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Original Article

Evaluation of Dentinogenisis of Bionanocomposite Scaffold versus Mineral Trioxide Aggregate as A Direct Pulp Capping Material for Vital Pulp Therapy: An Animal Study

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Abstract

Aim: The aim of this study was to evaluate the ability of bio nanocomposite material (Nano MTA/collagen) to induce dentin bridge formation in comparison to mineral trioxide aggregate material (MTA) when used as direct pulp capping materials.
Subjects and methods: A total of 32 incisors of rabbits were recruited. Traumatic pinpoint pulp exposure was done in all teeth. Teeth were randomly divided into two groups (n= 16 per group) according to the pulp capping materials. Group (1): innovative bio nanocomposite (NanoMTA/Collagen) and Group (2): conventional mineral trioxide aggregate. Materials were added as direct pulp capping materials and then sealed with conventional glass ionomer restorative material. Histological and Scanning Electron Microscopic examination with EDAX were used to evaluate dentin bride formation and mineral content after one-month time interval. Data were collected, tabulated, and statistically analysed using SPSS 20, Graph Pad Prism and Microsoft Excel 2016.
Results: Regarding histological evaluation, no significant difference was found between NanoMTA/Collagen and MTA in inducing dentin bridge formation. Tissue damage and Inflammatory response was higher in MTA compared to NanoMTA/Collagen. Regarding scanning electron microscope with EDAX, MTA group showed highest in calcium and phosphorus weight percentage, while Ca/P ratio was higher in NanoMTA/Collagen group.
Conclusion: Nano mineral trioxide aggregate/collagen (NanoMTA/Collagen) and mineral trioxide aggregate (MTA) both induce dentin bridge formation in direct pulp capping therapy. The induced reparative dentin of NanoMTA/Collagen was the closest in morphology and composition to normal dentin. NanoMTA/Collagen was biologically compatible when used as a pulp capping material.

Keywords: Dentinogenisis, Scaffold, Mineral Trioxide Aggregate, Pulp Capping, Vital Pulp Therapy

I. INTRODUCTION

Deep carious lesions are cavitated lesions that extend to the inner third of dentin ***(Bjørndal et al, 2019)***. Current conservative concepts support minimal invasive dentistry and preservation of pulp vitality. Healing of the dentin-pulp complex and maintaining pulp vitality occurs either by natural repair, which results in defensive hard-tissue formation, or

therapeutically regulated dentin regeneration ***(Ricketts, 2001)***. The aim of vital pulp therapy (VPT) is to maintain and stimulate the remaining healthy tissue to advocate the healing of the dentin-pulp complex and so preserve teeth.

Development of new pulp capping materials has provided a way to preserve pulp vitality as well as to enhance pulpal repair ***(Baum and Mooney, 2000)***. Mineral trioxide aggregate (MTA) is a bioactive material used to stimulate reparative dentin formation in pulp capping therapy. It is considered gold standard material nowadays ***(Shenkin and Wilson, 2019)***. However, MTA have some drawbacks that can lead to irreversible inflammation during dentinal bridge formation within the pulp, which then leads to endodontic treatment. In addition, MTA causes teeth discoloration, it is of difficult clinical handling due to its sandy consistency, it has prolonged setting time and finally it is of high cost ***(Parirokh and Torabinejad, 2010)***.

Recent biomaterials such as nano bioactive material (Nano MTA) and natural polymer (collagen) are developed to regenerate dental tissues thus preserving pulp vitality and enhancing pulp repair capacity following dental pulp injury ***(Moussa and Aparicio, 2019).*** Nano mineral trioxide aggregate (Nano MTA) is a calcium silicate-based sealer containing nano silica particles, which increase the active surface area for reaction thus lowering setting time and increasing flexural and compressive strength ***(Zanjani et al, 2018)***. Collagen is a known natural biomaterial that is widely used in tissue regeneration applications ***(Raddall et al, 2018)***. Collagen polymers are commonly used by aiding with other materials to form biocompatible cell scaffold, due to its structural properties and cellular interactions ***(Moussa and Aparicio, 2019).***

When inorganic nanoscale materials are incorporated into an organic matrix, it is called "polymer bio nanocomposites" ***(Lee et al, 2016)***. Thus, new bio nanocomposite biomaterial can be made by enhancing the collagen's mechanical properties when combining it with Nano Mineral Trioxide Aggregate (Nano MTA), so regulating odontoblastic cell proliferation and dentin regeneration. Therefore, it is beneficial to compare the innovative introduced bio nanocomposite (Nano MTA/Collagen) material with mineral trioxide aggregate (MTA) to test the null hypothesis that they both are able to induce dentin tissue formation when used as a direct pulp capping materials.

II. SUBJECTS AND METHODS

Ethical Statement:

Animal study was conducted in a UResearch Animal Facility after review and approval of the Institutional Animal Care and Use Committee (CU-IACUC) of Cairo University. Ethical approval was obtained prior to the start of the study; with approval number CU III F 37 22**.** Ultimate responsibility was done for all activities associated with the conduct of a research project including performing the procedures and care to the animals.

Study Design:

The current study was split mouth technique, animal study, double blinded, by which the outcome assessors and the statistician were blinded. It was not allowed amongst the examiners to exchange any information throughout the entire study period. Eight rabbits with total of 32 upper and lower incisors, were randomly assigned into two groups (n= 16 for each group).

- Intervention Group (1) received innovative bio nanocomposite (NanoMTA/collagen)
- Control Group (2) received conventional MTA as pulp capping material.

Self-cured Riva glass ionomer restoration was used in both groups as a final restoration to seal the cavities. The rabbits were followed up weekly and the study trial was evaluated using histological and scanning electron microscope with Energy-dispersive X-ray spectroscopy (EDAX) after 1 month ***(Gamal et al, 2017)***.

Experimental Animals:

Screening of rabbits was done in the veterinary center by the veterinarian according to the inclusion criteria mentioned in table (1).

Table (1): Rabbits Inclusion and Exclusion criteria.

Rabbits Inclusion Criteria	Rabbits Exclusion Criteria
Rabbits above 6 months	Molar teeth
Incisor teeth of rabbits	Premolar teeth
White Male Rabbits	Female Rabbits
Weight ranging from 2.5 – 3 kg.	Immature rabbits
New Zealand Rabbits	Diseased Rabbits

Once the rabbits were selected, the veterinarian randomly divided them into two equal groups of incisor teeth ***Figures (1 & 2)***.

