Biocompatibility of new cement based capping material prepared from eggshell and biopolymer (chitosan)

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Background and Objective: This study aimed to evaluate the biocompatibility of a newly prepared cement-based capping material made from eggshell and biopolymer (chitosan) with Mineral Trioxide Aggrigate MTA and Biodentine.

Methods: Fifteen male rabbits aged about 4-7 months of comparable weight more than 2.0 kg were selected. Animals were handled and maintained in accordance with international recommendations. The animals were divided into 3 groups, five rabbits for each group, for three time intervals (7, 14, and 21 days).Four specimens (4 mm long and 1 mm inner diameter) were inserted for each animal according to the following groups; Group 1: Empty pockets (control), Group 2: The Experimental material with a powder/liquid ratio of1 scoop: 8 drops by using glass slab with aid of cement metal spatula, Group 3: Mineral trioxide aggregate MTA which was mixed according to manufacturing instructions and Group 4: Biodentine which was mixed according to manufacturing instructions.

Results: The histological preparation in control group at 7 days time period demonstrated simple inflammatory reaction represented by slight looseness of the collagen fibers with increase the extracellular spaces between the fibers indicating mild edematous inflammation. For the Experimental group, MTA and Biodentine group there was great similarity in the tissue reaction when compared with control group. At 14 days time period, the edematous inflammatory reaction appeared to be reduced and the collagen fibers arranged closer to each other when compared with the previous time period this was the same for other the groups.At 21 days time period For control, Experimental, MTA and Biodentine group there were complete resolution of the edematous reaction with the formation of thick collagen bundles accompanied by typical fibroblast cells.

Conclusions: The implantation of the experimental material in the subcutaneous tissue of the rabbit's back demonstrated very limited inflammatory reaction that didn't differ from MTA and Biodentine group in all time intervals.

Key words: Egg shell, Biopolymer (chitosan), Biocompatibility, Capping material.

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Introduction

Natural tooth is considered as the best space maintainer. Therefore, it isconsequential to maintain the tooth in the dental arch till natural exfoliation takes place.[1,2] To retain every primary tooth as a fully functional component in the dental arch to allow for proper mastication, phonation, swallowing, preservation of the space required for eruption of permanent teeth and prevention of detrimental psychological effects due to tooth loss is the main objective of pulp therapy in the primary dentition. Pulpectomy is one of the treatment options for severely decayed primary teeth.[3,4]

Dental cements are the materials of comparatively low strength, but they are used extensively in dentistry when strength is not a prime consideration. Cements can be used as base material, temporary filling material and luting. There are also different types of cements developed to be used in orthodontic and endodontic treatments.[5]

Luting cements are used in adapting the tooth to indirect restorations prepared out of mouth, It protects the pulp from thermal, electrical and chemical effects.[6]

Cement may be permanent or temporary, depending on their physical properties and the planned longevity of the restoration. It is

still argued that there is no ideal cement answering all purposes yet, so different materials are required for the comprehensive patient treatment and it is not always that easy to make the best choice. Dental cements are based on mixtures of an acid and a base. The base is a solid, typically powdered zinc oxide or a special ion-leachable glass, whereas as the liquid is acidic solution, either of phosphoric acid or polyacrylic acid. The cements set by a process of a neutralization reaction to give a phosphate or polyacrylate salt matrix and leave some un reacted base to act as reinforcing filler.[7-9]

A remarkable biocompatible material, MTA with exciting clinical applications was pioneered by Dr. Mahmoud Torabinejad and co-workers in Loma Linda University.[10] MTA can be used in surgical and non–surgical applications, including direct pulp capping,[11] temporary filling material, Perforation repairs in roots or furcations,[12] apexification and root end fillings.[13,14] Despite the high clinical efficacy of this wonder cement, there were always some issues which prevented the clinicians to use it for many cases. The major ones being very long setting time and difficult manipulation.

Biodentina bioactive calcium silicate-based cement has been launched in the dental market as a 'dentin substitute'. This biologically active material aids its penetration through opened dentinal tubules to crystallize interlocking with dentin and provide mechanical properties. Biodentin has been formulated using MTA-based cement technology and hence some of the properties such as physical qualities and handling were improved to be applicable in a wide range of restorative dentistry applications like endodontic repair and pulp capping.[15] Thus, the purpose of this study was to compare the biocompatibility of newly prepared cement with MTA and Biodentinein subcutaneous connective tissue of rabbits.

