Journal of Oral Pathology & Medicine

Action of Anti-M3 muscarinic acetylcholine receptor IgG of primary Sjögren`s syndrome on the enzymatic antioxidant system in rat submandibular gland

Journal:	*Journal of Oral Pathology and Medicine*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Reina, Silvia; School of Dentistry, University of Buenos Aires, Pharmacology Unit Rodriguez, Marcelo; School of Dentistry, University of Buenos Aires, Pharmacology Unit Stranieri, Graciela; School of Dentistry, University of Buenos Aires, Pharmacology Unit borda, enri; universidad de buenos aires, facultad de odontologia
Methods:	Signal transduction
Keywords:	Salivary gland, Xerostomia, Oral mucosa
Abstract:	BACKGROUND: We demonstrate that serum immonuglobulin G (IgG) directed against glandular M3 muscarinic acethylcholine receptors (M3 mAChR) and pilocarpine triggers the increment of superooxidase dismutase (SOD) and catalase (CAT) and the production of nitric oxide (NO) and prostaglandin E2 (PGE2). METHODS: Enzyme-linked immunoabsorbent assay (ELISA) was performed in the presence of the human M3 mAChR synthetic peptide as antigen to detect in serum of pSS patients the autoantibodies. Further, SOD and CAT specific activity and NO were determined chemically in the presence of anti-M3 mAChR IgG and pilocarpine. The level of PGE2 generation in the presence of autoantibody and pilocarpine was determined by ELISA. RESULTS: An association between anti-M3 mAChR autoantibodies and pilocarpine given the increment of the specific activity of SOD and CAT in the serum of pSS patients and in the rat submandibular gland was observed. As a result of this action, M3 synthetic peptide and atropine abrogated the stimulatory action. The L-type calcium channel, calcium/calmodulin complex and COX-2 inhibitors selectively blocked the increment of the specific activity of SOD and CAT in the rat submandibular gland. An increased production of NO and PGE2 by the cholinergic autoantibody and pilocarpine was also been detected. CONCLUSION: On the basis of these results, the increment of the specific activity of SOD and CAT in pSS patients as compared to control healthy individuals may be seen as a defensive reaction to the increment of the amount of ROS in the body, which becoming uncontrollable, leads to

	irreversible cellular and tissue damage.

Action of Anti-M_3 muscarinic acetylcholine receptor IgG of primary Sjögren`s syndrome on the enzymatic antioxidant system in rat submandibular gland

Silvia Reina, Marcelo Rodríguez, Graciela Stranieri, Enri Borda

Pharmacology Unit, School of Dentistry, University of Buenos Aires, Buenos Aires, Argentina and National Research Council of Argentina (CONICET), Buenos Aires, Argentina.

Running Title: cholinergic autoantibodies and antioxidant enzymes

Correspondence to:
Enri Borda, M.D., Ph.D.
Pharmacology Unit, School of Dentistry
University of Buenos Aires.
Marcelo T de Alvear 2142 – 4 “B”
1122 AAH. Ciudad Autónoma de Buenos Aires – Argentina
Phone: +54-11-4964-1276
E-mail: enri@farmaco.odon.uba.ar

Abstracts

BACKGROUND: We demonstrate that serum immonuglobulin G (IgG) directed against glandular M_3 muscarinic acethylcholine receptors (M_3 mAChR) and pilocarpine triggers the increment of superooxidase dismutase (SOD) and catalase (CAT) and the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2).

METHODS: Enzyme-linked immunoabsorbent assay (ELISA) was performed in the presence of the human M_3 mAChR synthetic peptide as antigen to detect in serum of pSS patients the autoantibodies. Further, SOD and CAT specific activity and NO were determined chemically in the presence of anti-M_3 mAChR IgG and pilocarpine. The level of PGE_2 generation in the presence of autoantibody and pilocarpine was determined by ELISA.

RESULTS: An association between anti-M_3 mAChR autoantibodies and pilocarpine given the increment of the specific activity of SOD and CAT in the serum of pSS patients and in the rat submandibular gland was observed. As a result of this action, M_3 synthetic peptide and atropine abrogated the stimulatory action. The L-type calcium channel, calcium/calmodulin complex and COX-2 inhibitors selectively blocked the increment of the specific activity of SOD and CAT in the rat submandibular gland. An increased production of NO and PGE_2 by the cholinergic autoantibody and pilocarpine was also been detected.

CONCLUSION: On the basis of these results, the increment of the specific activity of SOD and CAT in pSS patients as compared to control healthy individuals may be seen as a defensive reaction to the increment of the amount of ROS in the body, which becoming uncontrollable, leads to irreversible cellular and tissue damage.

Keywords: autoantibodies, Sjögren syndrome, anti-M_3 peptide IgG, SOD, CAT, PGE_2, nitrites.

Introduction

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease. It is clinically characterized by dry eyes (keratoconjunctivitis sicca) and mouth (xerostomia) and histologically characterized by lymphocytic infiltration, the destruction of the salivary and lacrimal glands and their respective (1) multi-organ involvement (2).

The pathogenesis of SS is still unclear but our knowledge regarding the involvement of different cells (e.g. B- and T-cells, denditric cells, macrophages) and pathways (e.g. B-cell-activating factor (BAFF), interleukin, interferons, prostaglandins) is continually expanding (3-6). Many autoantibodies, including rheumatoid factor (RF), anti-nuclear antibody (ANA) and antibodies to SSA/Ro or SSB/La, have reportedly been detected in the case of both pSS and aSS (7, 8). Recent studies have proved that the antibody to M_3 subtype muscarinic acetylcholine receptors (M_3 mAChR) is a good serum marker in pSS (9- 11).

