The osteoinductive potential of collagen based scaffolds for human mesenchymal stromal cells depends on scaffold composition and on the source of the cells; the need for personalized tests

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Summary
The biocompatibility and the osteoinductive potential of scaffolds made of collagen, hydroxyapatite and sericin was tested in vitro employing human mesenchymal stromal cells (hMSCs) collected from four different patients. Cell proliferation was estimated by measuring the DNA amount, alkaline phosphatase activity by quantifying the dephosphorylated p-nitrophenyl phosphate, cell viability by MTT assay and assessment of the transcription level of osteocalcin and osteonectin genes by PCR assay. The results showed that all tested scaffolds were able to provide, to different extent, an osteoinductive potential, adherence and proliferation conditions for the cells of each patient, of bone matrix formation, led to the necessity to search for a personalized use of bioma-terials. However, notable differences were recorded for all measured parameters between patients regarding the cells behavior on scaffolds of identical composition. Furthermore, the relation between sericin concentration and alkaline phosphatase activity or between sericin concentration and osteocalcin gene expression level was not identical for all patients. These findings indicate that both, the scaffold composition and the cells’ origin (the donor) are important determinants in selecting the right scaffold for a specific patient. These data advocate for the necessity to test the suitability between the scaffolds and the MSCs of a particular patient prior to their possible clinical applications.

Introduction
Biomaterials science for bone implants has become an area of great interest especially nowadays with the increase in the population life span that brought to the forefront of biomedicine, the specific aging problems, such as those related to the skeletal system. Apart from this, repair of congenital or acquired bone defects requires appropriate and suitable biomaterials. However, the many failures of bone implants due to bone destruction around the implants or because of weak connection between implant and the bone due to the inhibition or poor stimulation of bone matrix formation, led to the necessity to
search for a personalized use of biomaterials. It is considered that cellularized scaffolds may provide better results for tissue integration than acellular scaffolds (Bhumiratana & Vunjak-Novakovic, 2012). Collagen is a good candidate for scaffolds intended for the repair of bone defects. Unlike inorganic scaffolds, made of fragile ceramics or bioactive glasses, and in contrast with artificial polymers scaffolds, which can be rejected as foreign body (Evans et al., 2006), the collagen scaffolds are more suitable for bone repair, because collagen is the natural support that osteoblasts mineralizes. Another advantage of collagen is that it has low antigenicity and immunogenicity. Although collagen has low mechanical resistance, compressive modulus (which reflects the compression elastic properties) can be increased by the addition of hydroxyapatite (Kane & Roeder, 2012) that is already used in clinical practice because of its osteoinductive properties (Qin et al., 2014).

Sericin, a 70-200 kDa molecular weight protein is produced together with fibroin by silkworm Bombyx mori. Sericin can be successfully employed to make biodegradable materials because it exhibits oxidation resistance, inhibits lipid peroxidation, is UV resistant and has antibacterial properties (Zhang, 2002).

We hypothesized that a good match between the composition of the scaffold (the biomaterial) and the cells to be attached to it is needed for a successful bone implant. Thus, we conducted this study having two aims: (1) to evaluate the biocompatibility and osteoinductive potential of six types of scaffolds made of collagen with or without hydroxyapatite to which various concentrations of sericin were added and (2) to compare the responses and the outcome of hMSC from different patients when grown on these scaffolds. We provide evidence that the tested scaffolds are suitable for osteogenic differentiation of human mesenchymal stromal cells. However, both the cells source (the donor) and the scaffold composition modulate, to various extent but significantly, the proliferation, viability, alkaline phosphatase activity and transcription rate of osteocalcin and osteonectin of hMSCs grown on these scaffolds. These findings suggest the need for biomaterial evaluation for each particular patient before considering or receiving a bone implant.

Materials and methods

Sericin, Hoechst 33258, Histopaque-1077, p-nitrophenyl phosphate, dinitrophenol, DMSO (dimethyl sulfoxide) and MTT tetrazolium salt were purchased from Sigma-Aldrich. Hydroxyapatite nanopowder was purchased from Aldrich and glutaraldehyde was purchased from Merck. Salmon DNA and Trizol reagent were from Invitrogen. Moloney murine leukemia virus reverse transcriptase (M-MLV) and Taq-Pol enzyme were from Fermentas. Human mesenchymal stromal cells were obtained from the bone marrow (approx. 4 ml) harvested, under general narcosis, from the posterior spine of the iliac crest of four patients (noted P1-P4), after informed consent. The patients were between 59 and 65 years and were hospitalized with osteoarthritis of the hip joint (coxarthrosis). Three of the patients (P1, P2 and P4) were women and P3 was male.

