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Developing Silicate Additive Antibacterial and Osteoinductive Injectable Bone Grafts

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***Abstract*— In recent years, a striking increase in the number of commercial products has emerged with the introduction of first injectable bone grafts. Bone substitutes developed as highly water retained hydrogel structures and calcium phosphate-based ceramic composites have gained popularity especially in orthopedics and maxillofacial applications. The fundamental requirement of these injectable bone grafts are having radiopaque characteristic that allows surgeons to detect graft material after the surgery, antibacterial efficacy to prevent infections and gaining partial osteoinductive characteristic to support bone tissue regeneration. Therefore, the aim of this study was to develop silicate additive biocompatible, radioopaque and partial osteoinductive synthetic injectable bone grafts that do not need mixing during surgical application.**

Keywords — Bone regeneration; Bone tissue engineering; Injectable Bone Substitute; β-TCP; Hydrogel

I. Introduction

Osteoporosis is becoming an extensive medical problem that causes an increasing possibility of bone fractures because of the accelerating population age. An important disadvantage of the existing orthopedic implant materials which are sintered solid and rigid form is their inadequate filling microcavities in the bone defect area. Shaping the graft material in the surgical area during the procedure extends the surgery time and increases the infection possibility [1, 2]. To overcome these problems, directly injectable biomaterials are needed with the development of minimally invasive surgical methods. Ideal synthetic bone substitutes should be injectable, shapeable by hand, promote bone adhesion and osteogenesis while they disintegrated by body fluids and cells [3, 4].

In recent years, injectable calcium phosphate-based (CaP-b) grafts have been approved as promising bone grafts by virtue of their resemblance to the mineral phase of the bone and promote in-situ mineralization. Previous studies reported that CaP-b grafts are biocompatible which do not display toxicological actions and stimulate foreign body reactions within the body [5]. Although CaP is partial radiopaque, it is not quite enough for surgeons to detect graft material after the surgery. Enriching graft material structure with inorganic non-metallic material not only gains radiopaque characteristics to the grafts but also brings in partial antibacterial efficacy.

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Moreover, silicate additive within the range of a clinically equivalent product can increase bone regeneration capacity of β-TCP based bone graft materials as depicted from the literature [6-9]. Within the scope of this study, prototype studies of the injectable β-TCP & Silicate based bone graft (Powerbone Putty Plus) which do not require extra mixing during the surgical application, were performed. The graft material used in the Putty Plus is a silicate enriched CaP-b grafts, which is designed as sterile, biocompatible and ready to use (no mixing required) bioactive injectable graft material. Due to it's dense, mouldable and excellent retention structure, it can fit on the defect region, stays there without membrane coverage, and enhance the amount of bone contact which also augment the cell migration into the graft. In addition to these, Powerbone Putty contains no tissue of human or animal origin, therefore, carries no risk of disease transmission.

II. Materials and Methods

The β-TCP synthesis was carried out by a chemical precipitation method according to the developed methodology by Bonegraft Co. [10]. The semi-synthetic cellulose-based polymer that provides an excellent bioactive environment for cells was used to obtain the hydrogel structure. After obtaining viscous polymer solution, a certain proportion of β-TCP powder was mixed in a special mixer and injectability and paste consistency was obtained. The obtained paste was filled into injectors and gamma sterilized.

The viscosity of the gels was measured at 22□ by using a Vibro Viscometer (SV100, A & D Company, Ltd., Japan). The measurements were recorded until the viscosity values were fixed.

