DNA damage in salivary gland tissue in patients with chronic kidney disease, measured by the comet assay

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Objective. The aim of this observational study was to investigate the relationship between DNA damage in minor accessory salivary glands, hyposalivation, and inflammation in patients with chronic kidney disease (CKD).

Study design. DNA strand breaks and oxidative DNA lesions in salivary glands, inflammatory markers, and uremic state were measured in 79 patients with CKD and matched controls.

Results. CKD patients not yet on dialysis had significantly more, and dialysis patients significantly less, DNA strand breaks in salivary tissue compared with controls. All measured inflammatory markers were higher in patients with CKD compared with controls. Salivary secretion rates were significantly lower in dialysis patients compared with controls. A high level of salivary secretion rate at rest significantly predicted a high level of DNA strand breaks in patients with CKD.

Conclusions. Dialysis patients had fewer DNA strand breaks in minor accessory salivary glands than controls, suggesting that peripheral tissue is differently affected by CKD than leukocytes. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:209-215)

Patients with chronic kidney disease (CKD) report mouth dryness as a discomfort and daily problem that affects speech, food intake, and medication, as well as oral disease development and oral disease progression.1 One underlying mechanism for oral disease might be oxidative stress and damage to salivary glandular tissue.2 Previous studies have shown that CKD patients are exposed to oxidative stress, which has been suggested to be related to the high morbidity and mortality in this patient group.3, 4 In addition, oxidative stress is interrelated to inflammation, which is another risk factor in CKD patients.5-7 The level of oxidative stress in cells can be estimated by analysis of sufficiently stable end products from oxidative processes, such as oxidatively modified DNA. Increased levels of DNA damage in peripheral blood mononuclear cells (PBMCs) of CKD patients have been observed in several studies using different methods.8-13 A sensitive method for measurement of DNA damage is the alkaline single-cell gel electrophoresis method (SCGE or comet assay). In its original form, DNA strand breaks and alkali-labile sites are measured, but by adding specific types of lesion-specific repair enzymes, other lesions, such as oxidative DNA lesions, can be measured.14 The comet assay is widely used in genetic toxicology, where DNA damage can be measured in practically any cell type. However, because of accessibility it is mainly applied to PBMCs. Increased levels of DNA strand breaks in PBMCs have been observed in CKD patients using the comet assay in several studies.8,9,11,12 Even though the high level of DNA damage in PBMCs in CKD patients is well established, DNA damage has not been measured with the comet assay in CKD patients’ tissue as far as the authors are aware. It is unclear whether the overall oxidative stress found in the circulatory system also applies to peripheral tissues. Patients with pronounced kidney failure, CKD stage 4 to 5, present an elevated level of high-sensitivity C-reactive protein (hs-CRP) and hypoalbuminemia causing oxidative stress.5 The type of renal replacement therapy and uremic toxins retained in the tissue affect oxidative stress.3 There are reports suggesting that oxidative stress might be related to the oral complications frequently observed in CKD patients. For example, the severity of periodontitis has been associated with an increased level of lipid peroxidation in saliva.2,15 A recent study found that the salivary content of urea and potassium was significantly higher in patients with moderate to severe CKD compared with healthy controls, suggesting that the accu-
mulation of uremic toxins in CKD patients also applies to saliva.\textsuperscript{16} Furthermore, significantly higher salivary concentrations of urea, creatinine, and potassium were observed in patients with end-stage renal failure.\textsuperscript{16}

The aim of this study was to investigate DNA damage in minor accessory salivary glands and changes that occur in the salivary gland tissue owing to the severity of CKD and oxidative stress. A secondary aim was to investigate a possible correlation between observed inflammation and DNA damage in salivary glands.

