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Effect of Zinc Supplementation on Urea Hydrolysis in an *In Vitro* Fermentation Using Rumen Liquor

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ABSTRACT

Rapid hydrolysis of urea in the rumen is the principle cause for urea (ammonia) toxicity. Efforts were directed to retard urea hydrolysis by supplementing zinc at gradual levels viz, 0, 5.0, 10.0, 15.0 and 20.0 ppm in one ml solution to 35 mg of urea in one ml solution, and in one ml distilled water with 40 ml buffered rumen liquor in 100 ml syringe fitted with butyl rubber cap and incubated at 39°C. The 35 mg of urea per 43ml of liquor in the in vitro batch culture is equivalent to toxic dose of 100 mg per 100ml of rumen liquor in adult cattle. The incubation was carried out in an anaerobic environment at pH of 6.8. Five replications were conducted twice, resulting in ten replications in each treatment. The residual urea retained in each tube at 0, 1, 2 and 3 hour intervals from the respective aliquot (1 ml) was measured at wavelength of 520 nm. Highest residual urea ($P<0.01$) was observed in 10 ppm zinc supplementation over the rest of the treatments imposed across incubating hours. The residual urea (mg/dL) at the end of 1, 2 and 3 hours of incubation were 28.99± 1.04, 18.33± 0.04 and 15.45± 0.18 respectively at 10 ppm of zinc compared to 18.95± 0.38,10.00± 0.16 and 7.48 ± 0.12 in control (0 ppm). The result divulged that 10 ppm zinc was able to effectively retard the urea hydrolysis up to 3 hours, reflecting its effect on the extent of duration. Though the results demonstrated the superiority of 10 ppm zinc treatment over the rest of the treatments in retarding urea hydrolysis, yet another experiment was conducted to further improve the precision at a pH of 7.4 that is considered to be favorable environment for ammonia toxicity. The second trial was conducted following the same procedure except that of the level of zinc supplemented. In this experiment, zinc was supplemented at 0, 7.5, 10.0 and 12.5 ppm to 35 mg of urea with 40 ml of buffered rumen liquor incubated for 0, 1, 2 and 3 hours. The intial pH was brought to 7.4 by addition of a suitable quantity of soda bicarbonate to simulate a conducive environment for free ammonia production. The results of this experiment further strengthened the previous experiment's results with a significantly ($P<0.01$) higher residual urea in 10 ppm against the rest of the treatments. Thus it can be concluded that supplementation of 10 ppm zinc delayed the hydrolysis of urea.

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INTRODUCTION

Low cost non-protein nitrogen (NPN) supplements provide an effective means of enhancing animal productivity through increased intake and utilisation of low quality roughages. The use of urea supplements has become more relevant and widely used because it is an economical source of non-protein nitrogen for conversion into protein by the rumen microorganisms. However, rapid hydrolysis of urea leads to faster rate of ammonia release, which can result in ammonia toxicity thereby, limiting its utilisation in ruminants (Doyle, 1987).

Many attempts have been made to improve the utilisation of urea by reducing its rate of ammonia production to match the rate of assimilation by rumen microbes. Decreasing the rate of ammonia release from urea can prove beneficial in two ways: 1) to avoid ammonia spikes and consequent loss from the rumen and; 2) to maintain ruminal ammonia at adequate levels for longer post-feeding time. The rate of ammonia release can be controlled either by decreasing the activity of rumen urease (by the use of the specific urease inhibitors), or by modification of urea into products which would release ammonia slowly.

Various methods to modulate urea degradation have been developed. These include complexing or coating urea with a variety of compounds such as oils, carbohydrates and treatments with formaldehyde or acids (Makkar and Negi, 1988; Jinderpal and Kaushal, 1993). Some of these studies showed inconclusive results or no marked difference compared with feeding untreated urea (Doyle, 1987). Hence the alternative approaches of decreasing the urease activity in the rumen are needed. One such alternative is based on the fact that an elevated concentration of zinc could inhibit ammonia accumulation from urea by inhibiting urease enzyme activity in the rumen (Spears and Hatfield, 1978). The objective of this study was to evaluate efficiency of zinc on urea hydrolysis and to find out the ideal level of zinc in inhibiting ammonia spikes from urea.

MATERIALS AND METHODS

The inorganic salt of zinc chloride was used to study the effect of zinc in retarding urea hydrolysis. The zinc content in all the rumen liquor used in the experiments was analysed by atomic absorption spectrometer (AAS) Model 3100 (PerkinElmer,Inc, USA) to judge the extent of zinc contribution.

