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ORIGINAL ARTICLE

Calcium signaling and cell volume regulation are altered in Sjögren's Syndrome

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Abstract

Objective. Sjögren's Syndrome (SS) is a chronic autoimmune disease, leading to deficient secretion from salivary and lacrimal glands. Saliva production is normally increased by cholinergic innervation, giving rise to intracellular calcium signaling and water transport through water channels (aquaporins, AQPs). The aim of this study was to investigate possible pathophysiological changes in cell volume regulation, AQP expression and localization, and intracellular calcium signaling in glandular cells from SS patients compared to controls. ***Materials and methods.*** A total of 35 SS patients and 41 non-SS controls were included. Real time qPCR was combined with immunohistochemistry to analyze the mRNA expression and cellular distribution of AQP1, 3 and 5. Cell volume regulation and intracellular calcium signaling were examined in fresh acinar cells. ***Results.*** We show for the first time a reduced mRNA expression of AQP1 and 5 in SS compared to controls, accompanied by a decrease in staining intensity of AQP1, 3 and 5 in areas adjacent to local lymphocytic infiltration. Furthermore, we observed that the SS cells' capacity for volume regulation was abnormal. Similarly, the calcium response after parasympathetic agonist (carbachol) stimulation was markedly decreased in SS cells. ***Conclusions.*** It is concluded that mRNA expression of AQP1 and 5, protein distribution of AQP1, 3 and 5, glandular cell volume regulation and intracellular calcium signaling are all altered in SS, pointing to possible pathophysiological mechanisms in SS.

Key Words: *aquaporins, calcium signaling, cell volume regulation, saliva, Sjögren's Syndrome*

Introduction

Sjögren's Syndrome (SS) is a chronic inflammatory, autoimmune disease affecting exocrine glands. Most commonly afflicted are the salivary and lacrimal glands, leading to reduced secretion and dry mucous membranes [1]. Current standards are based on the American–European Consensus Group (AECG) classification, including a total of six items [2]. Of these, two are subjective, i.e. dryness of mouth and eyes. The four remaining items are based on objective measures of oral or ocular hyposecretion, labial salivary gland lymphocytic infiltrates (to yield a focus score of ≥1) and the presence of Anti-Ro (SSA)/Anti-La (SSB) autoantibodies. A patient will be classified with SS either if three of the four objective items are fulfilled or four of all six items, requiring a positive salivary gland biopsy or positive serology with either SSA or SSB autoantibodies or both. When employing the AECG criteria, the prevalence of SS is estimated to be ~ 0.05%, with a higher incidence among adult women [3]. Moreover, SS patients can have oral manifestations, such as caries and fungal infections, non-specific symptoms, like fatigue and arthralgia, and an increased risk of non-Hodgkin's lymphoma. All of the above may contribute to a reduced quality of life [4–8].

The etiology of SS is debated, but is currently believed to depend on a complex combination of genetic predisposition, immune dysregulation and epigenetic factors [9]. The apparent lack of correlation between the degree of lymphocytic infiltration and secretory capacity in salivary glands indicates that changes in acinar cell function other than

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those directly caused by immune attack and inflammation may contribute to the observed hyposecretion [10].

As in other polarized epithelial tissues, salivary gland acinar cells depend on an apical-basal organization of membrane proteins in order to carry out their functions. For normal salivary secretion, proper calcium signaling, water transport, and cell volume regulation, upon autonomic nervous stimulation, are essential [11]. Cell volume and water flow over the cell membrane depend on proper functioning of water channels (aquaporins, AQPs). The AQPs are a family of water transporting proteins embedded in cellular membranes and to date the presence of AQP1, 3, 4, 5, 6 and 7 have been shown in human salivary glands [12–16].

In addition to the importance of structural integrity of acinar cells, salivary secretion depends on both parasympathetic and sympathetic signaling. Parasympathetic activation will lead to increased Ca^{2+} release from the endoplasmic reticulum, which will open apical Cl^- channels and basolateral K^+ channels [17]. The subsequent cellular efflux of Cl^- and K^+ leads to water flux out of the cell and, thus, cell shrinkage. The cell shrinkage is temporary and merely a response to an osmolarity imbalance. This perturbation is normally followed by a regulatory volume increase, which seems to depend in part on the intracellular free Ca^{2+}-levels and uptake of osmotically active particles such as K^+, Na^+ and Cl^- [18]. Intracellular Ca^{2+}-changes and KCl-efflux are also seen when cell swelling and regulatory volume changes are experimentally induced by exposing cells to water influx by hyposmotic solutions.

