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Induction of mineralized matrix production by recombinant human BMP-2 Immobilized in TEMPO-Oxidized Cellulose Hydrogel: a novel target for tissue repair

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Abstract: Bone morphogenetic proteins (BMPs) are potent promoters of osteogenesis, especially BMP-2, which has been highlighted for acting as a growth and differentiation factor that promotes new bone formation. There are several biomaterials that can be used to release bioactive substances, such as natural polymers. Cellulose has stood out for the possibility of its chemical modification using the reagent 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) to obtain a cellulose derivative (TEMPO oxidized cellulose nanofibers - ToCNF), which is shown to be a promising material for biological application. The objective of this work was to evaluate TEMPO cellulose immobilized with rhBMP-2 against the activity of inducing bone cell proliferation and differentiation in vitro, evaluating the ability to form bone matrix in pre-osteoblastic cell lineage of rats - MC3T3. Cell viability assays using resazurin were performed and for detection of mineralized matrix, Alizarin Red solution was used. The results reveal the good capacity of TEMPO cellulose functionalized with rhBM-2 in inducing the synthesis of mineralized bone matrix.

Keywords: Bone Morphogenetic Protein 2. Bone regeneration. TEMPO cellulose. Tissue engineering. Biocompatible materials.

Introduction

Bone morphogenetic proteins are a group of proteins belonging to the Transforming Growth Factor Beta (TGF-β) superfamily. BMPs are the most studied and most promising group of growth factors because they have a potent ability to promote bone and cartilage formation. The ability of BMPs to induce bone formation is called osteoinduction. Osteoinduction is due to the competence of this group of proteins to induce the undifferentiated mesenchymal cell, present in the receptor area, to transform into a bone-forming cell[1].

After the discovery of BMPs, several studies emerged on the osteoinduction capacity of these proteins, as they have become of great interest to several areas of medicine, especially regenerative medicine [2]. Some BMPs, such as BMP-2 (bone morphogenetic protein 2) have been highlighted for playing an important role in osteogenesis, and can be used as a powerful osteoinductive component in several tissue engineering products in the areas of orthopedics and dentistry[1,3].

BMP-2 has stood out for acting as a growth and differentiation factor in the body, which promotes new bone formation, acting extensively throughout the osteogenesis phase, both in vitro and *in vivo*[4,5]. Years of studies have been carried out on the application of different types of growth factors in bone regeneration. It is proven that, among all members of the BMP subgroup, BMP-2 is the most potent growth factor with positive effects on undifferentiated mesenchymal cell differentiation and osteogenesis[6]

The results of several animal experiments suggest a bright future for BMPs for bone reconstruction, bringing therapeutic benefits[7]. However, like other factors, BMPs require a transport system that releases them slowly and gradually, allowing an adequate condition for cell migration, proliferation and differentiation[6].

The use of BMPs in bone repair and regeneration is ushering in a new era for orthopedic and craniofacial reconstruction[8], as animal and human studies have already proven the ability of BMPs to promote bone formation[9]. Therefore, since their discovery, BMPs promise a promising future in the field of tissue engineering[10]. However, one of the biggest problems for researchers is to develop ways to deliver these osteoinductive factors and therefore achieve clinical success in humans[8,9].

Tissue engineering and regenerative medicine are constantly growing and have positive contributions to

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the advancement of various pathologies, especially on the world stage due to the low number of organs and tissues available for transplantation[11]. Tissue engineering is a multidisciplinary field that involves several areas of knowledge, mainly the medical, biological and engineering areas. It aims to improve new techniques and develop new biomaterials that restore, maintain or improve the function of different organs and tissues[12,13].

The choice of a biomaterial depends on the analysis of several conditions, such as biocompatibility, toxicity, biodegradability, as well as the material's degradation rate and biofunctionality. A biomaterial must also be able to retain the growth factor at the repair site for a period compatible with the time of tissue re-formation[9]. There are several biomaterials available, highlighting the natural polymers that can be of protein origin, such as collagen, and polysaccharide, such as cellulose, among others.

Hydrogels are three-dimensional polymeric networks formed by hydrophilic structure capable of absorbing and releasing water or biological fluid in response to environmental conditions[14]. The retention capacity of hydrogels is associated with the hydrophilic groups present in their composition (-OH-, -CONH-, -COO- and -SO2H-)[15] and other factors such as pH and temperature. While the expansion capacity is linked to the osmotic phenomenon[14]. There are several hydrogels with application in regenerative medicine, among them are cellulose-based hydrogels.

