Al-Nahrain Journal of Science
ANJS, Vol. 28(3), September, 2025, pp. 119 – 124

Biochemical Changes of Alkaline Phosphatase in Tissue Homogenate of the Developing Femurs in Chick Embryos after in Ovo Calcium Carbonate Nanoparticles Inoculation

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Article's Information

Received: 08.07.2025
Accepted: 03.08.2025
Published: 15.09.2025

Keywords:
Chick eggs,
Embryo,
Calcium carbonate,
Nanoparticles,
Femur,
Osteogenesis,
Tissue homogenate,
Alkaline phosphatase.

Abstract

This study is oriented to provide evidence related to the effects of calcium carbonate nanoparticles (CCN) on the osteogenesis of the developing femur in chick embryos. The 20 fertilized chick eggs used in this study were divided into two control and experimental groups. The control group of eggs was injected with normal saline during pre-incubation. In contrast, eggs of the experimental group were injected with 500 g/mL hydrocolloid of CCN in 0.2 mL volume per egg. The homogenate of the femur obtained from the control and experimental groups show statistically significant variations when comparing the level of alkaline phosphatase in the tissue of the control group at day 13 with that of the experimental group at day 13 and day 14. The alkaline phosphatase level as a marker of bone metabolism in the femur tissue homogenate of the experimental group revealed a highly significant increase in concentration compared to that of the control group at both day 13 and 14. This characteristic finding is associated with the effect of in Ovo inoculation of CCN on the femur tissue homogenate in pre-hatching chick embryos during osteoid formation and mineralization.

http://doi.org/10.22401/ANJS.28.3.15
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1. Introduction

The chick embryo model offers a valuable platform for studying early bone development, owing to its external development, accessibility, and well-defined timeline of ossification. In ovo manipulation, such as injection into the albumen or yolk sac, allows for the direct assessment of developmental effects of introduced compounds in a controlled embryonic environment [1,2]. Bone development, also known as osteogenesis, is a dynamic and highly regulated biological process that involves cellular differentiation, extracellular matrix secretion, and subsequent mineralization. It is essential not only during embryogenesis but also throughout life for growth, bone turnover, and fracture healing. Any disruption in this process may result in congenital skeletal anomalies or acquired metabolic bone diseases [3-4]. Alkaline phosphatase (ALP) is a critical enzyme in bone development and mineralization, primarily functioning by hydrolyzing phosphate esters to release inorganic phosphate. This phosphate plays a key role in the formation of hydroxyapatite, the mineral responsible for bone hardness. ALP is found in several tissues, including the liver, bile ducts, kidneys, and bones. It is widely recognized as an indicator of osteoblast function and bone generation [4, 5]. Bone-specific alkaline phosphatase (BALP), an ectoenzyme synthesized by osteoblasts, is particularly significant in bone mineralization. It attaches to bone matrix proteins and facilitates mineralization by activating pyrophosphate hydrolysis. The two primary isoforms of ALP, bone and liver isoforms, account for roughly 50% of total ALP activity [6, 7]. Humans possess four ALP genes, each coding for distinct isoenzymes: intestinal, placental, placental-like, and tissue non-specific ALP (TNAP), the latter being most relevant in bone, liver, and kidney tissues. Elevated ALP levels in the blood often signify increased bone activity, which can occur during periods of rapid

growth, fracture healing, or bone-related disorders like osteoporosis [3, 8]. Tissue-nonspecific alkaline phosphatase (TNAP) is densely localized at sites of skeletal mineralization. It drives hydroxyapatite deposition by breaking down inorganic pyrophosphate, a natural inhibitor of calcification, and elevating extracellular inorganic phosphate, which is critical for mineral crystal formation. [9]. Recent advances in nanotechnology have introduced nanoparticles as bioactive agents capable of modulating cellular functions. Calcium carbonate nanoparticles (CCN) are biodegradable and biocompatible particles that mimic physiological bone mineral components. Due to their physicochemical similarity to the inorganic phase of bone, CCNs are considered potential enhancers of osteoblast function and inducers of matrix mineralization [10-12]. Therefore, this study aims to investigate the biochemical effect of in ovo calcium carbonate nanoparticle (CCN) inoculation on the developing femur of chick embryos. This is achieved by measuring ALP activity in femur tissue homogenates at critical ossification days (13 and 14), to contribute foundational data for future bone tissue engineering and nanomedicine applications. The role of calcium carbonate nanoparticles in the development of bone in the chick embryo by ovulation alkaline phosphate enzyme level.

