10.21608/ADJC.2023.209192.1301 Advanced Dental Journal
Volume 5 (2023) | Issue 4| Pages 768-784

Original Article

A comparative study of the biological and histological response of eggshell nanoparticles, hesperidin, and calcium hydroxide as direct pulp capping materials: An in vivo study on rat molars

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Submitted: 7-5-2023
Accepted: 21-8-2023

Abstract

Aim: This study aimed to assess the biological and histological pulp response following direct pulp capping with eggshell nanoparticles and hesperidin compared to calcium hydroxide in rats' teeth.

Subjects and methods: Eighteen rats were divided into three groups according to pulp capping materials (eggshell powder nanoparticles, hesperidin, and calcium hydroxide). Each group was divided into two subgroups based on the time of sacrifice following the application of pulp capping materials (2 and 4 weeks). The pulp tissue of the maxillary first molars was exposed and directly capped by one of the three materials. Glass ionomer was then used to fill the cavities. At each interval period, animals were sacrificed, and their teeth were collected for histopathological analysis. The differences in the inflammatory response and dentin bridge formation of the exposed pulp between the three groups were statistically evaluated.

Results: The difference between all groups was statistically significant. The inflammatory cell count results revealed that eggshell powder recorded the lowest pulp inflammatory response at both intervals (2 and 4 weeks). Regarding the dentin bridge, at 1st interval, hesperidin recorded the most significant thickness, but at 4 weeks, the greatest thickness was recorded for eggshell powder. On the other hand, at 4-week intervals, in terms of pulp inflammatory response and calcified bridge formation, no significant differences were found between eggshell powder and hesperidin.

Conclusions: Eggshell powder nanoparticles and hesperidin have better dentin bridge formation and pulp preservation than calcium hydroxide. Moreover, eggshell powder led to faster hard tissue formation and less inflammation than hesperidin.

Keywords: Direct pulp capping, histological evaluation, Inflammatory response, Dental pulp, Dentin Bridge.

Introduction

Many researchers and clinicians are interested in the management of dental pulp exposures. [1] Direct pulp capping is a therapy protocol where a biocompatible pulp capping material is applied directly to the exposed site, followed by a restoration that creates a coronal seal to inhibit disease progression and aids in the healing and repair process. [2, 3] As a result, the best pulp capping material should have favorable biocompatibility, good sealing ability, potent antibacterial properties, and beneficial handling characteristics. [4] Calcium hydroxide ($Ca(OH)_2$) was introduced by [Hermann 1930] [5] in dentistry and quickly became the standard gold material for pulp capping in human teeth. It has powerful antibacterial effects and can stimulate hard tissue formation [6,7], but it has several disadvantages, such as weakness, cohesive strength, and poor adhesion to dentin. [8] Due to the reduced biological efficiency and hazardous nature of some capping materials, newer and safer alternatives are needed, particularly those possessing higher antimicrobial activity than their traditional counterparts. [9] One of the main objectives in dentistry is to create a natural bioactive capping material that is affordable, capable of remineralization, and adheres to the dentin via forming an interfacial layer rich in minerals and connected to dentinal tubules. Eggshell powder (ESP) is a good source of calcium. It includes 94% calcium carbonate, 4% organic matter, 1% calcium phosphate, 1% magnesium carbonate, [10] phosphorus, strontium, zinc, fluoride, and copper that could assist in remineralization, [11,12] improves bone density and regeneration of bone defects. [13] When examined in rats and humans, it shows an antirachitic effect. [14] In dentistry, ESP found an interest in treating enamel surface lesions, as this might enhance the remineralization potential comparable to the existing agents [15] and was used as toothpaste to treat dentin hypersensitivity. [16] On the other hand, hesperidin is a natural plant extract that regulates the immune response, reduces the production of free radicals, and inhibits the growth of bacteria and fungi, indicating that this ingredient naturally has anti-inflammatory, antioxidant, and immune-regulating properties. [17] It is a flavanone glycoside found as a byproduct in citrus food, making it an inexpensive product. It was found to effectively control bleeding and edema of the pulp by reducing the permeability of capillary walls. In addition to the properties mentioned above, hesperidin also has a remineralization effect by reducing the susceptibility of dentin lesions to demineralization with the potential to promote remineralization and reparative dentin formation. [18,19] So, this study aimed to investigate pulp tissue's biological and histological response after applying eggshell nanoparticles and hesperidin as capping materials on experimentally created pulp exposures in rat model teeth and compared them to calcium hydroxide-based agent conventionally used.

Subjects and Methods

Preparation method of eggshell nanoparticles:

The World Property Intellectual Organization's calcination protocol was used to prepare the chicken eggshell powder in Nano-Gate, Egypt (WO/2004/105912: Method of producing eggshell powder). [20] Chicken eggshells contain roughly 95% calcium carbonate, which, upon calcination, transforms into basic calcium oxide and causes a rise in alkalinity. [21] To make the membranes easier to remove, 12 chicken eggs were used, cleaned with distilled water, and placed in hot, boiling water for 10 minutes at 100°C. The eggshells were crushed into tiny particles using a sterile mortar and pestle. The tiny, crushed particles were kept in a muffle furnace (Neycraft Model JFF 2000) at 1200°C to ensure the finished powder was pathogen-free. Then the dried powder was milled using a

ball mill machine (planetary-ball-mill-pm-400) for 10h, speed 350 rpm, and at 3minutes intervals.

