Human postmortem lacrimal and submandibular glands stored in RNAlater are suitable for molecular, biochemical, and cell biological studies

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Purpose: Gene expression and protein analysis studies require high-quality human tissue which is a challenge and difficult to obtain through live human biopsies. Human postmortem lacrimal gland (LG) and submandibular gland (SMG) tissues have the potential to provide an invaluable source for studying the mechanisms involved in LG and SMG dysfunction. Therefore, we aimed to test the suitability of post-mortem LG and SMG for molecular, biochemical, and cell biological studies.

Methods: LG and SMG tissue from healthy donors was collected and immediately placed in RNAlater solution and then shipped overnight at 4 °C. After receipt, each gland was divided into three pieces for RNA, protein, and histological analysis, respectively. Total RNA isolated from each LG and SMG was analyzed for RNA integrity using an Agilent Bioanalyzer and reverse transcription–PCR (RT–PCR). For histology, tissues were embedded in paraffin and stained with hematoxylin and eosin. For protein analysis, lysates were prepared and processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting.

Results: When the LG and SMG samples were preserved in RNAlater, the RNA integrity number (RIN) values from the LG and SMG were >7.0 from all three donors, while the RNAs from tissue not preserved in RNAlater were of poorer quality. The gene and/or protein expression of E-cadherin, aquaporin 5, alpha-smooth muscle actin (α-SMA), β-actin, and GAPDH was preserved in all samples. In addition, histological analyses showed normal tubuloacinar structures of all glands with serous and mucous producing acini within lobules interspersed with adipose fat.

Conclusions: In this study, we determined that RNA, protein, and histological sections obtained from postmortem human LG and SMG tissue preserved in RNAlater were of high quality. This would provide a viable source of human LG and SMG tissue suitable for studies of diseases that affect these glands, such as Sjögren's syndrome.

Sjögren’s syndrome is a chronic autoimmune inflammatory disorder that affects mainly the moisture-producing exocrine glands, specifically the salivary gland and the lacrimal gland (LG) leading to dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca, KCS) [1]. Some of the hallmarks of Sjögren’s syndrome are the presence of focal lymphocytic infiltrates in the LG and the submandibular gland (SMG) and circulating autoantibodies [1,2]. The disease can be present alone (primary Sjögren’s syndrome) or along with an underlying connective tissue disorder, most commonly rheumatoid arthritis or systemic lupus erythematosus (secondary Sjögren’s syndrome). The etiology of Sjögren’s syndrome remains poorly understood, and the susceptibility to the disease can be attributed to the interplay among genetic, environmental, hormonal, and neuropsychological factors that underlie the complex mechanisms of this disease [1-3].

The mechanisms leading to dysfunction of the LG and the SMG in Sjögren’s syndrome remain elusive, and most of our understanding is based on studies that used animal models of this disease. Increasingly, human postmortem tissue is being studied to evaluate the cellular and molecular markers that play an important role in affecting the normal function of the LG and the SMG during disease conditions [4-6]. Although studies have been reported for several decades [5-9], present-day molecular and cellular techniques require increased tissue quality for improved sensitivity and specificity. Several studies from different human tissues have reported the importance of death to preservation (DP) time [10], postmortem interval [8,11], pH [12,13], and tissue preservation in RNAlater [10] for collecting human tissue of high quality.

Gene expression profiling is being used widely to study development, differentiation, and disease pathogenesis [8].
For a long time, the markers of tissue quality have been postmortem interval, agonal conditions, and donor age and health [14,15]. More recently, the important factors in collecting human tissues with high RNA integrity are postmortem time to preservation, the tissue’s metabolic profile, endogenous RNase activity, and degradation of RNA [8]. Several studies have been performed to evaluate optimal conditions to extract high-quality RNA from human ocular tissues. A study by Wang et al. reported that RNA quality from human ocular tissue is affected by DP time, preservation in RNA later, and overall RNA extraction technique [10]. In another study, it has been reported that vascular human ocular tissue with shorter DP time had higher RNA integrity [8]. This group also reported that non-vascular ocular tissues had higher-quality RNA compared to tissues with vasculature. However, to our knowledge there are no reports evaluating optimal protocols for extraction of RNA and proteins for human postmortem LG and SMG tissues.

