Original Article

Immunohistochemical study of mixing mineral trioxide aggregate with hyaluronic acid as a pulp-capping agent in dog teeth

Muthanna S. Ahmed, Mohammed G. Saeed[1], Nadia H. Hasan[2]

Specialized Dental Center, Ninawa Health Directorate, [1]Department of Pathology and Poultry Diseases, College of Veterinary, University of Mosul, [2]Department of Conservative Dentistry, College of Dentistry, University of Mosul, Mosul, Iraq

Abstract

Objective: The purpose of this study was to evaluate the immunohistochemical effect of hyaluronic acid (HA) on the mineralization rate of the reparative dentin when it is used as a mixing medium with mineral trioxide aggregate (MTA).

Materials and Methods: Direct pulp capping (DPC) was performed on 90 teeth from 10 dogs that had been experimentally exposed. The exposed pulps 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 HA (HCC-HA), Group III: MTA + high molecular weight HA (HMW-HA). After pulp capping, all cavities were restored with final restoration. The dogs were divided randomly into five groups (two dogs each) according to the evaluation periods (7, 14, 21, 30, and 60) days. At the end of the study, the dogs were euthanized, and the sampled teeth were processed for immunohistochemical investigation.

Results: Both types of HA (HCC-HA, HMW-HA) showed an increase in the expression of alkaline phosphatase (ALP) at a higher rate than using distilled water with MTA.

Conclusions: Within the limitations of this study, HA proved to be an effective additive to MTA for DPC.

Keywords: Alkaline phosphatase; hyaluronic acid; mineral trioxide aggregate; mineralization; vital pulp therapy

INTRODUCTION

The aim of vital pulp therapy (VPT) in teeth is to maintain dental pulp vitality and function.[1] The treatment of vital pulp exposure has shifted from using calcium hydroxide (CH) to using bioceramic materials like mineral trioxide aggregate (MTA). Compared to CH, they induce a more positive pulpal response and allowing for the healing of pulp by the formation of reparative dentin.[2,3]

Despite the clinical advantages of MTA, many drawbacks have been reported, such as the long time required for a complete setting, the possibility of teeth discoloration, difficult handling properties, and high cost.[4]

Previous attempts have been made to evaluate the clinical outcome of adding materials to modify MTA cements to enhance the physical and biological properties; however, the histological results of these trials were not supportive.[5,6]

Hyaluronic acid (HA) is the main naturally occurring carbohydrate constituent of the extracellular matrix in many tissues.[7] HA allows cell motility, regulates the adhesion of the cells to each others and to the matrix, it also enhances cell proliferation, and suppresses differentiation. It plays a major role during developmental and morphological activities; the material enhances the healing course and regulates inflammatory processes

Address for correspondence:
Dr. Muthanna S. Ahmed,
Specialized Dental Center, Ninawa Health Directorate, Mosul, Iraq.
E-mail: muthann. 21dep54@student.uomosul.edu.iq

Date of submission : 16.02.2024
Review completed : 14.03.2024
Date of acceptance : 25.03.2024
Published : 10.05.2024

Access this article online

Quick Response Code:

Website: https://journals.lww.com/jcde

DOI: 10.4103/JCDE.JCDE_88_24

How to cite this article: Ahmed MS, Saeed MG, Hasan NH. Immunohistochemical study of mixing mineral trioxide aggregate with hyaluronic acid as a pulp-capping agent in dog teeth. J Conserv Dent Endod 2024;27:485-90.

as it has anti-inflammatory effect and angiogenic properties.[8]

As the mechanical properties of commercially available HA have been enhanced by crosslinking technology, and they are readily available in controlled preparations.[9] They can be easily used and mixed with other materials and available in a standardized sterile dose.[10]

This study aimed to evaluate the effect of mixing MTA with commercially available HA instead of distilled water on the formation of reparative dentin.

MATERIALS AND METHODS

Ethical approval

This study obtained approval from the Ethical Scientific Board/Research Ethics Committee, College of Dentistry, University of Mosul, Mosul, Iraq, under the recorded reference number (UoM. Dent/H.DM.16/23). In addition, the study also followed the ARRIVE guidelines[11] and the (3Rs) rule that forms the basis of the regulations and ethical approval for animal use in scientific research.

Experimental design

Ten healthy, mature, local breed male dogs were obtained commercially from different areas in Mosul city. These dogs were weighed (20 ± 0.5 kg) and aged (12 ± 0.6 months) to ensure the complete histological development of the dental pulp. Each dog was housed in a separate cage and observed for 2 weeks with a periodic medical examination by a veterinarian consultant to exclude any diseased animal.

