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THE EFFECT OF BMP-4 LOADED IN 3D COLLAGEN-HYALURONIC ACID SCAFFOLDS ON BIOCOMPATIBILITY ASSESSED WITH MG 63 OSTEOBLAST-LIKE CELLS

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Numerous studies recommend collagen to be employed in constructing scaffolds for bone tissue engineering. The aim of this study was to test whether addition of hyaluronic acid (HA) and of human bone morphogenetic protein 4 (BMP-4) to collagen scaffolds improves its physical-chemical and morphological properties and biocompatibility with human MG 63 osteoblast-like cells. Type I fibrillar collagen, HA and BMP-4 were used to prepare 3D porous scaffolds by freeze-drying method. The structure of these natural polymers was stabilized employing 0.25% glutaraldehyde, a method that warrants that all the scaffolds components keep their structural integrity and biological properties. The physical-chemical properties were assessed using infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and thermogravimetry (TG/DTG); the morphological properties were evaluated by scanning electron microscopy (SEM) and water uptake. The biological properties were determined (in vitro) by collagenase degradation and by assessment of the biocompatibility with MG 63 osteoblast-like cells. The results demonstrated that the collagen scaffolds with HA and BMP-4 had good physical-chemical and morphological properties, promoted adhesion, maintained viability and sustained the migration of osteoblast-like cells into scaffolds.

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1. Introduction

Tissue engineering has emerged as a new solution for bone repair. A strategy in the construction of scaffolds for regenerative medicine is to fabricate an analog of extracellular matrix using collagen, proteins from extracellular matrix, and glycosaminoglycans (GAG) [1,2]. The biocompatible and biodegradable biomaterials are especially favored as scaffolds for the delivery of cultured cells or for three dimensional (3D) tissue reconstruction [3,4]. These scaffolds should provide structural support for cells and the new tissue being formed, function as delivery vehicles for growth factors, antibiotics, and chemotherapeutic agents, and act as a temporary extracellular matrix inducing the natural processes of tissue regeneration and development [5].

Natural polymers like collagen, gelatin and silk fibroin have been extensively used in tissue engineering. Collagen is considered an attractive biomaterial available for use as artificial extracellular matrix (ECM) in tissue engineering applications because of its excellent

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biocompatibility, low antigenicity, high biodegradability and good mechanical, haemostatic, and cell-binding properties [6-8]. Collagen is the major protein component of mature bone, and is the precursor matrix for direct bone repair. Hyaluronic acid, also referred to as hyaluronan, a high molecular weight GAG polymer composed of repeating units of D-glucuronic acid and n-acetyl-D-glucosamine, is a ubiquitous biopolymer in the mammalian body. HA is found in highest concentrations in cartilage tissue [9], vitreous humor [10], synovial fluid of joints [11,12] and umbilical cord [13], and is responsible for maintaining tissue homeostasis [14,15]. Of relevance to bone, HA has been found in high concentration in the early fracture callus [16], in lacunae surrounding hypertrophic chondrocytes in the growth plate [17], and in the cytoplasm of osteoprogenitor cells [17]. In addition, the carboxyl functional groups present in HA could be used for the modification of substrate with bioactive molecules.

The combination of osteoinductive growth factors with scaffolds could provides an appropriate osteoinductive environment for bone cells. Osteoinductive growth factors, such as bone morphogenetic protein (BMP), transforming growth factor b (TGF-□), and basic fibroblast growth factor (bFGF) have been reported to induce bone regeneration [18]. BMPs are members of TGF-□ superfamily, and have been implicated in the development of various tissues, most notably bone [19-21]; it is among the most promising factors for improvement of bone healing. In the context of bone regeneration, BMPs act as chemotactic agents by initiating the recruitment of osteoprogenitor and mesenchymal stem cells towards bone defect sites [19]. For these reason osteogenic BMPs, including BMP-2, BMP-4, and BMP-7, have been used to induce bone formation and to repair bone defects in various animal models [22–25]. A variety of BMPs have been isolated and cloned, including BMP-4. The combination of BMP-4 with ECM components involves two different functional concepts: the external pre-integration of osteoinductive BMP-4 and the possible interaction of ECM components with endogenous growth factors.

In previous studies, HA-collagen scaffolds were used for adipose tissue engineering [26], HA-BMP-2 scaffolds for bone regeneration [27] and a scaffold based on collagen HA was developed for brain tissue engineering [28]. Other composites based on collagen and HA were prepared and physical-chemical characterized [29-31].

