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Localization of TNF-a in Experimentally Induced Bone Defect Treated with Topical Application of Flavanoids Extract of Hibiscus Sabdariffa

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Abstract: ***Background**: Repair of bone defect is a multistep procedure involving migration, proliferation, differentiation and activation of several cell types. Many cytokines which play an important role in the regulation of bone repair, Tumor necrosis factor-a (TNF-a) is one of the several proinflammatory cytokines involved in bone remodeling (Osta et al., 2014). TNF_a possessed osteogenic differentiation effects, its determined promoter of osteoclastogenesis and inhibitor of osteoblastogenesis. The use of plants for healing purposes predates human history and forms the source of current modern medicine. This study was achieved to investigate effect of flavonoid extract on molecular events in bone healing process by expression of Tumer Necrosis Factor-alpha (TNF-α). **Materials and methods**: Immunohistochemica localization of TNF-a was investigated for healing of induced bone defects in tibiae of 24 adult New Zealand rabbits. Two holes were induced in rabbit tibia the left side left to heal normally as control, while, the other hole made on the right side filled with flavonoid extract regarded as experimental. Sacrifications of the animals were done according to the healing periods. Routine processing and sectioning technique was performed for immunohistochemical evaluation. **Results**: Findings obtained in this study showed decrease in percentage of positively stained bone marrow stromal cells (BMSC) between experimental and control groups with statically significant difference among (3days, 1, and 2weeks) durations; while, in 4 weeks duration there was no obvious difference and its statistically non-significant. Positive expression of TNF-α by osteoblasts and osteocytes was statistically significant at 1week, non-significant at 4weeks, however at 2weeks statically significant analysis was recorded with osteocytes and osteoclasts. **Conclusion**: The study revealed obvious effect of flavonoid extract in promoting bone healing process as demonstrated TNF-α expression by cells involved in the process in different durations.*

Keywords: bone healing, flavonoid extract of Hibiscus Sabdariffa, TNF-a

1. Introduction

Bone defect can result after a pathological process has destroyed vital components of the bone. Bone healing is a procedure of reconstruction of the bone tissue, which generally undergoes a multidimensional procedure with an overlapping timeline. Most of bone defects can heal spontaneously under suitable physiological environmental conditions due to the regeneration ability of bone that can be only expected after some time of rest and procedures of debridement. However, the healing process of bone defect is time consuming, and new bone generation takes place slowly due to the size of defects or unstable biomechanical properties, unfavourable wound environment, suboptimal surgical technique, metabolic factors, hormones, nutrition, and applied stress [1, 2]

Tumer Necrosis Factor-alpha (TNF-α) is a major inflammatory factor that is induced in response to injury, and it contributes to the normal regulatory processes of bone resorption, both in vitro and in vivo, The overproduction of TNF-α may have an important influence on bone metabolism, regulating different phases of bone remodeling cycle [3, 4].

The primary roles that TNF-alpha function contributes to in promoting postnatal fracture repair as well as that processes of skeletal tissue development and postnatal repair are controlled in part through the induction of osteoprogenitor cell recruitment or osteogenic cell activation in the context of intramembranous bone formation [5].

Naturally occurring flavonoids influence immunoregulatory activities against cytokine production in vitro and in vivo. Since tumor necrosis factor (TNF) -α is one of the major inflammatory cytokines, Flavones, flavonols, and chalcone are the most potent inhibitors of production of TNF-α [6].

2. Materials and Methods

The materials used in the present study were flavonoid extract 2 µml, anesthetic solution (Ketamine hydrochloride10% Woerden Xylazine 20 mg), formalin 10%, ethanol alcohol 96%, xylol, paraffin wax, and Hematoxylin and Eosin (H&E) stain, Anti-Tumor necrosis Factor-alpha Antibody (TNF-a) from Abcam company UK (ab212899), Detection Kits System, Abcam Company England (ab80436).

