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Toxicity of debris released from oral endosseous implants

Redouane Messous Haida

Dissertação conducente ao Grau de Mestre em

Medicina Dentária (Ciclo Integrado)

Gandra, 5 de junho de 2020

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Trabalho realizado sob a Orientação de "Prof Doutor Júlio C. M. Souza"

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O Orientador

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Many years have passed since I was born. From that moment and even before that, you were already looking for ways to offer me the best, you have worked hard, no matter if you arrived tired of your work, you always had a smile to offer me. The help you have given me has formed bases of great importance, now I am aware of that. Thank you very much parents my father Boujemâa and my mother Amina.

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Put your soul, your heart, your mind in everything you do therein lies the secret of success.

Redouane Messous

SUMMARY

Degradation particles released from commercially pure titanium (cp Ti) and Ti6Al4V alloys as a consequence of therapeutic treatment for periimplantitis and / or corrosion of titanium can release metallic ions and particles to peri-implant tissues. Micro- and nano-scale debris and ions induce inflammatory, mutagenic, or carcinogenic reactions depending on the content, size, and chemical composition of the particles.

This study was divided in two chapters. Chapter 1 deals with a systematic review on the adverse biological reactions of submicron and nano-scale cp Ti particles in contact with fibroblasts, osteoblasts, or mesenchymal cells. A PUBMED search was performed using the following key terms: cytotoxicity, mutagenic, apoptosis, dental implants, debris, nanoparticles. Chapter 2 comprises an *in vitro* study focusing on different content (10, 50, 100 μg/mL) of titanium particles at micro- and nano-scale in contact with fibroblasts' culture medium over a period of 7 days. Cp Ti particles were assessed by Field Emission Guns Electron Microscopy (FEGSEM), Scanning Transmission Electron Microscope (STEM), and Energy Dispersion Spectrometry (EDAX). MTT assays were carried out to determine the mitochondrial function of cells in contact with Ti particles. The in vitro study was part of an on-going project at CESPU (cod: IMPLDEBRIS-PI-3RL-IINGACTS-2019).

Selected studies from the literature assessed cp Ti particles ranging from 20 up to 100 nm and submicron particles between 0.25-1 μm although micro-scale particles were used for comparison. Results showed an increase of different cytokines (e.g. TNF-α, IL-6, and IL-1β) from the cells when in contact with the titanium debris. Thus, submicron and nano-scale titanium debris induced toxic effects and therefore a mutagenic effect can occur when the nano-debris are internalized into the cells.

Results from the present in vitro assays revealed the deagglomeration of the cp Ti nano- in protein-rich medium leading to the decrease of the titanium debris size down to around 60 nm and the increase of the specific surface area up to 57.3 m2/g. MTT assays showed an adverse effect of the content of titanium particles on the cell growth. For 3 days incubation, 50 nm and 1 μm size particles at concentration of 50 μg/ml and 100 μg/ml

decreased the proliferation of fibroblasts. Thus, the size and content of cp Ti particles negatively affected the viability of fibroblasts in cell culture medium.

KEYWORDS

Titanium debris; dental Implants; Ti nanoparticles; cytotoxicity; mutagenic.

RESUMO

Produtos de degradação libertados de estruturas de titânio comercialmente puro (cp Ti) e ligas de Ti6Al4V devido ao tratamento terapêutico para peri-implantite e / ou corrosão de titânio podem libertar iões e partículas metálicas nos tecidos peri-implantares. Iões e detritos micro e nano-métricos induzem reações inflamatórias, mutagênicas ou carcinogênicas, dependendo do conteúdo, tamanho e composição química das partículas.

Este estudo foi dividido em dois capítulos. O capítulo 1 trata de uma revisão sistemática sobre as reações biológicas adversas das partículas de Ti em escala submicron e nanométrica em contato com fibroblastos, osteoblastos ou células mesenquimais. Uma pesquisa no PUBMED foi realizada usando os seguintes termos-chave: cytotoxicity, mutagenic, apoptosis, dental implants, debris, nanoparticles. O capítulo 2 compreende um estudo *in vitro* sobre o efeito de diferentes conteúdos (10, 50, 100 µg / mL) de partículas micro- e nano-métricas de cp Ti em contato com meio de cultura de fibroblastos durante um período de 7 dias. As partículas de titânio foram avaliadas por Microscopia Eletrônica de Emissão de Campo (FEGSEM), Microscópio Eletrônico de Transmissão (STEM) e Espectrometria de Dispersão de Energia (EDAX). Foram realizados ensaios de MTT para determinar a função mitocondrial de células em contato com partículas de cp Ti. O estudo in vitro fez parte de um projeto em andamento na CESPU (cod: IMPLDEBRIS-PI-3RL-IINGACTS-2019).

Os resultados dos presentes ensaios in vitro revelaram a desaglomeração das partículas em um aumento na proporção de nanopartículas individuais até aproximadamente 60 nm, associado com um aumento da área superficial específica até 57,3 m2/g. Os ensaios de MTT mostraram um efeito adverso do conteúdo de partículas de titânio no crescimento celular. Após 3 dias de incubação, partículas de 50 nm e 1 µm de tamanho na concentração de 50 µg / ml e 100 µg / ml foram capazes de prejudicar a proliferação celular, com a valores estatisticamente significantes ($p <0,0001$).

PALAVRAS-CHAVE

Resíduos; Titânio; Implantes Dentários; Nanopartículas; Citotoxicidade; Mutagenicidade.

TABLE OF CONTENTS

CHAPTER 1

1.Introduction……………………………………………………………………………3

2.Method……………………………………………………………………………………5

3.Results and Discussion…………………………………………………………………5

4.Conclusions……………………………………………………………………………17

References………………………………………………………………………………19

Annex………………………………………………………………………………………29

CHAPTER 2

1.Introduction……………………………………………………………………………37

2. Materials and Methods ………………………………………………………………39

3.Results and Discussion…………………………………………………………………43

4.Conclusions……………………………………………………………………………50

References………………………………………………………………………………51

CHAPTER 1

Dental implant debris and their influences on peri-implant tissues.

Abstract

Purpose: The objective of this study was to perform a systematic review on the toxic effect of submicron and nano-scale cp Ti debris on cells of peri-implant tissues.

Materials and Methods: A systematic review was carried out on the PUBMED electronic platform using the following keywords: Ti "OR" titanium "AND" dental implants "AND" nanoparticles "OR" nano-scale debris "OR" nanometric debris "AND" osteoblasts "OR "Cytotoxicity" OR "mutagenic"

Results: Titanium nanoparticles in submicron- and nano-scale altered the behavior of cells in culture medium. An inflammatory response was triggered by macrophages, fibroblasts, osteoblasts, and mesenchymal cells and odontoblasts as indicated by the detection of several inflammatory mediators: IL-6, IL-1β, TNF-α and PGE2. The formation of a rich bioactive-complex composed of Ca and P on titanium nanoparticles allowed the binding to proteins leading the cell internalization phenomenon. The nano-particles induced mutagenic and carcinogenic effects into the cells.

Conclusion: The cytotoxic effect of debris released from dental implants depends on the size, concentrations, and chemical composition of the particles. A high concentration of particles on nanometric scale intensifies the inflammatory responses with mutagenic potential of the surrounding cells.

1.Introduction

The long-term success of the dental implants reveals a high rate of around 90%(1,2). However approximately 70% of the implant losses have occurred after due to prosthetic and abutment complications on loading and inflammatory reactions(1). The inflammatory reactions confined at soft tissues is named mucositis while the progression of the disease involving bone loss characterizes the peri-implantitis(3,4). The presence of metallic debris at peri-implant tissues also stimulate the migration of immune cells and inflamatory reactions(5). Ti ions and debris were detected in peri-implant tissues with different sizes and forms(6). The accumulation of Ti ions and debris take place due to the degradation of abutment and implant surfaces and implant-abutment connections(7,8). Also, therapeutic procedures by using acidic substances and fluorides or mechanical debridement (Implantoplasty) can corrode the titanium-based surfaces(9,10).

The degradation of the implant-abutment connection cause the increase in microgap size, materials loss and mechanical instability(7,8). The design of the implant systems plays an important role in the maintenance of implant-abutment microgap and mechanical stability(11–13). Another factor that can influence stability is the type of titanium Commercially pure titanium (cp Ti) grade IV and Ti13Zr are the most used for manufacturing endosseous implants while Ti6Al4V is used for abutments(14). The elastic modulus of the cp Ti and Ti13Zr is approximately 100-110 GPa while the Ti6Al4V has an elastic modulus of around 150 GPa that is significantly higher compared to the bone of (10-30 GPa)(15). Nevertheless, the tensile strength of both types of titanium has sufficient tensile strength values to withstand mechanical stresses in the oral cavity cpTi grade IV at 550 MPa, Ti13Zr and Ti6Al4V at about 940-1000 MPa(15,16).

The chemical composition of titanium-based materials is responsible for their chemical stability (resistance to corrosion) in different media, especially in contact with acidic and reactive substances(17,18). These properties are determined by the titanium oxide thin layer (passive film) that forms spontaneously on the surface of the cp Ti and Ti alloys when exposed to ambient environment due to the high affinity of Ti for oxygen. The passive film composed mainly of TiO_2 has a thickness of approximately 1.5-10 nm(17,18), which protects the titanium from aggressive environment such as lactic acid, citric acid, hydrofluoric acid (HF), microbial metabolites, fluorides(19,20). Nevertheless, the TiO_2 thin layer can be altered

and damaged due to oscillations in temperature, pH, oxygen, bacteria metabolites, and dietary. Fluoride-containing varnishes, gels, and oral rinses which are commonly used to prevent dental caries and tooth demineralization induce a progressive destruction of the oxide layer of titanium(9,14). A solution containing more than 30 ppm HF may promote the localized corrosion of titanium(21). The chemical decontamination of the implants with citric acid as a treatment for peri-implantitis are also considered aggressive processes that damage the surface of the implant and abutment(10,14). The destruction of the passive film cause the release of Ti, Al, V ions and titanium-based debris at micro- and nano-scale(9,19).

The metallic debris released from titanium implants reveal several morphological and chemical including organometallic complexes, free metallic ions, and inorganic metal oxides such as cp Ti or TiO_2 particles(22,23). Cp Ti and TiO_2 submicron and nanoparticles have been widely used to provide whiteness and opacity to products such as adhesives, toothpastes and sunscreen, and other cosmetics. In 1999, TiO_2 was considered as a group 3 carcinogen, has no carcinogenic effect on humans(22). In 2006, the International Agency for Research on Cancer (IARC) classified TiO_2 nanoparticles as an IARC Group 2B carcinogen ''possibly carcinogen to humans''(24). Also, several previous studies revealed the local and systemic toxic effects of TiO_2 nano-particles(25–27). Cp Ti and TiO_2 micro- and nano-scale particles can reach the blood stream, accumulating in lungs, liver, spleen, and bone marrow. That contributes to the development of nanoparticle-associated diseases in the respiratory or cardiovascular systems, which can lead even to malignant tumor(28,29).

The main aim of this study was to perform a systematic review on the toxic effect of submicron and nano-scale titanium debris released from dental implants in contact with peri-implant tissues as a consequence of wear and corrosion phenomena. It was hypothesized that micro- and nano-scale titanium particles induce inflammatory reactions and DNA damage on cells of peri-implant tissues.