In this study, we developed new scaffolds based on collagen, BMP-4 and hyaluronic acid using freeze-drying method. Evaluation of the physical-chemical and morphological characteristics as well as the *in vitro* biocompatibility with human MG-63 osteosarcoma cells demonstrate that the collagen-HA-BMP-4 scaffolds are suitable for bone tissue engineering purposes.

2. Materials and Methods

2.1. Materials

Type I collagen gel (Coll) at a concentration of 1.83% was obtained from calf hide by acid and alkaline treatments as previously described [32, 33]. Hyaluronic acid potassium salt from human umbilical cord and human BMP-4 were purchased from Sigma (Germany), Collagenase Type I, *C. histolyticum* was from US Biological (USA) and glutaraldehyde (GA) from Merck (Germany). Sodium hydroxide and phosphate buffer solution (PBS), pH 7.4 were of analytical grade.

Chemicals used for cell cultures were supplied by Sigma (Germany) and those used for electron microscopy were from Polysciences (USA), except sodium sulphate and lead citrate, which were purchased from Merck (Germany), and tannic acid from Mallinckrodt. Tissue culture flasks were from Nunc (Germany). The human MG-63 osteosarcoma cell line was obtained from the American Type Cell Culture Collection (ATCC).

2.2. Preparation of collagen scaffolds

Collagen gel (1.2%, 7.4 pH) – reference gel (Coll) - was the basic material for scaffold construction. HA was added to collagen gel at a ratio of 10:1 (Coll:HA) (w/w). An amount of 40 μg/mL BMP-4 was embedded in the collagen gel and Coll:BMP-4 = 50:1 (w/w) was obtained. The

same amounts of HA and BMP-4 were introduced in the reference gel in order to obtain Coll:HA:BMP-4 = 50:5:1 (w/w/w). The four gels obtained were cross-linked with 0.25% glutaraldehyde (GA) (reported to the weight of dry collagen), then cast in polystyrene dishes (3 cm diameter and 1 cm height) and kept at 4^0C for 24 hours. After the cross-linking process they were freeze-dried (48 hours) in order to obtain porous scaffolds, as follows: cooling to -40^0C (4 h), keeping up for 5 h, then freeze-dried at -40^0C and 0.12 mbar for 10 hours, then heating to $+20^0$C at a rate of 3^0C/hour (20 h) at 0.12 mbar, then heating (6 h) to 30^0C at a rate of 2^0C/hour and finally freeze-dried at same temperature at 0.01 mbar for 3 hours, using the Christ Model Delta 2–24 LSC freeze-dryer (Germany).

2.3. FTIR-ATR analysis

FTIR-ATR spectra were registered on a VERTEX 70 BRUCKER FT-IR spectrometer equipped with an attenuated total reflectance (ATR) accessory. All FTIR measurements were performed in the ATR-FTIR cell on Ge crystal, at room temperature. The FTIR spectra were recorded using 32 scans, with a resolution of 4 cm^{-1} in 600-4000 cm^{-1} wave number region.

2.4. Thermal analysis

The DSC curves of collagen matrices were recorded on a Netzch equipment, under nitrogen atmosphere, within the temperature range 25-250^{o}C, using a heating rate of 10°C/min. The TGA curves were registered on a Q500 TA instrument at 10°C/min heating rate, from 25°C to 150°C, under nitrogen atmosphere.

2.5. Scanning electron microscopy characterization

Morphological information including the internal structure was assessed by scanning electron microscopy (SEM) analysis of the gold-coated collagen scaffolds. Osteosarcoma cells were examined by SEM after 5 days in culture. The fixation with 2.5% v/v glutaraldehyde in 0.1% w/v cacodilate buffer (pH 7.4) for 1 hour was followed by rinses in 0.1% w/v phosphate-buffered solution containing 0.1 M sucrose, (3 × 5 minutes) and post-fixation in 1% v/v osmium tetroxide in 0.1% w/v phosphate buffer for 1 hour. The cell cultures were dehydrated in graded ethanol series, critical point dried, and gold-sputtered prior to observation. The analysis was performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG) with a resolution of 1,2 nm.

