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Hyaluronic Acid-Coated Carbon Nonwoven Fabrics as Potential Material for Repair of Osteochondral Defects

Abstract
Damaged articular cartilage is known to have poor capacity for regeneration. Carbon fibres (CFs) have been widely investigated as cellular growth supports in cartilage tissue engineering. However, the long duration of the process of cartilage restoration limits the applicability of CFs implants in the treatment of cartilage tissue defects. Hyaluronic acid (HA) plays a key role in cartilage tissue development, repair and function. In the present study we focused on the in vitro and in vivo evaluation of two types of carbon nonwoven fabrics: HA modified and non-modified carbon nonwovens. The results of in vitro studies showed that cells attached well and retained their good viability in the carbon nonwoven matrix. The incorporation of hyaluronic acid resulted in the enhancement of cell proliferation. The results of in vivo studies showed a faster process of tissue regeneration in the case of HA modified carbon nonwovens. The results presented indicated that HA-modified carbon materials seem to be a suitable material for the treatment of osteochondral defects.

Key words: *cartilage, carbon fibres, nonwoven scaffolds, hyaluronic acid, biomaterials.*

Introduction

When damaged, the articular cartilage has very little capacity for spontaneous healing because of the avascular nature of the tissue [1, 2]. Untreated cartilage defects lead to the degeneration of tissue. Even small chondral defects may necessitate surgical intervention. Therefore the major goals in cartilage tissue engineering is the development of new scaffold material that can fill the damaged area and integrate with the surrounding tissue, preventing further degeneration and consequent osteoarthritis [3]. Ideally scaffolds should possess a three-dimensional and highly porous microstructure with an interconnected pore network, mechanical properties that match those of tissue at the site of implantation, and should be easily processed to form a variety of shapes and sizes [4 - 6].

Carbon nonwoven fabrics have been used for the supplementation of cartilage losses as they have a unique ability among synthetic materials, which enables the restoration of cartilage tissue [7, 8]. The assumption that carbon as the fundamental element in biological tissue would not induce adverse reactions encouraged scientists to use carbon fibres in medicine as an implantable biomaterial [9]. The properties of carbon fibers important for medical applications are related to the physical and chemical structure of their surface [10]. Moreover carbon nonwoven fabrics present a remarkably increased surface area for cell attachment and a significantly improved interconnected pore architecture that provides easier pathways for the diffusion of gases, transportation of nutrients and migration of cells [11, 12]. The broad possibility of customising mechanical properties to be comparable with the tissues replaced make carbon nonwovens increasingly attractive for the treatment of cartilage defects [13]. Such material should direct tissue formation, while providing adequate structural support [14]. However, even though carbon nonwovens are biocompatible, they do not have sufficient biological activity enabling the stimulation of chondrocytes, but they can be subjected to various chemical and structural modifications. It is possible to modify their surface by coating them with films and layers.

Hyaluronic acid (HA) is an important component of the extracellular matrix of many types of tissue (i.e. cartilage) [15, 16]. HA is a naturally occurring, high molecular weight glycosaminoglycan (GAG) composed of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine [17 - 20]. HA plays an important role in lubrication, water sorption, water retention and a number of cellular functions such as attachment, migration and proliferation [21]. HA also presents unique advantages: it is easy to produce and modify, hydrophilic and naturally biodegradable [22]. When the hyaluronic acid chains are chemically cross-linked, the solubility and rheological properties of the material change to become more viscous and water-insoluble as gels [23]. It has been used for a long time by orthopedic surgeons for intra-articular injection [24]. HA derivatives, forming high viscosity solutions, have been used for the relief of pain in patients affected by osteoarthritis [25, 26]. Due to its high biocompatibility and low immunogenicity, HA is gaining popularity as a biomaterial for tissue engineering and cartilage regeneration [27]. Unfortunately, rapid degradation and accompanying changes in mechanical properties limit the direct clinical application of HA [28].

The purpose of this study was to consider the use of hyaluronic acid-coated carbon nonwoven fabrics as a material for cartilage defects or osteochondral defect treatment. The addition of HA should improve cell attachment, proliferation and differentiation, and the presence of carbon nonwoven fabrics (CNF) will provide a mechanical support. In the present study, we focused on the *in vitro* and *in*

vivo evaluation of pure carbon nonwoven fabrics (CNF) and carbon nonwoven fabrics modified with hyaluronic acid (CNF_HA).

