HELENA FRANSSON
ON THE REPAIR OF THE DENTINE BARRIER
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ABSTRACT

The overall aim of this thesis was to study some aspects of the repair of the dentine barrier, especially in conjunction with dental pulp capping. Understanding the events leading to the healing of the dentine and pulp, and hence successfully preserving the vitality and functions of the tooth, would lead to a scientific basis for a less invasive treatment of pulp exposures than performing root canal treatments.

The surfaces of the body have physiological barrier functions aimed at protecting the body from external noxious agents. In the tooth, the odontoblasts, which line the outermost part of the pulp and are responsible for the formation of dentine, play a central role in the barrier function and thus in the defence mechanisms of the tooth. The micro-organisms in the caries lesion can reach the pulp via the dentinal tubules. However, the barrier function helps to prevent microbial invasion and thereby avoid deleterious inflammation and subsequent necrosis of the pulp. Dentine repair is an important part of the barrier function. There are however doubts as to whether the repair also leads to restitution of the function and the ability to withstand bacterial influx over the longer term.

Pulp capping is a treatment method used when the pulp has been exposed in order to stimulate healing of the pulp and dentine. The evidence for repair of the dentine after pulp capping in humans has been studied by means of a systematic review. The focus of the literature search was studies performed in humans where hard tissue formation had been studied with the aid of a microscope. We con-
cluded, based on the limited evidence available, that calcium hydroxide based materials but not bonding agents promote formation of a hard tissue bridge. Scientific evidence was lacking as to whether MTA was better than calcium hydroxide based materials in this regard.

A gel (Emdogain® Gel) containing amelogenin, known to be involved in dentinogenesis, was evaluated with regard to formation of hard tissue in a clinical study. A greater amount of hard tissue was formed after application of the gel compared to the control. Characterization of the tissue concluded it to be dentine, based on its content of type 1 collagen and dentine sialoprotein, although it was not formed as a continuous bridge covering the pulp wound.

Beneath a deep caries lesion an important part of the barrier function is the odontoblasts’ response to bacteria with the formation of new dentine. A cell model with odontoblasts was used to study the effects of clinical isolates from a deep carious lesion on their viability and production of type 1 collagen, the major component of the dentine in the early stages of its formation. There were bacteria that negatively affected the viability of the odontoblast-like cells and different bacteria varied in their effects on type 1 collagen production, suggesting that some bacteria may have a direct influence on the odontoblasts’ ability to form dentine.

In summary; Emdogain® Gel initiated dentine formation, though not in a form that could constitute a barrier and there are indications that bacteria may differentially affect the odontoblasts’ ability to repair the dentine barrier.
Det övergripande målet för avhandlingen har varit att studera några aspekter av läkningen av tandens huvudsakliga hårdvävnad, dentinet. Vid mycket djupa kariesangrepp där dentinet förstörts och pulpan därmed blottats, rotbehandlas ofta tanden vilket innebär att pulpan tas bort och att rotkanalen fylls med ett rotfyllningsmaterial. Djupare kunskaper om dentinets läkningsförmåga kan leda till att andra mindre invasiva och kostsamma behandlingsmetoder än rotbehandlingar skulle kunna användas vid mycket djupa kariesangrepp.

Pulpaöverkappning är en behandling som används när pulpan blivit blottad i ett försök att bibehålla pulpans vitalitet och funktion. Faktorer som påverkar hårdvävnadsbildningen vid pulpaöverkappningar har studerats i en systematisk litteraturöversikt. Base-rat på det begränsade vetenskapliga stödet visade resultaten att kalciumhydroxidbaserade material men inte bondingmaterial ger en hårdvävnadsbildning som täcker pulpasåret då de används som överkappningsmaterial. Det finns inget vetenskapligt stöd för att kunna fastslå att mineraltrioxidaggregat (MTA) skulle ge mer hårdvävnadsbildning jämfört med kalciumhydroxidbaserade material när dessa används som överkappningsmaterial.

En gel (Emdogain® Gel) som innehåller amelogenin som man vet är inblandat i processen då dentinet börjar bildas, utvärderades i en klinisk studie med syfte att studera hårdvävnadsbildningen. En större mängd hårdvävnad bildades efter appliceringen av gelen jämfört med kontrollmaterialet. Hårdvävnaden kunde karakteriseras som att vara likt det ursprungliga dentinet, men den bildades inte i en struktur som skulle kunna utgöra en fysiologisk barriär.

Under ett kariesangrepp bör odontoblasterna svara på närvaro av bakterier med försvarsreaktioner såsom bildande av nytt dentin, men kvalitén på det dentinet verkar ibland bli sämre än det ursprungliga dentinet. Produkter från bakterier tagna från ett djupt kariesangrepp användes för att studera dess effekter på odontoblastliknande cellers aktivitet och förmåga att bilda en typ av kollagen som är den huvudsakliga beståndsdelens i nybildat dentin. Vissa bakterier hade en negativ påverkan på odontoblasternas aktivitet och bakteriernas effekt på kollagenproduktionen varierade, vilket skulle kunna tyda på att bakterier kan ha en direkt effekt på odontoblasternas förmåga att upprätthålla dentinetts barriärfunktion.

Sammanfattningsvis kan man säga att Emdogain® Gel initierade dentinbildning, men inte i en struktur som skulle kunna utgöra en barriär och det förefaller som om bakterier i olika grad kan påverka odontoblasternas förmåga att bilda en dentinbarriär.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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## THESIS AT A GLANCE

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<td>(I) Formation of a hard tissue barrier after pulp cappings in humans. A systematic review</td>
<td>To answer the questions: Can a pulp exposure heal? Under which circumstances does a hard tissue barrier form? What happens over time?</td>
<td>A literature search was performed and the publications included were assessed with regard to their level of evidence</td>
<td>The use of calcium hydroxide frequently results in the formation of hard tissue covering the pulp exposure (Limited scientific evidence)</td>
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<td>(II) Dental pulp capping: effect of Emdogain® Gel on experimentally exposed human pulps</td>
<td>To study the effect of EMDgel as a pulp capping agent on experimentally exposed human dental pulps with regard to hard tissue formation and pulp inflammation</td>
<td>9 pairs of premolars were treated randomly with EMDgel or calcium hydroxide. The teeth were extracted, prepared and subjected to microscopic examination.</td>
<td>Hard tissue in irregular masses and inflammation was observed in the wound area. The formulation with EMD in a PGA vehicle does not seem to be appropriate for treatment of pulp exposures.</td>
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<td>(III) Dentine sialoprotein and Collagen I expression after experimental pulp capping in humans using Emdogain® Gel</td>
<td>To characterize the hard tissue formed after pulp capping with EMDgel using DSP and Col I dentine markers</td>
<td>Immunohistochemistry was performed to stain histological sections of teeth treated with EMDgel or calcium hydroxide.</td>
<td>The newly formed hard tissue contained DSP and Col I, indicating that it is dentine rather than non-specific hard tissue.</td>
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<td>(IV) Effects of bacterial products on the activity of odontoblast-like cells and their formation of type I collagen</td>
<td>To investigate how products from biofilms of bacteria found in a deep caries lesion affect the activity of mouse odontoblasts and their formation of Col I</td>
<td>A cell-line was exposed to conditioned medium from biofilm cultures. Their activity was assessed using an MTT assay and Col I was determined by ELISA.</td>
<td>The activity and production of Col I varied, suggesting that the nature of bacterial species in a caries lesion may influence the formation of tertiary dentine.</td>
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There are several terms used in the literature concerning dentine formation and different modes of pulp capping. The following applies to this thesis:

<table>
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<td>Dentine</td>
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<tr>
<td>Primary</td>
<td>dentine formed by primary odontoblasts until the root is fully formed</td>
</tr>
<tr>
<td>Secondary</td>
<td>dentine that is formed at a highly reduced rate after the root has been fully formed</td>
</tr>
<tr>
<td>Tertiary</td>
<td>dentine formed in response to injury. This might further be characterized as reactive when the primary odontoblasts have been triggered or reparative when the primary odontoblasts have been replaced by odontoblast-like cells</td>
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<tr>
<td>Pulp capping</td>
<td>application of a material to an exposed pulp (direct capping) in order to allow the pulp to recover and maintain its normal vitality and function</td>
</tr>
<tr>
<td>Partial pulpotomy</td>
<td>a superficial pulp amputation and application of a pulp capping material as in pulp capping</td>
</tr>
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Indirect pulp capping: a treatment in which infected dentine is intentionally left under a restoration in order to avoid pulp exposure.

CENTRAL: the Cochrane Controlled Trials

Col I: type 1 collagen
DAB: 3,3-Diaminobenzidine
DMEM: Dulbecco’s Modified Eagle Medium
DSP: dentine sialoprotein
DSPP: dentine sialophosphoprotein
ELISA: enzyme-linked immunosorbent assay
EMD: enamel matrix derivative
EMDgel: enamel matrix derivative in a formulation with propylene glycol alginate
FBS: foetal bovine serum
LPS: lipopolysaccharide
LTA: lipoteichoic acid
MDPC-23: a spontaneously immortalized cell line from the dental papillae of mouse molars
MeSH: Index Medicus: Medical Subject Headings
MTT: methol-thiazolyl-diphenyltetrazolium bromide
PGA: propylene glycol alginate
RCT: randomized controlled trial
SE: standard error
SIBLING: Small Integrin-Binding Ligand N-linked Glycoprotein
TBS: tris-buffered-saline
VAS: Visual Analogue Scale
INTRODUCTION

The barrier function of dentine
The surfaces of the body including the skin, the gastro-intestinal tract and the oral mucosa have physiological barrier functions aimed at protecting the body from external noxious agents (Turner 2009; Elias 2008). In the tooth the enamel is, by virtue of its hardness and structure, an effective mechanical barrier while the physiological barrier function of the tooth lies mainly within the pulp-dentine complex.

In the pulp-dentine complex, the dentine is traversed by tubules filled with fluid in which the odontoblast processes are located. The odontoblasts are the cells responsible for the formation of dentine. They align at the periphery of the pulp in close proximity to the dentine with their cellular processes situated within the dentine tubules. As dentine is being formed, a layer of collagenous network with associated non-collagenous proteins is laid down by the odontoblasts. This zone of so-called “predentine” is thus to be found between the fully mineralised dentine and the odontoblast.

