ISSN: 2456-2912
VET 2024; 9(3): 100-104
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www.veterinarypaper.com
Received: 01-02-2024
Accepted: 07-03-2024

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Effect of inorganic and nano forms of copper and zinc supplementation on hematological parameters of GIR Calves

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Abstract
The present study was conducted to evaluate the effects of inorganic and nano forms of copper and zinc supplementation on growth performance and health status of Gir calves. Twenty-five Gir calves (1 month old) were distributed into five homogenous groups on the basis of their live body weight and sex in completely randomized design (CRD). The control (T_1) group was offered a basal diet consisting of concentrate, sorghum green and dry pasture grass without any additional supplementation as per ICAR (2013) feeding, while T_2, T_3, T_3 and T_4 groups were fed basal diet with inorganic Cu @ 20 mg/kg DM + inorganic Zn @ 40 mg/kg DM, inorganic Cu @ 20 mg/kg DM + nano Zn @ 20 mg/kg DM, nano Cu @ 10 mg/kg DM + inorganic Zn @ 40 mg/kg DM and @ 10 mg/kg DM + nano Zn @ 20 mg/kg DM, respectively, for a period of 150 days. Blood haematological parameters, Hb (g/dL) concentration, RBC count (10^6/µL), WBC count (10^3/µL), Blood platelet count (10^3/µL) and PCV (%) did not revealed ($p>0.05$) difference among the groups and found within the normal physiological range throughout the experimental period. Overall mean value of IJF- 1(ng/mL) was significantly higher T_5, T_4 and T_3 groups as compared to T_1 and T_2 groups. However, the serum copper concentrations (mg/L) was significantly ($p<0.05$) higher in T_4 and T_5 groups as compared to T_1, T_2 and T_3, while the zinc concentrations (mg/L) was significantly ($p<0.05$) higher in T_3 and T_5 groups compared with T_1 T_2 and T_4 groups. Thus, it can be inferred that nano zinc @ 20 mg/kg DM and nano copper @ 10 mg/kg DM should be added in the diets of growing Gir calves to maximize overall performance and thus to increase overall profit for well-being of farmers.

Keywords: Gir calves, economics, nano particles, immunity

Introduction
The potential for attaining optimum body weight of well grown dairy calves is an important factor that affects the economy and future success of a dairy farm. The replacement heifers and bulls are crucial for the profitability of dairy farms. Therefore, the success of dairy enterprises depends to a great extent on the proper rearing, care and management of the calves. Amongst all the measures nutrition play a very important role in calf rearing. A ration balanced in all nutrients i.e. energy, protein, minerals and vitamins is essential for growth, production and reproduction. Amongst the minerals both macro and micro minerals are needed for growth and development as they are essential components of many enzymes, vitamins, hormone, respiratory pigments, etc. and also act as cofactors in metabolism, catalysts, enzyme activators, important for immunity and reproduction. The most obvious functions of minerals are as components of body organs, tissues and to provide structural support. Trace minerals are the minerals, which are required in minute quantities (usually in microgram or milligram/day) and play a vital role in animal nutrition and are important for growth, immunity, reproduction and milk production in livestock. Nine trace minerals are known to be essential viz., Zinc (Zn), Copper (Cu), Chromium (Cr), Cobalt (Co), Iodine (I), Iron (Fe), Manganese (Mn), Molybdenum (Mo), Selenium (Se). Minerals, especially Cu and Zn are critical for young calves and as the calves get closer to weaning, milk and forage don't provide all of their nutritional needs.

In India, livestock do not get mineral supplementation, except for common salt. They depend upon forages for their mineral requirements. Soil concentration of minerals keeps changing due to many factors like crop yield, fertilizer application, rain and natural calamities. As a result, the mineral contents in feed and fodder may be altered which affects the mineral status of animals (Underwood and Suttle, 1999) [20]. Rarely forages can completely satisfy all the mineral requirements. The mineral requirements of animals are not being met by the forages which are grown on mineral deficient soils. Hence, supplementation of critical nutrients to the basal diet is necessary. The severity of the mineral deficiency in farm animals depends upon the type of diet, age, physiological status of the animals and the agro-climatic conditions of the region. Mineral deficiencies lead to diseases resulting in economic loss to the farmer. The performance of growing animals is severely affected even upon a minute deficiency of trace minerals. Therefore, getting the correct amount of trace minerals in the right ratios is the key to get optimum health and production. A number of trace elements have been shown to be important for adequate functioning of the immune system, among which Cu and Zn play a major role.

