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Whole saliva in X-linked hypohidrotic ectodermal dysplasia

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Background. X-linked hypohidrotic ectodermal dysplasia (HED) is the most common type of ectodermal dysplasia. Identification of female carriers of X-linked HED can be difficult because of varying degrees of clinical symptoms due to the X-chromosome inactivation. This is the first study about whole saliva flow and composition in males affected by HED and female carriers all confirmed by molecular genetic analysis.
Hypothesis and aim. As salivary glands derive from ectoderm, we hypothesized that whole saliva flow and composition are altered in males affected by HED and female carriers.
Design. Saliva flow and composition were examined in a group of affected males and in a group of female carriers, all confirmed by molecular genetic analysis, and compared with healthy male and female controls.
Results. Affected males and female carriers had reduced whole saliva flow and saliva with high concentrations of most inorganic salivary constituents as well as total protein. However, affected males and female carriers seemed to have reduced amylase activity and concentration relative to their total protein concentration.
Conclusion. Saliva flow and composition may be used as part of a comprehensive clinical examination to identify potential female carriers of HED.

Introduction

To date, more than 150 distinct conditions in which ectodermal derivated tissue is affected have been identified[1]. Most of these conditions are very rare and manifest variable defects in morphogenesis of ectodermal structures, such as hair, skin, nails, teeth, and glands. The most common type of ectodermal dysplasia is the X-linked recessive hypohidrotic ectodermal dysplasia (HED; OMIM#305100) where males are usually more severely affected, and female carriers show variable severity ranging from mild to severe because of X-chromosome inactivation[2,3].

The X-linked form of HED is caused by mutations in the *ED1* gene, located at Xq12-q13.1[4]. The *ED1* gene encodes the transmembrane protein, ectodysplasin-A, which is a member of the tumour necrosis factor (TNF) family. The gene is normally expressed in tissues derived from the ectoderm and several splice forms are known. To date, more than 81 different *ED1* mutations have been identified (Human Gene Mutation Database, Cardiff). The autosomal recessive and the autosomal dominant forms of HED are caused by mutations in the gene encoding the receptor for ectodysplasin-A (*EDAR*), located at 2q11-q13[5].

Salivary glands derive from ectoderm, developing as an interaction between the oral epithelium and the underlying mesenchyme. The presumed mouse model of HED, 'Tabby', which shares many of the clinical symptoms found in humans affected with HED, has shown hypoplastic submandibular glands[6]. Several clinical studies have suggested that the saliva secretion is reduced in ectodermal dysplasia[7–10]. Saliva composition has also been shown to be different in male and female subjects affected with ectodermal dysplasia compared with those of controls[10]. However, the differences in saliva composition were not as pronounced as the saliva flow. In all previous studies, data from both male and female subjects were pooled, and the ectodermal dysplasia diagnosis was based on clinical symptoms only.

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This study is part of a genotype–phenotype correlation study of Danish males and female carriers with a mutation in the *ED1* gene. The data on whole saliva flow and composition were analysed and compared with those of healthy controls. We hypothesized that both affected males and female carriers had impaired saliva flow rate with the most pronounced effect in the affected males and that both affected males and female carriers had an altered saliva composition that reflects the impaired saliva flow.

In a further perspective, it was anticipated that the study could contribute to the identification of salivary biomarkers observed from affected male and female carriers with a known mutation in the *ED1* gene and, together with other clinical signs, could aid the clinical identification of potential female carriers of HED.

Materials and methods

Study group

With the existence of a well-organized Danish community dentistry system covering all children in Denmark, it has been possible to include nearly all affected males and female carriers in Denmark. Families with a proband who was clinically diagnosed at their regular dental practitioner with HED were contacted and invited to be included in the study. Informed consent was obtained in all instances according to Danish law. Probands and possible female carriers were examined clinically and radiographically, and blood samples were taken for DNA and chromosome analysis. The DNA analysis was made by PCR, SSCP, and direct sequencing. Only affected males and female carriers with known mutation in the *ED1* gene were included. In this specific part of the study, only adults and adolescents were included, as they have reached their mature salivary gland function[11]. Elderly participants were also included because saliva flow rates have been shown not to decrease with age[12]. The participants completed a questionnaire about their subjective symptoms. Among other questions, they were asked if they had any problems with heat and about their ability to sweat. Each participant was also asked about daily symptoms of dry mouth according to the UKU side-effect rating scale item 3.3[13]. This scale includes four scores where 0 denotes no feeling of dry mouth, 1 denotes a slight feeling of dry mouth, 2 denotes a severe feeling of dry mouth, and 3 denotes troublesome feeling of dry mouth that makes speech and eating difficult. The questionnaire also included a general question about medication intake and type of medication.

