Nigella Sativa Oil as a Pulp Medicament for Pulpotomized Teeth: A Histopathological Evaluation

Omar OM * / Khattab NM ** / Khater DS ***

Purpose: *Concerns about the safety of formocresol (FC) as a pulpotomy agent in Pediatric Dentistry have lead to the search of new capping medicaments. Indigenous plant medicines such as Nigella Sativa (NS) have been the focus of many researches. Therefore the purpose of this study was to investigate histo-pathologically the pulp response to NS oil and FC in dogs.* ***Method:*** *Forty teeth in 4 male dogs of undefined breed aging 12-14 months were used in this study. Coronal access cavities were performed on the upper and lower premolars so that both medicaments were tested in the same animal in alternate sides of the mouth. Four weeks after treatment the animals were sacrificed, paraffin sections were prepared for histological, histochemical and immuno-histochemical staining.* ***Results:*** *specimens in the NS group showed mild to moderate vasodilatation. Few specimens showed scattered inflammatory cell infiltration and the odontoblastic layer was continuous. While the FC group showed moderate to severe vasodilatation with high inflammatory cell infiltrate and degenerative changes.* ***Conclusions:*** *NS possesses an anti-inflammatory effect and the pulp maintains its vitality after its application, which could qualify its use as a pulp medicament for pulpotomized teeth in clinical practice.*

Keywords: *Nigella Sativa oil, formocresol, animal study, pulp tissue.*
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INTRODUCTION

Pulpotomy is a common procedure in the treatment of primary teeth as well as young permanent teeth with open apices. It is a vital pulp therapy procedure which consists of amputation of the inflamed coronal dental pulp and leaving the radicular pulp tissues intact. Several techniques and various medicaments have been suggested for different clinical situations.[1] However the use of formocresol (FC) in the pulpotomy of primary teeth is widely accepted as the treatment of choice among pediatric dentists owing to its high long term success rate.[2,3]

On the other hand, concerns have been expressed about the safety of formocresol use in Pediatric Dentistry. Some studies have mentioned that formocresol possess local and systemic toxic side effects to cells and tissues, as the possibility of passage of the medicament through the apical foramen exist.[4,5] In addition, in-vitro and animal studies have suggested that formaldehyde a primary component in formocresol may have both genotoxicity and mutagenicity and it is also a probable carcinogen in humans.[6]

The search for a new medicament to replace FC became imperative after several negative reports questioning both its local and systemic side effects. Several alternative pulpotomy medicaments have been introduced such as glutraldehyde,[7] ferric sulphate,[8] calcium hydroxide, freeze dried bone[9] and mineral trioxide aggregate.[10]

Over the past decade, herbal medicine has become a topic of increasing global importance. A larger number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials.[11]

Nigella sativa Linn (family Ranunculaceae) commonly known as black seed or black cumin is an indigenous herbaceous plant traditionally used worldwide in herbal medicine. The seeds from this plant have proclaimed medicinal usage dating back to the ancient Egyptians, Greeks and Romans. *Nigella sativa* plant have been used to promote health and fight disease for centuries, especially in the Middle East and in Southeast Asia.[12] In Islamic medicine, however, the use of the seeds whose Arabic name is *alhabat-al-sauda* is recommended in daily use because it has prophesized cure for all known diseases.[13]

Many of the claimed folk medicinal use of this plant have been scientifically tested and some have been confirmed. In particular the black seed extract has been examined and have

* Ola Moustafa Omar, PhD, Associate Professor, Department of Pediatric and Community Dentistry, Faculty of Oral and Dental Medicine, Cairo University, Cairo, Egypt.
** Nagwa Mohamed Khattab, Associate Professor, PhD, Department of Pediatric and Community Dentistry, Faculty of Dentistry, Minia University, Minia, Egypt.
*** Dina Soliman khater, PhD, Lecturer Department of Oral Pathology, Faculty of Oral and Dental Medicine, Cairo University, Cairo, Egypt.

