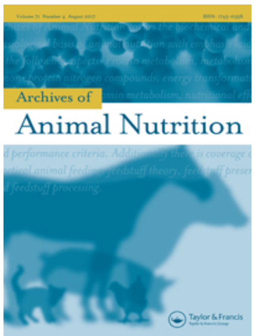

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Archives of Animal Nutrition

ISSN: 1745-039X (Print) 1477-2817 (Online) Journal homepage: http://www.tandfonline.com/loi/gaan20

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ARCHIVES OF ANIMAL NUTRITION, 2017
VOL. 71, NO. 4, 327–332
https://doi.org/10.1080/1745039X.2017.1331619

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RESEARCH NOTE

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Effect of copper nanoparticles administered *in ovo* on the activity of proliferating cells and on the resistance of femoral bones in broiler chickens

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ABSTRACT

The objective of this study was to evaluate bone resistance after *in ovo* administration of copper nanoparticles (NanoCu) and to determine the number of cells positive for proliferating cell nuclear antigen (PCNA) in the femoral bones of broiler chickens (n = 12 per group). The study demonstrated that femoral bones from the NanoCu group were characterised by a higher weight and volume and by significantly greater resistance to fractures compared to the Control group. NanoCu promoted the proliferation of PCNA-positive cells in the long bones of chickens. A significantly higher number of PCNA-positive cells in the bones of birds in the NanoCu group compared with the Control group (137 and 122, respectively) indicate a stimulatory effect during embryogenesis. Considering the improvement in bone resistance to fractures and the effect of NanoCu on the number of PCNA-positive cells in femoral bones, NanoCu may be an alternative agent to minimise the ever-present problem of weak bones in broiler chickens.

ARTICLE HISTORY
Received 17 March 2017
Accepted 15 May 2017

KEYWORDS
Bone density; bone formation; chickens; copper; *in ovo* administration; nanoparticles

1. Introduction

Today's conditions of rearing and nutrition as well as the genetic modification of broilers ensure, most of all, a high growth rate and muscle mass. However, rapid body weight gain and a lack of balance between muscle mass growth and bone mass increase the risk of deformations and fractures in various bones and cause health problems.

The resistance of bones is determined by many factors, such as genetics, age, sex, feeding, and flock management during rearing. Apart from the key impacts of calcium and phosphorus in the process of bone mineralisation, copper is an indispensable trace element that plays a significant role in maintaining healthy and strong bones.

Traditionally, inorganic copper salts have been used in feed formulation (Aksu et al. 2010), causing significant pollution to the soil and water (Jackson et al. 2003). The use

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of organic copper sources can reduce their excretion, due to increased stability in the upper gastrointestinal tract of the animal. Indeed, a number of trials have demonstrated greater bioavailability of organic copper compounds; however, the results are not consistent (Bao et al. 2009; Leeson 2009).

A proposed solution is the use of nanoparticles, which due to their physicochemical properties can be applied in much smaller doses than the bulk material. In the case of copper nanoparticles (NanoCu), it has been demonstrated that NanoCu administered *in ovo* decreases the metabolic rate of chicken embryos, but does not influence the immunity of hatchlings (Pineda et al. 2013). Furthermore, it has been shown that NanoCu stimulates the development of blood vessels in embryos to a greater extent than copper sulphate (Mroczek-Sosnowska et al. 2015). Recently, we have demonstrated that broilers that developed from embryos treated *in ovo* with NanoCu had a greater body weight gain and a better feed conversion rate than the control birds (Mroczek-Sosnowska et al. 2016).

NanoCu has different biological, chemical, and physical properties compared to the bulk form. The unique bioactivity of NanoCu is mainly due to the particle size and the large surface area. The size is important, for example, if penetration through a pore structure of a cellular membrane is required, thus affecting the number of proliferating cells (Salata 2004).

The objectives of this study were to evaluate bone resistance to fractures after *in ovo* administration of NanoCu and to determine the number of proliferating cells, indicated by proliferating cell nuclear antigen (PCNA) staining, in the femoral bones of broiler chickens.

