The Effect of Local Injection of Mesenchymal Stem Cells and IL-10 with Platelet Lysate on Fracture Healing in a Rat Model

Khaldoon Bashaireh [1], Nizar M. Abuharfeil [2], Ziad Oudat [1], Wael M. Hananeh [3]*

Abstract

Objective: This study investigates the effect of mesenchymal stem cells (MSC), an immune-privileged cell source for tissue repair, Interluken (IL-10), an immune inhibitory factor, and platelet lysate (PL) on the fracture healing process.

Methods: Forty adult male Sprague-Dawley rats were anesthetized, and left tibia mid shaft fractures were created. The rats were randomly assigned to 5 groups and received injections with saline (A); MSC and PL (B); IL-10 and PL (C); PL (D); and MSC, PL, and IL-10 (E). Lateral radiographs of four randomly selected rats in each group were taken at different days post-fracture. Four rats from each group were sacrificed at day twenty one post-fracture, with left tibiae harvested for histological analysis.

Results: At 7 days post-fracture, the highest level of callus formation and bone healing was observed in group E (treated with MSC, PL, and IL-10) followed by groups B (treated with MSC and PL). The same results were obtained at days 14, 21 and 28 post-fracture. At 21days post-fracture, histological analysis revealed that the callus of fractured tibiae in group A consisted mostly of chondrocytes, while in groups C and D, extensive remodeling with a limited amount of chondrocytes present at the fracture. Combination of MSC, PL, and IL-10was associated with the greatest fracture healing performance. After 3 weeks of treatment in group E and B, trabecular bone formation and cortex remodeling were observed.

Conclusions: The use of MSC in combination with IL-10 and PL is a promising alternative treatment to promote fracture healing.

Keywords: Bone regeneration, Mesenchymal stem cells, IL-10, Platelet lysate.

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Introduction

Bone is a unique physiological human tissue. There are many stages of fracture healing starting with hematoma and progressing to an acute inflammatory stage at the fracture site and then callus formation, ending with remodeling. Several factors are

1. **Department of Orthopedic Surgery, King Abdullah University Hospital, Jordan University of Science and Technology, Irbid, Jordan.**
2. **Department of Applied Biological Sciences, Jordan University of Science and Technology, Irbid, Jordan.**
3. **Department of Pathology and Public Health, Veterinary Medicine, Jordan University of Science and Technology, Irbid, Jordan.**

**** Correspondence should be addressed to:***
Khaldoon Bashaireh. E-mail: Bashaireh@just.edu.jo
P.O. Box 3030, 22110 Irbid, Jordan.

known to have a positive effect on the bone healing process. Platelets and platelet derivatives has acquired clinical relevance as a means of accelerating bone healing. Platelet beneficial effect is attributed to the release of growth factors and other bioactive substances able to wound and bone healing.

The process of bone healing is finely orchestrated by the action of locally released growth factors and cytokines at the site of fracture. Studies have documented the effect of several growth factors including PDGF, TGF-β, IGF, and b-FGF as well other hormones and cytokines on bone regeneration both *in vitro* and *in vivo*[1,2,3,4,5,6].

All of these growth factors and others are present at high concentrations in platelets. Platelet rich plasma (PRP) is defined as "the volume of plasma fraction of autologous blood having a platelet concentration above baseline"[7]. PRP has a wide range of applications, in particular for the treatment of muscular and skeletal injures[8]. Gandhi *et al*. demonstrated that PRP has the potential to serve as a concentrated autologous source of growth factors through local percutaneous administration of PRP at the site of fracture in a diabetic rat model of femur fracture[9]. Animal fracture models in both normal and osteoporotic rats treated with PRP reported increased callus to cortex width ratio and higher trabecular bone mass formation compared to control groups, indicating considerable enhanced healing over controls[3,10]. The presence of PRP not only accelerated fracture healing but also regeneration in bone defects in an ovariectomized osteoporotic rat model[11]. Early *in vitro* experiments assessing the effect of platelet lysate (PL) on bone marrow mesenchymal stem cell differentiation into osteogenic lineage revealed that PL increased cellular proliferation and expansion at the expense of cellular differentiation[12]. Subsequently, PL has been used as a serum substitute[13], and use of PL from the beginning of culture and during induction of the differentiation program resulted in superior osteoblastic differentiation and increased expression of osteogenic marker genes[14]. In addition, clinical trials have been conducted confirming the efficacy of administering PRP on alveolar bone regeneration following removal of the third molar[15], intra-articular calcaneal fracture healing in combination with allograft[16], and relieving early knee pain associated with osteoarthritis[17].

