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PREPARATION OF POROUS COLLAGEN MATRICES AND THEIR INTERACTION WITH DIFFERENT CELL TYPES

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ABSTRACT. The aim of our research was to obtain porous matrices from collagen and collagen-hyaluronic acid and to investigate their effect on two types of human cells: fibroblasts and osteoblasts. In order to fabricate the collagenous matrices, type I collagen solution and a mixture of type I collagen and hyaluronic acid solutions, in a weight ratio of 5:1 were conditioned as sponges by freeze-drying. ESEM examination revealed that the resulting matrices had a porous three-dimensional structure consisting of interconnected collagen fibers. The fine layers of hyaluronic acid coated these fibers and connected with them. All porous matrices were seeded with human dermal fibroblasts and with human osteoblasts and were maintained in culture for 7 and 21 days, respectively. Histological and immunohistochemical observations indicated that human fibroblasts and osteoblasts cultivated on porous collagenous matrices migrated and attached to them. We also noticed that collagen-hyaluronic acid sponges allowed a greater cellular invasion than the collagen matrices. The ability of these porous materials to act as a good support for fibroblast and osteoblast cell cultures make them convenient for obtaining tissue substitutes.

KEYWORDS. collagen, hyaluronic acid, human dermal fibroblasts, osteoblasts, porous matrices, cell proliferation.

INTRODUCTION

For a long time the extracellular matrix was considered a passive support essential for stabilisation of tissue structure. This concept was radically changed in

the last two decades. New techniques for identification of the connective tissue molecules showed that the type and the amount of proteins and polysaccharides secreted by the cells is different from one tissue to the other and, also, within the same tissue during development and healing processes [1].

Supramolecular assembly of connective tissue molecules in more or less organized networks is specific for each extracellular compartment [2]. Experimental data for cell cultures grown on different substrates demonstrated that the matrix biochemical and physical properties influence the cellular morphology, migration, proliferation and phenotype [3]. Collagen (COL), the main structural component of the vertebrate connective tissue is widely studied as a cell substrat. Depending on the preparation method, COL can be precipitated in fibrillar form or dried from acid solution in non-fibrillar form.

Another prevalent component of the extracellular matrix are the glycosaminoglycans (GAG) that interact with growth factors, receptors and adhesion proteins. Due to these properties the incorporation of GAG in the COL network could modify their bioactivity. The hyaluronic acid (HA), a component of GAG, has a high capacity of lubrification and water retention, and influences several cellular functions such as migration, adhesion, and proliferation [4]. Due to their biological functions COL and GAG had been extensively investigated as biomaterials for artificial skin, bones, tendons, cartilage and blood vessels [5]. An ideal material used for tissue engineering should possess the following characteristics: excellent biocompatibility, suitable microstructure, controllable biodegradability and suitable mechanical property [6].

In this work, the focus was on the fabrication of collagen and collagen-hyaluronic acid composite porous matrices for tissue engineering and to investigate their effects on two types of human cells: fibroblasts and osteoblasts.

MATERIAL AND METHODS

Preparation of COL and COL-HA matrices

Type I COL was extracted from calf skin by pepsin treatment and salt precipitation, as previously described [7]. The collagen solution was, then, dyalised against phosphate buffer saline (PBS), pH 7.4, and the resulting precipitate was centrifugated at 3,000 rpm, at 4^0C.

HA with average molecular weight of $3x10^5$ Da was extracted from porcine vitreous humour by papain treatment and cetylpyridinium chloride and ethanol precipitation.

In order to fabricate the porous matrices, the type I COL solution (0.7%, w/v) was mixed with a HA solution (0.3%, w/v) in a ratio of 5:1 (dry weight), at 20^0C, for 4 hours. Then, the COL solution (0.8%, w/v) and the COL-HA mixture were conditioned as sponges by frozen at -70^0C and lyophilization at – 40^0C for 48 hours.

The sponges obtained this way were sterilized by exposure to UV radiation for 4 hours.

Environmental scanning electron microscopy (ESEM)

Collagenous matrices were analyzed by SEM using an environmental scanning electron microscope ESEM, XL-30, FEI (Philips). Samples were processed in the 'low vacuum' mode and visualized as cross-sections.

Cell cultures

Human dermal fibroblasts were isolated from human dermis, after epithelial sheet removal by sequential trypsin and collagenase digestion. The cells were then cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% antibiotic mixture.

