Saudi Journal of Biological Sciences 27 (2020) 288–295

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Biosafety, and improvement of osteoporosis in cage layers through using chOPG protein

Lele Hou [a,b], Jiafa Hou [a,*], Zhenlei Zhou [a], Yifeng Deng [a], Dawei Yao [a]

[a] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210000, China
[b] Animal Husbandry and Veterinary Research Institute of Qingdao, Qingdao 266000, China

ARTICLE INFO

Article history:
Received 5 August 2019
Revised 11 September 2019
Accepted 12 September 2019
Available online 13 September 2019

Keywords:
pcDNA3.1(+)-chOPG
Bone metabolism
Performance
Cage layers

ABSTRACT

Thirty six 56-week-old ISA cage layers were divided into two groups randomly. The cage layers in control group (12 birds) and experiment group (24 birds) were respectively injected with 300 μL sodium chloride and 300 μg eucaryon recombinant plasmid pcDNA3.1(+)-chOPG. Eighty 56-week-old ISA cage layers were divided into group A, B, C and D randomly. Group A is for control group, while plasmid pcDNA3.1(+)-chOPG was injected to B, C, D groups in muscle at the dosage of 200 μg, 400 μg, 600 μg at 57, 59, 61, 63th weeks respectively. After the detection on the expression of chOPG protein after 3 h, it reached the peak at 7 d and then fell down. After 28 d, nothing was detected in the injected skeletal muscles. The other organs did not express exogenous chOPG protein. Plasmid in liver had the fastest metabolism. The pathological effects in main organs were not observed by histological section. The concentration of plasma calcium in B, C and D groups significantly decreased, while the activity of alkaline phosphatase was significantly improved, compared to control group. The total average value of abnormal and broken eggs of group C, D was significantly higher than those of group A. The bone biomechanical property and bone radiographic density of tibia and femur in experiment group were significantly higher than control group. Therefore, one conclusion is drawn that the expression of chOPG from foreign plasmid pcDNA3.1(+)-chOPG have contribute to bone formation, chOPG can increase bone density and strength by inhibiting bone resorption. Nevertheless, it was cleared out from cellular system in a short-term after intramuscular injection and cannot integrate into host chromosome genomic in cage layers. There were no pathological effects observed in the main tissues. It is believed that 200 μg plasmid pcDNA3.1(+)-chOPG should be within the safe range for application, because it can improve bone metabolism and will not affect the production of cage layer during the post cycle.

1. Introduction

Osteoporosis is one of common diseases in laying hens, which can lead to progressive losses of bones. Osteoporosis always result in increasing bone fragility and higher vulnerability to fracture, under commercial conditions fracture of laying hens induced over 30% in this period and commonly lead to depopulation (Whitehead and Fleming, 2000). Caged layer fatigue is an extreme consequence of structural bone losses. Sometimes the spinal degeneration can result in the paralysis of hens, but the transient irregularities of calcium metabolism in intracellular may inhibit muscular activity (Gao et al., 2017a, 2017b; Whitehead, 2004). Osteoporosis is one of significant problems in poultry farm and have caused lots of economic loss in yield of eggs and animal welfare. Even worse, the main motivation is to seek the interest in finding out some medicine and cytokines, which can increase mineral density and breaking strength instead of affecting laying performance. Some kinds of nutrition, such as macrominerals (Ca, P) and vitamin D3, were demonstrated that can influence quality of bone in many previous studies. As is known, mineralization of some enzymes related with microelements play important roles in bone formation, but there are only a few researches aimed to explore the relationship between trace elements and bones quality (Gao et al., 2017a, 2017b; Swiatkiewicz and Koreleski, 2008).

* Corresponding author.
E-mail address: houlele2006@126.com (J. Hou).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.09.010
1319-562X/

In recent years, the study of bone biology made a major advance in identifying the system as a dominant, final mediator of osteoclastogenesis (Feng et al., 2019; Alaam and Hussien, 2016; Han et al., 2015; Sacco et al., 2017). In many tissues and cells including osteoblasts, OPG is usually known to be expressed as a secreted soluble receptor. In osteoclasts, OPG is a decoy receptor for RANKL (receptor activator of NF-kappa B ligand) which can interact with RNAK and the interaction can be inhibited by OPG. OPG can further inhibiting osteoclasts differentiation, apoptosis and bone resorption (Findlay and Atkins, 2011; Boyce and Xing, 2008; Crockett et al., 2011). Therefore, OPG may be a promising drug for treating bone resorptive diseases like osteoporosis (Ulrich-Vinther et al., 2005). Therefore, OPG is used for studying new therapeutic strategies in a number of investigations. In osteopetrotic mice, OPG was confirmed that can inhibit osteoclastogenesis and tumor growth (Clohisy et al., 2000). In human, the bone resorption may be reduced when treated with human OPG in osteoporotic patients, as well as in patients with breast carcinomarelated bone metastases, multiple myeloma or prostatic cancer. (Bolzoni et al., 2013; Ney et al., 2016; Ibrahim et al., 2011). In other studies, OPG was believed that can relieve advanced bone cancer pain (Jung et al., 2001). In prostate cancer, serum OPG is also a marker to detect disseminating bone metastatic (Luger et al., 2001). However, in human clinical trials, the potential side effects and short half-life of OPG make which cannot play a great role in some human clinical trials. (Widschwendter et al., 2015). In prior studies, Adenoassociated virus (AAV) vector with OPG expression elements were used for delivering consistently at therapeutic levels for clinical therapy. (Yang et al., 2002; Ulrich-Vinther et al., 2002; Kostenuik et al., 2004).

So far, few reports have been reported on the use of OPG in the caged layer. Give the important role of osteopontin in bone metabolism, in this study, pcDNA3.1 (+)/chOPG expression vector was first constructed and introduced into chicken embryo fibroblasts. Cellular expression of chOPG had been demonstrated and were proven to inhibit osteoclast function as extracellular regulators (Hou et al., 2011). Our study aimed at studying the effects of pcDNA3.1(+)-chOPG on chOPG protein expression, biosafety, egg laying performance. Also bone metabolism in cage layers were examined after intramuscular administration in vitro. Also, it was used to determine a suitable dosage of the pcDNA3.1(+)-chOPG and to evaluate the possibility of gene therapy for cage layer osteoporosis.

