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VITALITY OF AUTOGENOUS BONE GRAFTS FOLLOWING DECONTAMINATION USING DIFFERENT ANTIMICROBIAL AGENTS. AN EXPERIMENTAL STUDY IN A RABBIT MODEL

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ABSTRACT

Aim of the study: The aim of the current study was to assess the vitality of autogenous bone grafts harvested from rabbits` calvaria following decontamination of the grafts using three different antimicrobial agents.

Materials and Methods: Autogenous bone grafts were harvested from the calvaria of twenty four white New Zealand male rabbits. The grafts were allowed a five minutes contact time with normal saline (Group I), 2% chlorhexidine (Group II), Clindamycine (Group III) and Oleozone gel (Group IV). The grafts were then reinserted into the previously created graft beds. Animals were sacrificed at 10 and 21 days post surgery, dual energy x-ray absorptiometry (DEXA) scans were performed for the skull of the rabbits and bone specimens were collected for histological examination.

Results: Histomorphometric analysis showed a superior results in favor of the ozone treated group represented as a significantly higher percentage of normal osteocytes and marked increase in area percentage of new bone formation. Additionally, DEXA scan revealed a significant increase in bone mineral density and bone mineral concentration of the ozone treated group compared to the other studied groups.

Conclusion: The ozone treated grafts showed a distinguished preservation of osteoblastic vitality, which may indicate a superior biocompatibility compared to chlorhexidine and clindamycin.

KEY WORDS: Autogenous bone grafts; Decontamination; Antimicrobial agents; Rabbit model.

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INTRODUCTION

Bone grafting is one of the most widely recognized and used maneuvers in oral and maxillofacial surgery for management of osseous deficiencies following removal of pathological conditions, sinus floor elevation and management of alveolar clefts.[1,2]

Autogenous bone grafts are considered the most superior and acknowledged type of graft material owing to its abundant content of viable osteocytes, osteoprogenitor cells and the readily available and essential chemical signaling substances such as bone morphogenic proteins (PMPs), in addition to absence of cross contamination and immunological reactions against the grafted material, Subsequently the autogenous graft is referred to as the gold standard for bone grafting procedures.[3,4] Contamination of the grafted bone are among the most damaging complication that may be challenged with autogenous bone grafts, thus, infection of the graft can have undesirable harmful consequences on augmentation outcomes and fate of the grafted bone. [5] Once infection is recognized, it commonly obliges additional surgical interference for elimination of the septic segment, radical debridement of the surgical area and a prolonged systemic antibiotic therapy with all of its recognized undesirable effects. Despite these trials, the result is frequently poor with significant patient morbidity. During a bone grafting procedure there is usually a pause between obtaining of the graft and its implantation, the delay may range from few minutes to several hours, which raised numerous realistic questions marks regarding the harmful effect of the delay on subsequent osteogenesis, probability of contamination of the graft and the optimal environment for preservation of the graft.[5,6]

The influence of exposure of bone –derived cells to different antimicrobials has been previously investigated with a controversy regarding the possible cytotoxic effects and consequent graft vitality.[7] The value of the use of various local antibiotics was verified in several studies.[8-10] However, their use were questionable as a result of their narrow specificity, potential cytotoxic effects and possible cross reactions. [10] Chlorhexidine, the widely used antiseptic was also used as a substitute for antibiotics in these situations, assuming that it has a broader therapeutic window and pathogens cannot develop resistance against it. In addition to, its low cost compared to antibiotics.[11] Ozone or trioxygen is an inorganic molecule that is normally present as a gas made of three atoms of oxygen in a cyclic structure.[12] Different forms of ozone had been thoroughly investigated in dentistry and maxillofacial surgery, most of the former studies have been focusing on their antimicrobial effects.[13,14] Though, there is insufficient evidence of the effect of ozone therapy on musculoskeletal regeneration.

It was reported that there were similarities of bone mineral density and the fracture toughness of bone between rabbits and human. In comparison with other species such as primates and some rodents, rabbit has faster bone turnover, in addition to being readily available, easy to house and handle. These characteristics make rabbits among the most commonly used animal model for in vivo skeletal investigation.[15,16,17]

In the current study, a rabbit model was used to compare the effect of three different antimicrobial agents, including ozone, on the vitality of bone grafts using a fixed contact time with the agents to eliminate the effect of exposure time.

MATERIALS AND METHODS

Experiment design

This study was conducted following the approval of the local ethical committee. Twenty four male white New Zealand rabbits (Oryctolagus cuniculus) of age 6 to 8-months-old with an initial weight of 3.0±0.50kg were used for this study. This species of rabbit was selected because of its small size, simple acquisition, reasonable cost, and convenient care in the laboratory. The rabbits were randomly assigned to one of the experimental groups.

A 5 mm diameter trephine bur was used to obtain a bone graft and creating a similar size defect in the midline of the calvarial bone of each animal.

A total of 24 grafts were harvested, each graft was crushed using a special bone mill device, After bone milling, each bone graft was collected in a special sterile container, Six grafts were mixed with normal saline solution (control group) and each one of the rest of the grafts were mixed with one of the examined antimicrobial agents. The contact time for each graft was five minutes during which the containers were kept at room temperature. The collected grafts were allocated equally to one of the following groups:

Group I: The grafts were soaked in 10 ml normal saline (control group).

Group II: The grafts were soaked in10 ml of 2% chlorhexidine.

Group III: The grafts were mixed with clindamycine powder (Dalacin-C 150 mg capsule - Pfizer pharmaceutical) and 2 ml saline.

Group IV: The grafts were mixed with 5 cc of Oleozone gel (Nour Advanced Technologies, Egypt).

