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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Tissue Remodeling After Implantation with Polymethylmethacrylate: An Experimental Study in Mice

Anália Cirqueira Milhomem[1] · Isabella Mendes de Souza Jorge[1] · Felipe dos Santos Arruda[1] · Fernanda Dias Tomé[1] · Eduardo Luiz da Costa[2] · Marina Clare Vinaud[3] · Jonathas Xavier Pereira[3] · Ruy de Souza Lino Júnior[3]

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Abstract Polymethylmethacrylate (PMMA) is a filler used for aesthetic and/or repair purposes. The response to the implantation of biomaterials varies according to factors related to the patient, the professional responsible for the application and the material used. In vitro and in vivo experimental models have been used to study aspects such as the organism/biomaterial interface and the role of macrophages, dendritic cells and neutrophils. This study aimed to characterize the inflammatory reactions related to polymer concentration, implantation depth and exposure time. Different concentrations of PMMA were implanted in different anatomical planes in mice. The consequences of contact with PMMA, from structural changes to the inflammatory characteristic of tissue damage, were histologically evaluated. The implantation interfered in the morphological structure of the region where it was implanted, expanding it and due to the inflammatory reaction generated, by the presence of the vehicle in the initial phase and by the collagen produced in the chronic phase. The 30% concentration of PMMA induced a greater presence of foreign body giant cells both subcutaneously, at 7, 30 and 90 days after implantation (DAI), and intramuscular at 30DAI. Tissue remodeling was more expressive in the subcutaneous region with significant density of the extracellular matrix at 90DAI. In conclusion, the foreign body reaction resulting from the implantation process acquires different characteristics depending on the anatomical plane and the concentration of implanted product, where the more superficial the implantation plane, the greater the inflammatory reaction. Moreover, PMMA concentration and the depth of implantation did not influence the collagen production.

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Keywords Polymethylmethacrylate · Foreign body giant cell · Experimental model · Collagen production

✉ Ruy de Souza Lino Júnior
ruy@ufg.br

[1] Tropical Medicine and Public Health Post-graduation programme, Tropical Pathology and Public Health Institute, Federal University of Goias, Goiania, Goias, Brazil

[2] Bioplastia Brasil, Goiania, Goias, Brazil

[3] Tropical Pathology and Public Health Institute, Federal University of Goias, Rua 235 s/n, Setor Leste Universitário, Goiania, Goias CEP: 74605-050, Brazil

Introduction

Polymethylmethacrylate (PMMA) is a highly malleable plastic polymer that was discovered by British chemists Rowland Hill and John Crawford in the early 1930s; then Otto Rohm, in 1934, used this polymer for the first time [1]. Currently, this biomaterial performs the most varied functions, from reconstruction of facial bone defects [2, 3], correction of atrophy due to hereditary diseases [4], facial lipoatrophy due to human immunodeficiency virus (HIV) infection [5], bone cement [6], dental applications [7],

intraocular lenses [8], even penis augmentation [9, 10] and nipple enlargement [11].

The implantation of PMMA is an alternative to surgical procedures, being popularly used for aesthetic and/or repair purposes, which has been widely performed around the world, and considered a minimally invasive intervention with satisfactory results [12–16].

When a biomaterial is implanted in the human organism, there is a cascade of reactions observed surrounding this substance. There are blood-material interactions, formation of provisional matrix, inflammation, establishment of granulation tissue, foreign body reaction and generation of a fibrous capsule surrounding the biomaterial [17]. The foreign body reaction is a layer formed by monocytes, macrophages and foreign body giant cells surrounding the material and is the most common one observed in response to PMMA implantation and is characterized by: adsorption of proteins on the surface of the biomaterial, acute inflammation, chronic inflammation, foreign body giant cell formation and finally, fibrosis or fibrous capsule formation. This mechanism results in isolation and stabilization of the permanent polymer [17–19].

This reaction may vary depending on some patient-related factors, such as autoimmune genetic predisposition, hypersensitivity and post-procedure behaviors [20, 21]; others related to the professional responsible for the application, such as the technique used, the volume and depth of implantation [12]; and others related to the material used, such as its topography, size and purity [22]. Bioplasty which is the implant of PMMA as a soft tissue filler is subject to these aspects, and its outcome may be considered satisfactory or in some cases there are adverse reactions that may or may not be transient, such as: granulomas [23–25], hypersensitivity reaction, necrosis, vascular changes, nodules, hematomas, edema, among others [26–29].

In order to better understand the interaction of the human organism to biomaterials, experimental models both in vitro and in vivo have been used. Several aspects have been described such as the migration of microspheres to distant organs [30–32], revealing the additional role of neutrophils in the foreign body reaction to biomaterials [33], comparing different implantation techniques [34] or even comparing the reactions to PMMA and other types of non-permanent fillers [35].

Given the many variables related to the development or not of adverse effects after PMMA implantation, the aim of this study was to characterize the inflammatory reactions and tissue remodeling that may be related to the following predictor variables: implantation depth, exposure time and PMMA concentration.

Materials and Methods

Ethical Considerations

This study was approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Goiás (UFG), under protocol number 027/2017.

Experimental Design

A total of 216 male BALB/c mice were used, whose matrices were kept in the Animals Facilities of the Tropical Pathology and Public Health Institute of UFG (IPTSP-UFG). They were 8 to 12 weeks old and weighed between 20 and 30 grams [36].

The experimental groups were designed as follows: 3 different concentrations (2%, 10% and 30%) of the commercialized form of PMMA (Linea Safe®) were tested, in 3 different depths of anatomical planes (subcutaneous, intramuscular and juxtaperiosteal), divided into 3 different experimental times (7, 30 and 90 days after implantation), which resulted in the formation of 27 experimental groups with 8 animals in each group (Table 1).

Linnea Safe® PMMA (Lots: 1608J, 1608G and 1608I) was implanted (1 μL) in the right hind leg of all mice (test group) varying the depth and concentration of the product. In the contralateral leg of these animals, sterile 0.9% NaCl (1 μL) was injected (control group) respecting the same depth of the PMMA implant. The control group was performed in the same animal in order to comply with the 3 r's principle of animal experimentation, ensuring that the reduction principle is applied [36]. Euthanasia was performed at 7, 30 and 90 DAI (days after implantation).

Experimental Procedures

The mice were initially anesthetized intraperitoneally with a solution of ketamine 100 mg/mL and xylazine 20 mg/mL at the proportion of 0.1 mL/10g of animal weight and later immobilized to allow the trichotomy in the caudal region of the hind limbs. A 0.40x12mm needle port was performed in the epidermis, dermis (for subcutaneous implantation) and skeletal musculature (for intramuscular and juxtaperiosteal implantation) for retro injection of PMMA with a blunt tip microcannula (21G X 25mm) and adjustable syringe applicator (pistol). This procedure ensured the formation of tunneling and subcutaneous, intramuscular or juxtaperiosteal implantation of the polymer. To provide a better distribution of the solution, a light and quick massage was performed in the region after the implant. The procedure was always performed by a physician specialized in the implantation of this product.

