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Epidermal growth factor enhances spinal fusion: Posterolateral lumbar fusion model on rats

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ABSTRACT

Objective: The aim of this study was to investigate the effects of human recombinant epidermal growth factor (EGF) on posterolateral lumbar fusion in a rat model.
Methods: 36 male Sprague Dawley rats underwent posterolateral fusion at L4-5 level. They were randomly assigned to 3 groups: 1- Sham control group where no local augmentation was made, 2- Local Hydoxyapatite β-tricalcium phosphate (HA/β-TCP) augmentation group and 3- Local HA/β-TCP + EGF augmentation group. Rats were euthanized at 8 weeks post-surgery. 6 rats from each group were selected for manual palpation examination, micro-computed tomography analysis and histologic analysis; and the rest was used for biomechanical analysis.
Results: Based on manual palpation, there was no fusion in the sham control group. Fusion rate was 33.3% in the HA/β-TCP group and 66.7% in the HA/β-TCP + EGF group ($p = 0.085$). Micro-CT results revealed that new bone formation was higher in the HA/β-TCP + EGF group (BV/TV: 40% vs. 65%) ($p = 0.004$). Histologically newly formed bone tissue was more pronounced in the EGF group and compacted and bridging bone spicules were observed. The median maximum bending moment values were 0.51 Nmm (0.42–0.59), 0.73 Nmm (0.49–0.88) and 0.91 Nmm (0.66–1.03) in the sham control, HA/β-TCP and HA/β-TCP + EGF groups, respectively ($p = 0.013$). The median stiffness values were 1.69 N/mm (1.12–2.18), 1.68 N/mm (1.13–2.74) and 3.10 N/mm (1.66–4.40) as in the previous order ($p = 0.087$).
Conclusion: This study demonstrates that EGF enhances posterolateral lumbar fusion in the rat model. EGF in combination with ceramic grafts increased the fusion rates. Our findings may provide insights to further studies, investigating EGF's clinical usage as an alternative fusion enhancer.

Introduction

Spine fusion is the preferred treatment method for many spinal diseases like trauma, degeneration, deformity, tumor and infection. For a reliable fusion, gold standard is the usage of autologous bone graft because of its unique microstructure and osteoconductive, osteoinductive and osteogenic properties.[1,2] However its limited supply, donor site morbidity and increased operation time are well known disadvantages.[3] Over the years some alternatives have been developed like allografts, demineralized bone matrix, ceramics, bone morphogenetic proteins, autologous growth factors, synthetic

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peptides, allograft cellular bone matrix and stem cell combinations. Despite all these innovations in graft field, literature still reports high rates of pseudarthrosis and the search for the perfect substitute is going on.[2,4]

Human recombinant epidermal growth factor (EGF) has been used in the treatment of diabetic foot ulcers for more than three decades. It has wound healing and tissue forming abilities. It stimulates proliferation of epidermal and epithelial cells, fibroblasts, and embryonic cells and chemoattractant for fibroblasts and epithelial cells. It also stimulates re-epithelialization, augments angiogenesis and influences the synthesis and turn-over of extracellular matrix.[5]

EGF has an important role in angiogenesis in local bone environment and facilitates bone remodeling. EGF receptors are expressed in different cell types including osteoblasts, osteoclasts and endothelial cells.[6] Literature has conflicting reports about the effects of EGF on osteogenic differentiation. It has been found that EGF both inhibit and enhance osteogenic differentiation in different, mostly in vivo, studies.[7] In a recent study, mesoporous bioactive glass adsorbed EGF was reported to accelerate bone tissue regeneration in vivo.[8] In animal models, EGF, carried by liposome, was found to enhance bone healing in rat tooth sockets.[9] EGF and rhBMP-2 combination was found to induce bone formation earlier compared to the single use of rhBMP-2 in rat calvarial bone defects.[10]

A quite number of papers have been published in literature regarding the promising effects of EGF on bone healing. Besides, current literature is lack of enough information about the effect of this protein on spinal fusion. Within this framework, we hypothesized that EGF may enhance the spinal fusion by its favorable effects on angiogenesis and osteogenic differentiation. As our knowledge EGF effect has not been studied in spinal fusion until now. In the present study, we aimed to investigate the effects of EGF on posterolateral lumbar fusion in a rat model.

