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Localization Of Procollagen Type I N-Terminal Propeptide in Bone Healing Treated by Local Application of Moringa Oliefera /Marine Collagen in Rats

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Abstract

Backgrounds: Bones healing is a complex multi steps process, Moringa Oliefera"s flavonoids and Marine collagen enhance minerals density of bones.Objectives: Differences in healing process in bones where evaluated in this study among bone defects that heal normally (control group) and (experimental groups) using Marine collagen (MC), Moringa oleifera extract (MO) and combination of (MO &MC). Material and Methods: in this study twenty albino rats, after anesthesia bone defects were created in both femurs by drilling. In ten rats, the right bone defects left to heal normally, and left MO added to the bone defects, in other ten rats MC added to the right bone defects, left defects treated with MO & MC. Rats were scarified after 2 and 4 weeks. Immunohistochemical analysis of bone marrow stromal cells, osteocytes, osteoblasts and osteoclasts done using Procollagen type I N-terminal Propeptide (PINP). Results: Immunohistochemical result revealed stronger positive expression of PINP in bone defects treated with (MO&MC) than that other groups Conclusions: Treatment of bone defects with MO and MC showed increase osteogenic capacity by increase the immunoreactivity of PINP.

Introduction:

Bone repair is a complex process appeared in many steps. Many types of cells undergo proliferation, migration, activation ,and differentiation, these cells include osteoblast, osteocyte, osteoclast and osteoproginetor cells (1). Bone has three healing phases: Inflammatory, Reparative and Remodeling Phases (2). Leafs, roots, flowers, and seed coats of Moringa oleifera (MO) contains many types of benefit flavonoids(3). Many studies evaluate hypocholesterolemic and anti-obesity activity of Moringa oleifera leaves(4). Favonoids in Moringa olifeira ethanolic extract have ability to induce osteogenic differentiation in mesenchymal stem cells(5) Soekobagiono study the effect of combination of demineralized freeze-dried bone bovine xenograft DFDBBX and Moringa leaf extract after teeth extraction in Cavia cobaya rats and found that sockets had decrease the number of receptor activator of nuclear factor kappa-B ligand (RANKL) expressions in on the s7th and 30th days (6).

Marine collagen (MC) that derived from fish, seaweeds, sponges, and jellyfish of marine offers benefits over mammalian collagen(7). In many biomedical researchers, MC has concidered as a substitute of mammalian collagen(8). Marine collagen effect on cartilage substance had been studied clinically and proven that MC lead to matrix synthesis enhancement and osteoarthritic pain reduction(9). In many studies, it had been revealed that biomaterial scaffolds base of MC could utilized as bone tissue substitutes and could enhance bone regeneration (10). MC promote bone formation significantly, and this approved by up regulated osteogenic markers expression (11).

Collagen synthesized in the form of pre procollagen. These precursor molecules are characterized by short terminal extension-peptides: the amino (N-) terminal propeptide (PINP) and the carboxy (C-) terminal propeptide (PICP) (12). Pro collagen type I N-terminal propeptides (PINP) cleave from the amino (N)-terminal end by action of procollagen N-proteinase resulting in mature type I collagen formation (13). The serum levels of PINP and PICP are considered an index of collagen synthesis and thus of bone formation because they secreted in a 1:1 ratio with new collagen molecules (14) .

many reviews of bone turnover markers have published by the International Federation of Clinical Chemistry and the National Bone Health Alliance, recommend PINP as the reference biochemical marker of bone formation(15) .

Expression of PINP peaks on day ten post-fracture in human. This suggests that PINP may play a more important role during fracture healing, since its level peaks during the critical phase of chondrogenesis (16)

Al-Ghaban and Jassem ,2020 study the immunohistochemical expression of PINP during bone healing process after red clover treatment and found that there was strong expression of osteoblast, osteocyte and osteoproginetor cells in almost all groups but the maximum expression found at 2 weeks duration(17) .

