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Safety Evaluation of Tricalcium Phosphate/Collagen Nanocomposite Scaffold in Bone Defect in New Zealand White Rabbit Model

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Abstract

Objectives: Bone loss with skeletal trauma or metabolic diseases usually will require a bone graft. In addition, medical devices used for replacement in tissues such as bones and cartilages for more than 30 days must be checked and controlled for biological safety.

Materials and Methods: New Zealand white rabbits were divided into two groups. The first group had no defects and was selected as the control group. In the experimental group, tricalcium phosphate/collagen (TCP/collagen) nanocomposite was utilized as the replacement tissue in the femoral defect site. Then, the factors of kidney, liver, and TCP/collagen biocompatibility were evaluated drawing on hematological quality. Free radicals are generated by the damaged tissue when there is a fracture in a bone. Oxidative stress is involved in this mechanism which is defined as the excessive imbalance of reactive oxygen species (ROS) and inappropriate antioxidant anti-mechanical mechanisms.

Results: In the treatment group, malondialdehyde (MDA) level increased postoperatively in the 15th and 30th days, but in due course, it reduced on days 45 and 60. Further, glutathione peroxidase (GPX) enzyme increased after the surgery on days 15 and 30 in the test group and superoxide dismutase (SOD) enzyme demonstrated a slight increase in 15th day. The hematologic investigations were all within a normal limit, including hepatic enzymes, alanine transaminase, aspartate aminotransferase, and alkaline phosphatase (ALP), which indicate the liver damage, as well as creatinine and urea levels displaying the renal function.

Conclusions: Overall, the results of the current study revealed that the oxidative stress factor in the treatment group was not higher compared to the control group, thus showing good biocompatibility of TCP/collagen nanocomposite.

Keywords: Tricalcium phosphate/collagen, Nanocomposite, Biocompatibility, Oxidative stress, Bone defect

Introduction

Tissue engineering is regarded as a potential method regarding providing novel therapeutic methods for large bone defects in animals (1). The treatment program should be checked for biological safety if it is designed in a way to implant a medical device for more than 30 days in a bone or cartilage. Therefore, several other factors should also be meticulously evaluated and controlled, including cytotoxicity, sensitization, and irritation, as well as genotoxicity, encapsulation in implantation, hemolysis, pyrogenicity, acute systemic toxicity, subacute systemic toxicity, chronic systemic toxicity, and carcinogenicity (2).

As regards the biocompatibility of a material or device, it is believed that it should be capable of preserving its chemical and biological inertia during the period of manipulation and forming its solid integrity with the surrounding tissues. The biocompatibility is often measured according to the toxicity level of the implant. The released specific chemicals primarily determine the toxicity effect of a material which thus may be responsible for systemic or local tissue reactions, as well as possible toxicological, allergic, carcinogenic, or mutagenic responses. Any harmful effect, imposed on an organism resulted from short-term or single exposure (between 24 and 96 hours), is defined as acute toxicity which may further lead to severe biological injury or even death. At the cellular level, measuring the enzyme activity of antioxidants can help evaluate the subacute toxicity. The examples of such enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX), reduced glutathione, glutathione reductase, along with main indicators such as malondialdehyde (MDA) and oxidative stress (3).

Thus, it seems that the application of nanotechnology

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in practical medicine is still uncommon due to possible undesirable outcomes which are enforced by potential toxicity or the long-term adverse effects of the treatment. According to previous research, nanoparticles increase the levels of reactive oxygen species (ROS) or anti-inflammatory mediators (4).

The third part is tricalcium phosphate (TCP) which is also known as the bone ash (Ca3 PO4). TCP is a rich source of calcium and phosphate and can be easily absorbed as well. In addition, TCP beta is environmentally friendly and improves a specific network of the defected site. Further, collagen is considered as a protein rich in the extracellular matrix (ECM) and is in the group of proteins with the fibrous structure that helps the extracellular structure.

