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Effect of ozone on dental caries and on cariogenic microorganisms

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Abstract

There are a number of unanswered questions regarding new caries preventive methods such as ozone treatment as well as novel caries detection methods that monitor and evaluate these preventive methods. This thesis seeks answers to some of these questions.

Aims: Paper I investigates the in vitro sealing capacity of a novel ozone delivery system and its re-suction capacity. Paper II studies the in vitro antibacterial effect of ozone on cariogenic bacterial species with and without the presence of saliva and its possible effect on the saliva proteins. Paper III assesses the in vitro validity of laser-induced fluorescence (LF) measurements, visual inspection (colour), and tactile examination (surface texture) on root caries lesions and correlates these with histopathological measurements of lesion depths. In addition, Paper III investigates how inter-device, inter-examiner, and intra-examiner levels influence LF reading agreements and whether lesion colour and surface texture influences LF readings. Paper IV evaluates the in vivo effect of ozone and fluoride varnish treatments on occlusal caries in primary molars.
Material and methods: Paper I: Full ozone application cycles, including the re-suction period, and interrupted cycles with displacement of the cup during the delivery cycle were studied using an ozone air analyser. Paper II: Ozone gas was exposed to the bacteria in the solution's buffer and saliva via a tube connected to the ozone generator for 10, 30, and 60 seconds. Paper III: Calibrated examiners assessed lesion colour and surface texture and performed measurements with two LF devices for three separate one-week intervals. Sections (300µm thick) of 64 out of 93 teeth were obtained and examined under a microscope. Lesion depth was assessed with two references: from the delineated borderline of the original exposed root surface (ref I) or, if loss of surface continuity, the absolute lesion depth (ref II). Paper IV: The split mouth study was conducted in two parts. The participants in part A were children (mean age 4.8 years) with medium-high caries risk. Inclusion criteria were bilateral matched pairs of cavitated or non-cavitated occlusal lesions in primary molars (Ekstrand index score ≤ 3). Children in part B (mean age 4.5 years) with low-medium caries risk had pairs of non-cavitated lesions only (Ekstrand index score ≤ 2a). The assessments and treatments with ozone and fluoride varnish were performed at baseline and at three, six, and nine months. At the 12-month follow-up, only assessments were performed. **Results:** Paper I: Ozone

leakage levels varied between 5.2 and 9.8 μg/m^3. Paper II: In the salt buffer, 92%, 73%, and 64% of the initial numbers of *A. naeslundii*, *S. mutans*, and *L. casei*, respectively, were killed after 10 s ozone exposure and approximately 99.9% of the bacteria were dead after a 60 s exposure. After 10 and 30 s but not after 60 s exposure to ozone, *S. mutans* and *L. casei* were less efficiently killed in saliva compared to in the salt buffer. Various saliva proteins were degraded by ozone after the 60 s exposure. Paper III: The correlation between LF readings and histological depth was low with values ranging from 0.22 ($p > 0.05$) to 0.31 ($p < 0.05$). The LF devices were significantly correlated with discolouration and with a surface texture denoted as hard. A significant correlation was found between colour and histological depth. No significant correlation was found between surface texture and histological depth. The reliability, evaluated as intra-class correlation coefficient, was 0.99 for intra-examiner, 0.97 for inter-examiner, and 0.98 inter-device level. Large differences were found between two consecutive measurements and high measurement errors indicated considerable deviation of individual measurements.
Paper IV: In the first 15 pairs of part A, eight lesions treated with ozone and nine treated with fluoride, including all cavitated lesions, progressed to failure, i.e., required operative treatment during the study time. Due to non-acceptable results, the sample collection was discontinued because of ethical reasons. In part B, of 35 pairs, one of the ozone treated lesions failed at 12 months. A small shift towards increased VI scores was recorded for both ozone and fluoride lesions in this second part.

Conclusions:

- The ozone delivery system can be considered a safe system with low leakage levels in air, also with accidental displacements.
- The cariogenic species *S. mutans, L. casei*, and *A. naeslundii* were sensitive to ozone gas treatment. The presence of saliva hampered the antibacterial effect of ozone.
- A low correlation between the LF readings and the histopathological depth of root caries lesions was shown. The LF device was found not to be appropriate for application to root caries diagnosis.
- Neither ozone nor fluoride varnish treatments arrested the progression of cavitated occlusal caries lesions. In low and medium caries risk children non-cavitated occlusal lesions remained mainly unchanged during the study period. No difference in the effect of ozone and fluoride varnish treatments on occlusal caries in primary molars was seen.

List of papers

This thesis is based on the following papers referred to in the text by their roman numerals I-IV:

I. **Elisabeth Johansson,** Annika Hagenbjörk-Gustafsson, Ingrid Andersson-Wenckert, Jan van Dijken.
Ozone air levels adjacent to a dental ozone gas delivery system.
Acta Odontologica Scandinavica 2007; 65: 324 - 330

II. **Elisabeth Johansson**, Rolf Claesson, Jan van Dijken.
Antibacterial effect of ozone on cariogenic bacterial species.
Journal of Dentistry 2009; 37: 449 - 453

III. Lena Karlsson, **Elisabeth Johansson**, Sofia Tranæus.
Validity of infrared fluorescence measurements on sound and carious root surfaces in vitro.
Caries Research 2009; 43: 397– 404

IV. **Elisabeth Johansson**, Lena Karlsson, Ingrid Andersson-Wenckert.
Treatment effect of ozone and fluoride varnish application on occlusal caries in primary molars: a 12-month study.
Submitted manuscript.

Reprints of paper I, II, and III were made with permission from the publishers.

Introduction

Dental caries

Dental caries is a term used to describe the results, signs, and symptoms of a localized chemical dissolution of enamel and dentin. The disease is one of the most prevalent infections of people worldwide [1, 2]. Throughout life, everyone is at risk of developing the disease [3]. The disease occurs both in enamel and dentin and the earliest visible sign of dental caries in the enamel is a white spot on the surface. Before the lesion is visible, the process has already started and a small demineralised area below the outer tooth surface has occurred. This white lesion is visible, provided the tooth is clean and dry, but histologically the lesion is halfway through the enamel. If the lesion progresses into the dentin, loss of tissue and cavitation occurs [4]. A decline of caries prevalence has occurred during the last decades, and progression of enamel caries is today slower, allowing time for preventive intervention before irreversible destruction of tooth substance [1]. This trend has increased the need for methods that detect incipient caries [5] as well as the need for preventive treatments to arrest or even re-mineralise the lesions.

Caries is a multifactorial chronic disease associated with life style-related factors, such as diet, oral hygiene, and use of fluoride products. In addition, socio-economic status and genetic predisposition are factors involved in the aetiology of the disease [2, 4]. However, the initiation and progression of caries requires biofilm to be present on the teeth [6, 7].

