COMMONLY USED METALLIC BIOMATERIALS MAY BE INVOLVED IN CANCER PROCESSES – *IN VITRO* STUDIES

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Abstract

There are reports available in the literature describing neoplastic changes around implants or at distant sites that temporally correlate with implantation, although they are not supported by sufficient clinical evidence. Such reports mainly concern the implantation of dental implants, which are performed in the largest number, and squamous cell carcinoma is one of the main types of cancer located in the vicinity of such implants. The occurrence of malignancies after hip arthroplasty has also been described in the vicinity of endoprostheses. At present, there are no indisputable data on the promotion of carcinogenesis by the implants used, and the problem of accelerated tumour induction in the area of implantation is still poorly understood and unclear. The aim of the study was a preliminary assessment of changes in the physiological processes of cells induced by metallic biomaterials intended for orthopaedic implants. A preliminary assessment of changes in the expression of cancer-promoting genes in chondrocytes exposed to metallic biomaterials was recently published. The current report is an analytical summary of changes in proliferation potential, DNA damage repair activity, and apoptosis level of primary and neoplastic cells (chondrocytes and osteoblasts) exposed to commonly used metallic biomaterials (AISI 316L, Ti6Al4V, Ti6Al7Nb, and CoCrMo). Immunofluorescence labelling techniques in flow cytometry were used for the study. The results obtained allow us to state that short-term (48 h) direct exposure to metallic biomaterials of osteoblasts and chondrocytes, both primary and cancerous, can cause significant changes in cell physiology, which may result in promoting the cancer process.

Keywords: *osteoblasts, chondrocytes, metallic biomaterials, proliferation, apoptosis, DNA repair*

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Introduction

Metallic biomaterials, mainly due to their good strength properties and relatively high biocompatibility, are still widely used, especially in the case of bone implants (mainly dental, orthopaedic, and neurological). Numerous attempts are being made to modify the surface of these biomaterials to improve their biocompatibility [1] or to give the surface desired features [2], which brings us closer to the strategy of personalized medicine, taking into account the needs of individual patients [3]. In this context, it is worth paying attention to the influence of metallic materials on causing or worsening metal allergies [4,5], but also on the potential possibility of causing cancer or promoting its recurrence. The analysis of the available literature shows that in the case of the most frequently performed dental implant procedures, neoplastic changes are observed in the immediate vicinity of the implant, as well as in regions distant from the implant, but with a strong time correlation [6]. Dental implants are not the only ones suspected of this unwanted effect [7]. Numerous clinical reports have noted the appearance of malignant tumours in the vicinity of endoprostheses, including lymphoma [8], osteosarcoma [9,10], and squamous cell carcinoma [11] after total hip or total knee arthroplasty. The etiological factors of these changes suggested by various authors include, among others, corrosion of the implant in the environment of body fluids, release of harmful ions into the surrounding tissues, production of Ti/Co/Cr/Ni micro- or nanoparticles due to friction or increased bacterial colonization [12]. However, so far no specific mechanism has been identified that could be responsible for promoting the carcinogenesis process. Recently, we have taken steps to at least initially assess the risk of causing or resuming cancer by analysing changes in the expression of genes involved in carcinogenesis or cancer progression in primary and cancerous cartilage cells because of exposure of these cells to metallic biomaterials [13]. The aim of the current report is a continuation of our observations, but focused on selected cellular processes that are important for the proper functioning of cells and whose disruption may support the processes of cancer transformation. These processes included proliferation [14,15], apoptosis [16,17], and DNA damage repair [18,19], and we observed them in cells exposed to direct contact with metallic biomaterials. In our current work, we applied commonly used alloys of medical steel (AISI316L), titanium (Ti6Al4V and Ti6Al7Nb) and an alloy containing cobalt, chromium and molybdenum (CoCrMo), while the biological material were primary and cancerous bone cells (HO-f and Saos-2, respectively) and chondrocytes (HC-a and SW1353, respectively). The choice of both osteoblasts and chondrocytes was dictated by their participation in the formation of bone tissue at the stage of both bone formation and fracture repair, as well as the presence of cartilage tissue in the articular area of the bone. Moreover, clinical data indicate that chondrocytes and cartilage tissue are mainly responsible for cancerous changes in bone [20], and some benign lesions, for example, enchondroma, may develop into chondrosarcoma over time [21]. However, the selection of primary and cancer cells was aimed at checking whether cells with a cancer phenotype show a changed proliferation potential, altered level of apoptosis and different activity of repairing damaged DNA compared to the same type of primary cells.

