Comparison between the Effects of Stem Cells and Lepidium Sativum on the Healing of Extraction Socket in Albino Rats (Histological and Immunohistochemical Study)

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ABSTRACT

Aim: to compare the bone marrow derived mesenchymal stem cells (BM-MSCs) and Lepidium Sativum (LS) in their potential accelerating actions on the extraction socket healing in albino rats. Subjects and Methods: Forty-five albino rats had their mandibular first molar extracted and divided into 3 groups. Group (1) received no treatment, group (2) received single dose of intravenous BM-MSCs and group (3) received daily oral dose of LS extract till sacrifice. Each group was divided to 3 subgroups A, B and C according to the days from extraction to sacrifice (7, 14 and 30 days). Molar regions were processed for light microscopic examination via Hematoxylin and Eosin (H&E) and anti-osteonectin (ON) immunostain. Bone area fraction in H&E sections and the number of osteoblasts per area in ON sections were statistically analyzed. Results: All groups had proper socket healing, but group 2 had superior histomorphometric values followed by group 3. The least values were of group 1. The studied periods showed ascending bone healing values from day 7 to day 30. All differences between subgroups were statistically significant (p value < 0.05). Conclusion: Both stem cells and Lepidium Sativum have accelerating potential for the extraction socket healing with slight preference of stem cells.

KEYWORDS

Stem cells, Lepidium Sativum, Extraction socket, bone marrow, bone healing
INTRODUCTION

The extraction of teeth initiates several events in both hard and soft tissues to allow for hard and soft tissue healing. Understanding the extraction socket-healing steps to obtain the proper management outcomes is clinically significant. Generally, there are three overlapping steps of socket healing, namely, inflammation, proliferation, and remodeling. One of the common drawbacks of socket healing is ridge reduction, which necessitates clinical steps to minimize this reduction for proper later tooth replacement procedures.\(^{(1)}\)

Several procedures and materials have been developed to accelerate bone repair such as bone grafts, which are used for ridge augmentation. However, bone grafts have some limitations and post-operative complications, extreme inflammation, or retarded healing.\(^{(2)}\) Thus, the innovation of bone healing-enhancing materials was of significant value. Recently, the use of stem cells received great interest due to their capacity to replicate extensively without losing their multi-potency to differentiate into other cells.\(^{(3)}\) Bone marrow-derived mesenchymal stem cells (BM-MSCs) are composed of a group of cells from the portion of bone marrow that controls hematopoietic cell formation. BM-MSCs are able to differentiate into several cell lineages including bone and cartilage forming cells.\(^{(4)}\)

BM-MSCs are the most commonly used bone-forming cells’ source in bone regenerative medicine. They are used to enhance the bone formation in the extraction sockets and resulted in advanced bone development with higher bone marrow amount.\(^{(5)}\) The mesenchymal stem cells accelerate the bone healing in the extraction sockets in rats with induced diabetes with the mature collagen fibers formation.\(^{(3)}\)

In addition to synthetic substances and agents, natural materials and extracts have increased attention recently. One of these natural substances is Lepidium Sativum (LS), an edible herb also named Halim, Holan, Chandrasura, and others. It is a member of the Brassicaceae family and grows easily in all climates and soil types.\(^{(6)}\) It is widely used as an analgesic, anti-spasmodic, anti-diarrhoeal, galactagogue, hepatoprotective\(^{(7)}\), antioxidant, and anti-inflammatory agent.\(^{(8)}\)

Several studies suggested the stimulatory effects of LS in bone fracture healing in rats with increased collagen and tensile strength.\(^{(9)}\) Furthermore, LS seed powder enhanced the bone fracture healing in rabbits associated with significantly high osteogenic markers.\(^{(10)}\) Although it is evident that LS has many beneficial health effects including wound healing, more comprehensive studies are necessary to clarify the biological mechanisms of its action.\(^{(11)}\) To our knowledge, there is no study comparing stem cell-based and natural herbs-based therapies in the extraction socket healing acceleration. Thus, the aim of this work is to compare the BM-MSCs and LS in their effects on the extraction socket healing in albino rats.

MATERIALS AND METHODS

Animals and bioethical considerations

Forty-five adult male albino rats of average weight 180-200 grams were used. Rats were housed in Faculty of Medicine Animal House, Ain Shams University under controlled temperature and humidity and diet content. The principles for ethical use of animals in testing were considered during the research. All means to decrease the pain or distress of the rats were followed. The Research Ethics Committee of Faculty of Dentistry Ain Shams University reviewed and approved the research (Approval number: FDASU-Rec PC022466).