The study described that different doses of plasmid pcDNA3.1 (+)-chOPG were injected intramuscular in layer hens. The transcription of chOPG gene and the expression of chOPG protein were detected by RT-PCR and Western Blot, respectively. PCR was used to test the distribution of plasmid pcDNA3.1(+)-chOPG. The tissue section was used to evaluate the histopathological changes. The effect of biochemical indicator, egg laying performance and bone metabolism were experimented by measure index. Results indicated that the appropriate dosage of chOPG (200 μg) could improve bone metabolism and not affect the performance of cage layer during the late cycle. The aim was to provide new solution to find the mechanism of cage layer osteoporosis.

2. Materials and methods

2.1. Cage layers, Husbandry, and treatments

Thirty six 56-week-old ISA cage layers (Animal Husbandry Industry Co., Nanjing, China) were divided into two groups randomly, 12 birds were in control group while 24 birds were in experiment group. Control repeats and experiment repeats were respectively injected with 300 μL sodium chloride and 300 μg eucaryon recombinant plasmid pcDNA3.1(+)-chOPG (Nanjing Agricultural University, Nanjing, China). After 3 h, 1 d, 7 d, 14 d, 21 d and 28 d, the injected skeletal muscles, contralateral skeletal muscles, kidney, spleen, liver and heart were separated and collected. Eighty 56-week-old ISA cage layers (Animal Husbandry Industry Co., Nanjing, China) were randomly assigned to 4 groups, each containing 20 hens. Group A is control, pcDNA3.1(+)-chOPG was intramuscular injected to B, C, D groups at the dosage of 200 μg, 400 μg, 600 μg at 57, 59, 61, 63th weeks. At 58, 59, 60, 61, 62, 63, 64, 65th week, 5 mL wing vein blood samples were taken. After taking the blood sample, heparin was immediately added. Then, the mixture of blood sample and heparin was sent for centrifuge, and serum was stored at −20 °C. Laying eggs were counted, the abnormal and broken eggs were recorded every day. Every week twenty eggs from each group were collected and the eggshell quality parameters including the shell thickness and density were measured. At last, cervical dislocation and femur, tibia were used for executing layers so as to clean off muscle and soft tissue and then were stored to be frozen (−20 °C). Throughout the experiment, the laying hens were fed with standard fowl diet (Animals were fed with diet of GB/T 5916-2004, G*eneral Administration of Quality Supervision*, Inspection and Quarantine of the People's Republic of China, Standardization Administration of the People's Republic of China). All protocols of animal experiments were supplied by the *Care and Use of Laboratory Animals*.

2.2. Transcript analysis of chOPG mRNA by RT-PCR

Total RNA was isolated from the injected skeletal muscles with the TRIzol® Reagent (Invitrogen, Inc. Carlsbad, CA, USA). Purity of isolated total RNA was detected according to the by the absorbance at 260 nm and 280 nm while 1% agarose/formaldehyde gel electrophoresis was performed to identify its integrity. To avoid contamination of genomic DNA, DNase I (1 U/μg) (TaKaRa Bio Inc.) were used for incubating with total RNA before RT. Then the RT reaction mixture were subject to RT by Moloney murine leukaemia virus (MMLV) reverse transcriptase (TakaRa Bio Inc. Japan) and maintained at 42 °C for 60 min and 70 °C for 15 min. According to the given nucleotide sequence of chicken OPG published (chOPG, DQ098013), primers P1 (5′-ATGAACAAGTTCCTGTGC-3′) and P2 (5′-TTAGACACATCTTACTTT-3′) were designed to amplify the ORF of chicken OPG (10–1218, 1209 bp). The target gene were amplified as the PCR procedure of denaturation, annealing and extension at 94 °C for 30 s, 47 °C for 45 s and 72 °C for 50 s for 30 cycles respectively. The PCR products were examined by Sanger sequencing by Invitrogen Inc. Based on the nucleotide sequence published (β-actin, L08165), Primers P3 (5′-CTTGGGTATG GAGTCCTGTGGT-3′) and P4 (5′-GGTTTAGAAGCATTTGCGGTGG-3′) were designed to amplify β-actin sequence (866–1199, 334 bp). The target gene was amplified as the procedure of denaturation, annealing and extension at 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 50 s for 30 cycles respectively. The PCR products were performed to Sanger sequencing by Invitrogen Inc.

2.3. Protein isolation and western-blot analysis of chOPG protein

After experiments, tissues were quick frozen and collected mechanically homogenized (about 100 mg) and being thawed in icy cold RIPA buffer (Nanjing Agricultural University) (50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium Deoxycholate, and 1% protease inhibitor PMSF/isopropanol). Then the samples were incubated on ice and centrifuged at 25,000*g* at 4 °C for 30 min. Then the protein concentrations in the supinate was quantified through using of the BCA Protein Assay Kit (Wuhan Boster Biotechnology Company, China). Then the total protein was mixed with SDS-PAGE loading buffer (Tiangen Biotech

Co., Ltd) and denaturation by 5 min boiling and 80 μg of total protein were subjected to electrophoresed on 4% and 10% SDS-PGE gel at 100 V 25–50 mA 4 °C for 80 min to separate the target protein. Then, proteins on the gel was transferred onto nitrocellulose membranes. The chOPG band (80 kDa-100 kDa) and β-actin band (37 kDa) were separated from the cell membrane by cutting the cell membrane according to prestained SDS-PAGE protein marker (TakaRa Bio Inc.). Then the chOPG protein was blocked with 5% skimmed milk in TBS (Tiangen Biotech Co., Ltd), the rabbit polyclonal anti-chOPG antibodies (1:200) (Nanjing Agricultural University) or mouse monoclonal anti-β-actin antibodies (1:10,000) (Wuhan Boster Biotechnology Company) were used for primary antibody incubation at 4 °C overnight. The membrane was washed10 min for 4 times with TBS-0.1% Tween 20 (TBS-T) (Tiangen Biotech Co., Ltd). After that, HRP-conjugated goat anti-rabbit IgG (1:5000) and HRP-conjugated goat anti-mouse IgG (1:10,000) (Wuhan Boster Biotechnology Company) were used for secondary antibody incubation for 1 h. Finally, after washing, the enhanced chemiluminescence (ECL) (Tiangen Biotech Co., Ltd) were used for visualizing the protein signals. The protein signals presented on X-ray film and been captured by using of Kodak 1D Analysis System for further anlysis.