MATERIAL AND METHODS

Study design

In this observational study patients with CKD stage 4 to 5, as well as age- and sex-matched healthy controls, were investigated. The CKD patients consisted of predialysis patients, i.e., CKD patients with a glomerular filtration rate (GFR) less than 20 mL/min/1.73 m\textsuperscript{2} who are not yet on dialysis, and dialysis patients. The approach was to measure the levels of DNA strand breaks as well as oxidative DNA lesions in blinded minor accessory salivary glands, using the comet assay. The potential influence on DNA damage by the following factors was investigated: salivary secretion rate (rest and stimulated), sex, age, smoking, diabetes, serum creatinine, serum urea, hemoglobin in blood, serum albumin, inflammatory markers in circulation (i.e., hs-CRP in serum, orosomucoid in plasma, haptoglobin in plasma, leukocyte particle concentration [LPC] in blood), and interleukin-6 (IL-6) in submandibular/sublingual (SM/SL) saliva. Unfortunately, IL-6 in SM/SL saliva was measured in only a limited number of patients. All appointments were conducted before noon and immediately before a dialysis treatment to avoid fluctuation in salivary secretion and to eliminate the risk of major differences in hydration status.

Subjects

We investigated 79 patients with CKD stage 4 to 5, of whom 25 were women and 54 men, with a mean age of 60 years (range 25-87). Ten were predialysis patients, 3 were peritoneal dialysis patients, and 66 were hemodialysis patients. In addition, we analyzed 79 age- and sex-matched healthy controls. The CKD group consisted of patients from the Karolinska University Hospital and from the dialysis units at Löwenströmska Hospital, Kungsholmsdialysen, and Sophiahemmet in Stockholm, Sweden. We included dental patients older than 18 years. The primary diagnoses causing the renal diseases are shown in Fig. 1. We excluded patients with active hepatitis and patients positive for methicillin-resistant staphylococcus aureus (MRSA). Patients involved in other studies were also excluded. The control group consisted of healthy dental patients referred for participation in this study by their public dental service clinic in Solna municipality. The patients were consecutively chosen from the patient data files. Controls with elevated levels of hs-CRP and creatinine were excluded.

Two ethical approvals for the study were obtained from the regional Ethics Committee for Human Research, Stockholm, complying with the Helsinki Declaration. All subjects were informed about the study both orally and by letter. The patients were also informed by telephone about the examination routines when their appointments were booked by a dental nurse. All subjects gave oral and written consent.

Measurement of salivary secretion rates

The subjects were assessed in either a dental setting or at bedside in the dialysis units. Salivary secretion rates of whole saliva in all CKD patients and controls were measured. Registrations of the secretion rates were conducted in accordance with the draining method and the masticatory method.\textsuperscript{17} The patients were instructed not to eat or smoke for at least 1 hour before the visit. All patients were instructed to rest for at least 10 minutes before the measurement to avoid influence of stress on the secretion rates. In the dental setting, the patients were instructed on how to lean forward and hold the funnel and test tube during the sample collection. When collecting nonstimulated saliva, the patients were asked to achieve a passive flow without masticatory movements for 15 minutes. To measure the stimulated salivary secretion rate, the patients were asked to chew on a paraffin capsule. The saliva produced during 5 minutes was continuously collected. The salivary secretion rates were expressed in mL/min.

Measurement of blood parameters

Venous blood samples were collected to evaluate levels of serum hs-CRP, plasma orosomucoid, serum albumin, blood LPC, blood hemoglobin, plasma haptoglobin, serum urea, and serum creatinine. The pro-
Collection of minor accessory salivary glands
To avoid influence of the dialysis treatment, sample collections were made before the dialysis session. The biopsy was collected after local anesthesia with Citanest Dental-octapressin 30% (Dentsply, Stock-holm, Sweden) by incision with a number 15 scalpel (Braun, Tuttingen, Germany) in the mucosa close to a minor accessory salivary gland on the inside of the lower lip. The biopsies were collected from the same region for each patient. The gland tissue was detached from the connective tissue and immediately placed in sterile physiological saline solution in a test tube and transported on ice, protected from light, to the Karolinska University Hospital, where the sample was frozen at −70°C. The incision was closed with an Ethicon suture 5.0 (Johnson & Johnson International, New Brunswick, NJ, USA) if needed.

Collection of control cells
To verify the stability of the comet assay, isolation of PBMCs from the blood of 1 healthy blood donor was performed on a Histopaque-1077 (Sigma-Aldrich, St Louis, MO) gradient. The mononuclear cell layer was washed with phosphate-buffered saline (PBS) solution, as instructed by the supplier. Aliquots of isolated PBMCs were slowly frozen to −80°C in freezing media consisting of 90% fetal bovine serum (FBS) and 10% sterile dimethyl sulfoxide (DMSO). One aliquot was analyzed on each day of analysis to control the method.

