DE GRUYTER OPEN

Folia Medica 2014; 56(4): 275-281

doi: 10.1515/folmed-2015-0008

ORIGINAL ARTICLES

Experimental Investigations

INVESTIGATION OF CHROMIUM-COBALT COATED SCAFFOLDS ON CELL CULTURES *IN VITRO*

Denitsa D. Kiradzhiyska[1], Rositsa D. Mantcheva[1]*, Detelina L. Mileva[2], Krasimira I. Draganova[3], Yana N. Feodorova[4], Victoria S. Sarafian[4], Marian M. Draganov[4;]

[1]Department of Chemistry and Biochemistry, Faculty of Pharmacy; [2]Department of Languages and Specialized Training, Section of Natural Sciences; [3]Department of Microbiology and Immunology, Faculty of Pharmacy; [4]Department of Medical Biology, Medical Faculty, Medical University, Plovdiv, Bulgaria

ИССЛЕДОВАНИЕ МЕТАЛЛИЧЕСКИХ ПЛАСТИНОК, ПОКРЫТЫХ ХРОМ-КОБАЛЬТОМ В СРЕДЕ КЛЕТОЧНЫХ КУЛЬТУР IN VITRO

Деница Д. Кираджийска[1], Росица Д. Манчева[1]*, Детелина Л. Милева[2], Красимира И. Драганова[3], Яна Н. Феодорова[4], Виктория С. Сарафян[4], Мариан М. Драганов[4]

[1]Кафедра химии и биохимии, Факультет фармации; [2]Департамент языкового и специализированного обучения, Раздел естественных наук; [3]Кафедра микробиологии и иммунологии, Факультет фармации; [4]Кафедра медицинской биологии, Факультет медицины, Медицинский университет, Пловдив, Болгария

ABSTRACT

Introduction: The use of diverse materials for medical purposes is continuously expanding. The modification of materials which are routinely applied in medical practice as well as the development and introduction of new materials require studies on their biological activity. The first steps in this process are the preliminary short-term screening tests for cytotoxicity and biocompatibility performed on cell cultures. **Methods:** Coating of stainless steel (316 L) scaffolds with chromium-cobalt was performed by electroplating using the non-standard electrolyte *Chromispel*. The process was carried through at different cathode current densities and deposition times. The modified surface of the metal scaffolds was studied for cytoxicity and cell vitality on the serum-free McCoy-Plovdiv and the immortalized PDL cell cultures. **Results:** Our results indicate no cytotoxic effect of the coated metal scaffolds. Even more, three of the samples stimulated the proliferation and growth of McCoy-Plovdiv cells. **Conclusion:** We have strong reasons to believe that chromium-cobalt coatings are promising for future studies and reliable for medical purposes.

Key words*: Cr-Co alloy, biocompatibility, cell cultures, McCoy-Plovdiv, PDL cell line*

Folia Medica 2014; 56(4): 275-281

РЕЗЮМЕ

Введение: В последнее время всё более широко распространяется применение разнообразных материалов в медицинских целях. Модификация материалов, традиционно применяемых в медицинской практике, а также разработка и внедрение новых таковых требуют проведения исследований их биологической активности. Первыми шагами в этом процессе являются предварительные краткосрочные скрининг тесты на цитотоксичность и биосовместимость, которые проводятся на клеточных культурах. **Методы:** Покрытие пластинок из нержавеющей стали (316 L) хром-кобальтом было осуществлено посредством электролиза с применением нестандартного хлоридного электролита типа Chromispel. Процесс осуществлялся при различной катодной плотности тока и различной продолжительности депонирования покрытия. Модифицированная поверхность металлических пластинок была исследована на цитотоксичность и на клеточную витальность в среде бессывороточных McCoy-Plovdiv и иммортализованных PDL клеточных культур. **Результаты:** Полученные нами результаты показывают отсутствие цитотоксического эффекта металлических пластинок. Тем более, в трёх из проб было установлено стимулирование пролиферации и увеличение количества клеток McCoy-Plovdiv. **Заключение:** Налицо серьёзные основания считать, что хром-кобальтовые покрытия являются перспективными для будущих исследований и надёжными для применения в медицинских целях.