Primary cultures of osteoblasts were obtained from human bone (femural head) by enzyme digestion. Briefly, the cancellous bone was minced into small pieces and bone fragments were extensively washed with PBS in order to remove blood cells and fat. Then, bone chips were treated with a mixture of enzymes (collagenase+trypsin) at 37^0C, for 20, 30, 40 and 60 min. Supernatants were collected and centrifuged at 1000 rpm, for 10 min. The cell pellet was seeded in DMEM supplemented with 15% FBS, 2 mM L-glutamine and 1% antibiotic mixture.

The culture media were changed twice a week and the cells were harvested by trypsin treatment. After detachment from the culture plates, fibroblasts and osteoblasts were cultured into COL and COL-HA sponges at a density of $5x10^4$ cells/cm^2 (for the histological studies). The cells were cultured for 7 or 21 days in DMEM with 10% FBS and antibiotics.

Toxicity assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were injected into COL and COL-HA sponges at a density of $1x10^5$ cells/cm^2 and placed in 24-well culture plates. MTT assay was carried out daily during 5 days and experiments were performed in quadruplicate. Each day, the medium was discarded and replaced with 5 mg/mL MTT in DMEM. After 3 hours incubation, the formazan crystals formed in viable cells were solubilized in isopropanol and the absorbance was monitorized at 570 nm on a UV-VIS spectrophotometer (Jasco V-530).

Histological examination of fibroblasts

After different times of cultivation, sponge samples were fixed in Bouin solution, embedded in paraffin and stained with Harris hematoxylin.

Histochemical detection of alkaline phosphatase

Samples of collagenous sponges were fixed in 4% para-formaldehyde buffered with 10 mM phosphate (pH 6.9) for 2h and washed in the same buffer. The sponges were then incubated with BCIP/NBT substrate (Roche), at 37^0C, for 1h according to the manufacturer's instructions. The sponges were refixed in 4% para-formaldehyde in phosphate buffer at 4^0C, for 2h and processed for embedding in paraffin. Paraffin sections of 8 μm thickness were counterstained with 1% Nuclear Red.

Immunohistochemistry

Sponges were fixed in Bouin solution and embedded in paraffin. Tissue sections were deparaffinized in xylene and hydrated through a graded ethanol series. Endogenous peroxidase activity was quenched with 3% H_2O_2, for 10 minutes.

Sections were then sequentially incubated with 2% BSA to remove non-specific background staining, rabbit polyclonal antibody to osteocalcin (Chemicon) diluted 1:100, overnight, at 4^0C, mouse anti-rabbit IgG peroxidase conjugated (Sigma) diluted 1:350, at room temperature, for 1h. Each incubation step was followed by four 5 min. washings in PBS. To visualize the immune complex the sections were incubated for 5-15 min. in a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.015% H_2O_2 dissolved in PBS. Following immunohistochemistry the sections were stained with Harris hematoxylin.

RESULTS

Preparation and structural characterization of porous matrices

Two types of three-dimensional COL matrices were prepared by lyophilizing a type I COL solution and a mixture of type I COL and HA solutions in a mass ratio of 5:1. The collagenous materials obtained as elastic sponges were easily processed into various sizes.

Our ESEM observations revealed that morphologically, the two types of sponges were similar, with a microporous structure. This microscopic appearance is a consequence of the intrinsic fibrillary structure of the non-denaturated protein. In the case of COL matrices (fig. 1A) we observed that the structure heterogeneity is larger than that of COL-HA materials (fig. 1B). All sponges had a honeycomb structure with pores of several microns in diameter. In the COL-HA sponges the presence of COL fibers coated and connected by fine HA layers was observed (fig. 1B).

Cytotoxicity evaluation and cell-sponges interaction

To evaluate the effect of these matrices on cell proliferation, human fibroblasts and osteoblasts were seeded into COL and COL-HA sponges. The porous matrices were maintained in culture for 7 and 21 days, respectively.

The cell proliferation was evaluated by the MTT assay which is based on the cleavage of tetrazolium salt by mitochondrial succinate dehydrogenase in viable cells. Figures 2A and 2B show the absorbtion values for both cell types during the 5 days culture period. The results showed that proliferation occured in both sponge types and the growth rate of cells seeded into COL-HA sponge was slightly higher than that of cells cultured into COL sponge ($p<0.01$). This enhance of the proliferation rate in COL-HA sponges was observed for both cell types (fibroblasts and osteoblasts cells) (figs. 2A, 2B).

