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Biological properties of carbon/carbon implant composites with unique manufacturing processes

Guo-Hui Wang · Shu Yu · Shai-Hong Zhu · Chang-Qing Gao · Yong Liu · Yun-Liang Miu · Bo-Yun Huang

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Abstract The goal was to manufacture carbon/carbon (C/C) composites through a unique procedure with improved biocompatibility and reduced debris release. C/C composites were prepared by chemical vapor deposition, and their biological properties were analyzed. With regard to mechanical properties, compressive strength/modulus was 219.1 MPa/9.72 GPa, flexural strength/modulus was 121.63 MPa/21.9 GPa, and interlaminar sheer was 15.13 GPa. Biocompatibility testing revealed: (1) the extract liquid from the C/C composites had no effect on cell proliferation; (2) the extract had no impact on micronucleus frequency as compared with the control groups ($P > 0.05$); (3) in vivo, there was mild tissue inflammation after implantation within the first 2 weeks, but there was no significant difference compared with the control group ($P > 0.05$); (4) the implants were well integrated into the host tissue, and debris was limited. The tested samples have excellent biocompatibilities and reduced release of debris. The demonstrated changes in manufacturing procedures are promising.

Guo-Hui Wang and Shu Yu these two authors contributed equally to this work.

G.-H. Wang · S.-H. Zhu (✉) · C.-Q. Gao · Y. Liu
Transplantation Medical Academy, Third XiangYa Hospital, Central South University, Changsha 410013, People's Republic of China
e-mail: zhushaihong@medmail.com.cn

S. Yu · B.-Y. Huang
State Key Laboratory for Power Metallurgy, Central South University, Changsha 410083, People's Republic of China

Y.-L. Miu
Tianniao High-tech Limited Company, Yixing 214216, People's Republic of China

1 Introduction

The utilization of artificial materials within the field of medicine has become increasingly popular over the past two decades. One such material, metal, has traditionally been widely used in orthopedics. However, metal materials have important disadvantages that must be considered. These include the diffusion of ions from metal implants into the surrounding tissues causing unwanted effects, the degradation of the implanted materials themselves due to corrosion in liquid, and other harmful complications such as atrophy of bone [1].

In contrast, carbon fiber reinforced carbon matrix composites, or C/C composites, are inert materials that are stable in the physiological environment. C/C composites have many advantages such as tolerance of high temperatures and resistance to corrosion. The rigidity of C/C composites is also less than that of metal, which increases its compatibility with bone. Its properties have led the C/C composite to be the most promising new alternative to substances that frequently cause bone erosion and resorption [2, 3].

Wider use of C/C composites, however, is currently limited by its biocompatibility and debris release. In order to increase biocompatibility and reduce the release of particles, polymers have been used to encase the C/C composites [4]. Nonetheless, the binding between C/C composites and their polymer covers is often not strong enough, resulting in detachment of the cover from the composite. While a polymer cover can act as a physical barrier to reduce the release of particles and circle the debris into the implantation area, improved biocompatibility has been found to be negligible [4–6]. A polymer cover also can increase the thickness of the connective tissue capsule surrounding the implant, implying that the polymer cover may hinder integration between host tissue

and the implant [5]. Therefore, adding polymer covers does not appear to be an effective means of increasing biocompatibility.

Mechanical and biological properties of C/C composites are determined by factors such as the type of carbon fibers, initial precursor of the carbon matrix, and the processing conditions of carbonization or graphitization [6]. Further studies in the manufacturing of C/C composites are therefore warranted with the hope of increasing their biocompatibility and reducing debris release. In this study, a patented method was used to synthesize a C/C composite, and the biocompatibility of the resulting product was evaluated.

2 Materials and methods

2.1 Preparation of materials

The C/C composites were produced by a chemical vapor infiltration and deposition process, which involved a preparation consisting of dual carbide deposition of fiber-reinforced composite materials (China patent 200610032336.1). The carbon felt was placed into a chemical vapor deposition furnace maintained below 2 KPa in pressure and between 700 and 1600°C in temperature. A carbon source gas, C_3H_6, and a carrier gas, N_2, were introduced into the furnace, followed by impregnation, curing, carbonization, and coating.

2.2 Cytotoxicity experiments

2.2.1 Reagents and instruments

L929 mouse skin fibroblasts were provided by the central lab of Xiang-Ya Medical School of Central South University, fetal calf serum was purchased from Si-Ji Qing Company (Hang Zhou, China), RPMI-l640 and trypsinase were obtained from GIBCO Company (Beijing, China), and propidium iodide (PI) was provided by Sigma Company (Beijing, China). Male New Zealand white rabbits, Kun Ming mice, and male Hartley-Dunn guinea pigs were delivered from the Experimental Animal Center of Central South University. All experiments involving animals were approved and monitored by the Academic Committee of Xiang-Ya Medical School of Central South University and conducted according to the institutional guidelines on the care and use of laboratory animals.

