Evaluation of nano zinc supplementation on growth, nutrient utilization and immunity in goats (*Capra hircus*)

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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IN

ANIMAL NUTRITION

By

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CERTIFICATE

This is to certify that the thesis entitled, **"EVALUATION OF NANO ZINC SUPPLEMENTATION ON GROWTH, NUTRIENT UTILIZATION AND IMMUNITY IN GOATS (*CAPRA HIRCUS*)"**, submitted by **Dr. PARTHA SARATHI SWAIN** towards the partial fulfillment for the award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL NUTRITION** of **ICAR-NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my guidance, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 7/9/2017

MAJOR ADVISOR

Dedicated to…..

Whole of my family,

(Especially my father and mother)

Also, my Teachers & Friends…

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(Partha Sarathi Swain)

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List of abbreviations

Abbreviation	Expanded form
%	Per cent
^{0}	Degree
ADF	Acid detergent fibre
BD	Basal diet
BW	Body weight
CP	Crude protein
DCP	Digestible crude protein
DCPI	Digestible crude protein intake
DM	Dry matter
DOMI	Digestible organic matter intake
EE	Ether extract
g/ kg $W^{0.75}$	Gram per kg metabolic body weight
g/d	Gram per day
H	Hour
IGF-1	Insulin like growth factor-1
IZnO	Inorganic zinc oxide
Kcal/ kg $W^{0.75}$	Kcal per kg metabolic body weight
Kg	Kilogram
M	Molar
MCal/d	Megacalorie per day
MCal/kg	Megacalorie per kg
ME	Metabolizable energy
MEI	Metabolizable energy intake
mL	Millilitre
NC	Negative control
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
NP	Nanoparticles
NZn	Nano zinc
NZnO	Nano zinc oxide
^{o}C	Degree celcius

OM	Organic matter
RL	Rumen liquor
SRL	Strained Rumen liquor
T_3	Triiodothyronine
T_4	Thyroxine
TDN	Total digestible nutrient
TDNI	Total digestible nutrient intake
Total CHO	Total carbohydrate
VFA	Volatile fatty acid
$W^{0.75}$	Metabolic body weight
WAR	Wistar albino rats
Zn-Gly	Zinc glycinate
Zn-Met	Zinc methionine
Zn-Nic	Zinc nicotinate
ZnO	Zinc oxide
ZNP	Zinc nanoparticles
Zn-prop	Zinc propionate
Zn-prot	Zinc proteinate

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ABSTRACT

A comprehensive study was conducted to evaluate the efficiency of nano zinc as feed supplement on growth, nutrient utilization and immunity in goats (*Capra hircus*). Initially, nano sized zinc was synthesized by pyrolysis of zinc acetate [Zn $(CH_3COOH)_2$. $2H_2O$] and sodium bicarbonate ($NaHCO_3$) and particle size was estimated to be 195.9 nm. Subsequently, in order to get still smaller nano Zn (NZn), chemical method was used, where NP were synthesized by using 0.45M zinc nitrate [$Zn(NO_3)_2.6H_2O$] and 0.9 M sodium hydroxide (NaOH). The particle size was reduced to 74 nm and then, it was confirmed to be NZn oxide by using TEM-EDAX. The synthesized NZn was fed to rats in graded doses to validate the effects of NZn and to select the dose for goat experiment. Wistar albino rats (WAR) (84, 53.6±0.65g) were distributed to seven groups of 12 animals (4 replicate each with 3 rats) each. NC (negative control): Basal diet (BD) + no supplemental zinc; IZn-25: BD+ 25 mg/kg Zn from inorganic ZnO; NZn-25: BD+ 25 mg/kg Zn from NZnO; NZn-12.5: BD+ 12.5 mg/kg Zn from NZnO; NZn-6.25: BD+ 6.25 mg/kg Zn from NZnO; NZn-3.125: BD+ 3.125 mg/kg Zn from NZnO; NZn-50: BD+ 50 mg/kg Zn from NZnO. After a feeding trial of 60 days, it was observed that total weight gain, average feed intake (g/d/rat) were similar across the groups. However, FCR was found to be significantly ($P<0.05$) different among the treatment groups with NZn-25 was lower than NC, NZn-6.25 and NZn-3.125. Intake and digestibility of DM, OM and EE were also similar among the groups, but the CP digestibility was higher ($P<0.05$) in NZn-25 than NC. NC rats had lower ($P<0.001$) zinc intake (µg/ rat/d) than all other groups as no extra supplemental zinc was provided. Zinc absorbed (µg/ rat/d) was higher in NZn-50 and NZn-25 than all other groups. However, NC group absorbed minimum zinc (mg/d) from feed as compared to IZn-25, NZn-25, NZn-12.5, and NZn-50. Zinc absorption (%) was higher ($P<0.001$) in NC, NZn-25, NZn-12.5, NZn-3.125 than IZn-25 and NZn-50. Albumin, globulin and total protein were higher ($P<0.05$) in NZn-50 and NZn-25 as compared to NC. Serum T_3, and IGF-1 were not affected ($P>0.05$) due to dietary treatments but T_4 and testosterone showed a dose dependant increase ($P<0.05$) in NZn supplemented groups. The cell mediated and humoral immune responses were found to be positively affected in NZn-25 and NZn-50 as compared to NC. The zinc content was observed to be significantly ($P<0.05$) higher in liver, bone, kidney and serum due to NZn supplementation where NZn-50 had highest zinc content and NC had the least. But Fe, Mn and Cu were not affected due to dietary Zn supplementation. Due to zinc supplementation, metallothionein and SOD-1 m-RNA expression was up-regulated in NZn-25 and NZn-50 as compared to IZn-25. NZn-25 and NZn-50 group of rats had improved immunity, FCR, zinc absorption, CP digestibility, serum albumin, globulin, total protein, zinc level in serum, liver, bone and kidney in rats. Consistently throughout the study, NZn-12.5 group was either comparable or better to IZn-25 in terms of growth, immunity, FCR, zinc absorption, CP digestibility, serum albumin, globulin, total protein, zinc level in serum, liver, bone and kidney suggesting its better

bio-availability and was better than NZn-6.25 and NZn-3.125 also. Therefore, half of ICAR recommendation was selected for goat trial as reduced level. Twenty four male goats (18.7±0.33 kg) were divided in 4 groups of six each and were supplemented with either basal diet (concentrate and finger millet (*Eleusine corocana*) straw @ 50: 50 ratio; BD) i.e. NC, BD with 50 mg/kg zinc from inorganic ZnO (IZn-50), BD with 50 mg/kg zinc from NZnO (NZn-50) or BD with 25 mg/kg zinc from NZnO (NZn-25). Feeding trial was conducted for almost 4 months. ADG, total weight gain and FCR was found to be better ($P<0.05$) in NZn-25 than NC, without affecting their feed intake. However, NZn-25 was at par with IZn-50 with respect to ADG, total weight gain and FCR. DM, OM, CP, EE, Total CHO digestibility, DCP, TDN (%) and ME (MCal/kg) did not vary ($P>0.05$) among treatment groups. The ADF and cellulose digestibility in zinc supplemented groups (IZn-50, NZn-50 and NZn-25), irrespective of the source of zinc (inorganic or nano) was significantly higher ($P<0.05$) than NC. Ca, P and Mg intake (g/d), absorbed (g/d) and absorption (%) were similar ($P>0.05$) among the treatment groups. The Zn intake (mg/d) in IZn-50 and NZn-50 was significantly higher than NZn-25 and NC. More absorption (mg/d) of zinc was evident in NZn-50 group compared to IZn-50, NZn-25 and NC. However, NZn-25 and IZn-50 retained similar levels of Zn indicating the better bioavailability of NZn. After 90^{th} day of experimental feeding, no change was observed in the serum zinc level across the groups but was found significantly higher in all the zinc supplemented groups when compared with 0 day serum zinc content. Haematological parameters were found to be unaffected. The globulin and total protein contents were significantly improved in NZn-50 than other groups. Among hormones, T3 and T4 were not affected due to zinc supplementation but IGF1 and testosterone were significantly higher in NZn supplemented groups than NC. Cell mediated immunity, humoral immunity and immunophenotyping confirmed better immune status of the goats of NZn-50 than other groups. Supplementation of graded doses of NZn did not alter the individual and total VFA profile in goats, but the soluble zinc content was higher in NZn-50 than NZn-25 and NC. Nano Zn supplementation at either dose did not alter the semen volume and total sperm production in goats. Supplementation of NZn at 25 and 50 mg/kg improved sperm motility and velocity parameters in goats as compared to IZn-50 and NC. Hence, NZn at ICAR recommended dose (50 mg/kg) improved animal growth, FCR, immunity, mineral retention and semen motility parameters as compared to IZn-50 and NC. With regards to animal health, production and reproduction NZn-25 (NZn at half of ICAR recommendations) performed equivalent to IZn-50, showing its efficiency as animal feed supplement in goats. Nano Zn can be supplemented at half the dose of inorganic Zn without affecting animal health, production and reproduction.

सारांश

नैनो जस्ते की फ़ीड अनुपूरण के रुप में दक्षता का वृद्धि, पोषक उपयोग और प्रतिरक्षा पर मूल्यांकन करने के लिए बकरियों (कैपरा हिर्कस) पर एक विस्तृत अध्ययन किया गया । हमारी प्रयोगशाला में आरम्भ में भौतिक विधि द्वारा जिंक एसीटेट और सोडियम बाइकार्बोनेट की पाईरोलिसिस से नैनो जिंक का संश्लेषण किया गया और औसत कण आकार 197 nm प्राप्त किया गया। इस से भी छोटे आकार के नैनो जस्ता कण पाने के लिए रासायनिक विधि का इस्तेमाल किया गया, जहां 0.45 M जस्ता नाइट्रेट और 0.9 M सोडियम हाइड्रोक्साइड का उपयोग करके नैनो जस्ता संश्लेषण किया गया और औसत कण आकार 74 nm तक कम हो गया, और बाद में TEM-EDAX का उपयोग करके नैनो जस्ता होने की पुष्टि भी की गई । संश्लेषित नैनो जस्ता (NZn) के प्रभाव को देखने के लिए और बकरी प्रयोग के लिए खुराक का चयन करने के लिए वर्गीकृत मात्रा में पहले चूहों पर परिक्षण किया गया । विस्टर एल्बिनो चूहों (84, 53.6 ± 0.65g) को प्रत्येक 12 चूहों के सात समूहों में विभाजित किया गया । कण्ट्रोल: बेसल आहार (बीडी) + कोई पूरक जस्ता नहीं; IZn-25: बीडी + 25 मिलीग्राम/ किग्रा जस्ता, अकार्बनिक ZnO से; NZn-25: बीडी + 25 मिलीग्राम/ किग्रा नैनो जस्ता, NZnO से; NZn-12.5: बीडी + 12.5 मिलीग्राम/ किग्रा नैनो जस्ता , NZnO से ; NZn-6.25: बीडी + 6.25 मिलीग्राम/ किग्रा नैनो जस्ता, NZnO से; NZn-3.125: बीडी + 3.125 मिलीग्राम / किग्रा नैनो जस्ता, NZnO से; NZn-50: बीडी + 50 एमजी / किग्रा नैनो जस्ता, NZnO से । साठ दिनों तक खिलाने के बाद कुल वजन, औसत दैनिक भोजन का सेवन समूहों में समान पाया गया। NZn-25 की FCR, कण्ट्रोल, NZn-6.25 और NZn-3.125 के तुलना में कम ($P<0.05$) पाई गई । DM, OM और EE की ग्राह्यता और पाचकता भी समूहों के बीच में समान थी, लेकिन CP की पाचकता NZn-25 में कण्ट्रोल से उच्च ($P<0.05$) थी । अन्य सभी समूहों की तुलना में कण्ट्रोल चूहों में ($P<0.001$) जस्ता का सेवन कम था क्योंकि कोई अतिरिक्त पूरक जस्ता नहीं दिया गया था। अन्य सभी समूहों की तुलना में अवशोषित जस्ते की मात्रा, NZn-50 और NZn-25 में अधिक थी । हालांकि, कण्ट्रोल समूह ने IZn-25, NZn-25, NZn-12.5, NZn-50 की तुलना में फ़ीड से न्यूनतम जस्ता अवशोषित किया था। कण्ट्रोल, NZn-25, NZn-12.5, NZn-3.125 में जस्ते का अवशोषण (%) IZn-25 और NZn-50 की तुलना में उच्च ($P<0.001$) था । NZn-50 और NZn-25 में एल्बुमिन, ग्लोब्युलिन और कुल प्रोटीन कण्ट्रोल के मुकाबले उच्चतर ($P <0.05$) थी । सीरम T_3 और IGF-1 आहार उपचार के कारण प्रभावित ($P> 0.05$) नहीं हुए थे, लेकिन NZn पूरक समूहों में T_4 और टेस्टोस्टेरोन में वृद्धि ($P<0.05$) दिखायी दी । कण्ट्रोल समूह की तुलना में NZn-25 और NZn-50 में सेल की मध्यस्थता और कोमल प्रतिरक्षा प्रतिक्रियाएं सकारात्मक रूप से प्रभावित हुईं। नैनो जस्ता पूरकता के कारण यकृत, हड्डी, गुर्दा और सीरम में अधिक जस्ता पाया गया, जिसमे NZn-50 में अधिकतम और कण्ट्रोल में सबसे कम ($P <0.05$) जस्ता देखा गया । लेकिन, आहार अनुपूरक के कारण Fe, Mn और Cu प्रभावित नहीं हुआ । NZn-25 और NZn-50 में जस्ता पूरक के कारण, IZn-50 की तुलना में मेटलोथिओनिन (metallothionein) और SOD-1 अभिव्यंजना बढ़ गई। NZn-25 और NZn-50 समूह के चूहों में प्रतिरक्षा, FCR, जस्ता अवशोषण, CP पाचकता, सीरम एल्बूमिन, ग्लोब्युलिन, कुल प्रोटीन, सीरम, यकृत, हड्डी और गुर्दा में जस्ता स्तर में सुधार हुआ । पूरे अध्ययन में, NZn-12.5 समूह विकास, प्रतिरक्षा, FCR, जस्त अवशोषण, CP पाचकता, सीरम एल्बूमिन, ग्लोब्युलिन, कुल प्रोटीन, सीरम, यकृत, हड्डी और गुर्दे में जस्ता स्तर, IZn-25 की तुलना में या तो तुलनीय या बेहतर था , जो की इसकी बेहतर जैव उपलब्धता का सुझाव देता है और NZn-12.5समूह , अन्य NZn-6.25 और NZn-3.125 से भी बेहतर पाया गया । इसलिए, ICAR सिफारिश की आधी जस्ते की खुराक बकरी परीक्षण के लिए चुना गया । चौबीस बकरीयों (18.7 ± 0.33 किग्रा) को 4 समूहों में विभाजित किया गया और बेसल आहार (दाने का मिश्रण और फिंगर मिलेट (एल्यूसिन कोरोकाना) पुआल @ 50: 50 अनुपात; बीडी) दिया गया । विभिन्न समूहों में को अलग अलग प्रकार आहार दिया गया, कण्ट्रोल: बीडी + कोई पूरक जस्ता नहीं; IZn-50: बीडी + 50 मिलीग्राम / किग्रा

जस्ता, अकार्बनिक ZnO से; NZn-50: बीडी + 50 मिलीग्राम / किग्रा नैनो जस्ता, NZnO से; और NZn -25: बीडी + 25 मिलीग्राम / किग्रा नैनो जस्ता , NZnO से । खिलाने का परीक्षण 4 महीने के लिए आयोजित किया गया था । बिना भोजन सेवन को प्रभावित किये हुए, ADG, कुल वजन और FCR में कण्ट्रोल के मुकाबले NZn-25 में बेहतर (P <0.05) पाया गया था । ADG, कुल वजन और FCR के आकलन में , NZn-25 और IZn-50 बराबर थे । DM, OM, CP, EE, कुल कार्बोहाइड्रेट, पाचकता, DCP, TDN (%) और ME (MCal/ किग्रा) में उपचार समूहों के बीच (P> 0.05) भिन्नता नहीं थी। जस्ता पूरक समूहों (IZn-50, NZn-50 और NZN-25) में NDF और सेल्यूलोज पाचकता, कण्ट्रोल के मुकाबले काफी अधिक थी (P< 0.05) । उपचार समूहों के बीच Ca, P और Mg का सेवन (जी / डी), अवशोषित (जी / डी) और अवशोषण (%) समान (P> 0.05) थे। IZn -50 और NZn -50 में जस्ता सेवन (मिलीग्राम / दिन), NZn-25 और कण्ट्रोल से काफी अधिक था । IZn-50, NZn-25 और कण्ट्रोल की तुलना में, NZn -50 समूह में अधिक जस्ते का अवशोषण (मिलीग्राम / दिन) हुआ । हालांकि, NZn-25 और NZn-50 ने शरीर में जस्ते का समान स्तर रखा, जो नैनो जस्ते की बेहतर जैव-क्षमता को दर्शाते हैं। प्रायोगिक खिलाने के 90 दिन के बाद, सभी समूहों में सीरम जस्ता स्तर पर कोई परिवर्तन नहीं दिखा, लेकिन 0 दिन के कण्ट्रोल सीरम जस्ता परिमाण की तुलना में 90 वें दिन के सीरम नमूने में ज़िंक सभी पूरक समूहों में काफी अधिक पाया गया। रुधिरविज्ञान संबंधी प्राचल समूहों मैं अप्रभावित पाया गया। अन्य समूहों की तुलना में NZn-50 में, ग्लोब्युलिन और कुल प्रोटीन के स्तर मैं काफी सुधार हुआ । जस्ता पूरण के कारण, T_3 और T_4 हार्मोन के स्तर प्रभावित नहीं हुए थे, लेकिन IGF-1 और टेस्टोस्टेरोन नैनो जस्ता पूरक समूहों में कण्ट्रोल से काफी अधिक थे । अन्य समूहों की तुलना में सेल मध्यस्थ प्रतिरक्षा, रुधिर प्रतिरक्षा और इम्यूनोफेनोटाइपिंग NZn-50 के बकरों में बेहतर प्रतिरक्षा स्थिति की पुष्टि की। NZn की वर्गीकृत खुराक की पूर्ति ने बकरियों में व्यक्तिगत और कुल VFA प्रोफाइल को नहीं प्रभावित किया , लेकिन NZn-50 की तुलना में NZn-25 और कण्ट्रोल में घुलनशील जस्ता सामग्री अधिक थी । नैनो जस्ता पूरक आहार ने बकरियों में वीर्य की मात्रा और कुल शुक्राणु उत्पादन में परिवर्तन नहीं किया । नैनो जस्ते के पूरण के कारण NZn-25 और NZn-50 में IZn-50 और कण्ट्रोल NZn-25 के मुकाबले शुक्राणु गतिशीलता और वेग मापदंडों में सुधार देखा गया। इसलिए, ICAR की सिफारिश की खुराक (50 मिलीग्राम/ किग्रा) में NZn-50 ने IZn-50 और कण्ट्रोल की तुलना में पशु वृद्धि , एफसीआर, प्रतिरक्षा, खनिज प्रतिधारण और वीर्य गतिशीलता मानदंडों में सुधार किया । बकरियों में पशु खाद्य पूरक के रूप में अपनी दक्षता दिखाते हुए, NZn-25 (ICAR की सिफारिशों के आधे खुराक में) IZn-50 की तुलना में, पशु स्वास्थ्य, उत्पादन और प्रजनन में समान प्रदर्शन दिखाया । इसलिए, नैनो जस्ते को पशु स्वास्थ्य, उत्पादन को प्रभावित किए बिना अकार्बनिक जस्ते की आधी मात्रा में पूरण किया जा सकता है।

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INTRODUCTION

Minerals are required in very small quantities as compared to other nutrients. But, their deficiency can predispose to poor animal health and production (Overton and Yasui, 2014). Deficiency of trace minerals in the diet can decrease the animal production by 20-30% (Mohanta and Garg, 2014). Feeds and fodders are the principal sources of minerals for livestock. The crop residues form the bulk of rations in India, are deficient in most of mineral elements especially Zn (Datt and Chhabra, 2005). Generally, trace elements have been supplemented in the animal feeds as their inorganic salts such as sulphates, oxides and chlorides, however, the bioavailability of these trace elements from inorganic sources is not at par with that of minerals from feed and fodder sources (Spears, 2003) thus necessitating alternate highly bio-available sources of minerals.

Zn is the second most abundant trace element in the animal body, which can't be stored (Zalewski *et al*., 2005) and thus regular dietary intake, is warranted to meet the physiological needs. Being a component of numerous enzymes and hormones, Zn is necessary for the proper physiological functioning (Prasad, 1991). For instance, alcohol dehydrogenase, alkaline phosphatase (ALP), aldolase, lactate dehydrogenase (LDH), RNA and DNA polymerases, reverse transcriptase and superoxide dismutase (SOD). Zinc is critical for normal growth (Case and Carlson, 2002), reproduction (Uchida *et al*., 2001), DNA synthesis, cell division and gene expression (Prasad, 1991), photochemical processes of vision (Suchý *et al*., 1998), wound healing (Zhao *et al*., 2014), ossification (Roughead and Kunkel, 1991), augmenting the immune system of the body (Zhao *et al*., 2014; Parashuramulu *et al*., 2015) through energy production, protein synthesis, protection of membranes from bacterial endotoxins and lymphocyte replication and antibody production (Nockels, 1994). Furthermore, Zn is a component of the free radical scavengers which are produced during different physiological processes (Zhao *et al*., 2014), and is also required for the normal condition of epidermis, epithelium, skin and hooves (Kruczynska, 2004). Zn plays an important role in the formation of insulin (Kruczynska, 2004). Role of Zn on livestock reproduction came into picture when Mussill (1941) reported that, sterility in heifers was attributed to insufficient Zn. It is having a synergistic effect on the reproductive performances of the animals.

Rats and humans are susceptible to even marginal Zn deficiency which reduces immune responses (Fraker *et al*., 1984) but in ruminants (Droke and Spears, 1993), marginal Zn deficiency does not impair cell-mediated or humoral immune responses (Spears, 2000). There is an increase in the immunoglobulin level in colostrum as well as in blood serum by supplementing organic Zn (Kinal *et al*., 2005). Thus Zn is routinely supplemented in diets for normal metabolic processes listed above for efficient production and reproduction of livestock.

Absorption of Zn in the body is very less and differs with the age of the animal and the sites in the gastrointestinal tract and varies from 12-55% highest being in young calves. This necessitates for zinc supplements which are highly bio-available. Zn can be incorporated in the diet as inorganic salts like Zn Oxide (ZnO) and Zn Sulphate ($ZnSO_4$) and as organic chelates such as Zn propionate and Zn acetate. Even though, the bioavailability of Zn in organic sources is higher than that of inorganic Zn salts, the use of organic Zn chelates in animal diets is limited due to its higher cost (Zhao *et al*., 2014). Added to this, higher levels of Zn excreted from the inorganic Zn supplemented animals have raised concerns pertaining to environmental pollution (Feng *et al*., 2009). This problem opens up requirement for better bio-available and cheaper Zn sources to reduce the supplemental dose of Zn to the animals.

Several technologies are adopted to increase the bioavailability of the minerals. Nano technology can be used for production of nano sized minerals for use in mineral mixtures. Nano minerals improve the bioavailability due to the increased surface area. Nano particles exhibit unique properties in terms of chemical, physical, photo-electrochemical and electronic properties when compared to their respective bulk materials. The use of Nano Zinc (NZn) has shown to produce better results as compared with conventional Zn sources and also micro Zn and is also less toxic (Wang *et al*., 2006; Sahoo *et al*., 2014b).

Very few reports are there on various effects of NZn in livestock and reports in ruminants is still scarce. As this nano science is at its infancy in the field of mineral nutrition, systematic studies are needed to understand the effect of nano minerals, on their exerted biological effects in the animal body. Along with this, the possible toxicological effects (if any) in both non-ruminants and ruminants with the higher doses are to be documented before regular incorporation in the normal ration. Thus, the present investigation is aimed to produce nano Zinc (NZn) particles in the laboratory, evaluating

effects of different doses of NZn in laboratory animals for fixing the dose for farm animals, goats in this case.

The study was conducted to know the effectiveness of supplementation of NZn as a substitute to inorganic Zn from ZnO in rats and goats with the following objectives.

1. To synthesize and characterize nano zinc.
2. To evaluate the effect of different doses of nano zinc on growth, nutrient utilization and expression of certain selected genes in rats.
3. To evaluate the effect of selected doses of the nano zinc on growth, immunity and nutrient utilization in goat.

REVIEW OF LITERATURE

Elements that generally occur at relatively low levels in living tissues are designated `trace elements' or 'micro-minerals' but, their deficiency results in poor animal health and production. Supplementation of trace elements in animal diets has long been practised in order to ensure optimum growth, boost reproductive performance and improve immune response (Overton and Yasui, 2014). Zn content in soils, feeds and fodders varies widely due to various factors such as topography, climatic condition, irrigation, fertilizers and type of soils. In general, oil cakes, brans, and legumes hold higher concentration of Zn followed by green fodders, cereal grains and cereal straws (Bedi and Khan, 1984). But, in Indian conditions, there is widespread deficiency of Zn and Cu (Datt and Chhabra, 2005) in feeds, fodder and soil.

2.1. Biological effects of Zn in livestock

Zinc (Zn) is one of the most important mineral for sound health, production and reproduction in animals. Zn is an essential trace element, which was discovered way back in 15th century. The essentiality of Zn in the life science had become prominent after its role was recognized in growth of *Aspergillus niger* (Raulin, 1869). Until 1934, the biochemical effect of Zn deficiency in animals was not clearly reported (Todd *et al.*, 1934). In 1939, Zn, recognised as an integral component of an enzyme, carbonic anhydrase, which is the first report of its biochemical role (Keilin and Mann, 1939). Later, the clinical symptoms of Zn deficiency in pigs were established with skin parakeratotic lesions (Tucker and Salmon, 1955). It is the second most abundant trace element in the animal body but can't be stored in the body (Zalewski *et al.,* 2005) thus regular dietary intake is required. Animal body contains about 10 to 50 µg of Zn/g, while plasma contains 12 to 16 µM/100ml.

Zinc is also essential for normal growth (Case and Carlson, 2002), reproduction (Uchida *et al.,* 2001; Campbell *et al.,* 1999), DNA synthesis, cell division and gene expression (Prasad, 1991), photochemical processes of vision (Suchý *et al.,* 1998), wound healing (Kietzmann and Braun, 2006; Zhao *et al.,* 2014), ossification (Roughead and Kunkel, 1991), augmenting the immune system of the body (Conway, 1988; Hosnedlová

et al., 2007; Feng *et al.,* 2010), especially cell mediated immunity in the animal (Prasad, 1991).

Zn to be the second most abundant micro mineral in the mammalian body, and also is a cofactor for more than 300 metalloenzymes (Coleman, 1992a, b; Vallee and Falchuk, 1993) and over 2000 transcription factors in microorganisms, plants, and animals (Jeong and Eide, 2013; Vallee and Falchuk, 1993). Zn is the only metal encountered in all 6 enzyme classes established by the International Union of Biochemistry, which included oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee and Falchuk, 1993). Zn participates in cellular processes, antioxidant defences, gene expression. As a component of numerous enzymes and hormones, and thus, Zn is necessary for the proper physiological functioning (Prasad, 1991; Vrzgula *et al.,* 1990) which includes alcohol dehydrogenase, alkaline phosphatase, aldolase, lactate dehydrogenase, RNA and DNA polymerases, reverse transcriptase, carboxypeptidase A, B, G (Bencko *et al.,* 1995) and superoxide dismutase (SOD) (Yo, 1994; Markiewicz *et al.,* 2005). Due to the vast role in enzyme systems, either requiring Zn as catalytic, co-catalytic, or as a structural component (Reilly, 2004), supplementation of Zn in ruminants diets is essential for maintaining normal growth, normal development and function of the immune system, and metabolism (NRC, 2000). Furthermore, Zn also improves amino acid utilization, having role in protein synthesis (Spears, 1989).

This multifaceted function is achieved by energy production, protein synthesis, and protection of membranes from bacterial endotoxins and lymphocyte replication and antibody production (Nockels, 1994). Along with this, Zn is a component of the free radicals scavengers which are produced during different physiological processes (Zhao *et al.,* 2014). For an instance, SOD has antioxidant effects and catalyses the conversion of superoxide anions to oxygen and hydrogen peroxide (Blander *et al.,* 2003). Zn is also required for the normal condition of epidermis, epithelium, skin and hooves (Kruczynska, 2004). Dietary Zn, at low level, can reduce the feed intake and growth performance of the livestock (Jia *et al.,* 2008). It has been observed that rats and humans are susceptible to even marginal Zn deficiency which reduces immune responses (Fraker *et al.,* 1984) but in ruminants (Droke and Spears, 1993; Droke *et al.,* 1993), marginal Zn deficiency does not impair cell-mediated or humoral immune responses (Spears, 2000). It was shown to have a critical role in proteolytic enzyme systems associated with muscle protein turnover (Hassan *et al.,* 2011). Case and Carlson (2002) reported a dietary level of 2,000-4,000 mg/kg of Zn can enhance animal growth.

Absorption of Zn in the body is very less and differs with both site of absorption in gastro intestinal tract and age of the animal. For an instance, Miller and Cragle (1965) reported the net absorption of Zn administered daily was different in mature cows (12%), 5 to 12 months calves (20%) and also in week-old calves (55%). In the animal's body, Zn is mostly absorbed from the abomasum and lower small intestine. Absorption below the cecum is insignificant and secretion of endogenous Zn occurred from the upper part of the small intestine (Miller and Cragle, 1965).

Numerous studies have been conducted to evaluate the site of Zn absorption and metabolism by ruminants. The proximal part of small intestine is generally regarded as the main site of Zn absorption (Miller and Cragle, 1965; Hiers *et al.,* 1968). Since Zn is a relatively small, hydrophilic, highly charged element that cannot cross biological membranes by passive diffusion, specialized mechanisms are required for both its uptake and release (McMahon and Cousins, 1998) which are called as active carrier mediated process (Davis, 1980; Suttle *et al.,* 1982). In ruminants, Zn absorption is mostly as per the need of animal. However, a mucosal induced metal binding protein known as metallothionine, influences Zn absorption.

It has been well known that under normal dietary circumstances, the faeces are the major route of Zn excretion in ruminants, as well as in other animals (Miller, 1969). Urinary excretion of Zn by calves and lambs is generally low (< 1 mg/d), with little effect on dietary Zn supply (Hambidge *et al.,* 1986). Though it is low compared with faecal excretion, urinary Zn excretion could be increased by feeding chelating agents like EDTA in combination with the mineral (McDowell, 1992).

After absorption, Zn in serum is bound predominantly to albumin (60%), macroglobulin (30%) and transferrin (10%) (Scott and Bradwell, 1983). Though plasma Zn pool is a minor pool, it is highly mobile and immunologically important. However, as there is no specialized Zn storage system in the body, there must be a daily intake of Zn to achieve a steady state.

2.2. Bioavailability of different sources of Zn for livestock

Trace mineral supplements are added to dairy cattle rations to prevent mineral deficiencies, and supplementation has traditionally been provided in the form of inorganic salts. The forms of Zn commonly fed to ruminants in the inorganic form consist of ZnO or $ZnSO_4$ (Underwood and Suttle, 1999). Zinc oxide (72 per cent Zn) and $ZnSO_4$ (36 per cent Zn) are the two most widely used feed grade sources of Zn in industry (Batal *et al.,* 2001)

mostly due to their cost and commertial availability (Kwiecień *et al.*, 2017). Normally zinc sulphate is available in a dry form but dissociates from the sulfate when hydrated in the rumen. Generally, dissociated trace minerals in the reticulo-rumen, omasum, and abomasum can form indigestible compounds that pass into the manure. For instance, minerals can form insoluble complexes with other minerals that precipitate out of digesta (Spears, 2003). Formation of such compounds in the reticulo-rumen, omasum, and abomasum reduces mineral absorption in the small intestine. It is been reported that, Zn present in ZnO and Zn-Met is absorbed to a similar extent, but Zn from these two sources appears to be metabolized differently after absorption (Spears, 1989). Thus it can be perceived that, utilization and metabolism of Zn is source dependant.

Bioavailability of Zn from different sources is an important factor affecting Zn absorption. In ruminants, among inorganic sources, sulfate form is more bio-available than oxides and carbonates, whereas organic Zn (Zn-Methionine) appeared to be having equal or greater bioavailability as compared to sulfate form (Wedekind *et al.*, 1992). The absorption of Zn from inorganic and chelated sources was almost 40%, but chelated form of Zn was retained better than the inorganic form of Zn (Lardy *et al.*, 1992). Even though the bioavailability of organic Zn is higher than that of inorganic Zn, due to its higher cost, use of organic Zn in animal diets is limited (Zhao *et al.* 2014). However, the higher levels of Zn excreted from the supplemented animals have raised concerns pertaining to environmental pollution (Buff *et al.*, 2005; Feng *et al.*, 2009). So this problem opens a window for better bio-available Zn sources and/or to reduce the supplemental dose of Zn to the animals.

Among all the probable approaches, use of nanotechnology to produce nano sized Zn called as nano Zn (NZn) is a potential alternative to both organic and inorganic sources as lesser dose is needed. The use of NZn is having better results as compared to conventional Zn sources and also micro Zn and is less toxic (Wang *et al.*, 2006; Sahoo *et al.*, 2014b). Workers have proved the growth promoting effect of ZnO nanoparticles (NPs) (Lina *et al.*, 2009 a,b; Mishra *et al.*, 2014). Zinc oxide is among the most commonly utilized group of nanomaterials, and has wide-ranging applications from paint formulation and ceramic manufacture, protective sunscreen in skin and hair care products (Fan and Lu, 2005). Zinc oxide NPs are being used in the food industry as additives and during packaging due to their antimicrobial properties (Gerloff *et al.*, 2009). Studies have already proven the dose dependant effect of ZnO NPs on growth performance in livestock and poultry (Hualing, 2006; Hongfu, 2008; Yang and Sun, 2006; Lina *et al.*, 2009a, b; Mishra

et al., 2014; Sahoo *et al.*, 2014a,b). ZnO NPs are also reported as immune-modulatory agent. For instance, supplementation of ZnO NPs reduced the diarrhoea incidence rate in piglets (Hongfu, 2008). Hongfu (2008) attempted to explore the feasibility of lesser doses of ZnO NPs as a substitute for higher doses of conventional ZnO sources. The added advantage is ZnO NPs can efficiently be synthesized by using any of physical, chemical or biological methods (Swain *et al.*, 2015) which are cheap and easy.

Among metal NPs annually produced (by volume), ZnO NP is the third highest globally produced nano metal after nano SiO_2 and nano TiO_2 (Piccinno *et al.*, 2012). Bondarenko *et al.* (2013) reported the worldwide annual production of ZnO NPs is between 550 (Piccinno *et al.*, 2012) and 33,400 tons (Research and Markets, 2012). The sudden rise in the demand in ZnO NPs is mostly attributed to better antibacterial properties than the conventional ZnO (Padmavathy and Vijayaraghavan, 2008).

2.3. Nano minerals

Nano minerals are stable under high temperature and pressure (Stoimenov *et al.*, 2002). By virtue of its small size, it can be taken up easily by the gastrointestinal tract, so are more effective than the larger size ZnO even at lower doses (Feng *et al.*, 2009). In the animal body, it results in better interaction with other organic and inorganic substances due to its more surface area (Zaboli *et al.*, 2013). ZnO NPs also have minimal adverse effect on human cells (Reddy *et al.*, 2007) which is an added advantage. Nano minerals have the capability to cross the small intestine and further distribute into the blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach (Hillyer and Albrecht, 2001). The intrinsic properties of a metal NP are mainly determined by its size, shape, composition, crystalline structure and morphology (Dickson and Lyon, 2000).The functional activities such as chemical, catalytic or biological effects of NPs are heavily influenced by the particle size of the nanometals (Lewis and Klibanov, 2005; Rosi and Mirkin, 2005). The ZnO NPs were mainly found to be retained in the liver after 14 day sub-acute exposure (Sharma *et al.*, 2012) and oral administration through gastro intestinal tract (Chen *et al.*, 2006; Cui *et al.*, 2011).

This multifocal use of NZn has created a huge demand, which leads to develop some effective and sensitive methods to synthesize nanoparticles with desired size and properties. The prime aim of synthesizing these nanominerals is to have a better control over particle size, morphology, purity, quantity, and quality (Swain *et al.*, 2015; Hahn, 1997). Nano Zn can be synthesized by physical, chemical, and biological methods. In

general, biological methods are safe to use and can be efficiently exploited without further experiment on the residual effect and are eco-friendly too (Swain *et al.*, 2015). For animal feeding, NZn can be produced by any of the above methods but thorough study including toxicological effect is advocated before using these particles in the ration of livestock and poultry on a routine basis.

2.3.1. Synthesis of nano minerals

2.3.1.1. Physical methods

Different researchers have mentioned a wide range of the physical methods for preparation of nano particles for instance, by evaporation–condensation, which might be carried out using a tube furnace at atmospheric pressure (Ingale and Chaudhari, 2013), evaporation-condensation and laser ablation (Iravani *et al.*, 2014) electric arc-discharge, laser ablation, chemical vapour deposition, gas phase synthesis and ball milling-annealing methods (Rajendran *et al.*, 2013c), physical vapour deposition (Cardenas *et al.*, 2007) etc. As a mechanical method to prepare the nano minerals, ball mill is used to grind materials into extremely fine powder of nano dimension for use in livestock industry (Koch, 1997; Siegel, 1991; Bakker *et al.*, 1995). Gas phase synthesis of nano materials involves atmospheric or low pressure evaporation of either powders or the pre formed semiconductor or the co-evaporation of the two elemental components, for illustration Zn and sulphur, but the limitation of this method is that it usually results in deposits of particles with larger size distributions, in some cases ranging from 10 to 200 nm. The absence of solvent contamination in the prepared thin films and the uniformity of NPs distribution are the advantages of physical synthesis methods in comparison with chemical processes (Iravani *et al.*, 2014).

2.3.1.2. Chemical methods

The chemical methods are having the upper hand in contrast to physical method in stabilization of nanoparticles from agglomeration, extraction of nanoparticles from solvent, surface modification and application, processing control and mass production (Rajendran *et al.*, 2013c). In this method, uniform nano sized particles can be produced (Lane *et al.*, 2002). Effective and controlled bulk production can be achieved by using the chemical method. Reduction of mineral salts by chemical methods is the most convenient ways to reduce the size of the particles (Rajendran *et al.*, 2013c). But in chemical method there is always a chance of toxicity due to use of hazardous chemical during synthesis. So

attempts are made to produce nanoparticles by using eco-friendly chemicals, plant and fungal origin as reducing agents (Oremland *et al.*, 2004) called as green chemistry method of nano particle synthesis. Eco-friendly as well as nontoxic chemicals such as glucose, starch, amino-acids and plant extracts are used to synthesis metal nanoparticles. The particle size of the minerals depends largely on the reduction capability of the reagents for an instance; a strong reduction reaction promotes a fast reaction rate and produces smaller nanoparticles (Rajendran *et al.*, 2013c). In the chemical method of nano particle synthesis, stabilizing agents and surfactants like cyclodextrin, poly vinyl pyrrolidone (PVP), poly vinyl alcohol (PVA), citrate or quaternary ammonium salts *etc.* are needed to prevent the agglomeration of the metal particles (Szczepanowicz *et al.*,2010; Rajendran *et al.*, 2013c). The stabilizers controls the growth of particles, check nanoparticle aggregation, control of growth rate, control of particle size and also allow particle solubility in various solvents (Yang *et al.,* 2004). Ligands like phosphines, thiols, amines, carbon monoxide etc. can also be used as stabilizers in nano particle production which occurs through coordination between the ligand moiety and metal nanoparticles (Rajendran, *et al.*, 2013c). Zhou (2005) reported that solvent molecules can stabilize the nanoparticles more efficiently.

2.3.1.3. Biological methods

The conventional methods are usually hazardous and energy consuming. This leads to focus on "Green synthesis" of nanoparticles which seems to be an easy, efficient and eco-friendly approach (Marye and Inbathamizh, 2012) and also minimize the toxicity (Narayanan and Sakthivel, 2010). Biosynthetic methods using either biological microorganisms or plant extracts have emerged as a simple alternative to physical and chemical methods which are hazardous and is proved to be an effective source of nano mineral synthesis (Sri Sindhura *et al.*, 2014). Over the past several years, plants, algae, fungi, bacteria, and viruses have been used for production of low cost, energy-efficient, and non-toxic metallic nanoparticles (Oremland *et al.*, 2004; Kaushik *et al.*, 2010, Sri Sindhura *et al.*, 2014). Various metal nanoparticles like silver, gold, cadmium, selenium, palladium, barium titanate and titanium has been successfully synthesized by biological methods (Sharma *et al.* 2007, Narayanan and Sakthivel 2010; Philip 2010) by using different plant materials but the biosynthesis of nano ZnO is still in infancy. Sri Sindhura *et al.*, (2014) prepared nano Zn by using leaves of *Parthenium hysterophorous*. Metal nanoparticles have been successfully synthesized from *Avena sativa, Azadirachta indica, Aloe vera, Alfalfa*, lemon grass, *Sesbania drummondii*, papaya fruit extract, latex of

Jatropha cutcas (Shankar *et al.*, 2004). In spite of having so many advantages, maintaining the culture and its condition, culture media, time period in formation of the nanoparticles and difficulty in product recovery are among the main drawbacks of biological method of nano particle synthesis using microorganisms (Narayanan and Sakthivel, 2010).

2.4. Effect of sources of Zn supplementation on animal performances

2.4.1. Non-ruminants

Mohanna and Nys (1999) studied the effect of dietary Zn content and sources in broiler chicks and reported that feed intake increased with the dietary Zn content till supplementation upto 25 mg Zn/kg (45 mg/kg total dietary Zn). The changes in feed intake and feed conversion were not affected by the source of Zn (Zn sulphate ($ZnSO_4$, $7H_2O$) or Zn methionine.

Hassan (2003) investigated some productive and physiological responses of excess dietary Zn oxide (0, 5000, 10000 and 20000 mg/kg) on laying hens and reported that adding 5000, 10000 and 20000 mg/kg zinc oxide decreased body weight and the feed consumption in laying hens at such higher levels.

Sahin *et al.* (2006) supplemented ten day old Japanese quail with dietary Zn piclonate (@ 0, 30, 60 mg of Zn/ kg of diet) and vit-E (@ 0, 125, 250 mg/kg) until 6^{th} week under different environmental temperature. They suggested no significant difference with respect to feed intake and FCR of the birds in thermo neutral as well as heat stressed condition although the group supplied with highest concentration of both Zn and vit-E showed the best FCR.

Supplementation of Zn at 10, 20, 40, 80, 160 and 320 mg/kg in form of $ZnSO_4.7H_2O$ to corn-soybean basal diet (BD) containing 29 mg/kg Zn had no effect on feed intake at 4 weeks of age in broiler chicks (Sunder *et al.*, 2008).

Rossi *et al.* (2007) reported that dietary supplementation of 0, 15, 30, 45 or 60 mg/kg of organic Zn (Bioplex Zn) to a BD containing 60 mg/kg Zn from an inorganic source did not influence the average daily feed intake in broilers. Whereas, Liu *et al.* (2011) compared inorganic ($ZnSO_4$) and organic Zn sources (Zn-aa, Zn-prot) at different levels (60, 120 or 180 mg Zn/kg) and observed higher average daily feed intake in broilers fed with Zn supplemented diets compared to those fed on BD (no Zn supplementation), with no effect of source and level of Zn supplementation. On contrary, Ao *et al.* (2006) in experiment on broilers compared organic (Zn-prot) and inorganic Zn ($ZnSO_4$) sources at

four supplemental levels of Zn (5, 10, 20 and 40 mg/kg) and reported improvement in feed intake ($P<0.05$) with Zn supplementation and concluded that lower inclusion levels of organic source was sufficient for optimum feed intake as compared with $ZnSO_4$ (inorganic source).

Supplementation of 1500mg/kg Zn sulphate (reagent grade) caused a depression in feed consumption and body weight gain in broiler (Henry *et al.*, 1987; Sandoval *et al.*, 1997) these detrimental effects were not observed while supplementation of ZnO at 1500 mg/kg (Sandoval *et al.*, 1997).

Sahin and Kucuk (2003) reported a hike ($P<0.01$) in feed intake in Zn supplemented quails (30 and 60 mg/kg) compared to un-supplemented group.

Nagalakshmi *et al.* (2012) reported a comparable feed intake on rats by supplementing $ZnCO_3$ (0, 12, 24, 36 and 48 mg/kg) at graded doses to the BD up to 4 weeks and whereas from 4^{th} ($P<0.01$) and 5^{th} ($P<0.05$) week the feed intake was higher in rats fed 12 or 24 mg/kg Zn compared to BD fed rats and this effect continued till the end of 16^{th} week. But, feed intake in rats fed on 48 mg/kg Zn diet was comparable to that of BD, but later it increased by the end of 16^{th} week which became similar to the intake of other Zn supplemented rats (12-36 mg/kg).

Sridhar *et al.* (2014) observed in broiler birds, Zn supplementation at 40 mg/kg $ZnSO_4$ or 30, 20 or 10 mg/kg as Zn-gly did not affect feed intake during starter and finisher phase. The FCR did not differ among the dietary treatments during the starter phase, but during the finisher phase the FCR improved with 30 mg/kg Zn from Zn-gly compared to other dietary treatments which was also reflected in total FCR.

Nagalakshmi *et al.* (2015b) observed that reducing the dietary Zn supplementation up to 6 mg/kg using organic source (Zn-Nic) did not affect feed intake of the rats, which was comparable with rats fed on diets supplemented with 12 mg/kg inorganic Zn salt ($ZnCO_3$).

Nagalakshmi *et al.* (2016) supplemented Zn from inorganic ($ZnCO_3$) and organic (Zn-Met) Zn in combinations (100:0, 50:50, 25:75 or 0:100) to the BD. An increase in feed intake with replacement of 50 and 75% of $ZnCO_3$ with Zn-Met during the 4^{th} ($P<0.05$), 5^{th} ($P<0.01$) and 6^{th} ($P<0.05$) weeks, but the intake with 100% Zn-Met was comparable to 100% $ZnCO_3$.

Many workers have demonstrated the growth promoting effects of NZn in various animals (Hualing, 2006; Hongfu, 2008; Yang and Sun, 2006; Lina *et al.*, 2009a; Mishra *et al.*, 2014; Sahoo *et al.*, 2016). Nano ZnO (ZnO NP) has been reported to enhance growth

performance, improve the feed utility and provides good economical profit in weanling piglets (Yang and Sun, 2006) and poultry (Mishra *et al.*, 2014; Sahoo *et al.*, 2016a). Hualing (2006) reported the maximum growth at the dose of 500 mg/kg of ZnO NP. Encouraging results in average daily gain (ADG) was obtained by feeding BDs supplemented with 200,400,600 mg/kg ZnO NP or 3000 mg/kg ZnO (Hongfu, 2008). ZnO NP has been found to promote the production performance and dressing per cent of broilers at the level of 40 mg/kg in the diet (Lina *et al.*, 2009a). Mishra *et al.* (2014) observed improved growth rate in layer chicks than inorganic Zn supplemented groups due to supplementation of NZn, particularly at 1/500th level of basal dose. In ruminants, no reports are found pertaining to higher doses of Zn supplementation acting as growth promoters, however, doses up to 3000 mg/ kg feed have been successfully proved to be growth promoting in pigs (Hongfu, 2008).

Sahoo *et al.* (2016) fed broiler chicken with different sources of Zn (inorganic- $ZnSO_4$; organic- Zn-Met and NZn- ZnO NP) for a period of 280 days. Birds supplemented with 0.3, 0.06 and 0.03 mg/kg of NZn had better FCR than $ZnSO_4$ supplemented groups (15mg/kg). Even NZn at 0.06 mg/kg had a better growth rate as compared to the $ZnSO_4$ and birds without Zn supplementation.

Khah *et al.* (2015) reported that dietary ZnO NP at (30, 60, 90 and 120 mg ZnO NP/kg of diet) increased ($P>0.05$) live body weight, dressing carcass weight, breast and thigh weight in broiler birds than ($P>0.05$) control. The highest live body weight, carcass, breast and thigh were observed in birds fed diet consisting of 60 or 90 mg ZnO NP.

Mohammadi *et al.* (2015) supplemented different Zn sources in Broiler birds, namely, BD (without Zn) and BD supplemented with Zn-sulphate, Zn-Methionine, Zn-nano-sulphate, Zn-nano-methionine and Zn-nano max (synthesised based on nano chelating technology) at 80 mg/kg of diet. A decrease in weight gain and feed intake in dietary Zn-nano-sulphate supplemented birds during 1–42 d of age was observed, but feed conversion ratio was not affected by these Zn sources. Carcass yield (%) of birds in both Zn nano-sulphate and control groups were significantly reduced at 42 d of age whereas, abdominal fat (%) increased in those two groups.

Zhao *et al.* (2014) supplemented ZnO to broilers in four groups namely 60 mg/kg ZnO (control) or 20, 60, or 100 mg/kg ZnO NP, respectively along with BD, and observed greater weight gains and better feed conversion ratios in ZnO NP groups as compared to the control after 14 days. But after 28 days, the body weight of birds in the 100 ZnO NP group was reduced.

Ahmadi *et al.* (2013) observed that in broiler ZnO NP significantly affected body weight gain ($P<0.05$) and feed intake among treatments from 1 to 21 d. FCR was lesser than the control in all the ZnO NP groups which was 1.30, 1.14, 1.17, 1.16, 1.25, respectively in control, 30, 60, 90 and 120 mg/ kg feed fed birds. The feed intake was significantly decreased at levels of 60 and 90 mg/kg of ZnO NP.

Lina *et al.* (2009a) studied the effects of ZnO NP on the production performance and dressing performance of broiler. They supplemented ZnO NP at doses of 0 (control), 40, 80, 120 mg/kg for 6 weeks to broiler birds. The effect of NZn was varied as per the period of feeding, i.e. for first 21 d, growth was significant. Production performances and dressing per cent of the broiler with ZnO NP at the level of 40 mg/kg was higher than the control group at 42 d.

Hongfu (2008) studied the effect of NZn on weanling piglets by feeding them in five treatment groups namely, BD, BDs supplemented with 200, 400, 600 mg/kg ZnO NP and 3000 mg/kg ZnO, respectively for 24 d. They reported a significant increase in ADG in the all NZn supplemented groups as compared to the BD. The BD supplemented with 400 mg/kg ZnO NP had the best impact on diarrhoea rate depression, ADG, average daily feed intake.

Hualing (2006) supplemented weanling pigs with ZnO at 3000 mg/kg and ZnO NP at 250 mg/kg and 500 mg/kg, respectively and reported that ZnO NP at 500 mg/kg enhanced growth performances, improve the feed utility and resulted in good economical profit in weanling piglets.

Yang and Sun (2006) divided weaned piglets into five groups and fed them with different levels of Zn in different forms. The groups were fed with ZnO at 100, 3000 mg/kg ZnO and ZnO NP at 250, 375 and 500 mg/kg ZnO NP respectively, along with BD and observed a significantly higher ADG in the piglets in ZnO NP as compared to the ZnO supplemented animals.

2.4.2. Ruminants

Henry *et al.* (1997) fed sheep with a BD (41, 30 mg Zn/kg DM) supplemented with 0, 500, 1000, 1500, 2000, or 2500 mg/kg Zn in phase I and 700, 1400, or 2100 mg/kg Zn as reagent grade $ZnSO_4.7H_2O$ in phase II reported no effect on feed intake among treatment groups in both the phases. Similarly, Spears (1989) did not notice any effect on feed intake in lambs fed a control diet (2.8 mg Zn/kg DM) supplemented with 5 mg Zn/kg DM as ZnO or Zn-Met. Further, in another experiment on heifers by same author found

that feed intake of heifers throughout 126 days of experimental period was similar, among BD (23.8 mg/kg) and BD supplemented with 25 mg/kg Zn in the form of ZnO or Zn-Met.

Further, several researchers reported that supplementation of Zn as Zn-Met had no effect on feed intake, in dairy cows (Hatfield *et al.*, 1995) or goats (Puchala *et al*, 1999). Similarly, Engle *et al.* (1997b) reported that beef heifers fed on diets (17 mg/kg Zn) supplemented with or without Zn @ 23 mg/kg from $ZnSO_4$ had no effect on feed intake among the treatment groups. They also observed no change in feed intake among calves fed on BD (17 mg/kg Zn) supplemented with 23 mg/kg Zn through $ZnSO_4$, Zn-Met or Zn-lysine. Further, Haryana calves fed on BD (35 mg/kg Zn) supplemented with 20, 40 and 60 mg/kg of Zn as $ZnSO_4$ had no effect on DMI due to treatments (Bedi and Sawhney, 1979).

In a 112 days of feeding trial on beef steers, addition of Zn @ 20, 100 and 200 mg/kg Zn as $ZnSO_4$ to a BD containing 70 mg Zn/kg DM resulted in linear decrease ($P<0.10$) in daily DMI with increasing Zn concentration in the diet (Malcolm-callis *et al.*, 2000). On contrary, Spears *et al.* (1991) reported that steers fed on BD (26.4 mg/kg Zn) supplemented with Zn-Met or ZnO @ 25 mg/kg had higher DMI than those fed on control diet. Further, Galyean *et al.* (1995) found higher DMI in steers fed supplemental Zn than those fed BD with no Zn supplementation.

Wright and Spears (2004) observed similar feed intake in Holstein calves that received BD with no supplemental Zn (control), BD plus 20 mg of Zn/kg of DM as $ZnSO_4$ or Zn proteinate (ZnProt), or BD plus 20 mg of Zn/kg of DM with 50% of the Zn supplied from each source for 98 day (as Phase-1) and calves continued to receive the same Zn source with half of the calves in each treatment group received 500 mg of Zn/kg of DM ($ZnSO_4$, ZnProt, Zn-Met) for 14 day.

Jadhav *et al.* (2008) fed Murrah buffalo calves a BD (35 mg Zn/kg DM) supplemented with 0, 35, or 70 mg/kg Zn from $ZnSO_4$ and reported DMI to be comparable among the three groups. Similarly Mandal *et al.* (2007) observed no effect on DMI in crossbred calves supplemented with 35 mg/kg Zn from either $ZnSO_4$ or Zn propionate compared to those fed on BD (32.5 mg Zn/kg DM). Spears and Kegley (2002) investigated in growing steers by supplementing 25 mg/kg Zn from ZnO or Zn prot A (10% Zn) and Zn prot B (15% Zn) to diets with 33 mg/kg Zn for period of 84 days and reported no effect on feed intake with Zn supplementation from either source throughout the growing phase.

Nagalakshmi and Himabindu (2013) reported in lambs that Zn supplementation at either dose (15, 30 or 45mg/kg) of Zn supplementation with BD,or from any source (Zn sulphate and Zn proteinate at above doses) did not affect the daily dry matter (DM) intake and its digestibility. But, Galyean (1996) reported higher DM intake (DMI) in Zn supplemented steers.

Zaboli *et al.* (2013) compared the effects of ZnO and ZnO NP, on growth and blood mineral profile of Markhoz goat kids. Zn was administered at zero, 20 and 40 mg/kg in ZnO, 20 and 40 mg/kg as ZnO NP along with BD (22.12 mg/kg) for 70 days. They observed a non-significant difference in feed intake and ADG among the treatment groups irrespective of the sources. Zn supplementation did not affect the blood mineral levels in kids except for plasma Zn concentration on day 35 ($P<0.05$), and it was concluded that, ZnO and ZnO NP at applied concentrations did not affect growth performance in Markhoz goat kids.

2.5. Effect of Zn on nutrient digestibility, balances and tissue distributions

In livestock and poultry, there is very scanty published literature on the effect of Zn supplementation on the nutrient utilization and those are reviewed here.

Mostly minerals are less absorbed the animal system, thus making it low bio-available to the animals and birds. Mostly, absorption is coined with availability because a trace mineral must be certainly absorbed before it could be utilized. But it is not always true that absorbed minerals are retained in the body making its bioavailability low. During digestion, the mineral ions from inorganic sources are released and may re-combine with other digested components in the intestine forming insoluble complexes and thereby excreted, reducing their absorption. In addition ionized minerals may interact with other minerals and affect their absorption and availability.

2.5.1. Non-ruminants

Heugten *et al.* (2003) reported that adding Zn @ 80 mg/kg to BD (104 mg/kg) either from organic (Zn-lys or Zn-Met) or inorganic ($ZnSO_4$) source did not affect the Zn concentration in the liver, pancreas and spleen in broilers. But in weaning piglets, Revy *et al.* (2002) observed that Zn concentrations in plasma, bone, liver and empty body increased ($P<0.01$) linearly with addition of Zn to BD (28mg/kg) @ 10, 20 or 30 mg/kg either from organic source (Zn-Met complex) or inorganic source ($ZnSO_4$). Ao *et al.* (2006) reported higher Zn deposition in tibia and liver with Zn supplementation and

deposition was more with organic (Zn-prot) than inorganic Zn ($ZnSO_4$) sources in broiler receiving four supplemental levels of Zn (5, 10, 20 and 40 mg/kg).

Ma *et al.* (2011) fed different diets by supplementing Zn @ 30, 60, 90 or 120 mg Zn/kg as Zn-gly chelate and another with 120 mg Zn/ kg from $ZnSO_4$ to BD (29.3 mg/kg) and observed increase ($P<0.05$) in serum Zn concentration by 17.55 and 10.77% in 21 day and 42-day broilers with 90 mg/kg Zn-gly compared to control (no Zn supplementation) and higher levels of dietary Zn addition (120 mg/kg Zn-gly or $ZnSO_4$) increased ($P<0.05$) faecal Zn concentration at 21 and 42 days. However, with different levels of Zn supplementation, either from $ZnSO_4$ or Zn-gly had no effect on the contents of Cu, Fe and Mn in serum.

Wang *et al.* (2010a) reported higher ($P<0.01$) Zn concentration in serum and longissimus dorsi muscle with supplementation of 100 mg/kg dietary Zn as Zn-gly chelate or 3000 mg/kg Zn as ZnO compared to BD (no Zn supplementation) and 50 mg Zn/kg as Zn-gly.

Nagalakshmi *et al.* (2015a) in Sprague Dawley female rats, observed higher Zn concentration in serum and liver of rats supplemented with 9 and 12 mg/kg of Zn- nic than those supplemented with Zn CO_3. But Zn deposition in pancreas, muscle and kidney was not affected with reducing Zn supplementation by 50% (6 mg/kg) Zn-nic compared to 12 mg/kg Zn supplementation as $ZnCO_3$.

