Effect of Milled Tooth Powder versus β-tricalcium phosphate as Bone Grafts on Healing of Tooth Extraction Sockets of Rabbits: *An Experimental Study*

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Abstract:

Aim: To compare the effects of milled tooth (MT) powder and β-tricalcium phosphate (β-TCP) on healing of extraction sockets in rabbits.

Methods: Sixty healthy adult male rabbits were divided into four groups (n=15). After anesthesia the right mandibular first molars were extracted and the treated materials (β-TCP or MT) were gently applied to the extraction sockets forming two groups. In the positive (+ve) control group, sockets healed spontaneously. In the negative (-ve) control group, jaws were left without any interference. Five rabbits from each group were euthanized 10, 20, and 30 days after surgery. Specimens were prepared for light microscopic examination using hematoxylin and eosin, Masson's trichrome staining, and osteopontin. ANOVA was used to assess the statistical significance of the difference between more than two group means. Tukey's multiple comparisons and post-hoc tests were used to explore differences between the means of multiple groups.

Results: Regarding osteoid tissue formation by Masson's trichrome staining, a highly significant difference recorded between -ve control, +ve control/10 d, β-TCP/20 d, MT/10 d, and MT/30 d groups. However, no significant differences were detected between the -ve control and +ve control, β-TCP/ 20 d, β-TCP/30 d, and MT/30 d groups. Higher expression of osteopontin were detected in the β-TCP/20 d and MT/30 d groups, and a highly significant difference was observed compared to the +ve control group.

Conclusions: MT powder is a biocompatible osteoconductive graft material that can be used as bone graft material in tooth extraction socket healing.

Keywords: β-tricalcium Phosphate, Milled tooth, Bone graft, Bone defects,

Introduction

Tooth extraction due to caries, trauma, or advanced periodontal disease results in a reduction in alveolar ridge volume, both in height and width, leading to morphological changes (1). After tooth extraction, the width of the alveolar ridge was to be reduced by 50 % during the first 12 months (about 5 to 7 mm), two thirds of this bone loss found to be in the first three months after tooth extraction, indicating that most of the dimensional changes of the alveolar ridge occurs within the first 3 months of healing with a corresponding vertical bone loss of 0.9 to 3.25 mm (2, 3). This is a challenging problem encountered during the placement of implants. Bone repair is an important process in defect site remodelling that reaches the original structure (4). The main goal of an ideal bone graft material is to serve as a scaffold, maintain space for bone ingrowth and blood vessel formation, support soft tissues, and improve the quality and quantity of the regenerated bone (5).

Many studies have been conducted to investigate alternative synthetic biomaterials, such as metals, ceramics, and polymers; however, these materials carry the risks of infection and structural failure. β-TCP. It is biocompatible with no inflammation, and no fibrosis capsule develops between the graft particles and the newly formed bone. β-TCP acts as an osteoconductive scaffold that promotes bone regeneration (6).

β-TCP is the most studied graft material because of its composition, which is similar to that of bone

(7-10). They can partially integrate into natural bone tissue, and their high affinity for proteins such as BMPs, can induce stem cell differentiation and growth, and therefore, new bone formation (11,

12). They can be prepared in granular, block, or putty formats and their high porosity facilitates the penetration and distribution of blood vessels (7, 8). β-TCP is easily resorbable and rapidly replaced by new bones (13,14). Although β-TCP appears to be a good graft material, the contradictory results obtained in animal studies require further research to clarify the potential of β-TCP for the regeneration of bone defects. However, β-TCP has some drawbacks such as brittleness, low resistance to fracture, and resorption within six weeks after implantation (15).

To overcome these problems, teeth have attracted increasing attention as an alternative to bioinert bone substitutes. It is composed of an organic matrix and inorganic reinforcing phase of hydroxyapatite (16). Autogenous tooth bone grafting is a grafting technique that utilizes extracted teeth from the same individual to prepare graft material. Extracted teeth of the same individual provide such a valuable source without causing any harm because the teeth have already been extracted; hence, the need for donor-site surgery is eliminated (17).

Zhang et al. (18) reported that powder-type graft materials composed mainly of inorganic enamel carry the capacity for osteoconduction and can maintain the bone volume after grafting. In addition, tooth powder is biocompatible and resorbs slowly to be replaced by new bone. Many researchers have examined tooth dentin as a potential carrier for human proteins and as a grafting material because its biological composition is very similar to that of the alveolar bone. Moreover, research has revealed that the dentin matrix contains BMPs, osteopontine, osteonectin, osteocalcin, dentin sialoproteins, bone connexins, and alkaline phosphatase. These proteins play a role in bone formation and calcification of bone (19).

Based on the above information, the current study aimed to evaluate and compare the effects of MT and β-TCP bone graft materials on bone healing in tooth extraction sockets of adult male rabbits through histological and immunohistochemical investigations. The null hypothesis suggested that there was no difference in the initiation of bone healing between the tested materials.

Materials and Methods:

The present experimental study followed the ARRIVE guidelines. The study protocol was approved by the Ethics Committee of the Animal House of the Faculty of Dentistry, Suez Canal University (no.261/2020).

Bone graft materials

β-tricalcium phosphate bone graft material particles (0.5-1.0 mm), sterilized using irradiation, biomaterial (Medbone Medical Device, Lda)

Preparation of milled tooth bone graft material

Sound teeth extracted for orthodontic reasons (premolars or third molars) were stored in normal saline and cleaned using blade no.15. 950 °C (1742F) for protein denaturation.

The tooth material was pulverized using a freezing mechanical grinder and SPEX sample prep 6870 freezer mill. It is a cryogenic laboratory mill that cools samples to remove all attached soft tissues. Then were heated in a furnace for 30 min at-195.80 c (320.4F) using liquid nitrogen and pulverized by magnetically shuttling a steel impactor back and forth against two stationary end plugs. The MT bone graft material was obtained from the College of Dentistry, King Saud University, Riyadh, Saudi Arabia (20).

Characterization of bone graft powder

X-ray diffraction and scanning electron microscopy analyses:

These two analyses were aimed at characterizing β-TCP and MT and correlating their biomaterial crystal structures, phase compositions, and morphologies with their respective biological behaviors. X-ray diffraction (XRD) spectra were obtained using Cu (kα1) radiation (XRD-D2 Phaser-Bruker-Germany) in the region $100<2\Theta<700$.

Scanning electron microscopy (SEM) (JEOL. Ltd, Akishima, Tokyo. Japan) was used for the structural and particle-size distribution analyses of the tested materials.

Animals and grouping:

Sixty healthy adult male rabbits with body weights ranges from 1.5-2 kg were chosen. All the animals were maintained in accordance with the guidelines for laboratory animal treatment and care. All rabbits were housed in colonies. Each cage was kept in an individual mesh cage hung at a height of 0.8 cm above the ground so that the excrement could fall into the collection trays. 12 to 14 h light/dark cycle with free access to a rabbit diet consisting of fresh hay, fresh vegetables, and water. Food consumption and fecal characteristics were routinely monitored (21).

