Biomechanical and biological properties of the implant material carbon-carbon composite covered with pyrolytic carbon

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The aim of this study was to test C/C material (carbonized, graphitized or covered with pyrolytic carbon) designated for the use in orthopaedic and bone surgery. Using an *in vitro* assay we confirmed, that the cell proliferation was exhibited the mostly on the C/C composite coated with pyrolitic carbon and afterwards polished. The two latest of subsequent water extracts of this material had a slightly inhibiting effect on the cells metabolic activity. Biocompatibility test *in vivo* performed subcutaneously on rats did not show big differences between three tested implants (C/C composite, epoxy resine, titanium alloy), on the other hand the plates tested on pigs demonstrated foreign-body reaction induced by wear C/C composite material. Such debris were found both in the neighborhood of the implant as well as in the lymphatic node.

1. Introduction

Carbon-carbon composites (C/C), or rather carbon-fiber-reinforced carbons, are playing a more and more important role in technical as well as medical applications [1, 2]. The requirements for these materials-bioinertness and nontoxicity—have been recently evaluated in various studies [3–8].

C/C composites consist of uni-, bi- or multidirectional carbon-fiber reinforcement embedded in carbon matrix. The resulted mechanical and biological properties are determined not only by the above mentioned macro-structure but also by the type of carbon fibers, the initial precursor of the carbon matrix and the process conditions of carbonization or probably graphitization [1]. It is known that in bone surgery the metal bone plates, mostly used for the fixation of fractures, exhibit, together with a satisfactory strength, also high rigidity from the side of the bones [9]. This can cause various complications, e.g. atrophy of bones. We tried to eliminate these disadvantages by using a bone plate made by C/C composites, which exhibit sufficient strength but also lower rigidity. Preliminary studies performed on cultured vascular smooth muscle cells showed that these materials are supportive for cell adhesion and growth [10]. On the other hand, these materials are limited by their fragility, which often leads to the release of microparticles into the surrounding tissue [11].

The aim of this study was to test C/C material (carbonized, graphitized or covered with pyrolytic carbon) for the use in orthopaedic and bone surgery. Using an *in vitro* assay, we followed: (a) the growth of human embryonal lung fibroblasts (LEP) in contact with C/C composites and (b) the influence of the medium prepared from subsequent extracts of the C/C composite on the metabolic activity of LEP cells. *In vivo* the biocompatibility of the C/C composite was studied by (a) subcutaneous implantation to rats and (b) by implanting into the vicinity of the artificial bone defect in pigs.

2. Material and methods

2.1. Implant materials

C/C composites were prepared from plain-woven cloth (Torayca Carbon Fibers T 800, Soficar, France) and phenolic resin (Umaform le, Synpo, Pardubice, Czech Republic). The prepregs were stacked in the necessary number of layers, cured, carbonized at 1000 °C in nitrogen, reimpregnated with phenolic resin and graphitized at 2200 °C in argon. Pyrolytic carbon was deposited from propane in a tumbling bed reactor as described [12]. The mechanical properties of the C/C composites are given in Table I.

From the tested materials small pellets ($24 \times 15 \times 1$ mm) were prepared for tissue culture assays and for subcutaneous implantation to rats, further bone plates ($65 \times 15 \times 5$ mm with 6 holes) for implantation to pigs. The materials were washed in distilled and deionized water and autoclaved before using.

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TABLE I Mechanical properties of C/C composite material used in the experiments

Material	Apparent density g/cm^3	Open porosity %	Flexural strength MPa	Flexural stiffness Nmm^2
Bone	1.85	18	100–150	50×10^6
C/C carbonized	1.30	29	97	—
C/C graphitized	1.40	15	180	—
C/C graph. + pyr.C	1.50	10	250	2.6×10^6

2.1.1. Extraction procedure of the implant material

The C/C material covered with pyrolytic carbon was repeatedly autoclaved in deionized water for 1 h at the temperature of 125 °C as extreme. Seven subsequent extractions were carried out.

