

Original Research Article

Effect of Mongolian medicine alcohol spray therapy on fracture healing in rabbits

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Abstract

Purpose: To evaluate the healing effect and mechanism of Mongolian medicine alcohol spray therapy on fracture healing process in rabbits.

Methods: Thirty New Zealand white rabbits were randomly divided into normal, fracture, and treatment groups, with 10 rabbits in each group. Fracture and treatment groups underwent bone-fracture modeling, with the latter receiving daily alcohol spraying and stroking for six weeks. Serum calcium and phosphorus were measured using an automatic biochemical analyzer. Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of BMP-2, ALP, and TGF- β . Biomechanical testing was performed to assess callus properties, and real-time polymerase chain reaction (PCR) and western blot were performed to assess BMP-2, Runx2, and p-Smad1/5 expressions in callus.

Results: There were low serum calcium, phosphorus, BMP-2, ALP, and TGF- β levels in fracture and treatment groups, compared to normal group. Similarly, treatment group showed significantly increased levels of serum calcium, phosphorus, bone morphogenetic protein 2 (BMP-2), alkaline phosphatase (ALP), and transforming growth factor- β (TGF- β) ($p < 0.05$) compared to fracture group. Biomechanical testing revealed that maximum load, stiffness, and deflection of callus in the fracture and treatment groups were significantly lower compared to normal group ($p < 0.05$). Expressions of BMP-2, Runx2, and p-Smad1/5 mRNA in the callus significantly decreased in both fracture and treatment groups compared to normal group ($p < 0.05$). However, treatment group showed significantly increased expression levels compared to fracture group ($p < 0.05$).

Conclusion: Mongolian medicine alcohol spray therapy promotes fracture healing in rabbits and increases maximum load of the callus, possibly through upregulation of the BMP-2 signaling pathway. Further research is necessary to confirm the exact mechanism by which alcohol spray therapy regulates BMP-2 signaling pathway.

Keywords: Mongolian medicine, Alcohol spray therapy, Fracture, Bone Morphogenetic Protein 2, Transforming Growth Factor- β

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INTRODUCTION

With the continuous advancement of the transportation industry and overall transportation infrastructure, fractures resulting from trauma

have progressively become prevalent clinical conditions. These fractures significantly impact the quality of life for affected patients [1]. In addition, due to aging, the incidence of fractures in the elderly is also increasing, and this has a

significant impact on quality of life, including limited mobility, long-term pain, increased health risks, and the need for assistance [2]. Therefore, the repair and healing of fractures have always been a debate for orthopedic surgeons. Fractures are often treated using a method called reduction and compression fixation [3], but this treatment approach is known to be time-consuming and highly restrictive for patients in terms of daily activity. As a result, it causes significant physical and psychological stress for patients.

Traditional osteopathy in Mongolian medicine is derived from Horqin grassland and has a very long historical and cultural background, which is a systematic and theoretical discipline of bone injuries [4]. Traditional osteopathy in Mongolian medicine is a discipline that involves treating fractures in the body through a series of therapeutic techniques, including reduction and fixation and alcohol spray massage [5]. This approach is characterized by its non-surgical nature, lack of scars, and minimal tissue damage. It has gained popularity among patients due to its ability to promote fracture healing, shorten hospital stays, and reduce medical costs [6]. However, the exact mechanism of how traditional osteopathy in Mongolian medicine promotes fracture healing is not yet fully understood. Therefore, this study aimed to investigate the effect and mechanism of alcohol spray therapy in traditional osteopathy in Mongolian medicine on fracture healing.

EXPERIMENTAL

Animals and grouping

Thirty male New Zealand white rabbits aged 10 - 12 months, weighing 2.5 ± 0.25 kg and 25 - 30 cm in length, were provided by Liaoning Changsheng Biotechnology Co., Ltd. (batch number SCXk 2015-001). Experiments commenced after 1 week of adaptive feeding in a standard animal room at room temperature of 25 - 27 °C, relative humidity of 60 % and a 12 h light-dark cycle. This study was approved by the Animal Ethics Committee of Inner Mongolia University for the Nationalities Animal Center (approval no. SCXk 2015-001). Experiments were performed in line with the principles for the use and care of laboratory animals in the European Economic Community guidelines [7].

Modeling and treatments

Thirty New Zealand white rabbits were equally divided into normal group, fracture group and treatment group, with 10 rabbits in each group. In

the fracture and treatment groups, anesthesia was induced by intravenous injection of 2 % pentobarbital into the otogenic vein. The left forelimb bone was then carefully chiseled using a microsurgical osteotome to create a fracture in the middle section, resulting in a forelimb bone fracture. Kirschner wires were used for fixation to stabilize the fracture. The stitches were removed 8 days after the operation, and internal fixation had no obvious displacement or detachment. Feeding was normal, and there was no local infection. Vital signs were normal as well, and the model was successfully established.

