Step-by-step protocol to perfuse and dissect the mouse parotid gland and isolation of high-quality RNA from murine and human parotid tissue

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Macroscopic identification and surgical removal of the mouse parotid gland is demanding because of its anatomic location and size. Moreover, the mouse parotid gland contains high concentrations of RNases, making it difficult to isolate high-quality RNA. So far, appropriate methods for optimal perfusion-fixation and dissection of mouse parotid glands, as well as the isolation of high quality RNA from this tissue, are not available. Here we present a simple, optimized, step-by-step surgical method to perfuse and isolate murine parotid glands. We also compared two common RNA extraction methods (RNeasy Mini Kit versus TRIzol) for their yields of high-quality, intact RNA from human and murine parotid gland tissues that were either snap-frozen or immersed in RNA*later* stabilization solution. Mouse parotid tissue that was perfused and immersed in RNA*later* and human samples immersed in RNA*later* exhibited the best RNA quality, independent of the isolation method.

The parotid gland (glandula parotidea) is one of the major salivary glands present in humans and rodents. The secretory cells of the parotid acini mainly produce serous saliva. Saliva acts as an antibacterial, buffering, and lubricating agent

(1). The basic functions of the parotid gland and its gene expression can be studied using animal models such as the mouse (2). However, no step-by-step protocol for perfusion-fixation and dissection of mouse parotid glands has

been published. Furthermore, proper quantitative gene expression analysis and many other molecular biology applications involving the parotid gland can only be achieved by isolating high-quality RNA. Interestingly, like the pancreas, the parotid gland contains several RNases and other digestive enzymes that are highly expressed, making isolation of intact RNA from this tissue challenging (2,3). Indeed, autolysis of the parotid may start immediately after its removal from the blood circulation. We therefore sought to develop an easy, standardized protocol for the optimal extraction of high-quality RNA from this organ. Furthermore, a method for perfusion-fixation of the mouse parotid gland that preserves RNA stability has not been described before. Here we report a reproducible, step-by-step protocol for the surgical preparation and perfusion-fixation of the murine head and neck region with subsequent dissection of the parotid gland and isolation of high-quality total RNA. We also compared two different RNA isolation techniques on fresh human, fresh murine, and perfusion-fixed murine tissue.

Twelve adult C57Bl/6J wild type mice (specific pathogen free; 6 male, 6 female; 22–23 weeks old) obtained from Charles River Laboratories (Sulzfeld, Germany) were used as approved by the governmental ethics committee for animal welfare. Mice were killed by cervical dislocation (Regierungspraesidium Giessen, Germany, permit number: V 54–19 C 20/15 c GI 20/23). The skin of the chest and head/neck region was opened by a medial incision starting from the epigastrium toward the limbs (Figure 1, A–C). The thorax was opened with a trapezoid cut (Figure 1, D–E). A small incision was made in the apex of the left ventricle, and the perfusion cannula (23 gauge) was inserted into the incision and gently pushed into the aortic arch (Figure 1F). Perfusion [4% paraformaldehyde (PFA) in PBS made with RNase-free

METHOD SUMMARY

The mouse head and neck region was perfusion-fixed through the aortic arch, after which the parotid glands were surgically removed. RNA isolation was performed from RNA*later*-immersed or snap-frozen human or murine tissues using the RNeasy Mini Kit or TRIzol according to the manufacturer's protocol. RNA quality was examined using an Agilent 2100 Bioanalyzer, and the isolated RNA was analyzed by qRT-PCR for 28S rRNA and *HPRT* mRNA.

water at pH 7.4] was performed for 2–3 min. For a detailed description of how to identify and separate the parotid gland from other tissue during the dissection procedure, see Supplementary Figure S1. The correctly identified parotid glands were lifted up with a pair of forceps to locate the borders of the organ and cut out, including a part of the main excretory duct. Afterwards the parotid glands were separated from the surrounding tissue (Figure 1, G–I) (4). Although the major lobe of the parotid gland can be divided using sialography into three lobes (superior, middle, and inferior lobes, extending toward the ear and the submandibular gland) (5), we did not identify these lobe borders by macroscopic inspection with the naked eye. Some isolated parotid glands were immediately immersed in *RNA/later* (Qiagen, Valencia, CA) and then frozen in liquid nitrogen. In contrast, other mouse parotid samples were directly excised (without perfusion) and either snap-frozen or immersed in *RNA/later* followed by snap-freezing. Human parotid gland tissue was excised following standard operating procedures during surgery on benign parotid gland tumors. One sample of the excised human tissue was directly snap-frozen, and another sample was immersed in *RNA/later* with subsequent snap-freezing. All tissue samples were stored at -80°C prior to further use. Tissue homogenization and subsequent RNA isolation were performed using the TissueLyser LT (Qiagen) in combination with either the RNeasy Mini Kit (Qiagen) or TRIzol (Life Technologies, Darmstadt, Germany), according to the manufacturer's instructions, including DNase digestion. The integrity of the isolated RNA was evaluated using the Agilent 2100 Bioanalyzer system and the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). RNA integrity numbers (RINs) (from 1–10) and electrophoresis results (fluorescence measurements and gel images) are shown in Figure 2 (6). Quantitative RT-PCR (qRT-PCR) analysis was performed on the samples with the highest RNA quality to demonstrate their suitability for downstream applications. The High Capacity RNA-to-cDNA Kit (Applied Biosystems, Weiterstadt, Germany) was used for reverse transcription, and qPCR was performed

