Effect of hyaluronic acid on osteoblast function in vivo and on fracture healing in an experimental rabbit model

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Research article

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Abstract

Background Fracture healing is regulated by endocrine hormones and biochemical and biophysical factors, including hyaluronic acid (HA), which is a component of the extracellular matrix. The prerequisites for biological responses that promote fracture healing, such as biomechanical conditions, and molecular factors, can be investigated in bone cell cultures. **Methods** In cell experiments, CCK-8 was used to detect the effect of different concentrations of HA on MC3T3-E1 cells after cell intervention. After 10% of HA interfered with MC3T3-E1 cells, they were cultured in induction medium and observed for their mineralization **Impact**. Total protein was extracted from cells to observe Runx2 and OCN protein expression. After successful modeling in animal experiments, X-rays of the fracture site were taken to determine bone density at the fracture end. After the fracture site was taken, histological examination was performed and the callus was analyzed quantitatively and qualitatively. **Results** In this study, HA promoted the formation of calcium nodules and the expression of runt-related transcription factor (Runx)2 and osteocalcin (OCN) proteins in MC3T3-E1 cells; In a rabbit tibial fracture model, HA promoted formation of larger calluses than those in control or sham-treated animals at 2, 4, 6, and 8 weeks ($P < 0.05$). Osteophytes were larger in HA-treated than in control animals at 4 and 6 weeks ($P < 0.05$). Bone mineral density was greater in HA-treated than in control animals at 4, 6, and 8 weeks ($P < 0.05$). **Conclusions** The results showed that HA promoted the formation of calcified nodules in bone cell cultures and healing of rabbit tibial fractures.

1. Background

Fracture healing is regulated by endocrine hormones, biochemical, and biophysical factors. The prerequisites of good biological responses promoting fracture healing, including biomechanical conditions, and molecular factors, can be investigated in bone cell line cultures. Hyaluronic acid (HA) is a polysaccharide polymer present in the extracellular matrix of vertebrate connective tissue, and was first isolated from bovine vitreous humor^[1,2]. HA is composed of repeating disaccharide units of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc), with formation of glycosidic bonds catalyzed by hyaluronan synthases^[3]. It has broad medical applications, including relief from osteoarthritis pain and improving joint function because it is biocompatible, hydrophilic, absorbable, and has anti-inflammatory activity^[4]. HA supports cell proliferation and differentiation in temporary scaffolds used to promote the growth and calcification of osteoblasts and new bone formation^[6]. There is extensive evidence of the effects of HA on osteoblasts. Injection of 5,000 kDa high molecular weight HA into the maxilla and mandibula of rats resulted in a significant increase in the osteoblasts present in the jaws and premaxillary suture^[7]. Sá found that low concentrations of HA (1, 10, and 100 ng/mL) had no effect on osteoblast activity but reported that methylthiazolyl tetrazolium bromide (MTT) and immunofluorescence assays found that a high concentration (1 g/L) for 48 h decreased osteoblast viability^[9]. Von Kossa calcium staining of osteoblast cultures showed that 100 ng/mL HA significantly increased the area of nodule formation and RT-PCR confirmed that that HA was associated with type I and III collagen, osteocalcin, and bone morphogenetic protein 2 and 4 expression^[11]. Injection of 1% HA into the alveolar

fossa after tooth extraction was found to increase the deposition of mineralized bone and osteoblast number^[8]. Another study found that HA did not influence cell proliferation, but alizarin red staining confirmed that it induced osteoblast differentiation. Formation of mineralized nodules increased with the expression of osteocalcin mRNA and the number of calcium nodules formed by osteoblasts increased with the increase of the molecular weight of HA^[10,12]. Other studies have confirmed the effect of HA on cell viability by an oxygen consumption assay and increased expression of type I collagen and osteonectin by immunohistochemistry^[13]. In an inflammation model induced by interleukin-1, HA increased metalloproteinase 3 and matrix metalloproteinase 13 expression and reduced the expression of ADAMTS-4 and -5, RANKL, CD44, and the intercellular adhesion molecule (ICAM)-1 gene^[14]. Boeckel reported that HA did not have a significant effect on osteoblast viability as assayed by MTT^[15].

This study investigated the effects of HA on the growth and differentiation of osteoblasts and promotion of fracture healing. The effect of HA on the proliferation of MC3T3-E1 cells was assayed by CCK-8 and alizarin red, formation of calcium nodules and expression of Runx2 and OCN proteins was assayed by immunoblotting. Bone histomorphometry was investigated by X-ray in a rabbit model of tibial fracture.

2. Material And Methods

Test cells: MC3T3-E1 cell line, purchased from Shanghai Zhongzhou Biotechnology Co., Ltd..

