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Microvasculatory reaction of skeletal muscle to Ti–15Mo in comparison to well-established titanium alloys

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Abstract Beta-titanium alloys such as Ti–15Mo are increasingly utilized for orthopaedic implant applications because of their excellent corrosion resistance and low elastic modulus. Particularly in osteosynthesis, where the biomaterial stands in direct contact to soft tissue, undesirable biologic reactions may have severe consequences especially in the vulnerable state of trauma and added iatrogenic damage to the microvascular system. In a comparative study we therefore assessed in vivo nutritive perfusion and leukocytic response of striated muscle to the biomaterials Ti–15Mo, Ti–6Al–4V and Ti–6Al–7Nb, thereby drawing conclusions on their short term inflammatory potential. Utilizing the well established skinfold chamber preparation in the hamster and intravital fluorescence microscopy, we could not demonstrate any significant discrepancies between the three alloys. All metals induced an initial moderate inflammatory response in skeletal muscle microcirculation. While recuperation of animals treated with Ti–15Mo and Ti–6Al–7Nb was prompt, we documented a slightly more sluggish recovery of Ti–6Al–4V treated animals. A gross toxicity was not observed for any of the alloys. Conclusively, Ti–15Mo, Ti–6Al–4V and Ti–6Al–7Nb induce an only transient inflammatory answer of the striated muscle microvascular system. Our results indicate that on the microvascular level the tested bulk Ti-alloys do not cause enduring biologic impairment in muscle.

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Introduction

Titanium alloys with an alpha-beta-microstructure like Ti–6Al–4V and Ti–6Al–7Nb are commonly used for a variety of orthopaedic and trauma implants because of their excellent combination of corrosion resistance and biomechanical properties [1–3]. Concerning their biocompatibility, numerous in vivo and in vitro studies have suggested that particulary degradation products of Ti–4Al–6V are capable of activating an inflammatory cascade which in turn may cause severe local tissue damage [4–7]. In a recent in vivo study using the well established skinfold chamber model we could demonstrate that bulk Ti–4Al–6V and Ti–4Al–7Nb implants showed good tissue biocompatibility with only little impairment of microvascular perfusion, leukocyte flow behaviour and change in vascular permeability [8].

Ti–15Mo is a relatively new beta-alloy composition that has been recently standardized in ASTM F 2066 for surgical implant applications. Main advantages of this alloy are the higher strength than commercially pure grades, excellent casting ability, low modulus of elasticity, low notch-sensitivity, and enhanced corrosion resistance [9]. In vitro and in vivo biocompatibility evaluations of

Ti–15Mo performed by Zardiackas et al. [9] revealed a satisfactory level of localized biological response. Nevertheless, Puelo et al. [10] observed a moderate cytotoxic reaction of ionic molybdenum to bone marrow cells similar to that of stainless steel and nickel ions.

Particularly in osteosynthesis, where there is direct contact between implant and skeletal muscle, possible impairment of striated muscle microcirculation by the biomaterial may have severe consequences. Taking into consideration that a long-bones nutrition is substantially dependent on the integrity of the surrounding soft tissue, it can easily be appreciated that after implantation impairment of skeletal muscle perfusion by an implant in this early vulnerable state can have significant clinical implications. Reparative processes may be delayed, osseous healing impeded, bone necrosis facilitated and the host's local defense mechanisms against microorganisms impaired.

Hence, the aim of this study was to elucidate the local microvascular response of striated muscle tissue to bulk Ti–15Mo implants compared to Ti–6Al–4V and Ti–6Al–7Nb, thereby drawing conclusions concerning their short term effects on striated muscle microcirculation.

Experimental procedure

Animal model

We used Syrian golden hamsters (*Mesocricetus auratus*) (6–8 weeks old, 60–80 g body weight), kept on standard pellet food and water ad libitum. National regulations for the care and use of laboratory animals were observed. For in vivo fluorescence microscopy a dorsal skinfold chamber was implanted. Containing striated muscle and skin the chamber permits repeated analysis of the microcirculation in the awake animal [11]. It also allows to perform quantitative assessment of microhemodynamic parameters and cellular mechanisms over a prolonged period of time. Furthermore, microvascular permeability can be analyzed by quantifying the extravasation of macromolecules [12].