Copper is an essential trace element of animals. It can effectively maintain the stability of the internal environment and is closely related with hematopoiesis, metabolism, growth, reproduction and other important life activities (Ognik *et al.*, 2016) [10]. Copper is part of the active sites of many enzymes including superoxide dismutase, ceruloplasmin, cytochrome oxidase, L-lysine oxidase, ascorbate oxidase, tyrosinase and dopamine beta-hydroxylase (Ognik *et al.*, 2016) [10].

Zn is essential for the body's proper physiological functions like growth, reproduction and health, DNA synthesis, gene expression and cell division, photochemical processes of vision, ossification, wound healing, augmenting the immune system of the body, through energy production, protein synthesis, protection of membranes from bacterial endotoxins and lymphocyte replication and antibody production (Zhao *et al.*, 2014) [19].

The bioavailability of minerals from their inorganic sources is quite low due to minerals being inert and having more interactions with other minerals, so absorption of other minerals is decreased. As a result, required minerals are needed to add 20-30 times more than an animal's normal requirement. Among all the probable approaches, use of nanotechnology to produce nano sized minerals is a potential alternative to both organic and inorganic mineral sources. Nanotechnology is defined as the technology of materials and structures where size is measured in nanometers. Those minerals having a particle size of 1-100 nm are known as nano minerals. They are stable under high pressure and temperature.

Nano Cu has benefits over inorganic copper sulphate including improved efficacy, reduced doses with improved results, no interference with other ingredients and less environmental excretion (Ognik *et al.*, 2016) [10]. Nano Cu has beneficial effects on the animal performance and could be used to replace copper sulphate.

Nano zinc improves feed efficiency and growth in sheep. It also improves the absorption capacity of intestines and stomach of livestock and poultry as well as absorption rate and speed, which reduce the ratio of feed to meat and feed to egg, thus reducing the cost of production. The use of nano zinc gives better results as compared to conventional Zn sources and it is less toxic (Sahoo *et al.*, 2014) [14]. Until date, not sufficient information is available on the efficacy and suitability of nanoparticles forms of minerals including that of Zn in the diet of animals.

Even though the basic information regarding the effects of nano Cu and Zn has been shown in ruminants, confirmation of the effect of nano Cu and Zn supplementation on metabolism of nutrients, enzymatic activity, antioxidant and immune status in dairy animals is still lacking. As this nanotechnology is in infancy in this field of mineral nutrition, systemic studies have to be carried out to understand the effects of nano minerals on the biological effects in the animal body. Therefore, keeping in view the above facts, the present study was therefore designed to investigate the evaluation of nano copper and nano zinc supplementation on hematology of Gir calves.

Materials and Methods

Location of the Experimental Station

The present research experiment was conducted on Gir calves of Cattle Breeding Farm, Kamdhenu University, Junagadh, Gujarat and their geographical location is on 21.29° N latitude and 70.27 ° E longitudes, at an average elevation of 107 meters (351 ft.) from the mean sea level. Present research experiment was approved with protocol No. KU-JVC-IAEC-LA-97-22, dated 08/07/2022 along with different procedures involved and total number of experimental animals.

Procurement of inorganic and nano form of copper and zinc

The source of inorganic cupric oxide, inorganic zinc oxide, nano cupric oxide and nano zinc oxide (Table 1) were procured from Sisco Research Laboratories Pvt. Ltd., (SRL) Mumbai, India.

Table 1: Quality specifications of inorganic and nano form of copper and zinc

Cupric Oxide (Copper (II) Oxide)	
Specifications	Purity - 79.00% minimum Molecular weight - 79.55 g/mol Product code – 51120 CAS No - 1317-38-0
Zinc Oxide extrapure AR	
Specifications	Purity - 99.50% minimum Molecular weight - 81.38 g/mol Product code – 67062 CAS No - 1314-13-2
Cupric Oxide (Copper (II) Oxide) nanopowder	
Specifications	Purity - 79.00% minimum Molecular weight - 79.55 g/mol Product code – 28954 CAS No - 1317-38-0 Average particle size - 40 nano meter
Zinc Oxide nanopowder (Type I)	
Specifications	Purity - 99.90% minimum Molecular weight - 81.38 g/mol Product code – 91148 CAS No - 1314-13-2 Average particle size - 30 nano meter