Materials and methods

Animal experiments

A total of 36 male 8-week-old Sprague–Dawley rats (weight range, 250–300 g) were used in the present study. Animals were kept in polycarbonate cages at room temperature and light–dark cycle of 12 h with access to food and water ad libitum. This research has been approved by the IRB of the authors' affiliated institutions. Animals were randomly assigned to 3 groups (12 animals in each one): Sham control group, Hydroxyapatite β-tricalcium phosphate (HA/β-TCP) group and HA/β-TCP + EGF group.

Surgical procedure

All rats were anesthetized with intraperitoneal injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). The lumbar region was shaved and cleansed with povidone iodine-soaked gauze. A dorsal midline skin incision was made from L3 to sacrum, followed by two paramedian incisions through the lumbar fascia 3 mm from the midline. The L4 and L5 transverse processes were exposed and decorticated with a high-speed burr. Nothing was implanted to sham control group. 0.3 cc 15% HA/85% β-TCP granules (Mastergraft granules, granule size between 1.6 and 3.2 mm, Medtronic Sofamor-Danek, Memphis, TN) and 10 × 5 × 5 mm saline soaked absorbable gelatin sponge (Spongostan, Ethicon, Somerville, NJ, USA) were implanted bilaterally between the transverse processes at the L4–5 level in HA/β-TCP group animals. 6.25 μg human recombinant epidermal growth factor (Heberprot-P, Praxis Pharmaceutical, Vitoria, Spain) soaked gelatin sponge with same amount ceramic graft as in the previous group, were implanted bilaterally in HA/β-TCP + EGF group animals. The dorsal lumbar fascia and skin were closed using 4.0 absorbable sutures. AP spine radiographs were taken immediately after the surgery to confirm the accuracy of the surgical level. Animals were placed in separate cages and their conditions were monitored daily. They were euthanized at 8 weeks post-surgery using an overdose of anesthetics. 6 rats from each group were selected for manual palpation examination, micro-computed tomography analysis and histologic analysis; rest of the rats was used for biomechanical analysis.

Outcome measures

Manual palpation

Harvested spinal segments manually tested for intersegmental motion. Three independent orthopedic surgeons (not familiar to the study) stressed the fusion site (L4–L5) in anteroposterior and left–right direction. Any motion detected on either side between the L4 and L5 facets or between transverse processes was considered as fusion failure. The absence of motion was considered as an indicator of fusion.[11] When all the three examiners indisputably agreed, the segment was accepted as fused.

Micro-computed tomography (micro-CT) analysis

Spinal segments were fixed in 10% formalin for three days and then kept in ethanol solution until the high-resolution micro-CT examination. They were scanned using SkyScan 1174 (SkyScan, Kontich, Belgium) with the following settings: X-ray energy 50 mV and 800 μA and exposure time of 2700 ms, 33 μm voxel size. Reconstructed images were obtained using NRecon software (NRecon version 1.6.9.4, SkyScan). CTAn software (CTAn version 1.13.5.1, SkyScan) was used to analyze the volume of interest (VOI), between the top the L4 transverse process and bottom of the L5 transverse process, lateral to the pars of the vertebrae (vertebral bodies were not included). Thickness of the axial slices was 33 μm. Tissue volume (bone and soft tissue, TV) and bone volume (in the fusion mass, BV) parameters were analyzed. Bone volume/tissue volume (BV/TV) value was used to assess new bone formation in fusion mass.

