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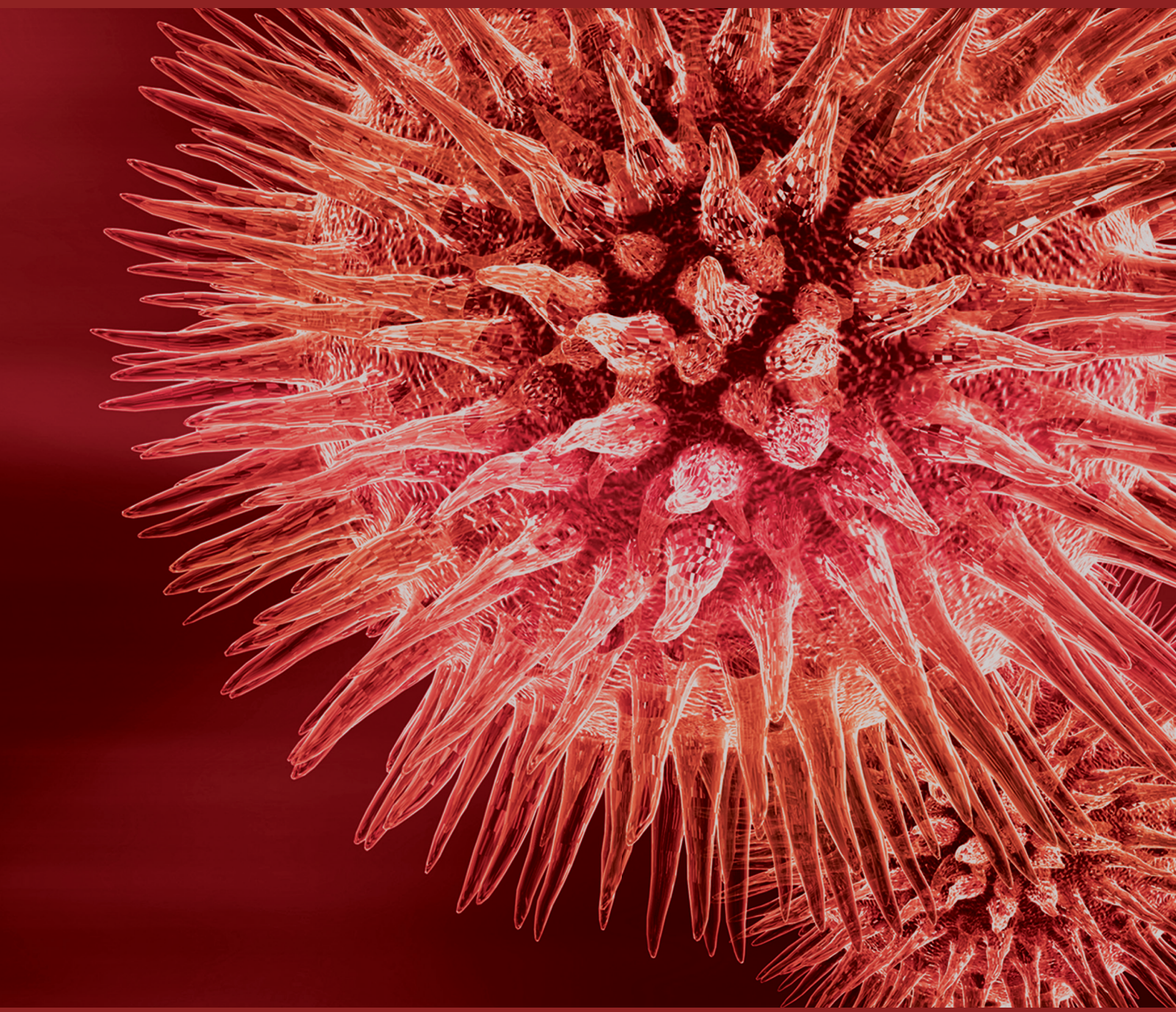
Trace Elements in Living Systems: From Beneficial to Toxic Effects

Guest Editors: Marcin Mikulewicz, Katarzyna Chojnacka, Beata Kawala, and Tomasz Gredes



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Editorial

Trace Elements in Living Systems: From Beneficial to Toxic Effects

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There are two faces of trace elements: beneficial and toxic. Trace elements although present in trace quantities can play an essential role in living organisms; for instance, the ions are frequently bound to the active sites of enzymes. For different branches of science, trace elements definition is diverse, for example, in living organisms or in soil [1]. In some cases the trace elements cover at the same time microelements and toxic elements. The role depends primarily on dose or concentration.

Living organisms are exposed to trace elements from different sources, for example, diet, environment (water, soil, and air), or biomaterials. Depending on the route of exposure (e.g., oral, dermal, or inhalation), the effect related with a given dose would be different. Also chemical form is significant: for example, the fact that Cr(III) is micronutrient versus the fact that Cr(VI) is carcinogenic and mutagenic agent. This multiplicity of roles and effects causes large variability of different topics and issues related with trace elements. There are several techniques to evaluate the potential toxicity of trace elements in living systems: in vitro and in vivo, from laboratory elution tests, through molecular biology trials, animal studies, and ending with human usage tests.

On the other hand, trace elements can be also considered nutritional elements, micronutrients. They perform many important functions in living organisms. Deficiency of those nutrients is called "hidden hunger" [2]. For this reason, fertilizers, feed, and food products should supply the required dose of microelements. Particularly designed formulations are elaborated and new strategies of feed and food biofortification are being implemented [3].

The effect of trace metals on living organisms can be investigated directly by using various biomonitoring techniques, for example, hair mineral analysis [4, 5] or analysis of other noninvasive matrices (urine and saliva) [6]. In some cases using invasive matrices seems essential, for example, blood.

In this special issue there are papers that explore various concepts related with advantages and disadvantages of the presence of trace elements in living organisms. On one hand trace elements are nutritive; on the other hand in excessive dose they can pose toxic effects. There are several sources from which living organisms can be exposed to trace elements: diet, environment, and biomaterials [5]. As a result, it is important to assess the exposure to trace elements by in vitro and in vivo approach.

Therefore, in this special issue, the subjects discussed are widespread. Some papers concern trace elements in agriculture, some others concern exposure of living organisms from the environment, and other papers concern aspects related with biomaterials containing trace elements.

Biomaterials are widely used in medicine because of their desired properties. They have found a wide variety of applications: from hip prostheses, orthodontic appliances, prosthetic restorations, implants, and metallic plates (fracture repair) to

surgical screws. Some of those materials are inserted for short period of time and others for a lifetime. Depending on the time of exposure and released doses, side effects related with insertion of a given biomaterial are different and determine its biocompatibility.

Biocompatibility is defined as the ability of a material to function in a specific application in the presence of an appropriate host response. Biocompatibility is assessed by appropriate test methods: in vitro and in vivo [7, 8]. In vitro methods are cost-effective and efficient [9] but do not fully reflect the real conditions in living systems. On the other hand, in vivo methods should be ethically accepted. For instance, determination of biosafety is important through assessment of the release of metal ions during orthodontic treatment [10].

It is known that there is no fully biocompatible material. Each alloy interacts with surrounding tissues, which may result in the release of trace element ions from metal alloys of biomaterials. An example of alloys used in biomaterials is orthodontic appliances.

Contemporary orthodontic treatment is based mainly on fixed orthodontic appliances. The main elements of those appliances (brackets, wires, and bands) are manufactured from metallic alloys such as stainless steel, nickel-titanium, and TMA (titanium-molybdenum alloy). Those alloys can be a source of exposure of metal ions, among others, Ni, Cr, and Cd, which have been proven to be mutagenic, cytotoxic, and allergenic [11]. Because a greater part of patients who undergo orthodontic treatment are children and teenagers previously mentioned issues are so important.

It has to be underlined that environment of oral cavity favors metal ion release from parts of orthodontic appliance. Saliva (pH), temperature, mechanical stress and/or damage, and bacterial colonization are all factors that initiate various types of corrosion processes (including deterioration) [12]. The other issue is the condition of enamel surface after debonding of the brackets/bands. Use of proper (advocated) technique and burs can reduce the surface damage.

The Guest Editors do hope that the present special issue would be interesting to investigators working in different branches of science related with trace elements and studying their both essential and nonessential or even toxic roles in living systems would find some useful information.

Marcin Mikulewicz
Katarzyna Chojnacka
Beata Kawala
Tomasz Gredes

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Research Article

Alterations of Hair and Nail Content of Selected Trace Elements in Nonoccupationally Exposed Patients with Chronic Depression from Different Geographical Regions

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The aim of this study was to determine if altered levels of selected trace elements manifest themselves during chronic depression. To identify elements strongly associated with chronic depression, relationships between the elemental contents of hair and nails and the interelement correlations were checked. Inductively coupled plasma mass spectrometry and ion chromatography were used to evaluate the contents of Zn, Cu, Co, Pb, Mn, and Fe in hair and nail samples from a total of 415 subjects (295 patients and 120 healthy volunteers). The study included logistic regression models to predict the probability of chronic depression. To investigate possible intercorrelations among the studied elements, the scaled principal component analysis was used. The research has revealed differences in TE levels in the group of depressed men and women in comparison to the healthy subjects. Statistically significant differences in both hair and nails contents of several elements were observed. Our study also provides strong evidence that the intermediary metabolism of certain elements is age- and gender-dependent. Zn, Mn, Pb, and Fe contents in hair/nails seem to be strongly associated with chronic depression. We found no statistically significant residence-related differences in the contents of studied elements in nonoccupationally exposed patients and healthy subjects.

1. Introduction

Depression is a very common condition in the general population, and it has serious health implications. According to the World Health Organization (WHO), 350 million people worldwide are affected by depression [1] and major depressive episodes may be foreshadowed by periods of dysthymia (chronic, mild depression) [2]. Patients with depression report a variety of symptoms that interfere with their day-to-day lives. Individuals suffering from depression will commonly lose interest in the activities which they would normally enjoy. Depressed individuals also lose their appetite, become sleepless, often suffer low self-esteem, and have difficulty in concentrating. Serious clinical depression may lead to a social withdrawal and self-neglect [3]. The cumulative, repeated episodes of depression may also contribute to developing some sort of inflammatory process in the body [4]. Depression is regarded as a multifactorial disorder with many causes [5]. Genetic, neurological, hormonal, immunological, and neuroendocrinological disorders have

all been implicated as important depression mechanisms. Gender and developmental factors can further alter these etiological factors [6].

Recently, numerous studies have provided valuable information regarding the involvement of essential chemical elements in psychiatric disorders [7, 8]. According to certain preclinical and clinical studies, such elements as zinc, magnesium, lithium, iron, calcium, and chromium are involved in the development of depression. It has been reported that supplementation of a low dose of an antidepressant with a specific chemical element can reduce unwanted side effects in different types of depression [9]. Although the exact mechanism of such an action is not fully understood, some chemical elements, such as magnesium, have long been successfully used as a supplement in the treatment of this condition [10]. The role of other chemical elements in depression is even less understood, although their importance in the physiology and pathology of the nervous system is indisputable [11]. Dysregulation of specific elements in the body often coincides with neurodegenerative and neuropsychiatric disorders. Frequently, this disruption in "normal" element content affects more than one element at a time, suggesting that the imbalance of one element must somehow affect that of others, upsetting the normal homeostasis [12]. Research findings indicate the warranted need of searching for trace elements (TE) biomarkers and mutual relationships between elements and etiology of a disease [13–15].

Analysis of TE in human tissues belongs to the challenges of today's analytical and clinical chemistry [16]. Such analysis is especially useful for quick detection of overall nutritional status and deficiency/toxicity studies. Hair and nail samples have been used frequently for the assessment of environmental and occupational metal exposure [17]. Nails provide an alternative sample medium. Both types of samples collected in a noninvasive way can serve as sources of valuable information about human metabolism. Validated procedures for the determination of elements in hair and nails are very scarce [18]. Due to the lack of standardised sample preparation procedures of hair/nail samples [19], there are still analytical problems that significantly impact the findings, which can lead to skewed results in publications and problems with comparisons between different studies [20]. Additionally, washing procedures used in hair elemental analysis can have significant effect on the internal elemental signal levels in hair [21].

Inductively coupled plasma mass spectrometry (ICP-MS) is the most versatile technique for determination of trace elements [22, 23]. Ion chromatography (IC) is an intensively developing technique for simultaneous determination of ions in samples of complex matrices [24].

The primary objective of this study was to evaluate the content of zinc, copper, manganese, iron, cobalt, and lead using optimised sample preparation method and two validated analytical methods (i.e., ICP-MS and IC). Both essential and toxic TE contents were evaluated in patients diagnosed with depressive disorder and compared with healthy subjects. We aimed to determine whether TE levels could be factors that significantly influence the risk for the development of depression in humans. An attempt was made to identify relationships between element content in hair and nails and interelement correlations to identify the elements and matrices that can be further used for the biological monitoring of depression. We also aimed to compare the content of selected trace elements in the samples taken from two populations coming from different geographical regions, that is, from Taiwan and from Poland.

Although many papers exist on toxic metals and microelements in human health and disease, studies on elements and patients suffering from persistent depressive disorder are sparse. Our study provides new data on toxic and trace elements that are associated with depressive disorders. We present here the studies replicated in two independent laboratories, what is more, with the use of two different analytical techniques, involving patients with the same disorder but coming from different geographical locations.

2. Materials and Methods

2.1. Patients and Samples. Our studies have been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study protocol was reviewed and approved by the Ethics Committee of the Medical University of Lublin, Room 128, Al. Racławickie 1, 20-059 Lublin, Poland, and Homu Clinic, No. 61, Dongping Rd., East Dist., Tainan City 701, Taiwan (KE-0254/293/2013).

Written informed consent was obtained from all participants. The study description and questionnaires were translated into the native language of the participants. Doctors performing sample collection and physical examination also gave verbal instructions. The diagnosis was made following the DMS-4 criteria [3]. Patients were diagnosed with dysthymic disorder (chronic, mild depression), currently replaced by *persistent depressive disorder* [25]. All laboratory data were anonymous.

Patients underwent an extensive clinician interview and comprehensive laboratory work-up and completed a questionnaire detailing their personal and medical history. The information required included gender, age, BMI, place of residence, hair colour, personal habits, occupation, possible metal exposure, and use of medical treatments. The participants were not on any special diets. They could not be on antidepressive medication or neuroleptics, nor could they be taking any supplements for the three months before samples were collected. Other exclusion criteria included a history of smoking, current pregnancy, acute and chronic infections, autoimmune, allergic, neoplastic, and endocrine diseases and other significant physical ailments or injuries, including surgery within the last six months. Volunteers for the control group were selected from patients who were at the clinic for routine check-ups and were healthy with no lifetime history or current diagnosis of any psychiatric disorders. The controls underwent the same procedures and exclusion criteria as the studied group.

Trace elements (Mn, Co, Cu, Zn, Pb, and Fe) were determined in samples of human hair and fingernails collected between 2013 and 2014 from 215 inhabitants of southern Taiwan region and patients of Homu Clinic, Tainan City,

Taiwan, and from 200 Polish inhabitants of Lublin region (south east region of Poland) who were enrolled in the study between 2014 and 2016. The entire analytical process was repeated twice.

Tests were performed in 415 adults who constituted the studied group (among 295 patients, 102 Taiwanese women and 53 men, age 33 to 73 y, the average age: 57.4 y, and 85 Polish women and 55 men, age 39 to 70 y, the average age: 59.2 y) and the control group, that is, 120 healthy volunteers (60 from Taiwan and 60 from Poland, among them 35 Taiwanese women and 25 men, age 29 to 71 y, the average age: 53.1 y, and 40 Polish women and 20 men, age 26 to 76 y, the average age: 49.3 y). Samples were collected by the use of stainless steel scissors. Hair was collected from the nape of the neck (less than 3 cm from the scalp) from patients who declared no occupational exposure to metals or metalloids. No samples were coloured or treated. The weight requested for each specimen was approximately 100 mg.

2.2. Sample Preparation Procedure. All samples of hair and nails underwent the washing procedure as follows: each sample was soaked in deionised water and later in acetone and again in deionised water with a resistivity of 18.0 MΩ cm (Millipore, Bedford, MA, USA). Each washing stage took 10 min. After drying in an oven (30 min at 100°C) they were weighed. To prevent possible loss of the sample, the weighing stage was after washing and drying. The samples collected from the participants of our study (approx. 100 mg) were divided (using stainless steel scissors) into 3 pieces to repeat the digestion procedure 3 times. The test portion mass was 30 mg. Next, samples were digested with 10 mL of digestion mixture, that is, 3 mL of 69.0% nitric acid (HNO_3) solution + 7 mL of 30% m/m (H_2O_2). Both reagents were of Suprapur grade (Merck, Darmstadt, Germany). The digestion was carried out in NovaWAVE Microwave Tunnel Digestion System (SCP Science, Canada) using Teflon® vessels. The microwave-assisted sample preparation was conducted in a closed system. To improve the recovery of analytes, a careful optimisation of the digestion procedure was performed. To limit the consumption and number of reagents, the following parameters were taken into account: composition and volume of reagents, time of the procedure, and temperature. Regardless of sample type, the time of the digestion procedure did not exceed 30 min, including the cooling stage. The temperature was set at 180°C. The applied conditions minimise the possibility of sample contamination and loss of analytes during the entire procedure.

Deionised water with a resistivity of 18.0 MΩ cm was used for dilution (1 : 10 v/v) of the sample solutions after digestion.

2.3. Analytical Methods of TE Determination. Currently, ICP-MS is the most common method utilised for analysis because it enables accurate, precise, sensitive, and rapid multielement analysis of samples with complex matrices. Unfortunately, ICP-MS is not free from interferences during the determination of certain elements, for example, Fe in the presence of multiple other elements. Because the role of Fe is relevant in depression [9], our study was expanded to include its determination using ion chromatography (IC) [26]. The quantitative analyses of metals and metalloids were carried out with an ICP-MS spectrometer (Elan DRC-e 6100 model, Perkin-Elmer, USA). The spectrometer was optimised daily with a 10 μg/L solution (Mg, Cu, Rh, Cd, In, Ba, Ce, Pb, and U) in 1% HNO_3 (Elan 6100 Setup/Stab/Masscal produced by Perkin-Elmer). The spectrometer was optimised to provide maximal intensity for ^{24}Mg, ^{115}In, and ^{238}U and minimal values for CeO/Ce (below 3%) and Ba^{2+}/Ba (below 3%). All solutions of multielemental (Merck, Germany) and monoelemental (Merck, Germany) ICP-MS standards were prepared daily by the dissolution of the reference materials in water and used for the calibration. One point calibration was performed. Standards, blanks, and samples were measured with ^{103}Rh as the internal standard (10 μg/L, Merck, Germany). 10 μg/L Rh solution was introduced into all solutions online, with the second tubing on the peristaltic pump.

HPIC analyses were performed on a Dionex DX-500 ion chromatograph (Dionex, Sunnyvale, CA, USA). Iron(III) cations were determined using an isocratic elution with 7.0 mmol of pyridine-2,6 dicarboxylic acid (PDCA), 66 mmol of potassium hydroxide, 5.6 mmol of potassium sulphate, and 74 mmol of formic acid as mobile phase, IonPac CS5A (250 mm × 4 mm ID, Dionex, Sunnyvale, USA) as the separation column and spectrophotometric detection (at 530 nm) after the postcolumn derivatisation with the use of 0.5 mmol of 4-(2-pyridylazo) resorcinol (PAR), 1.0 mol of 2-dimethylamino-ethanol, 0.3 mol of sodium bicarbonate, and 0.50 mol of ammonium hydroxide dissolved in deionised water. Appropriate concentrations of standards were prepared from 1 g/L stock standard solutions (Merck, Darmstadt, Germany). The operating parameters of IC and ICP-MS measurements are as follows:

HPIC

Stationary phase: ion exchange column IonPac CS5A (Dionex Co., Sunnyvale, USA), 2 × 250 mm

Guard column: IonPac CG 5A (Dionex Co., Sunnyvale, USA), 2 × 100 mm

Detection: absorbance, λ = 530 nm

Eluent flow rate: 0.3 ml/min

Postcolumn reagent flow rate [ml/min]: 0.15 ml/min

Sample loop: 25 μl

Temperature: 25 ± 1°C

Operating pressure: 1900 psi

Number of replicates: 3

ICP-MS

RF Power: 1125 W

Plasma gas flow: 15 l/min

Nebulizer gas flow: 0.76–0.79 l/min

Auxiliary gas flow: 1.15 l/min

Nebulizer: cross flow

Plasma Torch: quartz

Sample flow: 1 ml/min

Table 1: Validation parameters in applied analytical methods of trace elements determinations.

Analyte	Isotope	Operating range [μg/L]	V_R [%]	V_r [%]	LOD [μg/l]	LOQ [μg/l]	U [%]	Recovery NIST 1643-e [%]
Mn	55	0.20–100	10.4	6.3	0.033	0.198	11.1	108.1
Co	59	0.01–100	4.6	4.6	0.002	0.012	10.0	109.6
Cu	65	0.38–100	5.8	3.5	0.064	0.384	12.4	96.1
Zn	66	2.50–100	11.4	7.6	0.181	1.086	14.6	80.3
Pb	208	0.21–100	13.9	11.3	0.036	0.216	18.0	105.7
Fe*	—	0.30–5000	5.1	3.3	0.09	0.30	13.2	CRM NIES number 13 human hair 98.4

*Fe^{3+} determined by IC; LOD: limit of detection; LOQ: limit of quantification. V_R: relative standard deviation of reproducibility. V_r: relative standard deviation of repeatability. nd: no data in CRM certificate. U: expanded uncertainty.

Scanning mode: peak hopping

Dwell time: 100 ms

Sweeps/reading: 20

Number of replicates: 3

2.4. Validation Parameters. The detection limits were calculated as the average of six blank sample signals plus three times (and ten times for the limits of quantification) standard deviation of the signals obtained from blank samples. An acidified water sample was used to prepare calibration standards and dilution of all solutions and real samples. To assess the accuracy of the methods, the following certified reference materials were used: CRM NIES number 13 human hair and SRM 1643e-trace elements in water (National Institute of Standards and Technology (NIST) Gaithersburg, MD, USA). Table 1 presents the fundamental validation parameters in the applied analytical methods of trace element determinations.

Statistical analysis was carried out using R 3.1., an open source software using built-in routines and "rpart" package [27].

3. Results and Discussion

The normality of results was checked by the Shapiro-Wilk normality test. Almost all variables exhibited significant (at 95% level, with almost all at 99.9% level) deviance from normality, except for Cu content in nails. The distribution of the results was significantly positively skewed, which was confirmed by D'Agostino test for skewness under the null hypothesis of normality.

The correlation of elements' content with age was done by the Pearson correlation coefficient with the appropriate test for significant correlation. No significant correlations were found between element contents and age. The Spearman coefficient with the test gave consistent results.

The univariate difference of TE levels between healthy volunteers and patients (for each element separately) was checked using the Wilcoxon test. Significant differences were found for the elements' content in hair, namely, Co ($p = 0.017$), Cu ($p = 0.0016$), Fe ($p = 0.024$), Mn ($p = 2.7e-05$), and Zn ($p = 2.8e-08$), and for content in nails, namely, Cu ($p = 0.0072$), Fe ($p = 1.2e-08$), Mn ($p = 1.4e-05$), Pb ($p = 0.0020$), and Zn ($p = 2.4e-06$).

In an analogous manner, such differences between genders were tested. The content was found to be significantly different between genders for the elements Co ($p = 0.0025$), Cu ($p = 0.00021$), Zn ($p = 0.042$) in hair and Co ($p = 3.1e-06$), Cu ($p = 0.00095$), and Fe ($p = 0.032$) in nails. To investigate the possible interactions between depression, gender, and age, the appropriate linear models were built. These models applied the content of each element as modelled variables, whereas age, gender, and depression were used as predictors. The possible interactions were as follows: gender-age (difference in slope, when there is another gender), gender-depression (cross-difference in content level), and depression-age (difference in slope between the control and studied groups). Despite confirming earlier conclusions found by the Wilcoxon test, the following interactions were found: (i) For Fe content in hair, a significantly different dependence of element content on age between genders was found ($p = 0.016$); the content slightly increased with age in women, whereas it decreased with age in men. (ii) Significantly lower levels of elements in healthy men (gender-disease interaction) were found for content in nails of Co ($p = 0.018$) and Cu ($p = 0.0021$). The opposite behavior (higher levels in healthy men) was found (but significant at 90% level only) for the content in nails of Zn ($p = 0.030$). (iii) Depression caused a significantly lower increase with age for Fe content in hair ($p = 0.025$).

The significant elevation in the concentration of manganese and significant decrease in the concentration of zinc were observed in the hair and nail samples of depressed patients.

After we had run an ANOVA test and found significant results, we performed Tukey's HSD to find out which specific groups' means (compared with each other) were different. The test compared all possible pairs of means, that is, for depressed women, depressed men, healthy women, and healthy men. In hair, we found that cobalt in female group with depression was significantly decreased when compared to control group and depressed men. The opposite result was found for copper in hair (significantly increased in depressed female group). For iron and lead in hair there were no significant differences among each group, whereas for manganese and zinc significantly different contents were found between both control groups and between studied groups. In nails, significant differences between both sexes (each female and male group) were identified for cobalt. For copper in nails significant differences were found between healthy men and other groups. Iron content in nails was significantly different between each healthy and each studied

Table 2: The results of logistic regression (estimator value and its significance)*.

	Estimate	Std. error	z value	Pr(>|z|)
		Hair model		
(Intercept)	6.5	2.58	2.52	0.0119
Zn.H	−0.088	0.0274	−3.21	0.00131
Cu.H	0.204	0.0884	2.3	0.0213
Mn.H	1.71	0.692	2.47	0.0135
Co.H	−19.7	8.71	−2.26	0.0235
		Nail model		
(Intercept)	−6.65	3.52	−1.89	0.0591
Fe.N	0.112	0.0368	3.03	0.00247
Zn.N	−0.101	0.0338	−3	0.00274
Cu.N	0.38	0.141	2.69	0.00719
		Total model		
(Intercept)	−3.5	4.43	−0.791	0.429
Zn.H	−0.122	0.0408	−2.98	0.00286
Fe.N	0.0909	0.0293	3.1	0.00193
Cu.H	0.361	0.124	2.9	0.00373
Pb.N	2.7	1.08	2.51	0.012

*The modelled equation computes depression probability from the most significant elements determined in hair and nails.

group (higher in the group with depression), content of manganese in nails was significantly higher in group with depression, content of lead in nails was significantly different between healthy men and depressed men, and zinc content in nails was significantly different in each group (the lowest content was found in depressed women).

Figures 1(a) and 1(b) present the contents of the elements organised according to gender and studied versus the control group. The contents are presented in mg/kg dry mass.

To investigate possible intercorrelations between elements, scaled principal component analysis was used. This data mining method treats all patients as points in multivariate space and tries to find an angle of a two-dimensional plane (as a so-called subspace) to represent the highest variance of the data. Projection onto this plane, understood as a "shadow" of the points, is the best possible representation of objects (patients) on a two-dimensional plot. However, the quality of the two-dimensional representation depends on structure of the data; that is, the more the intercorrelations, the better the ability to compress. Only 18% of total variance was explained by the two first principal components. This proves that the levels of elements are very independent and it is difficult to find any multivariate intercorrelations or visible trends in these data. The subjects had no tendency to be clustered. The lack of intercorrelations can be proven by investigation of loading vectors, that is, the projection of original axes onto the two-dimensional plane found by the analysis. In this case, loading vectors were randomly and uniformly distributed in all directions.

Next, the multivariate discriminant models (equation differentiating persons with and without depression) were created with scaled PLS-DA (Partial Least Squares-Discriminant Analysis) for hair, nails, and the total dataset. This method is analogous to the LDA (Linear Discriminant Analysis) but instead of ordinary least squares regression uses the PLS (Partial Least Squares) regression. Having a matrix, containing n rows (subjects) and p columns (elements) and a column vector y containing 1 value for depression and −1 for control, the method tries to find an estimator for predicting this y vector from X. This estimator is then used for further prediction, and negative y means healthy, whereas positive indicates depression. In the case of multidimensional and intercorrelated data, it is the natural choice to use PLS regression instead of the classical one.

