

BIOCOMPATIBILITY OF NEEM OIL IMPLANTED IN RATS SUBCUTANEOUS TISSUE VERSUS DIFFERENT INTRACANAL MEDICATIONS USED IN REVITALIZATION OF NECROTIC TEETH

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ABSTRACT

Aim of the study: to investigate over the course of two time periods the biocompatibility through reaction of rats' subcutaneous tissue to three different intracanal medications (Neem oil, double antibiotic paste (DAP), and calcium hydroxide Ca (OH)₂).

Methodology: Forty-eight Albino rats were distributed into four groups according to the type of intracanal medicament (n = 12): Ca(OH)₂, Double antibiotic paste (DAP), Neem oil and a control group. Each group was further subdivided into two subgroups according to the time interval. Polyethylene tubes injected with one of the experimental medicaments were implanted into the dorsal subcutaneous tissues of the rats while empty tubes served as controls. After 7 and 21 days the implants with the surrounding tissue were excised. Qualitative and quantitative analysis were carried out for the stained histological sections. Statistical analysis was performed using Kruskal-Wallis test to compare between the groups and Wilcoxon signed-rank test to analyze the changes by time within each group.

Results: At 7 days: Ca(OH)₂ group was associated with a severe significant inflammatory reaction followed by Neem oil which had a moderate inflammatory reaction, the difference was statistically significant. At 21 days, the severity of inflammation decreased significantly in all groups. DAP had the lowest inflammatory reaction with no significant difference between it and the control group at 7 and 21 days.

Conclusions The severity of the inflammatory reaction decreased over time in all groups. DAP could be considered the most biocompatible agent followed by Neem oil.

KEYWORDS: Biocompatibility, Subcutaneous tissue reaction of rats, Calcium hydroxide, Double antibiotic paste, Neem oil, revitalization.

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INTRODUCTION

Revitalization is the currently advocated treatment alternative to Apexification for immature permanent teeth with necrotic pulp. Many clinical case reports have shown that revitalization prevents clinical symptoms, heals periapical lesions, and increases dentinal wall thickness and root length⁽¹⁾. Therefore, it is considered an ideal treatment to preserve the dental arch integrity, prevent apical periodontitis and vulnerability to fractures⁽²⁾.

The goal of revitalization of immature necrotic teeth is root maturation through vital tissue regeneration. However, histological studies have proven that the newly formed tissue inside the thoroughly disinfected root canal spaces is not truly pulp tissue but pulp like, periodontal like, or bone like tissue⁽³⁾. The reported studies where pulp tissue with odontoblast has been formed is the ones where remnants of dental pulp stem cells were preserved, thus the biocompatibility of the used chemicals inside the root canal of immature teeth is of paramount importance for preservation of stem cells either in the periradicular tissue or inside the pulp⁽⁴⁾.

Three-dimensional disinfection of the infected root canal is vital for successful revitalization procedure. Taking into consideration the thin root canal walls in immature teeth and the importance of preserving the remnants surviving stem cells. Numerous non-instrumental measures have been described to reduce the numbers of root canal microorganisms or totally eliminate them. This includes the use of various irrigation regimens, and intracanal medications.

Antimicrobial intracanal medicaments are also needed in between visits as an auxiliary tool to eradicate microorganisms from the root canal system in orthograde root canal treatment of mature teeth; since the chemomechanical step minimizes intraradicular infection, but microorganisms have the ability to compete with or remains hidden inside

the anastomosis or irregular hidden areas of the root canal system⁽⁵⁾.

The most commonly applied medicaments in regenerative endodontics are calcium hydroxide (Ca [OH]₂) and triple antibiotic paste (TAP) or double antibiotic paste (DAP)⁽⁶⁾. These medicaments may negatively affect the survival and differentiation potential of the DPSCs.

Neem oil is derived from Margosa tree, it has a strong antimicrobial effect suggesting its potential as an endodontic irrigant and intracanal medicament⁽⁷⁾. The use of neem in endodontics may be advantageous due to its antioxidant effect, and thus there is no risk of tissue toxicity on using it⁽⁸⁾.