Materials And Methods

Preparation of Moringa olifeira extract

Extract of Moringa olifeira was prepared in College of Education/ University of Samarra/ department of chemistry. 200 gm weight grinded dried MO seeds collected from local market, Extraction was done using Soxhlet extractor using 70% ethanol for about 72 hours, fallowed by using rotary evaporator to have thick jelly solution. MO extract saved in (5-10C) refrigerator until be used in vivo

Animal preparation

1. in this study, ethical approval of animal experiments of College of Dentistry/ University of Baghdad were used for experimental procedures. Animal house stuff in Collage of Farming/ University of Tikrit were responsible for supervision and nursing.
2. 20 males Albino rats, four to five months age with 350-450 gm weight were used in this experimental study.
3. After anesthetization of animels, both femurs on distal sides were prepared for surgery by shaving and exposure of femur bone (Fig.:1)

4. By intermitted drilling and continuous cooling with normal saline, intrabony defects were performed by microengine (2mm in diameter and 3mm in depth) in both right and left femurs (Fig.:2) 5. In ten rats the right bony defects were left to heal normally, while the left bone defects were treated with 0.5 ml of Moringa oliefera extract using micropipette. In the other ten rats the right bone defects were treated with 0.5 mg of Marine collagen and the left bone defect were treated with combination of 0.25 mg Marine collagen and 0.25 ml Moringa oleifera extract.
6. Animals were sacrificed by overdose anesthesia after two and four weeks of surgery (10 rats for each healing interval). Bone specimen were stored in 10% freshly prepared formalin.

Immunohistochemical preparation

Monoclonal antibody employed in this study for procollagen N terminal propeptide (PINP) from ABCAM company UK (ab64409).
10% freshly prepared formalin were used for fixation process for 24 hours, then decalcification process started using 10% formic acid for (3-8 days), paraffin wax used for embedding, bone blocks were sectioned by microtome for serial sections of 4μm was taken and placed on charge slide.
Immunohistochemical staining was done by using Procollagen type I N-terminal Propeptide.
Immunohistochemical analysis of PINP in bone marrow stromal cells ,osteoblast, osteocyte and osteoclast cells per mm2 were counted in both 2 and 4 weeks durations.
Scoring was done under light microscope by calculating the mean value of cell number of positive cells because the staining intensity was not uniform. This procedure was repeated for four sections of the each block then the mean value of the 4 sections and mean score were calculated at magnifying power lens X 40.The scoring was graded as follows:
0 – Negative scores of cells from 0-4 (-)
1- Weak scores of cells from 5-8 (+)
2- Moderate scores of cells from 9-12 (+ +)
3 –Strong positive scores of cells more than 12 (+ + +)

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 25 used for data analyzes. Analysis of Variance (ANOVA) and Independent t-test were used to compare the continuous variables accordingly. P-value less than 0.05 was considered significant.

Results

immunohistochemical finding of control group showed positive expression of Procollagen type I N-terminal Propeptide seen in bone marrow stromal cells, osteocytes, osteoblasts and osteoclasts, while negative expression seen in trabecular areas and in inflammatory cells Fig. (3 and 4). In MO group strong expression seen at 2 weeks duration and moderate expression observed at 4 weeks duration Fig. (5 and 6).
In MC group moderate expression observed at 2weeks while weak expression seen at 4 weeks duration Fig. (7 and 8). In combination group, in 2 weeks duration strong positive expression observed and moderate expression seen at 4 weeks duration ;Fig.s (9 and 10)

Statistical finding

Table:1 represented the LSD tests which were used for group comparison difference in positive expression of BMSCs number in each healing duration. The results revealed highly significant difference in mean number of positive expression of BMSCs between control and combination groups in four weeks healing duration. Also significant difference observed between Marine collagen and combination and between control and combination groups in two weeks healing duration Table:2 represented the LSD tests which were used to group comparison difference in positive expression of osteocyte number in two weeks healing duration. The results revealed high significant difference in mean number of positive expression of osteocyte between control and combination groups and significant difference between Marine collagen and combination groups. Table:3 described the statistical analysis of difference mean value of osteoblasts positive have expression to PINP antibody in each group between two and four weeks healing durations. Results showed high significant difference in all groups.

Table:4 described the statistical analysis of difference mean value of osteoclasts positive have expression to PINP antibody in each group between two and four weeks healing durations. Results showed significant difference in Moringa oleifera and combination groups.

Discussion:

Rats as experimental animals were selected in the present study since they were easy to handle and had metabolic responses similar to humans and have the ability to perform surgical procedures (18).