The study involved the use of 90 teeth (anteriors, canines, premolars) from the 10 dogs divided into three groups, each comprising 30 teeth according to the mixing media that was used with the MTA powder:

i. MTA + distilled water: Control group, three teeth of each dog were capped by MTA powder (MTA Repair HP, Angelus, Londrina, Brazil) mixed with distilled water as reported by the manufacturer
ii. MTA + hybrid cooperative complex HA (HCC-HA) Profhilo® (IBSANordic ApS): Three teeth of each dog were capped by MTA powder mixed with HCC-HA which is the first product developed by NAHYCO® Hybrid Technology that consists of monophasic HA, based on stable, cooperative, hybrid complexes high molecular weight HA (HMW-HA 1200 ± 100 kDa) and low-molecular-weight HA = 100 ± 10 kDa manufactured by using thermal management of HA to produce the hybrid cooperative complexes (HCCs) formula of HA.[12] With a concentration of 32 g/L: 32 mg H-HA + 32 mg L-HA in 2 mL volume, supplied in prefilled syringes
iii. MTA + HMW-HA Jalupro® HMW (PROFESSIONAL DERMA SA, ITALY): Three teeth of each dog were capped by MTA powder mixed with 20 mg/ml gel of noncross-linked HA (1200–1400 kDa).

These groups were further subdivided into five subgroups according to the evaluation period (7, 14, 21, 30, 60) days. Each period included 18 teeth from two dogs (nine teeth from each dog/three teeth for each group per dog) according to the postoperative observation period to get a total of (six teeth/group) for each evaluation period.

Experimental procedures

Anesthesia

After fasting the dogs for 12 h, the surgical operations were performed after induction of general anesthesia, which was administrated intramuscularly to the animals and included a mixture of 5 mg/kg ketamine Alfasan, Holland (concentration 10%), and 5 mg/kg xylazine Alfasan, Holland (concentration 2%) by weight, respectively.

Operative procedure

After 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 1–2 mm were prepared on the buccal aspect of the selected teeth using sterile tungsten carbide burs at high speed with copious sprays of air and water for cooling. The burs were for single use only to ensure the sterility of the prepared cavities and cutting efficiency. Preparation sites were located (2.5–3.5 mm) from the margin of the free gingiva in a parallel direction to the cemento-enamel junction. When the pulp shiny space was visible, the pulp was exposed by a sterile hand file (F5 ProTaper Universal Finishing file, DENTSPLY Maillefer, Switzerland) with a tip diameter of 0.50 mm and a taper of 5%, and the pinpoint orifices of the pulp were about 1 mm in diameter.

Sterile saline (2.5 mL) was used to rinse the cavities, then dried using light pressure on sterile cotton pellets to control any bleeding. The exposure sites were capped with one of the following capping materials according to the experimental groups (MTA + distilled water, MTA + HCC-HA, MTA + HMW-HA). Then, the cavities were immediately restored using glass hybrid restorative material (GH; EQUIA Forte Fil/EQUIA Forte Coat, GC). To minimize pain and discomfort, postoperative nonsteroidal anti-inflammatory ibuprofen medication (2 mg/kg) was administered.

Euthanizing of the animals

At the end of the study periods, the dogs were euthanized. The process followed the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA guidelines for the euthanasia of animals, 2020)[13] using 2.2 mg/kg ketamine intramuscularly, and 0.22 mg/kg xylazine-100 intramuscularly.

Samples preparation and immunohistochemical processing

The sampled teeth were sectioned from the dissected jaws. Then, specimens were fixed in 10% prepared neutral buffered formalin, followed by placing them in formic acid for 14 days, and then they were decalcified with 10% ethylenediaminetetraacetic acid decalcifying solution for 120 days.

An open processing system was used then in which the specimens were 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) were mounted to charged microscope slides.[14] The slides were then stained with ALP antibody using alkaline phosphatase polyclonal antibody (ALPL, IHC 1:50-1:200) (Cat≤#: E AB 93077, Elabscience®, USA). They were then examined under light microscopy for qualitative and quantitative analysis. Two calibrated examiners evaluated the slides according to Benetti *et al*.[15] scoring system [Table 1].