Table 1 Experimental groups design regarding the PMMA concentration, anatomical planes of implantation and time

Concentration	Anatomical plane		
	Subcutaneous	Intramuscular	Juxtaperiostal
Control group—NaCl 0.9%	CTS 7DAI	CTI 7DAI	CTJ7DAI
	CTS 30DAI	CTI 30DAI	CTJ 30DAI
	CTS 90DAI	CTI 90DAI	CTJ 90DAI
2%	2%S 7DAI	2%I 7DAI	2%J 7DAI
	2%S 30DAI	2%I 30DAI	2%J 30DAI
	2%S 90DAI	2%I 90DAI	2%J 90DAI
10%	10%S 7DAI	10%I 7DAI	10%J 7DAI
	10%S 30DAI	10%I 30DAI	10%J 30DAI
	10%S 90DAI	10%I 90DAI	10%J 90DAI
30%	30%S 7DAI	30%I 7DAI	30%J 7DAI
	30%S 30DAI	30%I 30DAI	30%J 30DAI
	30%S 90DAI	30%I 90DAI	30%J 90DAI

DAI days after implantation; *S* subcutaneous; *I* intramuscular; *J* juxtaperiosteal. *CTS* Control group of the subcutaneous region; *CTI* Control group of the intramuscular region; *CTJ* Control group of the juxtaperiosteal region

Euthanasia was performed using a lethal dose of anesthetic (ketamine 300mg/kg + xylazine 30mg/kg intraperitoneally) enabling the evaluation of pathological processes in the acute (7DAI), subacute (30DAI) and chronic (90DAI) periods.

Histopathological Analyses

After the mice were euthanized on predetermined experimental days, the left and right hind limbs were removed. The entire anatomical part was fixed in 4% paraformaldehyde for 24 hours and later transferred to a container containing 70% alcohol. The limbs that contained subcutaneous or intramuscular PMMA were dissected to discard the bony part. Meanwhile, the members that constituted the juxtaperiosteal group underwent a decalcification process with a solution composed of 91% distilled water, 5% nitric acid and 4% formalin. After 3 days immersed in this solution, the samples were transferred to a container with 70% alcohol.

All samples, decalcified or not, underwent histological processing, in which there was dehydration with alcohol, diaphanization with xylene and impregnation with paraffin.

The paraffin blocks containing the samples were cut in a microtome with a thickness of 4–5 mm, the fragments were captured on glass slides and stained by the techniques of hematoxylin–eosin (HE), Picrosirius red (PS) and Masson's trichomic (MT).

General pathological processes were observed, described and quantified semi-quantitatively. The following parameters were evaluated: Polymorphonuclear (PMN), mononuclear (MN) and foamy macrophage (fMO) inflammatory infiltrate, in addition to the presence of fibroblasts, fibrin, hemorrhage, foreign body giant cells (FGC), vascularization and bone remodeling. To allow a semiquantitative analysis, an intensity score was assigned as follows: score = 0 when there was no alteration observed; score =1 when the pathological alteration affected 1–25% of the tissue evaluated, score = 2 when 26–50% of the tissue was compromised, and finally, score = 3 when the presence of a certain alteration affected 51–100% of the analyzed area.

Subcutaneous thickening, spacing between muscle fibers, amount of collagen and extracellular matrix density around the PMMA microspheres were quantified. The first two were quantified manually using the LAS software (4.4.0 [Build 454], Copyright 2003–2013, Leica microsystem CMS GmbH) by measuring 5 random areas (in μm), covering the region of interest exclusively where microspheres were present. While the collagen quantification and extracellular matrix density, were quantified after staining with Picrosirius and MT, respectively.

After staining with Picrosirius, 25 photos were captured at 400x magnification on a Zeiss Axiostar Plus microscope with a Sony Nex-3 camera. The amount of collagen produced around the PMMA microspheres was quantified using the Image J software (Threshold color function). The assessment of extracellular matrix density was performed in the same way, however, the photos for this analysis were captured from the MT-stained slides, covering only the subcutaneous region where PMMA was present.

All analysis were performed blindly.

Statistical Analysis

The experiments were performed in triplicates. A data sheet was built in the Microsoft Excel program (16.0). The statistical analysis was performed using the GraphPad Prism software (7.0). The Kruskal–Wallis test followed by Dunn's post-test was used and differences were considered statistically significant when $p \leq 0.05$.

The statistical comparison was performed as follows: (1) between different depths of the same PMMA concentration and control group; (2) between different days of the same depth and same PMMA concentration and control group; (3) Between different PMMA concentrations in the same depth and control group.

Results

The Presence of the Biopolymer Alters the Structural Conformation of the Anatomical Plane

In the subcutaneous region, the presence of the biomaterial, regardless of its concentration, caused thickening of the epidermal region as a whole. This tendency to thickening was visible between the experimental groups and their respective control on the same experimental days. The epidermal thickening was more expressive in the 2% and 30% PMMA concentration groups in relation to their control at 7 DAI. When comparing the test groups with their respective control, the subcutaneous thickening was significant at 30DAI in PMMA 2% concentration and at 7 DAI in PMMA 30% concentration (Figs. 1 and 2).

The spacing of skeletal muscle fibers after PMMA implantation in the intramuscular region was significantly greater in the 30% PMMA concentration group when compared to the 2% and 10% groups at 30DAI. It was observed, at this depth, that the greater the concentration implanted, the greater the disruption and spacing between the muscle fibers (Figs. 1 and 2).

The contact of PMMA microspheres with the bone tissue of mice stimulated its remodeling, i.e., periosteum cells were surrounding the microspheres. Statistically significant bone remodeling was observed at 7DAI in the 2% and 30% PMMA concentration groups and at 30DAI in the 30% PMMA concentration compared to their respective controls (Figs. 1 and 2).

The Difference in the Concentration of Implanted PMMA Interferes with the Characteristics of the Inflammatory Response in Different Anatomical Planes

Subcutaneously, the implantation of 2% PMMA stimulated an inflammatory reaction with recruitment of both polymorphonuclear (PMN) and mononuclear (MN) cells in the acute phase (7 DAI) of the inflammatory process compared to the control group. In the subacute phase (30 DAI) there was significant infiltration of fibroblasts, fibrin deposition and vascularization in relation to the control group. While in the chronic phase (90 DAI) there was a significant presence of PMN, MN, fMO, fibroblasts and vascularization in relation to the control group (Table 2). When comparing the experimental days within the PMMA same concentration (2%) it was possible to observe that there were more foreign body giant cells ($p = 0.0016$) and vascularization ($p<0.001$) at 90 DAI than at 7 and 30 DAI.