In the treatment group, following reduction and fixation, binding and pressure were applied and alcohol spray (56 % vol 750 mL, Beijing Shunxin Agricultural Co., Ltd., Beijing, China) stroking was performed. This involved spraying a suitable amount of alcohol at the fracture site and utilizing a technique involving consistent application of force at the distal and proximal ends of the fracture for 1 to 2 min each time. This procedure was conducted once daily for 6 weeks. In the fracture group, only reduction and fixation were performed, and in the normal group, no operation was performed.

Preparation and storage of samples

At the end of the treatment, blood samples were collected from the hearts of rabbits in each group. The technique used for blood collection was venipuncture, where a needle was directly inserted into the heart to obtain blood samples. The blood was thereafter centrifuged (BG10-3A high-speed centrifuge, Beijing Era Beili Centrifuge Co., Ltd., Beijing, China) at 3500 rpm for 10 min. The resulting serum was then used to identify biochemical parameters and bone growth factors. Five rabbits were randomly selected from each group, and the entire left forelimb musculature and fascia were dissected, left forelimb bone was taken for biomechanical testing. In each group, the remaining 5 rabbits underwent dissection at the site of the left forelimb bone fracture. The muscle and surrounding fascia were carefully separated to expose the broken bone, and the fractured end as well as the surrounding callus were then divided into two parts and stored at -80 °C until further testing.

Assessment of blood biochemical indicators

Serum samples were collected to measure calcium and phosphorus concentration using an automatic biochemical analyzer (Changsha Xindi Medical Instrument Co., Ltd., Changsha, China).

Determination of serum bone growth factor

Serum samples were collected to measure the concentration of BMP-2, ALP and TGF- β levels using an ELISA kit (Wuhan Feinke Biotechnology Co. Ltd, Wuhan, China) following manufacturer's instructions. The absorbance of each sample was read at 420 nm using a microplate reader (LD-96B automatic microplate reader, Shandong Leonde Intelligent Technology Co. Ltd, Weifang, China), and concentration was determined by referencing the standard curve.

Biomechanical testing

The left forelimb bone of rabbits in 2.2 was embedded and fixed with denture base powder, and the left forelimb bone of the rabbits was loaded with a biomechanical material testing machine (8874 biomechanical material testing machine, Instron, USA) at a rate of 1.5 mm·min⁻¹. Data was collected and callus maximum load, stiffness, and deflection were recorded.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

A portion of the broken end of the fracture and surrounding callus stored at -80 °C was placed in a mortar, ground into powder after adding liquid nitrogen, and extracted with a bone tissue mRNA extraction kit (Shanghai Yisheng Biotechnology Co., Ltd., Shanghai, China). The extracted material was then mixed with reagents from a one-step reverse transcription amplification kit before being placed into a real-time PCR instrument (Bio-Rad, Hercules, CA, USA). Primer sequences of each target gene are shown in Table 1. β -actin was used as an internal reference gene.

Western blot

A portion of the fractured bone end and surrounding callus, which had been stored at -80 °C, was placed in a mortar and ground into a powder after adding liquid nitrogen. Total protein from the bone tissue was then extracted using a bone tissue protein extraction kit, and centrifuged at 12000 rpm for 20 mins. The supernatant was collected, and protein concentration was measured using bicinchoninic acid (BCA) assay. The extracted protein samples were loaded for

sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA), followed by transfer, washing, blocking, and overnight incubation with primary antibodies (Beijing All-Gold Biotechnology Co., Ltd., Beijing, China) (diluted 1:1000). After washing, the samples were incubated with the secondary antibody (HRP-conjugated goat anti-rabbit IgG antibody diluted 1:500) for 1 h and developed using an enhanced chemiluminescence (ECL) solution. Target protein bands were observed using a gel imaging analyzer (GE, San Francisco, CA, USA), and β -actin was used as an internal reference to calculate the relative gray values of each target protein.

Statistical analysis

Statistical analysis was performed using statistical software SPSS19.0 (Statistic Package for Social Science19.0, IBM, Armonk, NY, USA). Measurement data conforming to normal distribution was expressed as mean \pm standard deviation (SD). One-way analysis of variance was used for comparison of multi-sample measurement data, and LSD-t test was used for pairwise comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Serum biochemical parameters

According to results presented in Table 2, serum calcium and phosphorus levels of New Zealand rabbits in both fracture and treatment groups were significantly lower ($p < 0.05$) compared to normal group. Furthermore, serum calcium and phosphorus levels of New Zealand white rabbits in treatment group were significantly higher compared to fracture group ($p < 0.05$).