Materials and methods

Preparation of the samples

Hyaluronic acid sodium salt (HA, 1.4 MDa) was purchased from the company CPN Ltd. (Dolni Dobrouc, Czech Republic). Carbon nonwoven fabrics (CNF) were prepared from polyacrylonitrile precursor via a two-stage process, starting from thermal treatment in an air atmosphere at 150 - 280 °C, followed by carbonisation in an atmosphere of pure argon at 1000 °C [11, 14]. HA was immobilised onto the surface of macroporous carbon nonwoven fabrics. The samples were stored at room temperature in HA solution (0.5 g/100 ml H_2O) without employing any extraneous chemical cross-linking agents. After 3 days of incubation, the samples were dried at a temperature of 37 °C and sterilised with UV radiation for 30 min (both sides).

Characterisation of the samples

The microstructure of carbon nonwoven fabrics (CNF) and carbon nonwoven fabrics modified with hyaluronan (CNF_HA) were characterised using a Jeol, JSM-5400 scanning electron microscope. The diameter of the fibres was measured using a Lanameter MP-3 microscope. The average diameter of the fibres was determined by measuring the diameter at 100 different points. The pore structure of samples was evaluated by a mercury porosimeter (Carlo – Erba 2000).

In vitro studies

Cytotoxicity and immunogenicity tests

The cytotoxicity of the carbon nonwoven fabrics (CNF and CNF_HA) was determined in a culture of human lung adenocarcinoma cell line A549. For the cytotoxicity test, the A549 cells were seeded in a 24-well plate (Costar). 1×10^6 cells in 1 ml of the culture medium enriched with 2% calf serum, penicillin and streptomycin were deposited into each well. Samples of the tested materials in the amount of 10 mg were added to the plate wells, and incubated with cells at 37 °C in an air atmosphere containing 5% CO_2. The number of cells and changes in their morphology were estimated after 24 and 72 hours using an inverted microscope. Each sample was evaluated three times. Trypan blue staining was used for the

assessment of cell viability. Cellular cytokine production was used to assess inflammatory potential. The cytokine IL-6 levels produced by leukocytes in contact with modified and unmodified carbon nonwoven materials were investigated.

Cell adhesion and growth tests

Human osteoblast-like MG-63 cells (European Collection of Cell Cultures, Salisbury, UK) were used for testing the cell-material interaction *in vitro*. CNF and CNF_HA were inserted into polystyrene multidishes (24 wells, diameter 15 mm, TPP Company, Trasadingen, Switzerland), and seeded with MG-63 cells. Samples were cut into a round shape, to fully cover the bottom of the cell culture plates' wells. Each well contained 3×10^4 cells/well and 1.5 ml of Dulbecco's Modified Eagle Medium supplemented with 10% of foetal bovine serum and gentamicin (40 μg/ml). The microscopic glass coverslips served as a control material. Cells were incubated at 37 °C in air atmosphere with 5% CO_2. After 1, 3 and 7 days of incubation, the samples were rinsed in phosphate-buffered saline (PBS), fixed in 70% cold ethanol (-20 °C, 5 min) and visualised by staining with propidium iodide (concentration 5 μg/ml in PBS, incubation 5 min at room temperature). This dye stains nucleic acids, including those located in the cytoplasmic part of the cells, thus it can be used, at least partly, for evaluation of cell morphology [29]. The morphology and distribution of cells were evaluated and documented using an epifluorescence microscope IX 51 equipped with a digital camera DP 70 (Olympus, Japan). The effects of the materials tested on the adhesion, growth and morphology of cells on the carbon nonwoven fabrics were evaluated. The cells were detached with 0.1% trypsin solution in PBS (pH 7.35, temperature 37 °C, 5 min) and counted in a Burker's hemocytometer using an

inverted optical microscope (Olympus IX 51). The statistical significance of the differences between the experimental groups was estimated using ANOVA, the Student-Newman-Keuls method.

