Original Article

Histologic Comparison of Formocresol, Platelet-Rich Fibrin, and Hesperidin in Pulpotomy: A Randomized Trial in Dogs

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Abstract

Aims: To histologically assess and compare formocresol (FC), platelet-rich fibrin (PRF), and hesperidin (HPN) as pulpotomy agents in dogs. **Materials and Methods:** Pulpotomy was attempted from the buccal surface (class V) of 48 teeth in three mongrel dogs (*Canis Lupus*). Cavities were randomly allocated for three groups (n = 16) according to the pulpotomy agent used; (group I: FC (control), group II: PRF, and group III: HPN). All cavities were then sealed with zinc oxide eugenol followed by resin-modified glass ionomer restoration. Two months later, dogs were euthanized; the specimens were obtained and prepared for histological assessment followed by statistical analysis. **Results:** HPN specimens showed the best dentin bridge formation and the least inflammatory signs and pulp disorganization. Followed without statistically significant difference by PRF ($P \geq 0.05$). Both of HPN and PRF, however, showed a significant difference statistically ($P \leq 0.05$) to FC that showed no dentin bridging with more pronounced inflammation, necrosis, and pulp disorganization. **Conclusions:** For pulpotomy, HPN and PRF seemed histologically to be good substitutes for FC in the dog model.

Keywords: *Dentin bridge, formocresol, hesperidin, PRF, pulpotomy*

Introduction

Pulp vitality is important for long term maintenance and efficacy of the tooth against occlusal forces. Root completion in immature teeth requires vital pulp as well.[1] Pulpotomy is one of pulp therapy techniques, indicated in traumatic or cariously exposed pulps in asymptomatic primary molars and immature permanent teeth. In pulpotomy, only affected or infected coronal pulp is amputated, preserving the vitality and function of the radicular pulp using a pulpotomy agent.[2]

Formocresol (FC) is the standard pulpotomy agent for decades[2] with 70%–100% recorded clinical success. Its formaldehyde constituent is bactericidal, inhibits many inflammatory enzymes, and causes surface fixation, preventing further pulp injury. Unfortunately, FC preserves but never regenerates the pulp, and it was found to cause cancer and mutations at higher concentrations.[2,3]

Developing biocompatible materials that are additionally bioactive has recently revolutionized the concept of pulp regeneration rather than just preservation.[4] Of the most recent examples are platelet concentrates: platelet-rich plasma (PRP) and platelet-rich fibrin (PRF). They are derived from victim's own peripheral blood and, thus, represent autologous materials that can be used in dentin-pulp complex regeneration. Advantages include accessibility, biocompatibility, and being highly loaded with growth factors and immune cells.[5]

In PRF, centrifugal of autologous blood at different speeds separates more fibrin matrix with a larger number of immune cells especially leukocytes with more infection resistance and more growth factors than PRP. Also, in PRF, fibrin mesh entraps the growth factors, allowing for their sustained release (one week–28 days), which is excellent for regeneration.[5,6]

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Nowadays, natural agents gain a renewed attention in medicine.[7] Hesperidin (HPN) is a flavonoid derived from citrus fruits. It proved a wide range of pharmacological properties and medicinal uses such as anti-inflammatory, analgesic, anti-microbial, anti-oxidant, and anti-proteolytic effects.[8,9] Such properties suggest potential benefit of HPN in pulp tissue regeneration.

This study was held, therefore, to histologically evaluate PRF and HPN as pulpotomy agents in dogs and compare them to the gold standard FC.

Materials and Methods

Ethical approval

The protocol of this parallel randomized control study was approved by the Ethical Committee of the Faculty of Dental Medicine for girls, Al-Azhar University, Cairo, Egypt (REC-PD-21-01). Animal care and handling were performed in accordance with CPCSEA guidelines[10] and declaration of Helsinki.[11] This study followed the CONSORT statement.[12]

Sample size calculation

Using the statistical sample size calculator (WWW. Surveysystem.com), the confidence level was set at 95% with a confidence interval of 14.15% and a *P* value of 5%. In this software, it is instructed to keep the population size blank if the population is huge or unknown. The calculated sample size was 48 totally. Therefore, 16 teeth/group were designated for the study.