2.2.2 Preparation of extract liquid

The C/C composites were sterilized at 137.3 kPa and 121°C for 30 min and were then cultured in RPMI-l640 medium (composite to medium ratio of 0.2 g/ml) for 72 h in an incubator at 37°C with 5% CO_2. The extract liquid was then used to test the C/C composite cytotoxicity. RPMI-1640 medium alone, placed in the same conditions, was then collected as a negative control for later use.

2.2.3 Cell culture and flow cytometry assay

L929 mouse skin fibroblasts were cultured in RPMI-l640 medium supplemented with 10% fetal calf serum for 24 h. In the test group, the medium was then replaced by the extract liquid. RPMI-1640 medium alone, prepared in the same conditions as the extract liquid, served as a negative control, and RPMI-1640 with 0.64% phenol provided the positive control. All groups were subsequently cultured for 24 h. Cells were collected after digestion with trypsinase and washed twice with phosphate buffer solution (PBS). Cells were fixed at 4°C with 70% alcohol and washed twice with PBS, followed by staining with PI at a final concentration of 100 μg/ml. The fixed cells were placed in dark storage at 4°C for 30 min, followed by filtration through 400 meshes to exclude cellular debris and aggregates. The fixate was then analyzed using FACscan and CellQuest 3.1f software (Becton-Dickinson).

2.3 Micronucleus test

Twenty-four specific pathogen-free Kun Ming mice (12 females and 12 males with body weight 20–24 g,) were randomly assigned to three groups. The experimental group received the extract liquid of C/C composites described above at a dose of 50 ml/kg through the tail vein. The negative control group received normal saline of equal volume. Lastly, the positive control group received Cytoxan (CTX) solution at a dose of 40 mg/kg, followed by a second booster dose after 24 h. The experimental animals were sacrificed 6 h after the second injection, and bone marrow smears were stained with Wright–Giemsa stain. Under immersion objective, the marrow smears were observed for number of micronuclei per 1,000 polychromatic erythrocytes, and the chromatophile micronuclei frequency was calculated.

2.4 Implant experiment in vivo

Twenty specific pathogen-free New Zealand white rabbits (body weight 2.0–2.5 kg) were randomly assigned to four groups. The right lateral thigh was shaved and disinfected with 5% iodine in ethanol, after which the thigh bone was exposed under general anesthesia using 20% ethylcarbamate in sterile conditions. Two holes 2 mm in diameter and 2 cm apart were drilled into the thigh bone, and C/C composite samples were implanted into the holes. Titanium

alloy was implanted into the contralateral thigh bone of the same animal, serving as a control. Animals were then euthanized at 2, 4, 8, and 12 week intervals after the procedure. For light microscopy, the C/C composite samples along with 0.5–1.0 cm of surrounding tissue were removed and immersed in 10% formalin for 24 h. Implants and surrounding tissue were dissected and dehydrated with ethanol, embedded in paraffin, and cut into approximately l0 μm-thick sections. For each specimen, 10–15 sections were mounted and stained with haematoxylin and eosin (H&E), after which five randomly chosen sections of the soft tissue surrounding the implants were observed and photographed on color slides under light microscopy. The cell distribution around the implants was quantified by enlargement and projection of the color slides (original magnification of 100) onto a screen. Between 180 and 240 cells were counted for each specimen, and the percentages of the various cells were calculated. For electron microscopy, the implants removed from the animals were immersed in 3% glutaric dialdehyde for 2 h, washed three times with PBS, immersed in 1% osmic acid for 1 h, washed with PBS again, dehydrated with alcohol, then immersed in tertiary butyl alcohol, cool dried and spurted with gold, and finally evaluated with electron microscopy.

2.5 Statistics

Student's *t*-test, or Cochran & Cox's approximation *t*-test if variance or heterogeneity was present, was used for analysis. A *P*-value of less than 0.05 was considered to be significant.

3 Results

3.1 Mechanical properties of C/C composites

The C/C composites (Fig. 1) were composed of a three-dimensional, needled carbon fiber preform fabricated by needling a stack of alternating layers of non-woven carbon cloth and carbon felt, both of which were synthesized from chopped fibers. The densities of the C/C composites were 1.82–1.85 g/cm^3, and volume fraction of carbon fibers in the materials was 33–35%. The mechanical properties are shown in Table 1.

