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Changes in *Candida* spp., Mutans Streptococci and Lactobacilli following Treatment of Early Childhood Caries: A 1-Year Follow-Up

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Key Words

Candida spp. · Early childhood caries · *Lactobacillus* · Mutans streptococci · Oral microbiology

Abstract

Early childhood caries (ECC) is closely related to high numbers of mutans streptococci, lactobacilli and *Candida albicans*. Oral colonization of these microorganisms was monitored in a prospective clinical study in order to investigate the effect of comprehensive treatment under general anesthesia and the sustainability of microbial changes. Saliva samples were collected from 50 healthy infants with ECC before and in regular intervals up to 12 months after treatment. Microorganisms were detected by cultivation on selective agars (CRT[®] bacteria and Sabouraud/CandiSelect[™]) and scored. Additionally, plaque on upper front teeth and the dmft were recorded. Parents were repeatedly interviewed regarding the children's diet and oral hygiene, accompanied by corresponding advice. Plaque frequency and the numbers of mutans streptococci, lactobacilli and yeasts were significantly reduced as a result of treatment ($p < 0.0001$, Wilcoxon test). Nevertheless, this effect was not permanent. An ordinal regression model on the follow-up period revealed that the odds for bacteria and yeasts to reach a higher score increased linearly over time ($p < 0.01$) with an odds ratio of 2.244 per year. One third (34%) of the children developed new den-

tinal lesions within 1 year postoperatively. High scores of lactobacilli before treatment predicted caries relapse ($p < 0.05$). Nutritional and oral hygiene habits changed only slightly despite advising. Elimination and restoration of ECC lesions under general anesthesia proved to be an effective procedure in reducing cariogenic bacteria and yeasts. A satisfactory and sustainable success, however, could be achieved neither regarding microbiologic parameters nor with respect to the relapse rate. More suitable strategies are needed.

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In contrast to the successful decline of caries in the permanent dentition in many countries, early childhood caries (ECC) continues to be a serious global challenge in public health [Tinanoff and Reisine, 2009]. A key factor of this rampant form of caries is a frequent and prolonged dietary sugar exposure, commonly due to an inappropriate feeding behavior, especially the use of a sugar-containing liquid in a nursing bottle at night. The presence of fermentable carbohydrates in the mouth initially triggers an early oral infection with mutans streptococci by mainly vertical but also horizontal transmission [Poureslami and Van Amerongen, 2009]. By shifting the ecological balance of the dental biofilm towards an acidic environment, it moreover promotes the accumulation of aciduric and acidogenic species to cariogenic levels. In

further consequence the enamel is rapidly demineralized, which results in cavitation of tooth structure [Berkowitz, 2003].

Streptococcus mutans has shown to be highly associated with severe ECC [Parisotto et al., 2010; Tanner et al., 2011b]. The time span between the acquisition of mutans streptococci and caries initiation is approximately 13–16 months [Law and Seow, 2006]. Children who harbored *S. mutans* in their plaque at the age of 2 had the most pronounced caries activity by the age of 4 [Alaluusua and Renkonen, 1982].

Lactobacilli are considered to be participators in the caries process, playing an important role in lesion progression. The number of lactobacilli increases when mutans streptococci start to colonize the oral cavity, since they produce niches with a low pH. Lactobacilli were observed in higher proportions at the advancing front of carious lesions than in the overlying plaque [Beighton and Brailsford, 1998] or at sound surfaces [Matee et al., 1992].

