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Biological properties of the intervertebral cages made of titanium containing a carbon-carbon composite covered with different polymers

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Intervertebral cages are used in orthopaedics for stabilization of injured lumbar parts of vertebral columns. Our study provides preliminary results of tests of the biological properties of titanium cages with a variously modified carbon/carbon composite (C/C) core. This core was produced from a C/C composite modified by hydrogel materials based on poly(2-hydroxyethyl methacrylate) (HEMA) enriched with 1% collagen or 35% methylmethacrylate or 30% terc-butylmethacrylamide.

We evaluated the adhesion of the cells to the tested material coating using an *in vitro* study of the metabolic activity and cytokine production of the cells (TNF-α, IL-8). We studied the biocompatibility of intervertebral cages coated with different copolymers under *in vivo* condition and in an implantation experiment in the porcine femurs.

Both *in vitro* and *in vivo* results revealed favourable biotolerance of the use system. Modification of the composite HEMA with the use of collagen seems to have a more positive effect on the new bone tissue formed around the implanted devices than HEMA copolymerized with methylmethacrylate or terc-butylmethacrylamide.
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1. Introduction

The application of implants in orthopaedics and bone surgery has become widespread in the past ten years [1–3]. The interface between the bone and the synthetic material as well as the biomechanical properties of the implanted devices represent the focal point of research into and the development of new bone implants.

Intervertebral cages are used in the treatment of lumbar vertebral column injuries. Until now, cages based on titanium alloys have mostly been used [4, 5]. However devices prepared with polymer materials are more suitable because of (a) lower stiffness compared to metals, (b) X-ray, CT projection and magnetic resonance transparency [6]. Intervertebral cages have been launched on the market recently, e.g., cages based on composite polyetheretherketone-PEEK [3]. The surgical procedures that apply the cages use bone grafts to achieve the required integration of two adjacent vertebrae. Auto-spongioplasty applying the patient's own bone tissue is the safest and advantageous method from the standpoint of the healing process but it requires an extra-operating access, entailing the risk of complications [7]. Allo-spongioplasty utilizing bone grafts from a donor is more friendly for the patient but the risk of transfer of infection is quite high at present [8]. Financial and ethical considerations are also important.

The C/C composite was inserted to the titanium cage to test the biological behaviour of proposed implantable devices with perspective to exclude bone tissue graft from the system. This hypothesis is based on previous results where carbon-carbon composites exhibit a very good biotolerance because these composites are non-toxic and the soft tissue and bone integrate with them very well [9, 10].

Building on the long-standing research tradition in our laboratory dealing with carbon composite materials [11, 12], we studied the possibility of employment of these materials in the construction of an intervertebral cage. The first results demonstrating the biological behaviour of this device in animal experiment are shown

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in this study with aim to compare the influence of different polymer on reducing the release of composite debris and on the in-growth of hard tissue into the implant.

2. Materials and methods

2.1. Implants preparation

The preparation of carbon-carbon composites impregnated and covered with a pyrolytic carbon layer (C/C) was described earlier [11, 12]. We used these C/C composites infiltrated and coated with three distinct copolymers for intervertebral cages of a new type. The following modifications of C/C were prepared and analyzed:

(a) poly(-2 hydroxyethyl methacrylate) (HEMA) with 1% collagen (HEMA-C)

(b) HEMA copolymerized with 35 mol% of methylmethacrylate (HEMA-*co*-MMA),

(c) HEMA with 30 mol% of terc-butylmethacrylamide (HEMA-*co*-BMAA)

The preparation of copolymers of HEMA-C by additional crosslinking of the basic materials was performed with 0.25% (v/v) aqueous glutaraldehyde [13]. The copolymers HEMA-*co*-MMA and HEMA-*co*-BMAA were prepared with two different molar ratios by solution polymerization in ethanol at 70 °C for 4 h [14].

