Measurements of chlorhexidine, p-chloroaniline, and p-chloronitrobenzene in saliva after mouth wash before and after operation with 0.2% chlorhexidine digluconate in maxillofacial surgery: a randomised controlled trial

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Abstract

Chlorhexidine gluconate is used to prevent the accumulation of dental plaque and gingivitis, infection of the surgical site, and ventilator-associated pneumonia in maxillofacial surgery, but it is not clear whether the metabolites of chlorhexidine are detectable in the patient’s saliva at clinically relevant concentrations. Forty-three patients who had orofacial operations were randomised to use a 0.2% chlorhexidine gluconate (n = 23), or an octenidine-based, chlorhexidine-free (n = 20), mouthwash once preoperatively and three times daily for five postoperative days. After the first, 8.7 (23.3) mg/L chlorhexidine (0.7%-2.5% of the total amount used) was measured in saliva. The concentration increased to 15.2 (6.2) mg/L after the second rinse (first postoperative day), and peaked at 29.4 (11.2) mg/L on the fourth postoperative day. It remained detectable for up to 12 hours after the last one, but was not detectable in serum or urine at any time. The potentially carcinogenic metabolite p-chloroaniline was detectable in saliva at higher concentrations in the chlorhexidine group (0.55 mg/L) than the octenidine group (0.21 mg/L), and p-chloronitrobenzene was detected in both groups in only minimal concentrations (0.001-0.21 mg/L). Chlorhexidine gluconate mouthwashes do increase the concentration of p-chloroaniline, but a single use seems to be safe. Whether prolonged exposure over many years may have carcinogenic potential is still not clear. Based on the hitherto unknown kinetics of p-chloroaniline in saliva, the recent recommendation of the Federal Drug Administration (FDA) in the USA to limit the use of a chlorhexidine gluconate mouthwash to a maximum of six months seems to be justified.

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Introduction

Antimicrobial mouthwashes may often be used for long periods of time and, in vitro, there are mixed results for the mutagenicity of chlorhexidine digluconate.1-3 However, the incidence of micronuclei has been found to increase signif-
icantly in human lymphocytes after exposure to 0.5 mg/ml (0.05%). In further experimental studies, dermal application of an 0.5% solution 0.2 ml twice daily for 28 days induced a considerable increase in chromosomal aberrations in the bone marrow in mice, and daily oral use of 0.12% solution 3 ml for eight days in rats increased damage to DNA in white cells and kidney cells. Although this suggests that chlorhexidine gluconate has general mutagenic potential, we know of no specific clinical data in humans.

The antiseptics octenidine dihydrochloride and chlorhexidine are structurally identical with the exception of the substituent p-chloroaniline within the chlorhexidine molecule, and we know of no data that have shown that octenidine has any mutagenic potential. It is therefore possible that the experimental mutagenic and precarcinogenic potency of chlorhexidine gluconate or chlorhexidine is a result only of its metabolite p-chloroaniline, which is known to be a carcinogen. It has been detected in stored antiseptics that contain chlorhexidine gluconate, presumably through hydrolysis, but we know of no work that suggests that it is present in saliva after the use of a mouthwash that contains chlorhexidine gluconate. Because p-chloroaniline may be metabolised to p-chloronitrobenzene under oxidative conditions, the aim of this study was to measure both substances with an improved analytic method after the use of a chlorhexidine gluconate mouthwash.

**Methods**

After approval by the Ethics Committee of the University of Greifswald (Trial registry number: III UV 56/04) the study was done as a single-centre, blindered, randomised controlled trial using a chlorhexidine gluconate or an octenidine (control) mouth wash solution. After they had given written informed consent, adult patients over 18 years old of both sexes and with no ethnic limitations who were to have oral operations were included. Exclusion criteria were simultaneous participation in another study, pregnancy or lactating mothers, pre-existing or concurrent treatment with chlorhexidine gluconate, drug or alcohol misuse, or sensitivity to one of the test compounds.

The endpoints were defined as the occurrence of unwanted side effects or toxicologically relevant appearance of p-chloroaniline that exceeded the Biological Limit (Biologischer Arbeitsstoff-Toleranz-Wert, BAT) values for p-chloroaniline of > 1 mg/L of unbound substance in urine or > 100 mg/L in serum. Serum creatinine concentrations were measured to calculate the glomerular filtration rate in all patients.

