COMPOSITE SCAFFOLDS FOR BONE REGENERATION MADE OF COLLAGEN/HYDROXYAPATITE/EUCALYPTUS ESSENTIAL OIL

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Bone regeneration is a serious problem nowadays because of the increased number of people suffering from infections, arthritis and bond loss. The aim of the present study was to develop and characterize collagen – hydroxyapatite – essential oil matrices for bone regeneration. In our study we wish to develop biomaterials which mimic bone composition and prevent allergic or toxic effects. The matrices obtained by freeze-drying were characterized by FT-IR analysis, water uptake capacity and microbiological analysis. The results obtained from analyses confirmed that collagen–hydroxyapatite-essential oils matrices exhibit proper characteristics for bone regeneration.

Keywords: collagen hydroxyapatite, essential oils, bone regeneration.

INTRODUCTION

Bone grafting is ideal for bone defects reconstruction. Due to the fact that allografts can transmit certain diseases or may have problems related to sterilization, it has been chosen to be made by synthetic and natural materials. Increasing the osteointegration and antibacterial properties of these grafts and replacing them with the host bone is considerably important. Literature studies have also shown that various substituents, such as bone morphogenetic proteins, parathyroid hormones, or platelet-rich plasma, have been added to allografts or synthetic materials. Clinical applications of these substituents have shown good bone formation, but their subsequent application has been limited due to the high cost or potential adverse reactions (Habibovic, 2011). The alternative came from combining synthetic and natural materials like hydroxyapatite (HA) and collagen (COLL) with essential oils like eucalyptus essential oil (EEO). Hydroxyapatite, similar to chemical composition and morphology of bone apatite, can provide a good adhesion to the local tissue due to its surface and has been shown that it has the ability to enhance osteoblast proliferation and differentiation. Collagen is widely found in bone and skin (Type I), cartilage (Type II) and blood vessel (Type III), increasingly being used as a composition in artificial bone. As a natural polymer, it has excellent biocompatibility, which allow cell attachment and growth, and biodegradability in order to be easily absorbed by the body. Collagen-hydroxyapatite (COL-HA) composite imitates natural bone tissue (He *et al.*, 2017).

Essential oils from plant extracts are natural antimicrobial agents; incorporation of essential oil into synthetic materials may not only enhance the materials antimicrobial properties but also reduce water- solubility, vapor-permeability and slow lipid oxidation

of the product (Hafsa *et al.*, 2016; Stegarus and Lengyel, 2017). One example is eucalyptus essential oils which present several properties such as antimicrobial, antihyperglycemic, antioxidant and anthelmintic properties (Herculano *et al.*, 2014).

The aim of this study was to prepare and characterize some collagen – hydroxyapatite-eucalyptus essential oil scaffolds in order to facilitate bone reconstitution.

MATERIALS AND METHODS

Materials

The type I fibrillar collagen gel having a concentration of 2.54% (w/v) and acid pH, was extracted from calf hide using the protocol that has been previously described (Albu, 2011). Hydroxyapatite was purchase from Sigma Aldrich (Germany). Eucalyptus (*Eucalyptus globulus*) essential oil was obtained in Department of Medicinal and Aromatic Plants, Faculty of Agriculture, Mustafa Kemal University, Hatay, Turkey from wild plants from the province of Hatay in the period of their blooming, by hydrodistillation using a Neo-Clevenger apparatus. Glutaraldehyde (GA) was purchase from Merck (Germany). All the chemicals were of analytical grade and the water was distilled.

Preparation of Collagen Hydrogels/Composite Scaffolds

The concentration of each collagen gel was adjusted at 1,2% (w/v) and 7.4 pH using a solution of sodium hydroxide with 1M concentration (the pH of the physiological medium in human body). Hydroxyapatite and eucalyptus essential oil were added to collagen gel (w/v), in different proportions, and then the composite gels were cross-linked with 0.025% glutaraldehyde (GA) solution (reported to collagen dry substance) as Table 1 presents.

Table 1. Composition of collagen hydrogels

	COLL%	HA%	EEO%	GA%
1.C		-	-	
2.CE	1.2	-	0.5	0.025
3.CH		50	-	
4. CHE		50	0.5	

The collagen gels, in order to be analysed, were freeze-dried using Delta 2-24 LSC (Martin Christ, Germany) lyophilizer, using a 48 hours lyophilization programme and composite scaffolds were obtained.

Water Up-take Capacity

Water Up-take

The obtained collagen samples were tested by water up-take. They were firstly immersed in water at 37°C then withdrawn and weighed at fixed time intervals. The equation used (eq. 1) for water absorption determination was:

$$\% \text{Water up-take} = (W_t - W_d) / W_d \text{ (g/g)} \quad (1)$$

where W_t is the weight of the swollen samples at immersion time t , and W_d denotes the weight of the dry samples. All the samples were studied in triplicate.