2.Method

A bibliographic review was performed on PUBMED (via National Library of Medicine) using the following search items: "Ti" OR "titanium" AND "dental implants" AND "nanoparticles" OR "nano-scale debris" OR "nanometric debris" AND "osteoblasts" OR "cytotoxicity" OR "mutagenic" OR "apoptosis". A manual search of the reference lists in the selected articles was also performed. The inclusion criteria encompassed articles published in the English language, until January 16th, 2020, reporting the effect of nano-scale titanium particles as released from dental implants on the toxicity and damage of osteoblasts. The eligibility inclusion criteria used for article searches also involved: articles written in English; cell culture assays; *in vitro* characterization; meta-analyses; randomized controlled trials; animal assays; and prospective cohort studies. Two of the authors (JCMS, RMH) independently analyzed the titles and abstracts of potentially relevant articles written in English language. The total of articles was compiled for each combination of key terms and therefore the duplicates were removed using Mendeley citation manager. Selected articles were individually read and analyzed concerning the purpose of this study. The following variables were collected for this review: authors' names, journal, publication year, aims, content and size of titanium nanoparticles; study design, cell culture methods, and toxicity effects on osteoblasts.

3.Results and Discussion

The initial search in the available database yielded a total of 186 articles although 46 duplicate articles were eliminated. On the remnant 140 articles, the titles and abstracts were read seeking concordance with inclusion criteria of the present study and therefore 22 studies were discarded after because they were related only to TiO_2 or titanium alloys. The evaluation of titles and abstracts resulted in the selection of 14 potentially review articles as see in Figure 1.

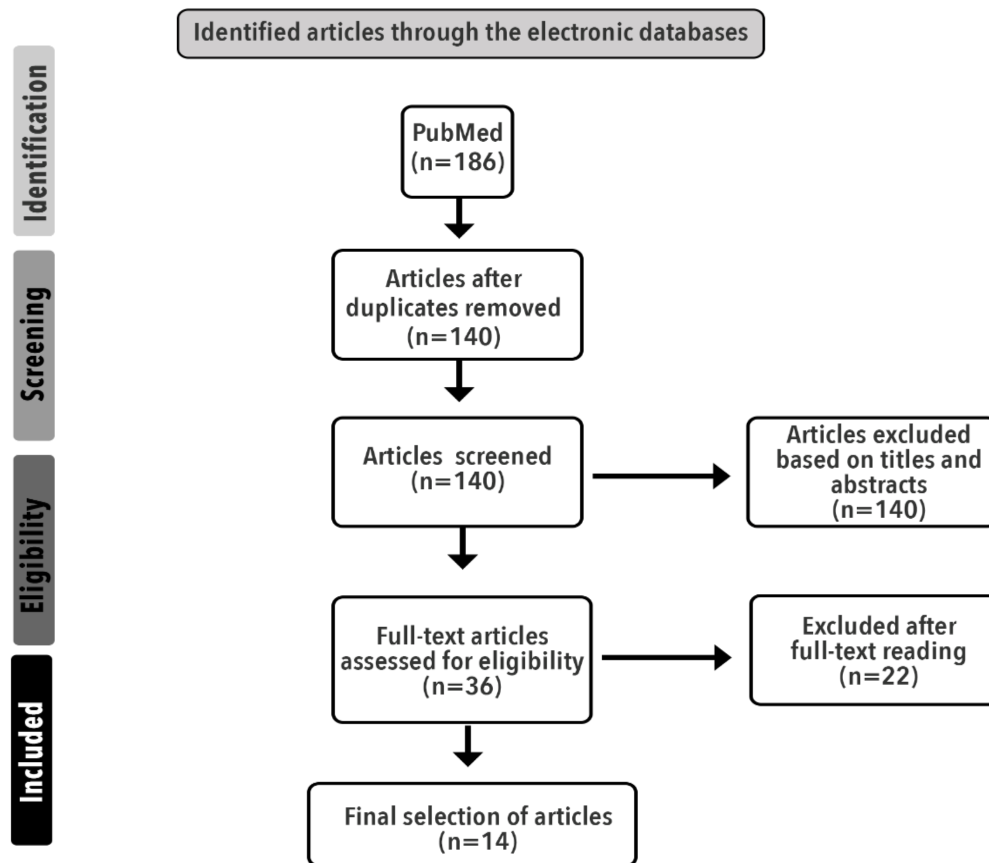

Figure 1. Flow diagram of the search strategy used in this study.

Of the 14 articles included in this review, three (21.42%) studies *in vitro* evaluated the toxicity induced by titanium particles in contact with osteoblast(30–32), in which two assessed human cell lines(30,32). Three *vitro* studies (21.42%) investigated the toxic effect of debris of titanium in human fibroblast(6,30,33), whereas one of them was in tissues from biopsies(33). One article evaluated the toxic effects of titanium debris released from dental implants on oral epithelial cells(34), while another one investigated the toxicity of titanium particles in culture of periodontal ligament with lentiviral gene transfer of human telomerase reverse transcriptase cells(35). One *in vitro* study examined the human Mesenchymal stem cells cytotoxicity upon exposure to submicron particles of titanium(36) . One *in vivo* study investigated the effects of titanium wear debris on the nutritive perfusion and leukocytic response in skeletal muscle of Syrian golden hamsters(37), while three studies (21.42%) performed in human participants reported the inflammatory reaction of macrophages and monocytes against titanium particles(38–40) considering one of them

inspected tissues from biopsies(39). Only, two articles (14.28%) described the mechanism of debris released from dental implants(41,42), in which one investigated the surface characterization of titanium healing abutments before and after placement in patients(41), and the other one reported the in vitro effect of ultrasonic scalers on titanium surfaces(42).

The main outcomes can be drawn as follow:

- The size of nanoparticles used in the different studies ranged from 20 up 100 nm(35,38), while other articles reported micrometric particles ranging from 0.25 up to 43 μm(6,30–32,35–40).
- Several proteins were involved in the toxicity induced by titanium particles according to the cell culture. *In vitro* studies in contact with macrophages revealed a high level of inflammatory mediators such as MyD88, TRIF and NF-kB in the presence of titanium particles size (≤ 20 μm)(30). In another *vitro* studies performed on human monocytes and macrophages, titanium particles with size ranging from (0.25 up to 7 μm) were able to induce a pro-inflammatory response characterized by the increased expression of transcripts and proteins linked to TNF-α, IL-1β and IL-6 cytokines(38–40). Protein expression was higher for IL1-β at all time points when compared to TNF-α and IL-6. IL-1β is the cytokine expressed to start the osteolytic process and plays an important role to TNF-α expression that modulates RANK-L production, leading to bone resorption(38–40).
- In osteoblast-containing medium, metallic wear debris (0.90-1.50 μm in size) resulted in changes in the expression of the markers of the ER stress apoptotic pathway, namely GRP78 and GADD153, and downstream caspase cascades, ultimately leading to cell apoptosis(31), in another *vitro* studies it was demonstrated that Ti particles (size 0.488 μm) caused up-regulation of zinc finger protein 467 (ZFP467), which is a regulator of osteoblast and adipocyte commitment indicating that a switch between osteogenic and adipogenic phenotypes had begun(6).
- Relatively to the toxic effect of titanium particles with size ranging from (0.25 up to <20 μm) in contact with fibroblasts the data showed high expression of inflammatory mediators, such as interleukin 6 (IL6) and IL1B, but also an increase in an anti-inflammatory one such as IL10, in addition to chemokine (C-X-C motif) ligand 2 (CXCL2), conversely (CXCL5) showed lower transcript levels(6,30,32). It was also observed high values of protein kinase C beta (PKCB), which is involved in

adipogenesis caused by titanium particles of size (0.488 μm)(6), and higher levels of phospho-FAK protein(33). Other study was performed on Normal oral keratinocyte spontaneously immortal-ized cells (NOK-SI) to evaluate if titanium debris release from different implant surfaces were biocompatible with oral epithelial cells or caused DNA damage, the results demonstrated that titanium particles from certain implant systems were able to activate CHK2 and trigger the recruitment of BRCA1 which are markers of DNA damage and genomic instability(34).

- One article related to this topic, made a comparision *in vitro* study of the toxicity and cellular uptake of three different titanium particles in culture of periodontal ligament with lentiviral gene transfer of human telomerase reverse transcriptase cells (PDL-hTERT), the results demonstrated that nanoparticles of titanium size (20-250 nm) induced higher cellular uptake efficiency and higher toxic potential than microparticles even nanoparticles were found in the nucleus(35).
- Another *vitro* study describe how titanium particles in submicron size (0.380 μm) in culture of human Mesenchymal stem cells (hMSCs) were able to affect adversely the cell viability through the induction of apoptosis, eliciting increased expression of the tumor suppressor proteins p53 and p73 in a manner dependent on particle dosage, and exposure time(36).
- An *In vivo* study in skeletal muscle in golden hamsters showed that titanium particles in submicron size (0,6 μm) caused an acute inflammatory process mediated by polymorphonuclear leukocytes with recruitment of leukocytes may at some stage culminate in a chronic inflammatory reaction(37).

The findings reported in the selected articles validate the hypothesis of the present study. Indeed, the size and content of titanium nano-debris released from implants negatively affect the osteogenic cell behavior, inflammatory response, and peri-implant tissues' health state. Parameters related to titanium, surface, release of debris, and toxicity are discussed as follow.

3.1. Titanium implants and abutments

Most endosseous implant systems for oral rehabilitation consist of a set of two major components including the implant and abutment for prosthetic rehabilitation. However, the connections between these components are subject to chemical degradation by corrosion and abrasion wear due to micro movements from mastication forces in contact with oral solutions(7,8). The degradation of the implant-abutment connection causes the increase in microgap size, materials loss, mechanical instability, and release of implant debris to the surrounding tissues(7,14). The design of the implant systems plays an important role in the maintenance of implant-abutment microgap and mechanical stability(11–13). The main types of implant-abutment connections are hexagon (external, internal) or Morse taper, as shown in Figure 2.

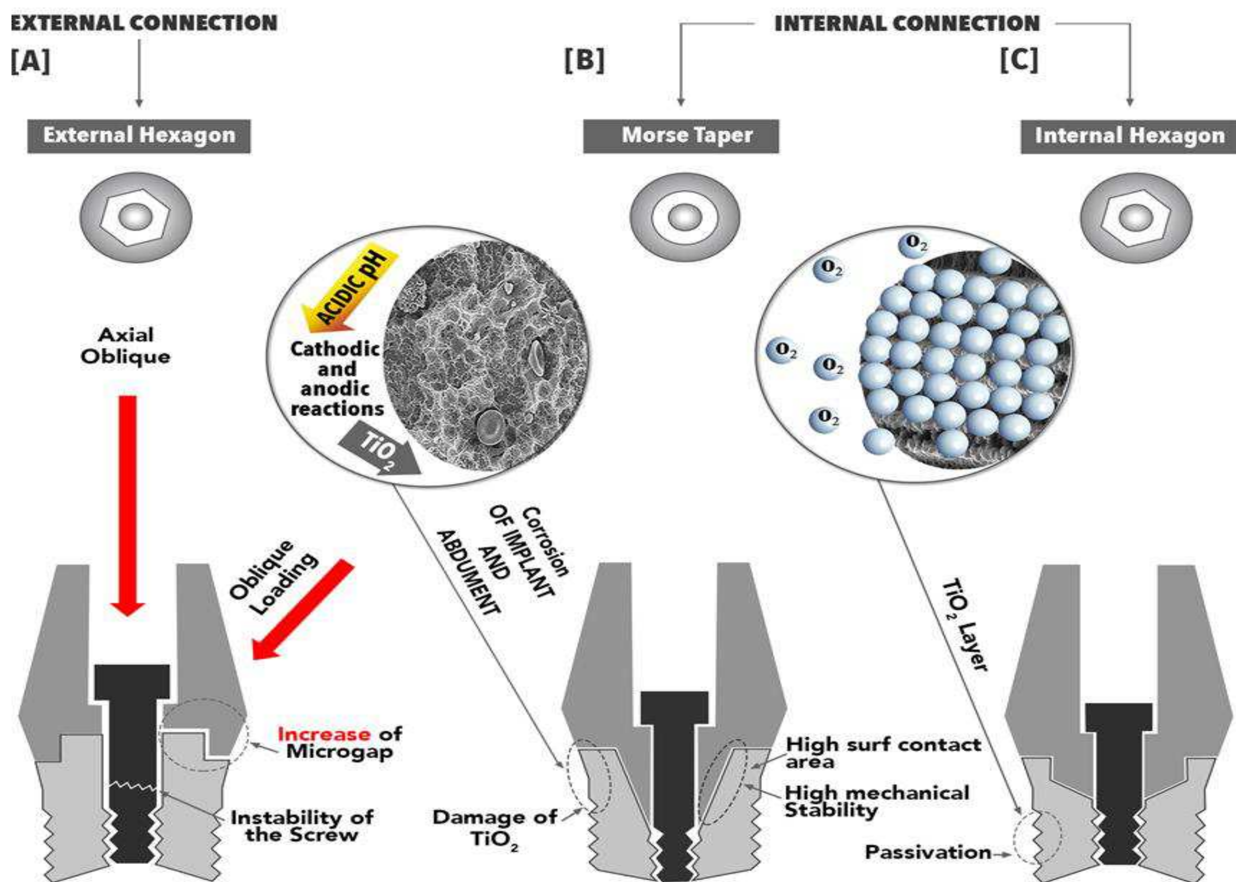

Figure 2. Schematics of different titanium implant-abutment connections.