3. Experimental Design

The total animals (24) were divided into four groups according to healing interval (3 days, 1, 2, and 4 weeks) six animals for each. A hole induced in left tibia as control was left to heal spontaneously, a second hole induced in right tibia where topical application of flavonoid extract was done as experimental side.

Surgical procedure
The surgical procedures were done under general anesthesia drugs by a mixture of ketamine hydrochloride 10% (50 mg/kg B.W) and xylazin 2% at a dose (5 mg/kg body weight) The surgery was performed in a well sterilized

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conditions and with gentle surgical technique, bur stopper was placed on surgical burs placed to recommended depth. After exposure of tibia, initial intermittent drilling was done to diameter of about 3mm and depth 3mm, vernia was used to check the depth and diameter of the holes [7, 8]. Topical application of flavonoid extract was done in the hole of right hole by pipette. The animals were scarified by an overdose of anesthetic solution at (3 days, 1, 2 and 4 weeks) healing intervals. Bone specimens were prepared by cutting the bone about 5 mm away from operation site with continuous irrigation with saline to avoid bone damage. Routine processing and sectioning technique was performed for immunohistochemical localization of TNF-α.The examination was done by using objective lens (power x 40) and the scoring done as follows: To evaluate the immunostaining of the antibodies, positively stained cells were counted at 5 representative fields (x40) for all healing periods. The percentage of cells positive for the protein of interest was scored and was estimated visually within the fields selected. Dividing the total number of stained cells by the total number of cells present and multiplying this value by 100 provided the approximate percentage of positively stained cells. The scores were: 0 (no stain), 1 (<25%), 2 (25-50%) 3 (>50 %) stained cells in two sections [9].

4. Results

Immunohistochemical results for TNF-α expression in different intervals

Three days duration

Immunohistochemical localization of TNF- α after 3days duration at defect site of control group was detected by positive staining of granulation tissue, progenitor cells, fat cells as seen in (figures1, 2). View of defect site of experimental group after 3days duration shows positive immunohistochemical localization of TNF- α, expressed by granulation tissue, fat cells, progenitor cells (Figures3).

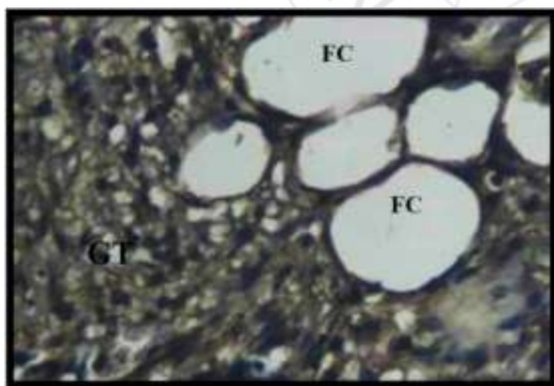

Figure 1: Positive localization of TNF-α in defect site of control group for 3days in fat cells (FC) granulation tissue (GT). DAB stain with counter stain hematoxylin X20

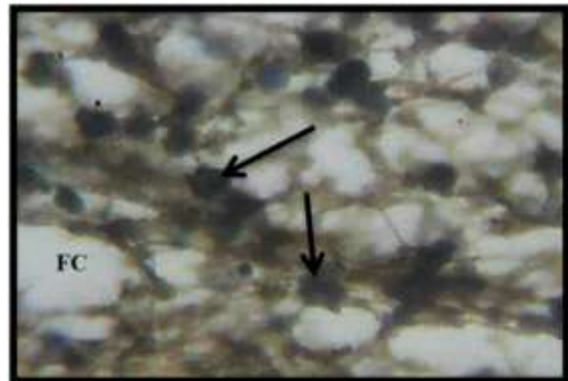

Figure 2: Positive localization of TNF-α in defect site of control group for 3days in fat cells (FC) progenitor cells (arrows). DAB stain with counter stain hematoxylin X40.

