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RESEARCH

Replacing Inorganic Source of Zinc with Zinc Hydroxy Chloride: Effects on Health Status, Hemato-biochemical Attributes, Antioxidant Status, and Immune Responses in Pre-ruminant Crossbred Calves

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Abstract

The current research aimed to assess the feasibility of using Zn hydroxy chloride (ZnOHCl) as an alternative to $ZnSO_4$ in pre-ruminant crossbred calves. Twenty-four male crossbred calves (Tharparkar × Holstein Friesian) were categorized into four groups according to body weight and age (body weight 31 kg; age 10 days). Experimental calves were kept on a similar feeding regimen except that different groups were supplemented with either 0 mg Zn/kg DMI (Zn-0), 80 mg Zn/kg DMI as $ZnSO_4$ (ZnS-80), 40 mg Zn/kg DMI as ZnOHCl (ZnH-40), or 80 mg Zn/kg DMI as ZnOHCl (ZnH-80). All the calves were fed for 90 days as per ICAR (2013) feeding standard to fulfill their nutrient requirements for growth rate of 500 g/day. The study observed the influence of different sources and varying levels of Zn supplementation over a 90-day experimental period on health status, hemato-biochemical attributes, antioxidant status, immune responses, and plasma minerals and erythrocyte Zn concentrations. The data was examined using a randomized complete block design (RCBD) with fixed effects of treatment, period, and their interaction. The results indicated that irrespective of the sources and levels of Zn, supplementation did not lead to significant changes in health status as assessed by fecal score, nasal score, ear score, and eye score. Hematological parameters remained unchanged following supplementation with different sources and levels of Zn. Zn-supplemented groups showed higher levels of total protein, globulin, and alkaline phosphates (ALP) compared to the non-supplemented group. However, no significant variations were detected within the Zn-supplemented groups. Zinc supplementation significantly increased total antioxidant capacity (TAC), antioxidant enzyme activity, total immunoglobulin (Ig), immunoglobulin G (IgG), cell-mediated immunity (CMI), and humoral immunity (HI); however, no significant variations were detected among Zn-supplemented groups. Zn supplementation enhanced plasma and RBC Zn concentration without affecting the plasma concentration of other minerals. However, among the Zn-supplemented groups, 80 mg Zn/kg DMI as ZnOHCl resulted in the highest RBC Zn concentration. The study results demonstrate that Zn supplementation enhanced biomarkers of zinc status, antioxidant levels, and immune responses in pre-ruminant crossbred calves. Nevertheless, no significant variations were observed between the different Zn sources ($ZnSO_4$ and ZnOHCl) utilized in this study. Research suggests that ZnOHCl could be a feasible alternative to $ZnSO_4$ in the diet of pre-ruminant crossbred calves.

Keywords Antioxidant enzyme · Crossbred calves · Hemato-biochemical · Immunity · Hydroxy trace minerals · Zinc sulfate

Introduction

Zinc (Zn) is essential for a wide range of functions in the body, including catalytic, structural, and regulatory roles. Its significance extends to essential metabolic processes including carbohydrate and nucleic acid metabolism, protein synthesis, and the maintenance of epithelial tissue integrity. The immune system of animals is highly dependent on Zn. Deficiency of Zn has become prevalent in soils and crops globally, making it the most prevalent micronutrient deficiency. In certain areas of India, high soil pH levels reduce the availability of Zn and its uptake by plants [1], resulting in the average Zn content in Indian fodders below the normal requirement to meet the nutritional needs of cattle [2, 3]. Supplementation with bioavailable Zn is vital in high-productivity animal systems, particularly in tropical regions where Zn deficiency is common,

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to prevent subclinical deficiencies and optimize animal health and production. Additionally, the source of the trace minerals should also be considered, as studies have suggested varying bioavailability for different sources of Zn [4, 5]. In animal nutrition, Zn is commonly supplemented in inorganic forms and $ZnSO_4$ is the most commonly used supplement owing to its widespread availability and cost-effectiveness. Recent studies [6] have indicated that higher levels of Zn may be required than the dietary recommended levels [7] for enhancement of immunity, particularly in pre-ruminant calves prone to infections with high mortality and morbidity rates. However, supplementing higher levels of Zn through inorganic sources can adversely affect the absorption of other minerals like copper (Cu) and iron (Fe). Therefore, to prevent interactions with other trace minerals, Zn sources with higher bioavailability are preferred. Organic Zn salts are favored for their higher bioavailability [8, 9]. However, studies comparing organic chelates to inorganic sources yield mixed results [4, 5]. Despite enhanced bioavailability, organic trace minerals are costlier, necessitating careful economic considerations in feed formulation decisions.