Under pentobarbital sodium anaesthesia (50 mg/kg body-weight, intraperitoneally; Merial, Halbergmoos, Germany) each animal was fitted with two symmetrical titanium frames, positioned on the dorsal skinfold, sandwiching the extended double layer of skin. One layer was completely removed in a circular area of ~15 mm in diameter. The remaining layers, consisting of striated muscle, subcutaneous tissue and skin were covered with a removable coverslip incorporated into one of the titanium frames. Fine polyethylene catheters (PE, Ø 0.28 mm ID; Neolab, Heidelberg, Germany) were inserted into the jugular vein. A recovery period of 72 h between implantation of the skinfold camber and the start of experiments was allowed to eliminate residual effects of anaesthesia and microsurgery on the microvasculature.

Implants

We used 4 mm^2 large samples with a thickness of 0.5 mm of either Ti–15Mo (ASTM F 2066), Ti–6Al–4V (ASTM F 136) and Ti–6Al–7Nb (ASTM F 1295). All implants were made from material identical to that widely utilised for osteosynthesis plates according to the ASTM-norm. Surface roughness values were measured with a confocal microscope (μSurf®, Nanofocus, Oberhausen, Germany) and calculated using surface analysis software (WinSAM, V 2.6). The implants were specifically produced, characterized and kindly supplied by the Dr. Robert Mathys Foundation (Bettlach, Switzerland).

Implantation technique

Implants were brought into position by removing the cover slip of the titanium frame and placing the metal plate directly onto the striated muscle and subcutaneous tissue. The thickness of the implant was chosen in such a way as not to irritate or mechanically impede the microcirculation after replacement of the cover-slip. No further manipulation of the skinfold chamber occurred after this initial implantation.

Experimental groups

The animals were randomly assigned to the following four groups: titanium-15molybdenum (TiMo; n = 7), titanium-6aluminium-4vanadium (TAV; n = 7), titanium-6aluminium-7niobium (TAN; n = 7) and controls (cntrl; no implant, n = 7).

Intravital fluorescence microscopy and microcirculatory analysis

For in vivo microscopic observation, the awake animals were immobilised in a plexiglas tube, and the skinfold preparation was attached to the microscope stage. By use of a modified Leica DM-LM-D microscope with a 100-W HBO mercury lamp, attached to a LU 4/25 fluorescence illuminator with an I_3 blue and $N_{2.1}$ green filter block (Leica Microsystems, Bensheim, Germany) for epi-illumination, microcirculation was recorded by means of a charge-coupled device video camera (FK 6990, Cohu; Prospective Measurements, San Diego, California) and images were transferred to a video system for frame-by-frame off-line quantification of the microhaemodynamics [13]. Leukocytes were stained in vivo with rhodamine-6G

(0.1 μmol/kg; Merck, Darmstadt, Germany) and classified according to their interaction with the vascular endothelial lining as adherent, rolling or free-flowing cells. Adherent leukocytes were defined in each vessel segment as cells which did not move or detach from the endothelial lining within a specified observation period of 20 s and were given as the number of cells per mm^2 of endothelial surface, calculated from diameter and length of the vessel segment studied, assuming a cylindrical geometry. Rolling leukocytes were defined as those white blood cells moving at a velocity less than two-fifths of the center-line velocity and were given as percentage of non-adherent leukocytes passing through the observed vessel segment within 20 s. Extravasated leukocytes, i.e. leukocytes which had migrated through the endothelial lining and were definitely no longer within a vessel, were expressed as the number of leukocytes per mm^2 of tissue.

Microvascular permeability was assessed after the intravenous injection of the macromolecular tracer fluoresceinisothiocyanate (FITC)-labeled dextran (Mr 150000, 100 mg/kg; Sigma Chemical, St. Louis, Missouri) into individual arterioles and postcapillary venules by quantifying extravasation of the marker by a computer-assisted image analysis system (Adobe Photoshop, Version 6.0; Adobe Inc, San Jose, California) using densitometric techniques [13, 14]. Baseline (BL) measurements were performed immediately after the intravenous injection of the fluorescent marker and subsequently after 30 and 120 min, 8 and 24 h and 3 as well as 7 days after plate implantation. For analysis of venular extravasation of FITC-dextran, grey levels were determined densitometrically in the tissue directly adjacent to the vessel wall (E_1), as well as in the marginal cell-free plasma layer within the vessel (E_2). Extravasation (E) was then calculated as $E = E1/E2$.