Cell volume regulation has been demonstrated to be dependent on AQPs [19]. Both AQP3 and 5 are known to be located in acinar cell plasma membranes, and particularly AQP5 has been shown to be important for saliva production [12,16,19–22]. In contrast, AQP1 is believed to be located in both capillaries and myoepithelial cells surrounding gland acini. Through contractile properties of myoepithelial cells, AQP1 may play a role in saliva secretion [23]; however, studies using AQP1 knock-out mice did not reveal altered salivary secretion [21]. The decreased secretory function in SS with regards to salivary gland physiology, AQPs and calcium signaling has been studied by some groups [23–29], but to our knowledge, the present study is the first to investigate AQPs, cell volume regulation and Ca^{2+} in SS, using fresh human labial salivary glands.

The aim of the present study was to examine AQPs, water transport and calcium responses in salivary gland cells from SS patients and controls. We hypothesize that alterations in basic cell functions may contribute to the decreased secretory functions seen in SS.

Materials and methods

Patients

All patients included in the study were referred to the Department of Oral Surgery and Oral Medicine, Faculty of Dentistry, University of Oslo, Norway, for a labial salivary gland biopsy as part of the diagnostic workup of SS. As the physiological experiments required fresh salivary gland tissue, the patients were asked to provide one or two glands in addition to those needed for diagnostic purposes. All patients except two agreed to participate in the study and two patients were excluded due to a previous history of sarcoidosis or rheumatoid arthritis. Informed consent was obtained from each patient. The study was approved by the Regional Committee for Medical and Health Research Ethics, South East, Norway. All biopsies were performed by the same oral and maxillofacial surgeon and were carried out between 2009–2013.

Inclusion criteria were: SS-patients fulfilling the AECG classification for SS; non-SS controls were patients evaluated for SS, but not fulfilling the AECG classification [2]. Importantly, none of the controls had a focus score ≥1 or positive anti-SSA/SSB autoantibodies and none of the patients included in the study suffered from secondary SS. Throughout the time course of this study, a total of 76 patients were included; 35 of these were SS positive and the remaining 41 served as non-SS controls. All experiments and data analyses were performed blinded.

RNA isolation, reverse transcription and quantitative real time polymerase chain reaction (qPCR)

Salivary labial glands from a total of 27 patients were used for qPCR; 12 of which were SS patients and 15 were non-SS controls (three of the latter were males). The fresh glands were placed in RNAlater (Sigma-Aldrich, St Louis, MO) diluted 1:2 in phosphate buffered saline (PBS) and kept at 4°C before being frozen at 80°C. RNeasy Mini kit (Qiagen, Valencia, CA) was used for total RNA extraction, according to the manufacturer's instructions, and RNA purity and quantification were measured with a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). A total of 200 ng RNA was used per 10 µl reverse transcriptase step. Complementary DNA (cDNA) was generated using the Reverse Transcription Core Kit (Eurogentec, Seraing, Belgium), with a mix of reverse transcriptase enzyme, RNAse inhibitor, MgCl, dNTP and 10× buffer. Each cDNA synthesis was performed in a total volume of 20 µl for 10 min at 25°C, then at 48°C for 30 min and terminated by incubation for 5 min at 95°C. Detection of AQP1, AQP3, AQP5, ubiquitin C (UBC) and glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was performed using Assay-on-Demand TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA). Each real-time PCR reaction consisted of 1× AOD mix, 1× qPCR MasterMix Plus (Eurogentec) and 10 µl cDNA (diluted 1:2.5 µl with H_2O) as the template. qPCR reactions were carried out on a Stratagene MX3005p (Agilent Technologies, Santa Clara, CA) for 40 cycles (95°C for 15 s and 50°C or 60°C for 1 min) after an initial 10 min incubation at 95°C. The data were normalized to GAPDH as endogenous control, and a Non Template Control (NTC) was included for each run to validate the assay. UBC was used as an additional control and only for AQP5. For each experiment each sample was run in duplicate. The relative amount of AQP1, 3 and 5 mRNA was standardized to that of GAPDH and UBC mRNA using $\Delta C_T = [C_{T(\text{target gene})} - C_{T(\text{reference gene})}]$ and displayed as $2^{(-\Delta Ct)}$.

