ANNEXURE - VII

INDIAN COUNCIL OF AGRICULTURAL RESEARCH

FINAL RESEARCH PROJECT REPORT (RPP- III)

(For Guidelines Refer ANNEXURE – XI(G))

1. Institute Project Code: **ANSC CIRG SI 2012 006 00217**

2. Project Title: **Effect of nutritional deficiency diseases on gene expression profiles in goats.**

3. Key Words: **Gene expression profile, goat, nutritional deficiency, nutrigenomics, pathology, selenium, Vitamin E, zinc.**

4. (a) Name of the Lead Institute: **Central Institute for Research on Goats, Makhdoom, Farah, Mathura (U.P.)**

   (b) Name of Division/ Regional Center/ Section: **Division of Animal Health**

5. (a) Name of the Collaborating Institute(s): Nil

   (b) Name of Division/ Regional Center/ Section of Collaborating Institute(s):

6. Project Team(Name(s) and designation of PI, CC-PI and all project Co-PIs, with time spent)

S. No.	Name, designation and Institute	Status in the project (PI/CC-PI/ Co-PI)	Time to be spent (%)	Work components to be assigned to individual scientist
	RVS Pawaiya, Principal Scientist (Vet Pathology)	Principal Investigator (PI)	30	Procurement of consumables/ biochemical/ labwares/ equipments etc., planning and designing of experimentation and implementation supervision, periodical examination of animals for the development of any deficiency lesions/ clinical signs, collection of biosamples from the experimental animals. Molecular and genomic studies and microarray studies and outsourcing of the services wherever required.
	UB Chaudhary Principal Scientist & Head, NFRPT (Animal Nutrition)	Co-PI	25	Procurement of consumables/ equipments etc., to provide and facilitate experimental animals of required age and sex groups, with proper housing, feeding and managemental care, formulation of Cu, Zn and Cu&Zn combined-deficient as well as –sufficient and balanced diets for the experimentation and feeding the same in precise manner for the successful running of the study. Molecular and microarray studies.
	Nitika Sharma, Scientist (Vet Medicine)	Co-PI	25	Collection of biosamples & AAS studies. Processing of the biosamples for the molecular biological studies and to help undertake the molecular biological and genomic studies and microarray studies.
	N Shivasharnappa, Scientist (Vet Pathology)	Co-PI (Discontinued from 21st April, 2015)	25	Periodical examination of animals for the development of any deficiency lesions/ clinical signs, collection of biosamples from the experimental animals. Carrying out pathological studies. Processing of biosamples, Molecular and genomic studies and microarray studies.

7. **Priority Area:** Flagship programme on nutrigenomics and immunogenomics.

8. Project Duration: Date of Start -4 Years; Date of Start – **October, 2012** Date of Completion – **September, 2016** (As per Director's instructions, to be closed by 31st March, 2015)

9. a. Objectives

1. Analysis of gene expression profiling of nutrient-deficient (Zn, Cu and Zn&Cu combined -deficient) goats.
2. Identification and characterization of genes specifically affected (upregulation and downregulation) by nutritional deficiency in goats.
3. To determine whether corrective measures to nutrient deficiency can restore the gene expression pattern comparable to healthy and control goats.

b. Practical utility

Nutrigenomics promise to provide the tools to understand the role of nutrition in gene expression; we will begin to appreciate the long-term effects of nutrition on animal diseases, reproductive and production performance. Gene expression studies will allow for the identification of pathways and candidate genes responsible for deficiency disease development and progression to overt sign.

It will also be possible to examine the tissue programming that is triggered by nutrition in goats when transcriptomic, proteomic and metabolomics studies are taken up after successful gene expression profiling. It will help in understanding and maneuvering the dietary manipulations and nutritional strategies as key tools for deficiency disease prevention and influencing goat production. Nutrition and genetic makeup both strongly influence the reproductive performance of milch animals. This is particularly important during the transition period and early lactation, when the animal is particularly sensitive to nutritional imbalances. Nutrigenomics may provide new tools that can be used to more clearly understand how nutritional management can be applied to address disease, performance and productivity in animals.

More importantly, the more precise measures of nutrient effects afforded by more reliable genomic markers and molecular profiling techniques will provide new tools for evaluating practical nutrient requirements and diet formulation strategies in a relatively short time. This will lead to improved livestock productivity, improved sustainability and the production of more wholesome animal products.

Using system biology approach (microarray) to analyse the gene expression profiling (molecular signature) of host following nutritional deficiency. Altered host gene expression following nutrient deficiency can be exploited as marker for facilitated/early diagnosis of the disease as well as marker for early prediction of the disease corrective (protective) measures. Finally analysis of differential molecular signatures of nutritionally deficient and healthy animals could contribute in our understanding about the molecular mechanism and pathways of micronutrient functions and utilization.

10. Final Report on the Project (materials and methods used, results and discussion, objective wise achievements and conclusions)

INTRODUCTION

Zinc and copper deficiency can occur in soil, plants, and animals. In animals, including humans, it is defined either qualitatively as insufficient zinc to meet the needs of the body and thereby causing clinical manifestations, or quantitatively as a serum zinc level below the normal range. Zinc and copper deficiency in humans/animals results from reduced dietary intake, inadequate absorption, increased loss, or increased use. The most common cause is reduced dietary intake; as much as 25% of the world's population is at risk. Zinc and copper plays an essential role in numerous biochemical pathways. It affects many organ systems, including the skin, gastrointestinal tract, central nervous system, and immune, skeletal, and reproductive systems. A lack of zinc and copper thus has numerous manifestations, the most common of which are increased rates of diarrhea and pneumonia. Copper deficiency may cause locomotor difficulties in goats in two distinct ways. Abnormal bone growth with increased bone fragility can predispose to fractures of long bones. Independently, a neurologic condition known as enzootic ataxia or swayback develops, in which copper deficiency of kids in utero or after birth results in permanent myelin degeneration in the spinal cord, leading to progressive incoordination and paralysis with failure of mobility. Dietary copper is not available in the hay or pasture in large enough quantities for goats. Thus copper must be added to the diet.

As biosystems are unable to store zinc, regular intake is necessary. Excessively low zinc intake can lead to zinc deficiency, which can negatively impact an individual's health (Prasad, 2013). The mechanisms for the clinical manifestations of zinc deficiency are best appreciated by recognizing that zinc functions in the body in three areas: catalytic, structural, and regulatory (Russel et al., 2002; cousins, 1994). Zinc (Zn) is only common in its +2 oxidative state, where it typically coordinates with tetrahedral geometry. It is important in maintaining basic cellular functions such as DNA replication, RNA transcription, cell division and cell activations. However, having too much or too little zinc can cause these functions to be compromised.

In its catalytic role, zinc is a critical component of the catalytic site of hundreds of metalloenzymes. In its structural role, zinc coordinates with certain protein domains; facilitating protein folding and producing structures such as 'zinc fingers'. In its regulatory role, zinc is involved in the regulation of nucleoproteins and the activity of various inflammatory cells. For example, zinc regulates the expression of metallothionein, which has multiple functions, such as intracellular zinc compartmentalization (Maret, 2003) and antioxidant function (Theocharis, 2003; Theocharis, 2004). Thus zinc deficiency results in disruption of hundreds of metabolic pathways, causing numerous clinical manifestations, including impaired growth and development, and disruption of reproductive and immune function (Yamada, et al., 2009; Kupka and Fawzi, 2002; Rink, 2000).

Minerals and vitamins are vital to the good health of goats. Most of the mineral- and vitamin-related problems in goats result from deficiencies. Although no single mineral can be singled out as more important than others, copper, zinc, selenium, and manganese levels are especially critical. The interaction of minerals is astoundingly complex. The most difficult task in raising goats is getting nutrition right, and vitamins and minerals are integral parts of proper nutrition. Most goat raisers (including me) don't know enough about nutrition to formulate our own feed ration. Zinc is needed in the synthesis of proteins and DNA and in cell division. Excessive salivation, deformed hooves, stiff joints, chronic skin problems, abnormally small testicles, and poor libido (reduced interest in mating) are some of the signs.

There is a broad spectrum of physiological signs of zinc deficiency given that zinc is involved in a great number of biochemical processes. Most commonly, zinc deficiency is associated with skin lesions, such as seen in *Acrodermatitis enteropathica*, an autosomal recessive metabolic disorder affecting the uptake of zinc. Additionally, growth retardation and hypogonadism in males were among the first and frequently reported clinical signs in zinc deficient patients (Prasad, 2013). However, apart from several other symptoms caused by zinc deficiency, such as poor appetite, delayed wound healing, cell-mediated immune dysfunction, and abnormal neurosensory changes (Prasad, 2013), behavioral alterations have been consistently reported. Zinc deficiency may result in depression, emotional instability, increased anxiety and aggression, irritability and deficits in social behavior.

Currently, an estimated 17.3% of the global population is at risk of developing zinc deficiency (Wessells and Brown, 2012) and a prevalence of inadequate zinc intake was estimated with a range from 7.5% in high-income regions to 30% in South Asia (Wessells and Brown, 2012). Zinc is an essential trace metal in the human body that contains about 2 g zinc stored mostly in muscle and bone tissue. However, the zinc content in the brain is surprisingly high. There, zinc is one of the most abundant trace metals and is enriched at the pre-synaptic compartment of nerve cells stored in specific vesicles together with the neurotransmitter glutamate, but also acts at the post-synapse (Bitanihirwe and Cunningham, 2009). Zinc is able to influence synaptic plasticity (Xie and Smart, 1994; Lu et al., 2000), to regulate post-synaptic proteins (Grabrucker, 2014) and has an important role in the formation and maintenance of the structure of the post-synaptic density (PSD), a network of proteins that links the neurotransmitter receptors to downstream signaling components and to the cytoskeleton (Jan et al., 2002; Grabrucker et al., 2011).

Zinc deficiency can be readily produced by dietary zinc restriction using special food. In rodents fed with a zinc deficient diet, signs of zinc deficiency occur very soon. For example, a typical reduction in food intake is observed within approximately 3 days (Evans et al., 2004). At the same time, serum zinc levels are decreased (Ohinata et al., 2009). However, zinc levels in the brain are affected only after chronic zinc deficiency. For example, 4-week zinc deprivation decreases hippocampal zinc concentrations in rats (Takeda and Tamano, 2009). In rodents, a diet that induced marginal/mild zinc deprivation contains 10 μg Zn/g. A zinc content of 5–7 μg/g is associated with moderate deprivation, and of <1–2 μg/g with severe deprivation (Golub et al., 1995). In monkeys, a marginal deprivation occurs at a zinc content of 4 μg/g, moderate deprivation at 2 μg/g and severe deprivation at <1 μg/g (with 50 μg Zn/g considered adequate).

Maternal zinc deficiency (prenatal zinc deficiency) produces effects ranging from growth retardation and teratogenesis to embryo/fetal death. Additionally, postnatal complications of maternal zinc deficiency such as

neurobehavioral and immunological abnormalities can occur. On the other hand, postnatal zinc deficiency in adult life may cause specific and distinct effects itself. Therefore, it is important to consider the different time-points of zinc deprivation, when investigating the effects of zinc deficiency.