Methods

In this study, four experimental groups were compared: New cement based capping material prepared from egg shells and the biopolymer chitosan (experimental material). The powder to liquid ratio was 1 spoon of powder to 8 drops of liquid; mixing time: 45 seconds, working time: 2 min, setting time: 5.45 minutes (each spoon was 0.2gm and each drop was 0.03ml) Mineral Trioxide Aggrigate (Rootdent,Tehnodent, Russia). Biodentine material (septodont, France).

Empty wound (Control group)

Preparation of the tested material

The powder

The powder of the material has several components. Calcium oxide is obtained from chicken egg shells. The egg shell is cleaned using tap water and then the internal protein layer is removed from the shell. The shell is then crushed and heated to 900° C for one hour in a furnace (Manfredi, Italy). At this temperature, the shell becomes porous, fragile, and very white in color. The egg shell CaCO3 decomposes during this decarbonation process and results in CaO and CO2, according to the reaction: [16]

$$CaCO3 \rightarrow CaO + CO2$$

One hundred grams of egg shell provides 56 g of CaO.

Hydroxy appetite (Chemical Point/ Germany), magnesium oxide (Chemical Point/Germany), bismuth oxide (Chemical Point/Germany), and calcium acetate (Chemical Point/Germany) are the other components.

Polyphosphonoic acid solution

The polyphosphonoic acid solution consists of 52% vinyl phosphonic acid, itaconic acid 2:1, and 5% malic acid.[17]

Chitosan solution

The chitosan solution (medium molecular weight) was purchased from (Sigma Aldrich, USA). This solution was made by dissolving 1g of chitosan powder in 100mL of acetic acid (0.1mol/L). The solution was then by stirred and heated at about 55°C in an oven overnight to form a clear homogenous 10-g/L chitosan solution.[18]

The powder of the test material was composed mainly of calcium oxide (70%) magnesium oxide (25%), hydroxyappetite(3%), calcium acetate (0.5%), and bismuth oxide (1.5%). The powder particle size was standardized using a 25-μm sieve, and the mixture was mixed using a grinder.[19]

The powder-to-liquid ratio was determined by trial using 1 spoon powder/8 drops liq-

uidand1spoon powder/10 drops liquid. For all liquid formulae, 1drop chitosan/8 drops polyphosphonoic acid, 1 drop chitosan/10 drops polyphosphonoic acid, and 1drop chitosan/20 drops polyphosphonoic acid were combined and the setting time and compressive strength test were used to evaluate the compound. The ratio of 1spoon powder/8 drops liquid for the formula to 1drop chitosan/8 drops polyphosphonoic acid was the best ratio that provided an acceptable working time and the hardest compound compared with the other formulae. Thus, this ratio was used in this study.

Study design

This study was performed to evaluate the biocompatibility and tissue damaging effects of the Experimental material implanted in the subcutaneous tissue of rabbits and compare it with tissue reaction of empty pockets, MTA and Biodentin.

Animal Selection

Fifteen male rabbits aged about 4-7 months of comparable weight more than 2.0 kg were selected. Animals were handled and maintained in accordance with international recommendations.

Rabbit housing and preparation

The rabbits had been housed and prepared in a custom made cage with dimensions of (2×1×1.25) Ms and elevated from the ground 20cms, in the Animal house at the College of Medicine, Hawler Medical University. The rabbits were under the direct observation. The cage with this dimensions provide good ventilation and space for rabbit movement, the cage has been cleaned twice daily, clean water provided continuously two to three times daily, supplied by antibiotic and multivitamins (with the consultation of the specialist veterinary), to prevent any infection and nutrition deficiency diseases, nutrition provided was composed of pellets and green hays and different types of graces and these two types provide good nutrition and prevent teeth over eruption to maintain the prepared section till examination.

Samples grouping

The specimens 1mm thick and 4mm inner diameter were used to carry the tested materials. The animals were divided into 3 groups, five rabbits for each group, for three time intervals (7, 14, and 21 days), with four implants per each animal according to the following groups.

