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EFFECT OF MESENCHYMAL STEM CELL AND ERYTHROPOIETIN COMBINATION IN A RAT SPINAL FUSION MODEL

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ABSTRACT

Objective: The benefits of erythropoietin (EPO) or mesenchymal stem cell (MSC) application during spinal fusion were discussed in the literature. Still, the effect of the combination may enhance favorable outcomes. This study compared the efficacy of MSCs and EPO treatments separately and together in a rat model.

Materials and Methods: This study was designed as an experimental, controlled animal study. The groups consisted of EPO or MSC application or both: PreS - EPO + MSC (EPO starting 24 h before the surgery) or PostS - EPO + MSC (EPO starting 72 h after the surgery) with control groups. Experimental posterolateral L4-L5 spinal fusion was performed. Plain radiographs and multi-detector computed tomography scans were performed for the rats preoperatively and at the 3rd and 6th weeks. Using the Mimics Innovation Suite, 3D models of the fusion site were reconstructed, volume analysis and volumetric changes in these periods were calculated. Manuel palpation assessment and histopathological analyses were also performed to assess the fusion.

Results: Radiologically, the fusion rate at weeks 3 and 6 were significantly higher in the EPO + MSC groups than that in the EPO group alone. The highest bone-volume increase was detected in the PostS - EPO + MSC groups. The PreS - EPO + MSC-3 group and the PostS - EPO + MSC-6 group had the highest fusion rates according to manual palpation (p=0.048, 71.4%). The EPO groups had lower fusion rates compared to those in the control and MSC groups (14.3 % both at the 3rd and 6th weeks). The PreS - EPO + MSC-3 group had the highest histological score among the groups. The EPO-6 and PostS - EPO + MSC-6 groups had the lowest scores with respect to histological examination.

Conclusion: The combination of EPO + MSC application showed additionally significant benefits according to radiological and histological examination, but EPO adversely affected the fusion.

Keywords: Sprague-Dawley rat, spinal fusion, erythropoietin, mesenchymal stem cell, MDCT, Mimics Innovation Suite

INTRODUCTION

Spinal fusion or spinal arthrodesis is a widely accepted and preferred surgical treatment for various spinal diseases. The risk of pseudarthrosis or nonunion is reported to be as high as 30% after spinal fusion, which is most likely caused by the procedure or approach used and patient-related factors such as osteoporosis, health status, and comorbidities[1,2]. For achieving a stable spine segment, and to increase the rate of complete fusion, autogenous bone grafts are held to be the gold standard, but alternative treatments have been explored due to the limited amount of grafts and donor site morbidity[3-5]. Mesenchymal stem cells (MSCs) have the potential to differentiate into osteoblasts and chondrocytes, so it was proposed as a reasonable option for utilization in spinal fusion[6,7]. In various studies, MSCs has been reported to increase the success in posterolateral spinal fusion with different scaffolds[8-17]. While there is a degree of consensus with respect to the benefits of MSCs, the therapeutic expectation did not occur as completely successful[2,18].

Erythropoietin (EPO) was formerly used in spinal surgery to reduce perioperative blood transfusion as a blood conservation therapy[19,20]. Rölfing et al.[21] first showed a significant enhancing effect of EPO on bone volume in a rodent spinal fusion model. Current reviews support the role of exogenous EPO during the signaling in bone remodeling and repair *in vivo* with increased osteogenesis, osteoclastogenesis, and angiogenesis. Controversially, the stimulatory effect of EPO on osteoclastogenesis and the stimulation of bone-resorbing activity *in vitro* (concentrations >100 mU/mL) are also widely accepted[22,23].

Simultaneous or sequential application of different growth factors has been considered to deliver the synergistic effect

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in spinal fusion. There is no experimental study (*in vitro* or *in vivo*) examining the effects of the combination of MSCs and EPO on the spinal fusion model. This study aimed to compare the mechanical, radiological and histopathological efficacy of MSCs and EPO treatments separately and together in a rat spinal fusion model.

MATERIAL AND METHODS

Study Design and Animals

This study was designed as an experimental, controlled animal study. Before the study, approval was obtained from the institutional review board (Gülhane Military Medical Academy Animal Care and Use Committee, approval no: 2013/26, date: 22.11.2022). Animal care complied with the guidelines of the institution and was conducted following the Animal Research: Reporting of *In Vivo* Experiments and Guide for the Care and Use of Laboratory Animals guidelines. The studies were conducted at the same Institution's Laboratory Animals Section of the Research and Development Center. Seventy three female Sprague-Dawley rats (18-20 weeks of age) weighing 253.2±32 g were included in the study. Three rats were used for MSC production and the remaining 70 rats were randomly divided into 10 groups. Study groups were briefly designed as "only EPO application", "only MSC application", "EPO and MSC application" and "control" groups. EPO and MSC application groups were further divided into "preoperative 24-hour" and "postoperative 72-hour" sub-groups concerning the starting time of EPO administration to assess the possible anti-inflammatory effects of EPO on fusion. Other groups were divided into subgroups in the 3rd and 6th weeks according to the time of sacrification of the rats. The interventions applied and respective groups are set out in Table 1.

