Possibilities of applying Ti (C, N) coatings on prosthetic elements – research with the use of human endothelial cells

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Purpose: The aim of our study was to examine the effect of prosthetic alloys with Ti (C, N) coatings on viability and pro life ration of human cells employing an MTT assay with the use of human microvascular endothelial cells derived from the skin – HMEC-1 (Human Microvascular Endothelial Cells-1). *Methods*: Cylindrical shape samples made of Ni-Cralloy were divided into S1-S5 groups and coated with Ti (C, N) layers with different content of C and N. S0 group – control group without layer. The alloys (S0-S5) were stored in an experimental medium (MCDB131 with antibiotics) for 30 days and then HMEC-1 cells were incubated in the alloy extract for 24 and 96 hours. Next, cell viability was determined using MTT method. *Results*: In the case of samples incubated for both 24 and 96 hours there are statistically significant differences (with *p*-value <0.05) between the uncoated samples (S0 group) and all the other Ti (C, N) coated samples. Higher absorbance values were observed in all coated groups than in the control S0 group, where cell growth was statistically significantly lower. *Conclusions*: During incubation of endothelial cells with coated samples the number of cells was significantly bigger than the number with uncoated alloys. The best viability of cells was obtained from the $S = 3$ (with 51.94% at. Ti, 28.22% at. C and 19.84% at. N) group of samples. Ti (C, N) coatings may be applied as protective components on prosthetic elements made of base metal alloys.

Key words: cellviability, microvascular endothelial cells, Ni-Cr, dental alloys, titaniumcarbonitride, Ti (C, N) coating

1. Introduction

Metals and their alloys have long been used in dental prosthetics. The side effects of using them could be adverse reactions of organism, e.g., allergy. The sources of the allergy are mainly corrosion products, as well as metal ions release during corrosion process that infiltrate surrounding tissues. Despite this fact, metal alloys are and very likely will be used in dental prosthetics for long time, since at the moment there are no alternative materials of comparable properties (resistance and durability, in particular). In order to increase their biological tolerance, different modifications of external layers are used for elements made of these alloys. This allows biocompatibility and increased durability to be achieved. In recent years, various deposited layers: CVD, PVD, sol-gel [8], [9], [21], [30] have been used more and more often. Out of many layers obtained using this method, most widely used are oxides, nitrides and carbides of metals [6], [12], [14], [15], [25]. Particular attention should be paid to titanium carbides and nitrides, because of great durability and high corrosion resistance [19].

Alloys with chromium and nickel or chromium and cobalt as basic components are widely used in prosthetics. However, they have relatively low corrosion resistance compared to noble metal alloys [11], [24]. Their wide use is determined by economic aspects. Improvement of their corrosion resistance by

covering with layers of titanium nitrides seems to be particularly purposeful [10]. Studies are conducted for modifying the technology of obtaining nitride layers in order to improve their properties that depend mainly on TiN to Ti_2N ratio in the layer. Second direction of research is obtaining layers of titanium nitrocarbide Ti(CN). As was proven during earlier studies [5], [8], [10], [13], [14] layers of Ti(CN) show higher corrosion resistance, significantly reduce amount of metal ions passing from the base and because of that they could potentially be used for covering prosthetic fillings.

In order to determine suitability of layers produced on the surface of alloys, it is necessary to complement the studies with biological investigation – influence of the layers with human tissues.

The aim of the study was to examine the influence of Ti (C, N) coatings of prosthetic alloys on proliferation and viability of human cells using the MTT assay with the application of human microvascular endothelial cells HMEC-1.

