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Functional and proteomic analysis of submandibular saliva in rats exposed to chronic stress by immobilization or constant light

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ABSTRACT

Objective: In this study, we have evaluated the effects of stress on functional and proteomic changes in submandibular saliva of rats.

Design: Male adult rats were divided in three groups: IMO (2 h/day of immobilization for 7 days), LL (constant light during 20 days), C (unstressed controls submitted to 14 h light–10 h dark cycle). Body weight, food intake and the dry weight of submandibular gland were recorded. Saliva samples, collected under anaesthesia following *i.p.* administration of isoproterenol and pilocarpine (5 mg/kg), were assayed for total proteins (TP), amylase activity and SDS-PAGE electrophoresis.

Results: Body weight, food intake and the dry weight of submandibular gland of IMO rats were lower than those of C and LL groups. The salivary volumes secreted in IMO and LL rats, were significantly higher than in controls. The TP output (μg protein/μg saliva/mg of dry tissue) and amylase activity output (AU/μg of saliva/mg of dry tissue) in IMO were significantly higher than in C and LL animals. The electrophoretic pattern of saliva proteins of LL rats, revealed the absence of a protein band of approximately 25 kDa. This band was composed by the common salivary protein-1 and a prolactin-induced protein as identified by peptide mass fingerprinting.

Conclusions: Differences in body weight and food intake between IMO and LL might be attributed to the sort and intensity of stressors stimuli. The changes in the volume of secreted saliva could be a compensatory mechanism in response to stressors. The increase of total protein in IMO rats and the absence of 25 kDa proteins in LL, would suggest that the submandibular glands respond to the sympathetic nervous system stimuli induced by the stress with an increase of activity of the sympathetic nerves in IMO and a reduction in LL rats.

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1. Introduction

Saliva plays an important role in the protection of the oral cavity and in the maintenance of the systemic health.[1] As other physiological processes, salivary secretion is not constant and the volume and composition changes in response to the requests of the organism. During chewing, the stimulated saliva, mainly from parotid gland, facilitates taste, bolus formation and swallowing. Between meals, non-stimulated saliva, mainly from submandibular glands, cleans, lubricates, and provides substances that contribute to repair and to maintain the integrity of oral tissues.[2]

Previous works suggested that changes in the volume and/or the flow rate of the saliva output, as well as its composition, affect microorganism proliferation and impact upon caries formation and periodontal diseases.[3–6]

The nervous control of salivary secretion is carried out by both branches of the autonomic nervous system working synergically, with the release of neurotransmitters and co-transmitters.[7] The intraperitoneal or intravenous administration of pilocarpine (cholinergic agonist) produces an increase of saliva output rich in water and electrolytes, whereas isoproterenol (β-adrenergic agonist) reduces the release of a saliva but increases markedly its protein content.[8]

The sympathoadrenal system (SAS) (sympathetic nerves and adrenal medulla) plays an important role in the adaptation of the organism to stress causing an immediate response to different metabolic, physiologic and physical challenges. Chronic stress situations such as immobilization (IMO) can produce an activation of both components of the SAS, with an increase in plasmatic adrenaline and noradrenaline.[9–12] In a previous work we found, by analyzing catecholamines synthesizing enzymes mRNA levels, that chronic stress by constant light (LL) produces an activation of the adrenal component but a decrease in the activity of the sympathetic nerves efferent to salivary glands.[13]

The purpose of this work was to study possible changes in output and some aspects of the protein composition in submandibular saliva of rats exposed to different environmental conditions that produce differential activation of SAS.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (300–350 g) were maintained in a temperature controlled room (24 ± 2.0 °C) and provided with food and water ad libitum, until 18 h before the experiments when food but not water was withdrawn. Animal care was provided according to ''The Guide to the Care and Use of Experimental Animals'' (DHEW Publication, NHI 80–23).