Send all correspondence to Dr. Ola Moustafa Omar, 12 Dar El-Shefa Street, Garden City, Cairo, Egypt,11451

Phone: +201006030036

E mail: omaromo@yahoo.com

shown to possess the following medical effects: bronchodilator,[14] immune-potentiating activity,[15] hypotensive,[12] analgesic,[16] antibacterial[17] and anti-inflammatory.[16,18]

Abu-Zinadah (2009) found that NS possess wound healing capacity as the peripheries of chemicaly induced wound of rabbit skin grows rapidly when treated with NS localy without any contamination by microbes. Thus, he concluded that Nigella sativa may contain proteins which may stimulate the dermal fibroblast to express fibronectin by human keratocytes and this might help in reducing the burn-wound.[19]

Black seed oil decreases the fibrinolytic potential of the human fibrosarcoma cell line (HT1080) *in vitro*, implying that inhibition of local tumor invasion and metastasis may be one such mechanism.[20]

The effect of NS on chemotherapy induced oral mucositis in albino rats was investigated by Lotfy and Zayed in 2009. The authors found that NS induced reduction of oral mucosal thickness and ameliorate oral mucosal damage induced by 5-fluorouracil in rats. Moreover, they concluded that NS might represent a promising prophylactic adjunct to conventional chemotherapy for reducing the severity of oral mucositis.[21]

Proven analgesic, anti-inflammatory and antibacterial action of *Nigella sativa* (NS) extract and oil encouraged its employment as a pulpotomy agent in the current study. As far as we are aware, the possible effect of this oil on vital pulp tissue has not been previously tested. Therefore the purpose of this study was to investigate histopathologically the response of the dental pulp in dog's premolars to *Nigella sativa* oil and formocresol.

MATERIALS AND METHOD

The experimental protocol was conducted in compliance with the specifications of the animal experimentation ethics of Helsinki and approved by the research ethics committee (REC) Faculty of Oral and Dental Medicine, Cairo University.

Forty teeth in 4 male dogs of undefined breed aging 12-14 months and weighing 7-10 kg were used in this experiment. IM injection of ketamine hydrochloride (5 mg/kg) and diazepam (1mg/kg) were used to sedate the animals before the operation. The dogs were anaesthetized by IV injection of xylazine 1 mg/kg body weight in combination with ketamine hydrochloride 2 mg/kg body weight.

Three mandibular and two maxillary premolars were chosen for this study. The teeth were disinfected using betadine and isolated using cotton rolls. Round burs at high speed with water coolant was used to perform coronal access cavity. The coronal portion of the pulp was removed using sharp spoon excavator. The pulp chamber was irrigated using saline then wet cotton pellets were placed on root stumps to control bleeding. Two opposing quadrants in each dog were assigned for each medicament (split mouth technique) so that both medicaments were tested in the same animal in alternate sides of the mouth. According to the capping medicament two groups were assigned.

Group I (experimental): a cotton pellet soaked with the nigella sativa oil (100% pure nigella oil extracted from fresh NS seeds stored in dark glass bottles at room temperature) was placed on pulp stumps for 5 minutes.

Group II (control): a cotton pellet damped with 20% Buckley's formocresol (1/5 dilution) was placed on pulp stumps for 5 minutes.

In both groups the pulp stumps were then sealed with a 2 mm Zinc oxide eugenol paste and the cavity was restored with glass ionomer filling material (Ketac Fill 3M ESPE).

After completion of the dental procedures the animals were taken care of according to the protocol of the animal house of the Faculty of Medicine Cairo University. They were kept in captivity under constant observation and receiving the standard diet and fresh water.

Four weeks after treatment the animals were sacrificed by vital perfusion with 10% formalin through the common carotid artery. The jaws were immediately dissected and the teeth with their surrounding structures were removed and placed in 10% buffered formalin.

