Local and systemic tissue response submitted to injection of 2 and 30% polymethylmethacrylate in rats' tongue

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Local and systemic tissue response submitted to injection of 2 and 30% polymethylmethacrylate in rats' tongue

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Local and systemic tissue response submitted to injection of 2 and 30% polymethylmethacrylate in rats' tongue

Introduction: Adverse effects on the oral mucosa after the use of dermal fillers have been reported due to their increased use for facial aesthetics.
Objective: This study aimed to evaluate, clinically and histologically, the local and systemic effects of two concentrations of polymethylmethacrylate (PMMA) dermal filler in rats.
Material and methods: Fifty-four female rats were allocated into three treatment groups (2% PMMA, 30% PMMA and 0.9% NaCl), according to the substance injected in the tongue, and three experimental periods: 7, 60 and 90 days. The rats were clinically evaluated and then euthanised, and their tongue and right kidney removed. The histological sections were stained with haematoxylin/eosin and picrosirius.
Results: Clinically, significant differences were found between test groups as to the occurrence of nodules (Kruskal–Wallis; $p < 0.001$). Histologically, there was greater inflammatory response in the PMMA compared with control (Kruskal–Wallis; $p < 0.001$). 30% PMMA had greater collagen formation (ANOVA mixed models; $p < 0.01$). No migration of the material towards kidney was found.
Conclusion: Polymethylmethacrylate induced intense reaction in the initial period of observation (7 days), followed by gradual decrease during the study, favouring the presence of fibroplasia adjacent to the material.

Keywords: dermal filler, polymethylmethacrylate, foreign body reaction, rats.

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Introduction

Injectable dermal fillers are commonly used by dermatologists and plastic surgeons to retard the effects of facial ageing, such as minimising wrinkles, increasing volume of lips and improving facial contour. However, there are very few studies related to the long-term physical, chemical and mechanical properties required for the functioning of these materials[1,2].

There are several of polymethylmethacrylate (PMMA) filling agents available on the market. These products are composed of a combination of PMMA microspheres and bovine collagen (Artefill®), colloidal medium of carboxymethylcellulose (Metacrill®) or hydroxyethylcellulose (NewPlastic®)[3].

PMMA is available for use at various concentrations, ranging accordingly to their respective indication. Usually, commercial concentrations of PMMA are 2, 10 and 30%. The 2% concentration is indicated for minimising thin wrinkles, especially in the lip region, while 10% PMMA is used for mobile areas, aimed at reducing the appearance of fatigue caused by flaccidity (nasolabial folds, expression marks, lips, back of hands). A concentration of 30% is indicated for increasing the volume of the region of interest, such as chin, malar, jaw line, nasal corrections, nasolabial folds, gluteus augmentation, chest, biceps and calves[1].

This type of treatment, which often replaces traditional surgical procedures such as rhytidectomies, provides satisfactory cosmetic results. It is

known, however, that there is a risk of undesired effects at the site of injection of the product or even at a distance. Dental literature reports numerous cases of orofacial injuries caused by the use of dermal fillers, reflecting a new reality in dental surgeons' practice[1,4].

The material microspheres remain in tissues and become encapsulated by connective tissue, which contributes to the formation of the effective volume of the material[5]. Granulomas or nodular lesions can occur up to 6 years after injection of PMMA[6]. The pathogenesis of adverse reactions for these products is still unknown. When exogenous materials are injected into tissues, a granulomatous reaction with the presence of macrophages and the formation of new collagen surrounding the area can be observed[7]. It is known that, during the initial period (4–6 months after injection), the PMMA spheres provoke a foreign body reaction and are later encapsulated by the collagen fibres of the tissue[8].

The objective of this study was to investigate the early and late effects, local or systemic, of the injection of two concentrations of polymethylmethacrylate in rats' tongue at different observation periods.

Material and methods

This research was performed after approval from the Scientific and Ethic Committee (protocol 0008/10) and then from the Ethic Committee for Animal Use (protocol 10/00150). A randomised longitudinal experimental study was carried out using 54 female Wistar rats (Rattus norvegicus) ageing 2 months, which were healthy, weighing approximately 200 g, and obtained from the same animal facility. Two PMMA's concentrations available on the market were used (NewPlastic®; Lebon Produtos Químicos e Farmacêuticos Ltda, Rio de Janeiro, Brazil) in the test groups: 2% PMMA and 30% PMMA. The rats were kept in an appropriate place, with ventilation, air filtration, controlled temperature of 22°C, and 12-h light–dark cycles, and they were fed with balanced rat chow and provided filtered water *ad libitum*.

