Minocycline-releasing PMMA for craniofacial bone reconstruction - *in vitro* characterization

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Introduction/Objective

The repair of complex craniofacial bone defects is challenging and a successful result depends on the defect size, the quality of the soft tissue covering the defect, and the choice of reconstructive method. [1] Defects or deficiencies of the craniofacial skeleton are quite common, and may be due to congenital or acquired causes. Common aetiologies include post-surgical defects following tumour resection or ablation, trauma, and a large number of congenital anomalies . [1, 3] These lesions can be quite complex for the development of infections, loss of local blood supply and massive tissue necrosis that reduce the tissue viability and difficult any kind of successful regeneration. [4-6] Generally, the treatment of those lesions is based on multiple reconstructive stages (staged repair) that despite the difficulties found (e.g., distortion of anatomic references, loss of volume defect and wound contamination) can produce a greater outcome in aesthetic/functional regeneration then the conventional approach of single stage procedure. With an objective to optimize the regenerative approaches and minimize the referred complications, a multistage approach is envisaged with the addition of a biomaterial that can be used as a space maintainer. [5, 7] The use of space maintainers based in polymethylmethacrylate (PMMA) has been applied successfully in craniofacial reconstructions.[8, 9] Successful space maintenance involve the prevention of soft tissue collapse, combined with mechanical support of the defect. [10]. Furthermore, cements can be combined with bioactive agents, for instance, antibacterial agents, in order to further display additional properties – in the case, antibacterial activity. Tetracyclines have been associated with distinct biomaterials for the management or prevention of biomaterial-associated tissue infections and to prospectively enhance the local bone tissue response upon implantation [11, 12].
In the present work, it is aimed the development and biological characterization of a poly methyl methacrylate (PMMA)-based minocycline delivery system, to be used as a space maintainer within craniofacial staged regenerative interventions.

Materials and Methods

Three different bone cement (BC) specimens were obtained: control BC and BC loaded with minocycline at 1% and 2.5%, relative to the weight of BC powder, hereinafter designated minocycline-loaded bone cement (MBC) 1 and MBC2.5, respectively. BCs were characterized for minocycline release and assayed *in vitro* for anti-bacterial and anti-inflammatory activity, and cytocompatibility with human bone cells.

Minocycline release: The release study was carried out in with a UV-HPLC system (Shimadzu system LC-6A, Shimadzu Corporation). Chromatographic analysis was performed at a detection wavelength set at 273 nm. The cumulative release (μg/mL) was expressed as the total minocycline released over time.

Anti-bacterial activity: Reference strains *Staphylococcus aureus* (ATCC® 25923), slime-producer-*S. epidermidis* RP62A (ATCC® 35984) and *Escherichia coli* (ATCC® 25922) were used and grown in Tryptic Soy Broth, at 37 ºC for 12 h. Bacteria were grown directly over material samples and assayed also on materials effluents, up to 72 hours. Quantitative evaluation were conducted with the reresazurin assay aiming the metabolic activity assessment (n=5). Qualitative evaluation was conducted following the Live/Dead® BacLight viability kit (Invitrogen, USA) staining and confocal imaging on a Leica SP5 confocal microscope (n=3).

Cytocompatibility assessment: MG63 human osteoblastic cells (ATCC® CRL-1427) were grown in α-MEM (Gibco, Germany) supplemented with foetal bovine serum (10% V/V), 100 IU/mL penicillin and 2.5 μg/ml streptomycin. Cultures were maintained at 37 ºC, in a 95% air and 5% CO2 humidified atmosphere. At adequate confluency, cells were harvested and seeded over the bone cement material samples, at a density of 10^5 cells/cm^2, for 7 days. Cultures were assayed for cell morphology and cell area determination (scanning electron microscopy and confocal microscopy following cytoskeleton and nucleus staining) n=3, metabolic activity (MTT assay) (n=5) and cell functionality (ALP activity) (n=5).

Anti-inflammatory activity: The macrophage cell line RAW 264.7 was grown in DMEM supplemented with foetal bovine serum (10% V/V), 2 mM L-glutamine, 100 IU/mL penicillin and 2.5 μg/ml streptomycin. Cultures were maintained at 37 ºC in a saturated-humid atmosphere of 95% air and 5% CO2. Cells were seeded at 2.5 x 10^5 cells/cm^2 and grown on the surface of the assayed bone cements (n=5), for 24 h. Cultures were stimulated with lipopolysaccharide 100 ng/mL (LPS, *Escherichia coli* serotype O128:B12, Sigma), and further maintained for another 24 h in the described conditions. Supernatants were collected for nitric oxide - NO (method of Griess - Griess Reagent System, Promega) and TNF-α (TNF-α ELISA Kit, Mouse, Thermo Fisher Scientific) determination, in accordance with the manufacturer's instructions.