The complexity of two PLS factors was chosen with cross-validation in all cases. In the case of hair, seven healthy persons were misclassified as sick and two persons with depression were misclassified as healthy. In the case of nails, five healthy persons were misclassified as sick and two persons with depression were misclassified as healthy, respectively. A total model resulted in the misclassification of two and one persons, respectively. The estimators of PLS-DA model are shown in Figure 2. Positive values mean an increase of the element content during depression, whereas negative means a decrease. It should be noted that the estimator values are scaled, so two similar values do not mean similar impact of the element, but similar impact scaled to its variance.

To obtain a formula for depression probability prediction, a logistic regression was used with stepwise variable selection against the Akaike information criterion (AIC). Three separate models were built and found: for hair, nails, and the augmented dataset. Table 2 presents the most significant variables (elements).

All of the methods detected similar variables, and this finding additionally confirms that these elements may, in fact, be relevant for chronic depression. It should be noted that the results do not predict the severity of depression.

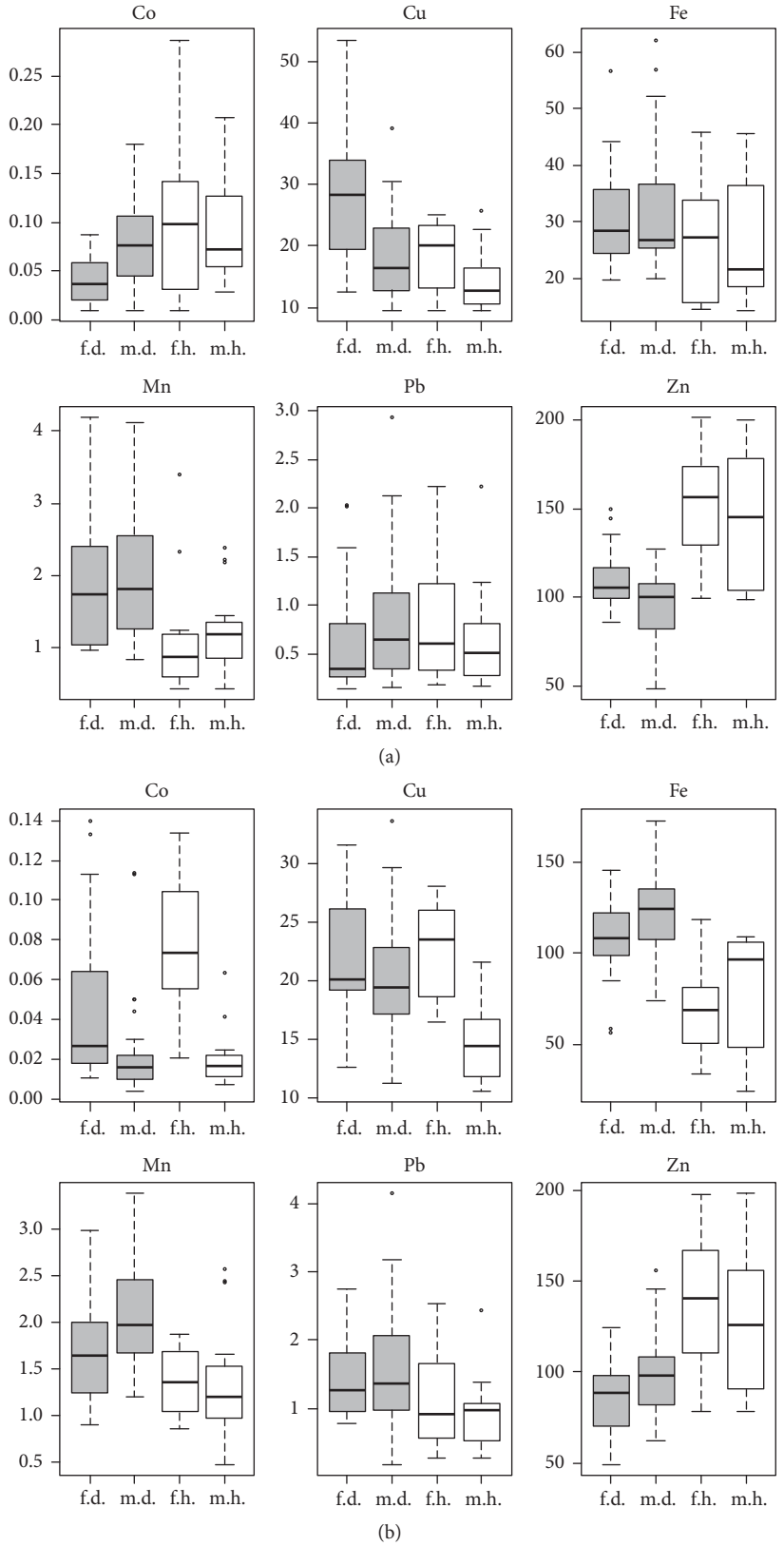

Figure 1: (a) Hair trace elements contents (mg/kg dry mass) in depressed (d) and healthy (h) women (f) and men (m). (b) Nail trace elements contents (mg/kg dry mass) in depressed (d) and healthy (h) women (f) and men (m).

Table 3: Contents of studied elements (in mg/kg on dry mass basis) in hair and nails of studied and control groups according to the place of residence ($N = 415$).

Element	Sample matrix	Patients ($N = 295$) from		Mann–Whitney test	Healthy volunteers ($N = 120$) from		Mann–Whitney test
		Taiwan (155)	Poland (140)		Taiwan (60)	Poland (60)	
		Mean ± SD*			Mean ± SD		
Zn	Hair	100.21 ± 31.56	99.16 ± 28.16	NS**	147.85 ± 38.22	150.11 ± 35.44	NS
	Nails	92.85 ± 29.22	90.11 ± 24.62		131.8 ± 30.57	129.8 ± 28.14	
Cu	Hair	22.05 ± 11.14	23.18 ± 9.98	NS	15.81 ± 6.68	16.81 ± 10.41	NS
	Nails	20.59 ± 10.23	22.13 ± 11.33		17.93 ± 10.89	18.23 ± 9.91	
Mn	Hair	1.92 ± 0.55	2.42 ± 0.48	NS	1.19 ± 0.42	1.22 ± 0.39	NS
	Nails	1.93 ± 0.62	1.69 ± 0.54		1.35 ± 0.63	1.39 ± 0.93	
Co	Hair	0.06 ± 0.02	0.05 ± 0.01	NS	0.09 ± 0.03	0.08 ± 0.04	NS
	Nails	0.03 ± 0.01	0.02 ± 0.01		0.04 ± 0.01	0.05 ± 0.02	
Fe	Hair	31.3 ± 9.23	33.7 ± 10.22	NS	26.60 ± 5.48	24.90 ± 4.22	NS
	Nails	115.9 ± 51.21	118.4 ± 41.11		75.32 ± 20.26	78.00 ± 18.16	
Pb	Hair	0.74 ± 0.28	0.82 ± 0.33	NS	0.75 ± 0.32	0.73 ± 0.45	NS
	Nails	1.48 ± 0.75	1.52 ± 0.84		1.00 ± 0.49	0.099 ± 0.36	

*SD: standard deviation; **NS: nonsignificant difference.

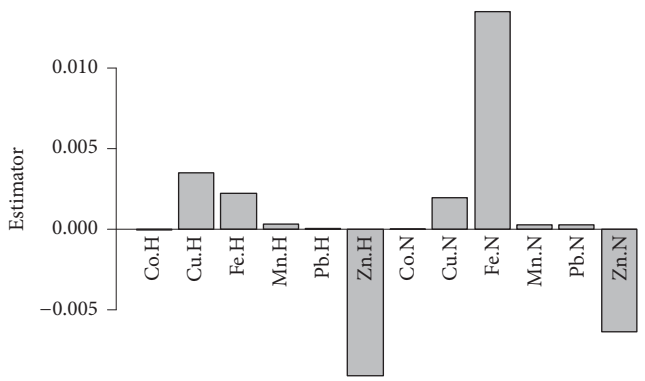

Figure 2: The values of PLS-DA estimator, discriminating subjects with disorder from control group, made with the whole dataset (H-hair, N-nails).

From our study, it is clear that there is a statistically significant difference in TE content of hair and nails between the depressed patients and the healthy subjects. It is important to take into consideration that all the participants were not on any special diets. Obviously the regular diet was different in the two countries, but the trend in altered trace elements profile was significant for dysthymic patients, regardless of their place of residence. It may suggest that not only nutritional factors are responsible for altered levels of investigated elements in hair and nails of dysthymic patients. Our findings may indicate a possible relationship between chronic depression and deficiency or excess of some TE as well as alterations in their metabolism.

The lack of strong correlation between TE contents and place of residence of our participants and similar trend in alteration of TE content in chronic depression indicate that trace analysis may serve as a useful tool for the purposes of prevention (Table 3).

Zinc deficiency can lead to numerous complications such as stunted growth, diarrhoea, impotence, hair loss, eye and skin lesions, impaired appetite, and depressed immunity. Some cross-sectional studies associate a low Zn intake with depression in women [28, 29], whereas a 20-year prospective follow-up study suggests that dietary Zn intake was not associated with an increased risk of depression in men [30]. Recent published findings [31] suggest such an association with a greater incidence of depression in both men and women. Very recently, Lehto et al. [32] have emphasised that Zn probably will not be the golden remedy for depression due to a high heterogeneity in the etiopathogenesis of depression. Reported clinical data indicate decreased serum Zn levels in human depression. Altered Zn levels could be a potential marker of depression [33–36]. Greater depression severity was associated with a relatively greater Zn deficiency [37]. Some studies suggested that low Zn levels could be related to the activation of cell-mediated immunity in depression [38]. Studies performed by Siwek et al. [39] suggest three possible reasons why lower Zn levels were observed in depression (i.e., nutritional deficiency, hyperstimulation of the hypothalamic-pituitary-adrenal axis and/or inflammatory/acute phase response, and oxidative stress). The authors stressed that serum Zn would not make a good specific marker of depression because it rather serves better as a marker of immune activation and oxidative stress. Ghanem et al. [40] found a significantly lower level of Zn in hair samples of depressed patients in comparison with healthy subjects, which is consistent with our results. According to our knowledge, there are no published studies of the Zn content in the nails of depressed patients. It is interesting that the difference in the decrease of Zn between nails and hair in both groups is so similar.

Certain studies imply that relatively low amounts of Mn exposure could affect mood because even low levels of exposure result in neuropsychological effects [41]. The issue of

Mn's role in psychiatric and neurological diseases is extremely important, because even after exposure has ceased, and serum, hair, and urine levels return to normal, the progress of neuropsychiatric symptoms can continue. It is suggested that Mn^{2+} and Mn^{3+} can react with dopamine to create reactive oxygen species, which is known to cause damage to the dopaminergic neurons [42]. The exact mechanism of Mn and dopamine interaction-induced cell death remains unclear [43]. It is known that disturbances in Fe homeostasis can contribute to Mn toxicity [44]. Fe is required for normal cellular function and structure, playing roles in DNA and neurotransmitter synthesis [45]. It was suggested that even in nonanaemic patients abnormal Fe levels may alter mood and behavior in a similar way to depressed subjects. Severe iron deficiency and resulting anaemia are associated with impaired cognitive functions and abnormal neuropsychological development [46]. According to Młyniec et al. [9] depression is a multifactorial disorder and Fe may have a positive or negative effect on depression. Psychological stress has been shown to cause Fe deposits in the brain. One study showed reduced release of Fe during inflammation and suggested that lower Fe levels may be a result of depression [47]. Our studies show that depressed patients can be expected to have an increase of Fe content in hair and nails even when no inflammatory state is present. Unlike with the proportional decrease of Zn in both matrices, the observed increase of Fe was not proportional between the hair and nail samples.

The mechanisms of Pb neurotoxicity and the effects of Pb on neuronal death, on intraneuronal regulatory mechanisms, on neurotransmission, on transmitter release, and on transport of thyroid hormone have been studied extensively [48]. Indirect neurotoxic effects of Pb include anaemia as a result of interference with heme synthesis and through decreasing Fe absorption from the gut [49].

As for the other elements that were quantified in our study, although no strong associations were found between their content and the prevalence of depression in the patients studied, it should be noted that our results found an increase of Cu in both types of samples. This observation is consistent with data of studies undertaken by Ghanem et al. [40]. Cu is involved in the absorption, storage, and metabolism of Fe; therefore higher Cu levels could be responsible for the higher levels of Fe in depression. Cu is required for dopamine synthesis, and dopamine in the brain plays an important role in depression [50].

The observed associations could provide insight into the etiology of depression, helping generate new hypotheses for further research and perhaps contribute to future healthcare planning. We realise that there is a need of a speciation analysis in relation to certain elements determined in biological matrices because various chemical species can indicate different biological effects. Unfortunately, some sample pretreatment procedures interfere with speciation studies, and therefore the final determination refers to "total" TE concentration in hair or nail samples. The ideal analytical strategy in which all species of all elements are determined simultaneously followed by the same pretreatment procedures still does not exist [20].

Our studies have important strengths, for example, application of the most suitable analytical techniques taking into account the complexity of samples' matrices. These techniques were evaluated with the appropriate reference materials to ensure high quality of the entire analysis. Moreover, in our study we used two analytical techniques which allowed us to perform a multielement determination, including Fe, which is usually underestimated in ICP-MS studies.

The determination of Fe by ICP-MS is not recommended, especially in samples with complex matrices because of polyatomic interferences from different isotopes produced by O, Ar, and Ca on ^{56}Fe ($^{40}Ar^{16}O^{+}$ and $^{40}Ca^{16}O^{+}$) and ^{57}Fe ($^{40}Ca^{16}O^{1}H^{+}$, $^{40}Ar^{16}O^{1}H^{+}$). The interferences caused by $^{40}Ar^{16}O^{+}$ and $^{40}Ar^{16}O^{1}H^{+}$ on ^{56}Fe and ^{57}Fe, respectively, may be reduced by using a shield torch, which unfortunately can produce other polyatomic interferences [51]. The next advantages that are not negligible include ease of collection and excellent stability of the samples.

We present the associations between hair and nail TE status after age and gender adjustments, and the exclusion criteria of our study limit additional factors (variables) that may affect the content of TE. Patients with other psychiatric pathologies were excluded in our study. Neither the participants nor the persons conducting the experiment had access to key information that could interfere with or influence the results.

There are different methods of analysis currently in use with different procedures used to collect and prepare samples. Widely divergent reference ranges are reported for the normal individual depending on which method of analysis is used, making it difficult to compare the results between different studies. External contaminants pose a significant concern and the proper pretreatment of samples should be used to limit such possibilities. There are a variety of factors that affect the content of TE in hair/nails in and of itself, namely, age, gender, diet, hair type, location of hair, and so forth. Despite these limitations, TE analysis of hair and nails remains an area of continuing interest and active research. However, the analytical data obtained must be interpreted with caution and forethought.

4. Conclusions

Despite a growing number of studies undertaken in the field of TE determination in various disorders, the role of TE in depression has not been thoroughly elucidated. A reliable analysis of easily accessed samples of hair/nail could provide an answer to the still unsolved question of whether deficiency of certain elements or excess toxicity of other elements manifests itself during depression. Mutual interactions between elements in this disorder should also be taken into consideration for useful diagnostic information.

The results of this study indicate various significant dependence between TE levels, the sample type, age, and gender. Zn, Mn, Pb, and Fe contents in hair/nails seem to be strongly associated with chronic depression. Further cohort studies could be useful in definitely elucidating whether TE are good predictors for the development of depression or whether depression's metabolic influences are the cause

of changes in TE levels. The significant elevation in the concentration of manganese and significant decrease in the concentration of zinc were observed in the hair and nail samples of depressed patients. For Fe content in hair, a significantly different dependence of element content on age between genders was found ($p = 0.016$); the content slightly increased with age in women, whereas it decreased with age in men. Significantly lower levels of elements in healthy men were found for content in nails of Co ($p = 0.018$) and Cu ($p = 0.0021$). The opposite behavior (higher levels in healthy men) was found (but significant at 90% level only) for the content in nails of Zn ($p = 0.030$). Depression caused a significantly lower increase with age for Fe content in hair ($p = 0.025$). The content of TE was found to be significantly different between genders for the elements Co ($p = 0.0025$), Cu ($p = 0.00021$), and Zn ($p = 0.042$) in hair and Co ($p = 3.1e - 06$), Cu ($p = 0.00095$), and Fe ($p = 0.032$) in nails. We found no statistically significant residence-related differences in the contents of Zn, Cu, Co, Mn, Fe, and Pb in nonoccupationally exposed patients with chronic depression and healthy subjects.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Enamel Thickness before and after Orthodontic Treatment Analysed in Optical Coherence Tomography

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Despite the continuous development of materials and techniques of adhesive bonding, the basic procedure remains relatively constant. The technique is based on three components: etching substance, adhesive system, and composite material. The use of etchants during bonding orthodontic brackets carries the risk of damage to the enamel. Therefore, the article examines the effect of the manner of enamel etching on its thickness before and after orthodontic treatment. The study was carried out in vitro on a group of 80 teeth. It was divided into two subgroups of 40 teeth each. The procedure of enamel etching was performed under laboratory conditions. In the first subgroup, the classic method of enamel etching and the fifth-generation bonding system were used. In the second subgroup, the seventh-generation (self-etching) bonding system was used. In both groups, metal orthodontic brackets were fixed and the enamel was cleaned with a cutter fixed on the micromotor after their removal. Before and after the treatment, two-dimensional optical coherence tomography scans were performed. The enamel thickness was assessed on the two-dimensional scans. The average enamel thickness in both subgroups was not statistically significant.

1. Introduction

Fixed braces are controversial because of the way they are attached to the tooth surface and a potentially devastating effect on the tooth enamel. Therefore, it becomes necessary to conduct research on the state of the tooth enamel after orthodontic treatment, depending on the used techniques and materials for fixing brackets. Research on this subject enables developing treatment procedures optimal for the enamel quality.

In clinical orthodontics, adhesive systems, whose structure is based on resin composites merging with enamel through an etching process, are most often used for bonding brackets. The purpose of etching is partial dissolution of the enamel minerals, which allows mechanical retaining of the orthodontic resin in the tissue pores created by an inorganic acid. It significantly increases the roughness of the enamel, enhancing the risk of plaque and sediments around the bracket, and reduces the hardness of the tissue and its resistance to external factors. Due to the effect of dissolving the enamel, it is very important to perform this procedure cautiously and skillfully and study possible alternatives to the above technique.

In the classic etching method, a relatively strong acid is used, which is usually a 35–40% solution of orthophosphoric acid. The solution is applied to the clean enamel surface during 15–30 seconds and then rinsed, and the enamel surface is dried using a strong air flow. The studies of Retief [1], Arakawa et al. [2], Asmussen [3], and Charbeneau Voss and Charbeneau [4] on the procedure of direct decalcification,

evaluated using an optical microscope, showed a penetration depth of the etching acid into the tissue ranging from 5 to 50 μm. In the course of the development of adhesive technology in dentistry, aiming to minimize the steps of attaching hooks, three separate elements were combined into two, combining the properties of the etchant and adhesive system [5–8]. Self-etching primers (SEP), owing to the presence of an acid primer, allow for the exclusion of the etchant [9, 10]. In the light of published studies [11], both ways of enamel etching show a similar pattern of the enamel porosity. The etching primer has a more classic pattern of etching [12–24] while maintaining an adequate, optimal bonding strength [8], similar to the strength generated using the classic method of enamel etching [15, 16].

The SEP bond strength, according to researches, ranges from 20 to 30 MPa [15], which shows a similar range of forces to the classical acid etching [16]. The big advantage of the system is the primers penetration on the entire depth of the generated pores of the enamel, which provides predictable, extremely durable mechanical fixation [17]. In the course of studies it has demonstrated that the extent of penetration of the glue is smaller using the same etching system than the normal etching. But this is not a disadvantage, because the greater hook in the enamel resin is, the greater risk of damage during removal of the debonding exists.

Many studies have shown that the extent of penetration of the glue is smaller using the self-etching primer than in the case of normal etching. However, this is not a disadvantage since the larger the resin hooks in the enamel, the greater the risk of its damage while debonding [18]. Considering this hypothesis, the presented article examined the effect of the method of enamel etching on its thickness before and after orthodontic treatment.

2. Material Methods

The study was carried out in vitro. The material comprised 80 teeth, divided into two groups of 40 teeth each. In the first test group, the orthodontic brackets were attached to the tooth surface using the fifth-generation adhesive system that uses the classic method of enamel etching with orthophosphoric acid. In the second group, the orthodontic brackets were attached to the tooth surface using the self-etching primer (seventh-generation system). In each group steel orthodontic brackets were used.

The experiment was carried out on the premolars, extracted for orthodontic and periodontal reasons. The exclusion criterion was defined by the following conditions: the presence of developmental defects of enamel, that is, hypoplasia, turbidity or discoloration, caries, and fillings on the vestibular surface.

The teeth qualified for research were stored for 30 days in demineralised water, with a crystal of thymol (0.1%) at room temperature.

Before fastening orthodontic brackets, the tooth surface was cleaned using a polisher (TopDental, Poland) with fluoride-free toothpaste Pressage (Shofu Inc., Japan) designed to prepare the enamel before fastening orthodontic brackets. Then, the tooth was washed with distilled water and dried with compressed air for 15 seconds. For fastening orthodontic brackets, an orthodontic composite material Transbond™ XT Light Cure Adhesive (3M Unitek, USA) was used, which requires the prior preparation of the enamel surface.

In the first group, the vestibular surface of the tooth was etched for 30 seconds with a 37% solution of phosphoric acid, Blue-Etch (CERKAMED, Poland), rinsed with distilled water for 15 seconds and dried using compressed air. The adhesive system OptiBond Plus Solo (Kerr, USA) was rubbed with an applicator into the etched enamel surface for 15 seconds; then the surface was dried under a gentle stream of air for 3 seconds and cured with a halogen lamp of the light intensity of 750 mW/cm^2 for 20 seconds. The orthodontic composite material Transbond XT Light Cure Adhesive was applied to the bracket surface. The hook was pressed against the enamel surface with commonly used tweezers. The orthodontic hook was placed in the middle of the mesial-distal axis of the tooth, moving its centre 3.5 mm away from the edge of the occlusal surface. The distance was measured using an orthodontic positioner. After proper placement of the hook, the material was subjected to polymerization with a halogen lamp for 40 seconds.

In the second group, the self-etching adhesive system G-Bond (GC, USA) was used. The self-etching primer when applied to the tooth surface using an applicator was left for 10 seconds, and then the excess was removed via an air stream for 5 seconds. After this time, the system was polymerized with a halogen lamp of light intensity of 750 mW/cm^2 for 20 seconds. The orthodontic composite material Transbond XT Light Cure Adhesive was applied to the surface of the hook. The orthodontic hook was placed onto the tooth surface using the above-described method.

The teeth with the fixed orthodontic brackets were stored in demineralised water at room temperature for 24 hours. After this time, the hooks were removed mechanically with pliers ix827 (Ixion Instruments, USA) designed for removing all types of hooks.

Residues of the adhesive material were removed from the enamel surface using a cemented carbide milling cutter H390.204 AGK (Komet URPOL, Poland) which has 8 notches, the size of 314.018, the length of 3.6 mm, and a diameter of 1/10 mm.

The enamel was processed with the use of a micromotor commonly mounted to a dental unit at a speed of 40000 revolutions/min with water cooling and pressure force of 1.0 N. The force was measured on a test stand consisting of scales, on which the processed tooth was placed.

The procedure of cleaning the enamel was considered to be finished on the basis of the naked-eye examination and by touching with the stylet 23 in the dental unit light. The assessment criterion was the smoothness of the tooth surface and the absence of the composite material residues.

2.1. Performance of Tooth Scans Using 3D-OCT. The area of the test teeth was imaged with a 3D-OCT camera (Topcon, USA, Figure 2) three times:

T0: imaging of the tooth surface before installing orthodontic brackets,

T1: imaging of the tooth surface after mechanical processing.

Each time, two-dimensional scans were performed allowing for a clear illustration of the enamel damage in a vertical plane. The procedure enabled showing the entire surface of the tissue and performing the subsequent comparative analysis of changes in its structure. The 3D-OCT device (Topcon, USA) in addition to CT has a coupled digital camera with a resolution of 16.2 Mpix, which provides highly accurate images of the test area with twentyfold zoom without losing image quality.

The technology of Fourier Domain OCT (S-OCT), which uses spectral analysis, provides very quick scanning (27000 A-scans/sec) and a high axial resolution of 5 μm and a horizontal resolution of 20 μm. The use of a pulsed light source, which is a superelectroluminescent diode (SLED) in the OCT, allows for better detection of low-contrast centres. The wavelength is 840 nm; the half-width is 50 nm. The 3D-OCT-2000 has a scanning range of 6 × 6 mm horizontally and 2.3 mm into the tissue. It is a device designed for ophthalmic diagnostics, whose system enables virtual segmenting of the retina into layers allowing for the assessment of the photoreceptors and pigment epithelium. The wavelength of 840 nm and the depth of penetration into the tissue also allow for imaging of the tooth enamel tissue through its entire thickness.

It was possible to obtain accurate scans of the surface and enamel structure of teeth with due repeatability during three examinations owing to a special matrix made for each tooth. The matrix allowed for repeatable tooth positioning in the frontal, sagittal, and horizontal plane relative to the optical axis of the OCT. The matrix was made of the c-silicone Zetalabor hard 85 Shore A (Zhermack, Italy), on the basis of the tooth impression in the long axis so that the vestibular surface of the crown remained above the silicone. The support for the silicone was a mould with fixed attachment with respect to the optical axis of the OCT.

The obtained OCT scans were subjected to an expert IT analysis. Image preprocessing involved automatic reading of the order of OCT images from the source file with the extension ∗.fds allowing for the development of matrices of individual images. Figure 1 shows the method of acquisition of OCT images of the teeth. Figure 2 depicts the reconstruction of the sequence of the OCT images. IT analysis, which was performed owing to a specially developed algorithm, was accurately described and published [25].

The results obtained in the study were statistically analysed. The Shapiro-Wilk test was used to verify the hypothesis of normality of variable distribution.

To verify the hypothesis of the existence and nonexistence of differences between the mean values for the independent variables, the median test and the Mann–Whitney U test were used. To verify the hypothesis of the existence or nonexistence of differences between the mean values for the dependent variables, the Friedman two-way analysis of variance and Wilcoxon matched-pairs signed-ranks test were used.

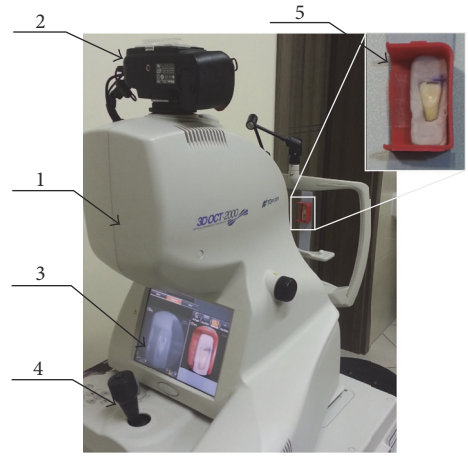

Figure 1: Image showing the method of acquisition of OCT images of the teeth. The following items are depicted: 1, OCT tomograph, 2, digital camera for taking images in visible light, 3, screen of the tomograph, 4, joystick enabling changing object position, and 5, method of attachment of the tooth in the device.

In order to assess the correlation between saccadic and qualitative variables, the chi-square test of independence, the chi-square test of independence with Yates' correction, and the Fisher's exact test were used. Maxwell's general principle was followed when using this type of tests.

The diversity of many variables in the categories determined by qualitative factors was analysed using models of univariate analysis of variance, ANOVA/ANCOVA. When verifying all hypotheses, the level of significance was $p = 0.05$.