The biological effect of the produced injectable bone grafts was tested in accordance with ISO 10993-5. The biological effect of the test sample was evaluated using the sample extract in L929 mouse fibroblast cell culture. Additives of Eagle's Minimum Essential Medium (MEM) (Sigma-Aldrich), and 10% Fetal Bovine Serum (Sigma-Aldrich St. Louis, MO) and 1% Penicillin-Streptomycin (Gibco, Grand Island, US) were used as the extract solvent. After adding the positive and negative control groups, sample extract on the monolayer cell layer was incubated for 24 hours at 37□ and 5% CO_2. Latex with toxic effect was used as positive control and High-Density Polyethylene (HDPE) without toxic effect was used as a negative control. Then, MEMs containing 10% MTT (Vybrant

® MTT Cell Proliferation Assay Kit-Invitrogen) were added to the wells and incubated for 3 hours and at the end of the incubation, the medium was replaced with DMSO and left in the shaker for 5 minutes to distribute homogeneously. The biological effect was evaluated on a spectrophotometer (Synergy ™ HTX, BioTek, Winooski, VT, USA) at 570 nm wavelength. Cytotoxic effect of the product on cells was evaluated by calculating viability.

In vitro micronucleus analysis for genotoxicity analysis was performed with peripheral blood lymphocytes from 3 healthy, young, non-smoker donors. Blood cells were cultivated with 20 µg/mL of PHA-additive cell culture medium at 37 °C and 5% CO_2. After 24 h the cell culture medium was removed and the cells were incubated with 5 mL of sample extracts and negative-positive controls for 48 h. Mitomycin C was used as a positive control and fresh medium was used as Negative Control. At the 44th hour of the culture period, 6 µg/mL of cytochalasin B (Sigma-Aldrich St. Louis, MO) was added to the culture tubes. After 72 hours, cells were fixed to microscope slides and Giemsa staining was performed. The number of micronuclei was analyzed microscopically in 1000 cells/slide.

Injectable prototypes were produced with an inorganic non-metallic material as a radiopaque agent at a concentration of 0%, 0,05% and 0,1% by mass, which do not leave at risk the graft materials at any biocompatibility point, respectively in order to enhance antibacterial efficacy and determine a radiopaque characteristic of the developed bone grafts. The produced prototypes were performed lyophilizing for 24 hours at -55□ using a freeze-drying machine after then characterized by µ-CT analysis as is seen in Figure 2.

The antimicrobial activity of a substrate-bound, non-leaching antimicrobial agent is dependent upon direct contact of bacteria with the active chemical agent. This test determines the antimicrobial activity of a treated specimen by shaking samples of surface-bound 1 gram of materials in a concentrated bacterial suspension for a one-hour contact time. The suspension is serially diluted both before and after contact and cultured for 24 hours. The number of viable organisms from the suspension is determined and the percent reduction (or log10 reduction) is calculated by comparing retrievals from 10^{-4} dilution samples.

III. Results And Discussion

The viscosity value of the β-TCP-Hydrogel-based hand-moldable paste-like grafts prior to gamma sterilization was 83.9 ± 2.4 mPa s. Post-sterilization values were recorded as 81.4 mPa s (± 1.9). In accordance with these values, it was concluded that gamma sterilization did not cause any change in the structure of the hydrogel.

In the cytotoxicity test results shown in Figure 1, when the Positive Control is taken as 100, the viability rate of the negative control expected to be toxic on the cell appears to be 5%, while the injectable grafts produced are around 98%. It was determined that the produced graft materials had no cytotoxic effect and did not cause cell death.

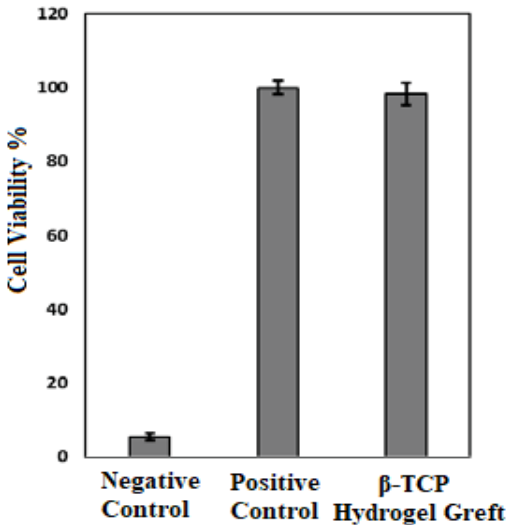


Figure 1. Cytotoxicity Test Results

When the results were evaluated in Figure 2, it was established that prepared prototypes at 0,1% inorganic non-metallic material concentration showed increased radiation on µ-CT imaging. When the relevant images were compared among them, it was found that inorganic non-metallic material increased the radiopacity clearly.

