Preclinical Evaluation and Clinical Trial of Chlorhexidine Polymer Scaffold for Vital Pulp Therapy

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Objective: *To evaluate the preclinical effectiveness and clinical efficacy of chlorhexidine polymer scaffold for vital pulp therapy.* ***Study design:*** *Polymer scaffolds loaded with chlorhexidine were prepared by electrospinning. The scaffolds were evaluated using four different tests: i) The release of chlorhexidine from the polymer scaffold was evaluated by Fourier-transform infrared spectroscopy (FTIR); ii) Biocompatibility of chlorhexidine scaffold was tested by subcutaneous implantation in rabbits; iii) The scaffolds were implanted into human molars for further ex vivo histological evaluation; and iv) The clinical efficacy of the scaffold was evaluated as a pulp dressing following vital pulp therapy, in comparison with MTA (control) in a clinical trial of forty primary molar teeth in 34 children aged 6 to 8 years.* ***Results:*** *The scaffold was antimicrobial to Streptococcus mutans, Lactobacilli and other facultative anaerobes. Fourier-transform infrared spectroscopy confirmed the presence of chlorhexidine and polyvinyl alcohol in the scaffold. The histological evaluation of the subcutaneous tissue of rabbits and ex vivo human molars provided an acceptable biocompatibility response to scaffold. The clinical trial showed that the efficacy of chlorhexidine loaded scaffold was 90% and comparable to MTA.* ***Conclusion:*** *The scaffold met acceptable standards in all the four tests that were performed, including the clinical trial. A larger clinical trial with chlorhexidine scaffold in adult teeth may be necessary to prove its efficacy. The preliminary clinical trial results demonstrated that the scaffold was beneficial in saving primary teeth that required pulpotomy as a vital pulp therapy.*

Key words: *Vital pulp therapy, polyvinyl alcohol, mineral trioxide aggregate, chlorhexidine.*

INTRODUCTION

Vital pulp therapy is a treatment which aims to maintain vital pulp tissue that has been compromised but not destroyed by caries, trauma or restorative procedures. Vital pulp therapy is particularly important in immature permanent teeth with incomplete apical root development, because it attempts to strengthen the tooth and resist fracture by promoting continued root development.

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Mineral trioxide aggregate (MTA) has become the most popular dental material for vital pulp therapy because of its biocompatibility, sealing ability that prevents microleakage and dentinogenesis promotion.[1] MTA is a successful pulp capping material for the pulpotomy of primary teeth, however MTA is expensive and it has difficult handling properties because of its slow setting time.[2]

The use of biomaterials with antimicrobial activity has become increasingly important in vital pulp therapy. Local delivery systems containing antimicrobial agent suppresses or eradicates the pathogenic microbiota or modulates the inflammatory response thereby limiting tissue destruction. They also maintain constant and prolonged concentration profiles of therapeutic agent.[3] Polymeric scaffolds have gained huge interest in local drug delivery system as they increase the surface area of drug carrier which enhances the drug dissolution rate. [4,5]

Chlorhexidine is a common dental disinfectant which is being used for many years in mouth rinses, gels, sprays, varnishes, chips and subgingival irrigation devices. Chlorhexidine is a popular and proven disinfectant to get rid of broad spectrum of oral microbiota.[6] In this study, we decided to load chlorhexidine into polymer scaffold by electrospinning and place it as a pulp dressing following vital pulp therapy.

Hence, the aim of this study was to evaluate i) The release of chlorhexidine from the polymer scaffold by Fourier-transform infrared spectroscopy (FTIR); ii) Biocompatibility of chlorhexidine scaffold is tested by subcutaneous implantation in rabbits; iii) The scaffolds are implanted into human molars for further ex vivo histological evaluation; and iv) The clinical efficacy of scaffold is evaluated as a pulp dressing following vital pulp therapy, in comparison with MTA (control) in a clinical trial of forty primary molar teeth in 34 children aged 6 to 8 years.

