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In vitro evaluation of different prevention and decontamination strategies used in peri-implant infections of titanium dental implants

PhD Thesis

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PUBLICATIONS RELATED TO AND INCLUDED IN THE THESIS

I. **R. Masa**, Á. Deák, G. Braunitzer, Zs. Tóth, J. Kopniczky, I. Pelsőczi-Kovács, K. Ungvári, I. Dékány, K. Turzó: TiO_2/Ag–TiO_2 Nanohybrid Films are Cytocompatible with Primary Epithelial Cells of Human Origin: An *In Vitro* Study. *Journal Of Nanoscience And Nanotechnology* 18 : 6 pp. 3916-3924. , 9 p. (2018), PMID: 29442727, doi: 10.1166/jnn.2018.15261

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II. **R. Masa**, I. Pelsőczi-Kovács, Z. Aigner, A. Oszkó, K. Turzó, K. Ungvári: Surface Free Energy and Composition Changes and Ob Cellular Response to CHX-, PVPI, and ClO_2-Treated Titanium Implant Materials, *Journal of Functional Biomaterials* 13 : 4 Paper:202,11p. (2022)PMID: 36412843,doi: 10.3390/jfb13040202

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I. HH. Niller, **R. Masa**, A. Venkei, S. Mészáros, J. Minárovits: Pathogenic mechanisms of intracellular bacteria. *Current Opinion in Infectious Diseases,* 2017; 30:309-315, doi:10.1097/QCO. 0000000000000363

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1. INTRODUCTION

The rehabilitation of partially or completely edentulous patients has undergone a significant transformation, shifting away from traditional treatments like bridges and removable dentures towards dental implants, thanks to their high success rate. Due to its exceptional physical-chemical and mechanical properties, titanium (Ti) and its alloys has become the preferred material in orthopedic and dental implantology. The screw-shaped implant body, the part that remains embedded in the bone, is usually made from commercially pure Ti.

The long-term success of a dental implant is mainly depending on the quality of osseointegration and the effective prevention of bacterial biofilm formation. Osseointegration is basically the biological process of new bone formation on and toward the implant side, regulated by osteoprogenitor and osteoblast cells. The establishment of a preferred gingival seal depends on the proper attachment and metabolic activity of gingival epithelial and fibroblast cells. Primary epithelial cells exhibit a preference for smooth surfaces (Ra < 0.5 μm) over rough ones, fibroblasts cells prefer machined (Ra: 0.5-1 μm), while osteoblast cells prefer moderately rough (Ra: 1-2 μm) titanium surfaces. Beside primary cells, immortalized cell lines are also frequently applied cell culture models of *in vitro* cytocompatibilty studies of biomaterials.

The same cell types participate in the connection of soft and hard tissues to the implant and to the tooth surface, but there are main differences too. In gingival attachment around implants, collagen fibers and fibroblast cells are oriented parallel to the implant or abutment surface, whereas in natural teeth, these fibers anchor perpendicularly into the cementum. Reduced cell number in peri-implant connective tissue and diminished blood supply can contribute to the faster and deeper progression of pathogenic bacteria.

Despite the high success rate of dental implants, challenges arise with failures becoming more frequent in parallel with the increasing number of implant placements. Changes in the oral microbiome, particularly an imbalance favoring pathogenic bacteria, can lead to inflammation in peri-implant tissues, similarly to gingivitis and periodontitis around natural teeth. In case of peri-implant mucositis, only the soft tissues are affected, while the more progressive form is peri-implantitis, described as an irreversible inflammation of both soft and hard tissues. Anaerobic Gram-negative bacteria dominate the biofilm in both cases. As there is currently no universally accepted gold standard treatment for peri-implantitis, prevention should be main priority.

Photocatalytic surfaces and coatings are promising candidates of antibacterial surface modifications of dental implants. They have the potential to prevent the attachment of pioneer colonizing bacteria, inhibit biofilm formation, and reduce pathogenic bacterial loads by releasing reactive oxygen species (ROS). Titanium dioxide (TiO_2) and silver-doped titanium dioxide nanoparticles (Ag-TiO_2) embedded in a polymer matrix can be applied as innovative antibacterial surface coatings on Ti surfaces. The addition of nano-silver to titanium dioxide can enhance its photocatalytic activity under visible light. The disinfectant effect primarily relies on the photocatalytic property of Ag-modified nano-hybrid films rather than the bactericidal effect of released silver ions. Based on our preliminary studies, we reduced the Ag content in the polymer films from 0.5% to 0.001% to improve cellular compatibility.