2.6. Water uptake

In order to determine the water absorption the scaffolds were first immersed in PBS at 37^0C. At scheduled time intervals, the samples were withdrawn, wiped (to remove the surface water) and weighed. The water absorption was calculated using the following equation: $water\ absorption = (W_t - W_d)/W_d$ (1), where W_t denotes the weight of the swollen samples at immersion time t, and W_d denotes the weight of the dry samples.

2.7. In vitro degradation by collagenase

Enzymatic degradation of collagen scaffolds was investigated by monitoring the mass loss of scaffolds as function of exposure time to a collagenase solution. Pieces of collagen scaffolds (1 x 1 x 0.4 cm) were accurately weighed (wet weight without excess of water), placed in a solution of PBS and collagenase (1μg/mL) at pH 7.4, and incubated at 37^0C. At regular intervals, the swollen scaffolds were removed from the collagenase solution, wiped and weight. The percent of degradation of matrices was determined by the following formula: $\%\ weight\ loss = (W_i - W_t)/W_i * 100$ (2), where W_i is the initial weight and W_t is the weight after time t. Each biodegradation experiment was repeated 3 times and the average values were used as the percentage of biodegradation.

2.8. Colonizing cell capacity

The colonizing cell capacity was tested using human osteosarcoma cell line (MG63) and the various types of prepared collagen scaffolds. The scaffolds were sterilized by electron beam irradiation with 25 kGy. The cells were grown in DMEM - 1‰ glucose medium supplemented with 10% fetal bovine serum, seeded on scaffolds at a density of 50.000 cells/mL. The scaffolds with cultured cells were maintained at 37°C in incubators with 5% CO_2 in air and high relative humidity (> 95%). All tests were done at five days after seeding. Scaffolds colonization was monitored by fluorescence microscopy (Hoechst staining), the cell viability by MTT assay and the morphology by scanning electron microscopy.

2.9. Scaffolds cell colonization

The cells were cultured on collagen scaffolds disks (5 mm in diameter and 2 mm thick) for five days, washed in PBS, fixed in 2% paraformaldehyde, and then cryoprotected. After washing in phosphate buffer (PB: 0,2 M Na_2HPO_4, and 0,2 M NaH_2PO_4) pH 7.2, the scaffolds were kept in a solution of PB (0.1 M + 5% sucrose) overnight at 4°C and then immersed in PB containing 5%, 10%, 20% and 50% glycerin at 4°C for 15 minutes, 1 hour, 10 hours. Then, the scaffolds were frozen in liquid nitrogen and sectioned with a Leica CM 1800 cryotome. Sections thicknesses were 4-6 μm. The cryosections were washed with PBS for 15 minutes, stained with Hoechst 33258 (a specific nuclear DNA staining) for 15 minutes, washed in distilled water, mounted in glycerol and examined with a Nikon microscope equipped with epi-fluorescence (triple band filter API/FITC/Rhodamine)

2.10. MTT assay

For MTT assay, all the scaffolds were transferred to a fresh plate, 200 μL of MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil tetrazolium bromide) were added to each well and incubated for 3 hours at 37°C. Cell cultures grown on scaffolds were incubated with 0.5 mg/ml of MTT during the last 4 h of the culture periods tested (24 and 72 h); then the medium was removed, formazan salts were dissolved with 200 μL of dimethylsulphoxide and the absorbance was measured at 570 nm in a 96-well microplate TECAN spectrophotometer reader. For each experimental condition, a minimum of three probes were used and the results were expressed as percentage viability.

3. Results and discussion

3.1. Preparation of collagen scaffolds

Bone regeneration could be promoted by combining osteoconductive porous devices with osteoconductive BMPs. For this reason, we investigated in this study the effect of BMP-4 added to the natural polymers, such as collagen and hyaluronic acid.. Although BMP-4 is considered an important factor of osteoinduction in bone tissue engineering, and has a crucial role in bone formation, it undergoes rapid degradation *in vitro*. In order to preserve the effects of BMP-4, it has to be freeze-dried.

In our experiments, porous 3D collagen scaffolds were prepared using a freeze-drying method that exhibits the advantage of maintaining the BMP-4, collagen and HA integrity with good biological effects and producing highly porous scaffolds with enough space to allow cell infiltration, growth and proliferation into the scaffolds.

