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Cytotoxicity and Mutagenicity of N2 Cement – Root Canal Filling Material

Cytotoksyczność i mutagenność cementu N2 – materiału do wypełniania kanałów korzeniowych

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Abstract

Background. Biocompatibility can be described as the ability of a material to function so as not to violate cell and tissue integrity when applied.
Objectives. The contemporary requirements for biocompatible materials evaluated according to "evidence-based medicine" induced the authors to evaluate the mutagenicity and cytotoxicity of dental materials (medical devices) authorized for sale in the EU. The use of some endodontic materials is questioned by many specialists.
Material and Methods. The Ames test was used to evaluate the mutagenicity of the most controversial endodontic cement, N2, according to the PN-EN ISO 10993-3:2008 norm. The cytotoxicity of this material was evaluated according to the PN-EN ISO 10993-5:2008 norm.
Results: The tested material did not show any mutagenic activity, but it was severely cytotoxic in the concentration range of 0.78–200 mg/ml.
Conclusions. In view of the broad availability of safe and effective alternatives, the use of paraformaldehyde-containing "aged" filling materials or root canal sealers is undoubtedly below the standard of care for endodontic treatment (**Adv Clin Exp Med 2009, 18, 6, 615–621**).

Key words: endodontics, root canal sealer/filling material, N2 cement, cytotoxicity, mutagenicity, Ames test.

Streszczenie

Wprowadzenie. Biokompatybilność można zdefiniować jako zdolność materiału, przy jego specyficznym zastosowaniu, do funkcjonowania w sposób nienaruszający zgodności tkankowej, tj. braku negatywnego, np. cytotoksycznego, wpływu na tkanki.
Cel pracy. Współczesne wymogi stosowania materiałów biozgodnych, ocenianych według kanonów *evidence based dentistry* skłaniają do prowadzenia rutynowych badań również mutagennego i cytotoksycznego oddziaływania materiałów stomatologicznych dostępnych na rynku UE. Zastosowanie niektórych materiałów w leczeniu kanałowym bywa kwestionowane wśród stomatologów.
Materiał i metody. Do oceny mutagenności najbardziej kontrowersyjnego z tego typu materiałów – pasty N2 wykorzystano referencyjny test Amesa, a ocenę cytotoksyczności przeprowadzono na podstawie metodyki normy PN-EN ISO 10993-5:2008.
Wyniki. Materiał N2 nie wykazał aktywności mutagennej, a w zakresie stężeń 0.78–200 mg/ml cechował się ostrą cytotoksycznością.
Wnioski. Ze względu na szeroką dostępność bezpiecznych i sprawdzonych klinicznie materiałów endodontycznych, zastosowanie uwalniających formaldehyd „historycznych" produktów do wypełniania lub uszczelniania kanałów korzeniowych odbiega od standardów leczenia stomatologicznego (**Adv Clin Exp Med 2009, 18, 6, 615–621**).

Słowa kluczowe: endodoncja, materiał do uszczelniania/wypełniania kanałów korzeniowych, cement N2, mutagenność, cytotoksyczność, test Amesa.

The root canal system is the hollow area within each tooth that normally encloses the pulp, i.e. the living part of the tooth that contains blood vessels, nerves, and connective tissue. If the pulp becomes diseased or injured, root canal therapy is required to save the tooth. It is performed by removing the pulp and filling (obturating) the canal with a proper substitute material. Most cases require one or two visits, after which the dentist places some kind of a restoration on the tooth to protect and restore its function. Thorough cleansing and shaping of the root canal system and placing a hermetic seal are the bases of success in root canal therapy. Although obturation may not necessarily be the most critical stage, it should be carried out to the highest clinical standards [1, 2].

Gutta-percha is a predictable and "user-friendly" material that has been used in dentistry for over 160 years. This naturally occurring polymer, altered by the addition of zinc oxide and other substances, undoubtedly requires the use of an efficient additional paste, i.e. a sealer. Root canal sealers work as lubricants for gutta-percha cones and adhesives for gutta-percha and dentine and assist in filling irregularities in canal walls and fill additional canals [1, 3].