Ключевые слова: *Cr-Co сплав, биосовместимость, клеточные куьлтуры, McCoy-Plovdiv, клеточная линия PDL*

Folia Medica 2014; 56(4): 275-281

Article's history: Received: 06 February 2014; Received in a revised form: 16 June 2014; Accepted: 24 June 2014
*Correspondence and reprint request to: *R. Mantcheva, Department of Chemistry and Biochemistry, Faculty of Pharmacy, Medical University, Plovdiv; E-mail: r_manch@abv.bg; Tel.:+359 887 64 19 72*
15A Vassil Aprilov St., 4002 Plovdiv, Bulgaria

INTRODUCTION

Metal biomaterials are often used to reinforce or replace components of the skeleton. They should have high tensile strength and fatigue and a greater resistance to fracture compared to ceramic materials. The choice of materials for medical implants is also based on properties such as corrosion resistance, biocompatibility, genotoxicity, carcinogenicity and cytotoxicity.[1,2]

The variety of materials and alloys used for the manufacture of implants is enormous. The most commonly used metal biomaterials are stainless steel 316 L, Cr-Co alloys, commercially pure titanium and Ti-6Al-4V alloy.[3-6]

Stainless steel used for medical implants is of austenitic type. It has no magnetic properties, and its disadvantages are associated with greater susceptibility to pitting corrosion and cracking.[3] Titanium alloys are also implicated in implant production but not in cases when hardness and resistance to wear are compulsory. It has been shown that their hardness is 15% lower than that of Cr-Co alloys.[7] In general, cobalt alloys can be characterized as non-magnetic, and resistant to corrosion, high temperature and mechanical load.[8] Since they are difficult to process, however, their use is limited. Therefore, new methods for specialized casting and selective laser sintering are currently in development.[9] One possibility to circumvent the difficulties in processing would be the electroplating of chromium-cobalt coatings on different scaffolds made of steel, copper, aluminium etc. Various *in vitro* and *in vivo* tests give evidence that cobalt alloys have good biocompatibility. For this reason they are used for the manufacture of surgical instruments, orthopaedic prostheses, fixtures, knee, hip and shoulder replacement prostheses.[10-12]

The results of electrochemical studies demonstrate that Cr-Co alloys have better corrosion resistance than nickel alloys.[13] The Ni-Co alloy has been shown to release toxic nickel ions in the body fluids as a result of corrosion.[14,15] In addition, *in vivo* tests have shown that the corrosion products of Ni-Cr alloys cause reduced cell proliferation.[16,17]

In addition, cytotoxicity tests of three types of dental alloys using the DNA Comet Assay in mice, detected cells with damaged DNA. The extent of DNA damage caused by Ni-Cr alloy is has been reported as heavier than that of gold alloy and Cr-Co alloy.[18] Therefore, Cr-Co alloy can be used as a substitute for gold alloys in dentistry.

The above mentioned results suggest that Cr-Co alloys are suitable for medical applications. Until now the deposition of these alloys by electroplating has been performed using standard sulphate electrolytes which have low effectiveness. For this reason we developed a method of electroplating using the *Chromispel* type electrolyte which is much more effective.[19] Since it contains chloride ions, however, it was crucial to test the biocompatibility of the coatings produced in this way.

AIM

The aim of the current study was to determine the biocompatibility of Cr-Co coatings deposited by a modified *Chromispel* electrolyte. The acquired data can be used to improve the electroplating technology so that these coatings can be applied in medicine and medical technology.

MATERIALS AND METHODS

Samples

Samples (MS1-7) are scaffolds of medical stainless steel 316 L coated electrochemically with Cr-Co. Coating was performed at 20 ± 2 ºC using a non-standard highly effective electrolyte of the *Chromispel* type at a standard concentration of 250 g/l CrO_3. Cobalt was imported into the plating bath by $CoCl_2.6H_2O$, and its concentration was 1 g/l. Process parameters of electroplating are given in Table 1. The samples were separated into two groups based either on the cathode current density or on the deposition time (Table 1). An uncoated steel scaffold was used as a control (Control, C). The thickness of the coating was measured with Fischerscope® XRAYXDAL (Fischer, Germany).