Allogenic Bone Marrow-Derived Mesenchymal Stem Cell Generation

Two biologists experienced in stem cell research generated the MSC using the technique of Nevruz et al.[24]. After sacrificing three rats with high-dose anesthetics, the tibia and femora of the rats were excised and bone marrow was aspirated from the medullary canal with an 18-gauge needle, collected in a centrifuge tube and diluted 1:2 with phosphate-buffered saline (PBS). In another centrifuge tube, 1:3 of the bone marrow volume was placed in Ficoll solution and the diluted bone marrow was added with a sterile pipette, layered, and then centrifuged at 1800 rpm for 30 minutes at room temperature. After centrifugation, MSCs in the middle layer were transferred to a new tube. Collected MSCs were centrifuged with 5 times the volume of PBS at least 2 times, at 1800 rpm for 5 minutes to remove the Ficoll. The cell pellet obtained at the end of the procedure was collected in a 25 cm^2 flask containing a medium consisting of 10% fetal calf serum, 6% 100 U/mL penicillin and 100 µg/mL streptomycin, and 1% L-glutamine and cultured at 37° C under 5% CO_2 pressure. Medium changes were made every 3 days. In the 7th-10th days, colonies began to form. On the 14th day, when 70% of the culture flask was covered, the cells were removed by trypsinization and placed in a 75 cm^2 flask for the 1st passage. After the 3rd passage, the cells were ready for use. To show that the passaged cells were MSCs, surface markers of CD45 (-), CD34 (-), HLA-DR (-), CD73 (+), CD90 (+), CD105 (+) were analyzed by flow cytometry.

Anesthesia Protocol

All radiological studies and surgical procedures were performed under general anesthesia. 10 mg/kg xylazine hydrochloride (Alfazyne 2%, Alfasan International B.V., Woerden, Netherlands) and 50 mg/kg ketamine hydrochloride (Brema-Ketamine 10%, Bremer Pharma, Germany) were used intraperitoneally. When necessary, 5 mg/kg xylazine hydrochloride and 10 mg/kg ketamine hydrochloride were used for maintenance.

Surgical Procedure

Experimental posterolateral L4-L5 lumbar spinal fusion was performed as previously described[25,26]. Prophylactic intraperitoneal cefazolin was administered at a dose of 22 mg/

Table 1. Characteristics of study groups

Group number	Group label	Group design
1	CT-3	Surgery, daily IP saline administration, sacrification at 3rd week
2	CT-6	Surgery, daily IP saline administration and sacrification at 6th week
3	EPO-3	Daily IP EPO starting 24 hours prior to surgery- surgery, sacrification at 3rd week
4	EPO-6	Daily IP EPO starting 24 hours prior to surgery- surgery, sacrification at 6th week
5	MSC-3	Surgery, MSC local application, IP saline, sacrification at 3rd week
6	MSC-6	Surgery, MSC local application, IP saline, sacrification at 6th week
7	PreS - EPO + MSC-3	Daily IP EPO starting 24 hours prior to surgery, surgery, MSC, sacrification at 3rd week
8	PreS - EPO + MSC-6	Daily IP EPO starting 24 hours prior to surgery, surgery, MSC, sacrification at 6th week
9	PostS - EPO + MSC-3	Surgery, MSC, daily IP EPO starting 72 hours after surgery, sacrification at 3rd week
10	PostS - EPO + MSC-6	Surgery, MSC, daily IP EPO starting 72 hours after surgery, sacrification at 6th week

IP: Intraperitoneal, Saline: 0.9% NaCl, equal to the volume of 500 IU/kg EPO dosage.
MSC application: Local application at decortication site, without osteoblastic differentiation and scaffold usage, approximately 1 million cells.
MSC: Mesenchymal stem cell, EPO: Erythropoietin, CT: Control

kg 30 minutes before the surgical incision. After the anesthesia, the lumbar region of the rats was shaved and placed in the prone position. The surgical area was cleaned with Octenisept solution (Schülke & Mayr GmbH, Norderstedt, Germany) and covered in a sterile manner. An approximately 4 cm midline skin incision between L4 and S1 was made and the lumbar fascia was opened using the Wiltse approach (Figure 1a, b, c). The paraspinal muscles were dissected laterally by blunt dissection (Figure 1d, e). The tendinous insertions of the longissimus lumborum muscles attached to the facet joints were released, revealing the L4 and L5 transverse processes (Figure 1f). The soft tissues on the transverse processes and facet joints were cleaned (Figure 1g, h). After irrigation of the surgical area with saline, the area was dried and the transverse processes, L4-L5 facet joints, laminae, and lateral surfaces of the spinous processes were decorticated using a burr at 10,000-15,000 rpm until punctate micro-hemorrhages were observed (Figure 1i, j). No further irrigation was made to preserve the bony fragments exposed during decortication. No significant bleeding was observed during the surgical procedure. In the MSC application groups, a suspension containing approximately one million MSCs, as Minamide et al.[27] suggested in their study, was applied to the decorticated area (Figure 1k) without using any scaffold. The lumbar fascia and the skin were closed (Figure 1l). In order not to trigger cannibalism, which is frequently seen in rats in the postoperative period, blood and tissue residues were cleaned from the incision area with saline. Antiseptic-disinfectant spray (Viocid®, Antiseptic Solution, Topical Spray, Provet®, Istanbul, Turkey) was applied to the incision area after the procedure.