Figure 1. Step-by-step protocol for perfusion and preparation of mouse parotid glands. (A) Mouse after cervical dislocation; dashed lines indicate where to cut the skin. (B) Mouse situs after the initial incisions. (C) Opened situs; the black arrow shows the location of the parotid gland. (D) Higher magnification of the opened situs; dashed lines mark the incision to open the thorax. (E) Opened thorax. (F) Perfusion through the aortic arch; the white arrow depicts the tip of the cannula, and the red box indicates the section magnified in the next panel. (G) Enlargement of the parotid region. P: parotid gland; *: neighboring tissue; S: sublingual gland. (H) Upper part: explanted parotid gland with neighboring tissue (*); lower part: dissected parotid and separated neighboring tissue (*). Scale bar is in cm. (I) Hematoxylin and eosin (H&E) staining of the parotid gland (P) with attached lymph node tissue (*).

using the SYBR Select Master Mix Kit (Life Technologies) and the StepOnePlus Real-Time PCR System (Life Technologies) according to the manufacturer's instructions. Primers were obtained from Eurofins (Friedrichsdorf, Germany) (Supplementary Table S1).

In general, RIN values of samples isolated with the RNeasy Mini Kit were slightly higher than with TRIzol but only exhibited a significant difference ($P < 0.05$) between perfusion-fixed mouse samples (Figure 2 and Supplementary Figure S2). Regardless of the RNA

Figure 2. RNA quality obtained using different isolation methods. (A) Comparison of the RNA integrity numbers (RIN) of murine samples that were (i) snap frozen; (ii) immersed in RNA*later* and subsequently frozen; or (iii) perfusion-fixed with a solution of 4% paraformaldehyde (PFA) in PBS, immersed in RNA*later*, and subsequently frozen. The graph presents RIN results as mean $\pm$ SEM. Significances were calculated among all groups (RNeasy Mini Kit versus TRIzol) by one-way ANOVA and then split in two graphs; the results for TRIzol are shown in Supplementary Figure S2. The perfusion-fixed samples showed a significantly higher RIN than the other 2 mouse samples ($***P < 0.001$; $n = 3$); NS: not statistically significant. (B) The human samples (frozen within 30 s after surgical excision) exhibited a significant difference between the snap-frozen and RNA*later*-immersed samples ($*P < 0.05$; $n = 3$). (C–G) Fluorescence intensity plots and gel images of the RNA samples isolated with the RNeasy Mini Kit from snap frozen (C,D), RNA*later*-immersed/frozen (E,F), and perfusion fixed/RNA*later*-immersed/frozen mouse (C,E,G) and human (D,F) parotid tissue. (H) Amplification plots of three murine and three human samples (with three technical replicates for each) subjected to real-time PCR for amplification of 28S rRNA (black) and human *HPRT* mRNA (red).

isolation method used, the RNA quality we obtained from differently pre-treated mouse parotid gland samples showed a significant difference between the perfusion-fixed group and the other experimental groups (Figure 2A and Supplementary Figure 2A). In contrast to the mouse samples, the quality of RNA isolated from human tissue either immersed in RNA/ater or snap frozen was excellent (Figure 2, B, D, and F). Mouse samples that were snap frozen exhibited almost no 18S and 28S bands and a strongly increased baseline signal (Figure 2C), whereas in human samples a clear banding pattern was detected (Figure 2D). Mouse RNA samples that were solely immersed in RNA/ater (without perfusion-fixation) showed a mean RIN of 3 and an increased baseline signal with multiple spikes indicating RNA fragmentation (Figure 2E), although no RNA degradation was observed in human samples (Figure 2F). The perfused mouse tissue that was subsequently immersed in RNA/ater showed the best results, exhibiting RIN values >7 and clearly visible 18S and 28S peaks/bands (Figure 2G). The amplification values for 28S rRNA and *HPRT* mRNA were used as indicators of the suitability of the isolated RNA for qRT-PCR (Figure 2H).

Based on these results, we suggest immediate perfusion-fixation with PFA before further preparation of the head and neck region of the mice in order to prevent degradation of parotid gland RNA. The lower RIN values of samples isolated from non-perfused mouse tissue were not significantly different between those that were snap-frozen versus those that were RNA/ater-immersed ($P = 0.41$). However, the RNA/ater-immersed samples exhibited a lower degree of RNA degradation. In contrast to the mouse samples, human samples were excised under continuous blood circulation, reducing the duration of hypoxia to a minimum prior to freezing, which might explain the higher RNA quality of these samples.

We recommend to perfuse mice via the left ventricle or aortic arch, dissect out the parotid glands quickly, and immediately immerse them in RNA/ater for the optimal preparation of high-quality RNA from mouse parotid

tissue. For human samples, immediate immersion into RNA/ater of tissue obtained in the operation room yields good RNA quality.

Author contributions

C.W., E.B.-V., and S.K. designed the research. C.W., K.P.V., S.K., and S.W. performed the research. S.W., C.W., J.P.K., E.B.-V., and S.K. contributed reagents and analytic tools. C.W., K.P.V., and S.K. acquired the data. C.W., K.P.V., S.W., E.B.-V., and S.K. analyzed and interpreted the data. C.W., E.B.-V., and S.K. wrote the paper. S.K. conceived of and supervised the study.

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Competing interests

The authors declare no competing interests.

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