Original Article

Histological evaluation of keratin and keratin with tricalcium phosphate as pulp capping agents: An animal study

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Abstract

Background: Keratin is a naturally derived biomaterial that is biocompatible and can enhance pulp healing by a reparative response. There are limited studies on the effect of keratin on the repair and regeneration of dental tissue. The present study was conducted to histologically evaluate the pulpal response and calcific barrier formation after using keratin, tricalcium phosphate (TCP), and keratin + TCP as a direct pulp capping agent on goat teeth after a 90-day experimental period.

Methodology: An *in vivo* animal study evaluated 36 teeth from 4 male goats (six lower incisors and Three lower premolars per goat) that were randomly divided into 3 groups ($n = 12$ per group). Class V cavities were prepared on each tooth, and pulp tissues were capped with keratin (Group 1), TCP (Group II), and keratin + TCP (Group III). After 90 days, the teeth were extracted and histologically compared for the formation of a calcific barrier and pulpal response to different pulp-capping agents. Chi-square test and one-way analysis of variance were used to analyze the data.

Results: Histological evaluation revealed no significant difference in the distribution of samples with reference to inflammation ($P = 0.71$), vitality ($P = 0.19$), and mineralization ($P = 0.35$). Both keratin and Keratin + TCP demonstrated a higher thickness of calcific barrier ($P < 0.05$) than TCP alone and had comparable results between them.

Conclusion: Keratin + TCP may serve as an alternative for a pulp-capping agent due to their ability to induce a calcific barrier compared to keratin and TCP alone.

Keywords: Keratin; pulp-capping agent; tricalcium phosphate; vital pulp therapy

INTRODUCTION

Vital pulp therapy aims to preserve the vitality of the tooth affected by caries or trauma by placing a medicament that facilitates the formation of reparative dentin, thereby eliminating the need for more invasive procedures like root canal treatment.[1] Recent strategies in pulp therapy include the introduction of synthetic materials such as mineral trioxide aggregate, calcium phosphate, hydroxyapatite, and bio-dentin that initiate pulp and dentinal regeneration.[2,3] Biological approaches also aim to develop a scaffold that would provide the framework for cell growth and differentiation, which in turn can trigger the sequence of regenerative events.[4]

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Keratin is a natural polymer that exhibits mechanical durability and biocompatibility. It is easily available and can regenerate tissues, including bone. Sharma *et al*. demonstrated that keratin enhanced the growth and differentiation of odontoblast-like cells.[5] In addition, since dentin resembles bone, both physically and chemically, it was hypothesized that these characteristics of keratin could be harnessed for the regeneration of dental hard tissues, especially in restorative dentistry.[6,7]

Tricalcium phosphate (TCP) is a potential biomaterial that has been extensively researched as a pulp capping agent due to its ability to convert into hydroxyapatite over time. It also exhibits excellent compressive strength and biocompatibility.[8,9] It has been postulated that this material has the potential for dentin regeneration by inducing the formation of a dentinal bridge without causing superficial necrosis and inflammation.[10]

There are limited studies on the effect of keratin on the repair and regeneration of dental tissue. The present animal study aimed to evaluate the pulpal response and formation of calcific barrier after using keratin, Tricalcium phosphate, and keratin with Tricalcium phosphate as a direct pulp capping agent on goat teeth.

METHODOLOGY

The study was approved by the Institutional Ethical Committee-Tamil Nadu Government Dental College, Chennai-3 (IERB Reference No: 4/IERB/2021). The study was carried out in the Department of Veterinary Surgery and Radiology, Madras Veterinary College, Chennai, Tamil Nadu, India.

Preparation of keratin

Pulverized raw hooves were obtained from a local slaughterhouse, washed using distilled water, and dried in an oven to be used as a raw material. The raw material (pulverized hoof sample) underwent the process of defatting using a Soxhlet's apparatus for about 2 days, followed by refluxing using a mixture of hexane and dichloromethane (1:1, v/v), which ensured the effective removal of fat from the hoof. Keratin was further extracted from the raw materials using an established technique.[11] The process of extraction of keratin is provided in a flowchart [Figure 1].