The external hexagon implant-abutment connection reveal disadvantages concerning the loosening of the abutment screw and increase in microgap size leading to lateral or oblique loading during mastication(8,43). The mechanical instability in external hexagon implant-abutment connections has been reported in clinical studies on the abutment torque inspection(44,45). The instability of the implant-abutment connections results in oblique or horizontal loads at higher magnitude when compared to axial loading. The internal implant-abutment connection emerged to solve the mechanical instability reported in may studies, thus seeking long-term mechanical stability, decrease in microgaps' size, and inhibition of biofilm accumulation(11,12). Internal connections comprise internal hexagon and Morse taper design (Fig. 2) though the Morse taper provides more intimate implant-abutment contact(13).

Commercial pure titanium (cp-Ti) grade IV has been considered the first choice for manufacturing dental implants while Ti6Al4V alloys are used to produce abutments(46) . Titanium and its alloys has a lower density (around 4.7 g/cm3) when compared to 7.9 g/cm3 stainless steel (AiSi 316 L) 316 and 8.3 g/cm3 Cobalt Chromium-Molybdenum (CoCr-Mo)(15). The tensile strength of cp Ti grade IV is lower (~550 MPa) when compared to that (950 MPa) recorded for Ti6Al4V(9) . However, the relationship of density and strength is more than enough for application as implant-abutment connections(46). The mechanical properties of titanium were improved by adding metal elements such as Al, V, Nb, Zr. CpTi consists of the α phase (hexagonal close-packed; HCP) while Ti6Al4V consists of both the α and β (body-centered cubic; BCC) phases stabilized by Al and V(15,46).

The high resistance to corrosion and biocompatibility of titanium-based materials is dependent on a surface thin film composed of titanium oxide, mainly TiO2(17,18). Such stable titanium oxide film is spontaneously forms at the titanium implant surface within a thickness between 1.5-10 nm when exposed to ambient air due to the high affinity of Ti for oxygen, known as passivation(17,18). Nevertheless, the titanium oxide layer can be altered and damaged by continuous corrosion and wear(47).Figure 2

Recent studies have reported local and systemic adverse biological reactions induced by titanium debris released from CpTi and Ti6Al4V surfaces(21). Aluminum (Al) and Vanadium (V) are considered toxic elements, and, therefore. Vanadium has revealed a mutagenic potential(48). For this reason, alternative titanium alloys have been developed involving

Niobium (Nb), Tantalum (Ta) and Zirconium (Zr), as β phase stabilizing elements, causing fewer toxic effects, and maintaining the corrosion resistance and mechanical properties(49).

3.2 Release of titanium debris from dental implants

The presence of titanium debris in the oral tissues begins from the moment of placement and friction of the implant into the surgical bone site(41,50). The rotating procedure on placing the implant into the bone cause the wear of the titanium oxide film and bulk material(9,10). Additionally, the surfaces of the implant connection and abutment will be continuously exposed to the oral cavity, as illustrated in Figure 3. In this way, the exposed surfaces are susceptible to the chemical effect of the acidic substances (e.g. lactic acid) from the oral fluids or microbial metabolism(14,47,51). Previous studies have reported an acceleration in the degradation of the protective titanium oxide layer (passive thin film) in the presence of lactic acid, hydrogen peroxide, citric acid, artificial saliva, or fluoride solutions(14,47,51).

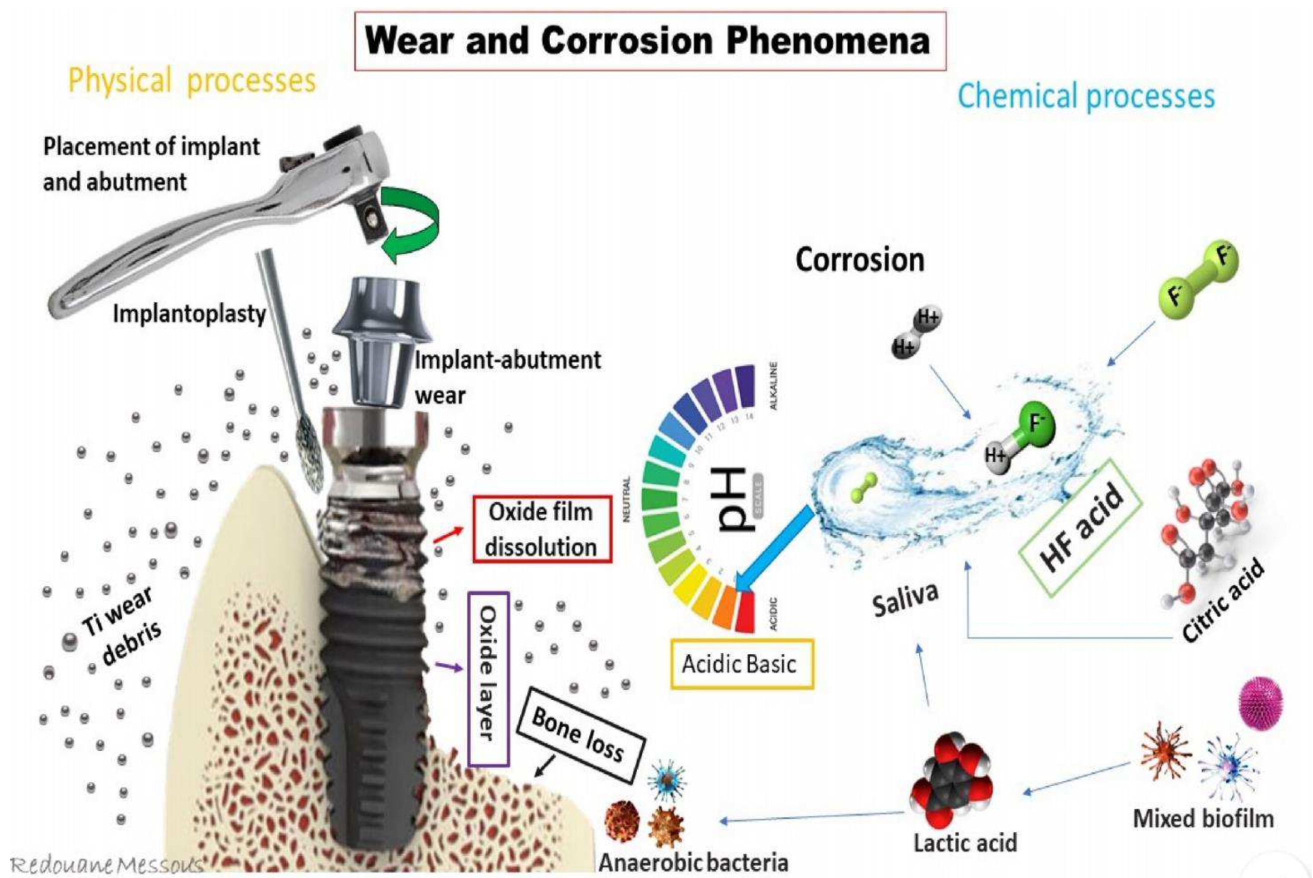

Figure 3. Degradation pathways of implant-abutment connections and consequent release of debris.

Corrosion and wear considered an inherent process in the oral cavity since the chemical reactivity of titanium metallic materials such titanium increases in contact with oral or therapeutic substances(52). The chemical reactivity of titanium significantly increased when immersed in therapeutic fluoride solutions or gels that are used by dentists and patients(9). In fact, high concentrations of F- promote an association between, H+ and F– ions, forming hydrofluoric acid (HF), which is corrosive to titanium(53). Even lower concentration of F– in acidic environment influences the corrosion resistance of titanium(52).

Corrosion occurs in contact with acidic substances from dietary, bacteria metabolism (e.g. lactic acid), and therapeutic treatment (e.g. fluorides, citric acid)(53,54). as illustrated in Figure 3. Wear starts on placement of abutment or healing caps over titanium surface connections due to the friction of the contacting surfaces (Fig. 3)(8,43). On mastication

loading, wear can take place by abrasion caused from the micro-movements on implant, abutment, and prosthetic contacting surfaces(55). The wear of titanium surfaces can remove the protective titanium oxide film and expose the bulk titanium to the acidic environment(14,47,51). In this case, the synergistic interaction between physical and chemical processes (wear and corrosion) can increase the degradation of the titanium surfaces (Figure 2 and 3) and increase the release of titanium debris and ions to the surrounding tissues(54). The fitting of the abutment and implant parts is crucial to decrease the micro-movements and metallic debris(55). The degradation of titanium-based surfaces leads to the materials loss and release of ions (Ti, Al, V) and metallic debris ranging from macro- to nano-scale size into the surrounding tissues(56,57). In an cross-sectional study, it was investigated the level of Ti in the peri-implant tissue, of 200 biopsies, which 160 from patients affected by peri-implantitis and 40 from patients healthy, the histological analysis showed presence of Ti and other metals (e.g. Zn, Al, Cu, and Ru) inside all samples affected by peri-implantitis(6). It was demonstrated that the interaction of Anatase Titanium Dioxide nanoparticles with calcium (Ca), phosphorous (P) and hydroxyapatite (HA) deriving from the medium culture due to the high reactivity of Anatase Titanium Dioxide. In FBS, protein corona adsorb on nanoparticles resulting in a bio-complex, serum albumin as well as other glycoproteins (ALB protein and Alpha 2HS) also were present(23). The role of the corona protein is creating an initial nano-bio interface that undergoes dynamic alterations as particles traffics onto or into cells(23). In previous studies showed that metallic particles released from dental implants can accumulate in the surrounding tissues(9,58). or even spread systemically, as reported in previous studies(59,60). contributing to chronic inflammation state in peri-implant disease(4,61).

