EVALUATION OF TISSUE RESPONSE OF ADULT MALE SPRAGUE-DAWLEY RATS TO AN EXPERIMENTAL CALCIUM SILICATE BASED CEMENT VERSUS ANGELUS WHITE MTA AND SINAI WHITE PORTLAND CEMENT

By

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ABSTRACT

Background: Calcium silicate cements are biocompatible materials, and can help in repair of osseous defects.

Objective: This study was conducted to evaluate and compare the tissue response to an experimental material (laboratory prepared) versus white MTA and white Portland cement implanted in a rat model. Materials and methods: The experimental highly purified calcium silicate based material was synthesized denovo in the lab from pure oxides, then tissue response was evaluated on adult Sprague-Dawley rats. Critical size bone defect was done at the middle third of the lateral surface of the right tibia in 75 rats. The bony defect was either left untreated to heal spontaneously in animals of the control group (15 rats) or filled with four different materials (15 rats each). At the end of the experimental periods for each subgroup tibia were dissected for histological analysis and evaluation of inflammatory reaction and newly formed bone. Data were collected, tabulated and statistically analyzed. **Results:** Within the limitations of this study, it was found that there was a decrease in inflammatory cell count as the wound healing process moves towards formation of granulation tissue and fibrous encapsulation of the different implanted materials, as well as deposition of newly formed bone throughout the experimental periods. Conclusion: The synthetic materials (laboratory manufactured) seemed to have comparable biological properties to those of commercially available bioactive materials. These experimental materials have an intense tissue inductive capacity, and also the incorporation of nanotechnology in the experimental material showed an intense biological effect in tissue regeneration.

INTRODUCTION

Calcium silicate based materials are interesting bioceramic products widely used in dentistry. These materials are broadly used in the field of conservative dentistry for regeneration, repair and reconstruction. These are available in different forms and compositions that act directly on vital tissue inducing its healing and repair (**Sonarkar and Purba, 2015**). Calcium silicate materials derived from the basic building material Portland cement were the first bioactive materials to appear for use in dentistry (Jefferies, 2014). Portland cement is a common cement used in civil engineering. The major constituents of ordinary Portland cement are similar to those of MTA. It was reported that their pH, antimicrobial activity. biocompatibility and low resistance to compression are similar (Silva Neto et al., 2011). Mineral trioxide aggregate is a biomaterial that is widely used in various conservative treatments due to its excellent biocompatibility, superior sealing and ability to set in the presence of blood (Suzuki et al., 2015). Many studies compared MTA with Portland cement, and indicated that they have similar chemical composition and biocompatibility. However, MTA is a quite expensive material (Hwang et al., 2009 and Yoshino et al., 2013). Therefore, Portland cement may be a possible substitute for MTA. From the disadvantages of MTA are its long setting time and the presence of bismuth oxide which alter its physicochemical properties. So, zirconium oxide promotes adequate radiopacity and biocompatibility when associated with Portland cement (Tanomaru-Filho et al., 2015). Nanohydroxyapatite (n-HAp) containing products with different formulations have been developed, and early data have suggested their remineralizing properties (Tschoppe et al., 2011).

MATERIALS AND METHODS

Highly purified calcium silicate based cement was prepared denovo in the

physical properties of building materials and refractories lab, department of Physics Faculty of Science, Al-Azhar University, from pure oxides with the same ratio as Sinai white Portland cement by sintering quenching technique and milling procedures. X-ray fluorescence elemental analysis was used to confirm the presence of oxides in the final compound of the calcium silicate prepared powder. Phase composition and microstructure were then characterized with an X-ray diffractometer (XRD) by placing the powder specimen into the sample holder for detection. To prepare the experimental material, 20 weight percent radiopacifing materials (10 weight percent zirconium oxide powder (Malkondu et al., 2014), and 10 weight percent titanium oxide powder (Hossienzade et al., 2016) were added to 80 weight percent experimental calcium silicate powder. Other group (Experimental material + nHAp) was prepared by adding 10 weight percent hydroxyapatite nanoparticles to a sample from the previously prepared experimental material (Guerreiro-Tanomaru et al., 2016). To prepare aqueous solution of calcium chloride, ten percent calcium chloride powder was added to distilled water (Camilleri et al., 2013) to decrease the setting time of the cement.