The exocrinopathology can be encountered alone (primary SS, pSS) or in association with other autoimmune disorders. The three most common ones are rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis (associated SS, aSS) (7).

Different histological scores have been employed to describe glandular involvement during pSS and aSS. The role of minor salivary glands histology is widely accepted and considered the "gold standard" for pSS diagnosis (8-15).

Under normal conditions, antioxidants are balanced with the formation of reactive oxygen species (ROS) in various tissues and fluids. This occurs at a level at which these compounds can play their physiological role without any toxic effects (16). In the normal ocular glands (17) and in the salivary glands (18) there is also a prooxidant/antioxidant balance at the ocular and oral surface. The danger to the eye and mouth appears when this balance is disturbed. ROS are produced in salivary glands under oxidative stress. The endogenous enzymatic antioxidant system is important to protect the organism against high concentration of ROS that has been

detected in other systemic diseases pathogenetically associated with oxidative stress (19, 20). This system is mainly composed of the enzymes superoxide dismutase (SOD) and catalase (CAT) that cleave ROS.

One aim of our study is to determine the specific activity of these antioxidant enzymes (SOD and CAT) measured in rat submandibular gland in the presence of anti-M_3 muscarinic acethylcholine receptor IgG (anti-M_3 mAChR IgG) from serum of pSS patients. Another aim of our study is to analyse the mAChR agonist pilocarpine. The third aim is to examine whether there is an association between the production of nitric oxide (NO) and prostaglandins (PGE_2) in this system or not.

Material and methods

Ethical approval of the study protocol

The study protocol complied with the tenets of the Declaration of Helsinki and accomplished with the rules established by the Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina). All subjects provided written informed consent.

Drugs

A 25-mer peptide (K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) corresponding to the sequence of the second extracellular loop of the human M_3 mAChR was synthesized by Peptido Genetic Research Company (Livermore, CA, USA) as previously described (10). Pilocarpine, atropine, verapamil and trifluoroperazine (TFP) were obtained from Sigma-Aldrich (St. Louis, MO, USA); 5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl)-thiophene (DuP697), methyl-isothiourea sulphate (methyl-U) and L-N^G-monomethyl arginine citrate (L-NMMA) were from Tocris Cookson (Ellisville, MO, USA). Stock solutions were freshly prepared in the appropriate buffers. The drugs were diluted in a water bath to achieve the final concentrations stated in the text.

Animals

Male Wistar rats weighing 250-300 g from the Pharmacologic Bioterium (School of Dentistry, University of Buenos Aires) were used throughout. The animals housed in standard environmental conditions were fed with a commercial pellet diet and water *ad libitum*. For surgical removal of submandibular glands, the animals were killed with an overdose of an i.p. ketamine/xilazine mixture (100 and 16 mg/kg respectively). The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

Preparation of submandibular gland acini

Submandibular gland acini were prepared from adult female Wistar-strain rats. Animals were used according to “The Guide to the Care and Use of Experimental Animals”

(DHEW Publication, NIH 80-23). Glands were dissected away from fat, connective tissue and lymph nodes and immersed in a tissue chamber containing Krebs-Ringer-bicarbonate (KRB) solution gassed with 5% CO_2 in oxygen and maintained at pH 7.4 and 37°C. All subsequent steps were performed at 4°C. Submandibular glands were minced and incubated in KRB supplemented with 10 mM Hepes and 5.5 mM glucose (KRB-Hepes) and 0.5% bovine serum albumin (BSA), pH 7.4, containing collagenase (150 U/ml). Gland lobules were subjected to gentle pipetting. The preparation was then filtered through nylon mesh (150 µm pore size) and the acini were pelleted with 2 min centrifugation at 50 g. The pellet was then washed twice by centrifugation (50 g for 2 min) through a 4% BSA solution made with KRB-Hepes buffer. The dispersed acini were allowed to recover for 30 min in 5 ml fresh KRB-Hepes buffer containing 0.5% BSA (21).

Patients

The subjects of this study were 25 pSS patients' anti-Ro/SSA positive and 25 healthy volunteers all female, (age 39-54 years) selected from the metropolitan area of Buenos Aires (Table 1). The diagnosis of pSS fulfilled the criteria described by Vitali et al. (12) and was given by means of a positive biopsy with a score focus of 3.8±0.07.

Purification of Human IgG

The serum IgG fraction from patients with pSS and from normal individuals (control) was isolated using protein G affinity chromatography as described elsewhere (22). Briefly, sera were loaded onto the protein G affinity column (Sigma-Aldrich, St Louis, MO, USA) equilibrated with 1 M Tris-HCl (pH 8.0) and the columns were washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine-HCl, pH 3.0, and immediately neutralized. The concentration and purification of IgG were determined using a radial immunodiffusion assay.

Serological Studies

Anti-Ro-SSA procedure: Saline-soluble extractable nuclear antigens (ENA) were obtained from human spleen in phosphate buffered saline (PBS) for anti-Ro. Patient

sera were tested undiluted and diffusion was carried out at room temperature in a humidified chamber for 48 hours. Precipitin lines were identified by comparison with reference sera. ELISAs for total anti-Ro (60kD and 52kD Ro-proteins) was performed with a commercial Kit based on purified antigens (Orgentec Diagnostika, Mainz, Germany) and the assays were carried out according to the manufacturer's protocols on an automated ELISA instrument (Radim, Pomezia RM, Italy). Values greater than 25 UI/ml were considered positive.

