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Evaluation of the pulp response following direct pulp capping with exogenous nitric oxide and Mineral Trioxide Aggregate (MTA) a histologic study

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ABSTRACT

Background: Recently, vital pulp therapy is widely used all over the world. It aims to maintain the viability and function of the injured dental pulp tissue. Direct pulp capping is recognized as one of the most common used techniques in this approach.
Aim of the study: we aimed to compare the histopathological response of the dental pulp following direct pulp capping using two different capping materials; the exogenous nitric oxide (NOC-18) and Mineral Trioxide Aggregate (MTA) in dogs' teeth.
Methods and materials: The pulp of thirty-two premolars and canines from four dogs (eight teeth per each dog) the pulp was exposed and treated with either exogenous nitric oxide (NOC-18) and MTA (four teeth in each group). The treated teeth were extracted and prepared for histologic evaluation after one month and three months, respectively. The histologic study evaluated the formation of dentine bridge, the degree of pulpitis, calcification of the coronal pulp tissue and odontoblasts layer activity. We have the ethical approval to achieve this research from the scientific committee in Faculty of Dentistry, Damascus University.
Results: The results showed that the active statue of odontoblasts layer in NOC-18 group was significantly more than in MTA group after a month of recall (*P: 0.003*). No significant difference was found between MTA and NOC-18 categories in the formation of dentine bridge after 3 months (*P: 1.000*).
Conclusion: Exogenous nitric oxide (NOC-18) maybe has a positive impact on formation of calcified bridge and efficacy of odontoblasts layer on directly capped dog's teeth. Exogenous NO donors might offer alternative to current pulp capping agents in Vital Pulp Therapy in endodontic.

1. Introduction

The ability of dental pulp to regenerate is far greater than had been traditionally believed, so amounted researches have studied how different dental materials could be used to reserve the vitality of dental pulp tissue [1]. Therapeutic strategies that consider dental pulp tissue preservation, are important when managing vital tooth with a pulp exposure [2]. However, these vital-pulp-treatments (VPTs) are considered as procedures that preserve the vitality of pulp tissue that has been compromised by extensive dental caries, trauma, and restorative materials or by iatrogenic pathogens [3]. Direct pulp capping (DPC) as one of the VPT methods, is a strategy

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used in reversible pulp injuries, which aims to keep both function and vitality of the dental pulp tissue. Much concern was given to develop or use specific dental materials that facilitate the process of healing within the exposure dental pulp. This could be explained by triggering the dental pulp to yield a reparative dentine, which is refer to in this process as dentine bridge, to conceal and close the area of exposure and support regenerative capacity of the pulp [4].

Various substances have been used for direct pulp capping since it was first performed as Pereira Paula et al. described [5]. Although calcium hydroxide-based cements have been the material of choice, it presents some disadvantages: bad adherence to dentin, dissolution over time and multiple tunnel defects in the formed dentin bridge [6]. With the development of other biomaterials, aggregate-based cements of mineral trioxides have emerged, which give higher success rates, form structurally more consistent dentin bridges and antimicrobial merits, they may be considered a suitable alternatives for vital pulp therapy [6–10].

Mineral trioxide aggregate are bioactive materials that create the appropriate environment to enhance tissue regeneration and healing. However, when it is applied in direct contact with tissues, it forms calcium hydroxide, which releases calcium ions, that are fundamental for cells adhesion and proliferation [11]. Additionally, it creates an antimicrobial miliue by increasing the pH (alkalizing the medium), modulates cytokine production, migration of cells that are capable of producing dentin or bone tissue, and forms hydroxyapatite or carbonate apatite on its surface, inducing a biological sealing [12]. However, MTA could state as an obstacle in some cases as to the high cost and long setting time [6,13]. However, to combat the drawbacks of MTA, Biodentine has been produced to marketing and which has shown to have promising results in vital pulp therapy and improved handling qualities compared to MTA [14].

Furthermore, Nitric oxide (NO) is an active gaseous mediator synthesized by nitric oxide synthase [15]. The Nitric Oxide Synthase (NOS) family including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [16]. NO is also an important transmitter involved in the pathological and physiological processes of multiple systems [17]. Nitric Oxide regulates the function and differentiation of several types of stem cells and also has a regulatory effect on cells in dental tissues [18].

