

Evaluation of tissue response and genotoxicity of poly(L/D-lactic acid) disks implanted in rat calvarium

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Summary. The aim of the present study was to evaluate local and cytotoxicity systemic tissue reaction in the skull of rats using the implantation of disks of poly (lactic L/D-acid) and evaluate its genotoxicity. 25 males Wistar rats were used, 20 animals underwent surgical procedures and had the discs implanted in the parietal bone, and 5 animals received postoperative medication in the same way, serving as a control group for genotoxicity. The results were subjected to statistical evaluation by analysis of variance (ANOVA). In histological evaluation, between periods of 90 and 120 days in the control group, a new formation at the edges of the defect was noticed. In the experimental group, there was new bone formation at the edges of the defect, migrating below the site occupied by the disk, an absence of inflammatory infiltrate. Regarding the evaluation of genotoxicity, a significant reduction in the frequency of polychromatic erythrocytes in relation to negative control or significant increase in the polychromatic erythrocytes with micronuclei was not detected. So, the material used in this study is biocompatible and well tolerated by the tissues studied, and found to be negative for chromosomal mutagenicity.

Key words: Bone regeneration, Genotoxicity test, Biocompatible material

Introduction

In 1960 research began on the first resorbable materials approved for use in the medical industry obtained from lactic acid and glycolic acid (Gilding and Reed, 1979). In oral and maxillofacial surgery, research

on resorbable materials, such as poly(L-lactic acid) (PLLA), for the internal fixation of fractures began in the 1970s (Cutright et al., 1971). Currently, biocompatible polymers based on polyesters derived from poly(α -hydroxy acids) are used in biological systems. The main polymers in this group are PLLA, poly(D-lactic acid), poly(DL-lactic acid), poly(glycolic acid) (PGA), polycaprolactone and poly(ϵ -dioxanone) (Chen et al., 2003; Santos and Wada, 2007).

Polymers initially have a stable molecular weight. During the degradation process, however, a polymer is broken down into smaller units through simple hydrolysis, with a consequent reduction in its molecular weight. The decomposition products are eliminated from the body through metabolic pathways, such as the citric acid cycle pathway, or directly through renal excretion (Santos and Wada, 2007).

PLLA was one of the first polymers researched and is used either in its pure form or combined with other polymers. It has a considerably long degradation time of two to six years (Pihlajamäki et al., 2006). Histological studies demonstrate that there is no statistically significant difference between PLLA screws and metal screws in terms of biocompatibility and no signs of inflammation or foreign-body reaction have been reported in follow-up periods of 36 to 51 months (Mollaoglu et al., 2003; Hochuli-Vieira et al., 2005; Pihlajamäki et al., 2006). When assessed clinically or radiographically, a PLLA-based fixation system exhibits the mechanical capacity to resist muscle forces, maintaining the fixation of fractured or osteotomized bone segments and allowing normal bone healing (Tams et al., 1995; Edwards et al., 2001; Suzuki et al., 2004; Costa et al., 2006; Laughlin et al., 2007). Low postoperative complication rates are reported with the use of resorbable materials – even lower than those reported with the use of metallic bone fixation (Ashammakhi et al., 2004; Eppley et al., 2004; Bhatt et

al., 2005).

However, the degradation time of PLLA implants, which was initially estimated at 12 to 18 months, has proven to be much longer (Tams et al., 1995). There are reports of adverse reactions to PLLA in humans, suggesting that this polymer has a second, slower degradation phase that is related to clinical complications. This phase is related to the crystallinity of the material and depends on the initial molecular weight and amount of residual monomers in the material, which varies between manufacturers (Walton and Cotton, 2007). Bergsma et al. (1995) reported that non-degraded PLLA particles can induce and maintain clinically detectable edema, suggesting that these particles may not be completely biocompatible.

Isama and Tsuchiya (2003) found that PLLA did not affect the proliferation of mouse osteoblast-like MC3T3-E1 cells in cultures, but low-molecular weight PLLA particles increased the differentiation of these cells, demonstrating that the osteoconductivity of implanted PLLA is based on the enhanced effect of these particles.