Cellulose is one of the most abundant polymers in nature, it is a polymer formed from the bonding of thousands of glucose units. This polymer can also be classified as a polysaccharide and is present in most plants, making up their cell walls. In the broad field of tissue engineering and biomedical applications, cellulose is recognized for having distinctive characteristics[16]. Although cellulose *in natura* does not present a degradation rate in time compatible with the repair time of most tissues, being a limitation for more complex biomedical applications[2]. However, cellulose has, among other characteristics, water retention capacity, hydrophilicity and biocompatibility[16]. In addition, it displays functional groups available for chemical modification. The oxidation of the glucose carbon-6 hydroxyl group with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) radical,generates the cellulose derivative (ToCNF or also called TEMPO cellulose) rich in carboxylic groups, highly hydrophilic, with the physical appearance of a viscous transparent hydrogel, formed by individualized nanofibrils[17].

Recent advances in bone regeneration have explored innovative biomaterials based on TEMPO-oxidized cellulose nanofibrils, as they have been shown to be resorbable in humans at physiological pH (~7.4). Approximately 90% by weight of the oxidized cellulose is solubilized within 21 days and converted into the sodium salt of polyglucuronic acid, which is easily eliminated by the body[18].

The study by Abouzeid et al.[19] evaluated the in situ mineralization of calcium phosphates (dicalcium phosphate dihydrate and hydroxyapatite) in hybrid hydrogels combining alginate, PVA, and TEMPO-oxidized cellulose nanofibrils (T-CNFs) under different pH levels, aiming at applications in bone regeneration and wound healing. The results showed that the hydrogels containing hydroxyapatite are more stable and promising for supporting the healing of both hard and soft tissues, demonstrating the potential of these materials in tissue engineering.

In line with this approach, the study by Gorgieva et al.[20] investigated gelatin scaffolds reinforced with TEMPO-oxidized cellulose nanofibrils modified with phosphonate groups to promote calcium deposition by mesenchymal stem cells. The modified scaffolds showed greater hydroxyapatite deposition and supported cell growth compared to unmodified scaffolds, demonstrating superior osteoinductive potential and standing out as a promising alternative for bone regeneration.

Salama et al.[21] then, assessed via calcium phosphates (CaP also developed a new biomaterial for bone regeneration composed of TEMPO-oxidized cellulose nanofibers (T-CNF) grafted with soy protein hydrolysate (SPH) and mineralized with calcium phosphate (CaP). This material demonstrated high biocompatibility, promoting the proliferation and differentiation of human mesenchymal stem cells and forming a mineralized matrix similar to bone apatite. These results indicate that the biomaterial is promising for bone tissue repair and regeneration, mimicking the composition and biological properties of natural bone.

Complementarily, the study by Yao et al.[22] developed biomimetic materials for bone regeneration using cellulose nanofibrils (CNFs) modified by TEMPO oxidation and chitin (ChNFs), which were enzymatically mineralized with alkaline phosphatase (ALP) to form calcium phosphate (CaP) deposits. The resulting materials demonstrated high stiffness and strength while maintaining flexibility and adjustable mechanical properties, showing great potential for applications in bone replacement and tissue regeneration with structural gradients.

Ingole et al.[23] developed and characterized hydroxyapatite (HA) nanocomposites reinforced with TEMPO-oxidized cellulose nanofibrils (TCNF) and cellulose nanocrystals (CNC) for bone regeneration applications. The composites containing TCNF exhibited superior mechanical properties, such as

compressive strength, elastic modulus, and fracture toughness, comparable to cortical bone. All HA composites, regardless of the type of nanocellulose, were biocompatible and promoted the viability of human osteoblasts, highlighting their potential for bone regeneration in load-bearing applications.

In the area of 3D printing, Im et al.[24] developed an osteogenic bioink composed of alginate, TEMPO-oxidized cellulose nanofibrils (TOCNFs), and polydopamine nanoparticles (PDANPs) for the creation of scaffolds intended for bone tissue engineering. The incorporation of TOCNFs and PDANPs increased printability, improved the mechanical properties and bioactivity of the bioink, and promoted the proliferation and osteogenic differentiation of osteoblastic cells.

