Dentine Permeability and Cytotoxicity of a Dentine Bonding Agent

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Declaration

I, Alexander Hermann du Bois, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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_____ Day of _____ of 2006

Abstract

In vitro cytotoxicity studies have gained tremendously in popularity in recent years. To make these *in vitro* studies more clinically relevant, diffusion experiments in an *in vitro* pulp chamber device (IVPC) using dentine discs as a barrier have been repeatedly advocated. The objectives of this study were to create an *in vitro* situation which closest resembled the clinical situation by inclusion of human dentine discs at a specified anatomic location as barrier and evaluating if comparable or graded perfusion could be identified, as well as whether a link existed between dentine thickness and permeability. The other leg of the study was to evaluate the cytotoxicity of Xeno III, one of the newly released "new generation dentine bonding agents", and to evaluate its cytotoxicity in an IVPC, determining which thickness of dentine would be able to protect the dental pulp from noxious substances in the bonding agent.

This aims at creating an *in vitro* model with strong clinical relevance, which could be used as benchmark in the assessment of biocompatibility of new materials, setting a platform on which different materials could be compared to each other internationally.

Materials and methods: Freshly extracted, intact human molar teeth were cut just coronal of the pulp horns creating discs which then were ground to a thickness of approximately 1000µm, 750µm, 500µm, 350µm, 200µm and 120µm respectively. To remove the smear layer, the discs were etched and washed in water. In the **perfusion experiments** the discs with thickness of 120µm, 200µm, 350µm and 500µm were placed in an *In vitro* Pulp Chamber (IVPC) under 300mm hydraulic pressure and the time was measured for 0.5µl to diffuse through the respective discs by monitoring the flow in a capillary connected to the system.

In the **cytotoxicity experiments** Xeno III was tested in extract tests using membranes, 120µm, 500µm, 750µm and 1000µm dentine discs. To produce the extract, Xeno III was

placed and cured on the different barriers in a modified IVPC and 24h allowed for the leachable components to be extracted. The extract was then added to 3T3 mouse fibroblasts in a strong growing phase and left for a further 24 hours. Cell viability was evaluated using MTT assays.

As part of this study the discs treated with Xeno III were studied with a **Confocal LASER Microcope** (CLMS) after the teeth were prepared accordingly. The cells which were exposed to Xeno III extract were studied with an inverted light microscope at 50 times magnification.

Results: When the relationship of disc thickness and **permeability** between discs of different thickness was studied, it was found that there was a large variation in the data at a specified thickness but still a clear link between thickness and permeability could be seen. For the permeability results of the 120µm, 200µm and 350µm discs compared to the 500µm discs, it was observed that the coefficient of determination is 39%, i.e. 39% of the variation could be explained by the difference in thickness. It was also established that the permeability became really evident at a dentine thickness of more than ~350µm. Statistical analyses demonstrated a significant link between thickness and perfusion. As a result a perfusion range could be predicted for any disc.

In the **cytotoxicity experiments** it was found that for the membrane group as well as for the dentine discs with a thickness of 120µm, cell viability was around 25% compared to the control which was 100%. When cytotoxicity was evaluated for the 500µm disc as barrier, cell viability was at 60.7%. Although this result was significantly higher, it still showed considerable cell death (39%). In the 750µm group cell viability was much higher at 75% but cell death was still noticed when compared to the control. Only at a disc thickness of 1000µm and above no cell death was observed. The **CLMS studies** showed deep penetration of Xeno III into the dentine tubules with marked resin tag

formation. When cells were studied under the **light microscope** cell death and changes in cell morphology were noticed in the cells exposed to Xeno III.

Conclusions:

Permeability: From these findings it appears that for standardization purposes in *in vitro* studies human dentine as barrier for cytotoxic tests is impractical, as too large variations in the perfusion results were found. Thus variation in data can easily be attributed to variation in dentine permeability. Furthermore, dentine permeability does not increase in a linear fashion but rather exponential to the increase in dentine thickness.

Cytotoxicity: Xeno III causes visible cell death and reduced cell viability in *in vitro* studies and is thus not biocompatible. Secondly, in the light of Xeno III, used in this study and dentine permeability, 500µm remaining dentine thickness can not be seen as benchmark to ensure that no pulp reaction takes place. From the results it can be concluded that it is unsafe to use Xeno III in cavities with a remaining dentine thickness of less than 1000µm. Therefore, it is suggested that an artificial membrane should be chosen as a barrier for these *in vitro* cytotoxic experiments. Artificial membranes are really standard, cheaper and setting a level by which materials can be tested and compared world wide.

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Chapter 1

Literature review

The placing of potentially cytotoxic materials like dentine bonding agents (DBA) (Camps, Tardieu, Dejou, *et al* 1997; Chen, Liu, Tseng, *et al* 2003; Huang & Chang. 2002; Szep, Kunkel, Ronge, *et al* 2002) or Composite restorations (Franz, Koning, Anglmayer, *et al* 2003) close to the pulp after the smear layer has been removed is a common practice in dentistry and is gaining popularity as a result of growing esthetic requirements (Szep, Kunkel, Ronge, *et al* 2002). As these materials have contact with various aqueous media *in vivo,* which may extract leachable components (Ratanasathien, Wataha, Hanks, *et al* 1995; Rawls, Marshall, Cardenas, *et al* 1992), the biocompatibility of these materials is of utmost importance (Geurtsen, Spahl, Müller, *et al* 1999). The optimal way to test and produce results would be doing *in vivo* tests where the system is used under clinical conditions; however this is seldom possible. As a result, considerable interest has recently been focused on cytotoxicity assays using cell culture to evaluate toxicity of dental materials.

Cell culture methods for toxicity testing of dental materials have important technical advantages compared with animal studies: they are easy to perform, inexpensive (Imazato, Tarumi, Ebi, *et al* 2000), simple, reproducible, relevant and suitable for the evaluation of basic biological properties of dental materials (Huang & Chang, 2002). Furthermore, Schamlz, Schuster, Koch, *et al* (2002) state that contradictory results have been reported on direct pulp capping with dentine bonding agents in animal experiments ranging from pulp necrosis to no damage and dentine bridge formation and laying down of collagen -1α (Demarco, Tarquinnio, Jaeger, *et al* 2001; Mc Lachlan, Smith, Sloan, *et al* 2003). Another problem is the fact that animal experiments are controversial in the

public mind and time-consuming, which puts another burden on these types of experiments (Schuster, Schmalz, Thonemann, *et al* 2001). Cell culture methods, on the other hand, are better standardized and reproducible as well as fast and easy to perform at relatively low cost.

Several *in vitro* methods have been developed in the past years to overcome the limitations of *in vivo* tests (Guigand, Pellen-Mussi & Le Goff. 1999; Hanks, Graig, Diehl, *et al* 1988; Schmalz 1994). In the early tests, materials were placed in direct contact with the cells in monolayer culture and cell number was used to monitor cytotoxic effects (Hanks, Anderson, & Graig. 1981). Placing materials directly on cell cultures was an appropriate test for pulp capping materials (Bouillaguet, Gysi, Wataha, *et al* 2001). They also stated that during experiments conducted they found variation in results obtained from direct contact tests and extract tests, with direct contact tests delivering more legitimate results but probably inappropriate for most dental materials placed on dentine, since the dentine markedly changes the biological response (Pashley, Derksen & Tao. 1988 and Imazato, Tarumi, Ebi, *et al* 2000). It was consistently demonstrated that dentine is an effective diffusion barrier, preventing pulp damage not only from toxic substances such as eugenol and phenol, but also from HEMA (Lonnroth & Dahl, 2001; Schmalz, Schuster, Koch, *et al* 2002). Hanks, Graig, Diehl *et al* (1988) showed that dentine can reduce the toxicity of resins by limiting diffusion of those substances from the cavity preparation to the pulp cavity. Abou Hashieh, Franquin, Cosset, *et al* (1998), found a clear link between dentine permeability and cytotoxicity and Prati, Venturi, Valdre, *et al* (2002) noted that tooth brushing could even alter dentine permeability where Poonam, Reinhardt & v Krell. (2000) stated that permeability values increased three times in the presence of a smear layer. Dentine probably absorbs substances in the tubules and also limits the traverse of substances through the dentine (Hanks,

Wataha, Parsell, *et al* 1994). According to Pashley, Derksen & Tao (1988), the main reason for the decreased cytotoxicity when using dentine discs is due to the large internal surface area of dentine, binding and trapping a variety of substances. Furthermore the barrier effect of dentine was clearly shown when dentine discs of increasing thickness reduced toxicity (Bouillaguet, Virgillito, Wataha, *et al* 1998; Hanks, Diehl, Graig, *et al* 1989; Schmalz, 1994; Schmalz, Schweikl, Esch, *et al* 1996). Vajrabhaya, Pasasuk & Harnirattisai (2003), also stated that dentine thickness influences the concentration and amount of bonding agents that penetrate through dentine into the pulp space. According to this author the diffusion rate should be inversely proportional to the dentine thickness and directly proportional to the fraction of the cross-sectional area of the dentine composed of dentine tubules. Ficks law states this as the rate of diffusion which is dependent on the applied concentration, but inversely proportional to the dentine thickness (Schmalz, Hiller, Nunez, *et al* 2001b). The surface area available for diffusion, the temperature and the chemical characteristics of the diffusing molecule all affect diffusion (Bouillaguet , Wataha, Hanks *et al* 1996).

Hanks, Wataha, Parsell, *et al* (1994), found that the concentration of Bis-phenol A diglycildyl-methacrilate (Bis-GMA) shown to be the monomer with the highest cytotoxicity, followed sequentially by urethane dimethacrylate (UDMA), triethylene glycol dimetacrilate (TEGDMA) and 2-hydroxyethyl methacrilate (HEMA), (Chen, Lui, Tseng, *et al* 2003; Abou Hashieh, Cosset, Franquin, *et al* 1999) was decreased by a factor of up to 1500 if it diffused across a dentine disc of 450µm resulting in a concentration too low to damage the pulp. Camps, About, van Meerbeek, *et al* (2002) also conducted tests on the cytotoxicity of resin-based desensitizing agents containing HEMA and found that the cytotoxicity was less than 20% (highest 12% for Seal and Protect and lowest for Isodan

at 0%). This is also confirmed by Vajrabhaya, Pasasuk & Harnirattisai (2003), who states that there is some controversy regarding whether pulp is damaged by diffusible hydrophilic resins, which is the main component of bonding agent, especially if the remaining dentine above the pulpal tissue is 500µm. In a similar study, Schmalz, Schuster, Koch, *et al* (2002) stated that low pH bonding agents (All-Bond 2, Prime&Bond NT, Syntac Classic and Prompt -L- Pop) did not show toxic reactions in a dentine barrier test and that pulp damage by the tested substances was unlikely if a dentine layer protects the pulp. On the other hand, de Souza Costa, Lopes do Nascimento & Teixeira (2002), found that moderate inflammatory response, disorganization of pulp tissue as well as deposition of a thin layer of reactionary dentine were observed in teeth in which the remaining dentine thickness was less than 300µm between Scotch Bond One (3M) and the pulp. As possible reason it is stated that this may be due to the deep infiltration of the resin tags and bonding agent diffusion through dentinal tubules. Hanks, Fat, Wataha, *et al* (1993) investigated the diffusion of hydrogen peroxide (H_2O_2) through 500µm discs in an IVPC and found the H_2O_2 being severely toxic through the barrier.

Another point seldom considered was that the cells must be grown directly on the dentine discs so that the effect of the dilution could be minimized. In these modified dentine barrier tests the diffusates from the material act through dentine on the cells with little dilution of medium. These results could thus coincide more closely with the clinical response of superficial pulp cells (Imazato, Tarumi, Ebi, *et al* 2000). Hanks, Diehl, Graig, *et al* (1989), found that cells on the pulpal surface of the dentine discs experienced more severe toxicity than cells on glass cover slips on the bottom of the chamber. This indicated that the medium in the chamber further diluted the toxin, and suggests that

cells deep in the dental pulp are much better protected from the direct effects of the toxic compounds than are the odontoblasts. However, as Hanks, Wataha, Parsell, *et al*. (1994) pointed out, the possible drawback of this modified method is the difficulty in achieving consistent plating of cells at reproducible concentrations on dentine discs due to the biological variability of dentine, and for large series of experiments, its availability is restricted. In their publication Vajrabhaya, Pasasuk & Harnirattisai (2003), state that unfortunately dentine permeability *in vivo* is not a constant. It can be changed when cavity preparation has been performed. It is a response of the pulpodentinal complex to irritation in a vital tooth. Large plasma protein in the pulpal fluid, such as fibrinogen, could be absorbed through the dentinal tubule wall. This will result in a decrease in dentine permeability.

Because filling materials are placed into cavities whose walls are composed of dentine, their direct toxic effect will be most likely on the pulp cells beneath the cavity (Schuster, Schmalz, Thonemann, *et al* 2001).

Therefore the choice of cell line for *in vitro* cytotoxicity screening assays also remains controversial, as the cell line selected for the test can significantly affect the apparent cytotoxicity of a material (Huang & Chang 2002). The use of permanent cell lines, such as transformed mouse fibroblasts (clone L-929) provides good reproducibility for *in vitro* cytotoxicity screening (Murray, Lumley, Ross, *et al* 2000; Schmalz, 1988). However, according to Szep, Kunkel, Ronge, *et al* (2002), human gingival fibroblasts proved more suitable than L929 cells for material testing. The Golgi apparati of L 929 cells were identified less frequently than those of primary human gingival fibroblasts and the author concluded that gingival fibroblasts were more resistant to environmental impact. After only 4h of exposure to the test materials, the organelles of the L-929 cells had been almost completely destroyed. It has been stated that primary cell cultures derived from

oral tissues seem to be more relevant to the clinical situation (Arenholt-Binslev & Horsted-Binslev, 1989; Seux, Couble, Hartmann, *et al* 1991; Szep, Kunkel, Ronge, *et al* 2002). Murray, Lumley, Ross, *et al* (2000) also found that the sensitivity of transformed cell types (when these are created to produce permanent cell lines) might deviate from the original primary cell. It was also stated that the results of biocompatibility tests obtained by various cell cultures could only be compared with restriction (Schuster, Schmalz, Thonemann, *et al* 2001) as dental pulp cells show different sensitivity to cytotoxic tests than murine cells (Schmalz, Schuster, Thonemann, *et al* 2001a) and donor age also influenced cell recovery (Shiba, Nakanishi, Rasi, *et al* 2003). Stable pulp derived cell lines with a metabolism close to odontoblasts are desirable. According to Schmalz, Schuster, Koch, *et al* (2002), IVPC tests should aim at combining the advantages of cell culture experiments (comparatively high degree of standardization) with an approach that includes dentine and target cells with a metabolism resembling that of pulpal cells in primary culture, and thus closely simulating the clinical situation in primary culture.

According to Freshney (1993), it must be kept in mind that *in vivo,* the target tissue is also influenced by a vast amount of chemical mediators. So for instance, Yang, Tsai, Huang, *et al* (2003) researched the role which the human pulpal fibroblasts (HPF) play during inflammatory processes and found that HPF additionally contribute to the orchestration of imuno- participant cells in the host defense network of pulpal disease, which could alter results. Adding to this, Trantor, Messer & Birner. (1995), found that substance P and calcitonin gene related peptide, which are secreted by the body as part of the normal inflammatory process, produced elevated levels of cell proliferation in human pulpal fibroblasts. Shiba, Nakanishi, Rasi, *et al* (2003) as well as Jukič, Prpic, Talan-Hranilovc, *et al* (2003) demonstrated the presence of estrogen receptors in dental

pulp and Dale, Sarich, Bretz, *et al* (2002) showed the presence of androgen receptors in the dental pulp and suggested that these may be altered by age and the presence of certain steroids and cytokynes. These as well as other receptors could alter the progression of tissue repair and the outcome of cytotoxicity. In an interesting and promising study, Magloire, Joffre & Bleicher (1996) conducted research on thick slices from human teeth drilled immediately after extraction and cultured for three days to one month. The results of their study showed that the damaged pulp beneath the cavity is able to develop some typical aspects correlating to tissue healing, evidenced by cell proliferation, neovascularization and the presence of functional cuboidal cells close to the injury area. After 30 days of culture, elongated spindle-shaped cells could be seen aligned along the edges of the relevant dentine walls, whereas sound functional odontoblasts were well preserved beneath healthy areas. In a similarly interesting study Calland, Harris & Carnes (1997) found that human pulpal cells in their 3rd to 8th passage responded to stimulation of calcitonin gene-related peptide *in vitro*, stimulating BMP-2 production which is a component of pulpal response to injury and associated with dentine formation.

Although it is suggested to use human pulpal fibroblasts to increase clinical relevance, it must be noted that there is variation in cell proliferation between different subjects' cells. Denholm, Moule & Barthold (1998) stated that considerable variation in the overall proliferative activity of the different pulp cell strains was noted with significant differences. It is proposed that the difference in proliferative activity is most likely attributable to the inherent variability within the established cell lines. As a result it is stated that the study confirms that when comparing findings of different *in vitro* studies involving human pulp cells, variations in experimental data can be strongly influenced by the pulp cell strain used and the culture technique employed. Pulpal regeneration and

recovery after injury is also influenced by the *in vivo* age of the pulpal cells. This was shown by Shiba, Nakanishi, Rasi, *et al* (2003) where growth rates and alkaline phosphatase (ALPase) activity of human pulpal cells decreased with increasing donor age. When damaged pulp tissue is recovered and mineralized tissue is formed to protect remaining pulp tissue, the general responses of pulp tissue after an adequate stimulus (pulp cell proliferation and activation of ALPase) are thought to be essential. The findings of this study suggest that impaired repair of pulp and dentine in aged patients is partially due to a decrease in the proliferative ability and ALPase activity in aged pulp cells. Out of the literature review above it can be seen that the usefulness of cytotoxicity data can only be dependent on the degree to which the cell culture assay can simulate the response of the oral cell populations to an introduced stimulus.

Chapter 2

Cell cultures

2.1 Background

The first tissue cultures were already done by Harrison in 1907 and Carel in 1912 (Carel, 1912), to study cells free of systemic influences. For about 50 years whole tissue was cultured instead of undisagregated fragments, hence the name tissue culture that still remains today.

According to definition an Organ Culture implies a three dimensional culture of undisagregated tissue retaining some histological features.

Cell Culture suggests cultures of dispersed cells taken from the original tissue, from a primary culture or cell line. A Histotypic culture implies that cells have been re-associated in some way to recreate a three dimensional tissue like structure.

At first cultures were done on frogs but soon it was progressed to more human like warm blooded mammals. Of these rodent tissue had the advantages of producing continuous cells lines.

The development of tissue culture owes much of its advances to production of antiviral vaccines and understanding of neoplasm. An additional force of increasing weight comes from the public's opposition to *in vivo* and the unnecessary use of experimental animals. Cell products such as human growth hormone and insulin have also been genetically engineered. Tissue culture has also been adopted into the routine application of modern medicine, by cellular analysis predicting disorders in a fetus as well as cell cultures for auto grafts in treatment of burn wounds.

2.2 Advantages of tissue culture

Control of the environment: The two major advantages being the control of the physiochemical environment (pH, temperature, osmotic pressure O_2 and CO_2) which can easily be controlled and the physiological conditions which may be kept fairly constant but can not always be defined. Most cell lines require supplementation, where the supplements are prone to batch variation and contain undefined constituents such as hormones and other substances (Maurer, 1992).

Characterization and Homogeneity of Sample: Tissue samples are inevitably heterogeneous and replicates even from one tissue can vary. After one or two passages, culture cell lines assume a homogeneous constitution, as cells are randomly mixed at each transfer. Hence, for each subculture the replicate sample will be identical and the characteristics of the line may continue indefinitely if the cells are stored in liquid N_2 (Bares & Sato, 1980).

Economy: Cells may be exposed directly to the reagent in a lower concentration with direct access to the cell. Thus much less material is needed than for *in vivo* experiments were 90% is lost to excretion. Screening tests with many replicates are cheaper and easier as well as side stepping moral, legal and ethical questions of animal testing.

2.3 Disadvantages of tissue culture

Expertise: Cell cultures are very technique sensitive and can easily be contaminated with other organisms. Cells from multi cellular organisms also require a very specific environment in order to exist independently.

Quantity: Very few cells can be produced in most laboratories and the cost of producing cells in culture is about 10 times that of using animal tissue. Thus providing large amounts of tissue (>10g) must be well justified. Costs for lesser amounts of tissue are readily covered.

Dedifferentiation and Selection: In the past problems were often encountered as undifferentiated cells overgrew in the culture compared to the differentiated cells (as it was wrongly believed the cells changed back to undifferentiated form hence the name). Due to new advances it is now possible to also reproduce the differentiated cells readily, so that the cells can keep their characteristics.

Origin of cell: If differentiated properties are lost, it is difficult to relate the culture cells to functional cells in the tissue from which they derived.

Instability: This is a major problem in a continuous cell line resulting from the capacity to differentiate within the population (Freshney, 1993).

