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## Biomimetic Implant Coatings: Do They Improve The Biological Performance Of Dental Implants? An Experimental Comparative Study Evaluating the Effect of Fibronectin versus Hyaluronate as Biomimetic Organic Coatings

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### ABSTRACT

**Background:** Biomaterial surface properties regulate cellular and host responses to implanted biomedical prostheses. Surface biomodifications of titanium implants using various modalities aim to improve the initial healing and promote biological performance of dental implants. **Objectives:** This study was conducted to evaluate hyaluronate versus fibronectin as bioactive implant coatings and to investigate their role in promoting the biological performance of dental implant. Material and methods: Twenty New Zealand white mature male rabbits weighing 2.5-4kg were divided equally into two groups and received implants in each tibia. In group I the implants in experimental side were coated with fibronectin whilst on the contralateral side was uncoated (control). In group II, the implants in the experimental side were coated with hyaluronate whilst on the contralateral side were uncoated control. Five rabbits for each group were sacrificed at 4 and 8 weeks. Following fixation and decalcification, the bone was embedded in paraffin, serial 6- $\mu$ m sections were obtained and stained with Hematoxylin and Eosin and Masson's Trichrome stain for histological examination. Histomorphometric estimation of the mean area percent filled by bone trabeculae was estimated using image analyzer computer system. **Results:** Histological examination revealed the superiority of hyaluronate over fibronectin and control at both observation periods revealing thick trabeculae of newly formed bone containing viable osteocytes and enclosing variable-sized marrow spaces with reversal lines denoting the rhythmic pattern of bone apposition. Histomorphometric estimation of the mean area percent filled by bone trabeculae 4 weeks post-operatively was greater in the hyaluronate group ( $63.68 \pm 1.68$ ) compared to the fibronectin group ( $60.75 \pm 5.37$ ). The control group revealed the lowest area % of bone trabeculae ( $54.83 \pm 4.94$ ). The same pattern was observed eight weeks post-operatively, where the area percent of bone trabeculae was greater in the group hyaluronate ( $87.93 \pm 3.18$ ) compared to the fibronectin group ( $82.04 \pm 4.12$ ), whereas the control group revealed the lowest area % of bone trabeculae ( $77.64 \pm 3.45$ ). **Conclusion:** Titanium surfaces passively coated with the hyaluronate-mimetic approach significantly enhanced osseointegration of titanium implants in rabbit cortical bone. This study established a biologically active and clinically relevant implant coating strategy that enhances bone repair and orthopaedic implant integration.

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## INTRODUCTION

The number of dental implant procedures has increased steadily worldwide per year. (Guehenec, Soueidan *et al.* 2007) After implantation, titanium implants interact with biological fluids and tissues. There are two types of response. The first type involves the formation of a fibrous soft tissue capsule around the implant. This fibrous tissue capsule does not ensure proper biomechanical fixation and leads to clinical failure of the dental implant. The second type of bone response is related to direct bone-implant contact without an intervening connective tissue layer. This is what is known as osseointegration. This biological fixation is considered a prerequisite for implant-supported prostheses and their long-term success. (Albrektsson, Branemark *et al.* 1981). The clinical success of oral implants is related to their early osseointegration. The osseointegration rate of titanium dental implants is related to their composition and surface roughness. Rough-surfaced implants favor

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both bone anchoring and biomechanical stability. (Steinemann 1998; Buser, Broggin *et al.* 2004). Various methods have been developed in order to create a rough surface and improve the osseointegration of titanium dental implants. These methods use titanium plasma-spraying (Rocuzzo, Bunino *et al.* 2001; Martini, Fini *et al.* 2003; Taba Junior, Novaes Junior *et al.* 2003; Ong, Carnes *et al.* 2004), blasting with ceramic particles (Abrón, Hopfensperger *et al.* 2001; Novaes, Souza *et al.* 2002; Piatelli, Scarano *et al.* 2002; Aparicio, Gil *et al.* 2003; Mueller, Gross *et al.* 2003) acid-etching (Cho and Park 2003; Papalexou, Novaes *et al.* 2004; Novaes, Papalexou *et al.* 2004; Zinger, Anselme *et al.* 2004) and anodization (Sul, Byon *et al.* 2004; Huang, Xiropaidis *et al.* 2005; Jungner, Lundqvist *et al.* 2005; Sul, Johansson *et al.* 2005; Xiropaidis, Qahash *et al.* 2005) and modifications of surface roughness at the nanoscale level for promoting protein adsorption and cell adhesion. (Bigerelle, Anselme *et al.* 2002; Zhu, Chen *et al.* 2004; Zinger, Anselme *et al.* 2004;) However, the Cochrane collaboration has not found any clinical evidence demonstrating the superiority of any particular implant surface. (Esposito, Coulthard *et al.* 2005).

The concept of functionalizing the implant surfaces with native or synthetic molecules based on proteins and growth factors emerged from the hypothesis that the ability of imitating the environment of bone, which is composed of an organic matrix (mainly collagenous proteins) and inorganic calcium phosphate, could enhance the implant surface performance and encouraging the initial biologic response. (Novaes, de Souza *et al.* 2010).

The osseointegration of titanium implants coated with biomimetic inorganic calcium phosphate has been investigated in pre-clinical comparative models. These studies have demonstrated a higher bone-to-implant contact for biomimetic calcium phosphate coatings than for uncoated titanium implants. However, the osseointegration of titanium dental implants coated biomimetically has not yet been compared with other surface treatments in pre-clinical models. (Barrere, van der Valk *et al.* 2003; Habibovic, Valk. *et al.* 2005).

Biomimetic organic coatings by proteins found in the extracellular matrix (ECM) such as collagen, fibronectin, osteopontin and bone sialoprotein, a part from cell attachment, they also act on cellular migration and proliferation events. Growth factors, mainly members of the transforming growth factor, bone morphogenetic proteins, platelet-derived growth factor and insulin-like growth factors are some of the most promising candidates for this purpose. (Tatakis, Koh *et al.* 2002; Boyne and Jones 2004; Stenport, Roos-Jansake *et al.* 2004).

Fibronectin is a major ECM component acting as a substrate for cell attachment. It is one of the growth-factors that induce cell proliferation, differentiation, and migration. (Hynes 1986) Moreover fibronectin belongs to a group of high molecular weight glycoproteins that exist on cell surfaces. It is found in connective tissues, basement membranes, as well as extracellular fluids, and is known to play a role in cell-to-cell and cell-to-substrate adhesion. (Tatakis, Koh *et al.* 2002) The first biological reaction at the interface between the biomaterial and tissue after implantation is the adsorption of body fluid proteins onto its surface, and subsequent biological responses at the interface are controlled by these adsorbed proteins. (Horbett 1993; Puleo 1999) The selective adsorption of beneficial molecules has been attempted by modifying the implant surfaces. It has been demonstrated that coating the surface with cell-adhesive proteins, such as fibronectin, collagen, and/or laminin, improved initial cell attachment, cell spreading, and cell activity. (Novaes, de Souza *et al.* 1995; Roehlecke, Witt *et al.* 2001).

In addition to the better-known biomaterials used as biomimetics & bioactive agents for implant coating, there are number of substances which are less used at present, and at the same time, have the potential to augment results of implant therapy. One such molecule is 'Hyaluronate'. (Moseley, Waddington *et al.* 2002). Hyaluronate is a polysaccharide present in the connective tissue of vertebrates, a polymer of glucuronic acid and N-acetylglucosylamine, and is a member of glycosamine family with a high molecular weight. It has many properties that make it a potentially ideal molecule for assisting wound healing by inducing early granulation tissue formation, inhibiting inflammation, promoting epithelial turnover and also connective tissue angiogenesis. (Moseley, Waddington *et al.* 2002).

Hyaluronate accelerates bone regeneration by means of chemotaxis, proliferation and successive differentiation of mesenchymal cells. It shares bone induction characteristics with osteogenic substances such as bone morphogenetic protein-2 and osteopontin (Embery, Waddington *et al.* 2000) It has been proposed that an accelerated wound healing in the bone matrix will occur due to stimulation of angiogenesis by hyaluronate, moreover High molecular weight of hyaluronate has shown to stimulate osteoinduction during wound healing (Sakasi and Watanabe 1995).

