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In Vitro Evaluation of Genotoxicity of a Commercial Polyaxial Pedicle Screw for Spine Surgery

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ABSTRACT

Biocompatibility, which shows the compatibility between the host and the biomaterial, is very important for the reliability of a biomaterial. It is a must for a newly produced biomaterial to meet the biocompatibility criteria, which are bound to certain rules by international organizations. One of the criteria of biocompatibility is genotoxicity. In this study, it was aimed to evaluate the genotoxicity of a commercial polyaxial pedicle screw *in vitro*. For this purpose, *in vitro* micronucleus test and bacterial reverse mutation test were performed. Extraction method was used for both tests. When the obtained results were compared statistically, it was concluded that the tested biomaterial was not genotoxic.

INTRODUCTION

Biocompatibility is a multidimensional concept and refers to the physical, chemical, and biological compatibility of a biomaterial with its host. Biocompatibility is an indicator of the host-material interaction. It refers to the optimum adaptation of a biomaterial to the mechanics and physiology of the host tissue. To explain it in more detail, biocompatibility can be defined as "the functionality of a material in the medical treatment, that is, its suitability for the targeted purpose, having the most appropriate cellular or tissue interaction for the host's condition, without causing any local or systemic side effects on the host, and having optimum clinical performance in that treatment" (Williams, 2008). Therefore, biocompatibility tests are a must for the development and approval of materials for clinical use, and biomaterials must meet the biocompatibility criteria determined by the International Standards Organization (ISO 10993-1, 2018).

One of the criteria for biocompatibility is genotoxicity, and the evaluation of genotoxicity is addressed in ISO 10993 section 3 (2018). Genotoxicity is a term that affects the genetic material (DNA) in the cell and covers various changes in DNA (DNA breaks, gene mutations, chromosome abnormalities, etc.) (Mohamed et al., 2017). Cells have some mechanisms to prevent genetic damage, but in case of genetic damage, they can prevent it from being transmitted to future generations by apoptosis. The degradation of genetic material in the cell can induce carcinogenesis, and damage to germ cell DNA can negatively affect reproduction or cause hereditary mutations (Elshahawy, 2011; Huzum et al., 2021). Genotoxicity tests are designed to evaluate two important endpoints: gene mutations and chromosomal damage.

Because there is no single mechanism that includes the pathway of action of genotoxic substances and they may act through various genotoxic mechanisms. Therefore, a standard genotoxicity assessment requires at least one *in vitro* test on mammalian cells and one *in vitro* test on bacteria to be designed (Assad and Jackson, 2019).

Spine diseases, which are problem that can reduce people's quality of life, are medical problems that are tried to be solved with various tools and medical applications in parallel with the advancement of biomedical applications and the development of medical techniques. The extension of the average life expectancy by medical technology has caused an increase in the activities of the older generation, and the increase in activity has led to an increase in lumbar stenosis, disc herniation and degenerative spine diseases in these individuals. In young people, similar spine diseases occur in people with high activity or posture disorders (Kwon et al., 2020). Individuals suffering from various spine diseases are usually treated using surgical techniques such as spinal decompression or spinal fusion. Pedicle screws are also one of the biomaterials used in spinal surgeries (Albanese et al., 2017). When associated with the purpose of use, it is inevitable for pedicle screws to remain in the patient's body for a long time. In such a case, although it is an inert metallic material, its long-term stay in the body brings the living tissue-material interaction to the fore. Continuous contact of living tissues with the surface of metal implants can lead to slow but continuous release of metal ions and accumulation in surrounding tissues, which can lead to toxicity, carcinogenicity, or delayed-type hypersensitivity reactions, leading to failure of the implant material (Gotman, 1997; Latka et al., 2024). In ISO 10993 part 1 (2018), the biomaterial contact location and duration in the patient are taken into account in the assessment of biocompatibility. Devices with longer patient contact and/or a more invasive contact area are classified in a high-risk category. Accordingly, genotoxicity assessment is not required for all medical devices (ISO 10993-1, 2018). However, it is important to evaluate the genotoxicity of biomaterials that have long-term patient contact, such as pedicle screws.

This study aimed to evaluate the genotoxicity of a commercial polyaxial pedicle screw *in vitro*.

MATERIALS AND METHODS

In this study, *in vitro* micronucleus test and bacterial reverse mutation test (Ames) were used to evaluate genotoxicity. A commercial polyaxial pedicle screw brought to Kırıkkale University Scientific and Technological Research Laboratory for testing was used in the tests.

