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Tissue reaction after subcutaneous implants of a new material composed of ethylene-vinyl acetate and starch for future use as a biomaterial

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Abstract: This study aimed to evaluate the tissue reaction of ethylene-vinyl acetate (EVA) in 4 different compositions and processing: EVA foamed at high pressure with ultrasound (EVACU); EVA with 15% starch foamed at high pressure with ultrasound (EVAMCU); EVA with 15% starch foamed at high pressure without ultrasound and EVA foamed at high pressure without ultrasound as future use as a porous scaffold. Scanning electron microscopy images showed the influence of starch, reducing the diameter of pores. The number of open pores was also reduced with the addition of starch. The ultrasound applied during the manufacturing of composites does not affect these characteristics. Eighteen rats were used to test the tissue reaction of materials and PTFE (polytetrafluoroethylene), proven biocompatible material. After 7, 15, and 60 days of surgery, the materials were removed and processed for microscopic evaluation and counting of the inflammatory infiltrate. The data shows inflammatory reaction similar to PTFE. However, in the quantitative analysis at 60 days, the EVACU and EVAMCU showed less quantity of mononuclear cells ($p < 0.05$). Thus, the results suggest that the use of ultrasound in the production method (EVA) seems to have improved cell behavior regarding the reduction of infiltration over the period, with tissue response equivalent to the PTFE. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

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INTRODUCTION

In adults only small bone defects are able to perform regeneration in a spontaneous way. It is known that after a tooth extraction, the alveolus, without the use of bone grafts or an immediate installation of osseointegrated implants, will lead to a process of horizontal and vertical bone resorption that may hamper future oral rehabilitation procedures. Another situation is found in the different patterns of bone loss in patients affected by periodontal disease, with an emphasis on vertical bone loss that requires specific techniques that aim at tissue regeneration.[1–3]

Although it is the gold standard, a treatment option for autogenous bones presents limiting reasons, in which besides the source of extraction being limited can cause complications in the place of the withdrawal. Therefore, the necessity for new materials of grafting in the present being them reabsorbed or not, for guided bone regeneration or as a scaffold, the latter modality aiming at an osteoconductive surface that allows the growth of cellular tissue within a matrix, is essential in the evolution to a good prognosis in certain cases of rehabilitation in dentistry.[4,5]

The architecture of the scaffold directly influences the functioning and performance of cells and substances for tissue growth. An interconnected structure will serve as a guide, for waste and degradation products, out of the scaffold. It is important, moreover, for it to be highly porous in order for cell penetration and adequate diffusion of nutrients to occur.[6]

The ethylene-vinyl acetate (EVA) is formed by the linking of random polyethylene and poly (vinyl) polyacrylate sequences with subcritical carbon dioxide and they are foam with a wide range of products, that is, manufactured in industrial scale that are presents in insulation materials, sports equipment, drug delivery systems, catheters, artificial organs and mouthguards.[7,8]

There are few reports in the literature on the use of EVA as biomaterial for regenerative processes. However, its use as

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drug delivery devices, such as a contraceptive intravaginal ring, stent coatings, intravitreal implants, among others, is carried out for a long time. It is a polymer approved by the U.S. Food and Drug Administration (FDA) for this purpose.[9] Biocompatibility tests have already been performed including cytotoxicity, sensitization, irritation, intracutaneous toxicity, acute systemic toxicity, genotoxicity, and implantation including into the eye and brain[10–12] and references listed. In a study with orthodontic movement, it was observed that the polymeric material (EVA) used as a drug delivery of substances that interfere bone remodeling, adjacent to maxillary molars in rats was efficient and did not cause inflammatory responses in the subcutaneous tissues during the total implantation period.[13]

The stability of EVA within the body maintaining function is also commonly reported in the literature. In one study, EVA was produced together with a metal material for functional bone rehabilitation in small animals, which served to release growth factors and was properly maintained within the body. Another application presented by this material together with extracellular matrix component resulted in attractions of nervous interactions in order to obtain regeneration of specific nerves.[14,15]

In an *in vitro* study with a polymer material similar to EVA, ethylene-vinyl alcohol (SEVA-C), processed by a fusion-based technology with a corn starch mixture revealed expression of osteopontin by cells indicating extracellular matrix deposition bone when observed by scanning electron microscopy (SEM) images concluding in the study that the presence of corn starch inside a scaffold should be considered as an alternative in the future of bone tissue engineering.[16]

In another study in rats, three polymeric scaffolds were used, one of them being corn starch and SEVA-C and concluding a favorable bone tissue response for the three types of materials in which there was formation of connective tissue (CT) rapidly around the scaffolds being this an early form of neoformed bone tissue.[17]

Starch is renewable biopolymer, derived from natural sources, cheap, and previous work showed possibility to forms blends with EVA.[7,18] The association EVA with starch makes the composite biodegradable, a characteristic that becomes interesting as a use as a biomaterial.[7] The presence of corn starch in this polymer material can present a satisfactory result in bone tissue regeneration so that the starch reabsorbs and the EVA integrates harmoniously with the bone tissue formed.