3. Results

The results of the statistical univariate analysis, evaluating the difference in the enamel thickness after orthodontic treatment depending on the adhesive system, have not confirmed the relationship between the thickness of the enamel tissue after completed orthodontic treatment and the adhesive system. To carry out the above analysis, average, minimum, and maximum values of the tissue thickness after treatment as well as average, minimum, and maximum differences between the initial and final enamel thickness were used. In the case of the fifth-generation system, the tissue thickness after treatment amounted to 472,75 μm, 128,18 μm, and 10093,62 μm, respectively, and the differences in thickness were 96,53 μm, 55,71 μm, and 432,69 μm. When the seventh-generation system was used, the tissue thickness after treatment amounted to 469,03 μm, 132,26 μm, and 1103,84 μm respectively, and the differences amounted to 90,93 μm, 50,15 μm, and 402,10 μm. Among these measurements the most reliable was again the difference in the average enamel thickness (Dif_Avg), which showed no statistically significant differences ($p > 0.407$).

Tables 1(a) and 1(b) show the values of the enamel thickness after the completion of orthodontic treatment depending on the applied adhesive system.

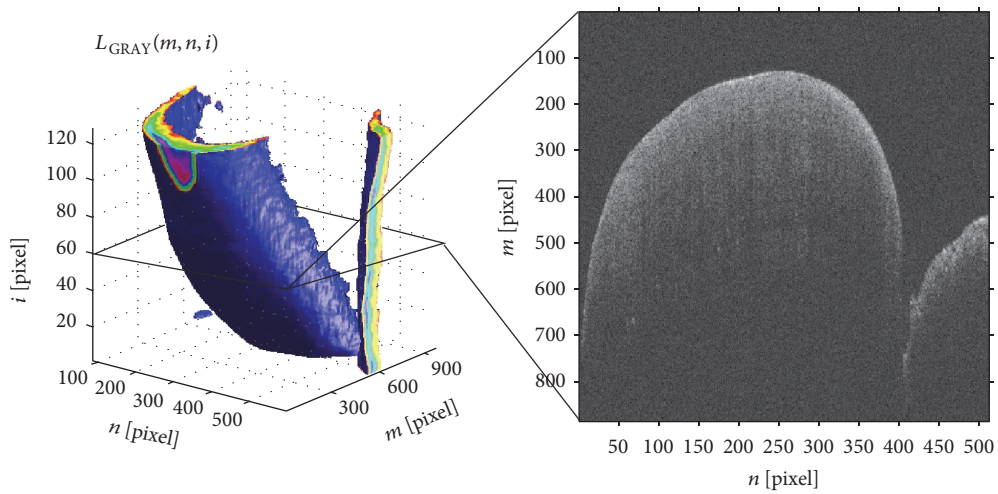

FIGURE 2: Reconstruction of a sequence of images $L_{GRAY}(m,n,i)$ for $M \times N \times I = 884 \times 512 \times 128$ pixels. The image shows the result of reconstruction of an image sequence with a sample B-scan obtained for $i = 60$. The analysis aims at automatic determination of the edge on a sequence of OCT images (B-scans) in order to determine enamel thickness.

TABLE 1: (a) The enamel thickness (μm) on the vestibular surface of the teeth after the completion of orthodontic treatment in the groups that used the fifth-generation system. (b) The enamel thickness (μm) on the vestibular surface of the teeth after the completion of orthodontic treatment in the groups that used the seventh-generation system.

(a)

Variables	n	M	Me	Min.	Max.	Q1	Q3	R	SD
V_Avg	40	472,75	453,60	259,16	743,87	376,28	564,13	187,85	117,25
V_Min	40	128,18	130,00	0,00	360,00	80,00	185,00	105,00	78,36
V_Max	40	1093,62	1040,00	450,00	2755,00	845,00	1255,00	410,00	411,96
Dif_Avg	40	96,53	76,50	−142,77	563,00	30,68	160,11	129,43	104,91
Dif_Min	40	55,71	55,00	−50,00	180,00	20,00	85,00	65,00	46,53
Dif_Max	40	432,69	180,00	−915,00	3060,00	15,00	700,00	685,00	665,75

The following symbols have been used in the table: n, number of samples; M, arithmetic mean; Me, median; Min–max, range of variation; Q1–Q3, first quartile, third quartile; R, interquartile range; SD, standard deviation; V_Avg, average enamel thickness prior to orthodontic treatment; V_Min, minimum enamel thickness prior to orthodontic treatment; V_Max, maximum enamel thickness prior to orthodontic treatment; Dif_Avg, difference in average enamel thickness prior to orthodontic treatment and after its completion; Dif_Min, difference in minimum enamel thickness prior to orthodontic treatment and after its completion; Dif_Max, difference in maximum enamel thickness prior to orthodontic treatment and after its completion.

(b)

Variables	n	M	Me	Min.	Max.	Q1	Q3	R	SD
V_Avg	40	469,03	439,72	172,14	844,79	367,49	570,38	202,95	130,18
V_Min	40	132,26	140,00	0,00	315,00	80,00	185,00	95,00	69,89
V_Max	40	1103,84	1030,00	460,00	2515,00	805,00	1330,00	400,00	432,52
Dif_Avg	40	90,93	65,15	−65,84	461,71	23,35	142,68	120,49	96,19
Dif_Min	40	50,15	40,00	−185,00	220,00	10,00	90,00	75,00	52,61
Dif_Max	40	402,10	265,00	−1415,00	3215,00	35,00	685,00	630,00	569,47

The following symbols have been used in the table: n, number of samples; M, arithmetic mean; Me, median; Min–max, range of variation; Q1–Q3, first quartile, third quartile; R, interquartile range; SD, standard deviation; V_Avg, average enamel thickness after orthodontic treatment; V_Min, minimum enamel thickness after orthodontic treatment; V_Max, maximum enamel thickness after orthodontic treatment; Dif_Avg, difference in average enamel thickness prior to orthodontic treatment and after its completion; Dif_Min, difference in minimum enamel thickness prior to orthodontic treatment and after its completion; Dif_Max, difference in maximum enamel thickness prior to orthodontic treatment and after its completion.

4. Discussion

The presented results show that the enamel thickness after completed treatment and its possible damage is not dependent in any way on the type of adhesive system. The studies by other authors, cited above, suggest a smaller impact of the self-etching system on the enamel and the performed experiment leads to the conclusion that the impact of the two systems on the enamel is similar. The methodology of the compared research is different. Our study focused on the

quantitative assessment of the enamel, while the previously mentioned experiments by other authors, Retief [1], Arakawa et al. [2], Asmussen [3], and Charbeneau Voss and Charbeneau [4], assessed the enamel quality. They measured the amount of dissociated calcium and depth of penetration of resin hooks. Therefore, it can be concluded that the results of the compared studies are not contradictory, since they measure different characteristics of the enamel. The use of an etchant does not reduce the enamel thickness due to the lack of abrasive abilities. The method of etching can only indirectly influence the final tissue thickness by substantial weakening of its structure, which increases the enamel sensitivity to operator intervention during debonding and cleaning. So far, the evaluation of the full tissue thickness has been difficult to carry out, so there are not many publications to refer to when discussing the results. Accordingly, in order to expand the available knowledge on this topic and objectify it, an attempt was made to use the OCT to assess the quality of the enamel after using various types of adhesive systems. A new application of the above-mentioned device was to evaluate the diversity of the image depending on the size and depth of the generated pores of the enamel, which affect the propagation of light waves in the tissue and the appropriate image registration. The result obtained has led to the conclusion that the use of self-etching systems is safe for the enamel.

Many independent studies describe the features of self-etching systems, which include small aggressiveness in relation to enamel. They result in substantially lower, than in the case of classic etching, irreversible changes in the tissue and affect the production of shorter resin hooks. However, they generate a sufficient bonding strength for the clinical procedure and there are rarer cases of bonding errors in the enamel-adhesive system phase than the classic etching method [26–28]. A significantly greater bonding strength of the self-etching system was confirmed in the studies of Bishara et al. and Buyukyilmaz et al. [17, 29]. These studies challenged the hypothesis of many critics such as Fjeld and Øgaard [30] and research groups led by the previously cited Bishara et al. [30–35], who hypothesized greater risks of self-etching systems in their experiments. It was associated with increased adhesion errors in the enamel-adhesive system phase. These errors increased the risk of cracks in the enamel. In this context, the performed studies have proven the superiority of the self-etching system over the classic one.

Such significant differences in assessing the strength of the adhesive system between many researchers may be related to the quality and type of selected test samples. Published studies were performed on extracted human or animal teeth, both front and back ones. Diversity of observations may be related to the method of testing, both in vitro and in vivo, as well as the preparation of the sample surface, the use of various orthodontic adhesive materials, debonding methods, the time after which the hooks were removed, and the conditions of storing samples.

The presented OCT method can be compared to other methods of imaging of the enamel layer. The known methods of tooth enamel analysis include assessment by means of an atomic force microscope (AFM) [36, 37] and a scanning electron microscope (SEM) [38].

The other known methods of enamel thickness analysis do not enable automatic, quantitative measurement of the enamel thickness present in the ROI and automatic comparison of image groups. This is the case in [39], where comparisons between specific areas of the tooth enamel were made manually in OCT images. Automatic measurement was presented only in [40]. However, it concerns polarization sensitive optical coherence tomography (PS-OCT) and is not related to the problem of overlap of individual images in the subsequent processing stages of the tooth as shown in this paper.

5. Conclusions

The range of variations in the enamel thickness after treatment with fixed thin-arched braces are not subject to modification of a factor such as the type of adhesive system.

The OCT is an effective diagnostic tool to evaluate the thickness of the enamel tissue before and after the completed orthodontic treatment.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Julia Seeliger and Monika Machoy contributed equally to this work.

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Research Article

Evaluation of Metal Ion Concentration in Hard Tissues of Teeth in Residents of Central Poland

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Objectives. The aim of the study was an assessment of the content of trace elements in enamel and dentin of teeth extracted in patients residing in urban and agricultural areas of Poland. *Methods.* The study included 30 generally healthy patients with retained third molars. 65 samples of enamel and dentin from individuals living in urban areas and 85 samples of enamel and dentin from individuals living in agricultural areas were prepared. The content of manganese, lead, cadmium, and chromium in the studied enamel and dentin samples from retained teeth was determined by Graphite Furnace Atomic Absorption Spectrometry. In the process of statistical hypothesis testing, the level of significance was assumed at $\alpha = 0.05$. *Results.* A comparative analysis of the data showed that enamel and dentin of inhabitants of industrialized areas contain significantly higher amounts of lead and cadmium than hard tissues of teeth in residents of agricultural areas and comparable amounts of manganese and chromium. *Significance.* It appears that hard tissues of retained teeth may constitute valuable material for assessment of long-term environmental exposure to metal ions. The study confirms that the risk of exposure to heavy metals depends on the place of residence and environmental pollution.

1. Introduction

Metals are elements widely found in the environment. They form natural deposits around the world and are widely used in many areas of life. Some of those elements, such as iron and copper, are essential for life and proper functioning of the body, playing an important role in metabolic processes. Other elements do not play a significant role in physiological processes; moreover, they can contribute to defects in organs as well as individual tissues, impair their function, and induce a broad spectrum of diseases in the mechanism of acute poisoning resulting from intensive supply and of chronic exposure to relatively low doses [1]. Due to their harmful biological effects, special attention is devoted to exposure and mechanisms of adverse action of trace elements such as lead, cadmium, manganese, and chromium on the human organism.

These metals can originate from industrial pollution including dust, sewage, combustion products of fossil fuels, paints and varnishes, tobacco smoke, everyday objects, and food contaminated with the above [2, 3]. The presence of metallic elements has been observed in all parts of the ecosystem including the water, the atmosphere, the soil, and plants.

Lead is commonly used in industry for production of batteries, cables, wires, or bearings; it may be a component of paint or varnish. It is also present in solders widely used in the electronics industry. Until recently, lead compounds which are contained in gasoline were one of the main sources of systemic exposure to the harmful element [4] and although in Europe and the United States unleaded fuel is commonly used, certain developing countries have not as yet introduced necessary regulations to eliminate the element from the production processes in the petrochemical industry.

Exposure to lead and its compounds during both the prenatal and postnatal periods causes damage to the nervous system and disorders of renal function or gastrointestinal tract. This element can also cause damage to liver or components of the hematopoietic system [5]. In the case of increased

concentration in the body, lead can be responsible for developmental disorders of the nervous system manifested by behavioral changes, in both children and adults [6, 7]. Lead has been classified as a potentially carcinogenic element [8] in the International Agency for Research on Cancer classification.

Cadmium is widely used in production processes of alloys including copper, zinc, and iron. It is also used in production of highly specialized electronic products such as microchips or motherboards of computers [9]. Therefore, it is a component of the so-called "e-waste," whose disposal is often carried out improperly or is associated with release of many biologically harmful chemicals into the environment.

According to IARC classification, cadmium is considered a dangerous carcinogenic factor [8]. It is also assumed that exposure to the element causes damage to kidneys and developmental disorders of the skeleton [10, 11]. Increased supply of cadmium during both prenatal and postnatal development can affect the development of the nervous system and consequently be the cause of concentration disorders and hyperactivity in children [12, 13]. It is also believed to have negative impact on spermatogenesis [14]. Apart from workers employed in heavy or electronic industries, also tobacco smokers and people in their vicinity are particularly exposed to the pathogenic effects of cadmium.

Manganese is used in the metallurgical, chemical, and ceramic industries. It is also used in the manufacture of dyes, pesticides, and fertilizers. Similar to lead and cadmium, it can be potentially harmful to health in case of increased supply. High exposure to manganese can cause damage to the respiratory system [15]. This element has a particularly negative effect on maturation and function of the nervous system [15, 16].

Chromium, similar to lead and cadmium, is classified by IARC as a factor inducing development of cancer [8]. It is used in production of dyes, tannins, and cements. This element is present in anticorrosion coatings obtained in the process of galvanization. Exposure to chromium can cause skin lesions and disorders of the respiratory and digestive systems [17]. It also demonstrated mutagenic and embryotoxic action [18–20].

The increasing pollution of the environment associated with the development of civilization makes us vulnerable to the rising supply of heavy metals.

Monitoring the content of substances potentially dangerous to health in the human body is essential for assessing health hazards for individuals and populations being at risk of exposure to heavy metals. Evaluation of the content of biologically harmful trace elements in the body may therefore be the starting point for the implementation of preventive and curative measures for the exposed individuals, as well as contributing to introduction of system solutions limiting the emission of heavy metals into the environment and minimizing the effects of occupational exposure.

Several methods for determination of metals in biological tissues are currently available, for example, inductively Coupled Plasma- (ICP-) Mass Spectrometry (MS) [21] or Ion Chromatography [22, 23]. In the present study the authors applied the Graphite Furnace Atomic Absorption Spectrometry (GFAAS) method, a sensitive and specific technique, which has been used by many clinical chemistry laboratories to determine the presence of trace metals in physiological fluids and in tissues.

2. Aim

The aim of the study was a comparative assessment of the content of trace elements in enamel and dentin of teeth extracted for surgical indications in patients residing in urban and agricultural areas of central Poland.

3. Material and Methods

The study included generally healthy patients living in the Mazowieckie province. Patients were qualified on the basis of the dental surgery health questionnaire and the clinical history card. Individuals working in heavy or chemical industries as well as tobacco smokers were excluded, as well as patients systematically taking vitamin-mineral preparations. A total of thirty generally healthy subjects were qualified, aged 26 to 37 years with fully impacted third molars, which had no contact with the environment of the oral cavity. Thirteen of the enrolled individuals, including seven females and six males, lived in the Warsaw metropolitan area, while seventeen patients, including nine females and eight males, inhabited rural areas of the Mazowieckie province.

Each subject underwent surgical extraction of one of retained third molars. The procedures were performed under local anesthesia and the teeth intended for analysis were removed entirely. In total 30 teeth were extracted from which samples of enamel and dentin were obtained for assessment in the study, five for each tissue type. Immediately after the treatment, the teeth were cleaned from soft tissue residue and rinsed with distilled water and then with HPLC-grade water. From the prepared teeth, samples of enamel and dentin were prepared with diamond drill bits under microscope control, in order to perform quantitative analysis of identified elements, independently for both fractions. After mechanical division of a tested tooth, enamel and dentin samples were left for 24 hours in 30% solution of H_2O_2 to remove organic contaminants, then washed with distilled water and high purity HPLC-grade water, dried at 80°C, and finally ground. Sixty-five samples of enamel and dentin from individuals living in the city of Warsaw were prepared (five for each of the thirteen patients), as well as eighty-five samples of enamel and dentin from individuals living in agricultural areas of the Mazowieckie province (five for each of the seventeen patients). The obtained material was analyzed for the content of lead, cadmium, manganese, and chromium, separately for enamel and dentin of the assessed teeth.

The assay method used for the content of manganese, lead, cadmium, and chromium in the samples of enamel and dentin of the retained teeth was Graphite Furnace Atomic Absorption Spectrometry (GFAAS). The obtained values are given in micrograms per one gram of assessed sample.

In all cases, a variant of the calibration curve was used, whose range matched the concentration of an assessed element in the analyzed material. The detection threshold of the

Table 1: Average concentration of cadmium in hard tissues of impacted teeth (μg/g).

Tissue	Enamel			Dentin			Total		
Place of residence	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$
Urban	0.020	0.002	$p = 0.0001$	0.046	0.008	$p = 0.0001$	0.033	0.005	$p = 0.0001$
Rural	0.015	0.002		0.025	0.009		0.020	0.005	

Table 2: Average concentration of chromium in hard tissues of impacted teeth (μg/g).

Tissue	Enamel			Dentin			Total		
Place of residence	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$
Urban	0.510	0.188	$p > 0.05$	0.640	0.1723	$p > 0.05$	0.575	0.180	$p > 0.05$
Rural	0.510	0.188		0.640	0.1723		0.575	0.180	

elements identified in the study ranged from 0.02 to 0.05 μg/l. The study used Avanta Ultra atomic absorption spectrometer by GBC, with PAL 4000 automatic sample dispenser and graphite furnace.

4. Ethical Considerations

The study was approved by the Bioethical Committee of the Medical University of Warsaw (approval number KB 219/2016). Participation in the study was voluntary, and the patients were informed about its course and assumptions and granted the right to withdraw from the study. Confidentiality of patient data with respect to the results was ensured by assigning numerical codes to samples of biological material subjected to analysis.

5. Methods of Statistical Analysis

Statistical analysis was performed using STATISTICA 8.0 software.

In the process of hypothesis testing the level of significance was assumed at $\alpha = 0.05$.

When the choice was determined by the researcher, conclusions were based on the two-sided critical region.

For each continuous variable, basic statistics were calculated: number (n), the arithmetic mean ($\overline{X}$), standard deviation (SD), median, minimum and maximum values, and indicators of skewness and kurtosis.

In the analysis of means for independent variables, in the case of two-point grouping variable, Student's t-tests or Cochran-Cox tests were used, depending on the result of F test with which the assumption of equal variances was tested. In the case of a three-point grouping variable, ANOVA was used. The assumption of equal variances was tested with Brown-Forsythe tests. Newman-Keuls tests were used as post hoc tests. For the analysis of dependent variables, Student's t-tests were used.

Correlation analysis was based on Pearson and Spearman correlation coefficients. The significance of correlation coefficients was tested with corresponding Student's t-test.

In regression analysis a linear regression model was used. The parameters were estimated by the least squares method.

6. Results

The average concentration of cadmium in the enamel of impacted teeth of residents of agricultural areas of Mazowieckie province equaled 0.015 μg/g and was significantly lower ($p \leq 0.05$) than the concentration of cadmium in the samples of enamel of Warsaw metropolitan area residents, averaging 0.020 μg/g.

The dentin of teeth extracted from residents of rural communities contained an average of 0.025 μg/g of cadmium, whereas dentin in Warsaw residents demonstrated an average of 0.046 μg/g of this element. These concentrations were statistically significantly different ($p \leq 0.05$).

The average concentration of cadmium in the hard tissues of teeth in residents of agricultural areas was 0.020 μg/g, while in the case of hard tissue obtained from the inhabitants of urban areas it was significantly higher, at 0.033 μg/g.

Average concentrations of cadmium in dentin and enamel are shown in Table 1.

The average concentrations of chromium in the enamel of teeth in residents of agricultural and urban areas remained at the same level and equaled 0.510 μg/g.

The average content of the element in the dentin tissue obtained from residents of both populations also assumed the same values and amounted to 0.640 μg/g. The average concentration of chromium in hard tissues of the impacted and extracted teeth was 0.575 μg/g, regardless of the individuals' place of residence.

Average concentrations of chromium in dentin and enamel are shown in Table 2.

The average concentration of manganese in hard tissues of retained teeth in residents of agricultural areas in Mazowieckie province was 0.485 μg/g: the enamel contained an average of 0.639 μg/g and dentin contained 0.320 μg/g of this element. The average concentration of manganese in hard tissues of teeth obtained from residents of Warsaw metropolitan area was 0.485 μg/g: enamel contained

Table 3: Average concentration of manganese in hard tissues of impacted teeth (μg/g).

Tissue	Enamel			Dentin			Total		
Place of residence	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$
Urban	0.640	0.117	$p > 0.05$	0.330	0.097	$p > 0.05$	0.485	0.106	$p > 0.05$
Rural	0.639	0.136		0.330	0.121		0.485	0.129	

Table 4: Average concentration of lead in hard tissues of impacted teeth (μg/g).

Tissue	Enamel			Dentin			Total		
Place of residence	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$	Concentration (μg/g)	SD	$\alpha = 0.05$
Urban	1.655	0.145	$p = 0.0001$	2.315	0.171	$p = 0.0507$	1.985	0.158	$p = 0.0010$
Rural	1.208	0.276		2.110	0.344		1.659	0.260	

on average 0.640 μg/g, while for dentine the value was 0.320 μg/g. As in the case of chromium content, manganese also demonstrated no differences in the concentrations of the element in the evaluated hard tissues of teeth in both populations of patients.

Average concentrations of manganese in dentin and enamel are shown in Table 3.

The average concentration of lead in the enamel of teeth in residents of agricultural areas averaged 1.208 μg/g and was significantly ($p \leq 0.05$) lower than the concentration in the tissue obtained from the inhabitants of urban areas, which amounted to 1.655 μg/g. The lead content in the dentine of teeth obtained from the rural residents did not differ significantly ($p \geq 0.05$) from the content of the element in the tissue of teeth of urban residents. These values averaged, respectively, 2.110 μg/g and 2.315 μg/g. In total, the hard tissues of retained teeth in residents of agricultural areas demonstrated significantly lower ($p \leq 0.05$) lead concentration at 1.659 μg/g, compared with enamel and dentine samples obtained from urban residents, which on average contained 1,985 μg/g of the element.

Average concentrations of lead in dentin and enamel are shown in Table 4.

7. Discussion

Lead, cadmium, manganese, and chromium are deposited in tissues and cause pathogenic action not only on individual organs but also on the whole body. In order to assess the concentration of trace elements in the organism, body fluids such as blood and urine are analyzed, as well as samples biopsied from individual organs including liver or kidney, bone fragments, and teeth [24].

Samples of blood and urine are easy to obtain. Their analysis, however, provides information only about recent and short-term exposure to harmful chemical substances. In this way the level of heavy metals in individual tissues is not detected either, which constitutes information about long-term exposure. Collecting biopsy samples from solid organs and bone tissue is technically difficult and is not a method used for screening.

The material, which is commonly available and at the same time easy to obtain, is hard tissue of teeth, that is, enamel and dentin. Because they are not as extensively metabolised as bone tissue [25], they constitute a reservoir of heavy metal ions, whose concentration informs us about the effects of long-term exposure to harmful elements in the context of health risk assessment. It is assumed that the level of metals observed in tissues of teeth correlates with their presence in the bloodstream, especially in the context of long-term exposure [26]. Deciduous teeth, which are lost naturally, constitute relatively easily accessible material for analysis. However, due to the processes of forming and mineralization of the enamel and dentin during the prenatal and postnatal periods, they provide information about exposure to heavy metals of fetuses and young children [27, 28]. It should also be noted that results of tissue analysis of deciduous teeth do not have to be one hundred percent reliable because of the placenta partially protecting fetuses against transmission of harmful elements from the mother's body and because children are usually protected against harmful environmental influences, which in adults can be associated with occupational exposure. Determining the concentration of harmful elements in the bodies of adults by analyzing the enamel and dentin refers to permanent teeth. Their development falls on the postnatal period and lasts—in the case of third molars—until about 20 years of age. Impacted teeth seem to constitute particularly valuable material for research, having no contact with the environment of the oral cavity even after the eruption period. They can provide research material on the basis of which we can assess the degree of prolonged exposure to heavy metals in adults.

The results of the present study indicate that hard tissues of evaluated impacted teeth contained significantly more lead and cadmium in the case of samples from the inhabitants of large urban areas than enamel and dentin of teeth extracted from the rural residents.

No studies in available literature describe the concentration of trace elements in enamel and dentin of the Polish population. Available literature describing studies from other parts of the world contains reports confirming the above observation.

A study by Costa de Almeida et al. [29], assessing the concentration of lead in the surface layers of tooth enamel, demonstrated significantly higher concentration of the element in samples from urban residents compared to samples of enamel in inhabitants of nonindustrialized areas. Similar observations were made by other authors [30, 31].

Prodana et al. [32] evaluated the concentration of cadmium in enamel and dentin of deciduous teeth of children living in industrialized and rural areas. The results reported by the authors from Romania indicate that the potentially harmful biological element was present in greater amounts in hard tissues of teeth of children living in highly industrialized areas at risk of environmental contamination.

Similar observations were made by authors from Spain [33] evaluating the relationship between place of residence and cadmium content in hard tissue of deciduous teeth, who confirmed the existence of a positive correlation between high environmental contamination and a higher concentration of a pathogenic element in enamel and dentin.

The above observations support the thesis, as reflected in the results of the present study, that concentration of lead and cadmium in hard tissues of teeth depends on the place of residence of the population whose samples were evaluated, and it is correlated with environmental pollution.

A different opinion was presented in the publication by Maah et al. [34]. The study evaluated cadmium content in hard tissues of permanent teeth extracted from residents of two different regions of Malaysia. The authors did not confirm the existence of a positive correlation between the concentration of the element and the place of residence; however, they noted that such a link exists in the case of cigarette smoking and suggested that the lack of significant differences could result from similar diet of patients living in both regions of the country included in the study.

Because the present study excluded patients declaring tobacco smoking, which is a major source of exposure to cadmium, it seems that the results are more meaningful in the context of exposure to the element resulting from environmental contamination.

In the available literature there are no publications correlating the concentration of manganese and chromium in hard tissues of teeth with the degree of environmental contamination. The study did not report the existence of any relationship between concentrations of these elements in enamel and dentin on one hand and the place of residence of the patients from whom tissue samples were collected on the other hand.

The reason for the lack of such correlation may be exclusion from the study of people working in heavy and chemical industries, which are predisposing factors for exposure to chromium and manganese in the context of occupational risk.

The literature provides relatively numerous reports assessing the concentration of metallic elements in hard tissues of deciduous teeth [29–40]. Unfortunately, the results published therein cannot be directly referred to the observations made in the present study because those publications evaluate the content of potentially harmful elements in deciduous teeth or, less frequently, in permanent teeth exposed to the oral environment.

Third molars, which were used in the present study, constitute a slightly different material for physicochemical analysis. It should be noted that they appear in the oral environment relatively late or remain in the bone tissue as impacted teeth, so they do not accumulate in themselves or accumulate in relatively small quantities, elements which have direct contact with their surface. Most often they are not exposed to masticatory forces; therefore, tooth crowns, which are used for samples to be analyzed, are not mechanically damaged. In the case of analysis of tissue composition of retained teeth, the influence of the external environment is eliminated, that is, drinks, food, temperature differences, and action of cariogenic microorganisms. Analyzed samples of third molars provide information about the influence of the external environment during the period from about seven to about eighteen years of age, when their buds are formed and mineralized. Therefore, they constitute a kind of 10-year carrier of data about diet, health, and human exposure to the effects of trace elements found in the external environment.

In a study by Liu et al. [41] the authors evaluated the content of phosphorus, calcium, strontium, barium, and lead in the enamel and dentine of third molars extracted for surgical indications. In their study the researchers from Taiwan used both impacted teeth and teeth in contact with the environment of the oral cavity of patients aged 17 to 68 years. The results presented by Liu et al. [41] allow identification of age groups 20–25 years and 25–30 years in the assessed population, roughly corresponding to the age group of patients evaluated in the present study.