Sridhar *et al.* (2015) observed similar effects on utilization of DM, OM, CP, CF, EE and NFE in broiler chicks receiving diets with different sources and levels of Zn (40 mg/kg $ZnSO_4$); 30, 20, 10 mg/kg Zn-gly). Replacement of 40 mg/kg Zn from $ZnSO_4$ with 30 mg/kg Zn as Zn-gly increased ($P<0.05$) Zn retention in kidney and pancreas without any adverse effect on the retention of other minerals. The Zn concentration in liver and tibia of birds was comparable with lower Zn supplementation (10-30 mg/kg) from Zn-gly than 40 mg/kg Zn from $ZnSO_4$.

Kwiecień *et al.* (2017) divided the broiler chicken five experimental groups and fed respectively, as without Zn, 100 mg ZnO, and 100, 50, and 25 mg Zn-Gly. An increase ($P < 0.05$) in the concentration of Cu and Zn was recorded in chicken livers after Zn-gly supplementation, irrespective of the level of Zn supplementation with the diet. Thus they concluded that addition of Zn-gly increased Zn and Cu storage in the liver; it also reduced the amount of Zn excreted with faeces and advocated Zn-gly to be an alternative to ZnO in providing a good source of Zn to enrich feed mixtures for broilers.

In broilers, Sahoo *et al.* (2014b) reported a higher Zn concentration in tibia, liver and serum of birds supplemented with 0.06 mg/kg NZn than birds supplemented with either no Zn supplementation, 15 mg/kg of $ZnSO_4$, 15 or 7.5 mg/kg organic Zn, 0.3 or 0.03 mg/kg NZn. But the muscle Zn content was reported highest in the birds supplemented with 15 mg/kg organic Zn which varied non-significantly with the NZn supplemented birds both at 0.06 or 0.3 mg/kg level.

Mohammadi *et al.*, (2015) fed broiler birds either with BD (without Zn) or BD supplemented with Zn-sulphate, Zn-Methionine, Zn-nano-sulphate, Zn-nano-methionine and Zn-nano max (synthesised based on nano chelating technology) at 80 mg/kg of diet. It was observed that tibia Zn concentration was higher in chicken fed on the diet supplemented with sources of NZn compared to the Zn-sulphate and without Zn supplemented birds ($P<0.001$). Thus they advocated that, NZn methionine and NZn max has positive effects on performance of broiler birds whereas, NZn sulphate affects adversely.

2.5.2. Ruminants

Zn is an important trace element, not only for host but also for microflora prevailing in rumen, thus this mineral influences the nutrient utilization by changing the rumen fermentation (Cecava *et al.*, 1993; Mandal *et al.*, 2007).

Kathirvelan and Balakrishnan (2008) reported that Zn supplementation reduces the in vitro dry matter (IVDMD) and neutral detergent fibre degradability. Zinc at 10 mg/kg depressed IVDMD by 12.65% and NDF digestibility by 13.82% at 48 h of incubation. They concluded that DM, and fibre degradability is inversely related to the Zn concentration in the feeds.

Arelovich *et al.* (2000) observed a drop in IVDMD by 2.01% in 10 mg/kg Zn supplemented group than the un-supplemented control group after 24 h or incubation. They hypothesized that decreased IVDMD and NDF digestibility might be due to selective inhibition of the growth and metabolic pathways of microbes by supplemental Zn. Matrinez and Church (1970) reported a drop in DM degradability. There was a fall in *in vitro* cellulose degradation by supplementing Zn at 20-30 mg/kg. Camberlain and Borrough (1962) reported that the DM degradability is inversely related to the Zn concentration in the feeds. However, Ivan and Grieve (1975) observed that Zn supplementation at 500 mg/kg did not affect the NDF digestibility in Holstein calves and

Daghash and Mousa (1999) reported that supplementation of Zn at 4.16, 12.98 or 32.28 mg/kg had no effect on the digestibility of nutrients in lambs.

Kegley and spears (1994) observed in lambs, fed on orchard grass hay (21.4 mg Zn/kg) had improved dry matter (DM) and neutral detergent fibre (NDF) digestibility, but decreased DM digestibility when fed tall fescue hay (14.7 mg Zn/kg) with 30 mg/d Zn supplementation) without affecting the urinary nitrogen excretion. But, Somers and Underwood (1969) reported that lambs fed on Zn deficient diet (2.4 mg/kg) excreted more nitrogen in urine, which indicated that Zn deficiency impaired the utilization of absorbed nitrogen thus affecting protein metabolism.

Pal *et al.* (2010) evaluated the effect of feeding organic Zn (Zn-Met + Cu Methionine) and inorganic Zn ($ZnSO_4$+ $CuSO_4$) on nutrient utilization and bioavailability in ewes. They reported that average daily feed intake and body weight gain did not get affected by the source of Zn, but the dry matter intake was lower in Zn-Met fed animals resulting in better feed: gain ratio. Supplementation of Zn from either source improved the Zn and Cu /Zn SOD status in the plasma. The gut absorption and liver Zn concentration was better in the animals supplemented with organic salts. They also reported less faecal Zn excretion in the organic sources indicative of its better bioavailability. Thus they concluded from better liver, plasma Zn concentration and from lesser faecal excretion, Zn-Met is better bio-available than $ZnSO_4$.

Supplementation of 0, 35 and 70 mg/kg Zn as $ZnSO_4$ to BD (35 mg Zn/kg DM) had no effect on DM, OM, CP, EE, NDF, ADF, cellulose and hemicellulose digestibilities in Murrah buffalo calves and the intakes of OM, CP, DCP, TDN were comparable (Jadhav *et al.*, 2008). Similarly, addition of 35 mg/kg Zn as $ZnSO_4$ or Zn-prop to a BD (32.55 mg Zn/kg DM) did not affect the intake and digestibilities of DM, CP, EE, NDF, ADF and balances of nitrogen, Ca, P in crossbred calves (Mandal *et al.*, 2007). Daghash and Mousa (2002) in buffalos, reported that supplementation of 50 and 100 mg/kg Zn through $ZnSO_4.7H_2O$ improved ($P<0.01$) the digestibilities of OM, CP and NFE in Zn supplemented groups while CF digestibility increased only with 50 mg/kg Zn supplementation in comparison to with those fed control diet.

In lambs, no significant difference was observed on intakes of DM, OM, CP, DCP and TDN between Zn supplemented (20 mg Zn/kg DM as $ZnSO_4$ or Zn-Met) and control (no Zn supplement) groups. But, digestibility of DM, OM, CP, EE, NDF and hemicellulose were comparable ($p>0.05$). However, digestibility of cellulose, ADF and Ca balance was significantly higher ($p<0.05$) in Zn-Met supplemented lambs. The retention of

Zn ($P<0.05$) and serum Zn concentration ($P<0.01$) were highest in Zn-Met group, followed by $ZnSO_4$ group and lowest in the control group, suggesting higher bioavailability of Zn from Zn-Met as compared to $ZnSO_4$ (Garg *et al.*, 2008).

Jia *et al.* (2008) found no differences in digestibilities of DM, CP, NDF and ADF with Zn supplementation (0 to 45 mg Zn/kg DM) when fed a BD (22.3 mg Zn/kg DM) supplemented with 0, 15, 30 or 45 mg of Zn/kg DM as reagent grade $ZnSO_4.7H_2O$ to Cashmere goats. Similarly, Zn supplementation had no influence on N balance or retention, but excretion of N in urine was lower with additional 45 mg Zn/kg DM diet than control.

Jia *et al.* (2009) reported that supplementation of 20 mg/kg of Zn as $ZnSO_4$ and Zn-Met to BD (22.3mg Zn/kg DM) had no influence on digestibility of DM, CP, EE and NDF. However, ADF digestibility improved with supplementation of Zn as Zn-Met. Similarly, DM digestibility in beef cattle was not affected with supplementation of 430 mg Zn/kg DM as $ZnCl_2$ (Arelovich *et al.*, 2008). Dietary Zn supplementation in the form of Zn-Met @ 0.1g/day in dairy goats increased digestibilities of DM ($P<0.05$), OM ($P<0.07$) and CP ($P<0.01$) with no affect on CF and NDF digestibilities (salama *et al.*, 2003).

Dietary supplementation of Zn at concentrations of 0, 15, 30 or 45 mg/kg as $ZnSO_4$ or Zn-prot to a BD (29.3 mg/kg Zn) in Nellore ram lambs had no affect on intake and digestibility of organic nutrients, fibre fractions and balance of nitrogen and energy (Nagalakshmi and Himabindu, 2013). They concluded that dose and source of Zn had no effect on weight gains, nutrient intake and efficiency.

Hassan *et al.* (2011) fed adult Barki sheep a BD (25 mg Zn/kg DM) plus 25 mg of Zn/kg DM as $ZnSO_4.7H_20$ in control group and 15 and 25 mg of Zn/kg DM as Zn-Met in other two groups, reported that sheep supplemented 15 mg Zn-Met showed higher apparent digestibility of DM, OM, CP, CF, EE, NFE, NDF, ADF and nutritive values (TDN and DCP) than those fed on basal and 25 mg/kg supplemented diet.

Supplementation of 5 and 10 g of Zn-Met per head per day in lactating Friesian cows fed BD containing 35.35 mg/kg of Zn significantly increased the nutrient digestibility and nutritive value of diets with no effect on feed intake. Further, cows fed 5 g of Zn-Met had the highest intake of TDN and DCP, followed by those fed 10 g of Zn-Met and control group, respectively ($P<0.05$; Gaafar *et al.*, 2011). Similarly, Balabánová *et al.* (2011) carried out an experiment on Holstein dairy cows to compare the effect of feeding inorganic (ZnO) and organic (Zn-Met) forms of Zn supplement at the rate of 139 to 166 mg/kg in three stages of reproductive cycle (14 d before calving, 30 and 60 d after

calving). The results revealed that regardless of stage of reproduction, cows supplemented with organic form of Zn had higher digestibility coefficients for CP, EE, CF, NFE, ash and Zn.

2.6. Effect of Zn supplementation on haematology

Effects exerted by Zn supplementation on the haematology depends both on the source of Zn and dose of its supplementation. Diversified results obtained in different animal models have been summarized in this section.

2.6.1. Non-ruminants

El Hendy *et al.* (2001) evaluated the effects of reduced graded Zn level (38 mg/kg diet, control; 19 mg/kg diet, 1/2 of control and 3.8 mg/kg diet, 1/10 of control) in growing male and female rats for 10 weeks. Hemoglobin (Hb), total erythrocyte count (TEC) and packed cell volume (PCV), serum total protein, globulin, glucose, and high density lipoprotein (HDL) were decreased ($P<0.05$) in a dose-dependent manner. Total leukocyte count (TLC) was increased in a dose-dependent manner as Zn deficiency increased ($P<0.05$)

Donmez *et al.* (2002) reported no effect on erythrocyte count (RBC), hemoglobin (Hb), hematocrit (Ht), total leucocytes and differential leucocyte count (DLC) with supplementation of 0, 125, 500 and 1000 mg Zn per kg of drinking water in broiler chicks. In contrary, Akbari *et al.* (2008) reported that addition of 60 mg Zn/kg BD from ZnO significantly ($P<0.05$) increased WBC and lymphocyte count with no effect on RBC count and Hb in broiler chicken (21 days).

Someya *et al.* (2007) studied the effect of Zn deficiency on white blood cells distribution in male Sprague Dawley rats for 26 days by feeding control food (CON: Zn=53.5mg/kg food) group and the Zn deficient food (ZDF: Zn=1.9mg/kg food). The number of basophils and eosinophils in ZDF group at the 14^{th} day was significantly higher than CON group. The number of total WBC and monocytes did not differ in both groups. The number of neutrophils in ZDF group at the 21^{st} and 26^{th} day was significantly higher than CON, where as the number of lymphocytes in ZDF group at the 21^{st} and 26^{th} day was lower than CON group. Thus they concluded that dietary Zn deficiency increased the number of basophils, eosinophils and neutrophils and decreased the number of lymphocytes, suggesting the change in white blood cell distribution.

Nagalakshmi *et al.* (2015b), reported that WBC, RBC, haemoglobin concentration, PCV, mean corpuscular volume, lymphocyte, monocyte, and granulocyte concentration, serum glucose, total protein levels were comparable among the rats fed inorganic (ZnCO3) and organic (Zn-nic; 6, 9, and 12 mg/kg) sources. Serum cholesterol reduced with organic Zn supplementation at either concentration (6-12 mg/kg). Serum globulin concentration reduced ($p<0.05$) with 6 mg/kg Zn-nic supplementation compared to other dietary treatments.

Kwiecień *et al.* (2017) fed broiler chicken respectively with no supplemental Zn, 100 mg ZnO, and 100, 50, and 25 mg Zn-Gly. There was a hike in the RBC values in Zn-gly at 100 and 50 mg/kg than un-supplemented and ZnO supplemented birds, where as a drop in MCHC in all the Zn-Gly supplemented birds as compared to the ZnO supplemented birds by 4.6, 4.9 and 5.4%, respectively.

2.6.2. Ruminants

El Hendy *et al.* (2001) evaluated the effects of reduced graded Zn level (38 mg/kg diet, control; 19 mg/kg diet, 1/2 of control and 3.8 mg/kg diet, 1/10 of control) in growing male and female rats for 10 weeks. Serum albumin, total lipids, cholesterol, triglycerides and low density lipoprotein (LDL) concentration were increased in a dose-dependent manner as Zn deficiency increased ($P<0.05$). However, Serum urea and creatinine were not affected ($P<0.05$) by Zn deficiency. Zn-deficient rats had lower serum concentrations of Zn, Cu and Fe.

Kegley *et al.* (2001) conducted two experiments, in experiment I on beef calves fed Bermuda grass hay (21 mg Zn/kg DM), supplemented with 360 mg Zn/d either as $ZnSO_4$ or Zn-amino acid complex, whereas in exp II, beef heifers were fed a control diet (38 mg Zn/kg DM) supplemented with similar concentration and sources of Zn and reported no difference in total white blood cell counts among the treatment groups in both the experiments.

Mandal and Das (2010) fed crossbred calves a BD (32.5 mg Zn/kg DM) supplemented with 35 mg/kg of Zn as Zn sulphate or Zn propionate and reported similar Hb concentration and packed cell volume (PCV) among the groups.

Sobhanirad and Naserian (2012) fed Holstein dairy cows on diets supplemented with 5 mg Zn/kg DM from either $ZnSO_4.7H_2O$ or Zn-Met and reported higher number of red blood cells, Hb concentration, packed cell volume, and mean corpuscular hemoglobin concentration in the Zn-Met than control and $ZnSO_4$ supplemented group.

2.7. Effect of Zn supplementation on blood biochemical constituents

Several researchers have reported effects of Zn supplementation at various concentrations and from different sources on various blood biochemical constituents such as glucose, total protein, albumin, globulin, cholesterol, creatinine and urea nitrogen (urea-N) along with activities of various enzymes like alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and super oxide dismutase (SOD) in different species. A brief account of literature available on this aspect has been reviewed and presented here.

2.7.1. Non-ruminants

Revy *et al.* (2002) reported that in weaning pigs, plasma ALP activity increased ($P<0.01$) linearly by addition of Zn to the BD (28 mg/kg) @ 10, 20 or 30 mg/kg either from organic or inorganic source ($ZnSO_4$) but activity of this enzyme was not affected by source. Similarly, Wang *et al.* (2010a) reported that supplementation of 100 mg Zn/kg as Zn-gly and 3000 mg Zn/kg as ZnO improved ($P<0.05$) the activity of ALP in pigs compared to unsupplemented ones and no difference in enzyme activity was observed between Zn-gly (100 mg/kg) and ZnO (3000 mg/kg).

Barman *et al.* (2008) reported that supplementation of 40, 60, 80 and 100 mg/kg of Zn in the form of ZnO had no effect on serum total protein in broiler chicks but glucose and cholesterol levels in the serum were higher in birds supplemented with 60 mg/kg Zn than other groups. Feng *et al.* (2010) in experiment on broilers added Zn @ 30, 60, 90 or 120 mg Zn/kg as Zn-gly and 120 mg Zn/kg from $ZnSO_4$ to the BD (29.3 mg/kg) and observed increased ($P<0.05$) serum total protein and calcium concentration with supplementation of 90 mg Zn/kg as Zn-gly with no effect on serum albumin concentration.

Al-Daraji and Amen (2011) reported that progressive addition of Zn (50, 75 and 100 mg Zn/kg diet) to the BD of broiler breeders resulted in significant ($P<0.05$) improvement in plasma cholesterol, protein, calcium and phosphorous concentration and alkaline phosphatase activity (ALP) compared to those fed only BD (without Zn). In contrary, Yalcinkaya *et al.* (2012) reported that addition of organic Zn (80 mg/kg) to the BD had no effect on serum AST, ALT, ALP and triglyceride levels in broilers.

Zhang *et al.* (2006) carried out an experiment to find out the effect of Zn on broilers at different supplemental levels (40, 80, 120, 160, 200 and 320 mg Zn/kg diet) and reported that dietary Zn significantly ($P<0.05$) elevated serum ALP activity and activity of this enzyme reached a peak with Zn added level being 80 to 120 mg/kg diet.

Idowu *et al.* (2011) observed improvement in serum ALP activity and protein concentration with Zn supplementation @ 140 mg/kg diet in laying hens at 10 weeks of age compared to control group (no Zn supplementation) but this improvement was significantly ($P<0.05$) higher with Zn supplementation from organic source (Zn-prot) compared to inorganic sources (ZnO, $ZnSO_4$, $ZnCl_2$ and $ZnCO_3$) and also observed no effect of Zn on serum glucose level.

Parak and Strakova (2011) observed that 100 mg Zn/kg diet supplementation from organic source (Bioplex Zn) reduced ($P<0.01$) the blood plasma cholesterol concentration at 15^{th} week age in breeding cocks compared to control group (no Zn supplementation) and other group of birds which were supplemented with same level of Zn from inorganic sources (ZnO, $ZnSO_4$), but Zn supplementation (organic or inorganic) at 20^{th} and 25^{th} weeks reduced the cholesterol level compared to control group, whereas blood plasma protein and glucose levels were not affected by Zn supplementation.

Nagalakshmi *et al.* (2015b) observed serum glucose, total protein levels were comparable among the rats fed Zn from $ZnCO_3$ and Zn-nic (6, 9, and 12 mg/kg) in rats. Serum cholesterol reduced with organic Zn supplementation at 6-12 mg/kg, where as serum globulin concentration reduced ($p<0.05$) with 6 mg/kg Zn-nic supplementation compared to other dietary treatments.

Kwiecień *et al.* (2017) fed broiler chicken with no supplemental Zn, 100 mg ZnO, and 100, 50, and 25 mg Zn-gly. No change in glucose, total protein, uric acid, ALP, ALT, AST and LDH was observed due to different doses and sources of Zn. Zn-gly at the level of 50 mg increased the concentration of Cu and Ca in chicken serum as compared to the no supplemental Zn group.

2.7.2. Ruminants

Huerta *et al.* (2002) did not find any change in plasma protein and blood urea-N concentration in beef heifers and steers with Zn supplementation at even 200 mg/kg. Dietary supplementation of 50 and 100 mg/kg of Zn increased serum retinal, total protein, globulin, glucose, albumin, alanine transaferase and aspertate transaminase in buffalo calves with decreased concentration of blood urea-N (Daghash and Mousa, 1999).

Malcolm-Callis *et al.* (2000) reported that supplementation of Zn at 20, 100 and 200 mg/kg DM to a BD containing 70 mg Zn/kg DM had no effect on serum cholesterol concentration and fatty acids profile in beef steers. Similarly, Whitman *et al.* (2007) reported no treatment effect on serum cholesterol concentration in steers supplemented (90

mg Zn/kg DM and 10 mg Cu/kg DM) with different combination of organic and inorganic source of Zn and Cu.

Supplementation of 75 mg/kg of Zn from $ZnSO_4$, Zn-Met or Zn-Prop to a control diet containing 52.5 and 50.5 mg Zn/kg diet during receiving and finishing period in beef heifers, respectively revealed no effect on serum Zn concentration (Nunnery *et al.*, 2007). Similarly, Salyer *et al.* (2004) also reported no differences among Zn sources on serum Zn concentration of newly received heifers supplemented with 75 mg of Zn/kg of dietary DM. Spears and Kegley (2002) observed no difference in serum Zn concentrations between steers supplemented (25 mg Zn/kg DM) with Zn proteinate or ZnO. Similarly, Malcolm-Callis *et al.* (2000) reported no differences in serum Zn concentrations measured on d 28 or 112 among steers supplemented with Zn sulfate, Zn polysaccharide, or Zn amino acid complex supplying 30 mg Zn/kg DM.

Organic Zn supplementation (Zn-Met) had no effect on plasma Zn concentration in lactating goats (Salma Ahmad *et al.* 2003). Contrary to this, Huerta *et al.* (2002) reported that the concentration of Zn in the serum of steers was affected by level of dietary Zn ($P<0.05$) and duration of Zn feeding ($P<0.01$). Steers supplemented with 200 mg Zn/kg diet had higher concentration of Zn in serum than steers fed control diet. They further reported that heifers fed diets with Zn-Met had higher ($P<0.05$) concentration of Zn in serum than heifers fed control or $ZnSO_4$ supplemented diets. Mandal *et al.* (2008) supplemented BD (33 mg Zn/kg) with 35 mg/kg Zn from $ZnSO_4$ or Zn-prop and reported no effect of Zn supplementation on ALP, ALT, AST and SOD activities and the concentrations of serum Zn, vitamins (retinol, ß-carotene and α-tocopherol) and hormones (T_3, thyroxin, insulin and testosterone).

Serum ALP is a Zn metalloenzyme that decreases in Zn deficiency and serum ALP activity is used as an indicator of animal Zn status (Miller *et al.*, 1965). Mandal *et al.* (2008) reported that Zn supplementation had no effect on ALP activity. Spears (1989) observed higher plasma ALP activity on Zn addition (48.8 mg Zn/kg) in heifers than control (23.8 mg Zn/kg diet). However, Hatfield *et al* (2002) did not find any increase in ALP activity with additional Zn (84 vs 65 mg Zn/kg diet) supplementation.

In case of Cashmere goats, Jia *et al.* (2009) fed a BD containing 22.3 mg Zn/kg DM and supplemented with 20 mg/kg Zn from either $ZnSO_4$ or Zn-Met and reported improved ($P<0.05$) plasma ALP activity by Zn supplementation, the activity being higher ($P<0.05$) with $ZnSO_4$ than the Zn-Met supplementation. Also, the plasma Zn concentration increased with Zn supplementation from both sources. Similarly, Garg *et al.* (2008)

reported higher plasma Zn concentrations in growing lambs fed BD (34 mg Zn/kg DM) supplemented with 20 mg Zn/kg DM ($ZnSO_4$ or Zn-Met) than control lambs. In contrast, Greene *et al.* (1988) measured serum Zn on 1, 28, 56, 84 and 112 day in heifers fed no supplemental Zn or Zn (360 mg/d) supplemented from ZnO or Zn-Met to BD containing 81 mg/kg DM and reported no difference among treatments. In earlier studies, supplementing Zn to lamb diets (34 mg Zn/kg DM) (Garg *et al.,* 2008) and crossbred calves diets (32.5 mg Zn/kg DM) (Mandal *et al.*, 2007) with 20 and 35 mg/kg Zn, respectively had no affect on plasma Cu concentration. Furthermore, Zn supplementation had no effect on plasma Fe and Mn contents. Kincaid *et al.* (1997) found no difference in serum Fe of Holstein calves with various levels and sources of supplemental Zn. Garg *et al.,* (2008) did not find any effect on serum Mn levels in lambs supplemented with 20 mg Zn/kg through different sources (Zn-Met or $ZnSO_4$) to the BD containing 34 mg Zn/kg DM. In another study, Cashmere goats fed on similar BD supplemented with 0, 15, 30 or 45 mg Zn/kg DM in the form of $ZnSO_4.7H_2O$ had no influence on plasma Zn concentrations among treatment groups at the beginning. However, plasma Zn concentrations on day 15, 30, 45 and 60 of the experiment were increased by all levels of Zn supplementation and were higher for the treatment groups supplemented with 30 and 45 mg Zn/kg DM compared with 15 mg Zn/kg DM (Jia *et al.*, 2008).

Nagalakshmi *et al.* (2009b) fed Nellore lambs a BD (29.28 mg Zn/kg DM) supplemented with 0, 15, 30 and 45 mg/kg of Zn from either inorganic ($ZnSO_4$) or organic (ZnProt) source and reported gradual increase in serum ALP activity with Zn supplementation in a dose dependent manner. At all the doses of Zn supplementation, the ALP activity was higher in lambs on organic compared to inorganic sources of Zn. Further the serum total protein in lambs was not affected by either dose or source of Zn in diet. The serum albumin concentration was not affected by dietary treatments except those fed 30 mg/kg Zn as inorganic form. The globulin concentration was higher in 30 mg/kg Zn supplemented lambs from $ZnSO_4$ and at 45 mg/kg Zn from either organic or inorganic source, compared to BD fed lambs.

Hassan *et al.* (2011) fed adult Barki sheep with a BD (25 mg Zn/kg DM) plus 25 mg of Zn/kg DM as $ZnSO_4.7H_2O$ in control group and 15 and 25 mg Zn/kg DM as Zn-Met in other two groups and reported that organic Zn (Zn-Met) caused a significant decline ($P < 0.05$) in glucose and urea compared to the inorganic Zn ($ZnSO_4 \cdot 7H_2O$). But inorganic or organic Zn did not cause any effect on the serum total protein, albumin concentration, globulin, creatinine, AST and ALT.

Supplementation of 5 and 10 g Zn-Met per head per day in lactating Friesian cows fed on BD (35.35 mg/kg Zn) observed that cows fed 10 g Zn-Met daily had highest plasma total protein, albumin and globulin concentrations and lowered activities of AST and ALT (Gaafar *et al.*, 2011). Similarly in buffaloes under cooling and non-cooling systems of maintenance, supplementation of Zn @ 5 g/head/d as Zn-Met did not affect the albumin and glucose concentration in plasma, but increased the total protein concentration in animals maintained under cooling system (Khalifa *et al.*, 2011). Supplementing 0.4 g Zn-Met per day per head in lactating goats fed on a control diet (24 mg Zn/kg DM), significantly increased serum Zn concentrations throughout the trial with no effect on levels of P, Cu and Fe in serum (El-Nour *et al.*, 2010).

2.8. Effect of dietary Zn supplementation on hormonal profile of livestock

Zn plays important role in hormone metabolism like production, storage, and secretion of hormones. Insulin, testosterone, thyroid hormones and adrenal corticosteroids are mostly affected by Zn deficiency (McDowell, 1992; McDonald, 2003).

2.8.1. Thyroid hormones

Zinc plays an important role in thyroid metabolism (Baltaci *et al.*, 2004). Zn is thought to play a role in conversion of T_4 to T_3 (Morley *et al.*, 1980) and also in binding of T_3 to nuclear receptors (Surks *et al.*, 1989; Miyamoto *et al.*, 1991). Zn also participates in the formation and action of thyroprotein releasing hormone (TRH; El-Sisy *et al.*, 2008). Pekary *et al.* (1991) has documented that the processing of pre and pro TRH to synthesis TRH is a Zn dependant process which is a done through translational processing enzymes like carboxypeptidase H. Plasma levels of T_3, T_4 and TSH reduced in rats fed with Zn deficient diets (Beresford *et al.*, 1984; Morley *et al.*, 1980; Pekary *et al.*, 1993). Zn deficiency can indirectly affect the thyroid hormone status by decreasing energy intake (Ruz *et al.*, 1999). But (Brandão-Neto *et al.*, 2006) reported that Zn supplementation had no effect on thyroid functions.

2.8.1.1.Triiodo-thyronine (T_3)

The active hormone is triiodo-thyronine (T_3) derived through peripheral deiodination from thyroxine (T_4), the major secretory product of the thyroid gland (Chesters, 1991). The T_3 receptor requires Zn for its biologically active conformation (Pathak *et al.,* 2011). Freake *et al.* (2001) did not observe any interaction between thyroid status and Zn status with respect to growth in rats. Pathak *et al.* (2011) reported a decline ($P>0.05$) in serum T_3 in $ZnSO_4$.$7H_2O$ supplemented (227 mg/L) rats (0.68 ng/mL) than control ones (0.73 ng/mL).

El-Sisy *et al.* (2008) observed a non-significant change in the serum T_3 levels (ng/mL) in Baladi male goats among the groups by feeding either 40 mg/kg Zn-M*et al*ong with the BD containing 24.53 mg/ kg DM Zn or BD alone or both at 3 months (0.78±0.18 in control *vs* 0.86 ±0.10 in Zn-Met group) and 6 months (0.67±0.22 in control *vs* 0.77 ±0.15 in Zn-Met group) of experimental feeding. However, there was a significant raise in T_3 levels as compared to pre-supplementation period in both the groups.

Supplementing 0.4 g Zn-Met per day per head in lactating goats fed on a control diet (24 mg Zn/kg DM), significantly increased serum T_3 concentrations throughout the trial (El-Nour *et al.,* 2010).

2.8.1.2.Thyroxine (T_4)

Pathak *et al.* (2011) observed a non significant rise in serum T_4 in Zn sulphate ($ZnSO_4$.$7H_2O$) supplemented (227 mg/L) rats (61.12 ng/mL) as compared to control rats (56.04 ng/mL) thus reported a non significant changes in the thyroid hormone levels between Zn supplemented and un-supplemented control groups.

El-Sisy *et al.* (2008) observed a non- significant change between the groups in their serum T_4 levels (ng/mL) in Baladi male goats by feeding either 40 mg/kg Zn-M*et al*ong with the BD containing 24.53 mg/ kg DM Zn or BD alone both at 3 months (40.86±1.60 in control *vs* 38.60 ±2.29 in Zn-Met group) and 6 months (40.90±0.35 in control *vs* 40.20 ±2.13 in Zn-Met group) of experimental feeding.

Supplementing 0.4 g Zn-Met per day per head in lactating goats fed on a control diet (24 mg Zn/kg DM), had no effect on serum T_4 level (El-Nour *et al.,* 2010).

2.8.2. Testosterone

Zinc has specific effects on testicular development and function, particularly the secretion of testosterone (Martin *et al.,* 1994). Liu *et al.* (2015) stated that plasma

testosterone concentration was significantly improved due to Zn supplementation ($p<0.05$) and the testosterone concentration was higher ($p<0.05$) in goats fed on the diet supplemented with 40 or 80 mg Zn/kg DM compared with those fed on BD. Xin *et al.* (2007) reported a highly significant effect on the serum testosterone contents in bulls by adding 110 mg Zn/kg DM in a BD of containing 27 mg Zn/kg DM. Kumar *et al.* (2013) also reported that serum testosterone concentration was higher in goat bucks, which were supplemented with 150 mg Zn/kg DM from Zn sulphate than in control goats grazing in pasture land (pasture grass containing 0.048 mg Zn/kg DM) and fed 250 g concentrate mixture (containing 0.094 mg Zn/kg DM) per animal daily.

Liu *et al.*, (2015) observed that plasma testosterone concentration (nmol/l) of control group (9.15) fed with BD without any supplemental Zn (BD containing of 45.9 mg Zn/kg DM basis) was similar ($P>0.05$) with 20 mg Zn/kg DM treatment group (9.31). They also observed a significant difference in plasma testosterone concentrations of goats supplemented with 80 mg Zn/kg than those in the 40 mg Zn/kg DM group.

El-Sisy *et al.* (2008) observed a significant raise in serum testosterone level (ng/mL) of Baladi bucks in the Zn supplemented (40 mg/kg Zn-Met) than control animals fed only with BD (24.53 mg/ kg DM Zn) without Zn supplementation for 3 months (4.00±0.70 *vs* 1.52 ±0.31) as well as 6 months (2.76±0.60 *vs* 1.02 ±0.35) of feeding.

It is been documented that Zn deficiency caused reduction in testosterone secretion and impaired the responsiveness to the Leydig cells to gonadotrophins (Martin *et al.*, (1994). El-Sisy *et al.* (2008) summarised the possible causes of declined testosterone secretion, which may due to lesion in the biochemical systems controlling steroid synthesis (Prasad, 1985) or damage to the smooth endoplasmic reticulum of the Leydig cells (Hesketh, 1982).

In the Zn deficient animals there is a decrease in testosterone secretion which might be attributed to the failure in complete development of the testis and its cells and tissues like Leydig cells (Martin *et al.*, 1994). They hypothesized the reduced testosterone secretion may in Zn deficient animals may be due to loss in the effectiveness of steroid binding and subsequent loss of key protein or enzyme mediating the response to LH, which in turn reduces the responsiveness to LH (Martin *et al.*, 1994)

2.8.3. Insulin like growth factor-1 (IGF-1)

Insulin-like growth factor-I (IGF-1) mediates a diversified of cellular events, like stimulation of amino acid and glucose uptake and regulation of the cell cycle; it associates

with a membrane-associated receptor, which possesses tyrosine kinase activity (De Meyts *et al.* 1994). Zn plays an essential role in the production and secretion of insulin, insulin-like growth factor 1 (IGF1) and growth hormones (Macapinlac *et al.*, 1966; Chen *et al.*, 2000). In the absence of adequate Zn, serum IGF-I concentrations are not maintained even when energy intake is adequate (Roth and Kirchgessner, 1994; MacDonald, 2000), hence Zn is essential to maintain normal plasma IGF-1 in rats. In humans, Zn deficiency decreased circulating IGF-I concentrations independently of total energy intake (Cossack 1991). Decreased hepatic production of IGF-I due to failure to respond to GH explains the growth failure observed in Zn deficiency (MacDonald, 2000). However, maintaining serum IGF-I levels by exogenous administration or by inducing food intake (Browning *et al.* 1998) in Zn-deficient rats does not correct the growth inhibition. Therefore, changes in the GH-IGF axis alone cannot explain the growth inhibition observed in Zn deficiency. Hence, Zn is required for some aspect of growth regulation at the cellular level that is independent of the effects observed on circulating IGF-I and GH (MacDonald, 2000). Zn deficiency in the rat is characterized by decreased food intake, decreased growth, low circulating levels of GH and IGF-I, decreased hepatic production of IGF-I (MacDonald, 2000).

Serum IGF-I was lower in rats fed a Zn-deficient diet for 2 wk compared with Zn-adequate controls (Bolze *et al.* 1987, Cossack 1984, Dorup *et al.* 1991), and the decrease in IGF-I corresponded to a decrease in serum Zn (Dorup *et al.* 1991). Tibial Zn concentration, which is a sensitive measure of Zn status, was positively correlated with serum IGF-I concentration (Cossack 1984). The decreased concentration of IGF-I in the serum of Zn-depleted rats was not corrected by a high protein diet, but the addition of Zn to a low protein diet increased serum IGF-I (Cossack, 1986). Freake *et al.*, 2001 reported a reduction ($P<0.001$) in serum IGF-1 level by reduced feed intake and Zn deficiency in rats.

Jafarpour *et al.* (2015) supplemented 58, 118, or 163 mg Zn/kg diet DM as Zn methionine to sheep and reported a linear increase ($P < 0.01$) in plasma IGF-1 concentration with increase in Zn concentration in feed (79.0 in 1.2 g/day Zn-Met vs. 72.3 in control).

2.9. Effect of Zn supplementation on immune responses

Immune system is a physiological system, which is influenced by the nutritional status of the animal. Micro nutrients like minerals such as Zn, copper, selenium, iron and

several vitamins play a pivotal role in maintaining the immune system. Among micronutrients, Zn is mainly responsible for maintaining healthy immune status of the animal. Zn being an important antioxidant, decreases reactive oxygen species (ROS) production (Fernandez *et al.*, 2003) which in turn improves animal health status and productivity. Irrespective of its source i.e. organic or inorganic (Nunnery *et al.*, 2007), Zn functions as a cofactor of SOD enzyme system (Markesbery *et al.*, 2001) which is responsible for the regulation of both extra cellular and intra cellular Superoxide radicals, reduces the ROS and thus improves the fertility (Andrieu, 2008) in farm animals. Zn also completes with iron and copper for binding with the cell membrane and thus decreases the production of hydroxyl radicals (Zago and Oteiza, 2001).

The immune response *i.e.*, both innate and specific immunity are influenced by Zn. Zn insufficiency impairs the activity of natural killer (NK) cell activity, phagocytotic activity of macrophage and neutrophil and oxidative burst (Allen *et al.*, 1983; Keen and Gershwin, 1990). Thus, Zn is needed to maintain the natural killer cell activity and Zn deficiency results in non specific killing activity and functional loss (Rink and Gabriel, 2000). Further, in addition to its role in innate immunity at early stage of immune response, Zn is also associated with the development of T-cells and is an essential cofactor for more than 300 metalloenzymes. Among the metalloenzymes, Zn is essential for functioning of DNA polymerase, thymidine kinase and DNA dependent RNA polymerase, which are involved in nucleic acid synthesis and also lymphoid-cell proliferation. Zn also influences the activity of the transcriptional regulator family, known as Zn finger DNA binding proteins.

Zn is necessary for the activity of thymic hormone, thymulin (Bach, 1981, 1983), which is secreted from thymus gland and induces differentiation in immature T-cells (Saha *et al.*, 1995). Further, Zn induces release of IL-1, IL-6, tumor necrosis factors and interferon (IFN)-α in polymorphonuclear cells (Salas and Kichner, 1987; Scuderi, 1990; Driessen *et al.*, 1994). In activation phase of immune responses, these cytokines stimulate the growth and differentiation of lymphocytes and effectors of innate and specific immunity to eliminate microbes and other antigens.

Birds fed on diets supplemented with a better available Zn source can potentially induce thymulin activity, and therefore promote immune responses through increased maturation of T-lymphocytes and activation of B-lymphocytes by T-helper cells (Moghaddam and Jahanian, 2009). So, higher bio-available Zn sources can enhance the immune reactions in the supplemented birds and animals.

In human and laboratory animals, it has been established that Zn deficiency reduces immune responses and disease resistance (Chesters, 1997). Zn is an important component of the antioxidant system in the body (Hassan *et al.*, 2011). The role of Zn as an antioxidant in the central nervous system (CNS), particularly the brain is gaining attention in recent times. The antioxidant properties of Zn were first demonstrated *in vitro* (Hassan *et al.*, 2011). Compared to other soft tissues, the human brain contains significant amounts of Zn. Among the essential trace elements, Zn is second only to iron in total concentration in the brain (Mittler *et al.*, 2004).

2.9.1. Non-ruminants

Research reports on human and laboratory animals indicate that severe Zn deficiency reduced the immune response and disease resistance (Chesters, 1997). Zn supplementation may be effective in cattle that are more prone to bovine respiratory disease (BRD) during transportation (Chirase *et al.*, 1991) or in recovery of inherited immunodeficiency disease of cattle known as bovine leukocyte adhesion deficiency disease (BLAD) (Arrayet *et al.*, 2002) or as prophylactic against coccidiosis in kids (Strnadova *et al* ., 2011).

Nagalakshmi *et al.* (2015b) studied the impact of feeding organic (Zn nicotinate and inorganic Zn (Zn carbonate) on rat immunity. Fourty eight rats were assigned to four different levels of Zn (12 mg/kg $ZnCO_3$; Control, Zn nicotinate at 6,9,12 mg/kg) along with the purified BD. Better immune responses were observed in the diets containing 12mg/kg Zn nicotinate than 12 mg/kg $ZnCO_3$ was recorded, however, Zn nicotinate (9mg/kg) was comparable to the control. The increase in immune response was attributed to higher bioavailability of Zn from Zn nicotinate than Zn $ZnCO_3$.

Nagalakshmi *et al.* (2016) investigated the effects of replacement of dietary Zn from inorganic ($ZnCO_3$) source with organic Zn sources (Zn-Met) on Wistar Albino rats. The control diet (AIN-76A) contained 12 mg/kg of Zn from ZnCO3 and in other groups, $ZnCO_3$ was replaced with Zn-Met at the rates of 50, 75 or 100%. It was reported that, the primary ($P<0.01$) and secondary ($P<0.05$) humoral immune responses increased quadratically with replacement of $ZnCO_3$ with Zn-Met. The cell mediated immune (CMI) response at 24 h post immunization was not affected by Zn-Met supplementation, and was comparable among the dietary treatments. On the contrary, at 48 h, CMI response improved linearly ($P<0.01$) by increasing the concentration of Zn-Met in diet.

Sridhar *et al.* (2016) evaluated the effect of organic Zn (Zn glycinate; Zn-gly) supplemented at lower levels (30, 20 and 10 mg/kg) on immune response in comparison to NRC (1994) recommended levels (40 mg/kg) of Zn supplemented from inorganic source (ZnSO4) in broiler chicks. The humoral immune response (antibody response against ND vaccine) improved ($P<0.05$) with 30 mg/kg Zn supplementation from Zn-gly compared to 20 or 10 mg/kg Zn supplementation from the same source but was comparable with control group supplemented with Zn Sulphate. The cutaneous basophilic hypersensitivity to PHAP indicative of CMI was significantly ($P<0.05$) higher at 30 mg/kg Zn supplementation from Zn-gly compared 20 and 10 mg/kg Zn from Zn-gly and control.

By supplementing BD with 400 mg/kg ZnO NP, diarrhoea rate was reduced up to 49.1 % and 21.6 % by providing the BD supplemented with 3000 mg/kg ZnO (Hongfu, 2008). Yang and Sun (2006) also reported a significant drop in the diarrhoea incidences by supplementing graded doses of ZnO NP in the diet. Significant improvement was observed in the health status (low blood cholesterol level and high SGPT) and improved immunity of the birds by supplementing NZn at 0.06 mg/kg to the BD of broilers in comparison to conventional dose of 15 mg/kg of organic and inorganic Zn (Sahoo *et al.*, 2014a,b).

In broilers, Sahoo *et al.* (2014b) observed higher ($P<0.05$) antibody titre levels against Sheep RBC in the treatments 15 mg/kg organic Zn group (8.832± 0.40) and 0.06 mg/kg NZn supplemented birds (8.83± 0.48) as compared to that of treatments no Zn supplemented as well as 15 mg/kg $ZnSO_4$ supplemented birds. The cutaneous basophilic hypersensitivity (CBH) response was higher in NZn supplemented birds as compared to no Zn and 15 mg/kg $ZnSO_4$ supplemented birds.

Mohammadi *et al.*, (2015) fed broiler birds with BD (without Zn) or BD supplemented with Zn-sulphate, Zn-Methionine, Zn-nano-sulphate, Zn-nano-methionine and Zn-nano max (synthesised based on nano chelating technology) at 80 mg/kg of diet and reported that addition of 80 mg Zn/kg diet significantly ($P < 0.05$) increased the weight of bursa fabricius, spleen (%) and antibody titre against NDV compared to birds without Zn supplementation

2.9.2. Ruminants

Droke *et al.* (1993) fed lambs on diet containing adequate (40 mg of supplemental Zn/kg) marginal (5 mg supplemental Zn/kg) and deficient (5.5 mg Zn/kg of diet) in Zn and reported no effect on lymphocyte blastogenesis in response to PHA, concoavalin A (Con

A) and PWM with dietary variations. Similar findings were reported by Droke and Spears (1993), where marginally deficient Zn (8.7 mg/kg) did not affect immune response of lambs. Kincaid *et al.* (1997) observed no significant effect of Zn supplementation on mitogen-induced lymphocyte blastogenesis, interleukin-2 production, lymphocyte cytotoxicity, phagocytic and intracellular killing ability in neutrophils in calves fed with calf starter diets containing 60, 150 or 300 mg/kg of Zn in the form of Zn-Met and Zn-Lys, or 300 mg/kg of Zn in the form of ZnO.

Supplementation of 23 mg/kg Zn as $ZnSO_4$, Zn-Lys, or Zn-Met in corn based diets (17 mg/kg Zn) of cross bred heifers resulted in comparable cell mediated immune response (CMI) against PHA-P in control and various Zn supplemented groups after 12 and 48 h of post injection, while after 24 h post injection, the CMI response was higher in Zn-Met and Zn-Lys compared to control and $ZnSO_4$ supplemented calves (Engle *et al.*, 1997a)

Droke *et al.* (1998) fed lambs a BD (27.6 mg Zn/kg) supplemented with 25 mg Zn/kg as ZnO or Zn-Met. On d 112 of experimental feeding, lambs were administered 100 IU of adrenocorticotrophin (ACTH) as stressor and feed was withdrawn for 48h. Lymphocyte blastogenesis antibody response to porcine red blood cells on d 112 (prior to ACTH injection) and d 114 was unaffected by dietary Zn supplementation. However, blastogenesis in response to pokeweed mitogen (PWM) was greater, whereas the response to PHA was reduced following ACTH administration.

Spears and Kegley (2002) investigated the effect of 25 mg/kg Zn supplementation as ZnO, Zn prot-A (10% Zn), or Zn prot-B (15% Zn) in steers and observed no effect ($P > 0.05$) on humoral immune response after infectious bovine rhinotracheitis (IBR) vaccination and CMI responses with Zn supplementation in steers, measured by an intradermal injection of PHA and *in vitro* response of lymphocytes to mitogen stimulation was comparable with the control (33 mg Zn/kg DM). Huerta *et al.* (2002) fed crossbred heifers (with or without growth implants) diets containing 64 and 84 mg/kg in the first and second experiment, respectively and in experimental groups control diet supplemented with 200 mg/kg of Zn as $ZnSO_4$ or Zn-Met. In first experiment, the concentrations of Ig G was greater in heifers fed $ZnSO_4$ supplemented diet, intermediate for control diet and lowest for Zn-Met supplemented diet. Serum titers against bovine viral diarrhea was lower in heifers fed diet supplemented with $ZnSO_4$ compared to heifers fed either Zn-Met or control diet. In second experiment, serum titer was higher in growth implanted steers fed Zn-Met compared to $ZnSO_4$ supplemented diet.

In another experiment on beef heifers, Nunnery *et al.* (2007) reported that supplementation of 75 mg/kg Zn supplementation from $ZnSO_4$, Zn-Met, or Zn prop to a BD (52.5 mg Zn/kg DM) did not influence the Ig G titers against ovalbumin. Similarly in crossbred calves fed on a BD (32.5 mg Zn/kg DM) supplemented with 35 mg/kg Zn from ZnProp had higher CMI and humoral immune response, while no effect on immune response was observed with $ZnSO_4$ supplementation (Mandal *et al.*, 2007)

Nagalakshmi *et al.* (2009) conducted an experiment in Nellore lambs fed a BD (29.28 mg Zn/kg DM) supplemented with 0, 15, 30 and 45 mg/kg Zn through $ZnSO_4$ or Zn Prot and reported no effect of Zn concentration or source on antibody titers against chicken RBC antigen. The cell mediated immune response assessed as delayed type hypersensitivity (DTH) response against PHA-P and *in vitro* lymphocyte proliferative response against Concanavalin-A was higher at 15 mg/kg Zn supplementation compared to BD fed lambs. The DTH response and antibody titres against *B. abortus* were higher on Zn prot compared to $ZnSO_4$ at 15 mg/kg Zn supplementation. However, supplementation of 45 mg/kg Zn had no positive effect on immune response.

Parashuramulu *et al.* (2015) studied the effect of dietary Zn supplementation ($ZnSO_4$) on immune responses in buffaloes, which were fed with BD (control; 29.72 mg/kg Zn from feed ingredients) alone or supplemented with 80 and 140 mg/kg Zn. Zn supplementation enhanced ($P<0.05$) the antibody titers against *B. abortus* antigen, and among the Zn supplemented calves the titers was higher with 140 mg/kg compared to 80 and 0 mg/kg Zn supplementation, and the CMI response was higher ($P<0.05$) in Zn supplemented calves at 24, 48 and 72h of post-sensitization

NZn has been reported to reduce somatic cell count (SCC) in subclinical mastitis cow and improved mastitis condition with increase in milk production than other larger ZnO sources (Rajendran, 2013; Rajendran *et al.*, 2013a, b). Rajendran (2013) advocated the supplementation of NZn in feed of dairy animal as a possible strategy to suppress of subclinical mastitis thus increase in milk production.

2.10. **Effect of Zn supplementation on rumen fermentation**

Zinc also influences the nutrient utilization in ruminants by altering the rumen fermentation (Cecava*et al.*, 1993). Optimal Zn concentration in rumen fluid is essential for optimizing number of cellulytic bacteria (Matrinez and Church, 1970) and intra cellular urease activity (Kathirvelan and Balakrishnan, 2006) in the rumen microbes. Zn also improves amino acid utilization, as it helps in protein synthesis (Spears, 1989). Eryavuz

and Dehority (2009) observed reduced (P<0.01) cellulose digestion with 50 µg Zn/ml rumen liquor while testing various concentrations (5, 10, 15, 20, 25 and 50µg/ml) of Zn additions. Chandanshive *et al*. (2007) also observed improved IVDMD and in vitro neutral detergent digestibility due to Zn supplementation at 50 and 100 ppm, respectively which decreased (P<0.05) at higher of doses Zn supplementation(150, 200, 250 and 300 ppm).

Ruminal soluble Zn concentrations were reported higher in steers supplemented with Zn proteinate than those supplemented with ZnO (Spears and Kegley, 2002) which suggests that along with Zn, its supplemental source also alters the ruminal fermentation characteristics.

The Zn source, as well as the achieved rumen concentration, the feeding procedures, the type of diet, and the interaction with other dietary components or additives may be some of the factors influencing the effect of Zn in the ruminal fermentation (Arelovich *et al*., 2008).

Zinc supplementation increased (P < .05) concentration of butyrate by13.5%, valerate by 7.1%, where as molar proportion of butyrate was increased by 11.7% and acetate and isovalerate were decreased (P <0.05) by1.9 and 6.5%, respectively in rumen (Froetschel *et al*., 1990). Thus, high dietary concentrations organic Zn (250- 1142mg Zn per kg) have also increased molar proportion of propionate [Froetschel *et al*., 1990; Arelovich *et al*., 2000]. Changes in isovalerate levels in rumen may indicate the extent of degradation of dietary protein as the capacity of ruminal bacteria to synthesize isovalerate from carbohydrates is limited (Bragg *et al*., 1986). Higher ruminal butyrate concentrations can be correlated with greater ruminal protozoal populations (Eadie *et al*., 1970).

Reports suggests that dietary addition of 250 to 400 mg Zn/ kg DM to low-quality forage alters rumen fermentation by retarding ammonia accumulation and increasing molar proportions of propionate (Arelovich *et al*., 2000). Zn, when administered at over 20 times the daily NRC (2000) requirement, could increase the concentration of rumen propionate (Arelovich *et al*., 2000) and decrease the acetate: propionate ratio (Bateman *et al*., 2004).

In ruminants, $ZnSO_4$ can be used as a defaunating agent at a dose levels greater than 1,000 ppm (Durand and Kawashima, 1980), and thus may affect the efficiency of dietary protein utilization as ruminal protozoa are involved in bacterial protein recycling (Veira, 1986). Extremely high concentrations (1142 mg Zn per kg) of ZnSO4 have been shown to affect ruminal protozoa numbers and degradation of feed protein and thus the rumen fermentation in steers (Froetschel *et al*., 1990).

Parashuramulu *et al*. (2013) observed by supplementing 0, 20, 40, 60, 80, 100, 120, 140 and 160 ppm of Zn from ZnSO4 to a sorghum based diet (29.72 ppm Zn), that the in vitro dry matter digestibility (IVDMD) increases with supplementation of Zn and was reported highest (P<0.01) in the diet containing 140 ppm. No further improvement in IVDMD was reported after 140 ppm of Zn supplementation. The mean gas volume, in vitro organic matter digestibility (IVOMD), metabolizable energy (ME) and short chain fatty acids (SCFA) contents were reported higher (P<0.01) in Zn supplemented diets than Zn unsupplemented diet which increased up to 140 ppm Zn supplementation in a dose dependent manner and again 160 ppm Zn supplementation, the values decreased but were comparable with that of 20 to 60 ppm Zn supplemented diet. The IVDMD, gas production, IVOMD, SCFA and ME content at 140ppm were though highest, was comparable with that of 80, 100 and 120 ppm Zn addition.

Hassan *et al*. (2011) reported a higher NH3-N (mg/100ml) concentration (P < 0.05) in ewes supplemented with 25 mg of Zn from ZnSO4 than either of 15 mg or 25 mg of Zn from ZnMet. But they observed that Sheep in the ZnMet treatments had higher (P < 0.05) total VFA concentrations than ZnSO4 supplemented animals. They hypothesized that, Zn from ZnSO4 may have been taken up by ruminal microorganisms to a greater extent, and this could explain the lower ruminal soluble Zn concentrations in steers fed ZnSO4.

Spears *et al*. (2004) reported that organic Zn (ZnMet and ZnGly) supplemented steers had higher ruminal soluble zinc than ZnSO4 supplemented steers when supplemented at 20 mg of Zn per kg diet. They hypothesized that the complexes might have interacted to a lesser degree than ZnSO4 in the rumen to form insoluble complexes, otherwise, possibly Zn from ZnSO4 may have been taken up by ruminal microorganisms to a greater extent, and this could explain the lower ruminal soluble Zn concentrations in steers fed ZnSO4. Other higher bio-available sources like Zn proteinate (Spears and Kegley, 2002) or a Zn polysaccharide complex (Kennedy *et al*., 1993) also had higher ruminal soluble Zn concentrations than those receiving inorganic Zn oxide.

There is always a change in the fermentation pattern with the change in the mineral sources and dose of supplementation. Addition of Zn to diets affected ruminal fermentation by shifting proportions of VFA in ruminal fluid and is more prominent in the initial hours of feeding (Bateman *et al.*, 2002). Report suggests that proportion of acetate was significantly increased by adding Zn to the diet during *in vitro* experiment (Bateman *et al.,* 2002). Supplementation of ZnO NP had a positive effect on the concentration of volatile fatty acid, improved growth of ruminal microorganisms, increased ruminal

microbial protein synthesis and raise the energy utilization efficiency in early phase (6 to 12 h) of incubation (Zhisheng, 2011). But the concentration of ammonia nitrogen and ratio of acetate to propionate were adversely affected by the supplementation of 100 and 200 mg/kg of ZnO NP at the 6th and 12th hour of incubation (Zhisheng, 2011). Some workers have different opinion as they reported a reduction in total VFA concentrations due to the inclusion of Zn in the diets (Bateman *et al.,* 2002) which may be attributed to the size of the Zn source. But studies may be framed to validate the impact of different sources of Zn on rumen fermentation pattern. Differences in results *in vivo* and *in vitro* is been observed by adding Zn to the diet. For an instance, there was a decline in the proportion of butyrate *in vitro*, but *in vivo* there was an increase in propionate (Bateman *et al.*, 2002). Zn is reported to improve the efficiency of dietary protein utilization. In growing steers, Froetschel *et al.,* (1990) reported that Zn chloride and Zn sulfate improved the efficiency of dietary protein utilization. Britton and Klopfenstein (1986) observed an increase in ruminal escape of soybean meal protein when soybean meal was fed after being treated with 1.5 to 2.0% Zn chloride. Zn sulphate can be used as a defaunating agent. At levels greater than 1,000 mg/kg in the ruminant ration was reported to defaunate the rumen (Bonhomme *et al.,* 1979; Durand and Kawashima, 1980).

2.11. **Effect of Zn supplementation on male reproduction**

Zinc plays an important role in prostate, epididymal, testicular functions (Ebisch wt al., 2003), development of seminiferous tubules, spermatogenesis, testicular steriodogenesis, androgenesis and interactions with streroid receptors (El-Sisy *et al*., 2008). Zn is also believed to regulate maturation of spermatozoa (Baccetti *et al.*, 1976). Zinc has been reported to influence the process of spermatogenesis (Wong *et al*., 2002), controls sperm motility (Wroblewski *et al*., 2003), stabilizes sperm membrane (Kendall *et al*., 2000), preserves the ability of sperm nuclear chromatin to undergo decondensation and modulates sperm functions (Suzuki *et al*., 1995). Thus overall, in male reproduction, Zn has a crtitical role in testicular development (Martin and White, 1992), sperm maturation (Martin and White, 1992; Martin *et al*., 1994) and testosterone synthesis (Root *et al*., 1979; McCIain *et al*., 1984; Prasad, 1985; Martin *et al*., 1994; Kumar *et al*., 2013). Zn is also involved in the activation and maintenance of the germinal epithelium of semineferous tubules and also stimulates production and secretion of testosterone, which influences spermatogenesis (Wong *et al*., 2002). Moreover, most important enzymes

involved in the process of spermatogenesis are sorbitol dehydrogenase and lactate dehydrogenase, which are essentially zinc metalloenzymes (Eggert *et al*., 2002).

During spermatogenesis, a functional locomotor apparatus is formed in spermatozoa (Mohri and Ishijina, 1989) and a considerable amount of Zn is incorporated into the spermatozoa (Parizek *et al.*, 1966). Flagellar Zn is located mainly within the outer dense fibers (Calvin *et al.*, 1973), where it is bound to the sulfhydryl groups of cysteine.

Zn content is reduced by approximately 60% during epididymal sperm maturation (Kaminska *et al.*, 1987) which stabilizes the outer dense fiber proteins by oxidation of sulfhydryl groups to disulfide bridges (Calvin *et al.*, 1973).

Hypozinkemia leads to gonad dysfunction, decreased testicular weight, atrophy of seminiferous tubules and complete cessation of spermatogenesis (Martin *et al*., 1994). Zn deficiency adversely affects sperm integrity (Merrells *et al.*, 2009). Particularly in young animals going through puberty, zinc deficiency completely arrests testicular growth and development (Martin *et al*., 1994). The production of sperm necessitates extensive cell division and Zn plays a significant role in it by influencing mitotic and meiotic cell divisions, along with synthesis of DNA and RNA by enhancing the activity of DNA polymerase and RNA polymerase, the two Zn containing enzymes (Kumar *et al*., 2006).

Martin *et al*., 1994 observed atrophy of of primary, secondary and accessory sex organs when rams were fed with zinc deficient diets, indicating the importance of zinc on growth and development of primary, secondary and accessory sex organs.

The main source of zinc in the semen is the prostate gland where the highest concentration of Zn has been reported, and it acts as a marker of prostatic functions (Elzanaty *et al*., 2004; Nagamine *et al*., 1990). Zinc is found in high concentration in the male reproductive tract as well as in semen (Chia *et al*., 2000).

Male goats are susceptible to maginal dietary zinc deficiencies (Haenlein, 1980) and required quantities of zinc must be supplied continuously to the animals as it is not stored in the body (Kumar *et al*., 2013). Zinc deficiency may impair the steroid synthesis (Lei *et al*., 1976) and development of smooth endoplasmic reticulum in Leydig cells, where testosterone is synthesized (Hesketh, 1982), or impair the LH receptor mechanism, controlling storage and release of testosterone (Kellokumpu and Rejaneimi, 1981).