The method was essentially adapted from Tilley and Terry (1963), wherein the incubation was carried out in 100ml glass syringes fitted with butyl rubber caps. The syringes were pre-warmed at 40°C prior to incubation and were incubated at 39°C in shaking water bath, specially designed to accommodate 100 syringes in vertical positions in a perforated stand. The perforated stands were made to shake 75 times/minute. The rumen liquor was obtained from three cattle maintained on grazing and pooled to ensure optimal cellulolysis. The ruminal fluid was filtered through four layers of cheesecloth, mixed with buffer solution (McDougal's buffer) in the ratio of 1:1 and bubbled with carbon dioxide. The entire laboratory handling of rumen fluid was conducted under continuous flushing with CO_2. The buffered rumen liquor pH was adjusted to 6.8. The effect of zinc at serial increments on 35 mg of urea was observed and the residual urea was measured at periodic intervals of 0, 1, 2 and 3 hours in two sequential experiments. In the *in vivo* experiments, 100 mg of urea/100ml of rumen fluid in adult cattle was considered to be toxic (Owens and Zinn, 1993). Considering the rumen volume, outflow rate and level of intake, the equivalent of a toxic dose in the rumen of the live animal at *in vitro* studies is 35 mg

of urea per 43 ml in batch culture (Arelovich *et al.*, 2000). The artificial saliva was constituted following McDougall's (1948) as follows:

Composition	**(g/litre)**
$NaHCO_3$	9.80
Na_2HPO_4 $2H_2O$	7.00
KCl	0.57
NaCl	0.47
$MgSO_4.2H_2O$	0.12
$CaCl_2$	0.04

The above chemicals, except $CaCl_2$, was mixed in about 800 ml of distilled water in a volumetric flask (1 litre) and stirred to dissolve, making up the volume. Calcium chloride was added just prior to use, kept at 39°C and carbon dioxide was passed through the solution. The pH was adjusted to 6.8.

EXPERIMENT 1: Studies on the effect of zinc at 0, 5.0, 10.0, 15.0 and 20.0 ppm on ruminal urea hydrolysis

In this experiment there were 5 treatments (0, 5.0, 10.0, 15.0 and 20.0 ppm of zinc) with 5 replications that were studied at one-hour intervals for 3 hours. Two runs were carried out, resulting in ten replications for each treatment. In each prewarmed 100 ml syringe fitted with butyl rubber caps on the nozzle, 40 ml buffered rumen liquor with 1ml distilled water (to accommodate additives for future experiments), 1 ml of urea solution (containing 35 mg of urea) and 1 ml of zinc solution (containing 0, 5, 10, 15 or 20 ppm of zinc) were added and incubated at 39°C in a shaking water bath, which was shaken horizontally at 75 times per minute. The butyl rubber cap on the syringe nozzle served to maintain an anaerobic condition within the syringe as well as facilitated the withdrawal of sub samples by piercing with 20 G needles fitted to another 2 ml syringe.

At the end of incubation, one ml of the aliquot was transferred into centrifuge tubes and immediately immersed in ice to stop fermentation. The samples were centrifuged at 13600 G for about one minute (Crocker, 1967) and refrigerated for urea analysis. Twenty microliters of the sample was analysed colorimetrically for residual urea using a blood urea-N kit in Perkin Elmer UV- Vis spectrometer at a wavelength of 520 nm. The data generated were also examined for the percentage of urea disappearance at cumulative hour intervals to study the extent of prolonged effect of zinc.

EXPERIMENT 2: Studies to identify the precise level of zinc required to retard urea hydrolysis at pH (7.4) susceptible to ammonia toxicity

In order to identify the precise level of zinc concentration required to retard urea hydrolysis, at a pH susceptible to ammonia toxicity (Bartly *et al.*, 1976; Hare Singh and Lewis, 1977), the zinc concentration at around 10 ppm was evaluated by initiating the incubation at pH 7.4. The initial pH was brought to 7.4 by adding a suitable quantity of sodium bicarbonate. This experiment was precisely similar to that of the previous experiment except that the concentrations of zinc added were at 0, 5, 7.5, 10, 12.5 and 15.0 ppm. Each treatment had five replications with two runs.

Statistical analysis

Two factor ANOVA with replication statistical design was used as per the procedure of Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The mean ± SE of zinc content in the rumen liquor collected for all the experiments was 0.23 ± 0.13 ppm, hence it did not contribute any significant amount to

the present study. Similar reports on the negligible quantity of zinc in rumen liquor has been reported by Arelovich *et al.* (2000).

EXPERIMENT 1: Studies on the effect of zinc at 0, 5.0, 10.0, 15.0 and 20.0 ppm in retarding urea hydrolysis at pH 6.8 (Normal rumen pH)

The residual urea concentration ranged from 7.48 to 51.81 mg/dl across treatments and periods studied (Table 1). The values reported in this study concurred with Arelovich *et al.* (2000), who recorded 14.77 to 40.34 mg /dl of residual urea.