Given the current scenario concerning studies on cellulose nanofibrils for bone repair, this study presents a novel approach by combining TEMPO-oxidized cellulose (ToCNF) with recombinant human bone morphogenetic protein BMP-2 (rhBMP-2).Therefore the objective of this work was to evaluate the ability of TEMPO cellulose hydrogel immobilized with human recombinant bone morphogenetic protein (rhBMP-2) to induce bone cell proliferation and differentiation *in vitro,* as well as the ability to form bone matrix in mouse pre-osteoblastic cell line - MC3T3.

Materials and Methods

Preparation and characterization of TEMPO-oxidized cellulose nanofibrils (ToCNF)

ToCNF was prepared in two steps, firstly, the bleaching, followed by chemical modification (oxidation). For bleaching, 10 g of milled sugarcane bagasse (~1 mm length) was extracted in a Soxhlet system using toluene/ethanol 2:1 (v/v) for 8 h and then dried overnight at room temperature. The dried fibers were dispersed in 1.3 % sodium chlorite solution (400 mL), the pH was adjusted to approximately 4 (± 0.5) with diluted acetic acid, the mixture was heated at 75 °C and stirred for 1 h, using a reflux condenser system. Then, the fibers were washed with deionized water up to neutral pH. The fibers were then dispersed in 2 wt% KOH aqueous solution (400 mL) and stirred at 85 °C for 2 h. Then, the fibers were again washed with deionized water up to neutral pH. The treatment with sodium chlorite solution (first step) was repeated. This was followed by treatment with 5 wt% KOH aqueous solution (400 mL) at 85 °C for 2 h, followed by washing with deionized water. For chemical oxidation, the bleached sugarcane bagasse suspension (10 g, 1 wt%), sodium bromide (1 mmol/g cellulose), and TEMPO reagent (0.1 mmol/g) were stirred together, and the sodium hypochlorite solution (5 mmol/g cellulose) was slowly dropped into the suspension. The pH of the suspension was maintained at ~10 by slowly dropping 0.1 M NaOH. After the pH was stable at ~10, the pulp was filtered and extensively washed with deionized water to neutral pH. The oxidized fibers were sonicated in an ultrasonicator (Hielscher, UP 400S, 400 W, 24 kHz) using an ice bath, until the formation of a transparent gel (1 min sonication, and 2 min interval, for about 10 min).

The ToCNF content in the hydrogel was determined by gravimetric analysis at 105 °C for 6 h. The carboxylic acid content in the structure of ToCNF was determined by conductometric titration[25]. The chemical modification was accessed by FTIR using a Perkin-Elmer Spectrum 100 FT-IR Spectrometer equipped with an ATR module with a selenite diamond crystal, the resolution being 4 cm^{-1} after 16 scans.

Cell culture

Human fibroblasts (GM07492) and osteoblast-like rat cells (OSTEO-1) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). The osteoblast precursor cell line derived from mouse calvaria (MC3T3) was cultured in α-MEM medium supplemented with 10% FBS. All cell lines were incubated at 37 ± 2 °C in an atmosphere of 5% CO_2 until reaching 80-90% confluence to be used in the experiments.

Cellularization and ToCNF cell viability assay

ToCNF membranes were inserted into the wells of a 96-well plate and then with $1x10^4$/well and kept in incubation. Growth was monitored for 72 hours by the resazurin viability method.

Resazurin is a blue, weakly fluorescent dye, reducible to resorufin, a pink dye with high fluorescence. The reduction of resazurin takes place by the dehydrogenase enzymes of the mitochondria of living cells, therefore the fluorescence generated by resorufin indicates cellular viability as it is a metabolite generated only in the presence of living cells.

Every 24 hours, 200 µL of the resazurin solution was removed and transferred to a 96-well plate, and the fluorescence measured in a plate reader (SpectraMax i3, Molecular Devices, USA) at 570 nm excitation and 590 nm emission length.

Detection of rhBMP-2 induced mineralization by Alizarin Red staining assay

The alizarin red staining was used to detect calcium deposits of mineralized extracellular matrix in Osteo-1 cell culture. Osteo-1 cells treated or not treated with rhBMP-2 were seed in a 24 wells plate ($1x10^5$ cells/well) and incubated in DMEM culture medium supplemented with FBS 10% at 37 ± 2 °C in 5

% CO_2 atmosphere for 7 days. The samples were fixed using ethanol (70 % v/v) and stained with alizarin red 1% (1 % w/v) for 2 min at room temperature followed by washing with distilled water until excess dyed is removed. The stained wells were photographed under light Zeiss microscope. After imaging the hydroxyapatite crystals were destained in 0.1% acetic acid (v/v) and a 100 µl aliquot was transferred to a 96-well plate for absorbance (540 nm) reading in Spectra Max i3 plate reader (Molecular Devices). The values obtained were plotted in a bar graph using the Graph Pad Prism program.