Dental biofilm

The mouth is the main passageway into the body for a huge number of bacteria that eventually colonize various internal sites [8]. Those bacteria that colonize the oral cavity have to withstand saliva flow and have the capacity to attach to surfaces covered with epithelium cells or to surfaces without this layer, such as the teeth. However, in addition to washing away some bacteria entering the mouth, the saliva also delivers proteins and glycoproteins that cover the teeth and provide sites for attachment of other bacteria [6]. The most important saliva components of this layer on the teeth, the pellicle, are the proline rich proteins (PRP:s): statherin, amylas, and the agglutinines [9]. After established, the pellicle is covered by bacterial species such as *Streptococcus mitis, Streptococcus oralis, Streptococcus gordonii* and *Actinomyces* species and other so-called early colonizers. In a subsequent step, called co-aggregation, a vast diversity of other bacterial species, the late

colonizers, bind to the early colonizers. Together with the saliva components, the bacterial colonizers and their metabolic products constitute the dental biofilm. Some bacteria that can induce caries may be hidden in the biofilm. In the presence of sugar, these caries-associated bacteria can have a deleterious effect on the teeth [6].

Caries-associated bacteria

These bacteria express specific virulence factors, i.e. factors that clearly link them to caries. Bacteria fulfilling these criteria are the mutans streptococci, lactobacilli, and *Actinomyces oris* (previously named *A. naeslunidii* genospecies 2) [10]. The associations of these bacteria to caries have been studied for decades [2]. More recently, *Bifidobacterium* and *Scardovia* species have been implicated in caries formation [11, 12].

The attack of the cariogenic bacteria on the teeth can be divided into two steps. First, these bacteria have to join the biofilm. *Streptococcus mutans (S. mutans)* and *Streptococcus sobrinus (S. sobrinus)*, the most important of the mutans streptococci, express the Antigen I/II on the bacterial surface. These adhesins mediate binding to receptors on the surface of the salivary protein GP340 [13]. In addition, mutans streptococci have the capacity to convert sucrose to extracellular polysaccharides, which contribute to the structural integrity of the biofilm [8].

A. oris adhere to the saliva-derived PRPs through type 1-fimbriae and to various *Streptococcus* and other bacterial species through type 2-fimbriae [14]. For some lactobacilli species, streptococci seem to be the binding target in the biofilm [15]. In addition to colonizing the biofilm, the cariogenic bacteria also have to produce high amounts of acid to be acidogenic and to express high acid tolerance (aciduricity), i.e., to be metabolic active in the acidic environment the bacteria create as the result of their own metabolism. However, fulfilling of these criteria is not enough for these bacteria to be considered cariogenic. To cause harm to the teeth, they also have to be present in enhanced proportions/amounts in the biofilm. This concept is called the ecological plaque hypothesis. Under some conditions, the metabolism of the biofilm bacteria is changed, which may result in an "ecological catastrophe" within the biofilm [8]. This phenomenon is called the "the sugar killing" and means that some of the bacteria in the biofilm could die when sucrose is present in high concentrations [16].

Actually the bacterial killing is due to the consequence of substantial fluctuations of the sugar concentration in the mouth. The mechanism can be described in detail as follows.

Many bacteria in the biofilm use salivary glucose as an energy source. The concentration of glucose is low, 5 – 40 μM, and streptococci use PEP-PTS (phosphophenol-phosphotransferase), a specific transport system, to bring the glucose into the cell, where it is eventually converted into pyruvate. By the activity of various enzymes, pyruvate is subsequently converted to formate, ethanol, and acetate, which are released extracellularly by the activity of the enzyme ATP-ase. During food intake, the sugar concentration may increase more than 1000 fold and the sugar may diffuse into the cell through specific permeases [16]. To avoid killing by a high concentration of the intracellular metabolic degradation products, two defence mechanisms are activated. First, some of these products are converted to intracellular polysacharides (IPS). Second, the enzyme lactate dehydrogenase (LDH) is activated, which more efficiently drains the cells from pyruvate than the other enzymes. As for the other enzymatic products, the LDH product lactic acid depends on ATP activity for extracellular release. Due to the release of metabolic end products, the pH of the extracellular environment decreases. However, while the ATP-ase activity of non-cariogenic bacteria is hampered by a low pH, corresponding activity of mutans streptococci and other cariogenic bacterial species remain high in a low pH. For the biofilm, the "sugar killing concept" means that bacteria's lacking the capacity to withstand the harmful effect of high amounts of sugar may die and cariogenic bacteria will increase in proportion/amounts. Increase from 0.001% of *S. mutans* in initial lesions to 45% in advanced lesions has been reported by Schupbach et al. [17]. The harmful effect of a cariogenic biofilm exposed to sugar is convincingly illustrated by the Stephan curve [18].

Prevention of caries

An erupting tooth is initially healthy but, depending on oral ecology, it may be at risk of developing caries. To prevent, reverse, or slow caries lesions, several factors have to be considered. The main factors are diet, oral hygiene, and fluoride use [19]. Another factor to prevent caries is fissure sealing on erupting molars. In children with high caries risk, the molars should be sealed as soon as possible [20]. These factors should not be looked on separately, but as highly interactive [19]. Fluoride has played and plays an important role in caries prevention and stimulates "self healing" of non-cavitated lesions by reducing the de-mineralisation process and promoting

the re-mineralisation process. The self care delivery of fluoride may be delivered by toothpastes, mouth rinses, or gels and the frequency of use is important [21]. Professional methods, such as applications of varnishes containing high concentration of fluoride, are the most common complementary treatment for caries-active patients in Sweden today. One meta-analysis of the fluoride varnish Duraphat® revealed an overall reduction of caries [22]. A systematic review concluded insufficient evidence concerning optimal application intervals [21].

Antibacterial substances are and have been used to reduce the level of cariogenic oral microorganisms. Chlorhexidine, distributed with different vehicles, is a well-established treatment regime for reducing the level of oral microorganisms [23] and may inhibit fissure caries development in children with low exposure to fluoride. In the elderly and in fluoride-exposed children, however, the evidence for an anti-caries effect of chlorhexidine has been inconclusive [21, 24]. One recently proposed caries preventive method is ozone therapy, which may help modify the biofilm and/or infected dentin to reduce the cariogenic challenge [25].

Ozone

Ozone, or trioxygen, is an unstable gas comprising three oxygen atoms [26]. Ozone, naturally present in the air, is a gas at room temperature. In the outer atmosphere, it exists as the ozone layer and is formed by the action of ultra-violet light and thereby has the capacity to absorb the ultraviolet rays present in the light spectrum from the sun. Ozone filters the light spectrum high up in the atmosphere and protects all life from the ultraviolet rays. Ozone also exists in small amounts on earth [26]. During the last decades, higher levels of ozone have been assessed on earth. Long-term exposures to increased amounts of ozone have shown negative effects on human health; these high levels are especially damaging to the pulmonary tract [27, 28] and it has been shown that asthmatic children are particularly vulnerable [29].

Ozone concentration is expressed as parts per billion (ppb) or $\mu g/m^3$. The factor is approximately two, which means 1ppb O_3 = 2 O_3 $\mu g/m^3$ and normally the levels of ozone in air are about 10 ppb. The World Health Organization (WHO), in published guidelines concerning air quality [30], recommends that people, especially the vulnerable, should limit their repeated daily exposure levels of ozone, not just limiting their exposure to occasional peak levels. The threshold level is expressed as mean during time

of exposure. The occupational exposure limit value is 100 µg/m^3, 8 h mean [30].