In this study, we evaluated the RNA quality and integrity, protein expression, and histological immunoarchitecture of postmortem human LG and SMG tissue from three donors. We chose to study the expression and cellular distribution of key proteins important for LG and SMG architecture and function. Namely, we studied E-cadherin (a protein crucial for cell–cell and cell–matrix interactions), aquaporin 5 (a water channel important for fluid secretion), and alpha-smooth muscle actin (α-SMA, a protein expressed exclusively by myoepithelial cells and shown to be important for contraction). The ability to extract high-quality RNA, protein, and histological sections was confirmed from tissue preserved in RNA later.

**METHODS**

Human LG and SMG tissue samples from three donors were obtained from Advanced Tissue Services (Phoenix, AZ). The study was reviewed by the Tufts Medical Center/Tufts University Health Sciences IRB and was determined to be exempt in accordance with 45 CFR.101(b)(4). Tissues (whole LG and whole SMG) were preserved immediately in RNA later (about 60 ml/container) and shipped on ice overnight. All donors were female, and their ages at time of death were 62, 84, and 90 years. The LG and the SMG were removed less than 24 h after death.

**Materials:** The miRNeasy isolation kit and the QuantiNova SYBR Green PCR mix were purchased from Qiagen (Valencia, CA). Running buffer, transfer buffer, NuPage 4–12% Bis-Tris gels, and Invitrolon polyvinylidene fluoride (PVDF) membranes (0.45 µm) were procured from Invitrogen (Carlsbad, CA) while the blocking buffer was from Li-Cor Biosciences (Lincoln, NE). Antigen retrieval solution and 4’,6-diamidino-2-phenylindole (DAPI) were from Vector Laboratories (Burlingame, CA), and the normal donkey serum was purchased from Jackson ImmunoResearch Laboratories (Westgrove, PA). The following primary antibodies were used for immunohistochemistry and western blotting: rabbit monoclonal antibody against aquaporin 5 (1:100 or 1:5,000; Abcam Inc., Cambridge, MA); mouse monoclonal antibody against E-cadherin (1:100 or 1:5,000; BD Biosciences, San Jose, CA), rabbit polyclonal antibody against α-SMA (1:100 or 1:5,000; Abcam Inc.), and mouse monoclonal antibody against beta actin (1:10,000; Sigma-Aldrich Co., St. Louis, MO). Fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TRITC) conjugated secondary antibodies (1:100, Jackson ImmunoResearch Laboratories) were used for immunohistochemical staining, while IRDye 680RD or IRDye 800CW secondary antibodies (1:5,000; Li-Cor Biosciences) were used for western blotting.

**RNA extraction and RT–PCR:** Total RNA was extracted using the miRNeasy isolation kit (Qiagen) according to the manufacturer’s protocol. Briefly, tissue was disrupted and homogenized in Buffer RLT. The tissue homogenate was centrifuged for 10 min at 5000 × g using an Eppendorf Centrifuge 5415D (Hamburg, Germany). Next, the supernatant was collected and mixed with 1 volume of 70% ethanol that was run through an RNeasy midi spin column at 5000 × g for 5 min. Buffer RW1 and buffer RPE (two times) were added one

| Gene name | Primers | PCR Product (bp) |
|-----------|---------|------------------|
| Aquaporin 5 | F: GCCGTGTTCGCAAGTTCTT  R: TGTGTTGTGTGACCGTGG | 340 |
| E-Cadherin | F: AAGAGAATGGAAGTGTCGA  R: GATCGAGAAGTGTCCCTG | 282 |
| GAPDH | F: ATGATTCCACCATGGGCAAA  R: TTCACACCCATGACGAACAT | 262 |

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; F, forward; R, reverse; bp, base pairs.
after another to the RNeasy MinElute spin column and centrifuged at 5000 × g for 5 min. Last, 250 µl of RNase-free water was added to the spin column, and the RNA was collected. RNA purity and quantity were analyzed using NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA). The samples were then stored at −20 °C until use. Next, the quality of the RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to determine the RNA integrity value before the downstream PCR analyses.

