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RESEARCH ARTICLE

Comparing the effect of different levels of zinc hydroxychloride with inorganic zinc sulfate on *in vitro* rumen fermentation parameters

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Abstract: Optimum productivity in ruminants is positively related to efficient rumen fermentation. Apart from major nutrients in diet, micronutrients (trace elements and vitamin B complex) play an essential role in maintaining proper rumen function; however the effect depends on source and level of micronutrients supplementation.Therefore, present study was conducted to compare the effects of different sources of hydroxy and inorganic zinc on *in vitro* rumen fermentation parameters. Different levels (0, 40, 80, and 160 ppm) of Zn as hydroxy zinc chloride ($Zn_5(OH)_8Cl_2$) and inorganic zinc sulfate ($ZnSO_4$) were added in substrate consisting of roughage (sugargraze fodder; a cross of sorghum and maize) and concentrate mixture in the ratio 50:50. Inclusion of Zn as zinc hydroxychloride and $ZnSO_4$ at different levels showed no change in total gas (mL/g DM) production. Similarly, supplementation of zinc either as zinc hydroxychloide or $ZnSO_4$ upto160 ppm supplementation did not affect (P>0.05) *in vitro* dry matter and organic matter digestibility (%) of the diet. Furthermore, no significant effect was observed in the CH_4 (%, mL/24h and mL/100mg DM) and NH_3-N (mg/dL) and individual fatty acid concentrations with variable sources and levels of Zn supplementation. It can be concluded from the present findings that supplementation of both hydroxy Zn and inorganic Zn up to 160 ppm had no adverse effect on *in vitro* rumen fermentation. Hence, hydroxy Zn can be used as an alternative source of Zn in the diet of ruminants to conventional inorganic sources.

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Introduction

Increased rumen fermentation efficiency leads to improved growth and production in animals. Various micronutrients affect rumen fermentation (Rodriguez et al. 1995; Arelovich et al. 2000). Especially, trace minerals are associated with several enzyme complexes and affect metabolic utilization of major nutrients like carbohydrates and proteins. Among the trace minerals, zinc (Zn) is vital for both animals and rumen microorganism for proper metabolic functions. Irrespective of sources, Zn at higher concentration affects cellulytic and proteolytic activity of rumen microbes (Eryavuz and Dehority 2009; Karr et al. 1991). Feeding high levels of Zn in the form of zinc sulfate decresed rumen fermentation and protozoa numbers in steers (Froetschel et al. 1990). Previous research has shown that rumen microorganism require much lower doses of Zn than those typically present in ruminant diets (Hubbert et al. 1958; Martinez et al. 1970). However, recent studies (Nagalakshmi et al. 2016) showed that host animal require higher dose of Zn (80- 140 ppm) than ICAR recommendation to improve health and immunity. Generally, trace minerals derived from feedstuffs and supplemental sources are soluble in rumen environment. Requirement of Zn for rumen microorganism is full filled by Zn present in feeds and fodders. However, solubility of trace minerals can affect the total concentration of available mineral to rumen microorganism because, only soluble minerals are available for use or interactions in rumen. Higher concentrations of soluble Zn (150 µg/mL) in the rumen can decrease cellulose digestion (Eryavuz and Dehority, 2009). Inorganic Zn sources are extensively soluble in rumen environment (Spears, 2013). Recently, hydroxy trace minerals are introduced as a new source of mineral in livestock feeding as they have low solubility in water (Cao et al. 2013) and rumen pH than inorganic source (Shaeffer, 2006). Hydroxy trace minerals are produced by crystallization process. Due to its crystalline structure it does not dissociate at rumen pH thereby reducing the chances of adverse effects on digestibility and rumen fermentation at higher doses. Recently, Caldera et al. (2019) reported that Zn from zinc hydroxychloride (ZnOHCl) had low rumen solubility and less tightly bound to ruminal solid digesta compared to Zn from $ZnSO_4$. However, lesser rumen solubility of Zn from ZnOHCl may affect rumen fermentation in a different way than Zn available from inorganic sources. Therefore, hypothesis of the present study was that Zn from hydroxy source will not cause any negative effect on rumen fermentation parameters and fibre digestion compared to inorganic source and till date no research has been done to assess the effect of hydroxy Zn on rumen fermentation pattern.Therefore, present experiment was conducted to evaluate the effect of ZnOHCl supplementation on *in vitro* rumen fermentation parameters compared to $ZnSO_4$.