In 1840, Christian Friedrich Schönbein discovered ozone when he passed an electrical discharge through water during which a strange smell was produced. Schönbein named it after the Greek verb *ozein* – "to smell" [31]. The production of ozone was achieved by a bond cleavage induced by the energy of the oxygen molecule when activated.

The antimicrobial effect of the ozone molecule is attributed to its oxidative properties and exerts antimicrobial effect both as a gas and as a liquid. Under normal conditions, ozone is a gas. However, dissolved in water, ozone can be more safely handled and is 10 times more soluble than oxygen. On the other hand, under these conditions ozone is short-lived due to degradation of the ozone molecule into oxygen [26].

Although it is well known that ozone has been shown to be a powerful antimicrobial, the killing mechanism is not fully understood [32-35]. However, some studies report that ozone induces cell wall disintegration and cell lysis resulting in inhibition of cellular activities [36, 37]. Furthermore, Gram-negative bacteria are more sensitive to ozone than Gram-positive bacteria [38]. This difference is possibly due to difference in cell wall thickness between these two types of bacteria. In addition, the cell membranes are damaged by ozone resulting in enhanced permeability to the ozone molecule and a subsequent oxidation of proteins and DNA [37]. Treatment with ozonated water results in pores and distortion in the membrane of *S. mutans* and complete membrane destruction can be seen after 120 s [39]. However, no structural changes of the cell membrane has been seen in any bacteria after ozone treatment for 60 s, irrespective of species [40].

Application of ozone in dentistry

By the 1930s, the use of ozone in dentistry was investigated by the Swiss dentist Edward Fisch who used ozone to treat infected wounds and chronic periodontal infections [41]. During the last decade, ozone has been evaluated in dentistry, mainly in vitro. Ozone has been used to disinfect equipment and dentures [42-48] and as an alternative treatment regime for different oral diseases. Ozone has been delivered as a gas, in water, in oils, or in gels depending on application areas and concentration requirements. For example, in addition to caries treatment, ozone has been used to disinfect root canals [49-58], as an antibacterial treatment for gingivitis,

periodontitis, and dental implants [59-67], and to reduce dentin hypersensitivity [68-70]. In addition, it has also been proposed for tooth bleaching [71-73]. Although promising results in the laboratory have been shown, there are conflicting results and insufficient evidence [74] in clinical practice with respect to the efficacy of using ozone for all these conditions.
Although some researchers have speculated that ozone could modify some physical properties on the tooth surface, no such changes or effects have been proven for enamel tissue or for dentin lesions [75, 76]. In restorative treatment, ozone has been proposed to disinfect the cavity before placing a restoration, and its influence on bond strength has been evaluated [72, 77-87]. No negative effect on the bond strength of resin composites in adhesive dentistry has been reported.

Ozone treatment of caries lesions

The ozone generator HealOzone™ 2130C (KaVo Biberach, Germany) (Figure 1) treats caries lesions in enamel and dentin.

Photo by Elisabeth Johansson
Figure 1. HealOzone™ 2130C

The device delivers ozone gas at a concentration of 2,100 ppm±10%. The hand piece is connected to the main unit by a hose and a single-use removable silicone cup is used, which is placed over the area of the lesion to be treated. The diameter of silicon cups, ranging between 3 and 8 mm, is chosen according to the size of the lesion to be treated. When the seal around the lesion is intact by the silicone cup, air is drawn through the hose, which in turn switches on the ozone production.
After the delivery of the ozone, a re-suction period is started, which, as claimed by the manufacturer, removes any remaining ozone during the following 10 s [31, 88]. As a complement to the ozone treatment, and applied immediately after treatment, an application of a solution containing fluoride, calcium, phosphate, zinc, and xylitol is recommended. Thereafter, the

patients should use a patient kit – toothpaste, mouth rinse, and spray with the same content as the topical solution used directly on the lesion after the ozone application. The manufacturer recommends patients to use this kit at least for the first four weeks after treatment [31].

Ozone treatment of caries-associated bacteria

The antibacterial effect on caries-associated bacteria have been evaluated using ozonated water in vitro [39, 89, 90] and in situ [91] as well as after using ozone gas in vitro and in vivo [40, 92-97]. The antibacterial effect depends on delivered ozone concentration, time, and the environment of the bacteria (e.g., in biofilm or dentin). Using ozonated water in primary caries root lesions [89], dental plaque [39, 90], and using ozone gas in primary root caries [92] have all shown to reduce significantly the amount of bacteria. However, some studies have reported conflicting results of the ozone effect on bacteria in a cariogenic biofilm [95] and in infected dentin [94].

One in vitro study [98] also presented the inability of *S. mutans* and lactobacillus to form a biofilm on dentin pre-treated with ozone. Furthermore, in a study comparing the effect of chlorhexidine with ozone, none of the treatment regimes produced significant immediate antibacterial effect in a superficial decayed layer [96].

Clinical studies of ozone treatment on caries

Only a few published longitudinal studies have investigated the effect of ozone treatment of early caries lesions. As mentioned earlier, the device delivers ozone at a fixed concentration for a specified time; these parameters can be changed so as to alter ozone concentrations and time of exposure. Ozone treatment times, follow-up periods, and intervals between the visits vary in different studies, an inconsistency that limits the ability to do comparisons. Two studies on root caries lesions, with follow-up times of six [99] and 18 months [100], respectively, showed arrested root lesions treated with ozone.

Three published studies have presented results of the effect on occlusal caries in children and adolescents with follow-up times of three [101], six [102], and eight months [103]. All three concluded that treatment with ozone reversed caries or reduced caries progression. One study has evaluted the caries protective effect on white spot formation in high risk patients during multi-bracket therapy [104]. This study could not show that ozone had any preventive effect on the development of white spot lesions. Furthermore, a Cochrane systematic review concluded in 2004 [105] that there was no

reliable evidence that the application of ozone gas to the surface of decayed teeth stops or reverses the decay process.

Detection of caries

Typically, caries lesions are detected by visual inspection, which might be supported by bitewing radiographs and tactile examination using a probe/dental explorer. When probing incipient lesions, it is important to avoid breaking the fragile surface layer. The appearance of a suspected lesion in enamel is judged by the colour (white or brown) lesion reflectance (shiny or dull) and surface texture (rough or smooth); these characteristics are used to decide whether the lesion is active or inactive [106].

One way to detect and classify caries (without using a probe) progression is to use the Ekstrand criteria [107] (Table 1). The criteria describe both non-cavitated (score 1-2a) and cavitated lesions (score 3-4). A modification of these criteria subdivides the non-cavitated lesions by colour and whether the lesion is visible on a wet or dry surface (score 1 and 2).

Table 1. Criteria visual inspection (VI), according to Ekstrand et al., [107].