Staining of the plasma by means of FITC-dextran furthermore allowed assessment of changes of venular diameter. The diameters of individual postcapillary and collecting venules were measured at baseline (BL) and these identical vessels assessed over the subsequent observation periods 30 and 120 min, 8 and 24 h and 3 as well as 7 days after plate implantation using the analysis-system DISKUS (Hilgers, Version 4.20; Koenigswinter, Germany). Fluctuations were expressed as venular diameter change in percent to BL-values.

Capillary density, which served as the measure of quality of microvascular perfusion, was defined as the length of all red blood cell-perfused nutritive capillaries per observation area and is given in cm/cm^2 (DISKUS Analysis System, Version 4.20; Hilgers, Koenigswinter, Germany). Capillary perfusion was expressed as a percentage reflecting the number of perfused capillaries to all visible capillaries in defined frames as mentioned above.

Experimental protocol

BL recordings for the analysis of leukocyte–endothelial cell interaction and assessment of the microvascular permeability included approximately 8–10 individual postcapillary and collecting venules. After BL recording, the plates were implanted, taking care to avoid contamination, mechanical irritation or damage to the chamber. Microcirculatory analyses were repeated at 30 and 120 min, 8 and 24 h as well as 3 and 7 days after implantation. In control animals identical frames were video-documented, thus allowing assessment of identical vessels throughout the experiment. In experiments with implants some individual blood vessels selected for microscopic study at BL were covered by the implanted material. We therefore had to select microvessels which were different from those analysed at BL. Identical microvessels were studied, however, at time of 30 and 120 min, 8 and 24 h as well as 3 and 7 days after implantation.

Statistical analysis

The statistical procedure included analysis of variance and Student's *t*-test for comparison between the groups. A paired Student's *t*-test was performed for analyzing differences within each group. Values are reported as the mean ± standard error oft the mean (SEM), and the level of statistical significance was set at $p < 0.05$.

Results

Roughness values of implant surfaces

Measurement of surface roughness revealed slightly higher values for TiMo (0.69 μm) compared to TiAlV and TiAlNb with 0.48 μm and 0.38 μm, respectively.

General characteristics

Of all animals treated, one died due to complications associated with the anesthesia during fitting of the dorsal skinfold chamber. Two animals had to be exsanguated due to complications associated with the skinfold chamber. These three animals were replaced so that 28 animals were finally evaluated. The implantation of the metal plate was tolerated well by the animals and was not associated with systemic effects. Assessment of the skinfold chambers was possible seven days after implantation in 6 of 7 animals for TAV and TAN implant groups and in 4 of 7 animals for the TiMo implant. Microcirculatory evaluation in the skinfold preparations of all animals without an implant (controls) was possible after 7 days.

Under BL conditions most leukocytes did not interact with the endothelial surface of the microvessels under study although in some cases ~10–20% of white blood cells showed spontaneous rolling along the endothelial lining of postcapillary and collecting venules. There was no marked leakage of FITC-dextran from venules before implantation of a plate, indicating that the microvascular endothelial lining was intact (Fig. 1).

Leukocyte–endothelial cell interaction

Within the first 30 min after plate implantation a notable increase of leukocyte rolling was observed in all groups. The amount of rolling leukocytes (%) reached a maximum at 8 h [TiMo: BL 6.0 ± 1.68 vs. 8 h 27.89 ± 2.62 ($p < 0.05$); TAN: BL 6.11 ± 0.77 vs. 8 h 28.34 ± 2.17 ($p < 0.05$); TAV: BL 6.49 ± 1.64 vs. 8 h 28.25 ± 3.06 ($p < 0.05$)]. After reaching this peak the fraction of rolling leukocytes fell in all implant-treated groups, most pronounced in those animals with TiMo and TAN-plates. Here almost baseline conditions were regained between 8 h and 24 h. TAV treated animals showed a moderate yet continuous recovery of the fraction of rolling leukocytes in the ensuing observation periods. In TAV-implants a slight, yet persisting increase of 10.5 ± 0.9% to baseline values was documented (Fig. 2a).

