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Hypoxia-Preconditioned MSCs for Enhanced Bone Regeneration in Ovine Fracture Case

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ABSTRACT

Background: Bone fracture is a common clinical issue that occurs in humans and animals. The fracture-wound healing process may have a variable duration since several conditions interfere with tissue regeneration. Mesenchymal stem cells (MSCs)-based therapy offers regenerative potential. **Objective:** This study aimed to evaluate the therapeutic effects of hypoxia umbilical cord mesenchymal stem cell (UC-MSC) secretome on fracture wound healing in sheep. **Methods:** A one-year-old male thin-tailed sheep (Ovis aries) weighing 25 kg had a fracture in the hindlimb digits. The sheep was treated with hypoxia MSC secretome injection thoroughly at the wound area. MSCs were isolated from the umbilical cord and cultured. The cell culture was conditioned in a hypoxic environment (1 – 5% O2). **Results:** The results showed that secretome application significantly accelerated the fracture wound healing process. **Conclusion:** This finding indicates that the hypoxia UC-MSC secretome has the potential as a non-cellular regenerative approach in animal orthopaedic cases.

Keywords: secretome, hypoxia, mesenchymal stem cell, fracture, wound healing

INTRODUCTION

Fractures are defined as bone integrity disruptions and classified by the type, location, and patterns[1]. Time required for fractures to heal cannot be determined with certainty. Fracture healing is complicated because osteogenesis taking time and being correlated with various variables[2]. Blood supply, stability, and inflammation rate affect osteogenesis. Despite these factors, failing risk still persists in 5 – 10%[3,4].

Fractures commonly occur as traumatic injury consequence in human and animal. Indonesia Ministry of Health[5] records that 5,8% of injured victims—approximately 8 million people—suffered fractures. Prevalency of anterior and posterior extremity fractures are 36,9% and 65,2% respectively. Animals are prone to fracture too due to traumatic responses. This is particularly evident in animals fracture cases, which are reported more than 50% were limb fractures[6]. These indicate fractures mostly occur in the skeletal system and will severely affect survivor life quality within disability. Therefore, study on fracture healing innovation to accelerate and reduce fracture complications is urged to be held.

The fracture treatment method chosen based on the tissue damage severity. When the fracture is not manageable with non-surgical methods, surgery is required. Surgery, as most well-known fracture treatment, should take place within not more than first 24 hours post-injury to prevent complications such as embolism, further infections, and pressure ulcers[7]. Orthopaedic surgery faces challenges related to cost, time, and risks, especially on animals. Recent research and case report highlight regenerative approach like mesenchymal stem cells (MSCs) for wound healing therapy. MSC secrete secretome that contain growth factors, cytokines, and extracellular vesicles which modulate the wound healing processes by regulating inflammation, angiogenesis, and extracellular matrix remodelling[8]. The MSCs secretome promotes healing without any cell-based therapies risk, such as immune rejection and tumorigenicity[9].

This report presents a fracture wound treating method in a thin-tail sheep. The secretome derived from umbilical cord mesenchymal stem cells (UC-MSCs). When a fracture is not manageable with conservative (non-surgical) methods, surgery is required. We outline the clinical findings, used treatment protocols, and final outcome for highlighting the potential of UC-MSC as one of therapeutic strategies in veterinary orthopaedic.

MATERIALS AND METHODS

Signalement, Anamnese, and Diagnosis

A 1-year-old thin-tailed sheep (Ovis aries) weighing 25 kg showed gait abnormality and isolated itself from herd. Physical examination was conducted on the sheep. The body temperature was in normal range (39.8°C) and the heart rate was at 86 beats per minute. Additionally, an open wound of about approximately 5 cm was identified. It showed exposed bone fragments on the right hindlimb at the phalanx bone. The wound looked swollen, showed hemorrhage and serosanguineous exudate. Periosteal tissue was visible throughout of the wound. The x-ray was not performed due to equipment limitation. However, the anti-inflammatory response in sheep following the UC-MSCs secretome treatment was characterized by measuring Interleukin 10 (IL-10) cytokine levels in treated sheep blood serum with enzyme-linked immunosorbent assay (ELISA). ELISA was performed using a Sheep IL-10 ELISA kit (FineTest, Wuhan Fine Biotech Co., Ltd.) and the optical density measured at 450 nm.

UC-MSCs Processing and Therapy

Wound sterilization, reposition and external stabilization were performed first. After examination and discussion between the veterinary and the sheep owner, the UC-MSCs secretome was chosen as the therapeutic intervention. Regarding the owner, they stated the inability to perform post-surgery aftercare and the productivity of the sheep following a surgical procedure.

