



Comparison of calcium absorption from nano- and micro-sized calcium salts using everted gut sac technique

RAKESH RANJAN¹, R K SAWAL², AMITA RANJAN³ and N V PATIL⁴

ICAR-National Research Centre on Camel, Jorbeer, Bikaner, Rajasthan 334 001 India

Received: 12 August 2018; Accepted: 19 September 2018

Key words: Absorption, Calcium, Nanoparticles, Poultry, Everted gut sac

Calcium (Ca) deficiency disorders, reported in almost all domestic animals occur when calcium present in the feed and fodder fails to meet their calcium requirement during different physiological status (Radostitis *et al.* 2000). Several calcium supplements are available in the market for treatment and prevention of these deficiency disorders. Nevertheless, therapeutic failure is a common complication perhaps, due to poor absorption efficacy of available calcium supplements. In cattle, oral calcium drenching has a mean efficacy of 50–60% in terms of milk fever prevention (Thilising-Hansen *et al.* 2002). Other disadvantages of available calcium supplements include high dose rate requirement, risk of change in gastric or ruminal pH and high cost (Vijaykumar and Balakrishnan 2014). Hence, developing an oral calcium supplement with high absorption efficiency, low cost and good patient acceptability is warranted.

Nanotechnology can help design drugs for many complex diseases that are otherwise difficult to cure till now (Convreur and Vauthier 2006). As nanoparticles can pass through biological membranes, high absorption efficiency of calcium nanoparticles as calcium supplement is expected. Calcium carbonate has the highest content of elemental calcium (40%) among all calcium salts (Hanzlik *et al.* 2005). It can serve as an excellent low cost oral calcium supplement. Phosphate supplements are common ingredients of mineral mixture available for veterinary use. The present study, therefore aimed to compare the absorption efficacy of nano and micro-sized calcium carbonate and calcium phosphate by everted gut sac technique.

The calcium carbonate nanoparticles were synthesized by controlled thermal decomposition of calcium oxalate as developed and standardized in our laboratory (Ranjan *et al.* 2018). The calcium phosphosate nanoparticles were synthesized by the method suggested by He *et al.* (2000).

Present address: ¹Senior Scientist (rakesh.ranjan@icar.gov.in), ²Principal Scientist (rksawal01@gmail.com), ⁴Director (nvpatil61@gmail.com). ³Assistant Professor (amita_pharma@rediffmail.com), Department of Veterinary Pharmacology and Toxicology, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan.

The synthesized nanoparticles were characterized by X ray diffraction (XRD) analysis, particle size analysis and scanning electron microscopy as per standard protocols. Micro-sized calcium carbonate (mean diameter 3752±637 nm) and calcium phosphate (mean diameter 4538±534 nm) were procured from SDFCL, Mumbai, India and characterized by XRD and particle size analysis.

White Leghorn broiler chicken (*Gallus gallus*), procured from the local market of Bikaner, Rajasthan were used in the experiment. Birds were fastened overnight, sacrificed and the intestine extending from the pyloric end to the ileocaecal junction was removed carefully. Everted intestinal sacs were prepared immediately after collection following the method described by Alam *et al.* (2012). The mesenteric attachments were removed and approximately 6 cm long duodenal segment was taken and flushed gently with ice cold saline. A plastic loop was pushed into the lumen of the intestinal segment until it appeared at the distal end. A knot was applied over intestinal segment on the loop and then the segment was slid down over the knot until it was completely everted. The empty sac was filled with 0.5 ml of Krebs-Ringer bicarbonate buffer (KRBB, pH 7.4) using a blunted syringe. The needle was then slipped off and the loose ligature on the proximal end was tightened.

Three filled everted sacs were then placed in KRBB (pH 7.4) added with either conventional or nanosized calcium carbonate or calcium phosphate at the rate of 2 mg/ml each and put over a stirring hot plate with temperature set at 40°C and aerated continuously using small air pump. The temperature was set 40°C to mimic the internal body temperature of broiler chicken. After a period of 15, 45 and 75 min of incubation, one sac each was removed, sectioned and the sac fluid was collected for analysis of calcium concentration. The experiment was replicated three times both for nano and micro-sized calcium carbonate and calcium phosphate. The calcium concentration in sac fluid before and after incubation was estimated by Arsenazo III colorimetric method, using kits supplied by Spinreact, Spain. Final Ca concentration in sac fluid was calculated as:

Ca conc. of sac fluid after incubation– Ca conc. in sac fluid before incubation].

The values (mean $\pm$ SE) obtained were compared by Student's T-test using Statistical Product and Service Solutions (SPSS), version 16.0 (SPSS Inc., China) statistical software. $P < 0.05$ was considered statistically significant.

