In this validation study, the section of the questionnaire concerned with dental health was mailed to 288 dentists randomly selected from 953 members of the Fukuoka City Dental Association from April through August 2008. One of the authors (TN) conducted all oral examinations, including CPI measurement, at the clinics of dentists who consented to undergo an examination.

Follow-up

The study participants are currently under observation for mortality and morbidity by their affiliated prefectural dental associations. They are to be followed until at least March 2009 (March 2010 in some late-starting prefectures) and, if permitted, the follow-up period will be extended. The prefectural dental associations operate their own fraternal insurance programs. When a JDA member dies or suffers a disease, the member or his/her proxy submits a death or medical certificate to claim the insurance benefit through the office of his/her prefectural dental association. Most events requiring hospital admission are recorded as part of this claims process. Mortal and morbid events in study participants are reported to the study office and coded according to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10). The cause of death is determined based on the death certificate. If a study participant resigns from their dental association, it is reported and treated as a loss to follow-up.

Ethical issues

All eligible respondents, ie, those who consented to participation in the study and the use of their insurance data, submitted consent forms to their prefectural dental association office. To assure strict confidentiality, the questionnaire was anonymized with an ID number identical to that used on the consent form. The outcome data of participants are also anonymized at the prefectural dental association offices using the same ID number and forwarded to the study office. The ethics committees of Nagoya University School of Medicine and Aichi Cancer Center (former affiliation of the principal investigator, [KW]) both approved the protocol of this cohort study (Nos. 33 and 8–21, respectively). All participants in the validation study of self-reported periodontal status provided written informed consent. The protocol of this validation study was approved by the ethics committee of Fukuoka Dental College (No. 77), where the study was conducted.

Statistical analysis

We tabulated the proportions, means, and/ or medians of respondents for selected baseline characteristics by sex and 10-year age subgroup. Body mass index (BMI) was calculated by dividing self-reported body weight (kg) by the square of the participant's height (m^2). Those who consumed alcohol at least once a month were classified as drinkers. Hypertension was defined as a self-reported systolic blood pressure of ≥ 140 mm Hg, a diastolic blood pressure of ≥ 90 mm Hg, or a prescription for medication to treat hypertension. Stroke referred to cerebral infarction, cerebral hemorrhage, and subarachnoid hemorrhage, and coronary heart disease referred to myocardial infarction and angina pectoris. The GOHAI questions were asked in 20 of the 46 prefectural dental associations.

In the validation study of self-reported periodontal status, the CPI score reported on the questionnaire was compared to that measured at an oral examination. We evaluated the consistency between the two sets of assessment of periodontal status by calculating the kappa statistic. In addition, we categorized the participants as those with or without a CPI score of ≥ 3, and computed sensitivity and specificity. A CPI score of ≥ 3 on an oral examination by one of the authors (TN) was defined as the reference, or "gold standard." The exact 95% confidence interval (CI) for sensitivity and specificity was estimated based on the binomial distribution. All the analyses were performed with the Statistical Analysis System version 9.1 (SAS Institute Inc., Cary, NC, USA).[22]

RESULTS

The questionnaire was delivered to 58 792 JDA members, 22 415 of whom returned it to the study office. We excluded 1074 respondents who did not submit the consent form to the prefectural dental association offices and 69 with missing information on age or sex, leaving 21 272 participants (36.2% of those receiving the questionnaire). The age distribution of the cohort peaked at 40 to 49 years (Table 1), with a mean age $\pm$ SD of 52.3 $\pm$ 12.3 years (range, 26–98 years). Women were 8.0% of the population ($n = 1700$). As of June 2007, 154 participants have resigned from their dental associations.

The proportion of participants without a spouse at baseline was higher among women than men (Table 2). More than 90% of men and 75% of women were independent operators of their dental offices. The mean length of career as a dentist was 26.4 years (SD, 12.2) in men and 26.5 years (SD, 13.3) in women.

The proportion of current smokers was higher among younger men as compared with elderly men: 39.0% of men in

Table 1. Sex and age distribution of study participants at baseline

Age	Men		Women	
	n	%	n	%
20–29	41	0.2	18	1.1
30–39	2455	12.5	210	12.4
40–49	6713	34.3	599	35.2
50–59	5695	29.1	467	27.5
60–69	2524	12.9	149	8.8
70–79	1572	8.0	178	10.5
≥80	572	2.9	79	4.6
Total	19 572	100.0	1700	100.0

Table 2. Selected baseline demographic, lifestyle, and medical characteristics of participants by sex and age

	Age at baseline							Total
	20–29	30–39	40–49	50–59	60–69	70–79	≥80	
Men								
Marital status								
Unmarried (%)	61.0	14.1	3.3	1.3	0.4	0.1	0.2	3.5
Married (%)	39.0	83.8	94.4	96.1	96.9	92.4	82.6	93.2
Widowed or divorced (%)	0.0	2.0	2.3	2.7	2.8	7.5	17.3	3.3
Employment								
Independent (%)	63.4	86.2	94.5	96.9	94.3	82.0	57.9	92.0
Employed by relatives (%)	26.8	10.5	4.0	1.7	2.1	6.4	7.0	4.2
Employed by others (%)	9.8	2.9	1.2	0.9	0.8	0.5	0.2	1.2
Not at work as a dentist (%)	0.0	0.0	0.0	0.1	2.2	9.6	33.7	2.1
Others (%)	0.0	0.4	0.3	0.3	0.6	1.6	1.3	0.5
Years practicing as a dentist (mean ± SD)	4.1 ± 1.4	10.8 ± 2.8	19.0 ± 3.6	27.3 ± 4.1	37.4 ± 4.6	48.3 ± 5.4	56.7 ± 6.7	26.4 ± 12.2
Smoking								
Current smokers (%)	39.0	39.8	32.3	29.7	25.1	21.3	14.5	30.2
Ex-smokers (%)	4.9	22.4	32.7	40.5	43.0	52.6	56.6	37.2
Alcohol drinking								
Current drinkers (%)	70.7	73.0	77.2	76.6	71.6	59.7	45.1	73.5
Ex-drinkers (%)	2.4	2.1	2.3	2.6	5.3	9.7	12.3	3.6
Body mass index ≥25.0 (%)	34.2	33.6	33.2	31.2	24.8	20.9	14.1	30.1
Hypertension (%)*	24.3	23.8	31.3	43.7	56.6	66.3	66.8	41.1
History of								
Stroke (%)	2.4	0.5	1.0	1.8	5.1	11.1	12.6	2.9
Coronary heart disease (%)	0.0	0.5	1.2	3.1	7.5	13.2	15.4	3.8
Diabetes (%)	0.0	1.4	3.8	9.3	15.5	17.5	13.8	8.0
Cancer (%)	0.0	0.3	1.0	2.6	6.9	13.7	17.0	3.6
GHQ-12[†] score [median (interquartile range)]	1 (0–4)	2 (1–4)	2 (0–4)	1 (0–3)	1 (0–3)	1 (0–3)	1 (0–3)	1 (0–4)
(n)[‡]	(41)	(2408)	(6591)	(5548)	(2417)	(1427)	(468)	(18 900)
Women								
Marital status								
Unmarried (%)	72.2	34.1	22.4	14.4	8.1	14.1	5.1	19.2
Married (%)	27.8	55.8	62.8	67.1	67.1	45.2	29.1	59.7
Widowed or divorced (%)	0.0	10.1	14.8	18.5	24.8	40.7	65.8	21.1
Employment								
Independent (%)	44.4	75.2	80.4	80.3	78.5	67.2	57.7	76.8
Employed by relatives (%)	33.3	21.4	16.6	15.2	15.4	13.6	10.3	16.3
Employed by others (%)	16.7	2.9	1.7	1.7	0.7	0.0	0.0	1.7
Not at work as a dentist (%)	0.0	0.5	0.5	1.5	4.0	18.6	32.1	4.4
Others (%)	5.6	0.0	0.8	1.3	1.3	0.6	0.0	0.9
Years practicing as a dentist (mean ± SD)	4.6 ± 0.8	11.5 ± 2.7	19.2 ± 3.8	26.7 ± 5.0	37.1 ± 5.1	49.8 ± 5.9	55.7 ± 7.9	26.5 ± 13.3
Smoking								
Current smokers (%)	16.7	12.0	13.7	10.0	8.1	5.2	3.9	10.7
Ex-smokers (%)	5.6	9.1	10.0	10.0	4.7	9.2	5.2	9.1
Alcohol drinking								
Current drinkers (%)	55.6	51.4	53.9	45.8	34.7	24.4	13.2	44.8
Ex-drinkers (%)	0.0	4.8	3.5	1.7	2.0	1.1	1.3	2.7
Body mass index ≥25.0 (%)	11.1	3.9	10.3	15.7	18.2	19.1	19.5	13.1
Hypertension (%)*	5.9	6.3	12.5	26.9	38.3	60.0	77.3	26.1
History of								
Stroke (%)	0.0	0.0	0.3	1.3	0.7	6.2	7.6	1.5
Coronary heart disease (%)	0.0	0.0	0.5	0.6	4.7	11.8	7.6	2.4
Diabetes (%)	0.0	0.0	1.2	1.7	7.4	7.3	6.3	2.6
Cancer (%)	0.0	0.5	4.2	5.1	7.4	11.8	7.6	5.2
GHQ-12[†] score [median (interquartile range)]	3.5 (1–6)	2 (0–4)	2 (0–4)	2 (0–4)	1 (0–4)	1 (0–3)	1 (0–3)	1.5 (0–4)
(n)[‡]	(18)	(209)	(579)	(455)	(146)	(160)	(61)	(1628)

*Self-reported systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or taking medication for hypertension.
[†]GHQ-12: 12-item General Health Questionnaire.
[‡]Number of participants with complete data.

their third decade of life smoked, as did 39.8% in their fourth decade and 14.5% in their ninth or tenth decade. However, male ex-smokers were more common in older age groups (Table 2). Approximately 10% of women smoked at baseline, and the trend decreased with age. At baseline, more than 70% of men and more than 40% of women were alcohol drinkers, but the prevalence of former drinkers was higher among the elderly in men. Obesity, defined as BMI $\geq 25.0 \mathrm{kg/m^2}$, was inversely associated with age among men; however, the association was positive among women. The prevalence of hypertension was clearly age-dependent in both sexes. Histories of stroke, coronary heart disease, diabetes, and cancer were also more prevalent among elderly participants and, with the exception of cancer, were more common in men than in women.