Surgical protocol

The animals were premedicated using midazolam (0.2mg/kg). General anesthesia was induced by intramuscular injection of ketamine (10 mg/kg of body weight), 2% xylazine (4 mg/kg), 0.2% acepromazine (0.15 mg/kg) and an intravenous propofol (2 mg/kg). Local anesthesia (mepivicaine 2% containing 1:100.000 levonordephrine) was infiltrated around the surgical site. Prophylactic enrofloxacin antibiotics were administrated via intravenous route. The skin at the operative site was shaved and scrubbed using 2% iodine solution. A midline incision from the frontal area to the occipital protuberance was made down to the osseous surface of the skull, and a full thickness flap was raised to expose the calvarial surface on both sides of the midline. A standardized bone graft was obtained from each animal using a trephine bur with an inner diameter of 5 mm mounted on a handpiece at 2,000 rpm under copious saline solution irrigation. The graft consisted of both the outer and inner calvaria cortical bone, which was approximately 3 mm thick with a diameter of 5 mm.

The obtained specimens were milled using a special bone mill machine (ACE Surgical Supply Company, USA), the milled grafts were then mixed with one of the four agents under investigation and allowed a contact time of five minutes at room temperature. The specimens were reinserted and gently packed into the created calvarial defects and the overlying skin was repositioned and sutured with (5-0) interrupted sutures.

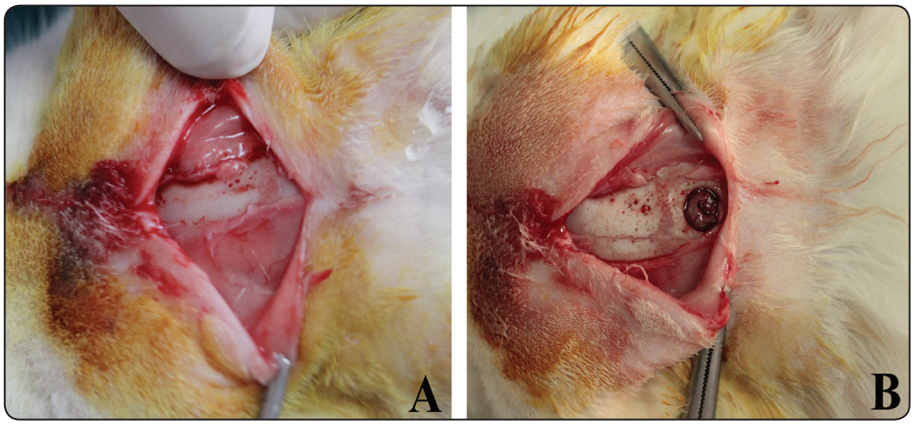

Fig. (1) Photographs showing the midline calvarial incision (A), The calvarial defect after taking the graft (B).

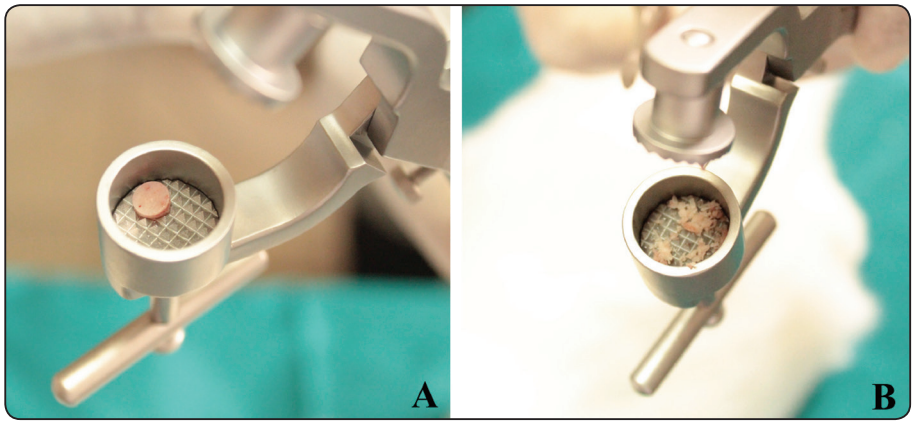

Fig. (2) Photographs showing (A) The graft within the bone mill, (B) The graft after milling.

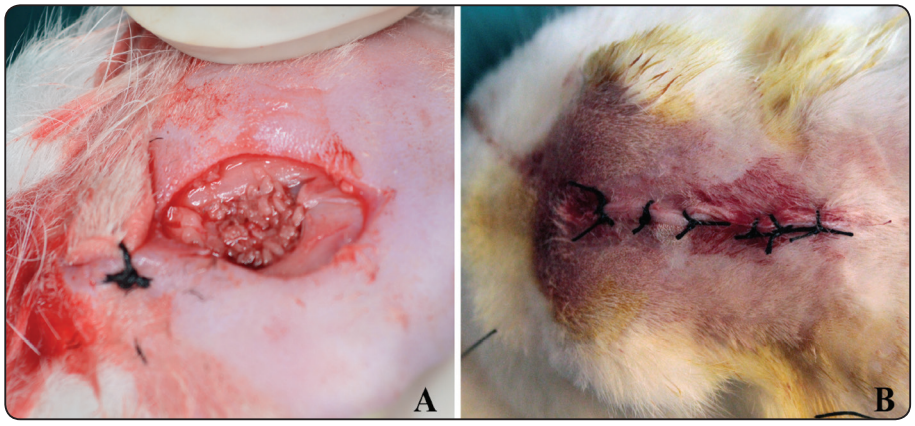

Fig. (3) Photographs showing (A) Re-insertion of the graft into the calvarial bed, (B) Closure of the wound.

Postoperatively the animals were administrated tramadol hydrochloride at 0.6 ml every 12 hours for three days and the enrofloxacin 2.5% at 0.6 ml every 12 hours for three days. The animals were kept in a temperature of 25°C, in a 12 h light/dark cycle with adequate ventilation and offered heavy ceramic bowels of water, fragrant herbs such as basil, fresh greens and hay.

