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In vivo Biocompatibility of Dental Scaffolds for Tissue Regeneration

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Abstract. It is important to develop scaffolds that resemble the extracellular matrix, thereby facilitating tissue regeneration. The objective of this work is to evaluate the biocompatibility of scaffolds of poly (L-lactide) with pure and grafted hydroxyapatite, at various concentrations of reinforcement, in Wistar rat tissues, to evaluate their potential application on tissue regeneration. The biocompatibility tests were carried out *in vivo* in Wistar rats by implanting the material into the subcutaneous tissue and muscle from 1 to 14 weeks and evaluating the surrounding tissue stained with hematoxylin-eosin. All variants of scaffolds provoked an inflammatory response from mild to moderate, without showing necrosis. These results show that even if there is recognition of the implanted materials by the immune system, it does not provoke a violent response that damages the surrounding tissue, implying that the materials to be implanted for use in hard tissue can cause a mild reaction and tolerable long term effect that does not prevent their later use in hard tissue regeneration.

Introduction

Tissue regeneration is required in situations that cause physiological or pathological tissue loss [1, 2]. Among the main methods found in tissue engineering is the "*in vitro*" growth of interest cells in a three-dimensional (3D) structure, shaped as the target organ or tissue. Most scaffolds for hard tissue regeneration use poly (L-lactide) (PLLA) due to its biocompatibility and biodegradative properties, and hydroxyapatite (HA) because of its osteoconductive characteristics. Electrospinning is a useful technique to prepare scaffolds that mimic bone extracellular matrix, with adequate mechanical properties, biodegradability and osteoconductivity [4, 5]. Vera-Graziano et. al. demonstrated that the grafted poly (L-lactide)-hydroxyapatite (PLLA-g-HA) scaffolds at different concentrations of reinforcement have good mechanical and *in vitro* biological properties. Especially the scaffold containing 4% of grafted HA, which has the best properties, in comparison with higher grafted concentration and the scaffolds made of pure HA [6]. This work was undertaken to evaluate the *in vivo* biocompatibility of microfibers made of mixtures of PLLA/HA and PLLA-g-HA (4-30% of HA) in two different tissues of Wistar rats with the objective of validating the non-toxicity of these materials in living tissues. Hopefully these results will lead to the third stage of biocompatibility

investigation as suggested by the current international toxicity standards, to evaluate the usefulness of the scaffold to regenerate *in vivo* bone and dentin tissues.

Materials and Methods

Materials. The PLLA was obtained by a non-catalyzed ring opening polymerization reaction and was grafted on the surface of the HA nanoparticles (Sigma Aldrich). Then pure PLLA was blended with grafted and pure HA at different concentrations (4–30% w/w) in solution in 2, 2, 2-trifluoreothanol. These blends were used to prepare porous fibrous scaffolds by electrospinning. The samples were named according to the concentration of pure HA: PLLA/HA4, PLLA/HA10, PLLA/HA20 and PLLA/HA30. PLLA grafted HA samples were named: PLLA-g-HA4, PLLA-g-HA10, PLLA-g-HA20 and PLLA-g-HA30, respectively. All materials were synthesized and mechanical properties characterized as previously reported [6].

Test Animals. Twenty four 5-week-old Wistar rats (*Rattus norvegicus*) with body weight of 100- 300 gr were used. The rats were housed one per cage and they had free access to tap water and standard pellet food (Furry Friends Pet Food®). All experiments were approved by the Bioethics Committee of the Medicine and Psychology Faculty, Autonomous University of Baja California, Mexico, with registration number IORG # IORG007487, of United States Department of Health and Human Services (HHS). Also, the experiments were conducted in accordance with the Mexican Legislation Standard of NOM-063-SSA1-1993, Ley General de Salud [7].