Characterization of Eggshell Nanoparticles:

X-ray Diffraction Analysis (XRD):

Diffraction patterns of the eggshell nanoparticles were determined using the XPERT-PRO Powder Diffractometer system, with 2 thetas (20^{O} - 80^{O}), with Minimum step size 2Theta: 0.001, and at the wavelength (Kα) = 1.54614^{O}. [22, 23]

Selected Area Electron Diffraction and Transmission Electron Microscope (TEM):

The particle morphology, size, and selected area electron diffraction (SAED) were examined by Transmission Electron Microscope (TEM). The HR-TEM is JOEL JEM-2100 operating at 200 kV and equipped with Gatan digital camera Erlangshen ES500. The prepared samples were prepared by depositing particles onto a holey carbon film-coated copper grid from a well-sonicated dilute suspension in acetone to minimize agglomeration. The specimens were then negatively stained with 2% w/w phosphotungstic acid and were left for 30 s. After removal of the excess stain, the samples were allowed to dry at room temperature for 10 min before investigation. [23]

Sample size calculation

Based on Salah et al. [24] and using the G power statistical power Analysis program (version 3.1.9.4) for sample size determination (25), A total sample size of n=18; (subdivided into 6 in each group).

Animals' selection and grouping:

Eighteen Sprague Dawley rats were chosen from the animal house of Ahram Canadian University. All experimental procedures were carried out according to protocols approved by the Ethics Committee of Research of the Faculty of Dentistry, Ahram Canadian University, with the approval number of (#A0001). The rats were about 150-200 grams in weight. Rats were individually housed in separate cages. Following the Canadian Council on Animal Care, a well-trained veterinarian assistant considered the temperature, humidity, ventilation, lighting, and chemical and microbiological control. The maxillary first right and left molars were checked to ensure there were no large cavities, pulp exposure, bleeding, or suspected fractures. The rats were divided into three groups, 6 in each, according to the material used for pulp capping. In the first group, the pulp was capped with Calcium hydroxide; the second group was capped with hesperidin; and the third group was capped with eggshell nanoparticles. Each group was further subdivided into two subgroups: each of three rats according to the scarifying time (two weeks, and four weeks).

Pre-operative Considerations and Anesthesia:

Each rat was anesthetized by administering an intraperitoneal injection of 5% pentobarbital sodium (at a dose of 40 mg/kg using a suitable gauge needle. Rats were fixed on an operating table; a jaw prop was used to keep the mouth open; 0.12% chlorohexidine disinfected the oral cavity (26). Cotton rolls and gauze swabs were utilized to attain an intra-oral dry field.

Pulp capping procedures:

Thirty-six right and left maxillary first molars in 18 rats were randomly allocated into three experimental groups. The maxillary right and left first molars were exposed using a sterile size 1 round tungsten carbide bur (Hager & Meisinger GmbH, Germany) with low-speed handpiece (NSK, Japan) under abundant sterile air water spray drilled into the middle of the cavity to expose the pulp in a diameter not exceeding 1 mm without invading the pulp tissue. Sterile cotton pellets soaked in saline for five minutes were used to control bleeding, apply pressure gently until physiologic hemostasis occurred. Then the exposure site was dried using sterile cotton pellets

. The capping materials were applied to the exposed pulp at the exposure site. The $Ca(OH)_2$ (Urbical – Promedica, Germany) was mixed according to the manufacturer's instructions, and a calcium hydroxide applicator (HuFriedyGroup, Germany) was used to apply it on the exposed sites. In the case of the eggshell and the hesperidin (SEDICO Pharmaceutical Company, 6th October City, Egypt) groups, the powders were mixed with distilled water that was used in the same method as the $Ca(OH)_2$. The $Ca(OH)_2$ was mixed according to the manufacturer's instructions and applied on exposure sites. The mixture was carried to the exposure site using a calcium hydroxide applicator. The hesperidin powder was mixed with distilled water in a ratio of 1:3 according to the manufacturer's instructions to obtain a homogenous mix, then a fine amalgam carrier was applied to carry the paste into the cavity. The Hesperidin mix was left for setting according to the manufacturer's instructions. [27] The ESP was mixed with distilled water to make a slurry, 1 mg eggshell powder was added to 5 μL distilled water, and the exposure site was covered with the slurry using a calcium hydroxide applicator. [28] The cavities were dried and then restored with glass ionomer. [27, 28] (High viscosity GIC capsule. GC Fuji IX GP fast, GC Corporation, Tokyo, Japan).

Post-operative procedures:

All rats were cared for in the animal house of Ahram Canadian University. The rats had a balanced, soft diet under constant temperature, humidity, and lighting conditions. Recovery after the procedure, rats were returned to their cages to recover from the anesthesia. A veterinarian checked post-operative signs of pain.

Sample preparation for histological examination:

After each study period (2 and 4 weeks), the rats were sacrificed with an intraperitoneal injection of a 5% pentobarbital sodium overdose. Following the sacrifice of the rats, the teeth and surrounding alveolar bone were dissected and immediately fixed in a 10% buffered formalin solution for at least one week. Samples were decalcified and placed in a 12% Ethylene Diamine Tetra-acetic acid (EDTA) solution changed every 48 hours for two weeks. The blocks were then embedded in blocks of paraffin. The embedded specimens were serially cut longitudinally in a mesiodistal direction through the prepared cavity and the pulp. Each section was 5μ thick, displaying the deepest part of the cavity and the underlying pulp. The sections were mounted on slides, and a staining procedure was achieved for standard histological analysis. [(29)]

The staining procedures:

The sections were stained with hematoxylin-eosin and examined with a light microscope to evaluate the histological changes of the pulp tissues, including the inflammatory cell infiltration and reparative dentin bridge formation within the groups. The findings were assessed according to the following criteria. [(30)]

Inflammatory cell infiltration

1. Absent or very few cells
2. Mild: Less than 10 cells
3. Moderate: From 10-25 cells
4. Severe: More than 25 cells

Reparative dentin formation

1. No formation of dentin bridge.
2. Initial formation of dentin bridge covering not more than one-half of the exposure site
3. Partial/incomplete formation of dentin bridge covering more than one-half of the exposure site
4. Complete formation of dentin bridge covering all of the exposure sites

Results

A-Characterization of Nanoparticles:

XRD Analysis reveals the XRD pattern of eggshell nanoparticles after thermal treatment at 1200 ^{o} c and milling as shown in Figure 1.

