ORIGINAL ARTICLE

Injectable Nano-Chitosan /$CaCO_3$ (NCsC) Composite as Novel Pulpotomy Paste in Partially Pulpotomized Rabbit Incisors

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Abstract

Purpose: Maintaining pulp vitality and functions are of paramount importance in different branches of modern dentistry like endodontic, conservative, pedodontics, and dental traumatology, the applying of tissue-engineering principles to create simple and effective scaffold material suitable for maintaining pulp vitality, represent a promising therapeutic option for treating immature and mature tooth with compromised pulp vitality.

To evaluate the pathophysiological response of the pulp to (nano-chitosan/$CaCO_3$) scaffold as a potential novel capping material on traumatically exposed pulp of rabbit incisors.

Materials and Methods: Twenty-four upper incisors of twelve completely dentulous healthy New Zealand male rabbits were used, subdivided into two groups of 12 teeth. In each group, six teeth were used as a control group, where the pulp was traumatically exposed and partially amputated, left free of capping material, and the other six teeth, used as an experimental group, where the amputated pulp was capped with an injectable (nano-chitosan/$CaCO_3$) composite. The cavities of both groups sealed with resin modified glass ionomer cement as the final restoration. Animals were sacrificed, and the teeth were collected for histological examination according to the sacrifice time (1 and 4 weeks).

Results: At 1 week, both groups showed a non-significant difference in inflammatory extent. Highly significant difference in calcific bridge formation (P=0.002), and dentin morphology (P=0.002). At a 4-week period, there is a non-significant difference in inflammatory response. High significant difference in dentine bridge formation (P=0.002). Non-significant difference in the dentin morphology (P=0.06). The nano-chitosan/$CaCO_3$ group showed faster dentin bridge formation in both periods compared to the control group.

Conclusion: (nano-chitosan/$CaCO_3$) composite is a promising novel pulpotomy material.

Keywords: Tissue Engineering; Scaffold; Nano-Chitosan; $CaCO_3$; Composite; Rabbit Incisors.

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1. Introduction

Traditional strategies for preserving pulp vitality involve the removal of inflamed or necrotic pulp tissue and replacing it with a synthetic capping biomaterial like zinc oxide Eugenol, ferric sulfate, formocresol, Calcium Hydroxide (CH), Mineral Trioxide Aggregate (MTA), biodentine, bonding resins system etc. [1] .All of these materials are suboptimal, showing many drawbacks and limitations, such as inducing chronic inflammation, poor sealing ability, formation of tunneled tertiary dentine, difficult handling, high cost, long sitting time, tooth discoloration, canal obliteration, and internal or external root resorption [2,3]. seeking the optimization of the outcomes and minimizing the drawbacks, innovative scaffold materials have been invented as a promising substitute to traditional capping material [4]. However, due to its antimicrobial, hemostatic, and safe, biocompatible profile, chitosan has gained great interest in the endodontic field [5,6]. Chitosan (CS) is a natural positively charged polysaccharide composed of repetition of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, resembling the component of the extracellular matrix ECM [7]. Chitosan is converted from a polysaccharide to a polycation in an acidic environment, where the amino groups (NH2+) protonate into (NH3+). responsible for the electrostatic attraction of anions and makes chitosan more biologically active [8]. Chitosan has multiple functional groups other than the amino group in its chain, such as hydroxyl [-OH] group, amide (-CONH-, -$CONH_2$), and carboxyl [COOH] [9]. It can be modified and used in liquid form, hydrogels, fibers, membranes, pastes, etc. [10]. Moreover, chitosan nanoparticle appears more promising than the base material in terms of mechanical stability, ion interaction, tissue adhesion, less body resistance, and more anti-microbial effectiveness owing to their high purity, extremely large surface area, and numerous functional groups which provide great potential for surface modification [11, 12]. As a mineralized tissue substitute, in vitro and in vivo studies demonstrated that the blending of nano-chitosan with other natural, synthetic polymers and/or bioceramics could efficiently increase their mechanical properties as well as the biological performance and clinical outcomes [13, 14, 15]. Within the same context, for dentin-pulp complex regeneration, adding of bioceramic as a mineral phase and/or bioactive molecules to chitosan scaffolds significantly enhance its osteogenic effects and greatly improves mechanical strength [16, 17, 18, 19].On the other hand, the addition of polymeric phase to bioceramics elaborates 3D biomimetic composite resembles the natural bone/dentin ECM with improvements of its injectability, setting time, and mechanical properties [20, 21, 22, 23]. As a mineral phase to be added to biopolymer, Calcium Carbonate ($CaCO_3$) is appealing due to its availability, biocompatibility, biodegradability, buffering action, easily incorporate to chitosan scaffold by simple mixing in situ, making the scaffold affordable, easily fabricated with low cost [24, 25]. Furthermore, in many in vivo animal studies, $CaCO_3$ has been described as an effective osteoconductive and biocompatible bioceramic [26, 27, 28]. Here in, we developing a nano-chitosan/$CaCO_3$ scaffold as potential novel pulpotomy medicaments, thus, present study aims to evaluate the pathophysiological response of traumatically exposed pulp of rabbit incisors to (nano-chitosan/$CaCO_3$) scaffold by histological analysis.