Histologic analysis

Spine segments were decalcified in formic acid–formalin solution, dehydrated through an ethanol series and embedded in paraffin wax. Paraffin blocks were serially sectioned at 4 μm thickness in the coronal plane. Slides were stained with hematoxylin–eosin (HE) and masson's trichrome (MT) stain for histological analysis. Slide images were obtained by using a computer assisted light microscope (Leica DM 4000B, Berlin, Germany) and analyzed with Leica QWin software. Randomly chosen twelve sections of each specimen were blindly evaluated by two observers. A scale of 0–3 points, modified version of Emery et al histological grading scale, was used to evaluate the new bone formation.[12] Samples were scored as follows: 0- empty cleft; 1- only fibrous tissue; 2- fibrous tissue is more than bone; and 3- bone is more than fibrous tissue (Table 1).

Table 1
Histological scoring scale.

Score	Tissue present
3	More bone than fibrous tissue
2	More fibrous tissue than bone
1	Fibrous tissue only
0	Empty cleft

Biomechanical analysis

Three-point bending tests were performed by using a servo-hydraulic 50 kN mechanical testing machine (UTM-8050, Utest, Ankara, Turkey). Harvested spinal segments were mounted onto supporting steel rollers (5 mm in diameter) with their posterior surfaces facing up. Distance between the two bearings was 10 mm. Tip of the bending apparatus (3 mm in diameter) was placed just on to the midline of the spine at the fusion level and 1 mm/min displacement force was applied until the breaking point (Fig. 1). The maximum bending moment (Nmm) and the stiffness (N/mm) were determined from the load–deformation curve. The slope of the first linear region of the curve was taken as the measure of stiffness.

Statistical analysis

Fisher's exact probability test was performed for analysis of manual palpation and histologic scores. Histologic scores 0 and 1 (no new bone formation in both) were combined and analyzed as a single parameter. Micro-CT data (differences in TV, BV and BV/TV) were analyzed by using Mann–Whitney U test. And finally, biomechanical data (differences in maximum bending moment and stiffness values) were analyzed by using Kruskal–Wallis test. One-way analysis of variance was used to determine significant differences between treatment groups. For multiple comparisons Dunn's test was performed. The results were presented as median, minimum and maximum values and percentages. The level of significance was set at $p < 0.05$. All statistical analyses were carried out using the NCSS 10 software program (2015. Kaysville, Utah, USA).

Results

Three rats (1 from each group) died on postoperative day 1. The remaining 33 rats had no complications and were sacrificed as planned. 18 rats were used for manual palpation examination, micro-computed tomography analysis and histologic analysis; remaining 15 rats were used for biomechanical analysis.

Manual palpation

Based on manual palpation, there was no fusion in the sham control group. Fusion rate was 33.3% (2/6 rats) in the HA/β-TCP group and 66.7% (4/6 rats) in the HA/β-TCP + EGF group. Statistical significance was found as $p = 0.085$ (Table 2).

Micro-CT analysis

The median TV values were 282.79 mm^3 (range, 169.02–484.48 mm^3) in the HA/β-TCP group and 451.31 mm^3 (range, 414.43–607.77 mm^3) in the HA/β-TCP + EGF group ($p = 0.025$). BV values were 115.41 mm^3 (range, 66.71–205.81 mm^3) and 304.37 mm^3 (range, 243.12–390.93 mm^3) as in the previous order ($p = 0.004$). The median BV/TV values were 0.40 (range, 0.38–0.43) in the HA/β-TCP group and 0.65 (range, 0.59–0.68) in the HA/β-TCP + EGF group ($p = 0.004$) (Table 3). No bony tissue regarding fusion mass could be detected with micro-CT examination in the sham control group rats; therefore this group was excluded from the statistical evaluation. Representative coronal micro-CT images of the spinal segments were shown in Fig. 2.

