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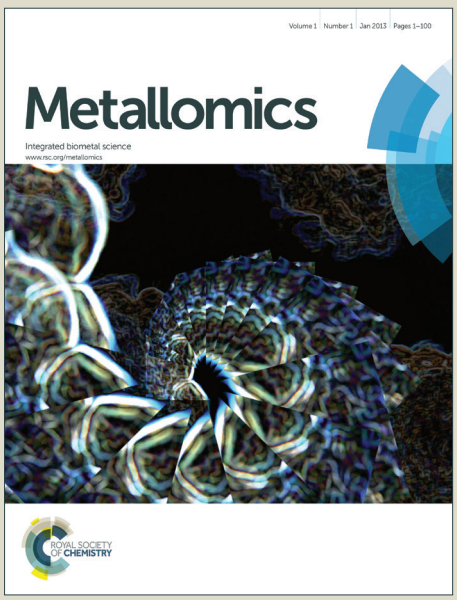

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Effects of vanadium (V) and magnesium (Mg) on rat bone tissue: mineral status and micromorphology. Consequences of V-Mg interactions ∫

Agnieszka Ścibior,*[a] Agnieszka Adamczyk,[b] Robert Mroczka,[c] Irmina Niedźwiecka,[a] Dorota Gołębiowska,[a] and Emilia Fornal[d]

[a] *Laboratory of Oxidative Stress, Center for Interdisciplinary Research, The John Paul II Catholic University of Lublin, Poland*
[b] *Laboratory of Physiology & Animal Biochemistry, Department of Zoology & Invertebrate Ecology, The John Paul II Catholic University of Lublin, Poland*
[c] *Laboratory of X-ray Optics, Center for Interdisciplinary Research, The John Paul II Catholic University of Lublin, Poland*
[d] *Laboratory of Separation and Spectroscopic Method Applications, Center for Interdisciplinary Research, The John Paul II Catholic University of Lublin, Poland*

Running title: V and Mg effects on bone - in vivo model

*Corresponding author:
Dr Agnieszka Ścbior
Laboratory of Oxidative Stress, Center for Interdisciplinary Research, The John Paul II Catholic University of Lublin, Kraśnicka Ave, 20-718 Lublin, Poland. E-mail: cellbiol@kul.lublin.pl;
Fax: +48 81 4454611; Tel.: +48 81 4454604

∫ Part of the results of this work was presented at the 9th International Symposium: "Trace Elements in the Environment", May 28-29, 2012, Olsztyn, Poland, [bone mineral status]; and at the 9th International Vanadium Symposium: Chemistry, Biological Chemistry & Toxicology (V9), June 29 – July 2, 2014, Padova, Italy, [bone micromorphology]

Abbreviations: AAS, atomic absorption spectrometry; ALP, alkaline phosphatase; BE, binding energy; BEOV, bis(ethylmaltolato)oxovanadium IV; BMOV, bis(maltolato)oxovanadium IV; CH, cerebral hemisphere; CRM, certified reference material; CsClLaCl, caesium chloride lanthanum chloride buffer; DFE, distal femoral epiphysis; DL (LOD), limit of detection; F, faeces; FAAS, flame atomic absorption spectrometry; FBSR, femoral bone surfae roughness; FCS, femoral cross-section; FD, femoral diaphysis; FOV, field of view; GFAAS, graphite furnace atomic absorption spectrometry; Ht, haematocrit; HA, hydroxyapatite; LPO, lipid peroxidation; MCV, mean corpuscular volume; Mg-HA, Mg-substituted hydroxyapatite; MS, magnesium sulphate; NCP, non-collagen protein; OP, optical profilometer; OS, oxidative stress; PCR, polymerase chain reaction; PFE, proximal femoral diaphysis; PGF_2, prostaglandin F_2; PO_4^{3-}, phosphate anion; PTH, parathyroid hormone; RBC, red blood cell count; RDW, red blood cell distribution width; Sa, the mean roughness; Sdr, the developed interfacial area ratio; Sdq, the root mean square surface slope; Si-HA, silicon containing hydroxyapatite; Sku, kurtosis; SMV, sodium metavanadate; SMV-MS, sodium metavanadate and magnesium sulphate; SPIP, scanning probe image processor; Ssc, the mean summit curvature; Ssk, skewness; Sq, the root mean square roughness; SIMS, secondary ion mass spectrometry; Sz, the ten point height; TGF-β1, transforming growth factor beta 1; TIBC, total iron binding capacity; TFM, teflon fluor modified; TS, transferrin saturation; VO_4^{3-}, vanadate anion; VO^{2+}, oxidovanadium(IV) [vanadyl]; 1,25-$(OH)_2$D, 1,25-dihydroxyvitamin D; XPS, X-ray photoelectron spectroscopy