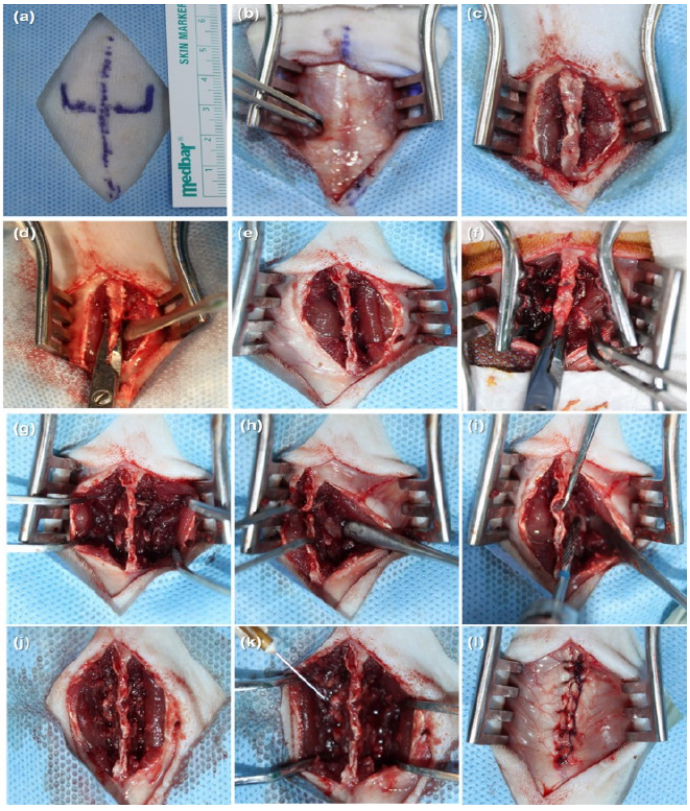

Figure 1. Posterolateral lumbar spinal fusion surgery for Sprague-Dawley rats, step-by-step procedure: Midline skin incision between L4 and S1 **(a)**. Note that marked iliac crests (caudal part of the rat) are at the top of the picture. Exposure of the lumbar fascia before **(b)** and after **(c)** the Wiltse approach. Blunt dissection **(d)** and retraction **(e)** of the paraspinal muscles. Exposure of the facet joint **(f)**. Cleaning off the soft tissues on the facet joint **(g)** and exposure of the transverse process **(h)**. Decortication of the desired fusion site using a burr **(i, j)**. Application of the mesenchymal stem cell suspension to the decorticated area **(k)**. The closure of the lumbar fascia **(l)** and the skin

Erythropoietin Administration

The EPO dose was calculated for each rat separately according to their weight. EPO alfa (Eprex; 4000 IU/mL, Santa Farma, Turkey) at a dose of 500 IU/kg/day was used intraperitoneally, as per Garcia et al.[28] in their study. To evaluate the possible anti-inflammatory effects of EPO in the inflammatory phase, which is the first stage of bone healing, the MSC and EPO groups were divided into two sub-groups, in which the EPO application started at the preoperative 24th hour (PreS - EPO + MSC groups) or postoperative 72nd hour (PostS - EPO + MSC groups). The groups that did not receive EPO were given a daily injection of saline (0.9% NaCl) in a volume equivalent to the EPO dose.

Outcome Parameters

Direct radiography: Using a digital mammography device (Selenia® Hologic, Inc. USA), a posteroanterior radiograph of the lumbar spine was taken at a dose of 30 kV 160 mAs, with a distance of 30 cm between the rat and the tube surface. Direct radiograms of the entire spine were obtained before the surgery (week 0) and at the 3rd and 6th weeks after the surgery for the designated groups. Three independent blinded observers evaluated the radiographs. For the evaluation, the commonly used criteria of Lenke et al.[29] were modified for our study. Radiographic fusion findings at the L4-L5 levels were divided into 5 stages and scored (Table 2) (Figure 2).

Computed tomography and volumetric measurement: A multidetector computed tomography (MDCT) device (Toshiba

Table 2. Modified Lenke radiological evaluation criteria used in our study

Points	Fusion status	Explanation
1	Definitely not solid	Obvious bone resorption at transverse processes bilaterally
2	Definitely not solid	No obvious fusion mass or bone resorption
3	Probably not solid	Small, thin fusion masses bilaterally
4	Possibly solid	Unilateral large fusion mass with contralateral small fusion mass
5	Definitely solid	Solid big trabeculated bilateral fusion masses

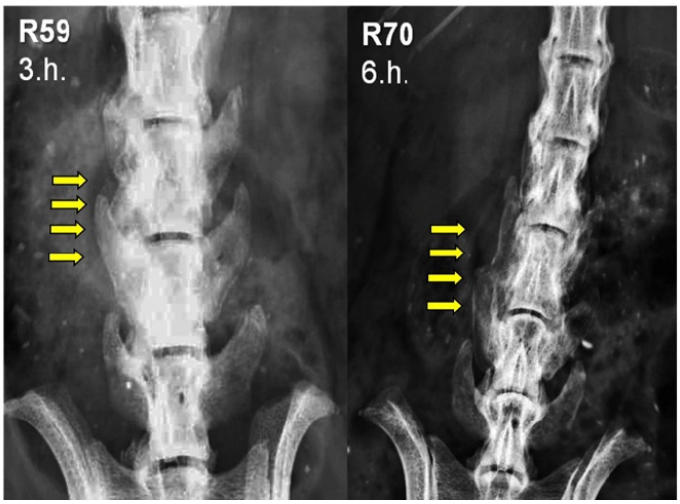

Figure 2. Direct radiography examples of two distinct rats in the third and sixth weeks that received 4 points based on our modified Lenke criteria. The new bone growth is denoted by yellow arrows

