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Evaluation of the Effect of Granulocyte–Macrophage Colony Stimulating Factor on Spinal Fusion in a Rat Model of Spinal Surgery

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ABSTRACT

AIM: To evaluate the effects of granulocyte–macrophage colony stimulating factor (GM-CSF) on spinal fusion through manual palpation, radiological examinations, and histopathological analyses in a rat model.

MATERIAL and METHODS: A total of 21 rats were evaluated in this study. The rats were divided into the following three groups, each consisting of seven rats: preoperative GM-CSF, postoperative GM-CSF, and a control group. L4–L5 posterolateral fusion was performed in all three groups. The preoperative GM-CSF group received 5 µg/kg GM-CSF subcutaneously for 5 days in the preoperative period, while the postoperative GM-CSF group received the same intervention in the postoperative period. No additional postoperative procedures were performed in the control group. All rats were euthanized at 6 weeks, and the fusion site was evaluated using manual palpation, radiological examinations, and histopathological analyses.

RESULTS: According to the classification of subjects according to manual examination, preoperative and postoperative GM-CSF groups had significantly higher rates of "single prominent callus formation + fusion" ($p<0.05$). When direct radiography scores were evaluated, the number of subjects with "unilateral solid new bone density – contralateral nonsolid bone density" was significantly greater in the preoperative GM-CSF group, while "bilateral solid new bone densities" was more prevalent in the postoperative GM-CSF group ($p<0.05$). In regards to histopathological scores, the number of subjects rated as "fibrocartilage tissue is more than bone tissue" was higher in the preoperative GM-CSF group, the number of subjects rated as"bone tissue is more than fibrocartilage tissue" was higher in the postoperative GM-CSF group, and the number of subjects rated as "fibrous tissue is more than fibrocartilage tissue" was greaterin the control group ($p=0.01$). Preoperative and postoperative GM-CSF groups had significantly higher manual examination, radiological, and histopathological scores and greater volume of new bone formation on 3D CT compared to the control group ($p<0.05$).

CONCLUSION: The results of our study demonstrated that preoperative and postoperative administration of GM-CSF had positive effects on spinal fusion in a rat model.

KEYWORDS: Spinal fusion, Granulocyte–macrophage colony stimulating factor (GM-CSF), Rat model

INTRODUCTION

Spinal diseases have become a significant health issue within societies (17). While the prevalence varies based on culture, developmental, and educational factors, approximately 24%–80% (17,30) of individuals experience low back pain at some point in their lives. Surgical intervention becomes necessary for 13%–18% of these patients (6,9,11,30). A 236% increase in the rate of patients undergoing spinal surgery was observed between 1998 and 2008, with the cost

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of these surgical procedures amounting to approximately 34 billion dollars in the Unite State (30).

The most common reasons for spinal surgery include disc hernias resulting from degenerative processes, spinal stenosis, spondylolisthesis, and spinal deformities (9,11,30). Spinal surgery may also be necessary due to tumors, trauma, or infections (10,15,37). While there are various pathologies that may necessitate spinal surgery, its primary goal is to alleviatenerve compression and achieve biomechanical stability in the vertebral segment. The ultimate objective of achievinga stable segment is fusion of the surgical site (15). In recent years, anterior lumbar interbody fusion, posterior lumbar interbody fusion, transforaminal lumbar interbody fusion, and posterolateral fusion methods have been used to achieve 360 degree fusion (5,23,28). Current surgical techniques involve bone grafting using fixation systems such as screws, rods, hooks, wires, plates, scaffolds, and cages to achieve adequate bone healing and solid fusion (32).

Despite an increased understanding of fusion biology and advances in surgical implants and techniques that have resulted in a substantial increase in fusion rates, pseudoarthrosis remains a serious complication. Pseudoarthrosis is noted in 15%–40% of patients undergoing spinal surgery for fusion purposes (7,33).

There are studies in the literature indicating that granulocyte–macrophage colony stimulating factor (GM-CSF) has a positive effect on bone healing when used in pseudoarthrosis surgeries (8,19,27). We also hypothesized that GM-CSF could play an active role in spinal fusion. However, no study related to this subject was encountered in our literature review.

The aim of this study isto evaluate the efficacy of GM-CSF on spinal fusion in a rat model.