Each bone marrow sample was centrifuged in a density gradient of Histopaque-1077 and the mononuclear cell fraction was harvested as described (Titorencu et al., 2007). The cells were placed in culture plates, in DMEM culture medium containing 4.5 % glucose and supplemented with 10% fetal bovine serum (FBS), sodium selenite 20 mg/l, ascorbic acid 30 mg/l, nonessential amino acids, HEPES 10 mM, antibiotics (penicillin 300
UI/ml, streptomycin 300 μg/ml and 150 μg/ml neomycin) and maintained in culture at 37°C, 5% CO2 concentration in air (v/v), relative humidity less than 95% and the pH 7.2 - 7.4. After 7-14 days (expansion time depends on the cells’ donor) the cells were cryopreserved in liquid nitrogen until use. For this, the cells were first exposed to trypsin concentrations of 1.25 ‰ in PBS (phosphate buffered saline). To avoid intracellular ice formation that can damage the cells, 10% (v/v) DMSO as cryoprotectant agent was used. The final cryopreservation medium also contained 20% fetal bovine serum. Before being placed in liquid nitrogen, the cells underwent a gradual decrease in temperature by ~1 degree/minute until attaining the final temperature of -80°C.

As biomaterial scaffolds, collagen, hydroxyapatite and various concentrations of sericin was employed. Type I fibrillar collagen was obtained as a 2.54% collagen gel from calf skin by alkaline and acid treatments as previously described (Albu, 2012). For pure collagen scaffolds, 1.2 % type I collagen in gel form was used. The lack of ash and fatty substances in this gel indicated a high degree of purity.

To obtain collagen scaffolds complexed with sericin and/or hydroxyapatite (Coll: Ser: HA) solutions of various concentration of sericin and hydroxyapatite were added to 1.2 % type I collagen gel at the concentrations that were previously established (Lungu et al., 2013). Thus, the composition of different scaffolds employed in this study were: 1:0:0 (Coll), 1:0.2:0 (Coll:Ser-20), 1:0.4:0 (Coll:Ser-40), 1:0:0.3 (Coll:HA-30), 1:0.2:0.3 (Coll:Ser-20:HA-30), 1:0.4:0.3 (Coll:Ser-40:HA-30). The gels were cross-linked with 0.5 % glutaric aldehyde (v/v) as previously described (Lungu et al., 2011). The cross-linking process is important because in its absence the scaffolds become hydrogels when hydrated (Glowacki & Mizuno, 2008). After the cross-linking process the gel composites underwent a freeze-drying process (liophylization) using the Christ Model Delta 2-24 LSC freeze-dryer (Germany) that lead to the formation of a porous structure. The freeze-drying process implied two operations in successive phases, namely sample freezing and sublimation of ice using high vacuum. As a result of these operations various size pores were formed instead of ice crystals, their size varying according to lyophilization conditions. The porous structure was analyzed by scanning electron microscopy (SEM) using a Quanta Inspect F SEM device equipped with a field emission gun with a resolution of 1.2 nm; gold coating was employed to enhance the surface conductivity.

In order to evaluate the scaffolds biocompatibility and osteoinductive potential hMSCs were seeded at a density of 50.000 cells/ml and allowed to grow on the scaffolds for 7 days.

Cell proliferation was determined by measuring the amount of DNA released from cells after the cells membranes were broken by exposing cells to large temperature variations using liquid nitrogen (Rage et al., 1990; Titorencu et al., 2007). Briefly, hMSCs were washed with PBS at 37°C and then liquid nitrogen was poured over the cells. After nitrogen evaporation and when the cells reached the room temperature (4-6 minutes) the process was repeated. After 4-5 freeze-thaw cycles, the cells were incubated for an hour at 37°C in a 10 μg/ml Hoechst 33258 fluorochrome solution made of 10 mM TRIS, 1 mM EDTA, 2M NaCl, pH 7.4 (TNE buffer). A standard curve was constructed using salmon DNA with concentration ranging between 0.5–100 μg/ml. After the incubation period, the fluorescence in the supernatants was measured employing an Tecan plate reader using λ= 350 nm wave length for fluorochrome excitation and recording the emission at λ= 460 nm. The DNA amount
released from the cells cultured on each scaffold was determined using the standard curve.