Recently, a novel source of trace element known as hydroxy trace minerals has emerged, with claims of possessing superior bioavailability compared to inorganic sources and potentially equivalent or even higher bioavailability than organic trace minerals, offering a more cost-effective option for enhancing animal production. Hydroxy trace minerals belongs to the category of inorganic trace mineral sources but share structural similarities with organic sources [10]. Their unique crystalline matrix structure and the presence of covalent bonds provide stability to the compound which prevents the metal from being released early and rapidly in the digestive tract, distinguishing hydroxy trace minerals from both inorganic and organic minerals [10, 11]. They are relatively insoluble in water or at weakly acidic pH, such as in rumen [12, 13], but soluble in acidic conditions, such as in the abomasum of ruminants. Hydroxy minerals can bypass the rumen due to their low solubility in the rumen's pH [14], reducing interactions with antagonists that normally occur in the rumen. This can explain the greater bioavailability (77 mg/day retained Zn) of hydroxy trace minerals compared to $ZnSO_4$ (34.7 mg/day retained Zn) sources in cattle [15]. Furthermore, recent research by Pal et al. [16] indicates that ZnOHCl (40 mg/kg DMI) can effectively serve as a Zn source for pre-ruminant calves at a lower dose than $ZnSO_4$ (80 mg/kg DMI). A study in non-ruminants also suggests that ZnOHCl is a more bioavailable source of Zn compared to $ZnSO_4$ [17]. Feeding ZnOHCl reduces environmental exposure of the dairy cow to *Treponema* spp. compared with $ZnSO_4$ [18]. Moreover, an in vitro study indicated that supplementing with Zn up to 160 ppm, in the form of either ZnOHCl or $ZnSO_4$, did not alter rumen fermentation characteristics [19]. We hypothesize that Zn hydroxy chloride ($Zn_5(OH)_8Cl_2 \cdot H_2O$) will improve the health, antioxidant status, and immune response of pre-ruminant calves with minimal antagonistic interaction with other dietary nutrients at lower dosages compared to sulfate forms. Therefore, the current study aims to examine the effects of supplementing Zn from Zn hydroxy chloride and Zn sulfate on the health status, hemato-biochemical parameters, antioxidant status, and immune responses in pre-ruminant crossbred calves.

Material and Methods

Animals, Experimental Design, and Treatment

The research was conducted at the Livestock Research Complex of ICAR-National Dairy Research Institute in Karnal, India, adhering to the regulations of the Institute Animal Ethical Committee (IAEC) with approval number 42-IAEC-18–17. The experiment followed the guidelines set by the Committee for the Purpose of the Control and Supervision of Experiments on Animals (CPCSEA), as mandated by Government of India. From the herd, 24 pre-ruminant healthy 10-day-old crossbred (Tharparkar × Holstein Friesian) calves were randomly selected and allocated to four groups in a randomized block design (RBD) with six calves (replicate) in each group considering their body weight (31.03 kg) and age (10 days). The animals were fed for 90 days to assess the treatment effects. Basal diet of calves, i.e., milk and a total mixed ration (TMR), was formulated to meet the growth rate of 0.5 kg of daily gain as per ICAR [3] recommendations. Total mixed ration (TMR) consisted of calf starter and sugar graze fodder in 50:50 ratio on dry matter (DM) basis. The formulation of the calf starter was 100 g of ground maize grain, 50 g barley grain, 25 g oat grain, 80 g wheat bran, 25 g mustard oil cake, 85 g groundnut cake, 80 g de-oiled soybean cake, 10 g mineral (without Zn) and vitamin premix, and 5 g common salt per kilogram of DM. The ingredients and nutritional content of the TMR provided during the experimental period are detailed in Table 1.

After 15 days of adaptation period, the experimental feeding regimen was implemented and continued for a duration of 90 days. According to the four treatment groups, the calves were allocated to receive a basal diet without Zn supplements (0 mg/kg DM) or supplemented with 80 mg Zn/kg DMI as $ZnSO_4$ (21% Zn, feed grade $ZnSO_4 \cdot 7H_2O$, Agri-Chem Industries Private Limited, Gujarat, India) or supplemented with 40 mg Zn/kg DMI as ZnOHCl (60.42% Zn, $Zn_5(OH)_8Cl_2 \cdot H_2O$, Anmol Chemicals, Mumbai, India) or

Table 1 Ingredient and nutrient compositions of TMR fed during the experimental period

Attribute	Content (g/kg DM)
Ingredient composition	
Sugar graze fodder	500
Maize grain	100
Barley grain	50
Oat grain	25
Wheat bran	80
Gram churi	40
Mustard oil cake	25
Ground nut cake	85
De-oiled soya bean cake	80
Mineral mixture[a]	10
Common salt	5
Zn sources	Variable[c]
Chemical composition of TMR	
Dry matter	588
Organic matter	919
Crude protein	156
Ether extract	29
Total ash	80
Carbohydrate	734
Non-fibrous carbohydrate	308
Neutral detergent fiber	460
Acid detergent fiber	215
Hemicellulose	245
Cellulose	128
Acid detergent lignin	48
Total digestible nutrient[b]	641
Metabolizable energy (Mcal/kg DM)[b]	2.47
Calcium	6.1
Iron, mg/kg DM	477
Zinc, mg/kg DM	32.92
Manganese, mg/kg DM	115.04
Copper, mg/kg DM	19.58
	Pal et al. [19]

[a]Per kg contained: vitamin A, 700,000 IU; vitamin D3, 70,000 IU; vitamin E, 250 mg; nicotinamide, 3.0 g; Ca, 190 g; P, 90 g; Mg, 19.0 g; Na, 50 g; Fe, 1.5 g; Zn, 0 g; Mn, 6.0 g; Cu, 1200 mg; I, 325 mg; Co, 150 mg; Se, 10 mg

[b]Calculated value [7]

[c]The calves were fed either basal ration (0) or basal ration supplemented with 80 ppm Zn ($ZnSO_4$), 40 ppm Zn (ZnOHCl), and 80 ppm Zn (ZnOHCl)

supplemented with 80 mg Zn/kg DMI as ZnOHCl. Dosage levels for different Zn sources were determined on the basis of available literature [1, 3, 5, 19]. The Zn supplements provided 100% of the calves' Zn requirements throughout the study, without accounting for contributions from other dietary ingredients.