2.2. Experimental groups

Control (C) group (photoperiod exposed group, $n = 12$): animals were kept to 14 h light/10 h dark cycle and not exposed to stress. The onset of the daily light phase (06:00 h) is defined as zeitgeber time 0 (ZT0). During the light period, light intensity was 150–200 lx at the level of the cages. In the other groups, rats were either continuously exposed to 150 lx for 20 days, LL group (constant light, $n = 12$) or submitted to immobilization stress (IMO group, $n = 12$). Immobilization stress was accomplished by taping the forelimbs and hind limbs of the rat in a prone position with surgical tape to metal mounts attached to a board for 2 h daily for 7 successive days as previously described [14,15] and were kept in the same photoperiod conditions of C group. The secretory response was tested 24 h after the last immobilization. The protocol and procedures used in this manuscript have been approved by the Ethics Committee of the School of Medicine, Universidad Nacional de Córdoba, Argentina.

2.3. Measurement of body weight and food intake

In all groups daily body weights and food intakes were recorded at 7:00 am. Food intake was measured to the nearest 0.1 g by weighing the food basket and the amount of spillage.

2.4. Secretory responses

In all groups the submandibular secretory response was analysed during the light phase (ZT2–ZT5) of the diurnal cycle. Rats were anaesthetized with chloral hydrate, (300 mg/kg body weight, *i.p.*). A tracheotomy allowed free pulmonary ventilation. Body temperature was measured with a rectal thermometer and maintained at 37.5 °C. Secretory ducts from both submandibular glands were exposed and cannulated with fine glass tubes that gave about 45 drops/ml of distilled water.[16,17] Since in salivary glands the nervous control of saliva secretion is carried out by both branches of the autonomic nervous system working synergically; to stimulate salivary output, pilocarpine was given and to induce discharge of stored proteins, isoproterenol was chosen. Secretory responses were obtained by injecting (*i.p.*) jointly isoproterenol and pilocarpine (5 mg/kg body weight of each one dissolved in isotonic saline), these dosages were comparable with those used by other authors in their studies of saliva secretion in rats[18–20]; the volumes injected ranged from 0.8 to 1.0 ml depending on the animal's body weight. Since the doses of agonists used and factors such as anaesthesia and surgical stress could alter the secretory response, the same experimental scheme applies to all groups studied. Saliva was collected for 20 min (from the moment the first drop appears at the tip of the cannula) into plastic tubes pre-weighed and kept on ice, the volumes of saliva secreted were determined assuming a specific gravity of 1.0 g/ml. The secretory response was expressed as μg of saliva per mg of dry submandibular gland tissue (μg saliva/mg of dry tissue). Saliva was stored at −20 °C until analysed.

2.5. Glandular dry weight

After 20 min of stimulation the submandibular glands were dissected, dried at 110 °C for 72 h and weighed.

2.6. Total proteins analysis

The amount of total protein in saliva was measured by Lowry's method and expressed as μg/μl.[21] Also the total protein output was calculated and expressed as μg of protein/μg of saliva per mg of dry tissue.

2.7. Amylase activity

Salivary α-amylase activity was assayed using Amilokit® (Wiener Lab Group, Rosario, Argentina) utilizing starch as substrate. The saliva sample was diluted (1:2) with distilled water before addition to the substrate for the assay. The enzyme activity as total amylase output was expressed as amilolytic units (AU/μg of saliva per mg of dry tissue).

2.8. Gel electrophoresis and mass espectrometry

SDS-PAGE was performed as described by Laemmli [22] using 12% acrylamide gels (Bio-Rad Laboratories, California, USA). The saliva samples containing 10 μg of total protein were denaturized and loaded into the gels. The electrophoresis was carried out at constant current amperage of 7.5 mA in the stacking gel and at 15 mA in the resolving gel. The gels were then stained with Coomasie Brilliant Blue R-250.

A protein band of 25 kDa present in samples of the control group was excised from the gel manually using a scalpel, and then transferred to a siliconized tube. Protein identification was made by peptide mass fingerprinting, using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in the Southern Alberta Mass Spectrometry (SAMS) Centre for Proteomics, University of Calgary, Calgary, Alberta.

2.9. Drugs

Chloral hydrate, isoproterenol and pilocarpine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.10. Statistical analysis

All numerical data are given as means ± SE. The data were compared by using one-way ANOVA followed by Tukey test using Graph Pad Prism 5.0. Statistical significance was set at $p < 0.05$. MALDI-TOF mass spectrometry data were analysed using standard procedures where: (1) the S = score, which is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ion scores >48 indicate identity or extensive homology; and (2) E = expectation values, which is directly equivalent to the E-value used in Blast searches, where a score of <0.05 is considered significant.