Statistical analysis

Micromorphometric measurements

A colored USB 2.0 digital image camera (Omax ToupView 9.0-Megapexil China) was used to measure the parameters. The camera was supplied with image processing software, which was calibrated to all lenses of Microscope-Olympus-CX31 with the aid of a 0.01 mm stage micrometer (ESM-11/Japan).

RESULTS

After 7 days

The ALP activity for both the control and HMW-HA groups demonstrated a weak score (1+). However, a moderate score was observed (2+) for the HCC-HA group for all samples [Figure 1]. A statistical difference was observed between the control group and the HCC-HA group ($P = 0.024$).

Table 1: Scoring system used during immunohistochemical examination of dental pulp for alkaline phosphatase expression

Score	Score to immunohistochemical expression
0	No immunolabeling (the extracellular matrix shows no labeling with a complete absence of immunoreactive tissue)
1	Low immunolabeling expression (the extracellular matrix shows weak labeling with approximately one-quarter of the immunoreactive tissue)
2	Moderate immunolabeling expression (the extracellular matrix shows moderate labeling with approximately one-half of the immunoreactive tissue)
3	Strong immunolabeling expression (the extracellular matrix shows strong labeling with approximately three-quarters of the immunoreactive tissue)

After 14 days

For the control group, the scores remained still weak (1+), while both the HCC-HA and HMW-HA groups achieved higher rates with a moderate score (2+) that exhibited an increase for the HMW-HA group [Figure 2]. A statistical difference was observed between both HA groups and the control group ($P = 0.020$).

After 21 days

For the control group, no change was observed in the ALP secretion rate after 21 days (weak, 1+). However, the HCC-HA group displayed an intense reaction (3+) which was higher than that in the previous period. The HMW-HA group expressed the same moderate reaction, similar to that observed during the 14-day evaluation period (2+) [Figure 3]. A statistical difference was noted between the HCC-HA group and the control group ($P = 0.008$).

After 30 days

The control group continued to express a weak positive reaction (1+). For group HCC-HA the expression rate reduced to a moderate value (2+). Group HMW-HA remained with the moderate value (2+) obtained during the previous evaluation period [Figure 4]. A statistical difference was noted between both the HA groups and the control group ($P = 0.038$).

After 60 days

A slight increase to a moderate value (2+) was evident in the ALP rate for the control group. The HCC-HA group demonstrated the same moderate value (2+) as that observed in the previous period. With the elevation in the value of the ALP expression

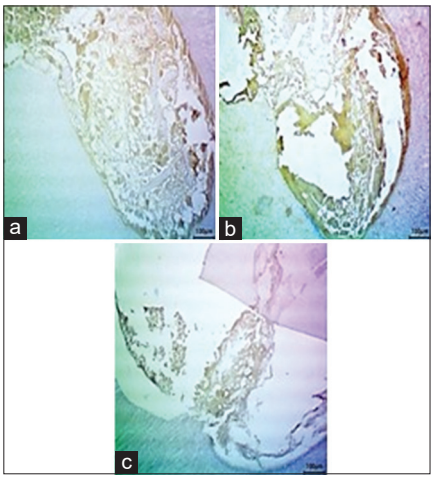

Figure 1: Histological section the dog's pulp-dentin area (7 days) immunohistochemistry expression for ALP. (a): Control group reveals weak positive reaction, (b): Hybrid cooperative complex hyaluronic acid group reveals moderate positive reaction, (c): High molecular weight hyaluronic acid group reveals weak positive reaction (positive reaction appeared dark brown color), ×100

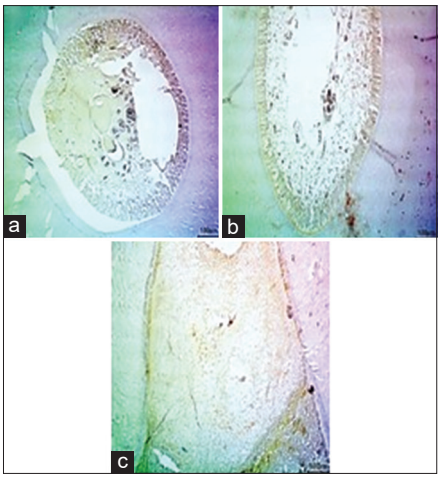

Figure 2: Histological section the dog's pulp-dentin area (14 days) immunohistochemistry expression for ALP. (a): Control group (hole without treatment) reveals weak positive reaction, (b): Hybrid cooperative complex hyaluronic acid group reveals moderate positive reaction, (c): High molecular weight hyaluronic acid group reveals moderate positive reaction (positive reaction appeared dark brown color), ×100