2.4 Major differences *in vitro*

Many of the differences stem from the removal of the cells from their three dimensional histological origin and placing them on a two dimensional substrate. Specific cell interaction characteristics are lost. When the cell line forms it can easily only represent one or two cell types.

The culture environment also lacks several components of homeostasis like the neural and endocrine systems.

Energy metabolism *in vitro* also differs as it relies mainly on glycolysis.

Although these differences can not be denied, it must be emphasized that many specialized functions are expressed. As long as the limitations are considered, cell culture can be a very valuable tool (Freshney, 1993).

2.5 Definition of types of tissue culture

Organ culture: *in vivo* characteristics are maintained at least in part on a 3D substructure with a liquid gas interface. These are the most expensive and difficult to standardize but give the truest results.

Primary explant culture: tissue is placed at a glass liquid interface and migration is promoted.

Cell culture: tissue or outgrowth from a host is dispersed into a cell suspension which may then be cultured as an adherent monolayer on a solid substrate, or as suspension in medium. A monolayer or suspension may be dispersed by enzymatic treatment or simple dilution and reseeded or sub cultured in fresh vessels and the daughter cultures are the beginning of a cell line. The formation of a cell line implies (1) an increase in total number over several generations, (2) cells with similar high growth capacity cause (3) a degree of uniformity in the cells (Schaeffer, 1990).

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2.6 Biology of the cultured cell

Most cells used originate from solid tissue. As a result after disaggregation or subculture they will need to attach or spread out on a substrate in order to proliferate. It is believed that cells secrete an extra cellular matrix and proteoglycans prior to adhesion. This matrix then attaches to the substrate and the cells then bind to the matrix via a specific receptor. Fibroblasts like cells do not seem to have to be in direct contact with each other on the substrate, however it appears that for epithelial cells this is the case.

2.7 Initiation of a cell culture

Cultures are derived by enzymatic or mechanical dispersal of tissue or by outgrowth of migrating cells from a fragment. Only the cells that survive disaggregation and adhere to the substrate or survive in suspension will form the basis of the primary culture. After a few hours the cells able to proliferate will increase in number as the next selection step. As a result the relative proportion of each cell type will change until all substrate is occupied. At confluence (once all available substrate has been used) cells are close together and cells which are sensitive to density limitations will stop growing. At this point the culture will show its closest morphological resemblance to the tissue of origin. Transformed cells which are insensitive to these limitations will continue growing until overgrowth may occur (This is often the case with mouse fibroblasts). To keep the numbers limited and prevent overgrowth of transformed cells, frequent subculture is used (Todaro & Green, 1963).

2.8 Evolution of cell lines

After the first subculture or passage of the cell, the primary culture becomes a cell line which may be replicated several times. Continuous replication causes the cells which proliferate the easiest to gradually dominate, diluting slowly proliferating cells. After the third passage the culture will become stable and dominated by hardy rapid proliferating cells (often mesenchymal cells from connective tissue or vascular elements) (Macpherson & Stoker, 1962). This has given rise to some very useful cell lines but gives rise to several problems of culturing slower growing more fragile cells.

2.9 The development of a continuous cell line

Most cell lines will either become continuous cell lines or otherwise die out. The alteration in a cell line giving rise to a continuous cell line is also coined "*in vitro* transformation". It is not clear whether the cells that become continuous cell lines are present from the beginning or if they only develop later due to transformation. The term "transformation" implies both a change in culture morphology as well as tumorigenicity. The transformed cells also share some other characteristics with malignant cells.

Many normal cells do not give rise to continuous cell lines but form finite cell lines. It could be that the potential to form infinite cell lines is a genetic trait (Freshney, 1993).

2.10 Dedifferentiation

Dedifferentiated cells lose their specialized properties. This could be caused by either undifferentiated cells that may overgrow or due to an absence of stimuli like matrix and hormones. It should be distinguished between dedifferentiation, deadaptation and selection. The first implies that specialized functions are permanently lost. Deadaptation implies that certain cell functions are under regulated control and can be recreated if these stimuli are reintroduced. Selection as explained earlier (Maurer, 1992) is the process of selection by which the conditions favor certain cells which will then form the main part of the culture.

2.11 What is a cultured cell?

Cell cultures are made up of an equilibrium of multipotent undifferentiated stem cells and mature differentiated cells. Routine passages at low density will result in a high proliferation of immature cells where passage at high density together with certain

hormones will promote differentiation and inhibit proliferation. Further, the tissue of origin determines the composition of the culture. Cells from embryonic origin as well as tissue that continuously regenerates *in vivo* will have much more undifferentiated cells and can easily be transformed into an immortal cell line. Cells from differentiated adult tissue however, which *in vivo* only proliferate under stress, will not render immortal cell lines. The identity of the culture is therefore determined not only by the origin of tissue but also the position in that lineage (i.e. stem cell, precursor cell or mature differentiated cell). Neoplastic cells need not adhere to these rules, but induction of differentiation may inhibit cell proliferation (Freshney, 1993).

2.12 Cell cycle control

Entry of cells into cell cycle is regulated by signals from the environment such as low cellular density and the presence of growth factors like epidermal growth factor and platelet derived growth factor. Intracellular control is mediated by positive factors like cyclins and negative factors like p53 or the retinoblastoma (Rb) gene. The link between intra and extra cellular factors which will finally determine whether proliferation takes place is made by cell membrane receptors.

For many years it has been recognized that specific cell functions are longer retained in three dimensional cultures like organ cultures. Unfortunately these must be prepared de novo for each experiment and are difficult to quantify. Many attempts have thus been made to grow cell lines on a three dimensional structure or matrix (like collagen gel). These techniques may hold considerable promise for the evaluation of tissue-specific functions.

2.13 Cell Transformation / Immortalization

A distinction must be made between cell transformation and transfection. The latter implies a change in phenotype, dependent on the uptake of new genetic material. Transformation is known as the process in which spontaneous or induced permanent phenotypic change occurs that does not necessarily involve uptake of new genetic material. Immortalization is the acquisition of an infinite life span, presumed to be due to the deletion or mutation of one or more senescence genes or over-expression of one or more oncogenes. Immortalization as such does not imply either failure in growth control or the development of malignancy (for instance the 3T3 cell lines are immortal but still maintain contact inhibition of cell motility and density limitation of the cell proliferation). However, abhorrent growth is often seen in immortal cell lines.

The third development is Malignancy (Freshney, 1993), which remains primarily an *in vivo* phenomenon. It implies that the cells have developed the ability to produce invasive tumors if transplanted in other hosts. As aforementioned the immortalized cell lines can sometimes give rise to malignancy. It is also seen that malignant cells can more readily give rise to immortal cell lines. This has significant implication for the study of cancer cells and the utilization of continuous cell lines, where quality assurance would prefer no relation to malignant cells.

2.14 Growth Cycle

The typical growth sequence for cells is comprised by the lag phase, the exponential or log phase, and the stationary or plateau phase. The log and plateau phase give vital information about the cell line, the population doubling time during the log growth, and the maximum cell density achieved in plateau. Measurement of the population doubling

time can be used to quantify cellular response to hormonal effects, toxic drugs and variations in nutrients (Stoker & Rubin, 1967).

The Population doling time (PDT) is not to be confused with the cell cycle time. The PDT is an average figure for the population and represents the total number of cells. It is therefore influenced by the number of new cells, non-growing cells and cell deaths. The cell cycle time is measured from one point of the cycle until that point is reached again.

2.15 The lag phase

This is the time during subculture and reseeding during which there is little or no evidence of an increase in cell number. It is a period of adaptation during which the cell replaces elements of the glycocalyx lost during trypsinisation, attaches to the substrate and spreads out (Stoker & Rubin, 1967).

2.16 The Log Phase

This is the period of exponential increase in the cell number after the lag period and ending one or two doublings after confluence has been reached. In the log phase there is a very high growth fraction of the culture (90%-100%) and the culture is in its most reproducible stage.

2.17 The Plateau Phase

Towards the end of the log phase the culture becomes confluent as the entire available surface is occupied and all the cells are in contact with surrounding cells. At confluence cell proliferation may nearly cease completely after the last one or two doublings. This is the point when the culture enters the plateau phase and the growth fraction falls to 0%-

10%. At this point cells will arrange in line to each other, and cells will reduce “ruffling” of the cell membrane and occupy less space. The aforementioned factors in combination with cell spreading as well as depletion of nutrients and growth factors which can inhibit cell growth is called density limitation of growth. Simple epithelial and endothelial cells will stop proliferating after confluence, creating a monolayer where most other cells will, however slowly, continue proliferation if the medium is replenished, forming multi layers well beyond confluence (Stoker & Rubin, 1967). It is important to consider that plateau does not imply a complete cessation of cell proliferation but rather represents a steady state where cell division is balanced by cell loss.

Figure 1: Growth curve of cell proliferation

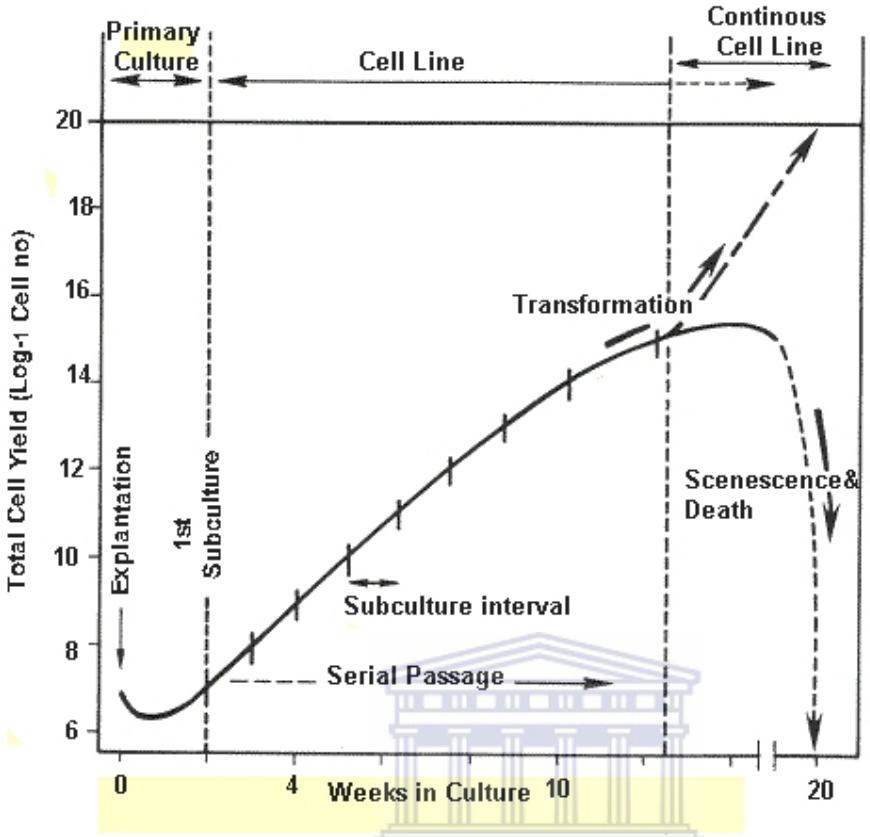


Figure 1 illustrates the increase in cell population as a progression of time

The graph in Figure 1 illustrates the growth curve of cells in culture. At zero weeks in culture the cells are explanted into dishes in which they start to grow. As long as there is space for the culture to grow, cell replication takes place exponentially until the maximum population is reached. After this point the population stops growing to either reach a plateau, transform into neoplastic cells which overgrow or the cell line comes to its end and the cells die. The period between the first passage and transformation or senescence is known as serial passage.

2.18 MTT Based Cytotoxicity Assay

Cytotoxicity is defined as the "capacity of substances to damage tissue cells (Szep, Kunkel, Ronge, *et al* 2002). One way of measuring cytotoxicity is by means of micro titration assays which have in recent years been dominated by the use of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetra zolium bromide (MTT) reduction in determination of the number of viable cells at the end of the assay. MTT is a yellow water-soluble tetrazolium dye that is reduced by live, but not by dead cells to a purple formazan product that is insoluble in aqueous solution. These tests can be used to evaluate a range of other treatments except cytotoxic drugs. However, it is important to ensure that the treatment itself does not reduce the cell's ability to metabolize the dye. Micro titration is not able to distinguish between differential responses between cells within a population and the degree of response in each cell, e.g. inhibition could mean that 50% of the cells respond or that each cell is inhibited 50% (Mossman 1983).

Chapter 3

3.1 Development of teeth: a brief summary

Teeth develop from both ectoderm and mesenchyme of mesodermal origin, the ectodermal component forming the enamel derived from the enamel organ and the mesenchyme producing the rest of the tooth. The enamel organ originates as cellular downgrowth of the oral epithelium (6th and 7th week of embryonic life), initially in the form of a cap-shaped epithelial tooth bud, connected to the overlying epithelium by the dental lamina. Beneath the epithelial tooth bud, a condensation of mesenchyme contributes to the rest of the tooth.

The cells of the epithelial tooth bud develop into the enamel organ by differentiating into a bell shaped structure (early bell stage) with a core of loosely arranged stellate cells (stellate epithelium) and a peripheral layer of cuboidal or low columnar epithelium. The outer cell layer on the convex surface is the external enamel epithelium, and the concave surface is the internal enamel epithelium.

The internal enamel epithelium differentiates into an outer layer of tall columnar ameloblasts and an inner layer called stratum intermedium (Ten Cate's, 2003 a).

Where the external and internal enamel epithelium meet is called the cervical loop. An extension downwards of cells of the external enamel epithelium forms Hertwig's root sheath which determines the final size of the root, being later replaced by cementum.

In the concavity of the enamel organ, the mesenchyme continues to condense to form the dental papilla, and a row of odontoblasts develops at its junction with the enamel organ, in contact with the ameloblasts.

During the late Bell stage odontoblasts begin to produce pre-dentine which stimulates the ameloblasts to produce enamel. Calcification of pre-dentine and pre-enamel begin

almost immediately, and enamel and dentine are laid down until the tooth form is completed. The dental papilla which is now enclosed by dentine forms the pulp. The non-ameloblast part of the enamel organ is progressively reduced until it finally atrophies and after the completion of enamel formation the ameloblasts degenerate to form a thin layer which disappears with tooth eruption.

The cells of Hertwig's sheath begin to degenerate as cementum is deposited by cementoblasts on the surface of the dentine of the tooth root. Partial development of the permanent tooth continues alongside the deciduous tooth in the same manner from a second early tooth bud of the dental lamina (Ten Cate's, 2003 a).

3.2 Enamel, Development and Anatomy/Composition

Although enamel has ectodermal origin, which is unlike the other calcified structures of the body, and a composition very high in minerals (96% of mass), enamel shows the same basic characteristics as other mineralized tissue. It is formed by a matrix produced by a cell layer, which then mineralizes with hydroxyapatite (Ten Cate's, 2003 a; Simmer & Hu, 2001).

The functioning ameloblast is a tall narrow cell, with its base attached to the cells of the stratum intermedium. The nucleus is located basally and basal cytoplasm contains abundant mitochondria and large active Golgi and abundant endoplasmic reticulum, together with micro tubules. At the upper pole the cell elongates into the Tomes' process and small neck processes which contain many microtubules and secretory vacuoles. The endoplastic reticulum synthesizes various proteins and glycoproteins (amelogenin and enamelin) which form the organic matrix of enamel (pre-enamel) and are packed by the Golgi into secretory vacuoles. These then move into the Tomes' and neck processes where they discharge their contents onto the surface. Mineralization of the matrix

proteins by hydroxyapatite takes place almost instantaneously producing small enamel crystals (at first needle shaped and later becoming 6 angular in shape) and with progressive mineralization forming the enamel rods and prisms. The compact structure enamel prisms are probably derived from the surface of the main Tomes' process, while the small amount of less compact interprism enamel, which has a larger organic matrix component, is probably derived from the small neck processes (Stevens & Lowe, 1993; Leblond & Warshawsky. 1979).

Chemical composition: According to volume, enamel consists of 86% inorganic material, 2% organic material and 12% water. The inorganic part is mainly made up of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) but fluoride ions may replace the hydroxyl group rendering flourapatite. The organic matrix is limited and fills the areas between the crystals (Simmer & Hu, 2001).

Structure: The crystals are arranged into prisms which stretch from the amelodentinal junction of the tooth surface. If sectioned, each prism has a "keyhole" appearance and are so arranged that they slot into each other snugly. The prisms do not always follow a straight path, but are often inter-woven especially close to dentine and the cusps of the tooth (Figure 2).

Enamel has several lines as part of its structure which are the Retzius lines and Hunter Schreger lines. These lines are formed due to the way the enamel is laid down during its formation (Ten Cate's, 2003 a).

Figure 2: Illustrating the Anatomy of Enamel

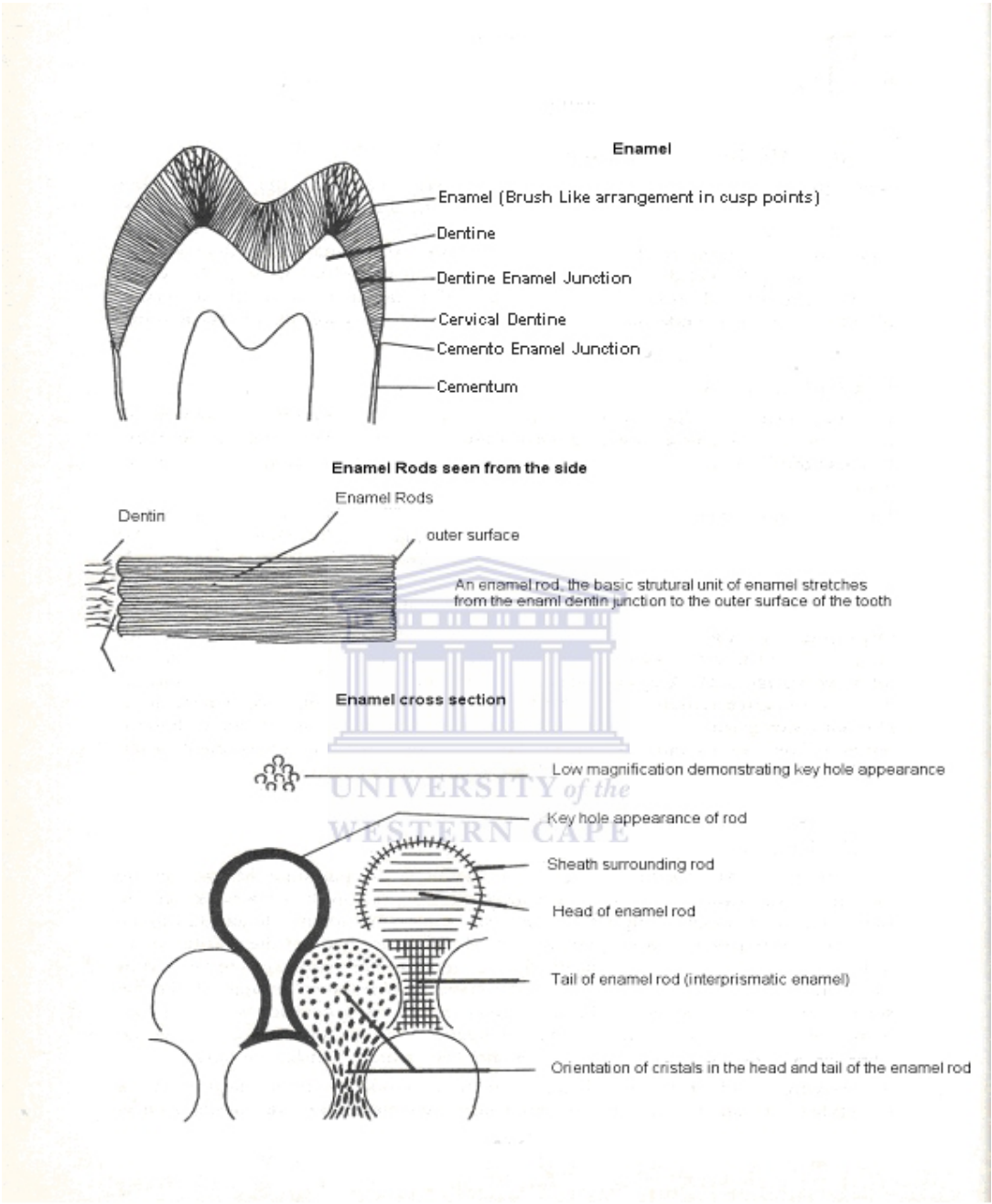


Figure 2: The top most illustration shows the cross section of the tooth, the middle illustration shows a parallel section with the enamel rods, and the bottom illustration shows a cross section and enlargement illustrating the anatomy of the enamel rods.

3.3 Dentine, Development and Anatomy/Composition

Initially all cells of the dental pulp look the same, but they soon differentiate. As differentiation progresses, pre-odontoblasts are formed which then later become odontoblasts. The pre-odontoblasts are short columnar cells and in the pre- odontoblast stage the cells already lose their ability to proliferate and predentine is produced.