- Group (1): 16 left incisors in total for pulp capping with bio nanocomposite group (NanoMTA (Nanogate, Egypt)/Collagen (Sigma-Aldrich, Germany)).
  - One upper left incisor tooth (#201) and one lower left incisor teeth (#301) in each rabbit.
- Group (2): 16 right incisors in total for pulp capping with MTA (Dentsply, Maillefer) group.
  - One upper right incisor tooth (#101) and one lower right incisor teeth (#401) in each rabbit.

Two incisors were taken to evaluate the histology of normal pulp.

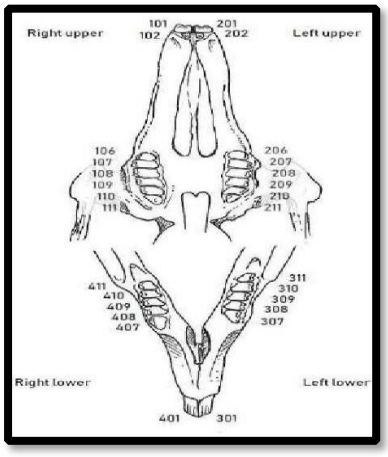

Figure (1): Schematic Diagram of tooth numbering in rabbits

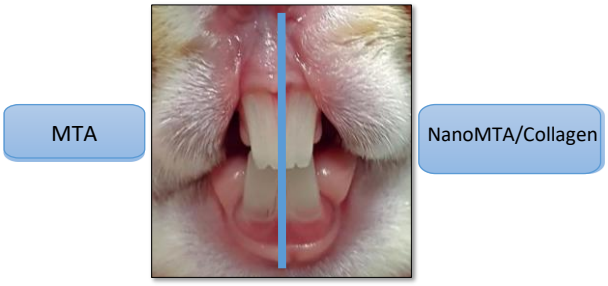

Figure (2): Rabbit's Dentition of upper and lower incisors

Sample Size Calculation:

Sample size was calculated depending on a previous study by **Faour et al. (2021)** as reference. The minimally accepted sample size were five rabbits, 20 teeth i.e. (n= 10) for each group, when the response within each subject group was normally distributed with standard deviation 3.4, the true mean difference was 6.50, the power was 80 % and type I error probability was 0.05. Sample size was increased by 60% to account for possible dropouts during follow-up interval to be total of eight rabbits, 32 teeth i.e. (n= 16) for each group.

Experimental Procedure:

Fabrication of Nano Mineral Trioxide Aggregate (NanoMTA):

The three primary mineralogical phases constituents of the white MTA (WMTA) are tricalcium silicate, dicalcium silicate, and tricalcium aluminate ***(Voicu et al, 2016)***. The two main components are the aluminates and silicates. These components were used in fabricating nano mineral trioxide aggregate by sol gel method ***(Voicu et al, 2012)*** resulting in the formation of a viscous gel. This gel was then dried at 120°C for 17 days and the final product was a white powder. The powder was pressed in pellets and thermally treated at 1200°C for 30 minutes. Rapid cooling of the thermally treated material was performed in air producing white spherical like shape Nano MTA powder with average size of 100 ± 10 nm ***Figure (3).***

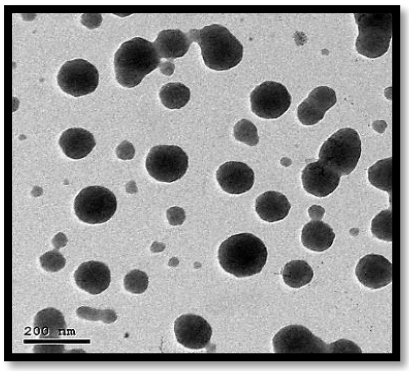

Figure (3): Transmission Electron Microscope (TEM) images of the prepared Nano MTA

Preparation of rabbits:

Rabbits were recruited according to the inclusion criteria mentioned in table (1). They were kept in cages with appropriate ventilation and food, which was 17% protein, 14% fibres, 2.5% fats to give 2550 kcal/kg for each rabbit for experimental measures.

All procedures were done under general anaesthesia. First, pre anaesthetic agent, meloxicam 1 mg/kg was subcutaneously introduced to the rabbits to act as a tranquilizer and a muscle relaxant. After about 5-10 minutes, the rabbit was consciously sedated. The anaesthetic agent which was combination of ketamine hydrochloride (HCL) 35 mg/kg and xylazine 3 mg/kg was intravenously introduced with the maintenance of propofol anaesthetic agent ***(Faour et al, 2021)***. Smooth technique and good degree of analgesia were done for smooth delayed recovery. Ophthalmic ointment; tramycin, was applied in both eyes throughout the procedure in order to prevent any corneal desiccation. No additional anaesthesia was needed throughout the procedure for any rabbit.

Cavity preparation:

After anesthetic administration, teeth surfaces of upper and lower incisors were first disinfected using 10% Betadine antiseptic solution and dried by cotton rolls ***(Faour et al, 2021)***. Cavity resembles class V cavity preparation was done using round carbide bur size 3 (1.2 mm in diameter and 1.1 mm in length) (RA# 3, ELA, Germany) with a low-speed contra-angle hand piece (EX203, NSK, Japan) with external water-cooling system. Cavity preparation was limited to just access the pulp chamber for pinpoint exposure on the gingival 1/3 without any special retention aids and no bevels. Traumatic/direct pulp exposure was made in each tooth in both quadrants (split mouth technique) ***(Faour et al, 2021)***. Proper haemostasis was done using cotton pellets moistened with sterile saline while applying light pressure ***(Safy and Ragab, 2019).***

Restoration Procedure:

After cavity preparation, irrigation of the pinpoint exposure using cotton pellet dipped in sterile saline and dryness of cavity was performed ***(Safy and Ragab, 2019)***. All restoration procedures were applied according to the respective manufacturer's instructions.

Intervention application (Bio Nanocomposite – NanoMTA/Collagen):

Innovative bio nanocomposite (Nano MTA/Collagen) mixture was composed of Nano MTA (powder form) and collagen solution (liquid form of Collagen type I alpha 1 chain (Col1a1)) were mixed in a ratio of 1:3 ***(Liu et al, 2020)*** using spatula and clean glass slab until a heavy suitable consistency was achieved, then applied over the pulp exposure site of the cavity using calcium hydroxide applicator (ARW2, 102-301, SedraDent, Spain).

