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EXPERIMENTAL STUDY

Histopathological evaluation of potential impact of β-tricalcium phosphate (HA+ β-TCP) granules on healing of segmental femur bone defect

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Abstract: Histopathological evaluation of β-tricalcium phosphate (HA+ β-TCP) granules demonstrated that it has properties to heal segmental femur bone defect in rat. In this study, 27 male white rats were examined. Rats were divided into tree groups. Surgical procedures were done after IP administration of ketamine 5 % and xylazine HCL 2 %. Then an approximately 5-mm long, 3-mm deep and 2-mm wide bone defect was created in the femur of one of the hind limbs using a No. 0.14 round bur. After inducing the surgical wound, all rats were colored and randomly divided into three experimental groups of nine animals each: Group 1 received medical pure β-tricalcium phosphate granules, group 2 received hydroxyapatite and third group was a control group with no treatment. Histopathological evaluation was performed on days 15, 30 and 45 after surgery. On day 45 after surgery, the quantity of newly formed lamellar bone in the healing site in β-TCP group was better than onward compared to HA and control groups.

In conclusion, β-tri calcium phosphate (β-TCP) granules exhibited a reproducible bone-healing potential *(Fig. 10, Ref. 28)*. Text in PDF *www.elis.sk.*

Key words: bone healing, β-tricalcium phosphate (β-TCP), hydroxyapatite (HA), histopathological evaluation, rats.

Calcium phosphate (CaP) ceramics are the most similar synthetic biomaterials of hard tissues of human body (1, 2). They consist of the same ions as the minerals in natural bones. This family of biomaterials is the most biocompatible material that has been known for hard tissue applications such as orthopedics, dental implants, alveolar bridge augmentation, maxillofacial surgery and drug delivery systems (3–5).

In dense and porous bulk materials, particulates, cements and coatings, CaP ceramics have been important biomedical materials in clinical hard tissue repairs and replacements. Porous CaP ceramics are only used as cancellous bone graft substitute materials in non-load bearing situations. Dense CaP ceramics have more excellent mechanical performance than porous ceramics (6–8).

The atomic ratio of Ca:P in calcium phosphates can vary between 2 and 1 to produce compounds ranged from calcium tetraphosphate (TTCP) $Ca_4P_2O_9$, through hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$, octacalcium phosphate (OCP) $Ca_8H_2(PO_4)_6.5H_2O$, and tricalcium phosphate (TCP) $Ca_3(PO_4)_2$ to dicalcum phosphate dihydrate (DCPD) $CaHPO_4.2H_2O$ or dicalcum phosphate anhydrus (DCPA) $CaHPO_4$. Small changes in Ca:P ratio have been shown to have a large impact on the biological response (9, 10).

Among calcium phosphates, HA and β-TCP have been receiving attention of researchers for many years (11, 12). HA with Ca/P ratio of 1.67 is the most thermodynamically stable phase of calcium phosphates in contact with body fluids. β-TCP with Ca/P ratio of 1.5 is biodegradable and more easily resorbed than HA. In an ideal situation, a biodegeradable bone substitute is slowly resorbed and replaced by natural bone. Under physiological conditions, calcium phosphates degrade via dissolution–reprecipitation mechanisms (13, 14).

Various techniques such as solid-state reaction, co-precipitation, andhyd rothermal method and sol–gel have been developed for the synthesis of CaP powders. Sol-gels are the most preferable to other methods such as high product purity, homogeneous composition and comparatively low synthesis temperature (15–17).

The aim of our study was to synthesize and characterize hydroxyapatite (HA) and β-tri calcium phosphate (β-TCP) granules by sol-gel methods and to compare their osseous regeneration in the healing of *in vivo* bone defects in a femoral model.

Methods

Samples preparation and characterization

In present research, analytical grade (Merck, Germany) phosphate pentoxide (P_2O_5) and calcium nitrate tetrohydrate ($Ca(NO_3)_2.4H_2O$) were selected for synthesis of CaP powders. In