The physiological barrier function of the pulp-dentine complex consists of several key elements:
- Dentinal fluid
- Formation of peri-tubular dentine
- Formation of tertiary dentine

There is a continuous outward flow of dentinal fluid through the dentine tubules and in exposed dentine, the outward fluid flow is
the first barrier against the inward diffusion of noxious agents such as bacteria or bacterial components (Pissiotis & Spångberg 1994). Consequently, any noxious agent migrating towards the pulp must do so against a pressure gradient (Mjör 2009) and in addition, the dentinal fluid dilutes the noxious agents. The dentinal fluid, which is considered to be plasma-derived, contains serum proteins and globulins which can agglutinate or bind noxious agents and thus play a protective role in the barrier function (Knutsson et al. 1994). A range of cytokines and chemokines such as IL-1β, IL-6, IL-8, IL-10 and TNFα can be found in the dentinal fluid (Geraldeli et al. 2012), though their role has not yet been elucidated. In addition, the dentinal fluid also contains beta-defensins which have antimicrobial properties and can kill bacteria directly (Dommisch et al. 2005). The substances found in the dentinal fluid do not fully correspond to those in plasma and thus dentinal fluid composition and flow thus appear to be regulated by the odontoblasts (Geraldeli et al. 2012).

The formation of peri-tubular dentine by the odontoblasts is another part of the physiological barrier function. This reduces the diameter of the dentinal tubules and reduces the chance of noxious agents penetrating into the pulp (Bjørndal & Mjör 2002). The secretion of peri-tubular dentine by the odontoblasts as well as the precipitation of crystals dissolved during the caries process may block the dentinal tubules, reducing the transport of fluids and matter (Tagami et al. 1992).

Slow formation of secondary dentine is seen throughout life. It is a continuum of the primary dentine, though the secondary dentine is formed at a much reduced rate once the root has been fully formed. In cases of disruption of the hard tissues, the odontoblasts may again, very locally, increase the rate of dentine production to form what is referred to as tertiary dentine. If the primary odontoblasts have been triggered, the tertiary dentine is denoted as reactive, whereas reparative dentine is the term used for tertiary dentine that has been formed by differentiated stem cells often called odontoblast-like cells, which have replaced the original odontoblasts. As reparative dentine is not formed by the same
odontoblasts as those which formed the primary dentine, the continuum of the tubules is interrupted and the structure of reparative dentine thus described as atubular (Barber & Massler 1964; Mjör 2009). The formation of tertiary dentine is part of the physiological barrier function through the increase of the dentine thickness between the site of the insult and the pulp. In case of reparative dentine, the atubular structure may reduce the transport of fluids and matter to the pulp. However the function of the dentinal fluid in the dentinal tubules and thereby the odontoblasts is affected.

These barrier functions of the dentine are considered to be under control of the odontoblasts. Strategically positioned, the odontoblasts with their processes within the dentinal tubules are the first cells to encounter and respond to noxious agents such as bacteria or bacterial components within the dentinal tubules. Odontoblasts are highly specialized cells and are involved in a similar defence system to that of epithelial cells regulating the host's response to injury (Dommisch et al. 2008; Goldberg et al. 2008). Apart from forming tertiary dentine, the odontoblasts are proposed to be involved in several defence mechanisms which protect the tooth from bacterial invasion. The odontoblasts are known to express several Toll-like receptors which recognise bacterial antigens (Staquet et al. 2011). Upon activation of Toll-like receptors the odontoblasts may release substances that will affect angiogenesis and the regulation of blood flow, key elements of the inflammatory process (Botero et al. 2006; Soden et al. 2009). Through activation of Toll-like receptors it seems possible that the odontoblasts, by releasing different substances, may negatively or positively regulate the inflammatory and immune response within the pulp and thus affect the formation of dentine (Durand et al. 2006; Farges et al. 2011; Hahn & Liewehr 2007). Odontoblasts may also release antibacterial peptides which can kill bacteria directly (Dommisch et al. 2005) and appear to amplify the responses to the presence of bacteria through a self-feedback system (Horst et al. 2011). To sum up; the physiological functions of the odontoblast-dentine unit play an important role in protecting the pulp from microbial influence when exposed to the oral environment and hence constitute an active barrier function as described for the skin and gastro-intestinal tract.
Injury to the dentine
Apart from trauma, attrition or restorative procedures, caries is the most evident process in which the dentine barrier function is activated and if overloaded, the dentine will eventually be destroyed leading to exposure of the pulp. Movement of bacterial products within the dentinal tubules will affect the odontoblasts, even though the bulk of the micro-organisms are situated some distance from the pulp.

Investigations of the micro-flora in deep caries lesions have proposed that the prevailing species are Gram-positive organisms such as *Lactobacillus*, *Actinomyces*, *Eubacterium*, *Propionibacterium* and *Streptococcus* species even though Gram-negative organisms including *Fusobacterium*, *Veillonella* and *Bacteroides* may be found at lower levels (Edwardsson 1974; Hoshino 1985). More recent studies using 16S rRNA gene sequencing have shown a dominance of Lactobacillus species in some caries lesions (Chhour et al. 2005; Munson et al. 2004). Bacteria have a strong tendency to attach to surfaces and form biofilms (Costerton et al. 1995) and several oral bacteria express surface adhesins which allows them to bind to collagen within the dentine matrix (McGrady et al. 1995; Love et al. 1997). Secondary colonizers that do not express these surface adhesins can co-aggregate to the primary colonizers allowing the formation of polymicrobial biofilms within the dentinal tubules (Love & Jenkansson 2002). Bacteria in biofilms may express phenotypes which differ from those of the same species growing in planktonic state due to the closeness of the bacterial cells and the presence of micro-niches in the biofilm (Stewart & Franklin 2008; Svensäter et al. 2001). Physiological adaptations to surface attachment include changes in the production of extracellular polymeric substances as well as modifications in cell morphology.

In *in-vitro* models of biological events within the pulp due to caries, it is common to use commercially available solutions of known bacterial pathogenic components such as lipoteichoic acid (LTA) or lipopolysaccharide (LPS) (Durand et al. 2006; Farges et al. 2011; Oliveira & Santos 2011; Soden et al. 2009; Telles et al. 2003). The use of such solutions may be beneficial as comparisons between
studies are made easy, but is unlikely to reflect the situation during the caries process as the pathogenic effect of the bacteria may not solely be attributed to LTA or LPS. There is scant information concerning the biological events within the pulp using fresh isolates in biofilm models.

Dentine reactions and repair
As mentioned above, the odontoblasts should be seen as a barrier entity such as the epithelium lining of the oral cavity, the gastrointestinal tract and the skin. The odontoblasts may be injured and die during restorative procedures or as a sequel to a rapidly progressing caries lesion. Unlike epithelial cells, odontoblasts are post-mitotic and are therefore incapable of cell division in order to replace the lost cells (Arana-Chavez & Massa 2004; Tziafas & Kodonas 2010). The fact that the odontoblast is post-mitotic might be of importance since the death of other post-mitotic cells within the brain or heart can lead to irreversible loss of function of the tissues. The ultimate aim of repair is to restore the original structure and the biological functions of the damaged tissues (Schmalz & Galler 2011); however as the dead odontoblasts need to be replaced by differentiated odontoblast-like pulpal stem cells, the repair process does not completely fulfil this ultimate aim. How the recruitment of the odontoblast-like cells actually takes place is not fully elucidated, though it seems likely that multipotent pulpal stem cells migrate to the site of the injury, where they proliferate and differentiate into odontoblast-like cells (Balic et al. 2010; Shi & Gronthos 2003). In cases of exposure of the pulp, healing with hard tissue has been observed although the morphology is similar to reparative dentine with an atubular structure and with cellular inclusions (Arana-Chavez & Massa 2004). It is not known whether this reparative hard tissue actually constitutes a functional recovery as it is questionable whether the odontoblast-like cells responsible for the formation of the hard tissue possess the same protective barrier functions as those seen in primary odontoblasts. Since the tubular structure of dentine is mostly missing, the pathway for agents to induce reactions in the odontoblasts is more or less hindered. Nevertheless, healing of the hard tissue structure with the physiological functions seems to be of great importance in keeping the integrity and vitality of the tooth.
The odontoblasts may be affected to various extents during the caries process. Theoretically this means that the dentine reaction beneath a caries lesion may involve anything from up-regulating primary odontoblasts to the replacement of the entire odontoblast layer. In the reaction to minor injuries, the pulp tissue is not exposed but dentine production is up-regulated underneath what is clinically observed as an intact layer of dentine. Beneath some caries lesions it seems to be rather similar to the primary dentine, whereas beneath others it appears to be structurally different or even absent (Bjørndal 2001). One explanation for these varying states might be that different consortia of micro-organisms lead to different degrees of repair. Thus invasion of the pulp by micro-organisms is probably dependent on both the nature of the bacteria present and the barrier function of the pulp-dentine complex. When the pulp has been exposed, and thus the odontoblasts have been destroyed, a pulp capping procedure in which a protective agent is placed on the pulp tissue may result in the recruitment of odontoblast-like cells and hard tissue repair (Nyborg 1958; Schröder & Granath 1972).

As reparative dentine is sometimes quite irregular with cellular inclusions and tunnel defects not seen in the primary dentine, it is sometimes referred to as “osteodentine”. As a consequence of the morphological differences observed, the use of the terms “dentine-like” and “odontoblast-like” have gained popularity when denoting hard tissue and cells involved in the repair, especially since the repair involves replacement of post-mitotic cells. Using a biochemical approach to characterize the odontoblast phenotype, a wide range of differentiation markers such as members of the SIBLING-family (Small Integrin-Binding LLigand N-linked Glycoprotein) has been suggested. Dentine sialoprotein (DSP) is a member of this family and together with type 1 collagen (Col 1) is suggested to be a late marker for odontoblast formation. These markers are frequently used to characterize the odontoblast and the dentine (Butler & Ritchie 1995; Nakashima et al. 2002; Narayanan et al. 2001). However members of the SIBLING-family are also expressed to some extent by osteoblasts (Qin et al. 2002). Using a marker for the odontoblast may be considered to be a complement
to solely morphological observations aimed at increasing the possibility of identifying whether newly formed hard tissue is dentine. However it does not actually elucidate whether all cellular functions are identical to those of the primary odontoblasts.