The appropriate sample size for our study was verified by Power analysis guided by similar studies\(^{(12,13)}\) to give a power of 80%, alpha level of 0.05 and effect size 0.490. The predicted total sample size was 45 (5 samples per subgroup) to assess the difference between subgroups.

Preparation of aqueous extract of Lepidium Sativum: The extract was prepared guided by previous study\(^{(14)}\) by mixing one gram of powdered seeds with 100 ml distilled water. The solution was
boiled for 10 minutes and left for 15 minutes at room temperature then was filtered using a sterile gauze.

**Stem cells:** BM-MSCs suspension (1x10^6 cells/0.1ml saline) was purchased as a cell-line from the Biochemistry Department, Faculty of Medicine, Cairo University.

**Teeth extraction**

All rats were fast twelve hours before the operation to avoid aspiration of gastric secretions while the rats are anaesthetized. Extraction was done under sterile conditions, and general anesthesia (intra-peritoneal injection of a 4:1 solution Xilazin (Syntec1, 20mg/kg) and Ketamine (Agener1, at 40mg/kg) at a dose of 0.015 ml/kg of body weight.\(^{15}\) The first mandibular molar was atraumatically extracted using gentle bucco-lingual movements. The rats were classified into 3 groups (15 rats each):

- **Group 1 (Control group):** Rats didn’t receive any local or systemic drugs.
- **Group 2 (Stem cells):** Rats were injected once intravenously with 1x10^6 cells/well bone marrow-derived mesenchymal stem cells (BM-MSCs) via tail vein by insulin syringe immediately after extraction.
- **Group 3 (Lepidium Sativum):** Rats received daily aqueous extract of Lepidium Sativum (LS) 20ml/kg body weight orally.\(^{16}\)

Each group was divided into 3 sub-groups according to the timing of sacrifice following the tooth extraction. Subgroups A, B and C (7, 14 and 30 days respectively).

**Sample preparation for routine histological examination:**

After rats’ sacrifice, the mandibular molar areas were dissected, fixed in neutral buffered formaldehyde solution for 72 hours. Samples were decalcified in 12% Ethylene Diamine Tetra-acetic acid (EDTA) solution which was refreshed daily. The samples were washed under running tap water then dehydrated with increasingly graded ethyl alcohol washes, then transferred to xylol to clear the specimens from alcohol. The samples were embedded in paraffin wax blocks oriented to cut sagittal plane of the jaw. The embedded specimens were sectioned by microtome to a thickness of 4-6μm, then transferred in decreasing alcohol concentrations ended by distilled water. Sections stained with Hematoxylin and Eosin (H&E) for routine examination by light microscope.

**Sample preparation for immunohistochemical staining**

The paraffin sections were stained by monoclonal anti-osteonectin antibody with labeled polymer reagent kit (Dako, Denmark) according to the manufacturer’s instruction. Sections of 4um thickness were mounted on positive glass slides. The slides were heated overnight at 37°C, de-paraffinized and hydrated in decreasing grades of alcohol solutions. Then the slides were heated with citrate buffered solution in a microwave oven at two cycles of 5 minutes each. The sections were then incubated at 4°C in a humidified chamber with the primary antibody at a dilution of 1: 20 overnight. The antibody was detected with the secondary biotin-conjugated antibody which was incubated for one hour at room temperature for 30 minutes. The slides were counterstained with hematoxylin. Positive immunoreaction was detected as a brown color and the cellular localization of this antibody is cytoplasmic. Osteonectin was used in the current study for the demonstration of active osteoblasts and young osteocytes.

**Histomorphometric and statistical analysis**

For each H&E section and positive anti-osteonectin section, three microscopic fields were selected. Photomicrographs were captured at a magnification of x200 for H&E and x400 for anti-osteonectin stained sections using digital camera (C5060, Olympus, Japan) which was mounted on a light microscope (BX60, Olympus, Japan). The images
were analyzed using Image J, 1.41a, (NIH, USA) image analysis software. The histomorphometric analysis was performed through:

1. Manual counting to the number of immunopositive osteoblasts on the newly formed bone margins per unit area in each field.

2. Calculating the area fraction (percentage) of the newly formed bone in each field.

Data were presented as mean and standard deviation. The differences between groups were considered significant when P values≤0.05. Statistical analysis was performed with SPSS statistics Version 20 for Windows.