The aim of the present study was focused on comparing the impact of fibronectin and hyaluronate in promoting the biological performance of dental implants.

## MATERIALS AND METHODS

The study was performed according to the experimental research ethics committee of the Animal house, Faculty of Medicine, Cairo University.

Twenty New-Zealand white mature male rabbits weighing 2.5-4kg were used in this study. During the experimental period each animal was kept in separate cage and they were fed on standard food, temperature and had free access to tap water.

#### **Study groups:**

The rabbit population was divided into two groups. Group I (n=10) one implant was inserted into each tibia; the implant in the right limb was coated with fibronectin (experimental side), whilst on the contralateral side was uncoated (control).

Group II (n=10) one implant was inserted into each tibia; the implant in the right limb was coated with hyaluronate (experimental side), whilst on the contralateral side was uncoated (control).

#### **1-Coating of implants:**

Forty titanium implants of 4.2 mm in diameter and 8.0 mm in length (Implantium, Dentium, Seoul, Korea) were used in this study. Twenty implants were implanted without coating in left limb of all rabbits, while the experimental condition was implanted by other twenty implants in right limb after coating with fibronectin and hyaluronate as follows: 10µg of fibronectin (Biochrom, Germany) and hyaluronate (Hyadent, BioScience GmbH, Germany) were dissolved in 1 ml of 0.9M phosphate buffered saline, pH 7.2. Then 10 implants were incubated for two hours in 300 µl of fibronectin solution and the other 10 implants were incubated for two hours in 300 µl of hyaluronate solution. The treated implants were removed from the coating solutions and allowed to dry under sterile conditions for 12 hours at room temperature. Thereafter, the coated implants were ready for implantation. (Wuttke, Müller *et al.* 2001; Petrie, Catherine *et al.* 2009)

#### **2-Surgical protocol:**

Under aseptic conditions the surgical procedure was carried out under general anaesthesia produced by an intramuscular injection of Xylazine (Chanazine, Chanelle Pharmaceutical, Ireland) 5mg/kg body weight and ketamine hydrochloride (Ketamine, Pharmazeutische Präparate, Germany). 30mg/kg body weight. Local anaesthesia with 1ml of 5% Xylocaine (Astra, Sweden) was administered to the tibial metaphysis where the implants were to be inserted. Once general anaesthesia was established, the medial aspects in the region of the proximal tibia were shaved; the skin was carefully swabbed with mixture of iodine and 70% ethanol. A 30 mm incision was made along the medial aspect of the proximal tibia and the wound advanced down to and through the periosteum. A subperiosteal dissection was then advanced up to the inferior attachment of the knee joint capsule and laterally to the full extent of the flat medial bone surface. Under continuous irrigation with sterile saline, the forty implants installation procedure in tibiae bone was carried out according to the manufacturer's instructions. The prophylactic administration of procaine penicillin (Wyeth Pharmaceuticals, Parramatta, New South Wales.) 60 000 units/kg intramuscularly was commenced during the surgery and continued for three post-operative days to reduce the potential for wound infection.

#### **3-Animal sacrifice:**

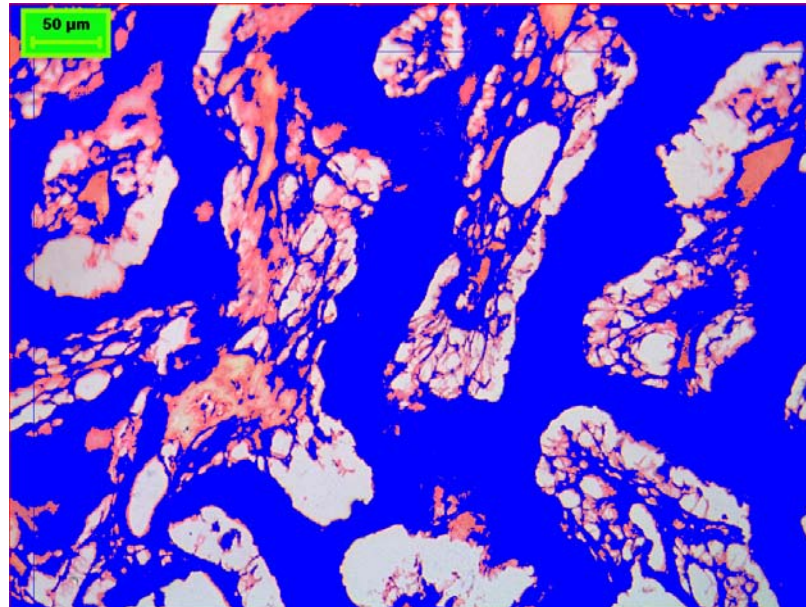
Five rabbits for each group were sacrificed at 4 and 8 weeks for histological and histomorphometrical studies using an intramuscular injection of sodium phenobarbitone (Phenobarbitone, Fawns & McAllan Pty Ltd, Melbourne, Victoria).

#### **4-Histological Examination:**

The tibia were dissected and fixed in 10 percentage normal saline for 48 hours. After that time, the specimens were decalcified with a 50 percentage formic acid and 20 percentage sodium citrate solution for 6 weeks. Eighteen days after the beginning of the decalcification process, the implant was removed from the bone tissue by an incision following the long axis of implant. Following fixation and decalcification, the bone was embedded in paraffin, serial 6-µm sections were obtained and stained with Hematoxylin and Eosin (H & E) and Masson's Trichrome stain.

#### **Measuring Of The Area Percent Of Bone Trabeculae:**

The area percent of bone trabeculae was estimated using Leica Quin 500 analyzer computer system, (Leica Microsystems, Swizerland). The cursor was used to outline the areas of bone trabeculae, which were then masked by a blue binary color that could be measured by the computer. The image analyzer is calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual 1micrometer units. The area percent of bone trabeculae was estimated in 5 different fields in each slide using magnification (x100), (Fig.1). Mean values and standard deviation were calculated for each group.



**Fig. 1:** Copy of the display seen on the monitor of the image analyzer revealing areas of bone trabeculae in the control group 4 weeks post-operatively covered by a blue binary color that could be measured by the computer system.

#### **Statistical Analysis:**

The data obtained from computer image analysis were presented as mean and standard deviation (SD), tabulated and statistically analyzed. Analysis of variance (ANOVA) test was used for statistical analysis of the difference between groups. Tukey's post hoc test was performed since ANOVA test revealed significant difference. Student's t-test was used for comparisons between the two different observation periods and for pair wise comparison between each 2 groups. P value  $\leq 0.05$  was considered statistically significant.

