Cytotoxicity and Cell Adherence Evaluation of 3D Orbital Polyamide Composite Customised Implant

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Abstract

Polymer composites based orbital floor implant namely 3D orbital polyamide customised composite (3D OPACC) has been successfully developed locally. The objective of this study was to evaluate the cytotoxicity and osteoblast cell adherence on the developed composite. The cytotoxicity of the 3D OPACC was evaluated by incubating the osteoblast cells in five different concentrations (100, 50, 25, 12.5, 6.25 mg/ml) of the composite extract for 72 hours. The cell viability was determined by MTS assay at a wavelength of 492 nm via a microplate reader. The cell adherence was evaluated by seeding the osteoblast cells on the 3D OPACC and incubated for 72 hours. Staining was performed using a live and dead cell imaging kit. The cell adherence was observed under a fluorescence microscope. In both experiments, commercial implants under the trade name of Synpor and MedPor were used as comparison. Regardless of the concentration, the viability of the cells treated with 3D OPACC were more than 80%. Besides exhibiting higher cell viability as compared to the commercial implant, the cells also adhered well to the 3D OPACC implant, which indicates that 3D OPACC could be a suitable host for osteoblast attachment and proliferation.

Keywords: cytotoxicity, cell adherence, polymer composite.

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Introduction

Orbital fractures are common in patients suffering from facial trauma due to the delicate anatomy, especially the medial wall and floor of the orbit. Repair and reconstruction are difficult due to its complicated anatomy. Surgical treatment is indicated for functional or aesthetic reasons with numerous techniques and materials. Implant materials include autologous graft, allogenic materials, and alloplastic materials, each with their advantages and disadvantages. Unfortunately, the ideal material for orbital floor fracture is yet to be discovered [1].

Ideal orbital implant materials should possess dimensional stability to support orbital content and related forces, as well as fixability to prevent migration. It should also be easily shaped to fit the complex and unique orbital anatomy of different individuals, which is easily achievable with the development of patient specific implants (PSI). The implants should also display good biocompatibility, allow drainage of orbital fluids, and avoid donor site morbidity, as well as being radiopaque. Lastly, implant materials should be readily available and inexpensive[2]. At present, frequently used orbital implant materials include titanium and porous polyethylene, but both at an expensive cost [2] and need to be trimmed according to the anatomical condition of the patient intraoperatively.

Polyamide, or nylon is a relatively new material that have gained attention in the biomedical field due to its potential use for bone tissue regeneration, thus suitable for fabrication of bone implants. Nylon foil has provided favourable results in preliminary non-comparative studies [3-5]. Besides displaying excellent mechanical properties, it has good biocompatibility because it is chemically similar to collagen proteins, hence possesses excellent stability in human body fluids[6, 7]. It is also inexpensive, reproducible and easy to manufacture. Compared to other biomedical polymers, it is also more compatible for 3D printing [8].

3D Orbital Polyamide Customized Composite (3D OPACC) is a 3D-printable composite that can fabricate customized patient specific implant. It contains a hybrid zirconia (ZrO_2), and beta-tricalcium phosphate (β-TCP) compounded with polyamide 12 [9]. It is cost-saving, without having to compromise the amazing feature of an anatomically accurate fit implant that allows easy placement without the need to reshape. This consequently aids surgical procedures in terms of operating time, patient's risk, and improves success rate with satisfying aesthetic outcomes [8].

To date, many studies have been carried out to evaluate the hybrid ZrO^2/β-TCP filled PA 12, which includes preliminary study of its tensile and thermal properties for 3D printing compatibility [10], mechanical and morphological properties [11], comparison of mechanical properties between fused filament fabrication and injection moulding [8], mechanical and physical properties of 3D OPACC via fused deposition modelling 3D printer [12], improvement of mechanical and thermal properties [13], and mechanical and cytotoxicity properties on fibroblast cells [9]. This study focuses on cytotoxicity and cell adherence of osteoblast on 3D OPACC.

Osteoblast cell was selected in this study because this material is intended to be used for bone replacement, osteoblast adhesion can enhance fixation of adjacent bone to the implant. It should be noted that osteoblast adherence is the initial step of osseointegration responding to bone implant materials, which produces downstream cell signalling pathways that regulate cell

spreading, proliferation, differentiation and migration [14]. Failure for osteoblasts to adhere, for example in cases of silicone implants, would cause migration and fibrous encapsulation of implant. Hence, having the implant directly attached to the bone is desirable [15].