In vitro micronucleus test

In vitro micronucleus test was performed by ISO 10993-3 and OECD 487 standards. The specified Chinese Hamster ovary epithelium (CHO) cell line specified in the standard was used as the cell line. (CHO-K1/An1, 95122902, Foot and Mouth Institute). The extraction method was used in testing the sample. The extraction process was performed by the ISO 10993-12 standard. The obtained extract was applied to the cells. The application was done both in the presence and absence of metabolic activation. First, 15x103 cells were seeded in 48-well well plates. The cells were left for incubation (37°C, 5% CO2) for 24 hours. At the end of incubation, the medium in the wells were discarded and three different concentrations of the prepared sample extract were applied as 1:1, 1:2, 1:4. For short application (3-6 hours), application was performed in the presence and absence of metabolic activation (medium containing 2% S9 enzyme), and for long application (24 hours) only in the absence of metabolic activation. For all applications, the test was performed in the presence of Cytochalasin B (3 µg/ml). For positive control, Mitomycin C was used in the absence of metabolic activation in short application, cyclophosphamide was used in the presence of metabolic activation, and colchicine was used in long application. A fresh medium was used for negative control. At the end of the application periods, the medium in the wells were discarded and 75 mM KCl was dropped into each well. Then, methanol: glacial acetic acid (3:1) was added to fixation the cells. Finally, the cells were stained with propodium iodide and mononuclear, binuclear and multinuclear cells were counted under a fluorescence microscope. Binucleated cells containing micronuclei were counted to determine the micronucleus ratio. Then, the Cytokinesis Block Proliferation Index (CBPI), % Cytostasis and % micronucleus ratio were calculated as specified in the standard.

Bacterial reverse mutation test (Ames)

The experiment was carried out under the guidance of the OECD 471 standard. For the experiment, Salmonella typhimurium (S. typhimurium) TA97a, S. typhimurium TA98, S. typhimurium TA100, S. typhimurium TA102 and S. typhimurium TA1535 strains recommended in OECD 471 standard were used. The strains were incubated in 30 ml nutrient broth in 250 ml erlenmeyer flasks for 10-15 hours at 37ºC. After incubation, measurements were made at 600 nm on a spectrophotometer, and the experiment was started by measuring absorbance values as 0.08-0.1. For the experiment, 5 different concentrations of the sample extracted for 3 days at 37ºC according to the recommendations of OECD 471 and ISO 10993-12 standards were used (1/1, 1/2, 1/4, 1/8, and 1/16). Each concentration and solvent control, negative and positive controls were tested in 3 replicates. For each replicate; 2 ml of histidine-biotin supplemented semi-melted (at 43-48ºC) top agar was transferred to the tubes and 0.1 ml of the sample concentration to be tested/solvent/positive control solution (mutagen)/negative control solution (phosphate buffer or sterile distilled water); 0.1 ml of the bacterial strain with determined concentrations and 0.5 ml of S9 mix and phosphate buffer instead of S9 mix for the 2nd series were added and vortexed and spread on minimal glucose agar. After the agar solidified, the petri dishes were turned upside down and incubated at 37ºC for 2-3 days. After incubation, the number of colonies in all petri dishes was determined and statistical calculations were made.

Statistical analysis

Student t-test was used in comparisons between groups, the difference was considered statistically significant when $p<0.05$.

RESULTS

In vitro micronucleus test results

The calculations made as a result of the in vitro micronucleus test are shown in Table 1 and Table 2. Additionally, photographs representing cell counts of the test groups are shown in Figure 1. As a result of the calculations, in the statistical comparisons of the negative

control and sample extracts with different concentrations in terms of *in vitro* micronucleus ratios, it was seen that the difference between the negative control and sample extracts was not statistically significant. In contrast, the difference between the positive control and sample extracts was statistically significant (Table 3). Therefore, when the results of the *in vitro* micronucleus test were evaluated, it was concluded that the tested sample was not genotoxic.

Table 1. Cell and micronucleus numbers of the short application

	Cell numbers and calculations					
	BN	**MN**	**MNC**	**Total cell count**	**CBPI**	**% Cyt**
Sample extract (1/1)	511	7	115	1111	1.66	6.33
Sample extract (1/2)	507	6	112	1064	1.68	3.51
Sample extract (1/4)	522	6	138	1151	1.69	2.63
Negative	512	5	157	1160	1.71	
Positive	500	68	117	1252	1.58	17.66

BN; Number of binucleate cells, MN; Number of micronucleus in binucleated cells, MNC; Number of multinucleated cells, CBPI; Cytokinesis-Block Proliferation Index, Cyt; cytostasis rate.

