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Influence of different grained powders and pellets made of Niobium and Ti-42Nb on human cell viability

Jana Markhoff [a,*], Markus Weinmann [b], Christian Schulze [a], Rainer Bader [a]

[a] University Medicine Rostock, Department of Orthopedics, Biomechanics and Implant Technology Laboratory, Doberaner Strasse 142, 18057 Rostock, Germany
[b] H.C. Starck Tantalum and Niobium GmbH, Im Schleeke 78-91, 38642 Goslar, Germany

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ABSTRACT

Nowadays, biomaterials can be used to maintain or replace several functions of the human body if necessary. Titanium and its alloys, i.e. Ti6Al4V are the most common materials (70 to 80%) used for structural orthopedic implants due to their unique combination of good mechanical properties, corrosion resistance and biocompatibility. Addition of β-stabilizers, e.g. niobium, can improve the mechanical properties of such titanium alloys further, simultaneously offering excellent biocompatibility. In this in vitro study, human osteoblasts and fibroblasts were cultured on different niobium specimens (Nb Amperit, Nb Ampertec), Nb sheets and Ti-42Nb (sintered and 3D-printed by selective laser melting, SLM) and compared with forged Ti6Al4V specimens. Furthermore, human osteoblasts were incubated with particulates of the Nb and Ti-42Nb specimens in three concentrations over four and seven days to imitate influence of wear debris. Thereby, the specimens with the roughest surfaces, i.e. Ti-42Nb and Nb Ampertec, revealed excellent and similar results for both cell types concerning cell viability and collagen synthesis superior to forged Ti6Al4V. Examinations with particulate debris disclosed a dose-dependent influence of all powders with Nb Ampertec showing the highest decrease of cell viability and collagen synthesis. Furthermore, interleukin synthesis was only slightly increased for all powders. In summary, Nb Ampertec (sintered Nb) and Ti-42Nb materials seem to be promising alternatives for medical applications compared to common materials like forged or melted Ti6Al4V.

1. Introduction

Constraint or loss of functions in the human body can be the result of tumors, fractures, injuries as well as chronic diseases, infections or simply aging [1]. Nowadays, maintenance or replacement of those functions can be achieved through the application of biomaterials mainly consisting of metals, ceramics or polymers [2–4]. Titanium and its alloys are the most common used implant materials in orthopedic surgery with an amount of 70 to 80% [5–7]. Their application ranges from load bearing areas like in orthodontics and orthopedics to gastroenterology as well as cardiovascular and reconstructive aspects. They possess appreciable mechanical properties, good corrosion resistance and biocompatibility [8–12]. The most frequently used titanium-based material is the titanium aluminum vanadium alloy Ti6Al4V (also referred to Ti Grade 5) [13–16]. However, by now the elemental component vanadium is proved to be toxic and aluminum is suspected to cause e.g. Alzheimer disease [7,17–19].

Titanium alloys in general crystallize in a hexagonal close-packed α-phase (stable at 25 °C) which at 882 °C reversibly transforms into a β-phase with body-centered cubic crystal structure. Addition of alloying elements may stabilize this phase or cause crystallization, i.e. stabilization of α + β mixtures [20]. The β–type titanium alloys possess significantly smaller Young's moduli compared with α- or α + β alloys. Some of the even build deformation-induced martensite structure possessing a shape-memory effect [21]. The formation of the β–type structure may avoid a mechanical mismatch in elasticity of bone and implant, causing stress shielding associated with implant loosening. Specific β-stabilizers for titanium alloys further improve the material properties [22–26]. For example, non-toxic and non-allergenic niobium represents a relatively new and promising implantable biomaterial, which is proved for its biocompatibility in vitro and in vivo. It has a corrosion resistance superior to titanium resulting from a self-passivating inert (native) oxide surface layer [27–32]. Furthermore, some niobium alloys offer a shape memory effect (SME) and possess superelastic properties, analogous to selected nickel alloys [7,32,33]. Nickel titanium alloys (NiTi) gained in importance in the last decades [34] being accounted for by its SME up to 8% [35], biocompatibility and mechanical properties (elastic modulus, compressive strength) which are close to bone. Even though NiTi is corrosion and wear resistant [35–38] there are concerns including toxicity, allergenic reactions and carcinogenic potential of dissolved nickel ions [27,39,40].

Niobium and Ti-Nb alloys or niobium oxide (Nb_2O_5) materials represent excellent alternatives for medical applications [31,39,41,42], even

* Correponding author.
E-mail address: markhoffj@gmail.com (J. Markhoff).

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though mechanical properties require for improvement [2]. Moreover, one of the problems of biomaterials used is the surface contamination with residues or particles within the production process and the formation of wear debris due to mechanical strain and friction of articulating implant components [43–45]. Particles and wear debris can accumulate in the periprosthetic tissue being size-dependent phagocytosed and activating or inhibiting the attendant cells. For example, osteoblasts, macrophages and osteoclasts are known to interact in various fashions [43, 45–47]. Those particles cause inflammatory reactions and moreover, lead to increased differentiation of bone resorbing osteoclasts and inhibition of bone forming osteoblasts. Finally, all these factors result in osteolysis and aseptic implant loosening [43,45,47].

The intention of the present study is to demonstrate the superior biocompatibility of Ti/Nb-based alloys compared with conventional metallic implant materials like Ti6Al4V. This includes biomedical issues, i.e. preservation of cell activities (osteoblasts, fibroblasts) and non-toxicity as well as (bio-) mechanical aspects, which are work in progress and will be published subsequently. The focus is not only on intrinsic material properties, but also on their morphologies, also considering 3D-printed bulk materials.

Therefore, in the present in vitro study, human osteoblasts and fibroblasts were cultured on two different niobium specimens (Nb Amperit, Nb Ampertec), Nb sheets as well as Ti-42Nb specimens (sintered and manufactured by SLM), compared with forged Ti6Al4V and referenced to tissue culture polystyrene (TCPS) as growth control. Furthermore, human osteoblasts were incubated with four particulates of the above mentioned groups to imitate influence of wear debris.

2. Materials & methods

Niobium powders and niobium sheet used in this study are commercial products produced and distributed by H.C. Starck Tantalum and Niobium GmbH, Goslar, Germany. Product data sheets can be obtained on request from www.hcstarck.com. Spherical Ti-42Nb powder was obtained by EIGA (Electrode Induction Melting Gas Atomization, EIGA) of Ti-42Nb rods. Appropriate SEM images are displayed in Fig. 1. Chemical analysis, particle size and abbreviations of the different powders used in this study are provided in Table 1. Additionally, chemical analysis of niobium sheet is given.

2.1. Preparation of test specimens

All specimens prepared for cell-biological investigations were cylindrical pellets with dimensions of d × l = 10 mm × 2 mm compounded from the above mentioned powders. Nb Amperit was uniaxially pressed at 25 °C using a lab toggle press applying a load of 65 bars. Nb Ampertec was uniaxially pressed at 25 °C using a lab toggle press applying a load of 65 bars and subsequently sintered in vacuum at 1200 °C for 10 min. Ti-42Nb powders could not successfully be compacted by uniaxial pressing due to their spheroidal shape. Accordingly, powder consolidation was performed pressureless by sintering at 1000 °C for 10 min of a 3 mm powder bed in Al_2O_3 sinter rings on Nb sheets. As-obtained specimens were machined to 2 mm height. Ti-42Nb specimens were obtained by additive manufacturing, i.e. selective laser melting (SLM) of Ti-42Nb (sieve fraction < 63 μm) using a TruPrint 1000 equipment (TRUMPF GmbH + Co. KG, Ditzingen, Germany) operated at Laserzentrum Hannover, Hannover, Germany. The following laser/scan parameters were applied: laser power 50 W, scan speed 400 mm/s, hatch 120 μm, powder bed feed rate 50 μm/layer. Surface roughness of the test specimens (Rz, Ra) was analyzed by means of a tactile measurement method using a Hommel-Etamic T1000 and a sampler (TKU300, probe tip radius 5 μm,) (both: Jenoptik, Jena, Germany). Thereby, a probe tip with defined radius slides across the surface of the test specimen permeating into the surface subject to the probe tip radius. The measuring range was 320 μm and the length of tactile measurement was 8 mm. Three surface points per specimen ($n \geq 3$) were swept (Table 2).

