An investigation on the effect of the Supernatant of *Porphyromonas gingivalis* exposed to Titanium on an *Ex Vivo* Mandible Culture Model

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Summary:

Oral Rehabilitation with titanium implants has become one of the most promising treatment modalities in dentistry. Although treatment with titanium implants is highly successful and predictable, it has been identified that chronic inflammatory diseases associated with bacterial colonisation, occur around the implant fixtures and may affect the supporting tissues of dental implants and their long-term success.. *Porphyromonas gingivalis*, a Gram-negative anaerobe, is one of the prime pathogens identified as implicated in the development of both these diseases.

An *ex vivo* culture model of 28-days old wistar rats mandible slices has been utilised to investigate the local response of the tissues to the supernatant produced by *Porphyromonas gingivalis*. To study the effect of titanium on virulence, three strains of *P. gingivalis* were cultured for 48 hours in the presence of smoothened and moderately roughened titanium specimens prior to supernatant collection. The *ex vivo* culture model was then exposed for a total of either 3 or 7 days to concentrations of 50μg/ml and 100μg/ml of supernatants from the three strains of *P. gingivalis* grown in the different conditions. The supernatants, which contain the extracellular produce of *P. gingivalis*, had an adverse effect in the periodontal connective tissues (ligament) of the mandible slices of this model, in that it caused extensive cell death and disorganisation of the matrix of these tissues and specific cellular responses. However, the presence of titanium (either smoothened or moderately roughened),whilst producing the supernatant did not appear to have an important additional effect on virulence in this model.

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List of Abbreviations

Al	Aluminium
ANOVA	analysis of variance (statistics)
Arg	Arginine
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
BCA	bicinchorinic acid
BHI	brain heart infusion
BIC	bone –implant contact
BMP	Bone morphogenetic protein
BMU	Bone multicelular unit
BSA	bovine serum albumin
C	Carbon
COX-2	Cyclooxygenase 2.
CP	commercially pure
DMEM	Dulbecco's Modified Eagle's Medium
DPX	Di-n-butyl-Phthalate in Xylene
ECM	Extracellular matrix
FAB	Fastidious Anaerobe Agar
FCS	foetal calf serum
Fe	Iron
fimA	Fimbrillin
H	Hydrogen
h	hours
H&E	Haematoxylin and Eosin
HbR	haemoglobin receptor protein
IL-1	Interleukin-1
IMS	Industrial Methylene Spirit
kDa	Kilodaltons (molecular weight).
kgp	genes encoding the gingipains of P.gingivalis
LPS	lipopolysaccharide

Lys	Lysine
M-CSF	macrophage colony stimulating factor
MPa	Megapascals
mRNA	messenger Ribonucleic acid
N	Nitrogen
NCTC	National Collection of Type Cultures
nm	nanometer
O	Oxygen
OPG	Osteoprotegerin
PBS	Phosphate Buffered Saline
PDGF	platelet-derived growth factor
PDL	Periodontal ligament
QS	quorum sensing
Ra	arithmetic average roughness
RANKL	Receptor Activator of Ng-kB Ligand
rgpA	gene encoding the gingipains of P.gingivalis
ROS	reactive oxygen species
S	Sulphur
Sa	average height deviation value
SDS- Page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T cell	Thymus cell
TEM	Transmission electron microscopy
TGF-ß	transforming growth factor beta
Ti	Titanium
TNF-a	tumor necrosis factor-a
TRAP	Tartaric Resistant Acid Phosphatase
V	Vanadium
WBM	working Broth Media
WR	working Reagent
Zn	Zinc
µm	micrometer

Chapter 1

Introduction and Literature Review

1.1 Introduction

Treatment with dental implants has profoundly changed the possibilities of oral rehabilitation. One of the biggest challenges in dentistry is the rehabilitation following loss of structural elements of the dentocraniofacial complex, the most common of which are teeth, as they simultaneously serve functional and aesthetic functions. Implants offer the possibility of provision of new rigid functional support in a predictable long-term manner that as a result opened new opportunities in oral reconstruction. Pioneer research by the Swedish Professor Per-Ingvar Branemark led to the introduction of treatment with implants as an accepted mainstream practice. The first modern titanium implant based on his concept was placed in his clinic in a human in 1965. Since then, numerous research studies have been undertaken in the subject, amongst others, expanding the use of titanium implants in other fields such as oral and maxillofacial surgery and orthopaedics. Research has focused mainly on their clinical use, and secondarily on understanding the underlying biological mechanisms responsible for their success. The use of implants in dentistry has very high long-term survival rates over 90%, however the maintenance of implants after placement is subject to multiple factors and failures do occur. Understanding the underlying complex mechanisms is subject for further research.

1.2 Implant-bone interface and Osseointegration

The success of treatment with implants is based on the rigid and load-bearing interface that is established between the bone and the implant material. This concept has been named 'osseointegration' and has been described by Branemark as a 'direct structrural and functional connection between ordered, living bone and the surface of a load-carrying implant' (Branemark, 1983) or later by Zarb and Albrektsson in 1991 as 'a process whereby the clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading'(Albrektsson T, Zarb GA.,1993) . The implant appears anchored and able to bear loads in the living bone tissue on a long-term basis, without deterioration, after an initial period of healing following the placement of the implant within the bone. This unique ability of an artificial material to be able to be reliably integrated within bone and with the absence of local or systemic adverse responses from the tissues that would lead to its rejection is shared by only a few materials. Materials that have properties of this kind include titanium, zirconium, tantalum, some polymers and some ceramics (Barradas et al, 2011). It is thought that this phenomenon is more likely to be due to the non-initiation of a negative tissue response, rather than a result of a positive interaction and is usually associated with specific properties of the material. (Stanford and Keller, 1991).

Bone is a specialised connective tissue, a composite of cells and extracellular matrix

(ECM). The ECM sometimes called osteoid consists of a relatively soft organic matrix, mostly from collagen fibers and other non-collagenous proteins, where a complex mineral substance largely composed from calcium, phosphate and carbonate is deposited. The main form of these mineral substances are the hydroxyapatite crystals (Ca_{10} $(PO_4)_6$ $(OH)_3$) (Cashman and Ginty, 2003). Bone's unique biophysical and biological properties are based on this specific architecture created between the cells, matrix and the minerals, that provide both elasticity and rigidity at the same time (Joos et al, 2006). Morphologically, there are two main forms of the bone tissue, cortical and cancellous bone, and these may interact slightly differently with implants (Gorski, 1998).

Bone is a dynamic tissue which, although not obvious macroscopically, is in a continuous complex remodelling process (regeneration). This process works through a balance of dissolution and formation of collagen-associated biominerals. At the same time it has the capacity to undergo repair ad integrum, which in contrast is dependent on a matrix vesicle-initiated mineralisation of newly synthesised extracellular matrix.

The implant-bone interface is, as a result. a dynamic interface developed through the initial healing process, in other words the process of the osseointegration. This is a complex cascade of cellular and extracellular biological events, that are similar to the osteogenic and bone healing processes, at least in the initial host response. These are regulated by growth and differentiation factors, and is not as yet completely understood (Davies, 2003). What modifies the response of the host to another surgical injury (such as an incomplete non-displaced fracture) is the presence of a rigid allogenic material, the implant, and a potential intra-operative heating injury from the procedure, which can

cause de-vitalisation to the tissue in an extent of 100-500μm (Franchi et al., 2005). The initial events that take place in the healing process around an implant are similar to wound healing processes and involve a vascular injury which leads to leakage of cellular and molecular elements and the release of numerous cytokines and growth factors. The phenomena observed are the inflammatory response and the clotting cascade. The clot and inflammatory infiltrate cytokines promote neo-vascularisation accompanied by proliferation and activation of cells and the formation of an immature fibrin matrix which acts as a scaffold for the migration of osteogenic cells. This sequence of events involves secretion and adsorption to the substratum of organic components, of which major proteins are osteopontin and bone sialoprotein. Osteogenic cells differientiate approximately 10-12 days following the initial surgical injury and a healing tissue appears within 3-5 weeks (Nefussi et al, 1998). These cells rapidly form woven bone (immature form of bone during healing and growth) to fill the initial gap at the implant-bone interface, which then slowly remodels to more mature (lamellar) bone, subsequently adapting to the local mechanical stresses and strains. It is thought that the ability of the implant surface to retain fibrin attachment is critical to maintain the osteogenic cell migration (Davies, 1998). The osteoblasts are the cells responsible for the synthesis of the bone matrix. Phenomena such as osteoinduction (implies the recruitment of immature cells and the stimulation of these cells to develop into preosteoblasts) and osteoconduction (ability of bone to grow on a surface) are critical to the way the tissue responds (Albrektson and Johansson, 2001). Around the implant, there are two main pathways of osteogenesis that have been proposed: distance and contact osteogenesis. Distance osteogenesis refers to the process where bone grows from the host bone towards the implant surface, whereas contact osteogenesis refers to the peri-implant bone grown

from the implant to the healing bone.

After this initial healing process which lasts two to four months, the remodelling process continues perpetually, as in the rest of bone tissue, through a group of cells called the bone multi-cellular unit (BMU). This process creates a balance between bone matrix resorbed and newly formed, maintaining the bone tissue in a functional state. The bone multi-cellular unit (BMU) comprise osteoclasts, cells that develop from hematopoeitic progenitors and osteoblasts, that derive from mesenchymal stem cells. The BMU action is thought to be controlled by local mechanical stimuli. The osteocytes, the basic cells found within the bone matrix, are thought to act as mechanosensors and their apoptosis can induce bone resorption.

It is understood that the osseointegrated bone-implant interface is a result of these processes and can be affected by factors interfering with the ossointegration process, as well as in the bone remodelling process. Factors that can positively or adversely affect osseointegration may be related to the implant characteristics, the status of the host bone and its healing potential. The initial mechanical stability and the loading conditions are also important as well as other simultaneous treatmens such as bone grafting, use of biological coatings and biophysical stimulation. Systemic conditions such as osteoporosis, rheumatoid athritis, diabetes, advanced age, renal insufficiency, nutritional deficiency, smoking and radiation therapy have been connected with reduced success rates in implant treament. (Mombelli and Cionca, 2006). In a systematic review by Strietzel et al (2009) out of 35 publications considered, most showed that smoking is a risk factor for dental implant treatment, however five studies revealed no significant

impact of smoking. The meta-analysis of 29 studies demonstrated an increased risk for smokers receiving implants with accompanying augmentation. Pharmacological agents such as cyclosporin, methotrexate, warfarin, low molecular weight heparins, non-steroid anti-inflammatories, especially COX-2 inhibitors have been found in studies to affect adversely bone healing, whereas others such as simvastatin and bisphophonates have been thought that they may enhance osseointegration in selected cases (Mavrogenis et al, 2009). However, an article by Zahid et al (2011) showed an association between bisphophonates and implant thread exposure in the loaded phase.

The exact nature of the implant-bone interface is subject to further research. Histologically, under optical microscopy a contact between the implant and the bone has been observed. In the case of titanium the ability of osseointegration has been connected with the passive surface oxide layer that it creates when exposed to the air (O_2). With transmission electron microscopy (TEM) an intimate contact between mineralised bone and the surface oxide of the implant has been recently identified, including bone growth into the nano-structured oxide of an apatite layer, however with different crystal orientation compared with the apatite in the bone tissue (Palmquist et al, 2011). Previous observations in earlier TEM studies, using different preparation techniques, showed an amorphous zone closest to the surface of retrieved oral implants from humans (Sennerby et al, 1991), bonding through a cement line-like layer (Davies et al, 1991) or a 20–50 nm electron-dense layer at the interface (Steflic et al, 1998)

This direct bone–implant contact (BIC) in a clinically successfully ossointegrated implant is not continuous, but in the range of 55% to 85% so there is histologically often

some inter-trabecular marrow spaces rich in blood vessels and mesenchymal cells and some fibrous tissue but inflammatory infiltrate is absent. (Franchi et al, 2005)

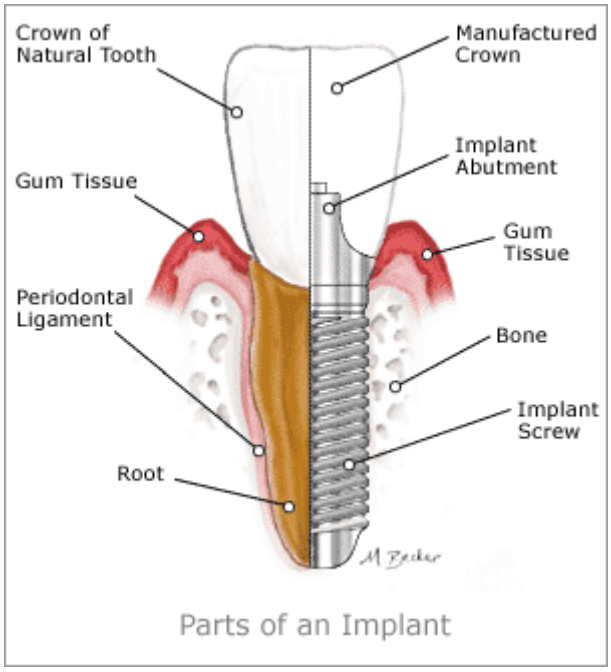

Fig 1.1 Dental implant versus tooth

Image adapted from http://www.atlanticdental.co.uk/dental_implants.htm

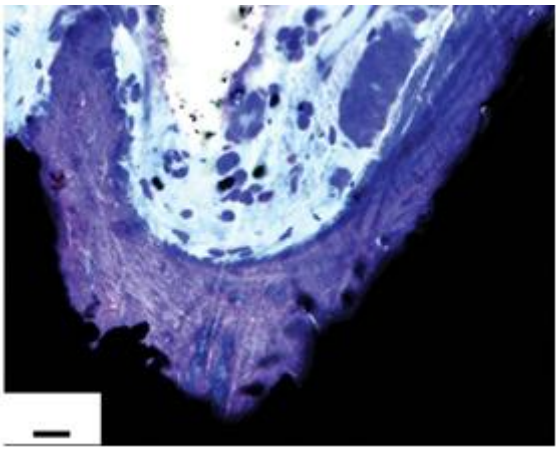

Fig 1.2 Photomicrograph of bone-implant interface taken by a light microscope at a highmagnification.
Image adapted from: Palmquist, A., Grandfield, K., Norlindh, B., Mattsson, T., Brånemark, R. & Thomsen P. (2011). Bone-titanium oxide interface in humans revealed by transmission electron microscopy and electron tomography. *J R Soc Interface*, Aug 17.

1.3 Dental Titanium Implants

1.3.1 Titanium characteristics

Titanium is a low density metal with very high resistance to corrosion. At the same time it is relatively strong, and due to its use in the airspace industry and the complex production process, it has been called the ‘space age metal’.

The most important characteristic of titanium of interest related to implants, to which much of its biocombatility is attributed, is the oxidation of its surface layer most commonly in the form of TiO_2 (Palmquist, 2010). The surface oxide is chemically stable and creates a corrosion-resistant surface under physiological conditions. The surface of Ti may contain a variety of other intentional or non-intentional impurities and organic molecules adsorbed from the air, packaging and other preparation processes, which may sometimes have an effect on the early cell reaction to the implant surface. The surface oxides can vary from a few nanometres up to several micrometers depending on the method of preparation of the surface and time exposed to an external enviroment. This is usually in the region of 10 to 100nm. The oxides can have an amorphous (non-crystalline) or crystalline microstructure and clinically available implants may have either surface, however its importance has not yet been fully investigated (Palmquist, 2010).

Titanium is offered commercially in different purities and alloys, which are classified in

grades. 39 grades are recognised by ASTM international (American Society for Testing and Materials) at the moment. In dentistry the most commonly used is the commercially pure grade 4 (which is the strongest of the unalloyed titanium grades (grades 1 to 4)) and grade 5 Ti which is an alloy. Grade 4 Ti is 99 % pure titanium containing also some other elements in very low concentrations (C max 0.1, Fe max 0.5, H max 0.015, N max 0.05, O max 0.4). The original Branemark implants were made from Ti grade 1 (Ti 99.5% and C max0.1, Fe max 0.2, H max 0.015, N max 0.03, O max 0.18) (and were as a result more prone to fracturing due to reduced strength). Some orthopaedics manufacturers prefer to use alloys such as grade 5 Ti (Ti-6Al-4V containing 90 % Ti, 6 % Al , 4%V and max Fe of 0.25 and O of 0.2%), as they have superior physical properties (greater yield strength and fatigue properties), although their biocompatibility has been questioned. In studies they form oxide layers like the commercially pure grades, and very few ions of the other metals are found released in the tissues, demonstrating good behaviour at least in the short-term.(Milosev et al, 2000, MacDonald et al, 2011)

The macrogeometry, microtopography (roughness), the chemical and phase composition of the Titanium surface has been thought to be of clinical relevance as affecting the biological performance of the implant *in vivo*. The wide variety of the commercially available implants are based on variations of these characteristics, as it is established that they can influence the cell reactions, tissue healing and implant fixation. A lot of these available implants have some documentation of their *in vitro* and *in vivo* performance from different experimental models. (Albrektsson & Wennerberg, 2004)

1.3.2 Macrogeometry of titanium implants

During the development of implant dentistry various forms of implants have been developed, with various success rates. It seems though that during the last few years there is an overall tendency from clinical and experimental data towards screw shaped implants. Geometrical designs of coated cylinders or hollow-basket or non-screw shaped implants have largely been abandoned, as their inherent problems caused them to be discontinued in this ever competitive market. For example, the hollow-basket implant seem to have had significantly higher rates of mechanical fractures and the coated cylinders although initially successful seem to have reduced ability to maintain bone-levels and were more susceptible to peri-implantitis.

The length and width of the implants and also the shape of the implant body seem to have substantial role in the treatment planning and the long-term outcome of the implant treatment. Due to the mechanical loading from the masticatory forces, and the consideration that the implant should be wholly surrounded by bone once integrated for its long-term viability, there has been limitations in the implant sizes due to anatomical and biomechanical reasons. Although the titanium is a metal, it is quite prone to fatigue fracture due its relatively low density and tensile strength. It has been found that implants from commercially pure titanium are recommended at the moment to have a minimum diameter of 3mm to have reduced fracture rates. The anatomy of the available bone, the space available between existing teeth, as well as aestheticconcerns place further restrictions on the implant width. For bone-physiological reasons, it has been found that

there is a minimum amount of bone needed between adjacent implants (3mm) or between adjacent implant and teeth (1.5mm) . (Tarnow et al, 2000; Vela et al, 2012) Wide implants over 5 mm have been introduced with varying degrees of success, possibly due to the specific reasons leading to their use (eg. Increased loading in the posterior mandible regions or use as an alternative following a failure of a previously placed implant). For the same reasons, the implant length is also an area of debate during treatment planning and can vary from 6 to over 18 mm. The traditional prosthodontic thinking approach based on previous experience on teeth and some reviews on the subject of the relation of the length of the implant to failures rates. (Renouand and Nisand, 2006), propose that a favorable crown to root ratio determines the minimum length required. It has been initially thought that shorter implants will have reduced success due to the distribution of the mechanical load, increasingly also where the bone quality seems to be compromised or when peri-implant bone loss may take place in the future. However, lately there is a tendency to propose implant as short as 6mm can offer successful clinical outcomes in carefully selected cases (Sun et al, 2011). The shape of the implant body can vary from more cylindrical to tapered designs to allow for the self-tapping of the titanium screw implants.

The implementations of threads and micro-threads of differing sizes is the norm in implant design nowadays as they provide more initial stability and biomechanical advantages for a more successful outcome and long term viability of the implant, as also demonstrated from various studies (Carlsson et al 1986, Gotfredsen, 1992). There have been several designs from different manufacturerers which aim to alter the distribution of

the biomechanical load and increase the primary stability of the implant at the time of insertion by altering the thread depth, thread thickness, thread face angle, thread pitch, and thread helix angle and also vary them through the implant body (Abuhussein et al, 2010). The primary stability is an important clinical parameter as it has been identified that displacement of over 150μm during the time of the healing lead instead of osseointegration to fibrous encapsulation of the implant (Moncler et al, 1998). As well as this, there is some evidence that micromovements up to 50 μm on the contrary were well tolerated (Piatelli et al, 1995). The concepts of early loading (immediate abutment restoration) is based on this latter evidence and studies have demonstrated that implants can become integrated under the influence of some loading (Piatelli et al, 1998). Following the initial integration process, bone loss of 1 mm in the first year and 0.2 mm in the subsequent years is regarded as acceptable (Albrektsson and Zarb, 1993). According to recent data, though only in 3-year studies, microthreads in the coronal part of the implant seem to prevent marginal bone loss (Lee et al, 2007; De Bruyn et al, 2008). Hansson (1999) suggested that retention elements such as threads would induce mechanical stimulation to maintain marginal bone. According to Frost (1989) over 3000-4000 microstrain units (stress around 45-60 MPa) cause microdamage in the bone that cannot be compensated by the bone remodelling processes and bone cells may trigger cytokines to begin a resorption process, although an adapted window between about 50 to 1500 microstrain units is required for the bone to remain in a steady state through continuous remodelling and not revert to atrophy.

1.3.3 Surface Topography of current implants

The Surface microtopography of the implant has been found to influence the capacity of the bone to heal and osseointegrate around the implant. The bone anchorage to the implant is also affected by the features of the surface, the microbial adhesion and associated disease seems to differ depending on different characteristics of the implant. Therefore, there has been an overwhelming amount of different surfaces aiming to address all these clinical problems and provide a more successful outcome. A systematic review by Esposito et al (2007) did not show any evidence that a particular type of dental implant has superior long-term success. However it included a limited number of RCTs with small number of patients and follow up of only 1 to 5 years . It is one of the main suggestion that RCTs with better design and longer follow up are needed to identify any true differences.

The predominant aims of altering surface topography are to accelerate bone healing and improve the bone to implant contact, and at the same time to increase any potential antimicrobial properties of the surface. There are some different strategies usually followed to achieve these aims. These strategies aim to improve the interface chemically by incorporating inorganic phases such as calcium phosphate or to improve the surface topography architecture physically, either on the micrometre level as roughened surface increases the biomechanical interlocking of the bone to the implant (at least up to a certain level) or lately on the nanometre level by changing the surface energy, which seems to improve matrix protein adsorption, bone cell migration and proliferation (Wennenberg & Albrektsson, 2010).

There have been numerous surfaces produced even following similar methodologies, just by changing few factors. In order to reduce confusion, Lang et al (2009) suggested to accept a classification primarily based on the roughness of the surfaces in 4 categories according to their absolute Sa (average height deviation value) as determined by optical interferometry using Gaussian filters. The 4 categories are:

- smooth surfaces with Sa value <0.5μm (eg. polished abutment surface),
- minimally rough surfaces with 0.5μm< Sa value< 1μm (eg. turned surface),
- moderately rough surface with 1μm <Sa < 2 μm (eg. most commonly used types),
- rough surface with Sa >2 μm (eg. plasma-sprayed surfaces).

The first surface used in implants was a turned surface (machined) introduced by Branemark and has a clinically successful record of over 20 years. This surface is obtained by machining of the surface with common metallurgical methods, as due to its reaction with oxygen, titanium cannot be cast easily. Therefore this (machining) is usually the method of producing all implant shapes from the raw material before treating the surface chemically or physically. Machining produces a surface with the roughness mainly due to the machining procedure and residual periodic microgrooves. (Lausmma et al, 2001). Although the clinical success of this surface has been well documented it has been found that there are ways to accelerate the osseointegration process and increase the bone to implant contact creating more stable implants that can withstand higher loads especially in areas with poor bone quality. Machined surfaces are therefore relatively rarely used today as they seem to provide poorer biomechanical fixation (Wennerberg et

al, 1996). The bone to implant contact (BIC) is one of the few histological criteria that have been identified in an attempt to measure the osseointegration process. There is evidence that before loading the implant there is a need of at least 40-50% bone to implant contact with a successfully integrated implant to have BIC in the range from 55% to 85% mostly depending on the surface microtopography, as previously mentioned. (Trisi et al, 2003)

There are several physical and chemical methods employed to achieve improved surface microtopography. The most common are acid-etching, grit-blasting, titanium plasma-spraying, anodizing or even combination of these methods, or in a different approach with the deposition of coatings (usually hydroxyapatite).

