

## IN VITRO TESTING OF MODIFIED COLLAGEN/ HYALURONAN/BETA-GLUCAN SCAFFOLD

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### ABSTRACT

Cells and scaffolds belong to main components of tissue engineering. In this study we have performed *in vitro* cytotoxicity and biocompatibility testing of modified collagen/hyaluronan/beta-glucan scaffold. We used direct contact assay as well as MTT test. Human adipose tissue-derived stem cells were used as biological model. According to obtained results, we can summarize, that modified collagen/hyaluronan/beta-glucan scaffold is non-toxic and biocompatible and after further testing it should be used as carrier of various types of cells.

**Keywords:** Somatic Stem Cells, Scaffold, Cytotoxicity, Biocompatibility

### 1. INTRODUCTION

Tissue engineering is relatively new multidisciplinary field aimed at producing tissue or organ substitutes by cultivation of cells on the various types of scaffolds. These scaffolds mimics native Extracellular Matrix (ECM) and provide not only mechanical support for cultured cells but also effect their attachment, proliferation and differentiation, both *in vitro* and *in vivo* (Alberti, 2012; Ge *et al.*, 2012).

Biodegradable polymers of natural or synthetic origin seem to be appropriate materials for preparation of ECM substitutes, because their interactions with cultured cells lead in to incremental biological degradation; and after their application there is no need to undergo other surgery to remove foreign material from patient's body.

ECM substitutes must possess specific biological and mechanical properties. They have to be non-toxic and biocompatible with cultured cells (Taylor, 2007).

Structure of surface, pore size, porosity and structural strength are also important characteristics, which may influence behavior of cells (Chung *et al.*, 2012). Another important advantage is fact that synthetic ECM allows cells to grow in three-dimensional structure, which can promote new tissue formation (Davies *et al.*, 2010).

Collagen and hyaluronic acid belong to natural biodegradable polymers, which are found in human body in large volumes. Their chemical and mechanical characteristics offer wide possibility to control and program their biodegradability (Kremer *et al.*, 2000). The problem of collagen antigen city can be overlapped by removal of the telopeptides from its molecules with specific enzymes or by chemical cross-linking (Ishihara *et al.*, 1994; Eriksson *et al.*, 1991). Beta-glucans are polysaccharides, which are abundant in plants. Moreover, they occur in the cell wall of yeasts, certain fungi and bacteria. Yeast and medicinal mushroom

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derived beta-glucans are notable for their ability to modulate the immune system (Ooi and Liu, 2000).

The main goal of present paper was preparation of collagen/hyaluronan/beta-glucan scaffold and its testing for cytotoxicity and biocompatibility by using somatic stem cells derived from adipose tissue.

## 2. MATERIALS AND METHODS

### 2.1. Scaffold Preparation

Hypro-Sorb (Hypro Otrokovice, Czech Republic) used as a source of collagen type I was cut into small pieces. Collagen (1.5 g) was mixed with 50 mL of distilled water. After that, 10 ml of 0.05 mol L<sup>-1</sup> acetic acid was added and mixed together. Then, 10 mL of distilled water was added to sodium hyaluronate (0.12 g). After 3 h, swelled sodium hyaluronate was carefully mixed with collagen. In the next step, obtained mixture was mixed with 2.3 mL of 1.3% dialdehyde starch. Finally, whole mixture was supplemented with beta-glucan (0.5 g) and was transferred to molds following by air drying at 37°C.

### 2.2. Morphology of Scaffold

The morphology of collagen/hyaluronan/beta-glucan scaffold was evaluated by Scanning Electron Microscopy (SEM). Before testing, scaffolds were gold-plated using a JFC 1100 ion sputter and were analyzed by JSM 840 JEOL (JEOL, Japan).

### 2.3. Isolation and Cell Culture

Human Adipose Tissue-Derived Stem Cells (ATSCs) were obtained from lip aspirates of healthy female individual undergoing elective suction-assisted lip aspiration always following patient's written and verbal informed consent in compliance with the Helsinki Declaration. The obtained samples were collected in sterile phosphate buffered saline (PBS; Oxoid, GB) with 100 U mL<sup>-1</sup> Penicillin, 100 µg mL<sup>-1</sup> Streptomycin and 250 ng mL<sup>-1</sup> amphotericin B (PAA, Austria) and stored at laboratory temperature overnight.