The age- and sex-matched control groups were recruited among staff and dental students at the School of Dentistry in Copenhagen. Subjects who used medication known to affect saliva secretion and composition[14] were excluded. Table 1 summarizes the anamnestic and clinical characteristics of the study group. The ethical committee of Copenhagen and Frederiksberg in Denmark approved the study.

Collection of whole saliva

Whole saliva was collected by the draining method[15] and all samples were collected between 09:00 and 16:00 hours. The reason for the wide timeframe was due to geographical distance. However, only two subjects had their saliva collected in the afternoon. Whole saliva was chosen, as it is an easy tool that can be used as part of an extensive clinical examination at a regular dental clinic, to detect possible female carriers of HED[10]. The participants were informed to refrain from eating and drinking for a period of 1 h before the test was performed. All were instructed in the procedure prior to the test and were told to refrain from any movement of the muscles around the mouth, including swallowing, during the test period. Before the test started, participants were informed to swallow once, after which the saliva was collected into a pre-weighted cup for 10 min. After collection, the weight increase of the cup was recorded, the saliva secretion rate was calculated in mL/min and the saliva was immediately stored at –80 °C for future analysis.

Saliva composition

The saliva concentrations of sodium and potassium were determined by atomic absorption spectroscopy (AAS) in the emission mode at 589.0 nm and 766.5 nm, respectively. The

saliva calcium concentration was determined by AAS in the absorption mode at 422.7 nm[16] with KCl and $SrCl_2$ in the matrix for reduction of oxysalts. Salivary chloride, total phosphate, total protein, and amylase activity were determined by colorimetric methods. Briefly, chloride was determined after the mercury–chloride/iron–TPTZ reaction at 610 nm[17], total phosphate after the molybdenum reaction at 700 nm[18], and total protein after the Coomassie blue reaction at 595 nm (Catalogue # 500-0006, Bio-Rad, Hercules, CA, USA). For total protein, the standard curve was obtained from lyophilized saliva proteins[19]. Amylase activity, which is the catalytic activity of the enzyme, was analysed with the Phadebas amylase test kit. This kit is based on the use of an insoluble, dyed starch substrate, which releases spectrophotometrically measurable dyed starch fragments upon amylase activity. All samples were measured at least twice. When a sample exceeded the calibrations, dilution of the sample was performed.

Protein profiles

Salivary protein profiles of the affected males, female carriers, and control groups were made by adding 2.5 μg of saliva proteins, determined by variable volumes of sample according to the actual protein concentration in each sample, to each well on 9% SDS polyacrylamide gels. The samples were processed this way in order not to lose any proteins during the process. The gels were fixed with a mixture containing 80% picric acid, 20% ethanol, and 0.05% glutaraldehyde and silver stained[20]. Gel images were made by scanning (300 dpi) and saved for analysis. The SDS gels were divided into four regions: proteins with high molecular weight (> 60 kDa), amylase (54–57 kDa), proteins with medium molecular weight (30–50 kDa), and proteins with low molecular weight (< 30 kDa). Two independent experienced examiners scored the colour intensity of the bands. The gels were presented to the examiners in a randomized sequence and a blinded setup. For this examination, the affected males and female carriers were pooled into one group and the healthy controls were pooled into one group to determine general trends in the protein profile between affected males and female carriers and healthy controls.