Abstract

The extent to which the 12-week separate and combined administration of vanadium (as sodium metavanadate - SMV, 0.125 mg V/mL) and magnesium (as magnesium sulphate - MS, 0.06 mg Mg/mL) affects bone mineral status and micromorphology as well as the alkaline phosphatase (ALP) activity in femoral diaphysis (FD) were examined in male rats. The bone chemical composition of SMV-exposed rats was also investigated. SMV alone or in combination with MS (as SMV-MS) reduced the levels of Mg_{FD} (by 21% and 20%) and P_{FD} (by 12% and 9%), lowered the Ca_{FD} content (by 7% and 10%), and caused a rise of the Fe_{FD} concentration (by 22.5% and 17%), compared with the control; SMV alone also reduced and enhanced the K_{FD} and Zn_{FD} concentrations (by 19% and 15%, respectively) but remained without significant effect on the femoral bone surface roughness (FBSR), whereas MS alone lowered the V_{FD}, P_{FD}, and Cu_{FD} levels (by 42%, 10%, and 20.6%), reduced FBSR, and created the regular femoral bone surface shape. The SMV-MS combination also induced a decline and rise in the levels of Cu_{FD} (by 30%) and Na_{FD} (by 15%), respectively, compared with the control and the MS-supplemented rats; elevated ALP_{FD} activity (by 24%, 35%, and 40%), compared with the control, SMV-exposed, and MS-supplemented animals; and increased FBSR. Relationships between the root mean square roughness (Sq) and skewness (Ssk): Sq [MS<SMV<Control<SMV-MS] <=> Ssk [SMV-MS>Control>SMV>MS], ALP_{FD} and Sq: ALP_{FD} <=> Sq [SMV-MS>Control>SMV>MS], and between other variables were demonstrated. A partial limitation of the drop in the P_{FD} and K_{FD} levels and normalization of the Zn_{FD} concentration were a consequence of the V-Mg antagonistic interaction whereas a consequence of the V-Mg synergistic interaction was the increase in the Na_{FD} level, ALP_{FD} activity, and FBSR. $Ca_{10}(PO_4)_5(SiO_4)(OH)$ was part of the inorganic component of bone of the SMV-exposed rats.

Key words: Bone, Micromorphology, Mineral status, Rats, Roughness, Vanadium-Magnesium Interactions, X-ray photoelectron spectroscopy

1 Introduction

Bone is a dynamic and highly specialized form of connective tissue, which undergoes a continuous remodelling process. It is rebuilt and degraded by tightly coupled actions of mesenchymal stem cell-derived osteoblasts and monocyte-derived osteoclasts (bone-forming and bone-resorbing cells, respectively) responsible for coordinating normal bone development.[1,2]

It is known that bone is very sensitive to alterations in mineral homeostasis and strongly influenced by the intake of several nutrients. Variations in the amount of minerals have been

reported to affect the properties and quality of this tissue. It is also generally accepted that the quality of bone is determined not only by its material composition but also by its structure.[1,3,4]

Beside sodium (Na), potassium (K), iron (Fe), zinc (Zn), copper (Cu), and a large amount of such elements as calcium (Ca), and phosphorous (P), bone also contains magnesium (Mg). This macroelement strongly influences bone final composition and crystal structure. Its deficiency has been suggested to be implicated as a possible risk factor for osteoporosis.[3-5] Bone is also the main tissue for storage of vanadium (V), a bone-seeking element,[6] nutritional and physiological role of which in enhancement of bone formation[7] and therapeutic application in osteoporosis[8-10] have been highlighted. Recently, it has also been reported that orthopaedic implants, if supplemented with V, might provide a source of a better model for bone formation and its turnover.[11] A wide role of V in the manufacture of hard tissue implants especially in bone tissue engineering is emphasized.[12]

In the literature, some aspects of action of V, the toxicological effects of which are well known to be dependent to many factors[13,14], and Mg on bone in *in vivo* and *in vitro* conditions have already been described. For example, studies on a rat model showed the effect of some V compounds [vanadium pentoxide (V_2O_5), sodium orthovanadate, bis(ethylmaltolato)oxovanadium IV (BEOV), and bis(maltolato)oxovanadium IV (BMOV)] on bone formation and mineralization, cortical bone toughness, osteoblast count and differentiation, the bone DNA content, the bone density and mechanical properties, and on bone metabolism in the femoral diaphysis.[7,15-18] The influence of V on skeleton in goats has also been described.[19] *In vitro* studies, in turn, showed the effect of orthovanadate, metavanadate, decavanadate and the influence of some oxidovanadium(IV) complexes on bone resorption, bone matrix formation and mineralization, cellular proliferation of normal and tumoural osteoblasts, cell differentiation of tumoural osteoblast, production of type-I collagen, macrophages, and on the bone DNA, collagen, and non-collagen protein (NCP) synthesis.[8,9,20-29] The influence of V^{5+} ions on formation of apatite has also been presented.[30] In addition, several studies on the effects of V on bone tissue, conducted both *in vivo* and *in vitro* conditions, yielded possible mechanisms of its action in bone.[7,9,10,20,21] As far as Mg is concerned, studies on an animal model showed the effect of this element on bone formation and mineralization, bone mass and the trabecular bone volume, the number of bone-resorbing cells, bone turnover, and on apatite crystal formation.[31-39] They also demonstrated that the long-term suboptimal Mg supply is beneficial for bone health, but long-term supplementation thereof may be deleterious.[40] Human studies, in turn, presented the effect of Mg on bone turnover, parathyroid hormone (PTH) secretion, and on the serum concentration of 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D).[41,42]

Despite the fact that some aspects of action of V and Mg on bone have already been recognized some of them have not been studied yet. For example, no data about the effects of administration of V and Mg individually or in combination on bone micromorphology are available.