MATERIALS AND METHOD

Polyvinyl alcohol polymer of molecular weight 1,24,000 g/mol (Sigma Aldrich, Mumbai), 2% chlorhexidine gluconate (Stedman pharmaceuticals, Chennai) and distilled water were used in the preparation of chlorhexidine loaded scaffold. Mineral trioxide aggregate (Angelus- White MTA, Brazil) was used as control in the study.

Preparation of chlorhexidine scaffold

Polymer solution was prepared by dissolving polyvinyl alcohol polymer and 2% chlorhexidine gluconate in distilled water. Polymer solution was placed on a magnetic stirrer for homogenous mix. Then the polymer solution was loaded into the electrospinning apparatus for preparation of scaffold.[7] Scaffold is collected on the aluminum foil and stored in UV chamber for 12 hours. Then the scaffold was cut into 1cm x 1cm and stored in disposable vials for single use (Fig 1).

Figure 1: Storage of chlorhexidine scaffold in a sterile vial for clinical use.

Fourier-transform infrared spectroscopy (FTIR) analysis

Fourier-transform infrared spectra were recorded with Fourier-transform infrared spectrometer (Bruker, Billerica, Massachusetts, USA) using potassium bromide pellets containing 1wt% of sample in an absorbance mode with resolution of 2 cm^{-1} interval in the wave number range of 4000–500 cm^{-1}. For each sample, the readings were recorded for three times to confirm the reproducibility and the presence of functional groups in chlorhexidine loaded scaffold.

Preclinical antimicrobial efficacy of scaffold

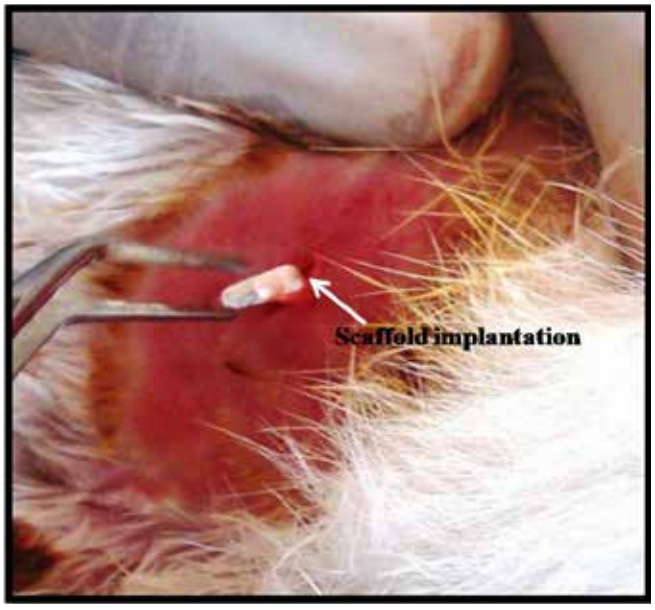
Infected carious dentin was isolated from carious tooth of a child and transported in phosphate buffer solution and inoculated using spread plate technique in *mutans sanguis* selective medium, blood agar and Rogosa agar. After incubation, colonies formed in the media were confirmed using histological staining. Bacterial sensitivity to the chlorhexidine scaffold was evaluated by placing 0.5cm x 0.5cm membrane into the selective media after subculture. The plates were incubated for 48hrs at a temperature of 37^0C, after which the zones of inhibition were measured in millimeters.

Subcutaneous implantation in rabbit

The experimental protocol was conducted in compliance with the specifications of the animal experimentation ethics of Helsinki and approved by the institutional research ethics committee. Two 8 week old male New Zealand white rabbits weighing 250-264 grams were selected and housed in animal unit at 25°C and on a 12-hour light- dark cycle. Rabbits had free access to water and low fat diet pellets and were allowed to acclimatize for one week prior to the experiment and were weighed on a regular basis. Prior to the implantation procedure, rabbits were anaesthetized by using ketamine (14units/kg) and xylazine (7units/kg). The back of the rabbits were shaved and swabbed with iodine (0.1%) and then two sagittal incisions (approximately 1 cm long) parallel to the midline were made on each side with a scalpel and subcutaneous pockets were made using blunt dissection. Then a chlorhexidine scaffold measuring 0.5cm x 0.5cm was placed in each pocket and sutured with nylon (Fig 2). The animals were monitored for local symptoms at the wound area on daily basis. At the end of 7 and 14 days of implantation, tissue surrounding the implantation site was taken for histological examination from one implantation site in each animal at a time.