2.2. Cell culture

Embryonal human lung fibroblasts LEP (24th–26th passage; SEVAC Prague, Czech Republic) were used in the experiments. The methods and the evaluation were performed according to the International Standard [13].

2.3. Animals

2.3.1.

Twenty seven adult Whistar rats, weight of 100 g, both sexes from Anlab Prague (Czech Republic).

2.3.2.

Six black Vietnamese pigs of both sexes, weight of 30–40 kg, age 2 years (Anlab Prague, Czech Republic).

2.4. Cytotoxicity tests *in vitro*

2.4.1. The direct contact: proliferation of the cells on the tested material

We tested with a cultivation method the effect of the surface quality (polished and untreated) of the implanted C/C composites: carbonized, graphitized and covered with pyrolytic carbon. The small pellet samples (see above) were placed on the bottom of plastic bacteriological grade Petri dishes, the diameter 60 mm (GAMA, České Budějovice, Czech Republic). The LEP were inoculated at the density of 15,000/cm^2 and cultured in MEM (SEVAC, Prague, Czech Republic), supplemented with 10% bovine fetal serum (Veterinary University, Brno, Czech Republic) penicillin 100 U/ml and streptomycin 100 µg/ml (SEVAC, Prague, Czech Republic). The medium was changed every 3 days while adherent cells proliferated. In the first week after the seeding, the cells were detached from the tested material by trypsinization and then they were counted. Plastic Petri dishes for tissue culture were taken as a positive control.

2.4.2. The extracts: the metabolic activity of cells cultured in the medium prepared by using the liquid extracts from C/C composite materials

The LEP cells were seeded into plastic cell wells (25,000 cells/well), (96-wells, Corning, New York, USA), and cultured in these composite material liquid extracts to which MEM components (see above) were added. MTT (dimethylthiazolediphenyltetrazolium bromide; Sigma, Prague, Czech Republic) was added after 48 h of cultivation [14] and after additional 6 h of cultivation the medium was discarded and formazan crystals were solubilized by 10% SDS (dodecylsulfate sodium salt; Serva, Heidelberg, Germany). The color reaction was measured with ELISA Reader (Bio-Rad Laboratories, CA, USA) at the wavelength of 580 nm.

Statistical evaluation was performed according to the Student's unpairent *t*-test.

2.5. Cytotoxity test *in vivo*

2.5.1. Rat

Three types of materials—C/C composite, epoxy resin (Type SVU, Polycom Ltd, Prague, Czech Republic) and titanium alloy Ti-6Al-4V (Poldi, Kladno, Czech. Republic) ($18 \times 8 \times 1$ mm) were implanted subcutaneously to the rat interscapular region under ether anaesthesia and aseptic conditions. The rats were divided into three groups according to the implanted material. After 5 days, 1 and 2 months of implantation, the implants were explanted together with the surrounding tissue. The formation and quality of connective tissue capsule including infiltration by inflammatory cells was studied.

2.5.2. Porcine bone

Under general anaesthesia (5% Narcamon; Léčiva, Prague, Czech Republic), C/C composite splints were implanted to the cover the porcine femur artificial defect. The splints were fixed by six metal screws. The animals were killed 5 or 12 weeks later and the femurs with capsule surrounding the bone plates were explanted.

2.6. Histological procedure

After fixation with Baker's solution the tissue specimens were routinely dehydrated by an increasing concentration of ethanol. The samples were embedded in parafin and they were sectioned (5 µm), stained with hematoxylin- eosin and mounted using Entellan (Merck, Darmstadt, Germany).