Surface decontamination of infected titanium implant materials is a huge challenge due to irregularities of the surface (macro and micro-topography). Beside various widely applied mechanical debridement techniques, chemical antiseptic solutions are preferred supplemental tools by most clinicians. The antimicrobial properties of these solutions are well known, nevertheless residues of these agents could alter the physicochemical properties of the titanium surface and subsequent cellular responses.

Chlorhexidine digluconate has been well-documented for its antimicrobial efficacy in both periodontal and peri-implant infections. However, recent studies have not recommended the use of CHX as an implant decontamination agent due to concerns about its unclarified cytotoxicity to osteoblasts. Povidone iodine (PVPI) is a wide-spectrum antibacterial and antiviral agent. It is a frequently used antiseptic in wound healing and in endodontics. Solumium is a recently developed antibacterial solution containing hyper pure chlorine dioxide (ClO_2). The penetration of this antiseptic into human tissues is limited, but it exhibits rapid bactericidal properties.

2. AIMS OF THE THESIS

The primary goal of my research was to investigate two different strategies employed against inflammation occurring around dental implants. In the first part of my dissertation, I examined two newly developed photocatalytic coatings (TiO_2 and Ag-TiO_2- copolymer). These polymer coatings could play a significant role in the prevention of peri-implantitis and the conservative (non-surgical) treatment of established inflammation. It is crucial for a biomaterial (such as a titanium dental implant) to maintain high biocompatibility even after surface modification. My colleague, Annamária Venkei, had previously confirmed the antibacterial effects of these polymer films, so our focus was on assessing the biocompatibility of the surfaces before proceeding to more complex animal experiments and clinical studies. Initially, we conducted a physicochemical analysis of the surfaces, followed by an examination of the adhesion and proliferation tendencies of two different cell types (epithelial and osteosarcoma cells).

In the second part of my dissertation, I examined the potential effects of disinfectants applied during the decontamination of titanium dental implants on the titanium surface. Various chemical agents (disinfectants) are routinely used in dental clinics, but the detailed interaction of these agents with titanium surfaces is not yet fully understood. We investigated two widely used solutions (Curasept and Betadine) and a recently developed one (Solumium). Initially, we examined the surface wettability, calculated the surface free energy, and assessed any possible changes in the chemical composition of the surfaces after a 5-minute treatment. To model the body's response, we evaluated the adhesion and proliferation of freshly isolated osteoblast cells.

Our research set out to achieve several key objectives. Firstly, we aimed to conduct a comprehensive physicochemical analysis of nanocomposite films. Secondly, we sought to assess the *in vitro* biocompatibility of these nanocomposite films by examining their interactions with epithelial and osteosarcoma cells. Additionally, we embarked on an investigation into the physicochemical properties of titanium surfaces that had been treated and came into contact with various chemical agents. Lastly, we focused on evaluating the biocompatibility of these chemically treated titanium surfaces, specifically their interactions with primary osteoblast cells.

3. MATERIALS AND METHODS

3.1 Biocompatibility of nanocomposite surfaces

- Commercially Pure grade 4 (CP4) polished titanium disks (1.5 mm thick and 9 mm in diameter) for epithelial cell culture were employed, while sandblasted and acid-etched (Ti_{SA}) disks were used for investigations with the MG-63 immortalized cell line.
- Disks were uniformly cleaned and sterilized and subsequently coated with polymer-based photocatalytic nanohybrid films: TiO_2 and Ag-TiO_2 (supplemented with 0.001 wt% plasmonic Ag). In order to achieve a partial photodegradation of the outermost layer of the polymer coatings, the disks were exposed to UV-C light for 60 minutes, which led to higher nanoparticle concentration on the surface.
- Scanning electron microscopy (SEM) was applied for capturing high magnification images.
- Surface roughness measurements were conducted using a profilometer. The average surface roughness (Ra, measured in micrometers - μm) of the disks was determined within 500×500 μm^2 areas.
- Two distinct cell culture models: primary human epithelial cells and MG-63 osteosarcoma cells were investigated.
- Plate wells and uncovered Ti disks were utilized as control surfaces. Cell attachment was assessed after 24 hours, while the proliferation rate was measured after 72 and 168 hours using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich GmbH, Germany).
- Fluorescent microscopy was applied for visualization of cellular behavior.
- The means ± SE (standard error of the mean) for Ra (μm) measured by the profilometer and OD_{540} values measured by the spectrophotometer underwent normality testing, and we conducted comparisons using a one-way analysis of variance (ANOVA), followed by post hoc tests including Tukey's HSD, LSD, and Scheffé. Statistical significance was set at $p < 0.05$.

