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Regenerative Potential of Bone Marrow and Breast Milk derived Mesenchymal Stem Cells Compared to Calcium Hydroxide in Direct Pulp Capping

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Abstract:

Purpose: This study aimed to compare the regenerative potential of bone marrow derived mesenchymal stem cells (BM-MSCS) and breast milk derived mesenchymal stem cells (BrM-MSCS) as pulp capping materials versus to calcium hydroxide (CaOH) at different time intervals.

Materials and Methods: 66 middle aged female rabbits were divided into 3 groups according to the pulp capping material (n=22) each group was subdivided into 2 groups according to the assessment time; at 2 weeks and 4 weeks. Group I: rabbits were treated with calcium hydroxide as control. Group II: rabbits were treated with bone marrow mesenchymal stem cells loaded on fibrin gel. Group III: rabbits were treated with breast milk derived mesenchymal stem cells loaded on fibrin gel. Histological examination was performed using light microscope to assess the effect of different pulp capping materials on pulpal regeneration at different time intervals. Data was collected, tabulated and statistically analysed.

Results: At two weeks as well as four weeks time intervals, there was a statistically significant difference between different pulp capping materials in percentage of rabbits numbers with different pulpal regeneration. There was a statistical significant increase in percentage of rabbits numbers with complete pulpal regeneration treated with stem cells groups.

Conclusion: BM-MSCs and BrM-MSCs loaded on fibrin gel were superior to calcium hydroxide in healing of pulp injury at different time intervals. Breast milk derived mesenchmal stem cells has the highest regeneration potential of pulpal tissue. Overall regeneration potential was better achieved at 4 weeks.

Introduction

The vitality of the pulp is crucial to the dental health. Maintaining the vitality of pulp tissues that have been exposed for any cause is the primary objective of vital pulp therapy [1].Trauma, dental cavities, and tooth malformation can all result in pulpal exposure. The pulp's vitality is maintained by the pulp capping agent's activity and the remnant coronal pulp's capacity to mend. Direct pulp capping is a useful technique to preserve vital pulp tissues when pulp exposure occurs. This therapy promotes reparative dentin growth. The development of dentin then takes place [2]. The usual substance for preserving pulp vitality was pulp capping, such as calcium hydroxide [3]. However, it has certain disadvantages, including inadequate dentin bonding, material resorption, and mechanical instability. Therefore, in the long run, it is not effective in preventing micro leakage [4].The drawbacks of using calcium hydroxide as a pulp capping material led to the adoption of alternatives such "mesenchymal stem cells," which are involved in the regeneration of pulpal tissue as well as the formation of hard tissue after installation.

Mesenchymal stem cells (MSCs) exhibit numerous attributes, including self-renewal, multipotency, and flexibility, that render them a prospective candidate for use in pulp capping. As MSCs can increase in number and differentiate to pulp tissues and many other types of cells as osteoblasts, chondrocytes, adipocytes, cardio-cytes, neural cells and hematopoietic cells according to the environment of application. MSCs produced from bone marrow are an excellent model because they can develop into many mesodermal lineages, which allows them to partially replace the damaged cells [5]. Although BM-MSCs is currently very important in regenerative uses, Breast milk (BrM-MSCs) showed more plasticty and form all three germinal layers [6], besides they are non-invasively accessed and non tumorgenic , providing a better and safer treatment option [7].

After pulp capping, the degree of pulpal regeneration was measured over different time intervals in a previous study, in which the regeneration of pulpal tissue and dentin bridge increased by time.[8] All these trials encourage us to use MSCs directly for dental pulp capping to get higher success rate in shorter time. Comparing the regeneration capacity of various stem cell sources in critical pulp therapy, however, is a lack luster area of research. This study aims to examine bone marrow and breast milk-derived mesenchymal stem cells' and calcium hydroxide's regeneration potential in direct pulp capping at different time intervals. The current study tested two null hypotheses: 1) There would be no difference in pulpal regeneration using different materials. 2) There would be no difference in pulpal regeneration between different time intervals.

OBJECTIVES:

To compare the regenerative potential of bone marrow derived mesenchymal stem cells (BM-MSCS) and breast milk derived mesenchymal stem cells (BrM-MSCS) as pulp capping materials versus to calcium hydroxide (CaOH) at different time intervals.

MATERIALS AND METHODS:

Study design

This trial was an animal study which was performed after the approval of legal ethical committee of Faculty of Dental Medicine for Girls Al-Azhar University. All animals were

maintained according to standard ethical guide lines of Institutional Animal Care and Use Committee. Approval of the Faculty of medicine AlAzhar University was obtained. Code:(REC-OP-24-01). In accordancewith ARRIVE guidelines

Rational**:** The rationale of this study is to compare different capping materials to a standard capping material which is calcium hydroxide.

The Experimental unit**:** individual animals were used in this study

Selection of experimental animals:

Sixty-six middle aged (1-2 years old) female rabbits Baladi Black type (2,500 and 3,500 g) were enrolled in this study. lactating rabbits were collected for breast milk stem cells group, non-lactating rabbits were collected for bone marrow stem cell group. 66 rabbits were divided into 3 groups each of 22 rabbits according to pulp capping material. Each group is then divided into 2 groups according to the time of evaluation of the degree of pulp healing after 2 and 4 weeks. Each rabbit had sound, non-carious, upper and lower molar teeth and healthy, non-inflamed gingivae. Rabbits with any systemic conditions such as papillomatosis, pneumonia, eye bulge, excessive thirst, urinary tract or bladder infection, convulsions, or excessive urine production were not allowed. This study was conducted according to the Institute Review Board Instruction of Care and Use of Laboratory Animals. The age of the rabbits at the start of the study was 1-2 years. Rabbits were fed complete pellets and water under a controlled temperature (28-30^{o}C) that is ideal for lactation.

Randomisation: No randomisation was done in this study.

Outcome measures: The outcome of this study was assessed by histological assessment.

Materials used:

Calcium hydroxide (Dycal; Dentsply, Caulk, Milford, DE, USA) was employed as a direct pulp capping material in group I (positive control).Mesenchymal stem cells (five times 106 cells/200 mg) generated from bone marrow were injected into the exposed area in group II. Group II and group III were put onto fibrin gel, and the exposed space in group III was filled with mesenchymal stem cells obtained from breast milk (5x106 cells/ 200mg).

1-Operative procedures:

A) Preparation of bone marrow mesenchymal stem cells:

Two intramuscular injections of ketamine (0.4 ml/kg) and xylazine (5 mg/kg) were used to anesthetize female rabbits. The dose of an anaesthetic solution used was calculated according tothe equation X= weight of each rabbit x dose of an anaesthetic solution/ 1000 [9]. After the animal skeleton was thoroughly cleaned with 70% ethanol, the surgical site was shaved using an animal clipper equipped with a size-40 blade. To create local hemostasis, 2% lidocaine was administered into the surgical site. Both femurs and tibias were removed, cut, and placed in sterile low glucose Dulbecco's Modified Eagle's Medium (DMEM 1.0 g/L glucose Lonza Bioproducts, Belgium) supplemented with 10% fetal bovine serum (FBS, Lonza Bioproducts, Belgium). After that, the femurs were split apart at both epiphyses using a bone cutter, and 10 ml syringes filled with DMEM-FBS were used to flush the contents into a 100 mm Petri dish that held 1 ml of heparin (heparin 2000 IU/0.2 ML). Next, a suspension of dispersed bone marrow cells was made.

The cell suspension was pipetted repeatedly to accomplish this. Gauge 18 needles were utilized to ensure adequate mixing, and a vortex was used to thoroughly mix the suspension. After that, centrifuged for ten minutes at 3000 RPM. Phosphate buffer saline (PBS) was used to wash and centrifuge the pellet twice in order to eliminate any residual fat or bone particles (Lonza Bioproducts, Belgium). Using PBS, the pellet was diluted [10].

