

Cytotoxicity of nickel ions for human osteoblasts in the context of orthodontic treatment in humans and animals

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Abstract: In the present work, the biocompatibility of orthodontic devices was tested by the propagation of human osteoblasts. A different methodological approach was applied. Since the materials used in human and veterinary orthodontics (not in implantology) had been tested, osteoblasts were not implanted on the surface of the biomaterial, but have been cultured in the medium containing the products of corrosion. In the present work, osteoblasts were propagated in the medium spiked with Ni(II) salt versus the control. Osteoblast cells (live, dead, and total) were counted under light microscope. The statistical significance of the differences between the experimental and control groups were evaluated ($P < 0.05$). Based on the number of live, dead, and total cells, the viability and proliferation were determined. A slight cytotoxic effect of Ni(II) ions at the studied concentration was confirmed. The mean viability was 7% lower in the experimental versus the control group (the difference was statistically significant), while the change of proliferation was not significant.

Key words: Nickel(II), cytotoxicity, osteoblasts, orthodontic appliance

1. Introduction

Cytotoxicity tests are undertaken in the investigation of the biocompatibility of biomaterials used in human and veterinary medicine to evaluate the possibility of toxic substance release. The selection of the type of cells for cytotoxicity assessments is dependent on the type of tissue the biomaterial is in contact with under *in vivo* conditions. Therefore, cytotoxicity tests on the osteoblasts (OBs) and fibroblasts are carried out to evaluate the biocompatibility of orthodontic implants or orthodontic appliances (1).

There is a need to elaborate on an experimental methodology of cytocompatibility testing of the materials used in medicine: the applied concentration of metal ions and the type of exposure, and culturing cells directly on the material or in media containing the products of its corrosion. In the case of implanted alloys, such as dental or hip implants, culturing on the surface seems to be more rational. However, cell cultivation in model media with a precisely estimated concentration of metal ions in preliminary experiments, e.g., in artificial saliva/

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biomaterial, seems more appropriate in cytotoxicity tests for dental materials. This is probably a more suitable method for the evaluation of cytotoxic effects of the materials of which orthodontic fixed appliances are made.

The orthodontic treatment of a human patient's existing malocclusion with the application of fixed appliances has been very popular since the late 1970s. Usually, a fixed appliance consists of a number of elements such as brackets, bands, wires, and ligatures. Most of those elements are manufactured from stainless steel, nickel-titanium, and titanium alloys (2–6). It is already clear that veterinary dentistry is one of the most interesting and important areas for veterinary clinicians (7). Similar techniques and materials to those used for humans are currently widely used in veterinary medicine in orthodontic treatment in dogs (8–11). Many metals such as nickel, chromium, and cadmium, which are the components of alloys, have been recognized as carcinogenic, cytotoxic, and sensitizing (12–16). Because a human treatment time with a fixed appliance lasts for

around 2 years, a question arises of whether metal ions are released in toxic doses in the corrosion process. It must be underlined that the oral cavity environment enhances the corrosion process (17,18). In dogs, the procedure is much shorter, but the corrosion process should be taken into consideration as well (11,19). Several experiments have been undertaken that investigated the quantity of metal ions released from orthodontic appliances under in vitro conditions (different media such as artificial saliva, sodium chloride, and organic acids). However, different materials and methodological approaches made the outcomes hard to compare. Authors in many papers have underlined that the element of major concern was nickel (2,20–22). Moreover, there have been several cell culture experiments described (different cell types such as human OBs, human OB-like cells, and animal OBs), in which the evaluations of the potential cytotoxicity of the chosen metal ions have been undertaken (23–28).

Although nickel is considered as a microelement, it is, on the other hand, also a toxic metal and its beneficial function in an organism has not yet been clearly explained. The majority of papers report the results of studies on OBs cultivated directly on the biomaterial surfaces. In the available literature, only Cortizo et al. (25) investigated OB culture in the presence of the products of corrosion of dental biomaterials. Moreover, in the present work, OBs were incubated in medium containing a concentration of nickel ions, as is done in metal release experiments conducted in the solution of artificial saliva. Such a procedure probably more faithfully reflects the conditions whereby orthodontic appliances are present in the oral cavity environment.