Recently, synthetic Nitric Oxide-releasing compounds, so called Nitric Oxide donors (such as NOC-18) have been applied in a variety of biological and medical fields [19]. NO is considered to have a key role in dental pulp tissue regeneration under physiological and pathological processes. An in vivo study revealed that NOC-18-application in a tooth cavity accelerated reparative dentin formation in the dental pulp tissues beneath the pulp exposure [20].

Reviewing the literature, you can find many researches that studied the efficacy of a variety of materials in pulp therapy and mainly as direct and in-direct pulp capping. Although the new direct pulp capping materials such as biodentine have promising results in vital pulp therapy and improved handling qualities compared to MTA [20]. This study suggested NOC-18 as a novel pulp capping agent and at the time of conducting this study not much publication had discussed the NOC-18 as effective dental material in pulp capping. So, in this study, we investigated NOC-18 as a suggestive material for vital pulp therapy. So, this study aimed to compare efficacy of exogenous nitric oxide (NOC-18) and MTA in direct pulp capping.

In this study, the null hypothesis was that there is no statistical significance in efficacy between MTA and NOC-18 as direct pulp capping agents.

2. Materials and methods

The protocol of this study was reviewed and approved by the Ethics Committee of Damascus University (NO: 3393). The maintenance and care of the animals complied at a clinic equipped to care for animals. The sample consisted of thirty-six teeth was obtained from four domestic saluki, (un threatened by extinction) dogs, (nine teeth from each one). Four incisors were positive control group (one teeth from each dog). The teeth included sound canines and premolars. The sample were randomly divided into two groups; NOC-18 group and MTA one (16 teeth in each group) as it is revealed in the following diagram (Diagram 1).

Ethical approval was obtained to achieve this research from the scientific committee in faculty of dentistry, Damascus University (NO:3393), according with American Psychological Association (APA) Guidelines [21]. In this study, the pulp was exposed after preparation class I cavity on the occlusal surface of premolars and on lingual surface of canines in each dog and then treated by NOC-18 (Santa Cruz Biotechnology) or MTA (Bio MTA, Cerkamed, Poland). The pulp was capped by NOC-18 in four teeth and by MTA in other four teeth in each dog. Then teeth in the first group were extracted after a month of direct pulp capping procedure and numbered according to the used material, whereas in the second one, the teeth were extracted after three months. However, central incisor was extracted as positive control sample in each dog. The dogs were anesthetized with ketamine HCl and xylazine under supervision of a veterinarian at a clinic equipped to care for animals. Local anesthesia was achieved with 3% mepivacaine without epinephrine. The oral cavity was rinsed with 0.2% chlorhexidine by wiping with sterile gauze. After isolation by rubber dam, in each tooth Class I cavity was made on occlusal surfaces of premolars and lingual surfaces of canines by diamond burs (Kerr, Canada) with low-speed hand piece (NSK, Japan) with copious water spray. A new bur for teeth preparation to avoid heat trauma to the pulp tissue. Pulp exposure was done in the center of class I cavities, the size of exposure point was about 1 mm and bleeding were controlled by mild pressure of sterilized cotton pellets. The exposure point was rinsed with sterilized normal saline, and dried by cotton pellets. The exposure pulp tissue treated with NOC-18 10 μM (NOC-18 powder mixed with 0.1 Naoh) or MTA (Bio MTA, Cerkamed, Poland). Finally, the cavities were filled with a glass ionomer cement (Fuji ionomer type I; GC Corporation, Tokyo, Japan) and composite resin (Colten, Brazil) based on manufacturer instructions; etching by phosphoric acid 37% for 30 s then rinse by water and drying then apply bonding agent (Colten, Brazil), then apply resin composite. The application of NOC-18, MTA and restorative procedures were done by an endodontist. The teeth were extracted after a month and three months in each group respectively; and kept in 10% formalin for 2 weeks. However, two teeth in the second group that had been treated with NOC-18, and 3 teeth in the first that had been treated with MTA, had been fractured, therefore it was excluded from the study. However, no abscess, fistula opening or any failure occurred during follow up

periods. Then the final sample consisted of 27 teeth. All teeth were fixed in neutral formalin solution (10%) to be prepared for the histologic study. After collecting the sample, teeth were removed from the fixative solution (formalin) to EDTA (PH: 7.3) to decalcified them. Then the sample was embedded in paraffin-wax to prepare the teeth to section in 5 μm thickness. The teeth were ready for the Hematoxylin and Eosin staining. Evaluations of the specimens were based on the following [22].