2. Materials and methods

Cylindrical shape samples of 8 mm in diameter and 10 mm in height made of Ni-Cr alloy were used as research material (Fig. 1). Initial composition of the alloy, determined by X-ray fluorescent analysis on SIEMENS XRS300 spectrometer, is presented in Table 1. Cylinders were divided into six groups with 8 samples in each group. Cylinders without covering were in the control group (S0). The remaining five groups were covered with Ti (C, N) layers of different C and N contents in the layer (Table 2).
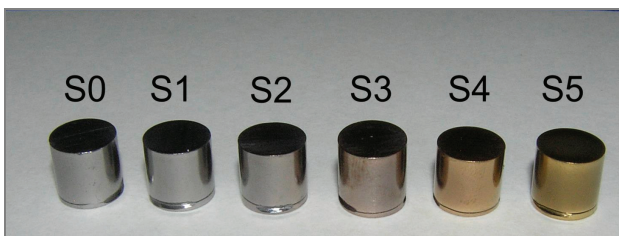

Fig. 1. Samples used for examinations

Table 1. Chemical composition of the alloy examined

Contents of elements – mass %						
Cr	Mo	Si	Fe	Co	Mn	Ni
24.79	8.89	1.57	1.33	0.17	0.12	Rest

Table 2. Chemical composition of the layers examined

Layer	Element content at.%		
	Ti	C	N
S1	53.50	48.50	0.00
S2	52.91	33.91	13.80
S3	51.94	28.22	19.84
S4	47.78	20.05	32.17
S5	56.79	0.00	53.21
Layer	Element content weight %		
	Ti	C	N
S2	80.18	19.82	0.00
S2	79.51	13.90	6.60
S3	78.76	11.67	9.57
S4	75.26	8.61	16.12
S5	79.78	0.00	20.22

Layers were deposited using magnetron sputtering method. Before applying a layer samples were cleaned mechanically and washed with detergent and acetone by ultrasonic method. Next, they were subjected to ion cleaning. For this purpose, they were placed in vacuum chamber, from which the air was pumped out until the pressure of approximately 0.0021 Pa. Ion cleaning was performed with ion gun using argon as working gas. Energy of ions reaching the sample base was regulated. Ions generated in the ion gun had energy of 4 keV. After 5 minutes of cleaning with these ions, a negative potential was applied to the bases in the following order: 800 V, 1000 V and 1500 V. Voltage was changed every 5 minutes. The total time of ion cleaning with Ar ions was 20 minutes. Then, the stage of cleaning and heating with chromium ions followed. Work parameters were: 55 A/1.7 kW; potential on the bases was set to 1250 V. Heating was conducted for 10 minutes. Layers were deposited by magnetron sputtering method on the samples prepared in such a way. In order to improve adherence of Ti(C, N) layers, first adhesive sublayer of pure titanium was deposited during 120 s with argon pressure equal to 0.24 Pa and with the following work parameters of magnetron: 3 kW/approximately 4.5 A. After two minutes reactive gas was slowly introduced: nitrogen, acetylene or their mixture. Deposition time of appropriate layer was the same for all processes and equal to 7200 s. Polarisation with constant voltage during deposition was – 100 V. Pressure of the process was 0.27 Pa in each case. The reactive gases and their flow are presented in Table 3. These were the only variables of the processes.

After deposition the vacuum chamber was cooled down and only then were the samples taken out.

Table 3. Reactive gas flow

Gas	Flow unit	Samples				
		S1	S2	S3	S4	S5
N_2	[sccm]	0	4	8	12	16
C_2H_2		8	6	4	2	0

In preclinical studies, cytotoxicity is determined using a variety of assays, dyes and methods. In most experiments, the viability of cells in direct contact with the surface of the substance after a few or tens of hours, assessing cell shape and the ability to create a process or proliferation capacity using microscopic techniques or indirectly with colorimetric assays.

2.1. Chemicals

MCDB 131 medium, fetal bovine serum, penicillin-streptomycin solution (5,000 units/ml penicillin and 5.000 μg/ml streptomycin sulphate in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsine-EDTA (0.25% trypsin, 1 mM EDTA-4 Na) were purchased from Invitrogen (Carlsbad, CA, USA). The thiazolyl blue tetrazolium bromide (MTT), human EGF and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, USA).

2.2. Cell cultures

HMEC-1 (human microvascular endothelial cells) were purchased from ATCC, catalog number ATCC-CRL-10636 (depositor Centers for Disease Control, Dr. Edwin W. Ades, Atlanta). For experimentation, the cells between passages 10–31 were used. HMEC-1 cells were cultured in 25 cm^3 flasks in MCDB 131 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone and penicillin-streptomycin solution, in a humidified atmosphere of 95% and 5% CO_2 at 37 °C. Cells were harvested every third day in a trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA). HMEC-1 cells were cultured according to the method described in the literature [1] and the author's own modification [29].