Cell cultures

Serum-free cell line McCoy-Plovdiv

Cells were cultured in medium DMEM/Ham's F-12 1:1 with 15 mM HEPES, 100 I.U. Penicillin and 100 μg/ml Streptomycin. The procedures for cell culturing and storing were performed according to.[20]

PDL cell line of immortalized precursor cells from periodontal ligament

Cells were cultured in medium DMEM/Ham's F-12 1:1 with 10 % FCS, 100 I.U. Penicillin and 100 μg/ml Streptomycin. The procedures for cell culturing and storing were performed according to.[21]

Biocompatibility testing

Samples were placed in 8-well glass slides with a lid for cell cultures. 6 x 10^4 cells were added to each well and incubated for 96 hours. The state of the cell cultures was inspected every 24 hours using an inverted microscope (Nikon Eclipse TS100).

Folia Medica 2014; 56(4): 275-281

Table 1. Description of the samples

Group No.	Sample name	Cathode current density ($A.dm^{-2}$)	Deposition time (min)	Average thickness of coating (μm)
-	Control (C)	untreated medical steel		
I	MS1	5	5	traces of coating
	MS2	10	5	0.15
	MS3	20	5	1.34
	MS4	30	5	1.70
II	MS3	20	5	1.34
	MS5	20	6	1.43
	MS6	20	7	0.81
	MS7	20	8	0.71

MS: metal scaffold.

Cell vitality and cytotoxicity

Cell vitality was determined in direct co-culture of McCoy-Plovdiv cells and the studied samples using the reagent WST-1 (Roche Diagnostics), which is reduced to a water-soluble product by cellular enzymes (mitochondrial dehydrogenases). Accumulation of the formazan product correlates directly with the number of metabolically active cells in the culture.

In the cytotoxic test McCoy-Plovdiv cells were treated with medium DMEM/Ham's F-12 1:1 which was pre-incubated with the samples for 24, 48, 72 and 96 hours. After 24 hours of treatment the cytotoxic effect was determined microscopically and by a cell vitality test using the reagent WST-1. The results were statistically analyzed.

Cells were incubated with the WST-1 for 4 hours at 37°C, after which the absorbance of the resulting colored product was measured at 450 nm using the ELISA reader *Sunrise* (Tecan).

Immunofluorescense

Samples were washed twice with PBS equilibrated at 37ºC. Cells were fixed in 4% Paraformaldehyde/PBS for 10 min and permeabilized with ice-cold acetone for 5 min. Incubation in 3% FCS/PBS was performed for 30 min to block non-specific binding. FITC-conjugated Phalloidin (1:40 in PBS) (Invitrogen) was used to stain actin and Hoechst 33342 (3 μg/ml) (Sigma) was used for nuclear staining. The results were observed and documented on the epifluorescent microscope (Nikon Eclipse TS100).

RESULTS

Figures 1 and 2 are microphotographs showing the state of McCoy-Plovdiv cell cultures after 72 hours of co-cultivation with the studied metal samples. In all samples from groups I and II cells have normal morphology and form a monolayer on the glass well surface. We did not detect any visual differences between cells from the control cultures (Fig. 1A, B; Fig. 2A, B) and the rest of the cultures (Fig. 1C-F; Fig. 2C-F). The microscopic evaluation was confirmed by the results obtained in the cell vitality test (Fig. 3). For samples MS1 and MS2 from group I and samples MS5 and MS6 from group II the measured values are close to those of the control. For the rest of the samples the cell vitality values are as follows: 113, 5% (MS3); 107, 3% (MS4) and 106, 4% (MS6) (Fig. 3). None of the studied samples showed a cytotoxic effect on McCoy-Plovdiv cells (data not shown).

Figures 4 and 5 are microphotographs of PDL cells co-cultured with the metal samples from both groups. We did not observe any inhibitory or toxic effect on the cell cultures. This can be easily seen in the light microscopy images showing a well formed cell monolayer (Fig. 4A-E; Fig. 5A-E). Cells on the opaque metal surfaces were visualized on an epifluorescent microscope. We assessed the state of the cell monolayer based on the presence of normal cell nuclei (Fig. 4A1-E1; Fig. 5A1-E1) and the distribution of cytoplasmic actin (Fig. 4A2-E2; Fig. 5A2-E2).