3.2 Biocompatibility of chemical agent treated titanium

- CP4 sandblasted and acid-etched titanium disks (Denti® System Ltd. Hungary) measuring 1.5 mm in thickness and 9 mm in diameter, were used in this study.
- Disks were cleaned and sterilized, then treated for five minutes with chlorhexidine-digluconate (Curasept ADS 220, 0.2%, Switzerland), povidone-iodine (Betadine, 10%, Switzerland), and chlorine-dioxide (Solumium dental, 0.12%, Hungary). Following the treatment, the disks were thoroughly washed with ultrapure water three times. In contrast, control disks were solely rinsed with ultrapure water.
- Surface wettability study was conducted by pure water (PW) and diiodomethane (MI) drops. The surface free energy (SFE), expressed in γ (mJ/m2), was determined using the Owen-Wendt-Rabel-Kaelble (OWRK) method.
- Chemical composition of the titanium surfaces was assessed through X-ray photoelectron spectroscopy (XPS-LEIS) analysis.
- Primary osteoblast cells were freshly separated and applied in viability studies.
- We employed standard colorimetric assays (MTT, alamarBlue®, lactate dehydrogenase (LDH) test) in the assessment of cytocompatibilty.
- Visualization was conducted with fluorescent microscopy.
- For contact angle measurements (Θ (°)), the means ± standard deviations (SD) of the groups were compared using an unpaired Student t-test. The means ± standard error of the mean (SE) were determined for each optical density (OD) value. Subsequently, after confirming normality through testing, the data were analyzed using the nonparametric Kruskal-Wallis test. Statistical significance was set at $p < 0.05$.

4. RESULTS

4.1 Structural characterization and cell viability studies of photoactive nanohybrid films

Scanning electron microscopy (SEM) images revealed significant differences in the surface morphology of the specimens, especially at higher magnification (x 5000). The polished surface was nearly flat, with some scratching originating from the polishing method. Ti_{SA} disks exhibited typical surface characteristics of uneven surface features. TiO_2-copolymer film exhibited an amorphous surface structure on the microscale, while on the silver-containing film characteristic aggregates of the nanoparticles appeared.

Profilometry measurements confirmed the above mentioned differences in surface morphology captured by SEM. Polished titanium exhibited the smoothest surface with a roughness value (Ra) of 0.037 μm. In contrast, both SA titanium and TiO_2-copolymer coated disks exhibited similar roughness values (Ti_{SA}: Ra = 1.26 μm; TiO_2: Ra = 1.79 μm). Due to the aggregation of nanoparticles, the Ag-TiO_2 coated samples displayed significantly rougher surfaces (Ag-TiO_2: Ra = 5.76 μm)

We observed a significant difference of epithelial cell attachment (24h) between the Ag-TiO_2 modified surface ($OD_{540nm,\ Ag\text{-}TiO2}$ = 0.08 ± 0.004) and the polished control Ti surface ($OD540_{nm,\ Ti\ (P)}$ = 0.05 ± 0.004). This positive cell response nearly reached the values seen on the control plate. No significant differences were found in epithelial attachment between the two copolymer-coated disks. Gingival epithelial cells exhibited similar proliferation rates on all investigated disks ($OD_{540nm,\ Ti\ (P)}$ = 0.07 ± 0.012, $OD_{540nm,\ TiO2}$ = 0.06 ± 0.002, $OD_{540nm,\ Ag\text{-}TiO2}$ = 0.07 ± 0.004) at 72h. Despite opposite tendencies after one week of incubation, no significant differences were observed between polished Ti and either of the nanohybrid films.