Since the last 50 years, zinc, from an ignored mineral, has now become so important that zinc deficiency is recognized as a major worldwide public health problem. In fact, zinc deficiency has been estimated to cause more than 450,000 deaths in children under the age of 5 (Fischer Walker et al., 2009) and about 800,000 deaths (about 1.5% of all deaths) (Nriagu, 2007) annually worldwide and is responsible for about 20% of perinatal mortality. Mild zinc deficiency is especially common in infants, children, women, and elderly people because of either high nutrient requirements or compromised digestion and absorption and contributes to impaired physical and neuropsychological function.

Zn deficiency in humans is widespread throughout the world. It is more prevalent in areas where the population subsists on cereal proteins. Conditioned Zn deficiency is seen in many disease states. Its deficiency during growth periods results in growth failure and lack of gonadal development in males. Other effects of Zn deficiency include skin changes, poor appetite, mental lethargy, delayed wound healing, neurosensory disorders, and cell-mediated immune disorders. Severe Zn deficiency, as seen in acrodermatitis enteropathica (a genetic disorder), is fatal if Zn is not administered to these patients. A clinical diagnosis of marginal Zn deficiency in humans remains problematic. Assays of Zn in granulocytes and lymphocytes provide better diagnostic criteria for marginal Zn deficiency than plasma Zn. Approximately 300 enzymes are known to require Zn for their activities. Zn is required for DNA synthesis, cell division, and protein synthesis. Recently, we learned that Zn-finger proteins are involved in genetic expression of various growth factors and steroid receptors. We suspect that several hundred Zn-containing nucleoproteins are probably involved in gene expression of various proteins.

MATERIALS AND METHODS

Level of copper more than 5mg/kg DM (preferably 7-12 mg/kg DM) is safe unless complicating factors cause secondary Cu-deficiency. Normal level of zinc requirement in ruminant/calves remains healthy when fed 40 mg/kg DM Zn in the diet. Grazing pastures containing 20-80 mg/kg (normal 93 mg/kg) Zn contents cause parakeratosis in calves (with Ca content 0.6 %). Experimentation started with the feeding of zinc deficient diets. For this experiment 18 male barbari, aged around 2 months were procured from barbari shed of CIRG, Makhdoom, Mathura. Before starting deficient diets, the animals divided into two equal groups A and B, each containing 9 animals. Group A served as control (give standard ration) and group B served as treatment group (give zinc deficient diet). In beginning 3 months of age they were given low zinc purified diet ad-libitum. The diets contained the following: Maize 542.50 g/kg; starch 200.00g/kg; casein 230.00 g/kg; mineral mixture 20.0 g/kg; vitamin premix 5.0 g/kg and sodium bicarbonate 2.50 g/kg, zinc 21.25 mg/kg. To induce more deficiency, add 40 mg/kg molybdenum (Mo) and 3 g/kg sulpher (S) into the ration. You can adjust the CP and TDN as per need by changing the composition. Give straw as roughage source instead of green forages. Considering the zinc content, better restrict the roughage portion and give more concentrate. In mineral mixture add Fe 1.5 to 2.0 of its requirement which may induce Zn deficiency. You may add little more calcium (Ca) than its requirement to inhibit the Zn absorption. In vitamin premix- Vitamin A, D & E. Make a premix of 5 g (ground maize based) to supplement Mo and S and add to the ration daily to individual animal.

Formulation of Low copper and Zinc diets:

S.No.	Ingerdients	g/kg
1.	Maize	542.5
2.	Starch	200.00
3.	Casien	230.00
4.	Mineral Mixture	20.00
5.	Vitamin Premix	5.00
6.	Sodium bicarbonate	2.50

- To induce more deficiency, add 40 mg/kg Molybdenum and 3g/kg Sulphur into the ration.
- You can adjust the CP and TDN as per need by changing the composition.

- Give straw as roughage source instead of green fodder. Considering the Cu and Zn content, better restrict the roughage portion and give more concentrate.
- In mineral mixture, add Fe 1.5 to 2.0 times of its requirement which may induce zinc deficiency. You may add little more Ca than its requirement to inhibit the zinc absorption.
- In vitamin premix: Vitamin A, D & E.
- Make a premix of 5g (ground maize based) to supplement Mo and S and add to the ration daily to individual animal.

Parameters studied:
The experiment was started on 2nd November, 2013. The deficient diet was given only up to 17th October, 2014. Thereafter, were fed with normal balanced diet to observe the restoration of health. Finally, the experimentation was terminated on 17th January, 2015.

Body Weight: The period of study was 420 days. The body weights were taken each after 15 days intervals upto end of experiment in all groups i.e. A, B, C & D.

Blood parameters: In this all the parameters related to blood were studied as total erythrocyte count, total leukocyte count, packed cell volume and hemoglobin etc. The blood samples were collected in EDTA at each 45 days interval from all the groups and kept at 4^0c for further analysis.

Copper and Zinc Concentration in plasma/Serum: The concentration of Cu and Zn was measured by atomic absorption spectroscopy in all the groups as A, B, C and D. The blood samples were collected at each 45 days interval from all the groups and kept at 4^0c for further analysis.

Patho-morphological Study: Both gross and microscopic changes observed in tissues collected at time of postmortem examination of dead animals during the period of study. The tissues collected at time of necropsy preserved in 10 % formalin for further histopathological study as per Luna et al., 1984.

RESULTS

The study was 14 months, in which copper and zinc deficiency diets fed for 03 (three) months and then animals observed for rest period of experimentation. During the experimentation the 6 animals died in group A (Copper deficiency fed diet), 8 animals died in group B (Zn deficiency fed diet), 8 animals died in group C (Copper and Zinc deficiency fed diet) and 5 animals died in group D (Control group i.e. standard diet fed). The detailed necropsy performed and different pieces of tissues of visceral organs with brain collected and preserved in 10 % formalin. Then, the histopathology performed with the collected tissues by standard procedure as per Luna et al., 1984.

Effect on body weight:

All the groups (A, B, C and D) of animals showed body weight increases with advancement of experimentation. The body weight in group A showed increase in body weight upto 180 days and remain upto 240 days of experiment then it was decreases after 270 days and then again increases from 390 to 420 days of experiment. The body weight in group B showed highest growth in body weight than all other three groups, in this group only decrease in body weight observed at 390 days then again increase at 420 days of experiment. Control animals (Gorup-D) were showed increase in body weight from start of experiment to end of this experiment. The group C showed least growth in body weight than all other three groups. (Graph 1).

Effect on blood parameters:

Effect on TEC: In group A, B and C, **t**he initial concentration of RBC was ($11.40x10^6$/µl, $13.05x10^6$/µl and 11.52 $x10^6$/µl) respectively, then increases the concentration of RBCs and then decreases from 135 days onwards upto 225 days then again increases after 225 days of experiment, in all the groups-A, B and C (11.6 $x10^6$/µl, 16.65 $x10^6$/µl, 13.83 $x10^6$/µl) respectively. There was significant variation, in RBC concentration between day 45, 90 with 270 day of experiment in group A, and day 0, 225 and 270 day in group B, and between days 0 to 135, 225, 270 day in group C. (Table 1, 2 & 3, and Graph 1, 2, 3 & 4)

Graph 1: Effect on body weight in different groups (A, B, C and D) of goats

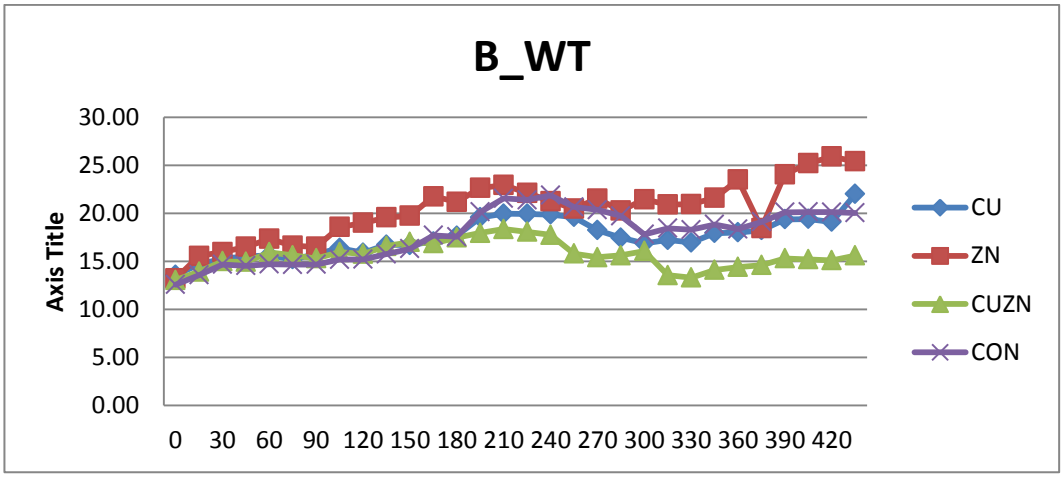


Table 1: RBC concentration in Cu deficient diet fed animals (Group- A).

Intervals	RBC Mean Concentration
0 days	11.4^{ab} x10^6/µl
45 days	12.7^{a} x10^6/µl
90 days	13.5^{a} x10^6/µl
135 days	10.4^{ab} x10^6/µl
225 days	6.7^{ab} x10^6/µl
270 days	11.6^{b} x10^6/µl

Graph 2: Effect on RBC concentration in copper deficient diet fed group (A) of goats.

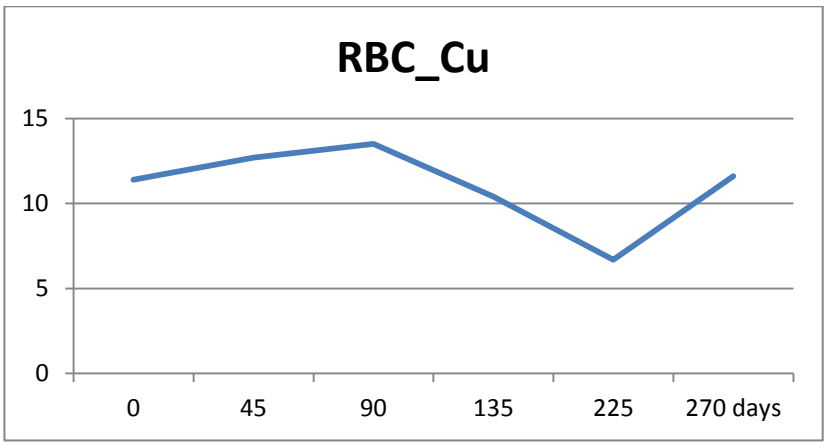


Table 2: RBC concentration in Zn deficient diet fed animals (Group B).

Intervals	RBC Mean Concentration
0 days	13.05^{b} x10^6/µl
45 days	14.26^{b} x10^6/µl
90 days	13.9^{b} x10^6/µl
135 days	12.3^{b} x10^6/µl
225 days	5.34^{c} x10^6/µl
270 days	16.65^{a} x10^6/µl

Graph 3: Effect on RBC concentration in Zinc deficient diet fed group (B) of goats.