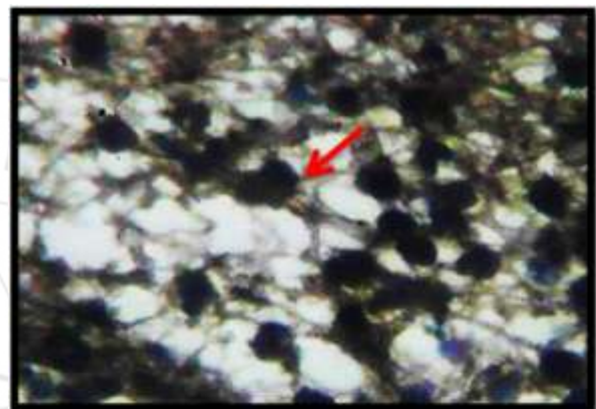

Figure 3: Positive localization of TNF-α in defect site of experimental group for 3days duration in progenitor cells (arrow), DAB stain with counter stain hematoxylin X40.

One-week duration

View of defect site in control group shows positive staining of marrow tissue, osteoblasts and osteocytes, osteoid tissue is negatively stained (Figure4). Immunohistochemical localization of TNF- α at one week duration is detected by positive staining of endothelial cells osteoblasts, osteocytes and osteoclast. Negative expression by osteoid tissue is seen (Figures 5)

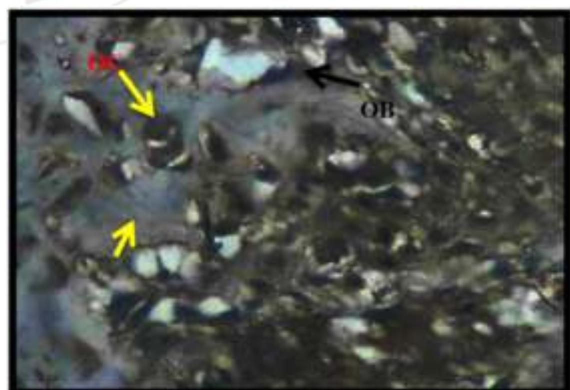

Figure 4: Positive expression of TNF- α, is detected by osteocytes OC), and osteoblasts (OB), negative staining of osteoid tissue (arrow). DAB stain with hematoxylin counter stain X40.

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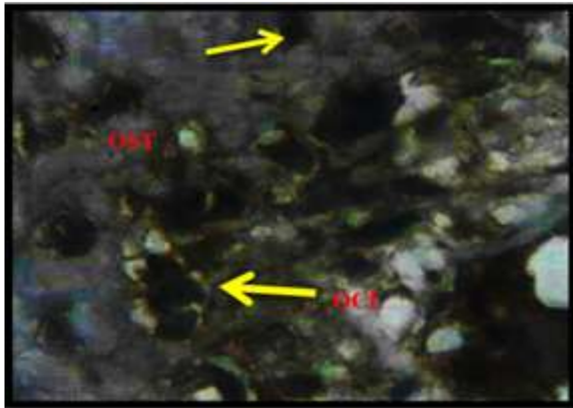

Figure 5: shows positive localization of TNF- α in osteocyte (arrow) osteoclasts (OCL), osteoid tissue is negatively stained (OST). DAB stain with hematoxylin counter stainX100.

Two weeks duration

View of defect area of control group after 2weeks duration shows positive localization of TNF- α in, osteoblasts, osteocytes, osteoclasts and negative expression of TNF- α is detected in bone trabeculae (Figure 6). View of defect area after 2 weeks after flavonoid application shows positive expression of TNF- α by osteoblasts, osteocytes, osteoclast (Figures 7).

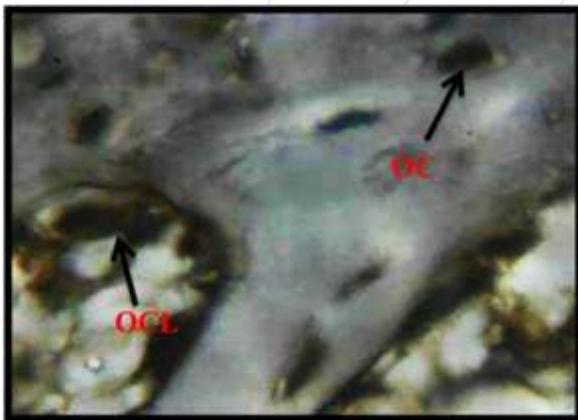

Figure 6: shows positive expression of TNF-α in osteocytes (OC), osteoclast (OCL), negatively stained trabeculae (BT). DAB stain with hematoxylin counter stain X40.