Aquilion One® 320-Detector Row CT, Toshiba Medical Systems, Tokyo, Japan) was used with a dose of 100 kVP, 200 mA for 500 milliseconds (0.5 mm slice thickness) for each rat (Figure 3). Digital Imaging and Communications in Medicine (DICOM) data were transferred to a biomedical engineering program, Mimics Innovation Suite® v16.0.0.235 (Materialise, Belgium), to reconstruct the L3-L6 segment. For all CT examinations performed at 0, 3, and 6 weeks, a 3D model was created and volume measurements were made from these models (Figures 4 and 5). The volumetric change between 0-3 weeks and 3-6 weeks was calculated for the groups.

Manual assessment of the fusion: After sacrification with high dose anesthetics at the end of the 3rd and 6th for the designated groups, the lumbar spines of the rats were en-bloc resected, and the soft tissues were stripped. Three independent blinded observers assessed the fusion site (L4-L5 segment) for intersegmental motion by using gentle movements in the coronal and sagittal planes. Any movement at any plane was considered non-fused. When all three observers agreed, the segment was considered completely fused.

Histologic evaluation: After the manual assessment, tissue samples were labeled and kept in a 10% formaldehyde solution (CH2O, MOS®, Moslab, Ankara, Turkey) for approximately 24 hours. Samples were then decalcified, the L3-L6 segments were prepared and the samples were placed in tissue-tracking cassettes. The cassettes were placed in an automatically closed system tissue-tracking device (Shandon®), dehydrated with alcohol and xylene series, and embedded in paraffin (Sasolwax®). Sections with a thickness of 4 micrometers were taken and deparaffinized by drying. Prepared slides were stained with hematoxylin-eosin in an automatic stainer (Sakura®, Tissue-Tek® Otostainer, DRSTM). Slides were evaluated by 2 independent observers using a standard light microscope (Olympus BX-51, Tokyo, Japan). The classification method defined by Emery and Murakami[30]. was used in the histopathological evaluation for assessment of the new bone formation at the L4-L5 level (Figure 6).

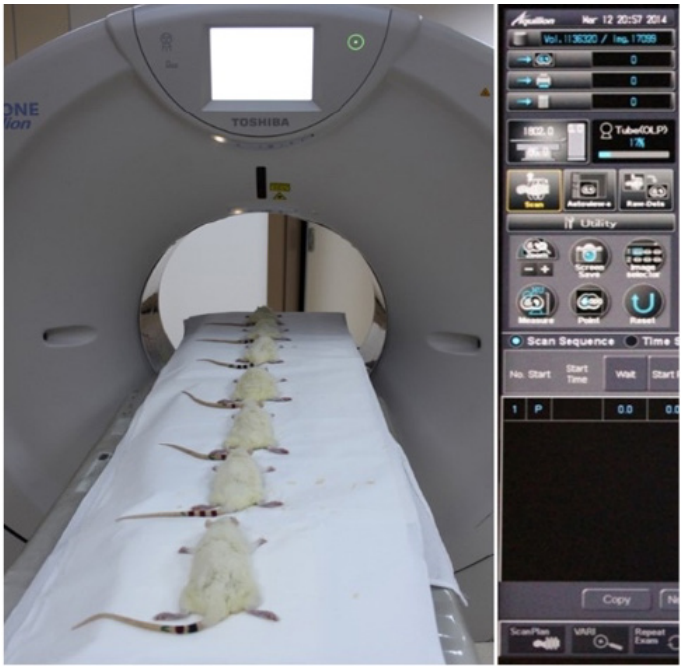

Figure 3. Utilizing a multi-detector computed tomography system to perform computed tomography scans for Sprague-Dawley rats

Statistical Analysis

SPSS 25.0 (IBM Corporation, Armonk, New York, United States) and PAST 3 (Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001. Paleontological Statistics) programs were used in the analysis of the variables. The conformity of univariate data to normal distribution was evaluated with the Shapiro-Wilk Francia test, while homogeneity of variance was evaluated with the Levene test. While the Mardia (Dornik and Hansen omnibus) test was used for the conformity of multivariate data to the normal distribution, the Box-M test was used for variance homogeneity. The one-way ANOVA (Robust test: Brown-Forsythe) test was used to compare the groups with each other according to quantitative variables, and the Tukey honestly significant difference and Games-Howell tests were used for post hoc analysis. Among the nonparametric tests, the Kruskal-Wallis H test, and Monte Carlo simulation results were used, and Dunn's Test was used for post hoc analysis. The Wilcoxon signed-rank test was tested using Monte Carlo simulation results to compare two replicate measures of quantitative dependent variables. The General Linear Model Repeated-Measures ANOVA test was used to examine the more than two repeated quantitative measurements of its variables and the interaction of these measurements according to the groups, while Fisher's Least Significant Difference was used for the post hoc test. Among non-parametric methods, Friedman's two-way test was tested using the Monte Carlo simulation method, while stepwise step-down comparison tests were used for the post hoc test. In the comparison of the groups according to the categorical variables, the Fisher-Freeman-Halton test was performed with the Monte Carlo simulation technique, and the comparison of the column ratios with each other was expressed with the Benjamini-

