Association of Syndecan 4 and Osteocalcin with Application of BMP2 and BDNF in Bone Healing of Osteoporotic Rats

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Abstract

Background: Bone tissue contains multipotent stromal cells, which have the ability to differentiate into different specialized cells. Induced stem cells have been developed with various growth factors using many biomaterials, which are the most common strategiesin tissue engineering. Syndecan 4 and osteocalcin expressions during healing of bone defect in osteoporotic rat, along with the application of bone morphogenic protein 2 (BMP-2) and brain-derived neurotrophic factor (BDNF), were evaluated in this study.

Method: Ten normal rats and 10 induced osteoporotic rats were used in this study. Three mm bone defects were created in the femur of each rat, one on the left side and another one on the right side. The left bone defects were left to heal spontaneously without any application, while the right ones were treated with a combination of BMP2 and BDNF. Immunohistochemical evaluation was done for positive expression of syndecan 4 and osteocalcin in bone tissue during healing periods.

Results: Application of BMP2 and BDNF for both normal and osteoporotic rats increased the expressions of syndecan 4 and osteocalcin. In addition, expression of Syndecan 4 was decreased with increment period, while Osteocalcin expression showed the reverse. Statistical analysis revealed a significant difference regarding both positive expression of Syndecan 4 and osteocalcin in the treated groups compared to their untreated counterpart.

Conclusion: Application of BMP-2 and BDNF enhances syndecan 4 and osteocalcin expressions in bone repair of osteoporotic rat.

Keywords: *BMP-2, osteoporosis, bone cells, bone healing, BDNF, Syndecan 4, Osteocalcin.*

Introduction

Bone healing is a physiological proliferate process resulting in new bone formation that fills the hole or the fracture site[1,2]. Osteoporosis is a public health problem associated with an increased risk of bone fractures and it was thought to delay or impair the regenerative response[3,4]. In bone repair of osteoporotic animals, changes in the expressions of bone sialoprotein (BSP), alkaline phosphatase, osteopontin, osteocalcin and bone morphogenetic protein (BMP) were observed. Their decreased expressions have been found to delay healing of osteoporotic bone fractures[5,6].

Combination of biomaterials with BMP2 and other growth factors, was reported in many advanced bone healing studies[7,8]. Brain-derived neurotrophic factor (BDNF) has an influence on bone innervation, and may modulate the proliferation or differentiation of

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the developing bone cells[9] by acting as osteogenic and angiogenetic factors[10]. Syndecan-4 considered as the best plasma membrane proteoglycan and was found on cell surfaces of fibroblasts and epithelial cells[11,12]. Osteocalcin is a vitamin K-dependent, bone-specific protein and considered as specific osteoblastic bone marker. Osteocalcin expression was observed during bone healing process at the periods when intense mineralization of the bone tissue occurs[13,14].

All those factors seem to be necessary and have important roles in bone healing .And because there are few studies concerning this field, therefore, current study was conducted to study the effect of local application of BMP-2 and BDNF with the expression of Syndecan 4 and Osteocalcin in repaired bone tissue.

Method

Animal Models: Twenty female Wistar rats, weighted 0.25-0.30 kg, aged 4-5 months were used and kept in the Animal Department of National Center of Drug Control and Research, Iraq at a constant humidity and temperature of 23°C. Animal care was furnished in accordance with the National Council's guide. After 2 weeks of acclimatization, rats were randomly allocated to 4 groups as follows, normal rats with bone defect left to heal spontaneously (group A), osteoporosis-induced rats with bone defect left to heal spontaneously (group B), normal rats with bone defect treated with application of BMP-2 and BDNF (group C), and osteoporosis-induced rats with bone defect treated with application of BMP-2 and BDNF (group D).

Induction of Osteoporosis: Ten rats were anesthetized by ketamine and xylazine mixture. The skin area was shaved, washed using chlorhexidine scrub and ethanol 70 % and disinfected by povidone iodine.

Bilateral ovariectomy was carried out by single sagittal medial laparotomy process. Then 2 weeks later the rats received a daily intra-muscular injection of methylprednisolone hemi succinate (MPH) at dose (1 mg/kg) for 4 consecutive weeks.

