# Addition of $Ca^{2+}$ to Titanium Plates by a Hydrothermal Method and the Effects on Human Gingival Fibroblasts

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# Abstract

**Background:** Human gingival fibroblasts (hGFs) have key roles in the formation of soft-tissue attachments around dental implants. We added calcium ions ($Ca^{2+}$) to the surface of titanium plates (TPs) to make it more conducive to the early adhesion and proliferation of hGFs.

**Methods:** Ca2+ was loaded onto the TP surface by a hydrothermal method. The morphology and composition of TP surfaces were determined by scanning electron microscopy and energy-dispersive spectroscopy. Proliferation of hGF-1 cells was measured by the CCK-8 assay. Immunofluorescence staining was done to detect adherent proteins on the TP surface. TPs were divided randomly into two groups: control and Ca.

**Results:** In the Ca group, irregular lamellar crystals were found on the surface of TPs; The percentage of hGF-1 cells adhering to TPs in the Ca group was significantly higher than that in control group ($P < 0.01$); The fluorescence of integrin-β1 and F-actin in the Ca group was stronger than that in the control group.

**Conclusions:** Our data suggest that $Ca^{2+}$ can be added to TP surfaces by a hydrothermal method, and can enhance hGF adhesion. This property may be beneficial if $Ca^{2+}$ is added to titanium surfaces before dental implantation.


# 1. Introduction

With the development of materials science, dental implants are being used increasingly in clinical treatment. Also, the long-term stability of dental implants is garnering attention [1,2].

In the past few decades, good osseointegration has been considered an important indicator to evaluate the success of dental implantation. In recent years, scholars have realized that the long-term stability of implants requires not only good osseointegration, but also good integration of soft tissue at the site of a transgingival implant [3,4]. The latter can separate the complex oral environment from alveolar bone, prevent the invasion of bacteria so as to protect the implant from bacteria and other inflammatory factors and, finally achieve long-term stability of the implant [5,6,7].

The soft tissue at the site of a transgingival implant is composed of epithelial tissue and its deep, dense connective tissue. A good combination of healthy connective tissue and implant abutment is particularly important [8]. Human gingival fibroblasts (hGFs) have a key role in the formation of soft-tissue attachments around an implant. They are the main cells in the connective tissue at the transgingival site, the main components that can synthesize the extracellular matrix (ECM), and participate in tissue repair/regeneration in the sealing of soft tissue [9,10]. Therefore, one must select suitable materials for the site of a transgingival implant and create them with suitable surfaces for the adhesion and proliferation of fibroblasts. This strategy facilitates early and rapid formation of good soft-tissue bonding around implants and reduces the risk of bacterial invasion so as to maintain the long-term stability of the implant.

Titanium is the main material for implant abutments due to its excellent biological properties. However, meeting the requirements of rapid integration with the surrounding soft tissue after repair to form an early soft-tissue seal using titanium is difficult. Therefore, several studies have treated the titanium surface to make it more conducive to the early adhesion and proliferation of fibroblasts so as to form good soft-tissue sealing faster [11,12,13,14].

The calcium ion ($Ca^{2+}$) plays a vital part in growth and development, osteogenesis, wound sealing, and antibacterial activities. $Ca^{2+}$, as a signaling molecule, can play a part in the differentiation, proliferation, and apoptosis of cells by regulating different signaling pathways[15,16]. Seo et al. found that different $Ca^{2+}$ concentrations in the cytoplasm can control ion release from intracellular storage by regulating the ryanodine receptor or inositol-1,4, 5-triphosphate receptor channel[17]. Giorgi et al. found that maintenance of the $Ca^{2+}$ concentration plays a crucial part in cellular operations, and that a change in $Ca^{2+}$ concentration can change the rate of apoptosis and, thus, affect cell death [18].

We added $Ca^{2+}$ to the surface of titanium metal by a hydrothermal method to make it more conducive to the early adhesion and proliferation of hGFs. We aimed to promote the formation of a good seal between a titanium-base platform and surrounding soft groups.

# 2. Results

2.1 SEM of the TP surface

SEM results of the TPs in the two groups are shown in Fig.1 (all images were taken ×800 magnification). TPs in the control group had a smooth and flat surface. In the Ca group, irregular lamellar crystals were found on the surface of TPs.

2.2 EDS of the TP surface

Surface elemental analysis of TPs in both groups as analyzed by EDS is shown in Fig.2. EDS of TPs in the control group showed them to contain oxygen (O) and titanium (Ti), whereas TPs in the Ca group contained O, Ti, and calcium (Ca).