The average concentration of lead in enamel of teeth in patients from Taiwan aged 20–25 years was about 0.53 μg/g, while in the group of 26–30-year-olds it equaled about 0.77 μg/g. The concentration of the element in the dentine of third molars evaluated by Liu et al. was 1.07 μg/g and 0.7 μg/g, respectively, for the studied age groups and it was significantly higher than its content in the enamel tissue.

The results of the present study confirm the reports by researchers from Taiwan [41] according to whom enamel has a lower potential to accumulate lead ions than dentine, which can be associated both with the period of development, during which both tissues are formed as well as with their chemical composition.

The results obtained by the authors from Taiwan [41] describing a lower concentration of lead in the hard tissues of teeth, compared to the concentrations noted in the present study, may indicate different environmental exposure to this element, which is potentially harmful to health with respect to both populations. Another reason for differences in results in terms of quantity could be the use of different analytical methods.

8. Conclusion

Despite an ever-expanding knowledge about the negative influence of heavy metal ions on the body, we still do not know the whole spectrum of their negative impact on human health. There is no doubt, however, that the risk of exposure to potentially harmful biological trace elements should be minimized, not only in particularly exposed occupational

groups, but also in whole populations. One of the components of health risk assessment is monitoring the content of harmful elements in the body. In contrast to hair or body fluids, impacted teeth, obtained as a result of routine dental surgical procedures, seem to constitute extremely valuable material that allows quantitative and qualitative assessment of long-term exposure of patients to chemicals potentially dangerous to health.

The results of the present study confirm that the risk of exposure to heavy metals depends on the place of residence and is associated with environmental pollution.

In order to ensure the highest reliability of further studies, it is necessary to include possibly the largest group of patients, strictly observing the inclusion/exclusion criteria.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

A Comparative Chemical Study of Calcium Silicate-Containing and Epoxy Resin-Based Root Canal Sealers

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Objective. The present study assessed the chemical elements in two novel calcium silicate-containing root canal sealers, BioRoot RCS and Well-Root ST, compared to a calcium silicate-containing root canal sealer that has been on the market for several years, MTA Fillapex, and epoxy resin-based sealer AHPlus. *Material and Methods.* The sealers were mixed and manipulated according to the manufacturers' instructions. Twelve cylindrical molds (inner diameter 4 mm; height 3 mm) were placed on a glass petri dish and packed with the materials. The dish was transferred to an incubator. After 72 h the molds were examined by scanning electron microscopy and energy dispersive X-ray microanalysis. *Results.* BioRoot RCS and Well-Root ST had high peaks of calcium, zirconium, oxygen, carbon, silicon, and chlorine. Well-Root ST also had sodium, magnesium, aluminum, and titanium peaks. MTA Fillapex and AHPlus had carbon, oxygen, calcium, titanium, and bismuth peaks. A silicon peak was also observed for MTA Fillapex, and zirconium and tungsten peaks for AHPlus. *Conclusion.* BioRoot RSC had the highest degree of purity. The clinical implication of metals contained in the other sealers needs to be investigated.

1. Introduction

Filling of the root canal involves the use of core material, such as gutta-percha, in combination with root canal sealer to provide an adequate seal. The primary role of the sealer is to obliterate irregularities between the root canal wall and the core material [1–4]. Root canal sealers, even if they will not be extruded beyond the apical foramen, are in direct contact with periodontal ligament and bone over extended periods of time and may release toxic elements, irritating these tissues and influencing the final outcome of the root canal. Therefore, a study of the chemical characteristics of root canal sealers is desirable [5].

Root canal sealers can be grouped based on their prime constituent or chemical structure, such as zinc oxide-eugenol, calcium hydroxide, silicone, glass ionomer, and epoxy or methacrylate resins. Recently, a new type of sealer containing mineral trioxide aggregate and calcium silicate has been developed. An advantage with these novel sealers is their potential bioactive properties. Similar to other silicate-containing materials, $Ca(OH)_2$ is produced upon reaction with water, leading to a high alkaline pH that activates and stimulates the expression of alkaline phosphatase, favoring the formation of mineralized tissue and having an antimicrobial effect. In addition, the alkaline pH could neutralize the lactic acid from osteoclasts and

prevent dissolution of the mineralized components of teeth [6–9].

One of the first mineral trioxide aggregate-containing root canal sealers introduced on the market was MTA Fillapex (Angelus, Londrina, Brazil). Because it has been available for 5 years, it is the most studied MTA-containing root canal sealer. MTA Fillapex is a paste-catalyst material. Paste A is composed of salicylate resin (methyl salicylate, butylene glycol, and colophony), bismuth oxide, and silica. Paste B includes silicon dioxide, titanium dioxide, and base resin (pentaerythritol, rosinate, and toluene sulphonamide), and 13.2% set MTA particles as filler. The working time is 23 min, with a complete set time of approximately 2 h. Several properties of this root canal sealer, such as flow and viscosity [10, 11], dimensional change [11], material porosity [12, 13], sealing ability [11], radiopacity, electrical conductivity [14], antibacterial effect [15], biocompatibility [16–20], cytotoxicity [19, 21–23], and genotoxicity [24, 25], have been investigated.

Another recently introduced sealer based on tricalcium silicate is Well-Root ST (Vericom, Gangwon-Do, Korea). This sealer is a premixed, ready-to-use, injectable bioceramic cement paste developed for permanent obturation of the root canal. The composition of Well-Root as described by the manufacturer includes zirconium oxide, calcium silicate, filler, and thickening agents [26]. The material is hydrophilic and uses moisture in dentinal tubules to initiate and complete its setting reactions. The setting time is 25 min, but in root canals the setting time can be more than 2.5 h. According to the manufacturer, the Well-Root ST should be used in conjunction with gutta-percha points. No information on the chemical composition and physical properties of this root canal sealer is available in the current scientific literature.

A new tricalcium silicate-based root canal sealer was introduced recently. BioRoot RCS (Septodont, Saint Maur-des-Fosses, France) consists of a powder and a liquid. The powder is composed of tricalcium silicate, zirconium dioxide, and povidone, and the liquid is composed of water, calcium chloride, and polycarboxylate. The BioRoot RCS has a minimum working time of 10 min and a maximum setting time of 4 h. This silicate-based root canal sealer has less toxic effects on human periodontal ligament cells than zinc oxide-eugenol sealer and induces a higher secretion of angiogenic and osteogenic growth factors than ZOE [27]. BioRoot RCS compared to contemporary root canal sealers (AHPlus, Acroseal, EndoRez, RealSeal SE, Hybrid Root SEAL, RootSP, and MTA Fillapex) has the lower cytotoxicity and genotoxicity [24]. The sealing properties of BioRoot RCS combined with gutta-percha are comparable to those of AHPlus, but microCT has revealed a higher void volume for BioRoot RCS than resin-based sealer, possibly due to the shorter working time and less flow than AHPlus [28].

AHPlus (Dentsply, DeTrey, Konstanz, Germany) is an extensively studied epoxy resin-based sealer and considered a gold standard endodontic sealer. The material is composed of epoxy resin, calcium tungstate, aerosil, iron oxide, adamantane amine, N,N-dibenzyl-5-oxanonane, TCD-diamine, calcium tungstate, and zirconium oxide.

Because of the good biocompatibility of bioceramic cements, calcium silicate-based root canal sealers are increasingly used for permanent root canal filling (BioRoot RCS, Septodont, Saint Maur-des-Fosses, France; Endo-CPM sealer, EGEO, SRL, Buenos Aires, Argentina; Endo Sequence BC Sealer, Brasseler Savannah, GA; iRoot, Innovative Bioceramix Inc., Vancouver, Canada, MTA Fillapex, Angelus, Londrina, Brazil; ProRoot ES Endo Root Canal Sealer, Dentsply Tulsa, Johnson City, TN; Tech Biosealer, Isasan, Rovello Porro, Italy; Well-Root ST, Vericom Co., LTD, Gangwon-Do, Korea). However, several studies have shown that some of these materials may cause cellular degeneration and delayed wound healing of periapical tissues [29, 30]. The cytotoxic effect of these endodontic sealers may be caused by heavy metals released from the set materials [11, 31].

Many studies have evaluated the chemical elements and heavy metals in MTA Fillapex and AHPlus [14, 29, 30] but, to the best of our knowledge, no studies have chemically analyzed the two new calcium silicate-containing root canal sealers, BioRoot RCS and Well-Root ST. The aim of the present study was to determine the chemical elements in these novel calcium silicate-containing root canal sealers. The results were compared to a calcium silicate-containing root canal sealer that has been on the market for several years MTA Fillapex and epoxy resin-based sealer AHPlus.

2. Material and Methods

The following root canal sealers were used in this study:

(1) BioRoot RCS (Septodont, Saint Maur-des-Fosses, France)

(2) Well-Root ST (Vericom, Gangwon-Do, Korea)

(3) MTA Fillapex (Angelus, Londrina, Brazil)

(4) AHPlus (Dentsply, DeTrey, Konstanz, Germany).

2.1. Sample Preparation. All sealers were mixed and manipulated according to the manufacturers' instructions. For scanning electron microscopy (SEM), twenty cylindrical molds with an inner diameter of 4 mm and height of 3 mm were placed on a glass petri dish and packed with the materials. Five homogeneous specimens were made for each studied material. The dish was then covered with wet gauze and transferred to an incubator (37°C, 95% relative humidity). After 72 h the specimens were ground by progressively finer diamond discs and pastes using a polishing machine.

2.2. Scanning Electron Microscopy and Energy Dispersive Microanalysis. The materials were examined using the following methods: SEM with a FE-SEM Hitachi SU-70 microscope, energy dispersive spectroscopy (EDS) X-ray microanalysis using NORAN™ System 7 UltraDry X-ray detector (Thermo Fisher Scientific). All analyses were performed at an accelerating voltage of 25 kV and electron beam current of approximately 3 nA. The "PROZA" correction method was applied for quantitative EDS analyses. The estimated uncertainty for EDS measurements was 0.1 wt.%. Samples

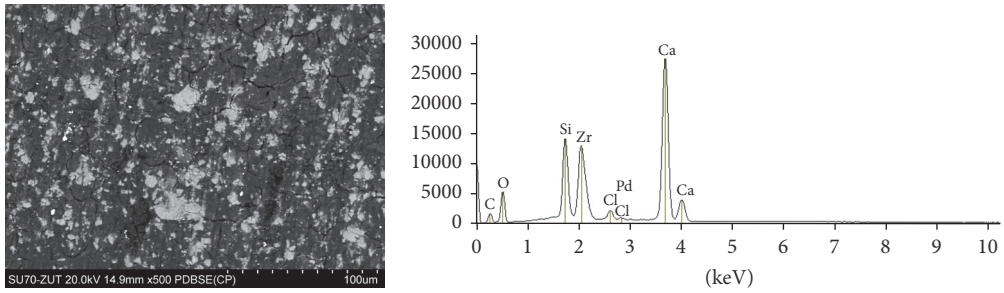

Figure 1: BioRoot RCS: backscatter scanning electron micrographs at 500x magnification (a); EDS X-ray microanalysis (b).

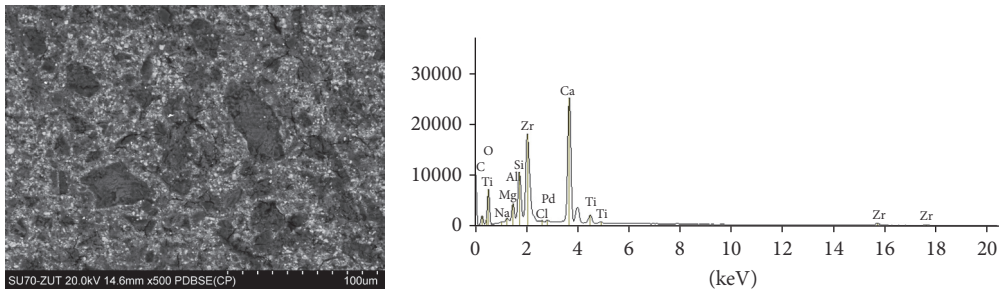

Figure 2: Well-Root ST: backscatter scanning electron micrographs at 500x magnification (a); EDS X-ray microanalysis (b).

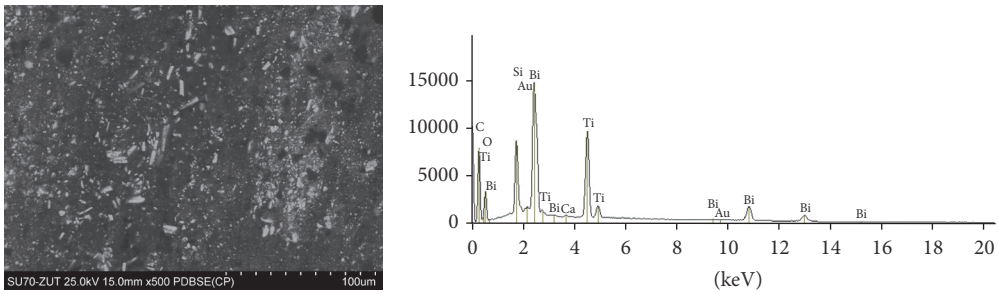

Figure 3: MTA Fillapex: backscatter scanning electron micrographs at 500x magnification (a); EDS X-ray microanalysis (b).

were coated with a very thin coating of gold-palladium alloy for electric conductivity. The metals used to sputter coat the specimens were excluded from the percentage found. Backscattered electron images in compositional contrast were acquired.

3. Results

The SEM images and EDS profiles for the randomly selected areas of equal sizes of the studied root canal sealers are shown in Figures 1–4. Quantitative results of elements according to microanalysis are described in Table 1. EDS microanalysis of BioRoot RCS and Well-Root ST revealed high peaks for calcium, zirconium, oxygen, silicon, carbon, and chlorine. For Well-Root ST, peaks were also present for sodium, magnesium, aluminum, and titanium. MTA Fillapex and AHPlus had peaks for carbon, oxygen, calcium, titanium, and bismuth. For MTA Fillapex, a peak was also present for silicon, and for AHPlus peaks were present for zirconium and tungsten.

EDS analysis performed for the components of investigated areas reveals that BioRoot RCS was composed of particles rich in zirconium, hafnium, and oxygen (marked (1) and (2) in Figure 5), and the cementation phase was composed of calcium, silicon, oxygen and carbon (marked (3), (4), and (5) in Figure 5). The size of the cement particles ranged from 5 μm to 30 μm. Well-Root ST had a similar composition. The particles were rich in zirconium, hafnium, and oxygen (marked (1) and (2) in Figure 6). However, in contrast to BioRoot RCS, zirconium was also found in the cementation phase (marked (3), (4), and (5) in Figure 6). In this phase,

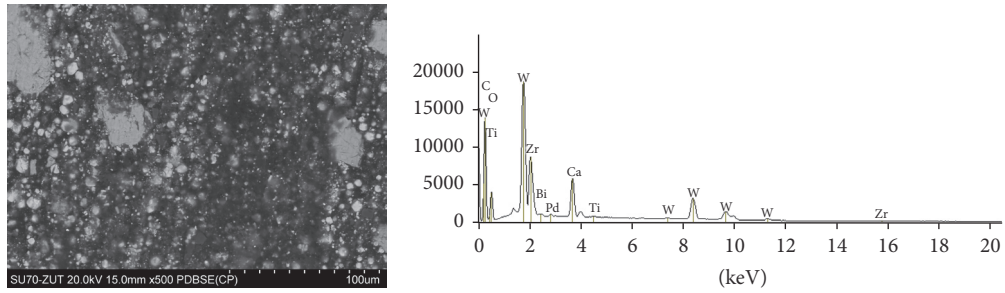

Figure 4: AHPlus: backscatter scanning electron micrographs at 500x magnification (a); EDS X-ray microanalysis (b).

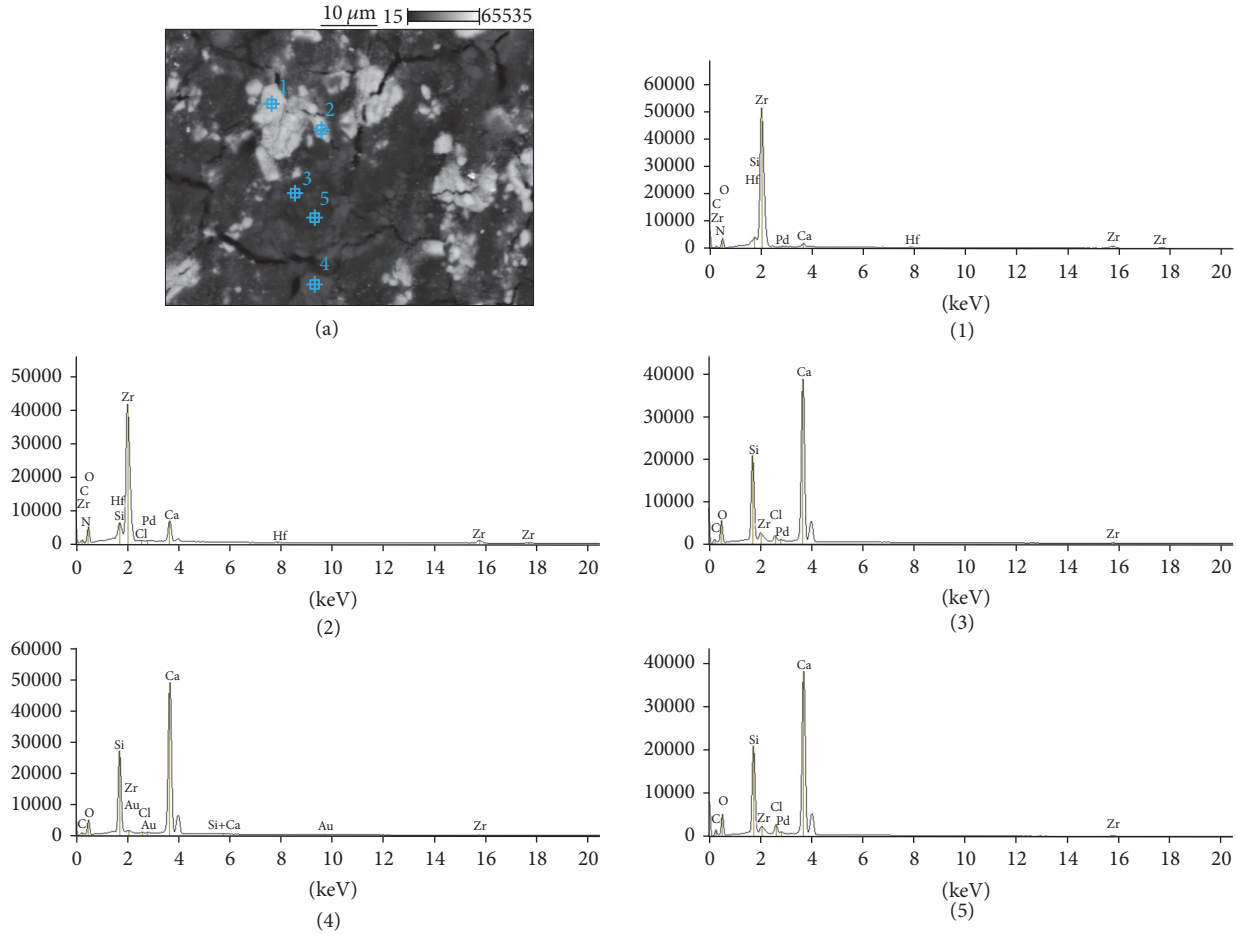

Figure 5: BioRoot RCS: backscatter scanning electron micrographs at 1000x magnification (a); EDS X-ray microanalysis of particles and cementation phase (1, 2, 3, 4, and 5).

peaks were also observed for oxygen, chlorine, silicon, aluminum, calcium, magnesium, and titanium. MTA Fillapex was composed of elongated particles approximately 10–15 μm long that exhibited peaks for bismuth, oxygen, carbon, and titanium (marked (1) and (2) in Figure 7) and roundish particles approximately 2-3 μm that exhibited peaks for titanium, bismuth, carbon, silicon, and oxygen (marked (4) and (5) in Figure 7). The cementation phase was rich in silicon, carbon, oxygen, titanium, and bismuth (marked (3) in Figure 7). AHPlus was composed of larger particles (marked (1) in Figure 8) rich in zirconium, hafnium, tungsten, carbon and oxygen, and smaller particles (marked (2), (3), and (4) in Figure 8) rich in tungsten, carbon, and calcium. Both particles were interspersed in the cementation phase composed of silicon and carbon, zirconium, and tungsten (marked (5) in Figure 8).

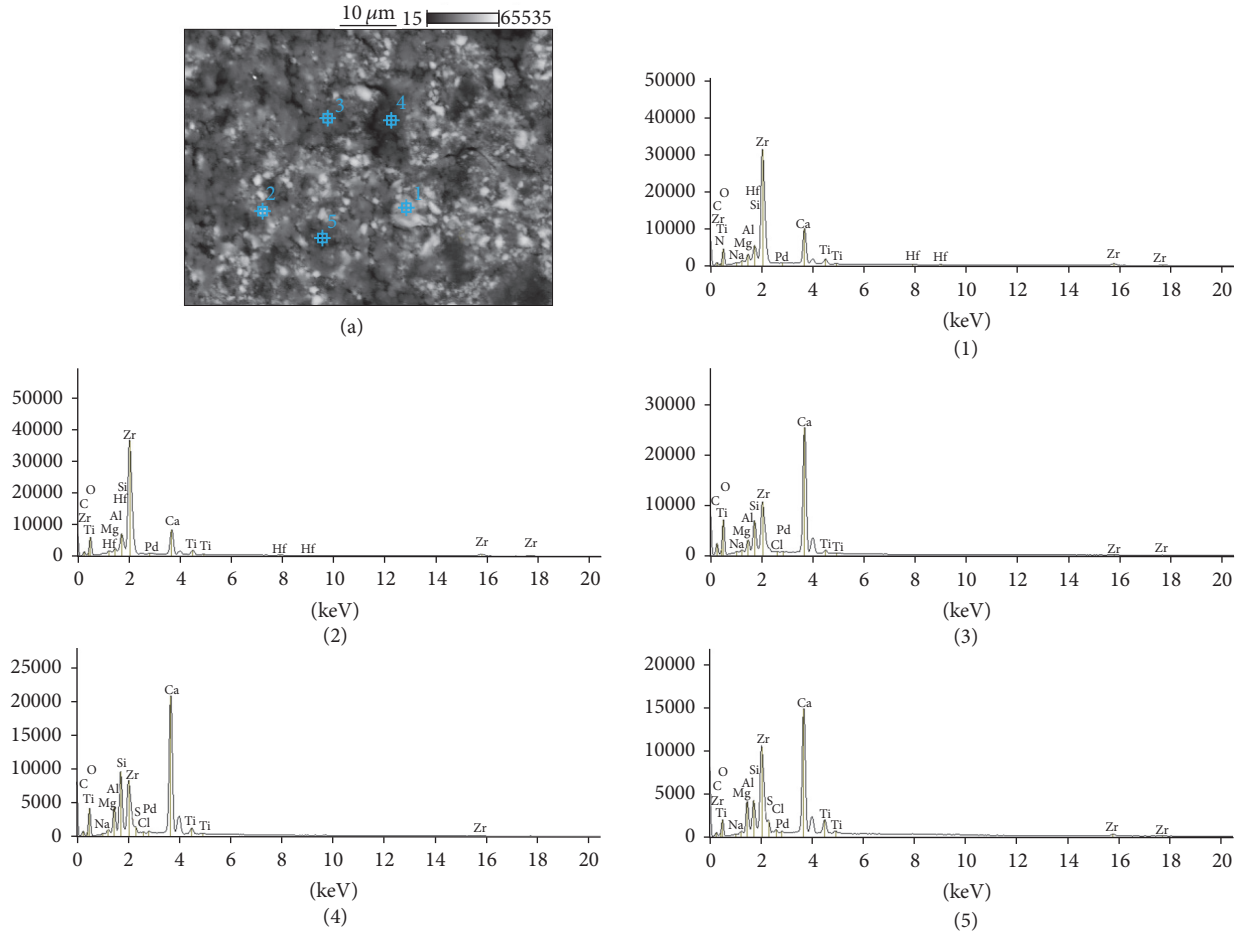

Figure 6: Well-Root ST: backscatter scanning electron micrographs at 1000x magnification (a); EDS X-ray microanalysis of particles and cementation phase (1, 2, 3, 4, and 5).

Table 1: The percentage (weight%) of elements in the tested root canal sealers.

Element	Root canal sealer			
	BioRoot	Well-Root ST	MTA Fillapex	AHPlus
C	5.6–6.2	5.4–6.0	20.0–21.9	31.4–34.5
O	35.1–37.1	37.9–39.3	21.3–22.8	19.2–21.2
Si	8.4–9.4	4.6–5.4	4.5–6.9	—
Cl	1.1–1.3	0.4–0.6	—	—
Ca	25.0–26.6	21.0–22.1	0.1–0.2	4.5–4.9
Zr	20.3–22.6	22.2–27.4	—	15.1–18.6
Na	—	0.3–0.4	—	—
Mg	—	0.5–0.6	—	—
Al	—	1.9–2.5	—	—
Ti	—	1.0–2.1	11.5–15.6	0.1–0.2
Bi	—	—	34.4–38.9	0.3–0.4
W	—	—	—	22.5–24.4

4. Discussion

In the current study, the chemical compositions of two new calcium silicate-containing root canal sealers, BioRoot RCS and Well-Root ST, were assessed and compared to the composition of extensively studied materials: calcium silicate-containing root canal sealer MTA Fillapex and epoxy resin-based sealer AHPlus.

EDS revealed that BioRoot RCS is mostly composed of calcium, zirconium, oxygen, carbon, silicon, and chlorine. No heavy metals or other toxic elements were found in this endodontic sealer. The elements observed in the present study were biocompatible with those given by manufacturer of BioRoot RCS and determined by Camilleri in an experimental tricalcium silicate-based endodontic sealer by Septodont [32]. However, the microanalysis revealed that Well-Root ST contained aluminum and titanium in addition to calcium, zirconium, oxygen, carbon, and silicon.

In both new silicate-based root canal sealers, the particles interspersed in the cementation phase were composed of zirconium, hafnium, and oxygen, making up the radiopacifiying material. Although zirconium oxide provides a lower contrast than other radiopacifiers, such as bismuth oxide, it seems to be more inert [33].