**Treatment of pulp exposures**

A pulp exposure is defined in the MeSH browser (Index Medicus: Medical Subject Headings) as the result of pathological changes in the hard tissue of a tooth caused by carious lesions, mechanical factors, or trauma, which render the pulp susceptible to bacterial invasion from the external environment. Two basically different types of treatment of teeth with pulp exposures are used in order to avoid extraction: pulp capping with the ultimate goal of preserving the pulp and thereby the vitality of the tooth and pulpectomy where the inflamed or injured pulp is removed and the root canal obturated with a root filling material.

During the pulp capping procedure, a protective agent is applied to an exposed pulp in order to allow it to recover and maintain its vitality and function. It is difficult to state the survival or success rate of teeth that have been pulp capped as most studies are performed retrospectively. The concepts “pulp survival” or “success” are typically used for teeth that are sensitive to electric pulp testing and show no signs of apical periodontitis. Partial pulpotomies, a type of deep pulp capping, have been carried out in traumatized teeth with complicated crown fractures with calcium hydroxide as a pulp capping agent and success was reported in 96% of the teeth after an average observation period of 31 months (Cvek 1978). Partial pulpotomies performed in young permanent teeth with carious pulp exposures were reported as successful in 93% of cases after an average observational period of 56 months (Mejàre & Cvek 1993). Al-Hiyasat et al. (2006) found a success rate of 92.2% after mechanical pulp exposures and 33.2% after caries pulp exposures at least three years after pulp capping with calcium hydroxide performed by dental students. In a study by Hørsted et al. (1985) it was demonstrated that the survival of the pulp after capping with calcium hydroxide in teeth that had pulp exposures mainly due to cavity preparation and some due to caries decreased over time. The
survival rate was 82% after 5 years and 73% after 10 years. Barthel et al. (2000) has published a study with a long observation period of pulp capping performed with calcium hydroxide in teeth that had carious pulp exposures. Five years after the pulp capping procedure had been performed there was a 37% success rate and after 10 years the success rate had decreased to 13%. Results from another recent study using MTA as a pulp capping material in teeth with carious pulp exposures show a one-year pulp survival of 67.7% and a two-year survival of 56.2% (Miles et al. 2010). In a prospective, multi-centre study in which patients with very deep caries (involving 75% or more of the dentine thickness) leading to pulp exposures were randomized to direct pulp capping or partial pulpotomy, After 1 year no difference in pulp survival (31.8% and 34.5% respectively) was seen (Bjørndal et al. 2010). The observation that the failure rate increases over time might imply that the newly formed hard tissue is not able to act as a functional barrier protecting the pulp against bacterial micro-leakage along the restoration margins (Barthel et al. 2000; Hørsted et al. 1985; Miles et al. 2010). The preoperative pulp status of the teeth also seems to influence the pulp survival since high success rates have been observed for pulp capping of traumatic or mechanical pulp exposures where most of the pulps are not inflamed, compared to some very low success rates observed for caries pulp exposures with sometimes severely inflamed pulps (Al-Hiyasat et al. 2006; Barthel et al. 2000; Bjørndal et al. 2010).

Pulpectomy means that pulp tissue is irreversibly removed and the root canal obturated with a root filling material. Because of the varying outcome of pulp capping, pulpectomy with a subsequent root filling that has a well-documented high success rate (Petersson et al. 1982; Gesi et al. 2006) is a common treatment for teeth with pulp exposures due to caries in adults. It is also the treatment recommended by The National Board on Health and Welfare in Sweden (National Guidelines for Adult Dental Care) in spite of this treatment being more invasive and more technically difficult to perform than pulp capping. Even if high success rates are observed for pulpectomy, the root filled tooth may be considered to be a tooth at risk of developing complications. Studies show that 10 - 19% of
root filled teeth will be extracted within 5 years after treatment (Alley et al. 2004; Tilashalski et al. 2004) and extractions of root filled teeth are more common than extractions of teeth without root fillings (Eckerbom et al. 1992).

Nearly a quarter of a million root canal treatments were reported to the Swedish Health Service (Försäkringskassan) during 2010 with reference costs from the Dental and Pharmaceutical Benefits Agency (Tandvårds- och läkemedelsverket) varying from 3180 to 5095 SEK per root filled tooth depending on the number of canals treated in each tooth. Thus, considerable resources are spent on root canal treatments in Sweden. A low estimation would mean that at least one billion SEK is spent solely on the fee for root canal fillings reported to the Swedish Health Service. Avoidance of some of these treatments in favour of pulp capping would be of benefit for the individual, oral health and society. Understanding the biological events leading to the healing of functional dentine and the successful preservation of the vitality of the tooth would give a scientific basis for a less invasive and more cost-effective treatment of human pulp exposures.

**Dentine repair by mimicry**

There have been many attempts to find a technique and an agent that will predictably induce repair of the hard tissue barrier (Hellner 1930; Nyborg 1958; Schröder & Granath 1972). Much attention has been given to calcium hydroxide as a pulp capping agent as it is considered to promote a superficial necrosis which will act as scaffold for the odontoblast-like cells (Schröder & Granath 1971). The cellular events in this process are poorly understood, but it is thought that bio-active dentine matrix components are released from the dentine in contact with calcium hydroxide which stimulate new hard tissue formation (Graham et al. 2006). There have been reports of irregularities in the newly formed hard tissue after pulp capping with calcium hydroxide (Schröder & Granath 1972). Cox has studied the formation of tunnel defects in dentine bridges on rhesus monkeys and reported that 90% of the bridges showed defects and subsequently inflamed pulps (Cox et al. 1996).
Much effort has been placed on finding strategies other than the ones in clinical use for the induction and regulation of the reparative or regenerative process after pulp exposure. These include the application of growth or cell differentiation factors to try to mimic dentinogenesis during embryonic development in the hope of achieving healing that resembles the original structure and functions of the lost dentine (Nakashima et al. 2002; Rutherford et al. 1993; Sloan et al. 2000; Smith et al. 2001).

Amelogenins and amelin are proteins that have been proposed to participate in the final differentiation of odontoblasts and subsequent dentine formation during dentinogenesis (Inai et al. 1991; Papagerakis et al. 2003; Spahr et al. 2002; Tompkins et al. 2005). Amelogenins are detectable in the odontoblasts during tooth formation at the time that they are synthesizing and secreting dentine sialophosphoprotein (DSPP), a protein involved in the mineralisation of dentine (Inai et al. 1991). An amelogenin-rich fraction of porcine enamel matrix derivative (EMD) that also contains amelin has been used to induce cementum formation and periodontal ligament regeneration in monkeys (Hammarström et al. 1997). The mechanism(s) by which EMD promotes periodontal ligament regeneration is still largely unknown, although the amelogenins are thought to self-assemble into nanospheres that create an extracellular matrix. Within the body, this matrix will slowly be digested by specific extracellular proteolytic enzymes (matrix metalloproteinases) releasing bioactive peptides to the surrounding tissues for weeks after application. These will stimulate the secretion of local growth factors and cytokine expression in the treated tissues, prompting a regenerative process mimicking odontogenesis (Lyngstadaas et al. 2009). Nakamura et al. (2001; 2002; 2004) used a formulation of EMD as a pulp capping agent in two studies performed on miniature swine. The amount of hard tissue formed in the EMD-treated teeth was twice that of the control group capped with calcium hydroxide cement. The biological responses to various treatments may differ between species, and prior to this thesis the effects of EMD used as a pulp capping agent on the formation of hard tissue and pulp status in humans had not been studied.
Today it is not known whether the unfavorable long-term results of pulp capping in teeth with deep caries are due to a dysfunctional barrier and a secondary infection at the exposure site or a withstanding inflammation originally caused by the caries lesion. Nevertheless, as the barrier function in the dentine seems to be important in protecting the pulp from microbial influence it would be beneficial to gain knowledge about factors that influence the repair of the dentine after dental pulp capping.
AIMS

Repair of the hard tissue after pulp capping has been observed in human experimental studies; however the clinical outcome of pulp capping is regarded as uncertain. The overall aim of this thesis was to study some aspects of the repair of the dentine barrier, especially in conjunction with dental pulp capping and to ascertain if, and under what conditions, the exposed pulp is able to heal with a hard tissue barrier. This, in order to obtain a scientific basis for a less invasive and perhaps more cost-effective treatment of pulp exposures.

The specific aims were:

- To evaluate the evidence for the formation of a hard tissue barrier after pulp capping in humans by conducting a systematic review
- To study the effect of an enamel matrix derivative (Emdogain® Gel) as a pulp capping agent on experimentally exposed human dental pulps with regard to hard tissue formation and pulp inflammation
- To characterize the hard tissue formed after pulp capping with Emdogain® Gel using DSP and type 1 collagen as dentine markers
- To investigate how extracellular products from biofilms of bacteria found in a deep caries lesion affect the activity of odontoblast-like cells and their formation of type 1 collagen.
MATERIALS AND METHODS

Systematic review (I)
A systematic review of the English literature on the formation of a hard tissue barrier after dental pulp capping was performed using specific indexing terms. In order to achieve a systematic approach, the literature search was conducted in four major steps using the method described by Goodman (1993). These steps were to: (1) specify the problem; (2) formulate a plan for the literature search; (3) conduct a literature search and retrieve publications; and (4) interpret and assess the evidence from the literature retrieved.

The following questions defined the problem:
- Can a pulp exposure heal, i.e. form a hard tissue barrier with pulp tissue that is free of signs of inflammation after pulp capping?
- Under which circumstances does a hard tissue barrier form?
- What happens to the pulp and the newly formed hard tissue over time?

Literature search
Publications were retrieved from PubMed with publication dates from 1 January 1966 to 1 January 2005. As several new articles on the subject have been published since the publication of the review in 2006, the systematic review was supplemented with a literature search extended until 1 October 2010. The searches were performed using the MeSH (Medical Subject Heading) term “Dental Pulp Capping” and was limited to publications with abstracts.
Animal studies and case reports were excluded. An additional search was performed in CENTRAL (Cochrane Controlled Clinical Trials Register) and the reference lists of included publications were hand searched to find additional original scientific articles that had not been found through PubMed.