2.3 Adhesion by hGF-1 cells

The CCK-8 assay was used to detect the adhesion of hGF-1 cells on the TP surface in the two groups (Fig.3). The percentage of hGF-1 cells adhering to TPs in the Ca group was significantly higher than that in control group at 1, 8, and 12 h ($P < 0.01$). After 1, 3, 5 days of culture, the proliferation trend of hGF-1 cells in the Ca group was significantly higher than that in the control group ($P < 0.01$).

2.4 Immunofluorescence staining for adherent proteins

hGF-1 cells were cultured on the TP surface of both groups for 3 days. Then, we observed the distribution of F-actin (red) and integrin-β1 (green) by fluorescence microscopy (Fig.4). hGF-1 cells on the TP in the

control group had short spindles, were small, spread over a small area, F-actin morphology was discontinuous, the range of integrin-β1 distribution was small, and fluorescence intensity was weak. In the Ca group, hGF-1 showed long spindles with a large area of spread, the fluorescence intensity of F-actin and integrin-β1 was wide, and the cell boundary was intact, continuous, and strong.

# 3. Discussion

The ECM–integrins–cytoskeleton is considered to be one of the most important signaling pathways [19]. Integrins act as protein receptors on cell membranes. Extracellular structures bind to proteins in the ECM. Intracellular structures bind to actin filaments on the cytoskeleton through actin, thereby realizing bidirectional transduction of intracellular and intracellular signals. Cells have transmembrane adhesion molecules on their surface (including integrin) that facilitate adhesion to the ECM surface [20,21]. Most human cells proliferate, migrate, and differentiate after adhering to the ECM through their adhesion proteins. We hypothesized that $Ca^{2+}$ addition to a titanium surface using a hydrothermal method would provide a favorable surface for ECM proteins to adsorb onto the titanium matrix.

In vitro studies, SEM, and EDS showed that $Ca^{2+}$ could be added to the surface of TPs by a hydrothermal method. Immunofluorescence staining revealed that, compared with the control group, hGF-1 cells on TPs in the Ca group grew spindles and had a wider area of spread; also, more hGF-1 cells were on the TP surface and they had more intercellular connections. F-actin is a basic cytoskeletal component involved in the movement and proliferation of cells. Therefore, $Ca^{2+}$ seemed to promote the adhesion of hGF-1 cells to the surface of TPs by enhancing F-actin expression.

Palaiologou et al. demonstrated in an in vitro study that an integrin-β subgroup binding different integrin-α subunits had the highest content on hGF membranes [22]. Hence, we selected integrin-β1 for observation. The cytoskeleton is a complex network comprising different protein fibers and various regulatory proteins, including microfilaments, microtubules, and intermediate filaments. Among them, microfilaments are the main purveyors of signal transduction in the cytoskeleton, and the latter is composed mainly of F-actin. Therefore, rhodamine was selected for F-actin-specific staining in the present study. Staining showed that integrin-β1 and F-actin had stronger fluorescence in the Ca group than that in the control group, which suggested that $Ca^{2+}$ adsorbed more ECM proteins, thereby promoting the formation of more adhesive plaques in hGF-1 cells. These results also suggest that $Ca^{2+}$ addition to TPs by a hydrothermal method can promote the adhesion and proliferation of hGF-1 cells on the TP surface.

The main limitation in our study was that we did not study the antibacterial properties of the TPs. In the mouth, implant abutments are surrounded by various types of bacteria.

# 4. Conclusion

$Ca^{2+}$ can be added to the surface of TPs by a hydrothermal method, and can enhance hGF adhesion. This property may be beneficial if $Ca^{2+}$ is added to titanium surfaces before dental implantation.

# 5. Materials And Methods

5.1 Titanium plates (TPs)

Commercially available plates made of pure titanium (15 mm in diameter and 1.5 mm in thickness) were used (Fig.5). TPs were divided randomly into two groups: control (Cont) and Ca (hydrothermal treatment with CaCl2 solution (20 mmol/L) for 10 h at 160°C).

5.2 Scanning electron microscopy (SEM) of the TP surface

After the two groups of TPs had dried naturally, the surface was sprayed with gold. We observed the morphology of each TP surface by SEM using an acceleration voltage of 15 kV and magnification of ×800.