A number of methods that detect damage in DNA have been used to identify genotoxic substances. The micronucleus test on polychromatic erythrocytes (PCEs) is a standard chromosome mutagenicity test. A micronucleus is the result of a chromosome not united with the mitotic spindle or a chromosome fragment without a centromere. Physical, chemical or biological processes that interfere in the binding of the chromosome to the microfibrils of the spindle (aneugenic) and those that break down chromosomes (clastogenic) induce the loss of genetic material, leading to genotoxicity or mutagenicity. Micronucleus analysis is highly correlated with the analysis of chromosome aberrations and, as PCEs have a precise cycle and limited age, the micronucleus in a PCE is always the result of a recent mitotic event (Schmid, 1976).

In the present study, poly(L/D-lactic acid) disks were inserted into the defects calvarium of rats in order to histologically assess the local tissue reaction, contact with the dura mater and cytotoxicity in organs (liver, kidneys and lungs), as well as chromosome mutagenicity through the induction of micronuclei in polychromatic erythrocytes in the bone marrow.

Materials and methods

This research was submitted to the evaluation and approval by the Scientific and Ethics, Faculty of Dentistry, Pontifical Catholic University of Rio Grande do Sul (FO-CCE-PUCRS) and filed under number 072/08, and by the Ethics Committee for Animal use of the Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS) under registration 08/00066.

Twenty-five male rats (*Rattus norvegicus albinus*, Wistar) eight weeks of age and weighing between 200 and 300 grams were maintained under controlled temperature ($24\pm 3^{\circ}\text{C}$), humidity and light conditions with free access to appropriate chow and water at the

animal lodging facility.

Assessment of histological reaction

For the assessment of the tissue reaction, 20 animals were randomly divided into four groups of five animals each based on the sacrifice period (15, 30, 90 and 120 days). Two circular osteotomies were performed with a trephine bit 4.1 millimeters in diameter in the calvarium, separated by the median parietal suture and maintaining the underlying dura mater intact (Fig. 1). The cavity on the left was filled with blood coagulate, serving as the negative control. A poly(L/D-lactic acid) (95%) disk (NeoOrtho[®], Curitiba, Paraná, Brazil) measuring 4 millimeters in diameter and 2 millimeters in thickness was inserted into the cavity on the right (Fig. 1) in contact with the underlying dura mater and overlying periosteum. After the implantation time (15, 30, 90 and 120 days), the animals were sacrificed through isoflurane inhalation. The calvarium was removed, fixed in 10% buffered formalin, de-calcified in 5% aqueous nitric acid, embedded in paraffin and processed for histological analysis, with serial cuts 5 micrometers in thickness stained with hematoxylin-eosin (HE). The sections were made transverse to the bone defect and in the center of the bone defect. Descriptive analysis was performed with regard to inflammatory infiltrate, polymorphonuclear neutrophils, vascular neof ormation, fibroplasia and bone neof ormation.

Cytotoxicity assessment

For the assessment of systemic cytotoxicity, the animals of the control group and the animals that received poly(L/D-lactic acid) disks were sacrificed through isoflurane inhalation and the liver, kidneys and lungs were removed. The organs were fixed in 10% buffered formalin for 48 hours and processed for histological analysis, with serial cuts 5 micrometers in thickness stained using the HE method.

The specimens were not embedded in paraffin. The histological analysis was performed with an optical microscope and alterations in the liver, kidney and lung were investigated, such as inflammatory infiltrate, hyperplastic or necrotic tissue, metaplasia and/or dysplastic transformation.

Genotoxicity assessment

For the assessment of systemic genotoxicity, the PCE micronucleus test was used on three groups of five animals. In the control group, the animals received the same preoperative and postoperative medications [hydrochloride of Ketamine (Dopalen[®]) 50 mg/Kg (0.5 ml/100g) and xylazine (Anasedan[®]) 5 mg/kg (0.025 ml/100g)] and underwent the same surgical procedures, but no test material was inserted. In the other two groups, poly(L/D-lactic acid) disks were inserted in the calvarium and maintained for 90 and 120 days. After the

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observation period, the animals were sacrificed, the femurs were removed and the bone marrow was extracted to slides containing a drop of fetal bovine serum. A smear was made, obtaining two slides for each femur. The slides were dried, fixed in methanol and stained. One slide from each femur received Giemsa stain and the other received Acridine Orange. The slides with Giemsa stain were analyzed with an immersion objective (magnification: x100) in an optical microscope for the examination of the PCEs in 1000 erythrocytes (500 per slide). The slides stained with Acridine Orange were analyzed in a fluorescence microscope, with 1000 PCEs examined on each slide, and the frequency of micronuclei recorded per slide and per animal.