One of the ways to alter the porosity of the foams is applying ultrasound during their manufacture.[19,20] The aim of this work was to investigate the properties of EVA with starch foamed with and without ultrasound and the tissue reaction after subcutaneous implantation, with the purpose of its use as future biomaterial.

MATERIALS AND METHODS

The present study was approved by the Ethics Committee of the Universidade Sagrado Coração—USC, Bauru, São Paulo State, Brazil and was conducted according to recommendations of the National Institute of Health.[21]

EVA materials

The EVA foams were prepared in the following forms: (1) EVA foamed at high pressure with ultrasound (EVACU), (2) EVA with 15% starch foamed at high pressure with ultrasound (EVAMCU), (3) EVA with 15% starch foamed at high pressure without ultrasound (EVAMSU) and EVA foamed at high pressure without ultrasound (EVASU).

The manufacturing process of EVA (100/0%) (PA-440, Repsol YPF 28%VA) and the EVA/starch (85/15%) (Starch from Syral, Zaragoza, Spain) was blend in an extruder. After, the blend and EVA 100% were introduced in a microinjection (Rheodrive 5000, Haake Fisions). The blend was injected in a mold with rectangular shape with dimensions 80 × 15mm and height 2 mm. The CO_2 treatments of samples were performed in an oxygen combustion bomb submitted at 40 bars, the absorption was carried out for 2 h and at room temperature until complete dissolution of CO_2 in its interior. Two groups of samples underwent two different foaming processes: with and without ultrasound exposure. After the treatment stage with CO_2, the samples were immersed in a water bath at (60°C) quenched in water (5°C). The second group of samples underwent the same thermal superimposed with ultrasound (UR1 with two sources of 240 W at 35 kHz supplied by Retsch®) exposure while in the hot water bath.

The materials were cut in circular shape with 5 mm diameter and sterilized with gamma radiation (25 kGy). The animals underwent subcutaneous implant surgery of the materials to be tested, as well as PTFE (Polytetrafluoroethylene, Bionnovation, Brazil).

SEM of EVA composites

Foam samples were cut at low temperature to provide a smooth surface that was vacuum coated with gold and examined using a JEOL JSM 820. The mean cell size in each foam direction (x, y, z) was estimated using the intersection method, described in Pinto et al.[22]

Open cells content in EVA composites

The density of the cell was measured using a gas pycnometer. The open cell content measurements were conducted according to the standard ASTM D2856-94 using gas Pycnometer, AccuPyc II 1340 provided by Micromeritics.

Density of EVA composites

Density measurements were performed by gas pycnometry using the density determination kit for the AT261 Mettler balance.

Dynamic mechanical analysis

The mechanical properties were studied by dynamic mechanical analysis (DMA) (DMA7, Perkin Elmer) using the compression technique with parallel plates of 15 mm diameter. The specimens were obtained by cutting the foams into prisms 8.5 × 8.5 × 4 mm. The tests were carried out at a constant temperature of 37°C using a static force of 500 mN and a dynamic force of 250 mN at a frequency of 1 Hz.