Experimental protocol

The animals were acclimatized for seven days before beginning the experiment. Sixty experimental rabbits were randomly divided into four groups (n=15).

The negative control group consisted of 15 rabbits that were left without any interference. They were used as a standard reference of normal bone for comparison of the newly formed bone quality.

Positive control group (clot group)

The mandibular right first molar was extracted and the socket healed spontaneously. Therefore, a topical treatment was administered to the extracted sockets.

β-TCP group

The lower right first molar was extracted. The socket was completely filled with β-tricalcium phosphate (β-TCP) bone graft material.

MT group

The lower right first molar was extracted. The socket was completely filled with milled tooth (MT).

Occasionally, owing to animal mortality or accidental mandibular fracture during the operation, the left side of the negative control rabbits was used to compensate for the lost samples.

Surgical extraction procedures

The general anesthetic procedure for each animal was performed by intravenous injection of 10% ketamine HCl (Alfasan, Woerden, and the Netherlands) at a dose of 50 mg/kg body weight and xylazine (Bayer, Munich, Germany) at a dose of 5 mL/kg body weight in the rabbit ear vein. For greater accessibility, a unilateral horizontal incision was made in the cheek and the mandibular right

first molar was extracted using a hand instrument. After extraction, treated materials (β-TCP or MT) were gently applied to fill the extraction sockets. Finally, the buccal mucosa and skin were sutured using a 2/sutured silk 0 sterile synthetic absorbable suture. Postoperative topical antibiotic spray was applied for infection control in addition to systemic antibiotics (intramuscular injection of ceftriaxone 100 mg/kg body weight once daily for 7 days) and anti-inflammatory agents (diclofenac sodium 15 mg/kg body weight IM twice daily for 3 days) (22). Gross examination of the sutured skin, head, and neck was also performed.

Tissue processing and histological staining

After 10, 20, and 30 postoperative days, five animals from each group were euthanized by veterinarians using sedation, followed by an injection of sodium pentobarbital (12). The right jaws were dissected, fixed in 10% neutral buffered formalin, washed, and decalcified with a 10% tetrasodium-EDTA aqueous solution. The molar regions were dissected and paraffin-embedded into buccolingual sections. Five to six microns thick) were cut and stained with hematoxylin and eosin for histological examination and with Maisson's trichrome stain for collagen evaluation.

Immunohistochemical staining

Tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, incubated with the primary antibody osteopontin (Calbiochem, Merck Group) overnight at 4°C, incubated with biotin-conjugated bridging antibody (1:150) in blocking solution (30 min, 37°C), and incubated with avidin-biotin-conjugated peroxidase (Vectastain Universal Elite Kit, Vector Laboratories) for 30 min. Immune reactions were visualized using romanulin (Biocare Medical) for 15 min at room temperature. The sections were counterstained with hematoxylin (5 min) according to the manufacturer's instructions (VWR, International GmbH). Osteopontin active sites appear brown in the cytoplasm and extracellular matrix (ECM) of cells (23).

Stained tissue sections were photographed with a Leica DM 1000 light microscope and camera using Leica Application Suite-LAS software at the Center of Excellence of Molecular and Cellular Medicine (CEMCM), Suez Canal University.

Histochemical and immunohistochemical analysis

Digital image analysis was performed using image analyzer computer system software (image J / Fiji 1.46). The area of the screen was measured by digitizing the slides under 200X objective magnification. The pixel intensity of the color, the osteopontin labeling index (the number of osteopontin positive cells divided by the total cells), was estimated and statistically analyzed.

Statistical analysis:

The collected data were revised, coded, tabulated, and introduced to a PC using GraphPad Prism Software 8.4.2 (San Diego, US). ANOVA was used to assess the statistical significance of the differences between the means of more than two study groups. Tukey's multiple comparison test is an integral part of ANOVA. After using ANOVA to test the equality of at least three group means, the statistically significant results indicated that not all the group means were equal. However, the ANOVA results did not identify significant differences between pairs of means. We used post-hoc tests to explore the differences between the means of multiple groups, while controlling for the experiment-wise error rate. p value: level of significance, $p>0.05$: non-significant, $p<0.01$: highly significant, $p<0.05$: statistically significant.

Results

1. Characterization of bone graft powder

X-ray diffraction and scanning electron microscopy (SEM)

The XRD pattern of the β-TCP bone grafting material revealed its presence as the main component, whereas minor amounts of other calcium phosphate phases were detected (Fig. 1, A).In contrast, the XRD pattern of MT corresponded to the hydroxyapatite pattern. The narrow sharp peaks indicate higher crystallinity, which may be due to high-temperature processing (Fig 1, B).

The SEM analysis of the β-TCP sample revealed the granular shape of the material. It exhibited a roughened surface without any significant porosity. In the MT sample, the presence of dentinal tubules indicated complete removal of the intertrabecular matter through deproteination.The diameter of the dentinal tubules was between 1.1 and 3.1µm.A normal dentine morphology was observed without structural deterioration. Compared with β-TCP, the surface exhibited higher roughness and porosity. β-TCP has a much larger particle size than MT with mean particle sizes of (925.3 µm, 27.5 µm) respectively (Fig 1, C & D).

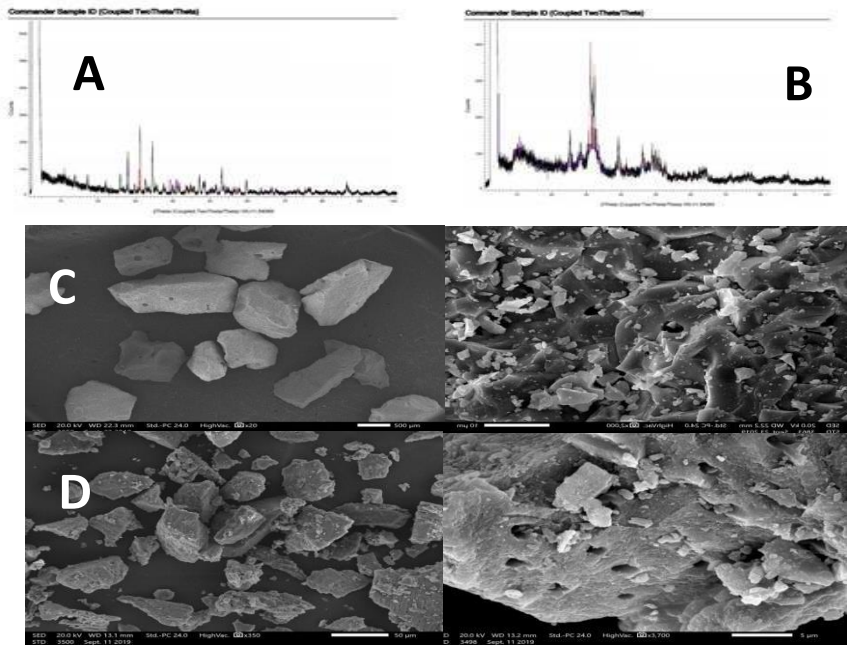

Fig. (1): XRD diffraction pattern of β-TCP (A) and MT (B), SEM microphotographs showing (C) β-TCP and (D) MT.

