ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΙΑΤΡΙΚΗ ΣΧΟΛΗ

ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ
<<ΜΟΡΙΑΚΗ ΚΑΙ ΕΦΑΡΜΟΣΜΕΝΗ ΦΥΣΙΟΛΟΓΙΑ>>

ΚΑΤΕΥΘΥΝΣΗ: ΒΑΣΙΚΗ ΕΡΕΥΝΑ

Free titanium particles act synrgistically with P.gingivalis lipopolysaccharide
An in vitro experimental study of periimplantitis

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ΑΘΗΝΑ 2021

Στους γονείς μου

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Preface

Ever since the genesis of osseointegration, in 1969, titanium implants have become one of the most rapidly and expandingly emerging concepts in the medical field. Had it not been for this scientific breakthrough, a significant portion of the human population would suffer from a severely compromised quality of living. It is, nowadays, estimated that approximately five million dental implants are annually placed in the United States of America solely. It is, therefore, more than evident how Implantology has literally taken over dental clinical practice, as well as the business industry, forming a very competitive market.

This wide use of dental implants, on the past decades, has brought up many challenges, as far as the esthetic, functional and biologically driven rehabilitation of a patient are concerned. The latter, which also refers to the long-term maintenance of any therapeutic outcome in a stable, healthy condition, has generated what is, perhaps, the most challenging concept in dentistry today. Similarly to the periodontal diseases, which have been known and well-studied for over a century, peri-implant conditions may severely affect the stability of an implant restoration and health of the peri-implant mucosa and underlying bone. What differs, however, is their rapid progression, as well as the unpredictable outcome of the established therapeutic interventions. Several questions naturally arise:

Why are we not able to treat periimplantitis as effectively as periodontitis?

Why does peri-implant mucositis, the equivalent of gingivitis, have a considerable recurrence rate?

It is believed that peri-implant diseases are primarily caused by the very same bacterial populations that are responsible for periodontitis. All means of therapy are, therefore, focused on effectively removing pathogenic biofilms, either mechanically or chemically. Implants are, of course, not teeth in any case and, the processing of their surface in different ways, disrupts the oxide layer, where successful osseointegration is attributed at. A more suitable scientific question may, therefore, be, how this disruption and release of tiny titanium particles affects peri-implant tissues and, is partially what this thesis is about.

This idea, that was briefly presented above, has a tremendous background of knowledge and experience of many people who, since the early steps of Implantology, have wondered and investigated what the possible complications following an implant placement could be.

Some of those people, I feel very privileged and fortunate to have met and, at this point, I would like to express my gratitude to those who have made the completion of this thesis possible:

Firstly, to Dr. Kyriaki Kyriakidou, PhD of the Ancona University of Italy, for being by my side on every step of this procedure, from the initial design of the experiment to the very last statistical analysis, for dedicating countless hours on guiding me, teaching me, offering me invaluable information on cell cultures and lab assays, sharing experience and, above all, for her consolation and reassurance through all the difficulties. I feel deeply grateful

and fortunate to call myself her student, among many who have seen her work be rendered possible because of her knowledge, experience and constant work.

To my supervisor, Dr. Anastasio Philippou, Associate Professor of Physiology at the Medical School of the University of Athens, whom I have first met during my undergraduate years and who truly stood out on his lectures, who taught me how to think on basic research and how to interpret results, who has cordially welcomed me in his laboratory and gave me the opportunity to complete my very first research attempt. I revere and deeply thank him for his patronage and moral support throughout the whole process, which has not always been easy.

To Professor and Lab Director of Physiology at the Medical School of the University of Athens, Michail Koutsilieri, I would like to express my most sincere thank you, for accepting me as a student in the Postgraduate Programme of Molecular and Applied Physiology, which has vastly expanded my horizons with cutting-edge and diverse knowledge and has been the place where I have met wonderful, hardworking people and teachers with exceptional ideas and, is what laid the foundation for any future scientific attempts of mine.

It is, at this point, where I would like to express a few words of gratitude to a very special teacher. To the teacher that altered my view on dentistry back at my fourth year of undergraduate studies, whom I have always admired and aspired to be like. To Dr. Ioannis Karoussis, Associate Professor of Periodontology at the Dental School of the University of Athens, I wish to

express my most sincere gratitude for being the strongest role model for me, the greatest teacher, the truest supporter, the person who taught me what it means to be a great clinician and restless mind, how to push oneself constantly towards excellence. I deeply thank him for posing this decisive role in my academic course, for always guiding me and for making this thesis possible in every way imaginable.

To my second mother, Stavroula Kousteni, Professor of Physiology at Columbia University, the one who has introduced me to research, has always inspired, always guided me at my very crucial decisions and helped my overcome my fears, I would like to deeply thank her for opening a sea of opportunity and inspiration for me and for showing me the true meaning of research, education and care.

Lastly, to my parents, Manoli and Rigopoula, I would like to express my deep love and my gratitude, since anything I have ever done, I owe to them. Their restless support, their most sincere concern, the principles, and morals they have bestowed upon me and their mere presence in my life have had the most catalytic effect on every step of the way. For this, and many more, I thank them truly.

'We cannot run before we learn how to walk.'

Nature Journal on proteomics, 2004

Introduction

Osseointegration of titanium implants

As Brånemark described in 1983, the term 'Osseointegration' is defined as 'the direct structural and functional contact between living bone and the surface of a loaded implant'.[1] Earlier, Schroeder had described the same process, characterizing it as 'functional ankylosis'.[2] After the bed preparation, that will receive the implant, and reflection of the mucoperiosteal flap, the trauma that is caused initiates the healing mechanisms, so that new osseous tissue can be formed. These mechanisms now seem to be led by the patient's immune system, through the complement cascade, while macrophages display a significant role.[3] Therefore, the appropriate 'stimulus' is the first fundamental element for the onset of the whole process.

Three main cell lines are implicated in the osseointegration process, these being *osteoblasts*, cells that compose bone at a rate of 0,17 mm/day, *osteoclasts*, cells that decompose bone at a rate of 100μm/day and finally, *osteocytes*.[4] The latter constitute the main stress sensors of the osseous tissue and dispose the dynamic to differentiate into osteoblasts or adversely, into fibroblasts. As a result, cells are the second fundamental factor for successful osseointegration.

The provision of all necessary nutrients for the optimal function of all the activities of the cell is the third and final, crucial element for osseointegration.

As far as the cortical bone is concerned, the speed rate of neoangiogenesis penetration is 0.05 mm/day, whereas for the trabecular (cancellous) bone, the same speed rate is 0.5 mm/day.[5,6] In cases of excess trauma or insufficient blood supply to a certain area, connective tissue intervenes between the living bone and the surface of the implant. **Consequently, the osseointegration process fails**.

Such events are classified as early failures of osseointegration, which are failures that occur prior to the functional loading of an implant. However, late failures are those that occur after the functional loading and compromise, or even destroy the aesthetic, biological and functional stability of implant restorations.

These late biological failures are peri-implant diseases, that being **peri-implant mucositis** and **periimplantitis**.

Peri-implant pathology and its treatment

Lately, the increasing number of titanium implants placed mainly in western societies is accompanied by a profoundly great incidence of peri-implant pathology.

In 2017, the American Academy of Periodontology, along with the European Federation of Periodontology have classified peri-implant diseases in two distinct entities: **peri-implant mucositis** and **periimplantitis**.[7] The main sign of peri-implant mucositis is bleeding on *gentle* probing, while inflammation signs may be present, such as erythema, edema, even

suppuration on probing or by gentle pressure on the soft tissues surrounding the implant.[8] The consensus clearly states that *'there is strong evidence from animal and human experimental studies that microbial plaque is the etiological factor for peri-implant mucositis'*.

Periimplantitis, however, is defined as a 'plaque-associated condition involving tissues around dental implants, with inflammation in the peri-implant mucosa and subsequent progressive loss of supporting bone'.[7] In other words, when all clinical signs of inflammation are present, but bone loss can also be appropriately justified, the diagnosis of periimplantitis is set and the microbial accumulation of periodontal pathogens is accused of it. Nevertheless, there are many more contributing, iatrogenic factors, such as residual cement, prostheses that do not favor cleansability, occlusion and the presence of foreign bodies. The prevalence of peri-implant mucositis reaches the 80% of the implant-bearing population, while the prevalence of periimplantitis reaches 28-56% of that population, depending on the diagnostic criteria applied.[6] These diseases are attributed to the microbial factor, that is common for periodontal diseases as well. What is different though, is that the disease progress is more rapid, the treatment very complex and, most alarmingly, with less predictable treatment outcomes. Back in 2004, the Cumulative Interceptive Supportive Therapy has been suggested as a detailed guideline for the treatment of peri-implant pathology by Niklaus Lang and Andrea Mombelli (Table 1).
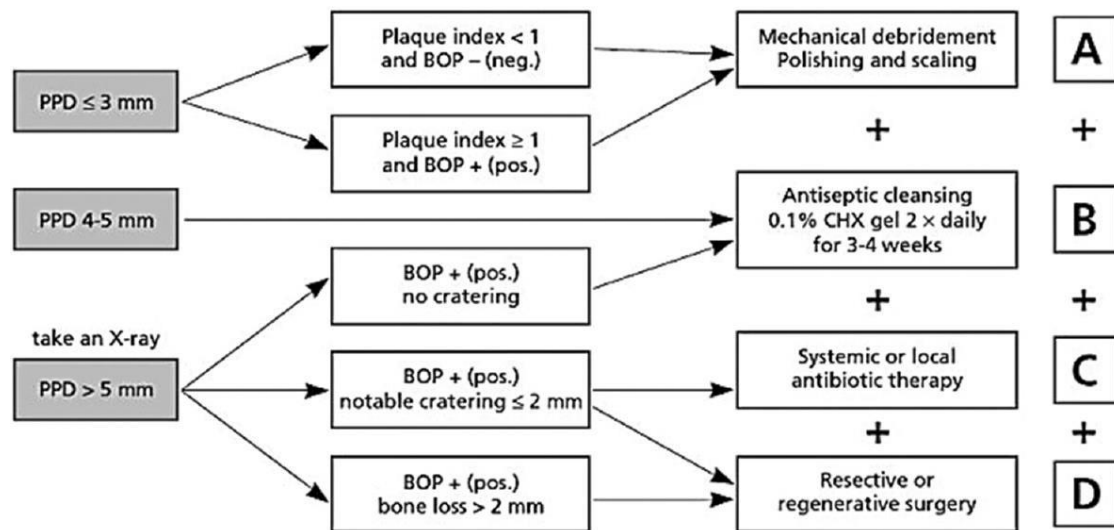
Table 1. CIST protocol. Lang and Mombelli, 2004

This protocol implies that the treatment of periimplantitis will end up in a surgical intervention. It is also clear that the initial step to treat any kind of peri-implant pathology is the '*m*echanical debridement'.

The past years, many different instruments were available in the market and thought to be appropriate for application on an implant surface, so long as they were not metallic, which are damaging to the titanium surface. As a result, Ultrasonic devices with Teflon-coated or plastic tips have been developed, as well as titanium or carbon-fiber scalers, all with a common cause: to remove microbial deposits without damaging the implant surface. Therefore, two factors are to be examined here: **effectiveness** and **safety.**

As far as effectiveness is concerned, current research suggests that carbon-fiber tips, as well as the Vector ultrasonic system, result in only minor improvements on bleeding on probing, which is indicative of inflammation

presence, and no changes in pocket depth, 6 months after non-surgical therapy.[9] Similar results were obtained from the comparison of titanium curettes with ultrasonic devices, that being no improvement on the probing depths.[10] These results favor the view that to effectively treat peri-implantitis, the clinician must be led to surgery, unless they use alternative means of non-surgical therapy. However, those instruments were not solely used during the initial phase of peri-implant therapy, where active disease is present, but also during the maintenance care phase, which is applied as a means of primary or, in treated cases, secondary prevention, where peri-implant health has been established and is to be preserved.

Now comes the safety factor. There is evidence that all those instruments severely affect the implant surface in different ways. Scanning electron microscopy images at a x1000 magnification reveal almost completely damaged sand-blasted and acid-etched (SLA) titanium surfaces that were previously treated with titanium brushes. Moreover, application of ultrasonic tips made of polyether ether ketone (PEEK) may cause only slight alterations to the titanium surfaces, however, detached pieces of material can be found on the surfaces. The damage is even greater when ultrasonic tips from synthetic materials (NSK) are used, with large pieces of the material integrated with the SLA surface.[11] Other studies have showed that these remnants are also very difficult to remove.[12]

Titanium instruments are also implicated in the surgical therapy of peri-implantitis, where non-regenerative implantoplasty is often performed, for exposed implant threads to be eliminated, the surface to be smoothened and all microbial deposits removed. This technique, according to another 2017

Consensus report, leads to a significant decrease in bleeding on probing and pocket depths and could result in improvement of clinical and radiographic parameters.[13] However, a recent prospective study has revealed an extremely high percentage of disease recurrence following implant surgery, that reaches 44% of the implants included.[14] What is very interesting is that Berglundh et al have found that implants with non-modified surfaces are less likely to present with residual deep pocket depths, bleeding on probing and bone loss after treatment, compared to modified surface implants.[15] Once again, that finding was attributed to the microbial accumulation and difficulty to remove deposits from modified surfaces. However, it should also be considered that the use of the 'implantoplasty' technique implies that a large quantity of titanium micro- or nanoparticles will be released on the adjacent soft and hard peri-implant tissues.

Slowly, a growing number of studies on the effect of those particles to adjacent and distant tissues emerges and their implication in peri-implant inflammation is under research.

Titanium corrosion

The term 'titanium corrosion' probably seems contradictory since titanium is a bioinert metal. In reality, that particularly high resistance to corrosion is attributed to the surface oxide layer that is formed during the contact of titanium with oxygen or water.[16] This layer consists of monoxide, dioxide or trioxide of titanium (TiO, TiO_2, TiO_3) in a crystalline form, whose thickness reaches up to approximately 50-100 nm.[17] Some important alloys have been developed to enhance corrosion resistance: grade 7 (Ti grade 2 + 0.12%− 0.25% Pd), grade 12 (Ti grade 2 + 0.2%−0.4% Mo, 0.6%−0.9% Ni) and grade 16 (Ti grade 2 + 0.04%−0.08% Pd). Yet, the most commonly used alloy is titanium grade 5, also known as Ti6AI4V, Ti-6AI-4V or Ti 6-4, containing 6% aluminum, 4% vanadium, 0.25% (max) iron and 0.2% (max) oxygen.[18] Alterations on this oxide layer may occur during the decontamination of the implant surface, during its placement, as well as during its functional loading.

Corrosive events related to dental implants

As far as the osteotomy part is concerned, titanium burs play a significant role in particle release. Specifically, Rashad et al proceeded to prepare implant wells with piezosurgery (group 1) and with rotating instruments (group 2), with common results for both groups: in the irrigation fluid, that they collected, residual titanium, zirconium, vanadium, iron and other metal particles were found. The authors recommended abundant irrigation during the well preparation for the removal of those particles.[19] However, Schwarz et al, who had also detected titanium micro- and nanoparticles in the subepithelial

connective tissue, after implantoplasty performance, reported their inability to remove them with irrigation, which was also histologically confirmed.[20] Additionally, Carvalho et al noticed that preparation burs used more than 10 times, presented with 17,86% deformation, while burs used more than 50 times, presented with 33,97% deformation.[21] Moreover, the means of decontamination, as well as the sterilization cycles, that instruments undergo, contribute to their additional corrosion, since after just 20 sterilization cycles, they presented with pitting corrosion.[22] Finally, zirconia burs have smaller deformation rates in comparison to the rest.[23]

Moreover, during its placement, the implant itself may be altered. During the implant screwing, the main aim is to acquire primary stability, which is solely mechanic, since there is zero degree of osseointegration at that initial point. The whole process creates stress areas within the implant, which results in the release of titanium particles of various sizes.[24] This event also depends on the quality of the surrounding bone, as well as the roughness of the implant itself.

As far as the superimposing implant restoration is concerned, the implant abutment plays a significant role in corrosive events. The past years, especially when it comes to the anterior esthetic zone, many clinicians prefer to use zirconia implant abutments, in order to improve the 'pink esthetics' score and avoid the grey shade that reflects through the peri-implant mucosa. However, that is not biologically favorable, since Klotz et al showed that zirconia abutments lead to a significantly greater mechanical abrasion of the implant, which is attributed to the four times greater roughness of zirconia, in comparison to 4th grade titanium.[25] On the other hand, if the abutment is made

of titanium of lower roughness than that of the implant, the first will undergo corrosion and, the result will, once more, be the same: the release of particles that either remain at the adjacent tissues or migrate to distant sites, *potentially* causing adverse immune reactions.[26] At the area of the implant – abutment connection, a phenomenon known as 'crevice corrosion' may occur. Specifically, in areas with restricted oxygen supply and acidic pH, chloride anions form titanium chlorides, which create an even more acidic environment. Such environments are abundant in hydrogen cations and thus, potential difference occurs, that leads to crevice corrosion. Finally, external connections of implants lead to the formation of bigger microgaps, therefore more micromovements of the system and mechanical abrasion and microbial penetration, that could potentially be the cause of a late failure of the implant.[27,28] In many cases, however, different kinds of corrosion coexist, rather than one distinct type solely.

Types of titanium corrosion

There are two basic types of titanium corrosion: **uniform** and **localized**. Localized corrosion can be distinguished into **crevice corrosion, pitting corrosion, hydrogen embrittlement, stress-corrosion cracking, fretting corrosion and erosion**.[29] According to the Pourbaix diagram of titanium in water, titanium is in passive conditions over a wide range of potentials but can be affected by strongly oxidizing conditions, where oxide is dissolved, or under strongly reducing conditions, where hydrides are formed. Uniform corrosion occurs in reducing environments, especially in acidic conditions.

high temperatures. Pitting corrosion can appear in severe working conditions, while the susceptibility to it is dependent on temperature and halide in concentration, the severity of which follows the order $F^- < Cl^- < I^- < Br$.[30] As it was described before, titanium may undergo crevice corrosion in hot chloride solutions. At temperatures higher than 70 degrees Celsius, crevice corrosion happens in many different solutions, including fluoride- and chloride-containing ones, only with crevice gaps that are narrower than 50 μm. Anodic dissolution of the tip of the crack begins at pits or crevices. In the presence of a corrosive environment and under the influence of a tensile force applied, the crack 'propagates' to the metal, causing mechanical failure. Hydrogen- assisted cracking, on the contrary, is based on the absorption of hydrogen atoms, that being the first step of the cathodic reaction of hydrogen, near the crack tip. The hydrogen permeates inside the titanium and promotes the embrittlement ahead of the crack tip.

In many studies, the term 'tribocorrosion' is used to describe the combination of debriding and corrosive events, which is a phenomenon influenced by mechanical and environmental factors.[31] Revanthi et al suggest that, through a certain load between two surfaces, with some sort of lubricant means, micromovement, particle release, re passivation and finally, dilution of the metal will occur, in one of the following types of corrosion:[32]

1. Microbial
2. Galvanic
3. Crevice
4. Fretting
5. Uniform

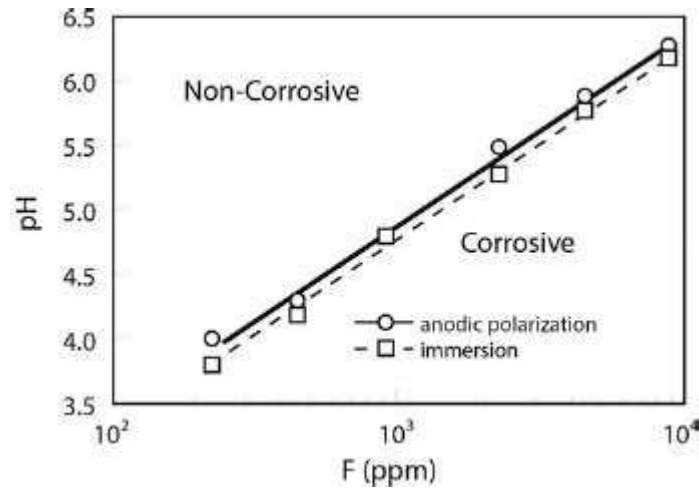However, Prando et al report that Microbial-induced corrosion does not affect titanium.