In the early 1950s, the Swiss dentist Angelo Sargenti began filling roots with just a paste that contained paraformaldehyde. Over the years, the formula's name and ingredients changed many times, but paraformaldehyde has always been included. The names used have included "Sargenti Paste", "N2", "N2 Normal", "N2 Medical", "N2 Universal", "N2 Apical", "RC-2B", "RC-2W", "TCM", "White One-Step Endodontic Formula", and "Endodilato". His followers claim that the paste, commonly referred to as N2, is easier and faster to place than gutta-percha and a sealer. However, when paraformaldehyde comes in contact with water, it forms formaldehyde. Sargenti's procedure may save a tooth, but it seems to be much less predictable than standard treatment. The pressure needed to reach the apex of the root can force the paste into the surrounding tissues, where it can cause serious and painful injury [2, 4–7]. The FDA has banned the interstate sale of Sargenti-type pastes, but pharmacists can legally prepare them for local use [9].

In 2004, when Poland joined the EU, N2 became officially available on the domestic market, being distributed by Hager Werken (Germany) and marked CE 0434. The material has never been tested at the National Institute of Medicines during the process of Marketing Authorization or any other studies. The availability of this material on the EU market came to the authors as a surprise. The cytotoxic, mutagenic, and carcinogenic potential of formaldehyde as well as its systemic effects have been demonstrated *in vitro* and *in vivo* [10–14].

The aim of biocompatibility testing is simulation of the biological response to the material placed in a direct contact with cells or tissue. Cell cultures provide a convenient, controllable, and reproducible instrument for the preliminary evaluation of biological response. It is worth noting that many endodontic materials show some cytotoxic properties [15–20]. The testing of mutagenicity and carcinogenicity, due to their extremely serious consequences, are gaining increasing public interest [6, 19]. In this study the authors evaluated the cytotoxicity and mutagenicity of N2 cement, a controversial root canal filling material authorized for distribution on the EU market.

Material and Methods

Tested Material

Endodontic Cement N2® is a root canal filling paste containing 50 mg of paraformaldehyde in 1 g of material. An extract of freshly prepared N2® material in 0.9% sodium chloride was prepared before each experiment and incubated in 37°C for 24 hours before testing.

Bacteria

Salmonella typhimurium strains: TA97, TA98, TA100, and TA102 were used. These are alimentary mutants requiring the presence of histidine and biotin for growth. The bacterial strains' characteristics, i.e. spontaneous reversion level, ampicillin resistance (R plasmid presence), crystal violet sensitivity (rfa mutation), and UV sensitivity (uvrB mutation), were checked before the study according to the procedure of Maron and Ames [21].

Cells

The L929 line (cells of the mouse connective tissue neoplasm C3H/AN) was from the American Type Culture Collection (ATCC). The cells were cultured as a monolayer in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere at 37 C and 5% CO_2. The cell culture was mycoplasma free.

Media

The media for the bacterial cultures were liquid broth medium (Nutrient Broth Oxoid), minimal agar medium (Bacto Agar Difco with Vogel

and Bonner salt and 40% glucose), and semi-liquid top agar (Bacto Agar; Difco). The media for the cell cultures were MEM medium, fetal bovine serum, and trypsin 0.5% + EDTA (Gibco) and PBS without calcium and magnesium ions (IITD, Wrocław) and antimycotic antibiotic (Sigma).

Controls

Positive controls (without metabolic activation) were 4-nitro-1,2-phenyldiamine (NPD) (Merck) at a concentration of 20 μg/plate for strains TA97 and TA98, sodium azide (Sigma) at a concentration of 1.5 μg/plate for strain TA100, and methyl methanesulfonate (MMS) at a concentration of 1.0 μg/plate for strain TA102. Positive controls (with metabolic activation) with 2-aminofluorene (2AF) (Fluka) at a concentration of 10 μg/ /plate for strains TA97, TA98, and TA100 and 9-aminoacridine (Sigma) at a concentration of 50 μg/plate for strain TA102.