In other words, collagen plays an important role in maintaining the biological order and the integrity and structure of the ECM. It is also a rich source for bones, cartilages, tendons, ligaments, blood vessels, nerves, and skin. Common structural proteins are of hard and soft tissues. Furthermore, this protein has several features for regulating morphology, adhesion, migration, and cell differentiation, and leads to immunogenic deficiency, porous structure, permeability, good biocompatibility, and biodegradability.

The biomaterials of nanocomposites or biological nanocomposites have more designing adaptability because of their better control over nanoparticles-polymer interaction.

Similarly, nanocomposite polymer biomaterials have greater mechanical features compared to macro- and micro-supplements. The findings of one study showed the effect of nanocomposite TCP collagen on the improvement of bones in femoral surgery in rabbits (5).

Given that the oxidative stress can lead to an accelerated healing period, the objective of the current study was to assess the oxidative stress levels imposed on tissues as a result of exposure to the aforementioned materials. To the best of our knowledge, this was the first study to apply TCP/collagen nanocomposite, which is used as a scaffold implant in live samples, in New Zealand white rabbits. TCP nanocomposite was also tested to examine histological biocompatibility features in vivo and cytotoxicity.

Materials and Methods

Preparation of Tricalcium Phosphate/Collagen Nanocomposite

First, collagen suspension was prepared in an alkaline solution having a pH of 12 and at room temperature. The glutaraldehyde solution was added as a cross-linker to the homogenous suspension and then the TCP powder was slowly added to 1:2 collagen suspension, followed by placing the mixture at -40°C for 5 hours. Finally, porous composites were obtained after further challenges in lyophilization (5).

Experimental Design

The present research was performed in accordance with the International Guiding Principle for Biomedical Research Involving Animals (2012). Furthermore, this study was conducted after obtaining the Ethical Approval of the Research Committee of the Faculty of Science and Research of the Islamic Azad University of Tehran, Iran (No. IR.IAU.SRB.REC.1395.15).

Twelve mature male New Zealand White Rabbits weighing 2.5-3 kg were randomly selected for the present study. All animals were selected from a common source for reducing genetic diversity. Each of the animals was separately placed in a cage and under a standard pellet diet and water ad libitum. Furthermore, the rabbits were kept under normal ambient conditions with the temperature, humidity, and lighting of 18±3°C, 60±5%, and 12 hours, respectively.

Then the rabbits were divided into 2 groups of six rabbits each. In the treatment group, the defect was filled by TCP/collagen while in the control group, the defect was empty. Before the surgery, full physical examination was performed and for the evaluation of biochemical profile and complete blood count, 2.5 mL blood was obtained from the jugular veins of the rabbits which were fasted for approximately 8 hours.

Surgical Procedure

Intramuscular ketamine 10% (ketamine hydrochloride, 50 mg/kg) and xylazine 5% (5 mg/kg) were used for the surgery. The surgical site was incised and cleansed with iodine surgical soap following aseptic methods. Then, a 5 cm incision was made at the beginning of the midline of the upper right limb and the average part of diaphyseal femur underwent surgery. The periosteum was removed and a cylindrical 5-6 mm bone defect was created on the thigh bone of one of the organs. Next, the osteotomy site was cleaned with 0.9% saline and the sternal periosteum was preserved with excessive muscle. A therapeutic protocol was utilized for every rabbit undergoing osteotomy operation.

Treatment

Antibiotic (penicillin G procaine 40 000 IU/kg IM, SID), dexamethasone (0.6 mg/kg, IM), and tramadol hydrochloride (5 mg/kg, IM, BID) were employed for 3 days after the surgery (6).

Observation and Clinical Examination

All animals were closely monitored during the study and reexamined on days 7, 15, 30, 45, and 60.