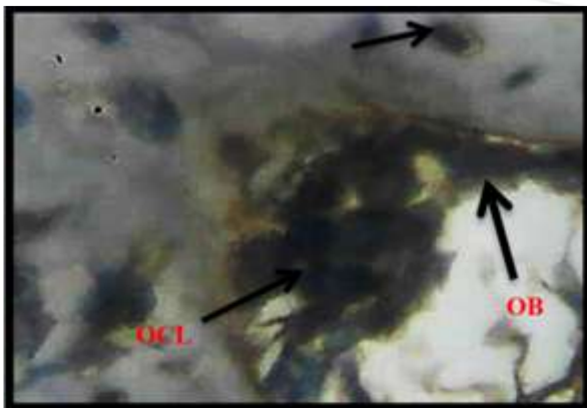

Figure 7: Magnified view of defect area of experimental group after2weeks shows positive staining of osteoblasts (OB), osteocyte (arrow) and osteoclast (OCL). DAB stain with hematoxylin counter stain X100.

Four weeks duration

View of defect area of control group at 4weeks duration shows positive immunohistochemical localization of TNF- α detected by marrow tissue, osteoblasts, osteocytes cells, whereas bone is negatively stained (Figure 8). Immunohistochemical positive localization of TNF- α of experimental group at 4 weeks duration is expressed by osteoblasts, havarsian canal, osteocytes and reversal line (Figures 8).

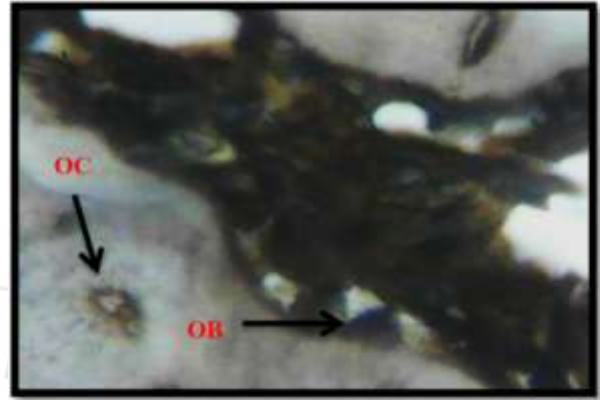

Figure 8: shows positive expression of TNF- α in osteoblasts (OB), osteocytes (OC). DAB stain with hematoxylin counter stain X40

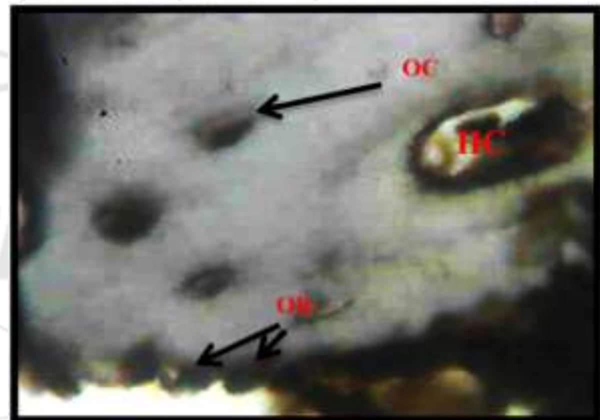

Figure 9: Magnifed view of 4weeks of experimental group shows positive expression of TNF- α in osteoblasts (OB), osteocytes (OC). DAB stain with hematoxylin counter stain X40.