2.3. MTT conversion

HMEC-1 (human microvascular endothelial cells) cells was measured using the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion method. The essence of the method is to measure the reduction (or change) of a yellow MTT liquid to a purple coloured formazan salt MTT-f (in the form of insoluble crystals). The reaction proceeds thanks to mitochondrial succinate dehydrogenase in living, metabolically active cells. If the cells are damaged by the factor tested, the reaction is less intense or may not occur at all, which can be seen in the colour change and spectrophotometric determination of absorbance (Optical Density, OD). The optical density corresponds to the number of surviving cells incubated in the test medium.

Cells were seeded (50 000 cells/well) into 96-well plates. The treated cells were incubated for 24 h or 96 h with medium in which prosthetic alloys were stored for 30 days or not (control group). After incubation, 50 μl MTT (1 mg/ml, Sigma) was added and the plates were incubated at 37 °C for 4h. At the end of the experiment, the cells were exposed to 100 μl isopropanol, which enabled the release of the blue reaction product: formazan. The absorbance was measured on an ELISA (ELx800, BioTek) microplate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm.

In our study, the selection of microvascular endothelial cells was dictated both by their presence in the oral cavity and the data acquired from the literature [16], [22]. Other works seem to confirm the choice of endothelial cells as an optimal model for testing cytocompatibility [27], [28]

Test selection was dictated by the fact that the MTT assay is one of the most widely used tests, which is defined as referential by international standard-setting organizations. This test determines the viability and proliferation of living cells with intact metabolism and respiration chain.

Average absorbance for each group under examination was calculated from 8 repetitions.

Statistical test selection was based on the distribution of normality (using the Shapiro–Wilk test) and equality of variance (Brown–Forsythe test). Having obtained positive results of the tests, the results were statistically analysed by using 1-way ANOVA ($\alpha = 0.05$). The null hypothesis was formulated as follows: Ho: μ1 = μ2 = μ3, ..., μi, assuming an absence of statistically significant changes among the analysed specimens. The null hypothesis was rejected when F > $F_{critical}$. If the test showed that this condition was met in the analysed case, then the null hypothesis was rejected. This indicated that statistically significant changes had occurred in the study group. Fisher's Least Significance Difference (LSD) test and Sheffé's test were used to determine differences between a pair of specimens.

3. Results

Examples of microscopic images of cells after contact with the extraction medium are shown in Fig. 2.

