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Comparative study of combined treatment with systemic substance P and local stromal derived factor 1 alpha on recombinant human bone morphogenic protein 2- induced ectopic bone formation in a rat model.

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- In situ bone regeneration
- Substance P
- Stromal Cell-Derived Factor-1 α
- Bone

Abstract

Introduction: Healing of bone defects represents clinical challenge due to limited effectiveness of available treatments. Enhancement of endogenous mesenchymal stem cells (MSCs) mobilization and homing is a promising approach for bone regeneration. The present study tested the hypothesis that stem cell mobilization and homing by systemic Substance P (SP) and local Stromal Cell-Derived Factor-1 (SDF-1α) treatment can augment low-dose recombinant human bone morphogenetic protein-2 (rhBMP-2) induced ectopic bone formation in a rat model. Materials and Methods: Rats received intravenous injections of either saline or SP in implantation day as well as postoperative day 1 of the subcutaneous implantation of absorbable collagen sponges (ACS's) loaded with saline or suboptimal dose of rhBMP-2 alone or suboptimal dose of rhBMP-2 and SDF-1α. At 28th days, bone formation in the explanted scaffolds was examined for alkaline phosphatase activity (ALP), osteopontin (OPN) assays as well as radiologically and histologically. Results: systemic SP and local SDF-1 α significantly enhanced ALP activity in the explanted bony nodules of the studied groups as compared to controls. Radiological and histological assessment were consistant with biochemical results showing much more mature bone formation. Conclusion: The study provides evidence of the effective treatment of systemic SP and local SDF-1 α in enhancement of in vivo trafficking of MSC's in low-dose rhBMP induced ectopic bone formation.

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INTRODUCTION

Bone tissue has limited intrinsic capability for restoration after large loss due to trauma, degenerative disease, or tumor resection. In vivo innovative bone tissue regeneration has a great impact on such clinical situations (1,2).

Bone regeneration is initiated by recruitment of mesenchymal stem cells (MSCs) and progenitor cells into the defect site. MSCs proliferate and differentiate into osteogenic cells and deposit mineralized extracellular matrix (ECM). There differentiation is guided by several molecular events. Bone morphogenic proteins (BMPs) are the most potent osteoinductive proteins in bone regeneration. Among them, BMP-2 is a well-known growth factor inducing osteogenic differentiation of stem cells and has been used clinically for bone regeneration (3,4). Despite its great osteoinductivity, clinical treatments require large doses of the expensive recombinant protein, which could potentially lead to several complications (5).

Regenerative medicine has adopted the concept of utilizing endogenous cells for in situ tissue regeneration, by the use of local bioactive delivery system on a target specific-scaffold to mobilize host MSC to a site of injury without the need for cell seeding. However, this approach has limited stem cell recruitment into the implants (6,7).

The use of this approach for treating bone defects has the potential to be advantageous over the use of allografts, which have complications related to poor integration with native bone (8). and autografts that require additional donor surgery with many morbidities. Furthermore, this customized and controlled trafficking of endogenous MSCs would eliminate the need for ex-vivo expansion, manipulation, and transplantation of autogenous MSCs, which often results in poor homing efficiency (9).

Substance P (SP) is a neuropeptide that functions as a neurotransmitter and neuromodulator. A function of SP was identified as an "endogenous wound healing mechanism" that call bone marrow mesenchymal stem cells (BMSCs) from bone marrow reservoir to be engaged in the tissue repair (10,11). Use of SP appears to be a cost-effective treatment because of high efficacy of host MSC mobilization even with a single injection (12).

Another promising factor to be incorporated in scaffolds to induce bone regeneration is stromal-derived factor 1 α (SDF-1α) (13). It is a chemokine signaling molecule that demonstrates various functions such as regulating cell migration, cell growth, angiogenesis and it stimulates homing of BMSCs in bone regeneration (14,15).

In this study, we tested the pharmacologic recruitment of endogenous MSCs to enhance bone formation in an established rat model of recombinant human bone morphogenetic protein-2 (rhBMP-2) induced ectopic bone formation.

AIM OF THE WORK

The aim of this study was to investigate the effect of systemic injection of SP and/or local use of SDF-1α, on ectopic bone formation induced by low-dose rhBMP-2 in a rat model.

MATERIALS AND METHODS

Animals

The study was conducted on 70 adult male Wistar albino rats, with a body weight range 100-150 g. The animals were purchased and kept under standard laboratory conditions, at the Laboratory Animal Services Research Facility at

the Medical Physiology Department, Faculty of Medicine, Alexandria University, Egypt. Animals were maintained on a 12-h light–dark cycle with free access to food and water and room temperature 20-24^{0}C. The research was conducted in accordance with the approved guidelines of the Research Ethics Committee of Alexandria Faculty of Medicine and the National Institutes of Health guide for the care and use of Laboratory animals (NIH publications No.80-23, revised 1978).

Scaffold and reagents: The scaffolds used in the study were the Helistat® hemostatic absorbable collagen sponges (ACSs) that were purchased from Integra Lifesciences Corporation (New Jersey, USA; Cat. No.1690-ZZ). Substance P was purchased from Tocris Bioscience (Bristol UK; Cat. No. 1156) and reconstituted according to manufacturer's instructions using sterile water at a concentration of 1 mg/ml. Recombinant rat SDF-1α and recombinant human BMP-2 proteins were purchased from PeproTech (New Jersey, USA; Cat. No. 400-32A and 120-02C respectively) and prepared according to manufacturer's instructions using sterile water at a concentration of 0.1mg/ml.

Growth factors loading into absorbable collagen sponges (ACSs)

ACSs were cut into pieces (12.5 mm x 6.5 mm x 7 mm) (1). The ACSs were soaked under sterile conditions with 100 µl of saline, low-dose of rhBMP-2 (2.5 µg) or both low-dose of rhBMP-2 and SDF-1α (1 µg) for 1 hour at room temperature (1,16) The sponge was stored overnight at 4 C and implanted the next day (1).

Rat surgery for in vivo ectopic bone formation assay model

The animals were generally anaesthetized with intramuscular injection of ketamine hydrochloride (5 mg/kg body weight). The skin of the upper back was shaved and scrubbed with iodine. A vertical incision was made 1 cm from midline. After flap reflection, a subcutaneous pocket was prepared by blunt dissection for placement of the scaffolds (1).

The experimental design:

Group I (Saline control group; n=14) Each animal received a sterile ACS loaded with normal saline (1) .

Group II (rhBMP control group; n=14) Each animal received a sterile ACS loaded with 2.5 µg rhBMP-2 (16).

Group III (rhBMP/SDF-lα loaded group; n=14) Each animal received a sterile ACS loaded with 2.5 µg rhBMP-2 and 1 µg SDF-1α (16) .

Group IV (rhBMP loaded SP treated group; n=14) Each animal received a sterile ACS loaded with 2.5 µg rhBMP-2 (16). Substance P, at a dose of 5 nmol/kg, was intravenously (IV) injected via tail vein on implantation day and on postoperative day 1 (11, 16, 17).

