**In vitro investigation of laser-induced microgrooves on titanium surface**

V P Veiko¹, Yu Yu Karlagina¹*, E E Egorova¹, E A Zernitskaya², D S Kuznetsova³, V V Elagin³, E V Zagaynova³ and G V Odintsova¹

¹ ITMO University, 197101, Saint-Petersburg, Russia
² Pavlov First Saint Petersburg State Medical University, 197022, Saint-Petersburg, Russia
³ Institute of Biomedical Technologies, Privolzhsky Research Medical University, 603005, Nizhny Novgorod, Russia.

*jujukarlagina@itmo.ru

**Abstract.** We developed a surface design that imitates the osteon structure of human bone tissue to improve the bio-integration of a titanium implant. Two types of subcellular-sized microgrooves were formed on VT6 (analogue 6Al-4V) titanium surface by laser processing: closed and open microgrooves. An *in vitro* study of the proliferation of human bone marrow mesenchymal stem cells on the formed structures was carried out, osteogenic differentiation was studied.

1. **Introduction**

In the course of research on the development of laser structuring technology of the titanium dental implant surface, we hypothesized that for better biointegration of a titanium implant into bone tissue, it is necessary to create a surface relief with the least external mechanical impact on the biological cell. To approve this hypothesis, we developed a special surface design imitating the osteon structure of human bone tissue (figure 1(a)).

The osteon (figure 1(b)) is the basic unit of bone at the microlevel. The Haversian canal, located in the center of osteon [1], surrounded by a concentric row of mineralized bone plates (lamellae). Osteon usually contains from 3 to 25 bone plates. Small spaces, called lacunas, are located along the boundaries of the lamellae, osteocytes (mature bone cells) are fixed in it.

Our idea is to form a system of concentric rings – small elongated spaces of the extracellular size on the surface of titanium by laser action, thereby simulating the natural structure of osteon. Osteocytes can potentially be located in these small spaces, forming reliable adhesion of the newly formed bone to the implant.

2. **Results and discussion**

Due to the laser action, it is possible to create grooves of two types: closed (figure 2(b), top), when the width of the groove is far less than the width of the rims, and open (figure 2(b), bottom), when the width of the groove is far more than the one of the rims. In the work, two scanning patterns were developed (figure 2(a)). We use fiber laser with $\lambda = 1.06 \mu m$, $P = 20 W$, $f = 60 kHz$, $d = 50 \mu m$ in ablation mode with a power density exceeding the boiling threshold of the VT6 titanium surface. The following
Laser parameters were used in closed grooves mode: power density \( q = 2.7 \cdot 10^{11} \, \text{W/m}^2 \), quantity of pulses in \( x \) axis \( M_x = 3 \, \mu \text{m} \), in \( y \) axis \( M_y = 40 \, \mu \text{m} \); and in open grooves mode: \( q = 5.7 \cdot 10^{11} \, \text{W/m}^2 \), \( M_x = 3 \, \mu \text{m} \), \( M_y = 40 \, \mu \text{m} \).

The period of closed microgrooves is \( 28 \pm 5 \, \mu \text{m} \), and the width is \( 25 \pm 5 \, \mu \text{m} \). The period of open microgrooves is \( 45 \pm 7 \, \mu \text{m} \), width is \( 50 \pm 5 \, \mu \text{m} \). Such parameters of the grooves are comparable with the sizes of osteoblasts (about \( 15 - 20 \, \mu \text{m} \) [2]) and osteocytes (length varies from 22 to 55 \( \mu \text{m} \), width - from 6 to 14 \( \mu \text{m} \) [2]).

![Diagram of osteons and osteocytes](image1)

**Figure 1.** (a) Schematic representation of osteons in compact bone [2]. (b) Design of osteon structure on a titanium implant surface.

![Diagram of laser pulses and microgrooves](image2)

**Figure 2.** (a) Scanning pattern by laser pulses. (b) 3D model of the developed reliefs with closed (top) and open (bottom) microgrooves. (c) SEM images of the surface of the samples at x1000 magnification.

Five samples of each type of microgrooves were prepared. Samples were placed in 48-well plates for *in vitro* study of proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells (hMSCs). hMSCs were cultured in MesenCult™ MSC Basal Medium, MesenCult™ MSC Stimulatory Supplement and 0.58 mg/ml L-Glutamine (PanEco), then transplanted onto the surface of
the samples and examined 1, 5, 10, 15 and 20 days after transplantation. For comparison, the same experiments were performed on a laser-untreated titanium surface.

After the first 24 hours of cultivation, living cells were detected on all types of samples (figure 3). On the surface of the closed grooves, single round hMSCs were found. This result indicates incomplete adhesion to the closed grooves surface. On untreated titanium and on a sample with open microgrooves, the cells were evenly layered and had a fibroblast-like elongated shape, which indicates their normal adhesion. Quantitative and qualitative analysis of proliferation showed an increase in the number of viable hMSCs on the surface of both type of samples by the end of the experiment, compared with the untreated surface. On the 20th day of cultivation, almost all cells on the surface of untreated titanium died. The best MSC proliferation was observed on the surface of the sample covered with open microgrooves.

![Figure 3](image)

**Figure 3.** Quantitative analysis of cell proliferation on the surface of samples before and after laser treatment. The scale bars in SEM images correspond to 300 microns.

The study of the early marker of hMSCs osteogenic differentiation - osteocalcin, showed that formation of osteocalcin occurs more actively on the surface of the sample with open microgrooves, compared with other samples. Figure 4A shows photographs taken on a Leica DM IL LED Fluo fluorescence microscope, green patterns indicate osteocalcin, blue pattern indicates cell nuclei. In support of this result, an analysis of alkaline phosphatase activity was carried out, another informative marker of osteogenic differentiation. Conducted using a Synergy™ Mx Multi-Mode Microplate Reader fluorometer, it was found that alkaline phosphatase is also more active in cells grown on the open microgrooves surface (figure 4(b)). In addition, it was noted that the size and shape of the cells and their nuclei for two different reliefs are different. Fibroblast-like cells have different orientations on the closed microgrooves (figure 4(a), left). The open microgrooves cells have an elongated along the grooves shape. The nuclei of these cells have a smaller size compared to the ones on closed microgrooves. This result indicates that the geometric shape of the surface affects the size, shape and orientation of the cells during the bone tissue cells proliferation process.

3. **Conclusion**

Thus, open microgrooves demonstrated the best proliferation and osteogenic differentiation. Based on the results obtained an osteon relief was created on the surface of VT6 titanium (figure 5) by laser
irradiation, which repeats the natural structure of osteon. In further studies, additional *in vitro* analysis and *in vivo* verification of the osseointegration of the formed osteon structure will be carried out.

**Figure 4.** (a) Analysis of osteogenic differentiation by expression of osteocalcin: green patterns - osteocalcin, blue patterns - cell nuclei; (b) alkaline phosphatase activity. The scalebar corresponds to 300 µm.

**Figure 5.** Laser-induced osteon structure on the surface of a titanium dental implant.

In vitro studies were carried out by specialists of the Research Institute of Biomedical Technologies of Privilzhsky Research Medical University of the Ministry of Health of Russia in Nizhny Novgorod according to the protocols of experiments approved by the Research Ethics Council of the Nizhny Novgorod State Medical Academy (PRMU, Nizhny Novgorod) and in accordance with the principles of the Helsinki Declaration.

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