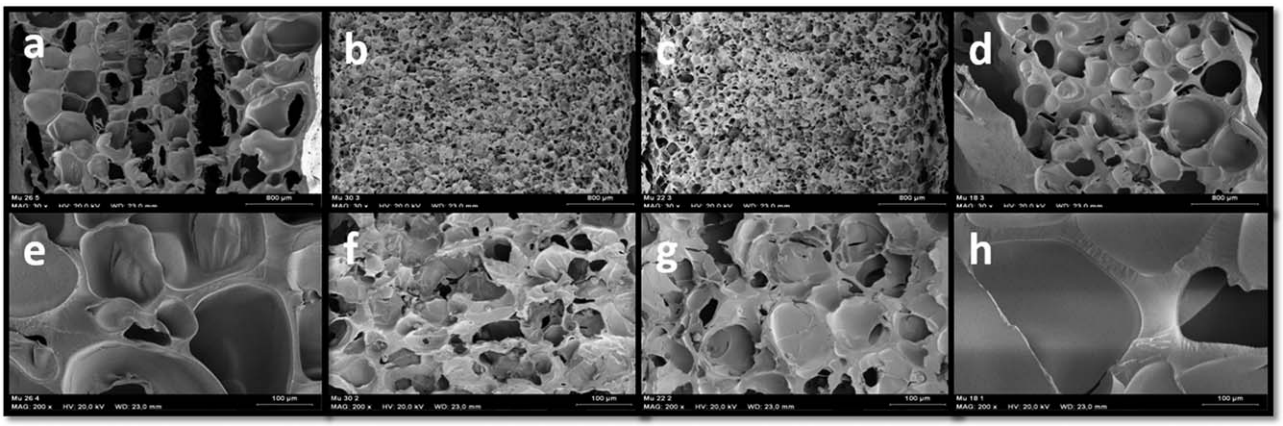

FIGURE 1. SEM images of materials EVACU (a,e), EVAMCU (b,f), EVAMSU (c,g), and EVASU (d,h). The porosity of the materials can be noted.

Subcutaneous implant of materials

Eighteen male *Wistar* rats, weighing approximately 300 g were used. During the experimental period, the animals were kept in plastic cages, fed with water and *ad libitum* feed, in an environment with controlled temperature and light (12 h cycles). Initially, the animals underwent deep sedation with preanesthetic intraperitoneal administration of muscle relaxant, xylazine chlorhydrate 10 mg/kg (Anasedan; Bayer, Brazil) and ketamine chlorhydrate 90 mg/kg (Dopalen; Vetbrands, Brazil).

This was followed by hair removal in the dorsal region and antisepsis. In each animal, five incisions of approximately 8 mm were made along the back, 3 being on the left and 2 on the right side, using sterile fields, followed by divulsion with straight surgical scissors. The animals received the materials to be tested: EVACU, EVASU, EVAMCU, EVAMSU and PTFE in the subcutaneous tissue of the back with the aid of a clinical clamp. In sequence, the suturing of the tissues with 4–0 silk suture thread was carried out. After 7, 15 and 60 days postsurgery, six animals were submitted to euthanasia through an overdose of barbiturates sodic thiopental 150 mg/kg (Thiopentax; Cristália, Brazil) associated with lidocaine chlorhydrate 10 mg/mL (Lidovet; Bravet, Brazil) by intraperitoneal administration. Thus, there are $n = 6$ samples of each material in each period of analysis.

After samples removal, they were processed by routine histological procedures. The pieces were embedded in paraffin and microscopic sections obtained in the longitudinal direction with 6 micrometers thickness and stained with hematoxilin and eosin.

Six images of each histological section were taken in a standardized way with a microscopic optics system (Nikkon H550L), with the following regions: upper left, lower left, lower middle, lower right, upper right and upper middle, with enlargements of 2× and 40×. Subsequently, ImageJ software[23] was used to perform the counting of inflammatory infiltrate cells (mononuclear, polymorphonuclear and giant cells) present in the images obtained at the 40× magnification. Nonparametric Kruskal Wallis test was used for comparison of data.

In this way, analyzing 36 images of each material in each period, performing the count of the inflammatory cells and subsequent statistical comparison with the standard material, we have a methodology has a reproducible methodology for analysis of the tissue reaction.

RESULTS

SEM of EVA composites

Figure 1 shows SEM images of materials EVACU (a and e), EVAMCU (b and f), EVAMSU (c and g) e EVASU (d and h). The porosity characteristic of the materials can be noted.

Table I shows the mean cell size of materials determined by image analysis techniques. A nucleating effect of starch and decrease of cell size due use of ultrasound are observed.

Open cells content in EVA composites

Table II shows the percentage of open cell in the materials obtained through gas picnometry. There are no significant differences due to use of ultrasound. The presence of starch in composition reduced the open cell content due to its nucleating effect.

Density

Table III shows the results of density of materials. Due to the fact that the foams were elastic the measurement error is high, however, it is to be expected that the value obtained is not very far from the actual density value. No significant differences in density were found for the use of ultrasound.

Dynamic mechanical analysis

Figure 2 shows the compression modulus obtained for studied materials. The presence of starch increases the modulus

TABLE I. Average of Cell Size in Materials EVACU, EVASU, EVAMCU, EVAMSU

Materials	Cell Size (μm)
EVACU	257 ± 18
EVASU	390 ± 27
EVAMCU	53 ± 4
EVAMSU	63 ± 4

TABLE II. Percentage of Open Cells in Materials EVACU, EVASU, EVAMCU, EVAMSU

Materials	Open Cell (%)
EVACU	72.5 ± 7.1
EVASU	64.1 ± 13.7
EVAMCU	46.6 ± 3.5
EVAMSU	45.8 ± 7.6

of compression of materials however no significant differences are found by the use of ultrasound.