Histological examination revealed that after 7 days of cultivation, *human dermal fibroblasts* were organized as cellular groups of various sizes, especially on the surface of the scaffolds (figs. 3A, 3B). Cells showed a normal morphology characterized by a round or oval nucleus containing granular cromatin. After 21 days of cultivation, human dermal fibroblasts were arranged unequally in the structure of porous matrices (figs. 4A, 4B). Frequently, cells filled the smaller pores of the sponges.

Human osteoblasts seeded into COL and COL-HA sponges and cultivated for 7 days were found predominantly at the edge of COL matrices (figs. 5A, 5B).

Osteoblasts had a cuboidal or fusiform shape with an euchromatic nucleus and an evident nucleolus. At both time points osteoblasts showed a high cytoplasmic activity of alkaline phosphatase. The number of osteoblasts increased in contact with the collagenous scaffolds in both material types after 21 days of cultivation (figs. 6A, 6B). Moreover, osteocalcin immunoreactivity was found in the cytoplasm of osteoblasts.

We also observed that the cell proliferation in COL-HA sponges was higher than that found in COL sponges for both types of cells.

DISCUSSION

In this study we describe the generation of two types of reconstituted matrices consisting either of COL alone or COL with HA. Type I COL was extracted from calf skin by a non-denaturing method and HA with an average molecular weight of $3x10^5$ Da was extracted from porcine vitreous humour. The COL and COL-HA matrices were designed as sponges with highly porous structures (figs. 1A, 1B), fabricated by lyophilizing of type I COL solution and a mixture of type I COL and HA solutions.

Previous studies showed that the size and morphology of the sponge pores are recognized to be dependent on the freezing temperature of the mixture before lyophilization [8]. In our study, the freezing temperature for COL and COL-HA solutions was -70^0C, value reported as optimal to obtain pore sizes that can provide appropriate space for cellular infiltration and proliferation [9].

Our ESEM studies revealed that obtained matrices had a three-dimensionally interconnected porous structure and therefore could be of interest for developing substrates for tissue engineering.

Another purpose of this work was to evaluate the *in vitro* behaviour of the porous matrices using two types of human cells: dermal fibroblasts and osteoblasts, by toxicity and proliferation assays. Toxicity was measured by the MTT assay which is very useful for determining the viability of the cells at early stages. According to the MTT assay, in the first five days, none of the porous materials elicited a toxic response (figs. 2A, 2B). In contrast, the contact of cells with COL and COL-HA sponges enhanced their proliferation.

Histological examination of COL and COL-HA sponges after 7 or 21 days of cultivation, respectively, in the presence of fibroblasts and osteoblasts indicated the migration and proliferation of the cultured cells at the surface and into the sponges. Osteoblastic phenotype was demonstrated by phosphatase alkaline and osteocalcin expressions, the last being a specific bone protein. Differentiating osteoblasts are known to synthesize and secrete alkaline phosphatase during post proliferative period of extracellular matrix maturation and the expression of osteocalcin, osteopontin and bone sialoprotein occurs later during while third period of extracellular matrix mineralization [10, 11]. Previous studies showed that the activity of alkaline phosphatase remained elevated and reached the peak value during the stage of extracellular matrix formation (day 14-16) and decreased markedly after day 26. For

this reason the alkaline phosphatase should be regarded as an early marker of osteoblast differentiation [12].

Previously, cellular interactions with collagens have been shown to be mediated by cell surface receptors that belong to the integrin family. The α1β1 and α2β1 integrins represent the major receptors for collagens, mediating the transmembrane signal transduction that affects cell functions such as cell proliferation and collagen synthesis [13]. The mechanism of cell adhesion in this case may involve the formation of focal contacts through a clustering on the cell surface of cell integrin receptors that interact with collagen [14, 15]. On other hand, it has been reported that cell adhesion to collagens is dependent of their native or denaturated conformation. The denaturation of the collagen triple-helical structure greatly affects cell adhesion level and disrupts the binding sites recognized by the integrins [16, 17]. These observations demonstrated the importance of the collagen conformation for its using in the field of biomaterials.

In this study, we used for porous matrices fabrication the non-denaturated type I COL which allowed a good proliferation of fibroblasts and osteoblasts. We have also observed that the cells infiltrated and proliferated much better into COL-HA sponges than into COL sponges.