Statistical analysis

The SAS 9.1 statistical analysis system (SAS Institute Inc., Cary, NC, USA) was used to analyse the results. Due to the small sample sizes and the non-normal distribution, the nonparametric approach was preferred (Kologemol–Smirnow test). Data are presented as medians with their 95% confidence intervals. Differences between two groups was analysed by a two-sided Wilcoxon rank sum test. Differences between affected males, female carriers, and healthy controls were analysed by the Kruskal–Wallis test. Differences in proportions (e.g., scores of dry mouth, staining on SDS-gels) between study and control groups were analysed using Fisher's exact test. Correlation between variables was analysed by the Spearman's rank correlation analysis. The level of significance was set at $P < 0.05$.

Results

Anamnestic and clinical data

Anamnestic and clinical data were compared for all four groups (Table 1). As shown, both control groups were matched, as close as possible, in age compared with the two HED groups. No male subject used medication, whereas four female carriers and two control females used medication. However, for the female control group, medication was limited to contraceptives. A majority of both affected males and female carriers had daily complaints of dry mouth in contrast to only one control male and one control female. Regarding hypodontia, it was clinically observed that all affected males (100%) had oligodontia (agenesis of six or more teeth) and 79% of the female carriers had hypodontia. In comparison with normative values, which are 7.5% for males and 9% for females[21], these findings are highly elevated.

Differences between affected males and male controls

The saliva flow and composition in males affected by HED and male controls are given in Table 2. The median value for the saliva flow of the

Table 1. Characteristics of the study group.

	HED affected males	Male controls	*P*-value	HED female carriers	Female controls	*P*-value
Number of subjects	11	15	–	28	15	–
Age in years (median and range)	26 (15–49)	30 (23–41)	NS	41 (13–75)	35 (25–75)	NS
Medication	0	0	–	4	2	–
Scores of dry mouth† (0/1/2/3)	7/2/1/1	12/1/0/0	0.130	16/9/3/0	14/1/0/0	0.038
Hypodontia (yes/no/NA)	11/0 (100%)	1/14 (7.5%)‡	0.000	19/5/4 (79%)	1/14 (9%)‡	0.000
Mean number of missing teeth (range)	22 (14–28)			4 (0–22)		
Subjective feeling of and problems with heat and/or sweating (yes/no)	10/1 (91%)			17/11 (61%)		

P-values obtained by the Fisher's exact test. †Scores of dry mouth: 0 denotes no feeling of dry mouth, 1 denotes a slight feeling of dry mouth, 2 denotes a severe feeling of dry mouth, and 3 denotes troublesome feeling of dry mouth that makes speech and eating difficult. NS denotes nonsignificance.
‡Denotes that the distribution of aplasia in the control groups was estimated according to normative values[20]. NA denotes not accessible for cases where the aetiology for missing teeth was unknown (agenesis, extraction).
With respect to dental and other oral findings see Lexner *et al*.[22].

Table 2. Saliva flow and composition in HED males and female carriers as well as healthy controls (median and 95% confidence interval).

	HED affected males (n = 11)	Male controls (n = 15)	*P*-value	HED female carriers (n = 28)	Female controls (n = 15)	*P*-value
Flow (mL/min)	0.09 (0.02–0.2)	0.34 (0.27–0.44)	0.000	0.14 (0.07–0.18)	0.34 (0.26–0.6)	0.000
Sodium (mmol/L)	8.5 (4.0–12.0)†	2.0 (2.0–4.0)	0.002	4.0 (4.0–5.0)¶	2.0 (2.0–3.0)	0.000
Potassium (mmol/L)	30.5 (25–55)†	18.0 (17.0–21.0)	0.000	29.5 (25.0–33.0)¶	20.0 (19.0–21.0)	0.000
Calcium (mmol/L)	2.1 (1.0–7.3)‡	0.5 (0.3–0.7)	0.000	1.1 (0.8–1.9)§	0.5 (0.4–0.6)	0.000
Chloride (mmol/L)	24.0 (14.0–76.0)‡	14.0 (12.0–17.0)	0.003	27.0 (21.0–30.0)¶	15.0 (13.0–17.0)	0.000
Phosphate (mmol/L)	14.2 (5.7–22.1)	4.3 (3.5–5.4)	0.000	7.7 (5.6–11.9)§	4.9 (3.4–6.5)	0.002
Protein (μg/mL)	2970 (928–5640)	1370 (1248–1588)	0.082	1905 (1460–2875)§	910 (728–1188)	0.000
Amylase activity (U/mL)	60 (9–720)†	96 (79–108)	0.622	105 (85–226)§	87 (45–139)	0.101