MATERIAL and METHODS

The study received approval from the Local Ethics Committee for Animal Experiments (University of Health Sciences, Fatih Sultan Mehmet Training and Research Hospital; Approval No.: 2021/47; Date: 05.08.2021). The study was conducted between November 2021 and February 2022. Twelve-week-old Sprague-Dawley male rats, weighing between 400–450g were used as experimental subjects.The sample size (n) was determined to be 7 in each group, and the minimum required number of subjects was 21. The effect size of the study was calculated to be 0.32, with 0.10, 0.25, and 0.40 being considered as small, medium, and large effect sizes, respectively.

The subjects were randomly divided into the following three groups, each consisting of seven rats, as follows: preoperative GM-CSF, postoperative GM-CSF, and the control group. Fusion was planned between the L4-L5 levels for all three groups.The preoperative GM-CSF group received 5 µg/kg (Neupogen, Amgen) GM-CSF subcutaneously for 5 days in the preoperative period, the postoperative GM-CSF group received the same intervention in the postoperative period, and the control group was not administered any additional medication. The doses of GM-CSF applied to the subjects were planned according to prior studies.

Surgical Procedure

The surgery was performed as described in previous studies on rat spinal fusion (26,31). General anesthesia (combination of 10mg/kg xylazine hydroxychloride and 50mg/kg ketamine hydroxychloride) and preoperative antibiotic prophylaxis (10mg/kg cefazolin sodium) were administered to all subjects. The L4–L5 level was identified by manually palpating the bone landmarks (31). Proper surgical preparation, including shaving the surgical site, sterilization, and draping, was performed. A longitudinal intermedian incision was made, extending 1.5 cm proximal and 1.5 cm distal to the previously determined level. After penetrating the subcutaneous tissues, the paravertebral muscle fascia was accessed. A 1.5 cm fasciotomy was performed approximately 0.5 cm lateral to the spinous processes on both sides of the identified level. Paravertebral structures were dissected. The L4–L5 facet joint and transverse and spinous processes were accessed and bilateral decortication was performed until punctate hemorrhages occurred (Figure 1). A micromotor (Saeshin Precision co. Ltd – Strong 90) operating at 8000 rpm was used for the procedure. After the decortication procedure, no grafting or scaffolding was applied. At the end of the procedure, the fascia was closed bilaterally with 4.0 Vicryl suture, and the skin was closed using a stapler. After 10% povidone iodine was applied as dressing, the wounds were left open for follow-up while the rats were placed in their cages.

Postoperative Follow-up

Wound dressing was performed using 10% povidone iodine solution daily in the first week and twice weekly in the second week. Paracetamol (Parol /Atabay) at a dose of 200 mg/kg was added to the rats' drinking water for pain relief. The sutures were not removed to avoid any damage to the fusion area and eliminate the need for additional general anesthesia. During this period, one rat in the control group developed superficial wound infection at day 5. The wound healed with routine dressing without the need for additional debridement. No deaths occurred during the follow-up period. Euthanization of the rats was planned at the end of 6 weeks.

Euthanization

Subjects were administered 150 mg/kg ketamine hydroxychloride 30 minutes prior to euthanization. Drug efficacy was assessed by monitoring heart rate and respiratory rate. Decapitation was not performed to avoid damaging the fusion site. After confirming the death of the subjects, a new incision was made by extending the previous surgical incision site proximally and distally. The paravertebral muscle groups were carefully dissected from the L2 level to the pelvis and the segment between these levels was removed as a whole (en-bloc) (Figure 2). Radiological images were obtained from the samples immediately before they were placed in a 10% formaldehyde solution for histological analysis.

Evaluation parameters

Manual palpation: Manual evaluation of the excised segment was performed according to the scoring system used by Azar (3). During the evaluation, extension, flexion, and lateral flexion

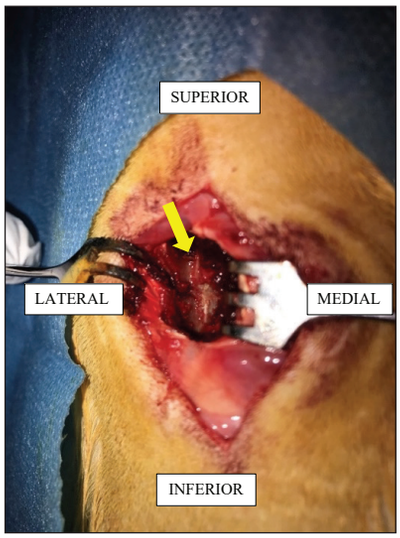

Figure 1: Facet joints and spinous and transverse processes after decortication.