Quantification of protein

The protein content was quantified using circular dichroism and Fourier Transform Infrared spectroscopy, and the molecular weight was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition, the thermal stability of keratin was assessed using Differential Scanning Calorimetry and Thermogravimetric Analysis, and the biocompatibility was assessed using the MTT assay.[11]

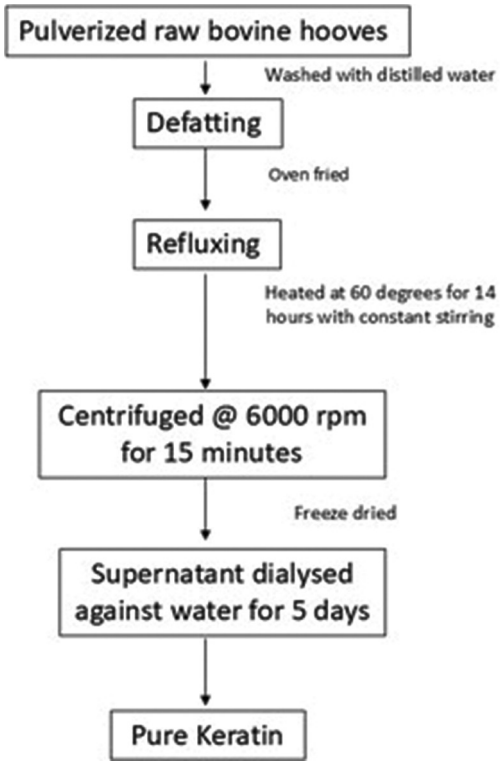

Figure 1: Preparation of keratin (flowchart)

Tricalcium phosphate

TCP, composed of nonresorbable hydroxyapatite (Hap) and resorbable TCP (α-TCP), was procured from Sisco Research Laboratories, Maharashtra.

Animal model

This *in vivo* study included four male goats aged around 4 years, weighing between 18 and 22 kg. The inclusion criteria were goats with intact and caries free lower incisors and premolars.

Procedure

The study was conducted at the Department of Veterinary Surgery and Radiology, Madras Veterinary College, Chennai, India. The goats were prepared for the procedure under general anesthesia by withholding feed for 24 h and water for 6 h. The animals were anaesthetized using injection butorphanol (1 mg/ml presentation) and injection diazepam (5 mg/ml) intravenously. Anesthesia was maintained using isoflurane. Upon induction, a long-bladed laryngoscope was used to keep the oral cavity open during the procedure. Six lower incisors and three lower premolar teeth per goat were used in this study. A sterile round diamond point dental bur (BR-46, Mani Inc., Japan) attached to a low-speed micromotor handpiece with an rpm of 50000 was used to prepare a Class V cavity of approximately 1.5 mm wide and 1 mm deep.[12] A straight fissure diamond point dental bur (SF-11, Mani Inc., Japan) was used to make a pinpoint pulpal exposure. Hemostasis was achieved using a sterile

cotton pellet soaked in saline solution. The cavity was then disinfected with 0.5% sodium hypochlorite (Prime Dental) for 60 s, followed by irrigation with saline, and then dried using a sterile cotton [Figure 2].

A convenience sampling method was used. The prepared teeth were divided into three groups ($n = 12$ teeth per goat) to receive respective direct pulp-capping agents as follows:

- Group 1 – Keratin powder was mixed with sterile distilled water to a paste consistency and was placed in the lower premolar and lower incisors of each goat using a sterile instrument
- Group 2 – TCP was mixed with saline (neutral carrier) by adding saline dropwise until a smooth paste consistency was obtained, and was placed in the lower premolar and lower incisors of each goat using a sterile instrument
- Group 3 – A mixture of TCP and keratin in a 3:2 ratio was prepared as part of a preliminary evaluation of this combination. The powders were blended with distilled water to obtain a paste consistency, which was placed over the pulp exposures of the lower premolars and incisors of each goat under aseptic conditions using a sterile applicator.

The test materials were placed over the exposed pulp in the prepared cavities using a messing gun to a thickness of approximately 0.5 mm, and were lined with type III Glass ionomer cement. After the placement of the respective test materials, the cavities were restored using Cention (Ivoclar Vivadent AG). The same operator prepared the cavity and restored the teeth. An immediate postoperative intra-oral radiograph was obtained after the procedures. All the goats were given cefotoxime 0.5 mg to prevent postoperative infection and meloxicam 0.2 mg intramuscularly for 3 days for postoperative pain relief.