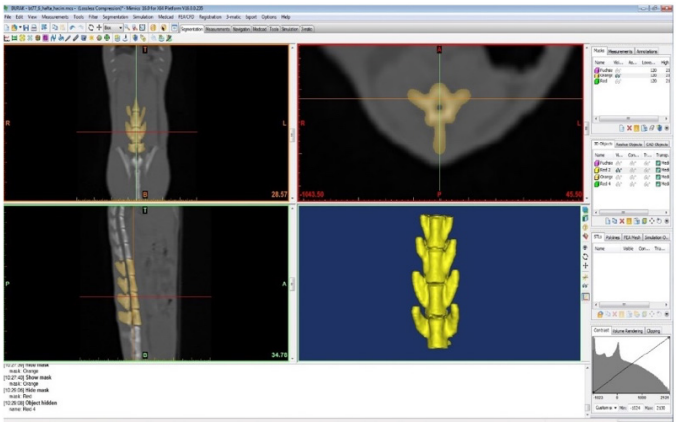

Figure 4. 3D reconstruction view of L3-L6 segment using the Mimics Innovation Suite®

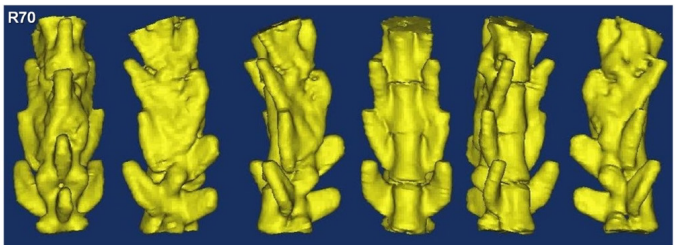

Figure 5. 3D model of a rat from MSC + EPO group in the sixth week. The model is rotated 180° and anterior, posterior, oblique and lateral images were obtained
EPO: Erythropoitein, MSC: Mesenchymal stem cell

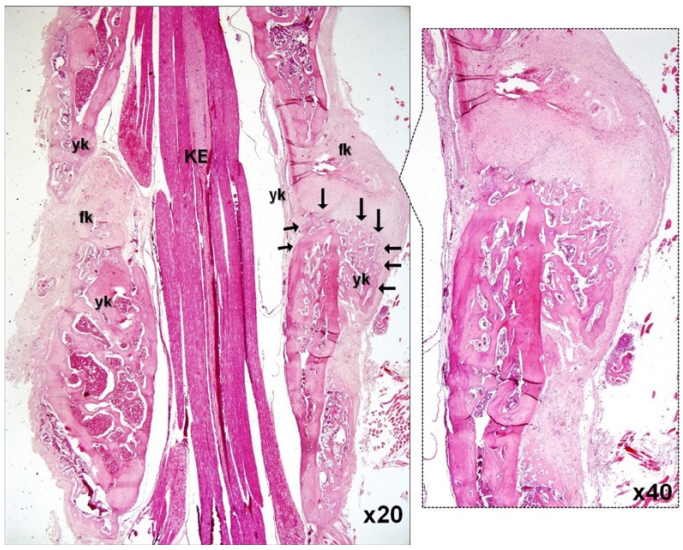

Figure 6. Histopathological specimen which received six points based on the Emery criteria. Note the new bone (yk) formation (black arrows) and a relatively small amount of fibrocartilage (fk) formation
KE: Cauda equina

Hochberg corrected p-value results. Quantitative variables were expressed as mean (standard deviation) and median (minimum/maximum) in the tables, while categorical variables were shown as n (%). The variables were analyzed at a 95% confidence level, and a p-value of less than 0.05 was considered significant.

RESULTS

Direct radiography: EPO + MSC groups had higher fusion rates than those in other groups at the 3rd and 6th weeks. In addition, the fusion rates at weeks 3 and 6 were significantly higher in the EPO + MSC groups than those in the EPO group alone (Table 3a). The highest increase in radiological scores was recorded in the PreS -EPO + MSC groups between the 0th and 3rd months (p=0.018), (Table 3b).

Computed tomography and volumetric measurement: While there was no significant increase in bone volume between 3 and 6 weeks in the EPO group, and between 0 and 3 weeks in the PreS - EPO + MSC groups, a significant increase in bone volume was detected in these intervals in the other fusion models followed for 6 weeks. In addition, the highest bone volume increase in the defined intervals was detected in the PostS - EPO + MSC groups (Table 4).

Manual palpation: The EPO groups had lower fusion rates compared to those in the control and MSC groups (14.3% at both the 3rd and 6th weeks). The PreS - EPO + MSC-3 groups and the PostS - EPO + MSC-6 groups had the highest fusion rates compared to those in the other groups (p=0.048, 71.4%), (Table 5).