Hematology

The blood samples were taken from jugular veins into ethylenediaminetetraacetic acid (EDTA)-contained test tubes. Using the Exigo-vet device, white blood cell

count, red blood cell count, hemoglobin concentration, hematocrit, and platelet count were measured before and 7, 15, 30, 45, and 60 days after the surgery.

Serum Biochemistry

To measure the biochemical factors, the blood samples were moved to the tubes containing anticoagulant and were allowed to coagulate at room temperature. They were then centrifuged and the blood serums were isolated and kept at -20˚C.

The blood urea nitrogen, creatinine, aspartate transaminase, alkaline phosphatase (ALP), and alanine transaminase were analyzed with an automatic chemistry analyzer (auto analyzer BT1500, Dialab Kit) before and 7, 15, 30, 45, and 60 days after the surgery.

To assess oxidative stress factors, MDA, SOD, and GPX were also measured by Zell Bio GmbH (Germany) kit and evaluated by a BIOTEK ELx800 ELISA plate reader.

Statistical Analysis

Statistical analysis was performed in SPSS software (version 22.0, SPSS Inc., Chicago, USA). Then, the one-sample Kolmogorov–Smirnov test was used to check data normality. Finally, ANOVA with repeated measure was applied to analyze the obtained numeric data. $P<0.05$ was considered statistically significant.

Results

All obtained values were normally distributed according to one-sample Kolmogorov–Smirnov. No statistical tests such as logistic regression were used because there was no mortality in the treatment and control groups of the current study. No behavioral changes or visible signs of physical impairment indicating systemic or neurologic toxicity were observed as well. Descriptive statics of the measured parameters in the studied population are summarized in Tables 1 & 2 and Figures 1 to 3. There was a significant difference in MDA level between the control and treatment groups ($P<0.05$) (Figure 1). In addition, a significant difference was observed in GPX level between the treatment and control groups ($P<0.05$) (Figure 2). Figure 3 depicts a significant difference in SOD level between the control and treatment groups ($P<0.05$) (Figure 3).

Discussion

Nanotechnology has been an attractive filed for researchers in the past decade due to its wide applications and the growth of nanomedicine. Moreover, recent studies have considered nanocomposites as an effective treatment modality of bone defects (7,8). Thus, investigations on their biocompatibility and oxidative stress, which is induced by nanocomposites, became popular. Although it is known that the increased levels of oxidative stress lead to implant rejection, at the lower levels of oxidative stress, tissue infection is expected as the result of defective defense mechanisms. Hence, to decrease the unfavorable outcomes, keeping the oxidative stress at a physiologic level is of great importance. In veterinary clinical practice, having a practical knowledge of biomaterials, particularly regarding biodegradable materials is essential respecting providing a useful tool for the proper handling of the bone defects. It should also be considered that better understanding of the cell-material interaction with regard to oxidative stress levels is mandatory for achieving more compatible biomaterials in the future (9,10).

Some previous studies confirmed the role of TCP-collagen nanocomposite in the humorous bone defects of

