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The hyaluronic acid gel promotes the formation of osteoblasts mineralized nodules and fracture callus by regulating the expression of Runx2 and osteocalcin

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ABSTRACT

This study aimed to investigate the effect of hyaluronic acid (HA) gel regulating the expression of Runx2 and osteocalcin (OCN) on osteoblast mineralization nodules and fracture callus formation. To achieve this aim, MC3T3-E1 cells were divided into two groups, the HA group and the control group, based on the intervention of HA gel. In addition, a fracture callus model was constructed to observe cell proliferation, cell mineralization, and fracture callus formation. Results showed that HA at different concentrations had no obvious outcome on the proliferation of MC3T3-E1 cells ($P>0.05$). The area of mineralized nodules in the HA intervention group (65.38±4.27) was higher than in the control (9.52±2.16, $P<0.05$). The expression levels of Runx2 and OCN in the HA intervention group were higher than control ($P<0.05$). The callus area in the HA group (110.05±4.16) and (143.16±8.84) was significantly higher as against control (72.51±6.32, 88.92±5.28) at 2 and 4 weeks after intervention ($P<0.05$). It was concluded that HA gel promotes the proliferation and differentiation of osteoblasts by regulation of Runx2 and OCN, and then promotes the formation of mineralized nodules of osteoblasts and fracture callus, thereby promoting fracture healing.

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Introduction

Fracture healing refers to the process of bone re-healing and regeneration through a series of biological processes after a fracture occurs (1). Under normal conditions, fracture healing is a complex process with multiple stages including blood coagulation, inflammation, chondrogenesis, bone formation, and remodeling (2,3). Among them, the inflammatory response is a key link. Through the role of inflammatory mediators, a large number of inflammatory cells and stem cells can be attracted to the fracture site to promote the proliferation and differentiation of cartilage and bone cells (4). In the process of chondrogenesis and bone formation, cells gradually differentiate into osteoblasts and chondrocytes, and synthesize bone matrix and cartilage matrix on collagen matrix, eventually forming mature bone tissue (5). However, due to the special nature of the fracture site, fracture healing may be affected by some adverse factors, such as poor local blood circulation, ischemia at the fracture site, infection, and surgical procedures. These factors may lead to the occurrence of complications such as prolonged fracture healing time and unstable or nonunion of the fracture (6,7). Therefore, the treatment of fracture requires comprehensive consideration of the type and degree of fracture, the age and health status of the patient, and the safety and effectiveness of treatment methods, etc., to select appropriate treatment programs and means to promote fracture healing and functional recovery.

Hyaluronic acid (HA) gel is a biodegradable polymer substance, which is a colorless and transparent viscous liquid mainly composed of HA molecules (8). HA is a polysaccharide molecule with good biocompatibility and biodegradability, which can be broken down and absorbed by the human body's enzymatic system, so it is widely used in medical and cosmetic fields (9,10). In recent years, HA gels have received extensive attention and applications in the field of orthopedics (11). HA gel can regulate the synthesis and secretion of bone matrix, and promote the mineralization of osteocytes and the regeneration of bone tissue (12,13). Therefore, HA gel can be used in fracture healing and bone defect repair and has good application prospects.

As a biomaterial, HA gel has the advantages of good biocompatibility, strong water absorption, and high viscosity, which can provide an ideal biological scaffold to provide the necessary support and growth environment for fracture healing (14,15). In addition, HA gel can also promote the proliferation, differentiation and mineralization of bone cells in a variety of ways, thereby accelerating fracture healing (16). Among them, regulating the expression of osteogenesis-related genes such as Runx2 and osteocalcin (OCN) is one of the important mechanisms by which HA gel promotes fracture healing (17). HA gel has potential advantages and application prospects in the treatment of fractures. Therefore, this article aimed to investigate the role of HA gel in the formation of mineralized nodules in osteoblasts and fracture callus and its related mechanisms, to provide new ideas and methods for the treatment of fracture.

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Table 1. Required materials and sources of materials.

Materials	Sources
The osteoblastic cell line MC3T3-E1	ATCC Corporation, USA
HA gel	Sigma-Aldrich (United States)
Calcium phosphate	Sigma-Aldrich (United States)
Alkaline phosphatase (ALP) kit	Nanjing Jiancheng Bioengineering Institute
α-MEM medium	Gibco (United States)
FBS	Gibco (United States)
Runx2 antibody	Cell Signaling Technology (USA)
OCNantibody	Abcam (UK)

Materials and Methods

Research materials

Table 1 gives the required materials.

