Effect of a Decellularized Omentum Scaffold With Combination of Mesenchymal Stem Cells and Platelet Rich Plasma on Healing of Critical-sized Bone Defect

Abdulsamet Emet (✉ drsametemet@gmail.com)

Hacettepe Universitesi <https://orcid.org/0000-0001-5415-218X>

Erdi Ozdemir

Hacettepe University: Hacettepe Universitesi

Duygu Uckan Cetinkaya

Hacettepe University: Hacettepe Universitesi

Emine Kilic

Hacettepe University: Hacettepe Universitesi

Ali Celalettin Sinan Yuruker

Usak University: Usak Universitesi

Ramin Hashemihasar

Istanbul Aydin University: Istanbul Aydin Universitesi

Egemen Turhan

Hacettepe University: Hacettepe Universitesi

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Abstract

Background: High-cost and the long time needed for the treatment of critical-sized bone defects is still major clinical concern in orthopedic surgery, therefore new research continues to identify more cost and time-effective methods. In this study, we aimed to investigate the effect of a decellularized omentum scaffold with a combination of platelet-rich plasma (PRP) and mesenchymal stem cells on the healing of critical- sized bone defects.

Materials and methods: Wistar albino rats (n=30) were investigated in five groups. Critical-sized bone defects were formed on bilateral radius shafts. To increase healing, no scaffold, decellularized omentum, omentum with with PRP and omentum mesenchymal stem cells was used in group 1 (control group) , 2, 3 and 4 respectively. In addition omentum with a combination of mesenchymal stem cells and PRP was used in group 5. After 6 weeks, both radiological and histological healing were evaluated comparatively among groups.

Results: After use of decellularized omentum scaffold, vitality of new cells maintained, new bone formation occurred. When compared to control group, radiological healing was statistically significantly better in omentum and omentum with PRP-treated groups ($p=0.047$). Furthermore histological healing was better in the omentum and omentum with PRP-treated groups than both control and omentum with mesenchymal stem cell-treated groups according to Salkeld Scoring System ($p=0.001$).

Conclusion: The use of decellularized omentum scaffold is suitable in the healing of critical bone defects.

1. Introduction:

The frequency of bone defect problems encountering by orthopaedic surgeons are increasing. High energy trauma, open fractures, bone tumors, infections, debridement of bone for any reason, metabolic diseases and massive osteolysis are the most common causes of bone defects [1]. Main problems in dealing with bone defects are high cost and long time needed for healing. Critical bone defect is more difficult situation and termed for defects when self-healing is not expected despite enough surgical stabilization [2]. In addition to appropriate surgical stabilization, critical bone defect treatment must include proper and functional segmental replacement regardless of the cause. In the literature, autologous bone grafts (ABG) are described as the gold standard among current treatment modalities [3]. However, due to some disadvantages such as limited availability and donor site morbidity, the efforts to find more appropriate and optimal scaffold options continue.

The bone scaffold is a 3D matrix that allows osteogenic cell adhesion, proliferation, and also stimulates cells [4]. Natural and synthetic polymers, ceramic-based scaffolds, metallic, and composite scaffolds have been tried so far but none of the used scaffolds contain all the properties of an optimal scaffold [5, 6]. Also, synthetic scaffolds are associated with rejection, allergic reactions and failure to integrate with host tissues. As a result, tissue engineering in current technological upgrades is seminal in bone defect treatment. Presently, natural extracellular matrix composite scaffolds are of interest because these are

accepted as most similar to the original tissue and allow cellular adhesion and proliferation, also the main advantage of this scaffold type is being non-antigenic to the native tissue [7]. The omentum has been used for defects of other organs because of its rich extracellular matrix, ability to adhere to local tissues and growth factors containment but it is generally used in reconstructive and cardiovascular surgery as a decellularized omentum scaffold. However, in orthopedic surgery, decellularized omentum scaffolds have not been used to date [8].