Odontoblasts are tall columnar cells with nuclei arranged basally near the pulp cavity and their long cell bodies lying in neat palisades towards the forming predentine. Their cytoplasm is rich in rough endoplasmic reticulum and contains prominent Golgi. At the dentinal border odontoblasts terminate in long cytoplasmic processes, the odontoblast processes, which extend into the layers of predentine and dentine, running in parallel narrow channels, the dentinal tubules (Thomas & Payne, 1983).

The first stage of dentine formation is the laying down of a matrix which consists of type 1 collagen fibers in a ground substance containing large amounts of glycoaminoglycans (mainly chondroitin-6-sulfate). The short cell processes of the early odontoblasts are found in this matrix. It appears that the odontoblasts are responsible for the laying down of the matrix while the sub-odontoblast cells are responsible for the formation of the von Korff fibers (Stevens & Lowe, 1993).

At first dentine is completely organic and inorganic crystals are only laid down as the dentine increases in width in the oldest part of the dentine. New predentine is thus always synthesized at the inner surface of the pulp cavity and the cavity slowly decreases in size throughout life. The layer of dentine next to the pulp remains unmineralized and is known as the odontogenic region. After the initial laying down of a thin layer of dentine, the dentinal tubules appear, containing the odontoblast processes. Mineralization is always delayed in relation to the production of the matrix. This results in the presence of a layer of unmineralized predentine (a layer of dentine that has not

yet been mineralized and is about 10-20µm thick and separates the odontoblasts from the mature dentine) between the odontoblasts and the mineralized dentine. Mineralization takes place next to the collagen fibers and around the dentine tubules (Stevens & Lowe, 1993; Ten Cate's, 2003 b).

The crystals grow individually and by fusion with other crystals until they form calcospherites. Although the crystals are arranged in a more complex manner they are much smaller than those found in enamel.

Composition: the organic component (18% of mass) is much larger than of enamel. It is formed out of 93% collagen, 0.9% citric acid, 0.2% protein, 02% mucopolysaccharides and 0.2% lipids. The inorganic fraction forms about 70% of the mass and the rest is water. The inorganic part consists mainly of hydroxyapatite crystals as well as amorphous calcium phosphates (Ten Cate's, 2003 b).

Structure: Dentine consists of cells, the odontoblasts lying on the periphery of the pulp chamber and intercellular tissue which is only intercellular by definition as it lies between the cytoplasmic processes of the odontoblasts. The dentine is pierced everywhere by microscopic tubules, the dentinal tubules, which contain the odontoblast processes. It is estimated that there are 65 000 tubules/mm² close to the pulp and about 15 000 tubules/mm² at the amelodentinal junction, with an average of 35 000 tubules/mm² in the middle of the dentine.

In man, odontoblast cytoplasmic processes extend only about 20-50% of the full length of the dentinal tubule, thus dentine close to the dentine-enamel border appears to contain empty tubules. In life these empty tubules may contain fluid.

Dentine tubules: The diameter of the tubules varies between 1-5 µm with 1,5 µm as average. The tubules are closer together close to the pulp compared to the periphery and as a result the amount of intertubular dentine varies greatly with more intertubular

dentine at the periphery. On their way to the periphery the tubules describe a soft S-curve in their shape (with a primary and secondary curve). The primary curve is in an apical direction and the final position of the tubule orifice is more coronal when compared to where it started. Close to where the dentine joins to the enamel the tubule splits into two or more terminal branches which communicate with each other forming a plexus (Mjor & Nordhal, 1996).

Peritubular dentine: Peritubular dentine surrounds the dentinal tubules and the rest of the dentine is intertubular dentine. The peritubular dentine is mineralized to a greater extent than the intertubular dentine. The peritubular zone is not found around the tubules in predentine and not all tubules are surrounded by peritubular dentine.

Interglobular dentine: At first the inorganic part of dentine exists as globules which later fuse to form homogeneously calcified dentine. In some regions however (especially close to the enamel-dentine junction) the globules do not fuse. This forms areas where there is only an organic matrix between the separate globules which is known as interglobular dentine (Ten Cate's, 2003 b).

Von Ebner lines: As is the case with enamel, dentine formation takes place in cycles and therefore lines within the dentine can be seen. These von Ebner lines are usually spaced about 5μm apart. A very pronounced line in this pattern is the neonatal line which is formed in the calcification period around birth and separates the pre and post natal calcification (Simmer & Hu, 2001).

Chapter 4

4.1 Bonding to enamel

To achieve proper bonding to enamel the surface is roughened by etching. After the etching with acid the soluble calcium salts are washed away, dried and a resin is applied which is then cured and forms a micromechanical bond by means of so called “resin tags”. Through this process bonding strengths of up to 26 MPa are usually achieved. In the past the clinical efficacy of etching with 37% ortho phosphoric acid has repeatedly been proven. The newer systems however make use of newer types of acid such as 10% citric acid (Superbond and Amalgabond plus (Parkell). However the shortened etching time and weaker acid have proven to give rise to higher micro leakage (Ferrari & Davidson, 1996; Owens, 1997) and decreased bonding strength.

4.2 Bonding to dentine

Producing the same repeatable results in bonding strength compared to enamel proved to be very difficult with dentine for many years. The main reason for this is that dentine is a more organic material (18% of mass especially collagen) than enamel (<4%) surrounded by hydroxy apatite. The hydroxy apatite made early attempts of chemical bonding difficult (Pashley, 1991) as well as the presence of dentinal tubules. The tubules are conically shaped with the greater diameter at the pulp and decreasing towards the dentine enamel junction. As a result the tubules occupy only about 1% of the surface area at the dentine enamel junction and approximately 22% close to the pulp (Pashley, 1992 a). This structural difference was listed as main cause in differences in dentine bonding, where better bonding was achieved close to the dentine enamel junction (Pashley, 1992 b). In their summary:” Dentine bonding agents – a

review of adhesion to dentine", Moodley, Grobler, Rossouw, *et al* (2000) wrote that initially bonding was achieved by the use of hydrophilic and hydrophobic coupling agents which contained phosphates as well as phosphonates which are thought to interact with the calcium ions in the dentine. These first and second generation bonding systems however only yielded low bonding strengths of between 2-7 MPa, which were too weak to counteract the forces of polymerization shrinkage of the composite resin resulting in marginal leakage. They were followed by the 3rd generation bonding agents which modified the dentine surface prior to bonding by means of a conditioning solution in conjunction with bonding agent. This conditioning solution either modified or changed the smear layer to interact with the superficial dentine layer. Initially a 2,5% nitric acid and 3% ferric oxalate solution was used and alternatively a system of EDTA and HEMA. In these systems adhesion was achieved by grafting to collagen (possibly on the hydroxyl, carboxyl, amino and amino groups). Again low bond strengths of about 10 MPa were reported with these systems. As a result micro leakage was reduced but marginal leakage was still present. Then the 4th generation or total etch systems were developed and *in vivo* and *in vitro* research back up their success. They are bonded in a three step system, namely: a) condition; b) prime; c) bond.

The conditioning, i.e. acid etching, removes the smear layer, decalcifies the intertubular and peritubular dentine, opens the dentinal tubules and increases dentine permeability. Removal of hydroxyapatite crystals leaves a collagen meshwork on the dentine surface. As a result of this superior bonding, bonding strengths dramatically increased to >15MPa. The next (5th) generation bonding agents also made use of this system but combined them into a more user friendly one bottle concept which also shows promising research results. Most recent developments aim towards self etching primers and adhesives that do not require rinsing and serve simultaneously as conditioner/primer.

Some adhesive bond strength and bonding properties of these systems seem comparable to older systems, but clinical trails are still being done (also known as 6th and 7th generation dentine boding agents). An example of these systems would be the dentine bonding agent under investigation, Xeno III (refer to Chapter 6) as well as the new bonding agent by 3M Prompt al Pop.

4.3 Dentine permeability in bonding

Dentine permeability is of utmost importance in bonding as contact of the bonding agents to the substrate is vital. During bonding the adhesive resin infiltrates vertically into porosities in the dentine created by acid etching. This, according to the article by Moodley, Grobler, Rossouw, *et al* (2000), was coined intertubular permeability. By demineralization of dentine the collagen fibers are exposed and bound by the agent. The adhesive replaces the mineral appatite and infiltrates the collagen matrix creating the so called hybrid layer. This zone of bound resin penetrated by collagen fibers forms a micro mechanical link between restoration and tooth, probably resulting in durable bonds. In the article it states that the deeper the acidic conditioners penetrate during etching, the deeper the adhesive must penetrate in order not to leave an underlying area of demineralized dentine with exposed collagen fibers which may result in a poor bond. The resulting leakage in this instance was termed nanoleakage by Sano, Takatsu, Ciucchi, *et al* (1995). The clinical significance of this is still not clear but it directly reflects the resin's inability to penetrate the dematerialized area sufficiently.

4.4 Role of Resin tags

Alongside resin infiltration into the intertubular dentine, infiltration into the intratubular dentine also occurs when the resin flows into the tubule lamina. As the resin penetrates the tubules it seals them off forming long resin tags. As a result of the dentine morphology these tags converge towards the pulp providing non-parallel tags.

As a result of etching, the smear layer is removed and the tubule openings are exposed to a depth of about 3-4µm which exposes collagen fibers continuous to the intertubular dentine along the tubule. The hybridization of collagen fibers in the orifices is known as lateral tubule hybridization (Van Meerbek, Perdigao, Lambrechts, *et al* 1998). This forms a three-dimensional network of interconnected collagen fibers which ads to bonding strength. As a result of this bonding process Moodley, Grobler, Rossouw *et al* (2000) reports that hermetical sealing of the pulpodentinal complex is achieved, thus preventing micro leakage. The role of the tags is to seal dentine, preventing breakage caused by shrinkage and add to bonding strength. The morphology of the dentine also plays a role by resulting in larger and smaller tags depending on the size of the tubules, which changes bonding strength.

4.5 Role of water

Water plays an important part in bonding and comes from several sources such as dentinal fluid, atmospheric water, rinsing procedures and from adhesive solutions. Originally water was seen as contamination. After etching dentine is rinsed with water removing the smear layer and soluble debris. As a result the spaces between the dematerialized dentine become filled with water, keeping the collagen in an expanded state, which is vital for sufficient resin penetration. Marshal, Marshall, Kinney, *et al*

(1997), is quoted as stating that demineralized dentine underwent dramatic collapse on drying until a dense collagen matt remained, through which the resin would first have to diffuse before it could infiltrate the demineralized dentine. If the desiccated dentine was left to rehydrate, it was interestingly found that the collagen would restore to its full height again. As a result Gwinnett & Kanca,(1992), suggested wet bonding. Their studies have demonstrated that bonding strength to moist dentine was higher than to dry dentine. Wet bonding implies that the dentine must not be dehydrated or desiccated following etching and rinsing. Too much water however will result in poorer bonds and the authors suggested the dentine be dried by blotting with a damp cotton pellet.

Adhesives in dentine bonding agents still need to displace water in order to be able to bond. This is achieved by the primer of the agent which contains both hydrophilic properties by displacing the water and hydrophobic properties to adhere to the adhesive. The primers are better termed adhesion-promoting resins containing monomers, acting as bifunctional resin. The organic solvents of primers (like acetone or ethanol) displace the water and occupy the spaces between the collagen fibers which prevents shrinkage and collapse of the collagen matrix (Moodley, Grobler, Rossouw, *et al* 2000). For strong bonding there must be good wetting of the adhesive agent and substrate i.e. the adhesive must easily spread across the surface. For this the surface tension of the agent must be equal or less than that of the hard surface. This is also achieved by the primers which have very low surface tensions.

4.6 The smear layer: definition and implication

The smear layer is formed by mechanically cut tooth tissue, blood, saliva and bacteria which adhere to the tooth with a bonding strength of about 5-6 MPa. It covers both tubular and intertubular dentine and primarily consists of dentine particles of between 0.05 to 10μm (Pashley & Carvalho, 1997). As it is made up of dentine, it resembles a layer of intertubular dentine. Smear layers created from caries-affected dentine may contain partially denaturated collagen (Eick, Gwinnett, Pashley, *et al* 1997). As with other collagen, overdrying of this layer will result in a matt like collagen layer which reduces primer permeation. In the past there was somewhat controversy on whether or not the smear layer hold should be removed. The arguments listed against the removal of the smear layer were that it prevents bacterial contamination; leakage of dentinal fluid (removal of the smear layer could result in pulpal damage) and prevents diffusion of toxic substances to the pulp. On the contrary there were arguments sited for the removal of the smear layer, namely that it does not protect but rather irritates the pulp and that it significantly reduces bonding strength. It is important to note that the permeability of dentine increases drastically after etching (based on observations and unpublished work) which increases the chances of pulpal irritation, which can clear up if the cavity is sealed properly and micro leakage is reduced. Another factor influencing the degree of pulpal damage is cytotoxicity of the material placed in the cavity.

With the new self etching primers the smear layer is now used as a bonding substrate as it is not washed off. The primers of these systems are considered acidic enough to mineralize the inorganic component of dentine. As they etch they also infiltrate the collagen matrix thus minimizing voids with an absence of dematerialized dentine not encapsulated by resin primer. Much has been written about the role of collagen fibers and their importance in the hybrid layer, with some authors suggesting that it plays no

role (Kanca & Sandrik, 1998) and that no difference with or without micro mechanical adhesion is found (Armstrong, Boyer, Keller, *et al* 1998), but may even prevent proper bonding**.** Vargas, Cobb & Armstrong (1997) also supported the theory that collagen may not be crucial to the mechanism of adhesion. Moodley, Grobler, Rossouw, *et al* (2000), found a clear link between bonding strength and depth of resin tags when a total etch system was compared to a self etching/ self priming dentine bonding agent. The total etch system produced deeper resin tags and stronger bond strength as well as no micro leakage opposed to the self etching primer system. A reason for this may be that the self etching primer is not acidic enough to dematerialize the dentine sufficiently.

Chapter 5

Aims and Objectives

In the past century amalgam was the filling of choice in Dentistry, with a long history of clinical success. However in a progressively emancipated population, in which increased importance was laid on aesthetics, amalgam fillings were soon found inadequate. Especially in the anterior and buccal as well as mesial interproximal areas, the dark, corroded amalgam restorations were deemed unattractive. In addition to this, the issue of possible mercury poisoning due to amalgam fillings has gained tremendous momentum in recent years, especially in the popular literature. Although it was soon found that little real risk of toxicity existed for the patient population, some cases of toxicity affecting dentists and auxiliary staff were identified. The only real aesthetic alternative was porcelain restorations which only gained in popularity in the last half of the century. The major drawbacks here, were the repeat visits required as well as material limitations, financial limitations and other impracticalities for smaller restorations like Black class I, class II and class III cavities. These shortfalls in treatment possibilities left a huge opening in the market, which was soon exploited and filled by the composite resins. At first these “white fillings”, which started as self cure or chemical cure fillings, had the advantage of resembling tooth structure but soon produced their own problems. Colour stability and micro leakage, as well as material and bonding strength posed the main obstacles to the early composites. These fillings were also found to be more time consuming, expensive and technique sensitive, compared to the amalgams. The growing demand in the market led to constant refining and improvement of the composites which led to generations of new material.

However, the ongoing evolution of the composite fillings soon led to the inclusions of monomers like Bis-phenol A diglycidyl-methacrylate (Bis-GMA) urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA). It was found that these substances produced allergic reactions as well as toxic reactions in many patients, especially when the composites were not fully cured, containing free monomers. As dentine is partially made up by permeable tubules, this was especially important in cavities close to the pulp, were adverse reactions could be anticipated. This resulted in an increase in cytotoxicity tests in order to evaluate and compare different filling materials. At first these tests were mainly based on in *in vivo* experiments, with *in vitro* experiments lacking clinical relevance. However, as *in vivo* experiments were expensive and time consuming, *in vitro* experiments were continuously refined to meet the clinical situation. This pursuit was amplified by growing public scrutiny and animal right campaigns which placed further stress on *in vivo* experiments.

The *in vitro* experiment which was found to resemble the clinical situation the closest was the "*in vitro* pulp chamber" (IVPC), which could be used to evaluate the cytotoxicity of materials in situations resembling the mouth. By using dentine as a barrier, materials could now be placed in indirect contact with cells and cell viability evaluated under conditions similar to the mouth. To make these experiments more clinically relevant the use of human dentine discs has repeatedly been advocated.

The objectives of this study were then to create an *in vitro* situation which closest resembled the clinical situation by inclusion of human dentine discs at a specified anatomic location as barrier and evaluating if comparable or graded perfusion could be identified as well as whether a link existed between dentine thickness and permeability. The other leg of the study was to evaluate the cytotoxicity of Xeno III,

one of the newly released “new generation dentine bonding agents”, and to evaluate its cytotoxicity in an IVPC, determining which thickness of dentine would be able to protect the dental pulp from noxious substances in the bonding agent.

This aims at creating an *in vitro* model with strong clinical relevance, which could be used as benchmark in the assessment of biocompatibility of new materials, setting a platform on which different materials could be compared to each other internationally.

Chapter 6

Xeno III

Self-etching adhesives make a separate etching step, typically applying a phosphoric acid gel followed by a rinsing and drying step, obsolete. The application of self-etching adhesives therefore simplifies the bonding procedure and thus reduces the technique sensitivity especially with regard to dentine bonding.

6.1 Product Description

6.1.1 Xeno III: Components and their Functions

Xeno III is a universal self-etching dental adhesive designed to bond light-cured restorative materials to the tooth substrate. Xeno III is a two-part single-step self-etching adhesive comprising a LIQUID A and a LIQUID B. The two liquids are mixed in a 1:1 ratio prior to application in order to obtain the self-etching adhesive which has three functions, effective in one application step: etching, priming and bonding. The Xeno III components and their specific functions are given in Tables 1 and 2 below.

Table 1: Xeno III Liquid A: components and their specific functions

Component	Function
2-Hydroxyethyl methacrylate (HEMA)	Primer
Purified Water	Solvent
Ethanol	Solvent
2,6-Di-tert-butyl-p-hydroxy toluene	Stabilizer
Nanofiller	Contributes to tackiness towards first layer of uncured restorative material

Table 2: Xeno III Liquid B: components and their specific functions

Component	**Function**
Tetrametacryloxyetyl pyrophosphate (Pyro-EMA)	Acidic, polymerisable monomer: etching and adhesive function
Pentamethacryloxyethyl cyclo-phosphazen monoflouride (PEM-F)	Fluoride releasing polymerisable monomer: Enhances etching effect by scavenging Ca-ions
Urethane dimethacrylate (UDMA)	Contributes to cohesive strength
2,6-Di-tert-butyl-p-hdroxy toluene (BHT)	Stabilizer
Campherquinone	Photo-initiator
p-Dimethylamino ethyl benzoate (EPD)	Co-initiator

Xeno III is a water/ethanol based self-etching adhesive. The two solvents are contained only in Liquid A. The resins in Liquid B are solvent-free and hence any hydrolytic degradation during storage can be ruled out. The use of water/ethanol as low volatile solvents has the advantage that solvent loss during storage or use is minimized, thus removing a source of variability.

The incorporation of specially treated nanofiller enhances the tackiness between the cured adhesive and the 1st layer of uncured restorative during placement.

Xeno III contains the mono- and bi-functional monomers HEMA and UDMA. The former is proven to be an excellent priming molecule and the latter contributes to cohesive strength. Besides these two well-known monomers, Xeno III contains two new monomers patented by Dentsply: Pyro-EMA and PEM-F. Both monomers contribute to the etching and adhesive function. Details and functionality of the latter monomers are described in the following chapter.

6.1.2 Xeno III: Working Mechanism

Both molecules Pyro-EMA-SK and PEM-F become active only when exposed to water (from Liquid A). The monomer Pyro-EMA (“masked acid”) forms free phosphoric acid groups immediately when contacted with water, as illustrated in Figure 3.