The activity of alkaline phosphatase was determined using p-nitrophenyl phosphate as a substrate for the enzyme and measuring the amount of substrate converted by the enzyme by reading the absorbance at a wavelength $\lambda = 405$ nm (Sabokbar et al., 1994). In essence, the cultured cells on scaffolds were gently washed with warm PBS at 37 °C and then the substrate solution, p-nitrophenyl phosphate 9.88 mM, dissolved in an alkaline buffer (pH = 9.86) was added. The buffer consisted in 0.08M Tris base and 0.5 mM anhydrous MgCl2. The cells and the substrate solution were incubated at 37 °C and after 60 minutes the supernatant was harvested. A standard curve was made using dinitrophenol at a concentration ranging between 20 μM and 200 μM. Both the supernatants from cells cultured on scaffolds and the standard curve absorbance at $\lambda = 405$ nm were measured. Using the absorbance values recorded for the calibration curve, the quantities of transformed substrate for cells cultured on each type of scaffolds were determined.

NAD(P)H dependent oxidoreductases reduce the tetrazolium salt MTT to an insoluble purple formazan. The cell viability assay was based on the colorimetric measurement of the amount of formazan product (Denizot & Lang, 1986). Briefly, after the culture medium was removed, the cells were washed with warm PBS (37°C) and then incubated in a solution of 0.5 mg/ml MTT made in DMEM culture medium without Phenol Red for 3 hours at 37 °C and 5% CO2 in air (v/v) and relative humidity less than 95%. The insoluble formazan formed was dissolved with 0.1 N HCl solution in anhydrous isopropanol. The color intensity of the resulting solutions was directly related with the total oxidoreductase activity and was determined by measuring the absorbance at $\lambda = 570$ nm wavelength from which the absorbance recorded at $\lambda = 690$ nm was extracted. Gene expression assay was done by PCR (polymerase chain reaction) with complementary DNA sequences obtained by reverse transcription as template for primers hybridization. The cells were washed with warm PBS (37°C) and lysed with Trizol reagent. Subsequently the total RNA was extracted as previously reported (Chomczynski & Sacchi, 1987). Using the reverse transcription enzyme M-MLV, the complementary DNA fragments were obtained. For amplification of these fragments Taq-Pol enzyme was used. The amplification products were visualized by agarose gel (1.5%) migration and the bands intensity was measured using Total Lab Quant software. Recorded value for each gene was normalized to the value recorded for "house-keeping" β-actin gene.

In order to perform hematoxylin-eosin staining, after the culture period the scaffolds were washed with warm PBS, fixed with paraformaldehyde 4% in PBS (v/v) and then frozen and sectioned (~7 μm thick sections) with a cryotome Leica CM 1850. The hematoxylin-eosin staining consisted in exposing the sections of the scaffolds for 10 minutes in hematoxylin solution, 7 seconds in the eosin solution, multiple washes with distilled water and covered with glycerol and coverslip. The images were captured with a MRc5 camera attached to an Axio Observer Microscope (Carl Zeiss).

**Results and discussions**

For all the studied scaffolds, SEM images showed a comparable porous structure with interconnected pores. The pore sizes ranged from 100 to 500 μm, enough to allow infiltration of cells and of the nutrients supply (Fig. 1).
**Fig. 1.** Scanning electron micrographs of scaffolds made of collagen (Coll) and containing various concentrations of Sericin (Ser) and hydroxyapatite (HA): (1) Coll; (2) Coll:Ser-20; (3) Coll:Ser-40; (4) Coll:HA-30; (5) Coll:Ser-20:HA-30; (6) Coll:Ser-40:HA-30.

The pore sizes of the scaffolds were measured by scanning electron microscopy. Addition of sericin produced dense structures, reducing the pores diameter from 90-150 µm that was determined for the sample without sericin to 50- 90 µm for the scaffolds with the highest sericin concentration. Hydroxyapatite did not substantially affected the pores size, the samples with or without hydroxyapatite having comparable values for pores diameter, that was between 45-115 µm. The hydrophilicity of the scaffolds directly correlated with the pores size, the lowest being recorded for the sample with smallest pores size and denser structure.