Bone marrow stromal cells (BMSC):

Statistical analysis of positively stained BMSC, measured at different healing periods (3days, 1, 2, and 4weeks) for all studied groups are shown in (Table 1), which shows slight decrease in percentage of positively stained cells between experimental and control groups with statically significant analysis in (3days, 1, and 2weeks) durations; while, in 4 weeks duration there is no obvious difference and its statistically non-significant. In linear coefficient correlation clarified in table (2) shows moderate to strong direct correlation in both experimental and control groups.

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Table 1: Control Vs experimental groups' comparison in median values for BMSC indices measured as percent of positively stained cells in different durations

Duration	*Variances*	*No.*	*Range*	*Median*	*Mean Rank*	*R (effect size)*	*P value*
3 days	Experimental group	6	66 to 77	74	4.4	-0.9	0.0277
	Control group	6	79 to 99	90	11.5		
	Mean count difference between experimental and control groups	6	-21 to 4	-17.5			
1 week	Experimental group	6	76 to 85	79	8.8	-0.82	0.0452
	Control group	6	80 to 88	83	4.6		
	Mean count difference between experimental and control groups	6	-6 to 1	-3.5			
2 week	Experimental group	6	83 to 90	88	15.5	-0.9	0.0269
	Control group	6	87 to 94	91	13.6		
	Mean count difference between experimental and control groups	6	-5 to -2	-3.5			
4 week	Experimental group	6	90 to 99	96.5	21.4	0.04	0.92[NS]
	Control group	6	92 to 98	96	20.0		
	Mean count difference between experimental and control groups	6	-4 to 2	0			

Table 2: linear correlation and p value for (Kruskall-Wallis) and (Mann-Whitney) for difference in median for BMSC indices measured as percent of positively stained cells in different durations

	Control groups	Experimental groups
Linear correlation with duration of follow ups	r=0.553 P=0.005	r=0.93 P<0.001
P (Kruskall-Wallis) for difference in median between all the 4 follow up intervals	0.003	<0.001
P (Mann-Whitney) for difference in median between:		
3 days X 7 days	0.003	<0.001
3 days X 14 days	0.09[NS]	0.045
3 days X 28 days	0.47[NS]	0.0039
7 days X 14 days	0.05[NS]	0.0039
7 days X 28 days	0.0064	0.008
14 days X 28 days	0.0038	0.0039

Bone cells

Statistical analysis of positively stained bone cells (osteoblasts, osteocytes and osteoclasts) are illustrated in table (4) for the healing periods of the studied groups (1, 2 and 4weeks).At one week duration of follow up osteoblast and osteocyte showed decrease in staining percentage values between experimental and control groups with statistically significant difference while for osteocytes it was non-significant. At 2 weeks duration of follow up osteoblast, osteocyte and osteoclast showed decrease in staining percentage between experimental and control groups with statically significant analysis except for osteoblast this change was statistically non-significant. After 4 weeks of follow up no obvious difference in percentage of positively stained osteoblasts and osteocytes between experimental and control groups however this change was statistically non-significant. These findings are demonstrated in figure (9).

In linear coefficient correlation clarified in this table (3) shows moderate to strong direct correlation in both experimental and control groups.

Table 3: linear correlation and p value for (Kruskall-Wallis) and (Mann-Whitney) for difference in median between all the 4 follow up intervals for bone cells indices measured as percent of positively stained cells in different durations

V.	Linear correlation of all duration		P (Kruskall-Wallis) for difference in median between all the 3 follow up intervals		P (Mann-Whitney) for difference in median between:					
					7 days X 14 days		7 days X 28 days		14 days X 28 days	
	C. groups	E. groups	C. groups	E. groups	C. groups	E. groups	C. groups	E. groups	C. groups	E. groups
O B	r=0.725 P=0.001	r=0.921 P<0.001	0.0102	<0.001	0.13[NS]	0.0039	0.0126	0.0039	0.0189	0.11[NS]
O C	r=0.228 P=0.28[NS]	r=-0.261 P=0.22[NS]	<0.001	<0.001	0.0038	0.0247	0.0038	0.0038	0.33[NS]	0.0039