**Interpretation and assessment**
The interpretation and the assessment of the quality of the studies were performed independently by the authors using pre-tested protocols. The quality of each included original scientific article was rated as high, moderate or low according to criteria modified after Guyatt *et al.* (1993; 1994). The data extraction and assessment were performed without blinding and disagreements were solved by discussion to reach consensus. The overall evidence grade, based on the quality of each included article, was rated to be strong, moderately strong, limited or insufficient as proposed by Centre for Evidence Based Medicine, University of Oxford.

**Clinical trial (II)**
Subjects and pulp capping procedure
The subjects were recruited from the Public Dental Health Service; Skåne County Council, Sweden and were eligible to be included in the study if they had contralateral premolars without clinically evident caries scheduled for extraction on orthodontic grounds. Eight subjects, aged between 12 and 16 years, with a total of 9 pairs of premolars were included in the study.

Following an experimental superficial pulp amputation, either Endogain® Gel (EMDgel) or a mix of calcium hydroxide and saline was placed at random in contact with the pulp wound. A disc of Teflon was placed over the pulp capping material and the tooth was restored with a temporary restoration. Each subject received both test and control in a split mouth design. After 12 weeks the teeth were extracted. Adverse events were recorded.
Outcome measures

- Frequency of any symptoms
- Evaluation of pulpal status
- Evaluation of hard tissue formation

The subjects made records of any symptoms using a Visual Analogue Scale (VAS) (Huskisson 1983) over the first 10 days after the pulp capping procedure. They were also asked to report any experience of spontaneous pain or use of analgesics. A blinded examiner carried out telephone interviews with the subjects about pain or discomfort. The structured interviews were performed at 1 day, 2 weeks, 6 weeks and 12 weeks after the treatment. At 12 weeks, just prior to extraction, the teeth were clinically examined for:
  - tenderness to percussion
  - tenderness to palpation
  - mobility

and the temporary filling was assessed for risk of leakage. The teeth had been examined similarly prior to the experimental treatment.

Histological preparations and analysis

The extracted teeth were fixed in formaldehyde for 7 days and thereafter demineralized in EDTA and subsequently embedded in paraffin. After longitudinal serial sectioning (5 μm), every fifth section was stained with haematoxylin and eosin. All stained sections covering the wound area were studied with the aid of a light microscope equipped with a camera and computer with a software programme in order to perform histometry. The sections were blinded regarding the subject and type of treatment that had been performed and were analysed by the operator performing the pulp capping procedure. The inflammation in the pulp was analysed and classified after criteria modified after Heyeraas et al. (2001) (Table 1). The inflammation was classified in three different locations within the pulp; in the central part, just apically to where the pulp had been amputated or within the proliferated pulp (if such a proliferation had occurred). The newly formed hard tissue was analysed with regard to: if and how it covered the wound area, the
area of hard tissue formed and the thickness of the hard tissue bridge if such a hard bridge had been formed covering the wound area.

Table 1 Criteria used for classification of the pulp status with regard to inflammation, modified after Heyeraas et al. (2001).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None: Fibroblasts but no inflammatory cells are found. Capillaries but no extravasated red blood cells can be found.</td>
</tr>
<tr>
<td>1</td>
<td>Slight: Increased number of cells, predominately fibroblasts. A few inflammatory cells are involved. An increased number of capillaries are noted, and a few extravasated red blood cells may be found.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate: Predominantly characterised by more cells in the area than in the slight reaction. Increased number of capillaries and vessels are found.</td>
</tr>
<tr>
<td>3</td>
<td>Severe: Marked cellular infiltration, including local abscess formation. Numerous blood vessels are found in the tissue surrounding the intense cellular infiltration.</td>
</tr>
<tr>
<td>4</td>
<td>Abscess formation or extended lesions not localised only to the tissue beneath the cavity floor.</td>
</tr>
</tbody>
</table>

Ethical approval and informed consent
The study was approved by the Committee on Investigations Involving Human Subjects at Lund University, Sweden (LU 297-01) and all the participants and their guardians completed a consent form.

Immunohistochemical studies (II and III)
Localisation of EMD in EMDgel treated teeth
As the control material in the clinical study was calcium hydroxide and not the propylene glycol alginate (PGA) which was used as the vehicle for EMD, there were concerns regarding whether the effects
seen could be attributed to EMD. In order to see if the hard tissue formed in the teeth treated with EMDgel was located in close proximity to the EMD, immunohistochemical localisation was carried out using a polyclonal rabbit anti-EMD antibody. The antibody has been described previously (Gestrelius et al. 1997b) and was supplied by Biora AB. Endogenous peroxidase activity was quenched and non-specific binding was blocked using normal goat serum. Endogenous biotin was blocked with the DAKO biotin blocking kit (Dako, Glostrup, Denmark) according to the enclosed instructions. Sections were incubated overnight at 4°C using the antibody. Antibody binding was visualized using DAB in TBS containing hydrogen peroxide and sections were counterstained with haematoxylin. Using a light microscope, evaluation was made by the same operator who assessed the histological sections stained with haematoxylin and eosin.

Localisation and assessment of DSP and Col I

In order to characterize the newly formed hard tissue in the teeth pulp capped with EMDgel or calcium hydroxide, two relatively specific markers for dentine were used; DSP and Col I. Representative sections from the clinical study were used, and at least one section from each tooth was used for each antiserum. The polyclonal DSP antibody, raised in rabbit, was provided by Dr. Qin, Department of Endodontics, University of Texas-Houston Health Science Center Dental Branch, Houston, TX, USA and the polyclonal rabbit anti-Col I was purchased from Nordic Biosite AB, Täby, Sweden. The same procedures were undertaken as for the localisation of EMD. Using a light microscope, two calibrated observers independently evaluated the reactivity of anti-DSP and anti-Col I in different locations within the area that had been pulp capped:

- newly formed hard tissue
- areas with less mineralised hard tissue, corresponding to predentine
- lining cells
- areas of inflammation

The immunostaining was classified as moderate when the intensity was comparable to the staining of dentine and predentine in the
root of the same tooth examined, as well as in a stained tooth which had been extracted prior to placement of a pulp capping material. In other cases the staining was classified as “not detectable” or “strong”. The sections were coded during the evaluation and disagreements among the observers were solved by discussion so that consensus was reached.

Staining of micro-organisms
In order to examine the possible occurrence of micro-organisms in the wound area, a modified Brown and Brenn technique (Churukian & Schenk 1982) was used to stain sections as well as the LIVE/DEAD® BacLight™ Bacterial Viability Kit according to the manufacturer’s instructions (Molecular Probes Inc., Eugene, OR, USA).

Cell culture study (IV)
An established cell-line of spontaneously immortalized cells from the dental papillae of mouse molars was provided by Professor Nör, University of Michigan, Ann Arbor, USA and was used in this study. The cells of MDPC-23 express both DSP and Col I and are therefore considered to be of the odontoblast lineage. The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) and supplemented with penicillin, streptomycin and L-glutamine at 37°C in a humidified atmosphere of 5% CO₂ in air.

Collection and isolation of bacteria
A dentine sample was taken from a tooth with a very deep caries lesion. Further excavation lead to a pulp exposure, with bleeding pulp tissue. Bacteria were cultured and identified on the basis of aerobic and anaerobic growth. Isolated strains were Gram stained and classified with regard to colony morphology into genus. Subculturing of Lactobacillus spp was done using Rogosa SL medium and the isolates were kept frozen. Further identification using 16S rRNA gene sequencing was done for the isolates used further in the study. The gene sequencing was performed by Associate Professor Ann-Catherine Petersson, Skåne University Hospital, Lund, Sweden.
Biofilm supernatants
Fresh isolates from the caries lesion (Lactobacillus rhamnosus, Lactobacillus casei and Shuttleworthia satelles) and from a clinical isolate of Enterococcus faecalis from an infected root canal were allowed to form biofilms in tissue flasks containing DMEM supplemented with 1% FBS and L-glutamine. After 96 hours of incubation in 5% CO₂ in air, the supernatants were collected and centrifuged to remove the bacteria. After concentration, the supernatant was filtered using a 0.2 µm filter. The total protein content was measured to lie between 2.8 and 3.2 mg/ml.

Cell activity
In order to investigate whether the biofilm supernatants influenced the MDPC-23 cells activity, a methol-thiazolyl-diphenyltetrazolium bromide (MTT) assay was used. MDPC-cells were grown and then exposed to the different biofilm supernatants or to the known bacterial antigens lipoteichoic acid (LTA) or lipopolysaccharide (LPS). After 96 hours of exposure, MTT was added. Yellow MTT is reduced to purple formazan crystals by enzymes in the mitochondria of cells and may therefore be used for assessment of metabolic activity but is also frequently used to assess viability or proliferation of cells. After incubation, the reduced MTT was dissolved and the absorbance was read. Absorbance values were divided by the value for the control and expressed as a percentage.

Col I production
To determine whether bacterial components affected the production of Col I, the major component of the non-mineralised matrix of tertiary dentine, MDPC-23 cells were grown as above. After 96 hours the cell layers were dissolved using guanidium hydrochloride, containing 0.1% CHAPS. The amount of Col I was assessed using enzyme-linked immunosorbent assay (ELISA) with a biotinylated monoclonal rat-anti-mouse Col I antibody (Chondrex Inc., Redmond, WA, USA). Absorbance values were divided by the value for the control and expressed as a percentage.
Statistical method
All data from study IV were analysed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). A one-sample t-test was used to determine whether the mean percentage changes were significantly different from the control value of 100%. Values p <0.01 were considered to be significant.
RESULTS

Systematic review (I)
The search strategy yielded 107 articles that were retrieved in full text of which 21 original scientific studies were included in the published systematic review. The reasons, in descending order, for exclusions were:

- animal experiment
- no histology
- full pulpotomy
- no pulp exposure
- review
- no light microscopic evaluation
- no intervention
- impossible to assess data
- no examination of the hard tissue
- case reports
- non-English language

The extended literature search is presented in fig 1. Twenty articles in full text were included in the extended literature. Hand searching of the reference lists of the reviews and articles included in this extended literature search did not result in any additional publications. Table 2-4 shows the summary of data from the extended systematic review, corresponding tables for the previously published systematic review can be found in paper I.
Figure 1 The extended literature search, reported according to PRISMA (Moher et al. 2009).