Materials and Methods

Materials

Material samples that were prepared include in-house produced 3D-OPACC and also porous polyethylene of Synpor (Art. No. 08.510.542S) and Medpor (CAT#8305). Both porous polyethylene were cut into 1cm x 1cm squares, while polyamide composites of 1cm x 1cm squares were fabricated using 3D printing method.

Alpha Modification Minimum Essential Medium, Alpha MEM (21444-05, Nacalai Tesque, Japan), Foetal Bovine Serum (10270, Gibco, USA) and Penicillin Streptomycin (15140, Gibco, USA) were purchased for culture medium. Live/dead Cell Imaging Kit (Catalog No: R37601, Invitrogen, USA) and CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, USA) were also purchased.

Human osteoblast cell lines, hFOB 1.19 (CRL11372) were thawed from existing cryopreserved passage 4 vial, and then cultured in α-MEM, supplemented with 15% foetal bovine serum (FBS) together with 1% Penicillin Streptomycin. The cells were then incubated in a 5% CO_2 37 °C humidified incubator (Sheldon, United States) and monitored closely for 24 hours. Using a laminar flow (ESC II Series, Germany), the aseptic work was maintained to avoid contamination of the cultured cells.

MTS Assay

3D-OPACC and porous polyethylene were cut into small pieces and weighed to make up 100mg. The extract solution with a concentration of 100mg/ml was initially prepared by incubating 100mg of each material with 1000µL of Alpha MEM for 72h at 37°C in a 5% carbon dioxide supplied incubator. HFOb cells were cultured at a density of $1x10^4$ cells/well (n=3/concentration) in 96 well plates and incubated for 24 hours prior to treating with extract. Serial dilution was done by adding Alpha MEM to extract solution to produce concentration of 50, 25, 12.5, 6.25 mg/ml. Previous medium was then removed and replaced with 100µL of each extract concentration, and 5 untreated control samples, which are then incubated for 72 hours.

After that, MTS assay was thawed approximately 90 minutes at room temperature. 20µl of MTS solution was pipetted into each well and incubated for another 2 hours in humidified, 5% CO_2 atmosphere. Absorbance were recorded at 490nm using a 96-well plate reader after shaking for 10 seconds. Results were analysed using IBM SPSS Statistics 24. Cell Viability (CV) was calculated by the following formula.

$$Cell\ Viability\ (\%) = \frac{Absorbance\ for\ treated\ cells}{Average\ absorbance\ of\ control\ cells} \times 100$$

Normality of the results were confirmed using Shapiro-Wilk test, and Kruskal Wallis Test was executed to analyse cell viability between different extract concentration of different materials.

Live-dead staining and cell attachment

Incubation with material composite

Medpor, Synpor and 3D OPACC with respective thickness of 1.0mm, 0.8mm and 2.2mm with approximately 1cm x 1 cm dimension, as well as round thermanox coverslips were placed into 24-well plates and pre-incubated for 30 minutes. The materials were then seeded with $5x10^4$ cells in 500 µL both on the top and around the materials and incubated for 15 minutes to aid attachment of cells on material, then another 500 µL medium was added to make 1000 µL. The cells were then incubated at 5% CO_2 37ºC humidified incubator for 72 hours. The cells attachment near the materials were verified every 24, 48 and 72 hours under inverted microscope.

Live-dead staining

At the end of incubation period, the materials were rinsed 3 times in PBS. Working reagent containing 1ml of supplied calcein AM and 1µl of ethidium homodimer-1 stock in 5ml PBS were added on materials and incubated 30 minutes at room temperature in the dark. Then the materials were taken out and mounted with antifade solution on the slide. The stained cells were then examined using fluorescence microscope under 200x magnification. Each image was then captured where green stains live cells and red stains dead cells. Cell viability were qualitatively compared between materials.

Results and Discussion

MTS Assay

The cytotoxicity of all materials with osteoblast cells were evaluated using MTS assay. Figure 1 illustrates the bar chart of cell viability against each extract concentration of Medpor, Synpor and 3D OPACC. From the graph, even at highest concentration of 3D OPACC, cell viability was above 80%. In comparison with commercially-used porous polyethylene, Medpor and Synpor shows similar cell viability percentage of 86% and 77% respectively, it is fair to consider 3D OPACC as non-cytotoxic towards osteoblast cells.

