The Dynamics of Subcutaneous Tissue Response to Microorganisms Associated with the Extract of Araçá (*Psidium cattleianum*): An Edemogenic and Microscopic Analysis

A Dinâmica da Resposta do Tecido Subcutâneo para Associação de Microorganismos com o Extrato de Araçá (*Psidium cattleianum*): Uma Análise Microscópica e Edemogênica

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ABSTRACT

Aim: The aim of the present study was to evaluate tissue reaction to the extract of araçá (*Psidium cattleianum*) associated with inactivated microorganisms. **Methodology:** For performing the investigation, a 0.1-mL suspension was used containing *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Peptostreptococcus micros*, and *Porphyromonas endodontalis*, which were inactivated by heat and mixed into a 1.0-mL physiological serum (control group), an aqueous solution, or a hydroalcoholic extract of araçá. Eighteen male rats (*Rattus norvegiccus*) under general anesthesia received an Evans blue intra shot at 1%. Thirty minutes later, 0.1 mL of one of the extracts or the serum (associated with the inactivated microorganisms) was injected into the animals' dorsal under-skin region. The animals were euthanized after 3 and 6 hours, and the materials obtained were placed in formamide for 72 hours. For the morphological analysis, 30 rats received polyethylene-duct implants with the extracts or the serum, in addition to the solution of inactivated microorganisms to the dorsal region. After 7 and 30 days, depending on the group, they were euthanized. **Results:** No significant difference ($P > 0.05$) was observed postoperatively in the amount of edema between groups. Results obtained from the reading of species in optical microscopy showed a repair in the 30-day period, which was significantly better when compared to the 7-day-period. **Conclusion:** Per the results, extracts of araçá (*Psidium cattleianum*) have no effect on the bacterial components. However, the extracts do not interfere in the process of subcutaneous tissue repair, showing good biocompatibility.

keywords: Anaerobic bacteria, Edema, Inflammation Plant extracts, *Psidium*

RESUMO

Objetivo: O objetivo do presente estudo foi avaliar a reação tecidual do extrato de araçá (*Psidium cattleianum*) associado com microorganismos inativados. **Metodologia**: Para a realização da investigação, uma suspensão de 0.1mL foi usada contendo *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Peptostreptococcus micros* e *Porphyromonas endodontalis* dos quais foram inativos por aquecimento e misturados a 1.0 mL de soro fisiológico (grupo controle), uma solução aquosa ou extrato hidroalcoólico de araçá. Dezoito ratos machos (*Rattus norvegiccus*) sobre anestesia geral recebida de uma injeção de Azul de Evans a 1%. Trinta minutos depois, 0.1mL de um dos extratos do soro (associado com microorganismos inativos) foi injetado em animais na região da pele sobre o dorso. Os animais foram eutanasiados após 3 e 6 horas, e os materiais obtidos foram colocados em formamida por 72 horas. Para as análises morfológicas, 30 ratos receberam implante em tubo de polietileno com os extratos ou soro, acrescentado a solução de microorganismos inativos na região dorsal. Após 7 e 30 dias, dependendo do grupo, eles foram eutanasiados. **Resultados**: Não houve diferença significante ($P > 0.05$) foi observado pós operatório quantidade de edema entre grupos. Os resultados obtidos da leitura de espécies em microscópio óptico mostrou um reparo no periodo de 30 dias, dos quais foram significantemente melhores quando comparados ao periodo de 7 dias. **Conclusão**: Para os resultados, o extrato de araçá (*Psidium cattleianum*) não tem efeito em componentes bacterianos. Portanto, os extratos não interferem em processos no reparo de tecidos subcutâneos, mostrando boa biocompatibilidade.

