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SHORT COMMUNICATION

Influence of nano zinc supplementation on digestibilty and rumen fermentation parameters under in vitro conditions

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Abstract: In vitro experiments were conducted to find out the effects of supplementation of nano zinc (nZnO) @ 0, 1, 5, 10, 20 and 40 ppm to substrate on digestibility and rumen fermentation parameters. The substrate consisted of concentrate mixture and maize fodder in the ratio of 40:60. The results showed that supplementation of Zn in the form of ZnO nanoparticle at 10 and 20 ppm level increased gas production, digestibility, ME values, acetate production and cellulose digestion under ***in vitro*** conditions.

Keywords: Nano Zn supplementation, in vitro digestibility, rumen fermentation

Among different essential trace minerals, zinc (Zn) is very important for all forms of life due to its role in gene expression, replication and part of many enzymes (Suttle, 2010). Bonhomme (1990) suggested that Zn is bound to the cell surface of rumen bacteria. Thus, Zn might be affecting the adhesion of microbial cells to cellulose particles. Martinez and Church (1970) showed Zn increased *in vitro* cellulose digestion. Woods (1965) suggested that the Zn requirement of rumen microorganisms for optimum cellulose digestion was less than 1 ppm. Protozoal growth (*Entodinium* sp.) was stimulated at Zn level of 5-10 ppm. Therefore, protozoa incorporated Zn easily and were intolerant to high Zn level, however, Zn did not penetrate bacterial cell readily and reduced metabolic activity. Eryavuz and Dehority (2009) found that 50 ppm level of Zn supplementation reduced cellulose digestion at 24 hours. Gupta (2016) reported that the bioaccessibility of Zn was highest in mustard seed cake (58.94 ppm) followed by cotton seed cake (38.93 ppm) and wheat straw (16.67 ppm) under *in vitro* conditions. In vitro DM digestibilty (IVDMD), *in vitro* OM digestibility (IVOMD), gas volume, metabolizable energy (ME) and short chain fatty acids (SCFA) contents were higher ($P<0.01$) in Zn supplemented diets (Parshuramalu et al. 2013). Aliarabi (2006) reported that Zn supplementation upto 120 ppm, either inorganic or chelated form, did not show significant effect on rumen fermentation parameters, however, negative effect was seen on IVDMD and IVOMD at 160 ppm level. Though study on different forms and sources of Zn are available but very few studies have been conducted regarding effect of nano Zn supplementation on digestibilty and rumen fermentation parameters in ruminants.

Samples of concentrate mixture and maize fodder were collected from Livestock Research Centre of ICAR-National Dairy Research Institute, Karnal, Haryana. The samples were dried in hot air oven at 65°C for 2 days and a constant weight was attained. The dried samples were ground through 1 mm sieve using electrically operated Willey mill. The basal substrate was prepared using concentrate mixture (% parts: maize 36, groundnut cake 10, full fat soya 15, wheat bran 18, de-oiled rice bran 18, mineral mixture 2 common salt 1) and maize fodder in 40: 60 ratios on DM basis. The proximate principles (DM, OM, CP, EE and total ash) in feeds were determined (AOAC, 2005) while cell wall constituents (NDF and ADF) were analysed as per Van Soest et al. (1991). The Zn contents in feeds were estimated using atomic absorption spectrophotometer (ZEEnit-700P) at ICAR-Central Soil Salinity Research Institute, Karnal, Haryana.

In vitro trials were conducted to estimate gas production (IVGP), true dry matter digestibility (TDMD), true organic matter digestibility (TOMD), microbial biomass production (MBP), pH,

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ammonia nitrogen (NH_3-N), individual volatile fatty acids (IVFAs) and cellulose digestion. The basal substrate used in this experiment consisted of dried ground maize fodder and concentrate mixture (% parts: maize 36, groundnut cake 10, full fat soya 15, wheat bran 18, de-oiled rice bran 18, mineral mixture 2 common salt 1) in the ratio of 60: 40. The substrate was supplemented with nano Zn (nZnO) @ 0, 1, 5, 10, 20 and 40 ppm in treatments T_1, T_2, T_3, T_4, T_5 and T_6, respectively.

For in vitro studies, rumen liquor was collected from 3 adult male Murrah buffalos maintained to meet the nutrient requirement (ICAR, 2013) before morning feeding and watering into a pre-warmed thermos flask and brought to the laboratory. Total gas production (Menke and Steingass 1988), true DM and OM digestibility (TDMD and TOMD) were estimated (Van Soest et al. 1991). Metabolizable energy (ME) of feedstuff was calculated using the prediction equation of Menke and Steingass (1988). The pH of strained rumen liquor was estimated (HANNA Instruments, USA).

