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Research Article

Effects of vertebral fusion on levels of pro-inflammatory and catabolic mediators in a rabbit model of intervertebral disc degeneration

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ABSTRACT

Objective: The aim of this study was to explore the alterations in levels of pro-inflammatory and catabolic mediators following vertebral fusion in a rabbit model of intervertebral disc degeneration.

Methods: In this study, 24 female New Zealand albino rabbits (aged 4 to 5 months and weighing 3 to 3.5 kg) were used. All the animals were randomly categorized into four groups, and dorsal spinal exposure of all lumbar vertebrae was routinely performed in each group. While disc degeneration was created in groups B, C, and D, spinal fusion was added to disc degeneration in groups C and D. Disc degeneration was typically created by puncturing the discs with an 18-gauge needle under the guidance of C-arm imaging. Fusion was achieved with posterior/posterolateral decortication and iliac bone grafts. The rabbits in groups A, B, and C were euthanized, and the discs were removed in the first week after the surgery. The rabbits in Group D were sacrificed, and the discs were harvested at 5 weeks after the surgery. The levels of Interleukin (IL)-1β, IL-6, Nitric Oxide (NO), Matrix Metalloproteinase (MMP)-3, MMP-13, and Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) in the discs were analyzed using enzyme-linked immunosorbent assay kits.

Results: Significant increase was observed in the protein levels of both pro-inflammatory and catabolic mediators in disc degeneration groups (Group B, C, and D) compared to Group A. In the fusion groups (Group C and D), these increased mediators decreased, compared to non-fusion group (Group B), (IL1-β $P = 0.017$, TIMP-1 $P = 0.03$, NO $P = 0.03$). However, there was no statistically significant difference in mediator levels between the short- and long-term fusion (Group C versus D).

Conclusion: The results of this study have shown that a significant decrease in pro-inflammatory and catabolic mediators may be expected after vertebral fusion whereas there may be no significant difference between the first and fourth week of fusion surgery. These findings may contribute to clarifying the mechanism of action of vertebral fusion in the treatment of low back pain.

Introduction

Low back pain is quite prevalent in the population and has negative effects on the economy and welfare by substantially limiting the functional capacity.[1] It is known that Intervertebral Disc Degeneration (IVDD), one of the major causes of low back pain, is the consequence of a series of biochemical and morphological changes, resulting in alterations of biomechanical characteristics of movement segment. Numerous studies have been conducted to define these biochemical changes and relevant treatment. It is emphasized that pro-inflammatory mediators, including leukotrienes, prostaglandins, Tumor Necrosis Factor-α (TNF-α), and Nitric Oxide (NO) have critical roles in low back pain mechanisms in IVDD.[2-6] Identifying these mediators and related factors have been the topic of many studies.[2-8] These studies were performed either as experimental animal researches[3,6,7] or using the samples obtained from human discs.[2,4,5,8,9] Moreover, studies focusing on the regulation of these mediators and accordingly controlling or reducing disc degeneration were also conducted.[9-11]

Surgical treatment is usually indicated in cases of low back pain with IVDD refractory to conservative therapy. Vertebral fusion with a posterior or anterior approach is among the various methods used in the surgical treatment of low back pain in IVDD. However, the action mechanism of vertebral fusion on pain-related inflammatory/catabolic mediators is not fully understood, and there is no study focusing on the biochemical changes after fusion.[12]

The posterolateral spinal fusion animal model is a successfully used model, which is first introduced by Boden[13] and later presented in a meta-analysis by Riordan et al.[14] We hypothesized that induced intervertebral disc degeneration in an animal model would cause an increase in pro-inflammatory and catabolic mediators in intervertebral discs, and posterior vertebral fusion would reduce these mediator levels. Therefore, the objective of this study was to determine the alterations of pro-inflammatory and catabolic mediators in degenerated intervertebral discs after posterior vertebral fusion. For this purpose, we selected a rabbit model of IVDD due to their high degree of homology

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to the human intervertebral disc in comparison to the other rodent models.[9]

Materials and Methods

Animal studies

This study was carried out in the Laboratory of Experimental Animals after obtaining the approval of the Local Ethics Committee for Animal Experiments. The study was performed in New Zealand albino female rabbits under standard laboratory conditions consisting of conventional 12-h day-night cycle, 16–22 °C room temperature, 50%–60% relative humidity, and 5–15 air change per hour. The rabbits were waited in the area of use for 1 week for inter-room adaptation, kept in standard cages in doubles, and fed with ad-libitum 3 mm pellet and fresh tap water over the study course. Twenty-four rabbits (4–5 months old and 3–3.5 kg) were randomly divided into four groups as follows:

Group A (Sham) (*n* = 6): Dorsal surgery procedure with no disc injury or fusion.