Immunohistochemistry (IHC)

Labial salivary glands were fixed in 4% formaldehyde solution prior to paraffin embedding and histopathological evaluation. Following diagnostic evaluation, 25 paraffin embedded glands were obtained and 15 of these were SS positive. The glands were cut in 5 µm thick sections using a Leica microtome (RM 2155, Leica Microsystems, Wetzlar, Germany) and mounted on SuperFrost® slides (Menze-Gläser, Braunschweig, Germany). Prior to immunostaining, the slides were deparaffinized and rehydrated. Between each step of the protocol the slides were washed in PBS at room temperature (RT). Slides were quenched in 0.3% H_2O_2 solution, followed by heat-induced antigen retrieval in citraconic anhydride (pH 7.4), using a pressure boiler (Biocare Medical, Concord, CA). To eliminate unspecific binding, all slides were incubated with 5% normal sera (Sigma-Aldrich, St Louis, MO), corresponding to the source animal of the primary antibodies (RT, 30 min). The following antibodies were used: rabbit IgG raised against amino acids 215–269 in the human origin of AQP1 (sc-20810, Santa Cruz Biotechnology Inc., Dallas, TX, 0.2 µg/mL), epitope affinity-purified rabbit Ig-antiserum against AQP3 (BT-BS3671, Bio-World Technology Inc., St.Louis Park, MN, 2 µg/mL) and rabbit monoclonal IgG1 targeting AQP5, clone EPR3747 (ab92320, Abcam, Cambridge, UK, 1:1800 dilution). For all primary antibodies, the slides were incubated overnight at 4°C. Secondary biotinylated antibodies (Vector Laboratories, Burlingham, CA, 7 µg/ml) were incubated for 30 min at RT, followed by horseradish peroxidase-conjugated ABC (ABC complex; Vectastain; Vestor Laboratories) and 3,3′-diaminobenzidine tetra hydrochloride (DAB; Ventana, Tuscon, AZ). Lastly, the slides were counterstained with hematoxylin, dehydrated with ethanol (70%, 90%, 100%) and xylene and mounted with coverslips. As all primary antibodies were derived from rabbit and since IgG is the dominant immunoglobulin in rabbit serum, we performed a negative control staining using Negative Control Rabbit Immunoglobulin Fraction (DakoCyotmation, Glostrup, Denmark), matching the highest concentration of the primary rabbit antibody used. In addition, another negative control was included by omitting the primary antibodies.

As a relative measure of IHC staining intensity, 10 acini were randomly selected for each biopsy. For each of these acini, five cells were randomly chosen. Color intensity on the apical, lateral and basal aspects was registered using a labeling index of 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) staining intensity. In addition, registrations for patients diagnosed with SS were performed in acini next to lymphocytic infiltrations and color intensity on the apical, lateral and basal side scored.

Cell volume measurements after hypo-osmotic challenge

For cell volume measurements after hypo-osmotic challenge, a total of 13 patients were included; five of these were SS patients, eight were non-SS controls (one of the latter being a male). Acinar cell isolation was performed as described by Valdez et al. [30]. Fresh salivary glands were mechanically minced for 5 min in 1 ml digestion medium containing 50:50 Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12) (GIBCO 11039, Life Technologies Corp., Oslo, Norway) and Dulbecco's PBS without Ca^{2+} and Mg^{2+} (PAA H15-002, GE Healthcare, Piscataway, NJ), 1% bovine serum albumin (Sigma-Aldrich), 100 U/ml collagenase IV (Worthington Biochemical Corp., Lakewood, NJ) and 1600 U/ml hyaluronidase (Sigma-Aldrich). Then followed a regime of gently pipetting the mixture, incubating the solution at 37°C with continued CO_2-gassing, centrifugation at 600 g for 5 min and resuspension in fresh digestion medium, as previously described [30]. After a total incubation period of 80 min, the gland fragments were centrifuged and washed 3 times in enzyme-free digestion medium. As described by Valdez et al. [30], we also found that the resulting suspension consisted of clumps of acinar cells and no or very few duct fragments. To validate this observation, immunocytochemistry was performed in chambered slides (Nalgene Nunc International, Naperville, IL) using the anti-AQP5 antibody described above. Here, cells were fixed with 4% formaldehyde for 30 min, washed in PBS and further incubated for 20 min in 110 mM glycine in PBS (Sigma-Aldrich). To permeabilize the cells, Triton-X 100 (Sigma-Aldrich) was added to the chambers for 5 min at 4°C. Subsequent steps of immunocytochemistry staining were identical to those described above for immunohistochemistry.