ATSCs were isolated according to method published by (Zuk *et al.*, 2002) with some modifications. Briefly, lipoaspirates were carefully washed with sterile PBS containing 100 U mL<sup>-1</sup> Penicillin, 100 µg mL<sup>-1</sup> Streptomycin and 250 ng mL<sup>-1</sup> amphotericin B to remove contaminating erythrocytes. Adipose tissue fragments were treated with 0.1% collagenase type II (PAA, Austria) at 37°C for 1 h, followed by centrifugation. Supernatant containing fat and floating

mature adipocytes was aspired and cell pellet was resuspended in Dulbecco's modified Eagle's medium (D-MEM; PAA, Austria) supplemented with 10% fetal bovine serum (FBS; PAA, Austria) and centrifuged. Final pellet was resuspended in complete culture medium (D-MEM +10% FBS +100 U mL<sup>-1</sup> Penicillin +100 µg mL<sup>-1</sup> Streptomycin +250 ng mL<sup>-1</sup> amphotericin B). Cells were counted and tested for viability by using trypan blue exclusion test and plated at density of 1×10<sup>6</sup> cells mL<sup>-1</sup> into 100 mm Petri dishes (TPP, Switzerland). Cultures were maintained in CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub> in the air). After 24 h, non-adherent cells were removed by aspiration medium. Culture medium was refreshed every 48 h. When the cells reached confluence they were detached with 0.25% trypsin (PAA, Austria) and re-plated at a dilution of 1:3. ATSCs were cultured up to the third passage under same condition.

### 2.4. Cytotoxicity and Biocompatibility Testing

The direct contact assay to evaluate biocompatibility and cytotoxicity was performed according to International Standardization Organization standard 10993-5 in triplicate. ATSCs in complete culture medium (50 000 cells mL<sup>-1</sup>) were seeded into 6-well plates with the collagen/hyaluronan/beta-glucan scaffolds. Their effect on the proliferation and morphology was evaluated by invert microscope Zeiss Axiovert 100 (Carl Zeiss, Germany).

Cytotoxicity of prepared materials was estimated according to proliferation tested by MTT-test. The tested scaffold was cut into small pieces and was eluted in complete culture medium for 24 h. Obtained eluate was filtered through the 0, 22 µm filter. Adipose derived stem cells were seeded into the 96-well microplate in amount of 5000 cells and cultured overnight. On the second day, eluates were added. Complete culture medium was used as negative control. After 24, 48 and 72 h, the eluates and control culture medium was aspired and CellTiter 96® Aqueous One Solution Reagent (Promega, USA) was added followed by 3 hours incubation. The cell proliferation was evaluated on ELISA microreader Bio Tek EL800 (BioTek, USA) by measuring the absorbance at 490 nm wavelength.

### 2.5. Immunohistochemical Evaluation

Scaffold with adipose tissue derived stem cells appointed for immunohistochemical evaluation were carefully rinsed with phosphate buffered saline and fixed in neutral formalin (Sigma-Aldrich, Germany) and embedded into paraffin. After that they were sectioned into 2 µm thick slices and stained by conventional staining methods. Immunohistochemical

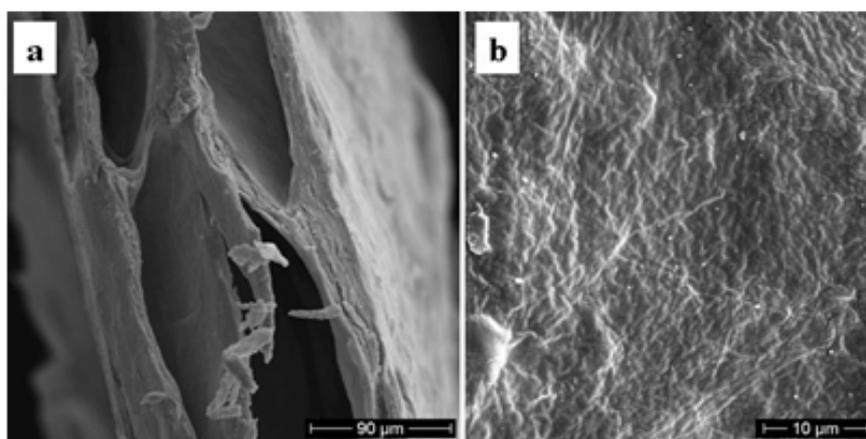
staining was performed using the biotin-avidin technique with primary mouse antibodies against vimentin (DAKO, Glostrup, Denmark). Visualization of the cells was developed with DAKO LSAB/HRP kit and DAKO En Vision/HRP kit (DAKO, Glostrup, Denmark). Observations were performed on the light microscope Nikon Eclipse 80i and camera Nikon DS-Fi1 (Nikon, Japan).