MG-63 osteosarcoma cells exhibited growing tendencies similar to epithelial cells. We did not find significant differences in MG-63 cell attachment between the coated surfaces and the control Ti surfaces ($OD_{540nm,\ Ti\ (SA)}$ = 0.06 ± 0.005, $OD_{540nm,\ TiO2}$ = 0.06 ± 0.003, $OD_{540nm,\ Ag\text{-}TiO2}$ = 0.07 ± 0.004). However, significantly more cells ($p = 0.03$) attached to the surface of nanosilver-coated disks compared to TiO_2-copolymer coated disks. Early proliferation rate of the cells was significantly higher on the control Ti surface than on the TiO_2-copolymer and nanosilver coated samples ($p < 0.001$). After 168 hours, a substantial increase in cell proliferation was observed on the uncoated Ti surfaces, whereas on the nanohybrid surfaces,

the cells did not exhibit any noticeable growth tendency ($OD_{540nm,\ Ti\ (SA)} = 0.28 \pm 0.022$, $OD_{540nm,\ TiO2} = 0.06 \pm 0.001$, $OD_{540nm,\ Ag\text{-}TiO2} = 0.08 \pm 0.005$).

The fluorescent composite images of the cells confirmed the MTT results.

4.2 Evaluation of the effect of different chemical agents used as supportive therapy

We compared the surface wettability properties of the chemical solutions with PW and MI drops. In the PW tested group the control titanium samples exhibited an average contact angle (θ) of 24.6 ± 5.4, almost identical with the contact angle observed in the PVPI treated group (θ = 24.9 ± 4.1). ClO_2 (θ = 39.2 ± 9.8) and CHX (θ = 47.2 ± 4.1) treated discs displayed significantly higher contact angle values ($p_{ClO2} = 0.012$; $p_{CHX} < 0.0001$) when compared to the control group. In case of MI drops the following contact angle values were recorded: $\Theta_{control\ Ti} = 20.1 \pm 2.1$; $\Theta_{PVPI} = 20.6 \pm 2$; $\Theta_{ClO2} = 21.1 \pm 2.3$; $\Theta_{CHX} = 24.3 \pm 1.7$. These measurements revealed relatively minor differences among the groups, with only the CHX treated group exhibiting a significantly higher Θ ($p = 0.003$) in comparison to the untreated Ti. Regarding SFE calculations, only Betadine treatment was able to preserve a similar SFE ($\gamma = 70.7\ mJ/m^2$) as the control titanium surface ($\gamma = 70.9\ mJ/m^2$).

The recorded X-ray photoelectron spectroscopy (XPS) spectra confirmed the presence of titanium (Ti), oxygen (O), and carbon (C) on both the untreated and treated titanium samples. These elements are typically observed on the surfaces of sandblasted-acid etched titanium disks. No other relevant element was identified. We concluded, that these chemical agents did not induce significant alterations in the surface chemistry of CP4 titanium models.

All of the cellular viability and cytotoxicity tests exhibited excellent osteoblast response to the treated disks. Initial cellular attachment (24h) of the primary osteoblast cells was significantly higher on the control plate ($p < 0.001$) compared to the Ti disks. However, no significant differences were observed between the control disk and the disks treated with the antiseptics ($OD_{570nm,\ Ti} = 0.031 \pm 0.001$, $OD_{570nm,\ CHX} = 0.034 \pm 0.002$, $OD_{570nm,\ PVPI} = 0.033 \pm 0.002$, $OD_{570nm,\ ClO2} = 0.032 \pm 0.001$). High rate of proliferation (72h) on both treated and untreated Ti surfaces was recorded, with no statistically significant differences ($OD_{570nm,\ Ti} = 0.142 \pm 0.014$, $OD_{570nm,\ CHX} = 0.147 \pm 0.021$, $OD_{570nm,\ PVPI} = 0.136 \pm 0.017$, $OD_{570nm,\ ClO2} = 0.143 \pm 0.017$).

The percentage reduction of alamarBlue® indicated high cellular viability in accordance with the MTT test. There were no significant differences observed between the control and treated groups, both at 24 hours and 72 hours.