Sample grouping and surgical procedures:

Approval for the animal use protocol was sought and given by the Animal Ethics Committee at Faculty of Dentistry, Al Azhar University Girls' branch. A total of 75 adult male rats each weighing from 300 to 350 g were used in this work. They were kept in cages (30 x 25 x 25) cm dimensions (2 rats per cage) at room temperature and normal dark/light cycle. They were fed on chow pellets and water ad lib. They were divided equally into 5 main groups according to the surgical procedure and the type of material used (A) Sinai white Portland cement (A1), Angelus white MTA (A2), experimental material (A3) and experimental material with hydroxyapatite nanoparticles (A4) and a control group (only critical size bone defect) (A0). Each group was further subdivided into 3 subgroups of 5 rats each the time interval according to of sacrification (B).Subgroup I: Rats were sacrificed 1 day post operatively **B1** (Rammelt et al., 2004), Subgroup II: Rats were sacrificed 1 week post operatively B2 (Dincol et al., 2016) and Subgroup III: Rats were sacrificed 1 month post operatively B3 (Dincol et al., 2016).

After one week of acclimation, rats of different groups were anesthetized by intraperitoneal injection of Ketamine Hydrochloride $10\%^1$ (35mg/ kg body weight) and Xylazine Hydrochloride $2\%^2$ (8 mg\ kg body weight) (Silva et al., 2015). All operative procedures were done under strict aseptic conditions and every effort was made to minimize animal discomfort. The surgical site (right tibia) was then shaved with One cream³ and

disinfected with Betadine solution⁴, and a linear 15 mm incision was made on the tibia and a full thickness skin muscle periosteal flaps were reflected. The anterior tibial muscle was retracted laterally in order to expose the tibial bone. A 4 mm bone defect (fig. 1) which is considered a surgical critical-size bone hole (Bernabe et al., 2012) was done at the middle third of the lateral surface of the right tibia (Nygern et al., 2014) using a sterile carbide dental round bur⁵ size 1/4 (4mm checked by periodontal probe) on a low speed hand piece⁶ with 25000 r.p.m under constant saline irrigation⁷ as a coolant. After performance of the surgical procedures the bony defect was either left untreated to heal spontaneously in animals of the control group or filled with different materials according to the different groups. After that the periosteum and muscles were repositioned properly over the defect and sutured with (2/0)catgut resorbable suture⁸ then the skin was repositioned properly and sutured with (3/0) silk suture⁹. Following the surgery the animals were allowed to resume normal activity immediately after surgery. Each rat received intramascular injection mg^{10} Cephotaxime 1000 (third of generation of cephalosporin antibiotics), and post-surgical pain was treated with

¹ Sigma-Tec Pharmaceutical industries-Egypt

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³ Eva Cosmetics company-Egypt

⁴ The Nile company for Pharmaceuticals and Chemical Industries-Egypt

⁵ ELA Carbide-Germany

⁶ Olympia-Japan

⁷ Egypt Otsuka Pharmaceutical Company

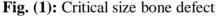
⁸ International sutures manufacturing Co. Egypt

⁹ KeeboMed- USA

¹⁰ Advocure Pharmaceuticals-Egypt

subcutaneous injection of Ketoprufen 75mg/ml¹¹ (antinflamatory and analgesic) postoperatively. Dose 3 days was calculated according to animal ethics committee pain control protocol. The animals were observed daily by the veteranian for any adverse effects related to the surgery. At the end of the experimental period, each subgroup rats was sacrificed by over dose of anesthesia. Tibiae were dissected free from any soft tissue carefully and the defect zone were defined as the area of interest.