The glass bottoms of flow-through cell chambers had been coated with Cell-Tak (BD Bioscience, San Jose, CA) according to the manufacturer's instructions. Acinar cells were plated on the chamber bottoms in iso-osmotic Ringer's solution containing (in mM): 116 NaCl, 1 $MgSO_4$, 5 glucose (all from Merck Chemicals, Whitehouse Stations, NJ), 4 KCl, 2 $CaCl_2$, 1 NaH_2PO_4 and 20 Na-HEPES (all from Sigma-Aldrich); 300 mOsm, pH 7.4. The chambers with cells were then placed on the stage of a Zeiss Axiovert 100 inverted microscope fitted with a Solent Scientific (Segensworth, UK) incubation chamber (37°C) and a HQ CoolSnap digital camera (Photometrics, Ottobrunn, Germany). The acinar cells were exposed to iso-osmotic solution for 3 min before changing the medium to a hypo-osmotic solution containing (in mM): 67 NaCl, 1 $MgSO_4$, 5 glucose, 4 KCl, 2 $CaCl_2$, 1 NaH_2PO_4 and 20 Na-HEPES; 200 mOsm, pH 7.4. Cell volume changes were measured as variations in cell areas using the MetaMorph 7.5.3 software (Molecular Devices, Silicon Valley, CA). Measurements were performed every minute in the isosmotic solution, then every 15 s for the first minute in the hyposmotic solution and then finally every minute for 14 more minutes in the hyposmotic solution.

Intracellular Ca^{2+} measurements

Glands were pre-treated as described for cell volume measurements above. After placing the acinar cell clumps in the glass-covered flow-through chambers, the cells were loaded with the fluorescent intracellular Ca^{2+}-binding dye fura-2-AM (1 μg/ml; Molecular Probes/Invitrogen, Carlsbad, CA) in an iso-osmotic Ringer's solution (30 min at 37°C, 5% CO_2). The cells were then washed 3× in fura-free Ringer's solution and allowed to equilibrate for another 30 min at 37°C. The cell-containing chambers were placed on the temperature-regulated stage of an inverted microscope. Cells were either (a) exposed to a hypo-osmotic challenge as described above for cell volume measurements or (b) stimulated by continuous exposure to a Ringer's solution containing 100 μM carbamylcholine chloride (carbachol, Sigma-Aldrich). For experiments described in (a), a total of nine patients were included and five of these were diagnosed with SS and four were non-SS and, in (b), 12 patients were included, five of these were diagnosed with SS, seven were non-SS (and one of the latter was male). To measure the free intracellular Ca^{2+} levels, fura-2 was excited at 510 nm and the ratio of emitted light at 340 and 380 nm was recorded with a HQ CoolSnap digital camera (Photometrics, Ottobrunn, Germany), using the MetaFluor 7.5.3 software (Molecular Devices). Intracellular free Ca^{2+} measurements were performed every 60 s in the 3-min pre-stimulation period, then every 5 s for 2 min, every 10 s for 5 min and every 30 s during the last 8 min of the experiment.

Statistics

Statistical analysis was performed using IBM SPSS 20 (IBM, Armonk, NY) and SigmaPlot 12.0 (Systat Software Inc, Chicago, IL). When data were normally distributed, independent sample *t*-tests were used, otherwise Mann Whitney U-tests were applied. Statistical significance was set to $p \leq 0.05$. When not specified, results are given as mean values with their standard error of the mean.

Results

The clinical characteristics of the 35 SS patients and the 41 non-SS controls are given in Table I. The five men included in the study all belonged to the non-SS group.

AQP1, AQP3 and AQP5 mRNA expression and IHC staining are decreased in SS

The relative mRNA expression of AQP1, 3 and 5 was decreased in labial salivary glands from SS patients compared with non-SS controls, significantly so for AQP1 and AQP5 (Figure 1). To verify the qPCR results, we included UBC as an additional reference gene for AQP5 and found nearly identical results as when GAPDH was used as a reference gene (data for UBC not shown).

Immunohistochemical staining of AQP1 was seen in myoepithelial cells surrounding the acini and in the capillaries (Figure 2A). AQP3 was located to the basolateral membranes of the acini (Figure 2B). AQP5 staining was confined to apical and basolateral membranes of the acini, with more intense staining of

Table I. Clinical features of patients included in the study.