No information about the influence of both elements administered in combination on bone mineral status has also been provided. In the literature, there is only a single report about the effect of administration of V alone on the rat femoral Zn, Cu, and Fe content[43] and few reports which describe the effects of separate administration of V and Mg on the femoral amounts of such basic minerals as Ca, Mg, and/or P under *in vivo* conditions.[7,44-49] Similarly, no information about the effects of the V-Mg interactions with regard to bone tissue has been reported either. Generally, the consequences of the V-Mg interaction for health are relatively little known, and the data referring to the interaction between the above-mentioned elements (except our studies)[50-56] are limited.[57-60]

It is known that recognition of interactions between metals (synergism/antagonism) may contribute to easier prediction of both the kind and the extent of outcomes evoked by them. It is also well known that the interactions may influence the morphology, ultra-structure, cell metabolism, and consequently they may affect the internal organs/tissues and whole organism functions. They may also be used to elucidate the cellular mechanisms of response to combinations of metals.

Taking all the above into account we undertook the study in order to expand the knowledge in the research field concerning the influence of V, as sodium metavanadate ($NaVO_3$, SMV), and Mg, as magnesium sulphate ($MgSO_4$, MS), on bone tissue in *in vivo* conditions drawing attention to the consequences of interactions between both the above-mentioned metals. The following specific aims have been taken into consideration a) evaluation on a rat model the femoral level of such basic elements as Ca, Mg, and P, and also the femoral level of other minerals such as K, Na, Fe, Zn, and Cu, needed for metabolic processes related to bone,[3,4, 61-63] under separate and combined administration of SMV and MS, as well as the activity of bone ALP, which plays a key role in bone formation being responsible for osteoblastic creation of the bone matrix and its mineralization, b) examination of the effect of the above-mentioned compounds on the micromorphology of the cross-section surface of rat femoral bone, and c) investigation of possible relationships between the chosen variables. We also wanted to d) recognize to what degree the effect of 12-week co-administration of SMV and MS differs from that found under separate application of both compounds, e) check in what way the indices presented in this report were modified under simultaneous SMV and MS administration (by an independent action of V, Mg, or/and by the V-Mg interaction), and f) describe the kind of interaction revealed between V and Mg, applied in combination as SMV and MS, respectively, with regard to parameters presented in this paper. Finally, we undertook an attempt in order to characterize the chemical composition of bone of the SMV-exposed rats with the X-ray photoelectron spectroscopy (XPS) method. Considering the importance of current bone related disorders the present study is important. According to our knowledge, no similar reports describing the changes in the parameters chosen for investigation in the rat bone tissue under separate and simultaneous SMV and MS administration in the selected *in*

vivo conditions have been published up until now. No reports that would show the character of the V-Mg interaction referring to the indices described in the present paper and relationships between them are available either.

2 Materials and methods

2.1 Reagents

$NaVO_3$ and $MgSO_4$ were obtained from Sigma Chemical (St. Louis, USA). Nitric acid (HNO_3, 65% suprapure, used for femur digestion) and diethyl ether ($C_4H_{10}O$, used for preparation of the femur for digestion) were acquired from Merck (Darmstadt, Germany), whereas hydrogen peroxide (H_2O_2; 30% pure P.A., used for femur digestion) was derived from POCH (Gliwice, Poland). The physiological buffered saline without calcium and magnesium ions (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4x12H_2O$, 1.5 mM KH_2PO_4; pH = 7.4 ± 0.2, used for preparation of the femur for digestion) was acquired from the Serum and Vaccine Factory (Biomed, Lublin, Poland). The caesium chloride lanthanum chloride buffer (CsClLaCl; Sigma Chemical, St. Louis, USA); stocks of V, Mg, Zn, and Cu (Inorganic ™ Ventures, Christiansburg, USA), and a stock of Ca (Spectracer, UK) atomic absorption standard solutions as well as a multielement standard solution for Na and K (Sigma-Aldrich) were used in the element analysis with the Atomic Absorption Spectrometry (AAS) method. The reagents for determination of the activity of alkaline phosphatase (ALP) and the level of P in bone were acquired from AlphaDiagnostics (Warsaw, Poland). All the chemicals were of the highest quality available.