Analysis of DNA damage with the comet assay
All used chemicals were of analytical grade. The Escherichia coli formamidopyrimidine DNA glycoylase (FPG) was kindly provided by Prof. Andrew Collins, Oslo University, Oslo, Norway.

The minor accessory salivary glands were thawed and homogenized in PBS using a Dounce B Pestle. The cell suspension was mixed with low melting point agarose (with a final concentration of 0.7%) and the comet assay was performed as previously described for the cell study by Johansson et al. A control sample of PBMCs was added to each analysis of salivary glands. An aliquot of the frozen control sample was thawed gently in a 37°C water bath, placed on ice, and cold RPMI 1640 medium with 10% FBS was added. The cells were centrifuged, washed in PBS, and analyzed by the comet assay.

In short, the cells were lysed at pH 10. To specifically measure oxidative DNA lesions, an additional step of digestion with FPG was added. FPG recognizes and removes specific oxidatively damaged purines creating an apurinic site, leading to a strand break either by the enzyme’s associated lyase activity or by the subsequent alkaline treatment. After treatment with an alkali solution, electrophoresis was performed in the same solution. The strand breaks, by their ability to relax DNA supercoiling, allow DNA fragments to migrate out from the nucleoids under the influence of the electric field. The DNA was stained, and comets with tails of mainly single-stranded DNA could then be seen with a fluorescence microscope. The comets were analyzed using computerized image analysis. The magnitude of the DNA tail (% DNA in the tail) provides information about the level of DNA lesions. For each sample, 105 cells were scored (35 cells from each of 3 fields on a microscope slide).

Statistical methods
Because most variables were not normally distributed, only nonparametric tests were used. The multivariate nonparametric quantile regression analyses were performed using Stata software (Stata Corp, College Station, TX). All other statistical analyses were performed using SPSS (SPSS, Inc., Chicago, IL). Whether or not the variables were normally distributed was determined using the Shapiro-Wilk test. Spearman’s correlation test was used for the correlation analyses and the Mann-Whitney test was used for comparisons between groups. Multivariate quantile regression was used to identify important variables affecting the median level of DNA strand breaks and oxidative DNA lesions, respectively, in minor accessory salivary gland tissue.

The covariates selected to be included in the model (age, sex, salivary secretion rate at rest, hs-CRP in serum, and creatinine in serum) were based on our hypothesis and on clinical relevance. Throughout this study, P values less than .05 were considered statistically significant.

RESULTS
DNA damage
Predialysis patients (n = 10) had significantly higher (P < .05) levels of DNA strand breaks compared with sex- and age-matched controls (Table I). In contrast, dialysis patients (n = 59) had significantly lower (P < .001) levels of DNA strand breaks compared with matched controls (Table II). In addition, the predialysis patients had a significantly higher level of DNA strand breaks (P < .001) compared with the dialysis patients. No statistically significant differences were observed in oxidative DNA lesions between the CKD patients and the controls (Tables I and II).

The stability of the comet assay was verified by analysis of mononuclear control cells. The level of...
DNA strand breaks in the control cells was 5.0 ± 0.9 and the level of oxidative DNA lesions was 12.9 ± 2.4 (n = 31).

Salivary secretion
Salivary secretion rates, at rest and stimulated, were significantly lower (P < .001) in the dialysis patients compared with controls (Table II). In addition, the dialysis patients had significantly lower salivary secretion rates, both at rest (P < .01) and stimulated (P < .05), compared with the predialysis patients.

The salivary secretion rate at rest significantly predicted the level of DNA strand breaks in all CKD patients in the multivariate quantile regression model (estimate = 3.5, P < .05, n = 68), with age, sex, salivary secretion rate at rest, hs-CRP, and creatinine in serum as covariates. Salivary secretion rates were not significantly correlated to either DNA strand breaks or oxidative DNA lesions (Spearman correlations). However, the correlation between salivary secretion was of borderline significance (r_s = 0.224, P = .066, n = 68).

There was a highly significant correlation between the salivary secretion rate at rest and the stimulated salivary secretion rate both in the CKD patients (r_s = 0.600, P < .001, n = 78) and in the healthy controls (r_s = 0.512, P < .001, n = 78).