Statistical analysis

The results of the micronucleus test were analyzed using the chi-square test. The negative control group was considered the normal pattern. The other groups were compared to the negative control group both separately and combined. Mutagenicity was recorded when the slides from the group with poly(L/D-lactic acid) exhibited double the frequency of PCEs with micronuclei in comparison to the negative control slides in at least one test group, or when the increase in mutations on the slides from the poly(L/D-lactic acid) group was statistically significant ($p < 0.05$).

Results

Histological assessment

In the 15-day observation period the bone defect was clear in both the control and experimental groups, with no bone neoformation. No episodes of necrosis or foreign-body reaction were observed around the poly(L/D-lactic acid) disk. Chronic inflammatory infiltrate and the formation of disorganized conjunctive tissue were observed between the disks and dura mater in the experimental group or filling in the bone defect in

the control group.

At 30 days bone defect was clear in both groups. In the control group there was no bone neoformation and in the connective tissue there was observed an organized and neovascularized tissue. In the experimental group there was bone neoformation on the margins of the defect, migrating below the site occupied by the disks and following the delimitation given by the dura mater.

At 90 and 120 days there was bone neoformation in both groups. In the control group bone neoformation resulted in a reduction in size of the defect, but not complete repair. The central area of the defect continued to be filled with conjunctive tissue. In the experimental group bone neoformation was observed migrating under the disks, with an absence of inflammatory reaction and foreign-body reaction. There was no reduction in the space occupied by the disks, demonstrating no degradation of the material tested at 120 days (Fig. 2).

Cytotoxicity assessment

Regarding the cytotoxicity assessment, no histopathological alterations were found in cells of the kidneys, lungs or liver in either the negative control group or the group in which the poly(L/D-lactic acid) disks were implanted in any of the observation periods (Fig. 3).

Genotoxicity assessment

The capacity of the poly(L/D-lactic acid) disks to induce chromosome mutations in rats was analyzed using the PCE micronucleus test on the bone marrow of the animals (Fig. 4).

Proportion of PCEs

One thousand erythrocytes from each animal were counted in order to determine the frequency of PCEs. A reduction in PCEs can signify toxicity in the bone

Table 1. Micronucleus test; number of PCEs in 1000 erythrocytes plus mean and standard deviation values per group.

Group	Femurs	Number of PCEs in 1000 erythrocytes					
		Individual		Mean \pm SD per group			
C -	A	359	300	330	303	281	626.4 \pm 40.4
	B	305	320	324	330	280	
	Σ	664	620	654	633	561	
90 days	A	270	300	340	321	-	617.0 \pm 46.8
	B	280	342	315	300	-	
	Σ	550	642	655	621	a	
120 days	A	375	350	378	290	300	668.2 \pm 50.1
	B	300	353	350	322	323	
	Σ	675	703	728	612	623	

a: spontaneous death one week prior to sacrifice.

Table 2. Micronucleus test; number of micronucleated PCEs (mPCEs) in 1000 erythrocytes plus mean and standard deviation values per group.

Group	Femurs	mPCEs in 2000 PCEs					
		Individual		Mean \pm SD per group			
C -	A	3	0	1	4	4	5.0 \pm 3.2
	B	4	1	2	5	1	
	Σ	7	1	3	9	5	
90 days	A	4	2	0	0	-	2.7 \pm 2.3
	B	2	1	1	1	-	
	Σ	6	3	1	1	a	
120 days	A	6	7	2	3	3	8.8 \pm 4.3
	B	5	7	4	0	7	
	Σ	11	14	6	3	10	

a: spontaneous death one week prior to sacrifice.

marrow but can also occasionally produce false negatives. Table 1 displays the numbers of PCEs in 1000 erythrocytes.