Anti-M_3 peptide IgG procedure: The IgG fraction from 25 patients with pSS and 25 healthy subjects was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described by Reina et al. (23). Briefly, the IgG fraction was loaded onto the affinity column equilibrated with PBS. The non-peptide fraction was first eluted with the same buffer. Specific anti-peptide antibodies were then eluted with 3 M KSCN and 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of non-anti-peptide antibodies and specific anti-muscarinic receptor peptide antibodies was determined by a radial immunodiffusion assay. Their immunological reactivity against muscarinic receptor peptides was evaluated by ELISA. The concentration of the affinity-purified anti-M_3 peptide IgG (1×10^{-8} M) increased optical density (mean OD ± SEM, 2.4±0.2). The non-anti-M_3 peptide IgG fraction eluted from the column showed OD values (0.27±0.06) similar to those of normal IgG from healthy individuals taken as control (0.26±0.05). The normal IgG fraction purified by affinity column chromatography gave a negative result (0.30±0.03). ELISA was performed as described previously (24).

ELISA

Fifty microliters of M_3 synthetic peptide solution (20 µg/ml) in 0.1 M Na_2CO_3 buffer, pH 9.6, was used to coat microtiter plates (NUNC, Kastrup, Denmark) at 4°C overnight. After blocking the wells, diluted sera from pSS patients and healthy individuals were added in triplicate and allowed to react with the peptide for 2 hour at 37°C. After the wells were thoroughly washed with 0.05% Tween 20 in a PBS, 100 µl of 1:6000

biotinylated goat anti-human IgG antibodies (Sigma Chemical Co., St. Louis, MO, USA) was added and incubated for 1 hour at 37°C. Then, a 1:6000 dilution of extravidin-alkaline phosphatase (Sigma) was allowed to react an extra 30 min at 37°C. After extensive washings, p-nitrophenylphosphate (1 mg/ml) was added as the substrate, and the reaction was stopped at 30 min. Finally, the plates were read at 405 nm and the results for each sample were expressed as the means ± SD of triplicate values.

PGE_2 procedure

Serum PGE_2 was measured by ELISA, carried out according to the manufacturer's protocols (Biotrack Enzyme Immune Assay System, Amersham Bioscience, Piscateway, NJ, USA). The OD cutoff value of PGE_2 was 4.4±0.33 ng/ml. All serum samples were frozen promptly after collection and kept at -80ºC until used for PGE_2 determination. The result is expressed as ng/ml.

Nitrate and nitrites assay

The 20 µl serum samples were mixed with an equal volume of 100 µl of Griess reagent (Ingredient A: 0.1% naphthalene diamine dihydrochloride at a final concentration of 5 mmol/l; Ingredient B: 1% sulfanilamide at a final concentration of 5 mmol/l in orthophosphoric acid) in a 96 well microtiter plate (NUNC, Roskilde, Denmark). Nitric oxide concentrations were determined by a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co, Ann Arbor, MI, USA) and measured spectrophotometrically at 540 nm using a microplate reader (Reader Model 230S; Organon Teknika, Boxtel, The Netherlands) following the criteria of Green et al. (25). The nitrate and nitrites values are expressed as µM/ml.

Biochemical analysis

For analysis of the activity of SOD the glands were homogenized at 10% (w/v) in PBS pH 7.2, and centrifuged for 10 min at 800 g (4°C). The supernatant was separated, sonicated at 300 W for 30 s and centrifuged at 20,000 g for 10 min (26). The supernatant was then used in analysis. The SOD enzyme activity was determined using the Superoxide Dismutase Assay Kit (Cayman Chemical Co, Ann Arbor, MI,

USA). The reactions were followed using a Beckman DU-68 spectrophotometer (Beckman Instruments, Fullerton, CA). The specific activity was expressed as U/ml/mg protein.

For analysis of CAT activity, the salivary glands were homogenized at 10% (w/v) in a phosphate buffer (KH_2PO_4 50 mM and K_2HPO_4 50 mM 1:1.5 (v/v)) pH 7.4. The homogenate was centrifuged at 1,500 g for 10 min and the supernatant used to assay CAT activity, which was measured in a medium containing 50 mM phosphate buffer, pH 7.0, supernatant and 100 mM H_2O_2. The catalysis of H_2O_2, which was observed spectrophotometrically, was shown by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity (27). The specific activity was expressed as nmol/min/ml/mg protein.

The protein concentration was measured using Folin's phenol reagent, as described elsewhere (28); bovine serum albumin was used as standard. The readings of the Beckman DU-68 spectrophotometer (Beckman Instruments, Fullerton, CA) were taken at 660 nm.

Statistical analyses

The Student's "t" test for unpaired values was used to determine the level of significance. If multiple comparisons were necessary, after analysis of variance, the Student-Newman-Keuls test was applied. Differences between means were considered significant if $P<0.05$.

Results

ELISA assays were performed to demonstrate the presence of serum IgG against M_3 mAChR using a human M_3 synthetic peptide as antigen in pSS patients compared to healthy individuals. Figure 1 shows the optical density (OD) values of the each serum of pSS patients and of the healthy subjects studied. The OD values obtained with pSS sera were always > 2 SD than those of healthy individuals. Anti-M_3 mAChR peptide IgG was significantly higher in pSS patients than in healthy individuals ($p<0.0001$).

We have previously demonstrated the existence of circulating antibodies against the submandibular gland membrane in SS patients, which are able to interact molecularly with human M_3 mAChR synthetic peptide (23). Knowing that the amino acid sequences of the second extracellular loop M_3 mAChR of rat and humans have strong homology (92%), we studied the mAChR-mediated effect of autoantibodies from pSS patients on the rat submandibular acini preparation. For this purpose, we used the affinity purified anti-M_3 mAChR peptide IgG from pSS patients using anti-M_3 mAChR peptide IgG from healthy individuals as control.