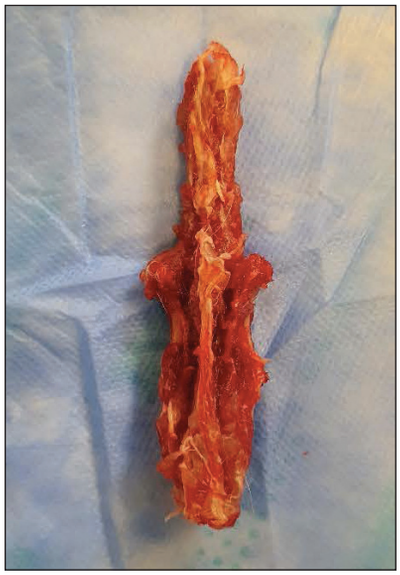

Figure 2: Bone block obtained after sacrification.

were performed on both sides. The surgically treated segment was compared with adjacent segments. Care was taken to avoid damage to the fusion areas during the procedure. The evaluations were performed by a blinded orthopedic specialist.

Radiological evaluation: Standard lumbar anterior–posterior (AP) radiographs were acquired usinga single-tube digital X-ray device (DR-RAD brand, X3C model) at a dose of 45 kVp and 5.5 mA for 23 milliseconds. The distance between the tube and the cassette was 90 cm and the subjects were in direct contact with the cassette as images were being acquired. The radiological fusion scoring system described by Lenke et al. was used for evaluation (25). The evaluations were performed by a blinded orthopedic specialist.

Axial, coronal, and sagittal cross-sectional computed tomography (CT) images were acquired using a flash CT scanner (Siemens Somatom Definition FlashCT device operated at a dose of 100 kvP, 200 mA following soft tissue protocols), and the images were converted tothree-dimensional (3D) images. CT assessment was performedas described by Park et al. (29). The volume of new bone tissue (cm^3) between L4–L5 was calculated using the 3D images. During measurement, the endings of the new bone tissue were used as the reference. In subjects with differingnew bone tissue formation on both sides, the measurement was made by considering the side with the most abundant bone tissue (Figure 3).

Histopathological Analysis: After the decalcification process, 5-µm-thick sections were obtained from the tissue samples and stained with Hematoxylin & Eosin. The sections were examined by a histologistusing a light microscope (Leica DM 6000) and photographed with the help of the Leica Application Suite program. Scoring was based on the classification method described by Emery et al. (14).

Statistical Analysis

Statistical analysis of the data was conducted usng the SPSS (Statistical Package for the Social Sciences) 25.0 software package program. Descriptive statistics included frequency,

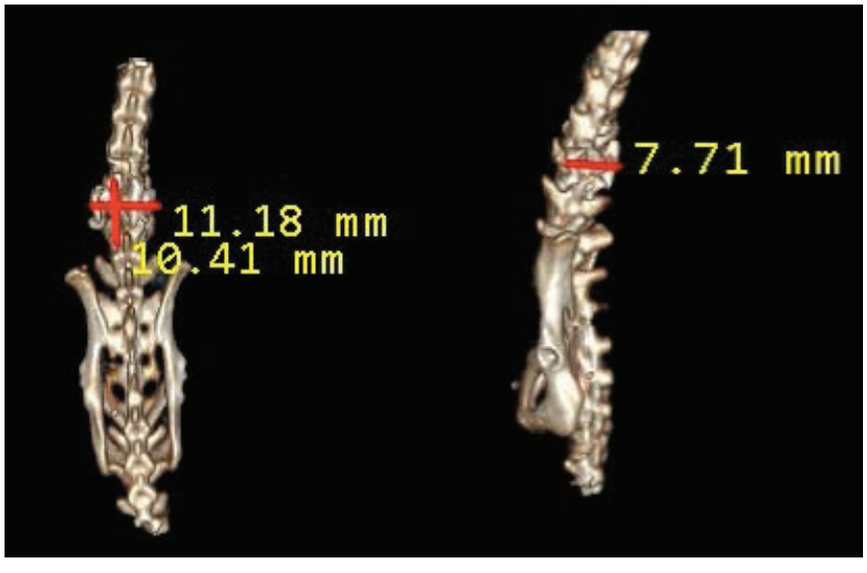

Figure 3: Measurement of new bone tissue volume in the coronal and sagittal planes via 3D reconsruction from CT images?

percentage, mean, and standard deviation were calculated. Kappa and intraclass coefficient (ICC) correlation tests were conductedto examine the level of agreement between assessment scores of two different evaluators. Chi-square test was used to investigate whether there were significant differences between the groups according to manual palpation,histopathological, and radiologicalassessments. Kruskall Wallis test was used to assess differences between the groups in regards to CT assessments and histopathological, radiological, and manual palpation scores. Mann Whitney U test was conducted to determine which groups contributed to the difference. Spearman correlation analysis was conducted to investigate the association between CT assessments, histopathological, radiological, and manual palpation scores. P values less than 0.05 were considered statistically significant.