2.4. PCR analysis of pcDNA3.1(+)-chOPG in tissues

According to the product manual, total DNA was isolated from injected skeletal muscles, contralateral skeletal muscles, kidney, spleen, liver and heart with TIANcombi DNA Lyse&Amp PCR Kit (Tiangen Biotech Co., Ltd). Primers P5 (5′-CTGGCTAACTAGAGAACC CAC-3′) and P6 (5′-TAGAAGGCACAGTCGAGG-3′) were used for amplification of pcDNA3.1 (+), PCRmix and DNA template were mixed and performed to PCR procedure, the temperature for denaturation, annealing and extension is 94 °C for 30 s, 49 °C for 45 s, and 72 °C for 50 s respectively. The PCR products were then Sanger sequencing were performed by Invitrogen Inc after 1% agarose electrophoresis.

2.5. Histological section

Tissues were fixed in 10% buffered formaldehyde (Bo Quan Sci&Tech. Co. Ltd. Nanjing, China) for 7 d at room temperature. PBS was used to wash tissues for twice and tissues were dehydrated through 50%, 75%, 85%, 95%, 100% and 100% ethanol (Bo Quan Sci&Tech. Co. Ltd) for 1 h each. Tissues were then incubated in xylol/ethanol 1:1 for 15 min, and pure xylol for 7 min at room temperature. After being transferred to oven at 60 C, paraffin/xylol ratio of 1:3, 1:2 and 1:1, and pure paraffin was applied, for one hour each. The tissues were then embedded in fresh paraffin and cooled down overnight at 4 °C. Serial sections of 5 μm were mounted on glass slides, then keep them to an oven at 60 °C for 2 h. Glass slides coated with 1% poly-L-lysine (Tiangen Biotech Co., Ltd) were used for improving adhesion between the sections and the surface. Mounted sections were incubated in two different xylols for 15 min and dehydrated through 100%, 95%, 85%, 75%, and 50% ethanol for 10 min each. Sections were histologically examined by conventional methods with hematoxylin and eosin (H&E) stain (Tiangen Biotech Co., Ltd).

2.6. Measure index

Plasma calcium, plasma phosphorus and plasma AKP were determined by kits (BioSino Bio-technology and Science Inc, Beijing, China). Bone biomechanical property were measured by three-point bending test (span = 60 mm). The strength of eggshell was measured by egg quality meter, and the average thickness of eggshell was calculated by vernier caliper. Bone radiography density method combined computer-assisted X-ray film was established for measuring layers bone volume.

2.7. Statistical analysis

Differences of normally distributed of all measured date was evaluated by one-way ANOVA test and Student's *t*-test. Results were shown as means ± the standard deviation (SD). The statistical difference was accepted when the $P < 0.05$.

3. Results

3.1. Transcript analysis of the chOPG mRNA

Total RNA was extracted from injected skeletal muscle, with OD 260 nm/OD 280 nm of 1.87. Denaturing gel electrophoresis showed 28S rRNA, 18S rRNA and 5S rRNA (Fig. 1a). The results suggested that the total RNA was integrated and highly pure. The results showed the transcription of chOPG mRNA was detected after transfection 3 h pcDNA3.1(+)-chOPG in the injected skeletal muscles. The maximum expression of chOPG appeared 7 d, and then fell off. Finally, it was not detected (Fig. 1b).

3.2. The chOPG protein expression

The results showed after 3 h transfection pcDNA3.1(+)-chOPG in the injected skeletal muscles, the expression of chOPG protein was detected. The maximum protein quantity appeared at 7 d, and then fell off. Finally, nothing was detected (Fig. 1c). The other organs (contralateral skeletal muscles, kidney, spleen, liver and heart) did not express exogenous chOPG protein (Fig. 1d).

3.3. The distribution of pcDNA3.1(+)-chOPG in tissues

After 1 d transfection pcDNA3.1(+)-chOPG, the injected skeletal muscles, contralateral skeletal muscles, kidney, spleen, liver and heart could amplify specificity chOPG. As time went by, the plasmid concentration in 14 d and 21 d after transfection was less than the plasmid concentration in 7 d after transfection. The plasmid was not detected in all tissues in 28 d. Plasmid metabolism in liver was the fastest (Fig. 2a).

3.4. Pathological change

PcDNA3.1(+)-chOPG transfection showed no pathological changes in the main organs and tissues (Fig. 2b).

3.5. Plasma calcium concentration, plasma phosphorus concentration and plasma AKP activity

The plasma calcium concentration (Fig. 3a) and plasma phosphorus concentration (Fig. 3b) showed a downward trend in different experiment groups. However, compared with the layers injected with 300 μL sodium chloride, the plasma calcium concentration in layers from experiment group showed significant difference ($P < 0.05$), while the difference of plasma phosphorus concentration had no significant ($P > 0.05$) statistically. In the experiment groups, average changes of AKP activity were observed to be positive in Fig. 3c. B, C, D groups were more obvious ($P < 0.05$) than in the control A group.

3.6. Egg laying quantity, the abnormal egg and broken egg quantity

There were no differences in the average number of weekly eggs laid in every experiment groups ($P > 0.05$) as shown in Fig. 4a.