2. Materials and methods

The institution's review board has considered the application for research ethics approval. The animal procedures, as well as the manipulation and sacrifice protocols used in this study, were approved by the Research Ethical Committee of Al-Nahrain College of Medicine (Approval No: 20250374) and are therefore in accordance with the Ethical Principles on Animal Experimentation. The fertilized chick eggs of (Gallus gallus domesticus) were obtained from a local hatchery during the period from October 2021 to October 2022. The eggs were kept at room temperature (approximately 25°C) for about few hours before being placed in the incubator (38°C).

2.1. Preparation of CaCO3 nanoparticles

The CaCO3 nanoparticles were prepared by mixing 0.1 M Na2CO3 and 0.1 M CaCl2 for 10 minutes, followed by centrifugation at 10000 rpm and drying at 80°C. The CaCO3 nanoparticles Components include:

- Nano Calcium Carbonate Powder 50-100nm CaCO3 Nanoparticles Nanopowder, Same Day Priority Shipping (500Gram)
- Brand: XFNANO, Manufacturer: Jiangsu XFNANO Materials Tech co., LTD. Particle size: 50-100nm, Content: > 95%, Bulk density: 0.45g/mL, PH: 9.5-10

2.2. Preparation of Embryos

The eggs were placed in an incubator and randomly divided into two groups, including:

- The control group; this group includes 10 eggs in ovo inoculation (IOI), the eggs of this group were injected with normal saline pre-incubation (before being incubated).
- The experimental group: this group includes 10 eggs (IOI), the eggs of this group were injected with 500 g/mL hydrocolloid of CCN in 0.2 mL volume per egg.

Two holes were made at the wider pole of each egg using a pointed instrument. The normal saline and hydrocolloid of CCN (for the control and experimental groups, respectively) were inoculated during the first day of incubation under sterile conditions into the albumen via the air sac of each egg through one of these holes using 27-gauge, 20-mm needles. The hole needs to be sealed using sterile tape, and the eggs then placed in an incubator (Memmert-Schutzart DIN 400050-IP 20) immediately after the injection [13]. The standard incubation conditions include the temperature 37.8C0, humidity 55%, and turning once per hour for the first 15 days [14]. The incubation period is determined till the beginning of ossification in the femur's cartilage for 13 and 14 days [15]. The region of the embryo in each egg was determined by a pencil using the handmade light box. The removal of the eggshell is followed by excision of the membranes. The excised embryo was extracted using fine forceps and a small spoon into a petri dish containing normal saline solution to clean the embryo from the surrounding yolk and the removed embryonic membranes [13].

2.3. Detection of alkaline phosphatase in femur tissue homogenate

The alkaline phosphatase investigated in this study is a biochemical marker for osteoblasts [16,17]. The evaluation of changes in this enzyme in this study was oriented for interpreting its concentration in femur tissue homogenate in relation to mineralization and bone formation during embryonic days 13 and 14 in both control and experimental groups. The fundamental basis reported in the biochemical methods of a study published in 1966 to evaluate alkaline phosphatase activity in limb tissue homogenate of chick embryos depends on the determination of the released phenol from phenyl phosphate. This principle was approved to be sensitive, rapid, and feasible for the analysis of

multiple samples [18]. According to this unchanging biochemical methodology, the current study adopted the principle for testing enzymatic concentration in the femur tissue homogenate, depending on the maneuver described for a reagent obtained from Liquicheck AGAPPE (https://www.agappe.com/media/catalog/product/file/11401001.pdf).

This biochemical method involved the catalysis of alkaline phosphatase for the hydrolysis of para-nitrophenyl phosphate, freeing para-nitrophenol and phosphate. The amount of catalytic alkaline phosphatase in the sample is directly correlates with the rate of para-nitrophenol production that was determined by photometry. The preparation of femur tissue homogenate begins after dissection and removal of the soft tissues adherent to the bone using micro-equipment under the dissecting microscope (Model 1310: Niko SMZ-2B).