Experiment animals

Three healthy male mongrel dogs (*Canis Lupus*) weighing 14–16 kg and aged 14–16 months were enrolled. Dogs were vaccinated and quarantined in separate cages under observation 21 days preoperatively. To ensure good care and handling of dogs, the study was held in Veterinary Surgery Department, Faculty of Veterinary Medicine, Ain-Shams University, Cairo, Egypt.

Grouping, randomization of teeth, and blinding

Three sound premolars and one first molar/quadrant in each dog were used in this study, summing up 48 teeth. After pulpotomy, teeth were divided randomly into three equal groups (n = 16) according to the pulpotomy agent used: group I (control group): formocresol (FC) (Prevest Denpro, Jammu, India); group II: platelet-rich fibrin (PRF) (prepared and applied only in relevant dog); and group III: hesperidin (HPN) (Sigma Aldrich co., St. Louis, MO, USA).

Block randomization sequence (RS) with block sizes of 2, 4, and 6 was computer-generated by an independent doctor using Excel 2007 (Microsoft, Redmond, WA, USA). RS was concealed in sealed opaque envelopes. The operator was informed about the RS after completing all pulpotomies and before materials application, while the histologist and data analyzer were blinded.

General anesthesia

For anesthetic induction, 0.05 mg/kg atropine sulphate (S/C) (atropine sulphate 1%; ADWIA, Egypt), 1 mg/kg xylazine HCl (IM) (Xylaject 2%; ADWIA, Egypt), and 10 mg/kg ketamine HCl (IM) (Keiran; EIMC Pharmaceuticals Co., Egypt) were injected. 25 mg/kg of 2.5% thiopental sodium solution (thiopental sodium, EIPICO, Egypt) was incrementally injected (IM) for anesthetic maintenance.[9]

Preparation of PRF

5 ml of venous blood from each dog were collected in anticoagulant-free glass tubes. After 1 min of rest (to prevent immediate coagulation on centrifugation), the tubes were centrifuged for 12 min at 1000 rpm.[6] PRF appeared as white jelly above the clot and was then isolated.

Operative procedures

Iodine solution (5%) was used for aseptic preparation of the mouth. In each tooth, the pulp was exposed buccally using no. 2 round carbide bur (SS White, Brazil) in high-speed hand piece with copious water spray, the access cavity was completed, the coronal pulp was extirpated, and bleeding was stopped using sterile moistened cotton pellets. Pulp stumps were then covered with FC in group I, PRF in group II, and HPN (mixed with distilled water in a ratio of 1:3 following manufacturer's instructions) in group III. All cavities were finally sealed with zinc oxide eugenol then resin modified glass ionomer (GC Corporation, Tokyo, Japan).

Histologic evaluation: After follow-up for two months, dogs were euthanized with an anesthetic overdose of 5% thiopental sodium IV (thiopental sodium, EIPICO, Egypt). Jaws were separated, and bone segments, including the control and experimental teeth, were resected. Teeth and surrounding periapical tissues were fixed using 10% buffered formalin solution (Research-Lab Fine Chem Industries, Mumbai, India) for 48 hr. Specimens were demineralized using (25% formic acid and 10% trisodium citrate solutions) for 6 months and then, dehydrated by passing them through ethyl alcohol (from 70% to 100% concentration). Specimens were later embedded in paraffin blocks and sectioned into mesio-distal sections of 6 μm thickness. Sections were stained using H and E dye and then histopathologically evaluated under a light microscope at 40× and 100×. Image analysis was performed using a Leica EZ4 HD digital image

analyzer (Switzerland). The evaluation was based on the classifications shown in Tables 1–3, according to the modified version of ISO 10993 and 7405.[13]

Statistical analysis

Data were analyzed using IBM SPSS software package version 20.0. (IBM Corp, Armonk, NY). Kolmogorov–Smirnov test was used to verify the normality of distribution of variables. Comparisons between groups for categorical variables were assessed using the Chi-square test (Monte Carlo). Kruskal Wallis test was used to compare different groups for not normally-distributed quantitative variables, followed by the *post hoc* test (Dunn's for multiple comparisons test) for pairwise comparison. Significance was judged at the 5% level.