With regard to oral health indicators (Table 3), more than half of men brushed their teeth at least 3 times per day, except for elderly men; women brushed their teeth more frequently. Tooth loss markedly increased with age, and the mean numbers of lost teeth were very similar between men and women in the same age categories. Interestingly, inter-individual variations, as shown in the SD values, were very large. The proportions of men and women with at least 20 teeth were greater than 95% for those under the age of 60 years, but dropped quickly as age increased, reaching 22.6% and 21.5% in men and women aged at least 80 years, respectively. The age-specific percentages of those with ≥ 20 present teeth differed little between sexes. The prevalence of periodontal disease with a CPI score of ≥ 3 also increased with age in men and women, and was approximately 10% overall. A history of periodontal diseases with pockets $\geq 4 \mathrm{mm}$ was more common in older participants. Some inconsistency was observed between the proportion of participants with a CPI score of ≥ 3 and the percentage of those with a history of periodontal disease with pockets $\geq 4 \mathrm{mm}$: the former was higher than the latter in men and women aged 20 to 29 years. The proportions, based on the response to the questionnaire, are shown in Table 3 without correction. An inverse correlation was found between age and GOHAI score, which implies that oral health QOL is poorer in elderly participants.

Validity of self-reported periodontal status

Of the 288 dentists who received the dental part of the questionnaire, 61 returned it and 50 (17.4% of the selected dentists) consented to undergo an oral examination. The mean age $\pm$ SD of the 50 participants was 53.3 $\pm$ 9.7 years (range, 33–73 years), and women accounted for 8.0% ($n = 4$) of participants. The mean interval $\pm$ SD between the reply to the questionnaire and the oral examination was 56 $\pm$ 36 days.

Table 4 shows the joint classification of CPI score as determined by oral examination and reported on the self-administered questionnaire. The consistency between the two sets of CPI scores was not high: the kappa coefficient was 0.53 (95% CI, 0.36–0.70). However, when the participants were dichotomized into those with or without a CPI score of ≥ 3 (Table 4), the kappa coefficient was 0.73 (95% CI, 0.53–0.93), with 87.5% sensitivity (95% CI, 61.7%–98.5%) and 88.2% specificity (95% CI, 72.6%–96.7%). Responses to the questionnaire indicated that periodontal status was assessed by a dental hygienist, another dentist, the respondent, or another person in 22 (44.0%), 14 (28.0%), 8 (16.0%), and 6 (12.0%) of the 50 participants, respectively. Even when the analysis was limited to assessments by the respondents themselves or another person, the kappa coefficient (0.85; 95% CI, 0.57–1.00) was not lower than that for assessments by a dental hygienist or another dentist (0.68; 95% CI, 0.43–0.93).

DISCUSSION

We have described the design of our nationwide prospective study of dentists and briefly presented the baseline lifestyle, clinical, and oral health characteristics of the participants. In addition, we validated the self-administered questionnaire on periodontal status by means of oral examinations. To our knowledge, this is the largest cohort study of dentists in Japan, a nation with relatively high mortality and incidence rates, among developed countries, for stroke and gastric cancer.[23,24] Much attention has recently been paid to the relations between oral health and the risks of both stroke[25] and gastric cancer[26,27]; information from the present study may increase our understanding of these relations.

To further characterize the cohort at baseline, we compared selected health-related characteristics of the cohort with those of the Japanese population by sex and age using data from available nationwide surveys, including The National Health and Nutrition Survey in Japan, 2004,[28] The Survey of Dental Diseases, 2005 (data available on the Internet at http://www.mhlw.go.jp/topics/2007/01/tp0129-1.html), and the nationwide survey of GOHAI, which establishes national norms for Japanese (available at http://www.i-hope.jp/2007/09/qol_06.html) (Appendix). In men in the present cohort, the proportion of current smokers was lower than in the general population, but the proportion of ex-smokers was higher. In particular, the percentage of male, current smokers younger than 60 years was more than 10 points lower than that of the general population. In women, however, smoking status among the cohort and general population was very similar, and the proportions of current drinkers were comparable, with the exception of middle-aged women. Obesity was similarly or less prevalent in the cohort, as compared with the general population, in middle-aged and elderly men and women. Hypertension was substantially more common in the younger age subgroups of the cohort, as compared with the general population.

The cohort members generally had better oral health than the general population: they had a more diligent hygienic routine, fewer lost teeth, fewer periodontal diseases, and

Table 3. Selected baseline oral health indices of participants by sex and age

	Age at baseline							Total
	20–29	30–39	40–49	50–59	60–69	70–79	≥80	
Men								
Brushing ≥3 times/day (%)	51.2	56.9	56.3	55.7	55.0	45.4	36.6	54.6
Filled teeth (mean number ± SD)*	7.9 ± 8.2	10.1 ± 6.4	10.5 ± 6.3	8.4 ± 6.0	5.2 ± 5.4	3.8 ± 5.1	2.8 ± 4.7	8.4 ± 6.5
Tooth loss (mean number ± SD)	1.0 ± 4.4	0.7 ± 1.9	1.2 ± 2.2	2.4 ± 3.7	5.3 ± 7.0	13.0 ± 10.0	18.6 ± 9.6	3.4 ± 6.4
Number of present teeth ≥20 (%)	97.6	99.6	99.3	95.5	80.7	44.4	22.6	89.3
Use of bridges (%)*	0.0	10.6	22.1	28.2	23.1	19.8	17.6	22.2
Use of partial dentures (%)	2.4	4.6	11.8	20.8	36.8	55.6	50.3	21.4
Use of full dentures (%)	0.0	0.0	0.0	0.9	7.0	30.2	53.6	5.1
CPI score ≥3 (%)†	7.7	5.5	7.8	12.9	19.7	15.8	10.0	10.9
CPI score =3 (%)	5.1	4.7	6.5	9.8	15.1	11.8	7.9	8.6
CPI score =4 (%)	2.6	0.8	1.3	3.1	4.6	4.0	2.0	2.3
No index teeth for CPI measurement (%)	0.0	0.0	0.0	0.4	4.7	23.8	47.5	3.3
History of periodontal diseases with pockets (≥4 mm) (%)*	0.0	10.0	13.4	24.1	39.2	51.1	42.5	23.0
GOHAI score (mean ± SD)‡	57.8 ± 3.7	56.0 ± 5.3	55.5 ± 5.4	54.5 ± 6.1	53.3 ± 6.9	51.5 ± 7.7	50.5 ± 8.3	54.5 ± 6.3
(Number of participants with sufficient data)	(19)	(1122)	(3158)	(2675)	(1265)	(772)	(241)	(9252)
Women								
Brushing ≥3 times/day (%)	83.3	73.8	72.8	78.9	60.3	59.3	47.9	71.2
Filled teeth (mean number ± SD)*	8.3 ± 5.5	11.5 ± 6.3	12.1 ± 5.9	10.8 ± 5.5	7.8 ± 5.1	4.7 ± 5.3	3.6 ± 5.8	10.0 ± 6.3
Tooth loss (mean number ± SD)	1.0 ± 1.7	0.9 ± 1.6	1.4 ± 2.1	2.0 ± 2.5	5.4 ± 6.6	13.1 ± 10.0	19.3 ± 9.6	3.9 ± 6.8
Number of present teeth ≥20 (%)	100.0	99.5	99.5	97.4	81.2	47.2	21.5	88.3
Use of bridges (%)*	18.2	15.3	21.7	35.8	38.5	32.8	22.4	27.5
Use of partial dentures (%)	5.6	7.7	11.4	21.5	43.6	58.2	43.0	22.9
Use of full dentures (%)	0.0	0.0	0.0	0.0	6.0	31.1	60.8	6.6
CPI score ≥3 (%)†	5.6	5.5	7.0	11.2	15.1	16.9	1.9	9.3
CPI score =3 (%)	5.6	4.0	6.3	8.1	13.0	13.9	1.9	7.6
CPI score =4 (%)	0.0	1.5	0.7	3.2	2.2	3.1	0.0	1.8
No index teeth for CPI measurement (%)	0.0	0.0	0.0	0.0	2.9	20.8	49.1	3.7
History of periodontal diseases with pockets (≥4 mm) (%)*	0.0	6.7	10.9	17.0	30.4	32.6	36.8	16.7
GOHAI score (mean ± SD)‡	56.0 ± 4.0	56.2 ± 4.7	55.8 ± 5.5	55.0 ± 5.3	54.1 ± 5.3	52.3 ± 7.7	52.7 ± 6.3	55.0 ± 5.7
(Number of participants with sufficient data)	(8)	(111)	(333)	(243)	(82)	(95)	(38)	(910)

*Surveyed in 31 of the 46 prefectural dental associations.
†CPI: Community Periodontal Index.
‡GOHAI: General Oral Health Assessment Index. Index was surveyed in 20 of the 46 prefectural dental associations.

