST values in treatment groups compared to control with best results in T3 group. So, it can be concluded that incorporation of different Se sources (inorganic, organic and nano) at 0.3 ppm level in diet increased plasma Se concentration, improved antioxidant, immunity, seminal attributes and reproductive hormones status. Moreover, nano Se seems to be more effective in improving fertility through enhancement of seminal attributes, antioxidant and Se status in bucks.

Introduction

CHAPTER-1

INTRODUCTION

Minerals are inorganic substances that cannot be synthesized in body, but are required in small amount to support different metabolic functions of body (Eruvbetine, 2003). 22 minerals are needed for animals. Out of these minerals, 15 are micro minerals and 7 macro minerals (Underwood, 1999). Trace minerals, though required in small quantities (less than 100 mg/kg dry matter) have critical roles in immune function, oxidative and energy metabolism in ruminants which are directly or indirectly involved in growth, production and reproduction (Bhalakiya et al., 2019). They include cobalt, copper, iodine, iron, manganese, molybdenum, Se and zinc (Soetan et al., 2010). Among these, Se is currently acknowledged to be an essential dietary trace element required for various body functions such as growth, reproduction, immune system and protection of tissue integrity (Pilarczyk et al., 2013).

The Se status of animals and plants varies markedly around the world as a result of different geological conditions and the concentrations of Se in plant material are highly correlated with those in the soil. Agricultural production system in developing countries is under pressure to fulfil the requirement of growing population, which has led to indiscriminate use of fertilizers resulting in severe deficiency of micro minerals in soil (Kumar et al., 2014). The deficiency of Se in soil and crop plants has been reported in many countries like India, China, Turkey and Pakistan (Rashid and Ryan, 2008). Se which was earlier classified as a toxic element, has now been proved to be an essential mineral required for proper health, immunity, and reproductive functions of animals. The essentiality of Se was proved for the first time from the work that liver necrosis in rats (Patterson et al., 1957) and exudative diathesis in chicks (Schwarz and Foltz, 1957) could be prevented by supplements of Se.Se is required to maintain normal physiological functions and provides a significant dietary source of antioxidant defenses (Sordillo, 2013). It is present in all cells and tissues and is necessary for maintaining the vital functions of humans and animals. The content of Se in the organism is naturally very low, the majority of Se being bound in tissues and blood in the form of selenoproteins. It is a component of at

least 25 selenoproteins with antioxidant, anti-inflammatory and chemoprotective properties (Pappas et al., 2008). The most important are glutathione peroxidases (GSH-Px-1, GSH-Px-6), thioredoxin reductases (TrxR1–TrxR3), iodothyronine deiodinases(ID1–ID3), selenophosphate synthetase, Selenop, and selenoprotein W. This element acts as a cofactor of the GSH-Px family of enzymes which protect against oxidative stress. Specifically, Se-dependent GSH-Px enzyme recycles glutathione, reducing lipid peroxidation by catalysing the reduction of peroxides, including hydrogen peroxide. In general, all these enzymes in their reduced state catalyse the breakdown of lipid hydroperoxides and hydrogen peroxides in cells (Navarro-Alarcon and Cabrera-Vique, 2008). GSH-Px and selenoprotein P are also involved in the regulation of the inflammatory response.

The ability of Se to improve the immune response in farm animals is well documented (Chauhan et al., 2014). Se deficiency has been reported to decrease humoral immune response and neutrophil killing activity in cattle without any clinical signs. (Hogan et al., 1990). Moreover, Hall et al. (2013) reported that dietary Se supplementation increases antibody titre, neutrophils killing activity and reduces morbidity and mortality in beef cattle. Se improves phagocytosis in white blood cell populations (Hogan et al., 1990). It might have been a boost in the passive immunity by enhancing immunoglobulin G (IgG) absorption in the new born lamb (Rock et al. 2001). The improvement of immune cell functioning is likely due to the enhanced antioxidant status (Rossi et al., 2017). Se performs significant functions in the male reproductive systems which are regulated by selenoproteins, especially GSH-Px4 (Glutathione peroxidase) and SELENOP (Selenoprotein-P) (Qazi et al., 2019). GSH-Px4 is distinctly expressed in testes and has both an antioxidant as well as a structural role; the latter context is evident from a fact that it constitutes over 50% of mitochondrial capsule (as an oxidatively inactivated protein) in midpiece of mature sperm (Foresta et al., 2002). In an early stage of spermatogenesis, GSH-Px4 is believed to protect the developing sperm from oxidative stress-induced DNA damage, however, in the later phase, through cross linkage with proteins in midpiece region, it provides the integrity to the sperm midpiece by becoming a structural component of mitochondrial sheath circumventing the flagellum, which is an essential component for sperm stability and motility.

SELENOP serves as a transport protein for Se and is also expressed in vesicle like structures in the basal region of the Sertoli cells (Olson et al., 2007). Male reproductive tissues are constantly exposed to reactive oxygen species (ROS) generated as products of normal metabolism, and during the fertilization process spermatozoa normally pass through an area of high oxygen level (Fujii et al., 2003). Mammalian spermatozoa membranes have a high content of polyunsaturated fatty acids and are highly susceptible to lipid peroxidation caused by reactive oxygen species (ROS) overproduction (Aitken et al., 1993). ROS induced damages decreased both semen quality and sperm function. High ROS concentrations lead to pathologic changes in sperm cells by intensifying lipid peroxidation, which eventually results in their motility and viability loss (Makker et al., 2009). Several defence mechanisms including antioxidant help in counteracting ROS detrimental effects and maintain sperm motility and viability (Aitken and Baker, 2004). The activity of the cytoplasmic antioxidant enzymes in sperm cells is very low (Bilodeau et al., 2000), and the small amount of the cytoplasm in their heads and tails makes them susceptible to oxidative stress (Irvine, 1996). Unlike spermatozoa, seminal plasma is a vital source of antioxidants, including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), uric acid and vitamin E (Raijmakers et al., 2003). Activity of Se-dependent phospholipid hydroperoxide GSH-Px (PH-GSH-Px, GSH-Px4) is very pronounced in late (meiotic) spermatogenic cells, where it acts as a structural protein in sperm heads, whereas the nuclear form GSH-Px4 contributes to chromatin condensation (Flohe, 2007).

Se also helps in testicular growth and development of seminiferous tubules, spermatogenesis, steroidogenesis in testes, synthesis and secretion of follicular stimulating hormone (FSH) and luteinizing hormone (LH) (Bedwal and Bahugana, 1994). Supplementation of Se has been found to improve semen quality by increasing antioxidative defence of seminal plasma in buck (Shi et al., 2010), boar (Marin-Guzman et al., 1997, 2000), Boer goats (Li-guang et al., 2010) ram (Kendall et al., 2000) and cockerels (Ebeid, 2012). Se deficiency has been linked to many health problems in young animals such as increased neonatal mortality, decreased sucking reflex, weakness, higher occurrence of infectious diseases and white muscle disease (Enjalbert et al., 2006). It may also result in immune and endocrine disorders, especially thyroid dysfunction. (Kohrle et al., 2005).

The bioavailability of Se is associated with its forms. Currently, sodium selenite and selenate are the usual inorganic Se source used as a supplement in animal feeds, and organic forms such as Se enriched yeast, seleno-cysteine or selenomethionine are the commonly used organic Se sources in animal feeds. Recently, elemental nano-Se has attracted a wide spread attention to its high bioavailability and low toxicity (Xu et al., 2003; Wang et al., 2007; Zhang et al., 2008). Nano minerals improve the bioavailability due to its novel characteristics such as high surface activity, a lot of surfaces active centers, strong adsorbing ability and high catalytic efficiency (Wang et al., 2013). Nano-Se has efficient functions on animal growth, reproduction and immunity systems (Shi et al., 2010). In sheep, Nano-Se had improved ruminal fermentation, nutrient digestibility (Shi et al., 2011). In addition, some reports on rats and mice demonstrated that Nano-Se had higher efficiency than sodium selenite and other Se sources in up-regulating selenoenzymes, exhibiting lower toxicity (Zhang et al., 2001). Subsequent studies also pointed out that Nano-Se has more beneficial effects to improve activity of glutathione peroxidase, blood biochemical indices with lower toxicity comparing with organic or inorganic Se sources (Yaghmaie et al., 2017).

Very few reports are there on various effects of different forms of Se supplementation in livestock and reports in ruminants is still scarce. Also, there is little information in the literature related to effects of different forms of Se supplementation on buck breeding and semen profile. Thus, the present investigation is aimed to compare the effect of dietary supplementation of inorganic, organic and nano Se on the performance of bucks with following objectives:

Objectives

i. To study the effect of different sources of Se supplementation on feed intake and nutrient utilization in bucks.
ii. To study the influence of different sources of Se supplementation on semen quality of bucks.
iii. To determine the haemato-biochemicals parameters and antioxidant status as affected by different sources of Se supplementation in bucks.

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

Adequate essential trace mineral intake and absorption is required for a variety of metabolic functions including immune response, reproduction and growth (Larson, 2005). Se is recognized as an essential trace element, and its deficiency has been associated with impaired growth, fertility, and health in farm animals (Khanal et al., 2010). Diets for ruminant animals are often of plant origin and the Se concentration within plants can be extremely variable. Consequently, concentration of dietary Se can be deficient, and Se supplementation may be required. Supplementation of trace elements in animal diets has long been practiced to ensure optimum growth production and improve immune response (Overton and Yasui, 2014). In recent years, there has been considerable interest in the use of Se in ruminant diets. Different forms of Se routinely used for dietary supplementation are inorganic, organic and nano Se. There are reports of improved growth, better production performance, reproduction and health in ruminants fed inorganic, organic and nano Se. Here upon, the literature pertaining to the dietary supplementation of different forms of Se in bucks has been reviewed under following sub headings:

1) Chemical nature and properties of Se
2) Sources of Se supplementation and their bioavailability
3) Requirements of Se in different species
4) Biological role of Se
5) Effect of Se supplementation on growth performance and nutrient digestibility
6) Effect of Se supplementation on haematological parameters
7) Effect of Se supplementation on blood biochemical constituents
8) Effect of Se supplementation on antioxidant status
9) Effect of Se supplementation on immune response
10) Effect of Se supplementation on hormonal profile
11) Effect of Se supplementation on seminal attributes
12) Effect of Se supplementation on milk production and milk quality
13) Effect of Se supplementation on Se concentrations in biological fluids.

2.1 Chemical nature and properties of Se

Se is a naturally occurring metalloid element that is essential to human and animal health in trace amounts but is harmful in excess. It was first identified in 1817 by the Swedish chemist, Jons Jakob Berzelius. It has chemical and physical properties intermediate between metals and non-metals and is similar to those of sulphur, arsenic and tellurium, all of which are in Group VI of the periodic chart of the elements (Verma et al., 2012). Like Sulphur, Se can exist in different oxidation states as selenide (2-), Se (0), selenite (4+) and selenate (6+), respectively (Khanal et al., 2010). It behaves antagonistically with Copper and Sulphur in humans and animals inhibiting the uptake and function of these elements (Netto et al., 2014).

2.2 Sources of Se supplementation and their bioavailability

The concentrations of Se in plant material are highly correlated with those in the soil. Fertilization of soil with Se increases Se concentrations in plants (NRC, 2001). The Se status in animals and plants varies markedly around the world as a result of different geological conditions. High Se concentrations are associated with some phosphatic rocks, organic rich black shales, coals, and sulphide mineralization, whereas most other rock types contain very low concentrations. Globally Se deficient soils are far more widespread than are seleniferous ones (Khanal et al., 2010). Animal health is affected by Se deficiency or excess in the diet, the intake of Se being dependent on the amount of Se taken up by plants as bioavailable Se (Fordyce, 2005).

Generally, Se is utilized as inorganic, organic and nano forms. Form of Se supplementation has an important bearing on its possible ameliorative effects and/or on general wellbeing and development of organisms (Pappas et al., 2008). Both form and the total intake of Se are equally important with regards to the potential health-related effects. It has been reported that organic forms of Se have greater bioavailability compared to the inorganic forms (Burk et al., 2015) because sodium selenite and sodium selenate must first be converted to hydrogen selenide and then to selenophosphate before they can be utilised in selenoprotein synthesis (Raymanet al., 2008; Kachuee et al., 2013). The most common inorganic Se sources are sodium selenite (SS) and sodium selenate, which are usually provided in mineral premixes or injected. Organic Se sources are seleno-amino acids (e.g. selenomethionine (Se-Met) and selenocysteine), which are found in Se yeast or in feeds grown on Se-rich soils.

Se from organic sources is assimilated much more efficiently than the inorganic forms, and could be expeditiously utilized for synthesis of seleno proteins in conditions of stress (Surai et al., 2016). The toxicity level of organic forms is also lower than that of inorganic selenite or selenate species, indicating that the higher bioavailability may be compensated by lower toxicity levels (Rayman, 2004). Usually, the organic forms of Se have higher bioavailability and antioxidant properties than inorganic forms (Wang et al., 2011). Moreover, it has been demonstrated that for modulation of antioxidant activities of GSH-Px's (glutathione peroxidases) under oxidative stress conditions, selenocysteine, derived from selenomethionine following B6-dependent-transsulfuration reactions, the organic Se is mineralized to selenide and re-synthesized into a seleno-cysteinyl moiety that regulates the gene expression and activates the GSH-Px system. In contrast, the metabolic pathway of inorganic Se is dissimilar i.e., it bypasses the B6-dependent-transsulfuration process for the modulation of GSH-Px system (Qazi et al., 2019).

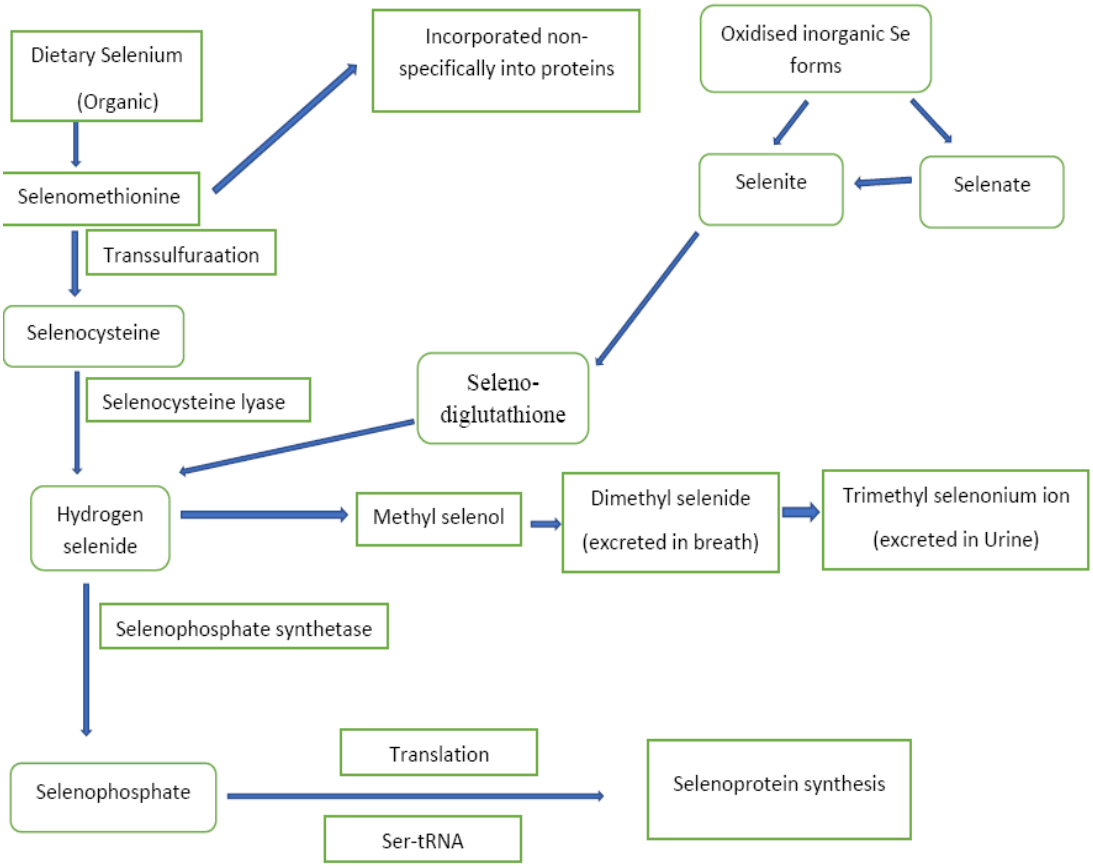


Fig. 2.1: Metabolic pathway of different forms of Se

Description of metabolic fate of different forms of Se is depicted in Fig. 2.1, which shows that Selenomethionine (SeMet) from organic sources (Se-yeast) and other food proteins undergoes trans-sulphuration reactions and is converted to selenocysteine (SeCys). Selenocysteine is then converted to hydrogen selenide (H_2Se); this reaction is catalysed by a substrate-specific enzyme selenocysteine lyase. Hydrogen selenide is converted to selenophosphate via selenophosphate synthetase, and following reaction with tRNA-bound serinyl residues, it produces a SeCys-bound tRNA from which SeCys is inserted co-translationally and further translated to selenoproteins (GSH-Px, SELENOP etc.). Hydrogen selenide could also be methylated and detoxified, and excreted in breath (as dimethyl selenide) and urine (as trimethylselenonium ion). Alternatively, selenomethionine may also be non-specifically incorporated into proteins such as albumin and haemoglobin in place of methionine. Inorganic forms of Se such as selenate and selenite are metabolized through thiol-dependent reduction reactions, producing hydrogen selenide via GS-Se-SG (selenodiglutathione), which is a starting point for the synthesis of selenoproteins.

The Se nano particles are a new form of Se supplementation. In the early stages of research, Se nano particles (SN), which are bright red, highly stable, highly water soluble and of nano-defined size in the zero redox state (Se_0), have been constructed for applications in nutritional supplements and in medical therapy (Davda and Labhasetwar, 2002). SN showed new characteristics of transport and uptake and exhibited higher absorption efficiencies than their conventional counterparts (Zha et al., 2008). It has been confirmed that SN have a greater effectiveness in up regulating selenoenzymes and reveal less toxicity than inorganic Se (Zhang et al., 2004). Owing to the boon of size effect and great surface reactivity, nano particles have been earlier utilized in pharmaceutical applications for enhancing the bioavailability of medicines and targeting remedial agents to specific organs (Davda and Labhasetwar, 2002). Subsequent studies also pointed out that Nano-Se has more beneficial effects to improve activity of glutathione peroxidase, blood biochemical indices with lower toxicity comparing with organic or inorganic Se sources (Yaghmaie et al., 2017). In spite of all advantages and opportunities which nano-materials use offer, it is still in the primary steps of its evolution and not practical throughout the world (Kachuee et al., 2019).

2.3 Requirements of Se in different species

Se concentrations in feedstuff range from 0.01- 0.3 mg/kg depending on Se content in the soil and pH (NRC 2007). Maximum tolerable level of Se in ruminant feed is 5 mg/kg diet. However, this value was set considering animal health (only) and lower levels are necessary to avoid excessive accumulation in edible tissues (NRC 2005). European Food Safety Authority suggests a maximum of 0.5 mg Se/kg diet as per EFSA Panel on Additives and Products or Substances used in Animal Feed 2016.

Recommended dietary allowance (RDA)

Species	**RDA**
Sheep and Goat	100–200 µg/kg dry matter of feed/day
Dairy cow	100 µg/kg dry matter of feed/day
Beef cow	300 µg/kg dry matter of feed/day
Bovine calf	100 µg/kg dry matter of feed/day
Horse	100 µg/kg dry matter of feed/day
Donkey	150 µg/100 kg BW
Pig	150–300 µg/kg dry matter of feed/day

(Qazi et al., 2019)

2.4 Biological role of Se

Se is an essential trace element for maintaining normal physiological processes in animals and humans (Sethy et al., 2014). Se exerts multiple actions on the antioxidant (Arthur, 2001), reproductive (Maiorino et al., 1999), endocrine (Beckett and Arthur, 2005), and immune systems (McKenzie et al., 1998; Beck et al., 2005). When ingested, it is incorporated into the functionally important group of selenoproteins, where, in combination with cysteine, a sulphur-containing amino acid, it is present mostly in the form of selenocysteine. (Khanal et al., 2010).

2.4.1 As antioxidant

Se is present within 25 selenoproteins in the form of the amino acid selenocysteine (Sec) (Papp et al., 2010). The enzyme glutathione peroxidase (GSH-Px) is one of the most important selenoproteins. GSH-Px contributes to the oxidative defense of animal tissues by catalysing reduction of hydrogen and lipid peroxides

(Barciela et al., 2014). This enzyme reduces peroxides in cells thus preventing oxidative injury to cells (Rotruck et al., 1973).

The most important amino acid in the active site of the enzyme GSH-Px is L-selenocysteine which is responsible for reducing hydroperoxides at the expense of the tripeptide glutathione GSH. The ability that the enzyme has to promote the reduction of peroxides lies in the redox properties of the selenol moiety of L-selenocysteine. Initially, the selenol functionality in the enzyme E-SeH (selenol, active catalytic site of enzyme) reacts with a peroxide molecule to generate the corresponding alcohol or water and E-SeOH (Selenenic acid). The latter then reacts with one equivalent of glutathione to produce water and the corresponding selenenyl sulphide E-Se-SG (Selenenyl sulphide). The last step is the reaction of glutathione with selenenyl sulfide producing the oxidized glutathione (GSSG) and regenerating the reduced enzyme selenol to resume the catalytic cycle (Alberto et al., 2010). Fig. 2.2 is depicting the redox cycle of glutathione peroxidase

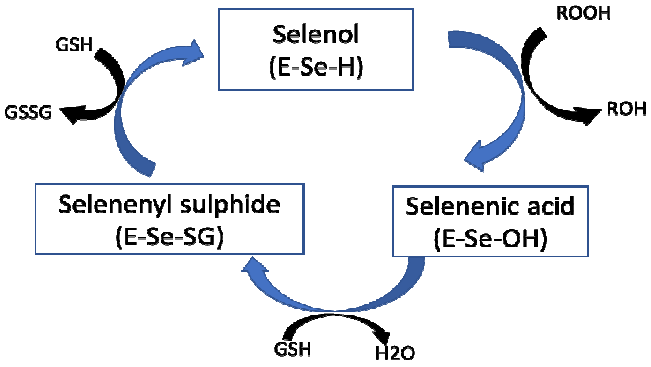


Fig.2.2: Redox cycle of GSH-Px (Alberto et. al., 2010)

The total process consists of the reduction of one equivalent of a reactive oxygen species at the expense of two equivalents of glutathione, producing two equivalents of water (when R = H) and oxidized glutathione (GSSG), as proposed by Alberto et al. (2010).

$$RCOOH + GSH\text{-}Px \xrightarrow{2GSH} ROH + HOH + GSSG$$

Where, R= H or Alkyl

2.4.2 As immune modulator

Oxidative stress affects health of animal, damaging the cells and tissues by free radicals or oxidants and if not controlled, it may impair the animal's immune system consequently lowering immunity of animals (Yatoo et al., 2013). Supplementation with Se can boost the immunity of the animals. Previous studies have demonstrated that the addition of Se in the diet improved both cell-mediated and humoral immune responses (Andrieu, 2008). Se increases antibody levels, enhances the phagocytic activity of neutrophil, granulocytes and macrophages and, when stimulated with myogens, increases T lymphocyte counts (Hoffmann 2007, Kamada et al., 2007). Se is indispensable in the production of the lymphocyte migration inhibition factor and interleukin 2 (Wintergerst et al., 2007), which accelerates the proliferation, maturation and activity of T lymphocytes (Shrimali et al., 2008). T cells are particularly sensitive to Se deficiency because their cell membrane contains lipids that are more readily oxidized than the membrane lipids of B lymphocytes (Arthur et al., 2003). Se deficiency lowers the count and cytotoxic activity of T lymphocytes, an effect which is accompanied by decreased lymphotoxin production (Hawkes et al., 2001).

The molecular mechanisms involved in the effect of Se on the immune system have not been fully elucidated. Se could exert its effect aided by selenoenzymes and thioredoxin reductase. Those selenoenzymes are responsible for maintaining thiol groups on the surface of lymphocyte membranes in a reduced state, which significantly enhances the lymphocyte proliferative response to myogens, increases immunoglobulin production and boosts the killing activity of lymphocytes (Akyol et al., 2007). Selenates increase the levels of reduced glutathione inside cells and maintain thiol groups in a reduced state (Qin et al., 2007). Se deficiency can aggravate an inflammatory process in the body, depressing the activity of selenoenzymes, which inhibit excessive synthesis of arachidonic acid from linoleic acid (Sakr et al., 2007). At normal concentrations of Se, GSH-px inhibits phospholipase A2 and lowers the levels of arachidonic acid and its metabolites, the products for eicosanoid synthesis, in particular leukotriene B4. the synthesis of prostacyclin from arachidonate is intensified, and it inhibits the lipoxygenase metabolic pathway (Johnson et al., 2000).

The effect of Se on the immune system could also be produced via a different pathway. The use of sodium selenate as an immune-stimulator influences the

expression of α and β subunits of interleukin 2 on the surface of activated T and B lymphocytes, natural killer 334 (NK) cells and lymphokine activated killer (LAK) cells, but it does not affect the endogenous concentrations of interleukin 1 (IL-1), interleukin 2 (IL-2) or interferon γ (IFN-γ). by binding to IL-2 receptors, IL-1 is internalized, and it induces the signal for the transition of activated cells from phase G1 (postmitotic) to phase S (DNA synthesis) of the cell cycle (Jozsef and Filep, 2003). Se deficiency inhibits neutrophil migration and disrupts the distribution of receptors on the neutrophil surface. The above can most probably be attributed to the oxidation of tubulin by excess H_2O_2 and the resulting damage to neutrophil microtubules (Haddad et al., 2002). Neutrophils sampled from Se-deficient animals were also characterized by impaired ability to produce and release free radicals for the extermination of foreign cells (Yang et al., 2004). Interestingly, high Se doses also attenuate the immune response. In an in vivo study, excessive Se concentrations inhibited the growth of cells in S and G2 phases of the cell cycle and decreased the synthesis of antibody proteins and prostaglandins (Zagrodzki, 2004).

2.4.3 In Endocrine metabolism

Se is also involved in proper functioning of some endocrine hormones (Lin et al., 2014). Proper dietary Se is required for triiodothyronine (T3) production. However, production of this hormone actually requires adequate supply of Se with sufficient iodine. Therefore, Se converts thyroxine (T4) to triiodothyronine (T3) regulated by thyroxin stimulating hormone (TSH). Thyroxine hormone is essential for controlling metabolic process in animals. Thyroxine hormone also influences testicular development and function (Wanger et al., 2008). In addition, T3 regulate the maturation and growth of testis controlling the sertoli cells and the leydig cells proliferation, testicular development in rats and other mammal species (Hosberger and Cooke, 2005).

Three differentially distributed selenocystein containing oxido-reductases (DIO1, DIO2, and DIO3) constitute the family of iodothyronine deiodinases (DIO). They catalyze the activation (DIO1 and DIO2) and inactivation (DIO3) of the thyroid hormones thyroxine (T4), 3, 5, 3'- triiodothyronine (T3), and reverse-3, 5, 3'-triiodothyronine (rT3) by removing distinct iodine moieties, as indicated in Fig. 2.3.

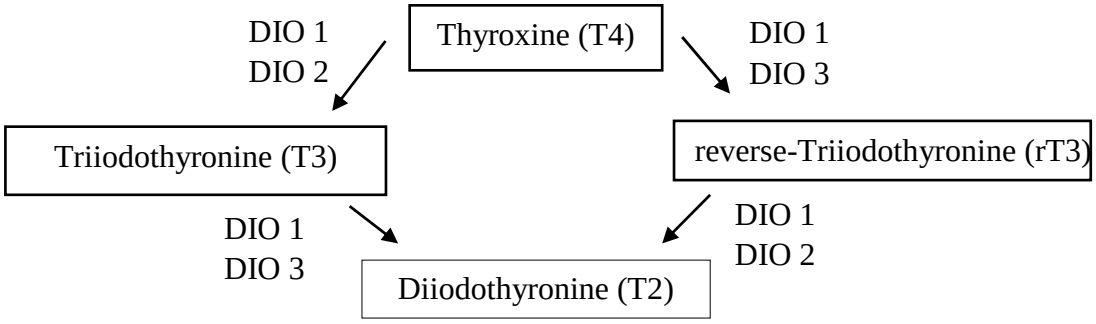


Fig. 2.3: Metabolism of thyroid hormones by types 1, 2, and 3 deiodinases (Bianco and Kim, 2006).

The three DIOs exert different actions as DIO1 plays a role in T3 production in the thyroid gland and controls the circulating T3 levels, whereas DIO2 and DIO3 are proposed to function in the local deiodination processes. Among various selenoproteins, DIOs rank high in the hierarchy of Se supply during Se deficiency, DIO1 expression being maintained or slightly increased in the thyroid gland. In the brain and placenta, DIO1 and DIO3 expression is maintained during Se restriction, whereas DIO1 expression decreases in other tissues. The mechanisms of DIOs regulation by Se availability is not fully understood, nevertheless, it appears to be tissue or organ specific. Additional regulators of DIOs expression are T3 and thyrotropin (TSH) (which act in a feedback loop), and cyclic adenosine monophosphate (cAMP) (Koenig, 2005).

2.4.4 In male reproduction

Se performs significant functions in the male reproductive system which are regulated by selenoproteins, especially Glutathione peroxidase 4 (GSH-Px4) and SELENOP **(**Qazi et al., 2019). GSH-Px4 is distinctly expressed in testes and has both an antioxidant as well as a structural role. It constitutes over 50% of mitochondrial capsule (as an oxidatively inactivated protein) in mid piece of mature sperm (Foresta et al., 2002). In an early stage of spermatogenesis, GSH-Px4 is believed to protect the developing sperm from oxidative stress-induced DNA damage, however, in the later phase, through cross linkage with proteins in mid piece region, it provides the integrity to the sperm mid piece by becoming a structural component of mitochondrial sheath circumventing the flagellum, which is an essential component for sperm stability and motility. SELENOP serves as a transport protein for Se and is also expressed in vesicle like structures in the basal region of the sertoli cells.

During the process of testosterone biosynthesis, ROS are generated and their excessive production contributes to male infertility (Nishimura et al., 2001). SELENOP may play a role as an intracellular antioxidant in Leydig cells. Besides, cytosolic GSH-Px was also implicated in counteracting the H_2O_2 generated as a result of testosterone biosynthesis, but its expression in testis was relatively lower (Nishimura et al., 2001). However, as for the effects of SELENOP on steroid biosynthesis in leydig cells, it was proposed that, in addition to its intracellular antioxidant role, SELENOP might also act as an extracellular antioxidant protecting Leydig cells from oxidative damage. It was suggested that SELENOP plays an important antioxidant role in protecting Leydig cells from the oxidative damage resulting from testosterone biosynthesis pathway (Koga et al., 1998). It has been suggested that blood testosterone concentrations have positive correlation with concentrations of Se. (Villaverde et al., 2014)

Se has also been implicated to play an important role in spermatogenesis. Watanabe and Endo (1991) reported that the ratio of abnormal sperm was high (6.8% to 49.6%) in Se-deficient group compared to the control group (4.0% to 15.0%). The morphological defects were more pronounced in sperm head compared to other regions i.e., mid piece and tail. However, the frequency of chromosomal abnormalities in spermatocytes (MI stage) was comparable between Se-insufficient and the control groups (Watanabe and Endo, 1991). The given fig.2.4 is depicting the role of Se in improving male fertility (Qazi et al., 2019)

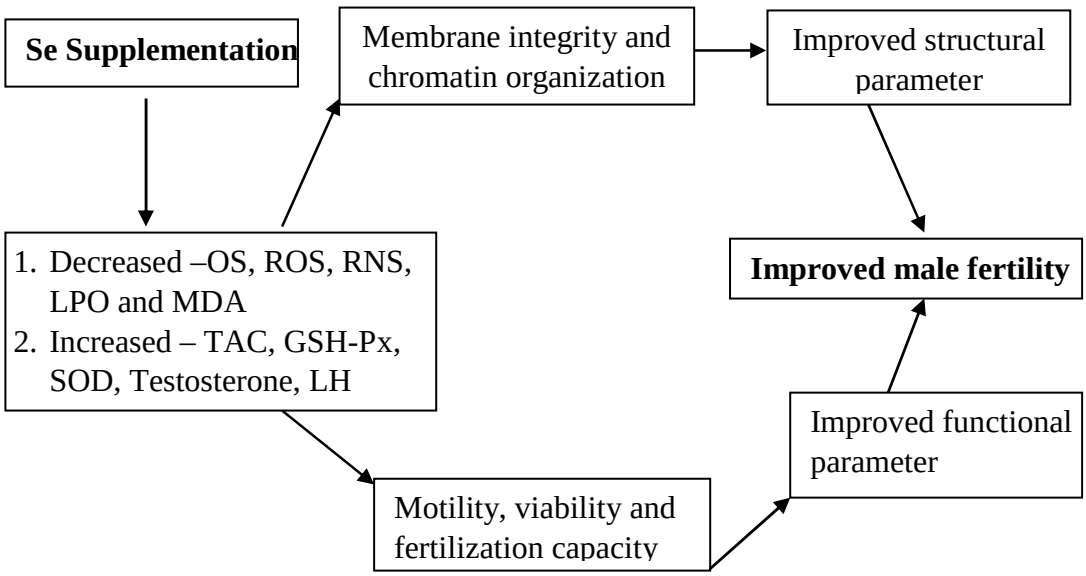


Fig.2.4: Role of Se in improving male fertility

Abbreviations used: OS: Oxidative stress, MDA: Malondialdehyde, TOAC: Total antioxidant capacity, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, LH: Luteinizing hormone.

2.5 Se deficiency

Low Se levels can lead to the development of nutritional muscular dystrophy, also known as white muscle disease, in lambs, kids, foals, calves and poultry from birth to 3 months of age (Muth et al., 1958). Se deficiency may also cause exudative diathesis in poultry as well as dietary necrotic liver degeneration and mulberry heart disease in pigs. Parturition problems resulting from reduced tension of the muscular layer of the uterus, post parturient paraplegia, placental retention and purulent inflammations of the uterine lining are also attributed to low Se levels (Rutigliano et al., 2008). Se deficiency contributes to the formation of ovarian cysts and increased embryonic mortality in the first 3-4 weeks after insemination. Se and vitamin E facilitate neutrophil migration to the mammary gland, and they enhance the bactericidal effects of neutrophils, thus shortening and alleviating the symptoms of clinical mastitis (Moeini et al., 2009). Se poisoning is rarely encountered, and it most often results from an overdose of Se supplements. The most common forms of selenosis are chronic selenosis, referred to as alkali disease, and acute selenosis, popularly known as blind staggers. (Zarczynska et al., 2013).

2.6 Effect of Se supplementation on growth performance and nutrient digestibility

Kumar et al. (2008) studied the effect of different levels of Se supplementation on growth rate and nutrient utilization in lambs. Eighteen male lambs were divided into three groups of six animals in each and fed a total mixed ration along with Se supplementation as sodium selenite at 0 (T1, control), 0.15 (T2), and 0.30 ppm (T3) levels. Supplementation of Se both at 0.15 and 0.30 ppm levels had no significant ($P>0.05$) effect on intake and digestibility of DM, OM, CP, EE, NDF, ADF, and hemicellulose; balances of calcium and phosphorus; and level and intake of digestible CP and TDN. The ADG was highest in group T3, followed by group T2, and lowest in the T1.

Wang et al. (2009) conducted a study to evaluate the effects of Se-yeast (SY) supplementation on feed digestibility in dairy cows. Twenty-eight multiparous

Holstein dairy cows were supplemented with 0 (control), 150 (Low Se-Yeast), 300 (Medium Se-yeast) and 450 (High Se-yeast) mg Se yeast. The DMI was not affected (P >0.05) but digestibility of DM, OM, CP, EE, NDF and ADF in the total tract were higher (P>0.01) for LSY and MSY than for control and HSY.

Alimohamady et al. (2013) evaluated the effects of different sources of Se on performance and nutrient digestibility in male lambs. The lambs were randomly allotted to five treatments groups and supplemented basal diet (containing 0.06 mg Se/kg DM;) along with (1) control without supplementary Se, (2) 0.20 mg/kg Se as sodium selenite, (3) 0.40 mg/kg Se as sodium selenite, (4) 0.20 mg/kg Se as Se yeast, and (5) 0.40 mg/kg Se as Se yeast. There were no significant effects for either source or amount of Se on average daily gain (ADG), average daily feed intake but the digestibility of DM, OM, CP, NDF, and ADF were significantly higher (P<0.05) by Se yeast supplementation.

Najafnejad et al. (2013) investigated the effect of sodium selenite, Se-enriched yeasts and Se nano particles on nutrient digestibility in lactating dairy cows. The treatments include basal diet without supplementary Se (Control), basal diet+0.3 mg/kg Se as sodium selenite (SeS), basal diet + 0.3 mg/kg Se as Se yeast (SeY) and basal diet + 0.3 mg/kg Se as nano particles (SeNP), respectively. The digestibility of dry matter, organic matter, non fiber, ether extract, neutral detergent fiber and non fiber carbohydrates increased significantly (P<0.05) by Se yeast and nano Se supplementation.