The teeth were fixed in 10% formalin solution for 48-72 hours. Decalcification of the specimens was carried out using 20% formic acid buffered with sodium citrate for 10 weeks. Following decalcification, the specimens were dehydrated in alcohol and embedded in paraffin; sections of 6µm were cut in a bucco-lingual direction and stained with the following methods:

I–Hematoxylin and Eosin stain

To demonstrate the histological and histopathological changes of the tissues examined. Under a light microscope five histological features were evaluated according to the criteria listed in Table 1 modified after Heyeraas et al., 2001.[22]

II–Histo-chemical staining

Masson's trichrome stain was used for detection of the collagen fibers.

Table 1. Criteria for histological features evaluation

Criteria	Grading
Inflammatory cell response	0: none or few scattered inflammatory cells adjacent to exposure site 1: slight inflammatory cell infiltration 2: moderate inflammatory cell infiltration 3: severe inflammatory cell infiltration or abscess
Vasodilatation	0: normal pulp vessels 1: mild vasodilatation 2: moderate vasodilatation 3: severe vasodilatation
Pulp fibrosis	0: none or slight 1: mild fibrosis 2: moderate fibrosis
Necrosis	0: none or slight 1: mild necrosis 2: moderate necrosis
Odontoblastic layer	0: continuous 1: interrupted

III–Immuno-histochemical staining:

For the immunohistochemical staining the paraffin embedded tissue sections were mounted on positive charged slides (SuperFrost Plus-Menzel GmbH). The sections were de-paraffinized and re-hydrated through a graded series of alcohols to water. To block non-specific binding of the immunoglobulin, sections were incubated with normal horse serum blocking solution for 30 minutes. Antigen retrieval was performed by boiling the slides in 10Mm citrate buffer, pH 6.0 for 20 minutes in a domestic microwave. Slides were left to cool for 30 minutes at room temperature and incubated with 0.6% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. The primary antibody used was the mouse anti-human ki-67 (B126, 1) (Santa Cruz Biotechnology, Cat. # sc-56317), for demonstration of proliferating cells. Tissue sections were incubated with the antibody for one hour at room temperature. The immunohistochemical staining was performed by the avidin-biotin peroxidase complex technique using the universal LSAB2 kit (Dako, Cat. # K0673). Detection was carried out using the relevant biotinylated secondary antibody, followed by incubation in peroxidase conjugated streptavidin. All stages were separated by phosphate buffer saline washes. Bound peroxidase was developed with 0.02% 3,3' diaminobenzidine in 0.1 mol/Tris buffer, PH 7.6, in 0.005% hydrogen peroxide for 5 minutes and counterstaining was performed with Mayer's hematoxylin (RE7107Novocastra, UK).

RESULTS

No adverse clinical signs as swelling or fistulas tracts were observed related to pulpotomized teeth in the experimental dogs.

Histological findings

Histological examination revealed empty pulp space in some specimens thus they were excluded and 12 specimens only in each group became included in this study.

Grading of the histopathology criteria manifested in both experimental groups is presented in Table 2 and Figure 1.

Group I (Nigella sativa group):

Most of the specimens showed pulp hyperemia (66.7%) in the form of mild vasodilatation of the blood vessels, whereas the odontoblastic cells of the pulp showed a continuous arrangement (Figures 2 and 3). Moderate vasodilatation (33.3 %) was also observed but without obvious inflammatory cell infiltration (66.7 %) (Figure 3). However, few specimens (33.3%) showed scattered inflammatory cell infiltration (Figure 3). The cellularity of the pulp and its fibrous elements more or less resembling normal pulp tissue; however slight fibrosis was noticed in most of the specimens (91.7%) (Figure 4).