Figure 3: Hydrolysis of Xeno III

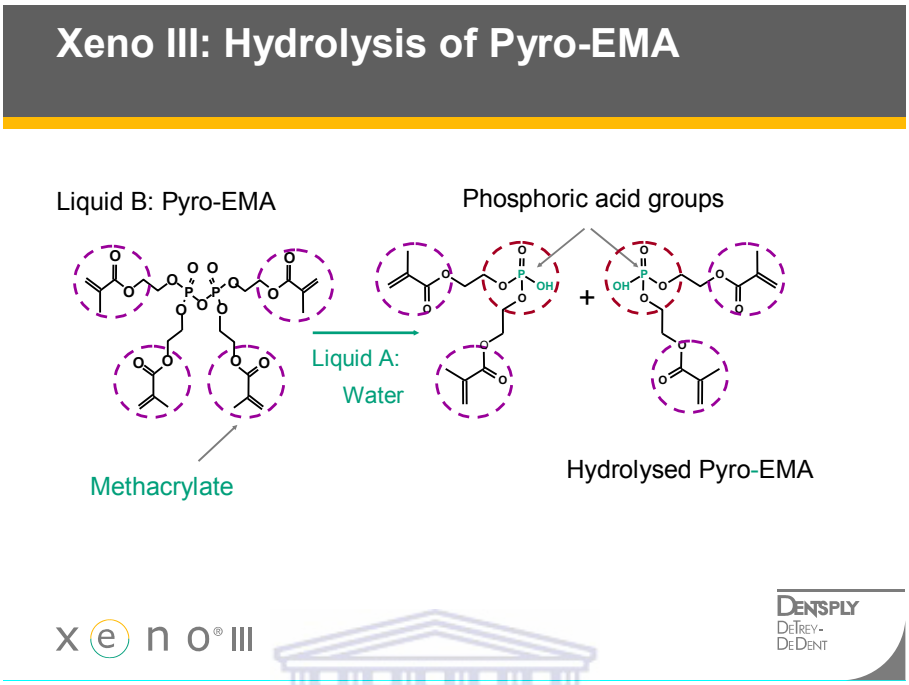


Figure 3 showing the formation of free phosphoric acid groups after contact of Pyro-EMA with water (from Liquid A)

Due to the formation of the free phosphoric acid groups, the mixture of Xeno III Liquid A and Liquid B is strongly acidic. The acidity of Xeno III has been compared with two commercial self-etching adhesives by measuring the respective pH values. Results (internal data) are given in Table 3.

Table 3: pH of Xeno III compared to other self-etching adhesives

Adhesive	**pH**
Clearfil SE Bond (Kuraray)	2.0
Prompt-L-Pop (3M)	1.3
Xeno III (Dentsply)	< 1.0

Note that Xeno III is the most acidic

Xeno III is more acidic than the two reference materials. Upon contact with the tooth substrate, the free phosphoric acid groups of the hydrolyzed Pyro-EMA demineralizes hydroxyapatite of the tooth substrate (etching). Calcium ions are released from the hydroxyapatite resulting in the complete neutralization of phosphoric acid groups via ionic interaction of the calcium ions with the phosphoric acid (Figure 4). After removal of solvent and light activation the neutralized Pyro-EMA is being co-polymerised via methacrylate groups.

Figure 4 Neutralization of hydrolyzed Pyro-EMA

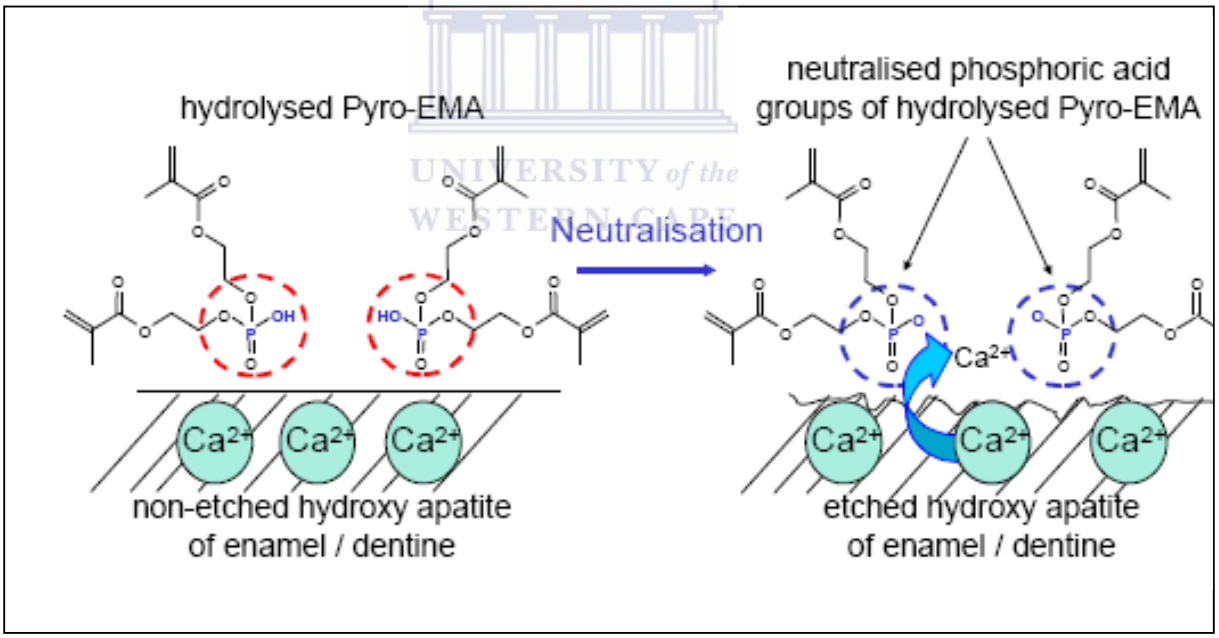


Figure 4 illustrating the neutralization of hydrolyzed Pyro-EMA. Note the reaction with Ca^{2+}

PEM-F is a multi-functional monomer. The molecular structure of PEM-F is given in Figure 5.

Figure 5: PEM-F

Figure 5: Molecular representation of Penta-methacryloxy-ethyl-cyclo-phosphazene-mono-fluoride (PEM-F)

According to the manufacturer (Dentsply), PEM-F (in Liquid A) releases fluoride ions which act as calcium ion scavengers, enhancing the etching effect of Pyro-EMA upon contact with water. In addition Dentsply states that PEM-F is a strong cross-linking monomer due to the five methacrylate groups attached to the phosphazene ring and thus also contributing to the cohesive strength of Xeno III.

6.1.3 Xeno III: Interaction with tooth substrate

The adhesion mechanism of Xeno III is illustrated based on its interaction with dentine (Figure 6-8).

Figure 6: Dentine prior to application of Xeno III

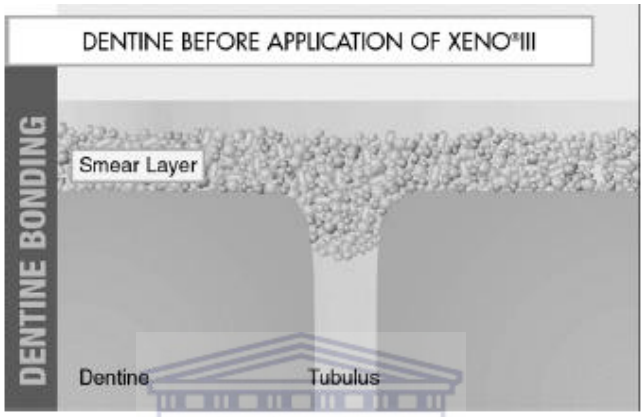


Figure 6: Note the obstruction of the dentine tubule prior to application of XENO III

Figure 7: Dentine shortly after application of Xeno III

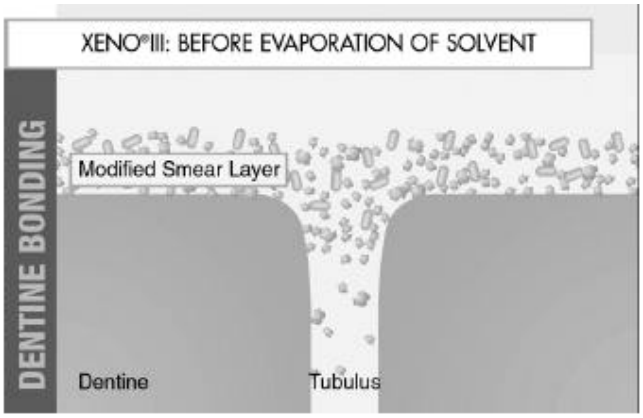
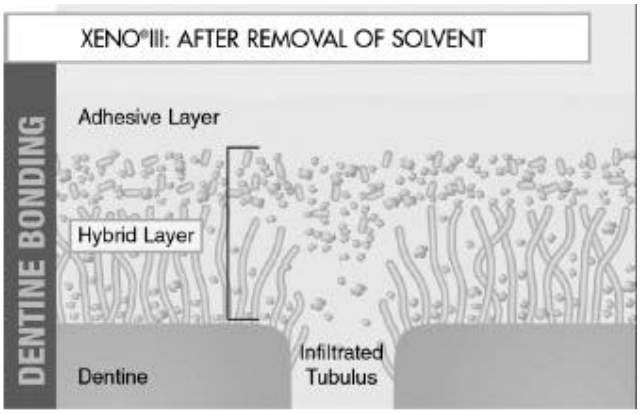


Figure 7: Note the dissolving of the smear layer and opening of tubule orifice

Figure 8: Dentine 20 seconds after the application of Xeno III



Once Xeno III has partially dissolved and penetrated the smear layer (Figure 7) it will then demineralize the dentine underneath the smear layer resulting in formation of a homogeneous hybrid layer (Figure 8).

6.2 Performance of Xeno III

6.2.1 Micro-Morphology Investigation

The mechanism illustrated is based on micro-morphological studies conducted by Pioch (Heidelberg) in his report to Dentsply 2002, using Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). The investigation of the interface between Xeno III and dentine using CLSM (Figure 9) and SEM (Figure 10) shows, that Xeno III is penetrating the smear layer as well as the smear plugs resulting in the formation of a homogeneous hybrid layer and complete formation of resin tags.

Figure 9: Resin Tags

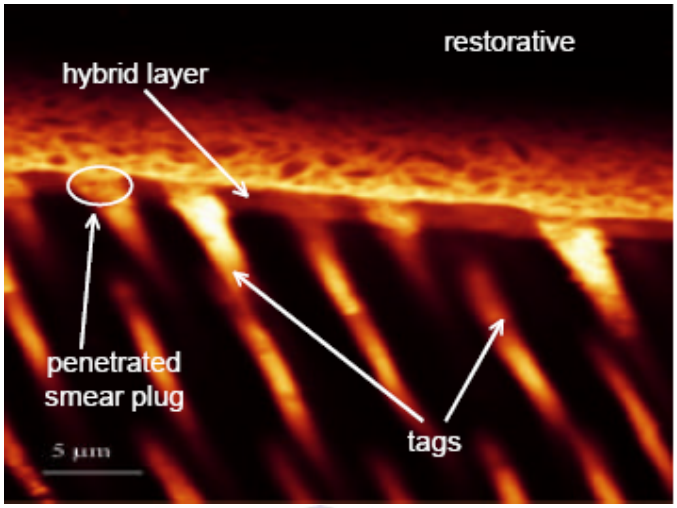


Figure 9: CLSM image of a cross-section of the interface between Xeno III and dentine. The hybrid layer appears homogeneously along the junction within the whole specimen. Tags are very well established.

Figure 10: Resin Tags

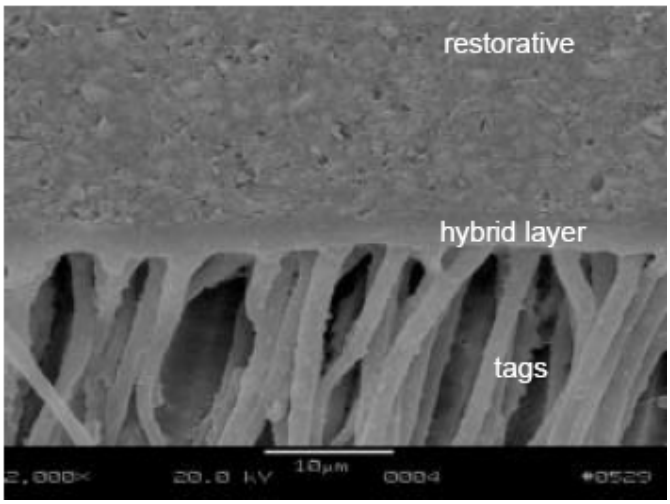


Figure 10: SEM image of a cross-section of the interface between Xeno III and dentine substrate. Tags are very well established.

The etching ability of Xeno III with regard to enamel is similar compared to phosphoric acid and results in a pronounced etching pattern as shown in Figure 11.

Figure 11: etching pattern Xeno III under SEM

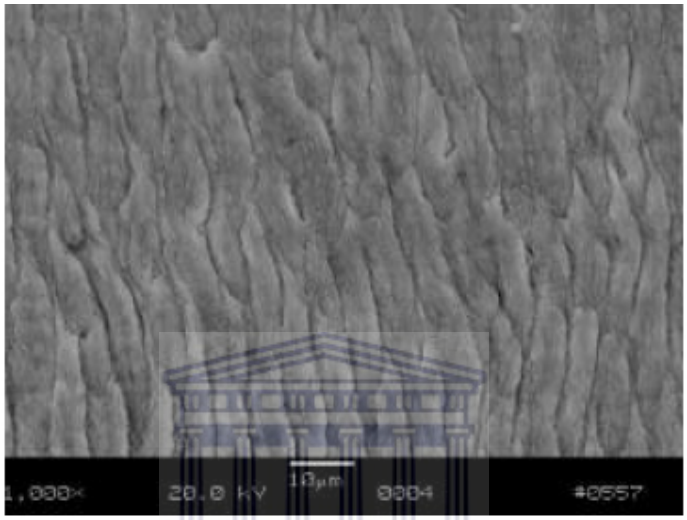
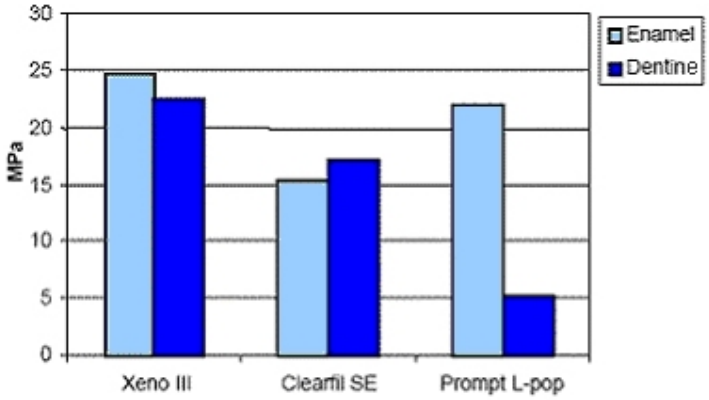


Figure 11 showing enamel treated with Xeno III (20 s) and then rinsed with ethanol. Etching pattern is similar to phosphoric acid treated enamel.

6.2.2 Adhesion Results

The adhesion performance has been investigated by external researchers (Bouillaguet, Gysi, Wataha, *et al* 2001; Cardoso, Braga & Carrilho, 1998; Frankenberger, Perdigao, Rosa, *et al* 2001; Rosa & Perdigao, 2000), and by Dentsply researchers. Xeno III gave better adhesion results in comparison to Prompt L-Pop and Clearfil SE (Figure 12). Especially with Prompt L-Pop the adhesion to dentine was very low.

Figure 12: Adhesion results



External dentine adhesion results (after 1800 thermocycles at 5 and 55 °C) of Xeno III compared to Prompt L-Pop

In addition, the adhesion of Xeno III has also been compared to other total-etch and self etch adhesives. The results are given in Figure 13.

Figure 13: Adhesion results

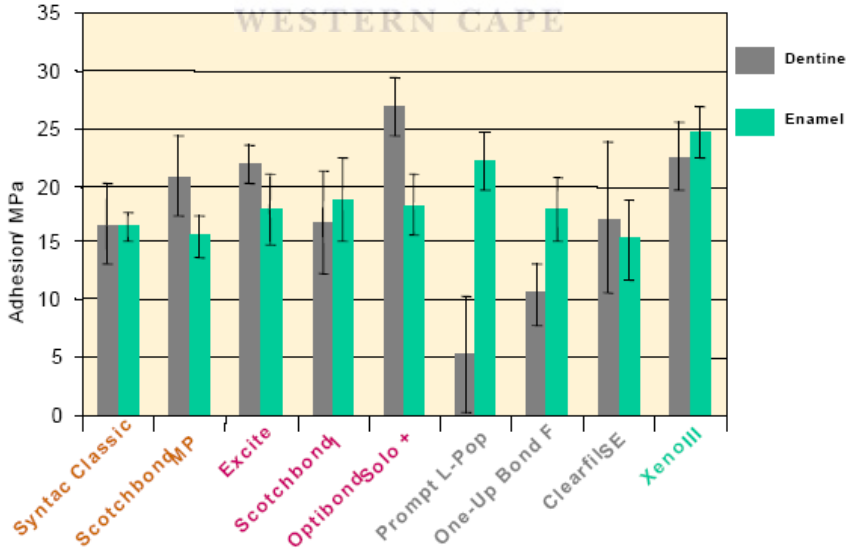


Figure 13: Internal adhesion results (after 1800 thermo cycles at 5 and 55 °C) of Xeno III compared to other total-etch and self-etch adhesives.

From the figure above it can be seen that Xeno III bonds the strongest to enamel and the second strongest to dentine compared to the other bonding agents tested.

6.2.3 Marginal Quality

The sealing ability of Xeno III in comparison to Prompt L-Pop and Clearfil SE Bond was evaluated by Dentsply in standardised Class V cavities. The specimen were thermocycled 250 times between 5ºC and 55ºC, immersed in a 0.5% water solution of basic fuchsin for 24 hours, embedded in acrylic resin and cut into bucco-lingual sections. The in-vitro microleakage of the occlusal and gingival cavity walls was evaluated using an optical microscope. The extent of microleakage along the restoration was ranked between 0 (hermetic seal) and 3 (massive microleakage). The results of this study are shown in Figure 14.

Figure 14 illustrating marginal integrity

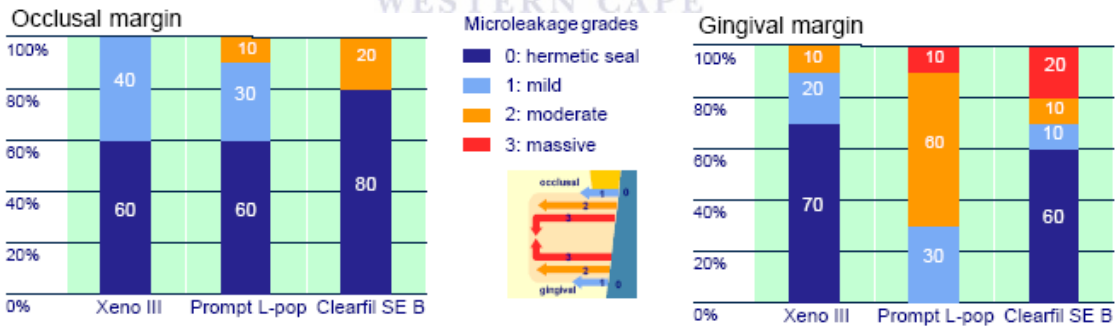


Figure 14: Marginal quality of class V restorations prepared with Xeno III compared to Prompt L-pop and Clearfil SE

The above results were obtained from Haller, Latta, Pameijer, Pioch, Powers, Rosales et al 2002 internal report to Dentsply DeTrey .

For all self-etching adhesives tested, the sealing obtained in the occlusal wall was better than in the gingival wall. Xeno III-treated occlusal cavity walls were – other than with Prompt L-Pop and Clearfil SE – hermetically sealed or showed only mild microleakage.

In the gingival wall, the best sealing was obtained for Xeno III, followed by Clearfil SE Bond. With Prompt L-Pop, the lowest sealing was obtained.

The above results however stand in strong contrast to research done by Grobler, Oberholzer, Rossouw, *et al* (2006). In their study of microleakage of Xeno III and Prompt L-pop on dentine, enamel and dentine as well as enamel alone, the authors found that microleakages between the enamel and dentine sides of teeth differed significantly for Xeno III, as well as Prompt L-pop while the microleakages between the dentine sides of Xeno III and Prompt L-pop did not differ significantly. The microleakage was found to be higher in enamel compared to dentine in both bonding agents tested with Prompt L-pop showing significantly higher microleakage (95% into the enamel) compared to 50% leakage into enamel in the Xeno III group. This is strongly contrasted to the manufacturer's report (Figure 14) which clearly states that less microleakage was found for the enamel group (occlusal wall) compared to the dentine (gingival wall). From their research Grobler, Oberholzer, Rossouw, *et al* (2006) concluded that both (Xeno III and Prompt L-Pop) single step self-etching dental adhesives gave micro leakage values for dentine which can be rated as low, with Xeno slightly better than Prompt L-pop. However, careful inspection of the enamel side indicated that sealing of enamel for both was poor and here Xeno III was the better of the two. From the above results the authors recommended neither of the two bonding agents for enamel sealing.

Chapter 7

Materials and methods

The experiment was subdivided into 4 sections, namely:

- 7.1 Dentine Permeability
- 7.2 Cytotoxicity
- 7.3 Confocal LASER Scanning Microscope studies
- 6.4 Light microscope studies

7.1 Dentine Permeability

Freshly extracted, intact human third molars (n=111) which were stored at 8°C with some thymol crystals added (approximate concentration 0,01%) in water to prevent bacterial growth were used for this study. All teeth were extracted due to clinical indications and written consent was obtained from the patients.

Figure 15: Cast teeth

Figure 15 showing the teeth cast in resin and ground until pulp horns are no longer visible.