Materials and Methods

Substrate composition

To assess the effect of supplementing different levels of ZnOHCl and $ZnSO_4$, a substrate was prepared withsugargraze and concentrate in the ratio of 50:50. Four levels of Zn i.e. 0, 40, 80 and 160 ppm was added in the form ZnOHCl and $ZnSO_4$ was added in graded levels for assessing comparative effect on rumen fermentation and DM digestibility under *in vitro* conditions. Proximate principles (AOAC, 2005) and fibre fractions (Van Soest et al. 1991) were determined for concentrate mixture and sugargraze fodder.

Total gas production and methane

For estimating *in vitro* gas production (Menke and Steingass, 1988), rumen liquor was collected from two fistulated buffalo bulls maintained on a standard diet of 50% roughage and 50% concentrate. About 200±10 mg of moisture free substrate containing sugargraze and concentrate mixture was incubated in glass syringes of 100 ml capacity along with 30 ml of buffered rumen inoculum for 24 h at 39±0.5°C. After incubation, total gas production was calculated and subsequent blank corrections were made. Subsequent blank corrections were made by subtracting the amount of gas produced from incubation of syringes consisting of buffered rumen fluid without substrate for correcting gas production from fermentation of endogenous substrates. For estimation of methane (CH_4), representative gas was sampled from the headspace by an airtight syringe and injected into gas chromatograph fitted with a flame ionization detector and stainless steel column packed with Porapak-Q. A standard mixture of 50/50 methane and carbon dioxide was used for calculating the concentration of CH_4 in unknown samples.

In vitro dry matter digestibility and microbial biomass production

For estimation of *vitro* true dry matter digestibility(IVTDMD), the pellets obtained after centrifugation of incubated samples werere fluxed with 40 mL of neutral detergent solution for an hour, filtered through G1 crucibles and residues were dried in hot air oven (80^{0}C). The loss in weight was considered as true dry matter digestibility. *In vitro* true organic matter digestibility (IVTOMD) was estimated by ashing the residue at 550°C in muffle furnace. The partitioning factor (PF) and microbial biomass production (MBP) were calculated based on truly degraded organic matter (TDOM) as described by Blummel et al. (1999) and Blummel et al. (2005), respectively. Where, TDOM was calculated by multiplying TOMD (%) by OM content (mg)of substrate. And PF was calculated by dividing TDOM (mg)/Net gas production (mL/24h) and microbial biomass production (MBP) from TDOM using equation:MBP (mg) = TDOM (mg) – (Net gas volume×2.20), where 2.20 is the stoichiometric factor.

Individual volatile fatty acids and ammonia nitrogen

After collectionof gas sample the contents of each syringe were centrifugedat 3000 rpm for 15 minutes to get a clear supernatant. An aliquot of the centrifuged supernatant along with equal volumes of 33% metaphosphoric acid was preserved at -20°C for determining individual volatile fatty acids (Erwin et al. 1961) using gas chromatograph fitted to a flameionization detector and stainless steel column packed with Chromosorb-101, whereas, another aliquot was used for estimation of ammonia nitrogen (NH_3-N) by Kjeldahl method (Sahoo et al. 2010).

Statistical analysis

The data of *in vitro* ruminal fermentation parameters was analyzed using one-way analysis of variance (ANOVA) by SPSS,16. In the case of significance (P<0.05) among treatments, Tukey's test was used to separate means.

Results and Discussion

Chemical composition of the substrate used as substrate has been presented in Table 1. All treatments had balanced CP and ME content except levels and sources of Zn. Four levels of Zn (0, 40, 80, 160 ppm) were added in the form of ZnOHCl or $ZnSO_4$. Effect of zinc hydroxychloride and $ZnSO_4$ on *in vitro* digestibility, *in vitro* gas production, pH, PF and MBP are presented in Table 2. No significant differences were observed in total gas production (IVTGP, mL/g DM), CH_4 (%), CH_4 (mL/100mg DM), IVDMD (%), IVOMD (%),PF and MBP (mg/g) among treatment groups. Supplementation of hydroxy Zn upto 160 ppm had no effect on NH_3-N and IVFA concentration (Table 3) and the results were similar to $ZnSO_4$.