Wei et al. (2019) studied the dose dependent effects of a hydroxy-analogue of selenomethionine (HMSeBA) on rumen fermentation, apparent nutrient digestibility, in mid-lactation dairy cows in comparison with sodium selenite (SS). Fifty mid-lactation dairy cows were randomly assigned 1 of 5 treatments. The cows were fed a basal diet containing 0.06 mg/kg DM of Se (control) or the same basal diet supplemented with SS, yielding 0.3 mg of Se/kg of DM (SS-0.3), or HMSeBA, yielding 0.1, 0.3, or 0.5 mg of Se/kg of DM (SO-0.1, SO-0.3, and SO-0.5, respectively), during the experimental period. Compared with SS, HMSeBA enhanced the apparent digestibility of CP, NDF, and ADF.

2.7 Effect of Se supplementation on haematological parameters

Faixova et al. (2007) evaluated the effect of Se-yeast supplementation on haematological parameters in lambs. Ten animals were divided into two groups and fed experimental diets for 3 months. The first group received a basal diet while second groups were supplemented with Se 0.3 mg/kg/DM in the form of Se-enriched yeast. After the experiment total erythrocyte count and osmotic resistance of red blood cells were significantly higher ($P<0.01$) in Se-supplemented animals.

Pisek et al. (2008) assessed the impact of Se supplementation on white blood cell parameters in the blood of ewes. The results of the experiment documented that the supplementation of different forms of Se did not markedly influence the dynamics of blood parameters in non-pregnant, pregnant and lactating ewes if the intake of vitamins and other essential microelements was adequate.

Alimohamady et al. (2013) studied the effects of different sources of Se on haematological parameters in male lambs. The lambs were randomly allotted to five treatments groups and supplemented basal diet (containing 0.06 mg Se/kg DM;) along with (1) control without supplementary Se, (2) 0.20 mg/kg Se as sodium selenite, (3) 0.40 mg/kg Se as sodium selenite, (4) 0.20 mg/kg Se as Se yeast, and (5)0.40 mg/kg Se as Se yeast. The mean RBC (10^{12}/L), PCV (%), Hb (g/dl), and WBC (10^{9}/L) values in all groups were found to be statistically ($P>0.05$) comparable among different treatments. The values of all these blood parameters were found to be within the normal reference range.

Shinde et al. (2009) studied the effect of supplemental vitamin E and/or Se on haematology of male buffalo calves. Twenty calves were divided into four groups of five calves each. Group I was control (without any supplementation), groups II, III and IV were supplemented with 0.3 mg Se, 300 IU of DL-α-tocopheryl acetate, and both 300 IU DL-α-tocopheryl acetate and 0.3 mg Se, respectively. No significant difference ($P>0.05$) in haematological parameters (haemoglobin, packed cell volume, red blood cell, white blood cell) due to the supplementation of vitamin E, Se or both vitamin E + Se buffalo calves was observed except for Significantly higher ($P<0.01$) concentration (mg/dl) of HDL cholesterol in Se and Se + vitamin E groups as compared to control and vitamin E supplemented groups.

Sethy et al. (2014) conducted a study to elucidate the effect of Se yeast and sodium selenite supplementation on haematology parameters in eighteen male kids. Group I was supplemented with basal diets and in addition, kids in group II and III were supplemented with 0.3 mg Se per kg DM as Se yeast and sodium selenite respectively. Non-significant differences were observed for haemoglobin content and packed cell volume among all the groups.

2.8 Effect on blood biochemicals parameters

Kumar et al. (2008) evaluated the effect of different levels of Se supplementation. 18 male lambs were divided into three groups. The animals in the control group (GrI) were fed a standard TMR (without Se), animals in Gr II and Gr III were additionally supplemented with 0.15 mg Se/kg of diet through sodium selenite (inorganic Se) and Jevsel-101 (organic Se), respectively**.** The results revealed that supplementation of Se had no effect on serum total cholesterol, total protein, albumin, globulin, albumin:globulin ratio.

Shinde et al. (2009) evaluated the effect of supplemental vitamin E and/or Se on their blood metabolic profile and serum enzymes in twenty male buffalo calves. Group I was control (without any supplementation), groups II, III and IV were supplemented with 0.3 mg Se, 300 IU of DL-α-tocopheryl acetate, and both 300 IU DL-α-tocopheryl acetate and 0.3 mg Se respectively. No significant change in concentration of glucose, total protein, albumin, globulin, urea, creatinine, total cholesterol, and triglycerides was found in the treatment groups. The activities of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, creatine kinase and lactate dehydrogenase remained unaffected in treatment groups.

Mahmoud et al. (2012) determined the effect of the combination of vitamin E (Vit E) and Se injection on some blood parameters in Ossimi rams. Fourteen mature healthy Ossimi rams were randomly divided into two equal groups control (CG) and treatment (TG). Rams of the CG were not supplemented with Se and Vitamin E but TG were treated twice weekly with 5 mg sodium selenite and 450 mg Vit E for 1 month. Treated rams showed higher values for blood parameters in terms of higher ($P<0.05$) serum total protein, albumin, globulin, glucose, total cholesterol and calcium in comparison with the control group.

Alimohamady et al. (2013) evaluated the effects of different sources of Se on blood biochemical parameters in male lambs. The lambs were randomly allotted to five treatments groups and supplemented basal diet (containing 0.06 mg Se/kg DM;) along with (1) control without supplementary Se, (2) 0.20 mg/kg Se as sodium selenite, (3) 0.40 mg/kg Se as sodium selenite, (4) 0.20 mg/kg Se as Se yeast, and (5) 0.40 mg/kg Se as Se yeast. There were no significant differences in serum activities of ALP, CPK, and AST between treatments (P>0.05).

Sethy et al. (2014) elucidated the effect of Se yeast and sodium selenite supplementation on blood biochemicals in male kids. Group I was supplemented with basal diets and in addition, kids in group II and III were supplemented with 0.3 mg Se per kg DM as Se yeast and sodium selenite respectively. No significant differences were observed for the serum glucose, total protein, albumin, globulin, A: G ratio, urea, creatinine and total cholesterol (P>0.05) among all the groups.

Ziaei (2015) conducted the study to investigate the effect of Se and vitamin (vit) E supplementation on biochemical metabolites in Raieni goats. Ninety adult Raieni goats were randomly allocated into nine groups of ten each. The experimental diets consist of three levels of Se (0.0, 0.3 and 0.5 mg/kg of feed) and three levels of vit E (0.0, 20 and 50 mg/kg of feed). The results of this experiment revealed that supplementation of diets with Se or vit E had no significant effect on biochemical metabolites.

2.9 Effect of Se supplementation on antioxidant status of animal

Pechova et al. (2012) compared the effect of supplementation of different forms of Se on the metabolism of kids at the time of weaning. The experiment was performed with forty-five kids whose mothers were supplemented with various forms of Se. Group C was control while the other four groups were supplemented with Se for six weeks before (0.3 mg/goat/day) and after parturition (0.9 mg/goat/day). Group Se-I received sodium selenite while the other groups received organic forms: Se-lactate-protein complex (Se-L), Se-proteinate (Se-P) and Se-yeast (Se-Y). Total antioxidant status in serum that was higher in the group Se-Y as compared with the group Se-I and the control group.

Alimohamady et al. (2013) evaluated the effects of different sources of Se on antioxidant status in male lambs. The lambs were randomly allotted to five treatments

groups and supplemented basal diet (containing 0.06 mg Se/kg DM;) along with (1) control without supplementary Se, (2) 0.20 mg/kg Se as sodium selenite, (3) 0.40 mg/kg Se as sodium selenite, (4) 0.20 mg/kg Se as Se yeast, and (5) 0.40 mg/kg Se as Se yeast. Dietary Se supplementation significantly improved ($P<0.001$) glutathione peroxidase activity in blood.

Ibrahim and Mohammed (2018) analysed the effect of different dietary Se sources supplementation on antioxidant status in sheep. Thirty-two Ossimi lambs were allocated into four equal groups. The lambs of control group were fed on basal diet containing 0.17 mg Se/kg dry matter (DM). The treated lamb groups fed on the same basal diet along with Se supplementation at the rate of 0.30 mg/kg DM was provided as sodium selenite (SS), Se yeast (SY) and Nano-Se particles (NS). The results obtained shows serum total antioxidant capacity (TAC) concentrations and glutathione peroxidase (GSH-Px) activity increased ($P<0.05$) for lambs fed SS, SY and NS vs. control. Higher ($P<0.05$) levels of TAC and GSH-Px levels were observed for lambs fed NS than those fed SS and SY.

Saba et al. (2019) studied the effect of dietary Se supplementation (organic or inorganic) on antioxidant status in late pregnant, lactating ewes and their suckling lambs. The control group fed the basal ration without any supplement, while the tested group (T1) fed 0.3 mg Sodium Selenate/kg diet and the tested group (T2) fed the basal ration + 0.3 mg Se yeast /kg diet. total antioxidant capacity was significantly higher in dietary treatments, (T1 and T2) compared with that of control group.

Raheem et al. (2019) performed a study to investigate the effects of pre and post-lambing intramuscular (IM) injections of vitamin E (VE) and Se on the antioxidant status of Ossimi ewes. The control group (CG) was administered 3 ml of normal saline. The treated group (TG) was administered a combination of 5 mg of sodium selenite and 450 mg of VE twice weekly for 2 weeks pre and post lambing. Treated ewes had higher antioxidant enzyme biomarkers as compared to control group. The MDA concentration (mmol/l) was higher ($P<0.05$) in control ewes compared with the treated ones. The blood concentration of TAC was significantly higher ($P< 0.05$) in the TG compared to the CG. Plasma levels of reduced GSH (U/g Hb) were significantly higher in the TG compared with the CG.

Rashnoo et al. (2020) conducted a study to assess the effects of supplementation of Se and iodine via slow-release boluses in late pregnancy on antioxidant status of goats. The boluses were administered four weeks prior to the expected kidding time to supply daily amounts of (1) 0 mg Se and 0 mg I, (2) 0 mg Se and 0.40 mg I, (3) 0.25 mg Se and 0 mg I and (4) 0.25 mg Se and 0.4 mg I. Regardless of I supplementation, whole blood GSH-Px activity were significantly greater ($P<0.05$) in goats received 0.25mg Se per day than in unsupplemented goats.

2.10 Effect of Se supplementation on immune response

Kumar et al. (2008) performed a study to evaluate the effect of different levels of Se supplementation on immune response in lambs. Eighteen male lambs were divided into three groups of six animals in each and fed a total mixed ration along with Se supplementation as sodium selenite at 0 (T1, control), 0.15 (T2), and 0.30 ppm (T3) levels. There was a significant ($P<0.05$) increase in the humoral immune response in the Se supplemented groups.

Kachuee et al. (2014) conducted study to determine the effects of organic and inorganic Se supplementation during late pregnancy on passive immunity in Merghoz goats. Four weeks before the expected kidding, the basal diets were supplemented with 0 (control), 0.3mg Se/head/day in the form of L-selenomethionine (Se-Met) and 0.3mg Se/head/day in the form of sodium selenite. The mean serum IgG levels was not different among goats and kids ($P > 0.05$). However, the WBC, neutrophil and lymphocyte counts were higher in the kids of goats in the Se-Met group compared with the control group on the day of birth and 7 days of age ($P < 0.05$). It seemed that Se-Met could have influenced the kids immunity at birth and 7 days of age while selenite has not affected the passive immunity in Merghoz goats.

Moeini and Jalilian (2014) investigated the effect of Se and vitamin E supplementation during late pregnancy on immune status of Sanjabi ewes and their lambs. Twenty-seven Sanjabi ewes were randomly assigned to three treatments groups. Four and two weeks before expected lambing, ewes were injected intramuscularly 0 ml (C) 5 ml (T1), 10 ml (T2) Se and vitamin E respectively. Each ml of the supplement containing of 0.5 mg Se as sodium selenite and 50 mg vitamin E as D, L-alpha-tocopheryl acetate. The results showed that the colostrum IgG concentrations at one hour postpartum were higher in T2 compared with controls

($P<0.05$). White blood cell counts were higher in lambs of T2 when compared with controls ($P<0.05$).

Erdogan et al. (2017) analysed the effect of supplementing organic Se at different levels in Norduz ewes in late pregnancy on its level of Immunoglobulin G (IgG). Group 1 was fed a standard pregnant sheep concentrate basal diet without any additional Se supplement (control). Experiment groups were fed diets supplemented with 0.150 mg/kg, 0.300 mg/kg, and 0.450 mg/kg organic Se to the same basal diet. IgG content of the serum was significantly higher in the ewes in late pregnancy regardless of Se intake. Lambs from Se supplemented ewes had higher serum IgG concentrations compared with lambs from the control group ($P<0.05$). Moreover, there was a strong positive correlation between serum IgG concentration of ewes and that of lambs ($r = 0.725$; $P<0.01$).

Zarczynska et al. (2019) performed a study on sixteen Holstein Friesian calves to evaluate effect of Se and Vit. E supplementation on IgG level. The first group was composed of calves whose mothers did not receive Se supplements (Se0) while second group mothers were administered intramuscular injections of a Se and vitamin E supplement containing 0.5 mg of sodium selenite/ml and 50 mg of tocopherol acetate/ml in a single dose of 30 ml (Se30) ml, 10 days before the expected parturition date. IgG concentration in the serum of the S30 group of calves was significantly higher during the entire experiment (21 days of calves' life) in comparison with the calves from the S0 group.

2.11 Effect of Se supplementation on hormonal profile

Kumar et al. (2008) performed a study to evaluate the effect of different levels of Se supplementation on thyroid metabolism in lambs. Eighteen male lambs were divided into three groups of six animals in each and fed a total mixed ration along with Se supplementation as sodium selenite at 0 (T1, control), 0.15 (T2), and 0.30 ppm (T3) levels. Se supplementation had no significant ($P>0.05$) effect on the tri-iodothyronine (T3), thyroxine (T4), and T4/T3 ratio; in the lambs.

Alimohamady et al. (2013) evaluated the effects of different sources of Se on thyroid metabolism in male lambs. The lambs were randomly allotted to five treatments groups and supplemented basal diet (containing 0.06 mg Se/kg DM;) along with (1) control without supplementary Se, (2) 0.20 mg/kg Se as sodium selenite, (3)

0.40 mg/kg Se as sodium selenite, (4) 0.20 mg/kg Se as Se yeast, and (5) 0.40 mg/kg Se as Se yeast. The mean serum T3 concentration was significantly ($P<0.05$) higher and serum T4 concentration were significantly ($P<0.05$) lower in treatment groups as compared to control but there were no significant treatment effects for either source or amount of Se on these serum hormones.

Erdogan et al. (2017) conducted a study to determine the effect of supplementing organic Se at different levels to Norduz ewes in late pregnancy on maternal thyroxin level. Group 1 was fed a without any additional Se supplement (control). Experiment groups were fed diets supplemented with 0.150 mg/kg, 0.300 mg/kg, and 0.450 mg/kg organic Se to the same basal diet. As Se level increased in basal diet, an upward trend was observed in maternal free thyroxine concentration. Likewise, serum free triiodothyronine and free thyroxine concentrations of lambs from the three treatment groups were significantly greater than of the control lambs.

Rashnoo et al. (2020) studied the effects of maternal supplementation of Se and iodine via slow-release boluses in late pregnancy on thyroid metabolism of goats and their kids. The boluses were administered four weeks prior to the expected kidding time to supply daily amounts of (1) 0 mg Se and 0 mg I, (2) 0 mg Se and 0.40 mg I, (3) 0.25 mg Se and 0 mg I and (4) 0.25 mg Se and 0.4 mg I. The serum T3 concentration was significantly greater in goats and their kids receiving 0.25 mg Se/day than goats received no supplemental Se ($P<.05$).

2.12 Effect of Se supplementation on seminal attributes

Shi et al. (2010) evaluated the short-term effect of dietary Se-enriched yeast on semen parameters in bucks during the breeding season. Seventy-two adult Taihang black bucks of proven fertility were randomly assigned to four treatment groups. They were fed the basal diet supplemented with 0 (control), 0.5, 1.0 and 2.0 mg of Se/kg DM (from Se-enriched yeast). The short-term dietary Se supplementation has both linear ($P<0.001$) and quadratic ($P<0.001$) effects on semen parameters (volume, motility, viability, concentration and abnormality).

Mahmoud et al. (2013) determined the effect of the combination of vitamin E (Vit E) and Se injection on semen quality of Ossimi rams. Fourteen mature healthy Ossimi rams were randomly divided into two equal groups. Rams of the treatment group (TG) were treated twice weekly with 5 mg sodium selenite and 450 mg Vit E

for one month. Semen quantity (semen volume, and concentration of the semen ejaculated) and quality mass motility and percentage of live and dead cells) were recorded twice weekly. Semen quality and quantity were significantly affected by treatments: the ejaculate volume, mass activity and sperm concentration increased ($P< 0.01$) in treated rams in comparison with control ones. The percentages of dead and abnormal spermatozoa were reduced in the treated groups.

Kumar et al. (2014) evaluated the effect of supplementation of zinc and Se on semen quality in twelve Barbari bucks of proven fertility. The experimental animals were randomly divided into two groups of six. They were fed without any supplementation (control) or with 150-ppm zinc sulphate and 0.50-ppm sodium selenate (treatment group). In treatment group significant ($P< 0.05$) increase in semen volume, progressive motility, sperm count, percent live spermatozoa, acrosomal integrity and hypo osmotic swelling test (HOST) responding spermatozoa and decrease in abnormal spermatozoa after 60 days of supplementation. Mass motility increased significantly ($P<0.05$) only at 105 days as compared to 0 day.

Piagentini et al. (2016) investigated the effects of different concentrations of oral supplementation with Se upon ram sperm parameters. Thirty rams were used and divided into five groups as follows: control group (G1) mineral mixture supplementation without Se, group 2 (G2) mineral mixture supplemented with 5 mg/kg Se, group 3 (G3) supplemented with 10 mg/kg Se, group 4 (G4) supplemented with 15 mg/kg Se and group 5 (G5) supplemented with 20 mg/kg Se. There was no statistical difference between treatment groups in relation to volume, mass movement, total motility, vigour, concentration, plasma and acrosomal membrane integrity ($P>0.05$). Sperm morphology was different between treatment groups, the G1 (0 mg of Se) had the highest percentage of major defects.

Lukusa and Lehloenya (2017) studied the effect of orally supplemented sodium selenite on semen attributes of Saanen bucks. Twenty mature healthy Saanen bucks were divided into two equal groups (10 bucks). The treatment bucks received sodium selenite at the dose rate of 0.34 mg/kg body weight at 10-day intervals for ninety days. The semen pH was significantly ($P<0.001$) different between the groups. Moreover, the ejaculate volume, sperm mass activity, progressive motility and normal morphology were higher ($P<0.001$) in Se supplemented group compared to the control.

Stefanov et al. (2018) evaluated effect of source of Se on semen parameters in merino rams. Fifteen animals were divided in three groups of five each. The rams from first group (G1) received a diet with supplementation of 4.0mg sodium selenite (Na_2SeO_3)/animal/day, while the animals of the second experimental group (G2) obtained diet with 1.83 g L-selenomethionine (Sel-Plex, Alltech, USA)/animal/day. Eventually, each animal from the G1 and G2 received 1.83g Se per day. The control group (GC) received a diet without supplementation of Se. Supplementation of ram studs either with inorganic and organic Se led to increase in the volume of the ejaculates, motility and survivability of the spermatozoa. The pH of the freshly obtained semen was not affected by Se treatment.

2.13 Effect of Se supplementation on milk yield and composition

Tufarelli and Laudadio, (2011) conducted a study to analyze the effect of dietary supplementation of Se and vitamin E (Vit E) on milk yield and composition in dairy Jonica breed goats. Dietary treatments included: (1) the control diet containing background Se and Vit E only, and (2) the experimental diet containing sodium selenite as the Se supplement in concentrate (0.20 mg/head/day of Na-selenite and 20 mg/head/day of Vit E). The goats fed Se-Vit E diet showed the higher milk production ($P< 0·05$), along with increased fat ($P< 0·01$) and protein ($P< 0·05$) percentages.

Kachuee et al.(2014) determined the effects of organic and inorganic Se supplementation during late pregnancy on colostrum and milk yield in Merghoz goats. Four weeks before the expected kidding, the basal diets were supplemented with 0 (control), 0.3mg Se/head/day in the form of L-selenomethionine (Se-Met) and 0.3mg Se/head/day in the form of sodium selenite. The results indicated that the colostrum and daily milk production were not affected by Se supplementation.

Bagnicka et al. (2017) conducted a study to determine the effect of Se in its inorganic form (sodium selenite) compared to organic form (selenised yeast, Saccharomyces cerevisiae) added to basal diet on milk yield, milk composition of dairy cows. Group 1 was supplemented inorganic Se (sodium selenite), while in group 2 Se included in the form of selenised yeast. No significant differences were observed between the treatments in daily milk yield or milk composition. However, the cows under the Se organic treatment had 20% higher overall milk production.

Saba et al. (2019) studied the effect of dietary Se supplementation (organic or inorganic) on antioxidant status in late pregnant, lactating ewes and their suckling lambs. The control group fed the basal ration without any supplement, while the tested group (T1) fed 0.3 mg Sodium Selenate/kg diet and the tested group (T2) fed the basal ration + 0.3 mg Se yeast /kg diet. Significantly higher milk yield observed between T2 group and the control one. The total solid and fat component were slightly increased but protein component was significantly higher in T2 as compared to control group.

Rashnoo et al. (2020) studied the effects of maternal supplementation of Se and iodine via slow-release boluses in late pregnancy on milk production and composition of goats. The boluses were administered four weeks prior to the expected kidding time to supply daily amounts of (1) 0 mg Se and 0 mg I, (2) 0 mg Se and 0.40 mg I, (3) 0.25 mg Se and 0 mg I and (4) 0.25 mg Se and 0.4 mg I. Milk production, milk fat percentage, daily production of milk components, milk Se and iodine concentrations were greater in goats received 0.25 mg Se/day than no supplemental Se ($P<0.05$).

2.14 Effect of Se supplementation on level of Se in different biological fluids.

Misurova et al. (2009) conducted a study to compare the concentration of Se in the whole blood of goats and their new born kids. Regression and correlation analysis produced regression line formulas and correlation coefficients that revealed a close, statistically significant relation ($p < 0.01$) between the concentration of Se in the blood of mothers and their kids were obtained. The relation between the concentration of Se in the blood (µg/l) of mothers and kids was $y = 0.484x + 15.55$; $r = 0.73$.

Kachuee et al. (2013) evaluated the effect of dietary Se supplementation to Merghoz goats at the last stage of gestation on serum Se status of goats and their kids. Three weeks before the expected kidding, the basal diet was supplemented with 0 (control) or 0.3 mg Se /head/day in the form of L-selenomethionine (Se-Met) and 0.3 mg Se /head/day in the form of sodium selenite (SS). The serum concentration of Se increased in supplemented does compared with the control ones. The Se concentration was also significantly increased in colostrum of treated does ($P < 0.05$) than control group but there were no differences between Se-Met and SS treatments.

Kachuee et al. (2014) determined the effects of organic and inorganic Se supplementation during late pregnancy on serum and colostrum Se status in Merghoz goats. Four weeks before the expected kidding, the basal diets were supplemented with 0 (control), 0.3mg Se/head/day in the form of L-selenomethionine (Se-Met) and 0.3mg Se/head/day in the form of sodium selenite. The serum Se concentration increased in supplemented goats compared with control. Se concentrations also increased significantly in the colostrum of treated goats ($P < 0.05$). Similarly, serum Se concentrations in kids of treated goats were higher at birth.

Erdogan et al. (2017) studied the effect of supplementing organic Se at different levels to Norduz ewes in late pregnancy on Se concentration in maternal serum, colostrum, and offspring serum. Group 1 was fed a basal diet without any additional Se supplement (control). Experiment groups were fed diets supplemented with 0.150 mg/kg, 0.300 mg/kg, and 0.450 mg/kg organic Se to the same basal diet. Addition of Se to gestating ewe diets increased Se in serum after one week of feeding. Ewes supplemented with 0.300 and 0.450 mg/kg Se had increased placenta, serum, and colostrum Se levels compared with those fed the control diet ($P<0.05$).Colostrum Se increased linearly with dietary Se in the treatment groups. At birth, lamb serum Se was affected by the Se concentration of the ewe diets, which indicated placental transfer of Se from the dam.

Kachuee et al. (2019) evaluated the effect of organic, inorganic, and Se nano-particle supplements at the final stage of pregnancy on Se concentrations in serum of goats and placental, colostrum, and milk transfer from goats to their kids. Animals were divided into four treatments groups including (1) no supplement (control), 0.6 mg Se /head/day of selenomethionine (SM), 0.6 mg Se /head/day of Se nano-particles (SN), and 0.6 mg Se head/ day of sodium selenite (SS), from 4 weeks before the expected day of kidding-to-kidding day. The whole blood and serum Se concentration was greater in Se supplemented goats compared with the control ($P < 0.05$). The total Se content of the whole blood and serum was higher in SN than in SM ($P < 0.05$) and SS goats ($P< 0.05$).

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

The aim of study was to evaluate the effect of dietary supplementation of different sources of selenium on the growth performance, nutrient utilization, seminal attributes, blood biochemicals, antioxidant status, and hormonal status of bucks. In this chapter, a brief description of material used and experimental techniques adopted during the study has been given in following heads.

3.1 Selection and grouping of experimental animals

The experiment was conducted in the Barbari buck shed at Goat Farm Complex, DUVASU, Mathura (Uttar Pradesh). A total twenty-four experimental bucks of 1.5 to 2 year age were selected from the herd maintained at Department of Physiology, DUVASU, Mathura (U.P.). Experimental bucks were randomly assigned into four groups (six bucks in each) on body weight basis. The experiment protocol was in accordance of standard of Institute Animal Ethics committee and the approval of experimentation was also obtained from the committee. The selection and grouping of experimental animals are presented Table 3.1.

Table 3.1 Selection and grouping of experimental animals

Group	S. No	Animal No.	Body weight (kg)
Control	1	BM004	27.30
	2	BM015	34.50
	3	BM031	34.60
	4	BM033	41.20
	5	BM034	40.70
	6	BM056	43.30
		Mean	**36.93**
T1	1	BM003	28.70
	2	BM006	31.80
	3	BM008	35.20
	4	BM014	39.80
	5	BM020	41.80
	6	BM024	44.20
		Mean	**36.92**

T2	1	BM007	28.80
	2	BM009	31.50
	3	BM011	35.40
	4	BM013	39.50
	5	BM017	42.00
	6	BM018	43.30
		Mean	**36.75**
T3	1	BM001	29.60
	2	BM002	30.40
	3	BM010	35.40
	4	BM021	37.70
	5	BM022	42.10
	6	BM047	42.50
		Mean	**36.28**

3.2 Housing and management of experimental animals

All bucks were housed in a well-ventilated individual sheds having the proper arrangement for feeding and watering. The buck shed was thoroughly cleaned to remove faeces and dirt. Deworming of all the animals was done with Albendazole before the start of the experiment.

3.3 Feeding of experimental animal

The bucks were fed basal diet comprising of concentrate mixture, gram straw and corn silage. The animals of each experimental group were maintained and fed individually on roughage and concentrate based ration to meet out requirement as per ICAR (2013) feeding standard. Diets were prepared by taking concentrate and roughage in the ratio of 40:60 respectively. The roughage part composed of gram straw (40%) and corn silage (20%). Concentrate mixture was prepared by mixing barley grain, wheat grain, wheat bran, gram chunni, mustard oil cake, and mineral mixture (without Se) in 15, 15, 25, 10, 33 and 2 parts, respectively. Clean and fresh drinking water was offered ad libitum twice to each animal daily. The duration of experiment was of 90 days. The animals were given an adaptation period of fifteen days, before the start of experiment. All the groups were kept on similar feeding regimen, except different sources of Se that was additionally supplemented to the treatment groups. The calculated amount of Se was premixed in barley flour that

differed in Se source and corresponded to three treatments; T1- Inorganic Se – 0.30 mg/kg Se as sodium selenite (Loba Chemie Maharashtra, India, Batch no. L156451908); T2-Organic Se – 0.30 mg/kg as Se-enriched yeast (Chaitanya Chemicals Maharashtra, Batch no: Yeast/Sel/10I/2020, India) and T3- nano Se – 0.30 mg/kg as Se Nano particles (Nano shel Punjab Stock No: NS6130-01-171, India). The premix was then mixed in concentrate mixture of each buck of respective group at the time of feeding to ensure the required intake. The animals of four different groups were subjected to the following dietary treatments:

Groups	**Diet**
Control	Basal diet only, with no additional selenium
Treatment 1 (T1)	Basal diet and sodium selenite @ 0.3mg/kg DM
Treatment 2 (T2)	Basal diets and selenium yeast @ 0.3mg/kg DM
Treatment 3 (T3)	Basal diets and Nano selenium @ 0.3mg/kg DM

Composition of feeds and mineral mixtures for different treatment groups are presented in Table 3.2. Half of total weighed amount of concentrate mixture, gram straw and corn silage were offered to the animals at 08.00 A.M. in morning hour and 4.00 P.M. in evening hour during entire trial period.

Table 3.2 Ingredient composition (%) of experimental diets

Ingredients	**Concentrate**		**Treatment**		
		Control	**T1**	**T2**	**T3**
Barley	15	6	6	6	6
Wheat grain	15	6	6	6	6
Mustard oil cake	33	12.8	12.8	12.8	12.8
Gram Chunni	10	4	4	4	4
Wheat bran	25	10	10	10	10
Mineral mixture and salt	2	0.8	0.8	0.8	0.8
Corn silage	-	40	40	40	40
Gram straw	-	20	20	20	20
Mineral mixture composition					
Calcium carbonate (%)		8.17	8.17	8.17	8.17
Dicalcium phosphate (%)		40.50	40.50	40.50	40.50

Magnesium sulphate (%)		23.21	23.21	23.21	23.21
Potassium sulphate (%)		15.09	15.09	15.09	15.09
Sodium chloride (%)		11.91	11.91	11.91	11.91
Cobalt chloride (%)		0.001	0.001	0.001	0.001
Zinc sulphate (%)		0.22	0.22	0.22	0.22
Copper sulphate (%)		0.087	0.087	0.087	0.087
Potassium iodide (%)		0.0014	0.0014	0.0014	0.0014
Ferrous sulphate (%)		0.65	0.65	0.65	0.65
Manganese Oxide (%)		0.17	0.17	0.17	0.17
Sodium selenite (ppm)		-	54.75	-	-
Selenium Yeast (ppm)		-	-	150	-
Selenium nano particles (ppm)		-	-		25

3.5 Growth performance indices

3.5.1 Body weight gain and dry matter intake

The animals were weighed before feeding and watering in the morning on two consecutive days at the start of experimental feeding and thereafter at fortnightly interval during experimental period of 90 days. Fortnightly weight gain was calculated by increase in body weight in one fortnight and ADG (g/d) was calculated by dividing the fortnightly weight gain with number of days (15). The feeds offered to the animals and residue left were recorded daily to find out the total DMI of the experimental animals. Intake of DM was calculated as the difference between the amount of DM offered and amount of DM left in residue.

3.5.2 Feed to gain ratio or feed conversion ratio (FCR)

Feed-to-gain ratio or FCR was calculated by the amount of DMI (kg) required for unit (per kg) weight gain by animals during the trial period.

3.6 Digestibility trial

To compare the efficiency of nutrient utilization in experimental bucks, a digestion trial for a period of 7 days was conducted at the end of the study. Bucks were weighed before start and at the end of digestion trial to find out body weight gain. Weighed amount of feeds and fodders was offered during digestion trial.

Representative samples of the feed offered and residue left were collected and analysed for chemical composition. Faeces voided during 24:00 hours were collected and measured daily for 6 days. About 1/10thof thoroughly mixed total faecal matter (as such basis) was taken for chemical analysis. Additionally, for N estimation, approximately 1/30th of total faecal sample was collected daily for 6days and stored in plastic containers having 25% sulphuric acid solution. Dried dung samples were grounded to pass through 1mm sieve size and analyzed for proximate principles and fibre constituents (NDF and ADF) as per standard techniques. The digestibility coefficient of nutrients was calculated from the nutrient intake and nutrient outgo in faeces during digestion trial:

$$\text{Digestibility (\%)} = \frac{\text{(Nutrient intake – Nutrient outgo in faeces)}}{\text{Nutrient intake}} \times 100$$

3.7 Chemical analysis

The representative samples of feeds and fodders offered and residue left and faeces were ground and analyzed for DM, OM, CP, EE, CF and total ash (AOAC, 2005). Fibre fraction of feed, fodder and faecal sample were analyzed by using detergent method of fibre estimation (Van Soest et al., 1991).

3.7.1 Dry matter (DM)

DM content in samples of feedstuffs and faeces was analyzed by using AOAC, (2005) method (id number 973.18c). 100 g sample was taken in a pre-weighed moisture cup or tray and it was placed in a hot air oven for 24:00 hours. The loss in moisture content after drying was estimated and DM was calculated as follows:

$$\text{DM (\%)} = \frac{\text{Weight of Sample after drying}}{\text{Weight of Sample taken}} \times 100$$

3.7.2 Organic matter (OM)

OM content of sample was determined by subtracting the total ash content from DM content of respective sample.

$$\text{OM (\%)} = \text{DM\%} - \text{Total ash (\%)}$$

3.7.3 Crude protein (CP)

Apparatus: Kjeldahl flasks, digester, Kjeldahl distillation apparatus, Erlenmeyer flasks, titration assembly.

Reagents: Digestion mixture (Na_2SO_4 and $CuSO_4$ in the ratio of 9:1), 40% NaOH solution (400 g NaOH pellets dissolved in distilled water and volume made to 1000 ml), Concentrated H_2SO_4 (98% purity and specific gravity 1.84), 4% boric acid indicator solution (40 g boric acid dissolved to 1 L and added with 10 ml 0.2% bromocresol green and 20 ml 0.1% methyl red indicators) and N/10 H_2SO_4 solution.

Procedure

A known quantity of sample (about 0.5-1.0 g) was taken in digestion tube and digested with 20-30 ml concentrated H_2SO_4 and 2-3 g of digestion mixture till the solution became colourless. After digestion, the contents were cooled and volume was made to 250 ml. 10 ml of aliquot was distilled in Kjeldahl distillation apparatus (KELPLUS Nitrogen Analyzer, Chennai) after adding 10-15 ml of 40% NaOH solution. About 60-75 ml of distillate (light green colour) was collected into an Erlenmeyer flask containing 10 ml of 4% boric acid indicator solution. The distillate was then titrated against N/10 H_2SO_4 solution and the end point was recorded when colour changed to slight pinkish. Volume of N/10 H_2SO_4 solution used in titration was recorded.

Calculation

$$\text{N (\%)} = \frac{0.0014 \times 0.1 \times \text{Volume of N/10 } H_2SO_4 \text{ used} \times \text{Volume made (ml)}}{\text{Aliquot taken (ml)} \times \text{Sample taken (g)}} \times 100$$

The CP (%) of sample was calculated by multiplying the N content with factor 6.25 (protein contains 16% nitrogen).

3.7.4 Ether extract (EE)

EE content in representative samples of feeds and fodders and faeces were analyzed by using AOAC (2005) method.

Apparatus: Socsplus extraction apparatus (Pelican Equipments), oil flask, thimble, hot air oven, desiccators, weighing balance.

Reagent: Petroleum ether (40-60°C).

Procedure

Rinse all the beakers and place them in oven with the temperature about 100^{o}C and also the samples. If all moistures were removed from the beakers, place them in desiccators about 5 to 10 minutes to bring them into room temperature. Weigh the empty beaker and let the weight be W_1. This is initial beaker weight (IBW). After that insert the thimble in the thimble holder and place it on the beaker and weigh the samples and transfer them to the thimble. Let the sample weight be (SW**)**, sample weight may be 2 to 3 grams, pour the solvent in the beaker, the volume may be 80 to 100 ml, load the beakers in the system. Switch on the system and set the boiling temperature as the solvent's maximum boiling point, leave the process for about 60 minutes. After the process time, increase the temperature to recovery temperature range of 160 to 180**º**C, then rinse about 2 to 3 times in order to collect the remaining fat that may present in the sample or in the thimble, take out the beakers from the system then remove all the thimbles from the beaker and put the beakers into a hot air oven at 100^{o}C, after 20 to 30 minutes, take out the beakers and place them in desiccators about 10 to 15 minutes for cooling up to the room temperature.Weigh the beakers, this is the final weight of the beakers (FBW), let the weight be W_2. By substituting SW, W_1 and W_2 in the following formula, the percentage of fat present in the sample can be calculated.

$$\text{EE (\%)} = \frac{\text{(Weight of oil flask with ether extract } (W_2) - \text{Weight of oil flask } (W_1)}{\text{Weight of sample (SW)}} \times 100$$

3.7.5 Total Ash (TA)

TA content in samples of feedstuffs and faeces was analyzed by using AOAC (2005) method (id number 942.05). A known quantity of sample (about 10g) was taken in pre-weighed silica crucible. After charring the sample on heater (till the smoke disappeared), the crucible was kept in muffle furnace for ignition at 550-600°C for 2-3 hours. Then the crucible was removed on cooling and kept in desiccator and weighed again to find out weight of ash. The ash content was calculated as given below:

$$\text{TA (\%)} = \frac{\text{Weight of crucible} + \text{ash after drying} - \text{Weight of crucible}}{\text{Weight of sample taken}} \times 100$$

3.7.6 Crude fiber (CF)

Apparatus: Fibra plus (Pelican Equipments), suction apparatus, Gooch crucible, hot air oven, muffle furnace, desiccators, weighing balance.