DISCUSSION

The demands of modern medicine constantly impose new requirements for scientific research in regard to the development of various and suitable materi-

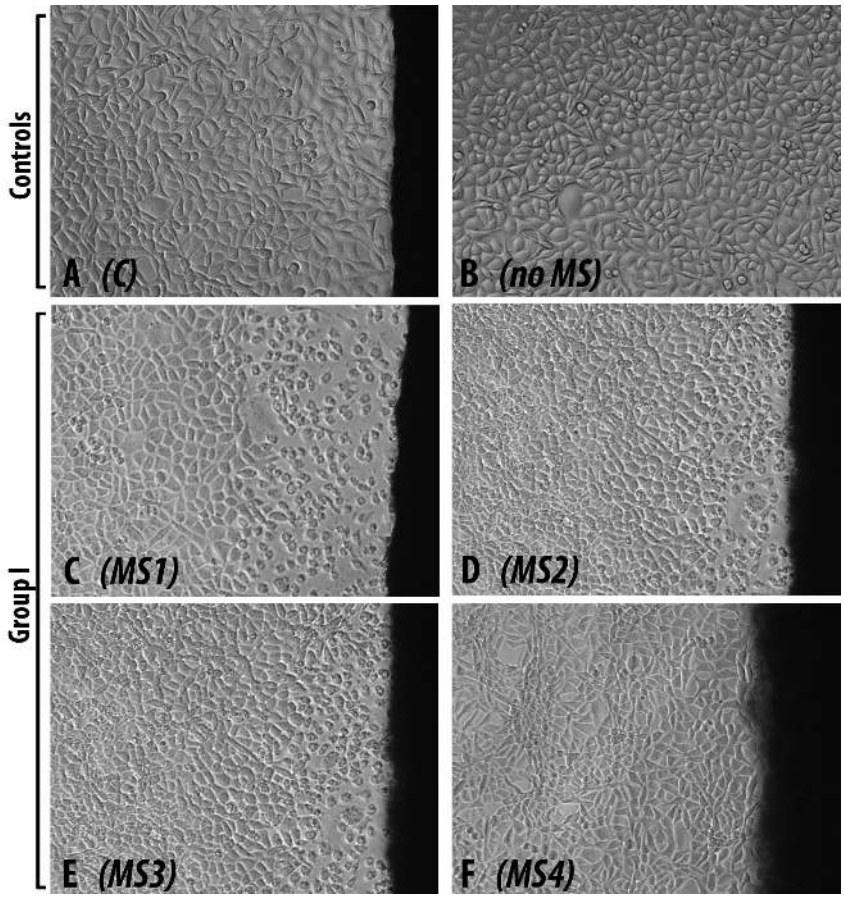

Figure 1. Microphotographs of the serum-free McCoy-Plovdiv cell cultures grown for 72 hours with metal scaffolds from group I: MS1 (C); MS2 (D); MS3 (E); MS4 (F); A – Control, untreated MS (C); B – cells grown without MS; magnification: 100x.