Liu et al. [18] reported that the combination of COL fibers and GAG polymers provides a composition closer to the native tissue that is more effective than COL alone in stimulating cell proliferation and differentiation. Our previous studies demonstrated that the presence of chondroitin sulphate in combination with COL stimulated the cellular invasion into the sponges [19].

This study suggests that the COL and COL-HA porous matrices we have obtained could be suitable materials for biomedical application.

CONCLUSION

- The COL and COL-HA-based biomaterials described in this study, designed as three-dimensional porous matrices were biocompatible and represented suitable substrates for human dermal fibroblasts and human osteoblasts adhesion and proliferation.
- The combination of COL with HA provided an ordered fibrillar structure which was more effective than COL alone in stimulating cell proliferation and migration inside the porous matrices.

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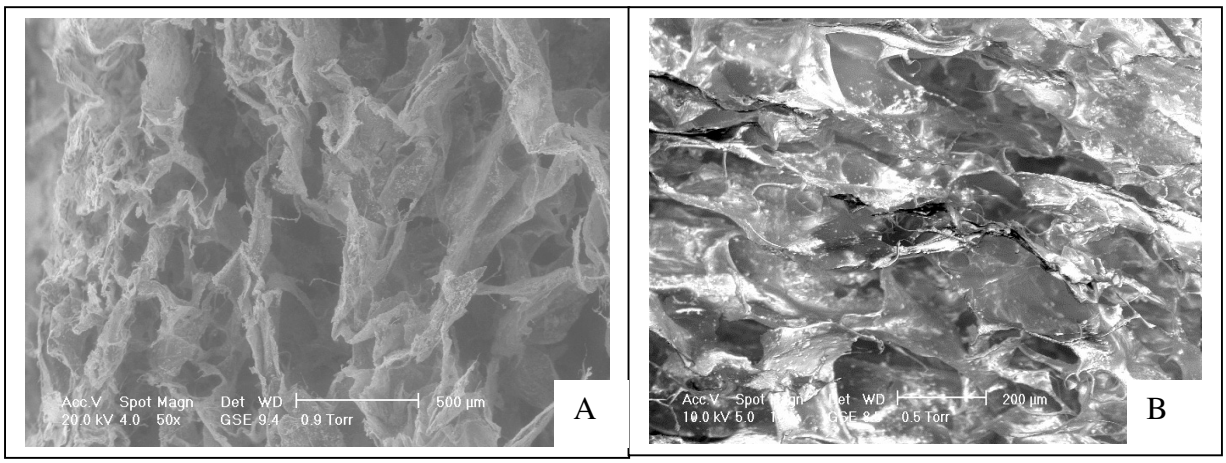

Figure 1 - Scanning electron microscopy (ESEM) images of COL sponge (A) and COL-HA sponge (B).

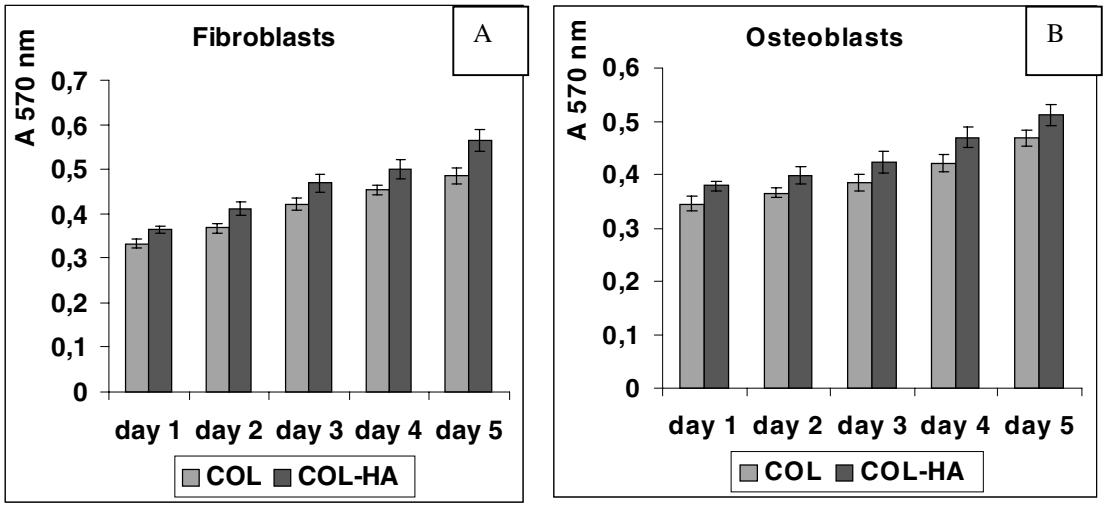

Figure 2 – Human dermal fibroblasts (A) and osteoblasts (B) proliferation in COL and COL-HA sponges monitored 5 days by MTT assay. The absorbance at 570 nm of formazan crystals formed in viable cells was expressed as a measure of cell viability. The initial seeded cell density was $1x10^5$ cells/sponge.