The acid-etching procedure can be performed with several acids or a combination of them in different temperatures or also in combination with other treatments (eg. grit-blasting). The acids usually used are HF, HNO_3 (eg. Astra), H_2SO_4, and HCl(eg. Osseotite, Biomet 3i). The procedure creates micro-pits in the surfaces in different sizes. These treatments have been shown to promote the adhesion of osteogenic cells and promote bone apposition (Davies, 1998) maybe by promoting the fibrin adhesion. The use of these different acids seems to provide different advantages. For example the use of fluoride has been suggested that it promotes the forming of TiF_4 species promoting better osteoblast behaviour (Cooper et al, 2006).

Grit-blasting is carried out by blasting with hard ceramic particles (usually silica (sand-

blasting), alumina, titanium oxide or calcium phosphate). The blasting conditions can vary from the size of the ceramic particles (usuallly though in the 25μm to 100μm range sometimes up to 250μm), velocities, and temperature in which this procedure is done. When using alumina or silica the procedure is usually followed by acid-etching as the surface is contaminated by the blasting particles and the etching procedure attempts to homogenise the microprofile of the implant and remove residual particles (eg. SLA-Straumann). Surfaces such as this have been found to reduce the clinical times needed before loading the implants (Cochran et al, 2002). The surfaces are often blasted with a biocompatible, osteoconductive, resorbable blasting material (coatings) such as calcium phosphates (Hydroyapatites), beta-tricalcium phosphate and mixtures of these. They seem to produce a higher early bone-to implant initial contact and promote bone apposition. It is however important that these coatings are very thin, as in the past there has been several problems, such as delamination with thicker coatings, and reduced clinical performance (Ehrenfest et al, 2009). Coatings nowadays are in the 20-50μm scale or even in the 20nm scale (eg. Nanotite implants). As a result, several other techniques have been developed to apply these coatings such as plasma-spray, sol-gel deposition, sputtering (Young et al, 2005) or ion-beam assisted deposition (Coehlo et al, 2009) and biomimetic precipitation with differing clinical results.

Titanium plasma-spraying is another technique used to produce more rough surfaces usually injecting Titanium powders in high temperatures forming a film almost 30μm thick. It has been shown that these extra-rough surfaces seem to offer reduced clinical results compared to moderately roughened surfaces and less bone to implant contact

(Guehennec et al, 2007) and therefore this method is not used widely.

Another method used is the anodization (eg. Nobel Implants) This method creates micro- or nano-porous surfaces by potentiostatic or galvanostatic anodization of titanium in strong acids (H_2SO_4, H_3PO_4, HNO_3, HF) at a high current density ($200A/m^2$) or potential (100V). The process is complex and several parameters can be altered such as the current density, concentration of acids, composition and electrolyte temperature. This can sometimes, under certain circumstances, create an ionic impregnantion of the TiO_2 layer and thicken it up to 1000nm (Sul et al, 2005; Sul et al, 2009). This moderately roughened surface has been proven to have better clinical results than a turned surface (Sul et al, 2002).

All these microstructures, on a microscopic level, increase the bone to implant contact area and the biomechanical interlocking by increasing the surface area available. From a bone biology perspective, it seems that for bone biological functions (bone formation, bone remodelling etc.) there is a need of spaces greater than 50μm between the bone and the implant (Ehrenfest et al, 2010) and as a result other parameters are investigated to understand better this bone-implant interaction. There is a tendency therefore to focus at the nanoscale level, as a more textured topography increases the surface energy. High surface energy increases the wettability and also the spreading and binding of fibrin and matrix proteins. This could improve cell attachment, tissue healing and influence cell proliferation and differentation (Ehrenfest et al, 2009). All surfaces have nanotopography but their nanostructures may not be significant enough to improve performance in a

controlled way. It seems that a repetitive and homogeneous nano-pattern is important to induce clinical differences.

There have been attempted organic modifications of the implant surface mostly on an experimental basis. This includes physicochemical and biochemical methods to provide the titanium with components of the ECM. These include immobilization of ECM proteins (such as collagen) or peptide sequences to improve bone cell adhesion, enzyme modified surfaces for enhanced mineralisation and deposition of cell signaling agents (eg. bone growth factors/). These sound promising but the host response cannot be usually manipulated in a predictable manner. For example experiments with BMPs (Bone morphogenetic proteins) have given contradiciting results, sometimes leading to bone destruction (Lang et al, 2009). Further experiments are therefore needed before considering them for clinical use.

1.4 Implant failures

Despite the high clinical survival rates of implants even in longitudinal studies, implant failures do occur with sometimes significant consequences. In many cases there may be substantial bone loss, which makes subsequent treatment and rehabilitation more difficult. There have been several .classifications of failures into different categories according to the criteria used, which aim to provide an insight into the reasons behind the implant failures. According to an article by Esposito et al (1998) two different

categorization of implant failures could be made (according to the mechanism or to the timing)

Implant failure can be divided according to the mechanism that leads to the failure of the implant. This can be a biological failure (related to biological processes), mechanical failure (which would include fracture of the implant, implant components, restorations, coatings) and iatrogenic failure (failures usually related to the planning of the surgical or restorative procedures such as malpositioning of the implant, damage to anatomical strucures and, failure to meet patient's expectations (aesthetically, phonetically).

Failures (mainly biological failures) can also be divided in early or late failures related to the ability of the implant to achieve osseointegration or maintain it. Early failures are related to primary failure of the implant to achieve adequate osseointegration for the prosthesis to be brought into function and is characterised by the fibrous encapsulation of the implant. Early failures are usually attributed to factors such as infection, surgical trauma, impaired healing ability of the host, and micromotion related usually to undesirable premature loading.

The late failures, classified by Heydenrijk et al (2002) can be divided into those occuring in the first year post loading and into those after the first year of loading. The main reasons for these failures, as summarised by Esposito et al (2000), are mainly attributed to overloading (in relation to the host implant site characteristics) and peri-implantitis (as a chronic inflammatory process thought to be related to bacterial contamination) or a

combination of these two factors. The clinical and radiographic manifestations related to these factors can be distinctively differentiated.

The long-term maintenance of an implant restoration is a primary goal of the implant treatment and peri-implantitis appears to be a clinical entity that poses certain defined risks to the survival of the implant. It is therefore of special interest to understand the biological processes related to it, in order to prevent it and if it finally occurs to treat it in a successful manner.

1.5 Peri-implant diseases

Peri-implant diseases are classified at the moment into two different entities: peri-implant mucositis and peri-implantititis. This is based mostly on the previous experience of the classification of the periodontal diseases around teeth.. According to a consensus report from the 1st European Workshop on Periodontology (EWOP) (Albrektsson & Isidor 1994), peri-implant mucositis was defined as a reversible inflammatory reaction in the

soft tissues surrounding a functioning implant, peri-implantitis described inflammatory reactions associated with loss of supporting bone around an implant in function.

These two entitites have been reported to have different histological characteristics as determined in analysis of human biopsies of related lesions. In sites with peri-implant mucositis the inflammatory cell lesion was dominated by T cells and had an apical extension that was restricted to the barrier epithelium (Zitzmann et al. 2001). In peri-implantitis, the lesion extended apical to the pocket epithelium and contained large proportions of plasma cells and lymphocytes but also polymorphonuclear cells and macrophages were found in high numbers (Gualini & Berglundh 2003; Berglundh et al. 2004) and the epithelial downgrowth is thought to be an important characteristic.

It is understandable that peri-implantitis can take place only where the bone implant interface has become functional and should be differientated from the inflammatory symptoms detected when there is primary failure of the implant to osseointegrate during the early phases of healing following the implant placement. Mobility to a detectable level is as a result not one of the criteria used as it indicates complete lack of osseointegration. If the implant has mobility then the implant needs to be removed and in peri-implantitis is the final result of a chronic process and not an ‘early’ indicator.

Radiographically, a periimplantitis defects usually have the appearance of a saucer- type defect around the implant and is well demarkated (Mouhyi et al, 2009). This is in contrast to the appearance of bony defects of periodontitis. As the lower part of the implant

remains osseointegrated, there is no mobility detected around the implant until there is overwhelming significant amount of bone lost. Signs of inflammation of the tissues accompanied by bleeding on probing and supuration are very common, although clinically usually the most alarming features is the radiographically identified bone loss, usually followed by the recession of the soft tissues when there has been significant peri-implant bone loss.

In order to detect inflammation, probing around the implant is needed to detect bleeding or supuration following mechanical challenge. The probing should be gentle and 0.25 to 0.5N force has been suggested (Heitz-Mayfield, 2008). For peri-implantitis radiographic evidence is required, however this should be distinguished from the process of bone remodelling that occurs in the early stages after the implant installation.

According to Roos-Jansaker et al (2006), the prevalence of peri-implant mucositis, using bleeding on probing as a criteria, with no bone loss, was identified as occurring in about 79 % of the subjects and 50% of implants. For the peri-implantitis, according to a systematic review by Zitzmann et al (2008), there are still only a small number of studies providing data, and the criteria used can vary between them. On the two cross sectional studies with large samples, peri-implantitis was identified in 56% of the subjects and 43% of the implant sites (Roos -Jansaker et al, 2006) (period of observation 9-14 years) and in Fransson et al (2005) in 28% of the subjects and 12.4 % of the implants (period of observation 5-20 years).

There seems to be several risk factors associated with a greater risk for peri-implantitis. In a review by Heitz-Mayfield (2008) poor oral hygiene, previous periodontal disease, diabetes and smoking were identified as associated with peri-implant diseases. There is conflicting evidence whether a rougher implant surface has a greater risk for peri-implant diseases, as one study by Astrand et al (2004) indicated that it has, but in another by Wennerberg et al (2004) it was suggested that there is no significant difference between roughened surfaced and turned surfaces.

The peri-implant soft tissues act as a barrier for the bone which sustains the implant. However, this barrier may be fragile in relation to external ingress. It is thought that in the event of breakdown of this attachment, through inflammation or other reasons, there may be a reduced protective mechanism for the underlying bone. Potentionally, this may make the implant more susceptible to ingress than would be the case with inflammation around natural teeth (Lindhe et al, 1992). The colonization of the peri-implant pockets by bacteria creates an imbalance between the microbial flora and the tissues, however this is not entirely predictive of the progress of the disease (Mouhyi et al, 2009).

It is unclear to what extent the biomechanical stress around the bone-implant interface (overloading) affects the way peri-implantitis progresses. It is well-known that excessive stresses causes strain on the bone-implant interface and the bone remodelling processess attempt to compensate for this. There has been little research done on whether these two factors act in a synergistic way or act in a totally independent way. That means whether the stress the tissues suffer from the bacterial contamination affect the bone remodelling

procedures secondary to biomechanical stress is an area that possibly needs to be further investigated. (Misch,2007).

It has been postulated that peri-implantitis is a pathology of osseointegration (Mouhyi et al, 2009). It is suggested that the peri-implant bone is lost due to the 'corrosion' and contamination of the implant surface rendering it less biocompatible. The periimplantitis can be thought of as being associated with the loss or reduction of Titanium's biocompatible interface, the titanium oxide layer. This may be related to surface contaminants, which make the adsorption of oxygenated derivatives and osseous glycoproteins impossible on the implant surface. A single layer of contaminants is sufficient to make a biomaterial unusable (Bjustern et al, 1990). The oxidative stress is a natural phenomenon which has been associated with ageing and its origin lies in the production of free radicals (hydroxyl groups OH) which can generate oxygenated derivatives (hydrogen peroxide H_2O_2 and superoxide radicals). In the event of aggression, oxygenated derivatives is a first natural defence, which in a case of a conventional wound are quickly deactivated by the cellular catalases. In comparison when a foreign body is introduced the free radicals are much more concentrated which cause tissue destruction and induction of the fibroplastic pathway to the detriment of the osteoblastic pathway in the case of bone.

In the case of Titanium, the overproduction of H_2O_2 allows the thickening of the oxide layer, which in turn allows the incorporation of calcium and phosphorous ions of the osseous matrix (Walivaara et al, 1994). It is thought that the establishment of this

dynamic interface forms the basis for the osseointegration process.

Therefore implant surface contamination may trigger a chain reaction leading to the dissolution of the titanium oxide layer. Increased production of free radicals can cause a reaction on the implant surface, inducing accelerated corrosion and a massive release of Titanium ions, which maybe the mechanism underlying peri-implantitis of bacterial origin. At the same time, the titanium oxide layer can be considered a relatively fragile ceramic prone to microfractures, which can also lead to a Ti dissolution and accelerated corrosion, in the presence of excessive biomechanical stresses.

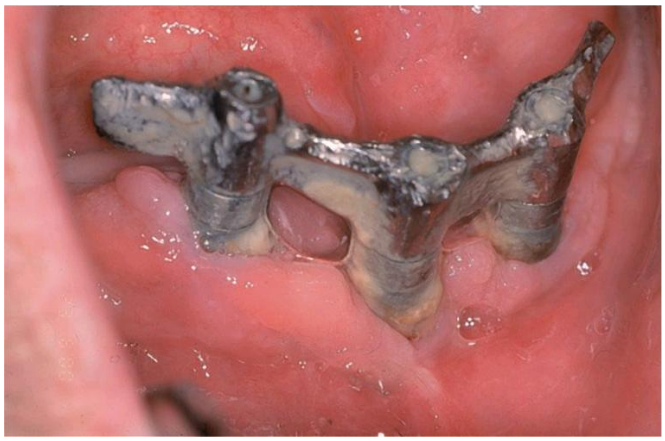

Fig. 1.3 Clinical manifestation of peri-implantitis

Figure adapted From Mosby's Dental Dictionary, 2nd edition. © 2008 Elsevier, Inc

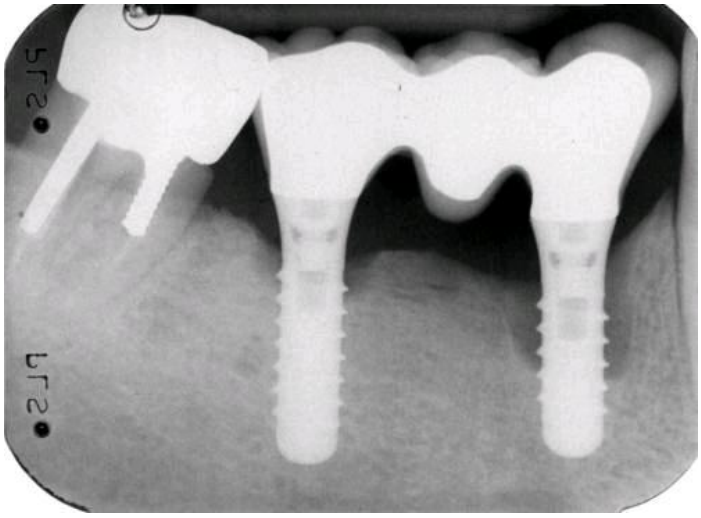

Fig. 1.4 Radiographic finding of peri-implantitis case

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1.6 Implant -Mucosal Interface

Although osseointegration is the basis of the success of the dental implants, it is also paramount that the transmucosal part of the implant is stable in the long term and free of inflammation and infection for the dental implant to be classified as successful. Most studies focus on the survival of the implant, as the determination of success would need to include the soft tissue response, that is difficult to define. The implant-mucosal interface and its stability is important as it acts as a barrier between the bone surrounding the implant and the external environment. This is because the bone as a tissue cannot act as an external barrier and exposure of the bone results in its resorption. Peri-implant health has functional and aesthetic purposes. The mucosal inflammation or recession may indicate evidence of imminent or progressive bone loss around the implant, which it can affect the long –term survival of the implant and causes aesthetic concerns.

Even though the use of dental implants aims to replicate the function and aesthetics of a natural tooth and its associated soft (gingival) tissues, they actually function in a different way. The implant lacks the ectomesenchymal attachment apparatus observed in teeth (alveolar bone, periodontal ligament, cementum) which ceases to exist with the loss of the tooth. The bone surrounding the implant contains hardly any periodontal structures and the mucosa surrounding an oral implant is different from the gingival tissues. The soft tissue surrounding the implant is a result of the wound healing of the epithelium in an attempt to cover the connective tissue surface that it has been severed. The epithelial cells at the mucosal wound respond aiming to restore the epithelial continuity. Due to the biocompatibility of the materials used (titanium, zirconia, gold) the epithelial cells seem

to have the ability to adhere to the surface of the implant components, synthetise basal lamina and hemidesmosomes and establish an epithelial barrier which has some features in common with the junctional epithelium observed around natural teeth. Below the epithelium the connective tissue also has the capacity to be uninflamed and form an attachment to the surface and offer support to the overlying epithelium, until where the bone commences (Lindhe and Berglundh, 1998).

The bone in successfully placed implants commences below the implant-abutment connection interface (microgap) which is of clinical importance. Listgarten et al (1992) found that in the collagen fibers in an intact connective tissue–implant interface were more or less parallel to the implant surface, which has since been confirmed from other studies. (Schierano et al,2002; Tete et al, 2009). Comparison between zirconia, titanium and gold however, seem to suggest slightly differing soft tissue healing (Welander et al, 2008), with gold offering less favorable soft tissue adaptation.

The existence of keratinised tissue in the epithelium as well as the biotype of the oral mucosa seems to affect clinical outcomes and the long-term health and stability of this interface. The fibroblast-rich layer adjacent to the titanium surface is thought to have a role in the maintenance of a proper seal between the oral environment and the peri-implant tissue (Moon et al, 1999). Studies from Van Drie et al (1988), Lindhe and Berglundh (1998) indicate that during wound healing the "apical part" of the supra-alveolar connective tissue is not recognised as a wound and there were no signs of inflammation in the area with the absence of round cells and foreign body giant cells. As

a result epithelial migration does not progress. It is thought that the healing of the soft tissues around an implant follows, as usual, the four stages of healing (haemostasis, inflammation, proliferation, and remodeling) (Werner et al, 2003), with slightly different events than in bone healing. Specific cellular components act through several mediators during these phases. The normal healing response begins the moment the tissue is severed. In the hemostasis phase, as the platelets come into contact with exposed collagen and other elements of the extracellular matrix. Components such as fibrin and fibronectin, clotting factors, essential growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-ß) are released. Following haemostasis, the neutrophils then enter the wound site and begin the critical task of phagocytosis to remove foreign materials, bacteria and damaged tissue and mediated by growth factors and proteases, macrophages and lymphocytes complement this action releasing also more PDGF and TGF-ß. Once the wound site is cleaned out, fibroblasts migrate in to begin the *proliferative* phase at about day 2 through to the third week post-operatively and deposit new extracellular matrix. The new collagen matrix then becomes cross-linked and organized during the final *remodeling* phase. In order for this efficient and highly controlled repair process to take place, there are numerous cell-signaling events that are required.

It was further thought that the peri-implant mucosa has a minimal "biological width", as also previously demonstrated in teeth, which is usually between 2 to 5 mm. Several studies have confirmed that changes to the height of the soft tissues (especially of the connective tissue) over the bone surrounding the implant can lead to reestablishment of

new level in the bone through resorption in a similar fashion like in teeth (Berglundh and Linde, 1992; Yeung, 2008,).

1.7 The Microbiology of Implants

1.7.1 Biofilm formation on dental implants

The biofilm is a sessile community of microorganisms embedded in a matrix of extracellular polymeric substances, mostly produced by the microbes, creating a substratum or interface for their growth (Subramani et al, 2009). Biofilms can be found on many different kinds of surfaces, including natural aquatic systems, living tissues, medical devices and implants. In the mouth, biofilms can be observed in the form of dental plaque on tooth surfaces and around implants and other artificial prosthesis. Biofilms in the oral cavity consist of polymicrobial communities embedded in a matrix of polymers, mostly of bacterial and salivary origin (Pratten et al, 2003). They have been identified as one of the primary causes of oral diseases, such as caries and periodontitis, although the presence of biofilms is just as necessary for the health of the oral cavity (Ruby and Barbeau, 2002). The biofilms in the oral cavity consist of microbial communities embedded in a matrix of polymers, mostly of bacterial and salivary origin (Pratten et al, 2003).

More than 500 bacterial taxa have been identified in the oral cavity and some of these

species are unique to this environment (Paster et al, 2001). The biofilm identified in the gingival crevice and in the periodontal pocket is extremely diverse with up to 100 culturable species from a single pocket (Haffajee & Socransky, 1994). Bacteria within biofilms are thought to act synergistically, possibly communicating using quorum sensing (QS), mediated through secreted molecules (Fuqua, et al, 1994). The most common in the oral cavity seem to be peptide auto inducers as between *Streptococcus gordonii* and *P. gingivalis* (McNab et al, 2003).

With the advent of confocal laser scanning microscopy it has been demonstrated that dental plaque develops not as a uniform layer of cells, as it was previously thought, but as discrete stacks of cells separated by channels (Pratten et al, 2003). Colonisation by bacteria seems to be affected by several factors, including the composition of a salivary pellicle that can promote the adherence and co-aggregation of the oral bacteria (Shupbach et al, 2001). Secondary colonisers can become co-adhered through bridging to the pioneer microoorganisms and promote anaerobic microenvironments, which can protect strict anaerobes and allow them to predominate.

The colonization by microbes of the peri-implant tissues is thought to be similar to that of teeth (Tanner et al, 1997), although some different patterns in the early events of the colonisation are evident. For instance, streptococci seem to initially colonise the surface, usually within 30 minutes after implant placement, followed by anaerobes after at least 48 hours (Furst et al, 2007).

In a systematic review on biofilms on dental implants by Subramani et al (2009)

identified 53 articles that describe or research the biofilm around implants. It was reported that biofilm formation is facilitated with an increase in the surface roughness and energy and at the same time the surface chemistry and the design features of the implant-abutment configuration also affect the biofilm formation. Furthermore, it has been even postulated that plasma proteins that may adsorp to Ti may enhance the affiliation of certain bacteria such as of *P.gingivalis* to the Titanium surface (Badihi et al, 2011)

. More recently, other characteristics such as wettability (the hydrophobic or hydrophilic properties of the surface) and surface free energy have been suggested to be more important. It has been shown that smooth surfaces below (Ra) <0.088 μm significantly inhibit biofilm formation, and as a result they are material of choice for an abutment. Design features of the implant and the abutment (e.g. overcontouring of the restorations) also seem to significantly affect the formation of the biofilm. The leakage at the implant-abutment interface (the microgap) is thought to influence the initiation of disease in the area and, as described before, changes to the design (e.g. platform-switching) seem to reduce the associated problems. (Serrano-Sanchez et al, 2011)

An experimental investigation on the nanophases of ZnO and TiO_2 nanocoating on implants seem to have caused reduced adhesion of microbial cells and increased osteoblast adhesion (Colon et al, 2006). Interestingly though, there does not seem to be a clinical correlation between the type of implant surface and the kind of bacteria found around them (Watzak et al, 2006).

1.7.2 Oral biofilms and peri-implantitis

Although the presence and action of the plaque (oral biofilm) is generally regarded as detrimental to the health of the periodontal and peri-implant tissues, it is recognised that the biological progression from health to disease is mostly due to the establishment and growth of a very few species of bacteria in the subgingival and submucosal areas (Holt et al, 1998). It is, however, more than likely that no single species is capable of all the destructive events observed in the inflammatory process of the soft tissues and bone (Holt et al, 1998), but it is a result of a synergistic, integrated action of several members of the local flora. It is also generally accepted that host cell interactions are also an important part of the way the diseases progress. While there are several bacteria that could be characterised as "putative" pathogens, few species have been shown to have a direct pathogenic effect to the host. The origin of the bacteria found in the peri-implant tissues seems to be also important, as differences between edentulous and partially-edentulous individuals have been identified, specifically more from periodontally involved sites around natural teeth (Kohavi et al, 1994, Mombelli et al, 1995).