No significant reduction ($p < 0.05$) in the frequency of PCEs in relation to the negative control was detected in either group (ANOVA, Dunnett's Multiple Comparison Test and Student's t-test).

Micronucleus assessment

Two thousand PCEs per animal (1000 per slide; two different slides per animal) were analyzed for the assessment of micronucleated PCEs. Table 2 displays the results.

No differences between groups were detected. No significant increase in micronucleated PCEs was detected ($p < 0.05$). The frequency in the experimental

groups did not double and there was no dose-response correlation (ANOVA, Dunnett's Multiple Comparison Test and Student's t-test).

Discussion

A large number of studies have reported positive results in relation to the use of resorbable materials for the internal rigid fixation of osteotomized bone segments. *In vitro* studies (Tams et al., 1995; Maurer et al., 2002; Mollaoglu et al., 2003; Hochuli-Vieira et al., 2005) and clinical and radiographic follow-up studies (Edwards et al., 2001; Ashammakhi et al., 2004; Eppley et al., 2004; Suzuki et al., 2004; Bhatt et al., 2005; Laughlin et al., 2007) have demonstrated the biomechanical qualities of these materials with regard to containing bone segments during the consolidation/

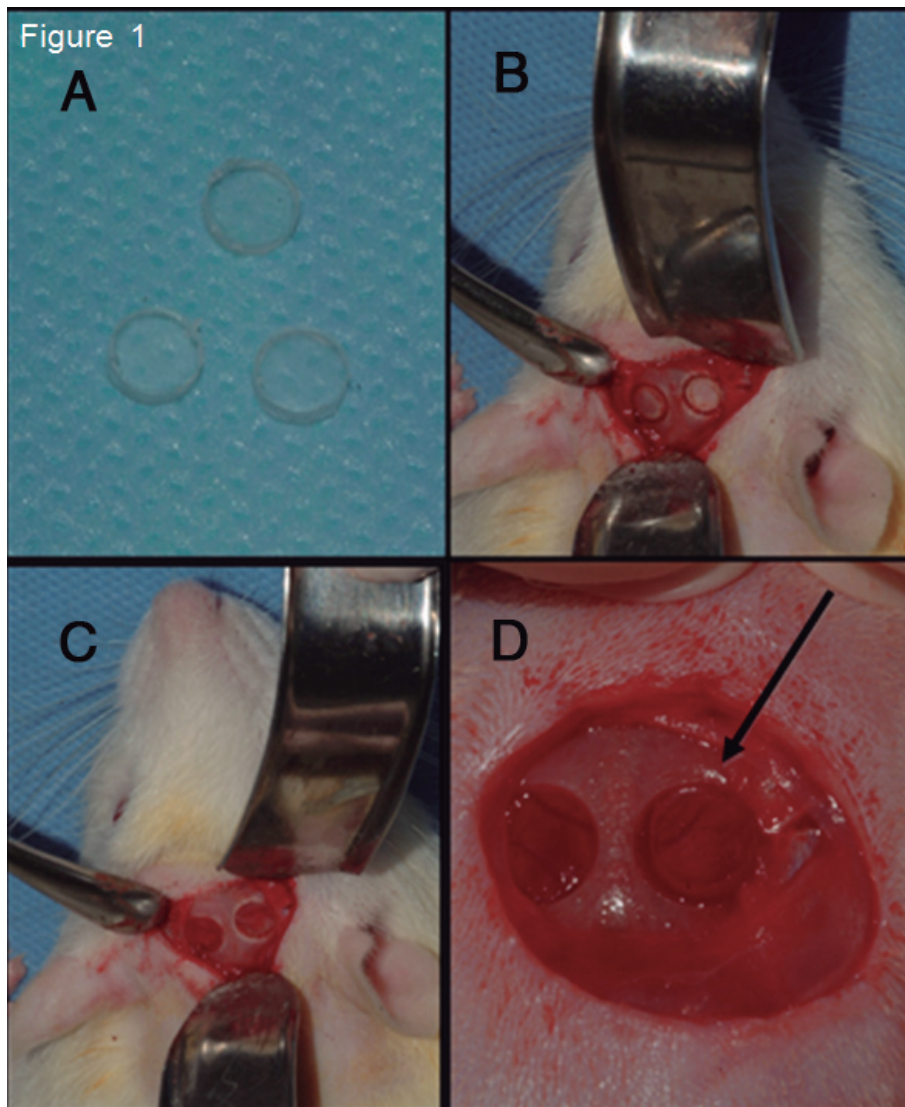


Fig. 1. A. Poly(L/D-lactic acid) disks (95/5%) (NeoOrtho®, Curitiba, Paraná, Brazil). B. Osteotomy. C. Bone defect created in rat calvarium. D. Right bone defect filled with poly(L/D-lactic acid) disks (arrow).