After excision, the tissue was fixed in 10% buffered neutral formalin for 2 days, embedded in paraffin and processed for histological examination. Sections of 4µm thick were prepared using microtome, stained with hematoxylin and eosin and examined under microscope.

Figure 2: Implantation of chlorhexidine scaffold into subcutaneous tissue of rabbit.



Ex vivo human molar study

Ex vivo experiment was carried out on 2 third molars which were indicated for extraction. Pulpotomy was performed under strict aseptic condition. Chlorhexidine scaffold was placed on the radicular pulp stumps and access was restored with glass ionomer cement and composite resin. After 14 days, the teeth were extracted and 10% formalin was injected into the pulp through apical foramen to fix the pulpal tissue. Specimens were decalcified with ethylene diamine tetra acetic acid and embedded in paraffin blocks. They were sectioned longitudinally, stained with hematoxylin and eosin and examined under microscope.

Clinical trial in primary molars

A clinical trial was carried out with an aim to evaluate the efficacy of chlorhexidine scaffold for vital pulp therapy in primary molars. MTA was considered as a control. The study design is a randomized controlled trial, approved by institutional review board (VDC/RP/2012-59) and was registered in Clinical Trials Registry–India (CTRI/2015/05/005746).

Clinical trial was conducted between december 2012 and december 2014 and the children were selected from outpatient clinic of pediatric dentistry. A total of 40 primary molars in 34 healthy children of 6-8 years indicated for pulpotomy were included. Written consent from the parents was obtained by explaining the purpose of the study, treatment protocol and materials being used. Teeth with spontaneous pain, swelling, tenderness to percussion, pathological mobility and unsuccessful hemorrhage control were excluded. Also the teeth showing radiographic signs of resorption, periradicular or furcal radiolucency, widened periodontal ligament space or physiological root resorption of more than two-thirds were excluded.

Forty primary molars in 34 children were randomly allocated into two groups by computer generated random numbers and 20 primary molars were included in each of the groups. After administration of local anesthesia and rubber dam isolation, caries was removed before entering the pulp chamber with a sterile high speed No. 330 diamond bur. Then pulp chamber was deroofed and coronal pulp tissue was removed with a sterile spoon excavator under continuous saline irrigation. Moist cotton pellet was placed on the radicular pulp stumps for 5 minutes to cease the bleeding. Chlorhexidine scaffold and MTA were placed on the radicular pulp stumps in test and control groups respectively. After the procedure, the access cavities were restored with glass ionomer cement followed by stainless steel crowns. All the children were recalled after 6, 12 and 24 months for clinical and radiographic evaluation. Parents were advised to report in case of any pain or discomfort. Follow-up evaluation was done independently by two blinded clinicians. Group allocation and subjects availability in follow-up visits are reflected in CONSORT flow diagram (Fig 3).

Radiographs of tooth 74 treated with chlorhexidine scaffold are illustrated in Fig 4; Fig 4a is pre-operative radiograph of tooth 74 showing deep carious lesion without any inter-radicular or periapical pathology; Fig 4b, 4c and 4d are 6, 12 and 24 months

Figure 3: CONSORT flow diagram showing group allocation and subjects availability in follow-up visits.