In fact, as extract concentration decreases, 3D OPACC demonstrates higher cell viability than both porous polyethylene. At 25, 12.5 and 6.25mg/ml extract concentration, 3D OPACC shows significantly higher cell viability as compared to Synpor. This could be attributed to the incorporation of beta-tricalcium phosphate (β-TCP) in 3D-OPACC, whereas Synpor is purely manufactured from ultra-high molecular weight polyethylene (UHMWPE) without additives.

β-TCP is a calcium phosphate-based material that is porous and easily dissolvable to release calcium and phosphate ions, which is of similar composition to natural bone, making it possible for bone tissue regeneration strategies. It is also said to elevate hydrophilicity, thus increasing water uptake through the pores and accelerate degradation[16]. Both calcium and phosphate are important in cell signalling process[17]. It has been shown that calcium ions enhance proliferation and morphological changes and osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells (hMSCs) [18]. Furthermore, inorganic phosphate ions have distinct anabolic effect on bone structure and metabolism by stimulating osteoblastic cell proliferation[19].

Cell viability in 3D-OPACC peaks at concentration of 12.5mg/ml followed by a slight drop at 6.25mg/ml, which might suggest an optimum extract concentration that is sufficient to induce maximum osteoblast cell proliferation.

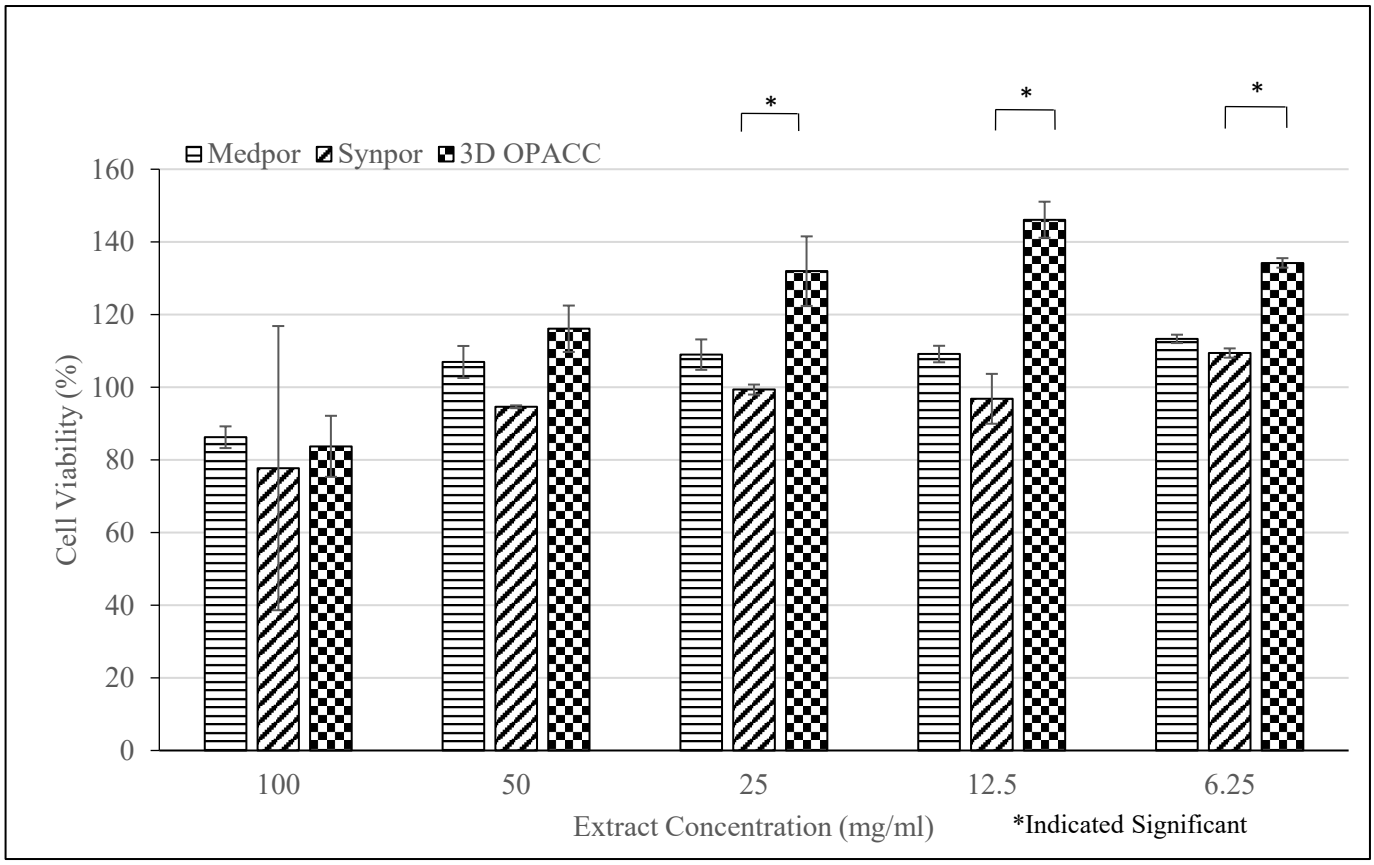

Figure 1: Mean Percentage of hFOB Cell Viability after 72 hours incubation in different concentration of Medpor, Synpor and 3D OPACC extract (n=3/concentration). Error bars represent standard deviation. Asterisks indicate statistically significant differences ($p<0.05$).

Osteoblast Morphology under Inverted Microscope

Direct contact experiments were then performed on the materials, using osteoblast in order to evaluate the cell/material interaction in terms of cell attachment and proliferation.

Figure 2 displays osteoblast cells in close proximity to the materials (dark area). Osteoblast attachment were evident around the margins of materials. Cell proliferation, as seen as increased cell density with incubation time was evident along the borders of the margin of 3D OPACC, as similar to both Synpor and Medpor. The cells were able to reach confluence at the end of 72 hour incubation without any sign of cytopathic morphology throughout the entire period. These interactions proves osteoconductive properties 3D OPACC and the results were in concordance with previous MTS assay results.