Materials and Methods

Metal alloys

The tests were carried out for medical alloys: medical steel AISI 316L (UGITECH ITALIA, Peschiera Borromeo, MI, Italy, ASTM F138-13/ISO5832.1 2016), titanium-aluminium-vanadium alloy Ti6Al4V ELI (VSMPO-AVISMA, Verkhnyaya Salda, Russia, ASTM F136-08e1/ISO 5832-3 1996), titanium-aluminium-niobium alloy Ti6Al7Nb (WOLF-TEN, Wroclaw, Poland, ASTM F1295/ISO 5832-11:2024), cobalt-chromium-molybdenum alloy CoCrMo (CARPENTER TECHNOLOGY, Philadelphia, PA, USA, ASTM F1537-11/ ISO 5832-12:2019).

Preparation of test samples

The test samples were prepared in accordance with the requirements of the ISO 10993-12:2021 standard. The initial material in the shape of rods with a diameter of 16 mm was cut into 3 mm thick disks, which were then ground and polished. The finished samples were washed and sonicated in 70% ethanol (15 min), then washed and sonicated twice in distilled water (15 min). Air-dried samples were sterilized with hot dry air at 180°C for 45 min using SRW 115 STD sterilizer (POL-EKO Apparatus, Wodzisław Śląski, Poland).

Biological materials

Primary cells: human osteoblasts isolated from the human femur (HO-f, Cat. No. 4610), and human chondrocytes isolated from human articular cartilage (HC-a, Cat. No. 4650) were provided by ScienCell Research Laboratories, San Diego, USA. Cancer cells: human osteosarcoma (Saos-2, Cat. No. ATCC HTB-85), and human chondrosarcoma (SW 1353, Cat. No. ATCC HTB-94) were from American Type Culture Collection, Manassas, VA, USA.

Chemicals

For the primary cell culture, the following chemicals were provided by ScienCell Research Laboratories, San Diego, USA: Fetal Bovine Serum (FBS), Trypsin/EDTA solution (T/E, 0.25%), Trypsin Neutralization Solution (TNS), Trypan Blue Solution (TB), Dulbecco's Phosphate-Buffered Saline (DPBS), Poly-L-Lysine (PLL), Chondrocyte Medium (CM), Chondrocyte Medium-phenol red free (CM-prf), Chondrocyte Growth Supplement (CGS), Penicillin/Streptomycin Solution (P/S), Osteoblast Medium (OM), Osteoblast Growth Supplement (OGS). For cancer cell lines, the following chemicals were provided by American Type Culture Collection, Manassas, VA, USA: Fetal Bovine Serum (FBS), Trypsin-EDTA Solution (T-E, 1X), Dulbecco's Phosphate Buffered Saline (D-PBS, 1X), Penicillin-Streptomycin Solution (P-S), McCoy's 5A Medium (MCM), Leibovitz's L-15 Medium (LBM). Apoptosis, DNA Damage, and Cell Proliferation Kit (Cat. No. 562253) was purchased from Becton Dickinson Biosciences, San Jose, CA, USA. This kit included: PerCP-Cy5.5 Mouse Anti-BrdU Antibody (Anti-BRdU), Alexa Fluor™ 647 Mouse Anti-H2AX Antibody (Anti-H2AX), PE Mouse Anti-Cleaved PARP Antibody (Anti C-PARP), BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution (Cytofix/Cytoperm), BD 10X Perm/Wash™ Buffer (Perm/Wash), BD Cytofix/Cytoperm™ Plus Permeabilization Buffer (Cytofix/Cytoperm Plus), BrdU (10 mg/ml), DNase (300 µl). Etoposide (Cat. No. E1383) and Hydrogen Peroxide Solution (30%, Cat. No. H1009) were from Sigma-Aldrich, Burlington, MA, USA. The remaining simple reagents were from Avantor Performance Materials Poland SA (formerly POCH), Gliwice, Poland.

Single-use plastics for cell culture

Tissue culture test plates (12 wells, Cat. No. 92012), tissue culture flasks (75 cm^2, Cat No. 90075) and syringe filter (22 µm, Cat. No. 99722) were from TPP Techno Plastic Products AG, Trasadingen, Switzerland. Sterile serological pipettes (5 mL and 10 mL), sterile conical tubes (15 mL and 50 mL), and sterile 1.5 mL microcentrifuge tubes were from SPL Life Sciences, Naechon-Myeon, Korea.

