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THE EFFECTS OF HYALURONIC ACID ON BONE-IMPLANT INTERFACE IN RABBITS (IMMUNOHISTOCHEMICAL STUDY FOR TNF-α)

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ABSTRACT
Various techniques of surface treatments have been applied to enhance surface texture of titanium implants, which support the mechanism of osseointegration. One of these techniques is by using natural polymers such as hyaluronic acid. The aim of the present study was to evaluate the effects of hyaluronic acid on bone-implant interface by immunohistochemical estimation of TNF- α in experimental and control groups. Sixty machined surface implants from commercially pure titanium rod will be inserted in thirty New Zealand rabbits. Two implants will be placed in both tibia of each rabbit. For all rabbits the right tibia's implant was control (uncoated) and the left one was experimental (coated with 0.1ml Hyaluronic acid gel). All rabbits were sacrificed at 1, 2 and 4 weeks post operatively (10 rabbits for each interval). Immunohistochemical test for the expression of TNF- α were performed on bone-implant specimens of both control and experimental groups at all healing interval. Immunohistochemical result revealed positive localization of TNF-α by BMSC especially in 2 weeks healing period and by bone cells include osteoblasts ,osteocytes and osteoclasts in different intervals in both groups with higher record scores in HA-coated group in almost all healing intervals than that of control one. Topical application of hyaluronic acid may act as an osteocoductive in the placement of endosseous implants thus accelerating osseointegration.

KEY WORDS: bone, implant, hyaluronic acid, osseointegration.

INTRODUCTION

Dental implant or fixture is a surgical component that interfaces with the bone of the jaw to support a dental prosthesis [1]. The basis for modern implants is a biologic process called osseointegration where materials, such as titanium, form an intimate bond to bone [2] . Osseointegrated implant is a type of implant defined as "an endosteal implant containing pores into which osteoblasts and supporting connective tissue can migrate. Applied to oral implantology, this refers to bone grown right up to the implant surface without interposed soft tissue layer[1,3]. Various modifications of surface treatments have been introduced to enhance surface properties of titanium implants, as a result supported osseointegration through encouraged bone formation and better implant stability [4]. Hyaluronan (hyaluronic acid) is considerd to be one of the fundamental constituent of connective tissue and bone marrow extracellular matrix. It mediates to chemotaxis, proliferation and successive differentiation of mesenchymal cells so it plays an essential function in regeneration and repair of tissue [5]. Due to its osteogenic induction ability, biocompatibility and non-immunogenic nature and believed to have angiogenic properties [6] This process increases the hydrophilic nature of the implant surface such that the growth factors and proteins necessary for osseointegration are more readily attracted to the implant surface and increase the rate at which the bone heals [7].

MATERIALS & METHODS

Sixty machined surface implants from commercially pure titanium rod will be inserted in 30 adult male New Zealand white rabbits aged from 10 - 12 months and their weights were between 1.5 - 2kgs. Two implants will be placed in both tibia of each rabbit, one in right tibia as control and another one in the left tibia as an experimental. The animals will be scarified at 1, 2 and 4 weeks after implantation (10 rabbits for each interval). The implants will be categorized as control group (30 uncoated implants), 10 implants for each healing intervals and experimental group (30 hyaluronic acid coated implants), 10 implants for each healing intervals. The sterilized implants were placed in the hole prepared in both rabbit's tibia. The insertion of the uncoated one was directly done in the right tibia, while the insertion of the coated implants was performed in the left tibia after the application 0.1ml hyaluronic acid gel inside the threaded part of implants.

All the bone-implant specimens, experimental and controls were fixed in 10% neutral formalin and processed in a routine paraffin blocks after complete decalcification of bone. Each paraffin-embedded specimen had serial sections were prepared as follows: 4µm thickness sections were mounted on clean glass slides for routine H&E staining procedure from each block of all studied specimens. Other 4 sections of 4µm thickness were mounted on positively charged microscopic slides for immunohistochemical localization of TNF-α. The procedure of the IHC assay was carried out in accordance with the manufacturer instructions

of Anti-TNF-α monoclonal antibody (ab212899) Abcam UK and Detection Kits System (ab80436) Abcam UK.