EDS analysis of MTA Fillapex revealed that the outer surface is rich in carbon, calcium, oxygen, silicon, titanium, and bismuth, whereas AHPlus is composed of carbon,

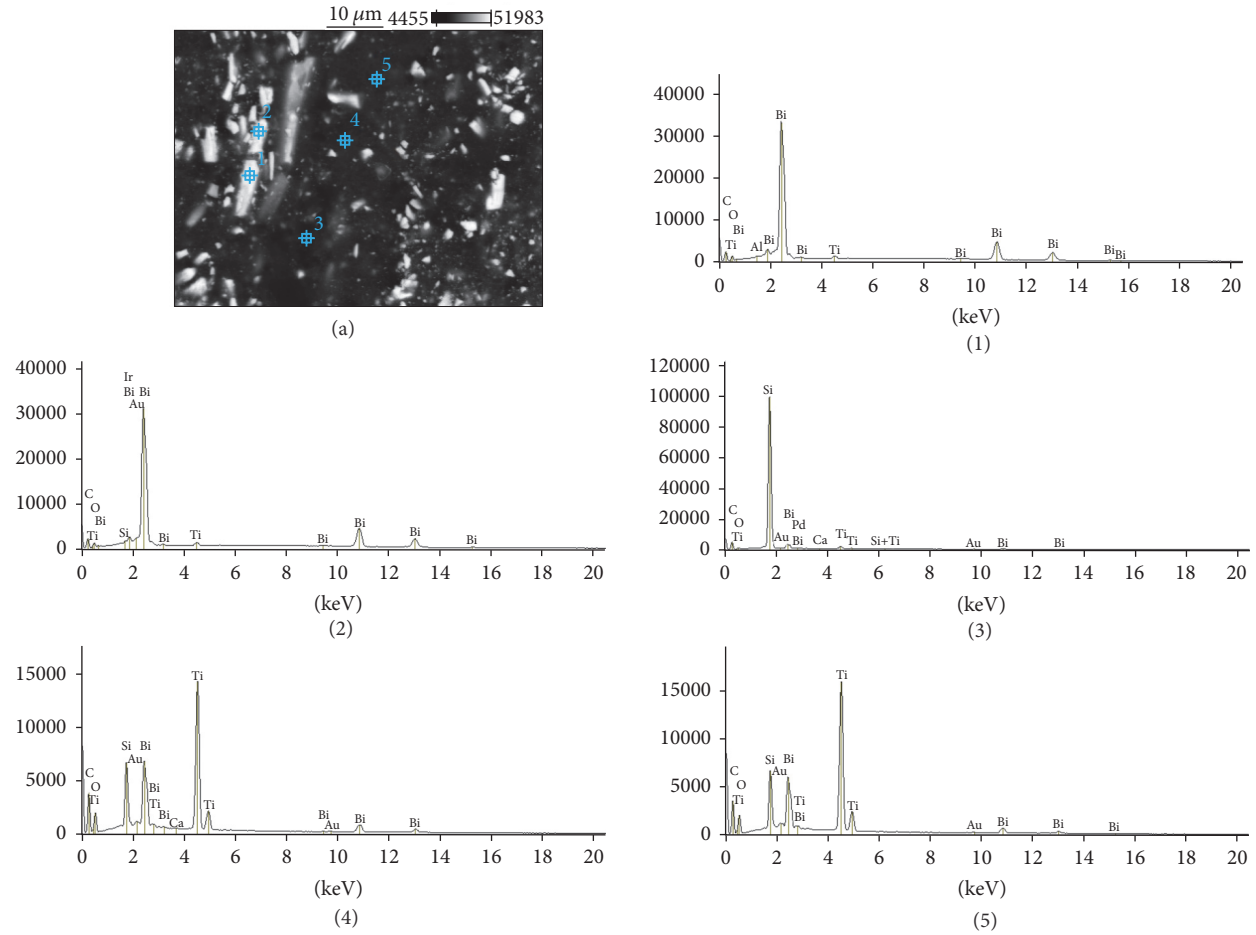

Figure 7: MTA Fillapex: backscatter scanning electron micrographs at 1000x magnification (a); EDS X-ray microanalysis of particles and cementation phase (1, 2, 3, 4, and 5).

oxygen, calcium, zirconium, and tungsten. These results are in accordance with previous reports [34, 35]. However, Gandolfi and Prati observed that MTA Fillapex also contains aluminum and sulfur, and AHPlus also contains aluminum and iron [36]. The differences between cited studies may be explained by variations in the experimental conditions.

When EDS was used to confirm the chemical composition of particles interspersed in the cementation phase, MTA Fillapex exhibited peaks for bismuth, titanium, and oxide, and AHPlus for zirconium, tungsten, and oxide. These elements (bismuth oxide and zirconium oxide) are added to improve the radiopacity of endodontic sealers. According to many researchers, bismuth is associated with the discoloration of bioceramic materials and tooth tissue [37–39]. However, Ioannidis et al. compared in vitro MTA Fillapex with Roth-811 cement and found that the application of MTA-based root canal sealer results in minimal color alterations of tooth tissues (not clinically perceptible discoloration), whereas zinc oxide-eugenol cement induces severe discoloration [40].

SEM and EDS are standard methods and have been utilized previously to assess the chemical composition of root canal sealers and other endodontic materials [14, 30, 32, 33]. These methods are relatively simple and not time-consuming. However, EDS microanalysis has some limitations, including the detection of light elements and an X-ray detection limit of ~0.1% depending on the element. Therefore, some authors suggest a qualitative and quantitative overview by SEM-EDS followed by more precise ICP-OES [41].

5. Conclusion

Among the materials evaluated in this study, BioRoot RSC represents the highest degree of purity. The clinical implication of heavy metals contained in Well-Root, MTA Fillapex, and AHPlus needs to be investigated.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

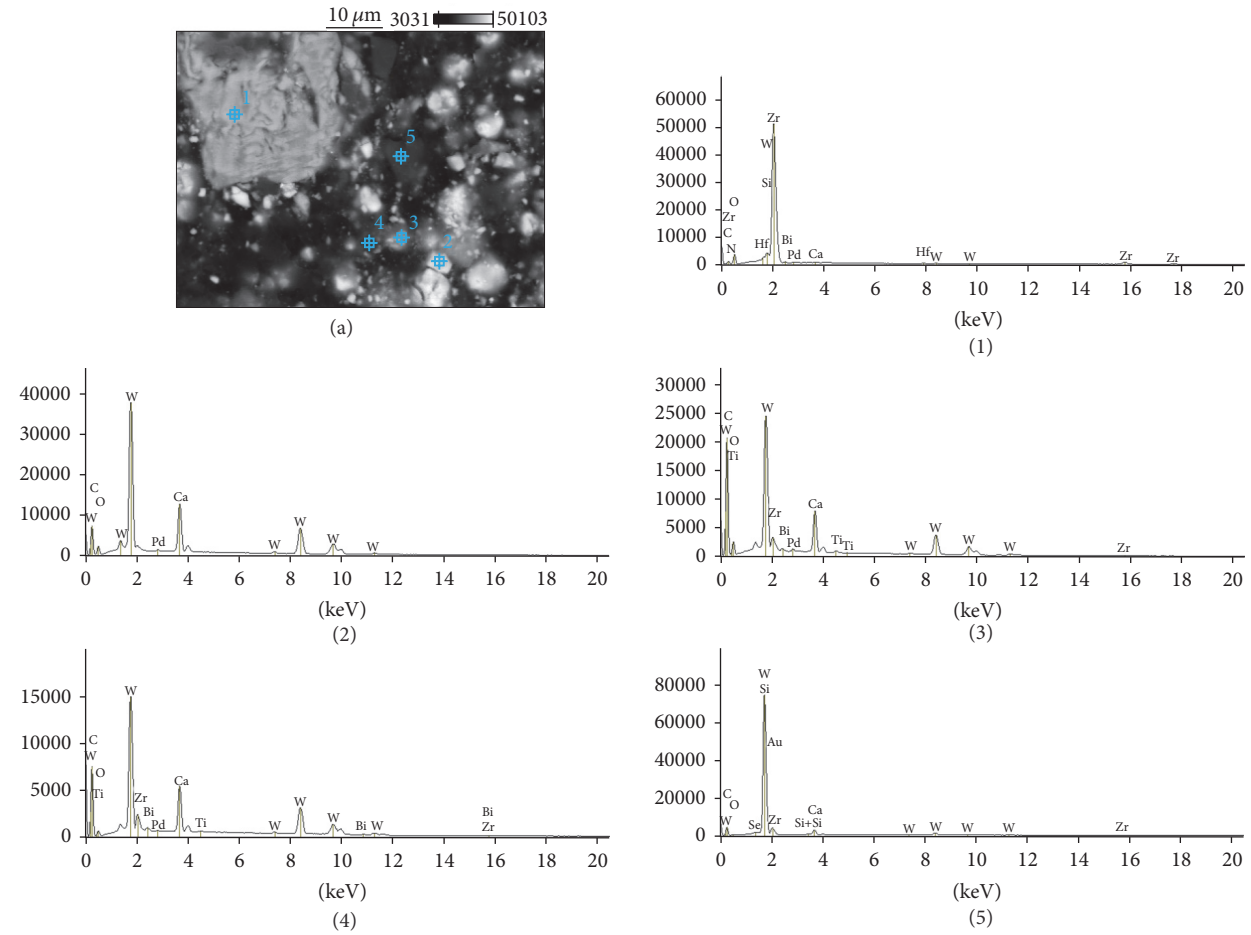

Figure 8: AHPlus: backscatter scanning electron micrographs at 1000x magnification (a); EDS X-ray microanalysis of particles and cementation phase (1, 2, 3, 4, and 5).

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Research Article

Do Mechanical and Physicochemical Properties of Orthodontic NiTi Wires Remain Stable In Vivo?

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Introduction and Aim. Exceptional properties of the NiTi archwires may be jeopardized by the oral cavity; thus its long-term effect on the mechanical and physiochemical properties of NiTi archwires was the aim of work. *Material and Methods*. Study group comprised sixty 0.016 × 0.022 NiTi archwires from the same manufacturer evaluated (group A) after the first 12 weeks of orthodontic treatment. 30 mm long pieces cut off from each wire prior to insertion formed the control group B. Obeying the strict rules of randomization, all samples were subjected to microscopic evaluation and nanoindentation test. *Results.* Both groups displayed substantial presence of nonmetallic inclusions. Heterogeneity of the structure and its alteration after usage were found in groups B and A, respectively. *Conclusions.* Long-term, reliable prediction of biomechanics of NiTi wires in vivo is impossible, especially new archwires from the same vendor display different physiochemical properties. Moreover, manufacturers have to decrease contamination in the production process in order to minimize risk of mutual negative influence of nickel-titanium archwires and oral environment.

1. Introduction

Since the 18th century when Edward Angle introduced orthodontic fixed appliances based on physical properties of wires inserted into bracket slots, they have been constantly improved, thus facilitating both the orthodontist's work and efficiency of the devices [1].

Considering composition of wires 3 major alloys may be currently distinguished: stainless steel, β-titanium, and—last but not least—nickel-titanium ones. Exceptional flexibility of the latter ones, resulting from their physical and chemical properties, technically allows application of nickel-titanium archwires thorough the whole therapy, often limiting number of used wires to 2-3 per treatment [1–4]. Nonetheless oral environment, namely, repetitive, wide-range changes of temperature; low pH; increased partial pressure of hydrogen ions as well as the microbe metabolism products, altogether may have vital effects on the wires via changing their physical and chemical properties [3, 5–9]. Our previous studies showed that the oral environment can change the mechanical properties of nickel-titanium wires within a period as short as 6–8 weeks [5]; that is why the obvious questions have risen. (1) Is the long-term influence of an oral environment capable of changing microstructure of nickel-titanium wires? (2) Do these changes possibly affect the release of microelements into the oral environment? Resolving those yet not answered issues might have brought scientific evidence supporting either health-care—in terms of prevention against undesirable chemical influences—or production of high quality archwires maintaining their properties thorough the orthodontic treatment course.

Thus this metallographic study was aimed at assessing the long-term effect of oral environment on NiTi wires in order to answer those above posted questions.

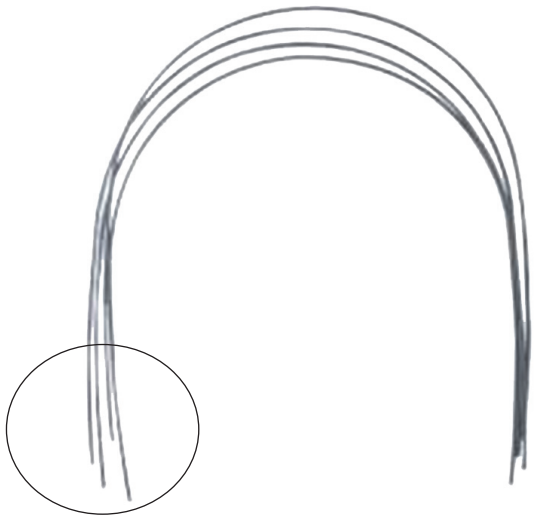


Figure 1: Orthodontic wires: location of the specimen at the distal free ends is marked with the circle.

2. Material and Methods

In order to increase homogeneity 60 orthodontic patients undergoing treatment with the products from only one company were enrolled in the project. Simultaneously the choice of one vendor allows obtaining preliminary data that will allow authors to expand research in the direction of cross-sectional studies on the wires of other brands. Study group (A) comprised 0.016 × 0.022 NiTi archwires randomly taken from 12 different packages, again originating from the same facility, passively, and piggyback ligated to the levelling archwires. Prior to ligation 30 mm long distal ends (Figure 1) were cut off from each archwire, inserted into the plastic bags, numbered consecutively from B1 to B60, and stored in the patient's paper file thus, composing control group B. After the first 12 weeks of orthodontic treatment 30 mm long pieces of each wire from group A were cut off and inserted separately into the plastic bags numbered from A1 to A60 according to the samples from group B.

Two envelopes containing all wires from both groups marked "A" and "B" for blinding the outcomes were sent to the laboratory. Successively the engineer further treated all the specimens grinding them in the longitudinal and transverse manner depending on the direction of plastic deformation and then polishing them mechanically. All samples were subjected to microscopic evaluation and nanoindentation test. Afterwards randomly selected half of the wires from group A and B separately underwent chemical etching with a mixture of hydrofluoric and nitric V acids (1.5 : 1 ratio) and were subjected to microscopic analysis.

Composition of the whole material is displayed in Table 1.

3. Microscopic Analysis

Visitron Systems integrated digital camera using NIS Elements BR software registered all specimen images further evaluated under NIKON ECLIPSE MA200 microscope with magnification ranging from 100 to 1000 times.

In order to assess the degree of contamination with nonmetallic inclusions the microscopic picture of the nonetched samples was compared with the norm PN-64/H-04510. Its scale contains 5 patterns, each of them divided into three variants (a, b, and c) based on the distribution of nonmetallic inclusions. When providing the final results only the highest scored pattern counts were registered separately for each type of inclusion. Thus the contamination is pronounced as the number of pattern and its variety.

Microscopic analysis also enabled us to determine crystal structure of all etched samples in the study material.

4. Nanohardness Analysis

Indentation Release Candidate "SBO" allowed measurement of the nanohardness based on maximum operating force and the maximal probe indentation (HV_{IT}) as well as on Instrumental Young's Modulus (E_{IT}) calculations. The measurement itself was nothing else but the maximum 250.0 mN loading of Berkovich indenter, lasting 15 seconds and resulting in the tetrahedron shape imprint. Both force values and the depth of penetration of the blade were recorded in the cycle of loading and unloading; an arithmetic mean was considered to be the end results. Loading factors as a function of penetration depth were determined for each cycle in three randomly selected locations of the wires from groups A and B separately.

Instrumental Young's Modulus was calculated using the Oliver and Pharr's method determining the force-displacement curve applying an appropriate formula:

$$\frac{1}{E_{IT}*} = \frac{\left(1 - v^2\right)}{E_{IT}} + \frac{\left(1 - vi^2\right)}{E_i}. \quad (1)$$

v is sample Poison's fraction, vi: Poison's fraction taking into account the indenter, and E_{IT}: Instrumental Young's Modulus.

5. Statistical Analysis

Normality and homogeneity of variance were preanalysed with Levene's test, which verified positive assumptions for implementation of parametric testing. The t-test applied was to evaluate intra- and intergroup differences of nanohardness in both groups.

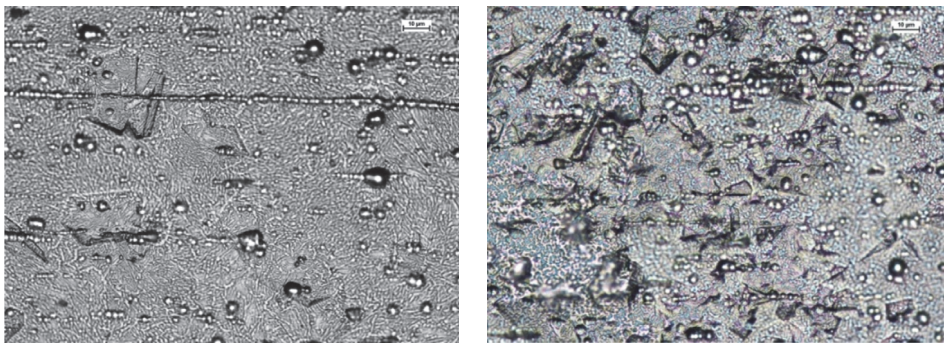
Statistical significance level was established at $p < 0.05$.

6. Results

6.1. Microscopic Analysis of Nonetched Samples. Microscopic images of the specimens showed substantial presence of nonmetallic inclusions, mainly in the form of silicates and oxides. The silicate inclusions were arranged in chains amounted from 1 to 3 and mainly displayed 3a pattern in group A and 1a and 2b patterns in group B. Oxidant impurities were arranged in dots amounted from 2 to 5 and mainly displayed pattern 5a in group A and 2a and 5a patterns in group B.

Table 1: Composition of the study material.

Group	Nonetched specimen (n)	Type of analysis	Etched specimen (n)	Type of analysis
A (used)	ANE (60)	Microscopic Nanoindentation	AE (30)	Microscopic
B (brand, new)	BNE (60)	Microscopic Nanoindentation	BE (30)	Microscopic



(a) (b)

Figure 2: Microstructure of etched material (a) used wires with predominant martensitic phase and (b) new wires with fine-grained austenitic structure.

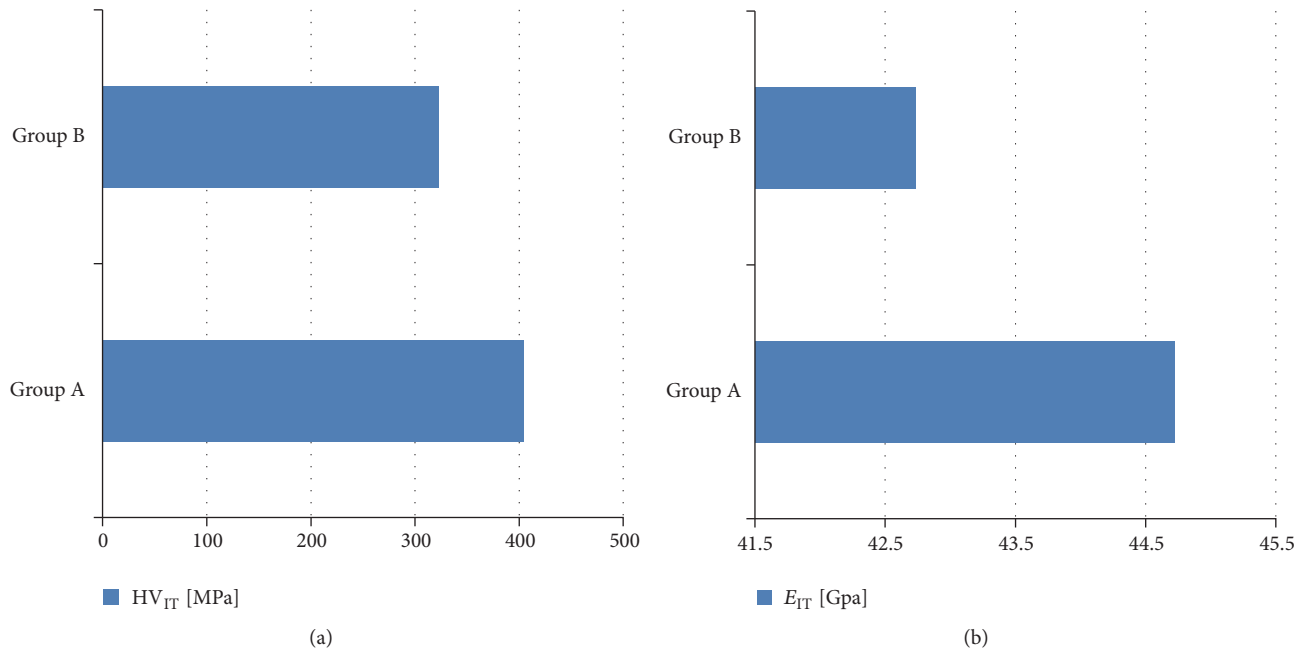


Figure 3: The values obtained in group A (used wires) and group B (new wires): (a) nanohardness (HV$_{IT}$) and (b) E_{IT}.

6.2. Microscopic Analysis of Etched Samples. The wires from group A presented combined structures, however, dominated by martensitic areas in all samples (Figure 2(a)). This arrangement of the microstructure indicates that the 12-week lasting influence of oral environment facilitates the transition of internal crystal structure into harder phase. The wires from group B showed austenite structure with grains diameter ranging from 0.5 to 1.5 μm (Figure 2(b)). Small martensitic areas are the evidence of partial transformation present already in the brand new products.

6.3. Nanohardness Analysis. The results were in accordance with microscopic evaluation. Statistically significant ($p < 0.05$) increase of nanohardness in the used archwires (group A) was apparent: mean HV$_{IT}$ and E_{IT} values exceeded the ones in group B by 100 MPa (Figure 3(a)) and nearly 3 GPa

Table 2: Results of t-test comparing the average value of nanohardness in groups A and B.

	Group A	Group B
HV_{IT} (MPa: mean value ± SD)	404.62 ± 40.3	323.18 ± 8.7
p (intragroup analysis)	0.03258	0.006258
t value	−7.01238	
p (intergroup analysis)	0.00	
E_{IT} (GPa: mean value ± SD)	44,72 ± 1,0	42,73 ± 1.8
p (intragroup analysis)	0.01246	0.00246
t value	3,70801	
p (intergroup analysis)	0.000996	

p: the level of significance, t value: the difference coefficient, and SD: standard deviation.

(Figure 3(b)), respectively. It is worth to emphasize that HV_{IT} and E_{IT} parameters in groups A and B and in different points of the measurements displayed significantly diverse values ($p < 0.05$). This may indicate both the heterogeneity of the single wire and the presence of individual components in the structure of the NiTi alloy.

Results of statistical analysis are shown in Table 2 and in Figure 4.

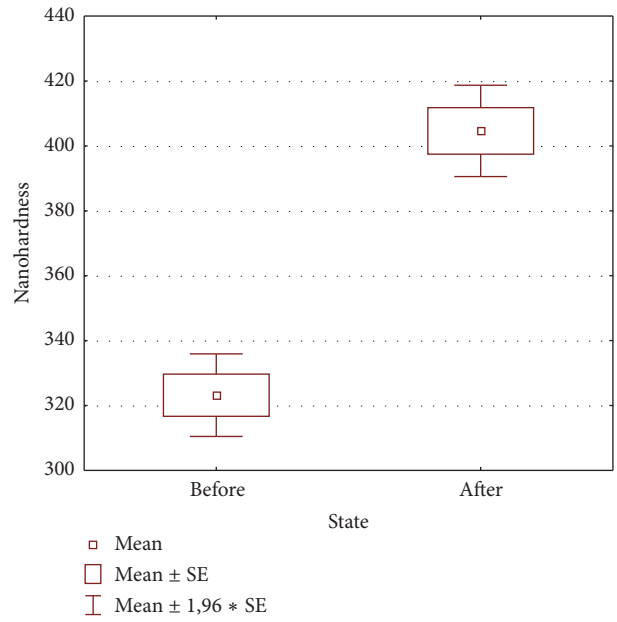


Figure 4: Box plot of nanohardness mean values in group A (used) and group B (new) wires; SE: standard error.

7. Discussion

Orthodontic biomechanics is based—among others—on the assumption that aligning and levelling archwires produce long-term, light, and constant force values [10]. Nonetheless our previous studies have already proved that those forces are likely to change with the function of time and are not always predictable [11]. Presented research was aimed at identifying alterations in the structure of orthodontic wires after their 12-week acting in vivo. It brought the evidence that oral environment evidently affects NiTi wires changing their structure and thereby their properties, which is in accordance with the results obtained by other clinicians [8, 9, 11].

Surprisingly the wires examined in the current research exhibited both uneven structure and mechanical properties already at the stage of their postproduction. Microscopic evaluation and nanoindentation tests showed that all new wires from control group presented with austenite and martensitic phases although the previous ones prevailed in all specimens. Such results allow assumption that since the internal configuration of new 0.016 × 0.022 NiTi wires of the same manufacturer varies from sample to sample and within the samples themselves, comparison with the wires produced in another facility may bring even more profoundly diverse results. It seems to be fully justified when analysing the results obtained by Nakano et al., Parvizi and Rock, and Nikolai who reported very high standard deviation in the force-deflection plot of brand new nickel-titanium wires from different vendors [12–14]. Due to the lack of studies evaluating nanohardness of nickel-titanium orthodontic wires one can only presume that their inconsistent crystal structure and diverse mechanical properties may subsequently affect biomechanics.

Such phenomena immediately arises further question: do NiTi wires of the same manufacturer change their properties and/or structure and do they change it in the same manner during an orthodontic treatment? Our results obtained in previous [11] and current studies allow answering both posted queries: the results of nanohardness significantly increased under influence of the oral environment ($p < 0.05$), although in the uniform manner ($p > 0.05$ for intragroup A comparison). Presence of permanently and diversely altered crystal structures within the same used wire is substantial. Such lack of uniformity in transformation of the internal net of atoms is an evident and major drawback impairing mechanical properties of the NiTi wires. It may be summarized that heterogeneous crystal structure negatively influencing mechanical properties, which exists already at the postproduction stage, is subjected to further negative changes under the influence of the oral environment, although significance and clinical impact of this finding require further investigation.

The presented results demonstrated no substantial differences between new and used wires in regards to the amount of nonmetallic inclusions, although impurities such as silicate and oxidant inclusions were present more frequently in group A. However, it is known already that their vestigial presence provokes deterioration of mechanical properties of wires and hence makes prediction of their exact mechanical and electrochemical behaviour technically difficult or even impossible [14–18]. Furthermore, since the orthodontic wires are used in the environment of the human body their composition should be extremely uniform and consistent with the data reported by the manufacturer [18, 19]. Whether the content of such inclusions results in an increased susceptibility to corrosion and/or cracking [20, 21] remains still an open question. However nonmetallic impurities present

already at the postproductive stage and increased under influence of the oral environment must not be neglected.

8. Conclusions

(i) The results proved that the NiTi wires produced by the same manufacturer do not have equal physical properties already at the stage of postproduction and that their structure is further altered by the long-term influence of an oral environment. Therefore reliable prediction of their biomechanics is impossible.

(ii) Since both used and new archwires contain a substantial amount of nonmetallic inclusions none of them meets the requirements for medical materials, since all impurities may be released to the oral cavity. The nonmetallic inclusions may also provoke corrosion or cracking of the wires, thus possibly affecting an orthodontic treatment; nevertheless solving this issue requires additional studies.

(iii) Manufacturers have to improve their production process in order to minimize mutual negative influence of nickel-titanium archwires and oral environment.

Competing Interests

The authors declared that they have no conflict of interests.

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Research Article

Assessment of Heat Hazard during the Polymerization of Selected Light-Sensitive Dental Materials

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Introduction. Polymerization of light-cured dental materials used for restoration of hard tooth tissue may lead to an increase in temperature that may have negative consequence for pulp vitality. *Aim.* The aim of this study was to determine maximum temperatures reached during the polymerization of selected dental materials, as well as the time that is needed for samples of sizes similar to those used in clinical practice to reach these temperatures. *Materials and Methods.* The study involved four composite restorative materials, one lining material and a dentine bonding agent. The polymerization was conducted with the use of a diode light-curing unit. The measurements of the external surface temperature of the samples were carried out using the Thermovision®550 thermal camera. *Results.* The examined materials significantly differed in terms of the maximum temperatures values they reached, as well as the time required for reaching the temperatures. A statistically significant positive correlation of the maximum temperature and the sample weight was observed. *Conclusions.* In clinical practice, it is crucial to bear in mind the risk of thermal damage involved in the application of light-cured materials. It can be reduced by using thin increments of composite materials.

1. Introduction

Light-cured materials are commonly used in dental treatment. The polymerization process takes place by activating a photoinitiator present in dental resin by using a light-curing unit. At present, a quartz-tungsten halogen (QTH) or a light-emitting diode (LED) light-curing units are used. Less popular solutions include the plasma-arc-source-PAC or the argon laser. Halogen lamps emit waves which are 360–560 nm in length, and the radiation flux density, also called light intensity of the majority of devices, ranges from 700 to 800 mW/cm^2 although in some cases it exceeds 1500 mW/cm^2. When compared with QTH, LED light-curing units are more efficient and they use less energy. The older generation of diode photopolymerization units emit light of wavelengths of approximately 468 nm which is of central importance for classical photoinitiators such as camphorquinone which are contained in the materials. The newer generation of LED (polywave) emits wavelengths of wider spectra (from 460 to 410 nm), as a result of which it is also effective for alternative photoinitiators [1–3].