Thus, there is a need to find the biocompatible antimicrobial alternatives to replace the synthetic intracanal medicaments. Subcutaneous tissue implantation of the investigated materials in small size animals has been widely employed to determine biocompatibility through preliminary in vivo studies. Among these animals, the rat (Albino) which is the most frequently used due to its dimensions that provides easy, safe handling, and faster metabolism when compared with other animals. Therefore, the subcutaneous tissue reaction of rats to an herbal antimicrobial agent (neem oil) was investigated and compared to DAP and Ca (OH)₂ in this study. The null hypothesis is that there is no significant difference between the tested groups.

MATERIALS AND METHODS

The methodology of the current study was carried out according to the Preferred Reporting Items for Animal studies in Endodontology (PRIASE) 2021 guidelines.

Study design

This study was single-blind clinical trial with 7- and 21-days follow-up, performed after the acceptance of the Research Ethics Committee (number 17/2017).

Sample size calculation

Forty-eight samples (12 samples for each group) determined G*Power software version 3.1.9.2 by were enough to detect the effect size of 0.25 according to **Cohen** ⁽⁹⁾, a power (1-β=0.80) of 80% at a significance level of p<0.05 partial eta squared of 0.06 ^(10,11).

Grouping

Group A: Neem oil (UpNature, Nicosia, Cyprus), Group B: Double antibiotic paste [1:1 mixture of Metronidazole (50 mg) (Sanofi-Aventis, Gentilly, France) and Ciprofloxacin (50 mg) (Bayer, Leverkusen, Germany) prepared with glycerol (50mL)⁽⁴⁾, Group C: Calcium hydroxide paste (Meta Biomed, Chungbuk, Korea), and Group D: Control group (empty polyethylene tubes) (**Table 1**) and (**Table 2**).

TABLE (1) The study Variables and time intervals.

Variable	Level	Referred to
Intracanal medication	A	Group(A): Neem oil The polyethylene tubes were filled with neem oil (n=12)
	B	Group (B): DAP The polyethylene tubes were filled with double antibiotic paste (n=12)
	C	Group (C): Ca(OH)₂ The polyethylene tubes were filled with calcium hydroxide (n=12)
	D	Group (D): Control (Negative control) The polyethylene tubes were kept empty (n=12)
Time (T)	T₁	Time of examination 7 days
	T₂	Time of examination 21 days

TABLE (2) Factorial design for inflammatory reaction assessment (n=12)

Variables	Type of intracanal medication				Total
	Group A	Group B	Group C	Group D	
Time (T)	T ₁ (n=6)	T ₂ (n=6)			
	A T ₁	B T ₁	C T ₁	D T ₁	24
	A T ₂	B T ₂	C T ₂	D T ₂	24
Total	12	12	12	12	48

Animal preparation

Forty-eight male healthy Albino rats (each weighing 180-200 g) were kept in separate numbered cages based on the group and study period. The cages were cleaned daily. The animals were monitored and received solid food and water ad libitum by the technician of the animal housing during the study, except for 12 h before surgery. They were kept in a temperature-controlled room (21- 25°C). All animals were in a good health without loss in the sample size.

Implantation procedure

Animals were anesthetized with 10% Ketamine Chloride (75 mg/kg) (Wallcur, USA) along with Xylazine (10 mg/kg) (Ranbaxy Pharmaceuticals, Haryana, India) by intramuscular injection. The back of animals was shaved and cleaned with betadine (MundiPharm, Egypt). Single (1cm) incision was made on the dorsal region with lancet no. 21 (HuaiAn TianDa medical instruments company, China). Then, sterile polyethylene tube (Nelco, USA) having the diameter of 1.3 mm and 10 mm length were filled with one of the intracanal medications, then they were subcutaneously applied, and the wound was sutured by 4-0 silk (International sutures manufacturing company, Egypt). After the specified time intervals (7 and 21 days), the animals were sacrificed by intravenous injection with of high dose of pentobarbital sodium (Nembutal, USA) and perfused with 10% buffered formalin (ChemLink,

Egypt). The tubes were excised with the surrounding tissues and kept in 10% neutral formalin (Chem-Link, Egypt) for 48 h for tissue preservation.

Histologic evaluation

Excised tissues around the tubes were dried with alcohol (Perfect chemicals, Egypt), embedded in paraffin (Bartoline, Beverley, England), then serially sectioned at 4 μ m and stained with hematoxylin and eosin (Abcam, USA). Afterward, samples were analyzed histologically by a single blind examiner who was unaware of the experimental groups to describe the samples qualitatively and quantitatively according to the following category ⁽¹¹⁾:

Grade I: no inflammation (similar thickness of the reaction zones, slightly larger than alongside tube, no or few inflammatory cells).