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the sol-gel process, designed amounts of P_2O_5 and $Ca(NO_3)_2.4H_2O$ were first dissolved in absolute ethanol with a pH of 10.5 to form 0.5 mol/l and 1.67 mol/l solutions. Then the obtained solutions were mixed with a Ca/P molar at the ratio of 1.5 (TCP) and 1.67 (HA) as an initial mixed precursor solution according to Ca/P ratio in tricalcium phosphate (TCP, $Ca_3(PO_4)_2$, Ca/P = 1.5) and hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$, Ca/P = 1.67). The mixtures were continuously stirred for 30 min at room temperature. Then they were heated in a water bath at 60 °C for 1 h in order to obtain a white transparent gel. The gel dried at 80 °C in 24 h in an air oven. Then it was calcined in a furnace at 700 °C for 3 h. The products were sintered for 2 h at 1200 °C, and then placed in air cooling to achieve ambient temperature. Finally, the sintered products were crushed into resultant granules using an agate mortar. The size of the resulting granules can be controlled by diameter of sieves. The specific surface area was determined by 15-point BET measurement (Micromeritics Gemini 2360). Powder X-ray diffraction (XRD) patterns were recorded using a Philips PW 1371 diffractometer with Cu Kα radiation. For FT-IR analysis, the cements were dispersed into pellet of KBr and the spectra recorded by Brucker IFS 48 were in the range 400 to 4.000 cm^{-1} with resolution of 5 cm^{-1}. Scanning electron microscopy (SEM) investigations were carried out using a Cambridge electron microscope, model Steroscan 360.

Experimental animals

The present study was conducted after obtaining the ethical approval from the Islamic azad University (Urmia branch) Research Committee. In the study we used 27 mature male Wistar rats (aged seven months and weighing 300–320 g). All animals were obtained from the same source and used in this study in order to decrease the genetic variability. The animals were housed separately (one rat per cage) and maintained on standard pellet diet and tap water. Animal houses were in standard environmental conditions at temperature of 22 ± 3 °C, humidity of 60 ± 5 %, and 12 h light/dark cycle. Lateral femoral osteotomies were performed surgically. Rats were divided into three treatment groups with 9 femur bones in each group.

Surgical procedures

Surgical procedures were done after IP administration of ketamine 5 % (85 mg/kg/; Alfasan International, Woerden, Holland) and xylazine HCL 2 % (25 mg/kg/; Alfasan International, Woerden, Holland), and an approximately a 5-mm long, 3-mm deep and 2-mm wide bone defect was created in the femur of one of the hind limbs using a No. 0.14 round bur. This osteotomy site was then irrigated with 0.9 % saline, but periosteum around the osteotomy site was preserved and retracted with the overlying muscles. The osteotomy site was then treated according to the treatment protocol for each rat (18).

Treatment

After inducing the surgical wound, all rats were colored with non-toxic color and randomly divided into three groups by 9. The segmental defects in first group were implanted with medical pure β-tricalcium phosphate granules (1–2 mm in diameter; Surgiplaster, Bio-Lok International Co.) and hydroxyapatite in the second group. The animals of third group were a control group and received no treatment. The periost and subcutaneous tissues were then closed primarily (18, 19).

A single dose of antibiotic (Gentamicin at the dose of 0.05 ml/kg) was injected immediately after surgery prophylactically. Experimental animals were kept in separate cages to prevent self-injury. After the procedure, daily observation was performed and evidence of infection or other abnormalities were noted. Three experimental subjects from each group were sacrificed at the end of follow-ups on days 15, 30 and 45 postoperatively by intra peritoneal injection of an overdose of pentobarbital sodium thiopental (200 mg/kg) (20, 21), and the femurs were extracted, placed in a container, filled with 10% formalin solution and then stored for histological examination.

Histopathological study

Slide preparation

For histological examination, the obtained tissues were decalcified with 10 % formic acid solution which was changed daily. The surgical specimens were submitted to decalcification