In vivo study

The knee cartilage of rabbits was used as model tissue for *in vivo* studies. Four rabbits for each group of materials were used in the study. The experiment was performed according to the EU ISO 10993-6 guidelines and the study protocol approved by the local ethics committee. The knee joints of rabbits were sterilely draped and opened by an anteromedial approach with the rabbits under general anaesthesia. The patella was dislocated laterally, and the femoral trochlea was exposed. Fullthick knee articular cartilage defects were produced through the articular cartilage and into the subchondral bone of the trochlear grooves of the knees with a 2-mm-diameter trephine. The holes were then carefully irrigated with saline to remove bone debris. The defects of 2 mm diameter and 5 mm depth performed were filled with equal portions of carbon nonwoven fabrics (CNF and CNF_HA). The implants were inserted press-fit without any additional fixation or glue. All animals survived the surgery. No wound healing complications were observed after the surgery and during the whole experiment. The animals were maintained under standard conditions with free access to food and water. After 2 and 6 months from the surgery, animals were sacrificed. Distal femora were removed, and areas of femoral trochlea that contained defects were dissected and placed in 4% neutral buffered formalin for 2 weeks. The specimens were subsequently placed into 15% ethylenediaminetetraacetic acid (EDTA) decalcification solution (pH 7.4) for 6 weeks at room temperature. Decal-
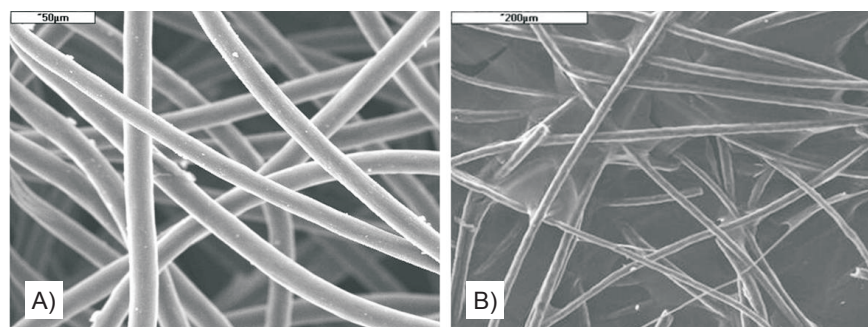

Figure 1. *Scanning electron microscope (Jeol, JSM-5400), images of carbon nonwoven fabrics: (A) before modification with HA (bar = 50 μm) and (B) after modification with HA (bar = 200 μm).*
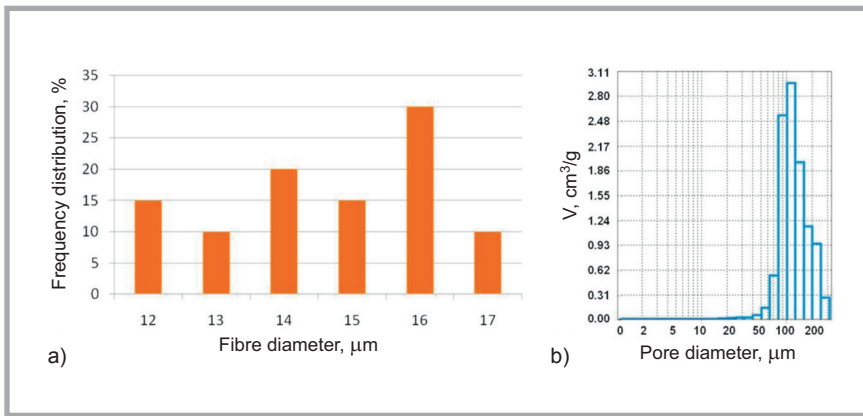
Figure 2. Characteristics of carbon nonwoven fabrics: (a) diameter distribution of CNF fibres and (b) pore size distribution for CNF.

cified specimens were embedded in paraplast and sectioned in a microtome into 9 - 15 μm thick slices. In order to evaluate the formation of cartilaginous repair tissue, histological analysis of the samples was performed. The sections obtained were stained by the Masson-Goldner method. Histological slides were examined and documented under a CX2 light microscope (Olympus, Germany).

■ Results and discussion

Material characterisation

Figure 1.A shows the morphology of the nonwoven fabrics (CNF) after the carbonisation process. CNF consisted of randomly oriented, smooth fibres of different diameters. The distribution of the fibre diameter and pore size are presented in ***Figure 2.A*** – ***2.B***, respectively.

The diameter of the fibres varied from 12 – 17 μm (***Figure 2.A***). According to the data obtained by means of a mercury porosimeter, the pore size distribution (20 – 250 μm) for the uncoated CNF sample was the result of the space between fibres. From the microscopic studies it follows that hyaluronic acid covered not only the CNF surface but it was also present among the fibres (***Figure 1.B***).