In parallel, analysis of venular leukocyte adherence to the endothelial surface of postcapillary and collecting venules after the implantation of the plates led to a marked rise of adherent cells within 120 min in all implant treated groups: TiMo: BL 30.62 ± 7.72 vs. 120 min 563.12 ± 33.74 ($p < 0.05$); TAN: BL 31.21 ± 5.97 vs. 120 min 575.49 ± 26.06 ($p < 0.05$); TAV: BL 31.47 ± 4.78 vs. 120 min 603.29 ± 23.08 ($p < 0.05$). After 120 min venular leukocyte adherence fell drastically in all implant treated animals. A statistical difference for this parameter was only observed between TAV and TAN groups at the observation period 8 h: TAV 313.7 ± 11.63 vs. TAN 263.23 ± 13.44 ($p < 0.05$). After 24 h the animals treated with TiMo and TAN implants had almost reached baseline values, while TAV treated animals demonstrated a slightly more sluggish decrease in venular leukocyte adherence compared to controls (Fig. 2b).

Leukocyte extravasation

Quantification of leukocyte extravasation into perivenular tissue showed a marked increase compared to BL values. The implantation of all materials led to a significant extravasation of leukocytes within the first 8 h (leukocyte extravasation (n/mm^2): TiMo BL 5.00 ± 1.59 vs. 8 h 73.86 ± 9.65 ($p < 0.05$); TAN BL 4.46 ± 2.07 vs. 8 h 68.81 ± 10.87 ($p < 0.05$); TAV BL 5.45 ± 1.26 vs. 8 h 64.06 ± 9.66 ($p < 0.05$). A moderate, yet significant increase of leukocyte extravasation compared to control animals was documented for all three implant groups over the whole observation period. For this parameter, a significant difference in leukocyte extravasation between the three titanium alloys could not be found (Fig. 3).

