Response of Exposed Pulp to Capping with Mineral Trioxide Aggregate Mixed with Hyaluronic Acid as a Water Substitute

Muthanna S. Ahmed 1, Nadia H. Hasan 2, Mohammed G. Saeed 3, Aous A. Abdalmajeed 4

1 Ministry of Health/ Nineveh Health Directorate
2 Department of Conservative Dentistry, College of Dentistry, Mosul University / Iraq
3 Department of Pathology and Poultry Diseases, College of Veterinary, University of Mosul, Mosul, Iraq
4 Department of General Practice, School of Dentistry, Virginia Commonwealth University, Richmond, Virginia, USA

Abstract
Aims: The study aims to evaluate the healing of exposed pulp immunohistochemically by using hyaluronic acid (HA) with MTA by evaluating collagen III expression rate.

Materials and method: Ninety teeth were used from 10 dogs to perform an experimental pulp exposure. The samples were divided into three groups according to the mixing medium with MTA: group I: MTA + distilled water (control group), group II: MTA + hybrid cooperative complex hyaluronic acid (HCC-HA), group III: MTA + high molecular weight hyaluronic acid (HMW-HA). After pulp capping, all cavities were restored with glass ionomer restoration. The dogs were divided randomly into five groups (2 dogs each) according to the evaluation periods (7,14,21,30,60) days. At the end of the study, the dogs were euthanized and the sampled teeth were processed for immunohistochemical investigation to evaluate collagen III expression rate by Kruskal-Wallis Test using Pairwise Multiple Comparison Tukey Test with significant level set on P ≤ 0.05.

Results: Both types of hyaluronic acid (HCC-HA, HMW-HA) showed a statistically higher expression rate of collagen III than using distilled water with MTA.

Conclusions: within the limitations of this study, using HA for mixing with MTA increased collagen III expression, which can be explained as an increase in the healing process. HA could be an effective water substitute for mixing with MTA for direct pulp capping.

الخلاصة
الأهداف: تهدف الدراسة إلى تقييم رد فعل اللثة نتيجة لتعتيم الفم بالتطبيقات التالية: المواد وطرق العمل: تم استخدام سبعين من 10 كلاب لإجراء تعبير لتعتيم الفم. تم تقسيم الحالات إلى ثلاث مجموعات وفقًا لمستويات الخلط مع مادة مختبطة مع نتائج الفم مع مادة مختبطة مع HA. بعد تقييم الفم، تم تقسيم الكلاب عشوائياً إلى خمس مجموعات (2 كلاب لكل مجموعة) وفقًا لفترات التقييم (7, 14, 21, 30, 60) يوماً. في نهاية الدراسة، تم الاقتنع بالجراحات، وتتطلب معالجة الأسئلة التي تم أخذ عينات منها من أجل التحقق من الكيمياء المتقدمة مع مادة مختبطة مع HA.

النتائج: كلا النوعين من HA كانا يزيدان من معدل التعبير على HA والكلاب مع HA. يمكن أن يكون مفيدًا فعالًا لحل خلط مع MTA لسد الفم بشكل مباشر.

DOI: 10.33899/RDENJ2024.147417.1250, © Authors, 2024, College of Dentistry, University of Mosul
This is an open-access article under the CC BY 4.0 license (http://creativecommons.org/licenses/by/4.0/)
INTRODUCTION

One of the main protocols in managing dental pulp injuries is Direct pulp capping (DPC) which is a part of Vital pulp therapy (VPT) protocols. It helps to preserve the functional properties and vitality of the pulpal tissue. In the last years, researchers have been focused on producing or developing dental materials that enhance the healing and repair of the exposed pulp (1). Since Pereira Paula et al. described the first pulp capping procedure, many materials have been applied for DPC (2). The use of calcium hydroxide (CH) has been reduced due to multiple drawbacks as poor adhesion sealing properties, the possibility of dissolution with time, and incomplete reparative dentin formation with multiple tunnel defects (3). Mineral trioxide aggregate (MTA) is one of the biomaterials that can replace CH for DPC with a higher success rate (3-7).