2. Histological results (hematoxylin and eosin staining)

- Negative control group

Examination of bone sections taken from the mandible of sham control animals showed normal histological features of lamellar bone, with Havarsian canals occupied by cellular elements and mature osteocytes embedded within their lacunae surrounded by mineralized bone matrix with osteoblastic rimming. The bone marrow consists of hematopoietic tissue islands and adipose cells. (Fig. 2, A & B).

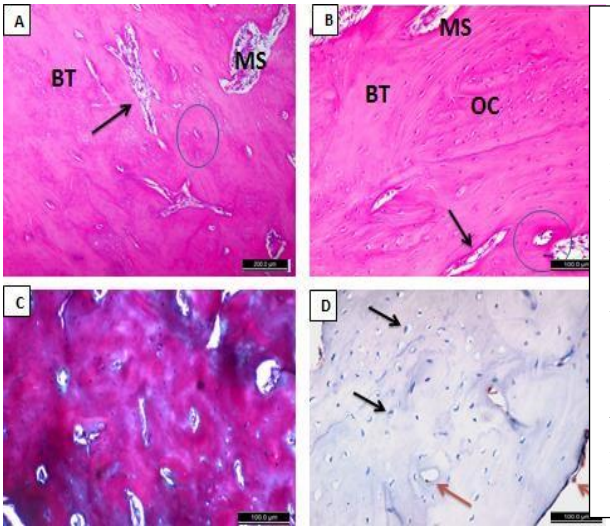

Fig (2): Photomicrographs of mandibular bone of control animals' (A& B) showing interlacing bone trabeculae (BT) with intervening bone marrow spaces (MS) and Osteocytes (OC) appeared in their lacunae. Havarsian canals (circle) as well as Volkman canals (arrow) were noticed (HX&E. orig. mag. 100, 200). C) the bone of control group showing strongly positive reactivity with Masson's trichrome stain (Masson's trichrome, Origin Mag. 200) D) Showing negative reaction of the osteocyte (black arrow) to osteopontin monoclonal antibodies white osteoblasts in the lining and periphery of bone reveal positive reaction (red arrow). (osteopontin, Origin Mag. 200).

- **Positive control (clot group)**

Ten days after extraction, the examined extraction socket of the clot group that was left to heal spontaneously showed granulation tissue formation. Newly developed areas of the bone matrix were observed to be connected to the old bone of the socket, entrapping new osteocytes and bordered by active osteoblasts (Fig. 3, A)

Twenty days after extraction, the bony defect showed an increase in the amount and thickness of the radiating bone trabeculae and their coalescence extended from the periphery of the socket to the center. Osteoblasts were arranged on the border of the newly formed bone and osteocytes appeared in their lacunae. Increased inflammatory cell infiltration and osteoclastic activity were also observed (Fig. 3, B).

Thirty days after extraction, the specimens showed interconnecting trabecular bones of various thicknesses extending from the periphery of the socket to the center. Osteoblasts are characterized by the presence of osteocytes at the border of the newly formed bone. Vascular proliferation, fewer inflammatory reactions, fibrosis, and no areas of bone resorption were observed (Fig. 3, C&D).

- β-TCP bone graft material group

Ten days after extraction, examination of the extraction socket revealed that the tricalcium phosphate bone graft material (β-TCP) completely filled the bone defect penetrated by the vessels. Numerous new blood vessels lined by squamous endothelial cells enclosed by RBCs, with spotty areas of woven bone entrapping new osteocytes and bordered by active osteoblasts were observed (Fig. 3, E). Twenty days after extraction, the newly formed bone was characterized by the presence of irregular osteocytes and rimming of osteoblasts. These trabeculae were interwoven and surrounded differently sized particles of bone graft material entrapped in small or large amounts, which were interpreted as remnants of the biomaterial (Fig. 3, F). A few layers of rimmed osteoblasts were observed in the area corresponding to the biomaterial, a few layers of rimming osteoblasts were seen. A foreign-body reaction is characterized by the presence of giant cells around remnants with mixed inflammation. Thirty days after extraction, most of the specimens in this group were similar to those of the previous period. The defect was partially filled with thick interconnecting trabecular bone. In the form of globules, many trabeculae were observed surrounding the remnants of β-TCP bone graft material (Fig. 3, G& H).

- **MT bone graft material group**

Ten days after extraction, granulation tissue and tooth ash partially filled the defect (Fig. 3, I). Twenty days after extraction, the newly formed woven bone displayed thin interconnecting bone trabeculae characterized by osteocytes within and filled with red bone marrow. Remnants of tooth ash with dentinal tubules were observed inside granulation tissues. Discrete and moderate inflammation were observed. Collagen deposition was intense and mature in the newly formed bone (Fig. 3, J).

Thirty days after extraction, the specimens showed that the bone defect was almost completely filled by thick interconnecting trabecular bone of various thicknesses, characterized by the presence of osteocytes in their lacuna and nonparallel basophilic lines. Occasionally, the trabeculae showed rimming osteoblasts, Havarsian canals, Volkman canals, and mature osteocytes that were embedded and widely separated by the bone matrix. (Fig. 3, K&L).

Bone densitometry analysis (Bone mineralization)

Each biopsy specimen was placed in 10% formalin and sent for histopathological slide preparation. The slides were examined histomorphometrically under high-power field. The histomorphometric slide clearly showed a dark osteoid seam covering the surface with scattered osteoblasts.
1. Osteoid seam width (OSW) (width of the osteoid-seam-lined bone trabeculae). Three measurements were taken from each slide and the average width was taken.
2. Osteoblast surface (OBS) (fraction of trabecular bone lined by osteoblasts). Three measurements were taken from each slide and the average width was recorded.
3. Osteoid surface (OS) (fraction of trabecular bone lined by osteoid seams).
The evaluation of bone healing around the bone graft material was quantitatively assessed .The percentage of bone formation was measured within a rectangular region of interest (ROI) with a length of 5x0.05 mm adjacent to the bone graft material. For defining a new bone formation, three factors “OSW, OBS, and OS” had predefined cut - off points (histological evidence of for new bone formation the specific parameter). New bone formation was diagnosed when the osteoid seam width was more than 8.8 m. If the specimen scored more than 20% on the remaining two characteristics, it was considered positive indicator for new bone formation. Sections were termed as having new bone formation at the bone graft material area if they displayed at least two out of three positive histological signs of bone remolding; this is known as the gold standard for bone healing. Table 1 and (Fig. 4) shows the histological characteristics of bone remolding in the studied groups. The new bone formation was measured by calculating the percentage of bone growth area (BGA %) within the ROI in the bone graft material area.