Palavras-chave: Bacteria anaeróbica, Edema, Extrato de Plantas, Inflamação, *Psidium*

INTRODUCTION

Bacteria are the primary cause of the development of necrotic pulps, periapical lesions, and post-treatment disease following root-canal treatment (Guneser et al., 2016). Hence, the eradication of microorganisms and their by-products from the root-canal system is needed for the success of the endodontic treatment (Guneser et al., 2016). Although mechanical instrumentation is one of the most important steps for controlling root-canal infection, it cannot achieve total elimination of bacteria when used alone. The complexity of root canals, which is attributable to the many cul-de-sacs, fins, and lateral canals, means that almost half of root-canal walls are left unprepared with only instrumentation. Therefore, the use of antimicrobial irrigation solutions has been advised as an adjunct to mechanical instrumentation (Guneser et al., 2016). Microorganisms with different characteristics (structural, metabolic, and pathogenic) reaching the periapical region stimulate the inflammatory and immunologic responses (Guneser et al., 2016). One option studied for bacterial control is the use of plant extracts in popular medicine (Machado and Oliveira, 2014). As presented in previous studies *in vitro*, these extracts have antibacterial potential (Machado and Oliveira, 2014).

Medicinal plants are an alternative and even support protocols to traditional treatment (Machado and Oliveira, 2014). In this sense, over the last few years, there has been a significant increase in scientific progress surrounding the pharmacological and chemical studies of medicinal plants aimed at obtaining new compounds with therapeutic properties (Cechinel Filho, 1998).

Recent studies using Brazilian Cerrado plant extracts provide evidence of antimicrobial activities, showing promising results for buccal microbial control (Sartoratto, 2004; Gaetti-Jardim Jr, 2010; Lima, 2006; Machado and Oliveira, 2014). Araçá (*Psidium cattleianum*) is a plant that has been studied and presented

promising results. Araçá, which belongs to the *Myrtaceae* family, is also known as araçá-do-campo and araçá-comum.

Despite the limited number of studies, it has proven antimicrobial action (Gaetti-Jardim Jr, 2010; Alvarenga et a., 2016) and possible anti-inflammatory potential, besides having proven biocompatibility through in vivo study (De Menezes, 2008).

Alvarenga et al., (2016) available the araçá's leaves rude extracts antimicrobial activity and these job results support to the use to the *P. cattleianum* leaves rude extract against the dental biofilm microorganism. The study extract it is to believe might have antibacterial potential to the use in formulation pharmaceutical or products improvement, like mouthwash, topic use ointment and addition in tooth paste formulation, to caries prevention (Alvarenga et al., 2016).

Gaetti-Jardim Jr (2010) used 22 plants species in popular medicinal use in Cerrado areas, which presented inhibitory activity against *Streptococcus mutans* ATCC 35688 and ATCC 1910 and against *Fusobacterium nucleatum* ATCC 10953 and ATCC 25586, as in planktonic condition as biofilm, evidencing positive results for extracts obtained from araçá leaves (*Psidium cattleianum*) for aqueous and hydroalcoholic solutions. The antimicrobial activity of araçá extract in hydroalcoholic and aqueous solution were also available for periodontopatogenic microorganisms *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 2564, *Actinobacillus actinomycetemcomitans* ATCC 33384, *P. gingivalis*, and 10 clinically isolated of *P. gingivali*, obtaining periodontal pockets of patients with chronic periodontitis and showing inhibitory effects in all tested microorganism such as planktonic method biofilm (Gaetti-Jardim Jr et al., 2010).

Crivelaro de Menezes et al. (2010) evaluated the influence of *P. cattleianum* sabine aqueous extracts on *S. mutans* counts and dental enamel micro-hardness of rats with caries. The extracts decreased *S. mutans* accumulation and enamel demineralization. De Menezes et al. (2010) also studied and corroborated that *P. cattleianum* aqueous extract significantly reduces the *S. mutans* counts and decreases the enamel demineralization rate. The leaf extract was also shown to exert anti-caries effects in rats.

MATERIAL AND METHODS

Animal

The research was approved by the Animal Experiment Ethics Committee, Araçatuba Dental School – UNESP (Process #2008 – 000165). Forty-eight Wistar (*Rattus norvegiccus*) male rats, 60 days old and 250g, were obtained from Araçatuba Dentistry School Vivarium – UNESP for this research.