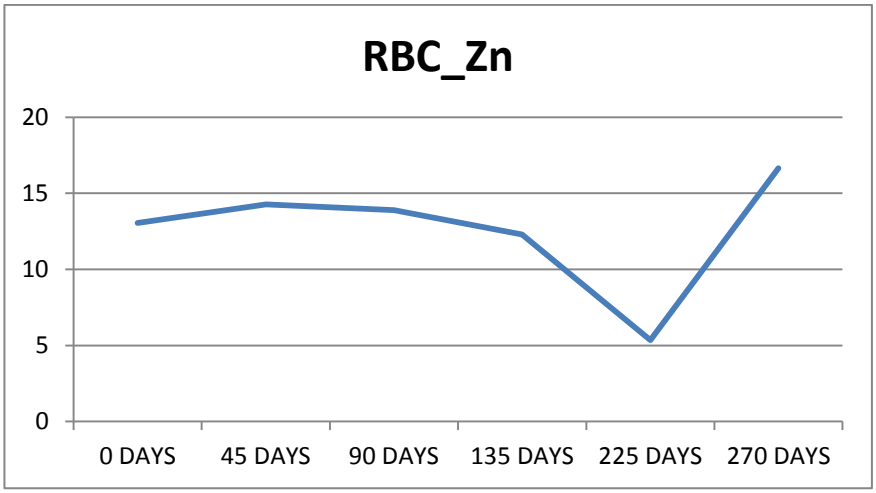
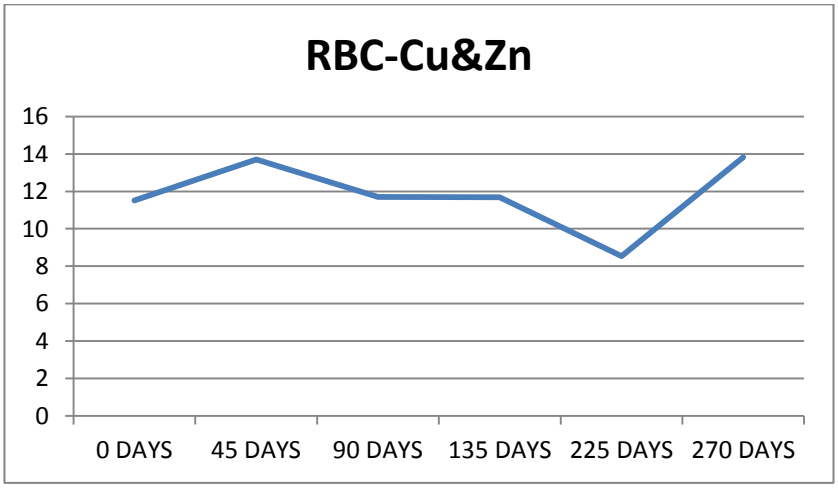


Table 3: RBC concentration in Cu and Zn deficient diet fed animals (Group C).

Intervals	RBC Mean Concentration
0 days	11.52^{a} x10^{6}/µl
45 days	13.71^{a} x10^{6}/µl
90 days	11.70^{a} x10^{6}/µl
135 days	11.69^{a} x10^{6}/µl
225 days	8.53^{b} x10^{6}/µl
270 days	13.83^{a} x10^{6}/µl

Graph 4: Effect on RBC concentration in copper and zinc deficient diet fed group (C) of goats.



Effect on WBC: In group A and C, the initial concentration of WBCs was (24.99 x10^{3}/µl and 23.00 x10^{3}/µl) respectively, then decreases in group A and C then increases from 90 days onwards upto 135 days in group A and 225 days in group C then again decreases after 225 days of experiment, in both these the groups- A (14.92 x10^{3}/µl) and C (13.39 x10^{3}/µl). But in group B initially the concentration of WBCs (14.81 x10^{3}/µl) increases then decrease after 45 days of experiment upto 135 days the again increases sharply in WBC concentration (17.00 x10^{3}/µl).There was significant variation, in WBC concentration between days 0 and 270 days of experiment in group C, other groups did not show significant variation. (Table 4, 5 & 6; Graph 5, 6 & 7)

Table 4: WBC concentration in Cu deficient diet fed animals (Group A).

Intervals	WBC Mean Concentration
0 days	24.99 $x10^3/\mu l$
45 days	17.70 $x10^3/\mu l$
90 days	20.80 $x10^3/\mu l$
135 days	29.30 $x10^3/\mu l$
270 days	14.92 $x10^3/\mu l$

Graph 5: Effect on WBC concentration in copper deficient diet fed group (A) of goats.

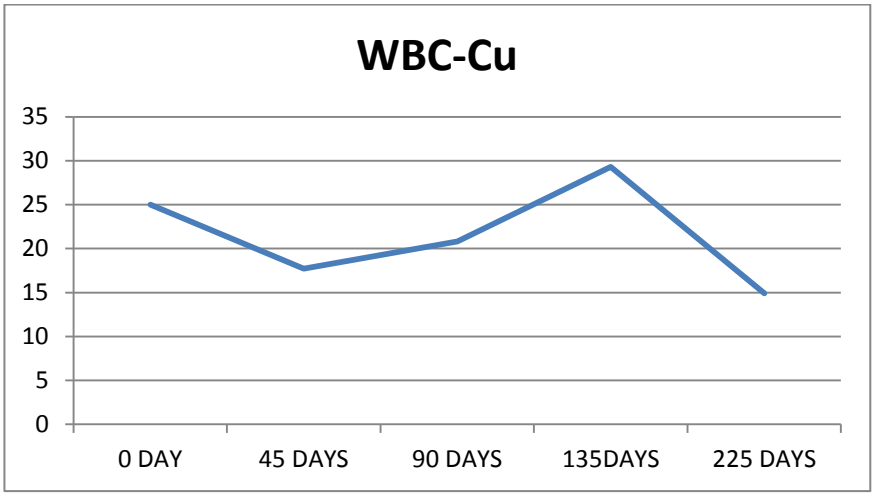


Table 5: WBC concentration in Zn deficient diet fed animals (Group B).

Intervals	WBC Mean Concentration
0 days	14.81 $x10^3/\mu l$
45 days	15.60 $x10^3/\mu l$
90 days	14.90 $x10^3/\mu l$
135 days	14.41 $x10^3/\mu l$
270 days	17.00 $x10^3/\mu l$

Graph 6: Effect on WBC concentration in zinc deficient diet fed group (B) of goats.

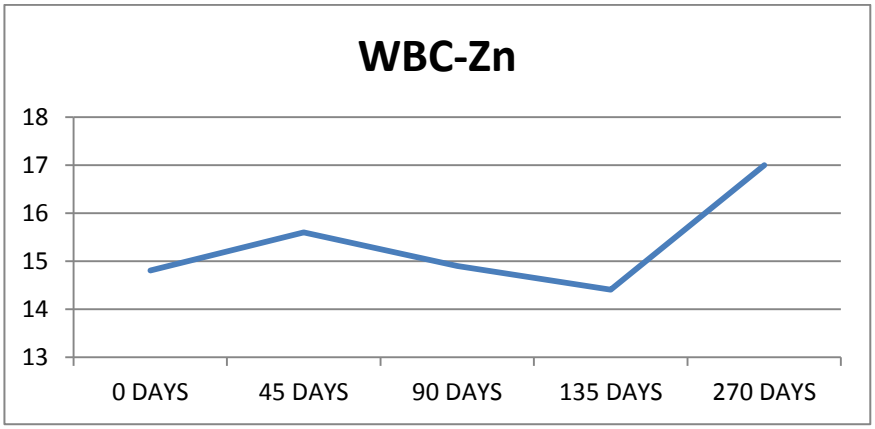
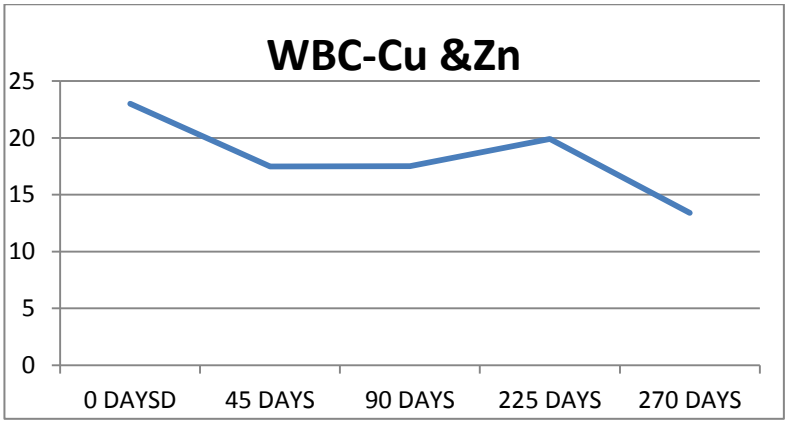


Table 6: WBC concentration in Cu and Zn deficient diet fed animals (Group C).

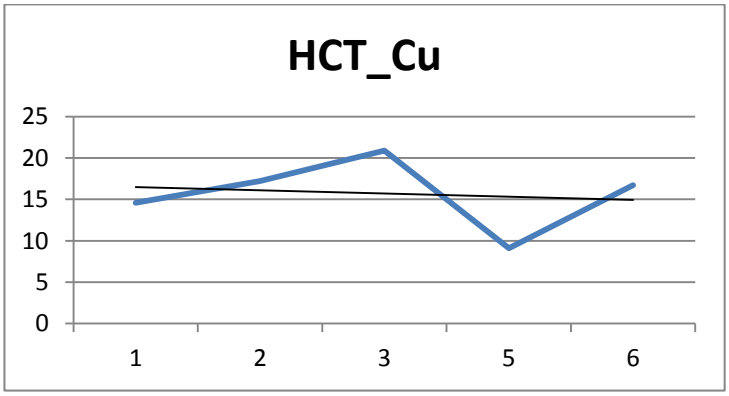
Intervals	WBC Mean Concentration
0 days	23.00^{a} x10^{3}/µl
45 days	17.5^{ab} x10^{3}/µl
90 days	17.53^{ab} x10^{3}/µl
225 days	19.9^{ab} x10^{3}/µl
270 days	13.39^{b} x10^{3}/µl

Graph 7: Effect on WBC concentration in copper and zinc deficient diet fed group (C) of goats.

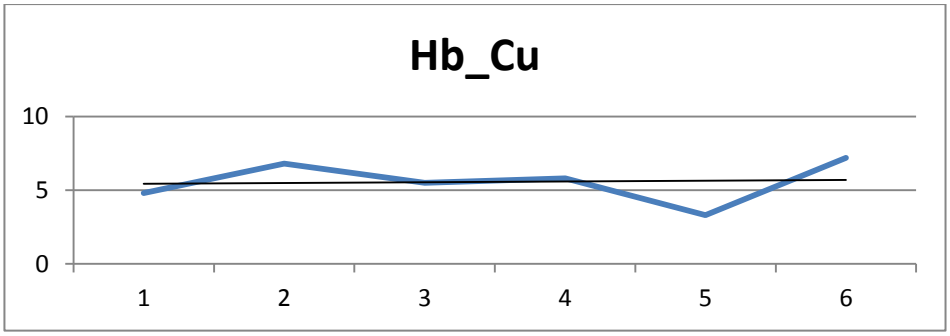


Effect on Hb and PCV: In all the groups values of HB ad PCV initially increases then decreases from 90 days onwards upto 225 days then again increases after 225 days of experiment, in all the groups-A, B, C and D. (Graph 8 & 9)

Graph 8: Effect on PCV concentration in copper deficient diet fed group (A) of goats.