Calcified bridge formation (CB): (0 = incomplete and 1 = complete);

Presence of inflammatory cells beneath the capping area (I): [0 = without inflammation, 1 = mild, 2 = moderate, 3 = severe];

Odontoblasts Layer Formation (OB): [0 = absence Odontoblastic Layer, 1 = presence of odontoblast cells layer];

Calcification (C): [0 = absence of Calcification in pulp chamber, 1 = presence of Calcification in pulp chamber].

The histologic slides were given a specific number referring to the group they are related to. The histologic examiner was blinded to the number and to the type of the capping agents as well. Two repeated histologic reading to the slides were achieved randomly with no differences between them. Intraexaminer variability was not detected.

All data was collected and analyzed using SPSS package program, version 20. Chicago, USA. The data in this study were ordinal and nominal date which are non-parametric data. To compare between MTA and NOC-18 groups, Mann-Whitney U tests were applied for all variables The level of significance was set at ($p < 0.05$).

The power of study was calculated using (Power and precision V4-USA) and the estimated effect size was calculated and revealed 36 teeth sufficient for the study.

3. Results

The following results were obtained from the qualitative values of pulp responses to DPC after follow-up periods, a month and 3 months respectively (Table 1). NOC-18 and MTA caused moderate inflammation in all specimens (Fig. 1), after a month of follow-up. MTA caused mild to moderate inflammation in all samples after 3 months (Fig. 2), Whereas, NOC-18 caused mild inflammation in two samples only. As to the formation of dentin bridge, NOC-18 and MTA caused formation of calcified bridge in all samples after 90 days (Fig. 3: a,b) histologic study revealed formation of the dentin bridge beneath the entire exposure in the roof of the pulp chamber, whereas there was no formation of dentin bridge after a month.

NOC-18 and MTA caused no calcification in the pulp chamber in all samples after, a month and 3 months respectively. Finally, NOC-18 stimulated Odontoblast-like cells Layer Formation in all samples after a month, 3 months respectively.

However, by comparing the groups that treated by NOC-18 or MTA after a follow-up period of a month, a statically difference has been found as to odontoblast layer formation ($p = 0.003$), which could mean that NOC- 18 could be stimulate odontoblast like cell differentiation faster than MTA (Fig. 4: a,b), (Tables 2 and 3).

Finally, by comparing between NOC-18 or MTA after a follow-up period of 3 months, a statically difference has been found as to the inflammation, the degree of inflammation in MTA group was less than in NOC-18 group ($p = 0.000$). (Fig. 5) (Tables 4 and 5).

4. Discussion

Dentists aim in their daily practice to maintain the vitality of the teeth in which pulp has been exposed due to trauma, carious lesions, or restorative procedures [11]. Management plans in this situation include direct pulp capping, pulpotomy. However, direct pulp capping is a protocol which is applied to stimulate the odontoblast-like cells to generate dentinoid materials or what is called dentin bridge and protect the exposed vital dental pulp [23].

Varieties of materials are suggested to be used as direct pulp capping agents. Calcium Hydroxide, Mineral Trioxide Aggregate and Biodentine are one of the most common agents, which are widely used in DPC. Regardless of all the advantages of these materials in vital pulp therapy, they have several drawbacks, that make us searching about new materials that can achieve the goal of vital pulp therapy [3].

Vital pulp therapy is used in the treatment of reversible pulpitis to maintain the viability and function of the injured dental pulp and direct pulp capping, is used for pulp exposure treatment. Recent advances in dental stem cell biology field have suggested a novel endodontic approach to regenerate the dentin-pulp complex [24,25]. The main goal of doing a regenerative technique for direct pulp capping is to reconstruct the normal dentin-pulp structure [26]. Our novel approach is based on an understanding of the molecular and cellular mechanisms regulating the tissue-specific repair process of reparative dentin formation by dental pulp stem cells. To achieve this therapeutic goal, we need to develop an alternative to the direct capping agents. Previous in vivo results indicated that NOC-18 induced the formation of newly differentiated odontoblasts or odontoblast precursor cells from the perivascular niche to the lesion under the prepared cavity [19]. However, at the same previous study, in *vitro* results showed that NOC-18-released NO induced

Table 1
The Frequency (%) of Calcified bridge formation (CB), Inflammatory Reaction (I), Odontoblasts cells Layer Formation (OB), and Calcification (C) in the Study Groups.