Group V (rhBMP/SDF-1α loaded SP treated group; n=14) Each animal received a sterile ACS loaded with both 2.5 µg rhBMP-2 and 1 µg SDF-1 α. Substance P at a dose of 5 nmol/kg was IV injected via tail vein on implantation day and on postoperative day 1 (11, 16, 17).

N.B. Animals in Groups I, II and III received systemic injections of normal saline.

At the 28th day of implantation (16). Rats were euthanized using cervical dislocation. A back incision and a subcutaneous skin flap were made, ACS implant were carefully trimmed from the surrounding and retrieved. Explanted bony nodules (70 nodules) were handled in 2 ways:

- 40 explanted bony nodules (n=8 out of 14 per Group) were washed with iced saline, weighed, homogenized in 10 times (w/v) Phosphate Buffered Saline (PBS) (pH 7.4), followed by centrifugation at 10.000 x g for 15 minutes (18). The resultant supernatant was used for the biochemical colorimetric assay of bone

alkaline phosphatase (ALP) (19). and ELISA Assay osteopontin (OPN) as markers of osteoblast differentiation (20).

- 30 explanted bony nodules (n=6 out of 14 per Group) were fixed in 10% neutral buffered formaldehyde and stored at 4 C for radiological evaluation using a high energy micro-CT system (Micro-CT 1173, Bruker micro-CT, Kontich, Belgium). Newly formed bone was confirmed by histological examination after μCT evaluation. the specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 1 week, embedded in paraffin, and cut into 5 mm sections. Sections were stained with hematoxylin and eosin to show the cellularity and the morphology.

Statistical analysis

Statistical analysis and generation of graphs were carried out with the statistics program SPSS (version 21.0; SPSS Institute Inc., Chicago, USA). Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Since biochemical data were abnormally distributed, the Kruskall-Wallis non-parametric test was employed. If it was significant the non-parametric Mann–Whitney U-test was performed to compare two groups of samples. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

I. Biochemical assessment of new bone formation

1. Bone alkaline phosphatase activity (ALP) in IU/mg protein (Table 1; Figure 1)

Bone ALP activity showed statistically significant difference between the different study groups ($p < 0.001$) with a median of 0.015, 0.055, 0.189, 0.939 and 0.704 IU/mg protein for the studied groups respectively as compared with group I. Mann-Whitney test indicated that alkaline phosphatase activity was significantly increased in Group III (p=0.040), in Group IV and Group V (p=0.001) as compared with Group I. As compared with Group II, its activity was significantly increased in Group IV (p=0.009) as well as in Group V (p=0.001).

Table (1): Bone Alkaline Phospatase Activity (ALP in IU/mg protein): Comparison between Groups III, IV, V and the Control Groups (Group I and II)

	Group I (Saline Control) (n= 8)	**Group II (rhBMP Control) (n= 8)**	**Group III (rhBMP+ SDF-1α) (n= 8)**	**Group IV (rhBMP+SP) (n= 8)**	**Group V (rhBMP+ SDF-1α+SP) (n= 8)**	**KWp**
Bone ALP (IU/mg protein)						
Min. – Max.	0.004 – 0.036	0.003 – 0.120	0.004 – 1.425	0.021 – 1.727	0.215 – 1.838	
Mean± SD.	0.015± 0.011	0.059± 0.037	0.311± 0.471	0.938± 0.559	0.825± 0.549	<0.001*
Median (IQR)	0.015 (0.01 – 0.02)	0.055 (0.04 – 0.09)	0.189 (0.02 – 0.31)	0.939 (0.45 – 1.34)	0.704 (0.42 – 1.28)	
P1			0.040*	0.001*	0.001*	
P2			0.226	0.009*	0.001*	

ALP: Alkaline Phosphatase Activity; IU: international unit, rhBMP: recombinant human Bone Morphogenic Protein, SDF-1α: Stromal Derived Factor 1α SP: Substance P

IQR: Interquartile Range ^{KW}p: p value for Kruskal Wallis test

Significance between groups by using Mann Whitney test p_1: p value for comparing between Group I and other groups p_2: p value for comparing between Group II and other groups

*: Statistically significant at $p \leq 0.05$

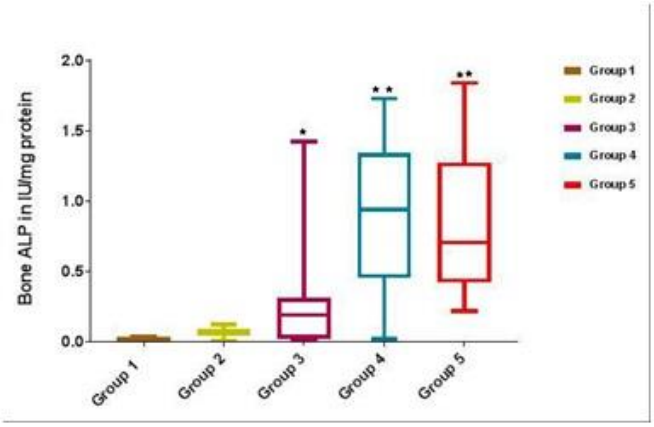

Figure (1): Box Plot of Bone Alkaline Phosphatase Activity (ALP) of different study groups;
The horizontal line indicates the median;
The box extends from the 25th to the 75th percentile
The whiskers indicate the largest and smallest observed values
IU: International Unit;
*: Statistically significant versus Group I;
**: Statistically significant versus Group I and Group II; $p \leq 0.05$;
Group I: Saline Control Group;
Group II: rhBMP Control Group;
Group III: rhBMP/SDF-lα loaded Group;
Group IV: rhBMP loaded SP treated Group;
Group V: rhBMP/SDF-1 α loaded SP treated

2. Bone osteopontin (OPN) assay in pg/mg protein (Table 2; Figure 2)

Regarding bone osteopontin level, there was a statistically insignificant difference between the different study groups (p=0.428). The median values in Group I, II, III, IV and Group V were 7.02, 7.36, 6.98, 8.6, and 8.62 pg/mg protein respectively.

II. Radiological assessment of new bone formation:

Group I (Saline Control group) was omitted from statistical analysis due to absence of bone formation.

A. Tissue mineral density (TMD) in mg Hydroxyapatite/cm^3(mgHA/cm^3) of the different studied groups (Table 3; Fig. 3)

The median TMD in Group II (rhBMP control group), Group III (rhBMP/SDF-lα loaded group), Group IV (rhBMP loaded SP treated group) and Group V (rhBMP/SDF-1α loaded SP treated group) were 20, 210, 440, and 400 mg HA/cm^3 respectively.

As compared with the Group II, TMD was significantly enhanced in Group III ($p \leq 0.024$), Group IV ($p \leq 0.006$) as well as in Group V ($p \leq 0.004$).

