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In vivo biocompatibility and fracture healing of hydroxyapatite-hexagonal boron nitride-chitosan-collagen biocomposite coating in rats

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Abstract: The biocompatibility of orthopaedic implants and their effects on fracture healing have key roles for success. In this study, it was aimed to investigate the effects of a novel biocomposite consisting of hydroxyapatite (HA), hexagonal boron nitride (h-BN), chitosan (Cs), and type 1 collagen (Ct1) on biocompatibility and fracture healing in rats. A total of 60 adult male Wistar rats weighing 300–500 g were used in the study. The rats were randomly divided into 2 groups named A (uncoated/control) and B (biocomposite coated). Biocomposite (HA/h-BN/Cs/Ct1) coated and uncoated stainless-steel implants were used as intramedullary pins. Groups A and B were divided into subgroups of A1 and B1 (15th day), A2 and B2 (30th day), A3 and B3 (45th day) according to the date of euthanasia. Clinical, radiographic, haematological, biochemical, and histopathological findings were evaluated by pairwise comparisons. The findings were consistent and similar. No statistically significant difference was found for a finding disturbing the biocompatibility. Histopathological examinations showed that coating biomaterials did not resorb over the course of 15, 30, and 45 days. It is thus revealed that the content is biocompatible. However, it has been concluded that it is necessary to increase the physical strength of the coating surface against sterilization and surgical procedures. As a result, based on the interpretations of the clinical, radiographic, haematological, biochemical, and histopathological findings, the biocompatibility of HA/h-BN/Cs/Ct1 biocomposite materials has been revealed.

Key words: Biomaterial, biocomposite, implant coating, boron nitride, electrophoretic deposition, experimental

1. Introduction

The success of orthopaedic implants may be affected by various factors, but their biocompatibility and effects on fracture healing have key roles. A number of events, such as blood-implant surface interaction, inflammatory processes, and primitive bone formation, are possible on or between surfaces of tissue and implant [1,2]. In the past, many coating biomaterials were used in order to improve the properties of orthopaedic implants and overcome unwanted features or results [2,3]. Many of them continue to be discussed and investigated in various regards. Implant surface properties have been examined in terms of chemical composition, biocompatibility, roughness, and such aspects, and coating design studies have been done in order to develop ideal implants [2,4,5]. However, optimizing the biological properties of coatings is still a challenge. In this field, the in vivo results of products developed by in vitro studies are important for new studies. In this context, our research hypothesis has been the effects of hydroxyapatite (HA)/hexagonal boron nitride (h-BN)/chitosan (Cs)/type 1 collagen (Ct1) biocomposite on

biocompatibility and fracture healing which was designed by Tozar in 2018 as a new implant coating biomaterial [6]. Each of these 4 different components was used alone or in different combinations in a large number of in vitro or in vivo studies [6–9]. In our study, we aimed to evaluate the in vivo biocompatibility and fracture healing effects of the newly designed biocomposite that contains these 4 components.

Hydroxyapatite (HA) has been used in a wide range of orthopaedics and dental applications for decades due to its high biocompatibility [10,11]. Due to its weak mechanical properties, it is generally not suitable for use alone as a biomaterial in applications that require resistance to load. Thus, it is generally used as a coating material on metal implant surfaces [12]. Due to its osteoconductive properties and similarity to bone tissue, it has widespread use as a coating material in order to increase the biocompatibility and bioactivity properties of metal implants such as stainless-steel, titanium (Ti), and alloys [7]. With advances in materials science and nanotechnology, many studies in which HA is combined with different strategies have

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been conducted. Thus, work is underway to produce new materials with the desired characteristics [6,13].

Chitosan (Cs), as a biomaterial, has been studied plentifully, especially in suture materials, dental-bone implants, and artificial skin applications [14]. Adsorption of biomaterials such as Cs is necessary to improve the biological interfacial properties of prostheses, orthopaedic devices, and tooth nests [15,16]. Among described approaches for biomaterials, chitosan is a highly promising option with its multifaceted and key roles [17,18].

In tissue engineering with type 1 collagen (Ct1), many studies have been conducted on tissue scaffolds, bone graft production, and wound healing improvement [19–21]. It is often used on biomaterial surfaces to increase cellular activities such as adhesion, proliferation, and differentiation [22]. Despite the potential benefits in stimulating new bone formation, there is insufficient information about the effect of Ct1 coating [23].

Scientific studies on the use of hexagonal boron nitride (h-BN) in biomedical products are progressing. A theoretical study on the encapsulation of anticancer drugs and an in vitro study on the production of biocomposite revealed no toxic effects [24]. It has been reported as biocompatible with muscle and neuroblastoma cells [25]. It is found in many areas of usage, from industry to cosmetics, but it is especially promising in medical applications with its versatile material properties and preventive properties of corrosion, oxidation, and chemical reactions [26]. In vivo studies of biomaterials containing h-BN are very limited. In particular, there is no study on the in vivo results of the biocomposite in our study. In this context, the biocomposite which constitutes

the working hypothesis will be the basis for further studies to present new information with the in vivo results of its biocompatibility and its effects on fracture healing as an orthopaedic implant coating biomaterial [6,27].

