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# Experimental long bone fracture healing in goats with cockle shell-based calcium carbonate bone paste

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A R T I C L E I N F O

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A B S T R A C T

Long bone fractures are common orthopedic conditions. There are numerous ways to repair these fractures. Bone grafting becomes necessary when a broken bone has a significant gap. However, due to insufficient donor volume and donor site morbidity, substitutes are required. In veterinary orthopaedics, calcium carbonate from cockle shells could be used as a bone biomaterial. We investigated its efficacy as a bone biomaterial repair for goat femoral fractures. The study included 10 healthy adult male Black Bengal goats weighing 8 kg and aged 12–13 months. The study includes control and treatment groups. Intramedullary pinning stabilized an 8-mm right femur diaphyseal fracture in the treatment and control groups. The treated group received 2 ml of bone paste in the fractured gap, whereas the control group left it empty. We examined all goats with X-rays on the 7th, 45th, and 60th days, followed by gross and histological findings. Due to callus bridging, radiographs revealed faster bone growth in the treated group than in the control group. Gross examination demonstrates the treated group had a larger fracture callus than the control group. Histopathology showed that bone formed faster and included more osteocytes, osteoblasts, osteoclasts, and bony spicules than in the control group. The treated group had more periosteum osteoblasts, while the control group had fibroblasts. These results showed that the treated group had more osteogenic activity than the control group. This study demonstrates the potential of cockle shell-based calcium carbonate bone paste as a synthetic biomaterial for healing long bone fractures in goats.

## 1. Introduction

In numerous clinical scenarios, bone regeneration is frequently compromised, particularly when large bone defects are formed, as in the case of severe trauma or following tumor resection. When this occurs, a more advanced approach to regeneration is necessary to facilitate bone healing and enhance its mechanical performance (Ni et al., 2023). Internal fixation involves surgically placing devices such as plates, screws, nails, wires, and pins to secure fractured fragments in their proper position during bone fracture. The specific type of internal fixator used depends on the location and type of fracture.

The practical application of regenerating and repairing large segments of bone is a difficult task that requires the use of autografts, allografts, and synthetic bone materials (Kim et al., 2020). Till now, the conventional approach has involved the utilization of autografts, which possess osteogenic cells that facilitate the generation of new bone tissue (Lippens et al., 2010). However, there are a number of drawbacks, including insufficient donor volume and donor site morbidity, necessitating the need for substitutes (Brandt et al., 2010).

For clinical repair of large bone defects, it is crucial to develop an artificial bone graft that possesses ideal osteoconduction and osteoinduction properties Zhi et al. (2022). Calcium carbonate ($CaCO_3$) finds widespread application in tissue scaffold construction, bone regeneration, and the development of state-of-the-art drug delivery systems (Hussein et al., 2020). Calcium carbonate ($CaCO_3$) occurs in three different polymorphs: calcite, aragonite, and vaterite (Bala et al., 2007). Aragonite is one of the most common biogenic polymorphs of calcium carbonate, widely used as a biomaterial for fracture bone healing (Islam et al., 2012). Due to their biocompatible characteristics, substantial research has been carried out on aragonites (Chen & Xiang, 2009).The aragonite polymorphs have many desirable properties for a biomedically important material (Wang et al., 2021).

Cockle (*Anadara granosa)* shell based nanoparticle bone paste rich in aragonite provides inexpensive, easily accessible calcium-based bone

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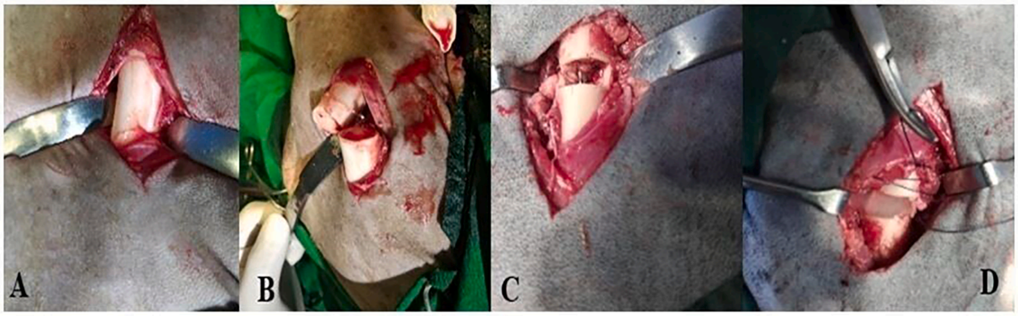