Table (2):

Bone Osteopontin (OPN in pg/mg protein): Comparison between Groups III, IV, V and the Control Groups (Group I and II)

	Group I (Saline Control) (n= 8)	**Group II (rhBMP Control) (n= 8)**	**Group III (rhBMP+ SDF-1α) (n= 8)**	**Group IV (rhBMP+SP) (n= 8)**	**Group V (rhBMP+ SDF-1α+SP) (n= 8)**	**KWp**
Bone OPN (pg/mg protein)						
Min. – Max.	3.22 – 8.92	3.21 – 10.7	3.11 – 10.12	3.86 – 11.39	4.93 – 11.3	0.428
Mean± SD.	7.02 ± 1.79	7.36± 2.62	6.98± 2.1	8.6± 2.24	8.45± 1.99	
Median (IQR)	7.02 (6.60 –8.59)	7.36 (5.41 –10.12)	6.98 (5.82 – 8.54)	8.6 (8.39 – 10.25)	8.62 (7.03 – 9.93)	

OPN: Osteopontin;
rhBMP: recombinant human Bone Morphogenic Protein
SDF-1α: Stromal Derived Factor 1α
SP: Substance P
IQR: Interquartile Range
KWp: p value for Kruskal Wallis tes

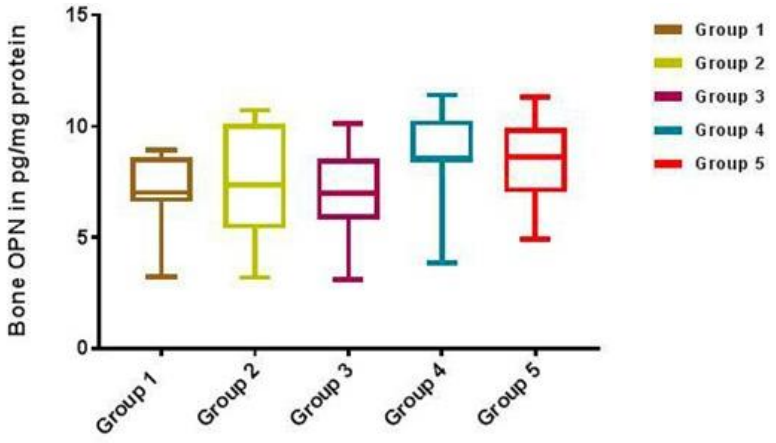

Figure (2): Box Plot of Bone Osteopontin (OPN) of different study groups;

The horizontal line indicates the median;
The box extends from the 25th to the 75th percentile
The whiskers indicate the largest and smallest observed values
Group I: Saline Control Group;
Group II: rhBMP Control Group;
Group III: rhBMP/SDF-lα loaded Group;
Group IV: rhBMP loaded SP treated Group;
Group V: rhBMP/SDF-1α loaded SP treated Group

Table (3): Tissue Mineral Density (TMD in mgHA/cm3): Comparison between Groups III, IV, V and Control Group II

	Group II (rhBMP Control) (n= 6)	**Group III (rhBMP+SDF-1α) (n= 6)**	**Group IV (rhBMP+SP) (n= 6)**	**Group V (rhBMP+ SDF-1α+SP) (n= 6)**	**KWp**
TMD (mgHA/cm3)					
Min. – Max.	0 – 170	30 – 350	160-460	340-450	<0.001*
Mean± SD.	40± 70	200± 110	390± 120	400± 30	
Median (IQR)	20 (0 – 80)	210 (120 – 260)	440 (330 – 460)	400 (380 – 420)	
P		0.024*	0.006*	0.004*	

TMD: Tissue Mineral Density; HA: Hydroxyapatite
rhBMP: recombinant human Bone Morphogenic Protein
SDF-1α: Stromal Derived Factor 1α
SP: Substance P
IQR: Interquartile Range
KWp: p value for Kruskal Wallis test
Significance between groups by using Mann Whitney test
p: p value for comparing between Group II and other groups
*: Statistically significant at $p \leq 0.05$

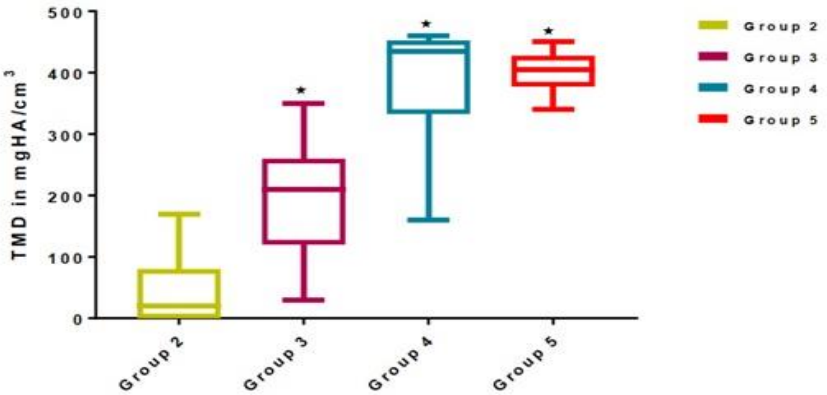

Figure (3): Box Plot of Tissue Mineral Density (TMD) of different study groups;

The horizontal line indicates the median;
The box extends from the 25th to the 75th percentile
The whiskers indicate the largest and smallest
*: Statistically significant versus Group II;
$p \leq 0.05$;
Group II: rhBMP Control Group;
Group III: rhBMP/SDF-lα loaded Group;
Group IV: rhBMP loaded SP treated Group;
Group V: rhBMP/SDF-1 α loaded SP treated

B. Bone volume (BV) in mm^3 of the different studied groups (Table 4; Figs.4-9)

There was no evidence of bone formation in 3 out of 6 bony nodules in Group II. Regarding the rest of the studied groups, the median values for BV in Group II (rhBMP control group), Group III (rhBMP/SDF-lα loaded group), Group IV (rhBMP loaded SP treated group) and Group V (rhBMP/SDF-1α loaded SP treated group) were 0.02, 0.18, 1.52, and 2.1 mm3 respectively.

As compared with Group II, SDF-1α loading (Group III) caused an insignificant increase in BV (p=0.076). However, SP treatment in Group IV and Group V resulted in a significant enhancement in BV (p=0.016 and p=0.004 respectively)

Table (4): Bone Volume (BV in mm^3): Comparison between Groups III, IV, V and Control Group II

	Group II (rhBMP Control) (n= 6)	**Group III (rhBMP+SDF-1α) (n= 6)**	**Group IV (rhBMP+SP) (n= 6)**	**Group V (rhBMP+ SDF-1α+SP) (n= 6)**	**KWp**
Bone volume (BV; mm3)					
Min. – Max.	0.0 – 0.59	0.07 – 2.06	0.03-5.4	1.13 -7.55	0.005*
Mean± SD.	0.12± 0.23	0.53 ± 0.78	2.04± 1.97	2.86± 2.43	
Median (IQR)	0.02 (0.0 – 0.24)	0.18 (0.07 – 0.99)	1.52 (0.51 – 3.68)	2.1 (1.29 – 4.10)	
P		0.076	0.016*	0.004*	

BV: Bone Volume
rhBMP: recombinant human Bone Morphogenic Protein
SDF-1α: Stromal Derived Factor 1α
SP: Substance P
IQR: Interquartile Range
KWp: p value for Kruskal Wallis test
Significance between groups by using Mann Whitney test
p: p value for comparing between Group II and other groups
*: Statistically significant at p≤ 0.05