Table 4. (a) Joint classification of CPI score as assessed by oral examination and reported on self-administered questionnaire among 50 participants in a validation study. (b) Categorization of participants with or without a CPI score of ≥3.

(a)

CPI score reported on questionnaire	CPI score assessed by oral examination					Total
	0	1	2	3	4	
0	8	0	2	1	0	11
1	4	4	0	0	0	8
2	4	0	8	1	0	13
3	3	0	1	10	2	16
4	0	0	0	0	2	2
Total	19	4	11	12	4	50

(b)

CPI score reported on questionnaire	CPI score assessed by oral examination		Total
	0–2	≥3	
0–2	30	2	32
≥3	4	14	18
Total	34	16	50

higher oral health-related QOL. In men, differences between the two populations in the mean number of teeth lost and the proportion of those with a sufficient number of teeth increased with age, but diminished in the very old. Thus, lifelong oral health was not easily attained, even among dentists with good oral hygiene.

Some methodological issues in the present study warrant consideration. First, the validity of self-reported oral health status needs to be confirmed. To address this concern, we conducted a validation study focusing on periodontal status that compared the responses on the questionnaire with the findings of dental examinations. The study demonstrated that the participants could be classified, with satisfactory validity, into those with or without periodontal pockets ≥4 mm in any of the index teeth used for CPI measurement (kappa coefficient, 0.73; sensitivity, 87.5% and specificity, 88.2%). The information collected with the self-administered questionnaire, therefore, should be useful when classifying respondents according to the presence of deep periodontal pockets. In the present baseline survey, periodontal status was not always assessed by a dental professional other than the participant: it was determined by a dental hygienist, another dentist, the respondent, or another person in 38.6%, 20.1%, 32.6%, and 8.6% of the participants, respectively. Although the respondents themselves and non-dental professionals cannot accurately measure CPI, they assessed periodontal status and classified it according to the criteria for periodontal status used for CPI scoring (score 0–4) in the present study. In terms of the assessment of periodontal condition, the validation study did not show lower validity for persons other

than dental hygienists or other dentists when classifying individuals as those with or without a CPI score of ≥3. Nevertheless, in analyses of the cohort data, subgroup analyses that account for the profession of the assessors may prove informative.

Second, our cohort was comprised of oral health professionals, and findings from such a highly specific population cannot be straightforwardly generalized. Although the participants were in better oral health than the general population, there was still considerable inter-individual variation in the indices; some respondents scored poorly on the indices (eg, many lost teeth, high CPI score). Therefore, we expect to be able to compare subsequent mortality and morbidity between those with better and worse scores on indices. In addition, the cohort is relatively homogeneous in terms of socioeconomic status, so the effect of this potential confounder[29,30]—a concern in other studies[26,27]—will be minimized. Third, lack of direct measurement of blood pressure, blood lipids, and glucose levels may be a limitation when attempting to determine the association between oral health and cardiovascular disease. Fourth, we have not analyzed some potential occupational risks, such as infections and exposures to asbestos and mercury, because of the difficulty in doing so with a self-administered questionnaire comprising a limited number of pages. We therefore must consider the extent to which residual or unadjusted confounding by cardiovascular risk factors or occupational risk affects the association between oral health and systemic diseases.

Finally, because the response rate was not high (36.2%), the respondents are not a representative sample of JDA members. This fact should be kept in mind when interpreting the baseline profiles in this article. In addition, in generalizing the findings from this study to a designated target population, differences in the background characteristics of the cohort and the target population must be identified, and the relevance of such differences should be carefully considered from the perspective of the underlying biological mechanisms.[31] Although dental status differed somewhat between the dentists and the general public, the relevant biological mechanisms are essentially identical among humans, and the associations between oral health and general health, as observed in dentists, should be applicable to the general population.

As of December 2007, the cohort members have been under observation for an average of 4 years. We expect the study to provide strong evidence for the link between oral health and general well-being.

ACKNOWLEDGMENTS

Appendix. Selected health-related characteristics of the Japanese general population, by sex and age (summarized data from nationwide surveys)

	Age						
	20–29	30–39	40–49	50–59	60–69	70–79	≥80
Men							
Current smokers (%)*	51.3	57.3	51.4	47.7	33.3	24.0	
Ex-smokers (%)*	6.9	11.3	19.3	27.2	32.2	38.1	
Current drinkers (%)*,†	64.6	72.0	75.8	76.5	73.0	57.2	
Ex-drinkers (%)*	0.5	1.1	2.1	0.6	2.3	4.9	
Body mass index ≥25.0 (%)*	19.9	28.9	32.7	30.8	29.7	25.5	
Hypertension (%)*,‡	7.3	16.4	44.0	53.7	67.7	72.9	
Brushing ≥3 times/day (%)§	9.4	9.0	10.7	10.8	15.2	18.4	18.7
Tooth loss (mean number)§,‖	0.2	0.5	2.0	4.6	8.1	14.2	18.4
Number of present teeth ≥20 (%)§	100.0	99.4	95.9	85.2	65.3	37.9	24.7
CPI score ≥3 (%)§,¶	20.3	29.4	36.3	53.8	55.5	46.6	34.4
GOHAI score (mean)**,††	54.3	53.8	53.6	53.1	52.8	50.4	NA‡‡
Women							
Current smokers (%)*	18.0	18.0	13.7	13.7	7.6	4.5	
Ex-smokers (%)*	5.2	7.8	6.0	2.7	3.8	4.0	
Current drinkers (%)*,†	51.0	49.6	47.2	38.3	29.4	17.9	
Ex-drinkers (%)*	1.9	2.8	1.0	0.5	1.4	1.7	
Body mass index ≥25.0 (%)*	5.4	8.3	17.9	24.1	29.9	26.7	
Hypertension (%)*,‡	1.1	6.5	21.9	38.8	55.9	75.2	
Brushing ≥3 times/day (%)§	30.8	28.9	27.1	26.0	27.5	26.8	17.9
Tooth loss (mean number)§,‖	0.3	0.7	1.7	4.2	8.9	15.4	21.0
Number of present teeth ≥20 (%)§	100.0	99.6	96.7	85.0	61.7	34.4	12.7
CPI score ≥3 (%)§,¶	11.2	20.8	35.8	40.6	46.1	44.6	27.3
GOHAI score (mean)**,††	52.3	54.8	53.7	51.3	52.4	51.2	NA‡‡

*National Health and Nutrition Survey in Japan, 2004.
†Current drinkers were defined as individuals who consume alcohol at least once a month.
‡Systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or taking medication for hypertension.
§Survey of Dental Diseases, 2005.
‖Maximum number of teeth was adjusted to 28 for comparison with figures from present study.
¶CPI: Community Periodontal Index.
**GOHAI: General Oral Health Assessment Index.
††National norm for Japanese (mean).
‡‡NA: not available.

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Tooth loss and intakes of nutrients and foods: a nationwide survey of Japanese dentists

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Abstract – *Objectives:* To clarify the association of tooth loss with dietary intakes among dentists, for whom sufficient dental care is available. *Methods:* We analyzed the data from 20 366 Japanese dentists (mean age ± SD, 52.2 ± 12.1 years; women 8.0%) who participated in a nationwide cohort study from 2001 to 2006. The baseline questionnaire included a validated food-frequency questionnaire to estimate intakes of foods and nutrients. We computed the geometric means of daily intakes by the number of teeth, adjusting for age, sex, smoking, physical activity, and history of diabetes. *Results:* The mean intakes of some key nutrients and food groups, such as carotene, vitamins A and C, milk and dairy products, and vegetables including green-yellow vegetables, decreased with the increasing number of teeth lost (P for trend <0.05). On the contrary, mean intakes of carbohydrate, rice, and confectioneries were increased among those with fewer teeth (P for trend <0.05). The difference in the geometric mean (%) between totally edentulous subjects and those with ≥25 teeth, that is [(Geometric mean for ≥25 teeth) — (Geometric mean for 0 teeth)]/(Geometric mean for ≥25 teeth) × 100, was 14.3%, 8.6%, 6.1%, and −6.1% for carotene, vitamin C, vitamin A, and carbohydrate, respectively. For food groups, it was 26.3%, 11.9%, 5.6%, −9.5%, and −29.6% for milk and dairy products, green-yellow vegetables, total vegetables, rice, and confectioneries, respectively. *Conclusions:* Tooth loss was linked with poorer nutrition even among dentists.

Attention has recently been paid to oral health in relation to overall well-being (1). Since the primary function of teeth is mastication, their loss will reduce masticatory ability (2, 3). Some authors (1, 4) have suggested that there are detrimental changes in dietary intakes that are due to impaired dentition, which they believe possibly to mediate the risk of systemic diseases. Even if the increase in health risks due to tooth loss may be small, the impact could be great because of the high prevalence of such losses. According to the National Dental Health Survey in 2005, 60.1% of Japanese aged 65 years or more were estimated to have <20 teeth (5).