Histologic analysis

Hematoxylin–eosin and masson's trichrome stained sections of sham control group did not show any evidence of new histological tissue formation. HA/β-TCP group showed, increased osteoblastic activity in newly formed fibrous tissue. Non-bridging dispersed local bone spicules and trabeculae were observed. Newly formed bone tissue was more pronounced in the HA/β-TCP + EGF group. Compacted and bridging bone spicules were observed in some areas (Fig. 3).

According to modified Emery et al histological grading scale, all sham control group samples got 0 points (empty cleft). 50% (n = 3) of the HA/β-TCP group samples were scored as 1 (only fibrous tissue) and 50% as 2 (fibrous tissue is more than bone). In the HA/β-TCP + EGF group, 50% of the samples got 2 and the rest got 3 (bone is more than fibrous tissue) points. Statistical significance was

Table 2
Assessment of spinal fusion by manual palpation.

Groups	Sham control	HA/β-TCP	HA/β-TCP + EGF
Fusion % (fused/total rats)	0% (0/6)	33.3% (2/6)	66.7% (4/6)

Fisher's exact probability test: $p = 0.085$.

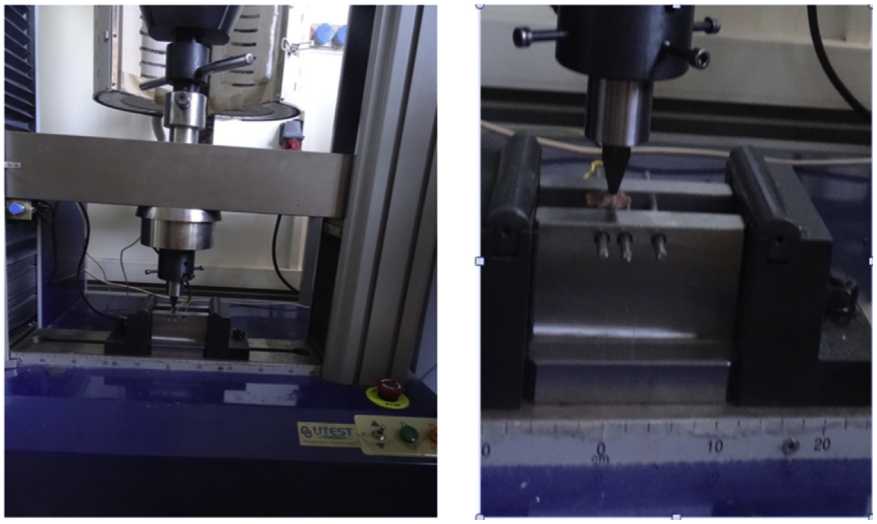

Fig. 1. Three-point bending test machine and setup.

Table 3
Micro-CT data of the study groups.

	HA/β-TCP	HA/β-TCP + EGF	*p* value
TV (mm^3)	282.79 (169.02–484.48)	451.31 (414.43–607.77)	0.025
BV (mm^3)	115.41 (66.71–205.81)	304.37 (243.12–390.93)	0.004
BV/TV (%)	40 (38–43)	65 (59–68)	0.004

Median (minimum–maximum) values were given. Mann–Whitney U test was used for the statistical analysis.

found as $p = 0.002$. Sham control group scores were statistically different than the others (Table 4).

Biomechanical analysis

The median maximum bending moment values were 0.51 Nmm (range, 0.42–0.59 Nmm) in the sham control group, 0.73 Nmm (range, 0.49–0.88 Nmm) in the HA/β-TCP group and 0.91 Nmm (range, 0.66–1.03 Nmm) in the HA/β-TCP + EGF group ($p = 0.013$). HA/β-TCP + EGF group showed significantly higher maximum bending moment values according to sham control group ($p < 0.05$). The median stiffness values were 1.69 N/mm (range, 1.12–2.18 N/mm), 1.68 N/mm (range, 1.13–2.74 N/mm) and 3.10 N/mm (range, 1.66–4.40 N/mm) for sham control, HA/β-TCP and HA/β-TCP + EGF groups respectively ($p = 0.087$) (Table 5).