2. Materials and Methods

2.1. Materials

Precipitated extra pure calcium carbonate $CaCO_3$ (india.mumbia). Glacial acetic acid was supplied from Merck (Germany). Chitosan (CASnumber 7631-86-9; MW=161, 50nm, degree of deacetylation more than 90% (Research Nanomaterial, USA). Light-cure resin-modified glass ionomere cement (RMGIC) (prime.dent.USA).

2.2. Methods

2.2.1. Acetic Acid Preparation

By adding 0.5 ml of glacial acetic acid to 99.5 ml of distilled water, (0.5%) of glacial acetic acid was prepared [29].

2.2.2. Preparation of the (Nano-Chitosan/$CaCo_3$) Paste

The injectable paste was prepared by mixing the liquid phase (0.5% acetic acid) with the powder phase,

the powder phase produced by admixing 1g of pure nano-chitosan powder with 1.5g of pure calcium carbonate powder to achieve an admixture of (60% $CaCO_3$ and 40% chitosan by weight) [30]. As an injectable material, to achieve the best handling properties of the paste, the powder-to-liquid ratio was chosen after a pilot study and determined by trial, using a (spoon of powder/drops of liquid) ratio [31]. Three drops of diluted glacial acetic acid (0.5%) mixed with one spoon of the admixture powder, each spoon of this admixture weighing 15 mg. The mixing is made on a cement slab manually and incrementally for 2 minute to achieve putty injectable consistency. By spatula, the paste is applied into a sterile disposable syringe (1ml). (ultra-dent.USA) to facilitate its injection into pulp chambers.

2.3. Design of Animal Experiments

2.3.1. Sample Size

This research is done according to ethical approval from the research ethics committee of college of Dentistry, University of Baghdad (Ref.no:462). Due to ethical and economical consideration, the least number of animals was used, and according to previous studies (6 teeth per group) is the minimal sample size for each outcome to be statistically reliable [32, 33].

2.3.2. Animal Grouping

Twenty-four incisors of twelve completely dentulous healthy New Zealand male rabbits weighing 2.5 Kg were used in the present study. The rabbits had been kept under constant conditions of balanced diet, temperature, humidity, and light. In each rabbit, two upper central incisors were selected to perform pulp exposure [34]. The teeth were randomly categorized into 2groups.

- Positive control group: teeth were exposed mechanically and the pulp partially pulpotomized without any capping material (n=12).
- Nano-chitosan/$CaCo_3$ group: teeth were exposed mechanically and the pulp partially pulpotomized, then capped with calcium carbonate-chitosan paste (n =12).