Study groups

The animals were randomly divided into three groups, according to the substance used: 2% PMMA (18 animals); 30% PMMA (18 animals); and 0.9% NaCl (control) (18 animals). Each group was subdivided into three observation periods according to the estimated time of euthanasia of the animals (7, 60 and 90 days).

Anaesthesia

The animals were handled in accordance with institutional guidelines or animal care and use. Initially, female rats were weighed on a digital balance so that the dosage of anaesthetic could be calculated. Anaesthesia was induced with an intraperitoneal injection of a mixture of xylazine hydrochloride (20 mg/ml) 0.05 ml/100 g with ketamine hydrochloride (50 mg/ml) 0.1 ml/100 g.

Substance injection

When sedation was observed, the animal was placed on a surgical table, in supine position and with paws tied using rubber bands. The rat's tongue was pulled out with tweezers, exposing the ventral tongue region. Using a disposable insulin syringe, 0.07 ml of each substance was injected in the middle third of the ventral tongue, 7 mm in front of the frenum. The needle was inclined as parallel as possible to the mucosa, with the bevel facing up, and inserted 7 mm deep, where this measure was standardised by an endodontic silicone stop.

Clinical analysis

After 7, 60 and 90 days and preceding euthanasia, the animals from each treatment group were sedated and the tongue was subsequently examined for possible tissue alterations.

Euthanasia

At the end of clinical analysis, euthanasia was performed by isoflurane inhalation. After necropsy, the animals were treated as biohazard waste, where they were frozen and collected in accordance with the regulations of the PUCRS animal facility.

Sample processing

After being euthanised, all animals were necropsied, where their tongue and right kidney were removed for microscopic analysis. Sample fixation was carried out with the use of 10% neutral buffered formalin for a minimum of 24 h. Samples of the tongue and kidney were sectioned longitudinally into two fragments. Tongue was embedded so that the edge of its long axis was parallel to the paraffin block section plane. For each specimen, there were two histological sections of 6 μm to prepare two slides, which were later stained with

haematoxylin and eosin (HE) and picrosirius red, the latter being a specific staining for collagen fibre analysis[9].

Histological analysis

The tongue slides were validated for histopathological analysis through the identification of the dermal filler. The analysis of the microscopic fields was made in the region adjacent to the filling material. In the control group, the observation fields were selected using the anatomical reference and the applied methodology. The analysis took place in the Oral Medicine Service Unit (São Lucas Hospital), using a light microscope (Zeiss® Axioskop 40, Zeiss, Oberkochen, Germany) at magnifications of ×40, ×100, ×200 and ×400. Slide evaluation was standardised by arranging a training session with an experienced pathologist. The intraexaminer calibration was performed with the reanalysis of each slide in a 7-day interval between observations. For slide analysis, the examiner was previously calibrated and blinded with the use of slide masks in all procedures.

Inflammatory reaction

For groups 2% PMMA and 30% PMMA, the histological evaluation was performed with an analysis of the microscopic fields adjacent to the dermal filler, choosing for analysis the ones that showed a higher intensity of inflammatory response. In the control group, the observation fields were selected using the anatomical reference where the 0.9% NaCl was applied. Thus, the absence or presence of lymphocytes, plasma cells, macrophages, neutrophils, eosinophils, giant cells, fibroblastic condensation and hyperaemia was determined. To standardise inflammatory scores, parameters previously validated[10–13] were used as follows: (i) Absence: absence of inflammation, (ii) Mild: sparse mononuclear cells, (iii) Moderate: infiltrate of mononuclear cells and/or sparse neutrophils and eosinophils and (iv) Severe: infiltrate of neutrophils and eosinophils.

Density of collagen fibres

Three to four areas (μm^2) that contained mostly connective tissue stained by picrosirius in a polarised light microscope (Zeiss® Axioskop 40 Zeiss) coupled to a camera (Cool Snap-Pro cf, Media Cybernetics, Bethesda, MD, USA) connected to a Dell® computer (Optiplex GX620 model, Round Rock, TX, USA) were selected using ×100 magnification. The chosen area should cover the largest amount of collagen fibres. Those images were exported to the Image Pro Plus® version 4.5.1 software (Media Cybernetics, Inc.; 2005), in which images obtained with polarised light were recognised and converted into shades of red (collagen representative area). Thus, the proportion of collagen fibres was determined by the calculation of the areas occupied by them in comparison with the total area of each field[9].