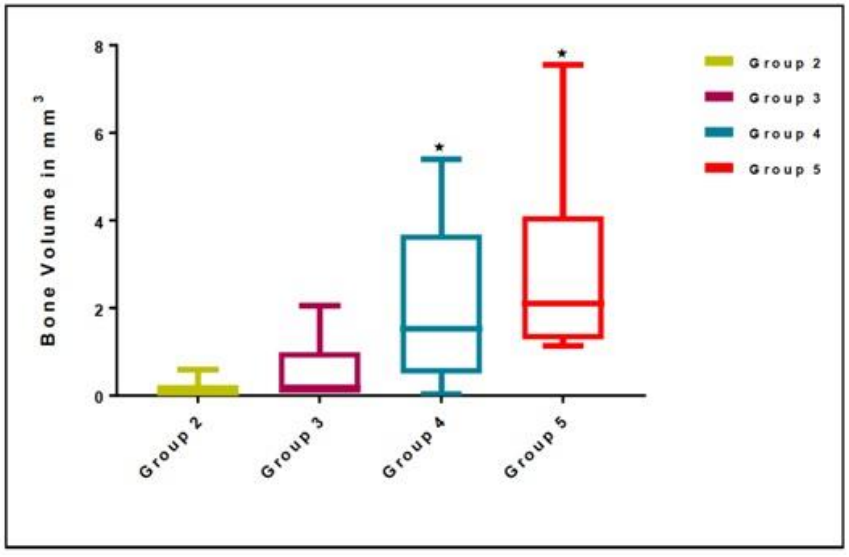

Figure (4): Box Plot of Bone Volume (BV) of different study groups

The horizontal line indicates the median;
The box extends from the 25th to the 75th percentile
The whiskers indicate the largest and smallest observed values
*: Statistically significant versus Group II;
p ≤ 0.05;
Group II: rhBMP Control Group;
Group III: rhBMP/SDF-lα loaded Group;
Group IV: rhBMP loaded SP treated Group;
Group V: rhBMP/SDF-1 α loaded SP treated

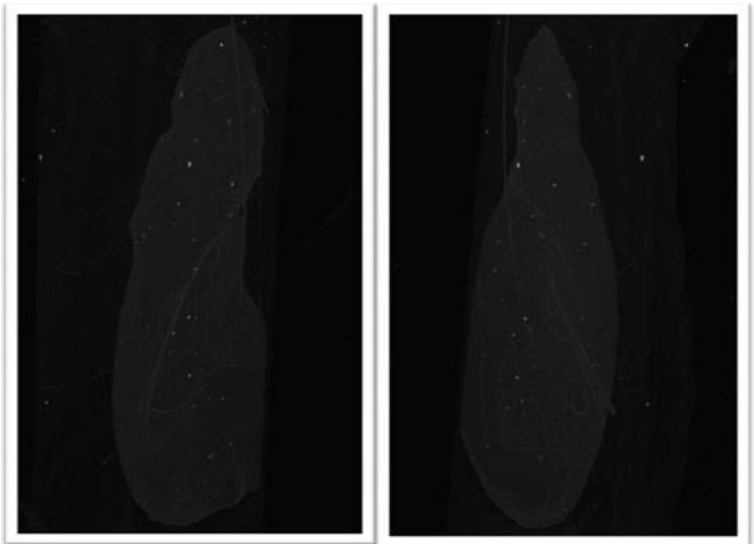

Figure (5): 3-D reconstruction of the implants; representative μCT image of Group I (Saline Control Group); different views; no evidence of bone formation

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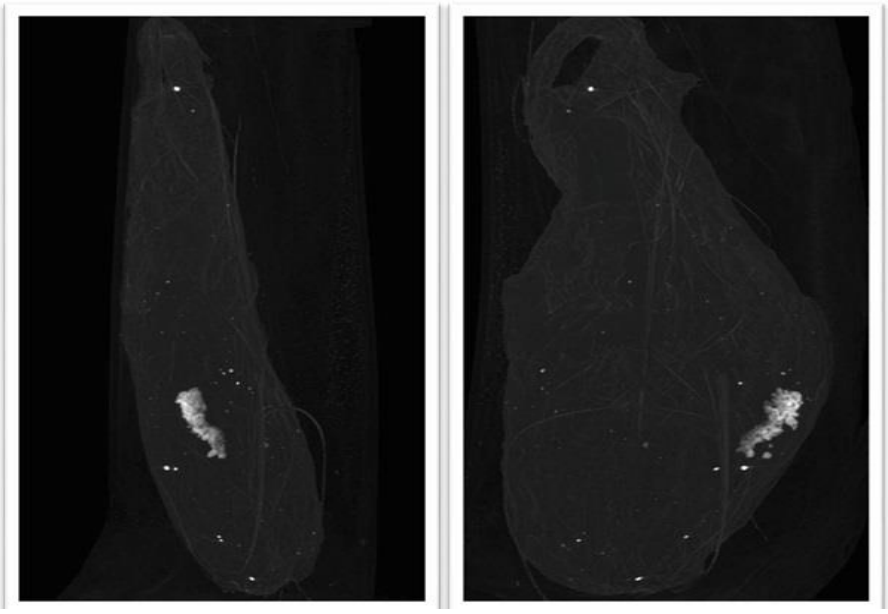

Figure (6): 3-D reconstruction of the implants; representative μCT image of Group II (rhBMP Control Group); different views; evidence of poor bone formation at the periphery of the implant

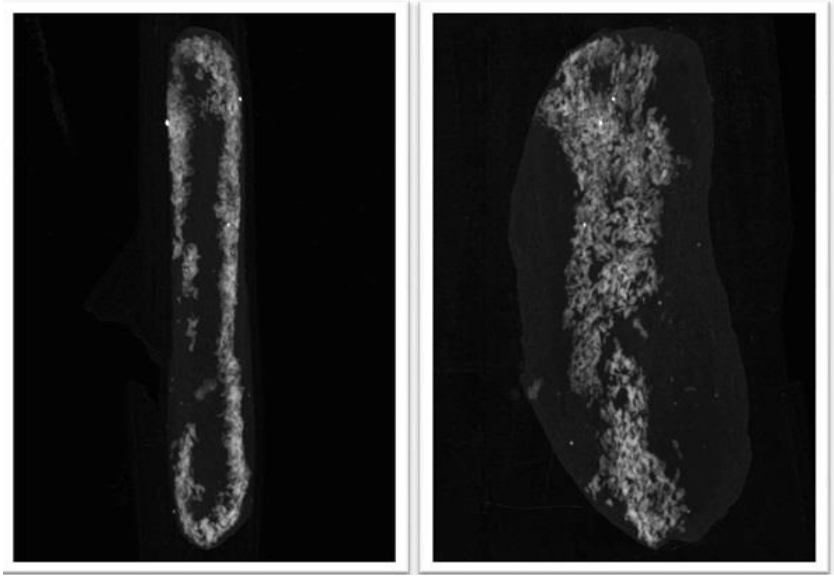

Figure (7): 3-D reconstruction of the implants; representative μCT image of Group III different views; evidence of bone formation bone formation mainly at the periphery of the implant