Assessment of quality
The results from the published systematic review are combined here with data from the new literature search. One of the studies included was judged to have a high quality (Olsson et al. 2005), six were judged to be of moderate quality (Accorinte et al. 2008; Hørsted-Bindslev et al. 2003; Kiatwateeratana et al. 2009; Lu et al. 2008; Min et al. 2008; Silva et al. 2006) and the remaining 34 studies were considered to have a low quality. The large number of studies with low quality was predominantly due to deficiencies in the study design, sample size or the manner in which the microscopic evaluation had been executed or reported.

Study designs
The studies were heterogeneous regarding their designs and the pulp capping procedures. Sixteen were classified as being prospective and randomised (Accorinte et al. 2009; Accorinte Mde et al. 2005; Brännström et al. 1979; deLourdes Rodrigues Accorinte et

Several studies had divided the sample size into, up to, 6 observational periods which meant that there were sample sizes ranging from one to 20 teeth per observational period.

Pulp capping procedures

Most studies used a direct pulp capping procedure, performed in younger healthy teeth without signs of caries, although there were reports of partial pulpotomy and a few studies were conducted in teeth with carious exposures.

A variety of materials was used for the pulp capping procedure; though the most frequent were calcium hydroxide based or resin bonding materials. The use of different calcium hydroxide based materials often resulted in hard tissue bridges, though observation periods of shorter than 3 weeks revealed no hard tissue bridging. However, the use of bonding materials as the pulp capping agent resulted in hard tissue formation only in single cases. In the studies where bonding material was compared to a calcium hydroxide based material, the calcium hydroxide based material was superior to the bonding material in all studies.
Table 2 Data from the extended literature search regarding studies using other pulp capping materials than bonding or MTA, or studying certain operative procedures.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Material</th>
<th>Reported results</th>
<th>Study quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accorinte Mde et al. 2005</td>
<td>CCT 60 days</td>
<td>Bleeding controlled with T1: Saline solution n=5 T2: Ferric sulfate n=5 T3: 2.5% NaOCl n=5 T4: calcium hydroxide solution n=5 followed by pulp capping with Scotchbond Multi Purpose Plus (3M ESPE, St Paul, MN, USA) C: Bleeding controlled with saline followed by pulp capping with calcium hydroxide powder covered with Dycal (Dentsply-Caulk, Milford, DE, USA) n=5 Subjects n=?</td>
<td>Dentine bridge formation in: T1: 0% T2: 0% T3: 0% T4: 0% C: 100% No inflammation in C, and inflammation to different extent in the majority of T</td>
<td>Low</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Observation intervals</td>
<td>Treatment 1</td>
<td>Treatment 2</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------</td>
<td>------------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>de Lourdes Rodrigues Accorinte et al. 2006</td>
<td>RCT</td>
<td>2 observation intervals: 30 and 60 days</td>
<td>T1: adhesive resin, rubber dam n=10</td>
<td>C1: adhesive resin, no rubber dam n=10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T2: Calcium hydroxide powder covered with Dycal (Dentsply-Caulk, Milford, DE, USA), rubber dam n=10</td>
<td>C2: Calcium hydroxide powder covered with Dycal, no rubber dam n=10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 of 10 of 10</td>
<td>No inflammation in T2 or C2 and some inflammation in the groups treated with adhesive resin</td>
</tr>
<tr>
<td>Parolia et al. 2010</td>
<td>CCT</td>
<td>2 observation intervals: 15 and 45 days</td>
<td>T: Propolis (Ecuadorian Rainforest LLC, USA) mixed with ethyl alcohol n=12</td>
<td>C1: ProRootMTA (Dentsply Caulk, Milford, DE, USA) n=12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: Dycal (Dentsply Caulk) n=12</td>
<td>Subjects n=12</td>
</tr>
<tr>
<td>Kiatwateeratana et al. 2009</td>
<td>RCT</td>
<td>6 months</td>
<td>T: Emdogain® Gel (Biora AB, Malmö, Sweden) n=15</td>
<td>C: Calcium hydroxide mixed with water n=15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Subjects n=15</td>
<td></td>
</tr>
<tr>
<td>Olsson et al. 2005</td>
<td>RCT</td>
<td>12 weeks</td>
<td>T: Emdogain® Gel (Biora AB, Malmö, Sweden) n=9</td>
<td>C: Calcium hydroxide mixed with saline n=9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Subjects n=8</td>
<td></td>
</tr>
</tbody>
</table>
Table 3 Data from the extended literature search regarding studies using mineral trioxide aggregate as the pulp capping material.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Material</th>
<th>Reported results</th>
<th>Study quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accorinte et al. 2009</td>
<td>RCT</td>
<td>T1: ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) n=20 T2: MTA (Angelus, Londrina, PR, Brazil) n=20 Subjects n=?</td>
<td>Complete hard tissue bridge at 30 days: T1: 5 of 8 T2: 5 of 8 Complete hard tissue bridge at 60 days: T1: 5 of 8 T2: 6 of 10 No difference in inflammatory response. Teeth in which microorganisms were found were excluded from the analysis</td>
<td>Low</td>
</tr>
<tr>
<td>Accorinte Mde et al. 2008</td>
<td>CCT</td>
<td>T: MTA (Angelus, Londrina, PR, Brazil) n=20 C: Calcium hydroxide powder covered with Life (Kerr, Romulus, MI, USA) n=20 Subjects n=?</td>
<td>T: 11 of 20 total dentine bridge. Less inflammation than in C C: 14 of 20 total dentine bridge</td>
<td>Low</td>
</tr>
<tr>
<td>Study Authors</td>
<td>Design</td>
<td>Observation Intervals</td>
<td>Treatment Details</td>
<td>Control Details</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
<td>-----------------------</td>
<td>-------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Accorinte et al. 2008</td>
<td>CCT</td>
<td>2 observation intervals: 30 and 60 days</td>
<td>T: MTA (Dentsply Caulk, Milford, DE, USA) n=20</td>
<td>C: Life (Kerr, Romulus, MI, USA) n=20</td>
</tr>
<tr>
<td>Caicedo et al. 2006</td>
<td>Series of cases</td>
<td>Typically after 6 months</td>
<td>ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) n=10</td>
<td>Subjects: n=12</td>
</tr>
<tr>
<td>Iwamoto et al. 2006</td>
<td>RCT</td>
<td>136 +/- 24 days</td>
<td>T: White ProRoot MTA (patent pending) mixed with sterile saline n=22</td>
<td>C: Dycal (Dentsply-Caulk, Milford, DE, USA) n=23</td>
</tr>
<tr>
<td>Min et al. 2008</td>
<td>RCT</td>
<td>2 months</td>
<td>T: MTA ProRoot (Dentsply, Tulsa, OK, USA) n=10</td>
<td>C: Dycal (Dentsply-Caulk, Milford, DE, USA) n=10</td>
</tr>
<tr>
<td>Nair et al. 2008</td>
<td>RCT</td>
<td>3 observation intervals: 1 week, 1 and 3 months</td>
<td>T: White MTA (Proroot®, Dentsply, Tulsa Dental, OK, USA) n=20</td>
<td>C: Dycal® Ivory (Dentsply Caulk, Milford, DE, USA) n=13</td>
</tr>
<tr>
<td>Sawicki et al. 2008</td>
<td>RCT</td>
<td>47-609 days, in average 138 days</td>
<td>T: White ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) n=32</td>
<td>C: Life n=16</td>
</tr>
</tbody>
</table>
Table 4 Data from the extended literature search regarding studies using bonding adhesives as pulp capping materials.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Material</th>
<th>Reported results</th>
<th>Study quality</th>
</tr>
</thead>
</table>
| Accorinte Mde et al. 2005 | RCT 60 days                 | T: different steps in Scotchbond Multi Purpose Plus (3M ESPE, St Paul, MN, USA) n=20 divided into 4 subgroups  
C: Calcium hydroxide powder covered by Dycal (Dentsply Caulk, Milford, DE, USA) n=5 Subjects n=7  | Complete bridging in:  
T: 0 of 20  
C: 5 of 5  
No or minimal inflammation in C, more inflammation in T  | Low            |
| Accorinte et al. 2008 | CCT 2 observation intervals: 30 and 60 days | T1: Clearfil Liner Bond 2V (Kuraray Medical, Osaka, Japan) n=12  
T2: Clearfil SE Bond (Kuraray Medical, Osaka, Japan) n=12  
C1: Dycal (Dentsply, Petrópolis, Rio de Janeiro, Brazil) covered by Clearfil Liner Bond 2V  
C2: Dycal covered by Clearfil SE Bond | At 30 days hard tissue bridge in:  
T1: 1 of 6  
T2: 0 of 5  
At 60 days hard tissue bridge in:  
T1: 1 of 6  
T2: 1 of 6  
C1: 4 of 5  
C2: 4 of 4  
No difference with regard to inflammation between T1 and T2, though more than in control groups | Moderate |
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Observation Intervals</th>
<th>Treatment A</th>
<th>Treatment B</th>
<th>Outcomes</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elias et al. 2007</td>
<td>RCT</td>
<td>30 and 90 days</td>
<td>Clearfil SE Bond (Kuraray Medical, Osaka, Japan) n=16</td>
<td>Calcium hydroxide powder n=10</td>
<td>At 30 days dentine barrier formation in: T: 0 of 8 C: 5 of 5 At 90 days dentine barrier formation in: T: 1 of 8 C: 5 of 5 Less inflammation in C than T.</td>
<td>Low</td>
</tr>
<tr>
<td>Ersin &amp; Eronat 2005</td>
<td>CCT</td>
<td>7 and 90 days</td>
<td>Prime&amp;Bond 2.1 (Caulk, Dentsply, Milford, DE, USA) n=10</td>
<td>Calcium hydroxide mixed with water n=10</td>
<td>At 90 days: T: 0 of 5 dentine bridge, inflammation C: 5 of 5 dentine bridge, no inflammation</td>
<td>Low</td>
</tr>
<tr>
<td>Lu et al. 2008</td>
<td>RCT</td>
<td>7, 30 and 90 days</td>
<td>Clearfil SE Bond (Kuraray Medical, Osaka, Japan) n=21</td>
<td>Dycal (Dentsply, Weybridge, UK) n=20</td>
<td>At 90 days: T: 0 of 7 full bridge C: 5 of 6 full bridge No difference in inflammation between T and C</td>
<td>Moderate</td>
</tr>
<tr>
<td>Silva et al. 2006</td>
<td>RCT</td>
<td>1, 3, 7 and 30 days</td>
<td>37% phosphoric acid n=26</td>
<td>10% phosphoric acid n=26 followed by pulp capping with Single Bond Adhesive System (3M Dental Products, St Paul, MN, USA)</td>
<td>At 30 days dentine bridge deposition in: T1: 0 of 5 T2: 0 of 5 C: 5 of 5 Somewhat more inflammation in T1 and T2 than in C</td>
<td>Moderate</td>
</tr>
<tr>
<td>Sübay &amp; Demirci 2005</td>
<td>CCT</td>
<td>40 days</td>
<td>Scotchbond Multi Purpose Plus (3M ESPE, St Paul, MN, USA) n=10</td>
<td>Dycal (Dentsply Caulk, Milford, DE, USA) n=6</td>
<td>Dentine bridge in: T: 0 of 10 C: 3 of 6 More inflammation in T than C.</td>
<td>Low</td>
</tr>
</tbody>
</table>
Concluding evidence grade
Combining the results from the published systematic review with the new literature search leads to these conclusions regarding the pulp capping materials:

- The use of calcium hydroxide based materials as pulp capping agents frequently results in the formation of hard tissue covering the pulp exposure (limited scientific evidence).
- The use of Emdogain® Gel as a pulp capping agent does not result in the formation of hard tissue covering the pulp exposure (limited scientific evidence).
- The use of bonding materials as pulp capping agent does not result in the formation of hard tissue covering the pulp exposure (limited scientific evidence).
- Scientific evidence is lacking as to whether teeth treated with MTA as a pulp capping material show hard tissue formation more frequently than calcium hydroxide based materials.
Effect of EMDgel on experimentally performed pulp exposures (II)

All subjects completed the study and no adverse events related to the treatment were recorded.

Symptoms and clinical assessment

No teeth were reported to give severe symptoms during the telephone interviews. The teeth treated with EMDgel gave fewer symptoms compared to the teeth treated with calcium hydroxide. At the examination, just prior to extraction, all temporary fillings were judged to be adequate. Two teeth treated with EMDgel were sensitive to percussion. The reported symptoms in each tooth are shown in table 5.

Table 5 Pulpal status with regard to inflammation, classified from 0 (None) to 4 (Abscess formation). The mean number of classifications from the representative central sections (n = 5) from each experimental tooth is given. The inflammation was judged above the exposure in the proliferated pulp tissue, directly below the exposure and in the centre of the pulp. Mild (●) and moderate (●●) symptoms reported at any of the telephone interviews after the pulp capping procedure. No severe symptoms were reported. At the clinical assessment only mild sensitivity to percussion was reported.

<table>
<thead>
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<th>Subject No</th>
<th>EMDgel</th>
<th>Pulp status</th>
<th>Symptoms telephone</th>
<th>Calcium hydroxide</th>
<th>Pulp status</th>
<th>Symptoms telephone</th>
<th>Clinical Assessment; tenderness</th>
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* There were difficulties in finding one representative area of the pulp, therefore the pulp was assessed in two different areas (n= 10).
** This tooth showed a total necrosis throughout the entire pulp.
Pulpal status
The pulp had proliferated into the space initially occupied by the EMDgel (fig 2). Therefore the soft tissue reactions were judged in three different areas of the pulp; the proliferated pulp, just below the amputation site and in the central part of the pulp. Within the proliferated pulp tissue there were areas where the inflammation was judged to be moderate or severe. One tooth showed an abscess. Two teeth showed abscesses or extended lesions not only localized to the proliferated pulp. In the calcium hydroxide treated teeth there was no proliferation of the pulp tissue (fig 3). The majority of teeth showed no or minimal inflammation, though one tooth exhibited total necrosis. The pulpal status in each tooth is shown in table 5.

Figure 2 Micrographs of teeth from subject no. 1 and subject no. 4a who twelve weeks earlier had received a partial pulpotomy and had been treated with EMDgel. Staining with haemotoxylin and eosin.
Figure 3 Micrograph of a tooth from subject no. 8 who twelve weeks before had received a partial pulpotomy and had been treated with calcium hydroxide. Staining with haemotoxylin and eosin.

Hard tissue formation
The hard tissue in the EMDgel treated teeth was not formed as a bridge covering the pulp tissue, but hard tissue was formed alongside the dentine walls which restricted the area which the pulp had proliferated into. There was also hard tissue formed in patches within the proliferated pulp tissue. In one tooth treated with EMDgel that showed an abscess, there was no hard tissue formed. In the calcium hydroxide treated teeth the hard tissue was formed as a bridge covering the pulp tissue.

The amount of hard tissue in the EMDgel treated teeth was considerably larger than in the teeth treated with calcium hydroxide. The thickness of the hard tissue bridge that had been formed in the calcium hydroxide treated teeth was on average 148 µm, though the dentine bridge was considerably less thick in the junction with the predentine of the primary dentine.
Localization of EMD
Detection of EMD was made in all EMDgel treated teeth. It was localized within the patches of newly formed hard tissue as well as alongside the dentine walls which restricted the area into which the pulp had proliferated.

Characterization of the newly formed hard tissue (III)
EMDgel treated teeth
In the teeth treated with EMDgel there was hard tissue formed alongside the dentine walls which restricted the area into which the pulp had proliferated, however this did not appear to be attached to the dentine wall. The areas corresponding to predentine in this hard tissue did appear to be continuous to the predentine and odontoblastic layer in the pulp. Staining with the DSP and Col I-specific antibodies revealed the proteins to be both present in the primary dentine and more abundantly in the predentine. They were also localized in diffuse areas of the newly formed hard tissue. The cells located in the close proximity to the hard tissue formed were also stained for DSP and Col I (fig 4, table 6).

Figure 4 Micrographs of a tooth from subject no. 7 who twelve weeks earlier had received a partial pulpotomy and been treated with EMDgel. (a) New hard tissue is observed alongside the exposed dentine surfaces and in isolated masses within the proliferated pulp (arrows). A dentine chip (DC) is seen below the amputation site. Staining with haematoxylin and eosin. (b) Detail of (a) at a higher magnification showing the dentine (D), predentine (PD), the hard tissue formed alongside the exposed dentine surfaces (HTA) and patches within the proliferated pulp (HTP). (c) DSP staining (brown) is observed in the newly formed hard tissue and is marked in areas corresponding to predentine (arrow). (d) Col I staining (brown) was observed in the newly formed hard tissue, especially in the areas corresponding to predentine (arrow). The dentine (D) and predentine (PD) were used as positive controls for each antibody in each tooth section.
Calcium hydroxide treated teeth
In the teeth treated with calcium hydroxide the same appearance regarding the staining with DSP and Col I in the root was seen; it was stronger in the predentine than in the primary dentine. Diffuse Col I staining was seen throughout the hard tissue bridge. The DSP staining was also observed in the bridge, though with a more patchy appearance and with the same intensity as seen in the predentine areas. A layer of columnar cells, positive for DSP and Col I lined the hard tissue bridge (fig 5, table 6).

No bacteria could be detected in the wound area after staining with the modified Brown and Brenn technique or with the LIVE/DEAD® BacLight™ Bacterial Viability Kit.

Figure 5 Micrographs of a tooth from subject no. 4a who twelve weeks earlier had received a partial pulpotomy and been treated with calcium hydroxide. (a) The hard tissue was formed as a bridge (B) covering the pulp. (b) DSP staining (brown) is observed in the bridge (B) and is marked in areas corresponding to predentine (arrow). (c) Col I staining (brown) was observed in the bridge (B). The dentine (D) and predentine (PD) were used as positive controls for each antibody in each tooth section.
Table 6 Evaluation of the staining of DSP or Col I antibody in the EMDgel or calcium hydroxide treated teeth. In two teeth, one treated with EMDgel and one with calcium hydroxide, it was difficult to find one section representing the pulpal status and two different sections were chosen. In all, a total of 40 sections from 18 teeth were used for immunohistochemical staining. The primary dentine in each section was used as reference standard (= moderate). The table presents number of sections with moderate or strong staining of total number of sections in which recordings were possible.

<table>
<thead>
<tr>
<th></th>
<th>EMDgel (n = 10)</th>
<th>Calcium hydroxide (n = 10)</th>
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<tbody>
<tr>
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<td></td>
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<tr>
<td>The newly formed hard tissue</td>
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<td>10/10</td>
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<tr>
<td>Areas corresponding to predentine</td>
<td>8/8 a</td>
<td>9/9 b</td>
</tr>
<tr>
<td>Cells lining the newly formed hard tissue</td>
<td>9/9 c</td>
<td>9/9 b</td>
</tr>
<tr>
<td><strong>Moderate to strong Col I staining within</strong></td>
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<td>The newly formed hard tissue</td>
<td>10/10</td>
<td>10/10</td>
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<tr>
<td>Areas corresponding to predentine</td>
<td>8/8 a</td>
<td>10/10</td>
</tr>
<tr>
<td>Cells lining the newly formed hard tissue</td>
<td>5/7 a</td>
<td>6/6 a</td>
</tr>
</tbody>
</table>

a In 2 of the 10 sections, no areas corresponding to predentine could be observed. 
b One tooth showed total necrosis, though a hard tissue bridge could be observed. 
c In one section, no cells lining the newly formed hard tissue could be observed. 
d In 3 of the 10 sections, no cells lining the newly formed hard tissue could be observed. 
e In 4 of the 10 sections, no cells lining the newly formed hard tissue could be observed. One tooth showed total necrosis, though a hard tissue bridge could be observed.
Effects of bacterial products on the activity of odontoblast-like cells and their formation of Col I (IV)

Identification of bacteria

The carious dentine sample contained in total 15 different bacterial strains. The predominant species were Lactobacilli. Gram-positive pleomorphic rods, most probably *Actinomyces* spp and Gram-positive cocci were also present. A selection was made to represent different strains according to morphology and growth conditions. The three strains that were able to grow in DMEM supplemented with FBS and glutamine were used in the experiments and were identified by 16S rRNA gene sequencing to be:

- *L. rhamnosus*
- *L. casei*
- *S. satelles*

Effects of bacterial products on the activity of MDPC-23 cells

When the MDPC-23 cells were exposed to the biofilm supernatants from the three strains (*L. rhamnosus, L. casei, S. satelles*) taken from the deep caries lesion, no significant effect on the activity could be noted. However, the root canal isolate *E. faecalis* reduced the activity of the MDPC-23 cells significantly (mean % of control ± SE = 80 ± 2.7; p < 0.05) (fig 6). Consequently, different bacterial species differentially affected the activity of odontoblast-like cells.
Figure 6 Effects of the biofilm supernatant on the activity of MDPC-23 cells over 96 hours. The absorbance values were divided by the absorbance of the controls and expressed as a percentage (100%). Thus values greater than 100% represent an increase and those below 100%, a decrease in activity. Values presented are mean % ± SE of values from three independent experiments (** = p < 0.01).