Earlier data indicated that treating mesenchymal stem cells with IL-10 suppressed alkaline phosphatase (ALP), osteocalcin, and matrix mineralization formation, and this inhibitory effect was mediated through the suppression of TGF-β[18,19,20]. However, IL-10 exerts this inhibitory function at the stage of stem cell specification to osteoblasts and is incapable of this effect in early committed osteoblastic cultures already expressing ALP, osteocalcin, and collagen type1[20].

Two recent lines of evidence have suggested that IL-10 plays a role in enhancing bone growth and healing. Toben *et al*. demonstrated that $RAG^{-/-}$ mice have enhanced bone growth, endochondral ossification, and remodeling. The cytokine profile in $RAG^{-/-}$ mice revealed decreased expression of pro-inflammatory cytokines including TNF and INF-γ and T-cell specific cytokines IL-2 and IL-4. By contrast, expression of the anti-inflammatory cytokine IL-10 was significantly higher[21]. Thus, it is highly possible that the shift in cytokine profile from pro-inflammatory to anti-inflammatory plays a crucial role in enhancing fracture healing, which highlights IL-10 as a central player in

accelerating fracture healing due to its immunomodulatory function. Iannone *et al.* have shown that treating osteoarthritis osteoblast cultures with extra-corporeal shock waves (ESW), which enhance osteoblast regeneration, enhanced the expression of osteoblastic markers such as CD29/β1 and CD105. In addition, along with the increase in cell surface osteogenic markers, IL-10 expression was significantly increased compared with control healthy osteoblast cultures[22].

Mesenchymal stem cells (MSC) have therapeutic potential to reduce the time of healing in patients with fractures[23]. It was demonstrated that MSC transplant was shown to induce a biomechanical improvement in the healing process associated with increases in callus volume and cartilaginous and bone content[24]. Intravenously administered MSC were capable of specifically homing to the fracture site and normalizing biomechanical, histological, and microcomputed tomography parameters of healing in animals[25].

The use of different local factors on the fracture to accelerate healing is attractive and could be implemented with adequate clinical evidence from clinical studies. In this study we aimed to analyze the effect of local injections of different combinations of MSC, IL-10, and PL on the fracture healing process.

Materials and Methods

Stem cells isolation and differentiation

Animal experiments were approved by Jordan University of Science and Technology Institutional Animal Care and Use Committee (IACUC).

Stem cells were isolated according to the method of Lennon & Caplan[26]. Briefly, four Sprague–Dawley rats were sacrificed, both tibial and femur bones were harvested, the marrow was flushed with 5ml basal α-minimal essential medium (MEM) (Hyclone, USA), and the cell suspension was centrifuged at 1000 rpm for 5 minutes. The cell pellet was re-suspended in α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin, penicillin, and amphotericin B (Hyclone, USA) and seeded at a cell density of 5000 cells/cm^2. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37ºC. Cells were allowed to adhere for 3 days; the first media change took place on the third day. Upon reaching 80% cell confluence, cells were passaged with 0.25% trypsin containing 1mM EDTA (Hyclone, US). Cells at passage three were used for downstream experiments.

Stem cells were characterized through verification of their multi-potent capacity. Cells were differentiated towards the osteogenic cells. MSC were seeded at a density of 5000 cells/ml, and after reaching 100% cell confluence, culture medium was switched to osteogenic differentiation medium (Hyclone, US) supplemented with 10% FBS and 1% streptomycin, penicillin, and amphotericin B. After 2 weeks, cells were washed with PBS, fixed with 10% formalin, and then washed twice with water. Alizarin red S staining was performer according to the manufacturer's protocol. Stained cells were observed under a phase-contrast microscope. After1 week of osteogenic induction, cells were trypsinized and prepared for injection.