To illustrate surface properties of the several metallic samples field emission scanning electron microscopic (FESEM) images were generated. The sample surface is gold-plated (~25 nm) using a sputter coater (Leica SCD004, Wetzlar, Germany) and figured with a Merlin VPcompact microscope (Carl Zeiss AG, Oberkochen, Germany) with a 50-fold magnification (Fig. 2).

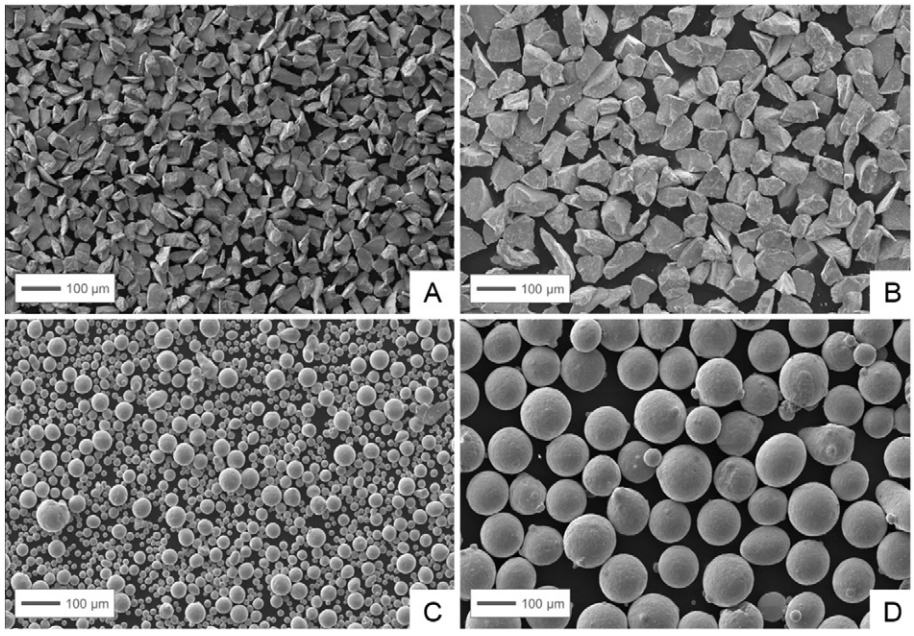

Fig. 1. SEM images of niobium powders recorded at 100× magnification. A: AMPERIT 160 NIOBIUM METAL 14–45 μm, B: AMPERTEC NIOBIUM EB MELTED 45–75 μm, C/D: AMPERTEC MAP Ti-42Nb powders - C: powder fraction <63 μm, D: powder fraction 103 μm - 350 μm.

Table 1
Particle size (PS) and chemical analysis of the powders used in this study. The chemical composition of the niobium sheet is provided for comparison.

	Abbreviation	PS (μm)	Nb (%)	Ti	O (ppm)	C (ppm)	H (ppm)	N (ppm)	Mg (ppm)
AMPERIT 160 NIOBIUM METAL	Nb Amperit	15–45	99.90%	n.d.	<2500	<100	<100	<150	<10
AMPERTEC NIOBIUM EB MELTED	Nb Ampertec	45–75	99.90%	n.d.	750	10	<10	41	<1
AMPERTEC MAP Ti-42Nb[a]	Ti-42Nb	<350	41.47	58.26%	2212	103	<10	51	<10
NIOBIUM SHEET	Nb Sheet	n.a.	>99.99%	20 ppm	90	30	10	24	n.d.

[a] AMPERTEC MAP Ti-42Nb was subsequently fractionized (<63 μm, 63 μm–105 μm, 105 μm–350 μm) by sieving.

Forged Ti6Al4V pellets, which served as bench mark material to compare with niobium (Nb) and Ti-42Nb samples, were provided by DOT GmbH, Rostock, Germany.

2.2. Cell isolation

Cell cultivation was carried out in an incubator (Binder GmbH, Tuttlingen, Germany) at simulated in vivo conditions at 5% CO_2, 21% O_2 and 37 °C with regular medium changes. Human osteoblasts were isolated after patient agreement from the spongiosa of patients undergoing primary total hip replacement as previously described by Lochner et al. [48]. Briefly, the spongiosa was mechanically removed from the femoral head, washed with PBS and enzymatically digested using collagenase A, culture medium without fetal calf serum (FCS) and dispase II. The gained suspension was sown out in cell culture flasks after removing spongiosa fragments. Cultivation was carried out in osteogenic cell culture medium (MEM Dulbecco, Biochrom AG, Berlin, Germany) with 10% FCS, 1% penicillin/streptomycin, 1% amphotericin B, 1% HEPES buffer (all: Gibco®-Invitrogen, Darmstadt, Germany) and additional osteogenic additives (dexamethason (100 nM), L-ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) (all from Sigma-Aldrich, Munich, Germany). The osteogenic character of the isolated cells was proved via alkaline phosphatase staining with fuchsin substrate chromogen (DAKO, Hamburg, Germany).

Human fibroblasts were isolated from skin biopsies (breast, eyelid) provided by a local aesthetic clinic. After excision of adipose tissue, the remaining tissue was cut in equal segments (2–3 mm edge length), which were then transferred to 6-well-plates (2–3 per well) with the epidermis upwards. After 20 min of surface drying, skin was overlaid with 3 ml DMEM-medium (with Glutamax, 10% FCS, 1% penicillin/streptomycin, 1% amphotericin B (all: Gibco®-Invitrogen, Darmstadt, Germany)) and cultured for three weeks. Subsequently, cells were transferred to tissue culture flasks and cryo-preserved after further confluence.

The test pellets were seeded with osteoblasts and fibroblasts in a density of 20.000 cells (third and fifth passage) per 48-Well in 500 μl culture medium. Ti-42Nb SLM specimen were tested subsequently and only used for human osteoblast cultivation. Cultivation on the test pellets was carried out for 96 h with an exchange of culture medium after 48 h. The Local Ethical Committee approved the use of the human cells for the experiments. The characteristics of the used human cells are listed in Table 3.

For incubation with niobium powders, the several powders were apportioned in amounts of 5 mg in glass jars and gamma-sterilized with 25 kGy. To produce a stock solution powders were re-suspended in 500 μl of PBS (= 10 mg/ml) and diluted with culture medium to get three working solutions (0.1/0.2/0.5 mg/ml). These concentrations were determined by means of appropriate pre-testings. First trials were performed on the basis of preliminary works of the working group regarding the influence of orthopedic wear particles on human cells [48–50]. Based on these results powder concentrations were adapted. The wide range was used to reveal concentration-dependent differences. Osteoblasts free of powder served as control group. Cultivation was carried out for four and seven days with exchanges of culture medium after 48 h and after 48 h and 96 h, respectively.