Group B (*n* = 5): Dorsal surgery with disc injury but no fusion.

Group C (*n* = 6): Dorsal surgery with disc injury and vertebral fusion.

Group D (*n* = 5): Dorsal surgery with disc injury and vertebral fusion (Table 1).

Procedures

Posterior spinal exposure of all lumbar vertebras down to spinous processes, lamina, and transverse processes under anesthesia was performed in all groups (Groups A, B, C, and D).

Spinal exposures were the same in length for each animal. Disc degeneration was established by percutaneous annular puncture technique using an 18-gauge angiography needle under c-arm guidance (Figure 1)[9] in Groups B, C, and D. Each rabbit was anesthetized by xylazine (5 mg/kg) and ketamine (35 mg/kg) administered via intramuscular route, and hair was shaved from the mid-back. Following the anesthesia, the rabbits were placed in a lateral oblique prone position. An alcohol sponge was used to sterilize the surgical area. Initially, the L5–L6 discs were identified by manual palpation of the interspinous space from the mid-back and iliac crest using fluoroscopy. After confirming the exact level, an 18G angiography needle was inserted into the disc space through 3–4 cm ventral aspect of the midline under fluoroscopic guidance. In each rabbit, L4–L5, L5–L6, and L6–L7 IVDs were punctured. Special care was taken to minimize damaging the periosteal tissues of the vertebrae as this could cause premature uncontrolled anterior fusion.

In Groups C and D, decortications of posterior elements were also performed for spinal fusion (Figure 2). Bone autografts of 1–1.5 cm^3, harvested from posterior iliac crest from the same posterior incision, were put over the decorticated area. Fusion was assessed both by inspection and by manual palpation. In the postoperative period, Pethidine (Meperidine) (Aldolan, Liba) was administered for analgesia at a dose of 5–10 mg/kg via a subcutaneous route at necessary intervals. Subjects in each group received first-generation cephalosporin (cefazolin sodium) at a dose of 50 mg/kg/day via intramuscular route both before surgery and for 2 days after surgery for the prophylaxis for infection. It was observed that the rabbits in Groups C and D (grafting groups) moved minimally or not at all until sacrifice.

The rabbits in Groups A, B, and C were euthanized, and the discs were removed in the first week after the surgery. The rabbits in Group D were sacrificed, and the discs were harvested at 5 weeks after the surgery to evaluate the effects of fusion.

Each rabbit was sacrificed using Pentothal (100 mg/kg) via IV route. The discs were obtained by removing the lower three vertebrae of each rabbit and stored at −80°C until the study (Thermo Electron Corporation; Beverly, USA).

Exclusion criteria were (i) the earlier than expected death of the rabbit, (ii) the detection of a vertebral or disc space infection during the sacrifice which may alter fusion or mediator levels, (iii) surgical complication, (iv) paraplegia, (v) decreased food and water intake, (vi) weight loss of 20% or more, and (vii) total protein concentration lower than 1 mg/mL.

Tissue processing and ELISA

One-hundred milligrams of intervertebral discs were disrupted in 1mL of ice-cold RIPA buffer with protease/phosphatase inhibitors (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1 mM PMSF, 2 μg/mL Aprotinin, 1 μg/mL Leupeptin, 1 μg/mL Pepstatin, and 10 mM NaF) using Tissue Lyser II homogenizer (Qiagen; Hilden, Germany) until the tissue was completely homogenized. Total protein concentrations of homogenates were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific; Rockford, USA) and total protein concentration over than 1 mg/mL indicated the high homogenization efficiency. NO, TIMP-1, MMP-3, MMP-13, IL-6, and IL-1β protein levels were measured in duplicate by ELISA kits, according to the protocol recommended by the manufacturer (Bioassay Technology Laboratory; Shanghai, China). Each data were normalized to protein concentration (1 mg/mL).