**RESULTS**

**Histological results (H&E)**

The control subgroup 1A (7 days) extraction sockets showed the initiation of bone healing represented by granulation tissue having cellular elements including spindle shaped fibroblasts and inflammatory cells. The cells were embedded in extracellular matrix containing collagen fibrils. Blood vessels were observed lined by squamous endothelial cells. There were occasional bone spicules connected with the original socket wall (Fig. 1a and 1b). Osteoblasts with variable outlines (oval, rounded and stellate) were bordering the bone spicules and the osteocytes were embedded in the bone matrix within lacunae (Fig. 1b). Subgroup 1B (14 days) showed the presence of bone trabeculae and marrow cavities.

**Fig. (1) Photomicrographs of group 1. (a): Subgroup 1A showing granulation tissue with blood vessels (yellow arrows) and bone spicules (black arrow). (B): subgroup 1A showing osteocytes in lacunae, with eccentric small nuclei (yellow arrows) or with larger central nuclei (blue arrow), osteoblasts with different outlines (black arrows), spindle fibroblasts (green arrow), inflammatory cells (red arrow) and collagen fibrils (white arrows). (c): Subgroup 1B showing bone trabeculae (arrows) and marrow cavities (MC) with no evidence of compact bone. (d): Subgroup 1C, showing bone trabeculae (white arrow), marrow cavities (MC), osteoblasts (yellow arrows), Haversian canal (red arrow) surrounded by osteocytes (blue arrows) and Volkmann’s canal (black arrow). H&E. (a:x100, b:x400, c:x100 d:x200).**
days) showed the developing bone with relatively more organized trabecular pattern with interconnected trabeculae enclosing medullary spaces with no evidence of bone compaction (Fig. 1c). Subgroup 1C (30 days) showed signs of bone maturation represented by apparently thick bone trabeculae with scattered osteocytes. Occasionally, areas of compact bone were noticed with well-defined Haversian canals surrounded by osteocytes and connected to each other by transverse canals in some regions. The osteoblasts were observed, forming a relatively more dense lining along the bone edges (Fig. 1d).

Extraction sockets of group 2 (stem cells) revealed relatively more evident bone healing rate than control group. Subgroup 2A showed new bone formation in a trabecular pattern enclosed in a granulation tissue which displayed clearly seen blood vessels in variable sizes (Fig. 2a). The blood vessels had well-defined endothelium in many regions, with occasional poorly developed endothelium in some blood vessels. The new bone showed clearly seen osteocytes in their lacunae (Fig. 2b). Subgroup 2B showed progress in the vascular network establishment and the anastomosing of the new bone trabeculae within a highly cellular granulation tissue. Occasional regions of bone rearrangement were observed as concentric rings around Haversian canal to form the primary osteon (Fig. 2c). The new bone deposition was also evident by the presence of osteoid entrapping the osteocytes and bordered by osteoblasts (Fig. 2d). Subgroup 2C sockets were almost filled with well-organized mature bone with concentric lamellae surrounding Haversian canals. Furthermore, bone marrow cavities were observed but were widely separated by the widespread of compact bone (Fig. 2e and 2f).
Extraction sockets of group 3 (Lepidium Sativum) revealed, in the subgroup 3A, patchy distribution of new bone masses within the granulation tissue (Fig. 3a). Subgroup 3B showed trabecular pattern of the new bone which occupied extensive area with several areas of compaction (Fig. 3b). The marrow cavities displayed scattered calcified masses (Fig. 3c). Subgroup 3C showed ingrowth of remodelled lamellar bone with evident osteon formation in a relatively less prevalence compared to subgroup 2C. Osteoblastic layer with several outlines were bordering the newly formed bone (Fig. 3d).

Fig. (3) Photomicrographs of group 3 (Lepidium Sativum). (a): Subgroup 3A showing patchy distribution of newly formed bone (arrows). (b): Subgroup 3B showing trabecular new bone (blue arrow) with areas of compaction (yellow arrow). (c): Subgroup 3B showing marrow cavities with calcified masses (yellow arrows) and osteocytes (black arrows). Subgroup 3C showing Haversian canals, bone trabeculae containing osteocytes (blue arrows) and marrow cavities lined by active osteoblasts (black arrow). H&E (a, b: x100. C, d: x400).
**Immunohistochemical results (Osteonectin)**

The extraction sockets of all groups revealed consistent distribution of the positive reaction among the cell types through the successive periods in the subgroups. The subgroup A and B in all groups (7 and 14 days respectively) showed immuno-positive reaction in the osteoblasts and the connective tissue cells (fibroblasts and blood cells) and occasionally the recently trapped osteocytes (Fig. 4a,b,d,e,g,f). While the subgroup C (30 days) in all groups showed immunopositive reaction in the osteoblasts and some connective tissue cells, with no observable positive reaction in the osteocytes (Fig. 4c,f,i).