2.3. Metabolic cell activity and live/dead staining

Metabolic activity of cells on the test pellets was determined via mitochondrial dehydrogenase activity (WST-1 test) (Roche, Grenzach-Wyhlen, Germany) after 96 h of cultivation. Activity of cells cultured with niobium powders was proven after four and seven days, respectively. The tetrazolium salt WST (water soluble tetrazolium) is transformed to formazan by mitochondrial succinate dehydrogenase from metabolically active cells. Thereby, adsorption is directly proportional to the metabolic cell activity and was measured at 450 nm in a Tecan reader (Infinite F200 Pro, Männedorf, Switzerland). Live/dead staining (Live/Dead Cell Viability Assay, Invitrogen, Darmstadt, Germany) was used to analyze qualitative cell viability. Therefore, adherent cells were simultaneously incubated with two fluorescence dyes after removing the culture medium. Vital cells were visualized green due to calcein AM (494/517 nm), which is embedded in the cell plasma. Dead cells get red by ethidium homodimer-1 (528/617 nm) interacting with the cell nuclei. Microscopic pictures were taken using an inverted microscope (Nikon TS 100, Nikon GmbH, Duesseldorf, Germany) with the appropriate filters for fluorescence images. All images were made using similar magnification and exposure time.

2.4. Enzyme-linked immunosorbent assays

Protein syntheses were quantified by means of enzyme-linked immunosorbent assays (ELISAs). Pro-collagen type I (Metra C1CP EIA Kit, Quidel, Buende, Germany) was chosen for human osteoblasts and fibroblasts. Therefore, protein content in the culture supernatants was determined according to the manufactures instructions after 96 h of cultivation with test pellets. CICP synthesis of osteoblasts cultured with niobium powders was measured after four and seven days, respectively.

Table 2
Dimensions and roughness values[a] of the test pellets.

Test pellet	Dimensions d × l [mm]	Ø Rz [μm]	Ø Ra [μm]
Ti6Al4V	10 × 2	15.71 ± 1.27	2.38 ± 0.15
Nb Amperit	10 × 2	7.04 ± 3.08	1.28 ± 0.88
Nb Ampertec	10 × 2	17.50 ± 8.95	2.49 ± 1.08
Nb sheet	10 × 1	18.14 ± 3.23	4.39 ± 0.38
Ti-42Nb sintered	10 × 2	41.65 ± 28.39	7.38 ± 5.86
Ti-42Nb SLM	10 × 2	46.83 ± 5.70	9.06 ± 1.28

[a] Rz - arithmetical mean deviation of the profile|Ra – average roughness of all individual measured values.

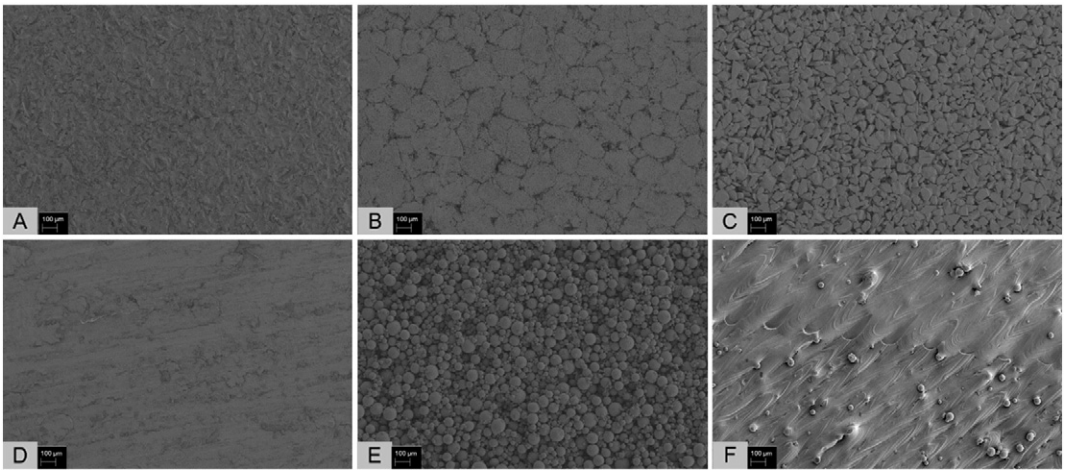

Fig. 2. FESEM images of the tested pellets. A – Ti6Al4V, B – Nb Amperit, C – Nb Ampertec, D – Nb sheet, E – Ti-42Nb sintered; F - Ti-42Nb SLM; magnification: 50×; bar: 100 μm.

2.5. Cytokine analysis

To verify a possible immune-stimulatory effect of the different niobium powders, a cytokine multiplex (interleukin (IL)-6, IL-8, monocyte chemotactic protein (MCP-1)) (Bio-Rad, Munich, Germany) was done according to the manufactures instructions. In brief, a multi-cytokine-detection in the culture supernatants was conducted by means of microsphere beads interlinking several cytokines via specific monoclonal antibodies and fluorescence dyes. The appropriate cytokine concentration was calculated based on a standard curve. Concentration of MCP-1 was mainly out of range.

2.6. Statistical analysis

The statistical significance was calculated with Mann-Whitney-*U* test using IBM® SPSS® Statistics Version 20 (IBM Corp., New York, USA). Data were shown as box plots or mean + SEM. Boxes denote interquartile ranges, horizontal lines within the boxes denote medians, and whiskers denote minimum and maximum values. Values of $p < 0.05$ were set to be significant.

3. Results

Human osteoblasts and fibroblasts were cultured on different niobium pellets for 96 h to verify their biocompatibility. The specimens were compared to Ti6Al4V pellets and referenced to TCPS as growth control. Metabolic activity of human osteoblasts was significantly decreased on Nb Amperit (−52%) and Nb sheet (−80%) (Fig. 3, left). Ti6Al4V (−29%), Nb Ampertec (−15%) and Ti-42Nb SLM (−8%) caused a slightly reduced activity compared with the reference. Against this, cultivation on Ti-42Nb sintered (+17%) significantly increased metabolic activity of human osteoblasts towards control.

Similar results were obtained if cultivating human fibroblasts on those surfaces. While Ti6Al4V (−68%) and Nb Amperit specimen (−52%) as well as Nb sheet (−92%) led to significant loss of metabolic activity, Nb Ampertec (−6%) and Ti-42Nb sintered (−13%) only slightly deviated from the reference. Accordingly, the metabolic activity of human fibroblast on these specimen was much higher than on the other samples (Fig. 3, right). These results are to a certain extend sustained by live/dead staining (Fig. 4). Thereby, results depended on the amount of metabolic cells as well as the specific level of cell activity.

