

The histopathological effects of calcium triglyceride bone cement and chitosan on the healing of experimental bone defects

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Background/aim: This study evaluated the histopathological effects of two different bone grafts, calcium triglyceride bone cement (CTBC) and chitosan, on the healing of experimental bone defects.

Materials and methods: Ninety-two Sprague Dawley male rats, with a weight of 240 ± 20 g, were used. Two experimental groups that consisted of 64 rats were divided into four subgroups due to the sacrifice days, which were 7, 14, 30, and 60. After creating an 8-mm-long and 1-mm-wide cortico-cancellous bone defect in the right tibia of each rat, CTBC and chitosan were applied to the bone defects. In the main group, after creating the bone defects in the right tibias, we kept those empty to serve as the control group. We evaluated inflammation, foreign body reaction, necrosis, fibrosis, new bone formation, and the residual graft material at 7, 14, 30, and 60 days.

Results: In both the CTBC and chitosan groups, the new bone formation was higher than that in the control group, whereas foreign body reaction and residual graft material in the CTBC group and fibrosis in the chitosan group were significantly higher. After evaluating the results, both materials were found not to be very useful in the bone healing process.

Conclusion: CTBC remained for a long time without being resorbed; it can be used as a placeholder in large bone defects, whereas the gel form of chitosan cannot be utilized for this purpose because it was resorbed in the first 7 days.

Key words: Bone cement, triglyceride, chitosan

1. Introduction

The problem of structural and functional reconstruction of bone defects in an oral and maxillofacial area that develop due to malformation, infection, trauma, or various operations has not been solved yet in a satisfactory manner (1,2).

Bone defects are defined as empty areas found inside or on the surface of the bone and that need to be filled with new bone. Although a part of these defects can recover, it may be necessary to aid these empty bone areas to restructure somehow to allow these defects that develop because of surgical excision of congenital, traumatic, or bone pathologies where this mechanism falls short to recover. For this purpose, bone grafts and several different materials have been used in the treatment of bone defects since the second half of the 18th century with the aim of providing new contours and supporting bone recovery (3–5). Bone grafts can be divided into groups as autogenous grafts, allografts, xenogenous grafts, and alloplasts.

Although autogenous cancellous bone graft is currently regarded as the gold standard in the treatment

of a bone defect treated surgically, due to reasons such as the requirement of more than one surgical region, inability to obtain sufficient substance, postoperative pain, patient discomfort, and duration and cost of surgery, research to find the ideal bone graft continues (6–8). Researchers have shown that natural and synthetic graft materials can be conveniently used in the restoration of deformities. Having advantages such as limitless availability, easy sterilization, and storage, synthetic or semisynthetic materials can be utilized by themselves or together with autogenous bone grafts (9).

The ideal bone graft material should be easy to obtain, osteoinductive, osteoconductive, easy to apply, and low priced. When deciding to use a bone graft, it is compulsory to consider the factors concerning the patient and the environment, the experience of the surgeon, and the economic aspects of graft use and to choose materials suitable for the subjects with suitable indications (10,11).

Experimental studies are needed to ascertain that osseous recovery is achieved in the best way with different applications in a critical size bone defect defined as the

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smallest intraosseous wound that does not heal by itself during the lifetime of the individual. To contribute to these studies, in our study, we aimed to histopathologically examine the short- and medium-term effects of locally applied calcium triglyceride-based bone cement and chitosan on the recovery of bone defects and to find an appropriate bone substitute for critical defects.

2. Materials and methods

The İstanbul University Institutional Animal Care and Use Ethical Committee approved all experimental procedures. The operations of the experimental animals and histopathological examinations of the bone defects were carried out at İstanbul University Experimental Medicine Research Institute and Oncology Institute Tumor Pathology and Oncologic Cytology Department.

Ninety-two Sprague Dawley strain male rats of 240 ± 20 g weight were kept at 21 ± 1 °C in an environment automated to provide the standard of 40%–60% relative humidity rate and a lighting period of 12-h light and 12-h dark in metal cages. The animals were fed tap water and laboratory feed. The right tibias of all the animals used in our study were included in the experimental protocol. This study involved three main groups and four subgroups arranged as per the sacrifice periods on days 7, 14, 30, and 60.