2.7. Immunocytochemistry

Tissue samples were embedded in the Tissue-Tek (Miles Scientific, Naperville, IL USA), frozen in liquid nitrogen, sectioned (5–6 µm) and fixed by 2% paraformaldehyde in PBS (pH 7.2–7.4). These samples were used for immunohistochemical detection of

fibronectin, chondroitin sulfate, ED1 and ED2 macrophage markers and cytochemical detection acid and alcaline phosphatase. The specific polyclonal anti-human fibronectin and monoclonal anti-chondroitin sulfate (Sigma, Prague, Czech Republic) antibodies and monoclonal antibodies ED1, ED2 (Serotec, Oxford, UK) were used as a first step. The peroxidase-labeled swine anti-rabbit conjugate for fibronectin assay and swine anti-mouse conjugate (SEVAC, Prague, Czech Republic) for chondroitin sulfate were used as a second step antibody. Control experiments were performed by the omission of 1st step antibody on its replacement with pre-immune serum. The slices were mounted in glycerine-gelatine. Acid and alcaline phosphatase were detected in frozen sections by the azocopulate method [15]. Briefly: the slices were fixed in 2% paraformaldehyde, washed in PBS (pH 7.2–7.4) and incubated (37 °C, 30–120 min). Incubation medium for the acid phosphatase assay: acetic acid buffer with pararosaniline (Sigma, Prague, Czech Republic) and naphtol AS-BI (Sigma) dissolved in dimethylformamide (Sigma). Incubation medium for the alcaline phosphatase assay: naphtol AS-BI in Tris buffer (pH 8.2–9.2). The omission of specific substrate was used to the control of the reaction specifity.

3. Results

3.1. Tests *in vitro*

3.1.1.

The materials with a lower degree of up-grading treatment (carbonization and graphitization) exhibit a lower increase in the cell population density in comparison with the pyrolytic-carbon coated C/C material. The untreated C/C composite materials with a rough surface can have a negative effect on the optimal adhesion of cells. The proliferation of cells on the C/C composite coated with pyrolytic carbon and with a smooth surface is the best of all in comparison with positive control (the plastic) (Fig. 1).

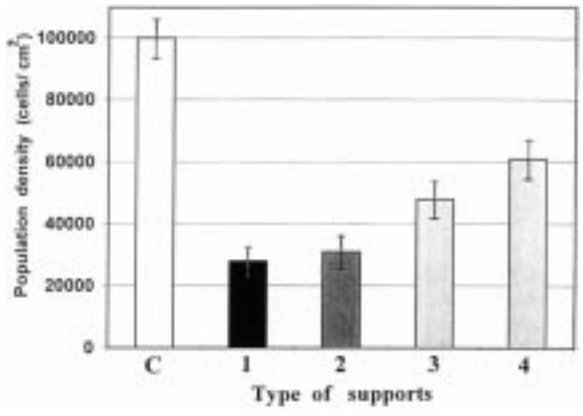

Figure 1 Influence of C/C composite samples on the growth of LEP cells. C/C composites: (1) carbonized, (2) graphitized, (3) covered with pyrolytic carbon, all materials a rough surface. (4) covered with pyrolytic carbon, with a smooth surface. Data are shown as number of cells per 1 cm^2 area of material. Each value represents the mean $\pm$ standard deviation of five samples.

3.1.2.

The first two extracts from C/C composite coated with pyrolytic carbon showed slightly inhibiting and the third extract manifested a slightly stimulating effect on the metabolic cell activity (non significant vs control). Further extracts significantly inhibited the metabolic activity of cells significantly at 0.01 level vs control (Fig. 2).

3.2. Biocompatibility tests *in vivo*

3.2.1. Rat

All types of implants induced the foreign-body reaction and they were surrounded by a connective tissue capsule. Almost the same histological feature was observed in the case of tested C/C composites and titanium implants. The epoxy resin implant was surrounded by a significantly thicker, highly vascularized capsule infiltrated with higher amount of leukocytes (Fig. 3).

The capsules surrounding both titanium and C/C composite were characterized by a mild infiltration by $ED1^+$ macrophages contrary to the epoxy implant, the capsule of which was infiltrated more distinably (Fig. 4). The connective tissue capsule, surrounding both C/C composite and epoxy resin, exhibited higher concentrations of chondroitin sulfate as well as fibronectin (Fig. 5).