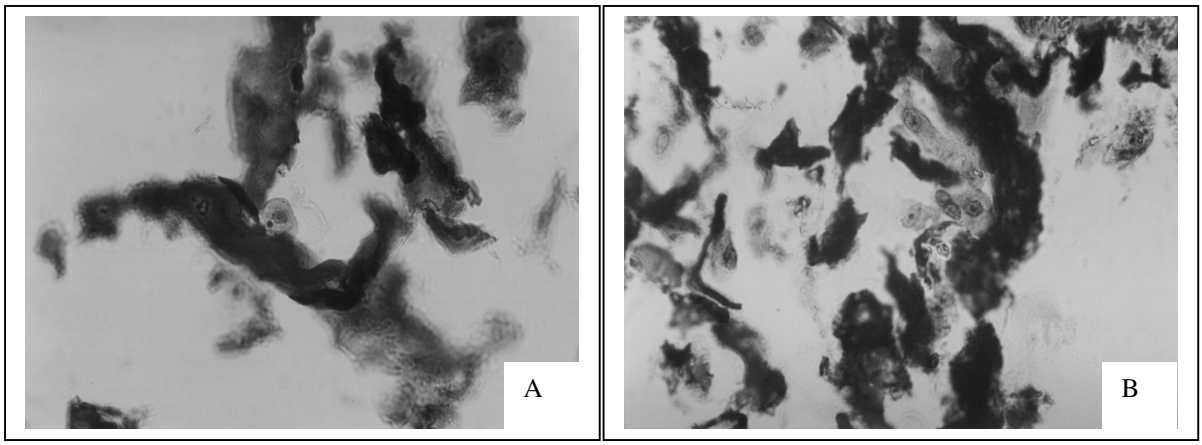

Figure 3 – Light micrographs of fibroblast cells cultured on the COL sponge (A) and COL-HA sponge (B) for 7 days; cells were organized as groups of various sizes especially on the surface of the sponges. (Hematoxylin Harris staining; A, x250; B, x400)

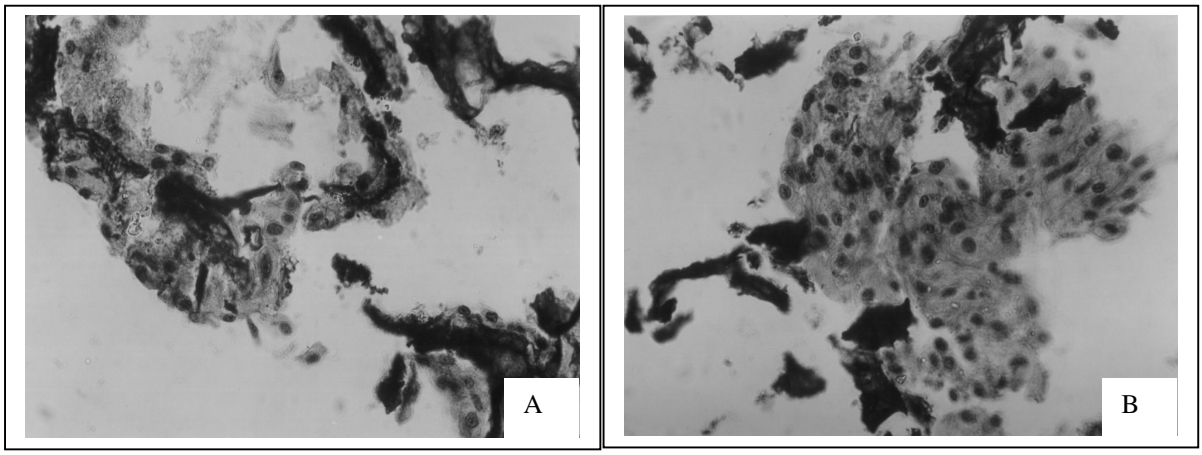

Figure 4 – Human dermal fibroblasts cultured on the COL sponge (A) and COL-HA sponge (B) for 21 days; cells were arranged unequally in the structure of porous matrices. (Hematoxylin Harris staining; A,B, x100)

