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Osteogenesis and cytotoxicity of a new Carbon Fiber/Flax/Epoxy composite material for bone fracture plate applications

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ABSTRACT

This study is part of an ongoing program to develop a new CF/Flax/Epoxy bone fracture plate to be used in orthopedic trauma applications. The purpose was to determine this new plate's in-vitro effects on the level of bone formation genes, as well as cell viability in comparison with a medical grade metal (i.e. stainless steel) commonly employed for fabrication of bone plates (positive control). Cytotoxicity and osteogenesis induced by wear debris of the material were assessed using Methyl Tetrazolium (MTT) assay and reverse transcription polymerase chain reaction (RT-PCR) for 3 osteogenesis specific gene markers, including bone morphogenetic proteins (BMP2), runt-related transcription factor 2 (Runx2) and Osterix. Moreover, the Flax/Epoxy and CF/Epoxy composites were examined separately for their wettability properties by water absorption and contact angle (CA) tests using the sessile drop technique. The MTT results for indirect and direct assays indicated that the CF/Flax/Epoxy composite material showed comparable cell viability with no cytotoxicity at all incubation times to that of the metal group ($p \geq 0.05$). Osteogenesis test results showed that the expression level of Runx2 marker induced by CF/Flax/Epoxy were significantly higher than those induced by metal after 48 h ($p = 0.57$). Also, the Flax/Epoxy composite revealed a hydrophilic character ($CA = 68.07° \pm 2.05°$) and absorbed more water up to 17.2% compared to CF/Epoxy, which reached 1.25% due to its hydrophobic character ($CA = 93.22° \pm 1.95°$) ($p < 0.001$). Therefore, the new CF/Flax/Epoxy may be a potential candidate for medical applications as a bone fracture plate, as it showed similar cell viability with no negative effect on gene expression levels responsible for bone formation compared to medical grade stainless steel.

1. Introduction

Femoral fractures at the tip of a total hip replacement occur in approximately 0.1%–6% of all arthroplasty patients [1–3]. This periprosthetic fracture is treated using a bone plate because intramedullary nailing is physically impossible [1,2,4]. Commercially-used bone plates are made of metallic materials, such as stainless steel, cobalt-chrome, and titanium-based alloys which have elastic moduli 5–10 times larger than human cortical bone [1,2,4]. This mismatch causes "stress shielding" with subsequent osteoporosis and bone atrophy mainly under the plate, potentially leading to refracture. In addition, the application of metallic materials for biomedical applications as bone implants is still a point of controversy due to their inherent disadvantages, such as corrosion and low fatigue strength. Moreover, metallic materials are not particularly compatible with modern medical imaging technologies like CT (computerized tomography) and MRI (magnetic resonance imaging), because they appear white on the exposed film due to their radiopaquecy.

To address these negative effects, polymer-based composite materials have been introduced for biomedical purposes because of their high strength-to-weight ratio, non-corrosiveness, tailorability, and radiolucency [5–10]. For these reasons, many kinds of polymer-based composite materials reinforced with carbon [6,11–19], aramid [20], glass [7,21], and natural fibers [22] have been explored for bone fracture plating. Regarding the matrix, biodegradable polymers have been introduced recently for bone fracture plates [23–25]. However, it is very difficult to match the degradation rate of the implants made of biodegradable polymers with the healing period of the fractures [24,25]. Moreover, their low mechanical properties, as well as histological response to degradation products, limit their employment as bone fracture plates [23,24]. With respect to the fiber itself, all the above-

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mentioned studies only used one type of fiber (synthetic or natural) to fabricate their bone implants. However, an ideal bone fracture plate should have low axial stiffness to decrease the "stress shielding" effect plus adequate bending stiffness to provide proper immobilization at the fracture site [2,4], which may not be achieved by using one type of material [2,4]. Thus, a hybrid composite made of two types of fibers may potentially be an optimal choice for manufacturing bone fracture plates, since it can be tailored to show desirable properties in different directions.

Consequently, some of the current authors have developed a new hybrid Carbon Fiber (CF)/Flax/Epoxy composite with a "sandwich structure" for bone fracture plate applications. The benefits of using the present composite structure over a clinically used metal plate for repairing femur fracture are its ease of fabrication, its lower cost since the majority of its composition is flax fiber which is abundant in nature, and its unique biomechanical properties. In terms of its static and dynamic mechanical properties, the current CF/Flax/Epoxy has a comparable elastic modulus (axial: 42 GPa, bending: 57 GPa) vs. human cortical bone (7–25 GPa) with superior strength (axial, 400 MPa; bending, 511 MPa; fatigue, 200–220 MPa) vs. human cortical bone (50–150 MPa) [11,26]. In addition, this composite retains its mechanical properties (i.e. stiffness) for almost 90% of its fatigue life, which is almost never the case for homogeneous metallic materials [26]. More importantly, as a consequence of its biomechanical properties, this composite can potentially minimize the common major problem of "stress-shielding", which is typically present when using metallic materials to fix bone fractures [27]. Also, this composite provides adequate immobilization that avoids any gross motion at the fracture site leading to proper healing of the fractured bone [27]. However, no prior studies exist which have assessed this unique composite material's cytotoxicity, its effect on osteogenetic differentiation necessary for bone fracture healing, and its wettability characteristics.