#### **Results:**

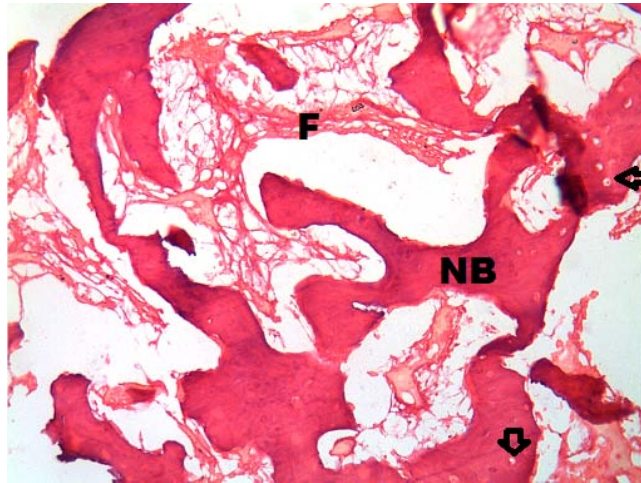
##### **A. Histological results:**

##### **1. Four weeks post-operatively:**

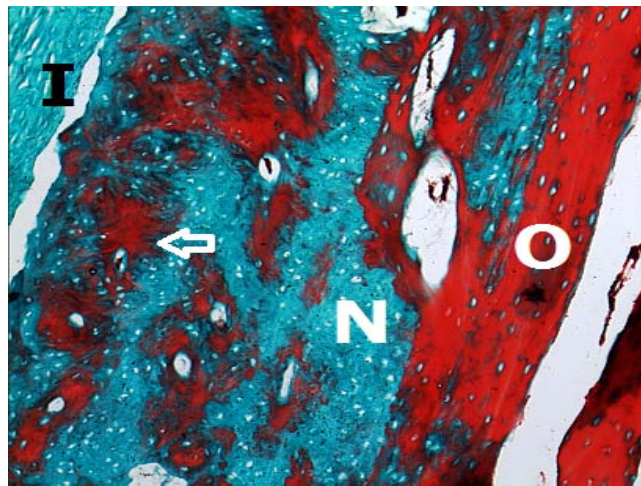
Microscopic examination of H& E and Masson's Trichrome stained section of the control group revealed the deposition of osteoid bone trabeculae surrounding the implant site. The newly formed bone trabeculae were irregularly arranged and separated by large marrow spaces. Collagen fibers of variable compactness and regularity were also demonstrated adjacent to the implant site. No inflammatory cell infiltrate was present (Fig. 2, 3&4).

In fibronectin group, dispersed irregularly arranged bone trabeculae separated by collagen fiber bundles and few normal sized blood vessels were demonstrated. The newly formed bone consisted mainly of woven bone with some more mature lamellar bone apparent. Large lacunae containing plump osteocytes were detected within the bone trabeculae. The evident merging of woven and mature bone trabeculae indicated that the maturation process was in progress. The implanted material appeared resorbed and replaced by the newly formed bone (Fig. 5, 6, 7&8).

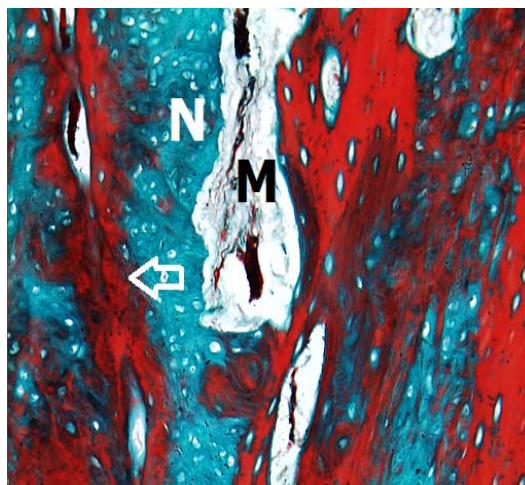
In hyaluronate group, microscopic examination revealed evident bone growth in the form of woven bone bordering the implant site. Osteoid bone trabeculae forming a lattice were dispersed within the bony defect and were separated by marrow spaces. Fibrous connective tissue showing fibroblastic activity and dispersed marrow spaces of variable size were seen adjacent to the newly-formed bone. The bone marrow comprised both haemopoietic cells and adipocytes (Fig. 9,10&11).



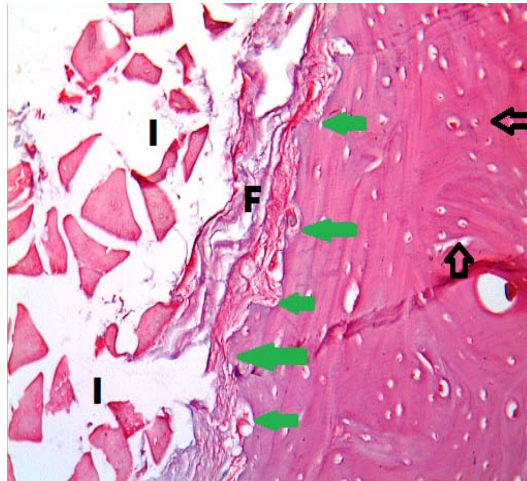
**Fig. 2:** Photomicrograph of control group 4 weeks post-operatively revealing, thin irregular newly formed bone trabeculae (NB) containing viable osteocytes (arrows) located adjacent to the implant site. Collagen fibers of variable compactness and regularity are also demonstrated (F), (H&Ex200).



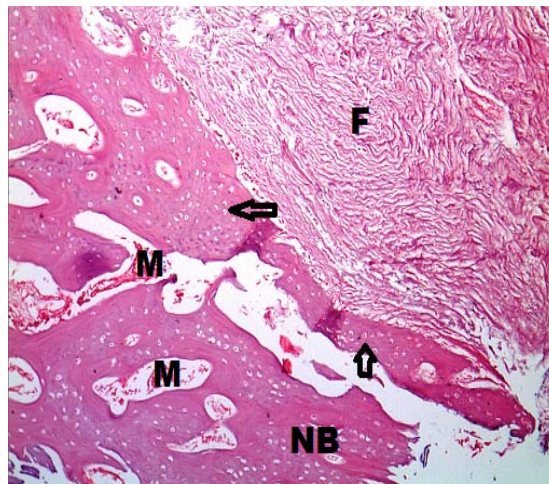
**Fig. 3:** Photomicrograph of control group at 4 weeks post-operatively, revealing an implant site (I) bordered by newly formed unclacified bone trabeculae (N) stained blue-green with Masson's Trichrome. Focal areas of calcification exhibiting red histochemical staining are seen within the newly formed bone. Original bone (O) is seen at the periphery, (Masson's Trichrome x100).



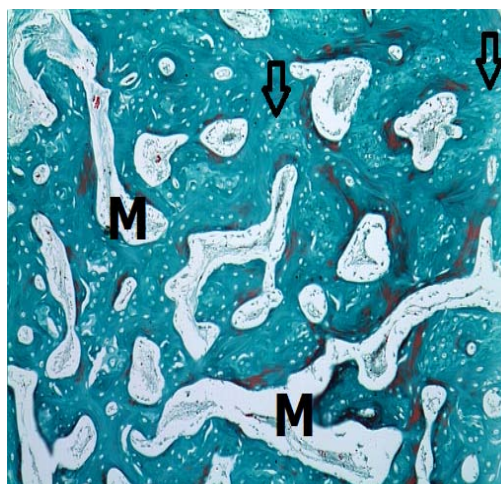
**Fig. 4:** Photomicrograph of control group at 4 weeks post-operatively, revealing newly formed unclacified bone trabeculae (N) stained blue-green with Masson's Trichrome. Areas of calcified bone exhibiting red histochemical staining are demonstrated (arrow). Large marrow spaces (M) are also demonstrated, (Masson's Trichrome x200).



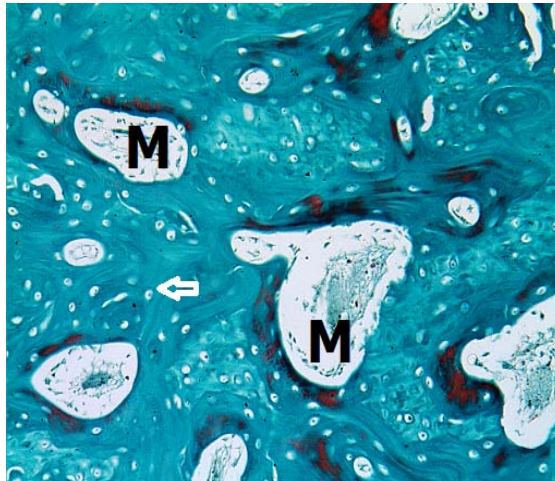
**Fig. 5:** Photomicrograph of fibronectin group 4 weeks post-operatively. Indentations created by the implant (green arrows) are seen on the surface of the bone adjacent to the implant site (I). Vitality of bone is confirmed by the entrapped osteocytes (black arrows). Parallel dense collagen fibers are also demonstrated (F), (H&Ex200).