2.2 Instrumentation

Samples of the femoral diaphysis (FD) for determination of selected elements were digested using a model Speedwave Four microwave digestion system* (Berghof, Germany) equipped with a temperature and pressure sensor in each vessel. V, Mg, Ca, Fe, Zn, Cu, K, and Na in FD (V_{FD}, Mg_{FD}, $Ca_{,FD}$ Fe_{FD}, Zn_{FD}, Cu_{FD}, K_{FD}, and Na_{FD}) were determined by Flame or Graphite Furnace Atomic Absorption Spectrometry (FAAS and GFAAS) using a SpectrAA Z2000 TANDEM atomic absorption spectrometer* (Hitachi, Japan) equipped with a Zeeman background corrector. The level of P and the activity of ALP in the FD homogenates (P_{FD} and ALP_{FD}, respectively) were assessed using an automatic biochemical analyser BS-120* (Mindray, China). Surface micromorphology studies of thin femoral cross-sections (FCS) obtained from all the rats were performed using an optical profilometer (OP) (WYKO NT9800, Veeco)*, whereas the chemical composition of selected rat bone was performed using a PHI 5000 VersaProbe™ (ULVAC-PHI) X-ray photoelectron spectrometer (Japan) with microfocused and monochromatic Al Kα radiation (1486.6 eV, 25 W,

100 μm beam size) equipped with a spherical capacitor energy analyzer with multi-channel detection within a 100 x100 μm area for XPS analysis.

2.3 Animals and experimental protocol

The biological material (femurs) used in this study was obtained from selected 44 outbred albino male Wistar rats used in our previous experiments, in which the animals were divided into 4 groups and received every day over a 12-week period: a deionised water to drink (Group I, Control); a water solution of $NaVO_3$ (Group II, SMV, 0.125 mg V/mL, pH = 7.2); a water solution of $MgSO_4$ (Group III, MS, 0.06 mg Mg/mL, pH = 5.7), and a water solution of $NaVO_3$ in combination with $MgSO_4$ (Group IV, SMV-MS, 0.125 mg V and 0.06 mg Mg/mL, pH = 7.1). All the rats were maintained individually in stainless steel cages under controlled conventional conditions and had *ad libitum* access to fresh fluids, deionised water, and a standard diet (Labofeed B, Fodder and Concentrate Factory, Kcynia, Poland). The intake of food, fluids, and deionised water was monitored daily and body weight was checked weekly throughout the experimental period. More details of the experiments conducted in accordance with the experimental protocol approved by the 1st Local Ethical Committee for Animal Studies in Lublin had already been provided in our earlier report.[54]

$NaVO_3$ and $MgSO_4$ were chosen for investigation. It is known that various compounds of the same element may respond differently. The V and Mg concentrations were selected on the basis of our previous experiments[52,54,56,64] and some literature data.[58,65] The dose of V is within the broad range of doses that were used for demonstrating the V antidiabetic activity[65,66] and for analyzing its pharmacokinetic behaviour[67] on an animal model. The concentration of Mg, in turn, was selected to be not too high, because Mg as $MgSO_4$ has been reported to induce dose-limiting diarrhoea.[68]

2.4 Preparation of femurs for digestion and profilometric evaluation

After the 12-week experimental period, the femurs were removed from all anesthetized rats. After removal of the overlying tissue with stainless steel knives, the femurs were washed in 0.9% NaCl, weighed, and stored frozen at –80 °C in a deep freezer HFU 486 Basic* (Thermo Fisher Scientific, Germany) until the time of mineralization and profilometric studies. Before digestion and profilometric measurements, all the femurs collected, thawed, cut (rotational speed 5000 rpm, clamping force 50 N, cutting speed 0.01 mm/s) at a temperature of 21 °C using a diamond disk saw (thickness =1 mm) cleaned with a corundum plate (Metkon Micracut 175)*, and cooled with ultra-pure water (resistivity = 18 MΩ, temp. 21 °C) obtained from an ultra-pure HLP Spring 5R water

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system* (Hydrolab, Poland) to separate the proximal and distal femoral epiphysis (PFE and DFE, respectively, regions of the trabecular bone) from FD (a region of the cortical bone). For profilometric analysis, FD obtained from each group of rats (Groups I-IV) were subsequently cut under the same parameters as mentioned above, at a temperature of 21 °C using the same diamond disk saw in order to obtain thin FCS, which were stored frozen at –80 °C after washing in ultra-pure water (resistivity = 18 MΩ, temp. 21 °C). After two weeks, when all the FCS for each group of rats were collected, they were used for profilometric analysis that was performed at a temperature of 21 °C. The details concerning the preparation of FD for digestion have not been described in this report because they had already been provided in our previous paper.[64]

2.5 Decomposition of FD for the measurements of V, Mg, Ca, Fe, Zn, Cu, K, and Na by the AAS method

The FD samples (~ 0.355 g) were wet-mineralized in Teflon Fluor Modified (TFM) covered digestion vessels. More details of the FD digestion had already been provided by us previously.[64] After mineralization, V_{FD}, Mg_{FD}, $Ca_{,FD}$ Fe_{FD}, Zn_{FD}, Cu_{FD}, K_{FD}, and Na_{FD} were determined. All the operating parameters of the SpectrAA Z2000 TANDEM atomic absorption spectrometer with details of measurements of V, Mg, Ca, Fe, Cu, K, and Na have already been provided in our previous papers.[54,64] Only the limits of detections (DL, LOD) for elements investigated in the rats' femur and the values determined by us in the selected bone Certified Reference Material (CRM), the analysis of which confirmed the reliability of the proposed approach, not mentioned previously, have been added in the present report and placed in the Results section.