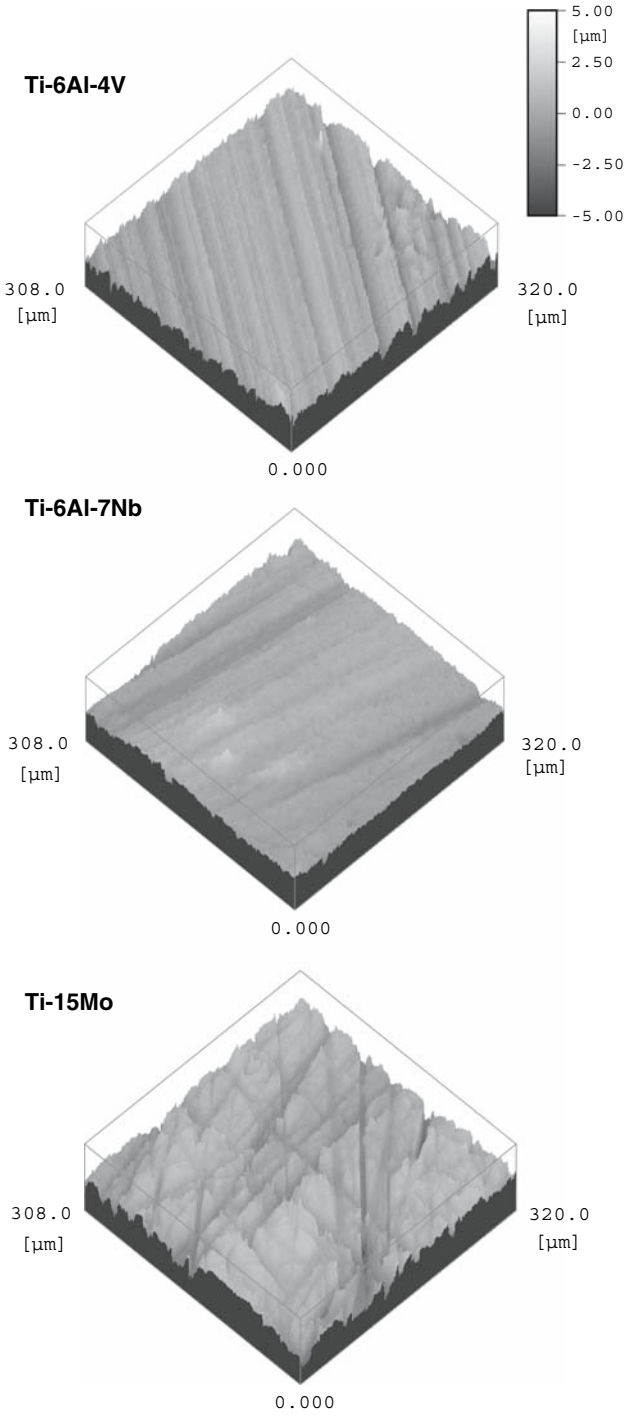

Fig. 1 Three dimensional projection of surface characteristics of TiMo, TAV and TAN

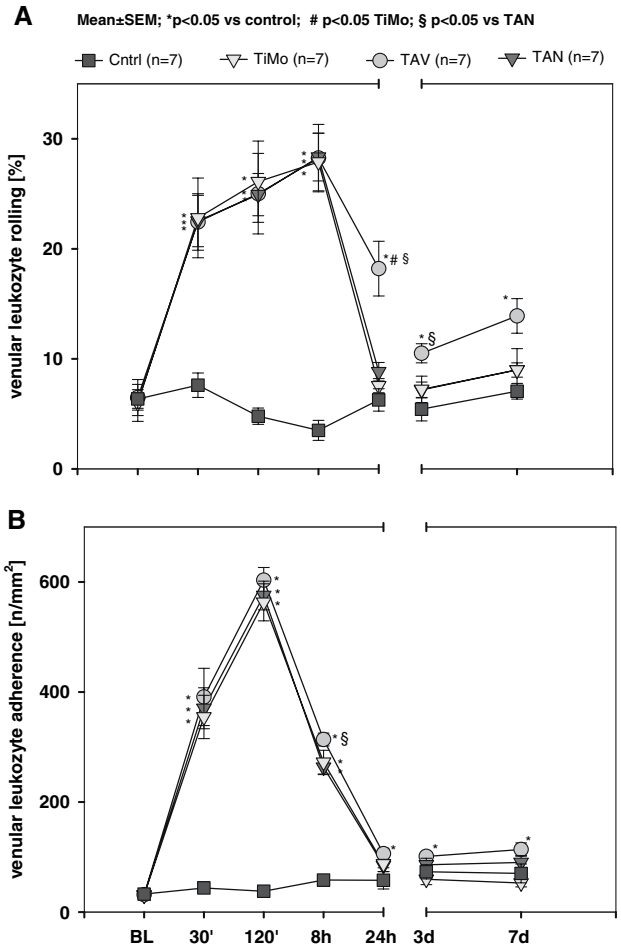

Fig. 2 (**A**) number of rolling leukocytes (given in % of non-adherent leukocytes) and (**B**) number of firmly adherent leukocytes (number of cells/mm^2 endothelial surface) in postcapillary and collecting venules of striated muscle in the hamster dorsal skinfold preparation before (BL, baseline) and 30 and 120 min as well as 8 h, 24 h, 3 and 7d after implantation of the metal plates. Means ± SEM. Unpaired Student's t-test for comparison between groups with $p < 0.05$. TiMo - BL: n = 7, 7d: n = 4; TAV - BL: n = 7; 7d: n = 6; TAN - BL: n = 7; 7d: n = 6; controls - BL: n = 7, 7d: n = 7. *vs control, #vs TiMo, §vs TAN

Microvascular permeability

In all implant groups a slight increase of macromolecular extravasation was measured over the whole observation period. Only at 3d animals with a TAV-alloy showed a marginally statistically significant increase of this parameter compared to controls (TAV: 3d 126.77 ± 7.01% to BL 100% vs. controls: 3d 100.47 ± 7.62% to BL 100%; $p < 0.05$). For all other groups the observed slight increase of microvascular permeability was not statistically significant. Overall extravasation of FITC-dextrane tracer in implant groups was low, indicating that the endothelial lining of postcapillary and collecting venules received little damage (Fig. 4).