Animal euthanasia

Animals were sacrificed at 10 and 21 days post-surgery (three animals from each group at 10 days and the rest of the animals at 21 days), using a combination of xylazine (20 mg/kg) and ketamine (50 mg/kg) administrated intramuscularly followed by 25 mg/kg sodium thiopental administrated intravenously in the ear vein.

Bone densitometry

The skulls of the animals were harvested, after animal scarification, and a dual energy x-ray absorptiometry (DEXA) scan was performed for each skull using a peripheral DEXA device (Norland XR-46, Cooper surgical company, USA). The area of interest was detected, centralized and scanned using an examination surface area of 4±0.5 cm^2. Two main modules were verified and represented as the bone mineral density (BMD) and the bone mineral concentration (BMC), the data for each group at 10 and 21 days were recorded in tables for statistical analysis. (Figure 4)

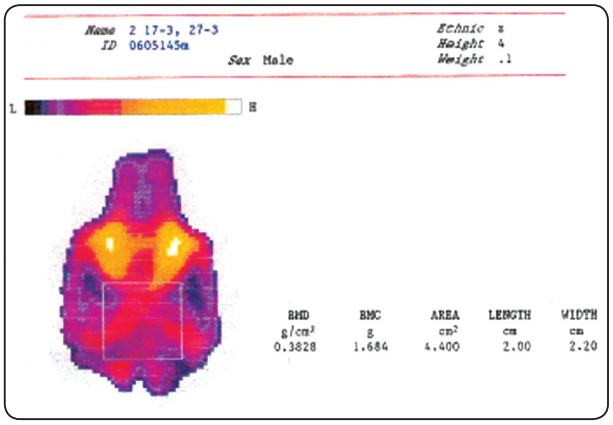

Fig. (4) A printout of the DEXA scan showing the area of interest and measurement of BMD and BMC

Histological sections

Once the animals were sacrificed, the specimens were collected and tissues were fixed in 10% buffered formalin for 48h hours. The specimens were then decalcified in 20% formic acid and sodium citrate for 10 days. After the initial decalcification, each specimen was divided longitudinally into two blocks along the center line of the original surgical defect. After complete decalcification, the specimens were washed with tap water, dehydrated and embedded in paraffin. Serial sections of 4 mm thickness were cut from each tissue block and stained with hematoxylin and eosin (H &E) for analysis. Depending on the size of the H & E section, three to five 1mm^2 areas within the healing area were selected randomly at magnification X 40 for evaluation. These areas subsequently were used for histomorphometric comparisons. Leica application suite (LAS V4) system (Switzerland) and Image J image analysis software were used to lock on these preselected areas for each histological section.

For each of the four studied groups a differential osteocyte count (normal osteocyte, abnormal osteocyte, empty lacunae) was performed for each section. The osteocytes were classified according to the morphological criteria established by Moura et al.,[18] those that occupied more than 50% of their lacunae were considered normal, and those that occupied 50% or less of their lacunae were considered abnormal. Empty lacunae were also counted.(Figure 5).

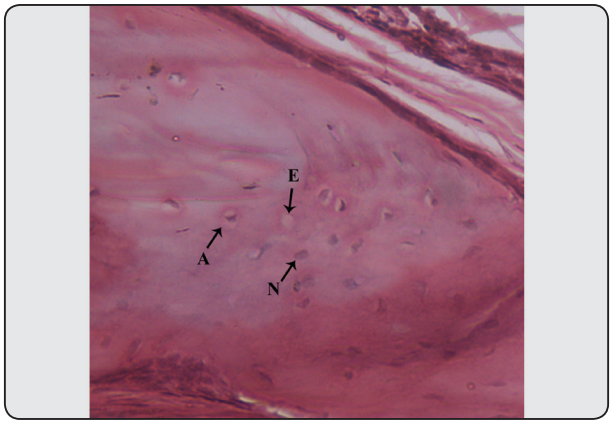

Fig. (5) A photomicrograph showing the criteria for histological evaluation, where normal osteocytes (N), Abnormal osteocytes (A), Empty lacuna (E).

The sequence of bone repair was observed histologically by examining the four groups at either 10 or 21 days by manually counting the area percentage of new bone and expressing it as areas (in mm2). To standardize our histomorphometric analysis, we based our measurements in part on the work of Messora et al.,[19] The total area (TA) to be analyzed corresponded to the entire area of the original surgical defect. The mineral deposition area (MDA) was delineated within the confines of the TA. The TA was measured in mm2 and was considered 100% of the area to be analyzed. The MDA was also measured in mm2 and calculated as a percentage of TA. The number of inflammatory cell infiltrate to the marrow spaces was also recorded for each histological sections obtained in the two time intervals.

RESULTS

Animal recovery

One animal from the chlorhexidine group was lost due to excessive dehydration and weight loss during the first postoperative week and was replaced with another one, the rest of the animals showed an uneventful postoperative healing period.

Descriptive histology

Histologically, bone matrix was secreted at day 10 and increased significantly at day 21, Osteoblastic cells appeared at the early stages of 10 days and matured over time. Osteogenic activity was detected directly at the interface. A higher degree of formation of vascularized tissues, of provisional matrix, and of bone remodeling activity at 10 and 21 days was recorded in the chlorhexidine and ozone groups as compared to the clindamycin group. Meanwhile, a higher number of normal osteocytes were detected in the ozone group.

At 10 days: Bone formation does not occur at a uniform rate across the entire defect. The initial synthesis of repair bone at ten days is woven and confined adjacent to the pre-existing lamellar cortical bone. Initially, new bone synthesis occurs peripherally restricted to areas that were close to the borders of the surgical defect. While the biggest central part of the surgical defect was occupied by connective tissue with collagen fibers parallel to the wound surface with a mild to moderate chronic inflammatory infiltrate. The complete closure of the defect was not observed at day 10. The repair process is associated with a rich vascular front, which primarily forms from the marrow and passages into the defect at right angles to the long axis of the bone (Fig. 6).