After sacrificing the animals, their tibiae were dissected and prepared for Paraffin sections. Finally, the sections were stained by hematoxyline and eosin stain (H&E) for routine histological examination under light microscope and Masson Trichrome special stain (MT) to detect areas of new collagen formation. Slides were digitized using Canon digital camera¹² mounted on Leica light

microscope¹³ using 40X objective lens for (H&E) slides (Silva et al., 2015) and 20X objective lens for (MT) slides (Saghiri et al., 2015). Images were then transferred to the computer system for detection of inflammatory reaction and the bone density analysis were carried out using Image J, 1.41a (NIH, USA) image analysis software. For inflammatory cell count to standardize the results, 5 fields for each section were taken at 400X. magnification For bone area fraction, 5 fields at magnification 200X were obtained for each section. Data were collected and tabulated for statistical analysis.

Statistical analysis was performed using IBM® SPSS® Statistics Version 20 for Windows. One-way ANOVA followed by Tukey post hoc-test which was used to compare between more than two groups in non-related samples. Repeated measure ANOVA and paired sample t-test were used to compare between related samples in two (Paired sample t-test) and more (ANOVA) groups. The significance level was set at $P \le 0.05$.

RESULTS

I- Inflammatory cell count:

Relation between different study groups and each period of implantation (Table 1):

After 1 day of sacrificing (Figures: 2ae): The highest mean value was found in Control group (172.00 \pm 11.22) while the least mean value was found in (Experimental material + CaCl₂ solution) (155.00 \pm 5.20) group. No statistically

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¹³ Germany

significant difference was found between Control, Sinai white Portland cement, Angelus white MTA, (Experimental material + CaCl₂ solution) and (Experimental material + nHAp + CaCl₂ solution) where (p=0.110).

After 1 week of sacrificing (Figures: 3 a-e): The highest mean value was found in Control (196.20 \pm 19.31) while the least mean value was found in (Experimental material + nHAp + CaCl₂ solution) (163.20 \pm 5.12) group. A statistically significant difference was found between Control, Sinai white Portland cement, Angelus white MTA, (Experimental material + $CaCl_2$ solution and (Experimental material + nHAp + $CaCl_2$ solution) groups where (p<0.001).

After 1 month of sacrificing (Figures: 4 **a-e):** The highest mean value was found in Control (159.20 ± 11.01) group while the least mean value was found in (Experimental material + nHAp + CaCl₂ solution) (92.00 \pm 15.68). A statistically significant difference was found between Control, Sinai white Portland cement, MTA, (Experimental Angelus white material + CaCl₂ solution and (Experimental material $+ nHAp + CaCl_2$) solution) where (p<0.001).

 Table (1): Inflammatory cell count of different study groups.

Inflammatory cell count	After 1 day		After 1 week		After 1 month		
Variables	Mean	SD	Mean	SD	Mean	SD	P-value
Control	172.00 ^{aA}	11.22	196.20 ^{abB}	19.31	159.20 ^{aC}	11.01	0.001*
Sinai white Portland cement	170.40 ^{aA}	11.41	190.80 ^{abB}	7.56	157.80 ^{aC}	13.07	0.001*
Angelus white MTA	168.20 ^{aA}	8.29	179.60 ^{bcB}	6.19	116.60 ^{bC}	11.39	<0.001*
Experimental material + CaCl ₂ solution	155.00 ^{aA}	5.20	166.60 ^{cB}	7.09	110.20 ^{bcC}	9.78	<0.001*
Experimental material +nHAp + CaCl ₂ solution	155.20 ^{aA}	7.43	163.20 ^{cB}	5.12	92.00 ^{cC}	15.68	<0.001*
P-value	0.110		<0.001*		<0.001*		

Mean values with different lower-case subscripts in the same column indicate statistically significance difference, mean values with different upper-case subscripts in the same row indicate statistically significance difference. *; significant