Group 1: Empty pockets (control).

Group 2: The Experimental material with a powder/liquid ratio of 1 scoop: 8 drops were mixed by using glass slab with aid of cement metal spatula.

Group 3: Mineral trioxide aggregate MTA which was mixed according to manufacturing instructions.

Group 4: Biodentine which was mixed according to manufacturing instructions.

Animal preparation and implantation procedure

An intramuscular injection of general anesthesia was given which contained a mixture of ketamine hydrochloride (50mg/kg) as general anaesthetic agent and Xylazine (10mg/kg) as sedative. To avoid any contamination, all procedures were done under aseptic conditions, the tested materials were mixed in aseptic conditions and left to set until fully hardened, the implants were kept in sterile; the containers were labeled each according to the material type.

The implantation procedure was as follow; the anaesthetized animal was laid on a sterile towel, then the hair was cut with a scissor and the dorsal skin was shaved and disinfected with iodine–povidine solution. Four pockets were created in the subcutaneous tissue of the rabbit, two pockets on each side of vertebral column with a distance of 10 cm between them.

Using no. 11 sterile surgical blade on a scalpel handle, a small longitudinal incision (about 5-7 mm) was made in the skin. The incision was made through the epidermis and dermis layers reaching to the hypodermis which is loosely connected to the dermis then using a scissor a pocket (approximately 15mm deep) was created in the subcutaneous layer (using a blunt dissection) by opening the attachment between the dermis and the hypodermis to accommodate the implant.

Then each prepared pocket receiveone specimen from group 2, group 3, group 4 and a fourth empty wound (group 1) that served as a control.

These specimens were transferred to the site with the aid of straight tweezers. The implants were carefully placed into the

pockets to a depth of 10-15 mm from the incision. After that each pocket was suture with 3.0 silk. All the surgical and implantation procedure were made by 2 operators

Specimens removal (biopsy)

The rabbits were sacrificed by anesthetic overdose intra-peritoneally in groups of 5 animals after 7, 14 and 21 days after implantation (five rabbits for each time interval). The areas were incised and removed in rectangular blocks to facilitate subsequent embedding and serial sectioning.[23] This procedure was repeated at each time interval. The tissue samples were fixed in 10% buffered formalin, subjected to routine histological processing in Vazhinhealth central laboratory in Duhok city.

Slides preparation

In order to obtain a qualitative estimation of the tissue reaction around the specimen, three sections belonging to the central areas of each specimen were obtained and examined). After that, the tissue samples were subjected to histopathological processing according to Suvarna et al. (2008).[20] After dehydration and clearing steps, the tissue samples were embedded in paraffin. The paraffin blocks were oriented parallel to the specimen long axis and approximately 5-µm-thick serial longitudinal sections were obtained from the central portion of each specimen with the aid of Microtome, and stained with trichrome and hematoxylin and eosin for 10 min, and then rinsed with distilled water. The slides were mounted on glass cover slips later on.

All specimens underwent blinded examination by a single histopathologist who did not know which material or which period was being examined.

The occurrence of inflammatory response were scored according to previously established scores[21-23] 0 (no reaction) for absence of inflammatory cells; 1+ (mild reaction) for presence of mild chronic inflammatory infiltrate, or few eosinophilic or giant cells; 2+ (moderate reaction) for presence of moderate chronic inflammatory infiltrate, or some eosinophilic or giant cells, or 3 + (severe reaction) for presence of an intense chronic inflammatory infiltrate, large number of eosinophilic or giant cells.

Results

At 7 days time period

A. Control

The histological preparation of the cutaneous tissue where the wound was made demonstrated simple inflammatory reaction represented by slight looseness of the collagen fibers with increase the extracellular spaces between the fibers indicating mild odeamatus inflammation.

B. Experimental material

For the experimental group, there was great similarity in the tissue reaction when compared with control group but the degree of oedematous inflammatory reaction was slightly more since the collagen fiber demonstrated looser appearance with large extracellular spaces between them (Figure 1).

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For the experimental group, there was great similarity in the tissue reaction when compared with control group but the degree of oedematous inflammatory reaction was slightly more since the collagen fiber demonstrated looser appearance with large extracellular spaces between them (Figure 1).