**Fig. 1.** Experimental femur fracture and making cortical bone gap.
Representative image of the experimental femur fracture in Black Bengal Goats. Normal femur bone (A), osteotomy in right femur (B), making 8 mm of bone gap and apposition of bone using intra medullary pinning (IMP) (C), apposition of bone by IMP and bone paste (D).

grafting materials that have been utilized as bone-filling materials in rabbits due to their high osteoconductive properties (Islam et al., 2011; Shafiu et al., 2013; Wang et al., 2005). The aragonite promotes the periosteal cells, which play a crucial role in the development of bone and cartilage within the callus during the healing of fracture (Colnot, 2009).

Based on the existing literature, there has not been conducted research to determine the efficacy of $CaCO_3$ bone paste made from cockle shells for the healing of long bone fractures in goats. Therefore, the objective of this study was to investigate the efficacy of calcium carbonate (CaCO3) derived from cockle shells for enhancing fracture healing in goats.

## 2. Materials and methods

### 2.1. Experimental goats

The research was carried out as a pilot study on 10 healthy male Black Bengal goats, with similar age of twelve to thirteen months, weighing 8 kg on average, and free from endo and ectoparasites, infectious and neurological diseases, or congenital abnormalities. The animals were divided into the treated group ($n = 5$) and the control group ($n = 5$). All goats were reared in standard housing with a 12-hour light-dark cycle and ad libitum access to balanced feed and water.

### 2.2. Cockle shell-based $CaCO_3$ bone paste preparation

In accordance with the previously published article (Islam et al., 2012, 2013), $CaCO_3$ bone paste was made. In brief, cockle shells were oven-dried at 50 °C for seven days after being cleaned, scoured to eliminate dirt, boiled, and then rinsed with distilled water. To create micron-sized powders, the shells were pulverized using a blender (Blender, HCB 550, USA) and sieved through a 90 μm pore stainless laboratory sieve (Endecott Ltd., London, England) to produce micron-sized powders. After being dried once more, the powders were crushed using a mortar and pestle and dried once more for seven days at 50 °C in an oven. Next, distilled water (HPLC-grade of resistance > 18 MΩ produced from a Milli-RO6 and Milli-Q-Water System, Organex) was used to prepare a 2 % glacial acetic acid solution (v/v). The chitosan aqueous solution was then made by adding 1.5 gram of chitosan (Sigma-Aldrich) to 100 ml of a 2 % glacial acetic acid solution. In order to make the bone paste, 8 g of calcium carbonate powder made from cockle shells were added to the chitosan aqueous solution, which was then stirred for five hours at 37 °C and 1000 rpm using a systematic Multi-Hotplate Stirrer. Next, the glass beaker with the paste was sterilized by radiation at 100 °C for 10 min in a micro-oven (Memmert, Germany). The paste was poured into glass petri dishes and dried for three days at 70 °C in an oven (Memmert UM500 GmbH Co., Germany) to make it suitable for usage.

### 2.3. Surgical approach for femur osteotomy and fracture management

Standard surgical procedures were followed. The surgical site was prepared aseptically by using 7.5 % providone iodine and 70 % alcohol after clipping and shaving. The goats were administered a sedative IV dose of 0.5 mg/kg diazepam (Injection Sedil®, Square Pharmaceuticals Ltd., BD). The operated site was desensitized by using 2 % lidocaine hydrochloride (Injections Jasocaine®, Jayson Pharmaceuticals Ltd. BD) in a line block at 1 ml/sq. cm.