Results

Histological results

FC (control) group

There is no evidence of dentin bridge in any specimen. The inflammatory infiltrate in different specimens ranged from mild to severe that spread apically with several areas of severe hyperemia, dilated blood vessels, irregularly distributed (PMNLs), and some proliferating fibroblasts among wavy course collagen fibers. The odontoblastic layer was discontinuous; areas of necrosis mainly at the pulp/FC interface, hyalinized connective tissue with different degrees, and disorganization of pulp tissue were observed [Figure 1a and b].

PRF group

Moderate basophilic dentin bridge was seen in half of specimens, and slight and intense deposition of hard tissue at the amputation site was also evident in others. Multiple calcified structures (bone or dentin-like structures) were scattered just beneath the dentin bridge. Predentin and line of demarcation between primary and reparative dentin were also observed. Complete absence of inflammatory features to mild or moderate

Table 1: Classification of reparative dentin formation

Reparative dentin formation	Characterization
Grade 0	Absence.
Grade 1	Slight deposition of hard tissue close to the amputation site.
Grade 2	Moderate hard tissue deposition immediately below the amputation site.
Grade 3	Intense deposition of hard tissue (complete bridging) immediately below the amputation site

Table 2: Classification of cellular inflammatory infiltrate

Inflammatory infiltrate	Characterization of inflammatory infiltrate
Grade 0	Absent or few inflammatory cells near the amputation site.
Grade 1	Light inflammatory cells infiltrate as polymorphonuclear leukocytes (PMNLs) and mononuclear leukocytes (MNLs)
Grade 2	Moderate inflammatory cells infiltrate.
Grade 3	Severe inflammatory cells infiltrate or abscess characteristics.

Table 3: Classification of pulp tissue disorganization

Pulp tissue disorganization	Characterization
Grade 0	Normal tissue.
Grade 1	Disorganization of the odontoblast layer only.
Grade 2	Total disorganization of pulp tissue morphology.
Grade 3	Pulp necrosis.

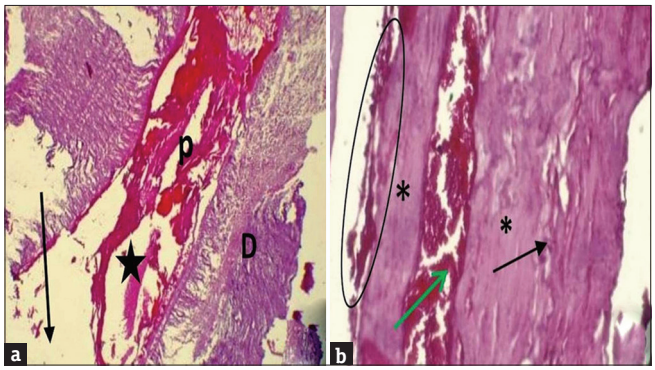

Figure 1: Photomicrograph of the pulp of formocresol group showing: (a) No evidence of the dentin bridge (black arrow), loss of normal architecture of pulpal tissue (p), and area of complete degeneration (star) and primary dentin (D) H and E, ×40. (b) Hyalinization of pulp tissue (*), proliferating fibroblast in between wavy course collagen fibers (black arrow), discontinuous layer of odontoblasts (circle), and dilated blood vessel engorged with blood (green arrow). H and E, ×100