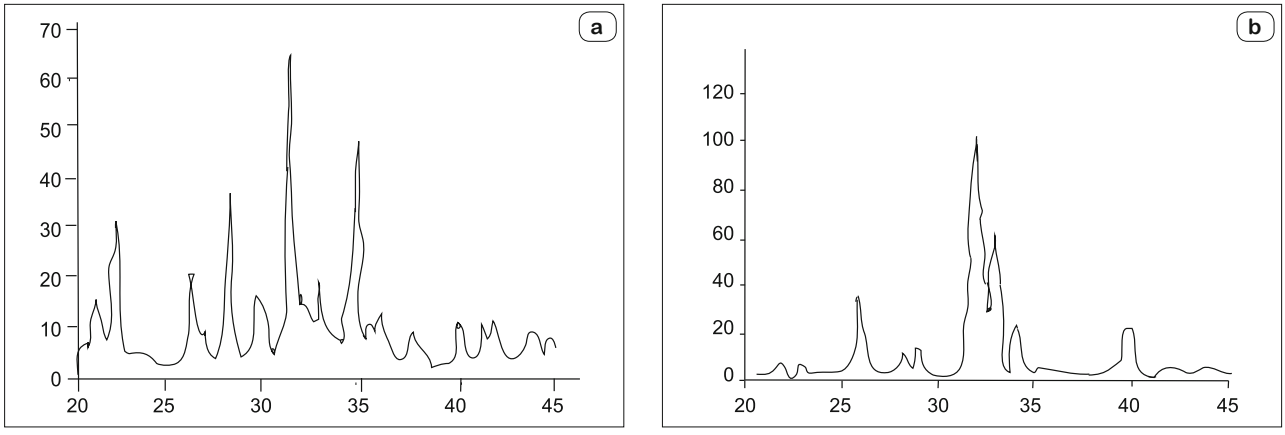

Fig. 1. XRD patterns of granules. a) sample with Ca/P; molar ratio of 1.5, b) sample with Ca/P; molar ratio 1.67.

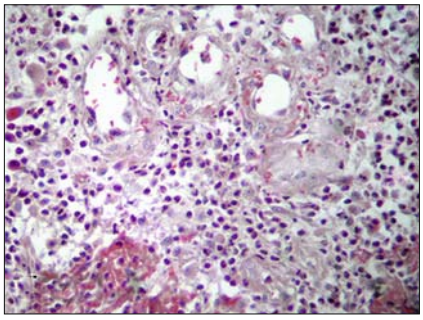

Fig. 2. Microscopic section from the healing site of control group on day 15 of healing shows the defect being filled with inflammatory cells infiltrate and immature granulation tissue consisting of newly formed vessels and plumpy fibroblasts dispersed among the inflammatory cells. Extravasations of red blood cells are obvious (H&E, x100)

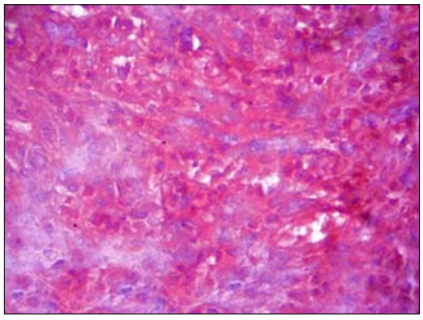

Fig. 3. Microscopic section from the healing site of HA-treated group on day 15 of healing. The repaired construct reveals an immature granulation tissue with large amounts of plumpy fibroblasts seated in implanted material (H&E, x250).

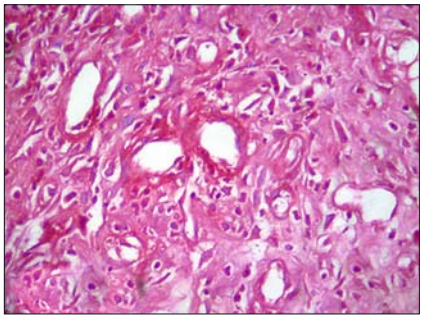

Fig. 4. Microscopic section from the healing site of TCP-treated group on day 15 of healing shows abundant capillary buds in relatively matured granulation tissue (H&E, x250).

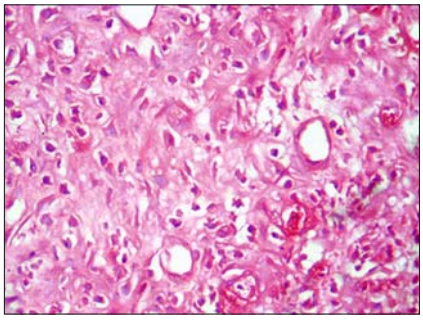

Fig. 5. Microscopic section from the healing site of control group on day 30 of healing. Well-matured granulation tissue is present in the bone defect. Infiltrate with only a few inflammatory cells is present in healing sites (H&E, x250).

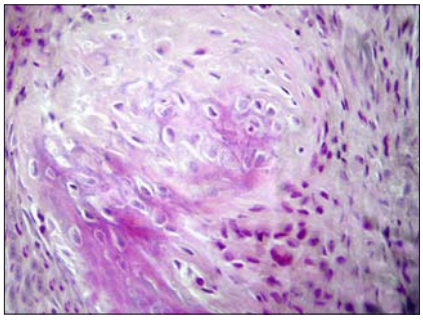

Fig. 6. Microscopic section from the healing site of HA-treated group on day 30 of healing. Cartilaginous nodule in repaired construct reveals chondrogenesis in fibrous tissue, which gives evidence of gradual substitution of granulation tissue with hyaline cartilage (H&E, x400).