Nucleated cells were extracted using a density gradient Ficoll/Paque (Lymphocyte Separation Medium 1.077, Lonza Bioproducts, Belgium). The same volume of Ficoll was gradually folded into 4 milliliters of diluted bone marrow. After that, the lymphocyte separation medium was spun for 20 minutes at 400 RPM in a cooling centrifuge at 20 ºC. Bone marrow mononuclear cell layer was collected from intermediate ring formed between two solutions (Bone marrow and Ficoll). This layer was centrifuged to get cell pellet, then washed by PBS to get rid of Ficoll traces then centrifuged for 5 minutes at 2000 RPM [10].

B) Preparation of breast milk mesenchymal stem cells:

Rabbit nipples were cleaned with soap before being handled with a cotton swab dampened with 70% alcohol for the pericarp region and the nipples themselves. Using a sterile syringe, milk was manually extracted from female rabbits and decanted by applying pressure to the mammary gland. It was a painless process which took within 1-2 minutes " a process imitating natural suckling [11]. 20 ml of milk was obtained in 1-2 minutes. Breast milk was diluted 1:2 using Dulbecco's modified Eagle's medium (DMEM) from Lonza Bioproducts, Verviers, Belgium. The mixture was then centrifuged at 400g for 10 minutes. PBS (phosphate-buffered saline) was used twice to wash the cell pellet (Lonza Bioproducts) [10].

C) Assessment of stem cells viability:

It was done using light microscope.The prepared stem cells were stained by trypan blue dye. While assessmentof size, shape and growth of stem cells was done using inverted image microscope [10].

D) Cell culture:

The final cell pellet was suspended in the entire culture media, which included high glucose DMEM (4.5 g/L glucose with l-glutamine, Lonza Bioproducts), 1% Penicillin Streptomycin Amphotericin B mixture (10 IU/10 IU/25 mg), and 10% fetal bovine serum (FBS). The cells were grown at 37 °C in 5% humidified CO2 in a CO2 incubator (Heraeus, Hanau, Germany) at a concentration of 5 × 106 per 25-cm2 culture flask. Changing the media every 48 hours for a duration of 12–14 days was the primary culture. When cells are 80% confluent, trypsin/ethylene diamine tetra-acetic acid (EDTA) is employed to passage them. Fourth-passage (p4) cells were used in the tests[10].

E) Characterization of stem cells:

An inverted microscope (Olympus IX51, Germany) was used to observe the fusiform, star-like, or spindle-shaped morphological appearance of MSCs in culture. A flow cytometer was used to quantify the expression of the MSC surface markers CD105 and CD34 [10].

F) Preparation of scaffold:

Two rabbits were given 20 milliliters of blood via heparinized falcon tubes. To properly separate the upper serum portion from the clot-forming portion of the blood, the whole blood samples were kept at 4oC for nine hours. Using a disposable pipette, the supernatant was carefully collected and placed into a different tube. The transferred supernatant was frozen at 20°C for eighteen hours in order to precipitate the fibrinogen later. For four hours, the frozen serum was gradually thawed at 4^{o}C. In order to obtain the fibrinogen pellet, the serum was finally centrifuged for 15 minutes at 400 rpm, after which the supernatant was discarded[10].

G) Pulp capping procedures:

Anaesthesia:

Rabbits were restrained securely, muscle mass to be injected was isolated then injection of the hind limb muscle mass was done using 900 ml ketamine and 300 ml xylazine [12]. A 0.12% chlorohexidine disinfectant (Ultra Dent, USA) was used to clean the oral cavity. Cotton rolls and gauze swabs were used to create an intraoral dry field [13].

Cavity preparation:

The pulps of the lower first molars were visible at the occlusal surface. The cavity was prepared using a sterile carbide fissure bur (#245, Hager& Meisinger GmbH, Germany) that was placed on a slow-speed contra-angle and thoroughly misted with sterile water. The pin point pulp was then intentionally exposed by inserting a sterile, tiny (#016) round diamond bur (Hager& Meisinger GmbH, Germany) in the center of the pulpal floor. The bleeding was controlled with cotton moisture pellets moistened with sterile saline and gently compressed to produce proper hemostasis. To prevent any potential cross-contamination, a sterile bur was used to prepare each cavity[13].

Application of scaffold:

Fibrin gel that was prepared as discussed before was injected as a scaffold using 25-gauge needles into the prepared pulp exposure sites in all tested molars[14].

Application of capping materials:

Calcium hydroxide was manipulated according to the manufactures' instructions. The previously prepared BM-MSCs and Br M-MSCs according to the divided groups were injected using 25-gauge needles untill the area of exposure was completely covered [14].

1- Final restoration placement:

As a permanent restoration, self-cured glass ionomer filling (Medifil: promedica, IX fort LC A2, Germany) was placed in each cavity as soon as the capping procedure was completed. The glass ionomer was manipulated according to manufacturer instructions, applied over the capping material [15].

2- Post-operative procedures:

Every rabbit received the treatment recommended by the Canadian Committee of Animal treatment convention. They were fed a particular soft food and kept in separate cages [13]. After two and four weeks' period, animals were sacrificed and extraction of their molars was done, and then histological examination using light microscope was performed to evaluate the effect of

different capping materials.

3- Histological assessment:

Using lower molar forceps (Surgical Excel #85-002) extraction of the molar teeth and collection was done under a strict sterile condition [12].Three findings were recorded according to the histological assessments after direct pulp capping; no regeneration which indicates presence of inflammatory cells, no dentin bridge formation and formation of dead space. Incomplete regeneration which indicates moderate dentin bridge formation and presence of dead space. Complete pulpal regeneration which indicates complete healing, fully formed dentin bridge and mature odontoblasts were found.

4- Statistical Analysis:

To get the overall P-value for intra- and inter-group comparisons, the Kruskal-Wallis test was employed. Pairwise comparisons between groups (inter) and subgroups (intra) were performed using the Mann-Whitney test. The following formula was used to determine the change %:

$$Percentage\ of\ change\ (\%) = \frac{No.\ of\ cases\ at\ 2\ weeks - No.\ of\ cases\ at\ 4\ weeks}{No.\ of\ cases\ at\ 2\ weeks}$$

The negative value of the percentage change indicates that the number of cases increased from 2 weeks to 4 weeks, while the positive value of the percentage change means the number of cases decreased from 2 weeks to 4 weeks.

Statistical evaluation was performed using the SPSS statistical package (version 25, IBM Co. USA).

RESULTS:

Histological results:

Cell viability test results:

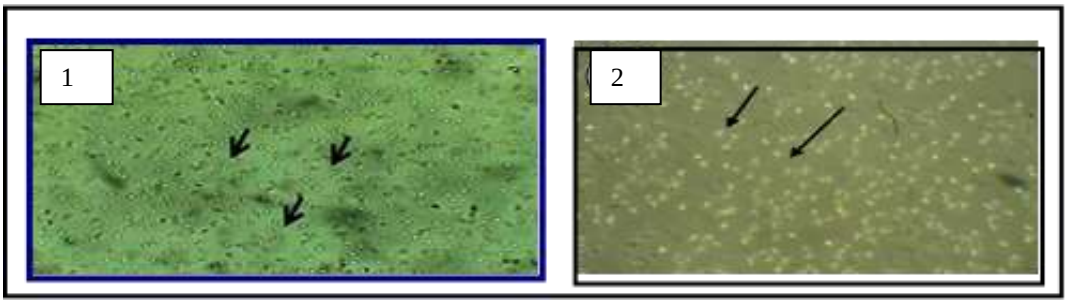


Figure (1): Bone marrow MSCs Black arrows indicate viable cells (transparent and shiny), (2) Breast-Milk MSCs Black arrows indicate viable cells.

Figures (1), (2) show results of light microscope images. Transparent and shiny cells are viable cells as represented by black arrows. While opaque cell are non viable cells.