The SOD specific activity in rat submandibular gland in the presence of $1x10^{-8}$ M of anti-M_3 mAChR peptide IgG showed an increment of approximately 29% ($p<0.05$) compared to the basal values (Fig. 2A). Anti-M_3 mAChR peptide IgG from normal subjects was ineffective in our submandibular glandular preparation (Fig. 2A). Pilocarpine (an authentic mAChR agonist) was used under identical experimental conditions and induced significant increment in SOD activity (36%, $p<0.05$) (Fig. 2A).

In order to assess the actions of pSS antibody on SOD activity pathways coupled to glandular M_3 mAChR the cholinoceptor antagonist and selective inhibitors of different enzymatic pathways commonly associated with glandular M_3 mAChR activation were studied. Figure 2B shows that the inhibition of glandular M_3 mAChR by atropine ($1x10^{-6}$ M), L-type calcium channel by verapamil ($1x10^{-5}$ M), calcium/calmodulin complex by TFP ($1x10^{-6}$ M), cyclooxygenase 2 (COX-2) by DuP697 ($5x10^{-8}$ M) and nitric oxide synthase by L-NMMA ($1x10^{-4}$ M), attenuated the increment

of SOD activity by anti-M_3 mAChR peptide IgG. All of these effects resemble those of the authentic mAChR agonist pilocarpine as shown in Figure 2C. Furthermore, the maximal increment of SOD activity induced by the autoantibody was neutralized after pre-incubating IgG with the M_3 synthetic peptide. Normal anti-M_3 mAChR peptide IgG was without effect (Fig. 2B).

An increment of 49% and 84% respectively ($p<0.0001$ versus basal values) was observed in the specific activity of CAT in submandibular gland in the presence of anti-M_3 mAChR peptide IgG and pilocarpine (Fig. 3A). The increment in CAT activity on submandibular gland pre-incubated with different inhibitors in the presence of anti-M_3 mAChR peptide IgG and pilocarpine as described above are shown in Figure 3B and 3C. The results obtained are the same as those previously described in Fig. 2B and in Fig. 2C.

A comparison between the mean levels ± SD of CAT and SOD enzyme activities in the serum of pSS patients and healthy individuals is shown in Table 2. A significant difference ($p<0.0001$) in both SOD and CAT mean levels was observed in pSS patients and healthy individuals (control). Table 2 also shows a significant difference in the levels of PGE_2 and nitrites in pSS patients and healthy individuals.

Figure 4 shows the ability of anti-M_3 mAChR peptide IgG ($1x10^{-8}$ M) (A) and pilocarpine ($1x10^{-7}$ M) (B) to stimulate nitrite production and the increment of PGE_2 generation. M_3 synthetic peptide was able to inhibit the action of anti-M_3 mAChR peptide IgG on nitrite levels; atropine was able to do so in pilocarpine stimulation; anti-M_3 mAChR peptide normal (n) IgG was ineffective in the studied system.

Discussion

This study examines antioxidant enzymes (SOD and CAT) in sera of pSS patients and in rat submandibular gland in the presence of anti-M_3 mAChR peptide IgG and pilocarpine. The results of biochemical analysis show both the enhancement of the specific activity of the enzymes in submandibular gland stimulated with the M_3 mAChR autoantibody and pilocarpine, an authentic cholinoceptor agonist, and in the sera of pSS patients.

This increment in enzyme activity is associated with the presence of anti-Ro antibody and anti-M_3 mAChR peptide IgG, and the symptoms of dryness (xerostomia, xerosphthalmia, xerodermia) in the sera of pSS patients. The specific activity of both enzymes, SOD and CAT, suffer no changes in the sera of healthy individuals.

Recently, it has been shown that the imbalance between the levels of ROS and the antioxidant enzyme system may play a key role in the salivary gland pathologies (20, 29). The alterations on rat salivary glands' function in the course of insuline resistance rats shows that parotid and submandibular glands react differently to an excess of ROS, which in turn leads to modifications in the expression of SOD and CAT (30).

The fact that M_3 mAChR autoantibody and pilocarpine stimulate the enzyme activity of SOD and CAT in the rat submandibular gland and in the sera of pSS patients, may be explained by the reason that the acinar machinery responsible for the increment of the specific activity of SOD and CAT damages the submandibular M_3 mAChR of the rat submandibular gland. This in turn, increases free radicals and initiates the oxidative stress with the modification of ROS molecules by alterations of the parasympathetic glandular system. The impairment of the action of the M_3 mAChR IgG and pilocarpine by atropine and by M_3 synthetic peptide corroborates this statement pharmacologically.

An increased amount of ROS species generated by polymorphonuclear leukocytes (31) and by xantine oxidoreductase / xantine oxidase released by the

conjuntival epithelium has been described in dry eye (32). Xantine oxidoreductase / xantine oxidase in turn generate ROS and may induce pro-inflammatory cytokines (33).

The results of our study point to the fact that the increment of the specific activity of antioxidant enzymes (SOD and CAT) contributes both to the imbalance between ROS molecules at the level of the pSS patients of the submandibular gland, and to the increment of nitrites/nitrates and PGE_2.

The formation of cytotoxic oxygen products and the presence of these inflammatory cytokines (Nitric oxide (NO) / PGE_2) provoke an oxidative tissue damage. Previous reports (34) demonstrate that NO and ROS participate in the immune signal; have the ability to kill pathogens and their enhancement in the downstream of pSS patients makes them complex modulators that act in the inflammatory process, worsen by the lesion of M_3 mAChR IgG and the generation of a large amount of NO and PGE_2.

In addition, there are specific oxidation events that modify intracellular signals at the level of M_3 mAChR salivary and ocular glands in SS patients, leading to the parasympathetic dysfunction observed in the course of the disease.

Taken together the increment of the specific activity of SOD and CAT in pSS patients as compared to control healthy individuals, may be seen as a defensive reaction to the increment of the amount of ROS in the body, which becoming uncontrollable, leads to irreversible cellular and tissue damage.