The disadvantages of employing collagen or collagen–HA composite as a scaffold for tissue engineering are their rapid biodegradation and relatively poor biomechanical strength. Hence, to stabilize the structure of these natural polymers various chemical and/or physical cross-

links are frequently introduced [34]. Thus, we employed glutaraldehyde at the concentration of 0.25% based on our previous studies that demonstrated that at this concentration GA was non-cytotoxic and had no side effects on attachment, migration and proliferation of cells on collagen biomaterials [35].

The prepared scaffolds were characterized by FT-IR spectroscopy, thermal analysis as well as for their morphology, biodegradability, water absorption and biocompatibility with osteoblast-like cells.

3.2. FTIR-ATR analysis

FT-IR is a useful method to monitor changes in secondary structure of a protein. The characteristic peaks identified in reference collagen scaffold (Coll) were: 3317 cm^{-1} (amide A), 2924 cm^{-1} (amide B), 1650 cm^{-1} (amide I), 1554 cm^{-1} (amide II) and 1240 cm^{-1} (amide III), results which are in agreement with the published data [36, 37].

The amide A band at 3317 cm^{-1} in Coll and Coll:HA samples was shifted to lower frequency such as 3315 cm^{1} for Coll:BMP-4 and to 3313 cm^{-1} for Coll:HA:BMP-4. These data indicated that addition of BMP-4 to collagen results in the formation of hydrogen bonds between the main-chain peptide groups involved in the interactions.

As shown in Figure 1 there were no significant differences between Amide I, II and III for all the studied scaffolds.

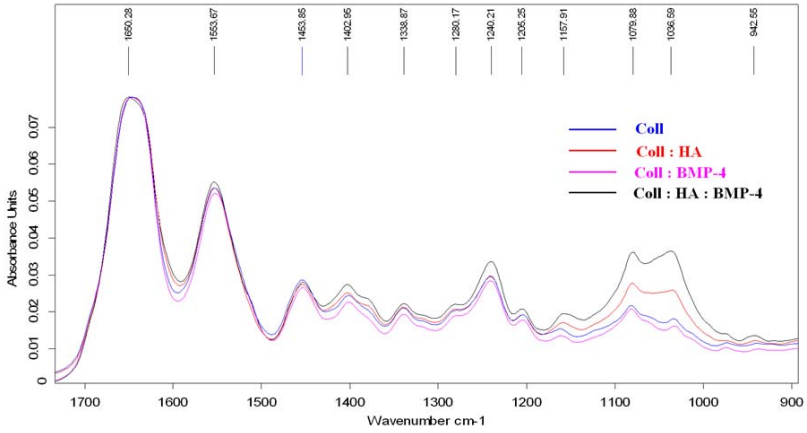

Fig. 1. FT-IR spectra of collagen scaffolds

In addition, for all scaffolds, the ratio of absorptions between 1240 cm^{-1} (Amide III) and 1453 cm^{-1} is higher than 1, demonstrating the integrity of the triple helix configurations [38]. Maintaining the collagen triple helical structure facilitates the interaction of a wide range of receptors in the regenerative cells.

The interaction of collagen with HA is put to evidence in Figure 1 in the region 1000-1100 cm^{-1}. Carboxyl groups of HA react with amino groups and hydroxyl groups from giving amide and ester groups.

3.3. Thermal analysis

The characteristic triple helical structure of collagen molecule is rigid due to the strong hydrogen bonds existing between the hydroxyl groups of hydroxyproline and amine groups of glycine which lead to the formation of a fibrous crystalline zone embedded into an amorphous matrix [39].

Considering the collagen biphasic structure, two successive endothermic processes take place when temperature increases: the first (I) consists in collagen dehydration, which takes place within the temperature range of 25-125^{o}C and refers to the amorphous zone, and the second (II) in crystalline (rigid) melting zone, occurring between ~ 210 and 250^{o}C, which is followed by the dried collagen thermal degradation or oxidation, [40] depending on the environment (inert or air) in which the heating process takes place.

Denaturation temperature measures the temperature of transition of collagen molecule conformation from triple helix to statistic coil [41].

Melting temperature (T_m) is a measure of the strength of bonds between the collagen triple helices, while melting enthalpy (ΔH) is a measure of the cross-linking degree (proportion of cross-linked collagen).

We have used DSC and TG/DTG measurements to determine the thermal stability of the collagen scaffolds. The parameters describing the thermal behavior of collagen scaffolds are shown in Table 1.