Polyphenols and flavonoids (phytochemicals) in Moringa oleifera proven stimulant effects on osteoblast differentiation and proliferation, besides inhibitory effect on osteoclasts. (19)

Collagen from marine animals had ability of modulating inflammatory processes after an injury, accelerating soft and hard tissue healing and stimulating new angiogenesis, thus it had been used as a promising material for tissue engineering (20) Grippingly, while the formation of bone at early steps of the modest healing course, heightest expression of osteogenic factors including PINP were vital proteins of osteoblasts to be differented during the reparative and physiological osteogenesis (21) Procllagen N- terminal propeptid (PINP) predominantly expressed in mesenchymal tissues and bone cells. Its produced by many cell types, including bone marrow cells, osteoblasts, osteocyte and fibroblasts, it is secreted in a latent form that must be activated to mediate its effects(22). In the present study immunohistological finding revealed that there was positive expression of PINP in osteoprogenitor cells, fibroblasts, osteoblasts and osteocytes but in different rates between groups. While negative expression seen in trabecular bone and inflammatory cells in almost all studied groups, this finding agree with (Al-Ghaban and Jassem) who study immunohistochemical localization of PINP during bone healing after treatment with red clover oil(17). Osteoblast and osteocyte showed strong positive expression for PINP after 2 weeks of treatment with Moringa oliefera groups when compared with control group. This suggesting the potential role of Moringa oliefera in modulating the healing responses. This finding agree with (Soekobagiono et al.) who reveal increase in collagen type I expression in osteoblasts in socket area after treatment with Moringa oliefera extract(6).

The current study showed higher mean value of positive expression of PINP by bone marrow stromal cells at 2 and 4 weeks in all experimental groups that treated by Moringa oliefera, Marine collagen and combination groups than that in control group. Maximum mean value of positive expression for PINP in BMSCs seen in combination group at 2 weeks healing duration but reduced in 4 weeks healing duration in almost all groups. This finding agree with (Al-Ghaban and Jassem) (17)

Maximum mean value of positively expressed osteoblasts to PINP observed in combination group in 2 weeks healing duration, this finding reveal that combined effects of flavonoids in Moringa Oliefera and Marine collagen in enhancing osteogenic activity. This finding agrees with (Ivaska et al.) who monitor PINP level after 2, 8 and 12 weeks after bone fracture and found that PINP serum level peak in 2 weeks post fracture accident, but it was decreased in the following 2–3 months and return to a stable level close to that before fracture.(23) This finding also agree with (Hitz et al.) who found that PINP serum level rise during the early 2 weeks after fracture, but it had returned to baseline after 4 months.(24)

At 4 weeks interval, the experimental and control group showed a positive PINP expression in osteoblasts and osteocytes that located within the new bone in addition to positive expression of bone marrow stromal cells to PINP. This agree with (Yan et al.) who study level PINP after hip fracture (3, 7, 14, 30 and 365 days) and found the percentage changes of median of serum PINP continuously elevated, peak values seen after 14 days, and PINP level decreased until day 365 (25) BMSCs expression to PINP was decrease with time at the defect area due to increase bone deposition and reduce bone marrow area. The result coincide with (Song) who found that the peak expression of PINP was seen at day ten post-fracture in human. This suggests that PINP may play a more important role during fracture healing,

since its level peaks during the critical phase of chondrogenesis. (16) Finally, after long web search for the last few years almost no previous study was found concerned with local use of combination of flavonoid extract of Moringa oleifera and Marine collagen for bone healing, so this study regard as the first one.

The results of this combination treatment illustrate enhancement of bone healing in the defect area by increase trabecular bone formation and mineralization due to increase in the osteoblast number and their activity.