Histopathology: The PreS - EPO + MSC-3 groups had the highest histological score among the groups (median=6). The EPO-6 and PostS - EPO + MSC-6 groups had the lowest score for histological examination with a median score of 3, although, there was no statistically significant difference between groups for bone healing (Table 5).

DISCUSSION

The main findings of the study showed that the highest increase in radiological scores was recorded in the PreS - EPO + MSC groups. In addition, the highest bone volume increase in the defined intervals was detected in the PostS - EPO + MSC groups. EPO groups had lower fusion rates compared to those in the control and MSC groups. The PreS - EPO + ESC-3 groups and the PostS - EPO + MSC-6 groups had the highest fusion rates with respect to manual palpation. In addition, the PreS - EPO + ESC-3 groups had the highest histological score among the groups. Briefly, intraperitoneal EPO application delayed the development of cartilage and bone tissue and adversely affected the fusion rates. MSC application alone increased fusion rates compared to the control and EPO groups. In addition, the combined application of MSC and EPO made a significant positive contribution to fusion rates compared to EPO or MSC applications alone. However, there is no significant difference between the applications of EPO at the preoperative 24 hours or the postoperative 72 hours.

EPO and EPO receptors were associated with the enhancement of osteogenic differentiation and mineralization in human and rodent bone marrow osteoblasts, especially in osteoblastic cell cultures with EPO doses between 10 and 100 U/mL[31,32], although, studies showed that EPO promoted *in vitro* osteoclastogenesis at

Table 3a. Radiological evaluation of fusion rates at different measurement points, in groups followed for 6 weeks with comparisons

	Radiography							Pairwise comparison for weeks		
	0. week	3rd week	6th week	Difference (0-3 weeks)	Difference (0-6 weeks)	Difference (3-6 weeks)	p-value			
	Med. (min/max)	Med. (min/max)	Med. (min/max)	Med. (min/max)	Med. (min/max)	Med. (min/max)		(0 vs 3)	(0 vs 6)	(3 vs 6)
2 CT-6	2 (2/2)	2 (1/3)	3 (1/3)	0 (-1/1)	0 (0/1)	1 (-1/1)	0.708[fr]	ns.	ns.	ns.
4 EPO-6	2 (2/2)	1 (1/2)	1 (1/3)	-1 (-1/0)	0 (0/1)	-1 (-1/1)	0.022[fr]	0.048	0.687	0.687
6 MSC-6	2 (2/2)	2 (1/4)	2 (2/3)	0 (-1/2)	0 (-1/1)	0 (0/1)	0.663[fr]	ns.	ns.	ns.
8 PreS - EPO + MSC-6	2 (2/2)	3 (3/3)	2.50 (2/3)	1 (1/1)	-0.50 (-1/0)	0.50 (0/1)	0.007[fr]	0.028	0.582	0.582
10 PostS - EPO + MSC-6	2 (2/2)	3 (3/3)	3 (2/4)	1 (1/1)	0 (-1/1)	1 (0/2)	0.003[fr]	0.023	0.098	0.999
P-value for groups	0.999[k]	0.005[k]	0.158[k]	0.005[k]	0.070[k]	0.158[k]				
2 vs 4	ns.	0.999	ns.	0.999	ns.	ns.				
2 vs 6	ns.	0.999	ns.	0.999	ns.	ns.				
2 vs 8	ns.	0.594	ns.	0.594	ns.	ns.				
2 vs 10	ns.	0.497	ns.	0.497	ns.	ns.				
4 vs 6	ns.	0.677	ns.	0.677	ns.	ns.				
4 vs 8	ns.	0.005	ns.	0.005	ns.	ns.				
4 vs 10	ns.	0.003	ns.	0.003	ns.	ns.				
6 vs 8	ns.	0.999	ns.	0.999	ns.	ns.				
6 vs 10	ns.	0.999	ns.	0.999	ns.	ns.				
8 vs 10	ns.	0.999	ns.	0.999	ns.	ns.				

[fr]Friedman test (Monte Carlo), Post hoc test: Stepwise step-down comparisons, [k]Kruskal Wallis test (Monte Carlo), Post hoc test: Dunn's test.
Med.: Median, min: Minimum, max: Maximum, vs: Versus, ns.: Not significant, MSC: Mesenchymal stem cell, EPO: Erythropoietin, CT: Control

Table 3b: Radiological evaluation of fusion rates at 0th and 3rd weeks in all groups with comparisons