The synthesized calcium carbonate nanoparticles were quasi-spherical in shape with mean diameter of 96.7 nm. The mean diameter of spherical calcium phosphate nanoparticles synthesized in the lab was 75.8 nm. Both were in dry fine powder form, stored at room temperature and used for the study within a week after synthesis.

The everted gut sac model is an efficient tool for studying *in vitro* absorption of nutrients and drugs (Alam *et al.* 2012). Calcium concentration in the sac fluid at different time intervals after incubation in KRBB added with calcium carbonate or calcium phosphate in micro-sized (group I) and nano-sized form (group II) is presented in Table 1. Ca concentration did not differ significantly between two groups after 15 min of incubation.

Table 1. Ca concentration in sac after incubation in micro- and nano-sized calcium carbonate and calcium phosphate

Time interval (min)	Calcium carbonate		Calcium phosphate	
	Gr I	Gr II	Gr I	Gr II
15	6.100 $\pm$ 0.416 ^{aA}	6.207 $\pm$ 0.241 ^{aA}	6.373 $\pm$ 0.418 ^{aA}	6.593 $\pm$ 0.377 ^{aA}
45	7.246 $\pm$ 0.035 ^{aB}	8.567 $\pm$ 0.260 ^{bB}	7.380 $\pm$ 0.232 ^{aAB}	8.893 $\pm$ 0.060 ^{bB}
75	7.477 $\pm$ 0.222 ^{aB}	9.070 $\pm$ 0.190 ^{bB}	8.480 $\pm$ 0.344 ^{aB}	9.763 $\pm$ 0.171 ^{bC}

Gr I, Micro-sized calcium salt; Gr II, Nano-sized calcium salt. Values with different superscript in small letter in a row (for a particular calcium salt) and capital letter in a column differ significantly ($P < 0.05$).

However, concentration was significantly higher in group II, 45 (18.23%) and 75 (21.39%) minutes after incubation as compared to corresponding values in group I. Likewise, Ca concentration in sac fluid after 15 min of incubation in micro-sized and nano-sized calcium phosphate solutions did not differ significantly. However, sac fluid Ca concentration in nano-sized calcium phosphate incubated sacs was 20.50 and 15.13% higher than those incubated in micro-sized calcium phosphate after 45 and 75 min of incubation, respectively.

Important calcium salts used as calcium supplements in veterinary and human medicine include calcium citrate, dicalcium phosphate, tricalcium phosphate, calcium carbonate and calcium formate. Cost of calcium supplements in poultry feeding is significant, hence various strategies have been explored in the recent past to reduce the calcium supplement cost in poultry and other livestock feeding. Increasing absorption efficacy of calcium supplements is one of the ways that can potentially reduce the input cost in poultry industry (Vijaykumar and Balakrishnan 2014). It is widely assumed that nanotechnological interventions can help enhance oral absorption efficiencies of nutrients and minerals. Several minerals in nanoform have been

synthesized and evaluated for their possible use in veterinary medicine (Swain *et al.* 2015). Calcium carbonate is a low cost calcium salt with highest percentage (40%) of elemental calcium among all other calcium salts. In a study, similar rise in serum calcium and an equivalent fall in serum parathyroid hormone was recorded after supplementation of tricalcium phosphate and calcium carbonate in both young and elderly individuals, suggesting calcium carbonate as calcium supplement is as good as tricalcium phosphate (Yang *et al.* 1994). Nano-sized calcium carbonate has very low toxicity potential as no-observed-adverse-effect levels of nanocalcium carbonate in mice was reported to be 1.3 g/kg body weight (Huang *et al.* 2009). Likewise, high dose level for 13 week repeated oral toxicity in rats was recommended as 1000 mg/kg (Jeong *et al.* 2015). Reducing the particle size will increase the surface area, solubility properties and dissolution rate according to the Ostwald-Freundlich equation, thereby increasing the bioavailability of the compound (Hassim and Rachmawati 2010). It was reported that food grade calcium carbonate nanoparticles had more efficient cellular internalization and elevated intracellular calcium ion levels in human intestinal epithelial cells than bulk- or reagent grade calcium carbonate (Kim *et al.* 2015). The authors further reported that calcium carbonate nanoparticles are more rapidly absorbed than food grade bulk calcium carbonate, but total absorption efficiency is not affected by the particle size. Recently, it has been reported that calcium absorption efficacy and bioavailability of calcium carbonate in ovariectomized (OVX) and OVX-osteoporosis rats can be enhanced by particle size reduction in nano forms (Erfanian *et al.* 2014). Likewise, calcium phosphate nanoparticles supplementation has also been reported to enhance the growth performance of broiler chicken (Vijaykumar and Balakrishnan 2014). However, the question as to whether the intestinal absorption of nanosized calcium phosphate and calcium carbonate are greater than those with larger particle sizes was not addressed in this report.