Statistical analysis: The non-parametric Kruskal-Wallis test was used to compare the results between samples on the same experimental day, using SPSS software (v 20.0 for Windows; SPSS Inc., Chicago, IL). For all tests, the level of significance was $p < 0.05$ and represented graphically as * - significant different from BC, $p<0.05$.

Results

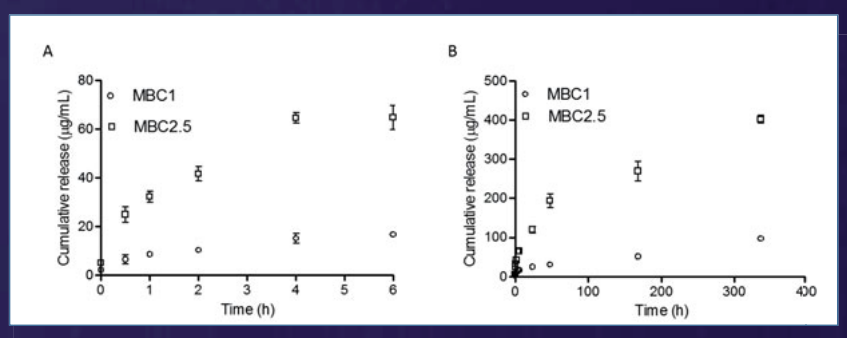

Fig. 1 - Cumulative minocycline release over time: A – 6 hours; B – 2 weeks.

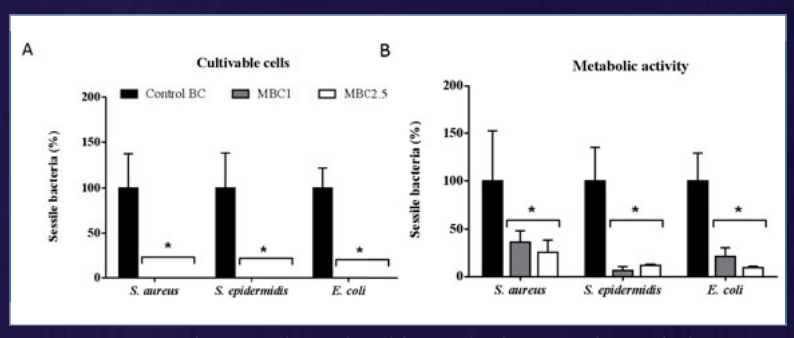

Fig. 2 – Grown bacteria by cultivable method (A) and metabolic active bacteria (B) on MBC, at 24 h of culture.

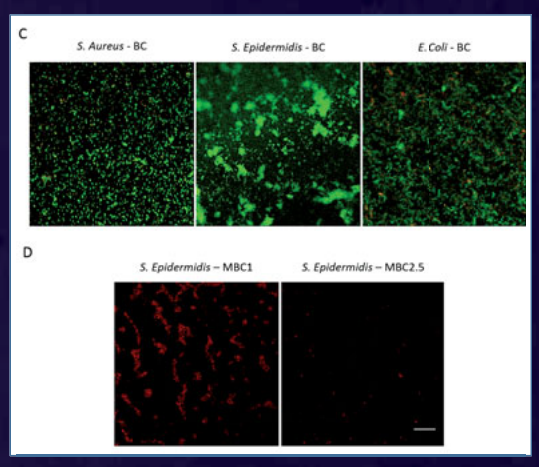

Fig.3 - C and D – Confocal images of bacteria grown on assayed formulations, following live/dead staining – viable bacteria stained green and non-viable bacteria stained red. Scale bar corresponds to 10 μm.