Similar to mutans streptococci and lactobacilli, yeasts of the genus *Candida* are frequently present in the oral cavity of children with ECC as well [Marchant et al., 2001; de Carvalho et al., 2006]. The predominant oral yeast in most populations is *Candida albicans*, but also other partly pathogenic *Candida* species including *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii* and *C. dubliniensis* are found in the oral cavity of children [Sánchez-Vargas et al., 2005; Jabra-Rizk et al., 2007]. Although *Candida* counts are generally low compared to caries-associated bacteria, the biomass proportion of the yeast is not negligible due to its large cell size. One colony-forming unit of *C. albicans* (yeast phenotype) proved to have a much higher dry weight of 17.4 pg compared to *S. mutans* (0.41 pg) or a selection of oral lactobacilli (3.5 pg) [Klinke et al., 2009]. The yeast displays characteristics related to cariogenicity [Klinke et al., 2009] and advanced occlusal caries lesions occurred when the dental biofilm of rats was associated with *C. albicans* [Klinke et al., 2011]. The relevance of *Candida* spp. to caries progression in humans is currently unknown.

Clinical studies of ECC have indicated that elimination of carious lesions by comprehensive dental treatment under general anesthesia results in a significant reduction of salivary mutans streptococci and lactobacilli [Chase et al., 2004; Morinushi et al., 2004; Tanner et al., 2011a]. However, this did not translate into acceptable clinical outcomes; the relapse rate was high and rapid [Almeida et al., 2000; Chase et al., 2004; Foster et al., 2006]. Compared to mutans streptococci, the decrease in lacto-

bacilli due to treatment was found to be more pronounced and durable [Wright et al., 1992; Morinushi et al., 2004]. This was related to the elimination of deep carious lesions, which are the predominant habitats for lactobacilli in the oral cavity [Matee et al., 1992; Wicht et al., 2004; Chhour et al., 2005; Kneist et al., 2010]. Similarly, *Candida* species have been isolated more frequently and in higher concentrations from carious dentin than from other oral sites [Wetzel et al., 1993; Sziegoleit et al., 1999]. Therefore it is conceivable that removal of caries will reduce the number of yeasts to a comparable extent.

Little is known about the long-time effects of dental treatment on the oral microflora in the case of ECC, and yeasts have not been included in related clinical studies so far. Accordingly, the present prospective study investigated the effect of full mouth rehabilitation under general anesthesia on oral *Candida* spp. as well as on mutans streptococci and lactobacilli in children suffering from ECC during a 1-year postoperative period. At defined intervals, the microbial load of saliva samples was investigated by cultivation on selective media. Yeasts were grown and quantified on Sabouraud agar while mutans streptococci and lactobacilli were evaluated using the commercial kit CRT[®] bacteria. Additionally, caries relapse and parameters of oral hygiene and nutrition were recorded.

Material and Methods

Subjects

A number of 50 infants, aged 1–5 years, with multiple caries lesions according to ECC types II or III [Wyne, 1999] were selected for this study. All children were physically healthy (ASA class 1), no antibiotics were administered in the previous 4 weeks and general anesthesia was indicated for dental treatment. Study design was approved by the Ethics Committee of the Medical Faculty Carl Gustav Carus, Dresden (No. 56032007).

Treatment and Follow-Up Schedule

Two weeks prior to treatment, the children and their parents were summoned into the Department of Paediatric Dentistry for a baseline examination. During that appointment they were given instructions regarding oral hygiene and they received diet counseling. A 0.2% chlorhexidine digluconate gel (CHX; Cervitec Gel, Ivoclar Vivadent, Liechtenstein) was handed out to the parents, who were briefed to use it for brushing of their children's teeth twice a day preoperatively. Dental treatment was performed under general anesthesia in one session and included extractions, composite resin restorations and tooth cleaning. In total four follow-up examinations were accomplished at time intervals of 2 weeks, 3, 6 and 12 months after treatment by two investigators (fig. 1). Only those children who attended at least the first two follow-up sessions were enrolled in the study.

Fig. 1. Flowchart of the study protocol.

