The Effect of Bone Morphogenetic Protein-2 and Hydroxyapatite Granules on the Incorporation of Autoclaved Femoral Autografts in *Sprague-Dawley* Rats

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ABSTRACT

Introduction. Numerous methods of limb salvage surgery have been utilized in musculoskeletal tumors, one of them being autoclaved autograft. Apart from killing tumor cells, autoclaving of autograft will also inevitably damage the inherent osteoblasts and bone morphogenetic proteins, thus impeding bone healing and graft incorporation. Bone Morphogenetic Protein-2 has been proven to accelerate fracture healing. Only few studies have investigated the effect of Bone Morphogenetic Protein-2 on the incorporation of autoclaved autografts. In this study we investigated the effect of Bone Morphogenetic Protein-2 on the incorporation of autoclaved autografts in *Sprague-Dawley* rats. The objective of the study is to evaluate the effect of Bone Morphogenetic Protein-2 with hydroxyapatite granules on healing or incorporation of autoclaved autografts.

Materials and methods. Twenty *Sprague-Dawley* rats were randomized into three groups: control group, hydroxyapatite group, and Bone Morphogenetic Protein-2 group. All animals underwent osteotomy of the femoral shaft, and the resulting autograft fragments were autoclaved for 15 minutes under a temperature of 134^{o} Celsius. Autoclaved autografts were then re-implanted and fixed with intramedullary Kirschner wires. hydroxyapatite granules were added into the fracture sites of animals belonging to hydroxyapatite group. In the Bone Morphogenetic Protein-2 group, hydroxyapatite granules that had been immersed in Bone Morphogenetic Protein-2 solution were added to the fracture sites. Meanwhile, rats from the control group received neither Bone Morphogenetic Protein-2 nor hydroxyapatite granules. Anteroposterior and lateral radiographs of the osteotomized femurs were taken eight weeks later, after which all animals underwent euthanasia. Osteotomized femurs were harvested and sent for histopathological examinations. Lane-Sandhu radiological score and Salkeld-Marino histological scores were calculated.

Results. The median distal radiological scores in control, hydroxyapatite and Bone Morphogenetic Protein-2 groups were 0.5, 1 and 2, respectively ($p=0.011$). Proximal radiological scores did not differ between groups ($p=0.160$). The median proximal histological scores in control, hydroxyapatite and Bone Morphogenetic Protein-2 groups were 3, 4 and 13, respectively ($p=0.004$); and distal histological scores were 3, 1 and 13, respectively ($p=0.001$). Post-hoc analysis revealed that significant differences in histological scores and distal radiological score were found between control and Bone Morphogenetic Protein-2 groups, as well as between hydroxyapatite and Bone Morphogenetic Protein-2 groups. Scores did not differ between control and hydroxyapatite groups ($p<0.05$). Proximal radiological and proximal histological scores were not correlated ($r = 0.267$, $p>0.05$). Distal radiological and distal histological scores, however, were moderately correlated ($r = 0.567$, $p<0.05$).

Conclusions. Bone Morphogenetic Protein-2 with hydroxyapatite granules accelerated the incorporation of autoclaved autograft in *Sprague-Dawley* rats, while hydroxyapatite granules alone did not.

Keywords: autograft, autoclave, Bone Morphogenetic Protein-2, hydroxyapatite

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Efek Pemberian *Bone Morphogenetic Protein-2* dan Granul Hidroksiapatit terhadap Inkorporasi Tandur Tulang Femur yang Dilakukan Autoklaf di Tikus *Sprague-Dawley*

ABSTRAK

Pendahuluan. Berbagai cara pembedahan preservasi tungkai di tumor musculoskeletal telah dikmebangkan, misalnya dengan menggunakan tandur tulang yang dilakukan autoklaf. Selain membunuh sel tumor, autoklaf juga menyebabkan kerusakan osteoklas dan *Bone Morphogenetic Protein-2* sehingga menghambat penyembuhan dan inkorporasi tandur tulang. *Bone Morphogenetic Protein-2* telah terbukti mempercepat penyembuhan fraktur di manusia dan hewan coba serta di tandur tulang yang telah mengalami radiasi ekstra korporal. Hanya beberapa penelitian yang telah mempelajari peran *Bone Morphogenetic Protein-2* terhadap inkorporasi tulang yang dilakukan autoklaf di tikus *Sprague-Dawley*. Tujuan dari penelitian ini adalah untuk mengevaluasi peran *Bone Morphogenetic Protein-2* dan granul hidroksiapatit terhadap penyembuhan atau inkorporasi tandur tulang yang dilakukan autoklaf.