Immobilization by adsorption of rhBMP-2 on TEMPO cellulose

In a 24-well plate, two wells were filled approximately with a volume of 0.3mL of ToCNF and 1 mL of PBS solution containing the purified rhBMP-2 at variable concentrations (mg/mL). aiming its immobilization on the surface of TEMPO cellulose by electrostatic attraction of oppositely charged , namely, the negative charges from TEMPO cellulose with the positive charges from the protein side chains. The plate was incubated at room temperature for 4 hours. Then, the solution from each well was removed using a micropipette for absorbance reading in Nano Drop (Thermo Fisher) - small volume spectrophotometer (1-2 µL).

Proliferation and Mineralization detection at the surface of ToCNF-rhBMP2

To evaluate the proliferation of cells with or without TEMPO cellulose, MC3T3 cells were seeded in triplicate in a 24-well plate previously filled or not with TEMPO cellulose, at a density of $5x10^3$ cells/well. The plate remained in an oven at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hours. Every 24 hours the medium was removed and a volume of 1mL of resazurin solution (10% in culture medium) was added to each well followed by 4 hours of incubation. After the incubation period, 200 µL of the resazurin solution was removed and transferred to a 96-well plate, and the fluorescence measured in a plate reader (SpectraMax i3, Molecular Devices, USA) at 570 nm of excitation and 590 nm of emission length.

After chemical bonding of rhBMP-2 on TEMPO cellulose MC3T3 cells were seeded in a 24-well plate ($2x10^4$ cells/well) whose wells were pre-filled with rhBMP-2 treated or untreated TEMPO cellulose. After 24 hours the medium was removed, the wells were washed with PBS Buffer and the differentiation medium (α-MEM supplemented with 10%FBS, 10 mM β-glycophosphate, 0.1 mM dexamethasone and 50 mM ascorbic acid) was added to the corresponding wells of cellulose untreated with rhBMP-2. The medium was changed every 3 days and the plate remained in an incubator at 37° C in a humidified atmosphere with 5% CO_2. After 14 days, staining with Alizarin Red was performed to verify the presence of mineralized bone matrix by t mineralization nodules detection. For staining the cells were fixed in 4% paraformaldehyde solution (m/v) at room temperature for 10 minutes followed by washing with 1X PBS. After washing, 1mL of Alizarin Red solution was added to each well and the plate was incubated for 30 minutes at room temperature. Then the dye was removed, the wells washed with distilled water and images were captured using a Nikon Eclipse T5100 inverted microscope.