Undoubtedly, introducing light-cured materials in clinical practice was a milestone in dentistry. However, these materials are not flawless. One of their drawbacks is the polymerization shrinkage which may result in marginal leakage of the restoration. In order to minimize the risk of the shrinkage, the composition of the materials is modified, special application techniques are introduced, and alternative light-curing techniques are recommended; for example, soft-start, pulsation, or pulsation soft-start are used. Another

significant issue is a temperature increase during polymerization process. On the one hand, it results from the curing unit emitting light energy; on the other hand, it is linked with the heat generated during the exothermic reaction [2, 4, 5]. It has been established that dentine has good insulation properties thanks to which the pulp tissue is protected from overheating [6]; however, many researchers emphasize the risk of its occurrence, particularly in deep cavities [7–12]. A temperature increase of 5.5°C may already cause tissue damage [13]. It has been determined that the threshold temperature value at which irreversible disturbances of the pulp circulation are initiated is 42.5°C [14, 15]. Therefore, it can be speculated that speed and duration of thermal stimulus as well as the extent of temperature rise play an important role in pulp damage and gradual temperature increase may raise the threshold temperature higher than 5.5°C.

The measurements of the temperature changes which occur in the light-cure materials during the polymerization process can be carried out by applying various methods, for example, using thermocouples [16–18] or thermography [19, 20]. The values of the temperature changes observed by different authors varied considerably, since they depended not only on the method of measurement but also on many other factors, such as the location of the measurement, the type and shade of the material, and the thickness of the light-cured increment.

The aim of this study was the comparative assessment of the maximum temperature rise and the duration of polymerization process of selected light-cured dental materials, as well as the analysis of the influence of their volume on thermal parameters. The hypothesis of this study predicts that the dangerous increase in temperature during the polymerization of light-cure composite materials has negative consequence for pulp vitality.

2. Materials and Methods

The following light-sensitive dental materials were involved in the study:

Composite Restorative Materials

(i) Te-Econom (hybrid, Ivoclar Vivadent, Schaan, Liechtenstein)

(ii) Filtek Supreme XT (nanofil, 3M ESPE, St. Paul, MN, USA)

(iii) Tetric EvoCeram (nanohybrid, Ivoclar Vivadent, Schaan, Liechtenstein)

(iv) Gradia Direct (microhybrid, GC Corp, Tokyo, Japan)

Lining Material

(i) Ionosit (compomer, DMG, Hamburg, Germany)

Bonding Agent

(i) ExciTE (V generation, Ivoclar Vivadent, Schaan, Liechtenstein).

Table 1: Types of materials, sample sizes, sample maximum temperature T_{max}, and time of reaching maximum temperature t_{max} (m: mass, d: diameter, h: height, and l: the distance between the polymerization unit and the sample).

Material	m (mg)	h (mm)	d (mm)	l (mm)	T_{max} (°C)	t_{max} (s)
Te-Econom A-1	70	2	4	0	40.5	7.6
	70	2	4	3	39.2	8.2
	150	5	4	0	39.8	17.5
	220	2	8	0	43.5	10.6
	520	5	8	0	52.5	20.6
Filtek Supreme XT A-1B	120	5	4	0	39.2	17.0
	490	5	8	0	41.3	23.4
Filtek Supreme XT A-3B	219	2	8	0	46.8	22.5
Filtek Supreme XT A-1D	69	2	4	0	31.4	9.3
	71	2	4	0	36.7	43.0
	123	5	4	0	37.1	49.8
	200	2	8	0	41.0	14.0
	200	2	8	3	40.8	14.4
Filtek Supreme XT A-4D	200	2	8	3	44.2	49.0
Filtek Supreme XT A-1E	68	2	4	3	36.4	10.0
	200	2	8	3	42.2	14.2
Tetric EvoCeram	69	2	4	0	31.1	22.9
	70	2	4	3	34.2	11.4
	140	5	4	0	38.5	21.8
	220	2	8	0	42.1	10.0
	210	2	8	3	39.3	43.1
	500	5	8	0	44.8	40.4
Gradia Direct A-3	60	2	4	0	36.8	44.6
	60	2	4	3	36.1	49.6
	92	5	4	0	51.6	41.4
	170	2	8	0	55.5	41.9
	172	2	8	3	62.5	42.0
	380	5	8	0	64.3	39.7
Ionosit	18	0.1	2.7	0	34.3	13.3
	19	0.1	3.0	2	35.4	12.9
ExciTE	22	0.1	6.0	0	30.2	3.1
	21	0.1	5.0	2	42.2	4.3
	22	0.1	5.5	5	39.4	4.9

E: enamel, D: dentine, and B: body.

The complete list of materials of various translucency and color intensity, as well as the size of the samples and polymerization conditions, is shown in Table 1. The research was conducted with the use of the Elipar II LED (3M ESPE) light-curing unit that emits waves of 400–515 nm in length, with the maximum light intensity of 800 mW/cm^2. The composite materials were light-cured by the polymerization unit for 40 s, lining material –20 s, and bonding agent –10 s. Samples of dental restorative materials were prepared in

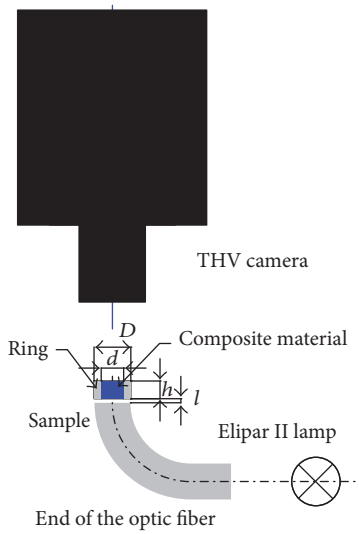

Figure 1: Measurement system diagram.

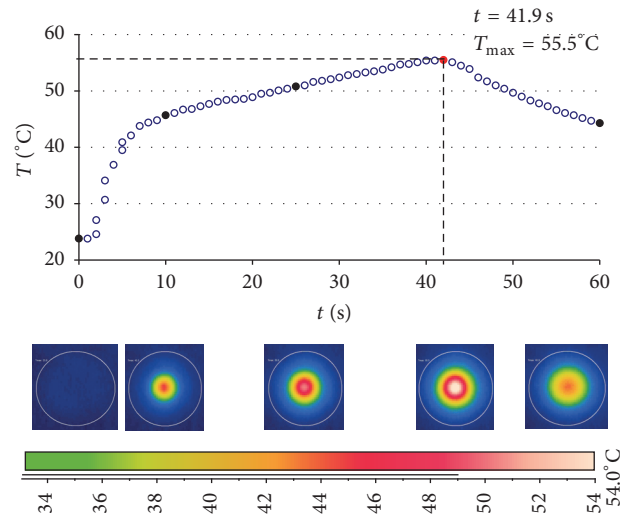

Figure 2: Example of temperature change in the Gradia A-3 sample (m = 170 mg; h = 2 mm; d = 8 mm).

form of cylinders which filled Teflon rings of various height (h) and internal diameter (d) and the external D = 10 mm (Figure 1). Samples of bonding agents and lining materials were placed on 0.5 mm thick transparent PC plates with the use of a pipette. Constant temperature measurement of the external surface of the samples was carried out using the Thermovision 550 thermal camera in an isolated, dark, and air-conditioned laboratory. The measurements were taken at a distance of 45 mm with the use of a wide-angle lens and a distance ring. As a result, thermograms in which samples fulfilled approximately 35% of the picture area were obtained.

The prepared samples were placed at an l distance from the tip end of the lamp optical fiber which was placed opposite the lens of the thermal camera. The l value was 0 or 3 mm. After the temperature of the sample has set at the level of approximately 24°C, the light-curing unit initiating the polymerization process and the program managing the acquisition of data from the thermal camera were simultaneously activated [21]. The measurements of the heat generated in the polymerization process were taken until the maximum temperature of the sample fell below 30°C (Figure 2).

The strength and direction of the relationship of the maximum temperature reached by the samples in the polymerization process, as well as their weight, were determined by calculating the value of the r Pearson correlation coefficient. The Kruskal-Wallis test was used to verify the hypothesis of lack of significant differences between the maximum temperature T_{max} and the time of reaching t_{max} and the type of material. The calculations were made with the use of the STATISTICA version 12 software.

3. Results

Examples of thermograms prepared at selected moments of the polymerization process of the Gradia Direct material samples (shade A-3) are shown in Figure 2. The maximum temperature values and the time of reaching them varied

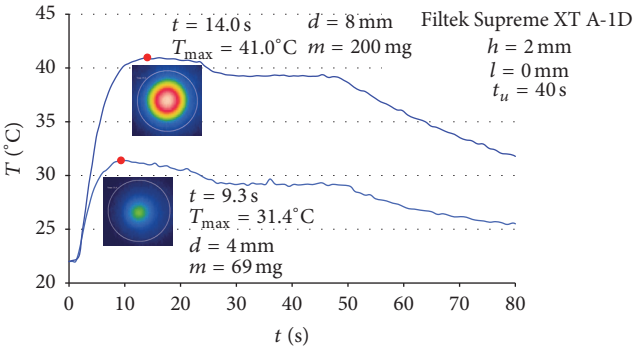

Figure 3: Example of temperature change during the polymerization of samples of Filtek Supreme XT A-1D of various weight and the time of exposure to the light of the light-curing unit: t_u = 40 s.

depending on the weight (Figure 3) and shade (Figure 4) of a given material.

The differences between the maximum temperatures reached during polymerization by some materials were statistically significant ($p < 0.05$). The *post hoc* test (multiple comparisons) confirmed that an average temperature T_{max} for Gradia Direct A-3 samples was significantly higher than that for Ionosit samples (51.5 versus 34.3; $p = 0.049$) (Figure 5). The differences between the time of reaching the maximum temperatures by some materials were also statistically significant ($p < 0.01$). The *post hoc* test confirmed that an average time t_{max} for samples made of Gradia Direct A-3 was considerably longer than that for samples made of Te-Econom A-1 (42.0 versus 10.6; $p = 0.009$) (Figure 6) and ExciTE (42.0 versus 4.3; $p = 0.005$).

A statistically significant positive correlation between the maximum temperature and the sample weight was observed (Figure 7). In some cases, placing the tip of the optic fiber at the shortest possible distance to the sample ($l = 0$) led

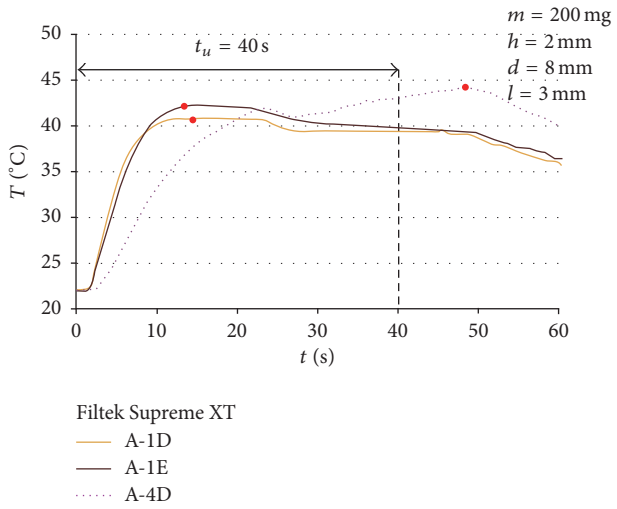

Figure 4: Temperature changes during the polymerization of samples of Filtek Supreme XT of various shades.

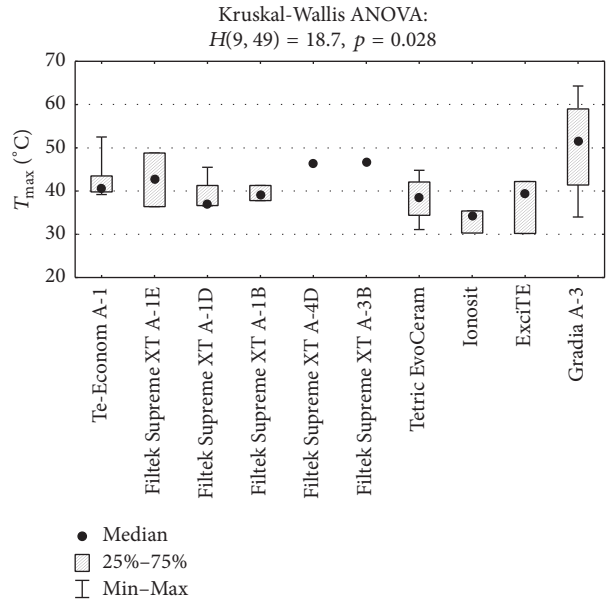

Figure 5: Comparison of the maximum temperature (T_{max}) of the examined materials and the Kruskal-Wallis test result.

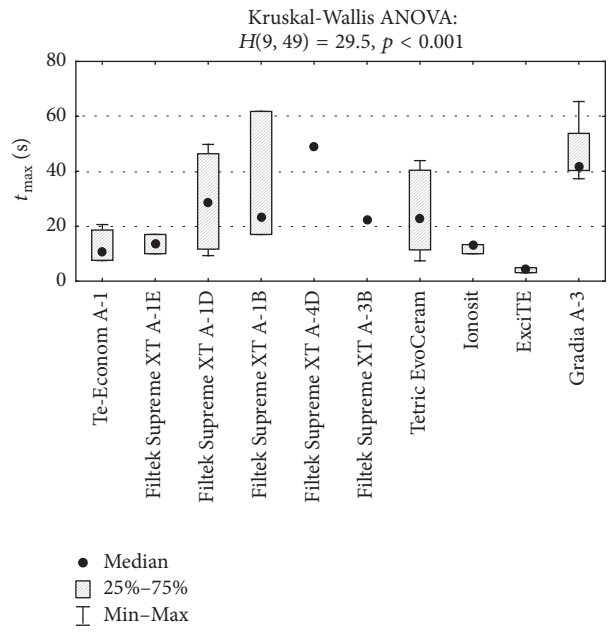

Figure 6: Comparison of time of reaching the maximum temperature (t_{max}) during polymerization of the examined materials and the Kruskal-Wallis test result.

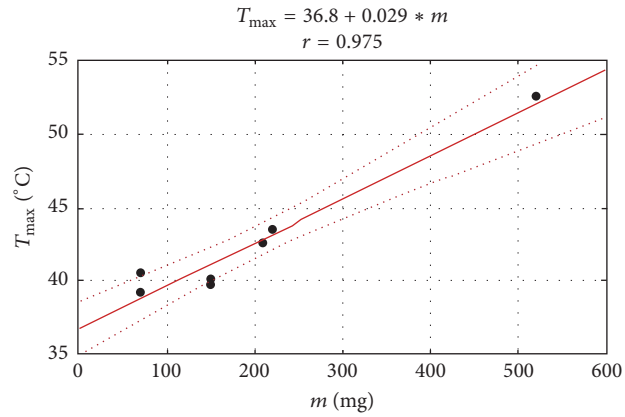

Figure 7: Example of a correlation diagram between the maximum temperature and the weight of a sample made of Te-Econom A-1.

to a dangerous increase in temperature. Placing the tip of the optic fiber in a 3 mm distance and reducing the sample weight resulted in the maximum temperature increments being reduced to safe values (Table 1).

4. Discussion

In all examined materials, the maximum temperature was positively correlated with the sample weight. Temperature increase and the time of reaching the maximum temperature also differed depending on the type of the material, its shade, and, to some extent, the distance from the curing unit. According to own research, samples with the Jonosit lining compomer and the ExciTE bonding agent did not cross the threshold of 42.5°C. When examining bonding agents of various generations applied in deep cavities, Khaksaran et al. [22] came to similar conclusions and they determined that the light-curing time for those materials followed in clinical practice, that is, 20 s, did not lead to any dangerous increase in temperature. It is an extremely essential conclusion, as the technique of working with a composite material requires placing the bonding agent directly on the dentine, in many cases in close proximity of the pulp.

The most considerable increase in temperature, that is, above 42.5°C, for samples weighing above 60 mg, was observed for a microhybrid material Gradia Direct. Crossing the 42.5°C threshold was also observed in the case of other

examined composite materials; however, it only occurred in heavier samples of more than 200 mg. Small samples measuring around 2 × 4 mm did not reach the temperature of 42.5°C for any of the examined materials. Single-time light curing of large amount of composite in a dental cavity may result in generating heat in an amount which may be harmful for the dental pulp. It is particularly risky in the case of the first layer of the material placed in a deep cavity. Similar conclusions were drawn by Kim et al. [23]. Interesting data were gathered by Chang et al. [19] who observed a significantly higher temperature inside the sample at the depth of 1–3 mm in comparison with the deeper layers and the external surface of the material. The authors suggest that the thickness of a light-cured layer cannot exceed 3 mm, as polymerization in the deeper layers may not be sufficient due to a deficient amount of light. Therefore, in order to protect the dental pulp from overheating and, at the same time, to ensure optimum polymerization, it is crucial to apply proper volumes of light-cured materials.

The results obtained by other authors confirm the differences in the changes of temperature depending on the type of material [4, 6, 16, 17]. Al-Qudah et al. [6] demonstrated the highest increase in temperature for flowable composites, hybrid composites, compomers, and composites with increased density (condensable), respectively. In the research conducted by Hubbezoglu et al. [4], the highest temperature was observed for ormocers and flowable composites and the lowest for nanofil ones. However, in no case was the critical temperature value for pulp exceeded. According to the authors, the unique structure of every type of material, as well as the content and type of filler and resin, has a significant influence on the amount of heat generated in the course of polymerization. This thesis is supported by Dąbrowski et al. [11] who observed an increase in temperature that might be dangerous for the dental pulp during polymerization of a material which contains epoxy resins. A considerable increase in temperature may also occur in the case of recently introduced composite materials, such as bulk-fill. Their advantage is reduction of clinical application time due to a possibility of placing thicker increments. However, it involves a risk of overheating the pulp, particularly in deep, extensive cavities [17]. According to Dąbrowski et al. [10], not only the maximum increase in temperature after the light-curing of a given material but also the curing time needed for it is significant. A significant amount of heat cumulated over a short period of time may be particularly dangerous for the pulp tissue. Based on own research, considerable high values of maximum temperatures were observed in multiple heavier samples over short periods of time.

The differences between the parameters in question were also observed among various shades of the same dental material (Filtek Supreme XT). The lightest shades, A-1E (used for enamel restoration) and A-1D (for dentine restoration), reached the maximum temperature within around 14 seconds; higher temperature of 42.2°C was observed in the case of A-1E. The darker shade (A-4D-dentine) reached the maximum temperature of 49°C; however, it lasted longer, that is, 44.2 s. All the compared samples were large and they weighed 200 mg each.

Zaborowski et al. [24] obtained ambiguous results: in the case of one composite material, the most significant increase in temperature was observed for the lightest shade and for the other two materials, for the darkest one. The differences of the temperature increments that depended on the shade of the material used were also observed by other authors [20].

The increase in the temperature of a material may also depend on the light-curing unit operation mode. The authors of this study used the standard operation mode which guarantees light emission with constant intensity. According to Hubbezoglu et al. [4], the most statistically significant highest temperature was generated when using the soft-start program, and the lowest was when using the pulsation operation mode. Both light-curing variants are used in order to reduce the polymerization shrinkage of materials. However, in no case did the increase in the temperature of the dentine exceed the value which had been determined as critical for the pulp (5.5°C). Chang et al. [19], in turn, did not demonstrate any significant differences in the increase in temperatures after applying various light-curing operation modes. Changes in temperature during polymerization are also influenced by the type of equipment used. Monowave light-emitting diode generates less heat in comparison to the halogen one [2, 11, 25]. Due to the high light intensity, the new generation of LED curing units produce the same heat as QTH, and similarly to QTH, they have incorporated filters. The argon laser has been reported to be an even safer solution in this regard [26].

Therefore, it can be stated that an increase in the temperature of light-sensitive materials in the polymerization process is influenced by many factors which depend on both the material itself and the type of light-curing unit used. The amount of energy applied together with the beam of light depends on the time of exposure, the distance between the tip of the optical fiber and the material, the type of device used, the light-curing operation mode, the light intensity, and the light wavelength. The amount of heat generated in a given material depends on its composition, the thickness of the polymerized layer, and its shade [4, 6, 8, 11, 14, 18, 20, 24, 27]. Consequently, the final increase in temperature during polymerization is a derivative of many factors. In clinical practice, the thickness of the dentine layer that separates the material from the pulp is also crucial, as the dentine layer is an important protective barrier against transmitting too much heat into the pulp chamber.

5. Conclusions

Dentists should be aware of the potential risk involved in the use of light-sensitive dental materials. In order to minimize it, it is necessary to follow the recommendations and instructions of the manufacturers of particular dental materials and photopolymerization units, as well as to apply appropriate volumes of these materials. One needs to be particularly cautious when restoring deep cavities, as it involves the most significant risk of overheat. This process may result in post-treatment hypersensitivity or even irreversible inflammation or dental pulp necrosis.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Brassinolide Increases Potato Root Growth *In Vitro* in a Dose-Dependent Way and Alleviates Salinity Stress

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Brassinosteroids (BRs) are steroidal phytohormones that regulate various physiological processes, such as root development and stress tolerance. In the present study, we showed that brassinolide (BL) affects potato root *in vitro* growth in a dose-dependent manner. Low BL concentrations (0.1 and 0.01 μg/L) promoted root elongation and lateral root development, whereas high BL concentrations (1–100 μg/L) inhibited root elongation. There was a significant ($P < 0.05$) positive correlation between root activity and BL concentrations within a range from 0.01 to 100 μg/L, with the peak activity of 8.238 mg TTC·g^{-1} FW·h^{-1} at a BL concentration of 100 μg/L. Furthermore, plants treated with 50 μg/L BL showed enhanced salt stress tolerance through *in vitro* growth. Under this scenario, BL treatment enhanced the proline content and antioxidant enzymes' (superoxide dismutase, peroxidase, and catalase) activity and reduced malondialdehyde content in potato shoots. Application of BL maintain K^+ and Na^+ homeostasis by improving tissue K^+/Na^+ ratio. Therefore, we suggested that the effects of BL on root development from stem fragments explants as well as on primary root development are dose-dependent and that BL application alleviates salt stress on potato by improving root activity, root/shoot ratio, and antioxidative capacity in shoots and maintaining K^+/Na^+ homeostasis in potato shoots and roots.

1. Introduction

Roots are fundamentally important for plant growth and survival because of their essential roles in water and nutrient uptake. As a serious stressful factor, salinity leads to growth arrest and crop yields decline [1]. Besides reduced nutrient uptake and adverse effects of growth and development, salinity stress can also result in osmotic stress and ionic imbalance [2]. Exposure to salinity enhances oxidative stress and the overproduction of reactive oxygen species (ROS), resulting in plant damage. ROS such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are highly reactive under NaCl stress and can alter normal cell metabolism through oxidative damage of membranes by lipid peroxidation [3]. To protect against oxidative damage, plants have evolved a complex antioxidant defense system which includes nonenzymatic antioxidants, such as glutathione, carotenoids, and flavonoids and several enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and peroxidases (POD) [4]. SOD is an intracellular antioxidant enzyme which combats oxidative stress by catalyzing the conversion of superoxide to H_2O_2 in organelles and cytosol. CAT scavenges H_2O_2 generated in photorespiration, which dismutates H_2O_2 into water and O_2. POD possess broad specificities located in vacuoles, cell walls, and the cytosol, which consumes H_2O_2 by catalyzing H_2O_2 to decompose other substrates [2, 5, 6]. Proline (Pro) is a major osmoregulatory substance and also known as a substance with nonenzymatic antioxidant properties [7]. It was reported that Pro might contribute to plant adaptive responses to salinity by regulating K^+ transport across the plasma membrane in barley [8] and exogenously supplied proline significantly reduced NaCl-induced K^+ efflux from barley roots in a dose-response manner and thus resulted in better K^+ in roots [9]. Malondialdehyde (MDA) content indicates the damage caused by ROS [10]. Salinity

leads to imbalanced ion ratio results from high Na^+ and Cl^- concentrations that are detrimental to plants, or decline of tissue K^+ content which is central to normal cell metabolism, or both [11, 12]. Numerous studies have reported that regulation of K^+ homoeostasis is a common denominator of plant adaptive responses to stress environment including drought, salinity, and oxidative stress [12, 13]. Thus, not only maintenance of a high K^+/Na^+ ratio in cytosol but also retention of absolute concentrations of K^+ is a biochemical strategy for plants growth in a saline environment.

Brassinosteroids (BRs), a group of naturally occurring steroidal compounds in plants, contribute to growth, vascular differentiation, development [14], and response to biotic and abiotic stresses [15–19]. These effects are mediated by BR-induced genes, including defense genes and genes that can detoxify ROS produced in plants experiencing abiotic stress [15]. Exogenous BR application enhanced salinity stress tolerance enhancement in a variety of crop species [14, 20]. Treatment with 24-epibrassinolide (EBL) reduced the salt-stress-induced inhibition of seed germination in *Arabidopsis thaliana*, *Brassica napus*, and *Cucumis sativus* [21, 22]. Presowing seeds with BR increased the growth vigor of *Medicago sativa* seedlings under saline stress [23] and reduced the deleterious effects caused by saline stress in *Pisum sativum* and *Cicer arietinum* [24, 25]. The application of EBL to leaves enhanced the photosynthetic capacity and regulated antioxidant enzymes of salt-stressed wheat, thereby increasing plant biomass and leaf area per plant [26]. Also, treatment with EBL downregulated the gene expression of *OsDWF4* and *SalT*, upregulated the gene expression of *OsBRI1*, and enhanced Pro content and antioxidant enzymes' activity under salinity stress in rice [27].

As an important staple crop in China, potato displays high sensitivity to excess soil salinity. Since *in vitro* culture systems have been successfully applied to produce virus-free seed potatoes, they could also be used to screen for salt tolerance. Although many studies demonstrated a positive effect of BRs application on plant tolerance to salt stresses [20, 28, 29], BRs stimulate root growth at low concentrations but are inhibitory at higher concentrations [30–32]. The roles of BRs, however, in adventitious root growth are rarely understood in tissue culture systems. Thus, it is necessary to determine the effects and optimal concentration of BRs for potato root formation and plant growth throughout the process of *in vitro* culture. In our study, an *in vitro* tissue culture system was used to examine the effects of brassinolide (BL) on the resistance of potatoes to salt stress. We focused on the physiological, K^+/Na^+ homeostasis and characterization of the antioxidant capacity of BL-treated plantlets cultivated *in vitro* under salt stress. The growth parameters, root activity, accumulation of MDA and Pro, the typical antioxidant enzymes' (SOD, CAT, and POD) activities, and mineral tissue (Na^+, K^+) content were evaluated for plantlets' tolerance to salt stress.

2. Materials and Methods

2.1. Plant Material and Experimental Treatments. Potatoes (*Solanum tuberosum* L. "Hui 2") were propagated *in vitro* from single-node cuttings on standard Murashige and Skoog (MS) medium containing 25 g/L sucrose solidified with 0.7% (w/v) agar. They were grown in a growth chamber under a 16 h photoperiod at 22°C. These plants were harvested 4 weeks later and used for subsequence experiments.

For root growth assays, MS medium was supplemented with 0, 0.01, 0.1, 1, 10, and 100 μg/L BL (Sigma Chemicals, USA). After 20 days, the root length (measured using vernier caliper), numbers of adventitious roots, root activity, and plant biomass were measured.

To examine the physiological role of BL in salinity stress, a 2-factor salinity and BL randomized block design was employed. Four levels (0, 50, 75, and 100 mM) for salinity (NaCl, Sigma Chemicals, USA) and 2 levels for BL (0, 50 μg/L) were designed. After 31 days of growth, 24 plants per treatment were collected to determine the plant biomass for shoots and roots. The stem fresh weight, stem length, root fresh weight, root length, and root activity were also measured. The remaining fresh samples were used for physiological analysis and determination of tissue K^+ and Na^+ content.