2. Materials and methods

2.1. Fabrication and electrophoretic deposition of the biocomposite material

Two different suspensions were prepared to contain biopolymers and ceramic particles forming the biocomposite material. Acetic acid (0.9 mL) was diluted in 20 mL of water to prepare the first (biopolymer) suspension. Cs (0.13 g) and Ct1 (0.0135 g) were completely dissolved in this solution and exposed to ultrasonic stirring for 5 min. For the second (ceramic) suspension, 0.4955 g of HA and 0.375 g of nano h-BN powder were mixed into 30 mL of ethanol and also stirred similarly. These 2 different suspensions were combined by ultrasonic stirring for 30 min and then allowed to magnetically mix for 24 h. With the final suspension stabilized at the end of this period, the surface coating processes of stainless-steel (Ø 1 mm) Kirschner (K) wires were performed at an electrophoretic deposition (EPD) mixing speed of 250 rpm with the electrophoretic deposition potential of 16.10 V and temperature of 34 °C [6]. After the coating process, the implants were subjected to gas sterilization with ethylene oxide and prepared for surgery by being packaged sterile (Figure 1).

2.2. Animals and surgical procedure

The study approval was obtained from the Local Ethics Board of Animal Experiments of Hatay Mustafa Kemal University (protocol number 2015/6–12). All of the

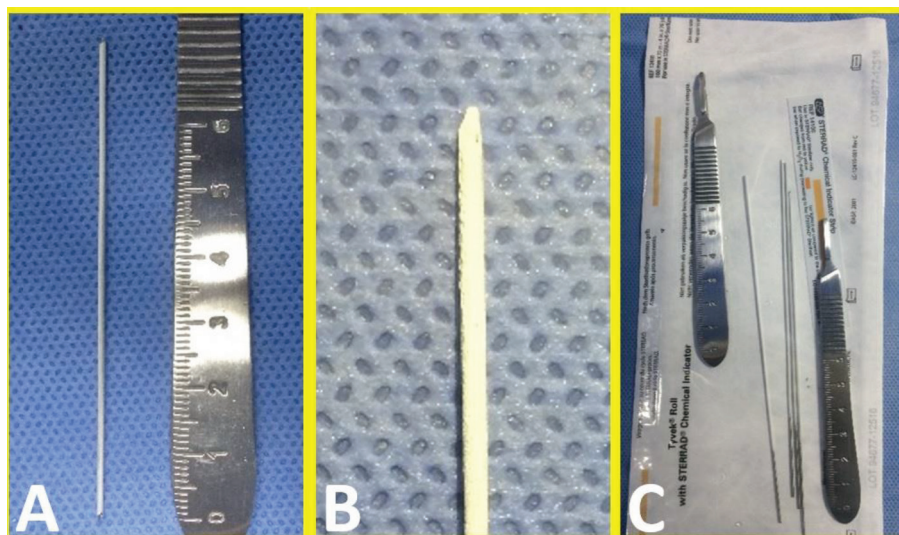


Figure 1. Biocomposite coated stainless-steel K wire (A) and zoomed view (B). The implants are sterilized with ethylene oxide gas sterilization and packaged (C).

applications for the experiment were conducted in accordance with the Turkish Code of the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes.

Sixty adult male Wistar Albino rats with an average weight of 300–500 g purchased from the Experimental Research and Application Center of Hatay Mustafa Kemal University were used in the present study. All rats were maintained in groups of 2 to 5 animals per cage on a 12 h light, 12 h dark circadian rhythm with water and food provided ad libitum. One week prior to the study, the animals were taken to the study place to undergo routine health checks and time for adaptation was provided.

Experimental animals were randomly allocated to 2 groups (n = 30 per group), whereby Group A received uncoated implants and Group B received biocomposite coated implants. Each group was divided into 3 subgroups (n = 10 per subgroup) according to the date of euthanasia. Subgroups to be euthanized on the 15th day postoperatively were A1 and B1, on the 30th day were A2 and B2, and on the 45th day were A3 and B3.

Rats were anaesthetized by an intramuscular injection (from the hindlimb, not operated) of xylazine (5 mg/kg) and ketamine (50 mg/kg) combination. [28]. The animals were prepared for surgery and positioned in lateral recumbency. The skin incision was made parallel to the femur. After the subcutaneous connective tissues, the intermuscular septum (that separating m. biceps femoris and m. gluteus superficialis) was incised and the femur was reached as described [28]. In order to create the fracture model, in this study, the femur was measured and the level of the fracture was stated. A diaphyseal transversal

fracture was created by rigorous cutting movements with a bone cutter. With the help of 2 small Halstead–Mosquito haemostatic forceps, distal and proximal fragments of fractures were held up and the reduction was achieved by retrograde intramedullary pinning of uncoated (for Group A) or biocomposite coated (for Group B) stainless-steel K wires (\varnothing 1 mm) for osteosynthesis (Figure 2). The surgical site was closed routinely with 2–0 poly (glycolide-co-lactide) sutures.

2.3. Postoperative procedure and clinical examination

Wound care, antibiotics (ampicillin-sulbactam combination, 250 mg/kg IM), and clinical examinations were performed daily for 7 days postoperatively. General findings such as habitus and food and water intake were followed. Local findings on the incision site, gait, and lameness were noted. Animals were weighed on the day of surgery and on the 7th day postoperatively.