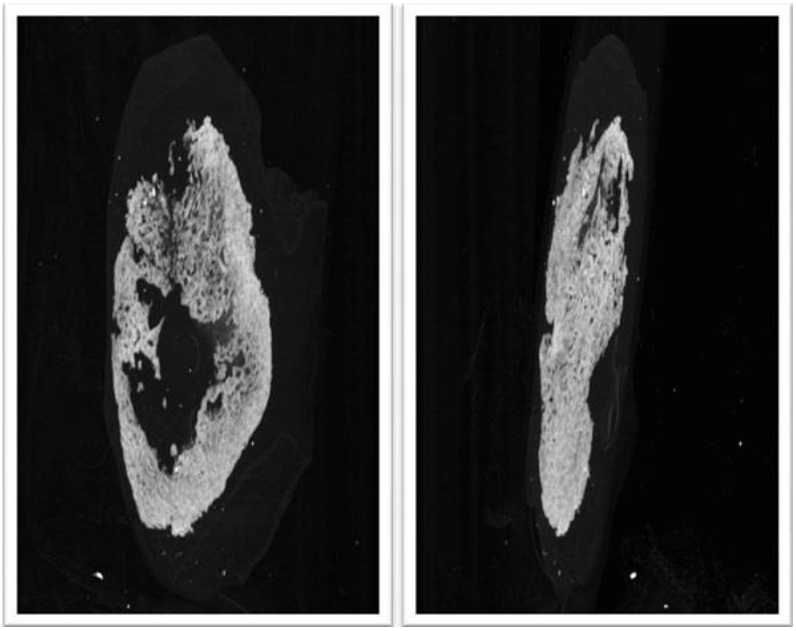

Figure (8): 3-D reconstruction of the implants; representative μCT image of Group IV; different views; evidence of bone formation occupying periphery as well as part of the implant center

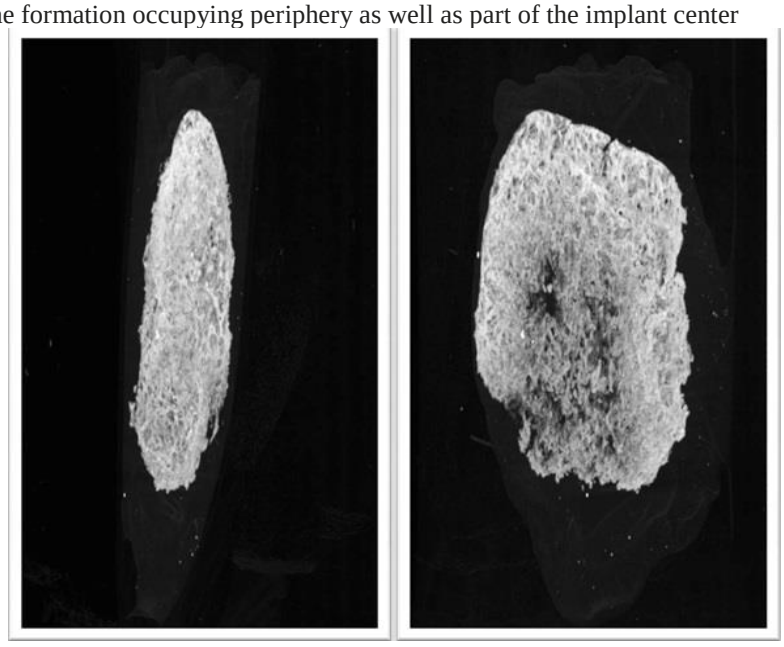

Figure (9): 3-D reconstruction of the implants; representative μCT image of Group V different views; evidence of bone formation occupying the whole implant (center and periphery)

C. Bone mineral content (BMC) in mg of the different studied groups (Table 5; Fig.10)

Median of BMC in Groups II, III, IV and V were 0.0, 0.04, 0.69, and 0.88 mg respectively. SDF-1α loading in Group III as compared with Group II, caused an increase in BMC that approached significance (p=0.053). However, SP treatments in group IV and V resulted in a significant augmentation of BMC (p=0.010 and 0.004 respectively).

Table 5 : Bone Mineral Content (BMC in mg): Comparison between Groups III, IV, V and Control Group II

	Group II (rhBMP Control) (n= 6)	Group III (rhBMP+SDF-1α) (n= 6)	Group IV (rhBMP+SP) (n= 6)	Group V (rhBMP+ SDF-1α+SP) (n= 6)	KWp
BMC (mg)					
Min. – Max.	0.0 – 0.1	0.0 – 0.73	0.0-2.49	0.44 -3.37	
Mean± SD.	0.02± 0.04	0.16 ± 0.28	0.92± 0.91	1.21 ± 1.12	0.003*
Median (IQR)	0.0 (0.0 – 0.04)	0.04 (0.01– 0.29)	0.69 (0.22 – 1.66)	0.88 (0.45– 1.75)	
P		0.053	0.010*	0.004*	

BMC: Bone Mineral Content rhBMP: recombinant human Bone Morphogenic Protein
SDF-1α: Stromal Derived Factor 1α SP: Substance P
IQR: Interquartile Range KWp: p value for Kruskal Wallis test
Significance between groups by using Mann Whitney test
p: p value for comparing between Group II and other groups
*: Statistically significant at p≤ 0.05

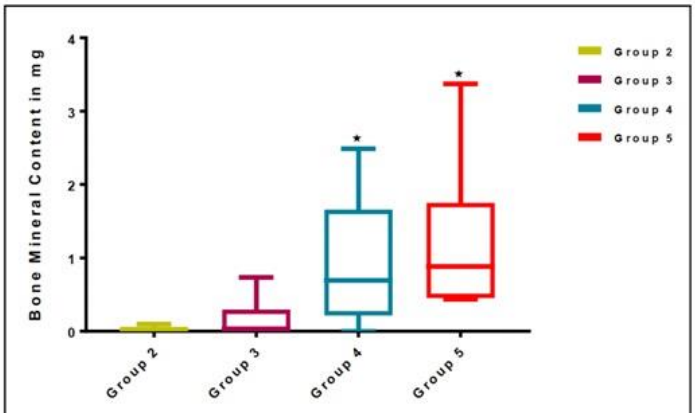

Figure (10): Box Plot of Bone Mineral Content (BMC) of different study groups
The horizontal line indicates the median; The box extends from the 25th to the 75th percentile
The whiskers indicate the largest and smallest observed values
*: Statistically significant versus Group II; p ≤ 0.05;
Group II: rhBMP control Group; Group III: rhBMP/SDF-lα loaded Group;
Group IV: rhBMP loaded SP treated Group; Group V: rhBMP/SDF- lα loaded SP treated Group

D. Trabecular thickness (Tb.Th) in µm of the different studied groups (Table 6) (Fig. 11)

The median values of the studied groups for Tb.Th were 0.01, 0.07, 0.08, and 0.08 µm in Group II, III, IV and Group V respectively.

As compared with the Group II (rhBMP control group), trabecular thickness was significantly increased in all test groups (p≤0.05).