C. MTA

The MTA group demonstrates oedematous inflammatory around the implant space with loose collagen fibers.

D. Biodentine

For Biodentine group, there was abundant inflammatory cells with histiocyte migration toward implantation space with loose connective tissue and collagen fibers.

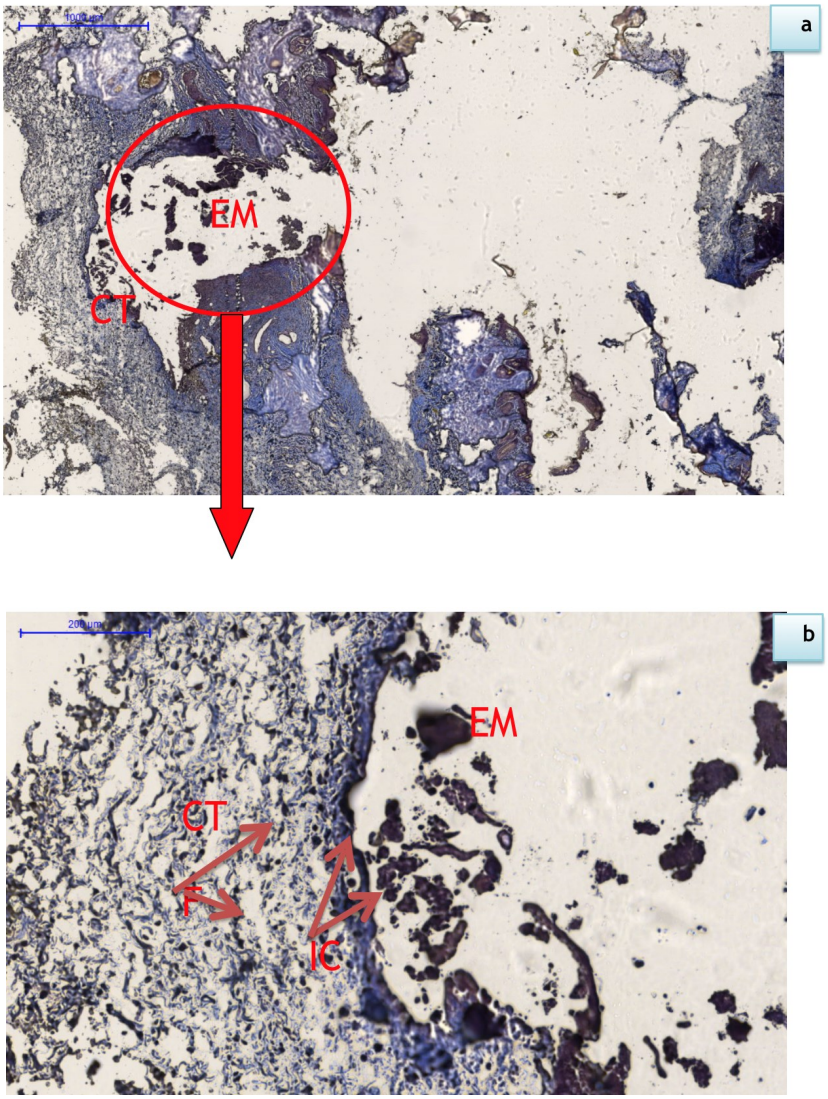

Figure 1. Micrograph of rabbit's cutaneous region (Experimental group) at 7 days time period, Trichrome stain, (a) demonstrates edematous reaction with large space between fibers (X50), (b) shows loose collagen fibers with extracellular matrix between them(X100).
(EM: Experimental material, CT: Connective Tissue, IC: Inflammatory cell)

At 14 days time period

A. Control

The odematous inflammatory reaction appeared to be reduced and the collagen fibers arranged closer to each other when compared with the previous time period.

B. Experimental material

The experimental group also demonstrated reduction of the odematous reaction and the collagen fibers arranged closer to each other but still demonstrated thinner bundles when compared with control group (Figure 2).

C. MTA

The MTA group demonstrates fibrosis around MTA space with thinner collagen fibers and still some extracellular spaces remained indicating incomplete healing.