3.2 Cytotoxicity experiments

As demonstrated in Table 2, the flow cytometry results demonstrate that the number of cells found in S-phase within the test group is not significantly different from those in the negative control group, whereas the number of cells in S-phase in both the test and negative control groups is significantly higher than that in the positive control group.

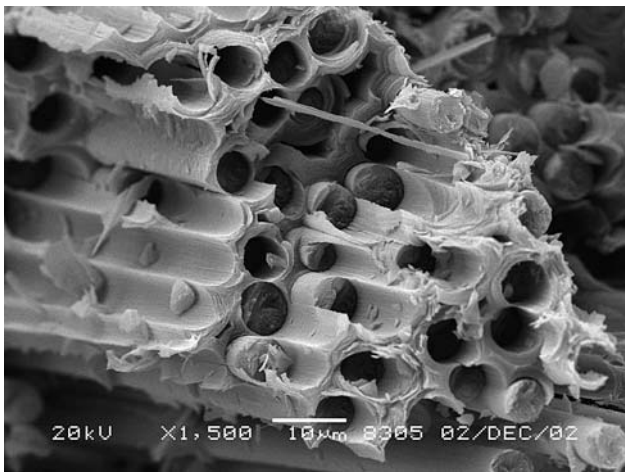

Fig. 1 The carbon/carbon composites were composed of a three-dimensional needled carbon fiber preform

Table 1 Mechanical properties of C/C composites

Strength/ modulus	Compressive (MPa/GPa)	Flexural (MPa/GPa)	Interlaminar sheer (GPa)
C/C composites	219.1/9.72	121.63/21.9	15.13

Table 2 Percentage of cells in S-phase

Group	Number of samples	Percentage of cells in S-phase (%) (mean ± SD)
Experimental group	8	38.47 ± 4.15$^{\Delta}$*
Negative control group	8	42.13 ± 3.53*
Positive control group	8	23.25 ± 3.42

$^{\Delta}$ $P > 0.05$ as compared with negative group

* $P < 0.01$ as compared with positive group

3.3 Micronucleus test

As shown in Table 3, the percentage of micronuclei in the experimental group (in which animals received C/C composites extract liquid) is approximately equal to that of the negative control group. Both percentages are significantly lower than that of the positive control group.

3.4 Implantation test

Animals started to eat and walk 24 h after the experimental procedure. No necrosis, pus, or bone fracture was noted, and no animal died during the experimental period. Nearly the same histological features were observed in both the C/C composite and titanium alloy implanted animals.

Table 3 Rates of micronuclei

Group	Number of samples	Polychromatic RBC count	Percent of micronuclei (%) (mean ± SD)
Experimental group	8	1000	$2.3 \pm 0.7^{\Delta *}$
Negative control group	8	1000	$2.1 \pm 0.8^{*}$
Positive control group	8	1000	30.2 ± 5.1

RBC indicates red blood cell

$^{\Delta}$ $P > 0.05$ as compared with negative group

* $P < 0.01$ as compared with positive control group

3.4.1 *Light microscopy*

Two weeks after implantation, tissues surrounding the implants were engorged, edematous, and moderately infiltrated with acute inflammatory cells (neutrophils and monocytes), and a thin layer of fibroblasts and fibrocytes was found to cover the implant (Fig. 2a). Eight weeks after implantation, engorgement and edema noticeably resolved in both groups, though mild inflammatory cell infiltration still existed. The fibrous tissue and fibrocytes covering the implants became thinner at eight weeks, as well. Twelve weeks after surgery, there is no significant inflammatory response within the tissue surrounding the implant, and the fibrous capsule is thinner still with more pronounced bone-like tissue proliferation (Fig. 1b). Degenerative tissue, necrotic tissue, cellular dysplasia, and cellular hyperplasia were not observed. From 2 to 13 weeks, cells around implants are mainly fibroblasts and fibrocytes.

3.4.2 *Electron microscopy*

Two weeks after implantation, electron microscopy reveals fibrous tissue adhering to the C/C composite surface, but the tissue is relatively loose and easily separates from the composite. A small number of cells can be seen within the spaces of the C/C composite (Fig. 3a). Four weeks after implantation, the fibrous adhesions are more integrated within the surface of the C/C composite and do not separate easily (Fig. 3b). Eight weeks after implantation, fibrous tissue and C/C composite are further integrated with more mature and thicker adhesions, and there is increased difficulty in separation (Fig. 3c). Twelve weeks after implantation, the fibrous tissue and C/C composites are fully integrated into one another (Fig. 3d). The same features are observed in the titanium alloy control implants.