2.1. Main groups and subgroups

Critical-size bone defects were left empty in the control group. Calcium triglyceride bone cement (CTBC) was used in the first experimental group and chitosan was used in the second experimental group. The control group comprised 28 experimental animals. The CTBC and chitosan bone graft groups both comprised 32 experimental animals. In the control group, each subgroup contained seven rats. In the other groups, each subgroup contained eight rats. The animals in each subgroup were sacrificed on days 7, 14, 30, and 60, respectively.

2.2. Surgical applications

General anesthesia was applied to the experimental animals by peritoneal injection of 5 mg/kg xylazine hydrochloride and 6 mg/kg ketamine HCl mixture. Medial surfaces of the right hind legs of the experimental animals, which were immobilized in standard posture, were cleaned and the surgery region was wiped with a povidone-iodine solution. The right and left legs were set in a flexion position and dermal, subdermal, and periosteum laceration of 20-mm length was performed in a longitudinal direction to reach the medial surface of the tibia. Using a one-diameter round-tipped stainless steel drill mounted on a handpiece connected to a micromotor, under irrigation of sterile serum physiologic solution, bone defects of 8-mm length and 1-mm width that include the cortex and medulla layers of the bone were created. Placement of chitosan and CTBC into the defect areas is shown in Figures 1 and 2. Postsurgical recovery was recorded by daily visual examination of the incisions and overall condition of the rats, daily food and water intake, and body weight gain.

2.3. Light microscopy monitoring and evaluation criteria

Sacrifications of the experimental animals in all the experimental and control groups were completed on days 7, 14, 30, and 60. After the sacrifice, the parts in the operative fields were found and removed. The material was fixed in 10% buffered formalin for 1 week. After fixation, all the material was decalcified in a solution prepared by a combination of 50% formic acid and 20% sodium citrate solutions one scale from each. After the decalcified parts had undergone routine tissue monitoring, cross sections of 5–7- μ m thickness obtained from prepared paraffin blocks were stained with hematoxylin-eosin (H-E) and examined under a light microscope. Cross sections obtained from all groups on days 7, 14, 30, and 60 were evaluated regarding inflammation, foreign body reaction, necrosis, fibrous tissue formation, new bone formation, and residual graft material. Evaluations of these parameters were conducted

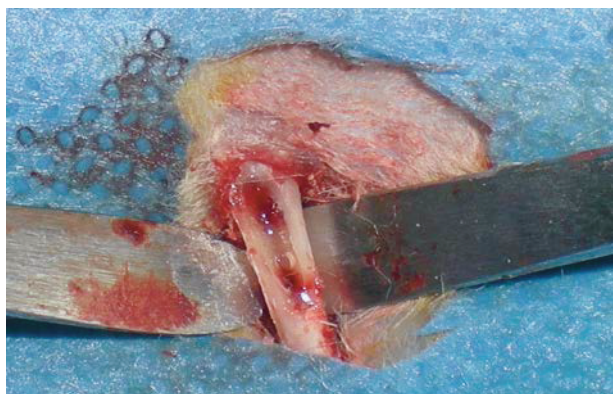


Figure 1. Placement of chitosan into the defect area.



Figure 2. Placement of calcium triglyceride bone cement into the defect area.

by their footprint. Parameters having a footprint of 0%–5% (–), 5%–30% (+), 30%–60% (++), or over 60% (+++) were evaluated.

2.4. Statistical evaluation methods

Statistical evaluations in this study were conducted at İstanbul University Biostatistics and Medical Informatics Department. Descriptive statistics were provided as distributions of percentage and frequency. Chi-square and Fisher’s exact tests were used for comparison of categorical data. Statistical significance was evaluated at P < 0.05 level. At least 8 subjects were used in each group at 80% strength and 95% confidence level so that a difference of 0.2 to 0.8 ratio (0% to 5%) of the foreign body reaction of chitosan and calcium triglyceride bone cement could be significant.

3. Results

Chitosan and CTBC placed into the critical-size defects were histopathologically evaluated on days 7, 14, 30, and 60 in terms of inflammation, foreign body reaction, necrosis, fibrosis, new bone formation, and residual graft material.

3.1. Control groups

Histopathological findings of the control groups on days 7, 14, 30, and 60 are shown in Tables 1–4, respectively, and Figure 3.

3.2. Experimental groups

Histopathological findings of the CTBC group sacrificed on days 7, 14, 30, and 60 are shown in Tables 1–4, respectively, and Figure 4.

Histopathological findings of the chitosan group sacrificed on days 7, 14, 30, and 60 are shown in Tables 1–4, respectively, and Figure 5.