Table 2. Scores assigned to histological features observed in investigated specimens

Histological Features	Score	Study Groups	
		Formocresol (%)	Nigella Sativa (%)
Inflammatory cell response	0	0.0	66.7
	1	8.3	33.3
	2	58.3	0.0
	3	33.3	0.0
Vasodilatation	0	0.0	0.0
	1	0.0	66.7
	2	66.7	33.3
	3	33.3	0
Pulp fibrosis	0	0.0	0.0
	1	25	91.7
	2	75	8.3
Necrosis	0	0.0	91.7
	1	33.3	8.3
	2	66.7	0.0
Odontoblastic layer	0	25	83.3
	1	75	16.7

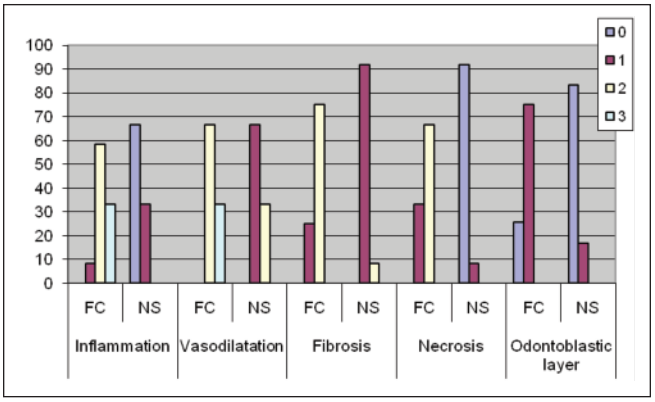

Figure 1. Scores assigned to histological features observed in investigated specimens

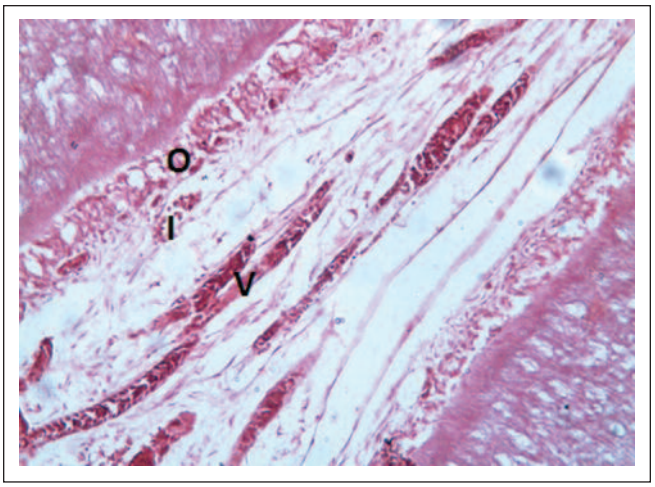

Figure 2. A photomicrograph of the pulp in NS group showing mild vasodilatation (V), mild inflammatory cell infiltrate (I) and intact odontoblastic layer (O) (H&E X200)

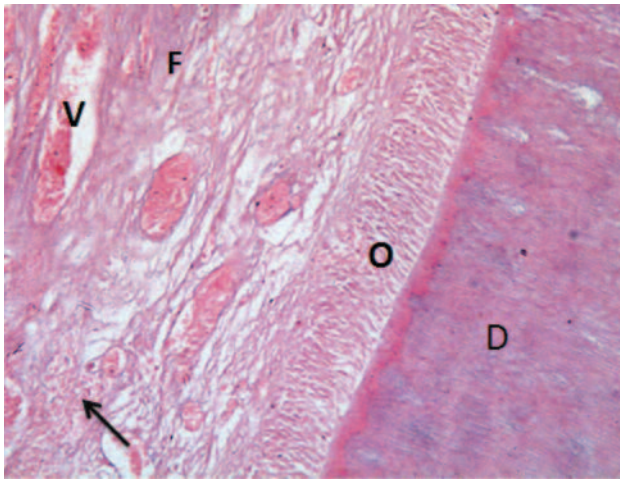

Figure 3. A photomicrograph of the pulp in NS group showing intact odontablastic layer (O), mild to moderate vasodilated blood vessels, moderate fibrosis (F) and few extravased Red.Blood.Cells (arrow) (H&E X200)