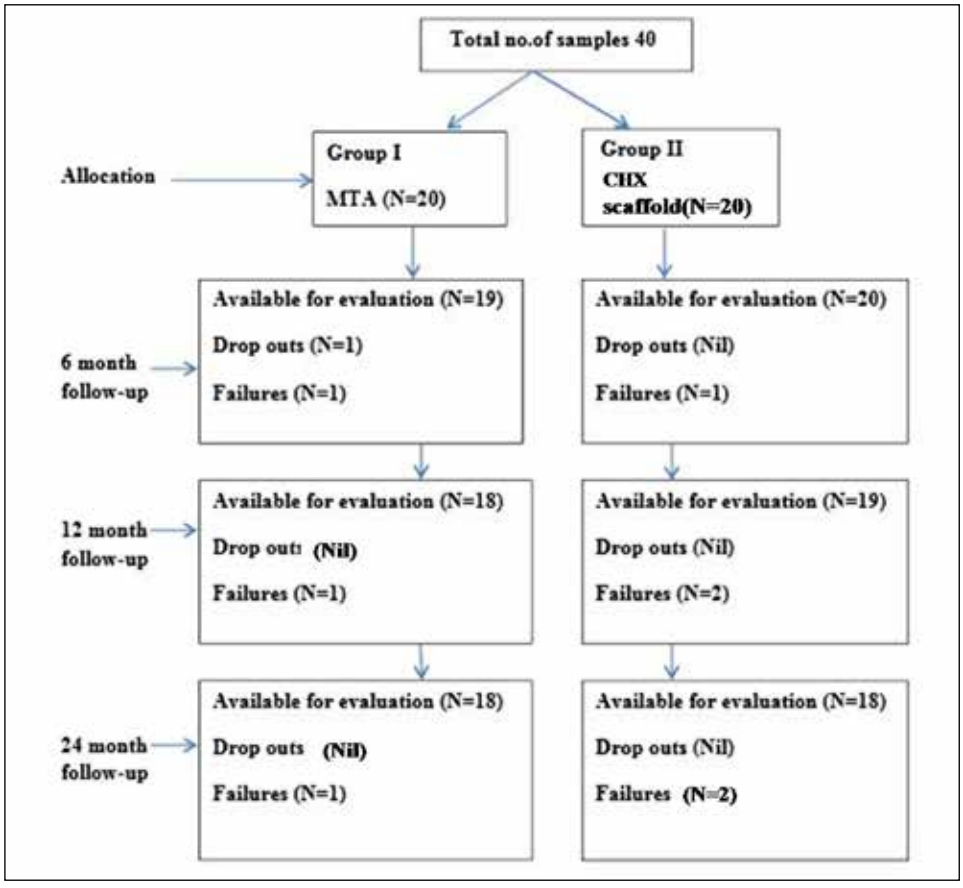


Figure 4: Radiographs of tooth 74 treated with chlorhexidine scaffold; (a) Pre-operative radiograph of tooth 74 showing deep carious lesion without any inter-radicular or periapical pathology; (b) 6 months follow-up radiograph showing no pathological signs; (c) 12 months follow-up radiograph showing no pathological signs ; (d) 24 months follow-up radiograph showing no pathological signs.

Figure 5: Radiographs of tooth 74 treated with MTA; (a) Pre-operative radiograph of tooth 74 showing deep carious lesion without any inter-radicular or periapical pathology; (b) 6 months follow-up radiograph showing no pathological signs; (c) 12 months follow-up radiograph showing no pathological signs ; (d) 24 months follow-up radiograph showing no pathological signs.

follow-up radiographs showing no pathological signs; Radiographs of tooth 74 treated with MTA are illustrated in Fig 5; Fig 5a is pre-operative radiograph of tooth 74 showing deep carious lesion without any inter-radicular or periapical pathology; Fig 5b, 5c and 5d are 6, 12 and 24 months follow-up radiographs showing no pathological signs.

Statistical analysis

The collected data was entered into microsoft excel sheet and was subjected to descriptive statistical analysis using chi-square test in SPSS (Version 17, IBM corporation, USA) software. Statistical significance was computed and p value ≤ 0.05 was considered significant.

RESULTS

Observations of Fourier-transform infrared spectroscopy (FTIR) of chlorhexidine scaffold

Functional groups present in the scaffold were studied using Fourier-transform infrared spectroscopy. Fourier-transform infrared spectra (X-axis represents wave number in cm^{-1} and Y-axis the transmittance) (Fig 6) showed the characteristic peaks in the range of 3267 to 2925 cm^{-1} indicating the presence of aromatic –NH, -CH, CH_2 groups and at the wave number of 1148 cm^{-1} confirming the presence of –OH group.[8]

Observations of preclinical antimicrobial efficacy of chlorhexidine loaded scaffold

Scaffold produced a significant inhibition zone of 16 ± 0.8 mm on mutans sanguis selective media (Fig 7a), 17 ± 0.8 mm on blood agar (Fig 7b) and 16 ± 0.8 mm on Rogosa agar (Fig 7c) containing *Streptococcus mutans*, facultative anaerobes and *Lactobacilli* respectively after 48 hours of incubation at 37^0C.