MSC proliferation on scaffolds was dependent on the cell’s source (the patient). The results recorded from the proliferation assay revealed significant differences as a function of the source of cells (the patients), i.e. between the amounts of DNA released from hMSCs harvested from different patients and grown on scaffolds of identical composition. Thus, the cells collected from patient P2 recorded the best proliferation that was superior to that determined for the cells collected from other patients, regardless of the scaffold composition (Fig. 2). The lowest proliferation rate was exhibited by the cells of P3. In general, large variations between the patients were recorded (Fig. 2). The data suggested that the cell proliferation is rather dependent on the patient than on the scaffolds composition.

**Fig. 2.** Proliferation assay of human MSCs collected from four patients (P1 – P4) and grown for 7 days on scaffolds of collagen (Coll) to which various concentration of sericin (Ser) or hydroxyapatite (HA) was added: (1) Coll; (2) Coll:Ser-20; (3) Coll:Ser-40; (4) Coll:HA-30; (5) Coll:Ser-20:HA-30; (6) Coll:Ser-40:HA-30.

Examination of hematoxilin-eosin stained cryosections by light microscopy showed that hMSCs were attached to the scaffold porous matrix. They were present both on the outside and within the scaffold structure. Figure 3 shows attachment to the scaffolds of the cells of one patient. Similar results were obtained with cells from the other three patients.

**Fig. 3.** hMSCs were attached on the outside and inside the scaffolds. Hematoxylin –eosin staining of cryosections shows the presence of cells on all sides of the scaffolds composed as described in figure 1: (1) Coll; (2) Coll:Ser-20; (3) Coll:Ser-40; (4) Coll:HA-30; (5) Coll:Ser-20:HA-30; (6) Coll:Ser-40:HA-30.

As in the case of the proliferation assay, we detected differences in the ALP activity as a function of the source of the cells. The highest ALP activities were recorded for the

cells provided by patients P1 and P4 and the smallest for the cells of P3 patient. ALP activity differences were also recorded as a function of the scaffold composition (Fig. 4).

**Fig. 4.** (A) Alkaline phosphatase (ALP) activity and (B) viability of hMSCs collected from patients P1-P4 and cultured on scaffolds made of: (1) Coll; (2) Coll:Ser-20; (3) Coll:Ser-40; (4) Coll:HA-30; (5) Coll:Ser-20:HA-30; (6) Coll:Ser-40:HA-30 (abbreviations as in Fig. 1)

It was also detected that the direct correlation between increased concentration of sericin and alkaline phosphatase activity level was not valid for all cells. Thus, it was observed, for the cells from P1 patient, a direct correlation between the sericin concentration and alkaline phosphatase activity for the cells grown on scaffolds with collagen-hydroxyapatite and sericin; however, for the cells from P4 patient cultured on the same scaffolds, it was found an inverse correlation between the two parameters.

Like ALP activity results, the viability of cultured hMSCs on scaffolds strongly depended on patient and to a lesser extent on scaffolds composition. Thus the best viability was recorded for hMSCs collected from P4 patient, which was higher than of all other patients’ cells. Some variations in viability were also recorded as a function of the scaffolds composition, but not as significant for all patients and not always in the same direction. Thus, if on the pure collagen scaffold the cells from P4 exhibited the highest viability, the cells obtained from P2 recorded the lowest viability on the same scaffold (Fig. 4).

Gene expression for osteocalcin and osteonectin of hMSCs attached to scaffolds depended both on scaffolds composition and the cells sources. For the hMSCs collected from P2 and P3 patients and cultured on scaffolds with sericin, a direct correlation between the sericin concentration and the transcription levels of osteocalcin and osteonectin genes was detected, irrespective of the presence and absence of hydroxyapatite. In contrast, in the case of the cells obtained from P1 patient and cultured on scaffolds with sericin, the presence or absence of hydroxyapatite in scaffold composition counted for the relation between sericin concentration and transcription level, both for osteocalcin and osteonectin genes (Fig. 5 columns 2, 3, 5 and 6).

**Fig. 5.** (A) The electrophoresis bands for osteocalcin (OC), osteonectin (ON) and GAPDH. The charts reflect the gene expression levels for osteocalcin (B) and osteonectin (C) in hMSCs from 3 patients grown for seven days on: (1) Coll; (2) Coll:Ser-20; (3) Coll:Ser-40; (4) Coll:HA-30; (5) Coll:Ser-20:HA-30; (6) Coll:Ser-40:HA-30; All the data are normalized to GAPDH expression.