The final C/C composite samples were impregnated and covered with the three mentioned copolymers in an autoclave by alternating a vacuum and a pressure of 0.4 MPa at 40 °C (see a), or by immersion into a solution only (see b, c). The probes were dried in vacuo at 50 °C for 24 h. The presence of modified HEMA-C in the open pores of the C/C composite was confirmed using the image analysis method in the LUCIA G system version 4.60, (Laboratory Imaging, Prague, Czech Republic) using the Nikon Optiphot 100s metallurgical microscope (Nikon, Prague, Czech Republic) and the infrared (IR) microspectra were measured using the Magna 750 spectrometer (Nicolet Instruments., Madison, USA) (Figs. 1 and 2).

All types of materials described above in the form of a block (3 × 6 × 4 mm) were inserted into a commercially available titanium alloy cage (5 × 6 × 7 mm) (Medin, Nové Město na Moravě, Czech Rep.) (Fig. 3).

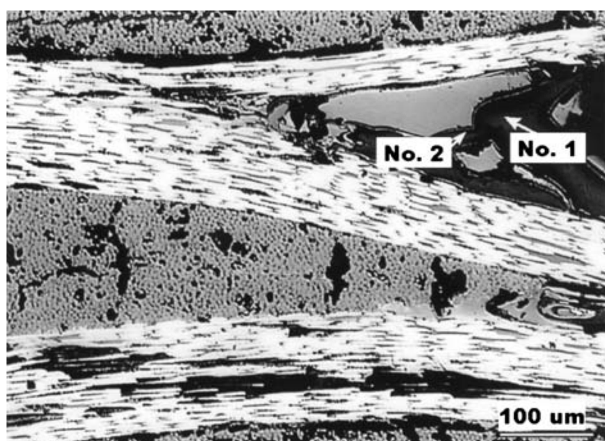

Figure 1 A micrograph of the polished section of the implant (composite C/C) infiltrated by the copolymer HEMA-C. Analysis of the pure components and of the areas No. 1 and No. 2 are presented in Fig. 2.

The prepared implants were sterilized using the STERRAD system (ASP c/o, J&J Medical, Wien, Austria) at 45 °C before the *in vitro* testing or implantation.

2.2. Cytotoxicity tests *in vitro*

2.2.1.

Small plates (20 × 15 × 1 mm) of the C/C + HEMA-C materials were used for *in vitro* testing. Human embryonal lung fibroblasts LEP (21st–22nd passage, Sevapharma, Prague, Czech Republic) were cultivated under standard conditions [11, 15].

2.2.2.

We used the methods described in our previous report [12] for assays of cell adhesion and metabolic activity. After the cultivation, LEP cells covering the surface of tested materials (C/C + HEMA-C) were stained with a 1% aqueous solution of Propidium Iodide (Sigma-Aldrich, Prague, Czech Republic). Subsequently, we inspected samples of the materials with cells in a fluorescence microscope (Olympus BH2, Prague, Czech Republic). The metabolic activity of these cells was estimated using MTT tests, such as those described [11, 12].

2.2.3.

The cultivation experiment was also performed for cytokine TNF-α and IL-8 detection using the IMMULITE analyzer (DPC, Los Angeles, CA, USA) [12]. The results were compared with measurements of the number and activities of the cells after cultivation on polystyrene of bacteriological as well as tissue-culture grade.

A statistical evaluation was performed using the Student's unpaired t-test.

2.3. Biocompatibility tests *in vivo*

2.3.1.

Seven Göttingen miniature pigs of both sexes, weight of 40–60 kg, age 6–12 months (BioTest Ltd. Pardubice, Czech Republic) were used.

The tested devices (see 2.1., Fig. 3) were implanted into the lateral epicondyle of the distal femur of both hindlimbs under sterile conditions for 3 and 12 months respectively. The animals were kept under standard conditions according to the rules for handling laboratory animals valid in the Czech Republic. The types and numbers of implants are summarized in Table I.