Serum cytokines

Serum levels of BMP-2, ALP, and TGF- β in fracture and treatment group significantly reduced ($p < 0.05$) compared to normal group. Furthermore, BMP-2, ALP, and TGF- β serum levels significantly increased in treatment group compared to fracture group ($p < 0.05$).

Table 1: Target gene primer sequences

Target gene	Upstream primer (5'-3')	Downstream primer (5'-3')	Length of primer (bp)
BMP-2	GGCAATTAGACCATAG	CAATAAGTAGCCATGA	500
RUNX2	GGGACTAACATAAGACC	CCAATGACATAGACATA	298
p-Smad1/5	TTACATAGACAATGA	GACCATAGACATAAG	275
β -actin	CCATTGACATAGCAATA	GGCAATAACACATAGA	409

Table 2: Levels of blood calcium and phosphorus in New Zealand white rabbits in each group (mean ± SD, n = 10)

Group	Blood calcium (mmol/L)	Blood phosphorus (U/L)
Normal	3.71±0.38	2.71±0.19
Fracture	3.16±0.19*	1.87±0.33*
Treatment	3.35±0.12**	2.21±0.32**

*P < 0.05 vs. normal group, #P < 0.05 vs. fracture group

Table 3: Level of BMP-2, ALP and TGF-β of New Zealand white rabbits in each group (mean ± SD, n = 10)

Group	BMP-2 (U/L)	ALP (U/L)	TGF-β (U/L)
Normal	54.74±10.81	141.78±29.07	771.31±111.35
Fracture	20.38±5.02*	75.11±13.24*	412.54±51.07*
Treatment	27.43±8.06*#	102.81±17.22**	463.71±50.83**

*P < 0.05 vs. normal group, #P < 0.05 vs. fracture group

Table 4: Level of maximum load, stiffness and deflection of callus in each group (mean ± SD, n = 5)

Group	Maximum load (N)	Stiffness (N*mm)	Deflection (mm)
Normal	417.25±25.41	425.14±29.07	4.71±0.54
Fracture	209.30±17.43*	155.74±11.45*	2.21±0.31*
Treatment	355.69±19.81**	184.08±14.77**	3.19±0.30**

*P < 0.05 vs. normal group, #P < 0.05 vs. fracture group

Biomechanical indices

Maximum load, stiffness, and deflection levels of left forelimb callus in both fracture and treatment groups significantly decreased ($p < 0.05$) compared to normal group. Maximum load, stiffness, and deflection levels of left forelimb callus in treatment group were significantly increased ($p < 0.05$) compared to fracture group.

Expression of mRNA levels of BMP-2, RUNX2 and p-Smad1/5

Expression of BMP-2, RUNX2, and p-Smad1/5 mRNA in the callus was significantly reduced ($p < 0.05$) in both fracture and treatment groups compared to normal group. Additionally, expression levels of BMP-2, RUNX2, and p-Smad1/5 mRNA in the callus were significantly increased in treatment group compared to fracture group ($p < 0.05$).

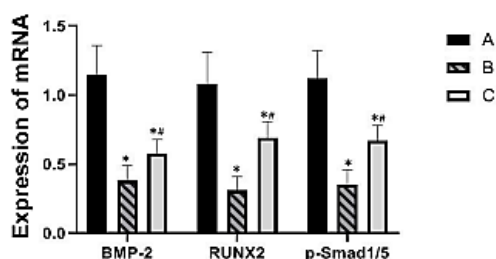


Figure 1: Expression of BMP-2, Runx2 and p-Smad1/5 mRNA in callus of New Zealand white rabbits in each group (A: normal group, B: fracture group, C: treatment group)

Protein expression levels of BMP-2, RUNX2 and p-Smad1/5

Relative protein expression levels of BMP-2, RUNX2, and p-Smad1/5 significantly decreased ($p < 0.05$) in both fracture and treatment groups compared to normal group (Figure 2). Moreover, relative protein expression levels of BMP-2, RUNX2, and p-Smad1/5 were significantly increased in treatment group compared to fracture group ($p < 0.05$) (Figure 3).