Table 2. Cell and micronucleus numbers of the long application

	Cell numbers and calculations					
	BN	**MN**	**MNC**	**Total cell count**	**CBPI**	**% Cyt**
Sample extract (1/1)	505	8	125	1177	1.64	9.41
Sample extract (1/2)	510	6	118	1115	1.66	5.51
Sample extract (1/4)	519	6	133	1145	1.68	3.18
Negative	503	5	167	1182	1.70	
Positive	506	74	107	1298	1.55	21.66

BN; Number of binucleate cells, MN; Number of micronucleus in binucleated cells, MNC; Number of multinucleated cells, CBPI; Cytokinesis-Block Proliferation Index, Cyt; cytostasis rate.

Table 3. Statistical comparison of the calculated micronucleus ratios (%) in the *in vitro* micronucleus test

	Micronucleus rates (%) short application	**Micronucleus rates (%) long applicaion**
Sample extract (1/1)	1.36[a]	1.58[a]
Sample extract (1/2)	1.18[a]	1.18[a]
Sample extract (1/4)	1.15[a]	1.16[a]
Negative control	0.98[a]	0.99[a]
Positive control	13.6[b]	14.62[b]

[a,b] Different superscripts in the same column indicate statistically differences (p<0,05).

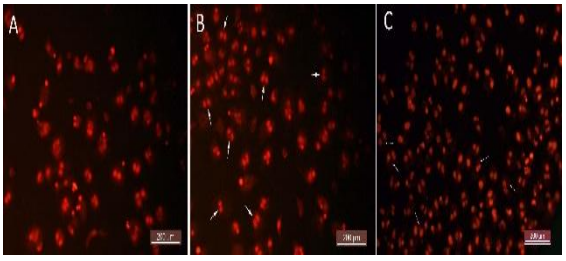

Figure 1. Representative photographs of test groups. A) negative control, B) sample extract applied, C) positive control. In B, arrows indicate binucleated cells, which lack micronuclei. In C, arrows indicate micronuclei in inucleated cells. Propidium iodide stain. Scale bar: 200 µm

Bacterial reverse mutation test (Ames) results

The application was carried out in 3 repetitions for each concentration. Negative and positive controls and sterility control petri dishes were interpreted. No incompatibility was found in positive and negative controls. Sterility control was successful. As a result of the Ames test scoring, no statistically significant genotoxic effect was determined for the dilutions of the sample subjected to the test (Table 4).

Table 4. Statistical comparison of Ames test results

Bacterial strains	With S9 mixture p value	Without S9 mixture p value	Result
S. typhimurium TA97a	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA98	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA100	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA102	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA1535	p>0.05	p>0.05	Not significant (All concentrations)

DISCUSSION AND CONCLUSION

Genotoxins cause DNA damage and can disrupt chromosomal structure in various ways. Efforts are made to prevent potential risks through genotoxicity studies. Because genotoxicity studies give an idea about whether a drug or medical material causes mutation or genotoxicity and tell us whether a developed material or drug is dangerous at an early stage.

In this study, it was concluded that the tested product has no genotoxic potential. These tests, which are performed in terms of chromosome damage and gene mutations, which are two important points for genotoxicity, can be attributed to the natural non-genotoxic structure of the produced biomaterial and the quality control measures in the production process. The absence of evidence that could cause genetic damage as a result of both *in vitro* cell culture and bacteria tests indicates the reliability of the tested material. However, if it is considered that it will interact and contact with the tissue for a long time, the genotoxicity of the wear particles may need to be evaluated (George et al., 2023). On the other hand, the evaluation of the contact surface, which plays a key role in the biomaterial-tissue interaction, and the determination of the concentrations of metal ions in body fluids such as blood and interstitial fluid are valuable in terms of evaluating the genotoxicity of the new biomaterials produced and predicting potential risks. In the study by Schliephake et al., (1993), high metal concentrations were observed in the lungs 5 months after the placement of metallic implant material. These accumulations prove that particles from the implant surface to erode and be transported hematogenous to distant regions. The biological interactions of such accumulations, even in non-toxic concentrations, should not be ignored (Ribeiro et al., 2007). Studies have also shown, for example, that exposure to nickel compounds is associated with various cancers, and similarly, the possible carcinogenic effects of cobalt or cobalt compounds (Merk and Speit, 1999).

As a result, it was concluded that the polyaxial pedicle screw tested in this study did not have a genotoxic effect. However, the possible negative effects of host-material interaction in the long term should not be ignored and it should not be forgotten that additional tests may always be needed for the reliability of biomaterials.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship contributions

Concept: Y.Ö., N.A.Ç., Design: Y.Ö., N.A.Ç., Data Collection or Processing: Y.Ö., N.A.Ç., Analysis or Interpretation: Y.Ö., N.A.Ç., Literature Search: Y.Ö, Writing: Y.Ö.

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