Reagents: 1.25% H_2SO_4 and 1.25% NaOH

Procedure

Take the moisture and fat free sample of known quantity (after ether extraction). Weigh the samples accurately and note down the weights (W),transfer the weighed samples into oven dried crucible, place the crucible into the metal adapters of Fibra Plus hot extraction unit and ensure proper sealing of crucible against the adapter rubber.

Acid wash: Pre-heat the prepared solution of 1.25% H_2SO_4 in FIBRA STAT unit, pour 150 ml of 1.25 % H_2SO_4 into the extractors from the top for each sample. Switch on the instrument and set the temperature to 500°C, after boiling starts reduce the temperature at 400°C, allow the samples to boil for 30 minutes in acid. After 30 minutes of boiling, switch off the unit and drain the acid and wash the samples twice or thrice with distilled water. During draining, ensure that the knob is in vacuum mode, if the draining is not effective due to clogging of sample in the crucible, then, keep the knob in pressure mode, press the pressure button twice or thrice and immediately turn the knob to vacuum mode**.**

Alkaliwash : Pre-heat the prepared solution of 1.25% NaOH in FIBRA STAT unit pour 150 ml of 1.25% NaOH into the extractors from the top for each sample. Switch on the instrument and set the temperature to 500°C, after boiling starts reduce the temperature to 400°C and allow the samples to boil for 30 minutes in alkali. After 30 minutes of boiling, switch off the unit and drain the alkali and wash the samples twice or thrice with distilled water. During draining, ensure that the knob is in vacuum mode, if the draining is not effective due to clogging of sample, then keep the knob in pressure mode and press the pressure button twice or thrice and immediately turn the knob to vacuum mode. After alkali wash, take out crucibles and dry them in hot air oven at 100°C until the crucibles are free from moisture after that cool down the hot crucibles to room temperature by using a desiccator. Weigh the crucibles and record the readings (W_1) after that place all the crucibles in the muffle furnace at 400°C for

ashing, cool down the hot crucibles after ashing to room temperature using a desiccator, now weigh the crucibles and record the readings (W2).

$$CF\ (\%) = \frac{\text{Weight of dried residue } (W_1) - \text{Weight of ash } (W_2)}{\text{Weight of sample taken}} \times 100$$

3.7.7 Nitrogen free extracts (NFE)

The NFE of sample was estimated by using following equation:

$$\text{NFE}\ (\%) = 100 - (\text{CP}\% + \text{EE}\% + \text{CF}\% + \text{TA}\%)$$

3.7.8 Estimation of cell wall constituents

The fractions of cell wall (fibre fraction) such as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were estimated as per methodology described by Van Soest et al. (1991).

3.7.9 Neutral detergent fibre (NDF)

Apparatus: Spoutless beaker, sintered crucible, vacuum pump, hot air oven, muffles furnace, weighing balance and desiccator.

Reagents: Neutral detergent solution (NDS), acetone, hot boiling water.

Table 3.3 Composition of neutral detergent solution (NDS)

Chemical	Amount
Sodium lauryl sulphate	30 g
Disodium ethylene diamino tetra acetate (EDTA)	18.61 g
Sodium borate decahydrate	6.81 g
Disodium hydrogen phosphate (anhydrous)	4.56 g
Triethylene glycol	10 ml
Distilled water	990 ml

Solution preparation

EDTA and sodium borate decahydrate were put together in a large beaker with some distilled water and heated on hot plate until dissolved. Similarly, sodium lauryl sulphate was dissolved in distilled water and triethylene glycol was added to it. The solution of sodium lauryl sulphate and triethylene glycol was added to the previous solution. Disodium hydrogen phosphate was taken in another beaker and some

amount of distilled water was added and the contents were heated until dissolved. Then, it was added to solution containing other ingredients and volume was made up to one litre with distilled water.

Procedure

A known quantity of ground sample (1.0 g) was taken in a spoutless beaker and to this add 100 ml NDS. The contents were heated to boil and refluxed for 60 min. The contents were filtered through a pre-weighed Gooch crucible (G1 porosity) under vacuum. The contents were given 3-4 washings with hot distilled water and a final washing of acetone. The crucibles were dried to a constant weight at 100º C and weighed. Cell wall contents or NDF was calculated as follows:

$$\text{NDF (\%)} = \frac{(\text{Weight of crucible with residue} - \text{Weight of empty crucible})}{\text{Weight of sample on DM basis}} \times 100$$

3.7.10 Acid detergent fibre (ADF)

Apparatus: Spoutless beaker, sintered crucible, vacuum pump, hot air oven, weighing balance, desiccator.

Reagents: Acid detergent solution (ADS), acetone, hot boiling water, 1N H_2SO_4 (Taken 26.63 ml of conc. H_2SO_4 and dissolved in 1litre of distilled water. This yield 1N H_2SO_4 solution).

Preparation of acid detergent solution (ADS): 20 g Cetyl trimethyl ammonium bromide (CTAB) was dissolved in one litre of 1 N H_2SO_4 (Taken 26.63 ml of conc. H_2SO_4 and dissolved in 1 litre of distilled water. This yield 1 NH_2SO_4solution)

Procedure

Approximately 1.0 g of sample was taken in a spout less beaker of 1 L capacity. To this, 100 ml acid detergent solution was added and the contents were refluxed for exactly 1 hour. After refluxing, the residue was filtered through pre-weighed sintered glass crucible using vacuum pump and washed with hot water 2-3 times followed by acetone to remove all salts. The crucible containing residue was dried in hot air oven (100 ± 5°C) and weighed again. The ADF was calculated as follows:

$$\text{ADF (\%)} = \frac{(\text{Weight of crucible with residue} - \text{Weight of empty crucible})}{\text{Weight of sample taken}} \times 100$$

3.7.11 Hemicellulose (HC)

Hemicellulose content was calculated as the difference between NDF and ADF.

$$\text{HC (\%)} = \text{NDF (\%)} - \text{ADF (\%)}$$

3.7.12 Acid detergent lignin (ADL)

Apparatus and reagents: same as that of ADF estimation.

Procedure

The procedure for estimation of ADL content was exactly same up to the filtering and drying of ADF contents of sintered crucible after treating with 72% H_2SO_4 (w/w) in the cellulose estimation procedure. Then the crucible with dry residue was kept in muffle furnace for ignition at 550^oC for 2-2.5 hours, cooled and weighed again. The acid detergent lignin was calculated as follows:

$$\text{ADL (\%)} = \frac{(\text{Weight of crucible with dry residue} - \text{Weight of crucible with ash})}{\text{Weight of sample taken}} \times 100$$

3.8. Calculation of nutrient digestibility coefficient

The nutrients (Dry matter, organic matter, crude protein, ether extract, NDF and ADF) digestibility coefficient was calculated from the nutrient intake and nutrient out go in faeces during digestion trial as follows:

$$\text{Digestibility (\%)} = \frac{(\text{Nutrient intake} - \text{Nutrient outgo in faeces})}{\text{Nutrient intake}} \times 100$$

3.9 Semen collection

Semen was collected twice a week using artificial vagina (length = 20 cm and diameter = 4.5 cm). The artificial vagina was prepared by filling with water at a temperature of 42- 45 ^0C and air through a valve to maintain optimum pressure. Just before collection the inner lining was lubricated with non-spermicidal lubricant followed by checking of temperature and pressure. A non-estrus doe was used for mounting of bucks and semen was collected into graduated tubes attached to one end of artificial vagina. After the start of experiment, the semen from each buck in a group were collected and pooled (to minimize individual variation). A total of six

pooled ejaculates from each group were evaluated at day 0 (from 0-21d) i.e., start of experiment and at day 90 (90-111d) i.e., end of experiment.

3.9.1 Semen samples

Semen of experimental bucks were collected with the use of sterilized artificial vagina and evaluated for various parameters.

3.9.2 Transportation of semen samples

Immediately after semen collection, samples were transported to Semen Analytical Laboratory, Department of Veterinary Physiology, DUVASU, Mathura for evaluation of various parameters.

3.9.3 Volume

The volume of semen was directly measured in milliliter (ml) from the graduated semen collection tube.

3.9.4 Colour

Semen sample were also observed for colour and consistency by direct visualization with naked eyes and any abnormalities in colour or consistency were treated as abnormal and the sample were discarded.

3.9.5 Mass motility

It was assessed by placing a small drop of semen of uniform size and thickness over a clean dry glass slide. The semen drop was examined under low power objective (10X) of microscope on a thermostatically controlled warm stage at 37°C. Nazir, (1988) reported that motility was rated according to the vigour wave motion on grade scale of 0 to 5 as given below.

Table 3.4 Mass motility scores

S. No.	Observation	Mass motility score (0-5 scale)
1	No motility	0
2	No wave but sperm movement evident	+1
3	Slow wave formation	+2
4	Relatively more wave formation with swirls	+3
5	Wave with swirls and eddies	+4
6	Wave with very rapid swirls and eddies	+5

3.9.6 Progressive motility

The progressive motility of the spermatozoa was observed under high power phase objective (40 X) on a thermostatically controlled stage maintained at 37°C. A small drop of diluted semen was put on a clean grease free slide and was covered with a cover slip. The slide was examined to observe vigorously motile spermatozoa exhibiting progressive path. The progressive motility of spermatozoa was then calculated as below

$$\text{Progressive motility (\%)} = \frac{\text{Number of progressively motile spermatozoa}}{\text{Total number of spermatozoa observed}} \times 100$$

3.9.7 Sperm concentration

The sperm concentration in semen samples were analyzed through flow cytometer (Easycyte II, IMV, France) using easykit (IMV Technologies, France) and using sperm guava easycyte software. To ascertain the sperm concentration in semen sample, 190 µL of Easy buffer B (IMV, France) was added in appropriate easykit well and mixed prior to semen dilution. 10µL of diluted sample was added to well. The sample was mixed in wells by pipetting up and down with a 200µL tip. The well was incubated at 37 °C for 10 minutes in the dark. The mixed samples in wells were transferred into tube before running the samples with appropriate setting. In the study, the sperm concentrations were estimated and are presented in results section. The gating of cells used in the flow cytometry is summarized in Fig. 3.1 (Fig 3.1.1 & 3.1.2).

Fig. 3.1: Dot Plot gating used in flow cytometry for estimation viability

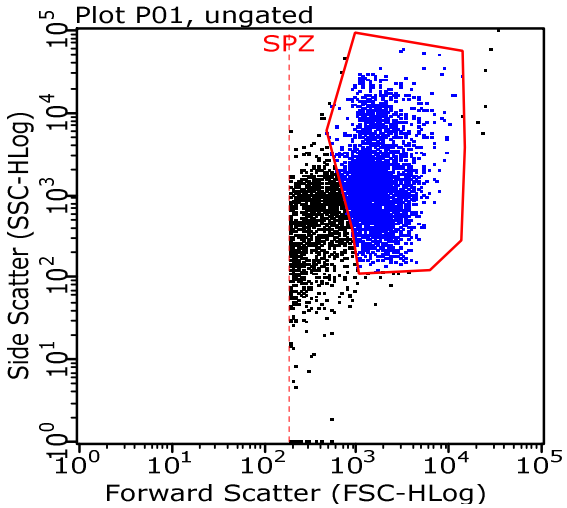


Fig. 3.1.1: Dot plot showing distribution of cell debris and cells

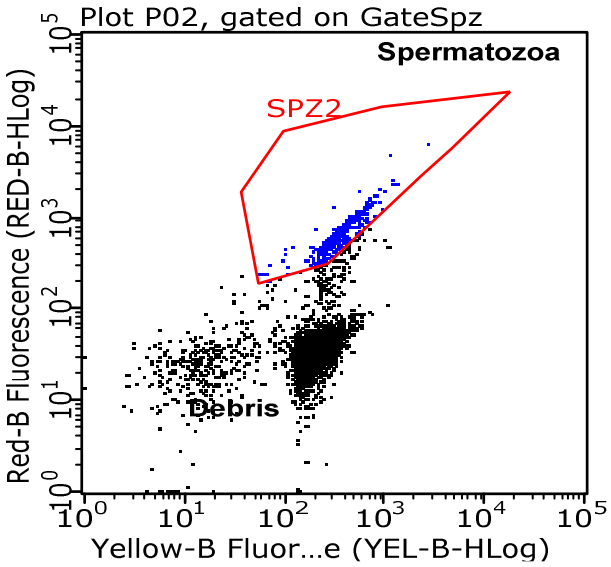


Fig. 3.1.2: Gating for evaluation of concentration of spermatozoa

3.9.8 Spermatozoa Viability

The percentage of live or dead spermatozoa in semen sample were analyzed through flow cytometer (Easycyte II, IMV, France) using easykit (IMV Technologies, France) and using sperm guava easycyte software. To ascertain the percent viable spermatozoa in semen sample, 190 µL of Easy buffer B (IMV, France) was added in appropriate easykit well and mixed prior to semen dilution. 10µL of diluted sample was added to well. The sample was mixed in wells by pipetting up and down with a 200µL tip. The well was incubated at 37 °C for 10 minutes in the dark. The mixed samples in wells were transferred into tube before running the samples with appropriated setting. In the study, live spermatozoa having intact membrane were estimated and are presented in results section. The gating of cells used in the flow cytometry is summarized in Fig.3.2 (3.2.1, 3.2.2 & 3.2.3).

Fig. 3.2: Dot Plot gating used in flow cytometry for estimation viability

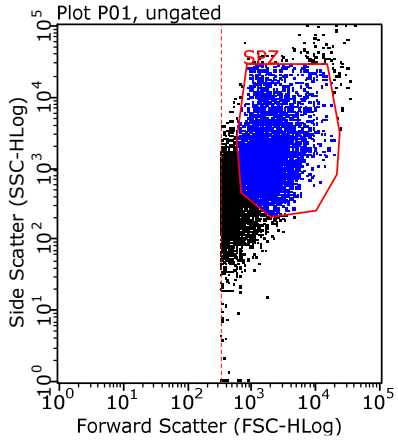


Fig 3.2.1: Dot plot showing distribution of cell debris and cells

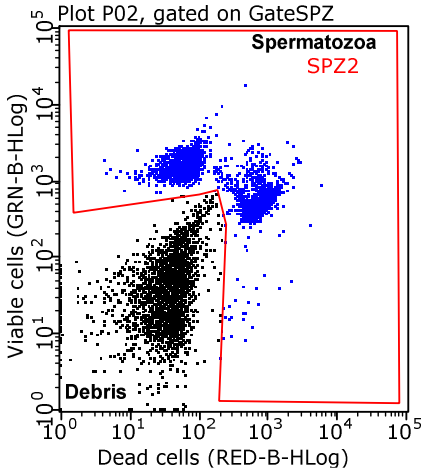


Fig 3.2.2: Gating for evaluation of viability of spermatozoa

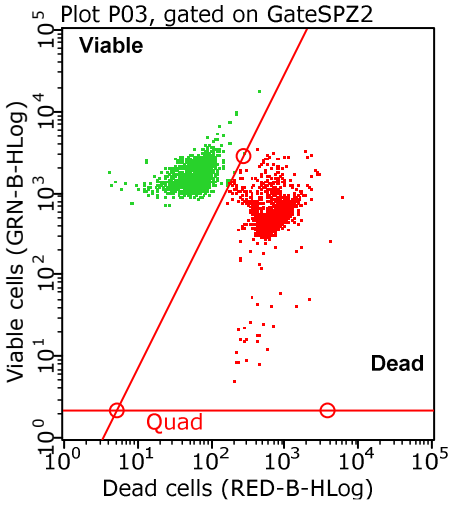


Fig 3.2.3: Dot plot (Green colour) representing the viable cells and dot plot (red colour) representing dead spermatozoa

3.9.9 Morphological Abnormalities

Live and dead count was also used for enumerating abnormalities. The classification suggested by (Lasley, 1951) was used for the study. The slides were observed under oil immersion in Phase Contrast Microscope. These studies were broadly classified in three groups as per abnormalities listed below in table 3.5.

Table 3.5 Sperm abnormality categories

S.No.	Head Abnormalities	Mid Piece Abnormalities	Tail Abnormalities
1.	Micro head	Abaxial attachment	Simple bent tail
2.	Macro head	Beaded	Coiled tail
3.	Giant head	Proximal protoplasmic droplet	Loose tail
4.	Loose head	Distal protoplasmic droplet	Tail stump
5.	Detached head	Corn screw mid piece	Double tail
6.	Pear shaped head (pyriform)	Bent mid piece	Corkscrew tail
7.	Bent head	-	-
8.	Knobbed acrosome detect	-	-

About 200 spermatozoa were counted in different fields and total abnormal spermatozoa were calculated as follows:

$$\text{Total Abnormalities (\%)} = \frac{\text{Total Number of abnormal spermatozoa}}{\text{Total Number of spermatozoa counted}} \times 100$$

3.9.10 Hypo-osmotic swelling test (HOST)

To analyze the functional integrity of plasma membrane hypo-osmotic swelling test was performed according to the technique described by Jeyendran et al. (1984). The protocol was modified in reference to incubation time (1 hour) and osmalality required of goat semen. Hypo-osmotic solution of 150 mOsm/l was prepared as follows.

Table 3.6 Composition of Hypo-osmotic solutions of 150 mOsm/l

Ingredients	Amounts
Sodium citrate	0.735 g
Fructose	1.351 g
Millipore/ double distilled water upto	100 ml

Procedure

One ml of HOST solution, having an osmotic strength of 150 mOsm/l was mixed with 0.1 ml of semen and incubated at 37^0C for one hour. Following incubation, a drop of well-mixed solution was taken on clean glass slide and covered with a cover slip. Sperm tail curling was recorded as an effect of swelling due to influx of water. A total of about 200 spermatozoa were counted in different fields under 40X phase objective under phase contrast microscope. The total proportion of swollen spermatozoa was calculated by dividing the number of reacted cells by the total spermatozoa counted in the same area and multiplying the figure by 100. These spermatozoa were classified in four different classes according to presence of following swelling pattern (Takahashi et al., 1990).

A. No swelling, no membrane reaction
B. Swelling of the tip of the tail
C. Different type of hair pin like swelling or swelling of mid-piece
D. Complete tail coiling

Spermatozoa showing B, C, D type of pattern was considered to be HOST positive.

$$\text{HOST positive Spermatozoa (\%)} = \frac{\text{Number of curled tail spermatozoa}}{\text{Total number of spermatozoa counted}} \times 100$$

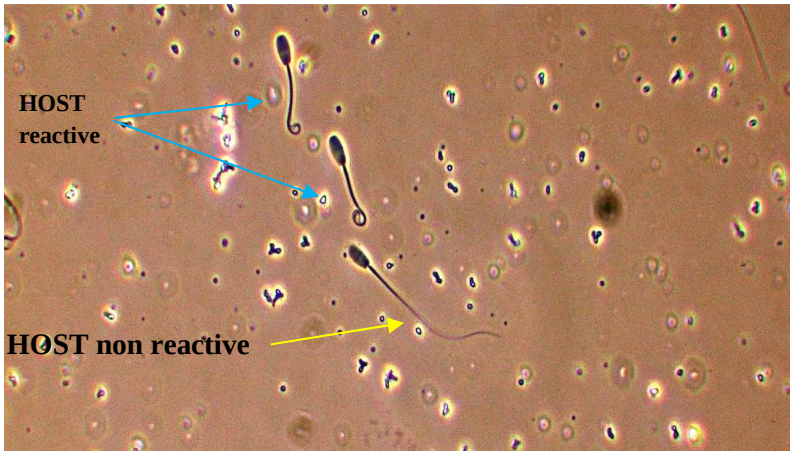


Fig. 3.3: Photograph showing hypo osmotic swelling test (HOST) reactive and non-reactive spermatozoa (magnification, 100X)

3.9.11Acrosomal integrity

Principle:

The fluorescent agent labels the ruptured acrosomes with green colour and a second fluorescent agent acted as a dead cell specific counter stain, labeling red spermatozoa.

Acrosomal integrity of spermatozoa in semen sample was evaluated using flow-cytometer (Easycyte II, IMV, France), specifically designed to evaluate the acrosomal structure spermatozoa.

Methodology:

To evaluate the acrosomal integrity of spermatozoa, 190 µL of easybuffer B was added in appropriate easy kit II well and mixing well. 10 µL of frozen thaw semen was then added in the well. The diluted semen was properly mixed. The well was incubated at 37 °C for 45 minutes in the dark. The mixed samples in wells were transferred into tube before running the samples with appropriated setting. The gating of cells used in the flow-cytometry is summarized in Plate 3 (Fig 3.7, 3.8 & 3.9).

Table 3.7 Different populations of sperm cells showing the status of acrosome

S No.	Type of spermatozoa population
1	Viable spermatozoa with intact acrosome
2	Viable spermatozoa with disrupted acrosome
3	Dead spermatozoa with intact acrosome
4	Dead spermatozoa with disrupted acrosome

Four different populations of spermatozoa were counted which are presented in the Table 3.7. The results of viable spermatozoa with intact acrosome are presented in the results section.

Fig. 3.4: Dot Plot gating used in flow cytometry for estimation of acrosome activity.

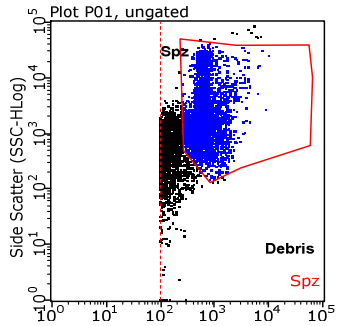


Fig. 3.4.1: Dot plot representing the cell concentration.

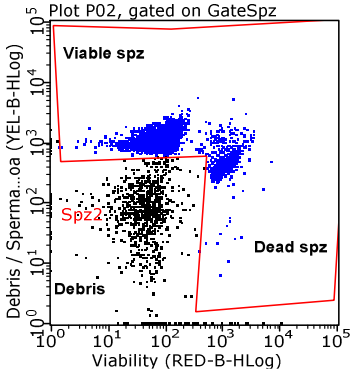


Fig. 3.4.2: Dot plot representing viable and dead sperm.

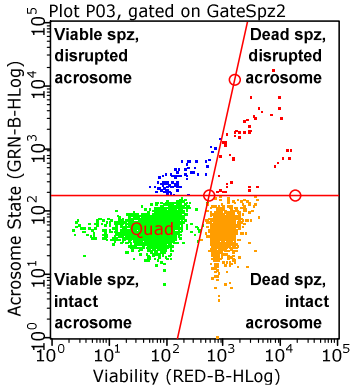


Fig 3.4.3: Gating for different populations of spermatozoa based on acrosomal integrity 1-Green colour dot plot representing the viable spermatozoa with intact acrosome, 2- Blue colour dot plot representing the viable spermatozoa with disrupted acrosome, 3- Orange colour dot plot representing the dead spermatozoa with intact acrosome, 4- Red colour dot plot representing the dead spermatozoa with disrupted acrosome

3.9.11 Reactive Oxygen Species (ROS)

Principle:

Viable spermatozoa with high level show ROS appeared green colour and dead spermatozoa with high level ROS appeared orange colour and viable spermatozoa with low level ROS appeared blue colour and dead spermatozoa with low level ROS appeared orange colour.

ROS of spermatozoa in semen sample was evaluated using flowcytometer (Easycyte II kit, IMV, France) specifically designed to evaluate the Reactive Oxygen species (ROS) in semen sample was utilized to study level of ROS.

Methodology:

To evaluate the level of ROS of spermatozoa, 195 µL of pre-warmed filtrated PBS was added in appropriate easykit II well and mixed well. 5 µL of diluted pooled semen was then added in the well. The diluted semen was properly mixed. The well was incubated at 37°C for 20 minutes in the dark. After incubation, 2µl of H_2O_2 at 39mM was added in the well and mixing and again incubated at 37°C for 40 minutes in the dark. The mixed samples in wells were transferred into tube before running the samples with appropriated setting. The gating of cells used in the flow-cytometry is summarized in Fig 3.5 (Fig 3.5.1 & 3.5.2).

Four different populations of spermatozoa were counted as summarized in Table 12. In the results section, viable sperm cells with high ROS are presented.

Table 12: Different patterns of spermatozoa showing ROS levels

S No.	Type of spermatozoa population
1	Viable ROS+
2	Dead ROS+
3	Viable ROS-
4	Dead ROS-

Fig.3.5: Dot Plot gating used in flow cytometry for estimation of ROS level

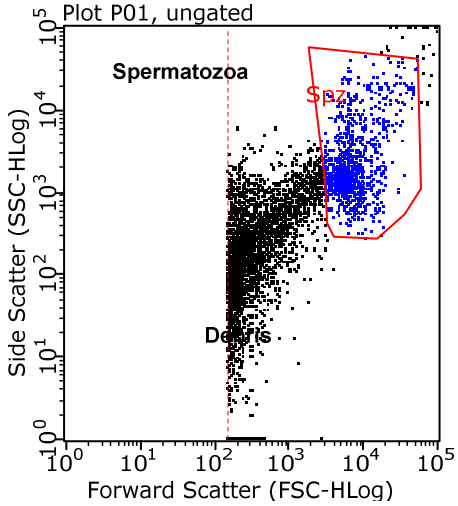


Fig. 3.5.1: Blue colour dot plot representing the cell concentration

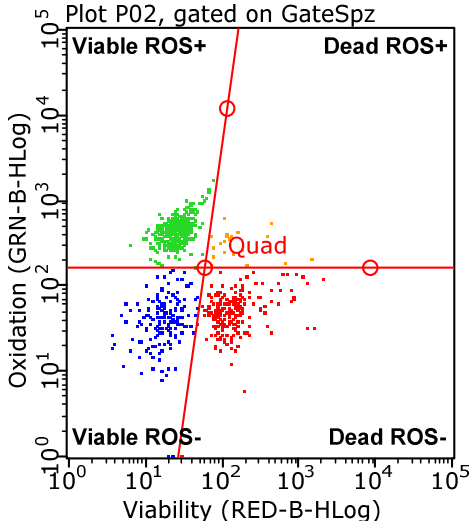


Fig 3.5.2: Dot plot showing different population of spermatozoa based of ROS reactivity: 1) Green colour dot plot representing the viable ROS+,

2) Blue colour dot plot representing the viable ROS-,

3) Orange colour dot plot representing the dead ROS-,

4) Red colour dot plot representing the dead ROS+

3.9.12. Membrane Fluidity

Principle:

Merocyanine 540 is a fluorescent lipophilic dye, which binds preferentially to membranes with loosely lipids, and as such stains apoptotic cells more brightly than normal cells and YO-Pro-1 dye fluorescent binds with the dead cells.

Membrane fluidity was evaluated through Merocyanine 540 and YO-Pro-1 dye using flowcytometer. Prior to the start of experiment stock solution of the dyes were prepared as follows:

Merocyanine 540 (Molecular weight: 569.67)
Stock solution 10X = 2.2 MG/0.704 ML DMSO
Working solution: 26µM in 200µL
1X = 1µL 10X + 9µL DMSO
YO-PRO-1 (Y3603, Invitrogen)
Working solution: 1Mm (tube 10µl à 1mM)
Final concentration: 25nM

Methodology:

To start the experiment, 190 µL of Easybuffer B was added in appropriate well. To the well 1µL of 1x working solution of Merocyanine and 10x working solution 0.1 Yo Pro was added and mixed. 10 µL of diluted pooled semen was added to the well and incubated at 37°C for 10 minutes in the dark. The mixed samples tube before running the samples with appropriated setting. In the results, the spermatozoa having higher membrane fluidity with intact membrane are presented. The gating of cells used in the flow-cytometry is summarized in Fig. 3.6 (Fig 3.6.1 & 3.6.2).

Fig.3.6: Dot-Plot gating used in flow cytometry for estimation membrane fluidity

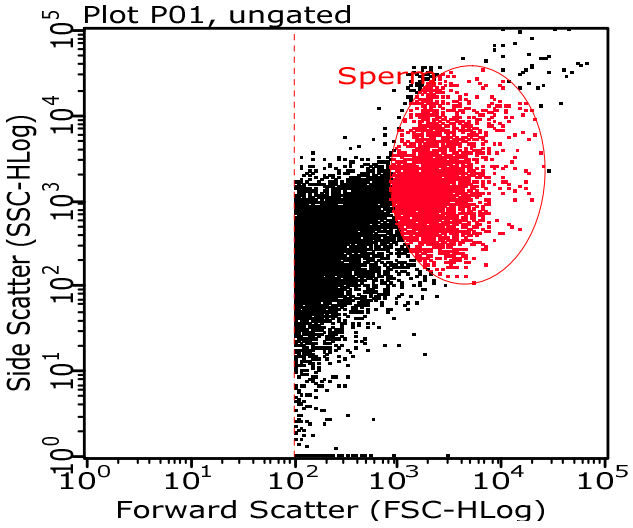


Fig. 3.6.1: Red dots plot representing the sperm cells

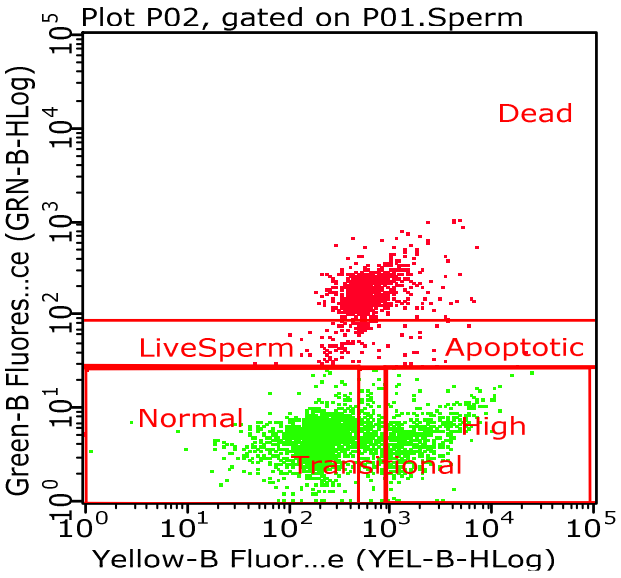


Fig. 3.6.2: Dot plot of different population of spermatozoa based on membrane fluidity: green plot exhibit different level of membrane fluidity in viable sperm membrane while red dot shows dead sperm

3.9.13. Mitochondrial Activity

Principle:

Active mitochondria (high membrane potential=polarized) will show orange fluorescence and mitochondria with (low membrane potential = depolarized) will show green fluorescence.

Methodology:

Mitochondrial activity was evaluated through flow cytometer using easy kit II. To evaluate the mitochondrial activity of spermatozoa in semen sample, 5 µL of DMSO was added in easykit II well and pipette up and down 3 times to re-suspend the dye. Then, 185 µL of easy buffer B (or EHS) and 10 µL of diluted pooled semen was added in well and properly mixed then incubated at 37°C for 30 minutes in the dark. The mixed samples in wells were transferred into tube before running the samples with appropriated setting. The gating of cells used in the flow-cytometry is summarized in Fig.3.7 (Fig 3.7.1 & 3.7.2).

Table 18: Different types of sperm population showing the mitochondrial activity

S. No.	Type of spermatozoa population	Mitochondrial Activity
1	Polarized spermatozoa	Spermatozoa with active mitochondria/ high potential
2	Depolarized spermatozoa	Spermatozoa with inactive mitochondria/ low potential

Two different populations of spermatozoa were evaluated and the results section summarizes the findings of polarized spermatozoa (spermatozoa with active mitochondria having higher membrane potential).

Fig. 3.7: Dot Plot gating used in flow cytometry for estimation of mitochondrial membrane potential

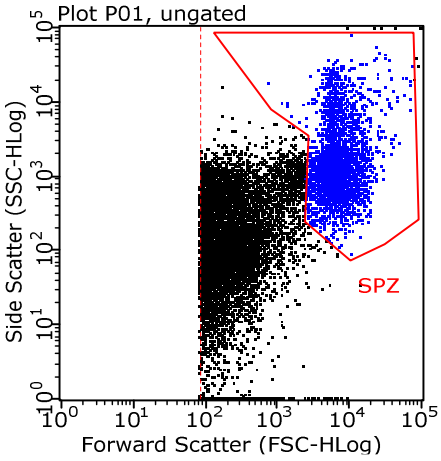


Fig. 3.7.1: Blue colour dot plot representing the cell concentration

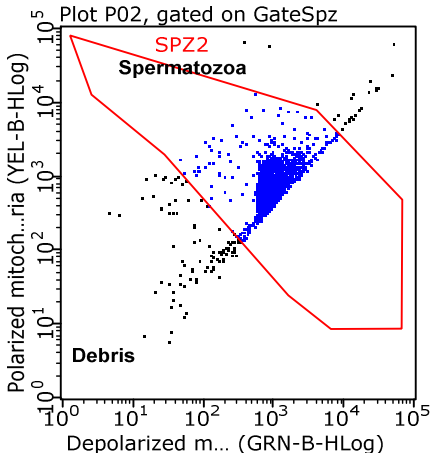


Fig. 3.7.2: Blue colour dot plot representing spermatozoa

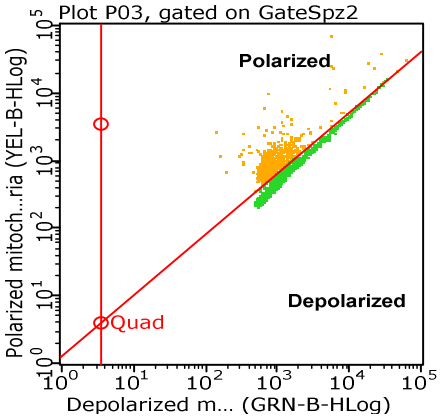


Fig. 3.7.3: Dot plot representing polarized and depolarized spermatozoa

3.9.14 Intracellular Calcium

Principle

The calcium assay was done using Fluo-4 Direct Calcium Assay Kits Invitrogen. Prior to estimation the solutions are prepared as follows:

1. Probenecid acid 250mM: 1 ml of assay buffer (C) added in probenecid (B) and mixed until complete mixing and stored at -20 °C
2. 2X dye: 5 ml of assay buffer (C) and 100 µL of probenecid (B) added in the bottle of A. Vortex for 1-2 minutes for complete mixing.

Methodology

The intracellular calcium in semen sample were analyzed through flowcytometer (easycyte II, IMV, France) using easykit (IMV Technologies, France) and using sperm guava easycyte software. Add 90 µL of assay buffer component C in appropriate wells, homogenize pre diluted semen and add 10 µL of diluted pooled semen in well and then add 100 µL of dye 2X. Homogenize the wells by pipetting up and down with a 200 µL tip. Now incubate for 30 minutes at 30 C in the dark. Homogenize the wells and transfer the wells into tubes before running the samples with appropriate settings. The gating of cells used in the flow-cytometry is summarized in Fig.3.8 (3.8.1, 3.8.2, & 3.8.3).

Fig. 3.8: Dot Plot gating used in flow cytometry for estimation of intracellular Calcium

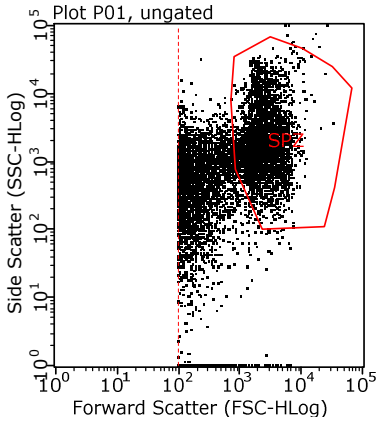


Fig. 3.8.1: Dot plot showing distribution of cell debris and cells

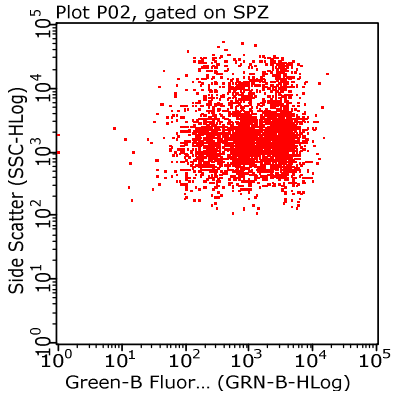


Fig 3.8.2: Red colour dot plot representing the cell concentration

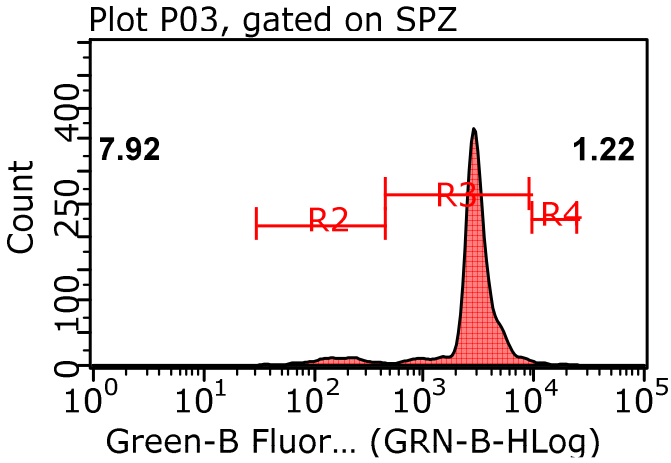


Fig. 3.8.3: Dot plot showing separation of spermatozoa based on concentration of calcium: 1. R2 shows spermatozoa with low intracellular calcium, 2. R3 shows spermatozoa with medium intracellular calcium, 3. R4 shows spermatozoa with high intracellular calcium.