Preoperatively, one of the two randomly-assigned antiseptic mouthwashes (15 ml) was used for a 30-second rinse. During the first five postoperative days, the same solution and procedure was used three times daily at 06:00, 12:00, and 21:00 hours.

**Test compounds**

The chlorhexidine gluconate mouthwash was 0.2% w/w chlorhexidine gluconate, 0.5% w/w peppermint essence, and 30% w/w sorbitol in purified water. Octenisept® (Schülke & Mayr GmbH, Norderstedt, Germany) contained 0.1% w/w octenidine hydrochloride and 2% w/w 2-phenoxyethanol, and served as the control mouthwash. They were placed into identical glass flasks by the same pharmacy to mask the study solutions.

**Preparation of the samples**

Samples of saliva were collected in 5 ml specimen tubes (Sarstedt AG & Co, Nümbrecht, Germany) before and after the preoperative oral rinse, and on the five postoperative days in the morning before the rinse, immediately after the rinses, and 30 minutes and 60 minutes thereafter. The final sample of saliva was obtained 12 hours after the last elective oral rinse on the morning of the sixth postoperative day with no further rinse. All patients were asked not to swallow for one minute after the rinse. Blood and urine samples were taken preoperatively and before each rinse on the mornings of the five following postoperative days. Samples taken before the preoperative rinse were used as the baseline measures.

**Analysis of the samples**

Chlorhexidine was analysed as triazine derivate using high performance liquid chromatography (HPLC, Liquid Chromatograph 1090 Series II with diode array detector, Hewlett Packard, USA). Chlorhexidine standard or sample 1 ml was added to 1 N sodium hydroxide 1 ml, followed by extraction with dichloromethane 4 ml. Trifluoroacetic anhydride 0.1 ml was then added to 3 ml of the extract and heated to 75 °C for one minute. The cooled content was then added to methanol/2% formic acid 100 μl, and 20 μl were injected into the HPLC after incubation for two hours at room temperature.

Both p-chloroaniline and p-chloronitrobenzene were analysed after derivatisation by gas chromatography-electron capture detection (GC-ECD, GC 5890 Series II, Hewlett Packard, USA). Gas chromatography variables were: column DB 624 (J&W Scientific, USA, Folsom), 30 m x 0.22 mm, 1.8 μm surface thickness; carrier gas: nitrogen 5.0; temperature 250 °C; temperature programme 100 °C (1 minute), 20 °C/minute up to 200 °C, 200 °C (14 minute); split off time 0.5 minute; column pressure 45 kPa; flow 1.16 ml/minute; septum wash 3.2 ml/minute; overall flow 51 ml/minute; aux gas 50 ml/minute; and ECD gas 6.3 ml/minute. Retention times were 12.3 minutes for p-chloronitrobenzene and 16.3 minutes for p-chloroaniline.

**Statistical analysis**

Power analysis was made using G-Power (Heinrich-Heine-University Düsseldorf, Germany). We aimed for a power (1-
Results

A total of 43 patients (25 male, 18 female) were enrolled in the study (chlorhexidine, n = 23, octenidine (control) n = 20). One patient in the control group withdrew consent, so was omitted from analysis. The patients were comparable (age in the chlorhexidine group 44 (20) and in the controls 52 (18) years). Two female patients had moderately decreased creatinine clearance at 55 ml/minute and 41 ml/minute, respectively, which were not considered to be clinically relevant.

The concentrations of p-chloroaniline in the chlorhexidine gluconate mouthwash were measured before use and set into relation to the manufacturing date. They ranged from 1.5, 2.68, 2.76, to 4.54 mg/L on days 3, 24, 36, and 64, respectively. None was detectable in the control solution at any time.