2.4. Histological investigation

Each implant was removed with the surrounding tissue and divided in two pieces (Fig. 4). Investigations were performed using two independent procedures. After fixation with Baker's solution, the piece of bone adjacent

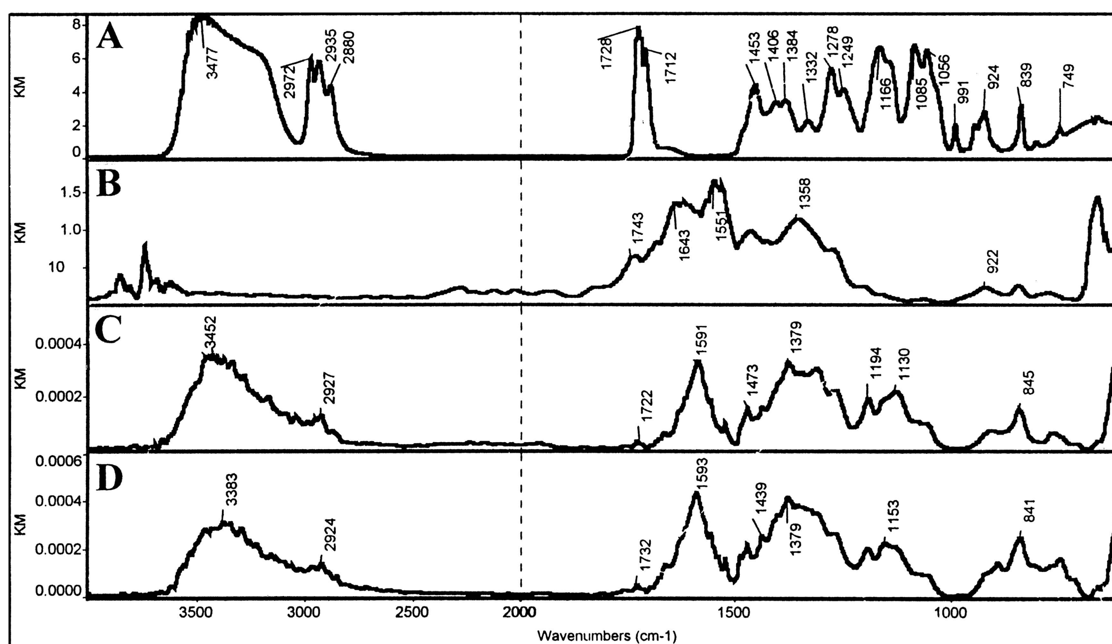

Figure 2 Infrared microspectra of the pure HEMA-C (graph A), glass–like carbon, matrix of the used composite (graph B), and analyzed areas marked in Fig 1., No. 1 (graph C), and No. 2 (graph D).

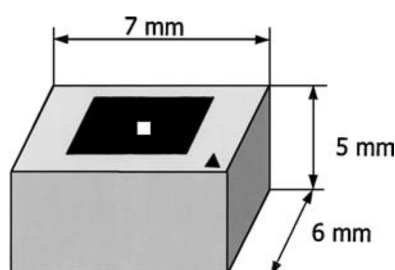

Figure 3 The intervertebral cage prepared for the pig bone embedding. C/C (□) coated by one of the copolymers. Titanium alloy (▲).

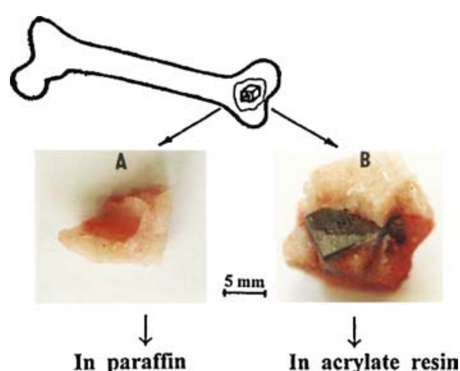

Figure 4 The preparation of samples for the histological procedure, (3 months after implantation.): (A) A part of the surrounding bone tissue from the porcine femur epicondyle region was removed and prepared for the paraffin embedding. (B) The implant with a piece of surrounding bone from the same region was embedded in the acrylate resin.

to the implant was decalcified with formic acid and sodium citrate (1:1) and rutinely embedded in paraffin (Fig. 4(A)). The 7 μm sections were stained with the hematoxylin-eosin or Masson trichrome procedure (Bio Optica, Milano, Italy). The implant together with the remaining surrounding bone tissue (Fig. 4(B)) was embedded in acrylate resin as described according to the Techniques of Biocompatibility Testing [16] and the 30 μm sections were stained with hematoxylin-eosin or with toluidine blue (Sigma-Aldrich, Prague, Czech Republic) (Fig. 5).