3. Results

3.1. Body weight and food intake

Results from measurements of body weight are shown in Fig. 1, 2 h of daily immobilization during 7 days reduced body weight, by 5–10%. These values were statistically significant since day 3. No significant changes were found in rats under LL (data not shown). Food intake in IMO group at the end of experiment is reduced by about 30%. These values were statistically significant since day 2 (Fig. 2). No significant changes were found in rats under LL (data not shown).

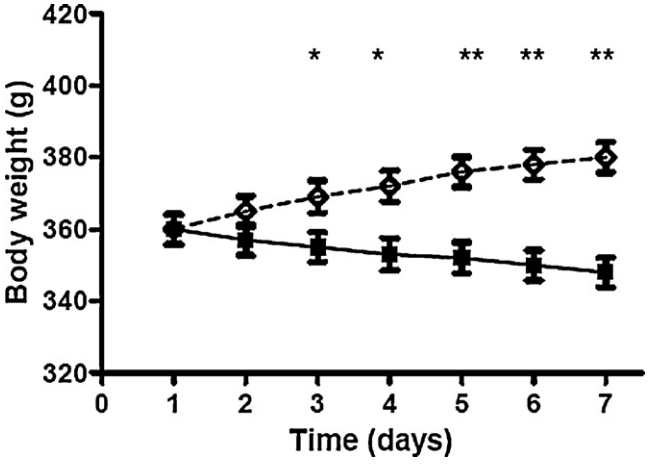

Fig. 1 – Effect of IMO on body weight (g) of rats. The values were expressed as the mean ± SE. $n = 12$ ($^{*}p < 0.05$, $^{**}p < 0.01$). (■) IMO; (◈) control.

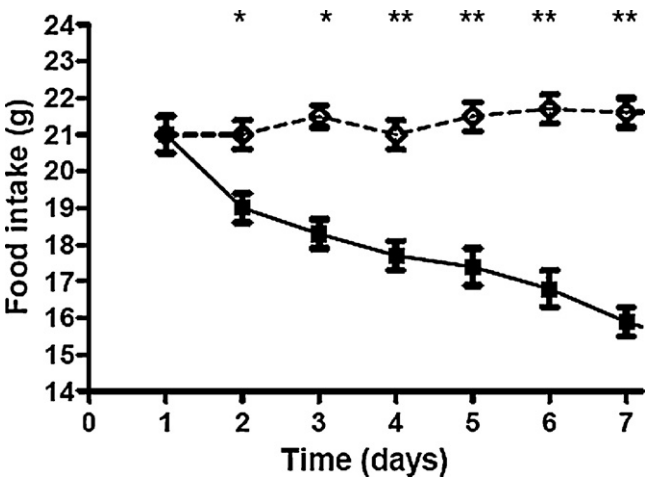

Fig. 2 – Effect of IMO on food intake (g) of rats. The values were expressed as the mean ± SE. $n = 12$ ($^{*}p < 0.05$, $^{**}p < 0.01$). (■) IMO; (◈) control.

3.2. Glandular dry weight

The dry weight of submandibular glands of IMO group were significantly lower ($p < 0.05$) than C and LL groups (IMO: 39.76 mg ± 1.72 ($n = 12$); C: 46.82 mg ± 2.02 ($n = 12$) and LL: 52.2 mg ± 1.85 ($n = 12$)).

3.3. Secretory responses

The secretion of saliva accumulated during 20 min in both chronic stress experimental groups increased significantly compared with controls (Fig. 3).