According to the LDH assay (24h) we observed the lowest cytotoxicity of the CHX treated group (2%) compared to the other antiseptics (above 5%). There was no significant difference in the number of damaged cells in the treated Ti samples (approximately 0% cytotoxicity) after 72 hours compared to the untreated control. This low release of LDH in the treated groups indicates that the titanium surfaces remained highly cytocompatible after a 5-minute treatment with the antibacterial solutions.

5. DISCUSSION

My doctoral work primarily focuses on improving the antibacterial properties of dental implants, further investigating two different strategies in the fight against bacteria: photo-activated nanohybrid coatings (for prevention and therapy) and the interaction of oral antiseptics (for therapy) with a Ti model surface. While antibacterial properties have been well described by other members of our research group, my goal was to examine the cellular response of various primary cells and cell lines to modified Ti surfaces.

Biocompatibility and surface characterization of the nanocomposite films

The first part of my thesis focuses on conducting a biocompatibility study involving two newly developed nanohybrid coatings: TiO_2-copolymer and $AgTiO_2$-copolymer films. These nanohybrid films hold potential for applications as thin coatings on the neck part of implants, primarily aimed at preventing or even treating peri-implant infections.

Surface roughness is a relevant topography feature of biomaterials regarding cellular response. Smooth titanium (Ti_P) served as the control for epithelial cells, whereas titanium-sandblasted-acid etched (Ti_{SA}) and titanium dioxide (TiO_2) copolymer disks exhibited moderately rough surfaces. It is widely accepted that epithelial cells tend to thrive on smooth, polished surfaces, while osteoblasts require surface irregularities to enhance osseointegration. Most *in vitro* studies have concluded that MG-63 cells exhibit a preference for rougher surfaces (with Ra values around 4-5 μm). Despite the R_a of AgNP-modified disks being close to the ideal range, MG-63 cells displayed a notably low proliferation rate. Similarly surprising was the relatively high attachment and survival of epithelial cells on the rougher Ag-copolymer modified surface. This unexpected response of the cells may be attributed to the chemistry of the polymer matrix or the presence of nanoparticles, and further molecular research is needed to elucidate the specific sensitivity of osteosarcoma cells in this context.

In contrast to findings in the existing literature, our results revealed a decrease in the quantity of epithelial cells and a low, albeit fluctuating, quantity of MG-63 cells on the TiO_2-copolymer modified disks. Recent publications have also demonstrated high proliferation rates of primary human gingival fibroblast cells on nanoporous TiO_2 coatings. The variation in epithelial cell proliferation observed in our study could potentially be attributed to size-

dependent toxicity of TiO_2NPs. Additionally, the specific sensitivity of cells to the polymer matrix (p(EA-co-MMA)) or the potential release of polyacrylate from the coatings may provide further explanations. Our MTT tests revealed that the attachment of gingival epithelial cells to the Ag-TiO_2-copolymer-coated disks was significantly higher when compared to the polished Ti control samples. Despite this promising attachment, these epithelial cells did not exhibit a significant growth trend on the AgNP-coated samples, and the relative cell mass remained stable throughout the duration of the study.

To assess the response of osteoblast cells, we employed the most commonly used immortalized cell line, as the separation of primary osteoblast cells had not yet been completed at the time of this study. MG-63 cells faced challenges in terms of survival on the photocatalytic films, with stagnant or even declining cell numbers observed. However, living cells were detected on both polymer coatings after one week of incubation, suggesting the possibility of delayed proliferation. Other researchers have also investigated the reduced viability of immortalized cell lines on surfaces incorporating silver nanoparticles (AgNPs). In recent years, the use of silver nanoparticles for cancer therapy has gained significant attention. Clinical studies involving silver nanoparticles (AgNPs) in conjunction with titanium dental implant surfaces remain limited, with the majority still in the pre-clinical phase. However, recent studies have shown promise in this regard.

These surfaces with reduced silver content demonstrated both acceptable biocompatibility and antibacterial activity simultaneously. Achieving such a balance would be desirable for various cell types in the future, promoting the safe and long-term utilization of nanomaterials in biomedical devices.