Migration

Migration or systemic distribution of injected material was evaluated microscopically in all study groups, based on the presence or absence of inflammatory response in the right kidney of each rat.

Statistical analysis

Statistical analysis was performed with the following softwares: SPSS 17 (SPSS Inc., New York, NY, USA) and SYSTAT 13 (Systat Software Inc., Chicago, IL, USA). Kruskal–Wallis tests with Dwass–Steel–Critchlow–Fligner post hoc tests were used for all pairwise comparisons, with the significance level set at 5% ($p < 0.05$). To analyse picrosirius red staining (which is a numeric variable), mixed models with Fisher's post hoc analysis were used with the significance level set at 5%. The fixed effects of the statistical model were substance, time, and time and substance interaction, whereas the random effect was the animal whose histological slide had multiple reading fields.

For the examiner calibration (re-analysis of each slide every 7 days), the concordance for the 52 observation pairs (Kappa value ± standard deviation) was 0.936 ± 0.044 ($p < 0.001$).

Results

During a 90-day experimental period, two animals died in the control group, resulting in a group of $n = 4$.

Clinical evaluation

Animals in the 2% PMMA and 30% PMMA test groups displayed clinical alterations including ulcers (Fig. 1a), white plaques and nodules (Fig. 1b). Control group, on the other hand, had no such features in any of the animals.

Clinical evaluation at 7 days, two rats subjected to application of 2% PMMA and one rat to 30% PMMA exhibited ulcerations. Within 60 days, two

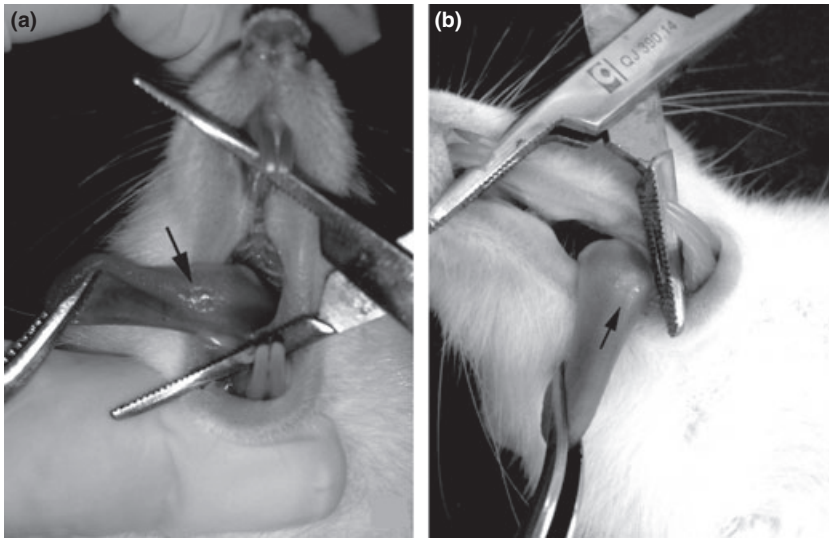

Figure 1 (a) Presence of ulcer in the ventral tongue, near the 2% polymethylmethacrylate (PMMA) injection site (7 days). (b) Presence of white nodule at the 30% PMMA implantation site (60 days).

animals from the 2% PMMA group and one from the 30% PMMA group showed white plaques at the injection site. There were nodular lesions with firm consistency in four animals from the 30% PMMA group after 60 days. After 90 days, no clinical changes were observed in the anatomical region where the material was injected.

Histological evaluation

In Fig. 2, the photomicrograph illustrates the PMMA histological pattern which consists of transparent microspheres, variable in size, distributed within the tissue.

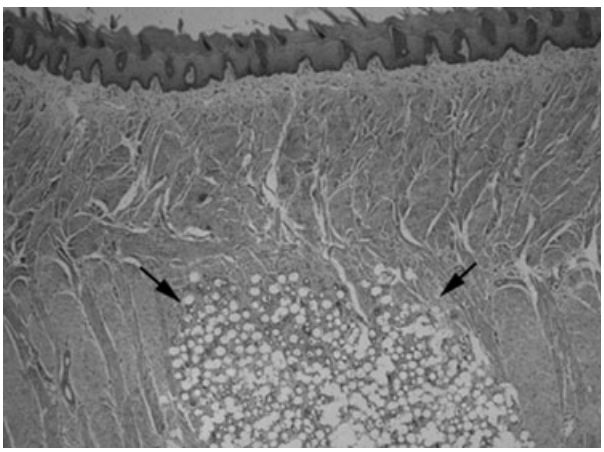

Figure 2 Photomicrograph of polymethylmethacrylate (PMMA) within the tissue. (HE, magnification: ×40).