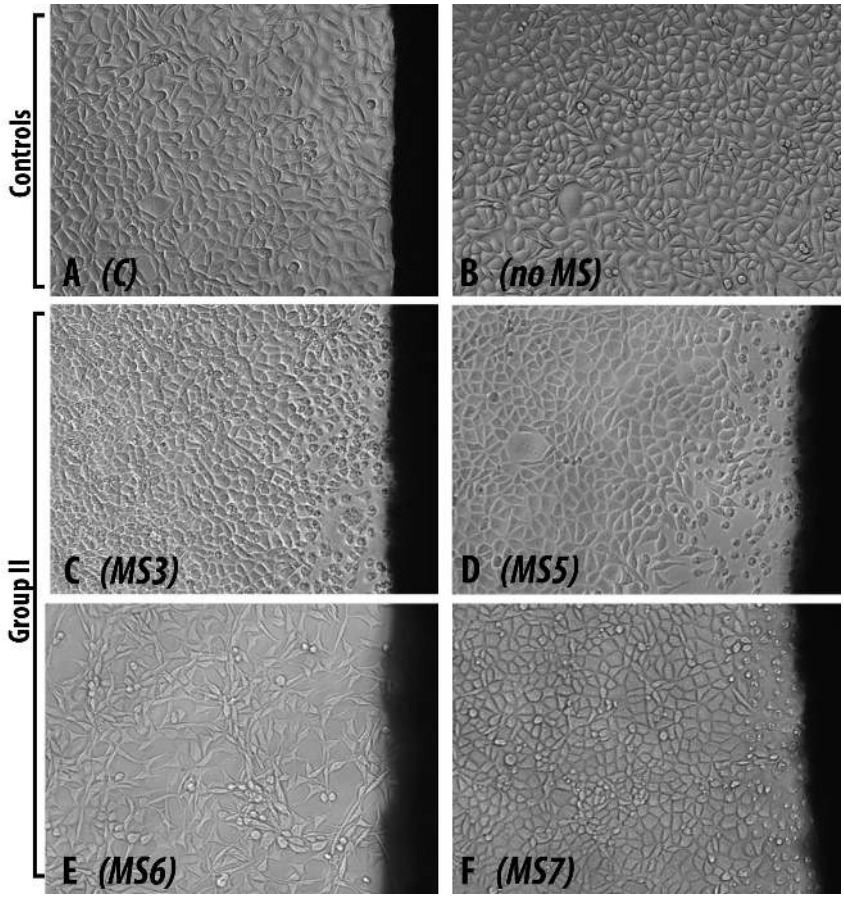

Figure 2. Microphotographs of the serum-free McCoy-Plovdiv cell cultures grown for 72 hours with metal scaffolds from group II: MS3 (C); MS5 (D); MS6 (E); MS7 (F); A – Control, untreated MS (C); B – cells grown without MS; magnification: 100x.

Folia Medica 2014; 56(4): 275-281

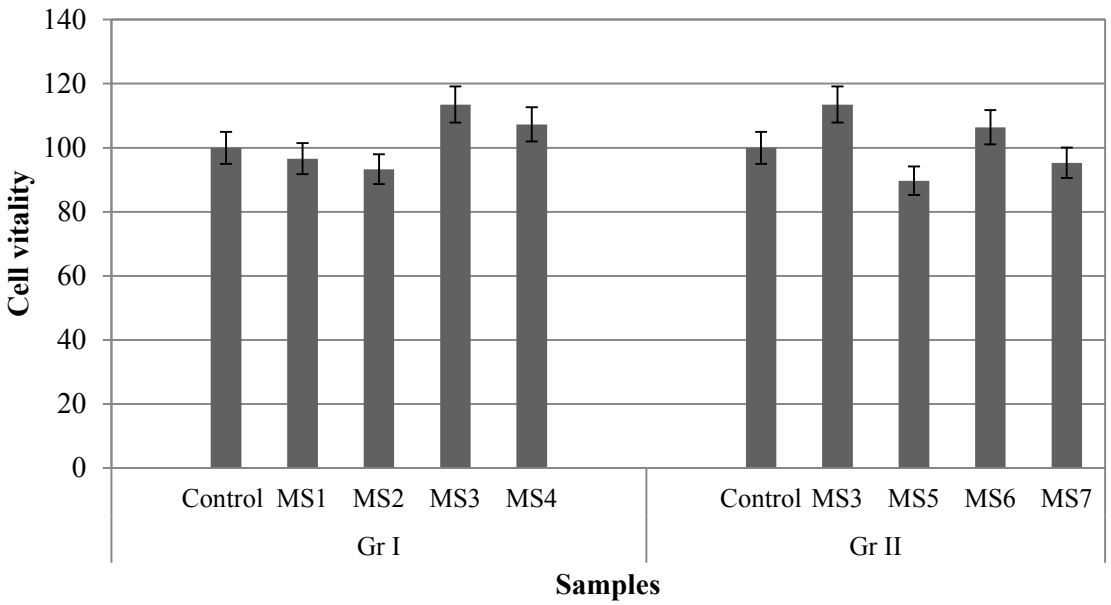

Figure 3. Cell vitality of McCoy-Plovdiv cell cultures determined with the WST-1 reagent. Cells were cultured for 72 hours in the presence of metal scaffolds: MS1, MS2, MS3, MS4, MS5, MS6, MS7. The results are represented as mean values of two independent experiments with three replicas per sample in each.