3. Histochemical results (Masson’s trichrome staining)

Masson’s trichrome staining was performed to determine the deposition and arrangement of the collagen secreted during socket healing. Blue indicates regenerated bone, newly formed (immature) collagen fibers, or osteoids. The red-stained area indicates mature bone. The examination revealed the following results.

Negative control group: mature bone and surrounding periosteum showing strong positive reactivity with Masson’s trichrome staining (Fig. 2, C).

Positive control group: Ten days after extraction, the expression of blue-colored areas of randomly arranged immature collagen, in which newly developed thin bone spicules stained blue at the beginning of maturation (Fig. 5, A). On days 20 and 30, maturation of the bone trabeculae showed moderate to strong positive reactivity with Masson’s trichrome staining (Fig. 5, B& C).

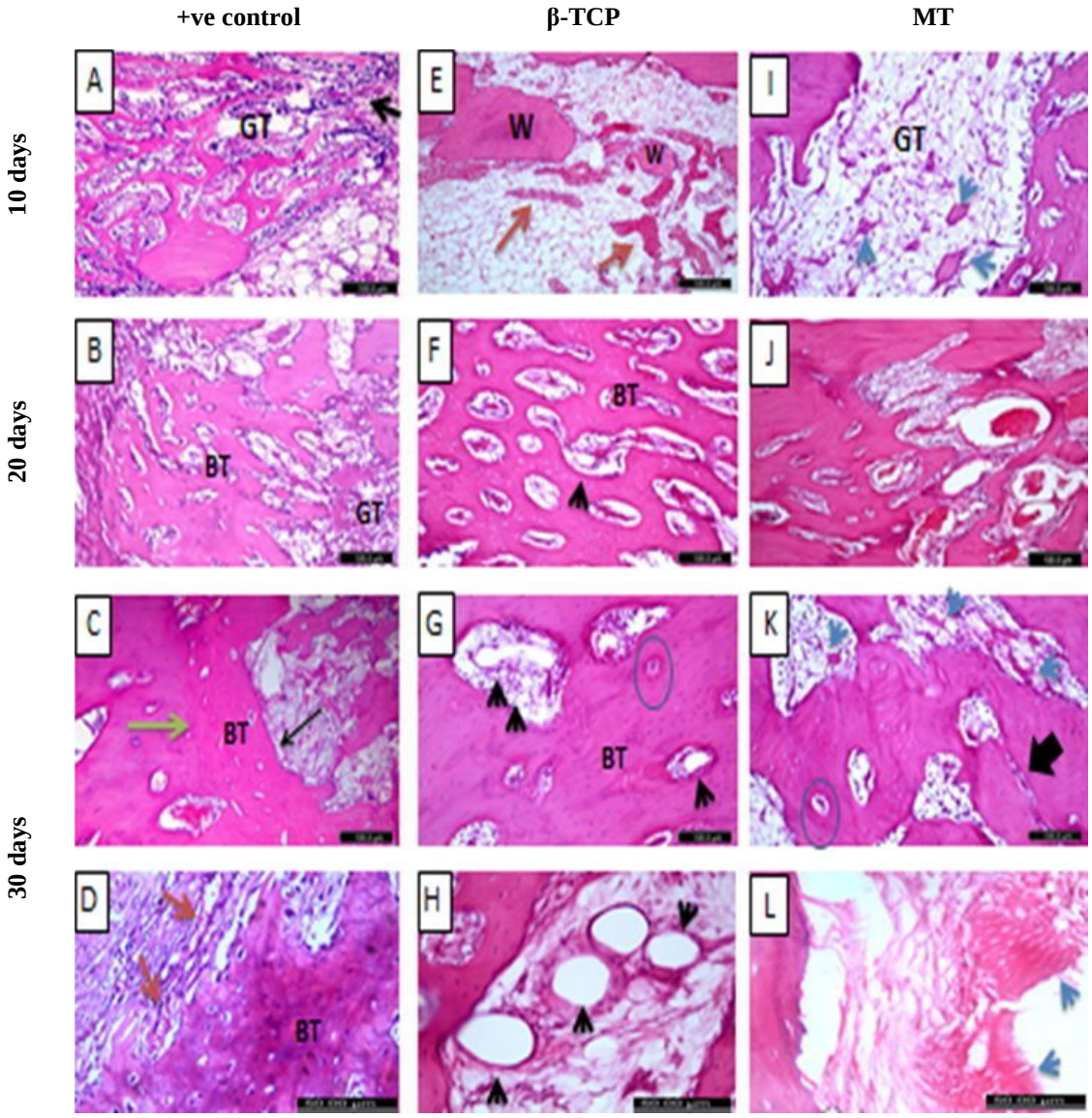

Fig. (3). Photomicrographs of the specimens taken from all experimental subgroups. A) showing socket of **clot** group filled with granulation tissue (**GT)**, hemorrhage areas (**short arrow)** and adjacent inflammatory cells, new blood capillaries and newly developed areas of bone matrix connected to old bone of the socket were seen (10 days after extraction). B) showing increased thickness and coalescence of the newly formed bone trabeculae (**BT)** (20 days after extraction). C& D) showing the socket almost completely filled by thick interconnecting trabecular bone (**BT)** extended from the periphery of the socket to the center characterized by the presence of osteocytes in their lacuna and osteoblasts were arranged on the border of the newly formed bone (**black arrow),** Cement line (**green arrow)** and vascular prolipheration (**red arrow)** were notice (30 days after extraction).

E) showing the extraction socket of **β-TCP** group completely filled with particles of tricalcium phosphate bone graft material (TCP) penetrated by new blood vessels (**read arrow)** enclosed RBCs, spotty areas of woven bone (**W)** (10 days after extraction). F) showing interwoven bome trabeculae (**BT)** surrounding different size particles of bone graft material entrapped in (**arrow heads)** (20 days after extraction). G&H) showing the socket partially to completely filled by thick interconnecting trabecular bone surrounding remnants of bone graft material tricalcium phosphates material (**arrow heads)** (30 days after extraction).

I) showing the extraction socket of **MT** group filled with MT presented with granulation tissue (**GT)** and tooth ashes (**blue arrow heads)** partially fill the defect (10 days after extraction). J) showing newly interconnecting bone trabeculae surrounding red bone marrow (20 days after extraction). K& L) showing the socket almost completed filled by thick interconnecting trabecular bone of varied thicknesses, characterized the presence of osteocytes in their lacuna and parallel basophilic lines. Remnants of tooth ash (**blue arrow heads)** with dentinal tubules were seen inside the granulation tissues. Areas of compact bone were evidence with appearance of Havarsian canals (**circle)** as well as Volkman canals (**bold arrow)** (30 days after extraction) (H&E, org. mag. 200, 400).

Table 1. The average of the three readings for bone mineralization assessment.