Chemical agents as part of the supplementary therapy of peri-implant infections

Antimicrobial oral solutions serve as widely employed adjunctive tools for both conservative and surgical interventions in case of peri-implant infections. The second part of my thesis focuses on a deeper understanding of the interactions between three distinct disinfectants and titanium models. This involved conducting studies on surface wettability and analyzing the elemental composition, followed by assessing the cellular viability of primary osteoblasts on titanium disks treated with the chemical agent for a duration of five minutes. This short time period is easily manageable even in private practice.

In general, hydrophilic surfaces facilitate cell adhesion and exhibit lower affinity for proteins, whereas hydrophobic surfaces exhibit the opposite behavior. The contact angle measurements of PVPI-treated samples closely resembled those of the control disc, while the contact angles of CHX and ClO_2-treated samples were similarly high. Notably, only the PVPI-treated samples were able to maintain the initial SFE of untreated titanium, suggesting the absence of any irreversible interaction between Betadine (PVPI) and titanium. Despite statistically significant differences in the wettability of the antibacterial agent-treated disks, all samples remained within the hydrophilic range ($\Theta < 90^{o}$).

The metabolic activity of osteoblasts on the treated samples was assessed using various bioassays, including MTT, alamarBlue®, and a cytotoxicity study employing lactate dehydrogenase (LDH). We observed no significant difference in the proliferation rate of osteoblast cells across the treated samples. The sole notable change was a higher number of damaged cells at the 24-hour mark, with CHX-treated samples appearing to exhibit greater cytocompatibility. Despite the anticipated substantial shift in surface free energy (SFE), which was expected to be a critical factor influencing biological responses, primary osteoblast cells demonstrated tolerance towards the higher contact angle (Θ) and lower SFE associated with CHX and ClO_2-treated disks. A limited number of publications exist concerning the biocompatibility of titanium biomaterials treated with povidone-iodine (PVPI) or chlorine dioxide (ClO_2). The significance of this study stems from the fact that primary osteoblasts displayed robust viability on these treated surfaces.

As antimicrobial resistance becomes increasingly prevalent, the demand for potent antibacterial enhancements of titanium surfaces and chemical disinfectants continues to grow. Visible light-induced Ag-TiO_2 nanohybrid coatings have exhibited promising responses from host cells, along with high bacterial toxicity. When used as supplemental therapy, solutions based on povidone-iodine (PVPI) and/or chlorine dioxide (ClO_2) have the potential to further reduce bacterial loads to non-pathogenic levels, thereby sustaining inflammation-free conditions over extended periods. To gain a deeper understanding of the response of the alveolar bone and gingival tissues as a complex system to these coatings and chemical antiseptics, further *in vivo* and clinical trials are essential.

6. SUMMARY AND CONCLUSIONS

Biomaterials offer a unique opportunity to restore impaired functions of the human musculoskeletal system. Among the most extensively researched biomaterials in healthcare, titanium dental implants have been a focal point of both industrial and dental research for decades. Bacterial adhesion to implant surfaces, either alone or in combination with excessive loads on implant-supported prostheses, can lead to severe peri-implant infections, ultimately resulting in implant failure.

The absence of a standardized, effective treatment protocol underscores the significance of any progress made in the prevention or treatment of implant-associated infections. The primary objective of my doctoral research was to investigate the biocompatibility of titanium surfaces treated with two nanoparticle-doped polymer films and three chemical disinfectants. This investigation encompassed the examination of various physicochemical properties, including roughness, wettability, and chemical composition, alongside *in vitro* cytocompatibility tests involving a range of cell types, such as MG-63 osteosarcoma cells, primary epithelial cells, and osteoblasts.

The main conclusions of the thesis are:

1. TiO_2-nanohybrid films exhibited similar roughness to the control Ti, whereas $AgTiO_2$-copolymer films displayed a significant increase in Ra.
2. The newly developed photocatalytic nanohybrid coatings demonstrated excellent attachment of human epithelial cells.
3. Despite the reduced Ag content (0.001%) of the polymer coating, suppressed proliferation of MG-63 cells was observed.
4. Treatment of Ti surfaces with PVPI maintained the wettability and surface free energy (SFE) at levels comparable to untreated Ti.
5. CHX and ClO_2 solutions led to higher contact angles and lower SFE compared to the control, although this shift still fell within the hydrophilic range.
6. Despite the significant alterations in wettability, primary osteoblast cells exhibited a high proliferation rate on all of the surfaces.

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