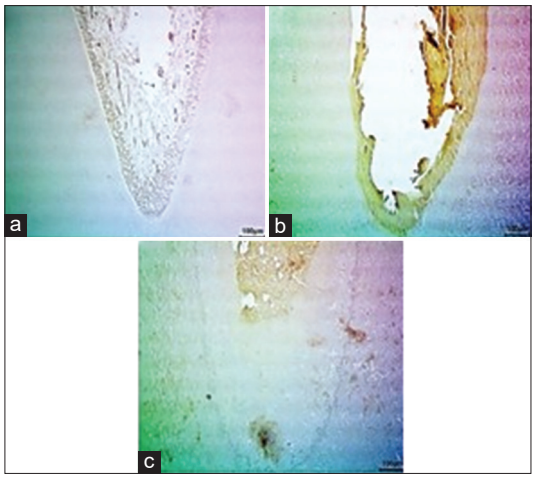

Figure 3: Histological section the dog's pulp-dentin area (21 days) immunohistochemistry expression for ALP. (a): Control group (hole without treatment) reveals weak positive reaction, (b): Hybrid cooperative complex hyaluronic acid group reveals intense positive reaction, (c): High molecular weight hyaluronic acid group reveals moderate positive reaction (positive reaction appeared dark brown color), ×100

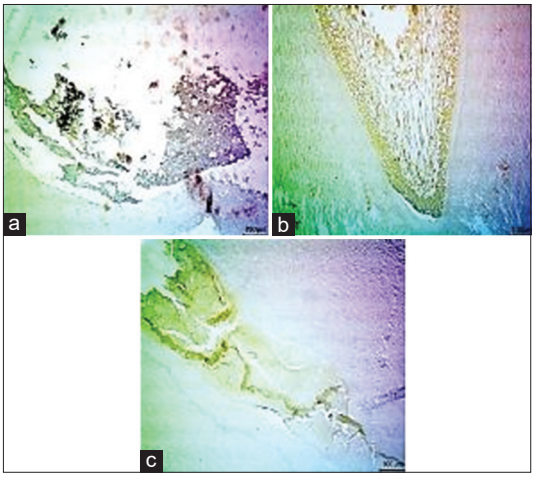

Figure 4: Histological section the dog's pulp-dentin area (30 days) immunohistochemistry expression for ALP. (a): Control group (hole without treatment) reveals weak positive reaction, (b): Hybrid cooperative complex hyaluronic acid group reveals moderate positive reaction, (c): High molecular weight hyaluronic acid group reveals moderate positive reaction (positive reaction appeared dark brown color), ×100

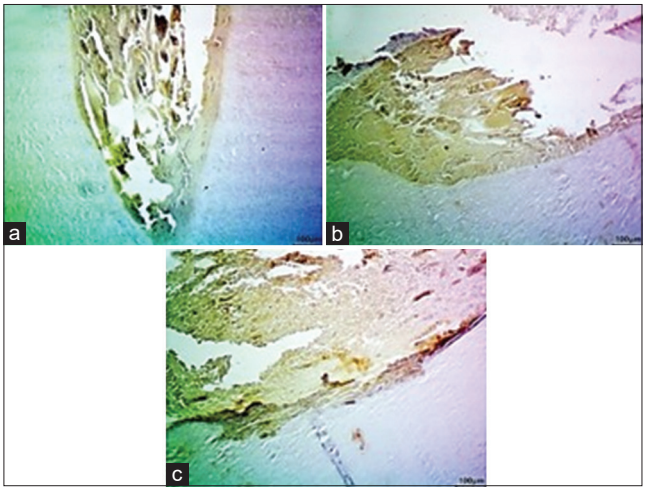

Figure 5: Histological section the dog's pulp-dentin area (60 days) immunohistochemistry expression for ALP. (a): Control group (hole without treatment) reveals moderate positive reaction, (b): Hybrid cooperative complex hyaluronic acid group reveals moderate positive reaction, (c): High molecular weight hyaluronic acid group reveals intense positive reaction (positive reaction appeared dark brown color), ×100

for the HMW-HA group to an intense level (3+) [Figure 5], a statistical difference was identified between the HMW-HA group and the control group ($P = 0.018$).

All the results are summarized in Figure 6.