RESULTS

Manual Examination

The classification of subjects according to the manual examination resultsis presented in Table I, and the mean scores of the groups are presented in Table II. Based on the results of classification of subjects according to manual examination, the number of subjects with"*single prominent callus formation + fusion*" was significantly higher in the preoperative and postoperative GM-CSF groups, while the number of subjects with "*minimal and moderate callus formation*" was higher in the control group, and this difference was statistically significant (p=0.04; Table I). When the groups were compared according to the manual examination scores, it was determined that the scores were lower in the control group than in the preoperative and postoperative GM-CSF groups. Furthermore, the manual examination scores were lower in the preoperative GM-CSF group than in the postoperative GM-CSF group (p<0.05).

The level of agreement between Evaluator 1 and Evaluator 2 was found to be generally high in the evaluation of control, preoperative GM-CSF, and postoperative GM-CSF groups (K=0.69, K=0.64, K=0.65).

Radiological Imaging

The distribution of subjects classified according to new bone tissue formation, which was assessed using direct radiographs, is presented in Table III, while mean scores are presented in Table II. The number of subjects with "*unilateral*

Table I: Classification of Subjects According to Manual Examination and Comparison Between Groups

	Manual examination classification categories				p
	There was no callus formation. Segment was mobile in all directions n (%)	**Minimal callus formation – Segment was mobile in all directions n (%)**	**Moderate callus formation – segment was partially mobile n (%)**	**Prominent callus formation + fusion n (%)**	
Preoperative GM-CSF group	0 (0.0)	2 (28.6)	2 (28.6)	3 (42.9)	
Postoperative GM-CSF group	0 (0.0)	1 (14.3)	2 (28.6)	4 (57.1)	**0.04***
Control group	1 (14.3)	3 (42.9)	3 (42.9)	0 (0.0)	

Significant difference at a p level of <0.05.* ***GM-CSF:* *Granulocyte–macrophage colony stimulating factor.*

Table II: Distribution of New Bone Tissue Formation Scores Based on Direct Radiographic Evaluation Across the Groups and Comparison Between Groups

		Radiological evaluation score				p
		No bilateral new bone density	**New bone densities that are not bilaterally solid**	**Unilateral solid new bone density**	**Bilateral solid new bone densities**	
Preoperative GM-CSF group	n	0	2	4	1	
	%	0.0	28.6	57.1	14.3	
Postoperative GM-CSF group	n	0	1	3	3	
	%	0.0	14.3	42.9	42.9	**0.03***
Control group	n	1	4	2	0	
	%	14.3	57.1	28.6	0.0	

Significant difference at a p level of <0.05.* ***GM-CSF:* *Granulocyte–macrophage colony stimulating factor.*

solid new bone density – contralateral nonsolid bone density" was significantly higher in the preoperative GM-CSF group, and the number of subjects with "*bilateralsolid new bone densities*" was significantly higher in the postoperative GM-CSF group (p=0.03; Figure 4). When the groups were compared according to their radiological scores, it was observed that the scores of the control group were significantly lower than those of the preoperative and postoperative GM-CSF groups. In addition, the radiological evaluation scores of the preoperative GM-CSF group were lower than those of the postoperative GM-CSF group (p<0.05).The agreement between Evaluator1 and Evaluator2 was generally found to be high in the evaluation of control, preoperative GM-CSF, and postoperative GM-CSF groups (K=0.74, K=0.62, K=0.68).

The meanvolume of new bone in the groups as measured using 3D CT is shown in Table IV. The volume of new bone tissue was found to be higher in the preoperative and postoperative GM-CSF groups than in the control group (p=0.01), but no significant differencewas observed between the preoperative GM-CSF and postoperative GM-CSF groups (p>0.05; Table IV, Figure 5).