Zinc supplementation is documented to enhance the sperm concentration, number, mass motility and individual motility per ejaculate in bulls (Kumar *et al*., 2006), men (Wong *et al*., 2002), rams (Kendall *et al*., 2000), bucks (Saleh *et al*., 1992) and rabbits (Tharwat, 1998), which is indicative of its essentiality for spermatogenesis. Zn also helps

in encoding a transcription factor involved in spermatogenesis (Bedwal and Bahuguna, 1994).

Sperm velocity and motility are the important parameters influencing fertility in animals (Aitken, 1990). Zinc supplementation to the rams with apparently normal zinc can improve the semen quality and sperm motility in rams (Kendall *et al*., 2000). Kumar *et al*. (2006) in bulls Kyanaston *et al*. (1988) in men and Liao *et al*. (1985) in boars did not find any influence of zinc supplementation on abnormal sperm per cent. Normal and abnormal morphology of sperm is completely dependent on the spermiogenesis phase of spermatogenesis, which in turn is regulated by sertoli cells (McDonald, 2003) and Zinc doesn't affect any structural and functional attributes of sertoli cells (Veda *et al*., 1991), which is presumed to be the cause behind similar abnormal sperm percentage in zinc supplemented and un-supplemented animals.

In bulls, Zn supplementation has been reported to have positive effects on ejaculate volume (mL), sperm concentration (million.mL^{-1}) and sperm number per ejaculate (millions) and is dependent on the dose of zinc supplementation (Kumar *et al*., 2006). Kumar *et al*. (2006) observed that mean ejaculate volume (mL), Sperm concentration (million.mL^{-1}) and sperm number per ejaculate (million) were significantly ($P < 0.05$) higher in Zn-supplemented groups than un-supplemented animals and the increases were in a dose dependent manner. Kumar *et al*. (2006) demonstrated that, irrespective of the source, zinc supplementation improves the semen quality of bulls as compared to the non-supplemented bulls.

In rams, Martin *et al*. (1994) observed that epididymal mass, testis mass, and testicular zinc concentration fed with 10, 17 or 27 µg Zn /g feed were significantly higher than control zinc deficient rams. However, mass of the testes in 17 and 27 µg Zn/g feed was higher than 10 µg Zn/g feed fed rams. Testicular tissue sections revealed poor tubule development and extensive interstitial cells in control groups than the zinc supplemented groups (10, 17 or 27 µg Zn /g feed).

Zn is also a scavenger of free oxygen radicals and protects sperm from oxidative damage and lipid peroxidation by inhibiting phospholipase (Eggert *et al*., 2002). Saleh *et al*. (1992) reported that Zn controls the motility of goat sperms by influencing development of flagellar system of sperm tail. Zn localizes in the sperm middle piece in association with lipoprotein fraction suggests that Zn is involved in catabolism of lipid, which is the principle source of energy required for movement of spermatozoa. High concentration of Zn in the spermatozoa is essential for viability and fertility of the buffalo

bull (Ahmed *et al*., 1997). Improved sperm motility can be attributed to the fact that, ATP is needed for the sperm flagellar movement and zinc controls the motility of sperm by controlling energy utilization through the ATP system, through regulation of phospholipid energy reserves and improving sperm oxygen uptake (El-Masry *et al*., 1994) or there might be an increase in activity of Zn containing enzymes (sorbitol dehydrogenase and lactate dehydrogenase) which play significant roles in sperm motility (Nagamine *et al*., 1990) or improved motility may be due to the enhanced anti-oxidative potential in the sperm due to zinc supplementation (Kumar *et al*., 2006). Kumar *et al*. (2006) in bulls, El-Masry *et al*. (1994) in rabbit and Omu *et al*. (1998) in man reported that zinc supplementation has improved the live sperm per cent. The effect of zinc in membrane stabilization in sperms, prevents the leakage of enzymes, proteins and other vital components of sperm, thus extending the functional life of sperm. Moreover, Bettger and O'Dell (1981) has documented that Zn also stabilizes ribosomes, lysosomes, DNA and RNA, which help in survival and normal functioning of the sperm. Improved livability of sperm may be due to the membrane stabilizing action of Zn, by virtue of which, it prevents leakage of enzymes, proteins and other vital components of sperm, thus extending the functional life of sperm. Moreover, Zn also stabilizes ribosomes, lysosomes, DNA and RNA, which help in survival and normal functioning of the sperm Bettger and O'Dell (1981).

As zinc enhances the free radical scavenging system, thus protects the sperm from free radical induced damages by scavenging excessive free radicals and thus improving sperm viability (Kumar *et al*., 2006). Zn, as a constituent of a large number of metalloenzymes, takes part in several enzymatic reactions related to carbohydrate, protein, lipid and nucleic acid metabolism, which may account for improved sperm livability (Bires *et al*. 1997). It is also been documented that zinc is responsible for production of an anti-bacterial substance from the prostate gland which is added into semen (McDonald, 2003), may be a cause for improved live sperm percentage.

Deficiency of Zn in the diets is the cause of a high incidence of abortions and stillbirths in the ewes (Campbell and Mills, 1979; Najafzadeh *et al.,* 2013). Supplementation in the form of NZn to animals can possibly eliminate these reproductive disturbances and thus improve the economics of farming. Very few reports are available on effect of NZn on male reproduction.

Abbasalipourkabir *et al*. (2015) administered 50, 100, 150 and 200 mg NZnO/kg body weight daily intraperitoneally for ten days to male rats, which resulted in a

significant decrease in the sperm count and vitality in all NZnO groups as compared to the control group. Furthermore, sperm motility decreased by increasing the concentration of NZnO, but the reduction was non significant at the 50 mg concentration. Thus NZnO injections at the above doses had a significant impact on sperm morphology and viability.

Talebi *et al*. (2013) administered 5, 50 and 300 mg/kg NZnO to the mice for a span of 35 days and observed that sperm number, motility and percentage of abnormality were significantly changed in 50 and 300 mg/kg NZnO treated mice ($p<0.01$). Histopathological section of testis were reported to have epithelial vacuolization, sloughing of germ and detachment were significantly increased in 50 and 300 mg/kg NZnO treated mice ($p<0.001$), whereas NZnO at 300 mg/kg induced formation of multinucleated giant cells in the germinal epithelium. A significant decrease in seminiferous tubule diameter, seminiferous epithelium height and maturation arrest ($p<0.001$) in NZnO at 50 and 300 mg/kg was reported.

2.12. **Effect of Zn supplementation metallothionein and SOD1 expression**

2.12.1. Metallothionein

Shay and Cousins (1993) suggested that metallothionein (MT), a protein involved in Zn homeostasis and its mRNA abundance is increased when Zn was supplemented to Zn deficient animals.

Cousins and Lee-Ambrose ((1992) reported that metallothionein expression was proportional to the dietary zinc intake and is tissue specific, which is maximam in kidney followed by liver, intestine, spleen and heart. However, they did not observe any appriciable change in thymus and lung mRNA expression.

Zn absorption requires Zn transport from the lumen of the intestine into a mucosal cell. A carrier-mediated transport mechanism is responsible for the transfer of Zn through the brush border, which likely includes an interaction with Zn in a chelated composition (Solomons and Cousins, 1984). The transport of Zn inside the intestinal mucosal cell is controlled by a metal-binding protein known as metallothionein, which is generated in the liver. Metallothionein is a low molecular weight cytoplasmic metalloprotein concerned in the metabolism of Zn (Richards and Cousins, 1976).

The synthesis of metallothionein is regulated by dietary Zn and plasma Zn concentrations, thus influencing the amount of Zn that enters the body, as result; it greatly contributes to Zn homeostasis (Cousins, 1978). Richards and Cousins (1976), in rats, Metallothionein bound -Zn in both liver and intestinal mucosal cytosol responded rapidly

to an altered dietary Zn level and serum Zn was found directly related to the appearance of Metallothionein. Zinc absorption also responded rapidly to a change in dietary Zinc. However, they reported that zinc absorption is inversely correlated with intestinal mucosal Metallothionein bound-Zn. Hepatic Zinc uptake appeared to be directly correlated with liver Metallothionein bound -Zn.

2.12.2. Superoxide dismutase-1 (SOD-1)

The SOD in conjugation with catalase and GPx scavenges both intracellular and extracellular superoxide radicals and prevents lipid peroxidation (Agarwal and Prabhakaran, 2005).

Jing *et al*. (2007) reported reduction in the Cu-Zn superoxide dismutase (Cu-Zn SOD) activity, in rats fed Zn deficient diet; whereas the rats fed zinc excess diet had higher Cu-Zn SOD activity. Kumar *et al*. (2011) reported improvement in SOD gene expression in the kids supplemented with 50 mg/ kg lead and 50 mg/ kg zinc along with basal diet than only lead exposed goat kids at 50 mg/ kg during a period of 90 days of experimental feeding, which indicated the positive effect of zinc on SOD gene expression in goat kids. Abbasalipourkabir *et al*. (2015) observed a rise in the SOD activity with increase in the concentration of NZnO, which did not vary statistically among the treatment groups and control.

Lee *et al*. (2014) reported that SOD levels were significantly higher in NZnO exposed cells, respectively. Up-regulation of these genes by NZnO could have increased the production of ROS and oxidative stress, and hence NZnO can potentially produce ROS through the perturbation of metabolic pathway, inducing oxidative stress. It was advocated that nanotoxicity `mechanism could correlate with the active oxygen production, oxidative stress, apoptosis, and antioxidant defence mechanisms.

However, Pal *et al*. (2010) reported higher activity of Cu/Zn-SOD in liver homogenate in ewes fed Cu and Zn-methionine in ewes supplemented with 50% more of Cu and Zn over a basal diet (9.8 mg of Cu and 39.33 mg of Zn per kg of DM) from inorganic (sulphates) or organic (methionine chelated) sources.

2.13. Effect of Zn supplementation on tissue architecture

The potential hazard of high concentrations of NZn as animal feed supplement is still unknown and their toxicological data are rather uncommon, but still, the toxicity of both conventional Zn and NZn compounds has been reported (Houston *et al*., 2001; Llobet *et al*., 1988; Lock and Janssen, 2003; Piao *et al*., 2003). The toxicological effects of ZnO NP are related to their dissolution and interference with Zn ion homeostasis. In Zn toxicity, pathological changes in the pancreas, kidney, liver, rumen, abomasum, small intestine and adrenal gland ware observed in sheep (Allen *et al*., 1983). Liver, spleen, heart, pancreas and bone are the target organs of ZnO NP on oral exposure (Wang *et al*., 2008). Due to the similarity in biochemical and physiological pathways with human metabolism, mostly toxicological studies are conducted on rodents (rats and mice) as in vivo models (Argmann *et al*., 2005). In the histopathological examination, ZnO NP has exhibited both dose and time dependent cytotoxicity and its mechanism is carried by oxidative stress, lipid peroxidation, cell membrane damage, and oxidative DNA damage (Lin *et al*., 2009; Xia *et al*., 2008; Najafzadeh *et al*., 2013). ZnO NP induced toxicity in cells resulted in the production of ROS, oxidative injury, excitation of inflammation and cell death (Xia *et al*., 2008). Toxic effects of the NPs are size-dependent that means, NZn will be more toxic than MZn at the same dose (Chen *et al*., 2007). ZnO NP tends to accumulate in the liver tissues thereby causing the toxicity. Najafzadeh *et al*. (2013) reported mild liver toxicity (edema and degeneration in the hepatocytes) and severe renal damage (multifoacal interstitial nephritis in 75% of animals) in lambs because of NZn feeding at a dose of 20 mg/kg body weight orally for a period of 25 days. In mice, mortality was not observed even by feeding 20 nm or 120 nm ZnO at 1 g/kg body weight orally (Wang *et al*., 2008). The proposed mechanism for NP toxicity may be may be as the particles are much more active and can be rapidly transformed into respective ions in gastric juice. So large amounts of metal ions are generated and subsequently brought to liver and kidney for metabolism and excretion, which cause damage to hepatic and renal tissues (Chen *et al*., 2007). By feeding ZnO NP at a dose of 300 mg/kg orally to mice for 14 consecutive days, accumulation of NPs in the liver was reported, which caused cellular injury leading to elevated ALT and ALP levels in serum and pathological lesions in the liver further, cytotoxic effect of feeding ZnO NP includes induced oxidative stress mediated DNA damage and apoptosis which is indicated by an increase in lipid peroxidation and accumulation of ZnO NP in liver was observed (Sharma *et al.*, 2012).

The toxicity of Zn is reported to be associated with the concentration of the free ion (Di Toro *et al.*, 2001; Lofts *et al.*, 2004; Thakali *et al.*, 2006; Kasemets *et al.*, 2009; Kool *et al.*, 2011). But ZnO NP are likely to have remained as NPs for longer duration, and thus are less toxic than the corresponding inorganic salts like $ZnCl_2$ (Hooper *et al.*, 2011). But reports suggests that toxicity of Zn in the acute tests was reported to be independent of particle size, coating of particles, aggregation of particles, the type of medium or the applied pre-treatment of the test dispersions (Wiench *et al.*, 2009).

Wang *et al.* (2006) studied the comparative toxicity of nano (58 ± 16 nm) and micro (1.08 ± 0.25 μm) Zn by feeding the adult mice orally at the dose of 5 g/kg. They observed lethargy, nausea, vomiting and diarrhoea at the beginning days in the group fed with NZn, but micro Zn (MZn) fed mice group did not show any abnormal symptoms. By histopathology, the damages in kidney, liver and heart were reported in both the groups but in MZn group it was very slight as compared to the NZn group. The histopathological changes observed were glomerulus swelling, proteinaceous casts in renal tubules, hydropic degeneration and necrosis in hepatic tissue and also fatty degeneration in cardiovascular cells.

Abbasalipourkabir *et al.* (2015) injected a dose of 50, 100, 150 and 200 mg ZnO NP/kg body weight intraperitoneally daily for ten days in Wistar rats. The histopathological sections of liver and kidney revealed toxicological changes in both the organs. The liver tissue exhibited increased Kupffer cells, congestion, inflammation in the liver parenchymal, ballooning, inflammation and chromatin condensation as a result of apoptosis. In kidney, pathological changes included proliferation of glomerular cells, inflammation of interstitial tissue and congestion of glomerulus in kidney of all rats treated with ZnO NP at concentrations above 50 mg/kg body weight.

Zn toxicity in sheep induced pathological changes in pancreas, kidney, liver, rumen, abomasum, small intestine, and adrenal gland (Allen *et al.*, 1983). In mice, Wang *et al.* (2017) observed that 250 mg/kg ZnO NP reduced the body weight from weeks 8 to 11 whereas, increased serum GPT activity, and Zn concentrations of the serum, liver, and kidney without affecting the relative organ weight, intestinal microbiota, and other mineral concentrations (Fe, Cu, and Mn) in the kidney, liver, and thigh muscle, whereas oral administration of $ZnSO_4$ at 250 mg/kg exhibited more severe and acute toxicity as they showed reduced body weight from 5 to 11 weeks, decreased relative pancreas weight, and increased serum glutamic-oxalacetic transaminase activity and intestinal enteric group.

Zhang *et al.* (2017) demonstrated in rats that ZnO NP is more effective than ZnO and $ZnSO_4$ in enhancing cell viability, lower cytotoxic, better uptake efficiency, and efficient transportation at lower concentrations, thus they advocated ZnO NP to be a newer and potential feed additive for Zn supplementation. They also observed that uptake efficiency in rat epithelial cell line IEC-6 is more in ZnO NP than $ZnSO_4$ and ZnO.

In a comparative study on ZnO NP, MZnO and Zn ions after long-term feeding with Zn replenished food (1600 mg Zn equivalent per kg food) for 270 days, It was observed that ZnO NP, MZnO and Zn ions were difficult to pass through the intestine barrier, and thus most of them were excreted mainly through faeces (Liu *et al.*, 2017). Further, they did not find any noticeable difference among the distribution profiles of ZnO NP, MZnO and Zn ions in mice. Zn tend to accumulated only in the digestive tract organs. The biomedical parameters and pathological investigations showed liver lesions induced by MZnO, but fewer by ZnO NP or Zn ions. Hence, ZnO NP as compared to the MZnO, are relatively better biocompatible as the nutritional feed supplement at the commonly used dose.

MATERIALS AND METHODS

A study was undertaken to assess the effect of graded levels of nano zinc (NZn) on growth, nutrient utilization and immunity in rats and goats. The experimental procedures and techniques adopted during the course of study are detailed in this chapter. The experiment was conducted at the Experimental Livestock Unit, ICAR- National Institute of Animal Nutrition and Physiology, Bengaluru, India. The study was conducted in three different phases namely, Phase-I Synthesis and Characterization of Nano Zinc (NZn) particles, Phase-II supplementation of graded levels of NZn particles to rats to study the effects on growth, immunity, blood biochemical profiles, nutrient utilization, mineral absorption so as to finalize the level of NZn particles for goat feeding experiment. Phase-III i.e. feeding of the synthesized NZn particles to goats on its impact in animal performance in terms of growth, immunity, nutrient utilization, blood biochemical and hormonal profiles and mineral absorption.

3.1. Phase I: Synthesis and Characterization of Nano Zinc (NZn) particle

3.1.1. Synthesis of NZn particle

The NZn particles were synthesized by using 2 different methods i.e. physical and chemical method.

3.1.1.1. Synthesis of NZn particles by physical method

Nano zinc (NZn) particles were synthesized by using zinc acetate (Zn $(CH_3COOH)_2$. $2H_2O$) and sodium bicarbonate ($NaHCO_3$) by following the method of Sabura Begum *et al*. (2008). Zinc acetate (Zn $(CH_3COOH)_2.2H_2O$) @ 10 mmoles and 23.8 mmoles of $NaHCO_3$ was taken in a 100 mL silica crucible and was ashed in muffle furnace under different temperature (300 and 400ºC) for various durations (3, 4 and 5h) to produce NZn particles. The produced NPs were washed with distilled water and filtered through Whatman No. 1 filter paper to separate the NZn particles. The filter paper along with the white coloured substances were dried at 60ºC for 8h and stored in airtight glass vials for further characterization.

3.1.1.2. Synthesis of NZn particles by chemical method

The NZn particles were synthesized following the procedure by Moghaddam *et al*. (2009) with some modifications (Fig 3.1.). One hundred mL of 0.45 M aqueous solution of zinc nitrate [$Zn(NO_3)_2.6H_2O$] and 100 mL of 0.9 M aqueous solution of sodium hydroxide (NaOH) were taken separately in 250 mL beakers. The aqueous solution of $Zn(NO_3)_2$ i.e. 100 mL was transferred into a burette and was added drop by drop at a speed of 40-45 drops per min to the beaker containing aqueous solution of 0.9M NaOH. The beaker containing aqueous solution of 0.9M NaOH was placed over a hot plate cum magnetic stirrer (with digital temperature and RPM display) maintained at 55^0C at high speed stirring (360 rpm). After complete transfer of $Zn(NO_3)_2$ into the beaker containing NaOH solution, while mixing, the beaker placed on hot plate was removed and kept undisturbed after covering with aluminium foil further for 2 h to facilitate precipitate formation and subsequently settling of the precipitates. The precipitated zinc oxide (ZnO) in the form of NZn particles were washed with HPLC grade water (milliQ) followed by ethanol for several times (5-6 times at least) until all the impurities are washed out. Then the sample was transferred into a petriplate and vacuum dried at 60^0C. The synthesized NZn particles were transferred and stored in airtight screw cap vials for characterization.

3.1.2. Characterization of NZn particle

The synthesized NZn particles were characterized to know the particle size at Department of Nano Science and Technology, TNAU, Coimbatore.

3.1.2.1. Particle size analysis

Particle size and Zeta potential and distribution pattern of synthesized NZn particles were determined by using Horiba Scientific Nanopartica SZ-100 (Nanoparticle analyzer), Japan. 0.5mg sample was weighed accurately and dispersed in 20 mL distilled water, subsequently sonicated for 15 min. Then, the suspension was analysed under dynamic scattering method using 90^o or 173^0 at 25^0C.

3.1.2.2. X- Ray Diffractometer (XRD)

X-Ray diffractogram was recorded on Powder XRD (Brucker D8 Advance powder X- Ray diffractometer, Germany), which uses Cu-Kα radiation (0.154 nm) for measuring the crystalline nature of the material (Toraya, 1986). The diffractograms were recorded with 2θ value ranging from 10 to 80 degrees at a scanning speed of 0.080 at a step time of 1 sec at $25\ ^0$C.

Fig 3.1. Synthesis of nanoparticles by using chemical method

(a) addition of 0.45 M $Zn(NO_3)_2.6H_2O$ to a solution containing 0.9M NaOH

(b) Stirring of the solution over hotplate cum magnetic stirrer at 55 ^{0}C

(c) Keeping the solution undisturbed for 2 h for precipitate formation

(d) drying of precipitate after washing with HPLC grade water and ethanol

3.1.2.3. Raman spectrophotometer

Raman spectrophotometer was employed to identify the composition of a mixture of unknown compounds based on elastic scattering. The wavelength of 785 nm monochromatic light was used in Roman spectrum Model –R- 3000-QE. Dried ZnO was spread to an extent of 1 cm^2 on polythene bag and exposed under dark room condition with Raman probe placed on the sample pockets without exposing the samples directly to the light.

3.1.2.4. Transmission electron microscope (TEM)

TEM FEI TECHNAI SPIRIT was used to analyze the chemically synthesized Zn NPs. Diluted suspensions of NPs (0.5 mg) in pure ethanol (15 mL) were prepared by ultrasonication. A drop of the suspension was placed on 300 mesh lacy carbon coated copper grid, dried and the images were recorded at different magnifications.

3.1.2.5. Energy dispersive X-Ray spectroscopy (EDAX)

EDAX is an analytical technique used for the elemental analysis or chemical characterization of a sample. It is one of the variants of X-ray fluorescence spectroscopy, which relies on the investigation of a sample through interactions between electromagnetic radiation and matter, analysing the rays emitted by the matter in response to being hit with the charged particles. Its characterization capabilities are due in large part to the fundamental principle that each element has a unique atomic structure allowing unique X-Rays that are characteristics of an elements atomic structure which differ from one another. The quantitative analysis of zinc NPs was done by EFI QUANTA 250 EDAX. About 1-2 g of sample was dusted on carbon conduction tape. Then the stuff was mounted on sample stage and the images were taken.

3.2. Phase-II: Supplementation of graded levels of NZn on growth, immunity, blood biochemical profiles, nutrient utilization, and mineral absorption in rats

An *in vivo* experiment on Wistar albino rats (WAR, *Rattus norvegicus)* was conducted in Laboratory Animal House, Experimental Livestock Unit, ICAR-NIANP, Bengaluru, India, after being approved by the Institutional Animal Ethics Committee (IAEC) (2/2014 of IAEC dated 12.04.2014) of ICAR-NIANP, Bangalore- 560 030. This experiment was carried out as per the guidelines of Committee for the Purpose of Control

and Supervision of Experiments in Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India, New Delhi, India.

3.2.1. Experimental animal and design

Eighty four weaned, apparently healthy WAR of uniform age, sex (male) having initial body weight (53.6±0.65g) were randomly divided into seven groups of 12 each (4 replicate in each group with 3 rats per replicate) on the basis of their body weight.

The rats were fed with basal semi-purified diet prepared as per ICAR (2013a) to meet their nutrient requirement except for that of Zn. The ingredients and their level of incorporation are denoted in Table 3.3.

Table 3.1. Experimental design

Particulars	**NC**	**IZn-25**	**NZn-25**	**NZn-12.5**	**NZn-6.25**	**NZn-3.125**	**NZn-50**
No. of Animals	4×3	4×3	4×3	4×3	4×3	4×3	4×3
Source of supplemental zinc	No Zn	ZnO	Nano ZnO	Nano ZnO	Nano ZnO	Nano ZnO	Nano ZnO
Zinc Level	Basal Diet	ICAR 2013a (25 ppm)	ICAR 2013a (25 ppm)	½ ICAR 2013a (12.5 ppm)	¼ ICAR 2013a (6.25 ppm)	1/8 ICAR 2013a (3.125 ppm)	Double of ICAR 2013a (50 ppm)

NC: Negative control

3.2.2. *Housing and management of WAR*

The rats were housed in polypropylene cages and maintained under similar management conditions in a well ventilated animal house (Fig 3.2.). The rats were kept in well aerated laboratory cages (43 X 27 X 15 mm), which were covered with stainless steel cage grill having provision to keep experimental feed pellets and water bottle. There was 12 h light and dark cycle with a light period between 06:00 and 18:00 h. Temperature and humidity was maintained at 23±2º C and 50 to 75%, respectively. The animals were fed with synthesized semi-purified pelleted feeds and offered purified RO water *ad libitum.*

Table 3.2. Composition of mineral mixture fed to different groups of rat (g/kg)

Chemicals (g/kg)	NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50
Calcium Phosphate (Dibasic) $CaHPO_4$	500	500	500	500	500	500	500
Potassium Citrate Monohydrate ($K_3C_6H_5O_7.H_2O$)	220	220	220	220	220	220	220
Sodium Chloride (NaCl)	74	74	74	74	74	74	74
Potassium Sulphate	52	52	52	52	52	52	52
Magnesium Oxide (MgO)	24	24	24	24	24	24	24
Ferric Citrate (16-17% Fe)	6	6	6	6	6	6	6
Manganous Carbonate (43-48% Mn)	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Zinc Oxide (ZnO)	0	0.9 Inorg.	0.9 (nano)	0.45 (nano)	0.225 (nano)	0.112 (nano)	1.8 (nano)
Chromium Potassium Sulphate [$CrK(SO_4)_2.12H_2O$]	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Cupric Carbonate (53-55% Cu)	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Potassium Iodate (KIO_3)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sodium selenite ($Na_2SeO_3.5H_2O$)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sucrose (Fine powdered)	119.63	118.73	118.73	119.18	119.42	119.52	117.83

3.2.3. Preparation of special mineral mixture

The rats were fed with the diet containing the specially formulated mineral mixture as per recommendations by ICAR (2013a) except zinc throughout experiment. The mineral mixture composition is depicted in Table 3.2.

Table 3.3. Composition of experimental rat feed

Ingredient	Quantity (g/kg)
Sucrose	450
Casein	200
Corn starch	150
Fine wheat flour (Maida)	50
Corn oil	50
Cellulose	50
Mineral mixture	35
Vitamin mixture *	10
DL-Methionine	3
Choline bitartarate	2

As per nutrient requirement of ICAR (2013a)

*Details of Vitamin Mixture are described in Table 3.4

Table 3.4. Composition of vitamin mixture

Vitamin	Quantity (per 200g)
Vitamin A (IU)	10×10^6
Vitamin D_3 (IU)	2×10^6
Vitamin K_3 (mg)	800
Vitamin E (mg)	8000
Vitamin B_1 (mg)	1000
Vitamin B_2 (mg)	4000
Vitamin B_6 (mg)	2500
Vitamin B_{12} (mcg)	9000
Niacin (mg)	12000
Pantothenic acid (mg)	6000
Folic acid (mg)	500
Biotin (mcg)	10000

3.2.4. Preparation of experimental rat feed

Different groups were fed with different diets which differ in the level and source of zinc (ZnO). First seven different minerals mixtures were prepared as per the composition denoted in Table 3.2. All the ingredients of the basal diet were weighed as described in Table 3.2. First the sucrose crystals were ground to fine powder in grinder cum mixer and later thoroughly mixed with mineral mixture and vitamin mixture to form a premix. Subsequently, casein, corn starch and cellulose were added to the premix in the order and were mixed thoroughly. Then, vegetable oil was added and again mixed uniformly. Then required quantity of water was added to the above mixture and mixed till the consistency is uniform for pelleting. Then pellets of each experiment diet were prepared by using manual hand operated pelletizer (Fig 3.3.). The pellets thus produced (Fig 3.3.) were subsequently dried at 60ºC for 24 – 48h and the pellets were stored in respective air tight containers. Feed pellets for different group was prepared, stored and handled separately and utmost care was taken to avoid ross contamination (Fig 3.4.).

Manual hand operated pelletizer was washed every time before and after preparation of each experimental diet. Maximum precaution was taken at each step of mixing, preparation, pelletizing and storage to avoid contamination and spoilage.

3.2.5. Experimental feeding of WAR

The rats were given access to *ad libitum* feed and water. Weighed amount of feed was offered daily per pen at 9.30 AM. After 24 h the residue was weighed to know the feed intake per pen. Thus daily feed offered and feed refusal was recorded to calculate feed intake. Animals were supplied with *ad libitum* wholesome, RO water throughout the feeding trial. The representative of feed offered and refusal were analysed for dry matter (DM) at weekly interval to calculate the DM intake by the animals.

3.2.6. Weighing of WAR

Weights of all the WAR were recorded initially and subsequently on weekly interval to know the growth of animals. The weighing of individual WAR was done before feeding and watering in electronic weighing balance with two decimal accuracy.

3.2.7. Digestibility trial on WAR

After a period of 30 days of experimental feeding, a digestibility trial was conducted by conventional total faecal collection by hand pick method for a period of 7 days including 3 days adaptation period and four days of actual collection period. As a routine procedure, feed offered and feed refusals were also recorded. Representative samples of experimental semi-purified diets and replicate wise residues from different pens of different dietary groups were pooled separately and stored at -20ºC in Deep freeze in self sealing cover for analysis of DM. Pooled diets and faeces samples during experimental period were dried and ground to pass through a 1 mm screen. The ground samples were preserved in moisture free self sealing covers in desiccators for further analysis. The faecal pellets were carefully handpicked quantitatively from uniformly spread bedding material. The total faeces voided from each replicate of different dietary treatments in 24h was separated, weighed and stored in a self sealing cover to prevent moisture loss. For nitrogen estimation an aliquot of $1/25^{th}$ of daily faeces voided was weighed, mixed with few drops of 1:4 sulfuric acid and preserved at -20^0C for further analysis. For DM estimation, the remaining faeces after aliquoting for nitrogen analysis was taken in a preweighed petriplate and dried at 80^0C in the hot air oven to a constant weight. After drying, the faecal samples were pooled in self sealing polythene bag for the

Fig 3.2. A view of hygienic and sanitary measures followed during rat feeding trial

Fig 3.3. Preparation of semi-purified synthetic rat diets with manual hand operated pelletizer

four days. After the completion of the trial the samples were ground to 1 mm size and preserved in self sealing covers for further analysis.

3.2.7.1. Estimation of proximate principles

The samples of feed offered, residue and faeces were analyzed for proximate principles and urine samples for nitrogen content as per standard procedures of Association of Official Analytical Chemists (AOAC, 1995). A brief description of the method employed is given as follows:

3.2.7.1.1. Dry matter (DM)

A known quantity of ground samples were taken in pre-weighed moisture cups; and these cups were placed in a hot air oven at 100 ± 2°C for 24 hours. The loss in moisture content after drying was estimated and dry matter was calculated as follows:

$$\text{DM (\%)} = \frac{Weight\ of\ the\ dried\ sample}{Weight\ of\ the\ sample} \times 100$$

3.2.7.1. 2. Organic matter (OM)

It was determined by subtracting the total ash content from 100.

$$\text{OM (\%)} = 100 - \text{Total ash (\%)}$$

3.2.7.1. 3. Crude protein (CP)

Crude protein was estimated as per Kjeldahl method. A known quantity of ground sample was taken in Kjeldahl flask and digested with concentrated H_2SO_4 and 2-3 g of digestion mixture (K_2SO_4 and $CuSO_4$ in the ratio of 9:1) till the solution became colourless. After digestion, the contents were cooled, transferred to volumetric flask after repeated washings and volume was made to 250 mL. 25 mL of aliquot was transferred into distillation tube in Nitrogen analyzer (Gerhardt make) which has provision for addition of 40% NaOH. The distillate was collected in 2% boric acid and auto titrat using standard sulphuric acid solution (0.01 N).

$$\text{Nitrogen (\%)} = \frac{0.014 \text{ x titer vol. x Normality x Vol. made}}{\text{Aliquot taken x Sample taken (g)}} \text{x} 100$$

The crude protein content of sample was calculated by multiplying the N content with factor 6.25.

3.2.7.1.4. Ether extracts (EE)

A known quantity dried (1-2 g) of ground sample was taken in Whatman thimble and extracted with petroleum ether (BP: 40-60°C) in a rapid fat extractor (Soxtherm-Gerhardt). After a series of operations like extraction, condensation the flasks were removed and excess of ether was dried in oven for overnight. Again, these flasks were cooled in a desiccator and their weights were taken. The difference in weights of oil flask before and after extraction gave the amount of ether extract or crude fat in the sample.

$$\text{EE (\%)} = \frac{Weight\ of\ the\ ether\ extract\ (g)}{Weight\ of\ the\ sample\ (g)} \times 100$$

3.2.7.1.5. Total ash (TA)

A known quantity of sample was taken in pre-weighed silica crucible. After charring the sample till the smoke disappears, the crucibles were kept in muffle furnace for ignition at 550°C for 2-3 hrs. The ash content was calculated as:

$$\text{Total ash (\%)} = \frac{Weight\ of\ ash\ (g)}{Weight\ of\ the\ sample\ (g)} \times 100$$

3.2.7.2. Apparent digestibility of nutrients

The apparent digestibility of DM, OM, CP, EE and CF were calculated from the difference in the quantity of a particular nutrient in feed and faeces of an animal. This is calculated by the following formula

$$\text{Nutrient digestibility (\%)} = \frac{Nutrient\ intake\ (g) - Nutrient\ excreted\ in\ faeces(g)}{Nutrient\ intake(g)} \text{ x } 100$$

Where,

Nutrient intake (g) = Nutrient in feed offered (g) – Nutrient in residual feed (g)

3.2.8. Study on immune responses in WAR

After 45 days of experimental feeding, the immune responses of WAR were assessed. The cell mediated immunity (CMI) was assessed by foot pad reaction test against sheep red blood cell (RBC) where as Humoral immunity (HI) was assessed by intra peritoneal injection of sheep RBC. The procedure is detailed below.

3.2.8.1. Cell mediated immune response

The CMI was assessed by foot pad reaction method in the end of the feeding trial. The WAR were injected with 100 µL of sheep RBC (0.025×10^9cells) diluted in phosphate

buffer saline (PBS) in the sub-plantar region of the right hind paw. The same region in the left hind paw was injected with 100 µL of PBS which was considered as control. The increase in paw thickness before and after injection was assessed after different time intervals (24 & 48 h). The mean change in the paw thickness was considered as delayed type hypersentivity (DTH) reaction and considered as an index of CMI (Nagalakshmi *et al*., 2015b).

3.2.8.2. Humoral immune response

On 45th day of experimental feeding, WAR were antigenically chellanged with 200 µL of 5% sheep RBC diluted in Alsevar's solution injected intra-peritonially to assess their humoral immune status. On 14th day post injection, animals were sacrificed and blood was collected by heart puncture, stored in sterilized vacutainer tubes for serum separation. Blood samples were kept undisturbed for 2 h to facilitate clotting and eventually centrifuged at 3000 rpm for 20 min at 4ºC. Then, carefully the supernatant clear serum was collected without disturbing the clot and stored at - 20ºC till further analysis. The haemagglutination inhibition (HI) antibody titre against sheep RBC was determined in serum to assess humoral immunity (Kamran Azad *et al*., 2009).

3.2.9. Sacrifice procedure of WAR

After 60 days of experimental feeding, ten rats from each dietary treatment were sacrificed by overdose of ether anaesthesia, and subsequently dissected to collect blood and vital organs (liver, kidney) and femur bone (Fig 3.4.). The samples were washed in phosphate buffer saline (PBS) and stored in 10% formal saline to study the histopathology of the organs. For mineral analysis organs were washed in PBS to remove the blood stains and kept in zip lock covers with proper labelling and stored in - 20ºC till further analysis.

3.2.10. Mineral analysis

The vital organs and bone for mineral analysis were washed in PBS and kept in zip lock covers with proper labelling and stored in - 20ºC till further analysis.

3.2.10.1. Preparation of mineral extract

3.2.10.1.1. Vital organs (liver, kidney), Muscle, feed and faeces

A weighed quantity of completely dried vital organs, feed samples and faeces were taken in pre- weighed silica crucible which was subjected to decarbonisation followed by ashing at 550- 600 ºC in muffle furnace for 5 h. The residue left in the crucible was

considered as total ash, which was digested with 5N HCl on a hot plate. After digestion, the digested samples were filtered through Whatmann filterpaper No. 41 in a volumetric flask depending on the sample, by repeated washing with milliQ water, till it was free from the acid and subsequently, volume was made.

3.2.10.1.2. Bone

Femur bones of the sacrificed WAR were boiled in distilled water at 80ºC for 30 min. and cooled. Then the attached muscles were manually removed from the bone and oven dried at 80ºC for overnight. Then the samples were solvent extracted in petroleum ether at 60ºC for 8 h and oven dried for overnight. Then the bones were subjected to ashing under 550-600ºC for 5h. After ashing, mineral extract was prepared by following the same procedure of vital organs as described above.

3.2.10.1.2. Serum

For the serum samples mineral analysis, 1 mL of the samples was taken and 1 mL of 5N HCl was added to it. Then the volume was made to 10 mL by adding double distilled water (1:10 dilution).

3.2.10.2. Estimation of mineral content by ICP-OES

Mineral contents in feed, faeces, liver, kidney, bone, and serum samples were estimated using Optima 8000 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Perkin Elmer, Shelton CT#064840, USA) as per the operating conditions mentioned in the following Table 3.5.

Minerals standard were prepared from ICP multi element standard solution and volume was made with Merck Millipore to give a concentration of 100 mg/L (stock solution) except for phosphorus, which was prepared from potassium dihydrogen phosphate (KH_2PO_4). The stock solution was used for further dilution to prepare at least 3 standards to know the concentrations for calibration. After calibration, one known sample was estimated to cross check the calibration. Then the unknown samples were analysed. After each sample sufficient flushing was done with Millipore water.

Fig 3.4. Collection of blood and organs from sacrificed Wister albino rats

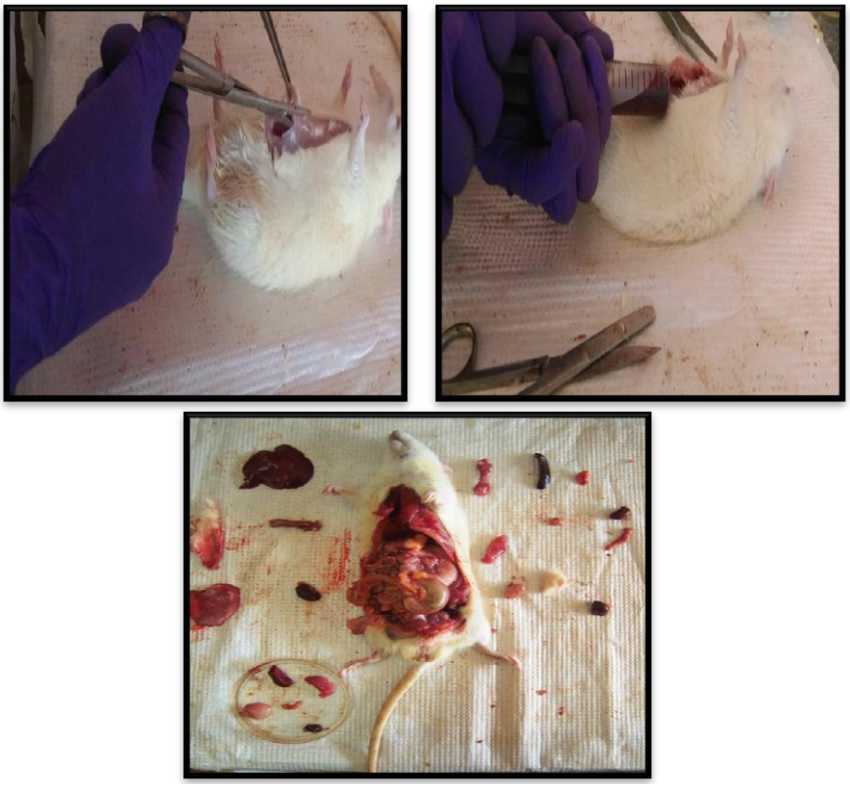


Table 3.5. Working conditions of optima 8000 ICP-OES.

Attributes	**Value**
Nebulization gas flow rate	0.55L/ min
Auxillary gas flow rate	0.2 L/min
Plasma gas flow rate	15 L/min
Sample flow rate	1.5mL/min
Operating power	1300W
View	Axial/ Radial
Sample volume uptake rate	1.0 mL/ min
Spray chamber	Cyclonic
Nebulizer type	Cross flowing
Replicates	2

Table 3.6. Mineral specifications of optima 8000 ICP-OES

Elements	**Wavelength**	**View**
Calcium	317.933	Radial
Phosphorus	213.617	Axial
Magnesium	285.213	Radial
Copper	327.393	Axial
Zinc	206.200	Axial
Manganese	257.610	Axial
Iron	238.50	Axial

3.2.11. Histopathology

A representative portion of liver, kidney, spleen and intestine of each rat sacrificed from different treatment groups were collected and then washed with PBS to wash out the blood stains and then kept in 10% formalin (10 mL of 40% formaldehyde added to 90 mL of distilled water). The tissues were fixed for 48 h and then washed for an hour under running tap water. Then the tissues were dehydrated by increasing concentrations of ethanol (70, 90 and 100%) each for an hour. Then the dehydrated tissues were cleared in xylene for 1 h and this step was repeated twice. Paraffin embedding was carried out by

keeping the tissues in melted paraffin at 56ºC for 3 changes. Longitudinal and transverse sections (5 µm) were made with semi-automatic microtome and were placed on glass slide coated with meyer's egg albumin. Tissue sections were dried by incubating them for 2 h at 40ºC. Rehydration of the fixed sections was carried out in the decreasing concentrations of the alcohol (100, 90, 70 and 50%) each for 1 h followed by water. The sections were stained by haematoxylin and eosin stain as per Bancroft *et al.* (1996) with some modifications. Then the sections were covered with DPX [(N, N-4-xylylene-bis (pyridinium); p-xylene-bis-pyridiniumbromide; p-xylene-bis (N-pyridinium bromide)] (SRL, India) mounting medium with cover glass and observed under light microscope (Nikon eclipse 80i, Japan) to study the histopathological changes.

3.2.12. Expression profiling of genes regulating zinc metabolism and immunity

The liver tissue from each sacrificed rats was collected and washed with DPBS (Sigma.USA). The samples were kept at 4ºC overnight in RNA later (Invitrogen, USA) and subsequently were removed from RNA later and stored in Deep freeze (-80ºC) till further processing.

3.2.12.1. Isolation of total RNA from liver tissues

Total RNA was extracted by using Trizol reagent (Invitrogen, USA) from approximately 50 mg of liver tissues by following the manufacturer's protocol (RNeasy minikit, Quagen, Netherlands). Liver tissues were homogenised using micropestle homogenizer (Tarson's, India) by adding 1 mL of Trizol. Then it was incubated for 5 min at room temperature. Then the homogenised mixture was centrifuged at 12000 rpm for 15 min at 4ºC. The clear aqueous phase was carefully collected without disturbing the sediment and the same was transferred into a micro-centrifuge tube. The volume of the aqueous sample was measured and equal quantity of 70% ethanol was added and mixed well. The whole volume was transferred into RNeasy spin column seated in a collection tube, spun for 30 sec. at 10000 rpm and the flow-through was discarded. Then, 700 µL RW1 buffer was added to spin the column and was centrifuged for 30 sec. at 10000 rpm and the flow-through was discarded. Again, to the RNeasy spin column 500 µL RPE buffer was added and again was centrifuged for 30 sec at 10000 rpm and the flow-through was discarded. Once again, to the RNeasy spin column 500 µL RPE buffer was added and centrifuged for 2 min. at 10000 rpm and the flow-through was discarded. The RNeasy spin column was transferred into a RNase free 1.5 collection tubes provided along with the kit.

RNase free water (30 µL) was added onto the column membrane. Then the sample was allowed to stand in room temperature for 1- 2 min. and subsequently, RNA was eluted by spinning for 1 min. at 10000 rpm. The isolated RNA was stored at -80 ºC until use.

3.2.12.2. Quantification of total RNA

The quantification and purity of total RNA was checked by Nano drop spectrophotometer (Eppendorf D30, Germany). Total RNA (1µL) was used to record the absorbance at 260 and 180 nm wavelengths against nuclease free water which is considered as blank. RNA samples having the ratio of absorbance at OD 260: OD 280 more than 1.8 was considered to contain no protein as impurity and was used for further studies. The samples having lower ratio were extracted once more as above and quantified eventually.

3.2.12.3. Confirmation of RNA by gel electrophoresis

The quantity and integrity of total RNA was checked by using denaturing agarose gel (2%) electrophoresis and visualized under UV light. Two intact bands of 28S and 18S with no smearing indicated good quality and intact RNA.

3.2.12.4. Synthesis of first strand cDNA

The first strand cDNA was synthesized from isolated total RNA. Constant quantity (2.5 µg) of isolated total RNA was reverse transcribed by using SuperScript® VILO ™ Master Mix (Invitrogen, USA). The cDNA synthesis was carried out in 20 µL reaction mixture. For cDNA synthesis, 2.5 µg of total RNA is taken into consideration. From the quantified RNA (done by using nanodrop in ng/µL), required volume of RNA was taken which contains 2.5 µg of total RNA. 4 µL SuperScript 2.5 µg of total RNA ® VILO ™ Master Mix was added to each reaction. Then the volume was made to 20 µL with DEPC treated water. Thus, in each reaction, total RNA and DEPC treated water are the variables. So the following components (Table 3.7.) are added for cDNA synthesis in a sterile PCR tube, kept over ice.

Table 3.7. cDNA synthesis

Sl no	Components	Volume (µL)
1	SuperScript® VILO ™ Master Mix	4
2	Total RNA (2.5 µg)	X
3	DEPC treated water	Y
Total volume		20

X= volume containing 2.5 µg of total RNA

Y=20-4-X i.e. the total volume of the reaction is adjusted to 20µL

The reaction mixture was gently mixed and incubated at 25ºC for 10 min. then, incubated at 42ºC for 60 min and terminated the reaction at 85ºC for 5 min in a thermocycler. The synthesized cDNA was stored at -20ºC until further use.

3.2.12.5. Quantitative Real Time PCR (qPCR)

3.2.12.5.1. Determination of efficiency of primers

The primer amplification efficiencies were determined by running a standard curve for easy assay prior to processing of experimental samples. A standard curve was obtained by serial dilution of the cDNA containing the template and a regression line equation in relation to the threshold value (Ct) was formulated. In order to obtain the best accuracy level, 6 serial dilutions of cDNA for gene under study was taken.

3.2.12.5.2. Protocol for the qPCR

Transcript abundance was measured for the genes under study, i.e. metallothonein (MT) and Superoxide dismutase-1 (SOD1) by using cDNA synthesized from liver tissues of WAR under different dietary treatments. All the samples were studied in triplicate by using qPCR. PCR analysis was done by using the STEP ONE PLUS real time PCR system (Applied Bio Systems, USA), Fast SYBR Green Master Mix (Applied Bio Systems, USA) and gene specific primers for both housekeeping and targeted genes **(Table 3.8)** were used.

Table 3.8. Gene specific primers for both housekeeping and targeted genes

Gene	Primer sequence (5' to 3')	Gene bank ID
GAPDH	F= GATGACATCAAGAAGGTGGTGA R= ACCCTGTTGCTGTAGCCATATT	AF178845.1
*SOD1	F= CTGCAGGACCTCATTTTAATCC R=CTTTCTTCATTTCCACCTTTGC	NM_017050.1
**MT	F= ACCCCAACTGCTCCTGCT R= CGCCTTTGCAGACACAGC	XR_001296756.2

*Superoxide dismutase 1; ** Metallothionein

For each sample the PCR reaction consisted of the following components (Table 3.9).

Table 3.9. Chemicals for Real time PCR reaction

Chemical	GAPDH	SOD1	MT
Sybrgreen	5	5	5
Primer Forward	0.5	0.5	0.25
Primer reverse	0.5	0.5	0.25
cDNA	1	1	1
RNAase free water	3	3	3.5
Total reaction volume	10	10	10

A negative control with no template RNA was also included. The reaction condition included an initial step of 95ºC (20 sec), followed by 40 cycles at 95ºC (03 sec) and 60ºC (25 sec) and a melt curve of 95ºC (15 sec), 60ºC (1min) and 95ºC (15 sec), respectively. The specificity of each PCR product was determined by a melt curve analysis and the amplicon size was determined in 2% agarose gel. qPCR data (Ct values) were analysed by using the $2^{-\Delta\Delta Ct}$ method (Livak and schmittgen, 2001). The ratio of target to reference RNA was used to determine the relative m RNA expression in rats.

3.2.13. Estimation of blood biochemical profiles

The serum samples collected after experimental feeding were analysed to determine the different blood biochemical constituents like ALP, blood urea nitrogen were

done by following the protocols of Erba diagnostic Mannheim GmbH (Germany) by using Alere (AM 2100) Micropate reader by following respective kit protocols (ERBA diagnostics Mannheim GmbH, Germany) and albumin, globulin, total protein, creatinine, AST, ALT done by using M/s. Span Diagnostics Limited, Surat, India. The serum biochemical estimations were carried out using Semiauto analyzer, Biosystems (BTS 320).

3.*2.13.1. Total protein and albumin*

Serum total protein (TP) and albumin were estimated by Biuret and BCG dye binding method (Dumas *et al*., 1971). Serum protein bound to copper ions in an alkaline medium of Biuret reagent and produced a purple colour complex, whose absorbance at 555 nm was proportional to protein concentration. Serum albumin bound to bromocresol green in acidic condition and produced green colour, whose absorbance was measured at 630 nm and the concentration, was expressed as g/dl blood serum.

3.2.13.2. Globulin

It was calculated by subtracting serum albumin from TP and expressed as g/dl blood serum.

3.2.13.3. Albumin to globulin ratio

It is mere the ratio of albumin and globulin in the blood of individual animal.

3.2.13.4. Blood urea nitrogen (BUN)

BUN level in the serum samples were determined by following the methodology of Talke and Schubert (1965) and Tiffany *et al*. (1972) and expressed as mg/dL. Urea is hydrolysed in the presence of water and Urease to produce ammonia and carbon dioxide. In the presence of Glutamate Dehydrogenase (GLDH) and reduced Nicotinamide adenine dinucleotide (NADH), ammonia combines with α-ketoglutarate to produce L-Glutamate. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm as NADH is converted to NAD. The rate of decrease in the absorbance was monitored at 340 nm and is directly proportional to urea concentration in the serum samples. The quantum of BUN in the serum samples were calculated by the following formulae.

Calculation:

$$\text{BUN}\left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\Delta A\ of\ test}{\Delta A\ of\ Standard} \times \text{Conc. of standard (mg/dL)}$$

Where,

$$\Delta A = Absorbance\ of\ test\ sample - absorbance\ of\ blank$$

3.2.13.5. *Creatinine*

Creatinine content in the serum, expressed as mg/dL, was determined by the alkaline picrate method of Bonses and Taussky (1945), where the creatinine in the protein-free solution was allowed to react with alkaline picrate to produce a red colour complex, which was subsequently measured colorimetrically at 520 nm.

3.2.13.6. *Alanine aminotransferase (ALT)*

ALT was estimated by the method described by Reitman and Frankel (1957) using diagnostic kit (manufactured by Span Diagnostic Limited, Surat, India). ALT catalyzed the transfer of amino group from L-alanine to a-ketoglutarate with formation of pyruvate and glutamate. The pyruvate so formed was allowed to react with 2, 4 dinitrophenyl hydrazine to produce 2, 4 dinitrophenyl hydrazone derivative, a brown coloured complex in alkaline medium. The absorbance at 505 nm of this hydrazone derivative is correlated to ALT activity by plotting a calibration curve using pyruvate standard.

$$\text{ALT (IU/L)} = \frac{Test\ absorbance - Control\ absorbance}{Standard\ absorbance - Blank\ absorbance} \times\ standard\ concentration$$

3.2.13.7. *Aspartate aminotransferase (AST)*

AST in blood serum was determined as per the method given by Reitman and Frankel (1957) using diagnostic kits manufactured by Span Diagnostic Limited, Surat, India. AST catalyzed the transfer of amino group from L-asparate to a-ketoglutarate with formation of oxaloacetate and glutamate. The oxaloacetate so formed was allowed to react with 2, 4 DNPH to form 2, 4 dinitrophenyl hydrazone derivative, a brown coloured complex in alkaline medium. The absorbance at 505 nmof this hydrazone derivative was correlated to AST activity by plotting a calibration curve using pyruvate standard.

$$\text{AST (IU/L)} = \frac{Absorbance\ of\ test - Absorbance\ of\ control}{Absorbance\ of\ standard - Absorbance\ of\ blank} \times\ Standard\ concentration$$

3.2.13.8. Alkaline phosphatase (ALP)

ALP (U/L) was estimated in the serum samples by using Wilkinson *et al*. (1969) which is a modification of Bessey *et al*. (1946) method. Alkaline phosphatase in the sample catalyzes the hydrolysis of colourless p-nitrophenyl phosphate to give p-

nitrophenol and inorganic phosphate. At the pH of the assay (alkaline), the p-nitrophenol is in the yellow phenoxide form. The rate of absorbance increase at 405 nm is directly proportional to the alkaline phosphatase activity in the sample. Optimized concentrations of zinc and magnesium ions are present to activate the alkaline phosphatase in the sample**.**

$$(\text{ALP})\text{IU/L} = \frac{(\Delta\text{A/min}) \times \text{TV} \times 10^3}{\text{SV} \times \text{Absorptivity} \times \text{P}}$$

Here,

TV= total volume of reaction (µL); SV= Sample volume (µL); Absorptivity = Milimolar absorptivity of p- nitrophenylphosphate at 405nm =18.8; P= Cuvette light path= 1cm.

So, the final equation was

$$\text{Activity of ALP at } 37^{o}\text{C (IU/L)} = (\Delta\text{A/min}) \times 2713$$

3.2.14. Estimation of hormones

The serum samples collected and preserved at -80 ^{o}C at the end of the experimental feeding were analysed subsequently for the quantity of metabolic hormones with the help of Radio Immuno Assay, using suitable kits. The hormones studied were triidiothyronine (T3), total thyroxine (T4), insulin like growth factor-1 (IGF-1), and testosterone.

The performance characteristics of the above hormone kits are depicted in the Table. 3.10.

Table 3.10. The performance characteristics of hormone kits used

Name of the hormone	Kit Code	Sensitivity		Specificity	Precision (Intra Assay)	Accuracy	
		Analytical	Functional			Dilution test	recovery test
T3	IM1699	0.26 nmole/L	0.49 nmole/L	Highly specific to T3	6.3%	83.6-110%	92.6-106%
T4	IM1447 IM3286	10.63 nmole/L	16.71 nmole/L	Highly specific to T3	3.29%	88.1-112%	81.0-107%
IGF-1	A15729	4.55 ng/mL	9.26 ng/mL	Highly specific to IGF-1	5.6%	87.9-110%	86.4-110%
Testosterone	IM1119	0.02 ng/mL	0.08 ng/mL	Highly specific to testosterone	5.6%	80.8-101%	89.3-106%

3.2.14.1. Estimation of total triidiothyronine (T3), total thyroxine (T4) and testosterone

The radio immune assay of T3, T4 and testosterone is a competition assay, where samples and calibrators are incubated with ^{125}I lebeled T3, T4 and testosterone respectively, as a tracer, in antibody coated tubes. After incubation, the liquid content of the tube is aspirated and the bound radioactivity is determined in a gamma counter. A standard curve is constructed and unknown values are obtained from the curve by interpolation.

Table 3.11. Working Procedure of T3, T4 and total testosterone estimation

Step-1 (Additions)	Step-2 (Incubation)	Step-3 (Counting)
Total triidiothyronine (T3) estimation		
25µL calibrator as the standard/ 25µL sample was added followed by 200µL of tracer was added to each antibody coated tubes and was mixed thoroughly by a vortex.	The Step 1 tubes along with the samples/calibrators were incubated for 1h at 20ºC at 300 rpm.	After step 2 the content in the antibody coated tube was aspirated carefully to remove the unbound tracers and allowed to air dry. Then after drying, the reading was taken in the gamma counter and was expressed as count per minute (CPM)
Total thyroxine (T4) estimation		
20µL calibrator as the standard/ 20µL sample was added followed by 500µL of tracer was added to each antibody coated tubes and was mixed thoroughly by a vortex.	The Step 1 tubes along with the samples/calibrators were incubated for 1h at 20ºC at 300 rpm.	After step 2 the content in the antibody coated tube was aspirated carefully to remove the unbound tracers and allowed to air dry. Then after drying, the reading was taken in the gamma counter and was expressed as count per minute (CPM)
Total testosterone estimation		
50µL calibrator as the standard/ 50µL sample was added followed by 500µL of tracer was added to each antibody coated tubes and was mixed thoroughly by a vortex.	The Step 1 tubes along with the samples/calibrators were incubated for 1h at 37ºC at 100 rpm.	After step 2 the content in the antibody coated tube was aspirated carefully to remove the unbound tracers and allowed to air dry. Then after drying, the reading was taken in the gamma counter and was expressed as count per minute (CPM)
Along with the calibrators, 2 tubes without antibody coating, 2 tubes with antibody coating were run parallel as total count and maximum count respectively, where only tracers are added in both the tubes. The total count tubes were kept in the refrigerator till counting was done whereas tubes for maximum counting were undergone Step 2 and 3 as discussed above.		

3.2.14.2. Estimation of IGF-1

The immunoradiometric assay (IRMA) of IGF-1 is different than other hormones assay in this study. Here the mouse monoclonal antibody is directed against 2 different epitopes of IGF-1 and hence noncompeting are used. In order to release binding IGF-1 from its binding proteins, a prior dissociation is necessary. Samples and calibrators are incubated in tubes coated in first monoclonal antibody in the presence of second monoclonal antibody lebeled with ^{125}I. After incubation the content of the tube is removed and the bound radioactivity is measured. Unknown values are determined by interpolation from a standard curve. The bound radioactivity is directly proportional to the IGF-1 concentration in the serum samples.

Reagents and their preparation

1. Reconstitution of calibrators and control: the contents of calibrators and control vials were brought to room temperature and then distilled water was added as per the indications provided in the manual. Then it was mixed properly without making any foam.
2. Wash solution: the wash solution (50 mL) was added to 950 mL of distilled water and was thoroughly mixed.

Sample processing

Before assay of the IGF-1 in the samples 25 µL of the sample was transferred to a sterilised new 2 mL eppendorf tube. Then, 0.5 mL of dissociation buffer was added and was mixed thoroughly by vortex type mixer.