The Zn supplements were administered through milk to individual calves once daily, before morning feeding at 08:00 h, ensuring they received the full treatment dose. The daily Zn doses were calculated by total DMI, including both milk and feed, and were adjusted weekly due to increasing age and subsequent increase in DMI of calves. Calves were provided milk twice a day, and clean drinking water without detectable Zn was available to them twice daily at 11:00 h and 19:00 h. All calves were individually housed in well-ventilated experimental sheds, equipped with individual feeding and watering facilities.

Observations Recorded, Sampling, and Analytical Procedures

Nasal score, eye score, ear score, and fecal score were recorded consistently throughout the experimental period. Fecal consistency in calves was observed using a standard protocol, following Larson et al.'s [20] 1–4 point subjective scale. Nasal discharge was graded on a scale of 1 to 4; 1 indicated normal serous discharge, 2 denoted a small amount of cloudy discharge in one nostril, 3 indicated cloudy discharge in both nostrils, and 4 represented copious mucopurulent discharge in both nostrils. Similarly, eye condition was scored from 1 to 4; 1 meant normal, 2 indicated a small amount of ocular discharge, 3 denoted moderate bilateral discharge, and 4 signified heavy ocular discharge. Ear condition was assessed on a scale of 1 to 4 as well; 1 indicated normal, 2 denoted ear flick or head shake, 3 represented a slight droop in one ear, and 4 indicated head tilt or drooping in both ears.

Feed intake of calves was monitored daily by recording the amount of feed offered and the residue left. The body weight of the calves was recorded biweekly over two consecutive days at 07:00 a.m. before providing feed and water. The average body weight for each fortnight was calculated based on the measurements taken over the two consecutive days. Samples of feeds and fodders offered and leftovers were dried in an oven at 80 °C until a constant weight was achieved. Weekly samples were pooled, ground to pass through a 1-mm sieve using a Wiley mill, and stored for chemical analysis, including DM, CP, EE, and total ash following standard methods (AOAC) [21]. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) levels were assessed using methods proposed by Van Soest et al. [22]. The nutritional composition of the experimental diet is presented in Table 1.

Blood samples were obtained from the jugular vein of the calf at 0, 30, 60, and 90 days after Zn supplementation. Heparinized vacutainers were employed for blood collection and the samples were gently mixed and stored in an icebox

before being transported to the laboratory. A portion of the blood (0.5 mL) was used to analyze hematological parameters such as total leukocyte count (TLC), red blood cell count (RBC), and hemoglobin (Hb) content using an Auto Hematology Analyzer (Model no. BC2800 vet). The RBC hemolysate was prepared from 2 mL of blood collected in a sodium citrate-containing microcentrifuge tube to analyze antioxidant enzyme activity. Enzymatic activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) were assessed in RBC hemolysate within 3 days of collection, following protocols described by Madesh and Balasubramanian [23], Aebi [24], and Paglia and Valentine [25], respectively. The RBC pellets (0.5 mL) were separated and stored with 0.5 mL of chilled distilled water for analysis of erythrocyte Zn content.

The blood samples were centrifuged at 3000 *g* and 4 °C for 20 min to separate plasma, which was then stored at − 20 °C for further analysis of various biochemical parameters, plasma minerals, antioxidant enzyme activities, and immunological attributes. Biochemical parameters were assessed using kits from Recombigen Laboratories Pvt. Ltd., New Delhi, India, based on colorimetric principle following the manufacturer's instructions. The parameters measured included plasma glucose (CAT No. GLU-L500), total protein (CAT No. TTP-L250), albumin (CAT No. ALB-L250), total cholesterol (CAT No. CHO-L500), and plasma enzymes: alkaline phosphatase (CAT No. ALP-L125), alanine aminotransferase (CAT No. ALT-L500), and aspartate aminotransferase (CAT No. AST-L500). The procedure involved mixing the sample and standard with their respective reagents separately and incubating them for a specific period of time. Subsequently, the absorbance of both the standard and test sample was measured against a blank using a spectrophotometer. Plasma globulin concentration was determined by subtracting the albumin concentration from the total protein concentration.

Total antioxidant capacity (TAC) was determined using the FRAP assay by Benzie and Strain [26], while lipid peroxidation (thiobarbituric acid reactive substance (TBARS)) was estimated following the method modified by Kaushal and Kansal [27] based on the Niehaus and Samuelsson [28] protocol. Total Ig in plasma was assessed using the Zn turbidity method by McEwan et al. [29], and IgG levels were quantified using a Bovine IgG ELISA kit (Catalog no. E0010Bo) from Bioassay Technology Laboratory, Shanghai, China. Sensitivity of assay was given as 1.03 µg/mL and precision, i.e., intra- and inter-assay coefficients of variation were < 8 and < 10% respectively. Cell-mediated immunity (CMI) was evaluated through delayed-type hypersensitivity (DTH) response against phytohemagglutinin-P (PHA-P), administered intra-dermally in the neck following the protocol outlined by Pattanaik et al. [30]. The thickness of the neck was measured at 0, 6, 12, 24, 36, and 48 h post-inoculation. Humoral immunity (HI) was assessed by challenging calves with sheep erythrocytes and analyzing antibody response using hemagglutination (HA) test on serum samples collected at 0, 7, 14, and 21 days post challenge.

For mineral analysis, 5 mL urine, 0.5 g feces, 1 g feed samples, 1 mL RBC pellets, and 1 mL plasma were digested using a 10 mL triple acid mixture (HNO_3:$HClO_4$:H_2SO_4-10:4:1) and analyzed for calcium (Ca), zinc (Zn), copper (Cu), manganese (Mn), and iron (Fe) concentrations using an Atomic Absorption Spectrophotometer (AAS, Model Z-5000, Hitachi High Technologies Corporation, Tokyo, Japan) after appropriate dilution with Millipore water.