Increasing the PMMA concentration to 10% induced a significant infiltration of PMN, MN, fMO and fibrin deposition during all the phases of the inflammatory process. Fibroblasts were significantly increased in the acute and chronic phases, i.e., 7 DAI and 90 DAI, respectively (Table 2). There were more foreign body giant cells at 90 DAI than at 7 DAI ($p<0.001$) and more vascularization at 90 DAI than on the other experimental days ($p<0.001$).

While the 30% concentration of PMMA in the subcutaneous region induced a significant increase in PMN, MN, fMO, fibroblasts infiltration and fibrin deposition in the acute phase (7 DAI). In the subacute phase (30 DAI) there was a significant increase in fMO and vascularization. In the chronic phase (90 DAI) there was a significant increase in fibroblasts. The presence of foreign body giant cells was significantly increased compared to the control group over the experimental days (Table 2). There were more foreign body giant cells ($p = 0.0019$) and vascularization ($p = 0.0016$) at 90 DAI than at 7 DAI.

The implantation of PMMA in the intramuscular region of mice resulted in the presence of less intense pathological processes when compared to the subcutaneous region due to the absence of PMN-infiltrated cells during the experimental days. Implantation of 2% PMMA induced migration of MN and fMO cells in the acute phase (7 DAI), of fibroblasts in the subacute phase (30 DAI) and of fMO, fibroblasts and foreign body giant cells in the chronic phase (90 DAI) (Table 2). The fMO inflammatory infiltrate was more intense at 7 DAI than at 30 and 90 DAI ($p = 0.0339$) and there were more foreign body giant cells at 90 DAI than on the other experimental days ($p = 0.0005$).

In the acute phase (7DAI), 10% PMMA stimulated the infiltration of MN cells in the intramuscular region; in the subacute phase (30DAI), in addition to these cells, it also

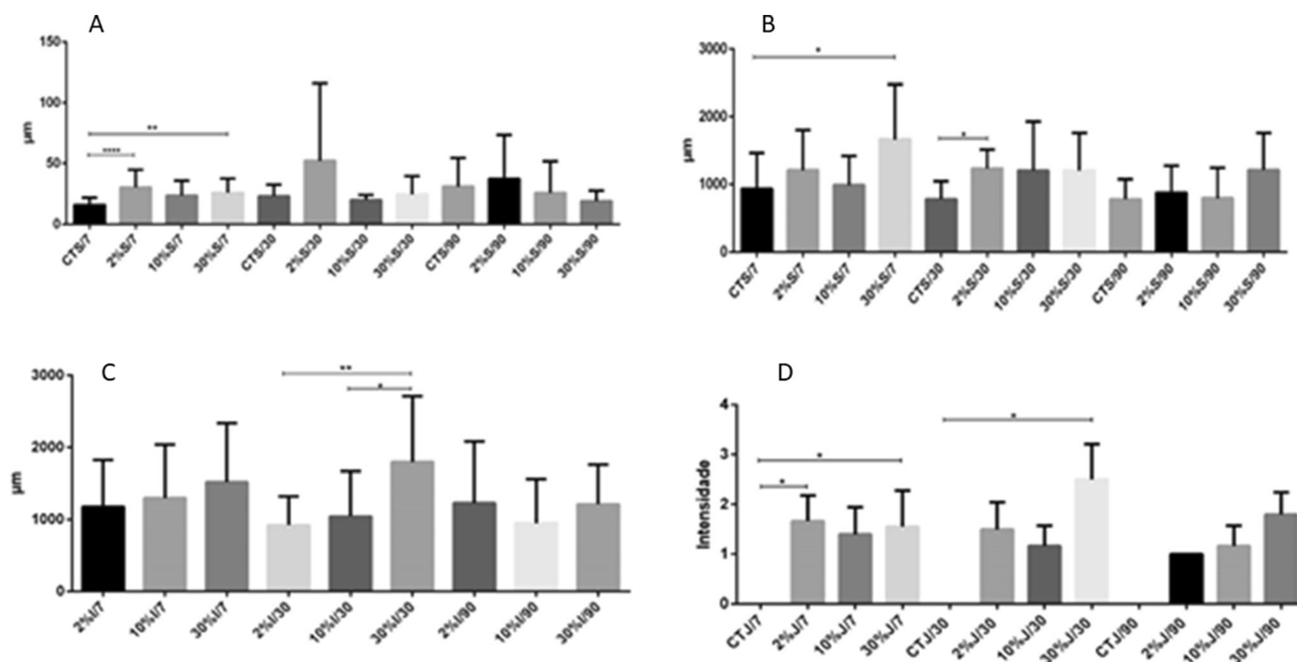

Fig. 1 Structural alterations of anatomical planes of mice, after implantation of Polymethylmethacrylate (PMMA) at different concentrations. Quantitative measurements of epidermal thickness (**A**), subdermal thickness (**B**), spacing between skeletal muscle fibers (**C**) and bone remodeling (**D**) in mice implanted with different concentrations of PMMA over time, 7, 30 and 90 days after implantation (DAI). The Kruskal–Wallis test followed by Dunn's post-test was used where the differences were considered statistically significant when $p \leq 0.005$(*). Values were expressed in micrometers (µm) or intensity of change. CTS7: control of subcutaneous inoculation 7 days after implantation; CTS30: control of subcutaneous inoculation 30 days after implantation; CTS90: control of subcutaneous inoculation 90 days after implantation. 2%S7: group implanted with 2% PMMA in the subcutaneous region 7 days after implantation; 2%S30: group implanted with 2% PMMA in the subcutaneous region 30 days after implantation; 2%S90: group implanted with 2% PMMA in the subcutaneous region 90 days after implantation. 10%S7: group implanted with 10% PMMA in the subcutaneous region 7 days after implantation; 10%S30: group implanted with 10% PMMA in the subcutaneous region 30 days after implantation; 10%S90: group implanted with 10% PMMA in the subcutaneous region 90 days after implantation. 30%S7: group implanted with 30% PMMA in the subcutaneous region 7 days after implantation; 30%S30: group implanted with 30% PMMA in the subcutaneous region 30 days after implantation; 30%S90: group implanted with 30% PMMA in the subcutaneous region 90 days after implantation. CTI7: control of intramuscular inoculation 7 days after implantation; CTI30: control of intramuscular inoculation 30 days after implantation; CTI90I: control of intramuscular inoculation 90 days after implantation. 2%I7: group implanted with 2% PMMA in the intramuscular region 7 days after implantation; 2%I30: group implanted with 2% PMMA in the intramuscular region 30 days after implantation; 2%I90: group implanted with 2% PMMA in the intramuscular region 90 days after implantation. 10%I7: group implanted with 10% PMMA in the intramuscular region 7 days after implantation; 10%I30: group implanted with 10% PMMA in the intramuscular region 30 days after implantation; 10%I90: group implanted with 10% PMMA in the intramuscular region 90 days after implantation. 30%I7: group implanted with 30% PMMA in the intramuscular region 7 days after implantation; 30%I30: group implanted with 30% PMMA in the intramuscular region 30 days after implantation; 30%I90: group implanted with 30% PMMA in the intramuscular region 90 days after implantation. CTJ7: control of juxtaperiosteal inoculation 7 days after implantation; CTJ30: control of juxtaperiosteal inoculation 30 days after implantation; CTJ90: control of juxtaperiosteal inoculation 90 days after implantation. 2%J7: group implanted with 2% PMMA in the juxtaperiosteal region 7 days after implantation; 2%J30: group implanted with 2% PMMA in the juxtaperiosteal region 30 days after implantation; 2%J90: group implanted with 2% PMMA in the juxtaperiosteal region 90 days after implantation. 10%J7: group implanted with 10% PMMA in the juxtaperiosteal region 7 days after implantation; 10%J30: group implanted with 10% PMMA in the juxtaperiosteal region 30 days after implantation; 10%J90: group implanted with 10% PMMA in the juxtaperiosteal region 90 days after implantation. 30%J7: group implanted with 30% PMMA in the juxtaperiosteal region 7 days after implantation; 30%J30: group implanted with 30% PMMA in the juxtaperiosteal region 30 days after implantation; 30%J90: group implanted with 30% PMMA in the juxtaperiosteal region 90 days after implantation