Prepared Extracts

The plants were collected in no deforested areas that are a permanent reserve in Carolina (MA) county rural property during the rainy season (December to February). The leaves used in the extracts were collected healthy plants and then dried at ambient temperature until dry and breakable (Matos, 2002) and by means of manual and grinding until powder. Araçá leaf (*Psidium cattleianum)* extract hydroalcoholic and aqueous solution was prepared. The hydroalcoholic solution was obtained according the Navarro et al. (1998). For the hydroalcoholic solution was used 20 gram leaves and 250 mL of the 80% ethanol (Gaetti-Jardim Jr et al., 2010).

Test of Linkage and Microorganisms

All microorganisms (Table 1) were provided by the Microbiology Laboratory in Araçatuba Dental School – UNESP.

The microbial containing all the bacteria was re-suspended in saline 1 mL (control) and araçá extract hydroalcoholic or aqueous solution 1mL. The experimental groups were:

1) Aqueous: bacteria pool ($5x10^6$ cel/mL to each reference strain) + 1 mL aqueous araçá extract.
2) Hydrohalcoholic: bacteria pool ($5x10^6$ cel/mL to each reference strain) + 1 mL de hydroalcoholic araçá extract.
3) Saline: bacteria pool ($5x10^6$ cel/mL to each reference strain) + 1 mL saline.

Edemogenic Test – Immediate Response

Eighteen animals were used and separated in groups by three to each available period (3 and 6 hours) and to each experimental group. The animals were injected with only one solution with araçá extract, aqueous or hydroalcoholic, added the microorganism solution, or with saline added to the microorganism solution to control group. In each group, 6 animals were injected for each period. The animals were submitted to general anesthesia with xylazine (Rompun 4 Bayer) to the surgery intercession at a ratio of 25 mg/Kg and ketamine (Francotar – Virbac), at a ratio of 50 mg/kg mixed in the same syringe, intramuscularly administered. Then, the animal received, in the penile vein, a 1% Evans blue intravenous (Evans Blue Difco Lab) (Canova, 2002; Takahashi et al., 2015). After 30 minutes a solution (0.1 mL of aqueous or hydroalcoholic) in each animal was injected in the dorsal area using the median line as a reference. The animals received an overdose of the same anesthesia after 3 and 6 hours, depending on the group. Subsequently, the animal's dorsal area was treated with a manual trichotomy; it was found that the edema area, characterized by a blue color zone, was removed by iron punches, with 23 mm diameter. The standard parts were perforated and kept in vials containing formamide 4 mL (Vetec 4 Química- RJ – Brasil), put in an oven at 45ºC

for 72 hours to stain extraction by the tissue dissolution and then the solution was filtered and analyzed in 630 nm spectrophotometer (Canova, 2002; Takahashi et al., 2015).

Implant in Rats' Subcutaneous Tissue – Later Reaction

Five animals for each group were used in this stage, aqueous, hydrohalcoholic, and saline, in 2 analysis periods, 7 and 30 days (Holland et al., 1999, 2001, 2002), involving a total of 30 animals. Each animal received two implants containing the same solution, with 10 implants added to each group.

The animals were subjected to general anesthesia with xylazine (25 mg/kg) and ketamine (50 mg/kg) mixed in the same syringe, intramuscularly administered. After trichotomy in the dorsal area and antisepsis with 1% polyvinylpyrrolidone (Riodente, Rioquímica, São José do Rio Preto, Brasil), a 2 cm longitudinal incision was made with a No. 15 scalpel blade following the median line with subcutaneous tissue. Then, the divulsion was done.

Before the implant were injected in the tubs the pool containing the aqueous solution, hydroalcoholic, or saline added to inactive microorganisms. Then, the polyethylene tubes containing the same solution (aqueous, hydroalcoholic, or saline) were implanted in the right and left side of the animals' subcutaneous tissue. The incision was later sutured with line of silk (4.0 Ethicon, Johnson & Johnson).

After at 7 and 30 days, the pieces containing the polyethylene tubes, around the adjacent conjunctive tissue, were obtained following the same surgical procedures.