The teeth where cast in clear casting resin (Clear Casting Resin, Resinate, 25 Webb street, Northmead, Benoni, South Africa) (Figure 15) to produce dentine discs of

approximately 120µm, 200µm, 350µm, 500µm, 750µm and 1000µm thick. This was done to evaluate dentine permeability of discs of various thicknesses as well as to compare the effect it may have on cytotoxicity. The discs were prepared in the following manner: after the resin had set, the teeth were ground with a grinding disc (50 grid, Double Disc Model Trimmer, BF Whemer, Franklin Park Illinois, USA) under constant water cooling, from apical until only the part of the crown remained that showed the pulp horns. The teeth were then further ground with 220 grid sand paper (PG2L, 639 3M) and water, as suggested by Chan, Franklin, King, *et al* (2003), up to the point were the pulp horns were no longer visible (Figure 16).

Figure 16: Origin of disc

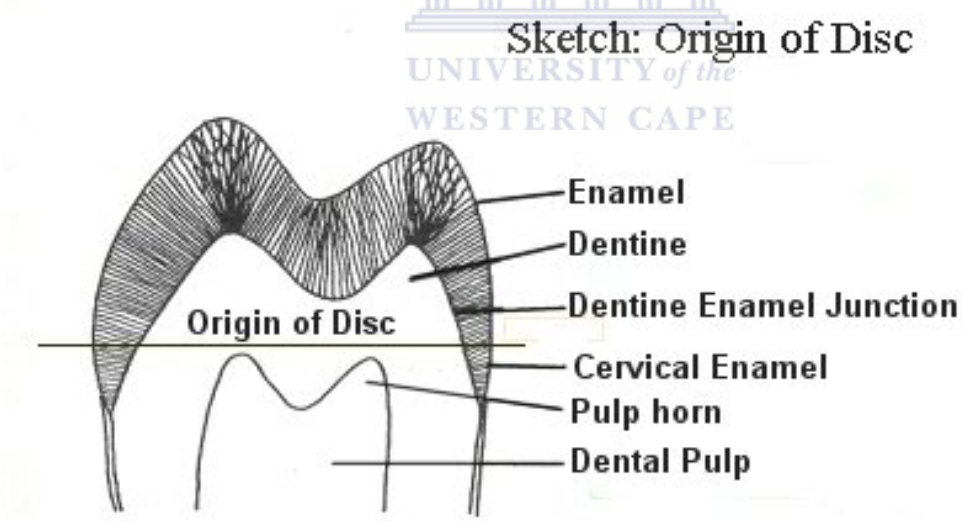


Figure 16 illustrates the origin of the disc as just coronal of the pulp horns

Subsequently the remaining tooth was sectioned just coronal of the pulp horns under constant water cooling by a 350µm thick slow speed diamond disc blade (Strues

Minitom Diamond wafering blade no 11-4244, Strues GMBH, Karl Arnold Strasse 13, D47977, Willich, Deutschland) (Figure 17) and

Figure 17: Strues Minitom

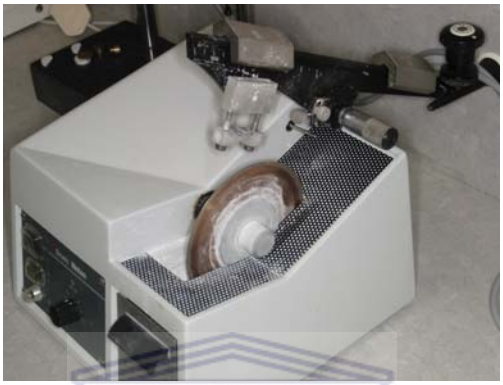


Figure 17: The Minitom with which the teeth were sectioned

ground with 220 grid sand paper (PG2L, 639 3M) and water on the coronal side until the desired thickness, measured with a Mitutoyo digital micrometer (Mitutoyo 965-Corporate Blvd, Aurora, IL 60504, USA) was obtained. This was done to ensure that the disc represented the section of the tooth just coronal of the pulp. The discs were stored in well plates in 70% alcohol at 8^0C to prevent bacterial and fungal growth. To avoid confusion, each thickness was kept in a separate well in the plates. To remove the smear layer, the discs were treated with 10% citric acid and 3% ferric chloride ($FeCl_3$) for 60 seconds (Cao, Huang & Jiang, 1992). To stop the etching process and to remove the dissolved appatite the discs were vigorously washed in three separate glasses containing distilled H_2O for 30 seconds each. Subsequently, after disinfecting (70% alcohol) the discs were placed in the special pulp chamber devices (20ml Micro reaction vessels by Supelco (Bellefonte, FA) between two rubber washers also supplied by Supelco. Inc. The washers have a hole of 5mm punched in the centre creating a

standard area for diffusion to take place and the system was tested to ensure that no leakage occurred, similar to Schmalz *et al* 2001b. In the perfusion experiments, the hydrostatic pressure of a 30cm water column was applied to one side of the dentine disc. A capillary with a known diameter was placed between the manometer column and the inlet. The movement of an air bubble in a capillary system was measured against time, where time was measured taken to allow the diffusion of 5μl through the standard opening on the dentine disc. The time the air bubble takes to move from one point to another represents the volume moving through the dentine by hydraulic conductance in that period of time (Figure 18). Thus, there will be a change in volume of X μL/cm^2/min for a particular height of water (Typical results expected for 0,5mm dentine discs may range from 0,01μL to 0,5μL) (Schmalz, Hiller, Nunez, *et al* 2001b). The temperature was kept constant at 23±1° C. After the perfusion for the discs had been measured and thus the first leg of the study completed, the tissue culture and cytotoxicity studies were started as the second leg of the study.

Figure 18: Diagram of apparatus used to measure hydraulic conductance

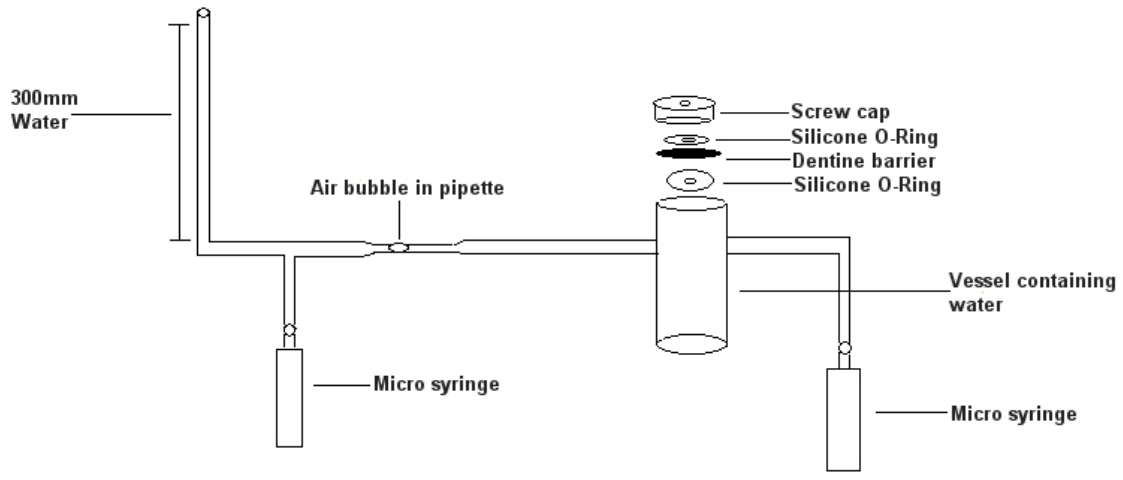


Diagram of apparatus used to measure hydraulic conductance. The flow of the bubble through the micro pipette towards the vessel represents the volume flow through the dentine barrier.

7.2 Cytotoxicity experiments

For the cytotoxicity tests Balb/c 3T3 mouse fibroblasts, which were obtained from the National Repository for Biological Materials (Sandringham, South Africa), in a strong growing phase were used. They were cultured in 5ml 5% Dulbecos Modified Eagle's Medium (DMEM) (Gibco BRL, Life Technologies, Paisley, Scottland) in Petri dishes (Figure 19).

Figure 19: Petri dishes containing DMEM

The Petri dishes containing DMEM in which cells were grown to near confluence

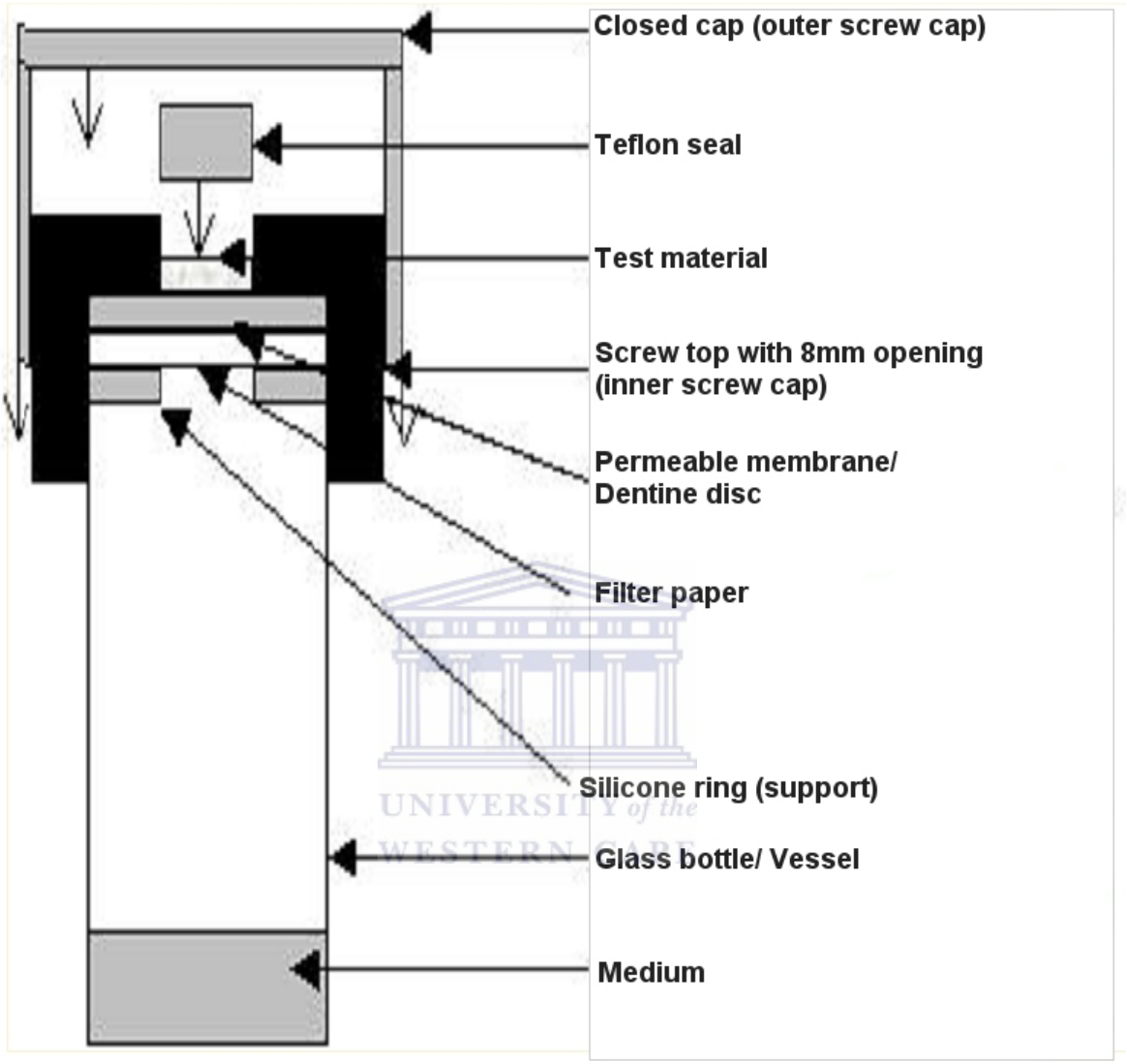
The medium was subsequently discarded and cells washed with 2,5ml phosphate buffer substrate (PBS) (ICM Biomedical Inc. Aura, Ohio). The PBS was then removed and 2,5ml trypsin (Trypsin/ Versene) added. The dishes with the cells were placed into an incubator (Labotec Incubators, Forma Scientific, 8008/848/3080, Box649 Manetta, Ohio, 457500, USA) (Figure 20) for 5 min

Figure 20: Incubator

Incubator in which cells were cultured and exposed to extract

at standard tissue culture conditions (5% CO_2 at 37^{o}C and 90% humidity) to break intercellular adhesion. An inverted light microscope (Olympus CK2, Wirsam Scientific, Po Box 130, Rondebosch, 7700, South Africa) was used to ensure that all cells were free in suspension. 2,5ml 5% Eagle's Medium was then added and the suspension centrifuged at 300 rpm for one minute to cause sedimentation of the cells. From this suspension 400μl was plated into well plates and cells were allowed to reattach to each other and the plates over a period of 24h in an incubator at standard tissue culture conditions.

Figure 21 : Modified *in vitro* pulp chamber device

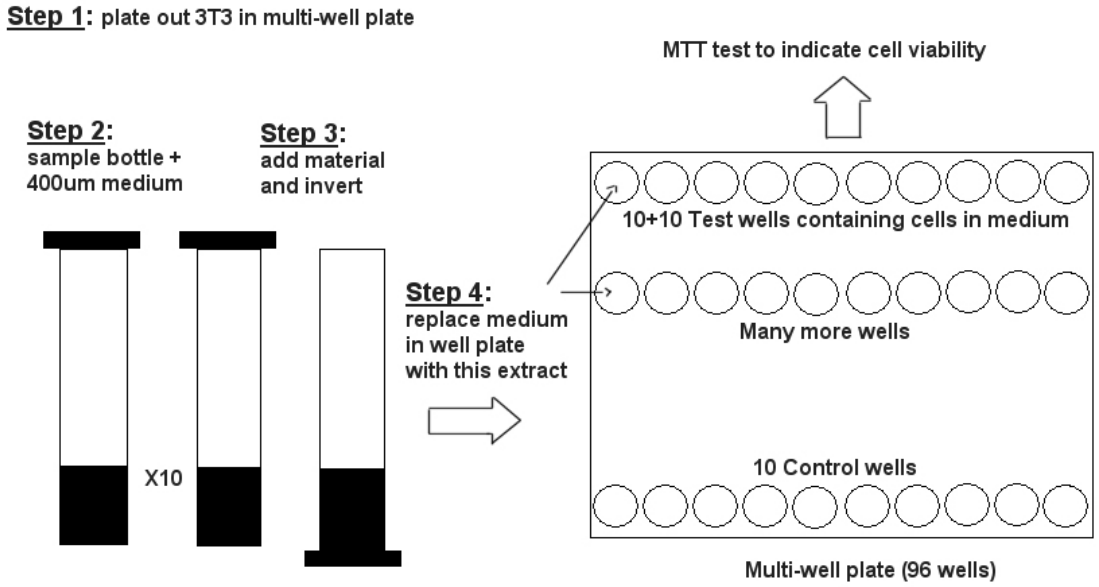


The disc or membrane is placed as barrier in front of the opening of a glass bottle containing Dulbecco's Modified Eagle's Medium. A silicon ring is placed under the barrier for support, from which it is separated by cellulose filter paper (Osmonics). A screw top with an 8mm opening (inner screw cap) is screwed down onto this and the bottle is inverted to ensure that no leakage occurs. Subsequently the material to be tested (in this case Xeno III) is applied to the top (coronal surface) of the barrier, sealed with a Teflon seal and a closed cap (outer screw cap) screwed down onto the external thread of the inner screw cap.

Xeno III (Dentsply) was then applied to the 120µm, 500µm, 750µm and 1000µm discs as well as to the membrane (Omonics MicronSep, Cellulosic, White, Plain, 45 Micron). The 120µm disc range was chosen to prove if Xeno III was toxic at all in extract tests. The 500µm disc range was used under the hypothesis that it could prevent cytotoxicity by forming a barrier for the toxic substances. The 750µm as well as 1000µm disc ranges were included to see at which thickness dentine really formed an adequate barrier for Xeno III. This led to the 200µm group being redundant and left out in the cytotoxicity experiments. Xeno III was applied, according to manufacturer's prescription, by dispensing an equal amount of Liquid A and Liquid B into a clean mixing well and the liquid mixed thoroughly for approximately five seconds with the applicator tip supplied. Eight micro liters (8µl) were then applied to the cavity side of the vessel (Figure 21) and left undisturbed for at least 20 seconds after which the adhesive was uniformly spread using a gentle stream of oil free air for 2 seconds until there was no more flow of the adhesive to ensure proper removal of solvent. As last step the adhesive was cured with a light-curing unit (Blue Light. LA 500. Input Rating 230V-0.5V, 50/65 Hz; APOZA Enterprise, 6F 657 Chung-Cheng Road, Hsin-Chuang Road, Hsin-Chuang City, 242 Taipei Hsien, Taiwan) for 10 seconds.

400µl Eagle's Medium was placed into the pulpal side (Figure 21 (p 63) and Figure 22 (p 65)) of the sterilized glass bottle and returned to the incubator with Eagle's Medium on the pulpal side, the bottle sealed and placed in the incubator.

Figure 22: Illustration of procedure



In Figure 22 the procedure is illustrated in 4 steps. Step one consists of the plating out of the cells in wells (96 well multi-well plates). In step two 10 bottles (like the one in Figure 21) are prepared as described, containing 400µl of DMEM. Step 3 was inverting the bottles after the material had been placed after which the inverted bottles were left for 24h. After 24h the medium containing the obtained extract was removed from the bottles and used to replace the medium in the well plates containing the cells (step 4). The cells were left in contact with the extract for 24h after which MTT cytotoxicity assays were done.

After the first hour in the incubator, once the bottles had warmed up, they were removed and the screw caps (Figure 20) were tightened, placed back into the incubator and left for an additional 30 minutes before inverting them in order to make sure that no leakage occurred. The sealed system was then left in the incubator under standard tissue culture conditions for 24h to allow components of the bonding agent sufficient time to be extracted and leach into the medium. After 24h in the incubator the bottles containing the extract, were removed. 400µl of this extract was then removed from each bottle, and used to replace the 400µl of medium in the well plates, containing the cells. The well plates containing the extract and cells were subsequently placed into the incubator for

further 24h under standard tissue culturing conditions. Cell viability was then measured after 24h using 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetra zolium bromide (MTT) assay and Photo spectroscopy as described in Mossman (1983). The medium containing the extract from Xeno III was removed from the wells and replaced with fresh medium to which the MTT was added. The plates were incubated in the dark for 4 hours, the medium and the MTT removed, and the water-insoluble MTT-formazan crystals dissolved in Dimethyl Sulfoxide (DMSO). Phosphate Buffer Substrate (PBS) was added to adjust the final pH and absorbance was recorded in an ELISA plate reader. The MTT assay is a sensitive, quantitative and reliable spectrophotometric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzyme in living cells to convert the yellow water soluble substrate (MTT) into a dark formazan product that is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cell lines.

7.3 Confocal LASER Scanning Microscope studies

In total seven teeth were sectioned into 500µm discs which were used for Confocal LASER Microscope studies. The teeth were cut and ground using 1200 grit silicon carbide paper (PG2L, 639 3M) by means of wet grinding to the desired thickness as described above, under 6.1. After grinding, three of the prepared discs were used to study the effect of different etching procedures. One disc was left unetched, one was etched with 10% citric acid alone for 60 seconds and the third one of this group was etched with 3% $FeCl_3$ as well as 10% citric acid for 60 seconds. All teeth were vigorously washed in three separate containers, each filled with double distilled water for 30 sec respectively, to stop the etching process. The surface of the teeth was coated with 0.07% Fluorescein (E. Merck, Darmstad, Germany) in 50% ethanol / 50% water to enable visualisation of the surface. Four of the seven teeth prepared, were used to study Xeno III, studying the disc after each relevant application step. Of these one was left as control i.e. untreated. In the next disc used to study Xeno III, Fluorescein dye was added to Xeno III to a final concentration of 0.07% in order to demonstrate penetration of the bonding agent into the dentine as well as to the surface and to create a 3 dimensional visualisation. Xeno III and 0.07% Fluorescein dye was applied to the third tooth but left uncured. The last tooth was treated with Xeno III and dye was applied and cured (Grobler, Oberholzer, Rossouw, *et al,* 2006).

7.4 Inverted Light microscope studies

After exposure to the extract and culture, cells in the 500µm dentine barrier were visually inspected using an inverted light microscope (Olympus CK2, Wirsam Scientific, Po Box 130, Rondebosch, 7700, South Africa). Cell morphology and viability were assessed and compared to the control at 50X magnification.