4 Discussion

In this study it was found that the extract liquid of C/C composite synthesized with this unique method has no impact on cell replication in vitro as demonstrated by

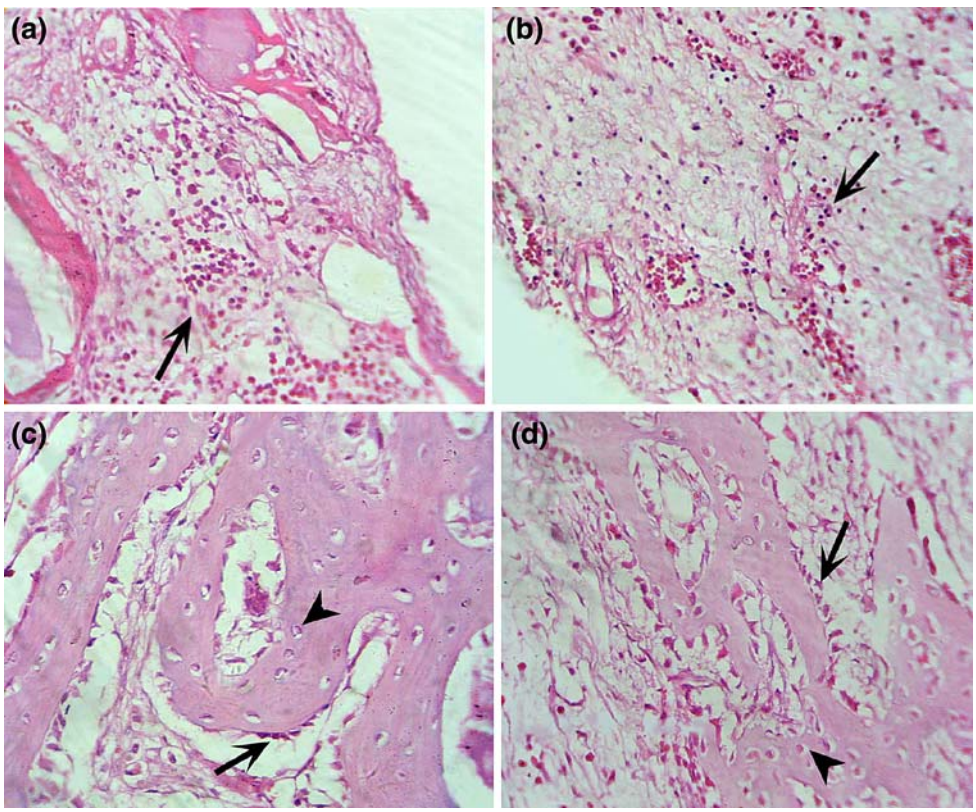

Fig. 2 Light microscopy of the implant and its surrounding tissue. **a** Two weeks after implantation of the C/C composites, tissues surrounding the implant are engorged, edematous, and moderately infiltrated with neutrophils and monocytes (*arrow*). **b** In the titanium group, tissues surrounding the implant are engorged, edematous, and moderately infiltrated with neutrophils and monocytes (*arrow*). **c** Twelve weeks after implantation of C/C composite, the fibrous layer begins to thin, and osteoblasts (*arrow*) and bone cells (*arrowhead*) become evident. **d** In the titanium group, osteoblasts (*arrow*) and bone cells (*arrowhead*) are also evident