Postoperatively, the animals were observed until recuperation of their physical activities and placed in individual rooms with no feeding restrictions. After the surgical procedure, all four goats were observed for any change in the feeding pattern till completion of the experimental period of 90 days. After 90 days, the goats were slaughtered for consumption, and the treated teeth were extracted for histologic evaluation. Immediately after extraction, the teeth were placed in 10% formalin for 96 h for fixation and decalcified with 2% nitric acid, dehydrated in increasing concentrations of aqueous ethanol, and embedded in paraffin wax.[13] Bucco-lingual sections of 4 μm thickness were cut longitudinally through the center of the exposure site with a soft tissue microtome (Leica Microsystems, Germany) [Figure 3]. The sections were then mounted on glass slides and stained with hematoxylin and eosin. The pulpal response was histologically evaluated concerning inflammation, vitality, and mineralization quantitatively [Table 1].[14] In addition, the thickness of the calcific barrier was categorized as: 1, no calcific barrier; 2, a calcific barrier of <0.1 mm; 3, a calcific barrier of 0.1–0.25 mm; and 4, a calcific barrier of more than 0.25 mm, respectively.

Statistical analysis

The data were analyzed using SPSS for Windows (ver. 26.0, IBM Corp., Armonk, NY, USA). Categorical data were analyzed using the Chi-square test, and continuous data were analyzed between the groups using the one-way analysis of variance (ANOVA) with *post hoc* Tukey's test. The level of significance was set at $P \leq 0.05$.

RESULTS

There was no statistically significant difference in the distribution of inflammatory cells ($P = 0.71$), vitality

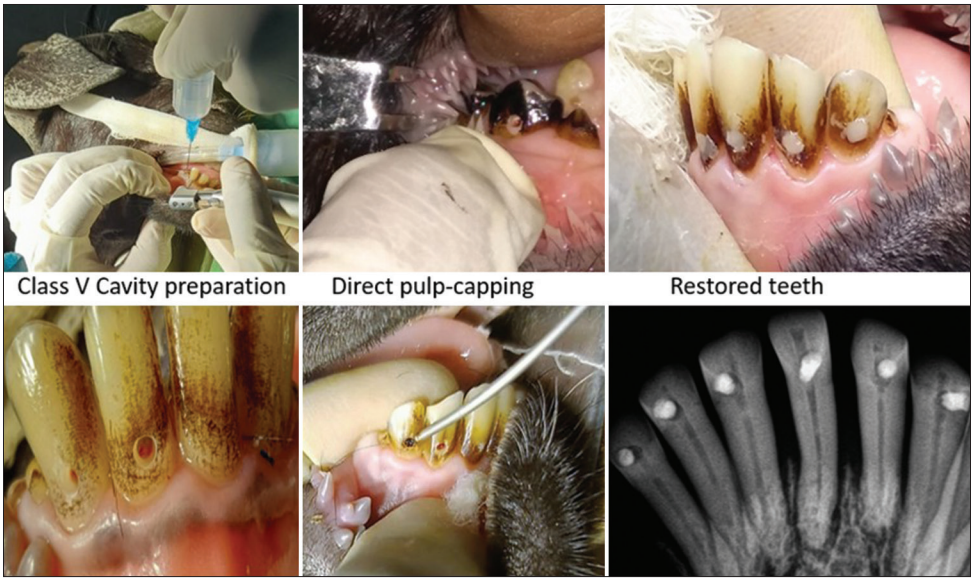

Figure 2: Class V cavity preparation, direct pulp-capping, and restored teeth

(P = 19), and mineralization (P = 0.37) between the groups [Table 2]. In addition, one-way ANOVA revealed that the highest thickness of the calcific barrier was observed in Group III (keratin + TCP). A *post hoc* multiple comparison test revealed that both keratin and Keratin + TCP demonstrated a significantly higher thickness of calcific barrier ($P < 0.05$) than TCP alone. However, the thickness was comparable between keratin and keratin + TCP (P = 0.39) [Table 3].

Table 1: Criteria for histologic evaluation

	Inflammatory cell response	
0	Absent	
1	Mild	<25 cells: A few inflammatory cells scattered throughout the pulp
2	Moderate	Moderate (25–125 cells): More than a few inflammatory cells scattered throughout the pulp
3	Severe	Severe (>125 cells): Dense collection of inflammatory cells scattered throughout the pulp
	Vitality	
0	No visible blood vessels	
1	Presence of blood vessels	
	Mineralization	
0	No trace of mineralization in the radicular pulp	
1	Increased deposition of dentin beneath the restoration section and/or along the root of the remaining pulp tissue	

Table 2: Distribution of teeth according to inflammation, vitality, and mineralization using histologic evaluation