Bahan dan cara kerja. Dua puluh ekor tikus *Sprague-Dawley* dibagi secara acak ke dalam tiga kelompok: kelompok kontrol, kelompok hidroksi apatit, dan kelompok *Bone Morphogenetic Protein-2*. Osteotomi dilakukan di semua hewan coba dan tandur tulang dilakukan autoklaf selama 15 menit pada suhu 134^{o} Celcius. Tandur tulang yang telah dilakukan autoklaf kemudian direimplantasi dan difiksasi dengan menggunakan *Kirschner wire*. Granul hidroksiapatit ditambahkan pada situs fraktur kelompok hidroksiapatit sedangkan granul hidroksiapatit yang telah direndam dalam larutan *Bone Morphogenetic Protein-2* diberikan pada kelompok *Bone Morphogenetic Protein-2*. Delapan minggu kemudian, dilakukan pemeriksaan ronsen. Hewan coba kemudian dikorbankan untuk pemeriksaan histopatolis. Skor radiologis Lane-Sandhu dan skor histologis kemudian dihitung.

Hasil. Nilai tengah skor radiologis pada kelompok kontrol, hidroksiapatit, dan *Bone Morphogenetic Protein-2* berturut-turut adalah 0,5; 1; dan 2 dengan nilai p=0,011. Skor radiologis proksimal tidak berbeda antar kelompok (p=0,160). Nilai tengah skor histologis proksimal pada kelompok kontrol, hidroksiapatit, dan kelompok *Bone Morphogenetic Protein-2* adalah 3, 4, dan 13 dengan nilai p=0,004 sedangkan skor histologis distal berturut-turut adalah 3, 1, dan 13 dengan nilai p=0,001. Analisis *posthoc* mendapati perbedaan signifikan skor histologis dan skor radiologis distal antara kelompok kontrol dan *Bone Morphogenetic Protein-2*, serta antara kelompok hidroksiapatit dan kelompok *Bone Morphogenetic Protein-2*. Tidak terdapat perbedaan yang bermakna antara kelompok kontrol dan kelompok hidroksiapatit. Skor radiologis proksimal dan skor histologis proksimal tidak berkorelasi (r=0,267, p>0,05). Terdapat korelasi sedang antara skor radiologis dan skor histologis distal. (r=0,567, p<0,05).

Simpulan. *Bone Morphogenetic Protein-2* bersama hidroksiapatit mempercepat inkorporasi tandur tulang yang dilakukan autoklaf di tikus *Sprague-Dawley*.

Kata kunci: tandur tulang, autoklaf, *Bone Morphogenetic Protein-2*, hidroksiapatit

Introduction

The advent of mega-prosthesis does not render the use of autografts in reconstruction following malignant bone tumor excision obsolete. In developing countries, where medical affordability and allograft supply are still limited, and in cases of tumors afflicting maxillofacial bones, autografts are still very much used. Some methods of autograft sterilization are employed: extracorporeal irradiation, autoclaving, pasteurization, and even simple boiling. While extracorporeal irradiation facility is

Table 1. Radiological union score according to Lane and Sandhu

Description	Scores
No callus	0
Callus formation present, but no evidence of union	1
Initiation of bony union present	2
Bony union present, fracture line no longer visible	3
Complete bony union	4

Table 2. Histological union score according to Salkeld et al

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

limited, autoclave, however, is ubiquitous and easily accessible.

Autoclave results in tumor cell necrosis. Unfortunately, autoclave also results in destruction of vascular structures, cellular components, collagen and osteoinductive factors that are critical in tissue regeneration, and thus also weakens the mechanical property of the bone.[1-4] Asmara and colleagues found a reduced Bone Morphogenetic Protein-2 (BMP-2) expression and osteoblast number in autoclaved *Sprague-Dawley* autografts.[5]

BMP-2 is a potent inducer of bone formation. It promotes the differentiation of fibroblastic cells into osteoblasts and chondroblasts. Administration of BMP-2 has also been proven to increase callus formation