The aim of the present work was to evaluate the cytotoxicity of nickel ions on human OBs. The concentration of metal ions in the model media used in the present study was obtained from the results of preliminary experiments in which a whole orthodontic appliance was incubated in a solution of artificial saliva (6). The concentration of nickel and chromium ions was 573 µg/L and 101 µg/L, respectively. The concentrations of other metal ions, released from the stainless steel, were at very low levels. Based on the comparison with the maximum recommended daily doses for humans, nickel was selected as the metal released in potentially toxic doses and, consequently, these ions were chosen for toxicological studies on OBs. The concentration of metal ions that underwent dissolution as the result of the corrosion process was used in the simulated model medium to investigate the cytotoxicity of nickel ions released from the appliance.

2. Materials and methods

Two series of experiments were performed: the control and experimental groups (each series was repeated 3 times). OBs were cultivated in the medium without (control) and with (experimental) nickel ions. The concentration of nickel ions was 0.01 mmol/L, as estimated previously in metal release experiments in which an orthodontic appliance was incubated in artificial saliva.

2.1. Evaluated materials

The evaluated materials were new parts of an orthodontic appliance, all manufactured from stainless steel: wires, braces, bands, and metal ligatures. The following elements were investigated: wires (American Orthodontics, catalog no. 853 – 224) with a slot size of 0.04318 × 0.0635 cm, bands (3M Unitek) of size 36.5, and brackets (Gemini APC II, 3M Unitek). Metal ligatures (American Orthodontics) were used to ligate the wires to the brackets. The simulated orthodontic appliance consisted of 20 brackets, 4 bands, 2 wires, and 20 metal ligatures. The appliances fulfilled the desired proportion of the quantity of elements. The average elemental composition of the appliance, determined using a JEOL JSM-5800 scanning electron microscope, was as follows: Fe, 71.23%; Cr, 19.40%; Ni, 8.12%; Si, 1.11%; and Mn, 0.13%.

2.2. Cell culture

The human OB cell line (HOB-c), purchased from PromoCell GmbH, was propagated in 75-cm² culture flasks (Nunc) in PromoCell OB growth medium containing supplement mix with 3% glutamine (Sigma-Aldrich), penicillin (104 U/mL), and streptomycin (10 mg/mL) in 5% CO₂ at 37 °C.

The medium was changed every 2 days. Cells were harvested by enzymatic digestion with trypsin for approximately 10 min. After digestion, the trypsin was neutralized with PromoCell Trypsin Neutralization Solution. The total amount, the number of dead cells, and the number of live cells of the harvested cells were determined under light microscope. The cells were treated with Trypan Blue and counted in a Bürker chamber. The viability was defined as the ratio of the number of live cells to the total cell number, while proliferation was defined as the ratio of the total number at the end of the cell culture to the number of the initial amount of cells at the beginning of the propagation.

The toxic influence of the nickel ions on HOB-c was determined in the culture in the presence of nickel salt NiCl₂•6H₂O (Sigma-Aldrich) after a 24-h preincubation. The cells were cultivated in a 24-well cell culture plate (Nunc). The initial concentration of the HOB-c was 0.375 × 10⁶/2 mL of specific medium, per well. The concentration of Ni(II) ions was 0.01 mmol/L. The cells were harvested and counted after 1, 2, 3, and 7 days of propagation. Cells in the control group were cultured under the same conditions, but without nickel salt.

2.3. Statistical methods

Statistical elaboration of the results was computed with Statistica 9.0 software. Descriptive statistics (mean values, standard deviation) were reported. For the assessment of significance of differences between the control and experimental groups, the Wilcoxon matched pairs test was used. Statistical significance level was assumed as $P < 0.05$.

3. Results

The results of the cell culture experiments are presented in Tables 1 and 2 and Figures 1–4 for live cells, dead cells, total number of cells, and viability and proliferation, respectively.