References

1. Jonsson R, Haga HJ, Gordon TP. Current concepts on diagnosis, autoantibodies and therapy in Sjögren's syndrome. *Scand J Rheumatol* 2000; **29**: 341-48.
2. Brito-Zerón P, Ramos-Casals M. Advances in the understanding and treatment of systemic complications in Sjögren syndrome. *Curr Opin Rheumatol* 2014; **26**: 520-27.
3. Voulgarelis M, Tzioufas AG. Pathogenetic mechanisms in the initiation and perpetuation of Sjögren's syndrome. *Nat Rev Rheumatol* 2010; **6**: 529–37.
4. Tatouli IP, Tzioufas AG. Pathogenetic aspects of humoral autoimmunity in Sjögren's syndrome. *Lupus* 2012; **21**: 1151–54.
5. Quartuccio L, Salvin S, Fabris M, et al. BLyS upregulation in Sjogren's syndrome associated with lymphoproliferative disorders, higher ESSDAI score and B-cell clonal expansion in the salivary glands. *Rheumatology (Oxford)* 2013; **52**: 276–81.
6. Passafaro D, Reina S, Sterin-Borda L, Borda E. Cholinergic autoantibodies from primary Sjögren's syndrome modulate submandibular gland Na+/K+-ATPase activity via prostaglandin E_2 and cyclic AMP. *Eur J Oral Sci* 2010; **118**: 131-38.
7. Srinivasan S, Slomovic AR. Sjögren syndrome. *Compr Ophthalmol Update* 2007; **8**: 205-12.
8. Fox RI. Sjogren's syndrome. *Lancet* 2005; **366**: 321-31.
9. Bacman S, Sterin┐ Borda L, Camusso JJ et al. Circulating antibodies against rat parotid gland M_3 muscarinic receptors in primary Sjogren's syndrome. *Clin Exp Immunol* 1996; **104**: 454-59.
10. Bacman S, Perez Leiros C, Sterin-Borda L, Hubscher O, Arana R, Borda E. Autoantibodies against lacrimal gland M_3 muscarinic acetylcholine receptors in patients with primary Sjögren's syndrome. *Invest Ophthalmol Vis Sci* 1998; **39**: 151-56.

11. Reina S, Passafaro D, Sterin-Borda L, Borda E. Atorvastatin inhibits the inflammatory response caused by anti-M_3 peptide IgG in patients with primary Sjögren's syndrome. *Inflammopharmacology* 2012; **20**: 267-75.
12. Vitali C, Bombardieri S, Jonsson R, et al. European Study Group on Classification Criteria for Sjögren's Syndrome. Classification criteria for Sjögren's syndrome: A revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002; **61**: 554-58.
13. Shiboski SC, Shiboski CH, Criswell L, et al. Sjögren's International Collaborative Clinical Alliance (SICCA) Research Groups. American College of Rheumatology classification criteria for Sjögren's syndrome: A data-driven, expert consensus approach in the Sjögren's International Collaborative Clinical Alliance cohort. *Arthritis Care Res (Hoboken)* 2012; **64**: 475-87.
14. Guellec D, Cornec D, Jousse-Joulin S, et al. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: A systematic review. *Autoimmun Rev* 2013; **12**: 416-20.
15. Risselada AP, Kruize AA, Goldschmeding R, et al. The prognostic value of routinely performed minor salivary gland assessments in primary Sjögren's syndrome. *Ann Rheum Dis* 2014; **73**: 1537-40.
16. Halliwell B. Reactive oxygen species in living systems: source, biochemistry and role of human disease. *Am J Med* 1991; (Suppl. 3C): 14-22.
17. Cejková J, Ardan T, Simonová Z, Cejka C, Malec J, Dotrelová D, Brunová B. Decreased expression of antioxidant enzymes in the conjunctival epithelium of dry eye (Sjögren's syndrome) and its possible contribution to the development of ocular surface oxidative injuries. *Histol Histopathol* 2008; **23**: 1477-83.
18. Zalewska A1, Knaś M, Zendzian-Piotrowska M, Waszkiewicz N, Szulimowska J, Prokopiuk S, Waszkiel D, Car H. Antioxidant profile of salivary glands in high fat diet-induced insulin resistance rats. *Oral Dis* 2014; **20**: 560-66.

19. Brik R, Rosen I, Savulescu D, Borovoi I, Gavish M, Nagler RM. Salivary antioxidants and metalloproteinases in juvenile idiopathic arthritis. *Mol Med* 2010; **16**: 122-28.

20. Al-Rawi NH. Oxidative stress, antioxidant status and lipid profile in the saliva of type 2 diabetics. *Diab Vasc Dis Res* 2011; **8**: 22–28.

21. Hodges RR, Dicker DM, Rose PE, Dartt DA. Alpha one adrenergic and cholinergic agonist use separate signal transduction pathways in lacrimal gland. *Am J Physiol* 1992; **262:** G1087-G96.

22. Reina S, Sterin-Borda L, Orman B, Borda E. Autoantibodies against cerebral muscarinic cholinoceptors in Sjögren syndrome: functional and pathological implications. *J Neuroimmunol* 2004; **150**: 107-15.

23. Reina S, Orman B, Anaya L, Sterin-Borda L, Borda E. Cholinergic Autoantibodies in Sjögren Syndrome. *Journal of Dental Research* 2007; **86**: 832-36.

24. Reina S, Sterin-Borda L, Passafaro D, Borda E. Anti-M_3 muscarinic cholinergic autoantibodies from patients with primary Sjögren's syndrome trigger production of matrix metalloproteinase-3 (MMP-3) and prostaglandin E_2 (PGE_2) from the submandibular glands. *Arch Oral Biol* 2011; **5**: 413-20.