3.3. Statistical results

Graphical comparisons of the control, chitosan, and CTBC groups are shown in Figures 6–11.

4. Discussion

Today, several different test methods are being used in researching the biological compatibilities of the materials used in dentistry. The autogenous cancellous bone graft is regarded as the gold standard (12,13). However, the obtained material is limited and the morbidity rate is

Table 1. Histopathological findings of the control, calcium triglyceride bone cement, and chitosan groups on day 7.

Day 7	Inflammation			Foreign body reaction			Necrosis			Fibrosis			New bone formation			Residual graft material		
	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan
Rat 1	++	++	++	-	-	-	-	-	-	+	++	++	+	++	++	-	+++	-
Rat 2	+	++	++	-	-	-	-	-	+	++	++	++	+	++	++	-	+++	-
Rat 3	+	++	+++	-	-	-	-	+	+	++	++	++	++	++	++	-	+++	-
Rat 4	++	++	+++	-	-	-	+	-	+	+	++	++	+	+	++	-	+++	-
Rat 5	++	++	++	-	+	-	+	+	-	+	++	++	+	++	++	-	+++	-
Rat 6	+	++	+++	-	+	-	-	-	-	+	++	++	++	+	++	-	+++	-
Rat 7	++	+++	++	-	-	-	-	-	+	++	++	++	+	+	+	-	+++	-
Rat 8		+++	++		+	-		-	-		+	++		++	++		+++	-

Table 2. Histopathological findings of the control, calcium triglyceride bone cement, and chitosan groups on day 14.

Day 14	Inflammation			Foreign body reaction			Necrosis			Fibrosis			New bone formation			Residual graft material		
	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan
Rat 1	+	+	+	-	+	-	+	-	-	++	+	++	+	+++	+++	-	+++	-
Rat 2	-	++	++	-	-	-	-	-	-	+	++	+++	+	++	++	-	+++	-
Rat 3	+	++	+	-	+	-	-	-	-	++	++	+	++	+++	+++	-	+++	-
Rat 4	-	+	+	-	+	-	-	-	-	++	+	++	++	++	++	-	+++	-
Rat 5	-	+	+	-	+	-	-	-	-	+	++	++	+	++	++	-	+++	-
Rat 6	+	++	+	-	+	-	-	-	-	++	+	++	++	++	+++	-	+++	-
Rat 7	+	++	++	-	-	-	+	-	-	++	++	++	++	+++	+++	-	+++	-
Rat 8		++	+		+	-		-	-		++	++		++	++		+++	-

Table 3. Histopathological findings of the control, calcium triglyceride bone cement, and chitosan groups on day 30.

Day 30	Inflammation			Foreign body reaction			Necrosis			Fibrosis			New bone formation			Residual graft material		
	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan
Rat 1	-	++	+	-	-	-	+	-	-	+	+	+	++	+++	+++	-	+++	-
Rat 2	-	++	+	-	+	-	-	-	-	+	+	++	++	+++	+++	-	+++	-
Rat 3	-	+	+	-	+	-	-	-	-	++	+	+	++	+++	+++	-	+++	-
Rat 4	-	+	+	-	-	-	-	-	-	-	+	++	++	+++	+++	-	+++	-
Rat 5	-	+	+	-	+	-	-	-	-	++	+	++	++	+++	++	-	+++	-
Rat 6	-	++	+	-	-	-	-	-	-	++	+	++	+++	+++	+++	-	+++	-
Rat 7	-	++	-	-	+	-	-	-	-	++	+++	++	+++	+++	++	-	+++	-
Rat 8		++	-		+	-						++	++	+++	+++		+++	-

Table 4. Histopathological findings of the control, calcium triglyceride bone cement, and chitosan groups on day 60.

Day 60	Inflammation			Foreign body reaction			Necrosis			Fibrosis			New bone formation			Residual graft material		
	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan	Control	CTBC	Chitosan
Rat 1	-	-	-	-	-	-	-	-	-	-	+	-	+++	+++	+++	-	+++	-
Rat 2	-	+++	-	-	-	-	-	-	-	-	++	-	+++	+++	+++	-	+++	-
Rat 3	-	+++	-	-	+	-	-	-	-	-	++	-	++	+++	+++	-	+++	-
Rat 4	-	+++	-	-	+	-	-	-	-	+	++	-	+++	+++	+++	-	+++	-
Rat 5	-	+++	+	-	+	-	-	-	-	-	++	+	+++	+++	++	-	+++	-
Rat 6	-	+++	-	-	+	-	-	-	-	-	++	+	+++	+++	+++	-	+++	-
Rat 7	-	++	-	-	+	-	-	+	-	-	++	-	++	++	+++	-	+++	-
Rat 8		++	-		+	-					+	-	++	+++	+++		+++	-