The goats were placed in a lateral recumbent position, with the right limb positioned dorsally. The incision was given craniolateral to the femur bone and the femur has been exposed. The incision of 5 cm allowed for a better view of the femoral diaphysis by separating the vastus lateralis muscle from the surface of the femur (Fig. 1, A). A right diaphyseal femur fracture was created using an osteotomy saw, followed by the creation of an 8-mm gap with the help of a measuring scale within the fracture site (Fig. 1, B, and C). Retrograde intramedullary pinning (IMP) was performed in both groups, and the gap was filled with 2 ml of bone paste in the treated group only (Fig. 1, D) and control group remain empty. The muscle was sutured with a simple continuous suture with a 1-number catgut, and the subcutaneous tissues were sutured into a simple continuous pattern using 1–0 cat gut. The skin was closed with an interrupted cross-mattress suture using 2–0 silk.

### 2.4. Radiographic evaluation

A SHIMADZU 500 mA, 3-phase, 6-pulse x-ray generator with a focal film distance of 100 cm and a 60 kVp (kVp), 250 mA (mA), and 22 mA-seconds (mAs) was used to take X-rays of the right limb at different times after surgery. These X-rays showed the fracture healing process in mediolateral and craniocaudal views of the limb.

### 2.5. Gross examination

On postoperative day 60, the goats were slaughtered, and the healed fractured femur bone was taken immediately. All the muscles attached to the fractured areas were cleaned and evaluated to see the bone healing progression. X-rays were taken for further study. For histological examination, only the healed fractured site was collected and immediately fixed in a 10 % neutral buffered formalin solution for 5 days.

### 2.6. Histological slide preparation

The preserved, healed bone was cleaned and cut into small pieces as a cross-section. Then it was immersed in a 10 % formic acid solution for decalcification. Subsequently, the tissue was dehydrated by a gradient concentration of alcohol, cleaned using xylene, and then embedded in a paraffin block. Then the block was cut at a 5 μm thickness, stained with hematoxylin and eosin, and observed under a microscope as described

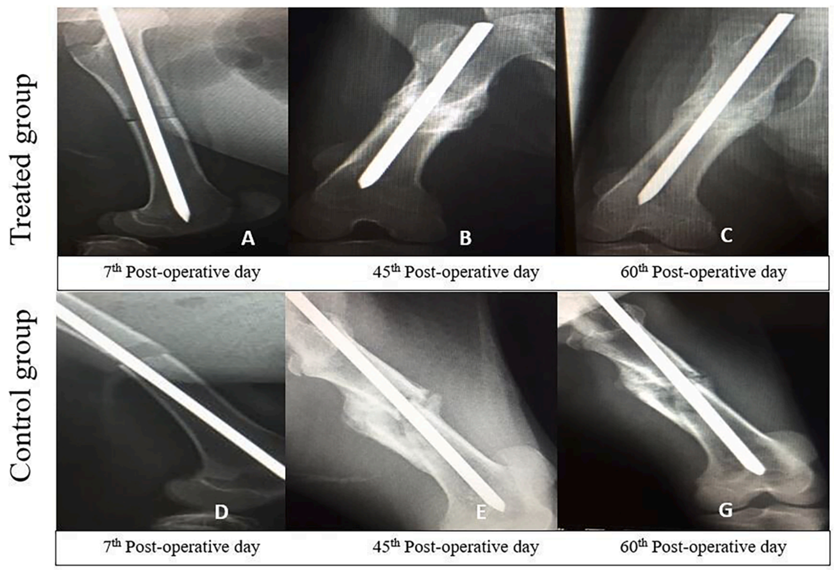


**Fig. 2.** Radiographic evaluation of fracture healing.
A representative image of the fracture healing was conducted at a certain time interval: bone paste group $A$ = day 7th, $B$ = day 45th, and C = day 60th, and control group $D$ = day 7th, $E$ = day 45th, and $F$ = day 60th.

