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# *BIOLOGICAL ANALYSIS OF SCAFFOLDS PRODUCED BY NANOTECHNOLOGY, TO BE USED TO CULTIVATE STEM CELLS FROM DECIDUOUS TEETH*

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**Abstract**: Bone loss represents a challenge to reconstructive surgery, where the use of different sources of bone grafts with inherent limitations is frequent. Currently, bioengineering studies are discovering new ways of tissue production, including their use in reconstructive surgery. The present study uses stem cells from the pulp of deciduous teeth, combined with biodegradable scaffolds produced using nanotechnology in order to evaluate their implantation with the aim of promoting bone tissue regeneration in rat calvaria. This work was conducted in four stages: characterization of cells obtained from deciduous teeth; characterization of nanofiber scaffolds for cell adhesion and proliferation; tests for interaction between cells and nanofibers and implantation of nanofibers seeded with stem cells in bone cavities in calvaria of mice. The results indicate that the cells collected from deciduous teeth have characteristics compatible with mesenchymal stem cells and the scaffolds have characteristics suitable for use in tissue engineering because the cells interact well when seeded onto these scaffolds.
**Keywords**: *Bioengineering, stem cells, nanotechnology, deciduous teeth.*

## Introduction

Tissue engineering combines the use of biomaterials with cell therapy and signaling factors, with the aim of using it as an alternative for tissue regeneration.Among the biomaterials used are the scaffolds, which can be used as molds and to simulate the extracellular matrix (ECM), in which cells can be cultured, promoting cellular adhesion and proliferation. While the cells grow, the biomaterial degrades and gives rise to the formation of new tissue [1, 2]. Among the polymers used to manufacture scaffolds, there is the co-polymer poly(lactide-co-glycolide) acid (PLGA), which is a biodegradable and biocompatible polymer which does not present any toxicity [3]. Scaffolds with more than 90% porosity can provide more space for cell accommodation, promoting more efficient exchange of nutrients and metabolic waste [4]. These scaffolds can be produced by the electrospinning method. One focus of bioengineering is the use of stem cells, such as those from the pulp of deciduous teeth [5,6], which represent a source of easy access. The aim of this study was to characterize PLGA scaffolds produced by the electrospinning technique in which stem cells from the pulp of deciduous teeth were seeded and evaluated as potential biomaterial for use in tissue

engineering in vitro, initially targeting their future use for clinical trials in bone regeneration.

**Experiment**

*Preparation of scaffolds by the electrospinning method*

The scaffolds were prepared using a poly(D,L-lactide-co-glycolide) (75:25) (PLGA) (P1941 Sigma-Aldrich). The polymer was dissolved in 1,1,1,3,3,3-Hexafluoride-2-propanol (Sigma Aldrich) to obtain a final concentration of 12% (w/v). The solution was kept in a magnetic stirrer for 24 h. The PLGA solution was placed in a 1 mL syringe needle equipped with an inner diameter of 0.8 mm. The polymer solutions were then submitted to the electrospinning process, using a voltage difference of 12 kV, a flow rate of 0.24 mL h-1 and a distance of 15 cm from the needle. All experiments were performed at room temperature (~ 25°C) and relative humidity (RH) of air around 35%.

*Evaluation of morphology and diameter of the fibers and the dimensions of the scaffolds*

The morphology of the PLGA scaffolds was evaluated by scanning electron microscopy (SEM) (JOEL - JSM 6060). The diameter of the fibers was evaluated by ImageJ software. The thickness of the matrices was measured with a Digimatic Micrometer MDC-25MY (Mitutoyo).

*Sample of pulp tissue from deciduous teeth*

To obtain and remove the pulp tissue from deciduous teeth, as well as to characterize their cells as mesenchymal stem cells, protocols already established by the group were used [7,8].

*Biological assays*

The cells were seeded onto the scaffolds at a density of 5 x $10^4$ viable cells in 1,000µl of culture medium and then incubated at 37˚C in a humidified atmosphere of 5% CO2.

The following biological analyses were carried out.

Evaluation of cell adhesion *on PLGA s*caffolds

After 6 hours of cultivation, the medium was removed and the samples were washed three times with phosphate buffered saline (PBS) 1X to remove the cells not attached to the scaffolds. The adhered cells were then fixed with 4% paraformaldehyde for 20 min and 0.5 µg/mL of 4,6-diamidino-2-phenilindole (DAPI) was used as a nuclear marker. From each sample, nine images (Olympus CX50, 400x magnification) were obtained, corresponding to nine different fields

randomly distributed to quantify the number of attached cells (number of cells/field). For the control group, the same procedures were carried out and the same quantity of cells was seeded onto 24-well plates without scaffolds.