TABLE I Types and numbers of implants

Type of the samples C/C modified core	Number of the implanted samples 3 month	12 month
C/C + HEMA-C	3	3
C/C + HEMA-MMA	3	–
C/C + HEMA-BMAA	3	–
C/C	2	–

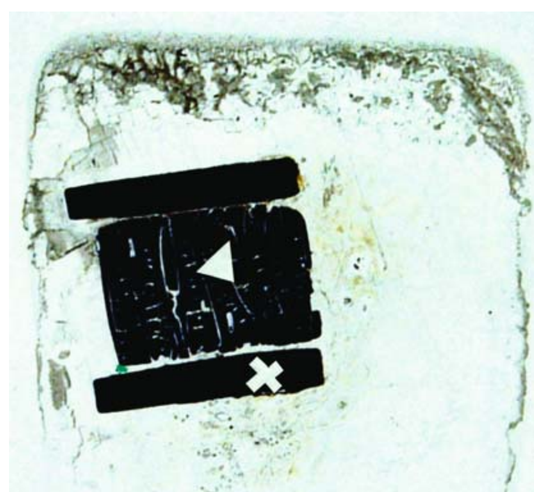

Figure 5 A section (30 μm) through the implant cage with a surrounding tissue, embedded in the acrylate resin. (▲) the C/C + HEMA-C core, (+) the titanium alloy cage. Unstained, magnification 2.3×.

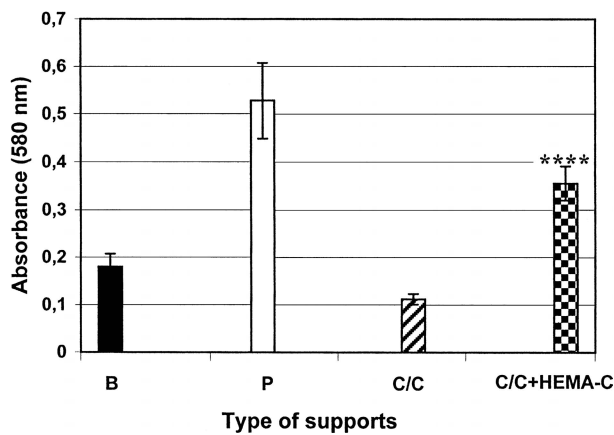

Figure 6 Proliferation of LEP cells, after 3 days of cultivation on the subsequent materials: bacteriological grade polystyrene (B), tissue culture grade polystyrene (P), C/C material, C/C + HEMA-C material (mean ± standard deviation of five samples). **** $P < 0.0001$ versus C/C.

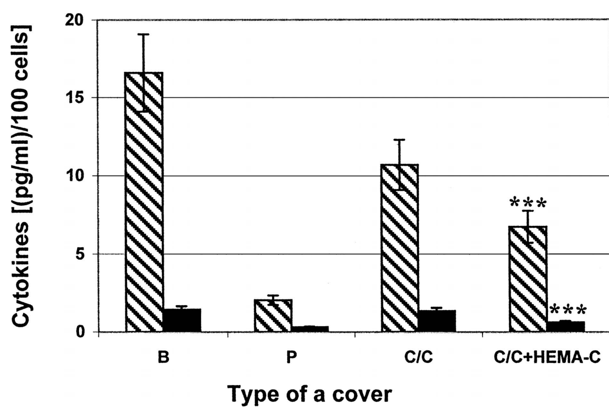

Figure 7 Production of TNF-α and Il-8 by LEP cells growing on the untreated and on HEMA-C treated C/C materials. Types of support: (B) bacteriological-grade polystyrene, (P) tissue culture grade polystyrene, C/C material, C/C + HEMA-C material. ■ TNF-α , ⧵⧵ Il-8, (mean ± standard deviation of five samples), *** $P < 0.001$ versus C/C.