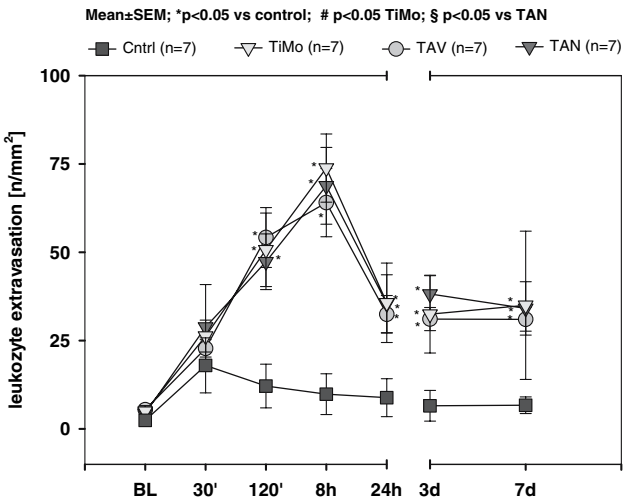

Fig. 3 Number of extravasated leukocytes (given as number of leukocytes/mm^2) outside of postcapillary and collecting venules of striated muscle in the hamster skinfold preparation before (BL, baseline) and 30 and 120 min as well as 8 h, 24 h and 7d after implantation of the metal plates. Means ± SEM. Unpaired Student's t-test for comparison between groups with $p < 0.05$. TiMo - BL: n = 7, 7d: n = 4; TAV - BL: n = 7; 7d: n = 6; TAN - BL: n = 7; 7d: n = 6; control - BL: n = 7, 7d: n = 7. *vs control, #vs TiMo, §vs TAN

Capillary density and perfusion

Capillary density (cm/cm^2) within the chamber preparations did not differ significantly between the three alloys. Under BL conditions capillary perfusion was also similar

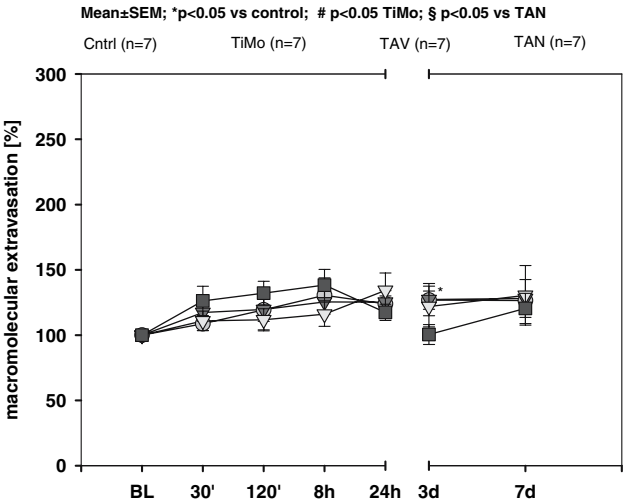

Fig. 4 Macromolecular leakage as a parameter of microvascular permeability in postcapillary and collecting venules of striated muscle in hamster dorsal skinfold preparation before (BL, baseline) and 30 and 120 min as well as 8 h, 24 h and 7d after implantation of the metal plates. Means ± SEM. Unpaired Student's t-test for comparison between groups with $p < 0.05$. TiMo - BL: n = 7, 7d: n = 4; TAV - BL: n = 7; 7d: n = 6; TAN - BL: n = 7; 7d: n = 6; control - BL: n = 7, 7d: n = 7. *vs control, #vs TiMo, §vs TAN