The experimental teeth were obtained at interval periods (7,28 days) following the pulpotomy procedure [34]. Six teeth from each group were examined histologically for each period [32, 33].

2.4. Pulp Capping Procedures

The rabbits were anaesthetized by intramuscular injection of Xylazine HCl 5 mg/kg, followed by Ketamine HCL 35 mg/kg in the quadriceps femoris muscle. The field of operation was disinfected with 10% povidin-iodin solution. class V cavity prepared by using a sterile size 0.8 mm carbide tungsten bur (Komet Dental, Lemgo, Germany) mounted on a high-speed handpiece (30,000 rev/min) under copious irrigation with sterile physiological saline. The bur was oriented 45-degree inclination apically at the cervical region of the buccal surface of the incisor, 1mm above the gingival margin, the drilling extended till reach the pulp, and the exposure was done by using a small spoon excavator, removing part of the pulp tissue. The cavity formed is approximately 2 mm^2 in depth necessary to apply capping material with final restoration [35], to achieve hemostasis and disinfection after pulp exposure, the cavity was rinsed with an antiseptic solution 5.25% NaOCl for 30 sec, then the cavity was washed with distilled water and dried by brief air blot [36]. The capping materials were prepared and injected according to the study design.

2.5. Sample Collection and Tissue Staining

By intravenous injection with an anesthetic overdose, 6 rabbits were euthanized at each interval. After rabbit scarification, teeth were fixed in 10% neutral buffered formalin solution and demineralized in 10% nitric acid for 3 days, dehydrated sequentially in graded alcohols, 70%, 80%, 90%, and 100%, then immersed in xylene, and finally embedded in paraffin blocks. The blocks were serially sectioned with an average thickness of 5 mm along the mesio-distal direction passing through the center of the pulp exposure site. The sections were stained with routine hematoxylin and eosin staining following a standardized protocol [37].

2.6. Histological Evaluation

All the representative sections were blindly examined and evaluated histologically by two experienced

investigators, using light microscopy (Leica microscope, Germany), which includes a digital camera mounted on it. The extent of the inflammatory reaction and mineralized substance next to the exposed area has been used for histological examination Table 1.

2.7. Statistical Analysis

The detailed description for each variable was made on the Statistical Package for Social Sciences (SPSS) version 26. Fisher's exact test was used to assess the relationship between categorical variables. A confidence level of 95% with a P-value equal to or less than 0.05 was considered significant.

3. Results

3.1. 1St Week Histological Observation

A. Control group: most of control specimens showed moderate inflammatory response where the extent of inflammatory cell infiltrations, tissue disorganization and dilated blood vessel extending to the middle part of the pulp Figure 1(a,b). Concerning dentin bridge formation and morphology, all of the six specimens showed diffuse, irregular scanty deposition of a tubular dentin on the wall of the exposure site with losing of odontoblast layer. Figure 1c.

B. Nano-Chitosan/$CaCo_3$ (NCsC) group: most of the specimens showed slight inflammatory cell infiltration, numerous congested blood vessels, while the middle part of the pulp showed normal architecture of odontoblastic layer Figure 2 (a,b). Concerning dentin bridge formation and morphology. Coronal pulp subjacent to capping material shows incomplete dentin bridge formation in the form of mixed irregular tertiary dentin with absence of odontoblastic layer. Figure 2c.