Furthermore, protein synthesis of matrix relevant pro-collagen type 1 (CICP) was determined using ELISA. CICP synthesis was significantly reduced in supernatants of human osteoblasts cultured on the metallic surfaces. Nb Amperit (−92%) and Nb sheet (−78%) resulted in the lowest synthetic activity, followed by Ti6Al4V (−58%), Ti-42Nb sintered (−45%), Ti-42Nb SLM (−39%) and Nb Ampertec (−26%) compared to TCPS (Fig. 5, white). Accordingly, Ti-42Nb sintered as well as Ti-42Nb SLM and Nb Ampertec revealed a significantly higher metabolic activity towards CICP compared with the other metals. Cultivation of human fibroblasts on Ti6Al4V and niobium surfaces, i.e. Nb Amperit (−69%) and Nb sheet (−54%) resulted in a distinct decrease of metabolic activity towards control (Fig. 5, gray). Ti6Al4V (−3%) and control medium displayed a similar performance in the Pro-collagen type I synthesis whereas CICP synthesis was noticeably increased on Nb Ampertec (+12%) and Ti-42Nb sintered (+9%)

In addition, human osteoblasts were cultured in the presence of different Nb powders using three concentrations (0.1/0.2/0.5 mg/ml) over four and seven days. The metabolic activity of the cells was significantly reduced compared with the control in all experiments and mostly concentration-dependent. To receive information of the influence of surface area of the powders on the metabolic activity, two sieve fractions of Ti-42Nb powder namely a fine-grained powder with $x < 63$ μm and a coarse powder with $103\ \mu m < x < 350\ \mu m$ were investigated.

Each experiment indicated a considerable impact of the powders on the cell metabolism, i.e. a decrease of activity after four and seven days of cultivation (Fig. 6). While the cell activity of Nb Amperit powder was reduced from day four to seven there was no time-dependent influence for Nb Ampertec detectable. However, the latter had the strongest impact on the cell metabolism and reduced the cell activity to <40% compared with the reference, for concentrations of 0.2 and 0.5 mg Nb/ml. In contrast, Ti-42Nb powders provoked higher activities after seven days than after four days. Interestingly, the coarse Ti-42Nb powder ($103\ \mu m < x < 350\ \mu m$) caused a slight concentration-dependent increase of metabolic activity at day four, which became significant at day seven. Table 4 depicts the significant differences between all tested powders at day 4 and 7, respectively.

To investigate the cell-powder interaction, light microscopic pictures were recorded using an inverted microscope (Nikon TS 100, Nikon GmbH, Duesseldorf, Germany). Those indicate up-take of small particulates (Fig. 7, right picture) and partially powder agglomeration.

Table 3
Characteristics of the human donors[a] used for the cell experiments.

Test material	Donors	Gender	Average age
Pellets (A-E in Fig. 2)	Osteoblasts ($n = 7$)	5♂/2♀	65.29 ± 14.47
Ti-42Nb SLM	Osteoblasts ($n = 4$)	4♂/-♀	61.25 ± 6.34
Pellets (A-E in Fig. 2)	Fibroblasts ($n = 4$)	-♂/2♀ (2 n/a)	40 ± 7.07 (2 n/a)
Powders (A-D in Fig. 1)	Osteoblasts ($n = 8$)	4♂/4♀	69.25 ± 8.46

[a] n = number of different donors.

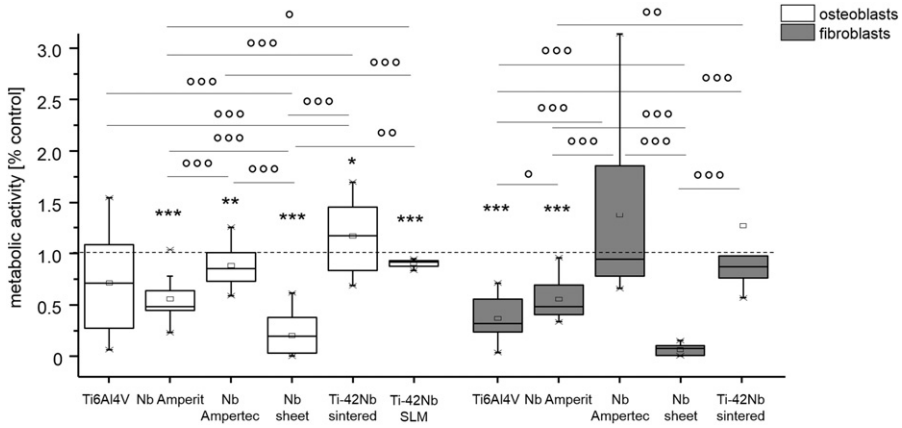

Fig. 3. Metabolic activity of human osteoblasts (left, $n \geq 4$) and human fibroblasts (right, $n = 4$) on different test pellets (Ti6Al4V, Nb Amperit, Nb Ampertec, Nb sheet, Ti-42Nb sintered, Ti-42Nb SLM) after 96 h cultivation. Boxes denote interquartile ranges, horizontal lines within the boxes denote medians, and whiskers denote minimum and maximum values. For statistical analysis Mann-Whitney-*U* test was conducted. Data were compared to the growth control (100%, *p) and against each other (°p). *$p < 0.05$, **$p < 0.01$, ***$p \leq 0.001$.

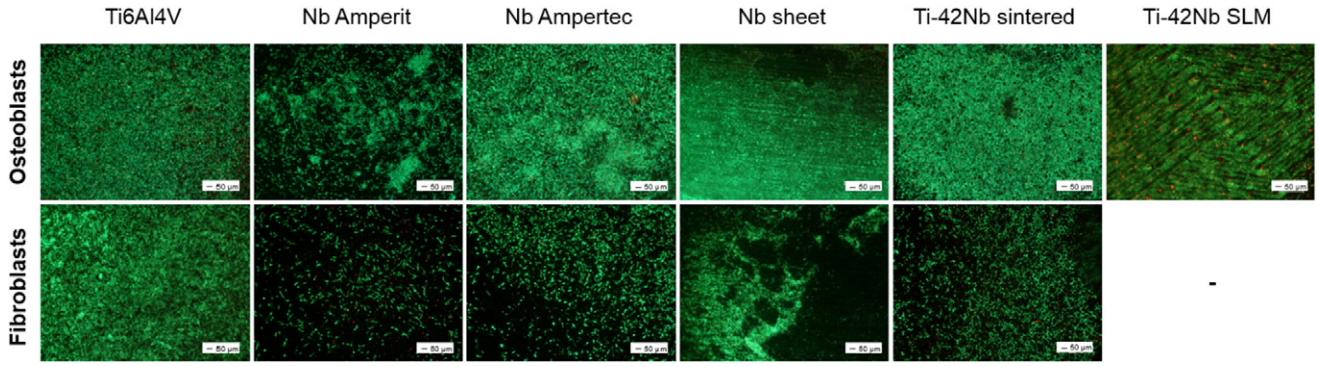

Fig. 4. Live/dead staining of human osteoblasts and fibroblasts on different test pellets (Ti6Al4V, Nb Amperit, Nb Ampertec, Nb sheet, Ti-42Nb sintered, Ti-42Nb SLM) after 96 h cultivation. Living cells are displayed in green, dead ones in red. Magnification: 40×, Scale bar: 50 μm.

After cultivation with several niobium powders live/dead staining was conducted which approved the results of the investigations on the metabolic activity (Fig. 8).

In addition, pro-collagen type I synthesis was analyzed. Data of collagen synthesis were relativized to metabolic activity and referred to powder-free control (Fig. 9). Again, concentration-dependent influence of niobium powders was clearly visible. While Nb Amperit powder resulted in a significant increase of collagen synthesis higher than control at nearly any point, Nb Ampertec and fine-grained Ti-42Nb revealed a decreasing pro-collagen type I synthesis with raising powder concentrations. Ti-42Nb powder (103 μm < x < 350 μm) only led to a negligible decrease of synthesis compared to control. Altogether, the pro-collagen type I syntheses increased with increasing time for either of the substrates in either concentration (Table 5).