Assay of IGF-1

Table 3.12. Working Procedure of IGF-1

Step-1 (Additions)	**Step-2 (Incubation)**	**Step-3 (Counting)**
300 µL tracer followed by 50 µL calibrator/ control/ sample was added to the antibody coated tubes. The content of the tube was mixed by a vortex.	The Step 1 tubes along with the samples/calibrators were incubated for 1 h at 22ºC at 190 rpm.	After step 2 the content in the antibody coated tube was aspirated carefully to remove the unbound tracers, and subsequently were washed twice with 2 mL of the wash solution prepared as above and were allowed to air dry. Then after drying, the reading was taken in the gamma counter and was expressed as count per minute (CPM)
Along with the calibrators, 2 tubes without antibody coating, 2 tubes with antibody coating were run parallel as total count and maximum count respectively, where only tracers are added in both the tubes. The total count tubes were kept in the refrigerator till counting was done whereas tubes for maximum counting were undergone Step 2 and 3 as discussed above.		

3.3. Phase III: Feeding of the synthesized NZn to goats on animal performance in terms of growth, immunity, nutrient utilization, blood biochemical and hormonal profiles and mineral absorption.

An animal experiment, on goats was conducted in Experimental Livestock Unit, ICAR-NIANP, Bengaluru, India. This experiment protocol was approved (F.No. 25/8/2016-CPCSEA (part-I) dated 20.05.2016 by Committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi after recommendation by Institutional Animal Ethics Committee (IAEC), ICAR-NIANP, Bangalore.. This

experiment was carried out as per the guidelines of CPCSEA, Ministry of Environment, Forests and Climate Change, Government of India, New Delhi, India.

3.3.1. Animal distribution, housing and management

Twenty four non-descriptive breed of goats of average body weight 18.7±0.33 kg were divided into four groups of six each based on their body weight. Goats were maintained under uniform management conditions throughout the experimental period. Goats were housed in a well ventilated shelter with individual feeding and watering facilities. All the goats were dewormed and vaccinated against enterotoxemia and *peste des petites ruminats* (PPR). The experiment was carried out for a period of 130 days (20 days adaptation period and 110 days feeding period). During this experimental period, growth rate, feed intake, digestibility trial, immunological studies were carried out and samples collected for haematological, blood biochemical and ruminal fermentation parameters as per the prescribed procedure

Table. 3.13. Experimental details of Goat trial

Attributes	NC	IZn-50	NZn-50	NZn-25
No. of Animals	6	6	6	6
Zn source	No	Inorganic ZnO	Nano ZnO	Nano ZnO
Zinc level	Basal diet (BD)	ICAR, 2013b (BD +50 ppm)		½ ICAR, 2013b (BD +25 ppm)

Initially, animals were individually fed with measured quantities of respective TMR (ICAR, 2013b) in the morning (9.30 AM) but as the residue was more, animals were fed with concentrate and roughage separately after calculating the DM requirement based on their body weight. Change of this feeding practice was followed by an adaptation period of 7 more days and thereafter the trial was initiated. Their rationwas composed of ragi straw and concentrate at 50:50 ratio. The residue was collected and weighed daily. A representative residue sample from each animal was pooled and DM was analysed every week to know the DMI. Animals were having access to *ad libitum* clean wholesome water throughout the experiment period.

3.3.2. Experimental feeding regime

3.3.2.1. Ration composition

An experimental concentrate was prepared by using the locally available feed ingredients (Table 3.14). The experimental ration consisted at 50% concentrate and 50% roughage. As the roughage source, ragi straw (finger millet straw) was chaffed and fed to the animal. All the animals were fed at 3% of their body weight throughout the experiment period, which was adjusted at every fortnight after weighing the animals. A mineral mixture (Table 3.15) was prepared as per the recommendations laid by ICAR (2013b) except that of the zinc. All the animals under different treatment groups were provided with the same experimental ration quantified as per their body weight, only variable being the source and quantity of zinc which was fed orally as a paper capsule (cellulose paper, 75 GSM).

Table 3.14. Composition of the concentrate mixture used for feeding goats

Ingredients	Quantity (kg/ 100kg)
Maize	40
Deoiled soyabean meal	35
Deoiled rice bran	22
Mineral Mixture*	2
Common Salt (NaCl)	1

*Mineral Mixture Composition- Table 3.15.

Table 3.15. Chemical composition of mineral mixture for experimental feeding

Chemicals	Quantity (kg/100kg)
Di-calcium phosphate (Ca_2PO_4)	50
Calcite ($CaCO_3$)	20
Magnesium sulphate ($MgSO_4$)	12
Manganese sulphate ($MnSO_4.H_2O$)	1.5
Zinc oxide (ZnO)	**
Copper sulphate ($CuSO_4.5H_2O$)	0.6
Cobalt chloride ($CoCl_2$)	0.01
Ferrous sulphate ($FeSO_4.7H_2O$)	1.3
Sodium selenite (Na_2SeO_3)	0.004
Common salt (NaCl)	14.59
Total	100.00

*** Zinc was provided as paper capsule to the experimental goats individually according to the experimental design*

3.3.2.2. Preparation of experimental ration

Ragi straw was chaffed with chaff cutter to 2-3 cm length, which was the only source of roughage to the goats. For preparing concentrate, maize was ground separately and mixed with the pre-weighed amounts of deoiled soyabean meal and deoiled rice bran (Table 3.14). Then adequate amount of mineral mixture and common salt was added to the ration, and then thoroughly mixed for 30 min with feed mill mixer available in the Experimental Livestock Unit, ICAR-NIANP. After thorough mixing the concentrate mixture was stored in airtight plastic tubs with a cover. The concentrate mixture was prepared in batches of 200 kg to reduce the possible sample variation. This concentrate mixture was fed to all the groups along with straw.

3.3.2.3. Experimental feeding practices

Twenty four goats were allotted to four different dietary treatments having six animals each (Table 3.15). Animals were offered with weighed amount of straw and concentrate to meet their nutrient requirements (ICAR, 2013b). The goats were individually offered a measured quantity of concentrate followed by ragi straw se in the morning (9.30 AM). Offered and refusals of ration from all the goats were weighed daily

and sampled at weekly intervals for subsequent analysis of dry matter to assess the average dry mater intake during the experimental period (Fig 3.5.). The ration requirement was adjusted with fortnightly record of body weight. Clean and wholesome drinking was made available to all the goats round the clock *ad libitum*.

3.3.3. Weighing of animals, average daily gain and feed efficiency

All the experimental goats were weighed initially to record the initial body weight (Fig 3.5.). Subsequently, throughout the experiment, goats were weighed at fortnight interval in the morning before feeding ration and watering in order to assess the change in body weight and average daily gain.

Absolute rate of growth was estimated by using the following formula

$$\text{Absolute rate of growth} = (W2 - W1)/T$$

where,

W2 = Final body weight,

W1 = Initial body weight, and

T = Duration of Experiment (in days)

The feed conversion ratio (kg DM intake/ kg live weight gain) was calculated for all the groups.

3.3.4. Digestibility Trial

After 75 days of experimental feeding, a digestibility trial of 8 days (3 days adaptation and 5 days collection period) was conducted to study the nutrient utilization in these animals (Fig 3.6.). During the collection period, animals were offered as usual at 9.30 AM. The daily feed refusals were weighed and representative sample was kept for DM analysis to accurately know the daily DM intake. The total amount of faeces voided per 24 h was collected quantitatively by using faecal bags tied to the individual animal, and then it was weighed and recorded. All the animals were weighed before and after the digestion trial.

3.3.4.1. Collection and processing of different samples

3.3.4.1.1. Feed offered and feed refusal

Representative samples of experimental ration (concentrate, straw) offered, feed refusal was collected and their DM was analysed daily. The dried representative samples stored in zip lock cover for determination of its nutrient content. At the end of the trial, all

Fig 3.5. Regular farm practices during goat feeding trial

a) Feeding of zinc oxide

b) Weighing of feed before offering

c) Weighing of animals

Fig 3.6. Practices during digestion trial on goats

a) Individual feeding of goats

b) Faecal bags for quantitative faecal collection

c) Quantitative collection of faecal matter

d) Sampling of faecal matter for analysis

the samples were ground to 1 mm screen size and preserved in self sealing covers for further analysis.

3.3.4.1.2. Faecal samples

Faecal sample from each animal was collected separately by using faecal collection bags. The faeces voided during 24h were collected in a self sealing zip lock cover to prevent the moisture loss. The total amount of faeces voided by each animal in 24 h duration was weighed at 9.30 AM every day. Then the samples were mixed thoroughly and ground to make a uniform sample. A representative sample was taken in a zip lock cover and $1/10^{th}$ of the total voided sample was kept for DM analysis and $1/100^{th}$ of total voided faeces was moistened by adding 20% of Sulphuric acid for estimation of total nitrogen (N), and subsequently stored in deep freeze (-20ºC) till further analysis.

For DM analysis $1/10^{th}$ of total voided faeces was taken in a pre weighed petridish and kept for drying at 80 ºC for 24 h in hot air oven. The loss in the weight referred to the moisture content of the faeces. Each day during the collection period, the dried faecal samples were pooled in a self sealing zip lock cover and stored for further analysis.

3.3.5. Estimation of proximate principles and nutrient digestibility

The proximate principles of feed and faeces were done as per the protocol described in 3.2.7.1. The apparent nutrient digestibility was measured by following the protocols described in the section 3.2.7.2.

3.3.5.1. Analysis of fibre fraction

Feeds and faeces samples were analyzed for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) as per the method of Van Soest *et al.* (1991).

3.3.5.1.1. Neutral detergent fibre (NDF)

About 1g of ground feed/faeces sample was taken in a 600 mL spoutless beaker. To it 100 mL of neutral detergent solution and 2mL decalin were added and refluxed for one hour after the initiation of boiling. Contents were filtered through pre-weighed sintered glass crucible (Grade-1) and washed repeatedly with hot water followed by 2 washings with acetone. The sintered crucible containing residue was then kept in hot air oven (100±5ºC) for overnight drying and weighed after cooling it in a desiccator. The

difference in the weight of crucible plusresidue and that of empty crucible was recorded as NDF and expressed on DM basis.

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

3.3.5.1.2. Acid detergent fibre (ADF)

About 1.0 g of dried ground feed/faeces sample was taken in a 600 mL spoutless beaker and to it 100 mL of acid detergent solution was added. Beaker contents were refluxed for 1h after onset of boiling. The contents of the beaker were then filtered through a preweighed sintered glass crucible and washed several times with hot water followed by two washings with acetone.The crucible was dried in a hot air oven at 100±5ºC overnight and weighed after cooling it in a desiccator to know the amount of acid detergent fibre (ADF).

$$\text{ADF (\%)} = \frac{\text{Weight of ADF}}{\text{Weight of sample (DM basis)}} \times 100$$

3.3.5.1.3. Acid detergent lignin (ADL)

In a sintered glass crucible, containing ADF, a small quantity of 72% H_2SO_4 was added. The lump formed by ADF was broken with the help of a glass rod to form a paste. Acid was allowed to drain through the crucible and after draining, it was refilled with 72% H_2SO_4 for 3 more times. The glass rod was kept in the crucible and stirring was done at hourly intervals. The contents of crucible were washed several times with hot water to make it acid free and then dried overnight in hot air oven at 100±5ºC. Crucible was weighed after coolingit in a desiccator. Then, it was ashed in a muffle furnace at 550-600^{0}C for 3 hrs, cooled and weighed. ADL (%) was calculated as follows:

$$\text{ADL (\%)} = \frac{\text{Weight of crucible and lignin- Weight of crucible and ash}}{\text{Weight of sample (DM basis)}} \times 100$$

3.3.5.1.4. Cellulose

Cellulose was determined by subtracting ADL from ADF.

$$\text{Cellulose (\%)} = \text{ADF (\%)} - \text{ADL (\%)}$$

3.3.5.1.5. Hemicellulose

Hemiellulose was determined by subtracting ADF from NDF.

3.3.6. Assessment of immunity in goats

After 75 days of experimental feeding, CMI was assessed in the goats through *in vivo* cutaneous DTH reaction against Concanavalin-A (Con A; Sigma C2010, 097K7670) and humoral immunity was done by antibody titre against the PPR vaccine. The detailed procedure is described below.

3.3.6.1. Cell mediated immune response (CMI)

The CMI was assessed in the goats through *in vivo* cutaneous DTH reaction against Concanavalin-A (Con A).

3.3.6.1.1. Preparation of mitogen (Con-A)

A solution of Con A was prepared by dissolving in autoclaved normal saline at a concentration of 150 µg/mL. The solution was stored in ice box before use. Each animal was injected with 30 µL of mitogen in one side and other side was injected with equal volume of normal saline water which served as blank.

3.3.6.1.2. Procedure

In the both side of neck of the goats were cleaned with soap, then shaved with the help of a razor 24 h before injecting the mitogen so that inflammatory swelling, if any, which might be due to abrasion or injury during shaving would subside. An area of about 1 cm^2 was encircled with a marker pen and a point was made at the site of injection on the both side of neck. The thickness of the skin was measured with the help of a vernier callipers, which would represent the 0h value. 0.2 mL (30 µg/mL of con A) was injected intra-dermally with the help of 1 mL intra-dermal needle in the left side of all the goats and 0.2 mL of normal was injected in the right side of neck which served as control. The thickness of the injected area was measured at 6 h, 12 h, 24 h and 48 h post injection. The change in the thickness of skin represented the CMI of the animal.

$$\text{Thickness (mm)} = \text{Thickness in ConA injected side} - \text{Thickness in NS injected side}$$

3.3.6.2. Humoral immune response (HI)

After 75 days of experimental feeding, all the goats were challenged with foreign antigen i.e. subcutaneous injection of PPR vaccine (Institute of Animal Health and

Veterinary Biological, Bangalore, Karnataka) to elicit the antibody production. Peste Des Petits Ruminants (PPR) vaccine (1 mL) containing 10^3 tissue culture infective dose 50 (TCID50) subcutaneous route was administered to all the Goats. After 14 days of injection blood was collected from jugular vein puncture in vacutainer tubes for separation of serum. Then the blood samples were kept undisturbed for 2 h to facilitate clotting, and then centrifuged at 3000 rpm at 4ºC for 20 min. the clear supernatant (sera) was collected and stored in deep freeze (-20ºC) till further use. Antibody titre against PPR vaccine was determined competitive enzyme linked immune sorbent assay (ELISA) kit of the Indian Veterinary Research Institute, Izzatnagar, UP, India (Singh *et al*., 2004). The values were expressed as per cent of inhibition value. The methodology and analysis was carried out as reported earlier (Krishnamoorthy *et al*., 2014).

3.3.6.3. Immunophenotyping of mice blood by flow cytometry

The T-lymphocyte subsets were distinguished using dual antibody analysis. The method followed was as per Miao *et al.* (2007) with slight modifications. The CD_4^+ and CD_8^+ T-lymphocytes in goat blood were determined by using flow cytometry FACS CANTO-II (BD Biosciences, USA).

3.3.6.3.1. Materials

1. The monoclonal anti-mouse CD_3 antibodies conjugated with eFlour 450, anti-mouse CD4 antibodies conjugated with Fluoroscein Isothiocyanate (FITC) and anti-mouse CD8a antibodies conjugated with Phylloerythrin (PE) were procured from eBioscience, USA.
2. Flow cytometry tubes (BD Biosciences, USA)
3. Sheath fluid (BD Biosciences, USA)
4. Lysis buffer – 1X (BD Biosciences, USA) – Prepared 10x lysis buffers by adding nine mL of distilled water to1 mL of 1x lysis buffer
5. Sterile distilled water
6. Sterile phosphate buffer saline (PBS) – pH 7.4
7. Micro pipette and micro tips (10 µL, 50 µL and 1000 µL)

3.3.6.3.2. Procedure

1. Taken 50 µL of dipotassium EDTA added blood in four flow cytometry tubes.
2. Added 10 µL anti-mouse CD3 antibodies conjugated with eFlour 450 in first tube, anti-mouse CD4 antibody in second tube, 10 µL anti-mouse CD8 antibody in third and 10 µL

of both anti-mouse CD3, CD4 and CD8 in fourth tube and the fifth tube was served as unstained control.

3. The above tubes were used for setting up the volts, compensation in flow cytometry for taking cell counts of test samples.

4. In the test sample tubes added 10 µL of anti-mouse CD3, 10 µL of anti-mouse CD4 and 10 µL of anti-mouse CD8 in each tube.

5. Incubated the tubes in dark at room temperature for 30 min.

6. Added 2 mL of 10X lysis buffer to each tube and mixed properly.

7. Incubated the tubes for 10 min at room temperature.

8. After incubation, centrifuged the tubes at 1200 rpm for 10 min.

9. Discarded the supernatant solution.

10. Added 2 mL of sterile PBS to each tube.

11. Centrifuged the tubes at 1200 rpm for 10 min.

12. Suspended the pellet in 400 µL of sheath fluid and recorded the cell counts in flow cytometry.

13. Data acquisition and post acquisition analysis was conducted using FACS Diva software version 6.1.3 (BD Biosciences, USA).

14. The percentage of CD4+ and CD8+ T-lymphocytes were calculated after counting 10,000 events or cells per sample by flow cytometry.

15. The CD4/CD8 ratio was calculated by dividing the percentage of CD4+ cells by percentage of CD8+ cells.

3.3.7. Haematology of goats

The blood was collected by jugular vein puncture before feeding on 90^{th} day of experimental feeding and 2 mL was transferred to a heparinised vacutainer tubes and 5 mL was transferred into a 10 mL vacutainer tube for separation of serum to assess the haematological parameters. Then the heparinised blood samples were analysed for its haematological parameters by using auto-analyser (Erba chem 5 plus, Germany). And the serum samples were prepared by following the standard protocol (discussed in the section 3.3.6) and stored in deep freezer (-80ºC) till analysis of blood biochemistry and hormones.

3.3.8. Biochemical parameters

The serum samples collected on zero and 90th day of experimental feeding were analysed to determine the different blood biochemical constituents by following the respective kit protocol as described in section 3.2.13.

3.3.9. Hormonal analysis of goat serum samples

The serum samples collected on 0 day and 90th day of experimental feeding were analysed for the total triidiothyronine (T3), total thyroxine (T4), insulin like growth factor-1 (IGF-1), and testosterone hormones by using Radio immune assay kits by following the protocols as described in section 3.2.14.

3.3.10. Rumen fermentation attributes

3.3.10.1. Collection of rumen liquor

From each animal, rumen liquor was collected on 0 day and 90th day of experimental feeding. The rumen liquor was collected by creating a negative pressure through suction by a hand operated pump. The animals were fed with their daily allowance early in the morning and had access to water. Two hours prior to collection the water sources were removed in order to prevent dilution of the ruminal contents and at the same time to give a uniform picture of rumen fermentation. One smooth and soft oesophageal tube was inserted into their mouth and negative pressure was created by hand operated vacuum pump. Due to negative pressure, the ruminal content was drawn out and was collected into the sample box attached to the hand pump. Then the sample was transferred to a clean and autoclaved container and stored in an ice box till further processing. Each time the sample collection box was rinsed with water before collection of each sample.

3.3.10.2. Ruminal pH

After the collection of the rumen liquor, it was analysed to know the ruminal pH at the earliest by using the Eutech digital pH meter after calibrating it. The pH of the rumen liquor was noted for 0 and 90th day samples.

3.3.10.3. Ammonia nitrogen content

The ammonia nitrogen (NH_3-N) was estimated in the collected rumen liquor samples by following the protocol described by Park *et al*., (2009) and Rao and Soren (2015). Determination of NH_3-N is based on the coloured reaction between phenolic derivatives/ phenol and ammonia through two steps. Finally ammonia reacts with

hypochlorite to convert into monocholoramine at optimal pH of 10. Then the sequence of continuous reaction between monocholoramine and phenolic compound occurs under suitable catalyst (nitropruside) to create strongly coloured Indophenols at the optimal pH in the range of 12 to13.

Reagents:

1. Solution A: 1.0 g of phenol and 5mg of sodium nitroprusside was added to distilled water in a 100 mL volumetric flask and the volume was adjusted to 100 mL with distilled water.
2. Solution B: 0.5 g sodium hydroxide and 0.84 mL of sodium hypochlorite was added with distilled water in two separate beakers and then transferred into a 100 mL volumetric flask and the volume was adjusted to 100 mL with distilled water.
3. Standard Solution: 0.048g of ammonium sulphate was dissolved in distilled water and transferred into a 100 mL volumetric flask and then the volume was adjusted to 100 mL with distilled water. This is called as stock solution having the concentration of 10mg NH_3-N per 100 mL solution.

From the above stock solution 10 mL was taken in a 100 mL volumetric flask and the volume was adjusted to 100 mL and called as working standard solution (WSS).

3.3.10.3.1. Protocol

The samples were vortexed vigorously to mix properly and subsequently were incubated at 39ºC for 15 min. for development of colour. Then the absorbance was recorded at 625 nm against reagent blank with the help of spectrophotometer (Hitachi, U2900). A calibration curve was plotted against standard NH_3-N concentration and NH_3-N concentration in the samples was calculated from the standard curve.

Table 3.16. Procedure for estimation of a*mmonia nitrogen content*

Attributes	**Standards**								**Sample**
Tube No	1 (Blank)	2	3	4	5	6	7	8	9 (Test)
Distilled water (µL)	500	475	450	400	300	200	100	0	490
WSS (µL)	0	25	50	100	200	300	400	500	-
Test Sample (µL)	-	-	-	-	-	-	-	-	10
NH_3-N (µg)	0.00	0.25	0.50	1.00	2.00	3.00	4.00	5.00	unknown
Solution A (µL)	2500	2500	2500	2500	2500	2500	2500	2500	2500
Solution B (µL)	2500	2500	2500	2500	2500	2500	2500	2500	2500

3.3.10.4. Individual volatile fatty acid profile

Rumen liquor was collected and filtered through four layer of muslin cloth and then 4 mL strained rumen liquor was treated with 1 mL of 25% meta-phosphoric acid in a centrifuge tube and kept for 30 min. at room temperature to precipitate the proteins. Thereafter, it was centrifuged at 10000 rpm for 20 min. at 40ºC and clear supernatant used for the individual VFA estimation using gas chromatograph (Agilent- 7890A) equipped with flame ionization detector (FID) and column (Agilent J&W DB-WAX GC Column 40m x 0.18mm x 0.18µm). Injector and detector temperatures were set at 2500ºC and 2800ºC, respectively. Initial and final column temperature was set at 500ºC and 2300ºC, respectively. The flow rate of carrier gas (nitrogen) through the column was 1 mL/min; and the flow rate of hydrogen and air through FID was 40 and 400 mL/ min., respectively. Vials containing the standard and the samples were placed into the sampler and the sequences were noted. Different VFA present in the samples were identified on the basis of their retention time and their concentration was calculated by recording the areas of

both the VFAs mixed standard and the sample, dilution factor (DF) and expressed as mM/L.

$$\text{Concentration of sample (mM/L)} = \frac{\text{Area of the sample x Concentration of standard x DF}}{\text{Area of the standard}}$$

3.3.*10.5. Rumen liquor soluble mineral content*

After the straining with double layered muslin cloth, 2 mL of rumen liquor was taken in a 2 mL eppendorf tube and centrifuged at 4ºC at 12000 rpm for 30 min. Then, carefully clear apical liquid was taken without disturbing the sediments. From this 1 mL of clear rumen aliquot was taken and 1 mL 2% HNO_3 was added and mixed well. Then final volume was made to 10 mL by adding double distilled water and again mixed to ensure thorough mixing. Then the mineral content was estimated by ICP-OES as described in the section 3.2.10.2.

3.3.11. Male reproduction parameters

The semen from the goats was collected by electro ejaculator method after 90 days of experimental feeding (Fig 3.7).

3.3.11.1. Collection of semen

The semen was collected by using electro ejaculator (Bailey electroejaculator, Western Instrument, Co, Colorado State, USA) in the morning (9-11 AM) before feeding and watering the animals. The hair around the genitalia was clipped and cleaned with rectified spirit before each collection to avoid cross contamination with debris and dust. Artificial stimulation was given to the animals to facilitate sperm collection. Then elector-ejaculator was inserted per-rectally and massaged to stimulate the animals. Then shock was given after a certain interval to collect semen. After collection, the semen is maintained at 37 ±0.5 ºC and transferred to the lab for further analysis of sperm kinetic and integrity parameters**.**

3.3.11.2. Assessment of sperm kinetic parameters

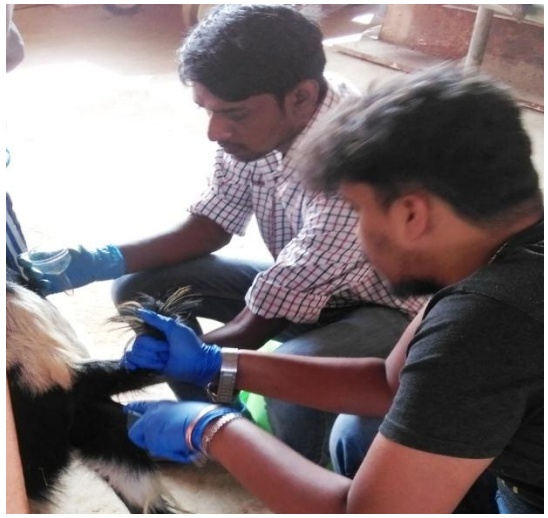
The sperm kinetic parameters of freshly collected semen were analyzed using computer assisted semen analyzer (CASA - Sperm Class Analyzer, Microptic, Barcelona, Spain). The conditions set for motility and velocity parameters were: static, <10; slow, >10 to <25; medium, >25 to <50 and rapid, >50 µm/sec; to obtain different sperm kinetic parameters. The semen samples were diluted in PBS (5-8 x 10^6cells/mL) and 5 µL was placed on to plain glass slide (Himedia, Mumbai, India) and covered with cover slip

(18x18 mm). Twenty five consecutive frames were captured under 100 x magnifications from different homogenous fields and analyzed for sperm kinetic parameters.

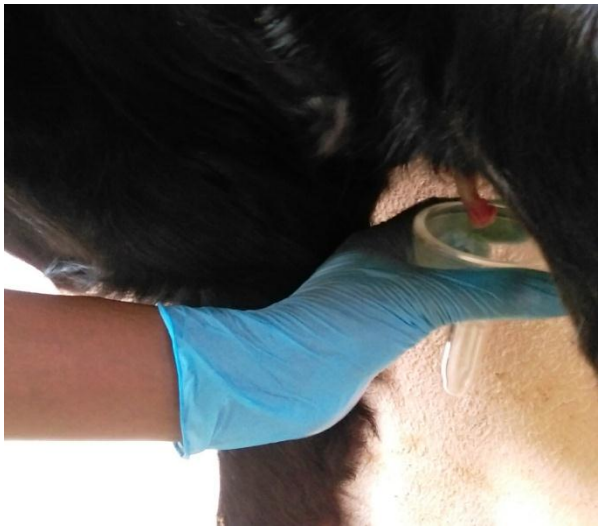
3.4. Statistical Analysis

Data obtained on various parameters were subjected to one way analysis of variance (Snedecor and Cochran, 1994). The statistical software SPSS (SPSS Inc., Chicago, IL, USA) was used for analysis of data and analysis of variance assuming for independent constant variance structure with post-hoc Duncan to find the pair wise significance between treatments. Results will be expressed as mean ± S.E. A P-value of less than or equal to 0.05 will be accepted to indicate statistical significance. Student's t-test was performed to compare the means of initial and final values of blood biochemical and hormonal analysis.

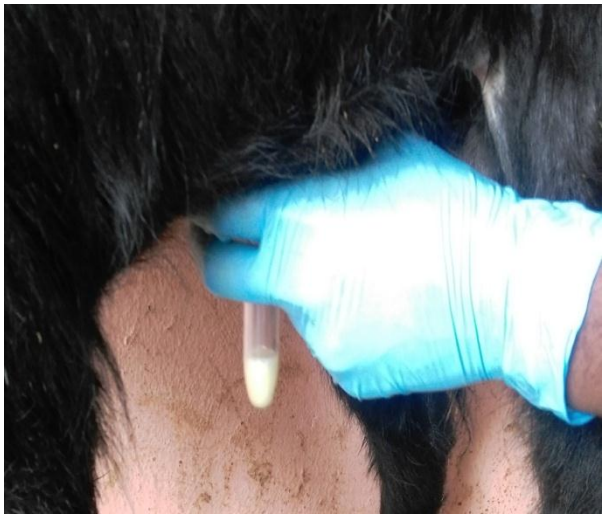
Fig 3.7. Collection of semen from goats by using elector-ejaculator



a) Cleaning of area around penis and massage with electo-ejaculator



b) Penile erection iduced during collection of semen



c) Quantitative collection of semen from doner goats for analysis

RESULTS

In this section, the results obtained during this study were divided into three phases. In Phase-I, results pertaining to characterization of synthesized zinc nanoparticles (NPs) is briefed whereas in Phase-II and Phase-III, results related to different biological effects of NP in rats and goats are described.

Phase-I. Characterization of nano zinc (NZn) particle

The synthesized NZn particles were characterized to know the particle size at Department of Nano Science and Technology, TNAU, Coimbatore.

4.1.2.1. Particle size analysis

The average particle size of synthesized NZn particles was found to be 195.9 nm in physical method (Fig. 4.1a.), whereas the same was found to be 129.5 nm in chemical method (Fig. 4.1b.). The particle size of inorganic commercial ZnO used during animal feeding was also characterised to know its particle size (Fig. 4.1c.) but it could not be detected by the particle size analyser as it was larger than its detection ability of 10000 nm.

4.1.2.3. X- ray diffractometer (XRD)

X-Ray diffractogram is represented in Fig 4.2a. The particle size was 74 nm from XRD.

4.1.2.4. Raman spectrophotometer

Raman spectrophotometer was employed to identify the composition of a mixture of unknown compounds based on elastic scattering. Thus this is an indicative of purity of the samples. Raman spectrophotometer graph is depicted in Fig. 4.2b.

4.1.2.5. Transmission electron microscope (TEM)

The TEM pictures of NZn are presented in Fig. 4.3a. This shows that the synthesized ZnO was rod shaped and has a dimension of around 40 nm scales.

4.1.2.6. Energy dispersive X-Ray spectroscopy (EDAX)

EDAX is an analytical technique used for the elemental analysis or chemical characterization of a sample. The graph (Fig 4.2b.) confirms that our synthesized products were zinc oxide and other above characterization confirmed it to be in nano scale. Thus we have successfully synthesized zinc NP using chemical method.

4.2. Phase-II: To evaluate the effect of different doses of nano zinc on growth, nutrient utilization and expression of selected genes in Wistar Albino Rats

In this phase an *in vivo* trial was conducted on rats to study the effect of supplementation of NZn synthesized in our lab and characterised thereof, on growth, nutrient utilization, immunity, expression of selected genes in Wistar Albino Rats.

4.2.1. Proximate and mineral composition

Proximate and mineral composition of semi-purified synthetic rat diet is presented in Table 4.2.1.

The dry matter (%) of the semi-purified synthetic diet was in a range of 96.8 to 97.3 in NZn-50 to NZn-25, respectively. The organic matter, crude protein, ether extract and total carbohydrate (%) was found to vary in a range of 97.17 (IZn-25) to 97.30 (NZn-3.125), 17.6 (IZn-25, NZn-3.125, NZn-50) to 18.5 (NC), 4.06 (NZn-6.25) to 4.49 (NZn-25), and 74.5 (NC) to 75.6 (NZn-3.125), respectively.

Macro-mineral i.e. calcium, phosphorus content (%) was found to be in the range of 0.62 (NC) to 0.69 (NZn-6.25), and 0.67 (NC, NZn-12.5, NZn-3.125) to 0.70 (NZn-6.25), respectively. However, magnesium content was found to be 0.07 % in all the groups.

The analytical level of zinc (mg/kg) in the diets of different groups was recorded to be 9.35 in NC, 34.3 in IZn-25, 34.1 in NZn-25, 22.2 in NZn-12.5, 16.1 in NZn-6.25, 12.6 in NZn-3.125 and 60.2 in NZn-50, respectively. Copper, manganese and iron content (mg/kg) in the rat feeds were recorded in the range of 4.70 (NZn-3.125) to 5.50 (NZn-25 and NZn-6.25), 48.4 (NZn-25) to 55.7 (NZn-3.125) and 71.8 (NZn-25) to 85.4 (NZn-6.25).

Fig 4.1. Characterization of synthesized NZn particles by particle size analyser

Measurement Results

Date	:	Tuesday, June 16, 2015 11:03:58 AM
Measurement Type	:	Particle Size
Sample Name	:	ZnO 1
Scattering Angle	:	90
Temperature of the holder	:	25.0 deg. C
T% before meas.	:	24281
Viscosity of the dispersion medium	:	1.083 mPa.s
Form Of Distribution	:	|Standard|
Representation of result	:	Scattering Light Intensity
Count rate	:	934 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	197.2 nm	15.5 nm	195.9 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	197.2 nm	15.5 nm	195.9 nm

Cumulant Operations

Z-Average	:	10645.1 nm
PI	:	10.130

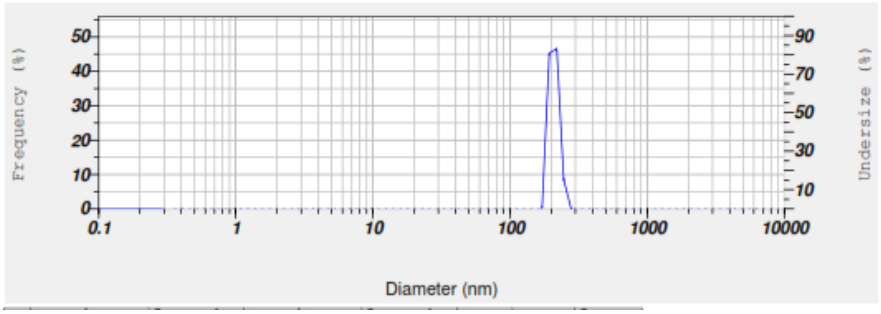


Fig 4.1. a. Particle Size measurements of Synthesized NZn by physical method

Measurement Results

Date	:	Wednesday, June 17, 2015 12:21:27 PM
Measurement Type	:	Particle Size
Sample Name	:	ZnO new 3
Scattering Angle	:	90
Temperature of the holder	:	30.0 deg. C
T% before meas.	:	22228
Viscosity of the dispersion medium	:	0.800 mPa.s
Form Of Distribution	:	|Standard|
Representation of result	:	Scattering Light Intensity
Count rate	:	782 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	131.1 nm	7.5 nm	129.5 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	131.1 nm	7.5 nm	129.5 nm

Cumulant Operations

Z-Average	:	12594.8 nm
PI	:	7.472

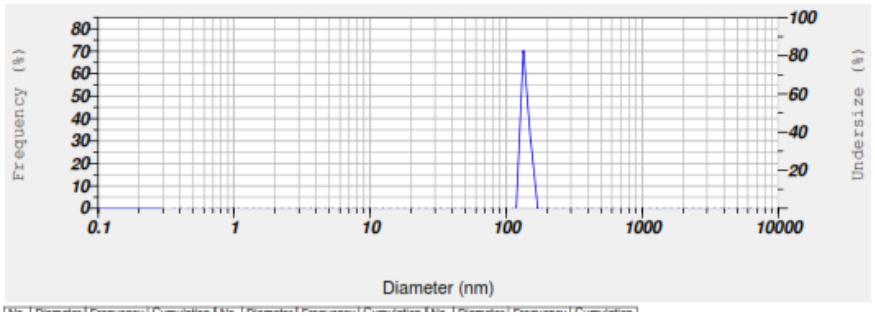


Fig 4.1. b. Particle Size measurements of Synthesized NZn by chemical method

Measurement Results

Date	:	Monday, June 15, 2015 3:50:19 PM
Measurement Type	:	Particle Size
Sample Name	:	ZnO 5
Scattering Angle	:	173
Temperature of the holder	:	25.2 deg. C
T% before meas.	:	0
Viscosity of the dispersion medium	:	1.080 mPa.s
Form Of Distribution	:	|Standard|
Representation of result	:	Scattering Light Intensity
Count rate	:	678 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	---	--- nm	--- nm	--- nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	---	--- nm	--- nm	--- nm

Cumulant Operations

Z-Average	:	16572.9 nm
PI	:	15.255

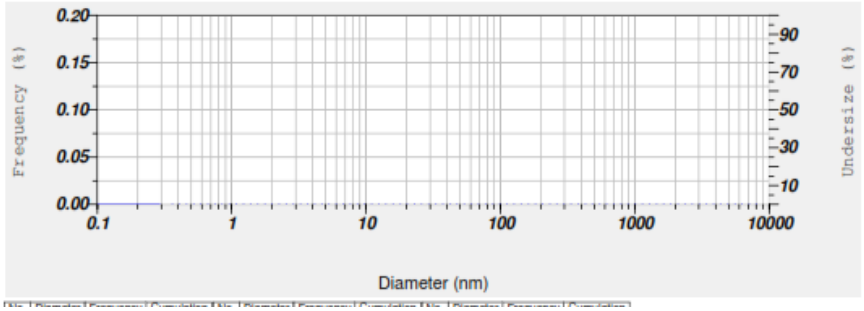
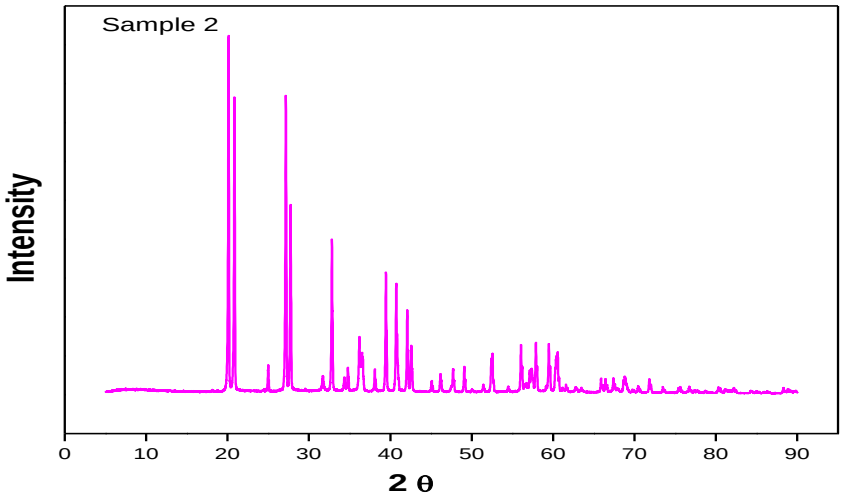
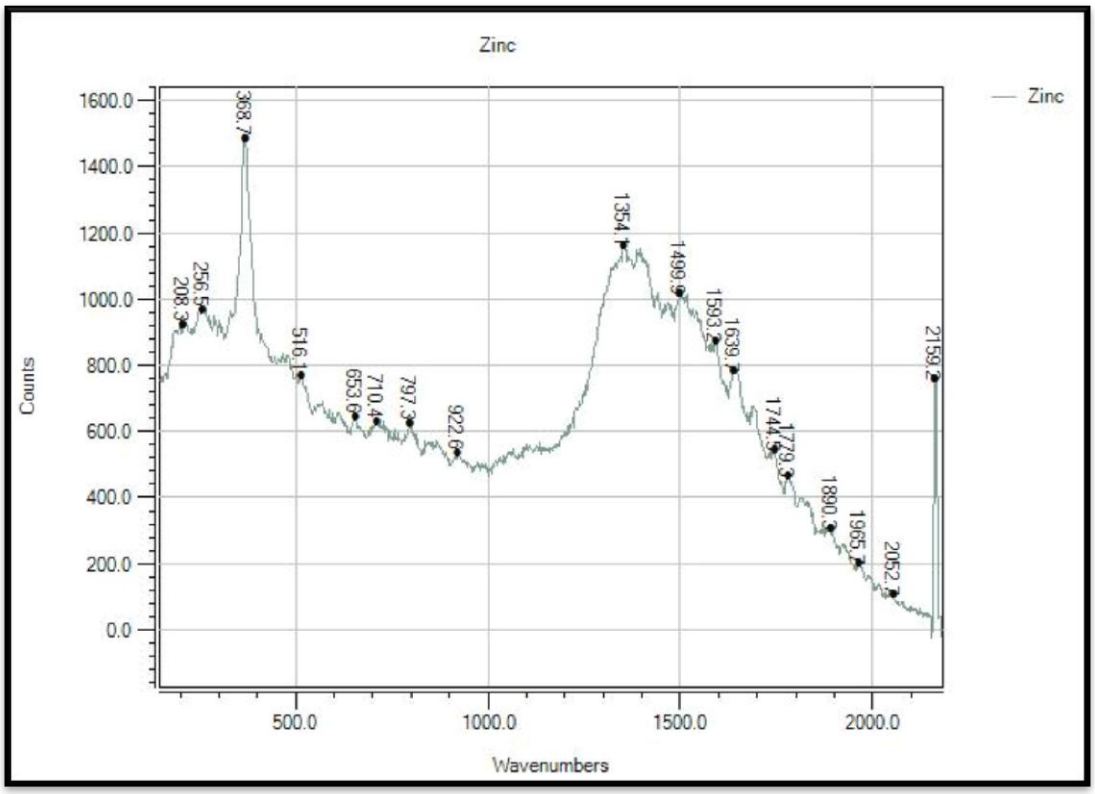


Fig 4.1(c). Particle Size measurements of commertial inorganic ZnO

Fig 4.2. Characterization of synthesized NZn particles by XRD and Raman spectroscopy

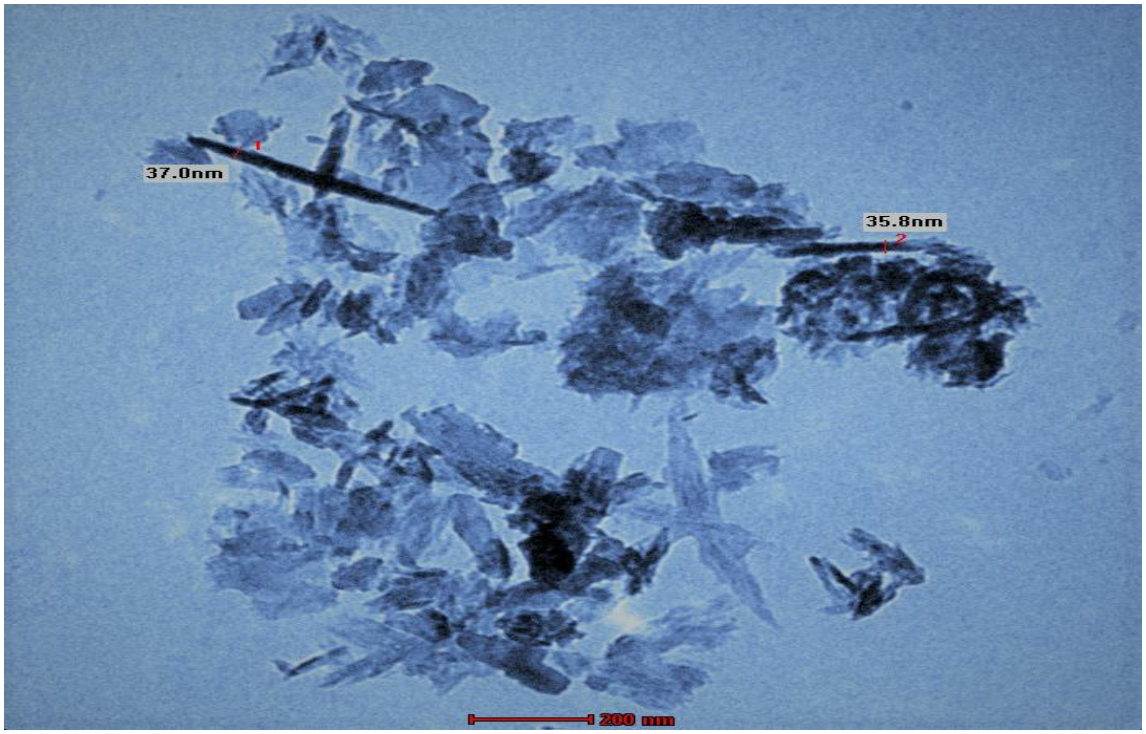


a) X- Ray Diffractometer graph of synthesized NZn particles

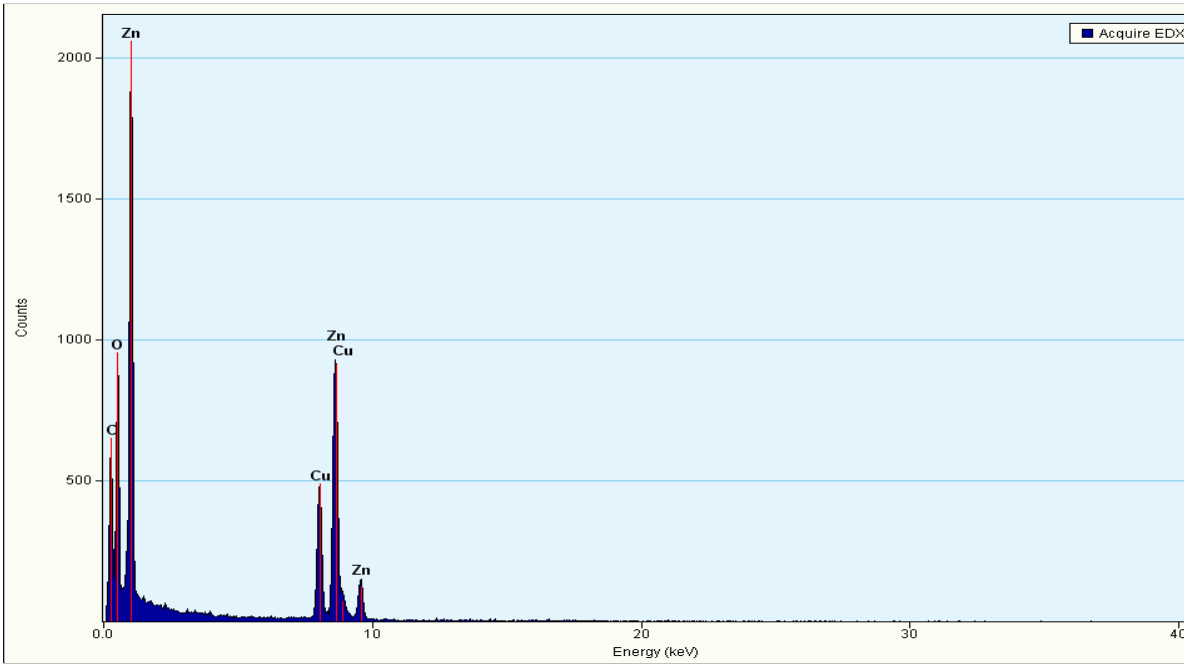


b) Raman spectrophotometer graph of synthesized NZn particles

Fig 4.3. Characterization of synthesized NZn particles by using TEM-EDX



(a) Transmission electron microscope picture of synthesized NZn particles



(b) Energy dispersive X-Ray spectroscopy (EDAX) of synthesized NZn particles

Table 4.2.1. Proximate and mineral composition of semi-purified synthetic rat diet

Attributes	NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50
Proximate principles (%)							
Dry Matter	97.1	97.0	97.3	97.0	96.9	96.8	96.9
Organic Matter	97.22	97.17	97.22	97.19	97.19	97.30	97.21
Crude Protein	18.5	17.6	17.7	18.3	17.9	17.6	17.6
Ether Extract	4.21	4.11	4.49	4.10	4.06	4.09	4.47
Total carbohydrates	74.5	75.5	75.0	74.8	75.2	75.6	75.1
Macro Minerals (%)							
Calcium	0.62	0.63	0.64	0.63	0.69	0.67	0.66
Magnesium	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Phosphorus	0.67	0.68	0.69	0.67	0.70	0.67	0.68
Micro Minerals (mg/kg)							
Zinc	9.35	34.3	34.1	22.2	16.1	12.6	60.2
Copper	5.25	5.24	5.50	5.02	5.50	4.70	5.16
Manganese	53.9	53.7	48.4	54.7	53.7	55.7	54.2
Iron	76.1	77.7	71.8	81.1	85.4	74.2	75.3

4.2.2. Growth performance and feed intake of Wistar albino rats

Effect of supplementation of graded doses of NZn on growth and feed intake of Wistar albino rats is depicted in table 4.2.2.

The average initial and final weight/ rat (g) was similar (P>0.05) among the treatment groups which varied in a range of 65.4±3.42 (NZn-3.125) to 72.8±2.31 (NZn-50) and 332.82±15.21 (NC) to 373.22±18.25 (NZn-25), respectively. Total weight gain in different treatment groups was non-significant (P>0.05), and varied from 261±15.4 (NC) to 308±18.8 (NZn-25). ADG (g) varied in a range of 4.14±0.24 (NC) to 4.89±0.30 (NZn-25), which was statistically similar in different groups (Fig 4.4.). Average daily intake (g/d/rat) was also found similar (P>0.05) among the treatment groups (Fig 4.5.) and varied within a range of 14.9±0.92 (NZn-25) to 16.0±0.42 (NC). However, FCR (Fig 4.6) was found to be statistically (P<0.05) differing among the treatment groups with NZn-25 (3.06±0.18) was more efficient than NC (3.88±0.17), NZn-6.25 (3.53±0.14) and NZnO-3.12 (3.53±0.13).

Thus NZn-25 group had significantly better (P<0.05) FCR compared to NC, NZn-6.25 and NZn-3.125 groups. NZn-12.5 group had comparable FCR with that of IZn-25, N Zn-25 and NZn-50.

4.2.3. Nutrient digestibility

Effect of supplementation of graded doses of NZn on nutrient digestibility in Wistar albino rats is presented in Table 4.2.3.

The DM intake (g/d), DM digested (g/d) and DM digestibility (%) in rats were similar (P>0.05) among the groups. The DM intake varied in a range of 15.0±1.25 (IZn-25) to 17.5±0.72 (NZn-50), whereas DM digested (g/d) varied in a range of 13.7±1.18 (IZn-25) to 16.0±0.68 (NZn-50). The DM digestibility (%) varied from NZn-3.125 (91.1±0.07) to IZn-25 (91.3±0.29). Similarly, OM intake (g/d), OM digested (g/d) and OM digestibility (%) varied non-significantly (P>0.05) among the dietary treatment groups which varied in a range of 13.4±1.12 (NZn-50) to 15.6±0.65 (IZn-25), 12.3±1.03 (IZn-25) to 14.3±0.61 (NZn-50) and 91.5±0.13 (NZn-3.125) to 92.1±0.21 (NC), respectively.

No significant variation was observed in CP intake (g/d), digested (g/d) in rats which varied between 2.63±0.22 (IZn-25) to 3.08±0.13 (NZn-50) and 2.43±0.21 (IZn-25) to 2.82±0.12 (NZn-50), respectively. However, the CP digestibility (%) was statistically higher (P<0.001) in NZn-25 (92.4±0.10) than NC (90.3±0.38). NC (90.3±0.38) had

Fig 4.4. Effect of supplementation of graded doses of NZn on average daily gain (g) in rats

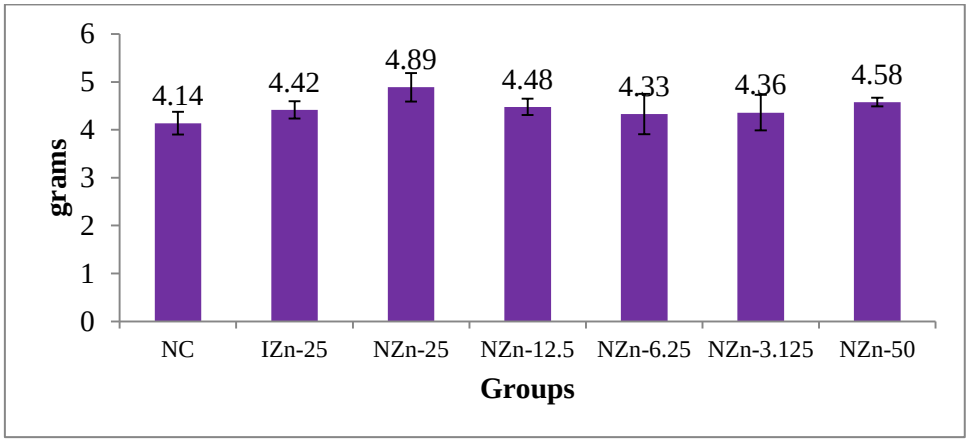


Fig 4.5. Effect of supplementation of graded doses of NZn on average daily feed intake in rats

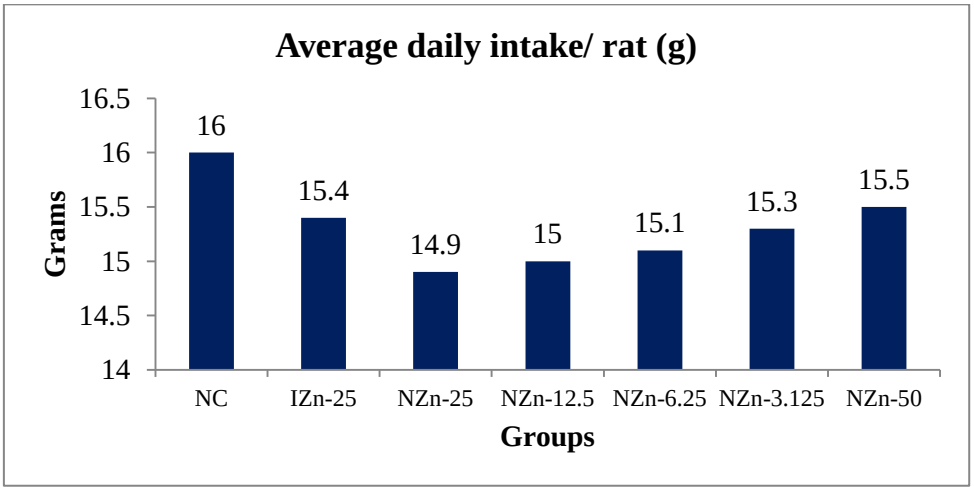
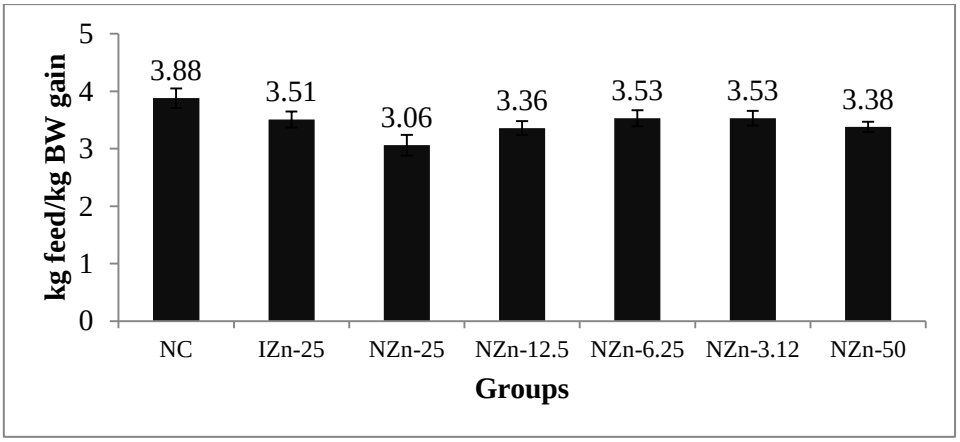


Fig 4.6. Effect of supplementation of graded doses of NZn on feed conversion ratio in rats



significantly lower protein digestibility than IZn-25 (92.1±0.22), NZn-12.5 (92.1±0.39) and NZn-50 (91.7±0.30).

The EE intake (g/d), digestibility (%) of offered to the rats were similar (P>0.05) among the groups whereas EE digested (g/d) varied statistically (P<0.05). EE intake (g/d) varied in a range of 0.62±0.05 in IZn-25 to 0.78±0.03 in NZn-50. The EE digested was higher in NZn-50 (0.72±0.03) than NZn-3.125 (0.72±0.03) and IZn-25 (0.57±0.05). Digestibility (%) of EE in the rat diets found to vary from 90.3±1.02 (NC) to 93.0±0.60 (NZn-12.5).

NZn supplementation at half dose (NZn-12.5) caused similar protein digestibility compared full dose (NZn-25.0) and double dose (NZn-50).

Table 4.2.2. Effect of feeding graded doses of NZn on growth and feed intake of Wistar albino rats

Attributes	NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50	SEM	P
Average initial weight/ rat (g)	71.8 ±0.22	72.0 ±0.91	65.4 ±3.17	66.0 ±2.99	67.9 ±0.91	65.4 ±3.42	72.8 ±2.31	0.98	0.100
Average final weight/ rat (g)	333 ±15.2	350 ±10.9	373 ±18.2	348 ±8.7	341 ±26.9	340 ±21.3	361 ±7.4	6.14	0.671
Weight gain (g)	261 ±15.4	278 ±11.2	308 ±18.8	283 ±11.0	273 ±26.7	275 ±23.0	289 ±5.84	6.36	0.644
Average daily gain (g)	4.14 ±0.24	4.42 ±0.18	4.89 ±0.30	4.48 ±0.17	4.33 ±0.42	4.36 ±0.37	4.58 ±0.09	0.10	0.644
Average daily intake/ rat (g)	16.0 ±0.42	15.4 ±0.15	14.9 ±0.92	15.0 ±0.29	15.1 ±0.90	15.3 ±0.82	15.5 ±0.24	0.22	0.895
Feed conversion ratio	3.88^{a} ±0.17	3.51^{abc} ±0.14	3.06^{c} ±0.18	3.36^{bc} ±0.12	3.53^{ab} ±0.14	3.53^{ab} ±0.13	3.38^{bc} ±0.09	0.06	0.027

$^{a, b,c}$ Means with different superscripts within a row differ significantly

Table 4.2.3. Effect of supplementation of graded levels of NZn on nutrient digestibility in Wistar albino rats

Nutrient		NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50	SEM	P
Dry matter	Intake (g/d)	17.0 ±0.63	15.0 ±1.25	16.7 ±0.92	16.7 ±0.25	16.5 ±0.83	15.9 ±0.98	17.5 ±0.72	0.32	0.501
	Digested (g/d)	15.5 ±0.56	13.7 ±1.18	15.2 ±0.83	15.2 ±0.24	15.1 ±0.74	14.5 ±0.89	16.0 ±0.68	0.29	0.507
	Digestibility (%)	91.2 ±0.19	91.3 ±0.29	91.3 ±0.14	91.2 ±0.23	91.2 ±0.16	91.1 ±0.07	91.3 ±0.21	0.07	0.976
Organic matter	Intake (g/d)	15.2 ±0.56	13.4 ±1.12	14.9 ±0.82	14.9 ±0.23	14.8 ±0.75	14.2 ±0.88	15.6 ±0.65	0.28	0.503
	Digested (g/d)	14.0 ±0.50	12.3 ±1.03	13.7 ±0.73	13.7 ±0.21	13.6 ±0.68	13.0 ±0.80	14.3 ±0.61	0.26	0.474
	Digestibility (%)	92.1 ±0.21	91.7 ±0.24	91.7 ±0.19	92.1 ±0.16	91.8 ±0.10	91.5 ±0.13	91.8 ±0.17	0.07	0.342
Crude protein	Intake (g/d)	3.05 ±0.11	2.63 ±0.22	2.96 ±0.16	3.04 ±0.05	2.96 ±0.15	2.79 ±0.17	3.08 ±0.13	0.06	0.365
	Digested (g/d)	2.75 ±0.10	2.43 ±0.21	2.74 ±0.15	2.80 ±0.04	2.70 ±0.14	2.54 ±0.16	2.82 ±0.12	0.05	0.412
	Digestibility (%)	90.3^{c} ±0.38	92.1^{ab} ±0.22	92.4^{a} ±0.10	92.1^{ab} ±0.39	91.2^{bc} ±0.45	91.1^{bc} ±0.24	91.7^{ab} ±0.30	0.17	0.002
Ether extract	Intake (g/d)	0.69 ±0.02	0.62 ±0.05	0.75 ±0.04	0.68 ±0.01	0.67 ±0.03	0.65 ±0.04	0.78 ±0.03	0.02	0.054
	Digested (g/d)	0.62^{abc} ±0.02	0.57^{c} ±0.05	0.70^{ab} ±0.04	0.64^{abc} ±0.01	0.61^{abc} ±0.03	0.59^{bc} ±0.04	0.72^{a} ±0.03	0.01	0.042
	Digestibility (%)	90.3 ±1.02	92.8 ±0.66	92.9 ±0.30	93.0 ±0.60	91.1 ±0.47	91.2 ±0.43	91.7 ±0.86	0.29	0.055

4.2.4. Blood biochemical and enzymes profiles

Effect of feeding graded doses of NZn on blood biochemical and enzymes profiles of Wistar albino rats is presented in the Table 4.2.4.