Chapter 8

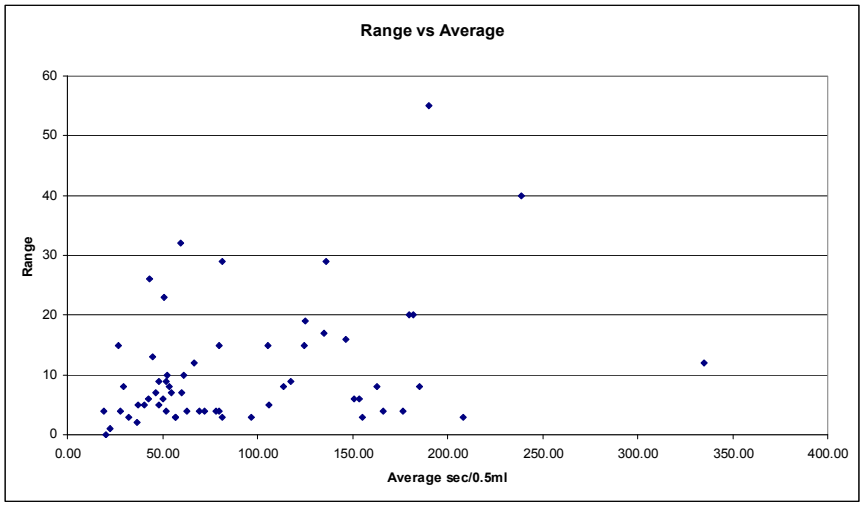
Results

- 8.1 Dentine Permeability
- 8.2 Cytotoxicity
- 8.3 Confocal LASER Scanning Microscope studies
- 8.4 Light microscope studies

8.1 Dentine Permeability

In the permeability experiments, 111 discs were used of which 73 for the 500µm disc group, 9 for the 120µm, 9 for the 200µm and 8 for the 350µm. Altogether 12 discs were discarded, as they did not meet the strict criteria set down to attempt standardization, out of the following reasons: 3 discs were dehydrated, in 3 the enamel layer was reached, 4 had a small disc diameter and leakage could not be excluded, one disc was too thin and one disc had a perforation. Of all discs 3 runs were done to measure the time taken for 0,5µl to diffuse. For the discs of approximately 500µm, it was found that spread of values considering the range was higher for higher perfusion times i.e. the variability increases as the perfusion time increased (heteroscedasticity was identified) (Graph 1).

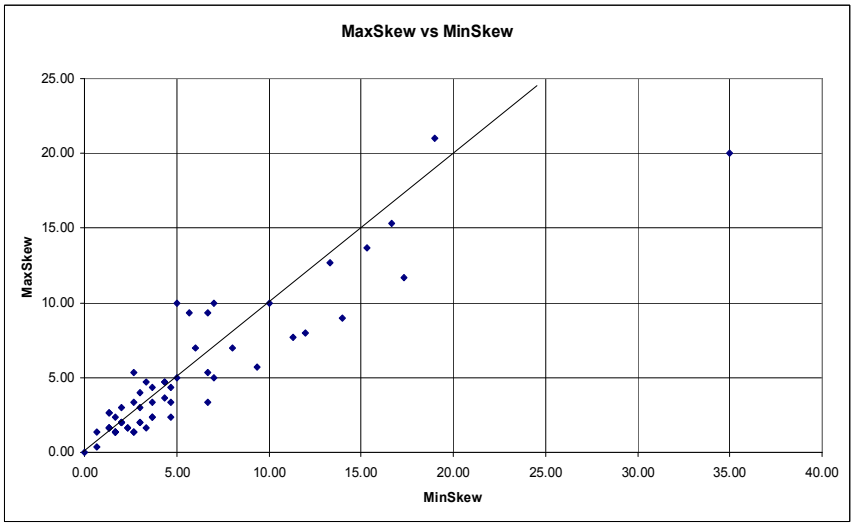
Graph 1: Range vs Average



In Graph 1 the X axis shows the average perfusion for each disc. The Y axis shows the range for each disc which was measured.

When plotted on a graph more values were lying underneath the 45% line than above it, suggesting a downwards skewness of values (Graph 2).

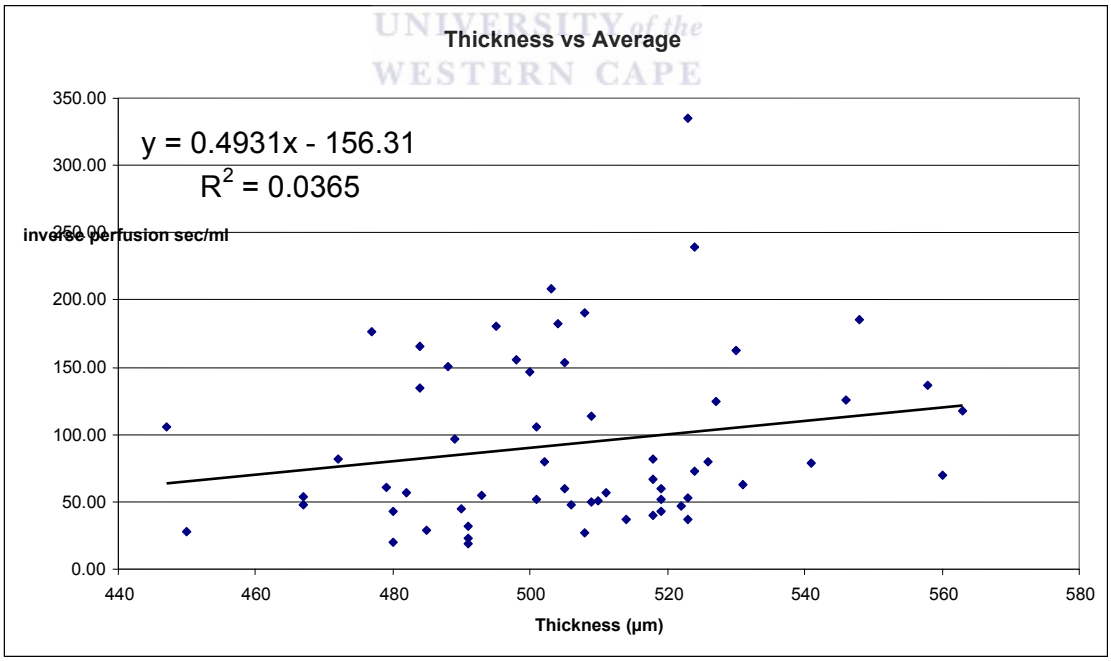
Graph 2: Skewness of values



The above Graph 2 illustrates downward skewness of values with the majority of values lying underneath the 45% line

With this in mind two outliers were removed and a linear regression line was fitted which demonstrated a statistical significant link between thickness and perfusion with the coefficient of determination (r^2) equal to 0.0365 (Graph 3) for disc with a thickness between 440-560µm i.e. under the assumptions of linearity and normality, 3.6% of the variation of the inverse perfusion is explained by the thickness. Both the inverse perfusion and the thickness were transformed by means of the square root transformation. In this case the coefficient of determination had improved to 3.65% from its value of 2.8% prior to transformation. As a result a perfusion range could be predicted for any disc by using the formula: Ln ("Inverse perfusion") = "thickness" x 0.00718 + 0.64807.

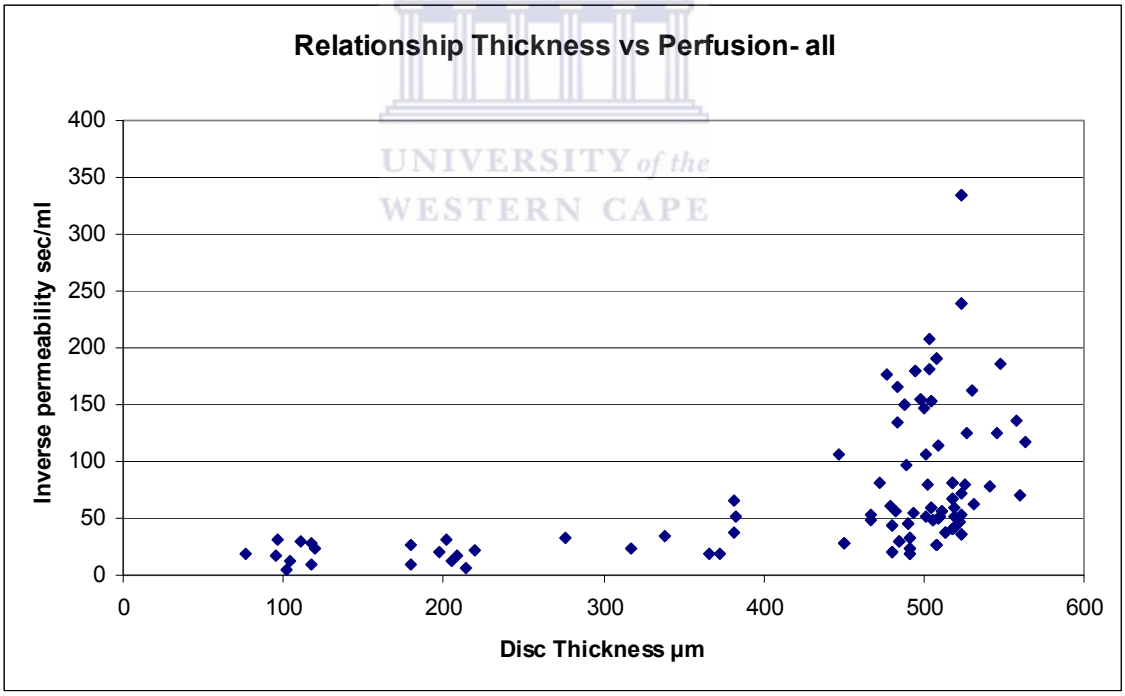
Graph 3: Thickness vs average perfusion



Graph 3 illustrates the relationship between inverse perfusion and thickness. Note that thicker discs resulted in higher inverse and therefore lower perfusion values.

When the relationship between discs of different thickness was studied it was found that there was a large variation in the data at a specified thickness, but still a clear link between thickness and permeability could be seen (Graph 4). For the 120µm, 200µm and 350µm discs compared to the 500µm discs, it was observed that the coefficient of determination is 39%, i.e. 39% of the variation could be explained by the difference in thickness. From the graph beneath, it can also be seen that the permeability became really evident at a dentine thickness of more than ~350µm. This definite link between disc thickness and perfusion has also been mentioned by others (Schmalz, Schweikl, Esch, *et al* 1996; Hanks, Diehl, Graig, *et al* 1989).

Graph 4: Thickness vs. Perfusion



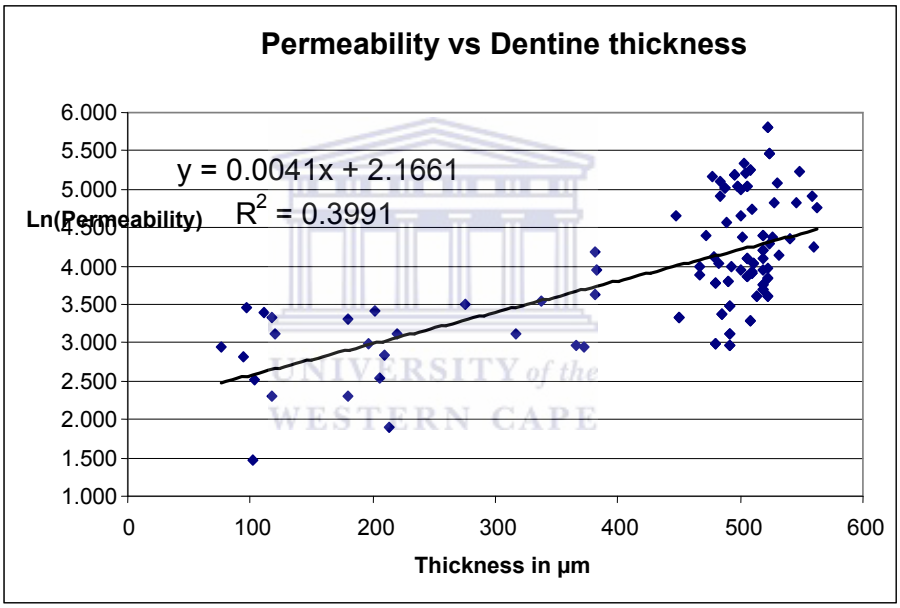
In Graph 4 it can be seen that the perfusion increases as the disc thickness increases.

In the above graph the X axis represents discs of various thicknesses and the Y axis represents the mean perfusion. It can clearly be seen that inverse permeability

increases mostly between c.a. 350µm -~500µm. It is however noted that even for the discs with a standard thickness of 500µm there is a large spread of values which can be attributed to individual and histological variations.

When the natural logarithm was applied to the above graph, a straight line could be fitted to describe the data (Graph 5). The coefficient of determination was very high, (39.1%) which could be explained by the difference in thickness.

Graph 5: Logarithmic transformation



Graph 5 illustrates that after logarithmic transformation there is a linear relationship between permeability and disc thickness. Here the coefficient of determination is 0.3991, i.e. 40% of the variation can be accounted for by the disc thickness.

8.2 Cytotoxicity experiments

As part of this study cytotoxicity tests were done on the newly released dentine bonding agent Xeno III by Dentsply. In these experiments permeable membranes (Omonics Micron Sep, Cellulosic, White, Plain, 45 micron) and alternatively dentine discs of ~120µm, ~500µm, ~750µm and ~1000µm where used as a barrier in extract tests. The discs were etched for 1 minute with 10% citric acid and 3%$FeCl_3$ (Cao, Huang & Jiang, 1992) and rinsed in water to stop all etching processes.

The results obtained showed that the cytotoxicity levels when using the membranes as barriers between the medium and Xeno III, compared to the 120µm discs etched with 3% $FeCl_3$ and 10% citric acid and used as barriers, were very similar. The ~120µm disc group etched with 3% $FeCl_3$ with 10% citric acid, produced 25.4% cell viability. The membrane produced similar cell viability at 22.4%, compared to the control which was 100%. Interestingly enough, it was found that the 120µm discs which were etched with 10% citric acid only, produced much less cytotoxicity (92% and 102% cell viability).

The ~500µm group showed an increase to 60.7% cell viability and the ~750µm group 72.9% cell viability. Only at a disc thickness of ~1000µm (100%cell viability) as barrier, no cell death was noted. The raw values obtained for the data can be seen in sheet 1 on p 74, with the standard deviation as well as mean percentage of cell viability for the respective groups below each column. Table 4 on page 75 summarizes the groups and expresses the mean percentage of cell viability to aide comparison between the groups. Table 4 also shows the number of experiments done in each group, as indicated by the letter “n”.

Sheet 1: Cell viability raw values obtained from Cytotoxicity studies: ~120µm, ~500µm, ~750µm, ~1000µm and membrane

Membrane

Membrane	control
0.173	0.89
0.174	0.807
0.165	0.795
0.153	0.752
0.175	0.649
0.143	0.873
0.172	0.75
0.187	0.833
0.165	0.6
0.163	0.68
0.174	

22.4%viable

Standard Deviation

0.011868

Dentine Discs ~500µm

500um	control
1.15	0.514
0.73	0.446
0.164	0.363
1.293	0.351
1.111	0.448
0.792	0.445
1.132	0.597
1.167	0.849
0.957	0.569
1.105	**0.968**
1.137	0.689

60.71% viable

Standard Deviation

0.317245

Dentine Disc ~1000µm

1000um	control
0.853	0.992
1.07	0.976
0.968	0.759
0.986	0.89
0.734	0.838
0.82	0.858
0.976	0.883
0.952	0.826
1.03	1.028
0.957	0.939

100%viable

Standard Deviation

0.102058

Dentine Disk ~120-200µm

120um	control
0.197	1.639
0.186	1.819
0.169	1.372
0.173	1.368
0.164	1.464
0.214	1.996
0.178	1.819
0.195	1.531
0.178	1.523
0.17	1.543
0.172	1.69
0.171	**1.543**

25.4%viable

Standard Deviation

0.014712

Dentine discs ~750µm

	750um	control
	0.662	1.046
	0.622	1.035
	0.664	1.037
	0.614	1.042
	0.8	1.033
	0.817	0.976
	0.756	1.074
	0.869	1.094
	0.82	0.956
	0.752	0.972
leaking	0.191	1.045
	0.177	1.041

72.9%viable

Standard Deviation

0.230702

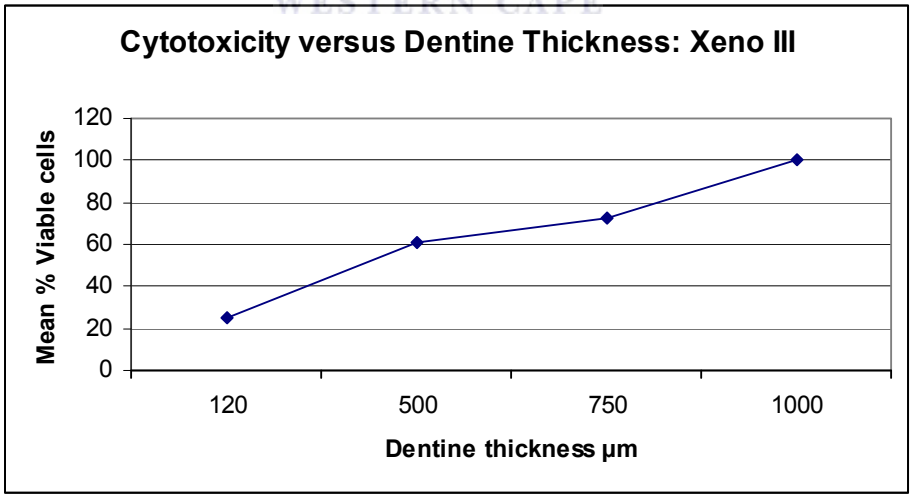
Table 4: Summary of cytotoxicity

Barrier Type	Membrane n=4x10	120µm n=3x10	500µm n=3x7	750µm n=3x7	1000µm n=3x7
Cell Viability	25.7%	25.3%	60.7%	72.9%	100%

The first row shows the barrier used in the experiment and the second row mean cell viability. It illustrates the mean cytotoxicity for Xeno III through each respective barrier. The letter "n" gives the number of experiments done in each group.

When evaluated on a graph (Graph 6) it can be seen that there is a non-linear relationship between cell viability and dentine thickness which becomes more relevant after a thickness of about 300µm. This was also found by Schmalz (1994), but contradicts the findings of Murray, Smith, Windsor, *et al* (2003) who states that no cell injury takes place in dentine thicker than 500µm.

Graph 6: Cytotoxicity vs Dentine Thickness

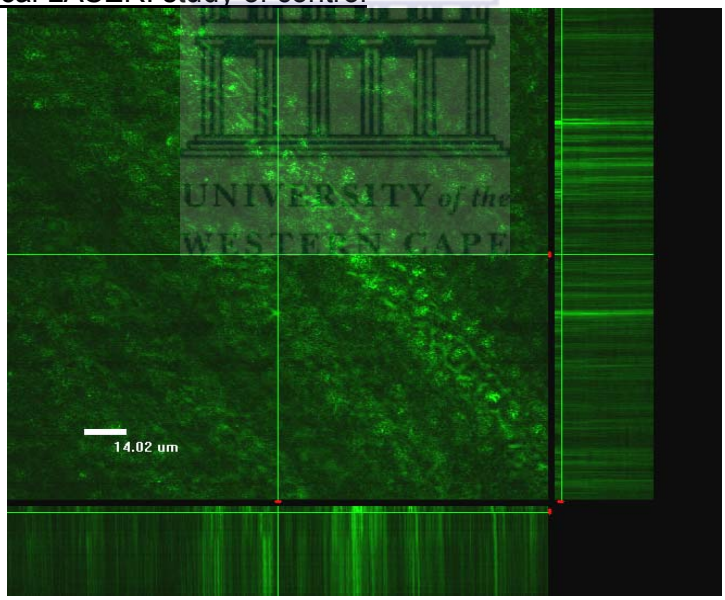


Graph 6 illustrating that cell viability clearly increases as result of an increasing in dentine thickness.

8.3 Confocal LASER studies showing the effect of etching on dentine

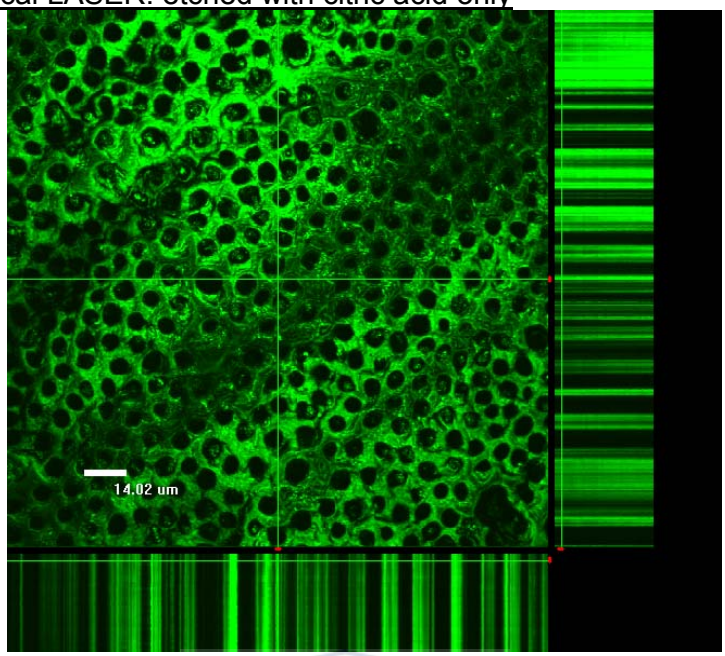
When the etched discs were studied under a Confocal LASER microscope a clear difference in the diameter of dentine tubules could be seen. Figure 23 shows dentine that was cut and left unetched and used as control. In this image obturation of the orifices of the dentinal tubules could clearly be seen. In comparison to this in Figure 24 the discs were etched with citric acid alone. In this figure partial obturation of the majority of dentinal tubules can still be seen. When compared to Figure 25, which shows a disc etched both with 10% citric acid as well as 3% ferric chloride ($FeCl_3$), it can be seen that in this image the orifices of most tubules are clearly open and enlarged.