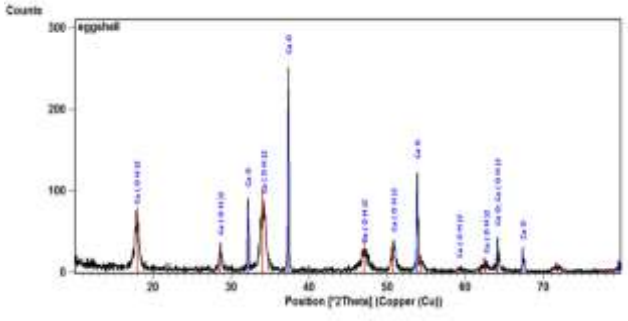


Fig.1: Shows the XRD pattern of eggshell nanoparticles after thermal treatment at 1200 o c and milling

The characterization results of the eggshell nanoparticles revealed the following criteria, the nanoparticles were in the form of white powder. The shape of the nanoparticles revealed by the transmission electron microscope was spherical-like, with an average size of 35-65 nm with the magnification as shown in Figure 2. The manufacture date of the nanoparticles was 7-2020 and expired by 7-2022.

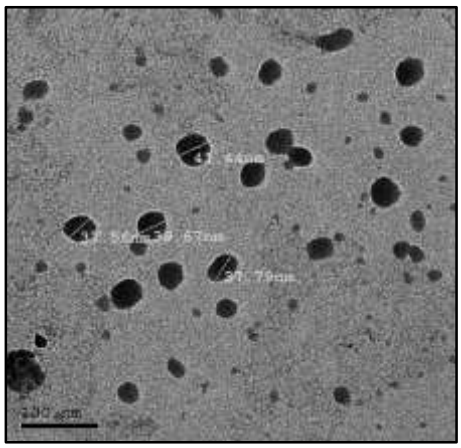


Fig 2: TEM image showing eggshell nanoparticles Characters (magnification x60000), (scale bar 100 nm).

Statistical results:

Capturing microscopic images using SOPTOP EX20 biological microscope (China), HD camera (model No. XCAM1080PHB), and Image view software.

Decalcified H&E-stained sections were examined using the image analyzer computer system applying Q path software for automated counting of inflammatory cells (lymphocytes and histiocytes). Dentin bridge thickness was measured in µm using Image View software.

1) Inflammatory cell count

Comparison between all groups at 2 weeks

The most significant cell count was recorded in the Ca $(OH)_2$ group, whereas the lowest value was recorded in the eggshell nanoparticles group. The difference between all groups was statistically significant ($P<0.001$), according to the one-way analysis of variance (ANOVA)

test. Tukey's post hoc analysis showed no statistically significant difference between the hesperidin and eggshell groups. In contrast, Ca $(OH)_2$ differed significantly from the other two groups (Table 1, Fig.3).

Comparison between all groups at 4 weeks

The most significant cell count was recorded in the Ca $(OH)_2$ group, whereas the lowest value was recorded in the eggshell nanoparticles group. The difference between all groups was statistically significant ($P=0.002$), according to (ANOVA) test. Tukey's post hoc analysis revealed no statistically significant difference between the hesperidin and eggshell groups. While the Ca $(OH)_2$ group differed significantly from the other two groups (Table 2, Fig. 4).

Table (1) Inflammatory cell count at 2 weeks in all groups and significance of the difference using (ANOVA) test.

P.O.C	$Ca(OH)_2$	Hesperidin	Eggshell
Mean	33.2^b	11.6^a	10^a
Std Dev	9.68	4.88	4.69
Std error	4.33	2.18	2.10
Max	43	17	16
Min	22	6	6
F-value			18.051
P-value			< 0.001*

*Significant at $p<0.05$

Tukey's post hoc test means sharing the same superscript letter is not significantly different.

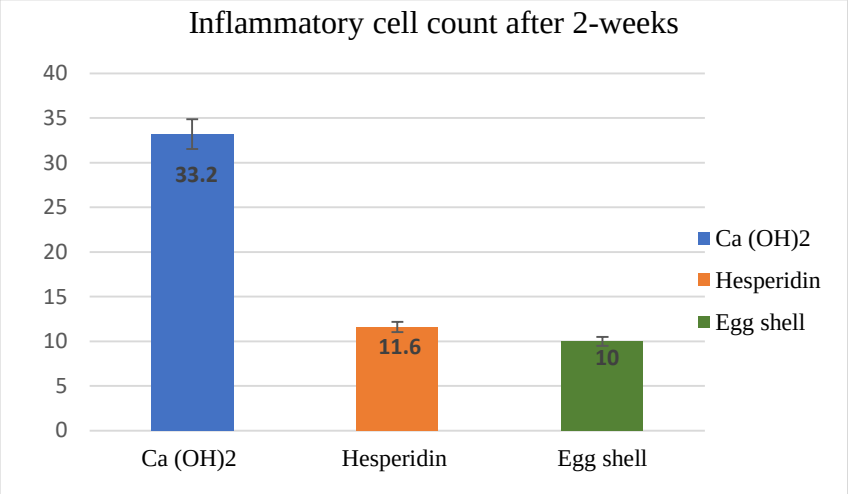


Fig. 3 Column chart showing 2 weeks mean inflammatory cell count in all groups.