Table 5. Absorbance results after 96 h

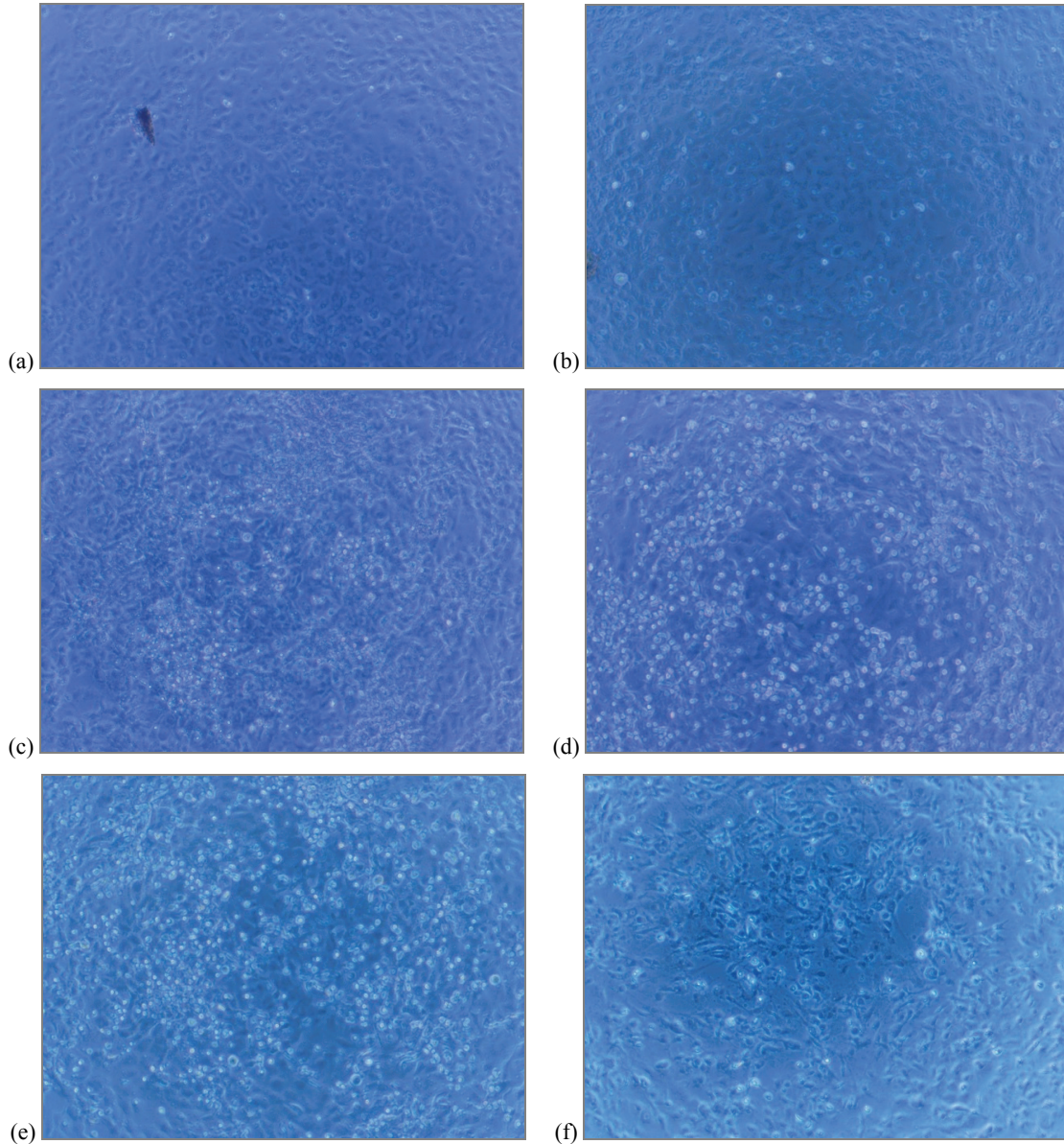| Sample	S0	S1	S2	S3	S4	S5
Mean	**0.32**	**0.38**	**0.47**	**0.53**	**0.47**	**0.47**
Standard deviation	0.03	0.01	0.04	0.03	0.01	0.03

Fig. 2. Examples of microscopic images of cells: (a) Sample S0, (b) Sample S1,
(c) Sample S2, (d) Sample S3, (e) Sample S4, (f) Sample S5

The mean values and standard deviations of absorbance measurements after 24 and 96 hours are shown in Tables 4 and 5.

Table 4. Absorbance results after 24 h

Sample	S0	S1	S2	S3	S4	S5
Mean	**0.27**	**0.35**	**0.39**	**0.52**	**0.41**	**0.37**
Standard deviation	0.02	0.02	0.02	0.05	0.04	0.05

In the pictures, we can see that the number of cells is different for each sample and the lowest number of cells can be observed on S0 (no coatings) and the highest on S3.

Absorbance examinations confirmed the observations. Both after 24 and 96 hours samples S0 demonstrate smaller absorbance than samples S1-S5 (covered with coatings). S3 coatings (with 51.94% Ti, 28.22% C, and 19.84% N) seem to be the most beneficial.

The results obtained were subjected to quantitative absorbance comprehensive statistical analysis. Both tests for samples examined after 24 and 96 hours showed statistically significant differences between groups S1-S5 and control group S0 (the first line in Tables 6–9).