stimulated the migration of fMO and fibroblasts to the implantation site. The chronic phase (90DAI) remained with the same pathological characteristics of the previous phase, with the addition of the presence of foreign body giant cells (Table 3). There was more intense inflammatory infiltration of fMO at 90 DAI than at 30 DAI ($p = 0.0058$) and more foreign body giant cells at 90 DAI than on the other experimental days ($p<0.0001$).

The implantation of 30% PMMA in the intramuscular region stimulated MN cells, fMO and fibroblasts, and the fibrin deposition in the acute phase (7DAI). In the subacute phase (30DAI) there was a predominance of MN cells, fibroblasts, fibrin deposition and foreign body giant cells in

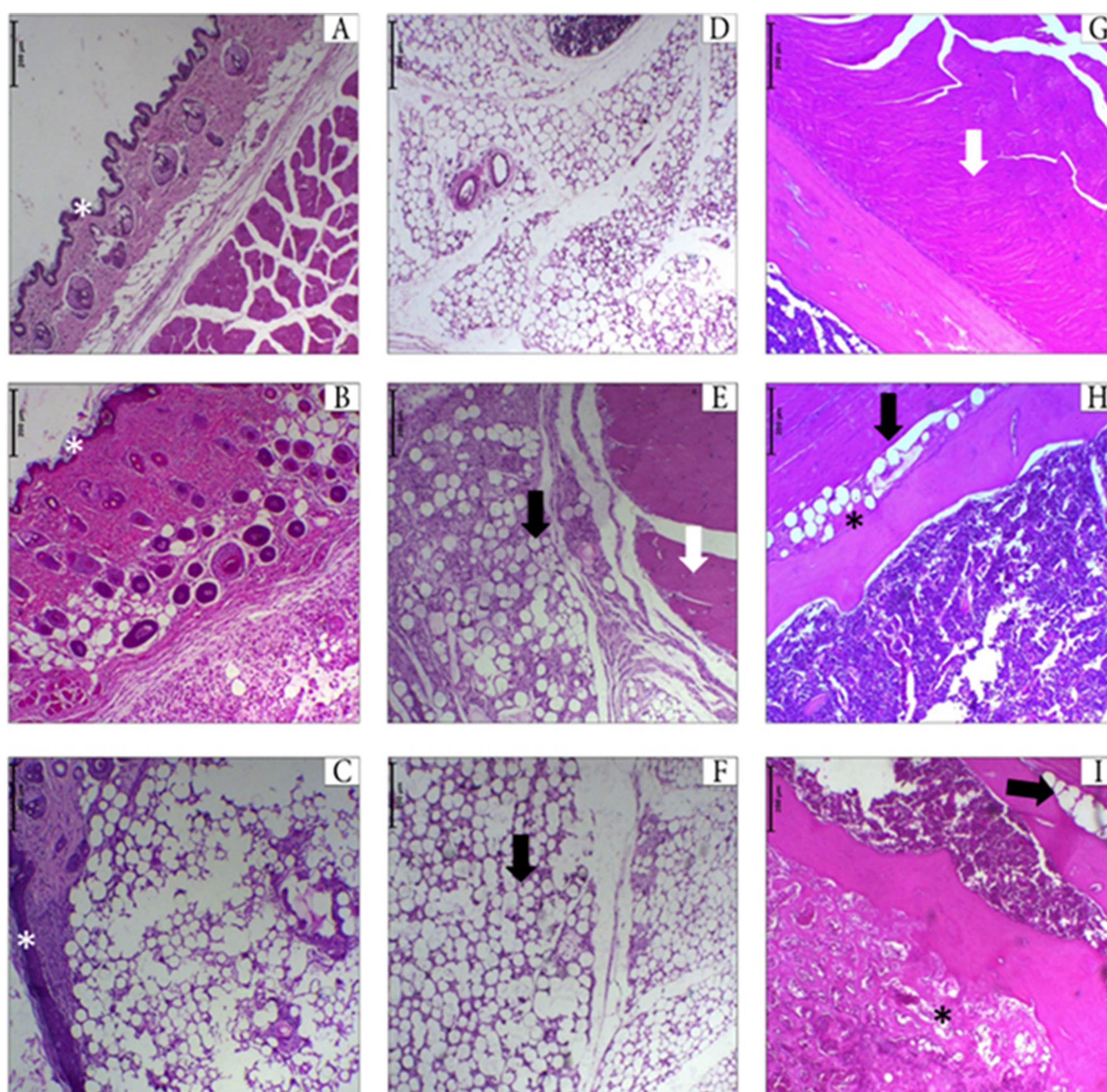

Fig. 2 Photomicrographs of mouse tissue inoculated with different concentrations of Polymethylmethacrylate (PMMA) microspheres. **A** Subcutaneous layer of the control group at 7 DAI (CTS7). **B** Subcutaneous layer of an animal from the group that received 2% PMMA implantation at 7 DAI (2%S7). **C** Subcutaneous layer from a mouse that received 30% PMMA at 7DAI (30%S7). **D** Intramuscular region without PMMA implantation at 7DAI (CTI7). **E** Intramuscular region after 2% PMMA implantation at 30DAI (2%S30). **F** Intramuscular region after implantation of 30% PMMA at 30 DAI (30%I30). **G** Normal juxtaperiosteal region of a mouse at 30 DAI (CTJ30). **H** Bone remodeling of the juxtaperiosteal region after implantation of 2% PMMA at 30 DAI (2%J30). **I** Juxtaperiosteal region of a mouse after 30% PMMA implantation at 7 days after implantation (30%J7). White asterisk: epidermis; Black asterisk: bone remodeling; Black arrow: PMMA microspheres; White arrow: skeletal muscle. Scale bar = 200μm

the inflammatory infiltrate. In the chronic phase (90DAI) it was possible to observe a significant increase in MN cells and fibroblasts in comparison to the control group (Table 2). In the chronic phase (90 DAI) there was a greater presence of fMO ($p = 0.0028$), fibroblasts ($p = 0.0002$). Also, the fibrin deposition($p<0.0001$) was greater at 90 DAI than in the acute phase (7 DAI). In addition, there were more foreign body giant cells at 90 DAI than on the other experimental days ($p = 0.0016$).