Inflammatory reaction

There was a predominance of moderate response with the two PMMA concentrations at the monitoring times. However, in the 30% PMMA group, after 7 days, there was a severe inflammatory response (Fig. 3).

Variables

In the test group samples, lymphocytes were present in 97%, plasma cells in 77% and macrophages in 100%. On the other hand, eosinophils were present in 16.6% of the 2% PMMA group (60 days), 100% of the 30% PMMA group (7 days) and 33.3% of samples of the 30% PMMA group (60 days). Eosinophils were absent in the test groups at 90 days and in the control group at all observation times. In the 2% PMMA group, neutrophils were observed in 50% of the samples at 7 days and in 16.6% at other times. In the 30% PMMA group, there was a gradual decrease in the number of neutrophils at different observation times, ranging from 100% (7 days) to 83.3% (60 days) and 33.3% (90 days).

In this study, the presence of giant cells (Fig. 4) and neovascularisation (Fig. 4) was clearly evident in both test groups at all times.

Density of collagen fibres

There was a slow and gradual appearance of newly formed collagen fibres in group 2% PMMA. In the 30% PMMA group, there was immediate and severe fibroplasia followed by stabilisation of the

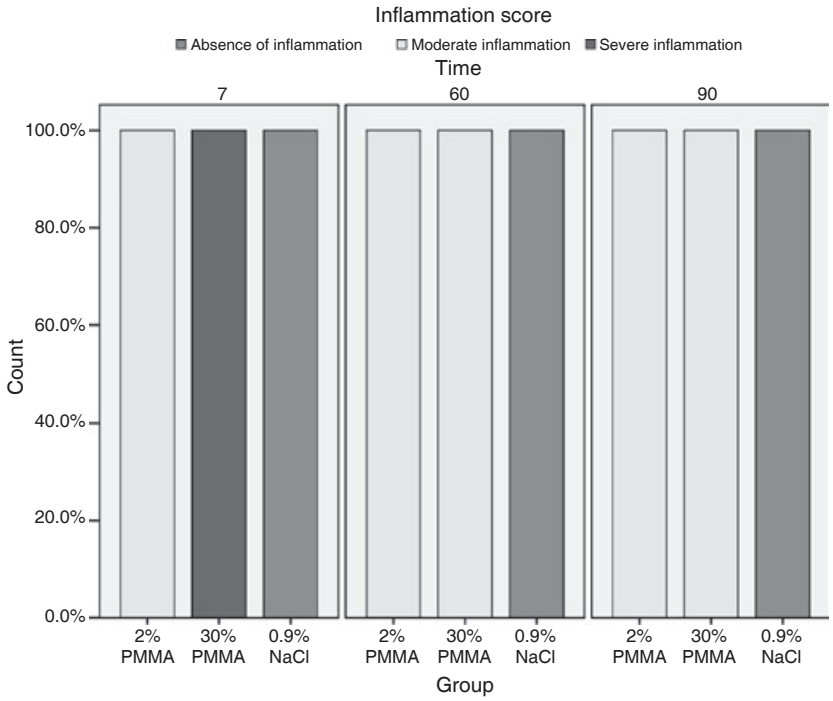

Figure 3 Score distribution of the inflammatory response among groups according to each observation period.

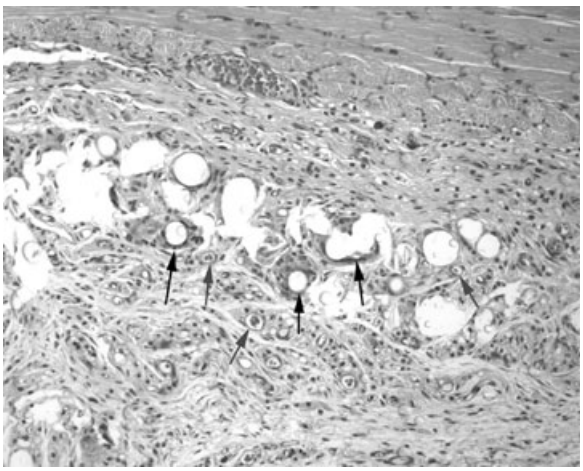

Figure 4 Presence of giant cells (black arrows) and newly formed blood vessels (grey arrows) after application of 2% polymethylmethacrylate (PMMA) (60 days). (HE, magnification: ×200).

process, whereas animals in the 0.9% NaCl group remained stable at all observation times, with total absence of this event (Fig. 5). Figure 6 describes the collagen formation around PMMA.

