Table 1. Characteristics for the subjects in this study

	SS	Non-SS
Number of Subjects	10	5
Sex (male/female)	1/9	0/5
Age	66.9 ± 9.4	69.8 ± 4.6
Gum test (ml/10 min)	3.59 ± 2.31	6.28 ± 2.05
Saxon test (g/2 min)	1.84 ± 1.56	2.01 ± 1.53

Values represent the mean ± SD. SS, Sjogren's syndrome; non-SS, dry mouth group.

Parkinson's disease were excluded from the study. The enrolled subjects had subjective dry mouth that was attributable to various factors (e.g., SS, adverse medication effects, mental stress, and depression). All 15 subjects provided informed consent. Each participant consumed one test tablet per day. The evaluation of the effects was performed by measuring salivary secretion and by a self-completed questionnaire (15 questions about oral and eye conditions) before intake and after 1 and 2 months of isoflavone intake.

Preparation of saliva. Tests were performed before intake and 1 and 2 months after oral intake of the isoflavone tablet. A piece of sterilized gauze was weighed before and after being chewed by a participant for 2 min (Saxon test). The difference between the two measurements (dry weight before chewing and wet weight after chewing) was regarded as the salivary secretion. The saliva samples were centrifuged at 10,000 rpm for 30 min and then passed through an ultrafiltration membrane (pore size, 0.22 μm).

Measurement of oxidative stress. The filtered samples were subjected to an enzyme-linked immunosorbent assay for the measurement of the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) and the lipid peroxidation markers hexanoyl-lysine (HEL) and propanoyl-lysine (PRL) using an anti-8-OHdG monoclonal antibody (N45.1; Institute for the Control of Aging, Shizuoka, Japan), an anti-HEL monoclonal antibody (Institute for the Control of Aging), and an anti-PRL monoclonal antibody (Healthcare Systems Inc., Aichi, Japan), respectively. The marker 8-OHdG in the sample was analyzed by an antibody chip[39] that was developed by Healthcare Systems Inc. As an antigen, 8-OHdG-BSA was spotted and immobilized onto the chip. The sample was then applied to the chip with a specific monoclonal antibody against 8-OHdG.[40] The chip was further treated with anti-mouse immunoglobulin antibody alkaline phosphatase labelled. The binding of the antibody was evaluated by chemiluminescence using CDP-Star. The amount of 8-OHdG was estimated by comparison with the standard curve of authentic 8-OHdG. HEL and PRL were tested in the same manner as 8-OHdG.

Measurement of isoflavones. The frozen saliva samples were thawed on ice. Three aliquots of saliva were transferred to sample tubes. The internal standard solution (genistein-d_4, daidzein-d_4, and equol-d_4) was added to each saliva sample (400 μl) to obtain a concentration of 125 pmol/ml. After the addition of ascorbic acid (50 μl of 0.1% phosphoric acid), the samples were hydrolyzed in the presence of the enzyme (60 μg) in phosphate buffer (pH 5.3, 500 μl) for 2 h at 37°C. After hydrolysis, the isoflavones were extracted with ethyl acetate (1 ml) twice and concentrated using a centrifugal evaporator. The dried extract was redissolved in 20 μl of 20% acetonitrile containing 0.1% acetic acid. A portion (5 μl) of the solution was subjected to LC-MS/MS analysis. An HPLC system (SI-2, Shiseido, Tokyo, Japan) connected to a quadruple MS/MS system API 4000 Qtrap (AB SCIEX, Santa Clara, CA) was used, and data acquisition and mass spectrometric evaluation were conducted using Analyst 1.5.1 software (AB SCIEX). The HPLC gradient conditions were as follows: the ratio of methanol containing 0.1% acetic acid (solution B) was increased linearly against the 0.1% acetic acid (solution A) after 4 min from 20% to 60% over 11 min and then to 90% over 5 min with a flow rate of 0.3 ml/min on a Zorbax Eclipse XDB column (2.1 × 150 mm, 5 μm, Agilent Technologies, city, CA) at 40°C. Selected reaction monitoring (SRM) was used to perform mass spectrometric quantification of isoflavones (precursor ion to product ion transitions from *m/z* 241/119 for equol, *m/z* 245/123 for equol-d_4, *m/z* 253/133 for daidzein, *m/z* 257/136 for daidzein-d_4, *m/z* 269/133 for genistein, and *m/z* 273/136 for genistein-d_4). The column eluent was introduced into the mass spectrometer using electrospray ionization in the negative-ion mode with a declustering potential of –90 V and ion spray voltage of –4,400 V. The temperature of the gas was 500°C. Nitrogen was used as the collision gas.

Statistical analysis. The results are expressed as the mean ± SD. Two-way repeated measures ANOVA was performed to test for the main effects of group ("SS" or "non-SS"), time ("before intake" and "after intake"), and their interaction. These analyses were performed using IBM SPSS (Statistical Package for the Social Sciences) Statistics ver. 19 (IBM Japan Inc., Tokyo, Japan). The data were analyzed for statistical significance, and the significance level was set at $p<0.05$.

Ethics. Informed consent was obtained from all subjects, and the Ethical Committee of Tsurumi University approved this study.

Results

Saliva flow rate. The saliva flow rate results are shown in Table 2. The Saxon test results showed a significant increase ($p = 0.005$) after 1 month (2.47 ± 1.66 g) and after 2 months (2.34 ± 1.65 g) compared with before intake (1.90 ± 1.50 g). No significant difference was found between the SS and non-SS groups.

Oxidative stress markers. The oxidative stress results for the 8-OHdG, HEL, and PRL levels are shown in Table 3. No significant differences were found among the measured data. For 8-OHdG, the comparison of the amount before intake (6.12 ± 7.79 ng/ml) with the amounts after 1 month (3.82 ± 3.21 ng/ml) and 2 months (3.87 ± 3.48 ng/ml) revealed an insignificant decrease. Meanwhile, HEL showed an insignificant decrease when the amount before intake (7.12 ± 8.35 ng/ml) was compared with that after 1 month (4.53 ± 5.83 ng/ml) or 2 months (4.71 ± 4.43 ng/ml). In general, the levels of 8-OHdG and HEL tended to decrease following intake. In contrast, for PRL, the comparison of the amount before intake (5.61 ± 9.92 ng/ml) with the amounts 1 month (8.86 ± 14.20 ng/ml) and 2 months (8.21 ± 13.36 ng/ml) after intake revealed an insignificant increase.

Table 2. Score for saliva flow rate pre- and post-intake of isoflavones for dry mouth patients

Item		Before	After 1 month	After 2 months	Results of two-way repeated measures ANOVA					
					Source of variation	SS (Type III)	DF	MF	F	*p* value
Saxon	Total	1.90 ± 1.50	2.47 ± 1.66	2.34 ± 1.65	Time	3.779	2.000	1.890	6.529	0.005*
	SS	1.84 ± 1.56	2.10 ± 1.59	2.10 ± 1.72	Time × SS or non-SS	1.495	2.000	0.747	2.583	0.095
	non-SS	2.01 ± 1.54	3.20 ± 1.71	2.84 ± 1.55	SS or non-SS	4.507	1.000	4.507	0.619	0.445

Results of two-way repeated measures ANOVA. Values represent the mean ± SD. Total, all subjects; SS, Sjogren's syndrome group; non-SS, dry mouth group; *Time, $p < 0.05$.

Table 3. Score for detection of oxidative stress in saliva pre- and post-intake of isoflavones for dry mouth patients

Item		Before	After 1 month	After 2 months	Results of two-way repeated measures ANOVA				
					Source of variation	SS (Type III)	DF	MF	F	*p* value
8-OHdG (ng/ml)	Total	6.12 ± 7.79	3.82 ± 3.21	3.87 ± 3.48	Time	61.042	2.000	30.521	1.628	0.216
	SS	5.77 ± 7.03	4.19 ± 3.07	4.16 ± 3.79	Time × SS or non-SS	9.474	2.000	4.737	0.253	0.779
	non-SS	6.83 ± 10.03	3.07 ± 3.73	3.29 ± 3.06	SS or non-SS	0.961	1.000	0.961	0.019	0.893
HEL (nmol/L)	Total	7.12 ± 8.35	4.53 ± 5.83	4.71 ± 4.43	Time	40.953	2.000	20.476	1.522	0.237
	SS	7.19 ± 9.11	3.79 ± 3.46	4.21 ± 3.46	Time × SS or non-SS	10.621	2.000	5.310	0.395	0.678
	non-SS	6.96 ± 7.56	6.01 ± 9.37	5.72 ± 6.32	SS or non-SS	13.617	1.000	13.617	0.131	0.723
PRL (nmol/L)	Total	5.61 ± 9.92	8.86 ± 14.20	8.21 ± 13.36	Time	42.960	2.000	21.480	0.280	0.758
	SS	7.82 ± 11.66	12.82 ± 16.17	11.73 ± 15.35	Time × SS or non-SS	49.170	2.000	24.585	0.320	0.729
	non-SS	0.35 ± 0.55	0.64 ± 1.21	0.31 ± 0.64	SS or non-SS	938.030	1.000	938.029	3.282	0.093

Results of two-way repeated measures ANOVA. Values represent the mean ± SD. Total, all subjects; SS, Sjogren's syndrome group; non-SS, dry mouth group; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; HEL, hexanoyl-lysine; PRL, propanoyl-lysine.

Table 4. Score for isoflavone concentrations in saliva pre- and post-intake of isoflavones for dry mouth patients

Item		Before	After 1 month	After 2 months	Results of two-way repeated measures ANOVA				
					Source of variation	SS (Type III)	DF	MF	F	*p* value
Equol (μmol)	Total	0.27 ± 0.45	0.48 ± 1.00	0.23 ± 0.53	Time	1.148	2.000	0.574	0.418	0.664
	SS	0.35 ± 0.55	0.64 ± 1.2	0.31 ± 0.64	Time × SS or non-SS	1.278	2.000	0.639	0.465	0.634
	non-SS	0.13 ± 0.05	0.18 ± 0.10	0.07 ± 0.05	SS or non-SS	0.131	1.000	0.131	0.086	0.774
Daidzein (μmol)	Total	32 ± 54	11 ± 9.8	45 ± 55	Time	8775.315	2.000	4387.658	3.452	0.047*
	SS	41 ± 65	12 ± 11	40 ± 55	Time × SS or non-SS	2960.434	2.000	1480.217	1.165	0.328
	non-SS	12 ± 18	9.7 ± 7.4	54 ± 61	SS or non-SS	383.663	1.000	383.663	0.102	0.755
Genistein (μmol)	Total	47 ± 70	20 ± 22	64 ± 72	Time	14901.865	2.000	7450.932	3.861	0.034*
	SS	63 ± 82	22 ± 25	59 ± 69	Time × SS or non-SS	6863.201	2.000	3431.601	1.778	0.189
	non-SS	13 ± 15	15 ± 13	73 ± 84	SS or non-SS	1933.870	1.000	1933.870	0.285	0.603

Results of two-way repeated measures ANOVA. Values represent the mean ± SD. Total, all subjects; SS, Sjogren's syndrome group; non-SS, dry mouth group; *Time, $p < 0.05$. Most of the equol scores were under the limit of detection.