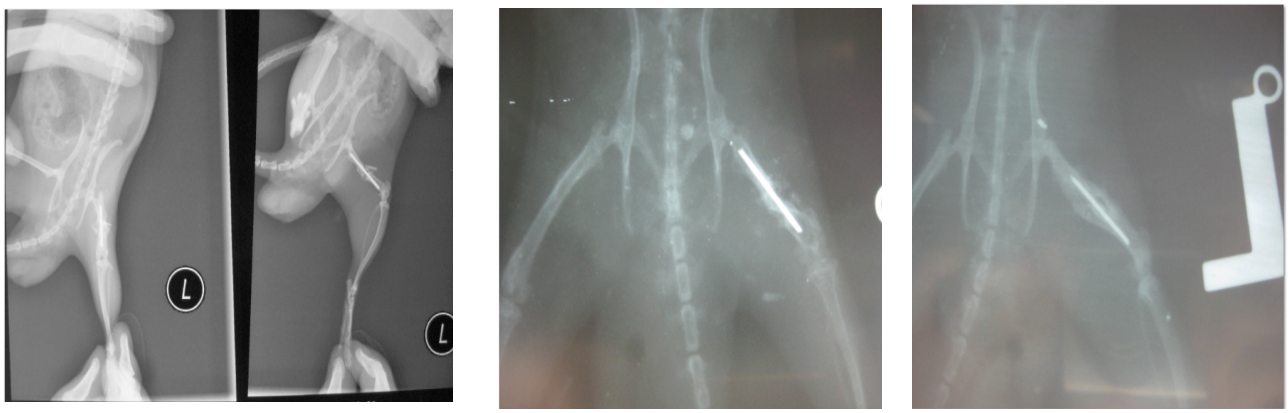

Figure 1. Radiographs of treatment group. Poor healing was seen in control group (left) and HA group (middle) while good healing was seen in BMP group (right)

in fracture healing[6,7] and clinically approved for the treatment of nonunion and lumbar spine fusion.[8,9]

To effectively exert its effects, BMP-2 needs a scaffold, or carrier. Naturally, BMP-2 is contained within extracellular matrix that consists of type-1 collagen, proteoglycan, glycosaminoglycan and hyaluronan. An ideal carrier resembles the properties of extracellular matrix. Hydroxyapatite, which contains calcium phosphate, resembles mineralized bone matrix, possesses adequate mechanical strength, high porosity and an optimal pore size that facilitates bony ingrowth as well as retains active substances including BMP-2s and osteogenic cells.[10-13] The combination of BMP-2 and hydroxyapatite has been shown to stimulate significantly greater trabecular bone formation compared to hydroxyapatite alone.[14,15] The purpose of this present study is to investigate whether administration of BMP-2 effectively enhances union of devitalized bone.

Materials and methods

A total of twenty healthy, male *Sprague-Dawley* rats were used in this study. They were all skeletally mature with an average body weight of 210 grams. The operative procedure and animal care were conducted in compliance with the regulations of the University's Committee of Research Ethics. A unilateral 5-mm segmental femoral shaft fracture was created in each animal. The resulting segmental fracture fragment was autoclaved and re-implanted and fixed with intramedullary Kirschner wire. The animals had been previously randomized into three treatment arms: those that received BMP-2 and hydroxyapatite (BMP-2 group), those that received