Figure 23: Confocal LASER: study of control



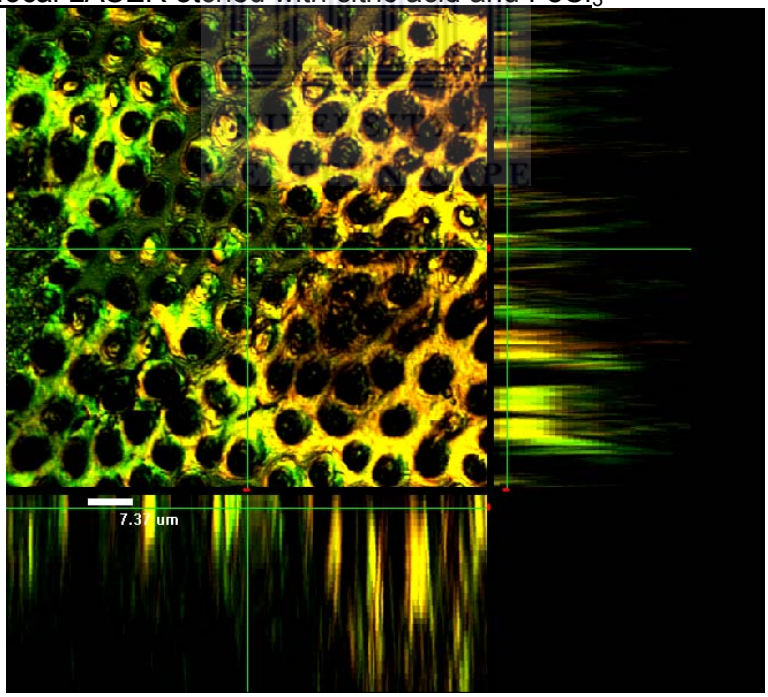
Confocal LASER studies of the control show marked obturation of the dentine tubules by the smear layer.

Figure 24: Confocal LASER: etched with citric acid only



Etching with citric acid left some of the dentine tubules partially occluded

Figure 25: Confocal LASER etched with citric acid and $FeCl_3$



Etching with citric acid and $FeCl_3$ resulting in large and open dentine tubules

Surface area plots of the above discs were drawn and also illustrated the differences. In Figure 26, which represents the control i.e. unetched discs, it can be seen that the surface plot is fairly even with a majority of peaks on the same level as well as very few broad troughs. When this is compared to the plot (Figure 27) of the discs etched with 10% citric acid alone, it can be seen that the peaks and troughs are more spread out. Figure 28 of the disc etched with 10% citric acid as well as 3% $FeCl_3$ shows the biggest spacing between the peaks with broad troughs. This is due to the fact that the tubule orifices are open and more penetration by the microscope could take place.

Figure 26: Surface area plot: control

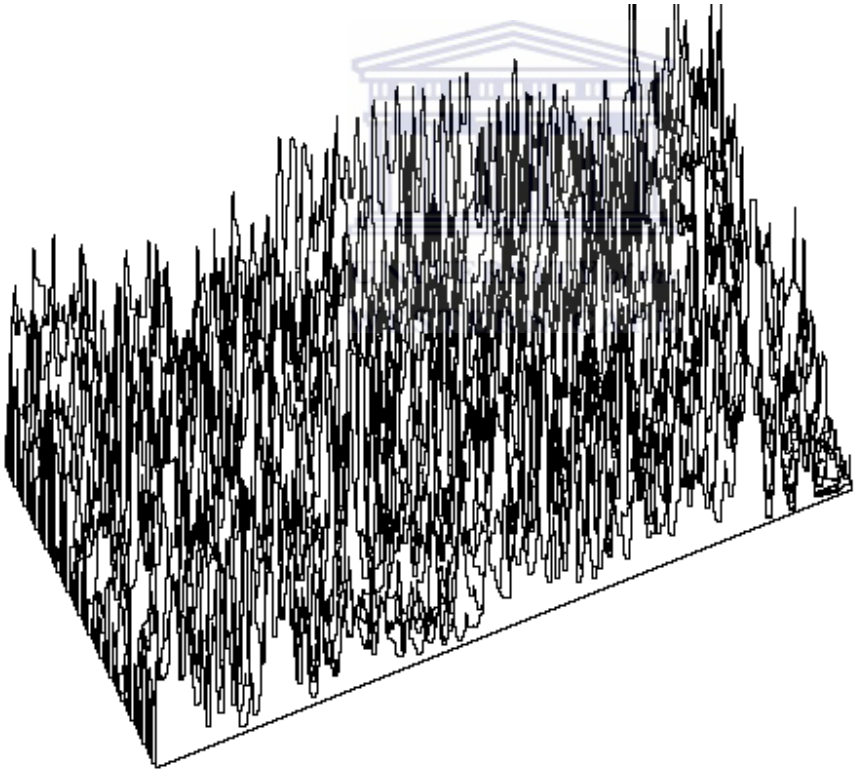


Figure 26: note uniform peaks and few deep troughs

Figure 27: Surface area plot: etched with citric acid

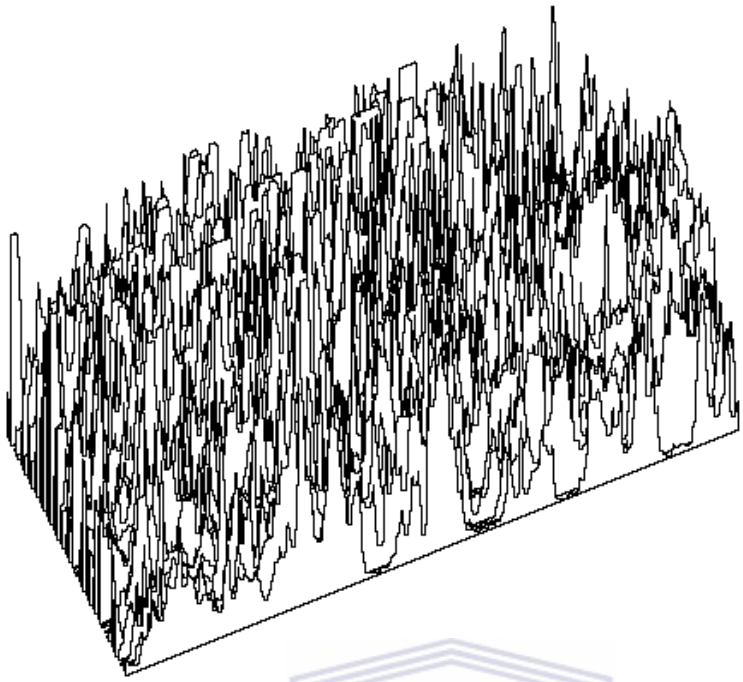


Figure 27: Deeper and clearer distinction between crest and troughs

Figure 28: Surface area plot: etched with citric acid and $FeCl_3$

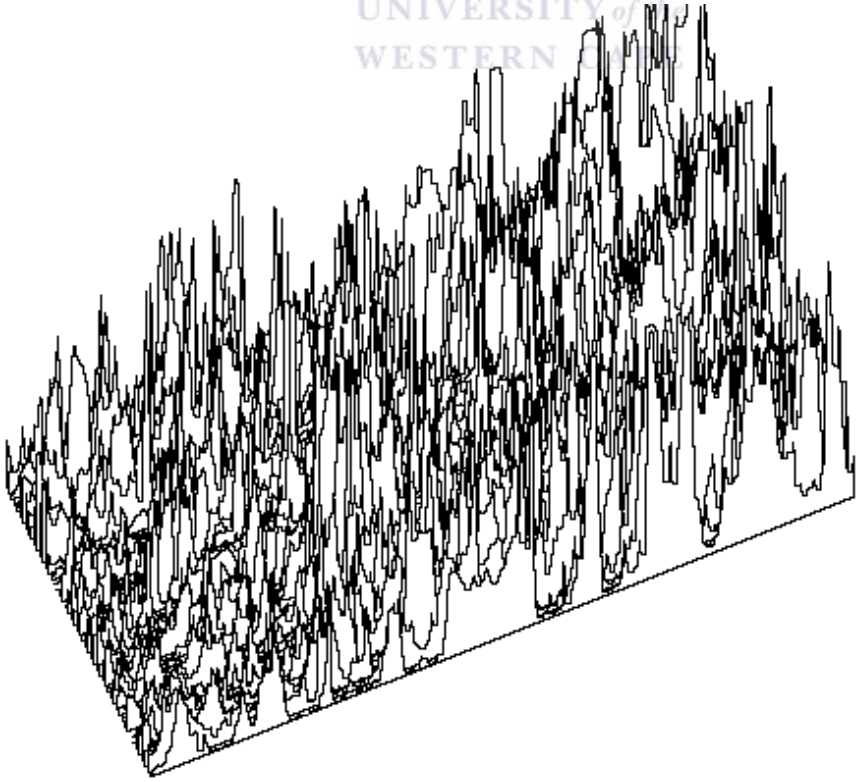
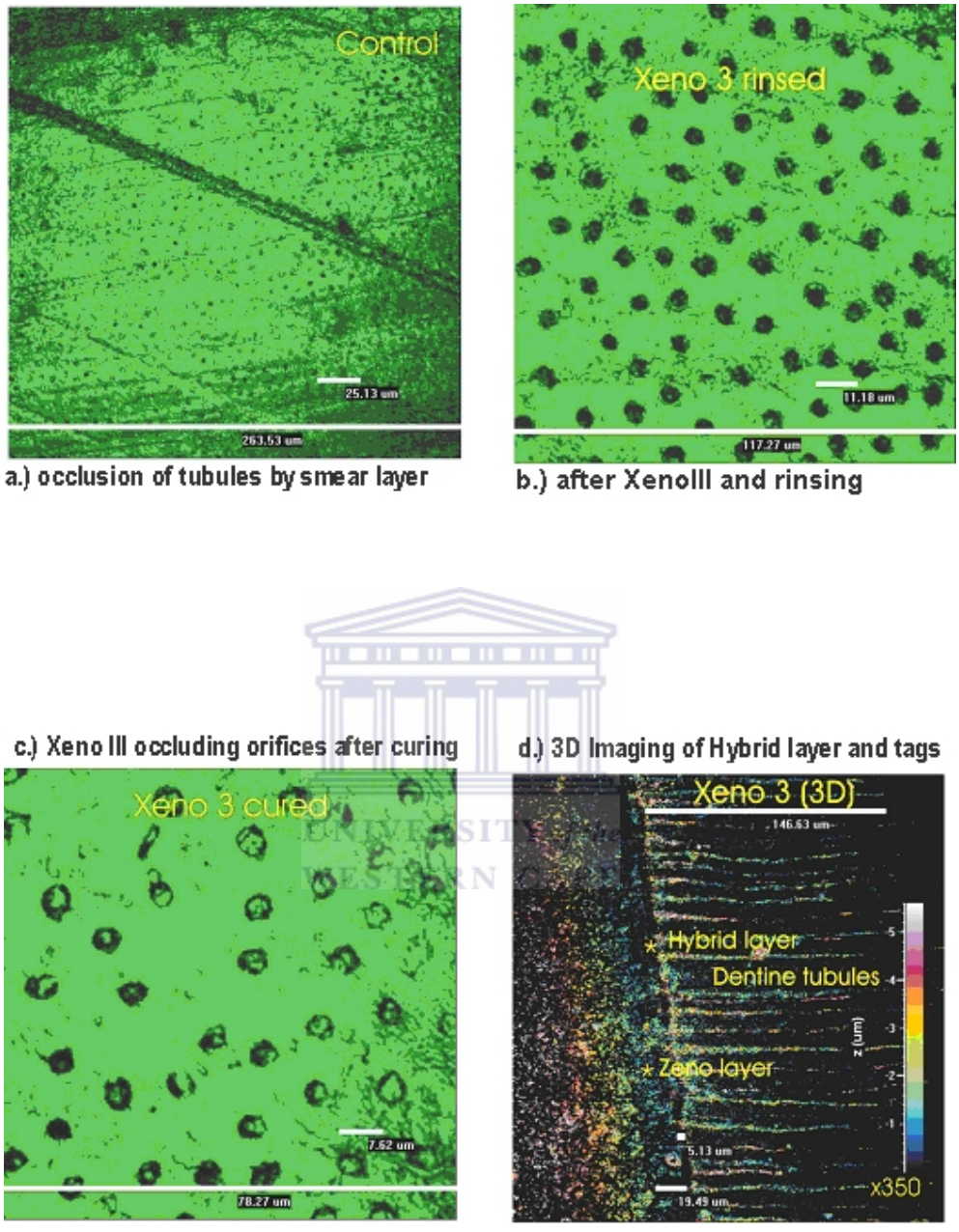


Figure 28: Wide crests and troughs as a result of deep penetration

Excessive penetration depth of Xeno III was also confirmed by 3D Confocal LASER studies (Figure 29). In images a, b, c and d it can be seen how at first the orifices of the dentine tubules are open and then subsequently obturated after curing of Xeno III. In the first image (Figure 29 a) of the sequence, the control can be seen with occlusion of the dentine tubules by the smear layer. In the second image (Figure 29 b) the dentine surface could be seen after application of Xeno III and rinsing. In this image the open dentine tubules can be seen as a result of etching. The third image (Figure 29 c) in the series shows the open dentine tubules with cured resin tags in them. Figure 29 d is a three dimensional recording which clearly illustrates deep resin tag formation and the hybrid layer.

Figure 29: Illustration of series microscope studies

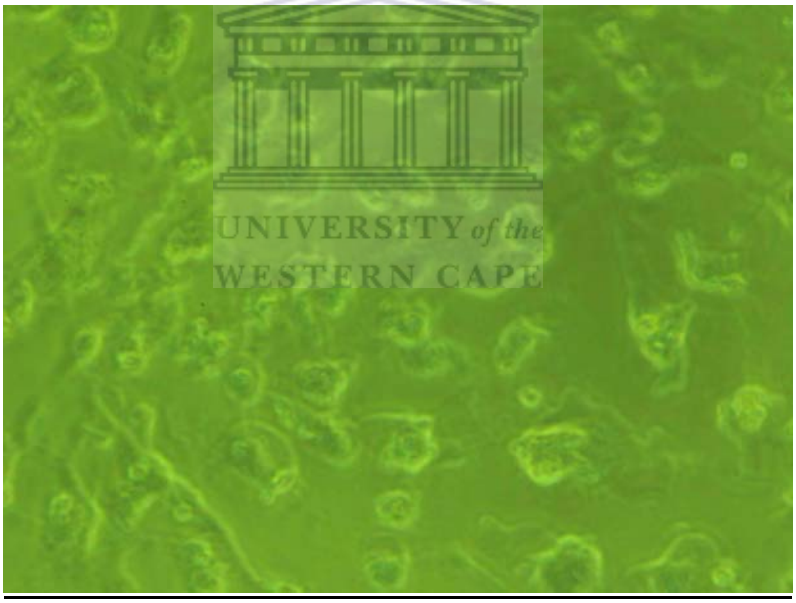


In the last (d) image in the sequence, the material layer, hybrid layer and dentine tubules with deep resin tag formation (~140µm) can be seen.

8.4 Inverted Light microscopic studies

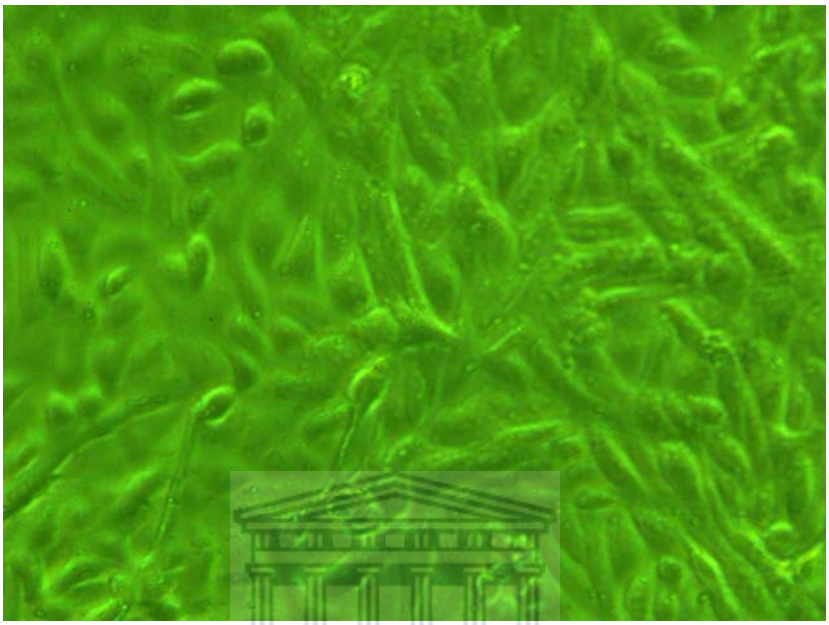
When the cell cultures were studied under an inverted light microscope at 50x magnification, it could be seen that massive cell death had taken place in the 120-350µm groups. The rupture of cell membranes had taken place, causing leaking out of cytoplasm and accumulation of cell organelles around the nucleus. The nuclei of the cells were clearly visible in the remains of the dead and rolled up fibroblasts (Figure 30). These stood in strong contrast to the cells at near confluence in the control which were classic spindle shaped fibroblasts with intact cell membranes and nuclei (Figure 31).

Figure 30: Xeno III group at 50X magnification



The figure above shows curled up fibroblasts.

Figure 31: Control group at 50X magnification



Control group showing healthy, spindle shaped fibroblasts.

Chapter 9

Discussion

During the preparation of the dentine discs in this experiment, the discs were prepared according to standards set by Cao, Huang & Jiang (1992). This was done to reproduce the smear layer equal to the one produced during cavity preparation. After cavity preparation the tooth is usually etched in order to prepare the area for bonding. As the scope of this experiment was to determine the permeability of different teeth as well as their effect on cytotoxicity, it was opted to remove the smear layer entirely by etching with 10% citric acid and 3% $FeCl_3$ for 1 minute, (Cao, Huang, Jiang, 1992). Discs were also etched with 10% citric acid only and left untreated as control. The discs were then subsequently studied under a Confocal Laser Microscope. It was found that when the discs were etched with 10% citric acid alone (Figure 24), the smear layer was not removed completely and dentinal tubules were only partially opened with debris clearly visible in the openings of the dentine tubules. However, if the discs were etched for one minute with 10% citric acid and 3% $FeCl_3$, the entire smear layer was removed (Figure 25), leaving the dentine discs completely open which resulted in tubular enlargement and removal of smear plugs was clearly visible. The effect hereof also had a direct impact on the surface plots.

Similar observations were made by Schmalz, Hiller, Nunez, *et al* (2001b). In their experiment they found that the acid etched radii of dentine tubules seemed to be at least 2-3 times larger than the unetched, thus etching enlarges the available area for diffusion. For both human and bovine dentine more tubules were observed on the pulpal side under SEM. For the perfusion experiments the coefficient of variation was 70% (SD divided by mean). These findings by Schmalz, Hiller, Nunez, *et al* (2001b) support observations made in the current investigation.

In the permeability experiments presented, variation was present although steps were taken to minimize this, by maintaining the anatomical position as well as repeating the preparation procedure carefully for each disc. This is also noted by Schmalz, Hiller, Nunez, *et al* (2001b) who found that for the perfusion experiments on dentine discs the coefficient of variation was 70%.

From these findings and the discussion above, it appears that for standardization purposes in *in vitro* studies, human dentine is not ideal as large variation in results can occur due to variation in dentine permeability. Although dentine discs have the advantage of directing towards the clinical situation (Pashley, Derksen & Tao, 1988; Imazato, Tarumi, Ebi, *et al* 2000), these high individual variations make it impossible to replicate *in vitro* experiments in order to compare the biocompatibility of different dental materials.