Statistical analysis

The sample size was determined on the basis of preliminary experiments to be at least four rabbits per group to detect

Table 1. Experimental Design of Rabbit Model

	Dorsal spinal exposure	IVDD with needle puncture	Posterolat grafting fusion w/Iliac autograft	Euthanized
Group A (*n* = 6)	+	None	None	1st week
Group B (*n* = 5)	+	+	None	1st week
Group C (*n* = 6)	+	+	+	1st week
Group D (*n* = 5)	+	+	+	5th week

HIGHLIGHTS

- Both pro-inflammatory (IL-1β, IL-6, and NO) and catabolic (MMP-3, MMP-13, and TIMP-1) mediator protein levels were significantly elevated in degenerated intervertebral discs.
- Protein levels of pro-inflammatory and catabolic mediators decreased after vertebral fusion in degenerated discs, however, there was no statistical significance between short-term and long-term effects of fusion
- These results indicate that vertebral fusion has significant effects on relieving lower back pain.

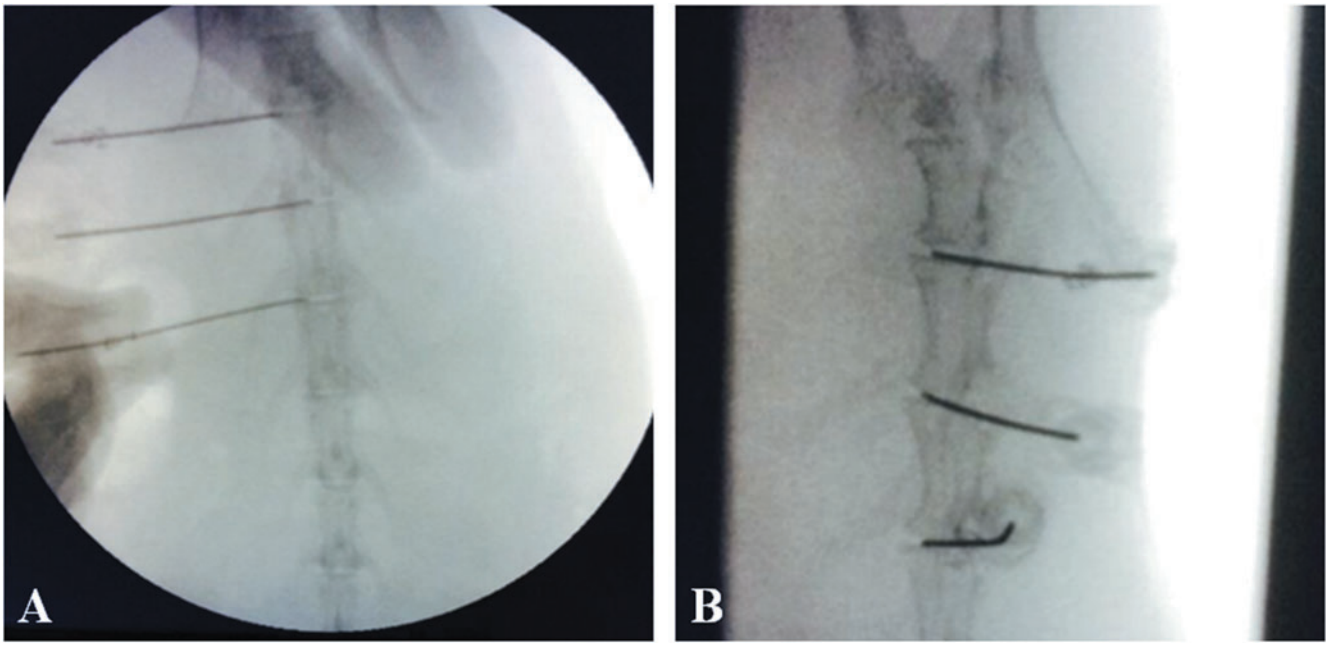

Figure 1. A, B. Percutaneous needle puncture into the rabbit discs under a fluoroscope. After localization of the exact levels of L4–L5, L5–L6, and L6–L7 using the fluoroscope, 18 G angiography needles are inserted into the discs under monitoring.