2.4. Haematological and biochemical examination

Blood samples were collected by intracardiac aspiration during euthanasia. White blood cells (WBC), lymphocytes, monocytes, granulocytes, red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and plateletcrit (PCT) values were examined by complete blood count (Mindray BC-2800Vet, Guangdong, China) and compared between subgroups. Additionally, with the measurements of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK),

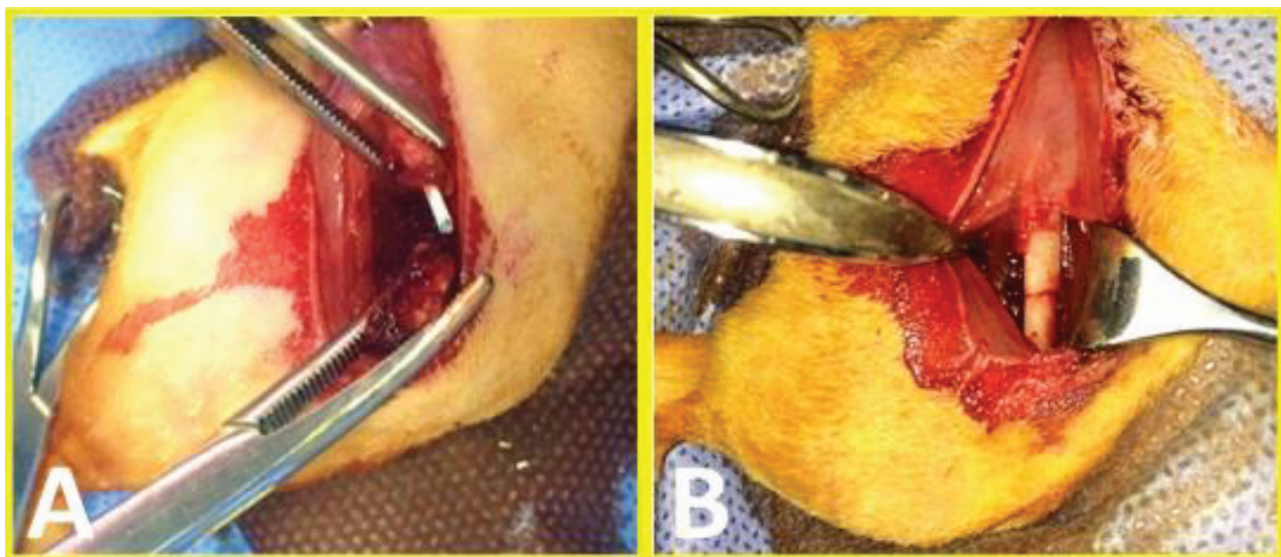


Figure 2. Application of biocomposite coated implant with retrograde intramedullary technique.

lactic acid dehydrogenase (LDH), total proteins (TP), creatine (Cr), total calcium (total Ca), and phosphorus (P) values, serum biochemistry profiles were examined (Gesam Chem 200, Campobello di Mazara, Italy) and compared between subgroups.

2.5. Radiographic evaluation

Radiographs were taken at 50 kV and 1 mAs (Intermedical, Basic 100-30, Italy) in mediolateral and anteroposterior positions. In the radiographic examination, callus formation and fracture line were evaluated. For this purpose, a radiographic union scale was adapted from a previous study [29]. On this radiographic union scale, the presence of callus formation and fracture line in the anterior, posterior, medial, and lateral cortices was scored. Each cortex was scored with 1 point if it still had a fracture line with no callus, 2 points if the callus and fracture line were both present, and 3 points if there was a callus with no fracture line. Thus, the totals of the 4 cortices were evaluated in the range of 4–12 points for each animal and compared between subgroups.

2.6. Histopathological study

The femur bones were separated from the soft tissues and calcified with nitric acid at a concentration of 3%. The fragments from the calcified bones were fixed in a 10% buffered formaldehyde solution. Tissues were subjected to paraffinization after being routinely washed in water and being passed through a series of graded alcohols and xylols. Paraffin blocks were obtained by embedding the tissue in paraffin. The sections were taken by a microtome (Leica RM2235, Heidelberg, Germany) in 5 µm thickness from these paraffin blocks and after drying in the oven, all of them were stained with haematoxylin and eosin (H&E) to be examined by binocular microscope (Olympus BX50-F4, Tokyo, Japan) [30]. The histopathological changes observed were scored as in previous studies by Allen et al., Estai et al., and Naddaf et al. [31–33]. Lesions such as bone (fracture) healing, inflammatory reactions, connective tissue proliferation, cartilage formation, and soft and hard callus formations were evaluated and scored according to their severity.

The absence of inflammatory cells was evaluated as 0, a small number of inflammatory cell infiltration as 1, a moderate number of inflammatory cell infiltration as 2, and infiltration of intense inflammatory cells as 3. Additionally, the scoring of the connective tissue and cartilage formation was evaluated according to the overall size of the callus. The scoring of soft and hard callus formations was 1 when cartilage and fibrous tissue increase was 10% or less, 2 if the increase was between 10% and 25%, and 3 if the increase was 25% or more. Bone healing was determined according to the scoring system of Allen's fracture healing [31]. Accordingly, the scores were considered as 0 in the presence of fibrous tissue alone, 1

for incomplete cartilage formation with fibrous tissue, 2 for cartilage formation, 3 for a slight trabecular bone formation with a majority of cartilage formation, 4 for an equal amount of cartilage and trabecular bone formations, 5 for a slight cartilage formation with a majority of trabecular bone formation, and 6 for complete compact bone ossification. Inflammatory changes were evaluated according to their severity; in the absence of lesions or cells it was scored as 0, mild lesions or small numbers of cells were scored as 1, moderate lesions or intermediate numbers of cells were scored as 2, and severe lesions or numerous cells were scored as 3. Statistical analyses and subgroup comparisons were made with these scores.