	Sjögren's syndrome	Non-Sjögren controls
Cohort characteristics		
Number, *n*	35	41
Age in years (mean ± SD)	59 ± 13	61 ± 10
Range (years)	36–92	39–79
Female:Male	35:0	36:5
Clinical features, *n* (%)		
SSB+ and/or SSA+	20 (57.1)	0 (0)
Xerostomia	31 (88.6)	39 (95.1)
Decreased salivary secretion (≤1.5 ml/15 min)	28 (80)	31 (75.6)
Pathological Schirmer's test	28 (80)	34 (82.9)
Eye dryness	32 (91.4)	39 (95.1)
Focus score		
FS ≥1	26 (74.3)	0 (0)

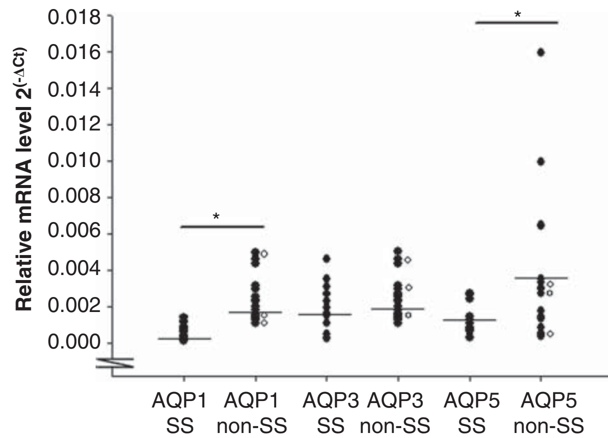

Figure 1. mRNA expression pattern of AQP1, 3 and 5 mRNA in labial salivary glands from SS patients ($n = 12$) compared to non-SS controls ($n = 15$) relative to the reference gene GAPDH. For both AQP1 and AQP5, the relative mRNA expression was significantly reduced in SS-glands compared to the control group ($*p < 0.05$). Individual $2^{(-\Delta Ct)}$ [cycle threshold (Ct)] from each patient is represented by a dot in the scatter plot and the horizontal lines represent mean values. The open circles represent individual values from males.