*Assay of Cell Proliferation and Viability*

The cell viability and proliferation were analyzed during the following periods of time: 3, 7, 14 and 21 days after seeding the cells, using the colorimetric assay with [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] MTT (thiazolyl blue). The metabolic activity measured by MTT assay is widely used in in-vitro evaluation of cell viability [9]. The number of cells in each scaffold was determined based on a calibration curve performed previously. For the control group, the same procedures were carried out and the same amount of cells were seeded onto 24-well plates without scaffolds.

*Confocal microscope analysis*

After 14 days of culture, the cells adhered onto the scaffolds were stained with DAPI, as previously described. Subsequently, staining was performed with 0.5 mg/ml conjugated with Rhodamine Phaloidina to mark the cytoskeleton. The images of the samples were obtained from an Olympus microscope SV1000.

**Results and Discussion**

By using images obtained by SEM (Fig. 1), it can be observed that the fibers formed were distributed randomly throughout the scaffold structure, which showed a large number of interconnected pores. The fibers showed a smooth surface and an average diameter of 315.9±70.2 nm (Fig. 2) and the scaffolds showed a thickness of 37±5.3 micrometers. The presence of beads in the nanofibers was also observed. The morphology of the nanofibers is the result of a combination of several factors, including intrinsic properties of the solution, such as the type of polymer, the concentration, elasticity and conductivity of the polymeric solution, as well as the type of solvents. Moreover, the morphology of the nanofibers is also influenced by conditions related to the technique of electrospinning, such as electric current, flow and distance between the needle and the collector plate. A small fiber diameter and a porous structure with a high surface area is reported as being beneficial for the application of nanofibers in tissue engineering [3]. In this study fibers with nanometer dimensions and scaffolds with interconnected pores and high surface area were produced.By staining the cell nuclei with DAPI, it was observed that the cells were able to adhere to

the scaffolds (Fig. 3 -A). The average number of adhered cells per field on the scaffolds in the group was similar to the control group (17.11 and 17.48 cells/field, respectively) (Fig. 3 - B). The capacity of the scaffolds to promote cell adhesion is a crucial feature for the applicability of the matrices in tissue engineering. Cellular adhesion is firstly measured by the adsorption of proteins from the extracellular matrices to the surface of the scaffolds.The assay showed that the average number of cells per field analyzed was similar, comparing the test and control groups. Thus, the PLGA nanofibers demonstrated good characteristics as scaffolds for cell adhesion.

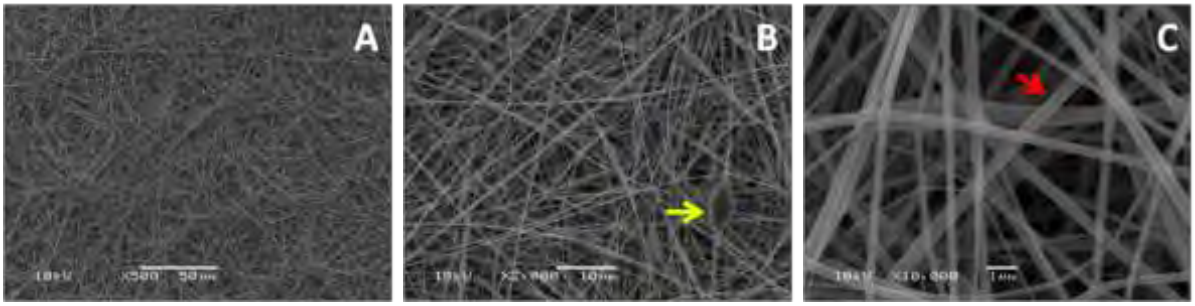


**Figure 1.** Analysis scanning electron microscopy (SEM). Electronic micrograph of PLGA nanofibers: 500x (A), 2000x (B), 10000x (C) of magnification. The yellow arrow (B) indicates a bead, and the red arrow (C) indicates a smooth fiber.