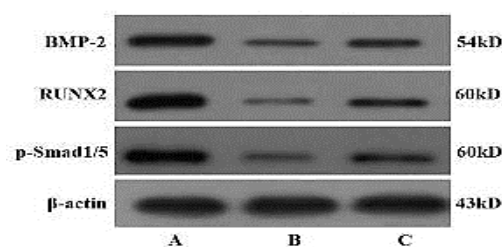


Figure 2: Expression of BMP-2, Runx2 and p-Smad1/5 protein bands (A: normal group, B: fracture group, C: treatment group)

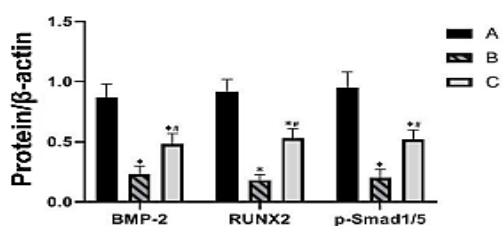


Figure 3: Relative expressions of BMP-2, Runx2 and p-Smad1/5 proteins (A: normal group, B: fracture group, C: treatment group)

DISCUSSION

Fractures are a common condition encountered in clinical practice, which may result in complete or partial fracture of the main phalanx. In the process of fracture treatment, poor healing or prolonged healing time is a major challenge that puzzles physicians and patients [8]. According to statistics, about 5 – 10 % of fracture patients suffer from delayed or non-healing of bone due to various reasons [9]. As a result, promoting fracture healing and shortening the healing cycle has become the focus of attention for professionals in the field. The process of fracture healing involves disciplines such as biology, histology, and endocrinology. Various factors such as bone growth factors, iatrogenic factors, and trace elements are also implicated in this process [10]. Callus formation is one of the markers of fracture healing [11], and its mechanism is important for clinical research in the field of fracture healing.

Mongolian osteopathy originated in the Corqin grassland of Mongolia, China, and encompasses unique concepts and techniques for fracture treatment. The ancient Mongolian nomadic lifestyle, characterized by horseback living, grazing, and hunting, led to frequent conflicts between tribes and a high incidence of fractures [12]. Over time, through careful observation and accumulated experience with fractures, a distinct treatment system emerged, which includes alcohol spray stroking, manual reduction, external fixation, and early mobilization [13]. Modern studies have indicated that the process of alcohol spraying during treatment serves multiple purposes. First, it facilitates heat dissipation and reduces swelling, as the alcohol quickly evaporates after being applied to the patient's fracture site. Additionally, the tissue stimulation caused by the alcohol promotes a stress response which activates the body's repair mechanisms [14]. Results of this study showed that serum calcium, phosphorus, BMP-2, ALP, and TGF- β levels significantly increased in the treatment group compared to fracture group. These results suggest that alcohol spray therapy induces stress response and activates the release of bone morphogenetic factors. Transforming Growth Factor- β (TGF- β) is a class of cytokines that regulates cell growth and differentiation and, like BMP, belongs to the TGF- β superfamily [15]. Bone Morphogenetic Protein 2 (BMP-2) is a major factor in BMP that regulates bone formation and plays a role in inducing bone formation [16]. Wang *et al* [17] showed that serum TGF- β and BMP-2 levels increased during bone healing in rabbits. In the results of this study, maximum load, stiffness,

and deflection levels of callus in the left forelimb of the rabbits in the treatment group were higher than those in fracture group. These findings suggest that alcohol spray therapy has the potential to enhance bone healing and improve the biomechanical strength of bones.

Furthermore, expressions of BMP-2, RUNX2 and p-Smad1/5 mRNA and their proteins were higher in treatment group compared to fracture group, suggesting that alcohol spray therapy may promote fracture healing by activating the BMP-2 signaling pathway. The RUNX protein is a transcription factor protein that plays an important role in bone growth and is an important protein in the RUNX protein family which is also widely expressed in osteoblasts and plays a crucial role in regulating osteoblast differentiation [18]. Also, Smad1/5 activates expression of downstream BMP-2 and TGF- β , thereby promoting osteoblast differentiation [19]. Once activated, BMP-2 binds to specific receptors on cell membranes and enters the cells, further activating Smad1/5. This forms a positive feedback loop that facilitates bone healing [20]. Ren *et al* [21] revealed that the fracture healing process in rats is related to the up-regulation of the BMP2/Smad1/Runx2 pathway, and it agrees with the findings of this present study. Thus, activation of BMP-2 promotes bone healing and fracture resolution.

CONCLUSION

The application of Mongolian medicine wine spraying therapy enhances fracture healing in rabbits and increases maximum load post-fracture healing. These effects are attributed to up-regulation of the BMP-2 signaling pathway. However, the exact mechanism by which alcohol spray therapy regulates the BMP-2 signaling pathway remains unclear, and further research is necessary for confirmation.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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