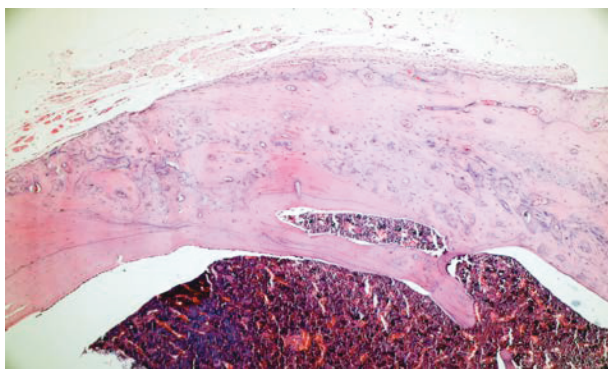


Figure 3. Lamellar cortical bone covering the defect that changed places with the reticular bone tissue in the defect area (H-E x100).

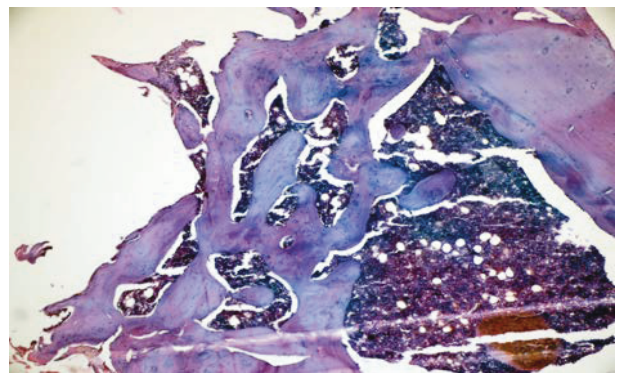


Figure 4. Trabecular bone tissue in which bone marrow was formed, filling the defect with the graft material maintaining its existence in the defect area (H-E x100).

high (14). In the present study, the responses of two different bone grafts (chitosan and CTBC) were evaluated. Assessment of the tissue responses in the defect area was carried out by evaluating the parameters of inflammation,

foreign body reaction, necrosis, fibrosis, new bone formation, and residual graft material. These parameters were assessed and compared to day 7, day 14, day 30, and day 60 separately.

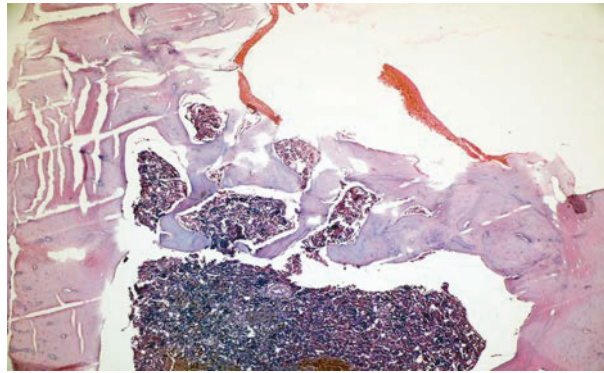


Figure 5. Bone marrow between the trabecular bone filling the defect area and replacement of this trabecular bone tissue by cortical bone in parts (H-E ×100).