The agreement between Evaluator 1 and Evaluator 2 was found to be high in the evaluation of control, preoperative GM-CSF, and postoperative GM-CSF groups (r_{ICC} = 0.78, r_{ICC} = 0.84, and r_{ICC} = 0.81, respectively, p=0.01).

Histopathological findings

The distribution of the groups according to their histopatho-

Table III: Evaluation of Scores According to Groups

Measurements	Group name			p	Difference
	Control	Preoperative GM-CSF	Postoperative GM-CSF		
	X ± ss	X ± ss	X ± ss		
Histopathological examination score	2.57 ± 0.79	4.57 ± 1.13	5.29 ± 1.11	**0.03***	1<2 = 3
Radiological evaluation score	2.14 ± 0.69	2.86 ± 0.69	3.29 ± 0.76	**0.01***	1<2<3
Manual examination score	1.29 ± 0.76	2.14 ± 0.9	2.43 ± 0.79	**0.01***	1<2<3

**Significant difference at a p level of <0.05.*

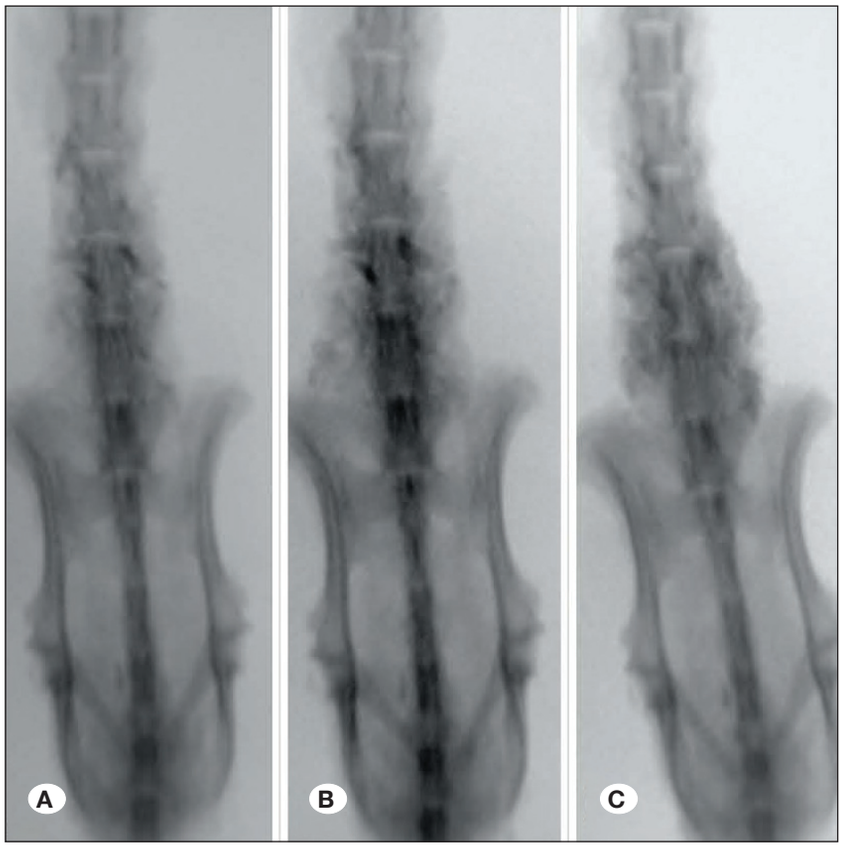

Figure 4: Samples of subjects with new bone density as evaluated via direct radiography **A)** Absence ofbilateral new bone density in a subject in the control group **B)** Formation of unilateral solid new bone density in a subject in the postoperative GM-CSF group **C)** Formation of bilateral solid new bone density in a subject in the preoperative GM-CSF group.

logical evaluation scores is presented in Table V, and the scores of the groups are presented in Table II. In the comparison between groups, it was found that the number of subjects rated as "*fibrocartilage tissue is more than bone tissue*" was higher in the preoperative GM-CSF group, the number of subjects rated as"*bone tissue is more than fibrocartilage tissue*" was higher in the postoperative GM-CSF group, and the number of subjects rated as "*fibrous tissue is more than fibrocartilage tissue*" was greaterin the control group (p=0.01; Table V, Figure 6). When the groups were compared according to their histopathological scores, it was determined that the scores were lower in the control group than in the preoperative GM-CSF and postoperative GM-CSF groups (p<0.05; Table V).