Control Application (Mineral Trioxide Aggregate - ProRoot MTA):

In the mineral trioxide aggregate group of teeth, the conventional Pro-Root MTA material was applied on the exposure site, following its manufacturer's instructions. MTA powder was mixed with water in a 3:1 ratio ***(Kadali et al, 2020)*** until reached suitable consistency using clean glass slab and spatula and then placed with MTA applicator on the exposure site.

Restoring Teeth with Riva Self-Cured Glass Ionomer:

Control and intervention cavities were then sealed with self-cured glass ionomer riva capsules (SDI, Australia) ***(Safy and Ragab, 2019)***.

Animal Care:

After the procedure was finished, rabbits took antibiotics; synulox and analgesic drug for three days' post-operative with daily

follow up and care. No extra antibiotics or analgesics were needed again. Rabbits were taken care of by the veterinarian with suitable nutrition of 2550 kcal/kg.

Sacrification of Rabbits and Teeth Preparation:

The rabbits were scarified after one month of the procedure ***(Faour et al, 2021)***. Exsanguination of rabbits was done under general anaesthesia. The incisors were extracted from the rabbit and examined for any germinative tissue. The teeth were then disinfected using 2% glutaraldehyde and then stored in 10% formalin for fixation process for later examination.

Outcomes:

The operator and the outcome assessors assessed the dentin bridge formation after one month ***(Faour et al, 2021)*** from administration of MTA and Bio nanocomposite (Nano MTA/collagen) mixture histologically and under scanning electron microscope with EDAX.

Preparation of Teeth Specimens for Histological evaluation:

Twelve teeth specimens of each group were fixed in 10% calcium formal solution for 48 hours and demineralized in 10% ethylene diamine tetra-acetic acid (EDTA) (El-Gomhouria co.) solution for 4-5 weeks. The specimens were subsequently dehydrated in ascending grades of alcohol as follows: 70% for 12 hr, 80% for 12 hr, 90% for 6 hr and 100% for 4 hr, cleared in xylol and then embedded in paraffin blocks ***(Bancroft et al, 2013)***. Serial 5-6 μm paraffin cross sections were cut with a microtome using diamond knife and mounted on clean positive charged glass slides, and finally stained with H&E (Epicophylline 39 Phenobarbitone, Eipico co.) stain for histological analysis of reparative dentin under light microscope (magnifications; x40, x100, and x200) with digital camera (Leica Application Suite (LASEZ) microscopy, version 3.0, Leica DM LS2 microsystem, Switzerland). The histological evaluation was done using the scoring system and criteria's provided ***Table (2) (Lu et al, 2008)*** and ***(Mahendran et al, 2019).***

Table (2): Scores of Histological Evaluation Criteria

Grading	**Characterization**
Score	**Inflammatory cell response**
1	None or a few (0-1 cell per high power field) scattered inflammatory cells present in the site of pulp exposure, characteristic of normal tissue
2	Slight inflammatory cell infiltration with polymorphonuclear or mononuclear leukocytes(2-5 cells per high power field)
3	Moderate inflammatory cell infiltration involving coronal pulp (6-15 cells)
4	Severe inflammatory cell infiltration (>15 cells) involving the coronal pulp or abscess present
Score	**Tissue damage**
1	Normal tissue
2	Odontoblast disorganization but central pulp normal
3	Total disorganization of the pulp tissue morphology
4	Pulp necrosis
Score	**Hard tissue formation**
1	Absent
2	Lateral deposition of hard tissue on the walls of the cavity of pulp exposure
3	Partial hard tissue bridge - little communication of the capping material with the dental pulp
4	Complete hard tissue bridge - Closure of exposure area

Scanning electron microscopic examination and EDAX analysis:

Eight teeth (n= four specimens from each group) were embedded in acrylic blocks and then sliced in bucco-lingual longitudinal direction into two sections (0.4 mm in thickness) through the centre of the prepared cavity using Isomet 4000 microtome (Buehler, German) ***(Gamal et al, 2017) Figure (4)***. Specimens were then subjected to morphological observation under scanning electron microscopy (SEM). Sections were subjected to elemental analysis using SEM (Quanta 3D 200i Field Emission Gun (FEI)) attached with Energy Dispersive Analytical X-ray (EDAX) Unit (Thermofisher pathfinder). The amount of calcium and phosphorous within the formed reparative dentin of the new bio nanocomposite mixture and mineral trioxide aggregate groups were measured and expressed as weight percentage (%) at the evaluated area ***(Abdelaz et al, 2019)***.

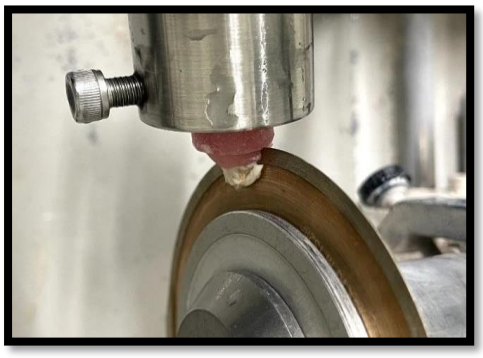

Figure (4): Longitudinal Cutting of Rabbits Tooth

Statistical methods:

Statistical analysis was performed with SPSS 20®, Graph Pad Prism® and Microsoft Excel 2016. All quantitative data were presented as mean and standard deviation. All data were presented in five tables and four graphs. The tests used were:

- Normality exploration of data by using Shapiro Wilk Normality test and Kolmogorov test.
- Comparison between Group (1): Bio Nanocomposite (Nano MTA/Collagen) and Group (2): Conventional Mineral Trioxide Aggregate (MTA) in histological findings was performed by using Wilcoxson Signed Rank test.
- Comparison between normal dentin, Group (1) and Group (2) was performed by using One Way ANOVA test followed by Tukey`s Post Hoc test for multiple comparisons.