Fig. 1 Corrosion of titanium as a function of pH and fluoride ion concentration (F): Circles refer to anodic polarization tests; squares refer to immersion tests; ppm = parts per million. *Prando et al, 2017.*

Corrosive events on other parts of the organism - Aseptic loosening

A great field to study titanium corrosion in the human body is orthopedics and total joint replacement. A major complication of total joint replacement is aseptic loosening, which is the main cause of arthroplasty failures in humans.[33] The onset of this complication can be put approximately five years after the implant placement. What makes it particularly interesting, compared to the oral cavity and dental implants, is the lack of the microbial factor, therefore inflammation in the periprosthetic tissues is attributed to mechanical

wear solely. The release of particles results in the stimulation of the immune system and subsequent proinflammatory cytokine production and secretion, such as interleukin-6 (IL-6), interleukin-1β (Il-1β) and tumour necrosis factor-a (TNF-a), focal bone loss (osteolysis), inhibition of bone formation and distorted osteoblast differentiation.[34,35,36,37] A recent experimental study has examined the potential effect of LPS endotoxin on aseptic loosening cases, since it may participate in the biological response caused by particles. Endotoxins may be found on areas of aseptic loosening since they are often present on the implant surface. Moreover, wear particles have high affinity for endotoxin and, that may lead to the accumulation of circulating endotoxin. Lastly, the most important source of endotoxin is probably the bacterial biofilms that are present on implants from patients with aseptic loosening. The results demonstrated that titanium-particle injections significantly increased LPS levels *in vivo*. Titanium particles also initiate the response and gathering of macrophages to produce cytokines, while there is a predominance (60-80% of the entire cell population) of these cells in the fibrous membrane around loosening implants.[38]

Generally, attenuation of the microcirculation in periprosthetic soft tissues seems to aggravate the whole situation, leading to early implant failure.

P. gingivalis LPS effect on human gingival fibroblasts

P. gingivalis is a gram-negative bacterial species and belongs to the red complex of oral bacteria, according to Socransky's classification in 1998, which is the most pathogenic of all when it comes to periodontal pathology.

Periodontopathic bacteria produce several virulence factors, one of which is lipopolysaccharide (LPS). These virulence factors induce host response with the production of pro-inflammatory cytokines. These cytokines are important in the pathogenesis of chronic diseases of the periodontal and peri-implant tissues. Human gingival fibroblasts are known to produce pro-inflammatory cytokines, such as IL-6 and IL-8 upon LPS stimulation. Therefore, these cells play a key role in regulating host response against gram-negative periodontopathogens, which are also implicated in peri-implant pathology.

Interleukin - 6 (IL-6)

Gingival fibroblasts are the most abundant cells in peri-implant connective tissue, since they constitute 65% of the entire cell population in the connective tissue. Apart from fibroblasts, the peri-implant connective tissue comprises inflammatory cells, such as multinuclear neutrophils, lymphocytes, macrophages, mast-cells, basophils and eosinophils).[39] Although the cells of the immune system and fibroblasts were considered mutually exclusive, it is now evident that they both significantly influence each other.[40] Fibroblasts produce and secrete a number of inflammatory cytokines, such as interleukin- 1 and interleukin-6.

Interleukin-6 is a soluble mediator with a pleiotropic effect on inflammation, immune response and hematopoiesis. Human Il-6 is made up of 212 amino acids and, its gene has been mapped to chromosome 7p21.[41] After Il-6 is

produced in the initial stage of inflammation in a local lesion, it transports to the liver through circulation and induces the production of several cute phase proteins, such as C-reactive protein (CRP) and fibrinogen. It also reduces the production of fibronectin, albumin and transferrin.
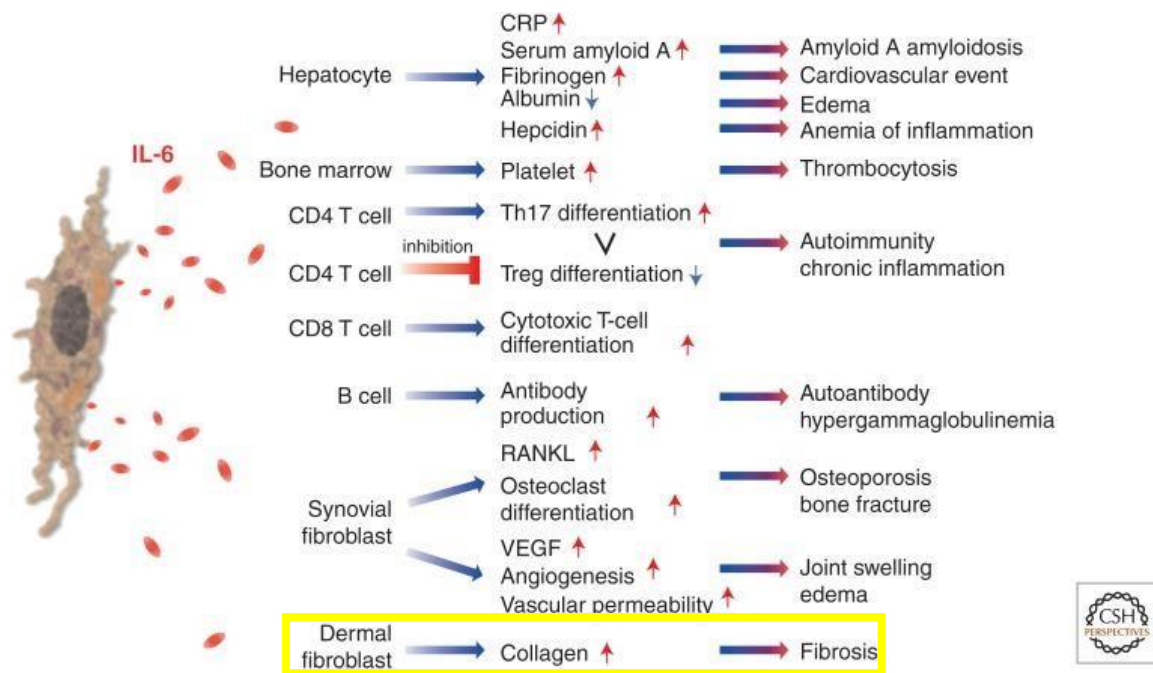

Image 1. IL-6 in inflammation, immunity and disease. Tanaka et al, 2014. Cold Spring Harb Perspect Biol.

It has been shown that IL-6, in combination with transforming growth factor (TGF)-β, is indispensable for Th17 differentiation from naïve CD4$^+$ T cells,[41] but that IL-6 also inhibits TGF-β-induced Treg differentiation.[42] Up-regulation of the Th17/Treg balance is considered to be responsible for the disruption of immunological tolerance, and is thus pathologically involved in the development of autoimmune and chronic inflammatory diseases.[43] The production of IL-6 by fibroblasts indicates that these cells have the potential to either regulate or exacerbate B-cell response seen in chronic inflammatory

situations, such as affected periodontal tissues. LPS seriously affects IL-6 production from gingival fibroblasts and interestingly, LPS derived from *P.gingivalis* produces the greatest amount of IL-6 activity at 50μg/ml.[44]

Figure 2. Effect of lipopolysaccharide from Salmonella enteritidis (Se), Actinobacillus actinomycetemcomitans (Aa) and Porphyromonas gingivalis (Pg).
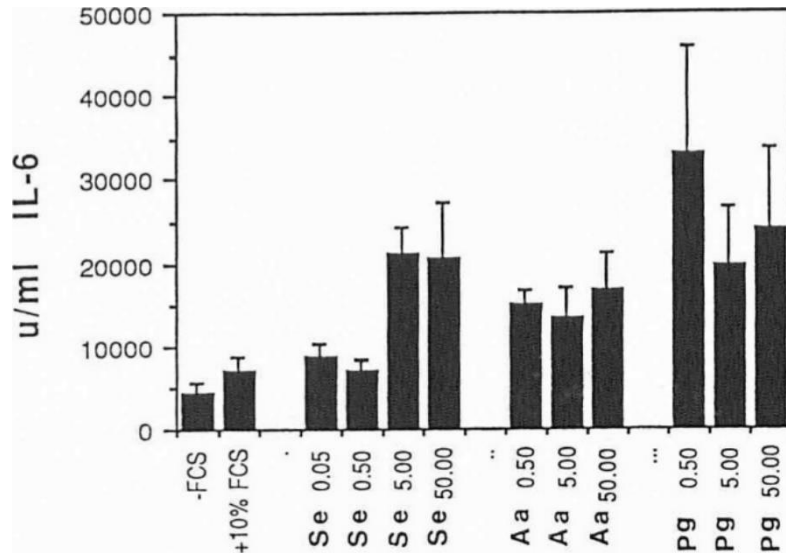
Cells were cultured in DMEM containing 10% FCS and exposed to varying concentrations of LPS. After 48 h in culture, IL-6 activity in the culture supernatants was determined. Data represent the mean and standard deviation of the mean from triplicate experiments. Bartold et al, 1991.

Interleukin-8 (IL-8)

Interleukin-8 is a chemotactic factor that attracts neutrophils, basophils and T cells during inflammation. It is involved in neutrophil activation and released by several types of cells, such as macrophages, bone marrow cells and monocytes. Apart from its role in inflammation, IL-8 is also involved in calcium

homeostasis, mitogenesis, inhibition of angiogenesis and neutrophil degranulation.

It is already mentioned that the peri-implant connective tissue contains numerous neutrophils. In gingivae, neutrophils are a very important feature in early stages of periodontal diseases. Human gingival fibroblasts have the ability to produce and secrete IL-8, after stimulation with LPS, derived from various pathogens. Il-8 produced from human gingival fibroblasts plays an important role in the early host response to bacterial invasion in the network of immune-participant cells. A very important difference between IL-6 and IL-8 is that the latter cannot stimulate IL-8 mRNA accumulation in gingival fibroblasts. On the contrary, IL-6 may stimulate its own production. What is also worth mentioning, is that interferon- γ (IFN-γ) inhibits the transcriptional stage of IL- 8, thus having down-regulatory effect on the production of IL-8 mRNA. Therefore, IFN-γ derived from T-lymphocytes present in the gingivae may be regulating neutrophil accumulation through the decrease in IL-8 production by gingival fibroblasts in later stages of inflammation.[45]

Type I Collagen

Type I collagen is a fibrillar type collagen, the most abundant and key structural composition of several tissues. It is expressed in almost all types of connective tissue and is the predominant component of the interstitial membrane. Type I collagen formation is associated with fibrosis and fibrogenesis.[46]

The genes encoding type I collagen are COL1A1 and COL1A2 and play a very important role in many genetic diseases since mutations in these genes

can lead to osteogenesis imperfecta, Ehlers-Danlos syndrome and Caffey disease.[47]

Regardless of the fibroblast type, LPS derived from different pathogens induces increased cell proliferation and collagen synthesis, by increasing the percentage of 'S' phase cells.[48]

Aim

The aim of this experimental study is to investigate the effect of titanium particles on human gingival fibroblasts, in comparison to the already known LPS effect. Specifically, the capability of those particles to induce the gene expression of pro-inflammatory cytokines IL-6 and IL-8, as well as type I collagen (COL1A1) is studied, as well as their effect on cell viability and proliferation. The possible synergistical activity of LPS and titanium particles is also investigated. Lastly, alterations in cell morphology is studied.

Materials   and   Methods

Specimens

The purpose of this experiment was the simulation of the conditions established in the oral cavity in cases of periimplantitis. For this reason, titanium disks with a sand blasted – large grid – acid etched surface were used (SLA disks, Straumann®). This is the optimal titanium surface for osteoblast adhesion and proliferation, but it does not come to contact with the soft tissues in a state of health, since it should be completely covered with bone. The diameter of each disk was 15 mm and the thickness was 1 mm. All disks were manufactured in 2012 and were autoclaved again before use, wrapped in sterilization pouches, at 121 degrees Celsius for 50 minutes (Runyes Medical Instrument Co.-SEA-17L-B-LCD).

Cell culture

Human Gingival Fibroblast (hGFs) cell line was used, purchased by ATCC® company, at Manassas, Virginia. For the cell culture, Dulbecco's modified Eagles Medium was used, complemented with 5% (v/v) Fetal Bovine Serum (FBS), which is the optimal and most used complement for cell cultures. The culture medium was also enhanced with 100 IU/ml penicillin and 100 mg/ml streptomycin, as well as 1% L-glutamine.

Cell thawing

The cryovial containing hGFs was removed from liquid nitrogen storage at 80 degrees Celsius. Immediately afterwards, it was placed into a 37-degree Celsius water bath for approximately 1 minute. This step can sometimes be avoided in order to avoid contamination of the cell line. The cryovial is then transferred under the flow hood after being thoroughly wiped with 70% ethanol. A small amount of pre-warmed (room temperature) complete culture medium was then dropwise put into the tube with thawed cells. The cells were not centrifuged, but the solution of thawed cells and culture medium was aspirated and resuspended several times quickly, until there was no more ice in the cryovial. The cells were then transferred to a 75 cm^2 vessel that contained 12 ml Dulbecco's culture medium (5% FBS). The vessel was appropriately labeled with the cell type, date, and current culture stage (p=20, I). Afterwards, the vessel was transferred to the CO_2 incubator, at 37 degrees Celsius, 5% CO_2 and 95% humidity. The first culture medium change was performed 24 hours after the thawing.

Cell preservation

The cell cultures were preserved in the CO_2 incubator, at 37 degrees Celsius, 5% CO_2 atmosphere and 95% humidity. Change of the culture medium was performed every 2 days, by the removal of existing supernatant medium and addition of 12 ml of fresh culture medium.

Cell split process

In order to estimate the proper time to split a cell culture, the state of cells must be assessed daily in an inverted optical microscope (Zeiss, Germany) and calculate the approximate confluence of the vessel surface. When the confluence of cells reaches 70-80% of the vessel surface, the culture can and should be split, to maintain cell health and proliferation. The split procedure is the following:

a. Determination of split-ratio.

b. Aspiration of the supernatant culture medium.

c. Rinsing of the vessel surface with 5ml Phosphate Buffered Saline (PBS) twice.

d. Addition of 4 ml Accutase into the vessel.

e. Placement of the vessel in the incubator for 3 minutes.

f. Check of the cell situation in the optical microscope. The elongated shape of cells should be gone and they should be seen 'flowing' in the fluid.

g. Addition of 8 ml Dulbecco's culture medium in the vessel, to inactivate the Accutase to avoid cytotoxic events.

h. Aspiration and resuspension of the cells several times in the same vessel to ensure that there will be no agglomerates.

i. Transfer of 6ml cells in new vessels that already contain 6 ml of fresh Dulbecco's modified Eagle Medium. Gently agitate each vessel to evenly distribute the cells on the vessel surface.

j. The new vessels are labeled with the cell passage code, date and cell type and transferred to the CO_2 incubator, at 37 degrees Celsius, 5% CO_2 atmosphere and 95% humidity.
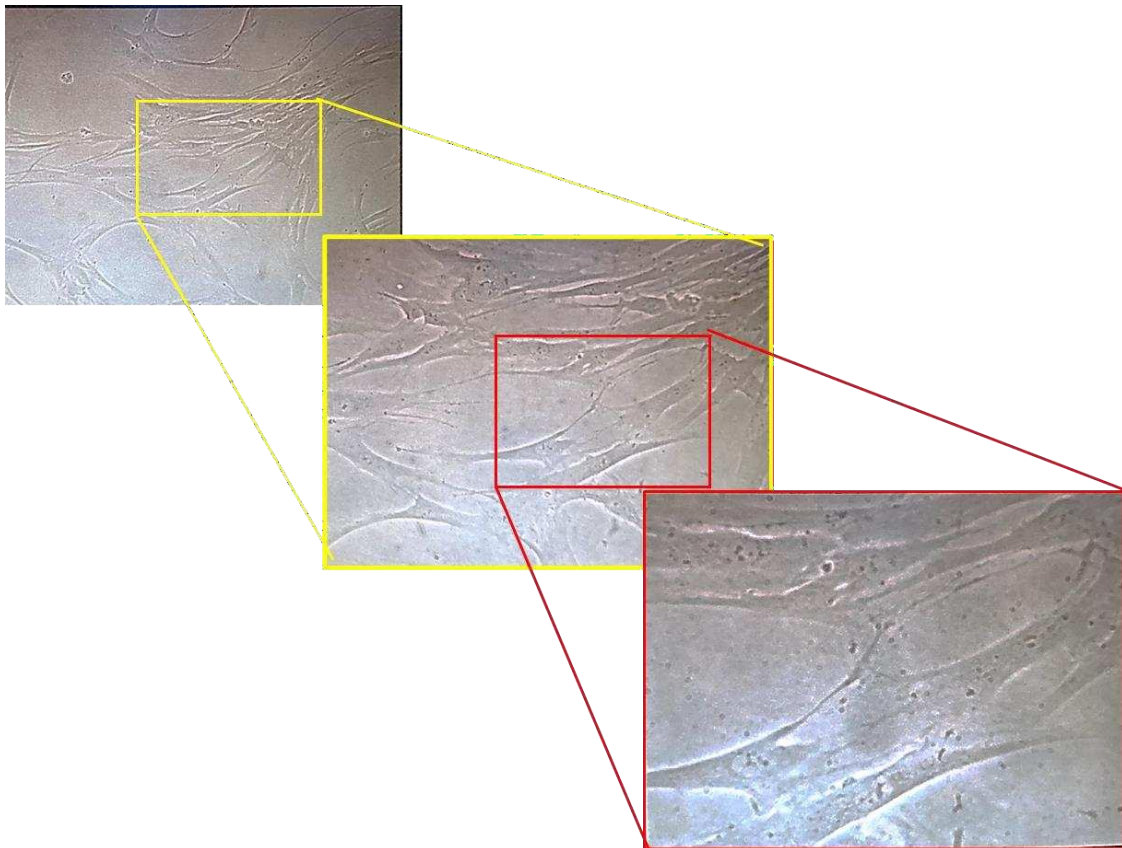
Image 2. Inverted microscopy image of human gingival fibroblasts at the 4th cell passage.