Group	Response	Group I, *n* (%)	Group I, *n* (%)	Group III, *n* (%)	*P*
Inflammation	Absent	5 (41.7)	6 (50)	4 (33.3)	0.71
	Present	7 (58.3)	6 (50)	8 (66.7)	NS
Vitality	Absent	3 (25)	2 (16.7)	0	0.19
	Present	9 (75)	10 (83.3)	12 (100)	NS
Mineralization	Absent	0	0	1 (8.3)	0.35
	Present	12 (100)	12 (100)	11 (91.7)	NS

NS: Not significant using the Chi-square test

DISCUSSION

This animal study aimed to assess the short-term pulpal response and hard tissue formation to keratin, TCP, and both keratin + TCP combination. In addition, the study also aimed to assess the pulpal response to the synergistic effect of both keratin and TCP. The findings of the study revealed that, though an inflammatory response was observed in the teeth of all the groups, the distribution of outcome (inflammation) was not statistically significant. In addition, the majority of teeth were vital and had evidence of mineralization in all the groups. Furthermore, a combination of keratin + TCP was found to induce a greater response, resulting in thicker hard tissue formation compared to only keratin and TCP.

Animal studies are widely used in endodontology, mainly to exclude the human confounding so that a clear biological and molecular scenario related to infection, inflammation, healing, and regeneration is understood before proceeding to human trials.[15,16] The selection of the goat as a model was considered suitable for the present study due to anatomical resemblance to human teeth.[17] In addition, a follow-up period of 90 days to assess the pulpal response was decided since, 90-days is sufficient for the formation of a dentin bridge from an induced pulpal response.[18] Furthermore, pulp exposure was appreciated via a class V cavity for the ease of procedure and to avoid technical difficulties.[12]

The present study investigated the inflammatory response, the vitality, and the mineralization ability of keratin, TCP, and keratin + TCP. On histological evaluation, it was found that pulp-capping with only TCP had the lowest inflammatory cells. TCP has been researched extensively as a biocompatible, moldable, and osteoconductive material.[8] The findings of the present study were consistent with other studies where TCP demonstrated minimal or no pulpal inflammation in almost 95.4% of samples.[19,20]

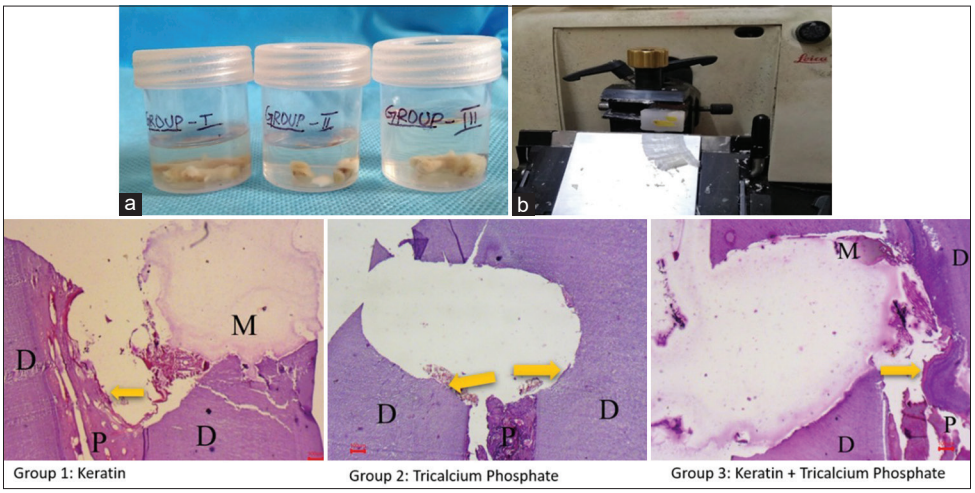

Figure 3: (a) Extracted goat teeth, (b) decalcification process and histological evaluation. (M: Test Material, P: Pulp, D: Dentin)

Table 3: Mean thickness of reparative dentin according to groups

	Number	Mean	SD	*F*	*P*	Multiple comparison	MD	*P*
Group I	12	9.1	1.9	9.101	0.001*	Group I versus Group II	1.96	0.02#
Group II	12	7.1	1.03			Group I versus Group III	−0.902	0.397
Group III	12	10.01	1.8			Group II versus Group III	−2.86	0.001#

*Statistically significant using one-way ANOVA, #Statistically significant using *post hoc* Tukey test. SD: Standard deviation, MD: Mean difference