Stem cell culture:

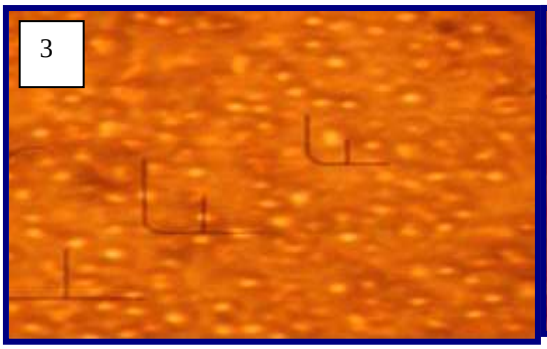


Figure (3): MSCs culture in the 1st day

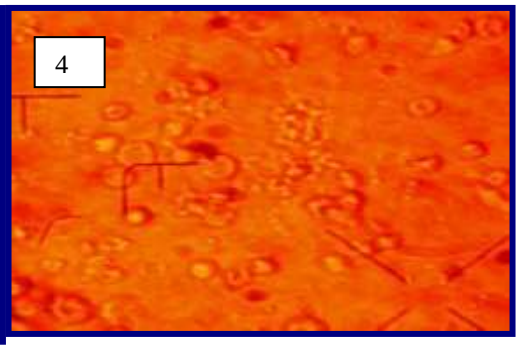


Figure (4): MSCs culture in the 1st week (black arrow indicate colony formation)

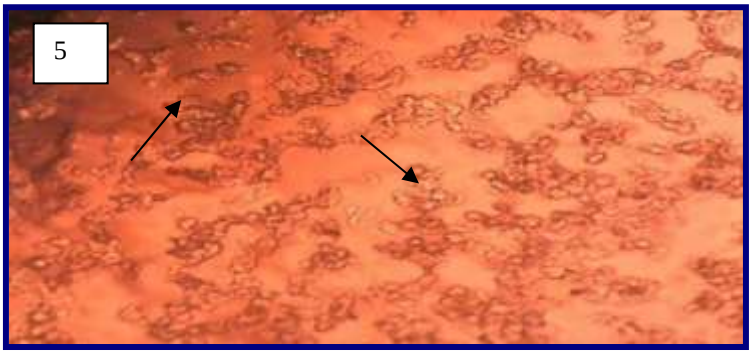
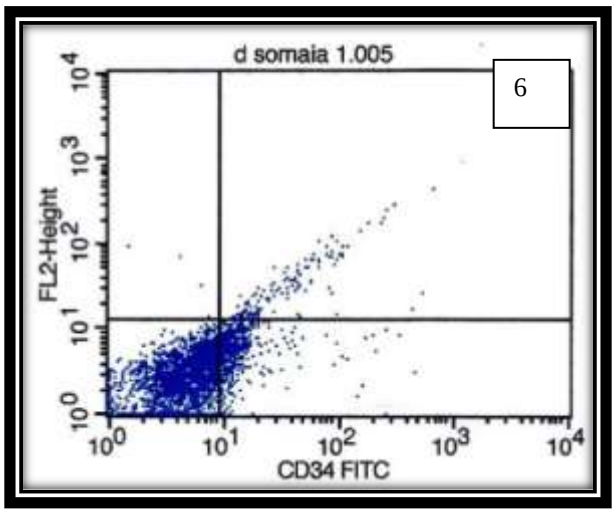
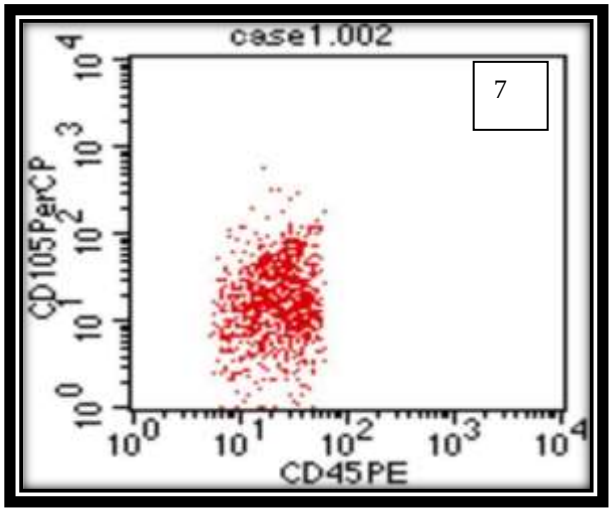


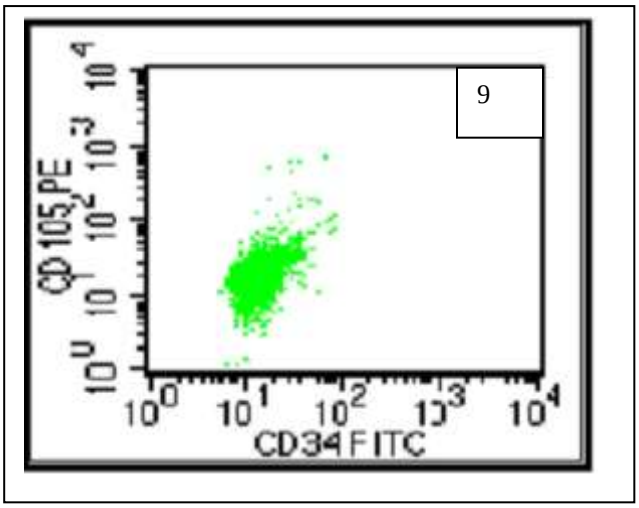
Figure (5): Mesenchymal stem cells culture in the 2nd weeks (Black arrows show viable stem cells colony)

Figures 3, 4 and 5 show results of inverted image microscope. Cells show variance in size, shape and growth (colony formation) at different time intervals at 1 day, 1 week and 2 weeks. Black arrows show colony formation.

Characterization of mesenchymal stem cells:







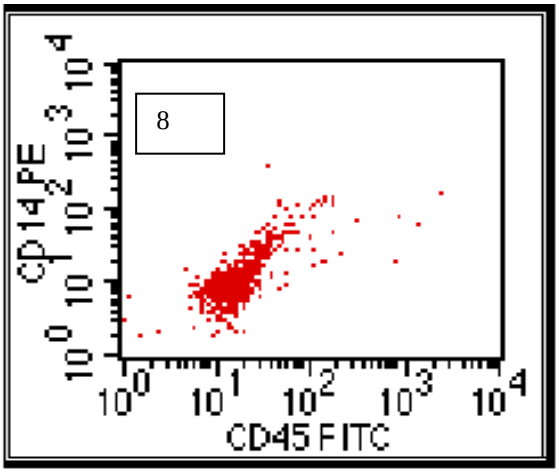


Figure (6): Fow cytometric pattern of BM-MSCs cells show negative expression of CD34.
Figure (7) BM-MSCs show negative expression of CD45 and positive expression of CD105.
Figure (8) BrM-MSCs show negative expression of CD34 and positive expression of CD105.
Figure (9) BrM-MSCs show negative expression of CD45 and positive expression of CD14.

Figures (6-7-8 and 9) show results of flow cytometric pattern. Bone marrow-derived mesenchymal stem cells (BM-MSCs) that express CD34 negatively (Fig. A). BM-MSCs with negative expression of CD45 and positive expression of CD105 (Fig. B). Breast milk mesenchymal stem cells (BrM-MSCs) with negative expression of CD34 and positive expression of CD105 (Fig. C). BrM-MSCs with negative expression of CD45 and positive expression of CD14 (Fig. D).

Histopathological observations:

At two weeks' time interval:

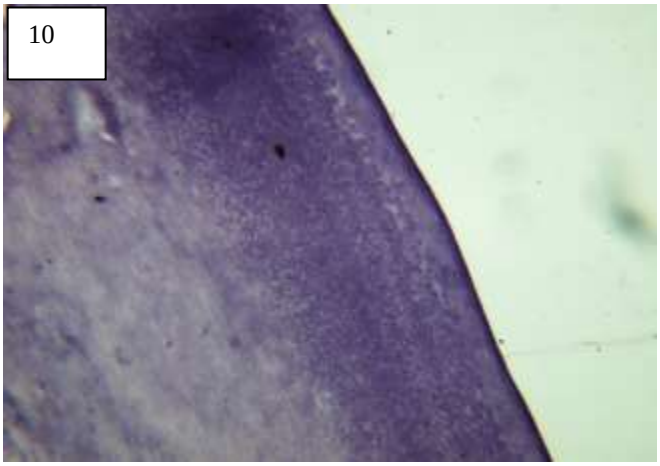


Fig (10): Light microscope photomicrograph for rabbit teeth pulp treated with calcium hydroxide showing mild regeneration of odontoblasts with incomplete formation of dentine bridge (black arrow) H&E X200.