After tissue removal, the animals received an overdose of anesthesia, and pieces were fixed in 10% buffered formalin solution for 48 hours and washed in water for 12 hours. Then, the pieces were dehydrated, clarified, and included in paraffin, oriented to permit implant histology by being cut longitudinally. The cuts,

semiserial and with a thickness of 6 μm, were stained with hematoxylin and eosin for microscopic analysis (binocular microscopes: Leica, Germany).

The results obtained for tissue response, induced for tested extracts, was compared to those of the control group. A descriptive analysis was done for the three experimental groups.

STATISTICAL ANALYSIS

The edemogenic test results, relative to edema quantitative, were available using variance analyses applied to 2 available standards (time and solution) using the software GMC 2002 (Campos, 2004). The obtained minimum grade was 5% significance.

RESULTS

Edemogenic Analysis

The results for edema quantification (Table 2) showed no statistically significant differences between the experimental groups ($P > 0.05$). Of the groups, the aqueous extract and saline presented less edema than the hydroalcoholic extract at 3 hours. At 6 hours, all groups showed similar values (Table 2).

Microscopy Analysis

Aqueous Araçá + Bacteria - Day 7: Here, inflammatory cells in large amount, with macrophage predominance, numerous lymphocytes, few leucocytes and other mononuclear cells, and chronic inflammation characteristics were observed. Some plasmocyte cells were noted. Some fibroblasts were seen around rare collagen fibers that presented complex arrangement (Figure 1).

Aqueous Araçá + Bacteria - Day 30: The microscopic image revealed a decrease in the inflammatory compared to the 7-day period. The cell inflammatory agglomerate

was substituted by a thin and parallel collagen fiber capsule, willing to the implant area. Some fibrocytes were identified possibly, as were some few fibroblasts around collagen fiber. In general, the macrophages layer was low profile, and the lymphocyte quantity and other mononuclear cells were significantly reduced. Blood vessels were fewer and less dense in the conjunctive tissue interior (Figure 1).

Hydroalcoholic Araçá + Bacteria - Day 7: Similar to the aqueous araçá group in the same period, there was rich macrophage quantity, lymphocytes, and few leukocytes and other mononuclear inflammatory cells. Some plasmocytes were observed. There was little fibroblasts predominance, and rare collagen fibers presented organized way. There was numerous blood capillaries diffused over the entire area (Figure 1).

Hydroalcoholic Araçá + Bacteria - Day 30: The collagen fibers were more organized and denser, at more advanced mature degree compared to the 7-day period. The fibroblasts present were thinner and had a linear nucleus (indicating a collagen metabolism decrease); in general, collagen fibers displayed parallel structure (Figure 1).

Saline + Bacteria - Day 7: Conjunctive tissue inflammation was observed near the implant. Fibroblast rare was present around few collagen fibers that performed complex and disorganized disposal. Dense blood-vessel networks were observed for all areas (Figure 1).

Saline + Bacteria - Day 30: This group presented major tissue organization relative to that of the 7-day period. An agglomerate macrophage surface was in contact with the implant area; some lymphocytes were present and other few inflammatory cells was observed. The lower caliber vessel around all area was verified capsule distant (Figure 1).

DISCUSSION

Bacteria are the primary cause of the development of necrotic pulps, periapical lesions, and post-treatment disease following root-canal treatment (Guneser et al., 2016). Hence, the eradication of microorganisms and their by-products from the root-canal system is needed for the success of the endodontic treatment (Guneser et al., 2016). Although mechanical instrumentation is one of the most important steps for controlling root-canal infection, it cannot achieve total elimination of bacteria when used alone. The complexity of root canals, which is attributable to the many cul-de-sacs, fins, and lateral canals, means that almost half of root-canal walls are left unprepared with only instrumentation. Therefore, the use of antimicrobial irrigation solutions has been advised as an adjunct to mechanical instrumentation (Guneser et al., 2016). Microorganisms with different characteristics (structural, metabolic, and pathogenic) reaching the periapical region stimulate the inflammatory and immunologic responses (Guneser et al., 2016). One option studied for bacterial control is the use of plant extracts in popular medicine (Machado and Oliveira, 2014). As presented in previous studies *in vitro*, these extracts have antibacterial potential (Machado and Oliveira, 2014).