Rat platelet lysate preparation

PRP was prepared according to the method of Ghandi *et al.*[9]. Briefly, whole blood was collected via cardiac puncture from 15 adult Sprague-Dawley rats in sodium citrate-treated tubes and centrifuged at 1800 rpm for 10 minutes. Plasma and buffy coats were pooled

in a 15-ml centrifuge tube and centrifuged at 3600 rpm for 10 minutes. A platelet pellet was formed, and the supernatant consisted of platelet poor plasma (PPP). At least half the PPP was discarded, and the rest was used to resuspend the platelet pellet to form PRP. PRP was subjected to 3-5 freeze-thaw cycles to break up platelets and release their contents, with freezing performed at -20ºC and thawing in a 37ºC water bath, followed by centrifugation at 4300 rpm for 15 minutes to remove platelet fragments. The supernatant contained platelet lysate.

Fracture model and treatment groups

Forty Sprague-Dawley rats (average weight 110g) were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (60 mg/kg body weight) and 2% xylazine (10 mg/kg body weight). The fracture site was labeled with a marker according to anatomical landmarks and left tibial midshaft fractures were created with a special device as described by Schmidmaier *et al*.[13]. The fractured tibia was stabilized with intramedullary 0.45 mm Kirschner wire.

Animals were divided into five groups: group A treated with 0.3 ml PBS; group B treated with MSC (5×10^5 cells suspended in 0.15 ml PBS) and 0.15ml PL; group C treated with 0.15 ml PL mixed with 75 µl IL-10 (10 ng/ml) and 75 µl PBS; group D treated with 0.15 ml PL and 0.15 ml PBS; and group E treated with MSC (5×10^5 cells suspended in 0.15 ml PL) followed by 75 µl IL-10 (10 ng/ml) and 75 µl PBS). Local injections at the fracture site were performed on day 0, 3, and 5 after induced fracture.

Radiographic analysis

Lateral radiographs of four randomly chosen rats in each group were taken at days 0, 7, 14, 21, and 28 post-fracture. Tibial radiographs were taken at 40 kV and 4 mA.X-ray meter and films were processed and developed using a Kodak RP X-omat Processor. Callus diameter was calculated at time of radiograph. Callus and tibia diameters were measured using Auto CAD software. Callus index, defined as the maximum diameter of the callus divided by the diameter of the bone, was calculated to stage the fracture healing.

Histological analysis

Four rats from each group were sacrificed at day 21 post-fracture, and the left tibia was harvested for histological analysis. Adequate biopsies were taken from fracture site and fixed immediately in 10% formalin solution for 24 hours. The tissues were then decalcified in 10% EDTA (pH 7.4) for 7 days, processed routinely, and stained with H&E and Safranin O stains. Examination was performed by a pathologist with no prior knowledge of the treatment groups. A modified Allen et al. grading system was adopted for histopathological union assessment[27]. The grade of histopathological change ranged from 0 to V as follows: 0, no healing or only fibrous tissue union; I, cartilage union with presence of fibrous tissues; II, only cartilage union; III, cartilage union with some bony ossification; IV, approximately equal amount of trabecular bone and cartilage union; and V, complete bony union

Statistical analysis

A two-tailed t-test was used to compare groups.

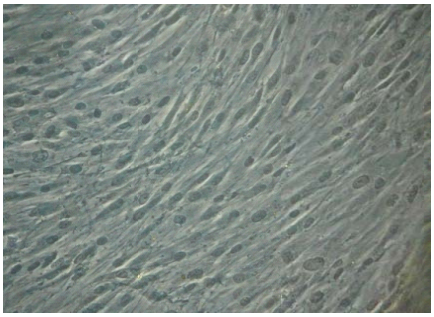
Results

Stem cell isolation and differentiation

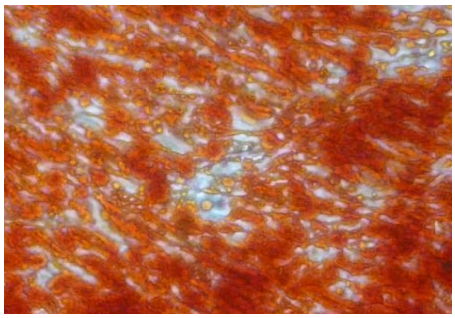
Stem cells cultures were initiated from rat

bone marrow. On the fourth day of culture stem cell colonies were observed, and at approximately 2 weeks the adherent monolayer reached 80% confluence (Fig. 1A). To assess the presence and differentiation capability of MSC, the cells were treated with osteogenic medium for 2 weeks, when the differentiated cells exhibited mineralized matrix on Alizarin Red S staining (Fig.1B).