Graph 9: Effect on heamoglobin (Hb) concentration in copper deficient diet fed group (A) of goats.



Effect on concentration of Copper in Serum estimated by AAS:

Ingroup A, initially the mean concentration of copper (0.11 ppm) was decreases upto 72 days then increases at peak 0.12 ppm on 87th day of experiment then again decreases at 172 days and remain at this level (0.04 ppm). In group B, the initial meanconcentrations of Cu (0.10) was increases to 0.85 ppm on day 57 then decreases on 72nd day and remain at this level (0.04 ppm)upto experimentation. In group C, the initial mean concentration of copper (0.192) was decreases from start of experiment then remain at this level with minor up and down in mean concentrations of copper (0.14 ppm). In group D, the initial mean concentration of copper (0.08) was initially increases then decreases then again increases (0.04) and so on. (Table 7, 8, 9 & 10; Graph 10, 11, 12 & 13).

Table 7: Copper concentration in Cu deficient diet fed animals (Group A).

Intervals	Cu Mean Concentration (ppm)
0 days	0.11 ppm
57 days	0.08 ppm
72 days	0.07 ppm
87 days	0.12 ppm
102 days	0.04 ppm
132 days	0.04 ppm
146 days	0.03 ppm
176 days	0.04 ppm

Graph 10: Effect on copper concentration in copper deficient diet fed group (A) of goats.

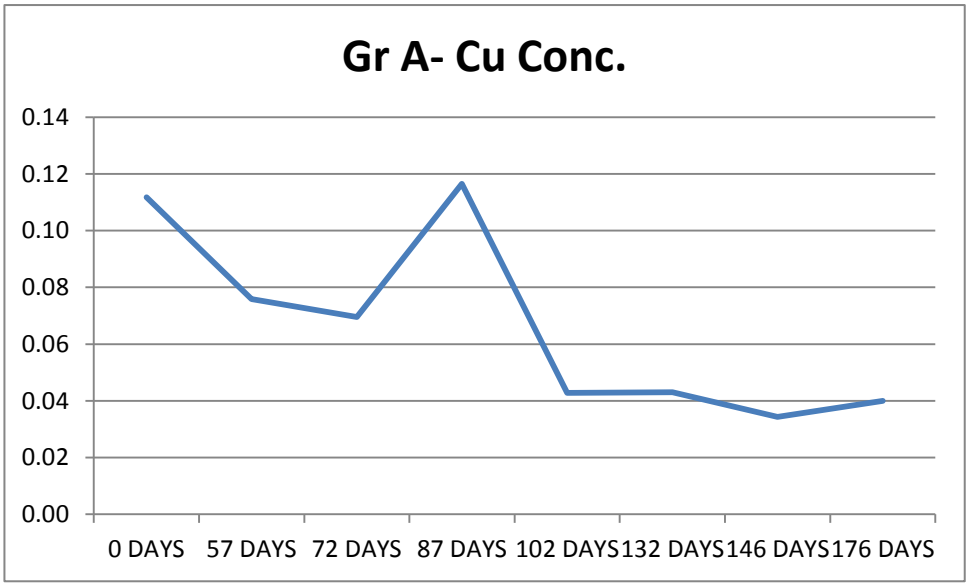


Table 8: Copper concentration in Zn deficient diet fed animals (Group B).

Intervals	Cu Mean Concentration
0 days	0.10 ppm
57 days	0.85 ppm
72 days	0.05 ppm
87 days	0.03 ppm
102 days	0.05 ppm
132 days	0.07 ppm
146 days	0.02 ppm
161 days	0.01 ppm
176 days	0.04 ppm

Graph 11: Effect on copper concentration in zinc deficient diet fed group (B) of goats.

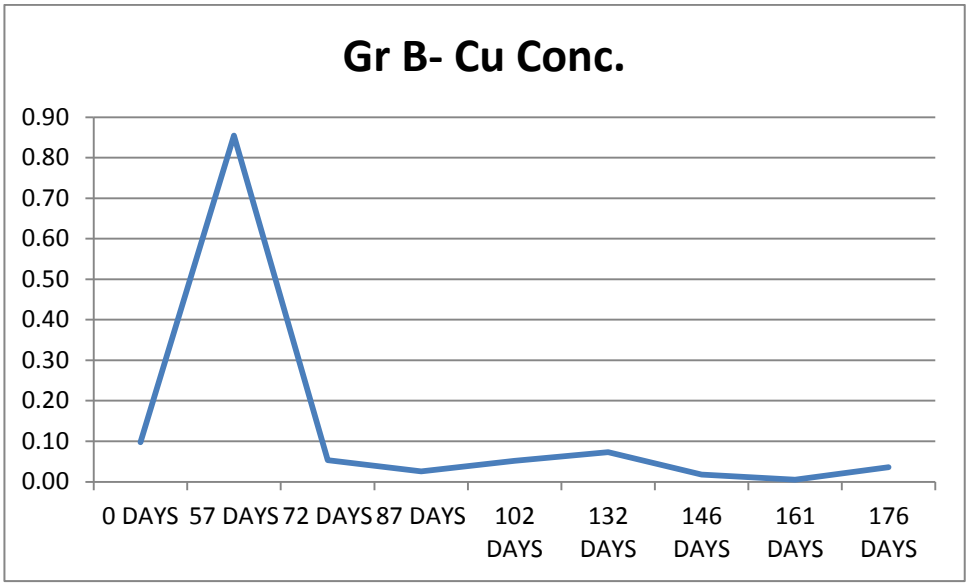


Table 9: Copper concentration in Cu and Zn deficient diet fed animals (Group- C).

Intervals	Cu Mean Concentration
0 days	0.19 ppm
57 days	0.11 ppm
72 days	0.08 ppm
87 days	0.02 ppm
102 days	0.03 ppm
132 days	0.043 ppm
146 days	0.03 ppm
176 days	0.14 ppm

Graph 12: Effect on copper concentration in copper and zinc deficient diet fed group (C) of goats.

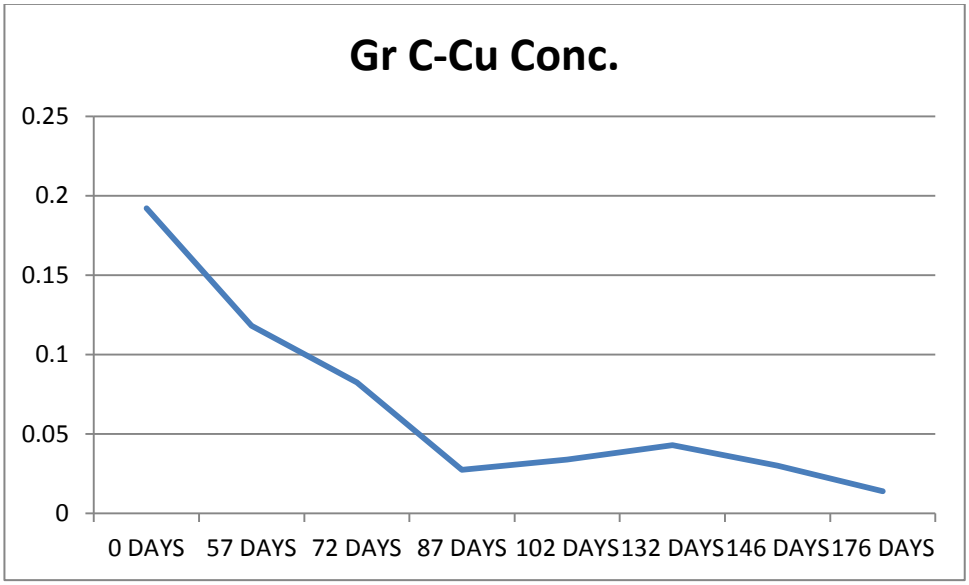
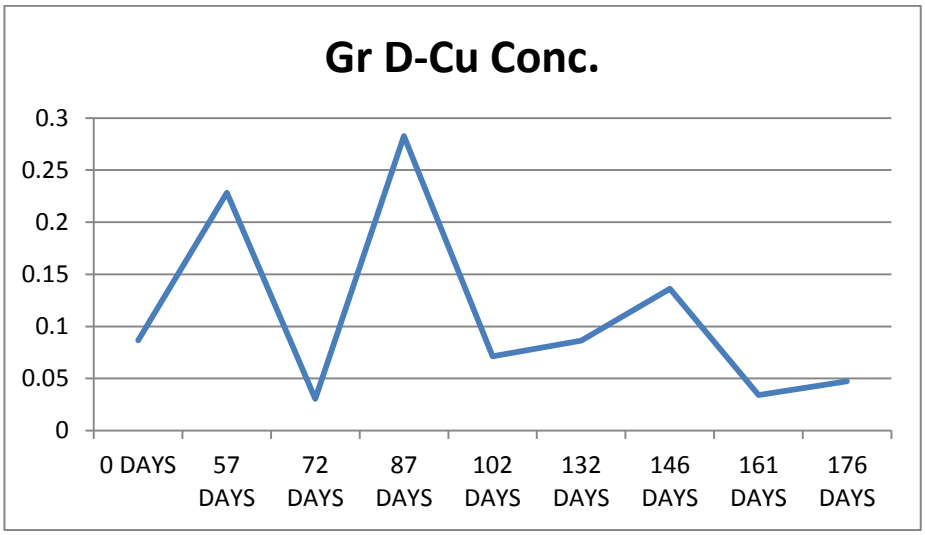


Table 10: Copper concentration in control group animals (Group D).

Intervals	Cu Mean Concentration
0 days	0.08 ppm
57 days	0.22 ppm
72 days	0.03 ppm
87 days	0.28 ppm
102 days	0.07 ppm
132 days	0.08 ppm
146 days	0.13 ppm
161 days	0.03 ppm
176 days	0.04 ppm

Graph 13: Effect on copper concentration in control group (D) of goats.



Effect on concentration of Zinc in Serum estimated by AAS:

In group A, initially the mean concentration of copper (1.02 ppm) was decreases upto57 days of experimentation then remain constant with few ups and down in mean concentration (0.34 ppm). In group B, the initialmean concentration of zinc (0.69 ppm) was decreasesfrom day 57 to 87 thenincreases upto102 days andand again decreases upto 132 days then increases 146 days and remain at this level upto experimentation (0.34 ppm). In group C, the initial mean concentration of zinc (0.63 ppm) was decreases from start of experiment then remain at this level with minor up and down in mean concentration of zinc (0.01 ppm). In group D, the initial mean concentration of zinc (0.52 ppm) was initially increases then decreases then again increases the mean concentration of zinc (0.36 ppm) and so on. (Table 11, 12, 13 & 14; Graph 14, 15, 16 & 17)

Table 11: Zinc concentration in Cu deficient diet fed animals (Group A).