Physical and chemical decontamination methods in the treatment of peri-implantitis can also induce the release of titanium debris to the surrounding tissues(57,62). Previous studies showed that sonic and ultrasonic scalers with metal tips cause considerable changes into implant surfaces(42,57). Implantoplasty should be highlighted as an aggressive process of release of titanium debris from dental implants (Fig.3). In peri-implantitis treatment, implantoplasty is also used by several dental practitioners as a mechanical therapeutic method for decontamination of dental implants by removing titanium layers from the rough implant surfaces(57). Figure 3. Nevertheless, such physical process results in the release of a high content of titanium debris and ions to the surgical

bone site and connective tissues(10). Also, the debris can be released from the burs used for implantoplasty(9).

3.3 The toxic effect of titanium particles on cells and tissues

Submicron and nano-scale debris released from dental implants as a result of degradation, are considered as foreign bodies which activate the human immune system. Several inflammatory mediators and cytotoxins associated with peri-implantitis disease and bone resorption are involved(30,33,39). as seen in Figure 4. As a first defense line of the immune system, the most representative leukocytes (54-65%) with the function of phagocytosis are neutrophils and macrophages(63).

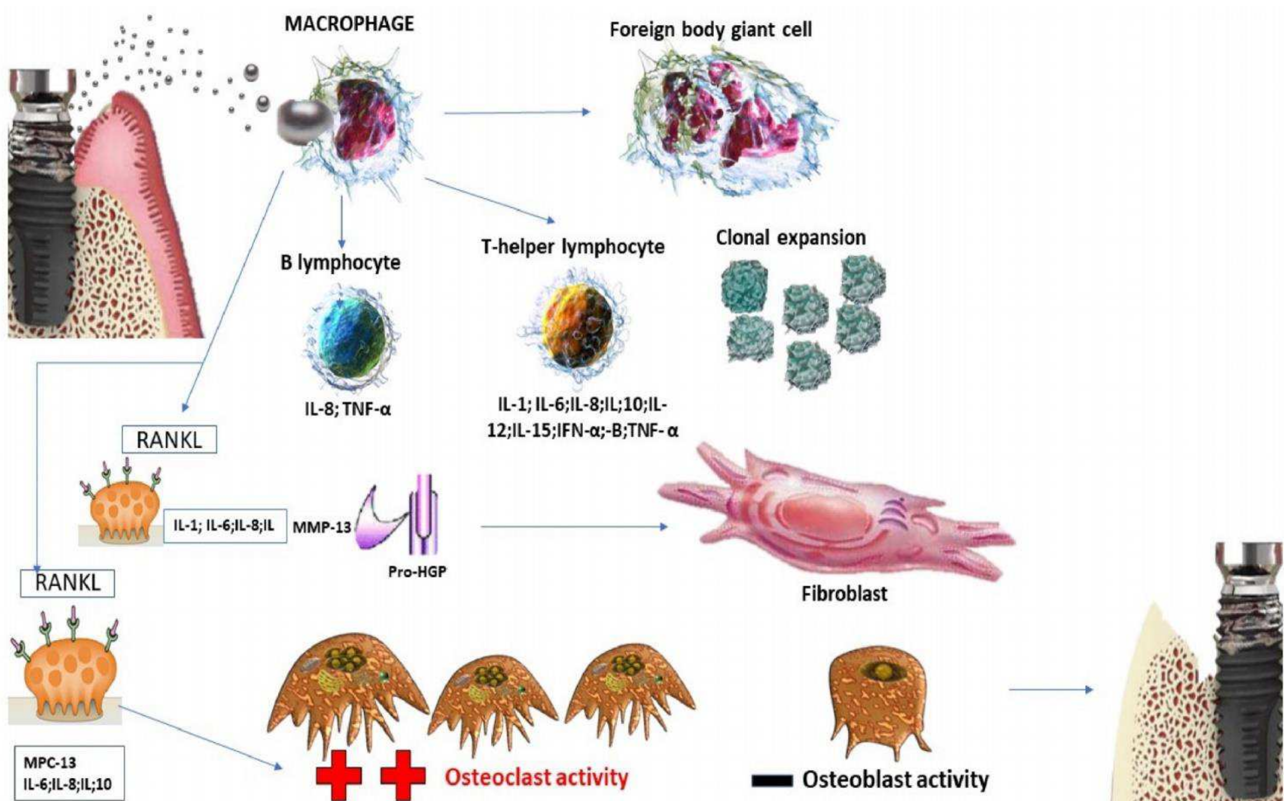

Figure 4. Schematics of the effect of cp Ti debris on surrounding cells and tissues.

In fact, cytotoxicity depends on the size, chemical composition, and content of metallic particles(63,64). Studies showed that neutrophils phagocytized Ti particles only when the particles were smaller than the cells (~5 μm)(63), while macrophages revealed a more

complex function(40). Monocytes and macrophages have a specific relationship with individual cytokines and respond to various cytokines secreted after neutrophils reaction with foreign bodies(63). Titanium particles ranging from 0.25 up to 7 μm in culture of macrophages could induce a pro-inflammatory response with an increase in expression of transcripts and proteins such as TNF-α, IL-1β, and IL-6 cytokines(38–40). Sub-micron and nanoparticles caused highest gene expression for all cytokines(65) probably because the cell internalization of the titanium particles(66). Therefore, micro and nano-particles of titanium induce an increase in secretion of IL-1β from macrophages leading to bone resorption by osteoclast activity via activation of RANKL expression(67,68). Titanium micro-scale particles size (≤ 20 μm) also induced the release of IL-6 and TNF-α from fibroblasts(69). An in vitro study with titanium and TiO2 particles size below 7 μm also revealed an augment in the amount of IL-6 and PGE2 from osteoblast(4,32). Thus, titanium particles promote a dual effect: first, the particles induce an inefficient bone formation due to the inhibition of osteoblast proliferation; and second, particles increased bone resorption by IL-6 osteoblast-mediated osteoclastogenesis(70).

An *in vitro* study assessed wear debris harvested from patients with aseptic joint loosening to ensure that the experiments more closely resembled the biological *in vivo* response. Titanium particles at a size of 0.90-1.50 μm were placed in culture of primary osteoblast from rats(31). The results indicated that reactive oxygen species (ROS) generation induced by metallic wear debris triggered endoplasmic reticulum (ER) stresses, mitochondrial dysfunction, and downstream caspase cascades, leading to cell apoptosis and consequently suppressing bone formation around the prosthesis(31).

In the presence of titanium particles, the inflammatory response can be progressive since a strong osteoclastogenesis process takes place due to the activation of mature osteoclast from macrophages(62,70). An *in vivo* study in skeletal muscle in golden hamsters showed that titanium particles (0.6 μm) caused an acute inflammatory process mediated by polymorphonuclear leukocytes with recruitment of leukocytes might at some stage culminate in a chronic inflammatory reaction(37). Another study analyzed the effect of 0.488 μm Ti particles against fibroblasts and mesenchymal stem cells and their subsequent distribution within peri-implant tissues. The results confirmed that titanium particles were embedded in all peri-implant tissue with different sizes and forms. Also, sub-micron

particles were internalized within cells(6). Submicron particles (0.380 μm) in culture of human mesenchymal stem cells (hMSCs) were able to affect adversely the cell viability by induction of apoptosis, eliciting increased expression of the tumor suppressor proteins p53 and p73(36). The adverse reaction magnitude was dependent on particle content and exposure time(36). A production of matrix metalloproteinase (MMP) was also reported with a reduction in the expression of their inhibitor TIMP1, which can be associated with tissue destruction(6). MMP are responsible for remodeling and degrading extracellular matrix molecules by cleaving components of cell-to-cell networking(71). The role of PGE2 in titanium particles-induced osteoclastogenesis and in osteolysis has been shown in an *in vivo* mouse calvaria resorption model(72). In previous studies, the increase in PGE2 have been detected in periprosthetic membranes of failed implants(73). In addition to direct effects of titanium particles on osteoblasts, particle-mediated induction of PGE2 contributes to decreased levels of OPG(32), which is as a potent inhibitor of osteoclast differentiation and activation. That acts as a decoy receptor for RANKL and prevents its interaction with the cognate receptor RANK expressed in osteoclasts precursors(74).

4.Conclusions

The present review analyzed the *in vitro* and *in vivo* toxic effects of titanium debris released from dental implants in contact with different cell lines or tissues. Within the limitations of the *in vitro* and *in vivo* studies, the following outcomes can be drawn as follow:

- Different mechanical and chemical processes are involved in the release of macro-, micro-, submicron, and nano-scale titanium particles or ions from dental implants and abutments into the surrounding tissues. Submicron and nano-scale particles are not easy to be detected by conventional physicochemical methods and therefore they can be internalized by the cells regarding the size.
- Micro-scale cp Ti particles assessed in the selected articles had a size ranging from 0.3 up to 20 μm, while the size of nano-scale cp Ti particles varied from 20 up to 100 nm. In vitro studies assessed commercially metallic powders to avoid the bias of results although one study assessed particles with mean of 0.90-1.50 μm in size retrieved from patients.
- CpTi micro-scale particles in contact with culture of fibroblast induced an increase in the amount of IL-6, TNF-α and PGE_2 cytokines. That corresponds to a chronic inflammatory response. CpTi particles in contact with culture of osteoblast also induced increase of IL-6 and PGE_2 with a decrease of OPG, which can lead to bone resorption.
- Micro and nanoparticles of CpTi in contact with culture of monocytes and macrophages induce an increase of for TNF-α, IL-6 and IL-1β which stimulates bone resorption via activation of RANKL expression. The increase in the content of nano-scale cp Ti particles negatively affect the immune response.
- Acute inflammatory process mediated by polymorphonuclear leukocytes with recruitment of leukocytes was noticed in an animal study. Such inflammatory process might result in a chronic inflammatory reaction that depends on the particle size and content.
- Despite the existing literature on the cytotoxicity of the debris released from the implants, few studies evaluate the cytotoxic effect of the CpTi particles on the peri-implant tissues, of which only one use particles in nano-scale, while the rest of the studies used particles in micro-scale. However, none of the studies investigated the

mutagenic or genotoxic effect that CpTi particles can induce. More studies are required to evaluate the effects of the content, size and chemical composition of CpTi particles and also more *in vivo and in vitro* studies are required in different types of cells (macrophages, fibroblasts, osteoblasts, mesenchymal cells) to clarify the mechanisms involved in the cytotoxicity and genotoxicity of CpTi particles.

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Annex

Table 1. Details on relevant previous studies regarding the effect of titanium debris to the surrounding medium.