Figure 1. Characterization of rat MSC confirming their multipotent potential



a. Confluent Primary MSC exhibiting spindle fibroblastoid morphology

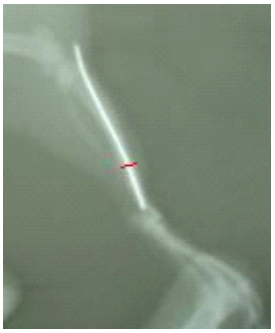


b. Alizarin Red S staining of Calcium deposits after 14 days of osteogenic differentiation

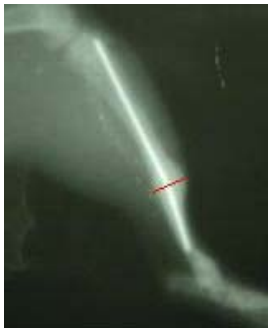
Radiological analysis

Callus formation, which is indicative of the bone healing process, was evaluated by lateral radiographs of fractured tibiae. Figure 2 contains representative radiographs showing the progression of callus formation at the fracture site after 14 days. All fractured tibiae achieved complete bridging by day 14 regardless of their group. Seven days post-fracture, group A had a visible fracture line and incomplete bridging, while in groups B, C, and D, (Figure 2) the fracture line was less apparent. However, group E showed complete bridging with no visible fracture line at day 7 (Figure 2). Calculated callus indices revealed an increase in control and treatment groups, reaching a peak at day 21 and decreasing afterwards (Table 1). Tibial callus index scores of treated animals in groups B, E, and C were significantly increased ($P<0.05$) at days 7 and 14 compared with the control group A. At 14 days post-fracture, the highest level of significance ($P<0.005$) was seen in group E (treated with stem cells, PL, and IL-10), followed by groups B (treated with stem cells and PL), C, and D (Fig. 3).

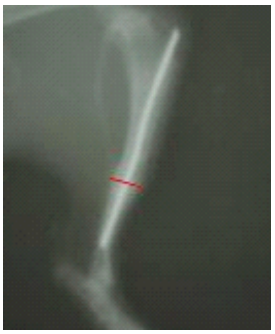
Figure 2. Representative radiographs of fixed fractures at day 14 post-fracture



a. Control group treated with PBS

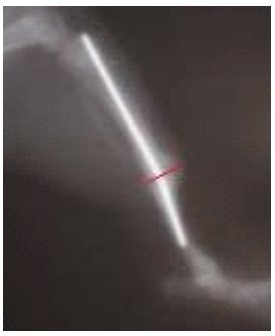


b. MSC and PL treatment group



c. PL and IL-10 treatment group

d. PL-only treatment group 2D



e. MSC, IL-10, and PL treatment group

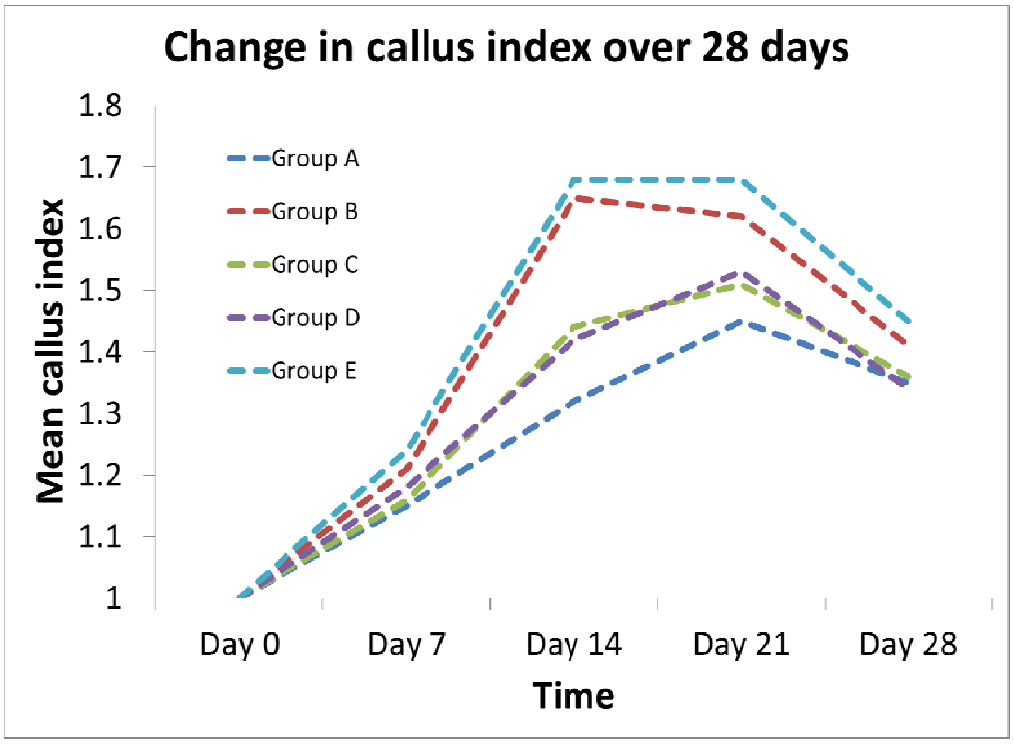
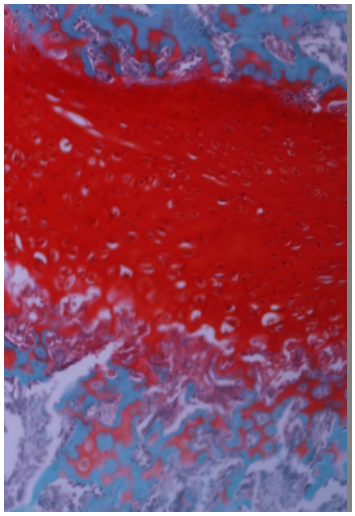