III. RESULTS

1. Normality test:

Exploration of the given data was performed using Shapiro-Wilk test and Kolmogorov-Smirnov test for normality. As Listed in ***Table (3)***, it was revealed that the significant level (P-value) was shown to be significant as P-value <0.05, which indicated that data originated from non-parametric distribution regarding histological findings, while in Scanning electron microscope with EDAX demonstrated insignificant as P-value >0.05, which indicated data originated from normal data.

2. Histological Analysis:

a. Normal Dentin:

Histological examination of the normal dentin specimens of the rabbit teeth using light microscope showed normal histological architecture of dentin, pre-dentin, well organized and intact odontoblastic layer and pulp ***Figure (5)*.**

Table (3): Normality exploration of histological findings and scanning electron microscope with EDAX in Normal dentin, Group (1) (NanoMTA/Collagen) and Group (2) (MTA)

		Normal dentin	Group 1 (Nano MTA/Collagen)	Group 2 (MTA)
Histological findings	Inflammatory cell responses	----	<0.05 *	<0.05 *
	Tissue damage	----	<0.05 *	<0.05 *
	Hard tissue formation	-----	<0.05 *	<0.05 *
Scanning electron microscope with EDAX		>0.05 ns	>0.05 ns	>0.05 ns

Ns; non-significant as P>0.05 (Normal data)

**Significant as P<0.05 (non-parametric data).*

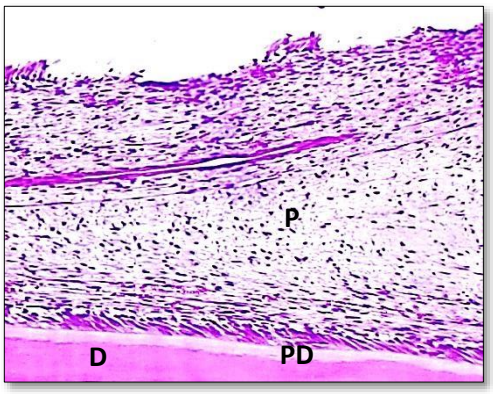

Figure (5a)

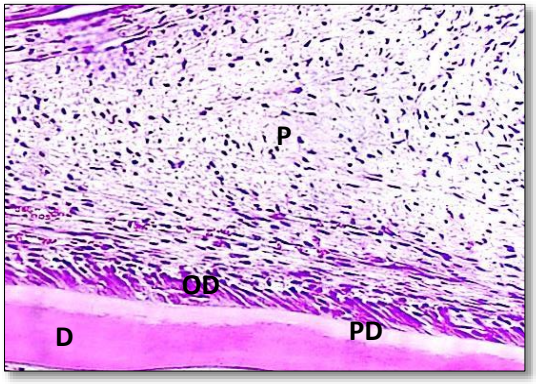

Figure (5b)

Figures (5a & b): A photomicrograph of normal histological dentin of rabbit teeth showing dentin (D), pre-dentin (PD), well organized intact odontoblastic layer (OD), and pulp (P) (H&E, Orig. Mag. x100 (5a) and x200 (5b))

b. Bio nanocomposite (Nano MTA/Collagen) Group (1):

Histological examination of the bio nanocomposite (Nano mineral trioxide aggregate/collagen) specimens of the rabbit teeth using light microscope showed lateral deposition of partial hard tissue bridge with little communication of NanoMTA/C (NanoMTA/Collagen) with the dental pulp ***Figure (6a)***. At magnification x200, mild inflammatory reaction was present in all samples ***Figure (6b)***. At higher magnification x400, numerous slight disorganized odontoblast cells with normal central pulp were present in almost all samples ***Figure (6c)***. No necrotic tissues were observed.

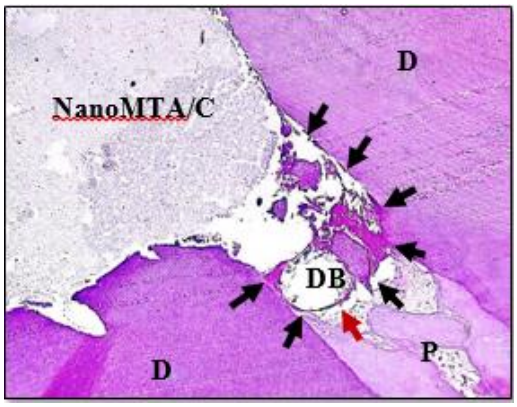

Figure (6a)

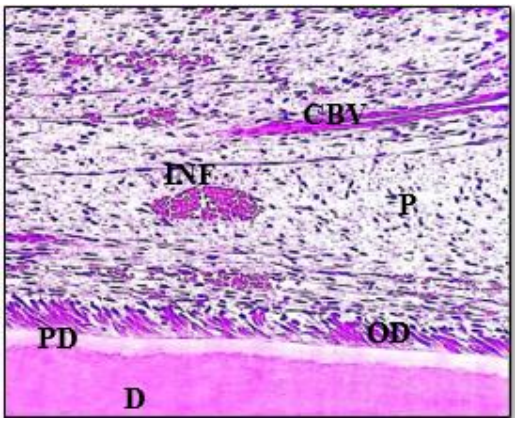

Figure (6b)

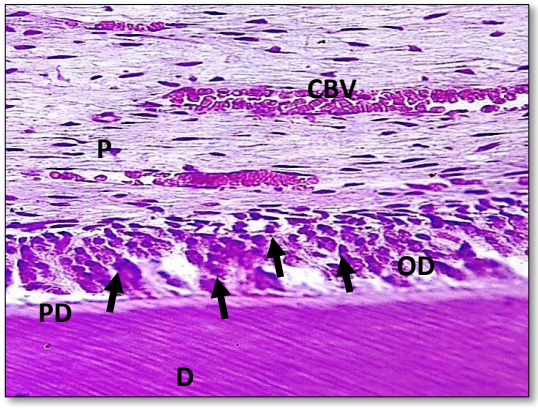

Figure (6c)

Figures (6): A photomicrograph of rabbit teeth specimen showing, a: partial hard tissue formation laterally (pointed black arrows) under NanoMTA/C capping material with thin communication of dentin bridge (DB) with pulp (red arrow) (H&E, Orig. Mag. X100); b: few scattered inflammatory cells (INF) and congested blood vessels (CBV) (H&E, Orig. Mag. x200); c: slight disorganized odontoblastic layer (OD) (pointed black arrows) with normal central pulp tissue (P) (H&E, Orig. Mag. X400)

c. Mineral Trioxide Aggregate Group (2):

Histological examination of the mineral trioxide aggregate specimens of the rabbit teeth using light microscope showed partial hard tissue formation in nine samples ***Figure (7a).*** At magnification (x200), inflammatory cells and dilated blood vessels were present in almost all samples ***Figure (7b)***. However, four samples presented moderate inflammatory reaction while the other eight samples presented severe inflammatory reaction of pulp. Two samples showed necrotic pulp. At higher magnification (x400), odontoblastic layer was visible. However, five samples revealed disorganized odontoblastic layer ***Figure (7c)***.