0	No or slight change in enamel translucency after prolonged air drying (>5 s)
1	Opacity (white) hardly visible on the wet surface, but distinctly visible after air drying
1a	Opacity (brown) hardly visible on the wet surface, but distinctly visible after air drying
2	Opacity (white) distinctly visible without air drying
2a	Opacity (brown) distinctly visible without air drying
3	Localised enamel breakdown in opaque or discoloured enamel and/or greyish discolouration from the underlying dentine
4	Cavitation in opaque or discoloured enamel exposing the dentine beneath

Another detection method is the International Caries Detection and Assessment System (ICDAS), which in 2003 was devised with the goal of designing an internationally accepted caries detection system that allow assessment of caries activity [108]. In the ICDAS I, there are seven steps as in Ekstrand and the visual examination is carried out on plaque free teeth after careful drying. Later, the criteria were modified and the ICDAS II was created. One of the changes consisted in a change of codes to ensure that the system would reflect increased severity [109].

Laser-induced fluorescence

It has become increasingly difficult to detect lesions before loss of substance, especially at early stages. The shortcomings of conventional caries detection and the need for supplementary methods have been acknowledged. Over the last two decades, two optical methods have been developed to complement visual examination, bitewing radiographs and tactile examination. Optical caries detection methods are based on observations of the interaction of energy, which is applied to the tooth, or observation of energy, which is emitted from the tooth [110]. One of these optical methods is laser-induced fluorescence (LF). Using red light (wave length of 655 nm) one can differentiate between sound tissue and carious lesions. In 1998, the use of red light (wave length of 655 nm) to differentiate between sound tissue and carious lesions was described and on this basis the device DIAGNOdent 2095™ (KaVo Biberach Germany, Figure 2) was developed [111, 112].

The LF device has been evaluated for longitudinal monitoring of caries preventive programs for quantification of changes in caries lesions [113-115] and has been suggested to be useful in both permanent and primary molars [116]. A recent review concluded that LF could be useful as an adjunct to visual-tactile and radiographic examinations, especially on occlusal surfaces in permanent and primary molars, but the evidence for this was limited [117]. The role of the LF device in detection of root caries lesions has not been extensively investigated and only two validity studies have been published [118, 119]. The LF device contains of laser diode detection fibres, digitally displayed on a screen from 0 to 99. Zero is sound enamel and 99 deep dentinal caries [112].

Photo by Lena Karlsson

Figure 2. The DIAGNOdent 2095™

Aims

There are several unanswered questions regarding new caries preventive methods such as ozone treatment as well as novel caries detection methods that are used to monitor and evaluate these preventive methods. This thesis seeks answers to some of these questions.

The specific aims of the papers included in the dissertation are listed below:

I. To investigate the in vitro sealing capacity of a novel ozone delivery system and its re-suction capacity during accidental displacement of the cup at different stages of the ozone delivery.

II. To study the in vitro antibacterial effect of ozone on cariogenic bacterial species with and without the presence of saliva and its possible effect on the saliva proteins.

III. To assess the in vitro validity of LF measurements, visual inspection (colour), and tactile examination (surface texture) on root caries lesions and to correlate these with histopathological measurements of lesion depths.

To investigate whether LF readings agree with inter-device, inter-examiner, and intra-examiner levels and whether lesion colour and surface texture influences LF readings.

IV. To evaluate the in vivo effect of ozone and fluoride varnish treatments on occlusal caries in primary molars during one year, monitored with visual inspection and with laser-induced fluorescence.

Materials and methods

This section describes the material and methods. An overview of the included studies is shown in Table 2. Further descriptions are presented in each paper.

Ethical approval and considerations

Two of the studies, III and IV, were approved by ethics committees. Study III was approved by the ethics committee at Huddinge University Hospital Huddinge, Sweden (2006/380-31/3) and study IV received ethical approval from the Regional Ethical Review Board, Umeå University, Sweden (§563/03, dnr 03-486 and dnr 07-189M).

In study IV, informed consent from the participating children's parents was obtained before the start of the clinical study. The parents were informed that participation was voluntary and at any time they could stop participating.

Table 2. Overview of the included studies in the thesis.

In vitro	Paper I, Evaluation of ozone air levels adjacent to a ozone device HealOzone™ 2130C
	Paper II, Evaluation of cariogenic microorganisms treated with ozone HealOzone™ 2130C
	Paper III, Detection of root caries lesions using the LF device DIAGNOdent™2095
In vivo	Paper IV, Evaluation the effect of ozone and fluoride treatment on occlusal caries in primary molars HealOzone™ 2130C DIAGNOdent™2095

Paper I. Evaluation of ozone air levels adjacent to an ozone device

Table 3. Flow diagram, paper I.

Flat metal surface*	Extracted molars** - buccal surface - occlusal surface	Extracted premolars** - buccal surface - occlusal surface

10 s ozone delivery cycles. No displacements. *
10 s ozone delivery cycles. Displacement before start of the re-suction period.*
10 s ozone delivery cycles. Displacement after 5 s during ozone delivery.*
20 s ozone delivery cycles. Displacement after re-suction period. **
60 s ozone delivery cycles. Displacements of the cup every 5 s during ozone delivery. **

At the beginning of study I, background ozone levels were measured once an hour for five days using a photometric ozone analyser, an instrument that measures ozone levels in the air. Before the ozone air levels were measured with the HealOzone™ 2130C (Kavo Biberach, Biberach, Germany), the background levels in the room were measured; thereafter, the levels were measured every ten minutes. To evaluate the device's sealing capacity, full ozone application cycles, including the re-suction period and interrupted cycles with displacement of the cup during the delivery cycle, were studied. In this context, displacement means the silicon cup was lifted above a surface before setting back in the same place within different time intervals.

Flat metal surfaces:
Preset delivery time was for 10 seconds of ozone delivery (10 seconds ozone–10 seconds of re-suction) and five cup sizes were used: 3-, 4-, 5-, 6-, and 8-mm diameter. Each cup was used continuously over the course of 30 ozone delivery cycles. Differences between the cup sizes were analysed before the start of the following evaluations with the metal surface. In this first part of the flat metal surface experiment described above, the 8 mm sized cups showed the highest leakage values and were thereafter used in the following evaluations on the flat metal surface:
- Displacements were performed directly after ozone delivery, but before the re-suction time.
- The cup was displaced after five seconds during the delivery of ozone.

The tooth surfaces:
Four molars and four premolars recently extracted for orthodontic reasons were used. They were placed in plaster and were thoroughly cleaned before the experiments and suitable silicon cups for all occlusal and buccal surfaces were used.
-Preset delivery time was 20 seconds of ozone delivery (20 seconds ozone - 10 seconds of re-suction). Displacement of the cup was performed after a full cycle (after the re-suction). The measurements were repeated one hundred times for each surface. Background ozone air levels were measured at the start and 10 minutes after the finish of each 10th application.
-Preset delivery time was 60 seconds of ozone delivery (60 seconds ozone - 10 seconds of re-suction). Displacements of the silicon cups were performed during the 60-second ozone delivery cycles. The cup was displaced every five seconds during the cycle, and ozone concentrations were measured during the following five seconds. One hundred measurements were taken for each tooth and surface. Background ozone air levels were measured at the start and 10 minutes after the finish of each 10th application.