***In vivo* scaffold implantation.** For implantation tests, the rats were divided into five groups in total. Two zones for implantation of the test materials were selected: the dorsum subcutaneous skin and the muscle area of the *Biceps Femoris*. For implant procedure, materials were cut with a diameter of 0.5 cm x 0.5 cm, blunt-tip, and were sterilized with ultraviolet exposition, then the animals were anesthetized and an incision of approximately 1 cm were made, the materials were introduced using dissecting forceps and placed into the appropriate tissue, finally, the incision was closed using surgical sutures of polyurethane, which has been shown that allows reepithelialization of the wound sutured and reduces scar formation. It was used sterile gauze serving as positive control. One to fourteen weeks after implantation, the rats were sacrificed, the implanted areas were dissected, and the implant-containing tissues were removed from the subcutaneous dorsum and the muscle of the *Biceps Femoris* of the rats. The tissues were immediately fixed with 10% formalin and embedded in paraffin wax, and then sectioned (4 mm) along the longitudinal axis of the implant. The sections were stained with hematoxylin and eosin; the slices were washed with PBS-T (phosphate buffered saline with Tween 20, 0.05%) and blocked with PBS containing 5% BSA (bovine serum albumin; Roche, Germany) for 1 h at 37 ^{o}C.

Statistics analyses. Statistical analyses were performed using SPSS ® (SPSS Inc., Chicago, IL). To evaluate significance differences among groups, analysis of variance was performed with post hoc pairwise testing. A α level of 0.05 was selected for significance for all statistical tests.

Results and Discussion

An scale for a qualitative evaluation of the inflammatory response in tissue, defining inflammatory response in levels from 1 to 4 has been proposed: level 1 corresponds to no inflammatory response is observed; level 2 when the immune response is smooth and implies the presence of macrophages and plasma cells; level 3 is for moderate immune response, in addition to macrophages and plasma cells, neutrophils and lymphocytes capsules are observed; and level 4 corresponds to severe inflammatory response and it includes areas of necrosis. Based on this evaluation scale, the inflammatory immune response caused by PLLA/HA and PLLA-g-HA scaffolds after 1 to 14 weeks of implantation ranges from level 2 to 3 (mild to moderate immune response) [8].

Implantation tests. After the test materials were extracted no signs of irritability or necrosis in the subcutaneous surrounding area were found. However, it was observed that all samples provoke immune system recognition during the first 5 days after implantation of the test materials; polymorph nuclear cells, lymphocytes and plasma cells were found. These cells are present in swollen areas and in the formation of a foreign body granulomatous swelling. However, after 5 days no presence of granulomas was found. At 33 days after implanting the materials, most samples showed adipose tissue. The presence of adipose tissue is related to the same immune system response [9]. In an analysis by Kaminski, et a [10], showed the non-reactive and biocompatible materials present a membrane encapsulation of adipose tissue, while incompatible materials did not present this phenomenon [10]. In our study all implanted materials were encapsulated with adipose tissue. According to the previous publications there is strong evidence that PLLA/HA and PLLA-g-HA scaffolds are biocompatible (Fig. 1).

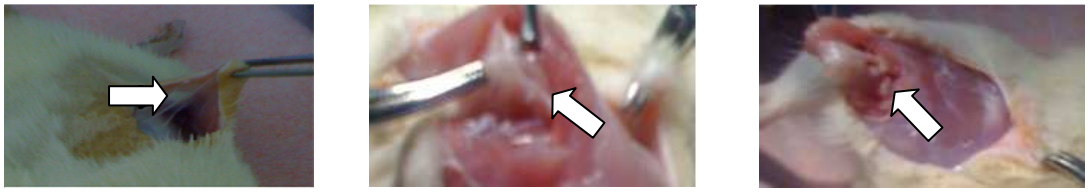
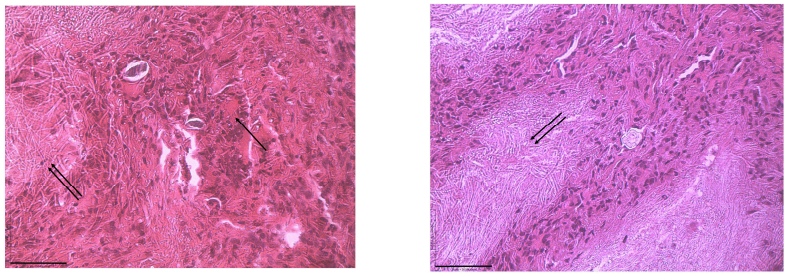
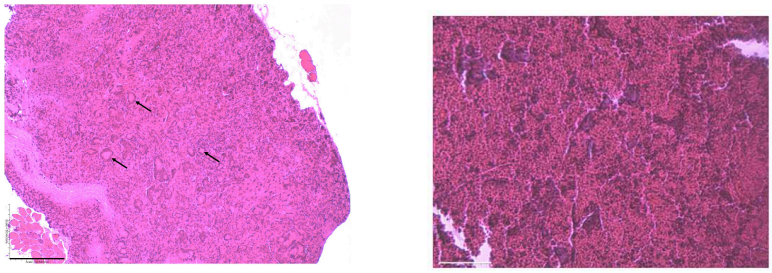