Haematoxyline and Eosin stain (H&E - 400X sections):

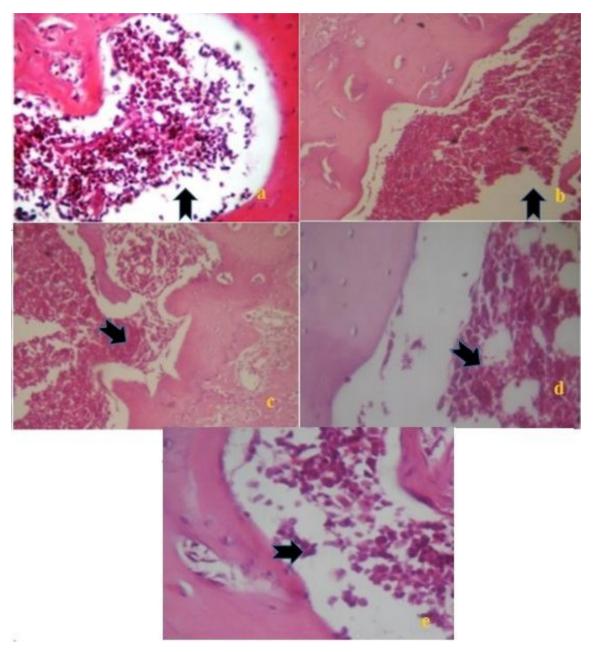
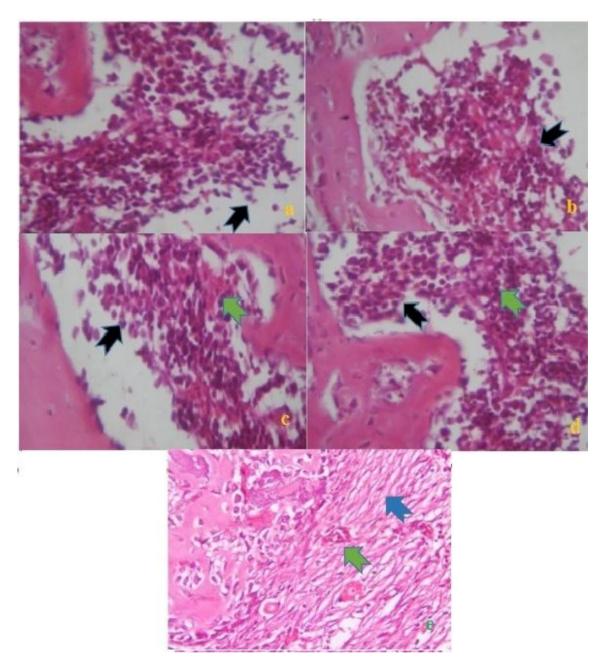


Figure (2 a-e): Photomicrograph after 1 day of sacrificing from different study groups showing the area of bone defect with extensive chronic and acute inflammatory reaction (black arrows).

- a- Control group (172 \pm 11.22)
- b- Sinai white Portland cement (170.40 ± 11.41)
- c- Angelus white MTA (168.20 ± 8.29)
- d- Experimental material + CaCl₂ solution (155.00 \pm 5.20)
- e- Experimental material + nHAp + CaCl₂ solution (155.20 \pm 7.43)

6



Haematoxyline and Eosin stain (H&E - 400X sections):

Figure (3 a-e): Photomicrograph after 1 week of sacrificing from different study groups showing the area of bone defect with extensive chronic inflammatory cells (black arrows), small foci of immature osteoid tissue matrix appear in groups (c&d) (green arrows) and immature osteoid tissue with dense and coarse collagen bundles in group (e) (blue arrow). a- Control group (196.20 \pm 19.31)

- b- Sinai white Portland cement group (190.80 ± 7.56)
- c- Angelus white MTA group (179.60 ± 6.19)
- d- Experimental material + CaCl₂ solution (166.60 \pm 7.09)
- e- Experimental material + nHAp + CaCl₂ solution (163.20 \pm 5.12)