Research methods

The experiment was divided into control and experimental groups. In the control and experimental groups, the cell culture, HA gel preparation, mineralized nodules of osteoblasts and fracture callus models were established. The cells in the control were cultured without HA, while the cells in the experimental group were cultured with HA. The total proteins of MC3T3-E1 cells before and after HA intervention were extracted and 15μL to 20 μL were loaded onto SDS-PAGE gel (10cm×10cm) for electrophoresis separation. The hybrid membrane of proteins was wet transferred in a bath, and then immunohybridization and color development were performed to analyze and compare the protein expression. CCK-8 assay was adopted to observe cell proliferation, qPCR analysis and fluorescence staining to detect the mineralization of osteoblasts, and qPCR and Western blot to detect the expression of Runx2 and OCN mRNA protein.

Cell culture

MC3T3-E1 cells were incubated in α-MEM medium (containing 10% FBS, 1% PS) and cultured at 37°C, 5% CO_2, and saturated humidity. The cell density was 1×10^5/cm^2, and the medium was changed every 3 days. The cells were passaged at a ratio of 1: 3 every 3-4 days, and the second to fourth passages of osteoblasts in the log phase were adopted as trial cells.

Preparation of the HA gel

(I) HA was added to 1×PBS buffer, pH to 7.4. (II) The solution containing HA was stirred in a water bath at 50°C for 1 h until HA was fully dissolved. (III) Gelatin was added to the HA solution and dissolved by adding dimethyl sulfoxide at the same time. The concentration of gelatin in the solution was 10 mg/mL, and the concentration of HA was 2%. (IV) The solution was stirred in the ice-water mixture for 10 min to form a gel. The cooled HA gel can be used for subsequent experiments.

Establishment of osteoblast mineralized nodule and fracture callus model

MC3T3-E1 cells were seeded in 96-well plates to form mineralized nodules. The cells were seeded on the 3D-printed artificial fracture callus to form a fracture callus model.

Staining of mineralized nodules

After cell culture was completed, the medium was taken, the cells were rinsed three times adopting 1×PBS buffer and were fixed by the addition of 4% formaldehyde for half an hour. The cells were rinsed adopting 0.1% formic acid and then stained with 2% acidic agarose. After precipitation with calcium-precipitating material, the cells were dissolved in a 2% potassium iodate solution to observe the formation of mineralized nodules.

Cell proliferation

MC3T3-E1 cells were seeded in cell culture plates, and then different concentrations of test substances, such as HA gels, were added at different time points. Subsequently, CCK-8 reagent was added, and after a certain period of culture, the cell proliferation ability was detected by detecting the absorption value of cell metabolites.

Statistical methods

SPSS 23.0 software was adopted for statistical analysis. The experimental results were compared by one-way analysis of variance at different time points in the same group, *t*-test was adopted for comparison between two independent sample groups, and analysis of variance was used for comparison among multiple groups. $P<0.05$ was considered statistically significant.

Results

The effect of different concentrations of HA on the proliferation of MC3T3-E1 cells

The effect of different concentrations (2μg/μL, 4μg/μL, 6μg/μL, 8μg/μL, 10μg/μL) of HA on the proliferation of MC3T3-E1 cells showed that 36 h and 72 h following the intervention, HA at different concentrations had no obvious effect on MC3T3-E1 cell proliferation, $P>0.05$ (Figure 1).

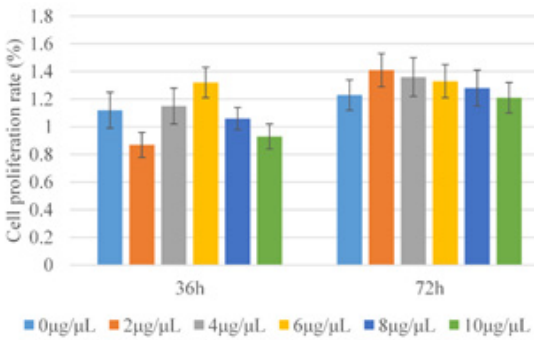

Figure 1. Effect of HA at different concentrations on MC3T3-E1 cell proliferation.