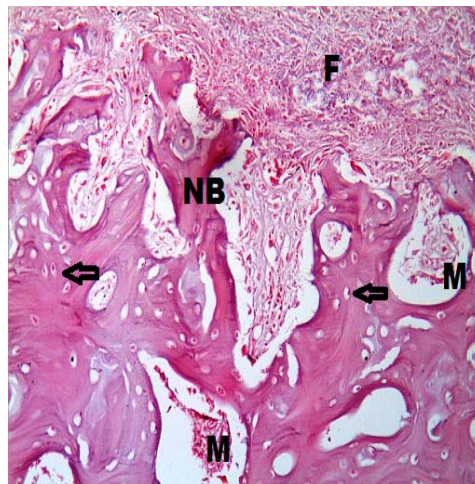
**Fig. 6:** Photomicrograph of fibronectin group 4 weeks post-operatively illustrating trabeculae of newly formed bone (NB) with entrapped osteocytes (arrows) enclosing marrow spaces (M) of variable sizes. Parallel dense collagen fibers are also demonstrated (F), (H&Ex200).



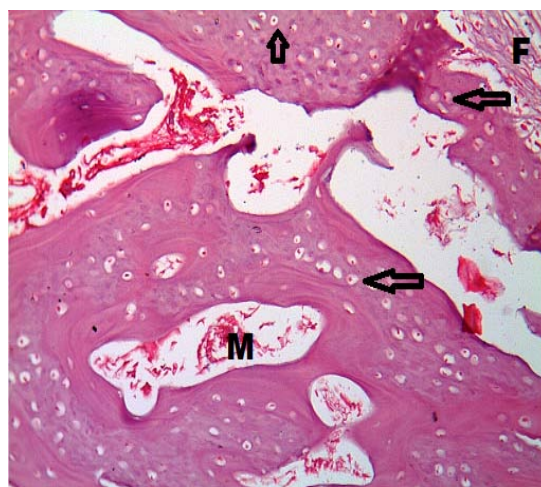
**Fig. 7:** Photomicrograph of fibronectin group at 4 weeks post-operatively revealing, filling of the implant site with newly-formed uncalcified woven bone (arrows) separated by wide marrow spaces (M), (Masson's Trichrome x100).



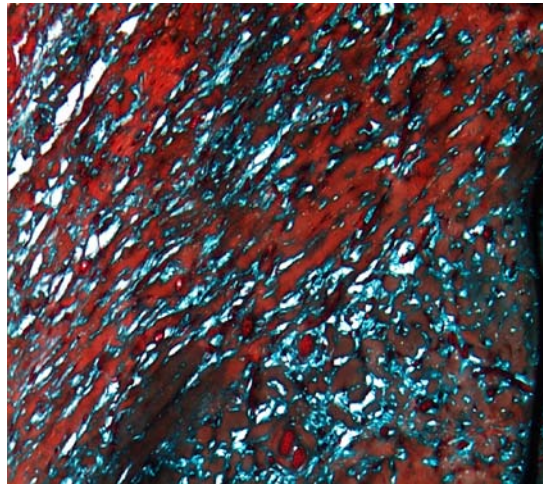
**Fig. 8:** Photomicrograph of fibronectin group at 4 weeks post-operatively revealing, filling of the implant site with newly-formed uncalcified woven bone separated by wide marrow spaces. Osteocytes are enclosed with lacuna denoting bone vitality (arrows), (Masson's Trichrome x200).



**Fig. 9:** Photomicrograph of hyaluronate group 4 weeks post-operatively showing irregular trabeculae of newly formed bone (NB) infiltrating dense collagen fibers (F). Osteocytes (arrows) and marrow spaces (M) are demonstrated within bone trabeculae, (H&Ex200).



**Fig. 10:** Photomicrograph of hyaluronate group 4 weeks post-operatively showing thick trabeculae of newly formed bone containing viable osteocytes (arrows) and enclosing variable-sized marrow spaces (M). Parallel dense collagen fibers (F) are seen adjacent to the implant site, (H&Ex200).



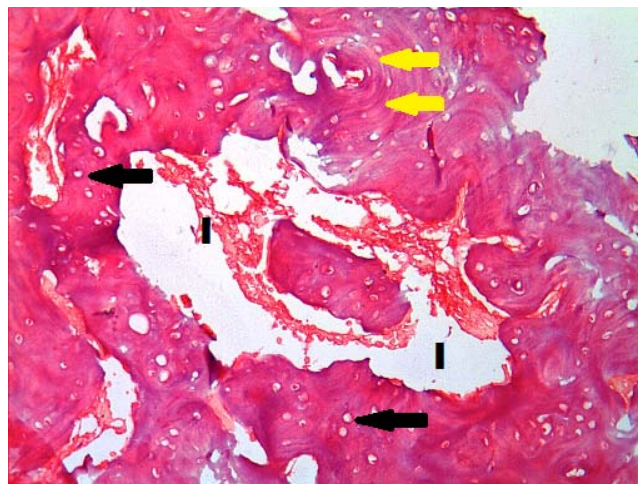
**Fig. 11:** Photomicrograph of hyaluronate group at 4 weeks post-operatively revealing Thin parallel trabeculae are dispersed by localized areas of calcified bone recognized by the red histochemical stain are dispersed within the new bone trabeculae, (Masson's trichromex100).

### **2.Eight weeks post operatively:**

Microscopic examination of H&E and Masson's Trichrome stained section of the control group revealed that the implant site was completely surrounded by thick bone trabecular, separated by a fibro-vascular stroma. Vitality of bone was confirmed by osteocytes enclosed in lacunae within bone. Reversal lines denoting the rhythmic pattern of bone formation were also demonstrated (Fig. 12, 13, 14 &15).

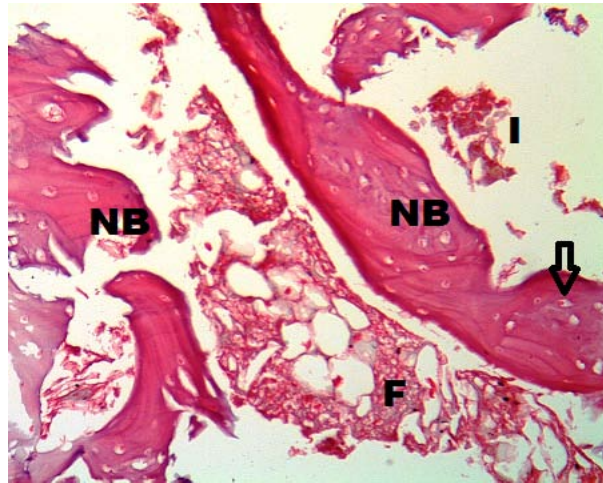
In fibronectin group, there was circumferential bone regeneration in the form of thick regular bone trabeculae, separated by marrow spaces. Randomly arranged osteocytes were embedded within the trabeculae denoting vitality and enhanced bone apposition. Variable-sized fibro-vascular marrow separated the newly formed bone trabeculae. Reversal lines denoting the rhythmic pattern of bone apposition were observed (Fig. 16, 17, 18 &19).

Microscopic examination of hyaluronate group revealed newly formed bone trabeculae that were mainly directed toward the implant site. Thick bone trabeculae were seen coalescing together in certain areas, but separated by marrow spaces in other sites. Osteocytes were embedded within the newly formed trabeculae confirming the maintained bone vitality. Reversal lines denoting the rhythmic pattern of bone apposition were observed. No inflammatory cells could be detected (Fig. 20, 21, 22, 23&24).