From the present study it can be concluded that calcium carbonate and calcium phosphate are more extensively absorbed in nano-sized forms than that in micro-sized forms. Hence, calcium salts in nano-sized forms may be more efficient for use in poultry and other livestock as oral calcium supplements.

SUMMARY

The present study aimed to compare the calcium absorption from calcium carbonate and calcium phosphate in nano and micro-sized forms using everted gut sac technique. It was found that calcium concentrations in sac fluid were higher after incubation in nano-sized calcium carbonate than micro-sized calcium carbonate by 18.23% at 45 min and 21.39% at 75 min. Likewise, Ca concentration in sac fluid was 20.50 and 15.13% higher in nano-sized calcium phosphate than those incubated in micro-sized forms at 45 and 75 min, respectively. Present study revealed that calcium carbonate and calcium phosphate are more

extensively absorbed in nano-sized forms than in micro-sized forms, suggesting their possible use as calcium supplements in poultry and livestock farming.

REFERENCES

- Alam M A, Al-Jenoobi F I and Al-mohizea A M. 2012. Everted gut sac model as a tool in pharmaceutical research: limitations and applications. *Journal of Pharmacy and Pharmacology* **64**: 326–36.
- Couvreur P and Vauthier C. 2006. Nanotechnology: intelligent design to treat complex disease. *Pharmaceutical Research* **23**: 1417–50.
- Erfanian A, Mirhosseini H, Manap M Y A, Rasti B and Bejo MH. 2014. Influence of nano-size reduction on absorption and bioavailability of calcium from fortified milk powders in rats. *Food Research International* **66**: 1–11.
- Hanzlik R P, Fowler S C and Fisher D H. 2005. Relative bioavailability of calcium from calcium formate, calcium citrate and calcium carbonate. *The Journal of Pharmacology and Experimental Therapeutics* **313**: 1217–22.
- Hassim A and Rachmawati H. 2010. Preparation and characterization of calcium carbonate nanoparticles. In: *Proceedings of the 3rd Nanoscience and Nanotechnology Symposium*, Bandung, Indonesia.
- He Q, Mitchell A R, Johnson S L, Bartak C W, Morcol T and Bell S J D. 2000. Calcium phosphate nanoparticle adjuvant. *Clinical and Diagnostic Laboratory Immunology* **7**: 899–903.
- Huang S, Chen J C, Hsu C W and Chang W H. 2009. Effects of nano calcium carbonate and nano calcium citrate on toxicity in ICR mice and on bone mineral density in an ovariectomized mice model. *Nanotechnology* **20**: 375102–08.
- Jeong A L, Kim M K, Kim H M, Lee J K, Kim Y R, Oh J M and Choi S J. 2015. The fate of calcium carbonate nanoparticles administered by oral route; absorption and their interaction with biological matrices. *International Journal of Nanomedicine* **10**: 2273–93.
- Kim M K, Lee J A, Jo M R, Kim M K, Kim H M, Oh J M, Song N W and Choi S J. 2015. Cytotoxicity, uptake behaviors, and oral absorption of food grade calcium carbonate nanomaterials. *Nanomaterials (Basel)* **5**: 1938–54.
- Radostits O M, Gay C C, Blood D C and Hinchcliff K W. 2000. *Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses*, 9th ed. W B Saunders Company Ltd, London.
- Ranjan R, Narnaware S D and Patil N V. 2018. A novel technique for synthesis of calcium carbonate nanoparticles. *National Academy of Science Letters* **41**(6): 403–06.
- Swain P S, Rajendran D, Rao S B N and Dominic G. 2015. Preparation and effects of nano mineral particle feeding in livestock: A review. *Veterinary World* **8**: 888–91.
- Thilsing-Hansen T, Jorgensen R J and Ostergaard S. 2002. Milk fever control principles: A review. *Acta Veterinaria Scandinavica* **43**: 1–19.
- Vijaykumar M P and Balakrishnan V. 2014. Effect of calcium phosphate nanoparticles supplementation on growth performance of broiler chicken. *Indian Journal of Science and Technology* **7**: 1149–54.
- Yang R S, Liu R K and Tsai K S. 1994. The acute metabolic effects of oral tricalcium phosphate and calcium carbonate. *Calcified Tissue International* **55**: 335–41.