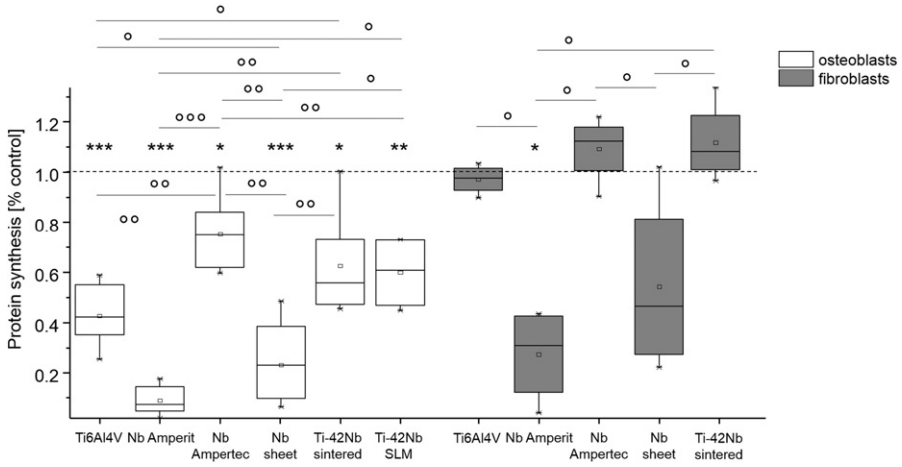

Fig. 5. Pro-collagen type I synthesis of human osteoblasts (white, $n \geq 4$) and human fibroblasts (gray, $n \geq 2$) on different test pellets (Ti6Al4V, Nb Amperit, Nb Ampertec, Nb sheet,Ti-42Nb sintered, Ti-42Nb SLM) after 96 h cultivation. Boxes denote interquartile ranges, horizontal lines within the boxes denote medians, and whiskers denote minimum and maximum values. For statistical analysis Mann-Whitney-*U* test was conducted. Data were compared to the growth control (100%, *p) and against each other (°p). *$p < 0.05$, **$p < 0.01$, ***$p \leq 0.001$.

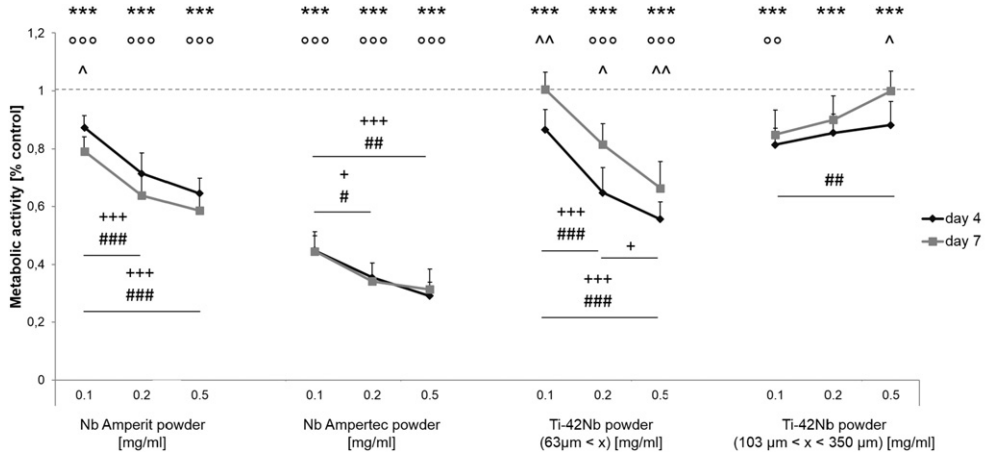

Fig. 6. Metabolic activity of human osteoblasts cultivated with different Nb and Ti-42Nb powders in three concentrations (0.1, 0.2 and 0.5 mg/ml) for four and seven days. Data are mean ± SEM ($n = 8$). For statistical analysis Mann-Whitney-*U* test was conducted. Data were compared to the growth controls (day 4 = *p/day 7 = °p) and between day 4 and day 7 (^p). Comparison of powder concentrations per niobium/Ti-42Nb powder is marked with +p (4 days) and #p (7 days); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$.

To evaluate an inflammatory response of human osteoblasts to four different niobium powders, interleukin syntheses were examined. IL-6 production was slightly increased after cultivation with Nb Amperit, Nb Ampertec and fine-grained Ti-42Nb powder at day four compared to control and raised further to day seven (Fig. 10). Dose-dependency is not that consistent between the powders. Coarse-grained Ti-42Nb caused a slight dose-dependent increased IL-6 synthesis being significantly higher than control at day four and decreasing with further cultivation until day seven.

Measurement of IL-8 synthesis revealed similar results compared to IL-6 synthesis for the several niobium powders. However, IL-8 was synthesized in a lower amount and was most widely equal to the control group (Fig. 11). There was no consistent trend perceptible between the several groups concerning dose- and time-dependency.

4. Discussion

In the present in vitro study, the biocompatibility of five different niobium-based metallic samples (Nb Amperit, Nb Ampertec, Nb sheet, Ti-42Nb sintered, Ti-42Nb SLM) towards human osteoblasts and fibroblasts is investigated and compared with Ti6Al4V pellets. In a previous work niobium exhibited better performance than titanium, using cell lines and testing proliferation, metabolic activity and maintenance of cell morphology [51]. Woo et al. [52] also used sintered Ti-42Nb, which was obtained by high energy ball milling, and demonstrated that mechanical properties and biocompatibility were superior to Ti6Al4V. An improved biocompatibility of coated Ti-Nb alloys has also been stated compared with pure Ti [53]. Moreover, shape memory effect and superelasticity are proved for Ti-Nb alloys [25,53]. Park et al.

Table 4
Appropriate significances to Fig. 5 comparing the different test powders. For statistical analysis Mann-Whitney-*U* test was conducted. Values of $p < 0.05$ were set to be significant. Significances for day 4 are above those of day 7, respectively.

	WST-1	Nb Amperit powder			Nb Ampertec powder			Ti-42Nb powder (63µm < x)			Ti-42Nb powder (103 µm < x < 350 µm)		
	[mg/ml]	0.1	0.2	0.5	0.1	0.2	0.5	0.1	0.2	0.5	0.1	0.2	0.5
Nb Amperit powder	0.1	n.s.	≤ 0.001 0.001	≤ 0.001 ≤ 0.001	≤ 0.001 ≤ 0.001	n.s.	n.s.	n.s. ≤ 0.001	n.s.	n.s.	0.040 n.s.	n.s.	n.s.
	0.2	≤ 0.001 0.001	n.s.	0.052 n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	n.s. 0.001	n.s.	n.s.	0.025 ≤ 0.001	n.s.
	0.5	≤ 0.001 ≤ 0.001	0.052 n.s.	n.s.	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	0.012 0.017	n.s.	n.s.	≤ 0.001 ≤ 0.001
Nb Ampertec powder	0.1	≤ 0.001 ≤ 0.001	n.s.	n.s.	n.s.	0.028 0.017	≤ 0.001 0.005	≤ 0.001 ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.
	0.2	n.s.	≤ 0.001 ≤ 0.001	n.s.	0.028 0.017	n.s.	0.076 n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.
	0.5	n.s.	n.s.	≤ 0.001 ≤ 0.001	≤ 0.001 0.005	0.076 n.s.	n.s.	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001
Ti-42Nb powder (63µm < x)	0.1	n.s. ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	n.s.	0.001 ≤ 0.001	≤ 0.001 ≤ 0.001	n.s. 0.006	n.s.	n.s.
	0.2	n.s.	n.s. 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	0.001 ≤ 0.001	n.s.	0.050 0.088	n.s.	≤ 0.001 n.s.	n.s.
	0.5	n.s.	n.s.	0.012 0.017	n.s.	n.s.	≤ 0.001 ≤ 0.001	≤ 0.001 ≤ 0.001	0.050 0.088	n.s.	n.s.	n.s.	≤ 0.001 ≤ 0.001
Ti-42Nb powder (103 < x < 350 µm)	0.1	0.040 n.s.	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	n.s. 0.006	n.s.	n.s.	n.s.	n.s.	n.s. 0.010
	0.2	n.s.	0.025 ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	≤ 0.001 n.s.	n.s.	n.s.	n.s.	n.s. 0.068
	0.5	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s.	n.s.	≤ 0.001 ≤ 0.001	n.s. 0.010	n.s. 0.068	n.s.

n.s. - not significant.