Table (2) Inflammatory cell count at 4 weeks in all groups and significance of the difference using (ANOVA) test.

P.O.C	$Ca(OH)_2$	Hesperidin	Eggshell
Mean	13^b	6.6^a	6.4^a
Std Dev	3	3.05	0.9
Std error	1.34	1.36	0.4
Max	16	10	7
Min	9	3	5
F-value			11.061
P-value			0.002*

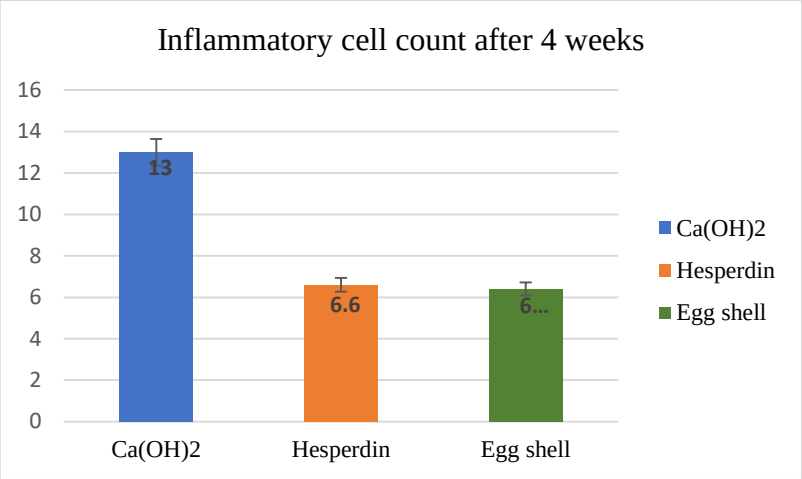


Fig. 4 Column chart showing 4 weeks mean inflammatory cell count in all groups.

2) Dentin bridge

Comparison between all groups at 2 weeks

The most significant thickness of the dentin bridge was recorded in the hesperidin group, whereas the lowest value was recorded in the Ca $(OH)_2$ group. The difference between all groups was statistically significant ($P<0.001$), according to (ANOVA) test. Tukey's post hoc analysis showed that all the groups were significantly different from one another. (Table 3, Fig.5).

Comparison between all groups at 4 weeks:

The greatest thickness of the dentin bridge was recorded in the eggshell nanoparticles group, whereas the lowest value was recorded in the Ca $(OH)_2$ group. The difference between all groups was statistically significant ($P<0.05$) using (ANOVA). Tukey's post hoc analysis showed no statistically significant difference between the hesperidin and eggshell groups. In comparison, Ca $(OH)_2$ differed significantly from the other two groups (Table 4, Fig. 6).

Table (3) Dentin bridge thickness at 2 weeks in all groups and significance of the difference using (the ANOVA) test.

P.O.C	**Ca $(OH)_2$**	**Hesperidin**	**Eggshell(µm)**
Mean	230^{c}	466^{a}	315.5^{b}
Std Dev	2.37	12.95	36.4
Std error	1.36	7.5	21
Max	231.5	479.6	356.7
Min	227.4	453.8	287.5
F-value			85.6
P-value			0.00004*

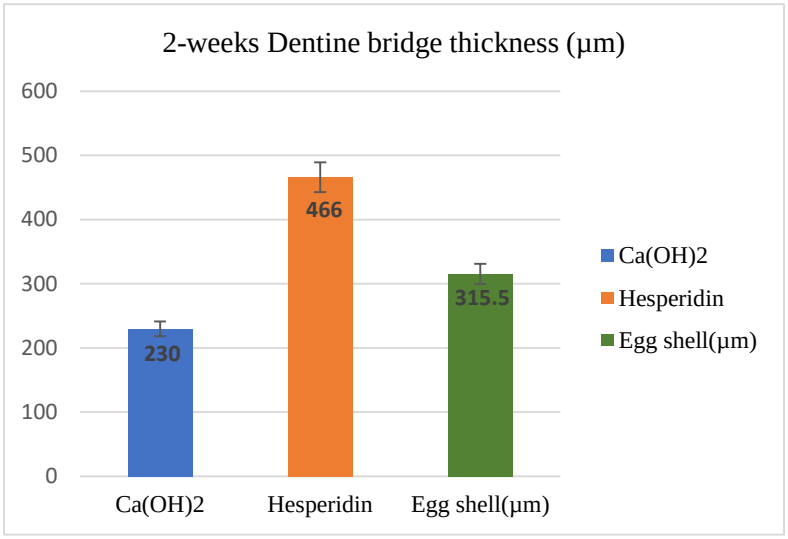


Fig. 5 Column chart showing 2-week mean dentin bridge thickness in all groups.

Table (4) Dentin bridge thickness at 4 weeks in all groups and significance of the difference using (the ANOVA) test.