3.10.1 Haematological attributes

3.10.1.1. Collection of blood

Peripheral blood samples were collected in heparinised vaccutainer tubes (BD Franklin, USA) by venipuncture of jugular vein at 0, 30, 60 and 90 days post supplementation. Collected blood samples were analyzed for haematological, blood biochemical, anti oxidant and hormonal attributes. A fraction of whole blood samples were used for haemoglobin (Hb) concentration and packed cell volume (PCV) estimation. Remaining amount of blood samples was centrifuged at 3000 rpm for 15 min to separate the plasma. Plasma samples were stored at −20°C until further analysis of metabolites.

3.10.1.2 Haemoglobin (Hb) concentration

Haemoglobin concentration was estimated in the fresh blood with the help of MS4Se autohaemoanalyser by Nexus medical solution 002296745-A.

3.10.1.3 Packed cell volume (PCV) or haematocrit (HCT)

PCV was estimated in the fresh bloodwith the help of MS4Se autohaemoanalyse rby Nexus medical solution 002296745-A.

3.10.2 Plasma attributes

3.10.2.1 Plasma Glucose

Plasma Glucose was estimated by “GOD-POD, End point assay test kit” supplied by Span Diagnosis Ltd. Glucose oxidase (GOD) oxidizes glucose to gluconic acid and Hydrogen Peroxide. In presence of enzyme Peroxidase, released Hydrogen Peroxide is coupled with phenol and 4-Aminoantipyrine (4-AAP) to form coloured Quinoneimine dye. Absorbance of coloured dye is measured at 505 nm and is directly proportional to glucose concentration in the sample.

Procedure

20 µl of plasma aliquot was pipetted in 10×75 mm tubes in duplicate, to which 1500 µl of working glucose reagent was added. Blank (20 µl distilled water) and standard (20 µl from standard 50 mg/dl) were pipetted in duplicate to which 1500 µl of working glucose reagent was added. The contents were mixed well and incubated at 37 °C for 10 min. 1500 µl of purified water was added to each tube. The contents

were mixed well. UV-spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured.

Calculation: Plasma glucose levels were calculated as per formula and expressed in mg/100ml:

$$\text{Glucose (mg/100ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

3.10.2.2 Plasma Cholesterol

Plasma cholesterol was estimated in plasma samples by "CHOD-PAP, End point assay test kit" supplied by Span Diagnosis Ltd. The principle of assay was cholesterol esters are hydrolyzed by cholesterol esterase to give free cholesterol and fatty acids. In subsequent reaction, cholesterol oxidase, oxidizes the 3-OH group of free cholesterol to liberate cholest-4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide couples with 4-amino antipyrine and phenol to produce red quinoneimine dye. Absorbance of coloured dye was measured at 505 nm and was proportional to amount of total cholesterol concentration in the sample.

Procedure

10 µl of plasma aliquots were pipetted in 10×75 mm tubes in duplicate, to which 1000 µl of working cholesterol reagent was added. Blank (10 µl distilled water) and standard (10 µl from standard 200 mg/dl) was pipetted in duplicate, to which 1000 µl of working cholesterol reagent was added. The content was mixed well and incubated at 37ºC temperature for 10 minutes. UV-spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 505 nm.

Calculation: The cholesterol concentration was calculated as per the formula and expressed in mg/100ml:

$$\text{Cholesterol (mg/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

3.10.2.3 Plasma Triglyceride

Plasma triglycerides were estimated in plasma samples by "End point assay test kit" supplied by Span Diagnosis Ltd. The principle of assay was triglycerides are hydrolyzed by lipoprotein lipase (LPL) to give glycerol and free fatty acids. In

subsequent reaction, glycerol 3-PO_4 oxidase, oxidizes the 3-PO_4 group of free glycerol to liberate dihydroxy acetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide couples with 4-aminoantipyrine and 4-chlorophenol to produce red quinoneimine dye. Absorbance of coloured dye was measured at 505 nm and is proportional to amount of total triglycerides concentration in the sample.

Procedure

10 µl of plasma aliquots were pipetted in 10×75 mm tubes in duplicate, to which 1000 µl of working triglycerides reagent was added. Blank (10 µl distilled water) and standard (10 µl from standard 200 mg/dl) was pipetted in duplicate, to which 1000 µl of working triglycerides reagent was added. The contents were mixed well and incubated at 37ºC temperature for 10 minutes. UV-spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 505 nm.

Calculation: The triglyceride concentration was calculated as per the formula and expressed in mg/100ml

$$\text{Triglyceride (mg/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

3.10.2.4 Plasma Total protein

Total protein was estimated in blood plasma samples by using "Modified Biuret, End point assay test kit" supplied by Span Diagnosis Ltd. The principal of the assay is that the peptide bonds of proteins react with cupric ions in alkaline solution to form a coloured chelate; the absorbance was measured at 578 nm. The absorbance of final colour is proportional to the concentration of total protein in the sample.

Procedure

10 µl of plasma aliquot was pipette in 10×75 mm tubes in duplicate, to which 1000 µl of working biuret reagent was added. Blank (10 µl distilled water) and standard (10 µl from protein standard 6.5 g/dl) was pipette in duplicates, to which 1000 µl of working biuret reagent was added. The contents were mixed well and incubated at room temperature for 1 minute. UV-spectrophotometer was blanked with reagent was added. The content was mixed well and incubated at room temperature for 1minute. UV- spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 578 nm.

Calculation: Total protein concentration was calculated as per formula and expressed in g/100ml

$$\text{Total protein (g/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 6.5$$

3.10.2.5 Plasma Albumin

Albumin was estimated in blood plasma samples by using "Bromocresol green, End point assay test kit" supplied by Span Diagnosis Ltd. It is based on the principal that albumin binds with anionic dye Bromocresol green (BCG) to form green colour complex, which is measured at 630 nm. Kit reagent were prepared and stored as per the instruction provided with the assay kit.

Procedure

10 µl of plasma aliquots were pipetted in 10×75 mm tubes in duplicate, to which 1000 µl of working albumin reagent was added. Blank (10 µl distilled water) and standard (10µl from standard 4g/dl) was pipetted in duplicates, to which 1000 µl of working albumin reagent was added. The content were mixed well and incubated at room temperature for 1 minute. UV-spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 630 nm.

Calculation: The albumin concentration was calculated as per the formula and expressed in g/100ml:

$$\text{Albumin (g/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 4$$

3.10.2.6 Plasma Globulin

It was determined by subtracting the albumin content from total protein content:

Plasma Globulin (g/100ml) = Total protein concentration – albumin concentration

3.10.2.7 Aspartate amino transferase (AST) activity

AST in plasma of bucks was determined by UV modified, kinetic assay AST test kit (Span diagnostic ltd. Surat, India).

Assay Principle

AST catalyses the transamination of L-aspartate and α-ketoglutamate and oxaloacetate. In subsequent reaction, malate dehydrogenase reduces oxaloacetate to malate with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and is directly proportional to AST activity in the sample. Lactate dehydrogenase is added to enzyme system to prevent endogenous pyruvate interference, which is normally present in the plasma.

Reagent	Composition	Concentration
1 (Buffer)	Tris buffer (pH 7.8)	80 mmol/l
	L- Aspartate	240 mmol/l
	MDH	≥ 600 U/l
	LD	≥ 600 U/l
2 (Substrate)	α- Ketoglutarate	12 mmol/l
	NADH	0.18 mmol/l

Procedure

Working AST reagent was prepared, as mentioned in test kit protocol, by mixing reagent 1 and reagent 2 in proportion of 1:4. 100 µl of plasma was mixed well with 1000 µl of working AST reagent and read absorbance at 340 nm in biochemical analyzer. Blank the analyzer with purified water. Read absorbance after 60 seconds. Repeat readings after every 30 seconds i.e. up to 120 seconds. Determine the mean absorbance change per minute (Δ A/minute).

Calculation

$$\text{AST activity (IU/l)} = \Delta\,\text{A/minute} \times \text{K}$$

Where; Δ A/minute is change in absorbance per minute and K is kinetic factor

3.10.2.8 Alanine aminotransferase (ALT) activity

ALT in plasma of calves was determined by UV modified, kinetic assay ALT (GPT) test kit (Span diagnostic ltd. Surat, India)

Assay principle

ALT catalyses the transamination of L-alanine and α- ketoglutarate to form pyruvate and L-glutamate. In subsequent reaction, lactate dehydrogenase reduces pyruvate to lactate with simultaneous oxidation of reduced NADH to NAD. The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and is directly proportional to ALT activity in the sample. Lactate dehydrogenase rapidly and completely reduces endogenous sample pyruvate during the initial incubation period, so that it does not interfere with the assay.

Reagent	Composition	Concentration
1 (Buffer)	Tris buffer (pH 7.5)	100 mmol/l
	L- Alanine	500 mmol/l
	LD	≥ 1200 U/l
2 (Substrate)	α- Ketoglutarate	15 mmol/l
	NADH	0.18 mmol/l

Procedure

Working ALT reagent was prepared, as mentioned in test kit protocol, by mixing reagent 1 and reagent 2 in proportion of 1:4. 100 µl of plasma was mixed well with 1000 µl of working ALT reagent and read absorbance at 340 nm in biochemical analyzer. Blank the analyzer with purified water. Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds. Determine the mean absorbance change per minute (Δ A/minute).

Calculation

$$\text{ALT activity (IU/l)} = \Delta\,\text{A/minute} \times \text{K}$$

Where; Δ A/minute is change in absorbance per minute and K is kinetic factor

3.10.3 Assessment of antioxidant status

Antioxidant enzymes (Superoxide dismutase (SOD), GSH-Px, ROS and Lipid peroxidation (LPO) were estimated to assess the antioxidant status. The SOD and LPO were estimated in blood lysate whereas GSH-Px and ROS were estimated in plasma.

3.10.3.1 Blood lysate preparation

Blood was taken in 2 ml micro-centrifuge tube and centrifuged at 3000 rpm in refrigerated centrifuge for 15 minutes. Plasma was taken off and the buffy coat was discarded and the compact RBC pellet was used for the preparation of the lysate. The pellets were washed thrice with normal saline (0.9% NaCl) solution and stored at -20^0C for further analysis. For SOD estimation, washed RBC pellets were first diluted to 33% with phosphate buffer saline [(PBS) (NaCl, 8g + KCl, 0.2 g +KH_2PO_4, 0.2g +Na_2HPO_4, 0.94g dissolved in about 800ml distilled water and final volume was made upto 1L)] and again diluted 1:10 with PBS and was stored at 4^0C till analysis.

3.10.3.2 Superoxide Dismutase (SOD) activity

SOD was estimated as per the method described by Madesh and Balasubramanian (1997). It involved generation of super oxide by pyrogallol autoxidation and the inhibition of super oxide dependent reduction of the tetrazolium dye MTT (3- (4-5 dimethyl 2-xl) 2, 5 diphenyltetrazolium bromide) to its formazan which was measured at 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazane formed. The colour evolved was stable for many hours and was expressed as SOD units (one unit of SOD is the amount (mg) of hemoglobin required to inhibit the MTT reduction by 50%).

Chemical and reagents

1) 100μM pyragallol (Sisco Research Laboratories Pvt. Ltd, India): 6.3 mg of pyragallol was dissolved in the 5 ml of distilled water. One ml from this solution was added to 100 ml of distilled water.
2) 1.25 mM MTT (Sigma-Aldrich, Missouri, USA): 2.58 mg MTT was dissolved in 5 ml of distilled water.
3) Phosphate buffer saline (PBS): PBS was prepared as described for separation of erythrocytes.
4) DMSO (Dimethyl sulfoxide)

Reagents	Sample	Control	Blank (duplicate)
PBS	0.65ml	0.65 ml	0.65 ml
MTT	30 µl	30 µl	30 µl
Hemolysate	10µl	--	--
Pyragallol	75 µl	75 µl	75 µl
Incubated for 5 min at room temperature			
DMSO	0.75 ml	0.75ml	0.75ml

The absorbance of sample was read at 570 nm against blank.

Calculation

$$\text{SOD (U)} = \frac{\text{mg of haemoglobin} \times 50 \times \text{Dilution factor}}{\text{Y \%}} \times 100$$

$$\text{(Y \%) Inhibition of MTT reduction by SOD protein (\%)} = \frac{\text{(OD of test)}}{\text{(OD of control)}} \times 100$$

3.10.3.3 Glutathione Peroxidase (GSH-Px)

GSH-Px was determined in the plasma of bucks by "Goat Glutathione Peroxidase ELISA Kit" (Catalog No. E0064Go) by Bioassay Technology Laboratory from Nanhu Dist, Jiaxing Zhejiang Province, China.

Assay Principle

This kit is an Enzyme-Linked Immuno sorbent Assay (ELISA). The plate has been pre- coated with Goat GSH-Px antibody. GSH-Px present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Goat GSH-Px antibody is added and binds to GSH-Px in the sample. Streptavidin-HRP is added and binds to the biotinylated GSH-Px antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of goat GSH-Px. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagents required

Components	Quantity
Standard solution(320ng/ml)	0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop solution	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash buffer concentrate (25x)	20ml x1
Biotinylated goat GSH-Px antibody	1ml x1

Reagent Preparation

All reagents should be brought to room temperature before use.

Standard Preparation

Reconstitute the 120µl of the standard (320ng/ml) with 120µl of standard diluent to generate a 160ng/ml standard stock solution. Allow the standard to sit for 15 min with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (160ng/ml) 1:2 with standard diluent to produce 80ng/ml, 40ng/ml, 20ng/ml and 10ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{O}C and used within one month. Dilutions of standard solutions suggested are as follows:

160ng/ml	Standard no.5	120µl Original standard + 120µl Standard diluent
80ng/ml	Standard no.4	120µl Standard no.5 + 120µl Standard diluent
40ng/ml	Standard no.3	120µl Standard no.4 + 120µl Standard diluent
20ng/ml	Standard no.2	120µl Standard no.3 + 120µl Standard diluent
10ng/ml	Standard no.1	120µl Standard no.2 + 120µl Standard diluent

Standard concentration	Standard no.5	Standard no.4	Standard no.3	Standard no.2	Standard no.1
320ng/ml	160ng/ml	80ng/ml	40ng/ml	20ng/ml	10ng/ml

Wash Buffer

Dilute 20 ml of Wash Buffer Concentrate 25X into deionized or distilled water to yield 500 ml of 1X Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Assay Procedure

1) Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2) Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3) Add 50µl standard to standard well.
4) Add 40µl plasma sample to sample wells and then add 10µl anti-GSH-Px antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5) Remove the sealer and wash the plate5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6) Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7) Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.
8) Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

Construct a standard curve by plotting the average OD for each standard on the Vertical (Y) axis against the concentration on the Horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

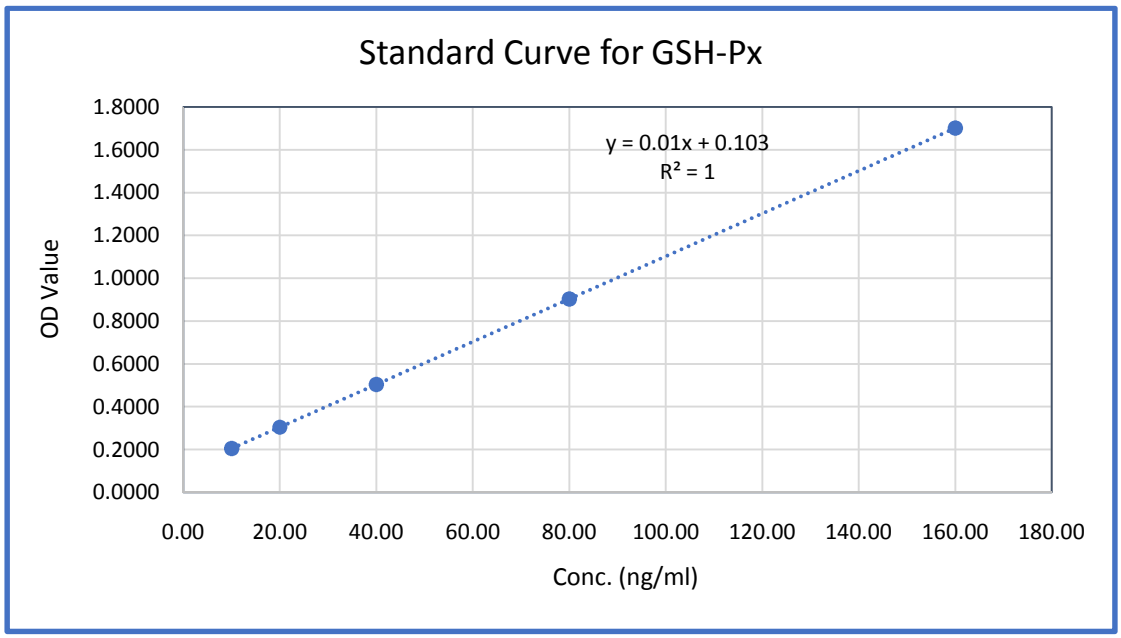


Fig. 3.9: Standard curve of GSH-Px

3.10.3.4 Lipid peroxidation (LPO)

MDA is the measure of lipid peroxidation. The extent of lipid peroxidation was evaluated in terms of malondialdehyde (MDA) production, which was determined by the method of Shafiq-U-Rehman (1984). 1.0 ml hemolysate (33%) was taken to which 1ml of 10% TCA was added and thoroughly mixed, vortex and centrifuged at 2000 rpm of 10 min. To 1.0 ml of supernatant liquid, an equal amount of 0.67% TBA was added and kept in boiling water bath for 10 min. The reaction mixture was cooled under running tap water and diluted with one ml distilled water. Absorbance was recorded ad 535 nm. Calculation was done by using the major extinction coefficient of MDA-TBA complex at 535 nm, i.e. 1.56×10^{8}M/cm. The amount of lipid peroxidation was expressed as nM MDA formed /ml packed RBCs.

LPO (nM MDA/ml packed RBCs = (OD/EC)×(Total volume of the reaction mixture/Amount of sample taken)×10^9×DF×2 (Incubation time)

Where, EC = 1.56 ×10^8M/cm molar extinction coefficient.

3.10.3.4 Reactive Oxygen Species (ROS)

ROS was estimated in terms of H_2O_2 in plasma sample as developed by Alberti et al. (2000) for a 96-well plate format.

Requirements:

1) Standard H_2O_2 solution
2) 0.1M acetate Buffer (0.1M, pH- 4.8)
   I. 0.1M Acetic acid
   II. 0.1M sodium acetate
3) 0.37M DEPPD (N, N′-diethyl-1, 4-phenylenediamine)

Protocol

To 0.2 ml of acetate buffer, 5 µl of plasma sample was added in micro plate. The plate was shaken for 1 min. on a thermo-shaker and then 5 µl of DEPPD was added. The plate was incubated at 37°C for 75 min. and shaking was done. A standard curve was prepared using different concentration of H_2O_2 in place of test samples. Then absorbance was read in spectrophotometer at 495 nm.

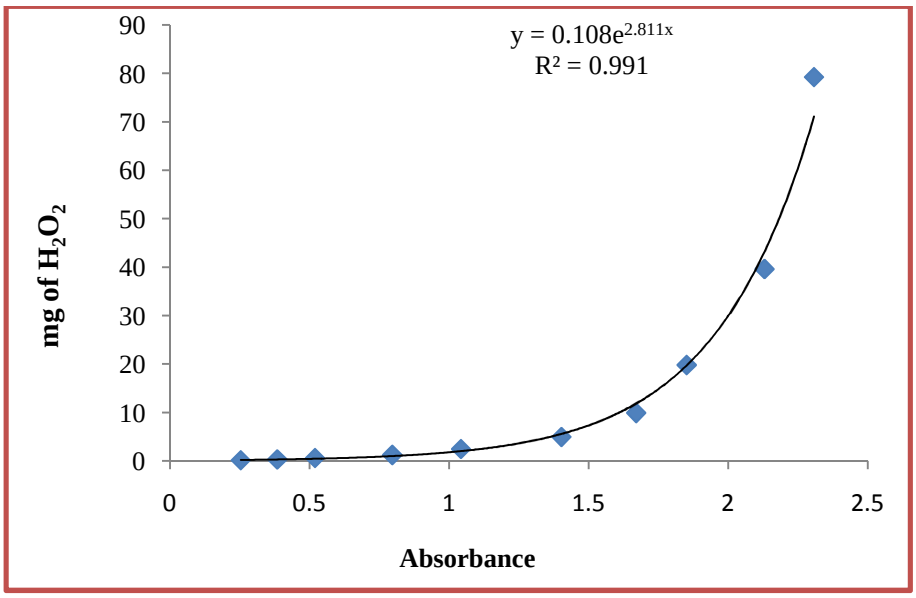


Fig. 3.10: Standard curve for the estimation of ROS activity

3.10.4 Assessment of hormonal status

3.10.4.1 Assay for plasma Testosterone

Testosterone was determined in the plasma of bucks by "Goat Testosterone ELISA Test kit" (Catalog No. E0026Go) by Bioassay Technology Laboratory from Nanhu Dist, Jiaxing Zhejiang Province, China.

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre- coated with Goat T antibody. T present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Goat T antibody is added and binds to T in the sample. Streptavidin-HRP is added and binds to the biotinylated T antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of goat Testosterone. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent required

Components	Quantity
Standard solution(32ng/ml)	0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop solution	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash Buffer concentrate (25x)	20ml x1
Biotinylated goat T antibody	1ml x1

Reagent Preparation

All reagents should be brought to room temperature before use.

Standard Preparation

Reconstitute the 120µl of the standard (32ng/ml) with 120µl of standard diluent to generate a 16ng/ml standard stock solution. Allow the standard to sit for 15 min with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (16ng/ml) 1:2 with standard diluent to produce 8ng/ml, 4ng/ml, 2ng/ml and 1ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{O}C and used within one month. Dilution of standard solutions suggested are as follows:

16 ng/ml	Standard no.5	120µl Original standard + 120µl Standard diluent
8 ng/ml	Standard no.4	120µl Standard no.5 + 120µl Standard diluent
4 ng/ml	Standard no.3	120µl Standard no.4 + 120µl Standard diluent
2 ng/ml	Standard no.2	120µl Standard no.3 + 120µl Standard diluent
1 ng/ml	Standard no.1	120µl Standard no.2 + 120µl Standard diluent

Standard concentration	Standard no.5	Standard no.4	Standard no.3	Standard no.2	Standard no.1
32ng/ml	16ng/ml	8ng/ml	4ng/ml	2ng/ml	1ng/ml

Wash Buffer

Dilute 20 ml of Wash Buffer Concentrate 25X into deionized or distilled water to yield 500 ml of 1X Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Assay Procedure

1) Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2) Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3) Add 50µl standard to standard well.
4) Add 40µl plasma sample to sample wells and then add 10µl anti-T antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells

(Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5) Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6) Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7) Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.

8) Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

Construct a standard curve by plotting the average OD for each standard on the Vertical (Y) axis against the concentration on the Horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

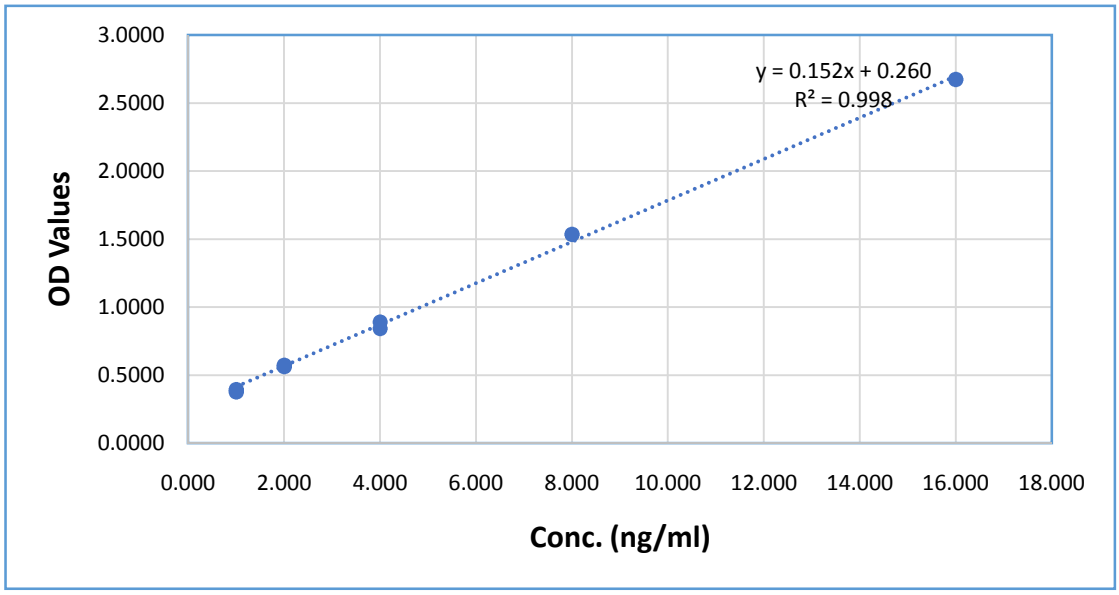


Fig. 3.11: Standard curve of Testosterone

3.10.4.2 Assay for plasma Luteinizing Hormone (LH)

LH was determined in the plasma of bucks by "Goat LH ELISA Test kit" (Catalog No. E0007Go) by Bioassay Technology Laboratory from Nanhu Dist, Jiaxing Zhejiang Province, China.

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre- coated with Goat LH antibody. LH present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Goat LH antibody is added and binds to LH in the sample. Streptavidin-HRP is added and binds to the biotinylated LH antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of goat LH. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent required

Components	Quantity
Standard solution(24mIU/ml)	0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash buffer concentrate (25x)	20ml x1
Biotinylated goat LH antibody	1ml x1

Reagent Preparation

All reagents should be brought to room temperature before use.

Standard Preparation

Reconstitute the 120µl of the standard (24mIU/ml) with 120µl of standard diluent to generate a 12mIU/ml standard stock solution. Allow the standard to sit for

15 min with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (6mIU/ml) 1:2 with standard diluent to produce 3 mIU/ml, 1.5 mIU/ml and 0.75mIU/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{O}C and used within one month. Dilutions of standard solutions suggested are as follows:

12 mIU/ml	Standard no.5	120µl Original Standard + 120µl Standard diluent
6 mIU/ml	Standard no.4	120µl Standard No.5 + 120µl Standard diluent
3 mIU/ml	Standard no.3	120µl Standard No.4 + 120µl Standard diluent
1.5 mIU/ml	Standard no.2	120µl Standard No.3 + 120µl Standard diluent
0.75mIU/ml	Standard no.1	120µl Standard No.2 + 120µl Standard diluent

Standard concentration	Standard no.5	Standard no.4	Standard no.3	Standard no.2	Standard no.1
24 mIU/ml	12 mIU/ml	6 mIU/ml	3 mIU/ml	1.5 mIU/ml	0.75 mIU/ml

Wash Buffer

Dilute 20 ml of Wash Buffer Concentrate 25X into deionized or distilled water to yield 500 ml of 1X Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Assay Procedure

1) Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2) Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3) Add 50µl standard to standard well.
4) Add 40µl plasma sample to sample wells and then add 10µl anti-LH antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5) Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6) Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7) Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.

8) Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

Construct a standard curve by plotting the average OD for each standard on the Vertical (Y) axis against the concentration on the Horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

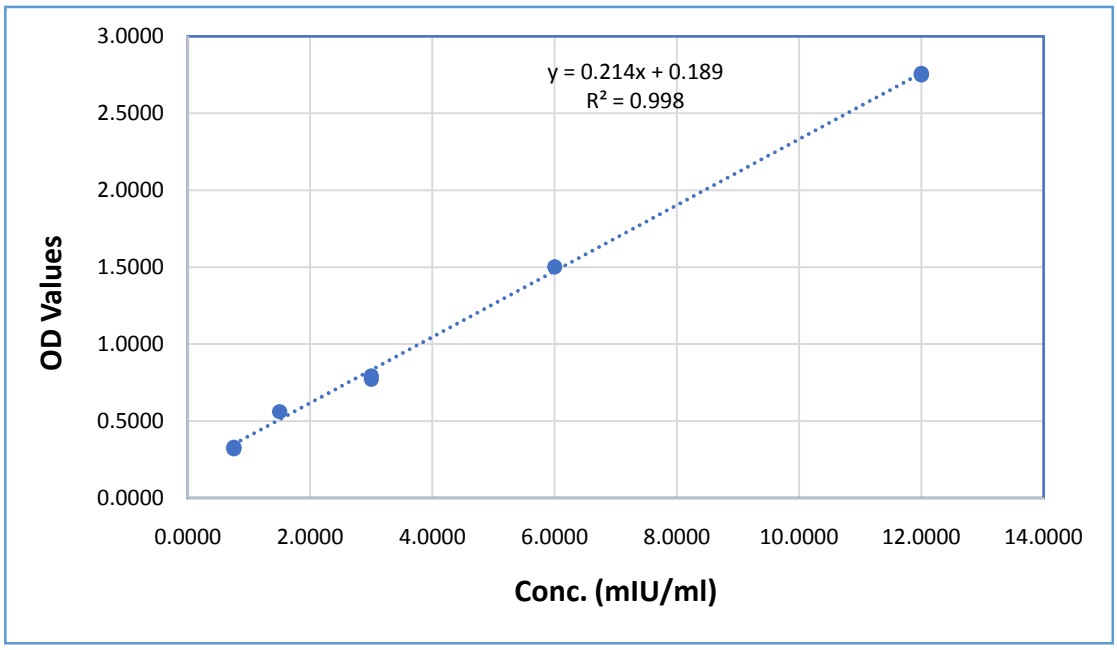


Fig. 3.12: Standard curve of LH

3.10.4.2 Assay for plasma IgG

IgG was determined in the plasma of bucks by "Goat IgG ELISA Test kit" (Catalog No. E0001Go) by Bioassay Technology Laboratory from Nanhu Dist, Jiaxing Zhejiang Province, China.

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Goat IgG antibody. IgG present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Goat IgG antibody is added and binds to IgG in the sample. Then Streptavidin-HRP is added and binds to the biotinylated IgG antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of goat IgG. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent required

Components	Quantity
Standard solution(240 μg/ml)	0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash buffer concentrate (25x)	20ml x1
Biotinylated goat IgG antibody	1ml x1

Reagent Preparation

All reagents should be brought to room temperature before use.

Standard Preparation

Reconstitute the 120μl of the standard (240 μg/ml) with 120μl of standard diluent to generate a 120 μg/ml standard stock solution. Allow the standard to sit for

15 min with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (120 μg/ml) 1:2 with standard diluent to produce 60 μg/ml, 30 μg/ml, 15 μg/ml and 7.5 μg/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{O}C and used within one month. Dilutions of standard solutions suggested are as follows:

120 μg/ml	Standard no.5	120μl Original standard + 120μl Standard diluent
60 μg/ml	Standard no.4	120μl Standard no.5 + 120μl Standard diluent
30 μg/ml	Standard no.3	120μl Standard no.4 + 120μl Standard diluent
15 μg/ml	Standard no.2	120μl Standard no.3 + 120μl Standard diluent
7.5 μg/ml	Standard no.1	120μl Standard no.2 + 120μl Standard diluent

Standard concentration	Standard no.5	Standard no.4	Standard no.3	Standard no.2	Standard no.1
240 μg/ml	120 μg/ml	60 μg/ml	30 μg/ml	15 μg/ml	7.5 μg/ml

Wash Buffer

Dilute 20 ml of Wash Buffer Concentrate 25X into deionized or distilled water to yield 500 ml of 1X Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Assay Procedure

1) Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2) Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3) Add 50μl standard to standard well.
4) Add 40μl plasma sample to sample wells and then add 10μl anti-IgG antibody to sample wells, then add 50μl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5) Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6) Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7) Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.

8) Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

Construct a standard curve by plotting the average OD for each standard on the Vertical (Y) axis against the concentration on the Horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

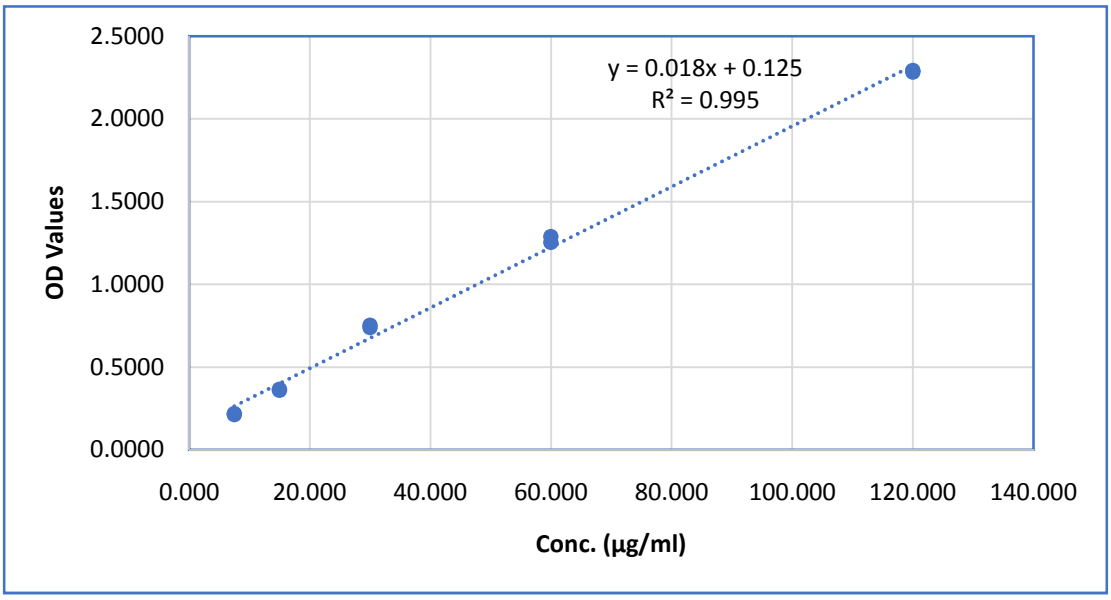


Fig. 3.13: Standard curve of IgG

3.4 Evaluations of the status of minerals in blood

Evaluation of blood minerals was done by using ICP- OES Techniques. Values were expressed in ppb and ppm.

3.4.1 Digestion of blood plasma sample

Plasma samples were digested by Microwave digestion method (Microwave digestion system: Multiwave 5000, Anton Paar, Virginia, USA) as per the procedure described by Rodushkin et al. (1999) used for ICP-OES techniques. 0.5 ml of plasma sample was taken into PFA digestion vessel, and 5 ml nitric acid (additionally purified), along with 0.5ml H_2O_2 were added to it. The vessels were closed tightly and place into the microwave oven and digested at 600 W powers for 1 hour. After digestion the vessel were removed from oven and cool to room temperature. Now the digest was transferred to a volumetric flask and diluted with MQ water to make final volume 50 ml for ICP analysis. For Selenium estimation using MSIS (Multimode Sample Introduction System), 2 ml of this sample were mixed with 2 ml of concentrated hydrochloric acid and incubated at 70°C for 10 minutes and then cooled at room temperature and used for analysis.

3.4.2 Estimation of minerals in plasma samples

The minerals namely, Calcium, Phosphorus and Selenium were analyzed by the inductively coupled plasma-optical emission spectroscopy (5800 ICP-OES, Agilent, CA, USA) facility at Animal Nutrition Department, DUVASU Mathura. The selenium was analyzed by hydride generation using Multimode Sample Introduction System (MSIS) using hydride generator assembly. The wavelengths (nm) used were 196.026 nm for selenium, 422.673 nm for Calcium, 213.618 nm for phosphorus. The instrument conditions were 12 L/min plasma gas flow, 0.7 L/min nebulizer gas flow, 1 L/min Aux flow and the viewing mode was axial at 8 mm height for analysis of the minerals. All the samples were run in triplicate.

For Calcium and Phosphorus estimation the standard prepared were 0, 1, 5, 10 and 20 ppm with ICP multi-element standard solution IV (Merck chemicals, Darmstadt, Germany), whereas for Selenium the standards prepared were 0, 10, 20, 50 and 100 ppb. From these standards, calibration curve was prepared for these minerals by plotting the absorbance against the concentration (Fig. 3.14, 3.15, 3.16). After plotting the calibration curve the concentration of the minerals in the sample

was calculated automatically by the system using ICP Expert software. Operating parameters for standards were similar as described for samples.

3.10.5 Statistical Analysis

The data was analyzed using the general linear model (GLM) procedure of Statistical Package for the Social Sciences (SPSS 2020 Inc., Chicago, IL, USA) as a randomized block design with animal as the experimental unit. The pair-wise comparison of means was carried out using "Tukey's honest significant difference (HSD) test". Significance was determined at $P<0.05$.