Group	OSW (µm)	Test result	OS (%)	Test result	OBS (%)	Test result	BGA%
Negative control	12	-ve	28	-ve	25	-ve	20
Positive control 30 d	7.5	+ve	25	-ve	15	+ve	15
Positive control 10 d	5.2	+ve	10	+ve	12	+ve	6
β-TCP/ 10 d	7.2	+ve	12	+ve	22	-ve	12
β-TCP/ 20 d	9.5	-ve	22	-ve	18	+ve	38
β-TCP/ 30 d	11.0	-ve	28	-ve	30	-ve	55
MT/10 d	7.5	+ve	20	+ve	10	+ve	27
MT/20 d	8.2	+ve	30	-ve	25	-ve	42
MT/30 d	12.6	-ve	28	-ve	30	-ve	70

OSW: Osteoid seam width "width of the osteoid-seam-lined bone trabecula", OS: Osteoblast surface "fraction of the trabecular bone lined by osteoblasts", OBS: Osteoblast surface "fraction of the trabecular bone lined by osteoblasts", -ve : negative, +ve: positive, BGA(%): Bone growth area within the ROI in the bone graft material area.

ANOVA	
F	57.33
P value	<0.0001
P value summary	****
R squared	0.96

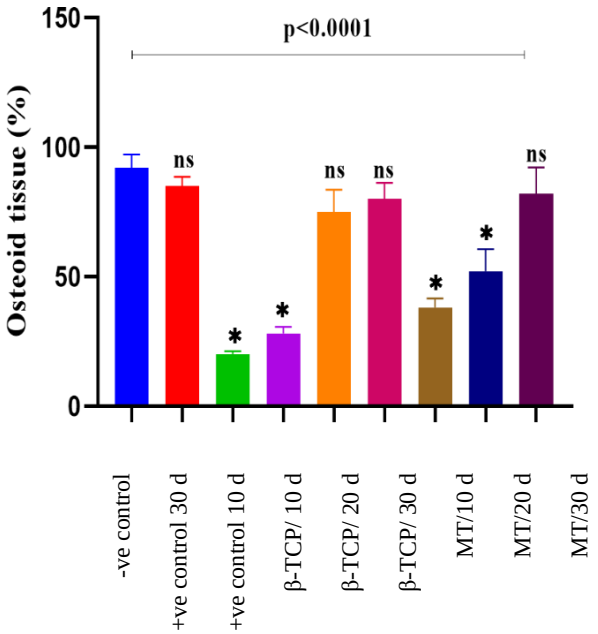

Fig. (4) Bar chart showed a high significant difference for the % of Osteoid tissue between the studied groups. Data are presented as mean and SD, The X axis is presented in mean percentage of osteoid tissue. *: statistical significance ($p<0.05$) compared to the negative control group, ns: no significant difference ($p>0.05$)

β-TCP group: 10 days, the woven bone revealed areas of maturation (red colored), while on the 20 and 30 day revealed more maturation of the developing bone was observed (Fig. 5, D, E& F). Moreover,

MT group:10 days showed apparent maturation as indicated by reddish staining. At 20 and 30 days, the bone tissue was clearly well-organized and matured compared to β-TCP. Collagen deposition in the bone trabeculae was intense and mature, showing moderate-to-strong positive reactivity to Masson's trichrome staining (Fig. 5, G, H& I).

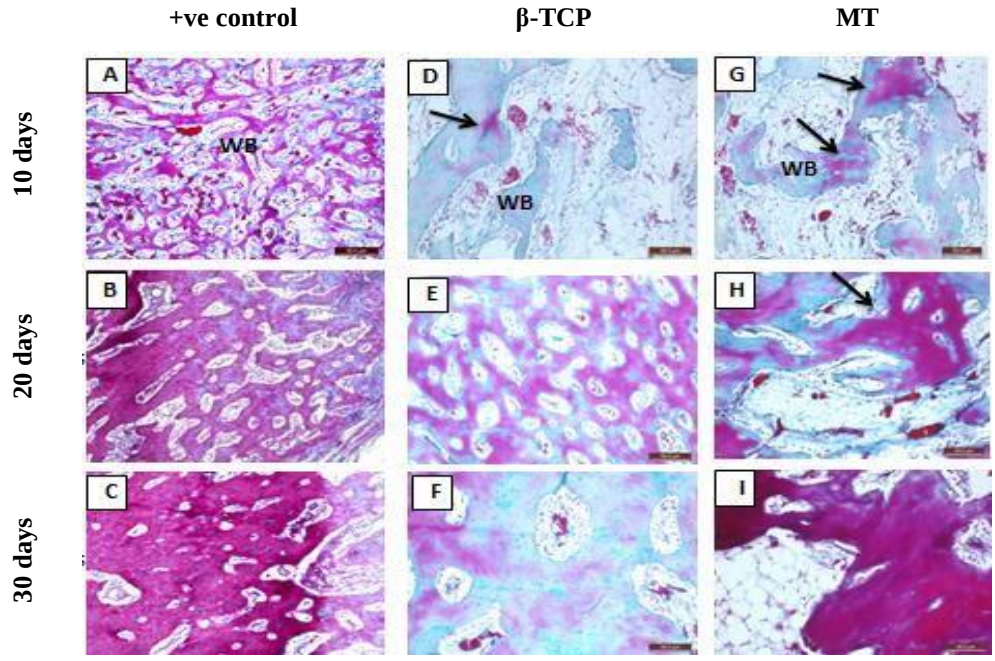

Fig. (5): Photomicrographs of the bone defects of all groups stained with the Masson's trichrome stain. A) **+ve control** group showing randomly arranged immature collagen in newly developed thin woven bone spicules which stained blue with beginning of maturation (10 days after extraction). B& C) the same group showing increased maturation of the bone trabculae with moderately to strongly positive reactivity to Masson`s trichrome stain (20 and 30 days after extraction). D, E& F) **β-TCP** group showing on the 10 days woven bone with areas of maturation (**arrow)** while on the 20 and 30 days showing more maturation of the developing bone. G, H, I) **MT** group on 10 days showing apparent maturation (**arrow)** of the woven bone while on the 20 and 30 days showing clearly well organized and matured bone tissue. (Masson's trichrome, Origin Mag. 100, 200).

Comparative analysis of the mean percentage of osteoid tissue in bone sections stained with Masson's trichrome among the studied groups

One-way analysis of variance (ANOVA) was used to compare the mean percentages of osteoid tissue in the rat bones of the studied groups and then compared to the negative and positive control groups. (Table 2). The results revealed a highly significant difference in the percentage of osteoid tissue between groups ($F=57.33$, $p<0.0001$). The results are shown in Fig. 4. Furthermore, Tukey's multiple comparisons test revealed a highly significant difference between the -ve control group and the +ve control/10 d, β-TCP/20 d, MT/10 d, and MT/30 d groups. However, no significant differences were detected between the -ve control and +ve control, β-TCP/ 20 d, β-TCP/30 d, and MT/30 d groups ($p>0.05$), table 2.