In the case of scaffolds without sericin, made of pure collagen or of collagen and hydroxyapatite, the hMSCs from P1-P3 patients behaved differently regarding transcription level. For example, addition of hydroxyapatite to the collagen scaffolds induced for P1 cells an increase of the transcription level of osteocalcin gene; in contrast, in the case of cells from P2 and P3
patients, hydroxyapatite decreased this level (Fig. 5 columns 1 and 4).

The PCR results on the gene expression of osteocalcin and osteonectin in human MSCs attached to scaffolds of various composition revealed a noticeable dependence of the gene transcription level both on scaffolds composition and cells sources.

Discussion
In this study we conducted experiments aimed at the evaluation of the biocompatibility and osteoinductive potential of scaffolds of different composition and to asses and compare the results of culturing human MSC collected from different patients on these scaffolds.

The new findings of this investigation are: (a) the scaffolds made of collagen, hydroxyapatite and sericin have osteoinductive potential allowing human bone marrow MSCs to attach and grow outside and inside the scaffold’s pores; (b) the concentration of sericin in the collagen and collagen-hydroxyapatite based scaffolds modulate, in part, the performances of human bone marrow MSCs and (c) the source of human MSCs (the donor) is particularly important for the behavior of the cells (especially for the elderly donors) when in contact with a biomaterial.

We have tested scaffolds of different composition and found that all had, to an extent, osteoinductive potential and exhibited appropriate conditions for the attachment and growing of human MSCs.

Scaffolds of various compositions were produced before. For example there are reports on scaffolds containing, besides collagen and hydroxyapatite, hyaluronic acid (Bakos et al., 1998), macroporous hydroxyapatite scaffolds (Li et al., 2002) with silk (Panas-Perez et al., 2013) and scaffolds with collagen and sericin (Lungu et al., 2011).

Some of the obtained results show that human bone marrow MSCs responses to culture condition on tested scaffolds depends on scaffold composition. These results are explicable if we take into account the different chemical compositions of the scaffolds, which can be also reflected in the different physical characteristics of scaffold-cells contact surface, scaffold porosity etc. Other studies have also shown that human MSCs behave differently depending on the scaffold type. For example it was shown that MSCs from humerus bone marrow of a 54 years old male patient respond significantly different to cultivation on various commercial scaffolds (Beitzel et al., 2014). Our findings revealed that not only the scaffolds composition counts for the cells responses, but, to a great extent, also the cell source (the donor patient). We found that cells collected from different patients responded differently when were in contact with scaffolds of identical composition.

The variability in the response generated by the cells source (patient) went so far as for different patients opposite correlations occurred between the sericin concentration and alkaline phosphatase activity or between sericin concentration and osteocalcin gene transcription level.

It was previously shown that scaffolds made of hydroxyapatite-collagen combination (30 wt.% hydroxyapatite) provides better outcomes for osteogenic differentiation than pure collagen scaffolds, when human MSCs from bone marrow of 6 healthy patients aged 20-24 years were culture on them (Zhou et al., 2011). However, our results are not consistent with these findings. The addition of hydroxyapatite to the collagen scaffolds did not increase the osteogenic markers for all patients’ cells. For example addition of hydroxyapatite to the scaffold composition increased the osteocalcin gene transcription level for P1 but decreased it for P2 and P3 patients (fig. 5 columns 1, 4). An explanation for these different results could be the different cell sources, which in our
case was represented by older patients (59-65 years old) all of them with osteoarthritis of the hip joint (coxarthrosis).

There is evidence that the number of osteoblast precursors in the bone marrow is age-related (Muschler et al., 2001). In our study the age range was quite small, only 6 years difference between patients. Even accepting that the number of osteoblast precursors substantially varied between patients, this cannot explain the distinct responses recorded for the cells from different patients. Thus, other factors may contribute to the recorded differences between the cells from various patients. These factor could be the existence of local or systemic bone disease, endocrine status, gender, the use of various drugs, nutrition, environment factors etc. all of them making difficult to estimate a standard response of MSC from a patient to a specific biomaterial.

Conclusions

Our results imply that, it could be of particular importance to perform biocompatibility and osteoinduction tests before a patient receives a scaffold implant, so as to assess the proper match between the donor cells and the biomaterial employed. These data show once more the importance of personalized medicine for the future treatments.

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