Table 1 DSC and TG/DTG analysis of collagen scaffolds

Collagen scaffold	DSC in N_2			TG/DTG in N_2	
	T_d (^{0}C)	T_m (^{0}C)	ΔH (J/g)	Δm (%)	T_{max} (^{0}C)
Coll	75.8	214.4	6.7	79.14	313.8
Coll:HA	82.7	214.4	6.5	70.64	314.7
Coll:BMP-4	75.7	214.8	6.7	62.66	315.3
Coll:HA:BMP-4	75.2	217.7	8.6	61.07	315.8

T_d, thermal denaturation temperature; T_m, melting temperature; ΔH, melting enthalpy, Δm, total mass loss at 700°C; T_{max}, maximum temperature of the thermo-oxidation process as measured from DTG curve. *Note:* each parameter is the mean of two tests.

It was reported that the denaturation temperature strongly depends on the water content in collagen and its degree of cross-linking between the chains [42]. The values of T_d indicated that the cross-linking process was enhanced in the presence of HA and decreased slightly when BMP-4 was added. These could be due to the hydrogen-bound water produced by hydroxyl groups from hyaluronic acid. Melting temperatures exhibited very close values except for the samples containing both HA and BMP-4, which had a value with about 3^{0}C higher. Also the ΔH for this sample was about 2 J/g higher than for the other scaffolds, which demonstrated a stronger crosslinking network.

The significant peaks on the DTG curves were between 313.8^{o}C and 315.8^{0}C. At 700^{o}C the total mass loss was the highest for Coll sample (79.14%) while the scaffold including both HA and BMP-4 showed the lowest value of the mass loss.

3.4. Scanning electron microscopy characterization

The collagen scaffold morphological structure is important because it affects the hydrophilicity, nutrients and cells diffusion through network, degradation properties and interaction with cells. Figure 2 shows SEM images of collagen scaffolds with and without HA and /or BMP-4.

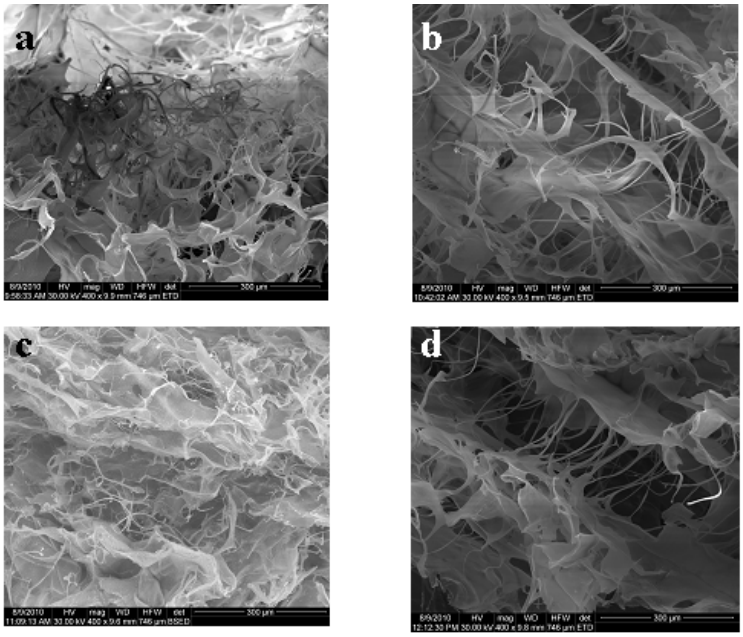

Fig. 2. SEM images for: a) Coll; b) Coll:HA
c) Coll:BMP-4; d) Coll:HA:BMP-4 scaffolds

The pore sizes estimated from SEM images by the measurement of pore zones with arbitrary shapes were in the range of 50÷150 μm for Coll, 100÷225 μm for Coll:HA, 40÷80 μm for Coll:BMP-4 and 100÷150 μm for Coll:HA:BMP-4 scaffolds. All the scaffolds revealed interconnecting pore morphology. Comparing the Coll and Coll:HA scaffolds (Figure 2 a, b), it was found that HA produces higher pores with an ordered structure. The scaffolds containing collagen and BMP-4 (Figure 2c) exhibited sheets and highly interconnected porous structures. Addition of BMP-4 to Coll:HA scaffolds (Figure 2d) induced the sheets formation in a more ordered structure and the sheets were interconnected by collagen fibrils.