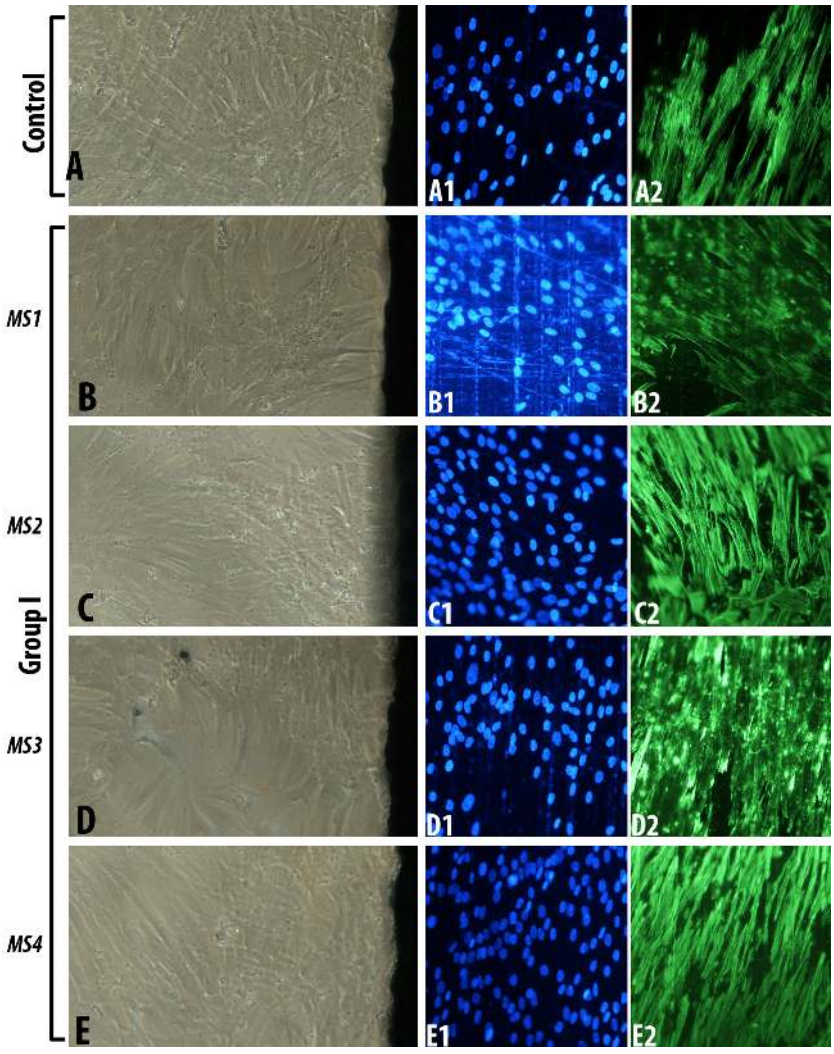

Figure 4. Microphotographs of PDL cell cultures grown for 96 hours with metal scaffolds from group I: MS1 (B); MS2 (C); MS3 (D); MS4 (E); A – Control, untreated MS. A-E – light microscopy; magnification: 100x. A1-E1 – Hoechst stained nuclei; fluorescent microscopy; magnification: 100x; A2-E2 – actin filaments stained with FITC-conjugated Phalloidin; fluorescent microscopy; magnification: 100x.