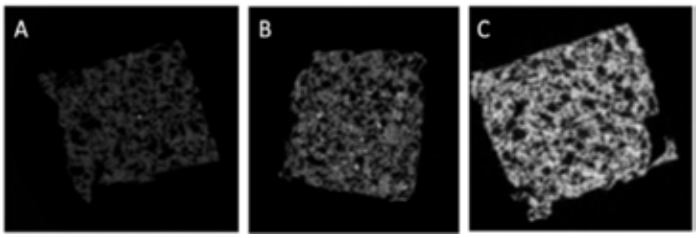


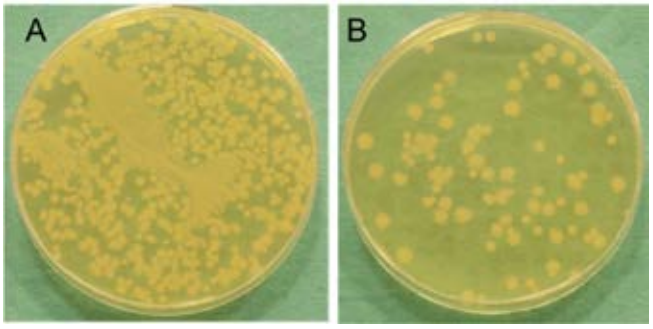
Figure 2. Inorganic non-metallic material as a radiopaque agent at a concentration of 0%, 0,05% and 0,1% by mass

Tracking bone graft substitutes after implantation into the defect area is crucial especially on spinal and dental operations where controlled volume of bone regeneration is required. It was demonstrated that inorganic non-metallic material additive bone graft substitutes showed radiopaque characteristics without any reported risks caused by inorganic non-metallic material [11, 12].

Table 1. Genotoxicity Test Results

Details	Micronucleus Percentage
Injectable Bone Graft	4.2 %
Negative Control	2.3 %
Positive Control	36 %

In genotoxicity analysis, micronucleus test was performed to determine the damage of genetic material in the cell. In the event of genetic damage, the complete nuclear division cannot take place and the presence of micronuclei near the cell nucleus is also detected. The results shown in Table 1 show that the micronucleus percentage of the produced grafts is in the desired range (>5). As a result, it was found that the material had no genotoxic effect.



Control (A)	Putty Plus (B)
Average Colony Forming Unit Number After Contact	Average Colony Forming Unit Number After Contact
$2.97x10^2$ CFU/ml (A)	$2.31x10^1$ CFU/ml (B)

Figure 3. Antibacterial efficacy of Powerbone Putty Plus.

Bacterial adhesion and colonization are considered to play key roles on formation of most of the infections in orthopedic, spinal, and dental surgeries. Presence of inorganic non-metallic material in graft enhance antibacterial activity by inhibiting adhesion mechanisms of bacteria on graft materials which prevents colonization and biofilm production of pathogen bacteria. It was previously indicated that addition of inorganic non-metallic material significantly decreased the bacterial adhesion and colony formation [13-15]. The antibacterial activity of inorganic non-metallic material additive β-TCP bone graft substitutes were assessed according to ASTM E2149-13a standard. It was reported that addition of inorganic non-metallic material to β-TCP bone graft substitutes manufactured by Bonegraft Biomaterials Co. gained antibacterial characteristics.

IV. Conclusion

Powerbone Putty Plus has pre-mixed structure which provide a comfortable application for the clinician. Putty has adequate injectability and can adapt to the defect shape. Antibacterial efficacy of Powerbone Putty Plus potentially prevents development of infection in the grafting area. Therefore, we predict that Powerbone Putty Plus will be widely preferred in dental, orthopedic, and spinal surgeries.

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