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The long-term administration of calcineurin inhibitors decreases antioxidant enzyme activity in the rat parotid and submandibular salivary glands

Luís C. Spolidorio [a,*], Bruno S. Herrera [a,b], Leila S. Coimbra [a], Cleverton R. de Andrade [a], Denise M.P. Spolidorio [a], Carlos Rossa Junior [a], Marcelo N. Muscará [b]

[a] Department of Physiology and Pathology, Dental School of Araraquara, State University of São Paulo (UNESP), Araraquara, SP, Brazil
[b] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, SP, Brazil

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ABSTRACT

Aims: Calcineurin inhibitors are widely used for prevention of graft rejection and treatment of autoimmune disorders, which result in increased longevity and enhanced quality of life for patients. Unfortunately, the toxic side effects of these drugs (mainly renal, hepatic and cardiac) limit their use. In this work, we studied the effects of long-term treatment of rats with the immunosuppressant cyclosporin (CsA) or tacrolimus (Tac) on salivation, saliva composition and on the major salivary glands (parotid and submandibular) in terms of histological alterations and oxidative stress, evaluated as lipoperoxidation (thiobarbituric acid reactive species — TBARS) and antioxidant enzyme activity contents (superoxide dismutase — SOD, catalase — CAT and glutathione peroxidase — GPx).
Main methods: Male adult rats were treated with either CsA (10 mg/kg/day) or Tac (1 mg/kg/day) subcutaneously for 30 or 60 days. At the end of the experimental periods, pilocarpine-stimulated salivary flow rate was measured, saliva samples were collected and the salivary glands were dissected for morphological and biochemical analyses.
Key findings: After a 60-day treatment with any of the immunosuppressants, the total protein, Ca^{2+} and Na^{+} saliva concentrations were decreased but salivary flow rates were unaffected. In addition, both parotid and submandibular glands showed decreased SOD, CAT and GPx activities, increased TBARS contents and histomorphological alterations involving the epithelium and acini.
Significance: Based on these results, we suggest that the systemic long-term administration of the calcineurin inhibitor CsA or Tac induces an impairment of the antioxidant enzymatic defense in the rat major salivary glands, which may, in turn, lead to altered saliva composition.

1. Introduction

In addition to prevention of graft rejection, calcineurin inhibitors such as cyclosporin (CsA) and tacrolimus (Tac) are commonly used for treatment of autoimmune disorders, resulting in increased longevity and enhanced quality of life of the patients [52]. CsA, a lipophilic 11-amino acid polypeptide isolated from the fungus *Tolypocladium inflatum*, has been used in clinical practice since the early 1980s. CsA exerts its immunosuppressive action by interfering with the activation and proliferation of the CD8+, CD4+, CD25+ and Th17 cells through calcineurin inhibition and the consequent blockade of nuclear factor of activated T cell (NF-AT) translocation to the nucleus [22,58]; as a consequence, several genes required for the proliferation of B-cells (such as interleukin [IL]-4 and CD40 ligand) or T-cells (such as IL-2), fail to be activated [25]. Tacrolimus (Tac; also known as FK-506 or Fujimycin), a macrolide immunosuppressant, is approximately 100 times more potent than CsA and acts by mechanisms similar to the latter, thus representing an effective alternative to CsA for primary and rescue therapies [50].

Unfortunately, the toxic side effects of these drugs (mainly renal but also affecting the liver and heart) limit their use, and oxidative stress is among the many proposed mechanisms [20,54]. In fact, an imbalance in favor of excessive production of reactive oxygen species (ROS) compared to their removal has been widely supported to play an important role in calcineurin inhibitor toxicity [9]. Little is known about oral lesions caused by these drugs, with the exception of gingival overgrowth and alveolar bone loss [31,45]. Indeed, gingival overgrowth is a well-documented side effect of CsA therapy, while Tac-induced gingival overgrowth appears to occur just after longer treatment periods [31,47]. A recently published study demonstrated that CsA significantly upregulated the intracellular ROS generation and transglutaminase (TGM^{-2}) expression in human gingival fibroblasts [24]. Only a few studies have shown that these drugs can increase the risk of the development of oral infections (such as candidiasis and herpes simplex

* Corresponding author at: Department of Physiology and Pathology, School of Dentistry at Araraquara, State University of São Paulo (UNESP), Rua Humaitá 1380, Araraquara, SP, Brazil.
E-mail address: lcs@foar.unesp.br (L.C. Spolidorio).

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infections), hairy leukoplakia [6,47] or lip cancer [21,47]. In addition, recent studies have shown that immunosuppressant therapy can induce xerostomia or hyposalivation [10,13,27,37]. It is thus worth considering that patients with hyposalivation have higher risk of infections, carious lesions and taste alterations which, in addition to an inadequate preparation for food digestion, leads to a significant decrease in the quality of life [34]. On the other hand, topical application of immunosuppressants (including CsA and Tac) at low doses has been used for the symptomatic treatment of eye dryness associated with primary Sjögren's syndrome, one of the most common autoimmune rheumatic diseases, clinically characterized by xerostomia and keratoconjunctivitis sicca [36].

Based on the considerations above, we decided to study the effects of the calcineurin inhibitors CsA and Tac on rat salivation and saliva constituents, as well as on parotid and submandibular glands, in terms of histopathological changes, lipoperoxidation and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

2. Materials and methods

2.1. Animals and treatments

All the protocols described in this work were approved by the local Ethics Committee of the School of Dentistry of Araraquara (protocol No. 26/2004). Ninety male Holtzman rats (155–200 g) were randomly distributed into three experimental groups of 30 rats/group. The rats were housed in polypropylene cages in groups of five animals per cage, at a controlled room temperature (23 ± 2 °C), humidity (55 ± 10%), and a 12/12 h light/dark cycle (beginning at 7:00 am), and received standard chow and tap water ad libitum.