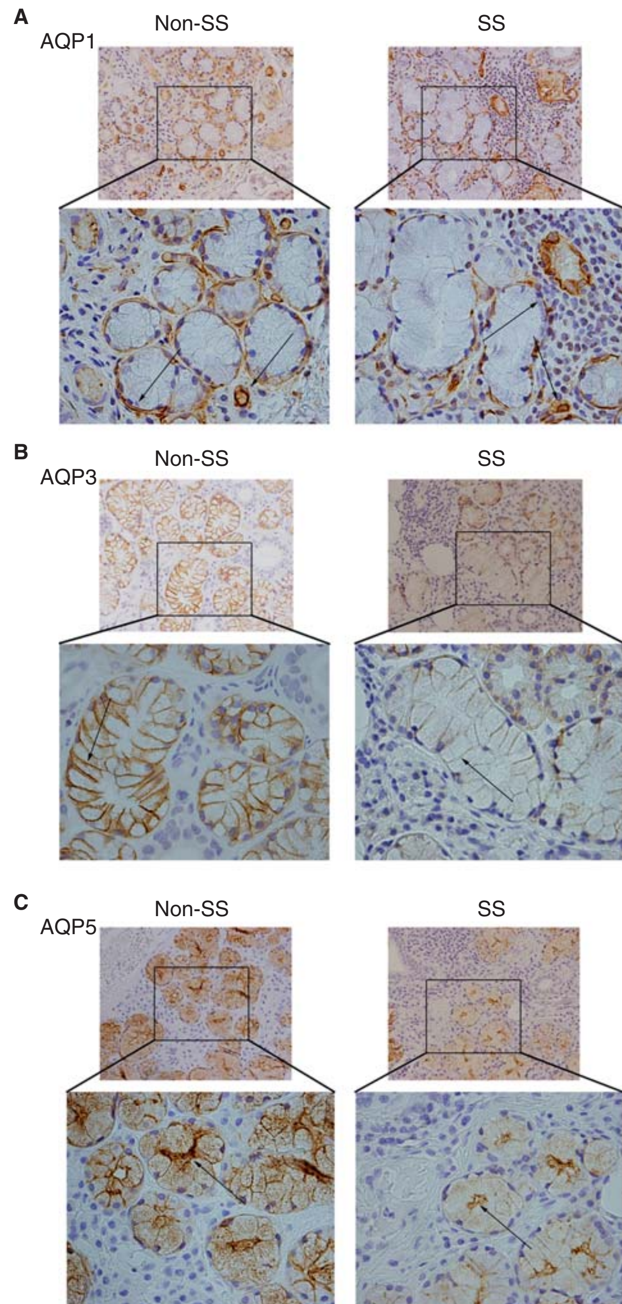

Figure 2. Immunostaining of labial salivary glands from SS patients ($n = 15$) and non-SS controls ($n = 10$). Brown color indicates positive immunostaining (hematoxylin was used as counterstain). Upper pictures: 20× magnification; lower pictures: 40× magnification. (A) Arrows: AQP1 is located in capillaries and in myoepithelial cells surrounding acini. (B) Arrows: AQP5 was observed at the basolateral and apical membrane of the acini. (C) Arrows: AQP5 was observed at the apical membrane of the acini. The magnified images show that reduced staining intensity for AQP1, 3 and 5 in SS positive glands is evident in regions adjacent to lymphocytic infiltrations (arrows).

the apical membrane (Figure 2C). The staining intensity was clearly fainter in labial salivary glands from SS patients when examining AQP1, AQP3 and AQP5 in acini adjacent to lymphocytic infiltrates (Figures 2A–C). When using our labeling index to compare randomly selected acini from non-SS controls to acini adjacent to lymphocytic infiltrations in SS patients, significant differences in staining intensity were found for all three AQPs (Supplemental Table 1A). However, no significant differences were found when comparing randomly selected acini from non-SS controls to randomly selected acini in SS patients (Supplemental Table 1B). Negative controls showed no staining.

Hypo-osmotic challenge leads to decreased regulatory volume response but not altered Ca^{2+}-responses in SS

Exposing fresh acinar cells to a hypo-osmotic solution (200 mOsm) showed that the regulatory volume decrease (RVD) response that follows cell swelling was significantly attenuated in SS cells, displaying a 6% (±1) reduction in cell volume after max swelling, as compared to 18% (±4) in non-SS controls. The swelling was slightly, but non-significantly, smaller in the SS group (SS group: max swelling after 60 s; max volume increase 12% (±3); non-SS controls: maximum swelling after 40 s; average volume increase 15% (±5) (Figure 3).

Exposing acinar cells to the hypo-osmotic solution (200 mOsm) led to a maximum increase in the free intracellular Ca^{2+} concentration for both groups within 20 s. Although no significant differences were observed in the magnitude of the Ca^{2+} response, non-SS controls had an average increase of 71% (±17) above baseline (t_0), vs 59% (±15) in SS cells (Figure 4). After reaching a maximum, the concentration decreased towards a plateau level and ended close to pre-stimulatory values for both groups after 15 min.

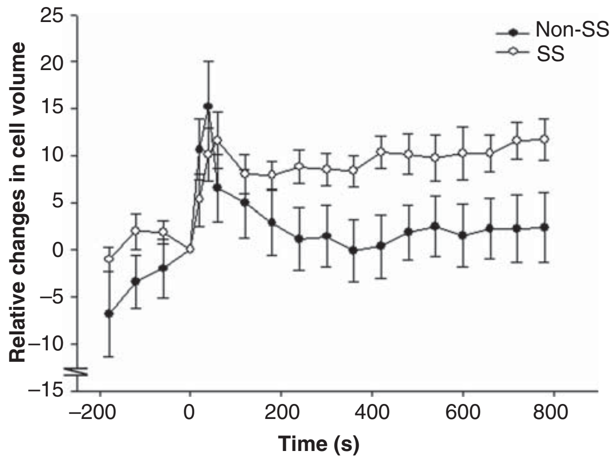

Figure 3. Cell volume regulatory response in cells exposed to hypo-osmotic solutions. Solution was changed from iso-osmotic (300 mOsm) to hypo-osmotic (200 mOsm) at t = 0. In non-SS controls ($n = 8$), a maximum swelling of 15% after 40 s was seen, compared to 12% after 60 s for SS patients ($n = 5$). Regulatory volume decrease (RVD) from max swelling to end of experiment was significantly less in acinar cells from SS patients (6% ± 1) compared to non-SS controls (18% ± 4) ($p < 0.05$).