Cell culture

Primary human cells: osteoblasts HO-f and chondrocytes HC-a were cultured in standard tissue culture flasks coated with poly-L-lysine using osteoblast medium (OM) and chondrocyte medium (CM) supplemented with osteoblast growth supplement (OGS) and chondrocyte growth supplement (CGS), respectively. The media were also supplemented with 5% of foetal bovine serum (FBS) and 1% antibiotics cocktail penicillin/streptomycin solution. Breeding was carried out at 37°C and 5% CO_2 in the atmosphere.

Cancer human cells: osteosarcoma Saos-2 cells and chondrosarcoma SW 1353 cells were cultivated at 37°C in standard tissue culture flasks using McCoy's 5A medium (MCM) and Leibovitz's L-15 medium (LBM), respectively. Both media were supplemented with 15% or 10% of foetal bovine serum (FBS), respectively, and 1% of the antibiotic cocktail (P-S). Saos-2 cells required 5% CO_2 in the atmosphere, whereas SW 1353 cells were cultured in free gas exchange with atmospheric air.

All cells intended for study were passaged no less than six and no more than twelve times with media exchange every three days. The cells were subcultured to 95% confluency.

Course of the flow cytometry experiment

For the purpose of the experimental approach, we used a commercially available apoptosis, DNA damage, and cell proliferation kit that measures three distinct markers of cell state. The test uses immunofluorescence staining and is intended for use with a flow cytometer [22]. To track the vital activity of cells expressed by their proliferation, bromodeoxyuridine (BrdU) is used. BrdU is an analogue of the precursor of thymidine – a nucleotide included in DNA. Cell culture in the presence of BrdU results in the incorporation of this molecule into the newly synthesized DNA, and this fact can be detected using specific anti-BrdU antibodies fluorescently labelled with PerCP-Cy5.5. In turn, poly(ADP-ribose) polymerase (PARP) is used in this kit to monitor the apoptosis process. In the early stages of apoptosis, caspase-3 is activated, which cleaves the PARP molecule, resulting in the formation of an 89-k fragment specifically recognized by the anti-cleaved PARP antibody fluorescently labelled with phycoerythrin (PE). To monitor the activity of repairing damaged DNA, the histone protein H2AX is used, which is quickly phosphorylated on serine 139 when DNA strand breaks occur, which in turn leads to the recruitment of proteins repairing DNA damage. The use of anti-H2AX antibodies (pS139) labelled with the Alexa Fluor 467 fluorophore allows for very effective monitoring of the DNA damage process.

Samples of the tested metal alloys were placed, each separately, in 12-well tissue culture test plates and flooded with a culture medium appropriate for the given cells. After 30 min pre-incubation, cells at the density of $1x10^5$ per well were seeded into wells with tested samples. The culture was carried out at a temperature of 37°C and an atmosphere suitable for the given cells for a 48-hour time exposure.

The negative control were cells cultured under the same conditions, but without contact with the tested samples of metallic biomaterials, while the positive control were cells cultured under identical conditions as the negative control, but with the difference that after 24 h of culture, etoposide (a cytostatic substance) was added to a final concentration of 5 µg/mL, and the culture was continued for another 24 h. After incubation (for a total of 48 h), the cells were washed with warm DPBS or D-PBS, depending on the cell type. Cells were then harvested from the surface using a trypsin solution (T/E, 0.25%) or (T-E, 1X), depending on the cell type. The trypsinization process was terminated by adding TNS (in the case of primary cells), or complete culture medium with FBS (for cell lines), and the obtained cell suspension was washed in DPBS or D-PBS by centrifugation (200xg, 5 min, room temperature (RT)), and then the cell pellet was suspended in 50 µL of Cytofix/Cytoperm buffer and incubated on ice in the dark for 30 min. In the next step, the cell suspension was washed in 500 µL of tenfold diluted Perm/Wash buffer (200xg, 5 min, RT). The obtained pellet was resuspended in 50 µL of cytofix/cytoperm plus buffer and incubated on ice in the dark for 10 min. Cells were washed again with tenfold diluted Cytofix/Cytoperm Plus buffer (500 µL, 200xg, 5 min RT). The cell pellet was fixed again in 50 µL of Cytofix/Cytoperm buffer (5 min, 4°C), then washed with 500 µL of tenfold diluted Perm/Wash buffer (200xg, 5 min RT). The cell pellet was suspended in 50 µL of freshly prepared DNase solution, and the cells were incubated for an hour at 37°C. Cells were then washed in 500 µL tenfold diluted Perm/Wash buffer (200xg, 5 min. RT). The obtained cell pellet was resuspended in 10 µL of tenfold diluted Perm/Wash buffer, and then 2.5 µL of mouse anti-BrdU/PerCP, 2.5 µL anti-H2AX/Alexa Fluor 647 and 2.5 µL anti-cleaved PARP/PE antibodies were added and incubated in the dark for 20 min at room temperature. After labelling, cells were washed in 500 µL of tenfold diluted Perm/Wash buffer (200xg, 5 min, RT). The pellet was resuspended in 200 µL of the same buffer and immediately analysed on the Accuri C6 flow cytometer (Becton Dickinson, Biosciences, San Jose, CA, USA). Each time, 5,000 objects were read in the P1 gate for a population of single cells and the percentage of expression of a given marker as well as the mean and median fluorescence intensity in the FL2 (anti - C-PARP/PE), FL3 (anti - BrdU/PerCP) and FL4 (anti - H2AX/Alexa Fluor 647) channels were analysed.