The number of live cells was evaluated (Figure 1). The average decrease of the number of live cells in the culture in the presence of Ni(II) was 10% (Table 2). The difference between the pairs of results (control and experimental) of the parallel series were evaluated using the Wilcoxon matched pairs test. The statistical significance of the differences was proven ($P = 0.0329$).

Figure 2 reports the number of dead cells over time. The Wilcoxon matched pairs test showed statistically significant differences between the pairs of results in the control and experimental groups. The average difference between the means was 18%. It can be concluded that the

number of dead cells exposed to the Ni(II) ions increased significantly. This showed the cytotoxicity of the ions at a given concentration.

Table 1 reports the total number of cells (live and dead) during the experiment. In the case of the total number of cells, statistical significance of the differences was not found; the average difference between the means was evaluated as 3% (Table 2), $P = 0.508$. The lack of significant differences in the overall number of cells between the groups was probably related to there not being an increase in the cell counts during the experiment. The duration of the test was 7 days, and an increase of cell numbers in both groups (control and experimental) was not observed.

Figure 3 reports the change in viability of OB cells over time. Viability was expressed as the ratio of live cells to the total number of cells (live and dead). The number of live cells in the experimental culture was 10% lower compared with the control; however, the number of dead cells was 18% higher, respectively. When adding data in the denominator (total number of cells), the increase of the value in the experimental group with relation to the control is obtained, while the value of the denominator increases. Such a definition of viability as a measure of the cytotoxicity of the nickel ions reflects the change of the ratio between the number of dead cells and all of the

Table 1. Total cell count in the OB culture: control and experimental (Ni).

Time (days)	Control					Ni				
	I	II	III	Mean	SD	I	II	III	Mean	SD
0	375,000	375,000	375,000	375,000	0	375,000	375,000	375,000	375,000	0
1	400,000	477,776	457,556	445,111	40,354	400,000	377,776	355,955	377,910	22,023
2	377,778	388,880	333,733	366,797	29,167	377,778	355,550	344,844	359,391	16,800
3	355,556	366,660	388,789	370,335	16,919	355,555	344,440	355,955	351,983	65,36
7	377,778	355,553	333,733	355,688	22,023	422,223	377,776	411,511	403,837	23,196

Table 2. Ratio between mean cell count (live, dead, and total number), viability, and proliferation.

Time (days)	Mean - control/mean - Ni				
	Live	Dead	Total	Viability	Proliferation
1	1.26	0.25	1.18	1.07	0.85
2	1.08	0.00	1.02	1.05	0.98
3	1.11	0.29	1.05	1.06	0.95
7	0.96	0.18	0.88	1.09	1.14
Mean	1.10	0.18	1.03	1.07	0.98

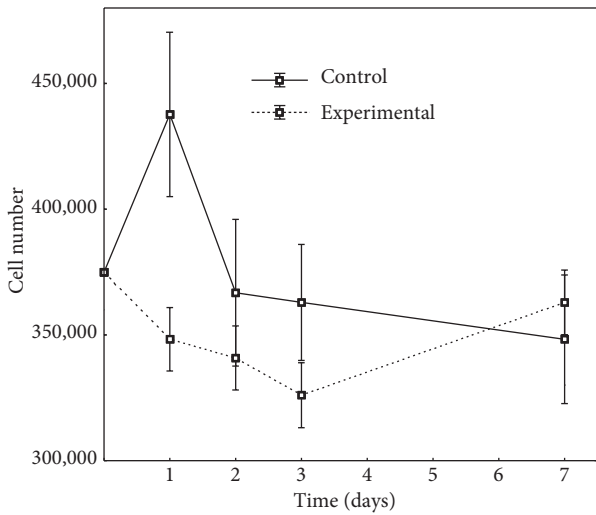


Figure 1. Live cell number in the OB culture: mean, □; line, mean ± SD.