Correlation of Scores According to Groups: A strong positive correlation was observed between volume of new bone tissue formation observed in CT, and histopathological, direct radiography, and manual examination scores in the preoperative GM-CSF and postoperative GM-CSF groups (p= 0.01). In the control group, there was no significant correlation between volumes of new bone tissue formationobserved in CTand histopathological, direct radiography, and manual examination scores (p>0.05; Table VI).

DISCUSSION

The results of our present study demonstrate that preoperative and postoperative administration of GM-CSF had a positive effect on spinal fusion in rats undergoing spinal surgery.

Table IV: Volume of New Bone Tissue Across the Groups

Measurement	Control Mean ± SD	Preoperative GM-CSF Mean ± SD	Postoperative GM-CSF Mean ± SD	p	Difference
Volume of new bone tissue as measured using CT	0.63 ± 0.06	0.72 ± 0.08	0.76 ± 0.09	**0.01***	1<2 = 3

Significant difference at a p level of <0.05.* ***GM-CSF:* *Granulocyte–macrophage colony stimulating factor.*

Table V: Evaluation of Histopathological Examination Scores Across Groups

	Fibrous tissue is more than fibrocartilage tissue n (%)	Fibrocartilage tissue is more than fibrous tissue n (%)	Only fibrocartilage tissue n (%)	Fibrocartilage tissue is more than bone tissue n (%)	Bone tissue is more than fibrocartilage tissue n (%)	p
Preoperative GM-CSF group	0 (0.0)	2 (28.6)	0 (0.0)	4 (57.1)	1 (14.3)	**0.01***
Postoperative GM-CSF group	0 (0.0)	1 (14.3)	0 (0.0)	2 (28.6)	4 (57.1)	
Control group	4 (57.1)	2 (28.6)	1 (14.3)	0 (0.0)	0 (0.0)	

Significant difference at a p level of <0.05.* ***GM-CSF:* *Granulocyte–macrophage colony stimulating factor.*

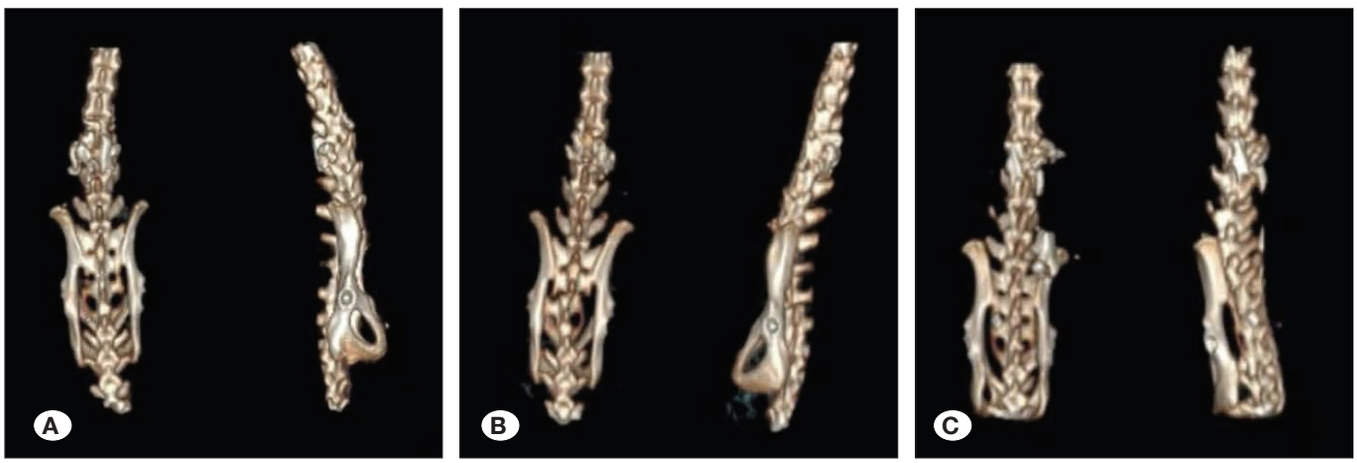

Figure 5: Samples of subjects undergoing the measurement of new bone volume using 3D CT **A)** Preoperative GM-GSF group, **B)** Control group, **C)** Postoperative GM-CSF group.