LTA, the cell wall component of Gram-positive bacteria, significantly reduced the activity of the MDPC-23 cells (mean % of control ± SE = 73.4 ± 3.5; p < 0.001). LPS, the cell wall component of Gram-negative bacteria did not influence the activity of the MDPC-23 cells.
Effects of bacterial products on the production of Col I by MDPC-23 cells

To evaluate whether the extracellular bacterial products can influence the production of Col I by the MDPC-23 cells, the cell layers were subjected to ELISA. The biofilm supernatant from *L. rhamnosus* as well as the *S. satelles* taken from the deep caries lesion had no significant effect on the Col I production by the MDPC-23 cells. The bacterial supernatant from the carious sample containing *L. casei* did however significantly increase the production of collagen (134% ±12.1%, p < 0.05) while the biofilm supernatant from *E. faecalis* caused an approximately 50% reduction in collagen production (p < 0.05). Again, different bacterial strains, as well as different bacterial species, differentially affected the collagen production.

LTA did not affect the production of Col I; however LPS significantly increased the production by almost doubling the amount compared to the control (195.4% ±12.2%, p < 0.01).
DISCUSSION

The aim of this thesis has been to study some aspects of the repair of dentine, especially in conjunction with dental pulp capping.

The main findings from the systematic review combined with the new literature search were (i) that based on the limited scientific evidence available the use of calcium hydroxide based pulp capping materials frequently results in the formation of hard tissue covering the pulp exposure and (ii) the use of Emdogain® Gel or bonding materials as a pulp capping agents does not result in the formation of hard tissue covering the pulp exposure. (iii) Scientific evidence is lacking as to whether teeth treated with MTA as a pulp capping material show hard tissue formation more frequently than calcium hydroxide based materials. The results from the clinical study testing EMDgel as a pulp capping agent showed that greater amounts of hard tissue were formed in the EMDgel treated teeth, although not in an arrangement that could constitute a barrier. The dentine functions seem to be important for the defence of the pulp tissue. Our studies revealed that the cells aligned with the newly formed hard tissue as well as the hard tissue itself contained DSP and Col I. This indicated that the cells were of the odontoblast lineage and the newly formed tissue could be regarded as dentine. The main findings from the experiments using a cell-line of mouse odontoblasts that formerly had been shown to express DSP and Col I, indicated that the responses with regard to activity and initial formation of tertiary dentine varied upon exposure to different exo-products from bacteria grown in biofilms.
Endpoint measures
As stated in the introduction, repair of the exposed pulp should ideally restore the original structure and the biological functions of the damaged soft and hard tissues, however this is a difficult task to evaluate and use as an end-point measure. Instead, different surrogate end-points such as whether the hard tissue covered the pulp wound and whether the pulp showed any signs of inflammation are often used and this is the case in this thesis. To the patient, the outcome of pulp capping that probably matters is to have a functional tooth without any symptoms and with a low risk for future pain. This is only an assumption, since no studies on patients’ preferences regarding pulp capping procedures currently exist.

The measures used here have primarily been based on histological studies and cell culture, even though some clinical information such as pain or discomfort from the treated teeth has been collected.

Systematic review
The systematic review included studies with a variety of study designs and only case reports were excluded since we wished to make an assessment of all available articles on the subject. In order to minimize the risk of missing relevant publications the search strategy used in PubMed was broad and the reference lists of included articles as well as reviews were hand searched in order to find articles that had not been identified by the search in PubMed. The fact that the new literature search only identified additional publications through the search in PubMed indicated that the search strategy was comprehensive. No additional publication could be found through the hand searching of reference lists or searching CENTRAL when performing the new literature search. We have chosen to include and present the results from all studies, even those with low quality, in order to aid the planning of future studies of pulp capping that will be assessed histologically.

The included studies were mostly performed in teeth without any signs of caries which is understandable since there may be difficulties in finding teeth and subjects that are suitable for such a study.
However choosing such a strategy affects the external validity of the results as the most common reason for exposure of pulps is caries. It is possible that different results may have been obtained if the experiments had been performed in teeth with pulps inflamed due to caries.

The majority of the studies included in the published systematic review were judged to have low quality. At the time of the publication of the systematic review only a few such reviews within the field of endodontics could be found through searches in PubMed, six years later an identical search yielded several hundred. The result of the systematic review was surprising at the time in that the quality of the retrieved publications was assessed to be so low. The same findings have however been the case in many systematic reviews. It is obvious that there is an evolving awareness of the importance of a proper study design and there was indeed a positive difference between the studies included in the published systematic review and those identified through the new literature search. The majority of the newer studies were performed as randomised controlled trials and 6 out of 20 were judged to have at least a moderate quality compared to 1 out of 21 studies in the published systematic review. Some of the improvement in the execution and reporting of the studies identified through the new literature search can probably be attributed to the growing awareness of evidence-based health care.

Nonetheless, there are still some major issues that should be considered; such as performing a power analysis prior to conducting the study based on the anticipated difference in effect that would be clinically significant. Such power analyses were lacking in the studies identified. Moreover, in many studies the material was divided into subgroups of different time intervals with very few teeth in each group, which means that an even larger sample size is needed to get valid data from the subgroups. In this context it is also obvious that the choice of control material affects the sample size. For instance; testing a MTA-based material and having a composite resin material as the control would mean that a very small sample would be needed to detect a difference since a consid-
erable difference can be anticipated. This is not to say that such a control material should be used. It seems most adequate to use a calcium hydroxide containing material such as a slurry of calcium hydroxide powder mixed in water, since calcium hydroxide has frequently been associated with hard tissue formation covering the pulp exposure and is the control material most often used in pulp capping studies. Another quality issue is the criteria used when evaluating the histological sections; many different criteria are used and if our hypothesis that healing with tissue with the functions of normal dentine is crucial for the long-term success of pulp cappings, a criterion such as “attempted bridging” seems difficult to assess. It is also obvious from the clinical study (II) within this thesis that serial sectioning and assessment of all sections is important since the hard tissue formation and the status of the pulp may vary throughout the exposure site of the pulp.

It is interesting to note the difference with regard to pulp tissue reactions seen in the human compared to other species. In the systematic review that was limited to human material, hard tissue formation was seldom observed in teeth that have been pulp capped with dentine adhesive materials, in contrast to the frequent hard tissue formation described in animal studies (Hafez et al. 2002, Kitasako et al. 1999; Olmez et al. 1998). A possible explanation for the occurrence of some cases of hard tissue formation in humans may be that the odontoblast layer has been left partially undisrupted during the pulp capping procedure.

Effects of EMDgel
In the literature there are reports of the use of different proteins such as growth factors in order to mimic dentinogenesis (Lovschall et al. 2001; Li & Sae-Lim 2007; Rutherford et al. 1993, Nakashima 1994, Decup et al. 2000, Zhang et al. 2008). These are all performed in animal models. Study I in this thesis identified two studies Kiatwateeratana (2009) and Olsson (2005) (the latter being Study II in this thesis), performed in humans in which dentine repair after pulp capping was induced by using material that would theoretically have the potential to mimic a part of the dentinogenesis process. These studies could be performed in hu-
mans since the experimental pulp capping material was already approved for use in patients with periodontal disease and had also been piloted in animal studies (Nakamura et al. 2001; 2002). The animal studies showed however a slightly different result to the ones performed in humans in that there was extensive hard tissue formation, not only localized to the pulp wound, but narrowing the whole pulp chamber.

Study II was designed as a pilot study, and had a limited sample size. However, care was taken to avoid unnecessary methodological faults. The materials were placed according to a randomization procedure that was performed immediately before placement of the pulp capping material. The person assessing the teeth for symptoms as well as the person assessing the histologic sections were blinded. On evaluation it was found that the pulp tissue reactions differed markedly in all EMDgel treated teeth from those in the control group treated with calcium hydroxide, and these reactions were therefore considered representative in spite of the limited sample size.

Calcium hydroxide should probably be the control material of choice in most pulp capping studies since the appearance in the histological sections treated with calcium hydroxide is quite characteristic when performed correctly. Nevertheless, in Study II it might be argued that PGA, which was the vehicle for EMD, should have been used as a negative control. Even more so since a similar appearance of the pulp and the newly formed hard tissue was seen in experimentally pulp capped monkey teeth after a disc of Teflon was placed on the pulp exposure. However there was no hard tissue formed in patches as seen in Study II (Heys et al. 1990). A study conducted in rats using PGA as a negative control showed that reparative dentine was formed both in the teeth treated with EMDgel and the negative control, though the formation was more extensive in the EMDgel group (Igarashi et al. 2003). We used an antibody to detect the EMD in the histological sections and it was present in close proximity to the newly formed hard tissue, giving some support to the idea of EMD being able to promote dentine repair in humans.
The hard tissue formed after placement of EMDgel cannot act as a barrier, as it was formed in patches and did not cover the pulp wound. The amount of hard tissue was however substantial and questions about how these patches of hard tissue would progress over time arose. In a similar study to ours, Kiatwateeratana et al. (2009) used EMDgel as a pulp capping material and had a longer observation period than the one used in Study II. Their results showed that the hard tissue formation was comparable to ours and even after 6 months the hard tissue masses did not close the pulp exposure.