Successful bone repair is based on combination of a suitable medium, stem cells and growth factors. Platelet-rich plasma (PRP), which has osteoinductive and osteoconductive properties is a good candidate to be added on a scaffold [9]. It is proven to have a number of growth and differentiation factors that play important roles in the intracellular signaling pathway for cellular proliferation, osteoid and extracellular matrix formation, and also callus formation in fracture healing [10]. Also, mesenchymal stem cells (MSCs) were shown to be involved in callus formation [11]. Both direct involvements in callus formation and inflammatory cytokines such as transforming growth factor (TGF)- β , insulin-like growth factor (IGF), and epidermal growth factor (EGF) production is stimulated by MSCs [12].

Therefore, we aimed to evaluate the effects of an decellularized omentum scaffold in critical bone defects. Also, the potential osteoinductive effects of the combination of PRP and MSCs were also investigated.

2. Methods:

The study was executed after receiving approval of the local ethics committee from Hacettepe University Medical Faculty Animal Research Laboratory, (Date: 25.07.2017, 2017/49 – 08)

2.1 Mesenchymal Stem Cell Isolation and In-vitro Expansion

Firstly, the permission was obtained from the local ethics committee (2016/03 – 01) for the use of mononuclear cells which were taken from 8 years old female bone marrow donors, isolated in our archive cryostocks and stored at -196°C until use. 20×10^6 isolated cells planted into the tissue culture medium in T-75 flasks with 10 ml of growth medium, 10 % fetal bovine serum (DMEM-LG) with 1% penicillin-streptomycin antibiotics. Incubation was performed at 37°C in 5% CO_2 . When cells reached 70–80% confluence at 7–12 days, medium were irrigated with a 0.25 % trypsin/EDTA solution for mobilization of non-adherent cells. Then adhesive cells were cultured in T-75 flask with of a density 2×10^3 cells/ cm^2 . In subsequent passages, cells were checked every day with an inverted microscope for non-adherent cells and the medium changed every three days to remove non-adherent cells. At 4th passage, adherent cells showed fibroblastic morphology and a homogenous cell population was obtained in all cultures. The cells demonstrated 95% positivity for stromal markers including CD29, CD44, CD73, CD90 and CD105 (*e-Biosciences, USA*). Hematopoietic markers, CD146, CD45 and CD34 (*e-Biosciences, USA*), were negative. The differentiation capacity of MSCs was confirmed by positive staining in osteogenic and adipogenic

differentiation assays after 21 days of culture. A 1×10^6 cell count for every rat in fetal bovine solution with 0.5 ml volume was prepared [13].

2.2 Decellularized Omentum Scaffold Preparation

For the preparation of the decellularized omentum, the omentum obtained from patients who had an abdominal surgery for non-tumorous surgeries were used after receiving ethics committee approval (2017/49 – 08). Omentum tissue was kept at -80°C , and dissolved gradually to $+4^{\circ}\text{C}$. First, tissues were washed with PBS solution for 24 hours in a 1 liter flask. After washing, tissues placed for intermittent shaking in a shaker for 24 hours to be smashed, then smashed tissues placed in 1% (w/v) sodium dodecyl sulfate solution and shaken at a constant shaking rate, also for 24 hours. The smashed pieces were washed out with PBS solution three times to remove the excess SDS remaining in the tissues. Tissues in PBS were replaced in separate tubes for 48 hours in isopropanol solution (%99.9) to remove the lipid tissues. The last part was the enzymatic release of the RNA and DNA, with RNase and DNase (*Sigma-Aldrich*) in 37°C incubation for 16 hours. The remaining decellularized omentum parts were washed out with PBS solution and placed into Petri dishes in a sterile conditions ready for use in surgical procedures [14].

2.3 PRP Preparation

Two rats were sacrificed through intracardiac blood aspiration, after an intraperitoneal injection of 50 mg/kg ketamine hydrochloride anesthesia. A sterile disposable monovette, containing 3.2% sodium citrate system, was centrifuged at 1800 rcf for 10 minutes. After the first centrifugation, two layers were seen in the monovette; the yellow layer consisted of PRP and the red layer of erythrocytes and leucocytes. After separating the two layers, the yellow layer was centrifuged at 4000 rcf for 10 minutes. The upper portion of the layer was platelet-poor plasma and a lower layer of 1 cm was PRP. The separated part of the lower layer was collected and transferred into an injector for use 30 minutes before starting the surgical procedure.