Cell population counting method using a hemocytometer

A hemocytometer (Neubauer®) was chosen to calculate the cell population at the 4th cell passage.
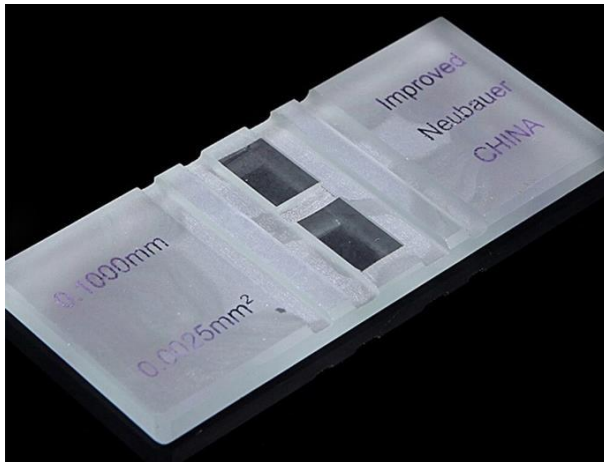
Image 3. Hemocytometer.
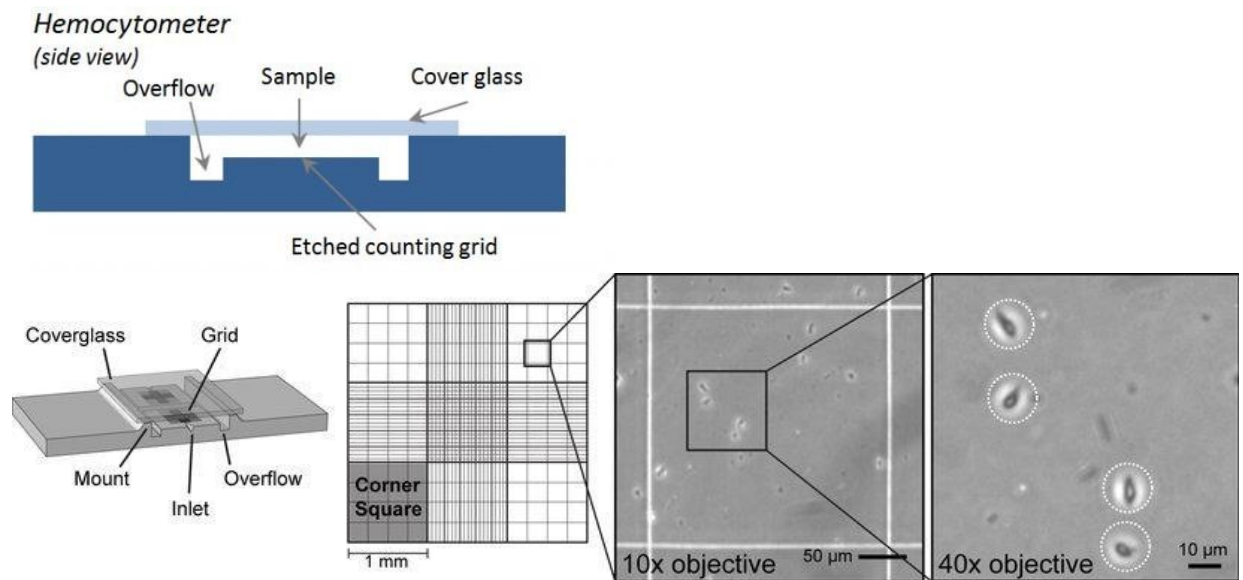
Image 4. Hemocytometer side view and square analysis.

The hemocytometer consists of 9 large squares (9 on the upper section and 9 on the lower). Each square may contain 10^{-4} ml of suspension. Cells are counted at the 4 corner squares and in the middle square. Both living and dead cells are counted.

The hemocytometer is peripherally cleaned with 70% ethanol and a new coverglass is placed on it. The cells are suspended in the inlets and counted according to the following equations:

a. **Percentage of viable cells** = viable cells/total cells x 100

b. **Average number of cells per square** = viable cells/5

c. **Dilution factor** = final volume/volume of cells

d. **Concentration** = average number of cells x dilution factor x 10^4

Cell treatment with LPS and titanium particles

Titanium particles were retrieved from an SLA disk with a fine grit diamond bur (45μm, red color coding) placed in an airotor handpiece under constant water irrigation. The reason why commercial titanium particles were not preferred is that there is evidence that it does not influence the tissues. Moreover, the particles during various surgical procedures are not even and uniform. Instead, their shape and size depend on the instruments used to modify the implant surface. Once gathered, the particles were decontaminated with absolute ethanol wash then resuspended in Dulbecco's culture medium.

The cells were placed in TCP (standard cell tissue culture treated polysterene surface, Corning®) wells and SLA titanium disks, at an initial density of 20.000 cells/cm^2 in DMEM 5% FBS. Cell cultures on both TCP and SLA surfaces remained in the incubator for 24 hours, in order to achieve cell adhesion on the surfaces. For both SLA and TCP surfaces, 4 groups were made:

a. Control groups (no treatment on either surfaces)

b. hGFs treated with LPS at a concentration of 10 ng/ml

c. hGFs treated with titanium particles at a concentration of 10 ng/ml

d. hGFs treated with LPS and titanium particles at a concentration of 10 ng/ml each
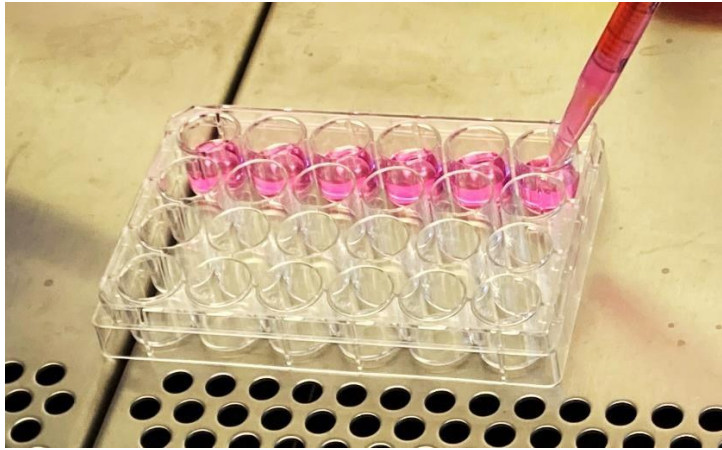
Image 5.1. hGFs cultured on TCP surfaces.
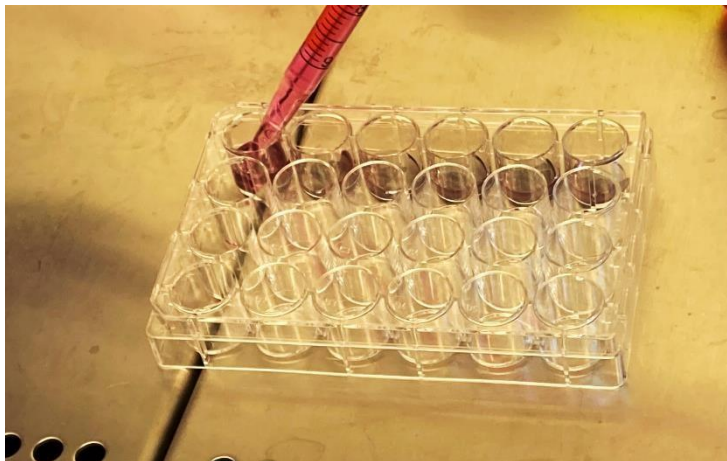
Image 6.2. hGFs cultured on SLA titanium disks.

24 hours later, the culture medium was removed from all groups, the cells were twice washed with PBS and fresh culture medium was added.

MTT assay was performed 24, 48 and 72 hours after cell treatment, as well as FDA/PI microscopy on the disks for all groups on the same time periods. For

the MTT assay, the experiments were performed in triplicates, in 3 independent runs. For the FDA/PI assay, one disk from each experimental group was used at each period. Molecular analysis was performed in 3 independent runs for each group, 5 and 7 days after treatment.

Image 6. Dispersion of titanium particles during their production from the modification of an SLA disk.

MTT assay

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Cells with a very low metabolism reduce very little MTT. On the contrary, rapidly dividing cells exhibit high rates of MTT reduction. As a result, the MTT assay is directly corelated to the mitochondrial activity and, as a

result, cell viability and growth rate.　　　　The optical absorption is recorded at

wavelengths between 570-600 nm.

MTT assay protocol:

a. Removal of all culture medium.

b. Addition of 200 µl of MTT diluted in 1800 µl Dulbecco's 5% FBS
   medium.

c. Incubation at 37 degrees Celsius for 4 hours.

d. Discard of the solution and dissolving of the formazan crystals in isopropanol 96%
   HCL 1N.

e. Calculation of absorption in an ELISA reader at 570 nm.

f. Quantification of the results, in comparison to the control group.

Images 7 a,b. MTT assay on 24 and 48 hours after treatment.
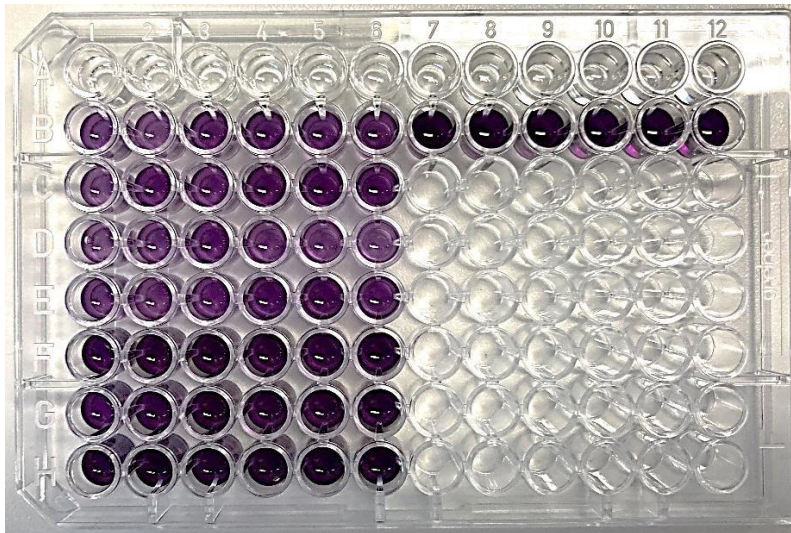
FDA/PI staining

Fluorescence-based live-dead assays can be used to evaluate the viability of mammalian cells. Simultaneous use of two fluorescent dyes allows a two color discrimination of the population of living cells from the dead population. FDA is taken up by cells that convert the non-fluorescent FDA into the green, fluorescent metabolite fluorescein. The measured signal is an indicator for viable cells, as the conversion is esterase dependent. On the contrary, the nuclei staining dye PI cannot pass through the viable cell membrane. It reaches the nucleus by passing through disordered areas of apoptotic cell membranes and intercalates with the DNA double helix of the cell.

FDA/PI protocol:

a. Fluorescein diacetate stock solution is prepared by dissolving 5 mg of FDA in 1 ml acetone (store at -20 degrees Celsius).

b. Propidium iodide solution is prepared by dissolving 2 mg of PI in 1 ml PBS (store at 4 degrees Celsius).

c. The staining solution consists of 5 ml PBS, 8 μl FDA and 50 μl PI.

d. Cell culture medium is removed from the cells.

e. Addition of staining solution.

f. Incubation for 4-5 minutes in the dark.

g. Sample washing with PBS.

h. Sample analysis with fluorescent microscopy.

Real-time Polymerase Chain Reaction (qPCR)

In conventional PCR, the amplified DNA product is detected in an endpoint analysis. In real-time PCR, however, the accumulation of amplification product is measured as the reaction progresses, in real time, with product quantification after each cycle.

Amplification reactions are set up with PCR reagents and unique (in this case) primers. Real-time detection of PCR products is enabled by the inclusion of a fluorescent reporter, in this case SYBR GREEN.

A basic PCR run can be separated into three phases:

a. **Exponential**: Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.

b. **Linear**: The reaction components are being consumed, the reaction is slowing, and products start to degrade.

c. **Plateau**: The reaction has stopped, no more products are being made and should PCR products remain long enough, they begin to degrade.

Measurements regarding quantification are made during the exponential phase of PCR. A very significant parameter of quantification is Ct (Cycle threshold), which is the number of reaction cycles required for the fluorescence value to reach a certain threshold. The threshold value is defined as the onset of the exponential phase. The Ct value is inversely proportional to the initial quantity of the substrate.

In this experiment, and in any experiment utilizing PCR, a reference gene is required. The reason for this is that all Ct values should be compared to the Ct values of the reference gene (in this case, GAPDH). The Ct values of all the targeted mRNA, including those of the reference gene, are regularized in relation to the reference gene values. The reason for this modification is to correct possible differences in the initial c DNA concentrations.

PCR Reaction Phases

PCR reaction:

(Genomic DNA) → (Denaturing step - 95C) → (Primer annealing - 55C) → (Elongation - 72C) → (Repeat cycle)

Scientific progress:

(Big question) → (Generate predictions) → (Experimental design) → (Collect and analyse data) → (Repeat cycle)
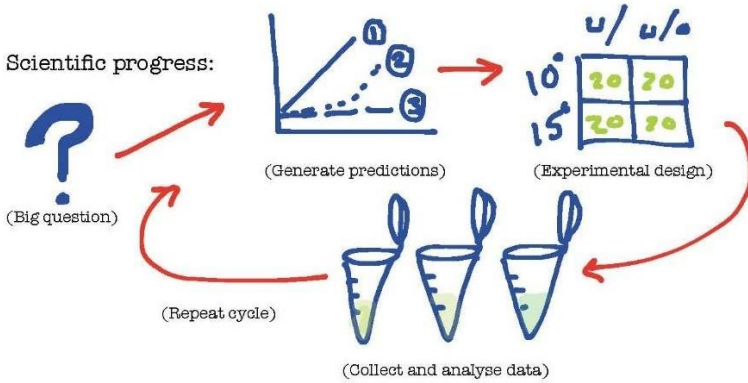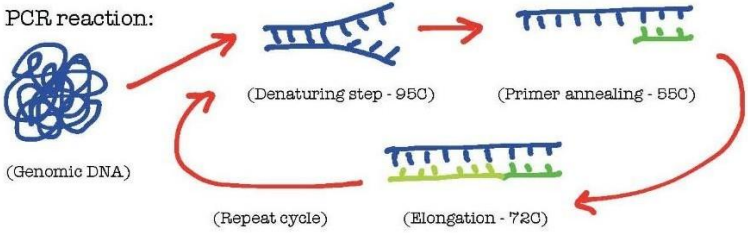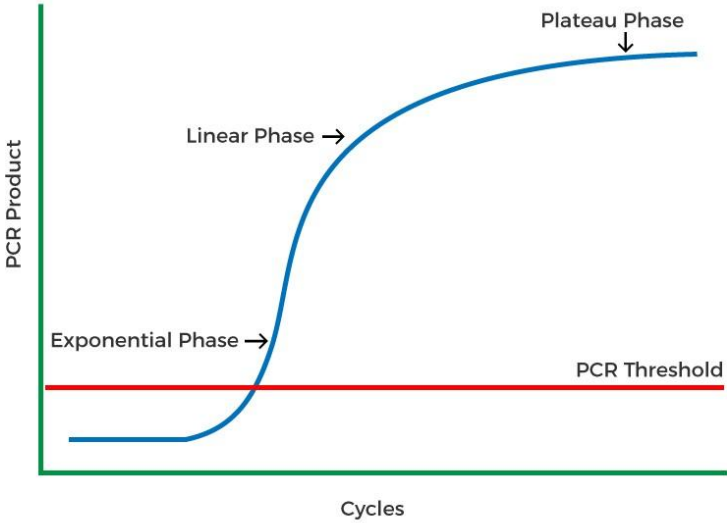
RNA isolation 5 and 7 days after treatment

Isolation of the whole RNA was performed with TRItidy G^{TM} (PanReac AppliChem).

TRItidy G^{TM} is a reagent, based on the Chomczynski method, with additional modifications that improve the purity of RNA.

TRItidy G^{TM} protocol:

a. Supernatant culture medium was removed from all groups, 5 and 7 days after the cell treatment.

b. 1ml TRItidy G reagent was added to each well to achieve cell lysis (1ml/10^2 cm, according to the company's protocol).

c. The samples were homogenized by pipetting the suspension up and down several times. A 10-minute incubation period also followed for the improvement of the separation of RNA/protein complexes.

d. 0,2 ml of chloroform were added to each well and, after mixture, another 10 minutes of incubation followed for the improvement for the purity of the RNA.

e. The lysates were centrifuged at 12.000 g for 15 minutes at 4 degrees Celsius.

f. Three separate phases could be distinguished in the Eppendorf tube afterwards. The aqueous phase was transferred to a new reaction tube and an equal amount of isopropanol was added to it. The RNA was precipitated on ice for 15 minutes.

g. The samples were centrifuged at 12.000g for 15 minutes at 4 degrees Celsius.

h. The RNA was washed with 80% ethanol by vortexing and subsequently with absolute (100%) ethanol.

i. The samples were centrifuged at 7500g for 10 minutes at 4 degrees Celsius.

j. The RNA was air-dried and dissolved in 10 µl of DEPC-treated water.

RNA quality and quantity measurement

NanoDrop Spectrophotometers are specially designed instruments for assessing RNA quantity and quality. Nucleic acids are exposed to ultraviolet light at a wavelength of 260 nanometres and a photodetector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration. As a result, less light will strike the photodetector and higher optical density will be produced. The amount of light absorbed can be related to the concentration of the absorbing nucleic acid with the Beer-Lamber Law. The optical density [4] is generated from equation:

Optical density= Log (Intensity of incident light / Intensity of Transmitted light)

In practical terms, a sample that contains no DNA or RNA should not absorb any of the ultraviolet light and therefore produce an OD of 0.

Optical density= Log (100/100)=0

One unit of optical density corresponds to 40 µg/ml RNA. In this experiment, Biospec-nano was used (Shimadzu Biotech, Japan). In order to assess the quality of the RNA acquired, the ratios A260/A280 and A260/A230 are used. Very pure RNA will have an A260/A280 ratio of approximately 2,1. Anything above 1,8 has satisfactory purity, whereas ratios <1,8 indicate DNA or protein contamination. A260/A230 ratio should ideally also be higher than 2,0. A low A260/A230 ratio indicates contamination with wash solutions, such as ethanol, phenols or proteins. It is very important to gently clean the instrument with a KimWipe after each use.

cDNA synthesis

The synthesis of the cDNA was achieved with the ProtoScript II First Strand cDNA Synthesis Kit (New England Bio Labs) protocol:

The kit includes:

Ingredients	Volume
Template RNA	1 µg per reaction
d(T)23 VN	2 µl
ProtoScript II Reaction Mix (2X)	10 µl
ProtoScript II Enzyme Mix (10X)	2 µl
Nuclease-free H_2O	20 µl

Protocol:

a. The RNA was left to thaw on the bench, along with all reagents of kit.

b. All tubes were carefully coded with the identification number of group of RNA.

c. 2 μl of oligoDTs were added to each tube.

d. The appropriate volume of RNA was added to each tube, so that tubes would contain 1000 ng of RNA in the end.

e. Incubation at 42 degrees Celsius for one hour for cDNA synthesis.

Image 8. cDNA samples ready to be incubated at 42 degrees Celsius for one hour.

Real-time PCR procedure

The gene primers selected for this experiment are presented in the following table:

GAPDH	REVERSE	GACGCCTGCTTCACCACCTTCTT
	FORWARD	GCGGGGCTCTCCAGAACATCAT
Interleukin 6 (IL-6)	REVERSE	CCATCTTTGGAAGGTTCAGGTTG
	FORWARD	ACTCACCTCTTCAGAACGAATTG
Interleukin-8 (IL-8)	REVERSE	AACTTCTCCACAACCCTCTG
	FORWARD	TTGGCAGCCTTCCTGATTTC
Collagen 1A (COL1A1)	REVERSE	CATGACCGAGACGTGTGGAAACC
	FORWARD	CATGACCGAGACGTGTGGAAACC

The master mix was prepared for each pair of primers. That contains the pair of primers (reverse-forward), the SYBR Green Master Mix and RNAase-free water, in a total quantity of 500nM. Those reactions take place in 96 well plates. Each well contains:

a. 15 μl of master mix and

b. 5 μl of cDNA, which corresponds to 50 ng of each cDNA sample.

Each reaction must be twice performed to ensure maximum reproducibility. The plate is covered with a transparent membrane that adheres on the plate's

surface (MicroAmp Optical Adhesive film PCR compatible DNA/RNA RNAase free, Applied Biosystems) and the plate is centrifuged for 3-4 minutes to ensure that the samples are adequately mixed and there is no sample on the walls of the wells rather than the bottom only.