The rats received daily subcutaneous injections of either cyclosporin (CsA, 10 mg/kg; Sandimmun, Novartis, São Paulo, Brazil), tacrolimus (Tac, 1 mg/kg; Prograf, Janssen-Cilag, São Paulo, Brazil) or vehicle (0.9% NaCl solution; control group), as previously described [19,43], and body weight was recorded twice weekly. According to Wassef et al. [55], 10 mg/kg of CsA provides plasma peak and therapeutic concentrations of 1000 and 750 ng/mL, respectively. Previous studies performed by our group show that this CsA protocol induces gingival overgrowth [44], alveolar bone loss [42] and increases salivary cytokine contents in rats [46]. Regarding Tac, the 1 mg/kg dose results in peak plasma concentrations of approximately 11.2 ng/mL in rats [18,53]. This protocol has been sufficient to achieve therapeutic Tac serum levels. It is clinically relevant within the range of doses used in studies on organ and limb transplantation as well as bone metabolism (between 0.6 and 1.0 mg/kg) in rats [53].

Thirty days after the beginning of the treatments, salivation rate was measured in 15 rats from each group (as described below), while the remaining 15 animals from each group were kept on the respective treatments during the following 30 days, when the salivation rate measurements were similarly performed.

2.1.1. Determination of salivation rate

At the end of each treatment period (30 or 60 days), and 3 h after the last dosing, all the animals from each group were anesthetized by intramuscular administration of ketamine (80 mg/kg; Francotar, Virbac do Brasil Ind. e Com. Ltda, Brazil) and xylazine (16 mg/kg; Kensol, Konig S.A., Brazil); additional doses of the anesthetics were administered 30 min later in order to keep the animals under complete anesthesia until the end of the experiments.

The animals were kept in the supine position, and were tracheotomized in order to facilitate respiration during the experiments. With the aid of a dissecting microscope, a 0.2 mm diameter polyethylene cannula was inserted into the Wharton duct in order to collect pure submandibular saliva [48]; parotid gland saliva was collected with a micropipette directly from the Stensen duct [59].

Pilocarpine hydrochloride (4 μmol/kg; i.p.) was then administered as the secretory stimulus and, after discarding the first two saliva drops, saliva was collected during the next 15 min into pre-weighed stoppered microtubes; at the end of this period, the tubes were weighed again and the amount of saliva secreted by each gland was recorded (in mg). At the end of these procedures, both the right and left parotid and submandibular glands were dissected free of connective tissues and had their weights recorded. Salivary flow rates were calculated by dividing the collected saliva weight value by the collection time (15 min) and normalized by the gland weights (in g), as previously reported [59].

The collected saliva samples were kept at −20 °C until analyzed for their protein and electrolyte contents. Total protein concentration was measured according to Lowry et al. [28] using bovine serum albumin as the calibration curve standard, and Na^+ and Ca^{2+} concentrations were determined by atomic absorption spectroscopy using a Shimadzu 680-AA spectrophotometer (Shimadzu, Kyoto, Japan), as previously described [60].

After these procedures, the animals were euthanized by anesthetic overdosing and had their parotid and submandibular glands removed for histological and biochemical analyses, as described below.

2.2. Histological analysis

The glands obtained from 5 rats of each experimental group were soaked in 10% formalin for 48 h and embedded in paraffin for both hematoxylin and eosin (H&E) staining and immunohistochemistry.

2.3. Immunohistochemistry

Immunostaining of Ki-67, a cellular marker for proliferation, was performed using the streptavidin–biotin peroxidase complex method. Briefly, after deparaffinization and hydration of the sections with successively decreasing concentrations of ethanol solutions, antigen was retrieved by using a 10 mM citrate buffer (pH 6.0) in a microwave oven. After washing with phosphate buffered saline (PBS), the sections were treated with 1% bovine serum albumin (BSA) for 1 h to block nonspecific binding of the primary antibody, and then incubated with the monoclonal mouse anti-Ki-67 antibody (clone MIB-5, 1:100 dilution; Dako; Denmark) followed by the ABC method (Vectastain ABC kit for mouse IgG; Vector®, Burlingame, CA, USA). Immunoreactivity was developed by incubating the sections with 0.6 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Co., St. Louis, MO, USA) containing 0.01% H_2O_2. Control reactions were performed by omitting the primary antibody. The percentage of nuclear-positive cells was calculated manually.

We selected 5 random semi-serial sections for counting of Ki-67 positive cells in each sample. These sections were equally spaced and spanned 525 μm of the whole specimen. Positive cells expressing Ki-67 were identified by a brown precipitate in the nucleus except in mitotic cells, where the chromosomes and the cytoplasm are labeled. Approximately 800 cells from the cell population were counted by one observer at a magnification of 400× (Olympus, Japan) and the percentages of Ki-67 positive cells were calculated. The level of Ki-67 expression was evaluated according to the scoring system of [40] The application of this system gives a score ranging from 0 to 3 for both degrees of positivity: percentage of positively stained cells [(absent: <1%), (mild: 1–10%), (moderate: 10–50%), (strong: >50%)].

2.4. Analysis of thiobarbituric acid reactive species (TBARS) contents and SOD, CAT and GPx activities

The dissected submandibular and parotid glands from the remaining 10 rats from each group were freshly divided into two for measurement of lipid peroxidation and antioxidant enzyme activities.