Test animals: New Zealand white rabbits, purchased from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.1. Cell culture

MC3T3-E1 cells were cultured in a high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum at 37°C, 95% relative humidity, and 5% CO₂. The cells were passaged every 3–4 days at 1:3. After two–four passages, osteoblasts in logarithmic growth phase were used in the experimental procedures.

2.2. Cell proliferation

A 1 mL suspension of $2 \times 10^7 \text{ L}^{-1}$ MC3T3-E1 cells was added to each well of a 24-well plate and 0.125 µg/µL, 0.25 µg/µL, 0.5 µg/µL, or 1 µg/µL HA in high-dose DMEM containing 10% fetal bovine serum was added. An equal volume of medium alone was added to control wells. The plates were incubated at 37°C, 95% relative humidity, and 5% CO₂ with a change in medium every 2 days. The cells were collected by trypsinization on day 1, 5, or 7, suspended in 1 mL high glucose DMEM containing 10% fetal bovine serum, and seeded in 96-well culture plates at 200 µL well with six wells per sample. After culture for 24 h, 10 µL CCK-8 was added and the absorbance was measured at 450 nm using a microplate reader after incubation for 30 min.

2.3. Western blot assays

Total protein was isolated from MC3T3-E1 cells before and after HA treatment and 15–20 μL samples were separated on 10 cm $\times$ 10 cm gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred by blotting onto membranes for immunological hybridization with anti-runt-related transcription factor 2 (Runx2) and anti-osteocalcin (OCN) primary antibodies and color development. The expression of Runx2 and OCN proteins in MC3T3-E1 cells before and after HA treatment were compared.

2.4. Mineralization of MC3T3-E1 cells

A 1 mL suspension of $2 \times 10^7 \text{ L}^{-1}$ MC3T3-E1 cells in high sugar DMEM was added to each well of a 24-well plate. HA in DMEM with 10% fetal bovine serum, $0.05 \text{ mmol} \cdot \text{L}^{-1}$ ascorbic acid, and 10 mmol β -glycerol was added. The plates were cultured at 37°C , 95% relative humidity and 5% CO_2 and the culture medium was changed every 3 days. After 21 days of culture and appearance of milky white spots, culture medium containing 50 mg/L alizarin red was added for 30 min. Cultures were then rinsed three times with phosphate-buffered saline, and then fixed with a volume fraction of 95% ethanol for 10 min.

2.5. Animal model

The institutional Ethics Committee approved the study. Eighty adult male white New Zealand rabbits 8–10 months of age and weighing 2,000–3,000 g were used and were handled following the Ethics Committee guidelines for the use of animals in experimental studies. Forty-eight rabbits were randomly divided into experimental, control, and sham operation groups of 16 each. After anesthesia induced by intravenous injection of 50 mg/kg sodium pentobarbital, the left rear leg was shaved, the skin was disinfected and surgically opened. The subcutaneous tissue was dissected to expose the tibia and an incomplete transverse fracture as made in the middle tibia with pendulum saw. The fracture width was about 2 mm and the depth was about one-third of the tibia diameter. The wound was sutured and penicillin was injected intramuscularly for 3 days after surgery to prevent infection (Fig. 1).

Rabbits in the experimental group were given a single 0.2 mL HA injection into the fracture region on the first day after surgery. Those in the control group were given normal saline, and sham operation rabbits were not given any treatment after surgery. Two, 4, 6, and 8 weeks after surgery, four rabbits in each group were randomly selected, anesthetized with 1% pentobarbital and sacrificed for evaluation of bone healing on X-ray films and bone histomorphometry.

All experimental animals were given an excessive intraperitoneal injection of pentobarbital to prevent pain and sudden death in rabbits, which was not ethical. The rabbits after the death were incinerated by the Animal Experimental Center of Lanzhou University.

2.6. Statistical analysis

The statistical analysis was performed with SPSS 10.0. Differences in general condition (wound oozing, redness and infection, daily feed consumption, activity level, and behavior) and histological findings (bony area and width of the epiphysis, and percentage of osteoid tissue) were compared. The paired-

sample t-test was used for within-group comparisons. The independent t-tests was used for between-group comparisons. The significance level was $P < 0.05$.

3. Results

3.1. Proliferation of MC3T3-E1 cells

When added to cultures of MC3T3-E1 cells at concentrations of 0.125, 0.25, 0.5, or 1 $\mu\text{g}/\mu\text{L}$, HA had no significant effect on proliferation ($P > 0.05$) after either 36 or 72 h of incubation. (Fig. 2).

Figure 2. (A) Effect of HA intervention on the proliferation of MC3T3-E1 cells after 36 h and (B) after 72 h.