Statistical Analyses

A two-way analysis of variance (ANOVA) was employed to analyze health status, hemato-biochemical parameters, antioxidant enzyme activities, immune responses, and plasma minerals. The factors considered were Zn supplementation levels (Zn-0, ZnS-80, ZnH-40, ZnH-80) and time intervals (0, 30, 60, and 90 days of dietary treatment). The model used for the analysis was as follows:

$$Y_{ijk} = \mu + A_i + B_j + C_{ij} + e_{ijk},$$

where;

Y_{ijk} = dependent variable (health status, hemato-biochemical parameters, antioxidant enzymes activities, immune responses, and plasma minerals), μ = total mean, A_i = mean effect of ith treatment, B_j = mean effect of jth time, Cij = effect of interaction between treatment and time of sampling, e_{ijk} = residual error.

Treatments were assessed through Tukey's test, with statistically significant differences defined at a significance level of $P < 0.05$ while P-values between 0.05 and 0.10 ($0.05 \leq P < 0.10$) were regarded as showing a tendency toward significance.

Results

Health Status

The supplementation of various levels and sources of Zn did not result in significant changes ($P > 0.05$) in fecal score, nasal score, ear score, and eye score in crossbred calves (Table 2). However, the incidence of diarrhea and nasal discharge was lower in the Zn-supplemented group compared to the non-supplemented group. Among the Zn-supplemented groups, calves supplemented with ZnOHCl showed fewer occurrences of diarrhea and nasal discharge in comparison

Table 2 Effect of Zn supplementation ($ZnSO_4$ vs. ZnOHCl) on health status of pre-ruminant crossbred calves

Attributes	Supplemental Zn				SEM	*P* value		
	0	ZnS-80	ZnH-40	ZnH-80		*T*	*P*	*T*×*P*
Fecal score (1–4 scale)	1.76	1.57	1.55	1.44	0.06	0.180	0.037	1.00
Nasal score (1–4 scale)	1.77	1.62	1.52	1.49	0.06	0.602	0.652	0.999
Ear score (1–4 scale)	1.63	1.55	1.49	1.45	0.05	0.602	0.652	0.999
Eye score (1–4 scale)	1.64	1.55	1.59	1.50	0.03	0.565	0.126	1.000

Level of significance ($P < 0.05$). The crossbred calves were fed either basal ration (0) or basal ration supplemented with 80 ppm Zn ($ZnSO_4$), 40 ppm Zn (ZnOHCl), and 80 ppm Zn (ZnOHCl).

SEM, standard error of the mean

to those supplemented with $ZnSO_4$, although these differences were not statistically significant ($P > 0.05$).

Hemato-biochemical Parameters

Dietary supplementation of various levels and sources of Zn did not affect hematological parameters. However, crossbred calves supplemented with Zn had higher ($P < 0.05$) total protein and globulin concentrations compared to the non-supplemented group. Among Zn-supplemented groups, those receiving 80 mg Zn/kg diet in the form of ZnOHCl had the highest concentrations of total protein and globulin concentration. Supplementation of Zn did not significantly influence the plasma albumin and glucose concentrations. However, notable variations were observed due to the period effect. The albumin to globulin (*A*:*G*) ratio was significantly reduced in the Zn-supplemented groups compared to the non-supplemented group. However, triglyceride and total cholesterol concentrations showed no significant changes throughout the study period. Supplementation of different levels and sources of Zn did not affect plasma enzyme activities such as AST and ALT. However, ALP activity showed a significant increase ($P < 0.05$) in Zn-supplemented groups compared to the non-supplemented group, with no significant differences observed among the Zn-supplemented groups. There were no significant interactions observed between treatment and period for hematological and biochemical parameters over the 90-day experimental period.

Antioxidant Status, Immune Responses, and Mineral Interaction

In the current study, GPx, SOD, and catalase were used as biomarkers for assessing the antioxidant status of calves (Table 3). Calves with supplementation of different levels (40, 80 ppm) and sources of Zn ($ZnSO_4$ or ZnOHCl) had higher ($P < 0.05$) activity of GPx, SOD, and catalase than other calves. However, there were no significant effects observed among the Zn-supplemented groups. Supplementation of different levels and sources of Zn did not result in a statistically significant change in plasma TBARS concentration, although there was a tendency for a decrease in concentration in Zn-supplemented groups compared to the non-supplemented group. However, TAC concentration was higher in calves fed on a diet supplemented with Zn compared to non-supplemented groups. Among the Zn-supplemented groups, TAC concentration was highest in calves fed on a diet supplemented with 80 mg Zn/kg DM as ZnOHCl group compared to those supplemented with 80 mg Zn/kg DM as $ZnSO_4$ and 40 ppm mg Zn/kg DM as ZnOHCl. Calves supplemented with 40 mg Zn/kg DM as ZnOHCl and 80 mg Zn/kg DM as $ZnSO_4$ showed similar TAC values.

Calves fed Zn-supplemented diet had significantly higher ($P < 0.05$) plasma concentrations of IgG and total Ig compared to non-supplemented calves. However, there were no significant differences found among the Zn-supplemented groups. The CMI assessed using the DTH response to PHA-P was significantly higher ($P < 0.05$) in calves fed Zn-supplemented diet compared to non-supplemented calves. However, there were no significant differences observed among the different Zn-supplemented groups. The HI assessed by measuring the antibody response to sheep-erythrocytes (SRBC) using the HA test showed significantly higher ($P < 0.05$) antibody titers in calves fed diet supplemented with Zn compared to non-supplemented calves, although there were no significant differences observed between the different Zn-supplemented groups (Table 4).