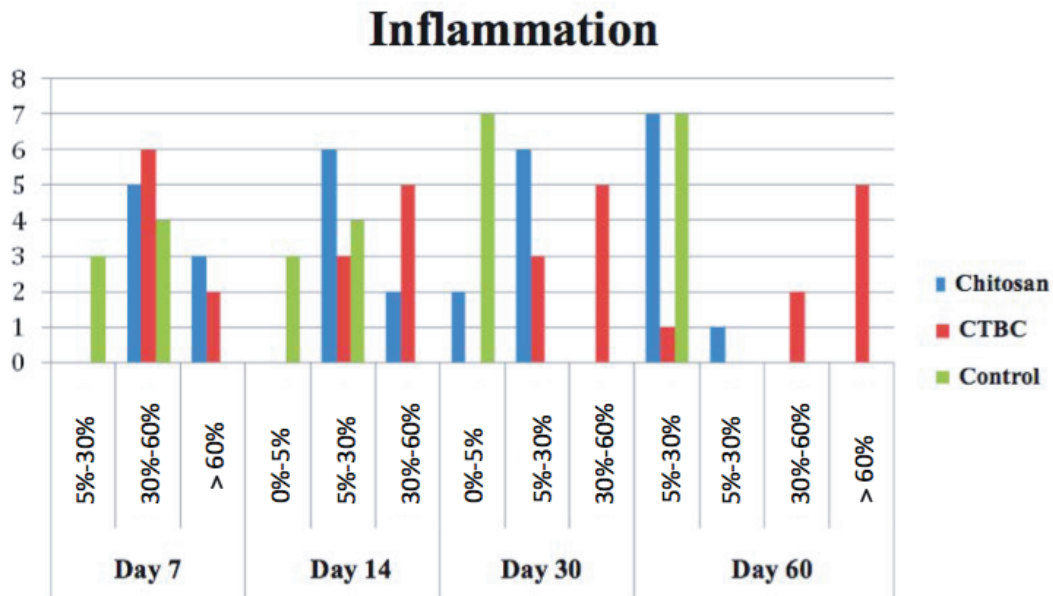


Figure 6. Graphical comparison of distribution of inflammation according to groups and days.

Chitosan is a reactive derivative of chitin. It is available on the market in several forms such as powder, film, fiber, and gel. In addition to its positive effects on wound recovery, chitosan also has analgesic, hemostatic, and antimicrobial effects (15–20). The gel form was used in this study for its ease of use considering these qualities of chitosan.

Tomihata et al. researched the relationship between the resorption time of chitosan films and lysosome. They indicated that the relation is directly associated with the deacetylation rate of chitosan (21). Rodríguez-Vázquez et al. stated that the sensitivity of chitosan having 70% deacetylation rate to lysozyme is maximum. They showed with studies that resorption of the chitosan with a deacetylation rate over 85% can thus last for months

(22). In the present study, chitosan was used with 86% deacetylation rate.

It was observed that pores of chitosan with a low deacetylation rate are more orderly and parallel. However, pore structures of chitosan with a high deacetylation rate are the opposite, and the connection between them is reduced. This porous structure of the material provides a suitable environment that facilitates cell migration into the tissue and exchange of metabolic products (23,24).

Chitosan and calcium triglyceride were statistically evaluated using the results obtained from the groups by comparing them both within themselves and with the control group and with each other. When the control group is examined, the new bone formation starts on day 7, gradually increased on days 14, 30, and 60, and the

Foreign Body Reaction

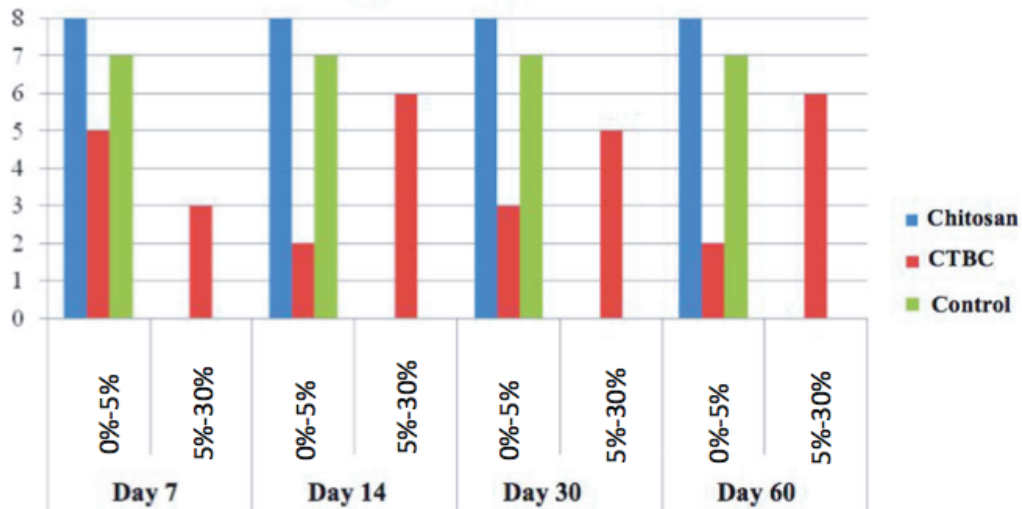


Figure 7. Graphical comparison of distribution of foreign body reaction according to groups and days.