Table 2. Multiple comparison test (Tukey's test), % of osteoid tissue

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
-ve control vs. +ve control 30 d	7.000	-10.79 to 24.79	ns	0.8920
-ve control vs. +ve control 10 d	72.00	54.21 to 89.79	****	<0.0001
-ve control vs. β-TCP/10 d	64.00	46.21 to 81.79	****	<0.0001
-ve control vs. β-TCP/20 d	17.00	-0.793 to 34.79	ns	0.0676
-ve control vs. β-TCP/30 d	12.00	-5.793 to 29.79	ns	0.3582
-ve control vs. MT/10 d	54.00	36.21 to 71.79	****	<0.0001
-ve control vs. MT/20 d	40.00	22.21 to 57.79	****	<0.0001
-ve control vs. MT/30 d	10.00	-7.793 to 27.79	ns	0.5803
+ve control 30 vs. +ve control 10	65.00	47.21 to 82.79	****	<0.0001
+ve control 30 vs. β-TCP/10 d	57.00	39.21 to 74.79	****	<0.0001
+ve control 30 vs. β-TCP/20 d	10.00	-7.793 to 27.79	ns	0.5803

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
+ve control 30 vs. β-TCP/30 d	5.000	-12.79 to 22.79	ns	0.9827
+ve control 30 vs. MT/10 d	47.00	29.21 to 64.79	****	<0.0001
+ve control 30 vs. MT/20 d	33.00	15.21 to 50.79	***	0.0001
+ve control 30vs. MT/30 d	3.000	-14.79 to 20.79	ns	0.9994
+ve control 10 vs. β-TCP/10 d	-8.000	-25.79 to 9.793	ns	0.8057
+ve control 10 vs. β-TCP/20 d	-55.00	-72.79 to -37.21	****	<0.0001
+ve control 10 vs. β-TCP/30	-60.00	-77.79 to -42.21	****	<0.0001
+ve control 10 vs. MT/10 d	-18.00	-35.79 to -0.206	*	0.0462
+ve control 10 vs. MT/20 d	-32.00	-49.79 to -14.21	***	0.0002
+ve control 10 vs. MT/30 d	-62.00	-79.79 to -44.21	****	<0.0001
β-TCP/7 vs. β-TCP/20 d	-47.00	-64.79 to -29.21	****	<0.0001
β-TCP/7 vs. β-TCP/30 d	-52.00	-69.79 to -34.21	****	<0.0001
β-TCP/7 vs. MT/10 d	-10.00	-27.79 to 7.793	ns	0.5803
β-TCP/7 vs. MT/20 d	-24.00	-41.79 to -6.207	**	0.0041
β-TCP/7 vs. MT/30 d	-54.00	-71.79 to -36.21	****	<0.0001
β-TCP/20 vs. β-TCP/30 d	-5.000	-22.79 to 12.79	ns	0.9827
β-TCP/20 vs. MT/10 d	37.00	19.21 to 54.79	****	<0.0001
β-TCP/20 vs. MT/20 d	23.00	5.207 to 40.79	**	0.0062
β-TCP/20 vs. MT/30 d	-7.000	-24.79 to 10.79	ns	0.8920
β-TCP/30 vs. MT/10 d	42.00	24.21 to 59.79	****	<0.0001
β-TCP/30 vs. MT/20d	28.00	10.21 to 45.79	***	0.0008
β-TCP/30 vs. MT/30 d	-2.000	-19.79 to 15.79	ns	>0.9999
MT/10 vs. MT/20 d	-14.00	-31.79 to 3.793	ns	0.1957
MT/10 vs. MT/30 d	-44.00	-61.79 to -26.21	****	<0.0001
MT/20 vs. MT/30 d	-30.00	-47.79 to -12.21	***	0.0004

CI: confidence interval, ns: no significant difference ($p>0.05$)

4. Immunohistochemical analysis (osteopontin expression)

Examination of osteopontin expression (brown staining) in bone sections obtained from the control mandible and incubated with primary monoclonal antibodies against osteopontin revealed a negative reaction for osteocytes, whereas osteoblasts in the lining and periphery of the bone showed a positive reaction (Fig. 2, D) Immunohistochemical examination of the experimental group showed OPN staining in osteoclasts, osteoblasts, fibroblasts, granulation tissue, and bone matrix in almost all specimens. (Fig. 6). Bone trabeculae and osteocytes exhibited weak to moderately positive reactivity. Examination of the bony defects at 20 and 30 days showed newly formed bony trabeculae with weakly positive osteopontin staining, whereas granulation tissue and osteoblasts on the border of the formed bone showed strong positive staining.

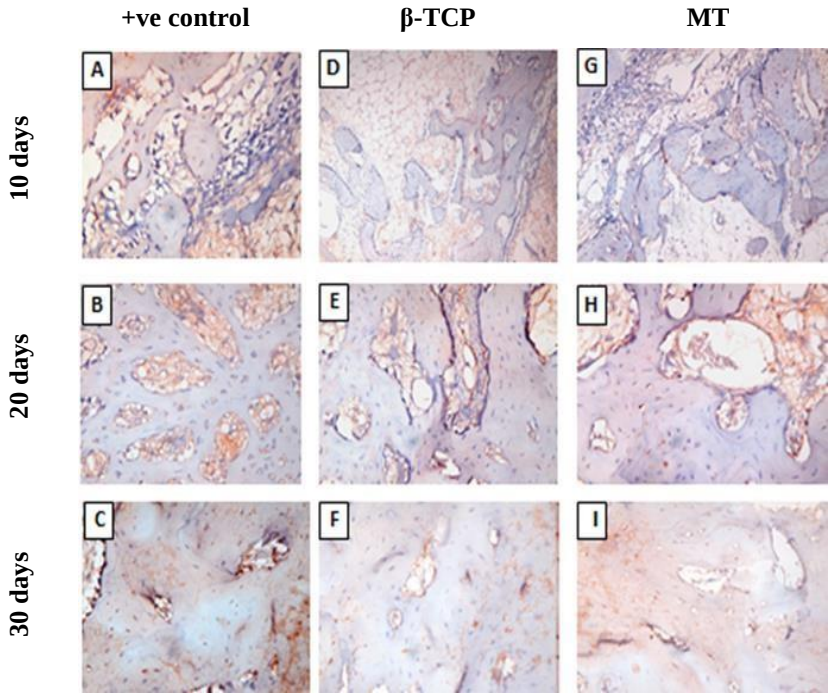

Fig. (6): Photomicrographs showing the immunohistochemical examination of the experimental subgroups stained with monoclonal antibody of osteopontin. OPN immunolabelling was observed in the osteoblasts, fibroblasts and the granulation tissue. The bone matrix also showed stained areas (osteopontin, 200, 400).