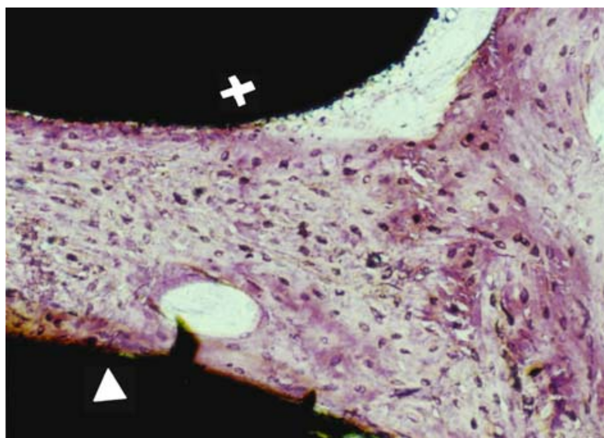

Figure 8 Section of the implant C/C + HEMA-C (▲), of the titanium alloy cage (+) and of the new bone tissue surrounding, embedded in acrylate resin, three months after the surgery. Pig, hematoxylin and eosin, magnification ×250.

3. Results and discussion

3.1. Tests *in vitro*

3.1.1.

Comparison of the adhesion and proliferation of cells growing on the C/C material and cells on C/C coated with HEMA-C material.

The extent of cell proliferation on the surface of the C/C implants was very good, similarly as we described previously [11]. HEMA-C infiltration of the carbon composite significantly stimulated the growth of cells on this type of surface (Fig. 6). No morphological signs of cellular degeneration as a result of material

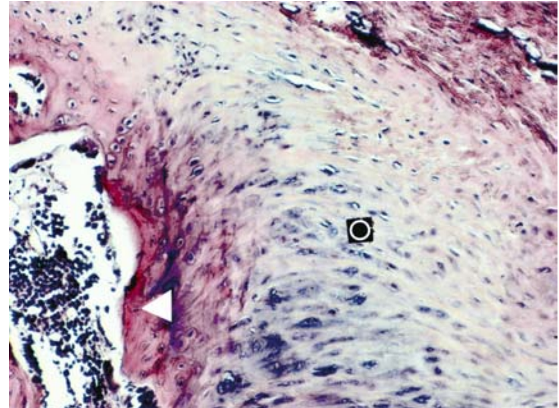

Figure 9 Paraffin section of the connective-tissue capsule surrounding the implants prepared from C/C+HEMA-C. (▲) the original bone trabecule, (◙) newly formed bone in the direction toward the implant. Three months after the surgery. Pig, hematoxylin and eosin, magnification ×160.

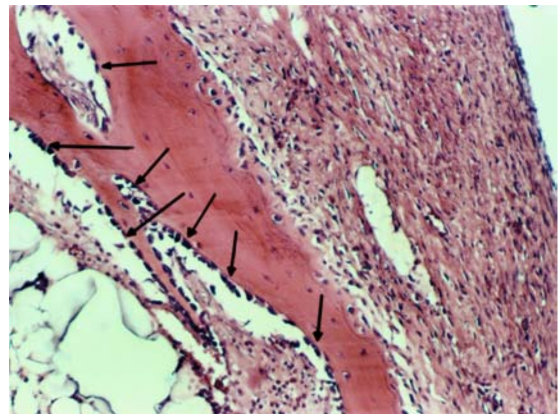

Figure 10 Osteoblasts on the surface of bone trabeculae (↓). Paraffin section of the connective-tissue capsule surrounding the implants prepared of C/C + HEMA-C. Three months after the surgery. Pig, hematoxylin and eosin, magnification ×160.