Figure 3. The line graph depicts the mean Callus Index (CI) in groups A, B, C, D, and E over 28 days

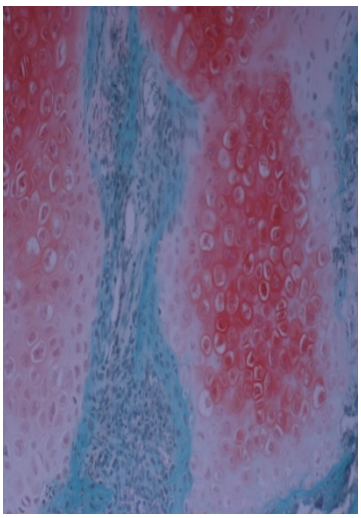
Histological analysis

Safranin O/methylene light green staining revealed that the callus of fractured tibiae in group A consisted mostly of chondrocytes at 21 days post-fracture (Figure 4A). In Groups C and D, extensive remodeling with a limited amount of chondrocytes present at the fracture and calcified tissue site was observed at 21 days post-fracture. The combination of stem cells, PL, and IL-10 had the greatest effect on enhancing fracture healing. After 21days of treatment in group E and B, trabecular bone formation and cortex remodeling were observed, surrounded by osteoblasts and resorptive osteoclasts (Fig. 4D and 4E). By contrast, groups C and D demonstrated the presence of woven bone and cartilage islands (Fig.4B and 4C).

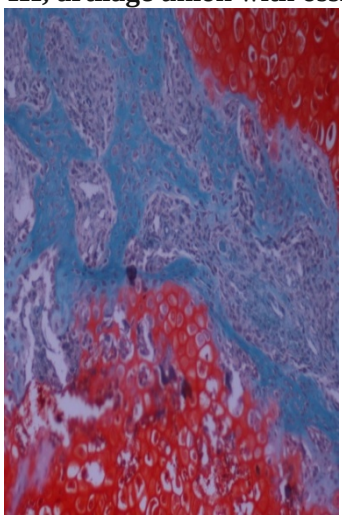
Figure 4. Safranin O staining at 21 days post-fracture