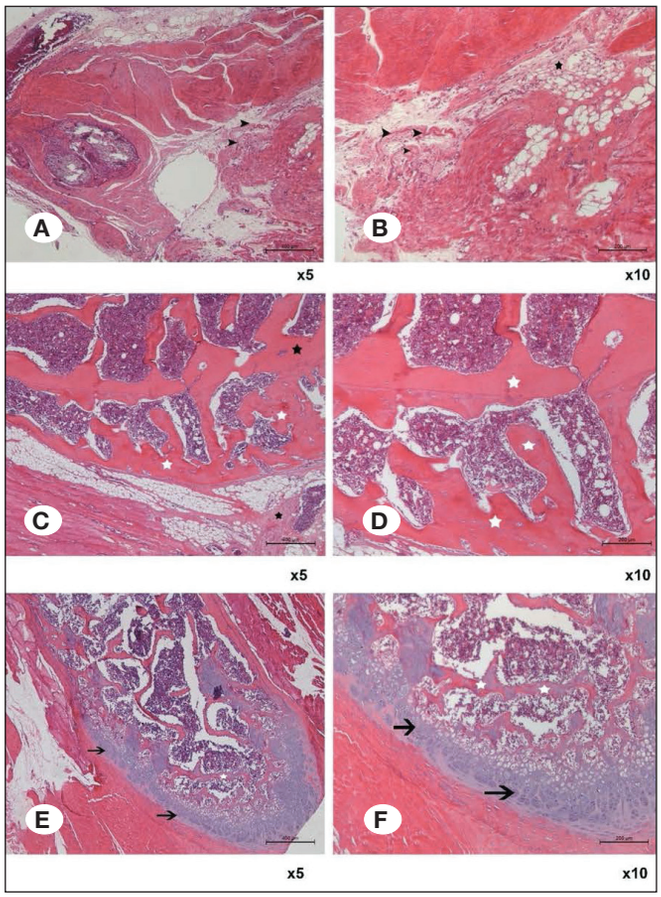

Figure 6: Histopathological images of samples of subjects in all three groups evaluated via Hematoxylin–Eosin staining. Images were taken at 5× and 10× magnification. The scale bar is 400 µm and 200 µm. **A-B)** Images of the control group, and collagen fibers are indicated using black arrowheads while connective tissueis indicated usingblack stars. Newly formed collagen fibers and connective tissue can be evidently seen in the images. **C-D)** The white star represents the bony areas and the black star represents the connective tissue areas. The bone tissue was widespread. **E-F)** Histopathological image of sample of a subject in the preoperative GM-CSF group, as evaluated via Hematoxylin–Eosin staining. The black arrows represent the endochondral ossification areas and the white stars represent the newly formed bone areas.

The main goal of the surgical treatment of spinal disorders is primarily focused on relieving nerve compression and achieving a biomechanically stable spinal segment. Fusion surgery remains the recognized gold standard, particularly in degenerative spinal disorders (24,30). Successful fusion rates in spinal surgeries range from 56% to 100% (2,12,21). There are essentially two surgical approaches in spinal fusion procedures. The anterior approach is associated with higher rates of comorbidities, complications, and extended hospital stays, yet it boasts a higher success rate compared to the posterior approach (13). In our study, we opted for the posterior approach, which has a lower complication rate and increased preference by surgeons in recent years.

Pseudoarthrosis is one of the most common causes of revisions following spinal surgeries and is a significant complication. A US-based study reported that revision spinal surgery costs ranged from $24,000–$64,000 per individual (16). In an analysis of 144 patients undergoing spinal fusion surgery, Kim et al. reported a 24% pseudoarthrosis rate (21). There are recent studies in the literature evaluating biological agents that can be used to increase the fusion rate (1,4,20,34). Our study aimed to investigate the efficacy of administering GM-CSF, which we hypothesized could influence the success of spinal fusion.

Recent rat studies have examined pharmacological agents that are speculatedto contribute to spinal fusion. The evaluation parameters used in these studies closely resemble those used in the present study. However, these studies failed to identify an agent that significantly impacts the success of spinal fusion (18,22,35). In our research, we observed a positive impact of GM-CSF on spinal fusion.