3.2 formation of calcium nodules in MC3T3-E1-cell cultures

Alizarin red staining after 28 days of cell culture with 1 $\mu\text{g}/\mu\text{L}$ HA revealed large numbers of calcified nodules. Mostly were round or elliptical, a few were irregular. Fewer nodules were seen in the control cultures (Fig. 3). Comparison of six randomly selected fields in each well for each treatment found that the area of the mineralized nodules in was significantly larger in HA-treated than in control cultures ($P < 0.05$, Fig. 3).

A:Formation of MC3T3-E1 mineralized nodules under optical microscope;B:Mineralized nodule area relative to control group after HA intervention

3.3 Expression of Runx2 and OCN proteins in MC3T3-E1 cells

Western blot results showed that both Runx 2 and OCN expression were upregulated by the induction medium and were further upregulated after adding HA. Protein expression was the highest at 1 $\mu\text{g}/\mu\text{L}$ HA and was significantly higher at that concentration than it was in the control group. ($P < 0.05$, Fig. 4)

3.4. Animal model

All 48 New Zealand rabbits were successfully modeled. No rabbits died, but their activity and food consumption decreased, and their mental state was impaired for at least 1–3 days. Rabbits in the sham operation group returned to normal after 4 days. Those in the experimental group returned to normal after 1 week. Animals in the control group were less active than those in the other two groups for 2 weeks. Most rabbits appeared healthy, but those in the sham operation, control, and experimental groups remained lame for about 1 month. Half the rabbits developed edema in the lower extremities that resolved within 2 weeks. One of the two rabbits that developed infections died.

3.5 Evaluation of fracture healing

X-ray films of rabbits in the control group at 2 weeks after surgery showed a sharp fracture edge, mildly reactive periosteum, a clear fracture line, discontinuous cortical bone, and swelling of the surrounding soft tissue. In the experimental group, the appearance of the fracture edge, periosteal cortical bone were similar to controls. The fracture line was clear but slightly blurred compared with the control group. There less swelling of the surrounding soft tissue than in the control rabbits (Fig. 5).

In X-ray films of control group rabbits 4 weeks after surgery, the fracture edge was not clear, the size of the callus had increased, the fracture line was blurred, the cortical bone was discontinuous, and the surrounding soft tissue shadow persisted. In the experimental group animals, the fracture edge was also blurred, with increased callus formation and discontinuous cortical bone, but the fracture end bone density was higher and the surrounding soft tissue shadow was reduced compared with control group rabbits (Fig. 6).

At 8 weeks after surgery, the fracture edge of the control group was blurred, the external epiphysis surrounded the fracture, the edge of the epiphysis was clean, and the fracture line was visible but fuzzy. The cortical bone was discontinuous. The bone density and blurring were greater in the rabbits in the experimental group compared with the control animals and the fracture line was nearly invisible. (Fig. 8)

Bone histomorphometry included measuring the total area of the epiphysis on lateral radiographs at 2, 4, 6, and 8 weeks after fracture. The osteophyte area was greater in the experimental than in the control rabbits at 2, 4, and 6 weeks, but the difference was no longer significant at 8 weeks because of increased size in the control group (Tables 1–3 and Figs. 9,10).

Table.1 bone metrology parameters in experimental group and control group after 2 week(n = 6)

parameter	Control group	test group	P value
Bone-like area(mm2)	71.48 ± 8.03	112.36 ± 6.52	P >0.05
Bone-like width(mm)	6.08 ± 0.39	6.14 ± 0.14	P >0.05
Period percentage(%)	2.51 ± 0.30	2.94 ± 0.61	P >0.05

Table.2 bone metrology parameters in experimental group and control group after 4 week (n = 6)

parameter	Control group	test group	P value
Bone-like area(mm2)	93.25 ± 6.61	158.10 ± 9.64	P >0.05
Bone-like width(mm)	7.13 ± 0.44	7.27 ± 0.31	P >0.05
Period percentage(%)	2.85 ± 0.40	10.01 ± 0.70	P >0.05

Table.3 bone metrology parameters in experimental group and control group after 6 week (n = 6)			
parameter	Control group	test group	P value
Bone-like area(mm ²)	173 ± 4.71	179 ± 8.36	P = 0.05
Bone-like width(mm)	4.93 ± 0.35	5.04 ± 0.42	P = 0.05
Period percentage(%)	2.18 ± 0.63	2.37 ± 0.36	P = 0.05

4. Discussion

The molecular formula of HA is (C₁₄H₂₁NO₁₁)_n; the structural formula is shown in Fig. 11. It is a linear polysaccharide composed of a repeating linkage of GlcA and GlcNAc by

β-glycosidic bonds. It has a negative charge and is present in the connective tissue of vertebrates. Unlike other glycosaminoglycans (GAGs) in connective tissue, HA is synthesized in the plasma membrane rather than the endoplasmic reticulum or Golgi body. It contains no peptides, is not linked to a core protein, is not sulfated, and has not undergone any postsynthesis modification. Like other GAGs, HA consists of a single polysaccharide chain that can have a molecular weight in the millions. Secondary hydrogen bonds that form along the axis give HA a rigid spiral columnar structure that is nonpolar and hydrophobic. The surface of the column includes hydroxyl groups with strong hydrophilicity. Its structure gives HA excellent rheological properties, lubricity, permeability, and water retention. When hydrated, HA is viscoelastic^[5]. HA has great potential for medical applications.