FOLLOW-UP PERIOD	NOC-18				MTA			
	(CB)	(I)	(OB)	(C)	(CB)	(I)	(OB)	(C)
A month	0	Grade 2 (100%)	8 (100%)	0	0	Grade 1 5 (100%)	1 (20%)	0
3 months	6 (100%)	Grade1 2 (33.33%) Grade2 4 (66.66%)	6 (100%)	0	8 (100%)	Grade1 8 (100%)	8 (100%)	0

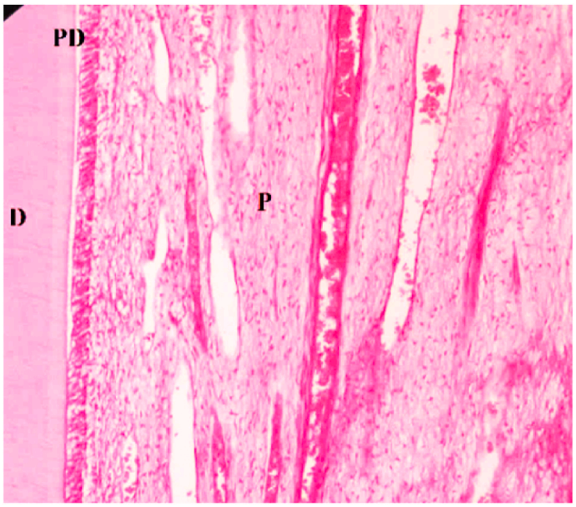

Fig. 1. a sample treated with NOC-18 after a month: hematoxylin and eosin and magnification 100, presence of inflammatory cells in the pulp; D: dentine, PD: predentine, P: Pulp.

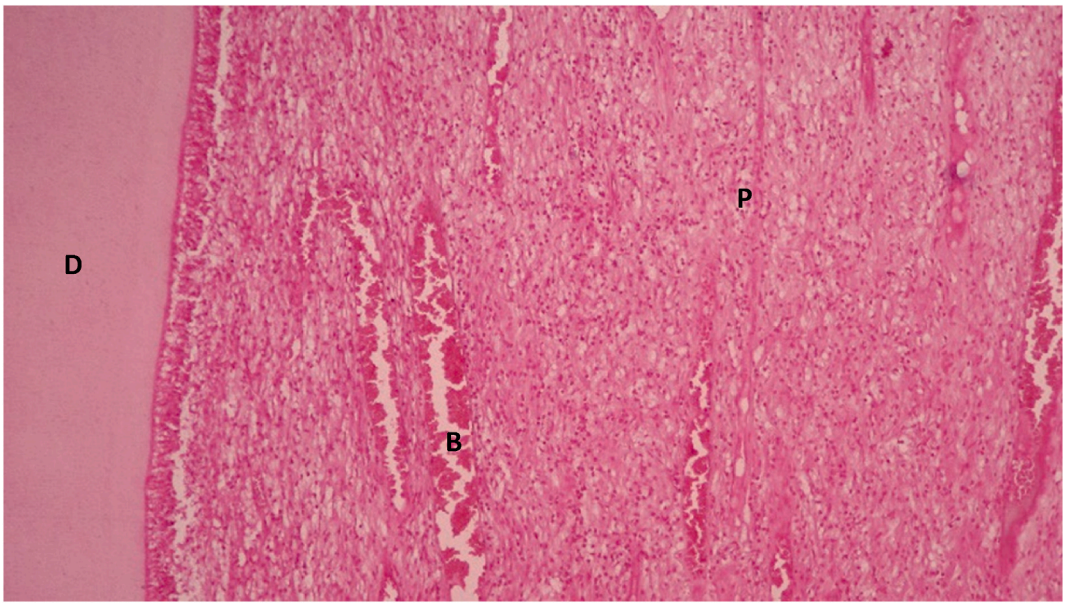

Fig. 2. a sample treated with MTA after a month: hematoxylin and eosin and magnification 400. Note the inflammatory cells in the pulp, dilation of blood vessels; D: dentine, P: Pulp, B: dilation in blood vessels.

odontoblast-specific genes, and activated reparative dentin formation [19]. Therefore, this study findings suggest that NOC-18 in the direct pulp capping releases gas-formed NO into the pulp to recruit and stimulate dental pulp stem cells from the perivascular niche into the dental pulp [27], suggesting NOC-18 as a novel pulp-capping agent for use in endodontics.