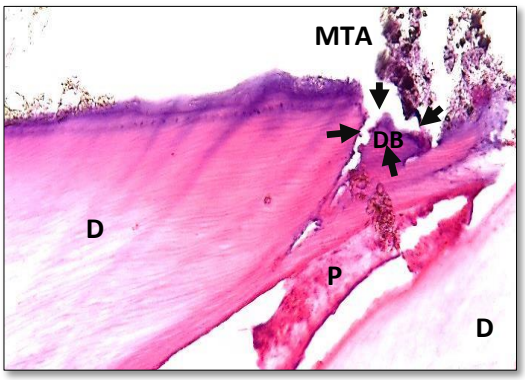

Figure (7a)

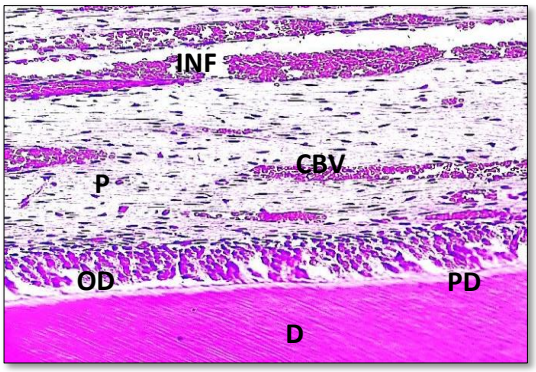

Figure (7b)

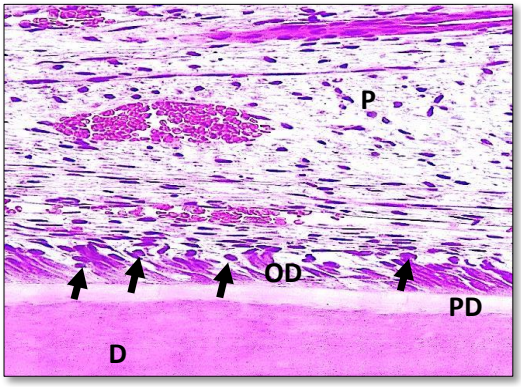

Figure (7c)

Figures (7): A photomicrograph of rabbit teeth specimen showing, a: partial dentin bridge (DB) (pointed black arrows) formation under MTA (H&E, Orig. Mag. x100); b: moderate inflammatory cells (INF) and congested blood vessels (CBV) (H&E, Orig. Mag. x200); c: disrupted odontoblastic layer (OD) (pointed black arrows) (H&E, Orig. Mag. x400)

Statistical Histological comparison between Groups (1) and (2):

Mean and standard deviation of all histological findings regarding both groups were presented in ***Table (4)*** and ***Figure (8).*** Comparison between them was performed by using Wilcoxson Signed Rank test which revealed that:

- In inflammatory cell response, Group (2): MTA (3.3 ± 0.48) was significantly higher than Group (1): NanoMTA/Collagen (1.7 ± 0.48) with (1.6 ± 0.22) difference between them as P=0.0001.
- In tissue damage, Group (2): MTA (2.9 ± 0.57) was significantly higher than Group (1): NanoMTA/Collagen (1.7 ± 0.48) with (1.2 ± 0.24) difference between them as P=0.0001.
- In hard tissue formation, Group (1): NanoMTA/Collagen (2.7 ± 0.48) with insignificant difference with Group (2): MTA (2.7 ± 0.48) as P=1.00.

Table (4): Mean and standard deviation of inflammatory cell response, tissue damage, and hard tissue formation in Group (1): NanoMTA/ Collagen and Group (2): MTA. Comparison between them using Mann Whitney's test:

Histological findings	Group 1 (NanoMTA/Collagen)		Group 2 (MTA)		Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		P value Mann Whitney's test
	Mean	Standard Deviation	Mean	Standard Deviation			Lower	Upper	
Inflammatory cell responses	1.70	0.48	3.30	0.48	1.60	0.22	1.15	2.05	0.0001*
Tissue damage	1.70	0.48	2.90	0.57	1.20	0.24	0.70	1.70	0.0001*
Hard tissue formation	2.70	0.48	2.70	0.48	0.00	0.22	-0.45	0.45	1.00

**Significant difference as P<0.05.*

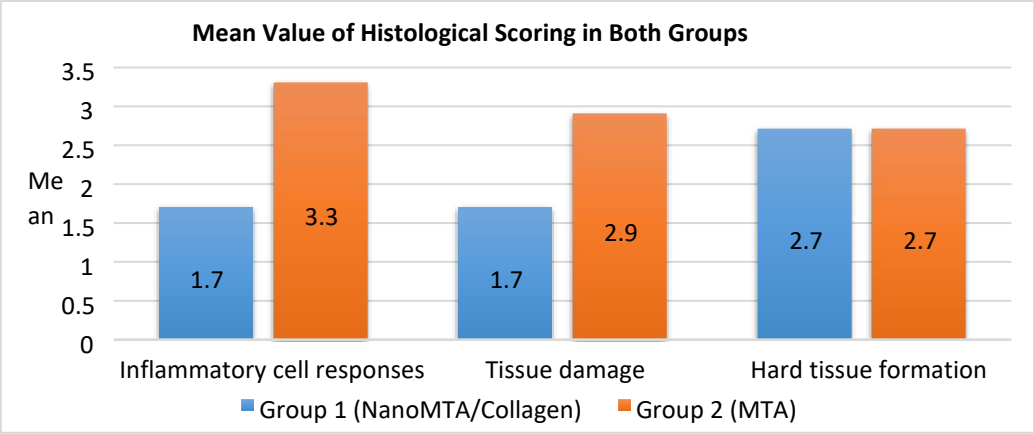

Figure (8): Bar chart illustrating mean value of histological scores comparison between both groups; NanoMTA/Collagen and MTA groups

3. Scanning Electron microscope with EDAX Analysis:

a. Normal Dentin Structure:

Normal dentin showed numerous non uniformly distributing dentinal tubules ***Figure (9a)***. The calcium and phosphorus elements are presented by weight percentage % in ***Table (5)***.