Paper II. Evaluation of cariogenic microorganisms treated with ozone

Table 4. Flow diagram paper II.

Lactobacilli casei** (NCTC 151)	Streptococcus mutans** (NCTC 10449)	Actinomyces naeslundii* (ACTCC 12104^T)
Exposed to ozone 10, 30, and 60 seconds in salt buffer and saliva** and in salt buffer only*		

The study included the bacterial strains *Lactobacillus casei* (NCTC 151), *Streptococcus mutans* (NCTC 10449), and *Actinomyces naeslundii* (ACTCC 12104^T). Three different cultivation media were used to estimate the number of the bacterial strains. Mitis salivarius agar supplemented with bacitracin and potassium tellurite (MSB)[120] were used to estimate the number of mutans streptococci. Rogosa selective lactobacilli agar (RSL) (Merck, Darmstadt, Germany) was used to estimate lactobacilli, and blood agar plates [121] were used to estimate the number of *A. naeslundii*. Aliquots

(concentrations adjusted to 10^{10}/ml) of 10 µl of the suspensions with the strains *L. casei* and *S. mutans* strain were added to a 990-µl salt buffer and saliva in tubes. Suspension of the *A. naeslundii* was added only to the salt buffer. The level of lactobacilli or mutans streptococci in the saliva (from one donor) before addition of prepared bacterial suspensions was below 1 x 10^4/ml, so these concentrations did not affect the results. The baseline value – i.e., cells of *S. mutans*, *L. casei* and *A. naeslundii* not exposed to ozone – contained the following numbers of living bacteria: 1.0 x 10^8 (SD 7.0 x10^5), 1.0 x 10^8 (SD 3.1 x 10^6), and 8.0 x 10^7 (SD 2.2x 10^7), respectively. The bacteria in the solutions were exposed to ozone that was delivered via a tube connected to the ozone generator. Equal samples were taken from the suspensions before and after the bacterial strains had been exposed to ozone gas for the time intervals 10, 30, and 60 seconds. Each bacterial species was tested nine times at all time intervals. Aliquots of the samples were spread on respective agar plates and thereafter incubated in 5% CO_2 and 5% air at 37°C for two days. The numbers of bacterial colonies (CFU) on the plates were counted and the killing rate for each bacterial species was calculated.
To evaluate a possible effect of ozone on saliva proteins, the same procedure as described above, but in the absence of bacteria, was performed. After the exposure with ozone, the saliva was mixed with an equal volume of a sample buffer and boiled for five minutes. Unexposed saliva was used as a control. The proteins in the samples were separated and the proteins in the gel were visualized by silver staining.

Paper III. Detection of root caries lesions using the LF device

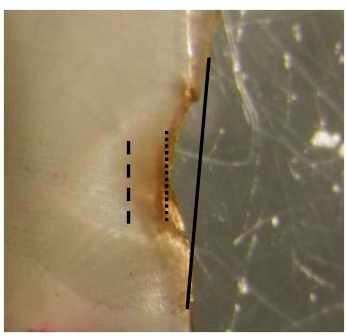
The study included 93 extracted teeth, nine with visually intact root surfaces and 84 with various stages of root surface caries lesions. The teeth were photographed to facilitate repositions at the test site, a reference that was also used for the subsequent tooth slice for histopathological analysis. Four calibrated operators assessed lesion colour and surface texture according to Table 5.

Table 5.Visual (a) and tactile criteria (b) for detecting toot caries lesions

a. Discolouration	b. Surface texture
Intact	Intact
Yellowish	Soft
Yellowish brown	Leathery
Brownish black	Hard

Thereafter, the operators performed measurements once a week with two LF devices (DIAGNOdent 2095™) with the flat tip in three separate series. The reliability was calculated with four operators, inter-operator agreement, and inter-device agreement. Intra-operator agreement was also calculated.
Each tooth was embedded in methyl-methacrylate and sectioned into 300 µm thick slices using a water-cooled diamond saw [122]. Due to saw machine failure, some slices could not be retrieved; 64 out of 93 teeth were obtained and examined in a light microscope at x16 magnification. Lesion depth was assessed with two references: from the delineated borderline of the original exposed root surface (Ref I), or, if loss of surface continuity, from the absolute lesion depth (Ref II) (Figure 3).

The validation



Reference I ——
Reference II ·····
Histopathological depth– – – –

Photo by Lena Karlsson

Figure 3. Lesion depth

Paper IV. Evaluation the effect of ozone and fluoride treatment on occlusal caries in primary molars

Table 6. Flow diagram paper IV.

Baseline	3, 6, and 9 months	12 months
2 operators VI and LF Ozone Fluoride	1 operator VI and LF Ozone Fluoride	1 operator VI and LF

A) children with a mean age of 4.8 years (range 3-6)
B) children with a mean age of 4.5 years (range 4-8).

This split mouth study was conducted in two parts: The inclusion criteria in first part (A) were one or two bilateral matched pairs of occlusal lesions in the first and/or second primary molar with a maximum score of three according to Ekstrand et al. [107] (Table 1, page 15). In part B, criteria were one or two bilateral matched pairs with only non-cavitated lesions, i.e., lesions with a maximum score of 2a. Exclusion criteria were health problems such as severe asthma, failure to cooperate, and teeth with obvious signs of enamel hypo-mineralisation or hypoplasia in the fissure.

The calculations for sample size were based on caries prevalence for four year olds in the region that was 46% in 2002 and 38% in 2007, including non-cavitated and cavitated lesions. Sample collection was performed between December 2003 (part A) and May 2008 (part B). To detect a significant difference between the two treatments with a two-sided significance level of 0.05 and a power of 80%, it was estimated that 40 pairs were needed. Primary results of part A showed high failure rates and the sample collection was discontinued after 15 pairs. At the end of the sample collection period of part B, 35 pairs fulfilled the requirements.

At baseline, anamnestic data were noted. At the regular check-up, the caries risk was assessed by the general dentist at the Public Dental Health Clinics on a three grade scale (low, medium, or high caries risk). At baseline, visual inspection (VI) was performed, the lesions were classified and described by Ekstrand et al. [107] (Table 1 page 15) and LF recordings were performed. The LF recordings were carried out with the same LF device throughout the study. The selected measurement points in the lesions, including the sound reference point, were noted graphically in a protocol so during the study assessments could be repeated.

The lesions were randomly assigned to ozone (test) or topical fluoride (positive control) by throwing a die. If two pairs were present, the second lesion treated with ozone was always on the same side as the first. The treatments were either 40 seconds of ozone or topical application of a fluoride varnish (Duraphat® 22, 6 mgF/ml).

The assessments and treatments were performed at baseline and at 3, 6, and 9 months. At the 12-month follow-up, only VI and LF were performed.

Ekstrand index score 4 was defined as failure and necessity of restorative treatment of the lesion.

Statistical analyses:

Paper I: Descriptive statistics were used to describe levels of ozone in air for each experimental period. Differences in leakage between the cup sizes on the metal surfaces were tested using the Kruskal-Wallis test and differences between normal and disrupted delivery cycles with the Wilcoxon Signed Rank test.