Immunohistochemical results for TNF-α

At 1 week duration

Control group

Immunohistochemical staining with TNF-α monoclonal antibody at one week duration revealed positive expression of TNF-α in fibroblasts, endothelial cells, osteoblasts and collagen fibers. Negative expressions were seen in proginator cells and osteoid tissue (Figures 1 and 2).

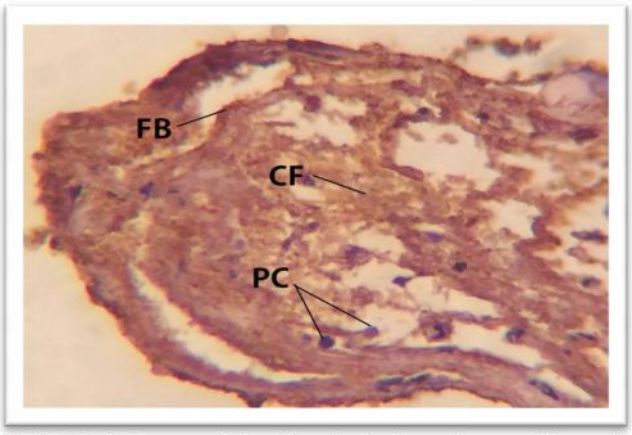

FIGURE 1: Immunohistochemical view of control 1 weeks shows positive expression of TNF-α in fibroblasts(FB) and collagen fibers(CF), and negative expression in proginator cells(arrows). DAB stain with hematoxylin counter stain X20.

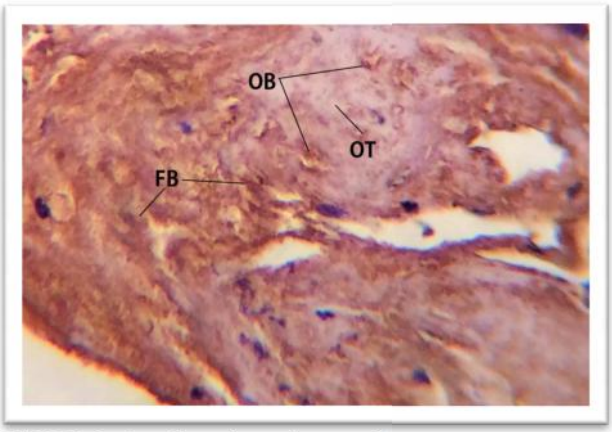

FIGURE 2: Another view of control 1 week shows positive expression of TNF-α in osteoblasts (OB), fibroblasts (arrows) and negative in osteoid tissue (OT).DAB stain with hematoxylin counter stain X40.

Experimental group

Immunohistochemical staining with TNF-α monoclonal antibody at one week duration showed positive brown stain in thread area which surrounded screw space. The high magnification of thread area revealed positive expression of TNF-α in fibroblasts, fat cells, osteoblasts, osteocytes, and woven bone. Negative expressions were seen in endothelial cells and progenitor cells (Figure 3 and 4).

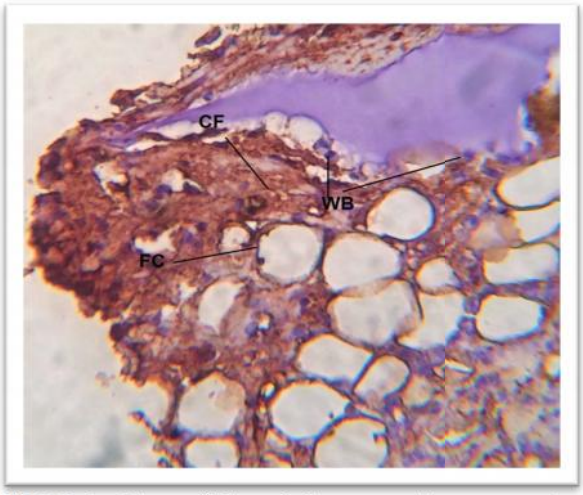

FIGURE 3: View of thread shows positive expression of TNF-α in fat cells (FC) and woven bone (WB), and negative in endothelial cells(red arrows). DAB stain with hematoxylin counter stain X20.