Live-dead staining

At the end of incubation period, live-dead staining was done and observed under fluorescence microscope to evaluate viability and attachment of osteoblast cells seeding directly onto surface of all materials. The cells were stained using Calcein AM and Ethidium homodimer, which stains viable cells as fluorescent green and non-viable cells fluorescent red respectively.

Results in Figure 3 depicts that the cells were viable and able to attach on the material surface. Osteoblast is an anchorage-dependent cell, hence cell adhesion to material surface is crucial for its survival [20]. In case of cytotoxic samples, osteoblasts are expected to exhibit zone of inhibition around materials or maybe even cell death [21]. However, this was not the case, as no red-stained cells were observed in all samples, indicating absence of non-viable cells. Images of viable cell attachment into 3D OPACC showed clear evidence that it is in fact not toxic to osteoblast cells.

Comparatively, more cells are seen on 3D OPACC than Synpor, Medpor and thermanox glass, which might further confirm osteoinductive properties of 3D OPACC due to favourable properties of β-TCP as described previously. Another possibility would be due to the porous 3D structure of polyethylene, in which the osteoblasts seeps within the porous gaps and were unable to be seen from the surface.

Upon introduction of implant into the human body, cell/material interaction will occur. Attachment, adhesion and spreading belong to the first phase of cell/material interactions and the quality of this first phase will influence the cell's capacity to proliferate and to differentiate itself on contact with the implant [22]. When cultured on a surface, the total area that the osteoblasts spread on the surface correlates with the amount of cell adhesion on that surface, and promotes cell proliferation and colonization. Hence, cell spreading and adhesion gives an indication on the cytocompatibility of different experimental substrates in vitro[20].

If cells have not adhered adequately, due to implant instability or the chemical and physical properties of the implant surface itself, fibrous encapsulation with liquid-filled void may form between soft tissue and implant [20, 23, 24]. This may cause further destabilization of the implant, inhibition of tissue regeneration and repair, and an increased risk of infection due to poor vascularization around implant and fibrous tissue [20, 23, 25, 26].