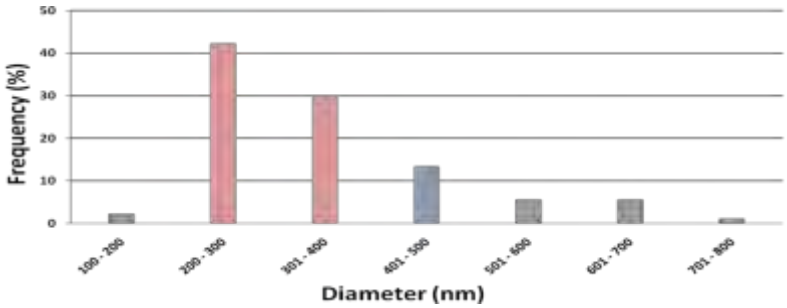


**Figure 2.** Distribution of the fiber diameters.

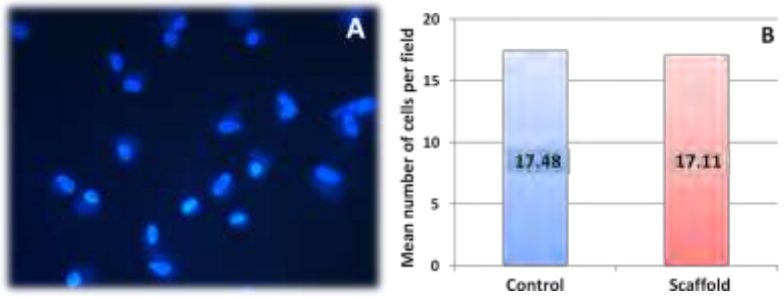


**Figure 3.** Image captured with a fluorescence microscope (400x of magnification). DAPI shows the nuclei of cells seeded onto scaffolds (A). The cell adhesion was similar between the control and test groups (B).

Cellular viability and proliferation analysis (Fig. 4) showed that, after 3 days of culture, the initial number of viable cells increased to approximately 1.2 x $10^5$ e 2.1 x $10^5$, in the test and control

groups, respectively. This find showed a proliferation of the cells which were initially seeded.

On day 7 of the cell culture, the number of viable cells in the test and control groups was approximately 1,6 x $10^5$ e 1,9 x $10^5$, respectively, demonstrating the continuity of cell proliferation in the scaffolds but with a decrease in the number of cells in the control group. Fourteen days after the initiation of the culture, the number of viable cells increased in both groups. On the other hand, on day 21 the number of viable cells decreased in both groups, as can be observed in figure 4.

This decrease in the number of cells can be attributed to the prolonged period in which the cells were maintained in the scaffolds. The high rate of proliferation could cause a confluence of cultivated cells higher than what is considered ideal for cell culture, making the environment hostile and damaging the cellular metabolism. Therefore, it can be suggested that the association between cells and scaffolds is favorable up to the 14$^{th}$ day after seeding.

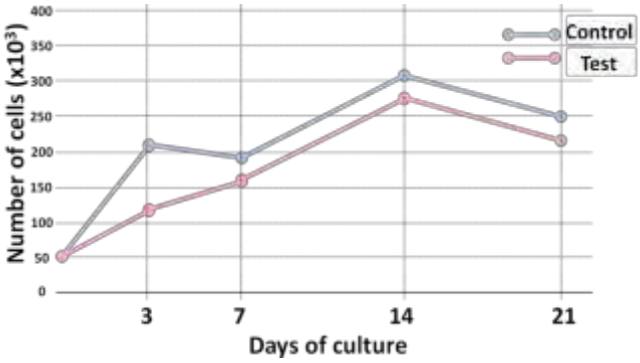


**Figure 4.** Progressive increase in cell number up to day 14 with decrease thereafter until day 21.

The images obtained with a confocal microscope show that 14 days after the cells had been seeded onto the scaffolds, they were distributed homogenously throughout the structure of the scaffold (Fig. 5 - A).

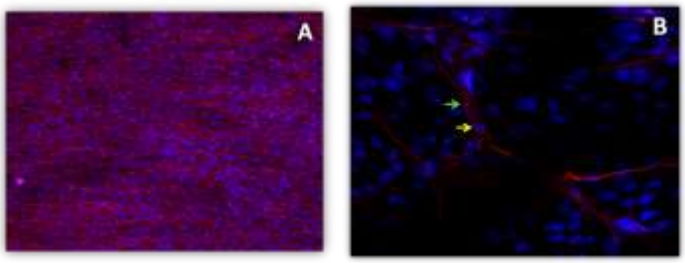


**Figure 5:** Image captured with a confocal microscope. The cell nuclei are stained in blue (yellow arrow) with DAPI. The cell cytoskeleton is stained in red (green arrow) with phaloidina/rhodamine (A). Increase of the region showing the nucleus and cytoskeleton (B).

It was found that, after 14 days of culture, the cells occupied all the structure of the scaffolds. The

entire structure of the cytoskeleton throughout the cytoplasmic extension was also observed, giving the cell a fusiform aspect, similar to fibroblasts. This cellular morphology was also found in the study of Miura and colleagues [5], which showed the same cellular aspect in cells isolated from the pulp of deciduous teeth in the resorption process.

## Conclusion

This study shows that the PLGA scaffolds are good biomaterial for cultivating stem cells from deciduous teeth and are sitable for use in tissue engineering.

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