No significant difference was observed among various levels of zinc at the start of the experiment (0 h). At the end of the first hour, the highest ($P<0.01$) amount of residual urea was recorded in 10 ppm and 15 ppm compared to 5, and 20 ppm respectively, which in turn had significantly ($P<0.01$) higher residual urea than the unsupplemented group. However, at the end of the second hour incubation, the 15 ppm of zinc had significantly ($P<0.01$) lower residual urea compared to 10 ppm zinc, but it was comparable to 5 ppm. At the end of the third hour of incubation, the highest residual urea was observed in the 10-ppm zinc, which was significantly ($P<0.01$) higher than 5 or 15 ppm zinc. However, the latter was in turn significantly ($P<0.01$) higher then 20 ppm zinc. Thus, it is evident that 10 ppm of zinc was effective in reducing urea hydrolysis. Similar observations were made by Arelovich et al. (2000), who reported that supplementation with zinc at 10 to 15 ppm concentration inhibited in vitro urea hydrolysis. Lowered and controlled urea disappearance is desired, as it would avoid ammonia spikes resulting in urea toxicity. Further excess of ruminal ammonia can decrease N retention, reduce productivity (Doyle, 1987) and is proven to be toxic (Froslie, 1977).

Table 1. The effect of zinc at various levels (ppm) on the residual urea (mg/dl) at three hourly intervals (Mean ± SE)

Zinc level	*0 hour* ^{NS}	*End of 1 hour*	*End of 2 hours*	*End of 3 hours*
Experiment 1 (pH 6.8)				
0	49.62 ± 0.45	18.95^{a} ± 0.38	10.00^{a} ± 0.16	7.48^{a} ± 0.12
5	49.75 ± 0.58	24.52^{b} ± 0.26	15.54^{c} ± 0.56	12.51^{c} ± 0.08
10	51.42 ± 0.36	28.99^{c} ± 1.04	18.33^{d} ± 0.40	15.45^{d} ± 0.18
15	51.17 ± 0.98	26.73^{bc} ± 1.31	14.85^{bc} ± 0.45	12.47^{c} ± 0.09
20	51.81 ± 0.91	24.46^{b} ± 0.91	14.01^{b} ± 0.29	10.83^{b} ± 0.09
Experiment 2 (pH7.4)				
0	49.96 ± 1.05	34.76^{a} ± 0.12	26.24^{a} ± 0.55	21.20^{a} ± 0.53
7.5	50.36 ± 0.35	39.07^{b} ± 0.17	29.10^{b} ± 0.86	26.85^{b} ± 0.45
10.0	50.54 ± 0.31	40.25^{c} ± 0.51	30.95^{c} ± 1.19	29.19^{c} ± 0.01
12.5	49.41 ± 0.24	39.20^{bc} ± 1.46	29.37^{c} ± 1.02	27.12^{b} ± 0.68

Note:

- Mean of five observations
- Means bearing different superscripts within a column differ significantly ($P<0.01$) for each treatment
- NS - Non significant

The mechanism by which zinc exerts influence to inhibit urease secretion by the microorganism has been attributed to the fact that urease produced by the rumen bacteria is considered to be a metal inhibitory intracellular enzyme. It has been postulated that zinc ion forms an active complex with the enzyme to inhibit its activity (Jones, Macheod and Blackwood, 1964). The gradual decline in retarding urea hydrolysis by higher concentrations of zinc could be attributed to its toxic effect on rumen microbes (Martinez and Church, 1970) or its modifying effect on enzymatic action (Hare Singh and Cole, 1988)

Hence, it is concluded that zinc at 10 ppm attains the effective concentration to achieve the desired effect of regulating ammonia spikes from urea. The results of this study concurred with Rodriguez *et al.* (1995) who showed that zinc intake to the equivalent of 13.95 ppm improved urea utilisation and nitrogen balance in sheep.

The percentage of urea disappearance as altered by various levels of zinc at cumulative hour intervals is presented in Figure 1.

The percentage of urea disappearance at cumulative hours was calculated to evaluate the extent of the effect of zinc in preventing urea toxicity. It was observed that 10 ppm zinc continued to retard urea hydrolysis until the end of 3 hours. Similarly, zinc at 5 or 15 ppm was able to retard urea disappearance better than 0 and 20 ppm. The graph reveals that zinc at 10 ppm was able to reduce urea disappearance to the extent of 18 per cent over control in the first hour, which is the crucial hour for ammonia toxicity. Rodriguez et al. (1993) reported that zinc at 600 ppm