It has been published in several studies that there are differences between the biofilms in health and disease around implants. In particular it is found that in healthy sites around implants the biofilms are dominated by dominated by Gram-positive facultative cocci and rods, although relatively low numbers of Gram-negative bacteria can be identified (De Boever et al, 2006; Furst et al , 2007 ; Leonhardt et al, 1999) . At the same time, in sites

with disease there has been found a lot of pathogens that have already been associated previously with periodontitis. (Tabanella et al, 2009; Maximo et al, 2009; Shilbi et al, 2008; Renvert et al, 2008; Botero et al, 2005; Takahashi et al, 2005; Rutar et al, 2001; Leonarht et al, 1999; Bower et al, 1989; Mombelli et al, 1987) These can be divided in categories of the red complex species (*Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*) and orange complex species (*Fusobacterium sp.* and *Prevotella intermedia*) .Other species of bacteria identified as part of a biofilm responsible for inflammation and destruction of peri-implant tissues have been found to be *Aggregatibacter actinomycetemcomitans, Bacteroides forsythus, Parvimonas micra, Campylobacter rectus, Eikenella corrodens* and *Selenomonas sputigena*. Interestingly, in areas of active infection microorganisms have been identified that could be thought of as opportunistic colonisers, such as *Staphylococcus* spp, *Enterobacteriaceae*, and *Candida* spp (Socransky et al, 1998, Leonarht et al, 1999).

Most of the studies identifying the pathogens in the peri-implantitis sites consistently find that *Porphyromonas gingivalis* is one of bacteria with direct association in implant sites of disease. . (Tabanella et al, 2009; Maximo et al, 2009; Shilbi et al, 2008; Renvert et al, 2008; Botero et al, 2005; Takahashi et al, 2005; Rutar et al, 2001; Leonarht et al, 1999; Bower et al, 1989; Mombelli et al, 1987) In particular in the study by Renvert et al (2008) with a sample of 213 individuals, where it was reported that the implant probing pocket depth at the implant site with the deepest probing depth was correlated with levels of *P. gingivalis* ($r=0.14$, $P<0.05$). In a study by Maximo et al (2009) it was also found that there was initial high counts of *P.gingivalis* in areas of peri-implant disease and how these were reduced after anti-infective treatment. As a result, *P. gingivalis* is thought of

as a putative pathogen in areas of peri-implantitis.

1.7.3 Virulence of *Porphyromonas gingivalis*

Porphyromonas gingivalis is a non-motile, non-spore forming, asaccharolytic, Gram-negative, black-pigmented, obligatory anaerobic coccobacilli that exhibits smooth, raised colonies when cultured on agar. The black pigmentation when grown on blood agar is due to the production of cell-associated protoheme, with the colour presenting in the later stages of growth, after the fourth day, starting from deep red to black. Initially the colonies are white to cream coloured (Hoover et al, 1989). *P. gingivalis* has been shown to adhere to various oral surfaces and to titanium (Cutler, 1995, Wu Juan et al, 1995). It has also been detected within gingival tissues *in vivo* (Papapanou et al, 1994), been found to invade oral epithelial cells *in vitro* (Sandros et al, 1994), and demonstrated to inhibit neutrophil migration through the epithelial layer (Madianos et al, 1996). Following these and other studies of models of periodontal disease (e.g. Holt et al, 1988; Yilmaz, 2008), *P. gingivalis* has been strongly associated as a putative pathogen and implicated in the progress of periodontitis and peri-implantitis.

Several mechanisms of action have been proposed. It has been found that *P. gingivalis* can produce a variety of enzymes, proteins and metabolic end-products that are active

against host proteins and provide protection against host immune defenses. These include proteinases, inhibitors, immunoglobins, iron-containing proteins, bactericidal proteins, extracellular matrix proteins and proteins involved in phagocytic functions (Potempa et al, 1995). The major fermentation end-products are n-butyrate, propionate and acetate, associated with the malodour of oral infections. Also iso-valerate, iso-butyrate, succinate and phenylacetate have been identified. (Holt et al,1999).

The molecules that might function in the inflammatory and destructive events in the host tissues, and can be classified as virulence factors, can be defined as either micromolecules or macromolecules. The macromolecules include those attached to the cell itself, such as the capsule, outer membrane and its associated lipopolysaccharides and fimbriae, and the extracellular produce, including proteinases and enzymes (Holt et al, 1998; Murakami et al, 2004).

The capsule of *P. gingivalis*, which varies between different strains, is composed mostly of mucopolysaccharides and is thought to have a significant effect on its ability to function as an oral pathogen (anti-phagocytic factor). The outer membrane is a complex multi-layered structure that contains the complex lipopolysaccharide (LPS), lipoproteins and peripheral and transport proteins and covered by numerous fimbriae. It is thought that some of the outer membrane proteins may play an important role in the formation and maintenance of the biofilm and also to the attachment to both soft and hard surfaces. As *P. gingivalis* has an absolute growth requirement for hemin, outer membrane proteins acting as a hemolysin have been identified and investigated (Choi et al, 1991; Bramanti et

al, 1992) and are thought to attack and hemolyse red blood cells during periods of active disease.

Lipopolysaccharide (LPS) has been investigated intensively as it has been suggested that it contributes significantly to the pathogenicity of *P. gingivalis* (Lamont et al 1998). LPS is composed of three covalently linked parts: lipid A embedded in the outer membrane, a core polysaccharide found at outer membrane, and the polysaccharide O antigen that extends from the outer membrane into the surrounding environment. These are large molecules with molecular weight usually greater than 10 kDa. *P. gingivalis* LPS possesses relatively low endotoxic activity in comparison to other species (Tanamoto et al, 1997), which may reflect its ability to colonise and grow in the tissues without severe host response (Darveau et al, 1998), but does have an ability to function as a cytokine and chemokine inducer and antagonist (Ogawa et al, 1994). It has been found to increase the release of inflammatory osteolytic factors from osteoblastic cells to stimulate bone resorption and may therefore indirectly hinder bone repair processes (Roberts et al, 2008).

Fimbriae are heat-stable, thin curly filaments, approximately 5 nm in width, that are arranged in a peritrichous fashion over the surface of the cells and comprise of at least 1000 monomeric subunits, known as fimbrillin. Generally, bacteria with fimbriae are capable of binding specifically to human salivary components as commensal bacteria (Amano 1994, 1998; Nakamura, 1999). *P. gingivalis* fimbriae have been proven to assist adhesion to host cells and sometimes even invasion of gingival epithelial cells (Lamont et

al 1995), macrophages (Takeshita 1998) and fibroblasts (Kontani 1997). They are also capable of eliciting host-associated responses, such as stimulating IL-1, neutrophil chemotactic factor, tumor necrosis factor-α from mouse peritoneal macrophages (Murakami et al, 1993), and IL-6, IL-8 and tumor necrosis factor -α in human peripheral blood monocytes (Ogawa et al, 1994). Their chemotactic ability could have an effect on the formation of inflammation as well as the progression of tissue destruction.

Murakami et al (2004) found that 80% of the fimbrillin remains attached to the cells, whereas 20% migrates to the supernatant. The gene encoding *P. gingivalis* fimA subunit has been cloned and characterised (Takahashi et al, 1992). Major fimbriae can be classified into six types (I to V and Ib) based on the variations identified in the fimA gene. In studies by Amano et al (2004) it has been found that the majority of the periodontitis patients harbor the type II fimA P. *gingivalis*, followed by the type IV fimA. The type II has revealed significantly greater adhesive and invasive capabilities to epithelial cells than other subtypes and it was postulated that minor fimbriae induced interleukin (IL-1α) IL-1b, IL-6, and tumor necrosis factor-a (TNF-a) cytokine expression in macrophages. This was strongly associated as a potent causative factor of alveolar bone resorption in animal models. However, further investigations should be considered as it seems that certain environmental conditions seem to alter *P. gingivalis* virulence. (Holt et al, 1999)

It has also been found that all *P. gingivalis* strains produce a large number of hydrolytic, proteolytic and lypolytic enzymes either exposed to the outer membrane or in outer

membrane vesicles, which are sloughed from the outer membrane during growth. These in particular include the putative *in vivo* virulence factors trypsin-, thiol-, and caseinolytic proteinases, peptidases, collagenases and aminopeptidases (Holt et al,1998). Of particular interest in the bibliography have been the trypsin-like arginine (Arg- 1 and Arg- 2) and lysine (Lys-) cysteine proteinases, which are also called gingipains (Imamura, 2003). The gingipains are responsible for at least 85% of the general proteolytic activity of *P. gingivalis* (Potempa, 1997). The genes encoding gingipains have been identified and referred to as rgpA, rgpB and kgp. The function of these proteinases include adherence to host cells, to other bacteria, supporting *in vivo* growth of the bacteria, inhibiting selected host defence mechanisms, like the polymorphonuclear leukocyte function (Kadowaki et al, 1994), degradation of components of the complement system, which prevents bacteriolysis by disturbing the neutrophil migration and rendering the leucocytes hyporesponsive to the bacterial lipopolysaccharides (LPS) (Sugawara et al,2000), and dysregulation of the cytokine network. They activate the kallikrein/kinin system (Imamura, 1995), and degrade the fibrinogen and fibrin (Pike et al, 1996) which may also explain the increased bleeding in periodontally affected sites. They also degrade the IL-8, IL-6 , IL-1β, tumor necrosis factor-α and interferon-γ affecting the inflammatory reactions and may explain the increased destruction of the periodontal tissue in areas of active disease. All these multiple pathogenic activities of the gingipain have placed them as potential targets in the therapy of periodontitis (Imamura, 2003).

It has also been investigated whether *P. gingivalis* can cause opportunistic infections in areas outside the oral environment, association with endocarditis or other potentially

dangerous diseases (Avila et al, 2009).

Several studies have investigated how *P.gingivalis* affects cells in the periodontium. In a study by Yamamoto et al (2006) it was found that human periodontal ligament cells produce many kinds of inflammatory cytokines (interleukin -1b, interleukin-6, interleukin-8 , tumor necrosis factor-a (TNF-a), receptor activator of nuclear factor-kB ligand and osteoprotegerin (OPG) mRNA after stimulation with *P.gingivalis*. It has also been reported that the release of interleukin-6 and interleukin-8 release from human periodontal ligament cells was induced by *P.gingivalis* and that may explain periodontal tissue destruction and alveolar bone resorption (Yamaji et al, 1995), and that the same inflammatory cytokines may also lead to the destruction and disintegration of the extracellurar matrix (Chang et al, 2002)

Moreover it has been found that periodontal liganment cells from donors with periodontitis are more responsive to aggregation with *P.gingivalis* than those from healthy donors (Scheres et al, 2011) . Another study by the same team (Scheres et al, 2009) identified that there is heterogeneity to the responsiveness of periodontal and gingival fibroblasts to live *P.gingivalis* , although there is strong response from both kinds of cells.

P.gingivalis has also been found to activate Toll-like receptors 2 and 4, and cytokine production in human periodontal ligament cells.(Sun et al, 2010) and regulate RANKL and OPG gene expression (Belibasakis et al, 2007)

Additionally, *P. gingivalis* has been shown to be susceptible to Penicillin, Clindamycin, Erythromycin, Metronizadole and Tetracycline, and less susceptible to Vancomycin, Spiramycin and Chloramphenicol and resistant to Gentamicin (Maryland and Holt, 1988)

1.8 Organ Culture

The understanding of the intricate physiological functions and responses of the human body is a field of further research. *In vivo* modelling is usually the best way to investigate these however there is a big amount of limiting, confounding factors which are very difficult to control. Alternative approaches have been developed in the field of tissue engineering in this continuous effort to reveal the mysteries of the nature and assist in battling and controlling several mechanisms in disease. A very interesting approach to the armamentarium of the researcher *in vitro* is the use of *ex vivo* modelling in the form of organotypic models.

The most commonly used are single cell or multi-cell type systems which in the field of the bone and periodontal –peri-implant tissue can usually be cells of the bone tissue or cell lines from the periodontal or epithelial barrier layers. While such models have given important information about the behaviour of these cells they are unable to recapitulate the spatial arrangement of the cells *in vivo* and their possible interactions. Models of this kind have been used also for the investigation of the response to the action of bacteria such as *Porphyromonas gingivalis*.

In that several organ culture systems have been developed from skin, lung, prostate,

salivary glands (Trowell, 1959), tumors, thymus (Moore et al., 1994), spleen (von Melchner, 1983) and others providing important information about the response of these tissues.

In 1998, Sloan and colleagues started developing an *ex vivo* tooth slice model , aiming to investigate initially dentine repair processes. Several investigations have been developed since to research on dentine repair processes (Sloan and Smith 1999; Murray et al., 2000; Gonçalves et al., 2007), the effect of orthodontic forces to the dentine-pulp complex (Dhopatkar et al., 2005), the effect of growth factors on fracture-repair processes (Smith et al, 2010), leading to the development of the mandible slice model. The model uses extracted tissues from the area of the mandible of young wistar rats, which are then cultured in special media following tissue engineering methods. This offers the possibility to model the response of the tissues for some time in an environment which closely resembles their *in vivo* arrangement. This offers the versatility and flexibility to the model, based on its *ex vivo* characteristics, to investigate the behaviour of the cells comprising the model in response to varying treatments, as the application of experimental agents can be a relatively easy process.

1.9 Aims

This is a study that aims to investigate the virulence of *P.gingivalis* grown in the presence or absence of titanium exposing its supernatant to an *ex vivo* tissue culture model of 28-day wistar rats mandible slices.

More specifically it aims to:

- Investigate whether this *ex vivo* mandible model is affected by the presence of the supernatant of a potentially pathogenic bacteria
- Examine the growth of three different strains of *P.gingivalis* in the presence of Titanium discs, with different surface topography (smoothened or moderately roughened) and its effect on its supernatant
- Investigate the behaviour of this *ex vivo* model when exposed to supernatants from different strains of *P. gingivalis*
- Investigate the behaviour of the *ex vivo* model when exposed to supernatant from *P. gingivalis* grown in the presence of Titanium discs with different surface topography
- Identify different cellular responses in the model as a result of the exposure to the supernatant

General Hypothesis:

The *P.gingivalis* supernatant of three different P.gingivalis strains grown in the presence or not of titanium discs with different surface topography (smoothened or moderately roughened) affects an *ex-vivo* tissue culture model of mandible slices from 28-day wistar rats

Chapter 2

Materials and Methods

2.1 Organ culture of mandible slices

2.1.1 Tissue culture reagents

The organ culture of the mandible slices has two stages. The first stage involves the preparation of the mandible slices from 28-day Wistar rats and the second stage is the actual culture of the mandible slices and experiments based on the culture. For the preparation and washing of the slices until their introduction to the culture environment, the washing medium was prepared. For the second stage of the experiment the culture medium was used as described by Smith (2009).

The washing medium consists of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co Ltd, UK) containing 1% concentration of Antibiotics (solution containing 10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK) and 200mM L-glutamine (Invitrogen, UK).

The culture medium is a mixture of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co Ltd, UK) containing 10% heat inactivated foetal calf serum (FCS) (Invitrogen, UK), 1% concentration of antibiotics (solution containing 10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK), 0.15mg/ml of ascorbic acid (Invitrogen, UK), and 200mM L-glutamine (Invitrogen, UK). Both of these medium were prepared by mixing the ingredients to the desired concentrations and preserved for use for a maximum of 3 weeks in a fridge (+4^{o}C) and were preheated to 37^{o}C before use in a water bath.

2.1.2 Preparation of the mandible slices

Twenty eight day old male Wistar rats were sacrificed by CO_2 asphyxiation to obtain their mandibles. Their mandibles were dissected within an hour of their sacrifice. All soft tissues (muscles etc. including the periosteum) were carefully removed from the surface of the mandible, and the mandibles bisected between the two incisors with a sterile scalpel and quickly placed in the washing medium. The mandibles were then cut with a diamond-edged rotary bone saw (TAAB Laboratories Equipment Ltd, UK) (disinfected with 70% ethanol) under constant cooling by the prepared washing medium. The molars, the coronoid process, condyle, ramus and erupted part of the incisor were firstly removed. Then 3-4 transverse slices of approximately 1.5-2 mm thickness of the mandible body containing the root of the incisor were obtained, washed with the washing medium and placed into fresh culture medium in 5 ml universal containers.

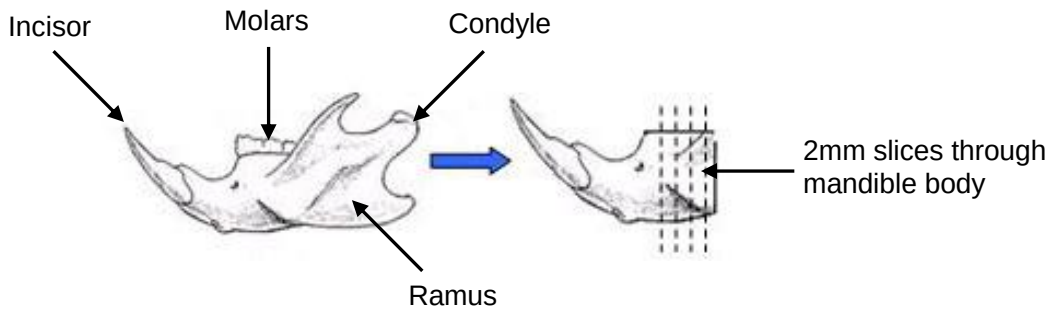


Fig. 2.1**:** Preparation of mandibles.

2.1.3 Culture method for the organ culture of the mandible slices:

For the experiments the mandible slices were cultured using the 'base type cultures' method as described by Smith (2009). The mandible slices were transferred from the universal containers into individual wells of a plastic 24-well plate (Greiner Bio-One, UK), and 2ml of culture media added to each well (Figure 2.2). Mandible slices were then incubated at 37^{o}C, in a humidified atmosphere of 5% CO_2 in air for 1 day then with the addition or not of the purified supernatant of *Porphyromonas gingivalis* strains for additional 3, 7 or 14 days.

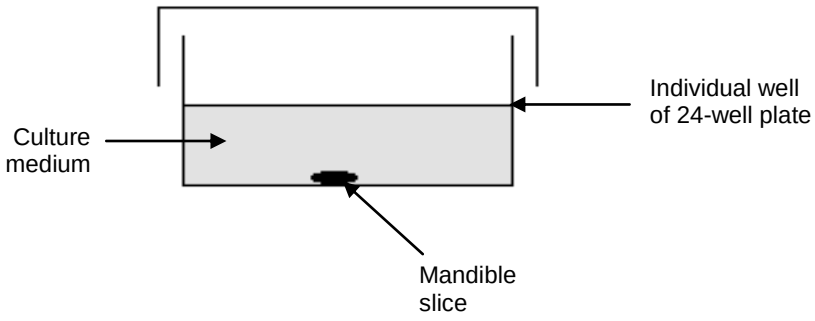


Fig 2.2 Diagrammatic representation of tissue culture conditions

2.2 Histological examination and TRAP stain

At the end of the culture period the mandible slices were prepared for histological examination with a process used for mineralised tissue. Once removed from the culture the slices were initially fixed in 10% neutral buffered formalin (v/v) for 24 hours at room temperature then demineralised in 10 % formic acid (v/v) at room temperature for 72 hours whilst gently agitated in a shaker and subsequently stored back in formalin for at least 24 hours before preparing them for processing. The slices were then placed in plastic cassettes and processed via an Automated Tissue Processor (Shandon Pathcentre (UK)) overnight through a series of 70,90 and 100% alcohol, cleared with xylene and finally in molten wax. Each slice was embedded in molten parafin wax on a stainless steel cassette mould and solidified by cooling in the embedding station (Leica EG1150H, Leica Microsystems Ltd. (UK).

The slices were then sectioned using a rotary microtome (Leica) to obtain sections of 6μm thickness at different levels within the slice, discarding the initial sections as the tissue may have been damaged by the process. Following this, the sections were carefully mounted on coated histological slides after floating them on a warm water bath (40^{o}C) and dried at 65^{o}C before staining to improve adhesion.

The sections were stained either with a standard H&E (Haematoxylin and Eosin) stain or TRAP stain (Tartaric Resistant Acid Phosphatase) procedures.

2.2.1 Haematoxylin and Eosin Stain (H&E)

The sections were stained with Haematoxylin and Eosin using an automatic staining machine (Shandon Linistain Linear Stainer, Thermo Scientific). This involves the section to be taken in a linear fashion initially through a series of solutions of xylene, series of graded alcohol, water, stained with Haematoxylin, washed and blued with Scott's tab water, then counterstained with Eosin, washed in water and alcohol and finally with xylene. Following this procedure the sections were fixed under a glass cover slip with the DPX (Di-n-butyl-Phthalate in Xylene) synthetic mounting medium.

2.2.2 Tartrate- Resistant Acid Phosphatase (TRAP)

For this stain the sections were processed using the Acid Phosphatase, Leucocyte (TRAP) kit (Sigma-Aldrich &Co.) for the detection of osteoclastic activity.

The processed sections were initially washed with two washes of xylene for 5 minutes to remove the parafin. The xylene was washed with Industrial Methylene Spirit (IMS) twice for 2 minutes. The sections were placed in a fixative solution containing 25 ml cirtrate solution, 65 ml acetone and 8 ml 37% formaldehyde for 30 seconds and then washed with distilled water. Following the sections were placed in a solution of 45 ml distilled water, 1 ml Diazotized Fast Garnet GBC solution, 0.5 ml Napthol AS-BI Phosphate solution, 2ml acetate solution and 1 ml Tartrate solution as required from the kit manufacterer within a Colin Jar in a water bath of 37^{o}C under light proof conditions and incubated for 1 hour or in the same solution without the Tartrate solution for the positive control. After the incubation the slides were removed, rinsed with warm water for 5 minutes, counter-stained with Haemotoxylin solution for 2 minutes and finally washed with tap water for 10 minutes. The procedure was finalised with dehydration in IMS and clearing with xylene for 1 minute before mounting with DPX synthetic mounting medium.

2.2.3 Photography and Analysis

All the slides were then examined on an Olympus AX70 microscope and micro-photographs were taken with a Nikon Digital Camera DXM 1200 through the Nikon Act-1 camera controller software.

The photographs were compared between each other and to the controls in order to draw conclusions about the way the tissue was affected from the different enviroments created through the experiments.

In order to quantify the results observed, from the photographs taken at 20x magnification, cell numbers within the PDL and the pulp were automatically counted using the Image ProPlus 6.0 software. Five randomly selected areas of size 100μm^2 within the PDL and the pulp from each section was selected and the programme counted cell nuclei above a certain preset size of parameters. Cell counts were obtained for the 7 days cultures and for the different exposures (supernatant in 2 concentrations and from different strains of the *P.gingivalis* and grown in the presence or absence of Ti). Mean values were analysed using one-way analysis of variance (ANOVA) to analyse differences between the cell numbers in different culture types and culture periods.

2.3 Preparation of the Titanium samples

All the titanium samples used in the experiments were prepared from 2 sheets of commercially pure (cp2-grade 1) Titanium 0.5 mm thick fully annealed and de-scaled (unalloyed Ti for medical applications (Goodfellow Cambridge Limited, Cambridgeshire, UK). The titanium sheets were cut with a spinning saw at constant temperature mediated by a jet of forced air in samples of 10mm x 10mm. In this study two types of Ti surfaces were prepared. A polished surface ,which is the surface of choice usually in the transmucosal part of the abutment of the implant,and a moderately roughened surface. The moderately roughened surface was chosen to be a sand-blasted acid etched surface, which is one of the most common roughened surface used nowadays from most implant companies (although there is continuous evolution in the surface topology and chemistry) and easier to reproduce with the equipment available.

2.3.1 Polished (smoothened) surfaces

In this study the CP titanium sample surfaces were all ground using the 320- and 600-grit silicon carbide metallographic paper and polished with the 0.5-μm diamond polishing paste at a polishing speed of 10 to 30 m/s. Then following the standard Branemark implant cleaning procedure (Machnee et al, 1993), the titanium sample was scrubbed with residue-free detergent, rinsed with distilled water and air dried. The samples were then placed in test tubes containing butanol and were ultrasonically cleaned for 10 minutes. They were further cleaned with two rinses of 99% ethanol and once again

ultrasonically cleaned for 10 minutes. The CP titanium samples were then handled with sterile titanium forceps to avoid contamination and placed in sterile plastic containers and left to dry in a vacuum desiccator.