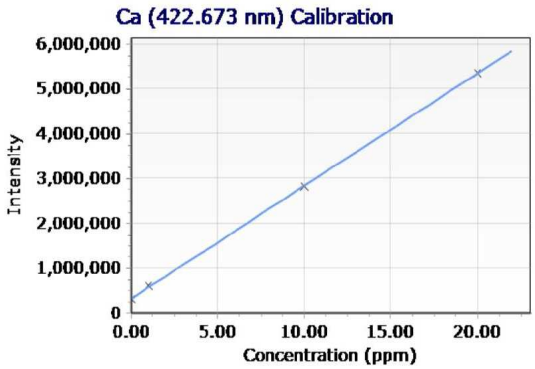


Fig. 3.14: Calibration curve of Calcium

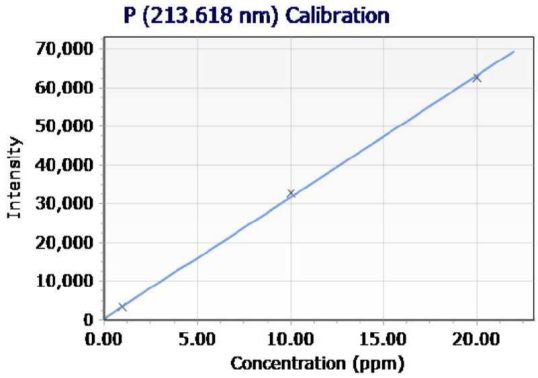


Fig. 3.15: Calibration curve of Phosphorus

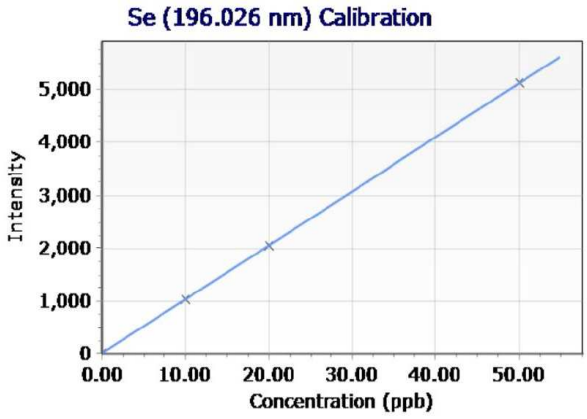


Fig. 3.16: Calibration curve of Selenium

Fig. 3.17: Shed of Bucks

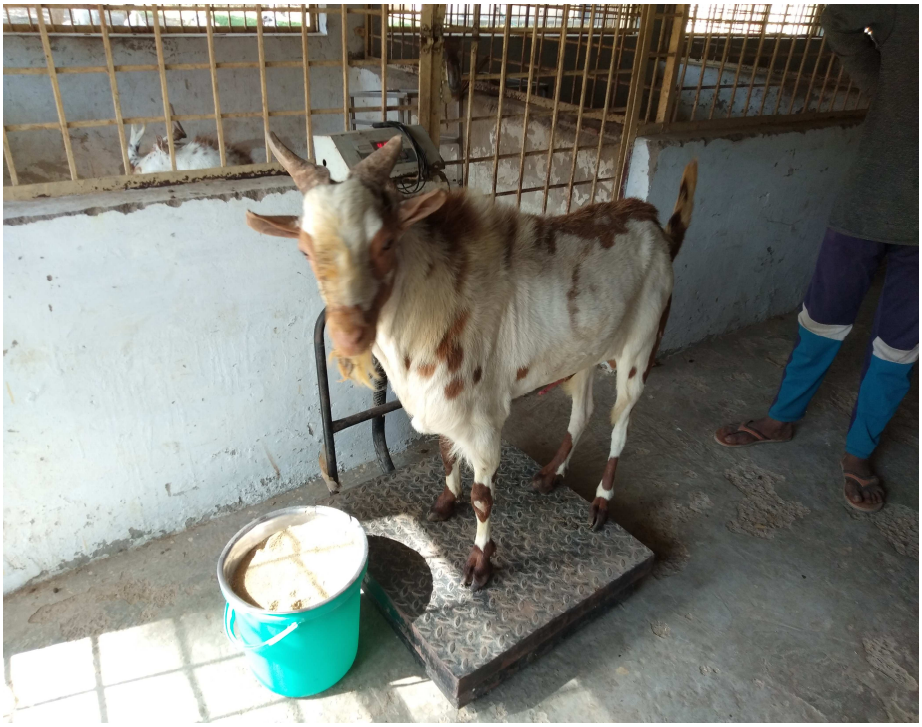


Fig. 3.18: Recording of body weight of buck

Fig. 3.19: Digestion trial

Fig. 3.20: Feeding of Selenium through concentrate

Fig. 3.21: Estimation of crude protein

Fig. 3.22: Haematological examination

Fig. 3.23: Mineral estimation by ICP-OES

Fig. 3.24: Hormone estimation by ELISA kit method

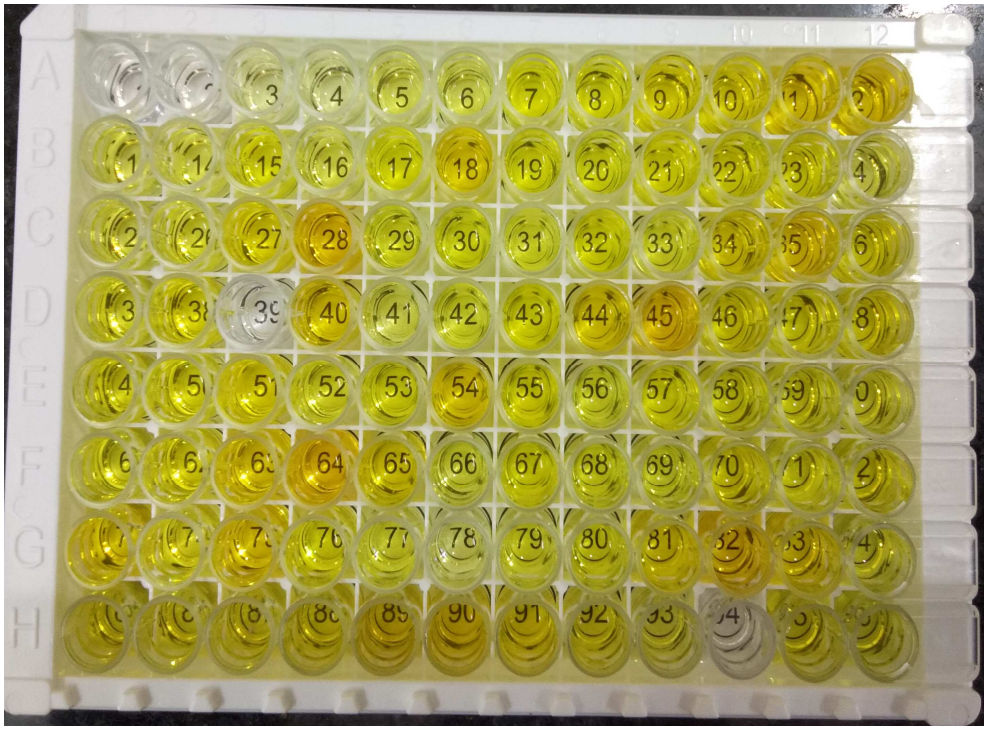


Fig. 3.25: ELISA Plate

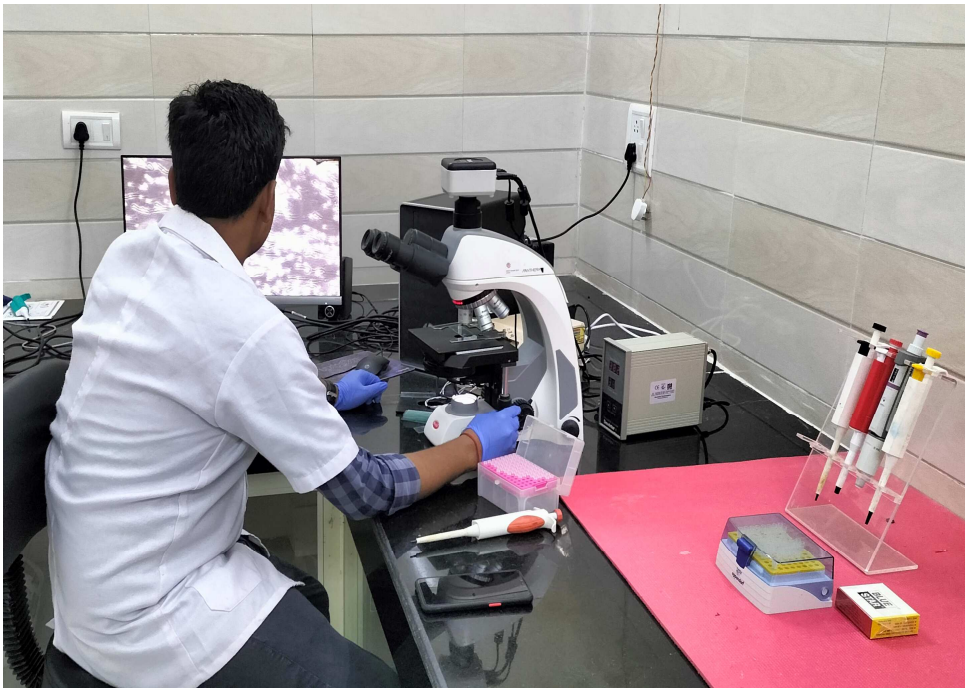


Fig. 3.26: Semen Evaluation through Microscope

Fig. 3.27: Evaluation of semen quality through Flow Cytometer

Results

CHAPTER- 4

RESULTS

The objective of the study was to evaluate the effect of dietary supplementation of different sources of Se on the animal growth performance, nutrient utilization, seminal attributes, blood biochemicals, antioxidant status, plasma mineral and hormonal status of bucks. In this experiment, inorganic, organic and nano Se were supplemented at level of 0.3 mg/kg DM offered in T1, T2 and T3 group respectively. The observations recorded on different parameters are presented in the following chapter.

4.1 Chemical composition (% DM basis) of dietary feed ingredients fed to bucks

The chemical composition of experimental diets and dietary components (on dry matter basis) are presented in Table 4.1. The proximate principle i.e., dry matter, organic matter, ether extract, crude protein, total ash, crude fibre, nitrogen free extract in concentrate mixture were found to be 91.32, 79.49, 3.34, 19.34, 14.83, 12.80, and 49.69 % respectively. The corresponding value for gram straw was 92.82, 82.75, 2.14, 8.21, 10.06, 31.42 and 48.16 % respectively. In case of corn silage was 34.33, 28.88, 3.8, 10.23, 5.45, 28.68 and 51.84 % respectively.

The percentage of cell wall constituent as per Van-Soest fraction i.e., NDF, ADF, ADL, cellulose and hemicelluloses in concentrate mixture was 31.58, 13.42, 1.60, 11.82 and 28.16 % respectively. The corresponding values in gram straw were 61.93, 41.36, 3.60, 37.76 and 20.57 % respectively. The values in corn silage were 57.4, 38.64, 2.80, 35.84 and 18.76% respectively.

Table 4.1: Chemical composition (%DM basis) of dietary feed ingredient fed to experimental bucks.

Sr. No	Item	Concentrate	Gram straw	Corn silage	TMR
1	DM	91.32	92.82	34.33	78.64
2	OM	79.49	82.75	28.88	68.23
3	EE	3.34	2.14	3.8	2.87
4	CP	19.34	8.21	10.23	11.99
5	ASH	11.83	10.06	5.45	10.4
6	CF	12.80	31.42	28.68	25.1
7	NFE	49.69	48.16	51.84	49.32
8	NDF	31.58	61.93	57.4	54.6
9	ADF	13.42	41.36	38.64	32.23
10	ADL	1.60	3.60	2.80	2.80
11	Cellulose	11.82	37.76	35.84	29.42
12	Hemicellulose	28.16	20.57	18.76	22.37
13	Se (ppm)	0.086	0.36	0.25	0.23
14	Ca (%)	1.85	0.31	0.74	1.01
15	P (%)	0.43	0.11	0.36	0.27

4.2 Different growth parameter in bucks supplemented with different sources of Se

4.2.1 Body weight (BW) and Metabolic body weight ($W^{0.75}$)

Average BW of experimental bucks of all four groups measured at fortnight intervals during the three months experimental period has been given in Table 4.2. Initially, average BW of bucks was 36.93, 36.92, 36.75 and 36.28 kg in Control, T1, T2 and T3 groups respectively. Final body weights of corresponding groups were 42.83, 43.35, 43.02 and 42.43 kg respectively. The average BW of all the fortnights was not significantly different ($P>0.05$) between groups. The overall BW (kg) was found similar in all the experimental groups.

Table 4.2: Body weight (kg) of bucks supplemented with different sources of Se

Days	Treatments				SEM	P Value
	Control	T1	T2	T3		
0	36.93	36.92	36.75	36.28	2.388	0.997
15	37.93	38.00	37.77	37.35	2.342	0.997
30	38.95	39.02	38.80	38.37	2.280	0.997
45	39.87	40.13	39.60	39.47	2.235	0.997
60	40.85	41.32	40.65	40.50	2.208	0.994
75	41.90	42.25	41.80	41.38	2.197	0.994
90	42.83	43.35	43.02	42.43	2.175	0.992
Average	39.84	40.14	39.77	39.40	0.853	0.941

Average metabolic body weight of bucks of all four groups measured at fortnight intervals during the three-month experimental period has been given in Table 4.3. Initially, average metabolic body weights ($W^{0.75}$) of bucks were 14.95, 14.94, 14.90 and 14.76 kg in Control, T1, T2 and T3 groups respectively. Final Metabolic body weights of corresponding groups were 16.72, 16.87, 16.78 and 16.60 kg, respectively. The average values of all the fortnights were not significantly different ($P>0.05$) within the groups.

Table 4.3: Metabolic body weight ($W^{0.75}$) of bucks supplemented with different sources of Se

Days	Treatments				SEM	P Value
	Control	T1	T2	T3		
0	14.95	14.94	14.90	14.76	0.732	0.997
15	15.26	15.27	15.21	15.08	0.713	0.997
30	15.57	15.58	15.52	15.39	0.689	0.997
45	15.84	15.92	15.76	15.72	0.670	0.997
60	16.14	16.27	16.08	16.03	0.657	0.995
75	16.45	16.55	16.42	16.30	0.649	0.994
90	16.72	16.87	16.78	16.61	0.639	0.993
Average	15.85	15.92	15.81	15.70	0.256	0.945

4.2.2 Dry matter intake (DMI)

Dry matter intake (kg/d) in Control, T1, T2 and T3 groups during different fortnights of experimental feeding has been presented in Table 4.4. At fortnightly interval DMI of control, T1 T2 and T3 ranged from 1.26 to 1.42, 1.29 to 1.42, 1.25 to 1.37 and 1.28 to 1.40 kg/d respectively. Overall DMI (kg/d) in present study did not differ significantly (P>0.05) between the groups. The percent DMI (kg/100 kg BW) in experimental animals in Control, T1, T2 and T3 groups during different fortnights of experimental feeding has been presented in Table 4.5. Overall percentage DMI (kg/100 kg BW) in present study did not differ significantly different (P>0.05) between the groups.

Table 4.4: Dry matter intake (kg/day) of bucks supplemented with different sources of Se

Days	Treatments				SEM	P value
	Control	T1	T2	T3		
15	1.26	1.29	1.25	1.28	0.071	0.978
30	1.31	1.34	1.30	1.32	0.074	0.984
45	1.31	1.34	1.30	1.32	0.074	0.984
60	1.35	1.38	1.35	1.35	0.073	0.989
75	1.38	1.41	1.36	1.40	0.069	0.96
90	1.42	1.42	1.37	1.40	0.063	0.939
Average	1.34	1.36	1.32	1.35	0.028	0.777

Table 4.5: Percent Dry matter intake (kg/100 kg BW/day) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
15	3.45	3.52	3.44	3.55	0.101	0.846
30	3.46	3.54	3.47	3.55	0.089	0.869
45	3.46	3.54	3.47	3.55	0.089	0.869
60	3.47	3.54	3.50	3.53	0.081	0.926
75	3.48	3.54	3.46	3.56	0.081	0.812
90	3.48	3.45	3.39	3.48	0.089	0.866
Average	3.47	3.52	3.46	3.53	0.034	0.268

4.2.3 Body weight gain

The fortnightly BW gain of experimental bucks of Control, T1, T2 and T3 groups have been presented in Table 4.6. Fortnightly body weight gain (kg) ranged from 0.92 to 1.05, 0.93 to 1.18, 0.8 to 1.22 and 0.88 to 1.1 kg in Control, T1, T2 and T3 groups respectively. The overall fortnightly BW gain (kg) in present study did not differ significantly (P>0.05) between the groups. Average daily gain (g/d) of bucks of all the three groups measured at fortnightly intervals is presented in Table 4.7. At first fortnight the average daily gain was 66.68, 72.22, 67.78 and 71.11 g/d in Control, T1 T2 and T3 groups, respectively. The overall average daily gain (g/d) did not differ significantly (P>0.05) between the groups.

Table 4.6: Fortnightly body weight gain (kg) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
15	1.00	1.08	1.02	1.07	0.087	0.89
30	1.02	1.02	1.03	1.02	0.116	0.999
45	0.92	1.12	0.80	1.10	0.126	0.258
60	0.98	1.18	1.05	1.03	0.119	0.678
75	1.05	0.93	1.15	0.88	0.095	0.22
90	0.93	1.10	1.22	1.05	0.133	0.522
Average	0.98	1.07	1.04	1.02	0.046	0.569

Table 4.7: Average daily gain (g) of bucks supplemented with different sources of Se

Days	Treatment				SEM	p value
	Control	T1	T2	T3		
15	66.67	72.22	67.78	71.11	5.80	0.89
30	67.78	67.78	68.89	67.78	7.74	0.99
45	61.11	74.44	53.33	73.33	8.42	0.26
60	65.56	78.89	70.00	68.89	7.95	0.68
75	70.00	62.22	76.67	58.89	6.30	0.22
90	62.22	73.33	81.11	70.00	8.89	0.52
Average	65.56	71.48	69.63	68.333	3.03	0.57

4.2.4 Feed conversion ratio (FCR)

The feed conversion ratio of experimental animals at different fortnight is given in Table 4.8. The FCR ranged from 20.40 to 26.32, 18.02 to 23.89, 18.07 to 38.56 and 18.52 to 24.68 in Control, T1, T2 and T3 groups respectively. The averages values of FCR of all the fortnights were not significantly different (P>0.05) between the groups. The Overall FCR was found similar in all the experimental groups.

Table 4.8: Feed conversion ratio (FCR) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
15	20.40	18.51	19.22	18.52	2.16	0.916
30	21.24	21.20	21.71	20.62	3.40	0.997
45	23.47	18.83	38.56	19.31	7.79	0.269
60	21.68	18.02	21.36	23.39	3.54	0.753
75	21.18	23.89	18.29	24.68	2.34	0.238
90	26.32	21.96	18.07	22.07	3.96	0.550
Average	22.38	20.40	22.87	21.43	1.76	0.764

4.2.5. Nutrient intake and digestibility in bucks supplemented with different sources of Se

The data pertaining to digestibility coefficient of Control and treatments group have been presented in Table 4.9. The DM digestibility was 60.83, 58.66, 61.42 and 62.02 in Control, T1, T2 and T3 groups, respectively. Whereas, the OM digestibility was 69.29, 68.18, 66.92 and 69.96 % in Control, T1, T2 and T3 groups, respectively. The statistical analysis of data on DM and OM digestibility coefficients revealed that there was no significant difference (P>0.05) between the groups. The CP digestibility coefficient in Control, T1, T2 and T3 groups were 66.11, 65.71, 68.03 and 66.64 %, respectively. The EE digestibility coefficient in Control, T1, T2 and T3 groups were 81.30, 82.13, 81.42 and 83.08 % respectively. The statistical analysis of data on CP and EE digestibility coefficient revealed that there was no significant difference (P>0.05) between the groups. The CF digestibility coefficient in Control, T1, T2 and T3 groups were 49.17, 46.05, 47.24 and 47.07% respectively. The NFE digestibility coefficient in Control, T1, T2 and T3 groups were 58.26, 56.54, 58.13 and 56.20%

respectively. The digestibility coefficient of CF and NFE were also found similar in Control and treatment groups. The NDF digestibility coefficient in Control, T, T2 and T3 groups were 56.38, 57.12, 57.68 and 58.06% respectively. The ADF digestibility coefficient in Control, T1, T2 and T3 groups were 51.19, 48.34, 47.49 and 46.17% respectively. Both NDF and ADF digestibility coefficient were found similar in Control and treatment groups. Digestible DM, OM, EE, NFE, CF, NDF, ADF and TDN intake were found similar in all the three groups (Table 4.10). Body weight and body weight gain was found similar in all the experimental groups during digestion trial.

Table 4.9: Nutrient intake and digestibility of bucks supplemented with different sources of Se

Attributes	Treatment				SEM	P value
	Control	T1	T2	T3		
Initial Wt.(kg)	41.20	41.69	41.30	40.67	2.23	0.991
Final Wt. (kg)	41.78	42.27	41.85	41.32	2.20	0.992
Wt. gain(kg)	0.58	0.58	0.55	0.64	0.067	0.803
DM intake kg/day	1.55	1.52	1.47	1.48	0.048	0.586
CP intake kg/day	0.17	0.16	0.16	0.15	0.008	0.532
DCP intake kg/day	0.11	0.11	0.11	0.10	0.007	0.788
DCP intake g/kg $W^{0.75}$	6.94	6.501	6.55	6.45	0.395	0.813
TDN intake kg/day	0.70	0.633	0.623	0.688	0.049	0.601
TDN intake g/kg $W^{0.75}$	43.02	38.91	38.44	42.77	2.72	0.502
Digestibility Coefficient (%)						
DM digestibility	60.83	58.66	61.42	62.02	1.87	0.615
OM digestibility	69.29	68.18	66.92	69.96	1.84	0.674
CP digestibility	66.11	65.71	68.03	66.64	2.01	0.857
CF digestibility	49.17	46.05	47.24	47.07	2.12	0.772
EE digestibility	81.30	82.13	81.42	83.08	1.973	0.914
NFE digestibility	58.26	56.54	58.13	56.20	2.88	0.937
NDF digestibility	56.38	57.12	57.68	58.06	3.01	0.981
ADF digestibility	51.19	48.37	47.49	46.16	2.20	0.439

Table 4.10: Digestible nutrient intake of bucks supplemented with different sources of Se

Attributes	Treatment				SEM	P value
	Control	T1	T2	T3		
DM	0.84	0.75	0.75	0.78	0.077	0.804
OM	1.25	1.22	1.18	1.17	0.067	0.813
CP	0.14	0.13	0.13	0.13	0.008	0.700
CF	0.15	0.13	0.13	0.13	0.013	0.553
EE	0.02	0.02	0.02	0.02	0.002	0.634
NFE	0.37	0.33	0.33	0.32	0.030	0.646
NDF	0.39	0.36	0.35	0.35	0.037	0.862
ADF	0.18	0.15	0.14	0.14	0.017	0.264
TDN	0.68	0.65	0.64	0.63	0.037	0.781

4.3 Seminal attributes

4.3.1 Ejaculated semen volume (ml)

Ejaculated semen Volume (ml) in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.11. At different collection interval volume of ejaculated semen (ml) of Control, T1, T2 and T3 groups ranged from 0.78 to 0.81, 0.77 to 0.79, 0.78 to 0.79 and 0.79 to 0.80 ml respectively. Overall ejaculated volume of semen (ml) in present study was found similar in all experimental groups.

Table 4.11: Ejaculated Semen volume (ml) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	0.79	0.77	0.78	0.79	0.007	0.61
90	0.81	0.79	0.79	0.80	0.015	0.909
Average	0.80	0.78	0.79	0.79	0.008	0.696

4.3.2 Colour of semen

Colour of semen in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.12. At different collection interval colour of semen is Creamy white colour.

Table 4.12: Colour of semen of bucks supplemented with different sources of Se

Days	Treatment			
	Control	**T1**	**T2**	**T3**
0 Day	Creamy white colour	Creamy white colour	Creamy white colour	Creamy white colour
90 Day	Creamy white colour	Creamy white colour	Creamy white colour	Creamy white colour

4.3.3 Seminal pH

Seminal pH in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.13. At different collection interval mean of seminal pH of all Control, T1, T2 and T3 groups were 6.75, 6.78, 6.79 and 6.77. Overall seminal pH in present study was found similar in all experimental groups.

Table 4.13: Seminal pH of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	**T1**	**T2**	**T3**		
0	6.72	6.78	6.80	6.77	0.02	0.092
90	6.78	6.78	6.78	6.76	0.018	0.883
Average	6.75	6.78	6.79	6.77	0.014	0.230

4.3.4 Concentration of spermatozoa (10^9/ml)

Concentration of spermatozoa (10^9/ml) in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.14. At different collection interval concentration (10^9/ml) of Control, T1, T2 and T3 groups ranged from 7.32 to 7.65, 6.97 to 7.42, 7.40 to 7.82 and 7.38 to 7.58

(10^9/ml) respectively. Overall concentration of spermatozoa in present study was found similar in all experimental groups.

Table 4.14: Concentration of spermatozoa (10^9/ml) of bucks supplemented different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	7.65	7.42	7.82	7.58	0.149	0.338
90	7.32	6.97	7.40	7.38	0.121	0.68
Average	7.48	7.19	7.61	7.49	0.108	0.058

4.3.5 Mass motility (0-5 scales)

Mass motility of sperms in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.15. At different collection interval mass motility of Control, T1, T2 and T3 groups ranged from 4.08 to 4.17, 3.92 to 4.08, 3.92 to 4.17 and 4.08 to 4.17 respectively. Overall mass motility in present study was found similar in all experimental groups.

Table 4.15: Mass motility of spermatozoa of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	4.17	3.92	3.92	4.08	0.128	0.432
90	4.08	4.08	4.17	4.17	0.132	0.939
Average	4.13	4.0	4.04	4.12	0.091	0.702

4.3.6 Percent progressive motility of spermatozoa

Percent progressive motility of spermatozoa in Control, T1, T2 and T3 groups during different collection interval of experimental feeding have been presented in Table 4.16. At different collection interval, percent progressive motility of spermatozoa of Control, T1, T2 and T3 groups ranged from 78.49 to 79.50, 78.78 to 82.51, 79.41 to 86.68and 77.15to 90.62% respectively. At 90 day collection percent progressive motility of spermatozoa count was significantly higher ($P<0.05$) in T3

group as compared to control group and comparable in T1 and T2 group as compared to control group. Overall percent progressive motility of spermatozoa count was found significantly higher (P<0.05) in T3 group animal as compared to Control group.

Table 4.16: Percent progressive motility of spermatozoa in bucks supplemented different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	78.49	78.78	79.41	77.15	1.12	0.913
90	79.50^{b}	82.51^{ab}	86.68^{ab}	90.62^{a}	2.00	0.012
Average	77.99^{b}	80.65^{b}	82.71^{ab}	84.97^{a}	1.19	0.033

Means with different superscript in row differ significantly (P<0.05)

4.3.7 Percent live spermatozoa

Percent live spermatozoa in Control, T1, T2 and T3 groups during different collection interval of experimental feeding have been presented in Table 4.17. At different collection interval, percent live spermatozoa of Control, T1, T2 and T3 ranged from 82.50 to 83.49, 82.79 to 86.52, 82.41 to 89.68 and 83.15 to 92.62 % respectively. At 90 day collection, percent live spermatozoa count was significantly higher (P<0.05) in T3 group as compared to control, T1 and T2 group. Overall percent live spermatozoa count was found significantly higher (P<0.05) in T3 as compared to Control group.

Table 4.17: Percent live spermatozoa in bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	83.49	82.79	82.41	83.16	1.122	0.913
90	82.50^{b}	86.52^{ab}	89.68^{ab}	92.62^{a}	2.003	0.012
Average	83.10^{b}	84.66^{ab}	86.21^{ab}	87.97^{a}	1.193	0.033

Means with different superscript in row differ significantly (P<0.05)

4.3.8 HOST reactive spermatozoa (%)

HOST reactive spermatozoa (%) in Control, T1, T2 and T3 groups during different collection interval of experimental feeding have been presented in Table 4.18. At different collection interval, HOST reactive spermatozoa (%) of Control, T1, T2 and T3 ranged from 78.17 to 79.33, 80.33 to 82.33, 78.33 to 82.00 and 81.33 to 85.17 % respectively. At 90 day collection HOST reactive spermatozoa count was significantly higher (P<0.05) in T3 group as compared to control, T1 and T2 group. Overall percent HOST reactive spermatozoa count was found significantly higher (P<0.05) in T3 group as compared to control group and comparable in T1 and T2 group.

Table 4.18: HOST reactive spermatozoa percentage of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	78.17	80.33	78.33	81.83	0.968	0.064
90	79.33^{b}	82.33^{ab}	82.00^{ab}	85.17^{a}	1.116	0.014
Average	78.75^{b}	81.33^{ab}	80.17^{ab}	83.50^{a}	1.116	0.031

Means with different superscript in row differ significantly (P<0.05)

4.3.9 Intact Acrosomal percentage

Intact acrosomal percentage in Control, T1, T2 and T3 groups during different collection interval of experimental feeding have been presented in Table 4.19. At different collection interval intact acrosomal percentage of Control, T1, T2 and T3 ranged from 72.69 to 73.69, 74.49 to 77.29, 74.27 to 83.52and 72.46 to 87.29 % respectively. At 90 day collection acrosomal percentage was significantly higher (P<0.05) in T3 group as compared to control, T1 and T2 group. Overall intact acrosomal percentage was found significantly higher (P<0.05) in T3 as compared to Control, T1 and T2 group bucks.

Table 4.19: Intact Acrosomal percentages of spermatozoa of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	73.69	74.49	74.27	72.46	2.011	0.893
90	72.69^{c}	77.29^{bc}	83.52^{ab}	87.29^{a}	2.193	0.001
Average	73.19^{b}	75.89^{ab}	78.97^{ab}	79.88^{a}	1.726	0.032

Means with different superscript in row differ significantly (P<0.05)

4.3.10 Total morphological abnormality percentage

Total morphological abnormality percentage in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.20. At different collection interval total morphological abnormality percentage of Control, T1, T2 and T3 ranged from 3.67 to 3.78, 3.50 to 3.60, 3.13 to 3.17 and 2.72 to 3.47 % respectively. At 90 day collection total morphological abnormality percentage was significantly lower (P<0.05) in T3 group and comparable in T1 and T2 group as compared to control group. Overall total morphological abnormality percentage of spermatozoa was found significantly lower T3 group and comparable in T1 and T2 as compared to control.

Table4.20: Total morphological abnormality percentage of spermatozoa of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	3.67	3.50	3.17	3.47	0.168	0.234
90	3.78^{a}	3.60^{ab}	3.13^{ab}	2.72^{b}	0.241	0.023
Average	3.72^{a}	3.55^{ab}	3.15^{ab}	3.07^{b}	0.150	0.008

Means bearing different superscript in a row differ significantly (P < 0.05)

4.3.11 Reactive Oxygen Species (ROS)

i. ROS positive

Total ROS positive spermatozoa percentage in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.21. At different collection interval total ROS positive spermatozoa percentage of Control, T1, T2 and T3 ranged from 11.42 to 11.59, 9.35 to 10.54, 8.05 to 11.53, and 6.97 to 10.08 % respectively. At 90-day collection total ROS positive spermatozoa percentage was significantly lower (P<0.05) in T3 and T2 group and comparable in T1 group as compared to control group. Overall total ROS positive spermatozoa percentage in present study was significantly lower in T3 and comparable in T1 and T2 as compared to control group.

Table 4.21: Total ROS positive spermatozoa percentage of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	11.42	10.54	11.53	10.08	0.958	0.667
90	11.59^{a}	9.35^{ab}	8.05^{b}	6.97^{b}	0.852	0.007
Average	11.51^{a}	9.95^{ab}	9.78^{ab}	8.53^{b}	0.657	0.024

Means bearing different superscript in a row differ significantly (P < 0.05)

ii. ROS negative

Total ROS negative spermatozoa percentage in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.22. At different collection interval total ROS negative spermatozoa percentage of Control, T1, T2 and T3 ranged from 64.64 to 65.64, 68.79 to 70.12, 67.08 to 73.91, and 67.11 to 78.78 % respectively. At 90 day collection total ROS negative spermatozoa percentage was significantly higher (P<0.05) in T3 group and comparable in T1 and T2 group as compared to control group. Overall total ROS positive spermatozoa percentage in present study was significantly higher in T3 group and comparable in T1and T2 group as compared to control group.

Table 4.22: Total ROS negative spermatozoa percentage of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	65.64	68.79	67.08	67.11	1.63	0.607
90	64.64^{c}	70.12^{bc}	73.91^{ab}	78.78^{a}	1.82	0.001
Average	65.14^{b}	69.45^{ab}	70.50^{ab}	72.95^{a}	1.55	0.009

Means bearing different superscript in a row differ significantly (P < 0.05)

4.3.12 Intracellular Calcium

i. Low Calcium

Total spermatozoa percentage with low intracellular calcium in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.23. At different collection interval total spermatozoa percentage with low intracellular calcium in Control, T1, T2 and T3 ranged from 7.67 to 7.81, 5.40 to 7.53, 3.04 to 7.04, and 1.04 to 7.45 % respectively. At 90 day collection total spermatozoa percentage with low intracellular calcium was significantly lower (P<0.05) in T3, T2 and T1 group as compared to control group. Overall total spermatozoa percentage with low intracellular calcium was significantly lower in T3 and T2 group and comparable in T1 as compared to control group.

Table 4.23: Total spermatozoa percentage with low intracellular Calcium of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	7.67	7.53	7.47	7.45	0.237	0.910
90	7.81^{a}	5.40^{b}	3.04^{c}	1.04^{d}	0.230	0.001
Average	7.74^{a}	6.46^{ab}	5.26^{b}	4.25^{b}	0.628	0.002

Means bearing different superscript in a row differ significantly (P < 0.05)

ii. Medium Calcium

Total spermatozoa percentage with medium intracellular calcium in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.24. At different collection interval total spermatozoa percentage with medium intracellular calcium in Control, T1, T2 and T3 ranged from 90.92 to 91.01, 91.00 to 93.16, 91.25 to 95.03, and 91.18 to 97.10 % respectively. At 90 day collection total spermatozoa percentage with medium intracellular calcium was significantly higher (P<0.05) in T3 as compared to control, T1 and T2 group. Overall total spermatozoa percentage with medium intracellular calcium was significantly higher in T3 group and comparable in T1 and T2 as compared to control group.

Table 4.24: Total spermatozoa percentage with medium intracellular Calcium of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	90.92	91.00	91.25	91.18	0.244	0.75
90	91.01^{d}	93.16^{c}	95.03^{b}	97.10^{a}	0.386	<0.001
Average	90.97^{b}	92.08^{ab}	93.15^{ab}	94.14^{a}	0.595	0.003

Means bearing different superscript in a row differ significantly (P < 0.05)

iii. High Calcium

Total spermatozoa percentage with high intracellular calcium in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has

been presented in Table 4.25. At different collection interval total spermatozoa percentage with high intracellular calcium in Control, T1, T2 and T3 ranged from 1.18 to 1.41, 1.44 to 1.47, 1.28 to 1.92, and 1.37 to 1.85 % respectively. Overall total spermatozoa percentage with high intracellular calcium in present study was found similar in all experimental groups.

Table 4.25: Total spermatozoa percentage with high intracellular Calcium of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	1.41	1.47	1.28	1.37	0.149	0.842
90	1.18	1.44	1.92	1.85	0.334	0.375
Average	1.29	1.45	1.60	1.61	0.185	0.593

Means bearing different superscript in a row differ significantly ($P < 0.05$)

4.3.13Mitochondrial Activity

Total spermatozoa percentage with polarized mitochondria in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.26. At different collection interval total spermatozoa percentage with polarized mitochondria of Control, T1, T2 and T3 ranged from 76.43 to 77.71, 78.04 to 80.44, 77.41 to 87.16, and 76.09 to 90.94 % respectively. At 90 day collection total spermatozoa percentage with polarized mitochondria was significantly higher ($P<0.05$) in T3 and T2 group and comparable in T1group as compared to control group. Overall total spermatozoa percentage with polarized mitochondria in present study was significantly higher in T3 group and comparable in T1 and T2 as compared to control group.

Table 4.26: Total spermatozoa percentage with polarized mitochondria of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	77.71	78.04	77.51	76.09	1.506	0.808
90	76.43^{b}	80.44^{ab}	87.16^{a}	90.94^{a}	1.102	<0.001
Average	77.07^{b}	79.24^{ab}	82.34^{ab}	83.52^{a}	1.617	0.029

Means bearing different superscript in a row differ significantly (P < 0.05)

4.3.14 Membrane Fluidity

i. Normal

Total spermatozoa percentage with normal membrane fluidity in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.27. At different collection interval total spermatozoa percentage with normal membrane fluidity of Control, T1, T2 and T3 ranged from 70.45 to 72.12, 70.49 to 71.49, 71.44 to 78.78, and 72.35 to 81.68 % respectively. At 90 day collection total spermatozoa percentage with normal membrane fluidity was significantly higher (P<0.05) in T3 and T2 group as compared to control and T1 group. Overall total spermatozoa percentage with normal membrane fluidity in present study was significantly higher in T3 group and comparable in T2 as compared to control and T1 group.