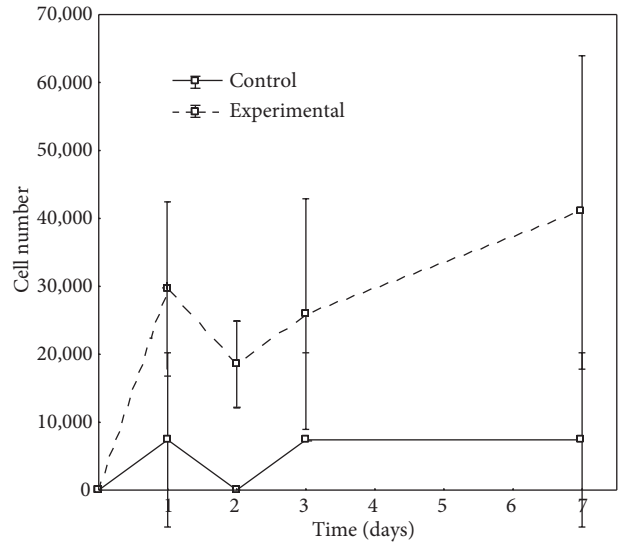


Figure 2. Dead cell number in the OB culture: mean, □; Line, mean ± SD.

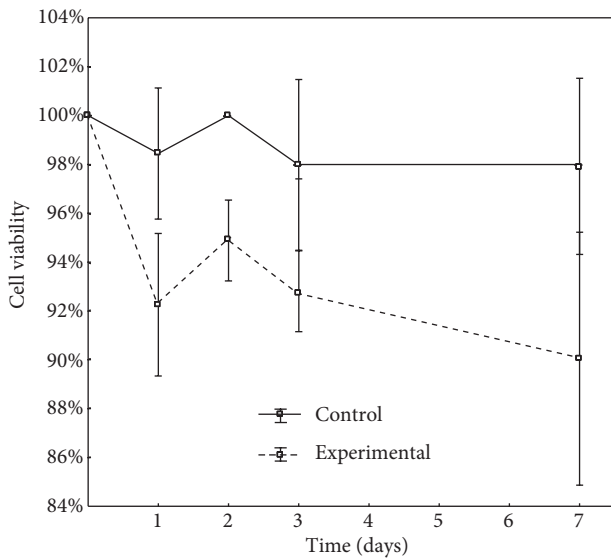


Figure 3. Cell viability of human OBs: mean, □; line, SD.

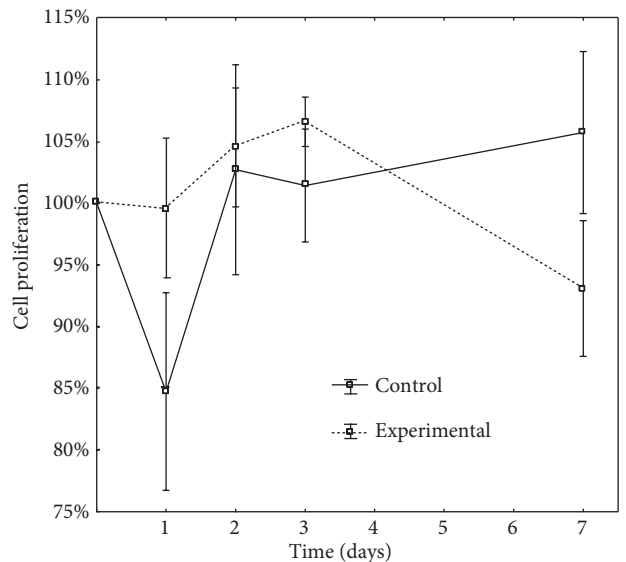


Figure 4. Cell proliferation of human OBs: mean, □; line, SD.

cells, independently of the kinetics of cell proliferation. Therefore, viability seems to be the proper measure in the assessment of the toxic effect of given concentrations of metal ions in OB culture. Consequently, the viability of the cells in the presence of nickel ions versus the control decreased by 7% (Table 2). The difference was statistically significant by the Wilcoxon matched pairs test, $P = 0.00604$.

On the other hand, the assessment of proliferation did not yield unique results for viability (Figure 4). Proliferation was defined as the ratio between the initial cell count and the number of cells during the culture.