2.4 Animal preparation and surgical procedure:

Thirty male, inbred Wistar-Albino rats weighing over 300 g were included in the study after acclimating the laboratory environment for 10 days. The rats had not been involved in any previous experiments and were also screened for any diseases. They were kept in metal cages with access to water and food ad libidum. The rats were maintained in $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ environmental conditions with 12 hours of light and 12 hours of darkness. The animals were fasted for 24 hours before the procedure.

Five groups were made randomly:

1. Control group (n = 6)
2. Scaffold group (n = 6)
3. Scaffold + PRP group (n = 6)

4. Scaffold + Mesenchymal stem cell group (n = 6)
5. Scaffold + PRP + Mesenchymal stem cell group (n = 6)

Before surgical procedure, decellularized omentum cut into 0.5 cm³ pieces and saturated with 0.5 ml PRP or 0.5 ml MSC or both PRP and MSC, outside the surgery site in a Petri dish and waited 5 minutes to adhere on scaffold. All rats were anesthetized with 50 mg/kg dose of intraperitoneal ketamine hydrochloride injection. All rats were monitored by a veterinarian during the surgery. The surgical position of rats was supine and both forelimbs were shaved. 0.5 cm³ sized bone defects were created in all rats' in both radius bones with a 5 mm Kerrison reuneur. Periosteal elevation and muscle retraction were not performed in any rats. The bone defect site was treated according to the following group protocol: group 1, bone defect left empty; group 2, previously prepared decellularized omentum sized 0.5 cm³ was placed to all rats' forelimbs; group 3, previously prepared decellularized omentum sized 0.5 cm³, saturated with 0.5 ml PRP; group 4, previously prepared decellularized omentum sized 0.5 cm³, saturated with 0.5 ml MSCs; and group 5, previously prepared decellularized omentum sized 0.5 cm³, saturated with both PRP and MSCs 0.5 ml. (Fig. 1) No fixation method was used for the bone. Wound was closed with non-absorbable sutures. After the surgical procedure, while rats were under anesthesia, 15 mg/kg tramadol was used for postoperative analgesia. Each group of rats was labeled and caged in a separate cage with no restriction of activities.

2.5 Radiologic Analysis

After waiting for 6 weeks of healing, the rats were euthanized and the forelimbs were disarticulated from the glenohumoral joint in order to obtain anterior-posterior X-rays. Two orthopedic surgeons who were blinded to the groups but was informed about the evaluation method performed in the radiologic assessment. Results were scored using the grading scale described by Cook et al. (Table 1) [15].

2.6 Histopathologic Analysis

After disarticulation of the forelimbs, the specimens were fixed in a 10% formaldehyde solution for 2 weeks. Then, samples were placed in 10% ethylenediaminetetraacetic acid solution for the decalcification process. Samples were embedded in paraffin blocks and 5-µm-thick sections were cut through the long axis from the bone defect zone and stained with hematoxylin and eosin (H&E) stain for light microscope analysis. The best sections of the specimens were evaluated by two histopathologists in Histology and Embryology Department, which were informed about the histologic grading scale described by Salkeld et al. (Table 2) [16].

2.7 Statistical Analysis

The SPSS software, version of 21.0 was used for statistical analysis. Descriptive statistics included median (minimum and maximum) values. Kruskal-Wallis analysis of variance (ANOVA) was used to compare the groups in terms of histologic, radiologic, and biomechanical results. After performing Kruskal-Wallis ANOVA, the Mann-Whitney U test was performed with Bonferroni correction for paired

comparison of groups. Results are expressed with 95% confidence intervals. Significance was defined as $p < 0.05$.

3. Results:

All 30 rats woke from anesthesia and no major wounds or other complications occurred. All rats were euthanized and radiologic and histopathologic studies were performed.

3.1 Radiologic Findings

A total of 60 sample assessments were performed according to the Cook scale. In the radiologic assessment, compared to control group, radiological healing was significantly higher in omentum and omentum with PRP-treated groups ($p = 0.047$ and $p = 0.047$). Also, the omentum and omentum and PRP groups had significantly higher healing than the omentum and mesenchymal stem cell group ($p = 0.047$ and $p = 0.047$) (Table 3). Radiologic data are shown in Fig. 2.