Many trials have been implicated to enhance the biological and healing properties of MTA by adding materials to modify the composition of MTA; despite the encouraging physical outcomes of these trials, the histological and immunohistological results were not encouraging (8).

Hyaluronic acid (HA) is a repeated disaccharide glycosaminoglycan (GAG). Native HA is found in the human body as a main compartment of the ECM and other vital tissues (9,10). In the tissues of the oral cavity, HA can be found in its native form throughout the periodontal ligaments, gingival tissues, alveolar bone, and cementum (9,11). Studies have reported that HA has an effective role in the regulation of cellular activities, differentiation, proliferation, migration, survival, and cellular motility (12,13). Because of its biological properties, HA is considered industrially an important biopolymer in biomedical research (14).

Dentin is a mineralized matrix composed of collagen that consists of approximately 30–50% by volume organic compound and about 20% by volume of water (15). In human teeth, collagen type I and III form the main tissue collagen in the pulp tissue. A large portion is type III collagen which is about (43%) of the total collagen amount (16). Expression of collagen III can be assigned as the first step during the initial phase of the healing process in hard and soft tissues (17). The mineralization of collagen fibrils in the presence of apatite forms the basis of the development of hard tissues such as dentin and bone (18,19). The mineralized ECM of dentin consists of complex macromolecules that arrange in the form of a 3D network, which consists of GAG, mineralized collagen fibrils, glycoproteins, proteoglycans (PGs), phosphoproteins, proteases, and growth factors (18).

The collagenous proteins, especially collagen I and III constitute the main macromolecules in the ECM of pulp tissue. While hyaluronic acid (HA) constitutes the main proteoglycan component (20). The immunohistochemical investigation by Magloire et al. (17) reported active expression
for collagen III antibodies in pulp tissues that produced reparative dentin.

However, to the best of our knowledge, to date, no study has evaluated in vivo the effect of hyaluronic acid as a mixing media for MTA, on collagen III rate after direct pulp capping. Therefore, this study aims to evaluate the effect of mixing MTA with hyaluronic acid instead of distilled water on reparative dentin formation by studying the immunohistochemical expression of collagen type III after direct pulp capping in dog teeth.

**MATERIALS AND METHODS**

**Ethical approval**

This study obtained approval from the scientific board, college of dentistry, university of Mosul, Mosul, Iraq (UoM.Dent/H.DM.16/23). It also followed the ARRIVE guidelines and the Three Rs (3Rs) rule that forms the basis of the regulations and ethical approval for animal use in scientific research.

**Experimental design**

For justification and standardization reasons, multiple teeth were used in the same animal. In this study, ten healthy male local breed male dogs were used. These dogs were weight (20± 0.5 kg) and aged (12± 0.6 months) old to ensure the complete histological development of the dental pulp. Each dog is housed in separate cages and observed for 2 weeks with a periodic medical examination by a veterinarian consultant to exclude any diseased animal.

The total sample size was 90 teeth randomly selected from anterior to premolar. The samples were divided into three groups of 30 teeth each according to the medium that was mixed with MTA:

I. **Control group:** MTA mixed with distilled water according to the manufacturer's instructions.

II. **HCC-HA group:** MTA mixed with HCC-HA.

III. **HMW-HA group:** MTA mixed with HMW-HA.

These groups were further subdivided into five subgroups according to the evaluation period (7, 14, 21, 30, 60) days. Each period included 2 dogs (9 teeth from each dog / 3 teeth for each group per dog) according to the postoperative observation period.

**Experimental procedures**

The split-mouth technique was applied in the current study so that all medicaments could be evaluated in the same animal with a similar environment on alternate sides of the mouth and jaws and for justification reasons.

Before starting the experiments, the selected teeth were radiographed using portable digital dental radiography (Rextar -X, posdion, South Korea) and digital radiograph sensor (Carestream RVG5200, Carestream dental LLC, USA) at 62kv and 10 mA with an exposure time of 0.04 s to ensure complete apical growth and the absence of any periapical pathology.
Anesthesia
After fasting the dogs for 12 hours, the surgical operations were performed after induction of general anesthesia that was administrated intramuscularly to the animals including a combination of xylazine (5mg/kg) (LIDXY, Alfasan, Woerden, Netherlands) (conc. 2%) and Ketamine (5mg/kg) (LIDXY, Alfasan, Woerden, Netherlands) (conc.10%) (21).