Grade II: mild inflammation (small reaction zone, < 25 inflammatory cells).

Grade III: moderate inflammation (greater reaction zone, 25–125 inflammatory cells infiltrate)

Grade IV: severe inflammation (zones of necrosis, >125 inflammatory cells).

Statistical analysis

By examining the distribution of the data and applying normality tests, numerical data were

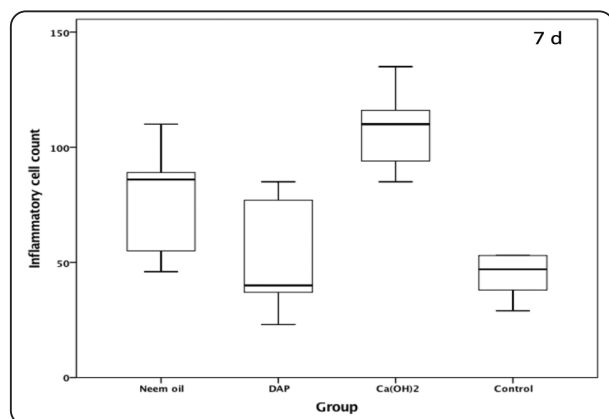


Fig. (1) Boxplot representing the inflammatory cell count after 7 days at different groups.

examined for normalcy (Kolmogorov-Smirnov and Shapiro-Wilk tests). The distribution of all the data was non-normal (non-parametric). The median and range values of the data were displayed. The Kruskal-Wallis test was employed to compare the groups. The Wilcoxon signed-rank test was used to analyze how each group's modifications over time changed. The cutoff for significance was chosen at P 0.05. With IBM® SPSS® Statistics Version 20 for Windows, a statistical analysis was carried out.

RESULTS

Quantitative evaluation:

A) Comparison between the biocompatibility of the three intracanal medications (Intergroup analysis)

A statistically significant difference emerged between experimental medicaments after 7 days (P-value 0.001, Effect size = 0.478). (P-value <0.001, Effect size = 0.478). When the groups were compared on a pairwise basis, Ca (OH)₂ demonstrated the highest significant mean value of inflammation, followed by Neem oil, which demonstrated a moderate level of inflammation. Although there was no discernible difference between the DAP and control groups, both demonstrated moderate inflammation. (Fig. 1). A statistically significant change emerged between all materials after 21 days (P-value 0.001, Effect size = 0.474). Comparing the groups in pairs showed that Ca (OH)₂ significantly differed from Neem oil and DAP. A moderate level of inflammation was characterized by Ca (OH)₂. While there was no discernible difference between DAP and Neem oil, both demonstrated a minimal amount of irritation. Additionally, there was no discernible difference between the DAP and control group. However, compared to the Ca(OH)₂ and Neem oil groups, the control group demonstrated the lowest mean value of inflammation. (Fig. 2).

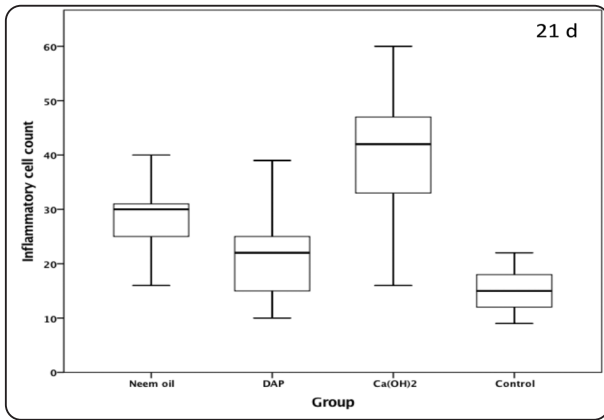


Fig. (2) Boxplot representing the inflammatory cell count after 21 days at different groups.

B) Comparison between the inflammatory reaction of each intracanal medication at different time intervals (7 and 21 days) (Intragroup analysis)

In all of the experimental groups, there was a statistically significant difference between the inflammatory response at the two-time intervals (7 and 21 days). The three medications' biocompatibility was inversely correlated with the passage of time, and as the passage of time passes, the degree of inflammation diminishes.(Fig. 3).