When used as a pulp-capping agent, TCP has also shown its marked ability to induce the formation of reparative dentin at the exposure site. TCP enhances the formation of mineralization nodules by expressing odontogenic-related markers such as dentin sialophosphoprotein, dentin matrix protein 1, and osteonectin proteins.[21] Lee *et al*. reported an elevation in the activity of such biomarkers in a study conducted on mongrels. This ability can be attributed to the ions released from the degradation of TCP, such as calcium and phosphate ions that promote the differentiation and mineralization of cells through ion-mediated reactions.[22,23] In addition, the alkaline environment created by TCP appears to stimulate the differentiation of cells, thereby resulting in the formation of reparative dentin.[24,25]

The present study focused mainly on the histologic evaluation of pulpal response to keratin. As mentioned earlier, keratin is a natural polymer, biocompatible, and can enhance the growth and differentiation of odontoblast-like cells. Kakkar *et al*. demonstrated that pure keratin can be extracted and characterized for biomedical applications.[11] A murine study by Sharma *et al*. found that keratin hydrogel is a stable, elastic, nontoxic, and biocompatible material that permitted the organization of pulpal tissue without any adverse reactions and encouraged pulpal healing by a reparative response.[5] In addition, the presence of disulfide bonds makes it an excellent scaffold. Keratin can function as an extracellular matrix due to its biodegradability and is therefore used in the study.[26] Though statistically not significant, when keratin was used alone, a higher percentage of teeth did not have any inflammatory response when compared to keratin + TCP; however, TCP (when used alone) was found to have a higher percentage of teeth without inflammatory response than keratin. When assessing the percentage of vital teeth (visible blood vessels histologically) among the groups, it was found to be the least when keratin was used alone compared to TCP and in combination with TCP, respectively.

In the present study, the thickness of reparative dentin formed was also evaluated. Keratin + TCP combination was found to have the thickest reparative dentin compared to keratin and TCP alone. This study is the first to investigate a standardized 3:2 ratio of keratin and TCP as a direct pulp capping material, demonstrating their synergistic effect on dentinal bridge formation. The 3:2 (w/w) ratio of TCP to keratin was chosen after initial optimization to achieve a balance between mineral availability and bioactivity, resulting in a paste that is manageable in clinical settings. TCP serves as a reservoir of calcium and phosphate, which are essential for hard-tissue development, while keratin contributes a protein-rich matrix that fosters cell adhesion and biological signaling. The 3:2 ratio ensures that there is an adequate amount of TCP to support mineralization, while also providing sufficient keratin to improve cellular interaction and handling properties. Additionally, this ratio resulted in a cohesive paste that offers acceptable working time and application characteristics suitable for direct pulp capping. The results can be attributed to a synergistic effect of keratin and TCP when used together, resulting in a thicker calcific barrier when compared to keratin and TCP alone. Furthermore, the delayed degradation of keratin ensured continued exposure of keratin to pulpal tissues, and the associated mechanism of action of TCP could be a reason for improved reparative thickness. Although the application of keratin in tissue regeneration looks promising, its application is limited due to poor mechanical properties and brittle structure. Thus, blending these natural polymers with synthetic materials is recommended to improve the bioactivity and mechanical strength of keratin. TCP was therefore added to keratin to improve its mechanical properties.[27]

The literature concerning the application of keratin as a direct pulp capping agent is notably limited. This study has proved that a natural polymer like keratin can facilitate pulpal healing by inducing the production and mineralization of reparative dentin. Furthermore, this study has demonstrated that *in vivo* formation of reparative dentin is possible with the use of keratin and TCP due to their ionic release and mildly alkaline environment. However, this study has a few limitations. First, these are animal studies, and the transition of such studies to human trials does not guarantee similar outcomes. Second, the exposure of pulp was made under ideal conditions without any preexisting inflammation by dental caries. It is recommended to conduct further studies and randomized clinical trials on carious teeth with longer follow-up times. The healing can be further validated using specific markers to assess odontogenic, angiogenic, and neurogenic potential and three-dimensional micro-computed tomography evaluation to assess the dentinal bridge formation. Future research incorporating these natural biopolymers with a combination of stem cells can also be studied.

CONCLUSION

Within the limitations of the present study, the findings suggest that the combination of keratin and TCP promotes

dentinal bridge formation more effectively than either keratin or TCP alone. Keratin, either independently or in combination with TCP, may therefore serve as a promising alternative biomaterial for vital pulp therapy.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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