Paper II: Differences in relative killing of the bacteria within each of the species and between the three ozone treatment times were tested with the Wilcoxon Signed Rank test. Differences in bacteria values between the species at each treatment time and between the solution buffer and saliva were tested using the Exact test.

Paper III: The correlation between LF measurements and the lesion depth, the correlation between lesion colour and surface texture with LF readings, and the correlation for ref I and ref II with colour and surface structure were analysed using Spearman's Rank Correlation coefficient (ρ). The same test was also applied to analyse the correlations between lesion colour and surface texture on the LF readings, as well as for ref I and ref II depths and correlation with colour and surface texture. Distribution of LF readings and lesion depth by colour and surface texture were described using descriptive statistics. The number of measurements per tooth was 48: four examiners x three weeks x two devices x two repeats. Mean values for LF readings per tooth were considered. Repeated-measurement ANOVAs were used to estimate sum of squares to calculate the intra-class correlation coefficient (ICC) and inter-device, inter-examiner, and intra-examiner agreement.

Paper IV: Descriptive statistics were used to describe the study groups concerning background data, VI, and LF values. Inter-operator agreement was assessed from values at the baseline visit using un-weighted Cohen Kappa (VI) and Spearman´s Rank Order Correlation tests (LF). A Bland Altman plot was used to identify systemic differences of LF values. Wilcoxon Signed Rank test was used to analyse differences between the study groups at baseline and, in part B, changes in LF values from baseline to 12 months.

In all the studies, the level of statistical significance was set at $p < 0.05$.

Main results

This section presents an overview of the main results. The results are briefly presented and detailed descriptions are presented in each paper.

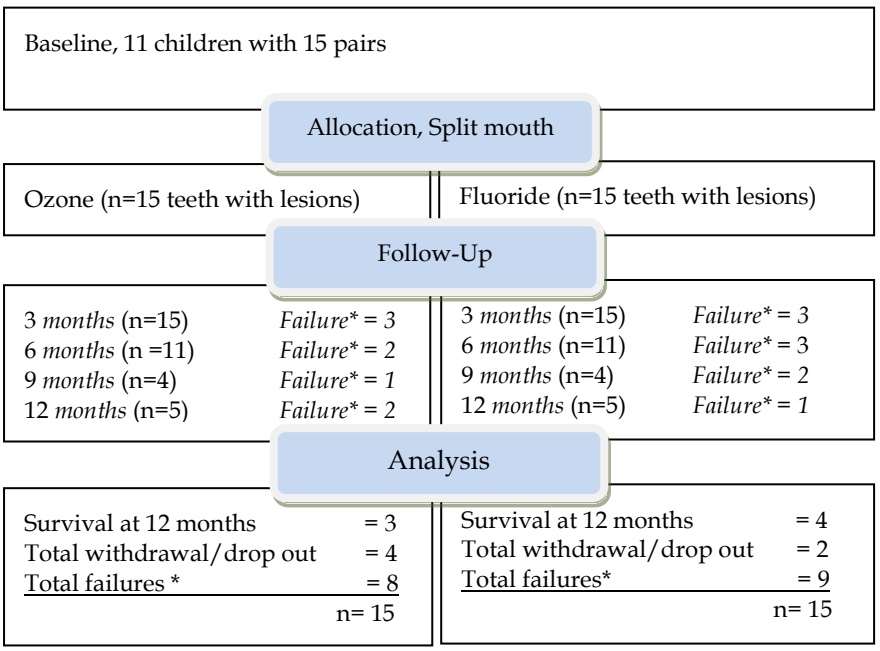
Paper I
Ozone air values varied for the flat metal surface between 8.0 and 166.0 µg/m^3 and for the tooth surfaces between 0.0 and 108.0 µg/m^3. Ozone leakage levels were 7.6 µg/m^3 for the flat and 7.4 µg/m^3 and 5.6 µg/m^3 for the buccal and occlusal surfaces, respectively, and 5.2 µg/m^3 and 9.8 µg/m^3 for the premolar and molar surfaces, respectively. Cycles with displacement showed leakage levels significantly higher than continuous complete cycles. All levels were well below the WHO occupational exposure limit value for ozone.

Paper II
In the salt buffer 92%, 73%, and 64% of the initial numbers of *A. naeslundii*, *S. mutans*, and *L. casei*, respectively, were killed after 10 s of ozone exposure, and approximately 99.9% of the bacteria were dead after a 60 s exposure. After 10 and 30 s, but not after 60 s exposure to ozone, *S. mutans* and *L. casei* were less efficiently killed in saliva compared to in the salt buffer. Various saliva proteins were degraded by ozone after a 60 s exposure.

Paper III
The correlation between LF readings and histological depth was low with values ranging from 0.22 ($p > 0.05$) to 0.31 ($p < 0.05$). The devices LF 1 and LF 2 were significantly correlated with discolouration ($\rho = 0.52$ and 0.46, respectively) and with surface texture classified as hard ($\rho = 0.34$ and 0.33, respectively). A significant correlation was found between colour and histological depth ref. I ($\rho = 0.51$) and ref. II ($\rho = 0.56$). No significant correlation was found between surface texture and histological depth. The reliability, evaluated as intra-class correlation coefficient, was 0.99 for intra-examiner, 0.97 for inter-examiner, and 0.98 for inter-device. Large differences were found between two consecutive measurements and high measurement error indicated considerable deviation of individual measurement.