Macroscopic aspect of materials after subcutaneous implant

Figure 3 presents the EVAMCU and EVAMSU materials in the implant region after 14 days postoperatively. The encapsulated material and the adjacent tissue demonstrate a normal aspect. The same features were observed for EVACU and EVASU.

Microscopic analysis

Initially, it was possible to observe that there was a fibrous capsule with adequate thickness around the implanted materials, as well as the normal appearance of the adjacent tissues (Figures 4 and 5).

At 7 days after the implant the fibrous capsule surrounding the materials had a mild to moderate mononuclear inflammatory infiltrate. CT is present with parallel bundles of collagen fibers as can be observed in EVACU and EVAMSU, also blood vessels and the polymorphonuclear cells in the EVAMCU and EVAMSU images can be seen.

At 60 days, organized CT with collagen fibers arranged parallel to each other in contact with materials are observed with a mild to moderate mononuclear inflammatory infiltrate. There is a decrease in the granulation tissue with persistence of mononuclear cells infiltration (Figures 6 and 7).

At 7 days, ultrasound materials (EVACU and EVAMCU) showed a lower inflammatory reaction in relation to materials that did not undergo this form of production (EVASU and EVAMSU). In this period the inflammatory reaction of the materials with ultrasound was similar to that of the PTFE. The observed infiltrate was composed essentially of mononuclear cells (lymphocytes and macrophages) with polymorphonuclear neutrophils, ranging from absent to discrete, among all groups (EVACU, EVAMCU, EVASU, EVAMSU and PTFE) (Table 4).

In the 15-day period, the EVAMSU material presented a lower amount of mononuclear cells in relation to the other materials. EVACU and EVAMCU materials, compared to the other materials, had a lower number of mononuclear cells at 60 days postoperatively, including PTFE. (Table 4) ($p < 0.05$, Kruskal Wallis).

Summarizing, comparisons of the amount of cells found in the inflammatory infiltrate adjacent to the materials within the same period resulted in values equal or lower than those found in the PTFE.

TABLE III. Density of Materials EVACU, EVASU, EVAMCU, and EVAMSU

Materials	Density (kg/m^3)
EVACU	433 ± 170
EVASU	425 ± 170
EVAMCU	353 ± 140
EVAMSU	363 ± 140

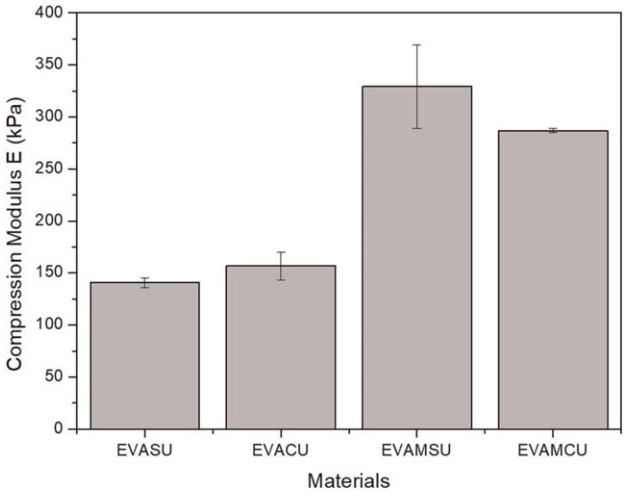

FIGURE 2. Compression modulus obtained for studied materials.

DISCUSSION

The presence of the corn starch associated with different types of material blends is present in the literature with the objective to behave as a resorbable material. For example, the presence of corn starch associated with polyvinylidene-trifluorethylene fluoride, which has piezoelectric properties, showed, in a study, that there was an evident increase in the number of pores in the material after 60 and 100 days postsubcutaneous implant in the back of rats.[24] Rodriguez-Perez et al.[7] showed the increase of the biodegradation with the amount of starch in the blends of EVA/Starch.

The biocompatibility of polymeric materials including EVA was evaluated in the back of rats in a variable long

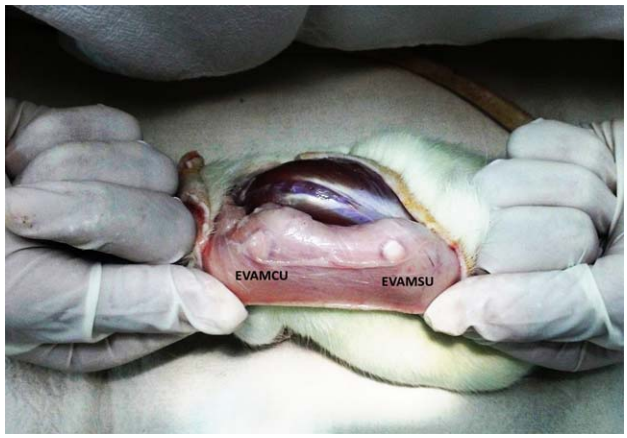

FIGURE 3. The materials EVAMCU and EVAMSU 14 days after surgery. The encapsulated material and the adjacent tissue can be seen presenting a normal aspect.