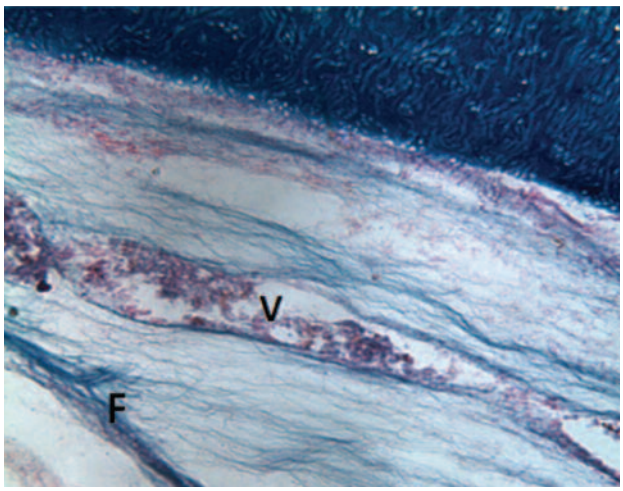

Figure 4. A photomicrograph of the pulp in NS group showing moderate vasodilated blood vessel (V) and mild fibrosis (F) (Masson trichrome X400)

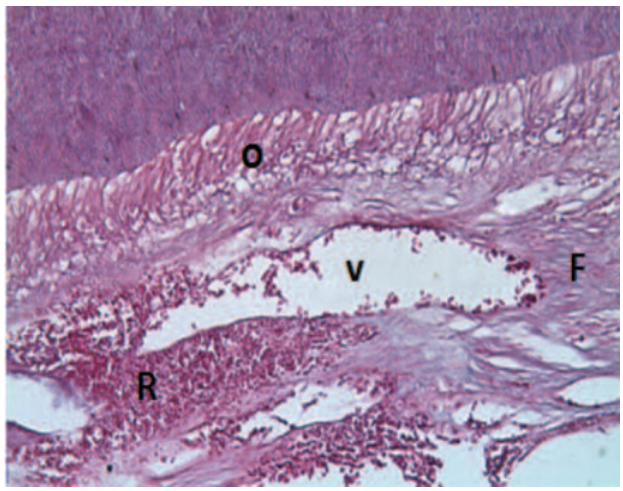

Figure 5. A photomicrograph of the pulp in FC group showing intact odontoblastic layer (O), extreme vasodilatation (V), extravased Red.Blood.Cells (R) and moderate fibrosis (F) (H&E X200)

Group II (Formocresol group):

Moderate to severe vasodilatation of the blood vessels and moderate inflammatory cell response (58.3%) was observed (Figure 5). The pulp showed vacuolated areas and degenerative changes (Figure 6). The odontoblastic layer was most commonly discontinuous (75%); in addition, moderate increase in the collagen fiber density was also demonstrated (Figure 6).Some specimens (33.3%) revealed abscess formation at the exposure site; the abscess cavity was lined by polymorph nuclear leucocytes (Figure 7).

Histo-chemical findings

Masson's trichrome stain:

Slight increase of the collagen fiber density was noticed in the NS group (Figure 4). Meanwhile, the FC group showed marked increase in the collagen fiber density (Figure 8).

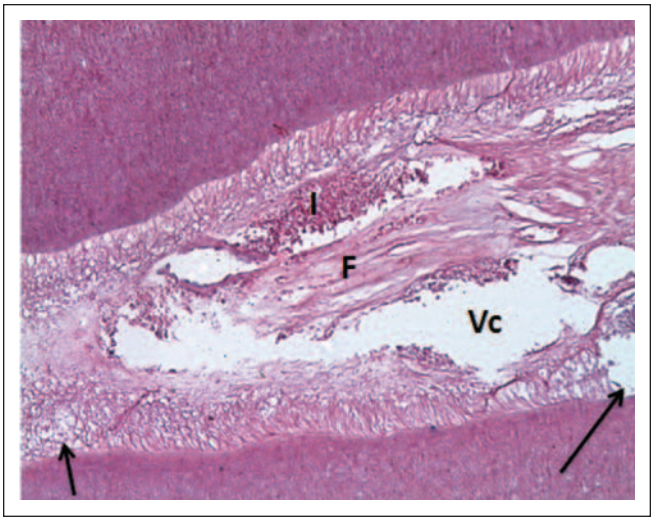

Figure 6. A photomicrograph of the pulp in FC group showing vacuolation (Vc), moderate fibrosis (F), inflammatory cell infiltration (I) and interrupted odontoblastic layer (arrows) (H&E X 200)