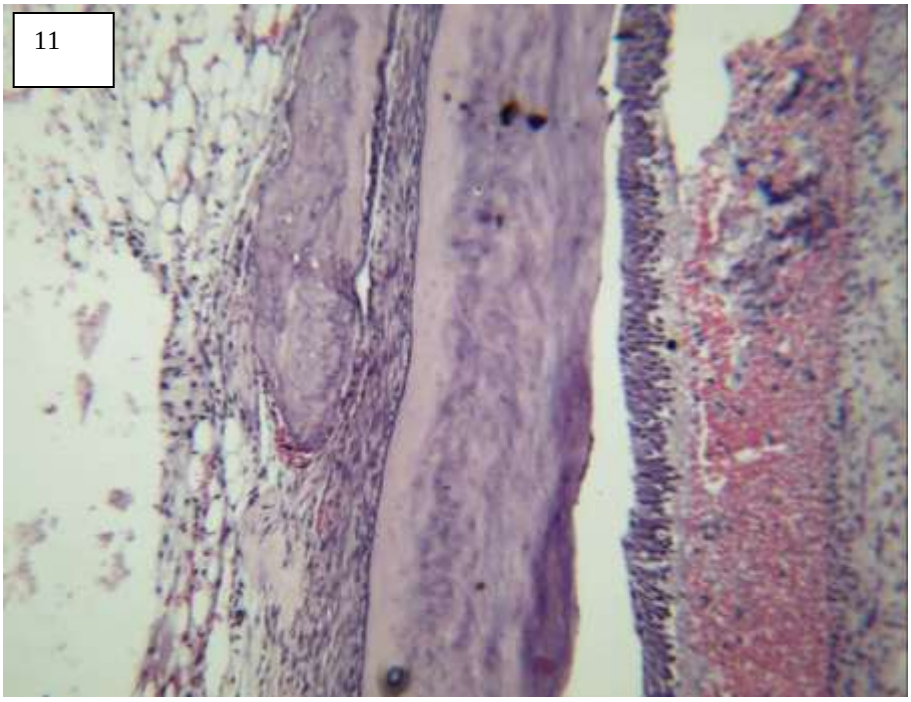


Fig (11): Light microscope a photomicrograph for rabbit teeth pulp treated with fibrin scaffold loaded with BM-MSCs showing regenerated odontoblasts (red arrow) and formation of dentine bridge (black arrow) H&E X200.

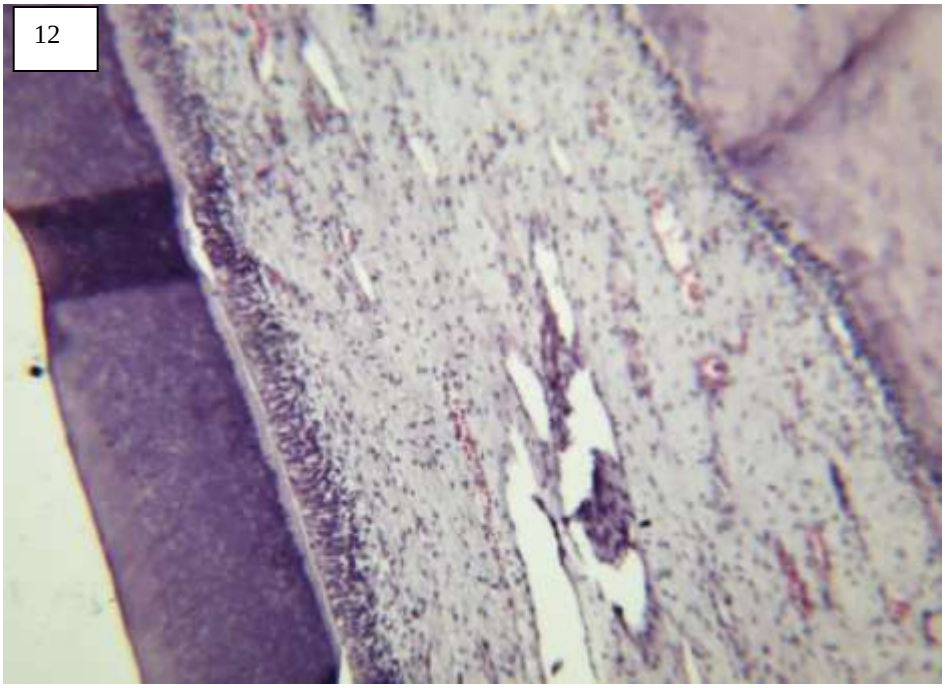
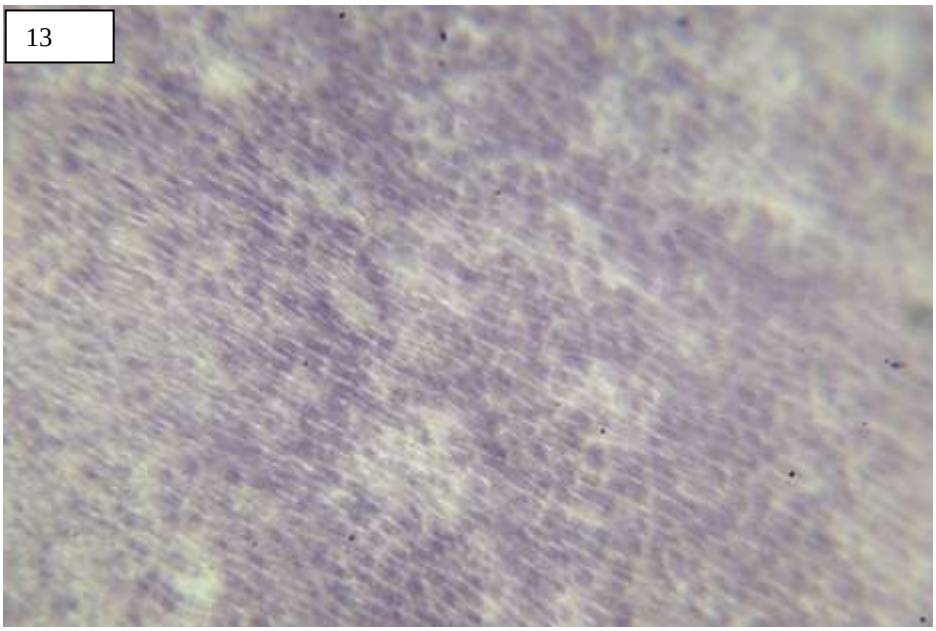


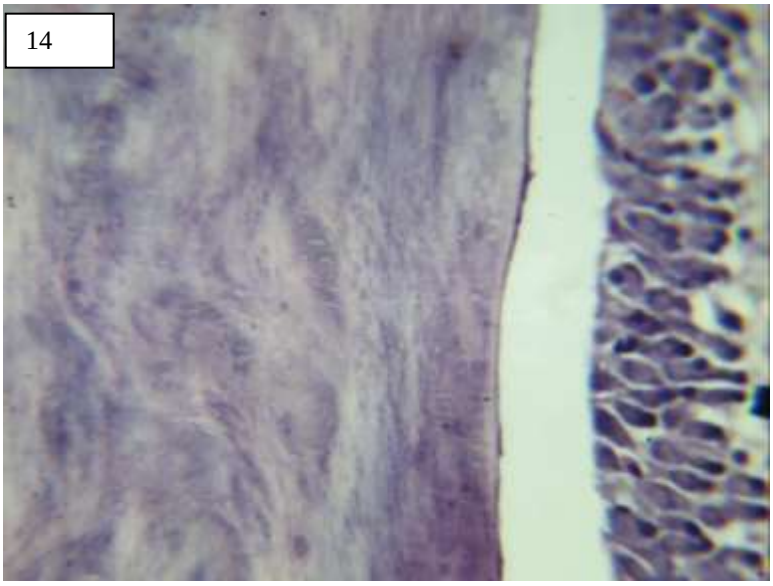
Fig (12): Light Microscope photomicrograph for rabbit teeth pulp treated with fibrin scaffold loaded with BrM-MSCs showing that the pulp cavity filled with regenerated cells (odontoblasts)(multipotent cells with high mitotic division) (red arrow) and formation of a layer of dentine bridge (black arrow)H&E X200.