Table (6): Trabecular Thickness (Tb.Th in µm) Comparison between Groups III, IV, V and Control Group II

	Group II (rhBMP Control) (n= 6)	Group III (rhBMP+SDF-1α) (n= 6)	Group IV (rhBMP+SP) (n= 6)	Group V (rhBMP+ SDF-1α+SP) (n= 6)	KWp
Tb.Th(µm)					
Min. – Max.	0.0 – 0.04	0.02 – 0.08	0.03-0.09	0.08 -0.09	
Mean± SD.	0.01± 0.02	0.06± 0.02	0.07±0.02	0.08± 0.0	
Median (IQR)	0.01 (0.0 – 0.03)	0_._0_7 _ _ (0.05- 0.07)	0_._0_8_ _ (0.06– 0.09)	0_._0_8_ _ (0.08– 0.09)	0.001*
P		0.010*	0.006*	0.004*	

TbTh: Trabecular Thickness rhBMP: recombinant human Bone Morphogenic Protein
SDF-1α: Stromal Derived Factor 1α SP: Substance P
IQR: Interquartile Range KWp: p value for Kruskal Wallis test
Significance between groups by using Mann Whitney test
p: p value for comparing between Group II and other groups
*: Statistically significant at p ≤ 0.05

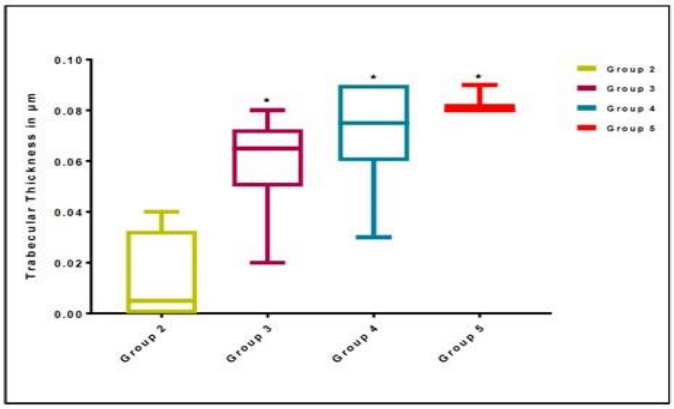

Figure (11): Box Plot of Trabecular Thickness (TbTh) of different study groups
The horizontal line indicates the median; The box extends from the 25th to the 75th percentile
The whiskers indicate the largest and smallest observed values
*: Statistically significant versus Group II; $p \leq 0.05$;
Group II: rhBMP control Group; Group III: rhBMP/SDF-lα loaded Group;
Group IV: rhBMP loaded SP treated Group; Group V: rhBMP/SDF- lα loaded SP treated Group

III. Histological assessment of new bone formation: (Fig. 12-16)

Microscopic examination of Group I revealed collagen fibers which were irregularly arranged with no evidence of formed bone. Noticeable amounts of engrafted inflammatory like-cells are also observed. (Fig. 12 a). Group II (rhBMP-2 control group) showed areas of cartilage formation marked by their purplish matrix and relatively large rounded chondrocytes within their lacunae. Few and thin acidophilic bone trabeculae lined by few flattened osteoblasts were also encountered (Fig.12 b, c, d). Group III exhibited more formation of bone trabeculae lined by cuboidal osteoblasts, while the osteocytes were also depicted within these trabeculae (Fig.13). Examination of group IV revealed more formation of bone trabeculae lined by cuboidal osteoblasts, while the osteocytes were also depicted within these trabeculae (Fig. 14). Multiple osteoclasts were also encounterd. Bone marrow-like tissue was noticed. (Fig.15) However, group V showed much more formation of bone trabeculae, delineating areas of bone marrow-like. Osteoblasts, osteocytes and osteoclasts were all encountered. An area of endochondral bone formation was also observed (Figs.16).

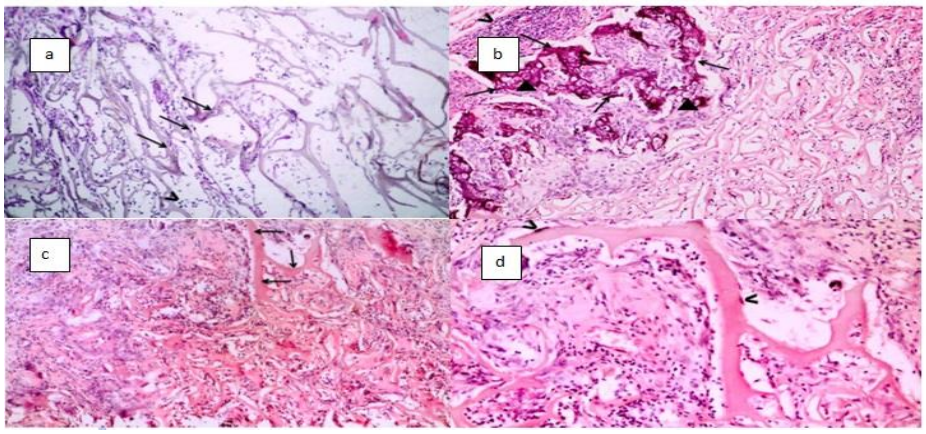

Figure (12): Photomicrograph of groups I & II**,** H&E stain.

- **a.** Group I (saline control) shows irregularly arranged collagen fibers (↑) without any evidence of new bone formation; inflammatory-like cells (^) are noticed; Mic. Mag. x 100
- **b.** Group II (rhBMP control) reveals areas of cartilage formation (↑). Noticeable amounts of engrafted inflammatory like-cells are also observed (^). Chondrocytes within lacunae (▲) are noticed; Mic.Mag. x 200
- **c.** Group II (rhBMP control) showing few and thin acidophilic bone trabeculae (↑);Mic. Mag. x 100
- **d.** Group II (rhBMP control) showing a few number of flattened osteoblasts (^) lying on the surface of these trabeculae; Mic. Mag. x 200

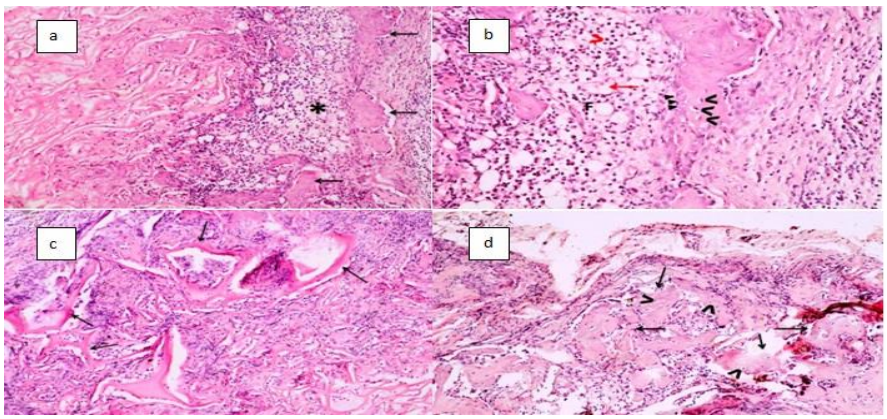

Figure (13): Photomicrograph of Groups III & 4**;** H&E stain

a. Group III (rhBMP/rhSDF-1α loaded group) exhibits bone trabeculae (↑) delinating an area of bone marrow-like tissue (*);Mic. Mag. x 100

b. A higher magnification photomicrograph of Group III (rhBMP/rhSDF-1α loaded group) shows cuboidal osteolasts (▲) lining the bone trabeculae while the osteocytes are seen within their lacunae (^) inside these trabeculae. Bone marrow-like tissue contains hemopoietic cells (red ^), fat cells (F) and blood vessels (red ↑); Mic. Mag. x 200.