Operative procedure
After the rubber dam application, nine teeth from each dog were polished slowly with a rubber cup and prophylaxis paste to remove any debris, then disinfected with povidone-iodine solution (10%). Class V cavities of diameter 1.5–2.5 mm that is located (2.5–3 mm) above the margin of the free gingiva in a parallel direction to the junction of cementoenamel area were prepared on the facial side of the teeth using sterile tungsten carbide round burs ISO #806 314 001534 012 and a straight fissure bur with ISO #500 314 107006 008 (Komet, Lemgo, Germany) mounted on an ultra-high-speed turbine that was cooled with copious air/water spray. To ensure sterility of the operated site, each bur is used for only 1 cavity. When the pulp chamber redness was visible, the pulp was exposed in a pin-point exposure by a sterile hand file (F5 ProTaper Universal Finishing file, DENTSPLY Maillefer, Switzerland) with a tip diameter of 0.50mm and a taper of 5%. When the hand file advanced 1mm from the roof of the shiny cavity, the diameter of the exposure sites was standardized to about (0.8-1) mm in diameter, sterile saline (2.5 ml) was used to rinse the cavities, then dried with light pressure on sterile cotton pellets to control any bleeding. Then the exposure site was capped with one of the experimental. The remains of the cavities in all groups were immediately restored by glass hybrid restorative material (Equia Forte HT Fil glass hybrid restorative material, GC, Tokyo, Japan). To minimize pain and discomfort, post-operative medication of nonsteroidal anti-inflammatory drug (2 mg /kg) of ibuprofen was administered.

Euthanizing of the animals
At the end of the study period, the dogs were euthanized following the protocol of the Panel on Euthanasia of the American Veterinary Medical Association with intramuscular injection of 2.2 mg/kg ketamine, 0.22 mg/kg xylazine-100, and 2 mL Beuthanasia-D (Merck Animal Health, Millsboro, MI, USA) (22).

Samples preparation and immunohistochemical processing
The maxilla and mandible were surgically removed then dissected and separated into four quadrants using an electric saw to accelerate the decalcification process. The sampled teeth were sectioned from the dissected jaws. The sections include the operated teeth and parts of the soft tissue surrounding them. Then they undergo a fixation process with 10% prepared neutral buffered formalin for 48 hours, followed by decalcification with formic acid for 14 days, then 10% EDTA at room temperature for 120 days. During that
period, the decalcifying solution was changed by a fresh mix every 48 hours.

The specimens were processed by using an open processing system in which the specimens were then dehydrated in ascending grades of ethanol and cleared in xylene. The specimens were then blindly coded and embedded in molten paraffin wax (56 °C) overnight. Consecutive block sections of 5 μm thickness (Richert – Jung,2030 –mot Biocut microtome.ss).

Immunohistochemistry

On charged slides, Paraffin-embedded slices were deparaffinized, dehydrated, and washed using PBS. Then the slides were incubated for 10 minutes in 3% hydrogen peroxide-methanol solution. Washed in PBS at pH 7.4. Then, 0.5% goat serum was used as a blocking agent. Then, the slides were incubated at 4°C overnight with primary antibody using collagen III canine Polyclonal (dilution: IHC 1:50-1:200; Elabscience®, USA).

Two calibrated examiners evaluated the slides according to Benetti et al. (23) scoring system (Table 1).

<table>
<thead>
<tr>
<th>Score</th>
<th>Score to immunohistochemical expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No Immunolabeling (the extracellular matrix shows no labelling with a complete absence of immunoreactive tissue).</td>
</tr>
<tr>
<td>1</td>
<td>Low immunolabeling expression (the extracellular matrix shows weak labeling with approximately one-quarter of the immunoreactive tissue).</td>
</tr>
<tr>
<td>2</td>
<td>Moderate immunolabeling expression (the extracellular matrix shows moderate labelling with approximately one-half of the immunoreactive tissue)</td>
</tr>
<tr>
<td>3</td>
<td>Strong immunolabeling expression (the extracellular matrix shows strong labelling with approximately three-quarters of the immunoreactive tissue).</td>
</tr>
</tbody>
</table>

Micro morphometric measurements

Color USB 2.0 digital image camera (Omax ToupView 9.0-Megapexil China) was used to measure the parameters. The camera was supplied with software for image analysis and processing. A stage micrometer (ESM-11 / Japan) of 0.01mm was used to calibrate the software of the camera to all lenses of Microscope Olympus-CX31 that was attached to the lens of the microscope.