25. Green LC, Ruiz de Luzuriaga K, Wagner DA, Rand W, Istfan N, Young VR, Tannenbaum SR. Nitrate biosynthesis in man. *Proc Natl Acad Sci USA* 1981; **78**: 7764-68.

26. Jung KaHW. Developmental changes of antioxidants enzymes in kidney and liver from rats. *Free Radic Biol Med* 1996; **20**: 613-17.

27. Wheeler CR, Salzman JA, Elsayed NM, et al. Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. *Ann Biochem* 1990; **184**: 193-99.

28. Simoes A, Nicolau J, de Souza DN, Ferreira LS, de Paula Eduardo C, Apel C. Effect of defocused infrared diode laser on salivary flow rate and some salivary parameters of rats. *Clin Oral Investig* 2008; **12**:25-30.

29. Zalewska A, Knaś M, Gińdzieńska-Sieśkiewicz E, Waszkiewicz N, Klimiuk A, Litwin K, Sierakowski S, Waszkiel D. Salivary antioxidants in patients with systemic sclerosis. *J Oral Pathol Med* 2014; **43**: 61-8.

30. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009; **417**: 1-13.

31. Augustin AJ1, Spitznas M, Kaviani N, Meller D, Koch FH, Grus F, Göbbels MJ. Oxidative reactions in the tear fluid of patients suffering from dry eyes. *Graefes Arch Clin Exp Ophthalmol* 1995; **233**: 694-98.

32. Cejková J, Ardan T, Jirsová K, Jechová G, Malec J, Simonová Z, Cejka C, Filipec M, Dotrelová D, Brunová B. The role of conjunctival epithelial cell xanthine oxidoreductase/xanthine oxidase in oxidative reactions on the ocular surface of dry eye patients with Sjögren's syndrome. *Histol Histopathol* 2007; **22**: 997-1003.

33. Page S, Powell D, Benboubetra M, Stevens CR, Blake DR, Selase F, Wolstenholme AJ, Harrison R. Xanthine oxidoreductase in human mammary epithelial cells: activation in response to inflammatory cytokines. *Biochim Biophys Acta* 1998; **1381**: 191-202.

34. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, Ridnour LA, Colton CA. Nitric oxide and redox mechanisms in the immune response. *J Leukoc Biol* 2011; **89**: 873-91.

35. Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol* 2004; **287**: C246-56.

Acknowledgement

Current study is founded by UBACyT 20020130100325BA and PIP 11220110100019 from Buenos Aires University and CONICET respectively, Buenos Aires, Argentina.

Conflict of Interest

The authors declare no conflict of interest.

Table 1

Characteristics of the study populations

Clinical characteristics		
	pSS patients	**Healthy individuals**
Number	25	25
Current age, mean years ± SD	41.4 ± 11.9	39.6 ± 10.2
Disease duration, mean years ± SD	6.88 ± 5.4	N/A
Female gender, *n* (%)	25 (100)	25 (100)
Organ/system involved		
Xerostomia, *n* (%)	25 (100)	N/A
Xerophthalmia, *n* (%)	25 (100)	N/A
Xerodermia, *n* (%)	25 (100)	N/A
Current antibodies		
ANA positive, *n* (%)	25 (100)	N/A
Anti-Ro/SSA positive, *n* (%)	25 (100)	N/A
Anti-M_3 synthetic peptide IgG, *n* (%)	25 (100)	N/A

Values are expressed as mean ± SEM. Values in parenthesis are the percentages.

ND: not detected.

Table 2

Comparison between mean levels ± SD of superoxide dismutase (SOD), catalase (CAT) enzymes and PGE_2 and nitrites in serum of patients with pSS and healthy individuals

Variable	SOD (U/ml)	CAT (nmol/min/ml)	PGE_2 (ng/ml)	Nitrites (µM)
healthy individuals (n=25)	3.38 ± 1.17	7.24 ± 1.74	4.4 ± 0.33	5.8 ± 0.51
pSS patients (n=25)	3.56 ± 1.10*	18.16 ± 8.22**	11.6 ± 1.10**	18.78 ± 2.7***

Values are mean ± SD. * significant differences with p=0.05; ** significant differences with p=0.0001; *** significant differences with p=0.006 between pSS patients and healthy individuals.

Legends of Figures

Figure 1

Detection of serum antibody titres; scatterogram showing the immuno reactivity of circulating IgG antibodies against M_3 mAChR synthetic peptide; individual optical density (OD) values for each serum (1:30 dilution) of 25 pSS patients and 25 healthy individuals (control); OD cutoff value 0.4 ± 0.01. $p<0.001$ between anti-M_3 mAChR synthetic peptide versus healthy individuals.

Figure 2

Specific activity of SOD in the rat submandibular gland; values of SOD activity in the presence of anti-M_3 peptide IgG ($1x10^{-8}$ M) and pilocarpine ($1x10^{-7}$ M) alone and anti-M_3 peptide normal (n) IgG (A); anti-M_3 peptide IgG alone (B) and pilocarpine alone (C) or in the presence of atropine ($1x10^{-6}$ M), verapamil ($1x10^{-5}$ M), TFP ($1x10^{-6}$ M), DuP697 ($5x10^{-8}$ M), L-NMMA ($1x10^{-4}$ M) and M_3 synthetic peptide ($1x10^{-5}$ M). As control, the effect of $1x10^{-7}$ M anti-M_3 peptide n IgG is also shown; mean values ± SEM of 12 pSS IgG, 10 normal IgG and 7 pilocarpine experiments in each group; *P<0.001 versus basal; **P<0.0001 pSS IgG or pilocarpine alone versus inhibitor agents.