Observations of biocompatibility test

Hematoxylin and eosin staining of 7 days specimens from rabbit (Fig 8a) showed connective tissue with dense irregular collagen fibers associated with dense chronic inflammatory cells, predominately lymphocytes. Few plasma cells and extravasated red blood cells were seen. Dilated blood vessels and engorged red blood cells infers the presence of chronic nonspecific inflammatory tissue response. No evidence of implanted material infers that implanted material was completely resorbed. In the specimens that were taken after 14 days of implantation, the connective tissue with loose irregular collagen fibers and fibroblastic proliferation was found and most part of the tissue was appearing normal confirming the drastic decrease in inflammation and signs of healing (Fig 8b).

Figure 6: Fourier-transform infrared spectra (FTIR) showing the characteristic peaks.

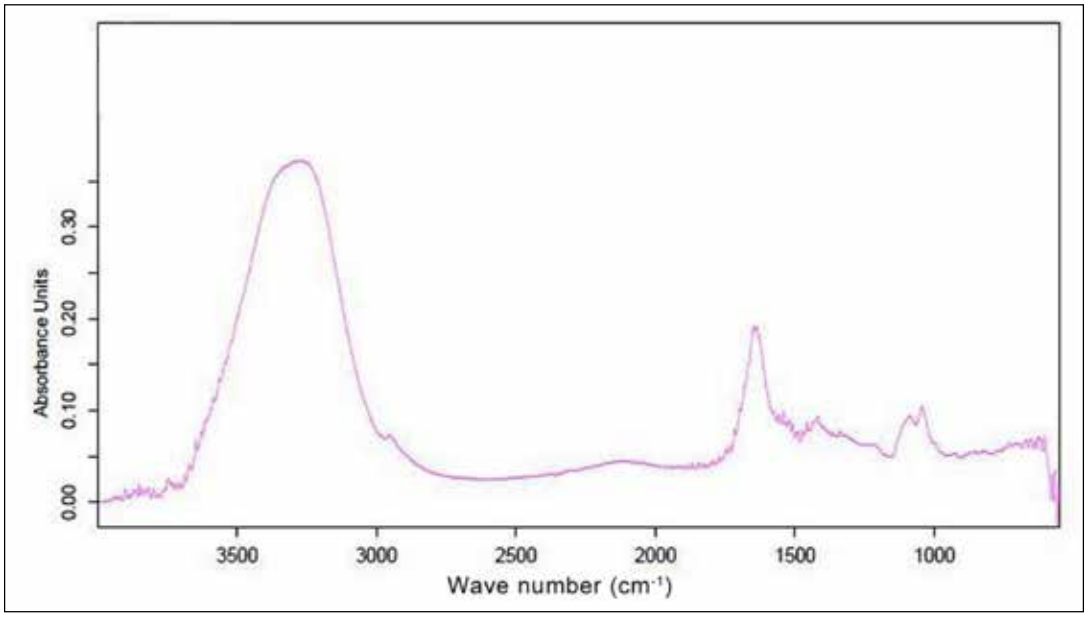


Figure 7: Demonstration of antibacterial activity of chlorhexidine loaded scaffold ; (a)Inhibition zone of 16 ± 0.8mm produced on mutans sanguis media containing *Streptococcus mutans*; (b) Inhibition zone of 17 ± 0.8 mm produced on blood agar media containing facultative anaerobes ; (c) Inhibition zone of 16 ± 0.8 mm produced on Rogosa agar media containing *Lactobacilli*;