Isoflavone concentrations in saliva. The concentration of each isoflavone (daidzein, genistein, and equol) is shown in Table 4. A significant main effect of time (after intake vs before intake) was noted for daidzein and genistein. Daidzein showed a significant increase when the amount before intake (32 ± 54 μmol) was compared with the amounts after 1 month (11 ± 9.8 μmol) and 2 months (45 ± 55 μmol), ($p = 0.047$). For genistein, a comparison of the amount before intake (47 ± 70 μmol) with the amounts 1 month (20 ± 22 μmol) and 2 months (64 ± 72 μmol) after intake revealed a significant increase ($p = 0.034$). For equol, a comparison of the amount before intake (0.27 ± 0.45 μmol) with the amounts after 1 month (0.48 ± 1.0 μmol) and 2 months (0.23 ± 0.53 μmol) revealed no significant difference. We speculate that the smaller than anticipated changes were due to the limits in the sensitivity of the employed tests.

Subjective measurements. The changes in the patient oral conditions are shown in Table 5. The results of the two-way repeated measures ANOVA showed the interaction between two main factors (time and SS vs non-SS) on "dry mouth" ($p = 0.031$) and "need water during eating" ($p = 0.020$). The condition of the eye regarding eyestrain, blurriness, dryness, and eye ache is shown in Table 6. No significant difference was found in any of the measured data.

Discussion

In a human intervention clinical trial, isoflavones were shown to be effective in the prevention and relief of menopausal symptoms.[41] Menopausal women with rapidly declining estrogen levels were reported to show decreased salivary secretion and intraoral discomfort.[42–44] In addition, considering reports that ERs are found in the salivary glands and that estrogen itself is secreted in the saliva, decreasing estrogen levels are assumed to affect salivary secretion. Furthermore, menopausal women on hormone replacement therapy showed improvements in a number of oral health-related complaints, such as dry mouth, glossalgia, periodontal disease, oral stickiness, and dysgeusia.[44,45] In this study, a significant effect was observed in the amount of saliva; oxidative stress levels showed a decreasing trend, and the interaction between isoflavone intake and the presence of SS was confirmed regarding the items "dry mouth" and "need water when eating" by the intake of 25 mg of soybean isoflavones (23 mg as aglycone) per day for two months in 15 subjects who recognized dry mouth symptoms. Therefore, the intake of isoflavones was thought to be effective in the relief of dry mouth that occurs in menopause and SS and of the general physical complaints of SS-affected individuals who presented with serious dry mouth.

As a result of measuring these three oxidative stress items before and after intake, 8-OHdG and HEL showed decreased levels and indicated the possibility that oxidative stress was reduced and the amount of saliva was increased by the intake of isoflavones. With regard to the antioxidant effect of isoflavones, the potential that the saliva secretional capacity was activated by eliminating oxidative stress and that the amount of saliva increased by activating the water secretion mechanism can be considered as an effect is expected in the improvement of the salivary secretion capacity by the continuous intake of isoflavones.

Furthermore, in a human study of isoflavone supplementation, a significant improvement in blood flow was found.[46] The components of saliva are derived from the blood and salivary glands, and the increase in salivary secretion was proposed to be due to "the increased blood flow". Thus, the possibility that the intake of isoflavones is useful for the recovery of salivary glands impaired by oxidative stress and for the improvement of blood flow is

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Table 5. Score for the subjective measurements of the oral condition pre- and post-intake of isoflavones for dry mouth patients

Item		Before	After 1 month	After 2 months	Results of two-way repeated measures ANOVA					
					Source of variation	SS (Type III)	DF	MF	F	*p* value
Dry mouth	Total	4.07 ± 1.44	4.00 ± 1.13	3.87 ± 1.06	Time	0.067	2.000	0.033	0.178	0.838
	SS	4.60 ± 1.26	4.30 ± 1.25	4.10 ± 1.20	Time × SS or non-SS	1.489	2.000	0.744	3.977	0.031*
	non-SS	3.00 ± 1.22	3.40 ± 0.55	3.40 ± 0.55	SS or non-SS	11.378	1.000	11.378	3.292	0.093
Have a cough or phlegm	Total	2.33 ± 1.05	2.53 ± 1.19	3.47 ± 4.96	Time	23.022	2.000	11.511	1.506	0.240
	SS	2.30 ± 1.16	2.50 ± 1.18	2.30 ± 1.06	Time × SS or non-SS	25.689	2.000	12.844	1.681	0.206
	non-SS	2.40 ± 0.89	2.60 ± 1.34	5.80 ± 8.56	SS or non-SS	15.211	1.000	15.211	1.420	0.255
Difficulty in chewing	Total	2.67 ± 1.40	2.73 ± 1.16	2.60 ± 1.30	Time	0.089	2.000	0.044	0.100	0.906
	SS	2.80 ± 1.62	3.00 ± 1.25	2.80 ± 1.48	Time × SS or non-SS	0.267	2.000	0.133	0.299	0.744
	non-SS	2.40 ± 0.89	2.20 ± 0.84	2.20 ± 0.84	SS or non-SS	3.600	1.000	3.600	0.860	0.371
Difficulty in swallowing	Total	3.00 ± 1.41	3.07 ± 1.16	2.80 ± 1.37	Time	0.467	2.000	0.233	1.000	0.382
	SS	3.30 ± 1.57	3.40 ± 1.17	3.10 ± 1.52	Time × SS or non-SS	0.022	2.000	0.011	0.048	0.954
	non-SS	2.40 ± 0.89	2.40 ± 0.89	2.20 ± 0.84	SS or non-SS	8.711	1.000	8.711	1.935	0.188
Difficulty in speaking	Total	2.67 ± 1.35	2.80 ± 1.08	2.53 ± 1.19	Time	0.200	2.000	0.100	0.239	0.789
	SS	2.90 ± 1.52	3.00 ± 1.05	2.50 ± 1.27	Time × SS or non-SS	1.267	2.000	0.633	1.515	0.239
	non-SS	2.20 ± 0.84	2.40 ± 1.14	2.60 ± 1.14	SS or non-SS	1.600	1.000	1.600	0.436	0.521
Worried about mouth or tooth condition	Total	3.40 ± 1.30	3.27 ± 1.49	2.87 ± 1.30	Time	1.867	2.000	0.933	2.220	0.129
	SS	2.60 ± 1.52	2.60 ± 1.82	2.20 ± 0.84	Time × SS or non-SS	0.089	2.000	0.044	0.106	0.900
	non-SS	2.60 ± 1.52	2.60 ± 1.82	2.20 ± 0.84	SS or non-SS	11.378	1.000	11.378	2.648	0.128
Have tooth or mouth sensitivity	Total	3.13 ± 1.46	3.07 ± 1.28	2.60 ± 1.40	Time	1.800	2.000	0.900	2.265	0.124
	SS	3.40 ± 1.26	3.20 ± 1.14	2.70 ± 1.42	Time × SS or non-SS	0.467	2.000	0.233	0.587	0.563
	non-SS	2.60 ± 1.82	2.80 ± 1.64	2.40 ± 1.52	SS or non-SS	2.500	1.000	2.500	0.485	0.498
Need water when eating	Total	3.87 ± 1.25	3.87 ± 1.30	3.67 ± 1.18	Time	0.022	2.000	0.011	0.056	0.945
	SS	4.20 ± 1.32	4.20 ± 1.40	3.70 ± 1.34	Time × SS or non-SS	1.800	2.000	0.900	4.558	0.020*
	non-SS	3.20 ± 0.84	3.20 ± 0.84	3.60 ± 0.89	SS or non-SS	4.900	1.000	4.900	1.203	0.293
Mouth feels pasty	Total	3.40 ± 1.30	3.33 ± 1.11	3.00 ± 1.31	Time	1.089	2.000	0.544	1.755	0.193
	SS	2.70 ± 1.49	2.90 ± 1.29	2.70 ± 1.34	Time × SS or non-SS	0.556	2.000	0.278	0.895	0.421
	non-SS	3.60 ± 1.34	3.20 ± 1.10	3.20 ± 1.30	SS or non-SS	0.178	1.000	0.178	0.041	0.842
Painful tongue	Total	2.80 ± 1.57	2.87 ± 1.41	2.80 ± 1.37	Time	0.000	2.000	0.000	0.000	1.000
	SS	2.70 ± 1.49	2.90 ± 1.29	2.70 ± 1.34	Time × SS or non-SS	0.356	2.000	0.178	0.937	0.405
	non-SS	3.00 ± 1.87	2.80 ± 1.79	3.00 ± 1.58	SS or non-SS	0.278	1.000	0.278	0.044	0.838
Worried about bad breath	Total	2.40 ± 1.06	2.53 ± 0.99	2.47 ± 0.99	Time	0.089	2.000	0.044	0.234	0.793
	SS	2.30 ± 0.95	2.50 ± 1.08	2.50 ± 1.08	Time × SS or non-SS	0.267	2.000	0.133	0.703	0.504
	non-SS	2.60 ± 1.34	2.60 ± 0.89	2.40 ± 0.89	SS or non-SS	0.100	1.000	0.100	0.034	0.856

Results of two-way repeated measures ANOVA. Values represent mean ± SD. Total, all subjects; SS, Sjogren's syndrome group; non-SS, dry mouth group; *Time × SS or non-SS, $p < 0.05$.

Table 6. Score for subjective measurements of the eye condition pre- and post-intake of isoflavones for dry mouth patients

Item		Before	After 1 month	After 2 months	Results of two-way repeated measures ANOVA					
					Source of variation	SS (Type III)	DF	MF	F	*p* value
Eyestrain	Total	3.13 ± 1.30	2.93 ± 1.39	3.00 ± 1.41	Time	0.622	2.000	0.311	1.411	0.262
	SS	3.10 ± 1.29	3.10 ± 1.52	3.10 ± 1.60	Time × SS or non-SS	0.622	2.000	0.311	1.411	0.262
	non-SS	3.20 ± 1.48	2.60 ± 1.14	2.80 ± 1.10	SS or non-SS	0.544	1.000	0.544	0.099	0.758
Blurred vision	Total	2.87 ± 1.46	2.87 ± 1.60	2.87 ± 1.46	Time	0.067	2.000	0.033	0.183	0.834
	SS	3.00 ± 1.63	2.90 ± 1.73	3.10 ± 1.60	Time × SS or non-SS	0.600	2.000	0.300	1.648	0.212
	non-SS	2.60 ± 1.14	2.80 ± 1.48	2.40 ± 1.14	SS or non-SS	1.600	1.000	1.600	0.236	0.635
Dry eye	Total	3.60 ± 1.40	3.47 ± 1.55	3.33 ± 1.45	Time	0.422	2.000	0.211	1.614	0.218
	SS	3.80 ± 1.48	3.70 ± 1.64	3.50 ± 1.51	Time × SS or non-SS	0.067	2.000	0.033	0.255	0.777
	non-SS	3.20 ± 1.30	3.00 ± 1.41	3.00 ± 1.41	SS or non-SS	3.600	1.000	3.600	0.560	0.468
Eye pain	Total	2.60 ± 1.64	2.47 ± 1.55	2.33 ± 1.54	Time	0.600	2.000	0.300	0.727	0.493
	SS	2.70 ± 1.77	2.60 ± 1.58	2.50 ± 1.72	Time × SS or non-SS	0.067	2.000	0.033	0.081	0.923
	non-SS	2.40 ± 1.52	2.20 ± 1.64	2.00 ± 1.22	SS or non-SS	1.600	1.000	1.600	0.225	0.643

Results of two-way repeated measures ANOVA. Values represent the mean ± SD. Total, all subjects; SS, Sjogren's syndrome group; non-SS, dry mouth group.

assumed in this study. Furthermore, the function of the salivary gland has likely been improved by the antioxidant effect of isoflavones against the oxidative stress of salivary glands, thereby improving the effect on blood flow. This potential mechanism may explain the significant promotion of saliva flow observed in our study. In the future, we aim to verify these results by analyzing the degree of the damage to salivary glands by oxidative stress and the direct effect of isoflavones on salivary glands.