Preparation of samples for SEM

Eight titanium disks, representing each treatment group, 5 and 7 days of the treatment were prepared for scanning electron microscopy. Firstly, the cells were fixed on the disks with 2% glutaraldehyde in a cacodylic acid buffer. After this initial fixation phase, the first wash follows, usually with saccharose. The reason for this is to remove any fixating component that has remain unbound. At the second phase of fixation, 1% osmium tetroxide is used in a cacodylic acid buffer. The cells must remain in this state for 60 minutes at 4 degrees Celsius, as higher temperatures could lead to the rapid reaction of the cells with the osmium tetroxide and the alteration of their morphology. A second wash, similar to the first one follows. The final step is the dehydration of the samples, that is gradually achieved by the replacement of water with ethanol. Every 10 minutes, the samples are immersed in increasing concentrations of ethanol (25%, 50%, 75%, 80%, 95%). After this, the samples are placed in envelopes made of filter paper and are soaked in 100% ethanol. The samples will remain at this solution for 12 hours, until all ethanol has evaporated.

Statistical Analysis

For the statistical analysis of all retrieved results, Graphpad Prism v5.2 has been used. The Confidence Interval for all experiments was 95%. A p value less than 0,5 ($p<0,5$) was set as statistically significant. For all data, a normality test was performed at first, which determined the appropriate statistical test that was applied. Paired t-tests and One-Way Anova were used for data that followed a normal distribution.

Results

MTT assay at 24, 48 and 72 hours after treatment with LPS, particles, LPS and particles combined

The diagrams bellow express the results retrieved from the collection and analysis of data derived from the MTT assay.

The assay was performed for treated cells on both SLA titanium disks and TCP surfaces. All pink columns represent groups of cells on SLA surfaces, while all blue columns represent groups of cells on TCP surfaces.

24 hours after the treatment of cells with LPS and particles, there is one main finding: SLA titanium surfaces have a significant effect on human gingival fibroblasts. Specifically, cells on SLA disks have a statistically significant difference in proliferation, compared to those cultured on TCP surfaces (control group). That is not just a random event, since hGFs on SLA disks treated with either LPS, Ti particles or both, exhibit a statistically decreased proliferation, in comparison to their corresponding groups on TCP surfaces. For the SLA groups, there is also a statistically significant difference between hGFs treated with LPS only and hGFs that received no treatment. (Diagram 1)

48 hours later, the same pattern can be observed. For all TCP groups, there is a statistically significant increase in cell proliferation, compared to the SLA groups. We also wanted to investigate whether any of the treatments applied could potentially arrest the progress of proliferation. From the statistical comparison for all groups between 24 and 48 hours, the result was a statistically significant increase in cell proliferation in one day. That implies

that, there is not only an increased metabolic activity of the cells, but also that none of the treatments are cytotoxic. (Diagram 2)

3 days after the treatment, there is still a statistically significant difference between the SLA and TCP groups for all treatment groups. There is still a statistically significant increase in proliferation between 48 and 72 hours for all groups, which confirms that the treatment does not negatively affect cell proliferation or, at least, not to a significant extent. In the 72-hours results, a statistically significant difference between the 'TCP + LPS' and 'TCP + particles' can be observed. For the SLA groups, a statistically significant difference between the 'particles' and 'LPS and particles' groups can also be observed. (Diagram 3)
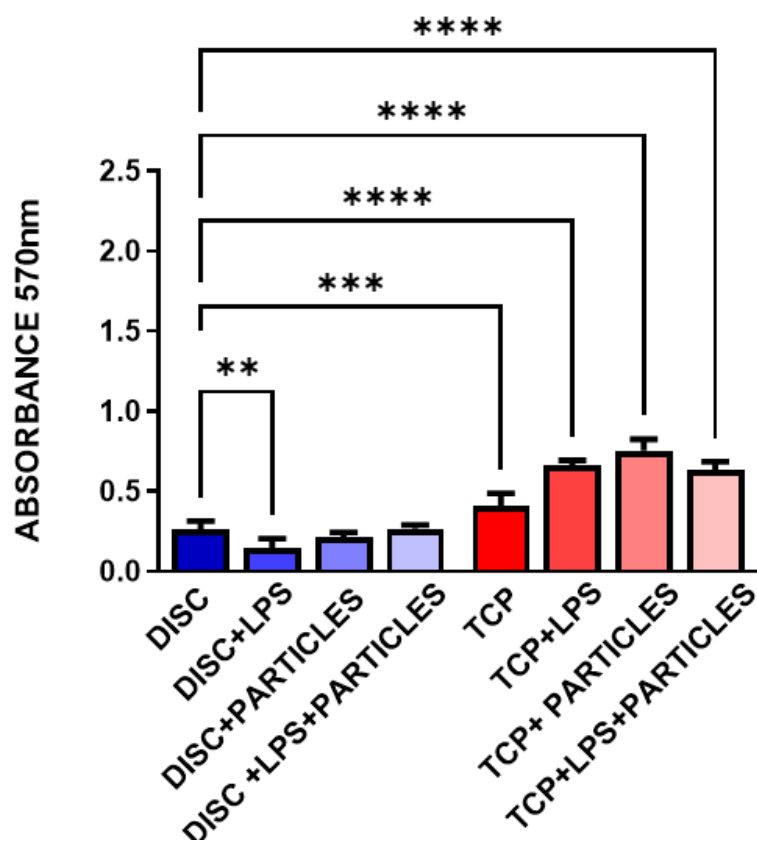
Diagram 1. MTT assay results after 24 hours.
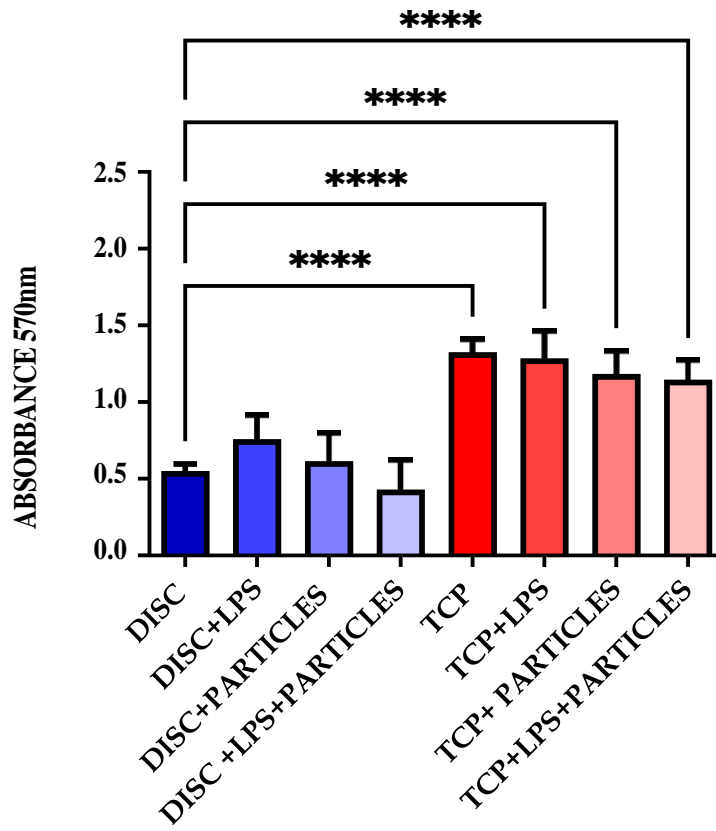
Diagram 2. MTT assay results after 48 hours.
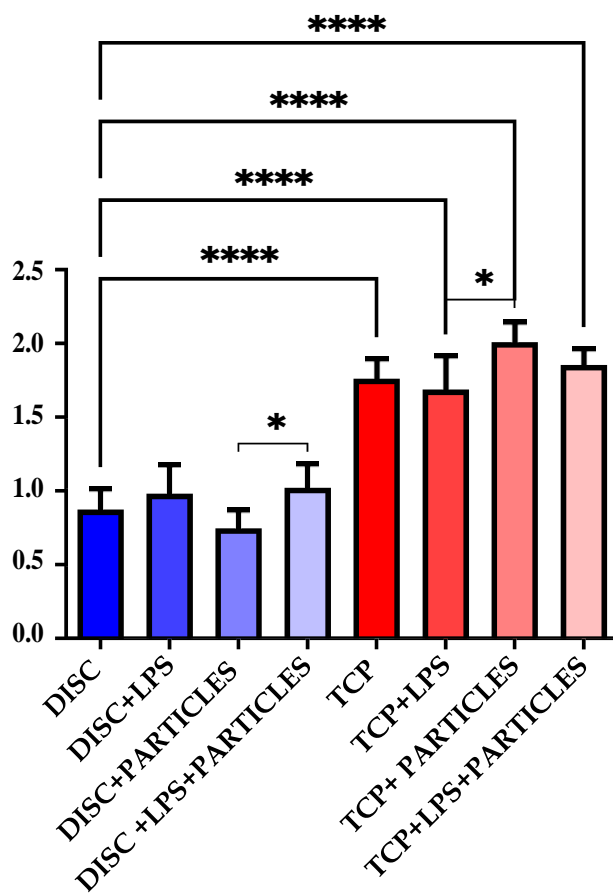
Diagram 3. MTT assay results after 72 hours.

Cell staining with FDA/PI 24, 48 and 72 hours after cell treatment with LPS, particles, LPS and particles combined

We examined cell viability and apoptosis of hGFs on SLA titanium surfaces, 24 hours after the cell treatment. For the group that received no treatment, substantial cell viability and no apoptosis can be observed for the first 24 hours. The cells have a rather elongated shape, with areas of high confluence. For all treatment groups, we could not detect significant apoptosis for the first 24 hours after treatment. As time after the treatment progresses, substantial cell apoptosis and less viable cells can be observed. There are also gap-junctions between living cells, which are more round than elongated.
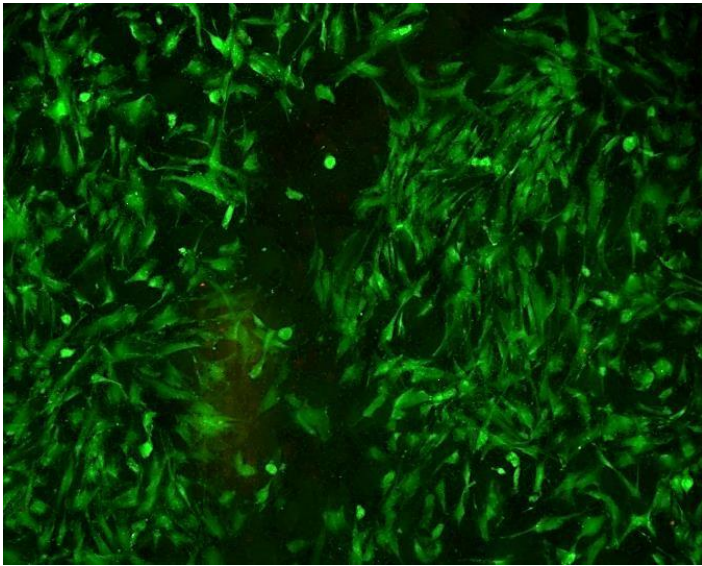
Image 9. 24 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. No treatment.
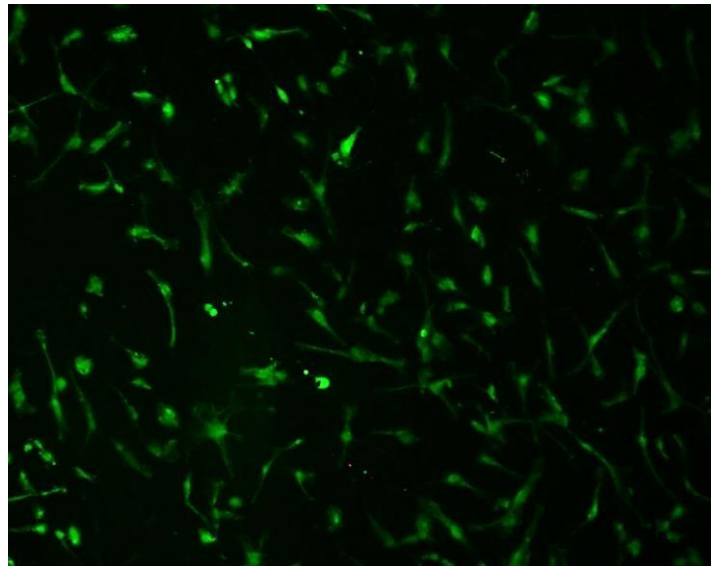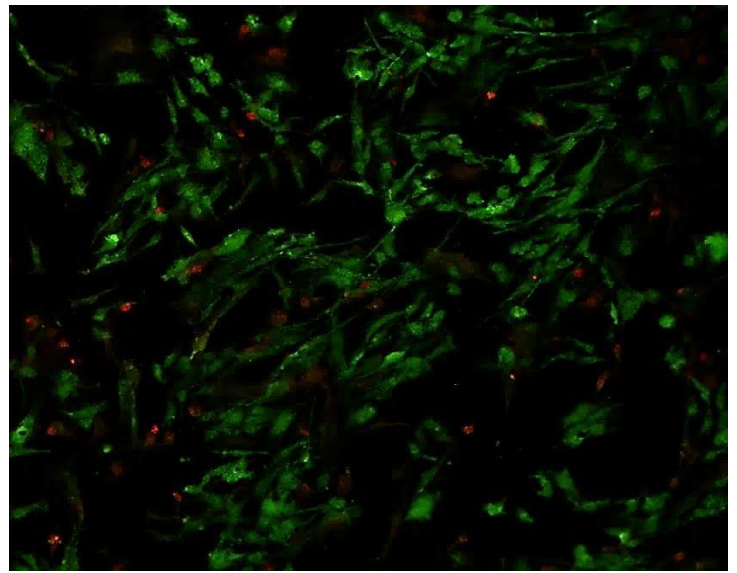

Image 10. 24 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with LPS.
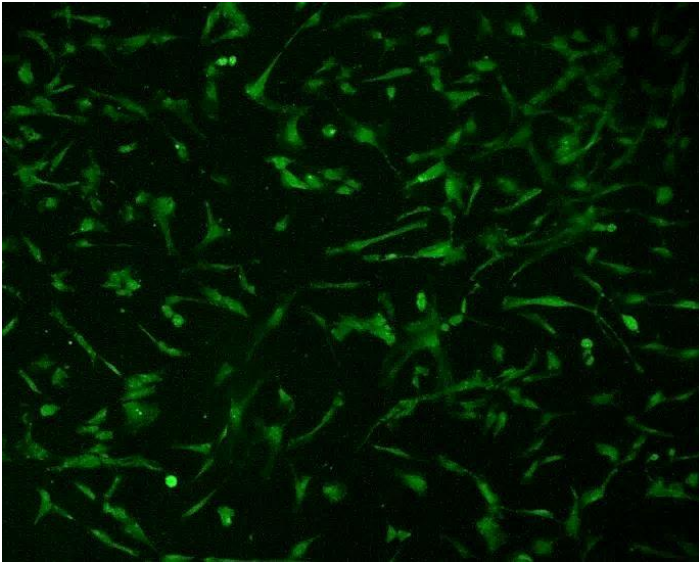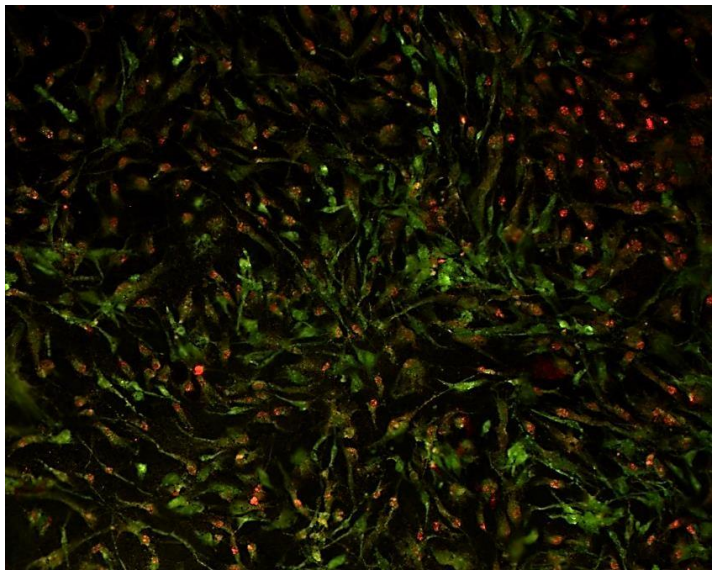