Table 4: Control Vs experimental groups' comparison in median values for bone cells indices measured as percent of positively stained cells in different durations

Duration	Variance	Groups	No.	Range	Median	Mean rank	R (effect size)	P value
1 week	OB	C.	6	73 to 91	85	5.3		0.0277
		E.	6	65 to 79	73.5	9.3	-0.9	
		MCD	6	-13 to -7	-9.5			
	OC	C.	6	61 to 74	70	3.5		0.06[NS]
		E.	6	55-71	66.5	4.2	-0.77	

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		MCD	6	-13 to 1	-4			
2 weeks	OB	C.	6	84 to 91	88.5	8.7		0.83[NS]
		E.	6	80 to96	85	16.8	-0.09	
		MCD	6	-10 to 11	-2.5			
	OC	C.	6	81 to 98	90	11.5		0.0277
		E.	6	61 to 89	78.5	8.8	- 0.5	
		MCD	6	-26 to-2	-0.9			
	OCL	C.	6	50 to 80	68	3.5		0.0273
		E.	6	33to 63	47	3.5	-0.9	
		MCD	6	-33 to -7	-18			
4 weeks	OB	C.	6	90 to 95	92	14.5		0.22[NS]
		E	6	90 to 97	93.5	20.2	0.5	
		MCD	6	-3 to 4	1.5			
	O C	C.	6	90 to 96	91.5	13.5		0.29[NS]
		E.	6	91 to 98	93	15.5	0.44	
		MCD	6	-1 to 4	0			