Haematoxyline and Eosin stain (H&E - 400X sections):

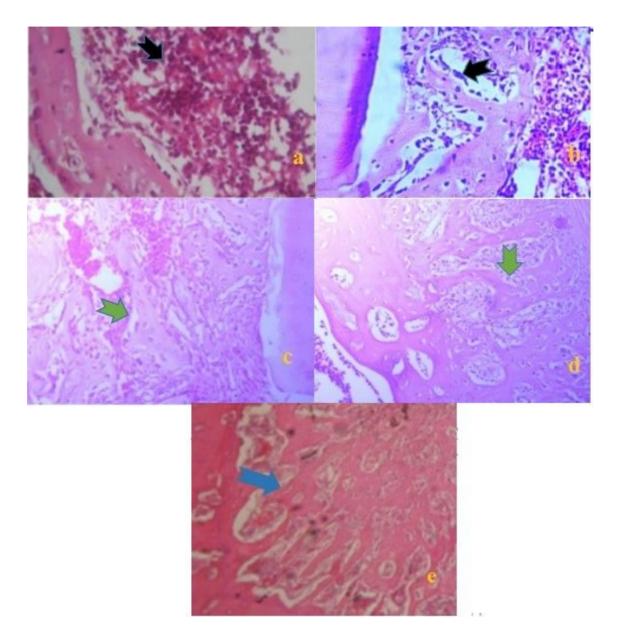


Figure (4a-e): Photomicrograph after 1 month of sacrificing from different study groups showing the area of bone defect with small foci of calcification admixed with moderate chronic inflammatory cells in groups (a&b) (black arrows), well-formed bone trabeculae in groups (c&d) (green arrows) and reparative mature osteoid tissue in group (e) (blue arrow). a- Control group (159.20 \pm 11.01)

- b- Sinai white Portland cement group (157.80 ± 13.07)
- c- Angelus white MTA group (116.60 ± 11.39)
- d- Experimental material + CaCl₂ solution (110.20 \pm 9.78)
- e- Experimental material + nHAp + CaCl₂ solution (92 \pm 15.68)

II- New bone formation:

Relation between groups in each time period (Table 2):

After 1 day of sacrificing Figures (5 ae): The highest mean value was found in (Experimental material + nHAp + CaCl₂ solution) (0.10 ± 0.02) while the least mean value was found in Control group (0.00 ± 0.00). A statistically significant difference was found between Control, Sinai white Portland cement, Angelus white MTA, (Experimental material + CaCl₂ solution) and (Experimental material + nHAp + CaCl₂ solution) where (p<0.001).

After 1 week of sacrificing Figures (6 ae): The highest mean value was found in (Experimental material + nHAp + CaCl₂ solution) group (1.78 ± 0.18) while the least mean value was found in Control (0.03 ± 0.01) . A statistically significant difference was found between Control, Sinai white Portland cement, Angelus white MTA, (Experimental material + CaCl₂ solution) and (Experimental material + nHAp + CaCl₂ solution) where (p<0.001).

After 1 month of sacrificing Figures (7 a-e): The highest mean value was found in (Experimental material + nHAp + CaCl₂ solution) group (3.14 ± 0.15) while the least mean value was found in Control (0.11 ± 0.05). A statistically significant difference was found between Control, Sinai white Portland cement, Angelus white MTA, (Experimental material + CaCl₂ solution) and (Experimental material + nHAp + CaCl₂ solution) where (p<0.001).