Six weeks after ovariectomy, surgical bone defect was done. The rats were anesthetized generally with a mixture of 50 mg/kg BW ketamine and 2.5 mg/kg BW xylazine. Surgical technique was performed in rat femur to prepare two 3 mm-drill-holes, 1 hole on the left femur and another hole on the right. Same surgical procedure was also performed for normal rats. The holes on left femur were considered for the A and B groups of normal/ osteoporotic rats. The holes left to heal spontaneously without any application, just washed with normal saline, dried gently and sutured. Meanwhile the right holes were considered for the C and D groups. The holes were treated with a combination of 0.5 microliter BMP2 (rhBMP-2, Medtronic Sofamor Danek, TN, USA) and 1 microliter BDNF (ab9794, Abcam, UK), dried gently and sutured. Determination of effective dose for BMP2/ BDNF depends on previous studies[15,16] .

The animals were sacrificed by an overdose of carbon dioxide gas after surgical operation at the 7th and14th day. Bone holes along with their surrounding bones were excised with a surgical saw right away following the euthanasia. The excess tissue were dissected and the specimens removed with a 5–10 mm margin of surrounding bone. The specimens immediately were fixed into the 10% formaldehyde solution. Then, the specimens decalcified, dehydrated, embedded in wax and sliced in serial with 4-µm thickness.

Immunohistochemistry: Then immunohistochemistry was performed with polyclonal anti-Syndecan-4 (ab24511, Abcam, UK) and anti-Osteocalcin (OC4-30, ab13418, Abcam) antibodies. Positive peroxidase staining produced brown color on light microscopy. The percentages of positively stained cells were counted at 5 representative fields with (X40) magnification.

Immunohistochemistry results were quantified by counting the positive cell in each 100 cells in five fields (X40) of different sections. Then the mean and scoring of positive cells was estimated for each sample. The scoring was score 0, none; score 1, <10%; score 2, 10-50%; score 3, 51-80%; and score 4, >80 %[17] .

Statistical analysis: All records were entered into Excel spread sheets for evaluation with the Statistical package deal for Social studies (SPSS) (Chicago, IL, united states of America). The data were analyzed using one-way ANOVA test with multiple comparisons of LSD.

Results

Syndecan 4 findings: The immuno-reaction for Syndecan 4 and Osteocalcin illustrated an increment in their expression with application of BMP2 and BDNF for both normal and osteoprotic groups. Positive expression of Syndecan 4 illustrated by osteoblast, osteocyte in

normal group while in osteoporotic group the expression mostly observed in osteoclast, inflammatory cell and mesenchymal cell (Figure 1). On other hand, minimal decrease in the scoring recorded in the 14th day period in all groups in comparison to 7th day period, which revealed mostly, score two and three. Statistic results revealed a significant different value of positive expression of Syndecan in treated groups in comparison to those untreated, in both studied periods, (Table 1).

Osteocalcin findings: Osteocalcin expressed by osteoblast and osteocyte in all groups but an intense brown stain observed in groups (C & D) that treated with growth factors application (Figure 2). On other hand, the expression increased with period and recorded mostly, score 2 and 3 in the 14th day period in all groups except for group B (untreated osteoporotic) that showed score 1 in both periods. Statistical analysis revealed a significant different value for positive expression of osteocalcin in treated groups in comparison to those untreated, in both periods. Groups (A & C) revealed a non-significant difference in the 14th day period, (Table 2).