3.5. Water uptake

The water uptake ability of scaffolds with spongious forms should be controlled mainly by means of two aspects: the hydrophilicity and the stability of porous structures in water [26, 43]. In our experiments, all the studied scaffolds absorbed water within 30 s and were saturated within 3 min. The results of water-uptake tests for collagen scaffolds after 5 min and 2, 4 and 8 hours are shown in Figure 3.

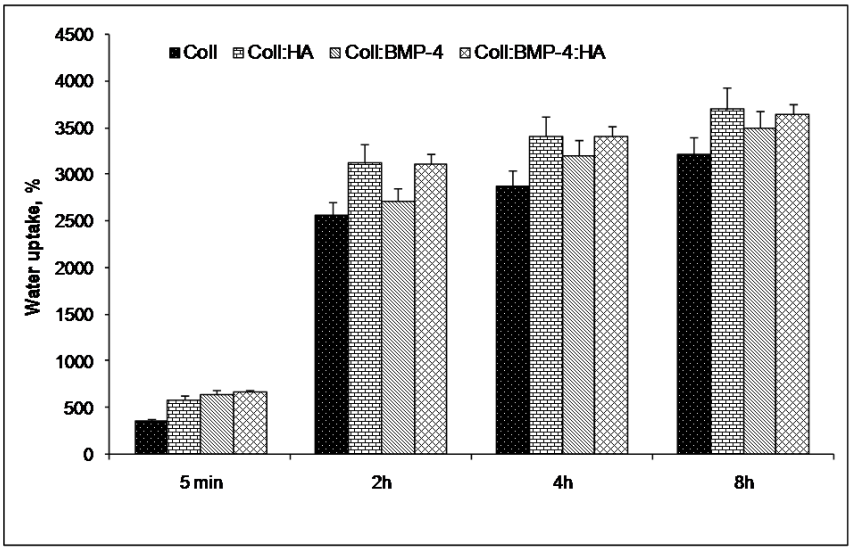

Fig. 3. Water uptake of scaffolds at different time intervals

The lowest water uptake was recorded for Coll scaffold. Comparing water absorption values for Coll and Coll:HA, it was found that the scaffold containing HA absorbed more water than the reference (Coll). We can safely assume that this is due to the carboxyl and hydroxyl groups of the HA molecules which may form hydrogen bonds with water molecules.

3.6. Degradation by collagenase

The control of degradation rate of the collagen scaffolds is an important feature, as the *in vivo* resorption influences tissue regeneration ability. Collagen can be fully digested only by collagenases that are distinctive enzymes since they are able to cleave collagen triple helical region under physiological conditions of pH and temperature. The cross-linking degree can also be indirectly evaluated by biodegradation, collagenase being able to cleave only the main chains of collagen and unable to cleave its intermolecular bonds.
Our experimental data are shown in Figure 4 as the dependence of percentage weight loss (%) on the degradation time (4, 8, 24, 48 and 72 hours) for all the studied scaffolds.