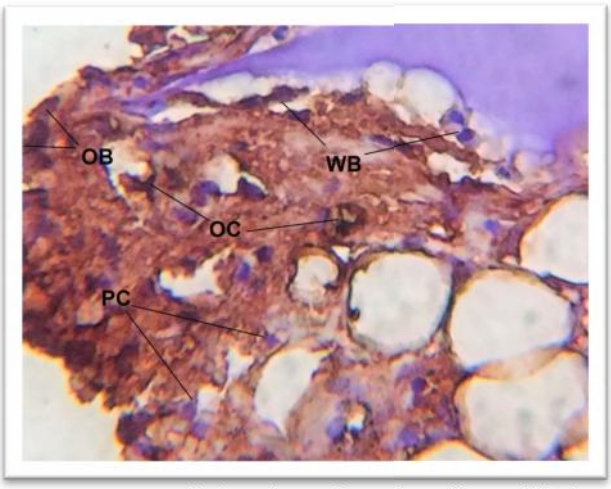

FIGURE 4: Magnifying view of previous figure (3) shows positive expression of TNF-α in woven bone (WB),osteoblast (OB) and osteocytes (OC), and negative in proginator cells (PC). DAB stain with hematoxylin counter stain X40.

At 2 weeks duration

Control group

Immunohistochemical staining with TNF-α monoclonal antibody at two weeks duration showed positive brown stain in high magnification of thread area revealed positive expression of TNF-α in BMSC, osteoblasts, osteocytes and osteoclast. Negative expression was seen in bone trabecullae (Figure 5 and 6).

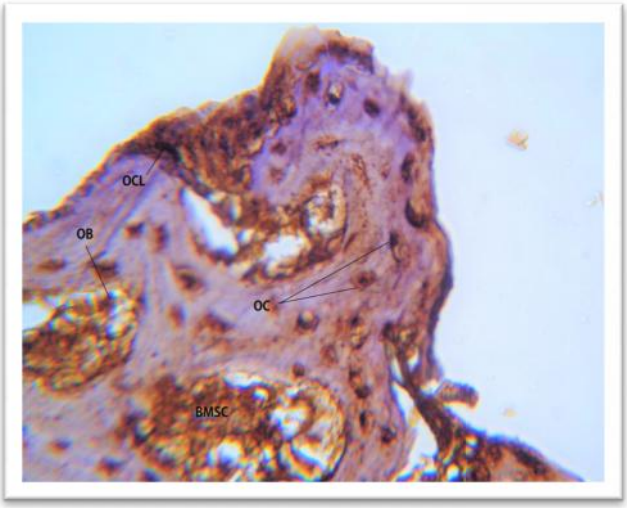

FIGURE 5: Shows positive expression of TNF-α in BMSC, osteoblasts (OB), osteocytes (OC) and osteoclast (OCL), and negative in osteoid tissue (OT). DAB stain with hematoxylin counter stain X40.