D. Biodentine

For Biodentine group, there was a fibroblast like tissue reaction with formation of abundant collagen fibers with giant cell reaction indicating the presence of inflammation in the area as a response for the foreign bodies.

D. Biodentine

Same as MTA group complete fibrous tissue is observed around Biodentine with thick collagen fibers indicating complete healing tissue.

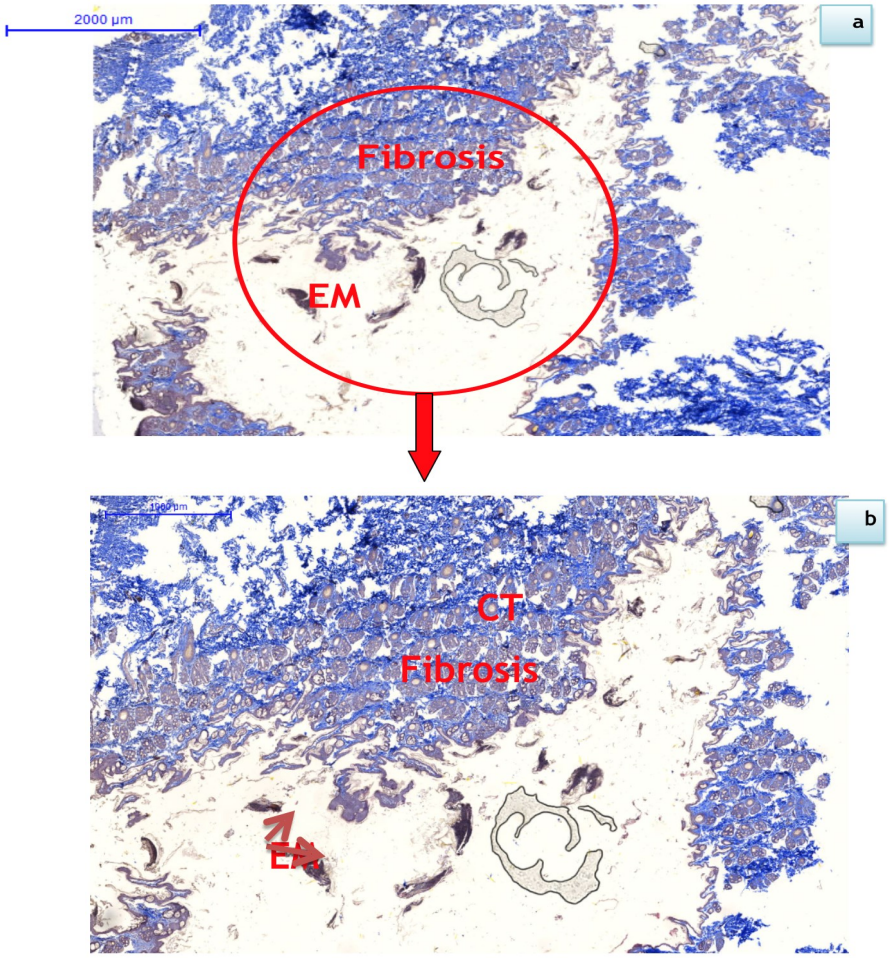

Figure 2. Micrograph of rabbit's cutaneous region (Experimental group at) 14 days time period, Trichrome stain, shows fibrosis and reduction in the extracellular spaces in area adjacent to the experimental material (a) X10 & (b) X20
(EM: Experimental material, CT: Connective Tissue)

At 21 days time period

A. Control

There was complete resolution of the odematous reaction with the formation of thick collagen bundles accompanied by typical fibroblast cells.

B. Experimental material

For experimental group, the resolution of odematous reaction was also observed with presence of thin collagen bundle accompanied by fibroblasts and presence of reasonable extracellular material (Figure 3).

C. MTA

A complete fibrous tissue is observed around MTA with thick collagen fibers Indicating complete healing tissue.