2.7. Statistical analysis

Statistical analysis of the study was done with the R 3.4.2. package program. Descriptive statistics for the variants are given with median and minimum–maximum values. The conformity of continuous variants to normal distribution was analysed by the Shapiro–Wilk test. The Mann–Whitney U test was used for independent 2 group comparisons and Kruskal–Wallis test was used for independent 3 group comparisons. The Bonferroni corrected Mann–Whitney U test was used for binary subgroup comparisons of significant variants. In all the statistical comparisons, comparisons with P-values below 0.05 were considered statistically significant.

3. Results

3.1. Clinical evaluation

The first week and the following weeks after the surgery, the general health of animals was good. The systemic clinical findings of the animals such as general condition, habitus, appetite, and feed and water consumption were normal. In the examinations that continued until the time of euthanasia, no systemic or local rejection complication, or any complication that negatively affected the general condition of the animals, was observed. In clinical examinations, a limited number of local complications such as wound dehiscence, pin migration, and subcutaneous granulation tissue formation were recorded similar for subgroups. There was no statistically significant difference between the subgroups in terms of systemic or local findings.

3.2. Radiographic evaluation

Statistical analyses of radiographic scores were performed using the Mann–Whitney U test. Median, minimum–maximum, and P-values of radiographic scores of all subgroups are presented (Table 1). There was no statistically significant difference between the subgroups. No significant adverse effects were observed in Group B that adversely affected biocompatibility or fracture healing. Radiographic images from subgroups are shown in Figure 3.

Table 1. Median, minimum–maximum, and P-values of radiographic scores of all subgroups.

| Subgroups | A1 | B1 | A2 | B2 | A3 | B3 |
|-----------------|----------|------------|----------|------------|----------|-------------|
| Score (min–max) | 9 (8–10) | 8.5 (8–10) | 8 (4–11) | 8.5 (8–10) | 9 (8–12) | 10.5 (8–12) |
| P | 0.853 | | 0.247 | | 0.280 | |

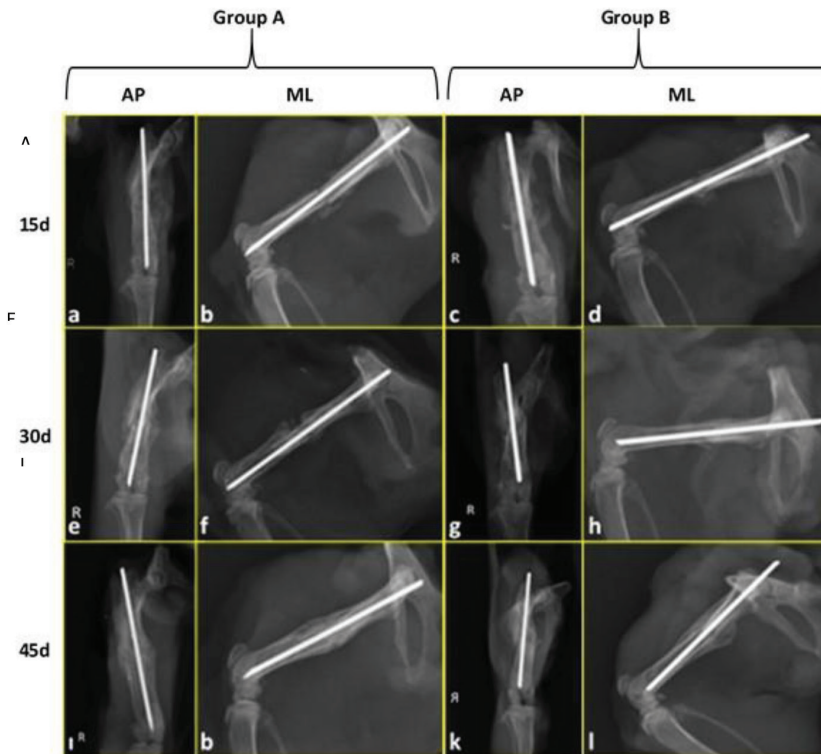


Figure 3. Anteroposterior (AP) and mediolateral (ML) radiographic samples from subgroups. (a) and (b) views are from A1, (c) and (d) views are from B1 subgroups (15 days). It shows that fixation persists with external callus onset in both A1 and B1. (e) and (f) views are from A2, (g) and (h) views are from B2 subgroups (30 days). It shows that fixation persists with callus formation in both A2 and B2. (i) and (j) views are from A3, (k) and (l) views are from B3 subgroups (45 days). It shows that fixation persists, callus formation has progressed, and the transition to the remodelling stage has begun in both A3 and B3. Radiographic results were similar for subgroups of the same days and no significant difference was observed for the evidence of impaired biocompatibility.

3.3. Haematological and biochemical results

Complete blood count results were evaluated by pairwise comparison of subgroups. Lymphocyte values were significantly higher in the A1 subgroup ($P = 0.043$) in comparison to subgroups A1–B1. In comparison of subgroups A2–B2, the MCV and MCH values were significantly higher in the B2 subgroup ($P = 0.003$ and $P < 0.001$, respectively). In comparison of subgroups A3–B3, the RDW values were significantly higher in the B3 subgroup ($P = 0.002$).