Microbial biomass production (MBP) was calculated using data of TDOM and net gas volume (Blummel et al. 1997; Blummel and Lebzien 2001). For estimation of NH_3-N, 5 mL of acidified supernatant was mixed with 10 mL of NaOH (1 N) and immediately steam distilled using KEL PLUS® - N analyzer (Pelican, India). The NH_3 evolved was collected in boric acid solution (20% w/v) having mixed indicator and titrated against N/100 H_2SO_4 (AOAC, 2005).

For analysis of individual fatty acids (IVFA), the *in vitro* rumen fermentation was arrested by chilling at 4°C and the syringe contents were then centrifuged at 3000 rpm for 10 min. A portion of 5 mL of supernatant was added to 1 mL of 25% metaphosphoric acid and kept overnight at 4ºC (Patra et al. 2006). The mixture was centrifuged at 3000 rpm for 15 min. and 2 mL of supernatant was taken and stored at -20ºC for VFA analysis. The individual VFA in the samples were determined using Gas Chromatograph (Nucon 5700, Nucon Engineers, New Delhi) equipped with flame ionization detector and stainless-steel column packed with chromosorb 101 mesh 80-100 (length 1.5 m; o.d 3.175 mm; i.d. 2 mm). Analytical conditions for fractionation of VFA were as follows: Injection port temperature 210ºC, column temperature 180ºC and detector temperature 230ºC. The flow rate of the carrier gas N_2 was 40 mL/min). Individual volatile fatty acids (Acetate, propionate and butyrate) in the samples were determined on the basis of retention time and their concentration was calculated by comparing the retention time as well as the peak area of the standard after blank correction.

In vitro cellulose digestion was done using a basal purified cellulose medium contained the following ingredients per 100 mL:

1. 15 mL each of mineral solutions I and II (Bryant and Burkey, 1953)
2. 0.1 mL of 0.1g/100 mL resazurin solution
3. 25 mL of a 3 g/100 mL suspension of cellulose (Sigma, St. Louis, MO, USA)
4. 40 mL of strained rumen fluid
5. 3.33 mL of 12 g/100 mL Na_2CO_3
6. 1.67 mL of 3 g/100 mL cysteine hydrocholoride

An aliquote of 8 mL was tubed under O_2-free CO_2 into 16 × 150 mm culture tubes closed with rubber stopper and autoclaved in racks at 121°C for 20 min. (Dehority, 1969). A solution of 1 mL of either sterile distilled water or different concentration of nano zinc solution was added at the time of inoculation to make the final volume to 10 mL. The cellulose concentration in the final medium was 0.075 g/mL. The mixture was agitated for 3 min. under a vigorous stream of O_2-free CO_2.

After incubation, the entire contents of the culture tubes were transferred to a previously weighed test tube and centrifuged at 1000×g for 10 min. at room temperature (20-23°C). The supernatant was decanted and 5 mL of acid detergent solution were added (Van Soest, 1963). The tubes were mixed and heated on hot plate for 1 h at 100°C. The insoluble residue was centrifuged as stated earlier and supernatant was discarded. The sediment was washed twice with boiled distilled water. The tubes were dried overnight in an oven at 100°C, placed in a desiccator and weighed (Hiltner and Dehority, 1983). The cellulose digestion was based on the difference between the weight of cellulose measured in the blank tubes (0 h) and other tubes after 24 h incubation time.

Statistical analysis of experimental data was analysed by one way analysis of variance (ANOVA) model as per Snedecor and Cochran (1994). This statistical ANOVA model was incorporated with General Linear Models procedure (SPSS, 2012, version: 20).

The contents of OM, CP, EE, NDF and ADF in concentrate mixture were 93.95, 20.45, 5.02, 30.42 and 12.27% (DM basis) with the corresponding values of 89.13, 11.02, 1.65, 56.43 and 30.53% for maize fodder. The Zn content in concentrate mixture and maize fodder were found to be 24.40 and 21.78 ppm, respectively. The effects of nano Zn supplementation on values of in vitro gas production (IVGP), true DM digestibility (TDMD), true OM digestibility (TOMD), partitioning factor (PF), short chain fatty acid (SCFA), microbial biomass production (MBP) and metabolizable energy (ME) in different treatments have been presented in Table 1. The IVGP was higher ($P<0.05$) in treatments T_4 and T_5 and the lowest value was observed in treatment T_1. In contrary to our findings, nZnO with 20 or 40 ppm (Zaboli and Aliarabi, 2013) had no significant effect on gas production. The highest ($P<0.05$) values of TDMD (%) were recorded in treatments T_4 and T_5 and the lowest in treatment T_1. Similar results were obtained by Ahmed et al. (2022) where maximum digestibility was obtained at supplementary level of 30 ppm Zn. Zinc supplementation in form of proteinate, propionate (Nagalakshmi