Table 4.27: Total spermatozoa percentage with normal membrane fluidity of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	72.12	71.49	71.44	72.35	1.69	0.975
90	70.45^{c}	70.49^{c}	78.78^{b}	81.68^{a}	0.571	<0.001
Average	71.28^{b}	70.99^{b}	75.11^{ab}	77.02^{a}	0.975	0.002

Means bearing different superscript in a row differ significantly (P < 0.05)

ii. Apoptotic

Total apoptotic spermatozoa percentage in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.28. At different collection interval total apoptotic spermatozoa percentage in Control, T1, T2 and T3 ranged from 4.25 to 4.45, 2.84 to 4.10, 1.21 to 4.38, and 2.23 to 4.06 % respectively. At 90 day collection total apoptotic spermatozoa percentage was significantly lower (P<0.05) in T2 and T1 group and comparable in T3 group as compared to control group. Overall apoptotic spermatozoa percentage in present study was significantly lower in T3 group and comparable in T2 and T1 group as compared to control group.

Table 4.28: Total apoptotic spermatozoa percentage of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	4.25	4.10	4.38	4.06	0.335	0.903
90	4.45^{a}	2.84^{b}	1.21^{c}	2.23^{ab}	0.399	<0.001
Average	4.35^{a}	3.47^{ab}	2.80^{ab}	3.15^{b}	0.384	0.039

Means bearing different superscript in a row differ significantly (P < 0.05)

iii. High Fluidity

Total spermatozoa percentage with high membrane fluidity in Control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.29. At different collection interval total spermatozoa percentage with high membrane fluidity of Control, T1, T2 and T3 ranged from 19.26 to 19.76, 20.55 to 22.05, 16.26 to 19.43, and 12.70 to 19.70 % respectively. At 90 day collection total spermatozoa percentage with high membrane fluidity was significantly lower (P<0.05) in T3, T2 and T1 group as compared to control. Overall total spermatozoa percentage with high membrane fluidity in present study was significantly lower in T3 group and comparable in T2 and T1 as compared to control group.

Table 4.29: Total spermatozoa percentage with high membrane fluidity ofbucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	19.76	20.55	19.43	19.70	1.481	0.956
90	19.26^{b}	22.05^{a}	16.26^{c}	12.70^{d}	0.616	<0.001
Average	19.51^{a}	21.30^{ab}	17.85^{ab}	16.20^{b}	0.967	0.004

Means bearing different superscript in a row differ significantly (P < 0.05)

iv. Transitional

Total transitional spermatozoa percentage in control, T1, T2 and T3 groups during different collection interval of experimental feeding has been presented in Table 4.30. At different collection interval total transitional spermatozoa percentage in Control, T1, T2 and T3 ranged from 4.99 to 5.49, 4.85 to 4.85, 3.95 to 5.12, and 3.41 to 5.08 % respectively. At 90 day collection total transitional spermatozoa percentage was significantly lower (P<0.05) in T3 and T2 group as compared to control and T1 group. Overall total transitional spermatozoa percentage present study was similar in all groups.

Table 4.30: Total transitional spermatozoa percentage of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	4.99	4.85	5.12	5.08	0.462	0.978
90	5.49^{a}	4.85^{a}	3.95^{b}	3.41^{b}	0.167	<0.001
Average	5.24	4.85	4.53	4.24	0.282	0.092

Means bearing different superscript in a row differ significantly (P < 0.05)

4.4 Haematological parameters

4.4.1 Haemoglobin concentration

Haemoglobin concentration (g/dl) of experimental animals at monthly interval is presented in Table 4.31. The overall concentration of haemoglobin at end of experiment was found to be 10.22, 10.67, 10.57 and 10.96 g/dl in Control, T1,T2 and

T3 groups, respectively. The overall haemoglobin concentration (g/dl) of treatment groups was found similar with Control group for whole trial period.

Table 4.31: Haemoglobin concentration (g/dl) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	11.68	11.37	11.10	11.60	0.494	0.839
30	11.10	10.63	10.62	11.17	0.506	0.797
60	10.68	10.45	10.47	10.38	0.363	0.942
90	10.22	10.22	10.08	10.68	0.363	0.669
Average	10.92	10.67	10.57	10.96	0.225	0.539

4.4.2 Packed cell volume (%)

The Packed cell volume (%) of experimental animals at monthly interval is presented in Table 4.32. The overall PCV percentage was found to be 30.9, 28.4, 28.20 and 29.17 % in Control, T1, T2 and T3 groups respectively. Overall packed cell volume (%) was found similar in all groups.

Table 4.32: Packed cell volume (%) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	30.78	28.383	28.25	29.13	1.004	0.289
30	29.22	29.25	28.22	28.90	1.002	0.875
60	29.65	28.05	27.88	29.07	0.947	0.514
90	30.90	27.92	28.47	29.57	1.101	0.263
Average	30.14	28.40	28.20	29.17	0.483	0.024

4.5 Blood Biochemical parameters

4.5.1 Plasma Glucose

The plasma Glucose concentration (mg/dl) of experimental bucks at monthly interval is presented in Table 4.33. The value ranged from 67.83 to 72.26 mg/dl in

Control, 66.37 to 71.29 mg/dl in T1, 66.69 to 73.70 mg/dl in T_2 and 67.88 to 71.04 mg/dl in T_3 groups. The overall glucose concentration (mg/dl) of treatment groups was found similar with Control group for whole trial period.

Table 4.33: Plasma Glucose concentration (mg/dl) in bucks supplemented with different sources of Se

Day	Treatment				SEM	P value
	Control	T1	T2	T3		
0	72.26	71.29	72.64	70.01	4.011	0.967
30	70.95	68.12	66.69	67.97	4.104	0.90
60	74.45	66.37	69.01	67.88	3.622	0.438
90	67.83	69.04	73.70	71.04	2.998	0.545
Average	71.37	68.71	70.51	69.22	1.787	0.71

4.5.2Plasma Cholesterol

The plasma cholesterol concentration (mg/dl) of experimental animals at monthly interval is presented in Table 4.34. The values ranged from 67.86 to 75.29 mg/dl in Control, 70.69 to 77.27 mg/dl in T1, 73.58 to 82.16 in T2 and 72.25 to 74.66 mg/dl in T3 groups. Overall cholesterol concentration of treatment groups was found similar with Control group bucks.

Table 4.34: Plasma Cholesterol concentration (mg/dl) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	71.59	73.82	73.58	72.25	3.419	0.961
30	70.43	73.37	82.16	74.66	4.163	0.261
60	67.86	70.69	76.23	75.10	4.757	0.581
90	75.29	77.27	79.41	72.77	5.349	0.839
Average	71.30	73.79	77.84	73.70	2.151	0.196

4.5.3 Plasma Triglyceride

The plasma triglyceride concentration (mg/dl) of experimental animals at monthly interval is presented in Table 4.35. The values ranged from 35.39 to 42.09 g/dl in Control, 31.28 to 53.38 mg/dl in T1, 33.86 to 45.53 in T2 and 34.17 to 39.54 mg/dl in T3 groups. Overall plasma triglyceride concentration of treatment groups was found similar with Control group bucks.

Table 4.35: Plasma Triglyceride concentration (mg/dl) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	35.39	31.28	33.86	34.17	1.298	0.185
30	42.08	53.38	45.53	39.06	3.557	0.054
60	41.96	31.59	39.27	37.13	3.878	0.306
90	37.82	37.68	41.49	39.54	2.077	0.542
Average	39.31	38.48	40.04	37.48	1.742	0.754

4.5.4 Plasma Total Protein

The plasma total protein concentration (g/dl) of experimental animals at monthly interval is presented in Table 4.36. The values ranged from 6.89 to 7.08 g/dl in Control, 7.25 to 8.40 g/dl in T1, 7.34 to 9.07 in T2 and 7.56 to 8.45 g/dl in T3 groups. Overall plasma total protein concentration of treatment groups was found significantly lower ($P<0.05$) in control group than treatment group bucks.

Table 4.36: Plasma Total Protein concentration (g/dl) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	6.96	7.25	7.34	7.56	0.391	0.757
30	6.93	7.34	7.91	8.45	0.608	0.34
60	6.89	8.30	9.07	8.26	0.538	0.065
90	7.08	8.40	8.53	8.31	0.501	0.183
Average	6.97^{b}	7.82^{ab}	8.21^{a}	8.15^{a}	0.258	0.003

Means bearing different superscript in a row differ significantly ($P < 0.05$)

4.5.5 Plasma Albumin

The plasma albumin concentration (g/dl) of experimental animals at monthly interval is presented in Table 4.37. The values ranged from 4.08 to 4.31 g/dl in Control, 4.03 to 4.42 g/dl in T1, 3.88 to 4.67 g/dl in T2 and 3.59 to 4.56 in T3 groups. Overall plasma albumin concentration of treatment groups was found similar with Control group bucks.

Table 4.37: Plasma Albumin concentration (g/dl) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	4.17	4.03	3.96	3.59	0.207	0.273
30	4.31	4.39	4.67	4.56	0.213	0.629
60	4.09	4.42	3.88	4.33	0.224	0.341
90	4.13	4.36	4.34	4.24	0.243	0.902
Average	4.17	4.30	4.21	4.18	0.117	0.868

4.5.6 Plasma Globulin

The plasma Globulin concentration (g/dl) of experimental animals at monthly interval is presented in Table 4.38. The values ranged from 2.62 to 2.95 g/dl in Control, 2.95 to 4.02 g/dl in T1, 3.24 to 5.18 in T2 and 3.88 to 4.08 g/dl in T3 groups. Overall plasma globulin concentration of treatment groups was found significantly higher ($P<0.05$) than control group bucks.

Table 4.38: Plasma Globulin concentration (g/dl) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	2.80	3.22	3.39	3.97	0.427	0.308
30	2.62	2.95	3.24	3.88	0.657	0.581
60	2.80^{b}	3.88^{ab}	5.18^{a}	3.93^{ab}	0.531	0.039
90	2.95	4.03	4.19	4.08	0.57	0.404
Average	2.79^{b}	3.52^{ab}	4.00^{a}	3.96^{a}	0.274	0.008

Means bearing different superscript in a row differ significantly ($P < 0.05$)

4.5.7 Plasma ALT activity

The plasma ALT activity (IU/L) of experimental animals at monthly interval is presented in Table 4.39. Mean plasma ALT activity at the beginning of experiment were 19.62, 18.45, 16.84 and 17.58 IU/L in Control, T1, T2 and T3 groups, respectively and corresponding values at the end of experiment were 22.14, 21.80, 22.67 and 20.09 IU/L respectively. Overall plasma ALT activity of treatment groups was found similar with Control group for whole trial period.

Table 4.39: Plasma ALT activity (IU/L) of bucks supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	19.62	18.45	16.84	17.58	1.704	0.691
30	21.62	16.38	21.57	16.22	2.037	0.114
60	22.25	16.62	17.50	22.23	1.661	0.042
90	22.14	21.80	22.67	20.09	1.989	0.815
Average	21.41	18.31	19.64	19.03	0.969	0.141

4.5.8 Plasma AST activity

The plasma AST activity (IU/L) of experimental animals at monthly interval is presented in Table 4.40. Mean plasma AST concentration at the beginning of experiment was 58.55, 61.26, 58.80 and 57.97 IU/L in Control, T1, T2 and T3 groups, respectively and corresponding values at the end of experiment were 59.12, 61.65, 57.92 and 60.26 IU/L respectively. Overall plasma ALT activity of treatment groups was found similar with Control group for whole trial period.

Table 4.40: Plasma AST activity (IU/L) of bucks supplemented with different sources of Se

Days	Treatments				SEM	P value
	Control	T1	T2	T3		
0	58.55	61.26	58.80	57.98	2.384	0.776
30	62.31	61.80	55.22	59.66	3.597	0.506
60	62.28	57.94	59.53	60.42	2.926	0.766
90	59.12	61.65	57.92	60.26	3.228	0.864
Average	60.57	60.66	57.87	59.58	1.464	0.506

4.6 Antioxidant parameters

4.6.1 Super oxide dismutase (SOD) activity

The SOD activity (U/mg Hb) of experimental animals at monthly interval is presented in Table 4.41. Mean SOD activity at the beginning of experiment were 22.49, 22.57, 22.53 and 23.02 U/mg Hb in Control, T1 and T2 groups, respectively and corresponding values at the end of experiment were 22.37, 27.42, 36.09 and 44.31 U/mg Hb, respectively. At 90 day the SOD activity U/mg Hb in T3 groups was found significantly higher (P<0.05) than Control group and comparable in T1 and T2 groups. Overall SOD activity of T3 group was found significantly higher (P<0.05) whereas of T1 and T2 was comparable than Control group bucks.

Table 4.41: SOD activity (U/mg Hb) of bucks supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	22.49	22.57	22.53	23.02	1.477	0.993
30	21.28	22.39	20.24	24.65	1.333	0.144
60	21.55	23.83	24.86	28.51	1.994	0.13
90	22.37^{b}	27.82^{ab}	36.09^{ab}	44.31^{a}	4.252	0.009
Average	21.92^{b}	24.15^{ab}	26.84^{ab}	29.21^{a}	1.646	0.014

Means bearing different superscript in a row differ significantly (P < 0.05)

4.6.2 Glutathione Peroxidase (GSH-Px)

The Plasma GSH-Px activity (ng/ml plasma) of experimental animals at monthly interval is presented in Table 4.42. Mean GSH-Px concentration (ng/ml plasma) at the beginning of experiment were 69.84, 72.17, 71.51 and 68.27 ng/ml plasma in Control, T1, T2 and T3 groups, respectively and corresponding values at the end of experiment were 67.42, 117.06, 133.60 and 168.81 ng/ml plasma respectively. GSH-Px activity in last fortnight of experimental period was significantly higher (P<0.05) in treatment groups as compared to Control group. Overall GSH-Px activity was found significantly higher (P<0.05) in treatment group bucks as compared to Control group bucks.

Table 4.42: GSH-Px activity (ng/ml) of bucks plasma supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	69.84	72.17	71.51	68.26	5.365	0.955
30	71.13	77.22	78.50	79.88	4.008	0.446
60	70.13^{a}	98.57^{b}	112.78^{bc}	132.62^{a}	5.89	0.001
90	67.42^{c}	117.06^{b}	133.60^{b}	168.81^{a}	8.597	0.001
Average	69.63^{c}	91.25^{bc}	99.10^{ab}	114.69^{a}	6.144	0.001

Means bearing different superscript in a row differ significantly (P < 0.05)

4.6.3 Lipid peroxidation (LPO) activity

Lipid peroxidation (nM MDA/ml packed RBCs) value of experimental animals at monthly interval is presented in Table 4.43. Mean lipid peroxidation (nM MDA/ml packed RBCs) activity at the beginning of experiment were 6.24, 6.11, 6.28 and 6.26 nM MDA/ml packed RBCs in Control, T1, T2 and T3 groups, respectively and corresponding values at the end of experiment were 6.12, 5.64, 4.67 and 4.23 nM MDA/ml packed RBCs respectively. Lipid peroxidation value in last fortnight of experimental period was significantly lower (P<0.05) in treatment groups as compared to control group. Overall lipid peroxidation value was found significantly lower (P<0.05) in T3 group bucks as compared to Control group bucks.

Table 4.43: Lipid peroxidation (nM MDA/ml packed RBCs) level of bucks supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	6.24	6.11	6.28	6.26	0.52	0.995
30	6.20	5.92	5.78	5.33	0.419	0.532
60	5.54	4.50	4.08	3.86	0.533	0.077
90	6.12^{a}	5.64^{ab}	4.67^{b}	4.23^{b}	0.375	0.0001
Average	6.20^{a}	5.61^{ab}	5.24^{ab}	4.82^{b}	0.264	0.003

Means bearing different superscript in a row differ significantly (P < 0.05)

4.6.4Reactive oxygen species

Reactive oxygen species (nM /ml) value of experimental animals at monthly interval is presented in Table 4.44. Mean Reactive oxygen species concentration (nM /ml) at the beginning of experiment were 0.618, 0.626, 0.616 and 6.12 nM/ml in Control, T1, T2 and T3 groups, respectively and corresponding values at the end of experiment were 0.626, 0.52, 0.46 and 0.40 nM/ml respectively. ROS value in last fortnight of experimental period was significantly lower (P<0.05) in treatment groups as compared to Control group. Overall ROS value was found significantly lower (P<0.05) in T3 group and comparable in T1 and T2 group bucks as compared to Control group bucks.

Table 4.44: ROS concentration (nM/ml) of bucks plasma supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	0.62	0.63	0.62	0.61	0.056	0.998
30	0.63	0.63	0.59	0.56	0.055	0.807
60	0.63	0.59	0.50	0.49	0.060	0.326
90	0.63^{a}	0.52^{ab}	0.46^{ab}	0.40^{b}	0.046	0.015
Average	0.62^{a}	0.59^{ab}	0.54^{ab}	0.52^{b}	0.028	0.030

Means bearing different superscript in a row differ significantly (P < 0.05)

4.7. Hormonal attributes

4.7.1 Plasma Testosterone

The plasma Testosterone concentration (ng/ml) of experimental animals at monthly interval is presented in Table 4.45. The value ranged from 3.08 to 3.18 ng/ml in Control, 3.25 to 3.63 in T1, 3.14 to 4.09 ng/ml in T2 group, and 3.12 to 4.53 ng/ml in T3 and group. In fourth and sixth fortnight the testosterone level was significantly higher in treatment groups than Control group. Overall plasma testosterone concentration was found significantly higher (P<0.05) in treatment group bucks as compared to Control group bucks.

Table 4.45: Plasma Testosterone (ng/ml) level of buck supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	3.18	3.25	3.14	3.13	0.245	0.983
30	3.12	3.33	3.30	3.35	0.252	0.917
60	3.10^{b}	3.46^{ab}	3.83^{ab}	4.16^{a}	0.237	0.028
90	3.08^{b}	3.63^{ab}	4.09^{ab}	4.50^{a}	0.329	0.036
Average	3.12^{b}	3.42^{ab}	3.59^{ab}	3.78^{a}	0.145	0.013

Means bearing different superscript in a row differ significantly (P < 0.05)

4.7.2 Plasma Luteinizing hormone (LH)

The plasma LH concentration (mIU/ml) of experimental animals at monthly interval is presented in Table 4.46. The value ranged from 2.48 to 2.49 mIU/ml in Control, 2.42 to 3.23 in T1, 2.44 to 3.64 mIU/ml in T2 group, and 2.41 to 4.02 mIU/ml in T3 and group. In fourth and sixth fortnight the LH level was significantly higher in treatment groups than Control group. Overall plasma LH concentration was found significantly higher (P<0.05) in treatment group bucks as compared to Control group bucks.

Table 4.46: Plasma LH concentration (mIU/ml) of buck supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	2.48	2.42	2.44	2.41	0.213	0.996
30	2.42	2.50	2.61	2.68	0.238	0.871
60	2.38^{b}	2.87^{ab}	3.15^{ab}	3.57^{a}	0.218	0.008
90	2.49^{b}	3.22^{ab}	3.64^{a}	4.02^{a}	0.274	0.005
Average	2.44^{b}	2.75^{ab}	2.96^{ab}	3.17^{a}	0.143	0.004

Means bearing different superscript in a row differ significantly (P < 0.05)

4.8 Plasma Immunoglobulins

4.8.1 Plasma Immunoglobulin G concentration (IgG)

The plasma (IgG) concentration (µg/ml) of experimental animals at monthly interval is presented in Table 4.47. The value ranged from 20.84 to 23.32 µg/ml in Control, 22.17 to 37.20 in T1, 20.33 to 38.73 µg/ml in T2 group, and 23.60 to 57.60 µg/ml in T3 and group. In fourth and sixth fortnight the plasma IgG level was significantly higher in treatment groups than Control group and is highest in T3 group. Overall plasma IgG concentration was found significantly higher (P<0.05) in treatment group bucks as compared to Control group bucks and significantly higher in T3 among treatment groups.

Table 4.47: Plasma IgG conc.(µg/ml) of buck supplemented with different sources of Se

Month	Treatment				SEM	P value
	Control	T1	T2	T3		
0	20.84	22.17	20.33	23.60	2.720	0.831
30	21.07	28.14	28.44	29.31	3.629	0.371
60	21.80^{b}	32.31^{ab}	27.69^{ab}	43.32^{a}	5.218	0.053
90	23.32^{b}	37.20^{ab}	38.76^{ab}	57.62^{a}	7.669	0.039
Average	21.76^{b}	29.95^{ab}	28.81^{ab}	38.46^{a}	2.914	0.002

Means bearing different superscript in a row differ significantly (P < 0.05)

4.9 Plasma mineral concentration

4.9.1 Se (µg/ml)

The plasma Se concentration (µg/ml) of experimental animals at monthly interval is presented in Table 4.48. The value ranged from 0.137 to 0.146 µg/ml in Control, 0.132 to 0.214 in T1, 0.127 to 0.286 µg/ml in T2 group, and 0.133 to 0.356 µg/ml in T3 group. At 60 and 90 day the Se conc. was significantly higher in treatment groups than control group. Overall plasma Se concentration was found significantly higher (P<0.05) in treatment group bucks as compared to control group bucks.

Table 4.48: Plasma Se conc. (µg/ml) of buck supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	0.137	0.132	0.127	0.133	0.006	0.763
30	0.130	0.147	0.156	0.160	0.012	0.359
60	0.126^{b}	0.179^{ab}	0.227^{ab}	0.249^{a}	0.026	0.015
90	0.146^{d}	0.214^{c}	0.286^{b}	0.356^{a}	0.015	< 0.001
Average	0.135^{b}	0.168^{ab}	0.204^{a}	0.219^{a}	0.014	<0.001

4.9.2 Mean Calcium concentration (mg/dl)

The plasma Calcium concentration (mg/dl) of experimental animals at monthly interval is presented in Table 4.49. The value ranged from 10.22 to 11.42 mg/dl in control, 10.90 to 11.15 in T1, 9.81 to 10.80 mg/dl in T2 group, and 10.89 to 11.21 mg/dl in T3 group. Overall plasma Calcium conc. of treatment groups was found similar with control group for whole trial period.

Table 4.49: Plasma Ca conc. (mg/dl) of buck supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	10.22	11.15	10.22	10.89	0.846	0.817
30	11.42	10.97	10.80	11.59	1.166	0.959
60	11.27	10.47	10.13	11.01	0.981	0.841
90	10.82	10.90	9.81	11.21	1.042	0.798
Average	10.93	10.87	10.24	11.17	0.479	0.56

4.9.3 Mean Phosphorus concentration (mg/dl)

The plasma Phosphorus concentration (mg/dl) of experimental animals at monthly interval is presented in Table 4.50. The value ranged from 4.70 to 5.40 mg/dl in Control, 4.78 to 5.22 in T1, 4.93 to 5.22 mg/dl in T2 group, and 4.27 to 5.05 mg/dl

in T3 group. Overall plasma Phosphorus conc. of treatment groups was found similar with Control group for whole trial period.

Table 4.50: Plasma Phosphorus concentration (mg/dl) of buck supplemented with different sources of Se

Days	Treatment				SEM	P value
	Control	T1	T2	T3		
0	5.40	5.12	5.22	5.05	0.336	0.896
30	4.74	5.22	5.10	4.53	0.294	0.341
60	4.70	5.04	5.22	4.27	0.306	0.171
90	4.91	4.78	4.93	5.01	0.247	0.935
Average	4.93	5.04	5.12	4.71	0.147	0.242

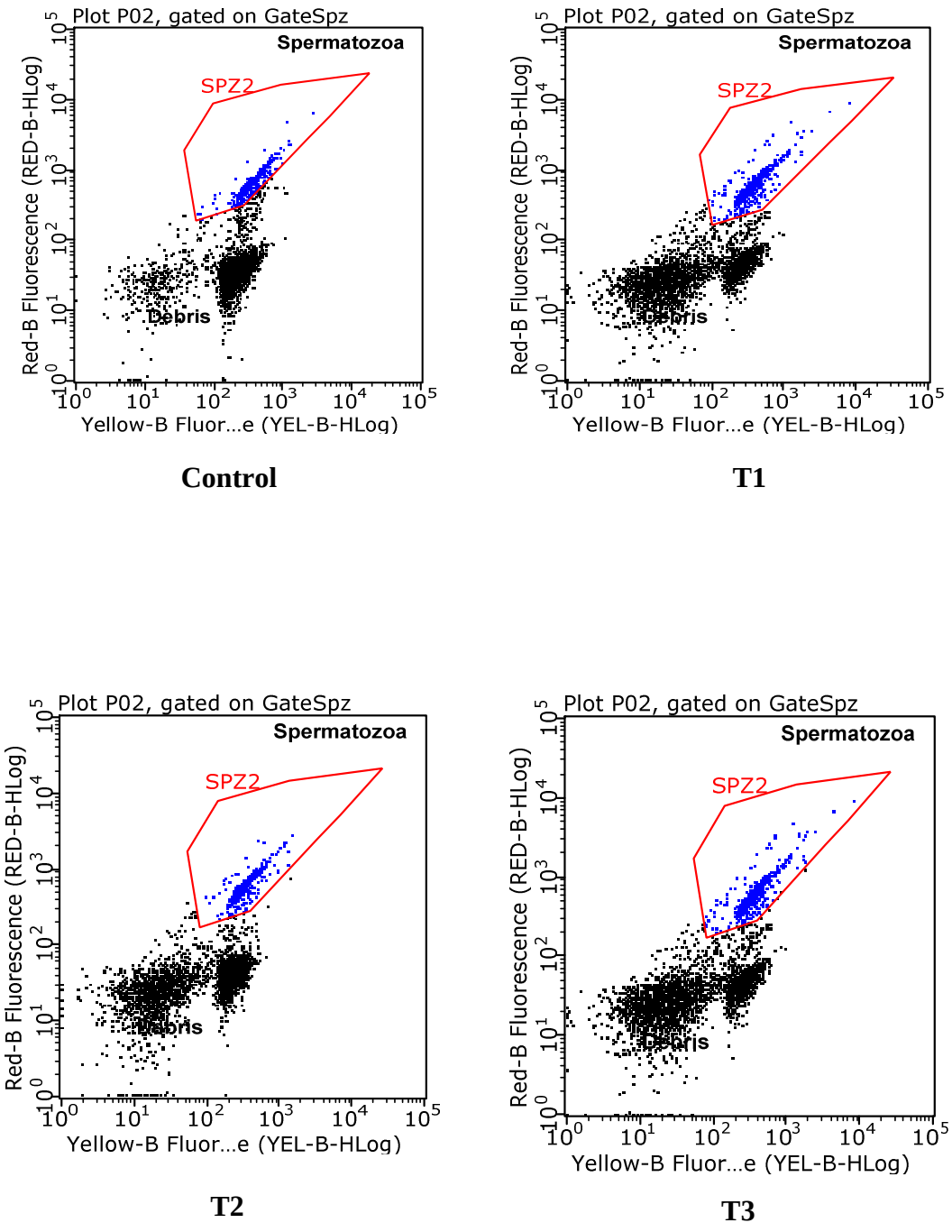


Fig. 4.1: Sperm concentration of different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks.

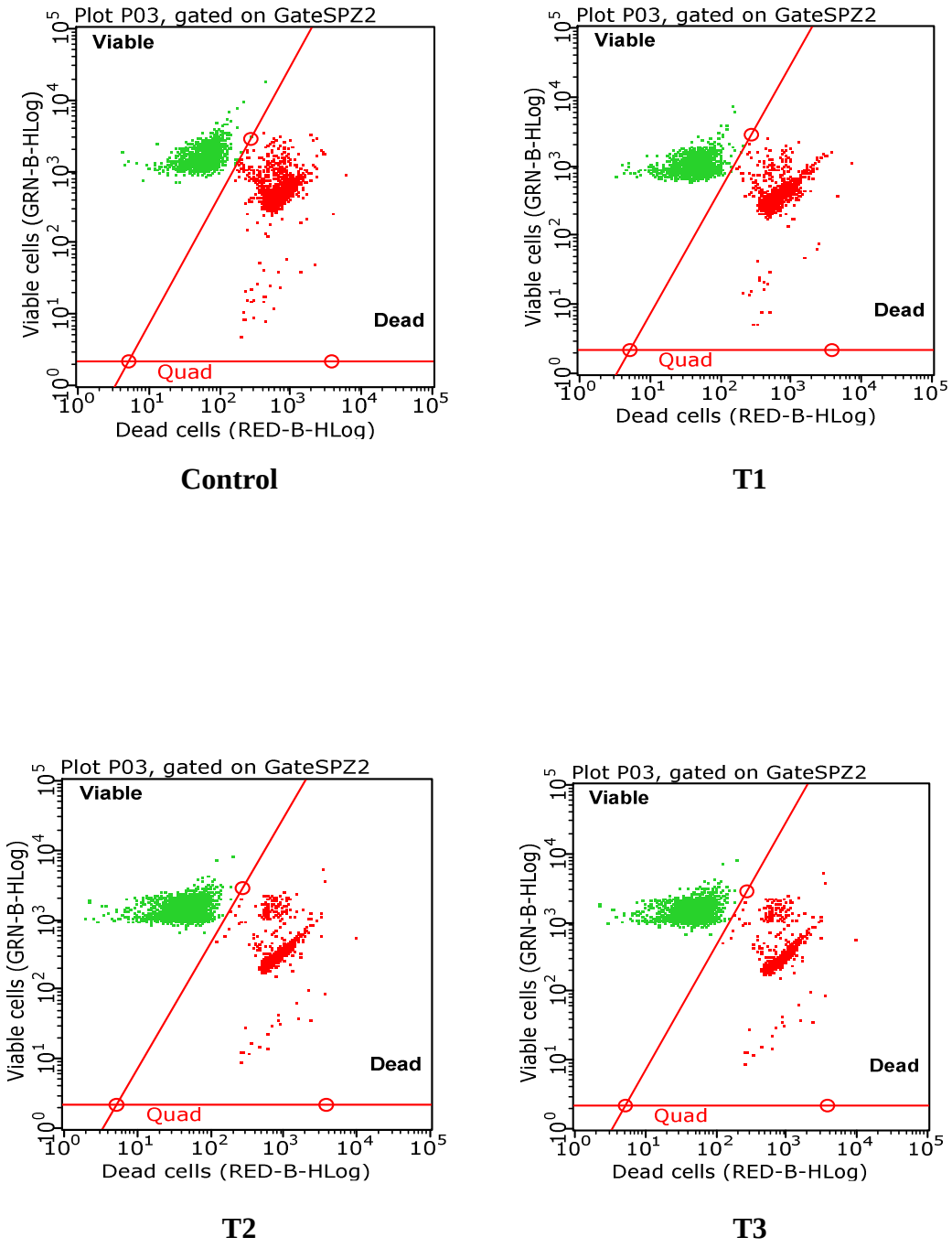


Fig. 4.2: Sperm viability of different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks.

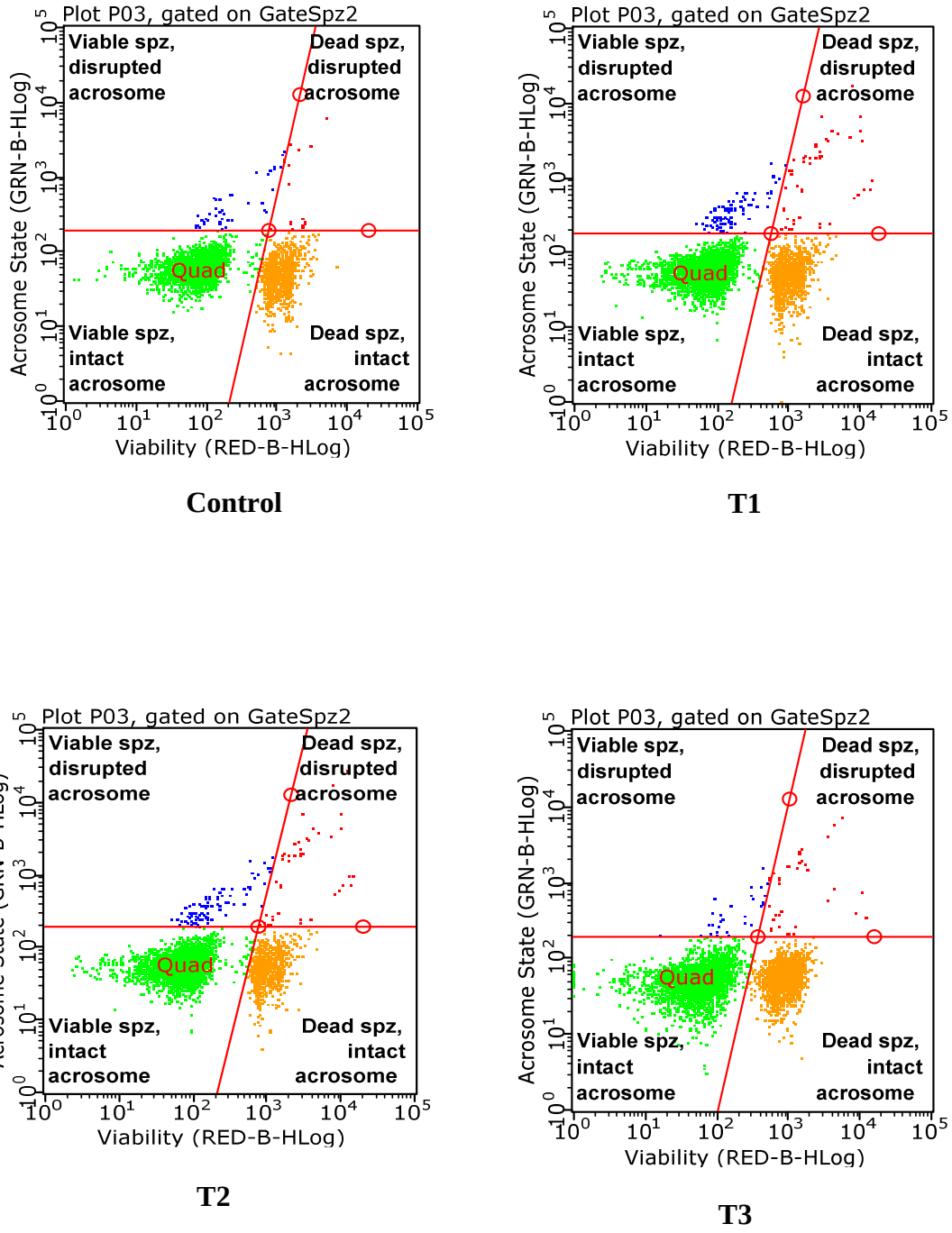


Fig. 4.3: Acrosomal integrity of spermatozoa in different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks

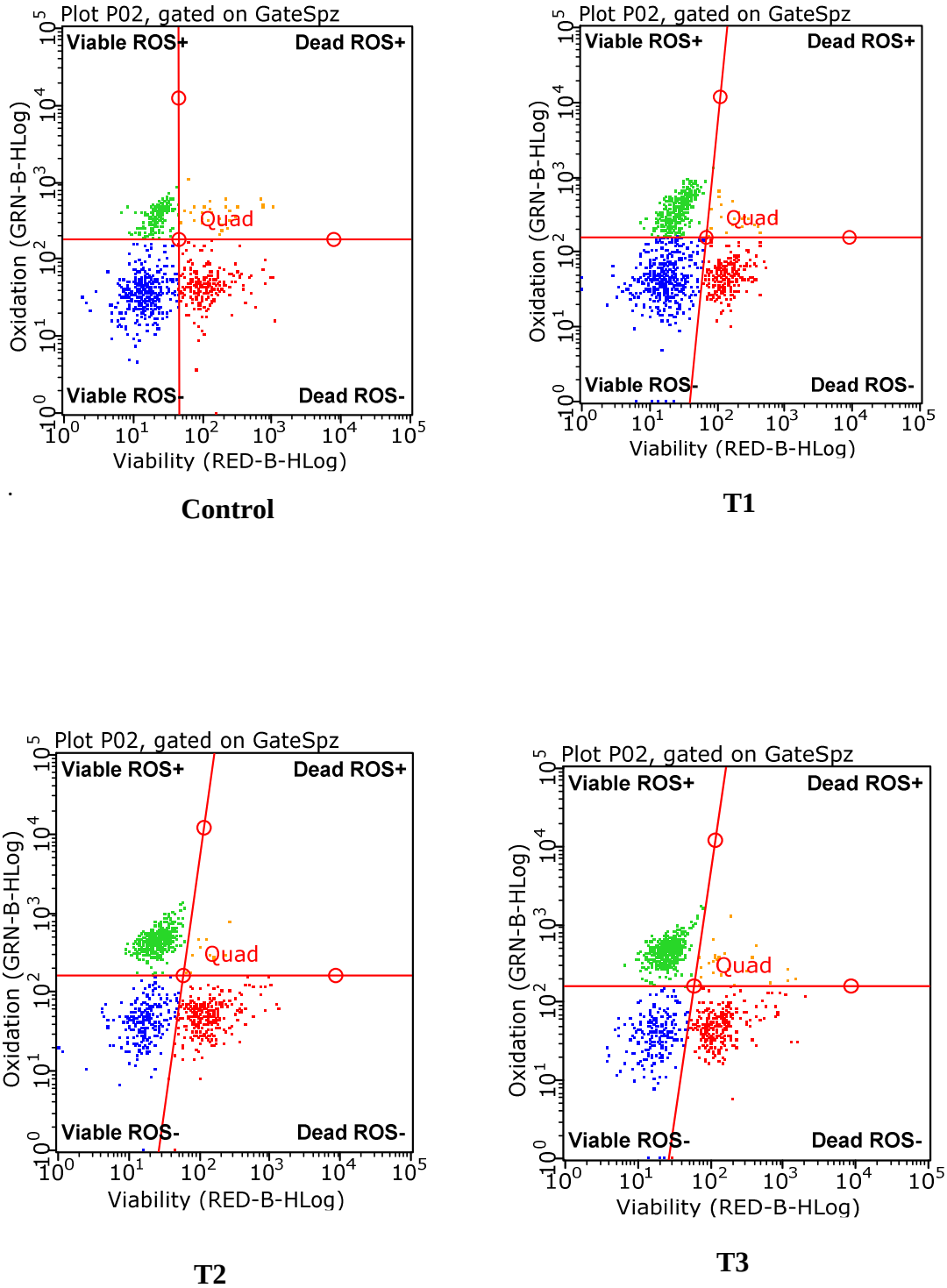


Fig. 4.4: ROS reactive spermatozoa in different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks

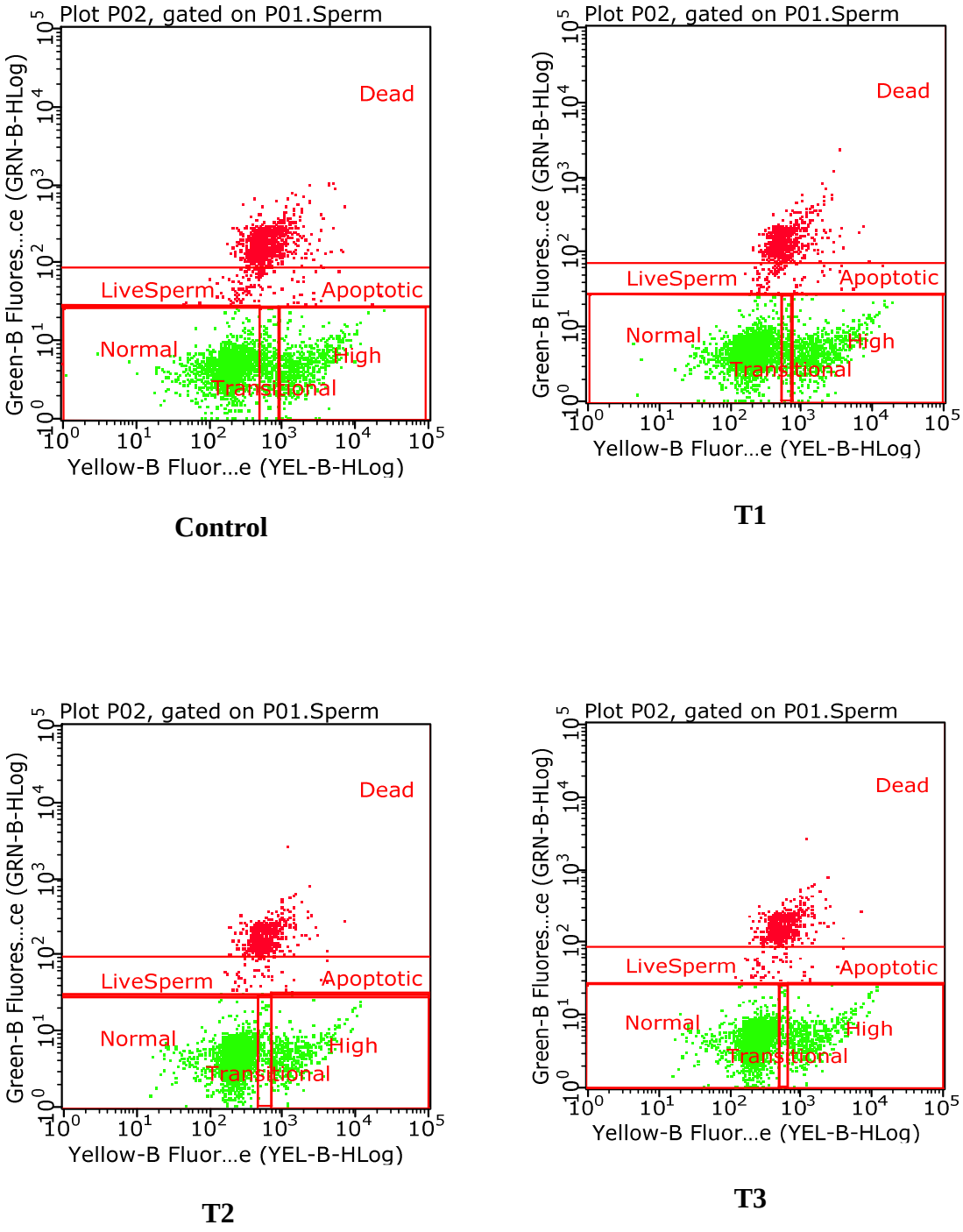


Fig. 4.5: Membrane fluidity of spermatozoa in different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks

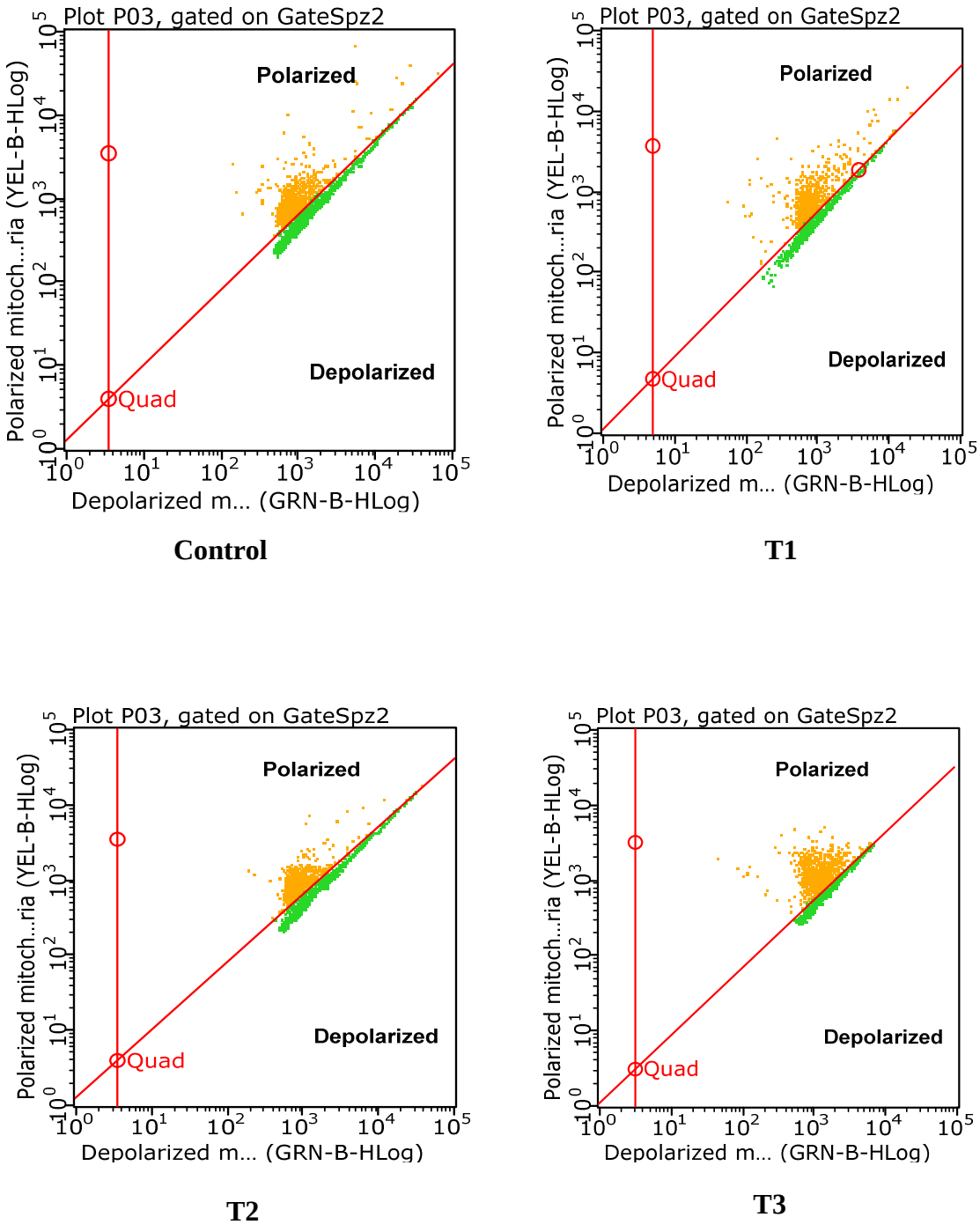
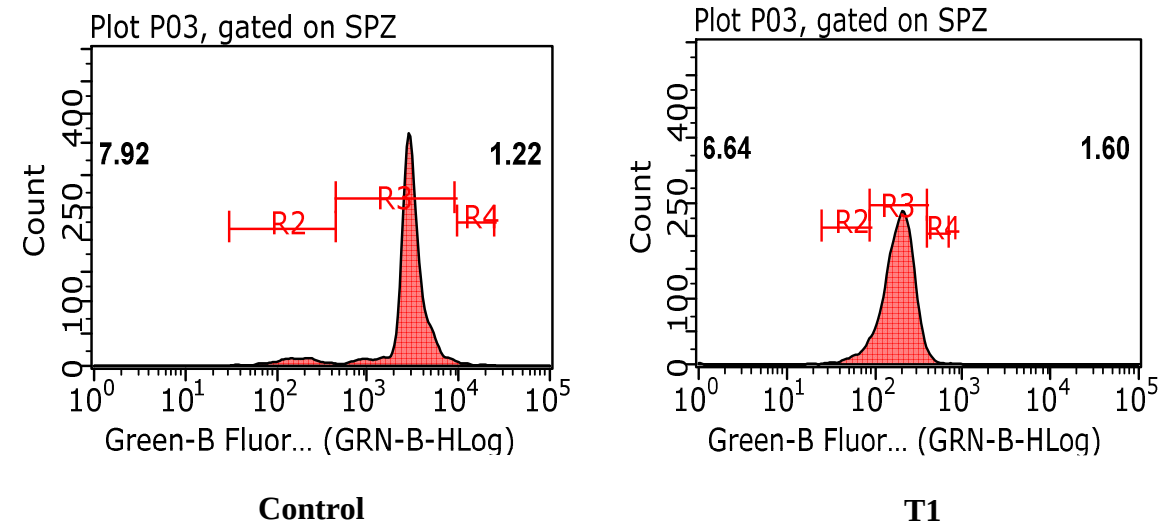
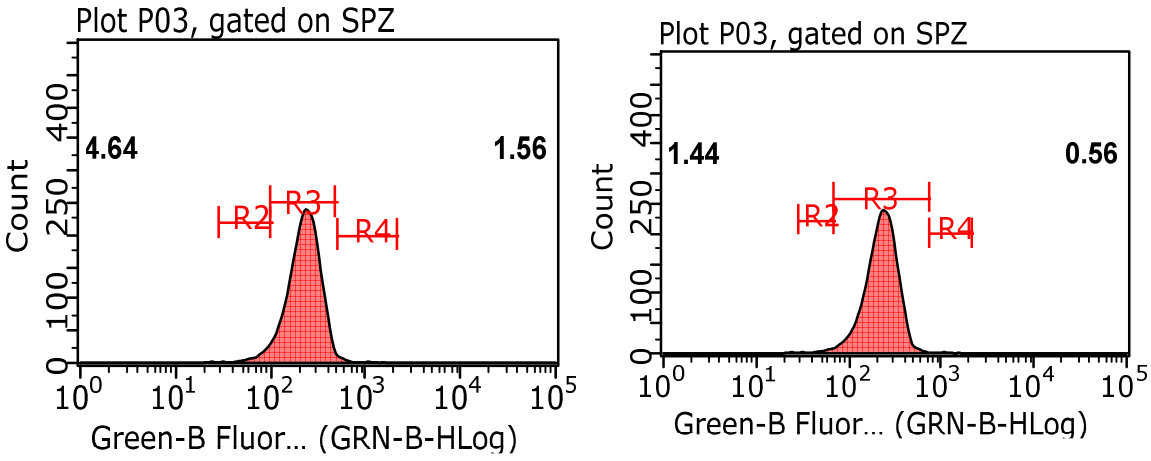


Fig. 4.6: Acrosomal integrity of spermatozoa in different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks



Control **T1**



T2 **T3**

Fig. 4.7: Intracellular Calcium level in spermatozoa of different groups (Control, T1, T2, T3) after supplementation with different sources of Se in bucks

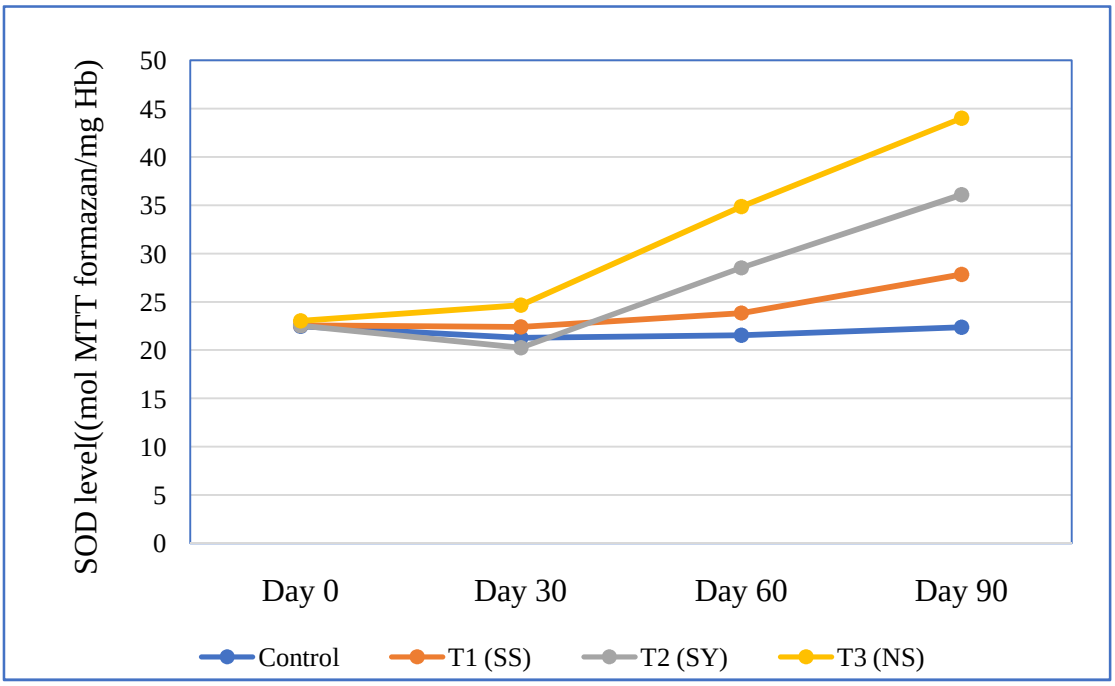


Fig. 4.8: SOD level of bucks supplemented with different sources of Se during 90 day study period

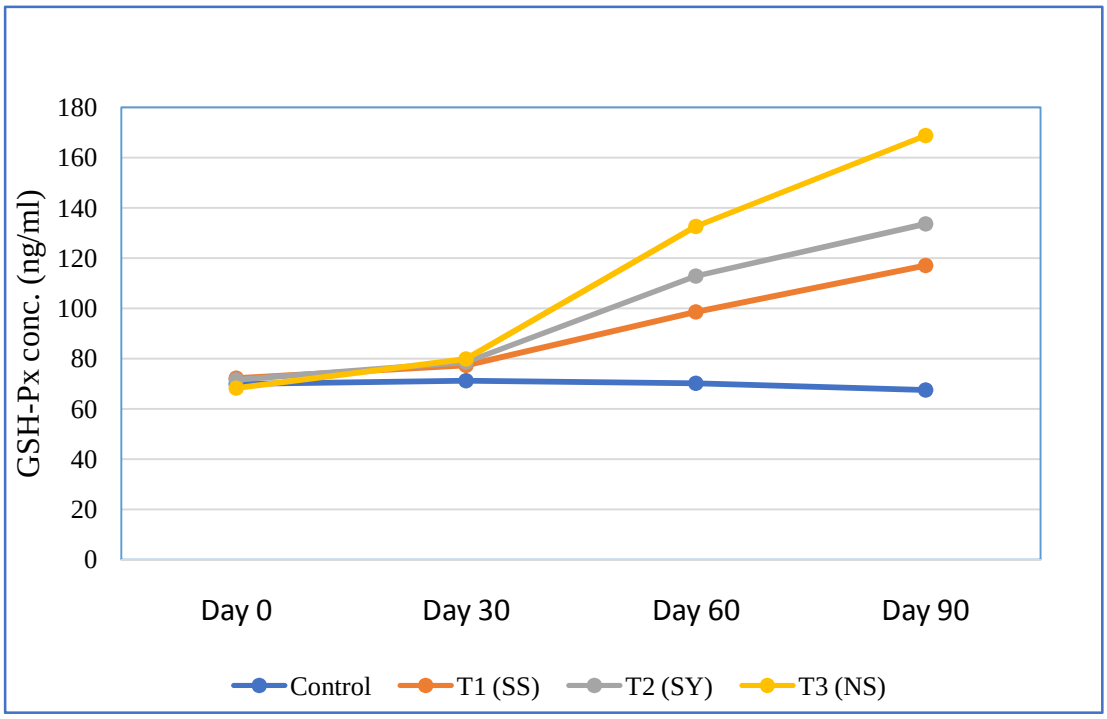


Fig. 4.9: GSH-Px concentration of bucks supplemented with different sources of Se during 90 day study period

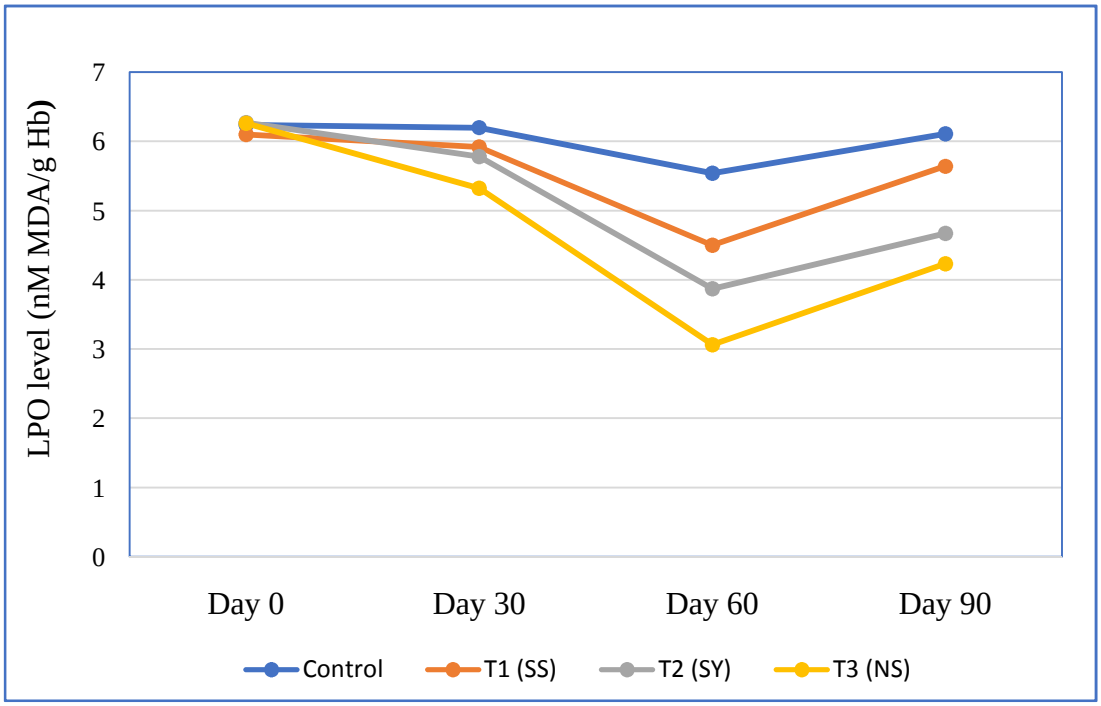


Fig. 4.10: LPO concentration of bucks supplemented with different sources of Se during 90 day study period

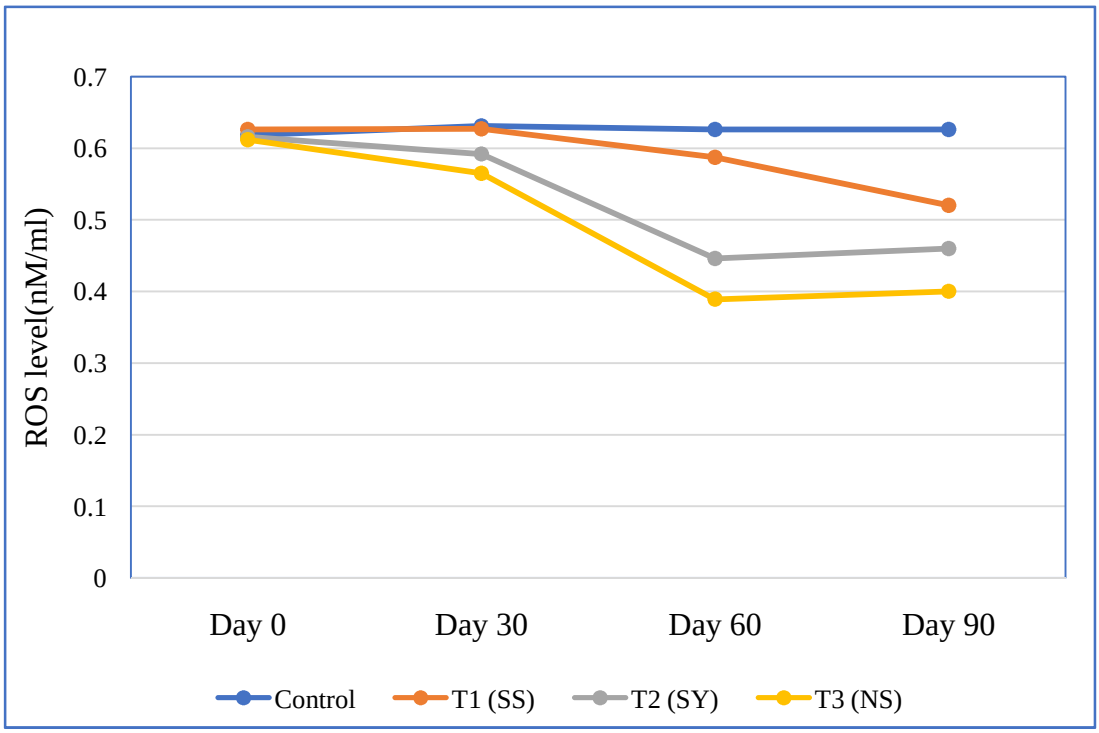


Fig. 4.11: ROS level of bucks supplemented with different sources of Se during 90 day study period

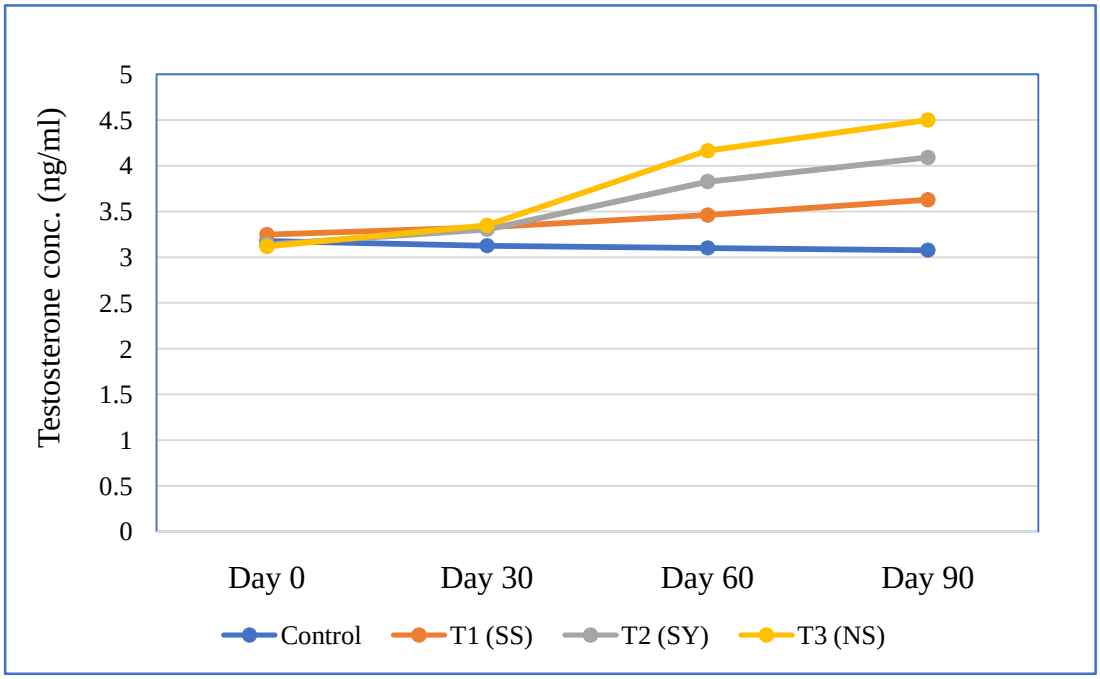


Fig. 4.12: Plasma Testosterone concentration of bucks supplemented with different sources of Se during 90 day study period

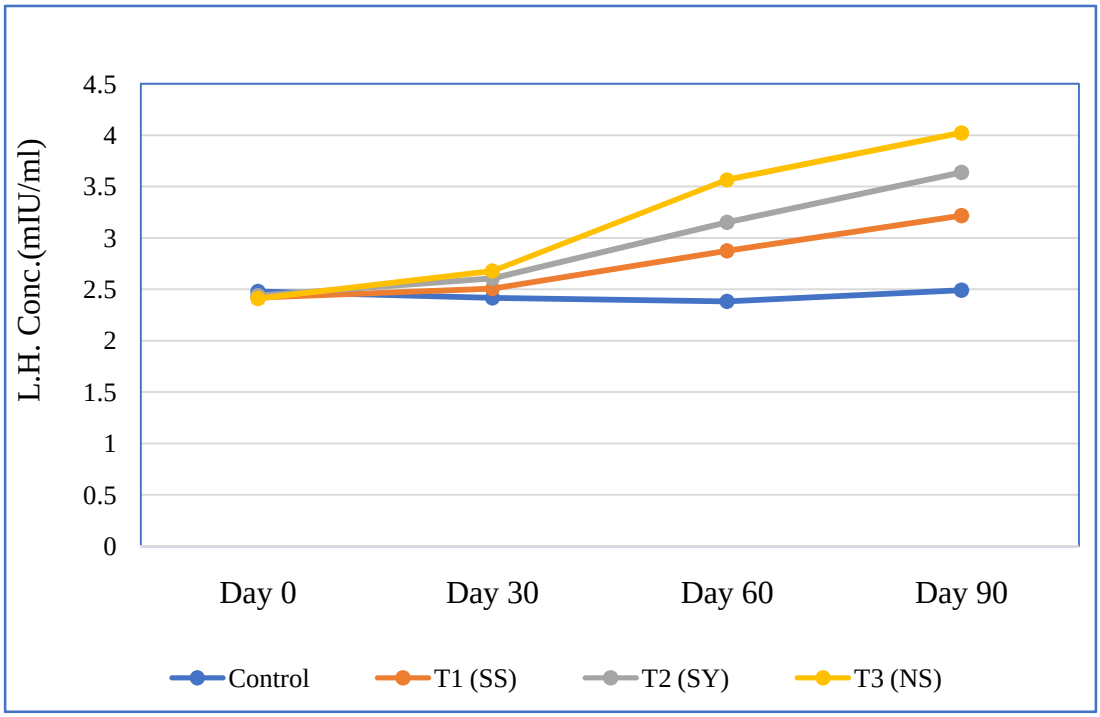


Fig. 4.13: Plasma LH concentration of bucks supplemented with different sources of Se during 90 day study period

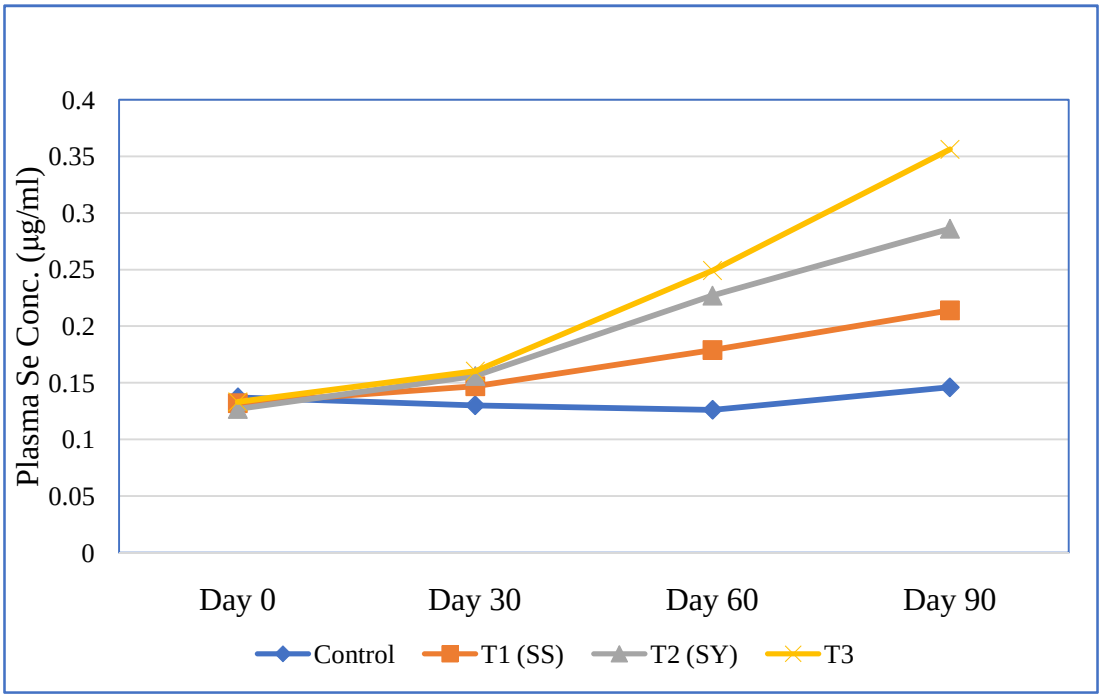


Fig. 4.14: Se concentration in plasma of bucks supplemented with different sources of Se during 90 day study period

Discussion

CHAPTER-5

DISCUSSION

The results obtained on supplementing different sources of Se on growth performance, nutrient utilization, seminal attributes, haematological parameters, blood biochemicals, antioxidant and hormonal parameters of Barbari bucks are discussed in following chapter.

5.1 Chemical composition of experimental diet

The chemical composition (on DM basis) of experimental diet offered to Barbari bucks during the experimental period was estimated. The ratio of concentrate and roughage in experimental diet was 40:60. Out of total 60 percent contribution of roughage, corn silage contributed 20 percent and gram straw contributed 40 percent dry matter. Similar roughage: concentrate ratio has been used by earlier workers (Saijpaul et al., 2005; Das et al., 2008). The CP content of concentrate mixture, corn silage and gram straw were found to be 19.38, 10.23 and 8.21 %, respectively. The Ca concentration was 1.08, 0.53 and 0.34 % while the P concentration was 0.75, 0.53 and 0.19% in concentrate mixture, corn silage and gram straw respectively. Se concentration in concentrate, corn silage and gram straw were found to be 50, 21.78 and 27.89 ppm, respectively. TMRs were also analyzed for nutrient specification (as specified previously) to give basal diet nutrient composition and final Se content was also analyzed. The Se concentration reported in basal diet was 0.23 ppm. In contrary to our results, Shi et al., (2011) noticed the Se levels of 0.033 mg/kg, 0.13 mg/kg (Vignola et al., 2009), 0.124 mg/kg DM (Aghwan et al., 2016) in the basal diet fed to experimental animals. However, the variations in the Se content of basal diet in results could due to variation in feedstuffs, soil or the environmental factors associated with the study.

5.2. Growth parameter and nutrient utilization in bucks supplemented with different sources of Se

5.2.1 Growth indices:

The experimental results revealed no significant ($P>0.05$) effect on growth performance (average body weight, overall fortnightly body weight gain (kg), average daily gain (g) and FCR) among the groups supplemented with different (inorganic,

organic and nano) forms of Se at 0.3ppm levels in Barbari bucks. The present results are in agreement with a previous work involving Korean native goats (Chung et al., 2007) and in lambs (Domínguez-Vara et al., 2009; Vignola et al., 2009). Many researchers also indicated that either levels or source of Se did not influence growth in cows (Gunter et al., 2003), pigs (Mahan and Peters, 2004), lambs (Juniper et al., 2009b) and chicken (Payne and Southern, 2005). Juniper et al. (2006), did not reported any significant results on rates of growth, feed intake or feed to gain ratio using organic and inorganic Se supplements in the diets of growing lambs for a period of 112 days. In addition, Lawler et al. (2004) observed that neither Se source nor dietary Se concentration affected the physical performance of finishing beef steers fed with supra-nutritional levels of organically bound Se. Skrivanova et al. (2007) did not report any influence of different Se levels or sources on the performance of growing calves. Sushma et al. (2015) reported that the gradual increase in Se supplementation from 0.0 to 1.8 ppm as sodium selenite had no significant ($P > 0.05$) effect on total weight gain, ADG), feed intake and FCR (kg DMI/kg gain) of lambs. No difference in performance of Han woo steers was observed with increasing levels of Se from 0 to 0.9 mg Se/kg (Lee et al., 2007).

Dominguez-Vara et al., 2009 noticed no effect of Se addition as Se enriched yeast at 0.3 ppm to basal diet on growth performance (final BWs and weight gains) of Rambouillet lambs. This might be due to availability of Se through basal diet, which may be sufficient enough to meet the nutrient requirement of sheep. However, contrary to present findings, some studies showed that feeding efficiency and ADG (Yue et al., 2009), and the final BW and ADG (Shi et al., 2011) of growing male goats supplemented with dietary Se were significantly higher compared with the control animals. Castellan et al. (1999) reported greater ADG and higher growth of calves receiving parenteral administration of Se having a low Se status. The inconsistency in responses may possibly be due to variable Se levels in the basal diets. In the present study, Se content present in the basal diet (0.23 mg/kg DM) was sufficient to meet the requirements that, if inadequate, could have adversely affected the growth performance. Se supplementation is not likely to influence growth rate unless there is an evident lack of the mineral (Johansson, et al., 1990). Se is involved in the metabolism of thyroid hormones. A Se-deficient diet causes a reduction of tri iodothyronine (T3) and an increase of the tetra iodothyronine (T4) and a decrease in

the ratio T3/T4 levels in blood (Thompson et al., 1995; Beckett et al., 1989). These effects can influence growth rates since T3 is an active form of T4, which is known to be involved in the growth mechanisms. Se is a component of enzyme 5-iodothyronine deiodinase that convert T4 to T3 and this Se-dependent selenoprotein is affected in the event of Se deficiency. This delay could explain the fact that several studies that have explored various ways of Se supplementation do not show any significant effect of Se supplementation on growth, weight gain of calves, lambs, kids, cows etc. Apart from Se status other factors contributing to variation in results might be differences in age, breed, assays used to determine Se status, ration composition and availability or concentration of other trace elements.

5.2.2 Nutrient utilization

Influence of Se supplementation on DM intake and apparent digestible parameters showed no significant difference on DMI, nutrient digestibility and as well as total digestible nutrients ($P > 0.05$). The mean daily DM, OM and CP intake was found to be similar among the control and Se supplemented groups, which indicated that supplementation of different sources of Se at 0.3 ppm level had no effect on palatability and feed intake pattern of the bucks. The results of most researchers are consistent with the present study results, where DMI remained unaffected due to supplementation of inorganic Se at the 0.3 ppm level in Holstein cows (Ivancic & Weiss, 2001), at the 0.38 ppm level in crossbred beef steers (Lawler et al., 2004) and at the 0.65 ppm level in Merino weathers (White and Somers, 1977). Similarly, supplementation of Se at the 1 ppm level in male Holstein (Fehrs et al., 1981), at the 0.3 ppm level in buffalo calves (Mudgal et al., 2008) also did not have any effect on DMI.

The digestibility of organic nutrients and TDN was also found to be similar ($P > 0.05$) among the treatment groups suggesting that supplementation of Se through different forms (inorganic, organic and nano) had no effect on the digestibility of these nutrients. In agreement with these observations supplementation of 1 ppm of Se had no effect on the digestibility of OM, CP and NDF in cattle calves (Nicholson et al., 1991). Similarly, there were no effect of 0.3ppm Se supplementation on intake and digestibility of organic nutrients in male buffalo calves (Mudgal et al. 2008) and lambs (Kumar 2006). However, Taheri et al. (2018) proved that adding Se yeast significantly improved digestibility of nutrients and dry matter intake in forage in

Iranian native goats. Wang et al. (2019) showed that the addition of Se yeast could improve digestibility of herbage and some nutrients. Chadio et al. (2006) studied the effects of Se supplementation on the level of thyroid hormone and Se enzyme activity in growth lambs, and found that Se affected thyroid hormone metabolism. The digestion and utilization of nutrients directly affect the growth of livestock (Feng et al., 2013). This suggested that Se regulates the nutrient digestibility of the body by regulating thyroid hormone. In the current study, it was found that supplementation of different forms of Se at 0.3 ppm level in the diet of bucks had no effect on the intake and digestibility of organic nutrients.