	Radiography			
	0th week	3rd week	Difference (0-3 weeks)	p-value
	Median (min/max)	Median (min/max)	Median (min/max)	(0 vs 3) week
1 CT-3	2 (2/2)	1 (1/2)	-1 (-1/0)	0.061[w]
2 CT-6	2 (2/2)	2 (1/3)	0 (-1/1)	0.999[w]
3 EPO-3	2 (2/2)	1 (1/2)	-1 (-1/0)	0.033[w]
4 EPO-6	2 (2/2)	1 (1/2)	-1 (-1/0)	0.033[w]
5 MSC-3	2 (2/2)	3 (2/4)	1 (0/2)	0.034[w]
6 MSC-6	2 (2/2)	2 (1/4)	0 (-1/2)	0.441[w]
7 PreS - EPO + MSC-3	2 (2/2)	3.1 (3/4)	1.1 (1/2)	0.018[w]
8 PreS - EPO + MSC-6	2 (2/2)	3 (3/3)	1 (1/1)	0.017[w]
9 PostS - EPO + MSC-3	2 (2/2)	3 (2/4)	1 (0/2)	0.033[w]
10 PostS - EPO + MSC-6	2 (2/2)	3 (3/3)	1 (1/1)	0.017[w]
P value for groups	0.999[k]	<0.001	<0.001	
1 vs 7	ns.	0.025	0.025	
2 vs 10	ns.	0.012	0.012	
3 vs 5	ns.	0.011	0.011	
3 vs 6	ns.	0.031	0.031	
3 vs 7	ns.	0.033	0.033	
3 vs 8	ns.	0.012	0.012	
3 vs 10	ns.	0.011	0.011	
4 vs 5	ns.	0.031	0.031	
4 vs 6	ns.	0.033	0.033	
6 vs 10	ns.	0.031	0.031	
7 vs 8	ns.	0.031	0.031	
All other pairwise comparison	ns.	ns.	ns.	

[k]Kruskal Wallis test (Monte Carlo), Post hoc test: Dunn's test, [w]Wilcoxon signed rank test (Monte Carlo).
min: Minimum, max: Maximum, vs: Versus, ns.: Not significant, MSC: Mesenchymal stem cell, EPO: Erythropoietin, CT: Control

Table 4. Final bone volume evaluation at different measurement points with group comparisons

	MDCT; bone volume						p-value	Pairwise comparison for weeks		
				Difference						
	0. week	3rd week	6th week	(0-3 weeks)	(0-6 weeks)	(3-6 weeks)		(0 vs 3)	(0 vs 6)	(3 vs 6)
P (volume*groups)=0.009[g]	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)				
2 CT-6	1281.4 (137.6)	1396.0 (161.8)	1425.8 (158.3)	114.7 (37.5)	144.5 (37.7)	29.8 (22.0)	0.001[g]	<0.001	<0.001	0.012
4 EPO-6	1376.3 (248.8)	1441.8 (267.0)	1477.4 (275.3)	65.5 (34.3)	101.1 (67.7)	35.6 (42.9)	0.015[g]	0.002	0.008	0.071
6 MSC-6	1414.7 (135.8)	1503.8 (122.3)	1551.0 (139.5)	89.1 (40.9)	136.4 (21.0)	47.3 (28.5)	<0.001[g]	0.003	<0.001	0.010
8 PreS - EPO + MSC-6	1560.8 (231.2)	1688.6 (343.0)	1754.7 (348.4)	127.9 (127.1)	193.9 (132.3)	66.0 (24.6)	0.020[g]	0.057	0.016	0.001
10 PostS - EPO + MSC-6	1299.2 (117.9)	1459.9 (107.7)	1551.0 (121.0)	160.7 (56.7)	251.8 (75.9)	91.1 (29.9)	<0.001[g]	<0.001	<0.001	<0.001
P-value for groups	0.086[a]	0.212[a]	0.157[a]	0.187[a]	0.028[a]	0.007[a]				
2 vs 4	ns.	ns.	ns.	ns.	0.597	0.996				
2 vs 6	ns.	ns.	ns.	ns.	0.987	0.843				
2 vs 8	ns.	ns.	ns.	ns.	0.892	0.240				
2 vs 10	ns.	ns.	ns.	ns.	0.052	0.007				
4 vs 6	ns.	ns.	ns.	ns.	0.696	0.959				
4 vs 8	ns.	ns.	ns.	ns.	0.564	0.404				
4 vs 10	ns.	ns.	ns.	ns.	0.015	0.017				
6 vs 8	ns.	ns.	ns.	ns.	0.823	0.826				
6 vs 10	ns.	ns.	ns.	ns.	0.035	0.105				
8 vs 10	ns.	ns.	ns.	ns.	0.871	0.593				

[g]General Linear Model Repeated ANOVA (Wilks' Lambda); Post hoc test: Fisher's least significant difference
[a]One-way ANOVA (Robuts Statistic: Brown-Forsythe), Post hoc test; Games Howell, Tukey HSD
SD: Standard deviation, vs: Versus, ns.: Not significant, MSC: Mesenchymal stem cell, EPO: Erythropoietin, MDCT: Multi-detector computed tomography, CT: Control, HSD: Honestly significant difference

Table 5. Final histopathological score and fusion rate according to manual palpation with group comparisons

	Rat weight (gr)	Histopathology score	Manual palpation	
			Nonunion	Complete fusion
	Mean (SD)	Median (min/max)	n (%)	n (%)
1 CT-3	244.66 (8.34)	5 (2/6)	7 (100)[B]	0 (0)
2 CT-6	240.71 (22.09)	5 (2/6)	4 (57.1)	3 (42.9)
3 EPO-3	247.83 (22.66)	5 (3/5)	6 (85.7)	1 (14.3)
4 EPO-6	245.84 (35.36)	3 (2/6)	6 (85.7)	1 (14.3)
5 MSC-3	254.10 (34.15)	5 (5/6)	3 (42.9)	4 (57.1)
6 MSC-6	253.29 (29.82)	5 (5/6)	4 (57.1)	3 (42.9)
7 PreS - EPO + MSC-3	279.69 (38.01)	6 (5/6)	2 (28.6)	5 (71.4)[A]
8 PreS - EPO + MSC-6	272.64 (42.45)	5 (2/6)	4 (57.1)	3 (42.9)
9 PostS - EPO + MSC-3	274.27 (29.32)	5 (2/6)	6 (85.7)	1 (14.3)
10 PostS - EPO + MSC-6	232.23 (17.75)	3 (2/6)	2 (28.6)	5 (71.4)[A]
p-value	0.062[a]	0.149[k]	0.038[f]	