3.4. Total protein analysis

Total protein concentration did not show significant differences among the groups, C = 35.61 μg/μl ± 1.7, IMO = 34.2 μg/μl ± 1.8 and LL = 30.7 μg/μl ± 1.43. However, since significant

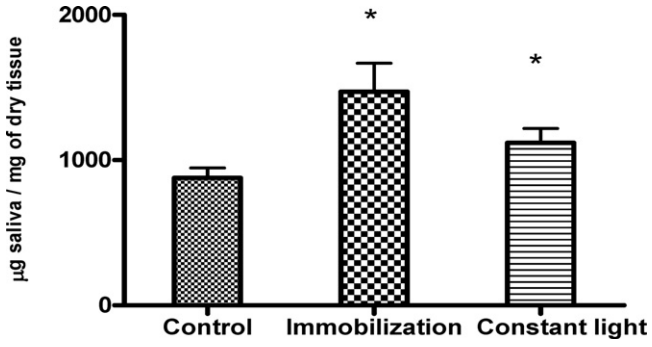

Fig. 3 – Saliva secreted during 20 min corrected by mg of dry tissue. The values were expressed as mean ± SE, $n = 12$ for each experimental groups ($^{*}p < 0.05$).

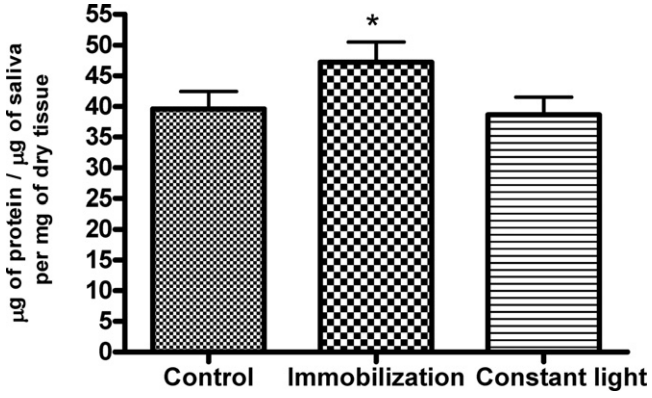

Fig. 4 – Effect of LL or IMO in the total protein output of rats' submandibular saliva (µg of protein/µg of saliva per mg of dry tissue). The values were expressed as the mean ± SE. $n = 12$ ($^{*}p < 0.05$).

changes in the volume of saliva secreted were seen, the amount of total protein was calculated to express "total protein output". Fig. 4 shows a significant increase in total protein output in IMO group compared to control and LL groups.

3.5. *Amylase activity*

Fig. 5 shows output of α-amylase activity (AU/µg of saliva/mg of dry tissue) in saliva samples of C, IMO, and LL. An increase

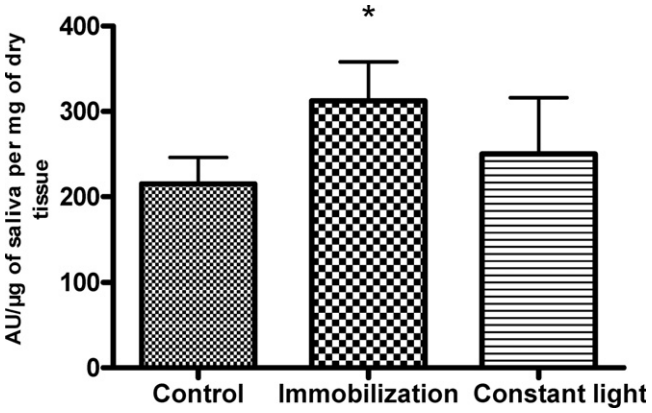

Fig. 5 – Effect of LL or IMO in the α-amylase activity output of rats' submandibular saliva (AU/µg of saliva per mg of dry tissue). The values were expressed as mean ± SE. $n = 12$ ($^{*}p < 0.05$).

was observed in IMO (312.6 ± 45.7) compared to C (215.2 ± 30.8) and LL (250 ± 66.1) ($p < 0.05$).

3.6. *Gel electrophoresis and mass spectrometry*

The most striking change in the SDS-PAGE electrophoretic mapping was the absence of the band of the 25 kDa protein in the LL group (Fig. 6). This band, present in the saliva of Control and IMO groups, was analysed by MALDI-TOF and two proteins were identified: common salivary protein-1 and prolactin-induced protein (PIP) (Table 1).

The band corresponding to α-amylase (45–50 kDa) was in very low concentrations in the three groups. No increase in saliva from IMO rats was detected (Fig. 6).