<table>
<thead>
<tr>
<th>Subgroup A (7 days)</th>
<th>Subgroup B (14 days)</th>
<th>Subgroup C (30 days)</th>
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<tr>
<td>Group 1 (Control)</td>
<td>Group 2 (Stem cells)</td>
<td>Group 3 (Lepidium)</td>
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<tr>
<td>A</td>
<td>D</td>
<td>G</td>
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<td>B</td>
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<td>C</td>
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Fig. (4) Photomicrographs of all subgroups showing the immuno-positive osteoblasts (black arrows), immunopositive connective tissue cells (yellow arrows). The osteocytes showed occasional immunopositive reaction in the subgroups (A and B) in all groups (Blue arrows) while in subgroup C in all groups, the osteocytes had no observed positive reactions (red arrows). Osteonectin (All figures:x400).
Statistical Results

Comparison between the periods subgroups regarding the area fraction of new bone and the number of immuno-positive osteoblasts in the unit area revealed that the 30 days’ subgroup (C) in each group showed highest mean value followed by 14 days’ periods (subgroup B) and the least values were those of the 7 days periods (subgroup A). The differences between the subgroups were statistically significant.

Table (1) The mean and standard deviation values of the area percentage of new bone and the number of osteoblasts per unit area in the studied subgroups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subgroups</th>
<th>Groups</th>
<th>P-value</th>
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<tr>
<td></td>
<td></td>
<td>Group 1 (Control)</td>
<td>Group 2 (Stem cells)</td>
</tr>
<tr>
<td>Area % of new bone</td>
<td>A (7 days)</td>
<td>25.7 ± 1.16</td>
<td>30 ± 1.07</td>
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<tr>
<td></td>
<td>B (14 days)</td>
<td>36 ± 3.02</td>
<td>55 ± 4.14</td>
</tr>
<tr>
<td></td>
<td>C (30 days)</td>
<td>67 ± 2.06</td>
<td>85 ± 1.13</td>
</tr>
<tr>
<td>Number of osteoblasts</td>
<td>A (7 days)</td>
<td>15 ± 0.70</td>
<td>20.5 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>B (14 days)</td>
<td>18 ± 0.42</td>
<td>23 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>C (30 days)</td>
<td>26 ± 0.57</td>
<td>31 ± 0.14</td>
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*: Significant at P ≤ 0.05.

The comparison between the groups in each duration revealed that group 2 (stem cells) had the highest mean value of bone area fraction in its three subgroups compared to the corresponding subgroups, followed by group 3 (Lepidium Sativum) while the least values were those of group 1 (control). Regarding the number of osteoblasts, subgroups 3B and 3C had more number than the corresponding subgroups in group 1 and 2. The differences between the subgroups were statistically significant (Table 1, Fig. 5).

Fig. (5) Bar chart representing the mean values of area % of new bone and the number of immunopositive osteoblasts.
DISCUSSION

Tooth extraction initiates a cascade of events involving both hard and soft tissues aiming to repair the injured tissues and regenerated the lost structures\(^1\). The ongoing study was designed for comparison between the potential effects of the bone marrow derived mesenchymal stem cells (BM-MSCs) and aqueous solution of Lepidium Sativum (LS) on the extraction socket healing of the rat mandibular molar. In the present work, we selected the rat as an animal model as the alveolar bone healing phases are similar in both human and rats.\(^{17}\)

We selected two different concepts of regenerative therapy, the stem cell based and the natural herbs based therapy. BM-MSCs are preferable for use due to their characteristics including the multi-potency, the evident role in bone formation in the extraction sockets and even the ability to ameliorate the adverse effects of diabetes on the bone healing\(^{3-6}\). The other material that was selected in our work was targeting another scope, the natural biomaterials that have received significant interest in the recent decades. Lepidium Sativum was reported to have proved medicinal effects, more detailed studies are necessary to clarify the mechanisms of its biological effects.\(^{11}\)

One of the limitation of the current study is the difference in the route of administration of the tested substances. However, this could be justified that the selected route is the suitable one for each drug to obtain the maximum predicted effect from them. The intravenous injection for BM-MSCs to mimic the pathway of the normal BM-MSCs through the blood stream to reach the target location.\(^{18}\) Also the intravenous BM-MSCs transfer primarily to the injured tissues.\(^{19}\) Besides, topical injection of BM-MSCs in extraction socket might induce inflammatory response, releasing cytokines, which may retard the healing. Moreover, in case of extensive wounds, the topical BM-MSCs might not act in a proper manner.\(^{20}\) Regarding the rout of administration of Lepidium Sativum in our work, the aqueous extract is inexpensive, safe and more effective than the methanolic extracts.