Comparative analysis of IRS Osteopontin protein expression in bone sections stained with an anti-OPN antibody between the studied groups

The expression of osteopontin protein in the bone tissue was measured by calculating the immunoreactive score, which reflects the combination of the percentage of positive cells and the intensity of expression in cells. Immune-reactive score (IRS) scores were compared between the different groups and -ve control group. According to the ANOVA results, a significant difference ($P < 0.01$) was detected between the studied groups (Table 6). Higher expression levels of osteopontin were detected in the β-TCP/20 d and MT/30 d groups, and a highly significant difference was observed compared to the -ve control group ($p<0.01$). However, no significant difference was detected between -ve control and +ve control/10 d, β-TCP/10 d, β-TCP/30 d, and MT/20 d ($p>0.05$). Table 6 presents the results of the multi-comparative analysis results are presented in Table 6 and (Fig. 7)

Table 6. Multiple comparison test (Tukey's test), Osteopontin protein expression

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
-ve control vs. +ve control 30	3.000	0.06162 to 5.938	*	0.0436
-ve control vs. +ve control 10	2.000	-0.9384 to 4.938	ns	0.3236
-ve control vs.β-TCP/10 d	0.000	-2.938 to 2.938	ns	>0.9999
-ve control vs. β-TCP/20 d	-5.000	-7.938 to -2.062	***	0.0005
-ve control vs. β-TCP/30 d	-2.000	-4.938 to 0.9384	ns	0.3236
-ve control vs. MT/20 d	-2.000	-4.938 to 0.9384	ns	0.3236
-ve control vs. MT/30 d	-5.000	-7.938 to -2.062	***	0.0005
+ve control 30 vs. +ve control 10	-1.000	-3.938 to 1.938	ns	0.9270
+ve control 30 vs. β-TCP/10 d	-3.000	-5.938 to -0.06162	*	0.0436

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
+ve control 30 vs. β-TCP/20 d	-8.000	-10.94 to -5.062	****	<0.0001
+ve control 30 vs. β-TCP/30 d	-5.000	-7.938 to -2.062	***	0.0005
+ve control 30 vs. MT/20 d	-5.000	-7.938 to -2.062	***	0.0005
+ve control 30 vs. MT/30 d	-8.000	-10.94 to -5.062	****	<0.0001
+ve control 10 vs. β-TCP/10 d	-2.000	-4.938 to 0.9384	ns	0.3236
+ve control 10 vs. β-TCP/20 d	-7.000	-9.938 to -4.062	****	<0.0001
+ve control 10 vs. β-TCP/30 d	-4.000	-6.938 to -1.062	**	0.0045
+ve control 10 vs. MT/20 d	-4.000	-6.938 to -1.062	**	0.0045
+ve control 10 vs. MT/30 d	-7.000	-9.938 to -4.062	****	<0.0001
β-TCP7 vs. βTCP/20 d	-5.000	-7.938 to -2.062	***	0.0005
β-TCP7 vs. βTCP/30 d	-2.000	-4.938 to 0.9384	ns	0.3236
β-TCP7 vs. MT/2 d	-2.000	-4.938 to 0.9384	ns	0.3236
β-TCP7 vs. MT/30 d	-5.000	-7.938 to -2.062	***	0.0005
β-TCP20 vs. β-TCP/30 d	3.000	0.06162 to 5.938	*	0.0436
β-TCP20 vs. MT/20 d	3.000	0.06162 to 5.938	*	0.0436
β-TCP20 vs. MT/30 d	0.000	-2.938 to 2.938	ns	>0.9999
β-TCP30 vs. MT/20 d	0.000	-2.938 to 2.938	ns	>0.9999
β-TCP30 vs. MT/30 d	-3.000	-5.938 to -0.06162	*	0.0436
MT20 vs. MT/30 d	-3.000	-5.938 to -0.06162	*	0.0436
-ve control vs. +ve control	3.000	0.06162 to 5.938	*	0.0436
-ve control vs. +ve control 10	2.000	-0.9384 to 4.938	ns	0.3236
-ve control vs. β-TCP/10	0.000	-2.938 to 2.938	ns	>0.9999
-ve control vs. β-TCP/20	-5.000	-7.938 to -2.062	***	0.0005
-ve control vs. β-TCP/30	-2.000	-4.938 to 0.9384	ns	0.3236
-ve control vs. MT/20	-2.000	-4.938 to 0.9384	ns	0.3236
-ve control vs. MT/30	-5.000	-7.938 to -2.062	***	0.0005
+ve control vs. +ve control 10	-1.000	-3.938 to 1.938	ns	0.9270

CI: confidence interval, ns: no significant difference (p>0.05)

ANOVA	
F	24.15
P value	<0.0001
P value summary	****
R squared	0.91

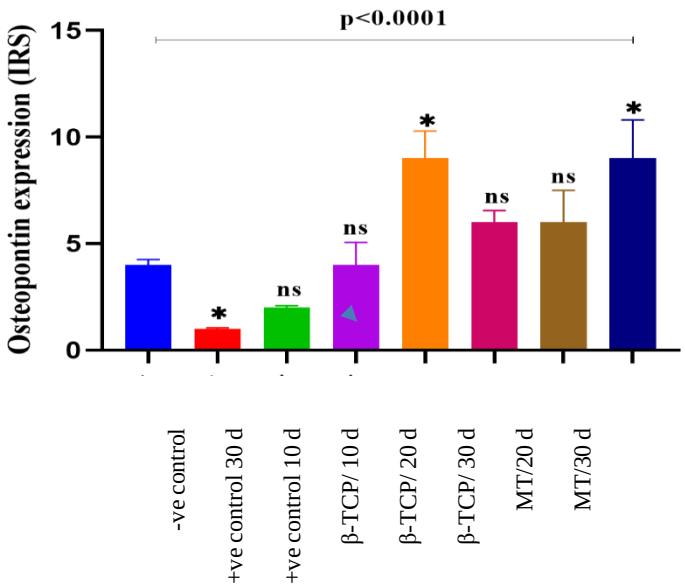

Fig. (7) Bar chart showed a high significant difference for the expression of Osteopontin protein. Data are presented as mean and SD, The X axis is presented in mean osteopontin expression (IRS). *: statistical significance (p<0.05) compared to the negative control group, ns: no significant difference (p>0.05)

Discussion

Bone grafting is recurrent procedure nowadays. Therefore, bone substitutes as β-TCP are being used abundantly. Teeth are considered a natural source of hydroxyapatite. β-TCP and hydroxyapatite are biocompatible, bioresorbable bone graft materials with osteoconductive properties (24).

Hydroxyapatite has slower resorption rate than β-TCP which may decreases the amount of new bone formation. Bone graft material should resorb with the same rate of bone formation (25).

During processing of MT heating of hydroxyapatite increases its crystallinity, indicated by sharper peaks in XRD pattern, which may further decreases the rate of desorption. However, both MT and β-TCP in this study produces comparable amount of bone formation. This may be due to that biological hydroxyapatite from natural source as MT is carbonated hydroxyapatite .The presence of CO_3^{2-} ions in the apatite structure affects its structure, morphology, and improves biochemical reactivity of bone minerals .It also increases the rate of resorption (26, 27)

The porous structure and smaller particle size of MT may be factors which further accelerate resorption (28). Moreover, the irregular and rough MT surface, as assessed through SEM, seemed to benefit the osteoconduction process (29).