5.3 Seminal attributes in bucks supplemented with different sources of Se

Imbalance of Se is known to impair the reproduction in male animals. It enhances testicular growth, development of the seminiferous tubules, testosterone biosynthesis and spermatogenesis (Shi et al., 2018). A deficiency of Se is reported to result in reduction of the antioxidant defense in farm animals, which in turn disrupts normal spermatogenesis (El –Mokadem et al., 2012). The present study indicated that the semen ejaculate volume, colour, pH and mass motility did not differ significantly in the Se supplemented as well as unsupplemented group. No significant difference in the ejaculate volume and spermatozoa concentration was similar to the findings of Petrujkić et al. (2014) and Jacyno and Kawecka (2002), who observed no effect of Se (inorganic or organic) on ejaculate volume in boars. Our results were comparable to findings of Bartle et al. (1980) and Marin-Guzman et al. (1997), who reported that Se injections did not significantly improve the semen production or quality in bulls, and that neither the supplementation of Se nor vitamin E had any effects on semen measurements in boars over a 16-week period. Ghorbani et al., (2018) also noticed that the semen characteristics were not affected by Se supplementation at 0.3 mg/kg DM in rams. Besides the sperm abnormality rate that was significantly higher in low-Se group, semen quality was not affected by Se supplementation (Shi et al., 2010).

The result of the current study revealed significantly ($P < 0.05$) higher values of progressive sperm motility and percent live spermatozoa in groups supplemented with Se compared to control. These results are in agreement with those reported in buck, bulls and rams (Lee et al., 2000; Xu et al., 2003; Kumar et al., 2014). Se supplementation improves the sperm motility and percent live spermatozoa. Se provided a sufficient protection to the spermatozoa as it reduces the number of sperm

with broken flagellum that in turns enables sperms to move. It also plays role as insulin mimetic (Stapleton, 2000). Se has been observed to stimulate the transport of glucose in the cell in a dose–dependent manner (Ezaki, 1990) and improves the uptake of glucose together with increased rate of aerobic and anaerobic glycolysis (Furnisin, 1996). It may be due to the positive effect of Se on interstitial cells of testes and integrity of these cells from damage (Marai et al. 2009). Thus, the improvement of the sperm motility can be associated with Se supplementation. The antioxidant protection of Se from oxidative damage allowed more survival of sperm cells.

On comparing the different forms of Se supplementation, the results showed that the progressive sperm motility and percent live spermatozoa were highest in nano Se supplemented group as compare to inorganic and organic group. The diluents containing 1 and 2 µg/ml nano Se significantly increased the progressive motility (PM), VAP, VSL, VCL, ALH, STR and LIN of sperm after freezing and thawing compare with the control group ($P < 0.01$) (Nateq et al., 2020) which may be due to notably higher plasma Se concentrations in nano group associated with highest activities of antioxidant enzymes GSH-Px, SOD that play important roles to eliminate reactive oxygen species, resulting in enhance motility and viability of spermatozoa.

Almost always, some spermatozoa from an ejaculate exhibit various form of deviation from normal morphology that, when present in great proportion, may adversely affect fertility (Segerson, 1981). Significantly ($P<0.05$) lower sperm abnormality was reported in Se supplemented groups as compare to control, it has been shown that when bucks are fed on a basal diet containing 0.23 ppm Se without additional dietary supplementation, the percentage of abnormal spermatozoa is 3.72% and following supplementation with an additional 0.3ppm Se in the form of inorganic, organic and nano, the percentage of abnormal spermatozoa was decreased to 3.55, 3.15 and 3.07 % respectively. Furthermore, among the treatment groups, nano supplemented group had reported significantly lower abnormality percentage than the inorganic and organic group. In agreement with our result, Nateq et al. (2020) also noticed that the addition of 1 and 2 µg/ml of Nano Se in the diluent significantly improved the sperm morphology and reduced the percentage of abnormal spermatozoa from the control group after freezing ($P < 0.01$) process in ram semen. The sperm abnormality observed was mostly abnormal heads, cytoplasmic droplets and broken tail. Theses occurs due to the destruction during spermatogenesis that

leads to release of the immature sperm, the GSH-Px, an enzyme made up of Se prevents the lipid attack thereby releasing the proportion of abnormal spermatozoa. The highest activity of GSH-Px in nano group might be related to lowest abnormality percentage in the group.

The obtained results of the present study revealed that the acrosomal integrity, HOST and mitochondrial activity were significantly higher in Se supplemented groups than control. These results corroborate with Marai et al. (2009) who reported a decline in acrosome damage when rams were supplemented with sodium selenite. Intriguingly, it is believed that sperm are more sensitive to oxidative damages; this is largely because of the biochemical composition of sperm i.e., it contains higher ratio of polyunsaturated fatty acids and low concentrations of cytoplasmic antioxidant enzymes compared to the somatic cells (Gharagozloo et al., 2016). In our experiment during semen collection and handling processes, stress is induced, which triggers LPO cascades and the plasma membrane, integrity is affected. Se is known to act nutritionally through its enzyme (cytosolic GSH-Px or phospholipid hydroperoxide GSH-Px) to protect germ cells, proteins, and organelle membranes from oxidative stress, to reduce peroxidated membrane phospholipids by oxidizing and to act as a structural protein of the spermatozoal mitochondrial sheath (Ursini et al., 1999). Se supplementation played a major role to provide a sufficient protection to the sperm membrane against lipid oxidation (Mahmoud et al., 2013). It may also act in the maintenance of mitochondrial structural integrity, which is reflected by increase in ATP of spermatozoon, thus causing an increase in sperm motility (Liang et al., 2007). In general, increase in acrosomal integrity and mitochondrial activity may be due to Se protection of lipid component of the plasma membrane over the entire sperm acrosome (Kumar et al., 2014).

However, the highest values of acrosomal integrity, HOST and mitochondrial activity were reported in nano Se supplemented group than inorganic and organic groups. This result is in agreement to that observed in rams by (Hozyen et al., 2019) who reported that supplementation of Se nano particles to extender significantly improved acrosome membrane integrity and plasma membrane integrity in ram semen (Nateq et al., 2020). Khalil et al. (2018) demonstrated that Se nano particles have membrane protective function in bulls. Supplementation of semen extender of rooster semen with Se nano particles successfully enhanced the post-thawing

quality as well as oxidative biomarkers (Safa et al., 2016). Also, oral supplementation of Se nano particles protected the quality of spermatozoa (motility, DNA integrity) and spermatogenesis against oxidative damage induced by Cisplatin, an anticancer agent with male reproductive toxicant properties (Rezvanfar et al., 2013). The improved acrosomal integrity, HOST and mitochondrial activity in nano Se group might be attributed to much smaller size of Se nano particles than its counter parts, which allow more surface area to react with free radicals and offers plenty of space to absorb oxygen (Safa et al., 2016). Se nano particles can also penetrate the cell membranes, which results in stimulation of the activity and expression of antioxidant and selenoenzyme genes (Surai et al., 2019). The glutathione peroxidase enzyme has the ability to scavenge all the reactive oxygen radicals thus preventing damage to sperm by protecting the sperm membrane integrity and maintaining its fluidity and stability (Peters, 2011; Rashad et al., 2018). Marin-Guzman et al. (2000) reported that ATP concentration was higher when boars were fed diets with added Se. It seemed that Se supplementation increases the ATPase concentration of spermatozoa indicating greater mitochondrial metabolism of spermatozoa that lead to increase sperm motility.

The present results showed that supplementation of Se has significantly reduced the ROS positive sperms, increased membrane fluidity and Intracellular Ca (medium Calcium) levels in the semen of treatment groups as compared to control. Though the highest response was reported in nano Se supplemented group. The nano group has shown minimum peroxidation; in terms of ROS generation and enhancing the antioxidant state which has prevented the oxidative damage and protected the sperm membrane integrity and fluidity. The reduced lipid peroxidation in the present study is agreed with findings of Safa et al. (2016); Hozyen et al. (2019) and Khalil et al. (2019) who reported reduced semen MDA when using Nano-Se.

5.4 Haematological parameters

The results of the blood chemistry parameters monitored showed no significant treatment effects of Se supplementation and the values were within the expected normal range (Beers and Berkow, 1998). Similar results were obtained for the number of red blood cells, white blood cells, hematocrit, and hemoglobin concentration, in calves receiving Se in the form of sodium selenate by Shinde et al. (2009), which is consistent with the results of our study. Also, an earlier study by

(Bednarek et al., 1996) found no effect of Se (and vitamin E) administration on RBC count and haemoglobin concentration in calves. There was no difference in hematological parameters in Se fed lambs in the study of (Mohri et al., 2011). In addition, supplementation with selenite triglycerides did not influence hematological parameters in calves, only physiological fluctuations in the values were observed (Zarczynska et al., 2021). However, Horton et al. (1978) observed increased Hb concentrations, erythrocyte counts and packed cell volume in control lambs than Se supplemented group. It might be due to deficient levels of Se in control group. Abnormal haemopoeisis in the bone marrow and a shortened erythrocyte life-span was reported on low-Se diet (Tucker, 1974). Normal values for Hb concentration and packed cell volume in present study indicated that there was no interference in erythropoiesis or normal erythrocyte destruction in any of the group

5.5 Blood biochemicals parameters

The results of the present study revealed no difference for the concentrations of glucose in treatment groups of bucks. Similar, results were also reported by Dominguez-vara et al. (2009) and Abbas (2002). Inorganic Se supplementation did not affect calves' blood glucose concentration (Shinde et al., 2009) or in adult cattle (Juniper et al., 2006). On the other hand, Se and vit E supplementation resulted in higher level of glucose than control lambs and ewes (Avci et al., 2000). In a study by Iukuza et al. (2010), 0.173 mg/kg of sodium selenite (Na2SeO3) was administered intra-peritoneally to diabetic rats for 14 days. The result was increased glucose uptake by peripheral tissues and adipocytes. The difference in results can be probably due to the action of Se as an antioxidant and insulin-mimetic nutrient, favoring the action of insulin by acting on the insulin receptor, that increases the sensitivity to insulin and alleviating hyperglycemia, for regulation of hepatic glucose production in diabetic subjects.

Significant effect of Se supplementation on total protein, albumin and globulin concentrations in the present study confirms the findings of Avci et al. (2000) who also reported higher levels of total protein following Se and vit E supplementation in pregnant ewes and lambs. In contrast with our results, Kumar et al. (2008) reported that organic or inorganic supplementation of Se had not any effects on the concentrations of serum total protein, albumin, globulin, and A/G ratio in lambs. Similarly, no difference in serum total protein, albumin and globulin was

observed due to supplementation of Se in steers (Arthur et al., 1988) and buffaloes (Singh et al., 2002). Similar result was also reported for the level of total protein in plasma of neonatal dairy calves (Mohri et al., 2005). The exact mechanism of higher serum total protein in supplemented animals was not clear but increased concentration of γ globulin could be considered as cause. However, a study in goats showed that Se might stimulate protein biosynthesis, and Se supplementation may increase this blood total protein concentration in ruminants, but such effects were observed after long-term dietary supplementation of Se (Żarczyńska et al., 2021).

The present findings indicated that either supplementation with Nano-Se or organic Se was more effective than inorganic Se (sodium selenite) to increase serum globulin concentrations. These results agree with similar response of Wistar male rats fed sodium selenite and Se nano particles at 150 ppb Se, where nano-Se was potent to increase serum globulin concentrations by 8.5 % than sodium selenite (Bunglavan et al., 2014). The presented results are also consistent with similar increased serum globulin levels in layer chicks fed Nano-Se at 0.3 ppm (Mohapatra et al., 2014), male buffalo calves supplemented with 0.3 ppm Se (Mudgal et al., 2008), and buffalo heifers supplemented with Se at 0.2 ppm (Ganie et al., 2012). In addition, serum globulin levels were increased ($P < 0.05$) concomitant with improving immune response and antioxidant function for growing rabbits fed organic Se at 0.3 ppm (Ebied et al., 2012). Thus, higher globulin content in the Se treatments suggests that dietary Se has positive effects on the immune status of animals, which is consistent with the previous reports in other species (Mudgal et al., 2008; Cai et al., 2012; Ashouri et al., 2015).

The lack of an effect of Se supplementation on blood total cholesterol and triglycerides concentration in bucks in the present study is in accordance with the findings of Singh et al. (2002) who found no effect of Se supplementation on blood total cholesterol concentration in calves, Shinde et al. (2009) found that Se administration increased the concentration of total cholesterol and its HDL fraction in calves. The authors explained this observation by the positive effect of increased blood Se concentration on pancreatic function, which facilitated the absorption and digestion of dietary fat. The failure of Se supplementation to alter physiologically normal serum triglyceride concentrations in the present study confirms previous findings on Se supplementation in calves (Sobiech et al., 2015).

Activity of liver enzyme ALT and AST is a sensitive indicator of potential Se poisoning in ruminants (Zaki et al., 2018). The data showed no significant differences in serum concentrations of ALT and AST enzymes activity in bucks fed supplemental Se sources of inorganic, organic and nano compared with control. These findings agree with similar trend of unchanged serum ALT and AST enzyme activities in lambs supplemented with organic SY at 0.3 mg/kg DM (Faixova et al., 2007). In the same way, these enzymes activity did not change in Merino lambs fed either inorganic SS (sodium selenite) or organic Se (Se-plex) at 0.3 mg/kg (Antunovic et al., 2014). Serum concentrations of ALT and AST enzyme activities did not change significantly in lambs supplemented with inorganic Se (sodium selenite), organic Se (Se-yeast) and Nano-Se particles (Ibrahim and Mohamed, 2018). Reported values of SGOT and SGPT concentrations were within the normal physiological range (Kaneko et al., 1997), suggesting that dietary supplementation of different source of Se at 0.3ppm dose level did not adversely affect liver function. Contrary to present findings, Singh et al. (2002) observed that buffalo calves fed high Se (8.54 ppm) had higher activity of plasma SGOT and SGPT. In an experimental study with buffalo calves, adverse effects appeared when the whole blood Se concentrations increased above 2000 µg/l, with mortality occurring when blood levels exceeded 3400 µg/l (Deore et al., 2002). Se levels in the present study were sufficient to maintain the normal enzyme activities of SGOT and SGPT and were below the values associated with clinical toxicity.

In summary, supplementation with inorganic, organic and nano sources of Se at the dose of 0.3ppm in bucks had no significant effect on haemoglobin concentration, PCV, glucose concentrations, liver enzymes and fat metabolism. However, total protein and globulins concentration were significantly higher in treatment group as compared to control.

5.6 Antioxidant parameters

Various physiological and environmental factors induce increased production of free radicals, which are counteracted by the antioxidant system to maintain homeostasis. However, when free radical production is exacerbated or the antioxidant is defective, it may cause DNA damage, protein cross linking, and lipid peroxidation, ultimately leading to oxidative stress (Dargel, 1992). The oxidative damage at the molecular level might alter physiological functions and affect

economically important phenotypes (Sharma et al., 2011). Se is an essential component of the antioxidant system, and its dietary supplementation enhances antioxidant capacity (Skrivan et al., 2012). The antioxidant enzymes SOD and GSH-Px, play important role to eliminate reactive oxygen species. SOD is not only a superoxide anion-scavenging enzyme, but also a major H_2O_2 producing enzyme, which plays a key role in the biological antioxidant system (Juniper et al., 2009). All kinds of antioxidants and antioxidant enzymes in the serum constitute the total antioxidant level. The level of lipid oxidation could be detected by measuring the level of MDA. Therefore, these indicators are indirect reflection of the effects of Se on the antioxidant capacity. GSH-Px was the first proven selenoenzyme that can prevent oxidative damage of the cellular membrane (Panev et al., 2013).

The present data showed that plasma SOD, and GSH-Px activity was significantly increased ($P<0.05$) with concomitant decrease in LPO and ROS concentration of the bucks fed diets supplemented with inorganic, organic and nano Se vs. control. Generally, the present results indicated that dietary supplementation at 0.3 ppm of nano Se to bucks was effective to significantly increase plasma SOD and GSH-Px activity, improving their antioxidant status. Similar results were obtained by Zhan et al. (2007), who reported that Se supplementation increased GSH-Px activity and decreased MDA content in the liver and muscles when compared with control. Likewise, supplementation of 0.3 ppm Se had an enhancing effect on RBC GSH-Px activity in male buffalo calves (Mudgal et al., 2008). Increased levels of ROS and LPO in the control group in our study might be associated with lower plasma Se concentration which could be the main reason of decreased GSH-Px and SOD activities in the control group. It might be indicated that lower plasma Se concentrations could more easily lead to lipid peroxidation. However, in treatment groups Se might resist peroxidation and decrease the free radical by enhancing levels of SOD and GSH-Px, the enzymatic antioxidant defense mechanisms

However, the highest activities of plasma GSH-Px and SOD were detected in nano Se supplemented group. To compare the potency of these Se sources in improving antioxidant function, nano Se was more effective than inorganic and organic by 21, 8.82 % with plasma SOD and by 25.7, 15.7 % with plasma GSH-PX activity, respectively. These findings strongly agree with similar observations of Shi et al. (2011b) who reported highest serum GSH-Px activity with feeding nano-Se than

sodium selenite and Se yeast on growing male goats at 0.3 mg/kg DM. Likewise Yaghmaie et al. (2017) reported significantly ($P<0.05$) highest GSH-Px activity and serum Se concentrations in nano-Se supplemented group than in sodium selenite group in Makuei lambs.

The bioavailability as well as the pharmacological and toxicological effects of Se in animals was associated with its chemical forms (Han et al., 2017). In comparison with inorganic Se, organic Se provided greater protection against oxidative damage and was less toxic (Li et al., 2017). Subsequent studies also indicated that nano-Se has more beneficial effects to improve glutathione peroxidase activity comparing with organic or inorganic Se sources (Yaghmaie et al., 2017). In addition, some reports on rats and mice demonstrated that nano-Se had higher efficiency than inorganic and other Se sources in up-regulating selenoenzymes, exhibiting lower toxicity (Wang et al., 2007). Nano forms have high catalytic efficiency, strong adsorbing ability and low toxicity. All these specific properties of nano-Se and the different absorption pattern may explain the greater bioavailability of nano-Se compared with organic or inorganic Se.

5.7 Hormonal attributes and IgG

The Plasma IgG, testosterone and LH concentration was significantly ($P<0.05$) higher in treatment than control group, and when compared with the forms of Se supplemented, nano Se exhibited highest concentration than its counter parts. Our results with IgG levels are supported by Ghany-Hefnawy et al. (2010) who reported that Se supplementation of cows induces a high concentration of IgG in the serum and colostrum. Higher levels of IgG in the serum were also recorded in their calves. Kamada et al. (2007) found that Se supplementation (selenite) of colostrum increases IgG absorption by new-born calves. Awadeh et al. (1998) also observed significantly higher concentration of serum IgG in beef cows having access to high Se salt mix. Similarly, Larsen et al. (1988) showed enhanced antibody response and total serum IgG concentrations in the Se supplemented lambs com-pared to control group. In a study, Swecker et al. (1989) observed that weaned beef calves supplemented with 80–200 mg Se/kg salt–mineral mixture had higher IgG titers to hen egg lysozyme (HEL) than calves receiving 20 mg Se/kg mineral mix. Mudgal et al. (2008) have also reported that supplementation of 0.3 mg Se/kg DM significantly increased the immune status in male buffalo calves compared to control group. As Se is an essential

micronutrient for various immune mechanisms the present findings are suggestive of its immunomodulatory effect.

Data presented showed that serum testosterone and LH concentrations were significantly ($P<0.05$) higher for bucks fed diets with supplemented Se than the control. Also, serum testosterone and LH levels were higher with feeding nano Se than inorganic and organic. The results indicated that serum testosterone level was increased by 9.52, 15.1 and 21.21% due to supplemental inorganic, organic and nano compared with control, respectively. Similar observations were reported in blood serum of bucks (El-Sisy et al., 2008), in lambs (Ibrahim and Mohamed, 2018). The beneficial effect of nano Se source in increasing serum testosterone levels, is shown in the present study could be associated with its significant effectiveness in enhancing serum GSH-Px activity. This also correlates with the increased uptake of the nano particles by the tissues (Khalaf et al., 2019). Se is an essential component of GSH-Px, an enzyme involved in detoxification of hydrogen peroxide and lipid hydroperoxides. The enzyme GSH-Px has been localized cytochemically in the cytoplasm of Leydig cells (Murakoshi et al., 1983). So, it is possible that the metabolic pathway of testosterone biosynthesis requires higher activity of GSH-Px to protect against peroxidation (Behne et al., 1996). Accordingly, the significant increases in serum testosterone concentrations for lambs fed different Se sources may be related to the significant concomitant increase in their serum GSH-Px activity, protecting the testes and Leydig cells against peroxidation and thus improving its steroidogenic function.

The results also revealed increase in plasma concentrations of LH in the group supplemented with Se. Similar results have been reported by Hezarjaribi et al. (2016) in male broiler breeders. It has been reported that testicular function is controlled by gonadotropin releasing hormones (GnRH) secretion, which is responsible for stimulating the gonadotrophes of the pituitary gland to secrete LH (Griswold, 1998). As Se is known to accumulate in the anterior pituitary (Thorlacius-Ussing and Jensen, 1988), it is possible that increase in plasma Se concentration may have activated GnRH receptors in the anterior pituitary gonadotrophes, leading to increased LH production in Se supplemented group (Ottinger et al., 2004).

5.8 Plasma Mineral concentration

In the present study, dietary concentration of Se at 0.30 mg Se/kg DM, in the different forms was fed to bucks and the plasma Se concentration of control, inorganic, organic and nano Se groups was (0.13, 0.16, 0.20 and 0.28) well within normal limits. The plasma Se concentration increased significantly ($P<0.05$) after 60 days period of Se supplementation in treatment groups than control thereby, indicating more absorption and retention from gut that is reflected in plasma. The present results were consistent with the findings of Shi et al. (2011) who reported that Se concentration in different tissues increased in Se supplemented group compared with the control bucks. Se concentrations in different organs were notably higher in Se supplemented animals compared with supplemented group (Juniper et al., 2009a). Our data also agree with the results of Kumar et al. (2008) who noticed significant increase in the plasma Se concentration with the duration as well as with the level of Se supplementation in the diet of male lambs. The reference values of Se in blood are different depending on the species and age of the animal, and also as per author's methodology. Constable et al. (2017) stated a range of 0.08–0.30 μg·mL−1 for blood serum in adult cattle. When comparing inorganic, organic and nano Se groups the results showed that the plasma Se concentration of nano supplemented group was significantly higher than inorganic and organic group. In agreement to our study Han et al. (2021) also noticed that Nano-Se supplementation significantly increased plasma Se levels, compared with the sodium selenite supplement in lactating dairy cows. Research with broiler chickens has also demonstrated that nano particles are more effectively absorbed in comparison with the usual dietary additive, sodium selenite (Hu et al., 2012). The difference is probably related to the different absorption processes nano particles were absorbed in duodenum by active transportation, and ruminants can absorb in the whole small intestine (Zhang et al., 2001). Furthermore, some reports indicated that organic or nano-elemental forms of Se are absorbed more readily by ruminants than inorganic forms (Xu et al., 2003; Gonzales-Eguia et al., 2009). However, the bioavailability of nano forms is highest. This may explain why nano Se supplemented Se group had showed highest plasma Se concentration than inorganic and organic source. The highest plasma Se concentration with nano form in the present study could be discussed in the light of the view that Nano-Se particles displayed a preeminent bioavailability because of its specific

properties such as high catalytic efficiency, strong adsorbing ability and low toxicity, explaining the greater bioavailability of Nano-Se when compared with organic or inorganic Se forms (Zhang et al., 2008).

Either form of dietary Se supplementation did not affect the levels of Ca, P or S reported in present study. Normal level of Ca and P were reported to be 9.8-11.59 and 4.27-5.39 (mg/dl) respectively. The data obtained from the study showed that concentration of plasma minerals (Ca, P and S) was in the normal range throughout the study and there was no significant effect of dietary Se supplementation on the plasma concentration of these mineral elements. It might be due to no interaction of Se with these mineral elements at the test level supplemented. It was also reported in studies that oral dosing of $MgSO_4$ and $CaSO_4$ (equivalent to 0.4% added dietary S) to dairy cows (diet contained 0.24 mg of Se/kg of DM) during the last 3 week of gestation did not influence plasma Se concentrations (Gant et al., 1998). Se status will be compromised when cows are fed Se deficient diets along with elevated dietary sulfate concentration for long periods because of reduced Se absorption from the gut (Ivancic and Weiss, 2001). In the current study, Se level was in normal range and dietary sulphur has not affected the Se status.

Summary and Conclusions

CHAPTER- 6

SUMMARY AND CONCLUSIONS

The aim of the study was to evaluate the effect of dietary supplementation of different sources of Se on the growth performance, nutrient utilization, seminal attributes, blood biochemicals, antioxidant and hormonal status of bucks. For this study, 24 bucks were selected from the herd maintained at Department of Physiology, DUVASU, Mathura (U.P.). The bucks were divided into 4 groups (Control, T1, T2 and T3) having six animals each group on body weight basis. The animals in Control group were fed on basal diet i.e., concentrate mixture, gram straw and corn silage without any supplementation of Se and the group T1, T2 and T3 were fed with basal diet along with additional supplementation of inorganic, organic, and nano Se at a level of 0.3 mg/kg DM offered respectively. Nutrient requirement of bucks was fulfilled as per ICAR (2013) feeding standard. The duration of experiment was 90 days. During the experimental period DMI, body weight of animal was taken fortnightly. At the end of experimental period, a digestion trial of seven days was conducted to assess the effect of dietary supplementation of different sources of Se on growth performance and nutrient utilization. Blood samples were collected at 0, 30, 60 and 90 days post supplementation. A fraction of whole blood samples were used for estimating haemoglobin (Hb) concentration and packed cell volume (PCV). Hemolysate was prepared with the part of freshly drawn blood sample for assessing the antioxidants parameters. The remaining amounts of blood samples were centrifuged at 3000 rpm for 15 min to separate the plasma. The blood biochemicals, minerals and hormonal estimation were done in blood plasma. The semen from each buck in a group were collected and pooled, a total of six pooled ejaculates from each group were evaluated at day 0 (from 0-21d) i.e., start of experiment and at day 90 (90-111d) i.e., post feeding.

6.1 Growth performance and nutrient utilization of bucks supplemented with different sources of Se

The average initial body weight of Control, T1, T2 and T3 groups were 36.93, 36.92, 36.75 and 36.28 kg in Control, T1, T2 and T3 groups respectively. Final body weights of corresponding groups were 42.83, 43.35, 43.02 and 42.43 kg respectively. The average BW and metabolic BW of all fortnights were similar ($P>0.05$) among the

groups. The DMI (kg/d), Fortnightly body weight gain (kg), average daily gain and FCR in present study did not differ significantly (P>0.05) between control and treatment groups. Buck supplemented with different sources of Se showed no significant (P>0.05) difference on nutrient digestibility in Control and Se supplemented groups. DM, OM, CP, EE, CF, NFE, ADF digestibility remain unaffected on Se supplementation. Dry matter intake (kg/day), CP intake (kg/day), DCP intake (kg/day, g/kg $W^{0.75}$) and TDN intake (kg/day, g/kg $W^{0.75}$) remained similar in all experimental groups.

6.2 Seminal attributes of bucks supplemented with different sources of Se

The mean of seminal parameters viz. semen colour, seminal pH, volume of ejaculated semen (ml), spermatozoa concentration (10^9/ml), and mass motility were not affected by Se supplementation and thus remained unaltered in Control and treatment groups. Whereas, percent progressive motility of spermatozoa and percent live spermatozoa was found significantly higher (P<0.05) in nano supplemented (T3) group as compared to Control group. The overall percent HOST reactive spermatozoa count was found significantly higher (P<0.05) in T3 group as compared to control group and comparable in T1 and T2 group. Viable intact acrosomal percentage was found significantly higher (P<0.05) in T3 as compared to Control and comparable in T1 and T2 group bucks. The total morphological abnormality percentage of spermatozoa was found significantly lower T3 group and comparable in T1 and T2 as compared to control.

Total ROS positive spermatozoa percentage in present study was significantly lower in T3 and comparable in T1 and T2 as compared to control group. Whereas, total ROS negative spermatozoa percentage in present study was significantly higher in T3 group and comparable in T1and T2 as compared to control group. Total spermatozoa percentage with medium intracellular calcium was significantly higher in T3group and comparable in T1 and T2 as compared to control group. Whereas, total spermatozoa percentage with low intracellular calcium was significantly lower in T3 and T2 group and comparable in T1 as compared to control group. Total spermatozoa percentage with polarized mitochondria in present study was significantly higher in T3 group and comparable in T1and T2 as compared to control group. Total spermatozoa percentage with normal membrane fluidity in present study was significantly higher in T3 group and comparable in T2 as compared to control and

T1group. Total apoptotic spermatozoa percentage and total spermatozoa percentage with high membrane fluidity in present study was significantly lower in T3 group and comparable in T2 as compared to control and T1 group. The overall total transitional spermatozoa percentage and overall spermatozoa percentage with high intracellular calcium in present study was found similar in all groups.

6.3 Haematological and blood biochemicals parameters of bucks supplemented with different sources of Se

Haematological parameters like blood haemoglobin concentration and PCV percentage of animals in present study were found similar in all groups throughout the study. The plasma glucose, cholesterol, triglyceride and albumin concentration did not change significantly ($P>0.05$) in the treatment groups. Plasma total protein and globulin concentrations were found significantly higher in T3 and T2 group and were comparable to T1 than Control group. Overall plasma ALT and AST activity of treatment groups was found similar with Control group for whole trial period.

6.4 Antioxidant status of bucks supplemented with different sources of Se

Mean SOD activity (U/mg Hb) and Mean GSH-Px concentration (ng/ml plasma) were found significantly higher ($P<0.05$) inT3 group bucks as compared to Control group bucks. Whereas, overall LPO (nM MDA/ml packed RBCs) and ROS concentration (nM /ml) in hemolysate was found significantly lower ($P<0.05$) in T3 group.

6.5 Hormonal status of bucks supplemented with different sources of Se

Overall plasma Testosterone and LH concentration was found significantly higher ($P<0.05$) inT3 group bucks as compared to Control group bucks.

6.6 Plasma IgG Level

Overall plasma IgG concentration was found significantly higher ($P<0.05$) in treatment group as compared to Control and significantly higher in T3 among treatment groups.

6.7 Plasma mineral concentration

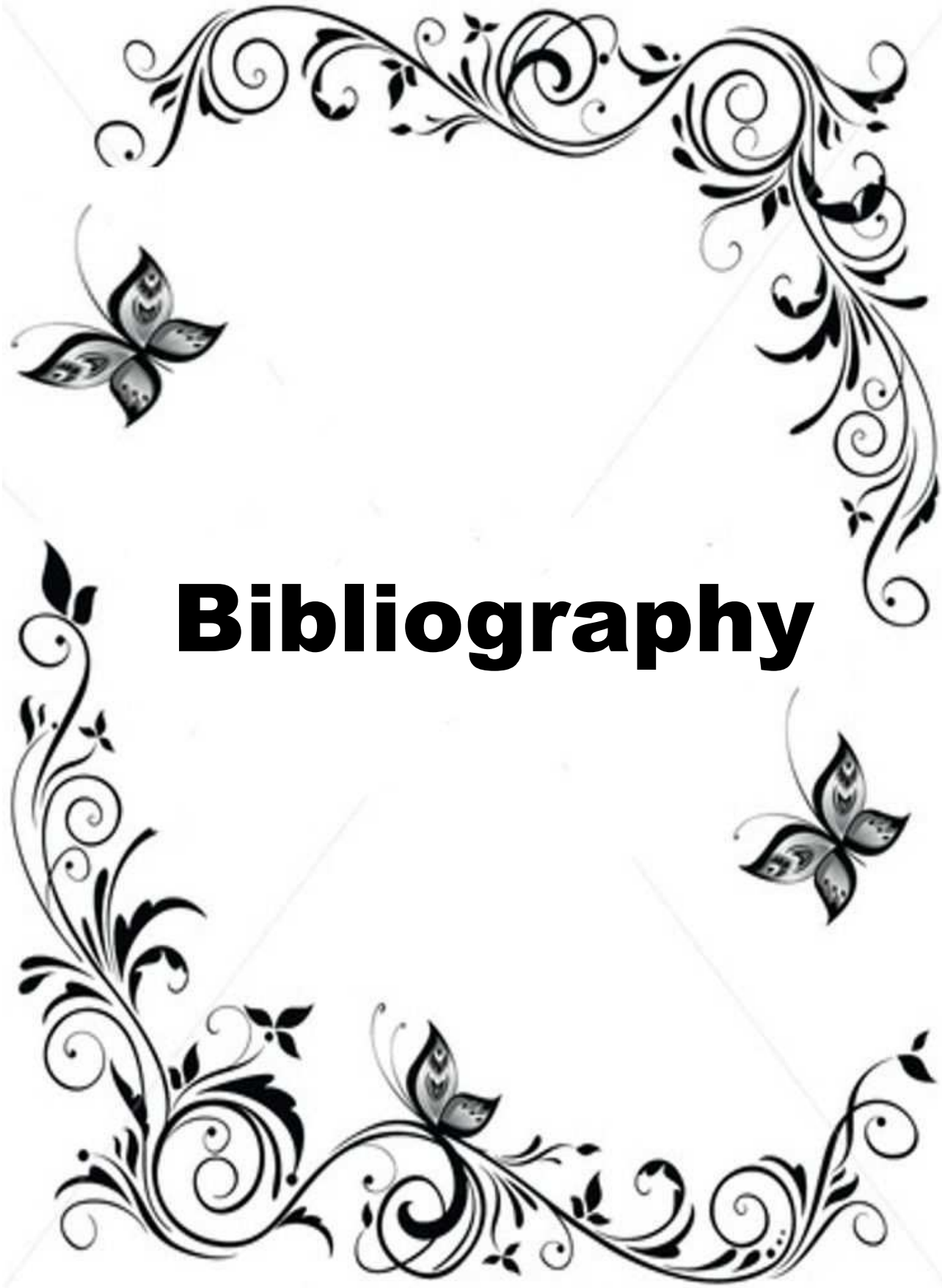
The plasma Se concentration (μg/ml) of experimental animals was found significantly higher ($P<0.05$) in treatment group as compared to Control group. The plasma Ca and P concentration did not change significantly ($P>0.05$) in the treatment group.

Conclusions:

The effect of supplementation of different sources of Se on the performance of bucks may be concluded as:

1) Dietary supplementation of Se from either source have no significant ($P<0.05$) effect on BW, ADG, FCR, intake and digestibility of nutrients, indicating no impact of Se supplementation on growth performance and nutrient digestibility of bucks.
2) Semen analysis showed significant ($P<0.05$) improvement in progressive motility, viability, Acrosomal integrity, ROS, membrane fluidity, intracellular Ca levels and HOST values in treatment groups compared to control. Though, the highest values were detected in nano Se supplemented group.
3) Plasma globulins and total protein concentration were significantly ($P<0.05$) higher in Se supplemented groups compared to control. However, the concentration of all other haemato-biochemical parameters monitored remained unaltered. Significant ($P<0.05$) increase in plasma SOD and GSH-Px activity with concomitant decrease in ROS and LPO levels was found in treatment groups as compared to control. However, compared with inorganic and organic sources, nano Se seems to be more effective in enhancing antioxidant status. Plasma Se concentration significantly ($P<0.05$) increased in treatment groups irrespective of source, However, nano Se resulted in higher plasma Se concentration compared with inorganic and organic sources at 0.3ppm level. The assay of IgG and the reproductive hormones (Testosterone and LH) significantly ($P<0.05$) increased in treatment groups with highest concentration in T3 group, suggesting effective role of nano Se in improving the reproduction performance and immune status of bucks.

Hence, it can be concluded that incorporation of different Se sources (inorganic, organic and nano) at 0.3ppm level in diet increased plasma Se concentration, improved antioxidant, immunity, seminal attributes and reproductive hormones status. Moreover, nano Se seems to be more effective in improving fertility through enhancement of seminal attributes, antioxidant and Se status in bucks in comparison to its organic and inorganic counterparts.



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PARTICIPATION

i Attended International conference of animal nutrition 2019 on **"Nutritional strategies for improving farm profitability and clean animal production"** organised by West Bengal university of Animal and Fishery Science, Kolkata in collaboration with Animal Nutrition Society of India on 17-19 December, 2019

ii Attended 1st Annual convention of Veterinary Internal and preventive Medicine society & National symposium on **"Sustainable improvement in Animal health and production- Bridging science and policy for upliftment of farmers"** organized by Department of Veterinary medicine, DUVASU, Mathura in collaboration with CIRG on 8th-9th November, 2019.

LIST OF PUBLICATIONS:

Research paper:
Submitted

Date: 23/10/21
Place: Mathura

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Signature

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