P-values obtained by Wilcoxon's rank sum test. For some HED males and female carriers, saliva flow was too low for obtaining the required amount for analysis; therefore †denotes n = 10, ‡n = 8, §n = 27, and ¶n = 26.

affected males was about one-fourth of the saliva flow of the controls ($P < 0.001$). Regarding the saliva composition, significant differences between affected males and controls were found for most salivary components, except for total protein concentration and amylase activity. All components, except amylase activity, were found to be elevated in the affected male group.

Differences between female carriers and female controls

The saliva flow and composition in the female carriers of HED and controls are given in Table 2. The median value for saliva flow in the female carrier group was about half the value found in the female control group ($P < 0.001$) and all saliva components, except for amylase, were significantly elevated in the female carrier group.

In order to determine if the differences in saliva composition in female carriers were related to the saliva flow rate, the group was dichotomized into two subgroups according to saliva flow rate with a cut-off point of 0.2 mL/min. Table 3 summarizes the data for the two subgroups. Most female carriers belonged to the group with saliva flow rates less than 0.2 mL/min. Saliva concentrations of potassium, chloride, and phosphate were all significantly

Table 3. Saliva composition in HED female carriers dichotomized according to saliva flow rate (median and 95% confidence interval).

	Female carriers < 0.2 mL/min (*n* = 21)	Female carriers > 0.2 mL/min (*n* = 7)	*P*-value
Flow (mL/min)	0.09 (0.05–0.15)	0.39 (0.24–0.70)	0.000
Sodium (mmol/L)	4.0 (4.0–7.0)‡	4.0 (2.0–7.0)	0.078
Potassium (mmol/L)	32.0 (26.0–37.0)‡	25.0 (18.0–31.0)	0.014
Calcium (mmol/L)	1.3 (0.8–2.0)†	0.9 (0.4–1.9)	0.080
Chloride (mmol/L)	28.0 (21.0–39.0)‡	15.0 (10.0–28.0)	0.020
Phosphate (mmol/L)	9.6 (6.7–13.0)†	5.6 (3.1–8.4)	0.007
Protein (μg/mL)	2241 (1460–3743)†	1870 (355–3115)	0.300
Amylase (U/mL)	113 (87–305)†	91 (47–232)	0.384

P-values obtained by Wilcoxon's rank sum test. For some subjects, the saliva flow rate was too low for obtaining the required amount for analysis; therefore †denotes $n = 20$ and ‡$n = 19$.

increased in the group with low saliva flow rate, when compared with the subgroup with higher flow rate. In contrast, no significant differences in the concentration of sodium, calcium, and protein and amylase activity were found between the groups.

In order to exclude any possible bias from the female subjects that used medication, which is known to influence saliva flow and composition, all the parameters were re-examined without the four female subjects using such medication for both the whole group and the dichotomized groups (data not shown). This analysis showed that the results did not differ from the ones obtained with the four female subjects using medication included. We also determined the relation between the number of missing teeth and saliva flow rate in the female carrier group and found a weak negative relation, although not significant.

Comparison of all groups

Figure 1, which is an overview figure, shows the distribution of saliva flow and composition (mean and SEM) in a combined female/male control group compared with the affected males and female carriers, respectively (for more details regarding the specific group sizes see Table 2). The two control groups could be pooled because no significant differences in saliva flow and composition were observed between them. In general, it was observed that for most parameters, the values for the female carrier group were placed between the values for the affected male group and the control group, respectively. Thus, there was a gradual reduction in the saliva flow from controls through female carriers to the male group, and a similar rise in the values for the saliva components.

When comparing the saliva flow rate and saliva components in the affected males and the female carrier group there was, however, only significant difference in the concentration of calcium ($P = 0.023$) and sodium ($P = 0.047$), which was higher in the males (data not shown). When comparing the affected male group with the reduced female carrier group (i.e., excluding the four that used medication that is known to affect saliva flow and composition) there was a significant difference in the concentration of calcium ($P = 0.012$), sodium ($P = 0.027$), as well as phosphate ($P = 0.027$).