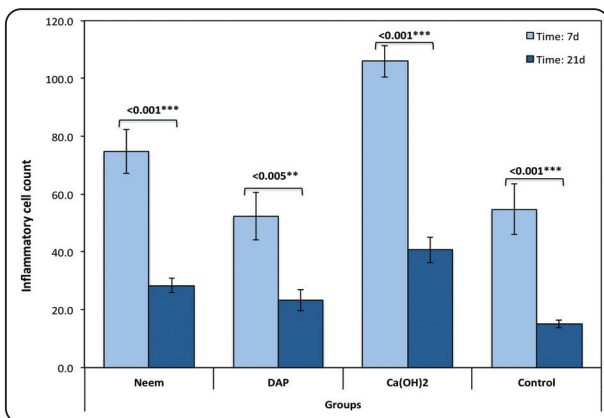


Fig. (3): Bar chart showing the difference in the inflammatory cell count after 7 and 21 days in each group.

Qualitative analysis (descriptive):

A) Comparison between the inflammatory reaction of the three intracanal medications after 7 days:

The histological analysis of the subcutaneous connective tissues of different groups disclosed the following:

- In control group, the subcutaneous connective tissues adjacent to the empty tube of control group showed formation of thin collagen fibers capsule with new formed blood vessels, moderate inflammatory cell infiltrate formed mainly of lymphocytes and macrophages (Fig 4.a).
- Adjacent to Neem oil material, areas of collagen fibers were merged with eosinophilic extracellular matrix representing zones of necrosis in addition to intense inflammatory cells infiltrate (Fig.4.b). Also, collagen fibers and zones of necrosis were evident in all samples adjacent to Ca (OH)₂ material. Severe inflammatory reaction composed mainly of lymphocytes, macrophages, and multinuclear giant cells were predominant (Fig. 4.c).
- While, adjacent to DAP material, organized thin collagen fiber capsule were formed with mild to moderate inflammatory reaction (Fig. 4.d).

B) Comparison between the inflammatory reaction of the three intracanal medications after 21 days:

- In control group, the histological analysis of the subcutaneous connective tissues adjacent to the empty tube showed thick capsular arrangement of the collagen fibers, which got interspersed with fibroblasts, minimal or no inflammatory cells were detected (Fig.5.a).
- Adjacent to Neem oil material, there was well organized fibrous capsule with moderate amount of collagen fibers, mild inflammatory cells infiltrate (Fig. 5.b).

- Collagen fibers were arranged in capsular arrangement with mild to moderate inflammatory reaction in all samples adjacent to Ca (OH)₂ material. Few multinucleated giant cells were present (Fig. 5.c).
- Adjacent to DAP material, well organized thick fibrous capsule of collagen fibers with fibroblasts arranged between them, minimal or no inflammatory cells were present (Fig. 5.d).