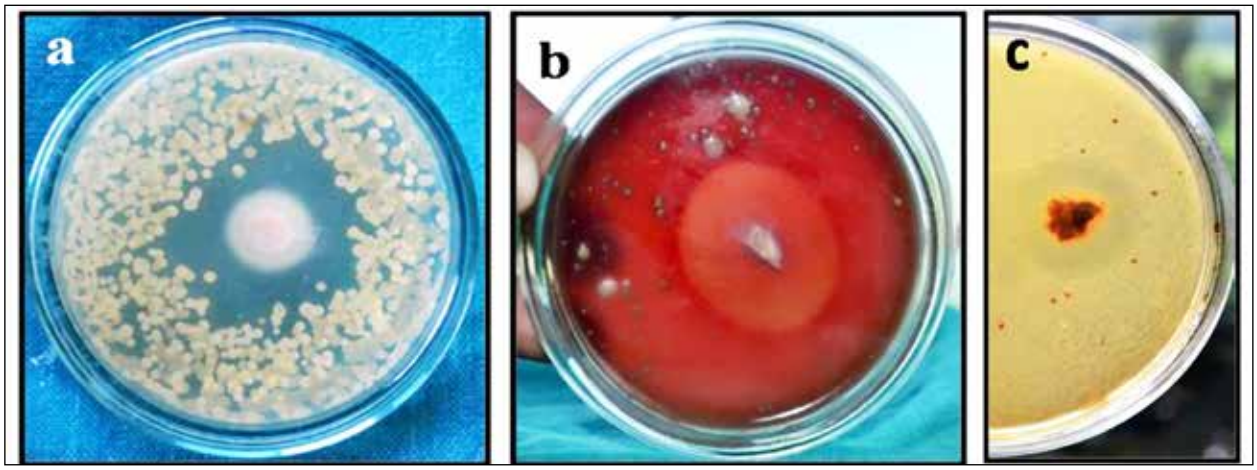
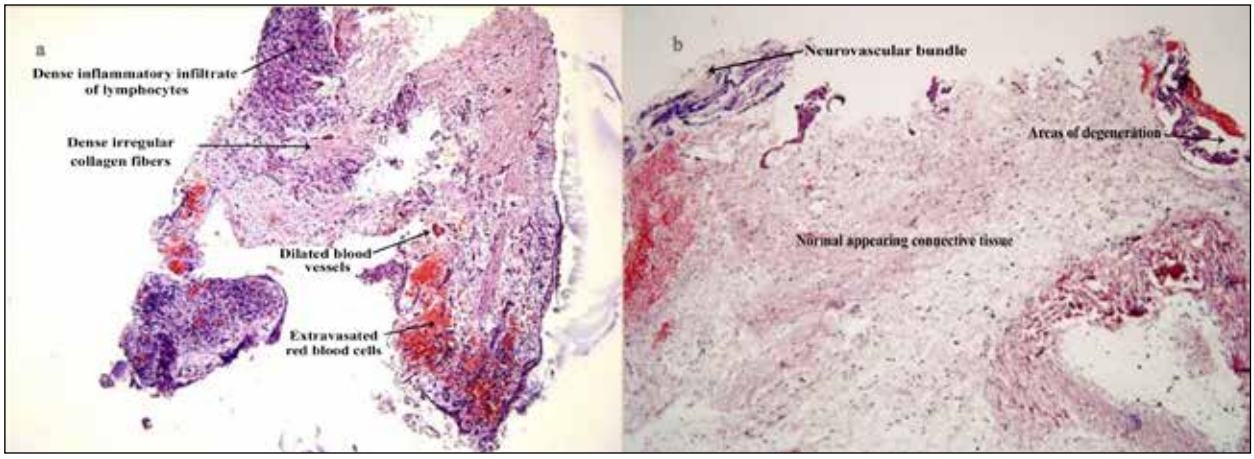


Figure 8: Histopathological sections of rabbit subcutaneous tissue (10X); (a) Specimen that was taken 7 days after implantation of chlorhexidine scaffold showing dense irregular collagen fibres, dense inflammatory infiltrate of lymphocytes, extravasated red blood cells and dilated blood vessels; (b) 14 days after implantation showing normal appearing connective tissue suggesting the healing pulp.