With respect to the human intervention clinical trial, many effective cases found that the volume of isoflavone aglycones that

should be ingested per day is at least 30 mg and that the ingestion period should be at least two weeks; in this study, the ingested amount was 25 mg, and the ingestion period was two months.[47–50]

The study by NIH reported that that "Isoflavone aglycone decreases the vasomotor symptom of menopause by 10–20%".[51] With regard to this study, menopause symptoms, with a focus on "hot flash" symptoms, are thought to be improved by the intake of 30–60 mg of soy isoflavone aglycone per day for at least two weeks in the United States and Europe. A study evaluating the effects of isoflavones reported that differences in the metabolism of isoflavones by *Enterobacteriaceae* affected isoflavone potency.[52,53] Because intestinal bacterial flora differs among individuals as shown in previous studies and the average age of the subjects was older than 60 years, the potential change in bacterial flora is thought to be affected by increasing age and a decrease in the number of bacteria. Furthermore, approximately 50% of Asians are equol producers and contain the intestinal bacteria required to convert daidzein into equol, which may have affected the efficacy of this evaluation of isoflavones.[54–58] Thus, we are planning to conduct the analysis with consideration of the equol-producing ability of individuals.

In this study, isoflavone intake is thought to act as an antioxidant in salivary glands impaired by oxidative stress, and the salivary function was likely improved by the increased blood flow. As the salivary secretion amounts of the subjects showed an increasing trend without any accompanying side effects, such as sweating and polyuria, which may be caused by salivary secretion promoters, an improvement in the QOL was confirmed. We aim to study the detailed mechanism in future studies.

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Abbreviations

ERs	estrogen receptors
HEL	hexanoyl-lysine
HRT	hormone replacement therapy
LDL	low-density lipoprotein
8-OHdG	8-hydroxy-2'-deoxyguanosine
PRL	propanoyl-lysine
QOL	quality of life
ROS	reactive oxygen species
SS	Sjogren's syndrome

Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

Efficient diagnosis of Sjögren's syndrome to reduce the burden on patients

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Abstract

Objective. The purpose of this study was to investigate the procedures for efficiently diagnosing Sjögren's syndrome to reduce patient burden.
Methods. This study analyzed data from 254 Japanese patients diagnosed with Sjögren's syndrome out of 4967 who visited our clinic complaining of xerostomia.
Results. Of the 254 Sjögren's syndrome patients, 140 fulfilled the criteria proposed by the Committee on Sjögren's Syndrome of the Ministry of Health and Welfare of Japan, 228 fulfilled the criteria proposed by the American-European Consensus Group, and 69 fulfilled the criteria proposed by the American College of Rheumatology. Numbers of definitive cases varied with each set of criteria. Logistic regression analysis was used to analyze useful examination items for definitive diagnosis of Sjögren's syndrome, demonstrating that anti-Ro/SSA (odds ratio (OR), 7.165), lip biopsy (OR, 4.273), sialography (OR, 2.402), and ANA (OR, 0.678) correlated significantly with definitive diagnosis of Sjögren's syndrome.
Conclusions. These results suggest that the following diagnostic procedure for Sjögren's syndrome would reduce burden on patients. When clinicians choose examination items for diagnosing Sjögren's syndrome, they should first select which criteria to use. Then, to minimize the number of examination items, examinations should be performed in order of anti-SSA antibody, lip biopsy, and parotid gland sialography.

Keywords

Anti-SS-A antibody, Diagnostic criteria, Lip biopsy, Parotid sialography, Sjögren's syndrome

History

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Introduction

Sjögren's syndrome is an autoimmune disease characterized by keratoconjunctivitis sicca and chronic sialadenitis. Postulated causes include genetic factors, immune abnormalities, and environmental factors, but the details remain unknown [1]. Treatment targeting the etiology of this disease is therefore lacking, and supportive treatment is currently given to alleviate the symptoms of dryness. Cevimeline hydrochloride and pilocarpine hydrochloride are indicated for treatment, but Sjögren's syndrome must be definitively diagnosed if these are to be used.

The diagnostic criteria for Sjögren's syndrome include the 1999 revised diagnostic criteria of the Ministry of Health, Labour and Welfare (MHLW) [2] and those of the American-European Consensus Group [3], which are generally used in Japan. In the absence of internationally consistent diagnostic criteria, the Sjögren's International Collaborative Clinical Alliance Project has gone ahead, with new criteria for classification recently proposed by the American College of Rheumatology (ACR) [4]. According to these criteria, Sjögren's syndrome is diagnosed if two of the following three criteria are fulfilled: 1) positive results for anti-SS-A antibody or anti-SS-B antibody or both, or alternatively positive results for rheumatoid factor together with an antinuclear antibody titer $\geq$320-fold; 2) ocular staining score $\geq$3; and 3) lip biopsy showing localized lymphocyte infiltration with $\geq$1 focus/4 mm^2 (Table 1). ACR criteria do not include the imaging tests used in the MHLW criteria or the American-European Consensus Group criteria.

The question of which criteria are most valid for diagnosis is a matter of debate [5–7]. In addition, many patients go untested and do not receive a confirmed diagnosis. The reasons for this may include the large number of tests required to fulfill the diagnostic criteria, the fact that many tests are invasive, and the fact that some patients do not want to be tested. In this context, and given this reasoning, the objective of this study was to ascertain how best to carry out the tests required for confirming a diagnosis of Sjögren's syndrome without imposing an excessive burden on patients. We investigated the status of testing and diagnosis in the Dry Mouth Clinic at Tsurumi University Dental Hospital, clarified some problems with the diagnosis of Sjögren's syndrome, and analyzed those test items believed to be most useful for diagnosing Sjögren's syndrome.

Subjects and methods

Study design and subjects

Of 4967 individuals examined in the Dry Mouth Clinic at Tsurumi University Dental Hospital between November 2002 and September 2013, 309 were diagnosed with Sjögren's syndrome. Among these, 55 patients were excluded from this study as they had been diagnosed at other hospitals and the details of their cases were unknown. A cross-sectional study was carried out on the remaining 254 patients diagnosed by our department (6.2% of the total number examined). A total of 245 patients who fulfilled at least one set of the MHLW, American-European Consensus Group, or ACR diagnostic/classification criteria were diagnosed

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Table 1. Criteria for Sjögren's syndrome.

	MHLW*	AECG**	ACR***
I. Ocular symptoms	N/A	Dry eyes for more than 3 months or sensation of sand or grit in the eyes or use of tear substitutes	N/A
II. Oral symptoms	N/A	Dry mouth for more than 3 months or swollen salivary glands or need to drink liquids to aid in swallowing	N/A
III. Ocular examinations	Schirmer test $\leq$ 5 mm/5 min and rose bengal staining $\geq$ 3 in van Bijsterveld's scale, or Schirmer test $\leq$ 5 mm/5 min and positive fluorescein staining	Schirmer's I test $\leq$ 5 mm/5min, or Rose bengal score or other ocular dye score $\geq$ 4 according to van Bijsterveld's scoring system	Keratoconjunctivitis sicca with ocular staining score $\geq$ 3
IV. Histopathology	Lacrimal or minor salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score $\geq$ 1 focus/4 mm^2	Minor salivary gland biopsy exhibiting focal lymphocytic sialadenitis with focus score $\geq$ 1 focus/4 mm^2	Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score $\geq$ 1 focus/4 mm^2
V. Salivary gland involvement	Sialography with diffuse punctate sialectasis, or decreased stimulated whole salivary secretion and decreased function by sequential salivary scintigraphy	Unstimulated whole salivary flow $\leq$ 1.5 ml/15 min or parotid sialography showing the presence of diffuse sialectasis or salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer.	N/A
VI. Serology	Positive serum anti-SSA/Ro or anti-SSB/La	Positive serum anti-SSA/Ro and/or anti-SSB/La	Positive serum anti-SSA/Ro and/or anti-SSB/La, or positive rheumatoid factor and ANA $\geq$ 1:320

*MHLW, a revised diagnostic criterion of the Ministry of Health, Labour and Welfare Japan;
Diagnosis of Sjögren's syndrome (SS) can be made when the patient meets at least 2 of the 4 objective items.
**AECG, revised version of the European criteria proposed by the American-European Consensus Group;
In patients without any potentially associated disease, primary SS may be defined as follows:
A. Presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (histopathology) or VI (serology) is positive.
B. Presence of any 3 of the 4 objective criteria items (i.e., items III, IV, V, or VI).
C. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological surveys.
For diagnosis of secondary Sjögren's:
In patients with a potentially associated disease (for instance, another well-defined connective tissue disease), presence of item I or item II plus any 2 from among items III, IV and V may be considered indicative of secondary SS.
***ACR, American College of Rheumatology classification criteria;
The classification of SS, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have at least 2 of the 3 objective features.
N/A, not applicable.

with Sjögren's syndrome in this study. All study protocols were approved by the Ethics Committee of Tsurumi University School of Dental Medicine (Establishment of Methods for the Diagnosis and Treatment of Dry Mouth, Receipt No. 31, approved March 24, 2003), and consent to the use of their clinical data was obtained in writing from all patients.

Methods

Descriptive survey of testing for and diagnosis of Sjögren's syndrome

We carried out a descriptive survey of which tests were used for the diagnosis of Sjögren's syndrome, as well as which diagnostic criteria were used as the basis for diagnosis.

Investigation of test items contributing to the diagnosis of Sjögren's syndrome

Logistic regression analysis was used to investigate which test items made the greatest contribution to the diagnosis of Sjögren's syndrome. Logistic regression is a type of probabilistic statistical classification model used to predict a binary response (dependent variable) from a binary predictor (independent variable). In this process, whether a patient did or did not fulfill the diagnostic criteria for Sjögren's syndrome was used as the dependent variable. Patients who fulfilled at least one of the three diagnostic/classification criteria (MHLW, American-European Consensus Group, or ACR) were diagnosed with Sjögren's syndrome (Table 1). Resting (unstimulated) saliva flow rate, stimulated saliva flow rate (gum test), anti-SS-A antibody, anti-SS-B antibody, antinuclear antibody, rheumatoid factor, Schirmer's test, ocular staining, lip biopsy, parotid sialography, and salivary scintigraphy were used as independent variables, and were treated as binary values depending on whether the test was performed. Significant variables were isolated using stepwise selection.

In the ACR criteria, total ocular staining score for each eye is the summation of the fluorescein score for the cornea and the lissamine green scores for the nasal and temporal bulbar conjunctiva. The ocular examination in this study was performed according to the methods indicated in the MHLW and AECG criteria, meaning that the ocular examinations recommended by the ACR were not performed in this study. Thus, all cases with positive ocular staining were regarded as positive cases when the ACR criteria were used in this study.