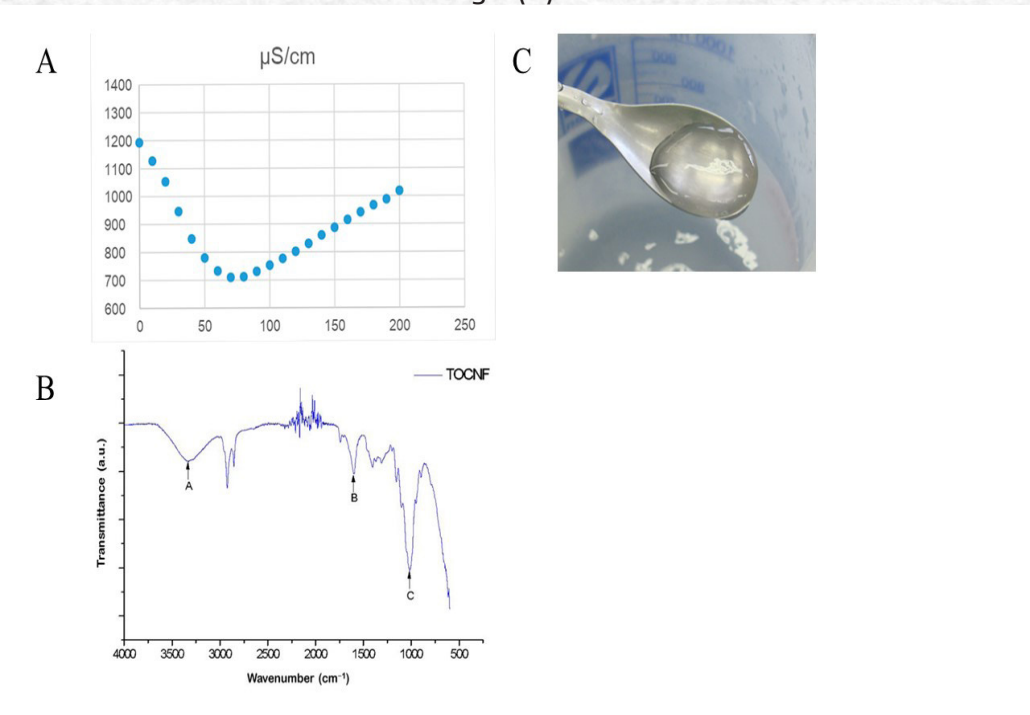
Results and Discussion

The curve of the conductometric titration of ToCNF is shown in Figure 1. The results indicated, by simple titration, that the number of free carboxylic acid in ToCNF was 0.25 mmol/g. These chemical modification was confirmed by FTIR, which results are shown in Fig. 1 B. ToCNF FTIR spectra bands at about 3300, 2900, and 1027 cm^{-1}, correspond to the vibrations of the -OH, C-H, and C-O-C groups, respectively, of the glucose structure, typical of cellulosic substrates. The strong peak at 1599 cm^{-1} is assigned to the asymmetric stretching vibration of carboxylate (C=O) formed after the oxidation of the C-6 primary hydroxyl groups of cellulose (26,27) with 2,2,6,6-tetramethylpiperidine-1-oxyl reagent (TEMPO. These results confirm the oxidation reaction of cellulose.

ToCNF membranes were inserted into the wells of a 96-well plate and then cellularized with 1x104/well and kept in incubation. Growth was monitored for 72 hours as shown in Photographs A, B, C, D and E in Figure 2.

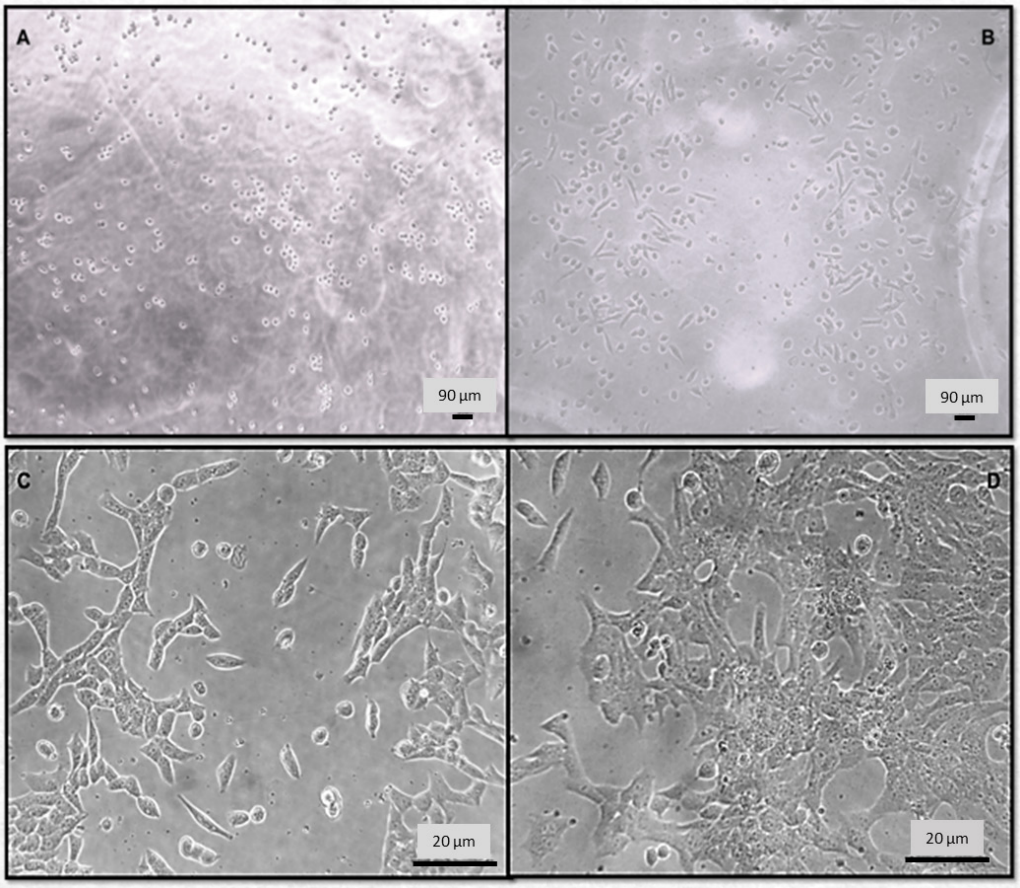
After cellularization of the ToCNF membranes, the resazurin method was adopted to verify the viability of the cells that were adhered to the cellulose membranes. Figure 3 shows the average of the fluorescence points measured at pre-established times. From these results it is possible to see that the ToCNF membranes had a lower cell viability compared to the control cells. However, there was a stabilization of cell growth demonstrating that ToCNF membranes were not cytotoxic (Figure 3).