4. Discussion

In this report two chronic stressors, immobilization and exposure to constant light, were found to have differential effect on body weight, food intake, dry weight of submandibular glands, stimulated salivary secretion and the profile of secreted proteins.

Exposing rats to immobilization causes a 5–10% body weight loss, about 30% reduction in food intake and a

Table 1 – Peptide mass fingerprinting, using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of the 25 KD band from SDS-PAGE runs of saliva samples from control (C), immobilized (IMO) and constant light (LL) exposed rats.

Common salivary protein 1 [*Rattus norvegicus*]			
Sequence	Control	Immobilized	Constant light
K.FGNNWSQEYGSSGR.A	S = 45; E = 0.013	S = 55; E = 0.0016	Not detected
Prolactin induced protein [*Rattus norvegicus*]			
Sequence	Control	Immobilized	Constant light
K.AYLISNTPVDGGFNYIQTR.C	S = 99; E = 3.9e–7	S = 88; E = 4.9e–7	Not detected

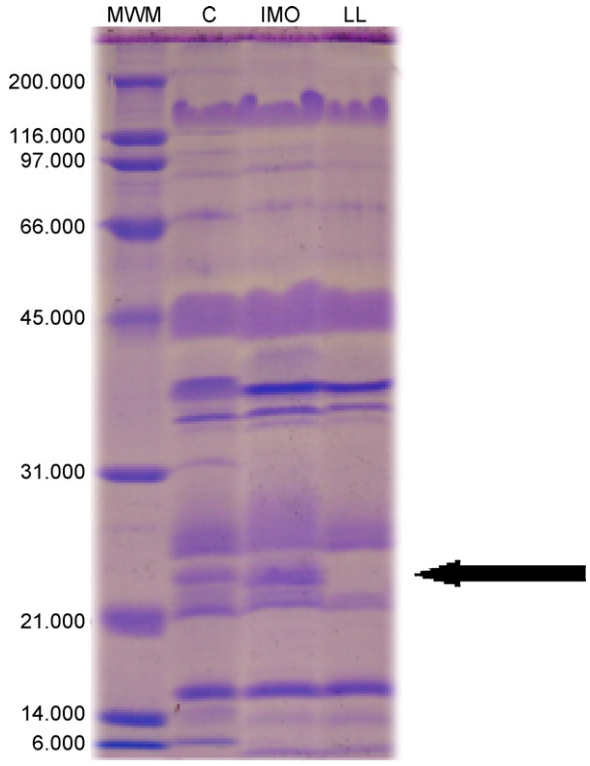

Fig. 6 – SDS-PAGE electrophoresis for submandibular saliva samples of C, IMO and LL rats. Samples equivalents to 10 μg protein were loaded in each lane. The molecular weight marker (MWM) has a range between 200,000 and 6000 kDa. The electrophoretic pattern shown is representative of 12 runs for each of the experimental groups. The arrow indicates the absence of the 25 kDa band in LL.

significant reduction in the dry weight of submandibular glands compared to constant light exposure or control rats. The effects of stress on body weight are determined both by the severity of the stress and by the individual's perception of the stress. In animals, mild stressors, such as tail pinch, increase food intake,[23] whereas more severe stressors, such as restraint or immobilization, inhibit food intake [24] and have long-term effects on body weight and behaviour.[25,26] In humans, extreme stress, such as combat, inhibits food intake,[27] but the chronic effects on food intake and body composition have not been determined. Elucidation of the mechanisms that cause this disruption of homeostasis would provide new information on the regulation of body composition and body weight.

The volume of saliva released by the submandibular glands was significantly increased by the combined stimulation of isoproterenol and pilocarpine in rats submitted to immobilization stress and constant light. This agrees with the results, of Fejerdy and coworkers,[28] who observed an increase in total saliva flow in patients under mechanical and heat stress. Previous studies in our laboratory demonstrated that both experimental conditions modify the sympathetic control of submandibular saliva secretion.[29–31] Several authors propose that sympathoadrenal system activation by either external or internal stimuli is accompanied by changes of the parasympathetic nervous system (PNS) activity in several organs.[32–35] It is also possible that the PNS participates regulating the output of saliva from the submandibular glands. To our knowledge, there are no studies addressing this issue in salivary glands.