Table 1. Scoring system for histological evaluation

scores	Inflammatory extent [38]	Hard tissue continuity [39]	Quality of dentin formation in the bridge [40]
1	Mild inflammatory cell infiltration confined to the area subjacent to the exposure site	hard tissue deposition as a complete and continuous dentin bridge	Regular homogeneous tubular dentin
2	Moderate: Inflammatory cells are observed in the middle part of the pulp	Incomplete and discontinuous	Mixed of Irregular &regular pattern of tubules
3	Severe: inflammatory cell infiltration seen in all parts of the pulp up to the apical region	a layer of scattered and foggy hard tissue deposition on the wall	A tubular and/or irregular dentin is present
4	Complete pulp necrosis	Absence	Absence

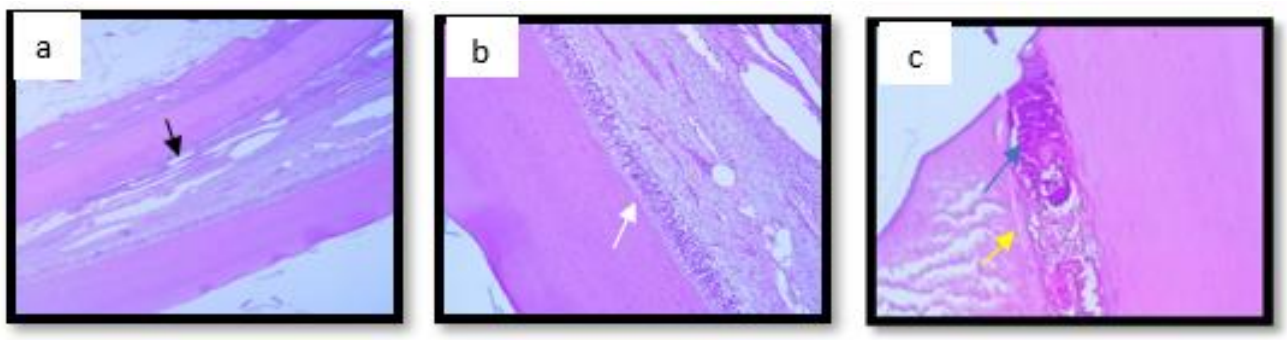

Figure 1. control group at seven days follow-up, stained with (H&E).(a)10x magnification, (b) 20x magnification, the middle part of the pulp, dilated and congested blood vessels(black arrow). odontoblastic layer (white arrow). (c) 40x magnification. Coronal pulp subjacent to empty cavity, a necrotic tissue, (blue arrow). atubular dentin formation (yellow arrow)

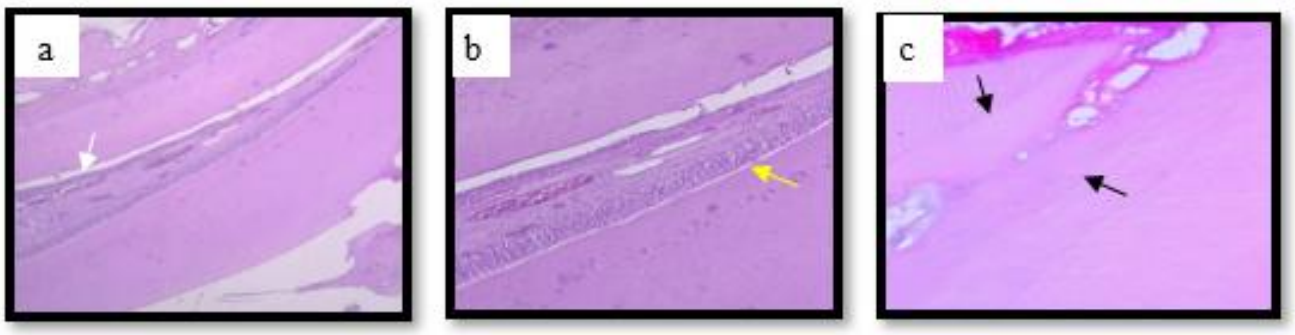

Figure 2. (NCsC) group at seven days follow-up, stained with H&E (a) 10x magnification, (b) 20x magnification. The middle part of the pulp, dilated and congested blood vessels (white arrow). odontoblastic layer (yellow arrow) (a) 40x magnification partially formed dentin bridge. calciotraumatic line (black arrow)

3.1.1. Results of Statistical Analysis of Histological Observation after one Week

Fisher's exact test shows no statistically significant differences between the groups in inflammatory response ($P > 0.05$). But high significant difference among groups in terms of bridge formation and morphology, as $p < 0.05$, with a favorable result of (Chitosan/$CaCo_3$) group as in Table 2.