2.6 Determination of the level of P in FD

0.150 g of FD was homogenized in 1 ml of ice-cold PBS using a BioGen PRO200 (ProScientific, USA) homogenizer to make tissue homogenates. Next, the samples were centrifuged with cooling (10 000*g*, 5 min, 4 ºC) using a centrifuge Heraeus Megafuge 11R* (Thermo Fisher Scientific, Germany). Afterwards, the samples were immediately frozen at -80 °C and stored in a deep-freezer HFU 486 Basic* until analysis. The level of P_{FD} was measured spectrophotometrically at 37 °C.

2.7 Surface analysis of the femur

The measurements were carried out at eighteen sample points (Fig. 1) with scanning dimensions 0.3 x 0.2 mm. The objective lens magnification was set to 20x, whereas the field of view (FOV) was set to 1.0x. The following surface roughness parameters, associated with peaks and valleys, were determined for each sample point: amplitude - the mean roughness (Sa), the root mean square roughness (Sq), skewness (Ssk), kurtosis (Sku), and the ten point height (Sz), and hybrid - the root

mean square of surface slope (Sdq), the developed interfacial area ratio (Sdr), and the mean summit curvature (Ssc), using the Scanning Probe Image Processor (SPIP) v. 5.1.4 software (Image Metrology A/S, Denmark). Formulas for each parameter have been attached in the form of a supplement provided at the end of the report. The amplitude parameters, which are generally considered as the most important property of the surface, were chosen for basic roughness characteristics. The hybrid parameters, in turn, which reflect slope gradients and combine amplitude and spatial analysis, were chosen in order to examine the distribution of slopes. Analysis of the distribution of slopes has been frequently used to differentiate between peaks and valleys along varying directions, and, thereby, it helps to define a surface accurately.[69-71]

2.8 Determination of the ALP activity in FD

0.150 g of FD was homogenized in 1 ml of ice-cold PBS using a BioGen PRO200 (ProScientific, USA) homogenizer to make tissue homogenates. Then the samples were centrifuged with cooling (10 000*g*, 5 min, 4 ºC) using a centrifuge Heraeus Megafuge 11R* and immediately frozen at -80 °C in a deep-freezer HFU 486 Basic* until analysis. The activity of ALP_{FD} was measured spectrophotometrically at 37 °C.

2.9 XPS measurements

A sample of bone of the SMV-exposed rats with a size of ca. 5x5 mm, which was previously measured by an optical profilometer, was heated in a load-lock vacuum chamber for 30 min in order to remove gas residues and then transferred to the analysis chamber. The X-ray beam was incident at the surface at an angle of 45^{o} with respect to the surface normal. The analyzer axis was located at 45^{o} with respect to the surface. For high-resolution XPS spectra, the analyzer pass energy was 23.5 eV and the energy step size was 0.1 eV. At this resolution applied, the line energy positions could be determined with accuracy equal to or better than 0.2 eV. The Casa XPS software was used to evaluate the XPS data. Deconvolution of XPS spectra was performed using a Shirley background and a Gaussian peak shape with 30% Lorentzian character. Spectra of the as-received sample were referenced to the C 1s line at the binding energy (BE) 285.0 eV. The atomic concentrations of carbon, oxygen, calcium, nitrogen, phosphorus, silicon, and magnesium were evaluated using the ULVAC-PHI MultiPak software (version 9.0.1).

2.10 Statistical analysis

The results were processed with the Statistica and IBM SPSS Statistics, version 9.0 PL and 21 for Windows, respectively. To assess the distribution patterns in the data, the values collected for each parameter (with the exception of XPS measurements) were evaluated using the Shapiro-Wilk's

normality test. When the data had a normal distribution, the homogeneity of variances was verified employing Levene's test. If the hypothesis of equal variances was rejected by this test, Hartley's Fmax, Cochran's C, and Bartlett's tests were carried out. If the resulting *P*-value of these tests was not less than 0.05, the two-way analysis of variance (2-way ANOVA) with V and Mg factors and the *F* test were employed to indicate the main effects of V and Mg, and the effect of the V x Mg interaction. The *F* values which had the *P* values smaller than 0.05 were considered statistically significant. If the 2-way ANOVA test demonstrated an interactive effect between V and Mg, subsequent calculations were done in order to describe the kind of the interaction that had been revealed. To this end, the sum of the effects of V and Mg action administered separately was compared with the effect of both co-supplied elements. Thus, when the co-administration of V and Mg produced a smaller (V + Mg effect < V effect + Mg effect) or greater (V + Mg effect > V effect + Mg effect) effect than the sum of the action of V and Mg applied separately, the interaction was recognized as antagonistic or synergistic, respectively.[72] Graphs confirming the interaction between V and Mg or the trend toward the V-Mg interaction were included in the paper. The post-hoc comparisons between the four individual groups were performed using Tukey's or T3 Dunnett's tests when the data had a normal distribution but the variances were or were not homogeneous, respectively. The differences were considered significant at *P* less or equal to 0.05. Pearson's correlation analysis for all the rats was performed to assess the relationships among the measured variables. In addition, correlation analysis was also conducted to reveal the possible relationships between the minerals in the femoral bone and parameters chosen for characterizing the femoral bone surface morphology at the microscale and between the level of V and Fe in femoral bone and other indices investigated by us previously. [54,55] The correlations were considered statistically significant at *P* less or equal to 0.05. All the results are expressed as means ± SEM.