2.3.2 Moderately Roughened Titanium samples

The samples were initially cleaned with residue-free detergent, rinsed with distilled water and ethanol and air dried to remove major contaminants. The surfaces of the sample were then sandblasted with 50μm aluminium oxide beads (Bracon Limited, Sussex, UK) in the hand operated S-U-Progressa 200 (Schuler dental GmbH & Co., Ulm, Germany) under pressure of 6.5 bars (as controlled by the manometer). The handpiece was maintained at an angle of 45^{o} and at a constant distance throughout the procedure to assure an even result. Then following a regime described by Nishimoto et al (2008) the sandblasted samples were thermally acid-etched in an ultrasonic bath with H_2SO_4 and HCL (4:1) at 80^{o}C for 5 minutes. The resulting acid-etched samples were washed from the acid by ultrasonic bath in double-distilled water three times for 5 minutes. Then handled with sterile titanium forceps the samples were placed in sterile plastic containers and dried in a vacuum desiccator and stored.

2.4 Preparation of the Supernatant of *Porphyromonas Gingivalis*

All the experiments were conducted using 3 strains of *Porphyromonas gingivalis*: The reference strain NCTC 11834 and 2 Clinical isolates, isolated from areas with active periodontal disease in patients at the Cardiff Dental Schoolnamed R17870 and R17988. Stock strains were stored on microbank beads (Protec, Ontario, Canada) at -80^{o}C in the Oral Microbiology Department at the Dental School of Cardiff. *Porphyromonas gingivalis* strains were routinely sub-cultured on blood agar plates (Fastidious Anaerobe Agar, LAB M, supplemented with 5% v/v sheep blood) in anaerobic conditions at 37^{o}C at 7 day intervals.

2.4.1 Growth of *Porphyromonas gingivalis* in liquid conditions

All 3 strains of *Porphyromonas gingivalis* had to be grown in liquid conditions in order to be able to obtain their supernatant (extracellular produce). Several media were attempted with a view to achieving optimal growth conditions, standardising concentrations and minimising the effect of the media to the culture. The test media used were: 100% Fastidious anaerobe broth, Fastidious anaerobe broth supplemented with cysteine (0.5 mg/ml), hemin (5 µg/ml), vitamin K (1 µg/ml) (*Porphyromonas gingivalis* growth factors), 20 % Fastidious anaerobe broth (v/v) and 80 % working media (v/v) (DMEM +

10% heat inactivated foetal calf serum without antibiotics) supplemented with cysteine (0.5 mg/ml), hemin (5 µg/ml), vitamin K (1 µg/ml) and Working media supplemented with cysteine (0.5 mg/ml), hemin (5 µg/ml), vitamin K (1 µg/ml). Each of the 3 Porphyromonas gingivalis strains were cultured in agar for 3 days and used to inoculate of each of the media types. The inoculated media was then vortexed for 2 minutes in order to disperse the bacteria evenly. A spectrophotometer (DU 800 spectrophotometer, Beckman Coulter) was used to calculate the relative numbers of bacteria in each the samples by measuring absorbance at a wavelength of 660 nm. An initial measurement with the spectrophotometer was taken to measure the concentrations achieved and dilutions were made as necessary to obtain similar starting concentrations. For each media, 3 containers of 20 ml were used. Sampling to measure concentrations was taken alternatively from each of the 3 containers every 6 hours for 120 hours in order to obtain the growth curves.

For the main experiment the following sequence was used. A small sample of *Porphyromonas gingivalis* on day 3 after subculturing was taken and inoculated in the working Broth media (WBM) (20% FAB(v/v)+80% working media (v/v) without the antibiotics supplemented with growth factors) for 24 hours initially under anaerobic conditions at 37^{o}C. After this 24 hour period the liquid culture was centrifuged (4000rpm /10 minutes) and the bacterial pellet obtained washed with PBS. The bacteria were then re-suspended in fresh WBM media to a standardised concentration, measured by the spectrophotometer (Absorbency: 0.1 at 660nm). For each strain (NCTC 11834, R17870, R17988) three distinct environments of growth were created using 4 ml of WBM media.

One where the strain would grow in 5ml universal plastic container in a planktonic fashion, one where a specimen of smoothed Ti would be added and one where a specimen of roughened Ti would be added. This would be further incubated for a period of 48 hours (highest point of growth curve) under anaerobic conditions at 37 ^{o}C. Following that the bacterial cells were removed by triple centrifugation of the media (7000rpm /15 minutes) and sterilised by double filter sterilisation. The resulting supernatant was stored in 0.5 ml beads in the freezer (-20 ^{o}C) for single use during the experiments. Some of the supernatant was used for determining an approximate concentration of the supernatant by BCA protein assay and evaluation of the protein content (banding) through SDS-page.

2.4.2 BCA protein assay

The BCA protein assay was used to determine the approximate concentration of protein in the supernatant as obtained following the incubation and removal of the bacteria content. This was necessary in order to have comparable concentrations used in all the experiments. Calculating the concentration of the protein in the supernatant is just an approximate method as it measures the whole protein content and not only the extracellular produce of the bacteria, which is the part of the protein content of interest. However as there is no easy method to measure the protein used from the bacteria for their intracellular needs and assuming similar growth has been achieved by the bacteria (that essentially belong to the same species and are usually expected to have more or less similar metabolic patterns), it was thought that the error in this comparison would be

minimal.

The BCA protein assay kit involved using a ready kit provided by Thermo Scientific, Pierce Biotechnology, Illinois, USA. This method exploits the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reduction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbency at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 μg/ml). Protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown before the concentration of each unknown is determined based on the standard curve. The micro-plate procedure was used as it affords the sample handling ease of a microplate and requires a smaller volume (10-25 μl) of protein sample. Firstly using a 1 ml ampule of 2.0 mg/ml Albumin Standard with a series of dilutions the working range of concentrations (20-2,000 μg/ml) was achieved. Then after calculating the total volume of the working reagent required, it was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). In a 96-well microplate 25 μl of each standard or unknown sample was pipetted and then 200 μl of the WR was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The microplate was covered and incubated at 37°C for 30 minutes and then cooled to room temperature. The absorbance

was read in the spectrophotometer at 562 nm on a plate reader. From the triplicate readings obtained the average 562 nm absorbency measurement of the blank standard replicates was subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates and a standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. This standard curve was finally used to determine the protein concentration of each unknown sample.

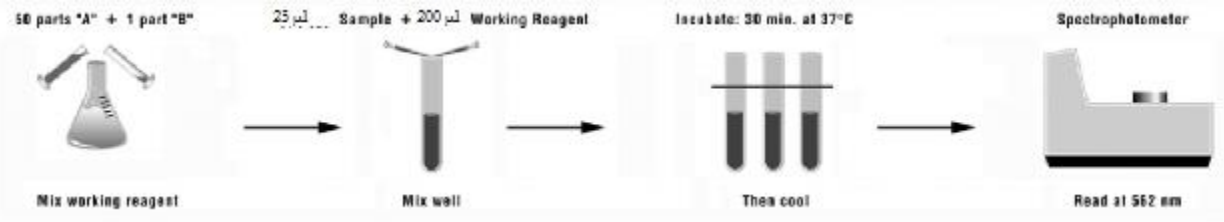


Fig 2.3 Graphic representation of BCA protein assay

2.4.3 SDS-Page Assay

The SDS-Page (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was used for visualisation of the protein composition of the supernatant, confirmation that the supernatant contained proteins produced from the *P.gingivalis* rather than those in the growth media and finally for a rough estimate of whether different proteins (protein banding) were produced between different strains of *P.gingivalis* and also between

different growth conditions (growth in the presence of Ti). The silver staining method was used which has a reported sensitivity of 0.3-0.5 ng of protein per band.

The SDS-page is a technique developed in the 1960s and 70s (Ornstein, 1964; Shapiro et al, 1967; Laemmli, 1970; O'Farrel, 1975) used to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). SDS is a powerful detergent, which has a very hydrophobic end (the lipid like dodecyl part) and highly charged part (the sulfate group). The dodecyl part interacts with hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends on interactions between hydrophobic amino acids in their core, the detergent destroys 3D structures, transforming what were globular proteins into linear molecules now coated with negatively charged SDS groups. After boiling in SDS proteins therefore become elongated with negative charges arrayed down them, so they will move towards a positive electrode. The largest extended molecules are generally retarded the most by polyacrylamide gels, and the smallest ones the least. Since some proteins have few or no hydrophobic residues it is also not surprising that such molecules don't run on SDS page in a fashion which accurately reflects their molecular weight. Finally cross-linked proteins don't run as their molecular weight would predict, generally running slower particularly on higher percentage gels. However a particular protein runs on a particular position on a particular percentage acrylamide gel in a reliable manner.

The procedure in this case was conducted with the use of the NuPage Electrophoresis® System by Invitrogen and specially the NuPAGE® Novex Bis-Tris [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl] Pre-Cast Gels for separating small to mid-size

molecular weight proteins. Following the kit's instruction the samples were initially prepared, Using the NuPAGE® LDS Sample Buffer (4X) for denaturing gel electrophoresis with the NuPAGE® Gels, under slightly alkaline pH conditions. Since the pH of the NuPAGE® LDS Sample Buffer is 8.4, sample reduction at this pH allows for maximal activity of the reducing agent (NuPAGE® Reducing Agent contains 500 mM dithiothreitol (DTT) at a 10X concentration). The samples were initially heated to 70^{o}C for 10 minutes for optimal results. Then all the samples were prepared by the use of the addition of the NuPAGE® LDS Sample Buffer (4X) 2.5 µl, NuPAGE® Reducing Agent (10X) 1 µl and Deionized Water to 6.5 µl.

Then for the denaturing electrophoresis the NuPAGE® MES SDS Running Buffer was prepared by reducing it in 950 ml of deionized water to a volume of 1000ml. These were mixed thoroughly and set aside 800 ml of the 1X NuPAGE® SDS Running Buffer for use in the Lower (Outer) Buffer Chamber of the XCell *SureLock*™ Mini-Cell. Immediately, prior to electrophoresis, 500 µl of NuPAGE® Antioxidant was added to 200 ml of 1X NuPAGE® SDS Running Buffer from Step 1 for use in the Upper (Inner) Buffer Chamber of the XCell *SureLock*™ Mini-Cell.The XCell *SureLock*™ Mini-Cell was then loaded with the 200ml in the Upper Buffer Chamber and 600 ml for the Lower Buffer Chamber. The NuPAGE® Gel was removed from the pouch, the gel cassette rinsed with deionized water and the plastic Buffer Dam replaced the second gel cassette. Then 10µl volume of each of the protein samples was loaded onto the gel, as well as the appropriate protein molecular weight markers. After connecting the electrodes, the NuPAGE® Novex

Bis-Tris Gels with MES SDS Running Buffer was run in a programme of voltage of 200 V,and current beginning at 110-125 mA/gel ending at 70-80 mA/gel for 35 minutes.

Once the electrophoresis was complete, the power was shut off, the electrodes disconnected and the gels removed from the XCell *SureLock*™ Mini-Cell. The gel was separated from the cassette, carefully removing and discarding the top plate, allowing the gel to remain on the bottom plate. The gel was then fixed and stained with the the SilverXpress® Silver Staining Kit.

Firstly the reagents were prepared as described below. For the fixing solution, Methanol 100 ml, Acetic Acid 20 ml and Ultrapure water 90 ml. For the sensitizing solution Methanol 100 ml, Sensitizer 5 ml and Ultrapure water 105 ml. For the staining solution, Stainer A 5 ml, Stainer B 5 ml, Ultrapure water 90 ml. For the developing solution, Developer 5 ml and Ultrapure water 95 ml as per the manufacterer's protocol.

The gel was fixed in fixing solution for 10 minutes, which was then decanted and the gel was incubated in two changes of the sensitizing solution for 10 minutes, which was again decanred and the gel rinsed twice with ultrapure water for 10 minutes again. The gel was followingly incubated in the Staining solution for further 15 minutes and after that rinsed twice with ultrapure water. The developing solution was the next step for 3-15 minutes with the stopping solution being used for 10 minutes once the desired staining intesity was reached. Finally the gel was rinsed three times with ultrapure water. Once the gel was fixed the bands were compared with each other to make conclusions and also with Novex® protein molecular weight standards to make rough estimates of the kind of the

proteins contained in the supernatant.

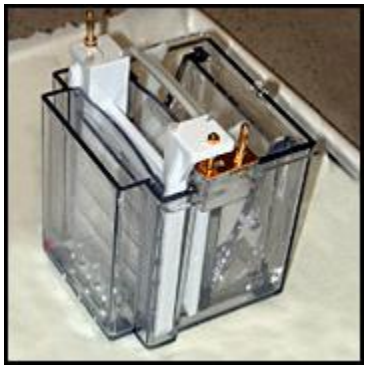


Fig 2.4 XCell *SureLock*™ Mini-Cell Device

2.4.4 Adherence assay of *P.gingivalis* to the titanium dics

In 5 ml containers *P.gingivalis* was incubated at 37^{o}C in the test media (20 % Fastidious anaerobe broth (v/v) and 80 % working media (v/v) (DMEM + 10% heat inactivated foetal calf serum without antibiotics) supplemented with cysteine (0.5 mg/ml), hemin (5 µg/ml), vitamin K (1 µg/ml)) for 48 h in the presence of smoothened and moderately roughened titanium discs as before. The titaniumsamples at the end of the incubation period were removed and washed in PBS buffer for 1 minute by agitation whilst submerged in the buffer, following a procedure in keeping with published methods (Mahmoud et al, 2012) The discs were then stained using the Live/dead BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen Detection Technologies, Oregon, USA). The preparation of the stain was performed by mixing 1 µl of component A (1.67mM propidium iodide, 1.67mM Syto 9) and 1 µl of component B in 1 ml of PBS

Buffer.

The discs were stained, keeping them in the dark at all times and then immediately viewed under fluorescence microscopy (Olympus AX70, Olympus Optical Co. Ltd, Southall, UK). Images were then captured by the attached Nikon Digital Camera DXM 1200 through the Nikon Act-1 camera controller software.

2.5 Addition of the supernatant to the mandible slice culture model

Following the preparation of the mandible slices as described before, the slices were initially incubated for 24 hours in the 24-well plates in 95% air 5% CO_2 at 37 ^{o}C with the culture medium, to allow for the superficial dead cells to precipitate and for the tissue to acclimatise. The culture medium was then changed every 2 days and to the medium there was addition of the supernatant of each of the strains (*P.gingivalis* NCTC 11834, R17988, R17870) grown under the 3 different conditions (planktonically, with the presence of smoothened or moderately roughened Ti specimen) as mentioned above in protein concentrations of the supernatant in the well of either 50μg/ml or 100μg/ml and for control the media in which the bacteria were grown (creating a total of 19 different combinations). For each combination of strain and growth condition 3 or 4 mandible slices were used, carefully chosen to be from different rat mandibles for randomising. These cultures were then incubated for further 3 or 7 days. The medium for the cultures containing the two different concentrations of the supernatant was changed every two

days throughout the culture period. The experiment was repeated three times for each of the combination of strain and growth conditions. At the end of each experiment the slices were then processed either for the H&E stain and few for the TRAP stain, photographed and assessed.

2.5.1 Statistical Analysis

The cell counts from the experiments were analysed and compared with the use of the one-way ANOVA test through the SPSS statistical software. The null hypothesis was that there is no difference in the means compared. The differences between each individual condition in the results were calculated by means of using post-hoc Tests (the Tukey's HSD (Honestly Significant Difference) test. Differences were reported at $p < 0.05$ level.

CHAPTER 3

The *Ex Vivo* Tissue Culture model and Characterisation of the *Porphyromonas gingivalis* Supernatant

3.1 Culture of mandible slices for up to 7 days (control)

The maintenance of the viability of the *ex vivo* organ tissue culture model for the duration of the experiment was the basis of this experiment. The inherent difficulties in maintaining tissue explants *ex vivo* are well known, especially in comparison with monolayer tissue culture models (Anderson et al, 1998). This study has been based on the development of the *ex vivo* mandible slice tissue culture model as described by Smith and colleagues (2010). The maintenance of the tissue architecture and viability was confirmed by histological examination of slices from the middle layers of the tissue stained with the Haematoxylin and Eosin (H&E) amongst others as previously proposed, using the techniques described by Smith (2009) . The general architecture of a mandible slice can be seen in Fig 3.1.

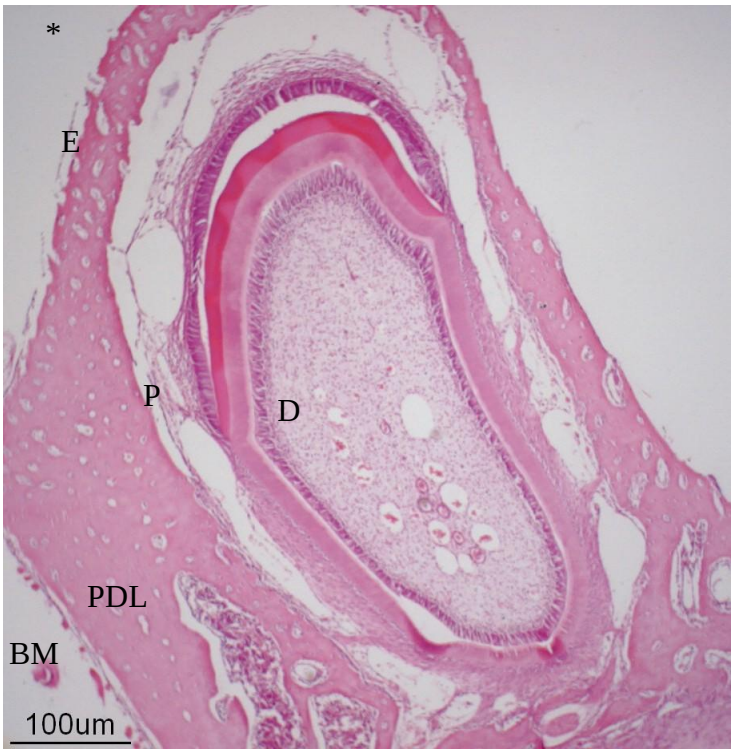


Fig 3.1 Mandible Slice stained with H&E stain
Legend: B: Bone
BM: Bone marrow
P: Pulp
PDL: Periodontal ligament
D: Dentine
E: Enamel
*: empty area – result of the processing

In this study, the control for the experiments was a mandible slice cultured in a similar manner as in previous experiments with the addition of the growth media used for the growth of *P.gingivalis* in the same concentrations as the supernatants. This control was selected as there was a need to identify the effect of the growth media used for the growth of *P.gingivalis*, and assess it against the effect seen on the main experiment. This was compared to specimens cultured in similar conditions without the addition of the growth media. The average cell counts of these base type cultures on a 100 μm^2 surface area in

the histological slides was 78.4 cell nuclei/100μm^2 in the periodontal ligament area (PDL) and 92.8 cell nuclei/100 μm^2 in the pulp area. In comparison, the average cell counts in specimens exposed to the growth media was 63.1 cell nuclei/100 μm ^{2} in the PDL area and 81.9 cell nuclei/100 μm^2 in the pulp area. As expected the addition of ingredients used for bacterial growth (growth media) would change the optimal conditions needed for the maintenance of the tissue and that would have an effect on the viability of the tissue. Through the histological examination, it was found that the tissue architecture though remained mostly the same, (although in the normal tissue culture conditions the cells seem arranged in a more orientated way) and the cells identified in the tissue were identical to those in the specimens grown in the more optimal conditions as can be seen in the histological images (Figure 3.2 and Figure 3.3).

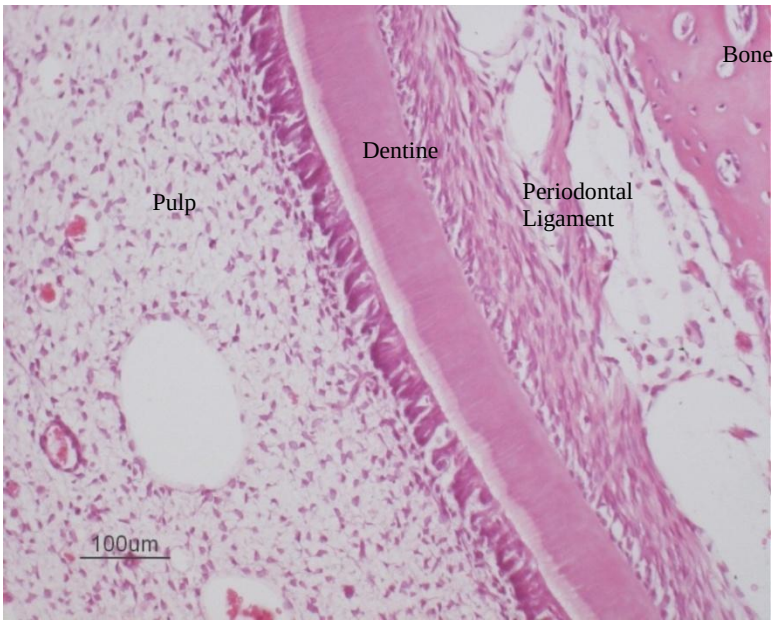


Fig 3.1 Slice of mandible grown in tissue culture media only (7 days)

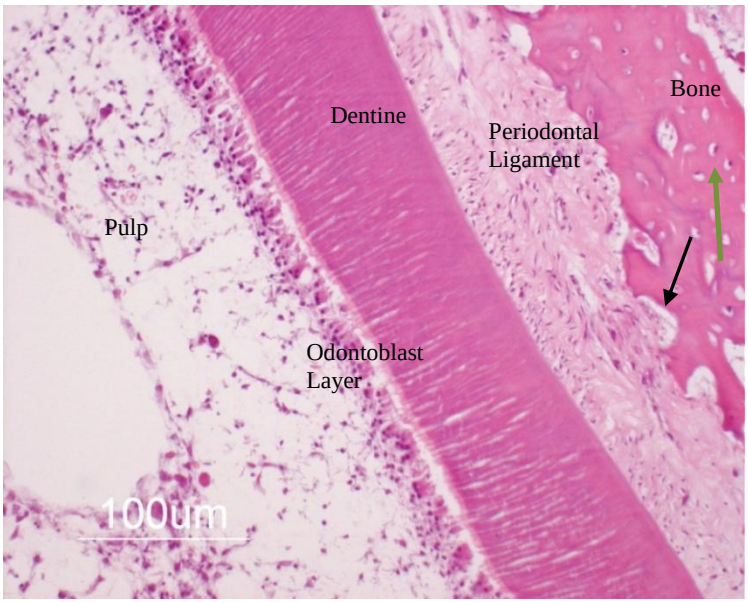


Fig 3.2 Slice of mandible grown in experimental conditions (control) (tissue culture media + 1.25 % (v/v) of bacterial growth media) (7days) (Black Arrow: Lacuna, Blue Arrow : Lacuna containing osteocyte)

3.2 Growth of planktonic *Porphyromonas gingivalis* in liquid media

The growth of *P.gingivalis* in planktonic culture has been identified as satisfactory in Fastidious Anaerobe Broth (FAB) producing a predictable growth curve. Growing *P.gingivalis* in broth only would have a detrimental effect to the mammalian tissue culture, so growth of *P.gingivalis* in the test situations outlined in the Materials and Methods was performed. From the growth curves produced it can be easily identified that using the 20% FAB + 80% DMEM combination gave typical bacterial growth for all 3 bacterial strains (Figures 3.3 - 3.4 – 3.5) beginning to enter the exponential growth phase

around the 16 hour mark and the stationary phase in/around 48 hours, which appeared similar to the one achieved when the bacteria were grown only in broth (supplemented with growth factors) and proved that a predictable growth of the bacteria could be achieved in these conditions. However, the growth of the bacteria was slightly different between the different strains.

The presence of Titanium did not affect the growth of the bacteria substantially, as this was previously demonstrated in an other study (Gao et al, 2002).