Decreased intracellular Ca^{2+} response in SS after agonist stimulation

Significantly less free Ca^{2+} was recorded in the SS cells after carbachol stimulation than in the non-SS controls (maximum intracellular Ca^{2+} increase: 46% (±11) in SS; 69% (±17) in non-SS; Figure 5). At the end of the experiment, the free intracellular Ca^{2+} concentration from maximum had decreased, with 54% in the SS cells and 31% in the non-SS controls. Moreover, a general observation of the response curves was that the SS-group had a shorter and more limited Ca^{2+} response than the non-SS controls.

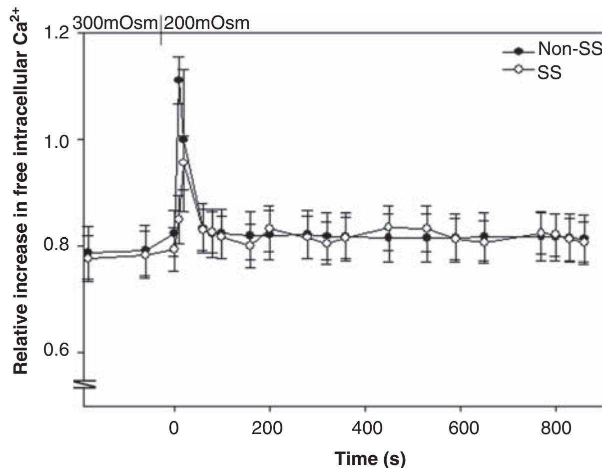

Figure 4. Intracellular free Ca^{2+} changes following hypo-osmotic exposure (200 mOsm). Max intracellular free Ca^{2+} increase for the non-SS controls ($n = 4$) occurred after 10 s and was 71% (±17) compared to 59% (±15) after 20 s in the SS group ($n = 5$). The amount of free Ca^{2+} increase in SS patients compared to non-SS controls was not significantly altered.

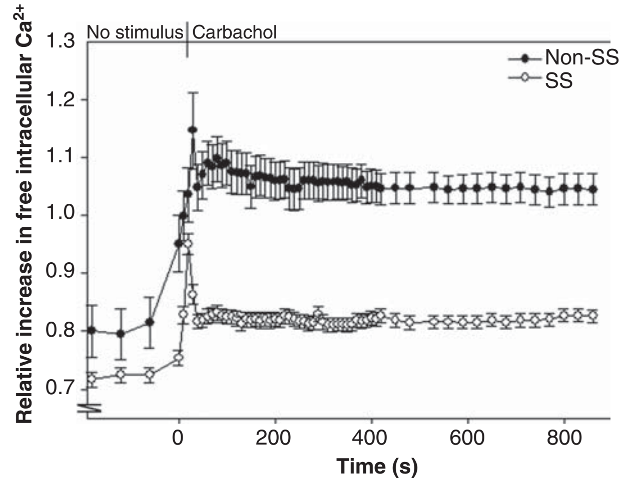

Figure 5. Intracellular free Ca^{2+} changes following carbachol stimulation. Exposing cells to 100 µM carbachol led to an increase in the free intercellular Ca^{2+} concentration in both groups. The maximum increase in free intracellular Ca^{2+} for the non-SS group ($n = 7$) occurred after 35 s and was 69% (±17) compared to a max increase of 46% (±11) after 20 s for the SS group ($n = 5$). Significant differences at all time-points after 20 s were observed between the two groups ($p < 0.05$).

Discussion

In this study, we have found a significantly reduced mRNA expression of AQP1 and AQP5 in labial salivary glands derived from SS patients. Furthermore, reduced levels of AQP1, 3 and 5 were observed in areas of the glands close to lymphocytic infiltrates. When investigating cell functions that are related to AQP physiology, we found that both cell volume regulation and intracellular calcium signaling were disturbed in SS cells.