Fig. (1) Muscle dissection & bone exposure

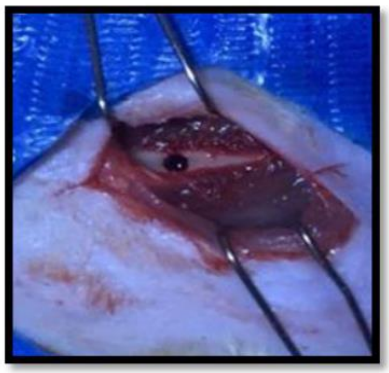

Fig. (2) Bone defect

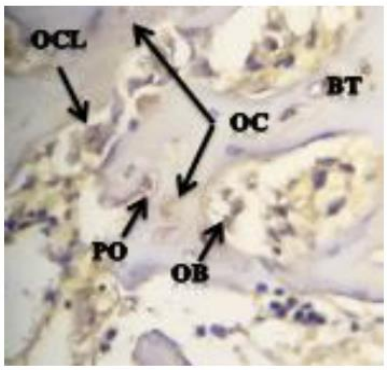

Fig. (3) View of control group showed negatively stained bone trabeculae (BT) and positively stained osteoclast (OCL), osteoblast (OB), osteocytes (OC), and preosteocyte (PO) . DAB stain with hematoxylin counter stain X 40 .

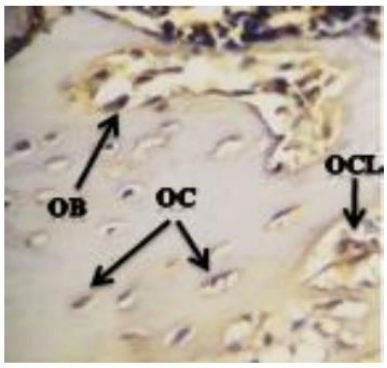

Fig. (4):- View of 4 weeks duration of control group showed positive expression of osteocytes(OC), osteoblast (OB) and osteoclast (OCL).DAB stain with counter stain hematoxylin x40

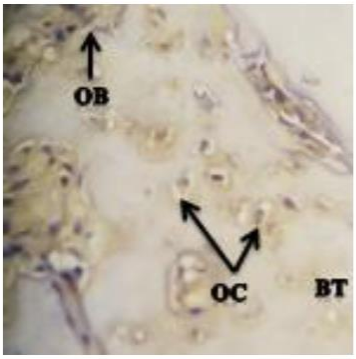

Fig. (5) Positive localization of PINP in (MO) group for 2 week in osteocyte (OC) and osteoblast(OB). Negative expression of trabecular bone(BT). DAB stain with counter stain hematoxylin X40.

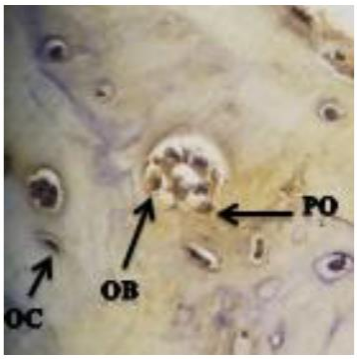

Fig. (6) View of 4 weeks duration of (MO) group showed mature bone contain positive expressed preosteocyte (PO) osteocytes(OC) and osteoblast (OB).DAB stain with counter stain hematoxylin x40

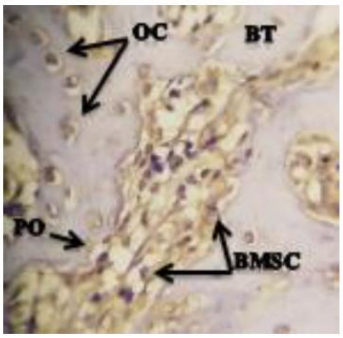

Fig. (7) View of 2weeks duration of (MC) group showed negatively stained woven bone (WB) and inflammatory cells (IC) and positively stained osteocytes (OC) and bone marrow stromal cells (BMSC). DAB stain with hematoxylin counter stain X 40 .

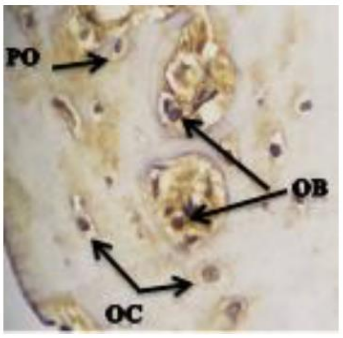

Fig. (8):- View of 4 weeks duration of (MC) group with positive expression of osteocytes(OC), preosteocytes(PO) and osteoblast (OB). DAB stain with counter stain hematoxylin x40.