[a]One-way ANOVA (Robuts Statistic: Brown-Forsythe), [k]Kruskal Wallis test(Monte Carlo), [f]Fisher Freeman Halton (Monte Carlo), Post hoc test: Benjamini-Hochberg correction
[A]Significant according to nonunion (manual palpation), [B]Significant according to complete fusion (manual palpation)
SD: Standard deviation, min: Minimum, max: Maximum, MSC: Mesenchymal stem cell, EPO: Erythropoietin, CT: Control

doses ranging from 5 to 20 U/mL or lower concentrations[23,31,32]. Recent reviews concluded that the EPO mechanisms producing beneficial effects on bone volume were unknown, and pointed to the different cell types with different responses to EPO during bone remodeling and repair, and concentrations of EPO[22,23]. Rölfing et al.[21] showed a significant increase in bone volume with subcutaneous injections of EPO compared to that in the control group in a rabbit posterolateral spinal fusion model. They also reported higher but not significant fusion rates in the EPO group, examined with MDCT, manual palpation, and X-ray, so they supported EPO as an autograft-enhancing factor[21]. Later, the same team reported that topical use of EPO with a collagen carrier significantly increased the median bone volume fraction by 1.06 compared to that in the control group in an animal study with adolescent pig's calvarial bone[33]. Omlor et al.[34] reported significantly increased bone formation and vascularization with local and systemic administration of EPO according to histomorphometric and radiological evaluation. In addition, they concluded that a direct local application of EPO (single dose) during surgery was sufficient to increase bone healing substantially[34]. Contrary to the majority of the current literature, in our study EPO has no additional benefit for bone volume compared to other groups, and had histologically lower fusion rates, although these were not significant. This supports both the osteogenic and osteolytic effects of EPO, which have been noted in systematic reviews previously.

Preclinical and clinical studies demonstrated that MSC improved successful spinal fusion with osteogenic and osteoinductive properties. The differing designs of existing studies, heterogeneous groups, the use of different animal models, various scaffolds, a combination of various growth factors, donor sites, and the harvesting and culturing mediums of MSC preclude the formation of a consensus for the development of a standard technique for MSC use[1,2]. Nakajima et al.[15] showed higher fusion rates in rabbit spines treated with MSC plus autograft compared to those in the control group. Minamide et al.[35] also reported increased fusion rates in the control group in rabbit models with bone marrow-derived MSC culture-supported growth factors, when compared with autograft. Additionally, adipose-derived MSC is beneficial with respect to fusion rates in both a rat and rabbit model of posterolateral fusion[11,36]. Current reviews indicate higher fusion rates of up to 100% with MSC application isolated from bone marrow harvested from the iliac crest or vertebral body intraoperatively and then transplanted[1,37]. However, due to the heterogeneity of the studies, valid comparisons cannot be made. Currently, randomized controlled studies are continuing with respect to MSC use and spinal fusion. In the present study, MSC administration achieved higher fusion rates and the combination of EPO + MSC application showed additionally significant benefits according to radiological and histological examination. The differentiation potential of MSC into osteoblasts may have been stimulated by EPO, and the results of this study support this hypothesis. The increased impact of MSC with BMP and the basic fibroblast growth factor has been demonstrated in an animal study. The stimulation of spinal fusion with various growth factors also continues to be explored and debated[38]. In spinal fusion, the current literature would indicate that it is possible to increase the success rate by combining different carrier elements with different biological agents. However, the presence of other factors, such as cost and patient selection, as well as treatment selection, will continue to be compelling factors for the establishment of standard approaches.

Study Limitations

The interaction of EPO with MSC treatment resulted in positive results at the macro evaluation, but the lack of an examination method such as flow cytometry and comparison with different growth factors are major limitations of the study.

CONCLUSION

MSC administration achieved a higher fusion rate and the combination of an EPO + MSC application showed further significant benefits according to radiological and histological examination. However, EPO confers no additional benefit for bone volume compared to other groups. The curative efficacy of MSC or EPO + MSC treatments in spinal fusion is confirmed by the literature and this study. However, the application of EPO alone has two-sided (benefit/harm) effects. On the other hand, the stimulation/direction of MSC with a growth factor such as EPO or the widely accepted BMP seems to be meaningful and more effective.

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Ethics

Ethics Committee Approval: This study was approved by Gülhane Military Medical Academy Animal Experiments Ethical Committee (decision no: 13/128, date no: 22.11.2013).
Informed consent: Experimental animal study.
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Authorship Contributions

Surgical and Medical Practices: A.B.B., Ç.N., Concept: A.B.B., Design: A.B.B., E.O., Data Collection or Processing: A.B.B., Ç.N., Y.E., Analysis or Interpretation: A.B.B., Y.E., Literature Search: A.B.B., Writing: A.B.B., Ö.E., E.O.
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