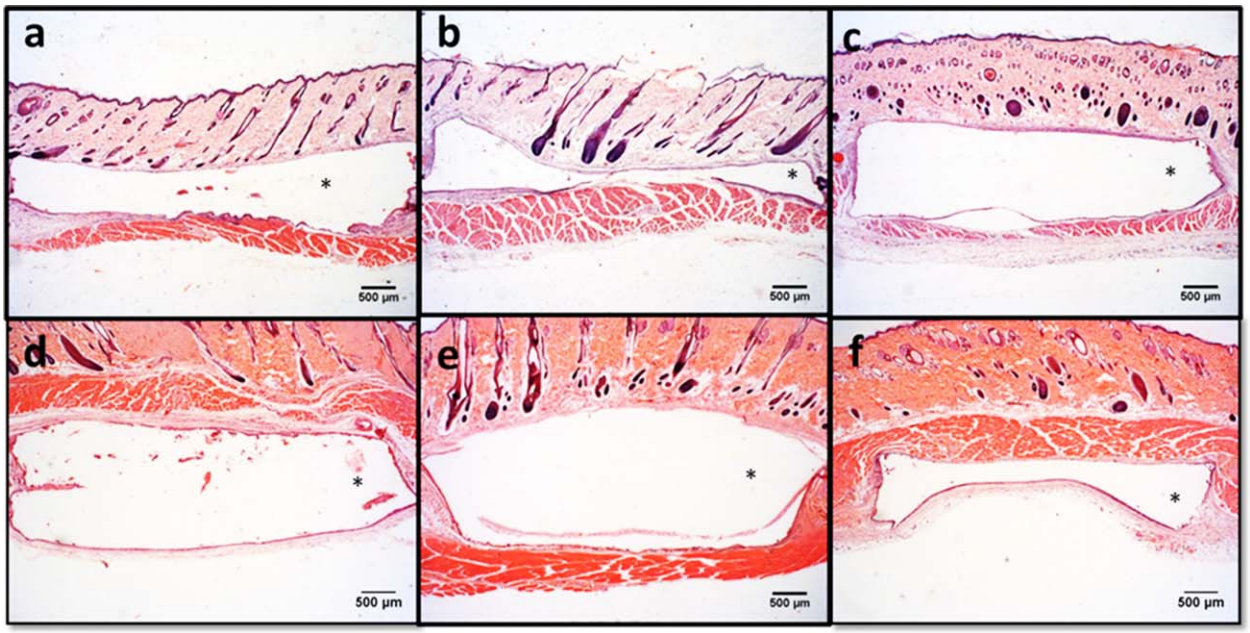

FIGURE 4. Microscopic images of the implantation region of the membranes, 7 (a–c) and 60 days (d–f) after surgery at higher magnification of EVACU (a,d), EVAMCU (b,e), and EVAMSU (c,f). Observe fibrous capsule with adequate thickness around the implanted materials, as well as the normal appearance of the adjacent tissues. The symbol (*) indicate the region of the EVA materials (hematoxilin and eosin staining, original magnification 2×).