New bone	After 1 day		After 1 week		After 1 month		-
formation Groups	Mean	SD	Mean	SD	Mean	SD	P-value
Control	0.000 dC	0.000	0.032 cB	0.015	0.110 dA	0.045	0.017*
Sinai white Portland cement	0.030 cB	0.023	0.102 ^{bc}	0.013	0.138 dA	0.036	0.006*
Angelus white MTA	0.054 bcC	0.011	0.184 ^{bc} B	0.011	1.318 cA	0.250	<0.001*
Experimental material + CaCl ₂ solution	0.072 _{abC}	0.008	0.228 bB	0.059	1.988 _{bA}	0.201	<0.001*
Experimental material + $nHAp + CaCl_2$ solution	0.098 aC	0.015	1.776 ^{aB}	0.176	3.142 _{aA}	0.145	<0.001*
P-value	< 0.001*		< 0.001*		< 0.001*		

Mean values with different lower-case subscripts in the same column indicate statistically significance difference, mean values with different upper-case subscripts in the same row indicate statistically significance difference. *; significant

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Masson Trichrome stain (MT - 200X sections):

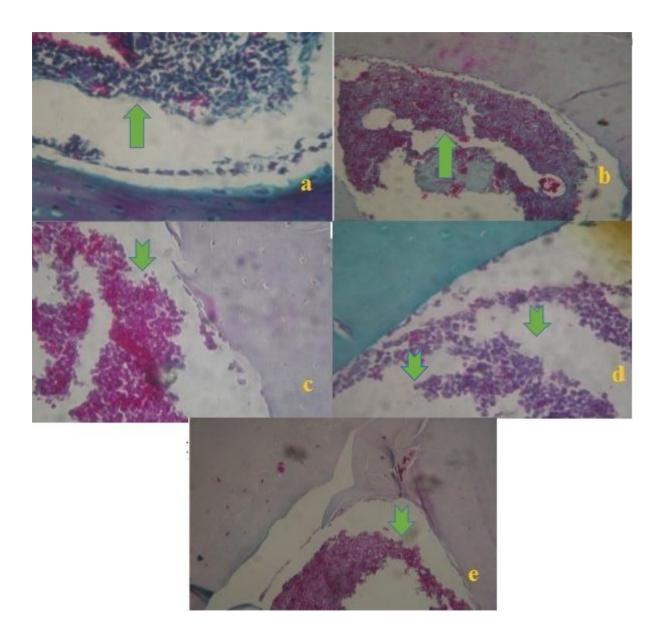


Figure (5 a-e): Photomicrograph after 1 day of sacrificing from different study groups showing the area of bone defect with intense inflammatory cells infiltrate in all groups (green arrows)

- a- Control group
- b- Sinai white Portland cement group
- c- Angelus white MTA group
- d- Experimental material + $CaCl_2$ solution
- e- Experimental material + $nHAp + CaCl_2$ solution

Masson Trichrome stain (MT - 200X sections):

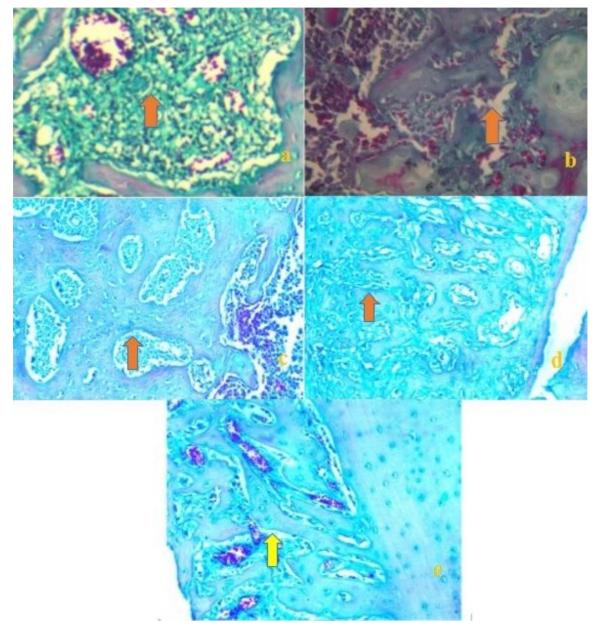
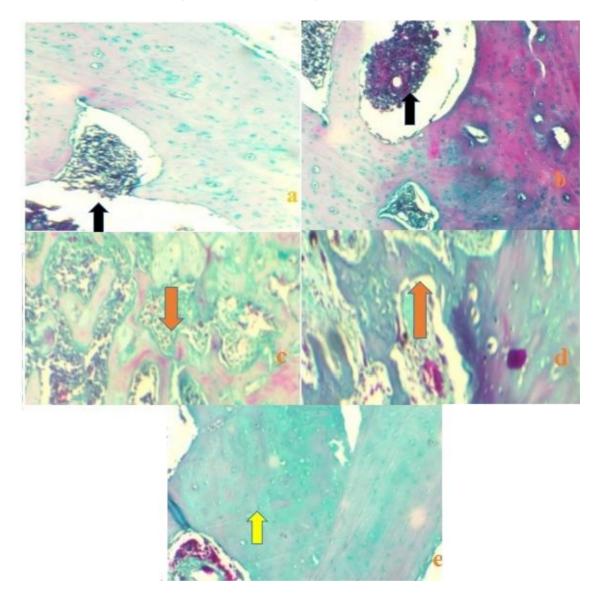