Figure 1 - Conductometric titration curve (A) and FTIR-ATR spectra of ToCNF (B) and the TEMPO cellulose gel (C).



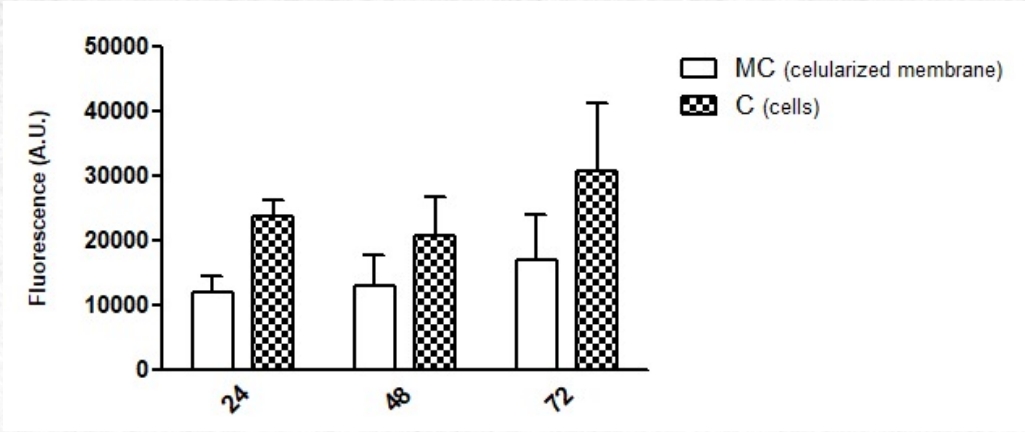
Source: Own authorship, 2024.

Figure 2 - Cellularization of the membranes (A – Cellulose membrane plus cells at time 0; B – Cellulose membrane after 4 hours; C – Cellulose membrane plus cells after 24 hours; D – Cellulose membrane plus cells after 48 hours).



Source: Own authorship, 2024.

Figure 3 - Cellular Proliferation of ToCNF membranes by Resazuirn Method.

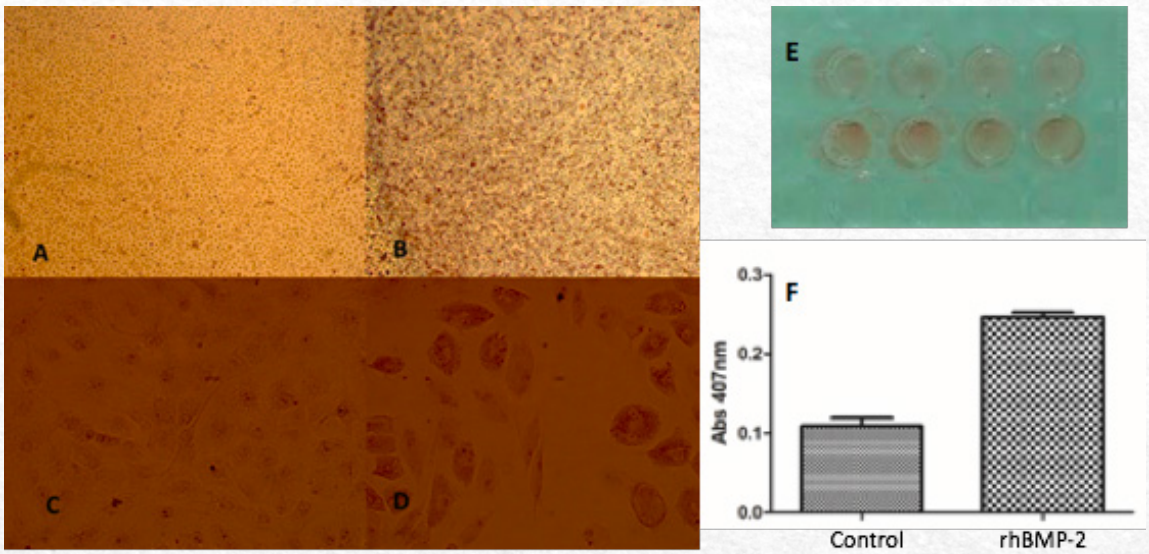


Source: Own authorship, 2024.