Table 1. Hematological Values of Rabbits

Days	Parameters					
	WBC (10^3/mm^3)	RBC (10^6/mm^3)	HCT (%)	PLT (10^3/mm^3)	Neutrophils	Lymphocytes
Day 0 control	6.51 ± 0.39	6.08 ± 0.14	36.03 ± 0.77	232.66 ± 8.75	1843.33 ± 33.26	4216.66 ± 470.81
Test	7.33 ± 0.08	6.48 ± 0.33	35.46 ± 0.76	260 ± 7.07	2700 ± 70.71	4363.33 ± 58.87
Day 7 control	8.5 ± 0.07	6.16 ± 0.16	38.18 ± 0.77	419.5 ± 12.86	2235 ± 39.37	5433.33 ± 382.97
Test	8.91 ± 0.08	6.38 ± 0.37	42.71 ± 0.4	319.16 ± 20.1	6200 ± 70.71	2366.66 ± 60.55
Day 15 control	4.4 ± 0.07	5.83 ± 0.16	39.08 ± 0.73	240.16 ± 7.22	2091.66 ± 80.1	2233.33 ± 225.09
Test	4.81 ± 0.08	5.800 ± 0.22	35.43 ± 0.59	270.83 ± 7.35	2600 ± 70.71	1951.66 ± 44.9
Day 30 control	5.2 ± 0.07	6.11 ± 0.11	38.31 ± 0.87	251.33 ± 7.78	1735 ± 39.37	3333.33 ± 186.18
Test	58.88 ± 0.08	5.85 ± 0.18	37.80 ± 0.58	349.16 ± 7.35	905.83 ± 46.41	4451.66 ± 353.29
Day 45 control	4.8 ± 0.07	5.91 ± 0.11	37.43 ± 0.91	311.83 ± 9.17	4458.33 ± 53.16	2858.33 ± 58.45
Test	5.01 ± 0.09	6.05 ± 0.18	36.50 ± 0.73	212.16 ± 6.79	1675 ± 82.15	3892.5 ± 643.1
Day 60 control	3.03 ± 0.08	6.18 ± 0.11	39.35 ± 0.75	267.33 ± 6.43	965 ± 28.8	2191.66 ± 257.71
Test	8.5 ± 0.07	7.23 ± 0.16	48.16 ± 0.79	246.66 ± 7.52	2363.33 ± 38.29	5975 ± 75.82

WBC: white blood cell; RBC: red blood cell; HCT: hematocrit; PLT: platelet.
Data are provided as means ±SD. There are highly significant differences in the values of hematology (i.e., RBC, WBC, neutrophil, lymphocyte, PLT count, and HCT) between treatment and control groups (P<0.01)

Table 2. Biochemical Values of Rabbits

Days	Parameters				
	BUN	SCR	ALT	AST	ALP
Day 0 control	21.5 ± 1.87	0.86 ± 0.04	58.33 ± 2.16	37.66 ± 5.46	34 ± 1.41
Test	25.63 ± 1.05	0.96 ± 0.05	65.83 ± 3.86	32.33 ± 1.21	21.16 ± 4.66
Day 7 control	26.75 ± 0.93	0.89 ± 0.06	71.83 ± 2.13	37.83 ± 12.3	46.83 ± 11.4
Test	30.7 ± 2.59	0.86 ± 0.12	76.66 ± 1.21	25 ± 1.41	37.5 ± 5.24
Day 15 control	25.5 ± 1.04	0.91 ± 0.02	56.83 ± 7.57	33.5 ± 4.76	50.83 ± 3.48
Test	26.81± 1.35	0.94 ± 0.03	47 ± 3.22	21.5 ± 1.87	57 ± 15.27
Day 30 control	27.75 ± 1.08	0.97 ± 0.05	59.16 ± 3.86	40.66 ± 4.08	26.16 ± 8.61
Test	26.65 ± 2.1	0.83 ± 0.09	76 ± 4.56	31.16 ± .16	32 ± 3.4
Day 45 control	26.66 ± 2.8	0.82 ± 0.05	73.66 ± 10.01	41 ± 11.98	36.66 ± 5.5
Test	31.1 ± 1.43	0.99 ± 0.05	92.5 ± 14.62	49.16 ± 2.31	39.83 ± 5.77
Day 60 control	29.71 ± 1.7	1.46 ± 0.04	59.66 ± 5.24	36.33 ± 2.16	38.33 ± 7.03
Test	23.95 ± 1.56	1.02 ± 0.15	62.83 ± 12.08	27.66 ± 8.06	44.66 ± 7.63

BUN: Blood urea nitrogen; SCR: Serum creatinine; ALT: Alanine transaminase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.
Data are presented as Means ±SD.
There was a highly significant difference regarding the values of serum biochemistry (i.e., BUN, SCR, ALT, and AST) from those of the control group in all days ($P<0.01$) while no significant differences were observed in ALP value between the treatment and control groups ($P>0.05$).

the rabbits. Thus, the novelty of the current study was to show the safety of this material in the body of a rabbit based on biochemical factors and the possibility of oxidative stress by TCP-collagen nanocomposite. In addition, there are reports on the safety of nanocomposites in vitro, in the tissue culture (11,12).