Plasma concentration of Zn was higher ($P < 0.05$) in calves fed on a diet supplemented with Zn compared to non-Zn-supplemented calves. The sources and levels of Zn supplementation showed no interaction with other minerals as evidenced by similar plasma concentrations of Ca, Cu, Fe, and Mn among all groups. Dietary supplementation of Zn significantly increased the concentration of Zn in RBCs compared to non-Zn-supplemented groups. However, among the Zn-supplemented groups, calves receiving 80 mg Zn/kg DM as ZnOHCl had the highest concentration of Zn in their RBCs.

Table 3 Effect of Zn supplementation ($ZnSO_4$ vs. ZnOHCl) on hemato-biochemical parameters of pre-ruminant crossbred calves

Attributes	Supplemental Zn				SEM	*P* value		
	0	ZnS-80	ZnH-40	ZnH-80		*T*	*P*	*T*×*P*
Days in trial	90	90	90	90				
Hematological parameters								
Hb (g/dL)	12.42	12.88	12.10	12.18	0.17	0.433	0.933	0.872
RBC ($\times 10^6$/μL)	10.90	11.20	10.59	11.10	0.13	0.369	0.218	0.765
WBC ($\times 10^3$/μL)	5.32	5.75	5.63	6.14	0.13	0.137	0.001	0.397
Lymphocyte (%)	66.27	67.83	68.34	68.42	0.60	0.541	0.008	0.816
Mix (%)	5.56	5.88	5.18	5.99	0.14	0.124	0.041	0.465
Neutrophil (%)	28.17	26.29	26.49	25.60	0.57	0.410	0.004	0.932
Biochemical parameters								
Total protein (g/dL)	6.12^{a}	6.52^{ab}	6.64^{ab}	6.79^{b}	0.17	0.023	0.016	0.478
Albumin (g/dL)	2.97	2.94	2.97	2.96	0.03	0.981	0.440	0.041
Globulin (g/dL)	3.15^{a}	3.58^{ab}	3.67^{ab}	3.83^{b}	0.08	0.022	0.016	0.728
Albumin:globulin	1.02^{b}	0.87^{ab}	0.85^{ab}	0.81^{a}	0.03	0.048	0.055	0.897
Glucose (mg/dL)	86.74	87.93	87.16	90.52	1.08	0.608	0.031	0.910
Triglyceride (mg/dL)	29.63	26.98	27.55	29.30	0.72	0.490	0.242	0.462
Total cholesterol (mg/dL)	108.62	107.86	106.91	105.55	1.16	0.839	0.975	0.999
Plasma enzyme								
ALP (U/L)	65.55^{a}	70.62^{b}	71.15^{b}	73.61^{b}	0.97	0.004	< 0.001	0.149
AST (U/L)	83.63	84.35	85.12	88.35	2.10	0.534	0.338	0.958
ALT (U/L)	49.74	53.85	52.41	52.30	0.95	0.517	0.122	0.993

Level of significance ($P < 0.05$). The crossbred calves were fed either basal ration (0) or basal ration supplemented with 80 ppm Zn ($ZnSO_4$), 40 ppm Zn (ZnOHCl), and 80 ppm Zn (ZnOHCl). Means with different superscripts across the row differ significantly ($P < 0.05$)

RBC, red blood corpuscles; *WBC*, white blood corpuscles; *Hb*, hemoglobin; *ALP*, alkaline phosphatase; *AST*, aspartate aminotransferase; *ALT*, alanine aminotransferase; *SEM*, standard error of the mean

Discussion

There is a paucity of research comparing the effect of $ZnSO_4$ and ZnOHCl on health status, hemato-biochemical parameters, antioxidant status, and immune responses of pre-ruminant calves. However, many studies have been undertaken to assess the comparative effects of the $ZnSO_4$ and organic Zn supplementation in animals. By exploring this comparison, we can gain insights into the potential differences in bioavailability between these $ZnSO_4$ and ZnOHCl. Any significant variations observed between the two salts could indicate a higher bioavailability of one over the other.

Health Status

Supplementation of different levels and sources of Zn did not significantly change fecal score, nasal score, ear score, and eye score in crossbred calves; however, the occurrences of diarrhea and nasal discharge were lower in the Zn-supplemented group compared to the non-supplemented group. Among the Zn-supplemented group, occurrences of diarrhea and nasal discharge were lesser in ZnOHCl-supplemented groups compared to the $ZnSO_4$-supplemented group. This finding aligns with a study by Lapierre [31], which also demonstrated a decrease in fecal score in the group supplemented with ZnOHCl compared to $ZnSO_4$. This difference might be due to higher bioavailability of ZnOHCl compared to $ZnSO_4$.