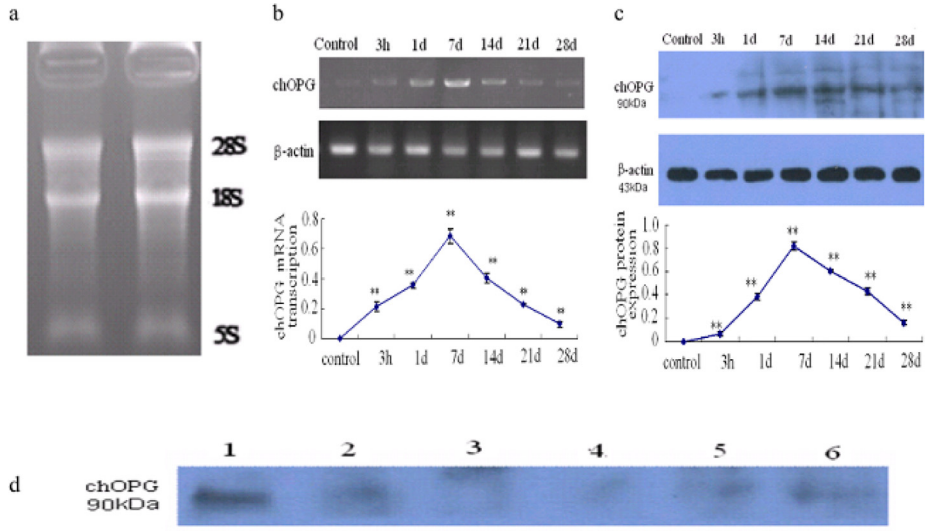

Fig. 1. a Denaturinggel electrophoresis of total RNA from the injected skeletal muscles. b Gel electrophoresis of chOPG and β-actin mRNA transcription from the injected skeletal muscles in different time; Fig. 2-2 Quantitative analysis of chOPG mRNA transcription from the injected skeletal muscles in different time. (Mean ± SD; n = 4). (** $P < 0.01$) c Immunoreactive bands for chOPG protein and β-actin protein expression from the injected skeletal muscles in different time; Fig. 3-2 Statistical analysis of chOPG protein values. (Mean ± SD; n = 4) (** $P < 0.01$). d Immunoreactive bands for chOPG protein from the main tissues. 1–6: the injected skeletal muscles, opposite side skeletal muscles, kidney, spleen, liver and heart.

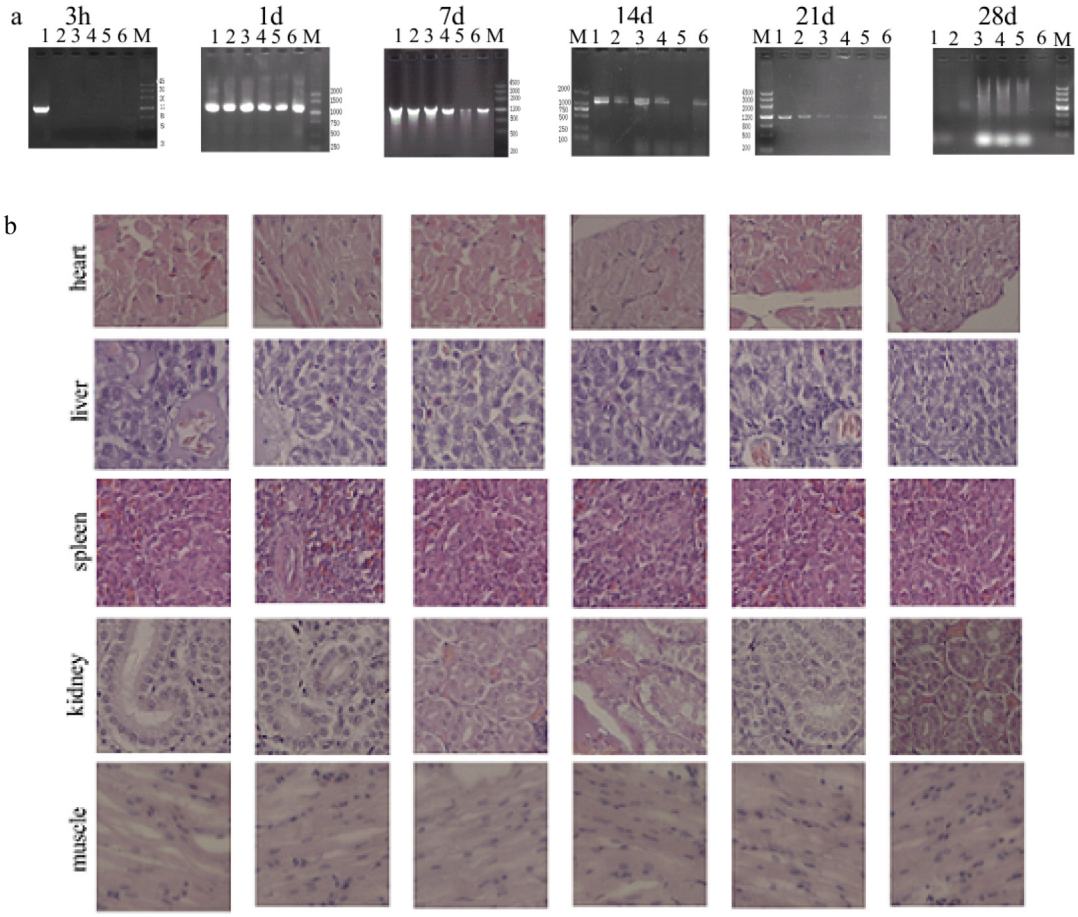

Fig. 2. a Result of amplification of genome from 3 h, 1 d, 7 d, 14 d, 21 d and 28 d in different tissues after transfection pcDNA3.1(+)-chOPG in different tissues. 1–6: the injected skeletal muscles, opposite side skeletal muscles, kidney, spleen, liver and heart. M: marker. b Pathological change from 3 h, 1 d, 7 d, 14 d, 21 d, 28 d in different tissues after transfection pcDNA3.1(+)-chOPG in different tissues. (×400).