The results of serum biochemistry profiles were also evaluated by pairwise comparison of subgroups. In group

A1, Cr was found to be significantly higher than in the B1 subgroup ($P = 0.003$). In subgroup A2, the ALP value was found to be significantly lower ($P < 0.001$) compared to subgroup B2, and additionally CK, LDH, Cr, and BUN values were significantly higher than in subgroup B2 ($P = 0.007$, $P = 0.002$, $P = 0.019$, and $P < 0.001$). When the A3–B3 subgroups were compared, none had any statistically significant difference.

3.4. Histopathological results

The removed femoral bones of 60 rats were examined microscopically. The obtained histopathological findings were scored according to their degree in terms of bone

healing, inflammatory reaction, connective tissue proliferation, cartilage formation, and soft and hard callus formations (Table 2; Figures 4–6). The data were analysed statistically by Mann–Whitney U and Kruskal–Wallis tests.

When the histopathological examinations were evaluated, mononuclear cell infiltration, fibroblastic proliferation, and the new granulation tissue formation mixed with collagen accumulation were similar around the fracture site in all subgroups (A1, B1, A2, B2, A3, and B3). In all subgroups, a small number of erythrocytes scattered across the connective tissue were seen at the ends of the fracture and just below the periosteum. In all subgroups of group B (B1, B2, and B3), dark brownish-black pigment accumulations were observed in the newly formed granulation tissue, especially in the medullary canal, between the bone tips, around the vessels, in macrophages, and in the osteoid tissue (Figure 7).

The abundance of hyaline cartilage formations, showing the early stage of endochondral ossification with a small amount of osteoid accumulation, was similar in both A and B groups. In Groups A and B, hypertrophic chondrocytes and hyaline cartilage formation between the ends of the bone were marked and intense. When the soft callus was evaluated, no statistically significant difference was found between the subgroups.

Osteoid mineralization and trabecular and lamellar bone tissue formations replacing soft callus in the late phase of bone healing did not show statistically significant difference between Groups A and B. The presence of diffuse hypertrophic chondrocytes in the osteoid mineralization tissue in new bone formations (hard callus) was not also statistically significant. Lamellar bone formation with hypertrophic chondrocytes in new bone tissue formation was prominent in both Group A and Group B.

By pairwise comparison of histopathological results and scores of subgroups, no significant differences were

found in bone healing, inflammatory reaction, connective tissue proliferation, cartilage, or soft callus and hard callus formations.

4. Discussion

Bone healing is a process that may be affected by various factors. Properties of the used biomaterials are crucial in this process and their biocompatibility is essential. In this context, in order to detect any possible negative effect on the fracture healing caused by inadequate biocompatibility of coating biomaterial, it was essential to see the results in vivo. In our study, the results clearly showed that the new biocomposite coating biomaterial is biocompatible and had no adverse effects on the fracture healing compared to the uncoated implant group. Thus, the results were consistent with previous studies and the proposed hypothesis [2,8,9,16,34–36].

HA/h-BN/Cs/Ct1 biocomposite coating designed by Tozar et al. was applied to the stainless-steel K wires by electrophoretic deposition [6,13]. Since it was the first in vivo study, stainless-steel K wire was preferred. Therefore, the intramedullary pin method was preferred for fixation of the experimental fracture. K wire and intramedullary pin methods have been used in many studies on fracture healing due to their application advantages [32,37].

Ethylene oxide was found to be suitable for sterilization of biocomposite coated K wires. It was determined that the heat sterilization methods were not suitable because of leading to peeling or the carbonization of the coating material. Therefore, studies are needed to increase physical resistance to the sterilization processes. The surfaces of biocomposite coated implants were found to be possibly damaged by the effect of implant-bone frictions during fracture fixation manipulations. Although it was biocompatible, it was determined that the physical strength of the coating biomaterial should be improved. With the improvement of the mechanical properties of

Table 2. Median, minimum–maximum scores, and P-values of histopathological results.

| | Bone healing | Inflammatory reaction | Connective tissue proliferation | Cartilage formation | Soft callus formation | Hard callus formation |
|----|--------------|-----------------------|---------------------------------|---------------------|-----------------------|-----------------------|
| A1 | 0.50 (0–1) | 1 (1–2) | 3 (1–3) | 1.5 (1–3) | 2.5 (1–3) | 1 (0–2) |
| B1 | 1 (0–3) | 1 (1–3) | 3 (2–3) | 1 (0–3) | 3 (1–3) | 1 (0–2) |
| P | 0.481 | 0.393 | 0.971 | 0.631 | 0.631 | 0.796 |
| A2 | 2 (0–4) | 2 (1–3) | 3 (1–3) | 2.5 (1–3) | 2 (1–3) | 1.5 (1–3) |
| B2 | 2 (0–4) | 2 (1–3) | 3 (1–3) | 2 (1–3) | 2.5 (2–3) | 1.5 (1–2) |
| P | 0.684 | 0.579 | 0.912 | 0.853 | 0.579 | 0.853 |
| A3 | 5 (3–5) | 1 (0–2) | 1 (1–3) | 2.5 (1–3) | 1 (1–3) | 3 (2–3) |
| B3 | 5 (3–5) | 1 (0–2) | 2 (1–3) | 2.5 (1–3) | 2 (1–3) | 2.5 (1–3) |
| P | 1.000 | 0.315 | 0.579 | 0.853 | 0.143 | 0.739 |