Several studies (4, 6–9) have found an inverse association between the number of teeth and intakes of several nutrients as well as fruits and vegetables, which are potentially important to reducing the risk of cardiovascular disease, cancer, and other systemic conditions (10, 11). The lower blood levels of some key nutrients such as retinol, β-carotene, folate, and ascorbic acid were reportedly associated with edentulousness (7, 12).

However, what if high standards of dental treatment are available? Is the association between tooth loss and dietary intake considerably weakened? Populations of dentists will be suitable to

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address this question. Dentists are expected to have more teeth than the general population (13). In addition, they have ready access to sufficient dental treatment that may result in better dietary intake despite tooth loss. On the contrary, if tooth loss is correlated with poor dietary intake even among dentists, it may imply that the deteriorated food intake due to tooth loss cannot be adequately recovered by dental treatment and will indicate the importance of preserving teeth for nutrition.

Because both diet (14) and tooth loss (15) are influenced by socio-economic status (SES) and educational attainment, SES may confound the association between tooth loss and dietary intake. If the association is examined in a population uniform for SES, such confounding will be well-controlled. Joshipura et al. (6) reported more pronounced differences in diet between edentulous participants and those with 25 or more teeth among non-dentists than among dentists in the Health Professionals Follow-up Study.

The aim of the present study was to further clarify the association of tooth loss with dietary intakes among a population homogeneous for SES, for which sufficient dental care is available. For this purpose, we analyzed the data from an ongoing cohort study among members of a dental association; the study was named LEMONADE (Longitudinal Evaluation of Multi-phasic, Odontological and Nutritional Associations in Dentists).

Participants and methods

Participants and baseline survey

The protocol of the LEMONADE study has been described in detail elsewhere (13). In brief, dentists joining the Japan Dental Association (JDA) were invited to participate in the study. A self-administered, paper-based questionnaire was mailed to all JDA members except those working in one prefecture at one time during the study period from 2001 to 2006. The survey covered self-reported oral health factors including oral hygiene habits, periodontal status, and the number of lost teeth (excluding third molars). The participants were also asked about their personal history of chronic diseases and lifestyle factors such as diet and smoking and drinking habits.

The dietary part of the questionnaire inquired as to the intake frequency of 97 modern Japanese foods or dishes consumed during 1 month just before the baseline survey. This food-frequency questionnaire (FFQ) was designed to estimate daily amounts of food-group consumption and nutrient intakes (16, 17). In the FFQ, we asked the participants about their intake frequencies of the 97 food items. The consumption of a food item was estimated by multiplying the standard portion size (i.e., average quantity of food per intake) by its intake frequency. For rice and beverages, questions on usual portion sizes were also included because they may greatly vary among respondents. Nutrient intakes can also be assessed by establishing a database on composition of the food items for target nutrients. The intake of a nutrient is calculated using the following formula for each item: (reported intake frequency per day) × (portion size in grams) × (nutrient content per 100 g)/100. The intake is then summed for all the 97 listed food items to obtain the intake per day. This food-frequency method has widely been applied to large-scale epidemiological studies and has proved to be quite useful (18).

Our FFQ was validated by referring to dietary records (16, 19). The de-attenuated correlation coefficients for energy-adjusted intakes between the FFQ and 16-day dietary records ranged from 0.42 to 0.83 for nutrients (median, 0.61) and 0.36–0.83 for food groups (median, 0.58) in this study. The correlation coefficients for nutrients and food groups of potential importance were as follows: 0.54 for vitamin A, 0.45 for carotene, 0.61 for vitamin C, 0.63 for dietary fiber, 0.50 for vegetables, and 0.80 for fruits. Consumption of fruit juice was not included in that of fruit in the FFQ. All the eligible respondents provided written informed consent for participation in the LEMONADE study. The ethics committees of Nagoya University School of Medicine and Aichi Cancer Center (the former affiliation of the principal investigator [KW]) approved the protocol of this study.

Statistical analysis

Body mass index (BMI) was computed by dividing self-reported body weight (kg) by the square of height (m^2). The subjects were a priori categorized into five groups according to the number of remaining teeth (calculated as 28 minus the number of lost teeth), that is, 0, 1–9, 10–19, 20–24, and 25–28 teeth (4, 6, 7, 9), irrespective of use of dentures. Background characteristics were compared between the groups after adjustment for age (10-year age group) and sex by the direct method for proportions and by the general linear model for means. The decreasing or increasing trend in

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proportion with the increasing number of remaining teeth was statistically tested with the Mantel extension test, as was such a trend in means with the general linear model. The categories of 0, 1–9, 10–19, 20–24, and 25–28 teeth were scored as 0, 1, 2, 3, and 4, respectively, and the score was included in the linear model as a single independent variable.

To examine the association of tooth loss with nutrient intakes (excluding those from supplements) and food-group consumption, we computed the means of intakes by the number of remaining teeth. The means were adjusted for age (10-year age group), sex, smoking (never, former, or current smokers), moderate to heavy physical activity (hours/day: five categories from the lowest quintile to the highest one), and history of diabetes (yes or no) by the general linear model. In the present analysis, SES was not considered because the population comprised of dental association members seems to be reasonably homogeneous for SES. Intakes of nutrients, energy, and food groups were $\log_e$-transformed to improve the normality in their distribution. The geometric means of intake were then calculated. Their 95% confidence intervals were estimated based on the standard errors derived from the linear models. The difference in the geometric means (%) between edentulous subjects and those with 25–28 teeth was defined as follows: [(Geometric mean for 25–28 teeth) − (Geometric mean for 0 teeth)]/(Geometric mean for 25–28 teeth) × 100.

The linear trend in the geometric means across the categories of the number of remaining teeth was assessed by incorporating the same score as the one used in the test for trend in means in background characteristics into a linear model. All P values were two-sided, and all the analyses were done using the Statistical Analysis System version 9.1 (20).

Results

The questionnaire was delivered to 58 792 members of JDA, 21 341 of whom returned it to the central office of the study. At the same time, each member submitted the consent form to the office of the prefectural dental association with which he or she was affiliated. We excluded 69 with missing data on age and/or sex, leaving 21 272 (36.2% of the dispatched questionnaires). For the present analysis, 411 participants with missing information on rice consumption were omitted since rice is still the essential staple among Japanese. In addition, we excluded 243 with implausibly high or low estimates of energy intake (participants with $\log_e$-transformed energy intake values beyond three SD from the sex-specific mean (21)), 90 lacking data on their number of missing teeth, and 162 without data on the covariates. The age distribution of the remaining 20 366 participants for our analyses peaked at 40 to 49 years and a mean age ± SD of 52.2 ± 12.1 (range, 26–98) years, with women accounting for 8.0% of them. A total of 5050 (24.8%) dentists reported that they had lost four or more teeth. Female participants, current smokers, and those with a history of diabetes were more common in those with fewer teeth (Table 1). The mean age decreased while physical activity increased with an increasing number of remaining teeth.

The mean intakes of carotene and vitamins A and C declined with the increasing number of teeth lost to constitute a significant trend (P for trend <0.05) (Table 2). On the contrary, mean intake of carbohydrate was increased among those with fewer teeth. The difference in the geometric mean (%) between totally edentulous subjects and those with ≥25 teeth ranged from −6.1% to 14.3%. It was greatest for carotene (14.3%) and vitamin C (8.6%)

Table 1. Background characteristics of study subjects by number of present teeth (n = 20 366)

	Number of present teeth					P for trend
	0	1–9	10–19	20–24	25–28	
n	554	627	959	2910	15 316	
Women (%)	10.5	8.0	8.1	10.5	7.5	<0.001
Age (years, mean ± SD)	75.2 ± 10.2	72.9 ± 9.9	68.1 ± 9.9	55.9 ± 11. 4	48.8 ± 9.5	<0.001
Current smokers (%)[a]	39.1	43.8	48.7	31.8	25.8	<0.001
Ex-smokers (%)[a]	30.0	30.4	34.0	34.8	35.2	0.41
History of diabetes (%)[a]	12.1	9.5	10.3	7.8	6.9	<0.001
Body mass index (kg/m^2, mean ± SE)[a]	22.1 ± 0.2	21.8 ± 0.1	22.0 ± 0.1	22.2 ± 0.1	22.2 ± 0.1	0.17
Moderate to heavy physical activity (h/week, mean ± SE)[a]	5.5 ± 0.3	5.5 ± 0.3	5.9 ± 0.2	6.0 ± 0.2	6.1 ± 0.2	0.004

[a]Adjusted for age and sex.

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and smallest for carbohydrate (–6.1%). Total energy intake was higher among the participants with fewer remaining teeth than among those with more teeth (P for trend =0.029). This trend appeared only after the adjustment for physical activity.

The mean consumption of noodles, milk and dairy products, and vegetables including green-yellow vegetables also decreased with the increasing number of teeth lost (Table 3). The consumption of rice and confectioneries, however, was lower in those with more remaining teeth; the difference in the geometric mean (%) between totally edentulous participants and those with ≥25 teeth was –9.5% and –29.6%, respectively. In contrast, the gap exceeded 10% for milk and dairy products (26.3%) and green-yellow vegetables (11.9%).

Discussion

In this cross-sectional analysis, even among dentists we found a decreasing trend in intakes of carotene and vitamins A and C with the increasing number of teeth lost. Similar trends were detected for the consumption of milk and dairy products and green-yellow vegetables, whereas the consumption of rice and confectioneries was inversely correlated with the number of remaining teeth. The associations of tooth loss with dietary intake were independent of age, sex, smoking habits, physical activity, or history of diabetes.