Author (Year)	Purpose	Study design	Physicochemical Characterization	Size, morphological aspects, and content of Ti particle (nm)	Biological response
Arndt Happe et al (2019)(30)	Evaluation of the effects of different titanium (Ti) particle concentrations on viability of human calvarial osteoblasts and human gingival fibroblasts	- In vitro - Dispersion of Ti powders in culture of Human calvarial osteoblasts (HCO) and Human gingival fibroblasts (HGF-1)	- MTT assay - SEM - ELISA	- Ti particles size (≤ 20 μm) - Ti particle concentrations (0.01 to 1.0 mg/mL)	Cell viability is negatively correlated with titanium concentration. Further, titanium debris might lead to an inflammatory biologic response of dental peri-implant tissue
Eriberto Bressan et al (2019)(6)	Identification of the role of titanium (Ti) nanoparticles released form the implants on the chronic inflammation and bone lysis in the surrounding tissue	- In vitro - Dispersion of Ti particles in culture of Human adult dermal fibroblasts (FU) and Mesenchymal stem cells (MSCs) isolated from human dental pulp extracted from healthy molar teeth	- MTT assay - ROS measurement	- Ti particles (size distribution: 0.2–0.8 μm; median: 0.488 μm; mode: 0.426 μm) were treated to remove >99.94% of adherent endotoxins. - Ti particle concentrations (100 to 150 particles/mL in growth medium)	The analysis of tissues affected by peri-implantitis revealed correlations between the presence of Ti nanoparticles and ROS
FEI YANG et al (2019)(31)	Expanding our understanding of the mechanism underlying metallic wear debris-induced aseptic loosening	- In vitro - Collected metallic wear debris (Ti,Co,Cr,Mo,Al,V) from 3 patients with aseptic loosening of hip joint prostheses in rat primary osteoblast	- Flow cytometry - ROS measurement - Determination of MMP - Analysis of extracellular activated caspases -Western blot analysis	- Metallic particles size (0.45-1.67 μm, with a mean size of 1.22 μm, and >90% of the particles were 0.90-1.50 μm in size. - Metallic particles concentrations (0.05 and 0.1 mg/ml)	- The ROS generation induced by metallic wear debris triggered ER stress, mitochondrial dysfunction and downstream caspase cascades, ultimately leading to cell apoptosis
Fernando Suárez-López del Amo et al (2017)(34)	Evaluation of the effect of titanium (Ti) debris release from dental implants and toxic effects particles on oral epithelial cells	- In vitro - Dispersion of Ti particles derived from 5 different Dental implants system with different surface treatments:	Indirect Immunofluorescence Method	N / A	Titanium (Ti) particles derived from implants containing (PETO, FM, GB) surface treatments were able to activate CHK2 and trigger the recruitment of BRCA1 (BRCA1 and

		.1 system: Biomet 3i, DAE surface (osseostite) .2 system: Astra, FM, surface (osseospeed) .3 system: Nobel, PETO, surface (TiUnite) .4 system: Straumann, SLA, HSLA, surface (SLA, SLActive) .5 system: Zimmer, GB, surface (MTX) in culture of Normal oral keratinocyte spontaneously immortal-ized cells (NOK-SI)			CHK2 are markers of DNA damage and genomic instability) in oral epithelial cells.
Cindy Goes Dodo et al (2017)(38)	Evaluation of inflammatory reaction of human macrophages in contact with micro and nanoparticles of titanium associated with Porphyromonas gingivalis lipopolysaccharide	- In vitro - Dispersion of MPs-Ti and NPs-TiO_2 associated with Porphyromonas gingivalis lipopolysaccharide (PgLPS) in culture of Human monocyte THP-1 cells	- MTS assay - SEM - qRT-PCR - ELISA	- MPs-Ti size (<20 μm, 93%) - NPs-TiO_2 (21nm) - Endotoxins <0.01 units/mL - 1 μg/mL of (PgLPS) in 50 ng/mL of titanium MPs - 1 μg/mL of (PgLPS) in 50 ng/mL of titanium oxide NPs	Titanium nanoparticles stimulate stronger pro-inflammatory response in macrophages, independent of their association with lipopoly-saccharide from Porphyromonas gingivalis
M. Pettersson et al (2016)(39)	Examination of the cytotoxicity and proinflammatory effects, even the activation of inflammation, provoked from debris released of dental implants, in vitro model, as well as from clinical tissue samples	- 2 Biopsies of deep soft-tissue at the clinically healthiest implant and at an implant showing signs of inflammation of each of the 3 patients. - Collected of crevicular fluid from the peri-implant pocket at the biopsy sites (PICF) - In vitro Dispersion of Ti particles in culture of Human monocyte THP-1 cells	- Neutral red uptake assay - ELISA - PCR - Inductively coupled plasma mass spectrometry	- Ti particles in plasma standard solution content hydrogen fluoride (HF) to stabilize in the ionic form - (Ti) solution was divided in two groups: one group was unfiltered with a concentration range (0-400 μm) and in the other group the solution was filtered through a (0,20 μm) sterile filter	Ti ions in physiological solutions stimulated inflammasome activation in human macrophages and consequently IL-1b release. This effect was further enhanced by macrophages that have been exposed to lipopolysaccharides. The proinflammatory activation caused by Ti ions disappeared after filtration (0.22 μm), which indicates an effect of particles. Ti ions alone did not stimulate transcription of the inflammasome components. The Ti levels of tissue samples obtained in

					the vicinity of Ti implants were sufficiently high (≥ 40 µm) to stimulate secretion of IL-1b from human macrophages in vitro
Xiuli He et al (2015)(35)	Comparision of the toxicity and cellular uptake of three different Titanium particles which can be released from dental implants	- In vitro - Dispersion of MPs-Ti (99% Ti) and MPs-NiTi (70% Ti,30% Ni) and NPs-Ti (98,5% TI) in culture of Periodontal ligament with lentiviral gene transfer of human telomerase reverse transcriptase cells (PDL-hTERT)	- XTT assay - Trypan blue staining - Comet assay - LSCM - TEM - SEM	- MPs-Ti size (0.3–43 µm) - MPs-NiTi size (0.7–90 µm) - NPs-Ti (20–250 nm) - THE EC_{50}: >999 mg/ml (Ti-MPs) 41.8 mg/ml (NiTi-MPs), 2.8 mg/ml (Ti-NPs)	Compared to Ti-MPs and NiTi-MPs, Ti-NPs induced higher cellular uptake efficiency and higher toxic potential in PDL-hTERT cells. Only Ti-NPs were found in the nucleus.
Vygandas Rutkunas et al (2015)(33)	Comparision of the viability and adhesive intensity of human gingival fibroblasts (HGFs) grown on several implant abutment materials	- In vitro - Dispersion of (Ti–P), (Ti–S), (ZrO–P), (ZrO–S), (Cr–Co), and (Au) in culture of Primary human gingival fibroblast (HGF) obtained from a healthy patient undergoing gingivectomy of the premolar region	- MTT assay - Cell-based ELISA - SEM	N / A	- There was a tendency of gingival fibroblast adhesion strength to decrease in the following order: (Ti–S), (Ti–P), (ZrO–S), (ZrO–P), (Au), (Cr–Co) - Higher levels of total and phospho-FAK protein were registered in (HGFs) grown on roughened titanium.
Gema Valle´s et al (2007)(32)	Comparison of the cytocompatibility of low content of (TIO2) and (CpTi) particles against primary cultures of human osteoblasts	- In vitro - Dispersion of TiO2 and CpTi powders in culture of Human osteoblastic cells (OB) obtained from fresh trabecular bone explants obtained from patients (aged 67 + - 6 years old) undergoing total knee arthroplasty	- Endotoxin test - Confocal microscopy - Cytotoxicity and metabolic activity assays - Immunoenzymatic assays	- Equivalent circle diameter (ecd) sizes of the particles were 0.45 +/- 0.26 µm for TiO2 (range was 0.1–1.5 µm, 92% were lower than 0.9 µm) and 3.32 +/- 2.39 µm for CpTi (range was 1–15 µm, 89% were lower than 7 µm) - The mean particle sizes either isolated or forming aggregates, were 1.43 +/-0.60 µm for TiO2 and 3.91 +/-3.29 µm for Ti	- Both titanium and rutile particles are efficiently internalized by human osteoblasts modulating the secretion of several mediators involved in bone resorption - Rutile showed a lower bioreactivity than titanium in osteoblast culture medium

Gema Valle´s et al (2006)(40)	Comparison of the cytocompatibility of low content of rutile and titanium particles on monocyte/macrophage and primary cultures of human macrophages	- In vitro - Dispersion of TiO2 and CpTi powders in culture of THP-1 cells differentiated to the monocyte–macrophage lineage as well as in human primary macrophages	- Endotoxin test - Cytotoxicity assays - Immunoenzymatic assays - Confocal microscopy	- Rutile (TiO2) of 0.9–1.6 µm Titanium (CpTi) particles of <20 µm - TiO2 particles are globular with a granulated surface structure - Most of Ti particles were round with a smooth surface, although the larger Ti particles were usually elongated and rough - The mean sizes of particles were 0.45 +/- 0.26 µm for TiO2 (range was 0.1–1.5 um, 92% were lower than 0.9 µm) and 3.32 +/- 2.39 µm for Ti (range was 1–15 µm, 89% were lower than 7 µm)	- Both types of particles were efficiently internalized in cultures of TPA-differentiated THP-1 cells as well as in primary cultures of human macrophages - Rutile particles stimulated the release of TNF-a, IL-6 and IL-1b to a lesser extent than titanium particles in these cellular models
Mark L. Wang et al (2003)(36)	Investigation of human Mesenchymal stem cells (hMSC) cytotoxicity upon exposure to submicron particles of commercially pure titanium (CpTi) and zirconium oxide (ZrO2)	- In vitro - Dispersion of CpTi and ZrO_2 powders in culture of human Mesenchymal stem cells (hMSCs) obtained from bone marrow aspirates of patients undergoing THA or TKA for primary osteoarthritis	- PCR - Western analysis of p53 and p 73 expression - TUNEL assay - Conditionel mediated assay	- The mean particle sizes of 0.939 # 0.380 µm for CpTi and 0.876 # 0.540 µm for ZrO_2 - endotoxin levels exceeding 0.06 EU/ml - The concentration of CpTi and ZrO_2 particles (500 particles/cell)	Direct exposure to CpTi and ZrO_2 submicron particles adversely affects (hMSCs) viability through the induction of apoptosis, eliciting increased expression of the tumor suppressor proteins p53 and p73 in a manner dependent on material composition, particle dosage, and exposure time
C. N. Kraft et al (2003)(37)	elucidation of the effects of titanium and stainless-steel wear debris on the nutritive perfusion and leukocytic response in skeletal muscle in vivo and comparison of the results with those of bulk implants of the same material.	- in vitro - CpTi and stainless-steel wear debris deposited directly on to the striated muscle and subcutaneous tissue of Syrian golden hamsters, *Mesocricetus auratus*	- Intravital fluorescence microscopy - Microcirculatory analysis	Particles were submicron in size: - Titanium: 83% <1 µm, mean diameter 0.6 µm - Stainless steel: 98%<1 µm, mean diameter 0.2 µm - The particles were not tested for endotoxin	- stainless steel induces a more pronounced inflammatory response than titanium, but this study was unable to show a difference between bulk and debris - Titanium wear debris induced a slightly more pronounced inflammatory reaction than its bulk product, especially in regard to extravasation of leukocytes

					- stainless steel wear debris, although initially slightly less inflammatory, had a more marked and persisting negative effect on leukocyte-endothelium interaction compared with its bulk product

Note: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM: Scanning electron microscopy; ROS: reactive oxygen species; MMP: mitochondrial membrane potential; MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; qRT-PCR: quantitative Reverse Transcription Polymerase Chain Reaction; LSCM: Laser scanning confocal microscopy; TEM: Transmission electron microscopy; CpTi: commercially pure titanium; Ti: titanium; Co: cobalt; Cr: chromium; Mo: molybdenum; Al: aluminum; V: vanadium; Au: gold alloy; TiO_2: titanium dioxide; NiTi: Nickel titanium; ZrO2: zirconium dioxide; Ti−P: polished titanium; Ti−S: sandblasted titanium; ZrO−P: polished zirconium oxide ceramic; ZrO−S: sandblasted zirconium oxide ceramic; DAE: Dual-acid-etched; FM: Fluoride-modified; PETO: Phosphate-enriched titanium oxide; SLA: Sand-blasted, large-grit, acid-etched; HSLA: Modified (more hydrophilic) sand-blasted, large-grit, acid-etched; GB: Grit-blasted; MPs: microparticles; NPs: nanoparticles; N/A: not available.

CHAPTER 2

Assessment of Murine fibroblast L929 interaction with commercially pure Titanium (Cp Ti) micro- and nano-scale particles

Abstract

Purpose: The main aim of this study was to evaluate the cell viability of commercially pure Titanium (Cp Ti) micro- and nanoscale particles and at three different concentrations in contact with fibroblasts.