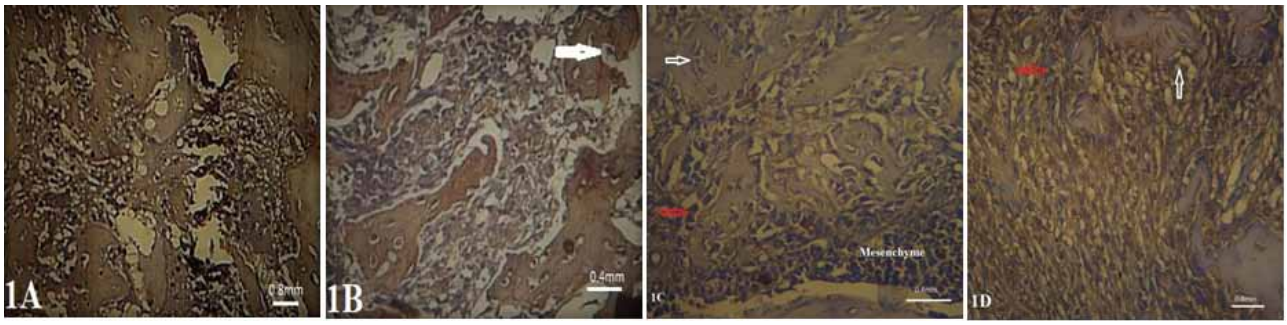

Figure (1). Positive expression of Syndecan 4. A: Immuno-reaction in normal group.DAB X10 (scale bar: 0.8mm); B: Immuno-reaction in osteoporotic group, osteoclast(arrow). DAB X20 (scale bar: 0.4mm); C: Immuno-reaction in normal group with application of growth factors osteoblast(red arrow), osteocyte(white arrow). DAB X20 (scale bar: 0.4mm); D: Immuno-reaction in osteoporotic group with application of growth factors, inflammatory cells (red arrow),osteoclast(white arrow). DAB X20 (scale bar: 0.4mm).

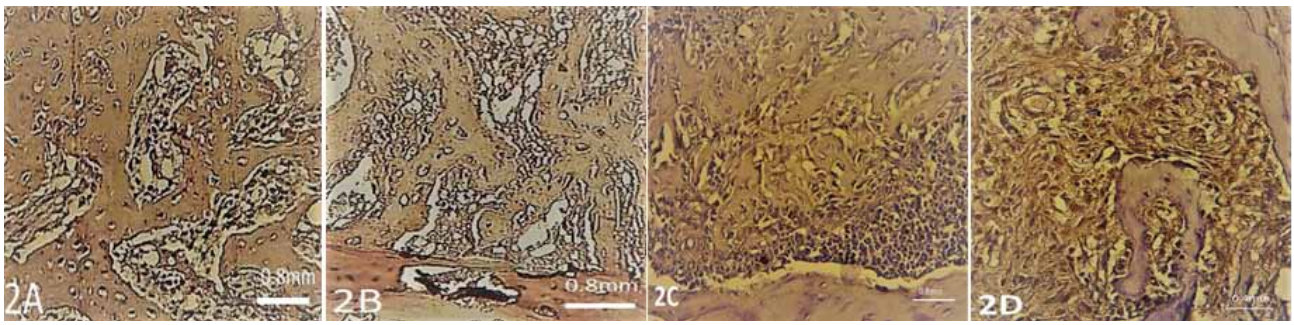

Figure (2). Positive expression of Osteocalcin. A: Immuno-reaction in normal group.DAB X10 (scale bar: 0.8mm); B: Immuno-reaction in osteoporotic group. DAB X10 (scale bar: 0.8mm); C: Immuno-reaction in normal group with application of growth factors. DAB X10 (scale bar: 0.8mm); D: Immuno-reaction in osteoporotic group with application of growth factors.DAB X20 (scale bar: 0.4mm).

Table (1): Observed Frequencies of the Studied immunohistochemical scoring of Syndecan 4 in different groups by different (S.O.V.) with LSD

Groups Score	7 Days	14 Days
Group A	1	2
Score - 1	2	2
Score - 2	2	1
Score - 3	0	0
Score - 4		

Groups Score	**7 Days**	**14 Days**
Group B	4	5
Score -- 1	1	0
Score - 2	0	0
Score - 3	0	0
Score - 4		
Group C	0	0
Score - 1	1	2
Score - 2	3	3
Score - 3	1	0
Score - 4		
Group D	2	3
Score -- 1	2	2
Score - 2	1	0
Score - 3	0	0
Score - 4		
LSD test		