Through our comprehensive literature review, we encountered numerous studies exploring the impact of biological agents, with a focus on mesenchymal stem cells, on spinal fusion. In a meta-analysis by Sandhu et al., it was reported that bone morphogenetic protein (BMP)-2 and BMP-7 alongside autogenous bone grafting contribute positively to spinal fusion, potentially enhancing its efficacy. Moreover, biological agents affecting bone metabolism couldfacilitate fusion in minimally invasive spinal surgeries (32). Bhamb et al.demonstrated that elevated serum vitamin D levels accelerate spinal fusion (4). Additionally, Yusupov et al. identified a positive correlation between vitamin D and GM-CSF levels in a prospective human study investigating the effect of vitamin D on serum cytokine levels (38). In a meta-analysis of 19 preclinical and 17 clinical studies, Stephan et al. revealed the positive effects of mesenchymal stem cells when used in conjunction with osteoconductive substances although their isolated use lacked efficacy, calling for more in-depth investigations (34). Furthermore, Kim et al. investigated the effects of subcutaneous GM-CSF injectionson mesenchymal stem cell mobilization from bone marrow to peripheral bloodin rats and observed an increase in peripheral blood mononuclear cells and fibroblasts from day 1 (20). This leads us tospeculate that the positive effect of GM-CSF on spinal fusion in our study may be attributed to its effect on mesenchymal stem cell release.

Existing literature on GM-CSF's influence on bone metabolism is relatively scarce, and no studies specifically investigating its effects on spinal fusion have been identified. Subasi et al. investigated the effects of osteomyelitis and GM-CSF on healing in rats, suggesting that GM-CSF enhances new bone formation and might bolster immune resistance against infections (36). On the other hand, Kaygusuz et al. reported that subcutaneous administration of GM-CSF had a positive effect on fracture healing in rats with tibial shaft fractures (19). The results of our study are consistent with those reported in the literature.

Our study also encompasses certain limitations. First and foremost, our study is an experimental animal study. We

Table VI: Relationship Between Examination Scores Across the Groups

Group name	Score		Histopathological examination	Radiological examination	Manual examination
Control	Histopathological examination	r	1	-	-
		p	-	-	-
	Direct radiography	r	0.75	1	-
		p	0.05	-	-
	Manual examination	r	0.52	0.73	1
		p	0.23	0.42	
	New bone tissue volumes on CT	r	0.52	0.73	0.73
		p	0.23	0.42	0.42
Preoperative GM-CSF	Histopathological examination	r	1	-	-
		p	-		-
	Direct radiography	r	0.974*	1	-
		p	**0.001**		-
	Manual examination	r	0.887*	0.844*	-
		p	**0.001**	**0.001**	-
	New bone tissue volumes on CT	r	0.848*	0.887*	0.911*
		p	**0.001**	**0.001**	**0.001**
Postoperative GM-CSF	Histopathological examination	r	1	-	-
		p	-	-	-
	Direct radiography	r	0.878*	1	-
		p	**0.001**	-	-
	Manual examination	r	0.979*	0.881*	1
		p	**0.001**	**0.001**	-
	New bone tissue volumes on CT	r	0.932*	0.892*	0.881*
		p	**0.001**	**0.001**	**0.001**

Significant correlation at a p level of <0.05.* ***GM-CSF:* *Granulocyte–macrophage colony stimulating factor.*

opted for the rat model due to the availability of numerous rat models related to spinal fusion, the establishment of precise GM-CSF dosages, their infection resistance, and the availability of defined models for surgical procedures (18,32,35,38). Furthermore, the absence of long-term follow-ups is noteworthy; although the processes of remodeling, resorption, and new bone formation sequentially occur and extend for approximately 6 months to 1 year in humans, this period is only 6 weeks in rats. Accordingly,rats were euthanized at 6 weeks (38). Moreoever, the high cost of GM-CSF can also be considered as a limiting factor.

CONCLUSION

Our study investigated the effects of preoperative and postoperative GM-CSF administration in rats undergoing spinal fusion surgery. The results demonstrated that GM-CSF had a positive impact on spinal fusion. These findings suggest that GM-CSF could serve as a promising biological agent for enhancing spinal fusion in cases requiring absolute fusion.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Ethics approval and consent to participate

The study was approved by the Acıbadem University Animal Experiments Local Ethics Committee (number: 2021/47; date: 05.08.2021).

AUTHORSHIP CONTRIBUTION

Study conception and design: MFA, AOA

Data collection: AOA, MFA, EB

Analysis and interpretation of results: AOA, BEK, MFA

Draft manuscript preparation: MFA, EB, BEK

Critical revision of the article: MFA, EB, BEK, AOA

All authors (MFA, EB, BEK, AOA) reviewed the results and approved the final version of the manuscript.

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