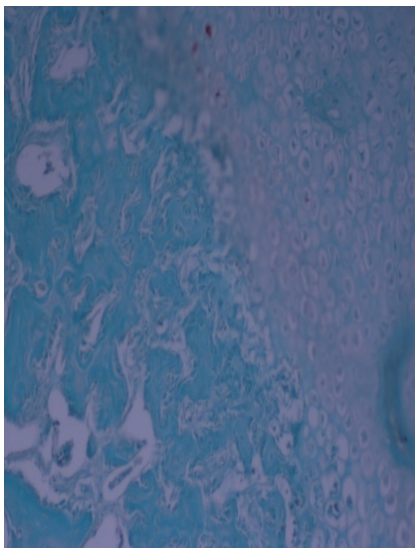
a. Score II, only cartilage union



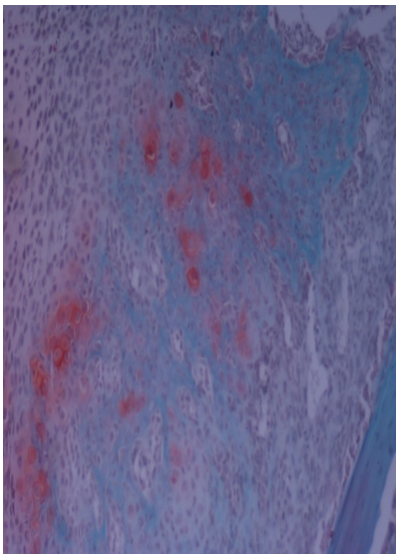
b. Score III, artilage union with ossification



c. Score IV, approximately equal amounts of trabecular bone and cartilage union



d. Score V, complete bony union



e. Score V, complete bony union

Discussion

Rat MSC, PRP, and the lack of adaptive immune response in association with the upregulation of IL-10 have been implicated in the acceleration of bone healing. In this study, the interplay of these factors and their combined effect on the process of fracture healing in a rat model were analyzed. The aims of this study were to generate a clinically relevant model of fracture and to evaluate the functional contributions of these factors to fracture repair after local injection.

Currently, use of stem cells has been a subject of special interest to improve the fracture healing process. MSC can differentiate into several different cell types. The ability of MSC to differentiate into bone tissue, which enhances fracture healing and bone regeneration, has been established through *in vitro* and *in vivo* studies.

Inflammation is the first reaction elicited from the immune system immediately after a

fracture occurs in order to combat pathogenic microorganisms when the fracture is associated with an infection. However, in cases where there is no simultaneous infection, this process is dispensable and in fact has been shown to be depressing bone regeneration[28]. RAG knockout mice exhibit enhanced fracture healing and bone regeneration that is coupled with lower pro-inflammatory cytokine secretion and higher anti-inflammatory cytokine production, most importantly of IL-10[21]. Several lines of evidence indicate that IL-10 is a positive regulator of bone regeneration. IL-10 influences the expression of genes that regulate osteoclastogenesis, thus exerting control over the process of bone resorption[29,30]. In addition, IL-10-targeted gene knockout mice demonstrate reduced mechanical strength of the bone, osteopenia, and defects in bone remodeling[31]. However, the role of IL-10 in the process of bone regeneration remains controversial, as *in vitro* studies conducted on bone marrow MSC revealed reduced expression of critical osteogenic proteins including ALP, collagen type I, and osteocalcin[18-20].

Recently, PRP has been shown to have an antibacterial effect: local application of PRP into the sternal wound in patients undergoing heart surgery reduced the incidence of both deep and superficial sternal wound infections[32]. On the other hand, the effect of PL on bone regeneration and the osteogenic differentiation of MSC is well established, and PRP is widely used in the treatment of muscular and skeletal injuries[8].

In this study, we found that using either PL alone or combined with IL-10 or MSC led to increased evidence of healing compared to the control group, with MSC and PL demonstrating the highest callus indices compared to other groups. MSC injected in combination with IL-10 and PL showed the highest callus-to-cortex index, an indication of enhanced fracture healing, compared to controls and all other groups. This confirms the synergistic effect exerted by MSC and IL-10 on the process of bone regeneration. This effect is either due to the anti-inflammatory properties of IL-10 or its effect on other processes such as bone resorption and callus mineralization.

Table 1. Callus indices of tibia fracture healing

	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Group A	1.00	1.15±0.23	1.32±0.29	1.45±0.20	1.35±0.21
Group B	1.00	1.21±0.26*	1.65±0.22*	1.62±0.20*	1.41±0.23*
Group C	1.00	1.16±0.28*	1.44±0.21*	1.51±0.26*	1.36±0.25*
Group D	1.00	1.18±0.24	1.42±0.28*	1.53±0.24	1.34±0.21
Group E	1.00	1.24±0.21*	1.68±0.24*	1.68±0.24*	1.45±0.22*

Date expressed as mean ± SD, n=4 per group
Group A: placebo, Group B: MSC and PL, Group C: IL-10 and PL, Group D: PL, Group E: MSC and IL-10
*P<0.05 vs. group A
N.S.: not significant