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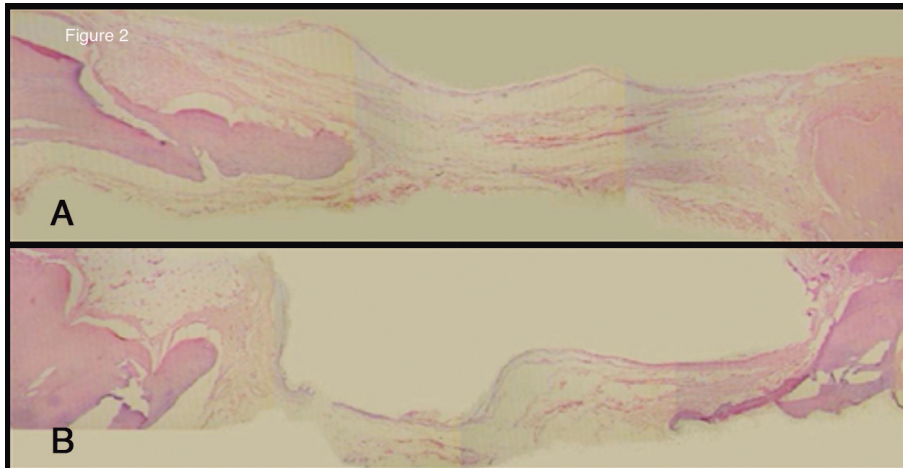


Fig. 2. Histological evaluation at 120 days. **A.** Defect in control group filled with conjunctive tissue and bone neoformation on margins. **B.** Space occupied by poly(L/D-lactic acid) disk, with formation of conjunctive tissue between space occupied by disk and dura mater. x 40

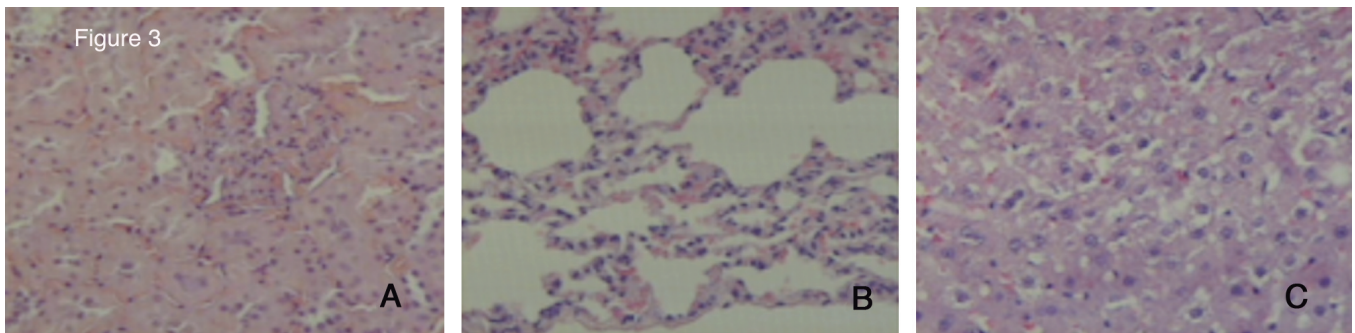


Fig. 3. Evaluation of systemic cytotoxicity in: **A,** kidney, **B,** lung and **C,** liver tissue 120 days after implantation of poly(L/D-lactic acid) disks. x 200