Purified total RNA (20 ng) was used for reverse transcription and PCR amplification with OneStep RT–PCR Kit using primers specific to aquaporin 5, E-cadherin, or GAPDH in a thermal cycler (2720 Thermal Cycler; Applied Biosystems, Foster City, CA). The primers shown in Table 1 were designed using NCBI / Primer-BLAST. The reverse transcription reaction mixture was prepared according to the OneStep RT–PCR kit (Qiagen). The reaction was cycled through the following conditions: 52 °C for 30 min followed by 15 min of hot start at 95 °C, and 25 to 30 cycles of denaturation for

![Figure 1](http://www.molvis.org/molvis/v22/1221) Analysis of RNA integrity in human LG and SMG tissues. Total RNA was extracted from lacrimal gland (LG) and submandibular gland (SMG) tissue from three donors (D1, D2, and D3) and used to investigate RNA integrity.

**A**: Representative Bioanalyzer 2100 traces from human LG and SMG total RNA depicting high RNA integrity number (RIN) values.

**B**: Extracted RNA was used for reverse transcriptase PCR analysis of E-cadherin, aquaporin 5, and GAPDH (the control housekeeping gene).
Figure 2. Histological analysis of human LG and SMG tissue. Postmortem tissue was fixed and processed for paraffin embedding followed by hematoxylin and eosin staining. Each column represents tissue from different donors. Scale bar represents 50 µm in A, C, E, G, I, K (magnification 200X) and 25 µm in B, D, F, H, J, L (magnification 400X). sA, serous acinus; mA, mucous acinus; Ad, adipocytes. Arrows depict examples of serous-mucous acini.
Figure 3. Immunohistochemical analysis of common LG and SMG cellular proteins. Tissue was fixed and processed for paraffin embedding. The lacrimal gland (LG) (A, C, E) and submandibular gland (SMG) (B, D, F) sections were stained with antibodies against E-cadherin, aquaporin 5, and alpha-smooth muscle actin (α-SMA) and then counterstained with 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Images are from a single representative donor. Scale bar = 25 µm. Magnification = 400X.
40 s at 94 °C, annealing for 40 s at 53 °C, extension for 1 min at 72 °C, and a final extension at 72 °C for 10 min. After amplification, the products were separated by electrophoresis on a 1.5% agarose gel and visualized by ultraviolet (UV) light after ethidium bromide staining.

**Immunohistochemistry:** Tissues were fixed overnight at 4 °C in 4% formalin made in PBS (containing, in mM, 145 NaCl, 7.3 Na$_2$HPO$_4$, and 2.7 NaH$_2$PO$_4$ at pH 7.2) and then processed for paraffin embedding. The embedded tissues were sectioned at 6-μm thickness and mounted on microscope slides. Slides were deparaffinized and rehydrated in graded alcohol solutions. For the histopathology experiments, the tissue sections were stained with Mayer’s hematoxylin and eosin (H&E) dye. For the immunofluorescence experiments, the slides were subjected to heat-mediated antigen retrieval (15 min). Following washes in PBS (3X), nonspecific binding sites were blocked for 60 min with 10% normal donkey serum. The slides were then incubated overnight at 4 °C with the appropriate primary antibody. Sections without a primary antibody were included as negative controls. Next, the slides were incubated for 60 min at room temperature with the appropriate secondary antibody. Finally, the slides were covered with mounting medium that contained DAPI. Sections were viewed using a microscope equipped for epifluorescence (Nikon UFXII 135 Epi-Illuminator, Melville, NY).

**SDS–PAGE and western blotting:** Tissues were homogenized in 0.4 ml of ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), supplemented with protease inhibitors). The supernatant containing proteins was collected by centrifugation at 24,000 × g for 30 min. Equal amounts of proteins were loaded and separated using NuPage 4–12% Bis-Tris gels. For Coomassie blue staining, the gel was stained in a staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid) for 60 min with gentle agitation. Then, the gel was destained in destaining solution (40% methanol and 10% glacial acetic acid) until the background of the gel was fully clear.