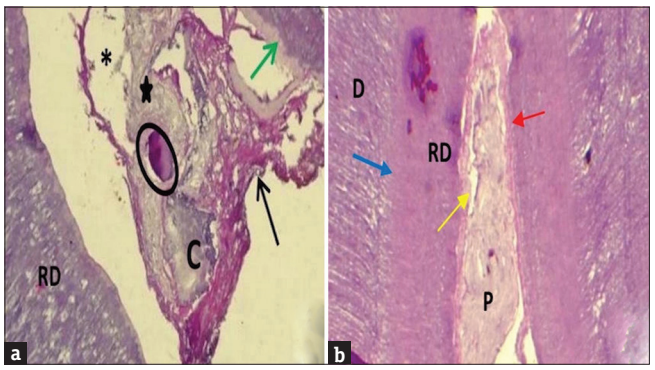

Figure 2: Photomicrograph of the pulp of the PRF group showing: (a) Newly formed basophilic dentin bridge nearly closes the orifice (black arrow), true dentin-like structure (circle), spot area of calcification (C), thick layer of predentin (green arrow), area of reparative dentin alongside the pulp (RD), and partial hyalinization of pulp tissue (star) H and E, ×40. (b) Normal pulp tissue (P), multiple successive layers of odontoblast (yellow arrow), predentin (red arrow), continuous variable thickened reparative dentin extending along the side walls of the pulp (RD), primary dentin (D), and line of demarcation separate between primary and secondary dentin (blue arrow) H and E, ×40

Table 4: Comparison between the percentages of the scores of the three groups

Score	Formocresol (FC) (*n*=16)	Platelet-rich fibrin (PRF) (*n*=16)	Hesperidin (HPN) (*n*=16)	χ^2	^{MC}P
Cellular inflammatory infiltrate					
0	0 (0%)	4 (25%)	8 (50%)	11.333	0.043*
1	2 (12.5%)	8 (50%)	6 (37.5%)		
2	8 (50%)	4 (25%)	2 (12.5%)		
3	6 (37.5%)	0 (0%)	0 (0%)		
Significance	$^{MC}P_1$=0.069, $^{MC}P_2$=0.019*, $^{MC}P_3$=0.671				
Pulp tissue disorganization					
0	0 (0%)	2 (12.5%)	8 (50%)	17.860*	<0.001*
1	2 (12.5%)	10 (62.5%)	8 (50%)		
2	2 (12.5%)	4 (25%)	0 (0%)		
3	12 (75%)	0 (0%)	0 (0%)		
Significance	$^{MC}P_1$=0.014*, $^{MC}P_2$=0.002*, $^{MC}P_3$=0.155				
Reparative dentin bridge formation					
0	16 (100)	0 (0%)	0 (0%)	17.073*	0.001*
1	0 (0%)	4 (25%)	2 (12.5%)		
2	0 (0%)	8 (50%)	8 (50%)		
3	0 (0%)	4 (25%)	6 (37.5%)		
Significance	$^{MC}P_1$=0.002*, $^{MC}P_2$=0.001*, $^{MC}P_3$=1.000				

χ^2: Chi square test MC: Monte Carlo. *P*: *P* value for comparing the studied groups. P_1: *P* value for comparing between FC and PRF. P_2: *P* value for comparing between FC and HPN. P_3: *P* value for comparing between PRF and HPN. *: Statistically significant at $P \leq 0.05$. *n*=number of specimens/group

Table 5: Comparison among the three groups according to the mean of different scores

Score	FC (*n*=16)	PRF (*n*=16)	HPN (*n*=16)	*H*	*P*
Cellular inflammatory infiltrate					
Mean±SD.	2.3±0.7	1±0.8	0.6±0.7	11.412*	0.003*
Median (Min.-Max.)	2^a (1-3)	1^b (0-2)	0.5^b (0-2)		
	P_1=0.014*, P_2=0.001*, P_3=0.429				
Pulp tissue disorganization					
Mean±SD.	2.6±0.7	1.1±0.6	0.5±0.5	15.008*	0.001*
Median (Min.-Max.)	3^a (1-3)	1^b (0-2)	0.5^b (0-1)		
	P_1=0.013*, P_2<0.001*, P_3=0.186				
Reparative dentin bridge formation					
Mean±SD.	0.0±0.0	2±0.8	2.3±0.7	15.217*	<0.001*
Median (Min.-Max.)	0^b (0-1)	2^a (1-3)	2^a (1-3)		
	P_1=0.002*, P_2<0.001*, P_3=0.634				