3.2 Histopathological Findings

Histologically, in the sections, the defect was still visible when looking at the control sample, and there were new bone islands and adipose tissue near the defective areas. In the omentum group specimen, the defect was completely loaded with new cells and the scaffold was organized. In the omentum and mesenchymal stem cell group specimen, the scaffold was still visible, meaning collagen was visible, most of the living cells and newly formed fat cells were in the scaffold with some bone islands and minimal calcification. In the omentum and PRP specimen, a reduction of defect size and increased calcification was observed with a fully organized scaffold with bone cells. In the omentum and mesenchymal stem cell and PRP group, there were increased vascular structures and fat tissue as well as increased bone islands and calcification compared to omentum and mesenchymal stem cell group sample. Histologic sections are shown in Fig. 3.

Forty samples were assessed according to the method of Salkeld et al. Evaluation was based on the following parameters: (1) quality of union, (2) cortical development and remodeling, and (3) new bone formation.

Between the five groups, there was a significant difference according to the Salkeld scoring system, for each parameter and total score. For the quality of the union, the control group was significantly lower than omentum and omentum + PRP used groups ($p = 0.004$ and $p = 0.016$). Also, the omentum + mesenchymal stem cell-treated group was significantly lower than omentum and omentum + PRP-treated groups ($p = 0.001$ and $p = 0.006$).

According to the cortical development and remodeling scores, the control group was significantly lower than the omentum and omentum + PRP-treated groups ($p = 0.001$ and $p = 0.001$). Also, the omentum + mesenchymal stem cell-treated group was significantly lower than the omentum and omentum + PRP-treated groups ($p = 0.001$ and $p = 0.007$).

According to the new bone formation scores, the control group was significantly lower than the omentum and omentum + PRP-treated groups ($p = 0.001$ and $p = 0.001$). Also, the omentum + mesenchymal stem cell-treated group was significantly lower than the omentum and omentum + PRP-treated groups ($p = 0.013$ and $p = 0.016$).

For total scores, the control group was significantly lower than the omentum and omentum + PRP-treated groups ($p = 0.001$ and $p = 0.001$). Also, the omentum + mesenchymal stem cell-treated group was significantly lower than the omentum and omentum + PRP-treated groups ($p = 0.002$ and $p = 0.003$) (Table 4) (Fig. 4).

After use of our decellularized omentum scaffold, the vitality of new cells was maintained, new bone formation occurred, the defect size became smaller and vanished, and also the scaffold was resorbed. With the combination of MSCs, new bone formation was diminished and bone healing was damaged. Decellularized omentum with a combination of PRP increased bone healing.

4. Discussion:

The present study is an experimental animal study, which evaluates whether the decellularized omentum could be used as effective scaffold in critical bone defect. In the previous literature it was well shown that decellularized scaffolds promote cellular adhesion and migration in addition to cell proliferation and differentiation [17]. We preferred omentum in our study because of its natural basis to increase the biocompatibility. For current literature, many studies showed that omentum provided support and growth of the cells as a bioreactor in soft tissues [18, 19]. However decellularized omentum has never been tried in orthopedic literature as a scaffold, our study for the first time showed that decellularized omentum could be used as an efficient scaffold. We investigate omentum in animal model despite its human origin but it should be kept in mind that decellularization process provides us to lose the antigenic activity of the omentum.

Histologic data showed that ossification was seen, the defect shrank, and resorption of the scaffold accrued. Also, groups in which the scaffold was used, it was seen that the decellularized omentum scaffold allowed cellular adhesion and proliferation and supported cellular vitality. This results made us think that the omentum is an inspiring scaffold that can be used in the treatment of critical bone defects.

The other goal of the study was to find a way to increase bone healing in addition to suitable scaffold. PRP were proven to increase bone healing in the literature previously [20]. In our study, histologically, PRP combination with omentum had higher organized ossification and bone mineralization than the omentum only group. Also, the PRP + mesenchymal stem cell and omentum combination group had higher bone islands than the mesenchymal stem cell and omentum combination group. These results shows that PRP increases bone union microscopically.