Although dentists have ready access to a high-quality dental care, the odontopathy-induced, compromised dietary intakes may not be sufficiently restored by such treatment. It has been indicated that loss of natural teeth causes reduced masticatory efficiency even after the replacement with dentures (22–24).

Many investigations have suggested a protective role for vegetable consumption against cardiovascular disease (25, 26), and to a lesser degree, against cancer (27). The lower intake of vegetables associated with tooth loss detected in our study may, in turn, lead to an increase in the risk of these diseases.

Our finding that the intakes of key foods and nutrients are positively associated with the number of teeth remaining is generally in line with those of previous studies. The association with intakes of carotene, fiber, and vegetables has been stressed (4, 6, 7, 12). In contrast, we found the intake of carbohydrate and consumption of rice and

confectioneries to be higher among subjects with fewer teeth, which is not surprising since these foods would be relatively easy to chew. The higher intake of confectioneries in those with tooth loss, however, might not be the consequence but rather the cause of their tooth loss.

In our dataset, the number of teeth lost was correlated with the self-reported frequency of difficulty in chewing; the Spearman correlation coefficient was 0.30 (P < 0.001) after adjustment for age, sex, smoking, physical activity, and history of diabetes. The difficulty in chewing was, in turn, associated with higher consumption of rice and confectioneries. Those who sometimes or more frequently experienced this difficulty consumed rice and confectioneries, on average, 334.7 and 8.3 g/day, respectively, but so did those who never or seldom encountered the difficulty 325.5 and 7.3 g/day (adjusted for age, sex, smoking, physical activity, and history of diabetes; P for difference, 0.021 for rice and <0.001 for confectioneries).

The large sample size enabled us to include a sufficient number of subjects with many teeth lost even in a population of dentists. Although the validity of responses to the number of missing teeth in our questionnaire was not evaluated in the participants, Buhlin et al. reported a good correlation between the self-reported number of teeth and the number detected at clinical examinations in a general population (28); the mean difference between the two numbers was 1.4 teeth. We may expect an even higher level of validity among dentists. Some other methodological issues, however, warrant consideration.

First, since our study population is comprised exclusively of dentists, it may differ considerably from more heterogeneous general populations. This, however, provided us a unique opportunity to examine the association between tooth loss and dietary intakes among subjects with ready access to sufficient dental treatment. In addition, the cohort appears to be relatively homogeneous in terms of SES. The potential confounding by SES (15), therefore, would be minimal.

The response rate of the present study was relatively low (36.2%). Accordingly, the non-responders may differ from the responders. To characterize the non-responders, we compared the 21 272 participants with all the JDA members (as of March 2005) in terms of age, sex, and geographical area and found that the age and sex distributions were very comparable except for the very old. Those aged 20–29, 30–39, 40–49, 50–59, 60–69,

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Table 2. Adjusted geometric means of daily nutrient intake by number of present teeth (n =20 366)[a]

Nutrients	Number of present teeth					P for trend	% Difference[b]
	0 Geometric mean (95% CI)	1–9 Geometric mean (95% CI)	10–19 Geometric mean (95% CI)	20–24 Geometric mean (95% CI)	25–28 Geometric mean (95% CI)		
Energy (kcal)	1770 (1716–1826)	1742 (1690–1795)	1752 (1706–1799)	1721 (1684–1758)	1716 (1683–1750)	0.029	-3.1
Protein (g)	64.1 (61.8–66.4)	63.6 (61.5–65.9)	63.0 (61.1–64.9)	62.7 (61.2–64.2)	63.1 (61.7–64.5)	0.63	-1.6
Fat (g)	45.1 (43.2–47.2)	46.1 (44.2–48.0)	45.3 (43.7–47.1)	45.6 (44.3–47.0)	45.9 (44.7–47.2)	0.44	1.7
Carbohydrate (g)	239.5 (232.4–246.8)	232.0 (225.4–238.8)	231.0 (225.2–236.9)	226.7 (222.1 –231.4)	225.7 (221.5–230.0)	<0.001	-6.1
Calcium (mg)	468 (445–493)	493 (469–518)	486 (465–507)	482 (466–500)	493 (477–509)	0.058	5.0
Iron (mg)	9.1 (8.7–9.4)	8.9 (8.6–9.3)	8.8 (8.5–9.1)	8.9 (8.7–9.2)	9.0 (8.8–9.3)	0.30	-0.2
Potassium (mg)	2470 (2371–2574)	2489 (2392–2590)	2467 (2382–2555)	2458 (2389–2528)	2483 (2419–2548)	0.60	0.5
Vitamin A (IU)	2046 (1900–2205)	2154 (2005–2315)	2107 (1978–2245)	2117 (2011–2228)	2180 (2080–2285)	0.046	6.1
Retinol (µg)	278 (255–302)	287 (265–312)	287 (267–309)	279 (263–296)	279 (264–294)	0.61	0.4
Carotene (µg)	1597 (1469–1737)	1746 (1610–1893)	1647 (1534–1769)	1747 (1648–1851)	1863 (1767–1965)	<0.001	14.3
Vitamin C (mg)[c]	93 (86–100)	97 (90–104)	95 (89–101)	97 (92–103)	102 (97–107)	<0.001	8.6
SFA (g)[c]	12.5 (11.9–13.1)	13.0 (12.4–13.6)	12.7 (12.2–13.3)	12.7 (12.3–13.1)	12.7 (12.4–13.1)	0.87	2.0
MUFA(g)[c]	15.5 (14.8–16.3)	15.9 (15.2–16.6)	15.6 (15.0–16.3)	15.8 (15.3–16.4)	15.9 (15.5–16.4)	0.28	2.4
PUFA (g)[c]	11.5 (11.0–12.0)	11.5 (11.1–12.0)	11.4 (11.0–11.9)	11.5 (11.2–11.9)	11.6 (11.3–11.9)	0.29	1.1
Cholesterol (mg)	227 (212–244)	239 (223–256)	227 (214–241)	233 (222–245)	235 (225 – 246)	0.33	3.5
Vitamin E (mg)	7.2 (6.9–7.5)	7.2 (7.0–7.5)	7.1 (6.9–7.4)	7.2 (7.0–7.4)	7.3 (7.1–7.5)	0.14	1.7
Dietary fiber (g)	11.9 (11.4–12.4)	11.8 (11.3–12.3)	11.6 (11.2–12.1)	11.8 (11.5–12.2)	12.1 (11.7–12.4)	0.051	1.3
Magnesium (mg)	253 (244–262)	250 (242–259)	250 (243–258)	248 (242–254)	250 (244 – 255)	0.88	-1.1
Zinc (µg)	7988 (7722–8262)	7904 (7650–8166)	7858 (7636–8087)	7801 (7621–7985)	7839 (7674–8008)	0.46	-1.9

[a]Adjusted for age, sex, smoking, physical activity (moderate to heavy), and history of diabetes.
[b](Geometric mean for 0 teeth — Geometric mean for 25–28 teeth)/(Geometric mean for 25–28 teeth) × 100.
[c]SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

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Table 3. Adjusted geometric means of food group consumption (g/day) by number of present teeth (n =20 366)[a]

Food groups	Number of present teeth					P for trend	% Difference[b]
	0 Geometric mean (95% CI)	1-9 Geometric mean (95% CI)	10-19 Geometric mean (95% CI)	20-24 Geometric mean (95% CI)	25-28 Geometric mean(95% CI)		
Rice	359.5 (343.7-376.2)	347.5 (332.7-363.0)	347.9 (334.8-361.5)	331.1 (320.9-341.6)	328.3 (319.0-337.7)	<0.001	-9.5
Breads	6.0 (5.0-7.2)	6.7 (5.6-8.0)	6.1 (5.3-7.2)	6.5 (5.7-7.3)	6.3 (5.6-7.1)	0.97	5.4
Noodles	44.1 (38.7-50.3)	39.5 (34.8-44.8)	39.4 (35.2-44.0)	46.6 (42.6-51.0)	45.9 (42.3-49.9)	0.039	3.9
Confectioneries	9.2 (7.9-10.6)	8.6 (7.5-9.9)	7.4 (6.6-8.4)	7.1 (6.5-7.9)	7.1 (6.4-7.7)	<0.001	-29.6
Pulses	52.6 (48.3-57.3)	54.5 (50.2-59.2)	53.8 (50.1-57.9)	52.5 (49.5-55.7)	55.1 (52.2-58.2)	0.088	4.6
Meats	38.2 (35.4-41.1)	38.6 (35.9-41.4)	37.5 (35.2-40.0)	39.2 (37.3-41.3)	39.5 (37.7-41.4)	0.14	3.4
Eggs	17.8 (16.0-19.8)	18.9 (17.0-21.0)	17.6 (16.1-19.3)	18.9 (17.6-20.4)	19.1 (17.8-20.5)	0.14	6.8
Milk and dairy products	60.0 (51.0-70.6)	84.8 (72.5-99.2)	82.8 (72.1-95.0)	79.7 (71.2-89.1)	81.4 (73.5-90.2)	0.033	26.3
Vegetables	188.4 (175.4-202.3)	197.5 (184.4-211.6)	189.7 (178.6-201.6)	194.8 (185.5-204.7)	199.6 (190.8-208.8)	0.040	5.6
Green-yellow vegetables	73.8 (66.8-81.6)	81.7 (74.1-90.0)	75.1 (68.9-81.7)	80.1 (74.8-85.9)	83.7 (78.6-89.2)	0.002	11.9
Other vegetables	96.6 (90.6-102.9)	100.5 (94.5-106.8)	98.2 (93.1-103.7)	98.7 (94.5-103.2)	100.5 (96.5-104.6)	0.18	3.9
Fruit	81.2 (70.7-93.3)	84.1 (73.6-96.2)	80.7 (71.7-90.8)	83.9 (76.2-92.3)	85.5 (78.3-93.3)	0.31	5.0

[a] Adjusted for age, sex, smoking, physical activity (moderate to heavy), and history of diabetes.
[b] (Geometric mean for 25-28 teeth — Geometric mean for 0 teeth)/(Geometric mean for 25-28 teeth) × 100.

