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EVALUATION OF PARTICULATE AUTOGENOUS BONE GRAFT HARVESTED BY AUTO CHIP MAKER VERSUS MILLING OF BLOCK BONE GRAFT (EXPERIMENTAL STUDY WITH BIOCHEMICAL AND GENETIC ASSESSMENT)

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ABSTRACT

Background: The mode of harvesting autogenous bone grafts can affect the quality of harvested bone and viability. The present study was carried out to evaluate cellular viability, differentiation and proliferative power in autogenous bone graft harvested by auto chip maker versus milled bone block.

Materials and Methods: 16 bone samples were harvested. In group I (n=8): auto chip maker (ACM) was used while in group II (n=8): bone blocks were harvested then milled. Flow cytometry, alkaline phosphatase activity, MTT assay, Alizarin Red Staining, and Real-Time PCR were performed.

Results: Group I had a significantly higher cell viability. MTT assay revealed a significant increase in cell proliferation in the ACM group. Both groups showed decrease in (RUNX2) gene expression over four days and increase in type 1 collagen alpha 1 and osteocalcin gene expression at 4th evaluation day with a statistical significant higher level of both markers in the ACM group.

Conclusion: ACM group showed better osteoblasts viability, proliferation, differentiation, and calcified tissue formation.

Keywords: Auto chip Maker, Bone mill, Osteoblast viability, Osteocalcin.

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INTRODUCTION

The gold standard of bone grafts is the autogenous bone due to its osteogenic (osteoprogenitor cells), osteoinductive (bone morphogenetic proteins), osteoconductive (scaffold) characteristics, and other growth factors. [1] Particulate bone graft permits better cellular attachment, cell migration, nutrient diffusion, and blood vessel ingrowth during the bone repair process. Moreover, the total surface area of particles is larger than that of a block graft. Consequently, osteoclastic activity is facilitated, resulting in enhanced resorption and regeneration. **(2,3)** Several studies have pointed to the fact that the harvesting technique has a significant influence on the viability of bone cells within the autografts **(4,5,6)** but to the best of our knowledge few data are available regarding how the harvesting technique affects the graft characteristics.

Aim of the study

The present study was carried out to evaluate osteoblasts viability, differentiative and proliferative power in particulate autogenous bone graft harvested by two different techniques; auto chip maker (ACM) versus milling of a bone block. The evaluation was based on biochemical and genetic assessment.

MATERIALS AND METHODS

Surgeries and Care of the study animals were performed at the Surgical unit, Faculty of Veterinary Medicine, Cairo University, Egypt. Power analysis of 16 samples (8 samples in each group) had 80% power assuming that the effect size was 1.51 using a two group t-test with a 5% two-sided significance level. Software used for sample size calculation was G power version 3.0.1 .[7] This was based on a study which evaluated cell viability in milled autogenous bone grafts. [5]

Harvesting particulate bone in group I was performed using a cylindrical trephine-like drill (Ø: 4 mm) with a hollow part in the center and a stopper to limit the depth of drill penetration up to 4 mm (Auto Chip Maker (ACM), Neobiotech, Seoul, Korea). While For Group II, trephine bur with an inner diameter of 5 mm (MCTBIO, Poland), chisel and mallet were used to harvest bone block that was then crushed to particulate bone by bone mill (BM) *(Helmut zepf, Germany)*.

Bone samples were obtained from male adult mongrel dogs (1-2 years old). The dogs were sedated by i.v. Xylazine HCl 2% at a dose of 1mg/kg (Xylaject 2%®, ADWIA, Egypt). Then, general anesthesia was induced using i.v. Ketamine HCl at a dose of 10 mg/kg (Alfasan®, International B.V. , Holland) and maintained by i.v. Thiopental sodium at a dose of 25mg/kg (Thiopental sodium®, EIPICO, Egypt). A flap was elevated at the lateral portion of the mandible using surgical scalpel and bone sample was collected. For GI, ACM was used at a speed and torque of 500 rpm 35Ncm. For GII, trephine bur was used at 2,000 rpm, 50Ncm. Bone samples were immediately placed in Alpha modified eagle medium (α-MEM) supplemented with 1% antibiotics-antimycotic (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin) then transported to the cell culture unit immediately. Blindness was ensured for the laboratory analysis as the ampoules containing the samples were coded with numbers then sent to the lab. Thus, the author responsible for biochemical and genetic analysis in the lab was unable to distinguish between samples of each study group. The surgical site was rinsed and sutured using absorbable #0 polyglactin suture (Vicryl®, India) in simple interrupted pattern. Postoperative ceftriaxone IM was administrated (5 days 30mg/kg once daily) and diclofenac sodium IM (3 days 1.1mg/kg twice daily).

