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Influence of particle size distribution on *in vivo* and *in vitro* limestone solubility

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Abstract

A study was conducted to determine the *in vivo* and *in vitro* limestone solubility of different mixtures of small and large particle limestone. Small (0 - 1.0 mm) and large (2.0 - 3.8 mm) particles were blended to obtain five distribution mixtures consisting of 0%, 25%, 50%, 75% and 100% large particles limestone. Thirty three individually caged Lohmann-Silver laying hens were randomly allocated to each of the five treatments for the determination of *in vivo* limestone solubility. A hydrochloric acid (HCl) solution was used as solvent for *in vitro* limestone solubility determination. An increase in the percentage large particle limestone resulted in a significant increased *in vivo* and decreased *in vitro* limestone solubility. A significant increased intestinal limestone content (ILC) and decreased faecal limestone content (FLC) were observed with an increase in the percentage large particle limestone. These results suggest that an increased percentage of larger particle limestone in distribution mixtures, resulting in a prolonged retention time in the gizzard, could provide more Ca^{2+} to laying hens due to the increased *in vivo* limestone solubility rate thereof.

Keywords: Limestone particles, retention time, intestinal tract, laying hens
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Introduction

Various factors such as calcium source, pH of the digestive tract, retention time in the gizzard, particle size and the interaction of other nutrients, influence the solubility of calcium in chickens, especially in laying hens (Ajakaiye *et al*., 1997). Rabon & Roland (1985) suggested that the solubility of similar size limestone particles from different sources could vary as much as 62%, and that solubility could therefore be an important factor influencing eggshell quality and bone status of laying hens (Roland, 1986; Cheng & Coon, 1990; Rao & Roland, 1990). Crystal (2000) reported that a staggering 14.3 - 21.3% of the total eggs laid are cracked, resulting in enormous financial losses to egg producers worldwide. These financial losses due to improper eggshell quality could partly be ascribed to the variability of solubility between various limestone sources (Leeson & Summers, 1997) and the subsequent bio-availability of Ca^{2+} to the laying hen for eggshell calcification.

Cheng & Coon (1990) indicated that the optimum *in vitro* limestone solubility for bone ash concentration lies between 11% and 14% and suggested that this solubility value could be obtained by mixing the correct proportions of different particle sizes of limestone. However, much controversy remains in literature regarding the ideal mixtures of small and large particle limestone for optimum solubility. Roland (1986) suggested that $CaCO_3$ mixtures should contain 33 to 66% larger particles for optimal eggshell quality, whereas Marangos (2004) concluded that large particles (2.0 - 4.0 mm) should contribute between 60 and 70% to the total limestone particle mixture for an increased retention time in the gizzard. The work of Scheideler *et al*. (2005) illustrated that a mixture of 50% small and 50% large particle limestone will meet the calcium needs for optimum egg production and eggshell quality of laying hens, although this ratio differs between young and older hens and it needs to be altered during different production stages. These controversial reports on the ideal mixture of different particle size limestone contribute to the uncertainties faced by feed manufacturing companies, especially when formulating diets for laying hens of different ages.

Most feed manufacturing companies, situated in the inland areas of South Africa use a specific calcitic limestone source in their poultry diets. This limestone is supplied in a fixed variety of particle sizes and the distribution mixtures of small and large particle limestone included in poultry diets differ considerably among feed manufacturers due to a lack of existing guidelines. Furthermore, no information regarding the Ca solubility of this specific limestone source, blended in different mixtures of small and large particles is currently available. The aim of the present study was to determine the *in vivo* and *in vitro* limestone

solubility of a specific South African limestone source, when blended in different distribution mixtures of small and large particle sizes.

Materials and Methods

Limestone (360 g Ca/kg DM) with a particle size of small (0 - 1.0 mm) was blended with that of a large (2.0 - 3.8 mm) particle size, to obtain five distribution mixtures consisting of 0%, 25%, 50%, 75% and 100% large particles limestone. Thirty three 37 weeks old Lohmann-Silver laying hens, caged individually in metabolism cages were randomly allocated to each of the five treatments (n = 33/treatment) for the determination of *in vivo* limestone solubility according to the procedures described by Rao & Roland (1989).