P.O.C	**Ca (OH)$_2$**	**Hesperidin (µm)**	**Eggshell**
Mean	246.4^b	459.9^a	514.8^a
Std Dev	92.2	23.9	24.9
Std error	53.2	13.8	14.4
Max	352.1	485.8	535.7
Min	182.5	438.8	487.2
F-value			18.6
P-value			0.0027*

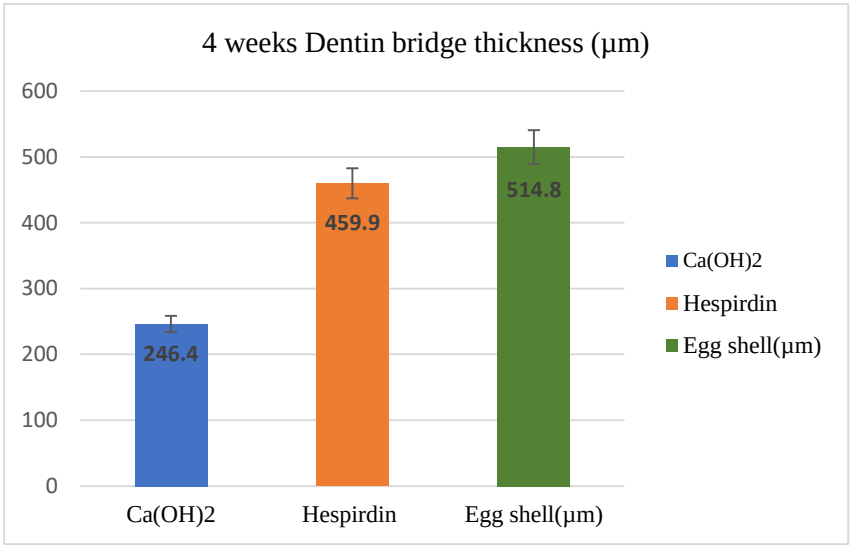


Fig. 6 Column chart showing 4 weeks mean dentin bridge thickness in all groups.

-Histological results:

1-Inflammatory response

Comparison of different groups over the same period: Data were collected, statistically analyzed, and displayed in Table (1,2). At the first interval (2 weeks), a group of ESP nanoparticles showed mild to moderate inflammation with a few numbers of inflammatory cell distributions near the exposure site and dispersed throughout the coronal pulp chamber (Figure 7A). The hesperidin group revealed moderate inflammation with the distribution of inflammatory cells detected below the exposure site, spreading to some extent in the pulp tissue and congested dilated blood vessels (Figure 7B). Areas of focal necrosis were identified near the exposure site of the Ca $(OH)_2$ group, showed many inflammatory cells around the exposure site and dispersed in pulp tissues, and severe inflammation (Figure 7C). At 4 weeks, the ESP nanoparticles group showed mild inflammation with a slight amount of scattered inflammatory cells in the pulp (Figure 8A). The hesperidin group revealed the persistence of mild inflammation with a few numbers of inflammatory cells and dilatation of blood vessels (Figure 8B). Most of the focal necrotic areas in the Ca $(OH)_2$ group had partially healed and revealed a moderate inflammation with some number of inflammatory cells still present (Figure 8C). Moreover, the odontoblast layers in the three groups showed disruption and non-organized near the exposed sites (Figure 8A, B, and C).

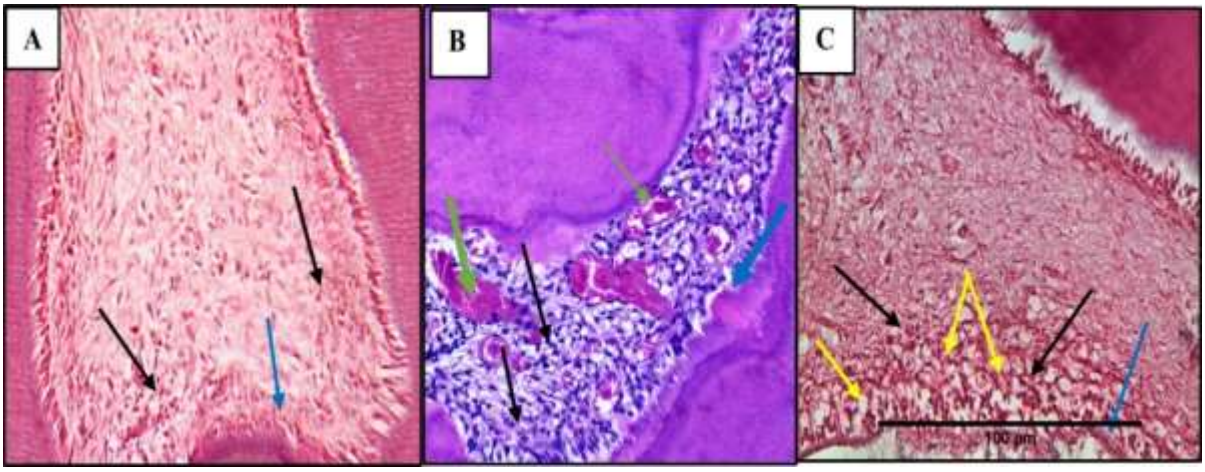


Fig. 7. Photomicrographs of L.S sections of coronal parts beneath the sites of exposure (A) ESP nanoparticles group demonstrated mild infiltration of inflammation cells scattered to some extent in the pulp tissue (black arrow). (B) Hesperidin group showed mild to moderate inflammatory cell infiltration (back arrow) near the exposure site and congested dilated blood vessels (green arrows). (C) Ca $(OH)_2$ group demonstrated severe inflammation and many inflammatory cells dispersed in the pulp tissue (black arrow) and areas of focal necrosis (yellow arrows). Odontoblastic layers were disrupted in three groups at the exposure site (blue arrows) (at 2 weeks) (H&E, Org. Mag. ×200, scale bar =100µm).