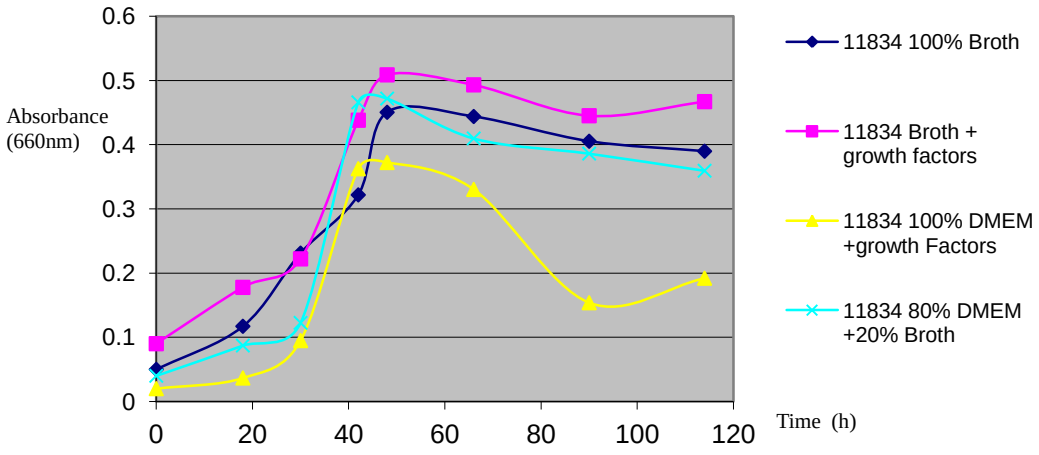


Fig 3.3 Growth Curve of *P.gingivalis* NCTC 11834 grown in different conditions

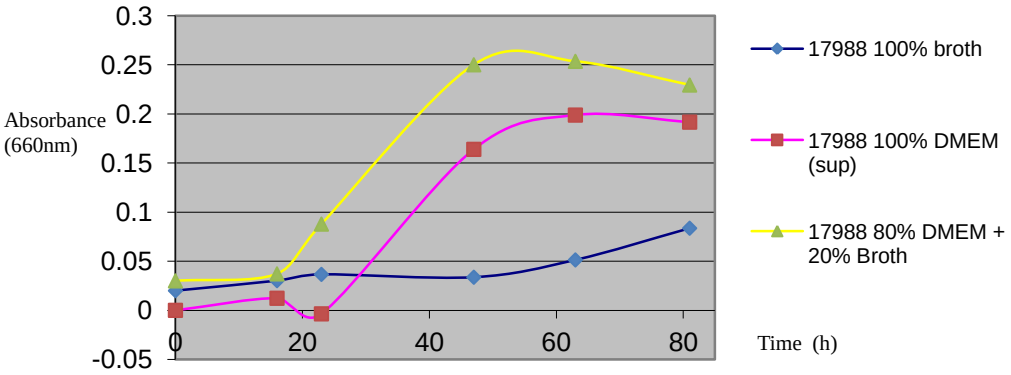


Fig 3.4 Growth Curve of *P.gingivalis* R17988 grown in different conditions

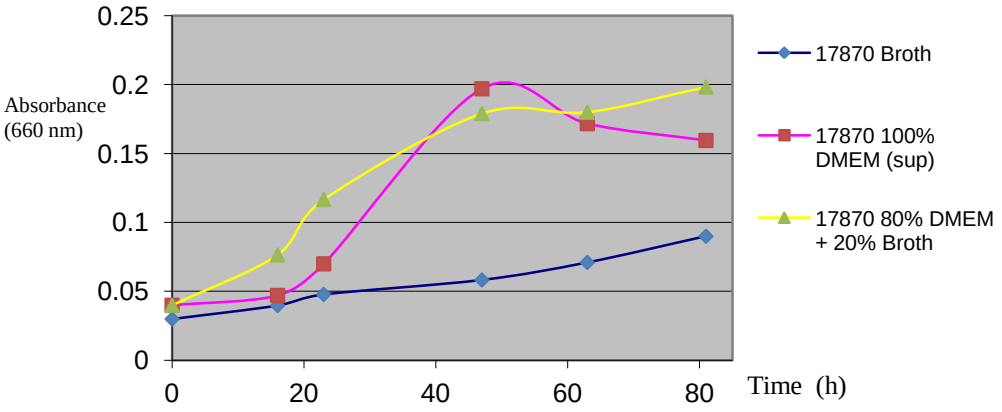
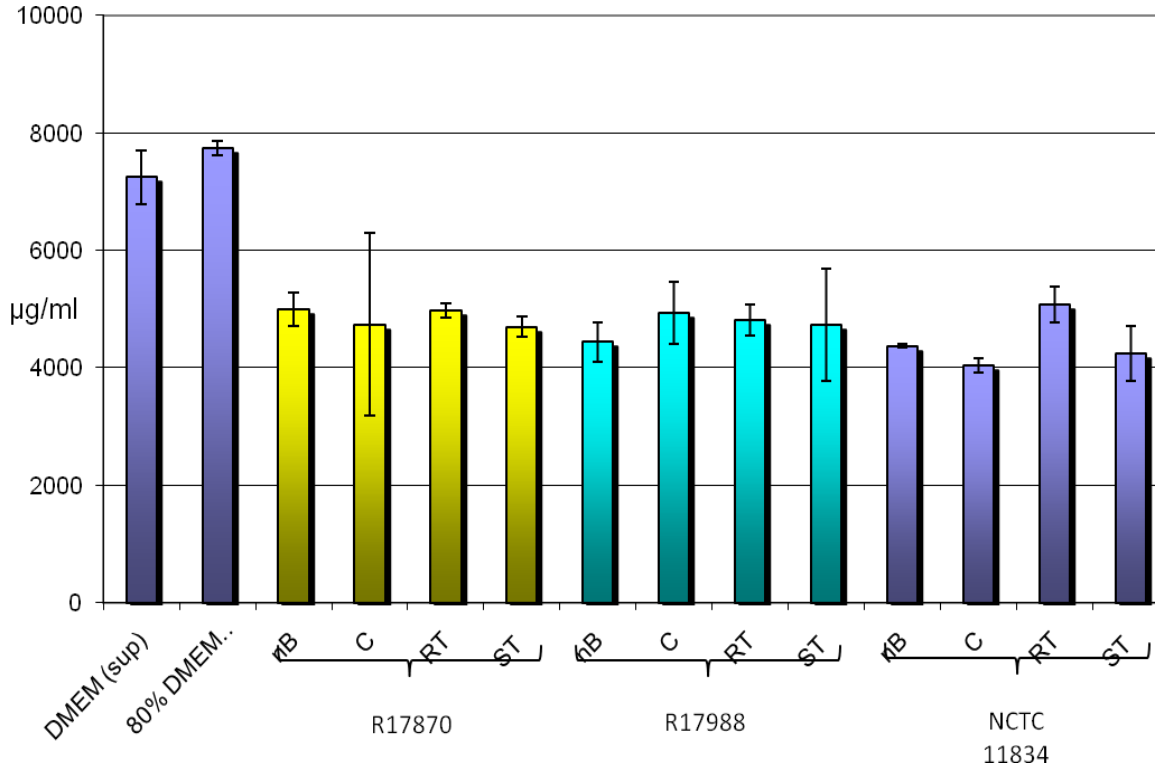


Fig 3.5 Growth Curve of *P.gingivalis* R17870 grown in different conditions

3.2.1 BCA Protein Assay

The results of the BCA Protein Assay are summarised in Figure 3.6. From these it can be assumed that, although minor differences in the extracellular proteins produced can be identified between the different conditions of growth, overall these differences appear to be minor. Therefore the amount of protein produced as extracellular matrix and consumed

by the bacteria for intracellular protein production and for their metabolism may be postulated to not differ significantly between the different strains and growth conditions, although through this method the whole protein is measured and it is not known how

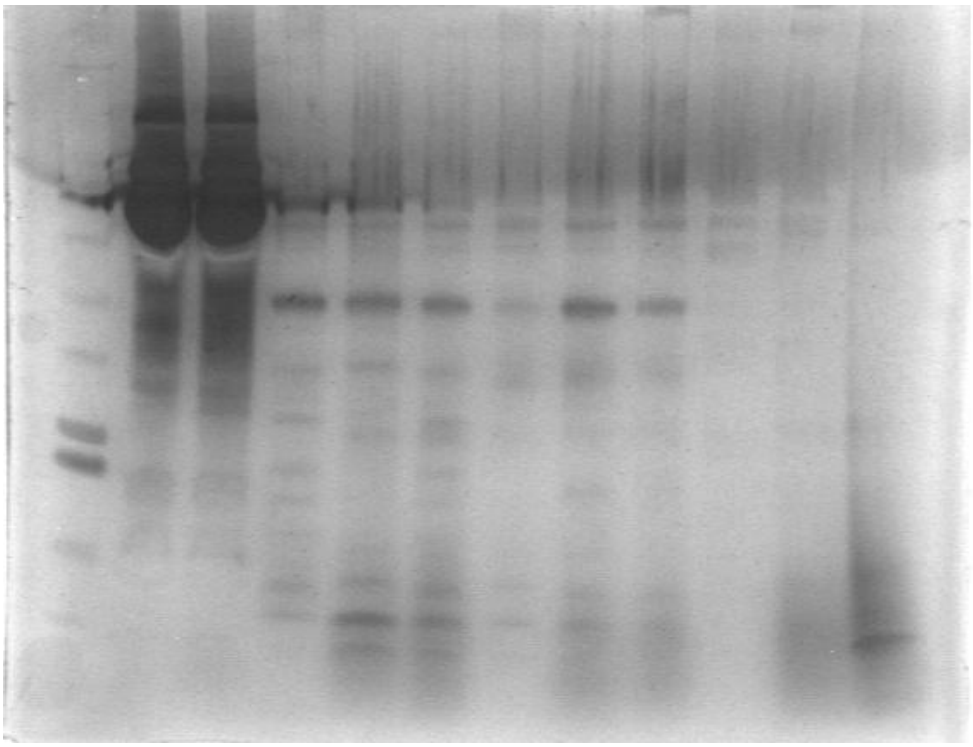


much unused protein (provided for the growth of the bacteria) may remain.

Fig. 3.6 BCA protein assay diagram showing approximate protein concentration in µg/ml of the supernatant of *P.gingivalis* various strains (R17870, R17988, NCTC 11834) and conditions of growth (nB: DMEM only, C: 80% DMEM + 20 % FAB (Broth), RT : 80% DMEM + 20 % FAB (Broth) in the presence of moderately roughened Ti, ST : 80% DMEM + 20 % FAB (Broth) in the presence of smooth Ti) and comparison to the initial protein content of the growth liquid media (DMEM or 80% DMEM + 20 % FAB (Broth)).

3.2.2 SDS-PAGE protein assay

The SDS-PAGE protein assay allows visulaization of the proteins produced by *P.gingivalis* when grown in the experimental conditions. The banding seen in the gel represents proteins with different molecular weights. Overall, it can be observed that in the two control samples there was an higher concentration of protein, as demonstrated in the BCA assay. Furthermore, the control samples (growth liquid media) appeared to differ significantly in the type of banding they produced when compared to the experimental supernatants, suggesting that the protein content of the media had mostly been metabolised by the bacteria. The banding identified from the different experimental supernatants appeared to represent expressed proteins of similar molecular weights, with only minor differences, as can been seen in Figure (3.7). It has been previously reported that *P.gingivalis* expression can change depending on the conditions of growth and on which media supplements are available for metabolism. For example, hemin-rich or hemin-deplete conditions has been seen to affect the proteins expressed by *P. gingivalis* (Daspher et al, 2009).



Indicator	DMEM onlly	80% DMEM +20% FAB (BOTH)	17988 DMEM	17988 BROTH	17988 mr- Ti BROTH	17870 DMEM	17870 BROTH	17870 mr-Ti BROTH	11834 DMEM	11834 BROTH	11834 mr-Ti BROTH

Fig 3.7 SDS-Page showing the protein banding found within the supernatants produced by the three strains of *P. gingivalis* grown in different conditions

The 50 kda mark is marked on the gel as it appears to have increased protein expression for the two strains R17988 and R17870.

3.2.3 Adherence of *P.gingivalis* to Titanium disks after 48 hours of incubation.

Both roughened and smooth titanium discs were stained with a dead-live stain as described in the Materials and Methods chapter to assess the adherence of *P.gingivalis* to the discs. Bacteria appeared to adhere to both roughened and smooth surfaces for the different strains as visual examination of the digital photos reveal.

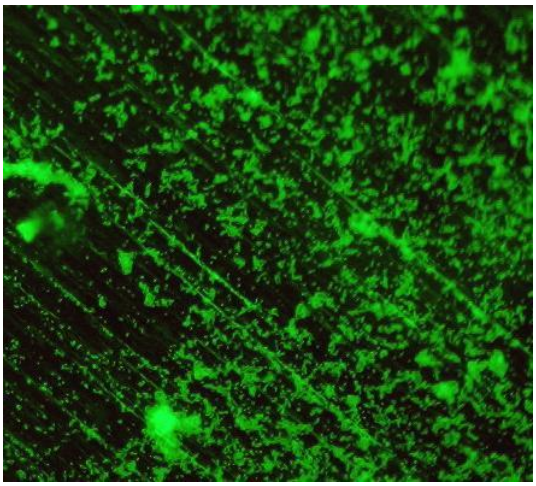


Fig. 3.8 Adherence of P.gingivalis onto smoothened titanium discs after 48h incubation (Green : live bacteria observed on the disc following washing out)

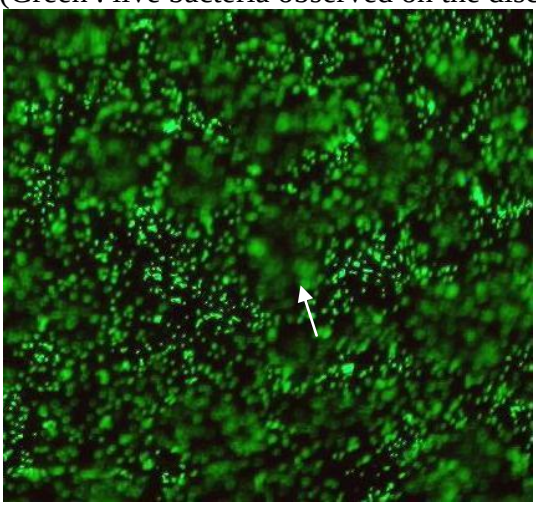


Fig 3.9 Adherence of P.gingivalis onto moderately roughened titanium discs after 48h incubation (Green: live bacteria found on the disc following washing out, White arrow: out of focus area possibly due to the roughness of the disc)

Chapter 4

Results of the Exposure of the mandible slices to the *P.gingivalis* Supernatant

4.1 Seven-day exposure of mandible slices to the *P.gingivalis* supernatant of 50μg/ml concentration

4.1.1 Seven-day exposure of mandible slices to the *P.gingivalis* NCTC 11834 supernatant of 50μg/ml concentration

After seven days of culture of the mandible slices in 50μg/ml of *P.gingivalis* NCTC 11834 supernatant cultured either in a planktonic fashion or in the presence of moderately roughened Ti or smoothened Ti (conditions), slices appeared to have maintained their general tissue morphology and similar appearances were observed for all 3 conditions. The general architecture of the tissues is maintained in comparison with the controls, however some of the characteristics in the microscopic level were different (Fig. 4.1). There was considerable reduction in the number of cells with distinct nuclei in the periodontal ligament. The periodontal ligament fibers appeared to be more randomly orientated and the matrix slightly thinner in comparison to the control. In the lacunae pattern at the edges of the periodontal ligament, osteoclast-resembling cells were not

easily identifiable, but this was similar to the controls. The bone marrow also appeared to have a reduction in cell number and the matrix in the bone marrow cavities was less organised. The osteocytes within the cortical bone did not appear to be considerably affected by the exposure to the supernatant at this concentration. The pulp tissue generally retained most of the architecture in comparison to the controls.

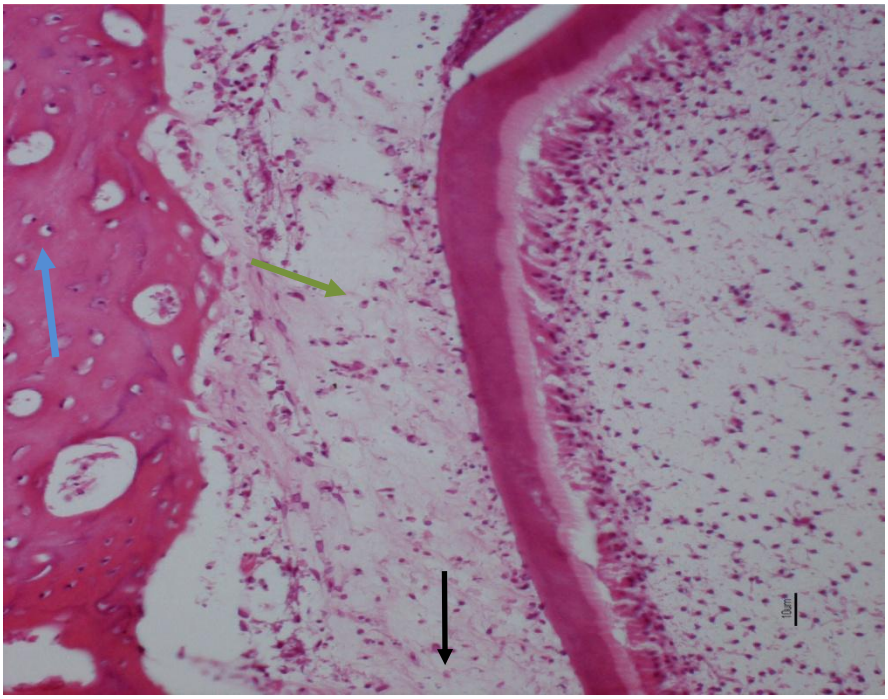


Fig 4.1 Mandible slice exposed to the supernatant of *P.gingivalis* NCTC 11834 (planktonic) at a concentration of 50µg/ml.

Osteocyte (Blue arrow), Anucleated cell (black arrow) Enucleated cell (green arrow)

The average cell counts in the periodontal ligament (PDL) and the pulp of the mandible slices exposed to the planktonically grown supernatant of the NCTC 11834 *P. gingivalis* were 48.8 and 75.5 per 100 μm^2 respectively. In the presence of moderately roughened

Ti. cell counts from the PDL and pulp were 42.8 and 71.8 and in the presence of smoothened Ti were 47.3 and 71.1 (Figures 4.2 and 4.3). A one-way ANOVA demonstrated a statistically significant difference between the controls and the slices exposed to the supernatant ($p<0.001$). A statistically significant difference in the PDL counts between the cultures exposed to the planktonically grown bacterial supernatant and the supernatant grown in the presence of moderately roughened Ti was also observed ($p<0,05$).

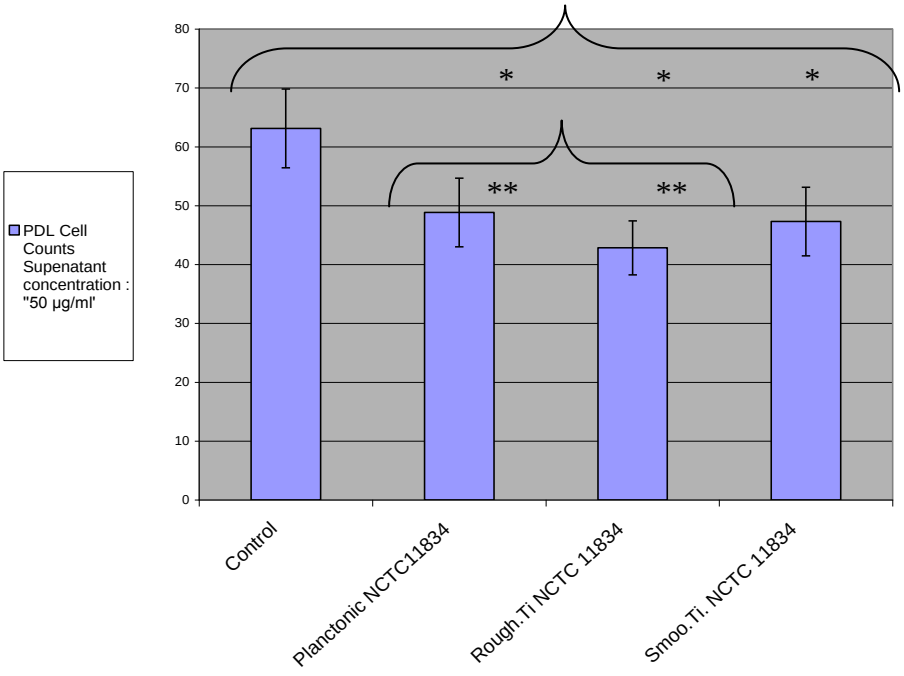


Fig 4.2 Average cell counts from the Periodontal ligament (PDL) of mandible slices exposed to a concentration of 50µg/ml of the supernatant of the *P.gingivalis* NCTC 11834 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$, (**) $p<0.05$

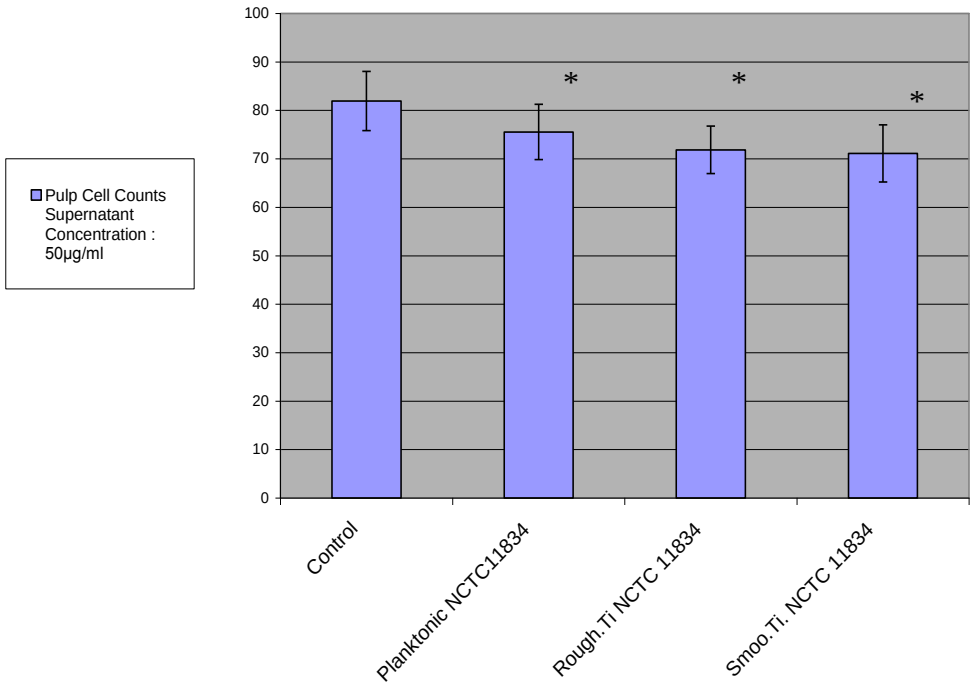


Fig 4.3 Average cell counts from the pulp of mandible slices exposed to a concentration of 50µg/ml of the supernatant of the *P.gingivalis* NCTC 11834 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

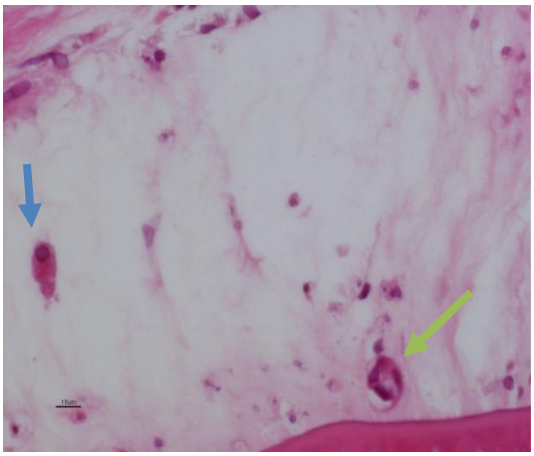


Fig. 4.3b Enlarged cell (blue arrow) and multinucleotic cell (green arrow)

An interesting observation was the presence of enlarged cells as well as multinucleotic-giant cells within the PDL (figure 4.3b). These were not identified in each and every slice observed, however the multinucleotic cells were routinely identified in mandible slices exposed to supernatants of all 3 conditions.