Necrosis

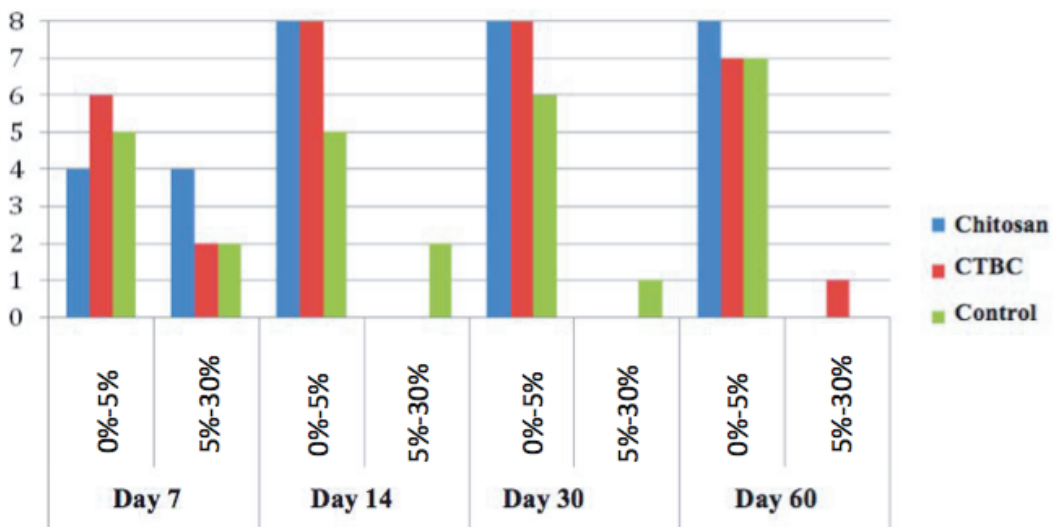


Figure 8. Graphical comparison of distribution of necrosis according to groups and days.

cavities, except for in two experimental animals, were filled with new bone on day 60. Distributions of inflammation, necrosis, and fibrosis gradually decreased in this group, and fibrosis was present in only one experimental animal on day 60. When this group is evaluated within itself according to days, these differences were not significant. This result is based on the natural increase in bone formation and a natural decrease in distributions in the other parameters (25).

The results of distribution on days 7, 14, 30, and 60 in the experimental group in which chitosan was used

show that the differences between them in all parameters are either negligible to be evaluated or insignificant. The necrosis seen in four experimental animals only on day 7 was not seen on days 14, 30, and 60 at all and the statistical significance of the difference between them was at $P = 0.077$ level, and there is a significant difference of $P = 0.041$ in the distribution of inflammation between days 30 and 60. Fibrosis seen on day 7 decreased gradually while new bone formation increased gradually and it significantly increased at $P = 0.026$ level especially between days 14 and 60. The significant difference observed in necrosis

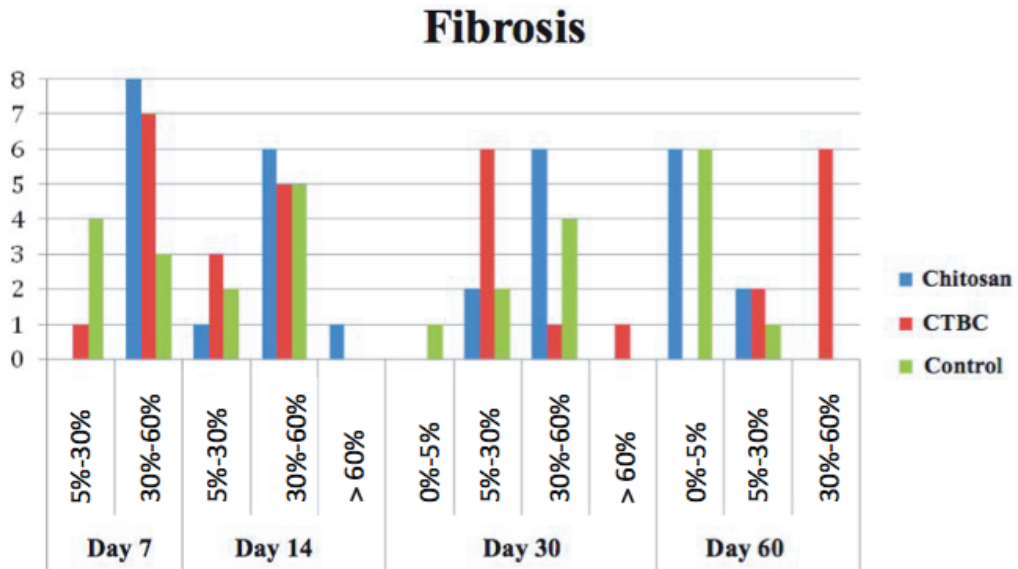


Figure 9. Graphical comparison of distribution of fibrosis according to groups and days.