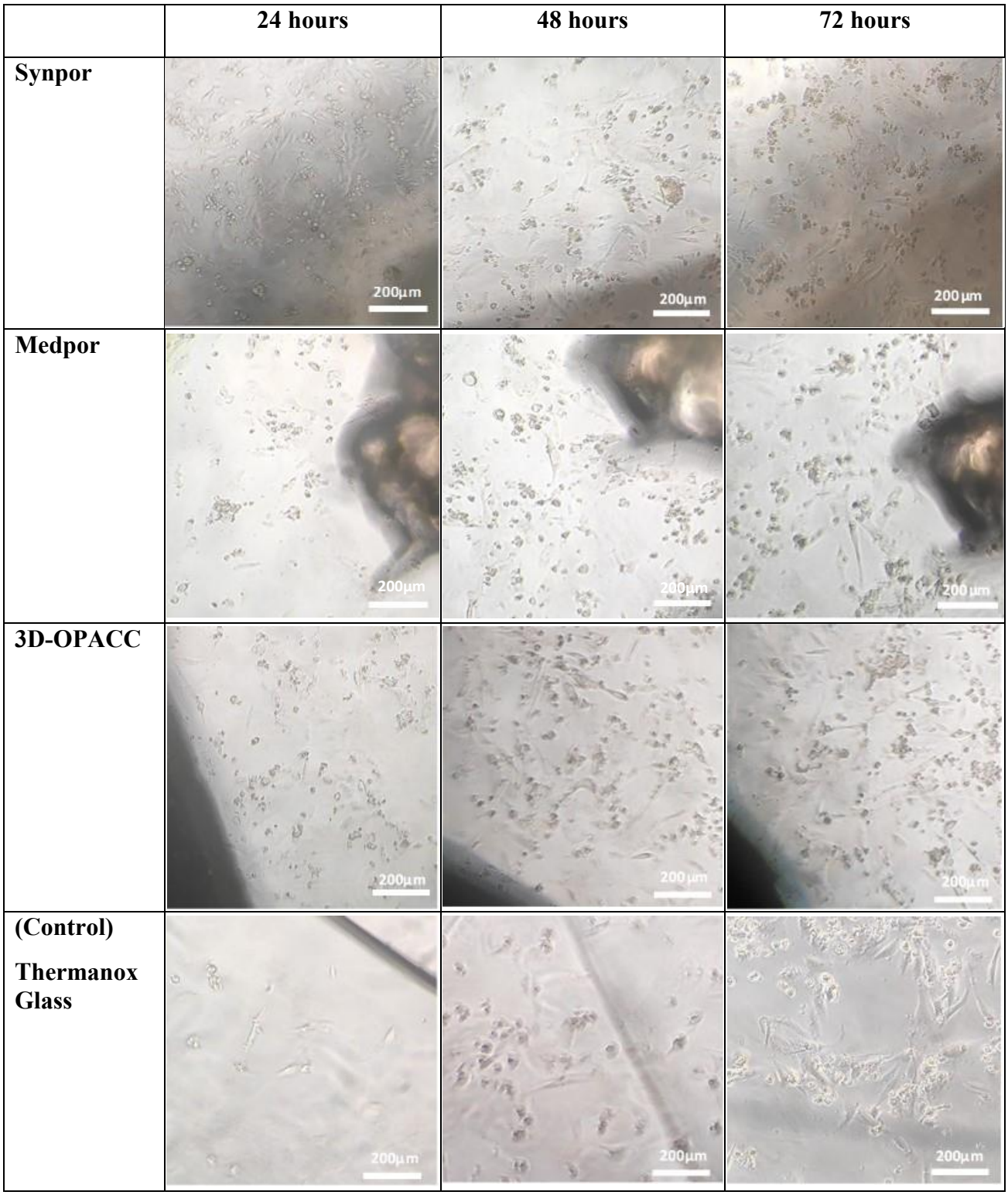

Figure 2: Inverted microscope images of osteoblast attachment near materials, which are Synpor, Medpor, 3D-OPACC at 24, 36, 72 hours. Thermanox Glass is used as a control.