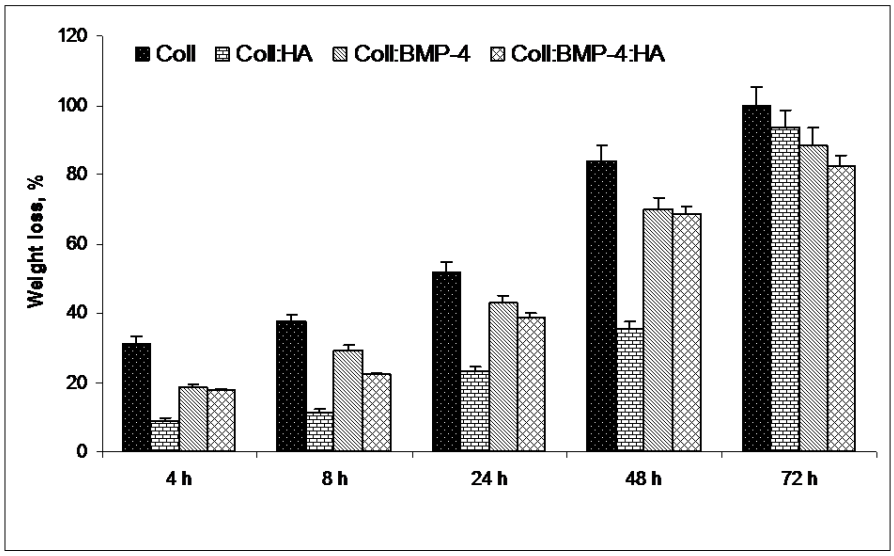

Fig. 4. In vitro enzymatic degradation of collagen scaffolds

The results revealed that the reference scaffold (Coll) was digested most rapidly. After 72 hours the Coll:HA scaffold was digested about 81%. The scaffolds containing BMP-4 in the presence or absence of HA were digested completely after 72 hours. The faster degradation of these scaffolds is most probably due to BMP-4 that is active in physiological conditions and performs itself the degradation during the release process. Comparing Coll:BMP-4 and Coll:HA:BMP-4, the degradation was slower for scaffolds containing HA than for the sample which includes BMP-4, only.

3.7. MG 63 osteoblast like-cell colonization on different collagen scaffolds

The Hoechst staining reveled that all scaffolds sustained three-dimensional cell growth at the surface and inside of the scaffolds and the cell migration within the structural pores (Figure 5).

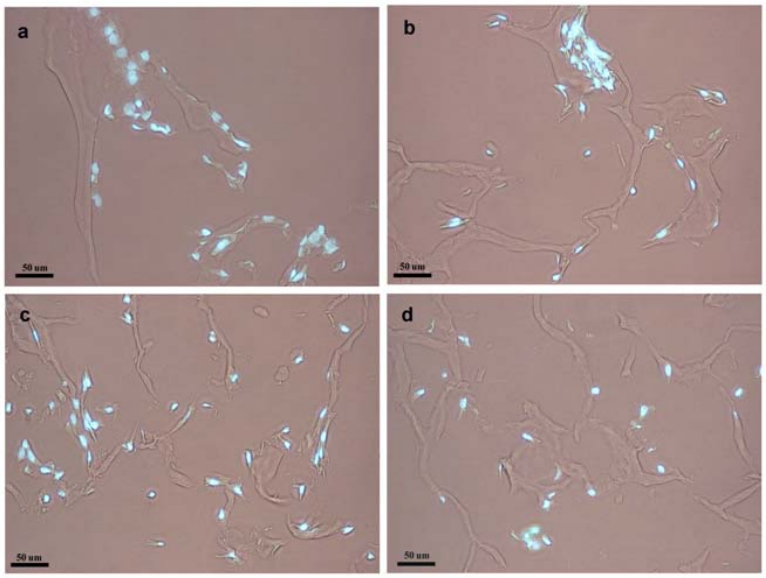

Fig. 5. The capacity of colonization of MG 63 osteoblast like cells grown for five days on: a – Coll, b – Coll:HA, c – Coll:BMP-4, d – Coll:BMP-4:HA

3.8. Viability assay

The cell viability was evaluated after five days after seeding on scaffolds. The percentage of viable cells in the analyzed scaffolds was determined having as reference the Coll scaffolds considered as 100% viability (Figure 6), and each samples were calculated as percent from the reference. We used scaffolds disks with 5 mm in diameter and 2 mm thick. As negative control, a collagen sponge with crystal violet (M) was employed.

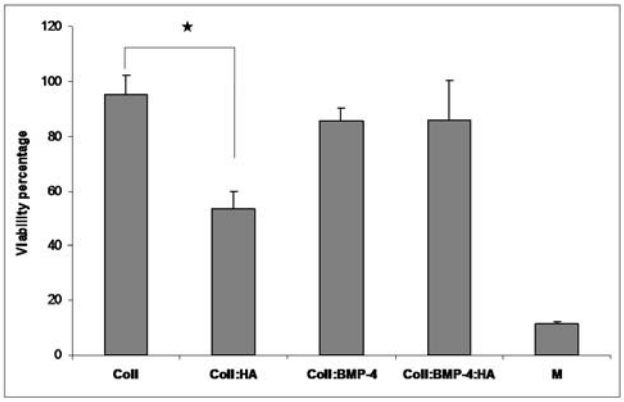

Fig. 6. Viability of MG63 osteoblast like-cells cultured on different collagen scaffolds

The values obtained by MTT assay were similar in reference scaffold (Coll) and Coll:BMP-4 and Coll:HA:BMP-4 scaffolds, suggesting an almost identical number of attached cells to these scaffolds (Figure 6). However, the reduction of cell viability in Coll:HA scaffolds was statistical significant (p= 0.029).