3 Results

3.1 Content/concentration of some elements in FD

In the SMV and SMV-MS co-administered rats, the V_{FD} content was significantly higher, compared with that found in the control animals (by 283- and 265-fold, respectively) and in those supplemented with MS (by 489- and 457-fold, respectively) (Fig. 2A). In turn, the V_{FD} content in the rats receiving MS alone was markedly decreased, compared with the control, SMV-exposed, and SMV-MS co-administered rats. In comparison with the Control, the V_{FD} content in the MS-supplemented animals was by 42% lower (Fig. 2A). The two-way ANOVA showed that the changes in the V_{FD} level in the rats exposed to SMV during the MS supplementation were only an effect of the independent action of V alone; the V-Mg interaction was not observed (Table 1, Fig. 3).

Biochemical examinations of the level of the selected macroelements in FD demonstrated that the content of Mg_{FD} in the SMV-intoxicated and SMV-MS co-administered rats markedly decreased, compared with the control (by 21% and 20%, respectively) and the MS-supplemented rats (by 24% and 23%, respectively) (Fig. 2B). However, the content of Mg_{FD} in the MS-administered rats did not change significantly in comparison with the control, whereas in comparison with the SMV-exposed and SMV-MS co-supplied rats, the Mg_{FD} level was markedly elevated by 31% and by 29.5%, respectively (Fig. 2B). The content of Ca_{FD} in the SMV-exposed and SMV-MS co-applied rats was also diminished, compared with the control (by 7% and 10%, respectively), but these differences were not statistically significant, whereas the content of this element in the FD of the MS-supplemented rats persisted within the range of the values represented by the control group (Fig. 2C). As revealed by the two-way ANOVA, the decrease in the Mg_{FD} and Ca_{FD} levels in the SMV-MS co-supplied rats was an effect of the independent action of V only; the V-Mg interactive effect was not found (Table 1, Fig. 3).

Further, the P_{FD} concentration in the rats receiving SMV and MS separately as well as SMV and MS in combination was lowered in comparison with the control (by 12%, 10%, and 9%, respectively) (Fig. 2D). The highest decrease in the P_{FD} concentration was found in the SMV-exposed rats. The two-way ANOVA demonstrated that the changes in the P_{FD} concentration in the rats exposed to SMV during the MS supplementation might result from the independent action of V alone as well as from the trend toward the independent action of Mg alone and from the V-Mg interaction (Table 1, Fig. 3, Fig. 4A).

In turn, no significant differences in the Na_{FD} concentration were found in the rats receiving SMV and MS separately or in combination, compared with the control (Fig. 2E). Only a visible tendency toward the increase in the concentration of Na in FD was demonstrated in the rats co-supplied with SMV and MS, in comparison with the MS-supplemented animals (Fig. 2E). The two-way ANOVA suggested that the rise in the Na_{FD} level observed in the SMV-MS co-administered rats might result from the clear trend toward the interaction of V with Mg (Table 1, Fig. 3, Fig. 4B).

Finally, the K_{FD} concentration in the rats exposed to SMV has been found to be lowered (by 19%), compared with the control, whereas the concentration of this macroelement in the FD of the MS-supplemented and SMV-MS co-administered animals has been observed to be unaltered markedly, in comparison with the control animals (Fig. 2F). The concentration of K in FD did not differ significantly between the SMV-exposed, MS-supplemented, and SMV-MS-co-administered animals either (Fig. 2F). The two-way analysis of variance indicated that the limitation of the fall in the K_{FD} level in the rats intoxicated with SMV during the MS supplementation might be due to the clear tendency toward the V-Mg interaction (Table 1, Fig. 3, Fig. 4C).

In turn, biochemical examinations of the level of the selected microelements in FD demonstrated that the Fe_{FD} concentration in the SMV-exposed and SMV-MS co-administered rats increased, by 22.5% and by 17%, respectively, compared with the control. The Fe_{FD} level in both the above-mentioned groups of rats was also higher, compared with the MS-supplemented animals by 30% and by 24.5%, respectively (Fig. 2G). However, the concentration of Fe in the FD of the MS-supplemented rats persisted within the range of the values represented by the control group, but compared with the SMV-exposed and SMV-MS co-supplied animals the Fe_{FD} level was lowered by 23% and by 19.7%, respectively (Fig. 2G). The two-way ANOVA indicated that the elevated Fe_{FD} concentration in the rats receiving SMV together with MS resulted from the independent action of V only; the V-Mg interaction was not revealed (Table 1, Fig. 3).