Therefore, numerical values in the formula of this measure for the control and experimental groups are the same in the numerator (initial cell count). The denominator signifies the total number, which, as is shown above, did not change during the culture (over time) and did not differ statistically significantly between the groups. Because the total number of cells did not increase in the experimental as well as in the control group, the interpretation of the results of proliferation did not provide any information about the cytotoxicity of the nickel ions. Such information could be obtained in the case of assuring conditions (if

possible), in which the measurable dynamics of OB growth would be reached. Similarly, as in the literature, in the present paper a statistically significant difference for cell proliferation between the experimental and the control groups was also not found; the rate between the average proliferation for the 2 groups was 2% (Table 2). The result was not statistically significant (Wilcoxon matched pairs test).

4. Discussion

Scientific papers have described many examples of investigations of human OB cell culture experiments regarding the materials (stainless steel) used in medicine and veterinary medicine (7,10,29–32). Because of their great importance in human medicine, human OBs are usually used in scientific investigations. In our opinion, the results on human OBs can be important for veterinary medicine, as well. The orthodontic and implantological techniques in veterinary medicine in the United States and West and Central Europe are important and similar. Only in 1 of the analyzed studies (29) was the viability of human OBs investigated, which was evaluated as 93.7% after 14 days of incubation. A similar result (90% after 7 days of incubation) was obtained in the present work. In other papers (30,31), the objective was the evaluation of cell proliferation. The following results (compared with the control) were obtained: 104.7% (30) and 109% (31). In the present paper, similar results were reached: 105.7% in the control and 93.07% in the experimental group. Hao et al. (32) also assessed the proliferation of human OBs. The aim was the assessment of the evaluation of the effect of the surface modification of the material (stainless steel) by CO₂ laser. In the results, the authors reported a significant increase of cell proliferation as the consequence of the modification of an alloy surface. Although the authors reported the results of proliferation for unmodified stainless steel, the control group was not included.

The results of experiments on human OB-like cells are coherent with the above assessed viability and proliferation of cells (24,33,34). The results were as follows: 95% and 102%, respectively. It seems better justified to use viability rather than a proliferation test to evaluate the cytotoxicity of alloys in studies on OBs. In 2 other papers (24,33), no disadvantageous effect of alloys on cell proliferation was found.

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On the other hand, the results of studies on animal OBs were not so unequivocal, perhaps because OBs derived from different species were used (mice, rats, and other murines). Those studies were carried out with the use of either OBs or OB-like cells (35).

In all of these works, the cells were cultivated directly on the surface of the evaluated materials. Similarly, as in the present paper, Morais et al. (36), in the preliminary stage of their study, assessed the concentration of metal ions (Ni, Cr, and Fe) in the solutions in which the materials were first incubated. Subsequently, the cell culture was undertaken in the medium containing ions of metals in the concentrations evaluated previously.

In the present work, cytotoxicity tests on human OBs were carried out to investigate the biocompatibility of the stainless steel elements of orthodontic appliances. Under in vivo conditions, OBs are not in direct contact with the stainless steel, but are in contact with the products of its corrosion. Therefore, in the first stage, the appliances were incubated in a solution of artificial saliva, and subsequently the OBs were propagated in a medium containing a level of toxic metals, as in metal release experiments. The results of this study can be important for both human and veterinary stomatology.

It seems that a methodological approach in which OBs are cultivated on the surface of materials in order to evaluate cytotoxicity is proper for the investigation of the biocompatibility of orthopedic or reconstructive materials, due to the direct contact of the material with cells in a human organism. In the case of the assessment of the biocompatibility of the materials used in orthodontics, the culture of OB cells in model media containing the products of corrosion based on simulated, previously determined levels is more justified.

This study has shown that orthodontic treatment with appliances made of stainless steel can lead to exposure to nickel on a cytotoxic level. This arose from the decrease of the viability of osteoblastic cells in the presence of Ni(II) ions. However, proliferation was not affected. These conclusions are coherent with other papers and point out the methodology of future studies on cytotoxicity.

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