Different limestone particle sizes were washed with deionized water to remove dust-size particles and dried overnight at 105 °C. Small and large limestone particles were thoroughly mixed by hand using 10 L plastic containers. To prevent segregation of particles, special care was taken by remixing small and large particles before each sample for the *in vivo* (10 g/sample) and *in vitro* (2 g/sample) study was weighed. Hens were fasted for 17 hours before the onset of the *in vivo* study. After fasting, five hens per treatment (n = 5/treatment) were randomly selected, sacrificed by cervical dislocation and their digestive tract contents were collected and individually stored at 5 °C. Digesta was examined to determine the intestinal limestone content that remained in the digestive tract after fasting.

On the first day of the experiment all the hens that ovipositioned between 7:00 and 9:00 (1^{st} oviposition) were intubated with a 10 g limestone sample/hen, using a plastic funnel attached to a polyvinyl tube. The limestone sample was gradually poured into the funnel and washed down with deionized water. After intubation, hens were supplied with a basal diet containing 211 g crude protein (CP)/kg, 14.27 MJ apparent metabolisable energy (AME)/kg and 4.3 g Ca/kg on dry matter (DM) basis for the duration of the trial. Excreta were collected in pre-weighed excreta trays after limestone intubation until the second oviposition period ended at 9:00 the following morning. On the second day of the experiment, all hens that ovipositioned between 7:00 and 9:00 (2^{nd} oviposition) were identified. Five hens per treatment (n = 5/treatment) with two consecutive ovipositions were randomly selected and sacrificed by cervical dislocation. Their digestive system contents were collected and individually stored for determination of intestinal limestone content (ILC). Excreta of the sacrificed hens were weighed, homogeneously mixed and sampled (40 g) for the determination of faecal limestone content (FLC).

The determination of ILC and FLC was done according to the decantation procedures described by Rao & Roland (1989). The digestive system contents were placed into a glass beaker with 250 mL deionized water and gently stirred until all clumps or aggregates were broken down. After the limestone particles settled at the bottom of the beaker, approximately two-thirds of the supernatant was removed. The beaker was refilled with deionized water to continue the stirring and decantation processes until all particles except the limestone were washed out of the beaker. The same procedures have been used to determine FLC of the excreta samples. Insoluble limestone particles (g) from the digestive tract and excreta were used to determine limestone solubility.

The procedures described by Zhang & Coon (1997) were used to determine the *in vitro* solubility of limestone. Pre-weighed glass flasks were filled with 200 mL of a 0.2N HCl solution to act as solvent for the limestone. Flasks containing the HCl solution were warmed for 15 min. at 42 °C in a water bath oscillating at 50 Hz until the temperature of the solution became constant. The limestone samples were poured into the solution and allowed to react for 10 min. After the 10 min. reaction time, limestone was filtered onto pre-weighed filter paper. Each treatment was replicated 19 times for statistical analysis. Limestone weight loss was used to calculate *in vivo* and *in vitro* solubility. Data was analyzed using a fully randomized one-way ANOVA design. The PROC ANOVA procedures of SAS (1999) were used to test for differences between treatment means. Tukey's studentized range (HSD) test was used to identify the differences between treatment means.

Results and Discussion

The effect of different distribution mixtures of limestone particles on *in vivo*-, *in vitro* solubility, ILC and FLC are presented in Table 1. An increased percentage of large particle limestone in the distribution mixtures resulted in an increased ($P < 0.05$) *in vivo* solubility. In contrast, the *in vitro* solubility of limestone decreased ($P < 0.0001$) with a proportionate increase in the percentage large particle limestone. An increased

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percentage large particle limestone also resulted in an increased ILC ($P < 0.0001$) and decreased FLC ($P < 0.0001$).

Table 1 The effect of different percentage large particle limestone on *in vivo*-, *in vitro* solubility, intestinal limestone content (ILC) and faecal limestone content (FLC) (Mean±s.e.)