Fig. 1. Implanted materials in the rat leg muscle (*Biceps Femoris*) after 47 days of implantation. (a) Sterile gauze as positive control, (b) PLLA/HA30, y (c) PLLA-g-HA30. In (b) y (c) can be observed white adipose tissue mass around the materials.

Fibrous tissue was found in PLLA/HA4, PLLA/HA10, PLLA/HA20, PLLA/HA30, PLLA-g-HA4 and PLLA-g-HA10 samples except for the PLLA-g-HA20 and PLLA-g-HA30 samples in subcutaneous skin tissues. These findings indicate that the tested materials are difficult to digest by the cells of the immune system. Finally, we found the presence of fibrous tissue in the PLLA/HA4, PLLA/HA10, PLLA/HA20, PLLA-g-HA4 and PLLA-g-HA20 samples except for the PLLA/HA30, PLLA-g-HA10 and PLLA-g-HA30 in rat muscle tissue. The presence of fibrous tissue, lymphocytes and giant cells suggest that the immune system recognize the PLLA-g-HA and PLLA/HA scaffolds similarly, and provoke the encapsulation of the material to try to phagocytize and degrade the implant. Some authors suggest that the presence of fibrous tissue in implanted materials does not always imply an incompatibility with the host tissues [7, 8]. In our study it was observed the presence of fibrous tissue in all samples at different times of material extraction. The above results suggest that the formation of fibrous tissue is a normal immune response to implanted materials; inert materials are considered biocompatible materials that do not cause tissue necrosis.





(d) (e)

Fig. 2. (a), (b) and (c): Microfiber scaffolds of PLLA-g-HA and PLLAHA in Subcutaneous tissue; all samples showed presence of polymorphonuclear cells and fibrous tissue. (a) PLLA/HA4 33 days after implantation (400X), (b) PLLA-g-HA4 33 days after implantation (400X), (c) PLLA-g-HA10 33 days after implantation (400X), (d) Sterile gauze as control, tissue with necrosis (400 X). Scale Bar= 50 microns, single arrow= giant cell, double arrows= microfibers.

Statistical analysis. Samples were compared across the box and whisker plot calculated by Minitab ® software (data not shown). Samples have no significant difference with each other sample for causing the tissue response as indicated by the ANOVA test ($P>0.05$). On the other hand, comparing to the negative control samples, the sample that caused the less immune response is the PLLA-g-HA30 and the one that caused a more evident immune response was PLLA/HA20. Notably, none of the variants of the scaffolds, even in any of the samples taken at different times and places of implantation caused apparent necrosis.

Conclusions

Immune responses from mild to moderate were observed in all samples, which included fibrous tissue: Nevertheless, there were no presence of tissue damage, necrosis or textiloma, this can be a good insight to decide the biocompatibility of the studied scaffolds. Also there is not a significant difference of reaction between the different porous fibrous scaffolds; the different proportion of pure and grafted hydroxyapatite apparently do not cause effect in the tissue reaction. These results provide valuable information for continuing studies to determine the effectiveness of the scaffolds for bone tissue regeneration.

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