The evaluation of cytotoxicity

An important aspect in the development of novel scaffold is the evaluation of cytotoxicity. According to the results presented in ***Figures 3*** and ***6***, non–cytotoxic effects could be noticed for both samples investigated (CNF and CNF_HA). After 24 hours all the A549 cells that were cultured in contact with the samples were properly adhered to the bed and had a proper morphological character.

The total number of cells cultured with the samples after 24 and 72 hours was higher than at the beginning of the experiment (***Figure 3.A***). Although the
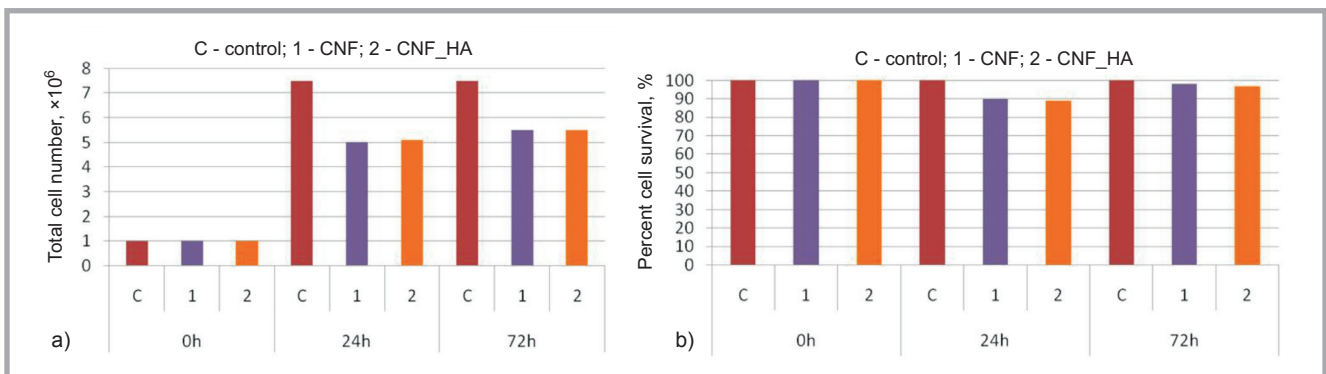

Figure 3. Comparison of cytotoxic effects of CNF (1) and CNF_HA (2) samples on lung cancer cell (A549): (a) total cell number; (b) percentage of cell survival. C indicates values obtained in cells cultured in wells without samples.
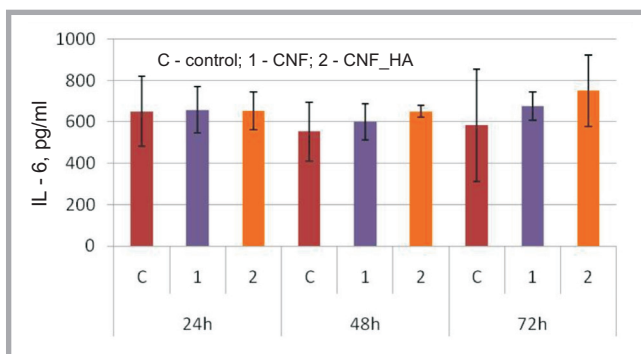

Figure 4. Cytokine IL-6 levels produced by leukocytes in contact with carbon nonwovens: 1) CNF, 2) CNF_HA.
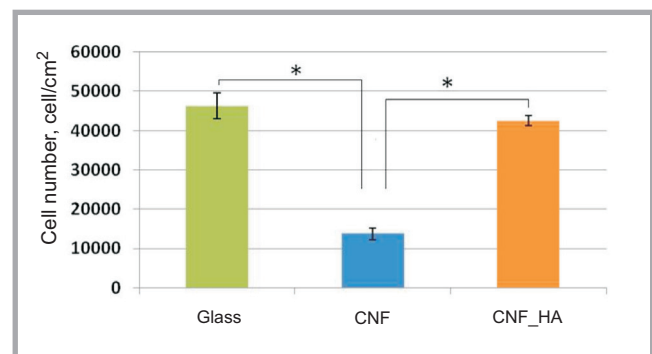