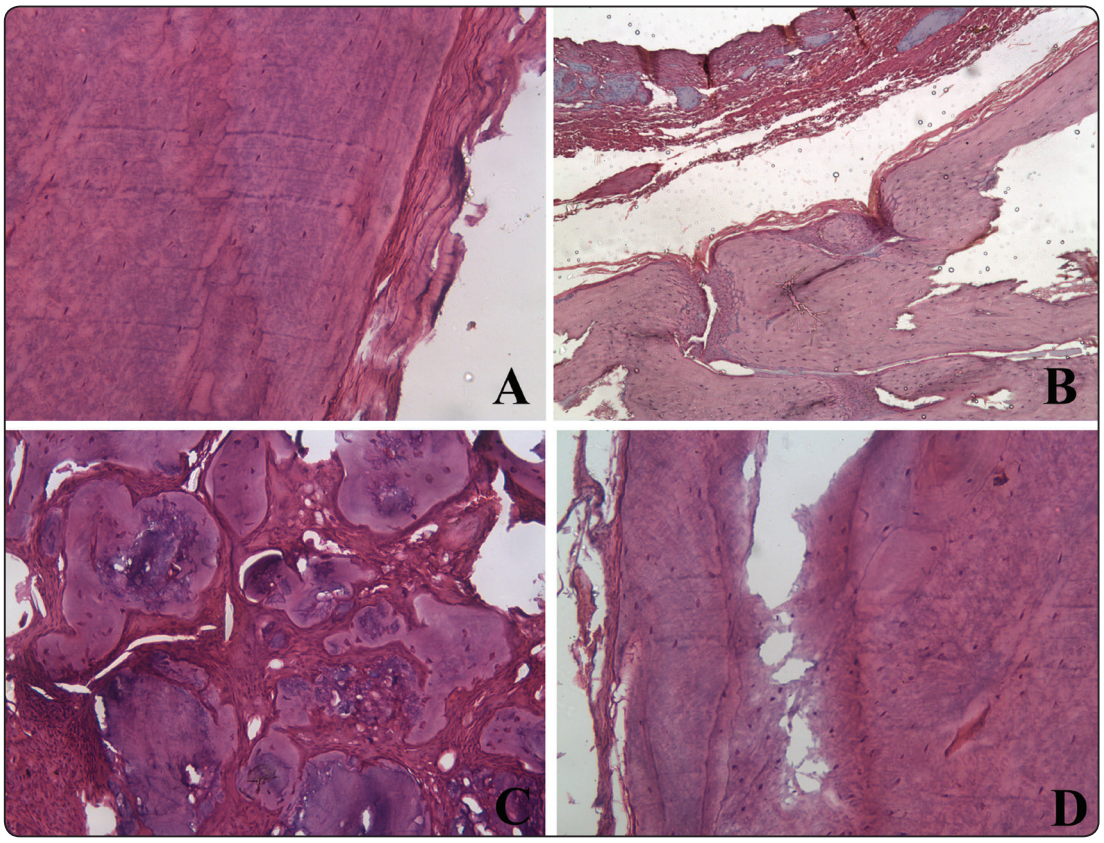

Fig. (6) Photomicrographs at 10 days (H&E × 200) :(A) Saline group showing the parallel arrangement of the collagen fibers in relation to the surface of the surgical defect, the collagen is restricted to areas that were close to the borders of the surgical defect while the center of the defect is still empty. (B) Chlorhexidine group showing parallel arrangement of the collagen fibers close to the surface of the surgical defect, that the biggest part of the surgical defect is occupied by connective tissue with few globules of woven bone. (C) Clindamycin group showing globules of bone with areas of mineralization in a connective tissue stroma, the biggest part of the defect was occupied by connective tissue. (D) Ozone group showing woven bone attempting to close the defect. Note that the newly formed bone was mainly restricted to areas close to the borders of the defect (top) although attempts of bridging are seen at the bottom of the photomicrograph.

At 21 days: All bone defects in the four groups healed with full regeneration of bone. The histological characteristics of the healing stages were very similar between the groups except for the ozone group which showed considerable faster healing at the end of the 21 days period together with decreased number of inflammatory cells. Bone at the periphery, which was originally woven was transformed into lamellar bone adjacent to the persisting cortices. Closer towards the center of the defect woven bone predominated. All the defects of all groups were mainly filled by newly formed woven bone with thin and irregular trabeculae surrounded by fibro-vascular tissue. The woven bone was rimmed by plump surface osteoblasts. The ozone group was bridged by mineralized bone with irregular shape and volume (Fig. 7).