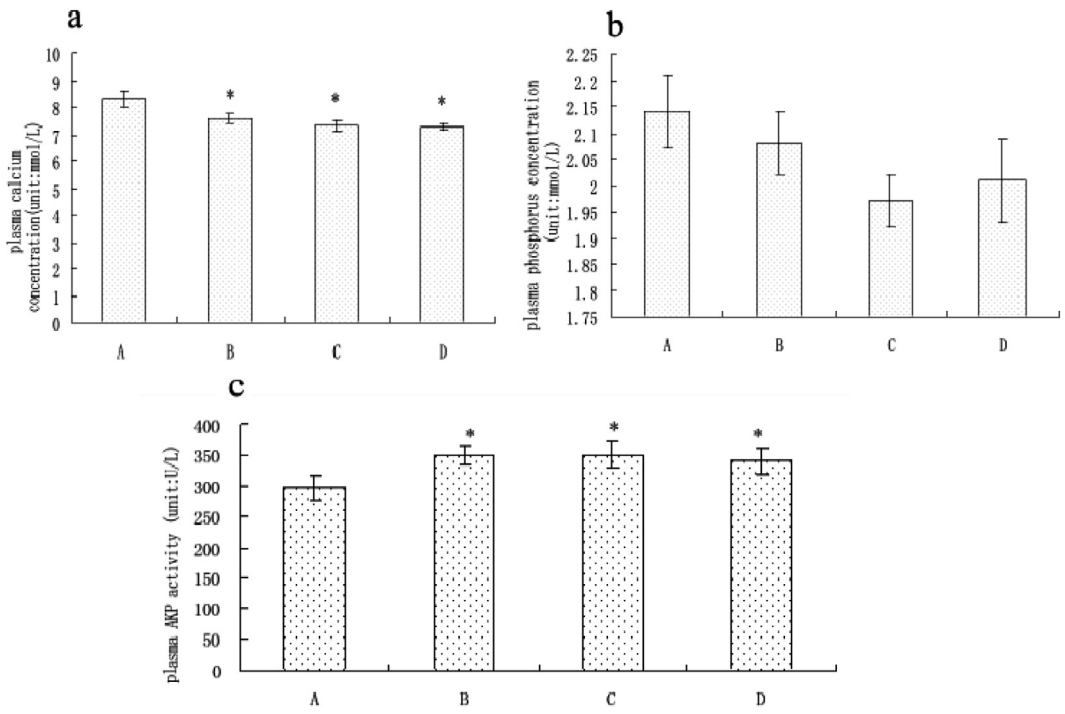

Fig. 3. a Total average of plasma calcium concentration in Group A, B, C and D (** $P < 0.01$, * $P < 0.05$). b Total average of plasma phosphorus concentration in Group A, B, C and D (** $P < 0.01$, * $P < 0.05$). c Total average of plasma phosphorus activity in Group A, B, C and D. (** $P < 0.01$, * $P < 0.05$).

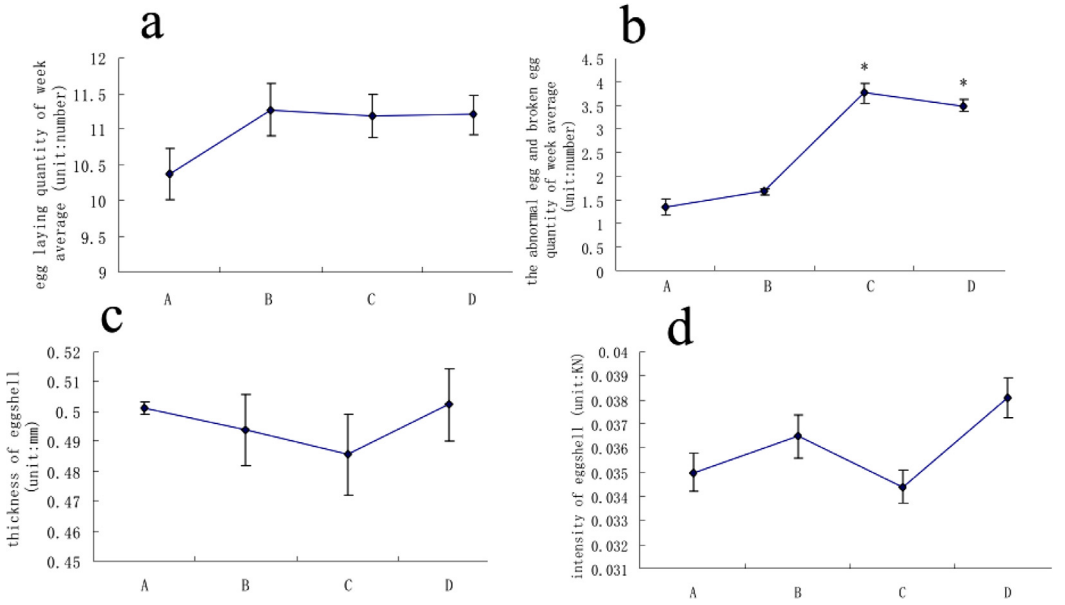

Fig. 4. a Total average week of egg laying quantity (** $P < 0.01$, * $P < 0.05$). b Total average week of abnormal and broken egg quantity (** $P < 0.01$, * $P < 0.05$). c Thickness of eggshell in Group A, B, C and D (** $P < 0.01$, * $P < 0.05$). d Intensity of eggshell in Group A, B, C and D (** $P < 0.01$, * $P < 0.05$).

Compared to the control group, the abnormal quantity of eggs and broken eggs increased ($P < 0.05$) in C, D group in Fig. 4b.

3.7. Eggshell thickness and intensity

The variation of eggshell thickness and intensity in different groups is demonstrated in Fig. 4c and d. The result showed there was no significant difference after intramuscular injection of different doses of plasmid pcDNA3.1(+)-chOPG during any experimental period ($P > 0.05$).

3.8. Bone biomechanical property

Fig. 5a showed the property results of bone biomechanical in different groups. The peak load represented bone intensity and the maximum energy absorption represented tenacity. As a result, the peak load and maximum energy absorption of tibia in experiment group were higher than those in the control group and the maximum energy absorption of tibia was also significantly higher in experiment group ($P < 0.05$). The result of femur was the similar to tibia.