H: H for Kruskal Wallis test, Pairwise comparison between each two groups was done using Post Hoc Test (Dunn's for multiple comparisons test). *P*: *P* value for comparing between the studied groups. P_1: *P* value for comparing between FC and PRF. P_2: *P* value for comparing between FC and HPN. P_3: *P* value for comparing between PRF and HPN. *Statistically significant at $P \leq 0.05$. Median with Common letters are not significant (i.e. Means with Different letters are significant).

inflammatory cells infiltration, including (PMNLs) and (MNLs), was seen as well [Figure 2a].

Preserved pulp tissue observed in some specimens, characterized by marked proliferating odontoblasts, appeared as multiple successive layers. Numerous collagen bundles were seen throughout the pulp with relatively few proliferating fibroblasts [Figure 2b].

HPN group

Marked complete dentine bridge closed the amputation site, ranging from thin, intermediate to intense thickness [Figure 3a]. The absence of inflammatory cells in the pulp area was noticed in half of specimens. Light to moderate cellular inflammatory infiltrate, however, was observed in others. Normal odontoblastic layer and normal pulp tissue were seen in most of specimens with evident collagen fibers. Disoganized odontoblastic layer and few edematous spaces were noticed in few specimens. Thick reparative dentin was seen along the lateral wall of pulp [Figure 3a and b].

Statistical results

Regarding the results, HPN specimens showed the least inflammatory signs and pulp disorganization with better

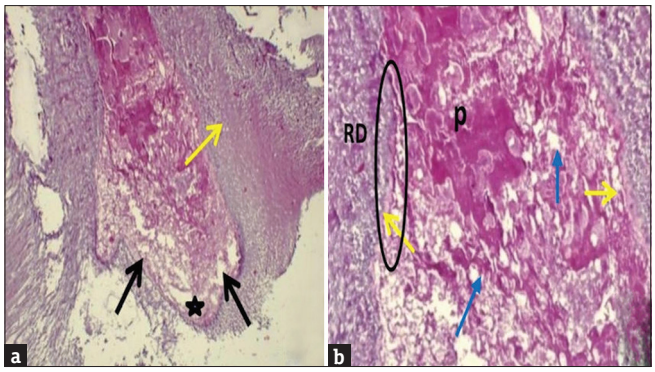

Figure 3: Photomicrograph of the pulp of the HPN group showing: (a) Marked complete reparative dentine bridge closes the orifice (black arrows). Normal pulp tissue with focal degeneration of pulp just beneath the orifice (star) and the thick layer of reparative dentin extending along lateral wall of the pulp (yellow arrow) H and E, ×40. (b) Normal pulp tissue (p), continuous layer of the odontoblastic layer (circle), few edematous spaces (blue arrows), predentin (yellow arrows), and thick layer of reparative dentin (RD) H and E, ×100

dentin bridge formation followed, without statistically significant difference, by PRF ($P \geq 0.05$). Both HPN and PRF, however, showed a significant difference statistically ($P \leq 0.05$) to FC that showed no dentin bridging with more pronounced inflammation, necrosis, and pulp disorganization. Comparisons between the three groups according to the percentages and mean of different scores are shown in Tables 4 and 5, respectively.

Discussion

Successful pulpotomy in primary dentition renders asymptomatic tooth with preserved vitality and function. In immature permanent teeth, pulpotomy permits root completion as well.[14,15] Success is related to pulp healing capacity and pulpotomy agent biocompatibility.[6] Despite being the golden standard, FC is widely criticized nowadays due to toxicity issues[16-18]; seeking other materials became mandatory. This study, therefore, had histologically investigated FC (as a control), PRF (as an autologous material known for its regenerative capacity), and HPN (as a natural flavonoid with antioxidant and anti-inflammatory effects) as pulpotomy agents in dogs.