Discussion

The results of the present study show that EGF enhanced posterolateral lumbar fusion in the rat model. When used in combination with ceramic (HA and β-TCP) grafts, EGF increased fusion rates according to controls.

Some special growth factors like bone morphogenetic proteins (BMP) have been widely used in spinal fusion procedures for long years. The most commonly used one, BMP-2, has been approved by FDA for use in anterior lumbar interbody fusion surgery. Despite this indication, high fusion rates provided by BMP-2 have led its widespread off-label usage. In the following years, BMP-2 has been associated with serious adverse effects; and the resulting safety concerns have reduced its usage.[13,14]

In order to meet the need for fusion enhancers, some other growth factors have been studied in animal and human studies. Such as autologous growth factors (protein rich plasma) and naturally occurring growth factors like Nell-like molecule-1 (Nell-1) and oxysterols (Oxy34 and Oxy49). Some of them showed promising features and found clinical usage.[2,4] EGF is also one of the natural growth factors. Its success in the management of diabetic foot ulcers has been known for long years but as our knowledge its effect on spinal fusion has not been studied until now.

The present study demonstrates its efficacy on posterolateral lumbar spinal fusion in a rat model. EGF and ceramic graft combination (HA/β-TCP + EGF group) showed a higher fusion rate in manual palpation test according to sham control and HA/β-TCP groups. Despite the low statistical significance of this finding it was clinically apparent and remarkable. Micro-CT analysis revealed that HA/β-TCP + EGF group had significantly higher bone and tissue volumes and higher new bone formation ratio according to HA/β-TCP group alone. Histologically bone tissue formation was more obvious when EGF used in combination with ceramic grafts. Biomechanically HA/β-TCP + EGF group had statistically higher maximum bending moment values according to other study groups but differences in stiffness values were not significant.

As aforementioned, EGF has not been studied in rat spinal fusion model; nevertheless our results are comparable with similar studies in which BMP was used. Buser et al reported, BV/TV value as 0.55 with low dose BMP-2 in a rat posterolateral fusion model,[15] which was 0.65 in our study. This value can be interpreted as a strong indicator of the positive effect on bone formation. Morimoto et al reported abundant bone formation with bridging between the transverse processes in rats treated with BMP-2/7.[11] Our histological findings were similar, we observed compacted and bridging bone spicules in the HA/β-TCP + EGF group. It was different from the dispersed bone formation in the HA/β-TCP group. Biomechanical testing is not a common method in rat spine studies, because of the small nature of the rat spines. Instead, the manual palpation test has been generally used for fusion analysis.[16] In the present study, we performed both tests. Maximum bending moment and stiffness values were higher in the HA/β-TCP + EGF group; which means more external force was necessary to bend the spine segment in EGF group. These results were consistent with manual palpation results.

There are many studies in the literature reporting that EGF has catabolic effects on bone tissue. It was shown that expression of epidermal growth factor in transgenic mice causes growth retardation and thinner bones were observed.[17] In another study, EGF at physiological dosage was shown to increase osteoclastic cell density and endosteal bone resorption.[18] Alves et al reported that local delivery of EGF–liposome stimulates osteoclastogenesis and tooth movement.[19] On the other hand, limited number of studies reported its positive effect on bone healing. Marques et al reported that liposome carried EGF enhanced bone healing of rat tooth sockets. They histologically demonstrated pronounced expression of fibronectin and collagen type III and an increased new bone formation.[9] Lee et al reported that EGF interacts synergistically