70-79, and ≥80 years accounted for 0.3%, 12.5%, 34.4%, 29.0%, 12.6%, 8.2%, and 3.1% in the responders and 0.1%, 10.8%, 32.4%, 29.4%, 12.4%, 9.5%, and 5.5% in all members. The proportion of female dentists was 8.0% in the former and 8.6% in the latter. The percentage of participants living in the metropolitan area, however, was lower than that of all the JDA members (18.7% versus 27.1%). The respondents, therefore, may have been less representative of urban areas.

Because the information was obtained through a self-administered questionnaire, there is a potential for reporting bias, particularly in relation to height, weight and dietary intake. Although BMI was scarcely correlated with the number of present teeth in the present analysis (Table 1), errors in self-reported height and weight could have somewhat masked a possible confounding. Participants with fewer teeth may have more under- or over-estimated their food consumption compared with those with more teeth. In addition, the relatively small number of younger participants with less than 20 teeth might limit the age adjustment in statistical considerations.

Finally, the cross-sectional design of this study prevents us from inferring a causal relationship between tooth loss and dietary intake. For instance, poor nutritional status for vitamins and minerals may exacerbate periodontal diseases and lead to tooth loss. Although the edentulism and/or tooth loss have been correlated with decreased blood levels of vitamin C, retinol, β-carotene, and folate (7, 12), some studies have reported associations of lower serum concentrations of vitamin C, folate, and calcium with periodontal disease progression (29-32). Prospective studies (4, 6, 8) will be needed to more clearly reveal the effect of tooth loss on dietary intake suggested in our study.

In conclusion, tooth loss was clearly linked with poorer nutrition even among dentists, for whom expert dental care is available.

Acknowledgements

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Neuroscience Letters

Ventromedial hypothalamic lesions change the expression of neuron-related genes and immune-related genes in rat liver

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ABSTRACT

There are no reports that hypothalamus can directly affect the expression of neuron-related genes and immune-related genes in liver. We identified genes of which expression profiles showed significant modulation in rat liver after ventromedial hypothalamic (VMH) lesions. Total RNA was extracted, and differences in the gene expression profiles between rats at day 3 after VMH lesioning and sham-VMH lesioned rats were investigated using DNA microarray analysis. The result revealed that VMH lesions regulated the genes that were involved in functions related to neuronal development and immunofunction in the liver. Real-time PCR also confirmed that gene expression of SULT4A1 was upregulated, but expression of ACSL1 and CISH were downregulated at day 3 after VMH lesions. VMH lesions may change the expression of neuron-related genes and immune-related genes in rat liver.

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A focus of attention among researchers has been the pathways that connect the nervous system and the gastrointestinal organs [4,5]. Liver is one of the autonomic nerve-enriched organs [4,5]. The hypothalamus plays a vital role in the integration of neurohumoral information and possesses autonomic centers that are connected to the viscera via the autonomic nervous system [4,5].

DNA microarray analysis is a powerful tool for detecting the characterization of mRNA expression pattern of a large number of genes. In the present study, we used DNA microarray analysis to identify genes for which expression profiles showed significant modulation and to investigate the cellular mechanisms of gene regulation in the rat liver at day 3 after VMH lesions, because it has been reported that cell proliferation in the liver increases and reaches a maximum at day 3 [7], and hyperphagia and the weight gains are induced obviously at day 3 [8], and real-time polymerase chain reaction (PCR) also confirmed a part of the results obtained by DNA microarray analysis.

Female Sprague–Dawley rats weighing 230–250 g were used in this study. They were maintained in a constant-temperature environment (23 ± 2 °C) in light-controlled cages with a 12-h light–dark cycle (lights on 7:00 AM) and were given free access to food and water. Tissue samples were taken from the liver of VMH lesioned rats and sham-VMH lesioned rats at day 3 after the operation ($n = 2$ in each group for DNA chips and $n = 3$ in each group for Real-Time Polymerase Chain Reaction).

VMH lesions or simulated operations were performed as previously described [6,7,9]. The stereotaxic coordinates were at bregma anteriorly, 0.75 mm lateral to the midsagittal line, and 1.0 mm upwards from the base of the skull, according to the atlas of De Groot [2]. Sham operations were performed in an identical manner except that no current was applied. After the operations, the rats were returned to their cages and given free access to food and water. Localization of the VMH lesions was verified by microscopic examination of the brain at the end of the experiment.

In two individual rats at day 3 after VMH lesioning and two sham-VMH lesioned rats, in order to circumvent RNA lysis by RNases that may be released in the rat liver when the animal is stressed, all procedures were conducted as swiftly as possible after each rat was sacrificed. The abdominal and chest cavities were opened. The samples of liver were quickly placed in 10 volumes of RNAlater® (Ambion, Inc., Austin, TX) at room temperature. The distance between the tissue surface, which is exposed to preservative, and the innermost regions of the fragment was minimized. We did this by cutting the tissues into 2 mm thick slices, thereby reducing the diffusion distance to 1 mm or less. RNA was isolated from the rat liver, using a commercially available kit (RNA easy Mini Kit, QIAGEN GmbH, Hilden, Germany). The RNA was quantified spectrophotometrically at 260/280 nm, and the quality of the isolated total RNA was determined by

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Table 1

Upregulated and downregulated neuron-related genes identified by DNA microarray analysis at 3 days after VMH lesioning.

Accession no.	Gene name	Fold change	Functions
(1) Upregulated genes			
1368562_at	SULT4A1	20.94	Sulfotransferase family; a brain-specific sulfotransferase involved in the metabolism of neurotransmitters
(2) Downregulated genes			
1369765_at	ASCL1	−2.76	Achaete–scute complex homolog 1. This gene encodes a member of the basic helix-loop-helix (BHLH) family of transcription factors. The protein activates transcription by binding to the E box (5′-CANNTG-3′). Dimerization with other BHLH proteins is required for efficient DNA binding. This protein plays a role in the neuronal commitment and differentiation and in the generation of autonomic neurons.
1369388_at	MUSK	−2.19	Muscle, skeletal, receptor tyrosine kinase. Members of the neural-specific TRK family that recognize nerve growth factor are absolutely required for the survival and development of discrete neuronal subpopulations, and the receptor tyrosine kinases play a critical role in the development of normal blood vessels.

Note: Fold changes in average difference values were calculated using Ingenuity Pathway Analysis.

electrophoretic separation on ethidium bromide-containing 1% agarose-gel. Regarding DNA microarray analysis, the preparation of cRNA was carried out by Ambion's MessageAmp® II-Biotin Enhanced and the target hybridization was performed according to the instructions provided in the Affymetrix GeneChip® technical manual. The double-stranded cDNA was synthesized from 5 μg of total RNA and hybridized Affymetrix GeneChip® arrays (Rat Genome 230 2.0, Affymetrix Japan Co., Tokyo, Japan) for 16 h at 45 C in GeneChip® Hybridization Oven 640. After washing and staining in GeneChip® Fluidics Station 450, hybridized cRNA was detected by GeneChip® scanner 3000. The digitized image data were processed using the GeneChip® Operating Software 1.4. The amount of probe-specific transcripts was determined based on the average of the differences between the perfectmatch and mismatch intensities. As replicate assays were not performed, a very stringent cutoff point was selected for the detection of significant upregulation or downregulation of the genes in the mRNA amount between the arrays. Using the signal intensity of selected genes that were up or downregulated compared to the sham-VMH lesioned control group, the analysis was performed using GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA) and Ingenuity Pathway Analysis (http://www.ingenucity.com/) (Redwood City, CA).

Regarding the real-time PCR analysis, RNA was stored at −70 °C until this analysis was performed. An aliquot (1 μg) of extracted RNA was reverse-transcribed into first-strand complementary DNA (cDNA) at 42 °C for 15 min, using 200 U/μl reverse-transcriptase (Takara Biochemicals, Shiga, Japan) and 10 mM of oligo (dT)-adapter primer (Takara Biochemicals) in a 2.0-μl reaction mixture.

Real-time PCR was carried out with a Terminal Cycle Dice TP800 (Takara Biochemicals) using the DNA-binding dye SYBR Green I

for the detection of PCR products. The reaction mixture (RT-PCR kit, Code RR043A, Takara Biochemicals) contained 12.5 μl SYBR Premix Ex Taq (2x) (Code RR041A, Takara Biochemicals), 10 μM PCR Forward Primer (0.5 μl), 10 μM PCR Reverse Primer (0.5 μl), and cDNA (2.0 μl) to give a final reaction volume of 25 μl. The sequences were obtained using Perfect Real-Time support system (http://www.takara-bio.co.jp/prt/imtro.htm). The PCR settings were as follows: the initial denaturation for 10 s at 95 °C was followed by 40 cycles of amplification for 5 s at 95 °C and 30 s at 60 °C, with the subsequent melting curve analysis increasing the temperature from 60 to 95 °C. Relative quantification of gene expression with real-time PCR data was calculated relative to GAPDH.