DISCUSSION

To the best of our knowledge, to date, no study has assessed the use of MTA mixed with HA as a pulp-capping material for VPT. Our results demonstrate that using HA provides better therapeutic outcomes on VPT through increasing the levels of inorganic constituents of the newly developed dentin.

To demonstrate the clinical effects of pulp-capping materials on exposed vital pulp tissues, human and animal teeth may be used.[16] The dogs were used in this study as animal

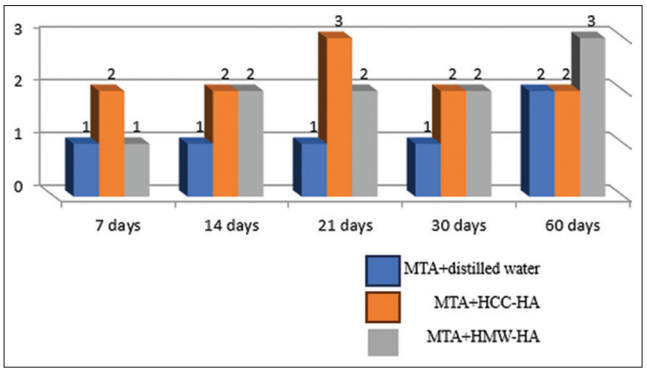

Figure 6: Histogram shows the scores of the immunohistochemistry expression of ALP. MTA: Mineral trioxide aggregate, HCC-HA: Hybrid cooperative complex hyaluronic acid, HMW-HA: High molecular weight hyaluronic acid

models because the reparative dentinogenesis mechanism in dog teeth is similar to that of humans, with a short time.

Immunohistochemical assessment of ALP enzyme activity used as ALP is a main marker of the early phase of odontoblast differentiation. It plays a major role in the mineralization of developed reparative dentin.[17]

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 clinical and immunohistochemical result. When using HA, higher ALP expressions were recorded. To complete the deposition of minerals, ALP is required in the sheets of dentin collagen.[18]

In 7 days, HCC-HA revealed a higher expression of ALP. This is in agreement with Umemura *et al.*[19] who reported that culturing human dental pulp stem cells (DPSCs) in HA reported an elevation in mineralization and ALP activity after 7 days. The results of our study are also consistent with the results obtained by Chen *et al.*[20] who stated that the application of HMW-HA demonstrated an elevation in mineralization rate on day 7 using the ALP activity test, and Nugraheni *et al.*[21] who stated that after 7 days of observation, HA application increased and stimulated odontoblast differentiation.

After 14 days, ALP activity continued to rise in both HA groups, while it remained weak for the control group. This is in agreement with Chen *et al.*[20] who stated that the application of HMW-HA showed an increased mineralization activity on day 14 using the ALP activity test. After 21 days, a higher expression rate of ALP was reported when using HCC-HA. Which can be related to a higher odontoblast differentiation rate. Nugraheni *et al.*[21] stated that HA stimulates odontoblast differentiation up to 21 days of observation.

After 60 days of the study, using HMW-HA instead of distilled water reported the highest levels of ALP expression during all the study periods. These levels could not be achieved with distilled water which reflects the efficacy of HA in comparison to distilled water. The HCC-HA group demonstrated statistical differences from the control group during all the experiment periods except at 60 days, while the HMW-HA groups were statistically different from the control group at days 14, 30, and 60.

Garimella *et al.*[22] reported that HA has no negative effect on the viability, proliferation activity, or differentiation potential of DPSCs.

The exact mechanism that explains the effect of HA on ALP expression rate is not clear, but it may be related to the higher rate of Ca^{+} ion release resulting from mixing MTA with HA, as reported by Ahmed *et al.*[23] which induce mineralization and formation of reparative dentin and elevation in ALP expression rate.

In vivo, experimental trials suggest that using HA alone or in combination with other bioactive materials potentiates a suitable environment for the formation of reparative tertiary dentin through the differentiation of mesenchymal stem cells.[24,25]

The HCC-HA represents a more stable formula with a slow degradation rate without using chemicals for cross-linking. Therefore, it delivers a higher amount of HA in comparison to the noncross-linked HMW-HA. HCC-HA protects the hyaluronan from degradation; therefore, it has a longer *in vivo* effect than HMW-HA.

CONCLUSIONS

The current study determined that HA expressed an improved mineralization potential and healing effect when mixed with MTA for direct pulp capping. Both types of HA (HCC, HMW) can serve as a scaffold for dentin remineralization with an early repair rate due to their readily available premixed formulation.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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