Statistical analysis was made and plots were calculated using SAS (Statistical Analysis Software, SAS Institute Inc., Cary, USA). Data were presented as mean (95% CI), where applicable. Descriptive statistics included median, range, 25 and 75 quantile, and interquartile range (IQR). The significance of differences between data sets were analysed using one-way ANOVA with multiple comparisons, and the significance of differences between the chlorhexidine gluconate and octenidine arms of the study by calculating surface integrals under the concentration-time-curve. Results were plotted as area under the curve (AUC) and integrals were compared using the Wilcoxon rank sum test. Probabilities of ≤ 0.05 were accepted as significant.

Concentrations in saliva

Chlorhexidine was not detectable in saliva in the control group at any time. Though none could be detected in saliva at baseline (before use) in the chlorhexidine gluconate group, samples contained 1.2 - 3.1 mg/L chlorhexidine before the next wash (postoperative days 1 - 5) and 8.7 - 29.4 mg/L immediately after the wash (days 1 - 5; Fig. 1). After rinsing, concentrations decreased to 4.7 - 7.4 mg/L (30 minutes) and 1.6 - 4.9 mg/L (60 minutes) on days 1 to 5, respectively. Maximum values were detected in single patients on postoperative days 2, 3, and 4 (27-97 mg/L) (Fig. 1).

In the chlorhexidine gluconate group, p-chloroaniline was not detected in any baseline samples of saliva (Fig. 2). Therefore, raised concentrations were detectable in 22 out of 23 patients. It was detected in 18.3% of the samples that were taken immediately before the morning rinse on days 1 to 5 postoperatively (means 0.001 - 0.02 mg/L, median 0.00 mg/L). Immediately after the rinse it was detected in 65% of the samples with the highest values during the course of the day (maximum values: 0.31, 0.28, 0.25, 0.55, and 0.2 mg/L on days 1, 2, 3, 4, and 5; medians: 0.04, 0.03, 0.02, 0.06,

![Fig. 1. Chlorhexidine concentration in saliva in the chlorhexidine gluconate group (mean 95% CI).](image-url)
and 0.00 mg/L on days 1, 2, 3, 4 and 5, respectively). It was detected in 27.8% of the samples at 30 minutes (maximum concentrations: 0.09 - 0.51 mg/L, median: 0.00 mg/L), and in 7.9% of the samples 60 minutes after the wash (0.01 - 0.08 mg/L, Fig. 2).

In the control group, p-chloroaniline was detectable at baseline in three patients (0.005, 0.009, and 0.011 mg/L), which did not differ significantly from the chlorhexidine gluconate group (Table 1 and Fig. 3). However, immediately after the rinse, the concentration in saliva differed significantly between the groups on postoperative days 1 to 5 (Table 1 and Fig. 3). During the next five days, six patients of the control group had concentrations in saliva that ranged between 0.003 mg/L and 0.21 mg/L (median: 0.00 mg/L).

In the chlorhexidine gluconate group, p-chloronitrobenzene was detectable in saliva of 6 out of 23 patients (26.1%) ranging above the detection limit of 0.002 mg/L (0.01 - 0.06 mg/L, medians: 0.00 mg/L). In the control group, p-chloronitrobenzene was detected in 10 of 19 patients (0.03 - 0.21 mg/L). There was, however, one patient in whom it was detected at baseline with a concentration of 0.02 mg/L. There were no significant differences between the samples taken at baseline, or on days 1 to 5.

Concentrations in serum and urine

Chlorhexidine was not detected in serum samples either from control patients or patients in the chlorhexidine glu-
conate group, and p-chloroaniline was measured in only one patient in the chlorhexidine group on postoperative days 4 (0.05 mg/L) and 5 (0.03 mg/L) before the rinse. At equal time points the same patient also showed concentrations of p-chloronitrobenzene (0.012 mg/L, on day 4 and 0.003 mg/L on day 5) in samples of serum. Neither was detected in samples from the control group. No patient yielded any measurable concentrations of any of the three substances in urine.