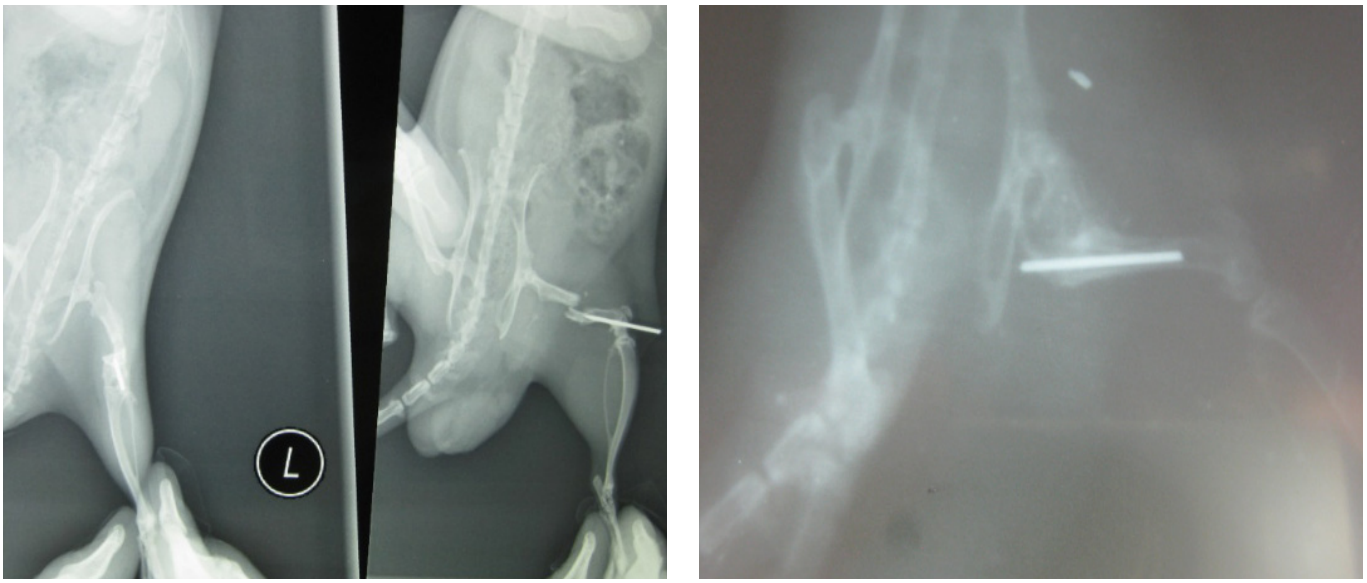

Figure 2. Fixation failure. Nonounion occurred in control group in case of fixation failure (left), while in BMP group, healing was achieved desoite fixation failure (right)

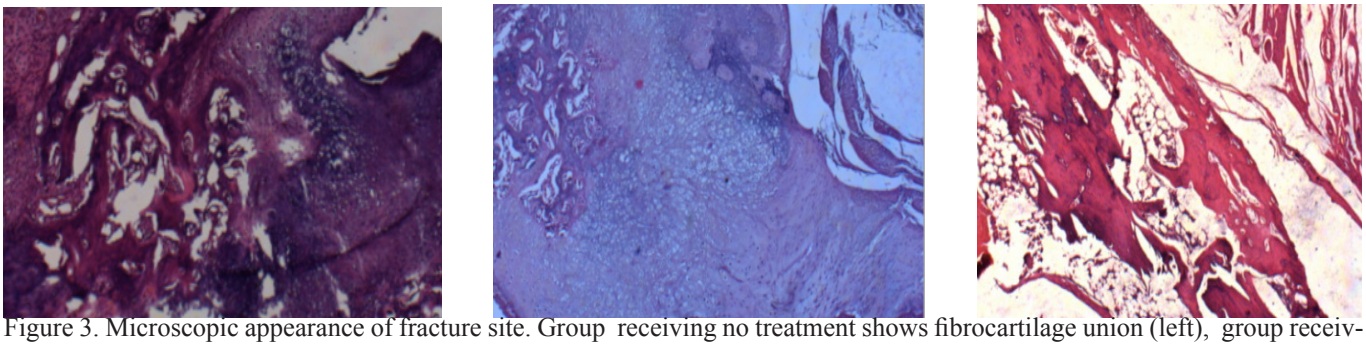

Figure 3. Microscopic appearance of fracture site. Group receiving no treatment shows fibrocartilage union (left), group receiving hydroxyapatite granules shows cartilage union(middle), and group receiving BMP shows complete cortical bridging (right)

hydroxyapatite only (HA group), and control group that received none.

We used Bongros® hydroxiapatite granules that came packed in vials. Each vial was allocated for 4 animals, and the amount of granules implanted was adequate to bridge the fracture sites. BMP-2.The BMP-2 used in this study was a recombinant-human BMP-2 from Daewoong Pharmaceuticals that came in the form of solvent (3 ml) and powder (3000 micrograms). The solvent and the powder were then mixed. Referring to studies by Zara[16], Tsuruga[17] and Brick[18], we determined that a dose of 150-200 micrograms of BMP-2 was needed for each animal, and calculated that this amount was equivalent to 0.15-0.2 ml of solution. Since one vial of hydroxyapatite granules was allocated for four animals, we injected 0.6-0.8 ml of BMP-2 solution into one hydroxyapatite vial and mixed them thoroughly.