The obtained results indicate a synergistic complimentary role of MSC and IL-10. Treatment of fractured tibia with stem cells in PL and IL-10 resulted in the most significant

improvement over the control group. MSC are known to have immunosuppressive and immunomodulatory characteristics due to their down regulation of the expression of costimulatory molecules as well as the production of anti-inflammatory cytokines, most notably IL-10[33,34]. Thus, we hypothesize that treatment of fractures with MSC provides cells that secrete IL-10, suppressing the deleterious inflammatory phase that at later stages affects osteoblast differentiation contributing to bone regeneration. In addition, angiogenic as well as morphogenic growth factors are provided at higher concentrations in the presence of PL.

Conclusions

The use of different local factors on the fracture to accelerate healing is attractive and could be implemented with adequate evidence from clinical studies. Factors that can play a significant role in enhancing fracture healing have been identified, with varying degrees of effect. This animal study revealed a positive and synergistic effect of MSC in combination with both IL-10 and PL on fracture healing. Of these factors, MSC was the most potent and promising alternative affecting fracture healing. Other factors like IL-10 and PL should also be considered for their positive synergistic effect. Further studies are needed to determine the optimal concentrations of different factors, especially IL-10, and optimal times for application that may lead to the use of such factors to enhance fracture healing.

Acknowledgments

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تأثير الحقن الموضعي للخلايا الجذعية والصفائح الدموية ومادة إنترلوكين 10 على شفاء عظمة الساق المكسورة عند الجراذين

خلدون بشايرة،[1] نزار أبو هرفيل،[2] زياد عودات،[1] وائل حننة[3]

1– قسم أمراض وجراحة العظام والمفاصل، مستشفى الملك المؤسس عبدالله الجامعي، إربد، الأردن؛
2– قسم الأحياء التطبيقية، جامعة العلوم والتكنولوجيا، إربد، الأردن؛
3– قسم الباثولوجيا والصحة العامة، الطب البيطري، جامعة العلوم والتكنولوجيا، إربد، الأردن.

الملخص

الهدف: تهدف الدراسة إلى فحص تأثير استخدام الحقن الموضعي للخلايا الجذعية والصفائح الدموية ومادة إنترلوكين 10 على شفاء الكسور في عظمة الساق عند الجراذين.

الطريقة: تم كسر عظمة الساق عند 40 من الجراذين بعد تخديرهم ومن ثم تم تقسيمهم إلى خمس مجموعات عشوائية وحقن كل مجموعة كالآتي: المجموعة الأولى: محلول ملحي طبيعي، الثانية: الخلايا الجذعية مع محلول الصفائح الدموية، الثالثة: إنترلوكين مع محلول الصفائح الدموية،الرابعة: محلول الصفائح الدموية، الخامسة: الخلايا الجذعية مع محلول الصفائح الدموية وانترلوكين 10 تم عمل صورة شعاعية جانبية لعظمة الساق وقت الكسر وعلى فترات مختلفة لمتابعة شفاء العظمة ثم تم اختيار أربعة جرذان من كل مجموعة بعد 21 يوما من عمل الكسر ودراسة التغير النسيجي في منطقة الكسر عند كل مجموعة.

النتائج: أظهرت النتائج وجود تكوين تكلسي واضح كدلالة للشفاء بعد مرور سبعة أيام من تاريخ الكسر في المجموعة الخامسة (الخلايا الجذعية مع محلول الصفائح الدموية وانتيرلوكين 10 ومن ثم المجموع الثانية (الخلايا الجذعية مع محلول الصفائح الدموية) مقارنة مع المجاميع الأخرى. نفس النتائج ظهرت بعد مرور أسبوعين وثلاثة أسابيع وأربعة أسابيع. كذلك الأمر أظهرت نتائج فحص النسيج وجود تغيرات تدل على حدوث التئام للكسر وتكلّس منطقة الكسر في المجموعتين الخامسة والثانية مع أفضلية للمجموعة الخامسة مقارنة بالمجاميع الأخرى.

الاستنتاجات: استخدام الخلايا الجذعية مع محلول الصفائح الدموية وانتيرلوكين 10 قد يكون خيار مستقبلي لعلاج الكسور العظمية.

الكلمات الدالة: شفاء الكسور، الخلايا الجذعية، إنترلوكين 10، الصفائح الدموية.