No literature is available on the effect of hydroxy Zn on total gas production, *in vitro* digestibility and rumen fermentation; however, studies are available on inorganic and organic sources of zinc (Parashuramulu et al. 2013; Wang et al. 2013)

Similarly, Zaboli and Aliarabi (2013) reported that addition of 20 or 40 ppm Zn as ZnO and nanoZnO did not affect total gas production. Mallaki et al.(2015) also reported that 20 ppm Zn as $ZnSO_4$ had no significant effect on gas production, however, Zn supplementation in higher forage diet (68% forage) increased gas production (Armijo et al. 2011). In present study 50% concentrate and 50% forage were used as a substrate. The differences observed between studies could be due to the different ratio of concentrate: forage used in the diet. Chanzanagh et al. (2018) reported that addition of nanoZnO up to 60 ppm in protein sources did not affect total gas production. Contrary to our result, Parashuramulu et at.(2013) found that gas production increased with increasing Zn up to 150 ppm. In present experiment, total gas production was not changed due to lower solubility of hydroxy Zn in rumen and thereby reducing the interaction between Zn and rumen microbes. Zn sulphate also did not change gas production in rumen might be due no effect of Zn level used in the experiment. Methanogens are responsible for the production CH_4 in animals (Hook et al. 2010). Methanogenic archaea in anaerobic condition utilize CO_2 and H_2 to produce CH_4. Contrary to our result, at higher level of nZnO (nano zinc oxide) supplementation (1000 μg/g) reduced the enteric CH_4 concentration (Sarker et al. 2018) due to inhibitory action of Zn on methanogens. In present experiment, no change in CH_4 production might be due to no effect of addition of ZnOHCl and $ZnSO_4$ (up to 160 ppm) on methanogens.

Table 1 Ingredient and chemical composition of the substrate

Ingredient	Dry matter basis (%)
Dry matter	59.11
Organic matter	90.79
Crude protein	14.40
Ether extract	3.00
Total ash	9.20
Neutral detergent fiber	43.16
Acid detergent fiber	25.46
Cellulose	13.40
Hemicellulose	17.67
Zn (ppm)	32.55

Substrate; Concentrate: Sugargraze 50: 50

Armijo et al. (2011) in a study on goats also observed no differences in IVDMD when ruminal fluid was used in *in vitro* ruminal fermentation. Wang et al. (2013) and Kathirvelan and Balakrishnan (2008) reported decreased IVDMD after addition of 20 μg/mL Zn from Zn sulfate and 10 mg/kg Zn from Zn chloride in *in- vitro* cultures with forage based substrate (concentrate and roughage; 32: 68 and 0: 100) . Arelovich et al. (2008) also observed that IVDMD was decreased on addition of 5, 10, 15, or 20 mg/kg inorganic Zn in substrate containing prairie hay, which might be due negative effect of Zn on cellulolytic enzyme produced by rumen bacteria. Eryavuz and Dehority (2009) reported that decreased cellulose digestion with increased supplemental Zn (50 ppm) might be due to negative effect of Zn on cellulolytic enzyme produced by ruminal bacteria which leads to decreased IVDMD. In the present study, IVDMD did not change which can be due to no effect of hydroxy Zn addition up to 160 ppm on rumen microbe's function as requirement of Zn for rumen microbe are fulfilled by Zn present in basal diet. There was no significant effect in PF value and MBP among treatment groups. All these parameters were comparable to that of Zn supplementation in the form of sulphate. PF is the ratio of substrate degraded (mg) to the volume of gas (mL) produced (Blummel et al. 1999). Partitioning factor (PF) is a reliable determination of true degradability of the substrate, a range of 2.74 to 4.41 is indicative of efficient rumen fermentation (Sarkar et al. 2018) which is also observed in the present study.