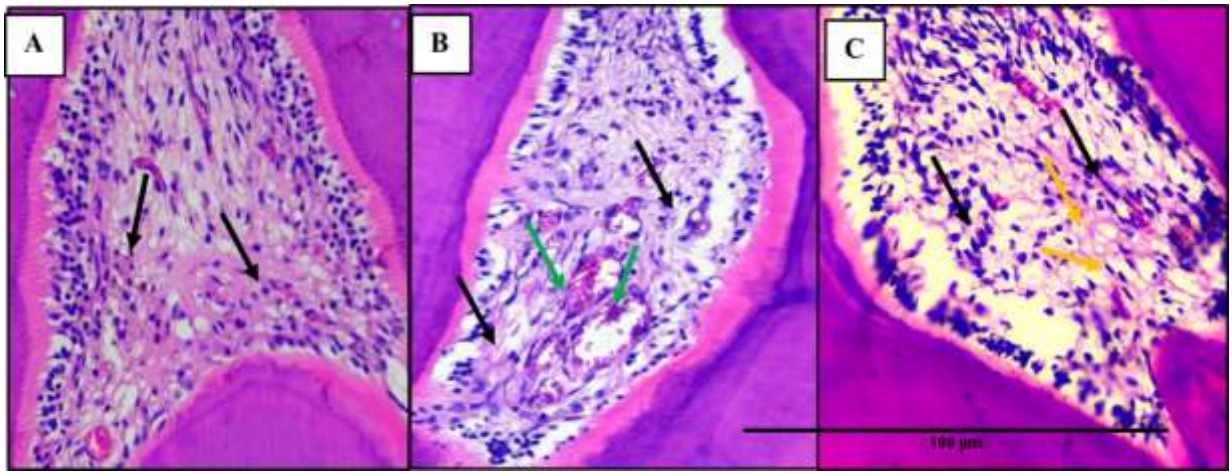


Fig. 8. Photomicrographs of L.S sections of coronals part beneath the sites of exposure (A) ESP nanoparticles group revealed a mild amount of dispersed inflammatory cells in the pulp (black arrow), (B) Hesperidin group demonstrated persistence of mild amount of inflammatory cells (black arrow), and blood vessels dilatation(green arrow), (C) Ca $(OH)_2$ group showed persistence of moderate amount of inflammatory cells (black arrow), with incomplete healing of most areas of focal necrotic(yellow arrows), (at 4 weeks) (H&E, Org. Mag. ×200, scale bar =100µm).

Dentine bridge formation

Comparative analysis of various materials in three groups during the same period

Data were collected, statistically analyzed, and displayed in Tables (3 and 4). At the first interval (2 weeks), a partially incomplete calcified bridge was noticed in the three groups. ESP nanoparticles and Hesperidin groups showed incomplete dentin bridge deposits at the site of exposure (Figure 9A, B). While the Ca $(OH)_2$ group showed thin initial calcified deposits with scattered globules around the capping material, demonstrating a feeble attempt at hard deposit formation. Some samples showed cellular incorporation that resembled bone lacunae in appearance (osteodentin) (Figure 9C). At 4 weeks interval, a complete amorphous non-tubular thick dentin bridge formation around the site of exposures of two groups (ESP nanoparticles and Hesperidin groups) (Figure 10A, B) with the greatest thickness observed in the ESP nanoparticles

group and lowest thickness in Ca (OH) $_2$ group and showed non-tubular, non-amorphous interrupted dentin bridge with tunnel defects (Figure 10C). Moreover, all groups showed new odontoblastic and predentin layers at the area of the newly formed dentin bridge at the exposure site.

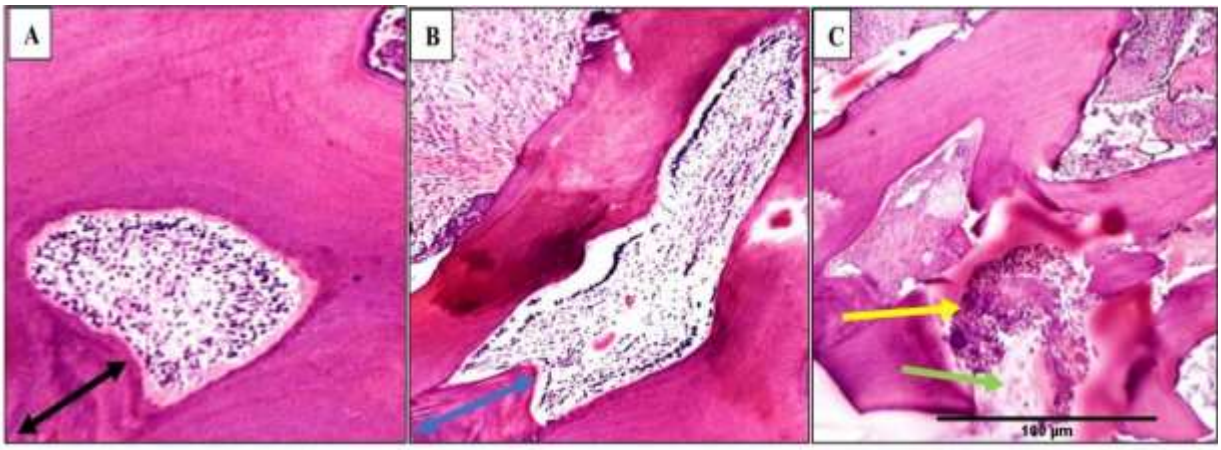
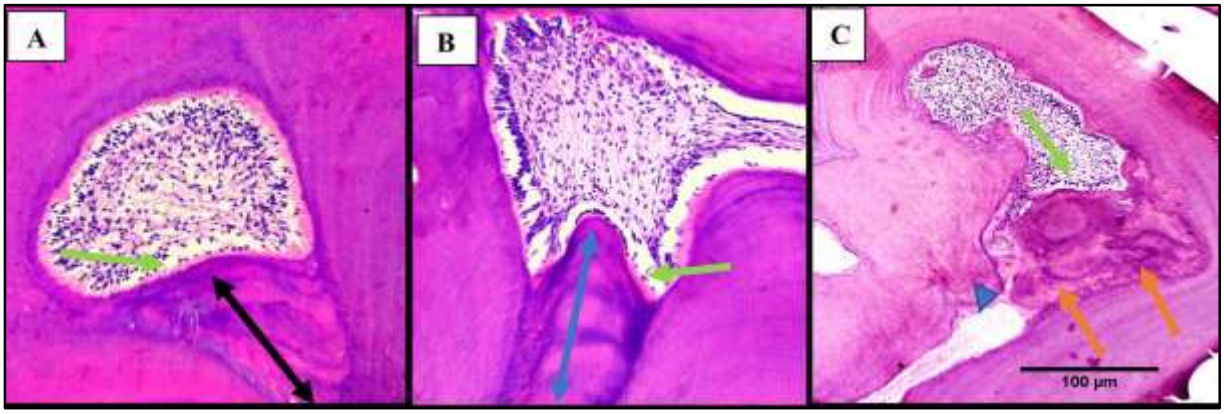