Hemato-biochemical Parameters

The current study suggests that Zn supplementation, whether in the form of $ZnSO_4$ or ZnOHCl, did not change hematological parameters such as TLC, DLC, RBC, and Hb in crossbred calves, with average values lying within the normal physiological range of dairy calves [32–34]. Similarly, Aliarabi et al. [35] noticed that supplementation of Zn (20–40 ppm) in lambs did not change RBC count, packed cell volume, and Hb. However, significant differences were noted in white blood cell and lymphocyte counts between the control group and Zn-supplemented groups. Similar to the present results for RBC, Zn supplementation also did not change the RBC number in men [36]. In contrast, Rupic et al. [37] reported that supplementation of Zn (84.3 ppm) as $ZnSO_4$ increased RBC count in pigs compared to the control and Zn methionine supplemented group, highlighting potential dose and species-specific variations in response

Table 4 Effect of Zn supplementation ($ZnSO_4$ vs. ZnOHCl) on antioxidant status, immune responses, and mineral interaction of pre-ruminant crossbred calves

Attributes	Supplemental Zn				SEM	*P* value		
	0	ZnS-80	ZnH-40	ZnH-80		*T*	*P*	*T*×*P*
Antioxidant enzyme activity								
GPX, µmol of NADPH oxidized/g Hb/min	25.18[a]	28.88[b]	28.98[b]	30.94[b]	0.66	0.001	< 0.001	0.104
SOD (U/mg Hb)	71.72[a]	79.77[b]	80.11[b]	82.07[b]	1.01	< 0.001	< 0.001	0.708
Catalase, µmoles of H_2O_2 consumed/min/g Hb	137.02[a]	144.53[b]	148.16[b]	150.24[b]	1.52	< 0.001	< 0.001	0.056
Antioxidant capacity								
TBARS, nmol/g protein	2.66	2.43	2.40	2.34	0.07	0.420	0.060	0.530
TAC, mmol/L	1.27[a]	1.39[ab]	1.40[ab]	1.51[b]	0.03	0.009	0.008	0.197
Immune response								
IgG, mg/mL	24.96[a]	27.89[b]	28.01[b]	29.59[b]	0.43	< 0.01	< 0.01	0.474
Total Ig, mg/mL	33.43[a]	36.67[b]	37.38[b]	38.02[b]	0.47	< 0.001	< 0.001	0.084
CMI, DTH response against PHA-P	6.21[a]	6.97[b]	7.26[b]	7.37[b]	0.20	< 0.001	< 0.001	0.96
CMI, DTH response against PHA-P, %	185.03[a]	207.90[b]	214.30[b]	221.71[b]	5.94	0.002	0.001	0.986
HI, HA titer, log 2 against sheep-RBC	2.34[a]	2.54[b]	2.60[b]	2.66[b]	0.04	0.001	< 0.001	0.925
Plasma mineral								
Zn (mg/L)	1.13[a]	1.45[b]	1.42[b]	1.60[b]	0.04	< 0.001	< 0.001	0.164
Ca (mg/dL)	12.37	11.29	12.49	11.26	0.21	0.055	0.081	0.584
Cu (mg/L)	1.07	0.97	1.09	0.98	0 0.02	0.050	0.362	0.515
Fe (mg/dL)	2.48	2.17	2.37	2.28	0 0.05	0.117	0.666	0.341
Mn (mg/L)	1.38	1.22	1.25	1.21	0 0.03	0.195	0.168	0.992
RBC Zn (mg/L)	3.70[a]	4.20[bc]	4.09[ab]	4.53[c]	0.08	< 0.001	< 0.001	0.120

Level of significance ($P < 0.05$). The crossbred calves were fed either basal ration (0) or basal ration supplemented with 80 ppm Zn ($ZnSO_4$), 40 ppm Zn (ZnOHCl), and 80 ppm Zn (ZnOHCl). Means with different superscripts across the row differ significantly ($P < 0.05$)

SOD, superoxide dismutase; *GPx*, glutathione peroxidase; *TBARS*, thiobarbituric acid reactive substances; *TAC*, total antioxidant capacity; *IgG*, immunoglobulin G; *Ig*, immunoglobulin; *CMI*, cell-mediated immunity; *HI*, humoral immunity; *SEM*, standard error of the mean

to Zn supplementation. Additionally, Zn deficiency in rats decreased RBC as reported by El-Hendy et al. [38], emphasizing the importance of adequate Zn levels for maintaining normal RBC count. Prasad [39] reported that a decrease in lymphocytes is the main cause of decreased host defense capacity in Zn-deficient rats.

The current study suggests that Zn supplementation did not significantly affect certain biochemical parameters such as glucose, cholesterol, albumin, and triglyceride. However, total protein and globulin concentration were significantly higher in the Zn-supplemented groups compared to the non-supplemented group. Average concentrations of total plasma protein, plasma cholesterol, and plasma triglyceride were found to be within the physiological range as reported by Das [40], Niwas et al. [41], and Kaneko et al. [42] respectively. Information on the effect of hydroxy Zn on biochemical parameters is meagerly available so discussion cannot be made with that perspective, but for critically analyzing the effect, other studies with supplementation of Zn with the variable bioavailable form are being discussed for the sake of comparisons. In concordance, Nobijari et al. [43] reported that supplementation of 150 ppm Zn as $ZnSO_4$ increased plasma total protein concentration. Similarly, Azizzadeh et al. [44] showed that supplementing calves with Zn (50, 100 ppm) as $ZnSO_4$ through milk for 14 days increased total protein and globulin, while glucose concentration decreased in Zn-supplemented groups. Another study [45] showed that supplementing calves with 80, 140 ppm Zn as Zn-proteinate increased serum total protein and globulin compared to $ZnSO_4$-supplemented groups. Nagalakshmi et al. [5] also reported that replacing inorganic Zn ($ZnSO_4$; 80 ppm) with lower level (75% of inorganic levels; 60 ppm) of organic Zn (Zn propionate (Zn-Prop)) did not alter serum albumin and globulin, while a higher ($P < 0.05$) serum total protein level was observed in organic Zn-supplemented calves. In the current study, plasma globulin concentration was higher in the hydroxy Zn-supplemented group compared to $ZnSO_4$ at the same level of supplementation indicating higher bioavailability of Zn from ZnOHCl compared to $ZnSO_4$. Contrarily, Elamin et al. [45] found that supplementing goat kids with $ZnSO_4$ (33 mg) significantly increased serum glucose level (77.48 mg/dL) compared to the control group (60 mg/dL). Nobijari et al. [43] also reported that supplementing with 150 ppm Zn as $ZnSO_4$ decreased