Saliva protein composition

SDS gels of salivary proteins from affected male and female carriers showed that the amylase band (54–57 kDa) was weakly stained compared with other proteins (PRP's) and compared with that of healthy controls ($P < 0.001$). These findings corresponded to a relatively low amylase activity relative to a high total protein concentration in the affected males and female carriers (Table 2).

Discussion

Due to a very inhomogeneous clinical picture in the female carriers of HED, there is a need

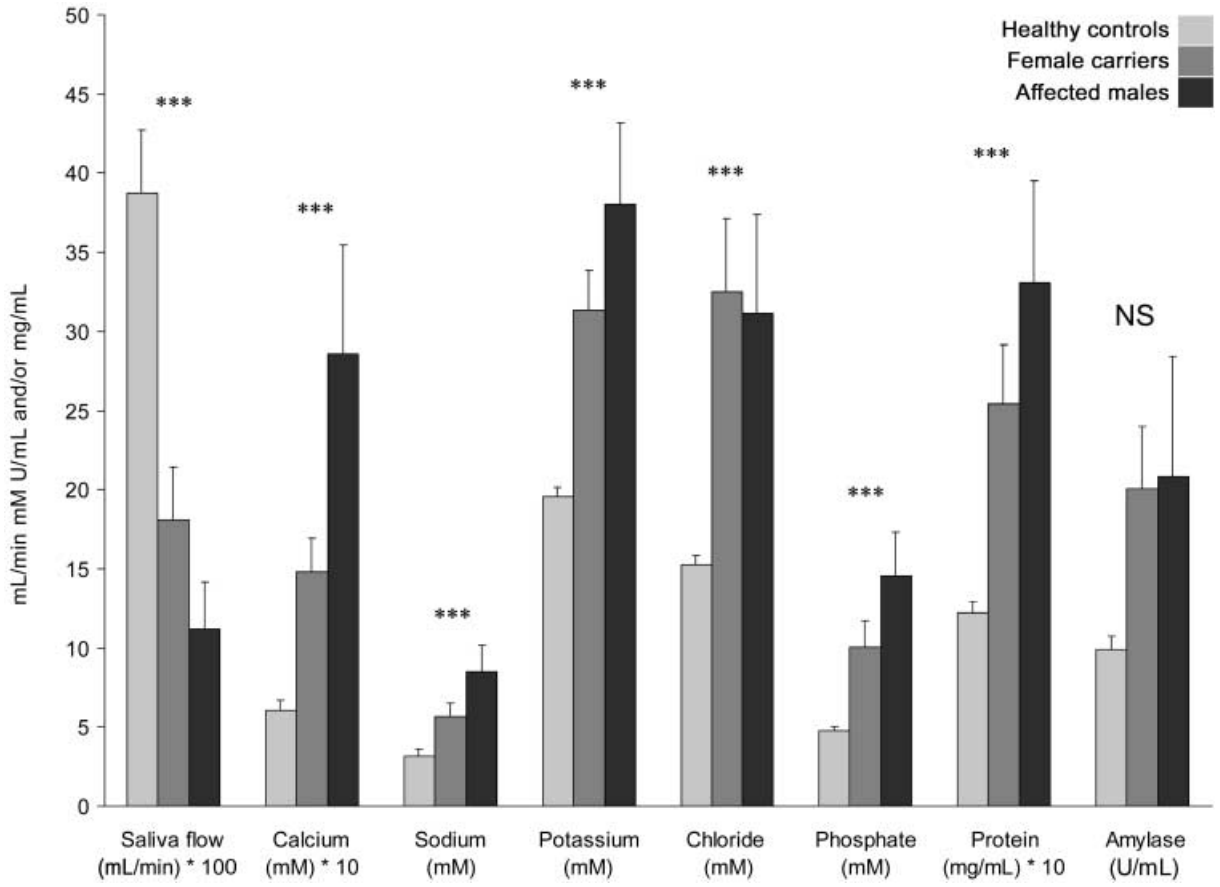

Fig. 1. Saliva flow and composition (mean and SEM) in a combined female/male control group compared with affected HED males and female carriers. For more details regarding sample size see Table 2. ***Denotes $P < 0.001$ and NS denotes not significant. *P*-values were obtained by the Kruskal–Wallis test.

to search for new methods to identify potential female carriers of HED clinically. Therefore, this study investigated salivary findings (flow and composition) in affected males and female carriers, where the mutation in the *ED1* gene was confirmed by mutation analysis. It is the first study to report, in detail, on these findings in a group with a known mutation in the *ED1* gene.