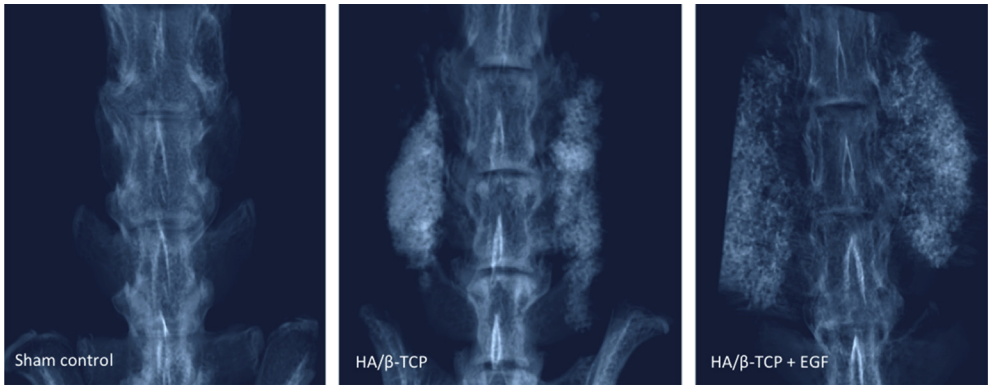

Fig. 2. Representative coronal micro-CT images of the spinal segments.