In the present study, three representative genes related to neuron and immune function were investigated by real-time PCR: (1) SULT4A1 (sulfotransferase family 4A, member 1) that be involved in the metabolism of neurotransmitters [10]; (2) ACSL1 (achaete–scute complex homolog 1) that plays a role in the neuronal commitment and differentiation and in the generation of autonomic neurons [1]; (3) CISH (cytokine inducible SH2-containing protein), one of cytokine-inducible negative regulators of cytokine signaling [11].

Results are expressed as the mean ± SEM. The mRNA levels were analyzed by the Mann–Whitney U test. Statistical analysis was conducted with SPSS version 11.0 statistical software. The differences between the groups were considered significant if the p value was <0.05 (two-tailed).

Among 31,099 probes, the expression of 203 probes (0.7%) showed at least a 2-fold upregulation (127 probes) or downregulation (76 probes) at day 3 after VMH lesioning as compared with sham-VMH lesioning. Table 1 shows the upregulated and downreg-

Table 2

Upregulated and downregulated immune-related genes identified by DNA microarray analysis at 3 days after VMH lesioning.

Accession no.	Gene name	Fold change	Functions
(1) Upregulated genes			
1375900_at	TNFRSF9	2.96	Tumor necrosis factor receptor superfamily, member 9. This receptor contributes to the clonal expansion, survival, and development of T cells. It can also induce proliferation in peripheral monocytes, enhance T cell apoptosis induced by TCR/CD3 triggered activation, and regulate CD28 co-stimulation to promote Th1 cell responses. The expression of this receptor is induced by lymphocyte activation.
(2) Downregulated genes			
1397271_at	TNFRSF13C	−4.42	Tumor necrosis factor receptor superfamily, member 13C. B cell-activating factor (BAFF) enhances B-cell survival in vitro and is a regulator of the peripheral B-cell population. The protein encoded by this gene is a receptor for BAFF and is a type III transmembrane protein containing a single extracellular cysteine-rich domain. It is thought that this receptor is the principal receptor required for BAFF-mediated mature B-cell survival.
1378586_at	CISH	−3.00	Cytokine inducible SH2-containing protein. The protein encoded by this gene contains a SH2 domain and a SOCS box domain. The protein thus belongs to the cytokine-induced STAT inhibitor (CIS), also known as suppressor of cytokine signaling (SOCS) or STAT-induced STAT inhibitor (SSI), protein family. CIS family members are known to be cytokine-inducible negative regulators of cytokine signaling. The expression of this gene can be induced by IL2 and IL3

Note: Fold changes in average difference values were calculated using Ingenuity Pathway Analysis.
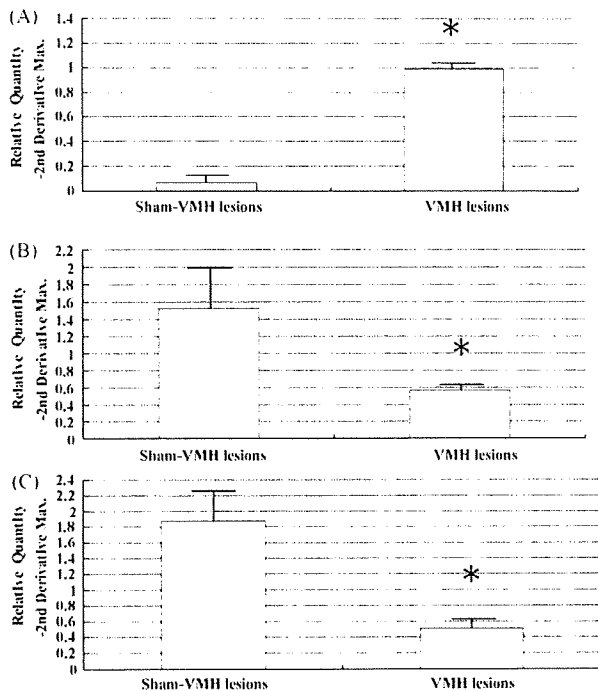
Fig. 1. VMH lesion-induced gene expression in real-time PCR analysis. Real-time PCR analysis of total RNA extracts was described in text. (A) SULT4A1 (sulfotransferase family 4A, member 1); (B) ACSL1 (achaete–scute complex homolog 1); (C) CISH (cytokine inducible SH2-containing protein). Values are the means SE of 3 different experiments. *<0.05 compared with sham-VMH lesioned rats.

ulated neuron-related genes identified by DNA microarray analysis. VMH lesions upregulated sulfotransferase family 4A, member 1 (SULT4A1), and downregulated achaete–scute complex homolog 1 (ACSL1) and muscle, skeletal, receptor tyrosine kinase (MUSK). Table 2 shows the upregulated and downregulated immune-related genes identified by DNA microarray analysis. VMH lesions upregulated tumor necrosis factor receptor superfamily, member 9 (TNFRSF9), and downregulated tumor necrosis factor receptor superfamily, member 13 (TNFRSF13C) and cytokine inducible SH2-containing protein (CISH). The expression of SULT4A1, ACSL1 and CISH were also examined by real-time quantitative analysis (Fig. 1). The gene expression of SULT4A1 was upregulated ($p < 0.05$), but expression of ACSL1 and CISH were downregulated at day 3 after VMH lesions ($p < 0.05$ and $p < 0.05$, respectively).

In the present study, we used the DNA microarray technique for mRNA expression profiling of mixture of rat liver cells to investigate cellular responses in response to VMH lesions. The present study showed that VMH lesions changed the neuron-related genes and immune-related genes in liver. Consistent with this, there are no reports that hypothalamus can directly affect the expression of neuron-related genes and immune-related genes in liver. There is a possibility that these changes may be based on the hyperphagia by VMH lesions, although there have been no reports describing that hyperphagia directly change the expression of neuron-related genes and immune-related genes in liver. Further investigations are needed to clarify whether VMH lesions *itself* or the hyperphagia by VMH lesions change these genes expressions.

As to the expressions of neuron-related genes, VMH lesions upregulated SULT4A1 gene, and downregulated ACSL1 gene. The encoded protein of SULT4A1 is a brain-specific sulfotransferase believed to be involved in the metabolism of neurotransmitters [10]. SULT4A1 is the only member of the SULT4 family. The fact that it is highly conserved and expressed primary in the brain suggests an important function has been identified for SULT4A1 gene [3]. Meanwhile, the protein of ACSL1 plays a role in the neuronal commitment and differentiation and in the generation of autonomic neurons [1]. Therefore, there is a possibility that VMH lesions may directly change the expression of the neuron-related gene families in liver. As to the expression of immune-related genes, VMH lesions upregulated CISH gene. CISH protein belongs to the cytokine-induced STAT inhibitor, and is known to be one of cytokine-inducible negative regulators of cytokine signaling [11]. Therefore, there is also a possibility that VMH lesions may directly change the expression of the immune-related genes families in liver.

In conclusion, although the networks of these genes involved in this process have not yet been elucidated sufficiently, and further investigations are needed to clarify what type of liver cells expressed the neuron-related genes and immune-related genes, this study is the first report to demonstrate that VMH lesions may cause changes of expression of neuron-related genes and immune-related genes in the liver.

Conflict of interest

All authors have stated that there is no conflict of interest to disclose the information regarding this manuscript.

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Effects of Gastric Vagotomy on Visceral Cell Proliferation Induced by Ventromedial Hypothalamic Lesions: Role of Vagal Hyperactivity

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Abstract In rats, ventromedial hypothalamic (VMH) lesions induce cell proliferation in the visceral organs (stomach, small intestine, liver, and pancreas) due to hyperactivity of the vagus nerve. To investigate the effects of selective gastric vagotomy on VMH lesion-induced cell proliferation and secretion of gastric acid, we assessed the mitotic index (the number of proliferating cell nuclear antigen (PCNA)-immunopositive cells per 1,000 cells in the gastric mucosal cell layer) and measured the volume of secreted basal gastric acid. Furthermore, to explore whether or not ethanol-induced acute gastric mucosal lesions (AGML) lead to ulcer formation in VMH-lesioned rats, we assessed the ulcer index of both sham-operated and VMH-lesioned rats after administration of ethanol. VMH lesions resulted in an increased mitotic index and thickness of the gastric mucosal cell layer and gave rise to the hypersecretion of gastric acid. Selective gastric vagotomy restored these parameters to normal without affecting cell proliferation in other visceral organs. Ethanol-induced AGML caused ulcers in sham VMH-lesioned rats, whereas VMH-lesioned rats were less likely to exhibit such ulcers. These results suggest that VMH lesion-induced vagally mediated cell proliferation in the visceral organs is associated with hyperfunction in these organs, and VMH lesion-induced resistance to ethanol may be due to thickening of the gastric mucosal cell layer resulting from cell proliferation in the gastric mucosa—this in turn is due to hyperactivity of the vagus nerve.