Figures (10-11-12): Represent light microscope photo-micrographs of pulpal regeneration at 2 weeks time interval in rabbit teeth treated with different capping materials: (Fig. 10) for Calcium hydroxide treated group which showed a very thin layer of regenerated odontoblasts (orange arrow), there was newly formed blood capillaries and progenitor cells which indicate signs of pulp tissue healing.(Fig. 11) for bone marrow stem cells: it showed signs of regeneration with presence of moderate layer of formative cells "odontoblasts" (green arrow), to regenerate dentin, "Fibroblasts" to regenerate collagen fibers and newly formed blood vessels indicating moderate healing and regeneration of normal pulpal tissue . (Fig.12) for breast milk stem cells which showed a thick layer of formative cells "odontoblasts"(blue arrow), to regenerate dentin, "Fibroblasts" to regenerate collagen fibers and newly formed blood vessels indicating healing and regeneration of normal pulpal tissue.

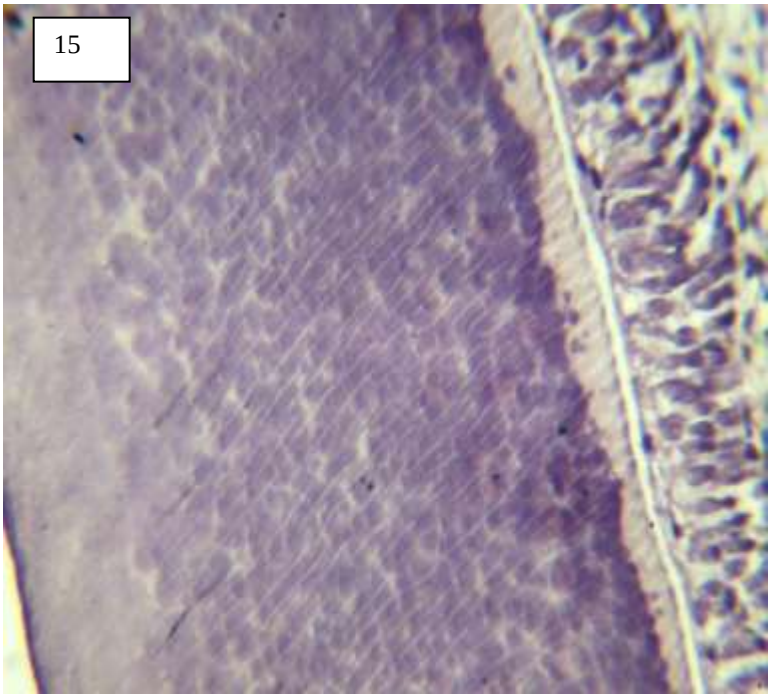
Four weeks time interval:



Fig(13): Light microscope photomicrograph for rabbit teeth pulp treated with $Ca(OH)_2$. It showed long odontoblastic processes with (red arrow) formation of reparative dentine (black arrow) H&E X400.



Fig(14): Light microscope photomicrograph for rabbit teeth pulp treated withBM-MSCs. showing regeneration of odontoblasts(red arrow) and formation of dentine (black arrow) H&E X400.



Fig(15):Light microscope photomicrograph for rabbit teeth pulp treated with fibrin scaffold loaded with BrM-MSCs showing highly dividing regenerated cells (multipotent cells with high mitotic division) (odontoblasts) (red arrows) with formation of new dentine (black arrow)H&E X400.

Figures (13-14-15) represent light microscope photo-micrographs of pulpal regeneration at 4 weeks time interval in rabbit teeth treated with different capping materials: (Fig. 13) for calcium

hydroxide which showed incomplete bridge formation which indicate moderate regeneration, there was a newly formed blood capillaries and progenitor cells which indicates signs of pulp tissue healing with inadequately regenerated odontoblast (orange arrow). (Fig. 14) for bone marrow mesenchymal stem cells: it showed complete regeneration with formation of dentin bridge, presence of well demarcated formative cells "odontoblasts" with highly divided regenerated cells (green arrow) to regenerate dentin, "Fibroblasts" to regenerate collagen fibers and newly formed blood vessels indicating full healing and regeneration of normal pulpal tissue. (Fig. 15) for breast milk stem cells: it showed complete regeneration which indicates formation of dentin bridge, presence of well demarcated formative cells "odontoblasts" to regenerate dentin, "Fibroblasts" to regenerate collagen fibers and newly formed blood vessels indicating full healing and regeneration of normal pulpal tissue. The pulp cavity filled with highly regenerated cells (odontoblasts) (green arrow).

Statistical results:

Effect of time on pulpal regeneration:

Table (1): Intra-group comparison between the two-time intervals for the three main groups:

		No Regeneration	Incomplete Regeneration	Complete Regeneration	P-value*
Calcium Hydroxide	2 W	72.7% (8)^{a}	18.2% (2)^{b}	9.1% (1)^{b}	<0.001^{HS}
	4 W	18.2% (2)^{b}	72.7% (8)^{a}	9.1% (1)^{b}	<0.001^{HS}
	P-Value**	0.001^{S}	0.001^{S}	1.000^{NS}	
	%	74.97%	-299.45%	0.00%	
Bone Marrow Stem Cell	2 W	36.4% (4)^{a}	27.3% (3)^{a}	36.4% (4)^{a}	0.654^{NS}
	4 W	9.1% (1)^{c}	27.3% (3)^{b}	63.6% (7)^{a}	<0.001^{HS}
	P-Value**	0.025^{S}	1.000^{NS}	0.025^{S}	
	%	75.00%	0.00%	-74.73%	
Breast Milk Stem Cells	2 W	36.4% (4)^{a}	36.4% (4)^{a}	27.3% (3)^{a}	0.654^{NS}
	4 W	0.0% (0)^{c}	27.3% (3)^{b}	72.7% (8)^{a}	<0.001^{HS}
	P-Value**	0.001^{HS}	0.265^{NS}	0.001^{HS}	
	%	100.00%	25.00%	-166.30%	

- Small letters for pairwise comparison between different regeneration types (Mann-Whitney test) and the means with different superscripts are statistically significant different at $P \leq 0.05$.
-* Overall P-value for Intra-group comparison between the three kinds of regeneration (Kruskal -Wallis test).
-** P-value for comparison between the two-time intervals
- HS= Highly significant at $P \leq 0.001$ - S= statistically significant at $P \leq 0.05$ - NS= Non-significant at $P > 0.05$
- The negative value of the percentage change number of cases increased from 2 weeks to 4 weeks, while the positive value of the percentage change means number of cases decreased from 2 weeks to 4 weeks.