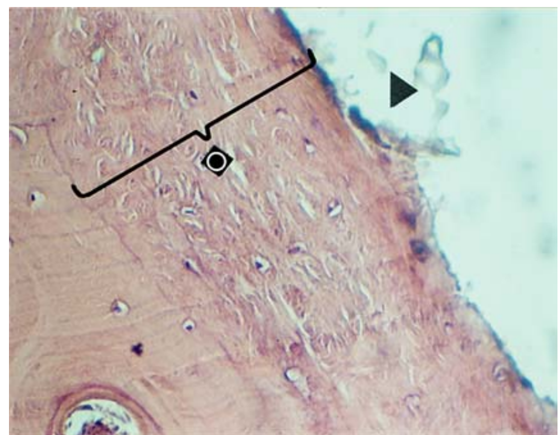

Figure 11 Paraffin section of the newly formed bone tissue (◙) surrounding the implants prepared from C/C + HEMA-C. The position of an implant (▲). Twelve months after the surgery. Pig, hematoxylin and eosin, magnification ×250.

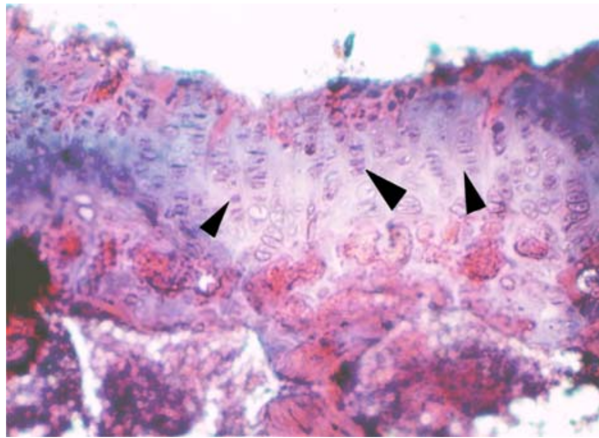

Figure 12 Longitudinally ordered cells of newly formed cartilage (arrows) in the vicinity of C/C + HEMA-BMMA, 3 months after the surgery. Pig, hematoxylin-eosin, magnification ×150.

cytotoxicity were observed in the cells covering these materials. By means of cell metabolic activity measurements using the MTT test it was confirmed that the growth of cells on C/C material coated with HEMA-C was three times higher than in the case of untreated pure C/C composite.

3.1.2.

Assay for detection cytokine production by cells growing on the tested C/C material and on treated C/C + HEMA-C material.

The production of inflammatory cytokines (Il-8 and TNF-α) was always lower in comparison with cells cultivated on the surface of the bacteriological-grade polystyrene and higher than in cells cultivated on tissue-culture grade polystyrene. These changes were pronounced in the production of inflammatory TNF-α than in the release of chemokine II-8. HEMA-C coating of C/C composite material resulted in a reduction of both evaluated inflammatory cytokines (Fig. 7). Because the studied cytokines are known to be proinflammatory agents [17], these findings seem to be important and they suggest a good immunotolerance of this material after the clinical implantation.

3.2. Biocompatibility *in vivo*

In the animal experiments, no principal differences were observed in the tissue/implant interface between the implant system prepared as the cage like composite and the pure-material implants. Almost no