Structural defects and abnormal distribution of AQP1 and 5 have previously been discussed in regard to SS. Steinfeld et al. [31] reported mislocation of AQP5 from apical to basal parts of the acini in SS. These findings were later confirmed by Tsubota et al. [29] in humans and by Konttinen et al. [28] in NOD mice, a well-known mouse model for SS. Similarly, Beroukas et al. [23,32] in two studies demonstrated selective down-regulation of myoepithelial AQP1, but found no abnormal translocation of AQP5 and AQP3. As we observed significantly decreased staining intensity for AQP1, 3 and 5 in regions close to lymphocytic infiltrates, qPCR was performed and showed significantly decreased mRNA expression of both AQP1 and AQP5 in SS patients compared to non-SS controls. To our knowledge, mRNA expression of AQPs in SS, using qPCR, has not previously been reported. It should be mentioned that the mRNA levels of AQPs may have been influenced by loss of secretory tissue with replacement of inflammatory or fibrotic tissue. However, if depletion of functional secretory tissue alone was the reason for the alterations found in AQP mRNA expression, one would expect significant differences to be found also for AQP3 (serving in sorts as an 'internal control').

In order to analyze whether decreased salivary secretion in SS was associated with cell volume regulation, fresh acinar cells were exposed to a hypo-osmotic solution. The average swelling was smaller in SS cells and the time to achieve maximum volume was longer than in non-SS derived cells, although these differences were not significant. We did, however, observe a significantly decreased ability to regulate cell volume back towards normal after peak swelling in SS compared to non-SS derived cells. These findings are in line with those of Krane et al. [19], who found that the water permeability in AQP5-deficient mice was greatly reduced. Consequently, we propose that, in SS patients, changes in expression and localization of AQP5 contribute to impaired water permeability and, thus, to delayed cell volume regulation.

Although no significant differences were observed in the magnitude of the Ca^{2+} response upon hypo-osmotic treatment of the cells, non-SS controls showed an average increase of 71% in the intracellular Ca^{2+} concentration from baseline, compared to 59% in SS cells, suggesting that the reduced ability in SS to regulate volume after hypo-osmotic exposure could still have been influenced by the reduced intracellular Ca^{2+} response. We cannot rule out, however, that other signaling mechanisms for cell volume regulation are affected in SS, or that the observed alterations arise from changes in cell–cell/cell–matrix contacts, abnormal cell cytoskeleton or intracellular signaling pathways, all of which might influence the function of the channels and transporters that carry out the unloading of osmotically active particles from the cells.

The SS cells displayed a significant decrease in the cellular response to carbachol. The reduced response to carbachol may result from receptor desensitization due to chronic muscarinic 3 (M3) receptor antibody exposure, as part of the autoimmunity disorder suggested by Cha et al. [33]. On the other hand, Beroukas et al. [25] suggested that circulating anti-muscarinic receptor antibodies may inhibit parasympathetic neurotransmission, in turn leading to a compensatory up-regulation of the inhibited M3 receptors. Circulating anti-lacrimal gland M3 receptor antibodies have in fact been observed in SS [24] and, rather than receptor desensitization, the response to carbachol stimulation may be due to anti-receptor antibodies. However, one would expect such antibodies to be removed during preparation of the glands for analysis and any compensatory up-regulation would have given the opposite results of the present ones. In line with the receptor desensitization hypothesis, Dawson et al. [26] reported that SS patients were only capable of proper Ca^{2+} response when a cholinergic agonist was administered at a maximum concentration. Contrary to the above findings, Pedersen et al. [34] reported on functional receptors with normal Ca^{2+} signaling in SS. The divergent results among these studies may be due to different methods being applied, to variations in inclusion criteria or to differences in disease manifestation.

The strength of this study is the use of fresh acinar cells from patients and in total a fair amount of patients was included. However, the access to human tissue is limited and the variations within the patient population are greater than in animals. Our control group was comprised of persons with symptoms of dryness and reduced secretion but not fulfilling the AECG classification for SS. Probably, even greater differences between test and control could have been demonstrated if our controls had not suffered from dryness symptoms. However, significant differences did exist between the non-SS and SS groups, underlining that the SS diagnosis is consistent with cell dysfunction.

Considering that the fresh glands had to be used for the physiological experiments prior to histological examination and because the access to fresh human material is limited, we experienced some difficulties in creating age- and sex-matched groups of SS and non-SS patients. Regarding age, no significant differences were found between the SS and non-SS group. Furthermore, although all men included belonged to the non-SS control group, no notable differences were found when male and female samples were compared within the non-SS group.

In summary, our study demonstrated changes in the localization and expression of AQPs in SS accompanied by decreased cell volume regulation and Ca^{2+} signaling. Thus, our results point to cellular and physiological mechanisms that may explain or contribute to the defective secretory capacity seen in SS.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Table 1.