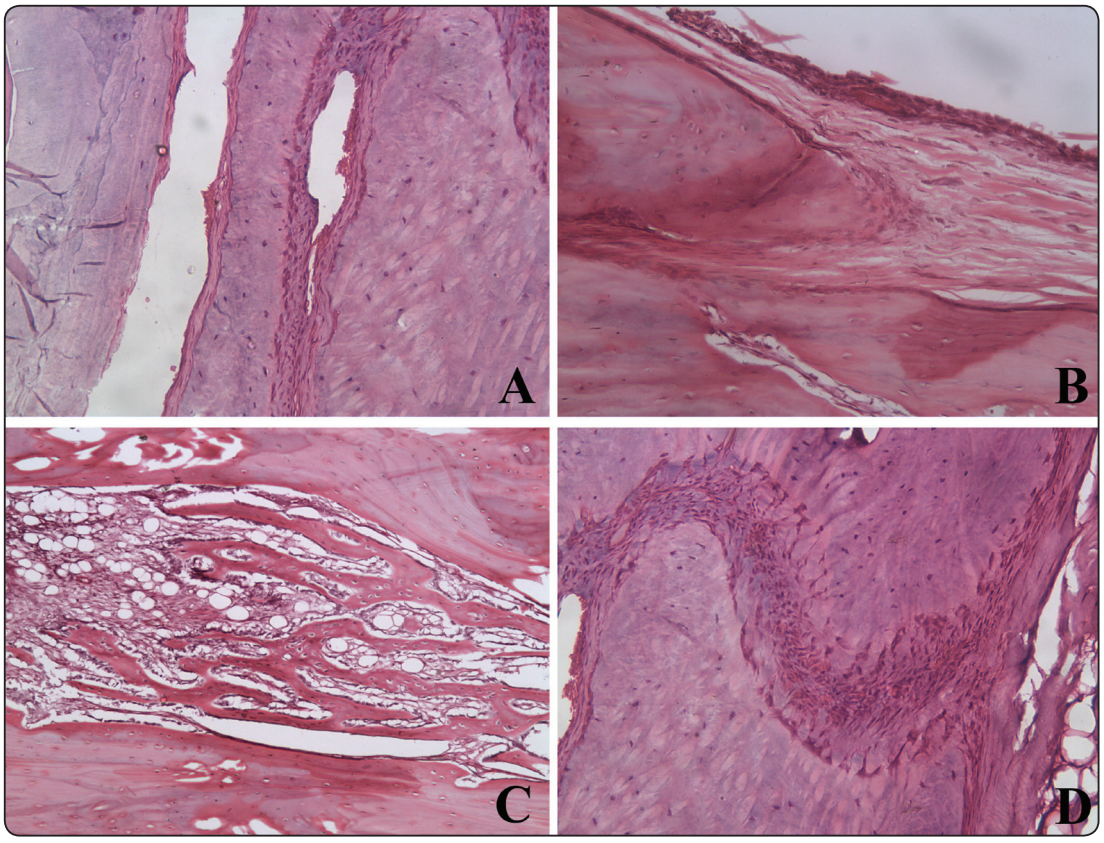

Fig. (7) Photomicrographs at 21 days (H&E × 200): (A) Saline group showing the longitudinal orientation of repair tissue (B) Chlorhexidine group showing initial foci of woven bone (deep color) surrounded by lamellar bone trabeculae (lighter pink) together with surface osteoblasts (C) Clindamycin group showing thin and irregular trabeculae in a fibro-vascular stroma., osteoblastic rimming clearly outline the bony trabeculae. (D) Ozone group showing complete closure of the surgical defect by mineralized bone trabeculae in a fibro-vascular stroma.

Statistical analysis

Records of all the studied parameters were presented as mean and standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that most of data were normally distributed (parametric data), so a parametric test (one way analysis of variance ANOVA test) was used to compare between groups. This was followed by Tukey's post hoc test whenever the difference was statistically significant.

Paired (t) test was used to compare between the two observation times (10 and 21 days). The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 16.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

It is worthily to mention that, paired (t) test revealed a significant increase of the means of the percentage of normal osteocytes, newly formed bone, bone mineral density and concentration, with a significant decrease in the mean number of inflammatory cells at 21 days in comparison to the same values recorded at 10 days in all the studied groups.

The greatest means of percentage normal osteocytes and newly formed bone were recorded in Group IV (Ozone), and group I (Control), with the least value recorded in group III (Clindamycin). The same pattern was observed at 10 days (Table 1) and at 21 days (Table 2). ANOVA test revealed an extremely statistically significant difference ($p<0.0001$) for both previously declared parameters. Tukey's post hoc test revealed a significant difference between each two groups (table 1, 2; Fig. 2). Regarding the mean of number of inflammatory cells in marrow spaces, the highest number was recorded in Group III, while the least value recorded in group IV and group I respectively. The same pattern was observed at 10 days (Table 1) and at 21 days (Table 2). ANOVA test revealed a statistically significant difference ($p<0.0001$). Tukey's post hoc test revealed a significant difference between each two groups (table 1, 2; Fig. 3).

TABLE (1) Difference between the four groups at 10 days regarding percentage of normal osteocytes, the area percentage of new bone and number of inflammatory cells in marrow spaces and significance of the difference using ANOVA test

At 10 Days	Group I		Group II		Group III		Group IV		F value	P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Percentage of normal osteocytes	38.40^{b}	0.92	32.84^{c}	1.75	22.92^{d}	1.59	48.36^{a}	0.97	307.2	<.0001*
Area percentage of new bone	45.80^{b}	2.82	38.84^{c}	1.06	29.08^{d}	3.25	54.62^{a}	4.14	63.53	<.0001*
Number of inflammatory cells	17.80^{c}	2.68	23.80^{b}	2.77	27.80^{a}	0.84	13.40^{d}	1.82	42.93	<.0001*

**** Significant at P<0.05***

Tukey's post hoc test: means with different superscript letters are significantly different

TABLE (2) Difference between the four groups at 21 days regarding percentage of normal osteocytes, the area percentage of new bone and number of inflammatory cells in marrow spaces and significance of the difference using ANOVA test

At 21 Days	Group I		Group II		Group III		Group IV		F value	P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Percentage of normal osteocytes	66.76[b]	8.81	52.36[c]	2.30	33.88[d]	1.91	80.22[a]	1.23	89.34	<.0001*
Area percentage of new bone	72.92	3.96	54.90	1.53	40.78	1.49	79.02	1.22	278.66	<.0001*
Number of inflammatory cells	11.00	1.00	14.80	2.77	20.20	1.64	5.80	1.79	50.64	<.0001*

significant at P<0.05 ***Tukey's post hoc test: means with different superscript letters are significantly different***