Materials and Methods: Cp Ti particles with 50 nm and 1 µm size were chemically and morphologically characterized by using a Field Emission Guns Electron Microscopy (FEGSEM), Scanning Transmission Electron Microscope (STEM) and Energy Dispersion Spectrometry (EDAX). Their cytotoxic effect of the particles at three concentrations (10, 50, 100 µg/mL) effect was analyzed by cell viability using an MTT assay in contact with Murine L929 fibroblast over a period of 1, 3, and 7 days.

Results: Physiochemical characterization of cp Ti nanoparticles revealed a mean size at 70 nm and specific surface area at around ~17.2 m^2/g. Cp Ti microparticles revealed size ranging from 0.3 up to 5.3 µm with a mean size at 1.4 µm at dry conditions. The optimized de-agglomeration of nano-particles resulted in an increase in the specific surface area up to 57.3 m^2/g. The chemical composition revealed the presence of (Na) sodium chloride, (K) potassium, and (Cl) deriving from the culture medium. MTT revealed the negative effect of cp Ti particles at 50 or 100 µg/ml on the fibroblasts over a period of 3 days ($p<0.0001$).

Conclusions: These results show that the toxic effect of the Ti particles does not depend only on the size of the particles but also on the time and concentrations that cells are exposed to.

Key words: cp Ti; titanium; nano-particles; cytotoxicity

1. Introduction

Although the success rates are high, 5-11% of dental implants fail within 10-15 years and must be removed(1–3). The loss of a dental implant is generally attributed to peri-implant inflammations leading to bone loss, namely peri-implantitis(4,5). The prevalence of peri-implantitis has been estimated at 22% (CI: 14-30%)(5). A complex co-aggregation of risk factors for implant failures take place in most cases(6,7). Recently, a concern is linked to the cyto- and geno-toxic potential of metal ions and debris released from dental implant and abutment structures in the peri-implant tissues has been reported(7–10). Ti, Al, and V ions and nanoparticles have been associated with to peri-implant inflammatory reactions leading to osteolysis(11–13).

Metallic micro and nano-particles released from dental implants as a result of degradation, are considered as foreign bodies which will activate the immune system. Micro and nano-size particles released from dental implant can accumulate in the surrounding tissues(14–16), or even spread systemically, as reported in previous studies(17,18) contributing to chronic inflammation state in peri-implant disease(19,20). Consequently, various inflammatory mediators and cytotoxins associated with peri-implantitis disease and bone resorption are activated(21,22). Neutrophils and macrophages are the primary defense cells representing 54% to 65% of leukocytes. Their function is to initially react non-specifically to foreign antigens and particles. Neutrophils are the non-specific defense cells against the titanium particles(23). In the continuous presence of titanium particles, the inflammatory response can be progressive since a strong osteoclastogenesis process takes place due to the activation of mature osteoclast from macrophages' response(24).

In fact, cytotoxicity depends on the size, chemical composition, and content of metallic particles(25,26). *In vivo* and *in vitro* studies reported engulfment of debris by macrophages stimulating the secretion of inflammatory cytokines such as TNF-a or IL-1b(27). In an *in vitro* study cp Ti and TiO2 nanoparticles induced a higher cell internalization when compared to cp Ti micro-particles when in contact with cells derived from the periodontal ligament(22). Other studies reported the mutagenicity and genotoxicity induced by TiO2 nano-particles in different cell lines involving Human Osteoblast Cells(18), in Chinese hamster lung fibroblast(8), or in the Brain of Mice(7).

The main objective of this work was to investigate the cytotoxic effect of micro- and nano-scale cp Ti particles at three different concentrations in contact with fibroblasts. A detailed physicochemical characterization of the Ti nanoparticles was performed prior to the cell culture. The working hypothesis was that high concentrations of Ti nanoparticles should significantly decrease the viability of fibroblasts.

2.Materials and Methods

2.1 Preparation of Ti stock suspension

Two groups of commercially pure titanium (cpTi) nanoparticles powder (SAT nano Technology Material CO., Ltd.) with particles size at 1µm or 50 nm (Product no. SAT-PWM200116) were assessed in this study. Stock solutions containing cpTi particles were prepared at a final concentration of 2 mg/mL (pH 4) in ultrapure water and 10% of fetal bovine serum (FBS; Gibco, USA). The sterilization of cp Ti nanoparticles suspension were controlled by using ultraviolet light for 20 min.

The agglomeration of Ti nanoparticles was decreased by adding FBS in the medium. After this procedure, an ultrasonic disintegrator (Vibra cell 75186, USA) equipped with a 19 mm Ti tip was used to disperse cp Ti nanoparticles until no further advance in the de-agglomeration process was achieved. Before setup the sonication protocol, a calorimetric method was used to measure the specific energy as well as the sonication power transferred to the suspension. Sonication was then performed at 130 W of delivery acoustic power on pulse mode (pulse on for 60 s and pulse off for 10 s) and 50% amplitude in ice bath to avoid temperature rise for 8 min.

2.2 Physicochemical characterization of Ti nanoparticles

Six groups were prepared for the physicochemical characterization of the cp Ti powder or 50 µg/ml particles in FBS or FBS and Modified Eagle's Medium (MEM; Gibco, USA), as follow: (group A) Cp Ti powder particles size at 50 nm or (group B) at 1µm in dry conditions; (group C) Cp Ti powder particles size at 50 nm diluted in FBS or (group D) in a mixture of FBS and MEM solution; (group E) Cp Ti powder particles size at 1 µm diluted in FBS or (group F) in a mixture of FBS and MEM solution. The specific surface area of cp Ti particle powder was determined by Brunauer-Emmet-Teller (BET) (Autosorb-1, Quanta Chrome Instruments).

Morphological aspects of the cpTi dry powder and particles' suspension in culture medium were inspected using a Field Emission Guns Electron Microscopy (FEGSEM; FEI Nova 200, USA) and a Scanning Transmission Electron Microscope (STEM; FEI Nova 200, USA). The chemical composition of cp Ti nanoparticles was confirmed by Energy Dispersion Spectrometry (EDAX; Pegasus x4M, USA) coupled to the FEGSEM. FEGSEM and TEM analyses were performed after cp Ti particles stabilization in the culture media for 24h. Then 10 µl

of the suspension of the groups C, D and E were transferred onto glass discs with 12 mm in diameter in triplicate. Particles were analyzed after drying at room environment and then sputter-coating with a 2 nm-thin AuPd layer. For nanoparticles, a drop of 50 µg/mL Ti suspension was placed onto a holey carbon-coated copper grid, air-dried, and inspected by STEM.

Size distribution of Ti particles was also investigated by dynamic light scattering (DLS) using a ZetaSizer Nano ZS (Malvern Instruments GmbH, Germany). DLS measurements were performed at 25 °C using standard 10 mm disposable optical polystyrene cuvettes. For each test condition, DLS measurements were performed in triplicate, with the test number and time for each run automatically determined by the apparatus's software. CpTi suspensions were homogenized by res-suspending the medium several times prior to filling the sample in the cuvette.

2.3 Cell growth conditions

Murine fibroblast L929 cells (kind gift from Dr Manuel Collado, Laboratory of Stem Cells in Cancer and Aging, Santiago de Compostela) were cultured in 25 cm^3culture flasks (VWR International, LLC, USA) containing MEM culture medium (Gibco, USA), and supplemented with 10% horse serum (HS), and maintained 37 °C in a 5% CO2 humidified incubator. After a 24h incubation period, culture medium was discarded as cells washed with PBS. Afterwards, cells were detached from the flasks using 0.025% trypsin (Sigma-Aldrich Co. LLC, USA) and were harvested for posterior assays. Cell morphology was regularly monitored, and all experiments were performed with healthy exponentially growing L929 cells with more than 90% viability as determined by trypan blue exclusion assay.

2.4 Cell viability assay

The cytotoxicity was assessed concerning the metabolic activity of L929 fibroblasts according to the ISO 10993-5. L929 cells were seeded in 96-well plates at a concentration of 5 x 10^4 cells/well in contact with three different concentration of particles: 10, 50, or 100 µl/ml. The cell metabolic activity of L929 cells in direct contact with the Ti nano particles was evaluated by colorimetric test using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Promega, USA), to assess the mitochondrial metabolic activity of the cells. MTT is a compound bio reduced by cells into a colored formazan product

(purple color), which is soluble in PBS. The number of living cells is directly proportional to the amount of formazan re-corded by spectrophotometry at 570 nm. The cellular metabolic activity was analyzed as a function of time over a period of 1, 3 or 7 days of cell culture. The MTT assay results assume that the metabolic activity increase is due to the higher number of cells. For each period of cell culture, the culture medium and samples were removed and then the cells were washed three times with PBS. Thereafter, 200 μL culture medium and 20 μL MTT reagent (1 mg/mL) were added in each well and the culture plates were incubated at 37 °C in an atmosphere of 5% CO_2 for 4 h. Afterwards, 100 uL of solubilization solution was added at each well to dissolve the formazan crystals and a 2h incubation period followed.

The absorbance was measured by spectrophotometry at 570 nm in a microplate reader (Biotek, Synergy 2). The fibroblasts grown without any cpTi particles were used as control for the normal proliferation activity of the cell line and all assays were performed in triplicate.

2.5 Statistical analysis

The statistical analysis of the experimental data was carried out using GraphPad prism 6 software (GraphPad Software, San Diego, USA). These results were compared by the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. It was considered statistically significant results for $p < 0.05$.