Area of mineralized nodules after HA intervention

10% HA was used to observe the formation of mineralized nodules in MC3T3-E1 cells. The presented results showed that the area of mineralized nodules in the HA intervention group (65.38±4.27) was obviously larger than the control (9.52±2.16, $P<0.05$) (Figure 2).

Effect of HA on Runx 2 and OCN protein expression in MC3T3-E1 cells

Western blot results suggested that the Runx2 and OCN in the HA gel group were higher as against control ($P<0.05$), indicating that HA gel could regulate the expression of Runx2 and OCN in osteoblasts (Figure 3).

Comparison of callus area and callus width between HA group and control after intervention

At 2 and 4 weeks after the intervention, the callus area of the HA group was 110.05±4.16 and 143.16±8.84, respectively, which was higher as against control (72.51±6.32 and 88.92±5.28), $P<0.05$. There was a similarity in the callus area between the two groups at 6 weeks following intervention ($P>0.05$). There was no statistically significant difference in callus width between both groups at 2, 4, and 6 weeks following intervention ($P>0.05$) (Figures 4 and 5).

Discussion

Fracture refers to the fracture of bone under the action of external force, which usually takes a certain time to heal (18). Fractures are usually divided into two types: closed and open (19). A closed fracture is a fracture of the bone but not of the skin; In open fractures, the skin is penetrated, leaving the bone exposed (20). Fracture healing is usually divided into three stages: the inflammatory stage, the repair stage and the remodeling stage (21). During the inflammatory phase, blood vessels and cells are destroyed, causing bleeding and an inflammatory response. During the repair phase, the periphery of the cartilage forms at the site of the fracture, forming what is known as the diaphysis. Next, the diaphysis is gradually remodeled by osteocytes to form mature bone tissue. The purpose of fracture treatment is to heal the fracture site and restore normal bone function. Treatment modalities usually include conservative and surgical treatment (22,23). Conservative treatment generally uses a fixed external fixator to stabilize the bone in the correct position and allow it to heal naturally. Surgical treatment includes internal fixation and external fixation. Internal fixation involves surgically fixing materials such as metal plates and screws to the fracture site to keep the bone in the correct position, whereas external fixation is fixed to the fracture site by inserting steel wires, hooks, etc. through the skin to stabilize the bone (24). Fracture healing is a physiological recovery process after bone tissue injury, including multiple stages such as mechanical stability, bone formation, and bone remodeling (25). The success of fracture healing is related to the recovery of daily life and labor ability of patients, as well as the maintenance of skeletal system function. Usually, fracture healing is achieved by both natural healing and surgical treatment. Natural healing requires certain time and conditions. If the conditions are not ideal, such as poor blood supply and unstable fracture, poor healing or delayed healing may occur (26). Surgical treatment can promote fracture healing by providing mechanical stability through external fixation or internal fixation. However, surgical treatment also has certain risks and complications, such as infection and nerve injury. Therefore, finding a simple, effective and safe treatment method to promote fracture healing has been a research hotspot in the field of orthopedics.

HA is a biomaterial commonly used in the medical field, which has good biocompatibility and degradability

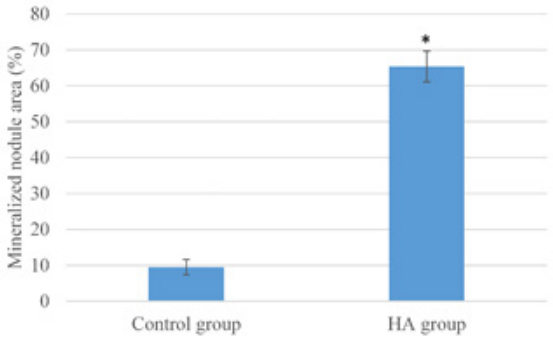

Figure 2. Contrast of the formation of mineralized nodules in MC3T3-E1 cells treated with HA at a concentration of 10%. Note: "*" indicates $P<0.05$ relative to the control.