For bone healing, the two methods, MSCs and PRP, were demonstrated to achieve improvement. Even though we prepared allogenic PRP, we could not prepare allogenic MSCs due to technical drawbacks.

However, previous animal studies that used human-origin MSCs with or without a suitable scaffold illustrated no systemic or local rejection reactions, which encouraged us to design our study [21, 22].

There are various studies regarding the effects of MSC usage in the healing of critical bone defects. In some studies, it was shown that MSC combinations increase osteoinduction and osteogenesis [23, 24]. CD44, CD45, CD73, CD90, and CD105 surface markers are essential for using of MSC in the orthopedic treatments to achieve osteogenic formation [25]. In our study, we used MSCs that express those surface markers. Although the decellularized omentum scaffold allowed cell adhesion and proliferation and the used MSCs had potential for osteogenic transformation, microscopic examinations and Salkeld scoring systems showed decreased bone healing and new bone formation.

There are studies claiming direct injection of MSCs into critical bone defect and non-unions impairs healing. When we looked at the mechanism which may have caused this, it was shown that bolus MSC could lead to reduction in gene expression of Runt-related transcription factor-2 (Runx2) and Osterix pathway via TNF- α which was responsible for the transformation of MSCs to osteogenic cells [26]. It is known that cumulative MSC expression causes an decrease in TNF- α [27, 28]. Scaffold application with low doses of TNF- α decreased osteogenic activation and high doses of TNF- α increased osteogenic activation in an experimental rat study [29]. The anti-inflammatory effect of cumulative MSC application might have decreased TNF- α levels in our scaffold and callus formation. This knowledge led us to speculate that this may be the reason for impairment of osteogenic formation with MSC application.

Using xenogenic mesenchymal stem cells could be another reason for decreased healing. Although there are many studies that used xenogenic mesenchymal stem cells that caused no local or systemic rejections, but also some controversial studies that describing human mesenchymal stem cell apoptosis and fragmentation in immune-competent mice [30]. It has been reported that xenogeneic, MSC-derived chondrocytes trigger T lymphocyte proliferation, cytotoxicity, increasing antigen presentation and further activation of the adaptive immune response [31]. Possibility of localized immune reaction and diminishing mesenchymal stem cells may also explain impairment of osteogenic formation [32].

Radiologic results showed a correlation to our histologic results. We think that these correlation empower our study. However, our scaffold was not fully ossified and radiologic healing was not seen completely. There is no similar study in the literature to discuss how much time would be needed for radiological healing.

One of the limitations of our study is the limited number of samples. Another limitation is that although PRP is allogenic, MSCs are not. Due to technical drawbacks, rat-origin MSCs could not be used in our study. In addition, we did not investigate the important features of our scaffold such as porosity, and micro, and nano construct.

5. Conclusion:

This research demonstrates that a decellularized omentum scaffold is suitable for critical-sized bone defects, and similar results in in-vivo tests and biomechanical studies highlights to usage of decellularized omentum being used as a scaffold in clinical practice for segmental bone defects.

Abbreviations:

ABG: Autologous Bone Grafts

PRP: Platelet Rich Plasma

MSC: Mesenchymal Stem Cell

Declarations:

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Authors' Contributions: AE made the animal preperation and surgical procedure, also analyzed and interpreted the data. EO and ET performed the measurements and scorings of radiologic data. ACSY and RH performed the histologic data analysis. DUC and EK prepared the mesenchymal stem cells. AE and ET reviewed the manuscript and plan the research. All authors read and approved the final manuscript.

Availability of Data and Materials: The data used and/or analysed during the current study are available from the corresponding author on reasonable request

Consent for Publication: Not applicable

Competing Interest: Authors have no competing interest to declare.

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Ethics Approval: Hacettepe University Medical Faculty Animal Research Laboratory, (Date: 25.07.2017, 2017/49-08)

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Tables:

Table 1
Radiographic grading scale for degree of healing (Cook et al.)