The teeth used in Study II were free from caries which means that at the time of the pulp capping procedure all pulps were considered to be free from inflammation. Thus, the inflammatory response seen in the histologic sections could be due to the pulp capping materials themselves or oral micro-organisms that reached the wound area through micro-leakage along the restoration margins. It should be stressed that the classification of the pulpal status was made in the area which showed the most severe class of inflammation. In the EMDgel treated teeth where the pulp had proliferated into the space initially occupied by the EMDgel there was also a higher density of cells resembling pulp fibroblasts with no other signs of inflammation. In an attempt to study if the presence of micro-organisms could be associated with the inflammation observed in some of the histologic sections, bacterial staining was carried out. In spite of the fact that some of the sections showed areas of necrotic pulp, in which one would have expected to detect bacteria with the techniques used, no micro-organisms were discovered. The reason for this is not known but it cannot be ruled out that bacteria have been removed as the temporary fillings were removed with a bur before serial sectioning or during the actual processing of the sections (Wijnbergen et al. 1991). Thus, no direct cause of the inflammation could be established in Study II.

The hard tissue was characterized using DSP and Col I. A wide range of differentiation markers has been suggested, but there seems to be some consensus among researchers dealing with formation of dentine that SIBLING proteins, such as DSP, in combi-
nation with Col I are suitable phenotypic markers for this tissue and the odontoblast (Chen et al. 2008, Hao et al. 2009). Staining with the DSP and Col I-specific antibodies was also present in diffuse areas of the newly formed hard tissue. The cells located in close proximity to the formed hard tissue were also stained with DSP and Col I indicating that they could be of the odontoblast lineage. These findings are in accordance to the ones reported by Nakamura et al. (2004) who had performed a similar study of pulp cappings in pigs. Finding a positive control may be difficult, but we had the advantage of being able to use primary dentine and odontoblast from other parts of each tooth section for this purpose. The staining with the DSP and Col I-specific antibodies revealed the proteins to be presented both in the primary dentine and more abundantly in the predentine, which perhaps could be explained by the higher content of extracellular matrix in the predentine. DSP can also be detected in bone, however in considerable smaller amounts than in the dentine (Qin et al. 2002). Since the staining of DSP in the newly formed hard tissue was equal to or stronger than the staining of the primary dentine in each section, we consider our results to be valid. Characterizing the hard tissue repair with regard to the morphology and the markers DSP and Col I is a way to approach the goal of evaluating whether the original structure and biological function of the dentine still remains.

Lesser symptoms were reported in teeth that had been treated with EMDgel than teeth treated with calcium hydroxide. The children who participated in the study were interviewed by a blinded examiner about any pain or discomfort from the teeth and they were also asked to report any pain on a VAS. The validity of VAS used in young children has been shown to be low (Shields et al. 2003). As reported by the Swedish Council on Health Technology Assessment (2010) the scientific evidence for the relation between symptoms and the assessments of inflammation in histologic sections is lacking, as only one study with moderate quality could be found (Hasler & Mitchell 1970). In the study by Hasler & Mitchell there is no direct correlation between symptoms and histological appearance and this was also the case in Study II. The reports of pain did not directly relate to the assessment of inflammation in the histo-
logic sections, though the two teeth classified with the most severe grade of inflammation also presented with tenderness on percussion.

Effects of bacterial products on odontoblast-like cells
In order to investigate how extracellular products from biofilms of bacteria found in a deep caries lesion affect the viability of odontoblast-like cells and their formation of Col I a fresh microbial sample was taken from a deep caries lesion. This sample was shown to be dominated by Gram-positive rods and the majority of the isolates were identified as Lactobacillus spp which is in accordance to others who have studied the microbiota of deep carious lesions (Chhour et al. 2005; Gross et al. 2010). Further identification using 16S rRNA of the three species used in the experiment showed that the identified lactobacilli both belonged to the L. casei group which together with L. salivarius and L. fermentum have previously been found to be prominent at the advancing front of a progressive caries lesion (Byun et al. 2004). Munson et al. (2004) identified L. casei and L. rhamnousus to be prominent in teeth with carious lesions that had spread to the inner third of the dentine as seen on x-ray by using 16S rRNA analysis. Lactobacilli have also been identified as prominent members of the microbial community in the initial stages of root canal infection (Nadkarni et al. 2010). The third species from the caries lesion was identified as S. satelles. The Shuttleworthia spp was identified in 2002 by Downes et al. amongst isolates from periodontal pockets and subsequently also been isolated from an infected root canal (Jacinto et al. 2007). Little is known about the role of this genus in the oral cavity. Shuttleworthia satelles have been identified as a pathogen as it was found forming a monoculture on a prosthetic mitral valve in a patient suffering from endocarditis (Shah et al. 2010) but has also been identified in carious lesions (Chhour et al. 2005; Gross et al. 2010; Munson et al. 2004). It is not solely Gram-positive species, as identified here, which may be found in deep caries. Gram-negative micro-organisms such as Prevotella intermedia and Porphyromonas endodontalis have also been found at low levels in deep caries (Massey et al. 1993, Martin et al. 2002). Therefore both LPS and LTA were used for comparison in the experiments.
The supernatants from the three strains isolated from the deep caries lesion and grown in biofilms had no effect upon cell activity. This suggests that under these experimental conditions, none of the strains released substances that led to cell death or cell cycle arrest in the odontoblast-like cells. In contrast, the supernatants from biofilms of the root canal isolate of *E. faecalis* caused a decrease in total activity of the MDPC-23 cells. Visual observation indicated that the number of cells present after exposure was lower than that in the control cultures and extracellular substances from biofilms of *E. faecalis* thus appeared to inhibit proliferation. *E. faecalis* have a number of virulence factors (Kayaoglu & Ørstavik 2004) that could cause the effects seen here.

The Col I production was used as a marker to study the effects of bacterial exo-products upon the odontoblasts synthesis of tertiary dentine since collagen is abundant during the formation of predentine. Cells exposed to the extracellular products from *E. faecalis* demonstrated a lower production of Col I, even though this effect can partially be attributed to a reduction in cell activity, *E. faecalis* appears to have a small inhibitory effect on the production of Col I. In contrast, the isolate of *L. casei* from the deep caries lesion caused a significant up-regulation of the production of Col I. The mechanisms underlying these effects are unknown but since no change was seen with LTA, it appears unlikely that activation of toll-like receptor 2 is involved in these processes. LPS had a significant up-regulatory effect upon the production of Col I, indicating that upon activation of toll-like receptor 4, the formation of tertiary dentine may be enhanced.

The cell line used in the experiments, MDPC-23, is a well-established model. The cells are deemed to be of the odontoblast lineage by virtue of their expression of both DSP and Col I (Hanks *et al*. 1998). As pointed out before, there seem to be a difference in pulp reactions between different species and it cannot be ruled out that other results may be acquired using human odontoblasts.
Future studies
During the work with this thesis several ideas of future work have been evoked, here a few brief thoughts are presented:

- Before conducting any other pulp capping studies using EMDgel, the formula of EMDgel should be altered as the formula with PGA does not seem to promote dentine repair which would be able to act as a barrier. One study of EMD used in conjunction with other pulp capping materials has recently been published (Al-Hezaimi et al. 2011).

- There is a lack of health economic evaluations within the field of endodontics. Root canal treatments seem to be difficult to perform in children and young persons (Ridell et al. 2006). Even though recent studies of pulp cappings in carious teeth (Bjørndal et al. 2010; Miles et al. 2010) have shown somewhat discouraging results, pulp capping procedures performed in children and young persons may have a better prognosis. This has generated an idea for a future study with a health economic evaluation of pulp cappings performed on caries exposures in children.

- Another area of interest would be to study the patients’ preferences. In the study of Bjørndal et al. (2010) a large proportion of teeth with very large caries lesions that received a pulp capping procedure did in fact develop painful conditions and the patients had to undergo emergency root canal treatment before the 1-year clinical evaluation. It would be valuable to know the patients’ treatments preferences in this perspective.

- As the result from Study IV indicates differences in how the odontoblasts are affected by the products from the biofilms, it would be interesting to develop this model further with polymicrobial biofilms under different environmental conditions.
CONCLUSIONS

Based on the limited scientific evidence available, calcium hydroxide based pulp capping materials frequently result in the formation of hard tissue covering the pulp exposure, something the use of Emdogain® Gel or bonding materials as a pulp capping agents do not. Scientific evidence is lacking as to whether teeth treated with MTA as a pulp capping material show hard tissue formation more frequently than calcium hydroxide based materials. In order to be able to detect treatment effects, future studies should be designed as RCTs with a prior sample size calculation.

Using EMDgel as a pulp capping agent in experimentally exposed human pulps gave greater amounts of hard tissue, but more pulp inflammation compared to the controls which were treated with calcium hydroxide. However the hard tissue was not formed as a bridge and thus could not provide a barrier in contrast to the hard tissue formed in the calcium hydroxide treated teeth in which the hard tissue covered the pulp wound.

The hard tissue formed after treatment with EMDgel or calcium hydroxide as well as the cells lining it, contained the dentine markers DSP and Col I, indicating that the hard tissue formed could be regarded as dentine and the cells to be odontoblasts.

The extracellular products from biofilms of bacteria found in a deep caries lesion did not affect the activity of odontoblast-like cells, as was seen with extracellular products from E. faecalis taken
from an infected root canal. However the synthesis of type 1 collagen was affected to various extents, suggesting that the bacterial species in a caries lesion may influence dentine repair.
CONTRIBUTORS TO THE STUDIES
Items according to International Committee of Medical Journal Editors requirements for authorship

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<th>Description</th>
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<td>(IV) Effects of bacterial products on the activity of odontoblast-like cells and their formation of collagen I</td>
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REFERENCES


Dental and Pharmaceutical Benefits Agency’s reference price list (Tandvårds- och läkemedelsverket)
http://www.tlv.se/tandvard/referensprislista/#500


Guyatt GH, Sackett DL, Cook DJ. Users’ guides to the medical literature. II. How to use an article about therapy or prevention. A. Are the results of the study valid? Evidence-Based Medicine Working Group. JAMA 1993; 270: 2598-601.

Guyatt GH, Sackett DL, Cook DJ. Users’ guides to the medical literature. II. How to use an article about therapy or prevention. B. What were the results and will they help me in caring for my patients? Evidence-Based Medicine Working Group. JAMA 1994; 271: 59-63.


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Li Z, Sae-Lim V. Comparison of acidic fibroblast growth factor on collagen carrier with calcium hydroxide as pulp capping agents in monkeys. Dent Traumatol 2007; 23: 278-86.


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