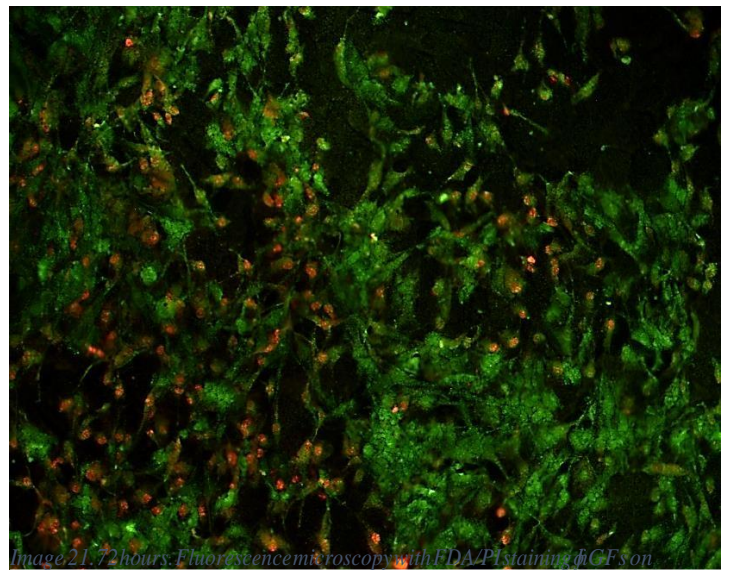*Image 11. 24 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with particles.*
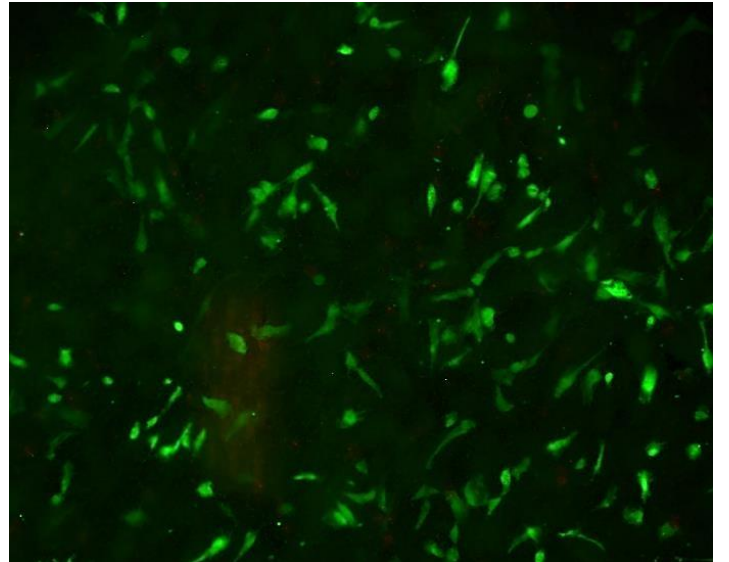
Image 12. 24 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with LPS and particles.
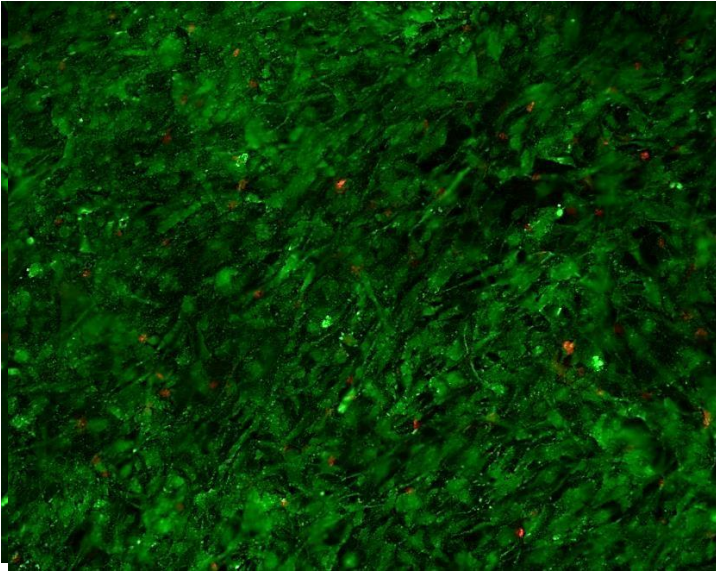
Image 13. 48 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. No treatment group.
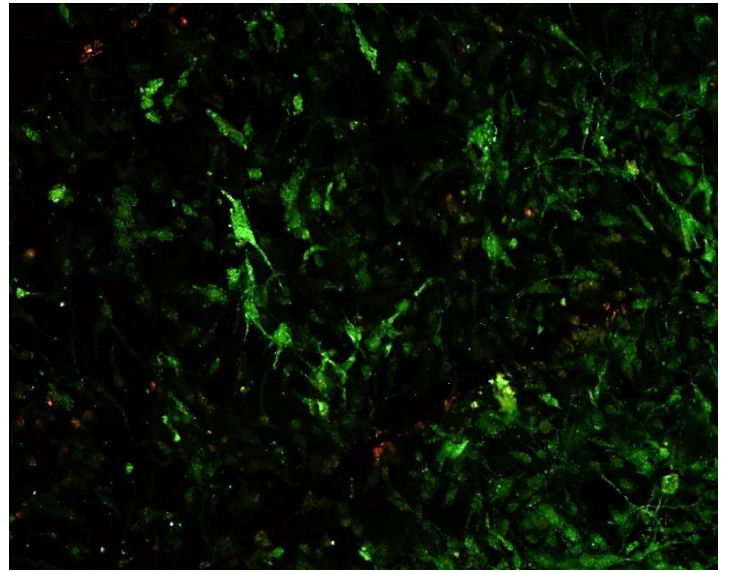
Image 14. 48 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with LPS.
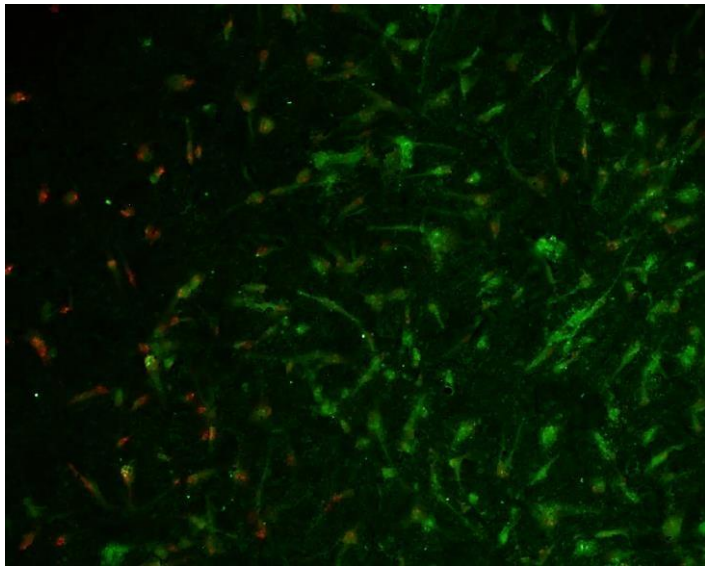
Image 15. 48 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with particles.
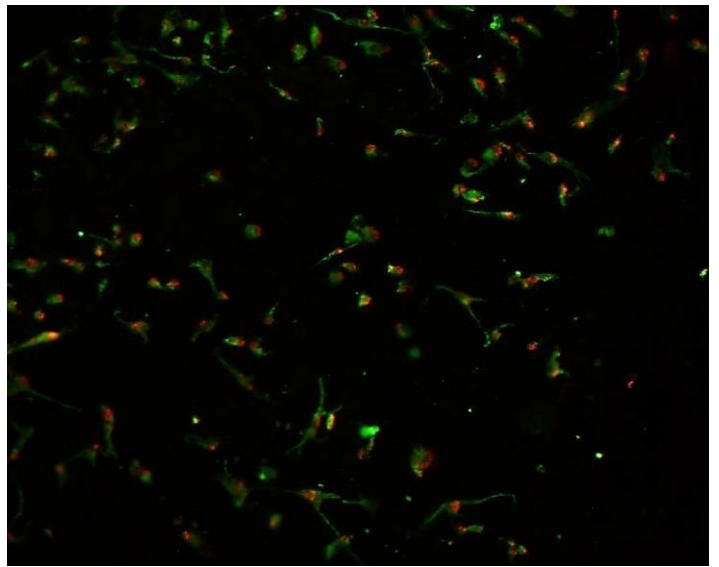
Image 16. 48 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with LPS and particles.
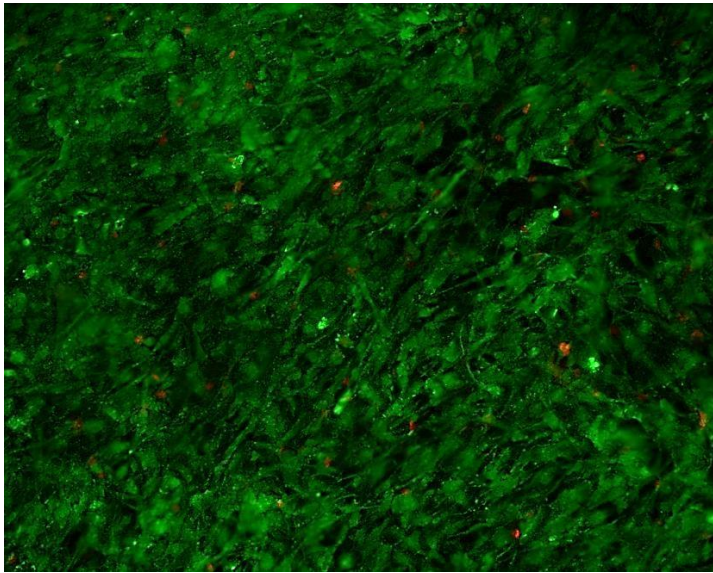
Image 18. 72 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. No treatment group.

Image 19. 72 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with LPS.

Image 20. 72 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with particles.

Image 21. 72 hours. Fluorescence microscopy with FDA/PI staining of hGFs on SLA surfaces. Treatment with LPS and particles.

Gene expression 5 and 7 days after cell treatment with LPS, particles, LPS and particles combined

On this experiment, we examined the gene expression levels of proinflammatory cytokines IL-6 and IL-8, as well as that of Collagen Type I(COL1A1) 5 days and a week after cell treatment, for hGFs cultured both on SLA and TCP surfaces.

There was a statistically significant difference in the expression of IL-6 between the 'LPS and particles' and 'LPS' groups on TCP surfaces, in favor of the first. This strengthens our view that LPS and titanium particles might have a synergistical effect, as far as the inflammation promotion is concerned. There was also a significant difference between the two 'LPS and particles' groups on SLA and TCP surfaces. From the diagram, we can also observe an increasing tendency of expression when hGFs are treated with particles, compared to being treated with LPS only, but not in a statistically significant manner. Moreover, in the SLA group, treatment with LPS seems to induce the greatest IL-6 expression, though not statistically significant.

IL-6 5 DAYS
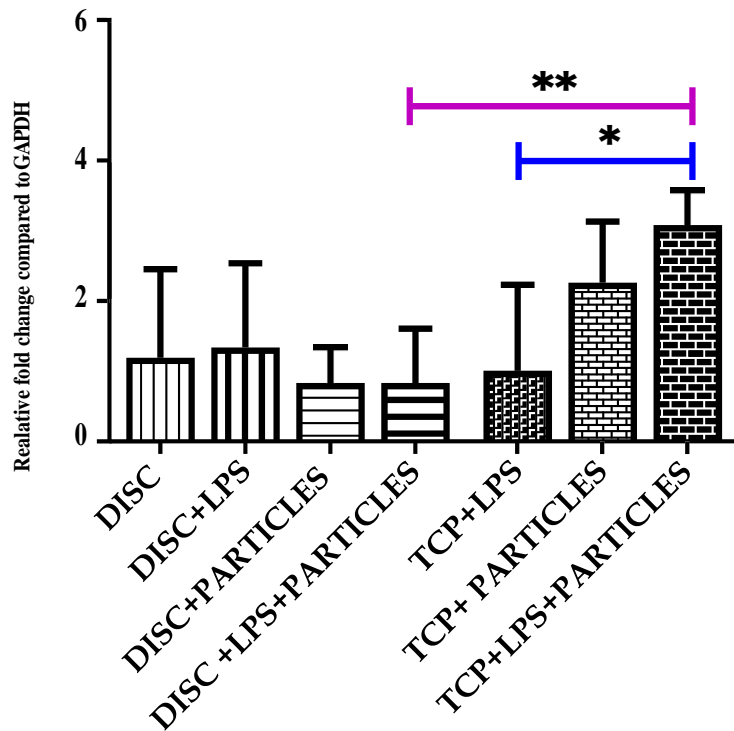
Diagram 4. Gene expression of IL-6, 5 days after the treatment.

As far as IL-8 expression is concerned, on 5 days, we can observe what maybe the **most important finding** of this experiment. There is a statistically significant difference in the expression of IL-8 between the 'LPS and particles' and 'particles' groups, as well as between the 'LPS and particles' and 'LPS' groups, for hGFs cultured on TCP surfaces. There is also a statistically significant difference between the 'particles' and 'LPS' groups, for hGFs cultured on TCP surfaces, in favor of the first. This enhances the former results regarding IL-6, despite them not being as strong. It also confirms that particles, either **individually or in combination with LPS**, induce a much **greater IL-8 expression than LPS only**, *in vitro*. The same results are no

observed for hGFs cultured on SLA surfaces, though a similar tendency is present.
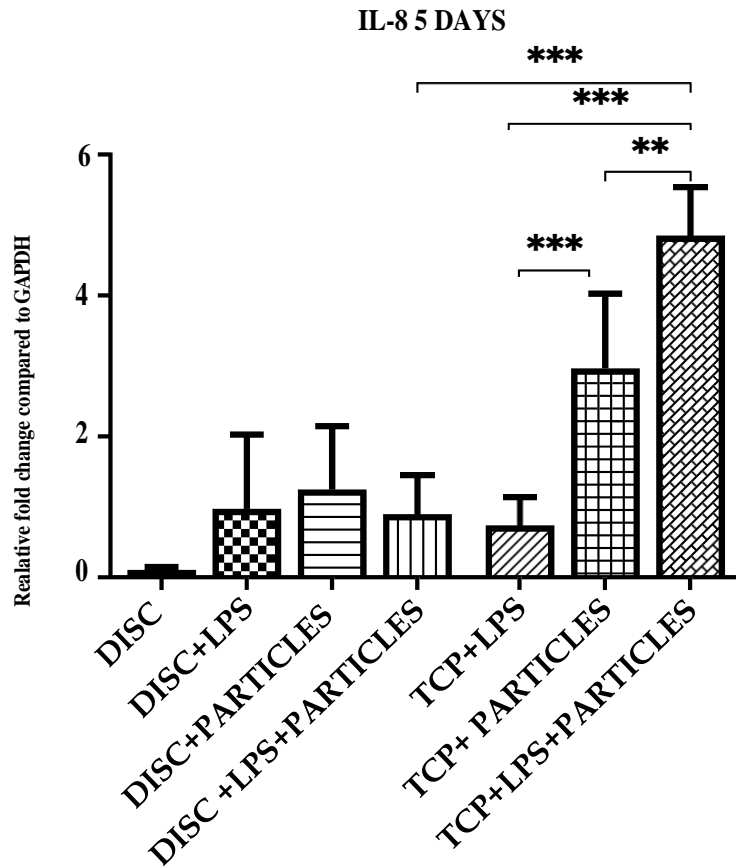
IL-8 5 DAYS

Diagram 5. Gene expression of IL-8, 5 days after treatment.

When it comes to Col1A1 gene-expression, for the TCP surfaces, there is a statistically significant difference between the 'LPS and particles' and 'particles' group, in favor of the first. There is also a statistically significant difference between the 'LPS' and 'LPS and particles' groups, in favor of the latter. This indicates that there is a higher collagen production in the presence of particles, simultaneously with the IL-8 and IL-6 production. For the SLA surfaces, there is a statistically significant difference between the 'LPS and

particles' and the group that received no treatment at all. There is also a similar tendency to that observed in the TCP groups, despite it not being confirmed statistically.

COL-1A 5 DAYS
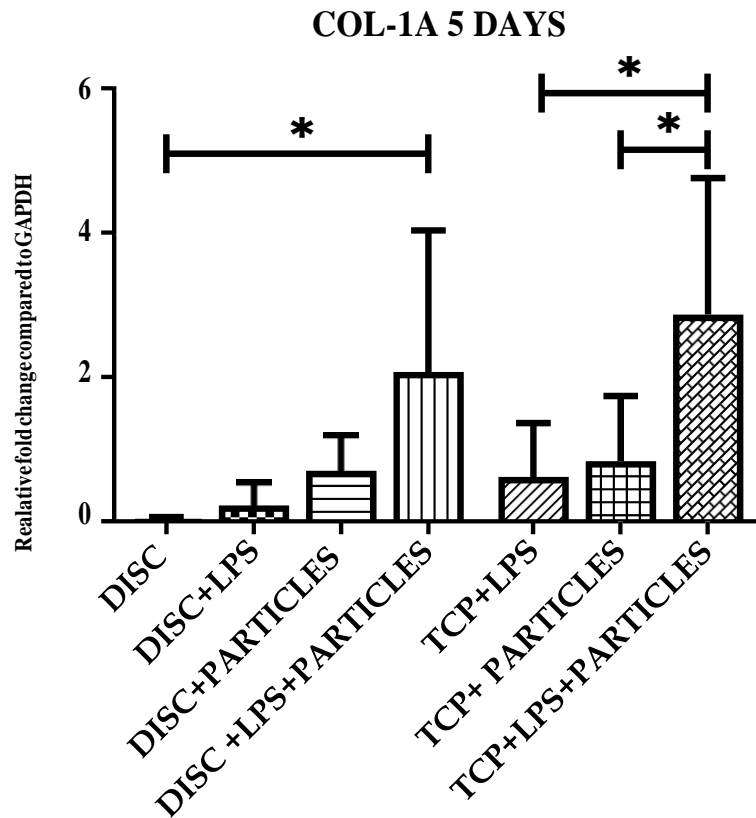
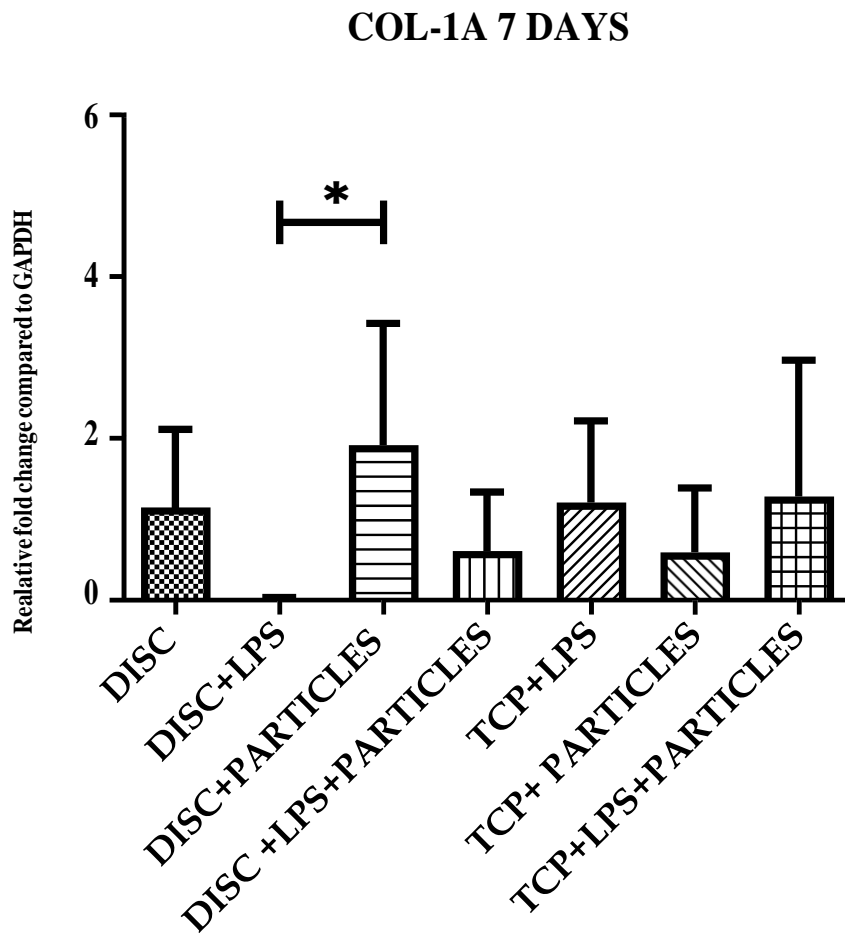*Diagram 6. Gene expression of collagen (COL1A1), 5 days after treatment.*

One week after the treatment, the levels of IL-6 expression for the 'particles' group is statistically higher than in the 'LPS' group, for cells on the SLA surfaces. There is no such tendency in the TCP group, neither are the gene expression patterns between the two different culture surfaces similar.
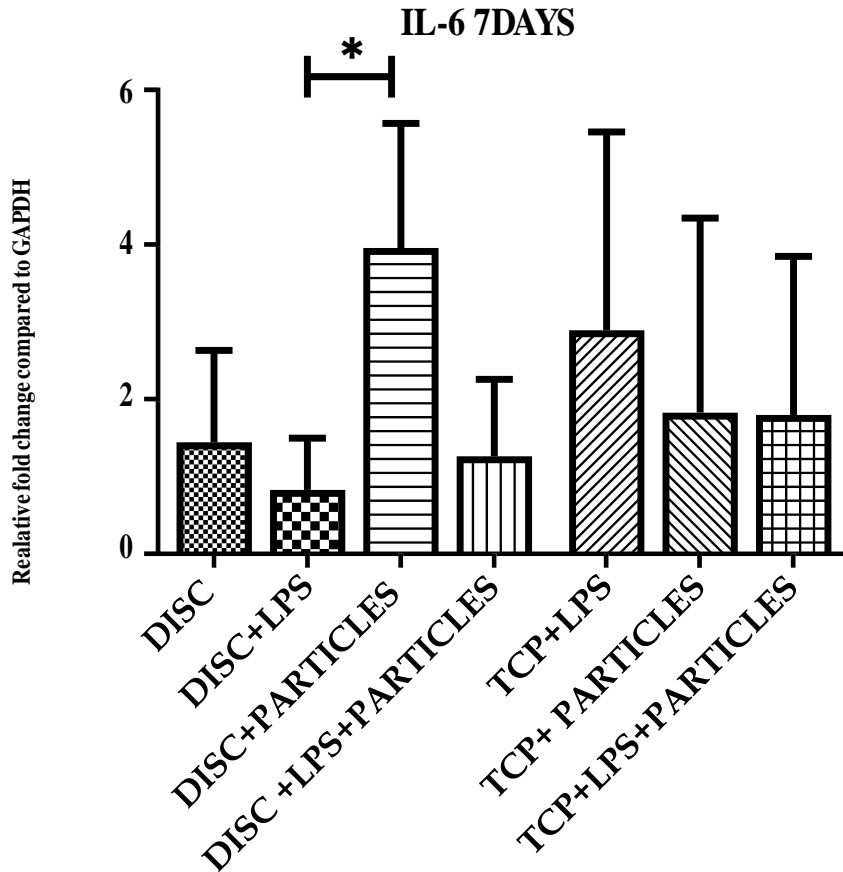
IL-6 7DAYS

Diagram 7. Gene expression of IL-6, 7 days after treatment.