Apical surgery may be the treatment of choice in cases of primary endodontic treatment failure or retreatment, apical surgery may be the treatment of choice. Additionally, enucleation usually results in bone defects. Bone formation after periapical surgery can be accelerated by inserting a bone graft into a bony defect. This type of treatment creates a favorable environment for periapical tissue healing (30). Biomaterials can induce healing and restore lost tissues within the biological environment. In dentistry, bone defects and injuries provoke an inflammatory response within 24 h after the injury and least for one week (31).

Rabbits were preferred in the present study because they are easier to handle than rats and mice are. Rabbits also have a Havarian system similar to that of humans, which is an important advantage (32).

The surgical procedure in the current study included cheek and skin incisions for easier accessibility, thereby decreasing trauma to the alveolar bone. Systemic postoperative antibiotics and anti-inflammatory drugs were administered to prevent post-operative infections and large traumatic bone defects. After extraction, the bone graft material was applied and gently adapted to cover the socket to ensure complete filling of the wound and decrease the chance of displacement during suturing. Biopsy samples were collected and examined, and data were analyzed. Our results showed that new bone formation was found only at the socket periphery in the clot control group, whereas bone trabeculae were more abundant around the graft material in the sockets treated with graft materials. The bone formed in patients treated with the tooth powder graft material was in close contact with the material, with minimal inflammation. This suggests that the tooth powder graft material undergoes complete resorption and replacement by mature bone.

No statistically significant difference was found between the groups in which the MT powder graft and β-TCP were used in the evaluations performed on Days 10 and 30. The new bone formation ratio in the clot control group was found to be statistically significantly lower than that in the other groups at both time periods. In a study published in 2018 by Calvo Guirado et al. (33), a histological and histomorphometric evaluation of the extraction sockets of bilateral premolar teeth of dogs was performed for the empty control group and dentin graft test group after 30 and 90 days. They observed a statistically significant difference in new bone formation between groups. At 30 days, bone formation in the test group was greater than that in the control group ($p < 0.05$) and less immature bone was observed in the test group (25.71%) than in the control group (55.98%). At 90 days, significant differences in bone formation were observed between the test and control groups. They concluded that tooth particles extracted from dog teeth could be considered as suitable biomaterial grafts for socket healing.

Similar results were observed in this study. The new bone formation ratios of the MT powder grafts in both time periods were found to be statistically significantly higher than those of the clot defect group. Our results are in accordance with those of other studies by Kim et al. (34), who reported excellent biocompatibility of the tooth bone graft material. In another study, Kim et al. (35) demonstrated that tooth bone graft material undergoes a gradual resorption process and is replaced by bone of good-quality, through both the processes of osteoinduction and osteoconduction, According to their study, fast and high amount of lamellate bone formation was observed in the healing process of autogenous tooth bone graft material (34). In a histological study, four months after grafting, the graft material directly fuses with the old bone and shows excellent vascularity, and the graft is completely replaced by normal bone in 12–15 months. Jun et al. (36) reported a mean bone density of 981 HU (D2 type) in a healed auto tooth bone graft versus 968 HU in Bio-Oss. Similarly, they reported an almost 60% proportion of new bone volume to the total bone volume with a tooth bone graft. Zhang et al. (18) reported that in addition to the high biocompatibility and convenient acquisition of tooth bone graft material, they do not require high temperatures or other processing procedures to change their internal structure; thus, they still preserve a large amount of organic matter to enhance bone regeneration. They have osteoinductive effects and dental tissues can act as scaffolds for bone repair.

Therefore, autogenous dental materials should be classified as novel bone substitutes for the grafts. In an osteoporosis model involving 60 rats, a mixture of dental ash and plaster accelerated new bone formation and was more stable than that in the control group (17). Gu et al. (37) used human dental ash to treat bone defects in dog alveolar bone and found the presence of osteoclasts in the surrounding tissue, indicating an active bone-remodeling process. Therefore, this material is simple, easy to obtain, convenient to use, and promotes bone healing.

In addition, in our study, the new bone formation ratios of β-TCP in both time periods were found to be statistically significantly higher than those in the positive control group. Several studies using β-TCP alone or in combination with other grafting materials for different surgical regenerative procedures have shown good results for bone filling and new vital bone formation, similar to those obtained using other bone grafting materials such as allografts and xenografts (38- 40). However, some studies contradicted these results. In a study by Snyder et al. (41), compared with other grafting materials, an inferior outcome was observed with β-TCP. Thus, conflicting results have been reported in literature. β-TCP is a resorbable bone graft material with osteoconductive properties (42). The ideal bone graft material should be resorbed at the same rate as bone formation, and slower resorption decreases the amount of new bone formation (43). Chazono et al. (44) and Shirasu et al. (45) have reported osteoblast proliferation and increased bone formation in the presence of β-TCP. They reported that osteoclasts surrounding the bone graft particles induced osteoblastic cell migration around the bone graft particles of β-TCP via cell-to-cell interactions. Wang et al. (42) suggested that β-TCP dissolution might provide high local concentrations of calcium and phosphate, thereby providing a good environment for new bone formation.

Our results are in agreement with Mohammed et al. (43) who examined the histological process of healing of bone defects in dog's mandibles. Histological analysis of β-TCP revealed that there was initial bone neoformation. Bone formation was well-organized at the end of 8th week. In addition, previous studies have recognized that purified β-TCP provides early bone conduction, followed by resorption and replacement of the newly formed bone (46).

In contrast, some studies have contradicted previous statements. Snyder et al. (41) reported an inferior outcome with β-TCP compared with other grafting materials. Thus, the literature shows conflicting results and there is a lack of information regarding the exact amount of BF associated with different surgical regenerative procedures using β-TCP.

In this study, histochemical analysis using Masson's trichrome staining for collagen fibers showed that all experimental groups exhibited new bone formation. At 30 days, the highest amount of newly formed bone was observed in the experimental group and the lowest value was observed in the clot control group. In the β-TCP group, collagen fibers were randomly arranged, representing embryonic bone formation, whereas in cases with tooth powder material, collagen deposition in the bone trabeculae was intense and mature, with alternating parallel layers. This was in accordance to Mohammed et al. (43).

Higher expression levels of osteopontin were detected in the β-TCP/20 d and MT/30 d groups, and a highly significant difference was observed compared to the +ve control group ($p<0.01$). However, no significant difference was detected between the two studied materials.

Conclusion:

The positive histopathological results of this study are promising for the use of MT powder as a bone graft in healing extraction sockets. It is a relatively new bone graft material with all the advantages of autogenous bone because of its biocompatibility, similar components to bone, and because it does not cause an immune response or a foreign body reaction. Its properties include osteoinduction and osteoconduction properties. Further in vivo and clinical studies are required to investigate the efficiency of the milled tooth powder as a bone graft material in tooth socket healing.

Conflicts of interest

The authors declare no conflicts on interest in relation to this study.

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