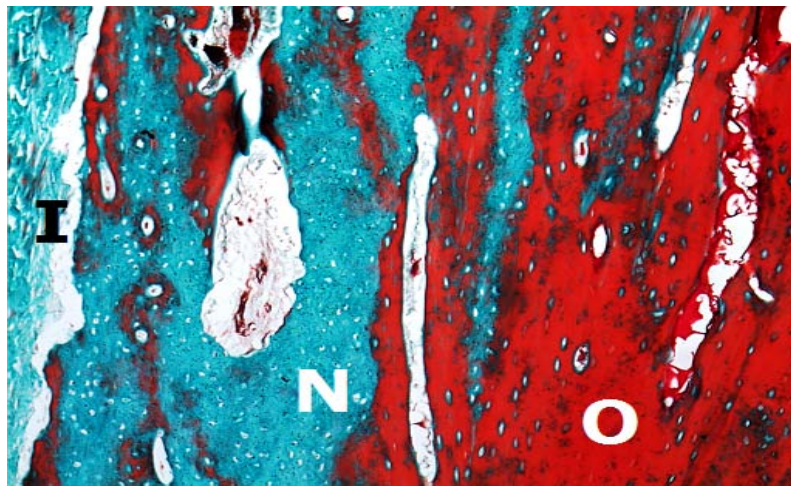


**Fig. 12:** Photomicrograph of control group 8 weeks post-operatively revealing thick trabeculae of newly formed bone trabeculae containing viable osteocytes (black arrows) surrounding the implant site (I). Reversal lines (yellow arrows) denoting the rhythmic pattern of bone formation are demonstrated, (H&Ex200).

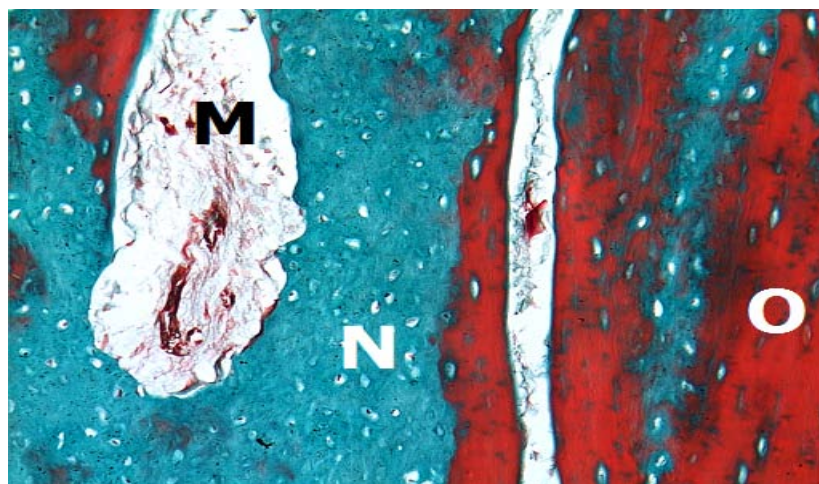




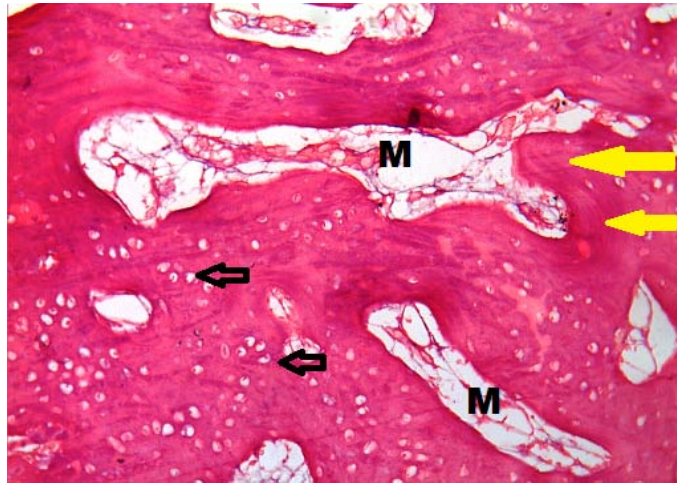
**Fig. 13:** Photomicrograph of control group 8 weeks post-operatively revealing ,elongated trabeculae of newly formed bone (NB) of varying thickness and containing viable osteocytes (arrow) surrounding the implant site (I). Dense fibro-vascular tissue (F) separates the newly formed trabeculae, (H&Ex200).



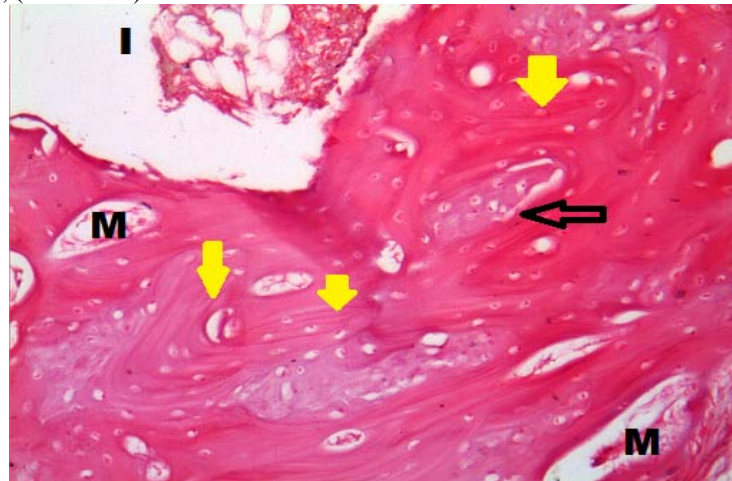
**Fig. 14:** Photomicrograph of control group at 8 weeks post-operatively, revealing an implant site (I) bordered by newly formed unclarified bone trabeculae (N). Original bone (O) is demonstrated at the periphery, (Masson's Trichrome x100).



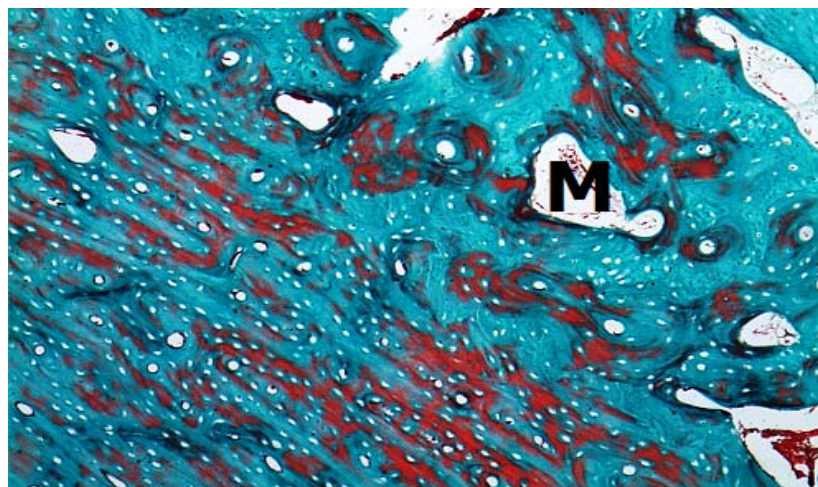
**Fig. 15:** A higher magnification of the previous photomicrograph of control group at 8 weeks post-operatively, revealing large marrow space (M) within the newly formed unclarified bone trabeculae (N). Original bone (O) is demonstrated at the periphery, (Masson's Trichrome x200).



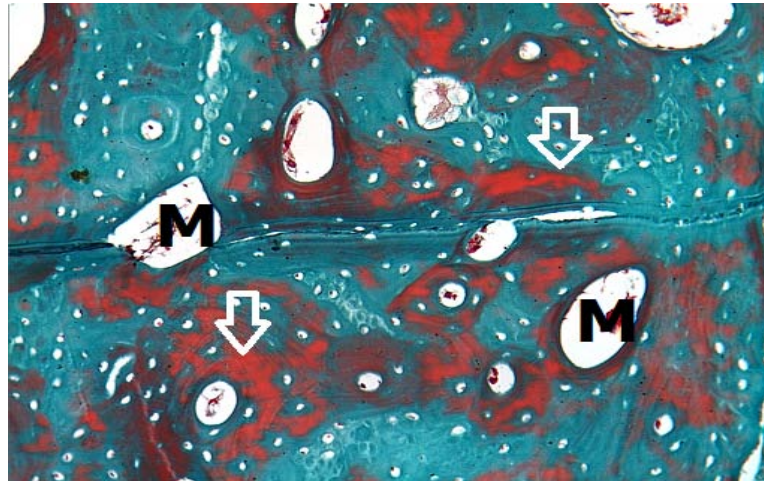
**Fig. 16:** Photomicrograph of fibronectin group 8 weeks post-operatively revealing thick trabeculae of newly formed bone containing viable osteocytes (black arrows) and enclosing variable-sized marrow spaces (M). Reversal lines (yellow arrows) denoting the rhythmic pattern of bone apposition are demonstrated, (H&Ex200).