The total protein (g/dL) varied significantly ($P<0.001$) among the treatment groups. Total protein was found to be higher in NZn-50(8.59±0.26), NZn-25 (8.62±0.23), NZn-12.5 (8.26±0.09) than NZn-3.125 (7.52±0.21), NZn-6.25 (7.47±0.22) and NC (7.09±0.11), whereas IZn-25 (8.08±0.24) was significantly higher ($P<0.001$) than NC but varied non-significantly with other groups. Albumin (g/dL) also followed the same trend across all the treatment groups with NZn-50 (4.38±0.11), NZn-25 (4.48±0.10) and NZn-12.5 (4.34±0.10) had significantly higher ($P<0.001$) albumin levels in blood than NC (3.75±0.14), NZn-6.25 (3.86±0.20) and NZn-3.125 (3.94±0.09). However, IZn-25 (4.21±0.14) had significantly higher ($P<0.001$) in albumin level than NC but was similar to all other groups. Globulin (g/dl) content was also statistically higher ($P<0.05$) in NZn-25 (4.14±0.25) and NZn-50 (4.21±0.24) as compared to NC (3.34±0.21) whereas other groups were found similar. The A/G ratio was found similar ($P>0.05$) across all the treatment groups which varied within a range of NZn-6.25 (1.09±0.09) to NC (1.16±0.11).

BUN (mg/dL) and creatinine (mg/dL) were not affected by zinc supplementation as they were statistically similar ($P>0.05$) across the treatment groups. BUN (mg/dL) fell in the range of NZn-25 (12.56±0.92) to NZn-6.25 (17.83±1.14) whereas, creatinine ranged from NZn-3.125 (0.43±0.02) to NZn-50 (0.52±0.04). The ALT (U/L) varied significantly ($P<0.05$) among the treatment groups with NZn-12.5 (37.9±1.30) recorded higher ALT than NZn-3.125 (29.6±1.85) and NC (29.1±1.59). AST and ALP (U/L) were found to be same ($P>0.05$) among dietary groups in a range from 76.4±1.59 (NZn-3.125) to 85.7±4.19 (NZn-50) and 146±14.6 (NC) to 162±22.4 (NZn-12.5), respectively.

Beneficial effects of NZn supplementation at graded levels (50, 25, 12.5 mg/kg) was documented in terms of increased total protein, albumin and globulin (g/dL) contents.

Table 4.2.4. Effect of feeding graded doses of NZn on blood biochemical and enzymes profiles

Parameters	NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50	SEM	P
Total Protein (g/dL)	7.09^{c} ±0.11	8.08^{ab} ±0.24	8.62^{a} ±0.23	8.26^{a} ±0.09	7.47^{bc} ±0.22	7.52^{bc} ±0.21	8.59^{a} ±0.26	0.11	0.000
Albumin (g/dL)	3.75^{c} ±0.14	4.21^{ab} ±0.14	4.48^{a} ±0.10	4.34^{a} ±0.10	3.86^{bc} ±0.20	3.94^{bc} ±0.09	4.38^{a} ±0.11	0.06	0.001
Globulin (g/dl)	3.34^{b} ±0.21	3.88^{ab} ±0.15	4.14^{a} ±0.25	3.92^{ab} ±0.15	3.61^{ab} ±0.20	3.59^{ab} ±0.16	4.21^{a} ±0.24	0.08	0.038
A/G ratio	1.16 ±0.11	1.15 ±0.03	1.11 ±0.08	1.13 ±0.07	1.09 ±0.09	1.11 ±0.05	1.09 ±0.04	0.03	0.988
BUN (mg/dl)	15.45 ±1.40	16.63 ±1.55	12.56 ±0.92	17.72 ±0.88	17.83 ±1.14	16.17 ±1.19	16.02 ±0.47	0.49	0.053
Creatinine (mg/dl)	0.48 ±0.02	0.44 ±0.04	0.47 ± 0.02	0.46 ±0.05	0.45 ±0.03	0.43 ±0.02	0.52 ±0.04	0.01	0.525
AST (U/L)	80.1 ±3.63	77.9 ±1.91	85.0 ±1.31	84.0 ±4.28	79.5 ±6.13	76.4 ±1.59	85.7 ±4.19	1.34	0.367
ALT (U/L)	29.1^{b} ±1.59	31.8^{ab} ±1.86	35.0^{ab} ±2.20	37.9^{a} ±1.30	34.7^{ab} ±2.40	29.6^{b} ±1.85	32.9^{ab} ±2.59	0.84	0.041
ALP (U/L)	146 ±14.6	157 ±11.2	148 ±19.2	162 ±22.4	155 ±8.28	162 ±12.9	158 ±16.8	5.51	0.986

^{abc} Mean with different superscripts in a row varies significantly (P<0.05)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline Phosphatase; A/G ratio: Albumin/globulin ratio; BUN: Blood Urea Nitrogen

4.2.5. Serum hormone profiles

Effect of supplementation of graded doses of NZn on serum hormone profile in Wistar albino rats is presented in table 4.2.5 and Fig 4.7.

Triiodothyronine (T_3) level (nmol/L) was similar (P>0.05) among the treatment groups which ranged from 0.72±0.22 (IZn-25) to 0.98±0.18 (NC), whereas Thyroxine (T4) varied statistically (P<0.01) among the treatment groups. T_4 (nmol/L) in NZn-50 (36.1±3.13) was significantly higher than NC (15.0±1.47), IZn-25(26.3±1.77), NZn-12.5 (25.8±2.79), NZn-6.25 (24.1±2.69) and NZn-3.125 (24.9±4.18), whereas similar to NZn-25 (33.5 ±4.71). Similarly, except NZn-6.25, NC had lower (P<0.05) serum T_4 levels than all other groups. Testosterone (ng/mL) level was altered significantly (P<0.05) due to dietary supplementation of zinc. NZn-50 (1.40±0.37) had significantly higher (P<0.01) testosterone level than NC (0.39±0.04), IZn-25 (0.70±0.06), NZn-12.5 (0.71±0.12), NZn-6.25 (0.57±0.05) and NZn-3.125 (0.43±0.13), whereas similar (P>0.05) to NZn-25 (1.16 ±0.36). In case of insulin like growth factor-1 (IGF-1), all the groups were similar statistically (P>0.05) and ranged from 69.5±7.36 in NC to 86.6±10.7 in NZn-25.

NZn supplementation at two levels i.e at 25 and 50 mg/ kg (NZn-50 and NZn-25 groups) had improved Thyroxine (T4) and testosterone concentrations than NC. Here, reduced dose of supplementation (NZn-12.5) had similar thyroxine (T4) levels compared to higher inorganic Zn (IZn-25) supplementation.

4.2.6. Immunity status

4.2.6.1. Cell mediated immunity

Effect of supplementation of graded levels of NZn on cell mediated immunity in Wistar albino rats measured as skin fold thickness (mm) is tabulated in Table 4.2.6.

The delayed type hypersensitivity (DTH) reaction, which is an indication of cell mediated immune response varied significantly among the treatment groups at 24 and 48 h post injection with sheep RBC (Fig 4.8). NZn-50 and NZn-25 had more (P<0.001) net skin thickness than NC (0.40±0.16), NZn-6.25 (1.00±0.10) and NZn-3.125 (0.72±0.08) at 24 h of injection where as NC showed least amongst all. After 48h of injection, NZn-25 (1.51±0.22) had statistically higher (P<0.001) foot pad thickness (mm) than NC (0.37±0.23), IZn-25 (0.98±0.19), NZn-6.25 (0.46±0.09) and NZn-3.125 (0.47±0.12), and is similar to NZn-12.5 (1.03±0.10) and NZn-50 (1.44±0.14). However, IZn-25 had statistically showed higher immune responses than NC (0.37±0.23), NZn-6.25 (0.46±0.09) and NZn-3.125 (0.47±0.12).

4.2.6.2. Humoral immunity

Effect of supplementation of graded levels of NZn on humoral immune response in Wistar albino rats measured as Haemagglutination titre (log_2) against Sheep RBCs is given in Table 4.2.6.

NZn-50 (3.87± 0.12) had higher (P<0.05) Haemagglutination titre (log_2) against Sheep RBCs compared to NC (2.83±0.17), IZn-25 (3.29±0.18), NZn-12.5 (3.27± 0.17), NZn-6.25 (3.17± 0.22) and NZn-3.125 (3.17±0.17). NZn-50 and NZn-25 groups had significantly higher (P<0.05) humoral immune response than NC.

Thus NZn supplementation at two levels (50 and 25 mg/kg) had resulted in significant improvement in cell mediated as well as humoral immune status in Wistar albino rats. Comparable responses in cell mediated immunity were also noted in rats received NZn at half dose (NZn-12.5) with that full dose IZn-25.

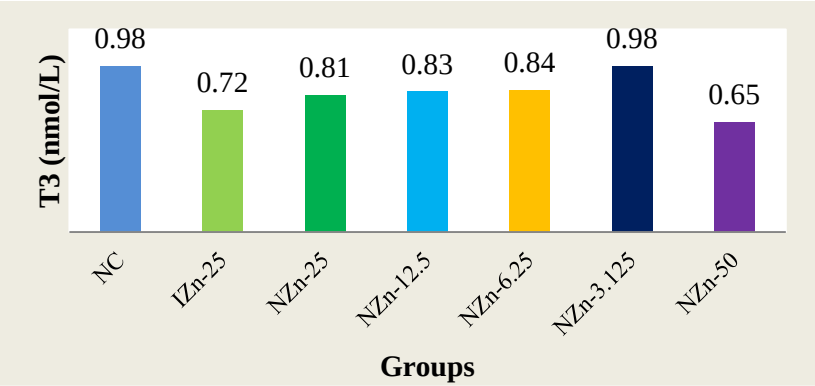
4.2.7. Micro mineral profiles of (mg/kg) visceral organ

Effect of supplementation of graded levels of NZn on visceral organ micro mineral profiles (mg/kg) of Wistar albino rats is presented in table 4.2.7 and Fig 4.9.

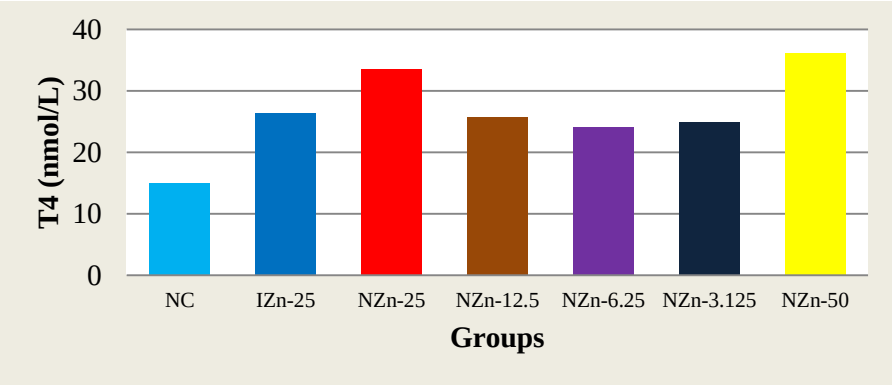
4.2.7.1. Liver

In liver, zinc content (mg/kg) varied significantly (P<0.05), whereas iron, manganese and copper were found similar (P>0.05) among the groups. The liver zinc (mg/kg) was higher (P<0.05) in NZn-50 (100±2.44) than NC (79.6±3.36), NZn-6.25 (86.0±3.77), NZn-3.125 (84.3±3.17) but was found similar to IZn-25 (90.4±4.39), NZn-25(96.5±6.65) and NZn-12.5 (91.5±3.30). Iron (mg/kg) varied (P>0.05) in a range of 262±21.2 (NC) to 374±39.6 (NZn-50). Manganese in liver also did not vary with the dietary treatment and ranged from 6.31±0.69 (NZn-25) to 7.62±0.83 (NC). Similarly, copper ranged from 10.73±1.80 (NZn-12.5) to 13.34±1.35 (NZn-3.125), which was similar (P>0.05) among the groups.

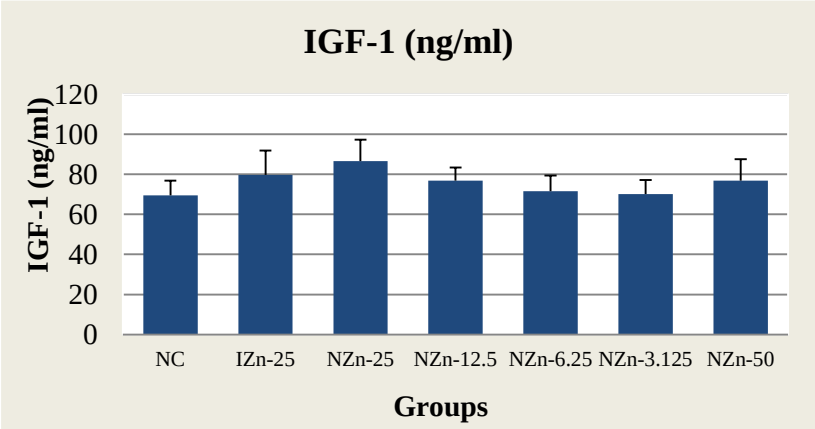
Fig 4.7. Supplementation of graded doses of NZn on zinc level on serum hormonal profile in rats



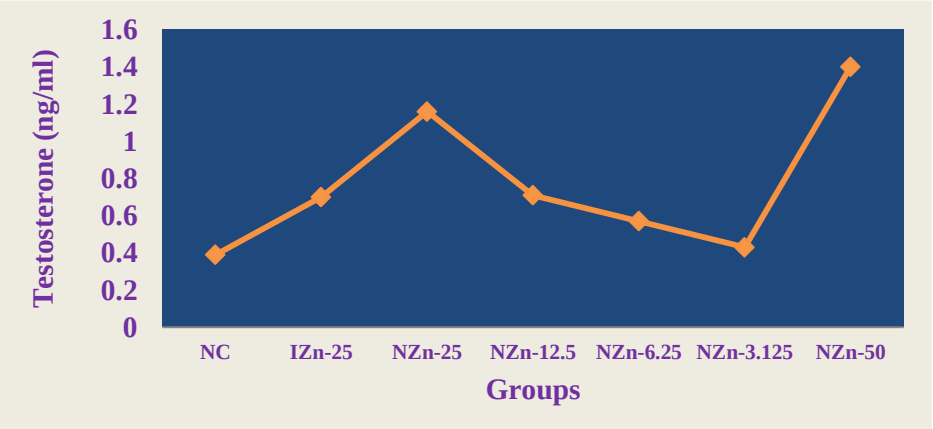
a) T_3 (nmol/L)



b) T_4 (nmol/L)

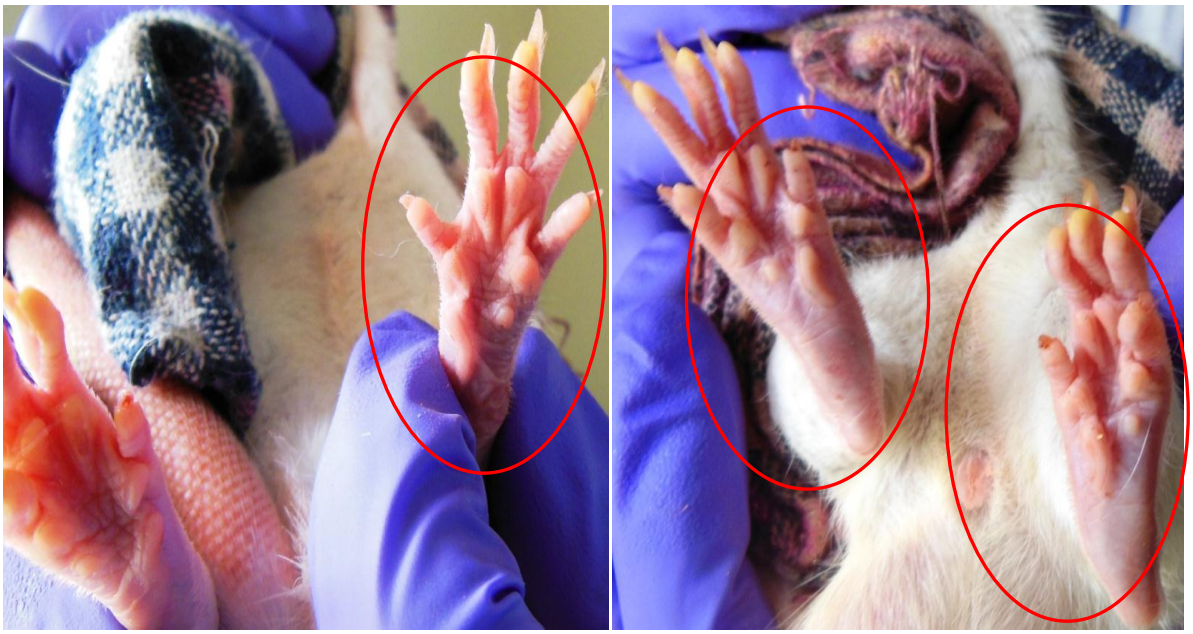


c) IGF-1 (ng/mL)

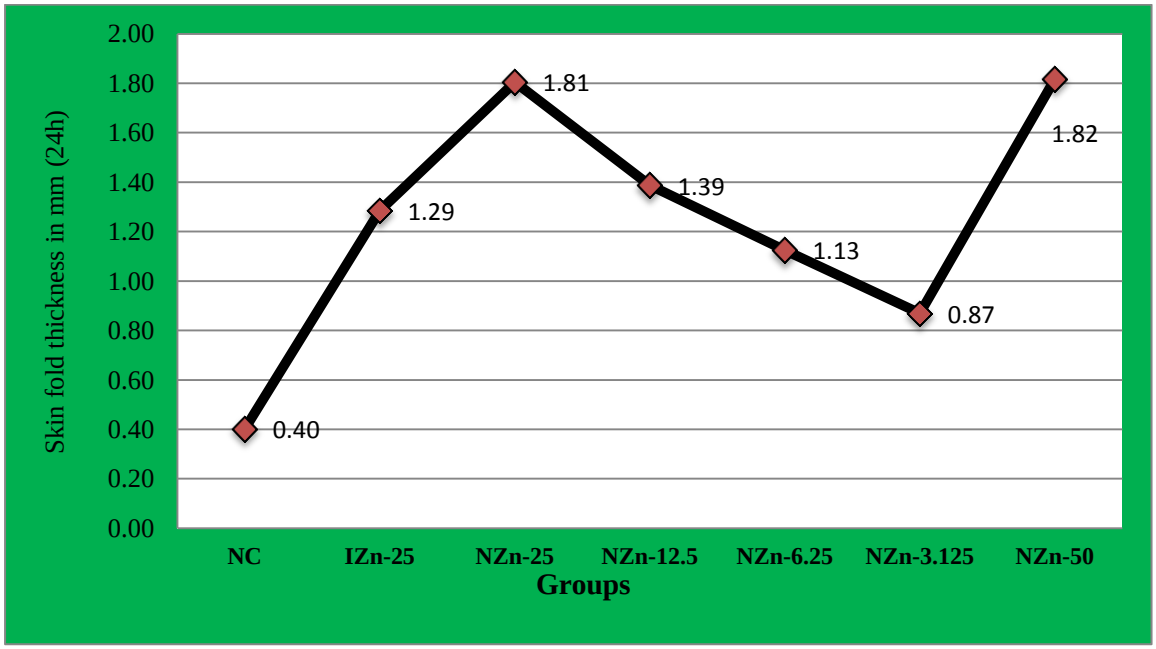


D) Testosterone (ng/mL)

Fig 4.8. Supplementation of graded doses of NZn on cell mediated immunity in rats measured by foot pad test



a) Before Injection b) After Injection



c) Change in Foot Pad thickness (mm) after 24 hours

Table 4.2.5. Effect of supplementation of graded levels of NZn on serum hormone profiles

Particulates	NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50	SEM	P
T3 (nmol/L)	0.98 ±0.18	0.72 ±0.22	0.81 ±0.08	0.83 ±0.13	0.84 ±0.23	0.98 ±0.16	0.55 ±0.08	0.06	0.548
T4 (nmol/L)	15.0^{c} ±1.47	26.3^{b} ±1.77	33.5^{ab} ±4.71	25.8^{b} ±2.79	24.1^{bc} ±2.69	24.9^{b} ±4.18	36.1^{a} ±3.13	1.54	0.002
Testosterone (ng/ml)	0.39^{c} ±0.04	0.70^{bc} ±0.06	1.16^{ab} ±0.36	0.71^{bc} ±0.12	0.57^{bc} ±0.05	0.43^{c} ±0.13	1.40^{a} ±0.37	0.09	0.016
IGF-1 (ng/ml)	69.5 ±7.36	79.7 ±12.1	86.6 ±10.7	76.9 ±6.48	71.6 ±7.81	70.2 ±6.90	76.8 ±10.7	3.30	0.841

^{a,b}Means with different superscripts in a row differ significantly (P<0.05)

Table 4.2.6. Effect of supplementation of graded levels of NZn on immune status

Attributes		NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50	SEM	P
Cell mediated immunity measured as skin fold thickness (mm)										
Duration after injection of sheep RBC	24h	0.40^{d} ±0.16	1.29^{ab} ±0.18	1.81^{a} ±0.12	1.39^{ab} ±0.14	1.00^{bc} ±0.10	0.72^{cd} ±0.08	1.82^{a} ±0.29	0.11	0.001
	48h	0.37^{c} ±0.23	0.98^{b} ±0.19	1.51^{a} ±0.22	1.03^{ab} ±0.10	0.46^{c} ±0.09	0.47^{c} ±0.12	1.44^{ab} ±0.14	0.10	0.001
Humoral immunity										
Haemagglutination titre (log_2) against Sheep RBCs		2.83^{c} ± 0.17	3.29^{bc} ± 0.18	3.67^{ab} ± 0.21	3.27^{bc} ± 0.17	3.17^{bc} ± 0.22	3.17^{bc} ± 0.17	3.87^{a} ± 0.12	0.09	0.003

$^{a, b, c, d}$ Means with different superscript in a row differ significantly (P<0.05)

4.2.7.2. Bone

Similarly, bone zinc content was higher (P<0.05) in NZn-50 (211±8.50) than NC (175±7.08), NZn-12.5 (190±2.84), NZn-6.25 (186±3.78), NZn-3.125 (186±5.34). However, the zinc levels were same in NZn-50, IZn-25 (196±11.7) and NZn-25(199±3.28). NC animals were having significantly lower zinc than IZn-25 (196±11.7), NZn-25 (199±3.28) and NZn-50 (211±8.50). Iron, manganese and copper (mg/kg) contents were similar in all the groups irrespective of the dietary treatment which varied in a range of 88.2±5.60 (NZn-12.5) to 128±30.8 (NZn-25), 7.22±0.42 (NZn-3.125) to 11.7±1.52 (IZn-25) and 2.30±0.41 (NZn-25) to 5.93±1.89 (NZn-3.125), respectively.

4.2.7.3. Kidney

In kidney, zinc content (mg/kg) was higher (P<0.05) in NZn-50 (127±5.76) than NC (108±3.24), NZn-6.25 (109±1.54) and NZn-3.125 (112±3.14), while NC (108±3.24) group showed significantly lower kidney zinc level than NZn-25 (126±5.01) and NZn-50 (127±5.76). Considering other trace minerals like iron, manganese and copper, a non-significant variation was observed among them which varied within a range of 306±26.1 (NC) to 394±39.1 (NZn-6.25), 7.07±0.50 (NZn-3.125) to 8.78±0.78 (NZn-6.25) and 29.4±1.86 (NC) to 42.1±3.45 (IZn-25), respectively.

4.2.7.4. Muscle

In muscle, all the trace minerals under study *viz.* zinc, iron, manganese and copper were similar irrespective of the dietary supplementation which ranged from 60.5±1.33 (NC) to 70.1±6.08 (NZn-50), 75.4±5.37 (NC) to 82.8±7.08 (NZn-25), 3.30±0.44 (NZn-6.25) to 5.45±1.10 (NZn-3.125) and 3.68±0.26 (NZn-6.25) to 6.63±1.54 (NZn-3.125), respectively.

Significant improvement in zinc concentration in liver, bone and kidney was observed in NZn-50, NZn-25 groups as compared to NC.

Table 4.2.7. Effect of supplementation of graded levels of NZn on visceral organ micro mineral profiles (mg/kg) of Wistar albino rats.

Particulates	NC	IZn-25	NZn-25	NZn-12.5	NZn-6.25	NZn-3.125	NZn-50	SEM	P
Liver									
Zinc	79.6^{c} ±3.36	90.4^{abc} ±4.39	96.5^{ab} ±6.65	91.5^{abc} ±3.30	86.0^{bc} ±3.77	84.3^{bc} ±3.17	100^{a} ±2.44	1.79	0.020
Iron	262 ±21.2	317 ±36.0	340 ±52.4	335 ±19.3	280 ±7.07	293 ±29.8	374 ±39.6	13.0	0.215
Manganese	7.62 ±0.83	7.29 ±1.07	6.31 ±0.69	7.21 ±0.69	7.06 ±0.62	6.68 ±0.48	7.19 ±0.79	0.28	0.916
Copper	12.61 ±0.75	10.74 ±1.04	11.39 ±2.03	10.73 ±1.80	10.87 ±1.15	13.34 ±1.35	13.07 ±1.51	0.51	0.668
Bone (femur)									
Zinc	175^{c} ±7.08	196^{ab} ±11.7	199^{ab} ±3.28	190^{bc} ±2.84	186^{bc} ±3.78	186^{bc} ±5.34	211^{a} ±8.50	2.76	0.012
Iron	128 ±16.5	118 ±11.2	128 ±30.8	88.2 ±5.60	91.2 ±6.98	110 ±8.20	113 ±17.7	6.15	0.431
Manganese	11.5 ±1.85	11.7 ±1.52	9.74 ±1.69	8.77 ±0.78	8.04 ±1.27	7.22 ±0.42	9.35 ±2.09	0.57	0.294
Copper	5.48 ±0.35	3.25 ±0.41	2.30 ±0.41	3.60 ±1.02	3.40 ±0.61	5.93 ±1.89	2.76 ±0.51	0.39	0.074
Kidney									
Zinc	108^{c} ±3.24	116^{abc} ±1.61	126^{ab} ±5.01	121^{abc} ±6.90	109^{c} ±1.54	112^{bc} ±3.14	127^{a} ±5.76	1.99	0.015
Iron	306 ±26.1	361 ±37.0	370 ±23.1	366 ±16.3	394 ±39.1	342 ±27.4	380 ±16.7	10.5	0.396
Manganese	7.95 ±0.27	8.10 ±0.56	7.68 ±0.43	8.59 ±1.09	8.78 ±0.78	7.07 ±0.50	7.79 ±0.67	0.26	0.615
Copper	29.4 ±1.86	42.1 ±3.45	30.7 ±2.51	30.6 ±2.66	30.6 ±2.93	33.0 ±3.36	29.8 ±3.68	1.22	0.153

Muscle									
Zinc	60.5 ±1.33	62.1 ±5.04	67.8 ±2.22	65.6 ±3.15	61.7 ±4.27	60.9 ±3.60	70.1 ±6.08	1.46	0.474
Iron	75.4 ±5.37	77.5 9.43	82.8 ±7.08	77.4 ±6.67	78.3 ±15.73	76.4 ±5.31	80.1 ±8.85	3.20	0.998
Manganese	5.38 ±0.83	4.24 ±0.96	3.69 ±0.65	3.47 ±0.82	3.30 ±0.44	5.45 ±1.10	3.55 ±1.03	0.33	0.310
Copper	5.90 ±0.56	3.89 ±0.42	4.90 ±0.75	5.37 ±1.11	3.68 ±0.26	6.63 ±1.54	4.83 ±0.83	0.36	0.265

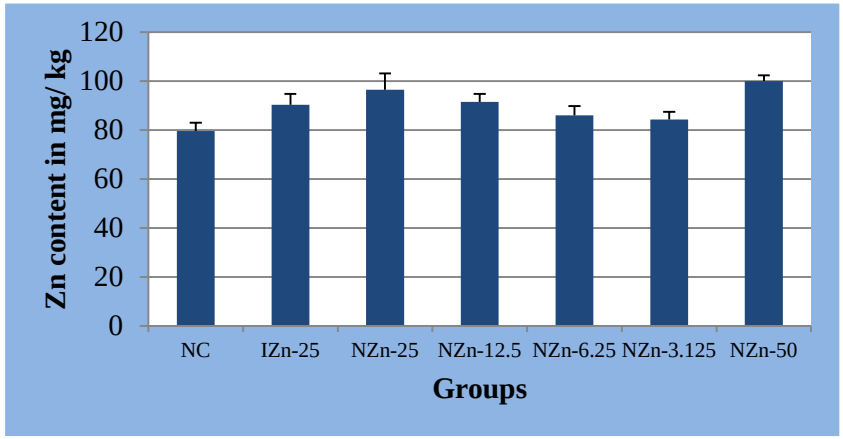
[a, b, c] Means with different superscript within a row differ significantly (P<0.05)

Table 4.2.8. Effect of feeding graded levels of NZn on serum minerals in Wistar albino rats.

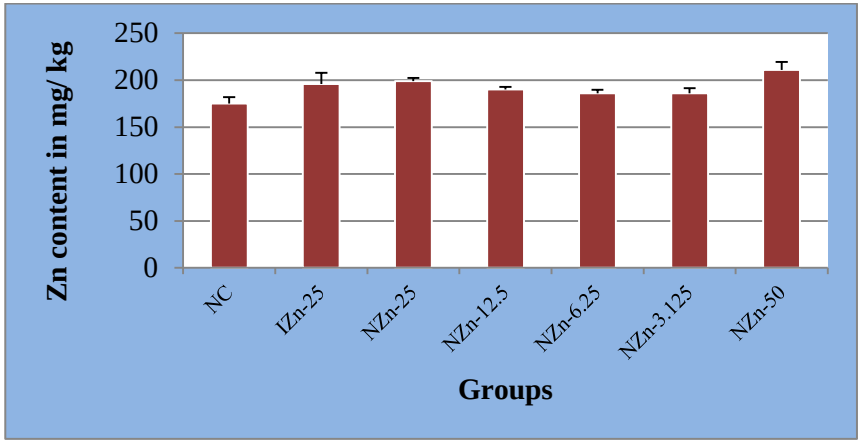
Mineral	**NC**	**IZn-25**	**NZn-25**	**NZn-12.5**	**NZn-6.25**	**NZn-3.125**	**NZnO- 50**	**SEM**	**P**
Calcium (mg/dL)	10.9 ±0.69	11.9 ±0.67	12.2 ±0.31	12.0 ±0.41	11.6 ±0.28	11.8 ±0.74	12.2 ±0.24	0.20	0.548
Phosphorus (mg/dL)	7.40 ±0.30	8.46 ±0.42	7.66 ±0.37	8.07 ±0.39	7.94 ±0.55	7.41 ±0.52	7.43 ±0.52	0.16	0.480
Magnesium (mg/dL)	3.07 ±0.20	3.05 ±0.21	3.06 ±0.25	3.01 ±0.32	3.19 ±0.43	3.23 ±0.23	2.79 ±0.11	0.09	0.941
Zinc (mg/L)	1.46^{c} ±0.05	1.68^{ab} ±0.03	1.79^{a} ±0.07	1.73^{ab} ±0.10	1.59^{bc} ±0.04	1.55^{bc} ±0.07	1.81^{a} ±0.05	0.03	0.001
Copper (mg/L)	1.85 ±0.09	1.87 ±0.17	1.99 ±0.10	2.01 ±0.10	1.87 ±0.18	1.78 ±0.03	2.06 ±0.11	0.04	0.620

[a,b,c]Means with different superscripts in a row differ significantly (P<0.05)

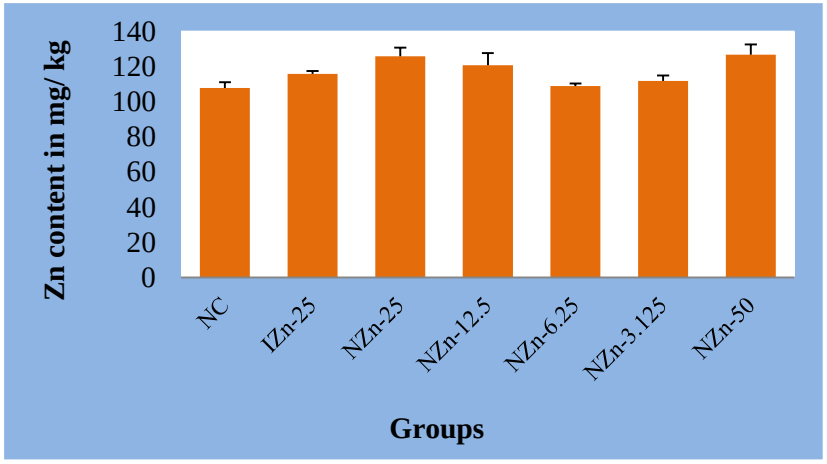
Fig 4.9. Supplementation of graded doses of NZn on zinc level in different organs



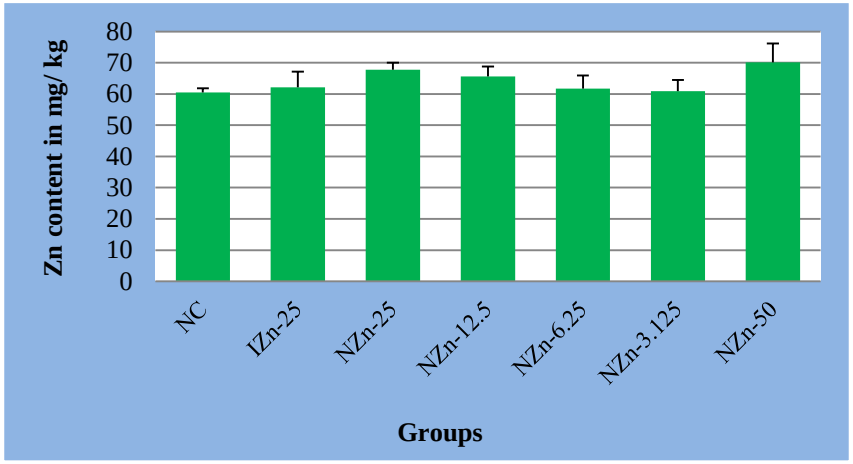
a) Liver



b) Femur



c) Kidney



D) Muscle

4.2.8. Effect of feeding graded doses of NZn on serum minerals in Wistar albino rats

Effect of feeding graded level of NZn on serum minerals in Wistar albino rats is depicted in Table 4.2.8.

Calcium, phosphorus and magnesium levels (mg/dL) were found similar in all dietary treatment groups and varied within a range of 10.9±0.69 (NC) to 12.2±0.31 (NZn-25), 7.40±0.30 (NC) to 8.46±0.42 (IZn-25) and 2.79±0.11 (NZn-50) to 3.23±0.23 (NZn-3.125), respectively. Zinc (mg/L) level was significantly higher in NZn-25 (1.79±0.07) and NZn-50 (1.81±0.05) as compared to NC (1.46±0.05), NZn-6.25 (1.59±0.04) and NZn-3.125 (1.55±0.07), whereas, NC group rats showed lower ($P<0.05$) serum zinc level than IZn-25 (1.68±0.03), NZn-25 (1.79±0.07), NZn-12.5 (1.73±0.10) and NZnO- 50 (1.81±0.05). On the contrary, serum copper content was not affected by the treatment and was found statistically similar among the groups which varied in a range of 1.78±0.03 (NZn-3.125) to 2.06±0.11 (NZn- 50).

Higher serum zinc levels were documented in NZn supplemented groups (NZn-50 and NZn-25) indicating its better bioavailability. Similarly, at reduced dose of NZn supplementation resulted in comparative serum zinc levels as that of IZn-25 supplemented rats.

4.2.9. Relative m-RNA expression in Wistar albino rats

The genes related to zinc metabolism like Metallothionein (Fig 4.11) and SOD-1 (Fig 4.12) were studied in liver and are presented below. The primer efficiency is denoted in Fig 4.10.

4.2.9.1. Metallothionein gene

The expression of metallothionein mRNA in liver of Wistar albino rats was presented in Fig 4.11.

The expression of metallothionein mRNA in liver of Wistar albino rats varied in a dose dependant manner among the NZn groups with maximum abundance in NZn-50 and minimum in NZn-3.125. The expression of metallothionenin mRNA was higher ($P>0.05$) in NZn-50 and NZn-25 as compared to IZn-25. However, similar metallothionein mRNA expression was observed in IZn-25 and NZn-12.5 groups.

4.2.9.2. SOD1 gene

The relative expression of SOD1 mRNA in Wistar albino rat liver is presented in Fig 4.12.

SOD1 mRNA expression in liver was similar (P>0.05) among the different treatment group but varied proportionallywith the dose of NZn supplemented to the rats. The expression of SOD1 mRNA was higher (P>0.05) in NZn-50 and NZn-25 as compared to IZn-25.

4.2.10. Tissue architecture

4.2.10.1. Liver

Histopathology of liver in different groups is presented in Fig, 4.13. The liver of rats in NC group showed moderate vacuolar degeneration which is evident by condensation of nuclei and vacuolated cytoplasm without pink staining with congestion of blood vessels. NZn-6.25 and NZn-3.125 groups showed mild vacuolar degeneration in the liver hepatocytes. This might be due to deficiency of zinc in the diet may led to the mild to moderate changes in the liver. The other groups IZn-25, NZn-25, NZn-12.5 and NZn-50 showed normal hepatocytes with vesicular nucleus stained blue and cytoplasm stained with pink colour.

4.2.10.2. Kidney

Histopathology of kidney in different groups is presented in Fig, 4.14. The kidney of rats in all the groups showed no observable changes.

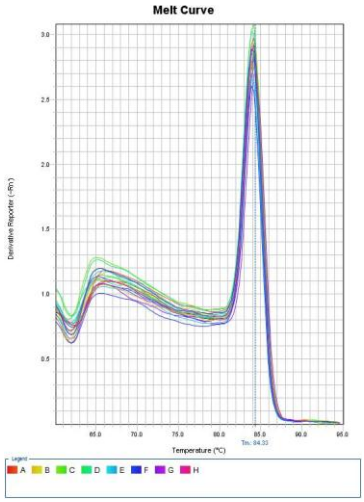
4.2.10.3. Spleen

Histopathology of spleen in different groups is presented in Fig, 4.15. The spleen of rats in all the groups showed no observable changes.

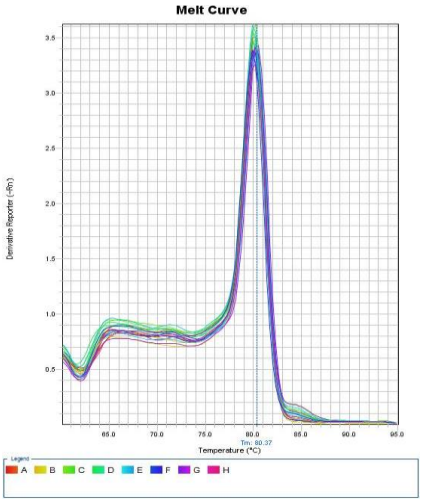
4.2.10.4. Intestine

Histopathology of Intestine in different groups is presented in Fig, 4.16. NC group showed normal columnar epithelial cells with few goblet cells with normal intestinal architecture but shortened villi. IZn-25 showing normal appearance of intestinal villi. NZn-25 showed the mild damage to the intestinal epithelial cells with elongation of intestinal villi (arrow) and partial fusion of villi with mild hyperplasia of goblet cells. NZn-12.5 group showed intestinal villi elongation and increase in goblet cells. NZn-6.25 showed intestinal epithelial cells and villi normal appearance but with partial fusion of villi. NZn-3.125 showed normal appearance of intestine architecture with partial fusion of villi. NZn-50 group showed moderate damage to intestinal villi and hyperplasia of goblet

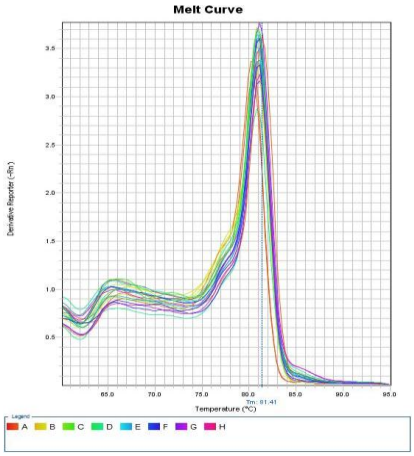
Fig 4.10. Melting curves and primer amplification confirmation by Gel-Doc.



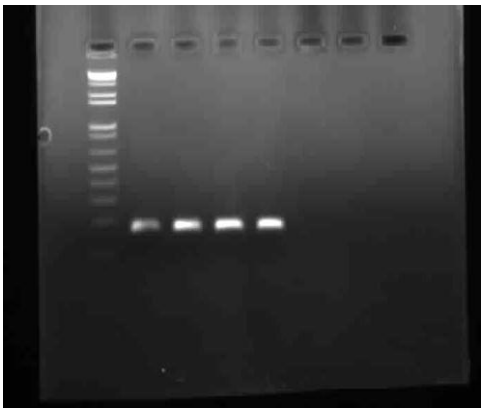
a) Melting curve of Metallothionein



b) Melting curve of SOD-1



c) Melting curve of GAPDH



d) Primer amplification confirmation by Gel-Doc (SOD-1 and Metallothionein)

Fig 4.11. Supplementation of graded doses of NZn on expression of metallothionein in rat liver

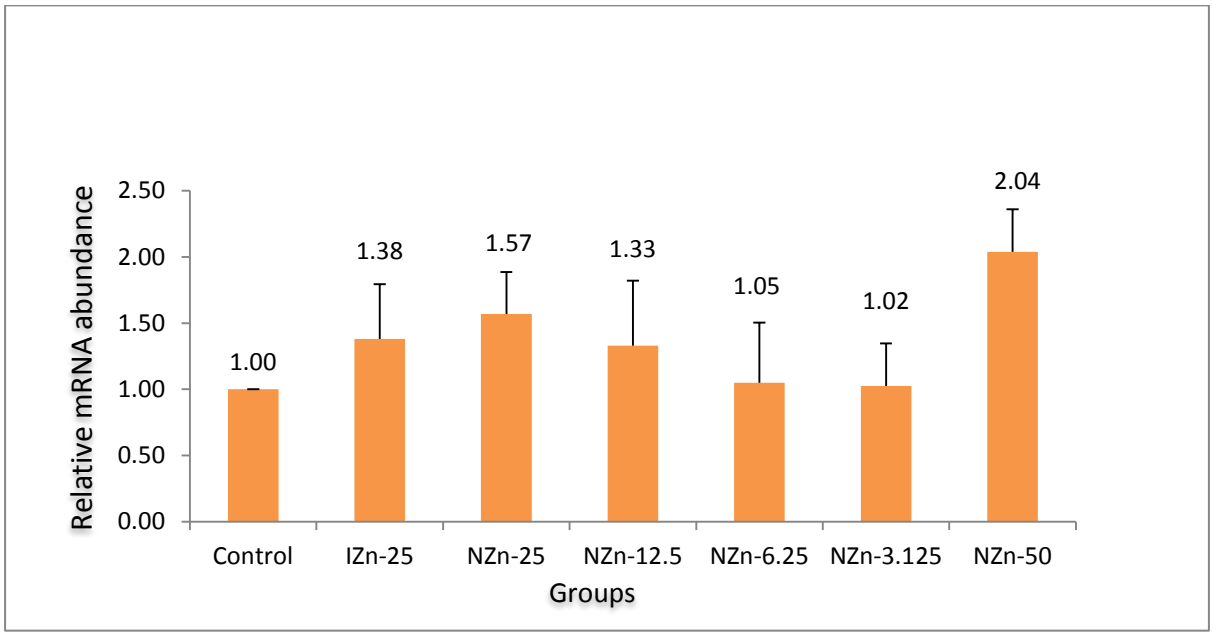


Fig 4.12. Effect of supplementation of graded levels of NZn on hepatic SOD1 expression in rats

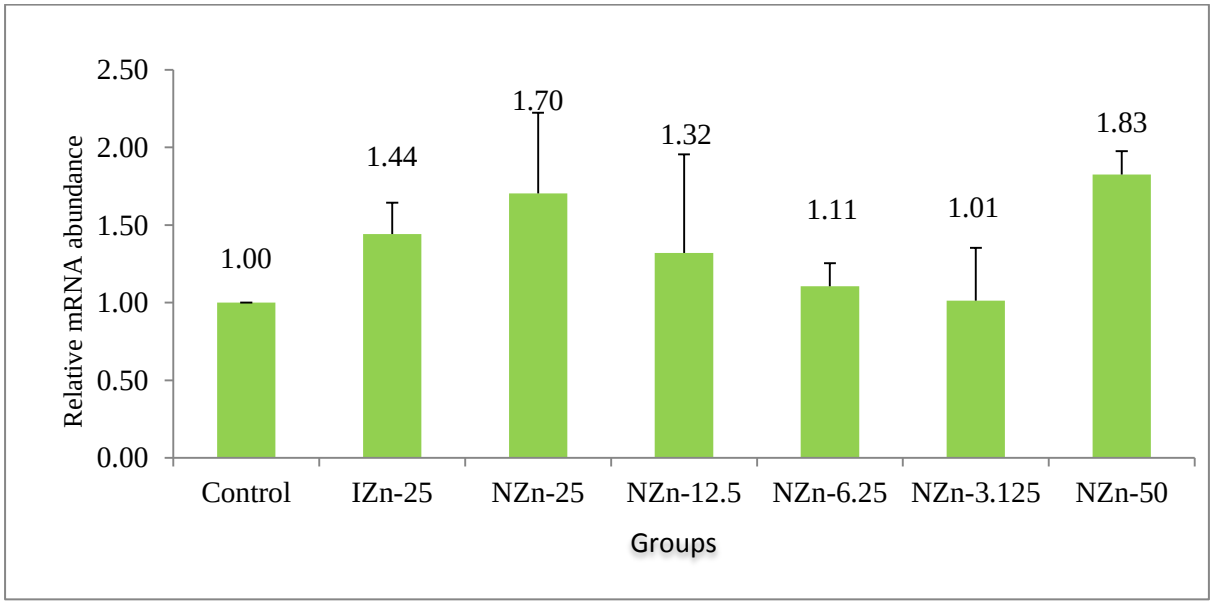
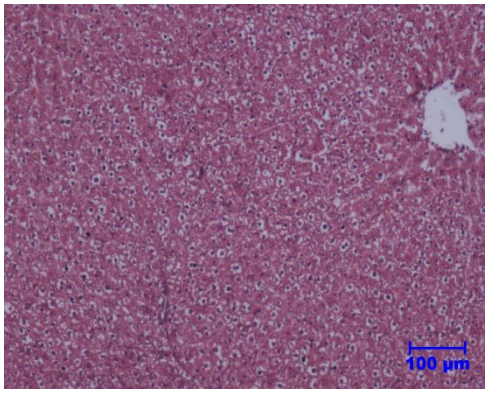
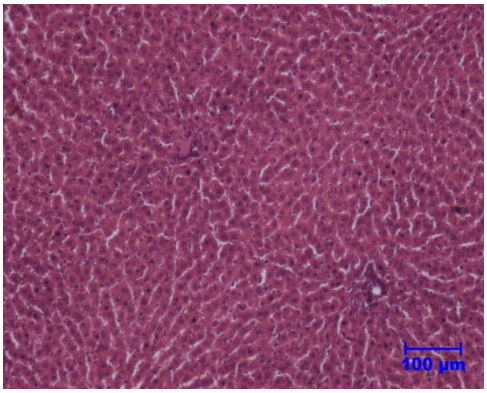


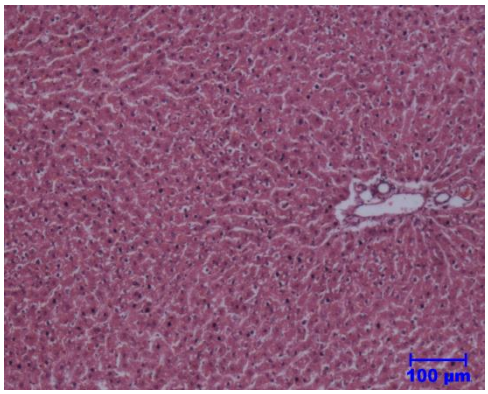
Fig 4.13. Supplementation of graded doses of NZn on liver architecture in rats



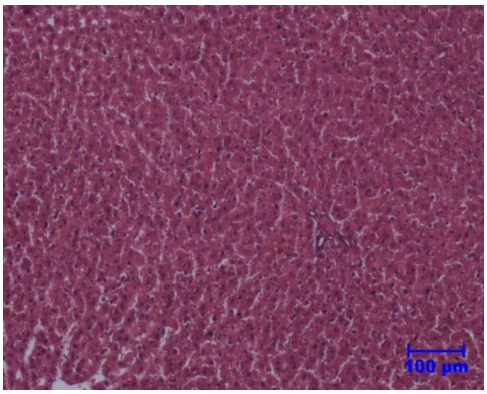
NC



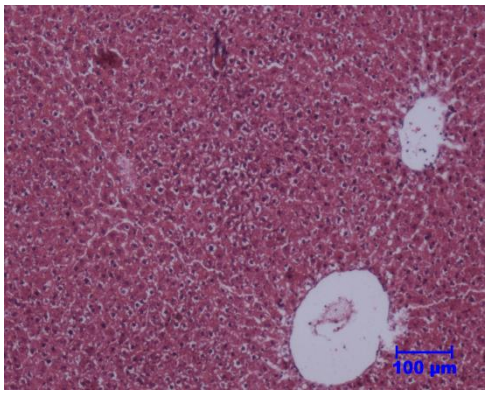
IZn-25



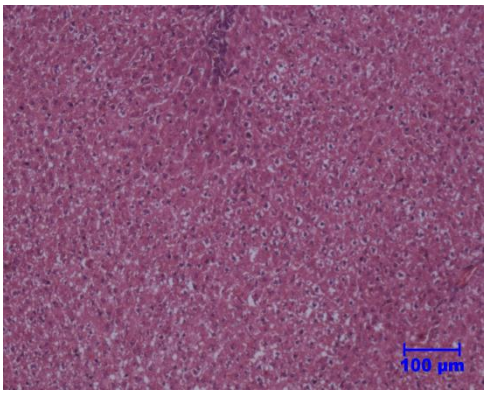
NZn-25



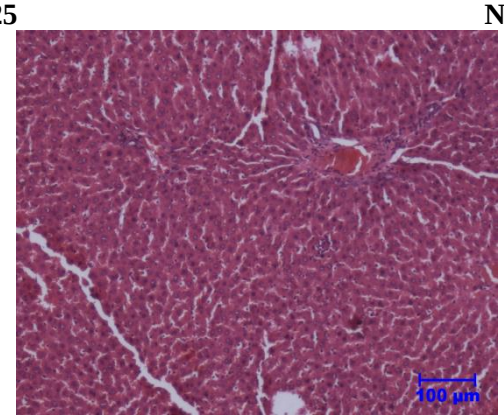
NZn-12.5



NZn-6.25

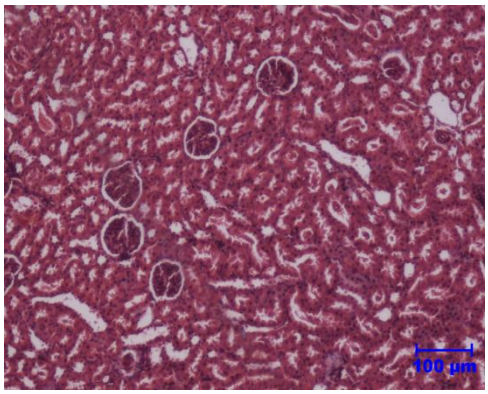


NZn-3.125

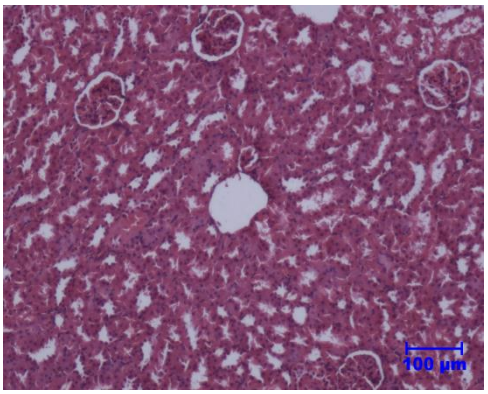


NZn-50

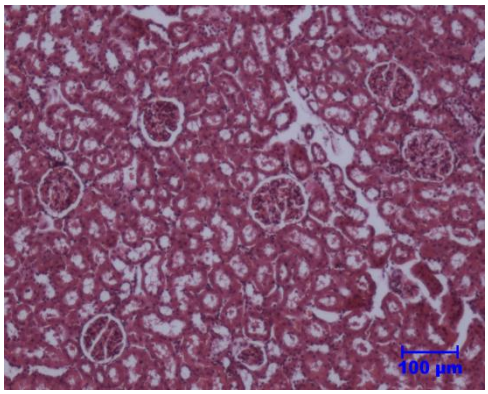
Fig 4.14. Supplementation of graded doses of NZn on kidney architecture in rats



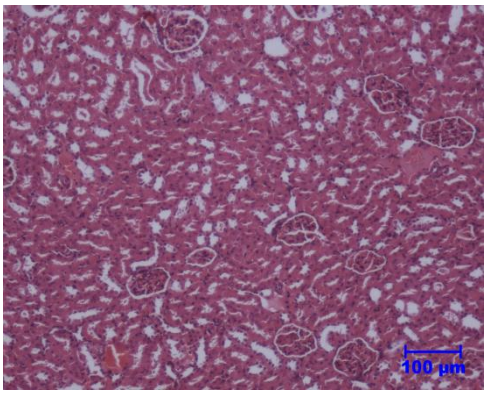
NC



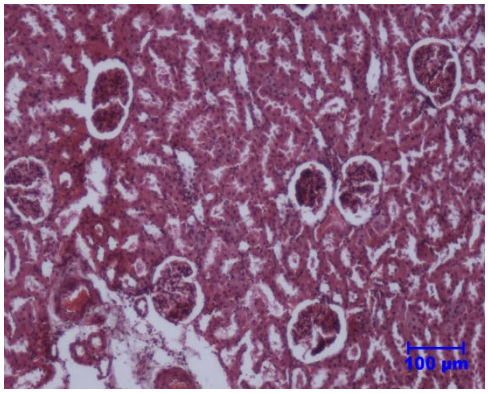
IZn-25



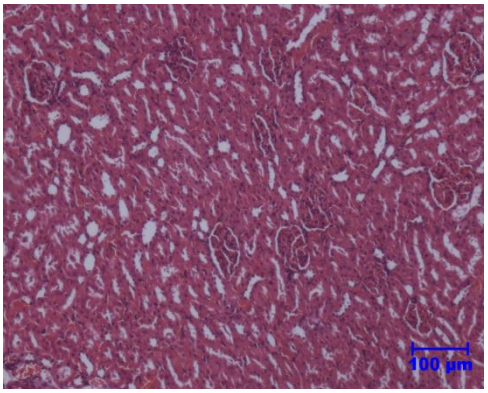
NZn-25



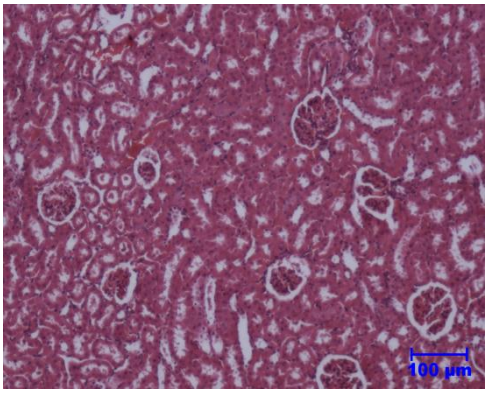
NZn-12.5



NZn-6.25

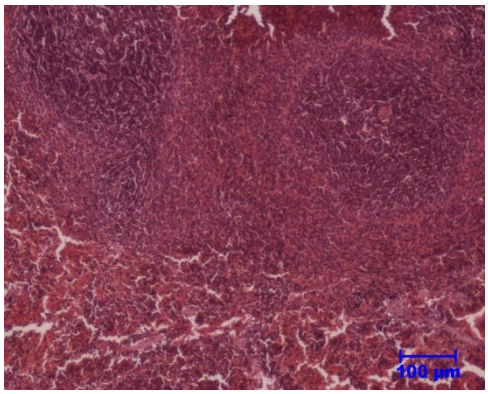


NZn-3.125



NZn-50

Fig 4.15. Supplementation of graded doses of NZn on spleen architecture in rats



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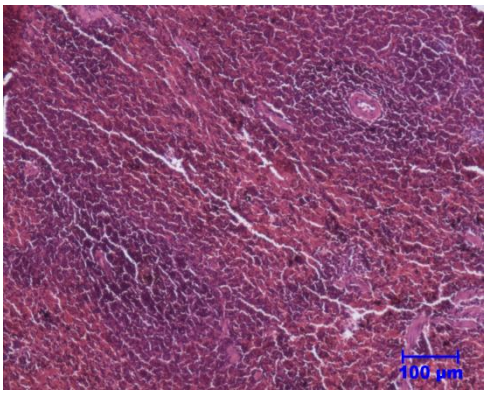
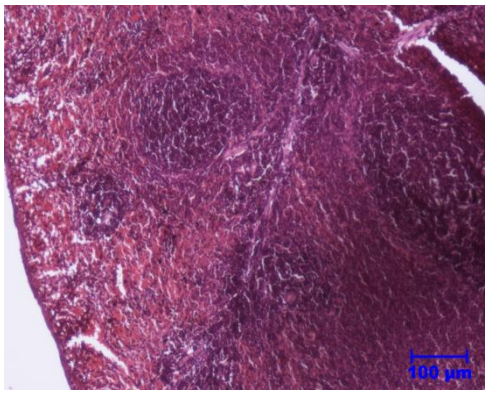
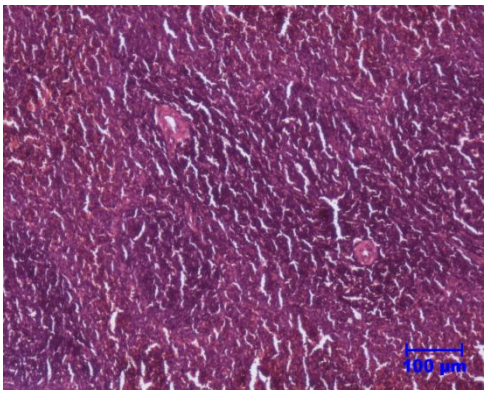


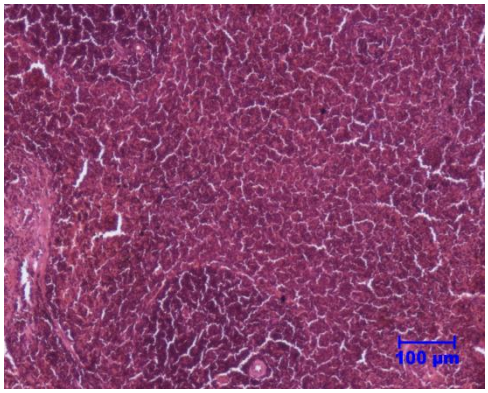
IZn-25



NZn-25



NZn-12.5



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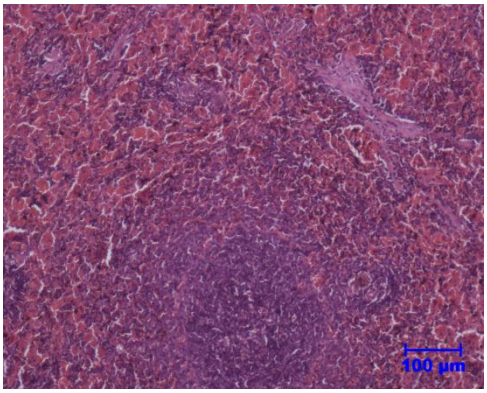
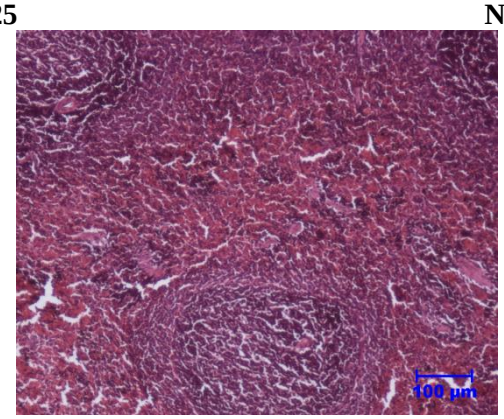
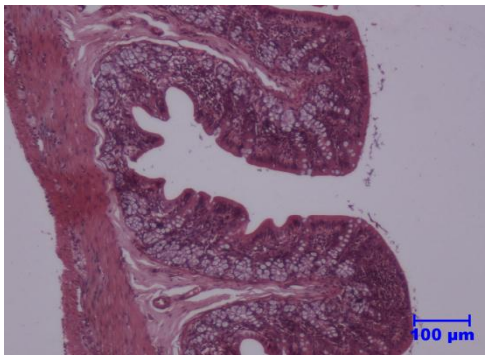


NZn-3.125

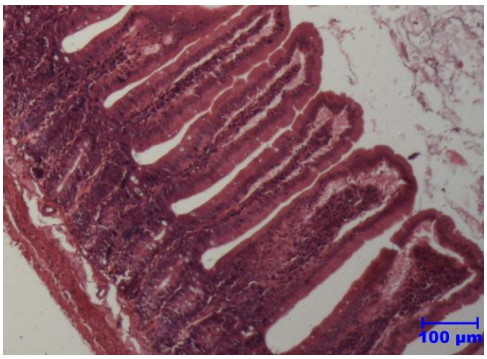


NZn-50

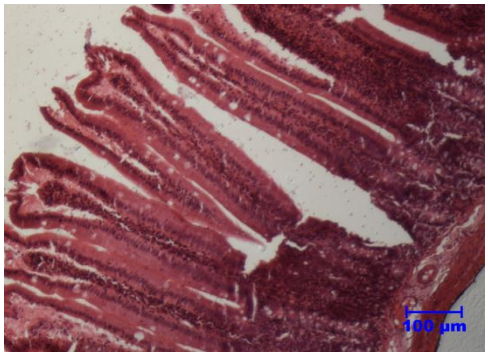
Fig 4.16. Supplementation of graded doses of NZn on intestine architecture in rat



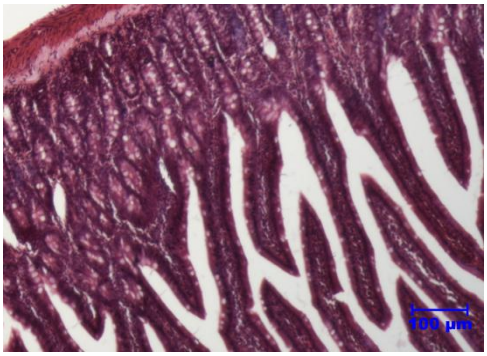
NC



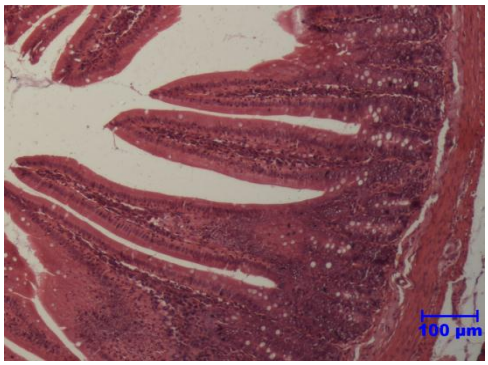
IZn-25



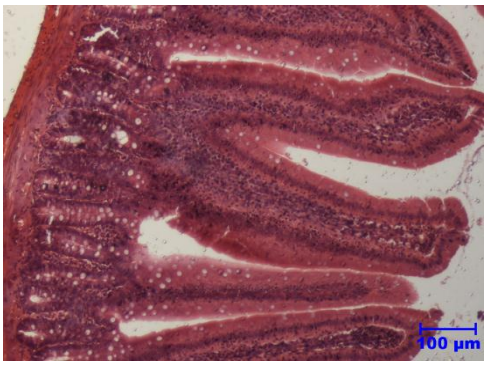
NZn-25



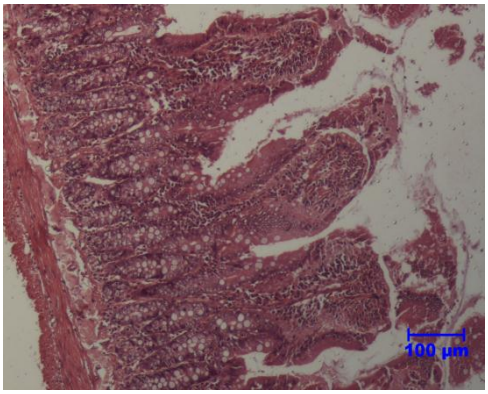
NZn-12.5



NZn-6.25



NZn-3.125



NZn-50

cells with fusion of intestinal villi (arrow). (Haematoxylin and Eosin staining with scale bar=100µm).