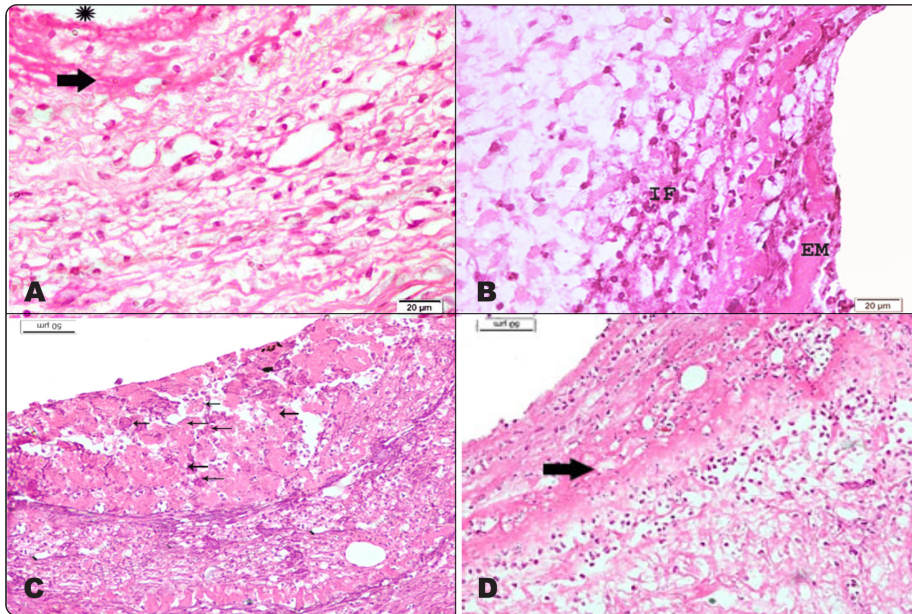


Fig. (4) Light micrograph of the different groups for the excised tissue adjacent to the tubes (*) at 7 days: a) negative control group, showing collagen fiber capsule with moderate inflammatory cells (arrows). b) Neem Oil group, showing collagen fibers with eosinophilic extracellular matrix representing zones of necrosis in addition to intense inflammatory cells infiltrate. c) Ca (OH)₂ group showing collagen fibers, zones of necrosis (small arrow) and severe inflammatory reaction. d) DAP group showing organized thin collagen fiber capsule (arrow), and mild to moderate inflammatory reaction [a, b bar 20/ c, d bar 50].

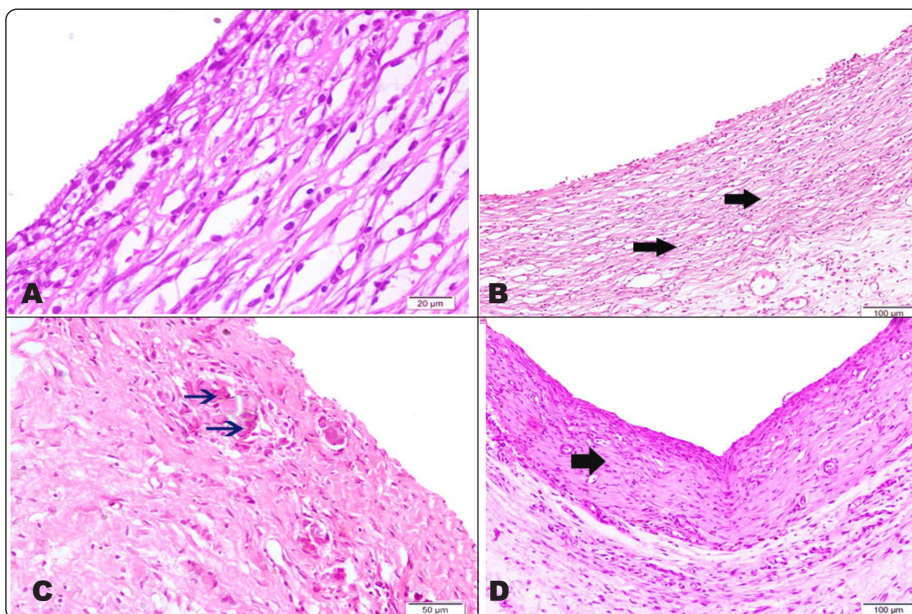


Fig. (5) Light micrograph of the different groups for the excised tissue adjacent to the tubes at 21 days: a) Control group showed thick capsular arrangement of the collagen fibers, with minimal inflammatory cells infiltrate. b) Neem oil group showed well-organized fibrous capsule with moderate amount of collagen fiber (arrow). c) Ca (OH)₂ group showed capsular arrangement of the collagen fibers, and mild to moderate inflammatory reaction with some noted giant cells (arrows). d) DAP group showed organized thick fibrous capsule (arrows), with minimal or no inflammatory cells [a bar 20; c bar 50 b, d bar 100].

AIM/HYPOTHESIS
to investigate over the course of two time periods, the reaction of rats' subcutaneous tissue to three different intracanal medications (Neem oil, double antibiotic paste (DAP), and calcium hydroxide Ca (OH) ₂).
ETHICAL APPROVAL AND COMPLIANCE WITH ANIMAL WELFARE STANDARDS
Research Ethics Committee acceptance (number 17/2017)
TYPE OF ANIMAL, weight
Albino rats weighing 180-200 g each.
SAMPLES
5 mm width of excised tissues around the implant was sectioned from each of the Forty-eight Albino rats
INTERVENTION / CONTROL GROUPS
N = 48: Group 1 - Ca(OH) ₂ (n=12), Double antibiotic paste (DAP) (n=12), Neem oil (n=12) and the control group(n=12). Two subgroups according to the time period (T1:7 days, T2: 21 days)
OUTCOMES ASSESSED
Biocompatibility of intracanal medication assessed by degree of inflammation of tissue surrounding the the polyethylene tube that was implanted in albino rats' subcutaneous tissue.
METHODS AND WHO ASSESSES OUTCOMES
Qualitative and quantitative analysis were carried out for the stained histological sections by a single blind histological examiner.
RESULTS WITH STATISTICAL ANALYSES
At 7 days: Ca(OH) ₂ group was associated with a severe inflammatory reaction followed by Neem oil which had a moderate inflammatory reaction, the difference was statistically significant. At 21 days, the severity of inflammation decreased significantly in all groups. DAP had the lowest inflammatory reaction without significant compared to the control group at 7 and 21 days.
ADVERSE EVENTS
One rat was died before starting follow-up and was replaced by another.
CONCLUSION(S)
The intensity of the inflammatory response faded over time in all groups. DAP could be considered the most biocompatible agent followed by Neem oil.
CONFLICTS OF INTERSET
Non of the author claims to have any conflicts of interest pertaining to this study