Paper IV

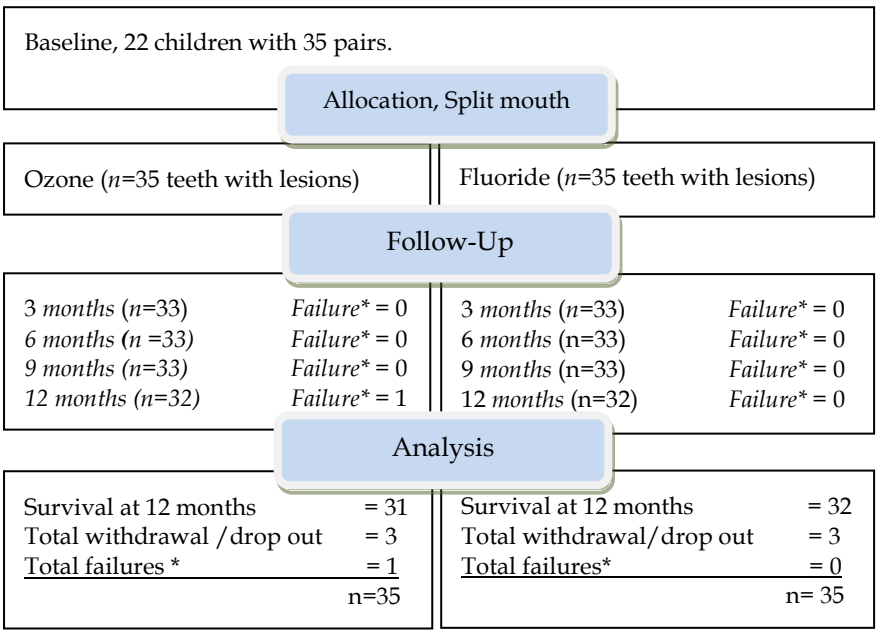
Table 7. Part A



**Failure* = necessity of restorative treatment

For the 15 pairs at baseline, before sample collection was discontinued because of non-acceptable clinical results, Ekstrand score of 3 was recorded on six lesions treated with ozone and five treated with fluoride. The other lesions scored from 1 to 2a. The median (min-max) LF values were 76 (0-99) and 69 (0-99) for ozone and fluoride lesions, respectively. The baseline values of both VI and LF did not differ significantly. At nine months, three pairs were not evaluated, and in total six lesions were not used as the other tooth in the pair failed. Eight lesions treated with ozone and nine treated with fluoride progressed to failure, i.e., a VI score of 4. All of the lesions with VI score of 3 at baseline, both in ozone and fluoride groups, failed during the study time. Two children had medium caries risk and nine children had high caries risk. In the high risk children only one lesion pair remained during the study time. Since only five pairs remained to be evaluated at the 12-month follow-up, no further statistical analyses were performed in part A.

Table 8. Part B



**Failure*=necessity of restorative treatment.

At baseline, none of the 35 pairs showed VI scores higher than Ekstrand index score 2a. The median (min-max) LF values were 21 (2-66) and 19 (2-69) for lesions treated with ozone and fluoride, respectively. The baseline values of VI and LF did not differ significantly.

At three months, two pairs dropped out due to relocation and at nine months one withdrawal due to repair of the proximal surface of one tooth. After 12 months, a small shift towards increased VI scores was recorded both for lesions treated with ozone and fluoride. One of the lesions treated with ozone failed at 12 months, reaching a VI score of 4 and necessitating an operative treatment. No other lesions in part B failed during the study time. At 12 months, median (min-max) LF values were 15 (4-99) and 17.5 (2-85), respectively. No significant improvement or difference in LF values was found over time between the groups. Sixteen children were assessed as having low caries risk and six as medium. The failed lesion was found in a child with low caries risk.

Discussion

During the early stages of dental caries, the process is reversible and avoiding tissue loss is possible. The major difference between non-invasive and invasive treatment is the long-term benefit of an un-restored tooth. There is an increased request for methods for both detection and monitoring of caries lesions and for successive preventive treatments of early caries lesions. Methods intended for clinical practice should be as least as effective as current methods, should meet safety regulations, should be cost-effective, and should be accepted by the patients. This thesis aims to evaluate the safety, the validity of a detection method, and the effect of a new preventive method. It includes three in vitro studies (papers I, II, and III) and one in vivo study (paper IV). The aims were to evaluate the safety of ozone treatment regarding ozone air levels adjacent to the ozone delivery device (paper I), its effect on cariogenic microorganisms in saliva or buffer (paper II), LF´s ability to detect root surface lesions (paper III), and the effect of ozone treatment on occlusal caries in primary teeth (paper IV).

Therapeutic use of ozone must be coupled with awareness of possible risk for the users and the patients. In paper I, we evaluated the HealOzone™ device´s ability to seal in vitro and used an ozone air analyser to measure ozone air levels. The results showed that cycles with displacement had significantly higher leakage levels than continuous complete cycles. Some moderate peak values were registered, but these were randomly dispersed among the readings. However, detectable ozone levels adjacent to the silicon cups never exceeded the WHO Guideline threshold value in any situation. Contact between the silicon cup and the teeth can easily be disturbed, caused accidently by the patient or the operator. Directly after loosening of the seal, the production of ozone gas is supposed to stop as a result of the negative pressure. During the evaluation of ozone levels at the laboratory, the series were performed in far longer sessions than would occur during treatment in the clinic. We investigated different cup sizes on flat metal surfaces using different application times with full contact or accidental loss of contact. These results were compared with Millar and Hodson [88] who also investigated the safety of the HealOzone™ but only concerning ozone air levels with completed delivery cycles. The manufacturer recommends regular service of the device. However, in the clinic, it would be desirable to

have an easy test available to control ozone concentrations between the annual services to enhance safety and control of the ozone concentration delivered at treatment.

Paper II showed S. *mutans*' sensitivity to the ozone exposure. In addition, the cariogenic species *L. casei* and *A. naeslundii* were efficiently killed by ozone. Based on the hampered bacterial killing in the presence of saliva and the observation that saliva proteins were alternated, probably degraded by ozone, it can be concluded that both bacteria and proteins are targets for the ozone molecules. Since the bacterial killing was just delayed, it was apparent that the targets in the saliva were readily saturated. The initial number of *S. mutans* was 100 million/ml. After approximately 10 mmol of ozone had been dispersed in the bacterial suspension for a 10 s period, approximately 75% of the initial number had been killed. In this situation, ozone had a powerful antibacterial effect on *S. mutans*. However, the bacteria were planktonic and in a more vulnerable environment than in a biofilm. This indicates that the present findings when applied to an in vivo situation should be interpreted with caution.
The antibacterial effect depends on delivered ozone concentration (as a gas or in water), delivery time, amounts of bacteria, and the environment of the bacteria, for example, biofilm or dentin tissue. After ozone delivery, it can be assumed that a caries lesion immediately after treatment will be covered by new salivary proteins. This will be followed by a subsequent re-establishment of the biofilm. Polydouro et al. [123], using a tooth cavity model, found a significant reduction of *S. mutans*. The antibacterial effect was seen after four and eight weeks, but a total elimination of the microorganisms was not seen and they concluded that using only ozone treatment was probably insufficient.

In paper III, we found a low correlation between the LF readings and the histopathological depth of root caries lesions. The LF readings generate a numerical value on the device´s display giving an indication of de- and remineralisation in enamel and dentin. Clinically, root caries lesions on accessible surfaces are often easy to detect by visual examination. However, an indication of the depth of the lesion would support the clinician in choosing the appropriate treatment, invasive or non-invasive. Such treatment decisions should be based on a valid, reliable diagnosis. The results in paper III did not demonstrate that the LF method can provide this

information. Our study applied essentially the same methods as Wicht et al. [118] and has overall recorded similar results. The LF readings obtained in our study, irrespective of device, showed a low correlation with the histological assessments (at best $\rho = 0.31$). With respect to validation of the LF method for root caries detection, the results were more discouraging than reported for occlusal caries.
However, an interesting finding from paper III was the test-retest exercises, which showed that repeated measurements are relatively stable with respect to lesion, instrument, and operator at group, but agreement at individual level was poor, with pronounced scattering of measurements. One potential weakness of the sample in paper III is that most of the sections (76%) had a histological lesion depth of 0–2 mm. A sample with a more even distribution of lesion depths would have been preferable. Furthermore, almost one-third of the original specimens were irreparably damaged during sectioning. This is probably caused because root tissue has a high organic and low mineral content and tends to dehydrate or absorb water readily, which makes it vulnerable to damage during sectioning.