et al. 2013) and Zn peptide (Mallaki et al. 2015) resulted in higher *in vitro* digestibility compared to $ZnSO_4$ addition (Arelovich et al. 2000) which might be due to more Zn and amino acid availability for rumen microbes. The TOMD values were lower ($P<0.05$) in treatments T_1, T_2 and T_3 compared to treatments T_4 and T_5. The enhancement in digestibility was reflected from increased total gas production. Similar findings were reported by Juncai et al. (2011) using nZnO upto 400 ppm, A significant increase in PF value was seen in treatment T_1 lower in treatments T_4 and T_5. The lower PF values indicate more gas production from the feed which signifies less DM intake by the animal with better performance (Blummel et al. 1997). The level of SCFA was significantly ($P<0.05$) higher in treatments T_4 and T_5 and lowest in treatment T_1. Blummel and Orskov (1993) observed a high significant correlation between SCFA and gas production. SCFA is directly related with gas production. Addition of inorganic Zn (Parshuramalu et al. 2013) and Zn peptide (Mallaki et al. 2015) increased short chain fatty acid (SCFA) level. The MBP value was lower ($P<0.05$) in treatments T_4 and T_5 compared to treatments T_1 and T_2. Microbial biomass is the major source of protein for the ruminant animals which is a source of truly available protein post ruminaly. *In vitro* gas production reflects primarily SCFA production and an inverse relationship exist between SCFA and microbial efficiency (Blummel et al. 1997). The ME value was the lowest in treatment T_1 and the highest in treatments T_4 and T_5. Supplementation of inorganic Zn (Parsurammalu et al. 2013), organic Zn (Nagalakshmi et al. 2013), Zn peptide (Mallaki et al. 2015) enhanced the ME value of the feeds.

The average values for pH, ammonia nitrogen (NH_3-N), individual fatty acids (IVFA; acetate, propionate and butyrate), IVFA (mol/100 mol) and A: P ratios have been presented in Table 2. The range of pH was found to be 6.70 to 6.83 in different treatments and the values were similar. Juncai et al. (2011) and Hassan et al. (2019) reported that supplementation of nano Zn had no effect on rumrn pH. The value of NH_3-N ranged from 23.38 to 24.47 mg/

Table 1: In vitro gas production, digestibility, microbial biomass production and ME values as affected by different levels of nano Zn supplementation

Parameter	Level of nano Zn (ppm)					
	T_1	T_2	T_3	T_4	T_5	T_6
$IVGP_{24h}$(mL/g)	$149.40^{c}\pm0.10$	$169.59^{b}\pm0.65$	$170.07^{b}\pm0.93$	$177.11^{a}\pm1.47$	$179.68^{a}\pm2.01$	$167.90^{b}\pm1.65$
TDMD (%)	$66.41^{c}\pm0.47$	$67.91^{b}\pm0.38$	$68.41^{b}\pm0.36$	$70.67^{a}\pm0.22$	$70.47^{a}\pm0.46$	$69.52^{ab}\pm0.49$
TOMD (%)	$69.24^{c}\pm0.49$	$69.49^{c}\pm0.10$	$69.94^{bc}\pm0.12$	$71.95^{a}\pm0.07$	$72.49^{a}\pm0.18$	$71.41^{ab}\pm0.19$
PF	$4.01^{a}\pm0.09$	$3.86^{ab}\pm0.05$	$3.75^{ab}\pm0.07$	$3.61^{b}\pm0.07$	$3.64^{b}\pm0.08$	$3.80^{ab}\pm0.02$
SCFA (mmol)	$0.70^{b}\pm0.02$	$0.75^{ab}\pm0.01$	$0.76^{ab}\pm0.02$	$0.80^{a}\pm0.02$	$0.79^{a}\pm0.02$	$0.76^{ab}\pm0.01$
MBP (mg/g)	$283.23^{a}\pm7.90$	$281.48^{a}\pm4.65$	$264.39^{ab}\pm6.47$	$255.07^{b}\pm8.03$	$255.40^{b}\pm8.69$	$273.93^{ab}\pm2.56$
ME (MJ/kg)	$7.53^{c}\pm0.12$	$7.86^{b}\pm0.06$	$7.91^{b}\pm0.10$	$8.17^{a}\pm0.09$	$8.24^{a}\pm0.08$	$7.81^{b}\pm0.03$

[a,b,c]Values bearing different superscripts in a row differ significantly ($P<0.05$)

Table 2: The effect of nano Zn supplementation on rumen pH, ammonia-N and volatile fatty acids under in vitro conditions