For immunoblotting, following electrophoresis the proteins were transferred on the Invitrolon PVDF membranes. The blots were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) diluted 1:1 in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 60 min at room temperature. Next, the membranes were

![Image](http://www.molvis.org/molvis/v22/1221/)

**Figure 4.** Analysis of protein expression in human LG and SMG tissue. Proteins from the three donors (D1, D2, and D3) were extracted using radioimmunoprecipitation assay (RIPA) buffer and processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or western blotting. **A:** Protein samples were used for SDS–PAGE and Coomassie staining to investigate the total protein expression and integrity. **B:** Western blotting was used to investigate the expression of aquaporin 5, E-cadherin, alpha-smooth muscle actin (α-SMA), and β-actin in the lacrimal gland (LG) and submandibular gland (SMG) samples.
incubated overnight at 4 °C with the appropriate primary antibodies prepared in diluted blocking buffer. After a series of washes with TBST (TBS plus 0.05% Tween-20), the blots were incubated for 60 min at room temperature with the appropriate secondary antibody diluted (1:5,000) in blocking buffer. After three washes with TBS, the membranes were scanned using an Odyssey infrared imaging system.

RESULTS AND DISCUSSION

Postmortem cellular death sets in and the rapid degradation of RNA begins to occur if it is not quickly counteracted. Figure 1A shows that the LG and SMG tissue preserved in RNAlater have clean bioanalyzer traces with minimal noise between the peaks corresponding to the 18S and 28S rRNA species. These samples and all other donor samples preserved in RNAlater solution displayed RNA integrity (RIN) values equal to or greater than 7.0. In contrast, the tissue not preserved in RNAlater solution had statistically significantly lower RIN values (data not shown), indicating poor RNA quality. However, although RIN values are a sensitive indicator of RNA quality, certain mRNAs may still be favorably degraded [16,17]. Thus, after we confirmed the ability to extract high-quality RNA from tissue preserved in RNAlater, we evaluated the ability to detect abundant genes of interest in the LG and the SMG: aquaporin 5, E-cadherin, α-SMA, and GAPDH. As seen in Figure 1B, all of these mRNA molecules were amplifiable and detectable at the correct size.

Clearly, RNAlater possesses the ability to preserve RNA quality in postmortem tissues as previously described [8,10] and as shown in Figure 1. However, the ability to preserve the histology and protein quality is just as important in studies of the LG and the SMG. The histology of postmortem LG and SMG tissue was therefore evaluated on H&E stained sections. As shown in Figure 2, the LG sections contained well-preserved lobules with serous acini and ducts while the SMG sections contained lobules with serous and mucous acini, as well as ducts. The presence of adipocytes and dispersed lymphocytes was also observed, especially in tissues from the oldest donor. These results suggest that storing tissues in RNAlater seems to preserve LG and SMG gross histology.

We next aimed to determine whether storing the LG and SMG tissues in RNAlater affected the expression and distribution of key proteins. First, immunohistochemical analyses were performed to determine the distribution of E-cadherin, α-SMA, and aquaporin 5, three major proteins important for tissue architecture and secretory function. Figure 3 shows that E-cadherin is expressed on the basolateral membranes (contact with the basement membrane and the extracellular matrix) as well as at sites of cell-to-cell contact. Aquaporin 5 expression was detected mostly on apical membranes similar to what was reported on biopsies or animal LG and SMG (Figure 3). Last, the expression of α-SMA, a protein expressed by the myoepithelial cells in the LG and SMG, was as expected detected around acini and ducts in both tissues (Figure 3). We subsequently performed SDS–PAGE on cell lysates prepared from the LG and SMG tissues. As shown in Figure 4A, the isolated proteins from both types of tissue showed a normal pattern when separated on SDS–PAGE. The proteins from the gels were then transferred to nitrocellulose membranes and immunoblotted with antibodies against E-cadherin, α-SMA, aquaporin 5, and β-actin. Figure 4B shows that all of these proteins were detectable at the right molecular weight in all LG and SMG samples. Together with the immunohistochemistry data, the SDS–PAGE and western blotting data indicate that shipping tissues in RNAlater solution seems to preserve protein integrity.

Taken together, the data suggest that postmortem LG and SMG tissue preserved in RNAlater is a viable source of high-quality RNA, protein, and histological samples. This is of great importance as the ability to obtain biopsies of LG and SMG from live human donors is not easily available. This will provide a great opportunity to look at potential markers that are altered during LG and SMG dysfunction in diseases such as Sjögren’s syndrome. Extracting high-quality protein and RNA samples is critical for in-depth studies of cellular and molecular markers of the transcriptome and proteome using techniques such as RNA-sequencing and mass spectrometry, which have become ubiquitous in disease-related research.

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