Figure 5. Number of MG-63 cells adhering and growing on carbon nonwoven fabrics and on the glass coverslips on day 7 after seeding. Glass: a control material, CNF: non-modified carbon nonwoven fabric, CNF_HA: carbon nonwoven fabric modified with hyaluronic acid. Mean ± S.E.M. from 4 - 12 measurements, ANOVA Student-Newman-Keuls method. *: $p \leq 0.05$ in comparison with the values obtained on control samples.
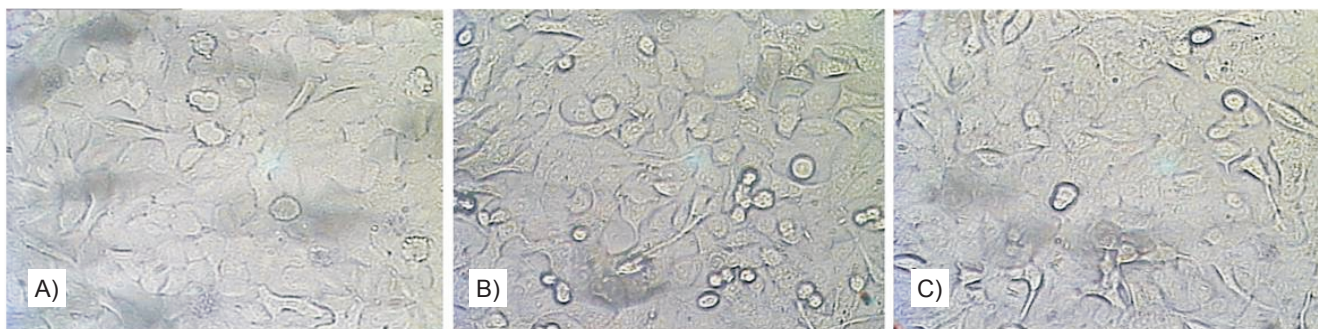
Figure 6. *Cytotoxicity test result in human lung adenocarcinoma cells line A549 after 72 h: (a) control, (b) CNF (c) CNF_HA (original magnification 40×).*
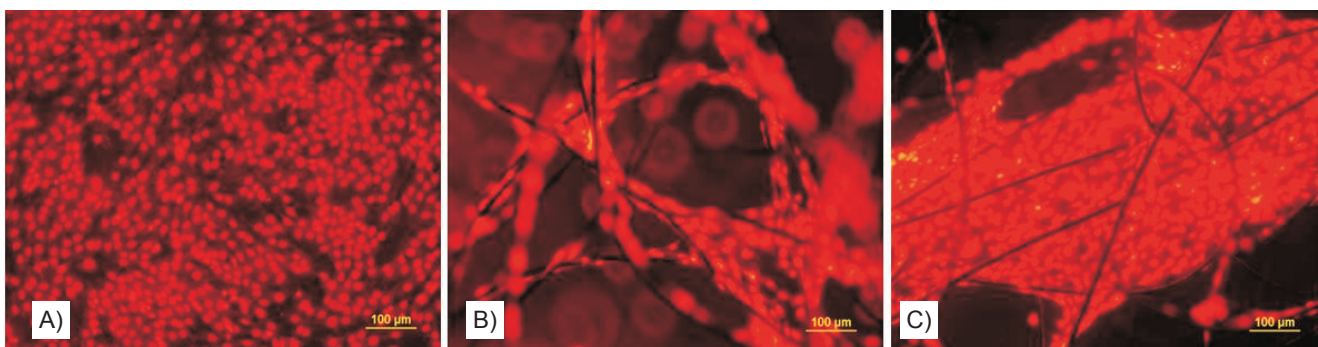

Figure 7. *Morphology of MG-63 cells seeded on carbon nonwoven fabrics after 7 days in culture: (A) cells on control glass coverslips, (B) cells on CNF, (C) cells on CNF modified with hyaluronic acid (CNF_HA). Stained with propidium iodide, microscope Olympus IX 51, digital camera DP 70, obj. 20×, bar = 100 μm.*

cell numbers in the presence of CNF and CNF_HA samples were lower than on the control wells, no significant differences were observed in the viability of cells cultured in the presence of both types of CNF samples (***Figure 3.B***). In addition, the cell viability was very high – after 24 hours of cultivation, it was approx. 90%, and after 72 h, it increased to almost 100% (***Figure 3.B***).