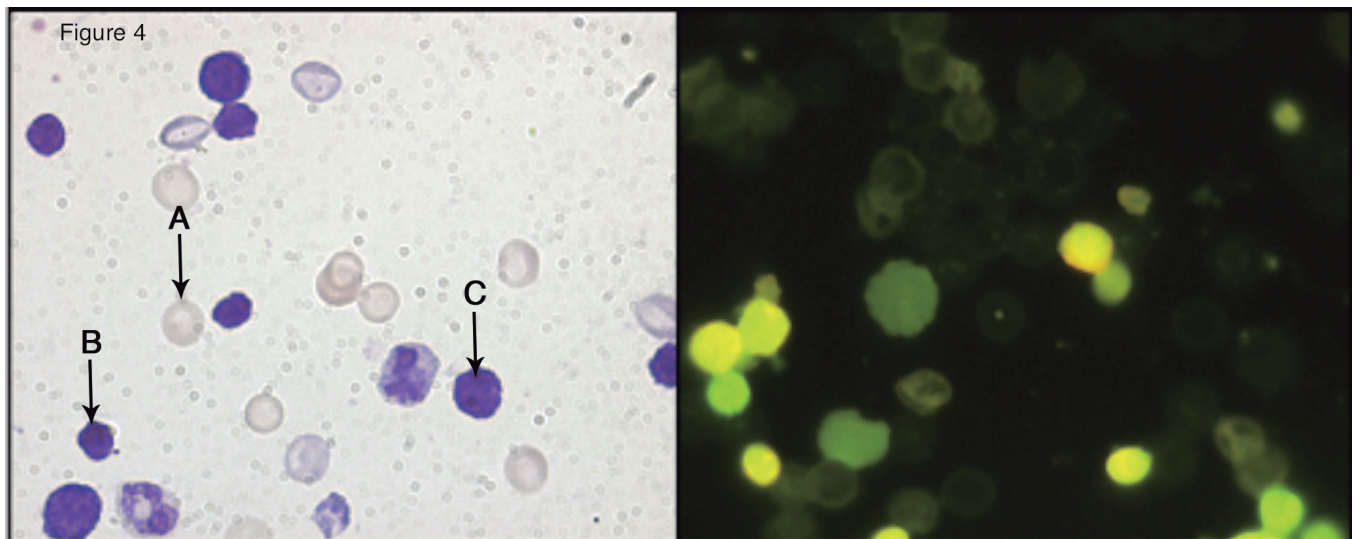


Fig. 4. Assessment of presence of PCEs in bone marrow of negative control group; slides stained with Giemsa. **A.** Normochromatic erythrocyte. **B.** Polychromatic erythrocyte. **C.** Micronucleated polychromatic erythrocyte. Optical microscope with immersion lens, x 200, and fluorescence microscope x 1000

healing period, with an absence of inflammatory tissue and foreign-body reactions. Studies on cytotoxicity and cell cultures have been carried out to understand the systemic effect of resorbable polymers, especially PLLA, at the cellular level (Schmid, 1976; Isama and Tsuchiya, 2003).

The use of resorbable plates and screws is mainly indicated for children and adolescents in the growth phase, as these materials eliminate the potential risks of metal and the need for a second procedure for the removal of the fixation device (Ashammakhi et al., 2004; Eppley et al., 2004; Bhatt et al., 2005). Numerous studies have documented the use of these polymers in children, especially in craniofacial surgery. Moreover, the number of documented complications in this population is small. In a retrospective study on 1883 craniostylosis surgeries in children up to two years of age, infectious complications occurred in 0.7%, primary instability due to trauma in the postoperative period occurred in 0.3%, limited local foreign-body reaction occurred in 0.7% and the rate of complications requiring reoperation was 0.3%. The study documented the safety of the long-term use of resorbable fixation and concluded that resorbable bone fixation for calvarium in rapid growth has a lesser potential for complications than the use of traditional metal plates, screws and wires (Eppley et al., 2004).

Different materials are available on the market for resorbable bone fixation, including a variety of combined polymers. Each combination has particularities regarding biomechanics and resorption time. Adverse reactions to PLLA were related to a possible slower degradation and the crystallinity of the material depend basically on the initial molecular weight and amount of monomers in the material, which varies between manufacturers (Walton and Cotton, 2007). It is therefore important to carry out exhaustive *in vitro* and *in vivo* studies on the degree of systemic toxicity of the materials to be used.