4.1.2 Seven-day exposure of mandible slices to the *P.gingivalis* R17988 supernatant of 50μg/ml concentration

The microscopic picture for the slices exposed to the supernatant of the *P.gingivalis* R17988 strain (cultured in the same 3 different conditions-planktonically, or in the presence of moderately roughened or smoothened Ti-) at the concentration of 50μg/ml shared common features with the one for the NCTC 11834 strain. The general architecture of the tissues was the same as in the controls, however there was similar reduction in the cells identified within the periodontal ligament and the bone marrow. The fibers and the matrix within the periodontal ligament appeared more disorganised in comparison to the controls. Enlarged cells with enlarged nuclei and multinucleotic cells were also identified in some of the slices within the periodontal ligament.

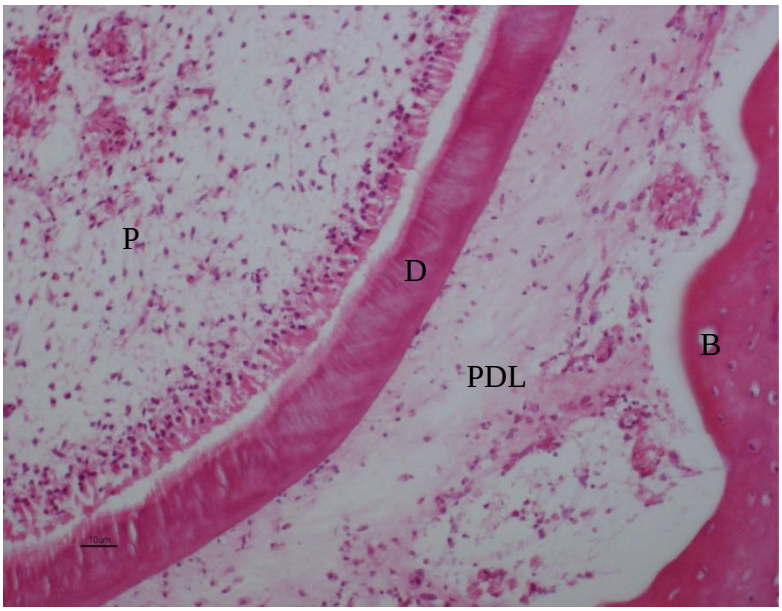


Fig 4.4 Mandible slice exposed to the supernatant of *P.gingivalis* R17988 (mod.rough.Ti) at the concentration of 50µg/ml. In this slide it is quite obvious the reduction in cells in the PDL (P: Pulp, D: Dentine, B: Bone, PDL: Periodontal ligament)

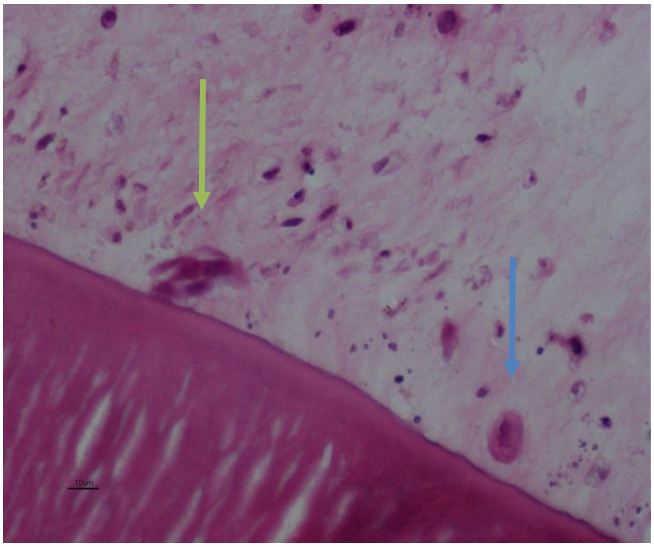


Fig 4.5 Detail showing enlarged (blue arrow) and multinucleotic cell (green arrow)

The average cell counts for the slices exposed to the supernatant of this strain according to whether they were exposed to moderately roughened or smoothened Ti or not was as following. The average cell counts in the periodontal ligament and the pulp of the mandible slices exposed to the planktonically grown supernatant of the R17988 strain were 44.2 and 68.3 respectively, to the ones grown in the presence of moderately roughened Ti were 45.4 and 70.3 and to the same strain grown in the presence of smoothened Ti were 45.7 and 72.1 (Figures 4.6 and 4.7). There was a statistically significant difference between the controls and the ones exposed to the supernatant of any of the 3 conditions ($p<0.001$).

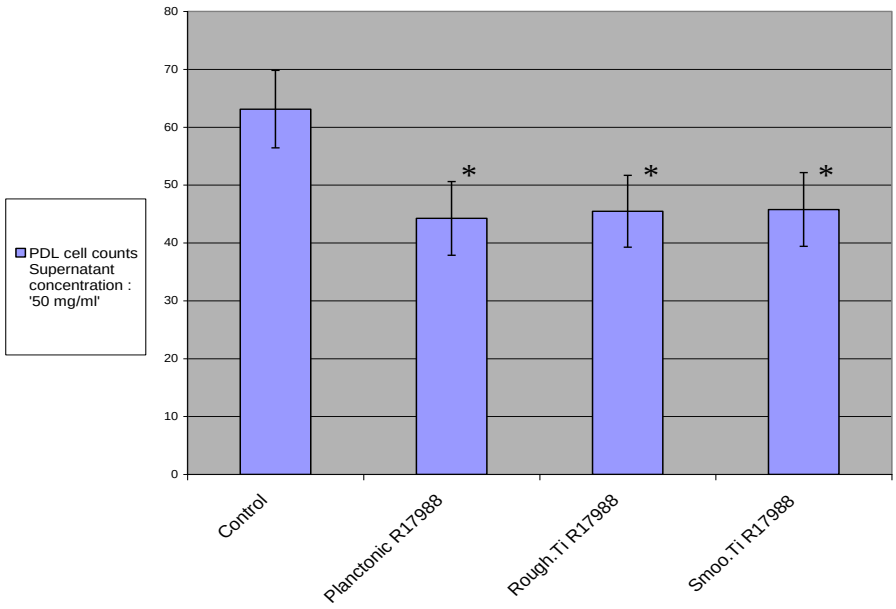


Fig 4.6 Average cell counts from the Periodontal ligament (PDL) of mandible slices exposed to a concentration of 50µg/ml of the supernatant of the *P.gingivalis* R17988 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

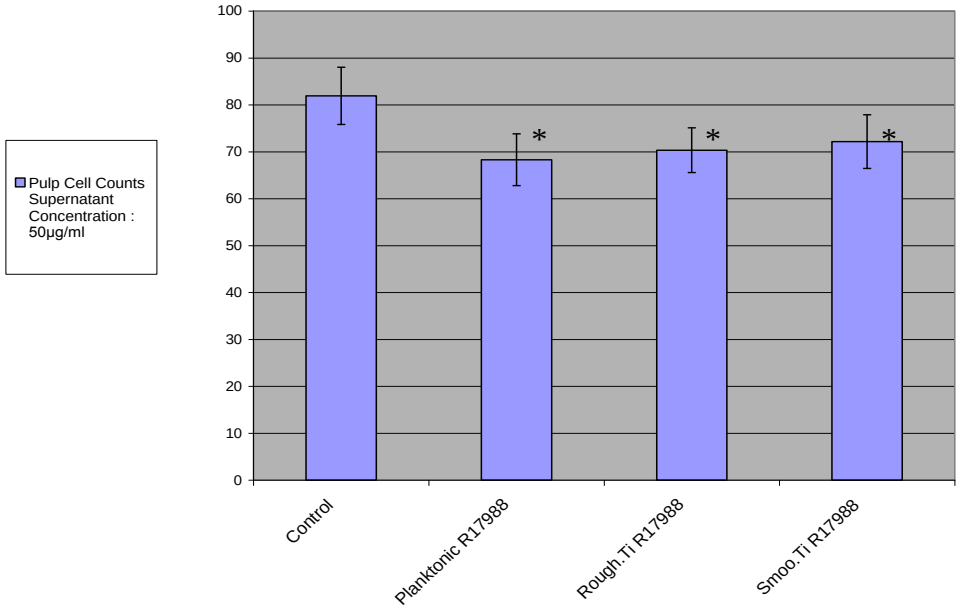


Fig 4.7 Average cell counts from the pulp of mandible slices exposed to a concentration of 50µg/ml of the supernatant of the *P.gingivalis* R17988 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti)
(*) $p<0.001$

4.1.3 Seven-day exposure of mandible slices to the *P.gingivalis* R17870 supernatant of 50μg/ml concentration

The same procedure was followed with the supernatant of the *P.gingivalis* R17870 strain grown in 3 different conditions. The microscopic picture obtained by observing slides from the mandible slices was similar to the other two strains and different to the controls. The architecture of the hard tissues was the same as in the controls, however the periodontal ligament had less organised fibers and matrix and fewer cells with distinct nuclei could be identified. The pulp tissue was relatively intact and the bone marrow seems to have lost cells and its organisation. Enlarged cells with enlarged nuclei were also found in these slices. Multinucleotic cells were also identified, however appearing less often than in the other strains, but this cannot be deemed as accurate as the multinucleotic cells were not identifiable in each and every slide produced and could be only a random observation.

The average cell counts for the slices incubated with the addition of the supernatant of *P.gingivalis* R17870 strain were as following. For the slices grown planktonically the counts for the periodontal ligament and the pulp were 47.2 and 70.8 respectively. For the slices that were cultured in the presence of supernatant of the R17870 strain grown in the presence of moderately roughened Ti the counts were 48.6 for the periodontal ligament and 72.5 for the pulp, and for the slices in the presence of smoothened Ti the results were 49.2 and 67.2 respectively (Fig. 4.10 and 4.11). There was a statistically significant difference between the controls and the experimental results for the R17870 strain as well ($p<0.001$).

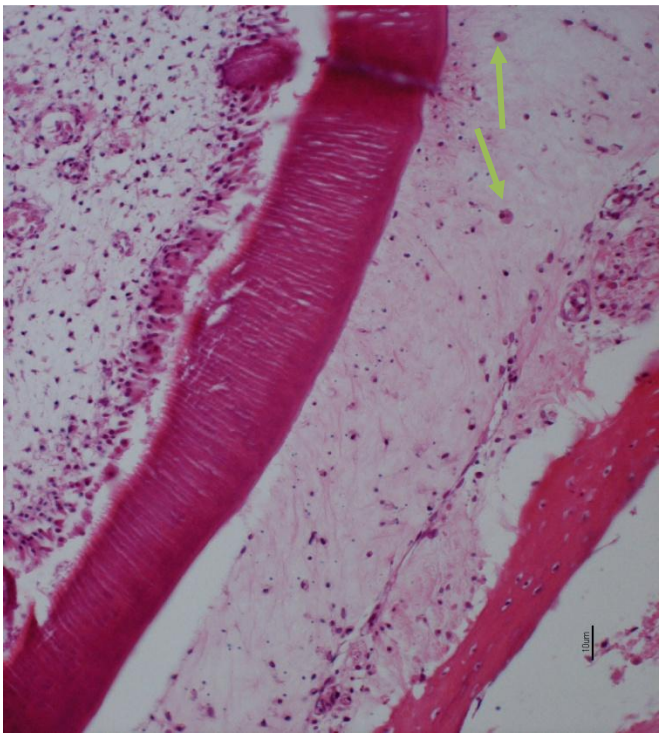


Fig 4.8 Mandible slice exposed to the supernatant of *P.gingivalis* R17870 (smoothened Ti) at the concentration of 50µg/ml (Green arrow: Enlarged cells)

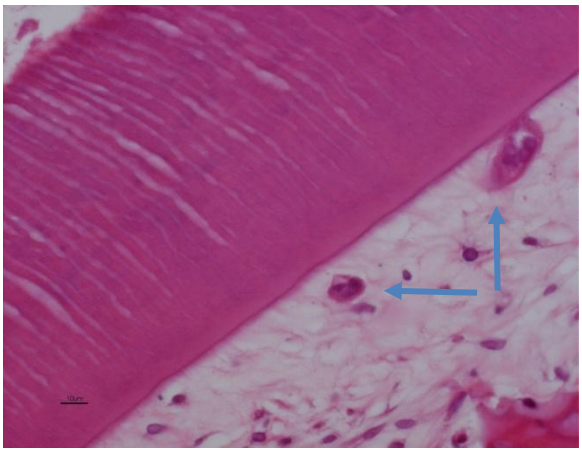


Fig 4.9 Multinucleotic cells in a slide exposed to the supernatant of the R17870 strain (Blue arrows)

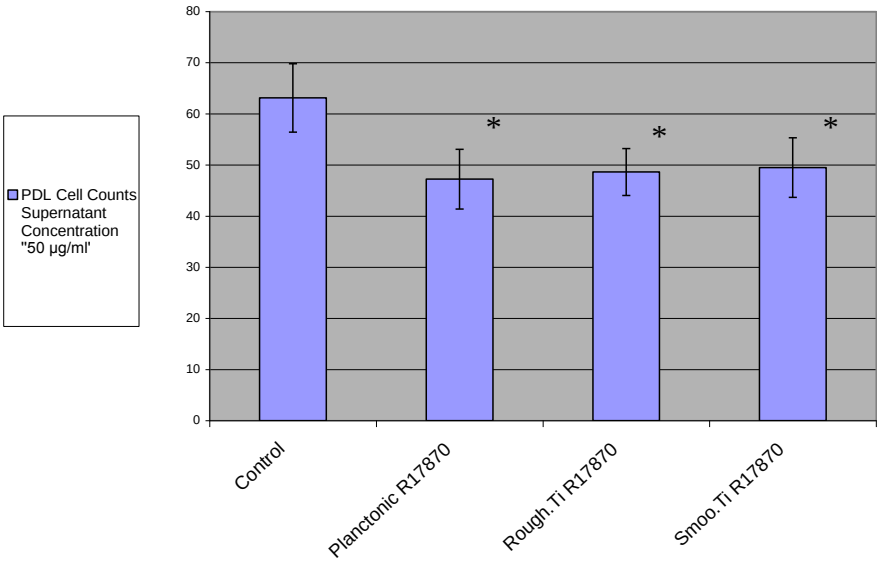


Fig 4.10 Average cell counts from the Periodontal ligament (PDL) of mandible slices exposed to a concentration of 50µg/ml of the supernatant of the *P.gingivalis* R17870 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

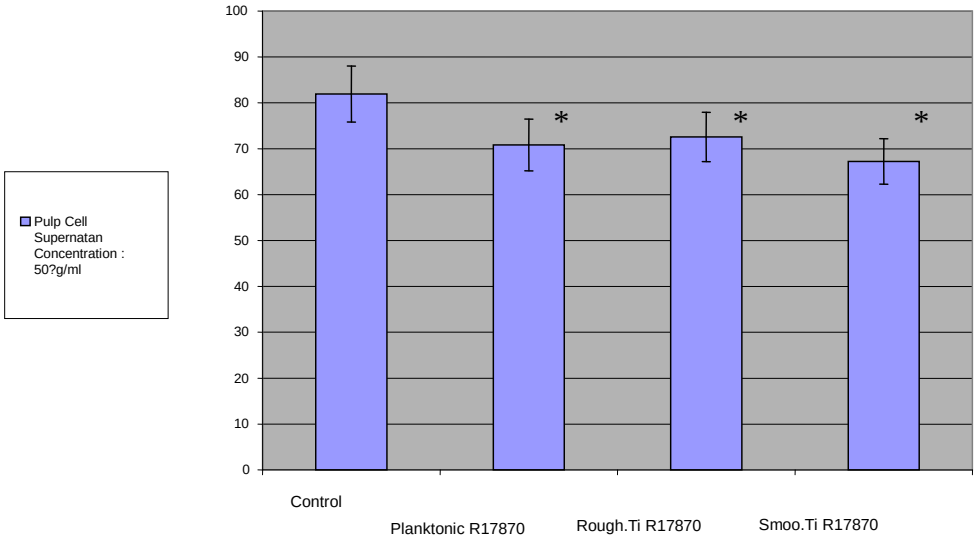


Fig 4.11 Average cell counts from the pulp of mandible slices exposed to a concentration of 50µg/ml of the supernatant of the *P.gingivalis* R17870 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti)) (*) $p<0.001$

4.1.4 Comparison of the effect to the mandible slices by the supernatant of the three *P.gingivalis* strains (grown in different conditions) at the concentration of 50µg/ml

The one-way ANOVA test did not demonstrate any statistically significant differences between the three strains. The histological picture of the mandible slices also appeared quite comparable between the three different strains without any exceptional differences.

4.2 Seven day exposure of mandible slices to the *P.gingivalis* supernatant of concentration of 100 µg/ml

4.2.1 Seven day exposure of mandible slices to the *P.gingivalis* NCTC 11834 supernatant of concentration of 100 µg/ml

Mandible slices were cultured in the same conditions as previously described; however the concentration of the supernatant was increased to 100 µg/ml. In these cultures, the tissue architecture and morphology was markedly different. The general architecture of the hard tissues did not appear to be altered however; the soft connective tissues appeared to be less cellular, although some cells with distinct nuclei were still identifiable in the PDL, pulp and bone marrow spaces. The PDL was observed to be disorganised with respect to tissue structure, with significant lack of organised fibers and matrix. Here, enlarged or multinucleotic cells were not commonly identifiable as previously observed in the concentration of 50 µg/ml. The bone marrow tissue also appeared severely affected by the supernatant with similar changes to the matrix as seen in the PDL, and the cells in

the bone marrow spaces appeared fewer in number and fewer cells had distinct nuclei, suggesting evidence of cell death. Osteocyte cells in the lacunae were often missing. The pulp tissue appeared to be less severely affected however, an altered architecture to the pulpal matrix was observed and cell numbers appeared reduced, with an increase in poorly stained cells lacking nucleus. This picture was similar in all slices irrespective of the conditions in which the *P.gingivalis* NCTC 11834 was grown into (planktonically or exposed to Ti –moderately roughened or smooth).

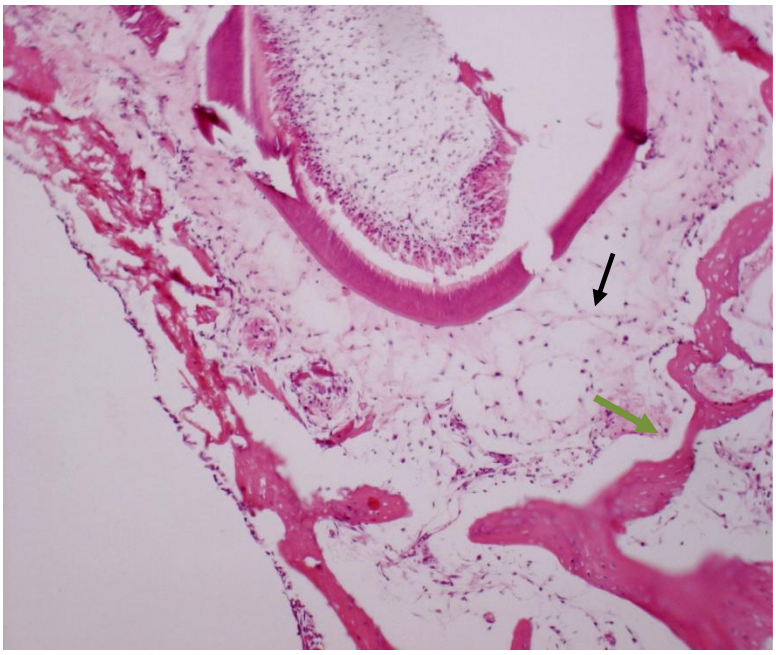


Fig 4.12 Slide stained with H&E from mandible slice exposed to the supernatant of *P.gingivalis* NCTC 11834 at the concentration of 100µg/ml. Tissue appeared more fragile and tended to tear whilst processing (Green arrow: Lacuna without osteocyte, Black Arrow: disorganised PDL matrix)

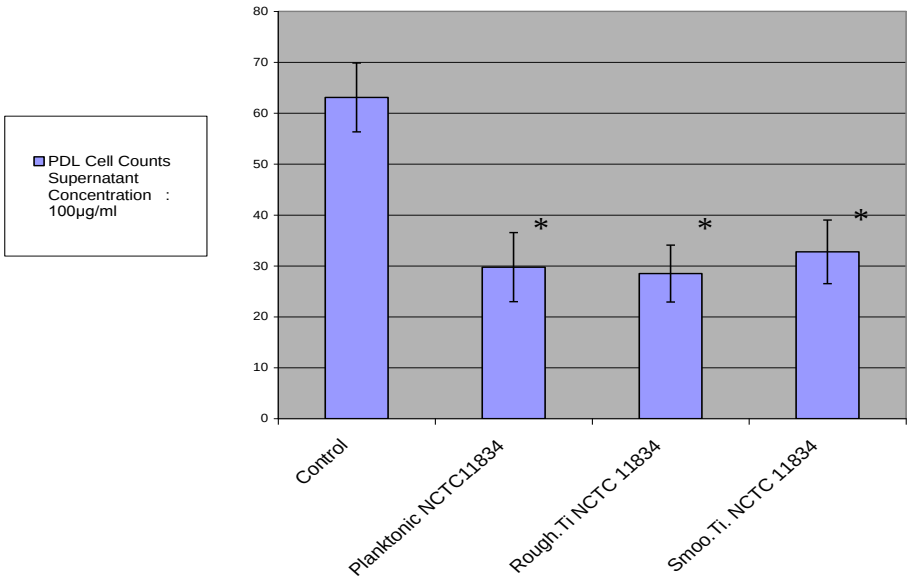


Fig 4.13 Average cell counts from the Periodontal ligament (PDL) of mandible slices exposed to a concentration of 100µg/ml of the supernatant of the *P.gingivalis* NCTC 11834 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

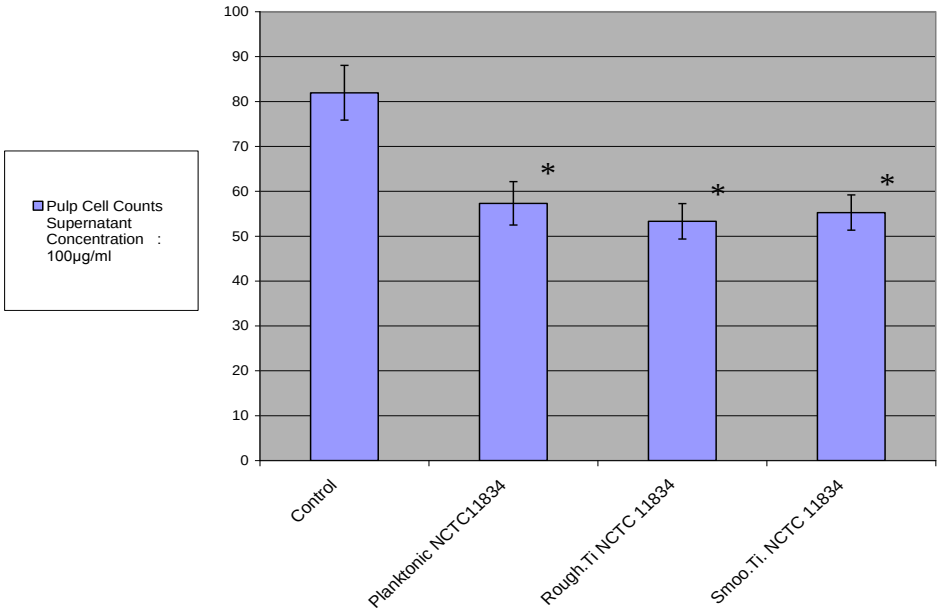


Fig 4.14 Average cell counts from the pulp of mandible slices exposed to a concentration of 100µg/ml of the supernatant of the *P.gingivalis* NCTC 11834 strain grown in the 3 different conditions (planktonik or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

The average cell counts in the PDL and the pulp for the slices exposed to the planktonically grown *P.gingivalis* NCTC 11834 were 29.7 and 57.3 respectively. Cell counts for those slices exposed to the NCTC 11834 grown in the presence of moderately roughened Ti were 28.5 and 53.3 and those of smooth Ti were 32.7 and 55.2 (fig. 4.13-4). Statistically significant differences between controls and the experimental slices of this strain were observed following one way ANOVA analysis ($p<0.001$).