5.3 Energy-dispersive spectroscopy (EDS) of the TP surface

After TPs in the two groups had dried naturally, the surface was sprayed with gold. We analyzed the elemental composition on the surface of each TP by EDS.

5.4 Culture of hGF-1 cells

TPs in both groups were cleaned ultrasonically with pure acetone for 20 min, anhydrous ethanol for 10 min, deionized water for 10 min, and then sterilized with ultraviolet light. hGF-1 cells ($2\times10^4$ cells/mL, CL-0356, Procell Life Science&Technology Co.,Ltd.) were inoculated on the surface of TPs and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was added. Culture was undertaken in a sterile environment at constant temperature (37°C) in an incubator containing 5%$CO_2$.

5.5 Adhesion of hGF-1 cells

Cell Counting Kit (CCK)-8 diluent solution (500 μL) was added to each TP, and the latter placed in an incubator at 37°C for 1 h away from light. The optical density of each well at 450 nm was measured, and the reference wavelength was 450 nm. The adhesion and proliferation of hGF-1 cells on the TP surface were detected at 1, 8, and 12 h, as well as at 1, 3 and 5 d.

5.6 Immunofluorescence staining for adherent proteins

TPs in both groups were cultured with hGF-1 cells for 3 days. Then, TPs were rinsed twice with phosphate-buffered saline, followed by addition with 4% paraformaldehyde, and fixed for 30 min at room temperature. Integrin-β1 (1:250 dilution) was added in a wet box at 4℃ and the TPs left overnight. TPs were rinsed thrice with PBS (5-min each time). A goat anti-rabbit secondary antibody solution (1:200 dilution) was added dropwise, and incubation allowed for 2 h at 37°C. A phalloidin solution (1:200

dilution) was added and allowed to act for 30 min at 37°C. TPs were placed in an oven at 60℃ for drying. After the tablets were sealed with anti-fluorescence quenching sealing agent, observed by fluorescence microscopy.

5.7 Statistical analyses

SPSS 23.0 (IBM, Armonk, NY, USA) was used for data analyses. All experiments were repeated independently three times. Data are the mean ± standard deviation. The experimental data of each group had a normal distribution and homogeneity of variance. One-way analysis with T-test significant difference test were also used for statistical analyses. $P < 0.05$ was considered significant.

# Abbreviations

hGFs: Human gingival fibroblasts;

$Ca^{2+}$: Calcium ions;

TPs: Titanium plates;

ECM: Extracellular matrix;

Cont: Control;

SEM: Scanning electron microscopy;

CCK-8: Cell Counting Kit 8

O: Oxygen;

Ti: titanium.

# Declarations

### Availability of data and material

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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### Consent for publication

Not applicable

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### Contribution

All authors have given approval to the final version of the manuscript. ZDF contributed to the design of the experiments, collection and assembly of all data, data analysis and interpretation, and manuscript writing. XDF conceived the idea and contributed to data interpretation. XHD participated in data collection and analysis.

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### Conflict of Interest

The authors declare no conflict of interests with this research.

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# Figures

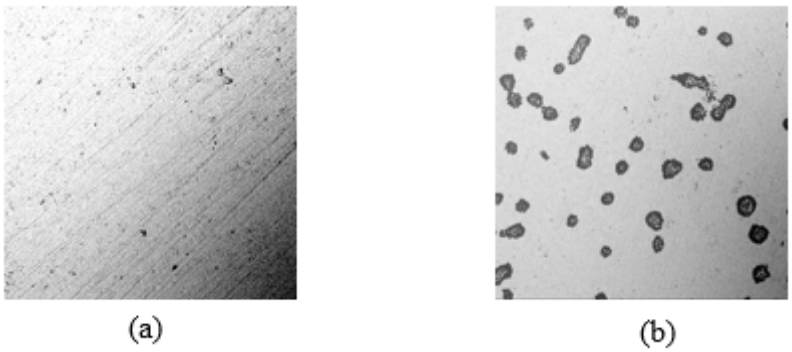


**Figure 1**

SEM results of the TPs in the two groups. (a) Control group had a smooth and flat surface; (b) Ca group, irregular lamellar crystals were found on the surface of TPs.