Statistical analysis

A computer package (Sigma Stat V12.0 / SYSTAT software) was used for immunohistochemical data analysis. The non-parametric data of the immunohistochemical scores were analyzed as median and IQR(Inter-Quartile-Range) by Kruskal-Wallis Test using Pairwise Multiple Comparison Tukey Test with a significant level set on \( P \leq 0.05 \).

RESULTS

Among the 90 teeth, 9 teeth were excluded from the analysis, comprising two from the control group, two from group II (30 days), two from group II, one from group III (60 days), and two from group III (60 days). The exclusions were either because of the loss of the coronal restoration, loss of the tooth as the...
dog tried to break the cage by biting the metal mesh, or an error during processing. **Collagen III expression**

**After 7 days**
The control and the HCC-HA groups showed negative reactions. While the HMW-HA group revealed a weak positive reaction (figure 1). There was a statistical difference between HMW-HA group and the control group ($p = 0.029$).

![Figure 1: Histological section of the dog's pulp-dentin area (7 days) immunohistochemistry expression for Collagen III. (A): control group (hole without treatment) reveals a negative reaction (0). (B): HCC-HA group reveals negative reaction (0). (C): HMW-HA group reveals weak positive reaction (1). (positive reaction appeared dark brown color). 100X.](image1)

**After 14 days**
The control group still represents a negative reaction (0). While the HCC-HA group expressed a moderate reaction. The maximum rate showed for the HMW-HA group that expressed an intense positive reaction (Figure 2). There was a statistical difference between both HA groups and the control group ($p = 0.018$).

![Figure 2: Histological section of the dog's pulp-dentin area (14 days) immunohistochemistry expression for Collagen III. (A): control group (hole without treatment) reveals a negative reaction (0). (B): HCC-HA group reveals moderate positive reaction (2). (C): HMW-HA group reveals intense positive reaction (3). (positive reaction appeared dark brown color). 100X.](image2)

**After 21 days**
An elevation in the expression rate for both the control group which expressed a moderate positive reaction and for the HCC-HA with an intense positive reaction. The rate of HMW-HA group declined to a moderate value (Figure 3). There was a statistical difference between HCC-HA group and the control group ($p = 0.004$).

![Figure 3: Histological section of the dog's pulp-dentin area (21 days) immunohistochemistry expression for Collagen III. (A): control group (hole without treatment) reveals moderate positive reaction (2). (B): HCC-HA group reveals intense positive reaction (3). (C): HMW-HA group moderate positive reaction (2). (positive reaction appeared dark brown color). 100X.](image3)
After 30 days
All groups revealed a moderate value in which the HCC-HA group declined in rate in comparison to the previous evaluation period (Figure 4). There was no statistical difference between all the groups.

Figure 4: Histological section the dog's pulp-dentin area (30 days) immunohistochemistry expression for Collagen III. (A): control group (hole without treatment) reveals moderate positive reaction (2). (B): HCC-HA group reveals moderate positive reaction (2). (C): HMW-HA group moderate positive reaction (2). (positive reaction appeared dark brown color). 100X.

After 60 days
Both the control and HCC-HA groups showed a moderate reaction as for the previous evaluation period with an elevation of the collagen III reaction for the HMW-HA group to an intense value (Figures 5,6). There was a statistical difference between the HMW-HA group and the control group ($p = 0.018$).

Figure 5: Histological section of the dog's pulp-dentin area (60 days) immunohistochemistry expression for Collagen III. (A): control group (hole without treatment) reveals moderate positive reaction (2). (B): HCC-HA group reveals moderate positive reaction (2). (C): HMW-HA group intense positive reaction (3). (positive reaction appeared dark brown color). 100X.