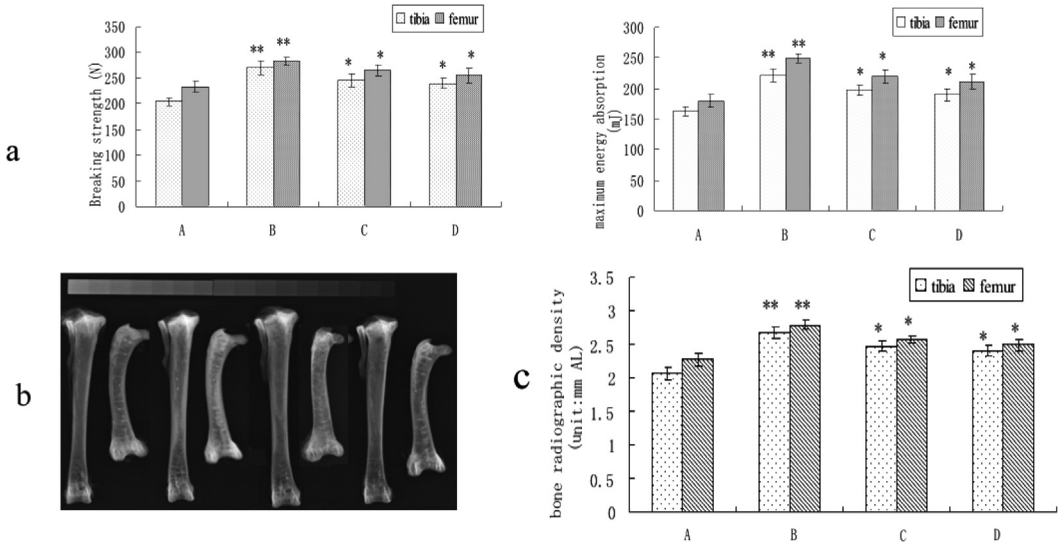

Fig. 5. a Bone biomechanical property (breaking strength and maximum energy absorption) of tibia and femur in Group A, B, C and D (** $P < 0.01$, * $P < 0.05$). b Bones X-ray film of cage layers. c Bone radiographic density of tibia and femur in Group A, B, C and D (** $P < 0.01$, * $P < 0.05$).

3.9. Bone radiographic density

X-ray film was established for measuring layers bone radiography density in Fig. 5b. It was a upward tendency of bone volume in tibia and femur. Bone radiography density of tibia and femur in layers injected 300 μg eucaryon recombinant plasmid pcDNA3.1-chOPG was higher than those injected with 300 μL sodium chloride as shown in Fig. 5c ($P < 0.05$).

4. Discussion

The severity of bone diseases in avian sometimes depend on peak structural of bone mass and the subsequent loss of bone structure, this is similar to women's postmenopausal osteoporosis. There are various drugs target to postmenopausal osteoporosis and had been utilized to treating osteoporosis in hens' laying stage (Zhou et al., 2009). It had been reported that OPG was effective in normal osteoclast development. Diseases characterized by the presence of decreased osteoclast activity, may be neutralized by rebalancing the RANKL/RANK/OPG system in bone environment in favor of decreased bone resorption (Hofbauer and Schoppet, 2004; Noor et al., 2018; Macari et al., 2018; Liu et al., 2017; Belibasakis et al., 2011). In osteoclast-dependent skeletal diseases including arthritis and osteolysis, expression of foreign OPG gene or delivery of OPG protein had been confirmed as an available therapy (Doran et al., 2004; Lubberts et al., 2003; Shafiey et al., 2018). However, due to the purify of protein always cost a lot, highly concentrated injections are need in treatment and it is difficult to maintain an effective concentration for long time, the administered protein was easily washed and restricted by circulating blood (Jin et al., 2003). In this work, the local OPG gene transfer rather than delivery of OPG protein directly were selected for relief of bone diseases. The prior experiment demonstrated that the construction and transfection of pcDNA3.1 (+)-chOPG were brought into chicken embryo fibroblasts and expressed bioactivity chOPG protein successfully, for inhibiting osteoclast function as extracellular regulators (Hou et al., 2011). In this study, RT-PCR was used for demonstrating transcription of the mRNA for chOPG in the recombinant plasmid pcDNA3.1(+)-chOPG injected skeletal muscles of cage layers. These data were consistent with the result: western-blot was used to demonstrate expression of the chOPG protein and the concentrations of chOPG could be sustained about three weeks after a single intramuscular injection of pcDNA3.1(+)-chOPG in the observation period. Regardless of blood circulation, expression of protein from local gene transfer can be prolonged and concentration of effective protein can maintain high enough. Also, the integral effects can be avoided by expression at the local site (Blesing and Kerr, 1996). Therefore, pcDNA3.1(+)-chOPG injected skeletal muscles of cage layers may prolong treatment intervals and decrease the number of repetitive treatments.

Although there are various viral and nonviral methods were used and improved for gene transfer (Marshall, 1995; Ledley, 1995), some viral vectors such as adenoviral for gene transfer, had been confirmed that have side effects including immunogenicity and cytotoxicity (Raper et al., 2003). Non-viral vectors usually have low cytotoxicity and immunogenicity *in vivo* or in vitro, however, these kinds of vectors have lower efficiency of gene transfer than viral vectors. The pcDNA3.1 vector is finally chosen because the cytomegalovirus (CMV) promoter contained in this vector can increase expression level of foreign genes in different types of mammalian cells. This vector also contains a polyadenylation sequence of bovine growth hormone (BGH) for post-transcriptional RNA stability. Thus, it has been confirmed that delivery of OPG gene mediated by pcDNA3.1 vector into mice can inhibit osteoclasteogenesis and reduced experimental tooth movement (Kanzaki et al., 2004). The current study demonstrates that the plasmid pcDNA3.1(+)-chOPG disappeared in all tissues at 28 d and no obvious pathological results were observed in the main organ tissues. The safety of pcDNA3.1 vector still need to be evaluated in further studies of animal models. There are two possible reasons maybe can explain this, one reason is that the blood flow may diffuse the vector solution The other reason is that the soluble OPG protein expressed by the pcDNA-OPG gene may be diffused by interstitial (Kanzaki et al., 2004).

Based on these in prior experiments, the different doses of plasmid pcDNA3.1(+)-chOPG were delivered into cage layers for further clarifying its effects on egg laying performance and bone metabolism in cage layers after intramuscular administration in vitro. The results of plasma calcium concentration and alkaline phosphatase activity revealed that obvious changes happened when plasmid pcDNA3.1(+)-chOPG was injected in muscles. In the later periods of egg formation in layers, Ca was transferred to the eggshell, which gradually depleted the calcium stores in layers (Clunies et al., 1992). During the process of egg formation,

pcDNA3.1(+)-chOPG could express chOPG protein which inhibits the activity of osteoclasts and promotes calcium absorption in the intestine, satisfying the Ca's needs for shell formation. The mechanism of this reaction is not clear at present, more researches are needed to clarify the relationship of OPG and Ca. Alkaline phosphatase, expressed in osteoblasts, can mineralize the matrix thus it is considered to be associated with osteoblastic activity in osteogenesis (Hsu et al., 1999). High level of plasma AKP reflected the increased osteoblastic activity in the experiment. We demonstrated that plasmid pcDNA3.1(+)-chOPG had no influence on egg production, eggshell thickness and intensity in laying hens. However, the abnormal egg and broken egg quantity were increased to over 400 μg plasmid, compared to control group.