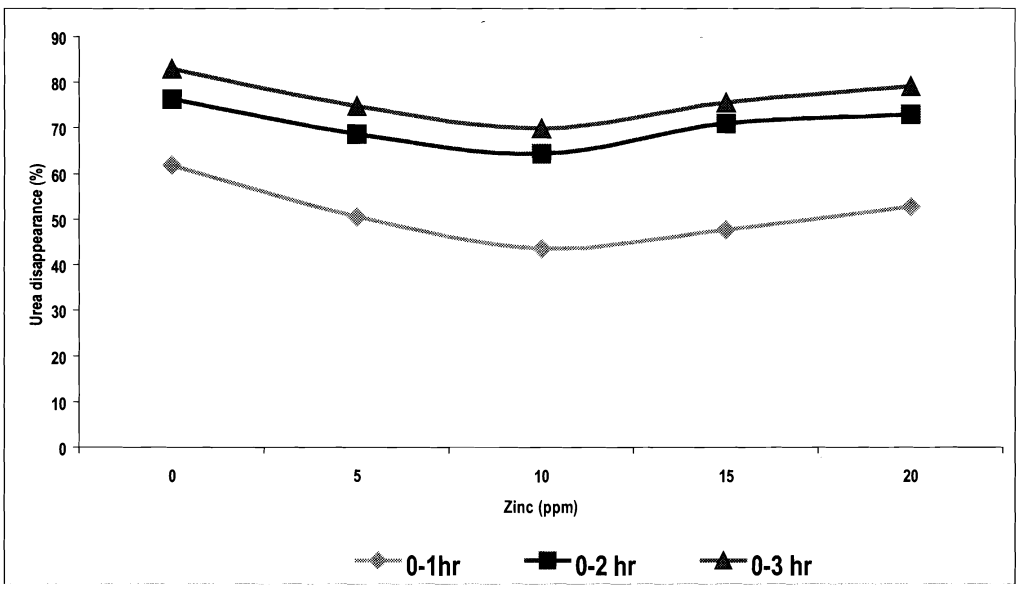

Figure 1. The effect of zinc at 0, 5, 10, 15 and 20 ppm on the percentage of urea disappearance at cumulative hour intervals in experiment 1

per day inhibits NH_3 production from urea to the extent of 24 per cent in in vivo incubation, which is equivalent to 13.8 ppm in in vitro study. However, further studies are required in order to pinpoint the precise level of zinc required to retard urea hydrolysis.

EXPERIMENT 2: Studies to identify the precise level of zinc required to retard urea hydrolysis at pH (7.4) susceptible to ammonia toxicity

Table 1 did not reveal any significant difference among different levels of zinc supplementation at the start of the experiment (zero hour). At the end of the first hour, zinc at 10 as well as 12.5 ppm significantly ($P<0.01$) retarded urea hydrolysis resulting in higher amounts of residual urea compared to 7.5 ppm of zinc supplementation. However, at the end of the second hour of incubation, zinc at 12.5 ppm and 7.5ppm had significantly ($P<0.01$) lower residual urea compared to 10 ppm of zinc supplementation. At the end of the third hour, the highest ($P<0.01$) residual urea was recorded in 10 ppm. The unsupplemented group (0 ppm) hydrolysed urea at a faster rate than the other levels of zinc supplementation.

It has been observed by Hare Singh and Lewis (1977) that rumen pH gets elevated to 7.41 in 60 minutes in toxic cases and is significantly higher then 6.8 to 7.0 for non-toxic cases. However, rumen ammonia concentration was the same in both toxic and non-toxic cases. Thus high concentrations of rumen ammonia obviously do not necessarily indicate ammonia toxicity. High rumen ammonia concentrations with high rumen pH would result in toxicity because the free ammonia concentration would be much higher at high pH, whereas at low pH ammonia is present as ammonium ion NH_4+. As the tissue membranes are permeable to the free NH_3 form and impermeable to the charged NH_4 form, higher levels of ammonia are absorbed at high pH than at low pH (Bartley *et al.*, 1976)

Lana, Russell and Van Amburgh (1998) reported that reduction in *in vitro* pH (6.8 to 6.0) also decreases ($P<0.001$) the rates of ammonia production. Similar in vitro results were also found by Van Kessel and Russell (1996)

Thus this experiment also demonstrated that 10-ppm zinc was effective in reducing the urea hydrolysis especially during the "golden hour" in urea toxicity even at the ammonia toxicity susceptible pH of 7.4.

CONCLUSION

The in vitro study conducted at equivalent toxic dose of urea revealed that 10 ppm of zinc supplementation to 35 mg of urea was capable of retarding urea hydrolysis to the extent of 18 per cent compared to 0 ppm zinc at the first hour of incubation, thus proving to be effective in handling ammonia toxicity. Zinc at 10 ppm level was able to demonstrate its superiority even at pH of 7.4 that is considered to be a favorable environment for ammonia toxicity.

Though 10 ppm zinc at in vitro is equivalent to a daily intake of 1.31 g in adult cattle to counter ammonia toxicity in vivo, further studies and validations are recommended.

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