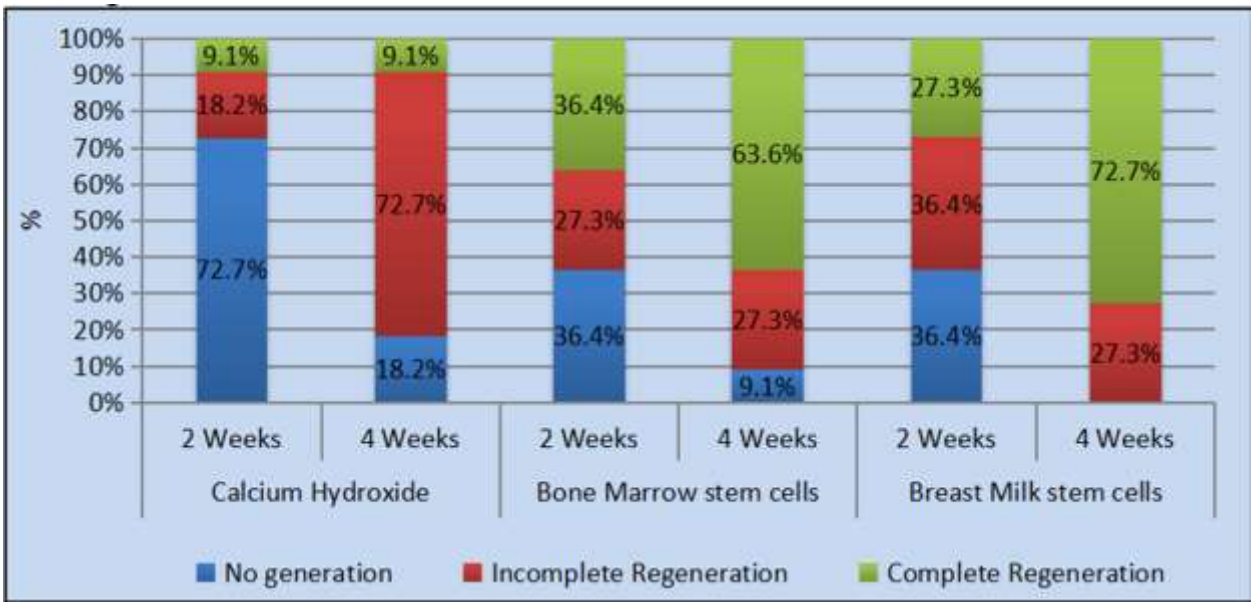


Figure (16): Pulp regeneration distribution (in %) between the two-time intervals for the three groups.

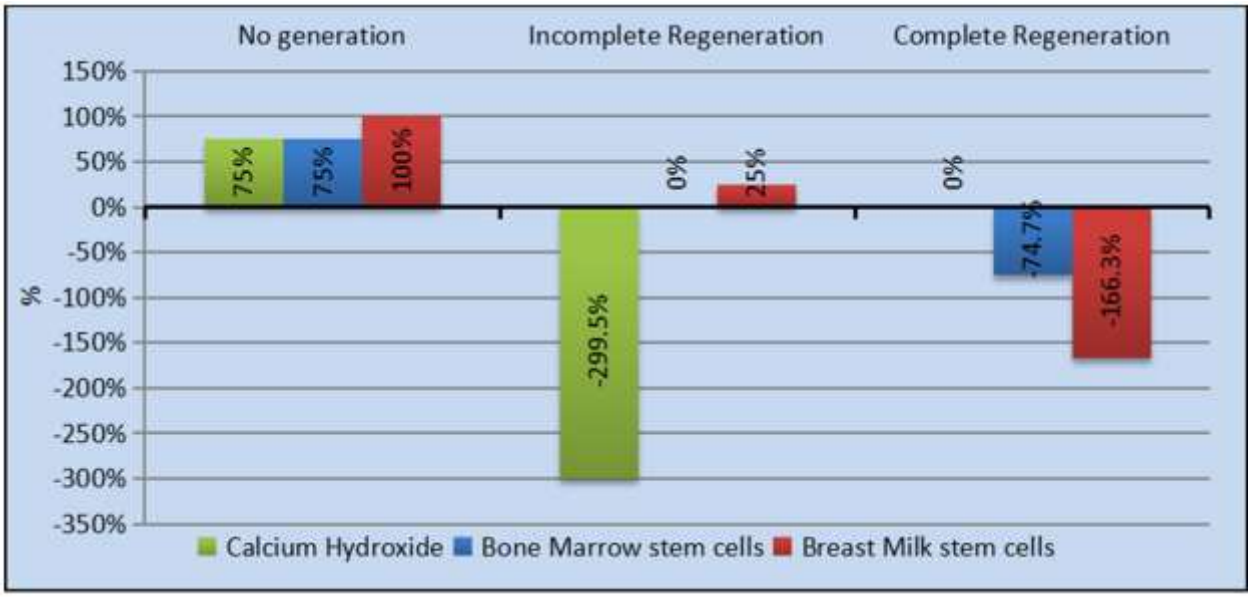


Figure (17): Bur chart representing the percentage of change in pulp regeneration for the three groups.

Table (1) and figures (16,17) show results of Kruskal-Wallis test for comparison between two time intervals in all groups and all types of regeneration within the same group.

A. Calcium Hydroxide group:

The percentage of cases that showed no regeneration decreased significantly from 72.7% after 2 weeks to 18.2% after 4 weeks and the percentage of change was 74.97%. Also, the incomplete regeneration cases percentage increased significantly from 18.2% after two weeks to 72.7% and

the percentage of change was -299.45%. Meanwhile, the complete regeneration cases percentage was 9.1% after 2 and 4 weeks without any significant changes between the two time intervals.

B. Bone Marrow Stem Cell group:

The percentage of cases that showed no regeneration decreased significantly from 36.4% after 2 weeks to 9.1% after 4 weeks and the percentage of change was 75%. While the incomplete regeneration cases percentage was 27.3% after 2 and 4 weeks without any significant changes between the two time intervals. Meanwhile, the complete regeneration cases percentage increased significantly from 36.4% after two weeks to 63.6% and the percentage of change was 74.73%.

C. Breast Milk Stem Cell group:

The percentage of cases that showed no regeneration decreased significantly from 36.4% after 2 weeks to 0% after 4 weeks and the percentage of change was 100%. The incomplete regeneration cases percentage decreased from 36.4% after two weeks to 27.3% and the percentage of change was 25%. Meanwhile, the complete regeneration cases percentage increased significantly from 27.3% after two weeks to 72.7% and the percentage of change was -166.30%.

Effect of materials on pulpal regeneration:

Table (2): Inter-group comparison of pulp regeneration for the three groups after 2 weeks

After 2 weeks	No Regeneration	Incomplete Regeneration	Complete Regeneration
Calcium Hydroxide	72.7% (8)^{a}	18.2% (2)^{b}	9.1% (1)^{b}
Bone Marrow Stem Cell	36.4% (4)^{b}	27.3% (3)^{ab}	36.4% (4)^{a}
Breast Milk Stem Cell	36.4% (4)^{b}	36.4% (4)^{a}	27.3% (3)^{a}
P-value**	0.000^{S}	0.027^{S}	0.009^{S}

- Small letters for pairwise comparison between different regeneration types between three groups (Mann-Whitney test) and the means with different superscripts are statistically significant different at $P \leq 0.05$.

-** Overall P-value for Inter-group comparison between the three groups at the same regeneration stage (Kruskal Wallis test) - S= statistically significant at $P \leq 0.05$

Table (3): Inter-group comparison of pulp regeneration for the three groups after 4 weeks

After 4 weeks	No Regeneration	Incomplete Regeneration	Complete Regeneration
Calcium Hydroxide	18.2% (2)^{a}	72.7% (8)^{a}	9.1% (1)^{b}
Bone Marrow Stem Cell	9.1% (1)^{b}	27.3% (3)^{b}	63.6% (7)^{a}
Breast Milk Stem Cell	0.0% (0)^{c}	27.3% (3)^{b}	72.7% (8)^{a}
P-value**	0.023^{S}	0.000^{S}	0.000^{S}

- Small letters for intra-group comparison between different thirds (Mann-Whitney test) and the means with different superscripts are statistically significant different at $P \leq 0.05$.