Microbial Investigations

At each session saliva samples were collected from the children; they served as a semiquantitative proof of caries-associated microorganisms. Mutans streptococci and lactobacilli were detected by cultivation using the CRT bacteria kit (Ivoclar Vivadent). The saliva was gathered with a tongue depressor, which was wetted with

saliva by turning repeatedly on the upper surface of the tongue. Immediately afterwards, the saliva was transferred onto CRT agar. After 48-hour anaerobic incubation at 37°C, the growth of colonies was evaluated by reference to model charts of the test kit and graded by four scores: 0 = not detected, 1 = sparse, 2 = moderate, 3 = numerous. In order to get scores analogous to mutans streptococci, scores 3 and 4 of lactobacilli were pooled and score 0 was added to represent lacking detection of lactobacilli. According to Richter [2004], the detection limit of mutans streptococci and lactobacilli was up to 1,000 colony-forming units (CFU) per milliliter of saliva, depending on the strain. Contamination of the CRT agar by yeasts was discerned by examining the appearance of colonies or by placing a drop of hydrogen peroxide onto the colony surface in order to demonstrate the yeasts' hydrogen peroxidase.

For the determination of *Candida* spp., saliva was absorbed from the sublingual area of the mouth with three sterile cotton-tipped applicators and was applied to three Sabouraud agar plates (glucose 20 g l⁻¹, agar-agar 17 g l⁻¹, peptone 10 g l⁻¹; pH 5.6). After 48 h of aerobic incubation at room temperature the plates were counted. The limit of detection for *C. albicans* was 250 CFU/ml as tested in preliminary experiments. In adaption to CRT, the averaged colony-forming units were allocated to four scores: score 0/not detected = 0 colonies; score 1/sparse = 1–9; score 2/moderate = 10–99, and score 3/numerous = more than 99 colonies per plate.

Yeast strains were identified employing CandiSelect™ 4 (BioRad, Munich, Germany) as well as by microscopic observation of colonies grown on rice extract agar. From every culture one colony of each morphotype was selected for differentiation. Additionally, a sugar assimilation test (Auxacolor™, BioRad, Munich, Germany) was used to identify *C. dubliniensis*. The quantitative determination of yeasts did not discriminate different *Candida* species.

Additionally, the presence of visible plaque on the labial surfaces of upper front teeth was recorded [Pieper, 2001].

DMFT and Questionnaire

At baseline as well as at the follow-up examinations, the dmft was recorded. Decayed teeth were classified using the levels d12 for precavitated or enamel lesions and d34 for dentinal or pulpal lesions [Marthaler, 1966, 1967]. An extensive questionnaire, including details about breast-feeding, drinking, meals and snacks, brushing habits and compliance was submitted to the parents or the chaperon at each examination except of the first follow-up (online suppl. material, www.karger.com/doi/10.1159/000351673). According to the outcome of the first questionnaire they were advised regarding diet and oral hygiene.

Statistical Analyses

Statistical analyses were performed with SPSS 17.0 (SPSS, Inc., Chicago, Ill., USA) and R 2.15.2 (R Foundation for Statistical Computing, Vienna, Austria). For pairwise comparisons of colony scores between different time points, Wilcoxon signed-rank test was used at a level of significance of $\alpha = 5\%$ (i.e. significant when $p < 0.05$) after Holm-Bonferroni correction for multiple testing. In a second step, longitudinal changes of colony scores after therapy were studied employing an ordinal regression model, namely a cumulative link mixed model. A logistic regression model served the identification of early predictors for caries relapse. Data on visible plaque over time were analyzed utilizing the exact McNemar test. Kendall's tau-b was calculated from ordinaly scaled data of the questionnaires in order to find correlations between microbial

Fig. 2. Quantities of *Candida* spp., lactobacilli and mutans streptococci in salivary samples of children with ECC at baseline (n = 50), after pretreatment with CHX on the day of treatment under

general anesthesia (n = 50) and at follow-up examinations 2 weeks (n = 50), 3 months (n = 50), 6 months (n = 44) and 12 months (n = 38) after treatment.

scores and caries recurrence, duration of daily nursing bottle use, frequency of intake of listed caries-promoting foods and beverages and also compliance in oral hygiene. Pairwise longitudinal comparisons of these data were performed with the Wilcoxon signed-rank test.