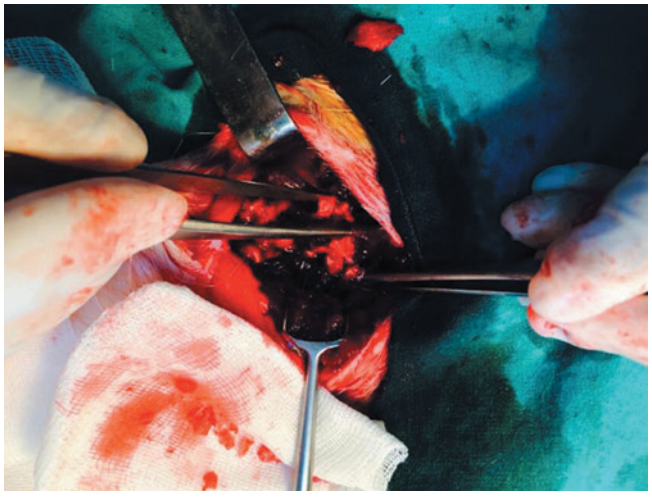

Figure 2. Autografts taken from posterior iliac crest (1–1.5 cm^3).

a difference between groups (given a 95% confidence level and 80% power) using "Openepi Version 3.01" software. Statistical analyses were performed by SPSS 15.0 software, and the graphs were generated with GraphPad Prism 8.0 software. The results were presented as mean ± SEM (Standard Error of the Mean). Kruskal Wallis Variance Analysis with the Bonferroni correction was used for the comparison between the groups and Mann Whitney *U*-Test for the subgroup comparison. The level of significance was determined to be $P < 0.05$.

Results

In this study, we analyzed the protein levels of pro-inflammatory (IL-6, IL-1β, and NO) and catabolic (MMP-13, MMP-3, and TIMP-2) mediators in rabbit intervertebral discs to evaluate the effect of posterior vertebral fusion on these mediators in IVDD. The mediator levels obtained from the rabbits in Groups A, B, C, and D are represented in Table 2. Two subjects from Groups B and D were excluded from the study due to low total protein concentrations. As shown in Figures 3 and 4, a significant elevation in IL-6 ($P = 0.004$), IL-1β

Table 2. The Protein Levels of Pro-inflammatory and Catabolic Mediators Obtained from the Intervertebral Discs of Rabbits of each Group

Rabbits		IL-1β (pg/mg protein)	IL-6 (pg/mg protein)	NO (nmol/mg protein)	MMP-3 (ng/mg protein)	MMP-13 (ng/mg protein)	TIMP-1 (pg/mg protein)
Group A	1	2.5	24.5	3.2	1.42	0.71	553.0
	2	2.4	19.5	3.0	1.10	0.68	424.7
	3	1.8	14.3	2.2	0.84	0.53	301.9
	4	1.3	7.1	1.7	0.58	0.38	186.4
	5	1.3	15.3	2.6	0.96	0.65	270.6
	6	2.2	17.1	2.1	0.88	0.50	268.7
Group B	1	4.6	34.2	5.3	2.21	1.07	639.1
	2	5.3	37.3	5.3	2.22	1.14	1047.2
	3	13.5	101.6	26.9	7.42	4.19	3703.4
	4	13.5	95.0	15.3	6.15	3.32	2239.8
	5	8.6	102.4	13.3	4.53	3.08	1905.5
Group C	1	6.6	82.6	17.8	6.81	4.11	2225.5
	2	2.8	20.7	2.8	1.28	0.63	435.2
	3	3.0	40.5	3.9	1.94	0.92	563.4
	4	2.8	15.6	2.5	1.43	0.61	391.0
	5	3.5	27.2	3.5	1.89	0.74	565.4
	6	3.3	25.5	3.1	1.76	0.88	507.9
Group D	1	6.2	49.8	5.4	2.99	1.55	990.8
	2	4.3	34.4	4.9	2.33	1.26	577.7
	3	2.2	19.8	2.1	1.44	0.50	349.0
	4	4.5	37.4	3.8	2.56	0.95	876.4
	5	2.5	19.1	2.5	1.14	0.62	328.9