2. Materials and methods

2.1. *Animals*

Eggs of 37-week old Ross 308 hens obtained from a commercial hatchery were randomly divided into two groups (2 × 120 eggs): Control (without injection) and NanoCu (injected with NanoCu). On the first day of incubation, 0.3 ml of experimental fluids were injected into the air cell of eggs from the NanoCu group using sterile syringes. Immediately after injection, the holes were sealed with sterile tape and the eggs were placed in an incubator. The eggs were incubated for 21 d under standard conditions. After hatching, standard assessment of 1-d chickens was performed by selecting at random healthy chickens with healed navels for rearing. In total, 160 broiler chickens were selected for further studies. They were allocated into two groups (Control and NanoCu), and kept on litter until 42 d of age. Stock density in the hen house was 11 birds per 1 m^2. A three-stage feeding programme was applied through rearing: starter (crumb), grower and finisher (granulate); the birds were fed *ad libitum*. Chickens of all groups were fed feed mixtures with the following composition: starter from d 1 to 14 (containing 22.2% protein, 0.36% lysine, 3.67% fat, 3.60% fibre, and 12.51 MJ metabolisable energy (ME); grower from d 15 to 35 (containing 20.8% protein, 0.34% lysine, 4.0% fat, 2.55% fibre, and 12.76 MJ ME); and finisher from d 36 to 42 (containing 18.5% protein, 0.28% lysine, 5.92% fat, 2.41% fibre, and 13.48 MJ ME). All diets contained about 30 mg copper from $CuSO_4$. For details of dietary composition confer Mroczek-Sosnowska et al. (2016). On d 42 of rearing, six hens and six cockerels with body weights similar to the mean body weight in the group were selected from each group. After slaughter, the femoral bones were dissected, cleaned mechanically from residues of meat tissue and prepared for further analyses.

2.2. *Experimental solutions*

The hydrocolloid of NanoCu, at a concentration of 50 mg/kg, was purchased from Nano-Tech, Warsaw, Poland, and was produced by a nonexplosive high-voltage method from high-purity metals (99.9999%) and high-purity demineralised water.

The zeta potential of the hydrocolloid was measured using a Nano-ZS90 Zetasizer (Malvern Instruments Ltd., Malvern, UK); the zeta potential of NanoCu was −28.1, indicating a stable solution. The shape and size of NanoCu were visualised using a JEM-2000EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV. The nanoparticles were spherical and rarely elliptical, rounded, with no sharp edges. The diameter of nanoparticles was on average 37.3 nm and varied from 15 to 70 nm.

2.3. *Analysis of parameters*

The bones were weighed, and the total length, the perimeter at ½ length and specified shear force were measured. The resistance to fractures was tested by a ZWICK type 1120 apparatus (Zwick Roell, Ulm, Germany), using a Warner–Bratzler type compressing element equipped in the head with a maximal force of 1 kN. The speed of the compressing element was 50 mm/min.

From the remaining part of the bones, specimens of osseous tissue were collected with a metal cutting saw and fixed in a solution of Schmorl's fluid for decalcification. Afterwards, the preparations were subjected to a standard procedure of embedding in paraffin. The embedded material was cut crosswise into 6–7-µm thick sections that were subjected to immunohistochemical staining with the use of an anti-PCNA monoclonal antibody, clone PC10 (DAKO, M0879, Glostrup, Denmark).

Microscopic measurements were taken using a Nikon Eclipse 90i light microscope with a Nikon Digital Sight DS-U1 camera (Nikon Corporation, Tokyo, Japan) and NIS-Elements AR 2.10 software (Nikon Corporation, Tokyo, Japan). Positively stained cells were calculated in each bird from both experimental groups (100 measurements). The positively stained cells were calculated based on a surface area of 20,400 μm^2 and then converted into 1 mm^2.

2.4. *Statistical analysis*

Data were normally distributed, and the results were analysed using a multifactor analysis of variance (least square method) using SPSS 21.0 software (SPSS, Chicago, IL, USA) at a significance level $p \leq 0.05$. The presented mean values are least square means.

3. Results and discussion

The present article is a continuation of the studies performed with embryos and growing chickens. Previously, it was demonstrated that *in ovo* administration of NanoCu did not influence embryo development. The body weight, organ weight and mortality of embryos were not different from the non-treated Control group (Mroczek-Sosnowska et al. 2015). Compared with the Control group, the chickens treated *in ovo* with NanoCu had higher final (d 42) body weight (2206 vs. 2000 g) and a better feed conversion rate (1.62 vs. 1.91). Furthermore, the content of breast muscles was significantly increased (Mroczek-Sosnowska et al. 2016).