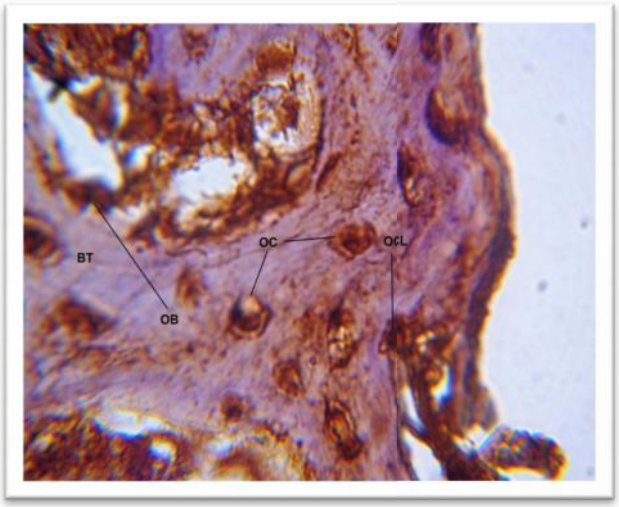

FIGURE 6: Magnifying view of previous figure (5) shows positive expression of TNF-α in osteoblasts (OB), osteocytes (OC) and osteoclast (OCL), and negative in bone trabecullae (BT). DAB stain with hematoxylin counter stain X100.

Experimental group

Immunohistochemical staining with TNF-α monoclonal antibody at two weeks duration in thread area revealed positive expression of TNF-α in BMSC, osteoblasts, osteocyts and osteoclast. Negative expression was seen in bone trabecullae (Figure 7 and 8).

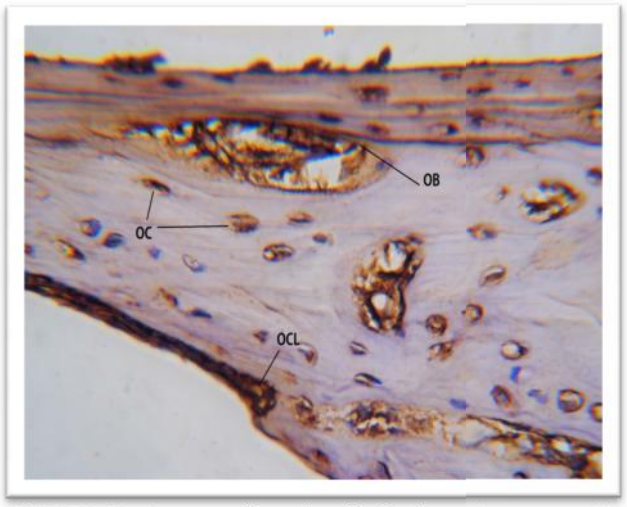

FIGURE 7: Immunohistochemical view shows positive expression of TNF-α in osteoblasts (OB), osteocytes (OC) and osteoclast (OCL. DAB stain with hematoxylin counter stain X40.

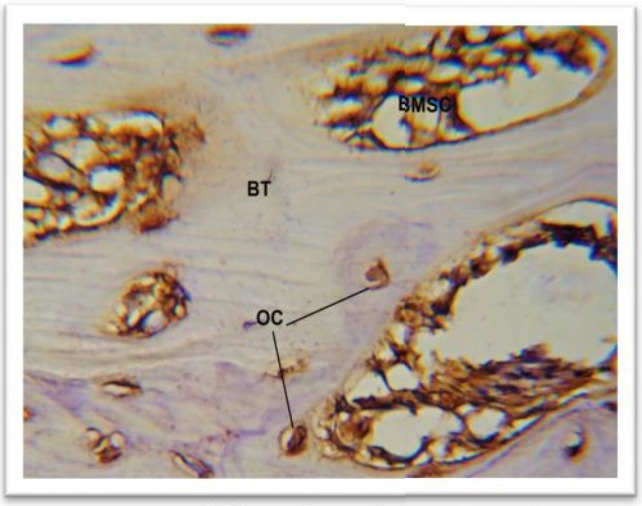

FIGURE 8: Magnifying view of experimental 2 weeks shows positive expression of TNF-α in BMSC and osteocytes (OC), and negative in bone trabecullae (BT). DAB stain with hematoxylin counter stain X100.