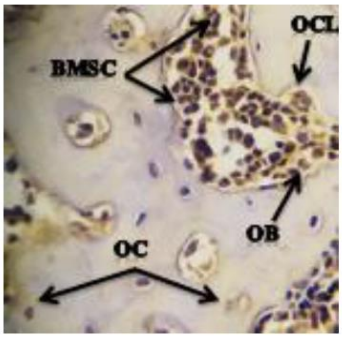

Fig. (9):- View of 2weeks duration of combination (MO & MC) group showed positive expression of osteocytes (OC), osteoblast (OB) and osteoclast (OCL). DAB stain with counter stain hematoxylin X40.

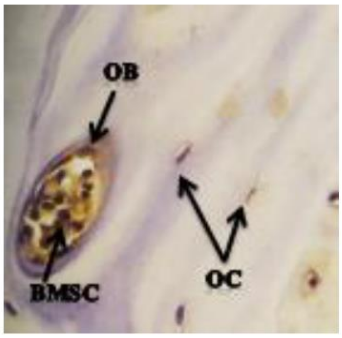

Fig. (10): View of 4 weeks duration of combination(MO & MC) group with positive expression of osteocytes(OC), osteoblast (OB) and bone marrow stromal cells (BMSC). DAB stain with counter stain hematoxylin x40

Table 1 The LSD test to confirm the differences between groups in BMSCs after 2 and 4 weeks

Duration	Study group				P – Value
	CONT Mean ± SD	MO Mean ± SD	MC Mean ± SD	MO & MC Mean ± SD	
2 weeks	9.4 ± 2.1	12.0 ± 2.5	-	-	0.233
	9.4 ± 2.1	-	11.2 ± 2.4	-	0.264
	9.4 ± 2.1	-	-	12.6 ± 3.5	0.012*
	-	12.0 ± 2.5	11.2 ± 2.4	-	0.256
	-	12.0 ± 2.5	-	12.6 ± 3.5	0.219
	-	-	11.2 ± 2.4	12.6 ± 3.5	0.026*
4 weeks	5. ± 1.7	6.0 ± 1.6	-	-	0.065
	5.0 ± 1.7	-	7.0 ± 1.6	-	0.308
	5.0 ± 1.7	-	-	6.2 ± 2.3	0.005**
	-	6.0 ± 1.6	7.0 ± 1.6	-	0.366
	-	6.0 ± 1.6	-	6.2 ± 2.3	0.236
	-	-	7.0 ± 1.6	6.2 ± 2.3	0.076

Table 2: The LSD tests to confirm the differences occurred between groups in osteocyte after two weeks

Mean of osteocyte after two weeks	Study group				P - Value
	CONT Mean ± SD	MO Mean ± SD	MC Mean ± SD	MO & MC Mean ± SD	
	5.8 ± 1.3	7.4 ± 2.3	-	-	0.143
	5.8 ± 1.3	-	6.6 ± 1.1	-	0.453
	5.8 ± 1.3	-	-	9.0 ± 1.6	0.007**
	-	7.4 ± 2.3	6.6 ± 1.1	-	0.453
	-	7.4 ± 2.3	-	9.0 ± 1.6	0.143
	-	-	6.6 ± 1.1	9.0 ± 1.6	0.035*

Table 3: Durations comparison difference by t-test for osteoblast in each group

Study Group	Duration	Mean ± SD	T test	P – Value
CONT	2 weeks	9.2 ± 1.9	0.98	0.004**
	4 weeks	4.8 ± 2.2		
MO	2 weeks	11.2 ± 2.6	1.25	0.003**
	4 weeks	5.6 ± 2.3		
MC	2 weeks	10.6 ± 1.3	1.21	0.003**
	4 weeks	5.8 ± 1.5		
MO & MC	2 weeks	13.0 ± 1.6	2.29	0.000**
	4 weeks	6.6 ± 1.8		

Table 4: Group comparison difference by ANOVA test for osteoclast in each healing duration

Duration	Study Group	Osteoclasts		
2w	Mean ± SD	Range	F value	P – Value
CONT	1.2 ± 0.54	1.0 – 2.0	3.333	0.046*
MO	0.6 ± 0.54	0 – 1.0		
MC	0.9 ± 0.83	0 – 2.0		
MO & MC	0.5 ± 0.44	0 – 1.0		
4w			0.098	0.96
CONT	0.25 ± 0.25	0 – 0.5		
MO	0.15 ± 0.13	0 – 0.25		
MC	0.25 ± 0.43	0 – 1.0		
MO & MC	0.15 ± 0.44	0 – 1.0		

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