As far as Collagen expression is concerned, there is a similar result to the IL-6 expression, 7 days after the treatment. There is a statistically significant difference between the 'particles' and 'LPS' groups, in favor of the particles, for the SLA surfaces. There are also equal expressions between the 'LPS and particles' and 'LPS' groups, for the TCP surfaces. Regarding the expression of IL-8 at 7 days, there were no statistically significant differences for any of the groups. There are, however, increased expression levels for both LPS

groups, as well as the rest. The results between the SLA and TCP surfaces are also more homogenous.

COL-1A 7 DAYS

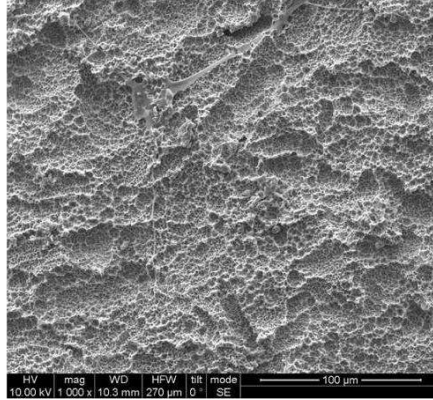*Diagram 8. Gene expression of collagen (COL1A1), 7 days after treatment.*
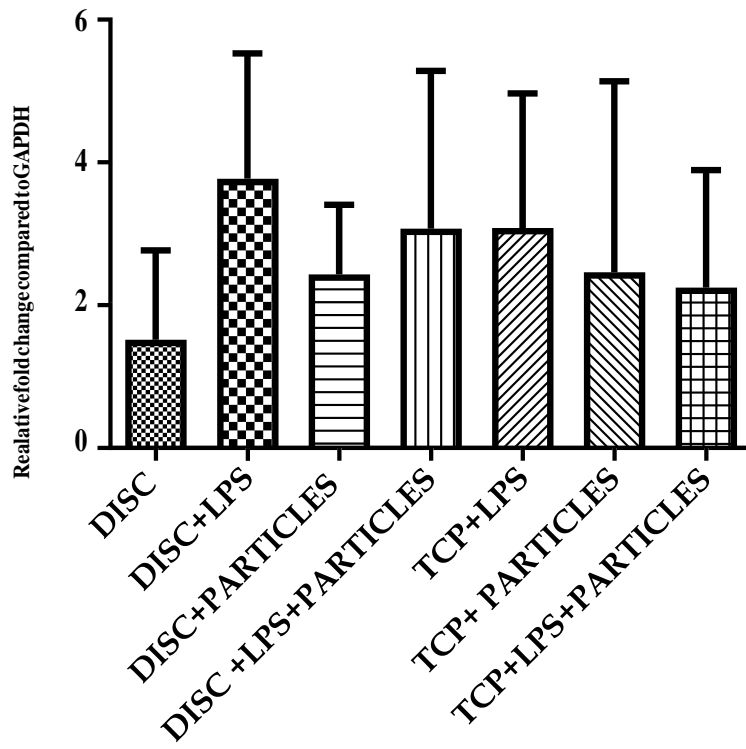
Diagram 9. Gene expression of IL-8, 7 days after treatment.
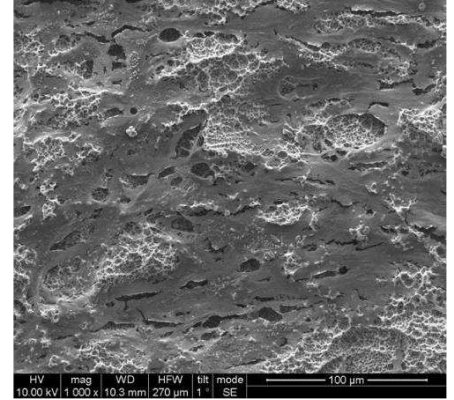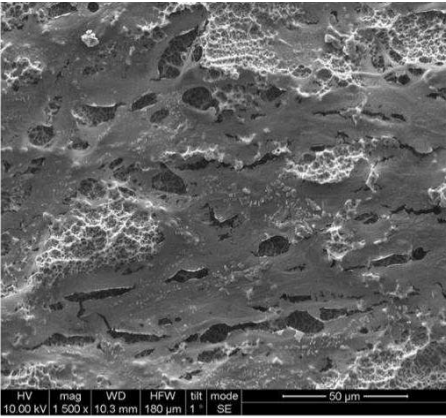
Scanning Electron Microscopy (SEM)

SEM microphotography of human gingival fibroblasts, 5 days after cell culture on SLA disks. x500 magnification
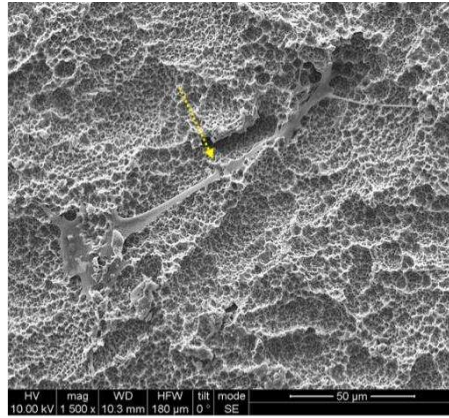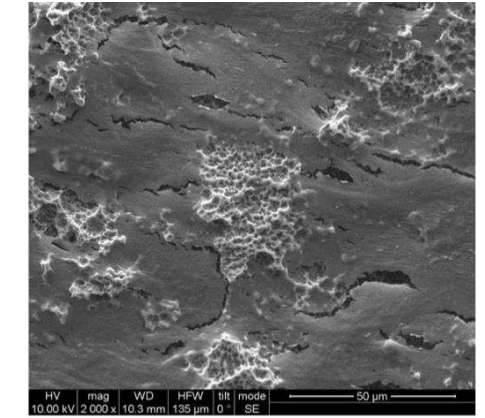

SEM microphotography of human gingival fibroblasts, 5 days after cell culture on SLA disks. x1000 magnification

SEM microphotography of human gingival fibroblasts, 5 days after cell culture on SLA disks. x1000 magnification
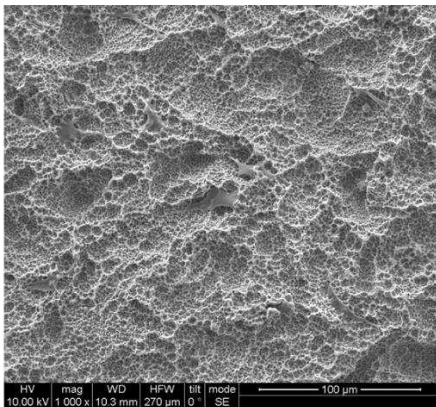

SEM microphotography of human gingival fibroblasts, 5 days after cell culture on SLA disks. x1500 magnification

SEM microphotography of human gingival fibroblasts, 5 days after cell culture on SLA disks. x1500 magnification
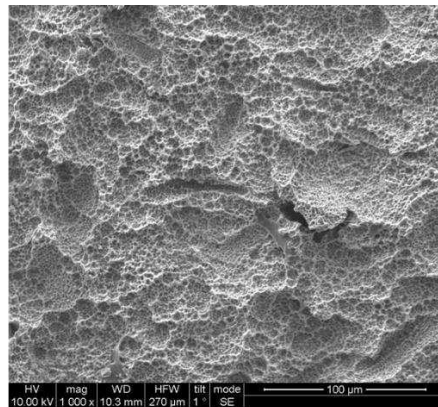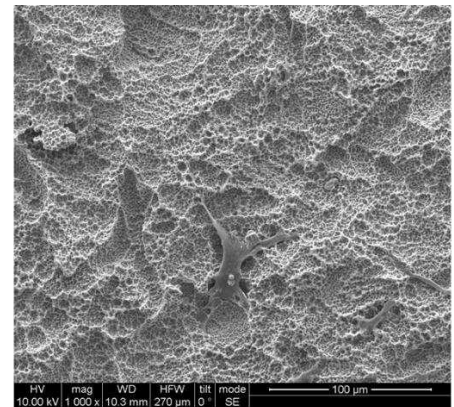

SEM microphotography of human gingival fibroblasts, 5 days after cell culture on SLA disks. x2000 magnification

SEM microphotography of human gingival fibroblasts treated with LPS, 5 days after cell treatment on SLA disks. x1000 magnification

SEM microphotography of human gingival fibroblasts treated with LPS, 5 days after cell treatment on SLA disks. x1000 magnification
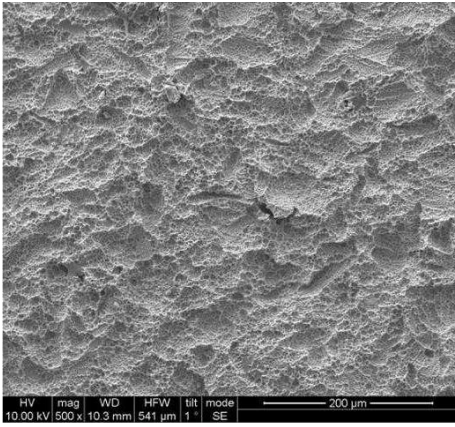

SEM microphotography of human gingival fibroblasts treated with LPS, 5 days after cell treatment on SLA disks. x1000 magnification

SEM microphotography of human gingival fibroblasts treated with LPS, 5 days after cell treatment on SLA disks. x500 magnification

SEM microphotography of human gingival fibroblasts treated with LPS, 5 days after cell treatment on SLA disks. x500 magnification
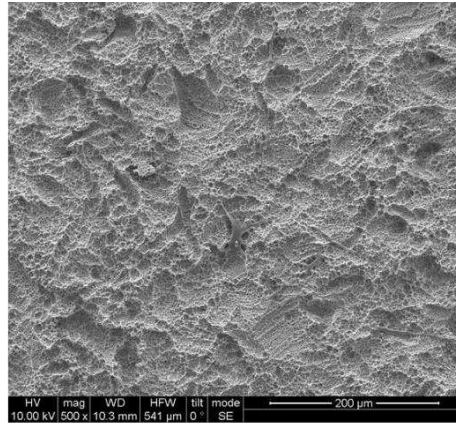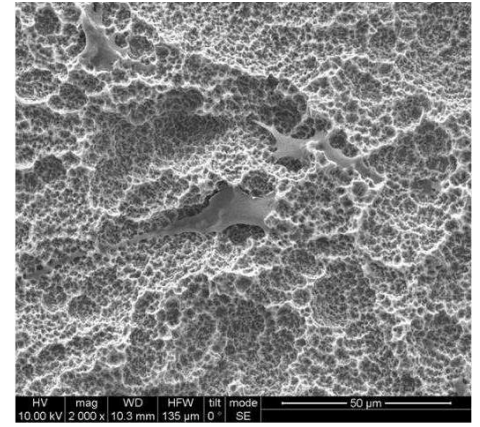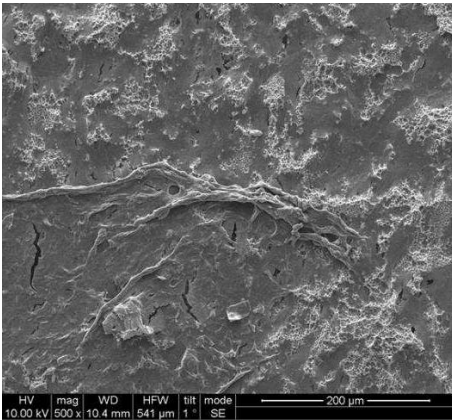

SEM microphotography of human gingival fibroblasts treated with LPS, 5 days after cell treatment on SLA disks. x2000 magnification

SEM microphotography of human gingival fibroblasts treated with Ti particles, 5 days after cell treatment on SLA disks. x500 magnification

SEM microphotography of human gingival fibroblasts treated with Ti particles, 5 days after cell treatment on SLA disks. x1000 magnification

SEM microphotography of human gingival fibroblasts treated with Ti particles, 5 days after cell treatment on SLA disks. x2000 magnification

SEM microphotography of human gingival fibroblasts treated with Ti particles, 5 days after cell treatment on SLA disks.
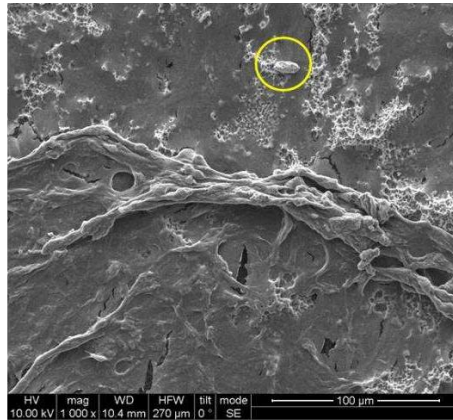x10.000 magnification

SEM microphotography of human gingival fibroblasts treated with Ti particles, 5 days after cell treatment on SLA disks.
x20.000 magnification

SEM microphotography of human gingival fibroblasts treated with LPS and Ti particles, 5 days after cell treatment on SLA disks.
x500 magnification

SEM microphotography of human gingival fibroblasts treated with LPS and Ti particles, 5 days after cell treatment on SLA disks.
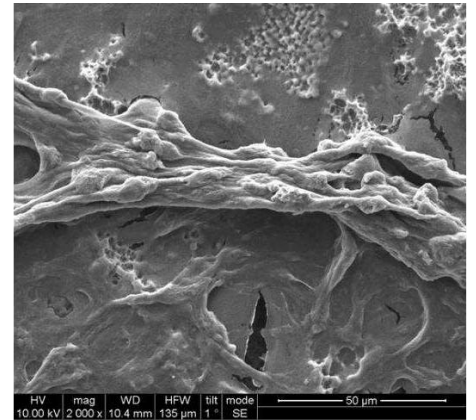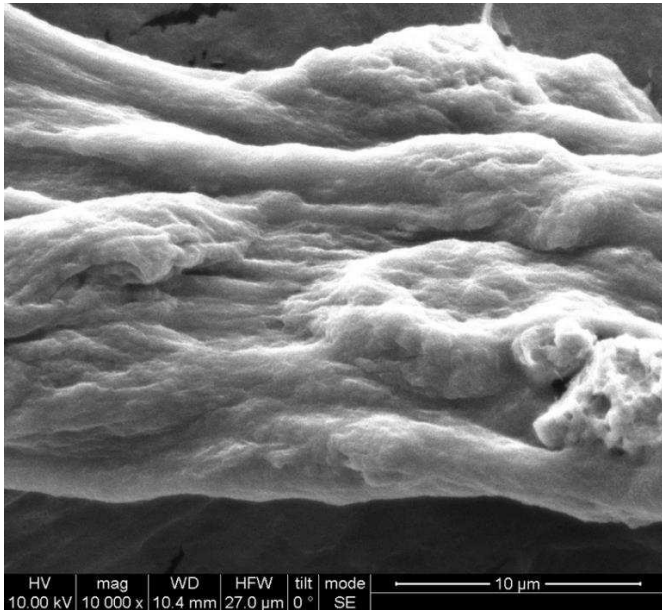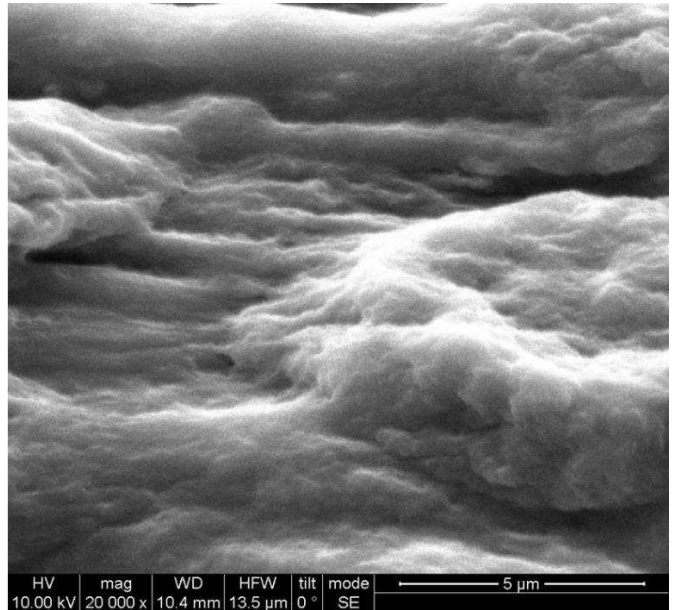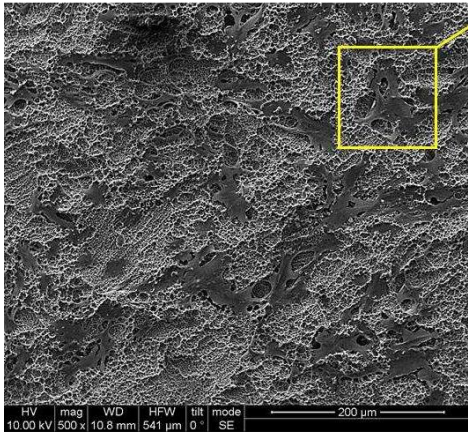x1000 magnification

SEM microphotography of human gingival fibroblasts treated with LPS and Ti particles, 5 days after cell treatment on SLA disks.
x2000 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell culture on SLA disks.
x500 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell culture on SLA disks.
x1000 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell culture on SLA disks.
x1000 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell culture on SLA disks.
x2000 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS.
x500 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS.
x1000 magnification

SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS.
x2000 magnification
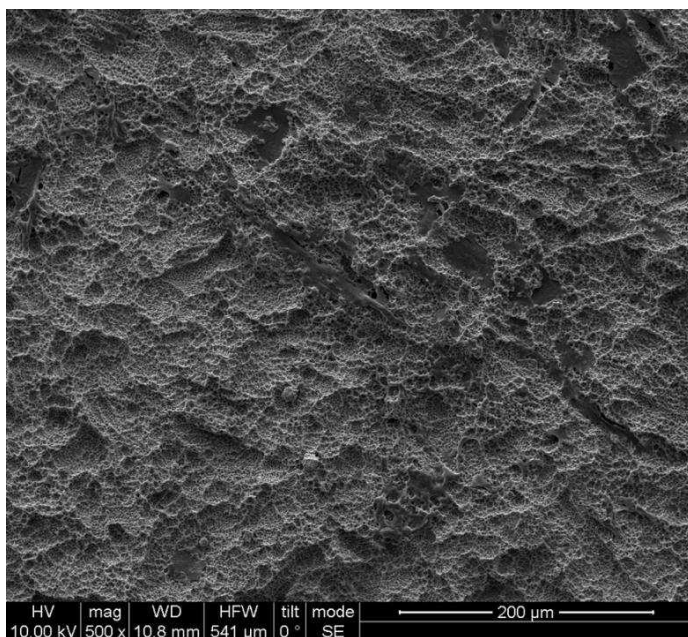
SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS.
x500 magnification
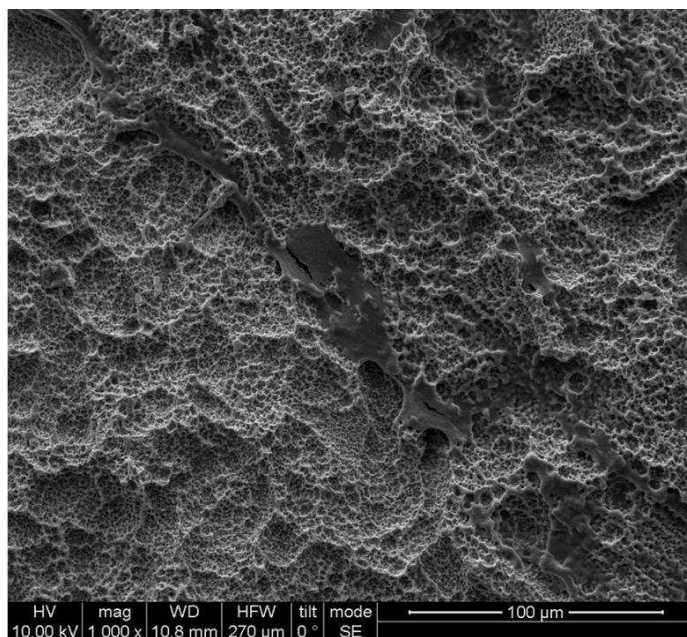
SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS.
x1000 magnification
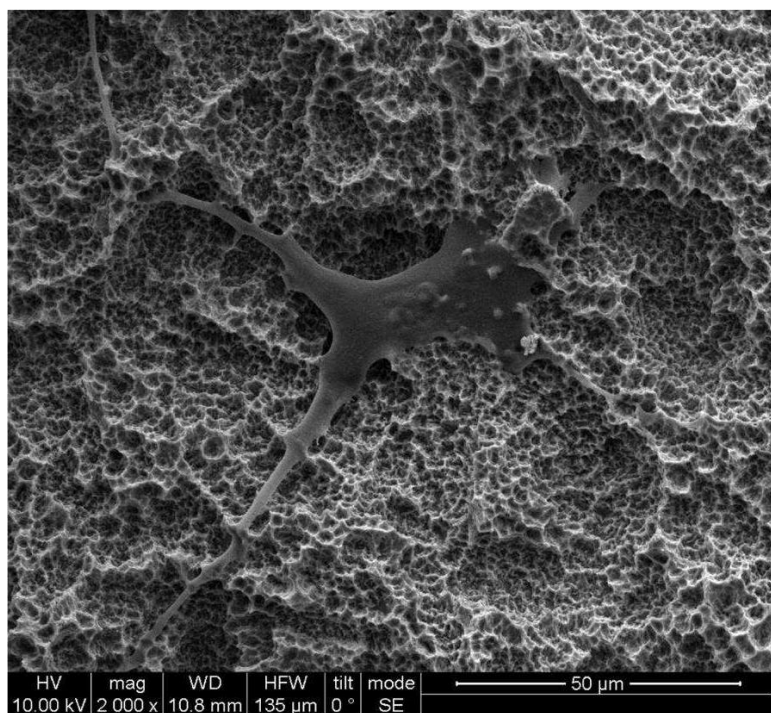
SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with Ti particles.
x1000 magnification
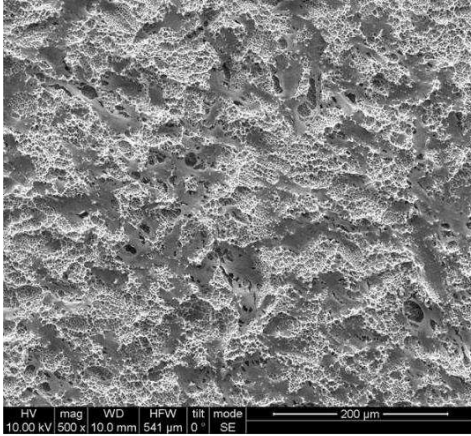
SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS and Ti particles.
x500 magnification
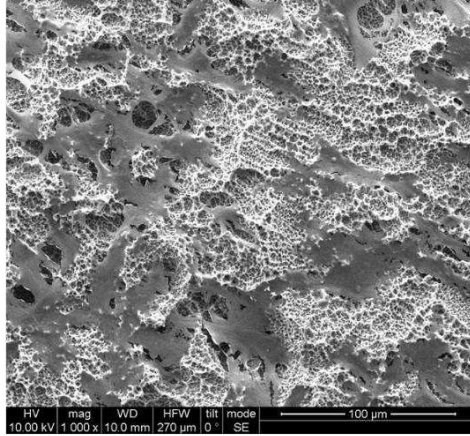
SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS and Ti particles.
x1000 magnification
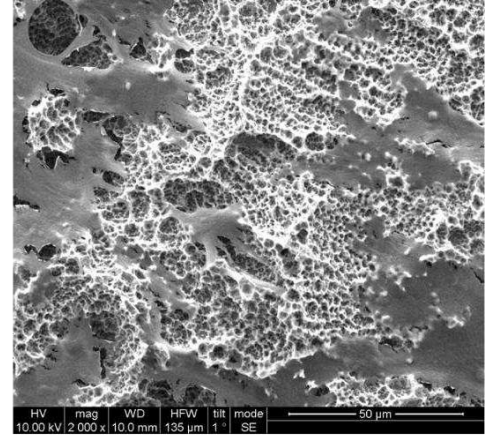
SEM microphotography of human gingival fibroblasts, 7 days after cell treatment with LPS and Ti particles.
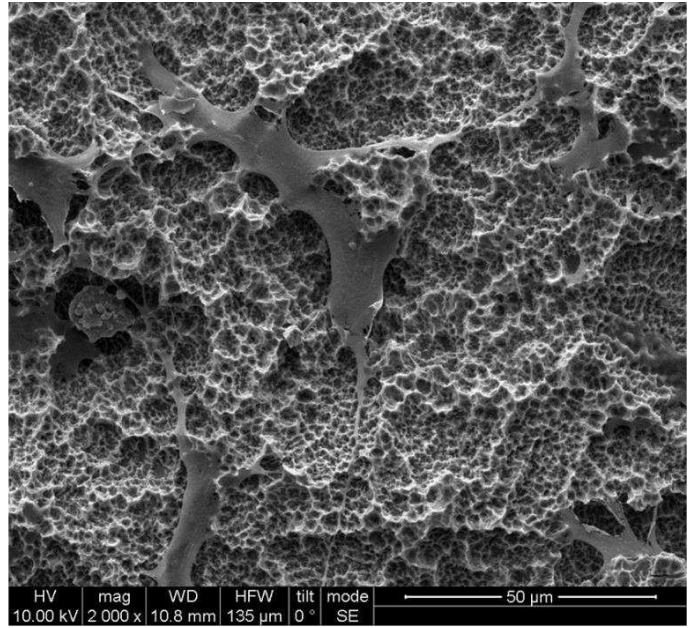x2000 magnification

Scanning Electron Microscopy of the SLA titanium disks, five and seven days after the cell treatment, has revealed a unique morphology of the human gingival fibroblasts, which were trying to adapt to the microrough titanium surface. Despite the fact that they appear to have an elongated shape with prominent filopodias, those filopodias are greatly extended, either in an attempt to gain contact with adjacent cells or to adhere on a surface whose 3D structure is full of grooves. The extracellular matrix is disrupted on some parts, yet present. It seems that the cell population is reduced in all treatment groups, however it is not exactly clear whether that can be attributed to the rough surface or mostly the treatment. Gingival fibroblasts also appear slightly detached from the SLA surfaces, though not round shaped. There are also

large zones with no cells. Titanium particles can be seen in some images (yellow circle, SEM image, treatment with particles, 5 days).

Discussion

The purpose of this experiment was to investigate the implication and effect of titanium particles on peri-implant inflammation and, specifically, to investigate their influence on pro-inflammatory cytokines and collagen gene expression *in vitro*, as well as their interaction with Lipopolysaccharide derived from *P. gingivalis*.

The limitations of this *in vitro* study is mainly the fact that we cannot make safe clinical interpretations of our results, since cell behavior is much different in a living organism, where all systems and different types of cell populations interact. It would also be beneficial to perform protein expression analyses, since over-expression of a gene, does not necessarily constitute protein production as well.

When this experiment was designed, little was known on the effect of SLA surfaces on gingival fibroblasts. In the recently published study of Maal et al the authors wanted to study the growth of human gingival fibroblasts on different titanium surfaces *in vitro*. Those surfaces were modified with different types of instruments since the experiment was a laboratory simulation of different ways to clinically perform implantoplasty. What was very interesting was that all modified surfaces facilitated the gingival fibroblasts' growth, except for the untreated SLA surfaces.[49] This is in accordance with our results from the MTT assay, where for all time periods and, regardless of the treatment, there was a decrease in the proliferation on SLA surfaces compared to the TCP surfaces. This is not just a short-term effect, as the authors of the other study have noticed a statistically significant difference

between proliferation on SLA surfaces and modified ones, even after 15 and

30 days of culture, when very few cells were left on the SLA surface, however, the fibronectin

remained. Apart from the proliferation parameter, there was also a statistically significant

increase of IL-6 for the SLA group compared to the modified surfaces. This is also in

agreement with our findings, but the extent of the effect of the surface on cells regarding this

interleukin is not clear, as it was also increased by the treatment provided. On dermal fibroblasts,

increase in the levels of IL-6 means fibrosis and we have indeed observed an increase of

collagen expression for the same groups.[49] Happe et al also studied the effect of

titanium powder on hGFs and osteoblasts in vitro. They report a negative correlation

between cell viability and proliferation and titanium particles accumulation. That, however, may

be associated to the profoundly increased concentrations of particle powder that were used

(0,01-0,1-1,0 mg/ml). They also report long-term (up to 21 days) IL-

6 expression on gingival fibroblasts, while osteoblasts only expressed this chemokine in the

first 24 hours. However, this expression of IL-6 for gingival fibroblasts was significantly different

than the control group only for the lowest concentration of particles. In the highest concentration,

the IL-6 expression levels were almost equal or smaller than the control.[50] This could be explained by

the cytotoxic effect this quantity of particles has on the cells.

The effect of titanium particles on different cell lines has already been studied. In osteoblasts, they

induce alterations on the levels of RANKL and OPG (osteoprotegerin). CCL-2 increase activates

macrophage infiltration, in the presence of LPS from *P.gingivalis*. Titanium concentration

of 9 ppm acts synergistically with the LPS, thus increasing RANKL, OPG and CCL2.

levels.[51] We also confirm that there must be a synergistical activity of titanium particles and LPS, when it comes to their effect on hGFs, since we detected greater expression levels of IL-8 in their presence, compared to when we treated the cells with either of them only.

Proper function of osteoblasts is critical to successful bone growth and, as a result, integration strength.[52] Osteoblasts treated with titanium particles do not have a clear nuclear region, but do have a disrupted focal adhesion. What is mostly engaging, however, is that particles of different sizes act through different mechanisms, all adverse. Particles with sizes between $(10 < x < 15) \mu m$ can physically prevent titanium integration, while small sizes $(<1,5 \mu m - 10 \mu m)$ cause phagocytosis events that result in smaller integration strength. Pro-inflammatory cytokines, such as IL-6, are released from macrophages after phagocytosis and after the development of an erosive pannus that stimulates bone resorption.[53] Metal-associate debris is also known to cause more extensive macrophage differentiation and bone resorption.[54,55]

A recent experimental study from Kotsakis et al has shown the effects of different cleaning instruments on titanium dissolution and biofilm removal from implant surfaces. The authors found that instruments with almost equal hardness to the titanium, such as titanium brushes, caused greater dissolution, without being more effective in terms of biofilm removal. It was also very interesting that titanium surfaces colonized by biofilms, also caused significant dissolution, without any surface treatment implicated. The effect of this dissolution on cells was also examined and, a 15% increase in dead cells was noted for the murine fibroblasts population.[56]

Titanium particles, either generated in the oral cavity or from any replaced joint, may also migrate to distant tissues and affect them. In vivo studies in mini-pigs have shown that titanium particles may migrate to the lungs of animals that had previously had implants placed in their mandibles. Weingart et al studied titanium accumulation on adjacent lymph nodes, following the placement of implants, and detected their vessels of transportation, which were lymphocytes.[57] However, they did not report any signs of inflammation or foreign-body reaction. Apart from the lymphatic path, particles can also be transferred through the blood circulation to the spleen or liver.[58]

Despite the continuously growing evidence that particles can indeed cause adverse tissue reactions, there is also strong scientific objection. Stavropoulos et al published, in 2019, a systematic review on the mechanical and biological complications after implantoplasty. The authors also refer to the issue of particle release and state that it is very difficult to control particle accumulation when clinically modifying an implant surface. That means that the quantity and sizes of particles vary significantly, depending on the case and instruments used.[59]

Could it be that there is also a critical mass of titanium particles, like with bacteria?

It is not yet known.

According to Siddiqi et al, cases of sensitivity reactions to titanium may be underreported since they are not usually included in potential causes of implant failure.[60] However, it is also stated that no clinical studies have mentioned any adverse effects and, inflammation induced in *in vivo* studies

was 'limited, low grade and chronic'. The 5th EAO Consensus analyzes the issue of particles, stating that there is weak evidence to prove hypersensitivity reactions to titanium particles. It also reports that despite the direct and indirect influence of particles on peri-implant tissues and different cell lines, there is not a simple cause-and-effect relationship between titanium particles and biological complications so far.[61] That, however, is difficult to prove in an environment so abundant in microbes that easily accumulate, form biofilms and cause inflammation.

Considering that gingival fibroblasts have immunoregulatory function, we wanted to examine the nature of response of the soft tissues to a potentially harmful agent. We have not only confirmed the synergistical activity of particles with LPS, but also the strong reaction that particles themselves can induce on gingival fibroblasts. IL-6 and IL-8 are major chemokines implicated in chronic inflammation and regulate neutrophil function. Collagen expression may not be a positive finfing, since it could clinically constitute fibrosis, especially in an inflammatory environment, where the organism tries to ringfence the lesion.

Future extensions of this study could be the investigation of molecular pathways that lead to overexpression of certain genes associated with inflammation, but also cell death. Moreover, protein detection assays could be very useful since the gene expression itself, does not necessarily mean increase of the quantity of the final product. Since fibrosis and chronic inflammation are strongly correlated to cellular senescence, the existence and influence of a senescent substrate on disease recurrence or deterioration

could also be examined. More *in vitro* and *in vivo* simulation studies will certainly be more enlightening on the subject.

Conclusions

This experiment was a simulation of the biological conditions present in cases of established periimplantitis. Human gingival fibroblasts were cultured on SLA or TCP surfaces and treated with P.gingivalis LPS, titanium particles and LPS with titanium particles combined. The effect of this treatment on two major pro-inflammatory cytokines (IL-6, IL-8) gene expression was studied, as well as on the collagen gene expression. Two of the most significant fibroblast functions were thus examined in response to an adverse factor. The results of the study, lead to the following conclusions:

1. **SLA surfaces are not an optimal surface for gingival fibroblast adhesion and proliferation**. However, the qualitative FDA/PI analysis revealed only a few apoptotic cells on SLA surfaces that received no treatment and a significant rate of proliferation every 24 hours, despite not being as increased as on the TCP surfaces.

2. The **synergistical action** of LPS and titanium particles is the **most important finding** of this study, since it induces greater responses than the mere action of particles or LPS alone, as far as the expression of proinflammatory cytokines is concerned.

3. **Collagen** expression is **increased** due to the synergistical action of B and particles, whereas in the LPS groups, there was almost no collagen expression. Further investigation of this phenomenon is certainly needed.

4. Cell viability is influenced by the treatment, since many apoptotic cells were present in all treatment groups, as was revealed by the FDA-PI qualitative staining.

5. The morphology and gap junctions of cells on SLA surfaces are seriously disrupted.

6. Clinicians must take great caution when treating peri-implant diseases or during the maintenance phase of implant-bearing patients, since iatrogenic particle release may, in combination with bacterial virulence factors, may jeopardize the long-term biological stability of an implant.

Literature

1. Branemark, P-I. "Osseointegration and its experimental background." *J prosthet Dent* 50 (1983): 399-410.

2. Albrektsson T, Berglundh T, Lindhe J. Osseointegration: Historic Background and Current Concepts. In: Lindhe J, editor. Clinical Periodontology and Implant Dentistry. 4th ed. Oxford: Blackwell Publishers Ltd; 2003. pp. 809–20.

3. Lee JWY, Bance ML. Physiology of Osseointegration. Otolaryngol Clin North Am. 2019;52(2):231-242. doi:10.1016/j.otc.2018.11.004

4. Jain, Rachna, and Daljit Kapoor. "The dynamic interface: A review." *Journal of International Society of Preventive & Community Dentistry* 5.5 (2015): 354.

5. Ιωάννης Κ. Καρούσης. <<Περιμεφυτευματίτιδα. Από τη Διάγνωση στην Αντιμετώπιση>>. Κεφάλαιο 2 - << Επούλωση των περιεμφυτευματικών ιστών μετά την τοποθέτηση εμφυτευμάτων>>. Εκδόσεις ΟΔΟΝΤΙΑΤΡΙΚΟ ΒΗΜΑ, Αθήνα 2020.

6. Zitzmann, Nicola U., and Tord Berglundh. "Definition and prevalence of peri- implant diseases." *Journal of clinical periodontology* 35 (2008): 286-291.

7. Ioannis K. Karoussis. Periimplantitis: From diagnosis to treatment. Chapter 7: Diagnosis of peri-implant diseases. Copyright 2020, Odontiatriko Vima, Athens.

8. Berglundh T, Armitage G, Araujo MG, Avila-Ortiz G, Blanco J, Camargo PM, Chen S, Cochran D, Derks J, Figuero E, Hämmerle CHF, Heitz-Mayfield LJA, Huynh-Ba G, Iacono V, Koo KT, Lambert F,

McCauley L, Quirynen M, Renvert S, Salvi GE, Schwarz F, Tarnow D, Tomasi C, Wang HL, Zitzmann N. Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Periodontol. 2018 Jun;89 Suppl 1:S313-S318. doi:10.1002/JPER.17-0739. PMID:29926955.

9. Karring ES, Stavropoulos A, Ellegaard B, et al. Treatment of periimplantitis by the Vector system. Clin Oral Implants Res. 2005;16:288–293.

10. Renvert S, Samuelsson E, Lindahl C, et al. Mechanical non-surgical treatment of peri-implantitis: A double-blind randomized longitudinal clinical study. I: Clinical results. J Clin Periodontol. 2009;36:604–609.

11. Ioannis K. Karoussis. Periimplantitis: From diagnosis to treatment.

    Chapter 9: Initial phase of therapy. Copyright 2020, Odontiatriko Vima, Athens.

12. Yang SM, Park JB, Ko Y. Use of confocal microscopy for quantification of plastic remnants on rough titanium after instrumentation and evaluation of efficacy of removal. Int J Oral Maxillofac Implants 2015;30:519–525.

13. Khoury, F., Keeve, P. L., Ramanauskaite, A., Schwarz, F., Koo, K., Sculean, A., & Romanos, G. (2019). Surgical treatment of peri- implantitis – Consensus report of working group 4. International Dental Journal, 69(S2), 18–22. doi:10.1111/idj.12505

14. Carcuac O, Derks J, Abrahamsson I, Wennström JL, Berglundh T. Risk for recurrence of disease following surgical therapy of peri-implantitis—

A prospective longitudinal study. Clin Oral Impl Res. 2020;00:1–6. https://doi.org/10.1111/clr.13653.

15. Berglundh, T., Wennström, J. L., & Lindhe, J. (2018). Long-term outcome of surgical treatment of peri-implantitis. A 2–11-year retrospective study. Clinical Oral Implants Research, 29(4), 404–410. https://doi. org/10.1111/clr.13138.

16. Ferguson, A.B.; Akahoshi, Y.; Laing, P.G.; Hodge, E.S. Characteristics of trace ion release from embedded metal implants in the rabbit. J Bone Jt. Surg. 1962, 44, 317–336.

17. Ioannis K. Karoussis. Periimplantitis: From diagnosis to treatment. Chapter 18 Titanium corrosion and peri-implant lesions associated with it. Copyright 2020, Odontiatriko Vima, Athens.

18. Sandvik Materials Technology. Material datasheets. 06 http://smt.sandvik.com/en/materials-center/material-data-sheets/.

19. Rashad, A.; Sadr-Eshkevari, P.; Weuster, M.; Schmitz, I.; Prochnow, N.; Maurer, P. Material attrition and bone micromorphology after conventional and ultrasonic implant site preparation. Clin. Oral Implant. Res. 2013, 24, 110–114.