S9 Mix Fraction

The mutagenic properties of many chemical compounds are revealed after metabolic activation. In the Ames test, the studied compound and bacterial cells were incubated in the presence of the S9 fraction (MP Biochemicals, Inc.), containing enzymes which are able to induce biochemical metabolism of the substance.

Cytotoxicity Test

The evaluation of cytotoxicity of N2 Endodontic Cement was performed on the basis of the norm PN-EN ISO 10993-5:2008. The samples were prepared according to the producer's instructions. The liquid and powder parts of the N2 cement were mixed to obtain a paste which, after setting, was placed in culture medium at 37 C for 24 hours. According to PN-EN ISO 10993-12:2008, the final concentration of the extract was 0.2 g of paste per 1 ml of culture medium. Further dilutions of the extract were also prepared using culture medium. The cytotoxicity test was carried out using four-well cell culture plates independently for every tested concentration to prevent the penetration of toxic formaldehyde vapors from the N2 material. The cytotoxic effect induced by the N2 Endodontic Cement was evaluated visually by microscopic observation using a cytotoxicity scale according to PN-EN ISO 10993-5:2008, i.e. 0 (none): discrete intracytoplasmic granules, no cell lysis; 1 (slight): not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells present; 2 (mild): not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells; 3 (moderate): not more than 70% of the cell layers contain rounded cells or are lysed; and 4 (severe): nearly complete destruction of the cell layers.

Ames Test

The mutagenic action of N2 Endodontic Cement was evaluated on the basis of the reference Ames test. The test was performed according to the procedure proposed by Maron and Ames [21] and PN-EN ISO 10993-3:2008. The test consists of evaluating the ability of the studied compound to induce reverse mutation in cells of auxotrophic *Salmonella typhimurium* strains (TA97, TA98, TA100, and TA102). The bacterial cells in which reverse mutation occurs show the ability to reproduce and form colonies on minimal medium lacking growth factor (histidine).

The test strains were cultured in liquid broth medium for 18 h in a water bath at 37°C under agitation. After incubation, 0.1 ml of bacterial culture and 0.1 ml of the studied compound were added to 2 ml of semi-liquid superficial agar containing 0.05 mM biotin and 0.05 mM histidine and poured onto the plate with minimal agar medium. For tests with metabolic activation, 0.5 ml of the S9 mix fraction was added. After 48–72 h of incubation at 37°C, His+ revertant colonies were counted on the plates. To confirm the validity of the test in each experiment, parallel positive controls were performed.

The results of the test are shown as the mean number of colonies (from two experiments done in triplicate) of histidine-independent revertants obtained for every tested concentration. The mutagenic action of a substance is confirmed by demonstrating a dose-response relationship between the concentration of the substance and the number of revertant colonies. According to the generally accepted procedure regarding this test, a substance in a concentration which causes a doubling of the number of His+ revertants in relation to the spontaneous reversion level is defined as mutagenic. A substance in a concentration which causes a gradual reduction or lack of bacterial growth is defined as toxic.

Results

The results of the cytotoxicity testing of N2 Endodontic Cement are presented in Table 1 and Figure 1. On the basis of the results it was found that the extract of the material in concentrations of

Table 1. Evaluation of the cytotoxic effect of N2 cement extract on L929 cell cultures

Tabela 1. Ocena działania cytotoksycznego wyciągu N2 w hodowlach komórek L929

Concentration of N2 (Stężenie wyciągu z N2) mg/ml	Cytotoxicity evaluation* (Ocena toksyczności*)	
	well with N2 extract	adjancent well**
200	3	3 3
100	3	3
50	3	3
25	2/3	3
12.5	2/3	0
6.25	3	0
3.13	3	0
1.56	3	0
0.78	3	0
0.39	1	0
0.185	0/1	0
0.0975	0/1	0
0.0488	0	0
0.0244	0	0
0.0122	0	0

* cytotoxic evaluation performed acc. to PN-EN ISO 10993-5:2008.
** adjacent well with cell cultures without the tested substance.