Nitric oxide (NO) is an essential molecule that plays important role in many cellular processes. Meanwhile, Nitric Oxide is generated mainly by dental pulp cells and immunocompetent cells. NO mediates inflammatory activities and signaling cascades that regulate tissue regeneration. Thus, nitric oxide as one of the modulators in dental pulp repair or regeneration in pathological process and physiological condition [24,29]. However, synthetic NO-releasing compounds, so called NO donors, were developed recently, and have been applied in a wide range of biological and medical fields [19]. An in vivo study revealed that exogenous nitric oxide donor (NOC-18) application stimulated reparative dentin formation, suggesting that exogenous nitric oxide might be effective for treating damaged dental pulp tissue as indirect pulp capping materials [20].

However, there was no study applied exogenous nitric oxide as direct pulp capping material in vivo studies, so this study hypothesis that NOC-18 could be an alternative or new material might be used as direct pulp capping. To date, there is no evidence to support using NOC-18 in humans, all researches are constructed to in vitro studies.

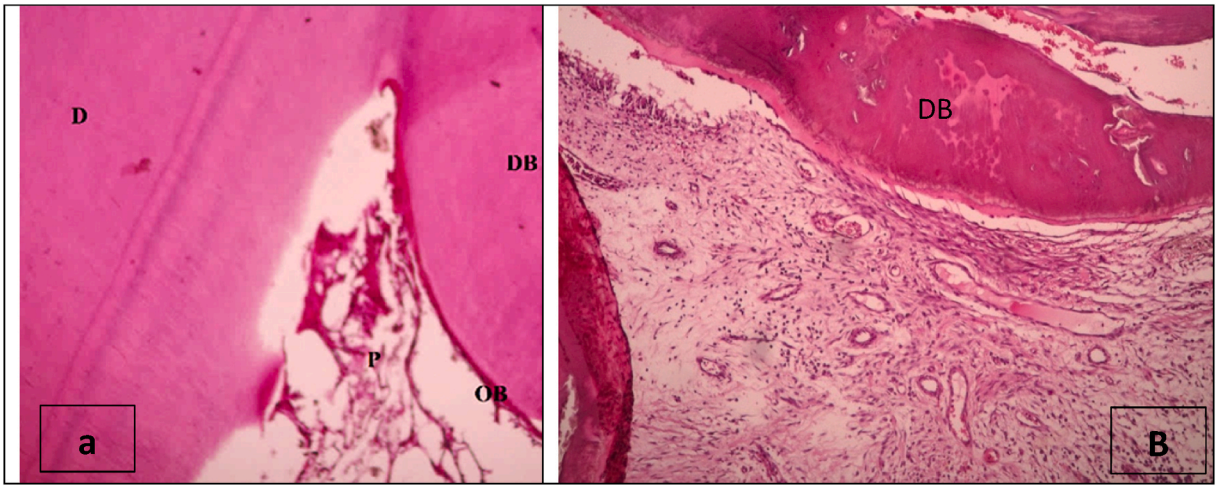

Fig. 3. (a,b) A sample treated with NOC-18 after 3 months: hematoxylin and eosin and magnification 400. Dentine bridge formation beneath the capping area. D: dentine, DB: dentine bridge, OB: odontoblasts, P: pulp (a: DB under a coronal pulp; b: DB under the entire exposure).

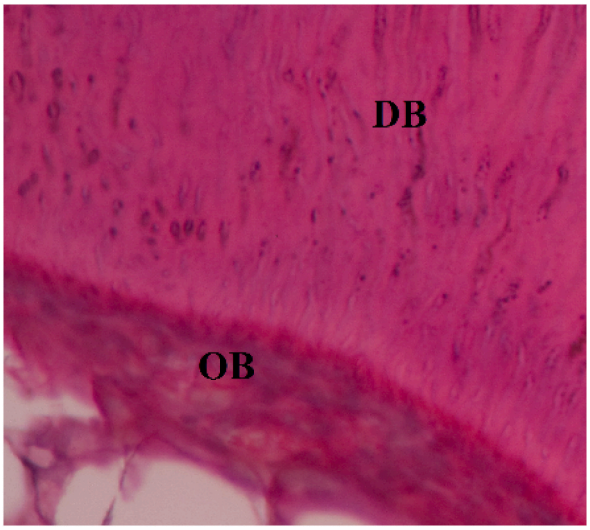

Fig. 4. Hematoxylin and eosin and magnification 400. Crowded odontoblasts because of the proliferation in a tooth treated with NOC-18. Note dentine bridge as well beneath the capping area.