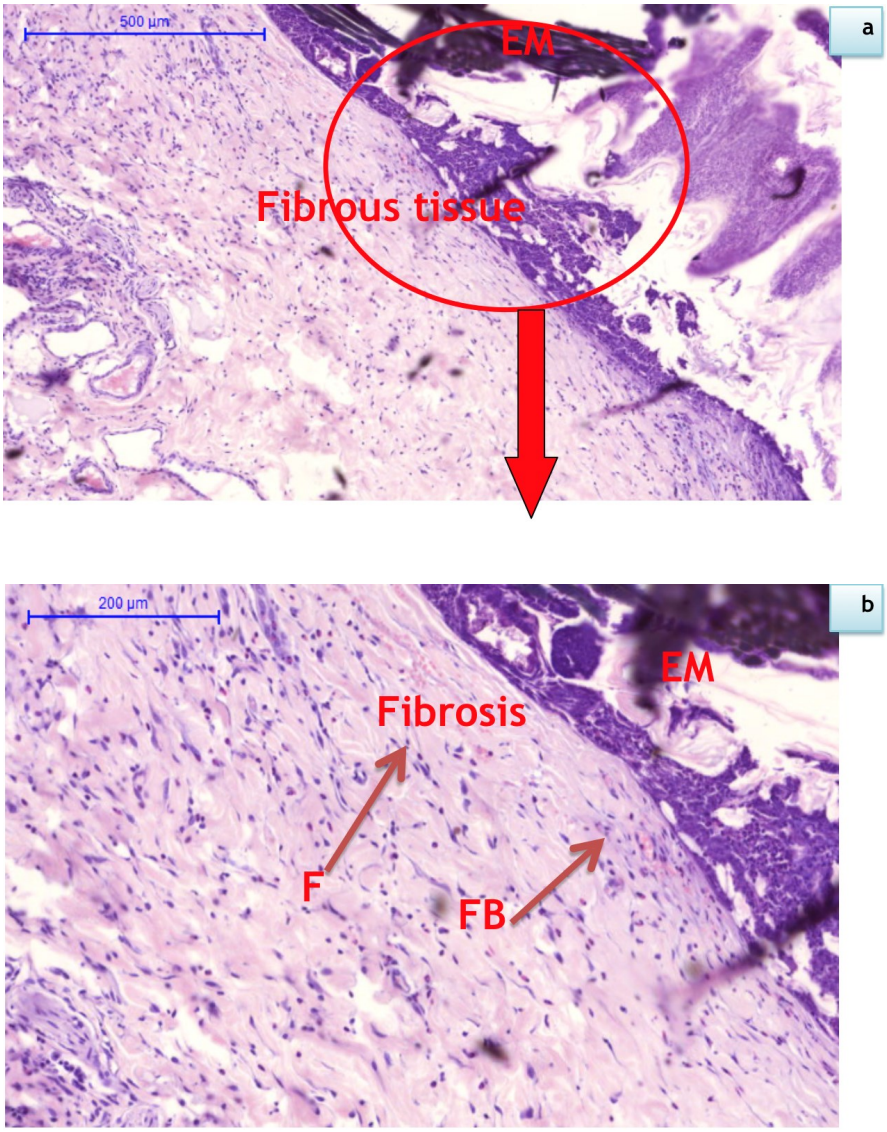

Figure 3. Micrograph of rabbit's cutaneous region (Experimental group at) 21 days time period,H & E, (a) demonstrates complete resolution of edematous reaction(X50),
(b) shows thin collagen bundle accompanied by fibroblasts and presence of reasonable extracellular material (X100).
(FB: Fibroblasts, EM: Experimental Material, F: Collagen fiber)

Discussion

The term biocompatibility is often described as the ability of a material to perform with an appropriate host response in a specific application.[24] Because of continuous introduction of new dental materials, evaluation of their biologic potential is necessary.[25] According to ISO-6876 and 10993-5 standards[26], tissue implantation of different materials in the body of laboratory animals has been proposed. Although data from laboratory animals could not be extended to human beings, it is considered as a valuable method to evaluate their biological properties.[27,28]

Also, biocompatibility of new materials should be evaluated to ensure that a new material does not cause irritation, unwanted reactions, or tissue necrosis compared with control groups. For this reason, histological investigations evaluate the inflammatory response adjacent to the materials.[29]