Migration

The kidney samples, in their entirety, showed neither evidence of inflammatory response nor traces of dermal filler.

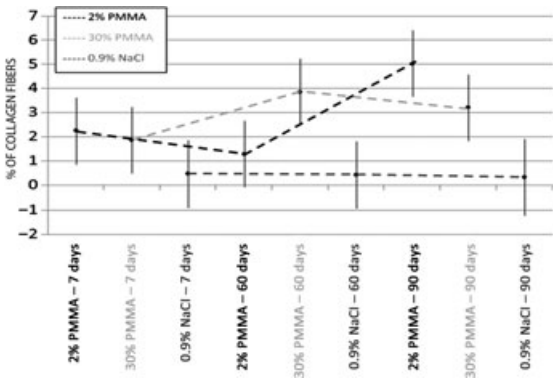

Figure 5 Distribution of fibroplasia in groups and its variation at different observation times.

Discussion

This research was prompted by the identification of oral lesions in patients, associated with adverse effects of dermal fillers for aesthetic purposes. It is believed that a high number of cases occur for different reasons, one of which is the excessive value placed on facial aesthetics for the purpose of preserving a young appearance. In addition, there is also the fact that these procedures are less invasive compared with traditional surgical interventions. Furthermore, they are low cost and can be easily accessed.

The intention of this study was to evaluate clinical and histological responses of PMMA,

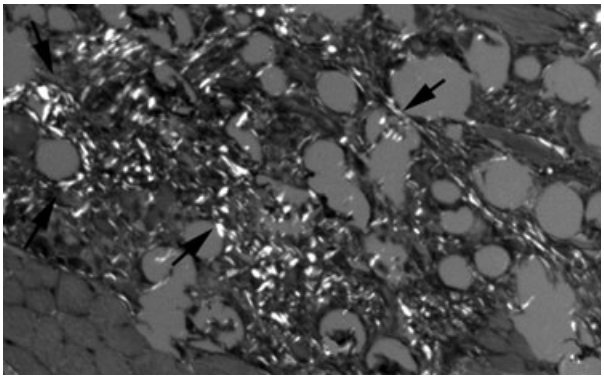

Figure 6 Photomicrograph showing characteristic collagen formation (arrows) around 2% polymethylmethacrylate (PMMA) (90 days) microspheres. (Picrosirius red, magnification: ×100).

considering the most commonly used aesthetic filling material nowadays. Extreme concentrations (2 and 30%) of this material were applied with the objective of assessing possible variations in the degree of tissue inflammatory response using the same product in different ways.

While these cosmetic products are not used intra-orally by dermatologists and plastic surgeons, the application of PMMA in the skin (where there are hair follicles and sebaceous and sweat glands) could complicate histological analysis. Thus, the tongue of rats was used and its ventral surface was chosen for the injection of substances with the intent to eliminate any interference with the results, as this region is free from trauma or chronic irritation[12,13].

These products have been increasingly utilised not only for anti-ageing purposes, but also for lip and malar augmentation and facial contour definition in young patients. Aiming to reproduce real-life clinical practice, both young and adult rats (3–6 months) were selected[14].

Responses triggered by the injection of dermal fillers illustrated the inflammatory process in all its stages. The presence of clinical alterations was observed in 27.7% of test groups, all at observation times of 7 and 60 days. In the first period (7 days), ulceration near the material injection was the most common finding. This could be explained by the volume increase with PMMA injection, which renders the tissue more susceptible to mastication trauma. This was confirmed with nodular lesions found in 22.2% of the samples of 30% PMMA group. This increase in volume was also associated with a chronic irritation and the development of white plaques, which were visible in 16.6% of the animals at 60 days. Loureiro Borghetti *et al.*[12] and Zimmermann and Clerici[15] made reference to hardening of tissue in the injected region, besides the appearance of nodules at the application sites, ranging from 3 to 24 months after the procedure. Christensen *et al.*[6] have mentioned that granulomas and nodules can appear up to 6 years after material injection.