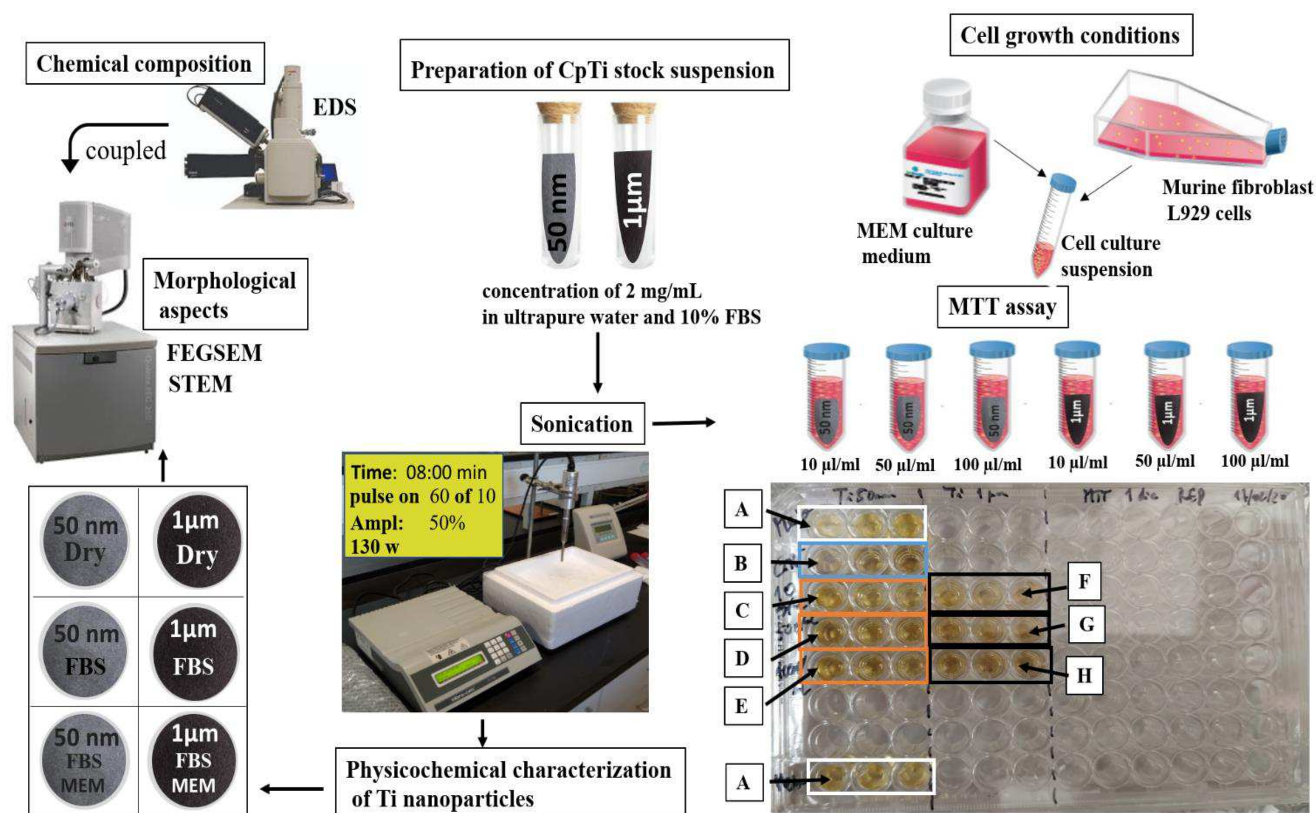


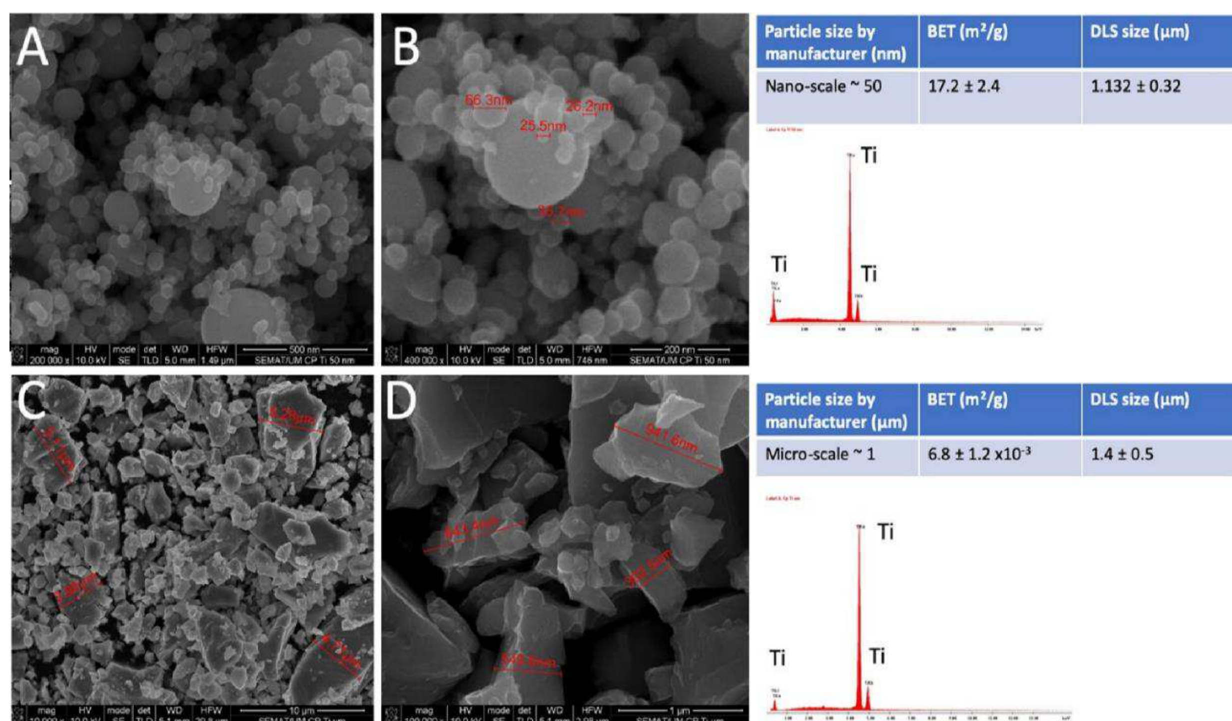
Figure 1. Schematic representation of the Physicochemical characterization of Ti nanoparticles and Cell viability assay. The Configuration of the 96-well flat bottom plates in which the cytotoxicity assay was carried out: **A** = Culture medium without cells, **B**= Culture medium with cells, **C** = Culture medium with cells and cp Ti 50 nm at 10 µl/ml, **D** = Culture medium with cells and cp Ti 50 nm at 50 µl/ml, **E** = Culture medium with cells and cp Ti 50 nm at 100 µl/ml, **F** = Culture medium with cells and cp Ti 1 µm at 10 µl/ml, **G** = Culture medium with cells and cp Ti 1 µm at 50 µl/ml, **H** = Culture medium with cells and cp Ti 1 µm at 100 µl/ml, all tested in triplicate.

3.Results and Discussion

Considering the effect of cp Ti particles on the cell viability, two different size (50nm and 1μm) and content (10, 50, and 100 μg/mL) of cp Ti particles were assessed in contact with L929 Murine fibroblasts by MTT assays over three different time points. The results partially validated the hypotheses since the 50 nm cp Ti particles at 50, and 100 μg/mL decreased the proliferation of fibroblasts. Thus, the cytotoxicity depends on the size, content, and chemical composition of the particles. In the culture medium, the minerals and proteins interacted with the micro- and nano-particles' surfaces that disturbed the cell perception on the titanium particles. The findings are described as follow.

3.1. Physicochemical characterization

Physiochemical characterization of cp Ti nanoparticles by BET revealed specific surface area at around ~17.2 m^2/g, as seen in Figure 2. Individual nano-particles at dry conditions were detected by FEGSEM and STEM at sizes ranging from 20 up to 90 nm with a mean size at 70 nm although particle agglomerates could be noted ranging from 150 up to 200 nm (Figure 2A and B). Cp Ti microparticles revealed size ranging from 0.3 up to 5.3 μm with a mean size at 1.4 μm at dry conditions, while particles agglomerates were noted at around 3μm after interaction with the culture medium (Figure 3 C and D).



Particle size by manufacturer (nm)	BET (m²/g)	DLS size (µm)
Nano-scale ~ 50	17.2 ± 2.4	1.132 ± 0.32

Particle size by manufacturer (µm)	BET (m²/g)	DLS size (µm)
Micro-scale ~ 1	6.8 ± 1.2 x10⁻³	1.4 ± 0.5

Figure 2. FESEM images of the cp Ti (A,B) nano- and (C,D) micro-particles. FESEGM performed at 10 kV on secondary electrons (SE) mode. On the right side, elements maps recorded by EDS analyses.

The optimized de-agglomeration of the nano-particles resulted in an increase in the individual nanoparticle proportion at around 60 nm leading to an increase of the specific surface area up to 57.3 m^2/g as seen in Figure 2. However, remnant particle agglomerates were still detected by FEGSEM and STEM that corroborated with the DLS results. High-resolution TEM images demonstrated that protein from FBS medium surrounded individual particles and aggregates since FBS was used as stabilization agent (Figure 3).

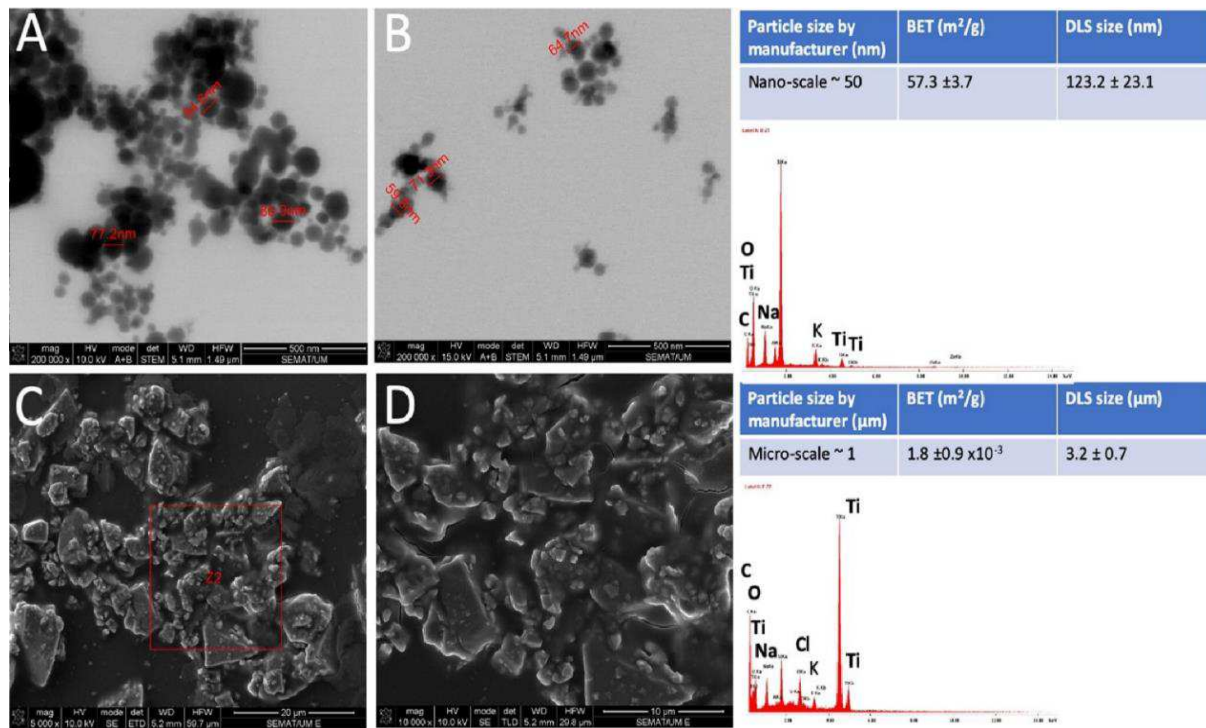


Figure 3. (A,B) STEM images of the cp Ti nano-particles performed at 10 kV. (C,D) FEGSEM micro-particles performed on secondary electrons (SE) mode at 10 kV. On the right side, elements maps recorded by EDS analyses.

EDS elemental maps recorded on the cp Ti particles are shown in Figure 3. In fact, the particles stablished interaction with the compounds deriving from the culture medium (MEM) as indicated by the presence of (Na) sodium, (K) potassium, and (Cl) chloride (Figure 2). The interaction of particles and salts occurs due to the chemical reactivity of the cp Ti particles to the medium since K^{+}, Na^{+}, Ca^{+2}, and PO^{-} form a stable layer on the cp Ti surfaces. In FBS, proteins adsorbed on the stable layers as represented by C or N, that results in a bio-complex with a cp Ti core that can influence the perception and interaction of the cells in culture medium. Cp Ti particles could effortlessly be internalized into the fibroblast considering the nano-scale size of the particles leading to genotoxic effects to the cells.