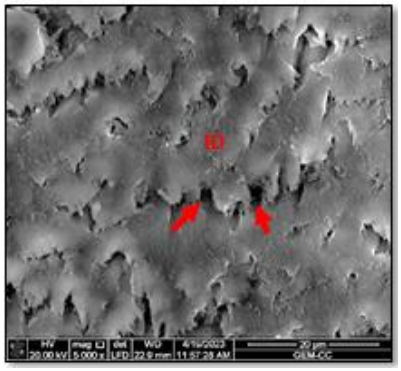

Figure (9a)

b. Bio Nanocomposite (Nano MTA/Collagen) Group:

The structure and EDAX elemental analysis of hard tissue formed in bio nanocomposite group was shown in ***Figures (9b)*** at magnification x5000. The morphology of the formed hard tissue was mesh like structure (containing matrix and mineral) which is closer to normal dentin structure. Tubular dentin was observed. The calcium and phosphorus elements are presented by weight percentage % in ***Table (5)***.

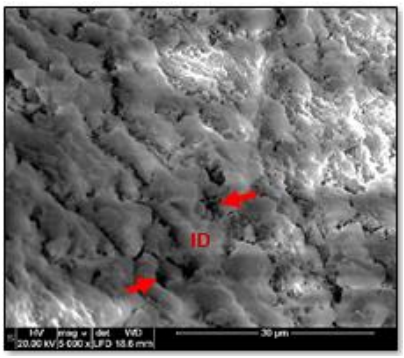

Figure (9b)

c. Mineral Trioxide Aggregate Group:

The structure and EDAX elemental analysis of hard tissue formed in mineral trioxide aggregate was shown in ***Figure (9c)*** at magnifications x5000. The MTA formed hard tissue crystalline barrier with high mineral content observed. The calcium and phosphorus elements are presented by weight percentage % in ***Table (5)***.

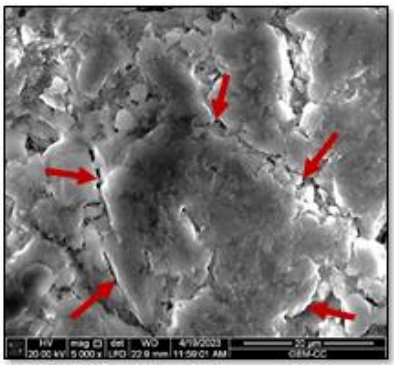

Figure (9c)

Figures (9): SEM analysis (Mag. x5000) showing, a: numerous non-uniformly distributed dentinal tubules (pointed red arrows) in between them are intertubular dentin (ID) of normal rabbit dentin; b: mesh like structure of dentinal tubules (DT) (pointed red arrows) and intertubular dentin (ID) are between the dentinal tubules of NanoMTA/Collagen Group; c: pointed arrows show crystalline hard tissue with amorphous pattern of MTA Group.

Mean and standard deviation of calcium, phosphorus, and calcium / phosphorus ratio regarding scanning electron microscope results in all groups were presented in ***Table (5)*** and ***Figure (10).*** Comparison between all groups was performed by using One Way ANOVA test which revealed significant difference between all groups as P<0.0001 regarding calcium and phosphorus only, followed by Tukey`s Post Hoc test for multiple comparisons which revealed that:

- In phosphorus, normal dentine (14.12 ± 0.03) was significantly the lowest, then group (1): NanoMTA/Collagen (14.34 ± 0.01), while group (2): MTA (15.75 ± 0.01) was significantly the highest.
- In calcium, normal dentine (28.57 ± 0.06) was significantly the lowest, then group (1): NanoMTA/Collagen (31.63 ± 0.05), while group (2): MTA (33.96 ± 0.05) was significantly the highest.
- In calcium/phosphorous ratio, normal dentine (2.01 ± 0.13) was insignificantly the lowest, then group (2): MTA (2.15 ± 0.18), while group (1): NanoMTA/Collagen (2.21 ± 0.43) was insignificantly the highest.

Table (5): Mean and standard deviation of scanning electron microscope with EDAX in normal dentine, Group (1): Nano MTA/Collagen and Group (2): MTA. Comparison between different groups using One Way ANOVA test, followed by Tukey`s Post Hoc test for multiple comparisons:

EDAX	NORMAL DENTIN		Group 1 (Nano MTA/Collagen)		Group 2 (MTA)		P value
	M	SD	M	SD	M	SD	
P	14.12%	0.03	14.34%	0.01	15.75%	0.01	<0.0001*
Ca	28.57%	0.06	31.63%	0.05	33.96%	0.05	<0.0001*
Ca/p	2.01%	0.13	2.21%	0.43	2.15%	0.18	0.27

*M: mean SD: standard deviation *Significant difference as P<0.05.*

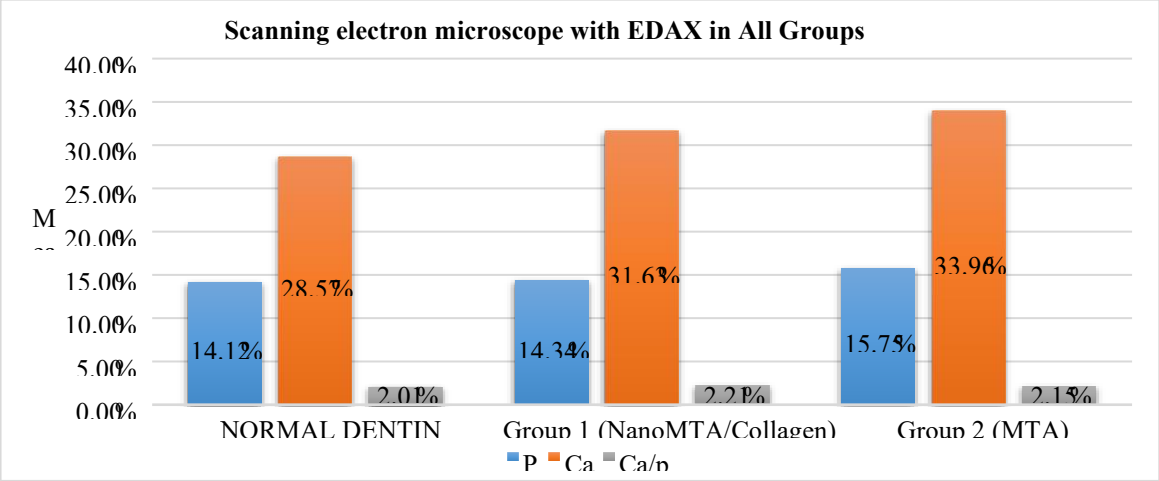

Figure (12): Bar chart illustrating mean value of scanning electron microscope with EDAX in normal dentine, Group 1 (Nano MTA/Collagen) and Group 2 (MTA)