Dog teeth closely mimic human ones histologically. They are big and numerous, limiting the total euthanized animals. Dog pulp is well developed between 1 and 2 years age.[9] Pulpotomies were attempted cervically for accessibility and avoidance of the effect of direct occlusal loading.[6] Two-months-assessment interval seemed suitable for pulp recovery after pulpotomy procedures and dentin bridge histological detection.[6,19]

Regarding the results, in FC (control) samples, histologic results came in accordance with several dog and human studies; El Meligy *et al*.,[20] similar to this study, had recorded complete absence of dentin bridging with FC. Notably, FC yields inert rather than regenerative pulp.[21] In contrary, Shulman *et al*.[22] had detected bridging with FC in the monkey model; possibly, monkey pulp is more reactive to irritation. Aldehyde in FC binds superficial pulp proteins causing fixation. Poisoning by formaldehyde explains subjacent necrosis. Inflammatory signs as hyperaemia and PMNs with pulp disorganization could be a sequlae of slow aldehyde diffusion.[23]

Dentin bridging appeared in all PRF samples. This coincides with Tabatabayi *et al*.[6] who detected dentin bridging in all dog incisors capped with PRF. PRF is rich in growth factors as platelet-derived, transforming, vascular endothelial, and insulin-like growth factors.[24,25] Huang *et al*.[26] recorded that PRF also increases the osteoprotegerin protein expression beside the alkaline phosphatase activity. Accordingly, even with few vital stem cells, proliferation, migration to the amputation site, and differentiation into new odontoblasts with subsequent dentinogenesis could occur. Fibrin mesh ensures continued release of its entrapped growth factors and cytokines. Fibrin scaffold microvascularization promotes further cell migration. Leukocytes in PRF provide infection resistance,[6] and this explains the mild to moderate inflammation and pulp disorganization noticed in some specimens.

HPN showed dentin bridging in all specimens comparable to PRF. This is compatible with Abo El-Mal *et al*.,[9] who proved more efficacy of HPN than calcium hydroxide in promoting odontoblast-like cells to differentiate and form dentin after four weeks from direct pulp capping in dogs. In the study of Parolia *et al*.,[27] propolis (a flavonoid comparable to HPN) and MTA had succeeded in better dentin bridging than calcium hydroxide in pulp capped human premolars.

HPN is known for being anti-inflammatory, causing down regulation of the hyperactive macrophages and up regulation of dysfunctional T lymphocytes.[28] However, HPN seemed not to negatively affect healthy tissues.[29] Furthermore, HPN has antioxidant properties probably due to its content of ascorbic acid (vitamin C) and trolox (vitamin E derivative), preventing or attenuating tissue damage caused by inflammation or toxins via direct scavenging of radicals, enhancing cellular antioxidant defense, and inhibiting the genesis of advanced glycation end products (AGEs). AGEs adversely affect the biological activity and are involved in aging and degenerative processes;[28] this could interpret pulp preservation in most of HPN samples.

Inversely, being crystalline with low water solubility, HPN is said to show low bioavailability (<25%).[30] Accordingly, the exact

bioavailable HPN fractions could not be determined as the pulp is highly hydrated.[17] This may explain signs of inflammation, and pulp disorganization appeared in some HPN specimens. Moreover, several studies recorded that HPN caused immunosuppression in some inflammatory models and immunopotentiation in others.[28] Therefore, further investigations of PRF and HPN with greater sample size and preferably on infected pulps are recommended.

Conclusions

Under limitations of this study, hesperidin and platelet-rich fibrin proved histologically far better than formocresol in pulpotomy, showing dentin bridge formation, less inflammation, and more preservation to pulpal tissues in dogs' teeth. These findings encourage more clinical studies concerning these bioactive materials, aiming at not only pulp preservation but also regeneration.

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Conflicts of interest

There are no conflicts of interest.

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