The UC-MSCs were already prepared before. The MSCs were isolated from human umbilical cord. The umbilical cord was collected in a sterile petri dish with 0.9% NaCl and processed immediately. Then, the umbilical cord was minced and cultured in a flask. The umbilical cord was submerged in complete medium which contained Dulbecco's Modified Eagle Medium and was supplemented with fungizone, antibiotic, and fetal bovine serum. The concentration of each supplement is according to a previou study[10]. Then, the flask was incubated in the incubator (37°C; CO2 5%). Cells would appear approximately 14 days after cell-culture process had begun. UC-MSC was cultured until 5th passage.

Cells were maintained until the confluence rate reached 80%. Then, UC-MSCs placed in hypoxic chamber (1 - 5% O2) for 24 hours. After 24 hours, the culture medium was collected and filtered through tangenital flow filtration to obtain the hypoxic MSC secretome. In order to separate cellular debris and yield the cell-free secretome, the culture medium were collected and processed through serial centrifugation. The culture medium centrifuged at 3,000 rpm for 15 min at 4 °C, followed by 10,000 rpm for 20 min at 4 °C. The secretome product was sterilized with 0.22 µm filter then stored at -80°C before the use.

All animal procedures were conducted at the Animal Research Centre of Stem Cell and Cancer Research Indonesia, adhering to established ethical guidelines. hUC-MSCs were administered via intramuscular into sheep by a veterinarian. The sheep received a single-dose of hUC-MSC of 6 x 106 MSC, based on a previous research (Yin et al., 2016), The hypoxic UC-MSCs secretome was injected locally at sterilized fracture wound area. Afterward, the wound was covered with sterile dressing and stabilized with orthopaedic casting tape. The wound healing progress and wound maintenance were monitored once every seven days throughout the study.

RESULTS

Macroscopic Findings

Some improvements were documented in Figures 1. On the first week of treatment, the wound exudate significantly decreased. Granulation tissue started to appear at the edge of the wound. Part of the phalanx bone still remained visible but more steady than before. There was barely any new muscle tissue covering the bone.

Following the initial evaluation, new epithelial tissue growth around the wound was observed one week later. The diameter of wound appeared have progressively decreased. The bone was no longer visible, unlike in previous observation. No evidence of hemorrhage and exudate. The sheep exhibited better gait.

The wound was well-healed on day 21th of treatment. The wound was completely closed by scar tissue. A few small strands of hair growth noted. The sheep did not showed pain or discomfort upon palpation of the healed wound area. Supporting clinical data on the progression of fracture healing with UC-MSCs therapy are summarized in Table 1.

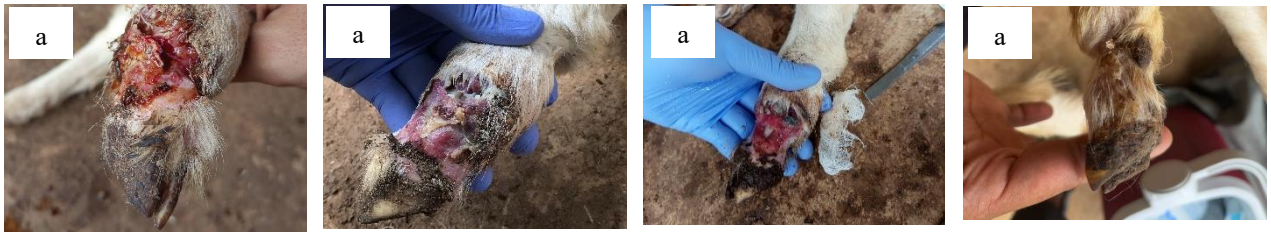

Figure 1. Fracture wound healing progress on thin-tailed sheep (**a**: day 0 treatment; **b**: day 7 treatment; **c**: day 14 treatment; **d**: day 21 treatment).

Table 1. Clinical data of the sheep during UC-MSCs treatment

Days of treatment	Body Temperature (°C)	Wound Diameter (mm)	IL-10 (pg/mL)
0	39.8	48	80
7	39.3	42	220
14	39.4	38	180
21	39.1	26	70

DISCUSSION

The UC-MSCs secretome has emerged as an impactful cell-free therapy to overcome the persistent clinical issues of delayed union and non-union fractues. Its powerful tissue-regenerative effect is driven by mixture of cytokines, growth factors, and extracellular vesicles (EVs). Hypoxic preconditioning of UC-MSCs mimicking the physiological low-oxygen environment of injured tissues to optimizes paracrine and immunomodulatory potential. Interleukins shifting are also vital in this process as dictates the immune environment, macrophage polarization, and the recruitment of reparative cells, which are necessary for bone regeneration.