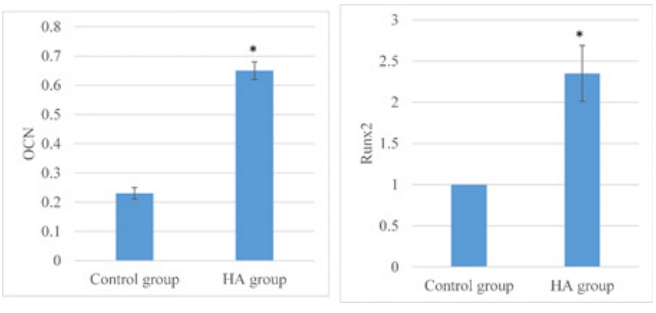

Figure 3. Effect of HA on Runx 2 and OCN protein in MC3T3-E1 cells. Note: "*" means $P<0.05$ relative to control.

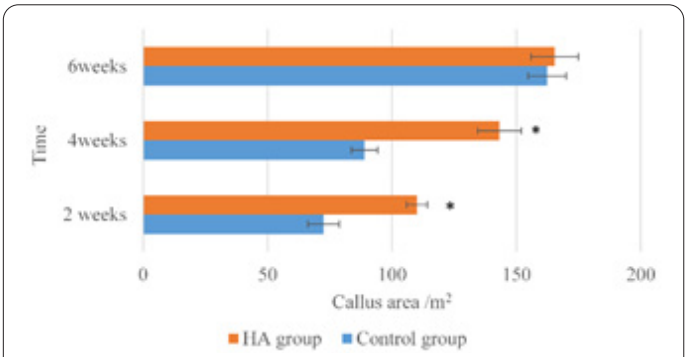

Figure 4. Comparison of callus area following intervention.

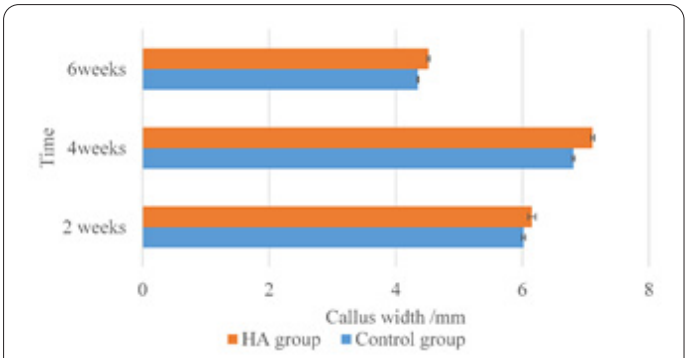

Figure 5. Comparison of callus width following the intervention.

and can mimic the function of extracellular matrix (27,28). HA gel can form a three-dimensional network structure to provide support and protection for cells, and can also release some bioactive substances, such as growth factors, to promote cell proliferation and differentiation (29). In terms of fracture healing, HA gel has also shown good effects (30). HA gel can promote the formation of mineralized nodules in osteoblasts and fracture callus, which is in line with some previous studies (31,32). HA gel may provide support and protection by mimicking the function of the extracellular matrix, and can also release some bioactive substances, such as bone morphogenetic proteins, to promote cell mineralization and bone tissue regeneration (33).

This article aimed to explore the mechanism of action of HA gel on fracture healing. The results showed that HA gel promoted the proliferation and differentiation of osteoblasts, as well as the formation of mineralized nodules of osteoblasts and fracture callus. HA gel was able to promote the proliferation and differentiation of osteoblasts, which is in line with the results of previous studies (34). The results revealed that HA gel had no obvious effect on the proliferation of MC3T3-E1 cells. Through the intervention of 10% HA gel in MC3T3-E1 cells, the area of calcified nodules was clearly larger as against the control, and the protein expressions of Runx2 and OCN were increased in this process. In addition, the callus area of the experimental group was larger as against control at 2-, 4-, 6-, and 8 weeks following intervention, and the callus area of the experimental group was larger as against control at 4- and 6 weeks following intervention ($P<0.05$), indicating that HA had a promoting effect on fracture healing.

In conclusion, the results demonstrated that HA gel promoted osteoblasts by regulation of Runx2 and OCN, which in turn promoted the formation of mineralized nodules of osteoblasts and fracture callus, thereby promoting fracture healing. It provides a theoretical and experimental basis for the development of new treatment methods for fracture healing. However, there are still some shortcomings, such as the small number of research samples and the volatility of experimental data, which need to be further strengthened.

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