Table 6. Fisher's Least Significance Difference (LSD) test results
for samples examined after 24 h
(marked differences are applicable with $p < 0.05$)

	S0	S1	S2	S3	S4	S5
S0		0.00	0.00	0.00	0.00	0.00
S1	0.00		0.04	0.00	0.00	0.26
S2	0.00	0.04		0.00	0.32	0.33
S3	0.00	0.00	0.00		0.00	0.00
S4	0.00	0.00	0.32	0.00		0.05
S5	0.00	0.26	0.33	0.00	0.05	

Table 7. Sheffé's test results for samples examined after 24 h
(marked differences are applicable with $p < 0.05$)

	S0	S1	S2	S3	S4	S5
S0		0.00	0.00	0.00	0.00	0.00
S1	0.00		0.49	0.00	0.10	0.93
S2	0.00	0.49		0.00	0.96	0.96
S3	0.00	0.00	0.00		0.00	0.00
S4	0.00	0.10	0.96	0.00		0.56
S5	0.00	0.93	0.96	0.0	0.56	

Table 8. Fisher's Least Significance Difference (LSD) test results
for samples examined after 96 h
(marked differences are applicable with $p < 0.05$)

	S0	S1	S2	S3	S4	S5
S0		0.00	0.00	0.00	0.00	0.00
S1	0.00		0.00	0.00	0.00	0.00
S2	0.00	0.00		0.00	0.94	0.70
S3	0.00	0.00	0.00		0.00	0.00
S4	0.00	0.00	0.94	0.00		0.76
S5	0.00	0.00	0.70	0.00	0.76	

Table 9. Sheffé's test results for samples examined after 96 h
(marked differences are applicable with $p < 0.05$)

	S0	S1	S2	S3	S4	S5
S0		0.02	0.00	0.00	0.00	0.00
S1	0.02		0.00	0.00	0.00	0.00
S2	0.00	0.00		0.03	1.00	1.00
S3	0.00	0.00	0.03		0.03	0.01
S4	0.00	0.00	1.00	0.03		1.00
S5	0.00	0.00	1.00	0.01	1.00	

One-way ANOVA statistical analysis showed that in the case of both samples incubated for 24 and 96 hours there are statistically significant differences (with p value <0.05) between the uncoated samples (S0 group) and all the other Ti (C, N) coated samples, as results from Tables 4–9. The absorbance value of S0 sample is significantly lower than that of the other samples, which indicates that the Ti(C,N) coating has a beneficial effect on fibroblast cells.

4. Discussion

The aim of our study was to resolve the issue concerning the reaction of microvascular endothelial cells to the extract in which Ni-Cr prosthetic alloy samples with titanium carbonitride coatings had been stored for 30 days, and to answer the question of whether the examined coatings exhibit cytotoxic activity. After 24 and 96 hours, higher absorbance values than in the S0 control group with significantly and statistically lower cell growth were observed. The absorbance values which reflect the proliferation and viability of cells after 96 hours were slightly higher than of those after 24 hours in all the groups examined. The best values were obtained after both 24 and 96 hours for the S3 coating (Figs. 3 and 4).

Our research results correspond to scientific reports of other investigators. Chien et al. [4]. Pointed out that the cytotoxicity of prosthetic alloys based on nickel can be reduced using a coating film of TiN and TiAIN. Chien conducted tests evaluating gingival fibroblast cell morphology after 3 and 24 hours with the use of immunofluorescent microscopy and an MTT assay after direct contact of the cells for 24 hours with the surface of prepared samples and substantiating the importance of the first contact of the cells with the surface being assessed. [4]. In Chien et al. study, the TiN coating did not cause cytotoxic irritation and, what is more, a significant increase in gingival fibroblast proliferation on the surface of the TiN coated nickel alloys was noted as opposed to the surface of the alloys without the TiN coating [4]. Although the basis and the biological test method used in the aforementioned and our experiment differ, the results of the tests determining metabolic activity with MTT are convergent. It would be difficult not to emphasize the beneficial effect of titanium nitride coating on living human cells in comparison with the uncoated alloys.