Regarding the subjective feeling of dry mouth in the affected males and female carrier group, no direct relation was obtained between the subjective feeling of dry mouth and the objective saliva flow rate. Thus, it is noteworthy that many of the affected individuals did not complain from dry mouth although their saliva flow rate was low. This finding corresponds to previous findings in published works[9]. The explanation could be that HED is present at birth and the affected males and female carriers therefore are adapted to the low saliva flow rate.

The significantly reduced saliva flow rate found both in the affected males and the female carriers is in agreement with the findings of previous studies of persons affected by ectodermal dysplasia[7–10]. Among the female carriers, the reduction in saliva flow rate seemed to be independent of their number of missing teeth. In contrast to previous studies, this study was performed on a more homogenous group as all affected males and female carriers had a mutation in the *ED1* gene and male and female subjects were studied separately. This made it difficult, however, to compare our findings directly to those of previous studies.

The finding of low saliva rates in female carriers of HED shows that these individuals have an increased susceptibly to oral diseases such as caries and oral candidiasis[23,24]. Thus, as 75%

had unstimulated saliva flow below 0.2 mL/min indeed oral health measures targeted at dry mouth patients should also be part of an individual treatment plan for these patients.

Regarding the saliva composition, an increased total saliva protein concentration was observed, which does not correspond with previous findings[10]. In this study, other saliva components were examined as well, and the results showed that in the affected male group, most of the saliva components were significantly elevated except for protein and amylase activity. The reason for the nonsignificance of those components could be that the variability in the values was large. Increasing the control group would not have changed the results, as the variance in these groups was already small. Comparing the groups of affected males and female carriers, we found that the male subjects generally had higher values than the female subjects.

To test if the saliva compositional differences in the female carrier group were flow related, the group was dichotomized into a low secretor and a normal secretor group according to the value of 0.2 mL/min for the unstimulated saliva flow rate suggested by Sreebny and Valdini[25]. The affected male group was not dichotomized like the female carrier group as only one male had a saliva flow over 0.2 mL/min. The high calcium, sodium, protein concentrations, and amylase activity in the female carriers did not seem to be related to their lower saliva flow rates. Thus, the changes in these saliva constituents seemed to be attributed to the disease *per se*. These changes could resemble a secretion that is dominated by a relatively large contribution from the minor salivary glands[26]. However, the changes in the whole saliva can also be the result of a general change in the secretions released by all salivary glands.

Examining the SDS gels, we found a weaker amylase band in the group of affected males and female carriers compared with that of the control group. Thus, although the affected males and female carriers had more than twice the concentration of total protein compared with those of controls, the amylase fraction compared with the total amount of protein was smaller than in controls.

In conclusion, we found that the affected males and female carriers had reduced saliva flow and saliva with high contents of sodium, calcium, and proteins as compared with that of healthy controls. Therefore, measurements of whole saliva flow rate and simple sialochemistry could be used as a part of a more extensive clinical examination and diagnosing together with other clinical signs, such as tooth hypodontia and tooth malformation, this giving the possibility of a more focused genetic diagnosing and counselling.

What this paper adds

- This is the first study that reports in details on saliva flow and composition in affected males and female carriers with a known mutation in the *ED1* gene.
- The sample sizes of the female carriers of HED are relatively large, compared with those of previous studies.

Why this paper is important for paediatric dentists

- Paediatric dentists are often the first medical personnel to suspect possible carriers of HED. It is therefore important to point out which clinical markers they can use to diagnose female carriers at an early age. Early diagnosis could improve treatment by referrals to a proper treatment unit enabling a long-term treatment plan and genetic counselling.

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