The ROS levels significantly increase when a bone fracture occurs, which is originated from the damaged

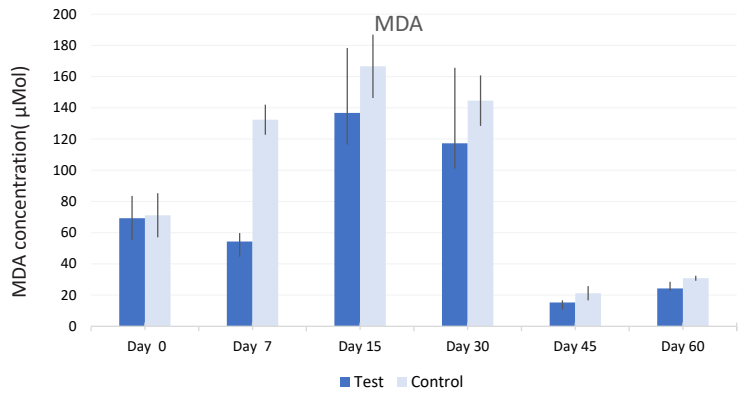

Figure 1. Malondialdehyde in Rabbits. *Note*. Data are demonstrated as means ±SD.

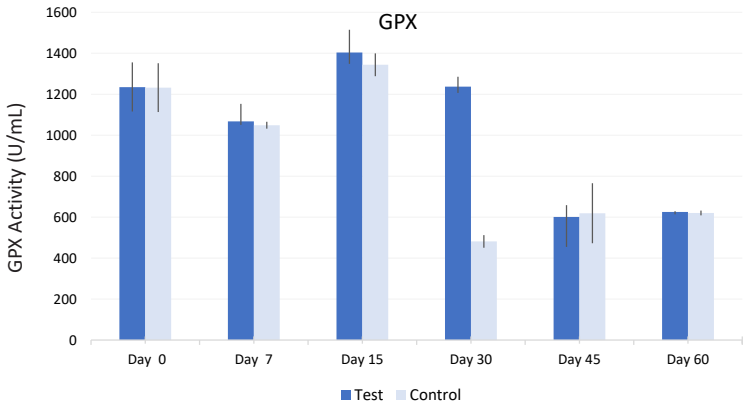

Figure 2. Glutathione Peroxide in Rabbits. *Note*. Data are demonstrated as means ±SD.

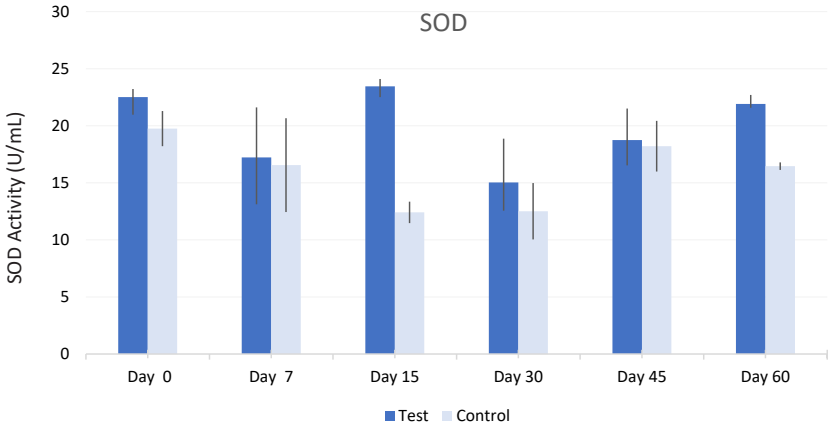

Figure 3. Superoxide Dismutase in Rabbits. *Note*. Data are demonstrated as means ±SD.

tissue. On the other hand, serum MDA level also increases due to osteoclastic activity, leading to the utilization of serum MDA. When the activity of antioxidant enzymes such as SOD and GPX is insufficient to keep pace with osteoclastic superoxidation, the defense mechanism fails, as was shown by the increased levels of serum MDA (13). In other words, excessive ROS production overwhelms antioxidant defense mechanisms, which results in the loss of the fundamental balance between them (14).