The objective of this study was to determine the effect of NanoCu on the resistance of femoral bones and the activity of proliferating cells in broiler chickens. The measured parameters were not different between males and females, and the presented results are mean values for all chickens. The application of NanoCu on the first day of incubation led to changes in many physical characteristics of the femoral bones (Table 1). Significantly higher bone weight, length, and circumference were found in the NanoCu group compared to the Control group. Considering the resistance of bones, expressed by the value of the shear force (Table 1), bones from the NanoCu group had significantly greater resistance compared with bones from the Control group (228 and 172 N, respectively). Similar results, but for copper sulphate (1 ppm), have been demonstrated for laying hens (Opsahl et al. 1982). The change in the mechanical properties of the bones can be explained by a decrease in the content of the amino acid hydroxyl lysine, which occurs only in proteins constituting connective tissue. It might be suggested that enrichment of the poultry diet with NanoCu at the commercial scale may bring economic benefits due to a reduced number of bone fractures, which are today one of the main factors responsible for bird elimination from the flock.

Tissue regeneration begins with cell proliferation; thus, the identification of proliferating cells may be equivalent to the detection of progenitor cells in bone or cartilage (Iwaki et al. 1997). Several studies have shown that PCNA-positive cells are clearly correlated with cell proliferation (Dietrich 1993; Paunesku et al. 2001). Consequently, the detection of PCNA can be used to localise and quantify proliferating cells in the growing tissues or in the bone healing process. The obtained results demonstrate that the injection of NanoCu colloid on the first day of incubation led to significant differences in the number of proliferating cells. The significantly higher number of PCNA-positive cells (+ 12%) in the bones of birds in the NanoCu group compared with the Control group indicates a stimulatory effect of the administered NanoCu during embryogenesis (Table 2, Figure 1).

In the present study, NanoCu was injected *in ovo* at the beginning of embryogenesis; however, the question remains whether it would be possible to enrich the diet or the drinking water of breeding hens with NanoCu and thereby naturally transfer nanoparticles to eggs. Furthermore, also other treatments could be considered. It has been demonstrated that the supplementation of NanoCu to diets of piglets (Gonzales-Eguia

Table 1. Physical parameters of femoral bones in chickens treated with copper nanoparticles (NanoCu) (n = 12 per group).

	Experimental groups		
	Control	NanoCu	SEM*
Perimeter [mm]	27.0^{A}	29.0^{B}	0.52
Shear force [N]	172^{A}	228^{B}	9.3
Bone weight [g]	14.3^{A}	16.8^{B}	0.44
Length [mm]	85.7^{A}	86.0^{B}	0.67

*SEM, standard error of the mean.
^{AB}Means with different superscripts differ significantly at $p \leq 0.01$.

Table 2. Number of proliferating cells in femoral bones in chickens treated with copper nanoparticles (NanoCu) (means ± standard deviation, n = 12 per group).

	Experimental groups	
	Control	NanoCu
Proliferating cells	122 ± 26.4[a]	137 ± 18.0[b]

[ab]Means with different superscripts are significantly different at $p \leq 0.05$.

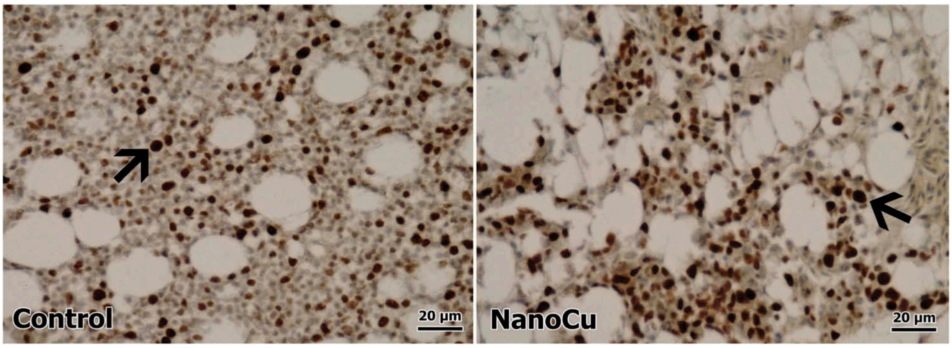

Figure 1. Section of bone marrow showing immunohistochemical detection of PCNA-positive nuclei (arrows).
Scale bars: 20 µm

et al. 2009; Wang et al. 2012), fishes (El Basuini et al. 2016), and broilers (Wang et al. 2011) improved animal performance. Moreover, the intramuscular injection of NanoCu enhanced the growth of broilers (Miroshnikov et al. 2015). Certainly, a follow-up research is necessary.

4. Conclusions

In ovo administration of NanoCu significantly improved the physical characteristics of bones. Furthermore, considering the increase in the number of proliferating cells in bones, NanoCu may be an alternative treatment to prevent the problem of weak bones in broiler chickens.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by grant 267659 “Gutfeed”, the National Centre of Research and Development, Poland.

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