Intervals	Zn Mean Concentration
0 days	1.02 ppm
57 days	0.42 ppm
72 days	0.45 ppm
87 days	0.49 ppm
102 days	0.43 ppm
132 days	0.51 ppm
146 days	0.39 ppm
161 days	0.36 ppm
176 days	0.34 ppm

Graph 14: Effect on zinc concentration in copper deficient diet fed group (A) of goats.

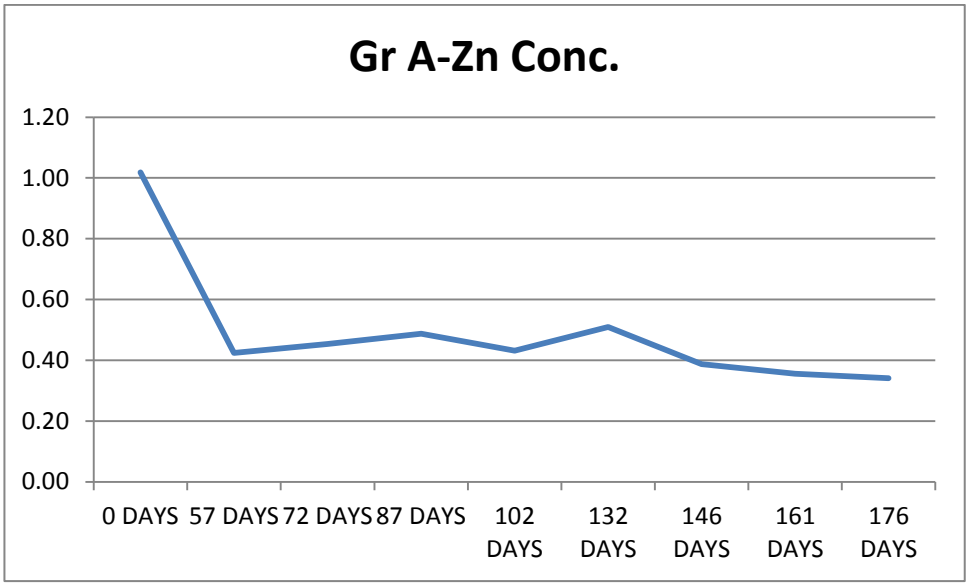


Table 12: Zinc concentration in Zn deficient diet fed animals (Group B).

Intervals	Zn Mean Concentration
0 days	0.69 ppm
57 days	0.62 ppm
72 days	0.44 ppm
87 days	0.36 ppm
102 days	0.44 ppm
132 days	0.24 ppm
146 days	0.33 ppm
161 days	0.35 ppm
176 days	0.34 ppm

Graph 15: Effect on zinc concentration in zinc deficient diet fed group (B) of goats.

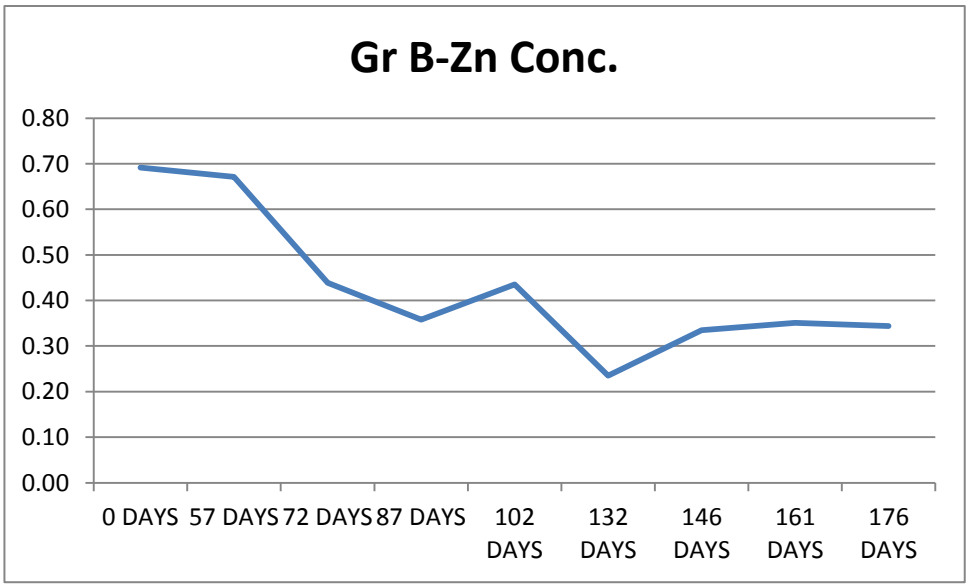


Table 13: Zinc concentration in Cu and Zn deficient diet fed animals (Group C).

Intervals	Zn Mean Concentration
0 days	0.63 ppm
57 days	0.58 ppm
72 days	0.42 ppm
87 days	0.40 ppm
102 days	0.44 ppm
132 days	0.46 ppm
146 days	0.37 ppm
161 days	0.22 ppm
176 days	0.01 ppm

Graph 16: Effect on zinc concentration in copper and zinc deficient diet fed group (C) of goats.

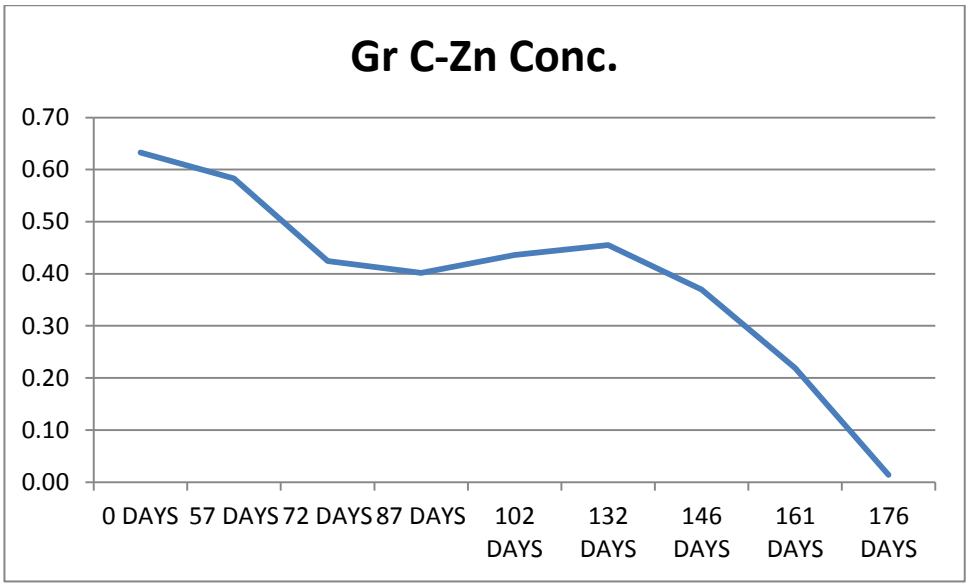
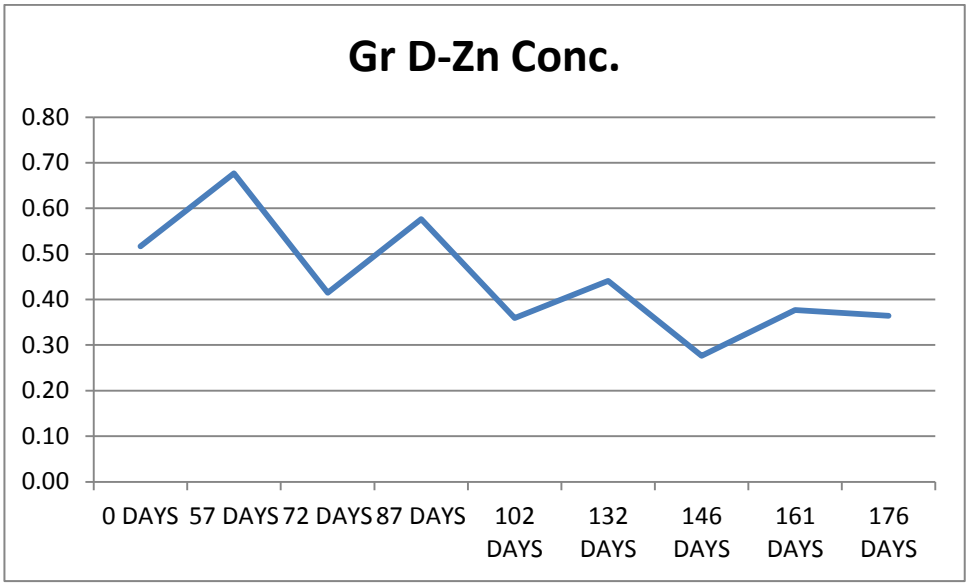


Table 14: Zinc concentration in Control group animals (Group D).

Intervals	Zn Mean Concentration
0 days	0.52 ppm
57 days	0.68 ppm
72 days	0.41 ppm
87 days	0.58 ppm
102 days	0.36 ppm
132 days	0.44 ppm
146 days	0.28 ppm
161 days	0.38 ppm
176 days	0.36 ppm

Graph 17: Effect on zinc concentration in standard diet fed group (D) of goats.



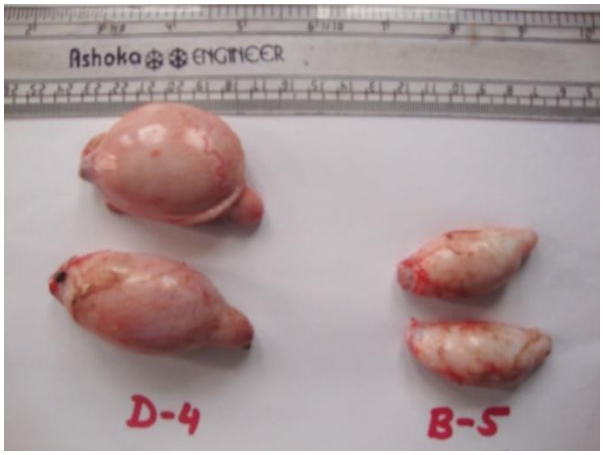
Clinical features:

Clinically, the animals started showing signs, especially of Cu-deficiency in the A group, from 60 days onwards with progressive roughness of hair coat and increasing tendency of coarseness of hairs till 165 days and then remained steadily consistent through 255 days and 270 days of experimentation as depicted in the figures below. Group B Zn-deficient animals; however, showed progressively and increasingly rough thickened dermal patches and scaly skin over the muzzle eye and other hairless areas. The animals started showing clear skin parakeratotic like lesions from 255 days onwards. The skin appeared hypertrophied, thickened in patches, alopecic, scaly and scabby with frequent desquamation and haemorrhages/ bleeding due to trauma during feeding etc. Group-C animals fed Cu & Zn combined deficient diet also showed tendency of rough hair coat at 180 days and also apparently roughened, thickened skin with increasing time, specifically by 255 and 270 days, however, the degree of changes were less intense compared to the group-A & B animals. Control (group-D) animals did not show any discernible changes in their skin hair coat.