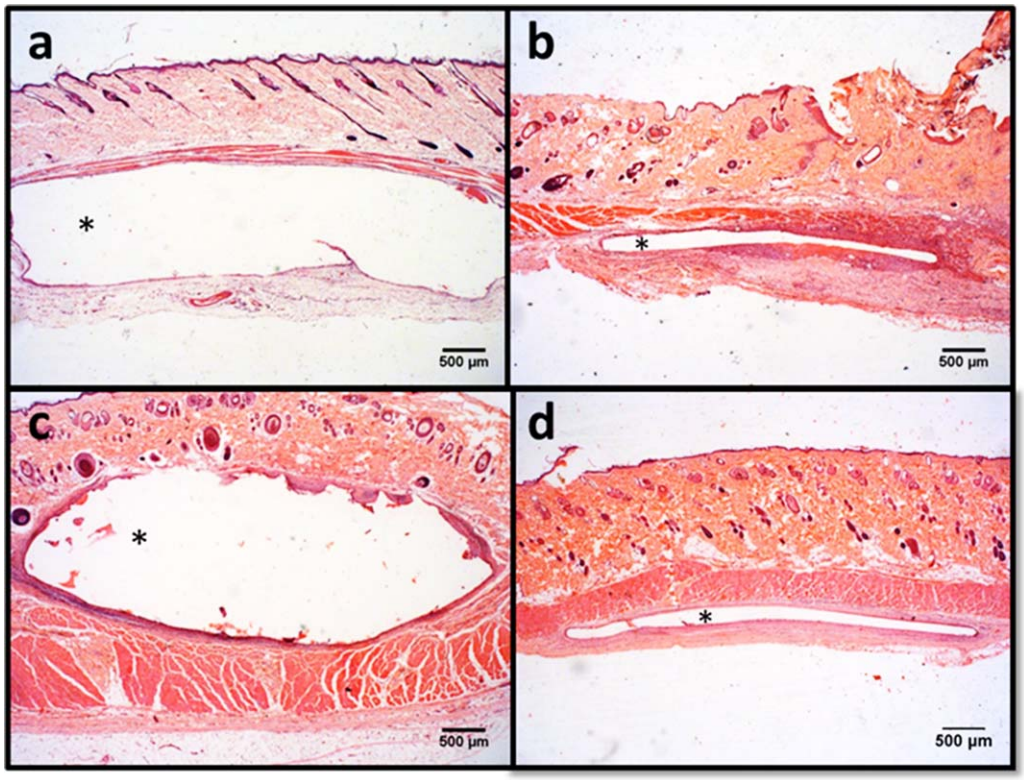

FIGURE 5. Microscopic images of the implantation region of the membranes, 7 (a,b) and 60 days (c,d) after surgery at higher magnification of EVASU (a,c), PTFE (b,d). Observe fibrous capsule with adequate thickness around the implanted materials, as well as the normal appearance of the adjacent tissues. The symbol (*) indicate the region of the EVA and PTFE materials (hematoxilin and eosin staining, original magnification 2×).

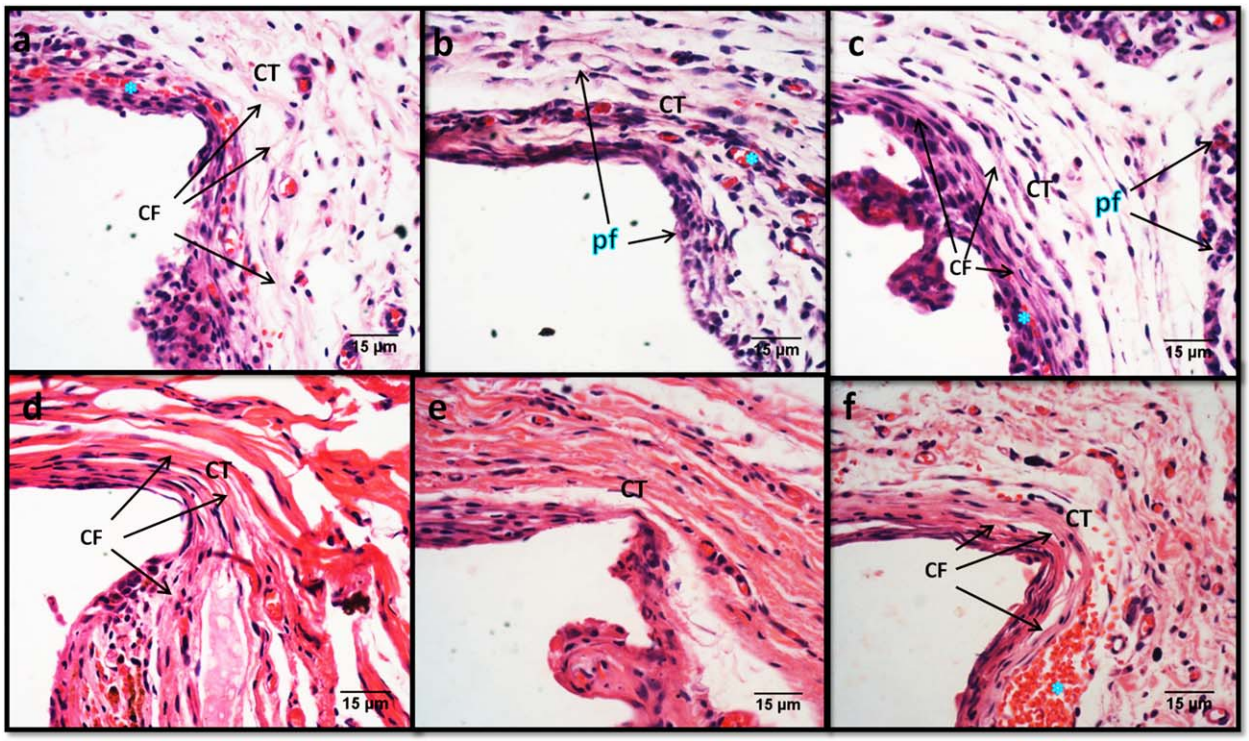

FIGURE 6. Microscopic images of the implantation region of the membranes, 7 (a–c) and 60 days (d–f) after surgery at higher magnification of EVACU (a,d), EVAMCU (b,e), and EVAMSU (c,f). At 7 days, a mild to moderate mononuclear inflammatory infiltrate in the CT is present. Parallel bundles of collagen fibers (CF) surrounding the materials are also observed. At 60 days, presence of a mild to moderate mononuclear inflammatory infiltrate, also organized CT with collagen fibers arranged parallel in contact with materials. pf, polymorphonuclear cells; * blood vessel (hematoxylin and eosin staining, original magnification 40×).