Experimental animals and diet

A total twenty-five healthy Gir calves around one-month age were randomly selected from cattle herd maintained at Cattle Breeding Farm, Kamdhenu University, Junagadh (Plate 1). Selected calves were randomly divided into five treatment groups with five calves in each group considering their body weight and sex in such a way that means average initial body weight under all the treatment groups remained non-significant. Since all the experimental animals were not available at a time, they were introduced in the experiment phase wise and the duration of experiment was 150 days. Experimental calves were randomly divided into five treatments group with five calves in each using completely randomized design (CRD). The control (T_1) group was offered a basal diet consisting of concentrate, sorghum green and dry pasture grass without any additional supplementation as per ICAR (2013) [6] feeding, while T_2, T_3, T_3 and T_4 groups were fed basal diet with inorganic Cu @ 20 mg/kg DM + inorganic Zn @ 40 mg/kg DM, inorganic Cu @ 20 mg/kg DM + nano Zn @ 20 mg/kg DM, nano Cu @ 10 mg/kg DM + inorganic Zn @ 40 mg/kg DM and @ 10 mg/kg DM + nano Zn @ 20 mg/kg DM, respectively, for a period of 150 days.

The nutrient requirements of experimental Gir calves in term of DCP and TDN were met by feeding of basal diets as a mixture of compound cattle feed, sorghum green fodder and mature pasture grass hay as per ICAR (2013) [6] feeding standards. All the feed ingredients were analyzed for proximate composition as per the methods described by AOAC (2023) [3] before the formulation of diet.

To ensure that each animal consumed the calculated amount of inorganic and nano form of copper and zinc was premixed with fine grinded maize flour as per the requirements in different groups. Freshly prepared 10 grams ball made with jaggery solution from prepared mixture was offered prior to providing the ration to calves in treatment groups. Freshly prepared simple mixture of fine grinded maize flour with jaggery solution without adding inorganic and nano form of copper and zinc was offered prior to providing the ration to calves in control group. Calculated amount of compound cattle feed was offered at 9 am.

Sorghum green fodder was offered maximum up to 2 kg at 11 am after chaffing, while mature pasture grass hay in the chaffed form was offered @ *ad lib* daily at 6 pm. All experimental calves were allowed to suckle their own dam up to 3 months of age with restricted time only for 1.5-2.0 minute at both the time of milking. Leftover of feeds and fodders for individual calf was recorded daily in the next morning at 8 A.M. Samples of compound cattle feed, sorghum green fodder and mature pasture grass hay were collected fortnightly and analyzed for proximate principles. Ration schedules were adjusted every fortnight according to changes in live body weight of each animal. The animals were provided with fresh and clean water free of choice thrice daily at 07.00, 14.00 and 18.00 hours.

Standard managemental practices

Deworming of all experimental animals with broad spectrum anthelmintic albendazole was carried out before start of experiment and regularly carried out as per schedule during entire experimental duration of 150 days. All the experimental animals were housed in a well-ventilated shed having tying arrangement for individual feeding and watering without having access to the other animal's diet. They were kept tied all the time and were let loose for two hours (7 to 9 am) in the morning for exercise (except during the period of digestion trial) in an open covered area (Plate 2). Each animal was given individual care.

Blood collection

At the beginning (0 day) and last day of experiment (150 day), approximately 5 ml whole blood was collected from jugular vein with all aseptic precautions from each calf from all the treatment groups in Na-EDTA vacutainer for hematological parameters. For separation of serum, 10 ml blood was collected in a tube without anticoagulant and kept in slating position. These tubes were incubated for 1 hour at 37 ºC. Blood clots were broken and tubes were centrifuged at 2800 rpm for 20 minutes. Serum was pipetted out and stored in properly sterilized 2 ml plastic storage vials at – 20 °C after adding a drop of methylate in it and stored in deep freezer until analysis.

Health and sanitation

The experimental shed area was thoroughly cleaned daily in the morning. Hygienic conditions were maintained during entire experimental period to prevent any incidence of infectious and contagious diseases. All the experimental animals were daily inspected thoroughly for detection of any health-related problems. The manger was cleaned twice, in morning at 8:00 a.m. and evening at 3:30 p.m. Dung was removed from the floor twice daily, in the morning and evening.

Hematological parameters measurement

Hematological parameters *viz*., hemoglobin (g/dl), packed cell volume (PCV) (%), RBC count ($x10^6$/µl), WBC count ($x10^3$/µl) and platelets count ($x10^3$/µl) of the blood samples were analyzed using auto hemato analyzer.

Statistical analysis

The data recorded were analyzed for descriptive statistics (mean and standard error). Treatment and period effects on different parameters, was analyzed by two-way analysis of variance (ANOVA). Pair-wise mean differences between groups were compared by DMRT test, and the mean differences were considered significant, if at $p<0.05$.