Table 2
Mann-Whitney *U* test to comparing the groups that treated by NOC-18 or MTA after a follow-up period of a month.

	Odontoblasts cells Layer Formation
Mann-Whitney U	4.000
p-value	0.003

Table 3
Comparing the groups that treated by NOC-18 or MTA after a follow-up period of a month.

Odontoblast layer formation	Material	N	Mean Rank
	MTA	5	3.80
	NOC-18	8	9.00
	Total	13	

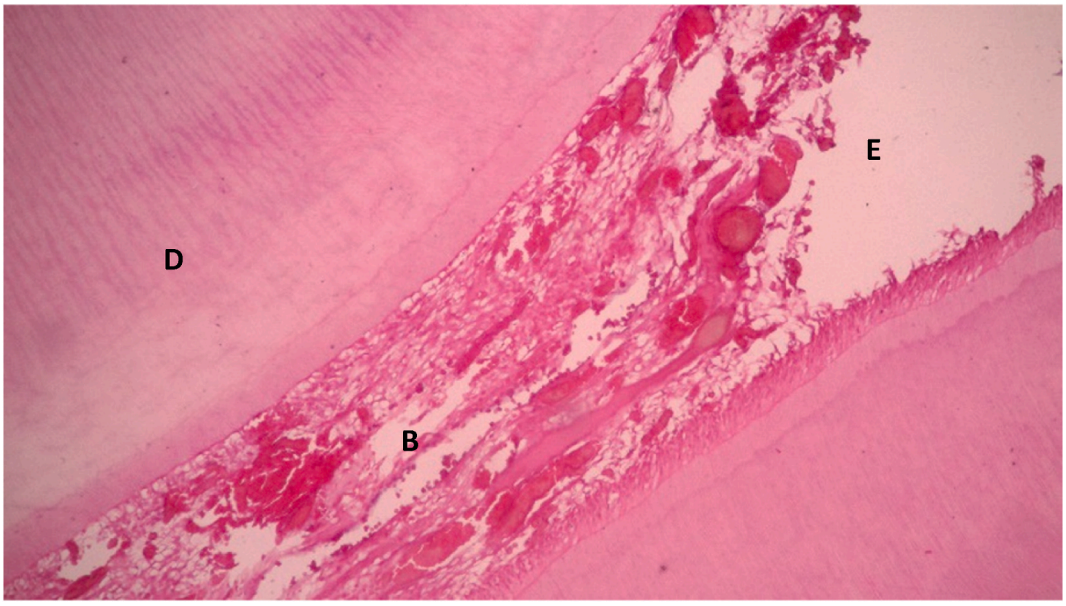

Fig. 5. a sample treated with MTA after a month: hematoxylin and eosin and magnification 400. Note the inflammatory cells in the pulp, the absence of dentin bridge in the exposure area; D: dentine, B: dilation in blood vessels, E: exposure area revealed no formation of dentine bridge.