Statistical considerations

The experiments for individual metallic biomaterials were repeated 3 to 11 times, and the results are presented as the mean and standard deviation of the mean (mean ± SD). The ANOVA test with Bonferroni correction of the *p*-values was applied for statistical analysis of the obtained results. Post-hoc analysis was conducted with the use of Tukey's test. It was assumed that if the value of statistical significance *p* was less than 0.05, then the results differ significantly. The significance levels are marked with asterisks as follows: * p<0.05, ** p<0.01, *** p<0.001.

Results

The results allow for statistical and comparative analysis of proliferation potential, apoptosis level and DNA damage repair activity in primary and neoplastic osteoblasts and chondrocytes subjected to direct contact with the tested metallic biomaterials (AISI316L medical steel, CoCrMo alloy and Ti6Al4V and Ti6Al7Nb titanium alloys) are presented below. Example results obtained for HO-f cells (primary osteoblasts) and for all types of tested samples and controls, presented in the form of dot plots, are shown in FIG. 1.

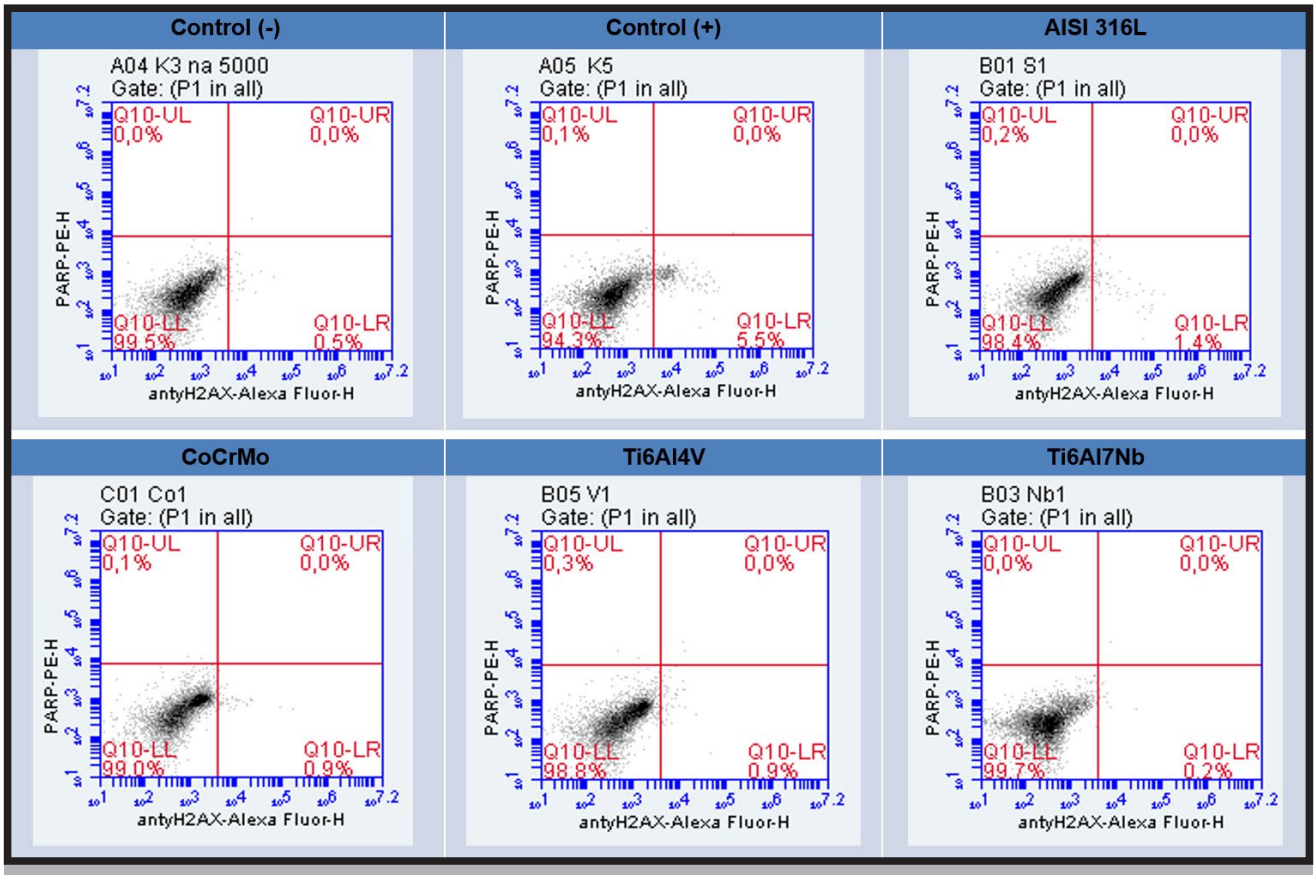

FIG. 1. Representative set of results fluorescence reading. The vertical axis shows the fluorescence intensity for C-PARP/PE antibodies, while the horizontal axis shows the fluorescence intensity of H2AX/Alexa Fluor antibodies. Control (-) and Control (+) are negative control cells and cells treated with etoposide, respectively.

Cell proliferation test

TABLE 1 contains the average values of fluorescence intensity read in the FL3 channel (anti-BrdU/PerCP) corresponding to the level of proliferation (% proliferation) in the tested cell population. The results apply to all types of cells used, i.e. osteoblasts and chondrocytes, both primary and neoplastic. For each type of cell and each type of biomaterial, mean values and standard deviations (mean ± SD) and the number of repetitions n of the experiment for a given cell-sample pair are provided. The last column and last row of the table show the levels of statistical significance of the differences in mean values, calculated using the ANOVA test, with the Bonferroni correction and Tukey's test. The entry "*** A-B" present in the first row of the last column of TABLE 1, regarding the negative control, should be read as a statistically significant difference at the level of $p<0.001$ between the mean values obtained for primary osteoblasts HO-f and neoplastic osteoblasts Saos-2, while the entries "** B-C" in the second row, and "* A-C" in the third row, should be read