Description	Score
No change from immediate postoperative appearance	0
A slight increase in radiodensity distinguishable from the graft	1
Recognizable increase in radiodensity, bridging of one cortex with new bone formation from the graft	2
Bridging of at least one cortex with material of non uniform radiodensity, early incorporation of th graft suggested by obscurity of graft borders	3
Defect bridged on both medial and lateral sides with bone of uniform radiodensity, cut ends of the cortex still visible, graft and new bone not easy to differentiate	4
Same as grade 3, with at least one of four cotices obscured by new bone	5
Defect bridged by uniform new bone, cut ends of cortex no longer distinguishable, graft no longer visible	6

Table 2
Histological grading scale for the degree of healing (Salkeld et al.)

Criteria	Description	Score
Quality of union	No sign of fibrous or other union	0
	Fibrous union	1
	Fibrocartilagenous union or cartilage union	2
	Mineralizing cartilage and bone union	3
	Bone union	4
Cortex development and remodelling	No cortex formed	0
	Formation of new bone along exterior borders	1
	Recognizable formation of both the outer cortex border and the medullary space	2
	Cortices formed but incomplete bridging	3
	Complete formation of cortices with bridging of defect	4
Bone-graft incorporation and new bone formation		
Decreasing graft, increasing new bone	No new bone, all or most of graft visible	0
	Graft present, some incorporation with new bone formation and small amount of new bone	1
	Graft present, some incorporation with new bone formation and moderate amount of new bone	2
	Graft present, some incorporation with new bone formation continuous with host bone and early remodelling changes in new bone	3
No graft visible, extensive new bone	Decreased amount of graft (compared with grade 3) good incorporation of graft and new bone with host and ample new bone	4
	Less amount of graft still visible (compared with grade 4), good incorporation of graft and new bone with host and ample new bone	5
	Difficult to differentiate graft from new bone, excellent incorporation and advanced remodelling of new bone with graft and host	6

Table 3
Radiological assessment scoring of groups according to Cook criteria

Group	N	Median	Minimum	Maximum	P < 0.05
Control	12	1.0	0	1	
Omentum	12	1.5	1	3	
Omentum + Mesenchymal stem cell	12	1.0	0	1	
Omentum + PRP	12	1.5	1	3	
Omentum + Mesenchymal stem cell + PRP	12	1.0	0	3	

* Data are given according to median (minimum-maximum)

Table 4
Histopathological assessment scoring of groups according to Salkeld et al.

	Quality of union	Cortical Development and Remodelling	New Bone Formation	Total Score	P 0.01
Control	2 (1-2)	0 (0-0)	1 (0-1)	3 (1-3)	
Omentum	3 (2-3)	3 (2-3)	3 (3-3)	9 (7-9)	
Omentum + Mesenchymal stem cell	1.5 (1-2)	0 (0-1)	1 (1-2)	2.5 (2-5)	
Omentum + PRP	3 (2-3)	2.5 (2-3)	3.5 (2-4)	8.5 (6-10)	
Omentum + Mesenchymal stem cell + PRP	2 (2-3)	2 (0-3)	2 (1-3)	6.5 (3-9)	

* Data are given according to median (minimum-maximum)

Figures

Figure 1

Surgical procedure. a) Bone defect b) Decellularized omentum scaffold with mesenchymal stem cells c) Bone defect treated with scaffold

Figure 2

Radiologic samples of the groups. a) Control group b) Omentum group c) Omentum and Mesenchymal stem cell group d) Omentum and PRP group e) Omentum and Mesenchymal stem cell and PRP group

Figure 3

Histopathologic sample of each group. (a) control group sample 4x magnification, defect still seen with new bone islands and fat tissue. (b) omentum group sample 4x magnification, defect fully loaded with new cells and scaffold was organized (c) omentum and mesenchymal stem cell sample 4x magnification, scaffold still seen with blue color with new formed fat cells within scaffold and minimal calcification d) omentum and PRP group sample 4x magnification, with shrinkage of defect size with full

organized scaffold, with bone islands e) omentum and mesenchymal stem cell and PRP group 4x magnification, increased vascular structures and fat tissue, also increased bone islands and calcification

Figure 4

Graphic of Salkeld scoring system