20. Schwarz F, Sahm N, Mihatovic I, Golubovic V, Becker J. Surgical therapy of advanced ligature-induced peri-implantitis defects: cone-beam computed tomographic and histological analysis. J Clin Periodontol. 2011;38:939-949.

21. Carvalho, A.C.; Queiroz, T.P.; Okamoto, R.; Margonar, R.L.; Garcia, I.R., Jr.; Filho, O.M. Evaluation of bone heating, immediate bone cell viability, and wear of high-resistance drills after the creation of implant

osteotomies in rabbit tibias. Int. J. Oral Maxillofac. Implant. 2011, 6, 1193–1201.

22. Chacon, G.E.; Bower, D.L.; Larsen, P.E.; McGlumphy, E.A.; Beck, F.M. Heat production by 3 implant drill systems after repeated drilling and sterilization. J. Oral Maxillofac. Surg. 2006, 64, 265–269.

23. Sartori, E.M.; Shinohara, É.H.; Ponzoni, D.; Padovan, L.E.M.; Valgas, L.; Golin, A.L. Evaluation of deformation, mass loss, and roughness of different metal burs after osteotomy for osseointegrated implants. J Oral Maxillofac. Surg. 2012, 70, e608–e621.

24. Guan, H.; van Staden, R.C.; Johnson, N.W.; Loo, Y.-C. Dynamic modelling and simulation of dental implant insertion process—A finite element study. Finite Elem. Anal. Des. 2011, 47, 886–897.

25. Klotz, M.W.; Taylor, T.D.; Goldberg, A.J. Wear at the titanium-zirconia implant-abutment interface: A pilot study. Int. J. Oral Maxillofac. Implant. 2011, 26, 970–975.

26. *Delgado-Ruiz, R., & Romanos, G. (2018). Potential Causes of Titanium Particle and Ion Release in Implant Dentistry: A Systematic Review. International Journal of Molecular Sciences, 19(11), 3585.*

27. Prado, A.; Pereira, J.; Henriques, B.; Benfatti, C.; Magini, R.; López-López, J.; Souza, J. Biofilm affecting the mechanical integrity of implant-abutment joints. Int. J. Prosthodont. 2016, 29, 381–383.

28. Alqutaibi, A.Y.; Aboalrejal, A.N. Microgap and micromotion at the implant abutment interface cause marginal bone loss around dental implant but more evidence is needed. J. Evid. Based Dent. Pract. 2018, 18, 171–172.

29. Davide Prando, Andrea Brenna, Maria Vittoria Diamanti, Silvia Beretta, Fabio Bolzoni, Marco Ormellese, MariaPia Pedeferri. Corrosion of Titanium: Part 1: Aggressive Environments and Main Forms of Degradation. Journal of Applied Biomaterials and Functional Materials, 2018, Volume: 15 issue: 4, page(s): e291-e302.

30. Liu J., Alfantazi A., Asselin E. Effects of temperature and sulfate on the pitting corrosion of titanium in high-temperature chloride solutions. J Electrochem Soc. 2015; 162(4): C189–C196.

31. Mathew, M.T.; Pai, P.S.; Pourzal, R.; Fischer, A.; Wimmer, MA Significance of tribocorrosion in biomedical applications: Overview and current status. Adv. Tribol. 2009, 2009, 1–12.

32. Revathi, A.; Borrás, A.D.; Muñoz, A.I.; Richard, C.; Manivasagam, G. Degradation mechanisms and future challenges of titanium and its alloys for dental implant applications in oral environment. Mater. S Eng. C 2017, 76, 1354–1368.

33. Gallo J, Vaculova J, Goodman SB et al: Contributions of human tissue analysis to understanding the mechanisms of loosening and osteolysis in total hip replacement. Acta Biomater, 2014; 10: 2354–66.

34. Zhao YP, Wei JL, Tian QY et al: Progranulin suppresses titanium particle induced inflammatory osteolysis by targeting TNFalpha signaling. Sci Rep, 2016; 6: 20909.

35. Lin TH, Tamaki Y, Pajarinen J et al: Chronic inflammation in biomaterial-induced periprosthetic osteolysis: NF-kappaB as a therapeutic target. Acta Biomater, 2014; 10: 1–10

36. Wang Z, Liu N, Shi T et al: ER stress mediates TiAl6V4 particle-induced periimplant osteolysis by promoting RANKL expression in fibroblasts. PLoS One, 2015; 10: e137774

37. O'Neill SC, Queally JM, Devitt BM et al: The role of osteoblasts in peri-prosthetic osteolysis. Bone Joint J, 2013; 95-B: 1022–26.

38. Guihua Wang, Pin Zhang, Jianning Zhao. Endotoxin Contributes to Artificial Loosening of Prostheses Induced by Titanium Particles. Med Sci Monit, 2018; 24: 7001-7006 DOI: 10.12659/MSM.910039.

39. Ιωάννης Κ. Καρούσης. <<Περιμεφυτευματίτιδα. Από τη Διάγνωση στην Αντιμετώπιση>>. Κεφάλαιο 1 - << Ανατομία περιοδοντικών και περιεμφυτευματικών ιστών>>. Εκδόσεις ΟΔΟΝΤΙΑΤΡΙΚΟ ΒΗΜΑ, Αθήνα 2020.

40. Page RC, Schroeder HE. Periodontitis in men and other animals. Basel: S Karger, 1982.

41. Korn T, Bettelli E, Oukka M, Kuchroo VK 2009. IL-17 and Th17 cells. Annu Rev Immunol 27: 485–517

42. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner H, Kuchroo VK 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441: 235–238

43. Kimura A, Kishimoto T 2010. IL-6: Regulator of Treg/Th17 balance. Eur J Immunol 40: 1830–1835.

44. Bartold PM, Haynes DR. Interleukin-6 production by human gingival fibroblasts. J Periodontal Res. 1991 Jul;26(4):339-45. doi 10.1111/j.1600-0765.1991.tb02072.x. PMID: 1831501.

45. Takigawa, M., Takashiba, S., Myokai, F., Takahashi, K., Arai, H., Kurihara, H., & Murayama, Y. (1994). Cytokine-Dependent Synergistic Regulation of Interleukin-8 Production From Human Gingival Fibroblasts. Journal of Periodontology, 65(11), 1002–1007. doi:10.1902/jop.1994.65.11.1002.

46. Biomineralization and Biomaterials, 2016

47. Biochemistry of Collagens, Laminins and Ela https://doi.org/10.1016/B978-0-12-817068-7.00001-X

48. Dipshikha Chakravortty, K.S.Nanda Kumar. Induction of Cell Proliferation and Collagen Synthesis in Human Small Intestinal Lamina Propria Fibroblasts by Lipopolysaccharide: Possible Involvement of Nitric Oxide. Biochemical and Biophysical Research Communications, Volume 240, Issue 2, 17 November 1997, Pages 458-463.

49. Beheshti Maal, M., Aanerød Ellingsen, S., Reseland, J.E. et al. Experimental implantoplasty outcomes correlate with fibroblast growth in vitro. BMC Oral Health 20, 25 (2020). https://doi.org/10.1186/s12903-020-1012-1.

50. Happe A, Sielker S, Hanisch M, Jung S. The Biological Effect of Particulate Titanium Contaminants of Dental Implants on Human Osteoblasts and Gingival Fibroblasts. Int J Oral Maxillofac Implants. 2019 Feb 26. doi: 10.11607/jomi.6929. Epub ahead of print. PMID 31170276.

51. Wachi T, Shuto T, Shinohara Y, Matono Y, Makihira S. Release of titanium ions from an implant surface and their effect on cytokine production related to alveolar bone resorption. Toxicology. 2015 Jan

2;327:1-9. doi: 10.1016/j.tox.2014.10.016. Epub 2014 Nov 3. PMID: 25446332.

52. Choi MG, Koh HS, Kluess D, O'Connor D, Mathur A, Truskey GA, Rubin J, Zhou DX, Sung KL. Effects of titanium particle size on osteoblast functions in vitro and in vivo. Proc Natl Acad Sci U S A. 2005 Mar 22;102(12):4578-83. doi: 10.1073/pnas.0500693102. Epub 2005 Mar 8. PMID: 15755807; PMCID: PMC555523.

53. Schwartz, Z., Lohmann, C. H., Wieland, M., Cochran, D. L., Dean, D. D., Textor, M., Bonewald, L. F. & Boyan, B. D. (2000) J. Periodontol. 71
, 586–597. pmid:10807123.

54. Kadoya, Y., Salmivirta, K., Talts, J. F., Kadoya, K., Mayer, U., Timpl, R. & Ekblom, P. (1997) Development (Cambridge, U.K.) 124, 683–691.

55. Sabokbar, A., Pandey, R., Quinn, J. M. & Athanasou, N. A. (98) Arch. Orthop. Trauma Surg. 117, 136–140. pmid:9521517.

56. Kotsakis, Georgios A., et al. "Effect of implant cleaning on titanium particle dissolution and cytocompatibility." Journal of periodontology (2020).

57. Weingart, D., Steinemann, S., Schilli, W., Strub, J.R., Hellerich, U, Assenmacher, J. & Simpson, J. (1994) Titanium deposition in regional lymph nodes after insertion of titanium screw implants in maxillofacial region. The International Journal of Oral and Maxillofacial Surgery 23: 450–452.

58. Heringa, M. B., Peters, R. J. B., Bleys, R. L. A. W., van der Lee, M. K, Tromp, P. C., van Kesteren, P. C. E., van Eijkeren, J. C. H., Undas, A. K., Oomen, A. G., & Bouwmeester, H. (2018). Detection of titanium

particles in human liver and spleen and possible health implications. Particle and Fibre Toxicology, 15(1), [15]. https://doi.org/10.1186/s12989-018-0251-7.

59. Stavropoulos A, Bertl K, Eren S, Gotfredsen K. Mechanical and biological complications after implantoplasty—A systematic review. Clin Oral Impl Res. 2019;00:1–16. https://doi.org/10.1111/clr.13499.

60. Siddiqi, A., Payne, A. G. T., De Silva, R. K., & Duncan, W. J. (2011). Titanium allergy: could it affect dental implant integration? Clinical Oral Implants Research, 22(7), 673–680.

61. Schliephake, H., Sicilia, A., Nawas, B. A., Donos, N., Gruber, R., Jepsen, S., … Stavropoulos, A. (2018). Drugs and diseases: Summary and consensus statements of group 1. The 5th EAO Consensus Conference 2018. Clinical Oral Implants Research, 29, 93–99. doi:10.1111/clr.13270.

Abstract

The substantial increase of patients bearing implants, the past two decades, brings the clinician before the prevention and treatment of peri-implant pathology, as well as the long-term maintenance of the therapeutic outcome. The past years, the research interest has been turned towards free titanium particles, that are the product of corrosive events or the mechanical debridement of the implant surface, during the surgical therapy of periimplantitis or during the maintenance care program with several types of instruments. The question is whether these particles are capable of inducing or worsen an already established inflammatory reaction that will certainly not improve the condition, despite the good will of the clinician.

Aim

The aim of the present study is to investigate the effect of titanium particles, on the same or maybe greater degree than P. gingivalis lipopolysaccharide, on the gene expression of two major pro-inflammatory cytokines, interleukin-6 and interleukin-8, as well as gene expression of collagen type I of human gingival fibroblasts. The possibility of a synergistical activity between titanium particles and LPS is also examined.

Materials and Methods

Human gingival fibroblasts were cultured on sand blasted and acid-etched (SLA) titanium and TCP surfaces for 7 days. 24 hours after the culture of cells on both surfaces, the cells were treated with either LPS, particles or LPS and particles combined. At 24, 48 and 72 hours after treatment, MTT assay was performed to assess cell proliferation. FDA/PI staining has also b

performed for the same time periods, in order to evaluate cell viability/apoptosis. At 5 and 7 days after the treatment, cell RNA was isolated and PCR was performed to assess gene expressions of IL-6, IL-8 and COL1A1. SEM microscopy was also performed five and seven days after the treatment for all groups on SLA surfaces.

Results

hGFs have shown a statistically significant difference in proliferation rates on SLA surfaces, compared to TCP, in favor of the latter. However, all groups, independently of the treatment kind, had a significant increase of the population between the time periods of examination. As far as the expression of interleukin levels is concerned, the combination of LPS and particles significantly increases the levels of interleukin-8 expression, compared to LPS or particles alone. On SLA surfaces, treatment with LPS and particles, induced a significant increase of interleukin-6 and collagen. FDA/PI microscopy has shown increased cell apoptosis for all treatment groups, as well as differences on cell morphology. SEM microscopy has revealed stressed cells with zones of no cells and a disrupted adhesion on SLA surfaces.

Conclusions

Rough titanium surfaces significantly affect the proliferation and morphology of human gingival fibroblasts, which contact such surfaces after bone resorption and pocket formation induced by inflammation. Moreover, the combination of titanium particles and LPS increase, in a statistically significant manner, the expression of IL-6, IL-8 and collagen. Taking into account the

immunoregulatory role of fibroblasts in the connective tissue of the peri-implant mucosa, it appears that particles arouse a similar cellular reaction as the bacterial endotoxin, while synergistically intensifying this reaction. Finally, since these cells are also responsible for collagen synthesis and extracellular matrix production, the increase in collagen expression, indicates an increased activity of cells towards the production of reactive tissue.

Key words: Titanium implants, titanium corrosion, experimental in vitro study, peri-implantitis, human gingival fibroblasts

Περίληψη

Τις τελευταίες δύο δεκαετίες, η σημαντική αύξηση των ασθενών που φέρουν εμφυτεύματα φέρνει τον κλινικό αντιμέτωπο με την πρόληψη και θεραπεία της περιεμφυτευματικής παθολογίας, καθώς και με τη μακροπρόθεσμη διατήρηση του θεραπευτικού αποτελέσματος. Τα τελευταία χρόνια, το ενδιαφέρον της ερευνητικής κοινότητας στρέφεται προς τα ελεύθερα σωματίδια τιτανίου, ως προϊόντα διαβρωτικών ή αποτριπτικών διεργασιών στην επιφάνεια του τιτανίου, είτε κατά τη χειρουργική θεραπεία της περιεμφυτευματίτιδας ή από διάφορες παρεμβάσεις κατά την εφαρμογή του προληπτικού προγράμματος. Το ερώτημα που τίθεται είναι, κατά πόσον αυτά τα σωματίδια είναι ικανά να προκαλέσουν ή να εντείνουν μια ήδη εδραιωμένη φλεγμονώδη αντίδραση των ιστών, γεγονός που σίγουρα δεν βοηθάει την ίαση μίας υπάρχουσας νοσολογικής οντότητας.

Σκοπός

Σκοπός της παρούσας διπλωματικής εργασίας είναι η διερεύνηση της επίδρασης των σωματιδίων στον ίδιο ή σε μεγαλύτερο βαθμό από το λιποπολυσακχαρίτη από P. Gingivalis, στη γονιδιακή έκφραση των προφλεγμονωδών κυτοκινών, ιντερλευκίνης-6 και ιντερλευκίνης-8, καθώς και του κολλαγόνου τύπου Ι σε ανθρώπινης προέλευσης ουλικές ινοβλάστες. Η πιθανότητα ύπαρξης μίας συνεργικής δράσης μεταξύ λιποπολυσακχαρίτη και σωματιδίων τιτανίου επίσης διερευνάται.

Μέθοδοι και Υλικά

Ανθρώπινης προέλευσης ουλικές ινοβλάστες καλλιεργήθηκαν σε αδροποιημένες και αμμοβολημένες (SLA) επιφάνειες τιτανίου, καθώς και σε

επιφάνειες πολυστυρενίου για συνολικά 7 ημέρες. Ένα εικοσιτετράωρο μετά την καλλιέργεια των κυττάρων στις επιφάνειες, πραγματοποιήθηκε η έγχυση λιποπολυσακχαρίτη, σωματιδίων τιτανίου ή και των δύο μαζί στις πειραματικές ομάδες. Μετά από 24, 48 και 72 ώρες, διενεργήθηκε δοκιμασία MTT για τον έλεγχο του κυτταρικού πολλαπλασιασμού. Επιπροσθέτως, για τις ίδιες χρονικές στιγμές, πραγματοποιήθηκε μικροσκοπία φθορισμού με χρώση FDA/PI για την αξιολόγηση της ζωτικότητας και απόπτωσης των κυττάρων. Στις 5 και 7 ημέρες, ολοκληρώθηκε η συνολική απομόνωση του RNA από όλες τις ομάδες για τον έλεγχο της γονιδιακής έκφρασης των ιντερλευκινών και κολλαγόνου, αλλά και η προετοιμασία των δίσκων για ηλεκτρονική μικροσκοπία σάρωσης.

Αποτελέσματα

Όσον αφορά στον πολλαπλασιασμό των κυττάρων, παρατηρήθηκε στατιστικά σημαντική διαφορά μεταξύ των δύο τύπων επιφανειών, με την αδρή επιφάνεια τιτανίου να ευνοεί λιγότερο τον πολλαπλασιασμό των ινοβλαστών. Παρόλα αυτά, ανεξάρτητα από το είδος της έγχυσης, όλες οι ομάδες παρουσίασαν στατιστικά σημαντική αύξηση του πολλαπλασιασμού μεταξύ των υπό εξέταση χρονικών στιγμών. Ως προς τη γονιδιακή έκφραση των ιντερλευκινών, ο συνδυασμός λιποπολυσακχαρίτη και σωματιδίων τιτανίου αυξάνει σημαντικά τα επίπεδα ιντερλευκίνης-8, σε σχέση με τη μονοθεραπεία με σωματίδια ή με ενδοτοξίνη. Επιπροσθέτως, στις επιφάνειες τιτανίου, η συνέργεια των δύο επέφερε σημαντική αύξηση τόσο της ιντερλευκίνης-6 όσο και του κολλαγόνου. Η μικροσκοπία σάρωσης αποκάλυψε την απώλεια συνδέσεων μεταξύ των κυττάρων, την επιμηκυμένη μορφολογία τους, καθώς και δυσκολία προσαρμογής τους στην επιφάνεια τιτανίου.

Συμπεράσματα

Οι αδρές επιφάνειες τιτανίου επιδρούν σημαντικά στον πολλαπλασιασμό και τη μορφολογία των ανθρωπίνων ουλικών ινοβλαστών, που υπό συνθήκες υγείας, δεν βρίσκονται σε επαφή με το αδρό τμήμα του εμφυτεύματος, παρά μόνο αν προκύψει οστική απορρόφηση. Ακόμη, ο συνδυασμός των σωματιδίων τιτανίου και του βακτηριακού λιποπολυσακχαρίτη επιφέρει στατιστικά σημαντική αύξηση των προφλεγμονωδών κυτοκινών, IL-6 και IL-8, καθώς και του κολλαγόνου τύπου I. Λαμβάνοντας υπόψιν τον ανοσορυθμιστικό ρόλο των ινοβλαστών στο συνδετικό ιστό του περιεμφυτευματικού βλεννογόνου, φαίνεται ότι τα ελεύθερα σωματίδια τιτανίου προκαλούν παρόμοια κυτταρική αντίδραση με μία μικροβιακή ενδοτοξίνη, ενώ συνεργικά εντείνουν αυτή την αντίδραση. Τέλος, καθώς τα ίδια κύτταρα είναι υπεύθυνα για τη σύνθεση κολλαγόνου και την παραγωγή της εξωκυττάριας θεμέλιας ουσίας, η αύξηση της έκφρασης του κολλαγόνου δυνητικά υποδηλώνει την αυξημένη δραστηριότητα των κυττάρων προς παραγωγή αντιδραστικού ιστού.

Λέξεις-κλειδιά: Εμφυτεύματα τιτανίου, διάβρωση του τιτανίου, περιεμφυτευματίτιδα, πειραματική μελέτη in vitro, ανθρώπινες ουλικές ινοβλάστες