c. Group III (rhBMP/rhSDF-1α loaded group) showing few bone trabeculae (black ↑); Mic. Mag. x 100

d. Group IV (rhBMP loaded SP treated Group) shows numerous bone trabeculae (black ↑) containing osteocytes within lacunae (^) Mic. Mag. x 100

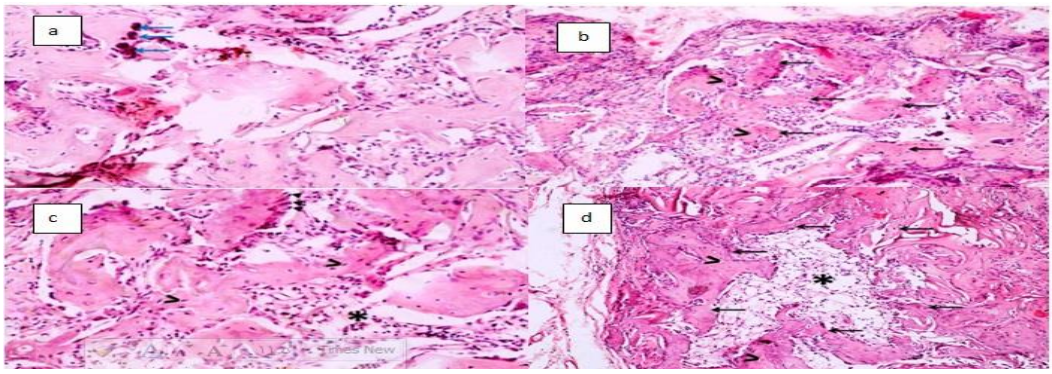

Figure (14): Photomicrograph of Groups IV & V; H&E stain

a. Group IV (rhBMP loaded SP treated Group) showing multiple osteoclasts (blue ↑); Mic. Mag. x 200

b. Group IV (rhBMP loaded SP treated Group) showing bone trabeculae (black ↑) containing osteocytes within lacunae (^) Mic.Mag. x 100

c. Group IV (rhBMP loaded SP treated Group) showing multiple cuboidal osteoblasts (▲) that are lining bone trabeculae and an area of bone marrow-like tissue (*) Mic. Mag. x 200

d. Group V (rhBMP/SDF-1α loaded SP treated group) revealing markedly obvious bone trabeculae (black ↑) containing osteocytes within lacunae (^) and surrounding an area of bone marrow-like tissue (*) Mic. Mag. x 100

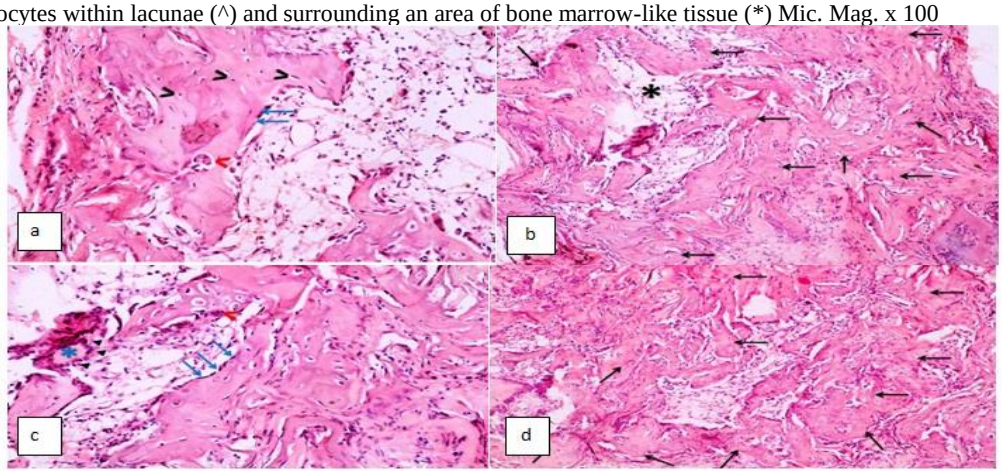

Figure (15): Photomicrograph of Group V (rhBMP/SDF-1α loaded SP treated group); H&E stain

a. showing flattened osteoblasts (blue arrow), osteocytes (^) as well as osteoclasts (red ^) Mic. Mag. x 200

b. showing numerous bone trabeculae (black ↑) and bone marrow-like tissue (*) Mic. Mag. x 100

c. showing flattened osteoblasts (blue arrow) lining the bone trabeculae while cuboidal osteoblasts (▲) are shown delineating an area of newly forming bone (blue *). Osteoclasts are also observed (red ^) Mic. Mag. x 200

d. showing markedly obvious bone trabeculae (black ↑) Mic. Mag. x 100

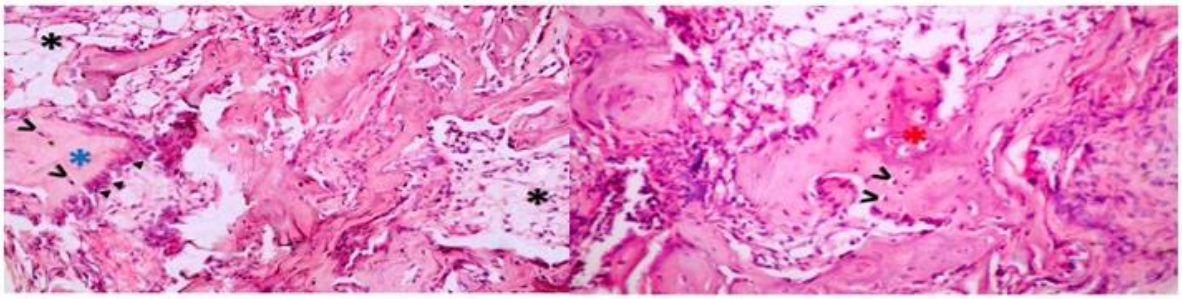

Figure (16): Photomicrograph of Group V (rhBMP/SDF-1α loaded SP treated group); H&E stain Mic. Mag. x 200

a. showing an area of a newly forming bone (blue *) demarcated by clusters of cuboidal osteoblasts (▲). Bone marrow-like tissue (*) are also noticed; Osteocytes within lacunae are noticed (^)

b. showing an area of endochondral bone formation (red *); Osteocytes within lacunae are noticed (^)

DISCUSSION

Bone tissue engineering (BTE) depend on: (a) osteogenic cells generate the bone tissue matrix, (b) a biocompatible framework, or scaffold that mimic the ECM deposition, (c) good vascularization and (d) morphogenetic signals to direct the cells (21).

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily, play a central role to induce bone growth, however, the dose-dependent side effects have prevented their widespread use. Combined use with other growth factors, that augment its osteogenic activity is an attractive solution to this problem (22,23).

Considering the indispensable role of SP in the osteogenic differentiation of MSCs (24,25). It was logical to speculate that its combined delivery with BMP-2 would enhance bone formation. Studies showed that the cytokine SDF-1α has potential to improve the bone regenerative effect of low BMP-2 concentrations. Notably, both proteins alone also provided a cumulative effect on MSC attraction toward the site of injury (26).