Results

Comparison of patients with confirmed diagnoses according to the three sets of criteria

Of the 254 patients, 140 met the MHLW criteria, 228 met the American-European Consensus Group criteria, and 69 met the ACR criteria (Figure 1). All 69 of those who fulfilled the ACR criteria also fulfilled the American-European Consensus Group criteria, while 65 fulfilled both the MHLW and American-European Consensus Group criteria.

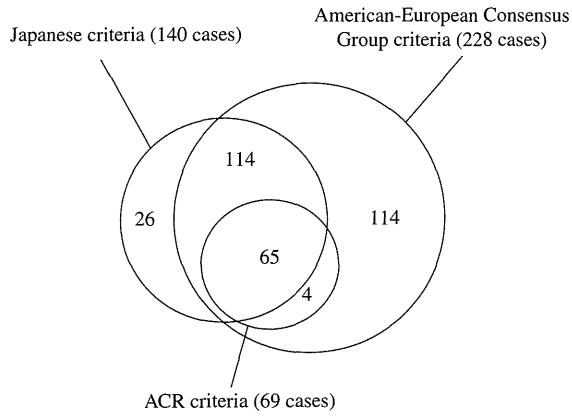

Figure 1. The number of patients with definitive diagnosis of Sjögren's syndrome. Area-proportional Venn diagrams visualizing interrelationships between the criteria proposed by the Committee on Sjögren's syndrome of the Ministry of Health and Welfare of Japan, the criteria proposed by the American-European Consensus Group, and the criteria proposed by the American College of Rheumatology (ACR). The diagram is based on results from 254 individuals.

Comparison of the proportions of positive test results that fulfilled the diagnostic criteria for the MHLW and American-European Consensus Group criteria

Of the 140 patients diagnosed with Sjögren's syndrome according to the MHLW criteria, 88 (62.9%) exhibited positive results from lip biopsy, 107 (76.4%) from sialography, 12 (8.6%) from ocular tests, and 108 (77.1%) from antibody tests (Table 2).

Of the 69 patients diagnosed with Sjögren's syndrome according to the ACR classification criteria, 61 (88.4%) exhibited positive results from lip biopsy, 69 (100%) from blood tests, and 12 (17.4%) from ocular staining.

Selection of examination items for diagnosis of Sjögren's syndrome and the percentages of positive results

The numbers of patients who underwent each type of test and the proportions of positive cases are summarized in Table 3. Of 4967 individuals examined at the Dry Mouth Clinic, saliva flow testing was performed in a majority of cases (4581/4967 at the resting [unstimulated] saliva flow rate, and 4473/4967 at the stimulated saliva flow rate), whereas ocular examinations were carried out in relatively few cases (a few percent).

Logistic regression analysis

Four test items were isolated as significantly associated with fulfilling the diagnostic criteria for Sjögren's syndrome: anti-SS-A antibody (odds ratio [OR], 7.165); lip biopsy (OR, 4.273); parotid sialography (OR, 2.402); and antinuclear antibody (OR, 0.678; Table 4).

Discussion

Numbers of confirmed diagnoses for each set of diagnostic criteria

Of the 4967 patients, 254 (6.2%) were tested and diagnosed with Sjögren's syndrome in our department, not a high figure. One reason for this is that dry mouth may have a wide variety of different causes, but this finding also illustrates the reality that sufficient testing is not being carried out. This can be extrapolated from the fact that although non-invasive tests such as saliva flow rate measurement were carried out for a high proportion of patients, lip biopsy and parotid sialography were much common.

Of the 254 patients, 140 fulfilled the MHLW criteria and 228 met the American-European Consensus Group criteria, showing a large difference in the number of patients diagnosed when different sets of criteria were used (Figure 1). The main difference between the MHLW and American-European Consensus Group criteria concerns items involving subjective symptoms. The inclusion of subjective symptoms in the classification criteria was one factor contributing to the increase in the number of patients diagnosed. The diagnostic criteria for Sjögren's syndrome include a large number of tests, and many patients may not want to undergo several different tests, meaning that diagnosis then becomes dependent on subjective symptoms. Performance of all the tests on every patient in everyday clinical practice is not possible, and the mindset that diagnosis should be reached on the basis of as few tests as possible comes into play on the part of both patients and doctors. The question as to whether subjective symptoms can be given the same weight in evaluation as objective tests has already been asked [7], and the number of patients diagnosed varies tenfold depending on the set of diagnostic criteria applied [8].

Neither the MHLW criteria nor the ACR criteria include subjective symptoms (Table 1). A comparison of the number of patients diagnosed according to these two sets of criteria showed that the numbers were 140 and 69, respectively, with a major difference again evident between the two. This may have been because results were affected by the different combinations of tests performed. Patients diagnosed with Sjögren's syndrome showed high rates of positive results for lip biopsy, sialography, and serological tests,

Table 2. Comparison of positive rates in examination for diagnosis of Sjögren's syndrome among three criteria.

Examinations	Criteria (number of cases fulfilled SS)	Number of cases performed test/Number of cases fulfilled SS (%)	Number of cases with positive result/Number of cases performed test (%)	Number of cases with positive result/Number of cases fulfilled SS (%)
Lip biopsy	MHLW* (140)	106/140 (75.7%)	88/106 (83.0)	88/140 (62.9%)
	AECG** (228)	120/228 (52.6%)	100/120 (83.3)	100/228 (43.9%)
	ACR*** (69)	61/69 (88.4%)	61/61 (100.0)	61/69 (88.4%)
Sialography	MHLW (140)	113/140 (80.7%)	107/113 (94.7)	107/140 (76.4%)
	AECG (228)	105/228 (46.1%)	81/105 (77.1)	81/228 (35.5%)
	ACR (69)	–	–	–
Ocular examination	MHLW (140)	23/140 (16.4%)	12/23 (52.2)	12/140 (8.6%)
	AECG (228)	26/228 (11.4%)	12/26 (46.2)	12/228 (5.3%)
	ACR (69)	14/69 (20.3%)	12/14 (85.7)	12/69 (17.4%)
Serological examination	MHLW (140)	130/140 (92.9%)	108/130 (83.1)	108/140 77.1%)
	AECG (228)	211/228 (92.5%)	187/211 (88.6)	187/228 (40.3%)
	ACR (69)	69/69 (100.0%)	69/69 (100.0)	69/69 (100.0%)

*HMLW, a revised diagnostic criterion of the Ministry of Health, Labour and Welfare Japan.
**AECG, revised version of the European criteria proposed by the American-European Consensus Group.
***ACR, American College of Rheumatology classification criteria.

Table 3. Selection of examination items for diagnosis of Sjögren's syndrome and the percentages of positive results.

Test items	Number of tests performed	Percentage of tests performed (%)*	Number of positive cases	Percentage of positive cases (%)
RSFR (≤ 1.5 ml/15 min)	4581	92.2	2625	57.3
SSFR (≤ 10 ml/10 min)	4473	90.1	2168	48.5
Serological examination	1355	27.3	267	19.7
Anti-Ro/SSA				
Anti-Lo/SSB	1092	22.0	87	8.0
ANA (≥ 320)	742	15.0	69	9.3
RF	682	13.7	164	24.0
ANA + RF	625	12.6	29	4.6
Ocular examination				
Shirmer's test	120	2.4	80	66.7
Ocular staining	64	1.3	23	35.9
Shirmer + staining	63	1.3	20	31.3
Salivary gland examination				
Sialography	334	6.7	146	43.7
Lip biopsy	343	6.9	126	36.7
Scintigraphy	2	0.0	1	50.0

*Percentage of tests performed is relative to a total of 4967 cases examined in this study.
RSFR, resting saliva flow rate; SSFR, stimulated saliva flow rate; ANA, anti-nuclear antibody; RF, rheumatoid factor

but low rates for ocular tests (Table 2). Patients are diagnosed as positive if they fulfill two of the four items from the MHLW criteria or two of three from the ACR criteria, and the low number diagnosed on the basis of the ACR criteria may have been because few patients tested positive in ocular tests and the ACR criteria do not include sialography. The combination of tests performed may thus influence whether patients fulfill the diagnostic criteria, suggesting that the diagnostic criteria used should be borne in mind when choosing which tests to perform.

Investigation of which test items are useful for diagnosing Sjögren's syndrome

Ascertaining which tests should be prioritized in order to obtain a diagnosis of Sjögren's system is important to reduce the burden on patients. This study investigated the contribution rates of test items for all patients diagnosed with Sjögren's syndrome. Logistic regression analysis was carried out with test items as independent variables, which were treated as binary values depending on whether the test was performed. The ORs thus obtained were regarded as values expressing the probability of obtaining a diagnosis of Sjögren's syndrome, depending on whether those tests were performed. Four test items were isolated as significant: anti-SS-A antibody; lip biopsy; parotid sialography; and antinuclear antibody (Table 4). The OR for antinuclear antibody was less than 1, meaning it did not actually contribute to the diagnosis of Sjögren's syndrome, but anti-SS-A antibody, lip biopsy, and parotid sialography made major contributions.

Both Anti-SS-A antibody and lip biopsy are included in all the various different sets of diagnostic criteria. Anti-SS-A antibody is an autoantibody, while lip biopsy examines lymphocyte infiltration. These tests both reflect immune abnormalities, a fact that reaffirms their validity. On the assumption that anti-SS-A and anti-SS-B antibody production and lymphocyte infiltration of the salivary glands are expressions of the basic pathology of Sjögren's syndrome, a positive result in either of these tests would be sufficient to diagnose the condition.

Parotid sialography is not included in the ACR classification, but rather than being excluded from the present analysis, it was actually selected as exhibiting a comparatively high OR. This suggests that parotid sialography may contribute to the diagnosis of Sjögren's syndrome [9]. Sialography offers good sensitivity and specificity in all the sets of diagnostic criteria [2], and should therefore not be excluded for statistical reasons. Unlike antibody tests and lymphocyte infiltration, sialography does not investigate immunological reactions directly, but rather reflects the acinar atrophy and ductal dilatation that occur as a result of autoimmune pathology, visualizing these morphologically. Parotid sialography is not without problems, however, including the painful injection of contrast medium and the risk of iodine allergy. Attempts are therefore being made to apply magnetic resonance imaging and ultrasonography as non-invasive forms of testing [10,11]. While acknowledging the value of diagnostic imaging, the ACR criteria have therefore gone so far as to select only one of either biopsy or imaging for salivary gland testing. These criteria may be viewed as balancing three different types of test: medical, ophthalmic, and dental. The greater the number of tests, the larger the burden on the patient, and this is another reason for caution when considering the inclusion of salivary gland imaging in diagnostic criteria.

A decrease in resting (unstimulated) saliva flow rate is closely associated with high-grade results from lip biopsy, the presence of anti-SS-B antibody, and age, while a decrease in stimulated saliva flow rate is associated with age and higher stage on parotid sialography [12]. This suggests that immunological factors are associated with resting saliva flow rate, whereas stimulated saliva flow rate is associated with parotid gland impairment. That Sjögren's syndrome causes a decrease in resting (unstimulated) saliva flow rate is beyond doubt. Nevertheless, this test was not selected as contributing to the diagnosis of Sjögren's syndrome (Table 4). Most patients who attended the Dry Mouth Clinic underwent saliva flow rate testing, but the positive result rate was also high (Table 3). A decrease in saliva flow rate may be caused by numerous other causes besides Sjögren's syndrome, including radiotherapy to the head and neck region, diabetes, side effects of regular medication, anxiety, and depression [13,14]. Given the large number of patients with decreased saliva flow rate due to causes other than Sjögren's syndrome, this test is not sufficiently specific, meaning that it was not judged as a required item for confirming diagnosis [15].