Cell isolation and culture

The particulate bone samples were transferred to sterile tubes to be rinsed with phosphate-buffered saline (PBS). After two times of washing, osteoblasts were isolated using a mixture of

collagenase/trypsin (3:1), where minced tissue was digested twice every 15 mins in a mixture of collagenase/trypsin. Viability of the isolated cells was assessed by flow cytometry. After that, the cells were placed in the culture flasks for two weeks of culturing, 75 cm^3 culture flasks contained 3mL of α-MEM supplemented with antibiotics (100 mg/mL penicillin G; 50 mg/mL gentamycin, 0.68mM l-glutamine, and 15% FBS). The flasks were incubated in 5% CO_2 at 37ºC and the medium was changed twice daily with no osteogenic factors added. During the cell culture process, the seeded cells were evaluated under inverted microscope to assess activity and microbial contamination. After two weeks, the seeded osteoblasts reached a confluence of 85 %. The seeded cells were detached from the bottom of culture flasks using EDTA-trypsin solution [0.25% trypsin, 0.1% glucose, PBS (pH 7.8)] . For cell counting, 20 µL of cell suspension was mixed with 20 µL of Trypan Blue and transferred to a hemocytometer chamber, and the viable cells were counted under a microscope. The osteoblasts in each flask were transferred to 96-well culture plates for analysis over four consecutive days. A total of $1x10^3$cell harvested from $75cm^3$ of culture flask were transferred to each per well of 96-well plate. Plates were used for alkaline phosphatase (ALP), cell proliferation assays (MTT), quantitative real-time polymerase chain reaction (qRT-PCR) experiments, and Alizarin Red staining.

Biochemical Analysis: ***Osteoblast viability Assay using Flow Cytometry:*** Analysis of cell viability was done on the day of the harvesting. Flow cytometers utilize lasers as light sources to produce both scattered and fluorescent light signals. If the cell population was negative Annexin V/ negative PI; it indicated healthy cells. Cell viability of both groups was analyzed with FACS using a flow cytometer instrument (BD Biosciences, San Jose, CA, USA). [8] ***Alkaline phosphatase (ALP) assay using ELISA technique:*** Seeded cells were rinsed with PBS to wash off the overlaying culture medium. Then, 500 µL of the assay buffer solution available in the ELISA kit was added to each well according to the manufacturer's instructions (Wuhan Fine Biotech, China). After centrifugation (13,000 rpm for 10 minutes), the supernatant was transferred to a 96-well plate and the protocol was followed. The resultant color change, having a direct correlation with the alkaline phosphatase (ALP) activity of GI and GII isolated cells, was measured by ELISA reader at absorbance 450 nm wavelength and reported as optical density (OD). [9]

Alizarin Red staining assay: It was used to measure quantitatively the calcium-containing osteoblasts in cell culture wells using 96-well plates. Mineralization analysis was performed after fluorescence labeling of the cells with Alizarin Red staining post-seeding over 4^{th} days. The stained cells detected more absorbance using (BMG Labtech, Germany).

Osteoblast proliferation (MTT assay): This was investigated post-seeding at an interval of 4 days. First, 20 µL MTT working solution was added to the medium and incubated 4 hours. The medium was then discarded, and 150 µL of dimethyl sulfoxide (DMSO) was added. The obtained solution was then transferred to a 96-well plate and the resultant color change (direct correlation with the metabolic activity) was measured by ELISA reader at absorbance (450) nm wavelength and reported as optical density (OD). The means and standard deviation were used to produce the cell growth curve after normalization . [10]

Genetic Analysis by qRT-PCR: Total RNA was extracted from the seeded osteoblasts of G I and GII using the Invitrogen RNA Purification kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Primer sequences used for amplification of the proposed genes were as follow: 5'-CAGACCAGCAGCACTCCATA-3' and 5'-CAGCGTCAACACCATCATTC-3' for Runt-related transcription factor 2 (Runx2),

5'-ACAGCCGCTTCACCTACAGT-3' and 5'-ATATCCATGCCGAATTCCTG-3' for Type I collagen, alpha1 (COL1α1), as well as 5'-AGCTGTGATGACAAGGACCC-3' and 5'-AGGGGTGAAATGGGCACTCT-3' for osteocalcin (OC). qRT-PCR was performed on DT-lite Real-Time PCR System (English/Russian system) using the MiScript SYBR Green PCR kit (Qiagen, Valencia, USA).

Statistical analysis:

Numerical data were presented as mean and standard deviation values. The data were tested for normality using the Shapiro-Wilk test. They were normally distributed so they were analyzed using an independent t-test for intergroup comparisons and repeated measures ANOVA followed by Bonferroni post hoc test for intragroup comparisons. Significance level was set at p≤0.05 within all tests. Statistical analysis was performed with R statistical analysis software version 4.1.2 for Windows.