term between 19 and 90 weeks postoperatively. The interstitial pressure measurement, X-ray photoelectron spectroscopy and histological tests used in the study concluded that there were no signs of incompatibility at the material/tissue interface and affirmed the biocompatibility of the polymers.[11] The use of EVA together with other compounds such as hydroxyapatite (HAP) has been reported as a bone substitute material. In a study that looked for bone tissue repair including greater gain in the final bone tissue resistance in the cranial calvarium for cranioplasties developed a scaffold material based on EVA and HAP and demonstrated biocompatibility in the intracutaneous irritation tests, histological analysis of the implantation in the paravertebral muscles and cell culture cytotoxicity *in vitro*. An increase in EVA content indicated a higher sintered density of HAP.[25,26]

In order to obtain a reconstruction of living bone tissue, in tissue engineering it is known that the use of porous biomaterials serving as a scaffold base allows for better proliferation and osteoblasts osteogenesis, while porosity is a crucial factor in the mechanical resistance of osteoblasts.[6]

In this work, the effect of corn starch addition and ultrasound on the structural formation process of EVA foam was investigated in relation to cell structure types (closed, partially interconnected and fully interconnected), pore sizes, compression modulus and tissue reaction, important properties related to use as biomaterial. Regarding to morphology of materials, it was found that the addition of starch reduced the cell size (Table I) and also the amount of open cells (Table II). This result was attributed to the nucleating effect of starch. The intention of the use of ultrasound was to increase the amount of open pores. Although the results found in this work did not present statistically significant difference due to the standard deviation they indicate that new methods of ultrasound application can be further investigated in order to achieve this objective (Table II). Some authors cite the importance of pore size emphasizing their need to be larger than 50 µm, thus favoring the promotion of new bone formation.[27,28] The studied materials are all in agreement (Table I). The application of ultrasound reduced the cell size but are still larger than 50 µm.

It was also found that the addition of starch reduced the density of materials (Table III) and increased the compression modulus (Figure 2). No significant differences in density were found with the use of ultrasound (Table III).

The tissue reaction through subcutaneous implant to the dorsum of rats was analyzed. The tissues adjacent to the all implanted material presented normal characteristics and absence of rejection or severe inflammatory response. The inflammatory cell count and its statistical analysis showed that the materials present the same inflammatory pattern found in PTFE since the amount of cells are equal or lower than those found in the PTFE. The microscopic analysis revealed a moderated inflammatory reaction in the initial period (7 days) of all materials, followed by a chronic