Thus the cytotoxicity test results indicate that the direct contact of carbon nonwoven fabrics with the human lung adenocarcinoma cells line A549 did not show any significant cytotoxicity effect. The cells cultured in the presence of both types of CNF retained their elongated or polygonal shape (***Figure 6***). The lower cell number observed in cultures with CNF and CNF_HA meshes could be explained by the mechanical obstruction posed by these meshes, which in fact covered the cells, rather than by the direct cytotoxic effect of these samples. The cell populations cultured in the presence of both types of meshes contained a slightly higher number of rounded cells, i.e. cells in mitosis or dying cells, which may be a sign of a higher turnover of cells (***Figure 6***).

Cellular cytokine IL-6 production by leukocytes after contact with hyaluronan-modified and unmodified carbon materials is shown in ***Figure 4***. The cytokine IL-6 amounts elicited by cells cultured in contact with carbon nonwovens materials were similar to those of the control group.

Adhesion and proliferation of osteoblast-like cells on HA modified and non-modified carbon nonwoven fabrics

The numbers of cells colonising the surface of CNF and CNF_HA samples and the control material (microscopic glass coverslips) 7 days after seeding are presented in ***Figure 5***. There were significant differences among the cell proliferation on the nonwoven materials. The MG-63 cells cultured in the presence of hyaluronic acid proliferated better than those in wells with non-modified carbon nonwovens.

Results of observations of the morphology of cells seeded on the surface of carbon nonwoven fabrics (CNF and CNF_HA) are presented in ***Figure 7***. The morphology of cells observed on both types of nonwovens was very similar to that

of cells incubated on the control glass surface, i.e. cells of both groups were mostly spindle-shaped (***Figure 7.A***). In the case of non-modified carbon nonwoven, cells (CNF) were uniformly spread along fibres and covered almost the whole of their surface (***Figure 7.B***). In the case of HA modified nonwovens (CNF_HA), cells covered the surface of the hyaluronan film formed among the fibres (***Figure 7.C***), which can explain the higher number of cell found on CNF_HA than on CNF only. Such a spreading of cells indicates their good adhesion. Cell counting and fluorescence microscopy observations confirmed the good biocompatibility of modified carbon nonwovens, which is substantiated with both the higher number of cells 7 days after seeding and the good spreading of cells.

***In vivo* results**

It is well known that fibrous matrices are highly promising for tissue regeneration by acting as a cell-supporting scaffold. It may be expected that the application of carbon nonwoven fabrics modified with HA would not only provide a mechanical function but also have regenerative

potential in the treatment of cartilage diseases.

Microscopic examination of cartilage defects filled with carbon materials (CNF, CNF_HA) 2 months after implantation showed that in both cases newly formed cartilage tissue is present in the outer layers of defects (*Figure 8.A - 8.B*). After 6 months from implantation, the processes of cartilage and bone tissue regeneration were more pronounced and observed also in deeper layers (*Figure 9.A - 9.B*).

In the Masson-Goldner-stained sections of both specimens, increased neovascularisation was noticed. Numerous capillaries present in deeper layers of treated defects allow to expect the proper re-construction of bone tissue. The results of the *in vivo* experiment showed that animals treated with carbon nonwovens both at 2 and 6 months revealed the presence of repairing mechanisms. Noticeable differences in tissue response to both types of implanted materials were observed after 6 months from implantation. In the case of the unmodified implant, a large majority of the implant area was filled with granulation tissue (*Figure 9.A*), whereas the faster growth of hyaline-like cartilage was observed in the presence of HA modified implants (*Figure 9.B*).

After 6 month from implantation, particles formed after the fragmentation of carbon nonwovens were observed (*Figure 9*).

The fragmentation of the carbon materials and toxic effects of fiber degradation products on the human body have been reported before [30]. The biological behaviour of carbon fibrous materials depends on the type of single fiber, its structure, chemical surface state and elemental composition [31]. It has been demonstrated that the cellular response to the fibrous carbon material depends on the degree of crystallinity of the material, therefore only selected types of carbon fibres are suitable for tissue treatment purposes [32]. Low-carbonised carbon fibres, used in this study, undergo partial fragmentation and react with the biological environment by being gradually resorbed at the implantation site [33]. Thanks to their amorphous structure the particles formed after defragmentation are acceptable for macrophages [34].