These findings from this case report indicate that UC-MSCs secretome has therapeutic potential, especially for treating open fracture case. An open fracture is a condition when the mucosa of the fracture area is broken and the fracture is able to directly or indirectly exposed[11]. The periosteum and soft tissue destruction resulting in blood circulation decrement, thus wound healing process can be prolonged. In the context of wound healing, the MSCs secretome already shown as dependable factor on wound healing phases, including hemostasis, inflammation, proliferation, and remodelling[12]. That influences on biological processes carried by MSCs secretion products such as cytokines, chemokines, growth factors, immunomodulatory molecules, micro RNA (miRNA), and EVs[13].

The MSCs secretome therapy comes up with bioactive factors and EVs ability for enhancing cellular communication and tissue regeneration[14]. In this case, hUC-MSCs were chosen as the usage of umbilical cord is considered more ethically acceptable[15]. The hUC-MSC secretome contains many of growth factors such as vascular endothelial growth factor (VEGF)[16]. The bioactive factors are important in angiogenesis, modulating immune response, and cell proliferation[17]. Moreover, hypoxic approach in MSCs culture aimed to increase VEGF, insuline like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β), and hypoxia-inducible factor 1-alpha (HIF-1α) production in secretome which enhance wound healing properties[18,19]. VEGF works by promoting angiogenesis, whilst IGF-1 and TGF- β stimulates fibroblast proliferation and migration[20]. Collectively, the explained facts before emphasize that MSCs secretome accelerate tissue repair and regeneration through collagen synthesis and re-epithelialization, as confirmed in this sheep fracture case.

Interleukin dynamics and immune modulation are key to the secretome function in fracture reapir. The hypoxia-conditioned UC-MSCs secretome effectively shifts the immune environment toward regeneration by increasing anti-inflammatory cytokine, IL-10 and growth factor, TGF – β levels and polarizing macrophages toward the M2 phenotype[21,22]. That mechanism also balances the Th17/Treg ratio, thus accelerating tissue repair and angiogenesis. In terms vascularization at the fracture site, IL-6 and IL-8 play roles in recruiting endothelial and progenitor cells. The combined

therapeutic pathways, supported by exosomal miRNA action on target cell apoptosis and migration. MSCs surpress inflammatory responses by secreting paracrine factors, which shift the tissue microenvironment from pro-inflammatory to anti-inflammatory state. This inflammation modulation prevents fibrosis and non-unions. According to Granero-Moltó *et al*[23]., MSCs reduced IL-6, IL-13, but not significantly for IL-10. Furthermore, the findings in this study show that IL-10 peaked on early healing stage after the treatment given and decreasing gradually from the second week post-treatment. Upon injection, MSCs secretome encounters acute post-trauma environment, characterized by high levels of pro-inflammatory cytokines, such as IL-1β and TNF-α[24].

In response to this milieu, MSCs secretome actively secrete large amounts of IL-10, which is the primary anti inflammatory cytokine. The elevated IL-10 serves as a crucial signaling cue to local immune cells, particularly local macrophages and regulatory B cells[25]. It promotes the rapid shift of pro inflammatory M1 macrophages toward reparative M2 maacrophage. This immunomodulation resolves acute inflammation, accelerating the clearance of damaged tissue and making a microenvironment conducive to subsequent bone formation and remodelling. By the end of the second week, acute inflammatory phase is largely resolved. As inflammation subsides, the population of M2 macrophages, which were responsible for cleaning up the debris, begins to decline. Since M2 macrophages are also IL-10 producer, decrease of M2 resulting in reduction of IL-10 concentration. The healing process shifts from immune regulation to tissue building. The dominant signaling molecules become pro-regenerative and osteogenic factors, including bone morphogenetic protein, TGF-β, and IGF-1. The injected MSCs simultaneously shift their focus from paracrine immune signaling toward differentiation into chondrocytes and osteoblasts to facilitate soft and hard callus formation. Tissue regeneration was deemed successful, as confirmed by both reduced wound diameter and improved stability of the sompromised bone structure.

CONCLUSION

The MSCs secretome has demonstrated significant therapeutic effects, including stimulating tissue repair and reducing inflammation. Specifically, a case study in an animal with a fracture resistant to standard care showed that ASC secretome treatment led to improved cell proliferation and reduction in curement time. These results, combined with previous animal studies shows accelerated healing, support the use of MSC secretomes as a safe and effective cell-free treatment for orthopedic injuries in animals.

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Authors’ contributions

OA as a veterinarian and post-graduate student, he is responsible for all of the animal treatment and basic concept of the analysis. BR as post-graduate student, she is responsible for data management and constructing the manuscript.

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