Results

A total of 50 patients was examined before and continuously up to 3 months after treatment. The third follow-up was attended by 44 patients and 38 of them kept the last appointment. This corresponds to a dropout rate of 12% after 6 months and further 14% after 1 year, respectively.

At baseline, *Candida* yeasts as well as mutans streptococci and lactobacilli were detected with high prevalence in the oral cavities of children with ECC (fig. 2). In 97% of the samples positive for yeasts, *C. albicans* was differentiated. Frequently, a combination with *C. tropicalis* (32%), *C. glabrata* (21%) and/or *C. krusei* (18%) was observed. Lactobacilli correlated weakly with *Candida* spp. ($\tau = 0.33$; $p = 0.006$), more distinctly after CHX pretreatment ($\tau = 0.46$; $p < 0.001$) and then accompanied by a weak correlation between lactobacilli and mutans streptococci ($\tau = 0.37$; $p = 0.003$). At the end of the study, yeasts correlated moderately with mutans streptococci ($\tau = 0.40$; $p = 0.008$).

Candida spp. as well as mutans streptococci and lactobacilli were significantly reduced by treatment (Wilcoxon

test, all adjusted $p < 0.001$). The decrease remained significant compared to the baseline data throughout the entire follow-up period (Wilcoxon test, all adjusted $p < 0.03$). Pretreatment with CHX had a limited antimicrobial effect which was statistically significant only for yeasts (Wilcoxon test, $p = 0.047$).

In the course of the 1-year follow-up period regrowth of all microorganisms occurred. The ordinal regression model revealed a significant ($p = 0.0036$) linear trend over time, i.e. the odds to fall in a higher colony score compared to the first follow-up after treatment increased with factor 1.070 per month (95% confidence interval, 1.022–1.119) or factor 2.244 per year, respectively. It is noteworthy that an interaction between time and type of microorganism was not significant, which indicates that all three microorganisms regrew similarly over time.

The number of subjects with visible plaque on the front teeth was reduced after treatment compared to the baseline data ($p = 0.0072$), but afterwards it increased again and 3 months later the differences were not significant anymore (table 1).

The average d34t at baseline was 10.1. Within the first year after treatment, 34% of the patients developed new dentinal lesions. Lactobacilli were found to be a positive predictor for caries relapse. An elevation of lactobacilli by one score at baseline was associated with an increase in the odds to develop dentinal lesions after 12 months by factor 2.247 (95% CI, 1.070–5.369; $p = 0.044$). Data on caries recurrence are given in table 1.

Table 1. Percentage of patients with visible plaque on upper front teeth at baseline, on the day of treatment and at follow-up examinations; mean cumulative number and percentage of teeth with new lesions/d34 lesions per patient as well as cumulative percentage of patients with new lesions/d34 lesions at follow-up examinations up to 12 months after treatment

Examination	Baseline (n = 50)	Treatment (n = 50)	2 weeks (n = 50)	3 months (n = 50)	6 months (n = 44)	12 months (n = 38)
Plaque on front teeth, %	65	57	35	46	56	51
Teeth with new lesions per patient				0.40	0.64	0.97
Teeth with new d34 lesions per patient				0.22	0.43	0.66
Teeth with new lesions per patient, %				2.5	4.0	6.0
Teeth with new d34 lesions per patient, %				1.4	2.7	4.1
Patients with new lesions, %				20	32	45
Patients with new d34 lesions, %				10	20	34

n = Number of patients.

Following the preoperation discussion with the parents or a chaperon, nutritional habits of the children in relation to cariogenic foods and beverages tended to improve up to the 3 months' follow-up examination, but then deteriorated again by the end of the study. The frequency of and willingness to dental home care did not improve. However, with increasing age the children more frequently attended a kindergarten where an additional brushing was performed. Correlations between oral hygiene behavior or nutritional habits and microbial counts or caries recurrence were not found.