* ocena cytotoksyczności przeprowadzona zgodnie z PN-EN ISO 10993-5:2008.
** sąsiadujące studzienki z hodowlami kontrolnymi bez substancji badanej.

0.0975–200 mg/ml was cytotoxic against L929 cells. At the concentration range of 0.78–200 mg/ml, cell damage was observed indicating moderate cytotoxicity (degree 3 according to the above cytotoxicity scale). At the concentration range of 0.0975–0.39 mg/ml, the tested substance produced slight cytotoxicity (degree 1 and 0/1). At the concentration range of 25–200 mg/ml, a severe cytotoxic effect against cells in the neighboring wells was also observed. No cytotoxic effect was observed in the succeeding wells. It should be assumed that this effect was due to formaldehyde vapor release from the tested substance and its toxic action against the neighboring control cell cultures.

The Ames test evaluating the potential mutagenic activity of the extract of N2 root sealing material showed that the four *Salmonella typhimurium* strains used in the test differed with respect to the type of mutation in the histidine gene, which results in different susceptibilities to the action of chemical mutagenesis and enables the determination of a compound mutagenic action evaluated as the induction of specific bacterial DNA changes. On the basis of the results obtained in the cytotoxicity test, the Ames test was performed using extract at concentrations of 0.1–0.4 mg/plate, which were assumed to be non-cytotoxic (1 and 0/1 degree of cytotoxicity). The results of the Ames test with and without metabolic activation are shown in Tables 2 and 3. The results indicate that the N2 root sealing material extract at the tested concentrations did not increase the number of revertants of *Salmonella typhimurium* strains both with and without metabolic activation. The number of revertants in the tested concentrations corresponded to the number of revertants in the control cultures.

Endodontic cement N2 exhibited cytotoxic activity against L929 cells at the concentration range of 0.0975–200 mg/ml. Its vapors contain formaldehyde, respectively from 25 to 200 mg/ml. This allows one to qualify the tested material to level 3, i.e. acutely cytotoxic, in the range of 0.78–200 mg/ml and mildly cytotoxic at concentrations of 0.0975–0.39 mg/ml. There was no cytotoxic effect on L929 cells at the lowest concentrations (0.0122–0.0488 mg/ml).

The results of Ames test showed that N2 is not genotoxic in the range of the tested concentrations (0.1–0.4 mg/ml). There was no relationship between the number of His^+ revertants and the rise in concentration of the tested substance. Doubling the number of His^+ revertants in relation to spontaneous reversion was not observed.

Discussion

Medical Devices Directive 93/42/EEC requires that companies wishing to sell medical devices within the EU must meet the requirements under the directive. In the case of endodontic materials, they should be submitted to biological testing according to EN ISO 10993-5 and EN ISO 10993-3. Especially worthwhile is evaluation of the materials with host DNA. The basic tests are cytotoxicity and genotoxicity.

In general, the toxicity of a root canal filling material is assessed using a three-step approach. The first step is to screen the candidate material using a series of *in vitro* cytotoxicity and genotoxicity assays. Then, if the material is deemed non-toxic *in vitro,* it can be implanted in subcutaneous tissue or muscle and the local reaction evaluated. Finally, the *in vivo* reaction of the target tissue vs. the test material must be evaluated in animals, then