Histopathological observations on liver, kidney, spleen and intestine indicated few changes in intestinal villi architecture due to feeding NZn may not be of consequence to the overall performance of rats.

4.3. Phase-III. Effects of two levels of NZn particles on growth, nutrient utilization, immunity, rumen fermentation, blood profiles and male reproduction in goats

In this phase an *in vivo* trial was conducted on goats to study the effect of supplementation of two levels of NZn on growth, nutrient utilization, immunity, rumen fermentation and male reproduction. The results are briefed as follows.

4.3.1. Chemical compositions of feeds

Chemical composition and mineral contents of the concentrate mixture and finger millet straw fed to goats during the feeding trial is presented in Table 4.3.1. The OM content of the concentrate and straw was 96.48 and 93.12%, respectively. The respective ash contents (%) were 3.52 and 6.88. EE (%) was found to 1.14 and 1.88 in concentrate and finger millet straw, respectively. The CP, T-CHO (%) content of the concentrate and finger millet straw was recorded as 20.49, 74.85 and 4.87, 86.37, respectively. NDF, ADF, hemicellulose and cellulose contents (%) of concentrate and finger millet straw were 27.96, 17.20, 10.76, 12.38 and 74.32, 44.75, 29.57, 35.72, respectively. Ca, P and Mg (%) in concentrate mixture and straw was 0.80, 1.00, 0.30 and 1.10, 0.40, 0.60 %, respectively. In respect of trace minerals, Zn, Fe and Mn (mg/kg) contents of concentrate mixture and straw were 22.34, 928.7, 127.8 and 12.33, 279.1, 108.7, respectively.

4.3.2. Growth performance

The results pertaining to growth performance of goats are presented in the Table 4.3.2 and Fig 4.17. The initial body weight (BW, kg) in goats ranged from 18.8±0.93 (IZn-50) to 19.7 ±0.93 in different treatment groups, which was found statistically similar ($P>0.05$). The final BW (kg) at the end of the experimental feeding of 112 days was 24.6 ±0.97, 25.1±1.07, 26.3±0.67 and 26.3±0.53 in NC, IZn-50, NZn-50 and NZn-25 groups, respectively. The total weight gain (kg) during this period was found to be highest ($P<0.05$) in NZn-25 (7.37±0.20) followed by NZn-50 (6.65±0.23), IZn-50 (6.38±0.46) and

lowest in NC (5.73±0.42). All the zinc supplemented goats exhibited significantly higher ($P<0.05$) body weight gain as compared to NC group. ADG (g) also followed similar trend as that of total weight gain i.e. NZn-25 (65.8±1.81) is being highest and NC (51.2±3.73) being the lowest. Irrespective of the treatment, the total DM consumed (kg) was similar ($P>0.05$) in all the groups which varied from 67.4 (NZn-25 and IZn-50) to 69.3 (NZn-50). The FCR (kg DM feed consumed per kg BW gain) also followed the same trend as ADG (g) and total BW gain (kg). The NC group (12.0±0.54) required significantly more ($P<0.05$) feed per kg BW gain than NZn-25 group (9.16±0.14). The FCR among zinc supplemented groups were found to be same ($P>0.05$).

Nano Zn supplementation at reduced dose (NZn 25) had increased body weight gain and ADG and improved feed conversion of goats during 112 day feeding period.

4.3.3. Nutrient intake

Effect of supplementation of NZn on nutrient intake recorded during digestion trial of goats is depicted in Table 4.3.3. Average body weight and metabolic body weight (kg) of goats was similar in different treatment groups. The concentrate intake expressed as g/d, % BW and g/$W^{0.75}$ was found to be 366±17.4, 1.62±0.01, 35.2±0.46; 356±16.1, 1.62±0.01, 35.0±0.43; 368±12.6, 1.64±0.01, 35.7±0.41; and 372±13.2, 1.63±0.01, 35.6±0.45, respectively in NC, IZn-50, NZn-50 and NZn-25.

Roughage DM intake in terms of g/d, % BW or g/ $W^{0.75}$ did not vary significantly ($P>0.05$) among the treatment groups. Similar trend was reflected in total DMI expressed as g/d, % BW or g/ $W^{0.75}$ (DMI g/d: 640±31.8 in IZn-50 to 671±37.4 in NC; DMI (% BW): 2.90±0.02 in IZn-25 to 2.96±0.05 in NC; DMI (g/$W^{0.75}$: 62.8±1.08 in IZn-50 to 64.5±1.51 in NC.

Table 4.3.1. Chemical composition of concentrate mixture and finger millet straw fed to goat during experiment

Chemical composition (%) +	**Concentrate Mixture**	**Finger Millet Straw**
DM	91.71	92.84
OM	96.48	93.12
Total Ash	3.52	6.88
EE	1.14	1.88
CP	20.49	4.87
T-CHO	74.85	86.37
NDF	27.96	74.32
ADF	17.20	44.75
Hemicellulose	10.76	29.57
Cellulose	12.38	35.72
Mineral Composition		
Calcium (%)	0.80	1.10
Phosphorus (%)	1.00	0.40
Magnesium (%)	0.30	0.60
Zinc (mg/kg)	22.34	12.33
Iron (mg/kg)	928.7	279.1
Manganese (mg/kg)	127.8	108.7

+ on dry matter basis except for dry matter

Table 4.3.2. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on growth performance of goats

Attributes	Treatment Particulars				SEM	P
	NC	IZn-50	NZn-50	NZn-25		
Initial BW, Kg	18.9 ±0.67	18.8 ±0.93	19.7 ±0.59	18.9 ±0.41	0.32	0.761
Final BW, Kg	24.6 ±0.97	25.1 ±1.07	26.3 ±0.67	26.3 ±0.53	0.42	0.402
Total Wt. gain, Kg	5.73^{b} ±0.42	6.38^{ab} ±0.46	6.65^{ab} ±0.23	7.37^{a} ±0.20	0.20	0.026
ADG (g)	51.2^{b} ±3.73	56.9^{ab} ±4.12	59.4^{ab} ±2.08	65.8^{a} ±1.81	1.81	0.026
Total DM consumed (kg)	67.8 ±2.86	67.4 ±3.45	69.3 ±1.85	67.4 ±1.28	1.18	0.937
FCR (Kg DM feed consumed per Kg BW gain)	12.0^{b} ±0.54	10.8^{ab} ±0.80	10.5^{ab} ±0.37	9.16^{a} ±0.14	0.32	0.009

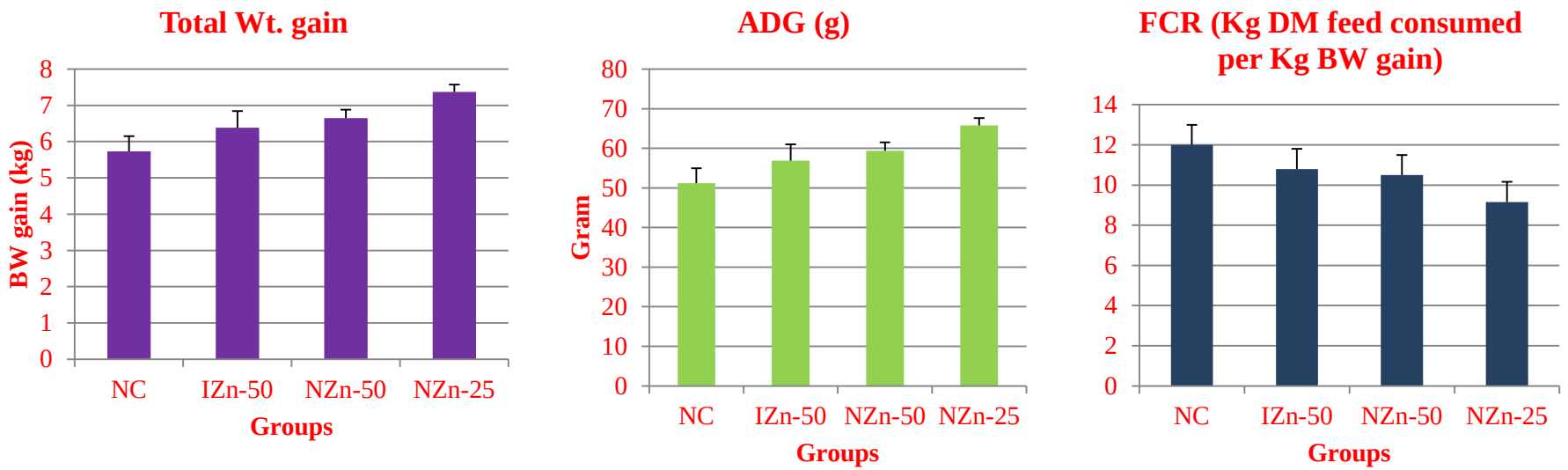
[a,b] Means with different superscript in a row differ significantly (P<0.05) ; Each value is an average of six observations.

The OMI, expressed either as g/d, % BW or g/ $W^{0.75}$, did not vary significantly (P>0.05) among the groups. The OMI g/d, % BW and g/$W^{0.75}$ was found to be 638±35.4, 2.81±0.04, 61.2±1.42; 608±30.1, 2.76±0.02, 59.7±1.01; 621±18.8, 2.78±0.02, 60.4±0.48; and 623±17.8, 2.74±0.04, 59.8±0.84, respectively in NC, IZn-50, NZn-50 and NZn-25.

The CP intake was comparable among the treatment groups. The CP intake (g/d) in NC, IZn-50, NZn-50 and NZn-25 was found to be 89.9±4.51, 86.8±4.05, 89.3±2.91 and 89.9±2.91, respectively. CPI (g/ kg $W^{0.75}$) ranged from 8.53±0.14 in IZn-50 to 8.68±0.08 in NZn-50.

The DOMI (g/d), DOMI (% BW) and DOMI (g/ $W^{0.75}$) were found to be same (P>0.05) among the treatment groups and DOMI (g/d) fell in the range of 395±25.9 (NC) to 410±12.8 (NZn-50), where as DOMI (% BW) ranged from 1.74±0.06 (NC) to 1.84±0.03 (NZn-50). DOMI (g/ $W^{0.75}$) in different groups was found 39.9±0.69 (NZn-50) followed by NZn-25 (39.2±0.66), IZn-50 (39.1±1.38) and NC (37.9±1.44). DCPI (g/d) was maximum (P>0.05) in NZn-25(64.9±3.26) and minimum in IZn-50 (59.3±4.07). DCPI (g/ kg $W^{0.75}$) was also similar in the treatment groups and numerically varied from 5.82±0.25 in IZn-25 to 6.21±0.17 in NZn-25. TDNI (g/d) and TDNI (g/ kg $W^{0.75}$) were found non-significant (P>0.05) in the treatment groups and ranged from 403±26.4 and 38.7±1.46 in NC to 419±13.1 and 40.7±0.71 in NZn-50. MEI (MCal/d) was also non

Fig 4.17. Supplementation of graded doses of NZn on animal performances



significant (P>0.05) among the groups and varied from 1.46±0.10 in NC to 1.52±0.05 in NZn-50. ME (Kcal/ kg $W^{0.75}$) also followed the same trend of NZn-50 (147±2.58) being the highest (P>0.05) and NC (140±5.29) being the minimum.

Intake of nutrients recorded during digestion trial was found to be same in all treatment groups with or without zinc supplementation as inorganic or nano forms.

4.3.4. Nutrient digestibility and nutritive value of diet

The results pertaining to effect of supplementation of two different sources (inorganic and nano) and two levels of nano zinc on nutrient digestibility in goats are presented in Table 4.3.4. DM, OM, CP, EE and Total CHO digestibility (%) did not vary (P>0.05) among different treatment groups. The DM digestibility (%) was found to be ranging from 59.8±1.24 in NC group to 64.3±0.95 in NZn-50. The OM digestibility (%) was highest in NZn-50 (66.1±0.94) followed by NZn-25 (65.6±1.32), IZn-50 (65.4±1.37) and NC (61.8±1.14) with minimum OM digestibility, whereas CP digestibility (%) was highest in NZn-25 (72.0±1.67) and least in IZn-50 (68.1±2.24) group. The EE digestibility (%) varied from 66.6±1.09 in NC group to 70.0±1.71 in NZn-50. The total CHO digestibility (%) ranged from 60.6±1.58 (NC) to 65.2±0.89 (NZn-50).

The NDF digestibility (%) was more (P>0.05) in NZn-25 followed by NZn-50, IZn-50 and NC with the values of 51.2±1.40, 50.5±1.65, 50.3±2.35, and 46.3±1.50, respectively. ADF digestibility (%) was significantly higher (P<0.05) in NZn-25(43.4±2.39) followed by NZn0-50 (37.3±1.90), IZn-50 (36.1±4.52) and NC (29.9±2.60). ADF digestibility (%) in NZn-25 was significantly higher (P<0.05) than that of NC but similar to IZn-50 and NZn-50. The cellulose digestibility (%) was highest (P<0.05) in NZn-50 (53.8±2.99) and minimum in NC group (41.8±3.05). The ADF and cellulose digestibilities in zinc supplemented groups (IZn-50, NZn-50 and NZn-25), irrespective of the source of zinc (inorganic or nano) were significantly higher (P>0.05) than NC.

DCP (%), TDN (%) and ME (MCal/kg) were similar (P>0.05) in different treatment groups. DCP (%) was ranging from 9.16±0.35 in NC to 9.89±0.32 in NZn-25; whereas TDN (%) was calculated to be in the range of 59.9±1.07 in NC to 64.0±0.91 in NZn-50. ME (MCal/kg) of the diet was found to be in the range of 2.17±0.04 in NC to 2.31±0.03 in NZn-50 and the differences among groups were non-significant.

4.3.5. Mineral absorption

Effect of NZn (50 and 25 mg/kg) on mineral absorption in goats is presented in Table 4.3.5 and Fig 4.18.

Irrespective of treatment, calcium intake was similar in the groups which were 0.70±0.03, 0.68±0.03, 0.70±0.02 and 0.71±0.02 g/d, respectively in NC, IZn-50, NZn-50 and NZn-25, respectively. Absorbed calcium (g/d) was also similar among the treatment groups varying from 0.19±0.03 (NZn-50) to 0.26±0.04 (NC). No significant variation was observed (P>0.05) in calcium absorption (%) among the groups due to dietary treatments which was 37.5±4.33 (NC), 38.3±2.67 (IZn-50), 27.2±3.64 (NZn-50) and 34.9±2.93 (NZn-25). Similar was the trend in case of phosphorus, where the intake (g/d), absorbed (g/d) and absorption (%) were similar (P>0.05) among the treatment groups. Phosphorus intake (g/d), absorbed (g/d) and absorption (%) was found to be 0.51±0.02, 0.27±0.02, 52.7±3.18 in NC, 0.50±0.02, 0.29±0.03, 57.3±4.68 in IZn-50, 0.51±0.02, 0.24±0.03, 46.8±4.43 in NZn-50 and 0.52±0.02, 0.28±0.03, 53.7±3.96 NZn-25. Magnesium intake (g/d), absorbed (g/d) and absorption (%) were similar (P>0.05) among the treatment groups. However, the absorption of magnesium in per cent was ranging in NZn-50 (44.4±2.89) to NZn-25 (50.7±3.04).

Table 4.3.3. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on nutrient intake of goats

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
Average body weight (kg)	22.7 ±1.05	22.0 ±1.00	22.4 ±0.75	22.8 ±0.70	0.42	0.934
Metabolic body weight ($W^{0.75}$) (kg)	10.4 ±0.36	10.2 ±0.35	10.3 ±0.26	10.4 ±0.24	0.14	0.932
DMI Concentrate g/d	366 ±17.4	356 ±16.1	368 ±12.6	372 ±13.2	7.07	0.901
DMI Concentrate % BW	1.62 ±0.01	1.62 ±0.01	1.64 ±0.01	1.63 ±0.01	0.00	0.225
DMI Concentrate g/ $W^{0.75}$	35.2 ±0.46	35.0 ±0.43	35.7 ±0.41	35.6 ±0.45	0.21	0.645
DMI Roughage g/d	305 ±20.9	283 ±16.2	286 ±7.90	284 ±8.84	7.01	0.675
DMI Roughage % BW	1.34 ±0.04	1.28 ±0.03	1.28 ±0.02	1.25 ±0.04	0.02	0.365
DMI Roughage g/ $W^{0.75}$	29.3 ±1.15	27.8 ±0.77	27.8 ±0.35	27.3 ±0.89	0.42	0.402
Total DMI g/d	671 ±37.4	640 ±31.8	654 ±19.7	655 ±18.6	13.3	0.886
Total DMI % BW	2.96 ±0.05	2.90 ±0.02	2.92 ±0.02	2.88 ±0.05	0.02	0.508
Total DMI g/ $W^{0.75}$	64.5 ±1.51	62.8 ±1.08	63.5 ±0.50	62.9 ±0.90	0.51	0.679
OMI g/d	638 ±35.4	608 ±30.1	621 ±18.8	623 ±17.8	12.6	0.888
OMI % BW	2.81 ±0.04	2.76 ±0.02	2.78 ±0.02	2.74 ±0.04	0.02	0.501
OMI g/ $W^{0.75}$	61.2 ±1.42	59.7 ±1.01	60.4 ±0.48	59.8 ±0.84	0.48	0.685
CP I g/d	89.9 ±4.51	86.8 ±4.05	89.3 ±2.91	89.9 ±2.91	1.73	0.919
CP I g/ kg $W^{0.75}$	8.64 ±0.14	8.53 ±0.11	8.68 ±0.08	8.63 ±0.09	0.05	0.814
DOMI (g/d)	395 ±25.9	399 ±26.8	410 ±12.8	409 ±16.3	10.0	0.943
DOMI (% BW)	1.74 ±0.06	1.81 ±0.05	1.84 ±0.03	1.80 ±0.02	0.02	0.400
DOMI (g/ $W^{0.75}$)	37.9 ±1.44	39.1 ±1.38	39.9 ±0.69	39.2 ±0.66	0.54	0.636
DCPI (g/d)	61.2 ±2.82	59.3 ±4.07	63.2 ±2.70	64.9 ±3.26	1.58	0.648
DCPI (g/ kg $W^{0.75}$)	5.89 ±0.15	5.82 ±0.25	6.14 ±0.18	6.21 ±0.17	0.10	0.413
TDNI (g/d)	403 ±26.4	407 ±27.3	419 ±13.1	417 ±16.7	10.2	0.945
TDNI (g/ kg $W^{0.75}$)	38.7 ±1.46	39.9 ±1.40	40.7 ±0.71	40.0 ±0.67	0.55	0.644
MEI (MCal/d)	1.46 ±0.10	1.47 ±0.10	1.52 ±0.05	1.51 ±0.06	0.04	0.941
ME (Kcal/ kg $W^{0.75}$)	140 ±5.29	144 ±5.06	147 ±2.58	145 ±2.43	1.97	0.645

Each value is an average of six observations.

Table 4.3.4. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on nutrient digestibility and nutritive value of diet in goats

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
Nutrient Digestibility (%)						
DM	59.8 ±1.24	63.4 ±1.43	64.3 ±0.95	64.0 ±1.42	0.71	0.070
OM	61.8 ±1.14	65.4 ±1.37	66.1 ±0.94	65.6 ±1.32	0.67	0.070
CP	68.3 ±2.11	68.1 ±2.24	70.8 ±1.79	72.0 ±1.67	0.98	0.439
EE	66.6 ±1.09	69.3 ±1.88	70.0 ±1.71	69.6 ±2.22	0.87	0.532
Total CHO	60.6 ±1.58	64.9 ±1.33	65.2 ±0.89	64.4 ±1.31	0.72	0.069
NDF	46.3 ±1.50	50.3 ±2.35	50.5 ±1.65	51.2 ±1.40	0.92	0.228
ADF	29.9^{b} ±2.60	36.1^{ab} ±4.52	37.3^{ab} ±1.90	43.4^{a} ±2.39	1.77	0.047
Cellulose	41.8^{b} ±3.05	51.6^{a} ±3.78	53.8^{a} ±2.99	52.7^{a} ±2.09	1.78	0.047
Nutritive value						
DCP (%)	9.16 ±0.35	9.25 ±0.31	9.66 ±0.27	9.89 ±0.32	0.16	0.340
TDN (%)	59.9 ±1.07	63.4 ±1.31	64.0 ±0.91	63.6 ±1.31	0.64	0.070
ME (MCal/kg)	2.17 ±0.04	2.29 ±0.05	2.31 ±0.03	2.30 ±0.05	0.02	0.074

[a,b] Means with different superscript in a row differ significantly ($P<0.05$) ; Each value is an average of six observations.

Table 4.3.5. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on mineral balance and its digestibility

Attributes		NC	IZn-50	NZn-50	NZn-25	SEM	P
Calcium	Intake (g/d)	0.70 ±0.03	0.68 ±0.03	0.70 ±0.02	0.71 ±0.02	0.01	0.903
	Absorbed (g/d)	0.26 ±0.04	0.26 ±0.03	0.19 ±0.03	0.25 ±0.03	0.02	0.355
	Absorption (%)	37.5 ±4.33	38.3 ±2.67	27.2 ±3.64	34.9 ±2.93	1.85	0.126
Phosphorus	Intake (g/d)	0.51 ±0.02	0.50 ±0.02	0.51 ±0.02	0.52 ±0.02	0.01	0.900
	Absorbed (g/d)	0.27 ±0.02	0.29 ±0.03	0.24 ±0.03	0.28 ±0.03	0.01	0.628
	Absorption (%)	52.7 ±3.18	57.3 ±4.68	46.8 ±4.43	53.7 ±3.96	2.07	0.367
Magnesium	Intake (g/d)	0.33 ±0.02	0.32 ±0.01	0.33 ±0.01	0.33 ±0.01	0.01	0.904
	Absorbed (g/d)	0.16 ±0.02	0.16 ±0.01	0.15 ±0.01	0.17 ±0.01	0.01	0.753
	Absorption (%)	49.2 ±3.79	49.2 ±2.70	44.4 ±2.89	50.7 ±3.04	1.54	0.526
Zinc	Intake (mg/d)	12.7^{c} ±0.60	44.3^{a} ±2.14	45.5^{a} ±1.41	29.3^{b} ±0.90	2.85	0.001
	Absorbed (mg/d)	5.26^{b} ±0.97	8.40^{b} ±0.61	12.3^{a} ±1.98	7.92^{b} ±0.29	0.75	0.001
	Absorption (%)	40.4^{a} ±6.20	19.0^{b} ±1.20	26.8^{b} ±3.88	27.1^{b} ±1.09	2.37	0.006

[a,b,c] Means with different superscript in a row differ significantly (P<0.05) ; Each value is an average of six observations.

The zinc intake (mg/d) varied significantly (P<0.001) due to extra supplementation of Zn in treatment groups. The Zn intake in IZn-50 (44.3±2.14) and NZn-50 (45.5±1.41) was significantly higher than NZn-25 (29.3±0.90) and NC (12.70±0.60). Intake of Zn in NC and NZn-25 also varied significantly (Fig 4.18). More absorption of zinc was evident in NZn-50 group (12.3±1.98) compared to IZn-50 (8.40±0.61), NZn-25 (7.92±0.29) and finally NC (5.26±0.97). However, the absorption (%) of zinc was significantly more (P<0.006) in NC compared to zinc supplemented groups.

Zn supplementation in the form of inorganic and nano form did not affect the absorption of Ca, P, and Mg. However, the net absorbed Zn (mg/day) was more in NZn-50 group compared to other groups.

4.3.6. Serum mineral profiles

Effect of supplementation of two levels of NZn on serum mineral profile in goats is presented in Table 4.3.6 and Fig 4.23.

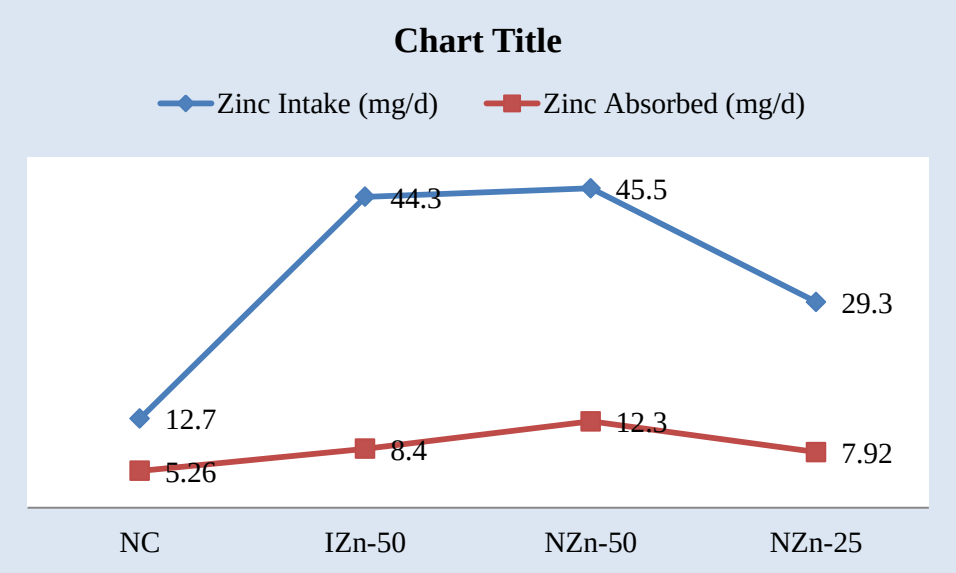
At the start of the experiment, serum Ca (mg/dL), Zinc, Iron, Manganese and Copper (mg/L) were found to be 9.19±0.46, 0.50±0.10, 3.39±0.46, 0.39±0.03, 0.96±0.07 in NC, 9.43±0.78, 0.41±0.07, 2.85±0.24, 0.33±0.00, 0.93±0.10, respectively in IZn-50, 9.77±0.52, 0.47±0.04, 3.55±0.24, 0.37±0.03, 1.01±0.05, respectively in NZn-50 and 9.76±0.64, 0.44±0.10, 3.40±0.29, 0.37±0.03, 0.94±0.11, respectively in NZn-25 group. On 90^{th} day serum samples Ca (mg/dL), Zinc, Iron, Manganese and Copper (mg/L) were 10.9±0.37, 0.65±0.07, 3.60±0.30, 0.43±0.02, 0.99±0.06, respectively in NC, 10.7±0.42, 0.83±0.08, 3.55±0.11, 0.45±0.00, 1.07±0.05, respectively in IZn-50, 10.9±0.48, 0.90±0.09, 3.73±0.11, 0.45±0.00, 1.13±0.05, respectively in NZn-50 and 10.4±0.37, 0.80±0.05, 3.73±0.06, 0.45±0.00, 0.98±0.04, respectively in NZn-25.

Initial (0 day) and final (90^{th} day) zinc concentrations were compared by using t-test. 90^{th} day zinc level was significantly higher than the initial serum zinc level in all the zinc supplemented groups, whereas in NC, the initial and final serum samples were statistically similar in zinc content. The NZn-50 group showed highly significance ($P<0.001$) whereas NZn-25 and IZn-50 were moderately significant in its initial and final serum zinc concentrations ($P<0.01$). Though the differences in serum mineral concentration across different groups are statistically non-significant ($P>0.05$) there is an increasing trend in zinc concentration in the NZn supplemented goats.

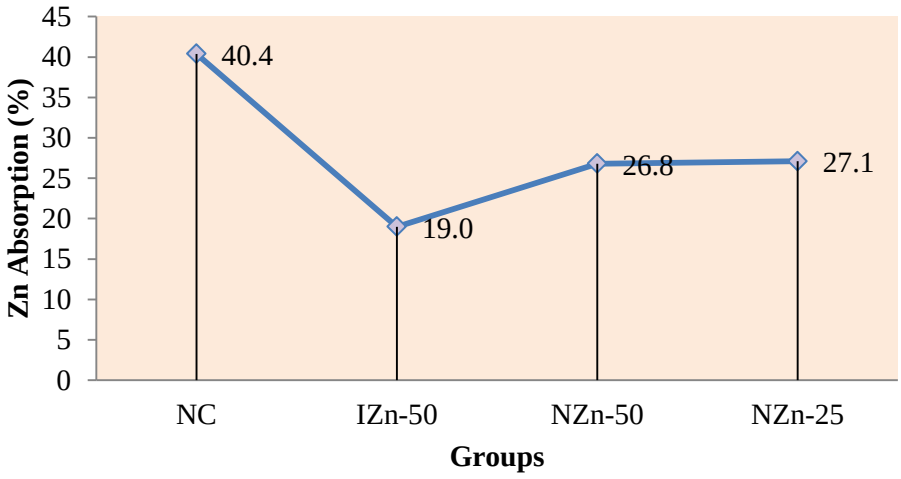
4.3.7. Haematological profiles

Effect of supplementation of graded doses of NZn on haematological profiles of goats is depicted in Table 4.3.7. It was observed that RBC (10^6/ µl), WBC(10^3/ µl), PCV (%), neutrophil (%), lymphocytes (%), eisonophil (%), monocyte (%) and haemoglobin (g/dL) level in the goat blood did not differ statistically ($P>0.05$) by IZn and NZn supplementation in goats. The RBC (10^6/ µl) was ranging from 17.4±0.55 (NC) and 17.4±0.60 (IZn-50) to 18.9 ±0.76 NZn-50. The WBC (10^3/ µl) count was 16.5±1.93, 15.0±1.98, 16.5±1.34 and 13.9±2.12, respectively in NC, IZn-50, NZn-50 and NZn-25 groups. The PCV (%) was found in the range of 27.2±0.64 (NC) to 28.1±0.81 (NZn-50). The differential count of WBC was also found to be same across the treatment groups ($P>0.05$). Neutrophils (%) were found in the range of 38.0±3.49 (NZn-25) to 52.4±8.45 (NC). Proportion of lymphocytes (%) ranged from 44.2±9.00 in NC to 58.2±3.48 in NZn-

Fig 4.18. Effect of supplemntation of NZn on zinc intake and retention



a) Absolute intake and absorption of zinc (mg/d)



b) Per cent absorption of zinc in different treatment groups

25. Eosinophil (%) ranged from 1.20±0.20 in NZn-50 to 3.80±1.59 in IZn-50. Monocytes (%) ranged from 0.80±0.37 in NZn-50 to 1.40±0.24 in IZn-50. The haemoglobin (g/dL) was found to be similar among the treatment groups within a range of 8.50±0.19 in NC to 8.90±0.21 in NZn-50.

Similar haematological profiles were recorded in all the treatment groups supplemented with or without inorganic and nano form of zinc.

Table 4.3.6. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on serum mineral profile

Mineral	Days of collection	NC	IZn-50	NZn-50	NZn-25	SEM	P
Calcium (mg/dL)	0^{th} day	9.19 ±0.46	9.43 ±0.78	9.77 ±0.52	9.76 ±0.64	0.28	0.873
	90th day	10.9 ±0.37	10.7 ±0.42	10.9 ±0.48	10.4 ±0.37	0.20	0.783
Zinc (mg/L)	0^{th} day	0.50 ±0.10	0.41^{a} ±0.07	0.47^{A} ±0.04	0.44^{a} ±0.10	0.04	0.879
	90th day	0.65 ±0.07	0.83^{b} ±0.08	0.90^{B} ±0.09	0.80^{b} ±0.05	0.04	0.184
Iron (mg/L)	0^{th} day	3.39 ±0.46	2.85 ±0.24	3.55 ±0.24	3.40 ±0.29	0.15	0.436
	90th day	3.60 ±0.30	3.55 ±0.11	3.73 ±0.11	3.73 ±0.06	0.08	0.832
Manganese (mg/L)	0^{th} day	0.39 ±0.03	0.33 ±0.00	0.37 ±0.03	0.37 ±0.03	0.01	0.416
	90th day	0.43 ±0.02	0.45 ±0.00	0.45 ±0.00	0.45 ±0.00	0.00	0.516
Copper (mg/L)	0^{th} day	0.96 ±0.07	0.93 ±0.10	1.01 ±0.05	0.94 ±0.11	0.04	0.915
	90th day	0.99 ±0.06	1.07 ±0.05	1.13 ±0.05	0.98 ±0.04	0.03	0.135

^{a,b,A,B} Means with different superscripts in a column differs significantly. ^{a,b,} Means shows a significance level P<0.001 and ^{A,B} Means defines shows a significance level P<0.05. Each value is an average of six observations.

Table 4.3.7. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on haematology of goats

Attributes	NC	IZn-50	NZn-50	NZn-25	Reference values*	SEM	P
RBC (10^6/ µl)	17.4 ±0.55	17.4 ±0.60	18.9 ±0.76	17.7 ±0.71	8-18	0.34	0.714
WBC(10^3/ µl)	16.5 ±1.93	15.0 ±1.98	16.5 ±1.34	13.9 ±2.12	4-13	0.88	0.344
PCV (%)	27.2 ±0.64	28.0 ±0.98	28.1 ±0.81	27.6 ±0.89	22-38	0.39	0.875
Neutrophil (%)	52.4 ±8.45	48.8 ±6.41	50.0 ±5.28	38.0 ±3.49	30-48	3.11	0.391
Lymphocytes (%)	44.2 ±9.00	46.0 ±5.81	48.0 ±5.62	58.2 ±3.48	50-70	3.15	0.423
Eisonophil (%)	2.40 ±0.98	3.80 ±1.59	1.20 ±0.20	2.60 ±0.51	1-8	0.49	0.344
Monocyte (%)	1.00 ±0.00	1.40 ±0.24	0.80 ±0.37	1.20 ±0.20	0-4	0.12	0.374
Haemoglobin (g/dL)	8.50 ±0.19	8.68 ±0.28	8.90 ±0.21	8.50 ±0.11	4-13	0.10	0.493

Each value is an average of six observations. *Feldman *et al*. (2002).

4.3.8. Blood biochemical profiles

The effect of supplementation of graded doses of NZn on blood biochemistry of goats is shown in Table. 4.3.8. ALT (IU/L) was found similar (P>0.05) in all the groups (16.0±3.76 in IZn-50 to 21.9±1.64 in NC). AST (IU/L) level in goats was also similar ((P>0.05) which varied from 197±5.14 (NC) to 229 ±17.6 (IZn-50). Similarly, ALP (IU/L) and creatinine (mg/dL) levels were also similar (P<0.05) among the treatment groups. ALP was more in NZn-50 (378±45.7) and minimum in NC (285±61.3). Creatinine level varied from 1.10±0.14 (NC) to 1.37±0.14 (NZn-25) among different treatment groups.

The blood albumin (g/dL) was found to be similar (P>0.05) among the treatment groups whereas, globulin (g/dL) and total protein (g/dL) varied significantly (P<0.01). Albumin level varied between 3.66±0.03 (NC) to 3.72±0.02 (IZn-50). The globulin level was more in NZn-50 (3.20±0.02) which varied significantly (P<0.01) with both NC and IZn-50. The NZn-25 (3.17±0.02) remained intermediate in globulin level. Similar to globulin, total protein (g/dL) was more (P<0.001) in NZn-50 (6.90±0.01) which varied significantly with NZn-25 (6.85±0.01) and NC (6.78±0.03), but non-significantly with IZn-50 (6.87±0.01). Albumin: globulin ratio was similar (P>0.05) in all the groups which ranged from 1.16±0.01 (NZn-50 and NZn-25) to 1.18±0.01 (IZn-50).

Table 4.3.8. Effect of supplementation of graded doses of NZn (50 and 25 mg/kg) on blood biochemical profiles in goats

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
ALT (IU/L)	21.9 ±1.64	16.0 ±3.76	19.9 ±0.66	17.3 ±1.89	1.17	0.298
AST (IU/L)	197 ±5.14	229 ±17.6	207 ±20.3	198 ±6.19	7.07	0.374
ALP (IU/L)	285 ±61.3	353 ±61.9	378 ±45.7	356 ±44.9	26.1	0.657
Creatinine (mg/dL)	1.10 ±0.14	1.16 ±0.10	1.21 ±0.09	1.37 ±0.14	0.06	0.413
Albumin (g/dL)	3.66 ±0.03	3.72 ±0.02	3.69 ±0.02	3.67 ±0.02	0.01	0.207
Globulin (g/dL)	3.12^{b} ±0.02	3.14^{b} ±0.02	3.20^{a} ±0.02	3.17^{ab} ±0.02	0.01	0.009
Total protein (g/dL)	6.78^{c} ±0.03	6.87^{ab} ±0.01	6.90^{a} ±0.01	6.85^{b} ±0.01	0.01	0.000
Albumin: Globulin	1.17 ±0.01	1.18 ±0.01	1.16 ±0.01	1.16 ±0.01	0.01	0.318

[a,b,c]Means with different superscripts in a row differs (P<0.05) significantly. Each value is an average of six observations.

4.3.9. Hormonal profiles

The serum T_3, T_4, Testosterone and IGF-1 profiles of goats under different dietary treatments are presented in Table 4.3.9. and Fig 4.19.

At zero and 90th day of experiment, T_3 (nmol/L) hormones were found to be 3.69±0.19, 4.17±0.20; 3.54±0.17, 4.29±0.19; 3.37±0.06, 4.41±0.14 and 3.45±0.09, 4.33±0.16, respectively in NC, IZn-50, NZn-50 and NZn-25. At zero and 90th day of experimental feeding, respective values of serum T_4 (nmol/L) hormones were found to be 106±13.3, 90.9±5.50; 104±10.1, 101±7.82; 99.5±6.40, 116±7.98 and 107±7.21, 111±15.1 respectively in NC, IZn-50, NZn-50 and NZn-25. Both the NZn groups showed higher T_3 and T_4 levels than NC and IZn-50 on 90th day samples.

Serum testosterone (ng/ml) in goats were statistically similar (P>0.05) in zero day samples which varied from 3.32±1.54 (NZn-50) to 3.72±1.49 (NC). But significant difference was observed in 90th day serum samples where both NZn-25 (7.48±0.54) and NZn-50 (6.79±0.37) groups exhibited significantly higher (P<0.001) testosterone levels than both IZn-50 (4.35±0.56) and NC (2.65±0.46). All the zinc supplemented groups had significantly higher (P<0.001) serum testosterone than the NC group animals. In all the zinc supplemented groups, there was a rise in testosterone level on 90th day than zero day,

whereas NC group had a decline in testosterone levels at 90th day of experimental feeding as compared to zero day.

IGF-1 (ng/ml) in zero day serum samples was similar (P>0.05) ranging from 166±26.0 (IZn-50) to 126±10.8 (NC), which varied significantly (P<0.05) in the 90th day samples. All the zinc supplemented groups *viz.* IZn-50 (356±30.6), NZn-50 (378±35.9) and NZn-25 (386±39.2) showed significantly higher IGF-1 level than the NC (248±28.5). Zinc supplemented groups (IZn-50, NZn-50 and NZn-25) had similar concentrations of IGF-1.

A positive effect on serum testosterone was noticed due to supplementation of nano Zn at two levels. IGF-I concentrations (ng/ml) were increased due to supplementation of zinc both in inorganic and nano forms.

Table 4.3.9. Effect of supplementation of two levels of NZn (50 and 25 mg/kg) on hormonal profile

Attributes		NC	IZn-50	NZn-50	NZn-25	SEM	P
Triiodothyronine (T_3; nmol/L)	0th day	3.69 ±0.19	3.54 ±0.17	3.37 ±0.06	3.45 ±0.09	0.07	0.417
	90th day	4.17 ±0.20	4.29 ±0.19	4.41 ±0.14	4.33 ±0.16	0.08	0.802
Thyroxine (T_4; nmol/L)	0th day	106 ±13.3	104 ±10.1	99.5 ±6.40	107 ±7.21	4.57	0.944
	90th day	90.9 ±5.50	101 ±7.82	116 ±7.98	111 ±15.1	4.62	0.206
Testoserone (ng/ml)	0th day	3.72 ±1.49	3.42 ±0.88	3.32 ±1.54	3.71 ±1.40	0.63	0.995
	90th day	2.65^{c} ±0.46	4.35^{b} ±0.56	6.79^{a} ±0.37	7.48^{a} ±0.54	0.50	0.000
IGF1 (ng/ml)	0th day (ng/ml)	126 ±10.8	166 ±26.0	131 ±26.1	141 ±30.5	11.8	0.672
	90th day (ng/ml)	248^{b} ±28.5	356^{a} ±30.6	378^{a} ±35.9	386^{a} ±39.2	20.1	0.037

^{a,b,c} Means with different superscripts in a row differs (P<0.05) significantly. Each value is an average of six observations.

4.3.10. Immunity status

Effect of supplementation of two levels of NZn (50 and 25 ppm) on different immune parameters were studied and described as follows.

4.3.10.1. Humoral immunity

Effect of supplementation of two levels of NZn (50 and 25 ppm) on humoral immunity status as measured by antibody titre expressed as per cent inhibition values against PPR virus in goats is depicted in Table 4.3.10. and Fig 4.20. Zero day (pre-immunization) PPR antibody titre was similar (P>0.05) which varied from 14.43 ± 0.98 (IZn-50) to NZn-25 (16.42 ± 0.50). In 21st day post vaccination serum samples, NZn-50 (86.0± 1.40) showed significantly higher (P<0.05) PPR antibody titre than NC (74.90±1.01). But all the zinc supplemented groups (IZn-50, NZn-50, and NZn-25) were statistically similar.

Table 4.3.10. Effect of supplementation two levels of NZn (50 and 25 mg/kg) on humoral immunity status as measured by antibody titre expressed as per cent inhibition values against PPR virus in goats

Parameter	Time	Treatment Particulars				SEM	P
		NC	IZn-50	NZn-50	NZn-25		
PPR antibody titre (HI titre Log_2)	Before vaccination (0 day)	14.96 ± 0.99	14.43 ± 0.98	14.46 ± 0.49	16.42 ± 0.50	0.742	0.263
	After vaccination 21 day	74.90^{b} ± 1.01	80.58^{ab} ± 2.36	86.01^{a} ± 1.40	81.33^{ab} ± 2.81	1.896	0.009

^{a,b,c} Means with different superscripts in a row differs (P<0.05) significantly; Each value is average of six observations.

4.3.10.2. Cell mediated immunity

Effect of supplementation of two levels of NZn (50 and 25 ppm) on cell mediated immunity status as measured by skin fold thickness (mm) of goats is presented in Table 4. 3.11. and Fig 4.22. The skin fold thickness at zero h was similar in all the treatment groups which ranged between 0.12±0.21 (NC) to 0.25±0.38 (IZn-50). But at 6h and 12h post sensitization by Con A showed significant increase in skin fold where all the zinc

supplemented groups *viz.* IZn-50 (2.08±0.18; 1.89±0.16) NZn-50 (2.58±0.18; 1.99±0.19) and NZn-25 (2.14±0.17; 1.85±0.20) showed significantly higher (P<0.05) thickness than NC (1.41±0.06; 1.27±0.04). However, at 24h, the skin thickness was same in different treatment groups varying from 1.14±0.04 in NC to 1.72±0.19 in NZn-50. But, after 24h the CMI followed the same trend as 6h and 12 h with zinc supplemented groups had better (P<0.05) CMI than the NC.

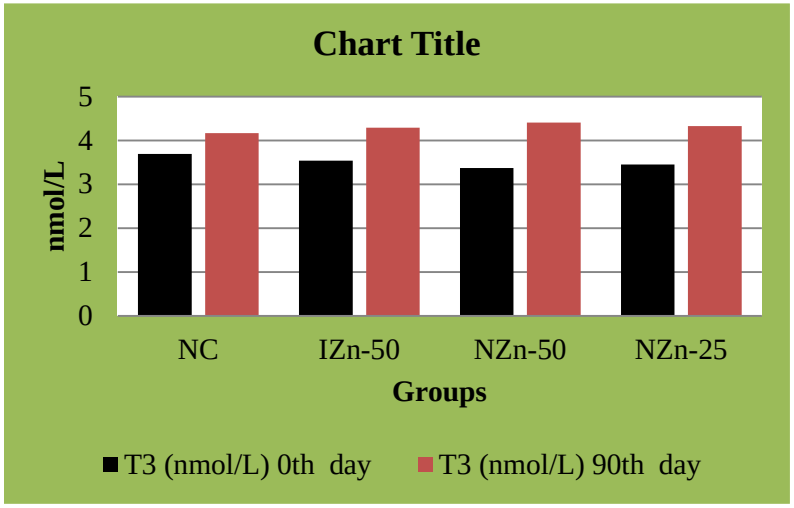
Table 4.3.11. Effect of supplementation of two levels of NZn (50 and 25 ppm) on cell mediated immunity status as measured by skin fold thickness (mm) of goats

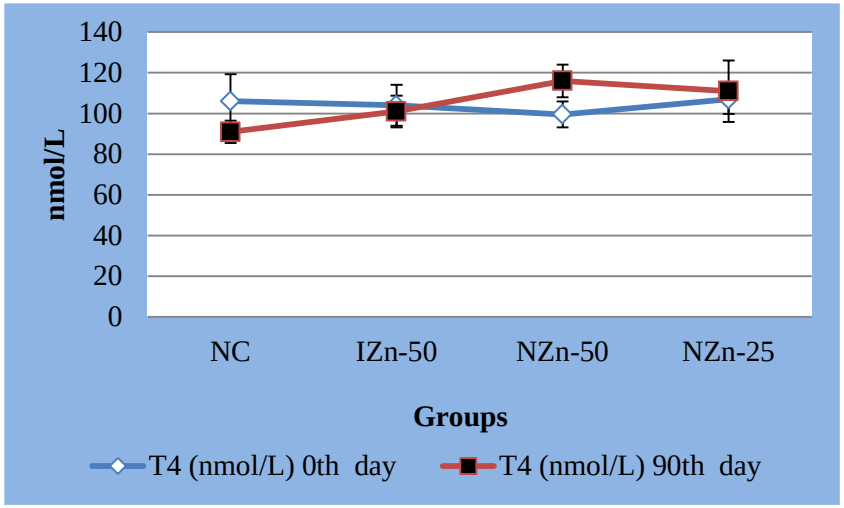
Parameter	Time of measurement	Treatment Particulars				SEM	P
		NC	IZn-50	NZn-50	NZn-25		
Skin thickness in mm	0h	0.12 ±0.21	0.25 ±0.38	0.13 ±0.09	0.14 ±0.28	0.16	0.992
	6h	1.41^{b} ±0.06	2.08^{a} ±0.18	2.58^{a} ±0.18	2.14^{a} ±0.17	0.13	0.002
	12h	1.27^{b} ±0.04	1.89^{a} ±0.16	1.99^{a} ±0.19	1.85^{a} ±0.20	0.10	0.044
	24h	1.14 ±0.04	1.55 ±0.15	1.72 ±0.19	1.60 ±0.25	0.10	0.169
	48h	0.87^{b} ±0.08	1.31^{a} ±0.15	1.41^{a} ±0.12	1.35^{a} ±0.14	0.08	0.040
CMI response after 24 h from 0h	Increase thickness (mm)	1.02	1.3	1.59	1.21	-	-
	%increase in skin thickness w.r.t. IZn-50	78.46	100	122.3	93.1	-	-
CMI response after 48 h from 0h	Increase thickness (mm)	0.75	1.06	1.28	1.21	-	-
	% increase in skin thickness w.r.t. IZn-50	70.75	100	120.7	114.1	-	-

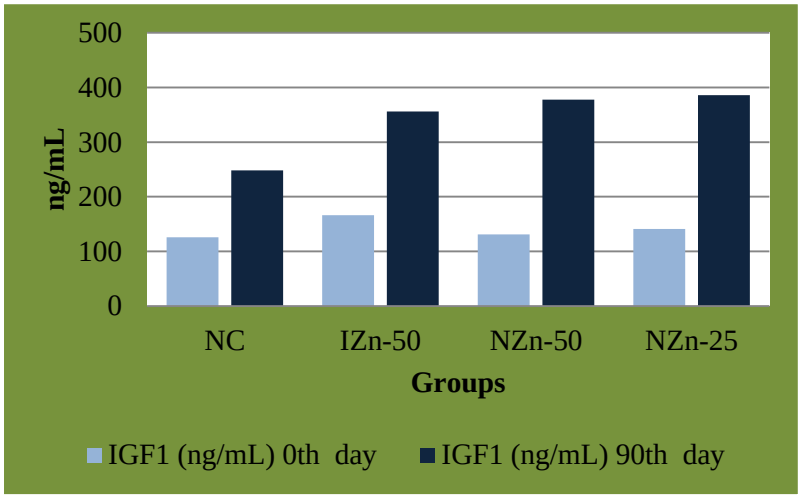
[a,b,c] Means with different superscripts in a row differs (P<0.05) significantly; Each value is an average of six observations.