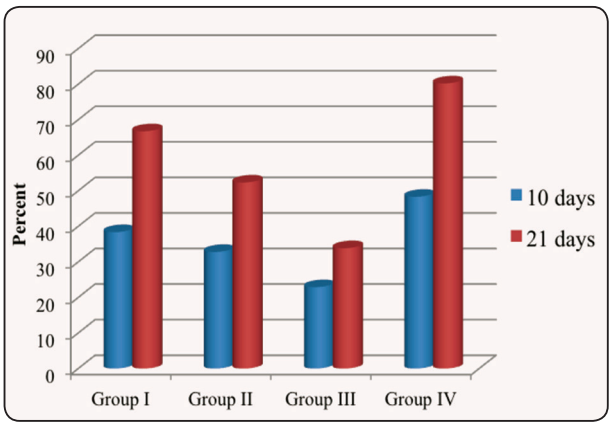

Fig. (8) Column chart showing mean percentage of normal osteocytes in all groups at 10 and 21 days.

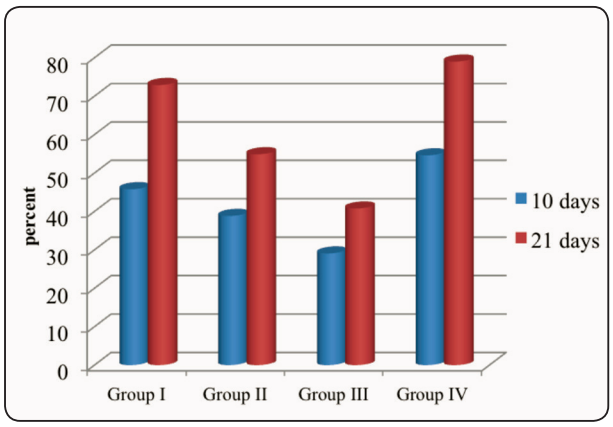

Fig. (9) Column chart showing mean percentage of newly formed bone in all groups at 10 and 21 days.

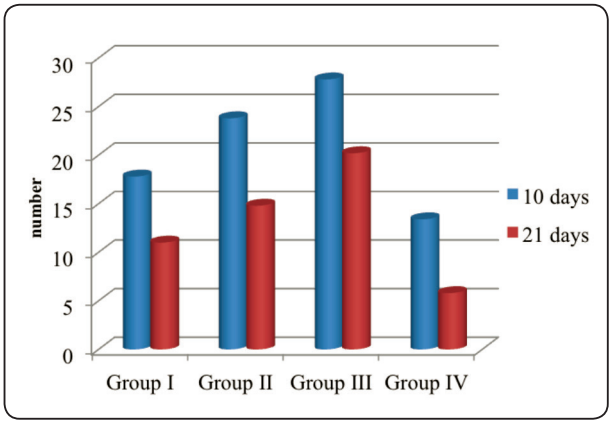

Fig. (10) Column chart showing mean number of inflammatory cells in marrow spaces in all groups at 10 and 21 days.

Concerning the Bone Mineral Density (BMD) and Bone Mineral Concentration (BMC), The greatest means were recorded in group I (control) and group IV, followed by group II with the least value recorded in group III. This pattern was observed at 10 and 21 days (Table 7). In both observation dates, ANOVA test revealed a statistically significant difference between groups (P<0.0001). Tukey's post hoc test revealed a statistically significant difference between group IV and groups II, III at 10 and 21 days, while no significant difference was verified between groups I and IV (Table 7, Fig.4). Moreover, paired (t) test revealed a significant increase in both BMD and BMC at 21 days in comparison to values documented at 10 days in all the studied groups.

TABLE (3) Bone mineral density (BMD) at 10 and 21 days (g/cm^2) and significance of the difference between groups using ANOVA test.

BMD	Group I (control)		Group II		Group III		Group IV		F value	P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
At 10 days	0.37^a	0.02	0.31^b	0.04	0.24^c	0.02	0.37^a	0.01	33.62	<.0001*
At 21 days	0.78^a	0.04	0.67^b	0.02	0.43^c	0.05	0.78^a	0.03	94.24	<.0001*

significant at P<0.05 ***Tukey's post hoc test: means with different superscript letters are significantly different***

TABLE (4) Bone mineral concentration (BMC) at 10 and 21 days (gm) and significance of the difference between groups using ANOVA test.

	Group I (control)		Group II		Group III		Group IV		F value	P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
At 10 days	0.84^a	0.04	0.3^c	0.06	0.61^b	0.03	0.82^a	0.04	168.47	<0.0001*
At 21 days	1.69^a	0.11	0.72^c	0.05	1.08^b	0.17	1.65^a	0.09	81.58	<0.0001*

significant at P<0.05 ***Tukey's post hoc test: means with different superscript letters are significantly different***

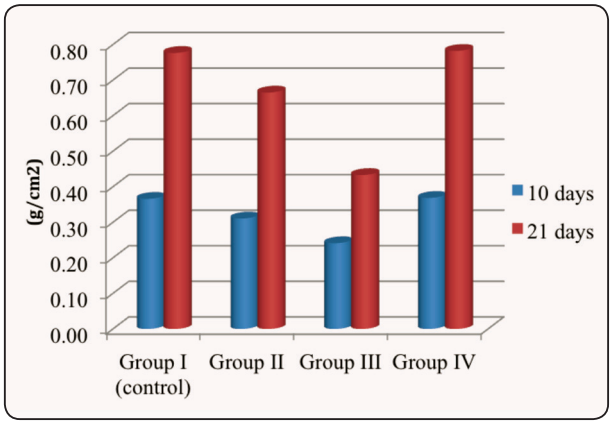

Fig. (11) Column chart showing mean Bone Mineral Density (BMD) in all groups at 10 and 21 days