Although few studies support the present study, ROS activity may escalate the complications of the replaced substance. For example, one study used four replacement substances (i.e., stainless steel, ceramic, titanium, and polyethylene) in order to compare antioxidant enzymes in the surrounding tissue. The analysis of glucose-6-phosphate dehydrogenase, glutathione reductase, SOD, GPX, and catalase (CAT) showed increased lipid peroxidation and decreased antioxidant enzymes in tissues that were in contact with ceramic and titanium. The results of this study suggest that polyethylene replacements yield better results compared to the others (15).

In vivo results of another study on the antioxidant effects of agarose-chitosan bone graft substitute revealed a rise in GPX, SOD, and CAT in the bone tissue 30 days after the replacement. MDA level was also found to raise in the treatment group. It seems that bone marrow transplant affects the bone through excessive intensification of oxidative processes and bone stimulation.

Based on the findings of other studies (16, 17), GPX also acts as a detoxifier in lipid peroxidation while SOD causes superoxide (-O2) to decompose into molecular oxygen (O_2) or hydrogen peroxide (H_2O_2).

Another research evaluated the time course of the changes in serum MDA levels and compared 2 different fixation techniques (i.e., intramedullary versus plate osteosynthesis) for performing experimental tibial osteotomies. The results demonstrated that MDA levels peaked at the end of the second week in the intramedullary osteosynthesis group, but in the plate osteosynthesis group, peak measurements were noticed at the end of the fourth week (18).

In our study, MDA level increased on days 15 and 30 postoperatively in the test group whereas it reduced on days 45 and 60. The MDA levels of the control group were more than that of the test group, which may be due to a more enhanced osteoclast activity and the empty defect. Contrarily, GPX enzyme level increased on days 15 and 30 in the test group. This enzyme is responsible for lipid peroxide detoxification, and together with CAT acts for H_2O_2 detoxification. Thus, when the MDA levels are high, GPX begins to increase as well. SOD enzyme levels slightly increased on day 15 after the surgery. SOD is responsible for the dismutation of O_2^- to molecular oxygen and H_2O_2.

In another study, the safety of a bioglass-polylactic acid composite scaffold in the skull bone defect of a rat was investigated by measuring biochemical, hematological, and free radical indicators (MDA & SOD) three months after the surgery and the results showed good biocompatibility of this biomaterial (17).

The blood chemistry profiles of the current study were also evaluated for the detection of possible abnormalities alongside the assessment of the biocompatibility of nanomaterial. Aspartate transaminase, alanine transaminase, and ALP, all the indicators of the liver damage, as well as creatinine and urea levels reflected renal function and were all within a normal range in all groups.

The upsurge, observed in ALP after the surgery, can be due to an increase in bone-specific ALP isoenzyme, which is the consequence of increased osteoblast activity in the reconstruction phase (6,19).

Hematologically, all the measured parameters were within the normal limit and white blood cell count indicated no sign of any systemic inflammatory reaction.

In conclusion, the results of the current study confirmed that TCP-collagen caused no tissue inflammation and organ damage. The results further revealed that the increase in MDA level in the test group was less than that of

the control group. Consequently, all these results suggested that TCP-collagen provide good biocompatibility and can be safely used as a reliable tool for treating bone defects in veterinary medicine.

Conflict of Interests

Authors have no conflict of interests.

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