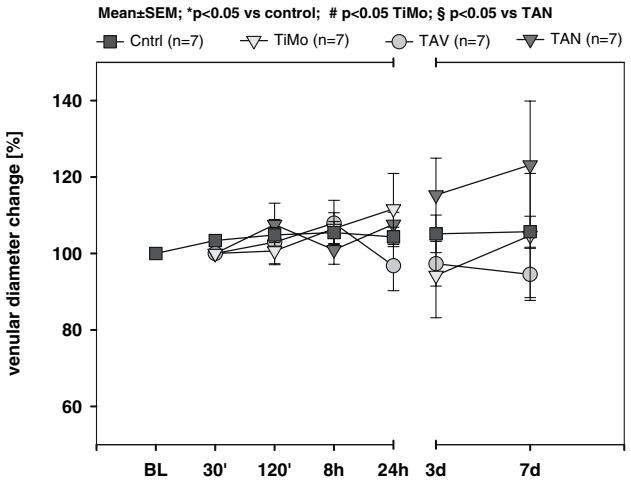

Fig. 5 Diameters (given as % change from baseline [BL] values) of postcapillary and collecting venules of striated muscle in hamster dorsal skinfold preparation before (BL, baseline) and 30 and 120 min as well as 8 h, 24 h and 7d after implantation of the metal plates. Means ± SEM. Unpaired Student's t-test for comparison between groups with $p < 0.05$. TiMo - BL: n = 7, 7d: n = 4; TAV - BL: n = 7; 7d: n = 6; TAN - BL: n = 7; 7d: n = 6; control - BL: n = 7, 7d: n = 7. *vs control, #vs TiMo, §vs TAN

in all groups with mean values of ~99% of perfused capillaries showing good perfusion. After local plate implantation there was no noticeable drop of capillary perfusion within the first 30 min, importantly indicating that the plate did not mechanically impede microvascular blood flow. TiMo, TAN and TAV showed a slight decrease in capillary perfusion over the observation period, yet without significant disparity between the three groups or compared to the control group.

Microvascular diameter

Overall we found a venular dilation of less than 10% compared to BL-values in almost all animals within the first 120 min after plate implantation. Further assessment of this parameter showed an increasing fluctuation of venular diameter, some animals demonstrating vasodilation, others vasoconstriction. At 3d there was a marked vasodilation for animals with TAN-alloys with maximum values reaching 123.14 ± 16.73% 7d after implantation of the plate. Compared among another and compared to the control group, these changes in venular diameter in implant animals were not significant. This data thus remains descriptive (Fig. 5).

Discussion

Particularly in osteosynthesis, be it for fracture management or other indications for internal stabilisation, plates, screws and rods stand in direct contact with the surrounding soft tissue. Impairment of tissue perfusion, which has been proven to have a crucial influence on reparative mechanisms [15], by a biomaterial may have a pivotal impact on healing. Particularly after initial and inevitable iatrogenic trauma the already vulnerable soft tissue may easily succumb by implanting a material that itself induces a massive inflammatory response. These concerns therefore justified the utilisation of an in vivo model to mimic the effects of frequently used bone implant materials on skeletal muscle in a standardised experimental setting [16–18].

We could show that bulk Ti–15Mo, Ti–6Al–4V and Ti–6Al–7Nb implants transiently influence the local microvascular system of striated muscle by inducing an inflammatory response with an increase of leukocyte–endothelial cell interaction only within the first 8 h after implantation. An almost total restitution to baseline values as measured in control animals was observed within 24 h in all implant treated groups. Macromolecular extravasation of FITC-dextrane tracer in all implant groups was low, indicating that the endothelial cell lining of postcapillary and collecting venules received no permanent damage.

Titanium alloys represent a major class of biomaterials that are utilized for orthopedic trauma applications because of their attractive biomechanical properties. Although particularly alpha/beta Ti–6Al–4V alloy is widely used as an implant material, studies have reported that the inevitable release of Al and V ions from the alloy might cause biological harm [7]. Therefore much research effort has been devoted to the study of titanium based alloys with improved corrosion resistance and better processability. Beta titanium-alloys like Ti–15Mo provide a unique combination of exceptional corrosion resistance, improved ductility and excellent casting ability [9].

Yet despite the fact that Ti–Mo is increasingly becoming popular as osteosynthetic material, particularly for the trauma setting, little information concerning the biocompatibility of this binary titanium alloy or even of molybdenum as such exists. The available data is in part ambivalent. Puleo et al. [10] could demonstrate that molybdenum, analogical to nickel and steel, is moderately cytotoxic in bone marrow cultures. Utilizing a bioluminescent assay and solutions with molybdenum Shettlemore and Bundy [19] could underline these findings. In contrast, Rae [20] as well as Schedle et al. [21] claimed that elementary molybdenum does not induce cell death. The latter studied the impact of various metal cations on fibroblasts and mast cells in the in vitro setting, the quintessence being that molybdenum-ions cause neither necrosis nor apoptosis. What was observed though was that molybdenum caused an increase of histamine release from mast cells above that induced by a cobalt suspension yet below that of mast cells confronted with mercury, nickel, chrome or

copper. Implantation of Mo into embryonic rat femora did not directly inhibit further bone growth, nonetheless hydrolysed Mo did marginally diminish development at the epiphysis [22]. In a large study, Hierholzer and Hierholzer [23] could show that molybdenum is principally well tolerated by humans. Patch-tests on 705 patients with the metal did not induce a noteworthy sensitisation or allergic reaction.