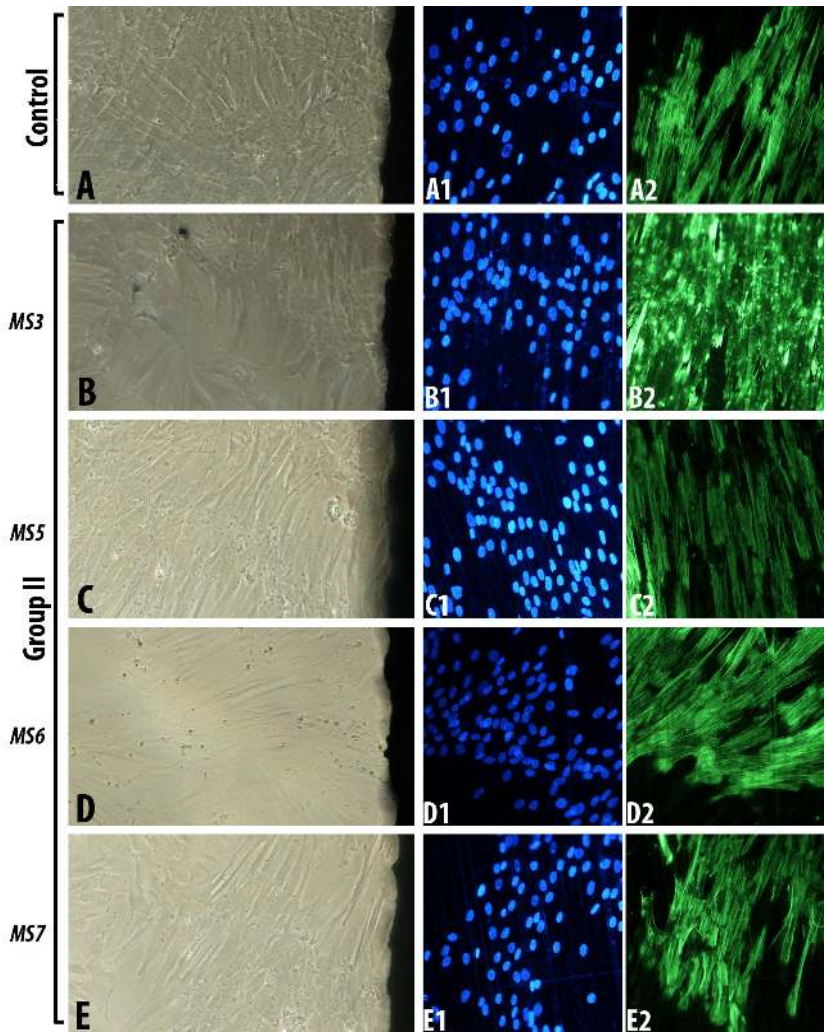

Figure 5. Microphotographs of PDL cell cultures grown for 96 hours with metal scaffolds from group II: MS3 (B); MS5 (C); MS6 (D); MS7 (E); A – Control, untreated MS. A-E – light microscopy; magnification: 100x. A1-E1 – Hoechst stained nuclei; fluorescent microscopy; magnification: 100x; A2-E2 – actin filaments stained with FITC-conjugated Phalloidin; fluorescent microscopy; magnification: 100x.

als for different therapeutic applications. There is an enormous variety of base-metal alloys used in dental implants.[22] Newly developed materials are subject to preliminary biological tests for cytotoxicity and biocompatibility. Stainless steel 316 L is a widely used material for medical purposes. In our experiments we modified the surface of this material by an electroplated Cr-Co coating in order to create a microenvironment (extracellular matrix) which facilitates better adhesion, spreading and proliferation of PDL cells. Our results show that the Cr-Co-coated metal scaffolds from groups I and II are biocompatible. This was confirmed in the cell vitality tests performed on the serum-free McCoy-Plovdiv cell culture. In group I the highest values for cell vitality were obtained for samples MS3 and MS4, whereas for samples MS1 and MS2 the values were lower. Samples MS3 and MS4 are characterized by a thicker Cr-Co coating than the other two samples. In group II the highest cell numbers were detected for samples MS3 and MS6. It is interesting that sample MS5, which has the thickest coating (1, 43 μm), causes a slightly stronger reduction in cell vitality than the other samples in group II. The number of living cells incubated with samples MS3, MS4 and MS6 was increased in comparison to the control cultures. This could be a result of a higher proliferation rate of cells in a co-culture with these samples. Our results demonstrate that the Cr-Co coatings of samples MS3, MS4 and MS6 favor cell adhesion and growth in *in vitro* conditions. It has been reported that cobalt-chromium alloy is well tolerated by a human osteoblast-like cell line[23] and does not cause cell injury in human oral fibroblasts.[24] It has been proven that the surface of metal implants plays a crucial role for the cell behavior and survival, the expression of specific molecules and cell differentiation.[21] The complex *reparative cells – biocompatible scaffolds* is one the most attractive approaches for tissue regeneration.[25]

CONCLUSION

Our results clearly demonstrate that Cr-Co coated metal scaffolds do not cause any cytotoxic effect on

McCoy-Plovdiv cells. They favor better cell growth and development of the PDL cell culture. These are strong reasons to believe that future *in vitro* and *in vivo* studies will support the introduction of these materials into medical practice.

ACKNOWLEDGEMENTS

The study is supported by grants NO 09/2012, NO 02/2013 and Young Scientist 05/2012 (Medical University-Plovdiv).