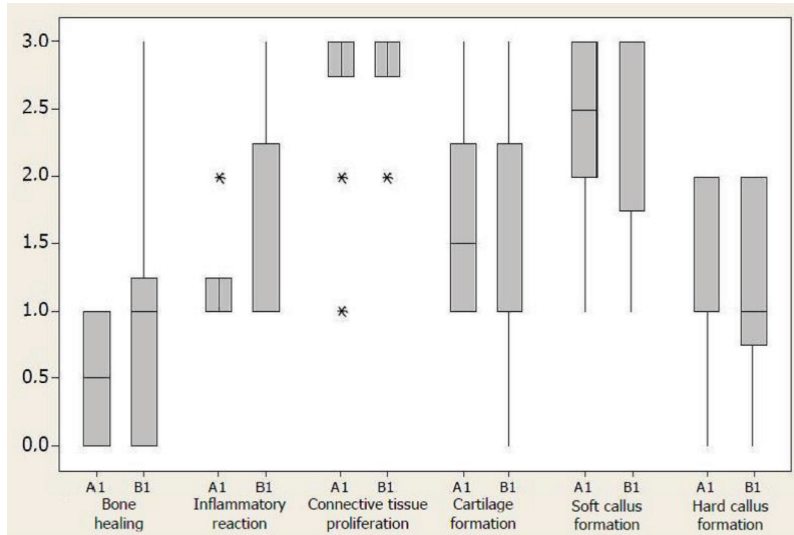


Figure 4. Score comparison of histopathological results of A1 and B1 subgroups.

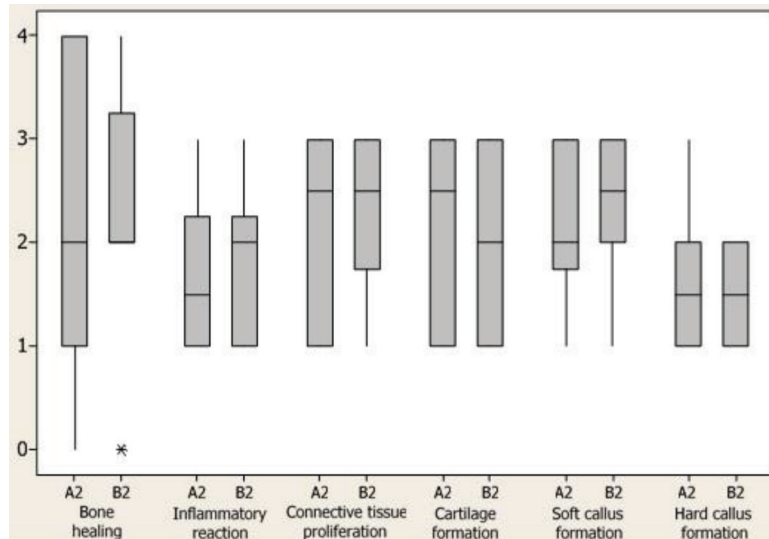


Figure 5. Score comparison of histopathological results of A2 and B2 subgroups.

the biocomposite coating, it is thought that further studies can be planned by coating other sorts of implants such as plates, screws, or prostheses. Also in further studies, more detailed monitoring of the fracture healing with micro-CT imaging devices is recommended [38].

When systemic complications such as foreign-body reaction, chronic inflammatory response, infection, septicaemia, toxicity, and allergic or hypersensitivity reactions are caused by implant coating biomaterial, it is likely to see one or more general clinical findings such as hyperthermia/hypothermia, respiratory or pulse increase, loss of appetite, cachexia, or dehydration [39,40]. In our study, no statistically significant difference was observed between Groups A and B in terms of these systemic clinical

findings. Local findings were also similar. Therefore, the similarity of general and local clinical findings indicates that the coating biomaterial is biocompatible.

In haematological evaluation, since there were no significant differences in other values, in spite of differences in MCV, MCH, and RDW, no reaction disrupting biocompatibility was observed. Biocomposite coated implants also do not have any negative effect on blood values. Based on this information, it is considered that the biocomposite coating is biocompatible [39,41]. From the blood serum biochemistry evaluation, it can be concluded that the Cr value was significantly higher in subgroup A1 compared to subgroup B1, and that postoperative muscle tissue healing was better in subgroup B1 since the Cr level

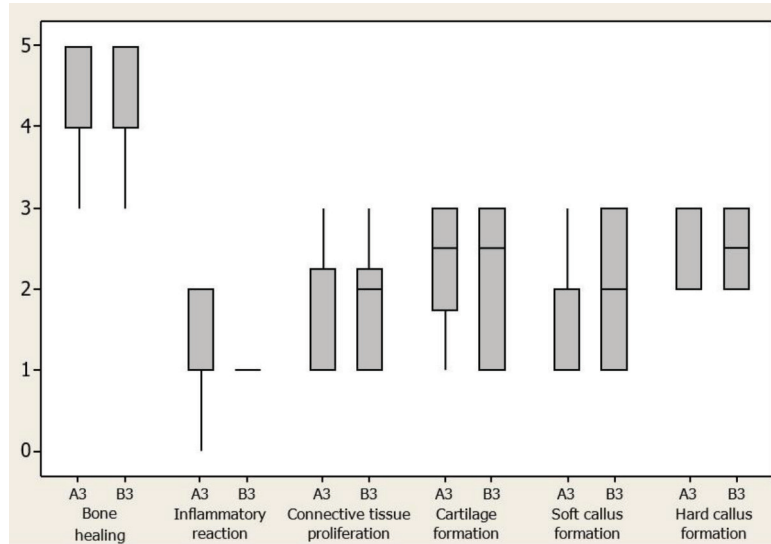


Figure 6. Score comparison of histopathological results of A3 and B3 subgroups.