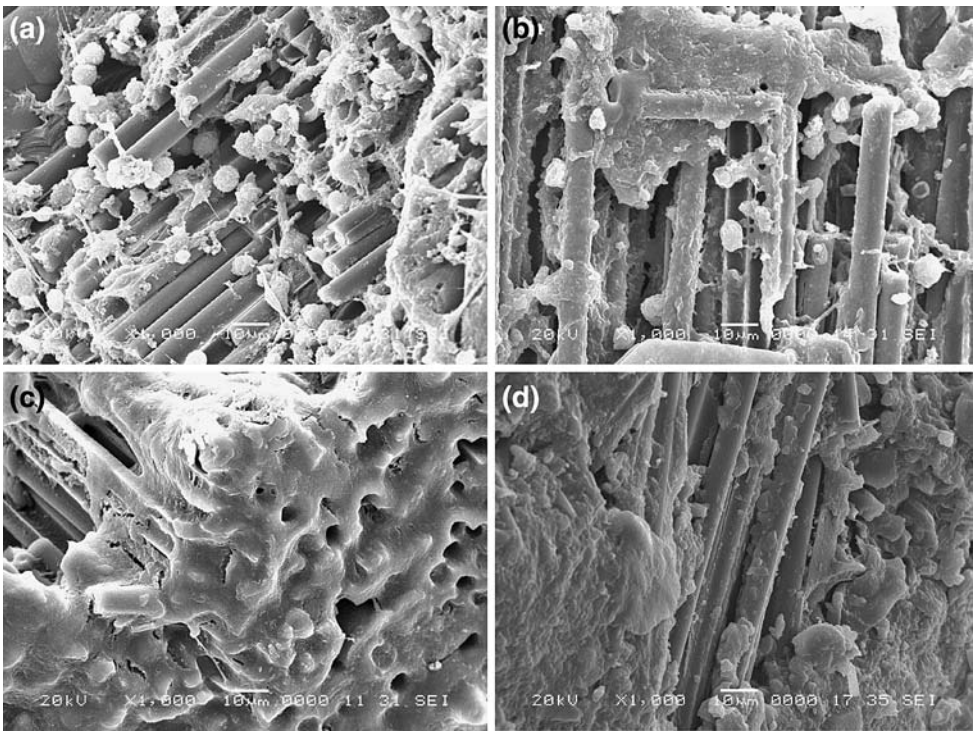

Fig. 3 Electron microscopy of C/C composites and fibrous tissue at 2 (**a**), 4 (**b**), 8 (**c**), and 12 (**d**) weeks after implantation. **a** Two weeks after implantation, fibrous tissue loosely adheres to the C/C composite surface with a small number of cells found within the space of the C/C composite. **b** Four weeks after implantation, the fibrous tissue adhering to the surface of the C/C composites is wide in range and not easily separated. **c** Eight weeks after implantation, fibrous tissue and C/C composites are closely integrated with more mature, thicker adhesions and are increasingly difficult to separate. **d** Twelve weeks after implantation, the fibrous tissue and C/C composites are fully integrated into one another

cytotoxicity experiments, and no genotoxicity exists as demonstrated by the micronucleus assay. In the in vivo study, the implant integrates well with the host tissue.

The density of the bone is 1.85 g/cm^3, and the flexural strength is 100–150 MPa. The density and flexural strength of the C/C composites synthesized in this study are nearly the same as that of the natural bone and better than that of the C/C carbonized composite used by Pesakova and colleagues. The density of Pesakova's C/C composites ranged from 1.30 to 1.50 g/cm^3, and flexural strength ranged from 97 to 250 MPa [6].

Compared to press fit carbon fiber prostheses with smooth surfaces as well as titanium alloy and stainless steel, the porous C/C composites have many apparent advantages. Previous in vitro studies have demonstrated that osteoblast cells can firmly attach to the porous materials [7]. The in vivo study showed that tissues could grow into the C/C composites through these openings and integrate well with the host tissue, preventing loosening, which is a major problem in the process of implantation [8].

However, if in addition to titanium, other materials such as the press fit carbon fiber composite with smooth surface were available and used as controls, more information surely would be revealed.

Pesakova et al. [4–6] have used human embryonic lung fibroblast LEP cells as a model by which to test the cytotoxicity of the extract liquid of C/C composites by observing adherence and cell proliferation. However, their in vitro results contradicted their in vivo outcomes. In this study, the L929 mouse skin fibroblasts were used as a model [9] by which to observe the effect of the extract liquid of the C/C composite on cell division by counting the percentage of cells at S-phase, resulting in an outcome that is in agreement with the in vivo observations.

The micronucleus assay, a sensitive marker of DNA damage, has been viewed as suitable for genotoxic risk assessment in regulatory scenarios [10–12]. The results of the micronucleus assay demonstrate that the extract liquid of the C/C composite has no influence on gene replication.

The in vivo study shows that acute reactions within the two thighs are nearly the same and are similar to what has been reported in the literature [4–6]. Inflammation within the first 10 days following surgery is mainly determined by the intrinsic properties of the surgical procedure itself [5], while later host reactions to the implant appear to be more meaningful. The morphological results at week 12 after surgery demonstrate that the implants integrate well with the host tissue.

Carbon debris was frequently observed by Pesakova et al. [5] in the case of pure C/C composite implants. Some debris was observed in this study, but infrequently, suggesting that the manufacturing process may have improved the quality of the C/C composite. As there is no "standard" or similar implant material that has been studied, one cannot draw a comparative conclusion that the current manufacturing method is better with respect to reducing the release of debris. However, it may be used as an in-house standard for future studies to improve the features of the C/C composite.

By modifying the manufacturing procedures, it was possible to synthesize a C/C composite that is very close to

natural bone with respect to its mechanical properties. Debris release from the implant likely was reduced, and there existed maintained, or even increased, biocompatibility of C/C composites, potentially providing a direction for future studies.

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