4.2.2 Seven day exposure of mandible slices to the *P.gingivalis* R17988 supernatant of concentration of 100 μg/ml

The doubling of the concentration of the supernatant of the *P.gingivalis* R17988 strain had also quite a similar effect to the mandible slices cultured, as it was for the previous strain. The tissues were quite severely affected by the supernatant in this concentration as a considerable reduction in the amount of cells with distinct nuclei was observed and the matrix of the periodontal ligament and the bone marrow appeared to be generally rather obviously disorganised. Osteocytes in the lacuna were not consistently present as previously and the pulp tissue was also quite affected. Enlarged or multinucleotic cells were not found in the same form as in the lower concentrations. The effect observed was similar for all of the mandible slices exposed to the supernatants of the R17988 strain grown planktonically or in the presence of moderately roughened or smoothened Ti.

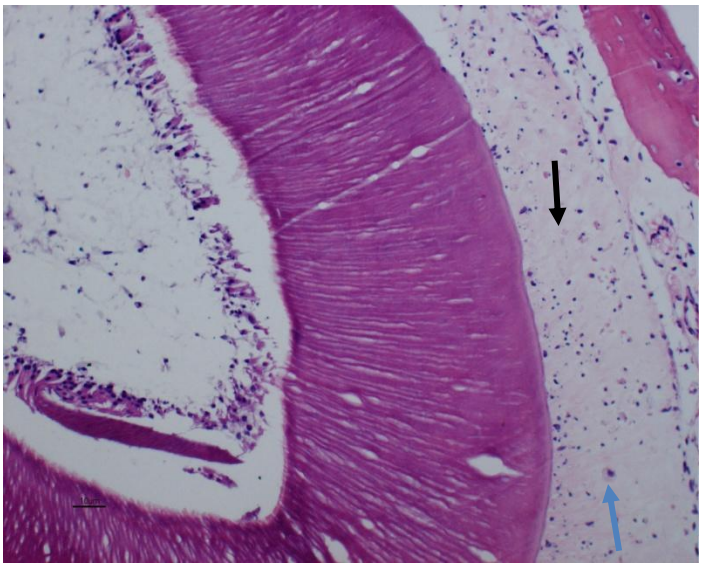


Fig 4.15 Mandible slice exposed to the supernatant of *P.gingivalis* R17988 at the concentration of 100μg/ml (Black arrow: Anucleated cell, Blue Arrow: Enlarged cell with disorganised cytoplasma)

The cell counts of the periodontal ligament and pulp of the slices exposed to a concentration of 100 μg/ml of the supernatant of the *P.gingivalis* R17988 grown planktonically were 27.5 and 52.4 respectively on average. The cells counts of those slices exposed to supernatant of the R17988 strain grown in the presence of moderately roughened Ti were 30.4 and 50.05, and the cell counts for those of the smoothened Ti were 54.1 and 32.1 (Fig. 4.16-4.17). As expected there was a statistically significant difference with the controls ($p<0.001$).

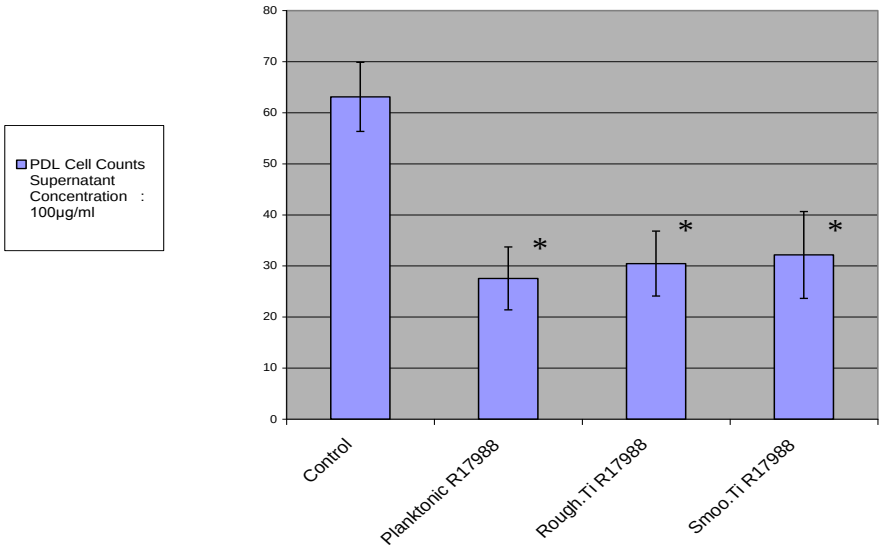


Fig 4.16 Average cell counts from the Periodontal ligament (PDL) of mandible slices exposed to a concentration of 100µg/ml of the supernatant of the *P.gingivalis* R17988 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

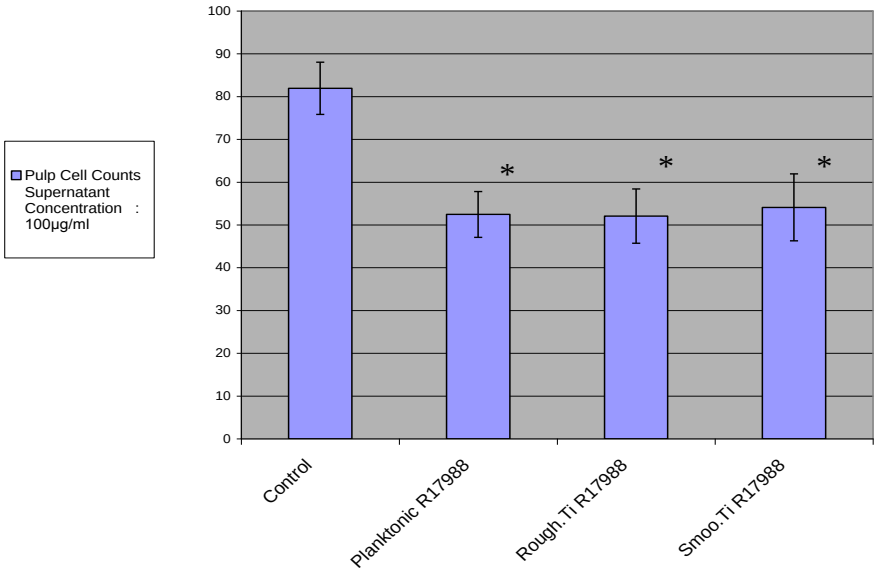


Fig 4.17 Average cell counts from the pulp of mandible slices exposed to a concentration of 100µg/ml of the supernatant of the *P.gingivalis* R17988 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

4.2.3 Seven day exposure of mandible slices to the *P.gingivalis* R17870 supernatant of concentration of 100 μg/ml

Culturing mandible slices with the addition of the supernatants of the *P.gingivalis* R17870 strain grown in the same 3 different conditions at a concentration of 100 μg/ml appeared to have a considerable effect in comparison to the controls. The periodontal ligament and bone marrow in the slices was more disorganized and with fewer cells with distinct nuclei in a similar way like for the other two strains. Enlarged or multinucleotic cells could not be identified and the pulp tissue was not as organised as in the control slides.

The average cell counts for those slices exposed to the supernatant of the R17870 strain grown in a plaktonic fashion were 31.8 (PDL) and 53.9 (pulp) respectively. For the slices exposed to supernatant of the moderately roughened Ti, the cells counts were 30.4 (PDL) and 56.7 (pulp), while the cells counts for the slices exposed to supernatant of the smoothened Ti were 33.05 (PDL) and 55.1 (pulp) (Fig 4.19-4.20). In this case there was also significant difference to the controls.

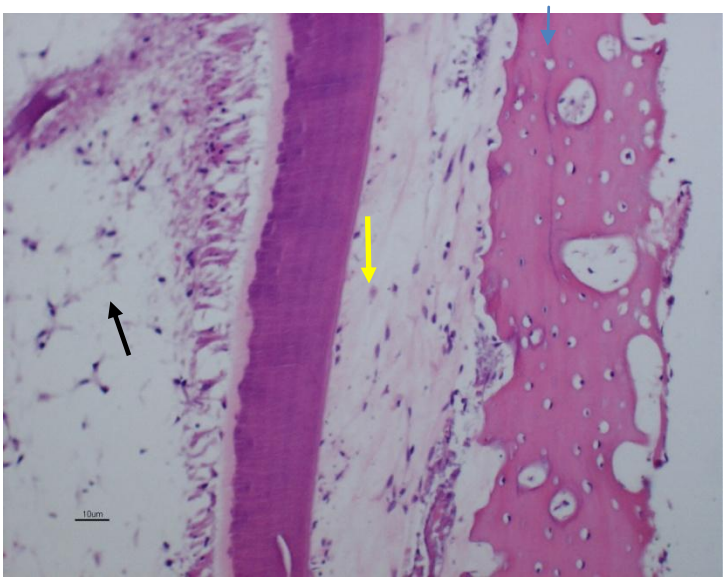


Fig 4.18 Slide stained with H&E from mandible slice exposed to the supernatant of *P.gingivalis* R17870 at the concentration of 100µg/ml (Black Arrow: disorganised pulp matrix, Yellow Arrow: Anucleated cell, Blue Arrow: Lacuna without osteocyte)

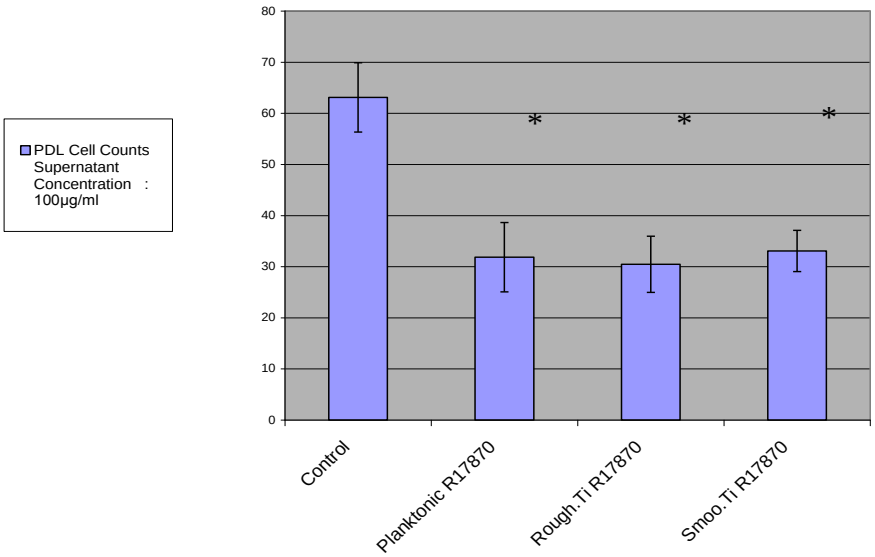


Fig 4.19 Average cell counts from the Periodontal ligament (PDL) of mandible slices exposed to a concentration of 100µg/ml of the supernatant of the *P.gingivalis* R17870 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

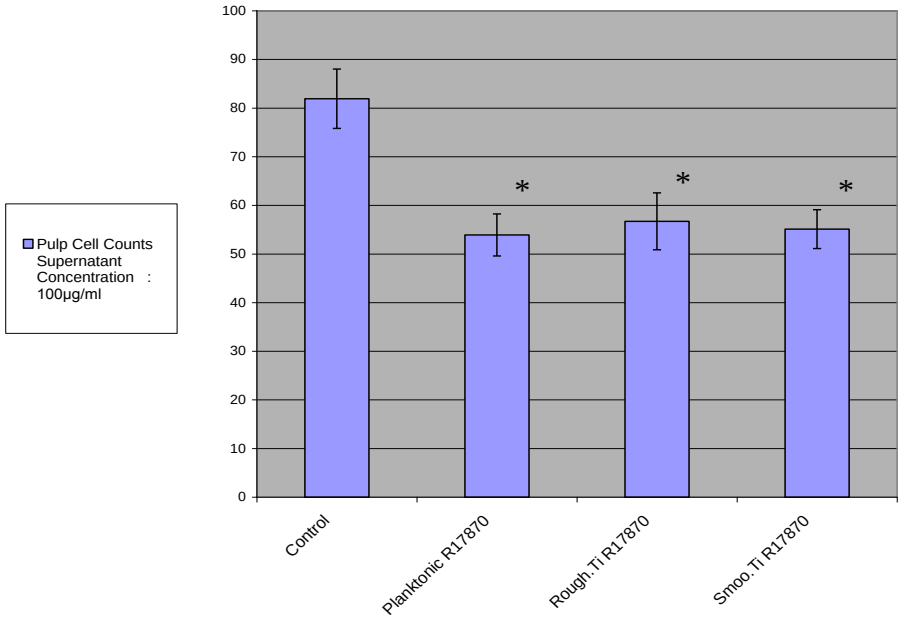


Fig 4.20 Average cell counts from the pulp of mandible slices exposed to a concentration of 100µg/ml of the supernatant of the *P.gingivalis* R17870 strain grown in the 3 different conditions (planktonic or exposed to moderately roughened or smoothened Ti) (*) $p<0.001$

4.2.4 Comparison of the effect to the mandible slices by the supernatant of the three *P.gingivalis* strains (grown in different conditions) at the concentration of 100µg/ml

The one-way ANOVA test did not demonstrate any statistically significant differences among the three strains. The general histological picture was in general similar, with similar features.

4.3 Three-day exposure of the cultured mandible slices to the *P.gingivalis* supernatant

In order to better understand if changes to the mandible tissues exposed to supernatants were time as well as dose dependent, mandible slices exposed to supernatants were removed from culture at the three-day point and compared with three-day cultured controls. After three days in culture, slices exposed to supernatants appeared to show signs of altered tissue structure. Fewer PDL cells were observed when compared with control cultures although the general tissue architecture was generally unaltered and was more similar to that in control cultures than to the experimental of 7 days, however some early signs of disorganisation was obvious. Some enlarged cells could be identified. The bone marrow was mostly intact and osteocytes were observed in the compact bone. The pulp tissue was mostly unaffected on this three-day culture point (Fig 4.21). In general, all tissues of the mandible slice exposed to supernatants, appeared healthier with respect to cell and tissue architecture when compared with 7 day cultures. The observations were consistently similar irrespective of the supernatant belonging to any of the three strains or the condition in which they were grown.

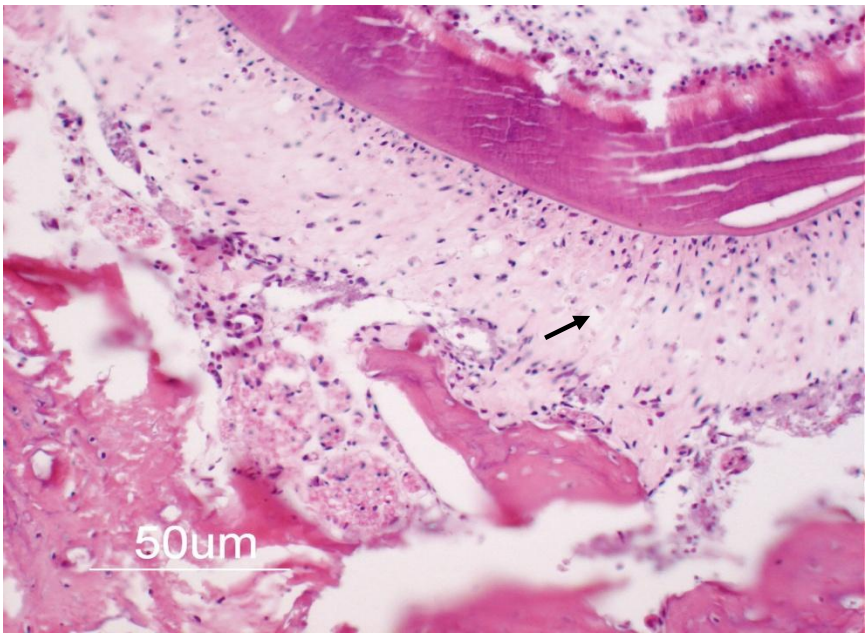


Fig 4.21 Image of mandible slice exposed for 3 days (R17870 strain) (Black arrow: anucleated cell)

4.4 Mandible Slices stained with TRAP stain

Some mandible slices were stained with the TRAP stain, as described in the materials and method chapter, in order to investigate whether any of the cells identified in the histomorphological analysis were expressing the Tartrate-resistant acid phosphatase (TRAP) histochemical marker, commonly expressed in cells of osteoclast/macrophage lineage. Mandible slices that were either cultured as controls or exposed to the supernatant of the *P.gingivalis* were stained. Positive TRAP staining was observed in enlarged cells and multi-nucleated cells which had been observed in previous H&E analysis. The cells identified were all within the periodontal ligament, and not exclusively in bone lacunae (Fig. 4.22-4.23). In the control cultures, such multi-

nucleated cells were found specifically within a lacuna at the edge of the ligament (Fig 4.24)

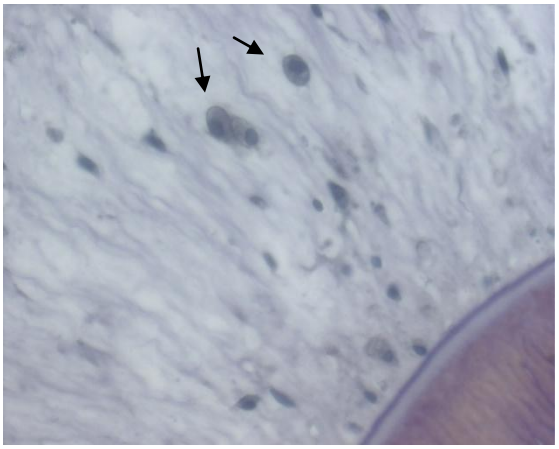


Fig 4.22 Enlarged Cells stained positive with TRAP stain (arrows)

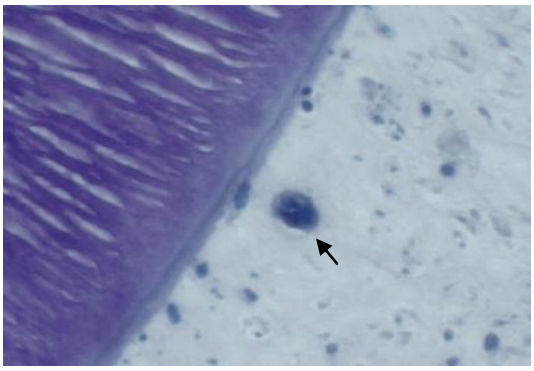


Fig 4.23 Multinucleotic Cell Stained with TRAP stain (arrow)

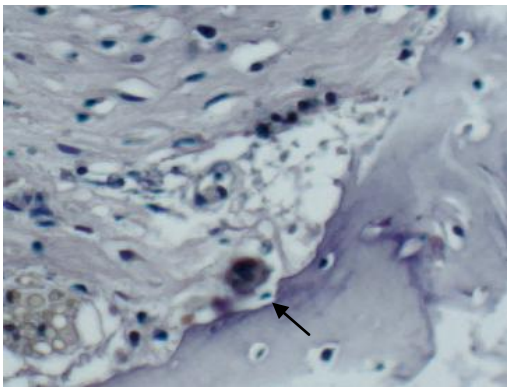


Fig 4.24 Positive Control of TRAP stain – multinucleotic cell (arrow) (3-day control)

Chapter 5

Discussion

5.1 Choice of Model

The model used for this study was the *ex vivo* mandible slice model described by Smith et al (2010). As an *ex vivo* tissue culture model it offers a lot of advantages but also has limitations. In comparison with monolayer tissue culture models, an organotypic culture model offers a 3D scaffold that represents what occurs *in vivo* in a more pragmatic way as it contains all the intricate architecture and variety of cells, along with their associated signalling, components and interactions. It offers the possibility to replicate a variety of parameters more consistently and has the benefits of *in vitro* experimentation; for example, it allows for direct application of agents and creation of environments that would be almost impossible to achieve *in vivo*. Several organ culture systems have been developed from skin, lung, prostate, salivary glands (Trowell, 1959), tumors, thymus (Moore et al., 1994), spleen (von Melchner, 1983) and others. Although these models offer, without parallel, opportunities for research there are certain limitations which can be difficult to overcome that also apply to this mandible slice model. There are limitations to the availability and viability of the tissue in culture. Also, there is large variability in the origin of the tissue and a lot of parameters cannot be controlled. There is no integration of the nervous and vascular systems and, as a result, the response of the tissues to experimental conditions is limited (Anderson, 1998). It allows, however, the

investigation of the direct response of the cells and the tissues without complex systemic influences.

In order to be able to interpret the results from the experiments it is very important to take into consideration the features of the mandible slice culture model from the 28-day male wistar rats. This is a murine model from still-growing rats which focuses on the apparatus surrounding a rodent incisor that is a continuously growing and erupting tooth. The osteogenic activity and the modelling and remodelling activity of the alveolar bone are increased at this stage as a result (Merzel and Salmon, 2008).

This model has been previously used to investigate dentine repair processes (Sloan and Smith 1999; Murray et al., 2000; Gonçalves et al., 2007), the effect of orthodontic forces to the dentine-pulp complex (Dhopatkar et al., 2005), the effect of growth factors on fracture-repair processes (Smith, 2009). Based on the results of Smith (2009) there were no significant differences between base type (no gel used) and trowel type cultures (mandible slices in gel). In this experiment base type model was preferred as the dispersal of the added ingredients would be more even.

The cellular viability in an explant model is expected to decline over time as a result of the conditions in which it is preserved. It has been proposed that this is associated with an increase in the levels of reactive oxygen species (ROS), especially during stress conditions (Stowe and Camara, 2009). It is also important to note that although this is a model that uses mandibles from young rats where the osteoclastic activity is increased,

confirmed by an increased presence of lacunae in the histological slices, osteoclasts could be scarcely identified and mostly in 3-day control slices. This can be explained by the fact that osteoclasts require the presence of osteoblasts and special treatment to be generated *in vitro* in co-culture models with the addition of different agents like vitamin D3 (Takajashi et al, 1998), parathyroid hormone (Yamashita et al, 1990), prostaglandin (Akatsu et al, 1991) or interleukins (Romas et al, 1996). However, there is a report where an osteoclast cell culture system was developed by using cells from minced mandibular bodies and erupting teeth of 9-11 day old mice without the addition of any of the previous growth factors, cytokines or hormones (Suda et al, 2003).

Additionally, for this study, the original model was not used as a control, but rather a model in which an amount of the bacterial growth media in concentration equal to the experimental conditions had been added. This was done in order to exclude this additional media as the reason for the effects seen. This had an additional effect to the cellular viability within the culture as noted in the results, comparing the initial model with the model used for this study. It was identified though that most of the structure has not been notably influenced in the 7 days of test period.

5.2 Porphyromonas gingivalis Growth

Porphyromonas gingivalis is a bacteria identified in areas of active periodontal and peri-implant disease. (Heitz-Mayfield and Lang, 2010; Shilbi et al, 2011) It is a versatile

bacterium, but its growth and virulence can be influenced by many factors. In order to simulate and investigate the activity of the extracellular products of *P. gingivalis*, it was necessary that the conditions in which *P. gingivalis* grew were as favourable as possible. The expression of virulence factors has been shown to be dependent on the environment in which *P. gingivalis* is growing (Masuda et al., 2006). The addition of essential growth factors in the form of hemin, cysteine and Vitamin K was therefore necessary for the optimal growth of *P. gingivalis*. In particular, the presence of heme has been proven important to the way *P. gingivalis* grows (Olczak et al., 2005) and different behaviour has been demonstrated in heme-deplete situations (Genco, 1995) as this leads to different patterns of gene expression and adaptation to the environment. *P. gingivalis* growth is dependent on Vitamin K (Wyss, 1992) and cysteine is an important growth factor for all anaerobes, necessary for its metabolism and for the production of cysteine proteinases.