The femur tissues were washed comprehensively with normal saline, and the homogenization was done mechanically. The mechanical methods of homogenizing the embryonic femurs do not introduce any chemicals or enzymes, but use manual force to smash the ten bones inside an Eppendorf tube by using a hard probe (Figure 1). The excessive forces during the smashing of the bony tissue were avoided in order not to damage the Eppendorf tube, and cooling of these samples to about 4 °C was considered to prevent the enzymatic damage by the temperature generated during homogenization. This experimental mechanical method of tissue homogenization had been constructed according to the previously reported theoretical concept to release the tissue components without impeding the enzymatic contents [19]. The tissue homogenate was immediately centrifuged (Model SL-12 GIMA/ Italy) at 3000 rpm for 5 minutes. Subsequently, 20 U/L of the resulting supernatant was aspirated. The total alkaline phosphatase activity in the homogenized tissue was determined using a reagent mixture composed of 500 µL of R1 and 500 µL of R2, mixed and incubated for 15 minutes. Then, 1000 µL of the mixture was placed in an incubator for 1 minute, and the absorbance was measured at a wavelength of 405 nm by a spectrophotometer (Model WP21B: GENEX Laboratories LLC/ USA).

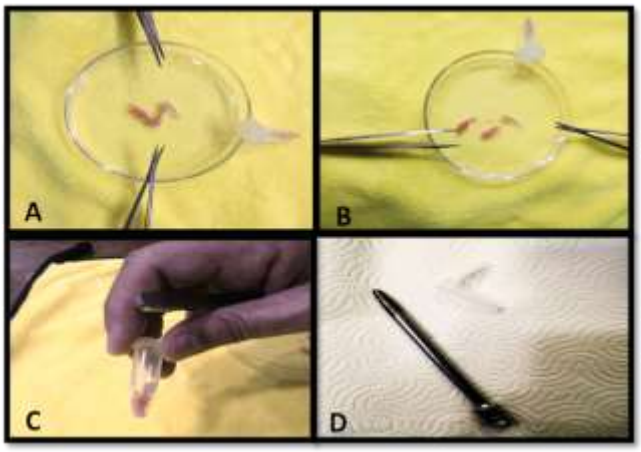

Figure 1: Method of sampling and homogenizing embryonic femur using manual force with a hard probe inside an Eppendorf tube.

3. Result & Discussion:

The developmental changes of the femur examined through histological sections from both control and experimental groups demonstrated notable progress in bone differentiation in the experimental group. This was evidenced by the progressive formation of mineralized bone trabeculae, which corresponded with a significant increase in alkaline phosphatase activity in femoral tissue homogenates (Table 1). This biochemical finding reflects the stimulatory effect of in ovo calcium carbonate nanoparticle (CCN) in ovo inoculation. Alkaline phosphatase (ALP) is a well-established biochemical marker of osteoblastic activity. Its expression is typically elevated during early stages of bone formation and tends to decline in later developmental phases, as reported by [20,21]. This pattern aligns with our findings, suggesting that the elevated ALP levels in the femoral homogenates on day 13 were driven by the physiological requirements for active bone development induced by CCN. Quantitatively, ALP levels varied significantly between groups, as illustrated in Figure 2. On day 13, the control group had a mean ALP level of 260.6 ± 19.17, while the experimental group showed a marked increase to 6019.8 ± 19.59 ($p < 0.0001$). This dramatic elevation suggests a peak in osteogenic stimulation, reflecting a robust phase of osteoid mineralization due to CCN exposure. This finding is consistent with the proposed mechanism by which ALP contributes to mineralization by hydrolyzing pyrophosphate and releasing inorganic phosphate to promote hydroxyapatite deposition [22]. Such an enzymatic surge on day 13 implies an enhanced mineralization response under the influence of CCN nanoparticles. On day 14, although the ALP levels in both groups declined, the experimental group maintained significantly higher values (166.23 ±

13.16) than the control (126.6 ± 10.02), with statistical significance ($p < 0.0001$), yet the degree of change was less prominent. This suggests that the enzymatic activity, while still elevated, begins to stabilize as bone maturation progresses. These observations are summarized in Table 1, which presents the comparison of ALP levels in femur homogenates between control and experimental groups at days 13 and 14.

Table 1. Alkaline Phosphatase Levels in Femur Homogenate: Comparison of Mean ± STD and P-Values for 13-Day and 14-Day Intervals

	Control Mean±STD	Experimental Mean±STD	P value
13 day	260.6±19.17	126.6±10.02	0.0001
14 day	6019.8±19.59	166.23±13.16	0.0001

P significant at level <0.05.