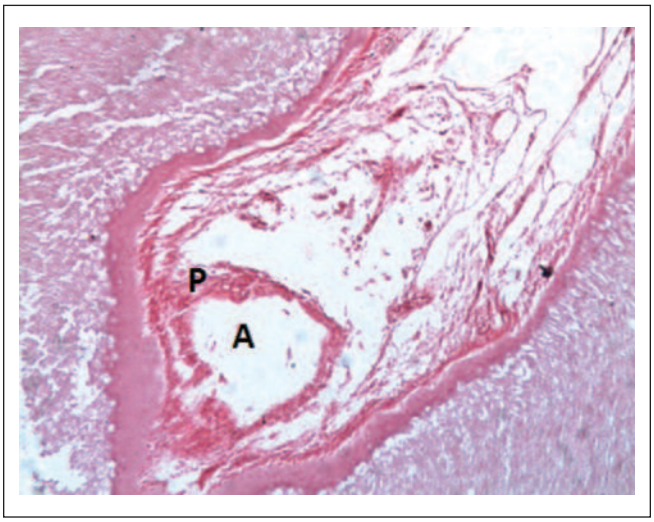

Figure 7. A photomicrograph of the pulp in FC group showing an abscess cavity (A) at the site of exposure surrounded by polymorph nuclear leucocytes (P) and degenerated pulp tissue (H&E X 200)

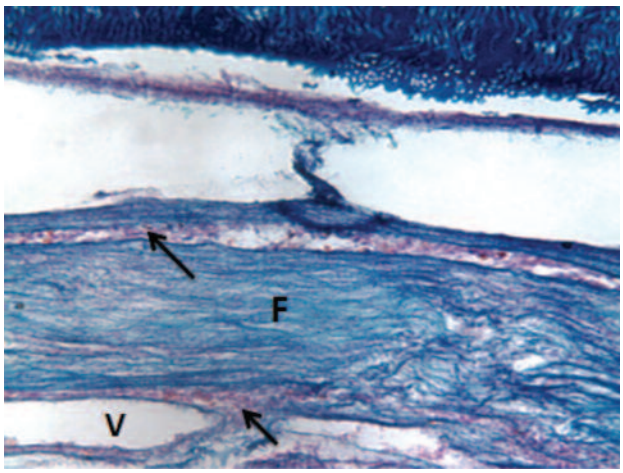

Figure 8. A photomicrograph of the pulp in FC group showing moderate fibrosis (F) with dilated blood vessels and extravased Red.Blood.Cells (arrows) (Masson trichrome X400)

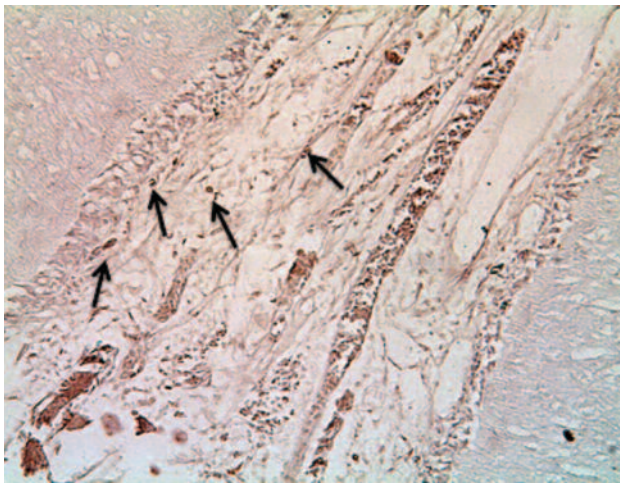

Figure 9. A photomicrograph of NS group showing ki-67 immunoexpression in the pulp core and the sub-odontoblastic layer (arrows) (DAB X200)