Periods	**Groups**		**Mean Difference**	**Sig.(*)**	**C.S.**
7 day	Group A	Group C	-0.81	0.610	S
7 day	Group B	Group D	-0.77	0.882	HS
14 day	Group A	Group C	-0.23	1.310	NS
14 day	Group B	Group D	-0.67	0.621	S

Group A= Normal; Group B=Osteoporotic;Group C=Normal with application; Group D=osteoporotic with application. P>0.05 non significant

Table(2) Observed Frequencies of the Studied immunohistochemical scoring of osteocalcin in different groups by different (S.O.V.) with LSD

Groups Score	**7 Days**	**14 Days**
Group A	4	1
Score - 1	1	3
Score - 2	0	1
Score - 3	0	0
Score - 4		
Group B	4	5
Score - 1	1	0
Score - 2	0	0
Score - 3	0	0
Score - 4		
Group C	1	0
Score - 1	2	1
Score - 2	2	2
Score - 3	0	2
Score - 4		
Group D	4	1
Score - 1	1	2
Score - 2	0	2
Score - 3	0	0
Score - 4		
LSD test		

Periods	Groups		Mean Difference	Sig.(*)	C.S.
7 day	Group A	Group C	-0.89	0.710	S
7 day	Group B	Group D	-0.57	0.782	HS
14 day	Group A	Group C	-0.63	0.910	HS
14 day	Group B	Group D	-0.47	0.721	S

Group A= Normal;Group B=Osteoporotic;Group C=Normal with application;Group D=osteoporotic with application. P>0.05 non-significant

Discussion

The present results showed an increment of the expression of both Syndecan 4 and Osteocalcin in treated groups with local application of growth factors in comparison to untreated. These results could be attributed to the cooperation of many factors related to the presence of BMP2 which enhanced many events, including the differentiation of mesenchymal stem cells (MSCs) into osteoblast and the enhancement of angiogenesis[18]. The BMPs potently record to induce osteogenic differentiation [6] and might be involved in the chemotactic recruitment of osteoblasts during new bone formation[5]. Moreover, BDNF has an effects on vascular endothelial growth factor (VEGF) that stimulated vascularization and promote bone formation[19]. A study revealed a delay in the healing process of osteoporotic rats in comparison to that in normal rats, where they detected disturbance of new bone formation accompanied with the reduction in bone resorption[6]. Other study used MBG particles loaded with BDNF filled fracture gaps in osteoporotic mice. The study showed increase in bone formation, and reported that this growth factor (BDNF) appears as potential drug suitable to stimulate bone formation during fracture healing in osteoporosis[20]. In the present study, the application of the growth factors seem to play an effect on the activity of cells related to bone healing, specifically in the osteoporotic rats that had a defects in differentiation, vascularization and in expression of many proteins including syndecan-4 and osteocalcin[21].

Furthermore, in the present results, the local application of combined BMP-2 and BDNF, might act as a bioactive molecules, stimulating bone cell proliferation that resulted in an increment of expression of Syndecan-4, specifically in the 7th day (the time of bone apposition). Moreover, an increment of Syndecan-4 enhanced the interaction of involved cells with the extracellular matrix, anticoagulants, and other growth-factors which activate cell adhesion and cell migration, and then enhance bone healing[22,23]. A study revealed that Syndecan-4 contains binding sites for various proteins, growth factors, and cytokines. In addition, it is involved in the influence bone structure and fracture healing[12] .

Osteocalcin considered as a marker of osteoblastic activity and specific marker of bone metabolism,therefore, the present results detected the expression of osteocalcin in osteoblasts and osteocytes. These cells may play a role in the remodeling of extracellular matrices with expression of syndecan 4 during healing of the bone defect. Osteocalcin expression observed to be increased in the 14th day, the period of mineralization of bone tissue during healing process[24,14]. A study observed that at seven days neo-formed trabeculae bone labelled with a small quantity of osteocalcin, while at 14 days a larger quantity of deposited trabeculae bone with higher osteocalcin values were recorded, our results were compatible with these findings[25].

Conclusions

Local application of BMP-2 and BDNF enhanced both the expression of syndecan-4 and osteocalcin of osteoporotic rats.

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Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq.

Conflict of Interest: None

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