IV. DISCUSSION

Pulp capping therapy is biological-based concept that has become the new era in conservative and restorative dentistry. It offers many advantages such as regeneration and preservation of the pulp vitality and tooth structure ***(Bjørndal et al, 2019).***

In the current study, the animal model that has been used was New Zealand white male rabbits. Female rabbits were excluded from the investigation due to their periodic hormonal imbalance and its potential for pregnancy ***(Faour et al, 2021)***. These species have pulpal tissues which are similar to those of humans and their tooth size are appropriate for restorative operations**.** The follow-up period was limited to four weeks long because rabbit teeth continuously erupt ***(Faour et al, 2021)***. However, dental abrasion from chewing a high-fibres diet keeps these eruptions in control ***(Gamal et al, 2017)*.** Moreover, the New Zealand white species have short lifespan compared to other rodents. **Gamal et al.** in **(2017); Faour et al.** in **(2021); Safy and Ragab** in **(2019)** have used similar follow up periods.

One criterion for evaluating a successful restoration has been the development of a dentin bridge or a calcified barrier, whose production can only be identified histologically ***(Emara et al, 2022)***. In the current study, a scoring system of histological criteria have been used to assess the hard tissue formation, inflammatory reaction, and tissue damage of both groups, MTA and bio nanocomposite groups. However, the composition and mineral content of hard tissue formation could not be performed histologically. Thus, scanning electron microscope with EDAX elemental analysis was conducted to analyze calcium and phosphorus minerals composition within limited regions of the tissue. Slight variations in the composition of the tissue or in the crystallinity of the minerals can have a significant impact on the function of the tissue.

Statistical analysis was performed using Wilcoxson Signed Rank test between both groups for histological outcomes and One Way ANOVA test followed by Tukey`s Post Hoc test for multiple comparisons of groups. The proposed hypothesis of this animal study was null hypothesis in the dentinogenisis of NanoMTA/Collagen versus mineral trioxide aggregate as a direct pulp capping materials for vital pulp therapy which was consistent with the results.

Upon acquiring data and analyzing the results statistically, it was found that regarding the histological outcomes in the term of hard tissue formation, both NanoMTA/Collagen and MTA groups showed insignificant difference between each other. The results were consistent to **Emara et al.** in **(2022)** who combined chitosan with MTA. Their animal study claimed that chitosan is a biomedical agent with good osteoconductivity and biocompatibility, aiding in hard tissue formation, as collagen that was used in the present study. **Abdelaz et al.** in **(2019)** and **Tran et al.** in **(2019)** are similar animal studies that have established mineralized tissue formation as a result of direct pulp capping with MTA**.** This was due to the release of hydroxide ions of MTA which interacts with tissue fluids forming calcium hydroxide that plays role in hard tissue formation between MTA and dentin ***(Kadali et al, 2020).***

Kavitha Sri et al. in **(2023)** and **Siswanto et al.** in **(2020)** in vitro studies claimed that the addition of nano particles acts as "pro-adhesive agents", thus improving the cells adherence, integration, differentiation, and viability. Results of the studies found that Nano hydroxyapatite/collagen scaffold was very effective in bone regeneration. This was due to bone morphogenetic protein 7 (BMP 7) and BMP 2 which are found in collagen, were shown to support the scaffold's osteoconductive properties.

Regarding inflammatory reaction, the present study showed significant difference between both groups. NanoMTA/Collagen showed mild inflammation compared to MTA which had moderate to severe inflammatory response with areas of blood congestion and necrotic tissue. Similar histological studies by **Eskandarizadeh et al.** in **(2011)** and **Manochehrifar et al.** in **(2016)** reported necrosis and inflammatory cell response in pulp

when capped with MTA. This could be due to bismuth oxide component in MTA, it was found to increase inflammatory reaction ***(Elkhashab et al, 2021)***. This was disagreed with **Safy and Ragab** in **(2019)** who revealed that MTA showed mild inflammatory reaction at two weeks and decreased at four weeks of direct pulp capping in rabbits. **Hosoya et al.** in **(2019); *Guerrero-Gironés* et al.** in **(2021);** and ***Elkaramany* et al.** in **(2023)** have shown similar study results that claimed mild or no inflammatory response due to the high alkalinity of MTA (10.2), which rises to 12.5 after three hours. In addition to providing efficient seal to MTA will decrease inflammatory reaction and increase the success of pulp capping procedure.

In comparison to MTA group, the results of NanoMTA/Collagen group were consistent with ***Guerrero-Gironés* et al.** in **(2021)** which showed no pulpal inflammation or necrosis in Beta Tricalcium phosphate/Collagen/Hydroxyapatite scaffold group. It was claimed that this is due to the materials biocompatibility. **Kavitha Sri et al.** in **(2023)** and **Thant et al.** in **(2023)** supported that collagen-based biomaterials have no cytotoxic effect on pulp cells, especially when used as scaffold with stem cells. However, when combining collagen with NanoMTA as done in the present study, mild inflammatory reaction appeared which was similar to the results of **Fayyad and ElBaz** in **(2018)** revealed that NanoMTA showed the highest mean of inflammation. It claimed that when silicon dioxide (SiO2) and titanium dioxide (TiO2) are in Nano size, it resulted in inflammatory activation and induction of pro-inflammatory cytokine interleukin (IL)-1β, while their micro-sized particles had been thought to be biocompatible and rarely induce inflammation. This was disagreed with **Elkhashab et al.** in **(2021)** which showed no significant difference between NanoMTA and MTA. It claimed that regarding the size of the particles, good sealing decreases any inflammatory reaction by time as MTA is considered a biocompatible material.