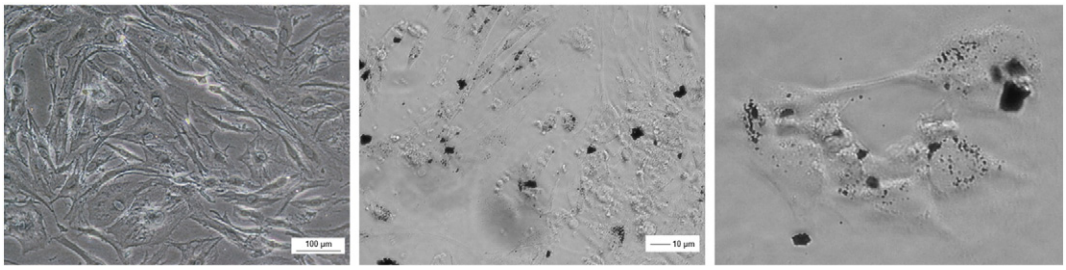

Fig. 7. Light microscopic pictures of control cells without powder (left, scale bar: 100 µm) and incorporation of powder particles by human osteoblasts at day four (Nb Ampertec (0.5 mg/ml), scale bar: 10 µm).

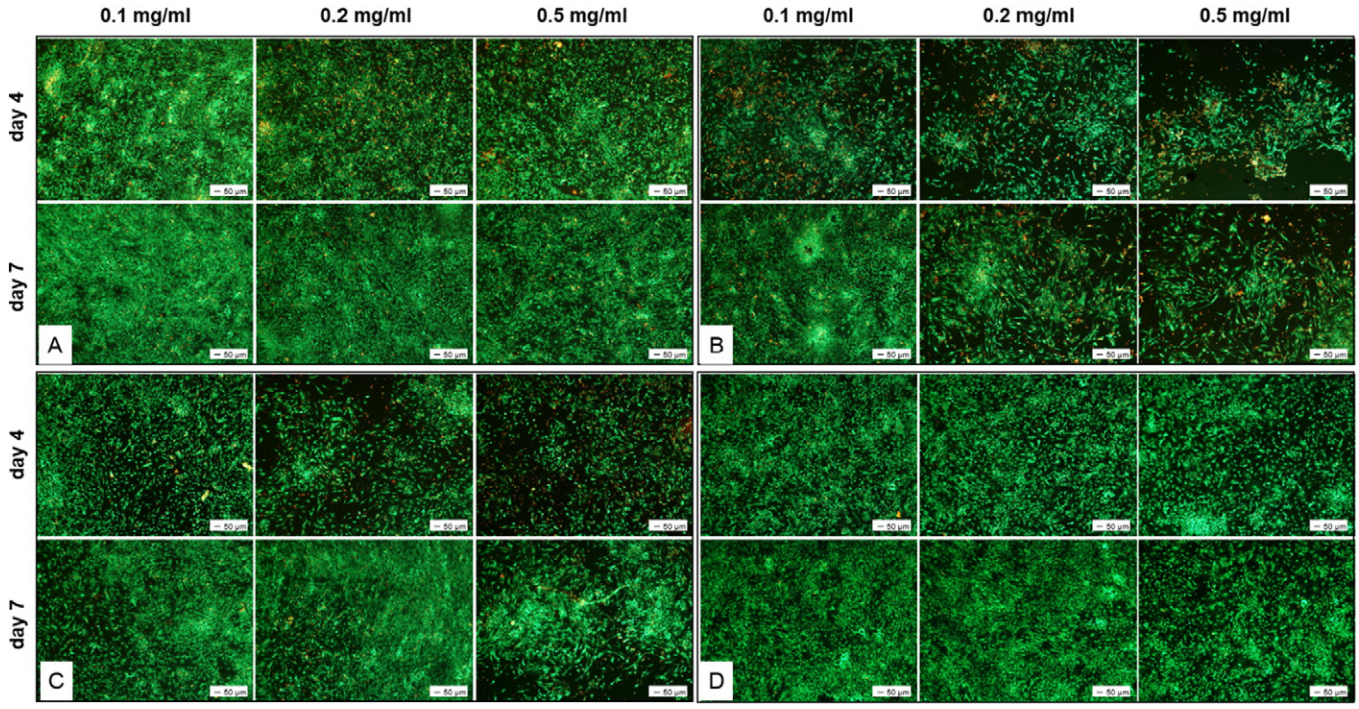

Fig. 8. Live/dead staining of human osteoblasts cultured with different Nb and Ti-42Nb powders in three concentrations (0.1, 0.2 and 0.5 mg/ml) after four and seven days. A – Nb Amperit, B - Nb Ampertec, C – Ti-42Nb powder (63 µm < x), D - Ti-42Nb powder (103 µm < x < 350 µm). Living cells are displayed in green, dead cells are displayed in red. Magnification: 40×, Scale bar: 50 µm.

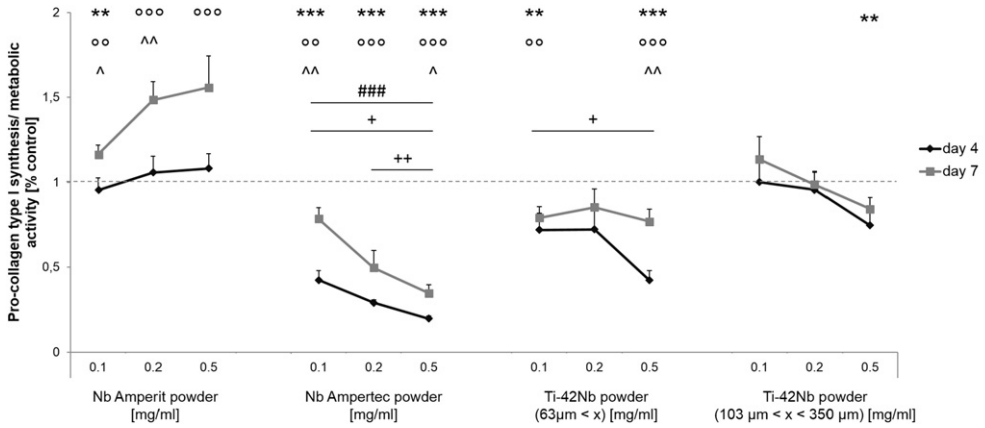

Fig. 9. Pro-collagen type I synthesis of human osteoblasts cultivated with different Nb and Ti-42Nb powders in three concentrations (0.1, 0.2 and 0.5 mg/ml) for four and seven days. Data are mean ± SEM ($n = 8$). For statistical analysis Mann-Whitney-*U* test was conducted. Data were compared to the growth controls (day 4 = *p/day 7 = °p) and day 4 towards day 7 (^p). Comparison of concentrations is marked with + p (4 days) and #p (7 days); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$.