3.2.2. Pig

The C/C composite material debris were found in a different amount in the vicinity of all samples. The debris were also located inside the compact bone which was in the neighborhood to the implant (Fig. 6). The implants and debris induced a foreign-body reaction, accompanied with an extensive infiltration with inflammatory cells. The tissue contained a higher number of acid phosphatase positive macrophages and foreign-body giant multinucleate cells. The destruction or degeneration of the neighbor muscle fibers were observed and macrophages invaded the fibers (Fig. 7). The connective tissue expansion around the implant was

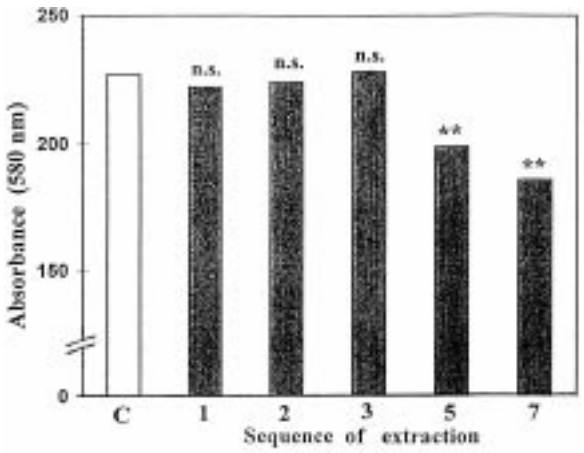

Figure 2 Influence of the water extracts from C/C implant material on the metabolic activity of LEP cells. C, control: H_2O_{deion}, 1–7 subsequent water extracts from C/C composite covered with pyrolytic carbon (**$P < 0.01$, n.s. versus control).

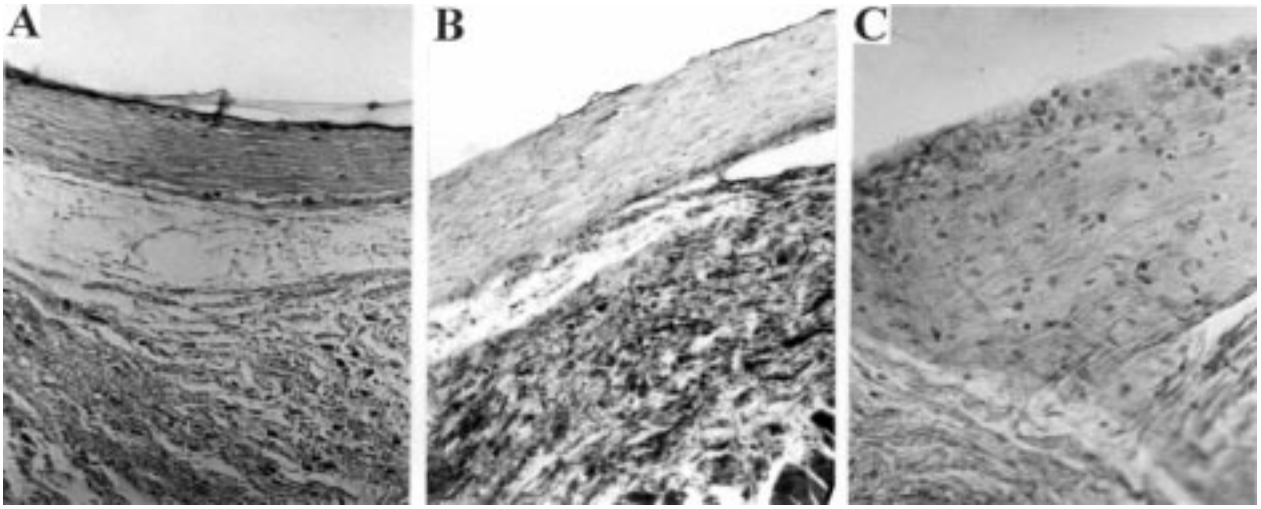

Figure 3 Cross section of the connective tissue capsule surrounding the implants prepared of C/C composite (A), titanium (B), epoxy resin (C), two month after the surgery. Rat, hematoxylin and eosin, magnification × 250.