cholesterol concentration. It can be summarized that supplementation with Zn hydroxy salt shows potential benefits, such as improving total protein and globulin level possibly due to enhanced Zn retention compared to $ZnSO_4$, which could have been utilized for different biological functions.

Supplementation of Zn did not alter plasma enzyme activity such as AST and ALT. However, it significantly increased ALP activity in the groups that received Zn supplementation, with all enzyme activity remaining within the normal physiological range [46]. ALP is a homodimeric enzyme with an active site comprising three metal binding sites (M1, M2, and M3); M1 and M2 are occupied by Zn ions while M3 is occupied by Mg^{+2} ions [47]. Thus, ALP activity was expected to be affected due to bioavailable Zn content as a direct component of the enzyme. ALP plays a crucial role in regulating cell division and growth [48], and its higher activity can be correlated to growth. Alterations in ALP activity in plasma are indicative of changes in plasma Zn concentration [49, 50]. In the present study, Zn content in basal diet (32.15 ppm) was insufficient to cause any change in plasma ALP activity, which remained similar throughout the study period. However, when the basal diet was supplemented with additional Zn, plasma ALP activity was increased. Similar findings were also reported by Nagalakshmi et al. [51] in buffalo calves, Aliarabi et al. [35] in kids, and Devi et al. [52] in lambs. Additionally, Alimohamady et al. [4] reported that supplementing 30 mg Zn/kg DM, whether from inorganic or organic sources, increased the ALP activity; however, they noted no significant differences among the Zn-supplemented group. Furthermore, Cho et al. [53] reported that animals deficient in Zn exhibited decreased ALP activity. The activities of AST and ALT in plasma are measured to assess liver and muscle lesions [54]. In a study by Daghash and Mousa [55], it was observed that supplementing buffalo bulls' diet with 50 or 100 ppm of Zn increased AST activity which is not in agreement with the present study. From the activity of these enzymes, it can be inferred that ALP activity can be taken as an indicator of bioavailable Zn, which further supports the efficacy of hydroxy Zn salt compared to $ZnSO_4$. Data further suggest the values were comparable even when a 50% reduction was made in supplementation, i.e., 40 ppm ZnOHCl proved as effective as 80 ppm of $ZnSO_4$, thus showing its potential benefit while assessing its biological usage. Meanwhile, other liver-related enzymes AST and ALT remained unchanged, suggesting that the supplementation levels were not sufficient to affect these enzyme functions.

Antioxidant Status, Immune Responses, and Mineral Interaction

To assess the impact of $ZnSO_4$ and ZnOHCl supplementation on antioxidant status, the activities of GPX, SOD, and catalase in erythrocytes, as well as TBARS and TAC concentration in plasma, were measured in calves kept on experimental diets. Results showed significant increases in erythrocyte SOD, catalase, and GPX activities, along with elevated plasma TAC concentrations in the Zn-supplemented groups compared to the non-supplemented group. However, there was no notable effect observed on plasma TBARS concentration. Similar to the present finding, Parashuramulu et al. [6] indicated that supplementation of 80 or 140 ppm Zn as $ZnSO_4{\cdot}7H_2O$ increased antioxidant enzyme activities such as GPx, GPx, and catalase activity. Similarly, Alimohamady et al. [4] observed that supplementing lambs' diet (19.72 mg Zn/kg DM) with 30 mg Zn/kg DM as either $ZnSO_4$, Zn-methionine, Zn-proteinate, or Zn-glycinate significantly increased erythrocyte SOD activity. However, GPx activity was notably higher in organic Zn-supplemented compared to inorganic and control groups indicating higher bioavailability of organic sources. Similar findings were also noted by Fadayifar et al. [56], who reported significant improvements in SOD activity in groups supplemented with either $ZnSO_4$ or Zn-proteinate compared to control groups.

Plasma IgG, total Ig, CMI, and HI were used to assess the comparative effect of $ZnSO_4$ and ZnOHCl on the immune responses of experimental calves. The current study suggests that Zn supplementation with either $ZnSO_4$ or ZnOHCl significantly increased plasma IgG, total Ig, CMI, and HI in pre-ruminant calves. These results are in line with those of Yasui et al. [57] who showed improved immune status and lower trace mineral feeding costs in cows fed hydroxy trace minerals compared to those receiving a combination of inorganic and organic trace minerals. Similarly, Dresler et al. [58] found that adding 30 mg Zn/kg DM (Zn-methionine) to weaned calves significantly boosted total Ig concentration. Parashuramulu et al. [6] also indicated that supplementing buffalo calves with 80 or 140 ppm Zn as $ZnSO_4{\cdot}7H_2O$ resulted in higher immune responses as evidenced by increased HI and CMI. Additionally, Nagalakshmi et al. [59] showed that Zn-proteinate supplementation led to superior immune response including antibody titers against *Brucella abortus* and chicken RBC, as well as enhanced in vivo DTH responses, compared to inorganic source. They also suggested that Zn supplementation could be reduced from 140 to 80 ppm as Zn-proteinate without adverse effects in buffalo heifers. However, Kincaid et al. [60] reported no difference in CMI among Holstein heifer calves when supplemented with various sources of Zn, including Zn oxide, Zn methionine, or Zn lysine. Similarly, Vilela et al. [61] found that different sources (Zn oxide, Zn amino acid, and Zn proteinate) and doses (200, 400, and 600 mg/kg DM) of Zn supplementation had no impact on plasma IgG in Santa Inês sheep.