Parameter	Large particles (%)	Mean ± s.e.	P	CV[1](%)
In vivo solubility (%)	0	56.13 [b] ± 1.17		
	25	57.04 [ab] ± 1.39		
	50	59.02 [ab] ± 1.72		
	75	61.77 [ab] ± 2.37		
	100	63.42 [a] ± 1.65	0.0324	6.43
In vitro solubility (%)	0	25.28 [a] ± 0.52		
	25	23.27 [b] ± 0.49		
	50	21.46 [c] ± 0.50		
	75	19.16 [d] ± 0.35		
	100	16.73 [e] ± 0.37	0.0001	9.31
ILC (g)	0	1.26 [d] ± 0.14		
	25	1.82 [cd] ± 0.10		
	50	2.23 [bc] ± 0.13		
	75	2.96 [ab] ± 0.17		
	100	3.59 [a] ± 0.38	0.0001	19.89
FLC (g)	0	3.68 [a] ± 0.12		
	25	3.01 [b] ± 0.08		
	50	2.56 [b] ± 0.11		
	75	2.03 [c] ± 0.12		
	100	1.64 [c] ± 0.15	0.0001	10.28

[a,b,c,d,e] Means ± s.e. within a column for the same parameter with different superscripts differ significantly at $P < 0.05$
[1] Coefficient of variation

Rao & Roland (1989) illustrated that the *in vivo* solubility of large particle limestone (2.0 - 5.0 mm) was significantly higher than the solubility of small particles (0.5 - 0.8 mm) limestone. Various other authors (Rao & Roland, 1990; Guinotte & Nys, 1991; Zhang & Coon, 1997) also reported that large (2.36 - 5.0 mm) compared to small (0.075 - 0.8 mm) particle size limestone resulted in a higher *in vivo* solubility. In the present study, the increased *in vivo* solubility observed with increased percentage of large particles could be ascribed to the prolonged retention time of larger particle limestone in the gizzard (Rao & Roland, 1990; Zhang & Coon, 1997). A longer retention time in the acidic conditions of the gizzard would ensure that more $CaCO_3$ dissipates into Ca^{2+} resulting in a higher *in vivo* solubility (Zhang & Coon, 1997; Jacob *et al*., 2003). In agreement to these findings, Hendrix-Genetics (2006) indicated that the bio-availability of Ca^{2+} at the end of the dark period is improved by using a larger particle size Ca source with a low *in vitro* solubility.

Guinotte *et al*. (1991) suggested that the surface area of large particle limestone is lower than that of small particles, resulting in a reduced reactive surface for HCl acid, therefore the proportionate decreased *in vitro* limestone solubility with an increase in percentage larger particles limestone. Iowa Limestone Corporation (ILC, 2005) stated that *in vitro* testing for limestone solubility will never completely simulate the *in vivo* solubility conditions, causing different solubility results for similar particle sizes of limestone. Factors such as retention time, physical movement of the gizzard, egg production status and Ca requirements of the hen as well as renal re-absorption of Ca cannot be simulated by *in vitro* techniques, contributing to the differences between *in vivo* and *in vitro* solubility results.

The increased FLC (3.68 ± 0.12 g) of birds consuming a distribution mixture containing 100% small (0 - 1.0 mm) particle size limestone was characterized by a decreased ILC (1.26 ± 0.14 g), illustrating the difference in retention time between small and large particle limestone. These results are in accordance with

that of Rao & Roland (1989), reporting that none of the small (0.5 - 0.8 mm) particles and 0.29 g of the large (2.0 - 5.0 mm) particle limestone remained in the digestive tract of birds after the first day of intubation.

Conclusions

In the present study, increased percentage large particles resulted in an increased *in vivo* and decreased *in vitro* solubility of limestone. These results confirm the negative relationship between *in vivo* and *in vitro* solubility of identical limestone particles, illustrating the differences between the respective techniques. Since *in vivo* solubility of limestone is a biological determinant, these results predict the effectiveness of limestone utilization by laying hens better than *in vitro* techniques. Results suggested that an increased percentage (±50 - 75%) of larger particle limestone (2.0 - 3.8 mm), with a prolonged retention time in the gizzard would provide more bio-available Ca^{2+} to laying hens, which could be used for shell calcification or bone mineralization. However, the effect of retention time and limestone solubility on egg production, eggshell quality and bone status of laying hens need to be further evaluated by a production study.

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