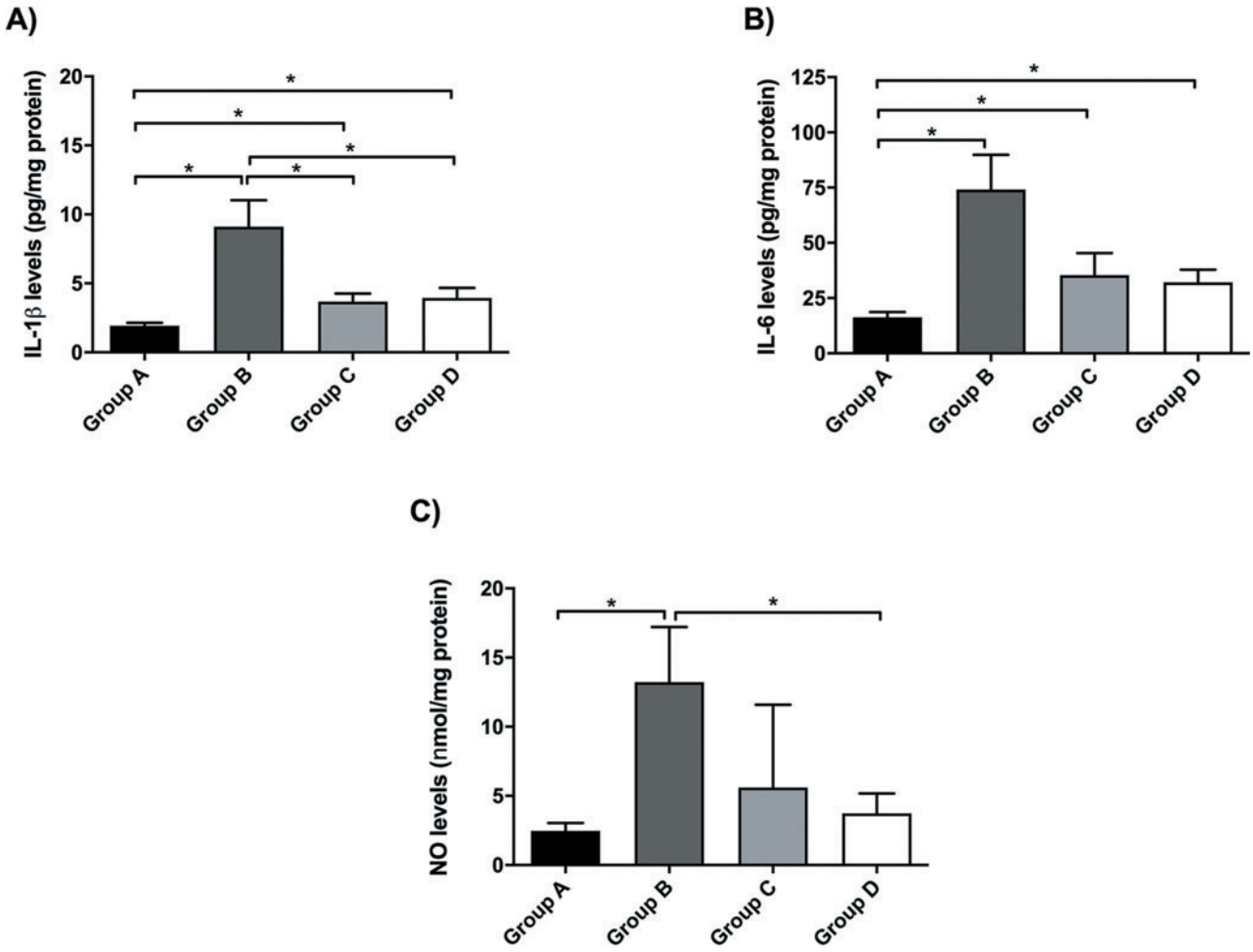

Figure 3. A-C. (A) IL-1β, (B) IL-6, and (C) NO protein levels in IVD of each group. Data are presented as mean ± SEM and $*P \leq 0.05$ is statistically significant.