-** Overall P-value for Inter-group comparison between the three groups at the same regeneration stage (Kruskal - Wallis test). - S= statistically significant at $P \leq 0.05$

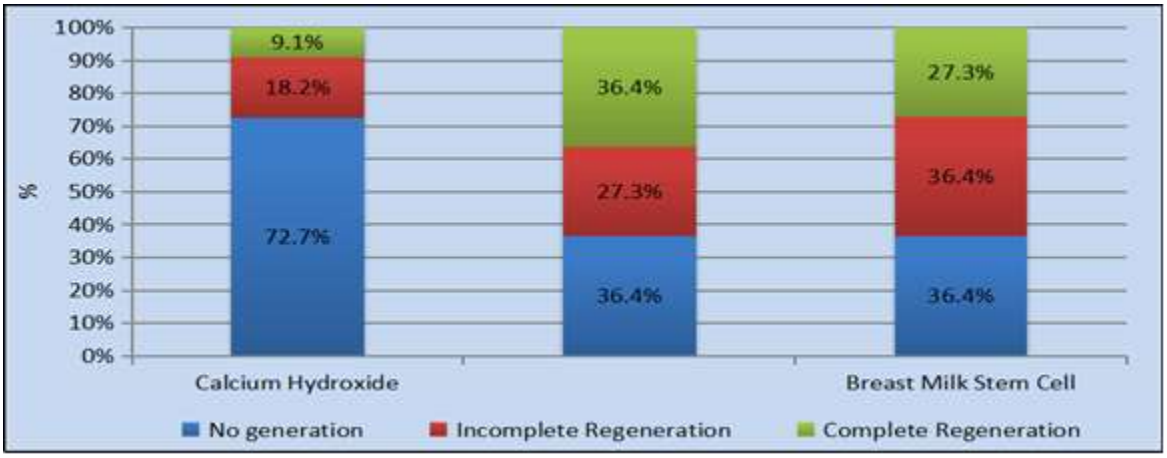


Figure (18): Pulp regeneration distribution (in %) between the three groups after 2 weeks

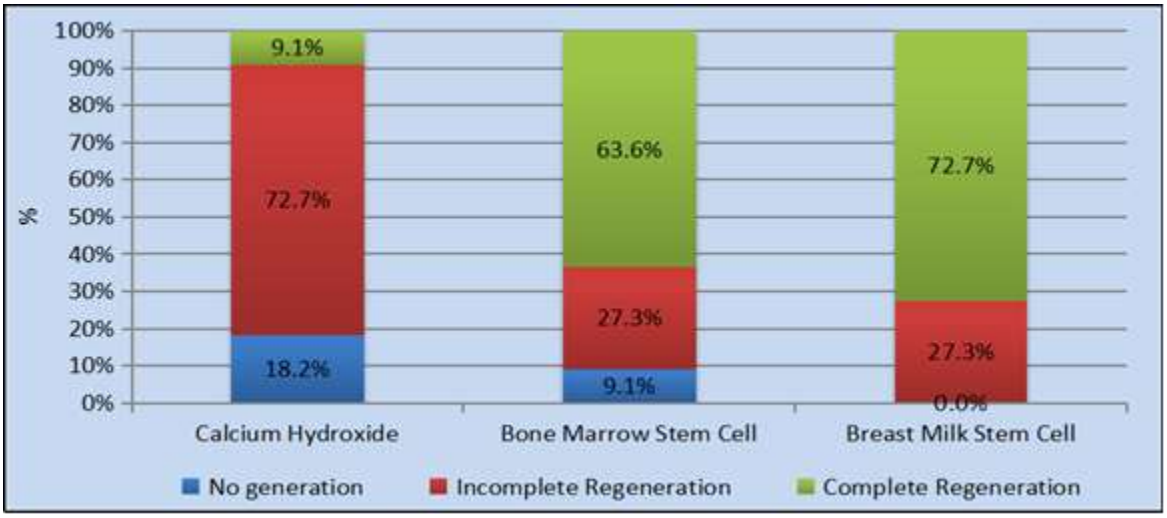


Figure (19): Pulp regeneration distribution (in %) between the three groups after 4 weeks.

Tables (2, 3) and figures (18,19) show results of Kruskal-Wallis test for inter group comparison at the same time interval and same regeneration type, the overall P-value was statistically significant different between different capping materials within the three kinds of regeneration at both time intervals.

After 2 weeks:

Calcium hydroxide group achieved the highest percentage of cases that showed no regeneration. While, breast milk stem cell group achieved the highest percentage of cases that showed incomplete generation. The Mann-Whitney test revealed the following differences between the three groups at each type of regeneration: for the no regeneration and complete regeneration types, the groups that received calcium hydroxide and the others did not differ significantly from one another, while the ones who received bone marrow and breast milk did. For partial regeneration, there was a significant difference only between the groups that received calcium hydroxide and breast milk, not between the groups that received calcium hydroxide and bone marrow or breast milk and bone marrow.

After 4 weeks:
Calcium hydroxide group showed the highest percentage of incomplete regeneration, While BM-MSCs group achieved the highest percentage of cases that showed complete regeneration. The following differences existed between the three groups at each type of regeneration, as determined by the Mann-Whitney test: The three groups differed significantly when there was no regeneration. However, the calcium hydroxide group differed significantly from the other groups, but not between the bone marrow and breast milk groups for the incomplete and complete regeneration types.

DISCUSSION:

Due to the high vascularization of dental pulp, certain conservative clinical dentistry procedures, such as direct pulp capping and pulpotomy operations, or inadvertent pulp exposure during caries excavation, might result in pulpal injury.
Dental pulp cells are critical for maintaining tissue homoeostasis and responsible for the defensive reactions which take place following injurious challenge. Thus, conservation of the pulp is critical.

Numerous academics and doctors have studied capping materials since choosing the right capping material is essential to achieving successful treatment outcomes. It was proposed that endothelial cell damage has a role in attracting odontoblast-like cells to the site of injury. This brings up the question of whether stem cells and signal molecules can be used to treat injured teeth and conservative pulp therapies.

The current study aimed to compare bone marrow and breast milk-derived mesenchymal stem cells with the regenerative capacity of calcium hydroxide in direct pup capping at different time intervals.

Calcium hydroxide Ca $(OH)_2$, which contains elements that encourage the migration, proliferation, and differentiation of pulp cells, is one material that is frequently used for pulp capping. The hydroxyl ions' chemical damage resulted in the positive benefits of calcium hydroxide, or Ca (OH)2. The essential pulp tissue was subjected to a limited necrosis. The pulp repair and minor discomfort were caused by the necrosis. Mesenchymal, endothelial pulp cells, and collagen production were regulated by the migration and proliferation of vascular and inflammatory cells. A reparative dentin bridge was formed and mineralized with the help of odontoblasts that underwent differentiation; direct pulp capping preceded the development of the dentin bridge [16].

Because bone marrow-derived stem cells are regarded as a standard form of stem cells with strong regeneration potential, they were injected into group (II). Because BM-MSCs demonstrated a notable pulp-dentin complex healing in direct pulp capping. Also, Bone marrow mesenchymal stem cells enhance the inflammatory response and induce the recruitment and proliferation of cells, required for repairing damaged tissues as it was reported by Radwan IA et al. That’s why BM-MSCs were employed as a pulp capping material [17]. In addition, the transplanted BM-MSCs show an ability to migrate to the site of injury and produce immunomodulatory cytokines and growth factors, helping local cells to recover and inducing the recruitment of new cells in the area As it was mentioned by Soliman et al [18]. Other types of

MSCs were reported to be of higher healing potential than BM-MSCs such as Dental Pulp derived Mesenchymal Stem Cells (DP-MSCs), However the difficulty and high cost of extraction of DP-MSCs is nearly equivalent to BM-MSCs. Added to that, many studies stated that DP-MSCs is more effective in direct pulp capping than BM-MSCs [19]. Whil[e Mohajeri](#) et al 2022 showed that the difference between BM-MSCs and DP-MSCs is negligible [20]. As it was reported that isolation of BM-MSCs is a difficult process using Ficoll reagent that is toxic to cell if not washed well, it is preferable to use BrM-MSCs in direct pulp capping as its separation is done without Ficoll and cells are separated in a simple easy procedure. Transplantation of BrM-MSCs in dental pulp injury was selected in group (III) following a previous study who reported that, BrM-MSCs, have a higher differentiation potential than BM-MScs and nearly equal to embryonic stem cells without having a teratogenic effect. BrM-MSCs are obtained by non-invasive method.
In the current study fibrin gel was used as a scaffold material since it aids in injection of ahigh number of the MSCs and maintains a good seeding of the cell. Added to that fibrin gel is good sealant for cell and growth factors [14].