Similar to saliva flow rate testing, measuring tears using Schirmer's test is also not specific for Sjögren's syndrome [4]. The ACR criteria, which are based on the concept of utilizing more objective tests, incorporate testing for keratoconjunctivitis by means of fluorescence staining and lissamine green [4]. Because this evaluation method differs in some ways from past methods, direct comparison is difficult, but in this study neither Schirmer's test nor ocular staining tests were judged to contribute to a diagnosis. Dry eyes are caused by either decreased tear production or tear evaporation [16]. Keratoconjunctive epithelial damage due to dry eyes may occur for many reasons other than Sjögren's syndrome [16,17], and this study judged ocular tests as not making a major contribution to the diagnosis.

Objective findings and subjective symptoms of dry mouth and dry eyes have low specificity as tests for Sjög

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Table 4. Useful examination items for definitive diagnosis of Sjögren's syndrome.

	Forced entry							Stepwise selection					
	B	SD	Wald	*P*	Odds ratio	Upper 95%	Lower 95%	B	SD	Wald	*P*	Odds ratio	Upper 95%	Lower 95%
Anti-Ro/SSA	1.756	0.233	56.978	0.000	5.789	3.669	9.133	1.969	0.176	125.053	0.000	7.165	5.074	10.118
Lip biopsy	1.427	0.211	45.771	0.000	4.164	2.755	6.295	1.452	0.21	47.965	0.000	4.273	2.833	6.444
Sialography	0.863	0.216	15.956	0.000	2.371	1.552	3.622	0.876	0.215	16.683	0.000	2.402	1.577	3.657
ANA	−0.331	0.248	1.777	0.183	0.718	0.441	1.168	−0.388	0.163	5.678	0.017	0.678	0.493	0.933
RSFR	0.387	0.523	0.549	0.459	1.473	0.529	4.104							
SSFR	−0.121	0.447	0.073	0.787	0.886	0.369	2.127							
Anti-La/SSB	0.393	0.227	2.997	0.083	1.482	0.949	2.313							
RF	−0.279	0.248	1.269	0.260	0.757	0.466	1.229							
Shirmer's test-L	−0.204	1.53	0.018	0.894	0.815	0.041	16.342							
Shirmer's test-R	−0.346	1.466	0.056	0.813	0.707	0.04	12.529							
Ocular staining-L	−0.32	0.486	0.434	0.510	0.726	0.28	1.883							
Ocular staining-R	1.853	0.538	11.875	0.001	6.378	2.223	18.297							
Scintigraphy	−20.429	28254.683	0	0.999	0.000	0								
Intercept	−4.389	0.351	156.451	0.000	0.012			−4.14	0.128	1042.721	0.000	0.016		

In logistic regression analysis, stepwise selection identified useful examinations for diagnosis of Sjögren's syndrome. Diagnosis of Sjögren's syndrome (definitive vs. non-definitive) was determined as a dependent variable, while the following examinations (performance vs. non-performance) were taken as independent variables: resting (un-stimulated) saliva flow rate (RSFR), stimulated saliva flow rate (SSFR), anti-Ro/SSA antibody, anti-La/SSB antibody, anti-nuclear antibody (ANA), rheumatoid factor (RF), Shirmer's test, ocular staining, lip biopsy, sialography, and scintigraphy

anti-SS-A antibody and lip biopsy are more useful. It is not possible to debate the diagnostic criteria on the basis of these results. In everyday clinical practice, a diagnosis must be clarified by administering pharmacotherapy, since consideration of the mental and physical burden on the patient means that it is not uncommon to limit test items without carrying out every test. Under such circumstances, in addition to investigating subjective symptoms, the most efficient way of proceeding while minimizing the burden on the patient is to carry out anti-SS-A antibody testing, lip biopsy, and parotid sialography, which show high ORs, in that order.

Conclusion

For the diagnosis of Sjögren's syndrome, it is important to decide which tests to perform on the basis of the set of diagnostic criteria being used. If only a limited number of tests are to be performed, proceeding in the order of anti-SS-A antibody testing, lip biopsy, and parotid sialography appears warranted.

Conflict of interest

None.

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ORIGINAL ARTICLE

Saliva as a potential tool for diagnosis of dry mouth including Sjögren's syndrome

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OBJECTIVES: Recently, the use of saliva as a diagnostic tool has gained considerable attention because it is non-invasive and easy to perform repeatedly. In this study, we focused on soluble molecules in saliva to establish a new diagnostic method for xerostomia.

MATERIALS AND METHODS: Saliva was obtained from 90 patients with Sjögren's syndrome (SS), 22 patients with xerostomia associated with neurogenic/neuropsychiatric disorders and drugs (XND), 30 patients with radiation-induced xerostomia (RX), and 36 healthy controls. Concentrations of helper T (Th) cytokines in saliva were measured by flow cytometric analysis. Concentrations of secretory IgA (SIgA) and chromogranin A (CgA) were measured by ELISA.

RESULTS: Unstimulated and stimulated whole saliva from patients with SS, XND, and RX was significantly reduced compared with controls. Th1 and Th2 cytokines from SS patients were significantly higher than controls. Furthermore, Th2 cytokines were closely associated with strong lymphocytic accumulation in salivary glands from SS patients, while Th1 and Th17 cytokines were negatively associated. SIgA levels were not significantly different between all patient groups and controls. CgA levels from XND patients were significantly higher than controls.

CONCLUSIONS: The measurement of cytokines, CgA, and SIgA in saliva is suggested to be useful for the diagnosis of xerostomia and also to reveal disease status.

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Keywords: xerostomia; Sjögren's syndrome; saliva; cytokine; secretory IgA; chromogranin A

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Introduction

Recently, heightened interest in oral health has led to an increase in patients complaining of dry mouth (xerostomia) (Navazesh and Ship, 1983; Greenspan, 1996; Guggenheimer and Moore, 2003). Xerostomia can be classified into the following three groups based on the cause: (1) xerostomia caused by dysfunction of the salivary gland, which can be due to Sjögren's syndrome (SS) and radiation-induced xerostomia (RX); (2) xerostomia associated with neurogenic/neuropsychiatric disorders and drugs (XND); and (3) xerostomia associated with systemic diseases or metabolic disorders such as diarrhea, dehydration, thyroid hyperfunction, diabetes mellitus (DM), kidney malfunction, and anemia (Bahn, 1972; Ettinger, 1981; Spielman *et al*, 1981; Billings *et al*, 1996; Greenspan, 1996; Nakamura *et al*, 1997; Guggenheimer and Moore, 2003; Matear and Barbaro, 2005; Scully and Felix, 2005; Ivanovski *et al*, 2012; Nonzee *et al*, 2012; Rahman *et al*, 2013). However, it is difficult to make a differential diagnosis of xerostomia objectively. Consequently, the diagnostic criteria for xerostomia remain to be established except SS. The criteria for SS are determined from the results of relatively complicated examinations including sialography, salivary gland scintigraphy, and labial salivary gland (LSG) biopsy, which are invasive and only performed in special facilities. Recently, the use of saliva as a diagnostic tool has gained considerable attention, as it is non-invasive and easy to perform repeatedly. Bigler *et al* reported that tumor markers could be detected in saliva from subjects with oral, lung, or pancreatic cancer (Zhang *et al*, 2012; Lau *et al*, 2013; Wang *et al*, 2013). In addition, secretory immunoglobulin A (SIgA) and chromogranin A (CgA) in saliva were recently reported the usability as stress-related substances (Bosch *et al*, 1998). In our previous studies, we focused on the involvement of Th cytokines in the pathogenesis of SS (Ohyama *et al*, 1996, 1998; Tsunawaki *et al*, 2002; Maehara *et al*, 2012a,b; Moriyama *et al*, 2013). In this study, we thus examined the diagnostic utility of saliva from patients with xerostomia including SS, RX, and XND.

Materials and methods

Patients

Ninety patients with SS (84 women and 6 men; mean age ± standard deviation (s.d.), 61.9 ± 12.6 years), 30 patients with XND (25 women and 5 men; mean age ± s.d., 69.4 ± 10.5 years), 22 patients with RX (7 women and 15 men; mean age ± s.d., 67.3 ± 9.8 years), and 36 healthy subjects (21 women and 15 men, mean age ± s.d., 42.4 ± 15.1 years) were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital between 2011 and 2013 and were included in the study. All patients with SS met the diagnostic criteria proposed by both the Research Committee on Sjögren's Syndrome of the Ministry of Health and Welfare of the Japanese Government (Fujibayashi *et al*, 1993) and the criteria proposed by the American–European Consensus Group criteria for SS (Vitali *et al*, 2002). The degree of lymphocytic infiltration in the specimens was determined by focus scoring (Greenspan *et al*, 1974; Daniels and Whitcher, 1994). One standardized score indicates the number of focal inflammatory cell aggregates containing 50 or more mononuclear cells in each 4-mm^2 area of salivary gland tissue (Szodoray *et al*, 2005). None of the patients had other autoimmune diseases and were not being treated with steroids or other immunodepressants. XND was diagnosed according to the following criteria: (1) failure to fulfill each of the above diagnostic criteria for SS; (2) taking drugs or diagnosed with depression in the Department of Psychosomatic Medicine; (3) decreased unstimulated whole salivary flow rate (UWS) (<1.5 ml 15 min^{-1}) or stimulated whole salivary flow rate (SWS) (<2.00 g 2 min^{-1}). XND patients were taking anxiolytics (n = 13), sleeping drugs (n = 11), antidepressant drugs (n = 1), and antihypertension drugs (n = 2). RX was diagnosed according to the following criteria: (1) failure to fulfill each of the above diagnostic criteria for SS; (2) having a history of radiotherapy to head and neck; and (3) decreased UWS (<1.5 ml 15 min^{-1}) or SWS (<2.00 g 2 min^{-1}). The disease duration was defined as the period from the initial observation of dry mouth to the first visit. The controls consisted of 36 healthy subjects who had no sicca or clinical or laboratory evidence of systemic disease. This study design was approved by the Ethics Committee of Kyushu University, Japan, and informed consent was obtained from all patients and healthy controls (IRB serial number: 25–287).

Saliva sample (stimulated)

Study participants were asked to refrain from smoking, eating, and drinking for at least 2 h prior to collection of samples. Patients rinsed their mouth with distilled water just before their saliva was taken. Stimulated whole saliva was collected by Salisoft tubes (Sarstedt, Nümbrecht, Germany) using polypropylene-polyethylene polymer swabs from the subjects' mouths following 2 min of chewing. Swabs with absorbed saliva were returned to Salisoft tubes and centrifuged for 5 min at 780 ***g***, yielding a clear saliva sample. Saliva was transferred to a microtube and stored at −80°C immediately.