The *in vitro* decrease in cellular viability on the Coll-HA scaffolds may be due to the anti-adhesive effects of HA [43]. The cells in the scaffold required additional ECM proteins for adhesion and proliferation. MG 63 osteoblast-like cells cultured for 5 days on the Coll-HA scaffolds had ~ 60% viability (Figure 6). Addition of BMP-4 to the Coll-HA scaffolds, increased the cell viability to ~85%. These results may be partially explained by the fact that BMP-4 acts as a promoter of osteogenesis procces, possibly influencing the induction of bone healing [44]. In addition, it may be due to the known functions of BMP-4 to act as chemoattractant and recruit precursor cells into the damaged areas, and as a differentiation factor for osteoprogenitor cells to drive bone formation [45].

3.9. Cell morphology

A large number of osteoblast-like cells were found to attach, spread and proliferate on all scaffolds tested. The cells appeared in close contact to the scaffold surface, adopting an extended morphology with numerous filopodia and lamellipodia. The cells established close contact with the biomaterials, showing flattened morphology and numerous filopodia, which anchored the cells to the scaffold surface (Figure 7).

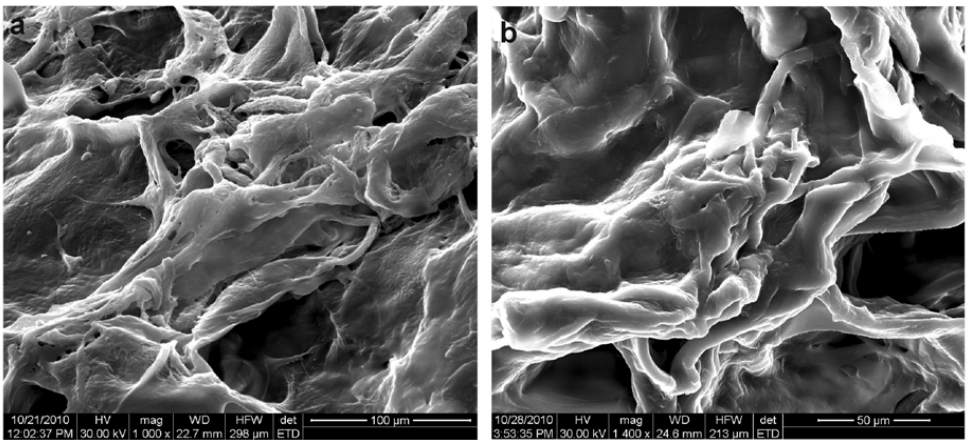

Fig. 7. Scanning electron microscopy revealing the morphology of MG-63 osteoblast-like cells cultured on (a) Coll:BMP-4 and (b) Coll: HA:BMP-4 after five days of cell seeding.

Differences were detected in degree of cell extension in Coll:HA:BMP-4 and Coll:BMP-4 scaffolds: the extent of cell coverage was higher for the latter (Figure 7a). On Coll:BMP-4 scaffolds, a large part of the surface was covered with well-spread stellate-shaped cells, bridging between the pores, with rough dorsal surfaces, a characteristic of active osteoblasts.

4. Conclusions

3D porous scaffolds based on collagen, collagen and HA, collagen and BMP-4 and collagen and HA and BMP-4 were prepared by freeze-drying method. The FT-IR analyses demonstrated the integrity of the triple helical structure of collagen for all studied scaffolds, a feature that may facilitate the interaction with the receptors of the regenerative cells. HA induced a higher thermal stability (as demonstrated by DSC and TG/DTG analyses) and a lower collagenase enzymatic degradation. The scaffolds exhibited a highly interconnected porous structure having

the pore sizes between 40 and 225 μm. The hydrophilicity was highest for scaffolds with HA which had the largest pore size.

The best cell viability was obtained on scaffolds that included BMP-4 in their preparation. SEM images revealed that these scaffolds had their surface covered with well-spread osteoblast like cells, connected between the pores, with a rough dorsal surface, characteristics for active osteoblasts.

Taken together the results show that in the construction of collagen scaffolds, HA improves their physical-chemical and morphological properties and BMP-4 enhances the cell biocompatibility. The combination of these components recommends these new scaffolds as a promising biomaterial for bone tissue engineering.

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