Fig. 9. Showing dentin bridge formation at 2 weeks, Photomicrographs of L.S sections of coronal parts around the exposure site (A) ESP nanoparticles group showing newly formed reparative dentin bridge (dentin bridge) (black arrow). (B) Hesperidin group showing dentin bridge (blue arrow). (C) Ca (OH) $_2$ group showed the incomplete formation of dentin bridge with dystrophic calcification (yellow arrows), and inclusion cells inside



newly formed reparative dentin bridge (green arrow) (H&E, Org. Mag. ×100, scale bar =100µm).

Fig. 10. Demonstrated reparative dentin bridge formation at 4 weeks around the exposure site Photomicrographs of L.S sections of coronal parts (A) ESP nanoparticles group showing thick amorphous dentin bridge (black arrow) and completely closing the exposure site., (B) Photomicrograph of Hesperidin group showing dentin bridge (blue arrow). (C) Photomicrograph of Ca (OH) $_2$ group showed a non-amorphous interrupted dentin bridge (yellow arrows) with tunned defect (arrowhead) and well-organized odontoblast cell and predentin layers (green arrows) in three groups (H&E, Org. Mag. ×100, scale bar =100µm).

Discussion

Biomaterial advancements are the focus of dental research. In recent years, several new biomaterials have been developed for direct pulp capping materials to maintain the health and functionality of dental pulp. Before moving on to human trials, a pulp capping agent must pass animal testing. [31] Thus, rat molars

were employed to evaluate our study's biological and histological results of direct pulp capping. Because of their anatomical and histological similarities to human molars, the process by which the rat pulp healed after direct pulp capping is similar to the mechanism in humans. [3, 32] The purpose of pulp capping material is to prevent bacteria from penetrating the pulp, improve dentin formation, and maintain healthy pulp tissue. Several factors influence healing after pulp exposure, including pulp vitality, bacterial invasion, the size of the injury, and the effect of capping materials. [33, 34]

We chose histological investigation to evaluate pulp capping results in this study because it is still the crucial and traditional method for assessing pulp capping results, as mentioned by several previous researchers [33-37]. Our study's principles for histological assessment included inflammatory response and dentin bridge formation as measured by Hematoxylin and Eosin stains. The first (2-week) period was chosen to assess the pulp tissue response. However, as forming a complete new dentine bridge takes longer than 2 weeks, the second period (4 weeks) was chosen to determine the hard tissue barrier formation. This study compared the effects of ESP nanoparticles and hesperidin as pulp-capping agents on pulp exposure of rat molars to the commonly used calcium hydroxide-based agent.

It has been debatable how inflammation affects pulpal healing and regeneration. [38-40] While some authors have previously claimed that inflammation only has a negative impact since it raises the possibility of pulp necrosis, others have discovered that inflammation plays a critical and beneficial role in tissue repair. [41] Therefore, any material intended to be applied immediately over pulp exposure should limit the inflammatory response and stop before pulp necrosis is established. [(34)] In ten-year follow-up studies, the success rate of cases directly capped with calcium hydroxide was 30-85 percent. [42] As a result, new capping materials were developed to overcome the disadvantages of calcium hydroxide.

In our study, the Ca $(OH)_2$ group had the highest pulp inflammation level at 2 and 4 weeks. Ca $(OH)_2$, a highly alkaline compound, causes a large area of inflammation and stimulates the pulp defense mechanism and repair. A necrotic area was observed two weeks after Ca $(OH)_2$ capping, lacking differentiated odontoblasts. Inflammatory processes were still visible after 4 weeks. As a result, distinct differences between the three capping materials were identified. These findings corroborated Schröder et al. observation [43] and Yasuda et al. [44], who found that histological investigations of Ca $(OH)_2$ capping material induce inflammation and tissue response begins with the migration of inflammatory cells and dilatation of blood vessels. In addition to the initial impact of Ca $(OH)_2$ on the exposed pulp is the development of superficial tissue necrosis due to chemical damage caused by hydroxyl ions. Moreover, Ca $(OH)_2$ has the disadvantage of providing a poor seal, and its self-cure preparations are soluble and susceptible to dissolution over time. [45]