**Fig. 17:** Photomicrograph of fibronectin group 8 weeks post-operatively revealing the implant site (I) separated by thick trabeculae of newly formed bone containing viable osteocytes (black arrows) and enclosing marrow spaces (M). Reversal lines (yellow arrows) denoting the rhythmic pattern of bone apposition are demonstrated, (H&Ex200).



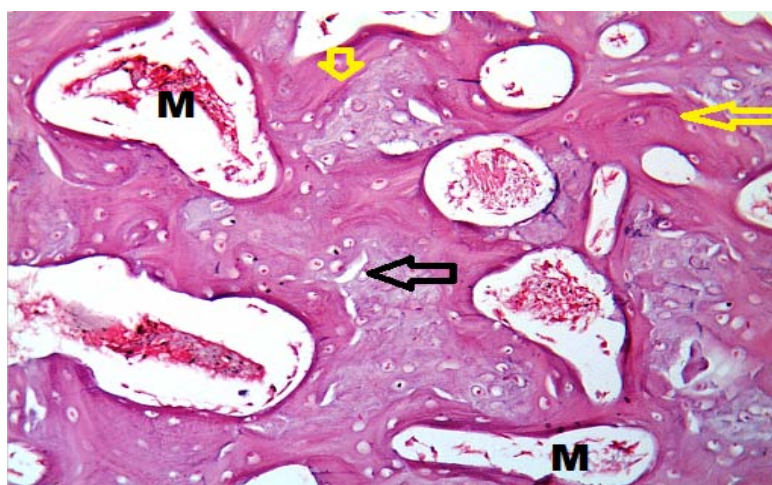
**Fig. 18:** Photomicrograph of fibronectin group at 8 weeks post-operatively revealing newly formed uncalcified bone trabeculae filling the implant site. Focal areas of calcified bone recognized by the red histochemical stain are dispersed within the new bone trabeculae. Wide marrow spaces (M) are demonstrated, (Masson's Trichrome x200).



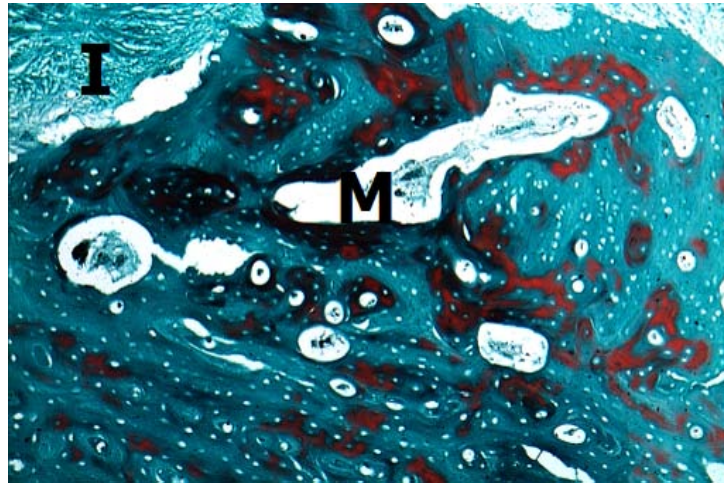
**Fig. 19:** Higher magnification of the previous photomicrograph of fibronectin group at 8 weeks post-operatively revealing newly formed uncalcified bone trabeculae filling the implant site. Focal areas of calcified bone recognized by the red histochemical stain (arrows). Marrow spaces (M) are demonstrated, (Masson's Trichrome x200).



**Fig. 20:** Photomicrograph of hyaluronate group 8 weeks post-operatively revealing thick trabeculae of newly formed bone containing viable osteocytes (black arrows) and enclosing variable-sized marrow spaces (M). Reversal lines (yellow arrows) denoting the rhythmic pattern of bone apposition are demonstrated, (H&Ex100).



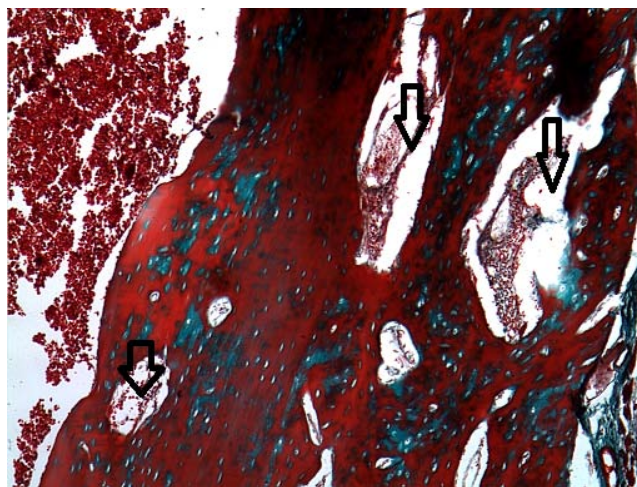
**Fig. 21:** Higher magnification of the previous photomicrograph of hyaluronate group 8 weeks post-operatively revealing thick trabeculae of newly formed bone containing viable osteocytes (black arrows) and enclosing fibro-vascular marrow (M). Reversal lines (yellow arrows) are observed, (H&Ex200).



**Fig. 22:** Photomicrograph of hyaluronate group at 8 weeks post-operatively revealing newly formed uncalcified bone trabeculae bordering the implant site (I). Focal areas of calcified bone recognized by the red histochemical stain are dispersed within the new bone trabeculae. Wide marrow spaces (M) are demonstrated, (Masson's Trichrome x100).



**Fig. 23:** Higher magnification of the previous photomicrograph of hyaluronate group at 8 weeks post-operatively revealing newly formed uncalcified bone trabeculae bordering the implant site (I). Focal areas of calcified bone recognized by the red histochemical stain are dispersed within the new bone trabeculae (arrows). Wide marrow spaces (M) are demonstrated, (Masson's Trichrome x200).



**Fig. 24:** Photomicrograph of hyaluronate group at 8 weeks post-operatively revealing calcification of the newly formed bone stained red, with localized areas of un-calcified bone stained blue-green with Masson's trichrome. Wide marrow spaces (arrows) are demonstrated, (Masson's Trichrome x200).

**B. Histomorphometrical results:****I-Comparison between control and experimental groups:**

Histomorphometric estimation of the mean area percent filled by bone trabeculae 4 weeks postoperatively was greater in the hyaluronate group ( $63.68 \pm 1.68$ ) compared to the fibronectin group ( $60.75 \pm 5.37$ ). The control group revealed the lowest area % of bone trabeculae ( $54.83 \pm 4.94$ ). Analysis of variance (ANOVA) test revealed that the difference between the 3 groups was extremely statistically significant ( $p < 0.0001$ ). Tukey's post hoc test revealed that the hyaluronate and fibronectin groups were not statistically different, as confirmed by Student's t test (Table 1, 2; Fig.25).

The same pattern was observed eight weeks post-operatively, where the area percent of bone trabeculae was greater in the group hyaluronate ( $87.93 \pm 3.18$ ) compared to the fibronectin group ( $82.04 \pm 4.12$ ), whereas the control group revealed the lowest area % of bone trabeculae ( $77.64 \pm 3.45$ ). Analysis of variance (ANOVA) test revealed that the difference between the 3 groups was highly statistically significant ( $p < 0.0001$ ). Tukey's post hoc test revealed that each two groups were statistically different, as confirmed by Student's t test (Table 1, 2; Fig.25).