Plasma mineral concentration indicates the mineral intake of animals [8]. The current study demonstrated that supplementation of different levels and sources of Zn increased plasma Zn concentration, though no significant differences

were detected among the Zn-supplemented groups. Previous research by Aliarabi et al. [35] and Garg et al. [62] highlighted similar ranges of plasma Zn concentration in lambs fed on Zn-supplemented diets. Notably, the observed plasma Zn concentration in our study, aligning with the range reported by Underwood [63] and NRC [64] in goats, supported these earlier findings. Limited research has explored the effect of hydroxy Zn on plasma mineral concentration; however, Shaeffer et al. [15] found a significant increase in plasma Zn concentration in the group supplemented with hydroxy Zn compared to those receiving $ZnSO_4$. Similarly, in our study, plasma Zn level was higher in the ZnOHCl-supplemented group compared to the $ZnSO_4$ group at the same level of supplementation (80 ppm), although differences were not statistically significant. Other studies [4, 56] have also emphasized the significance of Zn supplementation (20–300 ppm), whether inorganic or organic, in elevating plasma Zn levels. Mallaki et al. [65] specifically highlighted the superior bioavailability of Zn peptide (ZnP) compared to Zn sulfate. However, another study [66] on calves reported contradictory results, suggesting a potential role of homeostatic mechanisms in regulating plasma Zn levels [67] under specific conditions.

Plasma levels of other minerals were found within normal physiological range and were not altered due to supplementation of different levels and sources of Zn supplementation in calves. Across different studies, including those by Garg et al. [62], Mishra [68], and Kincaid et al. [60], it is evident that Zn supplementation, whether inorganic or organic, did not significantly alter Ca levels in cattle and goats. Similarly, plasma Cu concentrations remained stable with Zn supplementation, as indicated by studies conducted by Parashuramulu et al. [6] and Fadayifar et al. [56]. The Fe levels also showed no substantial changes due to Zn supplementation, aligning with the results obtained by Parashuramulu et al. [6] and Aliarabi et al. [35]. Furthermore, plasma Mn concentrations were unaffected by Zn supplementation, aligning with results reported in studies conducted on calves and cashmere goats [69, 70]. In contrast to the present finding on plasma Ca level, Daghash and Mousa [55] reported that serum Ca concentration decreased in buffalo supplemented with Zn. Similarly, Jia et al. [70] reported that supplementation of 20 ppm Zn as $ZnSO_4$ or Zn meth decreased plasma Cu concentration in cashmere goats. Attia et al. [71] also reported that serum Cu concentration was decreased in buffalo calves supplemented with ZnO (250 or 1000 ppm), potentially due to the high levels of Zn supplementation used in that study. However, in the current study, Cu concentration remained unchanged, possibly due to the absence of antagonistic effect of Zn on Cu levels and the presence of a sufficient amount of Cu in the basal diet. Contrary to the present result, Garg et al. [62] and Wieringa et al. [72] found that dietary Zn supplementation (20 ppm and 10 mg/day) decreased plasma Fe concentration in lambs and human infants respectively.

The present study revealed supplementation of Zn significantly increased ($P < 0.05$) RBC Zn concentration. Among Zn-supplemented groups, RBC Zn concentration was higher in 80 mg Zn/kg DMI (ZnOHCl) compared to 80 mg Zn/kg DMI ($ZnSO_4$) and 40 mg Zn/kg DMI (ZnOHCl). Existing literature lacks comprehensive information on the influence of hydroxy Zn supplementation on RBC Zn concentration. Moreover, the literature [73] suggests that RBC Zn concentration can be used as a novel biomarker for assessing Zn status, which seems to be strengthened by the present finding. The results indicate that the increased RBC Zn concentration due to Zn supplementation may be related to higher retention.

In conclusion, results revealed that supplementing a basal diet containing 32.92 mg Zn/kg DM with either 40 mg Zn/kg DMI through ZnOHCl or 80 mg Zn/kg DMI through ZnOHCl or $ZnSO_4$ improved biomarkers of Zn status, antioxidant status, and immune response in pre-ruminant crossbred calves. Although no significant differences were observed among the Zn-supplemented groups overall, ZnOHCl supplementation resulted in higher levels of globulin, total protein, TAC, and RBC Zn concentration compared to $ZnSO_4$ at the same supplementation level. These findings indicate that supplementing the basal diet with either $ZnSO_4$ or ZnOHCl can enhance the Zn status of calves, and a dosage of 40 mg Zn/kg DM of ZnOHCl was found to be sufficient to achieve these improvements.

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Author Contribution RPP: data collection, data analysis, and writing. VM: conceptualization, methodology, and supervision. SHM: methodology, review, and editing. AS: data analysis, review, and editing. SS: writing, review, and editing.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

The animal care procedures were approved and carried out in accordance with the specified regulations of the Institutional Animal Ethics Committee (IAEC), established as per Article 13 of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) rules set forth by the Government of India.

Conflict of Interest The authors declare no competing interests.

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