Discussion

Candida species are known to cause serious infections in immunocompromised persons. In healthy children, yeast abundance has been shown to be frequently related to extensive caries experience, particularly to ECC [Marchant et al., 2001; Raja et al., 2010]. Due to their pronounced acid production, *Candida* species are considered to be involved in caries progression [Sziegoleit et al., 1999; Signoretto et al., 2009; Klinker et al., 2011; Lai and Li, 2011]. Accordingly, a permanent reduction or even elimination of the fungus by caries therapy is desirable. To the best of our knowledge, the current study investigates for the first time changes in *Candida* load due to comprehensive treatment of ECC for a prolonged period of time.

Different sampling methods were used for bacteria and yeasts. Mutans streptococci and lactobacilli were collected by taking saliva samples from the upper surface of the tongue, as it is standard for the CRT bacteria

method. Applying the same procedure for the detection of *Candida* spp. would result in an overestimation, since the dorsum of the tongue is the most colonized mucosal area with respect to yeasts in the oral cavity [Arendorf and Walker, 1980] and gaining samples from this area would rather provide information about attached cells at this very site than illustrate the fungal content of saliva as a reflection of the infected dentition. The floor of the mouth, however, was found to be one of the locations with the lowest density of attached yeasts [Arendorf and Walker, 1980], which makes it a preferable harvesting site for salivary samples of *Candida* species. According to preliminary tests, the collection of unstimulated saliva from the sublingual pool turned out to be a reproducible sampling method for yeasts, applicable especially for less compliant toddlers and preschoolers.

The small volume of sublingual saliva gathered by a cotton carrier may have failed to capture yeast cells from sparsely colonized dentitions. Nevertheless, the prevalence of 68% for *Candida* spp. determined in this study corresponds to the results of comparable investigations on children with ECC. Wetzel et al. [1993] found a prevalence of 72% in saliva and 83% in samples of carious dentin; de Carvalho et al. [2006] and Marchant et al. [2001] isolated *Candida* spp. from carious dentin in 71 and 89% of cases, respectively.

The CRT bacteria test kit was chosen for the detection of mutans streptococci and lactobacilli combined with the tongue depressor method of sampling, because it is easily practicable even in uncooperative children. Though semiquantitative, the CRT enables appropriate results based on the immediate chair-side cultivation. Several in-

investigators found dip slide tests to be on a par with conventional cultivation methods and judged the results as reproducible [Jordan et al., 1987; Jensen and Bratthall, 1989; Jentsch et al., 1997].

Pretreatment with CHX did not substantially affect the oral microflora. Only for yeasts the suppression was statistically significant. Similar results were obtained by Signorello et al. [2009], but they observed an even lower decrease of *C. albicans* compared to lactobacilli with the highest effect on mutans streptococci by a CHX/fluorine rinse twice a day for 2 weeks.

In the present study, a considerable but not permanent decrease in microbial counts was accomplished by caries removal and extraction of teeth. Regarding mutans streptococci, this is in accordance with a comparable investigation by Litsas [2010], who found *S. mutans* to reappear already 3 months after therapy. Twetman et al. [1999] observed no significant changes in mutans streptococci or lactobacilli 5 months after the first posttreatment recall. The latter study, however, was conducted on preschoolers with severe dental caries, but not particularly with typical signs and a history of ECC. Oral yeasts were studied on Portuguese schoolchildren by Starr [2002]. The comparatively low prevalence of oral *Candida* (47%) decreased during and after a period of dental treatment and increased again at follow-up examinations without reaching the pretreatment level. This complies with the present results for children with ECC.