Discussion

Every mouthwash contained chlorhexidine 30 mg in 15 ml solution. According to Greenstein et al.\textsuperscript{16} about 4% (chlorhexidine 1.2 mg) of such solution remains in the oral cavity by absorption and swallowing after the solution has been spat out. Patients were advised not to swallow the minute before obtaining the sample of saliva, as humans produce about 1.0 – 3.5 ml saliva in one minute.\textsuperscript{17} A total concentration of chlorhexidine of 343 – 1200 mg/L could therefore theoretically be expected immediately after the first mouth wash (in 3.5 and 1 ml saliva, respectively). Concentrations of chlorhexidine peaked immediately after the rinse on the first postoperative day (8.7 (23.3) mg/L; 0.7% - 2.5% of the calculated total chlorhexidine concentration given in 0.2% chlorhexidine 15 ml, 343 – 1200 mg/L). According to Musteata and Pawliszyn,\textsuperscript{18} after a rinse with 0.1% chlorhexidine 10 ml, the amount of chlorhexidine 15 minutes after the wash was 295 mg/L, followed by 39.9 mg/L after one hour, 12.8 mg/L after four hours, and 2.0 mg/L after eight hours. The calculated amount of free chlorhexidine was therefore less than 2% (0.2% - 1.8%) of the total during the first hour, increasing to a maximum of 7% after eight hours, which is in line with our current results.

Concentrations of chlorhexidine in saliva increased immediately after the rinse on the second postoperative day to 15.2 (6.25) mg/L, and reached its highest concentration on the fourth (29.4 (11.2) mg/L) and fifth (27.7 (13.9) mg/L) postoperative day (Fig. 4). These results are in contrast to those of Tsuchiya et al.,\textsuperscript{19} who showed that it was detectable in saliva up to the 12th postoperative day, which fits with the reported duration of inhibition of plaque after use of a mouthwash containing chlorhexidine gluconate.\textsuperscript{20}

The lack of chlorhexidine in serum and urine mirrors the results of earlier studies on its absorption from intact skin,\textsuperscript{21} vagina,\textsuperscript{22} and in the continuous use of Perio-Chips in the oral cavity for nine days.\textsuperscript{23}

Kinetics of p-chloroaniline in saliva

To our knowledge this is the first report that has confirmed the presence of p-chloroaniline in saliva. Of all samples taken after the rinse in the chlorhexidine group, p-chloroaniline was found in 71% on day 1, followed by 70%, 63%, 76%, and 43% on subsequent days. Even 30 minutes and 60 minutes after the wash it could still be detected in 28% and 8% of all samples. In contrast to the kinetics of chlorhexidine in saliva, there was no evidence of accumulation of p-chloroaniline during the course of the study. The use of a mouthwash based on chlorhexidine gluconate and the associated accumulation of p-chloroaniline in the patients’ saliva was significant (Table 1).

Commercially available mouthwash solutions may contain up to 2.5 mg p-chloroaniline-L-chlorhexidine.\textsuperscript{14} This amount was never exceeded in saliva in our study. However, considering that the p-chloroaniline content measured at the beginning of the study was 0.075 mg within chlorhexidine gluconate100 mg, a maximum of p-chloroaniline 0.023 mg is taken up in chlorhexidine gluconate solution15 ml. After spitting out the fluid, an amount of about 4% (0.9 μg) p-chloroaniline was assumed to remain in the oral cavity, giving maximum concentrations in saliva of 0.25 mg and 0.9 mg/L.
in saliva 3.5 ml and 1 ml. At the end of the study period and storage time of 36 days, the concentration in the mouthwash solution was 0.14 mg/100 mg chlorhexidine gluconate, giving a theoretical concentration of 0.49 mg/L or 1.7 mg/L saliva. The highest detected concentration within the study was 0.55 mg/L. However, the study is limited in terms of whether it can confirm if exposure to a known carcinogen in the oral cavity might be potentially hazardous.

Conclusion
Chlorhexidine gluconate mouthwashes increase the concentration of p-chloroaniline, but single-uses in maxillofacial surgery seem to be safe. Whether prolonged exposure over many years may have carcinogenic potential is still not clear. Based on the hitherto unknown kinetics of p-chloroaniline in saliva, the recent FDA recommendation\(^3\) to limit the use of chlorhexidine gluconate mouthwashes to a maximum of six months seems to be justified.

Conflict of interest
We have no conflicts of interest.

Ethics statement/confirmation of patients’ permission
Approval was obtained from the ethics committee of the University of Greifswald. All patients gave their consent.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bjoms.2016.10.007.

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