3.2. 4th Week Histological Observation

A. Control group: The histological examination revealed that one third of specimens showed moderate inflammatory response, while the other two-thirds of specimens showed mild inflammatory response. The odontoblast cells showed palisade form, containing vacuoles, with diffuse pulp calcification and hyalinization. Concerning dentin bridge formation and morphology, all of the six specimens showed partially formed calcified bridges of tubular and tubular dentin, scattered by tunnel like defect and cell inclusions Figure 3(a, b, c).

B. Chitosan/$CaCo_3$(NCsC) group: All specimens showed slight inflammatory cell infiltration, few hyalinized blood vessels seen in most coronal part of the pulp and odontoblastic layer showed normal architecture , Concerning dentin bridge formation and morphology, complete dentin bridge with few cell inclusions seen in all samples , one third of samples showed bridge in the form of mixed irregular and regular dentin morphology while the other two thirds of samples showed bridge or regular secondary dentin confront the odontoblastic layer Figure 4(a, b, c).

Table 2. Difference between control, chitosan, and mixed groups regarding studied items at 1-week duration, Baghdad, 2023

Variable	Score 1		Score2		score 3		score 4		P value
	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	
Inflammatory cell extent									
Control	1	16.7	5	83.3	0	0.0	0	0.0	0.357
Chitosan/$CaCo_3$	4	66.7	2	33.3	0	0.0	0	0.0	
DB formation									
Control	0	0	0	0	6	100	0	0	0.000*
Chitosan/$CaCo_3$	0	0	6	100	0	0	0	0	
Morphology of DB									
Control	0	0	0	0	6	100	0	0	0.000*
Chitosan/$CaCo_3$-	0	0	6	100	0	0	0	0	

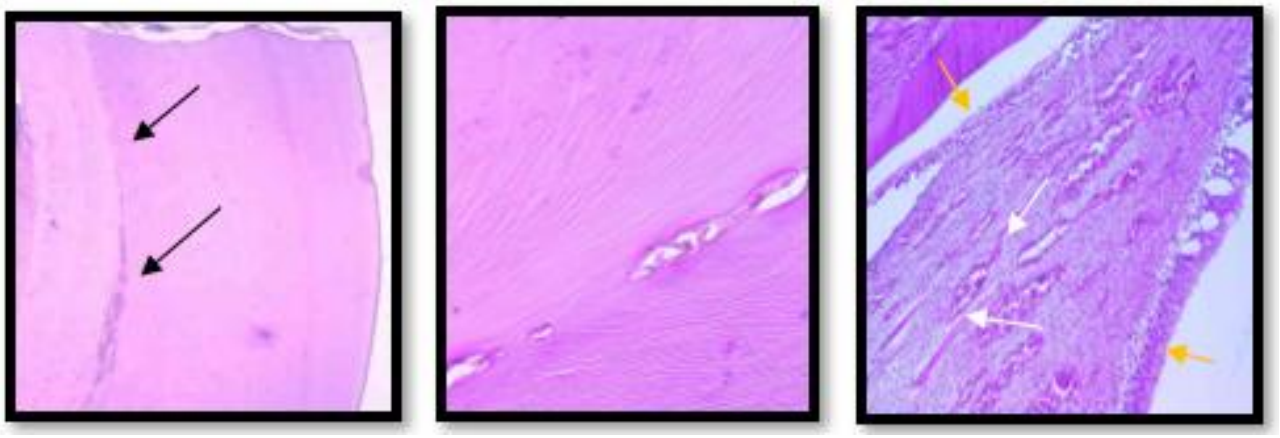

Figure 3. control group at twenty-eight days follow-up, stained with H&E(a) 10x magnification. shows partially formed dentin bridge (black arrow). (b) 40x magnification, calcified bridge with a mixed pattern of tubular and atubular dentin contains defects filled with fibrous tissue. (c) 20x magnification, odontoblast layer (yellow arrow), pulp calcification and hyalinization (white arrow)