Bone radiographic density reflected bone quality and the view presumed that an increased mass always bring about better mechanical properties. More recently, the conventional view has been questioned. However, plenty of investigations had shown that bone radiographic density exclusive standing for bone quality was not scientific. According to evidences, bone strength significantly declined, but bone radiographic density sustained constant in the late cycle of egg production in cage layers (Whitehead, 2004). In fact, in some bone diseases, there is a disorder between biomechanical properties and bone mineral content. Osteopetrosis was the typical example. Although bone mineral content was extremely high, vulnerability to fracture increased. In bones, some inorganic and organic elements will influence its biomechanical property (Oxlund et al., 1995). Under the same bone mineral content, the more reasonable bone trabecularism is, the better biomechanical properties are. Higher strength, more fracture resistance. In this study, bone quality was evaluated by the method of bone radiographic density related to biomechanical properties in cage layers after intramuscular administration plasmid in vitro. The result suggested effect of injected 200 μg pcDNA3.1 (+)-chOPG on bone radiographic density and biomechanical properties were the most significant.

In conclusion, this study demonstrates that chOPG protein could be expressed by intramuscular injection of the recombinant plasmid pcDNA3.1(+)-chOPG on cage layers, without bringing about any systemic effects. Considering egg laying performance, bone quality and bone metabolism, 200 μg plasmid pcDNA3.1(+)-chOPG was thought to be the appropriate dosage. Our results also demonstrated that pcDNA3.1 (+)-chOPG maybe a new target for studying the mechanism of bone metabolism regulation in osteoporosis.

5. Conclusions

Foreign plasmid pcDNA3.1(+)-chOPG can express chOPG which can promote bone formation, chOPG can increase bone density and strength, decrease bone resorption. Foreign chOPGwas cleared from cellular system in short time after intramuscular injection and cannot integrate into host chromosome genomic in cage layers. There were no pathological effects observed in the main tissues. It is believed that 200 μg plasmid pcDNA3.1(+)-chOPG should be within the safe range for application, because it can improve bone metabolism and will not affect the production of cage layer during the post cycle.

6. Contributions of authors

LH and JH participated in designing and coordinating the experiment. They also helped to writing the manuscript. ZZ recorded and collected the data. YL and JH fed the ISA laying hens and collected samples. LH and CZ participated in the other experiments. All authors have performed the writing of the manuscript and then read and approved the final manuscript.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

Supported by the National Natural Science Foundation of China (Project grant Nos. is 30972234 and 30671546), this work was a key program for the Education Ministry of China.

References

Alaam, M.M., Hussien, N.I., 2016. A comparative study between the effect of 17-β estradiol and angiotensin converting enzyme inhibitor on osteoporosis in ovariectomized rats. Gen. Physiol. Biophys. 35 (4), 433–441.
Belibasakis, G.N., Meier, A., Guggenheim, B., Bostanci, N., 2011. The RANKL–OPG system is differentially regulated by supragingival and subgingival biofilm supernatants. Cytokine 55 (1), 98–103.
Blesing, C.H., Kerr, D.J., 1996. Intra-hepatic arterial drug delivery. J. Drug Target. 3, 341–347.
Bolzoni, M., Storti, P., Bonomini, S., Todoerti, K., Guasco, D., Toscani, D., et al., 2013. Immunomodulatory drugs lenalidomide and pomalidomide inhibit multiple myeloma-induced osteoclast formation and the RANKL/OPG ratio in the myeloma microenvironment targeting the expression of adhesion molecules. Exp. Hematol. 41 (4), 387–397.
Boyce, B.F., Xing, L., 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch. Biochem. Biophys. 473, 139–146.
Clohisy, D.R., Ramnaraine, M.L., Scully, S., Qi, M., Van, G., Tan, H.L., Lacey, D.L., 2000. Osteoprotegerin inhibits tumor-induced osteoclastogenesis and bone tumor growth in osteopetrotic mice. J. Orthop. Res. 18, 967–976.
Clunies, M., Parks, D., Leeson, S., 1992. Calcium and phosphorus metabolism and eggshell formation of hens fed different amounts of calcium. Poult. Sci. 71 (3), 482–489.
Crockett, J.C., Mellis, D.J., Scott, D.I., Helfrich, M.H., 2011. New knowledge on critical osteoclast formation and activation pathways from study of rare genetic diseases of osteoclasts: focus on the RANK/RANKL axis. Osteoporos. Int. 22, 1–20.
Doran, P.M., Turner, R.T., Chen, D., Facteau, S.M., Ludvigson, J.M., Khosla, S., et al., 2004. Native osteoprotegerin gene transfer inhibits the development of murine osteolytic bone disease induced by tumor xenografts. Exp. Hematol. 32, 351–359.
Feng, W., Guo, J., Li, M., 2019. RANKL-independent modulation of osteoclastogenesis. J. Oral Biosci. 61 (1), 16–21.
Findlay, D.M., Atkins, G.J., 2011. Relationship between serum RANKL and RANKL in bone. Osteoporos. Int. 22 (10), 2597–2602.
Gao, W., Wang, Y., Basavanagoud, B., Jamil, M.K., 2017a. Characteristics studies of molecular structures in drugs. Saudi Pharm J. 25, 580–586.
Gao, W., Wang, Y., Wang, W., Shi, L., 2017b. The first multiplication atom-bond connectivity index of molecular structures in drugs. Saudi Pharm J. 25, 548–555.
Han, N.R., Kim, H.Y., Yang, W.M., Jeong, H.J., Kim, H.M., 2015. Glutamic acid ameliorates estrogen deficiency–induced menopausal-like symptoms in ovariectomized mice. Nutr. Res. 35 (9), 774–783.
Hofbauer, L.C., Schoppet, M., 2004. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. JAMA 292, 490–495.
Hou, L., Hou, J., Yao, J., Zhou, Z., 2011. Effects of osteoprotegerin from transfection of pcDNA3. 1 (+)/chOPG on bioactivity of chicken osteoclasts. Acta. Vet. Scand. 53, 21.
Hsu, H.H., Camacho, N.P., Anderson, H.C., 1999. Further characterization of ATP-initiated calcification by matrix vesicles isolated from rachitic rat cartilage: membrane perturbation by detergents and deposition of calcium pyrophosphate by rachitic matrix vesicles. Biochim. Biophys. Acta 1416 (1–2), 320–332.
Ibrahim, T., Sacanna, E., Gaudio, M., Mercatali, L., Scarpi, E., Zoli, W., et al., 2011. Role of RANK, RANKL, OPG, and CXCR4 tissue markers in predicting bone metastases in breast cancer patients. Clin. Breast Cancer 11 (6), 369–375.
Jin, Q.M., Anusaksathien, O., Webb, S.A., Rutherford, R.B., Giannobile, W.V., 2003. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. J. Periodontol. 74, 202–213.
Jung, K., Lein, M., von Hösslin, K., Brux, B., Schnorr, D., Loening, S.A., Sinha, P., 2001. Osteoprotegerin in serum as a novel marker of bone metastatic spread in prostate cancer. Clin. Chem. 47, 2061–2063.
Kanzaki, H., Chiba, M., Takahashi, I., Haruyama, N., Nishimura, M., Mitani, H., 2004. Local OPG gene transfer to periodontal tissue inhibits orthodontic tooth movement. J. Dent. Res. 83, 920–925.
Kostenuik, P.J., Bolon, B., Morony, S., Daris, M., Geng, Z., Carter, C., Sheng, J., 2004. Gene therapy with human recombinant osteoprotegerin reverses established osteopenia in ovariectomized mice. Bone 34 (4), 656–664.