Histologic observations of *ex vivo* human molar study

Decalcified bucco-lingual sections of molars which were extracted 14 days after vital pulp therapy with chlorhexidine loaded scaffold (Fig 9) showed radicular pulp which was fibro-cellular with collagen fibers, blood capillaries, active and inactive fibroblasts. Spindled and flattened odontoblasts with hyperchromic nuclei were evident surrounding the root canals suggesting the normal pulp in the root canals.

Observations of clinical trial

At 6 months follow-up, 39 pulpotomized teeth were available for evaluation. MTA and chlorhexidine loaded scaffold yielded a success rate of 94.7% and 95% respectively and the difference was statistically not significant (p=0.976). One failure was accounted in each group (Table 1).

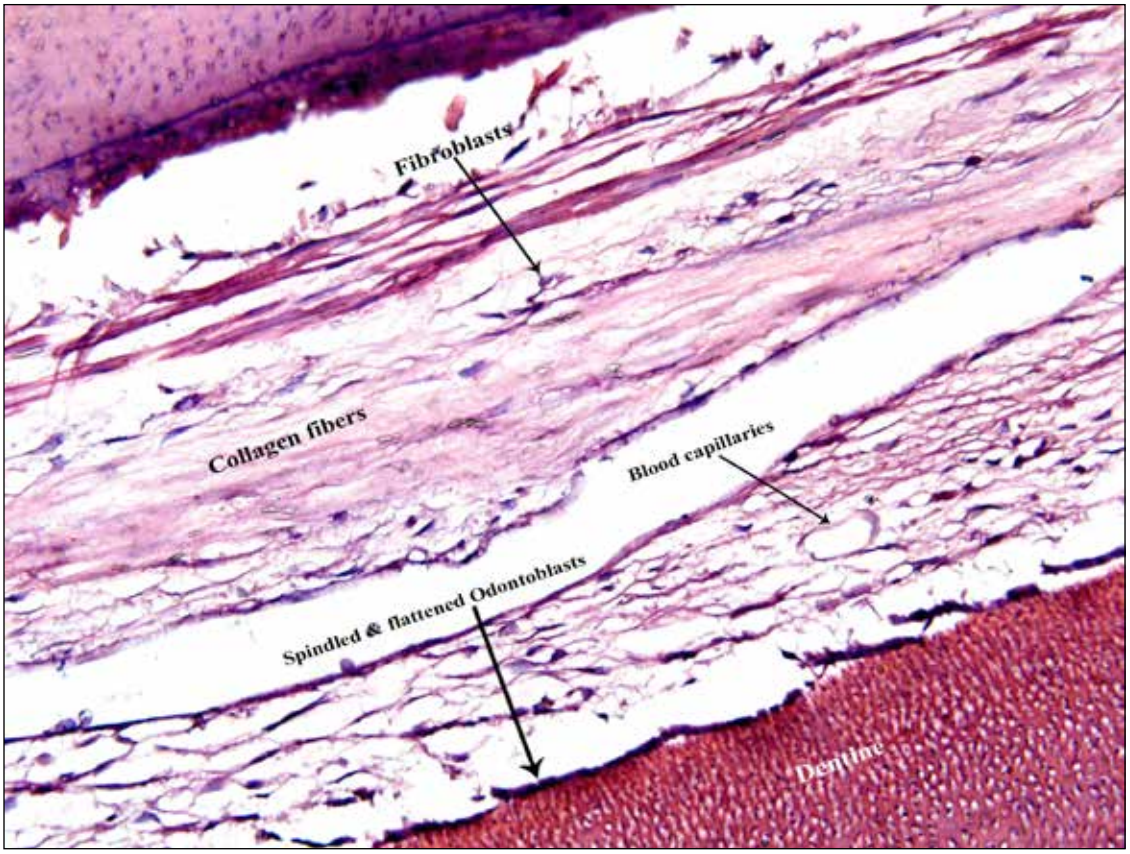
At 12 months follow-up, 37 teeth were available for evaluation. Success rate reduced to 90%, with one more failure in test group, whereas in control group no failures were seen and the difference was statistically not significant (p=0.579) (Table 1).