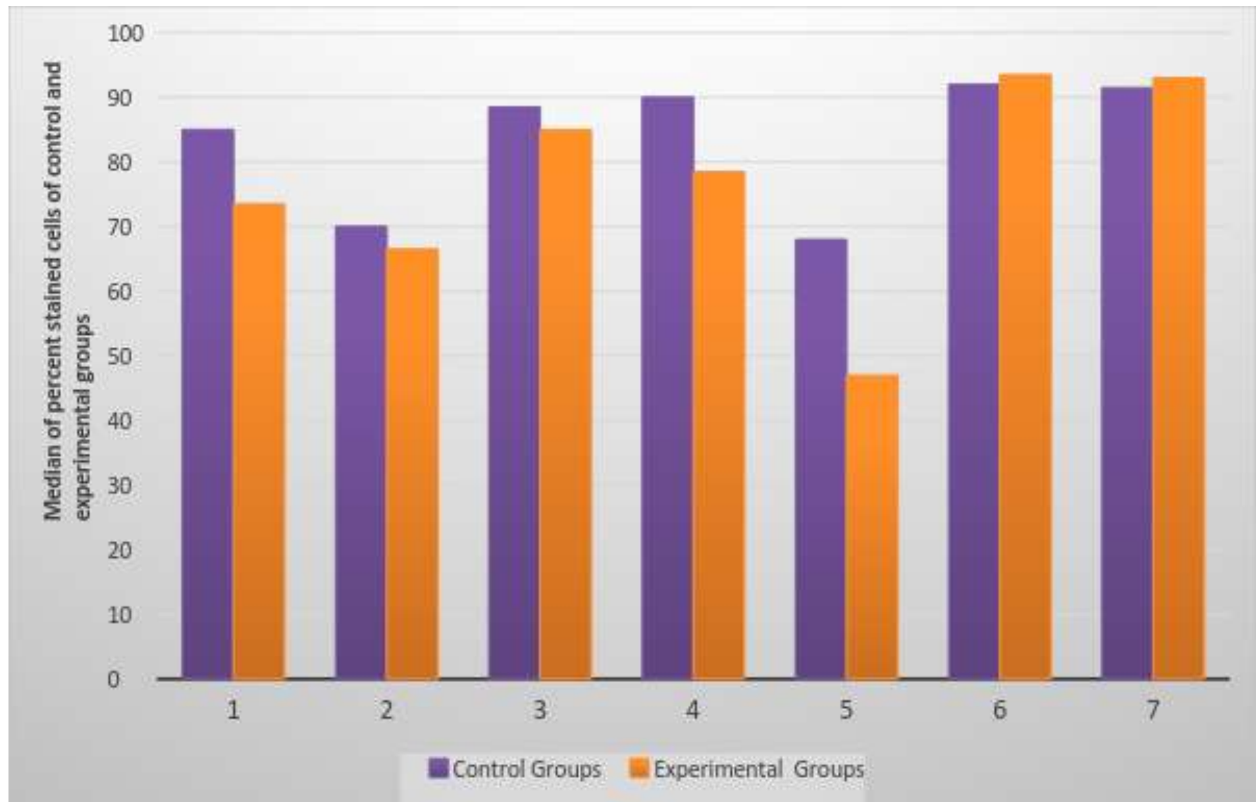

Figure 9: Control Vs experimental groups comparison in median values for bone cells indices measured as percent of positively stained cells in different durations

5. Discussion

Natural compounds act through gene expression and cell signaling pathways to activate enzymes that eliminate oxygen radicals that initiate inflammatory events [10]. The osteoblasts and osteoclasts activities are modulated by hormones, cytokines and growth factors. Previous studies have implicated certain cytokines in bone remodeling in vitro and in vivo, including Tumor Necrosis Factor-alpha (TNF-α) [11, 12].

It has been suggested that TNF-alpha plays a crucial role in promoting postnatal bone repair through the induction of osteoprogenitor cell recruitment or osteogenic cell activation in the context of intramembranous bone formation, TNF-a and other inflammatory molecules are resilient molecules that readily diffuse through the extracellular matrix and are thus ideally suited for transmission of signals guiding regeneration [5, 13].

Bone defect is an injury, and thus induce an inflammatory response, which reaches its peak at 24 h after the injury and is complete by the first week ;tumor necrosis factor-a (TNF-a) significantly elevated within the first few days inducting inflammatory cells and promote angiogenesis, it is mainly expressed by macrophages and other inflammatory cells, TNF-a concentration rises again approximately 2 weeks later, during this period, TNF-a is expressed by osteoblasts and other cells of mesenchymal origin. Signaling of TNFα is believed to induce the release of secondary signaling molecules and to exert a chemotactic effect, recruiting cells

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necessary for bone regeneration. in murine models, [5, 14, 15, 16] which matches with present results where immunohistochemical localization of TNF-α was detected by BMSC as percentage of stained cells was high at early period of healing process, and at 2weeks duration.

The study of Nair etal., 2006 [17] hypothesize that flavonoids exert anti-inflammatory effects by peripheral blood mononuclear cells, inhibiting the endogenous production of the proinflammatory cytokine TNF-α, suggesting that promoting inflammation, and specifically TNF-α, at the site of fracture or implant would promote bone formation and encourage a favorable clinical outcome. which may explain the significant decrease of TNF-a percentage of positive expression in experimental groups compared to control groups in the present study has proved the inhibitory effect of flavonoid on TNF-a.

Osteoprogenitor cells differentiate into osteoblasts, which express factors that promote osteoclast formation. The renewing and resorptive actions of these two cell types replace the initial woven bone with lamellar bone. This remodeling phase is regulated by several proinflammatory signals such as TNF-a, that are detectable in elevated levels at the injury site [15], in agreement with findings of this study where woven bone deposited during first week replaced by maturing bone throughout 2 and 4 weeks.

6. Conclusion

In conclusion, positive localization of TNF-α by cells involved in bone healing process indicates the effectiveness of flavonoid extract of hibiscuses in enhancement of healing procedure.