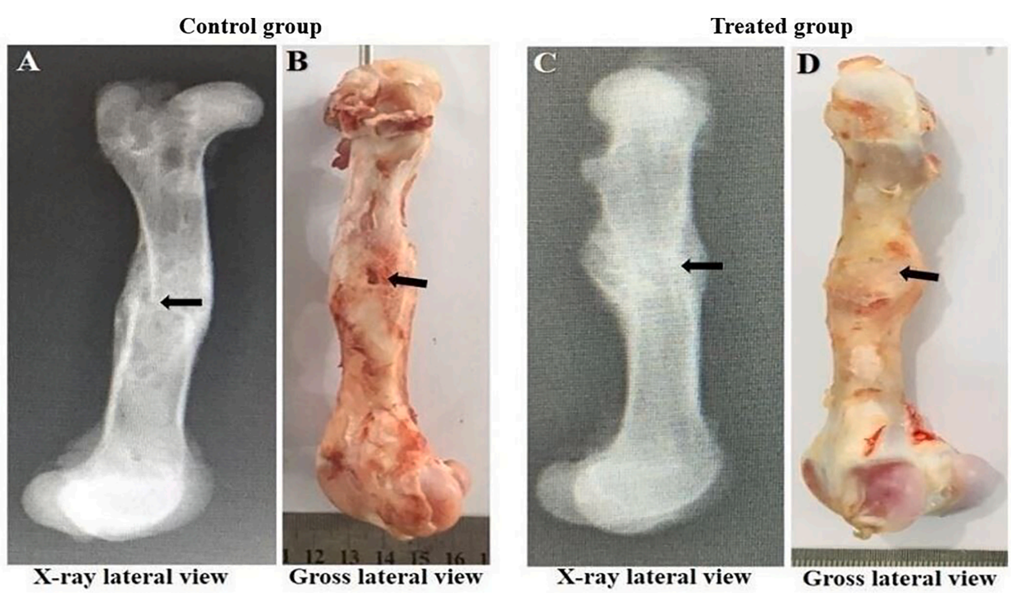


**Fig. 3.** $CaCO_3$ bone paste enhances bone healing.
Representative image of the gross lateral view of the bone healing. X-ray lateral view (A) and gross lateral view (B) of the partially healed bone in control group. X-ray lateral view (C) and gross lateral view (D).

previously (Karali et al., 2022).

## 3. Results

### *3.1. Radiographic observations*

Radiographic evaluation of the fracture healing was conducted at different time intervals. Radiographic examination revealed gradual progress of bone healing, such as bridging callus formation as well as no fracture gap in fractured areas at the 60th postoperative day in the treated group (Fig. 2. A, B and C) in comparison to the control group, where there were fewer bridging callus and less fractured gap (Fig. 2. D, E and F).

### *3.2. Gross observations*

The treated group of goats exhibited a considerably greater volume of callus formation at the fracture site (Fig. 3. D) compared to the control group (Fig. 3. B). In all instances, the treated group exhibited complete healing of the gap, but the control group showed the persistence of a gap, indicating a delayed process of bone formation.

### *3.3. Histological observations*

The bone cells in the treated groups were arranged normally, with more osteocytes and less osteoblastic activity than the control group, as shown in (Fig. 4. A, and B). During the bone regeneration process, the treated group exhibited endochondral ossification. In the treated group,