Figure (6 a-e): Photomicrograph after 1 week of sacrificing from different study groups showing the area of bone defect filled with a mass of granulation tissue in all groups (orange arrows) and marked collagen fibers around the newly formed immature bony matrix in group (e) (yellow arrow)

- a- Control group
- b- Sinai white Portland cement group
- c- Angelus white MTA group
- d- Experimental material + $CaCl_2$ solution
- e- Experimental material + nHAp + $CaCl_2$ solution



Masson Trichrome stain (MT - 200X sections):

Figure (7 a-e): Photomicrograph after 1 month of sacrificing from different study groups showing the area of bone defect filled with persistent chronic inflammation in groups (a&b) (black arrows), bony spicules enlarged maturated and coalesced with each other in groups (c&d) (orange arrows) and increased amount and organization of bone mass with marked maturity in group (e) (yellow arrow).

- a- Control group
- b- Sinai white Portland cement group
- c- Angelus white MTA group
- d- Experimental material + CaCl₂ solution
- e- Experimental material + nHAp + CaCl₂ solution

DISCUSSION

For microscopic evaluation of biocompatibility of dental materials, Federation Dentaire International (FDI) recommends inflammatory cell count in different areas of microscopic sections (Vosoughhosseini et al., 2012).

Monitoring of a specific response to the foreign body following the material implantation starts with inflammation continuing through the stages of wound healing with the involvement of various cell types specific for tissue repair, through which the total number of inflammatory cells decrease while the wound healing process moves towards formation of granulation tissue and fibrous encapsulation of the implanted material (**Opacic-Galic et al., 2017**).

Formation of calcium hydroxide is the cause of high alkalinity of MTA after hydration, which is considered an initial tissue irritant when MTA cement comes into contact with the tissue that provokes severe inflammatory reaction which subsides by time (Saghiri et al., 2013). The lower inflammatory reaction in MTA Angelus group compared to Portland cement group in different implantation periods could be related to the presence of some impurities and other heavy metals in non-dental cements (Minotti et al., 2015), but Portland cement exhibited other properties similar to MTA such as antiinflammatory effect, and this could be the reason of decreased inflammatory reaction in Sinai white Portland cement group than the control group in this study as Portland cement has an inhibitory effect on

prostaglandins (**Tenorio de France et al.,** 2010)[.]

An experimental bone wound used to asses repair should be large enough to preclude spontaneous healing. Also, for successful repair achieving and regeneration in the clinical treatment of critical-sized bone defects, the material should satisfy the following desirable criteria, ability to maintain in vivo mechanical stability at the defect site and withstand physiological loads, radiopacity for easy implant monitoring using noninvasive methods such as X-ray and micro-computed tomography $(\mu$ -CT). Bioactivity to promote integration with host bone, ability to degrade at a controlled rate that matches the rate of new bone formation and ability to allow easy handling and sterilization (No et al., 2017).