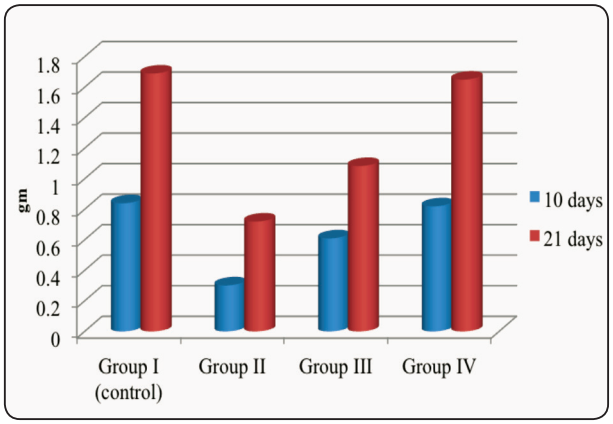

Fig. (12) Column chart showing mean Bone Mineral concentration (BMC) in all groups at 10 and 21 days

DISCUSSION

The rabbit model was used for this study because the bone repair process of rabbits, although faster, is physiologically similar to that of humans.[20,21] Furthermore, The use of cranial bone for grafting purposes has some significant benefits, such as a higher amount of surviving bone graft and a relatively short postoperative recovery period, Moreover, cranial bone harvesting is a safe procedure, with a low morbidity compared to iliac crest bone

harvesting.[22] Additionally, and most emphasized feature, is the embryologic, morphologic, and physiologic similarity of this bone to that in the maxillofacial region.[23]

The bacterial contamination of collected bone particles must be reduced because the implantation of contaminated bone particles may cause infectious complications at the host site augmented with this material. Therefore, decontamination methods should be considered to decrease the risk of augmentation failure due to bacterial contamination.

The efficacy of different antimicrobial agents in decontamination of bone grafts was extensively considered in the peer-reviewed literature. Among those agents, clindamycin[24] and chlohexidine[25] were specifically investigated.

Clindamycin is an antibiotic frequently used in different local application forms on osteoid tissue for the treatment of prosthetic joints infection or infection prophylaxis in bone cement. [26] Naal et al.,[27] demonstrated that clindamycin at lower concentrations stimulated the cell metabolism of human osteoblasts although higher levels had potential cytotoxic effect due to disturbed cellular energy supply by a clindamycin-induced mitochondrial inhibition.

Chlorhexidine soaks have become one of the more popular methods for decontaminating grossly contaminated bone and has both basic science literature and case reports to support its use.[11,28,29] Verdugo et al.,[5] provides a measure of how quickly and effective 2% and 4% chlorhexidine decreases the bacterial load on contaminated bone. Because of the reported detrimental effects that chlorhexidine has on cell viability, they recommended, based on there in vitro evaluation, the use of a 2% chlorhexidine solution soak for less than 20 minutes for decontaminated of bone to potentially minimize the reported cell viability concerns. Therefore, a concentration of 2% chlorhexidine was proposed in the current study.

The decontamination process may have a negative effects on bon vitality or healing of bone during regeneration owing to contact time alongside with concentration of the antimicrobials, In order to achieve more effective decontamination, Sivolella et al.,[30] left bone particles in contact with antibiotic solution for 10 minutes, on the other hand, Tezulas et al.,[31] compared chrohexidine and clindamycin with only a 3 minutes contact time with the bone graft and suggested that both of the agents effectively decontaminated collected bone particles. Though, none of the former studies investigated bone vitality or healing capacity of the decontaminated bone particles. As the main concern of this study was to evaluate the effect of the different antimicrobials agents on the cellular vitality, the agent-graft contact time in this article was fixed to five minutes which is more than that of Tezulas et al.,[31] but not as much as what Sivolella et al.,[30] did.

Ozone therapy can provoke several responses on the biological aspect of bone regeneration, such as improvement of the blood circulation in ischemic tissue by increasing oxygen delivery and enhancement of the general metabolism via mild activation of the immune system and upregulation of cellular antioxidant enzymes and growth factors.[32] Huth et al.,[33] compared the effect of aqueous form of ozone, as a possible antiseptic agent, with different types of antimicrobials such as chlorhexidine, hydrogen peroxide and metronidazole, they reported that ozone showed a significantly less cytotoxicity than those well-known antimicrobials. The histological findings of the current study revealed similar observations with an obvious enhancement in new bone formation and a marked reduction in concentration of inflammatory cells of the ozone treated specimens. Moreover, Bone densitometry measurements showed an understandable increase in mineral bone density and concentration within the ozone group, which may be considered as a additional confirmatory verdict to the distinct biocompatibility of ozone as a potential decontaminant for bone grafts.

CONCLUSION

Chlorhexidine, clindamycin and ozone gel did not interfere with potential new bone formation, However, they showed variable influences on the osteogenic activity of the autogenous grafts, taking into consideration, the limitations of the concentration of each agent and the restricted contact time that have been used in the current study. Moreover, the histomorphometric and bone densitometric analyses of the ozone treated group respectively showed a distinguished preservation of osteoblastic vitality alongside with an obvious increase in bone mineral density, which may indicate a superior biocompatibility of the ozone compared to the other antimicrobials.