Results and Discussion

Haematological Parameters

Blood Hemoglobin (g/dL): Average Hb concentration (g/dL) was observed among the groups and the Hb concentration (g/dL) was within the normal physiological range of the calves. Present findings align with findings of previous researchers; Gonzales-Eguia *et al*. (2009) [5] observed that Hb in piglets was not affected by nano-Cu supplementation. Datta *et al*. (2007) [4] also observed non-significant effect of source and dose of Cu on haemoglobin concentration in goat kids. Observed similar results for Hb in nano Cu supplemented weaned pigs. In contrast to the findings of the present study, Mondal and Biswas (2007) [9] reported higher Hb concentration in Cu-propionate groups as compared to CuSO4 supplemented groups. Observed higher haemoglobin levels in nano Cu and inorganic Cu groups than those in Cu-deprived goats. Significant increase in Hb values was observed in nano Cu treated broilers as compared to control and CuSO4 treated groups.

Consistent to present findings, Anil et al. (2020) [2] and Sethy et al. (2016) [16] observed a non-significant effect of zinc supplementation from either source on hemoglobin (Hb) concentration in crossbred calves and Black Bengal male goats, respectively. Similarly reported no effect of supplemental zinc on hemoglobin concentration in Goat kids. Corresponding observations were noted in abbits by Ismail and El-Araby (2017) [7], who revealed a non-significant difference in Hb concentration with dietary supplementation of zinc oxide nanoparticles. In contrast, Sobhanirad et al. (2014) [18] reported that zinc supplementation significantly ($p<0.05$) increased Hb concentrations in Malabari goat kids and Baluchi lambs, respectively.

Table 2: Effect of Inorganic and nano copper and zinc supplementation on hematological parameters in Gir Calves

Days	Treatment Groups					p value
	T_1	T_2	T_3	T_4	T_5	
Hb (g/dl)	10.23± 0.14	10.17± 0.21	10.48 ± 0.19	10.31 ± 0.30	10.32 ± 0.27	0.98
RBC count (106/μL)	8.50 ± 0.17	8.51 ± 0.10	8.43 ± 0.17	8.50 ± 0.12	8.60 ± 0.14	0.98
WBC count (103/μL)	9.85 ± 0.25	9.81 ± 0.38	9.68 ± 0.18	9.90 ± 0.16	9.73 ± 0.22	0.99
Platelet Count (103/μL)	444.70± 20.17	443.90± 18.59	446.90± 18.69	455.70± 17.86	449.50± 18.78	0.53
PCV (%)	31.43± 0.46	31.08± 0.38	31.63± 0.59	31.43± 0.61	31.40± 0.54	0.99

Blood RBC count (106/μL)

The overall mean RBC count (10^6/μL) for T_1, T_2, T_3, T_4 and T_5 groups was 8.50 ± 0.17, 8.51 ± 0.10, 8.43 ± 0.17, 8.50 ± 0.12 and 8.60 ± 0.14, 10^6/μL, respectively. The mean value of blood RBC count (10^6/μL) at day 150 was statistically non-significant among the groups, however, numerically higher values was observed in T_5 followed by T_2 and T_4, while T_3 group showed numerically lower value as compared to control. No any statistically significant (*p*>0.05) difference for overall average blood RBC count (10^6/μL) was observed among the groups and the blood RBC count (10^6/μL) was within the normal physiological range of the calves.

Similar to the results of the present study, RBCs in piglets were not affected by nano-Cu supplementation (Gonzales-Eguia *et al*., 2009) [5]. Datta *et al*. (2007) [4] also observed non-significant effect of dose and source of Cu on RBC in goat kids throughout the experimental period. also observed similar results for RBC in nano Cu supplemented weaned pigs. In contrary to the findings of the present study, Mondal and Biswas (2007) [9] noticed higher RBC in Cu-propionate groups than with CuSO4 supplementation. Kushwaha *et al.* (2021) [8], observed RBCs count in inorganic and nano- Cu supplemented groups was significantly higher (*p*<0.05) than non-supplemented group. Observed significantly higher erythrocyte count in nano Cu and inorganic Cu groups than those in Cu-deprived goats. Similar to the findings of the present study, no effect of Zn supplementation on values of RBCs in buffalo calves was observed by Ramulu *et al*. (2015) [11].

WBC count (103/μL)

The overall mean blood WBC count(10^3/μL) for T_1, T_2, T_3, T_4 and T_5 groups was 9.85 ± 0.25, 9.81 ± 0.38, 9.68 ± 0.18, 9.90 ± 0.16 and 9.73 ± 0.22, 10^3/μL, respectively. The mean value of blood WBC count (10^3/μL)at day 150 was statistically non-significant among the groups, however, numerically higher values was observed in T_3 followed by T_5 and T_2, while T_4 group showed same value as compared to control. Statistical analysis observed non-significant (*p*>0.05) difference for overall average blood WBC count (10^3/μL) at different stages of trial among the groups and the values was within the normal physiological range of the calves.