Salivary flow rate

Stimulated whole salivary flow rate was measured by the Saxon test. This test was performed by having subjects chew Surgeon® Type IV Gauze Sponge (Hakuzo Medical Corporation, Osaka, Japan) once a second for 2 min and measuring the weight of the gauze. If the increase in weight of the gauze was <2 g, the subject was classified as 'decreased' (Kohler and Winter, 1985). UWS was measured by a spitting test. This test was carried out by asking subjects to spit saliva into a paper cup for 15 min. The amount of saliva in the unstimulated condition (sitting on a chair and not moving) was measured. If the volume of saliva was <1.5 ml, the subject was classified as 'decreased' (Vitali *et al*, 2002).

Concentrations of cytokines in saliva by flow cytometry

The amount of saliva that could be taken from the xerostomia patients was very small, so we used Human High sensitivity Flex Sets of BDTM Cytometric Beads Array (CBA) system that allowed the measurement of many molecules at the same time from 50 μl of saliva (BD Biosciences San Jose, CA, USA). Saliva samples were thawed and centrifuged again, and the supernatant was measured. Soluble molecules including Th1, Th2, and Th17 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF, IFN-γ, and IL-17A) in saliva were quantitatively determined using antibodies from the multiplex Flex Set Cytometric Bead system® (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Serial dilutions (1/2 v/v) of the standards were prepared; saliva samples were (1/4 v/v) diluted using assay diluent and transferred to a 96-well plate. Then, 50 μl of mixed capture beads was transferred to each well. After a 3-h incubation period at RT, 50 μl PE detection reagent was added and samples were incubated for another hour at RT. 96-well plate was washed with wash buffer, and after final centrifugation at 800 rpm for 10 min, 150 μl wash buffer was added. Flow cytometric analysis was performed using the BD FACSVerse™ and CBA analysis FCAP software (BD Biosciences San Jose, CA, USA), which allowed measurement of cytokines only after passing the quality-control (QC) test including performance QC. In this study, % coefficient of variation (CV) of all samples was acceptance value (%CV was <3%) (Cook *et al*, 2001; LaFrance *et al*, 2008).

Measurement of SIgA and CgA in saliva by enzyme-linked immunosorbent assay

The concentrations of SIgA and CgA were measured by immunoreactivity in a double-sandwich enzyme-linked immunosorbent assay (ELISA) using a Salivary Secretory IgA Enzyme Immunoassay KIT® and YK070 Human Chromogranin A EIA KIT, as reagents to detect these molecules by cytometric bead array are not available. Saliva total protein concentration was measured using the QuickStart™ Bradford Protein Assay Kit (Bio-Rad, CA, USA). Quantity of CgA was calculated by dividing the concentration of CgA with the concentration of the saliva total protein. Concentrations of cytokines, SIgA, and CgA were evaluated by a researcher (O M) who was blinded to sample information.

Statistical analysis

The statistical significance of differences between groups was determined using the chi-square test, Kruskal–Wallis test followed by *post hoc* Steel's test, Mann–Whitney *U*-test, and Spearman's rank correlation. Values of $P < 0.05$ were considered statistically significant.

Results

Clinical findings

Table 1 shows the clinical characteristics (mean age, sex, disease duration, UWS, and SWS) of the patient and control groups. The mean amount of UWS from all patient groups was lower than the standard value, while the SWS of patients with SS and RX was lower than the standard value. In comparison with healthy subjects, UWS from all patient groups was significantly lower than that from healthy subjects. SWS from SS and RX patients was significantly lower than that from healthy subjects (Figure 1).

Cytokine and stress-related substances in saliva

Our previous studies demonstrated that Th1 cytokines were essential for the induction and/or maintenance of SS, whereas Th2 cytokines might be involved in disease progression, especially local B-cell activation (Ohyama *et al*, 1996, 1998; Tsunawaki *et al*, 2002; Maehara *et al*, 2012a, b; Moriyama *et al*, 2013). We thus focused on soluble molecules such as cytokines and examined their concentrations in saliva from patients with xerostomia. As cytokines and stress-related substance are soluble factors secreted in the salivary glands, we examined these molecules in the saliva using flow cytometry and ELISA. The concentrations of Th1 cytokines (IFN-©, IL-1®, TNF, IL-2, IL-6, IL-8, IL-12p70), Th2 cytokines (IL-4, IL-5, IL-10), and Th17 cytokine (IL-17) in the saliva from SS patients were significantly higher than the controls (Figure 2).

Secretory immunoglobulin A is the dominant immunoglobulin in external secretions that bathe mucosal surfaces (respiratory, intestinal, and reproductive), and salivary glands, where it plays a role in the immune system 'first line of defense' against microbial invasion. SIgA is reported to be a stress-related substance, and concentrations of SIgA are increased in patients with sialadenitis (Bosch *et al*, 1998). CgA is produced by salivary epithelial cells and is generally used as a novel mental stress marker (Nakane *et al*, 1998). Although the concentrations of SIgA from SS and RX patients were higher than in the controls, this was not statistically significant. Quantities of CgA in saliva from patients with XND were significantly higher than in the controls (Figure 3).

Association of saliva cytokines with the degree of lymphocytic infiltration in LSGs from SS patients

Sjögren's syndrome patients were divided into two groups: (1) those with weak lymphocytic infiltration of LSGs (focus scores ranging from 1 to 6, 55 women and 1 man; mean age, 48.7 ± 13.9 years) and (2) those with strong lymphocytic infiltration (focus scores ranging from 7 to 12, 29 women and 5 men; mean age, 62.9 ± 11.7 years). The concentration of IL-17 had no relationship with the degree of lymphocytic infiltration in the LSGs from SS patients. The concentrations of a non-specific inflammatory cytokine (IL-1®) and Th1 cytokine (IL-12p70) were significantly higher in LSGs with weak lymphocytic infiltration in comparison with those with strong lymphocytic infiltration. In contrast, the concentrations of Th2 cytokines (IL-4, IL-5) were significantly higher in LSGs with strong lymphocytic infiltration in comparison with those with weak lymphocytic infiltration (Figure 4). Furthermore, we examined the correlation between the focus score and cytokines with significant difference in Figure 4 and found that IL-4 showed significant positive correlation, while IL-1® and IL-12p70 had a negative correlation (Figure 5).

Discussion

Xerostomia is caused by numerous factors and complications. It manifests with various symptoms including mucosal atrophy, multiple caries, periodontitis and dysphagia because saliva serves a variety of functions in the oral cavity such as dissolving substances important for taste and acting as a lubricant for speaking, chewing, and swallowing. Saliva has buffering functions that neutralize acids formed during bacterial carbohydrate metabolism and contains minerals for tooth remineralization [1–12]. Saliva also contains bactericidal substances such as lysozyme,

Table 1 Patient profiles and clinical findings

	SS (n = *90*)	*XND* (n = *30*)	*RX* (n = *22*)	*Control* (n = *36*)
Men/women	6:84	5:25	15:7	21:15
Age (mean ± s.d. years)	61.9 ± 12.6	69.4 ± 10.5	67.3 ± 9.8	42.4 ± 15.1
Disease duration (mean ± s.d days)	52.8 ± 12.5	32.6 ± 10.2	18.4 ± 4.5	
UWS by spitting method [median (25th–75th percentiles) ml/15 min]	0.5 (0.1–1.0)	0.7 (0.2–1.0)	0.8 (0.5–2.0)	3.8 (2.9–5.1)
SWS by Saxon's test [median (25th–75th percentiles) g 2 min^{-1}]	1.22 (0.58–2.17)	2.11 (1.14–3.40)	1.98 (1.04–3.06)	5.06 (4.57–6.12)

SS, Sjögren's syndrome; XND, xerostomia associated with neurogenic/neuropsychiatric disorders and drugs; RX, radiation-induced xerostomia; SWS, stimulated whole salivary flow rate; UWS, unstimulated whole salivary flow rate; s.d., standard deviation.

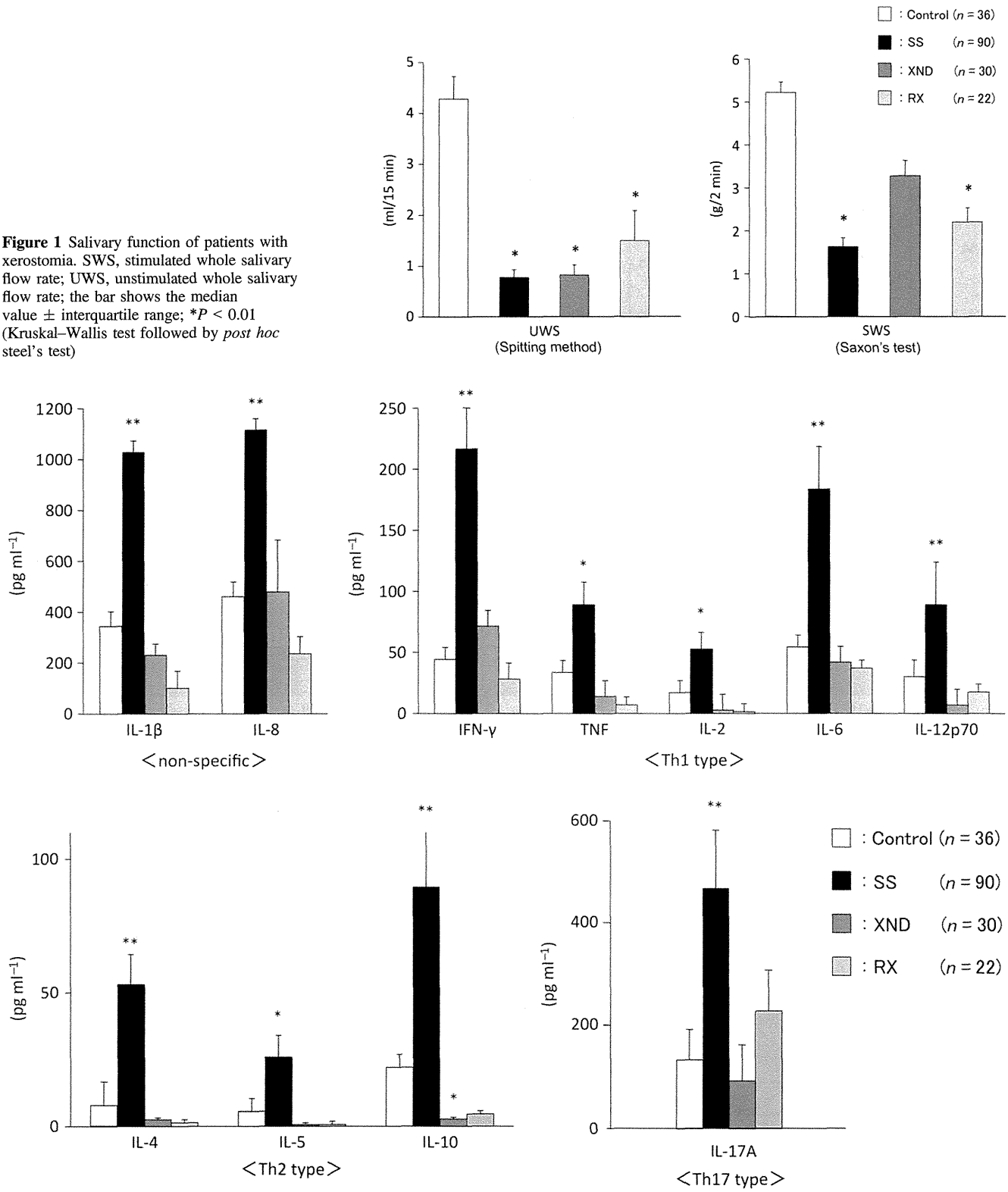

Figure 1 Salivary function of patients with xerostomia. SWS, stimulated whole salivary flow rate; UWS, unstimulated whole salivary flow rate; the bar shows the median value ± interquartile range; $*P < 0.01$ (Kruskal–Wallis test followed by *post hoc* steel's test)