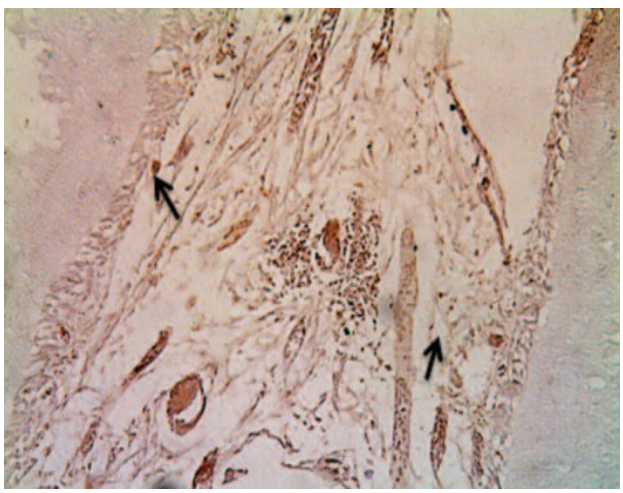

Figure 10. A photomicrograph of the FC group showing ki-67 immuno-reactivity in the pulp core and few cells of the sub-odontoblastic layer (arrows) (DAB X200)

Immuno-histochemical findings

In the NS group, the ki-67 immunoreactivity was evident in the subodontoblastic area and in some of the undifferentiated mesenchymal cells of the pulp core (Figure 9). In the FC group, the ki-67 immunoexpression was revealed in few cells of the subodontoblastic area and in the pulp core (Figure 10).

DISCUSSION

The success of vital pulp therapy is dependent upon the technique employed, the inflammatory status of the coronal and radicular pulp tissue and the type of pulp therapy agent used. The most important features of the successful pulpotomy treatment are lack of pain, maintenance of pulp vitality, minimal pulpal inflammatory response, the ability of the pulp to maintain itself without progressive degeneration, lack of internal resorption and radicular pathosis.

Formocresol continues to be the most widely used pulpotomy medicament. Concerns about its safety have led to several researches seeking better alternatives. Nigella sativa is a promising indigenous plant possessing several medicinal properties have been under extensive research in recent years. Careful scientific evaluation of the safety of essential oils derived from the seeds of indigenous plants is mandatory before they can be clinically applied. The use of the histological methods for the evaluation of the inflammatory response of the dental pulp both at primates and humans showed that they still are the most relevant. Experiments on animals for pre-clinical biocompatibility evaluation provide an accurate method of evaluating clinical response to dental materials.[23] Therefore this study was conducted to evaluate histopathologically the effect of NS oil on vital pulp tissue compared to FC in dogs.

Up to our knowledge the current study is the first study to test this material on vital pulp tissues, thus it can be considered a preliminary study. The follow-up period in similar studies ranged from four to eight weeks where most studies showed insignificant difference or similar results between the four and eight weeks period. Furthermore studies on materials inducing calcified bridge formation requires longer follow-up period while investigating inflammatory response requires less follow-up period. Thus the follow-up period in the current study was limited to four weeks which is considered a suitable period to show early pulpal response and in order to maximize the number of samples .

Histological results of the current study revealed mild vasodilatation (66.7%) in the pulp of teeth treated with NS whereas (33.3%) of specimens showed moderately vasodilated blood vessels and few extravasated RBCs; but without obvious inflammatory cell infiltrate in most of the specimens. This finding was supported by Ali and Blunden (2003)[18] who reported that thymoquinone, the major component of the essential oil of NS has an anti-inflammatory, analgesic and antimicrobial activity. Moreover, it was found that thymoquinone exerts its anti-inflammatory function through its effect on some mediators of inflammation as leukotriene.[24]

In the present study, formocresol was used as a gold standard pulpotomy agent. More pronounced changes in the pulp of the teeth treated with FC were observed. The pulp was highly vascularized with apparent chronic inflammatory cell infiltration, increased collagen fiber density and presence of numerous vacuoles. These findings are in agreement with El-Tawil *et al*. 2009.[25] Similarly, Salako *et al*, 2003[26] found that formocresol showed zones of atrophy, inflammation and fibrosis histologically. Abscess formation was also observed in specimens of the FC group at the site of the pulp exposure. Formocresol leaves the pulp chronically inflamed which increases the susceptibility to abscess formation.[27]