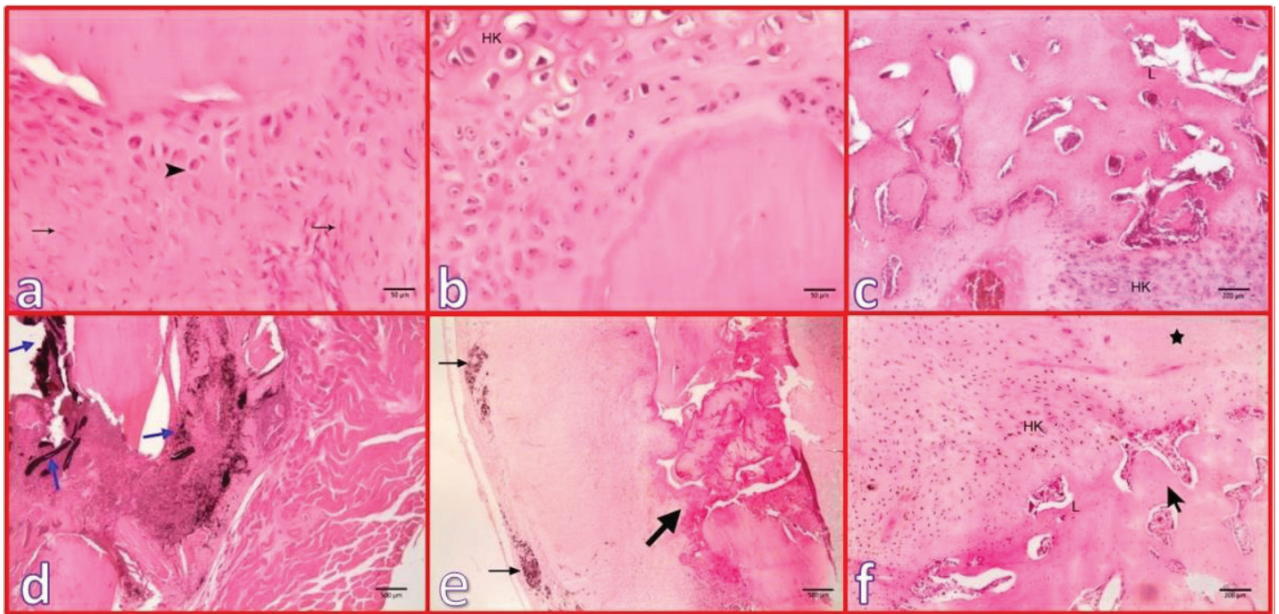


Figure 7. Soft callus in a section belonging to subgroup A1 (a): fibroblasts (thin arrows), hyaline cartilage, and chondrocytes (arrowhead). Bone end in a section of subgroup A2 (b): hyaline cartilage and hypertrophic chondrocytes (HK). In a section of A3 subgroup (c): hypertrophic chondrocytes (HK) and lamellar bone formations (L). In a section of B1 subgroup (d): medullary channel, pigment accumulations between the bone ends and in the granulation tissue (blue arrows). In a section of B2 subgroup (e): collagen deposition between the bone ends, erythrocyte deposits (thick arrow), black-coloured pigment deposits (thin arrows) around the veins in the newly formed granulation tissue and the connective tissue. Hard callus formation in a section from B3 subgroup (f): osteoid mineralization (star), hyaline cartilage (HK), and lamellar bone (L), newly formed bone formations (arrow).

is also an indicator for muscle tissue catabolic events [42]. The ALP and ALT values in subgroup B2 were higher than in subgroup A2, indicating that the mineralization and calcification might be increased in the period involving the callus formation since it is stated that ALP and ALT

enzymes are high in cases where mineralization and calcification events increase [43,44]. In subgroup B2, CK, LDH, Cr, and BUN values were significantly lower than in subgroup A2, which may indicate that muscle tissue healing is better in subgroup of B2, or that cellular damage

is less in subgroup B2 [42,43]. No significant differences were found in biochemical findings of subgroups A3 and B3. Thus, the biocomposite coating did not cause any effect that disrupted the biochemistry profile on day 45. Based on these findings, it was concluded that the biochemistry profile was consistent with the biocompatibility in all the periods of 15, 30, and 45 days. In addition, the differences in the B2–A2 comparison indicate the favourable effects especially at the time of callus formation.

The absence of a statistically significant difference in the radiographic findings of the A and B groups also showed that the biocomposite coating had no negative effect on the fracture healing and did not cause any biocompatibility disruptive effect. However, there is no evidence that it improves fracture healing.

Histopathological scores were compared pairwise with the subgroups showing the same fracture healing period; results were similar in terms of fracture healing, cartilage formation, inflammatory reactions, connective tissue proliferation, and soft and hard callus formations, and were consistent with other findings. Thus, the biocompatibility of biocomposite coated implants during fracture healing was determined and supported by the histopathological examination. However, based on these results, it cannot be said that it significantly increases or accelerates the fracture healing. It was determined histopathologically that the desired results were obtained in terms of biocompatibility in all 3 periods, but there was no additional superiority such as improving the fracture healing. The presence of dark brownish-black pigmentation points in histopathological examination, especially in the medullary canal and in some of the stated areas in subgroups B1, B2, and B3, is an indication that the coating biomaterial is not resorbed on the 15th, 30th, and 45th days.