Beneficial effect of these kinds of coatings was observed not only in reaction to metallic elements but also to polymers. Lehle et al. [16] used vascular endothelial cells in vitro to assess the cytotoxicity of different uncoated and coated with titaniumcarboxo-

nitride polymers to determine their potential use for the purpose of reconstructive surgery of the cardio-vascular system. After 4-day incubation, cell growth was not observed on polymers without titaniumcar-boxonitride layers and on silicone material, in addition, a limited growth was noted only on one polymer. In the case of polymers coated with titaniumcarboxo-nitride a typical standard cell growth as within the control group was observed and, what is more, in the case of polyurethane, polytetrafluoroethylene, poly-propylene polymers and silicone the titaniumcarboxo-nitride coating brought about a 7-fold increase in the number of cells [16].
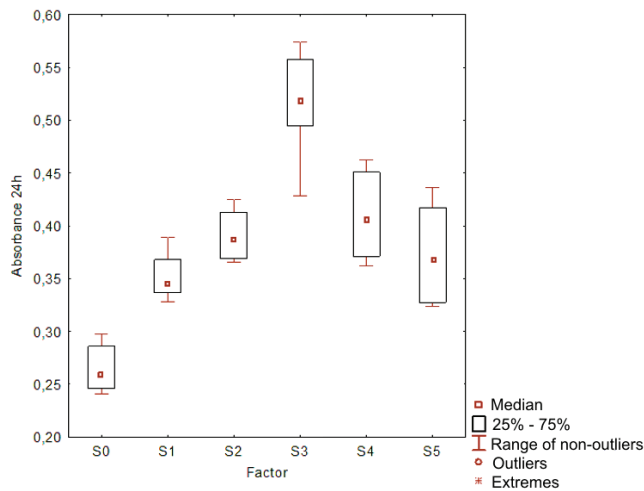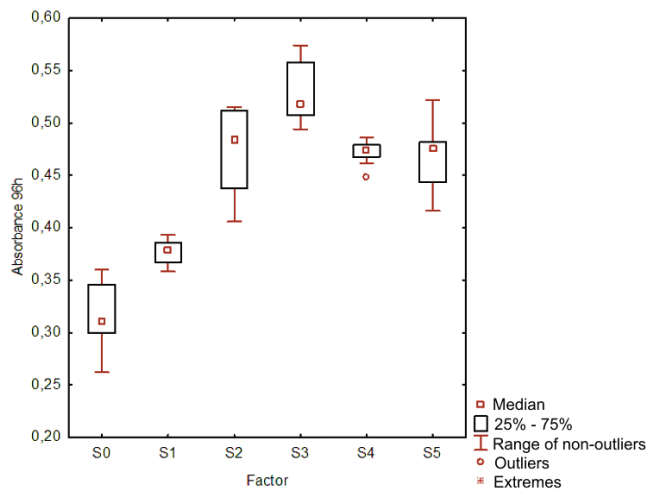

Fig. 3

Fig. 4

In another study, vascular endothelial cells were used to examine the adhesion, proliferation, meta-bolic and anti-inflammatory activity as well as anti-thrombogenic properties of polyurethane implants coated with titaniumcarboxonitride [Ti(C,N,O)] layer to determine their future use in heart and vascular

surgery [23]. The polyurethane coatings examined sig-nificantly improved proliferation and metabolic activity of human endothelial cells. After 72 hours, the coating proved to contribute to the increase of adhesion of endothelial cells, their spread and density to a form of a single organized layer of cells, covering the whole vascular section of polyurethane implants. The re-search of Riescher et al. [23] also suggest that these coatings exhibit anti-thrombocytopenic activity.

Riescher et al. [23] indicate a strikingly acceler-ated adhesion of vascular endothelial cells to titanium Ti(C,N,O) surfaces, which may be beneficial in clini-cal setting.

Referring to the aforementioned study [23], we found a similar trend in our research.

Continuing [17] the above-mentioned study, where the most promising in vitro results [16] were achieved, the experiment was conducted on rats using polymer and silicone implants (discs and meshes) coated as well as uncoated with titanium layers. Unfortunately, histological evaluation of implants (discs and meshes) installed subcutaneously under the light microscope gave inconclusive results. The titanium coating of polymers did not improve biocompatibility after sub-cutaneous implantation in rats. Although in the initial period after implantation the relative number of granulocytes and lymphocytes in the Ti covered im-plants was lower than within the group without the Ti coating, after 4 weeks each implant caused moderate inflammatory reaction thus presenting no difference between the coated and uncoated materials. The pres-ence of temperate inflammation within both coated and uncoated implants was, in the opinion of the authors, due to the observation time being too short, mechanical irritation of the moving subcutaneously implant during the movements of the animal, and probably heavy weight of the implant as well as meshes in discs being too small in diameter [17].