■ Conclusions

The results obtained in this study demonstrated that the 3D matrix of the HA modified carbon nonwoven was successfully prepared, and seemed to be a suitable scaffold for thetreatment of cartilage defects. The scaffolds prepared in this study were toxicity-free. In addition, the scaffolds modified with HA were non-immunogenic, as shown by the non-elevated production of interleukin 6 in *in vitro* experiments and by the absence of granulation tissue in experiments *in vivo*. The results of the *in vitro* studies not only showed that cells attached well and retained their good viability in the carbon nonwoven matrix but also suggested that the incorporation of hyaluronic acid resulted in the enhancement of cell proliferation. After the implantation of carbon nonwovens into the knee cartilage of rabbits, a faster process of tissue regeneration was observed in the case of carbon nonwovens modified with hyaluronic acid. The modification of carbon nonwoven fabrics using hyaluronic acid can lead to the development of a new material, which may be a good candidate for the manufacture of cartilage scaffolds.
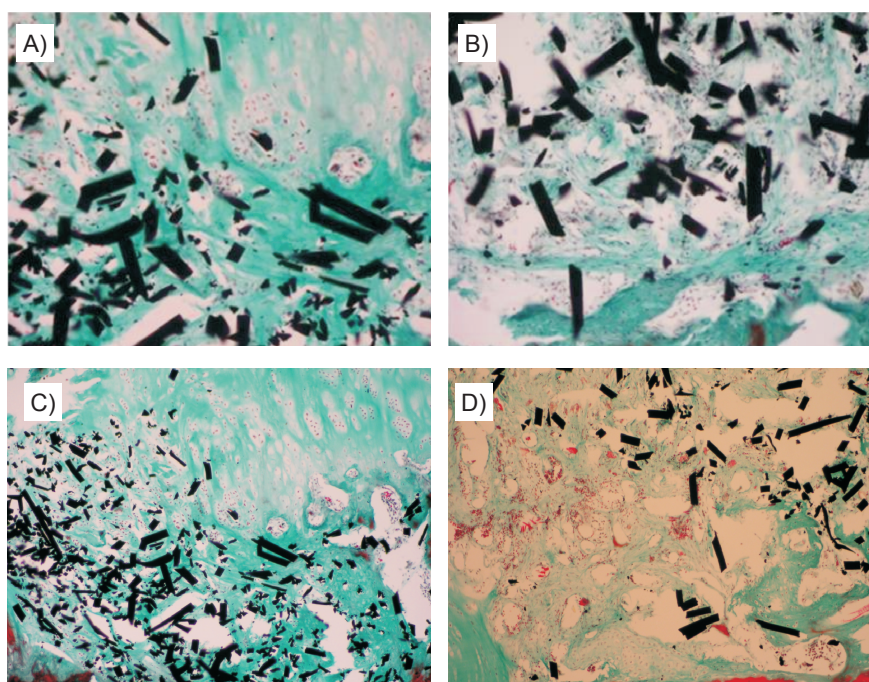

Figure 8. Defects filled with carbon materials 2 months after implantation: (A) unmodified carbon nonwoven (CNF) and (B) carbon nonwoven fabric modified with hyaluronic acid (CNF_HA). Masson-Goldner staining. ((A) and (B) magnification 20× and (c) and (d) magnification 10×).
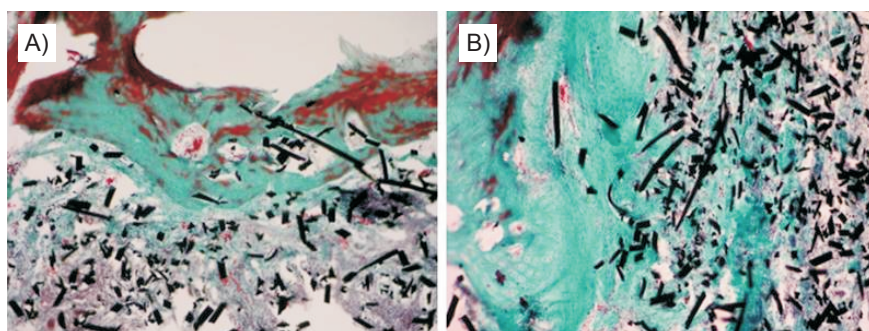

Figure 9. Defects filled with carbon materials 6 months after implantation: (A) unmodified carbon nonwoven (CNF) and (B) carbon nonwoven fabric modified with hyaluronic acid (CNF_HA). Masson-Goldner staining. Magnification 10×.

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