**Table 1:** Values of area % filled by bone trabeculae and statistical significance of the different between all groups at each observation period (ANOVA test)

Values	4 weeks			8 weeks		
	C	F	H	C	F	H
Mean	54.83 <sup>a</sup>	60.75 <sup>b</sup>	63.68 <sup>b</sup>	77.64 <sup>a</sup>	82.04 <sup>b</sup>	87.93 <sup>c</sup>
StdDev	4.94	5.37	1.68	3.45	4.12	3.18
Std Error	6.682	6.871	0.751	6.013	1.843	1.424
Max	74.26	71.681	65.497	92.569	87.005	93.295
Min	34.363	30.815	61.007	57.878	74.769	83.706
2-s Range	59.769	61.46	6.714	53.786	16.486	12.735
F value	10.877			20.510		
P value	<0.0001***			<0.0001***		

\*\*\*Highly statistically significant

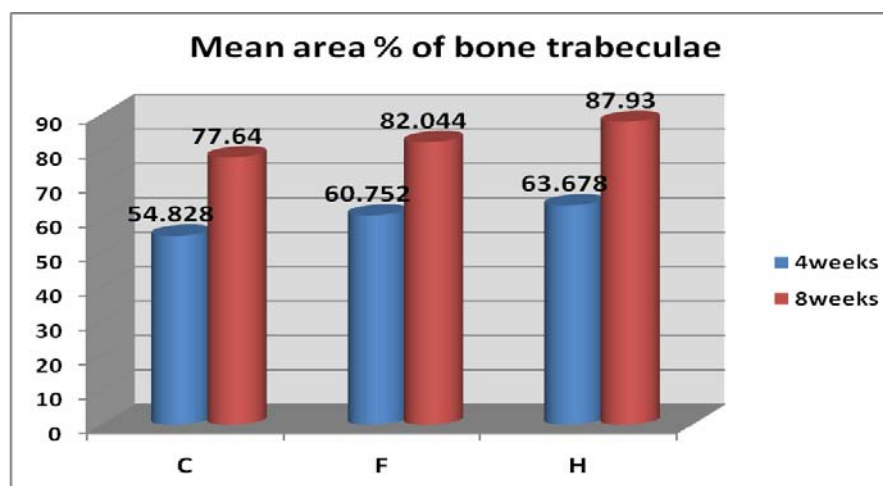
**Table 2:** Results of Student's t test used for pairwise comparison between groups

Observation time	Groups	t value	p value
4 weeks	C versus F	2.5657	0.0195*
	C versus H	5.3635	<0.0001***
	F versus H	1.6467	0.1170
8 weeks	C versus F	2.5893	0.0185*
	C versus H	6.9352	<0.0001***
	F versus H	3.5788	0.0021**

\*Statistically significant

\*\*Very statistically significant

\*\*\* Highly statistically significant



**Fig. 25:** Mean area % of bone trabeculae in all groups throughout the experiment

**II- Change By Time In Bone Trabeculae In Control And Experimental Groups:**

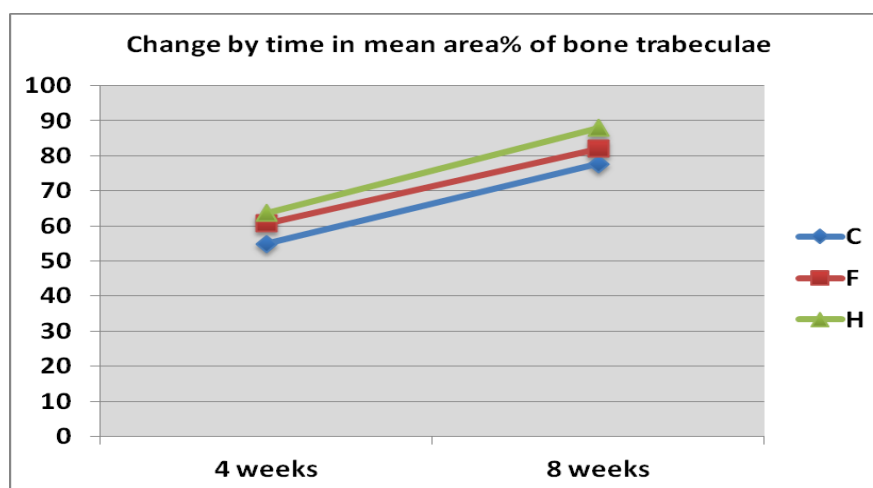
All groups exhibited an increase in the area percent occupied by bone trabeculae throughout time. Student's t test revealed that the difference in the mean area % throughout the experiment (four and eight weeks post-operatively) was extremely statistically significant ( $p < 0.0001$ ) in the control and experimental groups. The

percent increase was greater in the control group (41.60%), compared to the fibronectin group (35.05%), and hyaluronate (38.08%) (Table 3, Fig.26&27).

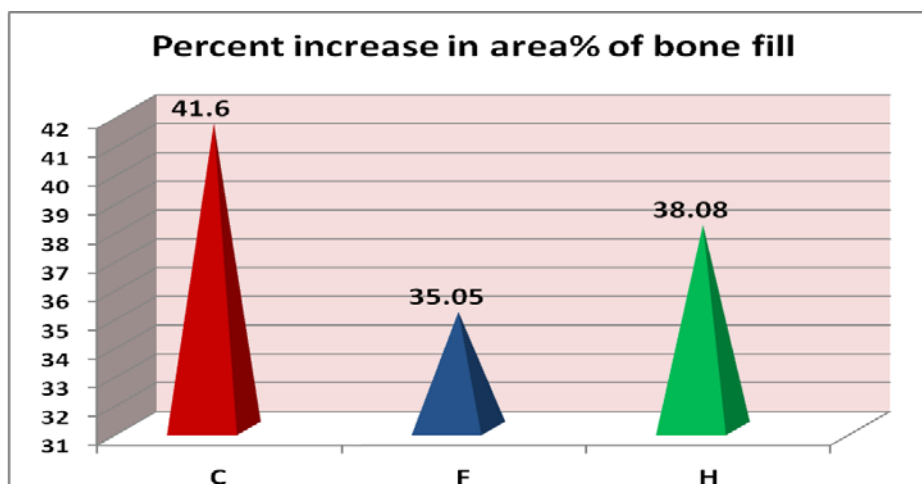
**Table 3:** Change by time in mean area % of bone trabeculae of each group and statistical significance of the difference (Paired Student's t test)

Groups Values	Control		Fibronectin		Hyaluronate	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
Mean	54.83	77.64	60.75	82.04	63.68	87.93
StdDev	4.94	3.45	5.37	4.12	1.68	3.18
% increase	41.60%		35.05%		38.08%	
t value	11.9711		9.9469		21.3222	
P value	<0.0001***		<0.0001***		<0.0001***	

\*\*\* Highly statistically significant



**Fig. 26:** Change by time in area % of bone trabeculae in all groups



**Fig. 27:** Percent increase in area filled by bone trabeculae throughout the experiment

### Discussion:

Surface modifications of titanium implants using various modalities aim to improve the initial healing and promote faster healing times. (Dohan, Coelho *et al.* 2010) With this surface modification, immediate or early loading has become a predictable treatment protocol. (Atieh, Payne *et al.* 2010) The future of dental implantology should aim to develop surfaces with controlled and standardized topography or chemistry. This approach will be the only way to understand the interactions between proteins, cells and tissues, and implant surfaces. The local release of bone stimulating or antiresorptive substances in the peri-implant region may also respond to difficult clinical situations with poor bone quality and quantity. These therapeutic strategies should

ultimately enhance the osseointegration process of dental implants for their immediate loading and long-term success. (Zhao, Schwartz *et al.* 2005)

The present study was designed to entail the tibial bone that constituted mainly compact cortical bone, with a structure comparable to that of human. Hence, the tibia was considered an ideal model for mandibular bone, this model was documented to provide ideal conditions for the investigation of bone regeneration and implant osseointegration. (Chong-Hyun and Dong-Hoo 1994)

The present research was focused on the development of surface modifications that mimic the existing biological situation by promoting specific cell attachment, and thereby enhancing osseointegration of the implant surface. To achieve this goal this study was conducted to characterize the osseointegration of fibronectin vs hyaluronate-coated titanium dental implants as a biologically active and clinically relevant implant coating strategy that enhances bone repair and implant integration.