Fig. (6) Preferred reporting items for animal studies in Endodontology (PRIASE 2021)- based flowchart.

DISCUSSION

Intracanal medications provide supplementary chemical disinfection to root canal irrigants; however, their use is limited to specific cases like weeping canals, aggressive flareup or persistence infection, in other word in cases which are contraindicated to single visit treatment. Furthermore, they are recommended to be used in necrotic immature teeth undergoing revitalization⁽⁸⁾ or MTA apexification. The biocompatibility of the applied intracanal medication is of high priority in immature teeth due to their wide-open apex that allow leakage of the medication into the periapical area and to preserve stem cells either those of the periapical papilla or other remnants stem cells responsible for healing and root maturation.

Calcium hydroxide is the most popular intracanal medication for necrotic weeping cases and in revitalization procedures followed by the triple antibiotic mix which gained popularity in early cases of reported revascularization of immature necrotic teeth, then it was replaced by the double antibiotic mix to avoid the discoloration of the minocycline ⁽¹²⁾.

To address the limitations of commercially available intracanal medications, a recent trend toward employing biologic medications derived from natural plants has grown quickly. These include cytotoxicity, microbial resistance, and their inability to penetrate the dentinal tubules. The major advantages of using herbal alternatives are their availability, lower cost and toxicity, increased shelf life and decreased microbial resistance ⁽¹³⁾.

Neem's ability to prevent the growth of the bacteria that cause infectious diseases has demonstrated its potency as an antimicrobial agent. It possesses an eminent anti-adherence activity, which modifies bacterial adhesion and its capacity to colonize the substrates⁽¹⁴⁾.

Subcutaneous implantation of polyethylene tubes into the Albino rat permits contact with the connective tissue just beneath the skin and

standardization for the medicaments volume and area of contact without interferences by the variability of volume and diffusion of the medicaments into the subcutaneous tissue. Thus, analysis of tissue reaction between the experimental groups could be accurately carried out ⁽¹⁵⁾.

Assessing the tissue reaction of rats at different time intervals (7 and 21 days) permits the observation of the histological reactions through both time periods, as the short and delayed response of tissues to certain material may differ substantially. Those specified time intervals were followed in previous studies that analyzed tissue response ⁽¹⁶⁾.

The histological analysis of the subcutaneous connective tissues adjacent to Neem oil revealed after 7 days, intense inflammatory cells infiltrate with zones of necrosis represented by collagen fibers merged with eosinophilic extracellular matrix were formed. This could be attributed to the alkaline pH (8.2) that induced a significant increase in the cytotoxic effect reflected by the intense inflammation ⁽¹⁷⁾.

This comes in harmony with previous studies, which found that Neem had high cytotoxic effect on cancer cells, and they referred that to its high alkaline pH. This alkalinity dramatically inhibited the multiplication of MDA-MB-231 cells, and they also died as a result.^(18,19) Unfortunately, toxicity to normal cells is unavoidable eventually causing cell death by mitotic catastrophe or by apoptosis. However, after 21 days subcutaneous connective tissues adjacent to Neem oil material showed great reduction in the inflammatory cells and well-organized fibrous capsule with moderate amount of collagen fibers arranged between them in an observed sign of healing. This could be assigned to the neutralization of the Neem alkaline pH ⁽²⁰⁾.

Regarding the subcutaneous tissue reaction adjacent to Ca (OH)₂, after 7 days, there was severe inflammatory reaction composed predominantly of lymphocytes, macrophages, multinuclear giant cells together with collagen fibers, zones of

necrosis. While, after 21 days, this inflammatory reaction was reduced in all samples and collagen fibers were organized in capsular arrangement with few multinucleated giant cells. These results were congruent with previous studies evaluating subcutaneous tissue reaction of rats to Ca (OH)₂. They showed that the inflammatory reaction noticed at 7 days could be related to the surface necrosis induced by calcium hydroxide, and due to its alkaline pH ⁽²¹⁾.