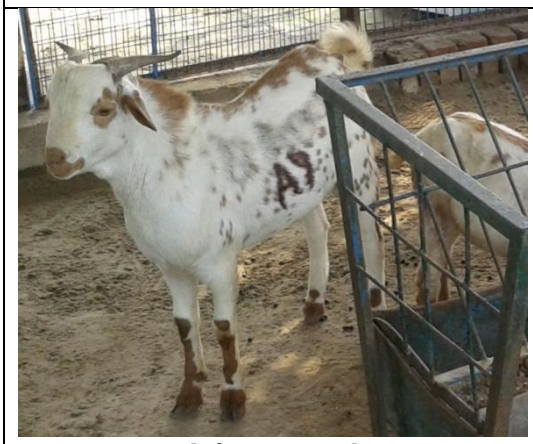
Pathology:

Gross lesions were not observed in an animal from Cu-deficient group-A which died at about 90 days of experimentation. However, the animal from Zn-deficient group-B died at about 105 days showed significantly atrophic testes in comparison to the control animal of group-D that was also died on the same day. The copper deficient diet fed goats (A) showed weak and aneamic with rough hair coat in most of the animals. The zinc deficient goats (B) showed hyperkeratinization at muzzle with scaling around mouth and loss of hairs around mouth. The gross lesions were more severe in combined deficient group (C) as compared to control group (D).The overall size and weight of both the testes of Zn-deficient animal was significantly decreased in comparison to the control animal as shown in the figure below. The size variables of testes in Zn-deficient (B-5) and Control (D-4) were as follow:

	B-5 (Zn-deficient)		D-4 (Control)	
	Weight (g)	**Length (cm)**	**Weight (g)**	**Length (cm)**
Right testicle	9.19	5.00	26.27	6.50
Left testicle	9.39	5.50	27.74	7.20

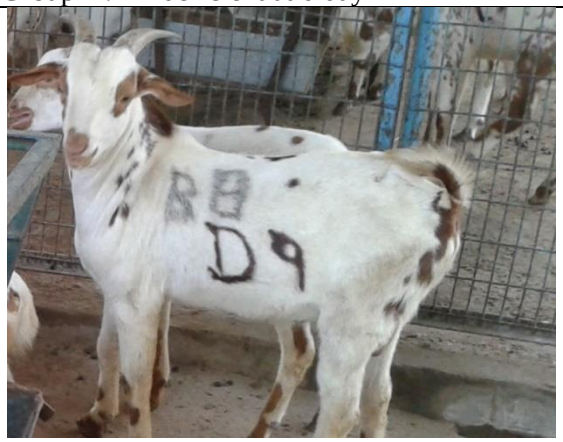


Gross photograph showing size difference in Zn-deficient (B-5) and control (D-4) group animals.

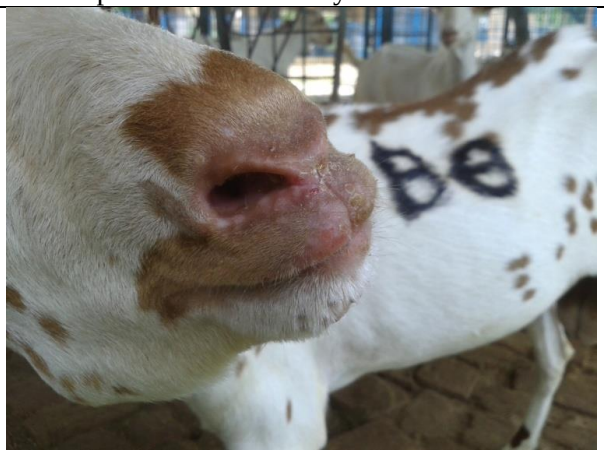

Group-A: Cu-deficient at 0 day


Group-B: Zn-deficient at 0 day


Group-C: Cu&Zn-deficient at 0 day

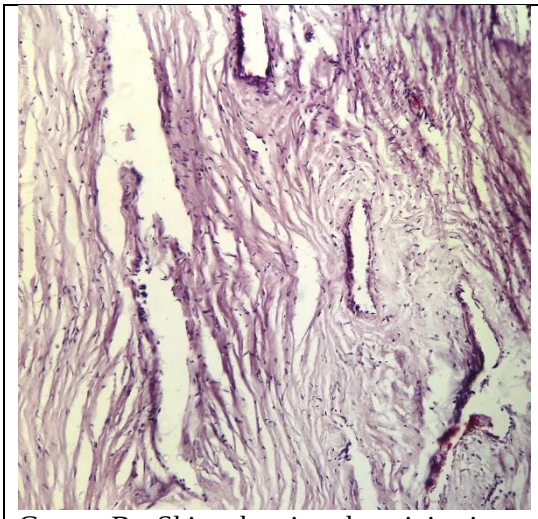
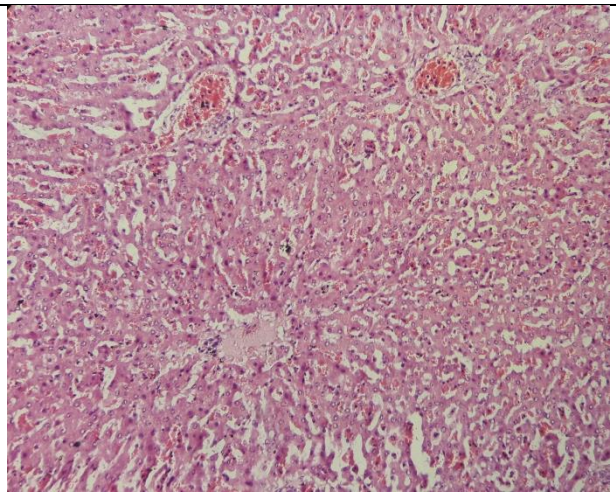

Group-D: Control at 0 day

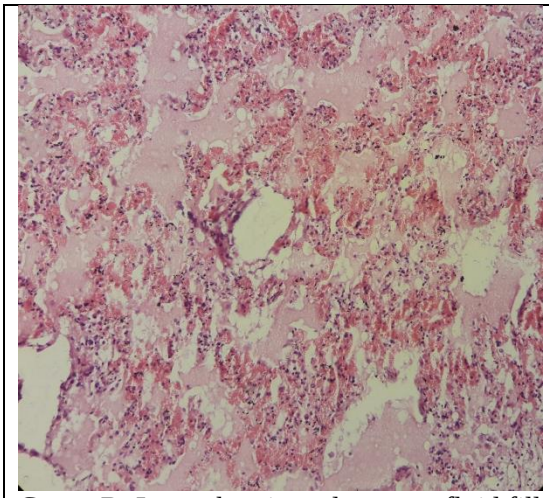

Group-A: Cu-deficient at 90 days showing rough


Group-B: Zn-deficient at 270 days. Note thickened

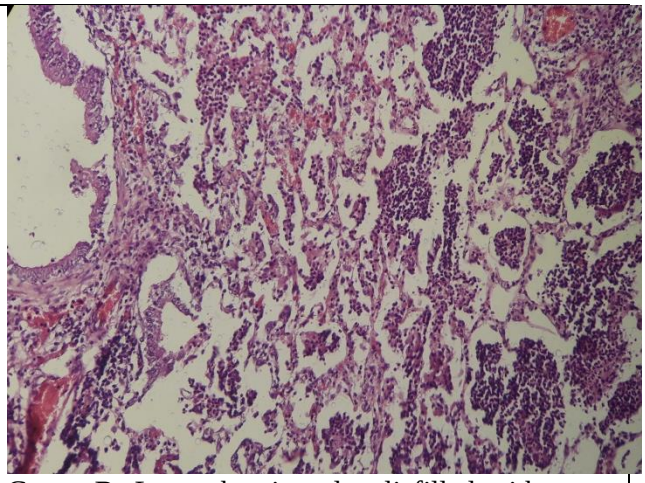
hair coat	scabby and scaly hyperaemic lesions in the skin around muzzle.
 Group-C: Cu & Zn-deficient at 270 days showing relatively rough and thickened skin of muzzle.	 Group-D: Control animals at 270 days showing normal smooth hair coat and skin.

Microscopic Study: Copper deficient and zinc deficient animals died during experimentation showed different histopathological changes as hyperkeratosis of skin epithelium, spleen showed depletion of lymphoid follicles in the germinal centers, lung showed edema fluid filled in the alveolar sacs with congestion of blood vessels in alveolar walls in zinc deficiency group. In copper deficiency showed severe congestion of central vein and sinusoids with degenerations of hepatocytes. These changes were same in combined (Cu and Zn group C) but more severe in nature. Figure as below-

 Group B: Skin showing keratinization of skin epithelium in Zn deficiency group. (H&Ex200)	 Group A: Liver showing severe congestion of sinusoids and central vein with hepatocytes degenerations. (H&Ex200)



Group B: Lung showing edematous fluid filled in Alveoli with severe congestion. (H&Ex200)



Group B: Lung showing alveoli filled with acute inflammatory cells with congestion of alveolar blood vessels. (H&Ex200)

Gene Expression profiling using Agilent Platform:

- RNA was isolated from blood and tissues for microarray analysis.
- **RNA Quality Control:** The RNA extraction from Goat Lung, Liver& uterus was performed using Trizol (Catalog: 15596018) method (Table 14) followed by DNAse treatment using Qiagen RNeasy Mini Kit (Catalog: 74106). The RNA integrity of the extracted RNA were analysed on the Bioanalyzer (Agilent; 2100 expert) (Grapah 18 & 19).
- **Labeling and microarray hybridization:** The samples for Gene expression were labeled using Agilent Quick-Amp labeling Kit (p/n5190-0442) and converted to double stranded cDNA. cRNA was generated by and the dye Cy3 CTP(Agilent) was incorporated.
- **Hybridization and scanning:** Labeled cRNA sample were hybridized on to a Genotypic designed Goat gene expression Microarray 8x60K arrays. Hybridization was carried out in Agilent's Surehyb Chambers at 65º C.
- **Feature Extraction:** Data extraction from Images was done using Feature Extraction software Version 11.5 of Agilent.
- **Microarray Data Analysis:** Images were quantified using Feature Extraction Software (Version-11.5 Agilent). Feature extracted raw data was analyzed using GeneSpring GX software from Agilent. Significant genes up regulated fold> 0.6 (logbase2) and down regulated <-0.6 (logbase2) in the test samples with respect to control sample were identified. Statistical student T-test p-value among the replicates was calculated based on volcano plot algorithm. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns. Pathway analysis for the differentially regulated genes was performed using Genotypic Biointerpreter-Biological Analysis Software. Genes were classified based on functional category and pathways using Biological Analysis tool DAVID (http://david.abcc.ncifcrf.gov/).

- Results showed 3855 genes were upreguluaed and 3819 genes were down regulated in the Zn-deficient group animals when compared to the control group animals (Grapah 20). Significantly expressed/ downregulated genes and the molecular pathways of their involvements are given in Table 15 & 16.