Pulpal inflammation can originate from many factors such as mechanical trauma and operative procedures, the toxicity of the capping materials as well as bacterial contamination at the material cavity wall interface.[28,29] Specimens treated with NS exhibited less inflammation compared to FC. This result could be attributed to the anti-inflammatory effect of NS[18,24] Furthermore this finding may suggest an antibacterial effect which agrees with Khattab and Omar 2006[30] who found that NS reduced the microbial flora of the infected root canals significantly. Thymoquinone, the volatile oil of NS has an antibacterial activity and antibiotics could potentiate its activity.[31]

Histochemical staining was performed using Masson's trichrome stain to detect collagen fibers content. The results of this work revealed that NS exerts an effect on the density of the collagen fibers of the pulp which showed a slight increase compared to normal. This might postulate that NS preserves the pulp vitality through decreasing the fibrotic replacement of pulp via granulation-tissue in-growth. Meanwhile, the FC group showed extensive fibrosis. This finding is consistent with other studies where extensive fibrosis in pulp tissue treated with FC was observed after 4 weeks.[25,26] This might indicate that FC induces pulp fibroblasts to synthesize more collagen as a part of the chronic inflammatory reaction.

In the present study, the odontoblastic cell layer was mostly intact in the NS treated group and interrupted in the FC treated group. This indicates that the pulp tissue in the NS group showed signs of reversible pulpitis which suggest high possibility of recovery and maintenance of pulp vitality. On the other hand, the loss of the odontoblastic cell layer in the FC group might be problematic as these cells cannot proliferate to replace any subjacent cells which become irreversibly injured.[32]

Ki-67, a proliferation specific antigen, is a nuclear and nucleolar protein that is tightly associated with somatic cell proliferation[33] It is a protein that is expressed in cycling cells whereas it is lacking in quiescent (G_0) cells. Our findings showed absence of Ki-67 immunoreactivity in the odontoblastic layers in both groups which confirms that the odontoblasts are post-mitotic terminally differentiated cells. On the other hand, positive immunoreactivity of Ki-67 was evident in the subodontoblastic layer in NS group. These immunoreactive cells are probably proliferating in order to replace the irreversibly damaged odontoblasts. Fitzgerald *et al*. (1990)[34] reported that at least two replications of DNA are required to have functional odontoblast-like cells at the site of the pulp exposure. The first replication takes place before cell migration and the second at the site of expression of the new odontoblast like phenotype.

The results of the present study confirmed the favorable outcome of NS oil when compared to FC in terms of lack of inflammation and abscess formation. Furthermore it may be considered a biologically accepted material since it is a natural oil which induced minimal inflammatory response, kept the pulp vital or capable of repair, in addition to being inexpensive and widely available. All These factors highlight the importance of conducting further studies on NS which has encouraging expectations as a pulp medicament in pediatric dentistry.

CONCLUSIONS

According to the methodology employed in this study and based on the results of the histological, histo-chemical and immuno-histochemical analysis, it could be concluded that:

1. NS oil induced a mild to moderate inflammatory response in dental pulp without inflammatory cell infiltration compared to moderate to marked inflammation in the FC group with apparent chronic inflammatory cell infiltration.
2. Dental pulp remains vital after 1 month of NS application with a minimal change in collagen fiber density compared to increased density of the collagen fiber in the FC group which suggest progressive pulp fibrosis.
3. Favorable histological results of NS oil could qualify its use as a pulp medicament for pulpotomized teeth in clinical practice.

RECOMMENDATIONS

1. Further studies should be done to investigate the effect of NS on the human dental pulp and confirm its antibacterial effect.
2. Short and Long-term clinical studies utilizing NS oil as a pulp medicament should be conducted on primary and young permanent teeth.

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