Further, the Cu_{FD} concentration in the SMV-exposed rats did not change significantly, in comparison with the control, whereas the concentration of this element in the FD of the MS-supplemented and SMV-MS co-administered animals markedly decreased, compared with the control (by 20.6% and 30%, respectively) (Fig. 2H). However, the Cu_{FD} concentration between the SMV-exposed, MS-supplemented, and SMV-MS-co-supplied rats did not differ significantly (Fig. 2H). As the two-way ANOVA suggested, the fall in the Cu_{FD} concentration in the rats exposed to SMV during the MS supplementation was induced by the independent action of Mg only; the V-Mg interactive effect was not observed (Table 1, Fig. 3).

The Zn_{FD} concentration, in turn, was significantly elevated in the SMV-intoxicated rats (by 15%), in comparison with the control (Fig. 2I). However, in the MS-supplemented animals and in those receiving SMV in combination with MS, the concentration of Zn in FD persisted within the range of the values represented by the control group (Fig. 2I). The Zn_{FD} concentration between the SMV-exposed, MS-supplemented, and SMV-MS-co-administered animals did not differ markedly (Fig. 2I). The two-way ANOVA showed that the return of the Zn_{FD} level to the control value range in the rats exposed to SMV during the MS supplementation might be due to the clear trend toward the V-Mg interaction (Table 1, Fig. 3, Fig. 4D).

The DL for Fe, Zn, Cu, Na, and K in FD was 5.86 μg/L, 10.86 μg/L, 2.21 μg/L, 0.21 mg/L, and 0.082 mg/L, respectively. The concentration of Fe, Zn, Cu, Na, and K (mean ± SD, $n = 5$) determined by us in the reference bone (Bone Ash 1400, NIST) (609.3 ± 39.1 μg/g, 179.30 ± 1.04 μg/g, 2.28 ± 0.47 μg/g, 6.242 ± 0.092 mg/g, 192.32 ± 1.04 μg/g, respectively) agreed well with the values provided by the producer (660 ± 27 μg/g, 181 ± 3 μg/g, 2.3 μg/g, 6 mg/g, 186 ± 8 μg/g).

3.2 Femoral bone surface roughness

The amplitude and hybrid parameters characterizing the femoral bone cross sectional surface area in all the tested groups of rats are shown in Fig. 5A-H. Fig. 6A-D, presents four micrographs of the

rat femoral bone cross sectional surface area at the microscale, which are the most representative samples selected from among all the samples collected for the four experimental animal groups.

In the rats intoxicated with SMV (Group II), neither Sa nor Sq, which characterize the surface based on the vertical deviations of the roughness profile from the mean line, altered markedly, in comparison with the control (Fig. 5A and 5B). The values of both the above-mentioned parameters in the SMV-exposed rats were observed to be slightly lower than those found in the SMV-MS co-administered animals (Group IV). The highest values of Sa and Sq were reported for the femoral bone surface of the rats exposed to SMV during the MS supplementation (Group IV), and this increase was statistically significant, compared with the SMV-exposed (Group II) and MS-supplemented rats (Group III) (Fig. 5A and 5B). Sa and Sq in the SMV-MS co-administered rats were elevated by 18% and 17%, respectively, in comparison with those found for the femoral bone surface of the SMV-exposed rats, and by 50% and 51%, in comparison with those demonstrated for the femoral bone surface of the MS-supplemented animals. The Sa and Sq values for the femoral bone surface of the SMV-MS co-supplied rats were also observed to be higher, in comparison with the control, but these differences did not turn out to be statistically significant (Fig. 5A and 5B). In turn, the femoral bone surface of the MS-supplemented rats were characterized by significantly lowered values of Sa (by 28%) (Fig. 5A) and Sq (by 28.4%) (Fig. 5B), compared with those found in the control rats. The Sa and Sq values in the MS-supplemented rats were also lower by 22% and 22%, in comparison with those demonstrated for the femoral bone surface of the SMV-intoxicated animals, and by 33.5% and 34%, in comparison with those observed for the femoral bone surface of the SMV-MS co-administered rats (Fig. 5A and 5B). The two-way ANOVA revealed that the changes in the Sa and Sq values in the rats receiving SMV in combination with MS resulted from the independent action of V and from its interaction with Mg (Table 1, Fig. 3, Fig. 4E and 4F).

Further, the distinctly lowered value of Sz, which is used to provide a stable peak-to-valley amplitude parameter for a given surface, was observed for the bone femoral surface of the MS-supplemented rats. This value decreased by 16% and 17%, compared with those found for the bone femoral surface of the control and SMV-MS co-administered rats, respectively. However, these differences were not statistically significant. The value of Sz for the bone femoral surface of the MS-supplemented rats was also lowered by 18%, compared with the SMV-exposed animals, and this difference was statistically significant (Fig. 5E). Elevated values of Sz (by 22% and 20%), were, however, observed for the femoral bone surface of the SMV-intoxicated and SMV-MS co-administered rats, respectively, in comparison with the MS-supplemented rats (Fig. 5E). The two-way ANOVA indicated that the alterations in the value of Sz in the rats receiving SMV in combination with MS were a consequence of the independent action of V and Mg; they also

resulted from the tendency toward the interaction between both the above-mentioned elements (Table 1, Fig. 3, Fig. 4G).