2.2. Root Vigor Assessments. Root activity in terms of 2,3,5-triphenyl tetrazolium chloride (TTC) reduction was measured as described previously [33]. About 0.5 g of fresh root samples were incubated for 2 h in a mixture of 5 mL 0.4% (w/v) TTC and 5 mL phosphate buffer (pH 7.5) at 37°C. The assays were terminated by adding 2 mL 1 M sulfuric acid to the reaction mixture. For triphenylformazan (TTCH, red product) extraction, the roots were removed and blotted dry on filter paper and ground in a mortar containing 3-4 mL ethyl acetate. The liquid phase was then transferred to a 10 mL stoppered test tube. Ethyl acetate was added to the 10 mL level, and the released TTCH was quantified photometrically at 485 nm. The TTCH reduction was calculated based on the slopes of the obtained curves, which were corrected for assay background slopes from "no-extract controls." The OD values were used to calculate the equivalent TTCH concentrations to determine the root vigor for each fresh root weight as follows: root vigor (TTCH $\mu g \cdot g^{-1}$ FW$\cdot h^{-1}$) = TTCH reduction (TTCH μg)/fresh weight (FW g)/time (h).

2.3. Measurement of MDA and Pro Content. Lipid peroxidation was estimated by measuring the level of MDA production using the thiobarbituric acid (TBA) method as described by Hodges et al. [34]. Fresh shoot samples (0.15 g) were crushed by grinding in a mortar containing 4.5 mL 10% TCA, after which the homogenized material was centrifuged at 5,000 ×g for 15 min at 4°C. The supernatant was transferred to a centrifuge tube and volume (V) was recorded. Then, 2 mL of supernatant was mixed with 2 mL 0.6% TBA, the homogenizing mixture was heated in boiling water for 20 min, and the reaction was terminated in an ice bath followed by the centrifugation at 5,000 g for 10 min. Approximately 2 mL (V1) of the supernatant was transferred to a cuvette. The absorbance of the supernatant was determined at 450, 532,

and 600 nm, respectively. The MDA content was determined as follows:

$$\begin{aligned}&\text{MDA concentration } (\mu\text{mol/L})\\&\quad = 6.45 * (\text{A532} - \text{A600}) - 0.56 * \text{A450},\\&\text{MDA content } (\mu\text{mol/g FW})\\&\quad = \text{C}\,(\mu\text{mol/L}) * \text{V}\,(\text{L}) * \text{V1}\,(\text{mL})\,/2\,\text{mL}\\&\qquad * \text{M}\,(\text{g FW}).\end{aligned} \tag{1}$$

The Pro content was estimated following the procedure given by Bates et al. [35]. Fresh shoot samples (0.15 g) were homogenized with 4.5 mL 3% (w/v) sulfosalicylic acid, and the homogenate was heated in a boiling water bath for 30 min then filtered through 0.2 μm filter paper, and the extract volume was marked as Vt. The supernatant was used for the Pro estimation. The reaction mixture was composed of 2 mL of plant extract and an equal volume of glacial acetic acid and acid ninhydrin. The test tubes containing the above mixture were heated in a boiling water bath for 30 min. The reaction was terminated in an ice bath followed by the addition of 4 mL of toluene. The contents were shaken vigorously and allowed to separate into phases. The chromophase containing the upper toluene phase with volume marked as V was carefully separated using a pipette; the absorbance was noted at 520 nm. The amount of Pro was calculated from the standard curve and expressed as $\mu g \cdot g^{-1}$ FW. The proline content was determined as follows: proline content (μg/gFW) = C * Vt/(V * W).

2.4. Determination of SOD, CAT, and POD. To estimate antioxidant activities, frozen shoots (about 0.5 g) were homogenized (1 : 9 w/v) in 0.1 M cooled phosphate buffer (pH 7.8) or normal saline. The homogeneous mixture was centrifuged at 12000 ×g for 20 min at 4°C and the supernatants were collected for determining antioxidase activity.

The activities of POD, CAT, and SOD in the shoot homogenate were examined by a reagent kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The principles of these kits are briefly described as follows.

POD activity was measured according to the reaction of hydrogen peroxide catalysis by POD, and we detected the changes of absorbance at 420 nm and calculated the POD activity. One unit of POD activity was defined as the amount of the enzyme in 1 g of fresh tissue that reduced 1 μg of H_2O_2 per minute at 37°C. CAT activity was measured according to the ammonium molybdate spectrophotometric method, where ammonium molybdate rapidly terminated the H_2O_2 degradation reaction catalyzed by CAT and reacted with the residual H_2O_2 to generate a yellow complex, which could be monitored by absorbance at 405 nm. One unit of catalase activity was defined as the amount of enzyme in 1 g of fresh tissue that reduced 1 μmol of H_2O_2 per minute at 37°C. SOD activity was determined using the xanthine oxidase method, based on its ability to inhibit the oxidation of hydroxylamine by the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the amount of the enzyme inhibiting the oxidation by 50%.

2.5. Measurement of Na^+ and K^+ Concentrations. The root and shoot tissues were harvested from the two-week-old potato plants grown under salt stress and the control, washed with distilled water for 2 times, dried at 65°C for 48 hours, and digested in HNO_3-$HClO_4$ solution. The final extracts volume was adjusted to 50 mL with 2% HNO_3 and filtered before the assay. Na^+ and K^+ concentrations were determined by using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent Technologies 7700x, Waldbronn, Germany) as previously described [36].

2.6. Data Analysis. All statistical analyses were performed with SPSS 20.0. Treatment means were separated using Student's *t* test or Duncan's new multiple range test at 95% or 99% level of probability. Microsoft Excel 2010 and Microsoft Office Visio 2010 (Microsoft Corporation, USA) were used to generate graphs.

3. Results

3.1. The Effects of BR on Root Development Are Dose-Dependent. BL promoted root growth significantly only at low concentrations (0.01 and 0.1 μg/L) in 20-day-old plantlets, and BL concentrations higher than 1 μg/L inhibited root elongation (Figure 1(a)). The length of roots of 20-day-old plantlets treated with 100 μg/L BL was lower ~53% than untreated roots of the *in vitro* cuttings and increased ~96% when treated with 0.01 μg/L BL (Figure 1(b)). BL-induced root shortening was coupled to a significant increase in adventitious roots number (Figure 1(c)) and root activity (Figure 1(d); $P < 0.05$ or 0.01). Although lower BL concentration promoted adventitious roots length growth, the number of adventitious roots decreased. However, when BL at higher concentrations inhibited the growth of adventitious root length, more adventitious roots were developed. Under the treatment of 0.01, 0.1, 1, 10, and 100 μg/L BL, the number of adventitious roots was 58 and 53% lower and 53, 174, and 242% higher than the control, respectively.

Generally, the root activity of all BL-treated plantlets increased with BL concentration. A maximum increase in root activity was observed in plants treated with 100 μg/L BL. Compared to those treated without BL (control), root activity at 0.01, 0.1, 1, 10, and 100 μg/L BL treatment increased by 6, 44, 52, 54, and 100%, respectively. The results show that BL can affect *in vitro* potato root growth in a dose-dependent manner and the application of BL can increase root activity and adventitious roots number.

Moreover, total plant fresh weight of all treatments except 0.01 μg/L BL showed a significant increase (Table 1; $P < 0.05$) compared to that of the control. As BL improved root absorbing ability and biomass of *in vitro* potatoes, it was of interest to determine the salinity response when BL was applied to MS medium. Given that significant increase of root weight, shoot weight, and root/shoot ratio was observed only at high concentrations of 100 and 10 μg/L BL (Table 1;

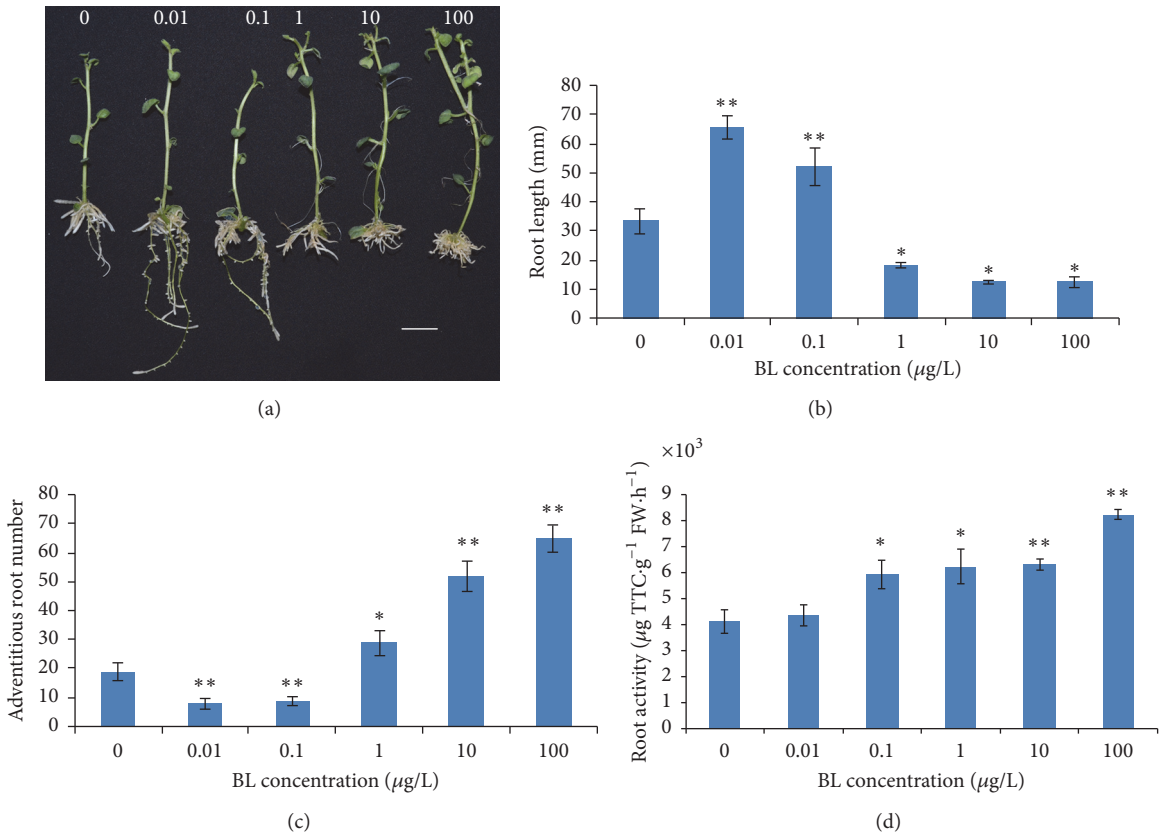

Figure 1: Brassinolide (BL) effect on the adventitious root growth of potatoes. (a) Phenotype of 20-day-old plants from left to right control (0 μg/L) and treated with 0.01, 0.1, 1, 10, and 100 μg/L BL. (b) Root-length measurements of various concentrations (μg/L) of BL-treated plants compared with that of the control. Values represent the mean of 43 measurements ± SD. (c) Adventitious roots numbers in BL-treated plantlets at different concentrations (μg/L) compared with that of control. Values represent the mean of 24 measurements ± SD. (d) Root activity of BL-treated plants at different concentrations (μg/L) compared with that of the control. Values represent mean ± SD of three biological replicates; asterisks indicate significant differences from the control: $*$ and $**$ indicate significance at 0.05 and 0.01, respectively.

Table 1: Effect of BL on biomass accumulation and root/shoot ratio of *in vitro* potato plants.

Treatments	Biomass accumulation (g)			Relative biomass (%)	Root/shoot ratio
	Root fresh weight	Shoot fresh weight	Total plant fresh weight		
0 μg/L BL	0.056 ± 0.004	0.129 ± 0.016	0.185 ± 0.011	100	0.437 ± 0.080
0.01 μg/L BL	0.064 ± 0.012	0.143 ± 0.029	0.207 ± 0.036	111	0.448 ± 0.071
0.1 μg/L BL	0.067 ± 0.062	0.155 ± 0.037	0.223 ± 0.036*	120	0.458 ± 0.102
1 μg/L BL	0.068 ± 0.008	0.156 ± 0.016	0.224 ± 0.020*	121	0.440 ± 0.065
10 μg/L BL	0.075 ± 0.009*	0.159 ± 0.011*	0.234 ± 0.023*	126	0.470 ± 0.090*
100 μg/L BL	0.093 ± 0.008*	0.181 ± 0.013*	0.274 ± 0.038*	148	0.526 ± 0.075*

Data represent mean ± SD of three biological replicates and were tested for significance by Student's t test ($P < 0.05$). Asterisks indicate significant differences from the control (0 μg/L BL).

$P < 0.05$), we applied 50 μg/L BL to carry out salt stress experiments.

3.2. BL Increases the Biomass of NaCl-Stressed Potato Plants. NaCl stress severely inhibited *in vitro* potato plantlets growth (Table 2 and Figure 2). The inhibition was more pronounced in roots than shoots, causing an overall decline in the root/shoot fresh weight ratio, whereas it showed the second-highest value in the treatment of 75 mM NaCl for the development of more and thicker adventitious roots. Compared with the control, application of NaCl at different concentrations (50, 75, and 100 mM) showed a distinct adverse effect on growth parameters of potato plantlets, such as shoot length, root length, and fresh biomass accumulation (Table 2 and Figure 2). Application of 50 μg/L BL increased the biomass of roots and shoots and increased the root/shoot fresh weight ratio via inducing more adventitious roots. The maximum enhanced effect of BL-treated plantlets weight in

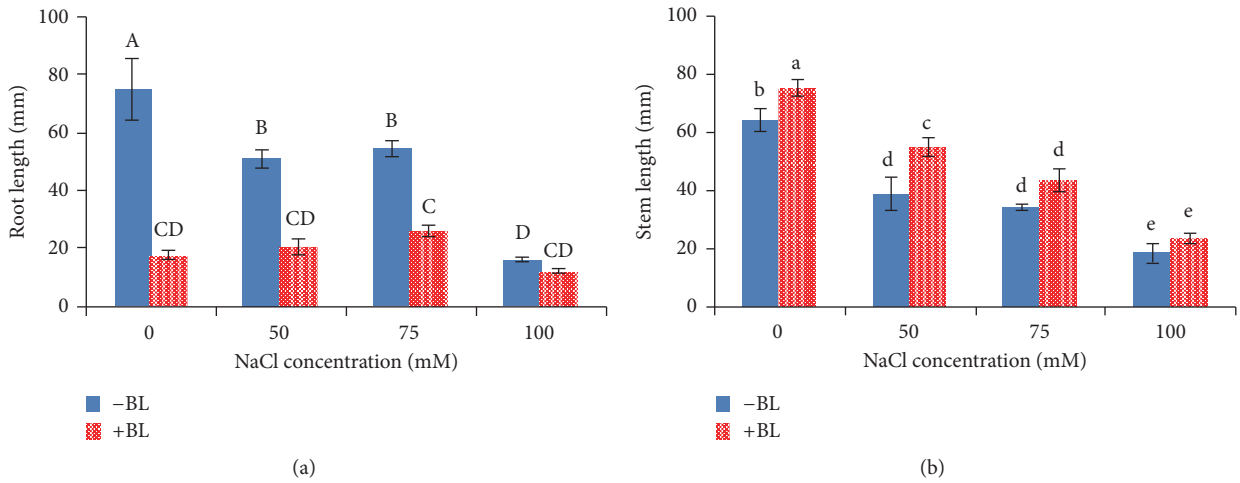

Figure 2: Effects of 50 μg/L BL on the growth of NaCl-stressed potato plants. (a) Root length and (b) stem length under 50, 75, and 100 mM NaCl and the control. Columns marked with different capital letters indicate significant differences by Duncan's new multiple range test at $P < 0.01$. Columns marked with different lowercase letters indicate significant difference at $P < 0.05$.

Table 2: Effect of BL on biomass accumulation and root/shoot ratio of *in vitro* potato plants under NaCl stress.

Treatments	Biomass accumulation (g)			Relative biomass (%)	Root/shoot ratio
	Root fresh weight	Shoot fresh weight	Total plant fresh weight		
0 mM NaCl	0.095 ± 0.017^{B}	0.217 ± 0.073^{B}	0.312 ± 0.009^{B}	100	0.438^{C}
0 mM NaCl + BL	0.167 ± 0.103^{A}	0.247 ± 0.089^{A}	0.414 ± 0.008^{A}	133	0.671^{A}
50 mM NaCl	0.057 ± 0.014^{C}	0.158 ± 0.045^{D}	0.215 ± 0.004^{D}	68.9	0.362^{F}
50 mM NaCl + BL	0.070 ± 0.027^{C}	0.173 ± 0.070^{C}	0.243 ± 0.003^{C}	77.9	0.407^{E}
75 mM NaCl	0.034 ± 0.022^{D}	0.078 ± 0.024^{G}	0.112 ± 0.002^{F}	35.9	0.440^{D}
75 mM NaCl + BL	0.050 ± 0.021^{C}	0.106 ± 0.022^{E}	0.156 ± 0.008^{E}	50.0	0.472^{B}
100 mM NaCl	0.009 ± 0.110^{E}	0.071 ± 0.045^{H}	0.086 ± 0.003^{G}	27.6	0.089^{H}
100 mM NaCl + BL	0.011 ± 0.065^{E}	0.093 ± 0.011^{F}	0.104 ± 0.001^{F}	33.3	0.118^{G}

Data represent mean ± SD of three biological replicates and were tested for significance by Duncan's new multiple range test. Columns marked with different capital letters indicate significant differences ($P < 0.01$).

roots, shoots, and total plants was 75.8 (0 mM NaCl), 35.9 (75 mM NaCl), and 39.3% (75 mM NaCl), respectively. NaCl decreased the length of stems and roots as compared to the control; however, it induced more and longer roots with the treatment of 75 mM NaCl than that of 50 mM NaCl. The 50 μg/L BL treatment increased the plant height but decreased the root length.

3.3. BL Increases Pro Content and Decreases MDA Content. Increasing the salt concentration significantly ($P < 0.05$) affected Pro and MDA content of shoots (Figure 3). A continuous increase in Pro content occurred with enhanced salinity level (Figure 3(a)) with a sharp increase of 343% at 100 mM NaCl. Application of 50 μg/L BL to MS medium enhanced Pro content, but a significant ($P < 0.05$) increase of Pro content was observed at high concentrations of 75 mM NaCl, and the maximum 41.6% increase was also observed at 75 mM NaCl. A similar trend in the MDA content occurred with increasing concentrations of NaCl. The MDA content was elevated to more than 164% at 100 mM NaCl than that of control plantlets. BL application significantly reduced the amount of MDA in the control and salt-stressed plantlets; however, the value in the salt-stressed plantlets was higher than that of the control. A maximum of 53% decline in MDA content was observed in plantlets growing in the combination of 50 μg/L BL + 100 mM NaCl than those growing in 100 mM NaCl alone.

3.4. BL Enhances Antioxidant Enzyme' Activity. In the present study, salt stress generally enhanced the activity of the typical antioxidant enzymes (Figure 4), whereas different antioxidant enzymes showed slight variations. Our results showed a significant increase ($P < 0.05$) in SOD activity in plants growing in 50, 75, and 100 mM NaCl compared to that of the control (Figure 4(b)). SOD, similar to POD and CAT activity, decreased at 100 mM NaCl but was still higher than that of the control. Treatment with BL resulted in significant increase in SOD activity at all concentrations but 50 mM NaCl.

An increase in POD (Figure 4(a)) and CAT (Figure 4(c)) activity is a typical response observed in plants grown under increasing of NaCl concentration, whereas, at 100 mM

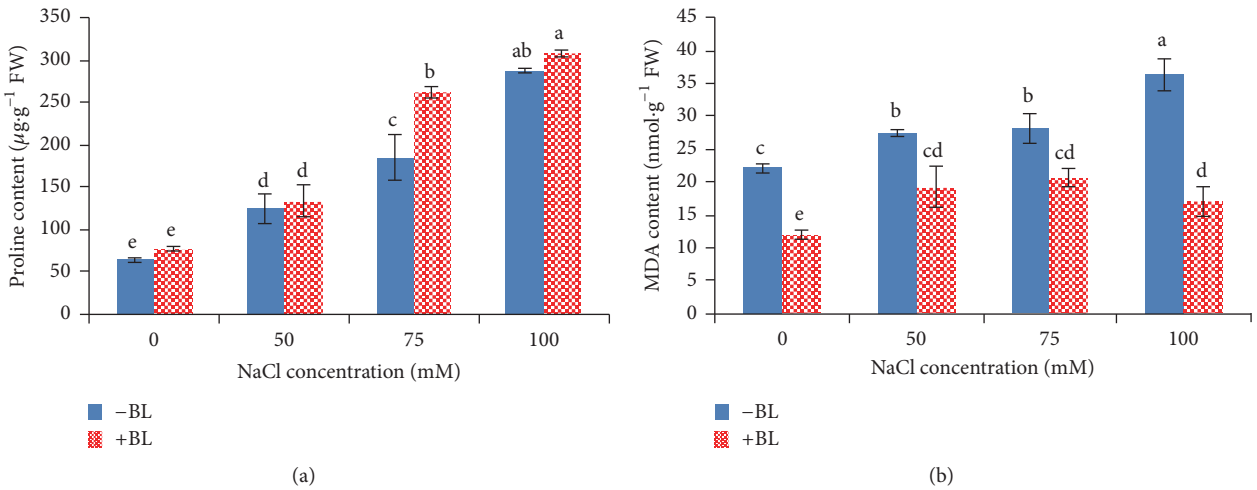

Figure 3: Effects of 50 μg/L BL on Pro (a) and MDA (b) content of potato shoots under salt stress conditions of 0 (control), 50, 75, and 100 mM NaCl. Columns marked with different lowercase letters are significantly different as determined by Duncan's new multiple range test at $P < 0.05$.

NaCl, activities of both enzymes declined and CAT activity decreased more sharply. CAT activity of 75 mM NaCl-stressed plants decreased less than that of 50 mM NaCl-stressed plants. Application of BL resulted in a considerable enhancement in the activity of both antioxidant enzymes.

3.5. BL Enhances Root Activity of NaCl-Stressed Potato Plants. NaCl stress significantly ($P < 0.05$) decreased root activity at 75 and 100 mM NaCl but significantly ($P < 0.05$) increased it at 50 mM NaCl (Figure 4(d)). The application of BL to MS medium enhanced the root activity in the control and in NaCl-stressed plants; and a maximum increase of 83% was observed under plants treated with BL only as compared to control plants. Compared with NaCl treatment alone, root activity of BL + NaCl treatment increased by 13, 6, and 14%, respectively. The results show that the influence of BL on root activity of NaCl-stressed plants is lower than that on the control.

3.6. BL Affects Na^+ and K^+ Homeostasis of Potato Plants under Salinity. To investigate the effect of BL on biochemical mechanism of ion homeostasis to salinity tolerance, we measured the concentrations of Na^+ and K^+ accumulation in tissues and also calculated the K^+/Na^+ ratio in potato roots and shoots, respectively. With the increase of the NaCl concentration, Na^+ accumulation exhibited a significant increase in both roots and shoots of potato plants especially in high concentration of NaCl. Under 75 and 100 mM NaCl, the roots Na^+ concentrations were 6.5-fold and 17.1-fold higher than that of control in roots and were 11.0-fold and 30.25-fold higher than that of control in shoots, respectively (Figure 5(a)). K^+ accumulations in both roots and shoots of *in vitro* potato were reduced under salt stress (except K^+ content in shoots under 75 mM NaCl); however, the decline of K^+ content was more moderate compared to the increase of Na^+ accumulation, and especially in shoots, K^+ accumulations were even higher than that of control level under 75 mM NaCl. Correspondingly, more Na^+ accumulation resulted in a significant decrease of K^+/Na^+ ratio in both roots and shoots under various NaCl treatments. Application of 50 μg/L BL significantly decreased roots Na^+ content of salt-treated and nontreated plants under saline conditions, while the decline in shoots under control and 50 mM NaCl was not significant. The effect of BL on K^+ accumulations showed more variations; significant increase and decrease were observed in tissue (Figure 5(b)). Compared with NaCl treatment alone, BL + NaCl treatment increased K^+/Na^+ ratio under 0, 50, 75, and 100 mM NaCl to 47.6, 55.9, 12.6, and 22.7% in roots and to 27.7, 21.4, 21.1, and 19.1% in shoots, respectively (Figure 5(c)). These results indicated that exogenous BL application reduced Na^+ accumulation and improved K^+/Na^+ ratio in salt-stressed tissue.

4. Discussion

4.1. BL Induced a Well-Developed Root System and Improved Root Absorption. In the present study, we demonstrated that the effects on *in vitro* potato adventitious root formation are strongly dependent on the BL concentration. Exogenous BL stimulated root growth at low concentrations (0.01 and 0.1 μg/L). Inhibition of root growth occurred at higher BR levels, which exceed a certain level of 1 μg/L. The BL dose-response phenotype in adventitious root development of potatoes was consistent with previous studies of seed root growth of *A. thaliana* [31]. The genetic analysis showed that both loss-of-function and gain-of-function BR-related mutants in *A. thaliana* have reduced meristem size and the dose-response analysis revealed that BRs promoted the exit of cells from the meristematic region and indicated that BRs promote cell elongation at the meristematic zone of the root. Thus, balanced BR signaling is required to maintain normal root growth rates through the control of the root meristem size. The root became curved at 1 to 100 μg/L BL, which could

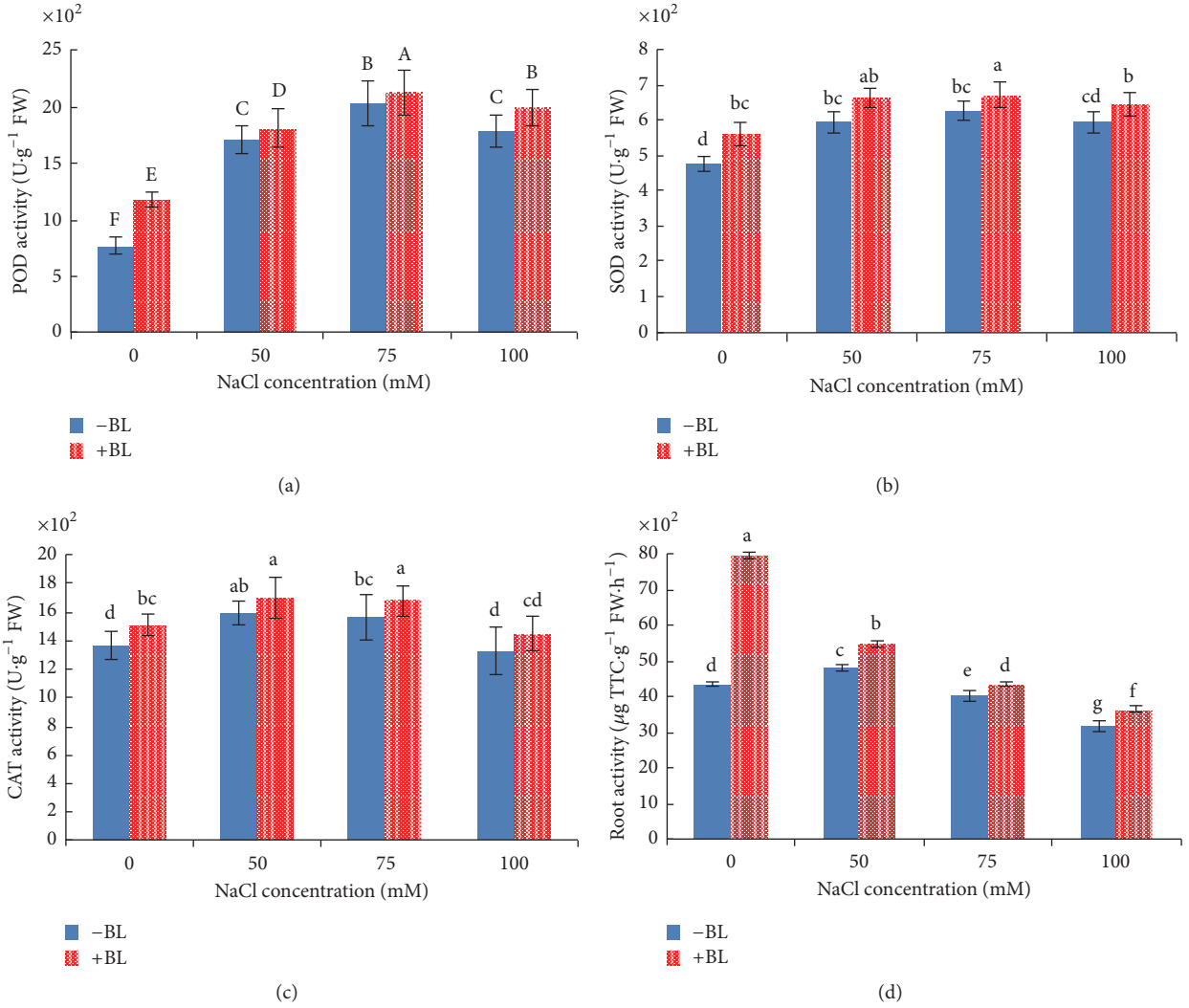

FIGURE 4: Effects of 50 μg/L BL on POD (a), SOD (b), CAT (c), and root activity (d) of potato shoots under salt stress conditions of 0, 50, 75, and 100 mM NaCl. Columns marked with different lowercase letters are significantly different by Duncan's new multiple range test at $P < 0.05$. Columns marked with different capital letters are significantly different by Duncan's new multiple range test at $P < 0.01$.

have resulted from unbalanced BL signaling inhibiting cell division and gravitropism.