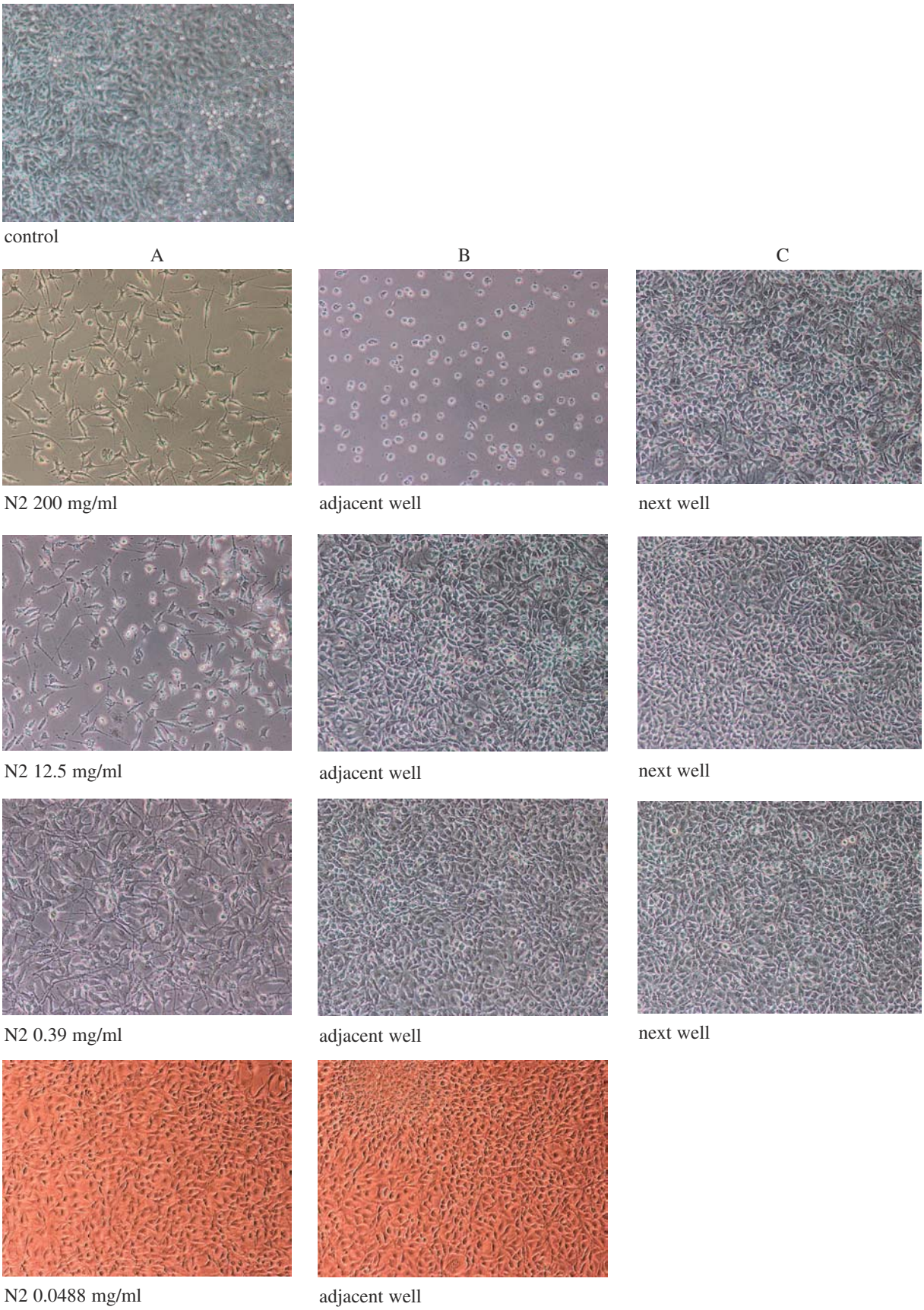

Fig. 1. Photographs (selected) of L929 cells after N2 extracts exposure: A) cells in cultures containing N2 cement extract at the concentration range from 0.0488 mg/ml to 200 mg/ml; B) control cell cultures without the tested substance (adjacent wells); C) control cell cultures without the tested substance (next wells)

Ryc. 1. Obraz mikroskopowy (zdjęcia wybrane) komórek L929 w hodowlach z wyciągiem N2: A) hodowle zawierające wyciąg N2 w stężeniu 0,0488–200 mg/ml; B) obraz hodowli kontrolnych w studzienkach sąsiadujących ze studzienkami zawierającymi wyciąg N2; C) hodowle bez badanego materiału (kolejna studzienka)