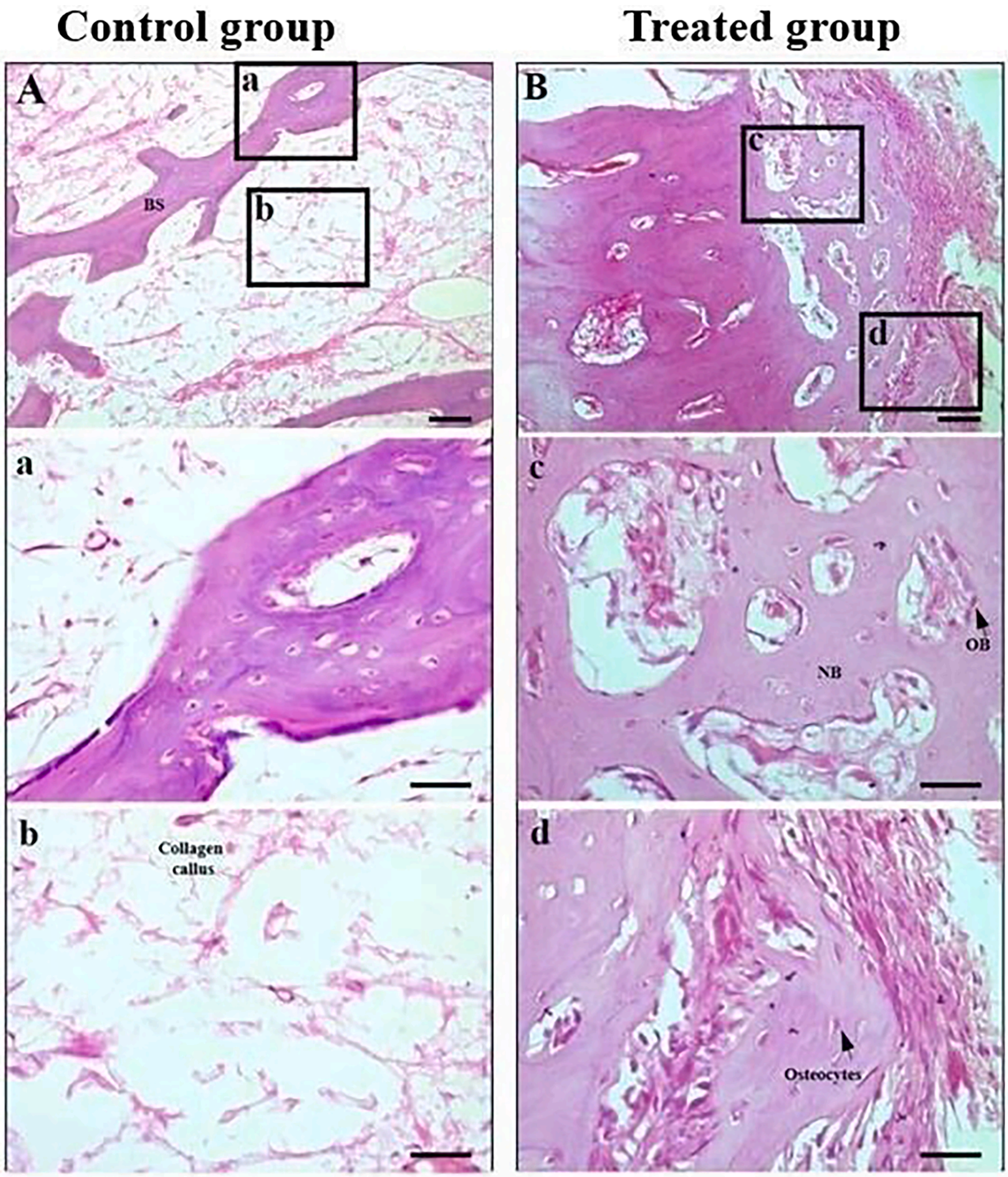


**Fig. 4.** $CaCO_3$ bone paste enhances osteogenesis.
Representative histomorphology image of newly formed bone from the middle section of repaired femur by random selection in control and experimental group (A, a, b and B, c, d) Hematoxylin and Eosin (H&E) staining showing newly formed bone (NB), osteoblasts (OB), bony spicule (BS) and osteocytes. Boxed area is enlarged in the below pictures. Scale for low magnification: 500 μm, for high magnification: 40 μm.

both the inside and outside of the fracture site have a large amount of newly formed bone tissue. Additionally, there were fewer osteoblasts near the periosteum in the treated group, whereas in the control group showed more fibroblasts. There was juvenile trabecular bone, fibrous connective tissue, and adipocytes in the bone of the control groups which healed incompletely. Histological observations thus corroborate the osteoconductive properties of the cockle shell-based $CaCO_3$ treated.