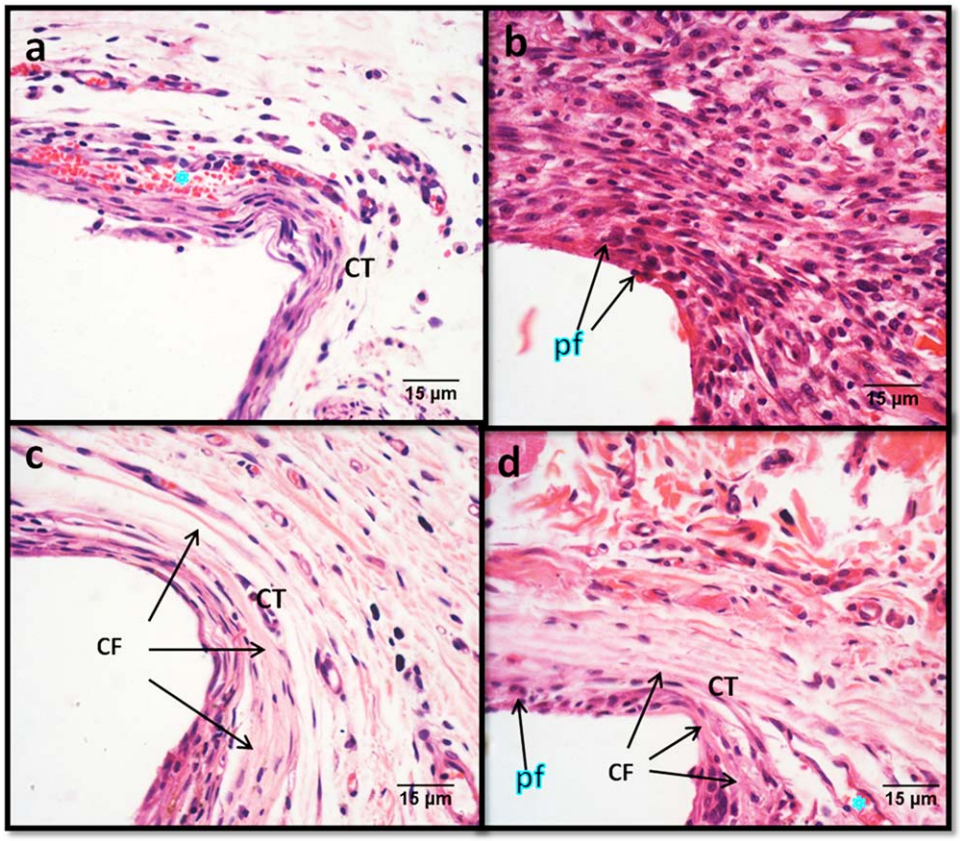

FIGURE 7. Microscopic images of the implantation region of the membranes, 7 (a,b) and 60 days (c,d) after surgery at higher magnification of EVASU (a,c), PTFE (b,d). At 7 days, a mild to moderate mononuclear inflammatory infiltrate in the CT is present. Parallel bundles of collagen fibers (CF) surrounding the materials are also observed. At 60 days, presence of a mild to moderate mononuclear inflammatory infiltrate, also organized CT with collagen fibers arranged parallel in contact with materials. pf, polymorphonuclear cell and * shows a blood vessel. (hematoxilin and eosin staining, original magnification 40×).

inflammatory pattern (60 days) that evolves during tissue regeneration.

In the first instance and analyzed separately, attention should be given to the production of EVA material with ultrasound that originates EVACU and EVAMCU. At 60 days postoperatively, the median of mononuclear inflammatory infiltrate (111 and 119.5) indicates a lower amount when compared to nonultrasound materials (173.5 and 158 cells) as well as PTFE (191.5 cells). Similar pattern is observed at 7 days (Table 4). Thus, the presence of ultrasound seems to have a tendency to act positively toward the reduction of inflammatory cells that, through apoptosis, would give rise to new biological events.[29]

TABLE 4. Median (First Line), Minimum and Maximum Values (Second Line) of Mononuclear Cells (M) polyphorphonuclear (P) and Giant Cells (G) According to the Period and the Implanted Material[a,b]

	7 Days			15 Days			60 Days		
	M	P	G	M	P	G	M	P	G
EVACU *Median*	105[b]	0	0	168[b]	0.5[a]	0	111[a]	0	0
Min-Max	21–342	0–5	0–2	65–265	0–7	0–3	25–244	0–6	0–2
EVAMCU *Median*	118[b]	2	0	162.5[b]	0[a]	0	119.5[a]	1	0
Min-Max	45–229	0–8	0–2	98–255	0–4	0–4	68–225	0–9	0–2
EVAMSU *Median*	168[a]	0	0	130[a]	0[a]	0	158[b]	1	0
Min-Max	122–335	0–7	0–2	58–193	0–3	0–3	18–285	0–4	0–2
EVASU *Median*	169[a]	0	0	169[b]	1[a]	0	173.5[b]	0	0
Min-Max	106–311	0–6	0–1	86–346	0–9	0–2	59–396	0–6	0–3
PTFE *Median*	87[b]	1	0	184.5[b]	2[b]	0	191.5[b]	2	0
Min-Max	40–152	0–3	0–2	340–611	0–7	0–1	93–334	0–4	0–2

[a] There are no differences statistically significant ($p > 0.05$, Kruskal Wallis) among materials in the same period.

[b] Different letters represent significant statistical differences ($p < 0.05$, Kruskal Wallis) in the comparison among the materials during the same period and type of cell.

The results indicate that EVA material with starch and with ultrasound in its conformation (Figure 1) would be able to direct bone neoformation as a scaffold, since at first they presented good inflammatory behavior. Thus, it is emphasized that more studies need to be performed with the objective of evaluating the response to these materials in bone tissue.

CONCLUSION

All the materials tested presented an inflammatory response similar to PTFE, therefore, favorable to the use as biomaterial. The starch in the composition of the EVA composite altered the density, the modulus of compression, the pore size and the amount of open pores. The use of ultrasound in the manufacturing process of the EVACU and EVAMCU composites did not affect these characteristics. However, the reduced amount of mononuclear cells indicates the tendency to improve the inflammatory response.

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