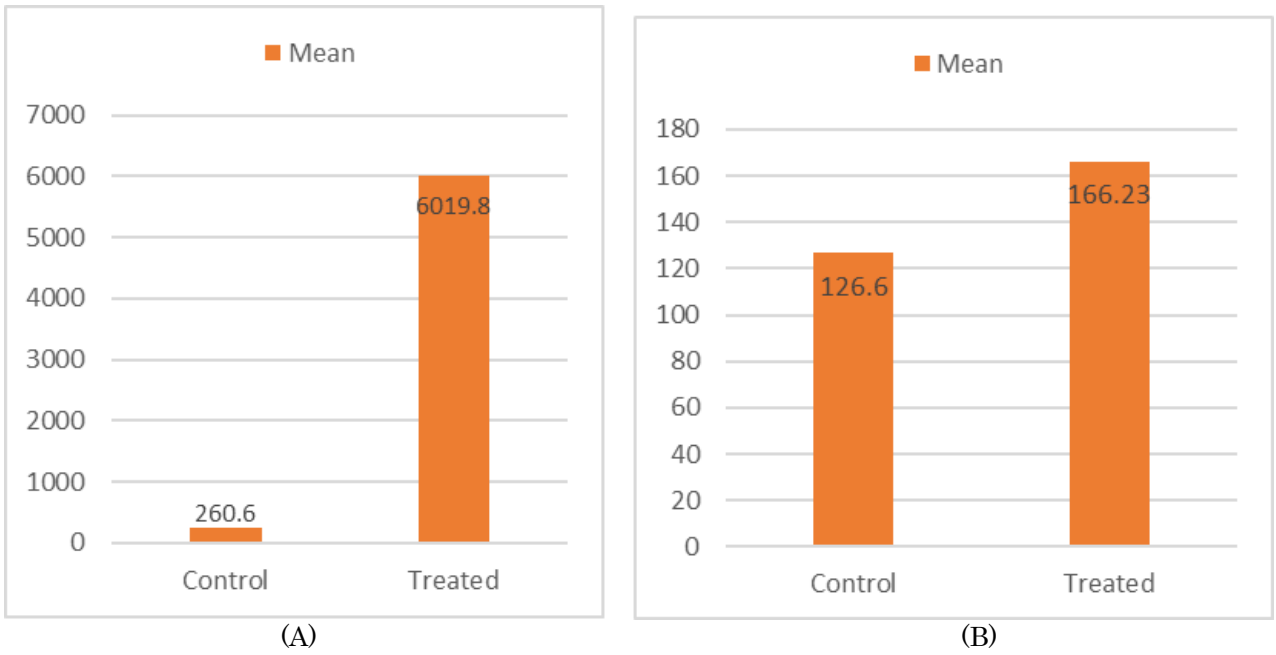

Figure (2): Comparison between the control group and experimental group for Alkaline Phosphatase reaction in the diaphysis of the femur at (a) day 13 and (b) day 14.

4. Conclusions

This study highlights the osteogenic influence of calcium carbonate nanoparticles (CCN) on femoral development in chick embryos. A marked elevation in alkaline phosphatase (ALP) activity was detected in femoral tissue homogenates from the CCN-treated group, particularly on day 13, signifying an upregulation in osteoblast function and active bone matrix formation. These outcomes imply that in ovo exposure to CCN may promote early mineralization and enhance the progression of bone tissue formation during embryogenesis.

Ethical approval:

All the procedures adopted in studies involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

Authors' contributions:

- Hayder J. Kadhim designed the study.
- Lamyaa Hadi worked the experimental laboratory work.
- Fatma Ayadi arranged the documentation of the results.

All authors read and approved the final version of the manuscript.

Funding: No funding has been received for this work.

Declaration of Competing Interest:
The authors declare that they have no conflicts of interest to dis- close.

Acknowledgments:
We would like to thank all the staff members of the College of medicine Al-Nahrain University and University of Sfax for the support provided in the study. My sincere thanking to Prof. Dr. Asmaa Hadi Mohammed and Assist. Prof. Dr. Marwa A. Hassan for deep help.

Conflicts of Interest: The authors declare no conflict of interest in this study.

Funding statement: The authors declare that no resources provide funding for this study.

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