Experiment Details

Slide Barcode	Samples hybridized	Analysis Plan
256481310002_1_1	D9_TR1	Control
256481310002_1_2	D9_TR2	
256481310002_1_3	Liver_B8_377th day_TR1	Treatment
256481310002_1_4	Liver_B8_377th day_TR2	

Summary of Differentially Regulated Probes

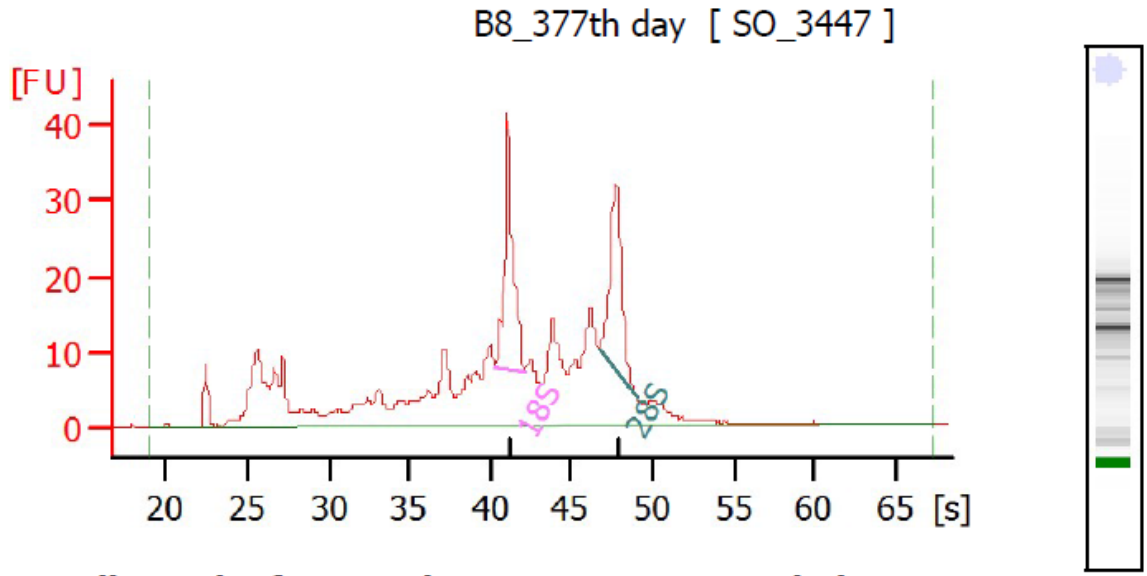
Samples hybridized	Up	Down
Liver_B8_377th day_TR1	3855	3819
Liver_B8_377th day_TR2		

Cut off used to filter up and down regulated genes

Upregulated **For filtering upregulation we consider Fold >=0.8 in the individual test samples & fold >=1 in the geomeanfold of the test sample.**
Downregulated **For filtering downregulation we consider Fold <=-0.8 in the individual test samples & fold <=-1 in the geomeanfold of the test sample.**

Expression fold values are provided in terms of log base 2 (Formating of color)

Graph 18. Electropherogram Summary of Bioanalyser profile of RNA extracted from Zn-deficient B group animal.



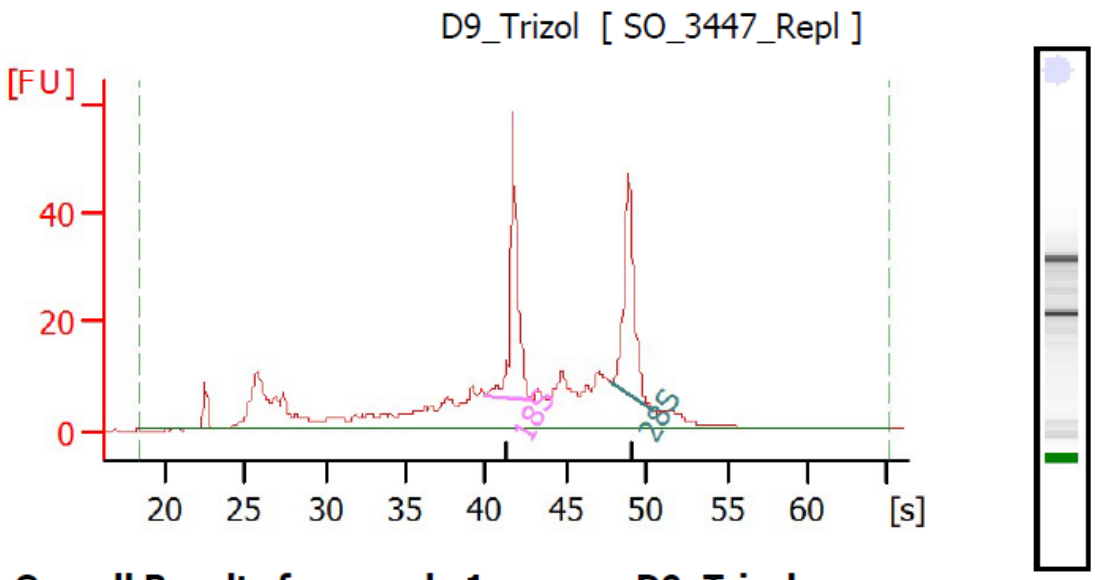
Overall Results for sample 2 : B8_377th day

RNA Area:	493.2
RNA Concentration:	499 ng/µl
rRNA Ratio [28s / 18s]:	0.9
RNA Integrity Number (RIN):	6.6 (B.02.08)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.60

Fragment table for sample 2 : B8_377th day

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.31	42.12	51.7	10.5
28S	46.66	48.95	44.2	9.0

Graph 19. Electropherogram Summary of Bioanalyser profile of RNA extracted from control D group animal.



Overall Results for sample 1 : D9_Trizol

RNA Area:	495.8
RNA Concentration:	526 ng/µl
rRNA Ratio [28s / 18s]:	1.0
RNA Integrity Number (RIN):	7.3 (B.02.08)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.30

Fragment table for sample 1 : D9_Trizol

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.99	42.83	61.9	12.5
28S	47.79	50.39	62.5	12.6

Table 14. RNA Concentration and Purity of samples estimated using Nanodrop Spectrophotometer.

RNA Concentration and Purity of samples estimated using Nanodrop Spectrophotometer:

Sl. No.	Sample Name	Absorbance value 260/280	Absorbance value 260/230	RNA Concentration ng/µl	Total yield in ng	QC Purity	QC concentration/yield	RNA Integrity Number	QC Integrity
	Goat Lung tissue								
1	SO_3447_Repl_LN-1_Trizol	1.84	2.47	2337.11	35056.65	Optimal	Optimal	3.6	Partially Degraded
2	SO_3447_Repl_LN-1_Trizol	1.87	2.36	2492.82	37392.3	Optimal	Optimal	3.1	Partially Degraded
3	SO_3447_Repl_LP1_Trizol	1.94	2.43	1092.82	16392.3	Optimal	Optimal	4.2	Admissible
4	SO_3447_Repl_LN-1_Trizol	1.93	2.31	2404.81	36072.15	Optimal	Optimal	4.5	Admissible
5	SO_3447_Repl_LP1_Isol	1.93	2.24	1310.22	19653.3	Optimal	Optimal	5	Partially Degraded
6	SO_3447_Repl_LP2_Isol	1.88	2.27	2516.56	37748.4	Optimal	Optimal	3	Degraded
	Goat Copper dificient, Copper+Zinc dificient & Normal								
1	SO_3447_Repl_D9_Trizol	1.92	2.3	2554.46	38316.9	Optimal	Optimal	7.3	Optimal
2	SO_3447_Repl_A2_Trizol	1.9	2.34	2323.09	34846.35	Optimal	Optimal	2	Degraded
3	SO_3447_Repl_C3_Trizol	1.01	1.77	3563.23	53448.45	Sub Optimal	Optimal	2.5	Degraded
4	SO_3447_Repl_A2_Trizol	1.91	2.24	3212.39	48185.85	Optimal	Optimal	2.1	Degraded
5	SO_3447_Repl_A2_Qiazol	1.95	2.36	2914.74	43721.1	Optimal	Optimal	2.6	Degraded
	Goat Uterus								
1	SO_3447_Repl_UN2_Trizol	1.9	2.1	1731.88	25978.2	Optimal	Optimal	4.3	Admissible
2	SO_3447_Repl_UE1_Trizol	1.88	2.42	961.74	14426.1	Optimal	Optimal	4	Admissible
3	SO_3447_Repl_UE1_Trizol	1.89	2.28	1914.85	28722.75	Optimal	Optimal	3.5	Partially Degraded
4	SO_3447_Repl_UE2_Qiazol	1.96	2.29	2743.05	41145.75	Optimal	Optimal	4.2	Partially Degraded

Bioanalyzer Profiles of RNA: Please refer to the accompanying file "Bioanalyzer profile_SO_3447.pdf" for Bioanalyzer profiles of the RNA samples.

Comments : The above highlighted samples are suitable for Microarray studies

OPTIMAL: Optimal purity (OD260/280 >1.7 and <2.2; OD260/230>0.5 and <2.5);

Sub-Optimal: Sub-Optimal purity (OD260/280<1.7 and OD 260/230<0.5 and >2.5);

Optimal: optimal concentration (>30 ng/microlitre and <2500 ng/microlitre);

Sub-Optimal: Sub-optimal concentration (<30ng/microlitre);