In paper IV, we investigated the ability of ozone gas to arrest occlusal caries lesions in primary molars in a split mouth study design with a conventional preventive treatment regime as a positive control. The study was conducted in two parts, A and B. The lesions to be treated were cleaned before the application of ozone to treat the lesion without the covering biofilm. In part A, lesions with localized enamel breakdown were included; in part B, only non-cavitated lesions were included. Neither ozone nor Duraphat® treatments could prevent progression of the cavitated lesions in part A. In part B, slightly increased VI scores were seen from baseline to the 12-month follow-up, i.e., low detectable progression of the lesions. Only one lesion required operative treatment.

The split mouth study design has both benefits and shortcomings. This design minimises the subject effect, for instance, differences in eating habits, salivary factors, oral hygiene (including use of fluoridated products such as toothpaste), and possible changes in these factors during the whole study period. However, the recruitment of patients to a split mouth study can be hampered because of the need of symmetrical disease patterns, and a restricted recruitment might limit the external validity of the results. When

assessing and interpreting the outcome, it is important to be aware of the difference between efficacy and effectiveness. Efficacy is used to describe how well a treatment performs under specific circumstances, is used as measurement in split mouth-studies, and makes it possible to decide if one treatment is more effective than another. The term effectiveness describes how well a treatment works under ordinary circumstances in general practice [124, 125].

Biased treatment efficacy is one of the risks with this study design and might occur when a local treatment also has a systematic effect, i.e., the active substance of an intervention may carry over to the other side of the mouth. To minimise this potential risk, only two types of treatments were evaluated in paper IV and only bilateral lesions in the fissures of the primary molars. Hence the lesions were as far from each other as possible. To avoid any issues associated with carry across effects, the fluoride varnish could have been applied on both sides, measuring additional effect of ozone treatment on one side. Including a negative control would mean not treating one lesion; this arrangement is not possible because it would be unethical to leave a lesion untreated.

In paper IV, we used Ekstrand's modified criteria [107]. A score of 4 was chosen as an outcome variable, i.e., failure and requiring operative treatment. As mentioned earlier, the ICDAS II criteria is used for detection of caries lesions. It has been slightly modified after Ekstrand et al. [107] and includes tactile feeling [106]. The ICDAS II criteria might have been preferable, but the criteria were not available at the start of our study.
To solely use visual inspection to measure small changes of caries regression or progression could have been of limited value, especially in non-cavitated lesions. This was the intent of using LF as a complement to detect and indirectly monitor changes, regression, progression, or no change to the lesion. However, observing the results in paper IV, the readings of LF showed large variations both within each visual category at baseline and for the individual lesions at the recall visits.

Treatment with the HealOzone with non-invasive ozone gas in order to reverse or arrest caries lesions was appealing when we started the study. Primary reports implied promising results after relatively short suggested

application times, easy to use, and good acceptance by patients [103]. However, today there are still questions about how to achieve optimal preventive effect of ozone. Which application time should be used, how often should it be applied, what is the duration of the effect, and how deep into the lesion does the ozone penetrate? Three other studies have presented results of the effect on occlusal caries in children and adolescents [101-103]. In paper IV, we treated the cavitated and non-cavitated lesions for 40 seconds every third month for one year. The other studies used 40 seconds of ozone on non-cavitated occlusal caries with three visits and follow-up after three months [101], 20 seconds every second month on excavated open lesions within eight months [103], and 40 seconds on non-cavitated lesions including four visits within six months [102]. The remineralisation solutions recommended by the manufacturer were omitted in our study, as in the studies by Huth et el. [101] and Dähnhardt et al. [103]. Atabek and Oztas [102] evaluated the efficacy of ozone alone or ozone in combination with the remineralisation solution. In the study by Huth et al. [101], after three months the ozone treated lesions in a high risk caries group showed more caries reversal or reduced progression than the control lesions, but longer follow-up times have not been presented. Dähnhardt [103] found significantly increased hardness values in the ozone group than in the controls and the LF values improved but were not statistically significant. In their study, the lesions were restored conventionally after completed observations. Atabek and Oztas [102] presented a 75% improvement in LF values in the group treated with ozone only, while 80% showed improvement of the LF values in the ozone combined with remineralisation solutions group. This was in a study population with a relatively high caries experience in the past.

Why was this temporary local antibacterial application insufficient in reversing or arresting the lesions as described in paper IV? During the evaluation of the results concerning parameters of importance for the outcome, several questions remained unanswered. After we considered the results from the first follow-ups in part A, we questioned whether the lesions had been too deep from the start, especially the cavitated lesions. Therefore, we decided to evaluate only non-cavitated lesions in part B. One possibility to further investigate would have been to prolong the delivery time and thereby increase the amount of ozone. It would have been interesting to follow-up the amount of bacteria in the lesion after treatment.

In addition, in part B, it is possible that a larger study group and/or a longer study period would have shown more distinct results.
The development of caries lesions is a dynamic process. As mentioned earlier, it can be assumed that the caries lesions immediately after treatment will be covered by new salivary proteins. This will be followed by a subsequent re-establishment of the biofilm including a re-colonisation of cariogenic bacteria. Would shortened intervals between the visits have been more effective? However, it can be questioned if this would be a practical and/or cost-effective preventive method.
Today there is no agreement about duration and frequency of ozone application in order to prevent or arrest caries progression, mainly due to lack of clinical studies. The evidence is still insufficient to conclude that ozone is a cost-effective method, a conclusion posited by Brazelli et al. [126].
However, in children with high caries risk, it is rather clear from paper IV that treatment of cavitated lesions with ozone or fluoride varnish as separate method is not sufficient.

In the future, the challenge is both to find new methods for detection of incipient caries and new ways for preventing the onset and progression of the disease. Appropriate detection methods must identify incipient caries lesions, their activity, depth, and extension. A quantitative detection method capable of measuring small changes would allow evaluation of treatment effect after shorter follow-up times, keeping in mind the risk of over-diagnosis. Furthermore, new preventive methods have to be accepted by patients, more effective and cost-effective than those available today.

Conclusions

In summary, using HealOzone™ is safe (paper I) and ozone has been shown to have an antibacterial effect on caries associated species (II). However, ozone gas application every third month was not shown to be an effective preventive method (paper IV). LF failed to be an appropriate detection method for root caries diagnosis (paper III).

The major conclusions of each paper in this thesis are listed below:

Paper I
The ozone delivery system can be considered safe with low leakage levels in air, even with accidental displacements.

Paper II
Like *S. mutans,* the cariogenic species *L. casei* and *A. naeslundii* were sensitive to ozone gas treatment. The presence of saliva hampered the antibacterial effect of ozone.

Paper III
A low correlation between the LF readings and the histopathological depth of root caries lesions was shown. The LF device was found not to be appropriate for application to root caries diagnosis.

Paper IV
Neither ozone nor fluoride varnish treatments arrested the progression of cavitated occlusal caries lesions. In low and medium caries risk children non-cavitated occlusal lesions remained mainly unchanged during the study period. No difference in the effect of ozone and fluoride varnish treatments on occlusal caries in primary molars was seen.

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