References

[1] Smrke D., Rožman P., Veselko M.and., Gubina B., Treatment of Bone Defects -Allogenic Platelet Gel and Autologous Bone Technique, Intech., 2013; (13) 326-340
[2] Li Y., Chen S, Li L, Qin L, Wang X, Lai Y., Bone defect animal models for testing efficacy of bone substitute biomaterials, Elsevier, Journal of orthopaedic translation, (2015) 3 (3), 95-104
[3] Lello santos F.R., Moyse´ s R., Montenegrof.M., Jorgetti V., and Noronha I., IL-1, TNF-, TGF-, and bFGF expression in bone biopsies before and after parathyroidectomy. Kidney International, Vol. 63 (2003), pp. 899–907.
[4] Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, Barnes GL, Graves DT, Einhorn TA. Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption, J Bone Miner Res. 2003 Sep;18 (9) :1584-92.
[5] Gerstenfeld LC1, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, Einhorn TA. Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. Cells Tissues Organs. 2001;169 (3) :285-94.
[6] Herath H.M., Takano-Ishikawa Y., and Yamaki K., Inhibitory Effect of Some Flavonoids on Tumor Necrosis Factor-α Production in Lipopolysaccharide-Stimulated Mouse Macrophage Cell Line J774.1., Journal of Medicinal Food. 2004, 6 (4) : 365-370
[7] Kadhim E.F. (2014) : Histological and Immunohistochemical Evaluation of the Effect of Local exogenous Application of VEGF /N-acetyl D-glucosamine on bone Healing (Experimental Study in rats).Phd thesis, collage of Dentistry, University of Baghdad.
[8] Razouki N.A. (2015) : Effect of Beta- Tricalcium Phosphate on Bone Healing In Alloxan -Induced Diabetes mellitus (Histological, Histomorphometrical and Immunohistochemical Study in Rabbits). M.Sc thesis, collage of Dentistry, University of Baghdad
[9] Hasina R., Whipple M., Martin L, Kuo W.P., Ohno-Machado L., and Lingen M., Angiogenic Heterogeneity in Head and Neck Squamous Cell Carcinoma: Biologic and Therapeutic Implications. Lab Invest. 2008 ; 88 (4) : 342–353
[10] Weaver MC., Alekel LD, Ward EW., and Ronis MJ., Flavonoid Intake and Bone Health, J Nutr Gerontol Geriatr. 2012; 31 (3) : 239–253
[11] Heymann, D and Ruusselle, AV. Gp130 Cytokine family and bone cells. Academic Press; Cytokine 2000, 12 (10), 1455-1468.
[12] Volejnikova, S, Marks Jr, SC and Graves, DT. Tumor necrosis factor modulates apoptosis of monocytes in areas of developmentally regulated bone remodeling. Journal of Bone and Mineral Research, 2002, vol. 17, p. 991-997
[13] Tsiridis, E., Upadhyay, N., and Giannoudis, P.V. Molecular aspects of fracture healing: which are the important molecules.Injury (2007). 38 (1), S11-25
[14] Dimitriou R, Tsiridis E, Giannoudis PV Current concepts of molecular aspects of bone healing. Injury: international journal of the care of the injured (2005) 36 (12) :1392–1404.
[15] Mountziaris PM, Mikos AG. Modulation of the Inflammatory Response for Enhanced Bone Tissue Regeneration. Tissue Engineering Part B, Reviews. 2008;14 (2) :179-186.
[16] Glass, G. E., Chan, J. K., Freidin, A., Feldmann, M., Horwood, N. J., & Nanchahal, J. (2011). TNF-α promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proceedings of the National Academy of Sciences of the United States of America, 108 (4), 1585–1590.
[17] Nair, M. P., Mahajan, S., Reynolds, J. L., Aalinkeel, R., Nair, H., Schwartz, S. A., & Kandaswami, C. (2006). The Flavonoid Quercetin Inhibits Proinflammatory Cytokine (Tumor Necrosis Factor Alpha) Gene Expression in Normal Peripheral Blood Mononuclear Cells via Modulation of the NF-κβ System. Clinical and Vaccine Immunology, 13 (3), 319–328.
[18] Osta, B., Benedetti, G., & Miossec, P. Classical and Paradoxical Effects of TNF-α on Bone Homeostasis. Frontiers in Immunology (2014) : 5, 48.

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