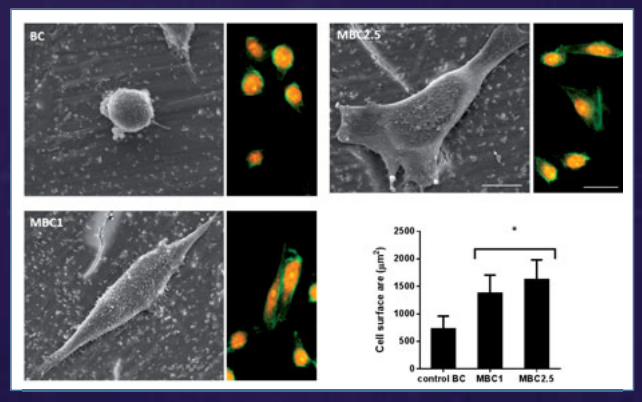

Fig. 4 – Osteoblastic cytocompatibility assessment by CLSM and SEM micrographs - cell area determination.

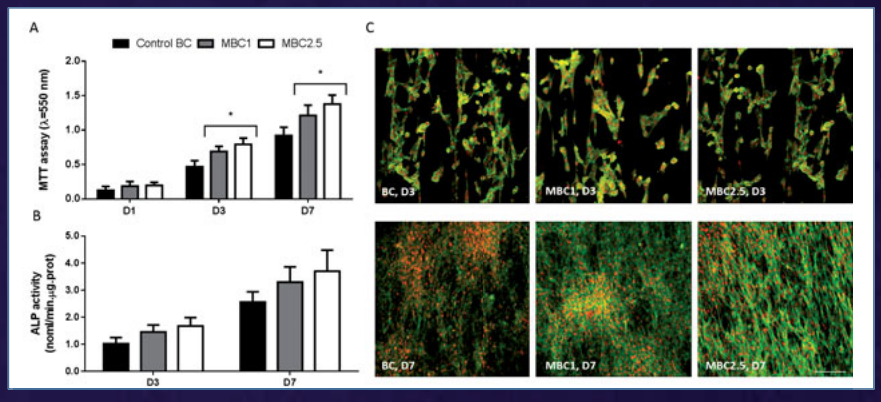

Fig. 5 – Osteoblastic cytocompatibility by MTT reduction, ALP activity and time course confocal imaging following cytoskeleton (green) and nucleus (red) staining.

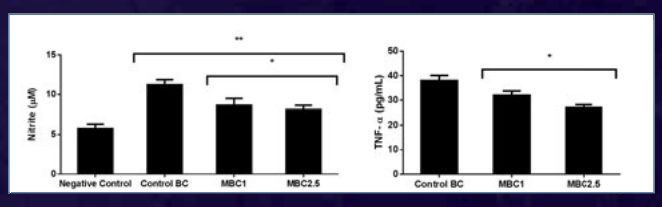

Fig. 6 - Determination of nitrite and TNF-α levels, following immune-inflammatory activation of RAW 264.7 macrophages.

Discussion

Attained results converge to support the possible efficacy of the developed MBC systems for the clinical management of complex craniofacial trauma, in which biomaterials with space maintenance properties are necessary for the management of staged reconstructive approaches, thus minimizing the risk of peri-operative infections and enhancing the local tissue healing. Regarding the release profile of minocycline, both MBC1 and MBC2.5 formulations showed an initial burst release, which is a convenient profile, thus minimizing the risk of infection in the immediate post-operative period. [13] The 72 hour release profile is of clinical relevance because it allows for an increased release within the perioperative period, granting an established antibacterial activity - with verified MICs (Minimum Inhibitory Concentration) for relevant pathogens, within early time points. In addition, minocycline was found to display cytoprotective effects over distinct eukaryotic populations, including those from muscle, microglia, neural tissue, liver, endothelium and kidney. [14] Presently, MBC endorsed an improved osteoblastic adhesion, with an increase cytoplasmic spreading and cell surface area, verified for both MBC1 and MBC2.5, in comparison to control BC. Also, increased metabolic activity and high ALP activity were verified for MBC formulations, sustaining an enhanced osteoblastic response. In terms of inflammation-modulatory capacity, MBC were found to significantly reduce the macrophage production of NO and TNF-α, following LPS activation. [15] Furthermore, no significant differences were found between the biological activity of MBC1 and MBC2.5 compositions.

Conclusion

The developed PMMA systems, with controlled release of minocycline, allowed for an effective antibacterial activity against strains relevant within trauma-related infections. Furthermore, an improved osteoblastic cell response - with enhancement of cell adhesion and cell proliferation – and increased anti-inflammatory activity were verified for MBC, as comparing to control BC.

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