Table 5
Appropriate significances to Fig. 8 comparing the several test powders against each other. For statistical analysis Mann-Whitney-*U* test was conducted. Values of $p < 0.05$ were set to be significant. Significances for day 4 are above those of day 7, respectively.

	CICP	Nb Amperit powder			Nb Ampertec powder			Ti-42Nb powder (63µm < x)			Ti-42Nb powder (103 µm < x < 350 µm)		
	[mg/ml]	0.1	0.2	0.5	0.1	0.2	0.5	0.1	0.2	0.5	0.1	0.2	0.5
Nb Amperit powder	0.1	n.s.	n.s. 0.059	n.s.	0.001 0.002	n.s.	n.s.	n.s. 0.002	n.s.	n.s.	n.s.	n.s.	n.s.
	0.2	n.s. 0.059	n.s.	n.s.	n.s.	0.001 0.001	n.s.	n.s.	0.093 0.003	n.s.	n.s.	n.s. 0.005	n.s.
	0.5	n.s.	n.s.	n.s.	n.s.	n.s.	0.001 0.001	n.s.	n.s.	0.001 0.001	n.s.	n.s.	0.021 0.001
Nb Ampertec powder	0.1	0.001 0.002	n.s.	n.s.	n.s.	n.s.	0.021 0.001	0.059 n.s.	n.s.	n.s.	0.001 0.036	n.s.	n.s.
	0.2	n.s.	0.001 0.001	n.s.	n.s.	n.s.	0.003 n.s.	n.s.	0.002 0.046	n.s.	n.s.	0.001 0.006	n.s.
	0.5	n.s.	n.s.	0.001 0.001	0.021 0.001	0.003 n.s.	n.s.	n.s.	n.s.	0.002 0.002	n.s.	n.s.	0.001 0.001
Ti-42Nb powder (63µm < x)	0.1	n.s. 0.002	n.s.	n.s.	0.059 n.s.	n.s.	n.s.	n.s.	n.s.	0.027 n.s.	n.s. 0.036	n.s.	n.s.
	0.2	n.s.	n.s. 0.003	n.s.	n.s.	0.002 0.046	n.s.	n.s.	n.s.	0.093 n.s.	n.s.	n.s.	n.s.
	0.5	n.s.	n.s.	0.001 0.001	n.s.	n.s.	0.002 0.002	0.027 n.s.	0.093 n.s.	n.s.	n.s.	n.s.	0.006 n.s.
Ti-42Nb powder (103 < x < 350 µm)	0.1	n.s.	n.s.	n.s.	0.001 0.036	n.s.	n.s.	n.s. 0.036	n.s.	n.s.	n.s.	n.s.	n.s. 0.093
	0.2	n.s.	n.s. 0.005	n.s.	n.s.	0.001 0.006	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	0.5	n.s.	n.s.	0.021 0.001	n.s.	n.s.	0.001 0.001	n.s.	n.s.	0.006 n.s.	n.s.	n.s.	n.s.

n.s. - not significant.

[22] cultured fibroblast cell lines on several pure metals and Ti alloys containing e.g. Nb, V or Al. WST-1 test showed good biocompatibility of pure Nb and the highest cell viability for Ti-10Nb. Those results are approved by de Andrade et al. [8] and Cremasco et al. [6] culturing rat osteoblasts and a fibroblast cell line on Ti-35Nb, respectively. Do Prado et al. [23] analyzed expression of relevant genes encoding for cell adhesion, differentiation and matrix synthesis in human osteoblasts. Thereby, Ti-35Nb revealed similar results to titanium. Bai et al. [31] used fibroblast and osteoblast cell lines to examine and prove cell proliferation and differentiation on Ti-45Nb. Biocompatibility of Ti–50Nb, Ti-30Nb, Ti-26Nb as well as Ti-15Nb, Ti-10Nb and Ti-5Nb with osteoblast and fibroblast cell lines was also attested [24,32,54–56]. Hofstetter et al. [57] also used human osteoblasts suggesting that Nb surfaces are more suitable than titanium. Likewise alloys containing Ti, Nb and a further element like e.g. tantalum or aluminum are proved to be suitable for medical applications [7,14,18,58–60]. Besides in vitro biocompatibility, niobium is also verified for in vivo use [27,61,62]. Nevertheless, a cell specific behavior on materials with different surface roughness is also stated. For instance, proliferation and differentiation of osteoblasts is proved to be roughness-dependent [63–66]. It is controversially discussed whether osteoblasts prefer rough surfaces [63,67] [10,68,69]. Our present study strongly supports these thesis, since the specimens with the roughest surfaces (both Ti-42Nb and Nb Ampertec) revealed the best results concerning metabolic activity and collagen synthesis. As described by Lohmann et al. [70], cell response of osteoblasts to surface roughness depends on their maturation state. In

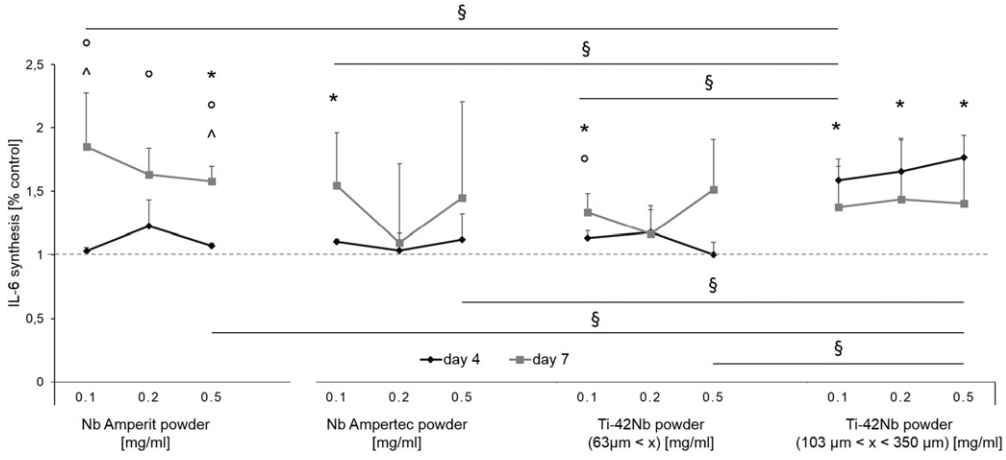

Fig. 10. IL-6 synthesis of human osteoblasts cultivated with different Nb and Ti-42Nb powders in three concentrations (0.1, 0.2 and 0.5 mg/ml) for four and seven days. Data are mean ± SEM ($n = 8$). For statistical analysis Mann-Whitney-*U* test was conducted. Data were compared to the growth controls (day 4 = *p/day 7 = °p) and day 4 towards day 7 (^p).). §p tags difference between the several niobium powders at day 7; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$.