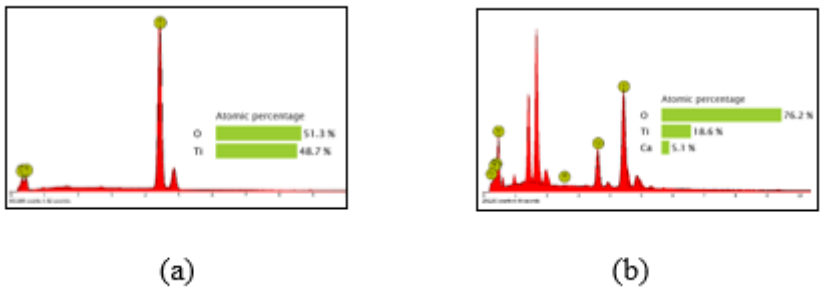


**Figure 2**

Surface elemental analysis of TPs in both groups. (a) Control group showed them to contain O and Ti; (b) Ca group showed them to contain O, Ti and Ca.

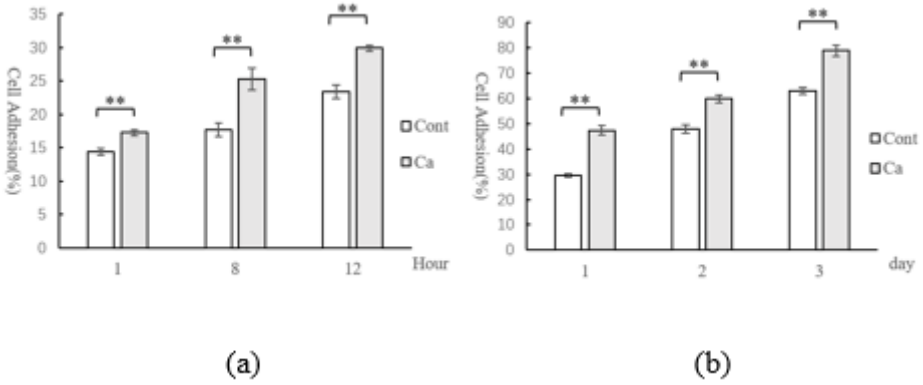


**Figure 3**

Adhesion ratio of hGF-1 cells. (a) The adhesion ratio on the TP surface in the two groups at 1, 8, and 12 hours. Each bar represents the mean ± SD. T-test; * * $p < 0.01$ between the indicated groups. (b) The adhesion ratio on the TP surface in the two groups at 1, 3, and 5 days. Each bar represents the mean ± SD. T-test; * * $p < 0.01$ between the indicated groups.

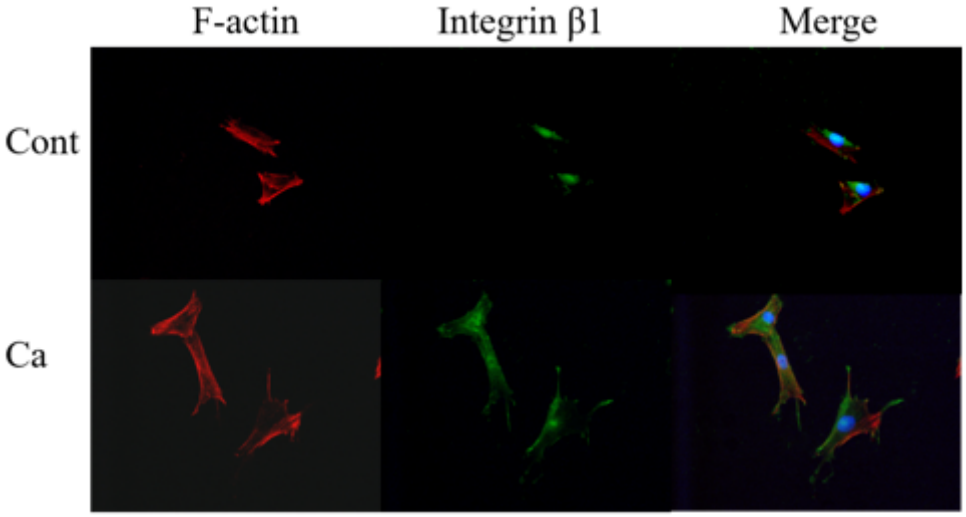


**Figure 4**

Immunofluorescence staining for adherent proteins. hGF-1 cells were cultured on the TP surface in the two groups for 3 days. Observed the distribution of F-actin (red) and integrin-β1 (green) by fluorescence microscopy.


**Figure 5**

(a) Control group (Cont); (b) Ca group (hydrothermal treatment with CaCl2 solution (20 mmol/L) for 10 h at 160°C).