## 4. Discussion

The fracture gap healed smoothly in the treated group and there were no signs of foreign body reaction, implant failure, or infection. According to Dizaj et al. (2015), the use of calcium carbonate as a bone substitute in human bone defects and tooth disorders revealed no signs of foreign body reaction or infection. Zhi et al. (2022) reported that in goats, a 3 cm-long bone defect was filled with a basic calcium phosphate (BCP) ceramic scaffold. The defect healed quickly and there were no signs of a foreign body reaction, implant failure, or infection after surgery. These finding are very similar to the present research work, where there was no evidence of post-operative complications.

A callus indicates the bone healing potential after a fracture (Inglis et al., 2022). In an X-ray image, the femur of a rat in the treated group with a fracture had a much greater callus volume than the control group, according to Shuid et al. (2010). This implies a higher healing score, indicating that a fracture is healing. In the later stage of fracture healing, bone remodeling usually replaces most of the callus with lamellar bone (Marsell & Einhorn, 2011). The present research demonstrates that the fracture in the treated group healed more quickly and had a larger callus volume than the control group.

In a study by Zhi et al. (2022) observed that the graft material at the femoral defect in the 9-month repair group was well integrated with the host bone, the new bone regeneration and construction were obvious, the implant site was significantly thicker, and bony callus formation was visible to the naked eye, but in the 18-month repair group, the implant site was more closely connected. This supports the present study that in the treated group the gap was filled with a more closely connected bone end, the new bone was further reconstructed, and the diameter tended to be similar to the middle and upper part of the normal femur, while in control group bone gap was visible, gap site was significantly thicker, and bony callus formation was visible to the naked eye. The complete bone healing within 60 days in present study equally agreed with the report of previous researchers following various implants for bone healing in rabbits (Marques et al., 2010; Oh et al., 2005) and humans (Hallman & Thor, 2008).

Histologically, endochondral ossification heals the gap with CaCO3 to generate lamellar bone. During bone regeneration, autogenous or allogeneic bone transplants cause endochondral osteogenesis (Dennis et al., 2015). Mesenchymal cells generate cartilage templates. Hypertrophic chondrocyte differentiation ensues and calcification of their matrix provides a pattern for osteoblasts and osteoclasts to produce new bone (Breur et al., 1991). This supports the present study's finding that this mechanism is identical to bone osteogenesis and development under physiological conditions in treated but not in control groups. The formation of periosteum from outer to inner indicates intramembranous osteoconductive bone production. In the earlier study by Gogolewski et al. (2000) reported that the periosteum formed from the implant's outer to inner surface and adipose and fibrous connective tissue filled the bone gap which are similar to the histological finding in the bone healing phase of the femur bone goats of our control group.

The design strategy successfully treats large segmental bone defects with calcium carbonate bone paste, demonstrating its ability to heal an 8-mm gap. This approach may be helpful for clinical application in treating such defects using calcium carbonate bone paste.

## 5. Conclusion

The complete bone recovery observed within 60 days of implantation of cockle shell-based calcium carbonate bone paste biomaterial is an indication that this new nanocomposite bone material has the potential to enhance fracture healing and bone regeneration, and such material should be utilized in veterinary practice and research.

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## Ethical statement

The study was conducted according to the guidelines provided by the ethical committee of the Chattogram Veterinary and Animal Sciences University, Bangladesh. (EC of CVASU Approval no: Memo no CVASU/ Dir (R&E) EC/2022/435(1)/5.

## CRediT authorship contribution statement

**Saroj Kumar Yadav:** Writing – original draft, Methodology. **Subrata Kumar Shil:** Writing – review & editing, Formal analysis. **Monoar Sayeed Pallab:** Writing – review & editing, Methodology, Investigation. **Kh. Nurul Islam:** Resources. **Bibek Chandra Sutradhar:** Supervision. **Bhajan Chandra Das:** Writing – review & editing, Writing – original draft, Supervision, Project administration.

## Declaration of competing interest

The nature of potential conflict of interest is described below:

No conflict of interest exists.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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