Figure 4. (NCsC) group at twenty-eight days follow-up, stained with H&E(a) 10x magnification. shows completely formed dentin bridge (black arrow).(b) 40x magnification, clarified bridge of tubular pattern .calciotruamatic line. (blue arrow) (c) 20x magnification, odontoblast layer (yellow arrow), pulp calcification and hyalinization (white arrow)

3.2.1. Results of Statistical Analysis of Histological Observation after Four Weeks

Fisher's exact test shows no statistically significant differences between the groups in inflammatory response and dentine bridge morphology. But high significant difference among groups in terms of bridge formation as $p < 0.05$ with a favorable result of (Chitosan/$CaCo_3$) group as in the Table 3**.**

4. Discussion

To verify the biological efficacy of innovative capping materials, a sequence of tests must be implemented, including in vitro cytotoxicity studies, in vivo animal experiments, and limited clinical usage tests [41, 42]. Animal studies are complementary to in vitro studies and mandatory to safeguard against potential side effects of dental materials [43]. In preclinical studies of dental pulp capping material, the histological evaluation represents the gold standard above the other evaluation measures like (radiographic or clinical evaluations, to assess the pathophysiological response of the pulp [38, 39]. However, due to ethical and economic considerations. Rodents' incisors (rat and rabbit), have been widely used as a model for preclinical pulpotomy experiments, despite the fact that they are much different from human teeth as they don't have a true anatomical root and continuously growing teeth. They have been validated as a model for pulpotomy,

Table 3. Difference between control, chitosan, and mixed groups regarding studied items at 4-week duration, Baghdad, 2023

Variable	Score 1		Score2		Score 3		Score 4		P value
	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	
Inflammatory cell extent									
Control	4	66.7	2	33.3	0	0.0	0	0.0	0.735
Chitosan/$CaCo_3$	6	100	0	0	0	0.0	0	0.0	
DB formation									
Control	0	0	6	100	0	0	0	0	0.000*
Chitosan/$CaCo_3$	6	100	0	0	0	0	0	0	
Morphology of DB									
Control	0	0	6	100	0	0	0	0	0.06
Chitosan/$CaCo_3$	4	66.7	2	33.3	0	0	0	0	

*Significant: Fisher's exact test used

because of their coronal pulpal histology are comparable with that of human pulp tissue and the effect of capping material may be extended up to most apical region as well as the main goal of most studies using rodent incisors, is the short term histological evaluation rather than long term clinical studies [44, 45, 46, 47]. Herein, two upper central incisors were used as an experimental model for capping procedures, all the medicaments tested in the same animal. The follow up period were of two different time points (one week for initial pulp response evaluation and four weeks for final pulp response evaluation), and these period chosen because of rabbits' incisors are continuously growing, and have been positioned by several previous studies [33, 46, 48]. In present study, a control group, free of capping material, was used as a baseline for evaluation and to distinguish between inherited pulp capacity to repair and the material-induced repair [39]. In the control group, although the RMGIC cements do not come in direct contact with the pulp tissue, their effects on the inflammatory process cannot be neglected. However, at the first week interval, the control group showed mostly moderate inflammatory response with superficial necrosis. There was an absence of total or partial necrosis. These histological features agreed with the histological results of a previous study using RMGIC as direct pulp capping and comparing it with calcium hydroxide in non-human primate teeth [49]. Also, consistent with invitro cytotoxicity test using human primary dental pulp fibroblast cells as a cell model, the results reported that the comparable biocompatibility of RMCIG with that of MTA [50]. However, the moderate inflammatory response in the control group, may be attributed to traumatic pulp exposure procedures [51] or the limited cytotoxic effect of RMGIC [52]. Meanwhile, in the (nano-chitosan /$CaCO_3$) group most specimens showed mild inflammatory response with less tissue disorganization and blood vessel congestion. These data agree with in vitro study to assess the biocompatibility of chitosan -$CaCO_3$ scaffold when cultured with osteoblast-like cells (MC3T3-E1) [53] or with skeletal muscle cells of mice [54]. Where the chitosan -$CaCO_3$ scaffold exhibits high cytocompatibility through cell survival and proliferation. Furthermore, similar results were reported when chitosan/ $CaCO_3$ hydrogels were used as a wound dressing for small cut incision on rat back [55]. Nevertheless, after 7 days post-capping period,there is no significant difference in inflammatory extent among the study groups, which may refer to the biocompatibility of the used capping material as well as the limited cytotoxic effect of RMGIC. However, on day 7, regarding calcified tissue formation, control group showed scanty calcified tissue formation at the periphery of the perforation sides on dentinal wall, these results are comparable to the results of previous studies [39, 46]. Meanwhile, in (Nano-chitosan /$CaCO_3$) group, all the specimens showed partial formation of dentin bridge with mixed (atubular and tubular dentin) morphology, which are