Inflammatory parameters
All measured parameters of inflammation in circulation (hs-CRP, orosomucoid, haptoglobin, and LPC), as well as IL-6 in saliva, were higher in the CKD patients compared with matched controls, although not all parameters reached statistical significance. The concentration of IL-6 in SM/SL saliva was higher in both predialysis patients and dialysis patients than in controls, but unfortunately IL-6 in saliva was measured in only a limited number of patients and the differences were not significant (Tables I and II). The concentrations of the inflammatory parameters hs-CRP and orosomucoid were significantly higher in both predialysis patients and dialysis patients compared with their respective matched controls (Tables I and II). Both dialysis patients and predialysis patients had higher levels of haptoglobin than their respective control groups but it was only significantly higher in the predialysis patients (Tables I and II). The LPC was higher in both dialysis patients and predialysis patients compared with their respective control groups, although this difference did not reach significance (Tables I and II). The predialysis patients had a significantly higher LPC compared with the dialysis patients (P < .05).

Relationships between DNA damage and inflammation were rare. Haptoglobin was significantly correlated with oxidative DNA lesions in dialysis patients (r_s =
hs-CRP significantly predicted the level of DNA strand breaks in the healthy controls in a multivariate quantile regression model (estimate $0.18$, $P < 0.05$, $n = 73$), with age, sex, salivary secretion rate at rest, hs-CRP, and creatinine in serum as covariates.

**Uremic state**

Urea and creatinine in serum were significantly higher ($P < 0.001$) both in predialysis patients and dialysis patients compared with their respective controls (Tables I and II).

**Albumin and hemoglobin**

The serum albumin concentration was significantly lower in the dialysis patients compared with their matched controls ($P < 0.001$).

Both predialysis patients and dialysis patients had significantly lower levels of hemoglobin compared with controls ($P < 0.001$).

Hemoglobin was significantly negatively correlated to the level of DNA strand breaks in healthy controls ($r_s = -0.294$, $P < 0.05$, $n = 73$).

**Patient characteristics**

Women on dialysis had a significantly higher ($P < 0.01$) level of oxidative DNA lesions compared with men in the same patient group (9.6 compared with 7.8 mean % DNA in tail). The same pattern was observed when looking at the whole CKD patient group, where women had a significantly higher level of oxidative DNA lesions in the multivariate quantile regression model with age, sex, salivary secretion rate at rest, hs-CRP, and creatinine in serum as covariates (estimate $2.2$, $P < 0.05$, $n = 68$). The level of DNA strand breaks was significantly higher ($P < 0.05$) among women compared with men in the predialysis CKD patients (11.9 compared with 7.6 mean % DNA in tail).

Age, smoking, and diabetes were not significantly related to DNA strand breaks or oxidative DNA lesions in any of the models.

**DISCUSSION**

In this study, we found that a high level of salivary secretion rate at rest significantly predicted a high level of DNA strand breaks in CKD patients in a multivariate quantile regression model. We also found that predialysis patients had significantly higher levels of DNA strand breaks (Table I) and dialysis patients had highly significantly lower levels of DNA strand breaks (Table II) in minor accessory salivary gland tissue compared with sex- and age-matched controls. Because of accessibility, DNA damage in humans is mostly measured in PBMCs. As PBMCs circulate throughout the body, they are believed to reflect the general state of DNA damage in the body. However, there is still much to
clarify when it comes to the levels of DNA damage in peripheral tissues. Roth et al. observed an increased level of micronuclei in buccal mucosa cells obtained by scraping of the oral cavity in CKD patients undergoing hemodialysis. However, as far as the authors of this paper are aware, this is the first time DNA damage has been analyzed with the comet assay in peripheral tissues, such as minor accessory salivary glands, in CKD patients. The observations in this study suggest that CKD might affect DNA in peripheral tissue differently compared with DNA in circulating PBMCs. Previous studies have demonstrated that predialysis patients have a higher level of DNA damage in PBMCs compared with healthy controls, and that dialysis patients seem to have even higher levels. Thus, we found that DNA damage in minor accessory salivary gland tissue does not follow the same pattern as DNA damage in PBMCs in this patient group. A possible explanation for the puzzling observations in this study is that the clearance of potential genotoxic substances during dialysis might be beneficial for the level of DNA strand breaks in peripheral tissue in the dialysis patients compared with CKD patients who have not yet been treated with dialysis. The significantly (P < .001) lower level of DNA strand breaks in dialysis patients compared with their matched controls is, however, more difficult to explain (Table II). Tomás et al. observed significantly higher salivary concentrations of both creatinine and urea in dialysis patients compared with healthy controls, which could reflect an accumulation of uremic toxins in saliva. One could speculate that the overall state of oxidative stress in the dialysis patients enhances the DNA repair and/or induces antioxidant enzymes. Herman et al. demonstrated that the dialysis session induces an increased DNA repair in PBMCs. Bibi et al. previously showed that peritoneal dialysis patients have a 15% higher level of salivary peroxidase (P = .01) and a 35% nonsignificantly higher level of salivary superoxide dismutase compared with predialysis CKD patients. Thus, an increased level of antioxidant enzymes in saliva and an increased DNA repair in the dialysis patients could be possible explanations for the significantly lower level of DNA strand breaks observed in minor accessory salivary glands in the dialysis patients, compared with the predialysis patients and matched controls.