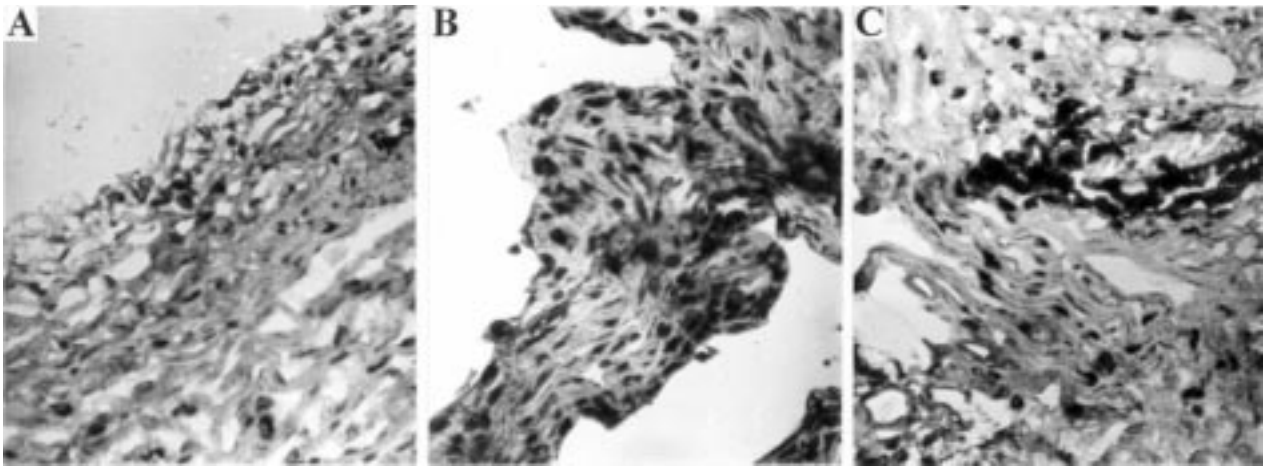

Figure 4 Infiltration of the connective tissue capsule surrounding the implant by $ED1^{+}$ macrophages, the fifth day after the surgery. (A) C/C composite, (B) titanium, (C) epoxy resin. Rat, specimens coulter stained with hematoxylin, magnification × 250.

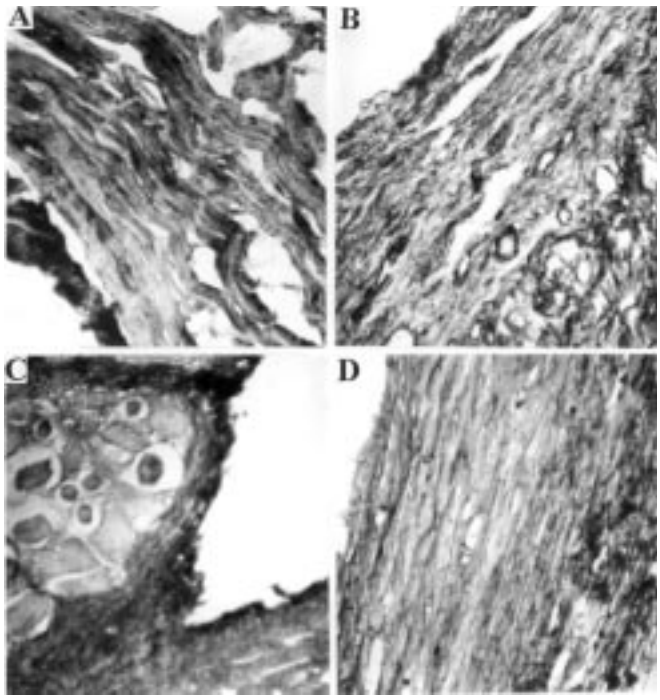

Figure 5 Detection of fibronectin (A,B) and chondroitin sulfate (C,D) in the rat connective tissue capsule surrounding the implant : C/C composite (A, C), epoxy resin (B, D), one month after the surgery. Stained with hematoxylin, magnification A,B,D × 250 and C × 130.