Hence, the objective of the current study was to investigate whether deliberate pharmacological stem cell mobilization by systemic SP and/or local SDF 1 α would enhance low-dose rhBMP-2 induced ectopic bone formation in a rat model.

In the present study, rats were treated with IV injections of either saline or SP on implantation day as well as day 1 postoperativly, of subcutaneous implantation of ACS's loaded with 2.5 μg rhBMP-2 or combination of 2.5 μg BMP-2 and SDF-1 α. After 28 days, bone formation in the explanted scaffolds was assessed biochemically for osteogenesis markers as well as radiologically and histologically.

In the present work, de novo mineralization and osteoblast differentiation were biochemically evaluated by bone alkaline phosphatase (ALP) activity assay due to the ease of measurement, cost efficiency and higher specificity in detecting small changes in bone formation (27). ALP activity was significantly increased with the IV SP treatment (Group III) as compared with the low-dose rhBMP-2 control group (Group II). This rhBMP dose would be too low to significantly induce bone formation according to Higashino et al (16). The mode of action of SP is apparently not only due to increased MSC mobilization but may be also due to an induction of osteoblast differentiation. ALP has crucial role in matrix mineralization and increase its activity denote differentiation to bone forming osteoblasts.

This finding is in accordance with previous studies of the effects of SP on osteoblast and osteoclast and reported that SP increases ALP expression and activity by stimulating osteoblast and osteoclast differentiation and function in vitro. It could potentially regulate local bone turnover in vivo (24, 28).

Osteoblastic differentiation was further evaluated by OPN expression using OPN quantitative ELISA assay. OPN levels showed insignificant changes between the study groups. Considering that OPN was reported in Group I, where bone forming activity is not expected to occur and Group II, where bone forming activity is supposed to be minimal, our finding implies that a physiological process other than bone formation may have been implicated in the uniform OPN signal.

OPN has been proved to be involved in inflammatory responses and is upregulated during wound healing(29). In the current work, considering the subcutaneous ectopic bone formation model as a healing wound as well, may further explain the uniform OPN signal that was reported in all study groups.

These OPN findings are comparable to the results of Kim et al (30). who reported an intense immunohistochemical staining for OPN near the newly formed bone. Importantly though, OPN also detected in spaces in which bone-forming activity was not observed histologically.

Ectopic bony nodules were assessed by μCT and revealed quantitative data about tissue mineral density, bone volume, bone mineral content as well as trabecular thickness. It revealed expectedly no evidence of bone formation in Group I (saline control group). In Group II (rhBMP-2 control group), 3 out of 6 implants newly formed bone was observed at the periphery indicating poor MSCs recruitment. This is consistent with Higashino et al (16). reported that 2.5 μg BMP-2 dose failed to make any bone. The μCT investigation showed that SDF-1α (Group III) did not potentiate suboptimal BMP-2 osteoinduction, insignificant improvement of BV and BMC. It showed bone formation on the superficial regions of the scaffold, imply the insufficient trafficking of MSCs into the implants and may explain the insignificant improvement of ALP activity of the same group. However, TMD and Tb.Th were significantly increased. This effect of SDF-1α on BV and BMC is in accordance with previous reports (1,31). Wise et al, (1). reported that co-treatment with 0.2 μg SDF-1α and 10 μg BMP-2 in rat did not yield elevated new BV and BMC compared with treatment with BMP-2 alone by 4 weeks.

In contrast to our results, a study reported that SDF-1α and a suboptimal dose of BMP-2 chemically conjugated on collagen scaffolds induced higher levels of ectopic bone formation. The different result is probably attributed to the type of bond (chemical conjugation in their study versus physical adsorption in the present study). The significantly enhanced TMD jn Group III may suggest that faster rate of mineralization by maturation of progenitor cells rather by increasing their number (32).

In the current study, μCT data revealed bone formation both on the superficial regions and internally with significantly enhanced TMD, BV, BMC and Tb.Th in Group IV and V as compared with Group II. This is in support of ALP activity results of the same groups. These suggested that systemic SP and systemic SP/local SDF-1α treatments efficiently augmented the recruitment

of MSCs into implants. Our findings are in agreement with other studies concluded that bone regeneration was significantly higher in the SP/BMP-2 dual delivery (33,34).

In the present work, when systemic SP was added to rhBMP-2/SDF-1α (Group V), the resulting osteoinduction was remarkable as proven by μCT (significantly enhanced BV and BMC), which is in accordance with our ALP activity assay testing. This synergistic effect can be explained by other studies indicating the efficiency of this system for MSCs recruitment, and vascularization to maintain cellular activity in tissue regeneration (6).

In view of our results and the previously mentioned information we suggest that, in Group V, systemic SP mobilized a large number of MSCs towards the implanted ACS because of the high concentration of SDF-1α resulting in promoting low-dose rhBMP-2 ectopic osteoinduction. In addition, considering the requirement of neovascularization occur before initiation of rhBMP-2 induced bone formation (35). We can postulate that neovascularization may have also been enhanced by SP/SDF-1α treatment.

H&E staining of Group I revealed expectedly no evidence of bone formation while Group II showed evidence of early, little bone formation as proven by areas of endochondral bone formation and few thin bone trabeulae lined by osteoblasts. In both groups, noticeable amount of engrafted inflammatory like-cells were observed, which may explain the strong OPN signals in these groups.

Histological examination of Group III showed evidence of active bone formation in the form of bone trabeculae lined by active cuboidal osteoblasts and contained osteocytes within lacunae. The trabeculae delineated fatty marrow-like tissue. These findings match the significantly enhanced TMD in this group and support our suggestion that SDF-1α accelerates the osteogenic differentiation of MSCs. On the other hand, some areas showed less newly formed bone which is in support of our BV and BMC results of the same group.

SP treatment in Groups IV and V resulted in even more new and active bone formation that was best when SP and SDF-1α were combined in Group V as evident by the numerous thick well organized trabecular masses lined by cuboidal osteoblasts and contained many osteocytes and the presence of fatty marrow-like tissue. These findings support our ALP activity results as well as our μCT investigation (significantly increased BV, BMC, trabeular thickness as compared to Group II).

In the present investigation, in addition to osteoblasts and osteocytes, osteoclasts were also encountered in SP treated groups implying that Hematopoietic Stem Cells (HSCs) are being mobilized into the circulating peripheral blood by SP and recruited to the ectopic implant site by SDF-1α. Osteoclastogenesis could then be happening in the absorbable collagen sponge due to differentiation of HSCs to osteoclasts under the influence rhBMP-2 induced osteoblastic differentiation of recruited MSCs.

To summarize, effective in situ bone tissue regeneration requires recruitment of enough numbers of host stem cells. In fulfillment of this intent, the current work provides biochemical, radiological as well as histological evidence of the efficacy of SP and SDF-1α combined treatment to stimulate trafficking of MSCs to an ectopic

implant site, in order to augment suboptimal dose of rhBMP-2 that alone would be insufficient for inducing full scale ectopic bone formation.

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