In this study, empty wounds as negative control group revealed no inflammation to mild inflammatory response, which is similar to previous studies.[23,30,31] Initial inflammatory response to empty wounds is probably the result of surgical process of tube implantation.[30,32] At 7 days, the MTA group displayed a mild-to-moderate inflammatory response which was reduced to a mild reaction after 21 days. This has been reported before by several studies showing the biocompatibility of MTA.[29,30,33-35]

In the present study, the tested experimental material, was placed in the subcutaneous pockets and the their histo-pathological response was studied in three time periods, (7 days, 14 days and 21 days) and compared to that of the empty subcutaneous pockets. The grouping of the rabbits according to these time intervals help us to estimate the early action of the material like acute inflammation, estimate the starting of the chronic inflammation, and the other period was to confirm the effect of time on the action of each material as the chronic inflammation might subside and healed.[36]

The results of this study showed that the empty wounds had simple inflammatory reaction at 7th day; however,the degree of oedematous inflammatory reaction was slightly more for the experimental material. The odematous inflammatory reaction appeared to be reduced at 14th day for other groups. There was complete resolution of the odematous reaction with the formation of thick collagen bundles accompanied by typical fibroblast cells, (active & inactive) at 21st day for control group, the same picture was observed for experimental material, although, they were not accompanied by typical fibroblast cells as in the control group.

The subcutaneous pockets that contain the experimental material which contain a mixture of the eggshell and chitosan exhibit a very little inflammatory response, the inflammatory response of the control group that was empty wounds was equal to a great degree to the tube containing the experimental material, that mean that the biocompatibility of newly prepared cement was not affected by the chitosan, however, the mild inflammatory response might be due to the anti-inflammatory mechanism of chitosan. Due to the acid hydrolysis of chitosan, into glucosamine hydrochloride or its sulfate, phosphate and other salt preparation by salt conversion. These monosaccharides are structural units of the proteoglycans contained in connective tissue and cartilage. These tissues can be repaired and regenerated by absorbing these monosaccharides when they are damaged or inflamed.[39]

The reactions to empty subcutaneous pockets in this study were similar to many authors[23,38,39] who found that empty wounds caused few or no reactions in subcutaneous connective tissues. They reported that there were some inflammatory symptoms in the regions where tubes were implanted until the end of the 2nd week, and this inflammatory infiltration subsided after the 3rd-week.This reaction might be the result of the trauma produced during the procedure.[39] However, Zmener et al.[40] showed that empty wounds were surrounded with fibrous tissue without inflammatory cells after 10, 30 and 90 days.

The histo-pathological response to experimental material was comparable to that of control, MTA and Biodentine group. The change in the kind of inflammatory cells from 7 days period to 21 days period of the experimental material took the same pattern seen in the other group with the degree of oedematous inflammatory reaction was slightly more for the experimental material.

This may be explained by the change of inflammation from acute type to chronic one by the progress in time. When assessing the biocompatibility of a material, later harmful effects are considered to be more important than its initial effects. Fibrous connective tissue surrounding the materials indicates that they were well tolerated by the tissue.[38]

The finding of experimental material in this study with mild inflammatory reactions agreed with that of others[23,41,42] who found that the inflammatory reaction to CEM was mild. The biocompatibility of CEM cement is related to its capacity of releasing calcium ions and, consequently, to the alkaline pH produced by the material. When exposed to a humid medium, CEM cement released calcium silicate and calcium oxide. The calcium oxide released produces calcium hydroxide after reacting with the tissue fluid, which in turn release calcium ions. The gradual release of calcium ion from the material, therefore may explain the mild inflammation around the material tubes in the present study, as the excess of calcium ions becomes irritant to tissues, resulting in an inflammatory process.[43,44]

The initial histo-pathological response of the experimental material may be due to chitosan addition. A study by Vande-Vord[45]indicated early neutrophil accumulation in subcutaneous tissue of mice around the chitosan scaffold (prepared in 0.2M % acetic acid) at 7 days, which resolved overtime. This could be explained by the chemotactic effect of chitosan on neutrophils. Neutrophilic migration to the material appears to be an inherent property of chitosan.[46]

Conclusions

The implantation of the experimental material in the subcutaneous tissue of the rabbit's back demonstrated very limited inflammatory reaction that didn't differ from the control, MTA and Biodentine group in all time intervals.

Conflicts of interest

The authors report no conflicts of interest.

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