as statistically significant differences in mean values with a significance level of $p<0.01$ and $p<0.05$ for the pairs Saos-2 and HC-a, and HO-f and HC-a, respectively. In turn, the last row of the table contains information about the statistical significance of the differences between the average values for one cell line exposed to contact with different tested samples. For example, the entry "*** a-b", present in the column for Saos-2 cells, means statistical significance at $p<0.001$ level between the mean values obtained for the negative controls and positive controls.

Cell apoptosis test

TABLE 2 contains average values of the percentage of apoptotic cells in the population of all cells corresponding to the fluorescence intensity read in the FL2 channel (anti-C-PARP/PE). All results and the level of statistical significance of the differences are organized in the same way as in TABLE 1.

DNA damage test

TABLE 3 contains the average values of fluorescence intensity read in the FL4 channel (anti- BrdU/PerCP), corresponding to the level of activity of repairing damaged DNA (% DNA damage) in the population of tested cells. All results and the level of statistical significance of differences are organized in the same way as in TABLES 1 and 2.

Discussion

Over the past few decades, there has been growing number of research reporting the occurrence of neoplastic lesions temporally associated with implantation procedures [7-12]. While such incidents have been documented across various types of implants, a significant portion of the literature has focused on dental restorations, likely due to their widespread use. Among the cancers reported in association with dental implants, squamous cell carcinoma has emerged as one of the most frequently observed malignancies in the vicinity of these prostheses [23-25]. Similarly, there have been reports of malignancies following hip or knee arthroplasty involving endoprostheses [12,20,26-28], and also breast implant-associated malignancies [29-31]. Despite these observations, the underlying mechanisms and causal relationships between implantation and tumour development remain poorly understood and the pathogenesis is not yet explained. The problematic nature of this topic is further enhanced by the fact that many recent studies based on a meta-analysis of data obtained from large cohorts of patients indicate a lack of correlation between the risk of cancer and the implantation of a hip prosthesis during short-term follow-up (up to 5 years after the surgical procedure) [32] or after long-term follow-up (10 years after surgery) [33].