Similar to the findings of the present study, Kushwaha *et al.* (2021) [8] reported WBCs count was unaltered in growing Sahiwal heifers supplemented with inorganic and nano-Cu. The WBC count in piglets was not affected by nano-Cu supplementation (Gonzales-Eguia *et al*., 2009) [5]. Datta *et al.* (2007) [4] also not observed effect of dose and source of Cu on WBC in goat kids. also observed a similar result for WBC in nano Cu supplemented weaned pigs.

Similar to current findings, Anil *et al.*, (2020) [2], Ramulu *et al*. (2015) [11] reported that dietary supplementation of different zinc sources and forms did not show any significant effect on WBC count in crossbred calves and Murrah buffalo. Found no significant effect of supplemental zinc WBC count in goat kids.

Blood platelet count (103/μL)

The overall mean blood platelet count (10^3/μL) for T_1, T_2, T_3, T_4 and T_5 groups recorded were 444.70 ± 20.17, 443.90 ± 18.59, 446.90 ± 18.69, 455.70 ± 17.86 and 449.50 ± 18.78, 10^3/μL, respectively. The mean value of blood platelet count (10^3/μL)at day 150 was statistically non- significant (*p*>0.05) among the groups, however, numerically higher values was observed in T_4 group, while numerically lower values was observed in T_3 followed by T_2 group as compared to control group. Non-significant (*p*>0.05) difference was observed for overall average blood platelet count (10^3/μL) at end of trial among the groups.

Similar to the findings of the present study, Gonzales *et al.* (2009) [5] observed that platelet count was non-significantly affected (*p*>0.05) by nano Cu supplementation. In contrast to the results of present study, Anil *et al.* (2020) [2] also reported statistically non-significant (*p*>0.05) difference with regard to platelet count among the groups.

Blood Packed Cell Volume (%)

The overall mean blood PCV(%) for T_1, T_2, T_3, T_4 and T_5 groups was 31.43 ± 0.46, 31.08 ± 0.38, 31.63± 0.59, 31.43 ± 0.61 and 31.40 ± 0.54%, respectively. Overall PCV(%) remained non-significant between treatment groups. The results of the present study are in accordance with the findings of Datta *et al.* (2007) [4] they did not observed any effect of dose and source of Cu on PCV in goat kids. In contradictory to present findings, Kushvaha *et al.* (2021) [8] reported that PCV % in inorganic and nano cu supplemented groups was significantly higher (*p*<0.05) than non-supplemented group. Shen *et al.* (2021) [17] also observed significantly (*p*<0.05) higher packed cell volume in nano Cu and inorganic Cu groups than those found in Cu-deprived goats. Supplementation of nano-Cu to groups of rabbit, showed significantly higher PCV value than the control group (Refaie *et al*., 2015) [12]. Similar to present findings, Anil *et al*., (2020) [2] and Sethy *et al.* (2016) [16] also reported non-significant effect of zinc supplementation on PCV concentration in crossbred calves and male black Bengal goats, respectively. While contrast to present findings, Sobhanirad *et al.* (2014) [18] reported that zinc supplemented diets increased the PCV concentration of Baluchi lambs. Sobhanirad and Naserian (2014) [18] also found that zinc supplemented diets significantly increased the PCV concentration of Holstein dairy cows.

Salama *et al.* (2003) [15] stated that zinc at higher concentrations tends to inhibit copper and iron absorption. Both copper and iron are essential for the proliferation and maturation of red blood cells (RBCs) and white blood cells (WBCs). In the present study, the hematological parameters were within the normal range. This could be attributed to the level of zinc inclusion in the study, which might not have interfered with copper and iron absorption to an extent that would affect the hematological parameters. The normal hematological parameters of all the experimental groups could be attributed to the adequate transfer of copper and zinc from the mothers to the calves, which might be a reason that copper and zinc supplementation failed to demonstrate a discernible impact on hematopoiesis.

Conclusions

In present study all the hematological parameters studied were non-significant among the treatment groups, and varied within normal range. Hematological parameters are indicators of the blood's cellular composition and function, which can be influenced by factors such as genetics, diet, overall health status, and environmental conditions. It is possible that the duration of the study or the specific dosages of nano zinc and copper supplementation may not have been sufficient to elicit significant changes in hematological parameters in Gir calves. Additionally, individual variability in response to supplementation and the specific mechanisms through which nano minerals interact with hematological processes may also play a role that remain unchanged.

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