Hesperidin-capped specimens in the current investigation showed moderate inflammation, which is less than Ca $(OH)_2$ at 2 weeks and 4 weeks, which was statistically significant ($P< 0.001$), where hesperidin is considered the most crucial constituent of propolis. The ability of propolis to reduce inflammation and support the immune system by encouraging phagocytic activities and increasing cellular immunity through the release of free radicals. It destroys the bacterial cell wall and cytoplasm and stops the division of bacteria. [46] Our findings were consistent with those of Abo El Mal et al. [47] demonstrated that 86 percent of samples covered with hesperidin capping material at both 2 and 4 weeks showed mild and moderate pulp tissue inflammation in the pulp chamber of dogs. The results of this study revealed that the eggshell group had mild pulp inflammation in both periods of the study compared to the Ca $(OH)_2$ group, with a highly significant difference between them. However, there is no

statistically significant difference between the hesperidin and eggshell groups. These findings could be accounted for by the relatively mild alkaline pH of ESP nanoparticles in contrast to the highly alkaline pH of Ca $(OH)_2$. The Ca $(OH)_2$ group experienced a more marked inflammatory reaction due to the high alkaline pH, which persisted throughout the study. Vuong et al. [48] also confirmed that decreasing the pro-inflammatory cytokine TNF-α mediates the ESP effect. It exhibited anti-inflammatory and immunomodulatory properties in lipopolysaccharide-activated human monocytes and macrophage-like cells via NF-κB intervention.

Our findings are consistent with those of Salah et al. [24] who revealed that rabbits' eggshell pulp capping specimens showed significantly less inflammation in all periods of the experiment compared to the Ca $(OH)_2$ group.

Regarding dentin bridge formation, in our study, partial dentine bridge formation was detected in the first interval (2 weeks) in all samples due to the short time for dentinogenesis, whereas an improvement in continuity and thickness by the second interval (4 weeks). This result agreed with the findings of Salah et al. [24] and Njeh et al. [49] On the other hand, Al-Sherbiny et al. [37] and Abo ElMal et al. [46] demonstrated that dentine bridge, whether partial or complete, was not detected before one month and showed improvement in continuity and thickness by 2 months. This result might be due to the variation in the animal model used for histopathological analysis; those authors used dogs in their research findings, whereas the current study used rodents (rats).

The current study's histologic data revealed ESP nanoparticle-capped teeth at the second interval (4 weeks) have the greatest calcific barrier thickness, surrounded by a well-organized odontoblastic layer with minimal inflammation. Moreover, the ESP group formed significantly thicker bridges in the first and second intervals than the Ca $(OH)_2$ group. The findings of the current study, supported by the previous study [24], suggested that the eggshell group formed considerably thicker bridges than the Ca $(OH)_2$ group at all-time intervals (1, 2, and 4 weeks). These results may be explained by the fact that ESP powder includes magnesium and tiny amounts of fluoride. Mg is necessary for cellular and enzymatic reactions; furthermore, in vitro and in vivo experiments revealed that Mg ions integrated into apatite crystals may help to facilitate the adhesion of osteoblasts to apatite and support the formation of bones. [50] Fluorine is a well-known essential trace element for developing teeth and skeletons. Fluorine can also substitute some hydroxyl groups, creating fluoridated hydroxyapatite, which has a more solid structure than hydroxyapatite and a slower dissolution rate than hydroxyapatite, thus improving physiological stability. [51]

The results of the hesperidin group at the first interval (2 weeks) showed the greatest dentin bridge formation between the three groups. The presence of hesperidin can explain that, as a significant component of propolis may promote the formation of hard tissue bridges by enhancing various enzyme systems, cell metabolism, circulation, and collagen synthesis. These effects have been linked to provitamin A and B complex, arginine, vitamin C, and trace minerals like zinc, copper, and iron. [52] The stabilized collagen matrix also served as a mechanical barrier to mineral diffusion, preventing demineralization and promoting remineralization. Dentin collagen acts as a scaffold for the deposition of minerals, so preserving and maintaining it may be essential during the remineralization process. [19] It also encourages stem cells to produce effective tubular dentin. [53] As a result, the current study hypothesized that hesperidin could be a beneficial natural material for pulp capping. While in the Ca $(OH)_2$ group, the formed reparative dentin bridge is not due to the effect of the Ca $(OH)_2$ capping material but rather as a result of the pulp's defense mechanism due to the severely irritating properties of Ca $(OH)_2$. [54] Because of its high alkalinity (pH 12.5), Ca $(OH)_2$ has been thought to exhibit its mechanism of action by forming a

layer of necrotic tissue and indirectly triggering an inflammatory response to establish a barrier of calcific material. [55] Furthermore, the dentin bridge was completed in the second interval (4 weeks) but with a tunnel defect. Also, $Ca(OH)_2$ capping promotes the formation of a heterogeneous reparative dentinal bridge as osteodentin, which encourages bacteria recontamination and produces a poor seal. As a result, the capped zone becomes exposed over time, stimulating cavity reinfection. [56]

Conclusion:

Under the conditions of the present study, ESP nanoparticles as pulp capping material resulted in a slightly better pulpal response and dentin bridge formation than hesperidin, even though there was no statistically significant difference between them. Hence, eggshell nanoparticles and hesperidin might present some clinical advantages over $Ca(OH)_2$ capping. So, they may be considered promising biocompatible materials for pulp regeneration.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

This study protocol was approved by the ethical committee of the Faculty of Dentistry, Ahram Canadian University, on the 2nd of October 2022, approval number (#A0001).

Conflict of interest:

No conflict of interest

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors**.**

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