After 48h, NZn-50 (1.41±0.12), NZn-25 (1.35±0.14), IZn-50 (1.31±0.15) groups showed better immune response than NC (0.87±0.08). The increase in skin thickness in

Fig 4.19. Supplementation of graded doses of NZn on hormonal profile in goats







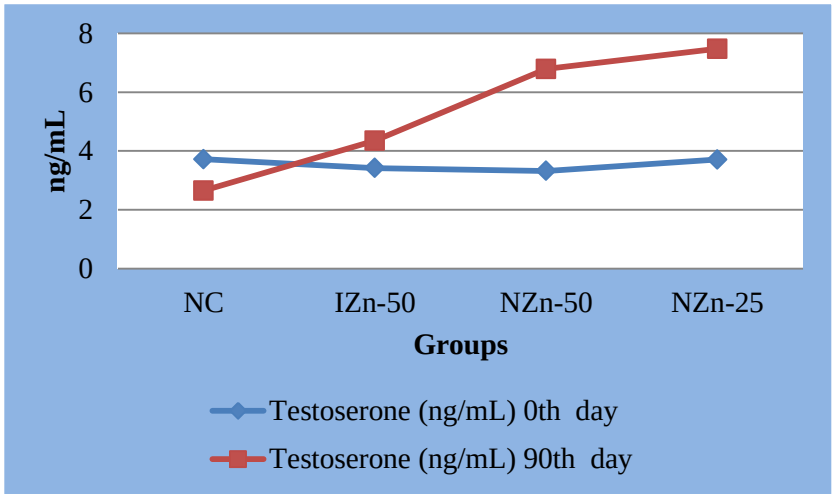


Fig 4.20. Effect of supplementation of graded levels of NZn on humoral immunity in goats

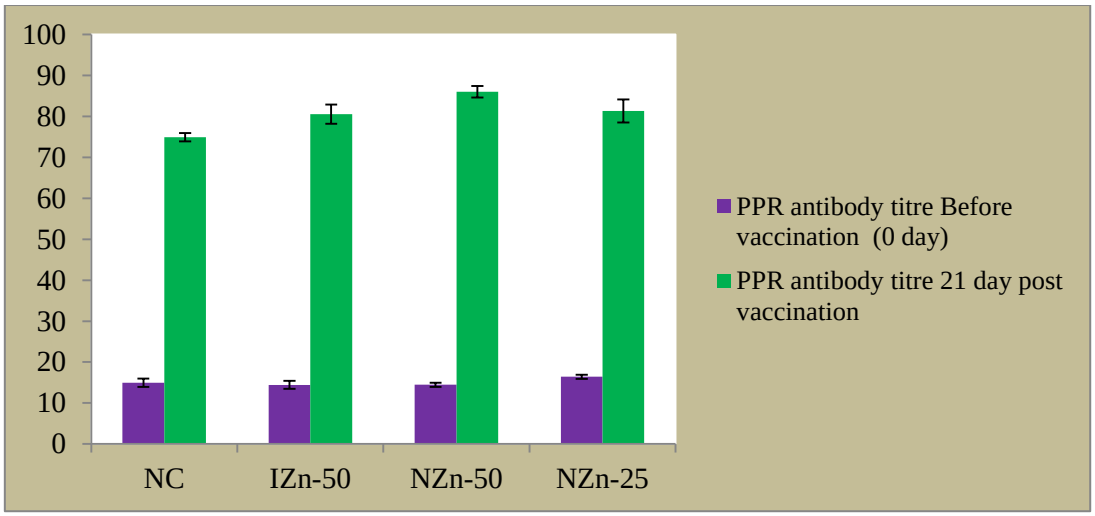


Fig 4.21. Effect of supplementation of NZn at graded levels on CD4 and CD8 cells

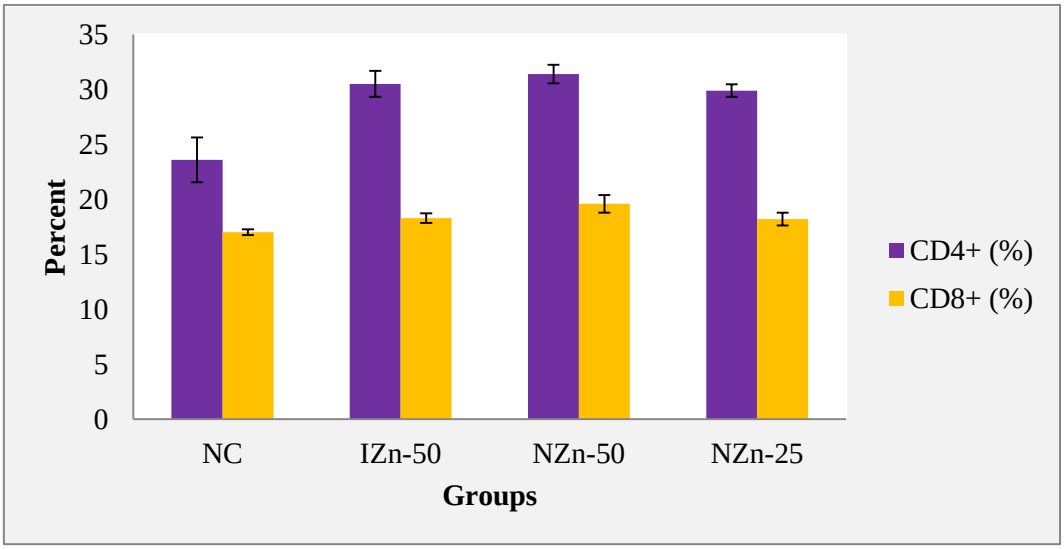
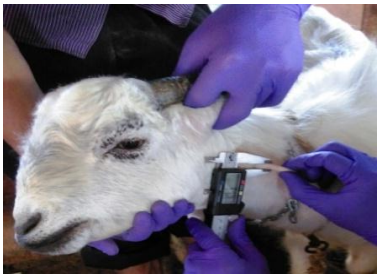
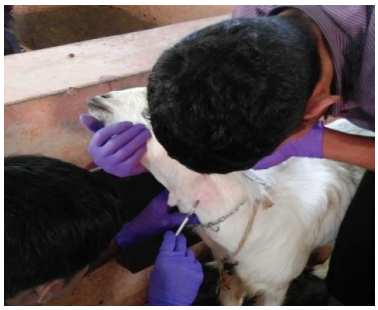


Fig 4.22. Delayed type hypersensitivity reaction to evaluate CMI in goats

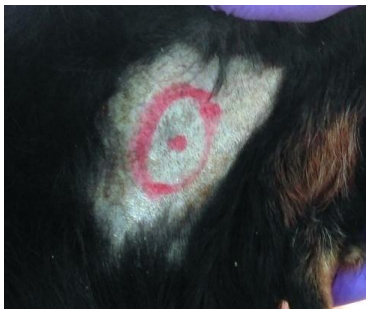
a) Demarkation of the area for injection



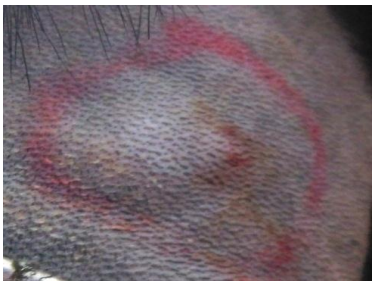
b) Skin thickness measurment before injection



c) Injection of antigen (Con-A)

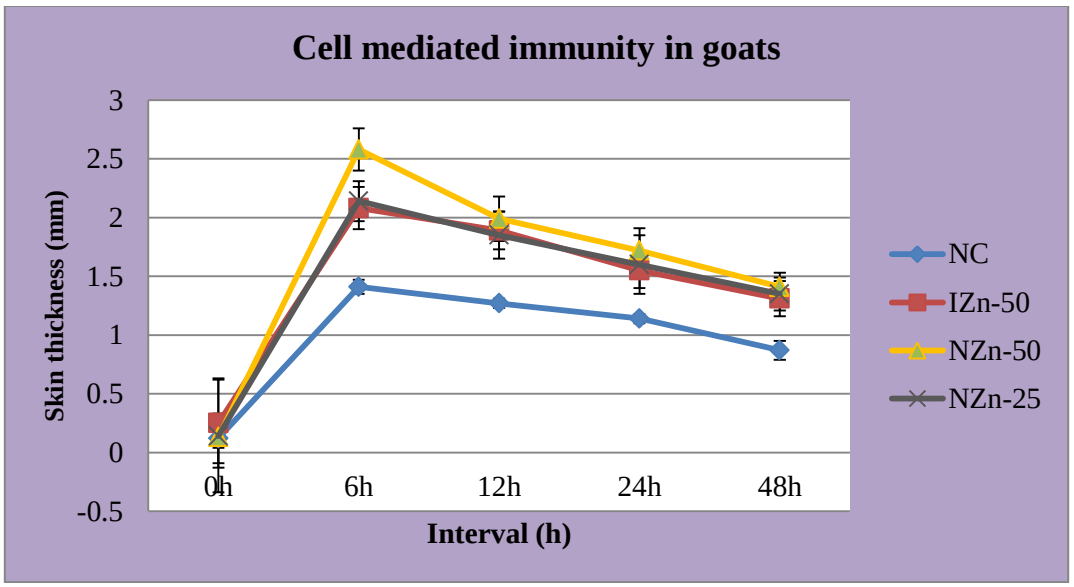


d) Swelling of the imjected area



e)Swelling showing the Cell meidated immunity

f) measurement of the swelling (mm)



g) Skin fold thickness recorded at different intervals to assess CMI in goats

NZn-50 was more (122.3% at 24h and 120.7% at 48h) as compared to IZn-50, indicating immune-modulatory effects of NZn.

4.3.10.3. Immunophenotyping in terms of $CD4^+$ and $CD8^+$ counts

Effect of supplementation of two levels of NZn (50 and 25 ppm) on proportion of $CD4^+$, $CD8^+$ and $CD4^+$:$CD8^+$ ratio of goats is summarized in Table 4.3.12. and Fig 4.21. The $CD4^+$ (%) was significantly high ($P<0.05$) in all the zinc supplemented groups than the NC group fed only with basal diet without any zinc supplementation. Among zinc supplemented groups, NZn-50 had highest $CD4^+$ (%) count with 31.4±0.84, followed by IZn-50 (30.5±1.18), NZn-25 (29.9±0.57) and NC (23.6±2.03). Like $CD4^+$, $CD8^+$ (%) also followed the same trend. NZn-50 (19.6±0.80) showed significantly higher ($P<0.05$) $CD8^+$ count than NC (17.0±0.26) and similar ($P>0.05$) to IZn-50 (18.3±0.43) and NZn-25 (18.2±0.58) groups. $CD4^+$: $CD8^+$ ratio varied non-significantly ($P>0.05$) among the treatment groups, where the ratio was highest in NZn-25 (1.71±0.09) followed by IZn-50 (1.69±0.08), NZn-50 (1.62±0.11) and NC (1.39±0.12).

Results indicated that NZn supplementation caused marked improvements in humoral immunity, cell mediated immunity and increased $CD4^+$ and $CD4^+$ counts.

Table 4.3.12. Effect of supplementation of two levels of NZn (50 and 25 ppm) on proportion of CD4, CD8 and CD4:CD8 ratio in goats

Parameter	Treatment Particulars				SEM	P
	NC	IZn-50	NZn-50	NZn-25		
$CD4^+$ (%)	23.6^b ±2.03	30.5^a ±1.18	31.4^a ±0.84	29.9^a ±0.57	0.98	0.004
$CD8^+$ (%)	17.0^b ±0.26	18.3^{ab} ±0.43	19.6^a ±0.80	18.2^{ab} ±0.58	0.34	0.045
$CD4^+$:$CD8^+$	1.39 ±0.12	1.69 ±0.08	1.62 ±0.11	1.71 ±0.09	0.06	0.153

^{a,b} -Means with different superscript in a row differ significantly ($P<0.05$). Each value is an average of six observations.

4.3.11. Rumen fermentation characteristics

Effect of supplementation of graded levels of NZn on rumen fermentation in goats in terms of rumen pH, ammonia nitrogen, volatile fatty acid profile and rumen soluble mineral profile is described below.

4.3.11.1. Rumen pH, ammonia nitrogen and volatile fatty acid profile

Effect of supplementation of graded levels of NZn on rumen fermentation in goats is summarized in Table 4.3.13**.** To assess the effect of zinc supplementation, samples were analysed on zero day (before start of experimental feeding) and on 90th day of experimental feeding. The pH on zero day was non-significant (P>0.05) among the groups varying from 6.58±0.07 to 6.72±0.05 in NZn-50 and NC respectively. But final pH varied significantly (P<0.05) among the treatment groups, with NC group exhibited significantly higher pH than that of IZn-50 and NZn-50. No significant difference (P>0.05) in rumen ammonia nitrogen (NH_3-N, mg/100mL) was observed on both initial and final rumen liquor samples. NH_3-N (mg/100mL) content in zero day rumen liquor samples were 15.4±0.47 in NC, 15.7±0.64 in IZn-50, 15.3±0.99 in NZn-50 and 15.7±0.77 in NZn-25 group. On 90th day of experimental feeding, NH_3-N in the treatment groups varied from 22.3±0.67 (NC) to 25.3±1.10 (NZn-50).

Acetate, Propionate, butyrate, valerate and Total VFA (mmol/L) content in RL were non-significant in zero and 90th day samples. Acetate content varied from 48.0±4.50 to 51.4±2.50 (NZn-50) (NC) in zero day samples. In 90th day samples the acetate concentration (mmol/dL) was highest in NZn-50 (66.6±2.78) followed by IZn-50 (65.2±1.89), NZn-25(63.8±1.87) and NC (62.8±2.76). Propionate concentration was highest in NZn-50 (12.7±0.40) in zero day and in NZn-25 (18.2±0.75) in 90th day samples. Propionate concentration was found lowest in NZn-25 (11.2±0.35) on zero day whereas, both IZn-50 (16.9±1.15) and NC (16.9±0.94) had lowest propionate on 90th day collected samples. In Zero day samples, concentration of butyrate was 6.69±0.24, 7.75±0.70, 6.89±0.30 and 6.73±0.30 whereas; in 90th day samples it was 7.95±0.45, 7.63±0.63, 7.60±0.33 and 8.79±0.81 in NC, IZn-50, NZn-50 and NZn-25 group, respectively. Likewise, valerate concentration varied from 0.45±0.02 (NC) to 0.53±0.07 (IZn-50) in zero day samples and 0.82±0.08 (NZn-25) to 0.70±0.05 (NZn-50) on 90th day samples. The total VFA (mmol/dL) under different treatments on zero day were 66.6±5.60, 71.3±1.14, 71.4±3.06 and 68.4±2.74 whereas, in 90th day the same was 88.5±3.28, 90.5±2.96, 92.2±3.30 and 91.6±2.66 in NC, IZn-50, NZn-50 and NZn-25 group, respectively.

Thus, results indicated that individual and total VFA did not differ significantly among the treatment groups on 0 and 90th day of experimental feeding.

4.3.11.2. Rumen soluble mineral profiles

Effect of supplementation of graded doses of NZn on rumen soluble mineral contents (mg/L) of strained rumen liquor of goats at 90th day of experimental feeding is presented in Table 4.3.14. and Fig 4.24. Zinc content in SRL varied significantly (P<0.01) among the treatment groups. The zinc content in SRL was highest (P<0.01) in NZn-50 (1.99±0.08) compared to NC, and NZn-25, whereas IZn-50 (1.81±0.12) had significantly higher (P<0.05) soluble Zn in SRL than NC (1.37± 0.09) However, NZn-25 did not vary significantly either with IZn-50 or with NC with respect to the rumen soluble zinc contents.

Table 4.3.13. Effect of supplementation of NZn (50 and 25 mg/kg) on rumen fermentation

Attributes		**NC**	**IZn-50**	**NZn-50**	**NZn-25**	**SEM**	**P**
Ruminal pH	0th day	6.72 ±0.05	6.60 ±0.06	6.58 ±0.07	6.59 ±0.05	0.03	0.282
	90th day	6.40^{a} ±0.05	6.24^{b} ±0.02	6.26^{b} ±0.05	6.31^{ab} ±0.03	0.02	0.036
NH_3-N (mg/100mL)	0th day	15.4 ±0.47	15.7 ±0.64	15.3 ±0.99	15.7 ±0.77	0.35	0.977
	90th day	22.3 ±0.67	24.2 ±1.03	25.3 ±1.10	23.9 ±2.63	0.77	0.572
Individual Volatile fatty acid profile (mmol/L)							
Acetate	0th day	48.0 ±4.50	50.6 ±0.54	51.4 ±2.50	50.0 ±2.77	1.35	0.862
	90th day	62.8 ±2.76	65.2 ±1.89	66.6 ±2.78	63.8 ±1.87	1.16	0.701
Propionate	0th day	11.4 ±0.98	12.3 ±0.78	12.7 ±0.40	11.2 ±0.35	0.34	0.399
	90th day	16.9 ±0.94	16.9 ±1.15	17.3 ±0.86	18.2 ±0.75	0.44	0.725
Butyrate	0th day	6.69 ±0.24	7.75 ±0.70	6.89 ±0.30	6.73 ±0.30	0.22	0.303
	90th day	7.95 ±0.45	7.63 ±0.63	7.60 ±0.33	8.79 ±0.81	0.29	0.448
Valerate	0th day	0.45 ±0.02	0.53 ±0.07	0.48 ±0.03	0.49 ±0.00	0.02	0.608
	90th day	0.74 ±0.03	0.75 ±0.02	0.70 ±0.05	0.82 ±0.08	0.02	0.433
Total VFA	0th day	66.6 ±5.60	71.3 ±1.14	71.4 ±3.06	68.4 ±2.74	1.66	0.725
	90th day	88.5 ±3.28	90.5 ±2.96	92.2 ±3.30	91.6 ±2.66	1.46	0.828

^{a,b} -Means with different superscripts in a row differs significantly (P<0.05). Each value is an average of six observations.

Table 4.3.14. Effect of supplementation of graded doses of NZn on soluble mineral contents (mg/L) in strained rumen liquor of goats at 90th day of experimental feeding

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
Zinc	1.37[c] ±0.09	1.81[ab] ±0.12	1.99[a] ±0.08	1.64[bc] ±0.12	0.07	0.003
Iron	3.17 ±0.12	3.21 ±0.15	3.21 ±0.14	3.21 ±0.17	0.07	0.997
Manganese	4.99 ±0.33	6.22 ±0.23	6.14 ±0.57	6.15 ±0.61	0.24	0.218
Copper	0.38 ±0.02	0.39 ±0.03	0.36 ±0.01	0.36 ±0.01	0.01	0.487

[a,b,c] Means with different superscripts in a row differs (P<0.05) significantly. Each value is an average of six observations.

No significant differences were observed in the rumen soluble iron, manganese and copper contents (mg/L) in the strained RL of goats at 90th day. The soluble iron was almost same in all the zinc supplemented groups, but in NC it was numerically less i.e. 3.17±0.12. Manganese (mg/L) in rumen liquor varied from 4.99±0.33 in NC to 6.22±0.23 in IZn-50. Likewise soluble copper (mg/L) in SRL varied from 0.36±0.01 in both the NZn supplemented groups (NZn-50 and NZn-25) to 0.39±0.03 (IZn-50).

Supplementation of inorganic and nano form of zinc exerted similar rumen fermentation pattern. However, it resulted in more soluble zinc contents due to supplementation of inorganic as well as nano zinc at higher level.

4.3.12. Supplementation of NZn (50 and 25 mg/kg) on male reproduction

The effect of supplementation of graded doses of NZn on male goat reproduction in terms of semen quality, sperm motility and velocity is described below.

4.3.12.1. Semen volume and total semen production

The effect of supplementation of NZn (50 and 25 mg/kg) on sperm quality is depicted in Table 4.3.15.. The semen volume (mL) did not vary statistically (P>0.05) due to the dietary treatment. The average semen volume was highest in NZn-50 (1.36±0.13) and minimum in IZn-50 (1.25±0.17). The total sperm production in millions was also similar (P>0.05) among the treatment groups which varied in a range of 2002±472 (IZn-50) to 2544±542 (NZn-25).

Fig 4.23. Effect of supplementation of graded levels of NZn on serum zinc profile in goats

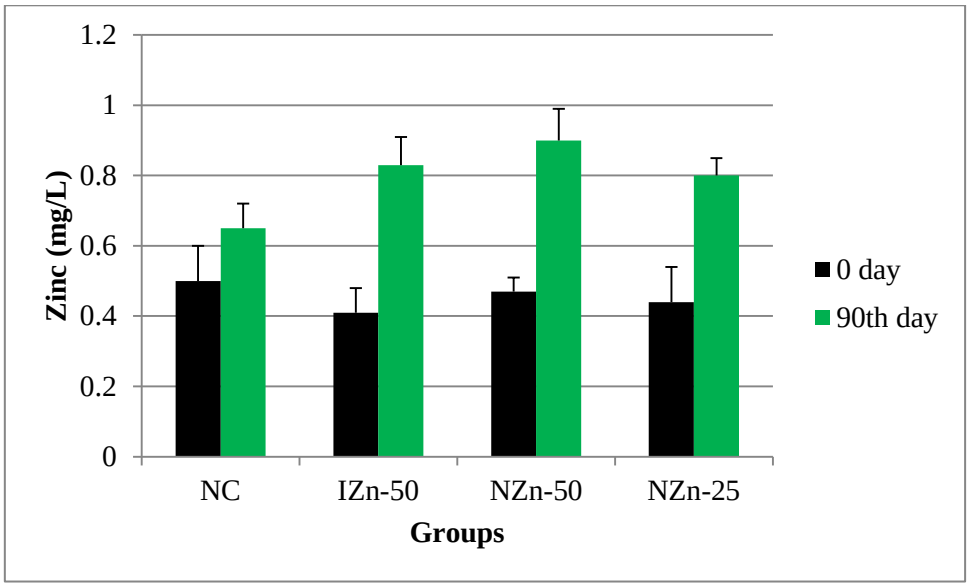


Fig 4.24. Effect of supplementation of graded levels of NZn on rumen soluble zinc in goats

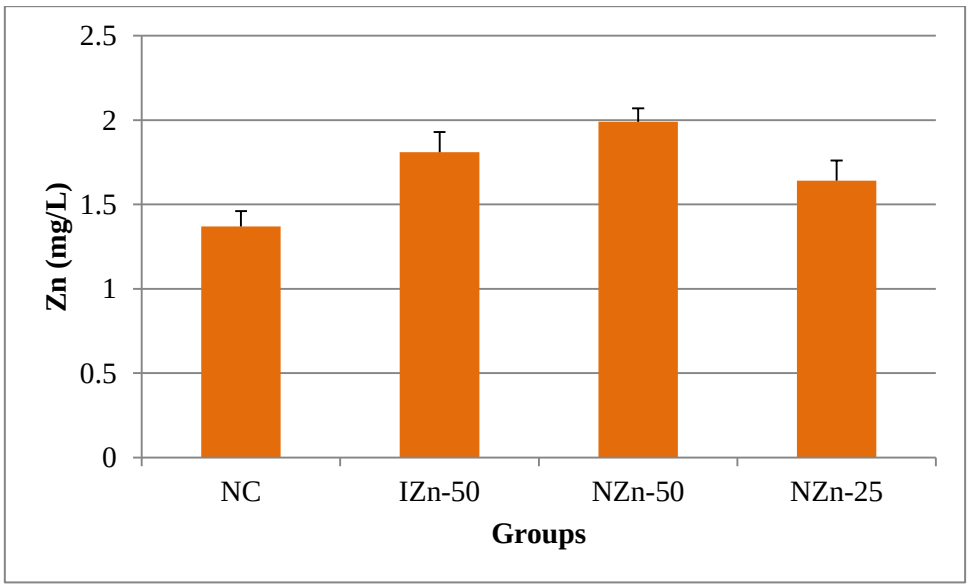


Table 4.3.15. Effect of supplementation of NZn (50 and 25 mg/kg) on sperm quality in goats

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
Semen volume (mL)	1.26 ±0.19	1.25 ±0.17	1.36 ±0.13	1.35 ±0.16	0.08	0.984
Total Sperm production (millions)	2537 ±430	2002 ±472	2015 ±167	2544 ±542	214	0.399

Each value is an average of six observations.

4.3.12.2. Semen motility

The effect of supplementation of NZn on sperm motility is presented in Table 4.3.16. and Fig 4.25. The total motile sperms were significantly higher (P<0.01) in NZn supplemented groups than that of NC. However, IZn-50 showed non-significant variation with both NC and NZn supplemented groups (NZn-50 and NZn-25). The total motile sperm (%) was highest in NZn-50 (81.7±1.61) and least in NC (64.7±2.35). Non-progressive motile sperm (%) did not vary statistically (P>0.05) among the treatment groups and varied in a range of 19.5±1.75 in NZn-50 to 26.9±2.29 in NC. But, progressive forward motile sperm (%) was significantly high (P<0.001) in NZn-50 (62.2±2.47) as compared to that of NC (37.9±2.81) and IZn-50(49.5±3.02), but was similar to NZn-25 (57.9±3.69). Even though NZn-25 was similar to both NZn-50 and IZn-50, it was significantly higher progressive motile sperms than NC. The rapid motile sperms (%), characterised by rapid velocity more than 50 µm/sec, were significantly higher (P<0.001) in NZn supplemented groups at either doses than both NC and IZn-50, which was found maximum in NZn-50 (75.8±1.96) and minimum in NC group (50.1±3.28). But, with respect to rapid sperms (%), a non significant relation was observed between NC with IZn-50 and NZn-50 with NZn-25 groups. Considering the medium motile sperms (%) i.e. with a velocity between 25 to 50 µm/sec, all groups were found similar (P>0.05) with highest in IZn-50 (8.50±2.87) and least in NZn-50 (3.48±0.38). Very high (P<0.001) slow motile sperms, (>10 to <25 µm/sec) were observed in NC (9.69±1.53%) which varied significantly with all the zinc supplemented groups namely IZn-50 (4.18±0.76%), NZn-50 (2.36±0.73%) and NZn-25 (2.17±0.49%). Static sperms (%, characterised by a velocity less than 10 µm/sec) were significantly high (P<0.01) in NC (35.3±2.35) and IZn-50 group (27.1±2.13) than NZn-50 (18.3±1.61) and NZn-25 (20.1±5.14). The hyperactivity (%) in

sperms was found non-significant (P>0.001) among the treatment groups which fell in the range between 19.4±2.40 (NC) to 26.0±2.09 (NZn-25).

Table 4.3.16. Effect of supplementation of NZn on sperm motility parameters in goats

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
Total motile (%)	64.7^{b} ±2.35	72.9^{ab} ±2.13	81.7^{a} ±1.61	79.9^{a} ±5.14	2.18	0.005
Non-progressive motile (%)	26.9 ±2.29	23.4 ±3.63	19.5 ±1.75	22.0 ±3.42	1.27	0.340
Progressive motile (%)	37.9^{c} ±2.81	49.5^{b} ±3.02	62.2^{a} ±2.47	57.9^{ab} ±3.69	3.69	0.000
Rapid (%)	50.1^{b} ±3.28	60.2^{b} ±4.45	75.8^{a} ±1.96	73.9^{a} ±5.53	3.12	0.001
Medium (%)	4.89 ±0.37	8.50 ±2.87	3.48 ±0.38	3.85 ±0.47	0.77	0.140
Slow (%)	9.69^{a} ±1.53	4.18^{b} ±0.76	2.36^{b} ±0.73	2.17^{b} ±0.49	0.77	0.000
Static (%)	35.3^{a} ±2.35	27.1^{a} ±2.13	18.3^{b} ±1.61	20.1^{b} ±5.14	2.18	0.005
Hyperactivity (%)	19.4 ±2.40	24.3 ±4.03	23.7 ±2.92	26.0 ±2.09	1.47	0.461

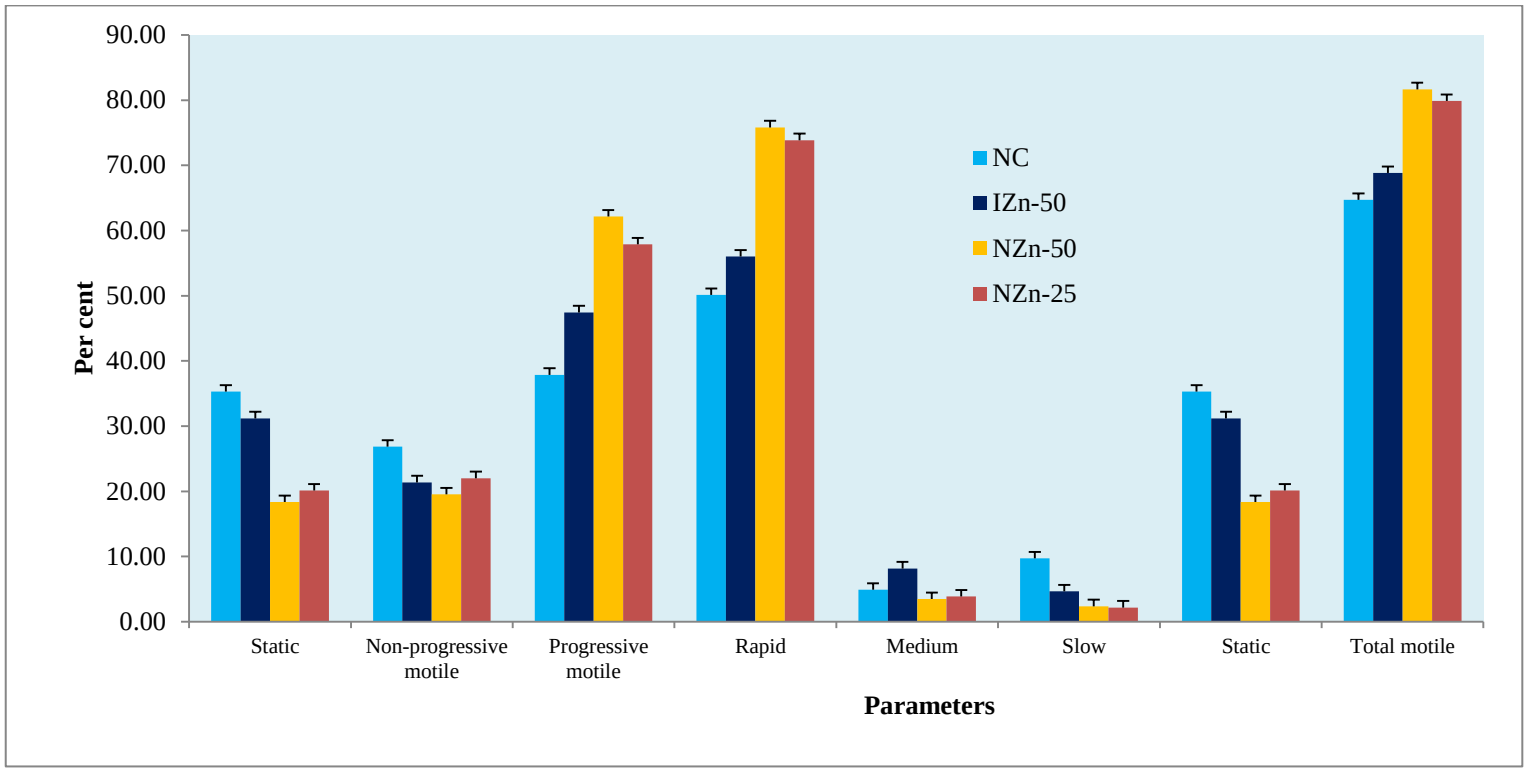
[a,b] Means with different superscripts in a row differs (P<0.05) significantly. Each value is an average of six observations.

4.3.12.3. Sperm velocity

Effect of supplementation of NZn (50 and 25 mg/kg) on sperm velocity is presented in Table 4.3.17. and Fig 4.26. There was a significant raise in fast progressive (Type A) sperms (%) in NZn-50 (59.8±2.41) and NZn-25 (55.2±3.95) as compared to IZn-50 (44.2±4.42) and NC (34.6±2.79), which were similar amongst them. but Slow progressive (Type B) sperms (%) were similar (P>0.05) among the treatment groups which was highest in NZn-25 (21.3±3.08) and least in NZn-50 (18.5±1.63). Regarding non-progressive (Type C) sperms (%), NC (11.3±1.24) had significantly higher P<0.001) Type C sperms than other three groups, whereas NZn-50 (3.44±0.72) and NZn-25 (3.37±0.47) groups had significantly lower Type C sperms than NC and IZn-50 (7.42±1.70). Type D or immotile or static sperms (%) were highest (P<0.01) in NC (35.3±2.35) followed by IZn-50 (27.1±2.13) which differed significantly with NZn-50 (18.3±1.61) and NZn-25 (20.1±5.14).

Head area (μm^2) was highest (P<0.001) in NZn-50 (32.6±0.47) which differed significantly with other three groups i.e. IZn-50 (30.5±0.56), NC (25.0±0.61) and NZn-

Fig 4.25. Effect of supplementation of graded levels of NZn on sperm motility parameters in goats



25(31.0±0.46). IZN-50 and NZN-25 were intermediate and varied significantly both with NC and NZn-50. Sperm of NZn-50 (8.95±0.60) had significantly lower (P<0.01) circular track (%) as compared to IZn-50 (18.3±2.62) and NC (18.3±2.66). However NZn-25 (12.9±2.02) was non-significant to NC, IZn-50 and NZn-50 also. Curvilinear velocity (µm/Sec) varied significantly among the treatment groups with NZn-50 (138±2.66) had higher curvilinear velocity of sperms than IZn-50 (113±7.14) and NC (118±2.29). NZn-25 (129±5.01) had significantly higher curvilinear velocity (µm/Sec) than IZn-50 but varied non-significantly with NZn-50 and NC. The straight-line velocity (µm/Sec) in NZn-50 (112±2.18) and NZn-25 (98.0±4.33) was more (P<0.001) which varied significantly with IZn-50 (78.7±7.72) and NC (79.2±4.77) groups.

The average path velocity (µm/Sec) and linearity (%) were statistically better (P<0.001) in sperm samples of NZn-50 (128±2.32; 80.8±0.63) and NZn-25 (114±5.09; 75.8±1.79) than that of IZn-50 (92.9±8.00; 68.2±2.77) and NC (96.1±4.43; 65.5±2.82). However, there was non-significant difference (P>0.05) in IZn-50 compared to NC and NZn-50 with NZn-25 groups with respect to the sperm average path velocity (µm/Sec) and linearity (%). No change was observed among the treatment groups with respect to the straightness (%) of sperms (P>0.05) which fell in the range of 81.6±1.70 (NC) to 87.2±0.79 (NZn-50). Wobble (%) in sperms of different treatment groups has significantly higher (P<0.001) in both the NZn supplemented groups (NZn-50 and NZn-25) as compared to both NC and IZn-50.

Head displacement (µm) was maximum in NC (3.17±0.08) group which varied statistically (P<0.01) with NZnO-5O (2.46±0.07) and NZn-25 (2.72±0.05).but no significance was observed between NC with IZn-50, NZn-50 with NZn-25 and NZn-25 with IZn-50 groups. Beat/cross frequency (Hz) and typa-A spermatozoa (%) was similar (P>0.05) among the treatment groups varying within a range of 8.58±0.27 (NZn-50) to 9.69±0.61 (IZn-50) and 18.5±2.38 (NC) to 25.3±2.05 (NZn-25).

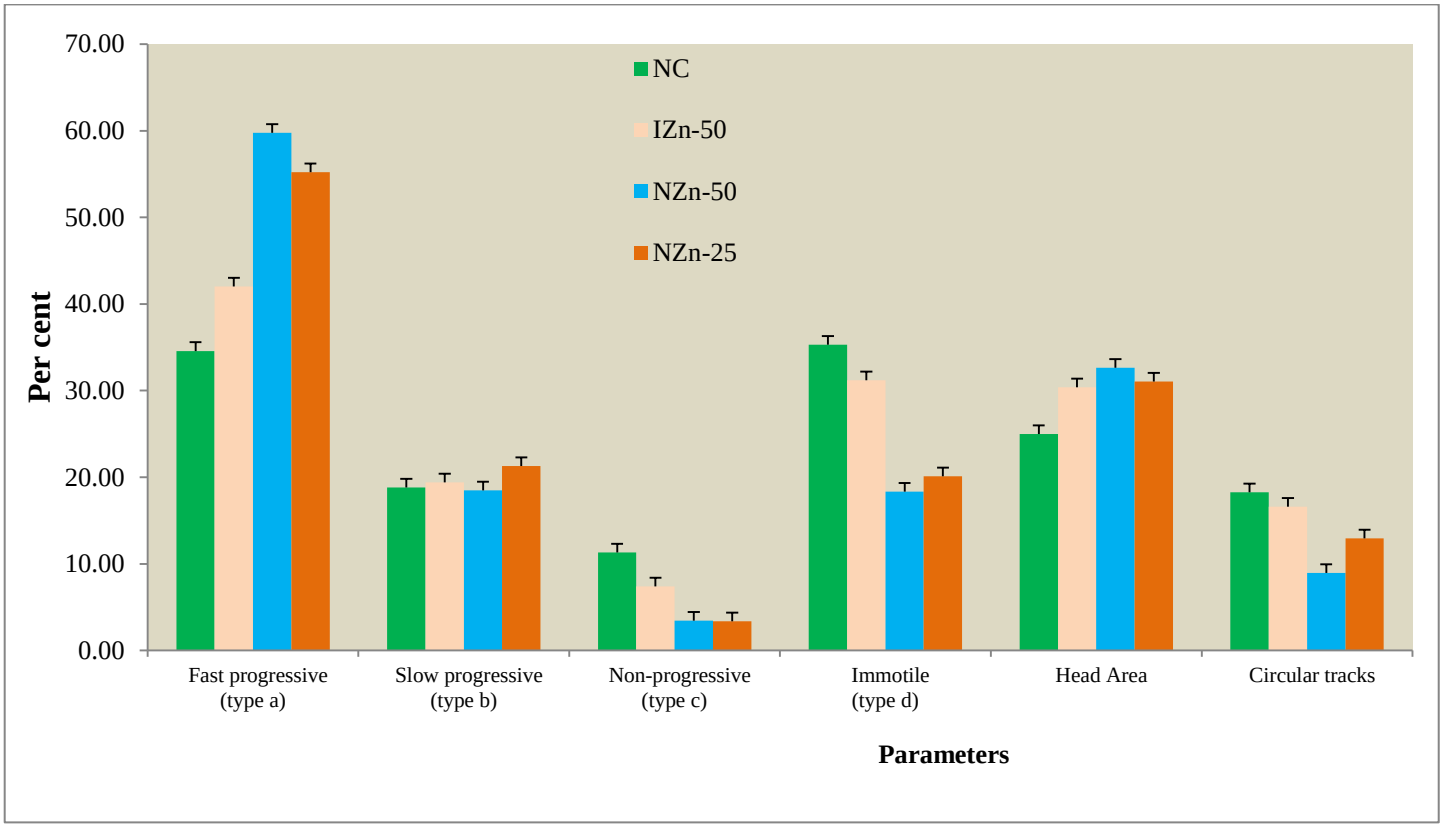
Zinc supplementation in nano form has a clear advantage in improving motility parameters in terms of motile, progressive motile and rapid (%) sperms and reducing slow and static (%) sperms. Further it increased various velocity parameters such as fast progressive (type a), head area (μm^2), curvilinear velocity, straight-line velocity (µm/Sec), average path velocity (µm/Sec), linearity (%), Wobble (%) and Type A spermatozoa (%).

Table 4.3.17. Effect of supplementation of NZn (50 and 25 mg/kg) on sperm velocity parameters of goats

Attributes	NC	IZn-50	NZn-50	NZn-25	SEM	P
Fast progressive (type a) (%)	34.6^{b} ±2.79	44.2^{b} ±4.42	59.8^{a} ±2.41	55.2^{a} ±3.95	2.80	0.000
Slow progressive (type b) (%)	18.8 ±1.98	21.2 ±3.05	18.5 ±1.63	21.3 ±3.08	1.04	0.799
Non-progressive (type c) (%)	11.3^{a} ±1.24	7.42^{b} ±1.70	3.44^{c} ±0.72	3.37^{c} ±0.47	0.86	0.000
Immotile (type d) (%)	35.3^{a} ±2.35	27.1^{a} ±2.13	18.3^{b} ±1.61	20.1^{b} ±5.14	2.18	0.005
Head Area (μm^2)	25.0^{c} ±0.61	30.5^{b} ±0.56	32.6^{a} ±0.47	31.0^{b} ±0.46	0.65	0.000
Circular tracks (%)	18.3^{a} ±2.66	18.3^{a} ±2.62	8.95^{b} ±0.60	12.9^{ab} ±2.02	1.18	0.009
Curvilinear Velocity (µm/Sec)	118^{bc} ±2.29	113^{c} ±7.14	138^{ab} ±2.66	129^{a} ±5.01	3.30	0.007
Straight-line Velocity (µm/Sec)	79.2^{b} ±4.77	78.7^{b} ±7.72	112^{a} ±2.18	98.0^{a} ±4.33	3.88	0.001
Average Path Velocity (µm/Sec)	96.1^{b} ±4.43	92.9^{b} ±8.00	128^{a} ±2.32	114^{a} ±5.09	4.10	0.000
Linearity (%)	65.5^{b} ±2.82	68.2^{b} ±2.77	80.8^{a} ±0.63	75.8^{a} ±1.79	1.61	0.000
Straightness (%)	81.6 ±1.70	84.0 ±1.87	87.2 ±0.79	85.7 ±1.67	0.77	0.060
Wobble (%)	79.5^{b} ±2.29	81.0^{b} ±3.28	92.8^{a} ±0.39	88.4^{a} ±0.90	1.48	0.000
Head displacement (µm)	3.17^{a} ±0.08	2.90^{ab} ±0.21	2.46^{c} ±0.07	2.72^{bc} ±0.05	0.08	0.005
Beat/Cross Frequency (Hz)	9.58 ±0.34	9.69 ±0.61	8.58 ±0.27	8.91 ±0.13	0.20	0.141
Type-A spermatozoa (%)	18.5 ±2.38	21.7 ±3.49	23.4 ±2.96	25.3 ±2.05	1.44	0.403

[a,b,c] Means with different superscripts in a row differs ($P<0.05$) significantly. Each value is an average of six observations.

Fig 4.26. Effect of supplementation of graded levels of NZn on sperm velocity parameters of goats



DISCUSSION

The results obtained during this study are discussed in this chapter by dividing this into three sections.

Phase-I. Characterization of nano zinc (NZn) particle

The nanoparticles (NP) were synthesized by both physical and chemical methods. The particles synthesized through chemical method were characterised by Particle Size analysis, X- ray diffractometer (XRD), Raman spectrophotometer, Transmission electron microscope (TEM) and Energy dispersive X-Ray spectroscopy (EDAX). The results confirmed that the Zn particles obtained from chemical method were of the dimension of 74 nm. The NZn particles were rod shaped (TEM picture). The averege NP obtained from physical method was 197nm.

Among metal NP annually produced, by volume, nano zinc oxide (NZnO) is the third highest globally produced nanometal after nano SiO_2 and nano TiO (Piccinno *et al*., 2012). The sudden rise in the demand in zinc oxide nanoparticles (ZnO NP) is mostly attributed to its better antibacterial properties than the conventional ZnO (Padmavathy and Vijayaraghavan, 2008). Zinc NP can be synthesized by physical, chemical or biological methods (Swain *et al*., 2015), out of which chemical method can produce uniform NP ealisy (Swain *et al*., 2016). Nanomaterials are having dimensions below 100 nm, are stable under high temperature and pressure (Stoimenov *et al*., 2002). By virtue of their small size, it is easier to be taken up by the gastrointestinal tract, so are more effective than the larger size ZnO at lower doses (Feng *et al*., 2009). In the animal body, nano minerals interact more effectively with organic and inorganic substances due to their larger surface area (Zaboli *et al*., 2013). A range of instrumentation is frequently used to measure NP physical attributes: X-ray analysis reports on NP crystallinity; electron- and scanning-probe microscopies reveal nanoscale structure (Dimkpa *et al*., 2012). These methods were followed to characterise the NP synthesized for animal feeding.

Han *et al*. (2011) also reported the particle size of NZn to be in the range of 40 – 80 nm from its oxide salt, which falls within our findings. Arabi *et al*. (2012) synthesized

the NZn particles by using zinc nitrate and sodium hydroxide and characterized by using both TEM and SEM at the scale of 20 nm and 10 nm scales. Even though method was same, our particles are bigger than the reported values of Arabi *et al*. (2012) which may be due to conditions while mixing the chemicals and speed at which particles of zinc nitrate was added to sodium hydroxide.

5.2. Phase II: To evaluate the effect of different doses of nano zinc on growth, nutrient utilization and expression of selected genes in Wistar Albino Rats

5.2.1. Proximate and mineral composition

The dry matter, organic matter, crude protein, ether extract and total carbohydrate (%), of the semi-purified synthetic diet was similar among the groups. Calcium, phosphorus, magnesium were also as per the recommended levels in all the groups. The diets were also found to contain sufficient levels of minerals as recommended for rats (NRC, 1978 and ICAR, 2013b) except for zinc (Ca %, 0.50; P %, 0.40; Mg %, 0.04; Copper mg/kg, 5; Manganese, mg/kg, 50; Iron mg/kg, 35).The analytical level of zinc (mg/kg) in the diets of different groups was recorded to be 9.35 in NC, 34.3 in IZn-25, 34.1 in NZn-25, 22.2 in NZn-12.5, 16.1 in NZn-6.25, 12.6 in NZn-3.125 and 60.2 in NZn-50, respectively which varied as per the experimental protocol. CP content in semi purified diets also satisfied the minimum assay of CP (16%) for rats given by American Institute of Nutrition (AIN) -76A but EE was found marginally less.

5.2.2. Growth performance and feed intake of Wistar albino rats

Few sporadic reports are available in the literature with regard to supplementation of NZn as feed supplement in animals. Hence, discussion was oriented comparing the results obtained in the experiment with studies conducted using organic or nano sources of Zn.

In the present study, no differences were observed in feed intake and growth of rats receiving different dietary treatments with positive effects on feed conversion in nano zinc supplemented group. Similar to our results, no effect of dietary dose as well as source of zinc (inorganic or organic) on feed intake and feed conversion of broilers (Mohanna and Nys, 1999; Rossi *et al*., 2007; Sunder *et al*., 2008), Japanese quails (Sahin *et al*., 2006) and goats (Zaboli *et al.,* 2013) were reported. In case of rats, effects of feed intake due to zinc

supplementation (0, 12, 24, 36 and 48 ppm) was not evident up to 4 weeks of age (Nagalakshmi *et al*., 2012).

Nano Zinc Oxide (NZnO) used in the present study, even at low levels of supplementation as well as double dose in the present study has not affected growth performance as well as feed intake of rats compared to rats received inorganic ZnO. Growth promoting effects of NZn in different animals (Hualing, 2006; Hongfu, 2008; Yang and Sun, 2006; Lina *et al.,* 2009a; Mishra *et al.*, 2014; Sahoo *et al*., 2016) have been demonstrated by different workers in weanling piglets and poultry. The beneficial effects of extreme lower doses of NZn reported by Mishra *et al*. (2014) and Sahoo *et al*. (2016) were not observed in the present study. However, recent studies have demonstrated beneficial effects of NZn even at higher concentrations ranging from 500 to 3000 ppm (Yang and Sun, 2006; Hualing, 2006; Hongfu, 2008 in weanling piglets). Thus, it is evident from the discussion that nano Zinc as a feed additive can be supplemented at varying levels also depending on purpose and duration of feeding. In the present study, NZn exerted beneficial effects on feed conversion of rats.

5.2.3. Nutrient digestibility and absorption

Intake of different nutrients such as DM, OM, CP and EE were found to be similar ($P>0.05$) among different treatment groups. The CP digestibility was found to be significantly higher in NZn supplemented groups (NZn-25, NZn-12.5, NZn-50 and IZn-25) compared to NC. Literature pertaining to effect of zinc supplementation on digestibility of DM, EE and CP in monogastric animals is very scanty. Hence it is attempted to compare the available literatures in calves and other ruminants.

Considering the nutrient intake, DM, OM, CP and EE intakes were similar in all the treatment groups which was also reported by Jadhav *et al*. (2008) who observed similar OM and CP intakes and Mandal *et al*. (2007) who observed similar DM, EE and CP intake in crossbred calves.

Sridhar *et al*. (2015) and Mandal *et al*. (2007) did not report any significant effect of supplementation of zinc on utilization of DM, OM and EE in broiler chicks receiving diets with different sources and levels of zinc (40 ppm $ZnSO_4$; 30, 20, 10 ppm Zn-gly), which was also observed in the present study in rats. Daghash and Mousa (1999) reported that supplementation of Zn at 4.16, 12.98 or 32.28ppm had no effect on the digestibility of nutrients in lambs.

Contrary to our findings, Arelovich *et al*. (2000) and Chamberlain and Borrough (1962) reported that DM digestibility is inversely proportional to the concentration of zinc in feeds, whereas Galyean (1996) reported higher DM intake (DMI) in Zn supplemented steers. In the present study, the CP digestibility was found to be improved by the zinc supplementation. Daghash and Mousa (2002), reported that supplementation of 50 and 100 ppm zinc through $ZnSO_4.7H_2O$ improved ($P < 0.01$) the CP digestibility in zinc supplemented groups (50 and 100 mg/kg) as compared to control diet fed buffaloes. But Sridhar *et al*. (2015) in poultry, Jadhav *et al*. (2008) in buffalo calves and Mandal *et al.* (2007) in crossbred calves did not obtain any effect of supplemental zinc on CP digestibility.

The intake (µg/ rat/d), absorbed (µg/ rat/d) and digestibility (%) of zinc offered to the rats were found statistically significant among different treatment groups as zinc was supplemented in graded doses. Zinc intake (µg/ rat/d) was higher in NZn-50 ($P<0.001$) than other groups, whereas IZn-25 and NZn-25 had higher intake than NC, NZn-12.5, NZn-6.25 and NZn-3.125. NC rats had lower ($P<0.001$) zinc intake than all zinc supplemented groups as their diet had no supplemental zinc. Zinc absorbed (µg/ rat/d) was higher in NZn-50 and NZn-25 than all other groups. However, NC group absorbed minimum zinc from feed as compared to IZn-25, NZn-25, NZn-12.5, and NZn-50, whereas, it was similar to NZn-6.25 and NZn-3.125. Zinc absorption (%) was higher ($P<0.001$) in NC, NZn-25, NZn-12.5, NZn-3.125 than IZn-25 and NZn-50.

The higher zinc absorption observed in NC might be due to sophisticated homeostatic mechanism mediated by numerous proteins including zinc transporters, zinc-responsive transcription factors and zinc-binding proteins, and affected by many factors such as diets, dietary zinc level, feeding regimens and the physiological state of the animal (Roohani *et al*., 2013). In human and animals, it has been observed that reduction in dietary zinc content results in a marked increase in intestinal absorption and decrease in intestinal zinc losses, with urinary losses that are low and refractory to zinc intake (Weigand and Kirchgessner, 1978; Taylor *et al*., 1991; Steel and Cousins, 1985; Mills, 1989; Lee *et al*., 1993; Jackson *et al*., 1984; Baer and King, 1984; Flanagan, 1984) which was also observed in the present study. The proximal part of small intestine is generally regarded as the main site of Zn absorption (Miller and Cragle, 1965; Hiers *et al.,* 1968). Since Zn is a relatively small, hydrophilic, highly charged element that cannot cross biological membranes by passive diffusion, specialized mechanisms are required for both its uptake and release (McMahon and Cousins, 1998) which are called as active carrier

mediated process (Davis, 1980; Suttle *et al*., 1982). However, a mucosal induced metal binding protein known as metallothionine, influences Zn absorption.

Similar to the results obtained, Kumar *et al*. (2006) in steers and Pal *et al*. (2010) observed better gut absorption in ewes and fed with organic zinc as compared to inorganic zinc fed animals due to better bio-availability of the organic sources of zinc. In the present study, it was observed that at the same dose of zinc from NZn-25 absorbed higher zinc and thus faecal excretion was less as compared to IZn-25, which could be due its better bioavailability. Considering IZn-25 and NZn-12.5, no significant difference ($P>0.05$) was observed in the absorbed zinc (µg/ rat/d) quantity, which also indicates the effectiveness of NZn supplemented at 12.5 mg/kg is equivalent to 25 ppm of IZn. It was been reported that, Zn present in ZnO and ZnMet is absorbed to a similar extent, but Zn from these two sources appears to be metabolized differently after absorption (Spears, 1989). Thus it can be perceived that, utilization and metabolism of zinc is source dependant, which may be the case with nano form of zinc.

5.2.4. Blood biochemical and enzymes profiles

Total protein, albumin and globulin (g/dL) were higher in NZn supplemented groups (50, 25, 12.5 mg/kg diet) with similar A/G ratio compared to other groups (NC, NZn-6.25, NZn-3.125). BUN (mg/dL), Creatinine (mg/dL) were not affected by zinc supplementation as they were statistically similar ($P>0.05$) across the treatment groups. AST and ALP (U/L) were similar across all the treatment group, however, ALT (U/L) varied in different groups. Similar our results, Idowu *et al*. (2011) observed improvement in serum protein concentration in broiler chicken supplemented with zinc at 140 mg/kg body weight. However, Nagalakshmi *et al*. (2015b) observed similar protein, glucose levels among rats fed Zn from $ZnCO_3$ and Zn-nic at 0, 9 and 12 ppm with reduced globulin levels at 6 ppm.

Scanty reports are available on exerted effects on biochemical profiles due to NZn feeding. Sahoo *et al*. (2014a) found levels of glucose, triglyceric acid, total protein, urea, albumin, globulin, BUN, SGOT, Ca and P in blood serum of the broiler birds were neither affected by different sources nor by the dose of supplemental Zn. Activities of serum ALT, AST, CK, and LDH in the NZn treated group significantly lowered in Wistar rats sprayed NZnO 2.5 mg/kg body weight into the nasal passage of Wistar rats for 3 days (Wang *et al*., 2010b). Similarly, hike in the levels of LDH, ALT, ALP, cholesterol esters (CHE), total proteins (TP), albumin (ALB), BUN, creatinine,

hydroxybutyrate dehydrogenase (HBD) were reported by Wang *et al*. (2006) when adult mice fed NZn at dose of 5g/kg body weight.

5.2.5. Serum hormone profiles

NZn supplementation at two levels i.e. at 25 and 50 mg/ kg (NZn-50 and NZn-25 groups) improved Thyroxine (T4) levels compared to NC with similar effects on T3 levels. Zinc plays an important role in thyroid metabolism (Baltaci *et al*., 2004). Zinc is thought to play a role in conversion of T4 to T3 (Morley *et al*., 1980) and also in binding of T3 to nuclear receptors (Surks *et al*., 1989; Miyamoto *et al*., 1991). Zinc also participates in the formation and action of thyroprotein releasing hormone (TRH; El-Sisy *et al*., 2008) and Pekary *et al*. (1991) has documented that the processing of pre and pro TRH to synthesis TRH is a zinc dependant process which is performed through translational processing enzymes like carboxypeptidase H. Pathak *et al*. (2011) observed a decline in T3 and elevation of T4 in rats due to zinc sulphate supplementation. Nano Zn at higher level of supplementation (25 and 50 mg), had elevated levels of serum testosterone indicating superiority over inorganic zinc. Zn has specific effects on the aspects of testicular development and function particularly the secretion of testosterone (Martin *et al*. 1994). In case of insulin like growth factor-1 (IGF-1), all the groups have shown similar ($P>0.05$) values and ranged from 69.5±7.36 in NC to 86.6±10.7 in NZn-25. This could be due to low requirement of zinc for growth in rats, thus zinc concentration in basal diet would have maintained IGF-I concentrations. Zinc plays an essential role in the production and secretion of insulin, IGF1 and growth hormones (Macapinlac *et al*., 1966; Chen *et al*., 2000). Very scanty literature is available on effects of supplementation of NZn on hormonal status to compare our results.

5.2.6. Immunity status

The delayed type hypersensitivity (DTH) reaction, which is an indication of cell mediated immune response varied significantly ($P<0.05$) among the treatment groups at 24 and 48 h post injection with sheep RBC. NZn-50 and NZn-25 had more ($P<0.001$) net skin thickness than NC, NZn-6.25, and NZn-3.125, at 24 h of injection where as NC showed least amongst all. After 48h of injection, NZn-25 had statistically higher ($P<0.001$) foot pad swelling (mm) than NC, IZn-25, NZn-6.25, and NZn-3.125, where as similar to NZn-12.5 and NZn-50. However, IZn-25 had statistically showed higher immune responses than NC, NZn-6.25 and NZn-3.125. In case of humoral immunity, NZn-50 (3.87± 0.12)

had higher ($P<0.05$) Haemagglutination titre (log_2) against Sheep RBCs compared to NC (2.83±0.17), IZn-25 (3.29±0.18), NZn-12.5 (3.27± 0.17), NZn-6.25 (3.17± 0.22) and NZn-3.125 (3.17±0.17). NZn-50 and NZn-25 groups had significantly higher ($P<0.05$) humoral immune response than NC.

Nagalakshmi *et al*. (2015b) reported better immune responses in rats by supplementing 12ppm zinc nicotinate than 12 ppm $ZnCO_3$, and they hypothesised the better responses might be due to the better bio-availability of zinc nicotinate than $ZnCO_3$. Similar to this in the present study, improved cell mediated immunity as well as humoral immunity was observed in the NZn supplemented groups than NC and IZn-25, which can be corroborated to its improved bio-availability. However, NZn at 12.5 also produced similar responses to that of IZn-25. Similar to present finding, Nagalakshmi *et al*. (2016) observed that the primary ($P<0.01$) and secondary ($P<0.05$) humoral immune responses increased quadratically with replacement of $ZnCO_3$ with Zn-Met. Sridhar *et al*. (2016) reported a dose dependant response of zinc towards immunity in broilers, where they observed that humoral immune response against ND vaccine was highest ($P<0.05$) in 30 ppm Zn supplemented group from Zn-gly compared to 20 or 10 ppm Zn from Zn-gly. Moreover, cutaneous basophilic hypersensitivity to PHAP indicative of CMI was significantly ($P<0.05$) higher at 30 ppm Zn supplementation from Zn-gly compared 20 and 10 ppm Zn from Zn-gly and control. Further, Engle *et al.* (1997a) also reported that, after 24h post PHA-P injection, the CMI response was higher in ZnMet and Zn-Lys compared to control and $ZnSO_4$ supplemented calves. Similarly, Parashuramulu *et al*. (2015) also observed enhanced ($P<0.05$) antibody titers due to zinc supplementation in buffaloes, and among the Zn supplemented calves the titers against *B. abortus* antigen was higher with 140 ppm compared to 80 and 0 ppm Zn supplementation, and the CMI response was higher ($P<0.05$) in Zn supplemented calves at 24, 48 and 72h of post-sensitization, which indicated the proportionate increase in immune response with the supplemental Zn dose, which was the trend in all the NZn supplemented groups.

Better bio-available sources will show improved immune responses at the same supplemental doses (Nagalakshmi *et al*., 2015b), which can be correlated to our study and thus it can be concluded that the enhanced immune responses in NZn supplemented groups could be due to more bio-availability of zinc, which is important for maintaining immunity and disease resistance of the animals (Chesters, 1997).

5.2.7. Micro mineral profiles of (mg/kg) visceral organ

In liver, femur bone and kidney, zinc content (mg/kg) was found to be significantly ($P<0.05$) higher due to supplementation of zinc in inorganic and nano forms (IZn-25; NZn 25, NZn 12.5 and NZn 50). Iron, manganese and copper levels were found similar ($P>0.05$) among the groups compared to un-supplemented NC.

The mineral contents of muscle were found to be same ($P>0.05$) across different treatment groups. Muscle zinc content was not affected even after reducing the dietary level up to 3.125 mg/kg level. Similar to our results, Revy *et al.* (2002) in piglets, Ao *et al.* (2006) in broilers and Nagalakshmi *et al.* (2015a) in Sprague Dawley female rats reported a rise in liver zinc content due to supplementation of higher level zinc in feeds which supports our findings. However, Heugten *et al.* (2003) reported no change in liver zinc content by supplementation of organic or inorganic zinc in broiler chicken, which is not in conformity to our findings. Idowu *et al.* (2011) also reported an increase in liver zinc content from un-supplemented control group by supplementing zinc from its different salts, which is also observed in the present study.