Two and four weeks time intervals were chosen in the current study, as it is the duration needed for inflammatory process followed by healing process to occur. As well as, it is the exact duration needed for mesenchymal stem cells to differentiate and form tissues in the functionally related region [8].

Rabbits were enrolled as the experimental subjects in this study becausetheir pulpal tissues are comparable with other mammals including human.They supply larger teeth models than rats do; also their pulps show a higher regeneration capacity. Their pulps show less wear rates than that of rats. Finally, they provide safer way of dental investigation to the dental staff than dogs do [21].

Hemetoxylin and Eosin was used to detect the degree of healing of pulpal tissue such as odontoblasts and other cell types included in the healing process(ref). Moreover injected stem cells either BM-MSCs or BrM-MSCs promote healing of original tissue (Pulpal tissue) and differentiate into the same cell types (odontoblasts not osteoblasts) [22].

There was a significant difference between the groups of calcium hydroxide and bone marrow stem cells, but not between the groups of breast milk stem cells and bone marrow stem cells, according to the current results regarding the effect of materials on pulpal regeneration. Thus, there was some acceptance of the initial null hypothesis.

Calcium Hydroxide group showed the highest percentage of cases that showed no generation and incomplete regeneration (18.2%) and (72.7%) respectively. On the other hand, breast milk stem cell group showed the highest percentage of cases that showed complete generation (72.7%), and then followed by bone marrow stem cell (63.6%), while calcium hydroxide showed percent of cases that showed complete regeneration (9.1%).This was explained by the detrimental impact of calcium hydroxide's hydroxyl ions, which caused chemical damage. Additionally, superficial necrosis and moderate pulp irritation may result from the higher alkaline pH of calcium hydroxide. Additionally, BM-MSCs show a unique ability to locate several tissues in response to injury indicators. Indeed, because of their identical perivascular niches and gene expression profiles, they can develop into dental pulp stem cells and periodontal ligament stem

cells.

The results of this study are in line with those of a previous investigation, which found that three main factors contributed to the failure of the pulp capping material—the dentinal bridge's porosity, its poor adhesion to dentin, and its inability to provide a long-lasting seal against microleakage-despite calcium hydroxide's reputation as the gold standard [15]. In animal models, stem-cell-based tissue engineering has produced a variety of tissue regeneration outcomes..

In agreement with a previous study which stated that application of BM–MSCs combined with Bio-dentin was more beneficial than Bio-dentin or Ca $(OH)_2$ monotherapy as the former reduces necrosis and inflammation, while promoting calcified tissue formation [15].

Parallel to this investigation, a prior study's histologic sections and longitudinal and cross-sectional radiographs demonstrated the creation of a noticeable calcific barrier following direct pulp capping with BMSCs. They came to the conclusion that following direct pulp capping treatments, autologous mesenchymal BMSCs could stimulate the creation of hard tissue [23].

Additionally, a small number of studies created an in vivo model for coronal pulp regeneration in which scaffolds containing BM-MSCs from the animal's bone marrow were placed inside the coronal pulp chamber of pulpotomized rats' maxillary first molars. This work aimed to investigate the in vivo destiny of mesenchymal stem cells (BM-MSCs) generated from bone marrow in the coronal pulp regeneration model. The findings demonstrated that the cells lived for two weeks after implantation and colonized the area of possible cyto-differentiation. Their findings suggest that bone marrow-derived mesenchymal stem cells may differentiate into cells that contribute to the synthesis of mineralized tissue in the functionally relevant area [24].

The ability of bone marrow-derived mesenchymal stem cells to regenerate themselves may be a factor in their effectiveness. Several studies have demonstrated that bone marrow-derived mesenchymal stem cells can migrate into injured and damaged dental tissue and differentiate into a form of cell that is compatible with that dental tissue when they are introduced into the pulp[20].

Additionally, a different study yielded similar results but assessed dental pulp stem cells (DPSCs) in comparison to calcium hydroxide $Ca(OH)_2$ and noted that while the calcified dentin in the calcium hydroxide group was lower than normal, the reparative hard tissue formed in DPSCs did not differ statistically from normal dentin. Their findings were explained by the ability of dental pulp stem cells, or DPSCs, to regenerate tissue as well as the promotion of vascularization and growth factor production [25].

Regarding the effects of bone marrow and breast milk-derived stem cells on full pulpal regeneration, no statistically significant differences were found between the two cell types, suggesting that they have almost equal differentiation capability. Stem cells from bone marrow and breast milk can both develop into odontoblasts and build dentin bridges of the same thickness within the same time frames. The ability of both kinds of stem cells to develop into three germ layers may be the cause of this [24]. Their similar differentiation mechanisms allow them to produce distinct tissues in functionally related regions. Stem cells produced from bone marrow

and breast milk have the ability to develop into calcified tissue [17,26].

Up to our knowledge, this study is the first study that used breast milk stem cells in pulp capping, not only breast milk derived stem cells but also that loaded with fibrin gel. The impact of stem cells produced from breast milk on the proper development and growth of numerous organs in humans and rabbits has not been extensively researched.

A previous study tracked breast milk derived stem cells integration after taken by oral feeding to new born rabbit. They discovered stem cell engraftment into the duodenum, liver, cartilage, and bone. Their impact on the formation and growth of these organs is demonstrated histologically and by increased expression of the markers of cell division and growth, cyclin D1 and proliferative cell nuclear antigen (PCNA) [27]. A 2018 study examined the ability of breast milk-derived stem cells to differentiate into osteoblasts, however it used a limited amount of milk and produced a high MSC yield [28].

Regarding the effect of time on pulpal regeneration using different pulp capping materials, the time factor affected the degree of pulpal regeneration.

Four weeks' time interval was more effective in complete pulpal regeneration than the two weeks' time interval. So, the second null hypothesis is rejected. This may be attributed to the need of more time for calcific barrier formation and maturation [29].

These results are consistent with those of a prior study that assessed pulpal regeneration at three separate time points: one, two, and four weeks. They discovered that while fibrosis and the production of calcific bridges dramatically increased at the 4-week mark, pulpal inflammation significantly decreased from 1 to 4 weeks [29].

Nevertheless, it was shown that after three months-longer than the current study's study period-bone marrow-derived mesenchymal stem cells considerably established a visible calcific barrier. This finding may be related to the fact that the study used dogs, which are larger animals and may heal more slowly than rabbits. [20].

Lastly, compared to Ca $(OH)_2$ pulp capping, mesenchymal stem cell transplantation from bone marrow or breast milk filled with fibrin gel shown considerable regeneration potential in the treatment of dental damage.

CONCLUSIONS:

At two- and four-week intervals, the regeneration capacity of bone marrow and mesenchymal stem cells generated from breast milk is superior to calcium hydroxide as pulp capping materials. Using fibrin gel as a scaffold is crucial for getting the best possible outcomes. Mesenchymal stem cells produced from breast milk demonstrated the pulpal tissue's highest capability for regeneration. Overall, a 4-week interval is preferable to a 2-week interval for achieving regeneration potential.

RECOMMENDATION:

Further studies should be performed to get the benefits of multipotency of stem cells and its ability to differentiate into progenitor cells to be used in direct pulp capping. So, impregnation of mesenchymal stem cells in capping materials should be done to obtain the best regeneration potential and capping results to maintain vitality of the tooth.

CONFLICT OF INTEREST:

The authors have no conflicts of interest relevant to this study.

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