REFERENCES

1. Brunski JB. Classes of Materials Used in Medicine. Metals. In: Ratner BD, Hoffmann AS, Schoen FJ, Lemons JC, eds. Biomaterials Science: An Introduction to Materials in Medicine. 2nd ed. London: Elsevier Academic Press; 2004: 137-53
2. Balazic M, Brojan M, Bombac D, et al. Titanium and Titanium Alloy Applications in Medicine. In: Jackson J, Ahmed W, eds. Surface Engineered Surgical Tools and Medical Devices. New York: Springer; 2007: 533-68
3. Sumita M, Hanawa T, Teoh SH. Development of nitrogen-containing nickel-free austenitic stainless steels for metallic biomaterials-review. Mater Sci Eng C Mater Biol Appl 2004; 24 (6-8): 753-60.
4. Annual Book of ASTM Standards. Section 13 - Medical Device and Services: Medical Devices; 1999.
5. Williams DF. Medical and Dental Materials. In: Cahn RW, Haasen P, Kramer EJ, eds. Materials Science and Technology: A Comprehensive Treatment. Vol. 14. Weinheim: Wiley-VCH; 1993, 1-27.
6. Breme J, Biehl V. Metallic Biomaterials. In: Black J, Hastings G, eds. Handbook of Biomaterials Properties. New York: Springer; 1998: 135-44.
7. Hallab NJ, Jacobs JJ, Katz JL. Application of Materials in Medicine, Biology, and Artificial Organs. Orthopedic Applications. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, eds. Biomaterials Science: An Introduction to Materials in Medicine. London: Elsevier Academic Press; 2004: 526-55.
8. Davis JR. Metals Handbook Desk Edition. 2nd ed. ASM International; 1998: 616-21.
9. Shetty RH, Ottersberg WI. Metals in Orthopedic Surgery. In: Wise D, Trantolo JD, Altobelli ED, Yaszemski JM, eds. Encyclopedic Handbook of Biomaterials and Bioengineering. New York: Marcel Dekker; 1995: 509-40.
10. Sury P, Semlitsch M. Corrosion behavior of cast and forged cobalt-based alloys for double alloy joint endoprostheses. J Biomed Mat Res 1978; 12 (5): 723-741.
11. Katti KS. Biomaterials in total joint replacement. Colloids Surf B Biointerfaces 2004; 39 (3): 133-42.
12. Long M, Rack HJ. Titanium alloys in total joint replacement - a materials science perspective. Biomaterials 1998; 19 (18): 1621-39.
13. Viswanathan SS, Han-Cheol C. Electrochemical behavior of Co-Cr and Ni-Cr dental cast alloys. Trans Nonferrous Met Soc China 2009; 19: 785-90.
14. Wylie CM, Shelton RM, Fleming GJP, et al. Corrosion of nickel-based dental casting alloys. Dent Mater 2007; 23 (6): 714-23.
15. Fossati A, Borgiolo F, Galvanetto E, Bacci T. Corrosion resistance properties of plasma nitrided Ti–6Al–4V alloy in nitric acid solutions. Corrosion Science 2004; 46 (4) :917-27.
16. Mulders C, Darwish M, Holze R. The influence of alloy composition and casting procedure upon the corrosion behaviour of dental alloys: an in vitro study. J Oral Rehabil 1996; 23 (12): 825-31.
17. Viennot S, Lissac M, Malquarti G, et al. Influence of casting procedures on the corrosion resistance of clinical dental alloys containing palladium. Acta Biomater 2006; 2 (3): 321-30.
18. Yong Z, Ji-ying W. Study on the cytotoxicity induced by ceramic alloys. Chinese Journal of Cellular and Molecular Immunology 2010; 26 (9): 929-30.
19. Mantcheva R, Gadzhov I, Nenov I, Inventor's certificate. № 85289, 1988.
20. Draganov M, Murdjeva M, Sarafian V. Guidelines for cultivation and preservation of the serum-free cell line McCoy-Plovdiv. Cytotechnogy 2003; 42 (3): 163-7.
21. Docheva D, Padula D, Popov C, et al. Establishment of immortallized periodontal ligament progenitor cell line and its behavioural analysis on smooth and rough titanium surfaces. European Cells and Materials 2010; 19: 228-41.
22. Geis-Gerstofer J. In vitro corrosion measurements of dental alloys. J Dent 1994; 22 (4): 247-51.
23. Allen MJ, Myer BJ, Millett PJ, Rushton N. The effects of particulate cobalt, chromium and cobalt-chromium alloy on human osteoblast-like cells in vitro. J Bone Joint Surg Br 1997; 79 (3): 475-82.
24. Arvidson K, Cottler-Fox M, Hammarlund E, Friberg U. Cytotoxic effects of cobalt-chromium alloys on fibroblasts derived from human gingiva. Scand J Dent Res 1987; 95 (4): 356-63.
25. Docheva D, Popov C, Mutschler W, et al. Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system. J Cell Mol Med 2007; 11: 21-38.