TABLE 1. Summary of the results of the values and fluorescence intensity read in the FL3 channel (anti- BrdU/PerCP) corresponding to the proliferation potential in the population of tested cells. The results were presented as the mean value and standard deviation of the mean (mean ± SD) of the percentage of the cell population in a state of cell division. The number of repetitions (n) of the experiment is also given. The levels of statistical significance are marked with asterisks as follows: * p<0.05, ** p<0.01, *** p<0.001, and the method of reading the level of statistical significance of differences between mean values is described in the text concerning TABLE 1. Control (-) and Control (+) are negative control cells and cells treated with etoposide, respectively.

	proliferation (%)				
Type of cells	osteoblasts		chondrocytes		
	A – HO-f primary	B – Saos-2 neoplastic	C – HC-a primary	D – SW1353 neoplastic	significance
a – Control (-)	0.89 ± 0.72 n = 9	23.20 ± 6.86 n = 7	3.50 ± 0.27 n = 5	5.45 ± 0.86 n = 3	*** A-B, B-C, B-D
b – Control(+)	0.73 ± 0.16 n = 7	1.23 ± 0.73 n = 9	0.40 ± 0.18 n = 9	0.40 ± 0.00 n = 3	** B-C
c – AISI316L	0.93 ± 0.36 n = 10	14.15 ± 7.60 n = 9	6.53 ± 0.23 n = 9	4.90 ± 1.43 n = 5	*** A-B; ** B-C, B-D; * A-C
d – CoCrMo	0.66 ± 0.34 n = 11	15.28 ± 8.22 n = 9	4.68 ± 0.78 n = 9	4.00 ± 1.52 n = 5	*** A-B, B-C; ** B-D
e – Ti6Al4V	1.15 ± 0.49 n = 11	19.15 ± 5.52 n = 9	5.55 ± 1.74 n = 9	1.45 ± 0.49 n = 5	** A-B; * B-D
f – Ti6Al7Nb	1.07 ± 0.62 n = 11	11.45 ± 9.28 n = 9	6.33 ± 0.93 n = 9	4.65 ± 0.67 n = 5	*** A-B
significance	ns	*** a-b, b-e ** b-d; *b-c	*** a-b, b-c, b-d, b-e, b-f; ** a-c, c-d; * a-e, a-f	* a-b, a-e, b-c, b-d, c-e, e-f	

TABLE 2. Summary of the results of fluorescence intensity values read in the FL2 channel (anti-C-PARP/PE) corresponding to the level of apoptosis in the population of tested cells. The results were presented as the mean value and standard deviation from the mean (mean ± SD) of the percentage of the cell population in the apoptotic state. The number of repetitions (n) of the experiment is also given. The levels of statistical significance are marked with asterisks as follows: * p<0.05, ** p<0.01, *** p<0.001, and the method of reading the level of statistical significance of differences between average values is described in the text concerning TABLE 1. Control (-) and Control (+) are negative control cells and cells treated with etoposide, respectively.

	apoptosis (%)				
Type of cells	osteoblasts		chondrocytes		
	A - HO-f primary	B - Saos-2 neoplastic	C - HC-a primary	D - SW1353 neoplastic	significance
a – Control (-)	0.57 ± 0.18 n = 9	1.60 ± 0.67 n = 7	5.00 ± 1.41 n = 3	0.30 ± 0.00 n = 3	*** A-C, B-C; ** C-D; * A-B
b – Control(+)	9.73 ± 11.18 n = 7	6.20 ± 1.69 n = 9	4.68 ± 4.23 n = 9	0.60 ± 0.00 n = 3	ns
c – AISI316L	2.44 ± 1.06 n = 10	2.24 ± 0,74 n = 8	2.97 ± 1.18 n = 5	1.40 ± 0.27 n = 5	ns
d – CoCrMo	2.54 ± 1.74 n = 11	3.38 ± 0.93 n = 9	4.59 ± 2.14 n = 5	0.15 ± 0.04 n = 3	* C-D
e – Ti6Al4V	3.00 ± 3.91 n = 11	3.75 ± 1.64 n = 9	3.56 ± 1.91 n = 5	0.45 ± 0.13 n = 5	ns
f – Ti6Al7Nb	5.16 ± 5.14 n = 11	2.28 ± 0.27 n = 7	2.66 ± 1.76 n = 5	0.35 ± 0.13 n = 5	ns
significance	* a-b	*** a-b, b-c, b-f; **b-d, b-e;	ns	***c-d, c-e, c-f; ** a-c, b-c	