PMMA is microscopically composed of transparent microspheres that are variable in size and distributed within the tissue. Histological examination at 7 days showed that the entire 30% PMMA group had an intense inflammatory response with the presence of infiltrate comprising neutrophils and eosinophils. Lemperle *et al.*[16] emphasised in a similar study that neutrophil infiltration reached its peak in the first 24 h, with an increasing inflammatory reaction. After 72 h, neutrophil migration ended, and macrophages began to accumulate at the implantation site, eventually becoming the predominant cell type. Due to the inability of tissue response to degrade the injected filling material initially, inflammation displayed chronic features at 60 and 90 days, represented by the presence of a lymphoplasmacytic infiltrate, featuring a moderate intensity response. Moderate alterations occurred in all samples of the 2% PMMA group and most of the 30% PMMA group, represented by infiltrate of lymphocytes, plasma cells and macrophages.

If the material implanted elicits an immunogenic response, the inflammatory response follows a different pathway characterised by the presence of giant cells originating from the fusion of macrophages. Foreign body giant cells were evident in 88.8% of samples of the test groups. PMMA is composed of microspheres of irregular surface and varying diameters, ranging between 30 and 80 μm[13], reasons why it is not capable of being phagocytised by macrophages. This material can only be degraded by giant cells, which are present at a later stage[17–20]. These results agree with those reported by McClelland *et al.*[21], as the authors mentioned that there is a strong presence of giant cells when dealing with PMMA implants. In line with our findings, Lemperle *et al.*[18] reported that after a period of 1–9 months, PMMA injection caused a moderate inflammatory response, with the presence of macrophages and foreign body giant cells. Moure *et al.*[13] also found a considerable number of giant cells in rats that received PMMA.

As to fibroplasia, this study showed a gradual formation of collagen fibres along the experimental periods with 2% PMMA and an immediate and intense formation of these fibres with 30% PMMA, followed by stabilisation at 60- and

90-day follow-up. According to Lemperle *et al.*[22], by 4 weeks, all injected microspheres have been individually encapsulated by fibroblasts and collagen fibres. The presence of macrophages was very small; however, capillary growth was evident. Zimmermann and Clerici[15] reported that monocyte invasion occurred 3 days after material implantation, with fibroblast differentiation in 6 days and that the microsphere interstitial space was filled by 9 days. After 2 months, each microsphere was surrounded by a thin fibrous capsule with the reduction in monocytes and histiocytes. After 3 months, all the injected collagen had been phagocytised by macrophages and the fibrous phase seemed to end in 4 months, with evidence of a stable condition. Christensen *et al.*[6] reported that between 8 and 10 weeks after the application of dermal filler, they found not only filling material, but also macrophages, lymphocytes and giant cells in the histological slides of adjacent tissues. Polymorphonuclear cells were absent in all cases. Alster and West[23] reported that after 12 weeks, collagen continued to undergo alterations, becoming denser and more resistant, leading to collagen formation and stabilisation of the inflammatory process.

In this study, neovascularisation was clearly seen in the test groups, possibly due to the need for generating a vascular network to allow the inflammatory reaction to occur[13]. In agreement with these results, several authors have reported that the presence of PMMA microspheres stimulates neovascularisation[13,23,24].

Regarding material migration or its systemic behaviour, findings obtained from microscopic analysis of rats' kidneys in this research did not show any alteration that could be related to this process. These results agree with previously published studies[12,13]. On the other hand, in the scientific literature, systemic migration cases showed the presence of hepatic and renal inflammatory infiltrates in rats submitted to PMMA injections of 0.05 ml in the ear[25]. Constant presence of periportal and intralobular infiltrates in the liver has been reported, as well as chronic pyelonephritis and interstitial nephritis. These alterations have been explained by the systemic distribution of implanted material, which could act over long distances, in a metabolism or excretion organ, as a chemotactic substance[25].

Based on these findings, it was concluded that both a low and high PMMA concentration induced inflammatory reactions with the strong presence of foreign body giant cells. Aesthetic procedures for the purpose of facial beauty have become popular. This should alert the dentist and oral surgeon to be aware of the possibility of adverse reactions from the use of these dermal fillers, occurring in oral and perioral tissues.

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