A 5-mm segmental fracture was created with osteotomy in each animal, and the segmental fracture fragment was then autoclaved for 15 minutes under a temperature of 134 degree Celsius. After being autoclaved, the fragment was re-implanted and fixed with intramedullary Kirschner wire in all groups. Control group received neither hydroxyapatite nor BMP-2. Dry hydroxyapatite granules were embedded in the fracture sites of rats belonging to HA group. Hydroxyapatite granules that had been mixed with BMP-2 were embedded in fracture sites of BMP-2 group animals. All animals then underwent surgical closure and were sent for recovery.

AP and lateral radiographs of operated femur were taken in all animals 8 weeks postoperatively. Lane-and-Sandhu radiological score (Table 1) was used to evaluate

Table 3. Results according to treatment arms and statistical analysis

	Treatment arm	N	Median	p (Kruskal-Wallis)	Posthoc Mann-Whitney
Proximal histological score	control	6	3.00	0.004	control VS HA : p=0.418
	HA	7	4.00		control VS BMP-2 : **p=0.002**
	BMP-2	7	13.00		HA VS BMP-2 : **p=0.013**
Distal histological score	control	6	3.00	0.001	control VS HA : p=0.557
	HA	7	1.00		control VS BMP-2 : **p=0.002**
	BMP-2	7	13.00		HA VS BMP-2 : **p=0.001**
Proximal radiological score	control	6	0.50	0.160	
	HA	7	1.00		
	BMP-2	7	2.00		
Distal radiological score	control	6	0.50	0.011	control VS HA : p=0.565
	HA	7	1.00		control VS BMP-2 : **p=0.028**
	BMP-2	7	2.00		HA VS BMP-2 : **p=0.003**

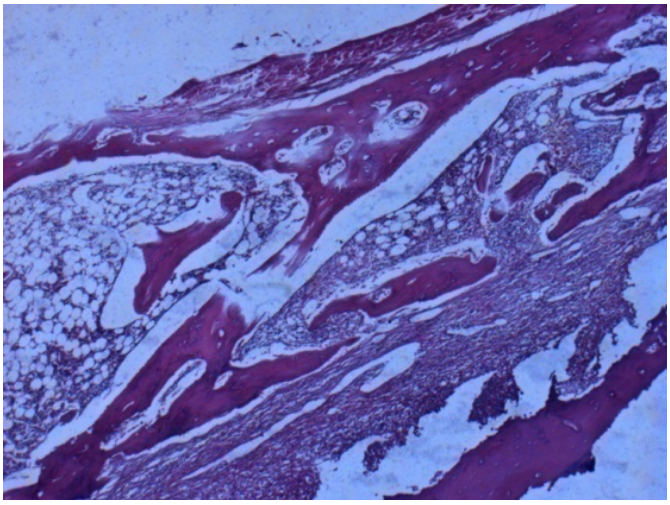

Figure 4. Microscopic appearance of an infected, yet healed autograft from BMP-2 group.

fracture healing. Radiograph analysis and scoring were conducted by certified radiologist (MP).

After being radiographed, all animals underwent euthanasia. Osteotomized femurs were harvested, preserved in 10% formaldehyde solution and sent to pathology for processing. Histological score according to Salkeld et al.[19] (Table 2) was used to evaluate healing. Histological analysis was performed by a certified pathologist (EK).

Nonparametric Kruskal-Wallis and Mann-Whitney posthoc tests were employed for statistical analysis, using SPSS16 software.

Results

All animals underwent uneventful recovery. Radiological and histological scores were determined for each fracture site (proximal and distal).

Proximal radiological scores and distal radiological scores were similar for each group, and an increment of score was seen from control to BMP-2 groups. Control, HA, and BMP group has a median score of 0.5, 1, and 2 respectively. There was no statistically significant difference of proximal radiological score among the three groups. Although the median of distal radiological scores was the same as that of proximal radiological scores, there was significant difference of distal radiological scores among the three groups. Posthoc analysis (Table 3) showed that differences were found between control and BMP-2 groups ($p=0.028$), and between HA and BMP-2 groups ($p=0.003$). Control and HA groups did not differ ($p>0.05$). Figure 1 represent radiographs from each treatment group.

Fixation was deemed failed if there was a complete displacement of the fracture site that rendered no contact between fracture fragments (figure 2). In the control and HA groups, fixation failures were seen in more than 50% of proximal fracture sites and more than 33% of distal fracture sites. Fixation failures were least in the BMP-2 group.