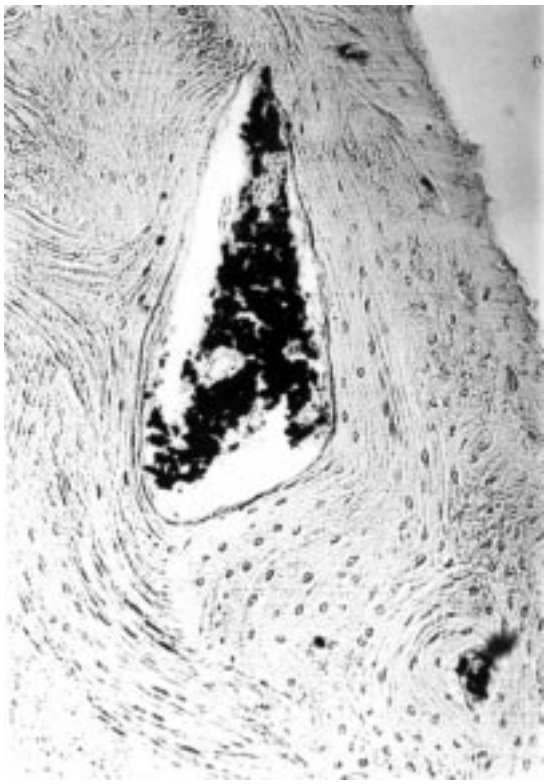

Figure 6 The debris of C/C composite material in the cystic dilatations in the compact bone. Porcine femur, three months after the surgery, decalcified specimen. Hematoxylin-eosin, magnification × 130.

clearly visible. Extracellular matrix of this connective tissue was highly positive for fibronectin and chondroitin sulfate (not demonstrated), similarly like in rats.

In inguinal lymph nodes exhibited significant activation with well developed germinative centers of lymphatic follicles. It is noteworthy, that deposits of implant debris were detected in the cortex of the lymphatic nodes (Fig. 8).

4. Discussion and conclusions

Adhesion of cells to a biomaterial surface is a important factor mediating its biocompatibility and can be influenced by factors such as surface charge hydrophobicity and topography of material [16]. The quality of the material increases when the C/C material was covered with pyrolytic carbon and polished (according to Bačáková, [10]).

For the cells metabolic activity *in vitro* test we used repeated extraction procedures, which attempted to exaggerate conditions in the living organism in order to define the potential toxicological hazard (one of recommended conditions according to [13]). The first three extracts had not significant influence on the metabolic activity of the cells. The further fifth and seventh extracts of this material exhibited a moderate but not drastic cytotoxic effect, which showes, that by extreme conditions a release some toxic substances is possible.

After preliminary assays *in vitro* only those implants of C/C material covered with pyrolytic