Concerning tissue damage, the present study showed significant difference between MTA and NanoMTA/Collagen groups. MTA showed disorganized odontoblastic layer compared to NanoMTA/Collagen which showed numerous organized odontoblasts. This was consistent with **Bae et al.** in **(2012)** in vitro study who claimed that nanobioactive glass/collagen scaffold are effective in odontogenic stimulation due to the existence of osteocalcin (OCN), alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP) that are key markers for odontoblastic cell differentiation and mineralization. Furthermore, **Alnour et al.** in **(2023)** showed improper or no odontoblastic layer in MTA at one-month time interval compared to nitric oxide. It claimed that MTA takes up to three months for proper odontoblastic organization with no inflammatory response, healing of injured pulp and full dentin bridge formation. Conversely, ***Guerrero-Gironés* et al.** in **(2021)** showed regular odontoblastic layers of both groups; MTA and Beta Tricalcium phosphate/Collagen/Hydroxyapatite scaffold. It claimed that proper sealing is essential for structured pulp with well-organized odontoblastic layer.

The histological dentin bridge formation of each group was confirmed by the scanning electron microscope results. In the terms of dentin morphology, a highly mineralized crystalline barrier was formed under MTA material. However, it was not similar to the morphology of NanoMTA/Collagen which resembles the morphology of normal dentin. **Kunert and Lukomska-Szymanska** in **(2020)** verified that transforming growth factor- beta 1 (TGF-β1) is secreted from pulp cells upon administration of MTA which affects the quality of the induced hard barrier. Furthermore, the formed layer in the interface between MTA and dentin was apatite in nature due to the release of high concentration of calcium which has been described as non-specified calcium phosphate that serves as a starting point for the synthesis of carbonated apatite. In contrast, **Tran et al.** in **(2019)** showed no difference in morphology of formed dentin of biodentin and MTA when compared to normal dentin, suggesting it to be similar to tubular dentin and its mineralization level was close to that of primary dentin due to MTA's bioactivity.

In contrast to MTA group, the NanoMTA/Collagen group showed reparative dentin bridge formation nearly similar in morphology to normal dentin which was

similar to **Sequeira et al.** in **(2023)** showed hard tissue formation with highly organized matrix when combing collagen with stem cells and growth factor compared to collagen alone and MTA**.** It claimed that collagen is similar to the normal collagen component of dentin. Collagen degrades by time; not less than six weeks, for tissue replacement of highly organized tissues with fibroblasts to resemble normal pulpal tissue. However, it mentioned that stem cells and growth factor have huge role in increasing collagen mechanical properties to aid in tissue regeneration. This can be explained that collagen scaffold can help in the migration of DPSCs, offer some mechanical and structural support, and have an impact on mineral deposition and odontogenic differentiation to resemble normal tissue formation **Whitehouse et al. (2021)**.

Regarding mineral content, MTA group showed significant higher level of calcium and phosphorus compared to NanoMTA/Collagen material. This was consistent with **Tran et al.** in **(2019)**, which showed no significant difference in mineral content of biodentin and MTA to primary dentin. It claimed that MTA is bio inductive bioactive material showed high calcium and phosphorus release which was comparable to primary dentin. In the present study, NanoMTA/Collagen had highest CA/P ratio compared to MTA. This can be explained that collagen becomes mineralized when combined with inorganic material as its mechanical properties increases. **Liu et al.** in **(2020)** showed high Ca/P ratio in hydroxyapatite/collagen scaffold. It claimed that the release of calcium and phosphate from collagen scaffolds mainly depend on the ratio or percentage of collagen within the inorganic material. The Ca/P molar ratio rises with increasing calcification. Scaffolds containing high collagen content permit the material to be internally replaced by new bone within period of time. Precipitates of calcium and phosphate in the collagen fibre structure after collagen degradation aid in osteoblastic differentiation of progenitor cells and promote osteogenesis. This could be the suggested explanation of the high Ca/P ratio of NanoMTA/Collagen.

One limitation of the current study is that it was limited to one-month time interval. However, this was due to the short life span of rabbits' animal model, few MTA samples did not resemble continuous dentin bridge formation which was similar to **Emara et al.** in **(2022)** who confirmed the presence of partial or complete dentin bridge formation following direct pulp capping with MTA in dogs at different time intervals (7, 21, and 60 days). It claimed that 60 days of evaluation period was enough to show complete dentin bridge and intact odontoblastic layer while less than that could be a short period for dentinogenesis. Direct pulp capping using nano biomaterials will aid in increasing the mechanical, biological, as well as the cellular activity thus aiding in enhancing tissue regeneration and preservation of pulp vitality.

V. CONCLUSION

Within the limitation of the current study, NanoMTA/Collagen and MTA both induce dentin bridge formation in direct pulp capping therapy. The induced reparative dentin of NanoMTA/Collagen was the closest in morphology and composition to normal dentin. NanoMTA/Collagen was shown to be biologically compatible when used as a pulp capping material.

Recommendations

Longer follow up period is strongly recommended to evaluate the time factor along with the quality and thickness of the formed dentin bridge. Moreover, In vitro studies are needed to assess the physical and mechanical properties of the Bio nanocomposite mixture (NanoMTA/Collagen).

Conflict of Interest:

The authors declare no conflict of interest.

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Ethics:

This study protocol was approved by the Institutional Animal Care and Use Committee (CU-IACUC) of Cairo university on: October 2022, approval number: CU III F 37 22

Data Availability:

Data will be available upon request.

Clinical trial registration:

The protocol for this study was not registered on clinicaltrials.gov as it is an animal study.

CRediT statement:

Author 1: Data curation, Writing - review & editing, Writing - original draft, Methodology, Conceptualization, Resources.

Author 2: Data curation, Conceptualization, Project administration, Supervision, Methodology, Writing - review & editing, Writing - original draft.

Author 3: Methodology, Writing - original draft, Writing - review & editing, Investigation, Formal analysis, Supervision, Data curation.

Author 4: Methodology, Writing - original draft, Writing - review & editing, Investigation, Formal analysis, Supervision, Data curation.

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