The dialysis session itself is likely to affect DNA in circulating PBMCs and peripheral tissues differently, because neutrophils are stimulated to release reactive oxygen species when they come into contact with the dialysis membrane. However, our samples were collected immediately before a dialysis session and the impact of the previous dialysis session was therefore expected to be low.

The increased level of inflammation in CKD is well established and several inflammatory markers have been associated with the increased morbidity and mortality in this patient group. In this study, all measured parameters of inflammation (IL-6 in saliva, hs-CRP in serum, plasma orosomucoid, plasma haptoglobin, and LPC in blood) were higher in the CKD patients compared with matched controls, although not all parameters reached statistical significance (Tables I and II). The only significant correlation between inflammatory markers and DNA damage in CKD patients was between haptoglobin and oxidative DNA lesions in the dialysis patients. The only inflammatory parameter measured in saliva, IL-6, was investigated in just a few patients. No significant correlations between IL-6 in saliva and DNA damage in CKD patients were observed in this study.

The dialysis patients had significantly lower salivary secretion rates, at rest and stimulated, compared with the matched controls (Table II), which is in accordance with a previous study by Gavaldá et al. In addition, the dialysis patients had a significantly lower salivary secretion rate at rest and stimulated, compared with the predialysis patients. There were no significant differences in salivary secretion rates between predialysis patients and matched controls (Table I). This observation was in agreement with observations by Tomás et al., who did not observe any significant differences in salivary flow between controls and predialysis patients with moderate to terminal renal failure. In the present study, salivary secretion rate at rest significantly predicted the level of DNA strand breaks in all CKD patients when adjusted for age, sex, hs-CRP in serum, and creatinine in serum in the multivariate quantile regression model. However, when analyzed with Spearman's correlation test, salivary secretion rates were not significantly correlated with either DNA strand breaks or oxidative DNA lesions in minor accessory salivary glands, although the salivary secretion rate at rest showed a trend in correlating positively with DNA strand breaks.

Women on dialysis had significantly higher levels of oxidative DNA lesions compared with men in the same patient group. When investigating the whole CKD patient group with the multivariate quantile regression model, women with CKD had a higher level of oxidative DNA lesions compared with men. Women in the predialysis patient group also had significantly higher levels of DNA strand breaks compared with men.

CONCLUSIONS

We found that dialysis patients had significantly fewer DNA strand breaks in minor accessory salivary
glands compared with age- and sex-matched controls. We also found that predialysis patients had significantly higher levels of DNA strand breaks in minor accessory salivary glands compared with age- and sex-matched controls, suggesting that DNA in salivary glands is differently affected by CKD compared with DNA in circulating PBMCs. In this study, the only correlation between either DNA strand breaks or oxidative DNA lesions in minor accessory salivary glands and inflammation in circulation observed in the CKD patient group was a significant correlation between oxidative DNA lesions and haptoglobin in the dialysis patients. No correlations were observed between salivary secretion rates and oxidative DNA lesions. However, salivary secretion rate at rest significantly predicted DNA strand breaks in minor accessory salivary glands when analyzed with a multivariate quantile regression model.

REFERENCES


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