Throughout the present study, low-intensity inflammatory infiltrate characteristic of normal bone repair processes was observed, as was also demonstrated in other studies involving resorbable fixation materials, which also report an absence of foreign-body reactions (Hochuli-Vieira et al., 2005; Pihlajamäki et al., 2006). In the present study, no multinucleated giant cells or macrophages were observed. There are reports that these cells are only observed at the end of the first year, since the implantation of the material, when the tissue reaction is associated to the initial signs of the structural degradation of PLLA (Walton and Cotton, 2007). The longest evaluation period in the present study was 120 days; therefore, no degradation of the material was observed. Previous studies report that degradation occurs after one year, when there is a reduction in the mass of the polymer, which is replaced with relatively avascular fibrous material containing macrophages and occasionally multinucleated giant cells on the surface of

the tested material (Bergsma et al., 1995; Walton and Cotton, 2007). Although biocompatible, fixation devices manufactured with PLLA alone degrade very slowly and the crystals resulting from this degradation exhibit excessive intracellular and extracellular degradation longevity. In previous studies, remnants of degraded PLLA were found surrounded by a dense fibrous capsule 5.7 years after implantation and the ultrastructural evaluation demonstrated PLLA crystals internalized by a number of cells (Bergsma et al., 1995; Walton and Cotton, 2007). However, the combination of PLLA with other substances or its laboratory modification has managed to solve this problem. Studies incorporating glycolic acid (PGLA) demonstrate a faster biodegradation rate. The incorporation of hydroxyl-apatite to PLLA is reported to result in the complete replacement of disks by bone in a period of four to six months (Shikinami et al., 2005). Moreover, the incorporation of bovine bone morphogenetic proteins to PGLA is reported to lead to complete replacement by bone after four weeks, which demonstrates the osteoinductive potential of this combination (Miki and Imai, 1996).

With regard to bone repair, bone neof ormation occurred on the margins of the defect, migrating under the disk in the later periods of the study (90 and 120 days), which corroborates the results of previous studies reporting that the material tested is surrounded by new bone in the first three months without causing adverse tissue reactions (Mollaoglu et al., 2003; Walton and Cotton, 2007). In the assessment of the contact between the poly(L/D-lactic acid) disks and dura mater, which is a critical tissue, there were no postoperative complications, such as convulsion, loss of motor capacity or eating disorders. The conjunctive tissue that formed in contact with the dura mater did not exhibit inflammatory infiltrate or a foreign-body reaction. These findings are in agreement with those reported by Peltoniemi et al. (1999), who analyzed the contact of PLLA and PGA screws with the dura mater and found that conjunctive tissue formed between the apex of the screws and the dura mater soon after implantation and remained throughout the study. This is a critical issue from the standpoint of the risk of death to the animal if the material is not tolerated biologically. Impurities on the ultrastructural level, pH and toxicity on the cellular levels could be determinants to the survival of the animals used in the present study, as the material tested was in direct contact with the dura mater.

Regarding the signs of possible genotoxicity of resorbable polymers, the present study found no significant reduction in the frequency of PCEs or any significant increase in the number of micronucleated PCEs in a period of 120 days since the *in vivo* implantation of the poly(L/D-lactic acid). This absence of genotoxicity is in agreement with findings described by Isama and Tsuchiya (2003), who found that PLLA did not affect the proliferation of mouse osteoblast-like

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MC3T3-E1 cells, but low-molecular weight PLLA particles increased the differentiation of these cells, demonstrating that the osteoconductivity of implanted PLLA is based on the enhanced effect of these particles.

The present study demonstrated that poly(L/D-lactic acid) disks in rat calvarium maintained for 90 and 120 days did not induce inflammatory signs or foreign-body reaction in the tissues in which they were implanted or systemic cytotoxicity in the kidneys, liver and lungs. Moreover, no genetic toxicity effect was found, as there was no reduction in the production of erythrocytes in the bone marrow, as determined by the frequency of PCEs. There was also no increase in the formation of micronucleated PCEs in any of the groups analyzed; the frequency of micronuclei did not double and there was no dose-dependent correlation. Based on the experimental design and micronucleus assessment, the material tested was considered negative for chromosome mutagenicity in the present trial.

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