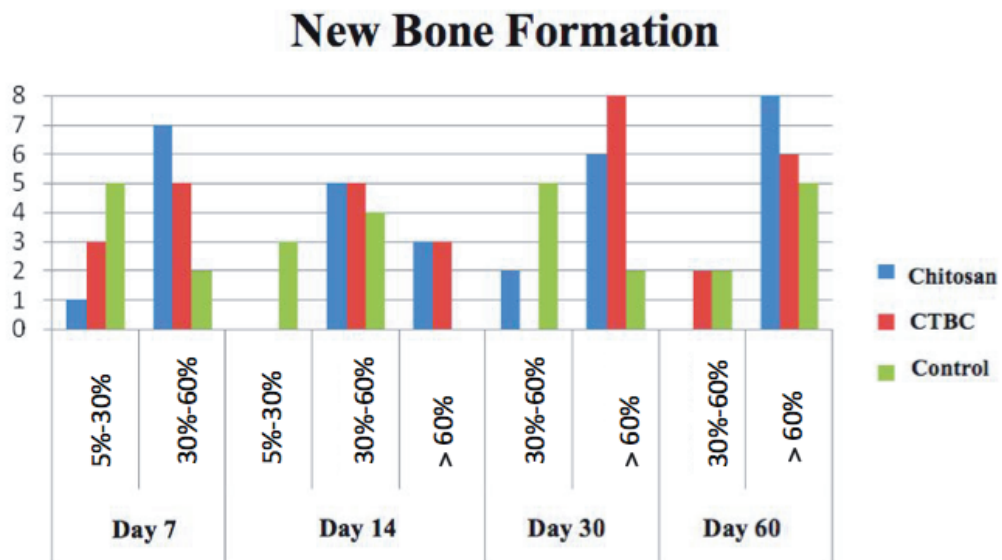


Figure 10. Graphical comparison of distribution of new bone formation according to groups and days.

distribution developed can be based on traumatic work occurring when the cavities were being prepared.

In all parameters in the CTBC group, the differences between day 7 and days 14, 30, and 60 were statistically insignificant. In this group, necrosis, which was seen at minor level in all experimental animals on day 7, was seen only in one on day 60 and not seen at all on days 14 and 30. Contrary to this, intermediate and slightly increasing inflammation was observed in all groups and experimental animals, minor foreign body reaction was seen in an increasing number of rats, intermediate fibrosis

was seen in all experimental animals, and a significant level of existence of residual graft material was determined in all groups and all experimental animals. The new bone formation was observed at a minor and intermediate level as of day 7; gradually increased on days 14, 30, and 60; and the existence of intensive new bone formation was observed on days 30 and 60. Mainly the difference in new bone formation between day 14 and day 30 was significantly high ($P = 0.026$). These results were obtained in the distributions of inflammation, foreign body reaction, fibrosis, and residual graft material from the