Excluding the failures, 36 teeth were available for evaluation at 24 months follow-up. No further failures were seen in either of the groups and the success rate remained same as 12 months interval. Overall success rate of vital pulp therapy with chlorhexidine scaffold and MTA was 90% and 94.7% respectively and this difference was statistically not significant (p=0.579) (Table 1).

Table1: Treatment outcome at three follow-up intervals

Follow-up Intervals	MTA group		Chlor-hexidine scaffold group		
	Success	Failure	Success	Failure	p value
6 months	94.7%(n=18)	5.3% (n=1)	95% (n=19)	5% (n=1)	0.976
12 months	94.7%(n=18)	5.3% (n=1)	90% (n=18)	10% (n=2)	0.579
24 months	94.7%(n=18)	5.3% (n=1)	90% (n=18)	10% (n=2)	0.579

Figure 9: Histo-pathological picture of decalcified bucco-lingual section of molars after two weeks of vital pulp therapy with chlorhexidine scaffold showing collagen fibers, fibroblasts, blood capillaries, spindled and flattened odontoblasts suggesting normal pulp (40X).



DISCUSSION

Vital pulp therapy is routinely practiced to manage primary teeth with carious exposure. Various materials have been tried as pulpal medicaments and mineral trioxide aggregate has been consistently shown to be the best material for vital pulp therapy in both primary and permanent teeth.[9, 10] The high clinical success with MTA is attributed to its biocompatibility, good sealing ability and unique property to stimulate high quality and a greater amount of reparative dentin formation.[1]

Recent trends in tissue bioengineering aims to replace or facilitate the regrowth of damaged or diseased tissue by applying biomaterials, cells and bioactive molecules. Biodegradable polymeric scaffolds have received much attention in tissue engineering as they provide temporal and spatial environment for cellular growth, tissue ingrowth and local drug delivery.[11] Polyvinyl alcohol is approved by Food and Drug Administration (FDA) and has good fiber forming property with electrospinning technique. Chlorhexidine gluconate (2%) was considered for loading with polyvinyl alcohol fibers because of its broad spectrum antibacterial activity.

Vital pulp therapy clinical trial in primary teeth using chlorhexidine scaffold as a pulp dressing material showed a comparable result with MTA at the end of 24 months. Prolonged concentration of chlorhexidine at the pulpotomy site could be the possible reason for the good clinical outcome with chlorhexidine scaffold. Chlorhexidine facilitates the decrease in the bacterial content and thereby decreases the inflammation of the underlying radicular pulp which helps in the healing. Chlorhexidine gluconate is water soluble and at physiologic pH it readily dissociates and releases chlorhexidine component.[12] Chlorhexidine is a synthetic cationic bis-guanide, positively charged hydrophobic and lipophilic molecule that interacts with negatively charged phospholipids and lipopolysaccharides on the microbial cell wall thereby altering the cell osmotic equilibrium. At 2% concentration, chlorhexidine is bactericidal and causes precipitation of the cytoplasmic contents, which results in cell death.[13]

Vital pulp therapy performed with MTA demonstrated superior results similar to previous studies.[1,14,15] These findings confirms its better biocompatibility and good sealing property. The drawbacks with MTA are its high cost and tedious handling properties.[2] The advantage with chlorhexidine scaffold is that it can be cut into required size, shape and can be easily placed in the pulp chamber. Also the preparation of scaffold is economical when compared to MTA. Thus chlorhexidine scaffold can be advantageous when used as vital pulp therapy medicament in primary molars. However, it is still premature to draw definitive conclusions and further long-term clinical trials with large sample size are warranted to reach sound conclusions.

CONCLUSION

The scaffold met acceptable standards in all the four tests that were performed, including the clinical trial. A larger clinical trial with chlorhexidine scaffold in adult teeth may be necessary to prove its efficacy. The preliminary clinical trial results demonstrated that the scaffold was beneficial for saving primary teeth that required pulpotomy as a vital pulp therapy.

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