From the growth curves, it was shown that *P. gingivalis* grew best in a combination of nutrients from Fastidious Anaerobe Broth (FAB) and Dulbecco's Modified Eagle Medium (DMEM) rather on DMEM alone. It has been shown previously that several strains of *P. gingivalis* are fastidious and require a protein or serum supplement for better growth (Wyss, 1992). *P. gingivalis* is asaccharolytic and produces aminopeptidases in order to cleave peptides into short-chain polypeptides, which are the most efficient energy sources for these bacteria (Oda et al., 2009).

The presence of titanium (smoothened or moderately roughened) during the culture of *P. gingivalis* did not seem to actively influence its growth although it could be argued that

it altered the environment in which *P. gingivalis* was incubated. This is in line with previous research by Gao et al., (2002) which had identified no statistically significant influence on the growth of subgingival bacteria, including *P. gingivalis*, or on the pH value of their culture environment, when grown in the presence of pure titanium (TA-2) or titanium alloy Ti-6Al-4V for 2, 7 or 14 days. However, an *in vitro* study by Bunetel et al (2001), using *P. gingivalis* in their exponential growth phase and cultured on Todd-Hewitt broth enriched with hemin and Vitamin K1, found that the presence of titanium enhanced bacterial growth ($p<0.05$), although it was found in the same study that titanium enhanced the antibiotic activity of metronizadole against *P. gingivalis*. These different findings may be attributed to the different growth phase, as well as the use of different broth as a growth media.

5.3 Porphyromonas gingivalis supernatant

The *P. gingivalis* supernatant used in the tissue culture experiment was the crude extract obtained after removing all the bacteria from the culture medium. As a result it was expected to contain the extracellular produce of *P. gingivalis*, which would be excreted proteins, released fimbriae and other molecules, extracellular membrane vesicles, by-products of *P. gingivalis* metabolism, and, to a lesser extent, lysis products from the intracellular or membrane environment from dead *P. gingivalis*. As the supernatant was obtained from near the beginning of the stationary phase of growth of the bacteria, the lysis products would be expected to be few and the supernatant expected to contain a lot of the major virulence factors of *P. gingivalis*. The BCA protein assay demonstrated that

P. gingivalis comparably reduced the protein content of the culture media, as expected for its intracellular growth, in line with what would be expected by its growth curve.

The following analysis by SDS-PAGE of the supernatant showed a variation of proteins produced and lines at the 48-50 kDa mark where some of the major virulence factors, the KGP gingipains, were expected (Oishi et al, 2010). It is clear that the supernatants contained completely different protein constituents to the media in which the bacteria were cultured, indicating that the protein content of them has been almost completely metabolised.

Some mild variations between the different strains can be visualised, as expected. At the same time the proteins produced by the same strain grown in different environments, such as culture media of DMEM only or 80% DMEM + 20% FAB, or in 80% DMEM + 20% FAB in the presence of moderately roughened Ti, do not have clearly distinguishable differences. This suggests that these three different conditions do not affect *P. gingivalis* growth and virulence in a considerable way. This probably can be attributed to the presence of the growth factors that, according to the literature, seem to regulate and control the way *P. gingivalis* reacts. (Genco ,1995)

The presence of titanium, smoothened or moderately roughened, does not seem to change considerably the proteins identified through the SDS-PAGE assay, although the banding patterns identified are not completely identical. However, it cannot be excluded that the changes may be proportionate, or that it may affect other products or pathways of its

metabolism, mostly intracellular or on the membrane. It was observed in this study that *P. gingivalis* adheres to the titanium surface directly, as has been reported previously (Yoshinari et al, 2000, Amoroso et al, 2006). This might have led to different expression, mostly on the membrane or intracellularly, which would not directly affect the extracellular produce obtained in this case.

5.4 Effect of the supernatant of concentration of 50 μg/ml to the culture

The addition of the supernatant to the tissue culture model had an obvious deleterious effect on the viability of the cells in the culture, by a statistically significant reduction in the cells ($p<0,001$) within the periodontal (PDL) ligament and also, to a lesser extent, to the pulp. It is obvious that the extracellular matrix has been affected in the PDL and the bone marrow cavities. In some slices enlarged cells could be identified, and also, more rarely, some multinucleotic cells. This observation was universal for all the strains and conditions in the concentration of 50μg/ml of the supernatant.

It is evident, therefore, that all these three strains of *P. gingivalis* cause similar effects to the culture. *P. gingivalis* pathological action is multidimensional. As an opportunistic pathogen, the bacterium's main aim *in vivo* is to survive by protecting itself from the host defense mechanism, attacking crucial intracellular pathways and manipulating host cell responses to favour its growth (Bhavsar et al, 2007). Different strains of *P. gingivalis* have been characterised with varying degrees of virulence. This has been attributed

mostly to their different invasive capabilities into host cells (Jandik et al, 2008), or, sometimes, it has been related to their diferent fimA protein production (Amano et al, 2004, Kato et al, 2007). Although all 3 strains used in this experiment could be categorised in the more invasive categories, originating as they did from infected periodontal sites rather than from healthy ones, in this experiment different invasive capabilities could not be examined as live bacteria were not used.

However, the factors contained in the supernatant have been thought to have an important role in virulence. The direct cytotoxic activity of culture supernatant from *P. gingivalis* has been demonstrated in the past for kidney and human lung fibroblast cells *in vitro* (Eke et al., 1989). This has been mostly associated with the byproducts of their amino-acid metabolism such as butyrate and propionate. It could be assumed that the reduction in the numbers of viable cells in this experiment could at least partly be attributed to this direct cytotoxic effect.

It has also been proposed that certain *P. gingivalis* proteinases, the gingipains, bind to collagen type V, fibronectin and laminin and have proteolytic activity against host cell surface matrix proteins. This cycle of binding and degradation can lead to cell death and tissue destruction (O'Brien Simpson et al., 2003) and at the same time contribute to the inflammation and vascular disruption processes. The disorganisation of the extracellular matrix evident in this study may be explained by the action of the proteinases contained in the supernatant, in addition to the disorganisation caused by cell death. At the same time, gingipains are thought to be important in mediating host cell responses and as a

result affecting the intracellular signalling (Kadowaki et al., 2009). All these effects can explain the reduction of the cells of the PDL and the bone marrow in the culture, as the cells become increasingly isolated, reducing their viability, although nutrients are supplied through the tissue culture system, On the contrary the pulp cells where the extracellular matrix is less severly affected, the histological picture gives the impression that the cells retain more their viability.

In a study by Belibasakis et al (2007) culture supernatants of *P. gingivalis* W50 and E8 and K1 mutant strains grown in brain heart infusion (BHI) broth supplemented with hemin were exposed to cultured human periodontal ligament cells from healthy teenage human biopsy specimens. It was found that *P. gingivalis* protein concentrations of ≤50μg/ml did not elicit strong cytotoxicity after a 6h bacterial challenge, and continued to be sub-toxic in longer exposures (up to 24 h). This is in line with the findings of this experiment, where cytotoxicity is relatively low for both the 3 and 7 day exposure.

It is important to notice that most of the virulence factors when researched are mostly thought to have an effect on the inflammatory and immune response of the host (Curtis et al., 2001). In this model, by its design, the systemic inflammatory and immune response could not be examined. Most cells of haemopoietic origin, including osteoclasts, could not routinely be identified or any of their expected interactions visualised. This may explain that the effects seen in this culture system are progressing slower than what possibly would be expected *in vivo.* There is no extensive inflammatory or immune response that are usually associated in periodontal and peri-implant disease with a lot of

the adverse clinical effects seen such as suppuration, erythema, bleeding and bone resorption. These are also later events secondary to some primary responses. This may explain the fact that in this experiment the tissue architecture has not been extensively altered, especially the hard tissues are not affected, although it is thought that osteoclastogenesis can be induced by *P. gingivalis* (Graves et al, 2001), but as discussed before osteoclasts are not favoured in this model.

At the same time it is interesting that in the slides enlarged cells were identified in both the 3 and 7 day slides in the concentration of 50μg/ml. These cells were rarely identified in the control slides in comparison, although even in the experimental slides these were not identified in each and every slide. As a result this can lead to the assumption that these cells may not be new cells that formed during the experiment, but rather cells for which their apoptosis was for some reason inhibited. Apoptosis is a process of cell death that is highly regulated and involved in the development, differentiation, homeostasis and the regulation of the immune response. There have been identified two major apoptotic pathways the mitochondrial-mediated or intrinsinc pathway and the death-receptor mediated or extrinsic pathway (Hail et al, 2006). *P. gingivalis* has been shown to be capable to alter apoptotic cell death in a variety of cells , sometimes acting pro- apoptotic and others anti-apoptotic (Mao et al ,2007) . Cells where it has been found that *P. gingivalis* can modulate apoptosis include human polymorphonuclear leucocytes (Preshaw et al, 1999 and Hiroi et al, 1998), neutrophils (Murray and Witon, 2003), monocytes (Ozaki and Hanazawa, 2001), fibroblasts (Graves et al, 2001), gingival epithelial cells (Brozovic et al, 2006), epithelial cells (Inaba et al,

2006), endothelial cells (Sheets et al, 2006). These activities are thought to partly explain the chronic inflammatory processes identified in chronic periodontitis. It has been thought that these effects are associated with control of mitochondrial permeability (Yilmaz et al, 2004) and sometimes with the action of the lipopolysaccharide (LPS) (Murray and Wilton, 2003). These different activities of *P. gingivalis* may also explain the high relatively survival of the periodontal ligament cells in the concentration of 50μg/ml.

5.5 Tartate-Resistant Acid Phosphatase (TRAP) activity

In order to investigate the enlarged cells and the multinucleotic cells some slices were stained with Tartate-resistant acid Phosphatase (TRAP) stain to identify whether these cells exhibited any TRAP activity. As it is shown in the results, these two kinds of cells demonstrated TRAP activity. This can lead to the assumption that the enlarged cells may be cells of monocyte/macrophage lineage, as their apoptosis has been found that it can be inhibited by *P. gingivalis*, as discussed before. By being stained with TRAP may also indicate that they are in the process of diferentiating. Their differentiation pathway may not be possible to identify due to the lack of the systemic influences, however it may imply that the expression of TRAP in this case may be connected in someway with the osteoclastogenesis pathway, as TRAP is one of the most common osteoclast markers. These cells were identified mostly within the periodontal ligament, an area where increased activity is expected for a 28-day old wistar rat, due to the continuous stress that

results from the normal function of the incisors, the inherent activity of continuous eruption of this tooth, and also because the growth of the rat has not yet been completed. The increased activity in young mice *in vivo* at this stage of development has been reported by Merzel and Salmon (2008).

Furthermore, the multinucleotic cells, as also demonstrating TRAP activity may be similar to the multinucleated giant cells of chronic inflammation (McNally and Anderson, 2011) as these cells are not identified near /inside lacunae, nor have characteristics typical of an osteoclast. The multinucleotic cells may either have been present in advance or may have formed from macrophages by fusion, secondary to the presence of the supernatant within the culture, or even by nuclear division without cytokinesis. A study from Holt and Grainger (2011) showed that multinucleated cells can be created *in vitro* from primary-derived murine fibroblasts in contact or not with macrophages mostly in long term cultures (5-30 days).

At the same time, in another study by Scheres et al (2010) where human periodontal ligament fibroblasts in an *in vitro* culture were exposed to dead or viable *P. gingivalis* grown in broth , it was found that the protein secretion of IL-1b, IL-6 and IL-8 was increased. Previous studies have found that *P. gingivalis* components may induce the expression of Receptor Activator of Ng-kB Ligand (RANKL), associated with bone resorption, in human periodontal ligament cells cultured *in vitro* ((Belibasakis et al, 2007; Yamamoto et al, 2006). Osteoprotegerin (OPG) (that blocks the RANKL action) mRNA expression on the contrary was reduced during this experiment, resulting in an increased

RANKL/OPG expression ratio. These findings may explain the cell fusion that may have led to the few multinucleated cells that where observed in this experiment, as it is thought that in humans multinucleated cells are formed by fusion of monocyte/macrophage precursors. In the canonical pathway, these precursor cells require the presence of RANKL and macrophage colony stimulating factor (M-CSF) (Knowles and Athanasou, 2009) In another study (Fujimura et al, 2006) it was found that the culture supernatant of *P. gingivalis,* and specifically the haemoglobin receptor protein (HbR), suppressed RANKL-induced osteoclastogenesis, thus explaining that no forming of osteoclast-kind of cells was identified, in addition to the finding that this kind of culture may not favour the survival of osteoclasts. Although these ways of expression have been identified in humans similar pathways may exist in rats.

5.6 Effect of the supernatant of concentration of 100 μg/ml to the culture model

In the increased concentration of 100 μg/ml the results showed increased cell death, disorganisation of the matrix and at the same time very fewer enlarged cells and possibly no multinucleated cells. This implies an increased cytotoxicity. This is in line with the findings of Belibasakis et al (2007) where after a 24 h challege of the cultured human periodontal ligament cells by a culture supernatant of *P. gingivalis* with protein

concentration of 100 μg/ml almost no viable cells were identified. The fact that in this experiment still some cells can be identified may be attributed to bigger variety of tissues. These tissues (and especially the bone) may reduce the permeability of the culture supernatant of *P. gingivalis* directly to the periodontal ligament cells, or to the different apoptotic pathway in an explant model in comparison with a monolayer model. Since the cytotoxic effect is considerably increased this may explain the further disorganisation of the matrix. An interesting finding is that the enlarged cells with the enlarged nuclei are very much reduced in this higher concentration of the *P. gingivalis* supernatant. It is possible that the cytotoxic effect of this concentration is so high that the anti-apoptotic agents cannot prohibit the cell death. Additionally, it has been proposed that in later stages of infection anti–apoptotic proteins subside and pro-apoptotic are more active thus leading to cell death (Urnowey et al, 2006). Although in this experiment the proteins in the supernatant are not altered by the concentration used, this finding may highlight an antagonistic effect between the cytotoxic agents and the anti-apoptotic agents.

These results demonstrate that the effects seen on the tissue culture by the supernatant are time and dose dependent.

5.7 Effect of titanium exposure to the supernatant on the culture

In this experiment all 3 strains of *P. gingivalis* were exposed to either smoothened or moderately roughened titanium, and the supernatant produced was added in both the concentration (50 and 100 μg/ml) to the mandible slice tissue culture model. Generally,

the histological picture remained consistently similar for all the strains and conditions grown at the same concentration of 50 or 100 μg/ml, as discussed previously.

The results generally -except for one case- did not show that the growth conditions for the same strain of *P.gingivalis* (exposure or not to treated Titanium discs) caused a statistical significant effect to the cell counts in the cultures. There was only one case where a statistical significant difference at the level of $p<0.05$ in the cell counts of the mandible tissue culture exposed to the supernatants of the NCTC11834 *P. gingivalis* strain at the concentration of 50 μg/ml between the planktonically grown and the one grown in the presence of moderately roughened titanium was identified. There was no difference though between the planktonically grown and the one grown in the presence of smoothened titanium or between the smoothened titanium and the moderately roughened titanium.

It has to be mentioned that during the BCA assay minor non-significant differences were found on the protein content of the supernatant. The BCA assay is an approximate method though where concentrations are found based on a standard curve. The concentration of the 50 μg/ml was obtained by making dilutions based on the measurements. As the sensitivity of this method is not high, there is a possibility that a slightly different concentration was finally managed between these two different supernatants. A discrepancy of this kind would possibly explain this different result, as from the previous discussion it is obvious that the effects seen on the culture are dose dependent. Otherwise, this difference may be attributed to a variety of other reasons. It

may be that the virulence of this strain is altered more than the other strains in the presence of moderately roughened titanium, maybe due to slightly different adherence levels, or a mechanism of interaction. It is known for example that different fimA changes the invasive capabilities and as a result different strains can express different virulence factors and may be more susceptible to different environments. It is therefore likely that a slightly different expression due to the presence of titanium may make the supernatant more toxic, although the SDS-page did not clearly demonstrate any major differences.

Another mechanism may be that this specific strain can in some way cause titanium ions to become detached from the titanium specimen. This would be easier in a moderately roughened surface rather than in a smoothened titanium surface due to the nature of its larger surface area and possibly more susceptible to corrosion due to an increased titanium oxide layer. It has been suggested lately that, although it is well known that titanium is biocompatible, titanium ions released may induce cell death and bone resorption (Sommer et al., 2005Vamanu et al., 2008;; Mine et al., 2010).

It has been also proposed that an increased concentration of titanium ions may act partly in a synergistic manner with *P. gingivalis* by elevating sensitivity of gingival epithelial cells to bacteria , as it was shown on a culture of gingival epithelial – like cells (Makihira et al., 2010).

Finally, it has to be mentioned that in peri-implant diseases the cells around the implant -

as it is known- are not periodontal ligament cells, but rather gingival epithelial cells, which usually exhibit slightly different behaviour. Additionally generally this is a murine model with slightly different behaviour than human cells.

5.8 General Discussion

A rodent model has been thought as suitable for studying how periodontal disease may be mediated by *P. gingivalis* as it offers similar characteristics to human processes, although it should be recognised that the inflammatory response is different. (Genco et al., 1998). In this model it was obvious that the supernatant of *P. gingivalis* had an effect on the viability of the culture and on the histological picture. The results of this study indicate that this observed influence should be attributed mostly to the presence of the supernatant acting to mediate the effect of the extracellular produce of *P. gingivalis*, rather than as a toxic agent that simply changed the circumstances in which the culture was maintained.

Periodontal and peri-implant diseases are quite unique in that they are demonstrated as a chronic inflammation of the tissues with gradual, slow progress in comparison with the acute inflammation processes seen in other diseases. A lot of research on *P. gingivalis* as a

primary periodontal and peri-implant pathogen has focused on this slow action and microorganism's long-term ability to survive in the tissues or even in the cells. In this experiment an interesting finding was that the action of the supernatant is time and dose dependent, affecting the tissues gradually which may be thought as consistent with its suspected chronic inflammatory action *in vivo*. The findings even led to the assumption that the supernatant may inhibit the apoptosis of some kind of cells. This assumption is consistent with other published experimental findings (Mao et al, 2007)

It has been demonstrated in research that many of the major actions of *P. gingivalis* as a pathogen mediate inflammatory and immune response, although admittedly not very acute .It has been shown that for example *P. gingivalis* LPS and FimA induce macrophage cytokine expression (Zhou et al., 2004) and that the lipid A molecule which is part of the lipopolysaccharide (LPS) has been reported as an active participant in endotoxic activation (Oqawa et al., 2007) as well as being a key inflammatory mediator (Bainbridge and Darveau, 2001). The gingipains have been shown to contribute to the bacterial evasion of host defence mechanism (Kadowaki et al, 2007) and at the same time promote the inflammatory response (O'Brien-Simpson et al, 2003)

It is generally acknowledged nowadays that some of the mechanisms of the immune response in periodontal disease play a significant role in the bone resorption processes (Taubman et al., 2005). For example, it has been suggested that bone resorption caused by periodontal pathogens *in vivo* in mice is mediated in part by prostaglandin (Zubery et al., 2008). However, with this model, where systemic influences could not be easily

simulated, these processes could not be investigated. Nevertheless, the identification of cells demonstrating TRAP activity may lead to the assumption that *in vivo P. gingivalis* virulence may have an important role in these processes. This is also confirmed by research where TRAP activity in murine models investigating *P. gingivalis* action is common. For example, in a murine abscess model where the mice were immunised by *P. gingivalis* ATCC33277 (Gemmel et al., 1997) acid-phosphatase-positive and phagocytic macrophages were found in the lesions.

Peri-implantitis as a disease process around a titanium implant has been becoming increasingly investigated (Sanz et al, 2011) with the wider use of dental implants in restorative dentistry. The different characteristics of the disease in comparison to periodontal disease, may be attributed to the lack of the periodontal apparatus that exists around a tooth, make its understanding more difficult. In this experiment the presence of the titanium had a small or controversial effect in the virulence of *P. gingivalis* supernatant against the tissue culture model. Titanium as a material has been mostly proven as inert *in vivo,* and the results of this study did not demonstrate a defined specific effect to the virulence of *P. gingivalis*. Titanium is used in many forms, and increasingly nowadays two main forms appear in dental treatment. A smoothened interface for the transmucosal part and moderately roughened (with several methods) for the part of the implant that integrates. These two different kinds of surfaces demonstrate quite different behaviour against the tissues and the bacteria alike. Affiliation of *P.gingivalis* to the smoothened interface is low and seems favourable for a transmucosal part, whilst for a moderately roughened interface is greater, which is favourable for the bone and also

inadvertently for the bacteria. There is contradicting evidence whether a moderately roughened interface can accelerate peri-implantitis, and whether this may be attributed to the inherent difficulties the surface creates or whether the bacteria's virulence may be altered. There was a finding in this experiment, although not universal among the three strains of *P. gingivalis* used that may imply a connection of virulence of *P. gingivalis* with the type of surface. This could be a chance finding due to the small extent of this experiment, as discussed already, but also a true effect cannot be excluded. In any case the effect may be very moderate, and clinical evidence implies some mild connection between surface and disease progression (Lang and Berglundh, 2011). However, these results should be viewed under the prism of a murine model as well as an effect on periodontal ligament cells rather than on gingival epithelial cells.

It is evident that the pathogenesis of periodontal and peri-implant diseases in relation to their microbial aetiology is not completely understood. The diversity and complexity in the subgingival microbiota seems to be a field of further research when aiming to gain better understanding of the disease processes (Sanz et al, 2011)

P. gingivalis as an identified primary pathogen is increasingly accepted that its virulence can be influenced by the presence of other bacteria, which can act antagonistically or mainly synagonistically. However, research with *P. gingivalis* has shown that on its own this bacterium has a wide range of mechanisms of action and can produce distinct host tissue responses. This experiment demonstrated that *P. gingivalis* supernatant can cause specific responses in this model.

Further work is needed to investigate these primary responses seen in this experiment and also the reproducibility of the results. The virulence of *P. gingivalis* supernatant is multifactorial and this was an initial experiment to investigate it through this model, and the different kind of cell response visualised proves the wide cascade of events taking place in the disease process. All these different factors need to be investigated individually in order to get a better understanding of the initiation and progress of the periodontal and peri-implant diseases and the specific role of *P. gingivalis* in them.

5.9 Conclusions

This was an exploratory study which intended with the use of an ex-vivo tissue culture model of 28-day wistar rats mandible slices to investigate the virulence of *P. gingivalis* supernatant exposed or not to Ti. *P. ginigvalis* is a bacteria very commonly identified in plaque biofilms in diseased areas of periodontis and peri-implantitis. It is one of the most researched bacteria in the periodopathogenic complex and and has been postulated that it plays a role in these disease processes. The *ex vivo* model used in this study offers a platform where cell-specific responses can be researched and the results of this study , investigating the effect of the crude extracellular produce of *P. gingivalis*, demonstrated that it has a direct effect on the model which affects the viability of the cells , and to a lesser extent the histological picture. This indicates that the supernatant of *P. gingivalis* has virulence factors that can cause specific responses from cells, without the presence of live bacteria. It appears also that the different strains used in the experiment yielded in general similar results in this model and the exposure to titanium caused only very moderate differences which may not be of clinical significance. The responses in the model were gradual and this may imply the virulence factors in the supernatant cause a slowly progressing process, which is in keeping with the observed *in vivo* slow relatively progress of periimplantitis and periodontitis. These results may therefore give an insight in the biochemical events and responses and possibly in the intracellular signalling that the virulence factors contained in the supernatant of *P. gingivalis* may cause to cells of the periodontal complex with the assistance of the *ex vivo* model. *P. gingivalis* is often a

member of the biofim in cases of periodontitis and peri-implantitis of varying severity is a versatile microorganism and further research in understanding its function may assist in revealing the intricate underlying mechanisms of periodontis and peri-implantitis and may assist identifying mechanisms of controlling their progress or preventative measures.

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