The results of the use of rhBMP2 into the mineralization culture medium are shown in Figure 4. The images of optical microscopy at two magnifications (A and B, and C and D) for the mineralized matrix free of rhBMP2 and in presence of rhBMP2 indicate that both the conditions led to the mineralization, however, with visual evidence of increased mineralization in the presence of the rhBMP2, evidenced by the intensity of the red color in this sample. These results showed the osteoinduction of rhBMP2, as it is one of its most important biological activities. Figure E shows the picture of the wells of the culture plate, with the mineralized culture replicates at the top and the mineralized culture replicates in presence of the rhBMP2 at the bottom. Figure 4 F shows the values of the absorbance of the mineralization tests carried out using the wells of Fig. 4 E, and the total agreement of the visual color intensity with the spectrophotometric reading.

In the experiments carried out to immobilize rhBMP-2 on TEMPO cellulose, it was possible to make an estimate in percentage of the concentration of protein adsorbed on the cellulose. In each experiment, a volume of 1 mL of solution was used, containing a certain concentration of rhBMP-2. After the incubation time applied in this work, the final reading of the protein concentration in each experiment was performed. Results were expressed as a percentage (Table 1).

Figure 4 - A and C shows the microscopy of the mineralized cells matrix formed in the medium free of rhBMP2, at two different magnification (20X and 40X); B and D shows the microscopy of the mineralized cells matrix formed in the media with rhBMP2, at two different magnification (20X and 40X); E shows the visual aspect of the plate wells with samples A and B; and F shows the spectrophotometric data reading of the test of mineralization for samples A and B.



Source: Own authorship, 2024.

Table 1 - Percentage of adsorption of rhBMP-2 on TEMPO cellulose.

Experiment	mg/mL (initial)	mg/mL (final)	%
1	0.225	0.110	48.8
2	0.244	0.120	49.1
Average	0.234	0.115	48.9

Source: Own authorship, 2024.

In the short incubation time adopted in this work, the results obtained in the two experiments gave a percentage of approximately 49% of adsorption of rhBMP-2 on TEMPO cellulose. In addition to being a low and affordable production cost strategy.

The cell proliferation assay was performed using resazurin. The blue, non-fluorescent resazurin is reduced to the pink, fluorescent resazurin, and its formation is directly proportional to mitochondrial metabolic activity and cell viability. For this work, readings of the times of 24 and 48 hours were made. The results were presented with the fluorescence values by arbitrary unit (AU), as shown in Figure 5.

The results revealed that the biomaterial used could promote viability and cellular proliferation by the increase in fluorescence.

The images in Figure 6 show TEMPO cellulose treated or not with rhBMP-2 before the induction of mineralized bone matrix synthesis, as well as revealing the synthesis of mineralized bone matrix stained with Alizarin Red. For TEMPO cellulose not immobilized with rhBPM-2, a culture medium containing osteoinductors (differentiation medium) was used. While for the TEMPO cellulose treated with the protein, there were no osteoinductive factors in the medium used.

Qualitatively, it is possible to observe that TEMPO cellulose - rhBMP-2 was able to induce the synthesis of mineralized bone matrix, as we can visualize calcium-alizarin crystals.

Figure 5 - Resazurin reduction at 24 and 48 hours, expressed as percentage reduction.