Previous studies have described the effects of HA on cell adhesion and migration, angiogenesis, morphogenesis, wound healing, inflammatory responses, and cancer metastasis. Preclinical studies of HA in orthopedics have included treatment of osteoarthritis, cartilage tissue engineering, and studies of bone morphogenetic protein carriers. Kim described the use of HA acrylate-based hydrogels as tissue engineering scaffolds to carry seed cells and active molecules to induce bone formation^[16]. Tissue engineering has achieved good early results in the repair of cartilage tissue. The use of stents to implant specific cell types and bioactive molecules has also been described^[17]. Martínez-Sanz described the preparation and use of noncytotoxic crosslinked aldehyde- and hydrazide-modified HA gels for controlled release of bone molecular protein (BMP)-2^[18]. In a rat calvarium model of bone formation, the hydrogels induced formation of new bone and increased osteocalcin and osteopontin expression. Bone cement composed of nanocrystalline hydroxyapatite crosslinked to an HA-tyramine conjugate was found to have a low heat release compared with conventional bone cement, produced little damage of surrounding tissues, and successfully repaired small bone and joint defects in mice^[19]. Exogenous HA oligosaccharides were reported to significantly inhibit growth of LM-8 and MG-63 osteosarcoma cells^[20].

Traumatic injury including severe fractures are increasingly common, and while treatment outcomes are generally favorable, 5–10% of patients experience fracture nonunion^[21]. Previous studies have not reported the promotion of fracture healing by HA in an animal model. Effective promotion of callus

formation, acceleration of fracture healing and overall patient healing are primary goals in orthopedic practice. HA did not significantly influence MC3T3-E1 cell proliferation, but it did promote the formation of mineralized nodules that was associated with increased Runx2 and OCN expression.

5. Conclusions

HA accelerated fracture healing by promoting the formation of osteophytes and relieving soft tissue swelling. It had a positive effect on the growth of osteoblasts, which is essential for bone healing.

Abbreviations

HA: Hyaluronic acid

OCN: Osteocalcin

GlcA: D-glucuronic acid

GlcNAc: N-acetyl-D-glucosamine

MTT: Methylthiazolyl tetrazolium bromide

GAGs: Glycosaminoglycans

DMEM: Dulbecco's Modified Eagle's Medium

Declarations

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Authors' contributions

Yongping Wang is the experiment designer and is responsible for experimenting with the entire project; critically modifying the manuscript of important knowledge content. Xiang Li: Participate in cell experiments, preparation of animal models; implementation of animal experiments; data collection; analysis and interpretation of data; statistical analysis; final approval of the version to be published. Ruimin Xie: Learning Concepts and Design; Preparation of Animal Models; Statistical Analysis; Drafting of Manuscripts; final approval of the version to be published. Xiong Wang: Preparation of animal models; data collection; final approval of the version to be published. Bohua LI: Data analysis and interpretation; final approval of the version to be published. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Ethics approval and consent to participate

The study was approved by Lanzhou University First Hospital Ethics Committee No. LDYYLL2019-219, and informed written consent was obtained.

Consent to publish

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

New Zealand rabbit tibia fracture model. (A) experimental, (B) control, and (C) sham groups.

Figure 2

(A) Effect of HA intervention on the proliferation of MC3T3-E1 cells after 36 h and (B) after 72 h.

Figure 3

Light microscopy ($\times 100$) of mineralized nodule formation in HA-treated and control cultures following alizarin red staining. (**** $P < 0.05$ vs control)

Figure 4

Effect of HA combined with the induction medium on Runx 2 and OCN protein expression in MC3T3-E1 cells.

Figure 5

X-ray films 2 weeks after surgery in the (A) experimental and (B) control groups.

Figure 6

X-ray films 4 weeks after surgery in the (A) experimental and (B) control groups.

Figure 7

X-ray films 6 weeks after surgery in the (A) experimental and (B) control groups.

Figure 8

X-ray films 8 weeks after surgery in the (A) control and (B) experimental groups.

Figure 9

Osteophyte areas in experimental and control group rabbits 2, 4, and 6 weeks after surgery.

Figure 10

Epiphysis width in experimental and control group rabbits 2, 4, and 6 weeks after surgery.

Figure 11

Structure of hyaluronic acid

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