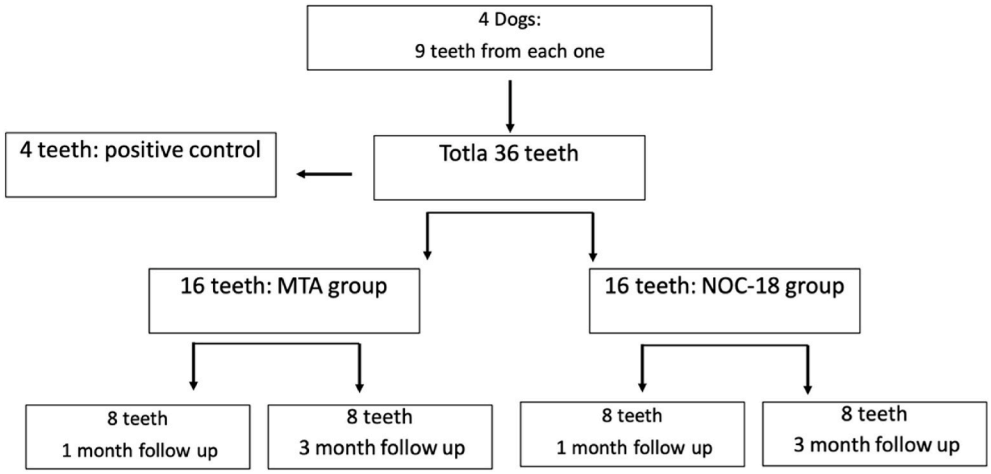

Diagram 1. flow of the sample.

Table 4
Mann-Whitney *U* test to comparing the groups that treated by NOC-18 or MTA after a follow-up period of 3 months.

	Degree of inflammation
Mann-Whitney U	0.000
p-value	0.000

In this study the sample consisted of four dogs (36 teeth, 9 teeth from each doge according to previous study), we applied noc-18 as direct pulp capping in four teeth, and MTA in other four teeth in each dog.

The follow-up periods were a month in the first group (to notice the healing of injured pulp tissue and absence of the inflammatory response) and three months in the second one (dentin bridge can be noticed histologically) [22] although some authors evaluated the pulpal effects of capping materials up to 150 days.

The occurrence of pulp inflammation was moderate (grade 2) in all samples, expect in two samples was mild (grade 1) in the second

Table 5
Comparing the groups that treated by NOC-18 or MTA after a follow-up period of 3 months.

	Material	N	Mean Rank
Degree of inflammation	MTA	8	4.50
	NOC-18	6	11.50
	Total	14	

group (3 months follow up period); however, there was no statically difference between the groups.

Several animal studies have reported inflammation in the pulps capped with MTA [22]. The results of our study were in accordance with the previous investigations regarding the presence of inflammation in the pulp space following pulp capping with MTA. However, there no studies evaluate NOC-18 in direct pulp capping, but by comparing NOC-18 with MTA, we found that the degree of inflammation was less in the MTA group after 3 months.

NOC-18 group, the first and the second follow up periods, all specimens showed formation of odontoblast-like cells layer that may be due to NOC-18 stimulation of odontoblast-like cells differentiation, which agree with the result of Sonoda et al. [20] However we applied NOC-18 as direct pulp capping material, whereas Sonoda et al. applied it on isolated dental pulp stem cells (in vitro). However, in MTA group, the present study showed that in the first follow-up period 20% of the specimens showed formation of Odontoblast cells Layer, whereas in the second follow-up period, all specimens showed formation of Odontoblast cells Layer.

In this study, All the samples showed formation of dentin bridge in NOC-18 and MTA groups in the second follow-up period (3 months). In NOC-18 group that may be due to NOC-18 stimulated the differentiation of dental pulp odontoblast-like cells, which agree with the result of Sonoda et al. However, in the present study we applied NOC-18 as direct pulp capping agent, whereas Sonoda et al. applied it as in direct pulp capping in rats, where his study showed formation of tertiary dentine under the cavities (in vivo).

Previous studies on MTA as a pulp capping material have shown formation dentin bridge under the MTA layer in comparison to other pulp capping materials such as calcium hydroxide [25,27]. Moreover, almost all specimens capped with MTA showed formation of dentin bridge [28,29].

The limitation of this study can be summarized into some points, the first one is that it is not in the human teeth and the second one is that this study did not include other materials in comparison. And finally, it did not include radiological evaluation of the efficacy of pulp capping process using the previous materials.

5. Conclusion

In the limitations of this study, exogenous nitric oxide (NOC-18) could be an alternative material of MTA during vital dental pulp therapy. Not ignoring the fact the MTA is still a golden stander in the VPT, but NOC-18 is to be considered as a suitable alternative with almost similar outcomes.

Author contribution statement

Ghassan AL Mohammad, PhD: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Anas Abdo, Assistant professor: Analyzed and interpreted the data; Wrote the paper.
Amirah Alnour, assistant prof: Contributed reagents, materials, analysis tools or data; Wrote the paper.
Kinda Layous, professor: Conceived and designed the experiments.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no conflict of interest.
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