### 3. RESULTS

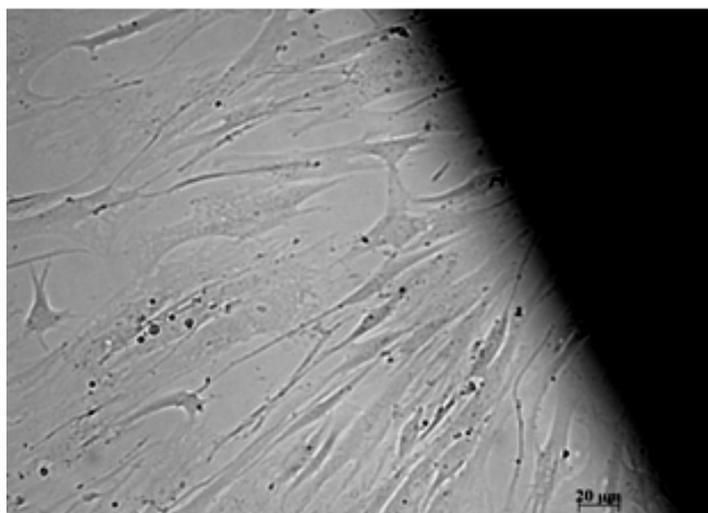
Prepared collagen/hyaluronan/beta-glucan scaffolds had the character of the membrane with a thickness of 2 mm. The SEM observation is shown in **Fig. 1**. The

scaffold had bubble-like structure; cavities size ranged from 50 to 100  $\mu\text{m}$  **Fig. 1a** and the surface was rough **Fig. 1b** to increase attachment of cultured cells.

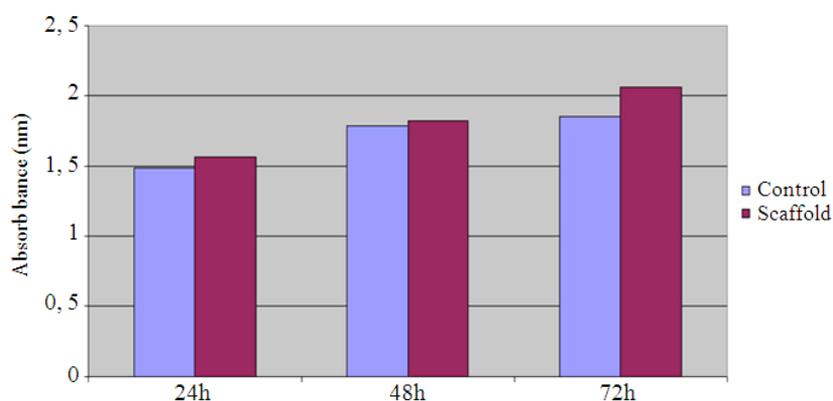
**Figure 2** shows the proliferation of ATSCs in the presence of collagen/hyaluronan/beta-glucan scaffolds. Growth inhibition as well as changes in morphology was not recorded. **Figure 3** shows results from MTT tests. Proliferation of ATSCs was not inhibited and was comparable with negative control. Obtained results indicated that collagen/hyaluronan/ beta-glucan scaffold is non-toxic and biocompatible with cultured cells. **Figure 4** presents proliferation of ATSCs attached on the surface of collagen/hyaluronan/beta-glucan scaffold.