Ledley, F.D., 1995. Nonviral gene therapy: the promise of genes as pharmaceutical products. Hum. Gene Ther. 6, 1129–1144.
Liu, X.Y., Xu, L., Wang, Y., Li, J.X., Zhang, Y., Zhang, C., et al., 2017. Protective effects of total flavonoids of Astragalus against adjuvant-induced arthritis in rats by regulating OPG/RANKL/NF-κB pathway. Int. Immunopharmacol. 44, 105–114.
Lubberts, E., van den Bersselaar, L., Oppers-Walgreen, B., Schwarzenberger, P., Coenen-de Roo, C.J., Kolls, J.K., et al., 2003. IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-κB ligand/osteoprotegerin balance. J. Immunol. 170, 2655–2662.
Luger, N.M., Honore, P., Sabino, M.A.C., Schwei, M.J., Rogers, S.D., Mach, D.B., et al., 2001. Osteoprotegerin diminishes advanced bone cancer pain. Cancer. Res. 61, 4038–4047.
Macari, S., Sharma, L.A., Wyatt, A., da Silva, J.M., Dias, G.J., Silva, T.A., et al., 2018. Lactation induces increases in the RANK/RANKL/OPG system in maxillary bone. Bone 110, 160–169.
Marshall, E., 1995. Gene therapy's growing pains. Science 269, 1052–1055.
Ney, J., Saar, M., Juhasz-Boess, I., Assmann, G., Kasoha, M., Solomayer, E.F., et al., 2016. 388 Genetic variations in the OPG and RANKL genes are associated with prostate cancer risk. Eur. Urol. Suppl. 15 (3), e388.
Noor, Z., Kania, N., Azharuddin, A., Budhiparama, N., Nurdiana, N., Hidajat, N.N., et al., 2018. The interaction of the active compounds of Labisia pumila on RANK–RANKL–OPG system. Clin. Nutr. Experim. 17, 23–27.
Oxlund, H., Barckman, M., Ørtoft, G., Andreassen, T.T., 1995. Reduced concentrations of collagen cross-links are associated with reduced strength of bone. Bone 17 (4), S365–S371.
Raper, S.E., Chirmule, N., Lee, F.S., Wivel, N.A., Bagg, A., Gao, G.P., et al., 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab. 80, 148–158.
Sacco, S.M., Saint, C., Longo, A.B., Wakefield, C.B., Salmon, P.L., LeBlanc, P.J., Ward, W. E., 2017. Repeated irradiation from micro-computed tomography scanning at 2, 4 and 6 months of age does not induce damage to tibial bone microstructure in male and female CD-1 mice. BoneKEy Rep. 6, 855.
Shafiey, S.I., Mohamed, W.R., Abo-Saif, A.A., 2018. Paroxetine and rivastigmine mitigates adjuvant-induced rheumatoid arthritis in rats: Impact on oxidative stress, apoptosis and RANKL/OPG signals. Life Sci. 212, 109–118.
Swiatkiewicz, S., Koreleski, J., 2008. The effect of zinc and manganese source in the diet for laying hens on eggshell and bones quality. Vet. Med.-Czech. 53 (10), 555–563.
Ulrich-Vinther, M., Carmody, E.E., Goater, J.J., Søballe, K., O'Keefe, R.J., Schwarz, E.M., 2002. Recombinant adeno-associated virus-mediated osteoprotegerin gene therapy inhibits wear debris-induced osteolysis. J. Bone Joint Surg. Am. 84, 1405–1412.
Ulrich-Vinther, M., Schwarz, E.M., Pedersen, F.S., Søballe, K., Andreassen, T.T., 2005. Gene therapy with human osteoprotegerin decreases callus remodeling with limited effects on biomechanical properties. Bone 37 (6), 751–758.
Whitehead, C.C., Fleming, R.H., 2000. Osteoporosis in cage layers. Poult. Sci. 79 (7), 1033–1041.
Whitehead, C.C., 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83, 193–199.
Widschwendter, M., Burnell, M., Fraser, L., Rosenthal, A.N., Philpott, S., Reisel, D., et al., 2015. Osteoprotegerin (OPG), the endogenous inhibitor of receptor activator of NF-κB ligand (RANKL), is dysregulated in BRCA mutation carriers. EBioMedicine 2 (10), 1331–1339.
Yang, S.Y., Mayton, L., Wu, B., Goater, J.J., Schwarz, E.M., Wooley, P.H., 2002. Adeno-associated virus–mediated osteoprotegerin gene transfer protects against particulate polyethylene–induced osteolysis in a murine model. Arthrit. Rheum. 46, 2514–2523.
Zhou, Z.L., Deng, Y.F., Tao, Q.S., Hu, Y.F., Hou, J.F., 2009. Effects of Gushukang, a Chinese herbal medicine, on bone characteristics and osteoporosis in laying hens. Poult. Sci. 88, 2342–2345.