Figure 3

Specific activity of CAT in the rat submandibular gland; values of CAT activity in the presence of anti-M_3 peptide IgG ($1x10^{-8}$ M) and pilocarpine ($1x10^{-7}$ M) alone and anti-M_3 peptide normal (n) IgG (A); anti-M_3 peptide IgG alone (B) and pilocarpine alone (C) or in the presence of atropine ($1x10^{-6}$ M), verapamil ($1x10^{-5}$ M), TFP ($1x10^{-6}$ M), DuP697 ($5x10^{-8}$ M), L-NMMA ($1x10^{-4}$ M) and M_3 synthetic peptide ($1x10^{-5}$ M); as control, the effect of $1x10^{-7}$ M anti-M_3 peptide n IgG is also shown; mean values ± SEM of 12 pSS IgG, 10 normal IgG and 7 pilocarpine experiments in each group; *P<0.001 versus basal; **P<0.0001 pSS IgG or pilocarpine alone versus inhibitor agents.

Figure 4

Effect of anti-M_3 peptide IgG ($1x10^{-8}$ M) and pilocarpine ($1x10^{-7}$ M) alone or in the presence of M_3 synthetic peptide ($1x10^{-5}$ M) and atropine ($1x10^{-6}$ M) on rat submandibular gland on the production of nitrites (A) and PGE_2 (B). As control, the effect of $1x10^{-7}$ M anti-M_3 peptide n IgG is also shown; mean values ± SEM of 10 pSS IgG, 9 normal IgG and 6 pilocarpine experiments in each group; *P<0.001 versus basal; **P<0.0001 versus pSS IgG or pilocarpine alone.

Table 1

Characteristics of the study populations

Clinical characteristic		
	pSS patients	Healthy individuals
Number	25	25
Current age, mean years ± SD	41.4 ± 11.9	39.6 ± 10.2
Disease duration, mean years ± SD	6.88 ± 5.4	N/A
Females gender, n (%)	25 (100)	25 (100)
Organ/system involved		
Xerostomia, n (%)	25 (100)	N/A
Xerophthalmia, n (%)	25 (100)	N/A
Xerodermia, n (%)	25 (100)	N/A
Current antibodies		
ANA positive, n (%)	25 (100)	N/A
Anti-Ro/SSA positive, n (%)	25 (100)	N/A
Anti-M_3 synthetic peptide IgG, n (%)	25 (100)	N/A

Values are expressed as mean ± SD. Values in parenthesis are the percentages. N/A: not detected.

Table 2

Comparison between mean levels ± SD of superoxide dismutase (SOD), catalase (CAT) enzymes and PGE_2 and nitrites in serum of patients with pSS and healthy individuals

Variable	SOD (U/ml)	CAT (nmol/min/ml)	PGE_2 (ng/ml)	Nitrites (µM)
healthy individuals (n=25)	3.38 ± 1.17	7.24 ± 1.74	4.4 ± 0.33	5.8 ± 0.51
pSS patients (n=25)	3.56 ± 1.10*	18.16 ± 8.22**	11.6 ± 1.10**	18.78 ± 2.7***

Values are mean ± SD. * significant differences with p=0.05; ** significant differences with p=0.0001; *** significant differences with p=0.006 between pSS patients and healthy individuals.

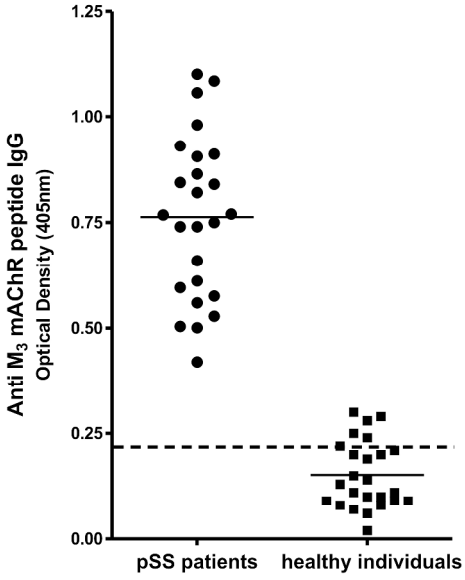

Figure 1
Reina et al

298x420mm (300 x 300 DPI)