significantly different than that of control group (P=0.002) , these histological findings agree with result of in vitro study of mesenchymal stem cells MSCs culturing on chitosan scaffold with different calcium carbonate content, where, on day 7 of post-culturing, the alizarin red staining shows significant deposition of mineralized nodule on scaffolds with 60% $CaCO_3$ content compare to that chitosan scaffold alone or with 20% content [56]. The significant difference in hard tissue formation among groups may attributed to the addition of $CaCO_3$ as mineral phase, which enhances the sustained release of Ca^{2+}ion, creating favorable environment for faster tissue mineralization [56]. Also, the buffering action of $CaCO_3$ will neutralize the initial acidic pH of chitosan scaffold [55] as well as the modification of chitosan scaffold surface chemistry (porosity and electrical charge) by the precipitation of $CaCO_3$ particles would favorable for protein adsorption, cell attachment and mineral deposition [56]. On day 28, the experimental group showed reduction of inflammatory extent with non-significant difference among them, and continuous formation of mineralized bridges. In the control group, the healing process and tertiary dentin formation may be attributed to the ability of the rabbit pulp to survive and induce hard tissue formation in the absence of microbial infection, similar to rats [57, 58]. However, the control group showed a partially formed dentin bridge comparable to that of a previous study using rat molar as the control group [39, 40]. Meanwhile, after 4 week (Nano-chitosan /$CaCO_3$) group exhibit complete dentin bridge formation, with high significant difference (P=0.00) to dentin bridge formation in control group, may owing to the adding of mineral phase to chitosan scaffold accelerating the osteogenesis and odontogenesis [59, 60]. Concerning dentin bridge morphology at this point of time, there is non-significant differences between groups. In (Nano-chitosan /$CaCO_3$) the presence of irregular dentine may be explained by the presence of chitosan, which may interfere with early mineral deposition [61, 62], stem cell migration and attachment [56, 63]. Consequently, it may postpone the formation of the dentin bridge with regular tubular morphology.

4.1. Limitation

One month period may be insufficient to reveal complete pulp healing, so long-term studies with other animal species are recommended. Other limitation should be considered and noted in this research is the serial longitudinal section are in parallel with the growth path of rabbit incisors which seems difficult to reproduce by other rabbit in the same group and difficult to reveal all the coronal and apical part of the same tooth (dentin and pulp) in one section, also the longitudinal section not reveal the complete topography of the formed dentin bridge as detected in previous study [35], we suggesting using of micro-CT to be complimentary examination in future studies.

5. Conclusion

Within the limitations of the present study, (nano-chitosan /$CaCO_3$) composite is suitable and effective to be used as pulpotomy paste.

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