Figure 6: Histogram shows the scores of the immunohistochemistry expression of collagen III.
DISCUSSION
The objective of the present study was to investigate the biological activity of replacing distilled water with HA for mixing with MTA by evaluating the collagen III expression rate. To the best of our knowledge, to date, no study has evaluated the use of MTA mixed with HA as a pulp capping material for VPT, with a limited clinical investigation that has evaluated the production of collagen type III as an organic constituent after direct pulp capping.

The dogs were used as animal models in the current study as the reparative dentin formation and healing mechanisms are the same as those in humans with a short time. In addition, the size of the pulp and pulp chamber in dog teeth is suitable to perform clinical, histopathological, and immunohistochemical evaluations.

In addition, a suitable number of teeth anterior and posterior helps to make a comparison of multiple capping materials within the same dog (24). The selection of class V cavities was to avoid dislodgment of the occlusal load on the tested materials and for easy standardization of the cavities location and materials handling (25).

Five evaluation periods were used (7, 14, 21, 30, and 60 days) to evaluate the primary, intermediate, late, and final tissue response to the capping materials and the mineralization rate of the tested materials as cross-linked-HA needs a long time to be degraded. Therefore, the evaluation periods were extended to 60 days to monitor the effect after the degradation process.

The extracellular matrix (ECM) of the dental pulp is a loose network composed of molecules such as collagen I and III (26,27). Which is required to support and preserve the texture composing the cells of the dental pulp, in addition to regulation and preservation of vital biological activities such as mineralization deposition (28).

The analysis of collagen III collagen expression and function in the human dental pulp has not yet been fully done. Most data related to the expression of collagen in dental pulp are about collagen I. Therefore, we performed an immunohistochemical investigation for collagen III antibody expression to demonstrate the effect of using HA as a water substitute on healing potential of MTA. During the initial phase of healing, collagen type III is the first substance that is synthesized in the wound area of various hard and soft tissues (29). Essentially, collagen III is expressed in tissues of the pulp (30) and they are often seen in the unphysiological state of dentin, for example, in reparative dentin (17).

Collagen III interacts with cell surface receptors like integrins to enhance cellular differentiation, migration, proliferation, and adhesion, which is required for odontoblast-like cell differentiation and migration towards the exposure site to produce reparative dentin bridge (31).

In this study, collagen III formation was interpreted as a positive sign of healing according to the
immunohistochemical results that showed an early activity of collagen III secretion for the HMW-HA group which refers to the beginning of a reparative phase in an earlier period than using distilled water for mixing. Asparuhova et al. (32) reported that HA has a direct role in increasing the expression of type III collagen genes encoding. This expression remains to elevate within the 14 days of the experiment for HA groups, while the distilled water group still shows no deposition of collagen III during this period. Previous research reported that HCC HA showed increases in the expression levels of collagen III that support the healing process (33,34).

The elevation in collagen III expression for the HMW-HA group continued even after 60 days due to the long time required for the degradation of HA giving it a longer effective time on the targeted cells. In vivo experiments suggest that using HA alone or combined with other biomaterials enhances the foundation of the environment required for the induction and development of reparative dentin by enhancing the differentiation of MSCs (35,36).

HA proved to have a major role during all steps of the wound healing process (37). It can be classified as a biomaterial that has no negative effect on the differentiation, viability, and proliferation potential of DPSCs (38,39). Damodarasamy et al. (40) reported that HA enhances wound repair and increases collagen III expression. HA increases the expression rate of collagen III and subsequently enhances pro-proliferative, pro-migratory, and pro-inflammatory factors in fibroblasts (41).

The immunohistochemical results of the current study showed that both types of HA can be effectively used with MTA as a mixing medium to substitute distilled water with a better immunohistochemical result. When using HA, higher collagen III expressions were recorded.

CONCLUSION

Conclusions of the current study found that using HA as a substitute for water for mixing with MTA resulted in higher expression of collagen III antibody, which is the marker of wound healing. Both types of HA (HCC, HMW) can be used as a scaffold for dentin remineralization with an early repair rate as it is readily available with a premixed ready formula.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

REFERENCES