Regarding the juxtaperiosteal implantation site, when comparing the test groups with their respective control, 2% PMMA induced a significant increase in the infiltration of MN, fMO and fibroblasts in the acute phase (7 DAI). In the subacute phase (30 DAI) there was an increase in fibroblasts and in fibrin deposition, while in the chronic phase (90 DAI) there was an increase in fibroblasts (Table 4). There was greater fibrin deposition at 90 DAI ($p = 0.0040$) than that observed at 30 DAI.

The implantation of 10% PMMA in the juxtaperiosteal region induced an increase in MN cells and fMO infiltration in the acute phase (7 DAI) and an increase in fibroblasts in the subacute phase (30 DAI). While in the chronic

Table 2 Histopathological analysis of inflammatory parameters in the subcutaneous region of mice after implantation of polymethylmethacrylate (PMMA) at different concentrations over time

Pathological processes	DAI	CTS	2%S	10%S	30%S	*p*	Dunn's
PMN	7	0/(0–0)	1/(1–1)	1/(0–1)	1/(1–1)	**=0.0010**	CTS<2%S/CTS<10%S/CTS<30%S
	30	0/(0–0)	0.5/(0–1)	1/(0–1)	0/(0–1)	**=0.0412**	CTS<10%S
	90	0/(0–0)	1/(1–1)	1/(0–1)	1/(0–1)	**=0.0120**	CTS<2%S/CTS<10%S
MN	7	0/(0–0)	1/(1–3)	2/(1–2)	1/(1–3)	**=0.0064**	CTS<2%S/CTS<10%S/CTS<30%S
	30	0/(0–0)	1/(0–1)	2/(1–3)	1/(1–1)	**=0.0005**	CTS<10%S
	90	0/(0–0)	2/(1–3)	1/(1–2)	1/(1–2)	**=0.0031**	CTS<2%S/CTS<10%S
MOf	7	0/(0–0)	1.5/(1–3)	3/(1–3)	2/(2–3)	**=0.0042**	CTS<10%S/CTS<30%S
	30	0/(0–0)	2/(1–3)	2.5/(2–3)	2/(1–2)	**=0.0008**	CTS<10%S/CTS<30%S
	90	0/(0–0)	2.5/(2–3)	2/(1–3)	2/(1–2)	**=0.0037**	CTS<2%S/CTS<10%S
Fibroblasts	7	0/(0–0)	1/(1–2)	1/(1–2)	2/(1–3)	**=0.0024**	CTS<10%S/CTS<30%S
	30	0/(0–1)	2.5/(2–3)	1.5/(1–2)	2/(1–2)	**=0.0009**	CTS<2%S
	90	0/(0–0)	2/(2–3)	2/(1–2)	2/(2–3)	**=0.0019**	CTS<2%S/CTS<10%S/CTS<30%S
Fibrin	7	0/(0–0)	1/(1–1)	1/(1–2)	2/(1–3)	**=0.0017**	CTS<10%S/CTS<30%S
	30	0/(0–0)	2/(1–3)	1/(1–2)	1/(0–1)	**=0.0006**	CTS<2%S/CTS<10%S
	90	0/(0–0)	2.5/(1–3)	2.5/(0–3)	2/(0–2)	**=0.0175**	CTS<10%S
FBGC	7	0/(0–0)	0/(0–1)	0.5/(0–1)	1/(0–1)	**=0.0258**	CTS<30%S
	30	0/(0–0)	0/(0–1)	2/(1–2)	2/(1–3)	**=0.0007**	CTS<30%S
	90	0/(0–0)	2/(1–2)	3/(2–3)	3/(3–3)	**=0.0006**	CTS<30%S
Vascularization	7	0/(0–1)	0/(0–0)	0/(0–0)	1/(0–1)	=0.0241	–
	30	0/(0–1)	2/(1–3)	1.5/(1–3)	2/(1–3)	**=0.0041**	CTS<2%S/CTS<30%S
	90	1/(0–2)	3/(3–3)	2.5/(2–3)	3/(2–3)	**=0.0062**	CTS<2%S

Results expressed in median/(minimum–maximum). *DAI* days after implantation, *CTS*: control group of subcutaneous implantation; *2%S*: group that received an implantation of 2% PMMA in the subcutaneous region; *10%S*: group that received an implantation of 10% PMMA in the subcutaneous region; *30%S*: group that received an implantation of 30% PMMA in the subcutaneous region. *PMN*: polymorphonuclear cells infiltration; *MN*: mononuclear cells infiltration; *MOf*: foamy macrophages infiltration; *FBGC*: foreign body giant cells

The bold indicate that there is a statistical significant difference between the analyzed groups

phase (90 DAI) it was possible to observe an increase in MN cells infiltration in relation to the control group (Table 4). There was a higher PMN cells infiltrate ($p = 0.0021$) at 7 DAI than those observed at the other experimental days. At 30 DAI, there was a greater amount of fibroblasts ($p = 0.0002$) than observed on the other experimental days.

While the implantation of 30% PMMA in the juxtaperiosteal region compared to their respective control groups induced an inflammatory infiltration of PMN and MN cells, and fMO in the acute phase; followed by an increase in MN cells, fMO and fibroblasts in the subacute phase. Whereas in the chronic phase there was an increase only in MN cells and fMO (Table 4). On the other hand, when comparing the evolution of the pathological processes throughout the experimental days, at 30 DAI there was more MN cells infiltrate ($p = 0.0003$) than at 7 DAI; more fMO infiltrate ($p = 0.0147$) than at the other experimental days and more fibroblasts (0.0027) than at 90 DAI.

When comparing the PMMA concentrations implanted within the same anatomical depths it was clear that all concentrations presented greater pathological processes than their respective controls, which is expected since there is a foreign body that the organism is trying to attack. But when comparing the inflammatory response generated by the PMMA concentrations within the same anatomical plane, there was no statistical difference between the intensity of the pathological processes, i.e., the PMMA concentration does not interfere in the intensity of inflammatory reaction induced in each depth (Tables 2, 3 and 4).

When comparing the inflammatory response between depths, at 90DAI, it was possible to observe some differences. In the subcutaneous group, there was more intensity in the infiltration of PMN cells, fibroblasts, fibrin deposition and foreign body giant cells than on the intramuscular and juxtaperiosteal groups, at all PMMA concentrations. Therefore, the inflammatory response in more superficial depths was more intense than in deeper ones (Tables 2, 3 and 4).