The treatment of open cavities, which are considered to act as an important oral reservoir for yeasts [Sziegoleit et al., 1999], gave reason to expect a lasting elimination of *Candida* spp. from the oral cavity on an individual level. However, 91% of children tested positive for *Candida* at baseline showed yeasts again at one or more follow-up examinations within 1 year. Just 9% of the individuals became enduringly free of yeasts, and all of these had only sparse *Candida* counts near the limit of detection at baseline. Therefore, despite the observed significant reduction in the prevalence of *Candida* spp. due to caries removal, permanent eradication of yeasts from the oral cavity cannot be expected. Admittedly, yeasts in low numbers are normal constituents of the oral microflora in healthy humans. Higher proportions of *Candida* may continue to be present on the basis of conditions which have not been changed by treatment. A significant link has been observed between oral abundance of *C. albicans* and sugar intake as well as acid oral pH [Moalic et al., 2001]. The yeast was only able to establish itself under conditions of glucose excess as part of a mixed community of oral bacteria in a chemostat [Basson, 2000]. In a study by de Car-

valho et al. [2006], *C. albicans* was isolated more frequently from the plaque of children with a specific clinical pattern of ECC and a history of prolonged breast-feeding and/or nursing bottle with fermentable carbohydrates, than from children equally affected by tooth decay but without specific ECC pattern and lacking a history of nursing bottle or prolonged breast-feeding. These observations underline the hypothesis that carbohydrate excess is more essential for oral *Candida* colonization than open cavities serving as a protected niche. Once present, cavities may promote in some way the retention of dietary sugars and the maintenance of a low pH. Consequently, removal of cavities alone will not sufficiently suppress *Candida*, if the etiological environmental conditions are not changed as well. In the present study, dietary and oral hygiene habits did not improve significantly. Instructions about oral hygiene and nutrition were not measurably successful despite the strengthening effect of multiple questionnaires.

It is an open question, if and how the presence of *Candida* has an impact on caries progression. According to the ecological plaque hypothesis, every acid-producing microorganism which prevails under caries-promoting local environmental conditions in the dental biofilm will contribute to the destruction of teeth [Marsh, 2006]. Depending on pH and substrate, *C. albicans* excretes mainly pyruvic ($pK_a = 2.39$) or acetic ($pK_a = 4.76$) acid [Klinke et al., 2009], whereas lactic acid ($pK_a = 3.96$) is the most important end product of cariogenic bacteria. Hence, the contribution of yeasts to hard tissue demineralization could be either promoting, indifferent or even inhibiting due to antagonistic effects [Klinke et al., 2011]. This aspect requires further research.

As there was microbial regrowth after therapy, caries risk remained high. However, the observed annual relapse rate of 34% was lower compared to similar studies [Graves et al., 2004: 37% within 6 months; Zhan et al., 2006: 60% within 12 months; Eidelman et al., 2000: 57% within 6–24 months, and Foster et al., 2006: 53% within 6–24 months]. This might be attributed to an inclusion criterion for the study cohort of at least two attended recalls, which strongly selected for compliant persons. In a study by Foster et al. [2006], children who attended their follow-up appointments were less likely to have developed new caries lesions than those who failed to attend reevaluation.

A high score of lactobacilli was the only significant early predictor for caries relapse. Lactobacilli correlate with the number of carious lesions in children and they are indicators of frequently occurring low pH levels due to

short-time repeated sugar intake [Larmas, 1992; Beighton and Brailsford, 1998]. Therefore they signify a high persisting caries risk irrespective of therapy if the conditions for caries do not change. Pienihäkkinen et al. [1987] observed an even higher predictive power of yeasts to indicate future caries, exceeded by the combined information of lactobacilli and yeasts. The present study does not corroborate this result.

In any case, visible plaque on the labial surfaces of primary maxillary incisor teeth is considered to be the best indicator for caries risk in young children [Alaluusua and Malmivirta, 1994]. The large number of positive cases observed in the present study at baseline and the rapid reappearance of plaque after treatment reflect the high persisting risk.

In summary, comprehensive treatment of ECC under general anesthesia is an effective regimen for reducing the numbers of oral *Candida* yeasts as well as of lactobacilli and mutans streptococci. However, in this study treatment of caries along with counseling of parents did neither maintain low levels of oral microorganisms nor prevent caries relapse sufficiently. The outcome of therapeutic interventions for ECC will not be permanent unless caries-promoting factors change substantially.

Disclosure Statement

There are no conflicts of interest for any of the authors involved in this study.

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