($P = 0.004$), NO ($P = 0.004$), MMP-13 ($P = 0.004$), MMP-3 ($P = 0.004$), and TIMP-1 ($P = 0.004$) levels were determined in disc degenerated group (Group B) in comparison to Sham (Group A). After posterior vertebral fusion (Group C), IL-1β ($P = 0.017$) and TIMP-1 ($P = 0.030$) levels revealed significant decrease according to Group B. Also, IL-6 ($P = 0.052$), MMP-13 ($P = 0.052$), MMP-3 ($P = 0.052$), and NO ($P = 0.052$) levels were dramatically decreased following posterior vertebral fusion, but these results were not statistically significant. Four weeks later, after posterior fusion (Group D), IL-1β ($P = 0.032$), NO ($P = 0.032$), and TIMP-1 ($P = 0.032$) levels were significantly reduced compared to Group B. In Group D, IL-6 ($P = 0.151$), MMP-13 ($P = 0.095$), and MMP-3 ($P = 0.222$) levels were also decreased; however, no statistical difference was found between Groups B and D.

We also compared the mediator levels between Group A and spinal fusion performed groups (Groups C and D) to evaluate short-term and long-term outcomes after fusion. IL-1β ($P = 0.002$), IL-6 ($P = 0.026$), MMP-3 ($P = 0.004$), and TIMP-1 ($P = 0.026$) levels were significantly higher in Group C, whereas MMP-13 ($P = 0.065$) and NO ($P = 0.065$) levels were also increased but these increments are not significant. Compared to Group A, in Group D, we detected increased levels of IL-1β ($P = 0.003$), IL-6 ($P = 0.03$), MMP-3 ($P = 0.009$), and TIMP-1 ($P = 0.052$). However, we recorded no significant difference in MMP-13 ($P = 0.177$) and NO ($P = 0.247$) levels between these two groups. In addition, no significant changes in mediator levels were observed between Groups C and D.

Discussion

Intervertebral disc degeneration is a multifactorial and chronic disease that is associated with alterations in structural and biochemical characteristics of the extracellular matrix and increased disc cell apoptosis.[11] Numerous studies have been conducted to explain the mechanism of pain in IVDD; for example, Burke et al. demonstrated that IL-6 and IL-8 levels were elevated in the degenerated human.[2] Besides, Kang et al. investigated a total of 18 lumbar disc samples from 15 patients after undergoing discectomy for lumbar disc hernia and determined significantly increased IL-6 and NO levels as compared to the control group.[4] When the disc samples from patients who underwent discectomy for discogenic pain were compared with those from individuals with no disc/spinal pathology causing pain, significantly higher IL-1β levels were found in patients with discogenic pain.[15] As these studies were all carried out in patients with various etiologies of IVD, we designed an experimental animal model to increase understanding in this area.