The medial proximal and distal histological scores were highest in BMP-2 group, in contrast to those of HA and control groups (Table 3, Figure 3), and there were significant differences found amongst the three treatment groups. Posthoc analysis showed that significant differences in proximal histological score were found between control and BMP-2 groups ($p=0.002$), and between HA and BMP-2 groups ($p=0.013$). No significant difference was found between control and HA groups. As with proximal histological scores, distal histological scores did not differ significantly between control and HA groups ($p>0.05$). Distal histological scores of BMP-2 group were significantly higher than control group ($p=0.002$) and HA group ($p=0.001$).

Spearman's correlation test was used to analyse the correlation between radiological and histological scores. Proximal radiological and proximal histological scores were not correlated ($r=0.267$, $p>0.05$). Distal radiological and distal histological scores, however, were moderately correlated ($r=0.567$, $p<0.05$).

Cases of osteomyelitis were found in 1 animal in control group, 2 in HA group and 3 in BMP-2 group. Infected animals in control and HA groups failed to achieve union (histological score less than 3), but two of the three infected animals in BMP-2 group achieved union with histological scores more than 12 (figure 4).

Discussions

Having been devitalized by various methods such as autoclaving or extracorporeal irradiation, autografts have limited osteoinductive capacity and their use in reconstructions following bone tumor excision has been problematic in terms of healing. BMP-2 has potent osteoinductive capacity. Geesink[20] found that bridging of defect was much faster in bones that received BMP-2. Salkeld[19] also found that there was more extensive new bone formation in allograft supplemented with BMP-2-7 than in autograft. In this present study BMP-2 has been demonstrated to be capable of promoting healing of devitalized autografts, as evidenced by the fact that radiological and histological scores of subjects receiving BMP-2 were significantly greater than those that did not receive BMP-2. The addition of hydroxyapatite only rendered no superior benefits to control group. Bony union, cortical bridging and good graft incorporation were seen in subjects that had received BMP-2, while in those

treated without BMP-2, regardless of hydroxyapatite addition, cortical bone formation and graft incorporation were minimal, and fibrous or fibrocartilaginous unions were the primary mode of healing. Our finding is in accordance to the findings reported by Hu[14], where defect that had received scaffold only healed with fibrous tissue, while defect that had received BMP-2 healed completely with higher bone density. Similar findings were also reported by Kamal [21,] who found a lower union score of autografts that had received hydroxyapatite compared to control group. Kamal hypothesized that this finding was due to the fact that hydroxyapatite crystals were foreign bodies that attracted giant cells that then exerted resoptive properties, hence healing impairment. Finkemeier[22] stated that superior results were obtained only when scaffolds were implanted in well-vascularized bed.

It is interesting to note that there was no statistically significant difference in proximal radiological score among 3 treatment arms. We hypothesized that this was accounted by the the presence of proximal fixation failure in more than 50% of subjects in each group.

Ideally radiological and histological scores are expected to be strongly correlated. In our study, however, radiological and histological score were not strongly correlated. While evaluation of histological union, aided by microscope, was much easier, the precision of evaluating radiological union was limited by the miniscule size of a rat femur as peered by the naked eyes. CT scan would've facilitated more accurate evaluation, unfortunately resources were lacking.

We considered several factors as the cause of fixation failures in our study. Postoperative immobilization in animals was not possible, hence early, unprotected weight bearing occurred. Technical errors such as too-short K-wires and osteotomy cuts that yielded certain degree of comminution also affected fixation failure. High temperature and pressure during autoclaving decreased mechanical strength and osteoinductive capacity. Fixation failures were seen more frequently in HA and control groups as many animals in these groups failed to reach union. As mentioned by Govender[9], hardware failure was less frequent in subjects that received rhBMP-2.

We did not venture to conclude that BMP-2 had any contribution to the development of infection, despite the finding that infection was most prevalent in BMP-2 group. However, two infected subjects (out of three) in BMP-2 group managed to reach union with high histological scores, while those in other groups did not. Whether BMP-2 promoted fracture healing independent of infection could not be determined, our finding concorded with those of Brick[18] and Chen[23,] who mentioned in their studies that rhBMP-2 effectively accelerated new bone formation regardless infection.

Conclusions

BMP-2 enhances incorporation of autografts that has been devitalized with autoclave, as reflected by superior union scores. Despite its promising role, BMP-2 safety profile in the presence of musculoskeletal tumor has not been established, thus further studies are needed.

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Disclosures

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