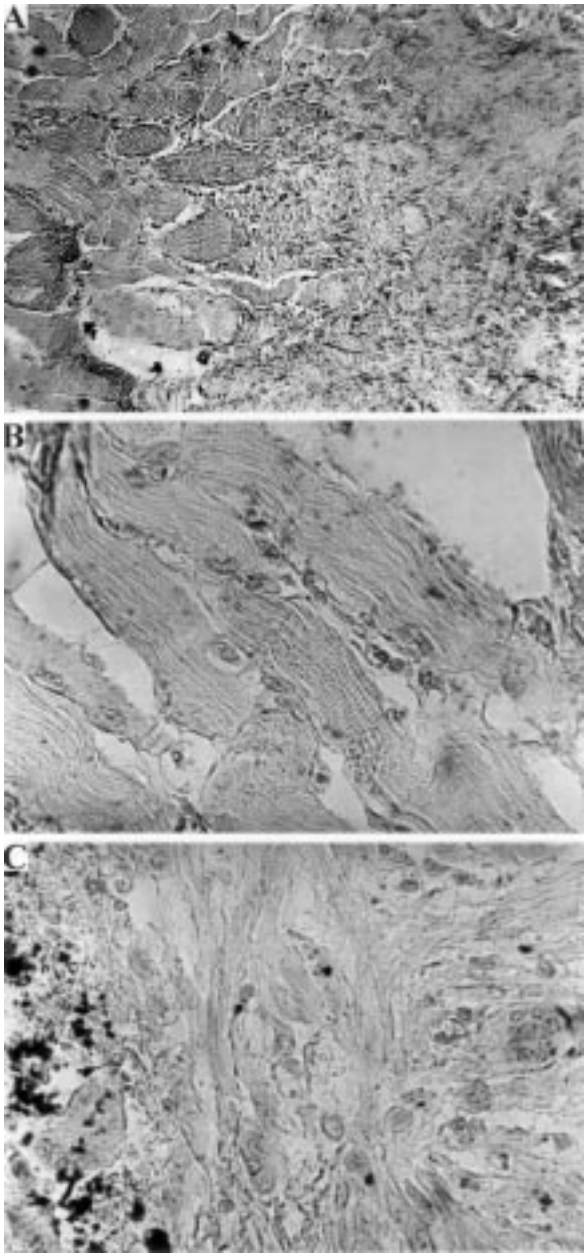

Figure 7 The invasion of connective tissue surrounding the implant into the muscle (A). The muscle fibers eroded and infiltrated by macrophages (B) and numerous foreign-body giant multinucleate cells in tissues in the vicinity of the implant (C). Porcine femur, three month after the surgery, hematoxylin-eosin, magnification A,C × 250, B × 380.

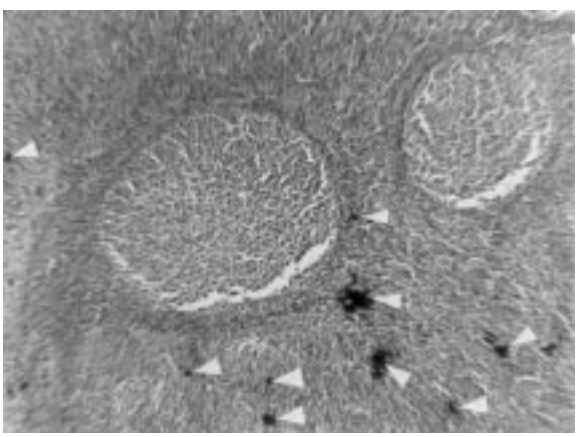

Figure 8 The debris of C/C composite localized in the porcine inguinal lymph node (arrow), three months after the surgery. Hematoxylin-eosin, magn. × 130.

carbon, which gave the best results were used in tests *in vivo*.

The process of a foreign materials save in living organism is very similar and therefore another materials procedure for the evaluation of implant biocompatibility were used [17]. From the immunohistological and histological results it is obvious that C/C composite implanted subcutaneously into rats induce a foreign-body reaction fully comparable with titanium alloy, the generally used biomaterial [18] in clinical practice. The influence of the epoxy resin implant was a little worse comparing to the C/C implant material. The epoxy resine material has been chosen as negative control in accordance with its possible non stability, when it releases agents [19].

The application of the C/C implant to the porcine bone was complicated by wear resulting of the high amount of material debris in the surrounding tissues. We tried to solve this problem by covering the release of carbon microparticles material surface by a thicker carbon biocompatible layer. However this wear could be also explained by the mechanical interaction of metal screws with the plate material, because rats tissue samples surrounding the subcutaneous implants did not contain the C/C material debris.

These results seem to be quite stimulation for the further development of devices prepared from the C/C composite covered with pyrolytic carbon for bone surgery.

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