When Ca (OH)₂ comes into touch with connective tissue, it creates a coagulative necrosis zone, which might explain why the inflammatory response to Ca (OH)₂ decreases over time. A variety of morphological alterations in living tissue brought on by the action of enzymes on cells that have been fatally wounded were referred to as coagulative necrosis. For a few days, the majority of necrotic cells still have the basic shape of the coagulated cells, because necrotic cells cannot retain the integrity of their membranes, their contents seep out, and cellular debris is then phagocytosed by an inflammatory cell infiltration (polymorphonuclear neutrophils, macrophages, and eosinophils) ⁽²²⁾. Then, healing takes place within 7 to 10 days through formation of collagen type I and fibronectin, both stimulate osteoprogenitor cells that form new mineralized tissue after contact of Ca (OH)₂ with connective tissue⁽²³⁾.

After 7 days, the histological analysis of the subcutaneous connective tissues adjacent to DAP showed mild inflammatory reaction together with thin organized collagen fiber capsule, that turned to well organized thick fibrous capsule of collagen fibers with fibroblasts arranged between them, with absence of inflammatory cells by the end of 21 days. The metronidazole that is used to prepare DAP has no cytotoxic effects, therefore it is thought to contribute to its biocompatibility ⁽²⁴⁾. The biocompatibility of DAP could be further attributed the glycerin involved in its formula as it was stated that glycerin is a biocompatible and well-known nontoxic natural metabolite ⁽²⁵⁾. Moreover,

the ciprofloxacin and metronidazole in DAP are already used in tablet form orally in much higher concentrations and dosage than that used inside the polyethylene tubes in the current study, which is considered another reason for the absence of inflammatory cells. The risk of developing adverse systemic side effects is reduced in clinical situation because the locally delivered small dose medication inside the root canals will leach out and reach the systemic circulation in much lower diluted dose.

Regarding the analysis of the influence of time interval on the three tested materials, there was a direct relation between the biocompatibility of the material and time intervals. This was in accordance with the previous studies ⁽²⁶⁾ that confirmed that most materials showed a considerable decrease in the inflammatory response over extended periods of time, thus increasing their level of biocompatibility. This could be partly related to the toxic effect of the material leached out from the implanted tubes. This causes a strong inflammatory response and the recruitment of macrophages and multinucleated giant cells which release inflammatory kinins, that induce fibroblasts to form the fibrous capsule to expand over time.^(27,28) This also could be attributed to the initial inflammatory response that was arisen from the aggression related to the surgical process and not only by the experimental medicaments. Same findings were reported by **Lalis *et al.***,⁽²⁹⁾ and **Silva-Herzog *et al.***,⁽³⁰⁾ which referred this initial inflammatory reaction to the surgical trauma effect that was reduced with time from 7-21 days with beginning of tissue repair.

A strength point of the present study is that the evaluation of the subcutaneous tissue implantation of the used intracanal medication results in as increased reproducibility of the study design. Nevertheless, Ex-vivo studies as the current one is a reliable indicator of the biological properties of intracanal medications that may come in contact with the viable cells during revitalization ⁽³¹⁾. However, as a limitation of the present work, is that in vivo clinical studies are needed to elucidate

the actual response and interaction of the applied intracanal medications with the surrounding tissue ⁽³²⁾, moreover the subcutaneous tissue reaction could be affected by the implant stability, host reaction, and the surgical procedure itself ⁽³³⁾.

Finally, adhering to precise reporting criteria improves the comparability of research using similar procedures. The goal of the current study was achieved by adhering to the recently approved PRIASE criteria.^(34,35) Accordingly, the main steps of this work have been depicted in the PRIASE 2021 flowchart (Fig. 6).

CONCLUSIONS

The intracanal medicaments used in revitalization must be decided based on their biocompatibility as well as their antibacterial characteristics. Based on the findings of the current study, DAP exhibited the least inflammatory tissue reaction and higher biocompatibility compared to calcium hydroxide and neem oil. Further in vivo studies should be conducted for better understanding of the tissue reaction to these medicaments and their influence on mineralization potential of stem cells to substantiate their clinical use.

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