REFERENCES

1. Goldberg V and Stevenson S. Natural history of autografts and allografts. Clin Orthop Rel Res. 1987; 22:7-16.
2. Ellis E. Surgical reconstruction of defects of the jaws. In: Peterson LJ, Ellis E III, Hupp JR, editors: Contemporary Oral and Maxillofacial Surgery. 3rd ed. St. Louis (MO): Mosby–Year Book; 1998. p. 680.
3. Finkemeier CG. Bone-grafting and bone-graft substitutes. J Bone Joint Surg [Am]. 2002; 84:454-455.
4. Bostrom MPG, Yang X and Koutras IK. Biologics in bone healing. Curr Opin Orthop. 2000; 11:403-412.
5. Verdugo F, Castillo A, Moragues MD and Ponton J. Bone microbial contamination influences autogenous grafting in sinus augmentation. J Periodontol. 2009; 80:1355-1364.
6. Hooe W and Steinberg B. Management of contaminated bone grafts: An experimental in vitro study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1996; 82:34-37.
7. Verdugo F, Saez-Roson A, Uribarri A, Martinez-Conde R, Jonathan Cabezas-Olcoz J, Moragues M, and Ponto J. Bone Microbial Decontamination Agents in Osseous Grafting: An In Vitro Study With Fresh Human Explants. J Periodontol. 2011; 82(6), 863-871.
8. Yarboro SR, Baum EJ and Dahners LE. Locally Administered Antibiotics for Prophylaxis Against Surgical Wound Infection. An in Vivo Study. J Bone Joint Surg (Am). 2007; 89:929–933
9. Kürkçü M, Öz IA, Köksal F, Benlidayi ME and Günesli A. Microbial analysis of the autogenous bone collected by bone filter during oral surgery: a clinical study. J Oral Maxillofac Surg. 2005; 63:1593-1598.
10. Zalavras CG, Patzakis MJ and Holtom P. Local antibiotic therapy in the treatment of open fractures and osteomyelitis. Clin Orthop. 2004; 427:86–93
11. Burd T, Conroy BP, Meyer SC and Allen WC. The effects of chlorhexidine irrigation solution on contaminated bone-tendon allografts. Am J Sports Med. 2000; 28:241-244.
12. Bocci VA. Scientific and medical aspects of ozone therapy. State of the art. Arch Med Res. 2006; 37:425-435.
13. Polydorou O, Halili A, Wittmer A, Pelz K and Hahn P. The antibacterial effect of ozone after 2 months of in vitro evaluation. Clin Oral Investig. 2012; 16: 545-550.
14. Baysan A, Whiley RA and Lynch E. Antimicrobial effect of a novel ozone- generating device on microorganisms associated with primary root carious lesions in vitro. Caries Res. 2000; 34:498-501.
15. Neyt J, Buckwalter JA and Carroll N. Use of animal models in musculoskeletal research. Iowa Orthop J. 1998; 18: 118-123.
16. Castaneda S, Largo R, Calvo E, Rodriguez-Salvanes F, Marcos ME, Diaz-CurielM and Herrero-Beaumont G. Bone mineral measurements of subchondral and trabecular bone in healthy and osteporotic rabbits. Skelet Radiol. 2006; 35: 34-41.
17. Li Y, Chen S, Li L, Qin L, Wang X and Lai Y. Bone defect animal models for testing efficacy of bone substitute biomaterials. J Orthop Transl. 2015; 3(3):95-104.
18. Moura CG, Betoni WJ and Dechichi P. Histomorphometric analysis of bone graft storage in physiologic solution [in Portuguese]. Cienc Odontol Bras. 2005; 8(1):23–27.
19. Messora MR, Nagata MJ, Mariano RC, Dornelles RC, Bomfim SR, Fucini SE. Bone healing in critical-size defects treated with platelet-rich plasma: a histologic and histometric study in rat calvaria. J Periodontal Res. 2008; 43:217–223.
20. Frame JW. A conventional animal model for testing bone substitute materials. J Oral Surg 1980; 38:176– 180.
21. Schmitz JP and Hollinger JO. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res. 1986; 205:299–308.
22. Fearon JA. A magnetic resonance imaging investigation of potential subclinical complications after in situ cranial bone graft harvest. Plastic Reconstr Surg. 2000; 56:1935–1939.

23. Jackson IT, Helden G and Marx R. Skull bone grafts in maxillofacial and craniofacial surgery. J Oral Maxillofac Surg. 1986; 44:949–955.

24. Tezulas E and Dilek OC. Decontamination of autogenous bone grafts collected from dental implant sites via osteotomy: a review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008; 106:679-84.

25. Veksler AE, Kayrouz GA and Newman MG. Reduction of salivary bacteria by pre-procedural rinses with chlorhexidine 0.12%. J Periodontol.1991; 62:649-51.

26. Mader JT, Wang J and Calhoun JH. Antibiotic therapy for musculoskeletal infections. J bone Joint Surg Am. 2001; 83A: 1878-1890.

27. Naal FD, Salzmann GM, Von Knoch F, Tuebel J, Diehl P, Gradinger R, and Schauwecker J. The effects of clindamycin on human osteoblast in vitro. Arch Orthop Trauma Surg. 2008; 128(3): 317-323

28. Mazurek MT, Pennington SE, Mills WJ. Successful reimplantation of a large segment of femoral shaft in a type IIIA open femur fracture: a case report. J Orthop Trauma. 2003;17:295–302.

29. Goebel ME, Drez DJ Jr, Heck SB and Stoma MK. Contaminated rabbit patellar tendon grafts. In vivo analysis of disinfecting methods. Am J Sports Med.1994; 22:378–387.

30. Sivolella S, Berengo M, Scarin M, Mella F and Martinelli F. Autogenous particulate bone collected with a piezo-electrical surgical device and bone trap: a microbiological and histomorphometrical study. Arch Oral Biol 2006; 51:883-91.

31. Tezulas E, Dilek O, Topcuoglu N, and Kulekci G. Decontamination of autogenous bone grafts collected during dental implant site preparation: A pilot study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009; 107:656-660.

32. Sagai M and Bocci V. Mechanism of action involved in ozone therapy: Is healing induced via a mild oxidative stress? Med Gas Res. 2011; 20:1-29.

33. Huth KC, Jackob FM, Saugel B, Cappello C, Paschos E, Hollweck R, Hickel R and Brand K. Effect of ozone on oral cells compared with established antimicrobials. Eur J Oral Sci. 2006; 114:435-440.