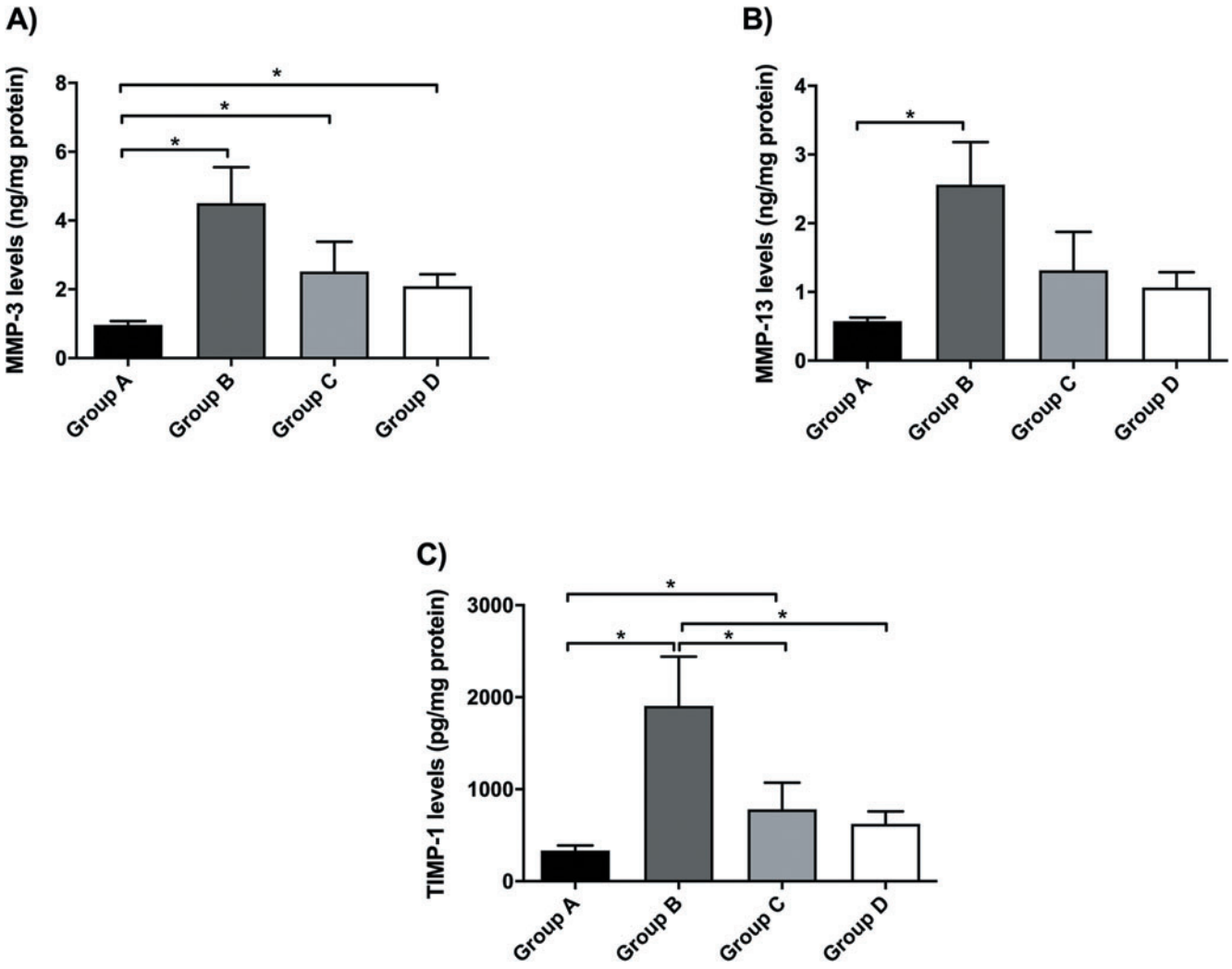

Figure 4. A-C. (A) MMP-3, (B) MMP-13, and (C) TIMP-1 protein levels in IVD of each group. Data are presented as mean ± SEM and $*P \leq 0.05$ is statistically significant.

Starting our trial, we aimed to create an optimal fusion considering all factors. For this reason, at the beginning of the study, we benefited also from the meta-analysis performed by Riordan et al., which comprises 56 studies conducted from 1995 to 2011 on posterolateral spinal fusion model in 733 rabbits. In this meta-analysis, Riordan et al. investigated the factors that are likely to influence the success of spinal fusion such as surgical technique, age, gender, body weight, and the size of graft-derived from the iliac crest.[14] According to this study, at least 5 weeks are required for a successful fusion; the age and weight of animal should be >6 months and >3 kg, respectively; the size of graft-derived from the iliac crest should be >1 cm^3, and the level should be L4–L5 or L5–L6. Since an iliac crest bone graft greater than 2 cm^3 enhances the morbidity as in humans, it needs to be between 1 cm^3 and 2 cm^3. This information can be used to optimize the posterolateral fusion not only in this study but also in future studies. According to this study as well, manual palpation during surgery is a gold standard clinical sign used to demonstrate pseudoarthrosis.[14]

Valdes et al. investigated the surgical technique in the rabbit model of posterolateral spinal fusion.[16] They established an experiment protocol by evaluating the complications that occurred in the first 48 (Group A) of the 77 New Zealand albino rabbits. The experiment was completed before the remaining 29 rabbits (Group B) developed any complication. The most common complications were determined to be nerve paralysis, anesthetic complications, wrong level fusion, and infection.[16] In this study as well, four rabbits died of anesthetic complications and bleeding; however, the study continued with four new rabbits after obtaining the approval of the local ethics committee again. Palpation is considered a valid and reliable method to assess the success of fusion.[13] In this study, the fusion has been achieved in all subjects of Group D by inspection, and successful posterior fusion was detected also by palpation. Boden's success rate remained at the level of 67%.