Table 2 Effect of supplementation of zinc hydroxychloride and zinc Sulphate on *in vitro* fermentation of the substrate

	Supplemental Zn (ppm)								
Attribute	0	ZnOHCl			$ZnSO_4$			SEM	*P* value
Net gas (ml/24h)	38.50	38.33	37.83	39.17	37.83	37.17	37.83	0.28	0.700
Total Gas (mL/g DM)	187.02	188.32	187.45	192.01	184.71	183.43	185.67	1.17	0.620
Methane (%)	32.12	31.48	31.02	31.72	31.07	30.90	32.41	0.24	0.620
CH_4 (mL)	8.57	8.28	7.96	8.64	7.96	7.69	8.48	0.10	0.050
CH_4 (mL/100mg DM)	6.75	6.45	6.23	6.70	6.34	6.11	6.73	0.07	0.050
IVDMD %	62.49	64.37	64.02	63.96	65.27	64.09	63.86	0.30	0.420
IVOMD%	63.74	65.59	65.14	65.39	66.50	65.41	65.31	0.29	0.360
pH	6.27	6.46	6.53	6.52	6.42	6.66	6.44	0.04	0.280
PF	3.20	3.21	3.21	3.18	3.20	3.24	3.21	0.01	0.730
MBP (mg/g)	36.71	36.92	36.14	36.28	35.85	36.61	36.30	0.24	0.950

Mean values with different letters in a row differ significantly ($P < 0.05$),
CH_4-methane, IVDMD-*in vitro* dry matter digestibility, IVOMD-*in vitro* organic matter digestibility, PF-partition factor, MBP-microbial biomass production.

Table 3 Effect of supplementation of zinc hydroxychloride and zinc sulphate on *in-vitro* NH_3-N and individual volatile fatty acid (IVFA) of substrate

Attribute	Supplemental Zn (ppm)							SEM	*P* value
	ZnOHCl			$ZnSO_4$					
	40	80	160	40	80	160			
NH_3-N (mg/dL)	12.83	12.37	12.13	12.13	12.60	11.20	11.67	0.21	0.480
Acetate (C2), mM/L	34.31	32.59	33.75	34.10	34.11	34.74	31.78	0.50	0.770
Propionate (C3), mM/L	17.65	17.46	18.39	18.49	18.19	19.00	18.42	0.23	0.690
Butyrate (C4),mM/L	9.95	9.89	10.09	10.01	10.77	9.14	10.01	0.22	0.730
C2 : C3	1.94	1.87	1.86	1.85	1.88	1.83	1.74	0.04	0.930

Mean values with different letters in a row differ significantly ($P < 0.05$),

NH_3-N-ammonia nitrogen, C2:C3-acetate propionate ratio.

Ruminal pH mainly depends on NH_3-N degradation and volatile fatty acid formation from fermented substrate. Ruminal pH was unaffected by different levels and sources of Zn. Bateman et al. (2002) showed that addition of 1,350 mg Zn/kg DM did not alter pH in continuous cultures. Production of ammonia N in rumen is related to type of diet and result from breakdown of protein and non-protein sources in feed. Minimum level of NH_3-N (mg/dL) required for the growth of rumen microorganism is 5 mg/dL (Satter and Slyter, 1974). In present experiment, level of NH_3-N (mg/dL) was higher (11.20 to 12.83) than the minimum requirement for growth of rumen microbes. In this study, no significant effect was observed in NH_3-N levels in different treatments which indicated the balance betweenNH_3-N produced and microbial protein synthesised. Wang et al.(2013) also reported that addition of Zn upto 20 ppm µg/mL did not change NH_3-N concentration.

IVFA production result from degradation of fiber and other carbohydrate is related to type of diet. Therefore, present result showed that addition of either inorganic and hydroxy Zn upto 160 ppm did not change IVFAconcentration. Similarly, Zn supplementation @ 430 mg Zn/kg DM had no effect on VFA concentrations in a diet consisting of 61% concentrate and 39% chopped alfalfa hay under *in vitro* condition (Arelovich et al. 2008). Spears et al. (2004) supplemented 20 mg Zn/kg DM from inorganic and organic sources and observed increased propionate and decreased butyrate in case of Zn propionate compared to control and other Zn sources even with so low level of supplementation. Furthermore, higher dietary concentrations (1142 ppm) of inorganic Zn increased molar proportion of propionate (Blummel et al. 2005).

Conclusions

It was observed from the present study that Zn in the form of ZnOHCl and $ZnSO_4$ upto160 ppm had no adverse effect on rumen fermentation. Results of zinc hydroxychloride is comparable to effects of zinc sulphate, however, further *in vivo* studies are required to be conducted to ascertain the efficacy of zinc hydroxychlorideas a possible Zn source supplementation in ruminants.

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