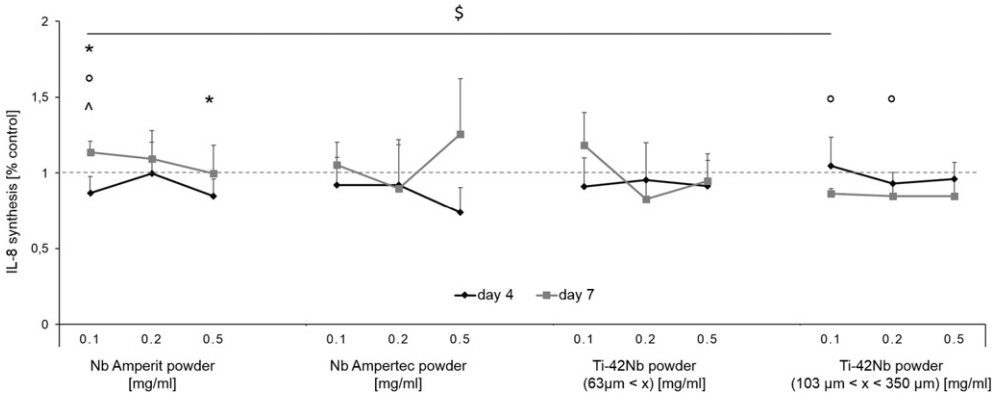

Fig. 11. IL-8 synthesis of human osteoblasts cultivated with different Nb and Ti-42Nb powders in three concentrations (0.1, 0.2 and 0.5 mg/ml) for four and seven days. Data are mean ± SEM ($n = 8$). For statistical analysis Mann-Whitney-*U* test was conducted. Data were compared to the growth controls (day 4 = *p/day 7 = °p) and day 4 towards day 7 (^p). $p tags difference between the several niobium powders at day 7; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$.

contrary, preference for smooth surfaces is well displayed for fibroblasts [67,68,71]. The presented data do not endorse these studies since behavior of human osteoblasts and fibroblasts was nearly similar indicating the best results for both Ti-42Nb surfaces and Nb Ampertec. Those surfaces differed strongly in their roughness values. Thus, the results might also be influenced by the surface material. Furthermore, Nb Ampertec and Nb sheet showed nearly identical coefficients of roughness, while Nb Ampertec resulted in great metabolic activity and procollagen type I synthesis for both types of cells. Hence, the adverse results for Nb sheets might be ascribed to production residues since those specimen were only stamped from on big sheet of metal without any further post-processing except ultrasonic bath and sterilization.

Furthermore, niobium powders (Nb Amperit, Nb Ampertec, and spherical Ti-42Nb in two particle sizes) were used for osteoblast exposition to imitate influence of particulate debris on potential osteolysis. The time- and dose-dependent influence of powders on the metabolic activity of human osteoblasts (Fig. 6) is supported by the live/dead fluorescence staining quite well (Fig. 8). A decrease of cell density and increase of dead cells is perceptible mainly for Nb Ampertec and Ti-42Nb powder (63 µm < x) with higher concentrations and cultivation time reflecting the results of metabolic activity. Nb Amperit und Ti-42Nb powder result in a slight decrease of cell density. Nevertheless, human cells are more sensitive than cell lines, but reacting in a donor-specific manner. Accordingly, metabolic activity is not reflected by live/dead staining, generally. For comparison it should be taken into account that pure powders (Amperit, Ampertec) and "alloy powders" (Ti-42Nb) had been used offering different chemical compositions. Apart from that particle size is one of the main factors influencing cell reaction. Particles of Nb Ampertec are larger than Nb Amperit ones, which might be a reason for the decreased metabolic cell activity. Since Ti-42Nb powders revealed an opposite result, here the chemical particle composition might be with higher influence than the particle size. As discernible in Fig. 1, titanium free niobium powders show a different particle morphology compared to Ti-42Nb ones. The particles are more edged than the spherical particles of the Ti-42Nb powders, which is proven to be an important factor for biological response. Dependency of cellular responses on the shape of particles is stated for at least polymer wear debris showing a decreased aggressive potential of spherical particles compared to other morphologies [72,73]. For a more detailed evaluation further investigations on this topic are required. A previous work investigating wear debris of Nb alloys like e.g. Ti-6Al-7Nb compared with common Ti6Al4V indicated a lower inflammatory response of monocytes cultured with Nb-containing particulates [27]. Kuroda et al. [74] examined influence of Nb and Ti wear debris in different concentrations over four and seven days on osteoblast cell lines and proved a dose-dependent influence on cell viability being depressed and lowest with the highest particle concentration. Thereby, viability of cells was still slightly higher using Nb debris. Similar experiments were done by Sakai et al. [46], who approved these results, but suggested that rather particulates wielded a slight effect on cells, but not the extracts. It is still proven that the effects of particulates on cells are caused by the direct contact of particulates with the cell membrane and phagocytosis of particles by cells [46]. Nevertheless, the recovery of viability is possible if the exposure level is below the "threshold particulate concentration value" [46]. Furthermore, it is well known, that cell viability is influenced by particulate size, concentration, composition and dissolved ions. Even contact with body fluid (blood, urine, saliva, serum) or culture medium results in corrosion processes of metals releasing ions to their environment [3,75], while intensity of ion dissolution is depending on medium properties (pH, temperature, chemical composition) [11,75,76] and the dissolution rate and roughness of the metal [77,78]. Caicedo et al. [79] published a comprehensive study concerning metal ion-induced cell effects. Additionally, in our present study random supernatants of the test specimens were analyzed after 72 h via Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES) for testing purposes using an ICP Optical Emission Spectrometers (Varian/Agilent 715-ES, Waldbronn, Germany). Sample digestion and dilution were done with HNO_3 and with H_2O_2 and H_2O, respectively. Due to technical incidents a high dilution of samples was necessary, so all tested supernatants were under the detectable limit. However, Obata et al. [80] demonstrated a concentration-dependent influence of Nb ions, which induced differentiation and mineralization of osteogenic cells rather than initial cell adhesion and proliferation. Niobium surfaces are well known to exhibit good resistance against corrosion in simulated body fluid and excellent biocompatibility [41].

There are no previous in vitro studies regarding the influence of niobium and Ti-42Nb powders on human cells examining cell viability, collagen and interleukin synthesis. Our results indicate a slight release of IL-6 after particulate exposure, while IL-8 synthesis is nearly unaffected. Therefore, further work concerning inflammatory influence should be done. In addition, gene expression analysis and measurement of other matrix relevant markers should be taken into account. Moreover, comparative analysis in terms of the influence of particle sizes to cell behavior are obligatory.

In summary, there are only few previous studies covering the in vitro biocompatibility of niobium and especially Ti-42Nb. None of them are dealing with spherical Ti-42Nb powders which are required for production of patient-specific implants using 3D-printing, e.g. SLM or EBM. In the present study, those materials show promising results with human osteoblasts and fibroblasts and might be an alternative for medical applications to common metallic implant materials like Ti6Al4V alloys.

5. Conclusions

Specimens and powders based on niobium (Nb Amperit, Nb Ampertec) and spherical Ti-42Nb (sintered, SLM) were determined in cell cultures of human osteoblasts and fibroblasts. Thereby, the roughest surfaces, both Ti-42Nb surfaces and Nb Ampertec specimen, revealed excellent results concerning cell viability and collagen synthesis compared to commonly used implant material Ti6Al4V. Nevertheless, the cell specific preferences for surface roughness known from literature could not be ascertained. Both cell types acted in a similar manner. Examinations with particulate debris disclosed a dose-dependent and partly time-dependent influence of all four powders, thereby Nb Ampertec showed the highest decrease of cell viability and collagen synthesis. Furthermore, interleukin synthesis was only slightly increased for all powders. In summary, Nb Ampertec and Ti-42Nb seem to be promising alternatives for medical applications compared to commonly applied implant materials such as Ti6Al4V.

Conflicts of interest

The authors declare no conflict of interest.

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