On the whole, the presence of a coating has a posi-tive effect on cell growth, which was confirmed by the study of Lehle et al. 16], Lehle et al. [17], Riescher et al. [23]. Differences in the number of cells may result, in the case of coatings, from different chemical com-position (in our study, there was no oxygen in the coating). Attention should also be paid to a com-pletely different sample material and therefore the lack of possibilities of direct comparison of research results.

In the study of Balázsi et al. [2], silicone alloys were coated with TiC and TiC,N layers. In this article, the authors nonetheless discuss the composite layers consisting of small inclusions of carbides or titanium carbonitrides in the graphite matrix, whereas our study

focuses on continuous layers. The similarity of Balázsi's [2] and our study can be marked in biological research. The culture of human osteoblasts revealed that the composite layers of TiC and TiCN deposited on silicon did not lead to an increase in cell number after 24 hours as compared to control cell culture (culture on a microscope cover slip). However, the viability of cells cultured in contact with TiC and TiCN after 7 days was better than within the control culture [2]. In our study, after both 24 and 96 hours, a better viability of vascular endothelial cells of examined layers than in control group was achieved. In the study of Balázsi et al. [2], better effects were obtained within the TiC layer deposited in argon, which, according to the authors' interpretation, explains the presence of thin layer of antibacterial TiO_2 on the surface of the TiC layer facilitating cell viability.

The coatings of titanium carbide and titanium carbonitride are considered as a layer on elements used as various kinds of implants. These studies also show their positive effect [3], [7]. Despite being conducted with different methods they are convergent to our observations when it comes to preserving the viability and proliferation of cells on TiC and TiN layers. Our studies cannot be compared, however, the trend is similar.

4.1. Summary

The results of our experiment in the assay for the activity of mitochondrial succinate dehydrogenase in the form of purple-coloured formazan being the effect of metabolic capacity of human microvascular endothelial cells showed significantly better properties of Ti(C,N) coated surface displaying higher absorbance values in comparison to a traditional nickel and chromium based alloy. Higher absorbance value resulted from a much larger number of proliferating human microvascular endothelial cells treated with extracts where layers of carbonitrides were applied. The most on Ti (63% C and 37% N). Accordingly, the titanium carbonitride coatings on alloy's surface can be considered as being more biocompatible and improving surface properties as compared to common prosthetic alloys. Our research showed that in the media which were in contact with coated alloys, the viability and proliferation of endothelial cells were greater than in the medium that was in contact with the uncoated alloys. The analysis showed that these differences were statistically significant. It can be assumed that all of the Ti(C,N) coatings have a beneficial influence on viability and proliferation of endothelial cells. Corre-

spondingly, there are indications for their use as protective components on prosthetic elements made of alloys causing an allergic reaction and may be a promising step to improve the biocompatibility of medical materials.

Our research results cannot be directly compared to the results of other researchers as there are differences in the composition of both the coating and substrate materials. It should, however, be noted that other studies have shown similar trends manifested by a favourable impact of these types of coatings in cell-based studies.

As shown by the tests there was no adverse impact of Ti (C, N) coatings on endothelial cells which have even better performance than Ni-Cr alloys without coatings. These are not the only positive properties. Previous studies have also shown that they also reduce bacterial adhesion to the metal components. This is particularly important in the case of dental and orthodontic appliances, in which it is difficult to maintain proper hygiene. The mechanical and tribological properties, and the adhesion of these layers are also satisfactory. In addition, damage of the ceramics is in fact among the most common and some authors argue that these types of coatings can improve the adhesion of dental veneering ceramic to metal substrate, which is essential for the viability of prosthetic components [18], [20].

5. Conclusions

1. During incubation of endothelial cells with coated samples the number of cells was significantly bigger than the number with uncoated alloys.
2. The best viability of cells was obtained from the $S = 3$ (with 51.94% Ti, 28.22% C and 19.84% N) group of samples.
3. Ti (C, N) coatings may be applied as protective components on prosthetic elements made of base metal alloys.

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