Recent studies using Brazilian Cerrado plant extracts provide evidence of antimicrobial activities, showing promising results for buccal microbial control (Sartoratto, 2004; Gaetti-Jardim Jr et al., 2010; Lima, 2006; Machado and Oliveira, 2014; Machado et al., 2016). Araçá (*Psidium cattleianum*) is a plant that has been studied and presented promising results. Araçá, which belongs to the *Myrtaceae* family, is also known as araçá-do-campo and araçá-comum. Despite the limited number of studies, it has proven antimicrobial action (Gaetti-Jardim Jr., 2010; Alvarenga et al., 2016) and possible anti-inflammatory potential, besides having proven biocompatibility through in vivo study (De Menezes, 2010).

The araçá leaves used for extracts in this study were the same as those used in previous studies that found evidence of antimicrobial action against buccal bacteria (Alvarenga et al., 2016; Gaetti-Jardim Jr et al., 2010) and biocompatibility (De Menezes et al., 2010), thereby avoiding any interference of biologic activity.

The microbiota used in the study was selected using isolated microorganism of the acute and/or refractory endodontic infection (Gomes Filho, 2008). Polyethylene tubes containing live microorganisms were implanted in rats' conjunctive tissue to determine tissue response (Gomes Filho, 2008).

The implantation of polyethylene-tube was performed following the literature, which showed material biocompatibility (Jeansonne, 1994; Holand, 2002; Yaltirik, 2004; Olsson, 1981; Gomes Filho, 2008; Torneck, 1967).

Compared with the results, the edema presented similarities in both time periods, independent of study group. Yet, for the 6-hour period, a slight increase in its quantity, though not significant statistically, was observed. We concluded that the inactive-microorganism presence did not interfere with the initial inflammatory response standard.

However, because it is inactive microorganism subproducts and products, it is not known how much time elapsed before inflammatory response was elicited.

About the edemogenic tests results, though not statistically significant, it was found that saline presented better values, cause edema smaller, follow by aqueous and hydrohalcoholic that presented higher initial edema. It was found that the inactive microorganism present did not drastically change the proportion of edema relative to pure materials, which were similar to results obtained by Machado (2012), but without inactive bacteria. The hydrohalcoholic general result was high due the observed edema in the 3-hour period, which we believe was intensified due to the ethanol presence. Both saline and aqueous groups presented a light increase of edema during the time, while

the hydrohalcoholic group presented a decrease. This fact can likely be explained, probably, due the ethanol annoying effect in the initial period, and its metabolism in organism in the 6-hours period. Therefore, in later research, can try to eliminate the ethanol before the experiment, once the same is a good extractor agent, but it could have masked or interfered in the effect of the extract active components.

According with to the obtained results, the later tissue response was observed, in all the available groups. This way, considering the intensity, noted a tissue response in the initial period (7-day) severe like the results the obtained by Machado (2012).

In the 7-day period, severe inflammatory response was observed, due, probably, to the inactive microorganism presence and its possible actions against the host. This finding supports the research of Machado (2012), whose results emphasized the presence of inflammation showed similar results on the 7-day period (Machado, 2012) but without the inactive microorganism presence.

In the 30-day period, there was an inflammatory process control in all available groups, with results very similar for each group. Thus, for the 3 groups in this period, inflammation level was reduced compared to initial period, and there was evidence of the repair process.

Machado (2012) performed a study with the aroeira (*Myracrodruon urundeuva* Allemão) plant aqueous extract without inactive bacteria, been more biocompatible than the hydrohalcoholic extract. Therefore, the hydrohalcoholic extract influence on lesion healing maintained the inflammation until advanced periods.

According to the results, the araçá extract (*Psidium cattleianum*) deserves more attention for future research, for this plant is unique practically in studies in odontology on account of its vast antimicrobial potential and demonstrated biological compatibility. However, need to be better available to obtain active compounds that can become part of future drugs for use in dentistry.

CONCLUSION

Per the results, extracts of araçá (*Psidium cattleianum*) have no effect on the bacterial components. However, the extracts do not interfere in the process of subcutaneous tissue repair, showing good biocompatibility.

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