The results of this study showed that all the tested cements are bioactive as they induced mineral deposition with differences in speed and intensity of deposition. This could be attributed to the calcium ions released from the tested cements that react with phosphate ions in resulting the tissue fluid, in the precipitation of carbonated apatite which is important to the formation and maintenance of the bone biomaterial interface (Dreger et al., 2012).

In all study groups, an increase of osteoid tissue from the period of one week up to one month was observed, the best results in terms of area of more osteoid tissue formed was in group (Experimental material + nHAp + $CaCl_2$ solution) after one month of implantation, that could be

related to the nano-hydroxyapatite which improve bone mineralization due to its chemical similarity to the mineral component of bone.

It was reported that the high level of calcium and phosphorus deposition when the calcium silicate based cements were associated with ZrO_2 nanoparticles indicates the formation of a layer of hydroxyapatite on the surface of the powder when comes in contact with buffered phosphate solution. thus reinforcing its bioactive potential (Bosso-Martelo et al., 2017).

Moreover, calcium fluoride (CaF₂) was found to have a synergistic effect on apatite forming ability of calcium silicate cements (**Gandolfi et al., 2011**). This is in agreement with the results of this study as the experimental material contained (0.3 weight percent) calcium fluoride that gave a more reactive osteoid tissue formation than the other two tested commercial materials.

Calcium sulphate, typically in the form of dihydrate (Gypsum) are widely recognized as safe and bioactive implant materials that have been successfully used as bone substitutes. Once it reacts with water, it creates a resorbable phase and the reaction results in the growth of interlocking needle like crystals that form the set cement. Also it is used as a drug delivery material (**Saghiri et al., 2015**).

In MTA Angelus group, deposition of newly formed bone throughout the experimental periods may be due to the presence of silicon oxide (SiO2) in the cement which acts as a suitable bed for apatite precipitation (**Hosseinzade et al.**, **2016**). The deposition of hard tissue on implantation of MTA Angelus could be related to its biocompatibility, alkaline pH, the presence of calcium and phosphate ions in its formulation and stimulation of expression of alkaline phosphatase by fibroblasts (**Silva et al., 2015**).

CONCLUSION

According to histological responses observed in this study that (Experimental material + nHAp + $CaCl_2$ solution) is a reliable material in bone defects which are of major concern in the restorative field.

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تقييم استجابة الأنسجة لأسمنت كالسيوم سليكات تجريبى فى مقابل ثلاثى أكسيد المعادن الكلى الأبيض و أسمنت سيناء البورتلاندى الأبيض

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خلفية البحث: أسمنت كالسيوم سليكات مادة ذات تقبل حيوى و تساعد في ترميم العظام.

الهدف من الدراسة: أجريت هذه الدراسة لتقييم و مقارنة إستجابة أنسجة جرذان التجارب لمادة معملية تجريبية في مقابل ثلاثي أكسيد المعادن الكلى الأبيض والاسمنت البور تلاندي الأبيض .

مواد و طرق البحث: تم تحضير المادة المعملية من الأكاسيد النظيفة. تم عمل فجوات في قصبة ساق الجرذ، و غرس مختلف المواد بها مع ترك مجموعة تحكم على حسب الفترات المحددة لإجراء الإختبارات ومختلف المواد المستخدمة في كل مجموعة عدا مجموعة التحكم .

النتائج: أسفرت نتائج هذه الدراسة عن زيادة الخلايا الالتهابية في كل مجموعات الدراسة بعد مرور يوم واحد على الإختبار، وقد إنخفضت بعد مرور شهر من إجراء الإختبارات، كما أظهرت نتائج الإختبارات تكوينات عظمية جديدة تزيد بزيادة الوقت المقرر لاجراء الاختبارات.

الإستنتاج: المادة المحضرة معمليا تشابه في تأثير ها المواد الحيوية التجارية.

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