Table 2. The influence of N2 cement extract (without the metabolic fraction S9) on the number of His+ reverants of *Salmonella typhimurium*

Tabela 2. Wpływ wyciągu N2 (bez frakcji metabolicznej S9) na liczbę rewertantów his+ *Salmonella typhimurium*

Strain (Szczep)	Concentration of NS extract in mg/plate (Stężenie wyciągu N2 w mg na płytkę)			
	0	0.1	0.2	0.4
TA97	101 ± 8	80 ± 6	76 ± 12	80 ± 7
TA98	34 ± 1	43 ± 5	32 ± 3	29 ± 3
TA100	116 ± 13	107 ± 9	116 ± 16	111 ± 15
TA102	318 ± 32	326 ± 46	314 ± 33	325 ± 25

Table 3. The influence of N2 cement extract (with the metabolic fraction S9) on the number of His+ reverants of *Salmonella typhimurium*

Tabela 3. Wpływ wyciągu N2 (z frakcją metaboliczną) na liczbę rewertantów his+ *Salmonella typhimurium*

Strain (Szczep)	Concentration of NS extract in mg/plate (Stężenie wyciągu N2 w mg na płytkę)			
	0	0.1	0.2	0.4
TA97	86 ± 5	105 ± 30	87 ± 18	86 ± 8
TA98	28 ± 5	24 ± 4	27 ± 7	32 ± 6
TA100	170 ± 53	145 ± 35	126 ± 23	146 ± 18
TA102	308 ± 67	349 ± 64	309 ± 67	326 ± 42

human subjects. [22]. The present study represents the first step of toxicity evaluation. The results of the cytotoxicity testing of N2 agree with previous studies and demonstrated its strong cytotoxicity [10, 11, 14, 19]. It is safe to say that if a material consistently induces a strong cytotoxic reaction in cell culture, it is very like to exert cytotoxicity in living tissue.

Genotoxicity/mutagenicity and carcinogenicity are important factors affecting the systemic compatibility of an endodontic material. In general, genotoxicity means the presence of a DNA-reactive component which may result in mutagenicity and carcinogenicity. Due to their serious and life-threatening consequences, mutagenicity and carcinogenicity are gaining increasing public interest.

The present results did not show a relationship between the amount of His+ revertants on the plate and increasing concentration of the tested substance. Duplication of His+ revertants with reference to spontaneous reversion was not found. The results showed no mutagenic activity of the tested material *in vitro*. It is worth noting that some kind of different outcome of the above test was expected. It must be considered that N2 cement has a strong cytotoxic activity and releases formaldehyde. Non-mutagenic materials presenting strong cytotoxic properties could induced tumors via indirect mechanisms. Permanent irritation of the oral tissue caused by formaldehyde may be seen as an additional risk factor and should be eliminated. These results confirm the opinion that a bacterial test system might not be the only basis for the assessment of the DNA-damaging activity of such a material. Therefore a combination of a bacterial and a eukaryotic test is necessary to gain more reliable results with respect to genotoxicity [19, 22, 23].

Extensive scientific research has proven unequivocally that paraformaldehyde-containing filling materials and sealers can cause irreversible damage to tissues near the root canal system, including destruction of connective tissue and bone, intractable pain, paresthesia and dysthesia of the mandibular and maxillary nerves, and chronic infections of the maxillary sinus [2, 6, 14, 17]. Moreover, scientific evidence has demonstrated that damage from paraformaldehyde-containing filling materials and sealers is not necessarily confined to tissues near the root canal. The active ingredients of these filling materials and sealers have been found to travel throughout the body and have been shown to infiltrate the blood, lymph nodes, adrenal glands, kidney, spleen, liver, and brain [8].

The present authors believe it is their responsibility to investigate *in vitro* and *in vivo* whether

current endodontic materials do or do not have the potential to generate systemic diseases or chronic local adverse effects over time. It is obvious that these important data are of great significance for the development of new biocompatible materials and thus for the future of "safe" dentistry.

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