FTIR-ATR Analysis

FT-IR spectral measurements were recorded by spectrophotometer Jasco FT/IR-4200. All the spectra were recorded at the following parameters: spectral range 4000-600 cm^{-1} , resolution 4 cm^{-1} with 30 acquisitions per each sample.

Microbiological Analysis

All the samples have been tested for antimicrobial activity against *Escherichia coli*, according to SR EN ISO 20645/2005 Control of antibacterial activity / Diffusion test on the gelled plate.

A volume of gelose (Tryptic Soya Broth) for the lower layer without bacteria was prepared. 10 ml of gelose was introduced into sterilized Petri dish and allowed the gelose to solidify. A volume of gelose for the upper layer was prepared and cooled to 45°C in a water bath. 150 ml of gelose was inoculated with 1 ml of bacterial solution of *Escherichia coli* ($1.5 \times 10^8 \text{ µg/ml}$). The container is vigorously stirred for the uniform distribution of the bacteria. 5 ml of solution was introduced in each Petri dish and allowed the gelose to solidify. The samples were placed on the surface of the nutrient medium and then incubated at 37°C between 18h and 24h.

RESULTS AND DISCUSSION

Figure 1 presents the water up-take during 24 hours for the studied samples:

Figure 1. Water absorption during 24 hours for collagen sponges

The crosslinked collagen (C) absorbed the higher amount of water than the others. The samples with crosslinked collagen, hydroxyapatite and eucalyptus essential oil (CHE) absorbed lower amount of water due to their more compact structure.

In figure 2 are presented the FT-IR spectra for collagen samples:

Figure 2. The FT-IR spectra of collagen samples: a-C sample; b-CE sample; c-CH sample; d- CHE sample

The spectrum of collagen sample (C) (Fig. 2 a) exhibited typical amide bands specific for collagen: 3295 cm^{-1} and 2091 cm^{-1} for amide A ($-\text{NH}-$ stretching) and B ($-\text{CH}_2-$) respectively, 1629 cm^{-1} was attributed to amide I ($\text{C}=\text{O}$ stretching), 1552 cm^{-1} to amide II ($\text{N}-\text{H}$ deformation) and 1444 cm^{-1} to amide III ($\text{N}-\text{H}$ deformation) (Albu, 2011).

In the case of sample with essential oil (CE) (Fig.2 b), in the FT-IR spectra it can be observe a displacement of spectral bands characteristic of the amide groups (I, II and III) because of essential oil, also were observed the bands of 1,8-cineole (a major component in eucalyptus essential oil composition) attributed to $\text{C}-\text{O}-\text{C}$ symmetrical (1116 cm^{-1}) and asymmetrical (1239 cm^{-1}) stretching vibrations as well as to CH_3 symmetrical deformation at 1386 cm^{-1} (Sirvaityte *et al.*, 2011).

In the FT-IR spectrum of the samples with hydroxyapatite (CH and CHE) (Fig. 2 c and d), it can be observed the spectral band characteristic for PO_4 groups of hydroxyapatite: 1035 cm^{-1} (CH sample) and 1039 cm^{-1} (CHE sample) (Chang, 2002).

The results of microbiological analysis are shown in Figure 3:

Figure 3. Microbiological results for collagen samples: 1-C sample; 2-CE sample; 3-CH sample; 4-CHE sample

The evaluation was based on the absence or presence of bacterial multiplication in the area of contact between the gelose and the sample and on the occurrence of a possible inhibition area around the samples, the results being shown in the Table 2:

Table 2. Effect of antibacterial treatment on collagen samples

Sample*	Inhibition area (mm)	Total number of aerobic germs (UFC/ cm ²)	Evaluation
1	0	7 UFC/ cm ²	Insufficient effect
2	0	Absent	Satisfactory effect
3	0	Absent	Satisfactory effect
4	0	Absent	Satisfactory effect

*1-C sample; 2-CE sample; 3-CH sample; 4-CHE sample

The result is considered to have a “satisfactory effect” if no bacterial growth is observed. The samples tested do not allow the development of aerobic germs for the tested bacteria, which prove the eucalyptus essential oil efficiency.

CONCLUSIONS

Type I collagen with hydroxyapatite and essential oil were used in order to obtain composite scaffolds for bone regeneration. The combination between components were highlighted by FT-IR spectra changes when hydroxyapatite and essential oil were added. The samples with crosslinked collagen, hydroxyapatite and eucalyptus essential oil absorbed lower amount of water due to their more compact structure and hydrophobic character of essential oil. The samples were tested against *E. coli* and the

results show that all the samples with eucalyptus essential oil present a satisfactory antibacterial activity.

The results showed that collagen-hydroxyapatite-essential oil matrices are potentially novel candidates as scaffolds for bone tissue engineering applications, but more tests will be performed on the samples.

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