TABLE 3. Summary of the results of the values and fluorescence intensity read in the FL4 channel (anti- BrdU/ PerCP) corresponding to the level of activity of repairing damaged DNA in the population of tested cells. The results were presented as the mean value and standard deviation of the mean (mean ± SD) the percentage of the cell population capable of repairing damaged DNA. The number of repetitions (n) of the experiment is also given. The levels of statistical significance are marked with asterisks as follows: * p<0.05, ** p<0.01, *** p<0.001, and the method of reading the level of statistical significance of differences between mean values is described in the text concerning TABLE 1. Control (-) and Control (+) are negative control cells and cells treated with etoposide, respectively.

	DNA damage (%)				
Type of cells	osteoblasts		chondrocytes		
	A - HO-f primary	B - Saos-2 neoplastic	C - HC-a primary	D - SW1353 neoplastic	significance
a – Control (-)	0.88 ± 0.73 n = 9	28.27 ± 2.21 n = 7	1.10 ± 0.80 n = 5	6.20 ± 0.34 n = 5	*** A-B, A-D, B-C, B-D; ** C-D
b – Control(+)	28.39 ± 21.55 n = 7	78.43 ± 11.12 n = 9	33.35 ± 13.82 n = 9	12.70 ± 0.00 n = 3	*** A-B, B-C, B-D
c – AISI316L	0.85 ± 0.16 n = 10	16.20 ± 7.58 n = 9	1.58 ± 1.29 n = 9	7.35 ± 0.85 n = 5	*** A-B, B-C, * B-D
d – CoCrMo	0.54 ± 0.15 n = 11	29.07 ± 4.84 n = 7	1.00 ± 0.69 n = 9	4.45 ± 0.04 n = 5	*** A-B, B-C, B-D; * A-D
e – Ti6Al4V	1.60 ± 1.29 n = 11	23.90 ± 5.62 n = 7	0.53 ± 0.36 n = 9	7.40 ± 1.16 n = 5	*** A-B, B-C, B-D; ** A-D, C-D
f – Ti6Al7Nb	0.83 ± 0.47 n = 11	17.18 ± 12.91 n = 9	0.55 ± 0.25 n = 9	7.15 ± 0.58 n = 5	*** A-B, B-C
significance	*** a-b, b-c, b-d, b-e, b-f	*** a-b, b-c, b-d, b-e, b-f	*** a-b, b-c, b-d, b-e, b-f	*** a-b, b-c, b-d, b-e, b-f; ** d-e, d-f	

Nonetheless, the complex interplay between host tissue responses, biomaterial properties, and the local microenvironment presents significant challenges in unravelling the molecular mechanisms causing potential tumour induction in implantation sites. Consequently, the problem of accelerated tumour induction in the vicinity of the implant remains a subject of ongoing investigation and debate within the scientific community.

Motivated by the need to address this knowledge gap, the primary aim of our study was to undertake a preliminary *in vitro* assessment of the potential carcinogenic risks associated with metallic biomaterials commonly employed in orthopaedic implants. Based on our recent publication investigating alterations in the expression profiles of tumour-promoting genes in chondrocytes exposed to metallic biomaterials [13], the present report attempts to provide a further comprehensive analysis of the cellular responses evoked by such biomaterials.

The process of cancer transformation is very complex and depends on numerous external factors [34], as well as on individual susceptibility to genetic damage and the accumulation of genetic changes [35]. Numerous metabolic and signalling pathways take an active part in the process of carcinogenesis [36]. Among them, it is worth mentioning an increased proliferation potential [37], altered activity of repairing damaged DNA [38], and a disturbed process of natural programmed cell death – apoptosis [17]. In this study, we decided to use a commercially available Apoptosis, DNA Damage, and Cell Proliferation Kit that allows for the assessment of the proliferation potential, level of apoptosis and DNA damage repair activity in a population of primary and neoplastic cells, including chondrocytes and osteoblasts, upon exposure to commonly used metallic biomaterials.