Another typical enhanced growth phenotype of shoot (data not shown) was accompanied by inhibition of root growth; thus, measurement of the root activity, adventitious roots numbers, and plant biomass were carried out. The results showed that increasing BL concentrations enhanced root activity and adventitious roots number. Under 0.01 and 0.1 μg/L BL-treatments, there were fewer adventitious roots than that of the control, whereas a well-developed root system was formed, with more lateral roots (data not shown) developing at the adventitious roots. These results concur with previous studies that showed in lateral root growth of *A. thaliana* [37]. In their study, exogenous BR promoted DR5::GUS expression in the root tips and lateral root development by increasing acropetal auxin transport. Thus, BRs are required for lateral or adventitious root development and BRs act synergistically with auxin to promote lateral/adventitious root formation. As one of the important physiological indexes of plant growth, root activity affects the growth and yield of overground portion [33]. Accordingly, increasing root activity with BL might result in enhanced root physiology.

4.2. BL Alleviated Salinity Injury of Potato In Vitro. The development of salt-tolerant crops is fundamental to alleviate salinity disadvantageous effect. However, achievements have been limited, due to complexity of the salt tolerance trait. Thus, understanding the physiological processes involved in salt tolerance is essential to improve the salt tolerance and develop biochemical strategies to enhance crop yields under salinity stress. The first *in vitro* selection of salt stress tolerant plant was reported in *Nicotiana sylvestris* [38]. Several studies have shown that BRs enhance plant tolerance and boost crop yield to a variety of environmental stresses and

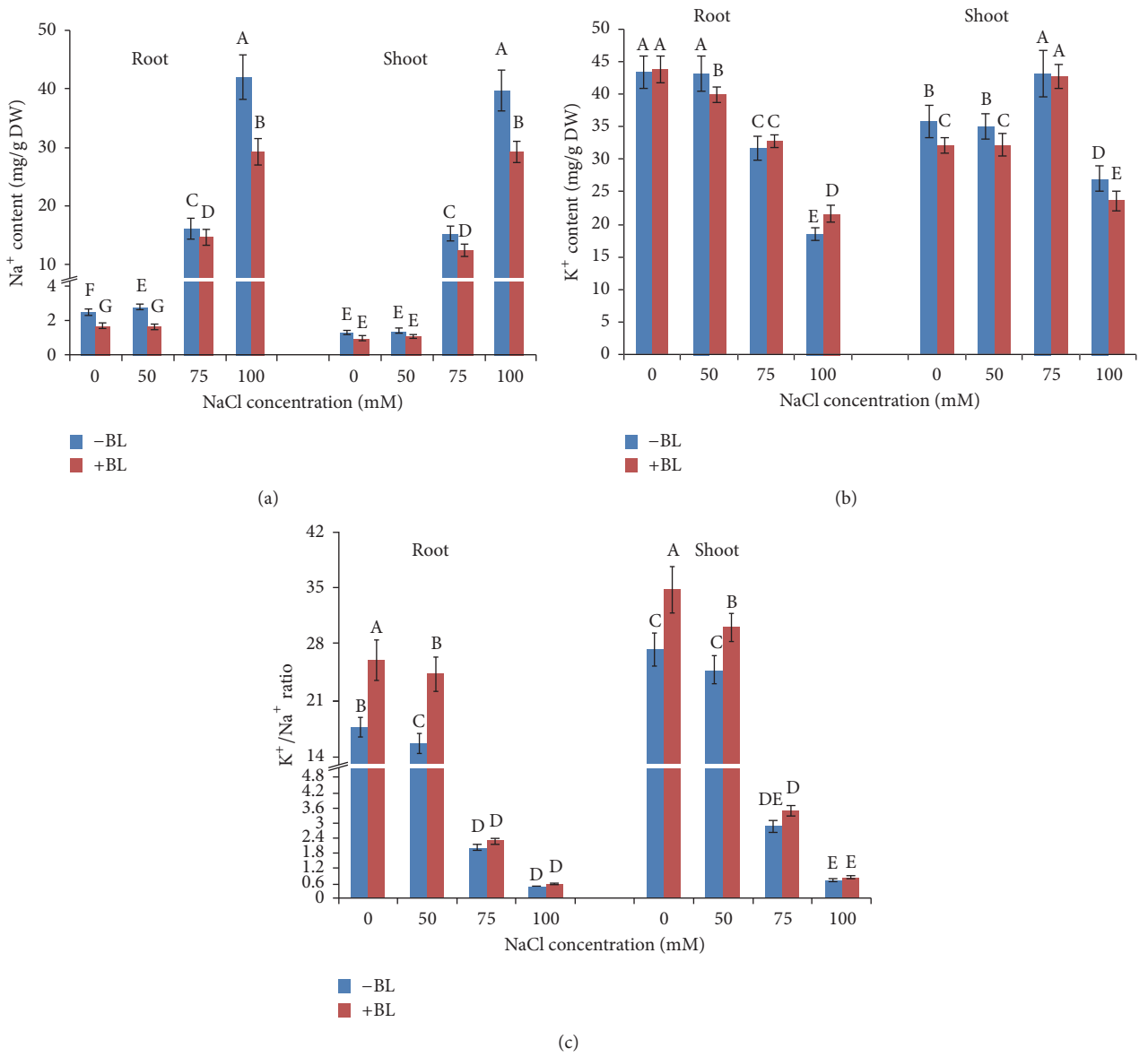

Figure 5: Effects of 50 μg/L BL on Na^+ (a) and K^+ (b) content and K^+/Na^+ ratio (c) of *in vitro* potato root and shoot under different NaCl treatments and the control for 20 days. Columns marked with different letters are significantly different by Duncan's new multiple range test at $P < 0.05$.

species [14, 22, 28, 39, 40]. Our results demonstrated that BL enhanced the biomass of NaCl-stressed potato plants and BL application enhanced the root/shoot ratio. The enhancement in biomass resulted from longer stem length and a well-developed root system. The longer stem length phenotype was consistent with the contribution of BRs in promoting the cell expansion of shoot organs [41]. NaCl resulted in a sharp overall reduction of growth indicators such as shoot height, root length, root fresh weight, shoot fresh weight, and root/shoot ratio; however, the value of root fresh weight and root/shoot ratio was higher at 75 than 50 mM NaCl. This is because longer and more adventitious roots were developed at 75 mM NaCl than at 50 mM NaCl. Application of BL alleviated salinity injury of potato *in vitro* by enhancing the root/shoot ratio, shoot length, shoot weight, root weight, and biomass.

To reveal physiological processes, we measured the content of Pro, MDA, root activity, and antioxidant enzymes' activity. Exogenous application of BL only significantly enhanced Pro content of NaCl-stressed potato plants under 75 mM NaCl. Thus, the osmotic adjustment may not achieve much by enhancing the production of osmoregulatory compounds of potatoes under salinity stress. Given the fact that accumulation of K^+ plays a pivotal role in this process, contributing on average between 35 and 50% of the cell osmotic potential in crops [13], we determined the tissue K^+ content. A continuous increase in the level of MDA content was observed with increased salinity levels, and application of BL

sharply decreased the MDA content. Enhanced MDA content indicates damage caused by ROS. It is necessary for ROS induced by NaCl to be scavenged by antioxidant enzymes for plant survival. The resulting decrease in MDA levels after BL treatment could therefore indicate the efficiency of BL-induced scavenging of ROS as a result of enhanced antioxidant enzymes' activity. These data suggest the adaptation and salt stress tolerance of plants with BL application. Various antioxidant enzymes help in maintaining the balance in ROS production and scavenging and it is believed that enhanced activity of these enzymes facilitates stress protection. A continuous increase in antioxidant enzymes' activity occurred with enhanced salinity level; however, antioxidant enzymes' activity declined at 100 mM NaCl; therefore, it is possible that the high NaCl concentration had severely damaged the lipid membrane, which was confirmed by the high MDA content. BL application resulted in an overall enhancement in the activities of SOD, POD, and CAT under varying degrees of salt stress, suggesting the presence of an effective scavenging mechanism to remove ROS from the plant system and a potential mechanism of plant salt tolerance. Similar results were reported for antioxidant enzymes' activity [20, 29, 42], while some researches showed that there was no or even negative correlation between antioxidant enzymes' activity and salinity stress tolerance [4, 43]. The variation may be caused by the specific variety, tissue, sampling time/observation period, and culture conditions. To make the conclusion more convincing, the amounts of Na^+ and K^+ accumulated in roots and shoots were measured.

Maintaining constant intracellular ion homeostasis, especially K^+ and Na^+ homeostasis, is crucial for plant adapting to saline environments [44]. In this study, the potato showed significant increase in accumulation of Na^+ and overall decline of K^+ in both roots and shoots under NaCl stress. Application of BL improved Na^+ exclusion in both shoots and roots of NaCl treated and nontreated plants, while the amounts of K^+ did not increase in all situations. Similar results were also observed in *Gossypium hirsutum* [20]. K^+/Na^+ ratio also increased when exogenous BL was applied, while it remains a very low value (0.44–0.81) under 100 mM NaCl compared to control plants. In fact, the growth of explant was seriously inhibited under 100 mM NaCl, and it takes a long time of more than 15 days to develop adventitious root, while the average time for NaCl nontreated plants is about 5 days, and two-thirds of the plants develop etiolation symptom. The serious inhibition of high NaCl caused by significant decline in K^+ content and K^+/Na^+ ratio concurred with previous studies that ion exclusion mechanism can provide a degree of tolerance to relatively low concentrations of NaCl but will not work at high concentrations of salt [45].

Roots uptake water and all inorganic nutrients for the plant and are the first organs to be affected by salinity stress; therefore, adaptation of roots to salt stress affects shoot response, physiological functions, and plant growth. BL-induced stress tolerance is a relatively complex process and involves the dynamics of several intrinsic factors. Thus, the growth-promoting effect of BL on salt-stressed potatoes could be attributed to its role in regulating osmotic pressure, ion homeostasis, lipid peroxidation levels, antioxidant enzymes activity, root activity, and adventitious root development. These results are important for the development of salinity-tolerant strains of potatoes that could be produced in more diverse environments, thereby enhancing global potato production.

5. Conclusion

The results presented in this report have clearly shown dose-dependent effect of BL on *in vitro* potato adventitious root growth. Although application of 1–100 μg/L BL inhibited the growth of root length, root number and biomass increased and root activity and root/shoot ratio improved. Thus, we applied high concentration of 50 μg/L BL to *in vitro* potato under salt stress. BL application resulted in an increased physiological action by maintaining K^+/Na^+ homeostasis in shoots and roots, improving root activity, root/shoot ratio, and antioxidative capacity in shoots.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

SEM-EDS-Based Elemental Identification on the Enamel Surface after the Completion of Orthodontic Treatment: *In Vitro* Studies

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Braces as foreign bodies in the mouth carry a risk of side effects and toxicity to the human body. This article presents the results indicating the possible toxic effects of tools used for cleaning the enamel after the completion of orthodontic treatment. The studies were carried out *in vitro*. The procedure of enamel etching, bonding orthodontic metal brackets, and enamel cleaning after their removal was performed under laboratory conditions. The enamel microstructure and elements present on its surface were evaluated using the scanning electron microscope (SEM). Silicon and aluminium were found in addition to the tooth building elements.

1. Introduction

Fixed orthodontic braces are increasingly being used to treat malocclusion in adolescents and adults. Clinical evaluation of the long-term presence of braces as foreign bodies in the mouth shows that they can be harmful to human health. Mucosal ulceration, acute irritation, and allergy to nickel and other alloying elements of orthodontic brackets and arches, mainly in the brazed components, caused by orthodontic braces can have damaging effects, both local and general [1–7].

The possible impact on human health of orthodontic resins and composite materials as well as their residues left on the tooth surface must be also taken into consideration [8]. All the materials used in dentistry have European authorization and overall safety certification. However, it does not exclude the possibility of cases of a negative impact of dental materials on health during their long-term presence in the mouth, depending on the sensitivity of individuals. Another stage, which can lead to exposure of the body to harmful materials, is the moment of cleaning the tooth surface from composite remnants. During the process of cutting or polishing, chemical elements are released from both cleaned and cleaning elements, which within the water spray allows for their immediate absorption through mucosa well supplied with blood and creates the risk of aspiration into the airways or ingestion of potentially harmful dust generated during cleaning. Teeth are typically cleaned with instruments comprising metallic alloys, which may contain heavy metals such as chromium, copper, lead, nickel, and zinc [9]. These metals bind with proteins in the body, replacing the naturally occurring macro- and microelements and causing dysfunction of cells. Therefore, they are toxic to them. Published studies have shown that damage to the oxidation of biological macromolecules results from binding of heavy metals with the protein of DNA and nucleotides [10]. Although it is known that metals, most of all aluminium, have a number of adverse health effects [11, 12], which persist for a longer period of time during and after exposure; the frequency of their use continues to grow. Their toxicity is a growing problem,

affecting the evolution, nutrition, and environment [13–16]. Therefore, in the present study, it was decided to examine the enamel surface after the completion of orthodontic treatment for the presence of elements recognized as toxic to the human body.

2. Material and Methods

The studies were carried out *in vitro*. The material consisted of 15 premolars extracted for orthodontic reasons. The following conditions defined the exclusion criteria: the presence of developmental defects of enamel, that is, hypoplasia, turbidity or discoloration, caries, and fillings on the vestibular surface.

The selected teeth were stored for 30 days in demineralised water, with a thymol crystal (0.1%) at room temperature.

Prior to the bonding of orthodontic brackets, tooth surfaces were cleaned using a polisher (Top Dental, Poland) with fluoride-free toothpaste Pressage (Shofu Inc., Japan) designed to prepare the enamel before fixing orthodontic steel brackets (Dentaurum, Germany). Then, the teeth were washed with distilled water and dried with compressed air for 15 seconds. For fastening brackets, orthodontic composite material Transbond™ XT Light Cure Adhesive (3M Unitek, USA) was used, which required prior preparation of the enamel surface. The vestibular surface of the teeth was etched for 30 seconds with 37% phosphoric acid, Blue-Etch (CERKAMED, Poland), rinsed with distilled water for 15 seconds and dried with compressed air. The dental adhesive OptiBond Solo Plus (Kerr, USA) was rubbed with an applicator into the etched enamel surface for 15 seconds. Then, the surface was dried with a gentle stream of air for 3 seconds and cured with halogen curing lamp light of the intensity of 750 mW/cm^2 for 20 seconds. The composite material Transbond XT Light Cure Adhesive was placed on the bracket surface. The bracket was pressed onto the enamel surface with commonly used orthodontic bracket tweezers. The orthodontic bracket was placed at the centre of the mesial-distal axis of the tooth, moving its centre 3.5 mm away from the edge of the occlusal surface. The distance was measured using an orthodontic positioner. Once the bracket was properly placed, the material was subjected to polymerization with halogen curing lamp light for 40 seconds.

The teeth with fixed orthodontic brackets were stored in demineralised water at room temperature for 24 hours. After this time, the brackets were removed mechanically with the ix827 pliers (IxionInstruments, USA) designed for removing all types of brackets.

The remains of the adhesive were removed from the surface using a micromotor, standardly mounted in a dental unit, at a speed of 40 000 revolutions/min with water cooling and pressure force of 1.0 N. Abrasive processing was applied using cup shaped polishers made of aluminium oxides and bonding silicone.

The enamel cleaning procedure was considered to be finished on the basis of the naked eye evaluation, without additional zooming, and touching with a dental probe in the unit lamp light. The assessment criteria were the smoothness of the tooth surface and the absence of residual composite material. After cleaning the surface, the tooth was washed with water spray using the air and water compressor attached to the dental unit.

Before treatment and after finishing the above cleaning procedure, the tooth surface microstructure was evaluated using the JEOL JSM6610LV scanning electron microscope with SEI and BEI detectors. Oxford's EDS (Energy-Dispersive X-ray Spectroscopy) was used to identify the elements, while data were analyzed in the Aztec Software. Microscopic studies were performed at the Institute of Technology of the Pedagogical University of Cracow. The analysis was performed in order to observe composite material remains on the enamel surface and evaluate other elements present on its surface and the surface of the tooth crown. The JEOL JSM6610LV scanning electron microscope is adapted to analyze highly developed surfaces. The microscope has high resolving power of at least 15 nm at a large depth of field, so it is possible to map the surface details of the test samples greatly enlarged. The test samples do not require special preparation of the surface, since the microscope is equipped with the so-called low vacuum allowing for observation of nonconductive specimens without the necessity of applying the conductive layer. The specimen was placed in the microscope chamber and then subjected to an electron beam generated by an electron gun and accelerated in an electric field of 1/30 kV. There is a deflection system in the path of the electron beam—deflection of the beam in the direction X-X and in the direction perpendicular thereto Y-Y. Then, the electron beam having typically a diameter of 10/200 nm moves to the specimen surface and is focused on it. The reaction of the beam with the observed specimen results in activation of electrons from its subsurface area, which are then trapped by the detector placed in the microscope chamber. The test specimens were analyzed using the secondary electron imaging (SEI) method, analysis of backscattered electron imaging (BEI), and EDS analysis. X-ray microanalyzer (EDS) enabled analyzing the chemical composition of the test sample in the selected micro area of its surface—in this case within the crown and the composite material.

Qualitative and quantitative analyses were made using a microprobe. The quantitative analysis involved plotting a graph of the distribution of elements along a specified line. The qualitative analysis shows the arrangement of the elements on the surface, wherein the amount of a given element is proportional to the brightness of the image in a given place.

The obtained results were subjected to statistical analysis, assessing the median of the set which was Q2 = 0.4. In order to assess the correlation between saccadic and qualitative variables, the Kruskal-Wallis test and U Mann Whitney test were used. When verifying the hypotheses, the level of significance was $p = 0.05$.

3. Results

SEM-EDS analysis of the teeth surface after finishing the enamel cleaning procedure showed that although the operator considered the tooth surface to be completely clean and smooth, there were still composite resin residues on the

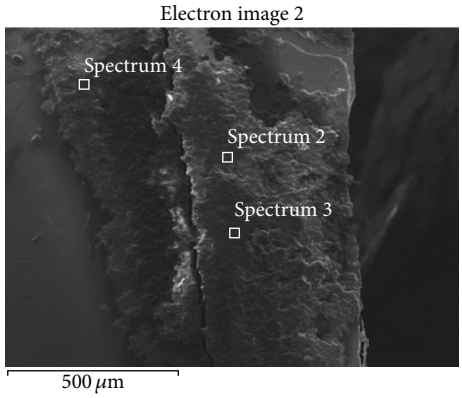
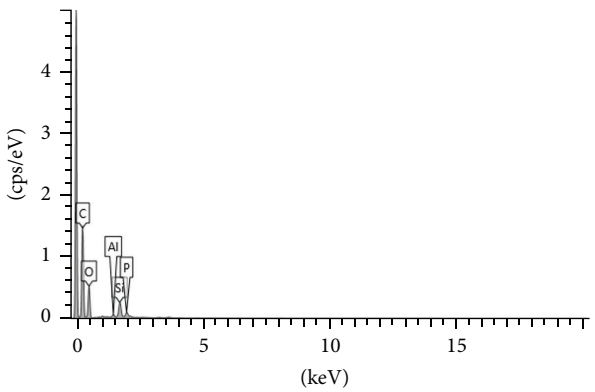


Figure 1: The residue of adhesive composite resins on the enamel surface left after tooth cleaning. Spectrums 2, 3, and 4 show the location of EDS analysis.

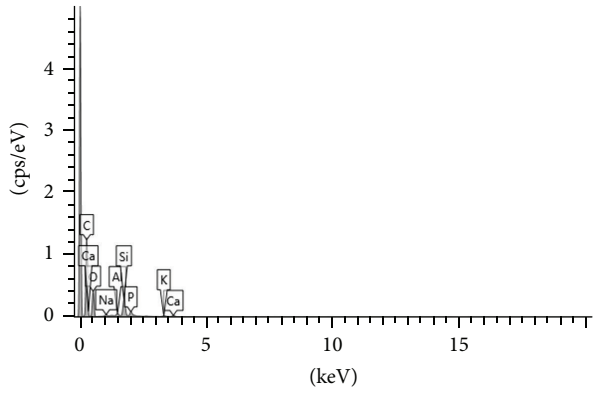


	Wt%	σ
C	63.5	0.8
O	32.6	0.8
Si	2.5	0.1
P	0.9	0.1
Al	0.4	0.1

Figure 2: The SEM-EDS analysis of spectrum 2 showed in Figure 1. The percentage range of the elements in the composite mass, visualized in the adequate voltage range, counted in seconds per electron-volt. keV: accelerating voltage range used for EDS analysis, kilo-electron-volt. cps/eV: counts per second per electron-volt.

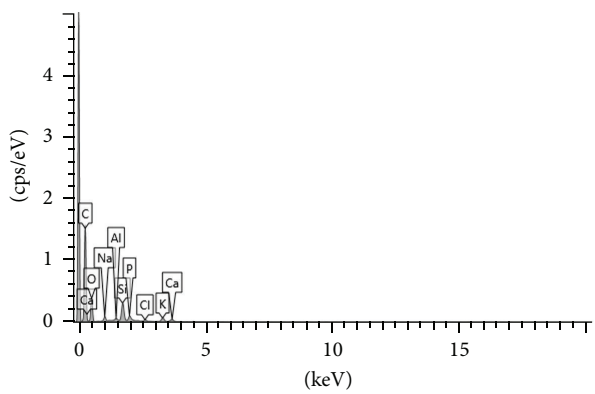
enamel surface. Elemental analysis of the material residues and the completely cleaned tooth surface revealed that, in both cases, in addition to the naturally occurring elements building the tooth tissue which were transferred from the enamel surface to the composite material during cleaning (oxygen, carbon, hydrogen, nitrogen, calcium, phosphorus, sodium, and potassium), there occur also other elements which do not build the tissue: silicon and aluminium oxides. Figure 1 shows an example of SEM image of the composite resin residues and the location of EDS analysis.

Figures 2, 3, and 4 present the example of the SEM-EDS analysis of spectrums 2, 3, and 4 showed in Figure 1. It shows the percentage range of the elements in the composite mass,



	Wt%	σ
C	64.9	1.1
O	29.5	1.0
Si	3.2	0.2
P	0.9	0.1
Ca	0.6	0.1
Al	0.4	0.1
Na	0.4	0.1
K	0.3	0.1

Figure 3: The SEM-EDS analysis of spectrum 3 showed in Figure 1. The percentage range of the elements in the composite mass, visualized in the adequate voltage range, counted in seconds per electron-volt. keV: accelerating voltage range used for EDS analysis, kilo-electron-volt. cps/eV: counts per second per electron-volt.



	Wt%	σ
C	67.2	1.0
O	25.8	0.9
Si	2.8	0.1
K	1.0	0.1
Na	1.0	0.1
P	0.8	0.1
Ca	0.6	0.1
Cl	0.4	0.1
Al	0.3	0.1

Figure 4: The SEM-EDS analysis of spectrum 4 showed in Figure 1. The percentage range of the elements in the composite mass, visualized in the adequate voltage range, counted in seconds per electron-volt. keV: accelerating voltage range used for EDS analysis, kilo-electron-volt. cps/eV: counts per second per electron-volt.

Table 1: The percentage of aluminium in the mass of orthodontic resin residues after completed enamel cleaning using silicone polishers with aluminium oxides. Wt%Al: percentage of aluminium by mass. SD: standard deviation.

Tooth number	Wt%Al	SD
1	0,4	0,1
2	0,3	0,1
3	0,5	0,1
4	0,4	0,2
5	0,4	0,2
6	0,1	0,1
7	0,3	0,1
8	0,4	0,1
9	0,5	0,2
10	0,4	0,1
11	0,3	0,1
12	0,2	0,1
13	0,4	0,1
14	0,5	0,2
15	0,3	0,1

Table 2: The percentage of aluminium in the mass of orthodontic resin residues before the orthodontic treatment. Wt%Al: percentage of aluminium by mass.

Tooth number	Wt%Al
1	0
2	0
3	0
4	0
5	0

visualized in the adequate voltage range, counted in seconds per electron-volt.

Table 1 shows the percentage of aluminium in the mass of orthodontic resin residues after completed enamel cleaning using silicone polishers with aluminium oxides. Table 2 shows the percentage of aluminium on the enamel surface in the beginning of the treatment. There were no aluminium ions on the enamel in the control group.

The difference between the two groups was statistically significant ($p < 0,05$).

4. Discussion

Aluminium is ubiquitous on the Earth's surface. It is a metal which is light and corrosion-resistant [17]. Because of these properties, aluminium has become widely used in daily life. However, its soluble form produced by the industry is a potential health hazard for the living organisms [18]. Nowadays, people are exposed to it through food, which contains chemical additives, cooking in aluminium pots, packaging lined with aluminium, some preparations shielding the digestive tract and neutralizing gastric acid, and water, to which aluminium salts are added in the purification process. Aluminium interacts with most physical and cellular processes in humans. The exact mechanism of aluminium absorption by the gastrointestinal system is not yet fully understood. Based on the available literature, it is difficult to give the exact period of time for the toxicity of aluminium, because some of its symptoms are detected within a few seconds and others within a few minutes after exposure [19]. The toxicity of aluminium is probably the result of the interaction between apoplastic and symplastic objects [20]. In the human body, Mg^{2+} and Fe^{3+} are replaced by Al^{3+}, which causes numerous disorders in intercellular communication, cell growth, and secretory functions. The changes that are caused by aluminium in neurons resemble degenerative changes observed in Alzheimer's disease. So far, reports have been published on the dissolution of metal ions from orthodontic braces and other dental materials in artificial saliva [21–24]. However, the enamel after the completion of orthodontic treatment has not been evaluated in this respect. Therefore, the comparison of the obtained results is not possible. In the present study, the most important and surprising observation is the presence of heavy metal ions on the enamel surface which was clinically assessed as cleaned. Qualitative assessment, which indicates the issue that needs to be examined, is more important than quantitative assessment.

The SEM-EDS analysis showed also the presence of silicone as a residue on the enamel after the orthodontic treatment. As silicone carcinogenicity end enzymes-inductive effects in mammals are lately brightly discussed and investigated [25, 26] it is very important to extend the researches in the dental industry. The silicones are regularly used in dentistry especially in endodontics as sealants, in prosthetics and orthodontics as impression materials, and in craniofacial surgery as internal implants. If the long-term studies give proof of silicone toxicity in human organisms it will be essential to change the whole methodology of treatment in many fields of medicine.

5. Conclusions

Metal ions, including aluminium, are present on the enamel surface after the completion of orthodontic treatment. The presence of aluminium was detected after cleaning the enamel using a polisher with aluminium oxides. Other methods for cleaning the enamel after orthodontic treatment should be assessed for the presence of residues of aluminium and other heavy metals. The problem of exposure of patients to adverse effects of heavy metals during and after orthodontic treatment should be explored and thoroughly investigated because of their potentially harmful effects on human health.

Competing Interests

There are no competing interests related to this paper.

Authors' Contributions

Monika Machoy and Julia Seeliger contributed equally to this work.

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