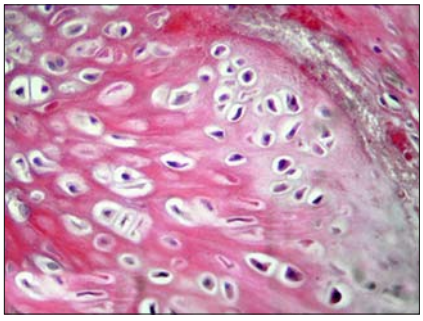

Fig. 7. Microscopic section from the healing site of TCA-treated group on day 30 of healing. Imperfect organic bone matrix, osteoid, deposition and calcification have lead to smaller improvement in bone formation (H&E, x400). The remnants of implanted material are visible around the repaired construct (H&E, x400).

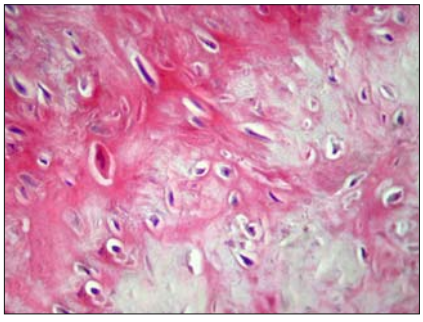

Fig. 8. Microscopic section from the healing site of control group on day 45 of healing. Bone deposition in healing site shows endochondral ossification. Owing to vital deposition and calcification of osteoid, this newly formed bone is more acidophilic (H&E, x400).

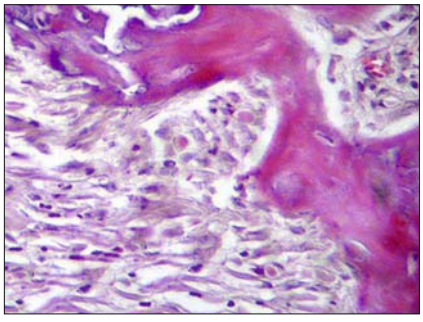

Fig. 9. Microscopic section from the healing site of HA-treated group on day 45 of healing. The defect is filled with fibrous connective tissue and newly formed trabecular bone (H&E, x400).

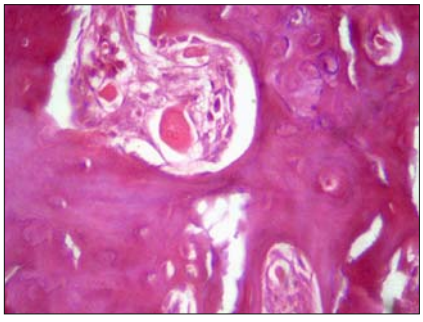

Fig. 10. Microscopic section from the healing site of TCA-treated group, 45 days of healing. The defect is almost filled with newly formed compact trabecular bone. Advanced stage of remodeling and consolidation developing haversian system is seen in repaired construct. The quantity of newly formed lamellar bone in healing site is better than that in HA-treated group (H&E, x400).

and routine histological processing for slide preparation and then embedded in paraffin blocks. Thereafter, they were sectioned at a thickness of 6 μm in a microtome using the largest diameter of the defect, stained with hematoxylin and eosin (HE) and analyzed under a light microscope by pathologist in a double-blind manner. Recorded factors from specimens were evaluated with a 10-point

histological grading scale described by Salkeld to determine the quality of the union, appearance and quality of the cortical and cancellous bone-remodelling, as well as to evaluate the degree of bone-graft incorporation and remodelling.

Results

Phase and composition of granules

The XRD patterns of the samples with Ca/P molar ratios of 1.5 and 1.67 are presented in Figures 1a and 1b. The pattern for sample with Ca/P molar ratio of 1.5 in Figure 1a shows well-characterized peaks of pure β-TCP (hereafter the sample with Ca/P molar ratio of 1.5 is referred to as β-TCP) and the peaks were indexed according to the standard pattern (JCPDS 09-0169). In Figure 1b, the peaks of the sample with Ca/P molar ratio of 1.67 were identified as corresponding to HA and indexed according to the standard value (JCPDS 09-0432). These results indicate that single phase of β-TCP and HA granules were successfully synthesized by sol-gel method. The specific surface area of the powders was same and about ~55 $m^2 g^{-1}$ (Fig. 1).