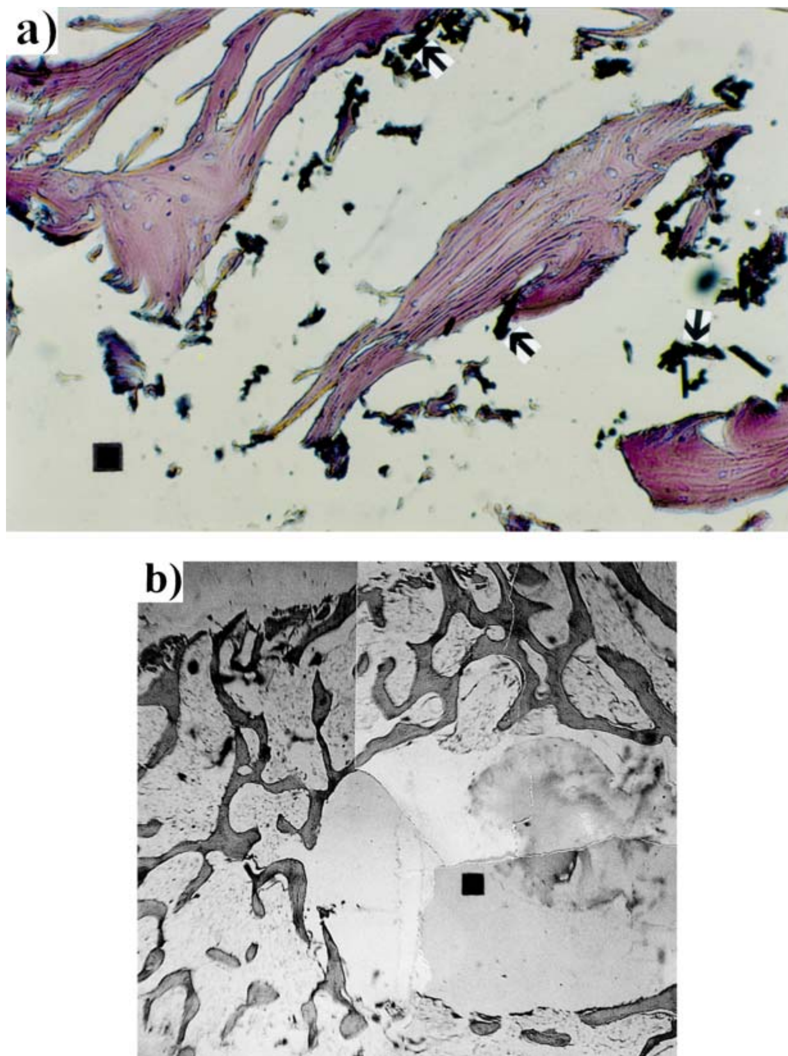

Figure 13 The place of the embedding C/C composite implants (■) to the spongious bone, 3 months after the surgery. Parafin section, pig, hematoxylin-eosin: (a) C/C without HEMA, (↓) carbon wear, magnification 280× and (b) C/C with HEMA, implant circled by spongiosis bone, without carbon wear, magnification 150×.

foreign-body reaction i.e., infiltration in the vicinity of the implant by inflammatory cells and extensive fibroproduction was visible in the tissue/implant interface in the case of all studied materials and implant systems. While the remarkable debris of a implant were observed in the implantation site if the pure C/C composite was used, the treatment of this material by HEMA, which biological properties are know, caused the significant reduction of material wear (Fig. 13(a) and (b)). Three months after the surgery, no signs of cytotoxicity i.e., osteonecrosis were detected.

The HEMA-C used as a glue for fixing the carbon composite core to the cage induced the observed significant ingrowth of bone tissue into the carbon/metal interface (Fig. 8). Signs of extensive production of new bone in the vicinity of this type of implant were visible (Fig. 9). However, the fibroproduction and formation of a connective tissue capsule were also more pronounced in this type of implant. Osteoblasts were observed on the surface of bone trabeculae (Fig. 10). Nine months later, the connective tissue capsule was even absent and the cytological appearance of the vicinity of the implants as well as bone trabeculae suggest a stabilization and a favourable internalization of the implants system into the bone (Fig. 11).

In the case of the implanted cages, where a carbon core was fixed with HEMA copolymerized with MMA or BMMA, we detected similar results as in the HEMA-C experiments. The connective tissue capsule was relatively thick three months after the surgery. However, the formation of a newly formed bone was detected. Interestingly, regions of the newly formed cartilage with longitudinally arranged cells were observed in the vicinity of this type of implant (Fig. 12).

In comparison with our previous results, mentioned pure C/C composite [11, 12] the polymer cover of the C/C composite significantly improved the biotolerance of tested materials. The difference between these two systems was shown (Fig. 13).

4. Conclusion

In agreement with our previous observation of the tissue tolerance for C/C composite materials, the studied system exhibited reproducible good results [12]. Our study of the biological behavior of devices composed of a C/C composite core adhering to titanium cages with various copolymers reveals signs of favourable biological tolerance. The polymer cover (C/C + HEMA-C based material) improved the biological properties of the studied samples. Both *in vitro* and *in vivo* experiments revealed highly satisfactory biotolerance of the used system. The modification of the composite HEMA by collagen seems to have improved effect on the form of the new bone tissue around implanted devices in comparison with HEMA copolymerized with methylmethacrylate or tercbutylmethacrylamide.

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