Introduction

Ventromedial hypothalamic (VMH)-lesioned rats are considered to be a representative model of hypothalamic obesity (Inoue 1992). In addition to the development of obesity, VMH-lesioned rats show physiological changes such as hyperphagia, autonomic derangements (hyperactivity of vagus nerve and reduced sympathetic activity), and biochemical changes including hyperlipidemia, hyperinsulinemia, and hyperleptinemia (Inoue et al. 1977a; Bray and York 1979; Yoshimatsu et al. 1984; Suga et al. 1999). In addition, we have found using the [^{3}H] thymidine uptake method that cell proliferation occurred in the visceral organs (liver, stomach, small intestine, and pancreas) and could be reversed by subdiaphragmatic vagotomy in the VMH-lesioned rats (Kiba et al. 1992, 1993; Kiba et al. 1996). This implies that VMH lesions induce cell proliferation in the visceral organs via hyperactivity of the vagus nerve (Inoue and Bray 1979; Yoshimatsu et al. 1984). Hypergastric acid secretion, which is considered to be attributed to vagal hyperactivity, was also noted in VMH-lesioned rats (Ridley and Brooks 1965; Tominaga et al. 1993). It is interesting to ponder whether selective gastric vagotomy would inhibit gastric cell proliferation and

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hypergastric acid secretion without affecting cell prolifera-
tion in the other visceral organs.

The integrity of gastric mucosa is maintained by an array of
defense mechanisms for protection against aggressive factors.
These defense mechanisms include HCO_3^- secretion, muco-
sal blood flow, mucus secretion, antioxidants, cell junctions,
apical resistance, enhanced thickness of the mucosal layer
due to cell proliferation or cell hypertrophy, and increased
mucosal cellular turn over (Yeomans et al. 1994; Gyires
2005; Ham and Kaunitz 2007, 2008). In a previous study, we
reported that gastric DNA synthesis in VMH-lesioned rats
was increased, peaked at 3 days, and then returned to almost
normal levels 7 days after the VMH lesions (Kiba et al.
1993). On this basis, we hypothesized that the enhanced
thickness of the gastric mucosal layer due to cell prolifera-
tion inhibits or attenuates the induction of acute gastric
mucosal lesions (AGML).

In this study, we examined whether or not selective gastric
vagotomy affects cell proliferation in the stomach and other
visceral organs. We also studied basal gastric acid levels in
VMH-lesioned rats to further explore the role of vagal
hyperactivity on cell proliferation and function of the visceral
organs of these animals. Moreover, we examined whether the
enhanced thickness of the mucosal layer due to gastric cell
proliferation exerts an effect against ethanol-induced AGML in
VMH-lesioned rats.

Materials and Methods

Animals

Thirteen-week-old female Sprague–Dawley rats weighing
260~280 g were used in experiments, all of which were
performed with the aim to minimize pain and discomfort to
animals.

Exp.1

Rats received a sham gastric vagotomy or selective gastric
vagotomy using the method of Tanaka et al. (Tanaka et al.
1987) for sectioning of the gastric branches of the vagus
nerve. As shown in Fig. 1, the right and left branches of the
vagal nerve run downward along the esophagus to the
stomach. With the aid of a dissection microscope to help
visualize the vagus nerve, the two gastric branches were
selectively sectioned. One week after the operation, sham
gastric vagotomized rats received sham VMH lesions
(controls) or VMH lesions (VMH with sham gastric
vagotomy), and vagotomized rats received VMH lesions
(VMH with gastric vagotomy; $n=7$ in each group).
Bilateral electrical VMH lesions were performed using the
coordinates of De Groot (Inoue et al. 1977b).
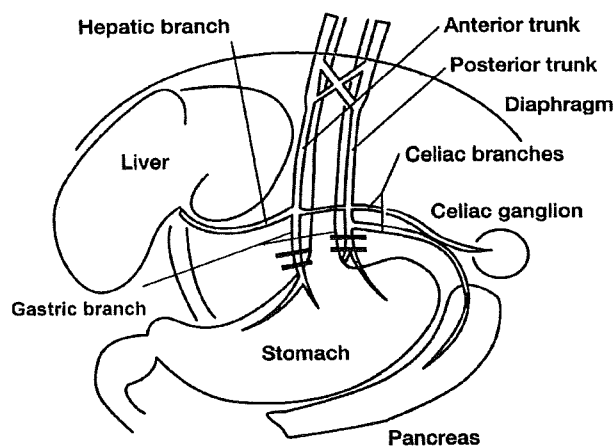
Figure 1 Schematic diagram of the abdominal vagal nerve below the
diaphragm in rat. *Double lines* represent the location of sectioning of
the gastric branch of the vagus nerve

Food intake and body weight were measured 5 days after
formation of the lesions. Under pentobarbital anesthesia,
gastric juices were collected through a gastric cannula from
overnight-fasted rats 7 days after the lesion formation, and
gastric acid levels were determined (Powley and Opsahl
1974). The liver, stomach, small intestine, and pancreas
were removed from sham VMH-lesioned and VMH-
lesioned rats, and paraffin sections were prepared from
these tissues. Stomach specimens were also subjected to
combined hematoxylin-eosin and immunostaining using an
anti-PCNA antibody (1:1,000, Dako, Kyoto, Japan) as a
marker of cell proliferation (Hall et al. 1990). The mitotic
index (number of cells with PCNA-positive nuclei out of
1,000 nuclei :‰) was determined in individual specimens
(Saftoiu et al. 2003).

Exp.2

Ten days after receiving sham VMH or VMH lesions, 24 h-
fasted rats were administered 60% ethanol (3 mg/kg)
through a gastric tube, and then 90 min later, animals were
sacrificed under ether anesthesia, and the stomach was
removed ($n=7$, sham VMH and VMH lesioned groups,
respectively). Each stomach was opened along the greater
curvature after infusion of 10 ml of saline and fixed with
10% formalin for 10 min. With the stomach placed under a
stereoscopic microscope (×10), the gastric mucosa was
carefully examined for lesions recognized as linear breaks
at the mucosal surface of the glandular part. The extent of
the lesion (ulcer index: millimeter) is expressed as the sum
of the length of these breaks per stomach (Ohta et al. 1999).
At the end of experiment, each stomach specimen was
subjected to hematoxylin-eosin staining for measurement of
the thickness of the mucosal layer.

Table 1 Comparisons of mitotic indices in liver, small intestine, endocrine pancreas and exocrine pancreas

	Stomach	Liver	Small intestine	Endocrine pancreas	Exocrine pancreas
Control	14.2±1.3	15.6±1.5	11.9±0.6	16.4±1.9	5.5±0.3
VMH with sham gastric vagotomy	22.7±3.8**	32.4±2.3**	36.0±1.9**	20.9±0.8*	36.4±1.2**
VMH with gastric vagotomy	15.4±0.9	37.9±1.5**	42.2±4.6**	19.6±1.3*	31.1±0.6**

Each group consisted of seven rats. *Control* sham VMH-lesioned rats with sham gastric vagotomy; *VMH* with sham gastric vagotomy1 VMH-lesioned rats with sham gastric vagotomy; VMH with gastric vagotomy: VMH-lesioned rats with gastric vagotomy. **P<0.05, **P<0.01 vs. Control.

Statistical Analysis

Results are expressed as mean values ± standard error (SE). One-way analysis of variance (ANOVA) was applied for comparison of the three groups, with verification using Tukey or Duncan multiple comparison tests. A value of $p <$ 0.05 was considered to indicate statistical significance.

Results

Exp.1

VMH-lesioned rats with sham gastric vagotomy or with gastric vagotomy exhibited a significant increase in food intake compared to controls (34.2±5 g/d, 30.8±4.1 g/d, and 13.9±1.3 g/d, respectively, both $p<0.01$ vs. controls) at 5 days after the lesion formation. Five days after VMH lesion, VMH-lesioned rats with sham or gastric vagotomy also exhibited significant increases in body weight (279.6± 2.3 to 332.8±3.2 g, and 275.1±5.0 to 338±7.38 g, respectively, both $p<0.01$), but controls did not show this increase (278.5±6.1 to 272.5±7.1 g, n.s.). VMH-lesioned

rats with sham gastric vagotomy showed significantly elevated mitotic indices in the liver, stomach, small intestine, and endocrine and exocrine pancreas (Table 1) compared to VMH-lesioned rats with gastric vagotomy, which exhibited a similar mitotic index in stomach tissue to that of control rats, without any change to the mitotic indices in other visceral organs (Table 1 and Fig. 2a). Consistent with these results, VMH-lesioned rats with sham gastric vagotomy had a significantly thicker gastric mucosal layer compared to VMH-lesioned rats with gastric vagotomy, the mucosal layer thickness in the latter being similar to that of control rats (Fig. 2b). The basal gastric acid levels in VMH-lesioned rats with sham gastric vagotomy were significantly higher than those of controls, whereas the gastric acid levels in VMH-lesioned rats with gastric vagotomy were similar to those of controls (Fig. 3).

Typical profiles of gastric mucosal layers in the three groups are shown in Fig. 4. VMH-lesioned rat that underwent a sham gastric vagotomy had a thicker mucosal layer and a greater number of PCNA-positive cells in the proliferative zone compared to control rat, whereas in VMH-lesioned rat with gastric vagotomy, the thickness of the mucosal layer and the number of PCNA-positive cells

Figure 2 Comparisons of the gastric mitotic index and thickness of the gastric mucosal layer. **P<0.01 vs. Control. *Control* sham VMH-lesioned rats with sham gastric vagotomy, *VMH with sham gastric vagotomy* VMH-lesioned rats with sham gastric vagotomy, *VMH with gastric vagotomy* VMH-lesioned rats with gastric vagotomy. a gastric mitotic index, b thickness of gastric mucosal layer