Table 3 Histopathological analysis of inflammatory parameters in the intramuscular region of mice after implantation of polymethylmethacrylate at different concentrations over time

Pathological processes	DAI	CTI	2%I	10%I	30%I	*p*	Dunn's
PMN	7	0/(0–1)	0.75/(0–1)	0/(0–1)	0/(0–1)	=0.2292	–
	30	0/(0–0)	0/(0–0)	0/(0–0)	0/(0–1)	=0.3340	–
	90	0/(0–0)	0/(0–0)	0/(0–0)	0/(0–0)	=1	–
MN	7	0/(0–0)	2.5/(1–3)	2/(1–3)	2/(1–3)	**=0.0068**	CTI< 2%I/CTI<10%I/CTI<30%I
	30	0/(0–0)	2/(1–2)	3/(1–3)	2/(2–3)	**=0.0023**	CTI<10%I/CTI<30%I
	90	0/(0–0)	1.5/(1–3)	2/(1–3)	2/(1–3)	**=0.0047**	CTI<10%I/CTI<30%I
MOf	7	0/(0–0)	2.5/(2–3)	2/(2–2)	2.5/(2–3)	**=0.0019**	CTI< 2%I/CTI<30%I
	30	0/(0–0)	2/(1–2)	2/(2–3)	2/(1–3)	**=0.0025**	CTI<10%I
	90	0/(0–0)	2/(1–2)	1/(1–2)	1/(1–1)	**=0.0010**	CTI< 2%I/CTI<10%I
Fibroblasts	7	0/(0–0)	1/(1–1)	1/(1–2)	2.5/(2–3)	**=0.0001**	CTI<30%I
	30	0/(0–0)	2/(1–2)	1.5/(1–2)	2/(1–2)	**=0.0046**	CTI< 2%I/CTI<10%I/CTI<30%I
	90	0/(0–0)	1/(1–2)	1/(1–1)	1/(1–1)	**=0.0004**	CTI< 2%I/CTI<10%I/CTI<30%I
Fibrin	7	0/(0–0)	1.5/(0–2)	0/(0–1)	2.5/(2–3)	**=0.0019**	CTI<30%I
	30	0/(0–0)	0/(0–0)	0/(0–0)	1/(0–2)	**=0.0013**	CTI<30%I
	90	0/(0–0)	0/(0–2)	0/(0–0)	0/(0–0)	=0.4180	
FBGC	7	0/(0–0)	0/(0–0)	0/(0–0)	0/(0–0)	=1	–
	30	0/(0–0)	0/(0–0)	1/(1–1)	2/(1–2)	**=0.0006**	CTI<30%I
	90	0/(0–0)	2/(1–2)	2/(1–3)	1.5/(1–2)	**=0.0070**	CTI< 2%I/CTI<10%I

Results expressed in median/(minimum–maximum). *DAI* days after implantation, *CTI* control group of intramuscular implantation; *2%I* group that received an implantation of 2% PMMA in the intramuscular region; *10%I* group that received an implantation of 10% PMMA in the intramuscular region; *30%I*: group that received an implantation of 30% PMMA in the intramuscular region. *PMN*: polymorphonuclear cells infiltration; *MN*: mononuclear cells infiltration; *MOf*: foamy macrophages infiltration; *FBGC*: foreign body giant cells

The bold indicate that there is a statistical significant difference between the analyzed groups

Table 4 Histopathological analysis of inflammatory parameters in the juxtaperiosteal region of mice after implantation of polymethylmethacrylate at different concentrations over time.

Pathological processes	DAI	CTJ	2%J	10%J	30%J	*p*	Dunn's
PMN	7	0/(0–0)	0/(0–1)	1/(0–1)	0/(0–0)	**=0.0089**	CTJ<30%J
	30	0/(0–0)	0/(0–0)	0/(0–0)	0/(0–1)	=0.01147	–
	90	0/(0–0)	0/(0–0)	0/(0–0)	0/(0–0)	=1	–
MN	7	0/(0–0)	1/(1–1)	1/(1–1)	1/(1–1)	**=0.0002**	CTJ<2%J/CTJ<10%J/CTJ<30%J
	30	0/(0–0)	1/(1–1)	1/(1–2)	2.5/(2–3)	**=0.0001**	CTJ<30%J
	90	0/(0–0)	1/(1–1)	1/(1–1)	1/(1–2)	**=0.0007**	CTJ<2%J/CTJ<10%J/CTJ<30%J
MOf	7	0/(0–0)	1/(1–1)	1/(1–1)	1/(1–1)	**=0.0002**	CTJ<2%J/CTJ<10%J/CTJ<30%J
	30	0/(0–0)	1/(1–2)	1/(1–2)	2.5/(1–3)	**=0.0011**	CTJ<30%J
	90	0/(0–0)	1/(1–2)	1/(0–1)	1/(1–1)	**=0.0031**	CTJ<2%J/CTJ<30%J
Fibroblasts	7	0/(0–0)	1/(1–3)	0/(0–0)	0/(0–3)	**=0.0025**	CTJ<2%J
	30	0/(0–0)	1/(1–1)	1/(1–2)	1/(1–2)	**=0.0012**	CTJ<2%J/CTJ<10%J/CTJ<30%J
	90	0/(0–0)	1/(1–1)	0/(0–0)	0/(0–0)	**=0.0002**	CTJ<2%J
Fibrin	7	0/(0–0)	0/(0–2)	0/(0–0)	0/(0–2)	=0.5834	–
	30	0/(0–0)	1/(1–1)	0/(0–1)	1/(0–2)	**=0.0050**	CTJ<2%J
	90	0/(0–0)	0/(0–0)	0/(0–0)	0/(0–0)	=1	–

Results expressed in median/(minimum–maximum). *DAI* days after implantation, *CTJ* control group of juxtaperiosteal implantation; *2%J* group that received an implantation of 2% PMMA in the juxtaperiosteal region; *10%J* group that received an implantation of 10% PMMA in the juxtaperiosteal region; *30%J* group that received an implantation of 30% PMMA in the juxtaperiosteal region. *PMN* polymorphonuclear cells infiltration; *MN* mononuclear cells infiltration; *MOf* foamy macrophages infiltration

The bold indicate that there is a statistical significant difference between the analyzed groups

The Implanted PMMA Concentration does not Interfere with Collagen Production

The production of collagen around the PMMA microspheres is a necessary mechanism to isolate the foreign body from the body, in addition to enabling its stabilization in the tissue. It was possible to observe that, regardless of the implantation depth, collagen production was more significant at 90 than at 30 DAI. Furthermore, it is observed that neocollagenesis is not proportional to a given concentration of PMMA, that is, there is no relation between the number of microspheres and the amount of collagen consequently produced (Fig. 3).

Regarding the density of the extracellular matrix in the subcutaneous region, it was statistically progressive over time among the groups that received 2% PMMA. However, corroborating our results regarding the quantification of the collagen concentration, this was not a finding that was repeated in the other polymer concentrations (Fig. 4).