In this study, we preferred to use a rabbit disc degeneration model, because this shows higher homology to the human intervertebral disc in comparison to other animal models. The formation of degeneration in animal models has been debated and investigated in many studies.[17] The refractory period for the onset of disc degeneration varied in these studies. Masuda et al. observed significant disc space narrowing at 2 weeks.[18] The reason for this long refractory period may be because radiological changes in IVDD become apparent after a longer period than the onset of alterations of pro-inflammatory mediators. This

hypothesis is supported by the findings in our study that a significant increase in all pro-inflammatory mediators was detected in Group B (spinal exposure surgery and degeneration with needle puncture) compared to Group A (only surgery, no degeneration).

Based on the literature review, this is the first study demonstrating the effect of posterior vertebral fusion on pro-inflammatory and catabolic mediators in IVDD. We demonstrated that pro-inflammatory (IL-1β, IL-6, and NO) and catabolic (MMP-3, MMP-13, and TIMP-1) mediator levels increase with disc degeneration. Miyagi et al. also created an IVDD model in Sprague Dawley rats and found a significant increase IL-1β in the acute phase of injury following dynamic IVD compression.[19] Similarly, Purmessur et al. found that IL-1β, MMP-3, and MMP-13 levels were elevated in bovine IVD culture after degeneration.[5] Kepler et al. suggested that pain in IVDD occurs due to pathological innervations and increased pro-inflammatory mediators including IL-1β and MMPs.[20] Consistent with our results, it is reported that TIMP-1 levels showed an increment along with MMPs after disc degeneration.[21–23] There are two possible explanations for this result: (1) TIMP-1 may increase to compensate for high MMP activity and/or (2) TIMP-1 may be involved in cell growth and apoptotic cell death.[24] Accordingly, to ensure lower back pain relief, we need effective treatments/therapies to reconstitute the extracellular matrix by reducing both inflammatory and catabolic processes.

The levels in Group D, in which the animals were sacrificed 4 weeks after Group C, indicated a lower than expected decrease. As a limitation, it may be speculated that Groups C and D had a longer and major operation for graft harvesting and decortication; thus, animals in Group C moved minimally or not at all in the first week. Therefore, Group C may be considered as an "immobilization group" that acts like a long-term fusion group or there may also be a fibrous callus in Group C that acted as fusion as Group D.

There are some limitations of this study. Long-term effects of vertebral fusion were detected in Group D, in which animals were sacrificed 4 weeks later than Group C. Although 5 weeks period is shown to be suitable for fusion to occur in rabbits, it may be speculated that alterations of levels may vary in a longer period. A group with a longer waiting period may be necessary to delineate the long-term effects of fusion. Another limitation is that we did not measure the long-term change in the mediator levels in a group of IVDD without fusion, which may indicate the alteration of mediator levels in the "natural history" of IVDD.

However, here, in this biologic, in vivo animal study, disc degeneration and fusion were achieved in groups and we indicated that both pro-inflammatory and catabolic mediators were reduced after spinal fusion (Groups C and D), nevertheless, remained higher than the sham group.

In conclusion, these outcomes provide further explanation for understanding the role of posterior vertebral fusion in relieving low-back pain. We emphasized that the disc degeneration and fusion model created in this study can be used in future studies that focus on the prevention of disc degeneration-associated low-back pain.

Ethics Committee Approval: Ethics committee approval was received for this study from the Local Ethics Committee of Dokuz Eylül University, School of Medicine, for Animal Experiments (18/2015).

Informed Consent: N/A.

Author Contributions: Concept - D.D., D.K.; Design - D.D., G.O., C.K.; Data Collection and/or Processing - D.D., D.K., G.O., C.K.; Analysis and/or Interpretation - C.B., S.S.; Writing - D.D., D.K., G.O., C.K.; Critical Review - D.D., D.K., G.O., C.K.

Conflict of Interest: The authors declare that there is no conflict of interest.

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