This modification on the topographic pattern of implant surface increases not only the bone-implant contact, but also the biomechanical interaction of that interface at early implantation periods. (Rocuzzo, Bunino *et al.* 2001)

Rocuzzo, Bunino *et al.* observed in an in vivo study that the topography is not the only surface characteristic capable of enhancing new bone apposition. (Rocuzzo, Bunino *et al.* 2001) Besides, Germanier, Tosatti *et al.* showed in an in vitro study that surface chemistry also influenced the attachment and morphology of cells. In general, an increase in cell number and more spread cells were observed on bioactive substrate compared to bio-inactive surfaces according to their findings. (Germanier, Tosatti *et al.* 2006)

However, Bioactive surface modification approaches, including calcium-phosphate ceramic coatings and macro/microporosity, have had limited success in promoting integration because of a chipping effect of apatite crystals from the surface, causing a phagocytic response by macrophages or even a foreign body reaction and peri-implantitis in the long run. (Schuler, Owen *et al.* 2006)

In attempts to avoid such effects and to improve osseointegration, titanium surfaces were biofunctionalized with ECM-mimetic peptides, this biomimetic implant coating is generated using a simple, single-step procedure that readily translates to a clinical environment with minimal processing and cytotoxicity concerns. (Iezzi, Pecora *et al.* 2009)

These findings were in consistence with the strategy of our study that combines the benefits of both topographic surface characteristics of sand blasted acid etched dental implant together with surface biomodification using two of the most important bioactive ECM proteins hyaluronate and fibronectin in an attempt to mimic the existing biological situation by promoting specific cell attachment, and thereby enhancing osseointegration of the implant surface.

Importantly, In this study, biomodification of implant surface utilized a simple, dip-coating method relied on physical adsorption of fibronectin and Hyaluronate to pre-sterilized titanium implants as a simple, clinically-translatable strategy to functionalize dental implants. This is in accordance with Petrie *et al* whom described this simple, one-step coating procedure that relies on the passive adsorption of an ECM peptide onto titanium implants to enhance osseointegration. (Petrie, Catherine *et al.* 2009)

On the other hand, the other approaches for implant coating and biofunctionalization, although are successful in many cases, they can be restricted by slow rates of osseointegration and poor mechanical anchorage, especially in challenging clinical cases, such as those associated with large bone loss and poor bone quality. (Catherine, Timothy *et al.* 2007) In addition, these surface modification approaches rely on costly and manufacturing-intensive processes. As an alternative to these surface technologies, emerging biomimetic strategies that focused on the presentation of extracellular matrix sequences and growth factors. (Dillow, Ochsenhirt *et al.* 2001) The general paradigm of these approaches is the covalent immobilization of the biological entities onto the underlying implant surface, which often involves multi-step procedures to render the surface suitable for biofunctionalization. (Chung, Gilbert *et al.* 2006) In contrast, the method used in our study for implant biofunctionalization ,relies on Petrie, Catherine *et al.* technique which offers a quick and versatile surface application conducted under physiological conditions that the surgeon can employ seconds before implantation. This single-step procedure, in turn, minimizes the chance of infection, reduces implant surface treatment variability, and minimizes cytotoxicity concerns inherent with covalent immobilization schemes, while maintaining the surgeon's dexterity. (Petrie, Catherine *et al.* 2009)

Clinical and histologic results revealed that all implants were clinically stable and non-mobile. The osseointegration of each implant was confirmed. Osteoblasts had actively deposited osteoid matrix on the implant surface. The newly formed bone in the interthread space was in close contact with the implant surface, without any gaps or dense fibrous connective tissue. No apical epithelial migration was found. No inflammatory cell infiltrate was present around the implants. Areas of bone remodeling with osteoblasts and osteoid matrix with reversal lines were observed specially in hyaluronate group more than in fibronectin while the least of these histological findings were observed in the uncoated control implants especially at the early healing period (4 weeks post-operative).

Histomorphometric estimation of the mean area percent filled by bone trabeculae was greater in the hyaluronate group ( $63.68 \pm 1.68$ ) ( $87.93 \pm 3.18$ ) at 4&8weeks post-operatively respectively compared to the fibronectin group ( $60.75 \pm 5.37$ ) ( $82.04 \pm 4.12$ ), while the control group revealed the lowest area % of bone trabeculae ( $54.83 \pm 4.94$ ) ( $77.64 \pm 3.45$ ) at the same observation periods. Analysis of variance (ANOVA) test revealed that the difference between the 3 groups was highly statistically significant ( $p < 0.0001$ ) at both estimation periods. While Tukey's post hoc test revealed that the difference between hyaluronate and fibronectin was not statistically significant at 4 weeks, however each two groups were statistically different at 8 weeks post-operatively, as confirmed by Student's t test.

Regarding Change by time in bone trabeculae in control and experimental groups, all groups exhibited an increase in the area percent occupied by bone trabeculae throughout time. Student's t test revealed that the difference in the mean area % throughout the experiment (four and eight weeks post-operatively) was highly statistically significant ( $p < 0.0001$ ) in the control and experimental groups. The percent increase was greater in the control group (41.60%), compared to the fibronectin group (35.05%), and hyaluronate (38.08%). This can be explained by the observation that the area % of bone fill was greater in experimental groups at an earlier time (in the first observation period), consequently, the percent increase was slightly lower in experimental compared to the control, which needed longer time for new bone formation.

Different mechanisms have been proposed to explain the effect of hyaluronate and fibronectin in accelerating wound healing and promoting osteogenesis. The biomimetic coating with fibronectin has been tested in previous studies. (Urban, Jacobs *et al.* 2000; Brett, Harle *et al.* 2004; Lutolf and Hubbell 2005) It is believed that application of fibronectin to the implant surface can enhance the osseointegration of dental implants to the bone. (Martini, Fini *et al.* 2003) This can be explained by the researches that demonstrated that coating the surface with cell-adhesive proteins, such as fibronectin, improved initial cell attachment, cell spreading, and cell activity. (Urban, Jacobs *et al.* 2000; Brett, Harle *et al.* 2004; Lutolf and Hubbell 2005) Others have documented that fibronectin is actively involved in cytoskeletal reorganization and bone tissue formation, in part by regulating the survival of osteoblasts. (Roccuzzo, Bunino *et al.* 2001) Moreover fibronectin is known to play a role in cell-to-cell and cell-to-substrate adhesion as well as an important role in osseointegration due to its capacity to make osteoblasts attach to ECM components. (Browne and Gregson 2000)

However, the present study supported the superiority of hyaluronate over fibronectin both qualitatively by the microscopic evaluation of the quality of the newly formed bone around hyaluronate coated implants vs fibronectin and quantitatively by histomorphometric analysis of the area percent occupied by bone trabeculae .

There are two mechanisms to explain these findings , firstly, Hyaluronate accelerates wound healing and promoting osteogenesis by means of chemotaxis, proliferation and successive differentiation of mesenchymal cells as well as by a certain role on hydroxyapatite crystal formation. (Pilloni, Rimondini *et al.* 2003) These important roles mediated by hyaluronate have confirmed by other investigators who documented that hyaluronate shares bone induction characteristics with osteogenic substances such as bone morphogenetic protein-2 and osteopontin. (Mendes, Silva *et al.* 2008)

Secondly, hyaluronate has anti-inflammatory properties through modulation of inflammatory cells, interaction with the proteoglycans of the extracellular matrix and scavenging of free radicals. Thus inhibiting tissue destruction and facilitates healing. (Moseley, Waddington *et al.* 2002), (Boskey and Dick 1991)

Based on our findings and on the previous mentioned studies it can be stated that hyaluronate-mimetic approach significantly enhanced osseointegration of titanium implants in rabbit cortical bone. This study establishes a biologically active and clinically relevant implant coating strategy that enhances bone repair and dental implant integration.

### **Conclusion:**

Biofunctionalization of implant surfaces is still a poorly investigated area. The enhancement of early osseointegration around the hyaluronate-coated surface is promising. Further in vitro and in vivo studies are necessary to elucidate how and in which extent biofunctionalization of the implant surfaces could represent an advantageous modification.

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