Parameter	Level of nano Zn (ppm)					
	T_1	T_2	T_3	T_4	T_5	T_6
pH	6.70±0.03	6.73±0.06	6.77±0.03	6.77±0.02	6.83±0.04	6.67±0.02
NH_3-N (mg/dL)	23.43±0.42	23.86±0.25	23.38±0.35	23.52±0.13	23.89±0.42	24.47±0.23
			IVFA (mM)			
Acetate	$39.13^{c}\pm1.06$	$45.65^{b}\pm1.20$	$54.47^{a}\pm0.76$	$57.57^{a}\pm1.14$	$57.90^{a}\pm1.24$	$56.24^{a}\pm0.99$
Propionate	15.93±0.55	16.68±0.72	17.00±0.70	17.20±0.64	17.92±0.62	18.35±0.65
Butyrate	6.60±0.40	6.61±0.42	7.77±0.32	7.37±0.19	7.52±0.87	7.72±0.37
A: P ratio	$2.46^{c}\pm0.07$	$2.75^{bc}\pm0.08$	$3.23^{ab}\pm0.13$	$3.37^{a}\pm0.17$	$3.25^{ab}\pm0.13$	$3.08^{ab}\pm0.14$
			IVFA (mol/100mol)			
Acetate	$63.44^{c}\pm0.70$	$66.24^{bc}\pm0.42$	$68.78^{ab}\pm0.61$	$70.08^{a}\pm0.96$	$69.48^{ab}\pm1.32$	$68.32^{ab}\pm0.85$
Propionate	$25.81^{a}\pm0.56$	$24.18^{ab}\pm0.65$	$21.43^{bc}\pm0.70$	$20.95^{c}\pm0.79$	$21.48^{bc}\pm0.58$	$22.29^{bc}\pm0.75$
Butyrate	10.75±0.75	9.58±0.50	9.79±0.29	8.97±0.22	9.04±1.08	9.39±0.49

[a,b,c,d] Values bearing different superscripts in a row differ significantly ($P<0.05$)

dL in different treatments. Similar report (Hassan et al. 2019) exist using sheep rumen liquor under in vitro. Juncai et al. (2011) found that the concentration of NH_3-N decreased ($P<0.05$) with the supplementation of 100 ppm nano ZnO. In contrary to this, addition of Zn decreased the NH_3-N released in vitro (Arelovich et al. 2000) and rumen fluid of sheep (Rodriguez et al. 1995). At the 6 and 12 h of incubation in vitro, the supplementation levels of 100 and 200 mg/kg of nano-zinc oxide considerably ($P<0.05$) reduced the concentration of NH_3-N and the ratio of acetate to propionate (Chen et al. 2011)

The values of acetate production increased in treatments T_3, T_4, T_5 and T_6. Juncai et al. (2011) also reported that supplementation of nano ZnO under in vitro rumen conditions increased VFA production. Chen et al. (2011) showed that VFA production increased ($P<0.05$) with the supplementation levels of 100 and 200 mg/kg of nano-zinc oxide at the 6 and 12 h of incubation in vitro. In contrast, Aliarabi (2006) and Hassan et al. (2019) reported that Zn supplementation either in inorganic or chelated and Nano Zn form, respectively did not show significant effect on rumen fermentation parameters. Propionate production ranged from 15.93 to 18.35 mM and value of butyrate production varied from 6.60 to 7.72 mM in different treatments. Spear et al. (2004) reported that propionate was higher ($P<0.05$) and butyrate was lower ($P<0.05$) in steers fed Zn-Met compared to $ZnSO_4$ diets. A significant ($P<0.05$) increase in the A: P value was seen in treatment T_4 and lower value in treatment T_1. However, Juncai et al. (2011) reported that at 50 ppm and Hassan et al. (2019) reported that at dose from 20 to 60 ppm of nano ZnO supplementation A: P ratio was same but higher than control group.

In vitro cellulose digested was lower ($P<0.05$) in treatment T_1 and higher at 10 and 20 ppm level of supplementation. Similar value of pH was seen in different treatments. Addition of Zn upto 10 ppm increased *in vitro* cellulose digestion (Martinez and Church 1970; Little et al. 1958). Further supplementation of 20 and 30 ppm of added Zn resulted in a decrease ($P<0.05$) in cellulose digestion. Eryavuz and Dehority (2009) found that 50 ppm Zn supplementation to the cellulose media reduced cellulose digestion. The adhesion of cellulolytic bacteria to cellulose is a critical early step in cellulose fermentation. Bonhomme (1990) suggested that Zn is bound to the cell surface of bacteria. Thus, Zn might be affecting the adhesion of microbial cells to cellulose particles.

Conclusion

Therefore, inclusion of Zn in form of ZnO nanoparticle @10 and 20 ppm of basal substrate showed enhancement ($p<0.05$) in acetate production digestibility and ME contents.

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