In our study, control cells (negative control) showed a statistically significant difference in the level of apoptosis between osteoblasts and chondrocytes, both primary and cancer. The addition of etoposide (positive control) resulted in a statistically significant increase in the level of apoptosis in osteoblasts, both primary and osteosarcoma, but not in chondrocytes (TABLE 1). No significant changes in the level of apoptosis resulting from contact with the tested samples were observed, with the exception of cancer chondrocytes, where contact with medical steel significantly increased apoptosis, both in relation to the negative control and in relation to the remaining tested samples, and even the positive control. *In vitro* studies investigating the direct effects of medical steel implants on apoptosis in cartilage cells (chondrocytes) are rather limited. However, some evidence suggests that materials used in orthopaedic implants, including medical steel, and their wear debris, can induce cellular responses that may lead to apoptosis under certain conditions [39-41]. Apoptosis is a natural and necessary process crucial for the development, growth, and maintenance of cartilage, as it helps shape tissues and remove damaged or aged cells. However, excessive apoptosis can lead to cartilage degradation, impair the tissue's ability to self-repair, and contribute to degenerative diseases like osteoarthritis [42]. Our observations indicate that while medical steel does not induce significant apoptosis in normal chondrocytes, it can cause a significant increase in apoptosis in cancerous chondrocytes even in short-term exposure (48 h). In the study of Li et al., the authors also observed an increased level of apoptosis in HUVEC cells after exposure to different stainless steel substrates that correlated with a notable up-regulated expression of pro-apoptotic genes [39]. In another study, the authors highlighted that the imbalance between proliferation and apoptosis can lead to hypercellular neointima formation after stent implantation which is a risk for restenosis [43]. In the case of cancerous cells, the increased level of apoptosis caused by medical steel is rather beneficial as evading apoptosis is one of the hallmarks of cancer. Our preliminary observations need further research to explain why medical steel stimulated apoptosis only in neoplastic chondrocytes but not in osteosarcoma.

In our study, we observed that all tested cells of different origin (negative control) had statistically significant differences in terms of apoptosis, proliferation potential and DNA damage (TABLES 1-3), while the addition of etoposide significantly reduced the proliferation potential in cancer cells, while in all cells it caused a very strong increase in DNA damage (TABLES 1 and 3). This increase in DNA damage and apoptosis and the reduction in proliferation is due to the properties of etoposide, which is a cytostatic substance used clinically in the treatment of cancer, which causes DNA damage and acts actively in the interphase of the cell cycle [44]. It is also worth noting that control Saos-2 cells (osteosarcoma) were characterized by a very high proliferation potential compared to other cells. This observation is consistent with the aggressive nature of osteosarcoma cells, which are characterized by rapid proliferation and resistance to apoptosis, contributing to their malignancy and difficulty in treatment.

In terms of tested biomaterials, medical steel and titanium alloys enhanced proliferation in primary chondrocytes, but vanadium alloy decreased proliferation in neoplastic chondrocytes (TABLE 1). The increased proliferation of primary chondrocytes on the surface may have both potential benefits and associated risks. Increased proliferation of primary chondrocytes may promote cartilage regeneration, which is desirable in the cases of damage or during healing processes after surgery, and may also contribute to better integration of the implant with surrounding tissues, which increases the stability and functionality of the implant. On the other hand, excessive proliferation can lead to uncontrolled tissue hypertrophy, which can lead to complications [42]. Attention should also be paid to the effect of the CoCrMo alloy on a significant reduction in the level of apoptosis in neoplastic chondrocytes when compared to primary cells (TABLE 2). In terms of DNA damage, in our study, none of the metallic samples caused an increased percentage of cells with disturbance in DNA, but in each case of contact with metallic biomaterials, cancer cells showed significantly higher activity in repairing damaged DNA compared to primary cells (TABLE 3).

Our findings are preliminary and need further investigation, but they underscore the importance of monitoring cellular responses to implant materials. Still, *in vitro* evaluations do not cover all aspects connected with pathological processes that can lead to cancer initiation, and also *in vivo* observations are not fully understood [45]. Explaining the molecular mechanisms underlying changes in apoptosis, proliferation potential and DNA damage in cells near implants can aid in developing strategies to mitigate risks connected with cancer formation.

Conclusions

The obtained results allow for the thesis that short-term (48 h) direct exposure to metallic biomaterials of osteoblasts and chondrocytes, both primary and cancerous, may cause significant changes in cell physiology, which may result in promoting the cancer process. However, our research, although of significant pioneering importance, allows us to draw only very limited conclusions about the inertness of metallic biomaterials due to the short observation period compared to the time of exposure of tissues of people permanently using implants, which is still a challenge for modern implantology [46].

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