A lot of research has been done on the protective properties of dentine in the past years (Abou Hashieh, Franquin, Cosset, *et al* 1998; Abou Hashieh, Cosset, Franquin, *et al* 1999; Chen, Liu, Tseng, *et al* 2003; Hanks, Graig, Diehl, *et al* 1988; Hanks, Wataha, Parsell, *et al* 1994; Pashley, Derksen & Tao, 1988; Schmalz, Schuster, Koch, *et al* 2002; Vajrabhaya, Pasasuk & Harnirattisai, 2003). In all this research the protective properties of dentine in terms of preventing cell damage caused by dental materials is well documented. Already in 1994, Hanks (Hanks, Wataha, Parsell, *et al* 1994) stated that if the remaining dentine thickness (RDT) between test material and cell would be 500µm or more, the dentine would protect the cells sufficiently by reducing the concentration of noxious materials reaching the cells to prevent cell death. This statement is contradicted by the results obtained in the present study. Supporting the present results, Vajrabhaya found (Vajrabhaya, Pasasuk & Harnirattisai, 2003) only 58% cell viability for Single Bond, 57.41% for Prime and Bond 2.1 and 59.1 % for Syntac Single component on a

500µm disc in their experiment, which are similar results compared to the 60,7% cell viability found for Xeno III in this study (Table 4). This contradiction can partially be attributed to the large variation found in the perfusion values for 500µm dentine discs in the current experiment, as well as in Schmalz, Hiller, Nunez, *et al* (2001b).

As it can be seen, this large variation can easily result in large variation in test results as no two discs were found with the same perfusion values. Another factor which must be kept in mind is the depth of penetration of the material, where modern materials as used here and by Vajrabhaya, Pasasuk & Harnirattisai (2003) may have deeper penetration depths and thus are more likely to produce a cytotoxic effect. Here once again this is supported by the 3D laser studies done in our study on the resin tag penetration of Xeno III. In the image (Figure 29d.) it can clearly be seen that the resin tags penetrate to a depth of ~140µm. Such deep penetration would have as result that the leachable components of the material would be placed much closer to the pulp than previously speculated. Interestingly enough, the 100µm membranes and the 120µm discs etched with 3% $FeCl_3$ and 10% citric acid produced very similar results. From these results it can be seen that the Xeno III is still cytotoxic even after it has reacted with dentine (see Chapter 6), changing from its acidic form to a neutral form after reacting with the mineral content of dentine. The vast difference in cell viability between the 3% $FeCl_3$ / 10% citric acid group and the 10% citric acid group can be explained by the smear layer and its removal (Figure 23-25). During classic cavity preparation a lot of debris is created. This material obstructs the orifices of the dentine tubules (smear layer) causing partial or total occlusion and thus influencing permeability. As an explanation for these similar results in cell viability between the 100-300µm discs as well as membranes, it can only be said that to produce the same amount of cell death in the experiments, an equal amount of material must have leached into the imitated pulp chamber. Since the volume of applied

material was kept constant, the leachable components of the material must have completely penetrated in the 120µm as well as the membrane experiments and some even past a thickness of 500µm (Graph 6), still causing cytotoxic effects at 750µm as already suggested by Chen, Liu, Tseng, *et al* (2003) and confirmed by *in vivo* experiments by Murray, Smith, Windsor, *et al* (2003). It was found that only at a thickness of 1000µm no cytotoxic effect was seen, which suggests that Xeno III should not be used in teeth with a RDT of less than 1000µm.

In correlation to this Murray, Smith, Windsor, *et al* (2003) found that the number of odontoblasts beneath cavity preparations was highly correlated to the remaining dentine thickness. With decreasing RDT, underlying odontoblast numbers decreased in an exponential manner rather than linear manner. They found that compared with unaffected odontoblasts, the numbers were reduced by 13.6% beneath a RDT of 2,5-0,5mm, 33,7% beneath a RDT of 0.5-0.01mm and 99% beneath pulp exposures. In our study we found an exponential rather than linear correlation (expressed by the strait line on the logarithmic graph, Graph 5) between the thickness of dentine discs and perfusion rather than a linear correlation. This would make sense to impact on cytotoxicity as well as cellular response in the Murray study as decreased permeability results in lower concentrations of noxious substances reaching the pulp. This is also reflected in the data derived in this study, which shows a nonlinear relationship between dentine thickness and dentine permeability (Graph 6).

Chapter 10

Conclusion

Permeability

1. From these findings it appears that for standardization purposes in *in vitro* studies human dentine as barrier for cytotoxic tests is impractical to use, as too large variations in the perfusion results were found. Thus variation in data can easily be attributed to variation in dentine permeability.
2. Dentine permeability does not increase in a linear fashion but rather exponential to the increase in dentine thickness.

Cytotoxicity

3. Xeno III causes visible cell death and reduced cell viability in *in vitro* studies and is thus not biocompatible.
4. In the light of Xeno III, used in this study and dentine permeability, 500µm remaining dentine thickness can not be seen as benchmark to ensure that no pulp reaction takes place. From the results it can be concluded that it is unsafe to use Xeno III in cavities with a remaining dentine thickness of less than 1000µm.
5. It is suggested that an artificial membrane should be chosen as a barrier for these *in vitro* cytotoxic experiments.
6. Artificial membranes are really standard, cheaper and setting a level by which materials can be tested and compared world wide.

Ethical acceptability of project

The methods used in these experiments are intended as an *in vitro* simulation of filtration and diffusion of materials from the dental cavity preparation to the dental pulp, thus it does not involve human or animal studies. Furthermore, it will be an expansion of the "Dentine barrier cytotoxicity test" named in Annex A and Annex C of ISO 7405: 1997 (E), entitled "Dentistry-Preclinical evaluation of biocompatibility of medical devices used in dentistry-Test methods for dental materials".

Future extensions

Potential future extensions of this project would be to determine the effect on a cell line of a series of concentrations of solutes of dental restoration materials on one of several biologic functions such as DNA synthesis, or stimulation of functions such as gene regulation.

References

- Abuo Hashieh I, Franquin JC, Cosset A, Dejou J, Camps J. Relationship between dentine hydraulic conductance and the cytotoxicity of four dentine bonding resins *in vitro*. Journal of Dentistry 1998; 26:473-477
- Abou Hashieh I, Cosset A, Franquin JC, Camps J. *In vitro* Cytotoxicity of one-step dentin bonding systems. Journal of Endodontics 1999; 25(2):89-92.
- Arenholt-Binslev D, Horsted-Binslev P. A simple method for evaluating relative toxicity of root filling materials in cultures of human oral fibroblasts. Endodontic Dental Traumatology 1989; 5:219-226.
- Armstrong SR, Boyer DB, Keller JC, Park JB. Effect of hybrid layer on the fracture toughness of adhesively bonded dentine-resin composite joint. Dental Materials 1998; 14:91-98
- Bares D, Sato G. Methods for growth of cultured cells in serum free medium. Annals of Biochemistry. 1980; 102:55-270
- Bouillaguet S, Wataha J, Hanks C, Ciucchi B, Holz J. *In vitro* cytotoxicity and dentin permeability of HEMA. Journal of Endodontics 1996; 22(5):244-248
- Bouillaguet S, Virgillito M, Wataha J, Ciucchi B, Holz J. The influence of dentine permeability on cytotoxicity of four dentine bonding systems *in vitro* Journal of Oral Rehabilitation 1998; 25:45-51.
- Bouillaguet S, Gysi P, Wataha JC, Ciucchi B, Cattani M, Godin C, Meyer JM. Bond strength of composite to dentine using conventional, one-step, and self-etching adhesive systems. Journal of Dentistry 2001; 29(1):55-61.
- Calland JW, Harris SE, Carnes DL. Human pulp cells respond to calcitonin gene-related peptide *in vitro*. Journal of Endodontics 1997; 23(8):485-489.

- Camps J, Tardieu C, Dejou J, Franquin JC, Ladaique P, Rieu R. *In vitro* cytotoxicity of dental adhesive systems under simulated pulpal pressure. Dental Materials 1997; 13:34-42.
- Camps J, About I, van Meerbeek B, Franquin JC. Efficiency and cytotoxicity of resin-based desensitizing agents. American Journal of Dentistry 2002; 15:300-304.
- Cao Z, Huang L, Jiang J. A scanning electron microscopic observation of inner carious dentin after cleansing and of the dentin-resin interface. Quintessence international 1992; 23(6):439-444.
- Cardoso PEC, Braga RR, Carrilho MRO. Evaluation of micro-tensile, shear and tensile tests determining the bond strength of three adhesive systems. Dental Materials 1998; 14:6;394-398
- Carel A. On permanent life of tissue outside the organism. Journal of Explant Medicine.1912; 15:516-528
- Chan KM, Franklin R, King NM, Imazato S, Pashley D. Bonding of mild self-etching primers/ adhesives to dentin with thick smear layer. American Journal of Dentistry 2003; 16(5)72-76
- Chen R, Liu C, Tseng W, Jeng J, Lin C. Cytotoxicity of three dentin bonding agents on human dental pulp cells. Journal of Dentistry 2003; 31:223-229
- Dale JB, Sarich SL, Bretz TM, Hatton JF, Zachow RJ. Hormonal regulation of androgen receptor messenger ribonucleic acid expression in human tooth pulp. Journal of Dental Research 2002; 81(5):360-365
- Demarco FF, Tarquinnio SB, Jaeger M, de Araujo VC, Matson E. Pulp response and cytotoxicity evaluation of 2 dentine-bonding agents. Quintessence International 2001; 32(3):211-220

- Denholm IA, Moule AJ, Barthold PM. The behavior and proliferation of human dental pulp cell stains *in vitro*, and their response to the application of platelet derived growth factor-BB and insulin-like growth factor -1. Internationl Endodotic. Journal 1998; 31(4):251-258.
- de Souza Costa C, Lopes do Nascimento X, Teixeira H. Response of human pulps following acid conditioning and application of a bonding agent in deep cavities. Dental Materials 2002; 18(7):543-551.
- Eick JD, Gwinnett AJ, Pashley DH, Robinson SJ. Current Concepts on adhesion to dentin. Critical Review Oral Biology and Medicine 1997; 8:306-335.
- Ferrari M, Davidson CL. Selaing Performance of Scotchbond Multi-Purpose-Z100 in Class II restorations. American Journal of Dentistry 1996; 9:145-149
- Frankenberger R, Perdigao J, Rosa BT, Lopes M. 'no-bottle' vs 'multi-bottle' dentin adhesives - a microtensile bond strength and morphological study. Dental Materials 2001; 17:(5)373-380.
- Franz A, Koning F, Anglmayer M, Rausch-Fan X, Gille G, Rausch W, Lucas T Sperr W, Schedle A. Cytotoxic effects of packable and nonpackable dental composites. Dental Materials. 2003; 19(5):382-392
- Freshney R. Biology of the cultured cell. In: Culture of animal cells, A manual of basic technique, Third edition, Wiley-Liss and sons, Inc., Publication New York 1993, 3-257.
- Geurtsen W, Spahl W, Müller K, Leyhausen G. Aqueous extracts from Dentin Adhesives containing cytotoxic chemicals. Journal Biomedical Materials Research (Appl Biomater) 1999; 48:772-777.

- Grobler SR, Oberholzer TG, Rossouw RJ, van Wyk Kotze TW. Microleakage and confocal laser studies of two single step self-etching bonding agents/systems.Quintessence International, Currently in press 2006.
- Guigand M, Pellen-Mussi P, Le Goff A,. Evaluation of the cytocompatibility of three endodontic materials. Journal of Endodontics 1999; 25(6):419-423.
- Gwinnett AJ, Kanca JA. Micromorphology of the bonded dentine interface and its relationship to bond strength. American Journal of Dentistry 1992; 5:73-77
- Hanks CT, Anderson M and Graig RC. Cytotoxic effects of dental cements on two cell culture systems. Journal of Oral Pathology 1981; 10:101-106
- Hanks CT, Graig RG, Diehl ML and Pashley DH. Cytotoxicity of dental composites and other materials in a new *in vitro* device. Journal of Oral Pathology 1988; 17:396-403.
- Hanks CT, Diehl ML, Graig RG, Makinen PK and Pashley DH. Characterization of the "*in vitro* pulp chamber" using the cytotoxicity of phenol. Journal of Oral Pathology 1989; 18:97-107.
- Hanks CT, Fat JC, Wataha JC, Corcoran JF. Cytotoxicity and dentine permeability of carbamide peroxide vital bleaching materials, *in vitro*. Journal of Dental Research 1993; 72(5): 931-938
- Hanks CT, Wataha JC, Parsell RR, Straw SE. Permeability of biological and synthetic molecules through dentin. Journal of Oral Rehabilitation. 1994; 21:475-478.
- Huang F, Chang Y. Cytotoxicity of dentine-bonding agents on human pulp cells *in vitro*. International Endodontic Journal 2002; (35):905-909.

- Imazato S, Tarumi H, Ebi N, Ebiso S. Cytotoxic effects of composite restorations employing self-etching primers or experimental antibacterial primers. Journal of Dentistry 2000; 28:61-67.
- Jukič S, Prpic G, Talan-Hranilovc J, Miletic I, Segovic S, Anic I. Estrogen receptors in the human pulp. Oral Surgery, Oral medicine, Oral Pathology, Oral Radiology & Endodontics 2003; 95(3):340-344.
- Kanca JA, Sandrik J. Bonding to Dentine. Clues to the mechanism of adhesion. American Journal of Dentistry 1998; 11:154-159.
- Leblond CP, Warshawsky H: Dynamics of enamel formation in the rat incisor tooth. Journal of Dental Research 1979; 58(b):950-958.
- Lonnroth E, Dahl JE. Cytotoxicity of dental glass ionomers evaluated using dimethylthiazol diphenyltetrazolium and neutral red tests. Acta Odontoligy Scandinavia 2001; 59:34-39.
- Macpherson I, Stoker M. Polyoma transformation of hamster cell clones- an investigation of genetic factors affecting cell competence. Virology. 1962; 16:147-149
- Magloire H, Joffre A, Bleicher F. An *in vitro* model of human dental pulp repair. Journal Dental Research 1996; 75(12):1971-8.
- Marshal GW, Marshall SJ, Kinney JH, Balooch M. The dentine substrate: structure and properties related to bonding. Journal of Dentistry 1997; 25:441-448
- Maurer HR Towards chemically defined serum-free media for mammalian cell cultures. In Freshney RI (ed): “Animal cell culture, a practical approach” second edition, Oxford, IRL Press at Oxford University Press 1992; pp15-46

- Mc Lachlan JL, Smith AJ, Sloan AJ, Cooper P R. Gene expression analysis in cells of the dentine- pulp complex in healthy and carious teeth. Archives of Oral Biology. 2003; 48:273-283.
- Mjor IA, Nordhal I. The Density and branching of dentinal tubules in human teeth. Archives of Oral Biology 1996; 41:401-405
- Moodley D, Grobler SR, Rossouw RJ, Oberholzer TG, Patel N. *In vitro* evaluation of two adhesive systems used with compomer filling materials. International Dental Journal 2000; 50:400-406
- Mossman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Imunological Methods. 1983; 65:55-63
- Murray PE, Lumley PJ, Ross HF and Smith AJ. Tooth slice organ culture for cytotoxicity assessment of dental materials. Biomaterials 2000; 21:1711-1721
- Murray PE, Smith AJ, Windsor LJ, Mjor IA. Remaining dentin thickness and human pulp responses, International Endodontic Journal 2003, 36: 33-43
- Owens BM. Microleakage of cervical restorations etched with weak organic acid. Journal of the Canadian Dental Association 1997; 63:445-449
- Pashley DH, Derksen CD, Tao L. The effects of multi-step dentin bonding system on dentin permeability. Dental Materials 1988; 4:60-63
- Pashley DH. Dentine Bonding: Overview of the substrate with respect to adhesive material. Journal of Esthetic Dentistry 1991; 3:46-50
- Pashley DH. Dentine bonding agents. Orthodontics and Pedodontics 1992a; 2:46-51
- Pashley DH. Smear layer: Overview of structure and function. Proc Finnish Dental Association 1992b; 1:215-224

- Pashley DH. Carvalho RM. Dentine Permeability and dentine adhesion. Journal of Dentistry. 1997; 25:355-372
- Poonam J, Reinhardt JW, v Krell K. Effect of dentin desensitizers and dentine bonding agents on dentin permeability. American Journal of Dentistry 2000; 13(1): 21-27.
- Prati C, Venturi L, Valdre G, Mongiorg R. Dentin morphology and permeability after brushing with different toothpaste in the Presence and Absence of smear layer. Journal Periodontology 2001; 73(2):183-190.
- Ratanasathien S, Wataha JC, Hanks CT, Dennison JB. Cytotoxic Interactive Effects of Dentin Bonding Components on Mouse Fibroblasts. Journal of Dental Research 1995; 74(9):1602-1606
- Rawls H, Marshall M, Cardenas H, Bhagat H, Cabasso I. Cytotoxicity evaluation of a radiopaque resin additive- triphenyl bismuth. Dental Materials 1992;8:54-59
- Rosa BT, Perdigao J. Bond strengths of nonrinsing adhesives. Quintessence International 2000; 31:(5)353-358.
- Sano H, Takatsu T, Ciucchi B, Horner JA, Matthews, Pashley DH. Nanoleakage:Leakage Within the Hybrid Layer. Operative Dentistry 1995; 20:18-25
- Schmalz G. Agar overlay method. International Endodontic Journal 1988; 21:59-66.
- Schmalz G. Characterization of an *In vitro* Dentin Barrier Test Using a Standard Toxicant. Journal of Endodontics 1994; 20(12):592-94.
- Schmalz G, Schweikl H, Esch J, Hiller K-A. Evaluation of a dentin barrier test by cytotoxicity testing of various dental cements. Journal of Endodontics 1996; 22: 112-115.

- Schmalz G, Schuster U, Thonemann B, Barth M, Esterbauer S. Dentin Barrier tests with transfected bovine pulp-derived cells. Journal of Endodontics 2001a; 27(2):96-102.
- Schmalz G, Hiller K A, Nunez L J, Stoll J, Weis K. Permeability characteristics of bovine and human dentin under different pretreatment conditions. Journal of Endodontics. 2001b; 27(1):23-30.
- Schmalz G, Schuster U, Koch A, Schweikel H. Cytotoxicity of low pH- dentin bonding agents in a dentin barrier *in vitro*. Journal of Endodontics 2002; 28(3): 188-192.
- Schuster U, Schmalz G, Thonemann B, Mendel N, Metzl C. Cytotoxicity testing with three- dimensional cultures of transfected pulp-derived cells. Journal of Endodontics 2001; 27(4):259-265.
- Seux D, Couble ML, Hartmann DJ, Gauthier JP and Magloire H. Odontoblast-like cytodifferentiation of human dental pulp cells *in vitro* in presence of a calcium hydroxide-containing cement. Archives of Oral Biology 1991; 36:117-128.
- Shiba H, Nakanishi K, Rasi F, Mizuno N, Hino T, Ogawa T, Kurihara H. Proliferative ability and alkaline phosphatase activity with *in vivo* cellular in human pulp cells. Journal Endodontics 2003; 29(1):9-11
- Simmer J, Hu J. Dental Enamel Formation and Its Impact on Clinical Dentistry. Journal of Dental Education 2001; 65(9):896-905
- Stevens A, Lowe J Development of teeth. In: Histology, First Edition, Mosby-Year book Europe- Ltd, Lynton House, 7-12 Tavistock Square, London, England,1993; 143-154
- Stoker M, Rubin H. Density dependant of cell growth in culture. Nature. 1967; 215:171-172

- Szep S, Kunkel A, Ronge K, Heideman D. Cytotoxicity of modern Dentine Adhesives- *in vitro* testing on gingival fibroblasts. Journal Biomedical Material Research 2002; 63(1):53-60
- Ten Cate's. Enamel: Composition, Formation, and Structure. In: Oral Histology. Development, Structure, and Function. Mosby. Sixth Edition. Mosby 11830 Westline Industrial Drive, St Louis, Missouri 63146, 2003a; 145-191.
- Ten Cate's. Dentine Pulp Complex In: Oral Histology. Development, Structure, and Function. Mosby. Sixth Edition. Mosby 11830 Westline Industrial Drive, St Louis, Missouri 63146, 2003b; 192-239.
- Todaro GJ, Green H. Quantative studies of the growth of mouse embryo cells in culture and their development into established lines. Journal cell biology. 1963; 17:299-313
- Thomas HF, Payne RC. The ultrastructure of dentinal tubules from erupted human premolar teeth. Journal of Dental Research 1983; 65:532-536.
- Trantor IR, Messer HH, Birner R. The effects of neuropeptides (calcitonin gene-related peptide and substance P) on culture human pulp cell. Journal Dental Research 1995; 74(4):1066-71
- Vajrabhaya L, Pasasuk A, Harnirattisai C. Cytotoxicity Evaluation of single component Dentin Bonding Agents. Operative Dentistry 2003; 28(4):440-444.
- Van Meerbek B, Perdigao J, Lambrechts P, Vanherle G. The clinical performance of adhesives. Journal of Dentistry 1998; 26:1-20
- Vargas MA, Cobb DS, Armstrong SR. Resin-dentine shear bond strength and interfacial ultra structure with or without hybrid layer. Operative Dentistry 1997; 22:159-166

- Yang L-C, Tsai C, Huang F, Liu C, Lai c, Chang Y. Induction of interleukin-6 gene expression by pro-inflammatory cytokines and black pigmented Bacteroides in human pulp cell cultures. International Endodontic Journal 2003; 36:352-357.