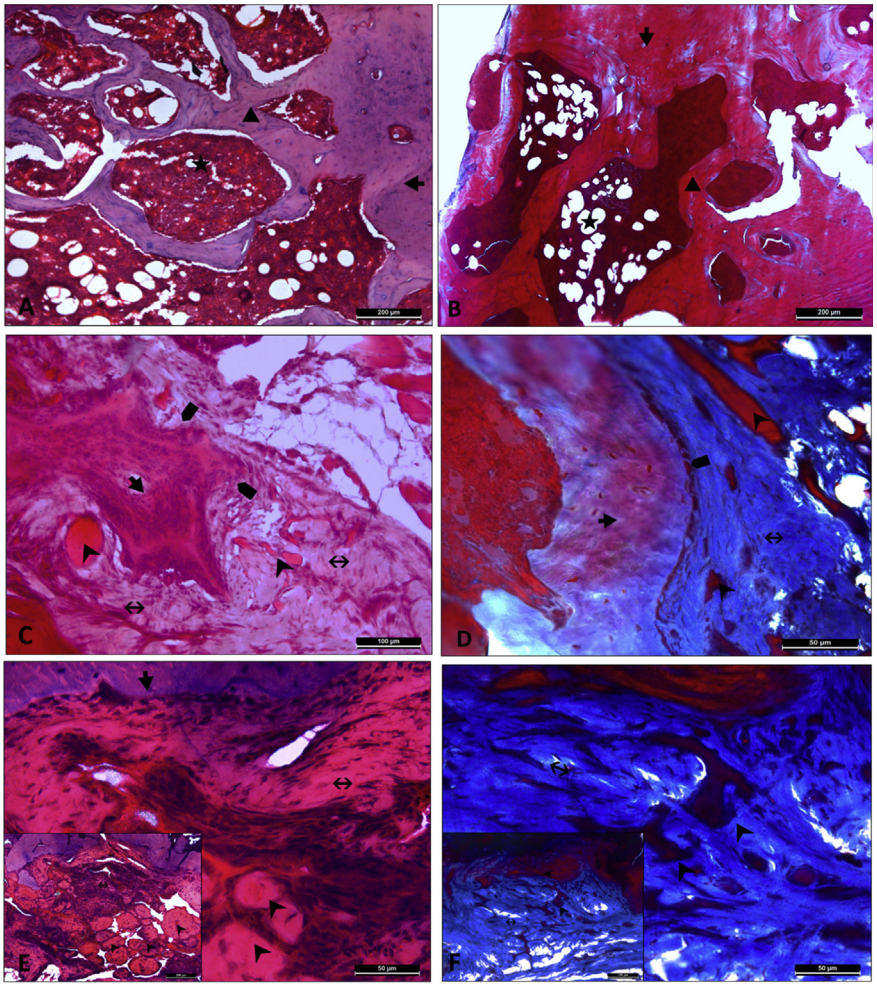

Fig. 3. A, B: Spine sections of sham-control group. Host bone tissue, bridging bone trabeculae (▲) and bone marrow (★) were observed. There was not any evidence of new tissue formation. (A: HEx100; B: MTx100). **C, D**: HA/β-TCP group. Host bone (◀), Fibrous tissue formation in the fusion area (↔), increased osteoblastic activity (◀) and non-bridging dispersed local bone spicules (➢) in fibrous tissue. (C: HEx400; D: MTx400). **E, F**: HA/β-TCP + EGF group. Host bone (◀), compacted and bridging newly formed bone spicules (➢) in fibrous tissue (↔). (E: Inset: HEx100, HEx400; F: Inset: MTx200, MTx400).

Table 4
Results of histological evaluation according to modified version of Emery et al histological grading scale.

Histological score	Sham-control	HA/β-TCP	HA/β-TCP + EGF
	% (n)		
0- empty cleft	100% (6)		
1- only fibrous tissue		50% (3)	
2- fibrous tissue is more than bone		50% (3)	50% (3)
3- bone is more than fibrous tissue			50% (3)

Fisher's exact probability test: $p = 0.002$ (Scores 0 and 1 combined and analyzed as a single parameter).

Table 5
Biomechanical data of the study groups.

	Sham-control	HA/β-TCP	HA/β-TCP + EGF	*p* value
Maximum bending moment (Nmm)	0.51 (0.42–0.59)	0.73 (0.49–0.88)	0.91 (0.66–1.03)	$p = 0.013$
Stiffness (N/mm)	1.69 (1.12–2.18)	1.68 (1.13–2.74)	3.10 (1.66–4.40)	$p = 0.087$

Median (minimum–maximum) values were given. Kruskal–Wallis test was used for the statistical analysis. Dunn's test was used for multiple comparisons ($p < 0.05$ for maximum bending moment differences of sham-control and HA/β-TCP + EGF group).

with *Escherichia coli* derived BMP-2 and accelerates protein induced bone formation and healing in rat calvarial bone defect model.[10] Our study supports the previous studies reporting that EGF has positive effects on bone healing. Micro-CT and histological analysis reveled that EGF increased new bone formation in rat spinal fusion model.

Another important point to mention is the possible side effects of EGF treatment on rats. EGF treatment for diabetic foot ulcers in

humans was reported to have some adverse effects like tremors, chills, pain and burning at the site of administration. Those were generally mild to moderate adverse effects and did not necessitate treatment cessation.[20] Occasionally, antihistaminic medication may be necessary in clinical practice. No serious adverse effects have been reported in the use of EGF in rats.[21] We did not observe any allergic reactions and serious adverse effects in EGF group rats. Additionally we did not use any medication to prevent the potential adverse effects.

A limitation of this study is that the absence of pre-tested controlled release system for EGF. Since EGF has a short half-life, its administration by a controlled release system could provide a more stable concentration of EGF in the tissue. Nevertheless EGF was administered within the gelatin sponge in the present study. Collagen and gelatin sponges were commonly used materials for administration of bioactive agents and this method has been reported to be effective in many studies.[22,23] Another limitation may be absence of auto-graft group animals. Its comparison with gold standard fusion application might give valuable information. Due to ethical factors, serious attention was paid to minimizing the number of animals and minimum number of animals was selected for each group. For this reason auto-graft group was not included in the study. The local ethics committee for animal experiments set the total number as 36 in this study which was lower than the ideal sample size for 80% power.

Conclusion

This study demonstrates that human recombinant epidermal growth factor enhances posterolateral lumbar fusion in the rat model. EGF in combination with ceramic grafts increased the fusion rates. Our findings may provide insights to further studies, investigating EGF's clinical usage as an alternative fusion enhancer.

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