Figure 2 Cytokine in saliva from patients with xerostomia. Th T-helper cell; the bar shows the mean value ± standard deviation (s.d.); $*P < 0.05$, $**P < 0.01$ (Kruskal–Wallis test followed by *post hoc* Steel's test)

lactoferrin, and antibodies, and SIgA is especially important for the defense systems of mucosal membranes. In this study, we first focused on the differences of salivary flow rates (SWS and UWS) among each patient group including SS, XND, and RX. The SWS from all patient groups was significantly lower than in controls, while the

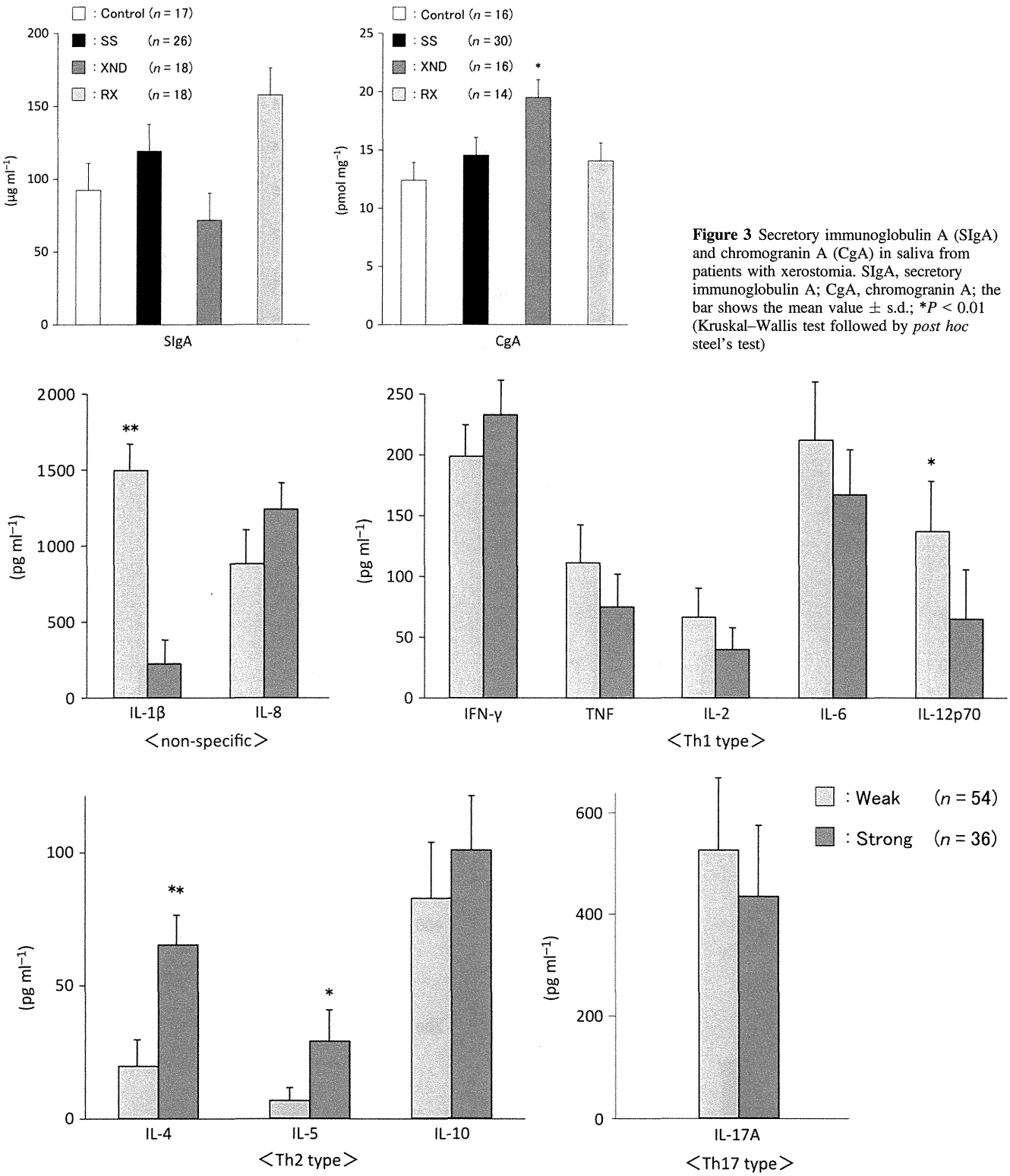

Figure 3 Secretory immunoglobulin A (SIgA) and chromogranin A (CgA) in saliva from patients with xerostomia. SIgA, secretory immunoglobulin A; CgA, chromogranin A; the bar shows the mean value ± s.d.; $*P < 0.01$ (Kruskal–Wallis test followed by *post hoc* steel's test)

Figure 4 Association between cytokines and degree of lymphocytic infiltration in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). The degree of lymphocytic infiltration in LSGs was graded from 1 to 12 by focus scoring and was then divided into groups: (i) those with focus scores ranging from 1 to 6 were categorized as having weak infiltration and (ii) those with scores from 7 to 12 were categorized as having strong infiltration; the bar shows the mean value ± s.d.; $*P < 0.05$, $**P < 0.01$ (Mann–Whitney *U*-test)

UWS from SS and RX patients was significantly lower than in controls. However, patients with XND showed no statistically significant difference with the controls. Several studies indicated that the decrease in SWS and UWS in SS and RX patients was caused by the physical destruction of salivary glands and that the decrease in UWS

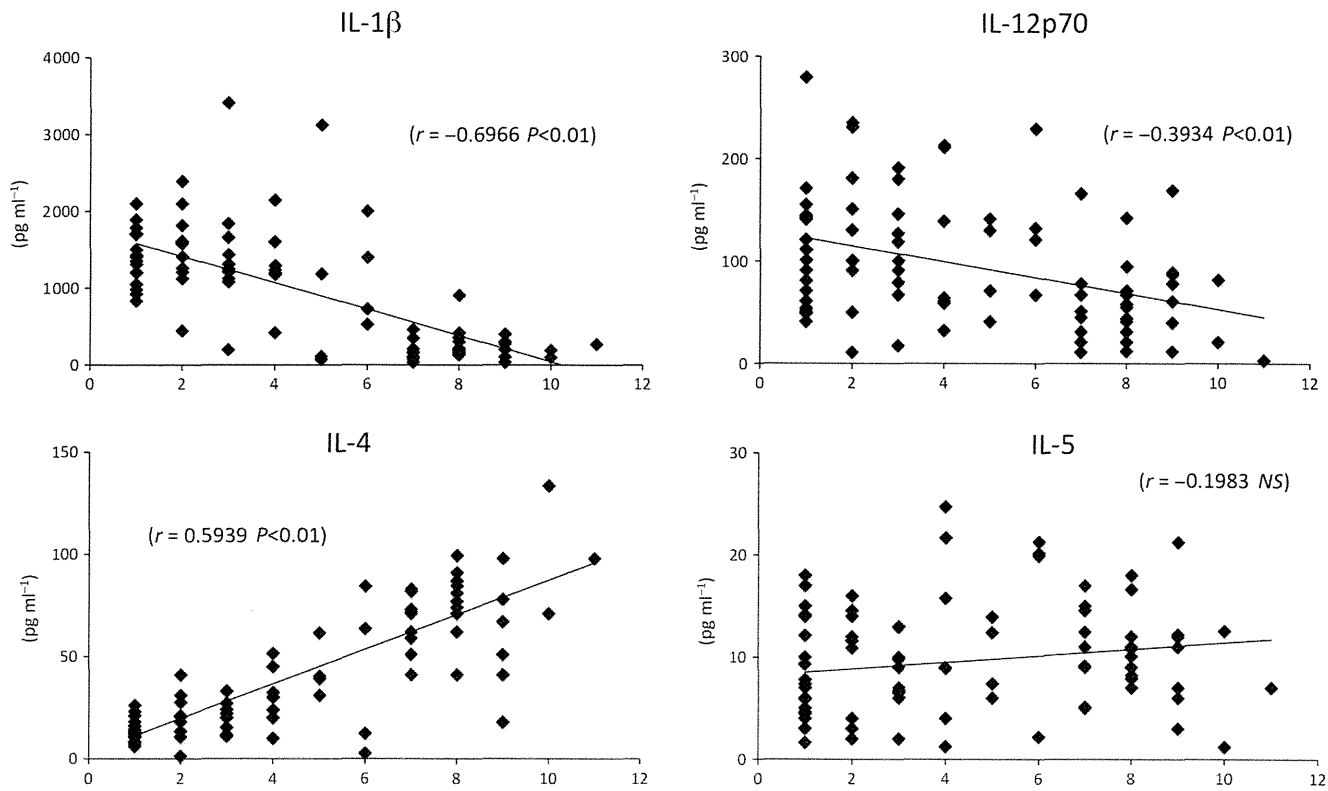

Figure 5 Correlation between cytokines and focus score in LSGs from patients with SS. Statistical significance of the differences between groups was determined by Spearman's rank correlation; NS, not significant

alone in XND patients was caused by suppression of the salivary secretory nerve system (Matear and Barbaro, 2005; Scully and Felix, 2005; Nonzee *et al*, 2012). When considering normal SWS in patients with XND, this suppression might be inhibited by stimulation of food intake (Matear and Barbaro, 2005; Scully and Felix, 2005; Nonzee *et al*, 2012). The results of the present study concerning salivary flow rates are consistent with these reports. However, it is difficult to diagnose xerostomia only using SWS and UWS because measurements of salivary flow rates often vary depending on the patient's condition and thus lack accuracy. In addition, the Saxon test often causes nausea. To overcome these issues, we examined soluble proteins such as cytokines and stress-related substances in saliva samples. However, it was difficult to collect the saliva required for analyses of multiple cytokines, especially in patients with xerostomia. Therefore, we used the 'CBA flex system' to measure many soluble factors from a single small-volume sample with high reproducibility. It is generally accepted that cytokines produced by Th cells play a crucial role in the pathogenesis of SS (Dhabhar *et al*, 2009). The results of the present study concerning cytokine concentration in the saliva from SS patients were consistent with this model. However, little is known concerning the association of cytokines in saliva with RX and XND. In this study, there was no significant difference in the levels of all tested cytokines between patients with RX and controls, indicating salivary RX dysfunction was caused by radiation-induced destruction without autoimmune reaction via cytokines. Only levels of IL-10 from XND patients were significantly lower than controls. Although it is unlikely that anti-inflammatory cytokines such as IL-10 are related to the inhibition of salivary secretion nervous system, Dhabhar *et al* (2009) reported that patients with depression had decreased serum IL-10, consistent with the results of this study.