In this study, the fibrous capsule was formed around small groups of microspheres, with projections of bundles of collagen fiber around each of them, and that, with the passage of experimental time, these fibers matured, becoming denser and possibly becoming type I (Fig. 5).

Discussion

The histopathological characteristics of the foreign body reaction to PMMA vary according to the concentration and depth of its implantation. Furthermore, there is probably no relationship between the polymer concentration and the density of the extracellular matrix in contact with it, or even with the amount of collagen produced around the microspheres.

Fulfilling the objective of being a tissue filler, PMMA altered the natural structural conformation of the tissues where it was implanted. The increase in volume was not proportional to the polymer concentration; however, this finding is attributed to the standardization of the implanted volume and the micro spreading of the biomaterial over time, which we attribute to the location close to muscles [13]. In addition, especially in the subcutaneous region, the thickening observed in the region occurred not only due to

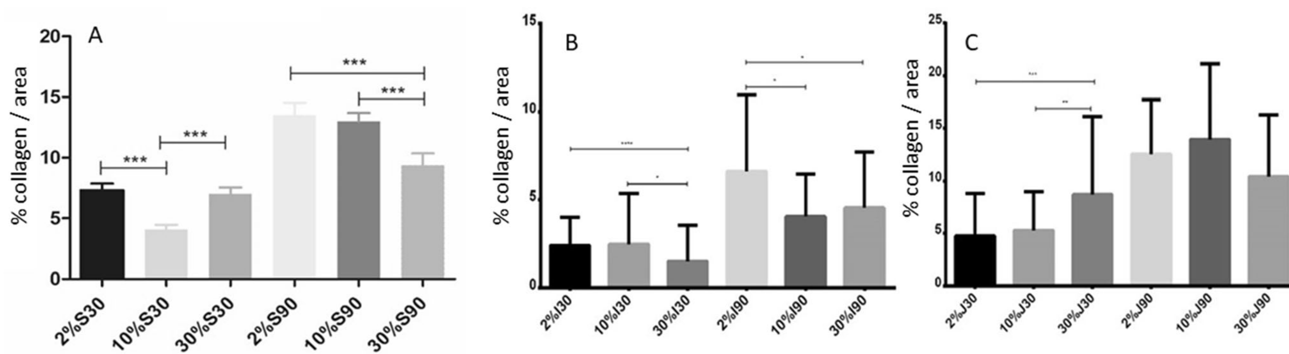

Fig. 3 Quantitative assessment of the concentration of collagen produced around Polymethylmethacrylate (PMMA) microspheres implanted in different anatomical planes of mice over time. **A** Subcutaneous inoculation plane; **B** Intramuscular inoculation plane. **C** Juxtaperiosteal inoculation plane. * $p<0.05$; ** $p<0.005$; *** $p<0.0001$. 2%S7: group implanted with 2% PMMA in the subcutaneous region 7 days after implantation; 2%S30: group implanted with 2% PMMA in the subcutaneous region 30 days after implantation; 2%S90: group implanted with 2% PMMA in the subcutaneous region 90 days after implantation. 10%S7: group implanted with 10% PMMA in the subcutaneous region 7 days after implantation; 10%S30: group implanted with 10% PMMA in the subcutaneous region 30 days after implantation; 10%S90: group implanted with 10% PMMA in the subcutaneous region 90 days after implantation. 30%S7: group implanted with 30% PMMA in the subcutaneous region 7 days after implantation; 30%S30: group implanted with 30% PMMA in the subcutaneous region 30 days after implantation; 30%S90: group implanted with 30% PMMA in the subcutaneous region 90 days after implantation. 2%I7: group implanted with 2% PMMA in the intramuscular region 7 days after implantation; 2%I30: group implanted with 2% PMMA in the intramuscular region 30 days after implantation; 2%I90: group implanted with 2% PMMA in the intramuscular region 90 days after implantation. 10%I7: group implanted with 10% PMMA in the intramuscular region 7 days after implantation; 10%I30: group implanted with 10% PMMA in the intramuscular region 30 days after implantation; 10%I90: group implanted with 10% PMMA in the intramuscular region 90 days after implantation. 30%I7: group implanted with 30% PMMA in the intramuscular region 7 days after implantation; 30%I30: group implanted with 30% PMMA in the intramuscular region 30 days after implantation; 30%I90: group implanted with 30% PMMA in the intramuscular region 90 days after implantation. 2%J7: group implanted with 2% PMMA in the juxtaperiosteal region 7 days after implantation; 2%J30: group implanted with 2% PMMA in the juxtaperiosteal region 30 days after implantation; 2%J90: group implanted with 2% PMMA in the juxtaperiosteal region 90 days after implantation. 10%J7: group implanted with 10% PMMA in the juxtaperiosteal region 7 days after implantation; 10%J30: group implanted with 10% PMMA in the juxtaperiosteal region 30 days after implantation; 10%J90: group implanted with 10% PMMA in the juxtaperiosteal region 90 days after implantation. 30%J7: group implanted with 30% PMMA in the juxtaperiosteal region 7 days after implantation; 30%J30: group implanted with 30% PMMA in the juxtaperiosteal region 30 days after implantation; 30%J90: group implanted with 30% PMMA in the juxtaperiosteal region 90 days after implantation

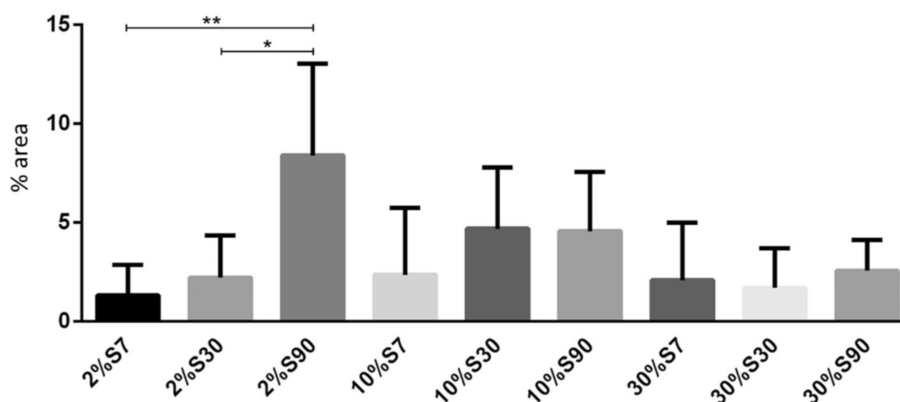

Fig. 4 Extracellular matrix density in the subcutaneous region of mice implanted with different concentrations of Polymethylmethacrylate (PMMA). 2%S7: group implanted with 2% PMMA in the subcutaneous region 7 days after implantation; 2%S30: group implanted with 2% PMMA in the subcutaneous region 30 days after implantation; 2%S90: group implanted with 2% PMMA in the subcutaneous region 90 days after implantation. 10%S7: group implanted with 10% PMMA in the subcutaneous region 7 days after implantation; 10%S30: group implanted with 10% PMMA in the subcutaneous region 30 days after implantation; 10%S90: group implanted with 10% PMMA in the subcutaneous region 90 days after implantation. 30%S7: group implanted with 30% PMMA in the subcutaneous region 7 days after implantation; 30%S30: group implanted with 30% PMMA in the subcutaneous region 30 days after implantation; 30%S90: group implanted with 30% PMMA in the subcutaneous region 90 days after implantation. * $p<0.05$; ** $p<0.005$; *** $p<0.0001$