Histopathological evaluation results

Histopathological evaluation was performed. The healing site of control group on day 5 of healing showed the defect to be filled with inflammatory cells infiltrate and immature granulation tissue consisting of newly formed vessels and plump fibroblasts dispersed among the inflammatory cells. Extravasations of red blood cells were prominent (Fig. 2). The healing site of HA-treated group at this time showed that the repaired construct was filled with immature granulation tissue consisting of large amounts of plump fibroblasts seated in implanted material (Fig. 3). The healing site of TCP-treated group on day 5 of healing showed abundant capillary buds in relatively matured granulation tissue (Fig. 4). The histopathological examination revealed the healing site of control group on day 30 of healing to contain well-matured granulation tissue in the bone defect. The infiltrate contained only a few inflammatory cells in healing sites (Fig. 5). The healing site of HA-treated group at this time showed the presence of cartilaginous nodules in the repaired construct indicating that chondrogenesis in fibrous tissue was taking place and granulation tissue was being gradually substituted with hyaline cartilage (Fig. 6). The healing site of TCA-treated group on day 30 of healing showed imperfect osteoid deposition and calcification leading to improvement in bone formation. The remnants of implanted material were visible around the repaired construct (Fig. 7). Microscopically, the healing site of control group on day 45 of healing indicated bone deposition in healing site suggesting endochondral ossification. Owing to vital deposition and calcification of osteoid, this newly formed bone was more acidophilic (Fig. 8). The healing site of HA-treated group at this time was filled with fibrous connective tissue and newly formed trabecular bone (Fig. 9). The healing site of TCP-treated group on day 45 of healing was nearly filled with newly formed compact trabecular bone. It was obvious that the repaired construct showed an advanced stage of remodeling and consolidation with a developing haversian system. The quantity of newly formed lamellar bone in the healing site was better than those in the HA-treated group (Fig. 10).

Discussion

Bone defects and injuries are a cause of one of the most agonizing suffering in humans. Two of the major clinical procedures for bone defects were autotransplantation and allotransplantation. Less than 10 percent of transplantations in bone defects were operated with the use of synthetic materials in year 2000 (22). A lot of disadvantages of auto and allograft repairs forced scientists to seek for new graft material. In autograft the need of another site of operation, confined amount of the required bone, bleeding in the second operation site, etc are considered to be an impediment. In case of allograft, the finding of compatible person, disease transfer risk, and immune rejection are obstacles in facing the operation. In this regard the current investigation was conducted in order to gather sufficient information with previous studies to take further steps for developing this material.

The main stages of repairing bone tissue are cell adhesion, proliferation and differentiation of newly developed cells into osteoblasts. The entrapment of bone growth factors (BMPs), and minerals and protein adhesion to bioceremics accelerate these stages. Apatites showed to be facilitating the protein adhesion in use of chromatography (23). On the other hand, TCP is believed to increase the bone formation rate by entrapping the osteoprogenitors in site of injury. This ability is referred to as osteoinductivity which is described as a capability of some materials to induce new bone formation in an absence of osteogenic factors. In contrast to osteoinductivity, the expression of osteoconductivity comes with the definition of ability of the material to act as a scaffold for conducing newly forming bone structures. The organizing of newly entrapped osteoblasts by bioceremics, especially CaPs, in the site of injury prepares a suitable place for proliferation and differentiation. In the last phase of bone healing (remodeling), competent biomaterial has to be substituted with bony tissue (24). The cavities filled with TCP showed this fact on day 15 after surgery, while on day 30 after surgery it is observed in the Ha-treated group. The results of study performed by Edela Puricelli et al and Toquet J, et al verify our findings regarding this issue. Some differentiations in the observation time may be due to structural distinctions in biomaterial arrangement.

Beside these factors in the nature of bioceramics, biodegradation within the body is one of the most important factors that need to be considered. Acidic environment in the site of injury dissolves the CaPs to its ions, namely Ca^{2+} and PO_4^{3-}. XRD is a simulation of body fluid for detecting the range of this index. Investigation shows that TCP has a higher rate of degradation when compared to apatite (25). The reduction in TCP solubility has a direct proportion to its Mg^{2+} or Zn^{2+} compositions, and in case of apatite, its reduction has a direct proportion to F^- in the composition of apatite (26, 27). As a result, it is expected that TCP will be resorbed by the tissue and therefore mineralization (28) would be occuring rapidly and efficiently and lead to better repair. This is obvious from results of this study. SUN Jiao et al reported the same finding as that in our

study when they compared HA to TCP with 75% of porosity and same dimensional structure in femoral bone of rabbit.

In conclusion, the results of this study show a promising potential for TCP to be used widely in grafting for bone fracture healing. Nevertheless, two major facts should be considered in this regard. Firstly, biodegradation, reaction of body and other factors affecting bioceramic's capability is under the influence of body's environment. Therefore human trail studies are strongly encouraged. The second fact in concern of TCP and even HA is finding the best porosity rate and tridimensional structures of these bioceramics that would work in the best interest of defective bone. In conclusion, it can be stated that TCP has an important role in the reconstruction of bone defects.

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