Also important in clinical settings is the long-term follow-up for SS patients because SS is progressive and causes the gradual reduction of salivary functions. However, it is extremely difficult to perform serial LSG biopsy for patients with high invasiveness. We thus examined cytokine concentrations in the saliva, which could be collected repeatedly. Our previous studies of salivary glands suggested that Th1 and Th17 cells are involved in the early stages of SS, while Th2 and follicular T cells are associated with germinal center formation in the late stages of disease (Maehara *et al*, 2012a,b; Moriyama *et al*, 2012). In this study, we found that the concentrations of Th2 cytokines in saliva were closely associated with strong lymphocytic infiltration, which is consistent with previous reports in salivary glands.

We also focused on stress-related soluble proteins in saliva such as SIgA and CgA. SIgA is a soluble immunoglobulin produced by plasma cells and is increased during salivary gland inflammation by physical and emotional stress via sympathetic reaction of the sympathetic–adrenal–medullary axis system (Spangler, 1997; Deinzer *et al*, 2000; Ring *et al*, 2000; Bosch *et al*, 2002). In this study, we observed no significant difference in salivary SIgA levels between the patient groups and controls, whereas SIgA levels in RX and SS patients were higher than in controls. SIgA in RX and SS patients might be produced

by salivary gland ductal epithelium via inflammation caused by the physical destruction of salivary glands. However, the accumulation of cases and further examinations are required to elucidate the involvement of SIgA production in SS and RX. In contrast, CgA is localized in the salivary gland ductal epithelium and released into the saliva by autonomic nerve stimulation, indicating CgA might be useful as a novel mental stress marker (Kanno *et al*, 1998; Nakane *et al*, 1998, 2002). This study demonstrated that the quantity of salivary CgA from patients with XND was significantly higher than in the controls. Twenty-five of 30 patients with XND took anxiolytic, antidepressant or sleep-inducing drugs for a long period. These results are in accordance with a previous report that salivary CgA was secreted owing to chronic psychological stress.

In conclusion, this study indicated that the measurement of cytokines, CgA, and SIgA in saliva can be useful for the differential diagnosis of xerostomia and could be used to monitor pathological states in SS by collecting saliva over time. However, evaluating greater numbers of saliva samples is required to set the reference value of these molecules, which might eventually lead to diagnostic tool for xerostomia.

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Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

KO and MM designed the study; KO, SS, SF, MO, and YI collected samples; KO, AT, and TM performed the experiments; KO, MM, JNH, and AT analyzed the data; MM and SN drafted the paper.

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Oral Medicine/Original Research

Differences of stimulated and unstimulated salivary flow rates in patients with dry mouth

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ABSTRACT

Purpose: The purpose of this study was to clarify the usefulness of noninvasive examination items such as sialometry and Visual Analog Scale (VAS) in distinguishing Sjögren's syndrome (SS) in dry mouth patients from neurogenic/neuropsychiatric disorders and drugs (DND).
Patients and methods: The study cohort comprised 50 patients with SS and 28 patients with DND. The gum test and Saxon test for stimulated salivary flow rate (SSFR), the spitting test for unstimulated salivary flow rate (USFR) and VAS were performed in all the patients with dry mouth.
Results: In SS patients, the SSFR (mean: gum test, 6.34 mL/10 min; Saxon test, 1.19 g/2 min) and USFR (0.61 mL/15 min) were decreased. In DND patients, the SSFR (gum test, 16.35 mL/10 min; Saxon test, 3.58 g/2 min) was within the normal range, but the USFR (0.90 mL/15 min) was decreased. In VAS, SS patients scored significantly higher in the items of "water-drinking at meals", "difficulty in swallowing", and "taste abnormality", while significantly lower in the item of "oral pain".
Conclusion: These results suggest that the SSFR, USFR and VAS could be useful in distinguishing DND from SS.

1. Introduction

The number of patients complaining of dry mouth has increased recently, because of raised awareness of oral health [1–4]. Xerostomia is defined as a subjective complaint of dry mouth, and is caused by the evaporation and/or hyposalivation of saliva. Evaporation of saliva is mainly caused by mouth opening or mouth breathing, which often occurs during the night without an apparent decrease in the salivary flow. Hyposalivation occurs due to various causes, including radiation therapy to the head and neck, the use of medications, and certain systemic conditions and diseases such as diarrhea, dehydration, hyperthyroidism, diabetes mellitus, kidney malfunction, anemia, and Sjögren's syndrome [5–10]. Hyposalivation can be divided into two groups according to the mechanism of disorder: a destruction of the secretary cells of the salivary glands and a dysfunction of the autonomic nervous system which stimulates saliva secretion. One of the causes of the former disorder is Sjögren's syndrome (SS), and the latter is caused by anxiety, depression, and medications such as antidepressant, antiemetic, antihistamine, and antihypertensive. The term "dry mouth associated with neurogenic/neuropsychiatric disorders and drugs (DND)" is proposed for the latter disorder by the Japanese Society of Oral Medicine in 2008 [11]. SS and DND compose a majority of the patients with dry mouth.

SS is diagnosed based on the diagnostic criteria including the 1999 revised diagnostic criteria of the Ministry of Health, Labor and Welfare (MHLW) and those of the American-European Consensus Group, which are generally used in Japan [12–14]. However, a limitation of the criteria is that many patients go untested and do not receive a confirmed diagnosis. The reasons for this may include the large number of tests required to fulfill the diagnostic criteria, the fact that some tests are invasive (especially lip biopsy and sialography), and the fact that some patients do not want to be tested. On the other hand, there are no diagnostic criteria for DND. Thus, it is

☆ AsianAOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants.

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necessary to ascertain how best to carry out the tests for diagnosis of SS and DND without imposing an excessive burden on patients. In this context, the purpose of this study was to clarify the usefulness of noninvasive examination items such as sialometry and Visual Analog Scale (VAS) in distinguishing DND from SS. Differences of stimulated and unstimulated salivary flow rates and score of the VAS were compared between the patients with SS and DND.

2. Patients and methods

2.1. Patients

Fifty patients with SS (48 women and 2 men; mean age, 62.6 ± 10.5 years) and 30 patients with DND (25 women and 5 men; mean age, 53.9 ± 8.8 years) referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital from 2009 to 2013 were included in the study. All the patients presented with subjective complaint of dry mouth and decreased USFR flow rate (<1.5 mL/15 min) or SWS flow rate (<2.00 g/2 min). SS was diagnosed according to both the Research Committee on SS of the MHLW of the Japanese Government (1999) [12] and the American–European Consensus Group criteria for SS [14]. None of the patients had other autoimmune diseases and were treated with steroids or any other immunodepressants. DND was diagnosed according to the following criteria: (1) fail to fulfill each of the above-mentioned diagnostic criteria for SS and (2) taking drug or diagnosed with depression in Department of Psychosomatic Medicine. The patients with DND were taking antidepressant drug ($n = 8$), sleeping-inducing drug ($n = 8$), antihypertension drug ($n = 6$), and other oral medicines with side effect of dry mouth ($n = 10$). In contrast, three patients with DND were diagnosed with depression by the physicians in our hospital but were not taking any drugs. Informed consent, which was approved by the Ethics Committee of Kyushu University, Japan, was obtained from all the patients, and healthy controls were included in the study (IRB serial number: 25-287).

2.2. Measurements of salivary flow rates

The gum test was carried out by asking the subjects to chew gum for 10 min. The saliva secreted during that time was collected in a cap and its volume measured. If the volume of the saliva was <10 mL, the subject was classified as "decreased" [12–14]. The Saxon test was undertaken by having the subjects chew Surgeon® Type IV Gauze Sponge (Hakuzo Medical Corporation, Osaka, Japan) once a second for 2 min and measuring the weight of the gauze. If the increase in weight of the gauze was <2 g, the subject was classified as "decreased" [12–14]. The spitting test was carried out by asking subjects to spit saliva into a cup for 15 min. The amount of saliva in the unstimulated condition (sitting on a chair and not moving) was measured. If the volume of saliva was <1.5 mL, the subject was classified as "decreased" [13,14].

2.3. Subjective symptoms of dry mouth

The subjective symptoms and major complaints of dry mouth were ascertained from the medical interview. Additionally, a VAS was used for quantifying the subjective symptoms of dry mouth. The scale was from 0 mm to 100 mm. A reading of 0 mm was designated as "do not feel any symptoms" and that of 100 mm as "feel significant symptoms". Patients were asked to mark their subjective feeling between these two points arbitrarily, and the distance from 0 mm to their mark was measured. With this VAS method, six items of the symptoms of dry mouth (xerostomia, feeling hyposalivation, oral pain, water-drinking at meals, difficulty in swallowing, and taste abnormality) were assessed [13,14].

2.4. Statistical analyses

The Mann–Whitney *U*-test, chi-square test, and Pearson's product-moment correlation coefficient were used for statistical assessments. $p < 0.05$ was considered significant.

3. Results

3.1. Differences in subjective symptoms between SS patients and DND patients

In terms of major complaints, SS patients complained of "dryness of eyes" significantly more often than DND patients, whereas DND patients complained of "feeling oral pain" significantly more often than SS patients (Table 1). According to the comparisons between patients with SS and DND in the VAS, SS patients scored significantly higher in the items of "water-drinking at meals", "difficulty in swallowing", and "taste abnormality", while significantly lower in the item of "oral pain". There was no significant difference in the items of "xerostomia" and "feeling hyposalivation" between SS and DND patients (Fig. 1).

3.2. Salivary flow rates of patients with dry mouth

The SSFR of SS patients (mean: gum test, 6.34 mL/10 min; Saxon test, 1.19 g/2 min) was decreased significantly compared

Table 1
Frequency of major complaints with dry mouth patients.

	SS ($n = 50$)	DND ($n = 26$)
Xerostomia (%)	84	100
Hyposalivation	42	58
Dryness of eyes	60	27
Feeling oral pain	18	65
Drinking excess water at meals	52	77
Difficulty in swallowing	44	38
Abnormality of tasting	60	62
No complaint	12	0

χ^2 test.
SS, Sjögren's syndrome; DND, dry mouth associated with neurogenic/neuropsychiatric disorders and drugs.
* $p < 0.05$
** $p < 0.01$.

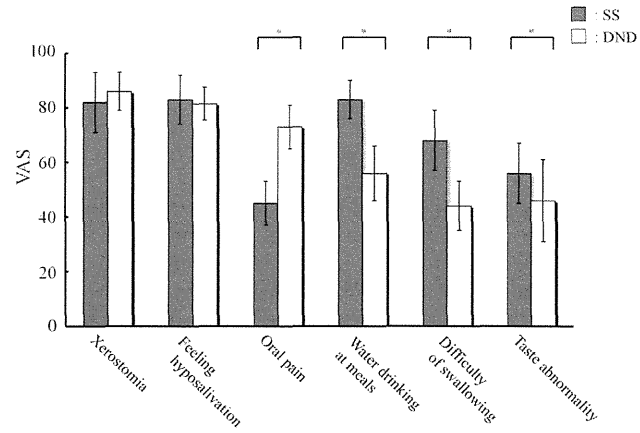

Fig. 1. Visual analog scale value of patients with dry mouth. Results of the visual analog scale (VAS) values of patients with Sjögren's syndrome (SS) and dry mouth associated with neurogenic/neuropsychiatric disorders and drugs (DND). The patients of both groups complained strongly in the items of xerostomia, feeling hyposalivation and oral pain. Additionally, the VAS values of water-drinking at meals difficulty in swallowing, and taste abnormality of DND patients were lower than those of SS patients (Mann–Whitney *U*-test, *$p < 0.05$).