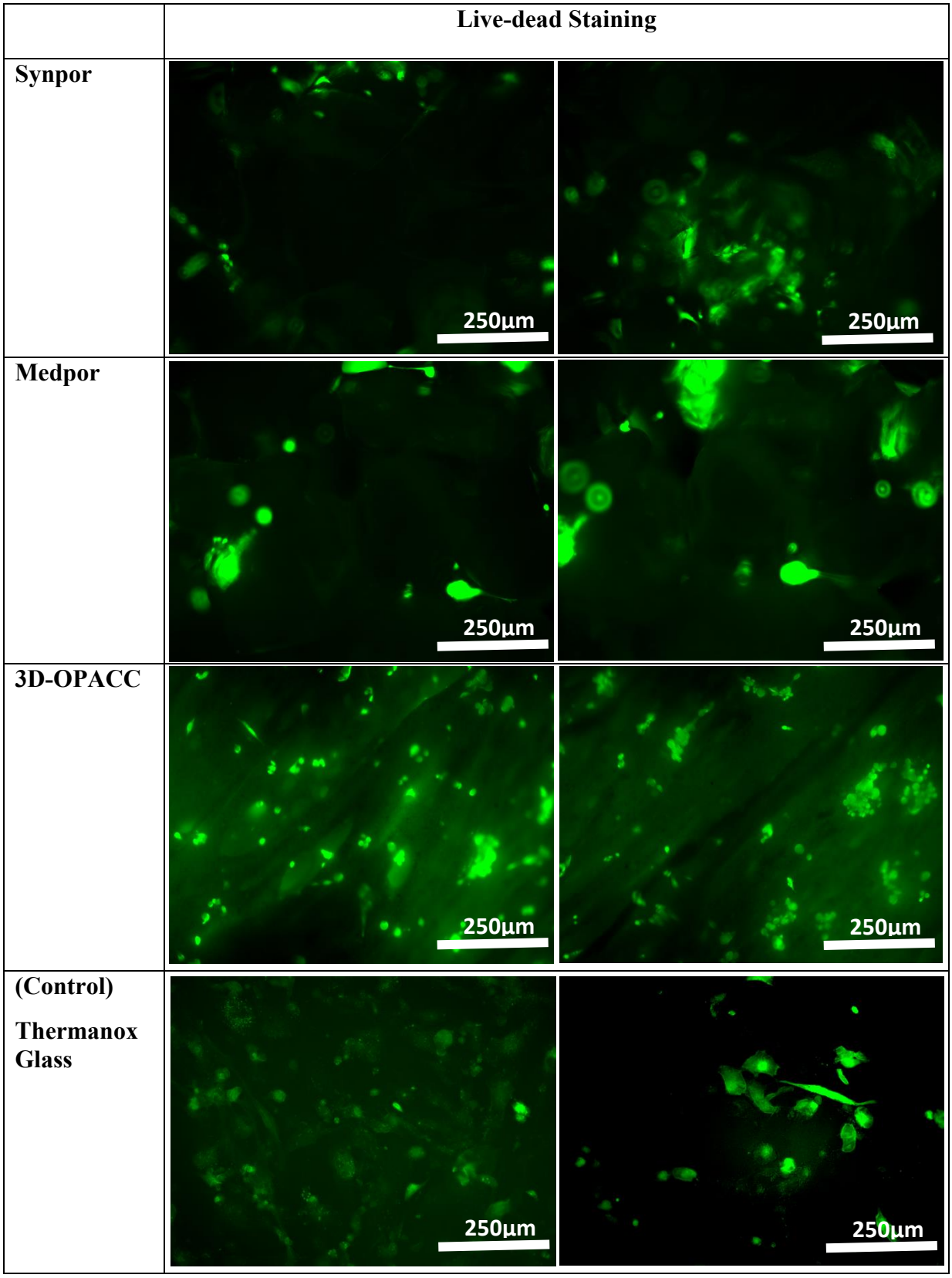

Figure 3: Live-dead stained osteoblasts after 72 hours incubation on Synpor, Medpor, and 3D OPACC. Thermanox Glass is used as control. Green fluorescence represents live cells. 1st and 2nd column are selected images from different samples of the same material.

All results show that 3D OPACC exhibits similar, or possibly better biocompatibility than porous polyethylene in terms of cell viability and attachment, without any sign of cytotoxicity. This finding further supports the continuation of research for better material development in hope to pursue the ideal implant material to provide the best surgical outcome possible for patients with orbital fracture.

In vitro tests are crucial as a quick, simple, inexpensive, controllable screening tool for materials. However, full evaluation of biocompatibility for biomaterials require further testing such as in-vivo and clinical trials[21]. The results provided evidence that 3D OPACC is not cytotoxic to osteoblasts, however further studies are needed to fully understand the biological behaviour of 3D OPACC. For more improvements, a clearer immunofluorescent image may be obtained using Confocal Laser Scanning Microscope for better visualisation of osteoblast morphology upon attaching to surface of 3D OPACC.

Conclusion

In conclusion, MTS assay quantitatively evaluated cytotoxicity of 3D OPACC relative to Synpor and Medpor, which are both commercially used porous polyethylene. Overall, cell viability are above 80% for 3D OPACC and displays similar percentage cell viability to both Synpor and Medpor at high extract concentrations of 100 and 50 mg/ml, while displaying significantly higher percentage cell viability than Synpor at extract concentrations of 25, 12.5 and 6.25 mg/ml.

Furthermore, no zones of inhibition of osteoblast cells attachment were seen at close proximity to material margins and cell proliferation was evident along the material borders. Direct cell viability and attachment on material surfaces were further evaluated by live-dead staining fluorescence microscopic images, where viable cells on top of 3D-OPACC were clearly visible, with more cells observed as compared to Synpor and Medpor.

This shows that 3D OPACC is equally good, or potentially better than porous polyethylene in terms of osteoblast cell viability, attachment and proliferation, thus proving itself as a favourable implant material.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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