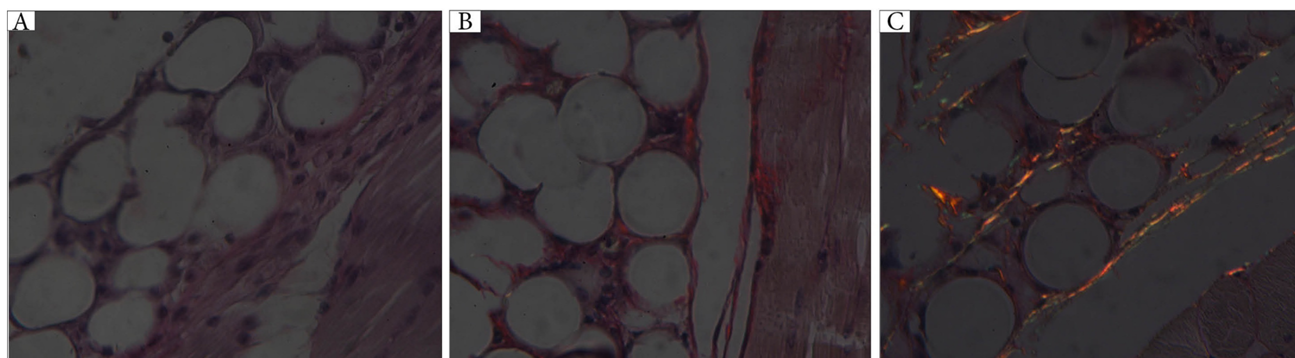

Fig. 5 Photomicrographs of BALB/c mouse intramuscular region inoculated with Polymethylmethacrylate (PMMA) at 30% concentration (**A**). The microspheres of PMMA are surrounded by inflammatory cells at 7DAI (days after inoculation) (**B**). The microspheres of PMMA in the beginning of the formation of the collagen fibers bundle and decrease in the inflammatory reaction at 30DAI (**C**). Fibrous capsule surrounding the PMMA microspheres and absence of inflammatory reaction at 90DAI. Picrosirius stain. 40x magnification in polarized light microscopy

the presence of the biopolymer, but also to the large number of inflammatory cells recruited to the implantation site, the products of these cells, cellular debris and the vehicle administered with the biomaterial, which is still present in the initial acute phase of the response [22, 37].

Usually, the implantation of non-phagocytic biomaterials triggers the development of a foreign body reaction, which, in general, is initially characterized by the adsorption of proteins, mainly blood, on the surface of the biomaterial; then there is the migration of neutrophils and the differentiation of macrophages from infiltrating monocytes; continued by the degranulation of neutrophils and secretion of NETs, resulting in the formation of a pro-inflammatory microenvironment. The persistence of the biomaterial stimulus, caused by its presence, stimulates the change in the inflammatory profile, causing the fusion of macrophages to form foreign body giant cells, transformation of fibroblasts into myofibroblasts, formation of extracellular matrix and, finally, formation of fibrous capsules [18].

It was observed that the difference in the depth of PMMA implantation triggered an inflammatory response with different characteristics. The PMMA used in this study has homogenously sized microspheres, around 36–45 µm in its majority, which prevents its phagocytosis [30]. The more superficial the implantation, the more expressive the inflammatory reaction generated, which corroborates with Lemperle (2018) [22]. Only in the subcutaneous region did PMMA trigger a typical acute inflammatory response, with a predominance of PMN cells, recruitment of MN cells and fibroblasts since the initial phase of the

response. The microspheres presence in the subcutaneous and intramuscular regions stimulated macrophage fusion and, consequently, the formation of foreign body giant cells.

In this study, the "gold standard" technique was used for PMMA implantation. This technique is called tunneling and avoids vascular injuries, rupture of muscle fibers and allows deep application of the biomaterial [12]. In the intramuscular and juxtaperiosteal regions, there was a foreign body reaction with the presence of MN cells, fMO, fibroblasts and foreign body giant cells. The latter are formed from the fusion of "frustrated macrophages" that failed to phagocytize the foreign body [38]. Its presence in the foreign body reaction cannot be considered good or bad, purely, it must be defined whether its profile comes from M1 or M2 macrophages [39]. In this study, the presence of foreign body giant cells was evident throughout the experimental period in the subcutaneous and intramuscular implantation sites.

Varying the experimental day and/or the depth at which PMMA was implanted, the collagen concentration produced changes in a non-standard way. This corroborates our assessment of the density of the extracellular matrix around the PMMA microspheres in the subcutaneous region, which mainly depends on the greater or lesser presence of collagen, that is, the thicker and more mature extracellular matrix also had a higher concentration of collagen fibers, however these findings do not correlate with a higher or lower PMMA concentration.

The tissue remodeling process activated by the presence of an implant involves a proliferative phase followed by the remodeling phase itself, where there is the involvement of several factors that alter and organize the conformation of the extracellular matrix depending on the stimulus, such as proteins and enzymes. It is interesting to note that collagen deposition around the foreign body and the potential for its subsequent encapsulation depends on its size [17]. In the proliferative phase, granulation tissue is formed, which is composed of macrophages, fibroblasts, neovascularization and collagen [12, 35]. Over time, the change in stimuli in this inflammatory microenvironment signaled a change in the inflammatory profile, causing greater activation of M2-type macrophages, fusion of foreign body giant cells, release of TGF-β and greater collagen production [18]. Macrophages and foreign body giant cells are proven to be the cell types responsible for most expressively stimulating the formation of the fibrous capsule around the biomaterial [35, 40].

Therefore, it is concluded that (1) the inflammatory reaction of a foreign body to PMMA is not standardized, limited or predictable, the cellular and molecular components stimulated by the presence of this biomaterial are changeable depending on the anatomical plane analyzed and the concentration of implanted product; (2) collagen production and, consequently, the density of the extracellular matrix around the PMMA microspheres is not related to its concentration in the tissue and also varies throughout the experimental period.

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Declarations

Conflict of Interest The authors declare that they have no conflicts of interest to disclose.

Human and animal rights Animal rights were completely respected and this study followed the regulations of the ethical committee in animal use, approval protocol number 027/2017 (CEUA/UFG).

Informed consent For this type of study, informed consent is not required.

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