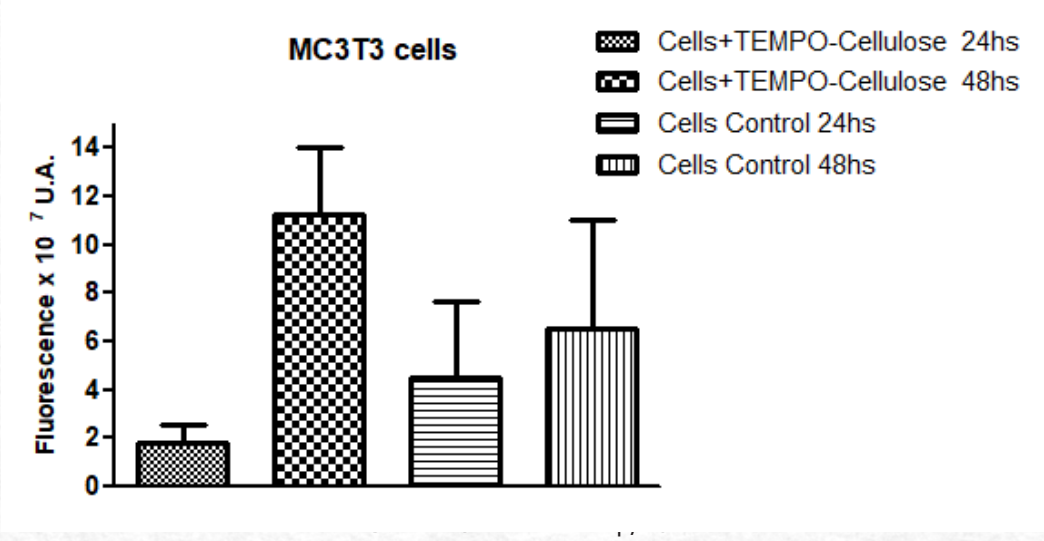
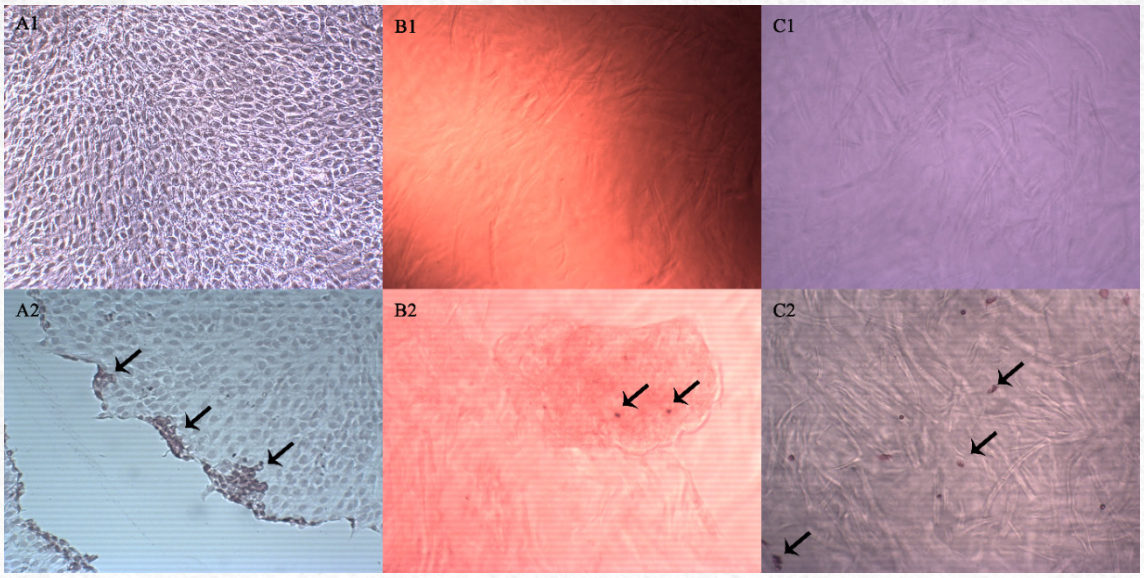


Figure 6 - TEMPO cellulose treated or not with rhBMP-2– Images captured at 10x magnification by optical microscopy. Arrows indicate the presence of mineralized bone matrix. Images A1, B1 and C1 show, respectively, the cell culture (MC3T3) without TEMPO cellulose, the cellularized TEMPO cellulose and treated with rhBMP-2 and the cellularized TEMPO cellulose and not treated with rh-BMP-2 before the induction of synthesis. of mineralized bone matrix. A2, B2 and C2 reveal the synthesis of mineralized bone matrix stained with Alizarin Red.



Source: Own authorship, 2024.

Conclusion

The cell viability results of cellularized ToCNF membranes indicate that the modified cellulose provides a stable environment for cell maintenance, corroborating the results of detection of proliferation and mineralization on the surface of ToCNF-rhBMP2. Overall, this study demonstrates that TEMPO cellulose functionalized with rhBM-2 has significant potential for use as a biomaterial in bone tissue repair. New studies should be encouraged to confirm the experiences found.

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