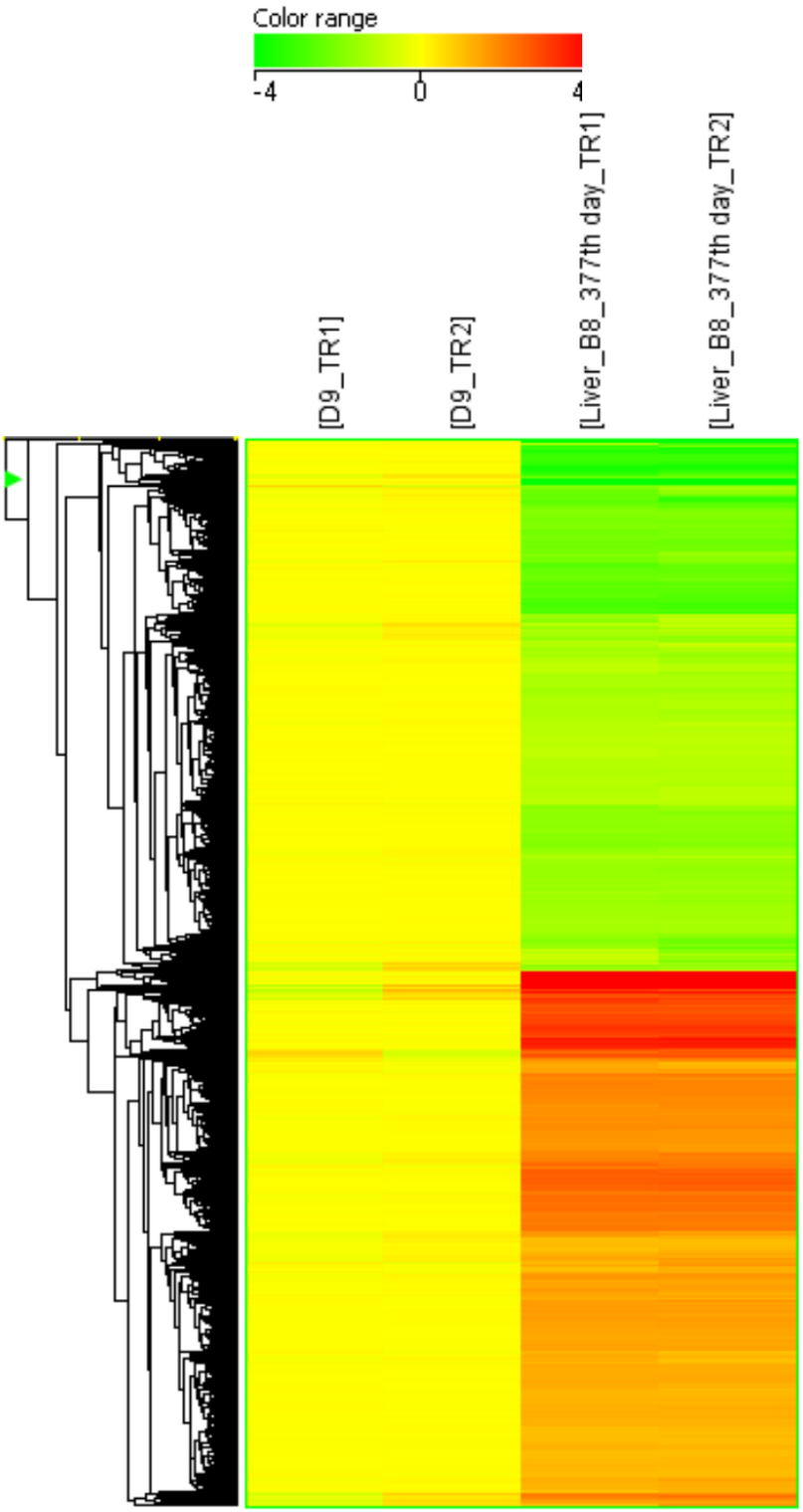
Note: Sub-optimal purity could lead to failed labeling reaction and sub-optimal concentration can lead to sub-optimal yields of labeled samples.

Table 15. Significantly upregulated genes and pathways of their involvement.

S. No.	Molecular Pathway	Genes
1.	P00031:Inflammation mediated by chemokine and cytokine signaling pathway	IFNAR2, PTGS2
2.	P00005:Angiogenesis	PDGFB
3.	P00047:PDGF signaling pathway	PDGFB
4.	P00053:T cell activation	CD247
5.	P00033:Insulin/IGF pathway-protein kinase B signaling cascade	IGF2
6.	P00019:Endothelin signaling pathway	PTGS2
7.	P00032:Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	IGF2
8.	P00026:Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	ADORA3
9.	P00011:Blood coagulation	SERPINC1
10.	P00027:Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	ADORA3
11.	P02776:Serine glycine biosynthesis	SHMT1
12	P00029:Huntington disease	CAPN3
13.	P00036:Interleukin signaling pathway	IL2RA
14.	P00051:TCA cycle	IDH1
15.	P00054:Toll receptor signaling pathway	PTGS2

Table 16. Significantly downregulated genes and pathways of their involvement.

S. No.	Molecular Pathway	Genes
1.	P00046:Oxidative stress response	TXN, MYC
2.	P00034:Integrin signalling pathway	CAV1, ASPM
3.	P05911:Angiotensin II-stimulated signaling through G proteins and beta-arrestin	AGTR1
4.	P04398:p53 pathway feedback loops 2	MYC
5.	P05917:Opioid proopiomelanocortin pathway	POMC
6.	P04380:Cortocotropin releasing factor receptor signaling pathway	POMC
7.	P00005:Angiogenesis	CRYAB
8.	P00030:Hypoxia response via HIF activation	TXN
9.	P00006:Apoptosis signaling pathway	BCL2L1
10.	P00031:Inflammation mediated by chemokine and cytokine signaling pathway	CCR3
11.	P00047:PDGF signaling pathway	MYC
12	P00036:Interleukin signaling pathway	MYC
13.	P00026:Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	PYGL
14.	P00057:Wnt signaling pathway	MYC



Graph 18. Overview cluster of differentially expressed genes in Zn-deficient and control group animals. Red colour indicates upregulated genes; green colour indicates downregulated genes; yellow colour indicates control. Data analysis was done using GeneSpring GX version 12.6 and Microsoft Excel. Fold cut-off used: Up Regulation: Fold value >=1 Down Regulation: Fold value -<=-1.

CONCLUSIONS

The effects of Cu and Zn deficiency diets in goats causes decrease in body weight, blood parameters and concentration of copper and zinc in plasma/serum in goats. The gross as well as microscopic lesion in copper deficient group (A), zinc deficient group (B) and combined Cu and Zn deficient group (C) also showed changes in different organs as well as at skin as compared with control animals. The zinc deficient group also showed testicular atrophy. Due to this the overall effects of minerals deficiency cause growth as well as reproductive and health problems. Because of theses both minerals require for many metabolic enzyme activity as well as cell functions. Gene expression studies showed that 3855 genes were upregulated and 3819 genes were downregulated in the Zn-deficient group animals when compared to the control group. It gave identification of pathways and candidate genes responsible for deficiency disease development and progression to overt sign.

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11. Financial Implications (`in Lakhs)

11.1 Expenditure on

(a) Manpower

(b) Research/Recurring Contingencies

(c) Non-Recurring Cost (Including cost of equipment)

(d) Any Other Expenditure Incurred

11.2 Total Expenditure

12. Cumulative Output

a. Special attainments/innovations:
b. List of Publications (one copy each to be submitted if not already submitted)
   i. Research papers
   ii. Reports/Manuals
   iii. Working and Concept Papers-

RVS Pawaiya, UB Chaudhary, Nitika Sharma and N. Shivasharanappa. 2014. Nutrigenomics – exploiting the system biology for goat health. Lead paper. In: Compendium of national seminar on 'Sheep and goat biodiversity and breeding policies: Issues and prospective' held at Shirwal, Maharashtra during 21-22 February, 2014. LP-1-6. pp. 130-139.

   iv. Popular articles
   v. Books/Book Chapters
   vi. Extension Bulletins
c. Intellectual Property Generation
   (Patents - filed/obtained; Copyrights- filed/obtained; Designs- filed/obtained; Registration details of variety/germplasm/accession if any)
d. Presentation in Workshop/Seminars/Symposia/Conferences-

RVS Pawaiya, UB Chaudhary, Nitika Sharma and N. Shivasharanappa. 2014. Nutrigenomics: a system biology tool for animal health. Presented in the 2nd International Conference on Animal & Dairy Sciences held from 15-17 September, 2014 at Hyderabad.

   (relevant to the project in which scientists have participated)
e. Details of technology developed
   (Crop-based; Animal-based, including vaccines; Biological – biofertilizer, biopesticide, etc; IT based – database, software; Any other – please specify)
f. Trainings/demonstrations organized
   Nil
g. Training received- Nil
h. Any other relevant information

13. (a) Extent of achievement of objectives and outputs earmarked as per RPP-I

Objective wise	Activity	Scientist(s) responsible	% of activity envisaged to be completed as per RPP-I	% achieved as targeted
1. Analysis of gene expression profiling of nutrient-deficient (Zn, Cu and Zn&Cu combined -deficient) goats.	Procurement of consumables/ biochemical/ labwares/ equipment etc., planning and designing of experimentation, animal allocation for experiment	RVS Pawaiya & UB Chaudhary	20	70
	Housing, feeding and managemental care of expl animals; Formulation and preparation of Zn, Cu and Zn&Cu combined -deficient and –sufficient diets and balanced	UB Chaudhary, Nitika Sharma & N Shivasharnappa	20	100

	diets for various animal groups,			
	Examination of animals for the development of any deficiency lesions/ clinical signs, collection of biosamples	RVS Pawaiya & UB Chaudhary & Nitika Sharma & N Shivasharnappa	20	100
	Processing of the biosamples and to undertake the molecular biological and genomic studies and microarray studies.	RVS Pawaiya & UB Chaudhary Nitika Sharma & N Shivasharnappa	20	70
	Tissue Cu & Zn analysis	RVS Pawaiya & UB Chaudhary & N Shivasharnappa	20	50
	Analysis of gene expression profiling of nutrient-deficient (Cu, Zn and Cu&Zn deficient) goats	RVS Pawaiya & UB Chaudhary	20	0
2. Identification and characterization of genes specifically affected (up-regulation and down-regulation) by nutritional deficiency in goats.	DNA isolation, purification, and Gene sequencing	RVS Pawaiya & N Shivasharnappa	20	20
	Outsourcing services for gene sequencing	RVS Pawaiya & N Shivasharnappa & UB Chaudhary	20	0
	Gene sequence alignment using specific software	RVS Pawaiya & N Shivasharnappa	20	0
	Compilation and documentation of results	RVS Pawaiya & UB Chaudhary & Nitika Sharma & N Shivasharnappa	20	0
3. Determining whether corrective measures to nutrient deficiency can restore the gene expression pattern comparable to healthy and control goats.	Comparison of gene expression profiles of nutrient deficient goats and recovered goats	RVS Pawaiya & N Shivasharnappa & UB Chaudhary	20	0
	Comparison of gene expression profiles of nutrient deficient goats and healthy control goats	RVS Pawaiya & N Shivasharnappa & UB Chaudhary	20	0
	Compilation and documentation of results	RVS Pawaiya & N Shivasharnappa & UB Chaudhary	20	0

(b) Reasons of shortfall, if any

The microarray analysis could be done of limited samples only due to funds crunch. The project is slated to be closed by 31st March, 2015 as per the directions form the Chairman, IRC and Director of the Institute. However, the substantial data of the research experimentation remains to be analysed, specifically differential gene expression profile of goats in different experimental groups, due to the want of funds.

14. Efforts made for commercialization/technology transfer

15. (a) How the output is proposed to be utilized?

To keep animals healthy and productive in their life span.

(b) How it will help in knowledge creation-

It will help in to assess the impact of minerals deficiency in goats.

16. Expected benefits and economic impact (if any)-

It will help in diagnosis of Cu and Zn deficiency in goats in terms of health and growth of animals.

17. Future line of research work/other identifiable problems-

18. Details on the research data (registers and records) generated out of the project deposited with the institute for future use- Yes

19. Signature of PI, CC-PI(s), all Co-PIs

(Dr RVS Pawaiya, PI)

18/12/17

18/12/17

(Dr UB Chaudhary, Co-PI) (Dr Nitika Sharma, Co-PI) (Dr N Shivasharanappa, Co-PI)

Transferred

18/12/2017

20. Signature of Head of Division

21. Observations of PME Cell based on Evaluation of Research Project after Completion

22. Signature (with comments if any along with rating of the project in the scale of 1 to 10 on the overall quality of the work) of JD (R)/ Director

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