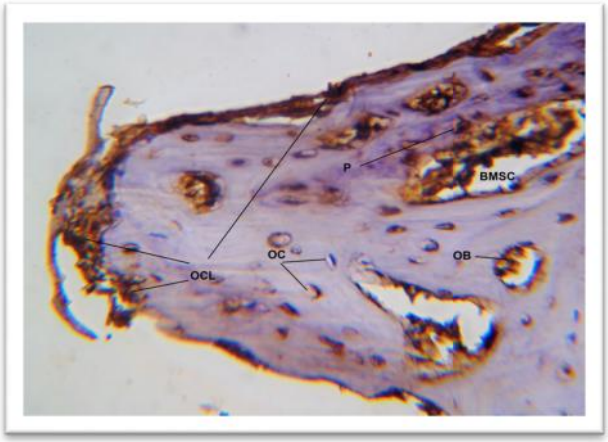

FIGURE 9: Immunohistochemical view of 4 weeks control group shows positive expression of TNF-α in BMSC, osteoblasts (OB), preosteocyte (POC), osteocytes (OC) and osteoclasts (OCL). DAB stain with hematoxylin counter stain X40.

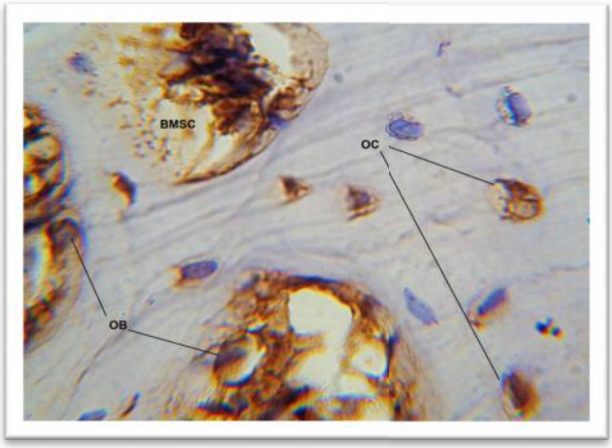

FIGURE 10: Magnifying view of 4weeks control group shows positive expression of TNF-α in osteoblasts (OB), osteocytes (OC) and in bone marrow stromal cells (BMSC). DAB stain with hematoxylin counter stain X100.

At 4 weeks duration

Control group

Immunohistochemical staining with TNF-α monoclonal antibody at four weeks duration showed positive brown stain in thread area. The high magnification of thread area revealed positive expression of TNF-α in osteoblasts, osteocytes and osteoclasts. Negative expressions were seen in new bone matrix (Figure 9 and 10).

B-Experimental group

Immunohistochemical staining with TNF-α monoclonal antibody at four weeks duration showed positive brown stain in thread are. The high magnification of thread area revealed positive expression of TNF-α in osteoblasts, osteocytes and BMSC. Negative expressions were seen in new bone matrix (Figure 11 and 12).

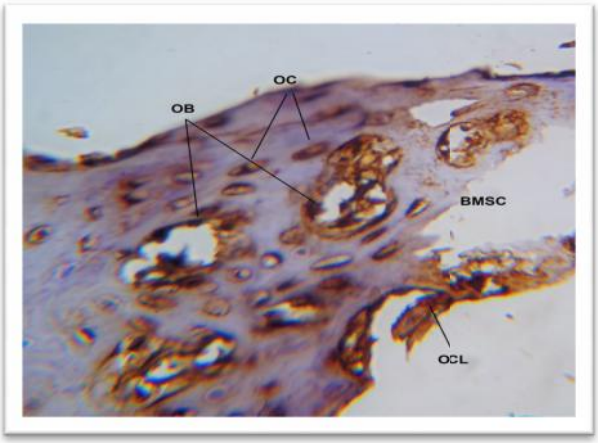

FIGURE 11: View of 4 weeks experimental group shows positive expression of TNF-α in BMSC, osteoblasts (OB) and osteocytes (OC), and osteoclasts (OCL). DAB stain with hematoxylin counter stain X40