In the comparison of the subgroups that belonged to Groups A and B (A1-A2-A3 and B1-B2-B3), the clinical, radiographic, haematological, biochemical, and histopathological findings are found to be consistent with the fracture healing time course. This consistent course is indicative of the biocompatibility of the biocomposite coated group during the fracture healing. In addition, to elaborate on the evaluation in further studies, supplementary methods are recommended such as immunohistochemistry or biomarkers [33].

There are numerous studies in which HA, h-BN, Cs, and Ct1 are used in biocomposites as double or triple components [8,14,22,26,36,45–52]. However, the *in vivo* results of a biocomposite with these 4 components were investigated in our study for the first time. It is thought by many researchers that HA/Cs composites are suitable for bone regeneration and repair in orthopaedic surgery thanks to their superior properties [51,53]. For example, in one study, a hybrid gel was produced by distributing

nano-HA particles into Cs hydrogels in order to increase the mechanical and bone compatibility characteristics and it was reported to have potential for use as an injectable bone scaffold [54]. Many articles on the production of similar HA/Cs hybrids or composite materials for orthopaedic use can be found in the literature [55–57]. In one study, HA/Ct1/poly (1-lactic acid) biocomposites were coated by the method of spin coating on the magnesium alloy of AZ31 and the bonds of HA/Ct1 macromolecules were suggested to resemble the natural bone [45]. In another study, the Ct1/HA biocomposites were coated on orthopaedic Ti implants by the plasma spray method and it was suggested that the Ct1 infiltrated porous HA coatings were very superior materials that allowed cell attachment, proliferation, and transformation, and that may be useful [34]. In one study, a team claimed that they obtained HA/Ct1 nanocomposites for the first time by electrodeposition in a thick nanoporous structure that had high biocompatibility with *in vitro* osteoblast culture experiments [46]. In a study in which electrochemical mineralization of HA was examined on Ti substrates together with Ct1 and Cs, it was stated that tighter mineralization was formed than with pure HA coatings; thus, biocompatibility and cell proliferation were more effective [58]. Our study also clearly demonstrates that the *in vivo* results are consistent with previous studies, and the biocompatibility of biocomposite coating containing HA/h-BN/Cs/Ct1.

In order to improve the mechanical properties of biomaterials and increase their biocompatibility, h-BN has been used in many studies as a biocomposite component [8,9,36,48–50,59,60]. For example, in one study, it was demonstrated that some particular mechanical properties of HA were improved with the addition of boron nitride nanotubes, and it was reported that there was no negative effect on the viability and proliferation of osteoblast cells [9]. In another study, boron nitride nanotubes composed of h-BNs were reported to be good candidates for the development of the properties of polymers, biocomposites, and tissue scaffolds due to their low toxicity, high mechanical strength, and chemical stability properties [49]. It was reported that boron nitride is biocompatible in a study where it was used in combination with gelatine and the denaturing form of collagen [60]. Also, HA/h-BN was applied by Atila et al. to experimental femoral defects and there was no increase in serum boron levels. Therefore, it was stated as promising [50]. Göncü et al. reported that they successfully applied h-BN/HA coating to Ti implants. They stated that they expected this coating to support cell proliferation and have high bioactivity [48]. However, in another study, Cs/h-BN and Cs/h-BN/TiO₂ biocomposite coatings were applied to stainless-steel (316L) implants by EPD and it was stated that they may

be used to give antibacterial properties but not bioactivity [8]. According to the results of our study, although the biocomposite coating material including the h-BN component is biocompatible, it cannot be said that any increase is demonstrated in bioactivity. When the studies are evaluated, it is seen that the *in vivo* research results of the biocomposites containing the h-BN component are limited compared to HA, Cs, and Ct1 components. In this respect, it is of particular importance that the h-BN component is present in the biocomposite coating used in our study.

In light of the data revealed above by a large number of researchers and the results of our study, the biocomposite coating with HA/h-BN/Cs/Ct1 content was found to be biocompatible and nontoxic, consistent with previous studies. In addition, it is seen that most studies are limited to the investigation of the *in vitro* effects. In this respect, it is considered that the *in vivo* results will provide scientific contributions and a basis for further studies.

5. Conclusions

This study has shown that the biocomposite coating material with the content of HA/h-BN/Cs/Ct1 was biocompatible in an *in vivo* experimental rat model and did not cause any negative effects to disrupt the fracture healing. However, there was no significant increase in the fracture healing or acceleration. Especially in the period of callus formation, which is important in the fracture healing, biochemical results are noteworthy considering their positive effects. It was determined histopathologically that the coating biomaterial was not resorbed at the 15th, 30th, and 45th

days. In addition, some physical disadvantages resulting from the coating method were determined. Further studies are needed to develop the biocomposite coating and obtain more detailed results. In new studies, it may be aimed to increase the fracture healing by changing the concentration and/or amounts of the biomaterial components used in the coating, or by changing the coating method. Studies intended to increase osteointegration for different implants such as screws or prostheses can be designed. With this purpose, further studies are recommended with biomechanical tests and micro-CT which allow for more detailed monitoring of the fracture healing. Immunohistochemistry, biomarker, or toxicological examinations are also recommended.

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