RESULTS

Biochemical findings

Osteoblasts viability: Flow cytometry analysis showed that Group I (ACM) had a significantly higher (95.88±1.53) mean value for cell viability (%) than group II (BM) (59.36±4.44) (P<0.001).

Alkaline phosphatase (ALP) activity*:** Mean and standard deviation (SD) values for normalized alkaline phosphatase (ALP) levels for both groups are presented in **(Table 1)**. At day 0, both groups had the same mean value (1.00±0.05) while at 1st, 2nd and 3rd days, group I (ACM) exhibited a significantly higher mean value of ALP activity than group II (BM) (P< 0.05). ***Alizarin Red staining (ARS): The absorbance values (%) for both groups are presented in (**Table 1).** At day 0, both groups had the same mean value (1.00±0.05) while at 1st, 2nd and 3rd days, group I (ACM) exhibited a significantly higher mean value of Alizarin Red staining than group II (BM) (P< 0.05). **Osteoblast proliferation:** Mean & standard deviation values for relative proliferation rate (%) for both groups are presented in (**Table 2).** At day 0, both groups had same mean value (100.00±5.00) while at 1st, 2nd & 3rd day, group I (ACM) exhibited a significantly higher mean value of osteoblast proliferation than group II (P< 0.05).

Genetic findings:

Runt-related transcription factor2 ***(Runx2):*** Mean and standard deviation values for (Runx2) relative mRNA expression for both groups are presented in (**Table 2**). At 1st day, both groups had the same mean value (1.00±0.05) . At 2nd and 3rd days, group I showed non-significant higher mean

TABLE (1) Mean and standard deviation values for ALP and ARS (%) levels

Time	ALP levels		P-value	Relative ARS (%)		P-value
	Group I	Group II		Group I	Group II	
Day 0	1.00±0.05	1.00±0.05	1ns	100.00±5.00	100.00±5.00	1ns
Day1	2.02±0.10	1.75±0.08	<0.001*	185.65±9.28	132.06±6.60	<0.001*
Day2	2.12± 0.10	1.76± 0.08	<0.001*	190.41±9.52	155.85±7.70	<0.001*
Day3	2.66± 0.13	1.83±0.09	<0.001*	309.96±15.49	206.17±10.30	<0.001*
Day4	3.50 ±0.17	2.17±0.10	<0.001*	504.13±25.20	208.80±10.44	<0.001*

****: significant (P < 0.05); ns: non-significant (P>0.05). ACM: auto chip maker; BM: Bone mill***

value than group II (P>0.05). At 4th day, group II (BM) exhibited a significantly higher mean value than group I (ACM) (P< 0.05). **Type 1 collagen alpha1 (COL1α1) :** Mean and standard deviation values for (COL1α 1) type 1 collagen relative mRNA expression for both groups are presented in (**Table 3).** At day 1, both groups had the same mean value (1.00±0.05) while at 2nd, 3rd and 4th days, group I (ACM) exhibited a significantly higher mean value of COL1α1than group II (BM) (P< 0.05). **Osteocalcin (OC)**: Mean and standard deviation values for OC relative mRNA expression for both groups are presented in (**Table 3).** At day 1, both groups had the same mean value (1.00±0.05) while at 2nd, 3rd and 4th days, group I (ACM) exhibited a significantly higher mean value of OC than group II (BM) (P< 0.05).

TABLE (2) Mean and standard deviation for relative proliferation rate & Runx2 relative mRNA expression

Time	Relative proliferation rate (%)		P-value	(Runx2) relative mRNA expression		P-value
	Group I	Group II		Group I	Group II	
Day 0	100.00±5.00	100.00±5.00	1ns			
Day1	193.17±9.60	145.69±7.30	<0.001*	1.00±0.05	1.00±0.05	1ns
Day2	202.22±10.11	160.00±8.00	<0.001*	1.20±0.06	1.13±0.06	0.071ns
Day3	250.47±12.50	174.16±8.70	<0.001*	0.91±0.04	0.94±0.05	0.278ns
Day4	296.190±14.00	188.05±9.40	<0.001*	0.77±0.03	0.85±0.04	0.003*

: significant (P < 0.05). ns: non-significant (P>0.05). ACM: auto chip maker; BM: Bone mill

TABLE (3) Mean and standard deviation values for COL1α 1 & OC relative mRNA expression

Time	COL1α1 relative mRNA expression		P-value	OC) relative mRNA expression		P-value
	Group I	Group II		Group I	Group II	
Day1	1.00±0.05	1.00±0.05	1ns	1.00± 0.05	1.00± 0.05	1ns
Day2	3.40±0.17	1.70±0.08	<0.001*	7.20±0.36	6.70±0.33	0.031*
Day3	5.30±0.26	2.50±0.12	<0.001*	11.50±0.57	9.30±0.46	<0.001*
Day4	9.50±0.47	4.00±0.20	<0.001*	15.70±0.78	10.00±0.50	<0.001*

: significant (P < 0.05). ns: non-significant (P>0.05).