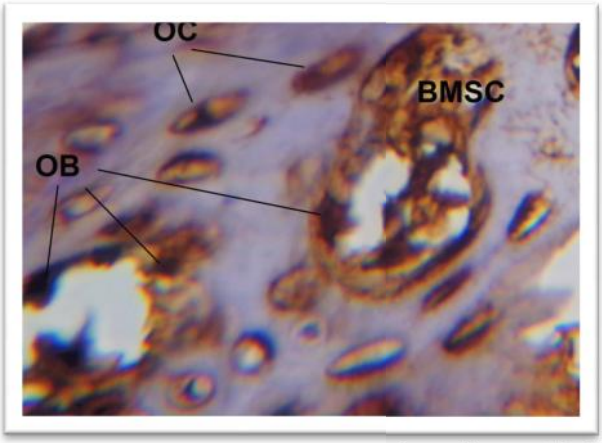

FIGURE 12: Magnifying view of previous (figure 11) shows positive expression of TNF-α in BMSC, osteoblasts (OB) and osteocytes (OC). DAB stain with hematoxylin counter stain X100.

Immunohistochemical score of TNF in studied groups

Bone marrow stromal cells (BMSC)

The descriptive statistics that measured at 1, 2, and 4 weeks for both control and experimental groups are listed in (Table 1). Depending on the obtained data, there was apparent decline in TNF-α positively stained BMSC mean values with time, and noticeable increased HA coated mean values in comparison to that in control group. According to the T-test (Table 2) which illustrates a significant difference between experimental and control groups in positive expression BMSC for TNF in week 4 interval.

Bone cells

The descriptive statistic measured at different healing periods for both groups for positive osteoblasts, osteocytes and osteoclasts counting for TNF-α are listed in (Table 3). The highest mean value for positive expressed osteoblasts and osteocytes were seen in the 4 weeks for HA treated group .While the highest mean value for positive expressed

of osteoclast was seen in the 2 weeks for both treated and control group. According to ANOVA test there are highly significant differences among all duration for positive expression of osteocytes for TNF-α in both experimental and control groups and significant only among treated group for positive osteoblasts (Table 4).

Table 1: Summary statistics of the studied Positive BMSC expressed TNF for both groups in different duration

Variables	Duration	Control group						Experimental group					
		N	Mean	S.D.	S.E.	Min.	Max.	N	Mean	S.D.	S.E.	Min.	Max.
Bone Marrow	1 week	5	13.63	2.5	0.88	10	18	5	16	3.2	1.13	11	20
Stromal Cells	2 weeks	5	20.6	14.2	5.05	6	50	5	23.3	14.3	5.05	9	45
(BMSC)	4 weeks	5	12.2	2.8	1.01	10	17	5	16.2	2.55	0.9	12	20

TABLE 2: Groups' comparison for Positive Stromal Cell expressed TNF-α in each duration

Variables	Duration	Groups' Comparisons d.f.** = 14	
		t-test	p-value*
Bone Marrow	1 week	1.65	0.121 (NS)
Stromal Cells	2 weeks	0.385	0.706 (NS)
(BMSC)	4 weeks	2.94	0.011 (S)

***** HS: highly significant, S: significant, NS: non significant, **d.f. = degree of freedom

TABLE 3: Descriptive statistics of positive expression of bone cells for TNF-α in both groups

Variables	Duration	Control group						Experimental group					
		N	Mean	S.D.	S.E.	Min.	Max.	N	Mean	S.D.	S.E.	Min.	Max.
Osteoblasts	1 week	5	4.88	2.58	0.91	2	10	5	7.25	1.38	0.49	6	10
	2 weeks	5	11.25	4.62	1.63	5	19	5	15.13	5.27	1.86	10	25
	4 weeks	5	28.88	34	12	9	110	5	13.88	8.32	2.94	8	31
Osteocytes	1 week	5	0	0	0	0	0	5	0.38	0.52	0.18	0	1
	2 weeks	5	7.25	4.06	1.43	3	13	5	9	4.27	1.51	3	15
	4 weeks	5	9.5	4.1	1.45	3	15	5	10.5	4.59	1.62	3	17
Osteoclasts	1 week	5	0.25	0.46	0.16	0	1	5	0.38	0.52	0.18	0	1
	2 weeks	5	1.38	1.5	0.53	0	4	5	1	1.41	0.5	0	4
	4 weeks	5	0.88	0.83	0.29	0	2	5	0.88	0.64	0.22	0	2