Since saliva and its proteinaceous components are important for the maintenance of the systemic and oral homeostasis,[5,36] total protein output was measured in submandibular saliva samples. We found an increase in total protein output in IMO rats. High-intensity intermittent exercise in humans also produces an increase of the concentration of total protein in total saliva.[37]

A good correlation exists between the α-amylase concentration in total saliva and catecholamines in blood.[38] Alpha-amylase appears a good stress index since its levels increase faster than that of cortisol to physiological stressors.[39] The activity of α-amylase in total saliva increases in humans under hard exercise.[37] Our results indicate that the enzyme also increases its activity in submandibular saliva of rats exposed to immobilization. The observed differences in the response to IMO and LL exposure could be explained by the fact that the sympathoadrenal system is activated in different ways depending on the sort and intensity of the applied stimulus.[10,12]

In vitro studies [40] indicated that amylase released by the submandibular glands is constitutive, non-regulated. The increase observed by us after IMO stress would suggest some kind of regulation by the SAS. However, the increase of α-amylase activity produced by immobilization may not be a good index to assess the effect of stressors since the amount of the enzyme in saliva from those glands represents only a minor fraction of the total present in mixed saliva. This is confirmed by the electrophoretic patterns which did not show any change in the region where amylase should migrate. It is possible that the changes of the enzyme in so low concentrations are functionally non significant.

The most important change in electrophoretic patterns was the absence of a 25 kDa band in the LL saliva, which could be explained by a less intense activity of efferent sympathetic nerves to submandibular salivary glands.[13] The 25 kDa proteins present in saliva samples of control animals and absent in saliva of LL rats, were identified as the common salivary protein-1 (CSP-1) and prolactin-induced protein (PIP) by MALDI-TOF. Although glands from IMO rats did not show marked changes in the electrophoretic pattern of proteins, other studies noted a marked increase in brain-derived neurotrophic factor (BDNF) expression in ducts cells of the submandibular glands after 60 min of immobilization stress.[41]

Prolactin-induced protein is found in tears, saliva, sweat, seminal fluid, plasma, submucosal gland of the lung, and amniotic fluid and is involved in mucosal host defence via high-affinity interaction with CD4 and IgG.[42,43] PIP also binds several oral and non-oral bacterial species,[44] and is down-regulated in nasal fluid from allergic individuals.[45] The decrease of PIP in saliva may contribute to compromised formation of dental enamel,[44,46] and reduced innate immune phagocytic function [47] and respiratory burst activities [48] of neutrophils observed with constant light. Although CSP-1 is found in all salivary glands,[49] its functions remain largely unknown, but it may display antimicrobial, and lubricating/coating activity in the oral cavity, trachea, and prostate,[50]

although recombinant human CSP-1 was recently found to enhance bacterial adherence to experimental salivary pellicles.[51] Common salivary protein-1 is expressed in stressed tissues, for example, pancreatic cancer,[52] and skin compression,[53] and the loss of this protein in saliva of LL rats is further suggestive that saliva proteins are involved in responses to systemic stress. Apparently photoendocrine mechanisms also participate in the role that submandibular glands play in regulating systemic responses to stressful and inflammatory stimuli, such as allergy, injury, trauma and infections.[54,55]

5. Conclusions

The increase of total proteins in IMO saliva samples could be due to an enhancement in the activity of the sympathetic nerves efferent to the submandibular glands. The absence of the protein band of 25 kDa in saliva samples of LL rats observed in SDS-PAGE gels may occur as a result of a lesser activity of the sympathetic nerves in that stressing condition. The changes in the secreted saliva by submandibular gland of rats under different stressors could result in modified oral and systemic health. Further studies are required to determine the role of constant light on synthesis and secretion of common salivary protein-1 and prolactin-induced protein.

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Competing interest

None declared.

Ethical approval

The protocol and procedures used in this manuscript have been approved by the Ethics Committee of the School of Medicine, Universidad Nacional de Cordoba, Ref.#: AOJ039.

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