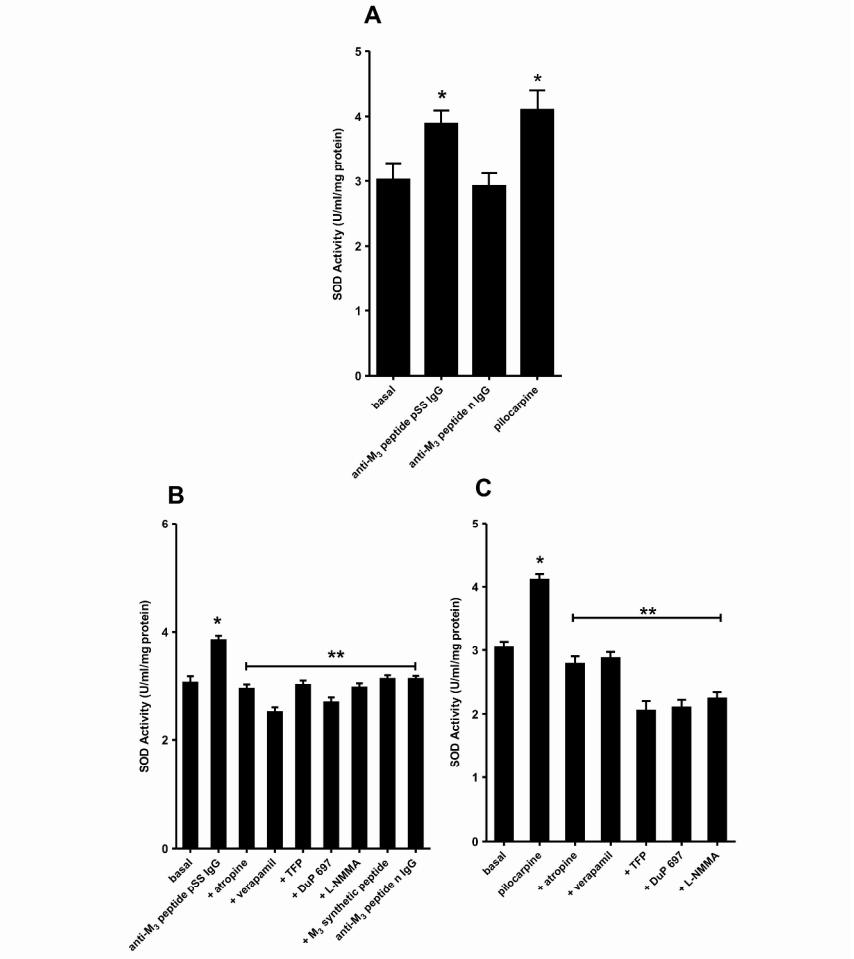

Figure 2
Reina et al

298x420mm (300 x 300 DPI)

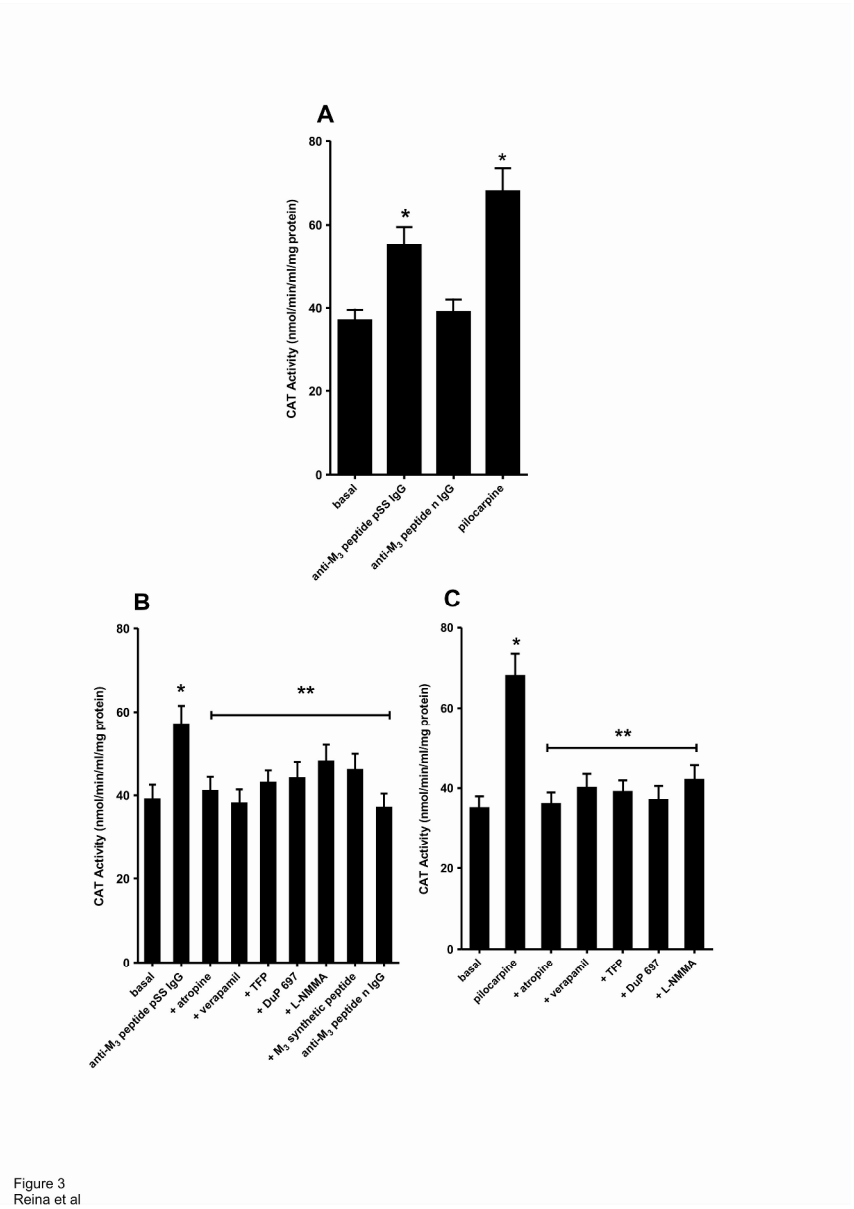

Figure 3
Reina et al

298x420mm (300 x 300 DPI)

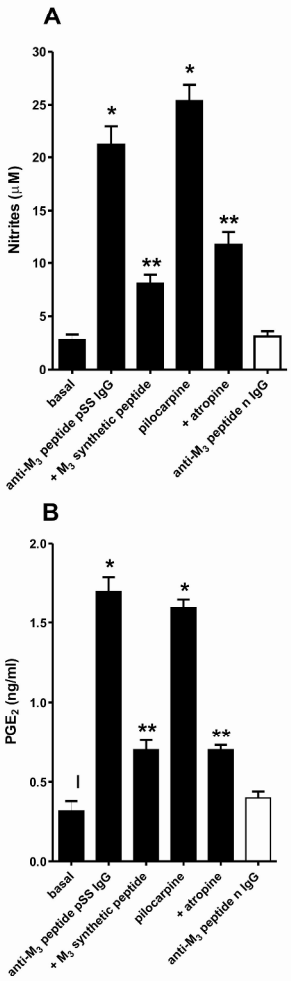

Figure 3
Reina et al

298x420mm (300 x 300 DPI)