TABLE 4: Durations' comparison using ANOVA test for Positive Bone cells expressed TNF-α in both groups

Variables	Control group		Experimental group	
	F-test	p-value*	F-test	p-value*
Osteoblasts	3.128	0.065 (NS)	4.34	0.026 (S)
Osteocytes	17.73	0.000 (HS)	18.05	0.000 (HS)
Osteoclasts	2.399	0.115 (NS)	0.98	0.392 (NS)

***** HS: highly significant, S: significant, NS: non significant

DISCUSSION

The goal of present study was to evaluate the effects of hyaluronic acid on bone-implant interface. Hyaluronan is considerd to be one of the fundamental constituent of connective tissue and bone marrow extracellular matrix. it plays an essential function in regeneration and repair of tissue[5]. Due to its osteogenic induction ability, biocompatibility and non-immunogenic nature led to its use in a number of clinical applications, such as fabricating and/or coating an implant or other structure to be inserted into bone or osseous tissue and to facilitate the healing and regeneration of bone [7]. Tumour necrosis factor-alpha (TNF-α) are shown to play a role in initiating the repair cascade. It carries out central functions in the induction of downstream responses to injury by having a chemotactic effect on other inflammatory cells, enhancing extracellular matrix synthesis, stimulating angiogenesis, and recruiting endogenous fibrogenic cells to the injury site [8]. The cell types in which TNF-α could be detected immune-histochemically varied with time: first inflammatory cells, then cells in late hypertrophying then osteoblasts and bone marrow granulocytes stained for TNF-α [9]. The current results showed that BMSC cells had a highly positive expression for TNF-α monoclonal antibody at early stage of bone healing (1 and 2 weeks), and the expression decreased in late stage (4 weeks) in both groups. Also there was increase in BMSC score mean values of positively stained cells for TNF-α , during the 1, 2 and 4 weeks of healing intervals in implant site coated with hyaluronic acid in rabbit's model. TNF-α is produced chiefly by activated macrophages, although it can be produced by many other

cell types such as lymphocytes, neutrophils, mast cells, eosinophils, and endothelial cells throughout the healing process[9]. This explains the increase of TNF-α concentrations within the first 2 weeks after implantation in the present study.

TNF-α has an inhibitory effect during various stages of osteoblast differentiation and can act on osteoblast precursor cells during the early stages of differentiation to inhibit insulin-like growth factor 1, which increases the differentiation of osteoblast precursor cells from stem cells suggesting a potentially critical function of TNF-α in the regulation of bone formation and repair. Mean value of positive osteoblasts number expressed TNF-α was higher in 2 weeks especially in HA treated group than control one but in 4 weeks showed a markedly decrease in number. These results agree with[10], which showed osteoblast positive expression of TNF-α in different osseointegretion intervals around Titanium (CPTi) implants were placed in rabbit's tibia. There was high mean value of positive osteocytes that expressed TNF-α were seen in HA treated group at 2 weeks when compared with week 1, with slight increase in the mean number of positive osteocytes was recorded in HA treated group after 3 weeks of healing period. These results agree with [11], who conclude that the raise in TNF-α positively stained osteocyte number in early stages of bone healing caused by osteoblastic overexpression of TGF-β. Osteoclast cells were found to be decreased in number of positive expressed cells for TNF-α with time in parallel with the increased bone formation .These findings were in line with [12], who suggested that TNF-α had stimulatory effects on early osteoclast differentiation. In contrast, TNF-α inhibits later stages of osteoclast differentiation

CONCLUSION

Results obtained in this study have shown that the increased positive expression of TNF-α in the experimental group at early stages of implantation give as indicator for acceleration of the osseointegration around titanium implant by hyaluronic acid coating material.

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