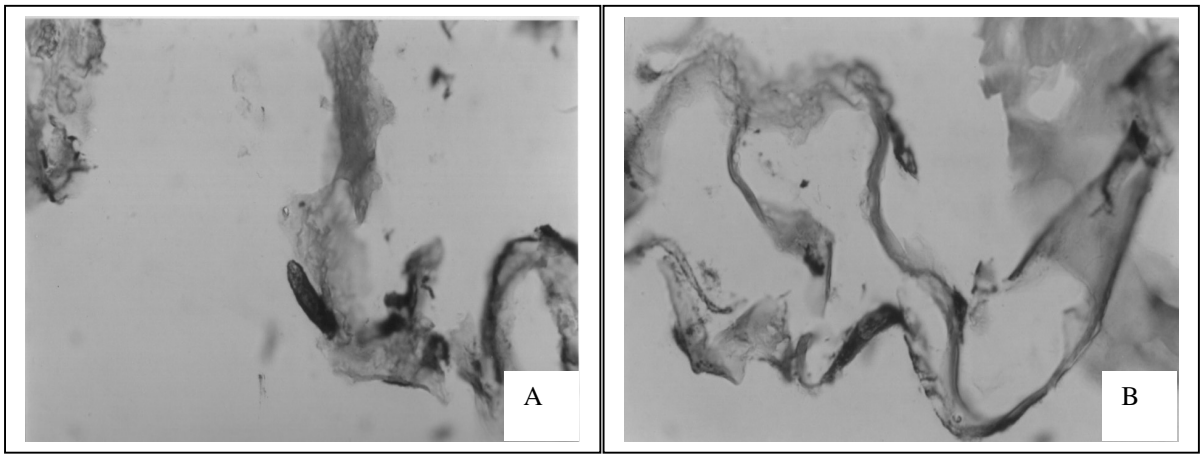

Figure 5 – Phosphatase alkaline detection in human osteoblasts cultured on COL sponge (A) and COL-HA sponge (B) for 7 days; cells were preferentially disposed at the edge of the sponges. (counterstaining with Nucler Red; A, B, x400)

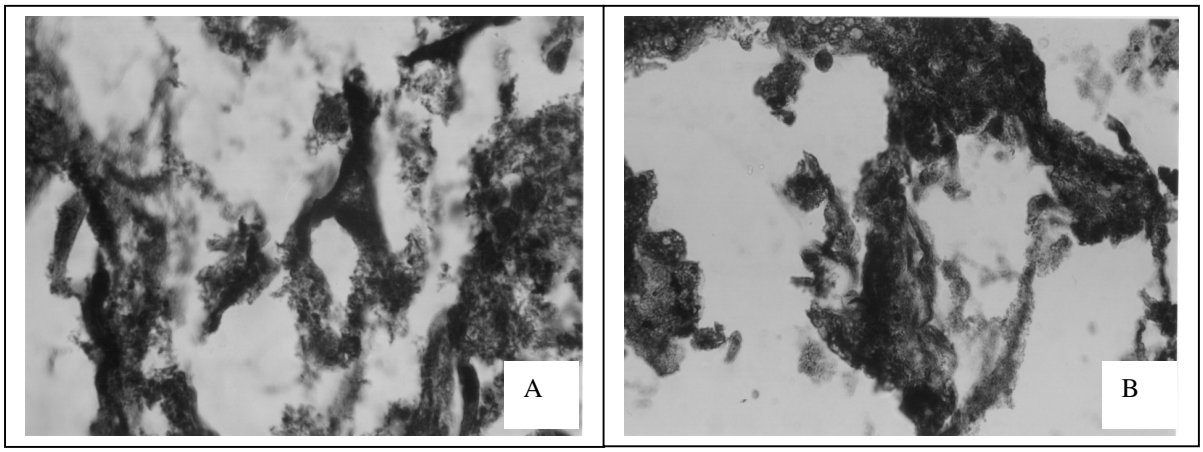

Figure 6 – Osteocalcin detection in human osteoblasts cultured on COL sponge (A) and COL-HA sponge (B) for 21 days; cells had migrated into the sponges and attached to them. (A, x400, B, x250)