The aim of the current study, therefore, was to determine this new CF/Flax/Epoxy's cytotoxicity and its effect on the osteogenesis (i.e. new bone formation by osteoblast cells) process vs. a medical grade metal obtained from a commercially-used clinical bone fracture plate. It was hypothesized that this new CF/Flax/Epoxy composite material would show good biocompatibility without any negative influence on the osteogenesis process.

2. Materials and methods

2.1. General approach

CF/Flax/Epoxy specimens were compared to metal specimens manufactured from a fracture plate commonly used clinically. In addition, CF/Epoxy, Flax/Epoxy, and CF/Flax/Epoxy composites were tested for their wettability properties by contact angle (CA) and water absorption tests. The aim was to determine whether the CF/Flax/Epoxy composite material had acceptable biological properties based on the current in-vitro test, in order to be used for biomedical engineering applications like orthopedic long bone fracture plating.

2.2. Specimens

The CF/Flax/Epoxy composite was manufactured using 16 layers of Flax/Epoxy laminae, as well as 2 layers of CF/Epoxy laminae added to the outer surface of the composite resulting in a "sandwich structure". The CF/Flax/Epoxy composite was manufactured in-house using a compression-mold machine at 500 kPa and 150 °C for 1 h. The Flax/Epoxy laminae (Lineo NV, Dendermonde, Belgium) had a density of 1.3 g/cm^3, a volume fraction of 58–60%, and a unidirectional fiber orientation, whereas CF/Epoxy laminae (Hexcel® Composites, Stamford, CT, USA) had a density of 1.56 g/cm^3, a volume fraction of 57%, and unidirectional fiber orientation. This composite plate is unique compared to previous proposed composite materials for orthopedic trauma applications [6,7,12–14,16,22,28] because it is the first to use a "sandwich structure" in which a rigid external layer of synthetic fibers (carbon) surrounds a softer internal matrix of flexible natural fibers (flax). This mimics the overall structure of human bone, i.e. a hard external cortical shell and a spongy internal cancellous matrix. To see the effect of each material on the cell behavior separately, Flax/Epoxy and CF/Epoxy specimens were also manufactured using compression molding and autoclave techniques, respectively. The final composites were cut using a circular saw to create 15 mm long × 5 mm wide × 4 mm rectangular specimens. The metal specimens (316L stainless steel) were obtained from a commercially-available clinical bone fracture plate (Zimmer, Warsaw, IN, USA) and were cut to the same dimensions as the composite specimens.

2.3. Sterilization and cell cultures

All specimens were prepared for culture by autoclave (Heidolph, USA) sterilization using 121 °C at 200 kPa for 45 min. Tissue cultured plastic plates (TCP), used as a standard dishes for cell growth, were cut to the same dimensions as other specimens and were sterilized using high power microwave sterilization for 15 min [29]. Following sterilization, specimens were handled according to standard sterile culture protocols. A rat osteoblast-like cell line (UMR-106 osteosarcoma, American Type Culture Collection, USA) was used, since this type of cell allows for the study of certain common cellular functions like viability, membrane state, proliferation, and attachment, which are factors associated with basal cytocompatibility of biomaterials [30,31]. In addition, this type of cell is widely employed as a human osteoblastic model [30,31]. Cells were grown at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco Minimal Essential Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin (PS), and 2 mM L-glutamine at 5% CO_2 and 37 °C.

2.4. Assay preparation and testing

2.4.1. Extract preparation

Each specimen was immersed in DMEM as an extracting medium with an extraction medium volume-to-surface area ratio of 1.25 cm^2/ml at 5% CO_2 and 37 °C [32]. Extraction was completed over 24 h, at which point the extraction medium was removed and centrifuged before being applied to cell cultures (Fig. 1).

2.4.2. MTT assay-indirect test

The cytotoxicity of Flax/Epoxy, CF/Epoxy, and CF/Flax/Epoxy composite extracts was evaluated against UMR-106 osteoblast-like cells using MTT assay in 96-well plates. The MTT is a salt that can be enzymatically converted by living cells, resulting in a color change from yellow, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, into purple formazan crystal. The intensity of the color produced is directly proportional to the number of viable cells and can be measured by reading absorbance at 590 nm on an enzyme-linked immunosorbent assay plate reader. Briefly, UMR-106 cultured cells were seeded at 1×10^4 cells/ml in a 96-well plate and allowed to adhere for 24 h at 37 °C in a 5% CO_2 humidified atmosphere, before being media aspirated. DMEM supplemented with FBS was added to cultures every second day. Extraction cultures were maintained at 37 °C in a 5% CO_2 atmosphere. Control samples consisted of UMR-106 cells grown on TCP supplemented with complete DMEM that was not exposed to extracts. After 24, 48, and 72 h of incubation, extracts were removed and each well was treated with MTT solution at 5% CO_2 and 37 °C for 4 h (Fig. 1.a). The medium was removed and then 100 μl of dimethyl sulfoxide (DMSO) was subsequently added to each well. The plate was then shaken for 5 min before reading the optical density at 590 nm with a corrected wavelength of 690 nm. Cytotoxicity was calculated as the percentage of control cell viability.