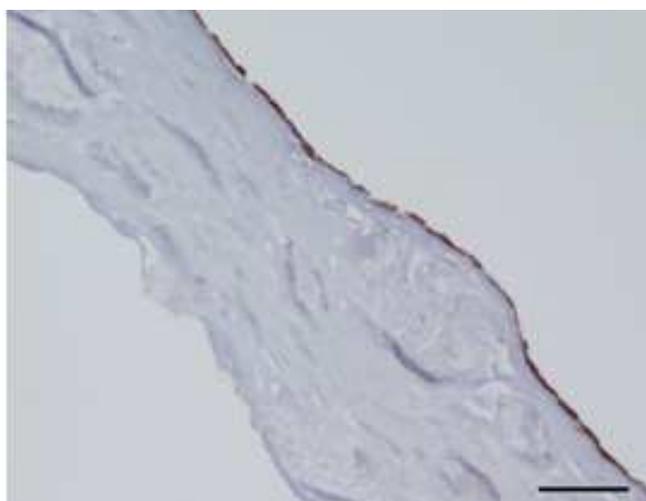
**Fig. 1.** Sem micrographs of collagen/hyaluron/beta-glucan scaffold



**Fig. 2.** Morphology of ATSCs in the presence of collagen/hyaluron/beta-glucan scaffold



**Fig. 3.** Proliferation of ATSCs in the presence of collagen/hyaluron/beta-glucan scaffold



**Fig. 4.** Proliferation of ATSCs on the collagen/hyaluron/ beta-glucan scaffold

#### 4. DISSCUSSION

In this study, novel scaffold composed of collagen type I, hyaluronan and beta-glucan was fabricated and characterized. The main objective of this study was to assess cytotoxicity and biocompatibility by using somatic stem cells derived from adipose tissue as in pertains to the potential usage in the tissue engineering and regenerative medicine.

Biodegradable polymers of natural origin, based on collagen and hyaluronic acid belong to widely used. They should be prepared by various techniques, including slow drying at laboratory temperature or freeze-lyophilization, that lead in the production of pores. Scaffold porosity, pore size and their distribution have important effect on the cell adhesion and

proliferation as well as differentiation (Kang *et al.*, 2009). It was also shown that pore size of 100-150  $\mu\text{m}$  is appropriate for uniform cell penetration and migration. Moreover, it positively influences the production of ECM (Ma *et al.*, 2003).

The tested collagen/hyaluronan/beta-glucan scaffold had bubble-like structure with rough surface to increase attachment of cells. Similar structural characteristics were observed by other authors in various composite scaffold based on the collagen and hyaluronic acid but also collagen and chitosan (Arana *et al.*, 2013; Lin *et al.*, 2009).

The crucial part of characterization of scaffolds appointed for utilization tissue engineering and regenerative medicine is their cytotoxicity and biocompatibility testing. Main components, collagen,

hyaluronan and beta-glucan are non-toxic, but the cytotoxicity of modified biomaterial depends on residual cross-linking agents or specific processing method (Ben *et al.*, 1988). For this reason, we used direct contact assay and MTT test. As a biological model, we chose somatic stem cells derived from adipose tissue. Obtained results indicate that collagen/hyaluronan/beta-glucan scaffold is non-toxic and fully biocompatible with ATSCs. Moreover, higher levels of MTT reduction after 48 and 72 h, suggesting that ATSCs not only adhered to scaffold but start to proliferate. Similar results were obtained by other authors (Arana *et al.*, 2013; Cheng *et al.*, 2012). This finding supports their usage as a three-dimensional carrier of various types of cultured stem cells, because the three-dimensionality is essential for fabrication of artificial tissues, including skin, cartilage, bone (Tang *et al.*, 2007; Harvanova *et al.*, 2009; Chen *et al.*, 2011).

Biocompatibility and non-toxicity was also proved by immunohistochemical analysis. Seeded ATSCs, attached on surface of scaffold and grow in confluent layer, which is again in accordance with the above literature.

## 5. CONCLUSION

Based on the obtained results, we can summarize, that modified collagen/hyaluronan/beta-glucan scaffold is non-toxic and biocompatible with adipose tissue-derived stem cells and after performing further tests it should be used as carrier of various types of cells in the tissue engineering as well as in the regenerative and substitutive medicine.

## 6. ACKNOWLEDGEMENT

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