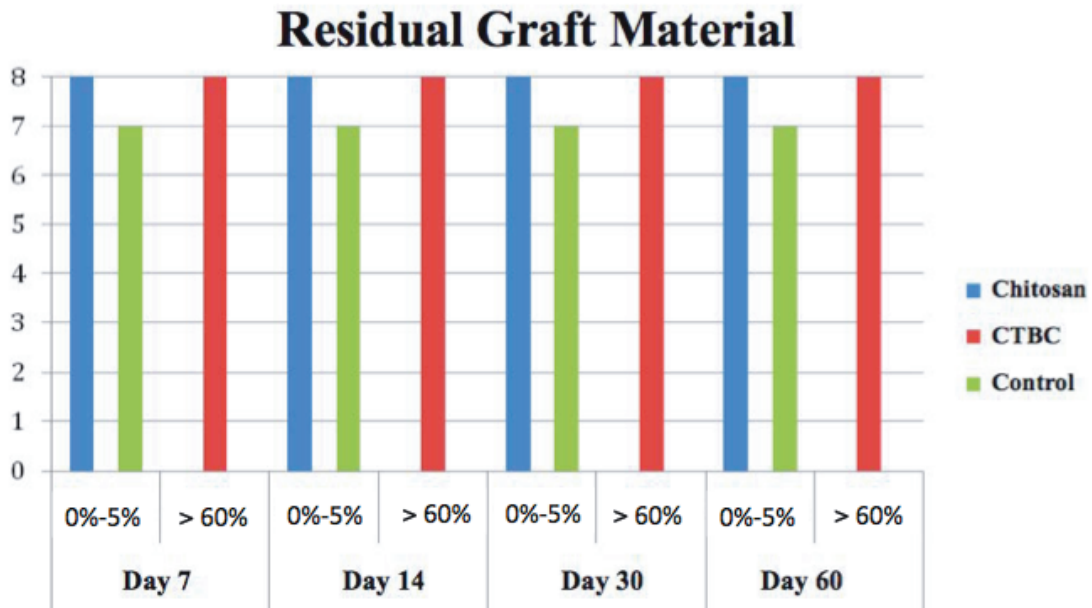


Figure 11. Graphical comparison of distribution of residual graft material according to groups and days.

physical structure of the material that becomes as hard as bone within 25–30 min.

Due to a lack of similar studies, the results of this study cohere with the results of studies indicating that CTBC can be used for different purposes. The fact that a minor foreign body reaction was observed in the study even at the end of day 60 does not comply with the results reported by researchers asserting that a foreign body reaction was not observed.

Except for necrosis distribution, the differences between the chitosan and control groups were not computable in any group on day 14. Although the difference in necrosis distribution was slightly higher in the control group, it was insignificant ($P = 0.200$).

On day 14, a significant difference was not found between the two experimental groups in new bone formation ($P = 1.000$) or in the distribution of inflammation although it was slightly higher in the calcium triglyceride group ($P = 0.315$). However, foreign body reaction was found to be significantly higher ($P = 0.007$) in the group to which CTBC was applied.

A significant difference between the experimental groups was not found regarding new bone formation, while the distribution of inflammation in the CTBC group and the distribution of foreign body reaction were significantly higher. This result contradicts the results of the researchers asserting that CTBC can be used without problems and creates a foreign body reaction based on clinical applications, while it is in conformity with the results of those asserting that chitosan plays a more

effective role in new bone recovery (26). The excess in the distribution of necrosis seen in the control group can result from traumatic reasons.

Distribution of inflammation in the chitosan group on day 30 was significantly higher in the experimental group ($P = 0.007$) compared to the control group. On the other hand, although the difference in new bone formations was higher in the experimental group ($P = 0.132$) compared to the control group, it was not significant.

The distribution of necrosis is higher in the control group on day 30 ($P = 0.200$). In the CTBC experimental group, distributions of foreign body reaction ($P = 0.026$) and new bone formation ($P = 0.007$) were significantly higher. When these results are evaluated all together, although it is seen that the distribution of new bone formation is significantly higher in the CTBC group compared to the control group, its not having a significant difference from the chitosan group and showing higher foreign body reaction give the idea that there are question marks about this graft material. These results we concluded are also in conformity with the positive results obtained from the studies concerning chitosan (27).

We may have studied the biocompatibility, and the effects of the biomaterials (inflammation, foreign body reaction) used in large quantities in bone defects at critical dimensions can be approximated to the human model using bigger experimental animals than rats.

When the results from the control, chitosan, and CTBC groups are evaluated all together, neither material has a very efficient role in bone recovery; CTBC can be

used as a space maintainer in large defects and in cases where bone contour is required to be preserved since CTBC maintains its existence without being resorbed for a long time. Chitosan in gel form, which was used in this study, was resorbed and completely disappeared within the first 7 days, showing that this material cannot be used in bone defects as a space maintainer or with the purpose of preservation of the bone contour.

Chitosan in gel form can be used in various surgical practices in cases where short-term space maintainer material is required and in replacement of resorbable membranes. It will be appropriate for new studies to be conducted to determine chitosan in powder, film, and fiber forms differently from the gel form. It seems quite

promising to examine the applicability and the effect on bone defects of different chitosan forms, which remains our next focus.

This study is the first in the literature in which chitosan and CTBC were compared. Although new bone formation is higher both in the chitosan group and the CTBC group compared to the control group, foreign body reaction is significantly higher in the CTBC group while fibrosis is higher in the chitosan group.

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