In a previous study, the size of cp Ti nanoparticles used was around 100 nm although Ti particles/agglomerates were detected by SEM above 100 nm were found(28). An *in vitro* study Physiochemical characterization of TiO2 indicated that their specific surface area was ~61.14 m^2/g and that the individual particles were approximately 25 nm in size, the

optimized de-agglomeration of the nano-particles resulted in an increase in the specific surface area with a mean hydrodynamic size of 142.1 ±5.6 nm due to the formation of a bio-complex made up of calcium (Ca), phosphorous (P) and proteins (e.g. Albumin) deriving from the medium culture(18). On the other hand, studies reported no agglomeration of cp Ti micro-particles particles since the size and morphological aspects of the particles are maintained in culture medium(27,29). Thus, the agglomeration of nano-particles occurs and the de-agglomeration must be performed prior to the culture medium to avoid bias of results. Since most of the studies have evaluated the effect of titanium oxide (TiO_2) nanoparticles, the agglomeration and de-agglomeration processes have been reported prior to the culture medium(6–10,18). There is lack of information on the morphological aspects, chemical composition, and size of cp Ti nano-particles regarding the agglomeration and de-agglomeration phenomena(22,30). However, the agglomeration of submicron particles (0.2-1 μm) has been inspected by FEGSEM and STEM in literature(15,16,31–33).

3.2. Cell viability

The principle of this test consists in the reduction of tetrazolium salts to formazan via mitochondrial reductase (Fig. 4).

Regarding the 50 nm particle size, a concentration of 10 μg/ml caused a lower decrease in the optical density of approximately by (8.57%) when compared to 50 μg/ml cp Ti nano-particles (9.28%) and 100 μg/ml (20%) for 1 day incubation. On the incubation for 3 days, the decrease in cell viability was significantly higher at 100 μg/ml cp Ti nano-particles (43%) when compared with 50 μg/ml (17%) although 10 μg/ml cp Ti nano-particles did not affect the cell behaviour ($p \leq 0.0001$). The cell proliferation significantly over 7 days incubation that MTT was not accurate to detected differences in cell viability among the three different content of cp Ti nano-particles as show in Figure 4.

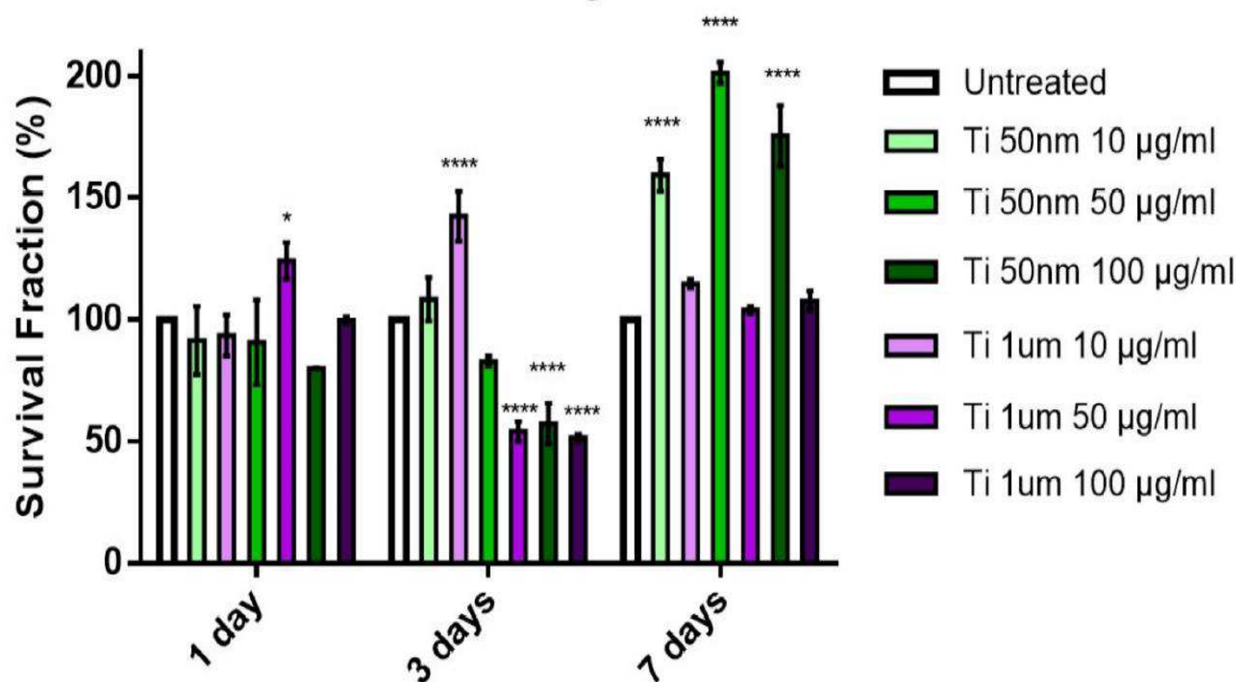


Figure 4. Schematic representation of the cell viability values obtained from the MTT assay. * $p \leq 0.05$; **** $p \leq 0.0001$.

Concerning cp Ti particles with 1 µm size, a slight decrease in cell viability by (6.4%) was recorded at 10 µg/ml particles although no effect was noted at 50 µg/ml (Fig. 4). Therefore, an increase in cell viability was noticed at 100 µg/ml of cp Ti particles ($p<0.05$). On the incubation for 3 days, a decrease in cell viability by (46%) was recorded at 50 µg/ml and by (48%) at 100 µg/ml cp Ti micro-particles ($p<0.0001$). The cell proliferation significantly increased over the seventh day of incubation and therefore the cell viability was not affected by the presence of cp Ti micro-particles as shown by MTT assays (Fig. 4).

Table 1. Statistic values obtained from the two-way ANOVA with Tukey's multiple comparison test. n.s. – no significance; * p ≤ 0.05; **** p≤0.0001.

Two-way ANOVA with Tukey's multiple comparisons test		Concentration of 10 µg/ml			Concentration of 50 µg/ml			Concentration of 100 µg/ml		
		Mean Difference	95% CI of difference	significance	Mean Difference	95% CI of difference	significance	Mean Difference	95% CI of difference	significance
1 Day	50 nm	8,57	-14,47 to 31,61	ns	9,28	-13,76 to 32,32	ns	20,02	-3,017 to 43,06	ns
	1 µm	6,44	-16,60 to 29,48	ns	-24,17	-47,21 to -1,133	*	0,26	-22,78 to 23,30	ns
3 Day	50 nm	-8,45	-31,49 to 14,59	ns	17,05	-5,987 to 40,09	ns	42,72	19,68 to 65,76	****
	1 µm	-42,46	-65,50 to -19,42	****	45,91	22,87 to 68,95	****	48,52	25,48 to 71,56	****
7 Day	50 nm	-59,33	-82,37 to -36,29	****	-101,4	-124,5 to -78,38	****	-75,54	-98,58 to -52,50	****
	1 µm	-14,78	-37,82 to 8,257	ns	-3,81	-26,85 to 19,23	ns	-7,7	-30,74 to 15,34	ns

To the best of our knowledge, the effect of cp Ti nanoparticles has not been studied in contact with fibroblasts(21,22). In a previous in vitro study, metallic ions released from stainless steel, nickel-free alloys, and titanium orthodontic alloys were placed in culture of human fibroblasts to evaluate the cell viability and DNA damage. MTT test results showed that the cell viability of fibroblasts in culture with Ti ions was higher when compared to stainless steel and nickel-free alloys(34). However, the toxic effect of cpTi particles was reported against other cell lines such as monocytes and macrophages(16,28,33). An *in vitro* study reported cpTi microparticle <20 µm in size in culture of human monocytes (THP-1 cells) over a period of 12, 24 and 48 h(16). MTS assays showed that titanium microparticles did not affect the proliferation of cells for 48 h incubation, but qRT-PCR demonstrated that cp Ti microparticles induced a proinflammatory response in macrophages characterized by increased expression of TNF-α and IL-6 cytokines known to stimulate bone resorption(16,33).

The toxic effect of 5 nm TiO2 nano-particles at different content (from 3 to 600 μg/mL) against Murine fibroblast L929 was reported by a previous study(35). MTT test indicated that cell viability was markedly reduced as the culture time and the concentration of TiO2 nano-particles increased(35). Another previous study reported the adverse osteoblast response when in contact with 25 nm TiO2(18). The bio-complex formed on TiO2 nanoparticles was composed of calcium (Ca), phosphorous (P) and proteins (albumin, glycoproteins-ALB protein and Alpha 2HS) deriving from the medium culture. That allowed the internalization of TiO2 nano-particles by osteoblast cells like a Trojan-effect(18). Previous findings suggested that TiO2 nanoparticles induced an adverse effect on the osteoblast that is dependent on the time and concentration of internalization. Morphological analysis of cell organelles indicated cell adverse response, and DNA analysis showed extensive fragmentation that may disturb gene expression and cause mutagenesis in cell replication(7–10,18).

Regarding cp Ti micro-scale particles, a previous *in vitro* study revealed the cytotoxicity of Cp Ti particles of ≤ 20 μm at concentrations of 0.01 to 1.0 mg/ml in culture of human gingival fibroblasts (HGF-1) and human calvarial osteoblasts (HCO) at three time points for 1, 7, and 21 days(31). MTT testing showed a decrease in metabolic activity only for 1-day incubation and at the highest cp Ti concentrations. The long-term toxic effect was slightly noticeable, although human gingival fibroblasts secreted interleukin 6 over the entire cell culture incubation. Such Interleukin stimulates the differentiation of macrophages and osteoblast precursor cells into mature and active osteoclasts(31). In contrast, another *in vitro* study was performed to determine the proliferation rate of human fibroblast cells in presence of cp Ti micro-particles at 0.488 μm size for three time points (1, 3, and 7 days)(32). MTT results showed that there was an inhibition of the metabolic activity and cell viability decreased progressively over time(32). Another *in vitro* study demonstrated that Ti6Al4V micro-particles (20 μm size) are also cytotoxic to Rat2 fibroblasts and therefore the MTT results showed a correlative relationship between cytotoxity and particle concentrations(12). It was also shown that Ti6Al4V particles at 20 μm size in culture of human osteoblast were able to induce cytotoxic effects. MTT assays were performed at four time points 1, 2, 3 and 7 days at concentrations of 100 , 50, 10, 1, 0.5, and 0.1 μg/mL. Those results showed that cellular viability decreased at high concentrations of micro-scale Ti particles(13).

4. Conclusions

Within the limitation of an *in vitro* study on physiochemical analyses of titanium nano-particles and their cytocompatibility, the main conclusions can be drawn:

- The mean size of cp Ti nanoparticles were at 70 nm with contacting surface area at around ~17.2 m^2/g although agglomerates of cp Ti were detected which decreased the surface contact area. After de-agglomeration cp Ti nanoparticles revealed size of 60 nm with surface area up to 57.3 m2/g, with protein from FBS medium surrounding individual particles. Cp Ti particles showed regular dimensions while cp Ti microparticles had an elongated morphological aspect and a mean size at 1.4 µm.
- Regarding the 50 nm particle size, The results from the MTT assay, used for studying the expression of mitochondrial enzymes, showed that there were marked reductions in the viability of cells cultured in media containing concentrations at 100 µg/ml only on 3 day.
- Concerning particles of 1µm, the MTT assay showed , a decrease in the metabolic activity of cells cultured in media containing concentrations at 50 and 100 µg/ml only on 3 day.
- Nevertheless, both nano- and micro-scale cp Ti particles could induce severe damage in cell DNA at high concentrations of (e.g. 100, 150, 200 µg/mL). It can occur due to the internalization of Ti nano-particles and ions which are chemically reactive to the cell organelles.
- Future studies are required to clarify the toxic effect of the Ti nano- and submicron particles on the cells and tissues surrounding the implant. Studies should focus on the inflammatory mediators and cytokines such as toll-like receptor 2 ligands in the immune response. Different cell lines such as macrophages, fibroblasts, osteoblasts, keratinocytes, mesenchymal cells should be assessed using different methods (e.g. comet assays, Reactive Oxygen Species (ROS) Measurements, Real-Time PCR).

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