DISCUSSION

Mongrel dogs were selected as an animal model in the current study due to their similar bone composition and metabolism as humans . [11, 12] they were less susceptible to genetic diseases than other breeds of dogs. [13] 1-2 years old to exclude age or systemic related diseases and to facilitate the healing of the soft and hard tissues . After harvesting the grafts, the samples were maintained in α-MEM supplemented with penicillin, gentamycin, and FBS, which are not harmful to the cells when used at certain concentrations as mentioned before .[14,15] It is important to notice that the seeding period in the present study was two weeks and analysis of

osteoblast differentiation was conducted 2 weeks post seeding differentiated bone tissue is formed after 2 weeks in culture, whereas mineralization of the tissue occurs after 3 weeks . [16] Regarding the analysis techniques used in the present study; Flow cytometry has become the methodology of choice for the quantitative analysis of cell viability, apoptosis, and necrosis. [4] ALP has an important role in mineralization and its expression coincides with the synthesis of COL1α1. Positive ALP activity indicates that survived osteoblasts are still vital after the harvesting procedure [17]. The MTT assay is a colorimetric assay for the assessment of cell proliferation by measuring oxidative-reductive mitochondrial enzymes. [18] qRT-PCR is a routine tool in molecular biology with a wide range of applications. Runx2 is considered crucial for osteogenic differentiation of progenitor cells by inducing the expression of osteoblast specific genes, such as ALP, OC, and COL1α1 . [19]

COL1α1 is an early marker of osteoblast differentiation and its expression is observed during transformation of osteoprogenitor cells to pre-osteoblasts. Induction of extracellular matrix (ECM) has a major activity on osteogenic cells that occurs due to the activity of COL1α1. [20, 21] OC is a small calcium-binding protein found predominantly in the ECM of bone and is considered a late marker for osteogenic differentiation and most specific marker. It's essential that the outgrowing cells in culture are mature osteoblasts to produce mineralized bone matrix. [17]

The ACM had a significantly higher percentage of cell viability (95.9%) than BM technique (59. 3%). This could be attributed to heat temperature generated during drilling force and its potential damage to bone cells. [22] Additionally, the degree of crushing is an important factor in bone viability and clinical predictability of autogenous bone grafts. [23] Intra group statistical analysis of ALP activity revealed significant increase by time in both groups. This indicates that the osteoblasts survive and have activity. [24] Intergroup statistical analysis revealed better ALP activity in the ACM group.

This is in agreement with the results of **Liang et al. (2017)** [25], who found a significantly higher ALP activity in the low-speed technique. There was a significant increase in MTT osteoblast proliferation in both groups by time in accordance to previous studies that used the trephination technique of harvesting bone and revealed a significant increase in proliferation by time [18, 25]. Concerning MTT intergroup comparison, significant higher values were obtained in the ACM group over the four consecutive assessments. These could be due to the differences in the speed and torque of bur used in each technique. The ACM bur is less thermal and mechanically traumatic to harvested tissue, which may have a positive effect on viability and cell proliferation. Results from the analysis of the expression of osteoblast markers support the findings regarding the assessment of the cell viability, activity, and cell proliferation. The results revealed that the ACM group demonstrated a significantly higher percentage of cell differentiation compared to the BM group. In both groups, runt-transcription factor Runx2 gene expression showed a statistically significant decrease from day 1 to day 4. This results is in accordance with previous studies.[26, 27] Inter group statistical analysis of COL1α1 and OC genes revealed significantly higher levels of both genes in the ACM technique compared to the BM technique. Taking into consideration the role of OC and Col1α in osteogenic differentiation, this means that the ACM technique results in higher cell differentiation and viability over time than BM technique. These is in accordance to a study which demonstrated that particulate bone obtained by low-speed drilling has superior biological properties. [28] Alizarin Red staining test demonstrated that the ACM group revealed a significantly higher percentage of mineralized tissue formation compared to the BM group. The calcified materials in the specimens

absorb the dye and appear red under the microscope. Observing the calcified materials produced by the transplanted cells means these cells are viable and functional. [29]

CONCLUSIONS

Particulate autogenous bone harvested by ACM drill showed better osteoblasts viability, proliferation, differentiation, and calcified tissue formation.

Declaration of conflicting interests: All authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics statement: This study was approved by the research ethics committee at faculty of dentistry, Ain Shams University (854-2020), Egypt.

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