While results on the biologic implications of pure molybdenum are inconsistent, the scarce data concerning the alloy Ti–15Mo seems homogenous. Zardiackis et al. [9] were not able to show a marked biologic activity of Ti–15Mo in L 929 fibroblasts over an observation period of 48 h. The same authors used a variety of in vivo models to prove that the mid-term implantation of Ti–15Mo does not cause significant cellular damage in host tissue [9]. Our results underline this reported moderately good biologic acceptance of Ti–15Mo. On the microvascular level we found little impairment of host tissue by the alloy. In parallel to Ti–4Al–6V and Ti–6Al–7Nb, Ti–15Mo induced a transient inflammatory response of striated muscle microcirculation with good recuperation after 24 h. A virtually non-discrepant increase of inflammatory parameters was observed for all 3 tested alloys within the first 8 h after implantation, a phenomenon we interpret as the organism's primary unspecific reaction to a foreign body stimulus. It seems likely that by up-regulation of a number of intracellular and vascular adhesion molecules (ICAM-1, VCAM-1) and selectines leukocyte adhesion and extravasation is mediated. The stimulus inducing this up-regulation can be specific or unspecific. In view of our prior studies where implantation of foreign material always induced a very rapid inflammatory answer in the same time period, the reaction within the first 8 h after implantation seems to be a consistent pathophysiological response. Only after this time does the inflammatory potential of the material as such play a part as to whether restitution takes place or the inflammation subsists [8, 16–18].

In this study animals treated with their corresponding titanium alloys showed that after an initial moderate inflammation lasting approximately 8 h restitution did take place. Yet in comparison to Ti–15Mo and Ti–6Al–7Nb, Ti–6Al–4V induced a slight persistence of leukocyte–endothelium interaction with prolongated recuperation.

In a recent in vitro study assessing toxicity of metal ions to osteoblasts, fibroblasts, and lymphocytes Hallab et al. [7] observed the highest cytotoxic potential for vanadium and cobalt ions. Microscopically we did not find severe or permanent damage to the microvascular system induced by Ti–6Al–4V implants. Due to the fact that our in vivo model mirrors the short term effects of bulk biomaterials on the microvascular system our results are not directly comparable to previous studies, mainly concentrating on the biologic implications of particles or ions [24–27]. Perhaps our model is not sensitive enough to compare biomaterials where only very discrete discrepancies, possibly even only on the molecular level, are anticipated. Thus, further in vitro cytokine and chemokine secretion studies are certainly necessary to elucidate the ambivalent data concerning the biomaterial Ti–6Al–4V.

Surface roughness has been reported to influence reactions between an implant and the biological environment [28]. Refai et al. [29] reported that murine macrophages significantly increased their secretion of proinflammatory cytokines when attached to rough surfaces. In our study the determination of surface roughness values of the utilized alloys investigated by confocal microscopy revealed only slight differences (range 0.38–0.69 μm). We believe that it is unlikely that the biologic reactions we observed in this study can be solely explained by surface characteristics of the metals, though admittedly they may well play a role. Also, it seems questionable as to what extent our in vivo model is able to pick up discrepant biologic reactions to surface characteristics when roughness values of the tested materials are as close as they were. Before further studies concentrating only on different surface variables are performed, influences of surface characteristics on skeletal muscle microcirculation remain speculative.

Conclusion

Conclusively this study revealed good short term response of the striated muscle microcirculation to the beta alloy Ti–15Mo and the alpha/beta alloys Ti–6Al–4V and Ti–6Al–7Nb indicating that they were well tolerated by the organism under the chosen test conditions.

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