Tibial zinc concentration is considered as sensitive measure of zinc status (Cossack, 1984). Concurrent to our findings, Revy *et al.* (2002) observed a linear increase in bone Zn concentrations ($P<0.01$) with addition of Zn to basal diet either as Zn-met complex or $ZnSO_4$ in weaning piglets. Similarly in broilers, Ao *et al.* (2006) and Idowu *et al*. (2011) in layers documented higher Zn deposition in tibia bones due to supplementation of zinc sources from its inorganic salts.

More Zn levels were observed in bone and liver in NZn-12.5 than IZn-25 groups in the present study was in accordance with results of Sridhar *et al*. (2015) who observed that the Zn concentration in liver and tibia of birds was comparable with lower Zn supplementation (10-30 ppm) from Zn-gly than 40 ppm Zn from $ZnSO_4$. Further, Sahoo *et al*. (2014b) reported a higher Zn concentration in tibia, liver of birds supplemented with 0.06 mg/kg NZn than birds supplemented with no Zn supplementation and Mohammadi *et al*. (2015) observed that tibia Zn concentration was higher in chicken fed on the diet supplemented with NZn sources compared to the Zn-sulphate and without Zn supplemented in broiler birds ($P < 0.001$).

Sridhar *et al*. (2015) reported increased ($P<0.05$) Zn retention in kidney without any adverse effect on the retention of other minerals. Similarly, Nagalakshmi *et al*. (2015a) in Sprague Dawley female rats demonstrated that Zn deposition in kidney and

muscle was not affected by reducing Zn supplementation by 50% (6 ppm) in Zn-nic compared to 12 ppm Zn supplementation as $ZnCO_3$. This finding supports the results of our study where similar levels of zinc were obtained with IZn-25 and NZn-12.5.

5.2.8. Serum mineral profiles

Calcium, phosphorus, magnesium (mg/dL) and copper (mg/L) were found similar in all dietary treatment groups. Zinc (mg/L) level was significantly higher in NZn-25 and NZn-50 as compared to NC, NZn-6.25 and NZn-3.125, whereas, NC groups rats showed lower ($P<0.05$) serum zinc level than IZn-25, NZn-25, NZn-12.5 and NZnO- 50.

In the present study, the supplementation of zinc in nano form than inorganic ZnO has shown better picture in terms of serum zinc content which is in agreement with Sahoo *et al*. (2014b) who reported a higher Zn concentration serum of broiler birds supplemented with 0.06 mg/kg NZn than birds supplemented with no Zn. Similarly, Nagalakshmi *et al*. (2015a) in Sprague Dawley female rats, observed higher Zn concentration in rat serum supplemented with 9 and 12 ppm of Zn- nic than those supplemented with $ZnCO_3$. Similarly, Wang *et al.* (2010a) and Ma *et al.* (2011) reported higher ($P<0.01$) Zn concentrations in serum in zinc supplemented groups than un-supplemented animals. Further, Jing *et al*. (2015) and Jing *et al*. (2009) also obtained a positive correlation between serum and tissue Zn concentrations which were both decreased in the Zn-deficient rats and elevated in the Zn-supplemented rats.

Revy *et al.* (2002) reported a linear increase in plasma zinc concentration due to supplementation of zinc to basal diet (28mg/kg) @ 10, 20 or 30mg/kg either from Zn-met or $ZnSO_4$, indicating the level of zinc in serum or plasma depends on dietary zinc supplementation. Similarly, we observed a proportionate change in the serum zinc concentration in all the NZn supplemented groups with increase in the dietary NZn levels. Pal *et al*. (2010) reported improved Zn and Cu /Zn SOD status in the plasma of ewes supplemented with Zn-Met than $ZnSO_4$ supplemented ones and corroborated better liver, plasma Zn concentration and lesser faecal excretion to better bio-availability of Zn met than $ZnSO_4$, which can be related to our findings and thus we can conclude that NZn is better bio-available than IZn.

In the present study calcium, phosphorus, magnesium and copper levels were similar across the treatment groups suggesting that supplementation of NZn upto 50 ppm level did not alter their serum levels. Similar to our findings, Ma *et al.* (2011) reported in broilers that different levels of Zn supplementation, either from $ZnSO_4$ or Zn-gly had no

effect on the contents of Cu, Fe and Mn in serum. Jing *et al*. (2015) in rats, observed that serum and femur zinc concentrations, respectively were 83% and 58% lower in zinc deficient group (<1 mg Zn/kg), and 49% and 62% higher in 300 mg Zn/kg supplemented group compared to control group with 30 mg/kg ($P < 0.001$). Kidney Zn levels were decreased by 15% in zinc deficient group and increased by 24% in 300 mg Zn/kg group compared with control group with 30 mg Zn/kg ($P < 0.001$); liver Zn concentrations in treatment group300 mg Zn/kg were 13% greater than control group (30 mg Zn/kg; $P < 0.001$), but no significant changes were found between zinc deficient group and control group (30 mg Zn/kg). Higher serum zinc level were documented in NZn supplemented groups (NZn-50 and NZn-25) indicating its better bioavailability. Similarly, at reduced dose of NZn supplementation resulted in comparative serum zinc levels as that of IZn-25 supplemented rats.

5.2.9. Relative m-RNA expression in Wistar albino rats

5.2.9.1. Metallothionein gene

The expression of metallothionein mRNA was higher ($P>0.05$) in NZn-50 and NZn-25 as compared to IZn-25. However, similar metallothionein mRNA expression was observed in IZn-25 and NZn-12.5.

Our findings are in accordance with the reports of Shay and Cousins (1993), who suggested an increase in metallothionein mRNA abundance when Zn was supplemented to Zn deficient animals. Cousins and Lee-Ambrose (1992) reported that metallothionein expression was proportional to the dietary zinc intake, which is also reflected in our results. The increase in metallothionein expression in liver might be due to better bioavailability of zinc from NZn sources as transport of Zn inside the intestinal mucosal cell is controlled by a metal-binding protein known as metallothionein (Richards and Cousins, 1976), which is generated in the liver. Thus more bioavailable zinc sources will be absorbed better and thus results in more metallothionein production from hepatic tissues for its transport. The synthesis of metallothionein is regulated by dietary Zn and plasma Zn concentrations, thus influencing the amount of Zn that enters the body, as result; it greatly contributes to Zn homeostasis (Cousins, 1978). As more zinc is absorbed from NZn-50 and NZn-25 compared to IZn-25 could have positively affected metallothionein synthesis in liver, leading to higher hepatic metallothionein mRNA expression. Similarly, absorbed zinc from IZn-25 might be equal to NZn-12.5, which produced almost same expression of metallothinein. Thus this indicates the effectiveness

of NZn at 12.5 is almost similar to that of IZn at 25 mg/kg indicating better bioavailability of NZn. Similarly, Richards and Cousins (1976) reported that hepatic Zinc uptake appeared to be directly correlated with liver metallothionein bound zinc, which can be related to the better bio-available of the NZn sources, and in the present study significant increase in liver zinc level was also an indicative of this.

5.2.9.2. SOD1 gene

The mRNA expression of hepatic SOD1 gene was found to be positively affected due to zinc supplementation, which varied ($P>0.05$) directly to the dose of supplemental zinc. Expression of SOD1 mRNA was highest in NZn-50 as compared to all other groups. Jing *et al*. (2007) reported a reduction in the Cu-Zn superoxide dismutase (Cu-Zn SOD) activity in rats fed Zn deficient diet, whereas the rats fed zinc excess diet had higher Cu-Zn SOD activity, which is in accordance with the present results as deficient groups were less mRNA abundance and NZn-50 was having maximum hepatic mRNA abundance for SOD1 gene. Again, there was an increase in SOD1 mRNA expression was observed with the increase in zinc level in all NZn groups. Kumar *et al*. (2011) also reported an increase in SOD1 mRNA expression in lead exposed kids when they were supplemented with zinc at 50mg/kg. In our study, with incorporation of zinc there was an improvement in SOD1 mRNA expression which varied in a dose dependant manner. Pal *et al*. (2010) reported higher activity of Cu/Zn-SOD in liver homogenate in ewes fed Cu and Zn-Met supplemented with 50% more of Zn over a basal diet. This gives an indication regarding the positive effect of zinc on SOD1 which could be correlated to the better SOD hepatic mRNA expression in rat liver. Lee *et al.* (2014) reported that SOD levels were significantly higher in NZnO exposed cells. Up-regulation of these genes by NZnO is also observed in our study. Similar to present results, Abbasalipourkabir *et al*. (2015) observed a rise in the SOD activity with increase in the concentration of NZnO, which did not vary statistically among the treatment groups and NC.

5.2.10. Tissue architecture of some organs in Wistar albino rats

Histological examination of liver in NC groups showed moderate vacuolar degeneration which is evident by condensation of nuclei and vacuolated cytoplasm without pink staining with congestion of blood vessels. Similar to NC, NZn-6.25 and NZn-3.125 groups exhibited mild vacuolar degeneration. The other groups IZn-25, NZn-25, NZn-12.5 and NZn-50 revealed normal hepatocytes with vesicular nucleus stained

blue and cytoplasm stained with pink colour. Kidney and spleen section of rats in all the groups showed normal structure without any observable changes.

Histology of intestine in NC group revealed normal columnar epithelial cells with few goblet cells with normal intestinal architecture but shortened villi. In case of IZn-25 and NZn-12.5, healthy architecture with normal appearance of intestine was noticed. NZn-6.25 and NZn-3.125 showed intestinal epithelial cells and villi normal appearance but with partial fusion of villi. However, rats of NZn-25 and NZn-50 have shown mild to moderate damage to the intestinal epithelial cells with elongation of intestinal villi and partial fusion of villi with hyperplasia of goblet cells. Hence, our study indicated that the NZn can be fed to rats at half the ICAR recommended dose without any observable changes in the liver, kidney, intestine and spleen, however NZn-25 and NZn-50 caused moderate intestinal damage .

The potential hazard of high concentrations of NZn as animal feed supplement is still unknown and their toxicological data are rather uncommon, but still, the toxicity of both conventional Zn and NZn compounds has been reported (Houston *et al.,* 2001; Llobet *et al.,* 1988; Lock and Janssen, 2003; Piao *et al.,* 2003). The toxicological effects of NZnO are related to their dissolution and interference with Zn ion homeostasis. In Zn toxicity, pathological changes in pancreas, kidney, liver, rumen, abomasum, small intestine and adrenal gland were observed in sheep (Allen *et al.,* 1983). Liver, spleen, heart, pancreas and bone are the target organs of NZn on oral exposure (Wang *et al.,* 2008). In the histopathological examination, NZn has exhibited both dose and time dependent cytotoxicity and its mechanism is carried by oxidative stress, lipid peroxidation, cell membrane damage, and oxidative DNA damage (Lin *et al.,* 2009; Xia *et al.,* 2008; Najafzadeh *et al.,* 2013). Toxic effects of the NPs are size-dependent that means, NZn will be more toxic than macro Zn at the same dose (Chen *et al.,* 2007), this can be corroborated to our findings as NZn at 25 mg/kg showed moderate changes of intestinal villi whereas, IZn at 25 mg/kg dose showed normal intestinal architecture.

In the present study, no toxicity in liver, spleen and kidney was observed due to feeding of NZn upto a dose of 50 mg/kg. Contrary to our findings, Najafzadeh *et al.* (2013) reported mild liver toxicity (edema and degeneration in the hepatocytes) and severe renal damage (multifocal interstitial nephritis in 75% of animals) in lambs because of NZn feeding at a very high dose of 20 mg/kg body weight orally for a period of 25 days. Wang *et al*. (2006) studied the comparative toxicity of nano (58 ± 16 nm) and micro (1.08 ± 0.25 μm) Zn by feeding the adult mice orally at the dose of 5 g/kg body weight and reported

damages in kidney, liver and heart in MZn group compared to the NZn group and reason could be due to higher doses of NZn compared to our study.

The proposed mechanism for NP toxicity may be the particles are much more active and can be rapidly transformed into respective ions in gastric juice. This could be a possible cause of intestinal lesions observed particularly in NZn-50 and NZn-25 groups. Jafarpour *et al*. (2015) observed that supplementation of Zn-Met (0.8 or 1.2 g/day) in the diet significantly increased villi height (linear, $p = 0.01$) and crypt depth (linear, $p = 0.03$) in the jejunum ($P < 0.05$) and thus affected intestinal architecture positively.

By feeding NZnO at a dose of 300 mg/kg body weight orally to mice for 14 consecutive days, accumulation of NPs in the liver was reported (Sharma *et al*., 2012), along with cellular injury leading to elevated ALT and ALP levels in serum and pathological lesions in the liver. In the present study, no elevation in ALP, AST and ALP confirmed that animals' liver was sound in NZn fed rats at 50 ppm.

The toxicity of Zn is reported to be associated with the concentration of the free ion (Di Toro *et al.,* 2001; Lofts *et al.,* 2004; Thakali *et al.,* 2006; Kasemets *et al.,* 2009; Kool *et al.,* 2011). These ions might have caused damage to the intestinal tissues which is the first line of contact for this active nano particles fed through feeds. NZnO are likely to have remained as NPs for longer duration, and thus are less toxic than the corresponding inorganic salts like $ZnCl_2$ (Hooper *et al.,* 2011). Very recently, Zhang *et al*. (2017) and Liu *et al*. (2017) demonstrated in rats that, NZn is more effective than IZn and $ZnSO_4$ in enhancing cell viability, better uptake efficiency and efficient transportation at lower concentrations with less cytotoxicity and liver lesions compared to IZn. These studies supported our finding as NZn was found to be better retained in the body than IZn and it did not affect the tissue architecture of liver, spleen and kidney of rats.

5.3. Phase-III. Effects of two doses of NZn particles on growth, nutrient utilization, immunity, rumen fermentation, blood profiles and male reproduction in goats.

5.3.1. Chemical composition of feeds

The analytical composition of concentrate mixture and finger millet straw are found to be within normal ranges and as per the formulation. The diets were formulated to support nutrient requirements except for zinc of male growing goats for achievable ADG of 50-75 g (ICAR, 2013b).

5.3.2. Growth performance of goats

In the present study, significant improvements in total body weight gain and ADG ($P<0.05$) were obtained in NZn supplemented group at half dose (NZn-25) compared to NC goats where there is no supplementation of additional zinc. The other two groups (IZn-50 and NZn-50) had intermediary body weight gain and ADG. However, DMI (kg) during the entire experiment remained same in all treatment groups resulting better FCR in NZn-25 group compared to NC group. This might be due to the fact that nano forms of zinc has got more bioavailability than ZnO, as a result, there might have been more absorption, distribution and uptake of Zn in the NZnO supplemented groups, which would have accounted for its better effect over inorganic ZnO, which was also postulated by Kumar *et al*., (2006) while studying the effects of Zn propionate and Zn sulfate in bulls, where the better responses in animal performances in Zn propionate was attributed to its superior bioavailability over Zn sulphate.

Very few references are available in documenting effects of NZn on performance of animals. Zaboli *et al*. (2013) observed non-significant differences in ADG, DMI and FCR of Markhoz goat kids were supplemented 20 and 40 ppm of NZnO to a basal diet containing 22.12 mg of Zn/ kg DM for 70 days. Similar to our results Spears (1989), Puchala *et al*. (1999) and Jafarpour *et al*. (2015) reported significant increase in ADG due to supplementation of dietary zinc, but the reports of Martin *et al*. (1987), Stobart *et al*, (1987), Greene *et al*. (1988) contradicted our findings. In the present study, DMI was not affected by Zn supplementation ($p>0.05$), which is in line with Puchala *et al*. (1999) who found that DMI of Angora goats was unaffected by Zn supplementation. Similarly, supplementation of Zn to a basal diet containing more than 25 mg Zn/kg DM had no effect on DMI in dairy goats (Salama *et al*., 2003), growing lambs (Garg *et al*., 2008) and beef steers (Mandal *et al*., 2007). Similarly, others also reported that supplementation of zinc as Zn-Met did not affect DMI in steers (Greene *et al*., 1988). Contrary to our findings, Jafarpour *et al*. (2015) reported improved DMI in sheep by supplementation Zinc as Zinc-Met .In pigs, Borah *et al*. (2014) observed improved total body weight gain, ADG, FCR in the zinc supplemented groups (100 and 500 mg Zn/ kg) than the deficient groups which were supplemented with 100 mg/kg Zinc and $CaCO_3$ @1.5% of DM, which supports our findings in goats.

The impact of zinc supplementation may be corroborated with the fact that Zn plays a significant role by influencing mitotic and meiotic cell divisions, along with

synthesis of DNA and RNA by enhancing the activity of DNA polymerase and RNA polymerase, the two Zn containing enzymes, thus acting as a growth promoting agents. Additional dietary Zn in weaning diets for piglets has also been reported to result in improved growth rate and increased serum IGF-I (Carlson *et al*. 2004), which was reflected in our results. Due to the vast role in enzyme systems, requiring zinc either as catalytic, co-catalytic, or as a structural component (Reilly, 2004), supplementation of Zinc in ruminants diets is essential for maintaining normal growth, normal development and function of the immune system, and metabolism (NRC, 2000). Furthermore, zinc also improves amino acid utilization, having role in protein synthesis (Spears, 1989). Nano Zn supplementation at reduced dose (NZn-25) had increased body weight gain and ADG and improved feed conversion of goats during 112 day feeding period due to growth promoting effects of nano Zn for the reasons explained above.

5.3.3. Nutrient intake

In the present study, non-significant differences ($P>0.05$) were observed in intake of different nutrients (DM, OM, CP, DOMI, DCPI, TDNI (g/d and g/ kg $W^{0.75}$) and MEI (MCal/d and Kcal/ kg $W^{0.75}$) due to supplementation of inorganic or nano form of zinc compared to NC with no zinc supplementation. Similar to the results obtained in the present study, no effect of zinc supplementation on feed intake was noticed by Spear (1989) in lambs fed control diet (2.8 ppm zinc) and supplemented diet (5 ppm zinc as ZnO or ZnMet) or heifers fed basal diet supplemented 25 ppm zinc (ZnO or ZnMet). An Indian study conducted on Hariana calves also documented similar results on feed intake due to supplementation of zinc at 20, 40 and 60 ppm of Zn as $ZnSO_4$ (Bedi and Sawhney, 1979). On the similar lines, Jadhav *et al*. (2008) in Murrah buffalo calves (supplemented with 0, 35 or 70 ppm to a basal diet of 35 mg Zn/kg DM), Mandal *et al*. (2007) in crossbred calves (supplemented with 35 ppm of Zn as $ZnSO_4$ or Zn propionate to basal diet of 32.5 ppm) and Nagalakshmi and Himabindu (2013) in lambs (15, 30 or 45ppm Zn supplementation with basal diet, or from any source (Zn sulphate and Zn proteinate at above doses) did not observe any significant effect on intake of nutrients. However, few workers found negative effect on dry matter intake due to feeding of high levels of zinc. Malcolm-callis *et al*. (2000) observed a linear decrease ($P<0.10$) in daily DMI with increasing Zn concentration (20, 100 and 200 ppm Zn as $ZnSO_4$ to a basal diet containing 70 ppm) in the diet of beef steers.

Intake of nutrients was found to be similar in all treatment groups received with or without zinc supplementation as inorganic or nano forms.

5.3.4. Nutrient digestibility and nutritive values of diet

The key findings in this study are NZn-25 supplementation caused similar DM, OM, CP, Total CHO, NDF digestibility in the goats. However, ADF digestibility in NZn-25 was significantly ($P<0.05$) higher than NC and similar to IZn-50 and NZn-50. However, there is a clear improvement of cellulose digestibility in zinc supplemented groups (IZn-50, NZn-50 and NZn-25) over unsupplemented NC. However, nutritive value of diet in terms of DCP, TDN (%) and ME (MCal/kg) also did not differ ($P>0.05$) among different treatment groups. Similar to the results obtained in the present study, Mandal *et al*. (2007), Garg *et al*. (2008) and Jia *et al*. (2008) did not observe any effect on digestibility of DM, CP, EE and NDF due to Zn supplementation from organic and inorganic sources in steers, lambs and Cashmere goats respectively. In agreement with the present study, an increase in digestibility of ADF and cellolose was also found by Jia *et al*. (2008) and Garg *et al*., (2008) in Cashmere goats and Muzaffarnagari lambs. DM digestibility in beef cattle was not affected with supplementation of 430 mg Zn/kg DM as $ZnCl_2$ (Arelovich *et al*., 2008). Very recently, Nagalakshmi and Himabindu, (2013) found that dietary supplementation of Zn at concentrations of 0, 15, 30 or 45 ppm as $ZnSO_4$ or Zn Proteinate to a basal diet (29.3 ppm Zn) in Nellore ram lambs had no affect on intake and digestibility of organic nutrients, fibre fractions and balance of nitrogen and energy. On the contrary, Hassan *et al.* (2011) reported in sheep supplemented 15 mg ZnMet showed higher apparent digestibility of DM, OM, CP, CF, EE, NFE, NDF, ADF and nutritive values (TDN and DCP) than those fed on basal and 25 ppm supplemented diet. Similarly, Gaafar *et al*. (2011) and Balabánová *et al.* (2011) observed increase in nutrient digestibilities due to supplementation of organic form zinc compared to inorganic form in lactating Fresian cows and Holstein dairy cows respectively. Daghash and Mousa (2002) in buffalos, also reported improved ($P < 0.01$) the digestibilities of OM, CP and NFE in zinc supplemented groups while CF digestibility increased only with 50 ppm Zn supplementation in comparison to with those fed NC diet. The variation in results due to species, production level etc.

Increased cellulose and ADF digestibility (%) was observed due to feeding of NZn -25 followed by NZn 50 and IZn 50 compared to NC.

5.3.5. Mineral absorption

Intake, absorption and absorption (%) of Calcium, Phosphorus and Magnesium were found to be same in zinc supplemented groups and NC. However, Zinc intake (mg/d) varied significantly ($P<0.001$) due to additional supplementation of Zn in treatment groups. The Zn intake in IZn-50 (44.3±2.14) and NZn-50 (45.5±1.41) was significantly higher than NZn-25 (29.3±0.90) and NC (12.70±0.60). Intake of Zn in NC and NZn-25 also varied significantly. Net absorption of zinc (mg/day) was more in NZn-50 group (12.3±1.98) compared to IZn-50 (8.40±0.61), NZn-25 (7.92±0.29) and NC (5.26±0.97). However, the absorption (as % of intake) of zinc was significantly more ($P>0.006$) in NC compared to zinc supplemented groups.

Similar to the present results, Jia *et al*. (2009) reported higher absorption of zinc in un-supplemented control compared to the groups which received zinc supplementation in Cashmere goats. Further, it is well documented (Miller, 1970) that Zn absorption greatly increased in Zn deficiency condition. Though, Zinc is under homeostatic control, but due to deficiency of zinc in control diet, it might have altered the homeostatic control and continued to absorb dietary zinc during progression of experiment. Net absorbed zinc (mg/day) was higher in NZn-50 compared NZn-25 and IZn-50 groups. Thus, bio-availability of nano ZnO is superior to IZn-50. Even at half dose, the net absorbed zinc is comparable to IZn-50. Though, net absorbed is higher, absorption (%) was similar across both inorganic and nano form of zinc. Zinc uptake from ZnMet and $ZnCl_2$ by intestinal tissue was also found to be similar using *in vitro* procedures utilizing noneverted intestinal sacs from pigs and chicks (Hill *et al*., 1987a) and everted duodenal sacs from rats (Hill *et al*., 1987b). So far we could not come across any report on absorption of nano ZnO in goats. Thus, nano ZnO is comparable to any organic Zn in terms of bioavailability.

Jia *et al*. (2008) reviewed the apparent absorption of Zn in ruminants and reported that, it ranges from 12.1 to 36.7%, which supports our findings. Differences observed, if any, might be due to animal status, age and dietary factors including Zn concentration in the basal diet, source, level of supplementation, interactions of Zn with other minerals such as Fe, Ca and Cd. The apparent absorption of NZn in NZn-50 and NZn-25 follows the findings of Reid *et al*. (1987) which indicated a decrease in the percentage of absorbed Zn with increasing dietary Zn level.

Zn supplementation in the form of inorganic and nano form did not affect the absorption of Ca, P, and Mg. Net absorbed Zn (mg/day) was more in NZn-50 group compared to other groups indicating more bio-availability.

5.3.6. Serum mineral profiles

Serum Calcium (mg/dL), Iron, Manganese and Copper (mg/L) estimated at 0, 90 day of experiment was found to be same across different treatments (NC, IZn-50, NZn-50 and NZn-25). Compared to initial values, serum Zn levels increased significantly in NZn-25, IZn-50 ($P<0.05$) and NZn-50 ($P<0.01$). Similar to the results, Spears (1989) in heifers; Nunnery *et al*. (2007) in finished beef steers; Salyer *et al*. (2004) in heifers; Spears and Kegley (2002) in steers; Mandal *et al*. (2008) in crossbred calves; Salama *et al*. (2003) in goats have reported similar blood zinc concentrations in animals fed inorganic or organic sources of zinc .

However, positive effect of zinc supplementation on plasma zinc status was obtained by other workers (Jia *et al*., 2009 in Cashmere goats; Garg *et al*., 2008 in lambs). Nano Zn supplementation at 40 ppm increased plasma Zn at 35 day after supplementation but not at day 70 day in Markhoj Goats (Zaboli *et al*., 2013). The non-significant levels obtained in present study due to homeostasis of zinc levels by 90 day of experiment.

It was observed that there was a significant increase in the serum Zn levels on 90^{th} day as compared to initial values in all supplemented groups, which indicated the positive effect of zinc supplementation on serum mineral profiles. This might be due to the fact that NZn has got more bioavailability than IZn , for which, there might have been more absorption, distribution and uptake of Zn which accounts for its better effect over ZnO as suggested by Kumar *et al*. (2006) who reported the superior bioavailablity of zinc propionate over $ZnSO_4$.

Though the serum mineral concentration across groups remained similar but increased significantly at 90 day of experiment.

5.3.7. Haematological profiles

Results indicated that supplementation of zinc did not affect ($P>0.05$) the haematological profiles of the goats compared to NC. PCV (%), eosinophil (%), monocyte (%) and haemoglobin (g/dL) were found in the normal reference range given by Feldman *et al*. (2002), whereas RBC (10^6/ µl) was within the normal range in NC, IZn-50 and NZn-25, but NZn-50 showed marginally higher RBC than the reference values by Feldman *et*

al. (2002). WBC (10^3/ µl) was found to be marginally higher than the reported values by Feldman *et al*. (2002). Lymphocytes (%) in NC, IZn-50 and NZn-50 were lower than reported values of Feldman *et al*. (2002), whereas NZn-25 was within the range.

Nagalakshmi *et al*. (2015b) reported similar WBC, RBC, haemoglobin concentration, PCV, mean corpuscular volume, lymphocyte, monocyte, and granulocyte concentration, among the rats fed inorganic ($ZnCO_3$) and organic (Zn-nic; 6, 9, and 12 ppm) sources. Kegley *et al.* (2001) also reported similar total WBC by supplementing 360 mg Zn/d either as $ZnSO_4$ or Zn-amino acid complex along with either Bermuda grass hay (21 mg Zn/kg DM) or control diet (38 mg Zn/kg DM) in beef calves and heifers. Mandal and Das (2010) reported similar haemoglobin concentration and packed cell volume (PCV) in crossbred calves after supplementing 35 mg/kg of Zn as zinc sulphate or zinc propionate to the basal diet (32.5 mg Zn/kg DM). Donmez *et al*. (2002) also reported that supplementation of 0, 125, 500 and 1000 mg Zn per kg of drinking water in broiler chicks had no effect on erythrocyte count (RBC), hemoglobin, hematocrit, total leucocytes and differential leucocyte count (DLC), which is in accordance with the present findings in goats.

On the contrary, Sobhanirad and Naserian (2012) reported higher number of RBC, haemoglobin concentration, packed cell volume, and mean corpuscular hemoglobin concentration in the Zn-Met than control and $ZnSO_4$ supplemented group after supplementing 500 mg Zn/kg DM from either $ZnSO_4.H_2O$ or ZnMet in Holstein cows. Akbari *et al.* (2008) observed that addition of 60 mg Zn/kg basal diet from ZnO significantly ($P<0.05$) increased WBC and lymphocyte count with no effect on RBC count and haemoglobin in broiler chicken (21 days). It has been reported that dietary zinc deficiency increased the number of basophils, eosinophils and neutrophils and decreased the number of lymphocytes, suggesting the change in white blood cell distribution (Someya *et al*., 2007).

Haematological profiles recorded in different treatment groups were similar found to be in normal ranges.

5.3.8. Blood Biochemical Profiles

In the present study, blood enzymes such as ALT, AST and ALP (IU/L) were found to be similar in all the groups. The values obtained in the present study are in physiological ranges suggested by Kaneko *et al*. (2008). Results obtained in the present study are in concordance with Mandal *et al*. (2008) in cross bred calves, Hassan *et al*.

(2011) in adult Bakri sheep and Kwiecien *et al*. (2017) in broiler chicken due to supplementation of zinc. Contrary to the present findings obtained in the study, few workers like Spears (1989) in heifers, Jia *et al*. (2009) in Cashmere goats, Nagalakshmi *et al*. (2009 b) in Nellore lambs suggested increase in ALP whereas, Gaffar *et al*. (2011) reported decrease of ALP due to supplementation of graded levels of zinc from organic or inorganic sources. Serum ALP is a Zn metalloenzyme that decreases in Zn deficiency and serum ALP activity is used as an indicator of animal Zn status (Miller *et al*., 1965). The reason for non-responsive of zinc supplementation to blood enzyme status might be due to advanced age of the goats used in the experiment. Creatinine (mg/dL), total protein, albumin (g/dL) obtained in the present study are in physiological ranges suggested by Kaneko *et al*. (2008). There is no effect of treatment on creatinine levels obtained in the study. Total Protein and globulin (g/dL) levels were found to be more in NZn supplemented at 50 mg/kg feed. Similar to findings obtained in the present study, Daghash and Mousa (1999) in buffalo calves observed increased protein levels. However, Nagalakshmi *et al*. (2009b) observed similar protein levels and increased globulin levels in lambs fed inorganic or organic zinc sources at 30 ppm. Huerta *et al*. (2002) did not find any change in plasma protein and blood urea-N concentration in beef steers with zinc supplementation even at 200 ppm. Similarly, Hasssan *et al*. (2011) in adult Bakri sheep found similar serum total protein, albumin and creatinine.

Very scanty literature is available on effects of feeding NZn as feed supplement. At higher doses, serum ALT, AST and ALP contents were elevated in mice with NZnO treated groups than control (Jung *et al*., 2010; Sharma *et al*., 2012). The proposed mechanism may be due to the fact that, the NZn is much more active and can be rapidly transformed into respective ions in gastric juice. So large amounts of metal ions are generated and subsequently brought to liver and kidney for metabolism and excretion, which might cause damage to hepatic and renal tissues (Chen *et al.*, 2007).

Thus, results indicated that supplementation of zinc especially NZn caused improvement in total protein and globulin concentrations without affecting albumin level in goats.

5.3.9. Hormonal profiles

There is no effect on T3 and T4 due to supplementation of zinc in inorganic or nano form in goats in present study. Similar to the findings, El-Sisy *et al*. (2008) observed

a non-significant change in serum T3 and T4 levels in Baladi male goats fed with or without 40 mg/kg Zn-Met .

Positive effects on serum testosterone were noticed due to supplementation of nano Zn at two levels for a period of 90 days. Zn has specific effects on the aspects of testicular development and function, particularly the secretion of testosterone (Martin *et al*., 1994). Similar to the results obtained in the present study, Xin *et al*. (2007) in bulls and Kumar *et al*. (2013) in bucks observed elevated serum testosterone levels due to supplementation of zinc.

IGF-I concentrations (ng/ml) were increased due to supplementation of zinc both in inorganic and nano forms for a period of 90 days. Zinc plays an essential role in the production and secretion of insulin, IGF1 and growth hormones (Macapinlac *et al*., 1966; Chen *et al*., 2000). Similar to the results obtained, linear increase in plasma IGF-I concentrations were documented in sheep supplemented graded levels (58, 118 or 163 mg Zn/kg diet DM as Zn Methionine) (Jafarpour *et al*., 2015).

A positive effect on serum testosterone was noticed due to supplementation of nano Zn at two levels. IGF-I concentrations (ng/ml) were increased due to supplementation of zinc both in inorganic and nano forms.

5.3.10. Immunity status

Immune system is a physiological system, which is influenced by the nutritional status of the animal. Zinc is mainly responsible for maintaining healthy immune status of the animal. Zinc being an important antioxidant, decreases reactive oxygen species (ROS) production (Fernandez *et al*., 2003) which in turn improves animal health status and productivity. Zinc insufficiency impairs the activity of natural killer (NK) cell activity, phagocytic activity of macrophage and neutrophil and oxidative burst (Allen *et al*., 1983; Keen and Gershwin, 1990). In human and laboratory animals, it has been established that Zn deficiency reduces immune responses and disease resistance (Chesters, 1997).

Similar to the results obtained in the present study, unsupplemented diets to lambs (29.28 ppm Zn in basal diet) had reduced cell mediated immune response assessed as delayed type hypersensitivity (DTH) response against PHA-P compared to $ZnSO_4$ supplemented (15 ppm) diets (Nagalakshmi *et al*., 2009b). Better immune response, in terms of CMI (skin fold thickness) and humoral immunity (Improved HI titres against PPR vaccine) was evidenced in the present study in NZn supplemented groups (50 ppm followed by 25 ppm) and IZn supplemented groups compared to un-supplemented NC.

No literature reports are available on effect of nano zinc supplementation on CD4+, CD+8 counts in livestock. However, few references exist on zinc supplementation alleviating CD4+ counts in human patients suffering from AIDS. Independent of disease progression, oral zinc supplementation led to an increase of the CD4 count and a reduced incidence of opportunistic infections in case of HIV infected human subjects (Mocchegiani *et al*., 1995 and Isa *et al*., 1992).

Better bio-available Zn sources can enhance the immune reactions in the supplemented birds and animals (Moghaddam and Jahanian, 2009). Nano zinc supplementation has been reported to reduce somatic cell count (SCC) in subclinical mastitis cow and improved mastitis condition with increase in milk production than other larger ZnO sources (Rajendran *et al.*, 2013a, b), higher ($P<0.05$) antibody titre levels against Sheep RBC in 0.06 mg/kg NZn supplemented birds (Sahoo *et al*., 2014b). Recently, Mohammadi *et al*., (2015) reported that better bio-available zinc sources (zinc-methionine, zinc-nano-sulphate, zinc-nano-methionine and zinc-nano max (synthesised based on nano chelating technology) at 80 mg/kg of diet) have significantly ($P < 0.05$) increased the weight of bursa fabricius, spleen (%) and antibody titre against NDV compared to birds without zinc supplementation.

Thus, present results clearly demonstrated nano zinc at two doses (50 and 25 ppm) has considerably improved the immune status of goats.

5.3.11. Rumen fermentation characteristics

Rumen fermentation pattern as measured by ammonia-N, Acetate, Propionate, butyrate, valerate and Total VFA (mmol/L) were similar among different treatment groups in the samples collected at 0 day and 90 day. The pH of rumen liquor collected at 90 day is significantly lower ($P<0.05$) in NZn-50 and IZn-50 compared to NC. Very scanty literature is available on exerted effects of NZn on rumen fermentation. Supplementation of NZnO had a positive effect on the concentration of volatile fatty acids, improved growth of ruminal microorganisms, increased ruminal microbial protein synthesis and raised the energy utilization efficiency in early phase (6 to 12 h) of incubation (Zhisheng, 2011). Addition of Zn to diets affected ruminal fermentation by shifting proportions of VFA in ruminal fluid and is more prominent in the initial hours of feeding with reduction of total VFA concentrations (Bateman *et al.*, 2002). But the concentration of ammonia nitrogen and ratio of acetate to propionate were adversely affected by the supplementation of 100 and 200 mg/kg of NZnO at the 6th and 12th hour of incubation (Zhisheng, 2011).

Some workers have different observations reduction in total VFA concentrations due to the inclusion of Zn in the diets (Bateman *et al.,* 2002) which may be attributed to the size of the Zn source.

Significant increase in soluble zinc contents of rumen liquor due to supplementation of IZn and NZn at 50 mg/kg body weight compared to NC and NZn-25 which is quite expected. The increased zinc levels might have resulted in more circulating levels of zinc and exerted some beneficial effects on the immunity and reproduction parameters.

5.3.12. Male reproduction-Sperm quality, motility and velocity parameters

In the present study, clear cut benefits of NZn supplementation were obtained on semen parameters. Zinc supplementation in nano form has a clear advantage in improving motility parameters in terms of motile, progressive motile and rapid (%) sperms and reducing slow and static (%) sperms. Further, it increased various velocity parameters such as fast progressive (type a), head Area (μm^2), curvilinear velocity, straight-line velocity (µm/Sec), average path velocity (µm/Sec), linearity (%), wobble (%) and Type-A spermatozoa (%).

It was already documented by many researchers that zinc supplementation enhanced mass and individual motility, sperm concentration and sperm number per ejaculate, sperm motility in bulls (Kumar *et al*., 2006), rams (Kendall *et al*., 2000), bucks (Saleh *et al*., 1992) and rabbits (Tharwat, 1998), which is indicative of its essentiality for spermatogenesis. Sorbitol dehydrogenase and lactate dehydrogenase, which are essentially zinc metalloenzymes are involved in the process of spermatogeneis (Eggert *et al*., 2002). No reports of NZn supplementation on semen motility and velocity parameters were available. At higher dose levels (50, 100, 150 and 200 mg NZnO/kg body weight daily intraperitoneally for ten days) in male rats caused decreased sperm count and vitality in all NZnO groups and decreased sperm motility at 100, 150 and 200 mg NZnO/kg body weight (Abbasalipourkabir *et al*., 2015). Similarly, Talebi *et al*. (2013) observed that sperm number, motility and percentage of abnormality were significantly ($P<0.01$) changed in 50 and 300 mg/kg NZnO administered mice for a span of 35 days compared to 5 mg/ NZnO to the mice.

In the present study, significant increase in serum Zn concentration at 90 day of experiment in NZn-50 might have resulted more availability of Zn for spermatogenesis and in turn increased all the sperm quality, motility and velocity parameters.

SUMMARY AND CONCLUSIONS

Zinc (Zn) is the second most abundant trace element in the animal body, which can't be stored and thus regular dietary intake is requisite for normal physiological functions. In Indian context, there is widespread deficiency of Zinc (Zn) and Copper. Mostly Zn is supplemented to the animals in its inorganic form i.e. ZnO or $ZnSO_4$. Better bio-available sources are required for supplementation to reduce to dose of supplementation and subsequently improve the animal production. Nano Zn (NZn) can be a potential replacement of the traditional inorganic sources, as they are hypothesized to be more bio-available due to their smaller particle size, which in turn increase the surface area. Hence, this study was planned to evaluate the effect of NZn supplementation on animal health and performance.

Nano Zn was synthesized in the laboratory by using both physical and chemical methods. In physical method, NZn particles were synthesized by ashing of 10 mmoles zinc acetate ($Zn(CH_3COOH)_2.2H_2O$) and at 23.8 mmoles sodium bicarbonate ($NaHCO_3$) at 300º C for 4 h and the average particle size obtained was 197 nm. Chemical method was employed to produce NZn where 100 mL of 0.45 M zinc nitrate ($Zn(NO_3)_2.6H_2O$) and 100 mL of 0.9 M sodium hydroxide (NaOH) were precipitated at 55 ºC and 360 rpm, resulted in NZn with average particle size of 74 nm. The produced nano particles are confirmed to be zinc oxide using Transmission electron microscope (TEM) and Energy dispersive X-Ray spectroscopy (EDX).The synthesized NZn was fed to rats in graded doses to validate the effects of NZn and to select the dose for further experiment on goats. Rats were divided into seven groups *viz.* NC: Basal diet (BD; semi-purified synthetic diet)+ no supplemental zinc; IZn-25: BD+ 25 mg/kg Zn from inorganic ZnO; NZn-25: BD+ 25 mg/kg Zn from NZnO; NZn-12.5: BD+ 12.5 mg/kg Zn from NZnO; NZn-6.25: BD+ 6.25 mg/kg Zn from NZnO; NZn-3.125: BD+ 3.125 mg/kg Zn from NZnO; NZn-50: BD+ 50 mg/kg Zn from NZnO and experimental feeding continued for 60 days. The dry matter (%) of the semi-purified synthetic diet was in a range of 96.8 to 97.3 in NZn-50 to NZn-25, respectively. The organic matter, crude protein, ether extract and total carbohydrate

(%) was found to vary in a range of 97.17 (IZn-25) to 97.30 (NZn-3.125), 17.6 (IZn-25, NZn-3.125, NZn-50) to 18.5 (NC), 4.06 (NZn-6.25) to 4.49 (NZn-25), and 74.5 (NC) to 75.6 (NZn-3.125), respectively. Macro-mineral i.e. calcium, phosphorus content (%) was found to be in the range of 0.62 (NC) to 0.69 (NZn-6.25), and 0.67 (NC, NZn-12.5, NZn-3.125) to 0.70 (NZn-6.25), respectively. However, magnesium content was found to be 0.07 % in all the groups. The analytical level of zinc (mg/kg) in the diets of different groups was recorded to be 9.35 in NC, 34.3 in IZn-50, 34.1 in NZn-25, 22.2 in NZn-12.5, 16.1 in NZn-6.25, 12.6 in NZn-3.125 and 60.2 in NZn-50, respectively. Other trace minerals were found almost same among the groups.

The average initial, final body weight/ rat (g), total weight gain (g), ADG (g) and average daily intake (g/ rat) were found similar ($P>0.05$) among the treatment groups. However, FCR was found to be statistically ($P<0.05$) differing among the treatment groups with NZn-25 (3.06±0.18) showed better efficiency than NC (3.88±0.17), NZn-6.25 (3.53±0.14) and NZn-3.12 (3.53±0.13).

The intake (g/d), digested (g/d) and digestibility (%) of DM, OM in rats were similar ($P>0.05$) among the groups. Even though no significant variation was observed in CP intake (g/d), digested (g/d) in rats, the CP digestibility (%) of NC (90.3±0.38) was significantly lower than IZn-25, NZn-12.5, NZn-25 and NZn-50. The EE intake (g/d), digestibility (%) in the rats were similar ($P>0.05$) among the groups whereas EE digested (g/d) varied statistically ($P<0.05$). Digestibility (%) of EE in the rat diets found to vary from 90.3±1.02 (NC) to 93.0±0.60 (NZn-12.5).

The total protein (g/dL), albumin (g/dL) and globulin (g/dl) varied significantly ($P<0.001$) among the treatment groups. Total protein was found higher in NZn-50 (8.59±0.26), NZn-25 (8.62±0.23), NZn-12.5 (8.26±0.09) than NZn-3.125 (7.52±0.21), NZn-6.25 (7.47±0.22) and NC (7.09±0.11). Serum albumin (g/dL) level in NZn-50 (4.38±0.11), NZn-25 (4.48±0.10) and NZn-12.5 (4.34±0.10) had significantly higher ($P<0.001$) albumin levels in blood than NC (3.75±0.14), NZn-6.25 (3.86±0.20) and NZn-3.125 (3.94±0.09). Globulin (g/dL) content was also statistically higher ($P<0.05$) in NZn-25 (4.14±0.25) and NZn-50 (4.21±0.24) as compared to NC (3.34±0.21) whereas other groups were found similar. The A/G ratio was found similar ($P>0.05$) across all the treatment groups. Other blood biochemical parameters like BUN (mg/dL), creatinine, AST and ALP (U/L) were not affected by zinc supplementation as they were statistically similar ($P>0.05$) across the treatment groups. The ALT (U/L) varied significantly

($P<0.05$) among the treatment groups with NZn-12.5 (37.9±1.30) recorded higher ALT than NZn-3.125 (29.6±1.85) and NC (29.1±1.59).

No significant change was observed in serum thyroid (T_3) and IGF-1 hormone levels in rats due to supplementation of zinc from either sources. T_4 (nmol/L) in NZn-50 (36.1±3.13) was found to be higher ($P<0.05$) than NC (15.0±1.47), IZn-25 (26.3±1.77), NZn-12.5 (25.8±2.79), NZn-6.25 (24.1±2.69) and NZn-3.125 (24.9±4.18), whereas similar to NZn-25 (33.5 ±4.71). However, serum testosterone (ng/mL) varied significantly with dietary NZn supplementation, where NZn-50 (1.40±0.37) had significantly higher testosterone level than NC (0.39±0.04), IZn-25 (0.70±0.06), NZn-12.5 (0.71±0.12), NZn-6.25 (0.57±0.05) and NZn-3.125 (0.43±0.13), whereas similar ($P>0.05$) to NZn-25 (1.16 ±0.36).

Significant variations in cell mediated immune response observed among the treatment groups at 24 and 48 h post injection with sheep RBC. NZn-50 and NZn-25 had more ($P<0.001$) skin thickness than NC (0.40±0.16), NZn-6.25 (1.00±0.10) and NZn-3.125 (0.72±0.08) at 24 h of injection where as NC showed least amongst all. After 48h of injection, NZn-25 (1.51±0.22) had statistically higher ($P<0.001$) foot pad thickness (mm) than NC (0.37±0.23), IZn-25 (0.98±0.19), NZn-6.25 (0.46±0.09) and NZn-3.125 (0.47±0.12) where as similar to NZn-12.5 (1.03±0.10) and NZn-50 (1.44±0.14). NZn-50 (3.87± 0.12) had higher ($P<0.05$) haemagglutination titre (log_2) which indicated the humoral immunity against Sheep RBCs compared to NC (2.83±0.17), IZn-25 (3.29±0.18), NZn-12.5 (3.27± 0.17), NZn-6.25 (3.17± 0.22) and NZn-3.125 (3.17±0.17). NZn-50 and NZn-25 groups had significantly higher ($P<0.05$) humoral immune response than NC.

In liver, femur bone, and kidney, the zinc content (mg/kg) varied significantly ($P<0.05$), whereas iron, manganese and copper were found similar ($P>0.05$) among the groups. In case of muscle, Zn, iron, manganese and copper levels were found to be same across different groups. The liver zinc (mg/kg) was higher ($P<0.05$) in NZn-50 (100±2.44) than NC (79.6±3.36), NZn-6.25 (86.0±3.77), NZn-3.125 (84.3±3.17) but was found similar to IZn-25 (90.4±4.39), NZn-25(96.5±6.65), NZn-12.5 (91.5±3.30). Similarly, bone zinc content was higher ($P<0.05$) in NZn-50 (211±8.50) than NC (175±7.08), NZn-12.5 (190±2.84), NZn-6.25 (186±3.78), NZn-3.125 (186±5.34). NC animals were having significantly lower zinc than IZn-25 (196±11.7), NZn-25 (199±3.28) and NZn-50 (211±8.50). In kidney, zinc content (mg/kg) was higher ($P<0.05$) in NZn-50 (127±5.76) than NC (108±3.24), NZn-6.25 (109±1.54) and NZn-3.125 (112±3.14), while NC

(108±3.24) group showed significantly lower kidney zinc level than NZn-25 (126±5.01) and NZn-50 (127±5.76).

Serum calcium, phosphorus and magnesium (mg/dL) and copper (mg/L) were similar in all dietary treatment groups. Zinc (mg/L) level was significantly higher in NZn-25 (1.79±0.07) and NZn-50 (1.81±0.05) as compared to NC (1.46±0.05), NZn-6.25 (1.59±0.04) and NZn-3.125 (1.55±0.07), whereas, NC groups rats showed lower ($P<0.05$) serum zinc level than IZn-25 (1.68±0.03), NZn-25 (1.79±0.07), NZn-12.5 (1.73±0.10) and NZn-50 (1.81±0.05).

Supplementation of NZn at graded levels has improved ($P>0.05$) the hepatic metallothionein and SOD1 expression in rats in a dose dependant manner. The expression was prominent in NZn-50 and NZn-25.

Histopathological examination of liver showed moderate vacuolar degeneration which is evident by condensation of nuclei and vacuolated cytoplasm without pink staining with congestion of blood vessels in NC rats, NZn-6.25 and NZn-3.125 groups showed mild vacuolar degeneration in the liver hepatocytes where as IZn-25, NZn-25, NZn-12.5 and NZn-50 groups showed normal hepatocytes.

From the results obtained with supplementation of graded levels of NZn on rats, it was observed that, IZn-25 and NZn-12.5 groups performed similar in all the respects. Hence, as described in the objective, out of the reduced doses, half of ICAR recommendation was selected for goat trial.

In the goat trial, 24 goats (18.7±0.33 kg) were divided into 4 groups of six each and were supplemented with either basal diet (concentrate and finger millet straw @ 50: 50 ratio; BD) i.e. NC, BD with 50 mg/kg zinc from inorganic ZnO (IZn-50), BD with 50 mg/kg zinc from NZnO (NZn-50) or BD with 25 mg/kg zinc from NZnO (NZn-25), and the trial was conducted for almost 4 months. The OM content of the concentrate and straw were 96.48 and 93.12%, respectively. The respective ash contents (%) were 3.52 and 6.88. EE (%) was found to 1.14 and 1.88 in concentrate and finger millet straw, respectively. The CP, T-CHO (%) content of the concentrate and finger millet straw was recorded as 20.49, 74.85 and 4.87, 86.37, respectively. NDF, ADF, hemicellulose and cellulose contents (%) of concentrate and finger millet straw were 27.96, 17.20, 10.76, 12.38 and 74.32, 44.75, 29.57, 35.72, respectively. Ca, P and Mg (%) in concentrate mixture and straw was 0.80, 1.00, 0.30 and 1.10, 0.40, 0.60 %, respectively. In respect of trace minerals, Zn, Fe and Mn (mg/kg) contents of concentrate mixture and straw 22.34, 928.7, 127.8 and 12.33, 279.1, 108.7, respectively.

The initial body weight (BW, kg) and final body weight was found statistically similar (P>0.05). The total weight gain (kg), ADG (g) and FCR during this period was found to be highest (P<0.05) in NZn-25 followed by NZn-50, IZn-50 and lowest in NC. However, the total DM consumed (kg) was similar (P>0.05) in all the groups. The NC group (12.0±0.54) required significantly more (P<0.05) feed per kg BW gain than NZn-25 group (9.16±0.14). The FCR among zinc supplemented groups were found to be same (P>0.05).

Average body weight, , OM intake, CP intake, DOM intake of goats in different treatment groups was similar in different treatment groups. The concentrate intake and roughage DM intake expressed as g/d, % BW and $g/W^{0.75}$ was found to be similar as well, indicating no impact of zinc supplementation on DMI in goats.

DM, OM, CP, EE, Total CHO and NDF digestibility (%) did not vary (P>0.05) among treatment groups. The DM digestibility (%) was found to be ranging from 59.8±1.24 in NC group to 64.3±0.95 in NZn-50. CP digestibility (%) was highest in NZn-25 (72.0±1.67) and least in IZn-50 (68.1±2.24) group. The EE digestibility (%) varied from 66.6±1.09 in NC group to 70.0±1.71 in NZn-50. The total CHO digestibility ranged from 60.6±1.58 (NC) to 65.2±0.89 (NZn-50). The ADF and cellulose digestibility in zinc supplemented groups (IZn-50, NZn-50 and NZn-25), irrespective of the source of zinc (inorganic or nano) were significantly higher (P>0.05) than NC. DCP (%), TDN (%) and ME (MCal/kg) were similar (P>0.05) in different treatment groups.

Non significant variations were observed (P>0.05) in calcium, phosphorus and magnesium intake (g), absorbed (g/d) and absorption (%) among the groups due to dietary treatments. The Zn intake in IZn-50 (44.3±2.14) and NZn-50 (45.5±1.41) was significantly higher than NZn-25 (29.3±0.90) and NC (12.70±0.60). More absorption of zinc (g/d) was evident in NZn-50 group (12.3±1.98) compared to IZn-50 (8.40±0.61), NZn-25 (7.92±0.29) and NC (5.26±0.97). However, the absorption expressed as proportion of intake (%) of zinc was significantly more (P<0.006) in NC compared to zinc supplemented groups mostly due to homeostatic NC of zinc absorption.

Non significant differences were observed in the serum Ca (mg/dL), Zinc, Iron, Manganese and Copper (mg/L) among the treatment groups in both zero and 90^{th} day samples. However, zinc level at 90^{th} day was significantly higher than the initial serum zinc level in all the zinc supplemented groups, whereas in NC, the initial and final serum samples were statistically similar in zinc content. Though the difference in serum mineral

concentration across different groups are statistically similar (P>0.05) there was a numerical increasing trend in zinc concentration in the NZn supplemented goats.

Supplementation of zinc from either inorganic or nano Zn had no effect (P>0.05) on RBC (10^6/ µl), WBC (10^3/ µl), PCV (%), neutrophil (%), lymphocytes (%), eisonophil (%), monocyte (%) and haemoglobin (g/dL) level of goat blood. The blood albumin (g/dL) and A/G ratio was found to be similar (P>0.05) among the treatment groups whereas, globulin (g/dL) and total protein (g/dL) varied significantly (P<0.01). The globulin level was more (P<0.01) in NZn-50 (3.20±0.02) compared to both NC and IZn-50. Total protein (g/dL) was more (P<0.001) in NZn-50 (6.90±0.01) which varied significantly with NZn-25 (6.85±0.01) and NC (6.78±0.03), but non-significantly with IZn-50 (6.87±0.01).

No change was observed in thyroid hormone status on zero and 90^{th} day of experimental feeding. But supplemental of NZn at 50 and 25 mg/kg improved (P<0.05) serum IGF-1 and testosterone levels after 90^{th} day of experimental feeding in goats.

Zinc supplementation improved the humoral immunity in all the groups irrespective of the source of zinc and NC showed minimum HI titre against PPR virus. Similarly, CMI measured by skin fold thickness after injecting Con-A, was also improved in zinc supplemented groups than NC at 6, 12 and 48 h of incubation. In all the cases, NZn-50 animals shown highest HI titre as well as skin thickness. The $CD4^+$ (%) was significantly high (P<0.05) in all the zinc supplemented groups than the NC group fed only with basal diet without any extra zinc supplementation. NZn-50 (19.6±0.80) showed significantly higher (P<0.05) $CD8^+$ count than NC (17.0±0.26) and similar (P>0.05) to IZn-50 (18.3±0.43) and NZn-25 (18.2±0.58) groups. $CD4^+$, $CD8^+$ ratio varied non-significantly (P>0.05) among the treatment groups.

Supplementation of NZn in graded doses did not affect the ammonia nitrogen as well as total and individual volatile fatty acid profiles in rumen liquor. There was a significant drop in rumen pH due to supplementation of zinc and higher dose of zinc made more acidic rumen than lower level of zinc. The soluble zinc content in rumen liquor was also affected in a dose dependant manner. The zinc content in SRL was highest (P<0.01) in NZn-50 (1.99±0.08) compared to NC, and NZn-25, whereas IZn-50 (1.81±0.12) had significantly higher (P<0.05) soluble Zn in SRL than NC (1.37± 0.09). However, NZn-25 did not vary significantly either with IZn-50 or with NC with respect to the rumen soluble zinc contents. There was no significant difference in case of rumen soluble iron, manganese and copper contents (mg/L).

Supplementation of NZn at graded doses of 50 and 25 ppm did not affect (P<0.05) the semen volume and sperm production in goats, but sperm motility and velocity parameters were significantly improved with NZn supplementation at 25 and 50 ppm levels in goats. The total motile sperm (%) was highest (P<0.01) in NZn-50 (81.7±1.61) and least in NC (64.7±2.35). Non-progressive motile sperm (%) did not vary statistically (P>0.05) among the treatment groups and varied in a range of 19.5±1.75 in NZn-50 to 26.9±2.29 in NC. But, progressive forward motile (%) was significantly high (P<0.001) in NZn-50 (62.2±2.47) as compared to that of NC (37.9±2.81) and IZn-50 (49.5±3.02). The rapid motile sperms (%) were significantly higher (P<0.001) in NZn supplemented groups at either doses than both NC and IZn-50. Static sperms were significantly high (P<0.01) in NC (35.3±2.35) and IZn-50 group (27.1±2.13) than NZn-50 (18.3±1.61) and NZn-25 (20.1±5.14). The hyperactivity (%) in sperms was found non-significant (P>0.001) among the treatment groups which fell in the range between 19.4±2.40 (NC) to 26.0±2.09 (NZn-25). Zinc supplementation in nano form has a clear advantage in improving motility parameters in terms of motile, progressive motile and rapid (%) sperms and reducing slow and static (%) sperms. Further it increased various velocity parameters such as fast progressive (type a), head area (μm^2), curvilinear velocity, straight-line velocity (µm/Sec), average path velocity (µm/Sec), linearity (%), wobble (%), Type-A spermatozoa (%).

From the above results, it can be concluded that NZn at half dose i.e. 12.5 ppm in rats and 25 ppm in goats performed equivalent to inorganic Zn as ZnO at 25 and 50 ppm, respectively. Thus, it can be recommended that NZn at half the dose of ICAR (2013) recommendation is sufficient enough in terms of animal health, production and male reproductive performances in goats. However, further studies are warranted to elucidate the different beneficial and harmful effects of NZn at different doses before using as feed supplement in goats at field level.

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M.V.Sc. (Animal Nutrition)	College of Veterinary Science, Rajendranagar, Hydrabad	Sri Venketeswara Veterinary University, Tirupati	2012	8.88
Ph.D. (Animal Nutrition)	ICAR-National Dairy Research Institute, Karnal, Haryana		2017	8.30

Dissertation

1) **Ph.D. Research**: **"**Evaluation of nano zinc supplementation on growth, nutrient utilization and immunity in goats (*Capra hircus*)"
2) **Master's Research**: **"**Chemical and *in vitro* evaluation of common pulse chunies"

Academic Achievements

- ✓ Qualified ICAR-NET in animal nutrition in 2016
- ✓ Awarded Institutional Research Fellowship by Indian Council of Agriculture Research (2013-17) for pursuing Ph.D degree (Animal Nutrition) at ICAR-NDRI.

Awards and Recognitions

- 3rd (poster presentation): Effect of feeding graded doses nano Zinc Oxide (nZnO) on rat immunity and intestinal architecture. Partha Sarathi Swain, D. Rajendran, SBN Rao, P. Krishnamoorthy, George Dominic, N.M. Soren. ANACON-2016. Tirupati.
- 2nd (oral presentation): Nutritional evaluation of different ayurvedic medicinal residues as livestock feed. George Dominic, K S Prasad, NM Soren, SBN Rao, Partha Sarathi Swain. ANSICON-2016