The oral route of administration resembles the normal ingestion of LS in human.\(^{21,22}\)

In the current study, the control group showed normal socket healing sequence as the rats didn’t receive any treatment. Yuan et al.\(^{23}\) explained this response in extraction socket healing that there is stem cell type in the PDL that are activated by the teeth extraction, then remain in the socket and differentiate into osteoblasts to form the new bone.

In the present work, H&E stain revealed relatively more enhanced healing in the rats treated with stem cells with and rapid rate of bone development and significant increase in the new bone formation area. This agreed with Mahmoud et al.\(^{19}\) Who reported an increase in the area fraction of trabecular new in the extraction sockets of the rats treated with BM-MSCs. Furthermore, the bone healing acceleration could be explained by the ability of BM-MSCs to differentiate into bone forming cells.\(^4\)

The prominent feature in the stem cell group was the presence of new blood vessels with well-defined endothelium. This finding coincides the study conducted by Mohammed et al.\(^{24}\) who reported that BM-MSCs promote healing in rats, with increased angiogenesis because of the raised level of growth factors and the endogenous signals that accelerate the healing. Moreover, BM-MSCs are capable of generating a huge number of endothelial cells both in vitro and in vivo.\(^{25}\)

Subsequently, we observed that the osteonectin in all samples had positive reactions in osteoblasts and other connective tissue cells. This was in accordance with Yang et al.\(^{26}\) who reported that osteonectin is produced by osteoblasts and other cells. A detailed explanation of the role of osteonectin in the healing regions was conducted by Marquez et al.\(^{27}\) who reported that in the wound healing, fibroblasts produce several proteins, including osteonectin, which contribute in the extracellular matrix structure and its vascularization.

Although Lepidium Sativum treated rats showed relatively less improved results in comparison to stem cell treated rats, but they were more developed in healing process than the control group.
This finding comes in agreement with Elshal et al. (28) who observed significant increase in alkaline phosphatase concentrations and the trabecular bone regained its normal structure in osteoporotic rats treated with Lepidium Sativum. This bone healing stimulation by LS could be due its calcium content of LS which was reported by Gokavi et al. (29) who emphasized that the positive effect of LS on bone healing is due to its rich content of calcium.

In the current study, we noticed that the number of osteoblasts was the highest in the subgroups sacrificed 14 and 30 days after extraction in the Lepidium Sativum. These findings were supported by studies reported that Lepidium Sativum stimulates bone healing and causes significant increase in bone markers. (9,10) Also this coincides with a previous study conducted by El-Haroun et al. (30) who reported that Lepidium Sativum ameliorated serum alkaline phosphatase level and induce the osteoblast proliferation.

Furthermore, the increased cellularity with LS treatment This could be attributed to that LS has antioxidant, anti-inflammatory, and anti-apoptotic effects (31) that could add values in the process of wound healing and promote the cellular viability. More detailed studies are needed to explain the molecular background behind the proliferative potential of osteoblasts in the late healing phases in association with LS treatment.

CONCLUSIONS

Within the limitations of the current study, it could be concluded that bone marrow derived mesenchymal stem cells and Lepidium sativum improve the extraction socket healing during all of the healing phases. Bone marrow derived mesenchymal stem cells are relatively more favorable than Lepidium sativum in the healing enhancement. Lepidium sativum improved the osteoblastic activity more than stem cells during the late phases of extraction socket healing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Comparison between the Effects of Stem Cells and Lepidium Sativum on the Healing of Extraction Socket in Albino Rats (Histological and Immunohistochemical Study)


Comparison between the Effects of Stem Cells and Lepidium Sativum on the Healing of Extraction Socket in Albino Rats (Histological and Immunohistochemical Study)

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The purpose of this study was to compare the effects of mesenchymal stem cells derived from the bone marrow with Lepidium sativum on the healing of extraction socket in albino rats.

Materials and Methods: A total of 30 rats were randomly divided into three groups. Group A received a daily oral dose of Lepidium sativum, Group B received a single injection of stem cells through the vein, and Group C did not receive any treatment. Each group was then divided into three subgroups according to the time of extraction.

Histological examination was performed by hematoxylin and eosin staining and immunohistochemical staining for osteonectin.

Results: The results showed a significant increase in bone healing rates in the stem cell and Lepidium sativum groups compared to the control group. The healing process was faster in the stem cell group, with significantly higher bone healing rates compared to the Lepidium sativum group.

Conclusion: Both stem cells and Lepidium sativum have the potential to accelerate bone healing after tooth extraction, with a slight preference for stem cells.