The values of other amplitude parameters such as Ssk and Sku did not differ significantly between the four experimental groups (Fig. 5C and 5D, respectively). In the case of Ssk, it should be highlighted that even though the two-way ANOVA did not show any significant effect (Table 1), the highest differences in the values of Ssk were observed between the MS-supplemented rats and those receiving SMV in combination with MS (Fig. 5C). Therefore, this aspect has been widely described in the discussion section. Ssk and Sku are sensitive to even an individual large number of peaks and valleys, and they allow classification of the surface to the Gaussian or non-Gaussian type. Ssk is also sensitive to even a low number of deep valleys or high peaks and it offers a convenient way to illustrate how peaks and valleys are affected by load during bone cutting. A positive Ssk value refers to peaks emerging as elevations from a relatively flat surface, whereas a negative Ssk value refers to wide plateaus that are eroded by deep valleys. Sku, in turn, determines the deviation of amplitude distribution for a surface from a normal distribution (the Gaussian type of surface), which may be used to detect anomalies on a surface. Distributions with negative or positive excess kurtosis have been called platykurtic (Sku <3, where distribution has relatively few high peaks and low valleys) or leptokurtic (Sku >3, where distribution has relatively many high peaks and low valleys), respectively.[69]

As far as the hybrid parameters are concerned, such indices as Sdq, Sdr, and Ssc have also been determined. Generally, Sdq gives information about the corrugation of the surface. This parameter is related, inter alia, to hardness and elasticity. Therefore, it can be an indication of crushability of the surface. Sdr, in turn, provides information about the percentage enlargement of the surface area due to roughness in percent. However, Ssc may help predict the degree of elastic and plastic deformation of a surface under different loading conditions. No significant alterations were, however, observed in the values of Sdq and Sdr for the femoral bone surface of the examined groups of rats (Fig. 5F and 5G, respectively). As far as the Ssc value is concerned, a clear trend toward the decrease in the values of this parameter has only been found in the MS-supplemented and SMV-MS co-administered animals in comparison with the control. In both the above-mentioned groups of rats, Ssc was lower by 11% and 11.5%, respectively, compared with the control animals (Fig. 5H). The two-way ANOVA showed that the changes in Ssc in the rats receiving SMV in combination with MS were due to the independent action of Mg only; the V-Mg interactive effect was not observed (Table 1, Fig. 3). Moreover, the values of Sdq, Sdr, and Ssc in the MS-supplemented and SMV-MS co-administered rats remained at a similar level (Fig. 5F-H), whereas the values of Sa and Sq in the same groups of rats were completely different (Fig. 5A and

5B, respectively). Therefore, this aspect has been described more widely in the discussion section as well.

3.3 FD ALP activity

The exposure of the rats to SMV, likewise the supplementation of the rats with MS, did not cause significant changes in the activity of ALP_{FD}, compared with the control. However, the administration of SMV in combination with MS markedly elevated the activity of ALP_{FD}, by 24%, 35%, and 40%, in comparison with the control, SMV-intoxicated, and MS supplemented rats, respectively (Fig. 7). As indicated by the two-way ANOVA, the changes in the ALP_{FD} activity in the rats exposed to SMV during the MS supplementation mainly resulted from the interaction between V and Mg (Table 1, Fig. 3, Fig. 4H).

3.4 Correlations between the measured variables

Positive correlations were revealed between Mg_{FD} and Ca_{FD}, Fe_{FD} and V_{FD}, Zn_{FD} and V_{FD}, Mg_{FD} and P_{FD}, Na_{FD} and K_{FD}, Na_{FD} and P_{FD}, as well as between K_{FD} and P_{FD}, whereas negative correlations were found between Ca_{FD} and V_{FD}, Mg_{FD} and V_{FD}, V_{FD} and P_{FD}, Mg_{FD} and Fe_{FD}, and between K_{FD} and Zn_{FD} (Table 2). In addition, the tendencies toward both positive and negative correlations between some elements in FD were also observed (Table 2).

Positive correlations were also found between Sa and Sq, Sa and Sz, Sz and Sq, Sku and Sz, Sdq and Sdr, Sdq and Ssc, Ssc and Sdr, as well as between Sdq, Sdr, Ssc and Sz, whereas negative correlations were demonstrated between Sz, Sdq, Sdr and Ssk (Table 2). Moreover, a trend toward positive and negative correlations was observed between Sku and Ssc as well as between Ssk and Ssc, respectively (Table 2).

Significant positive correlations were also revealed between V_{FD} and Sa, V_{FD} and Sq, and between V_{FD} and Sz, but significant negative correlations were found between Ca_{FD} and Sa, Ca_{FD} and Sq, Mg_{FD} and Sa, Mg_{FD} and Sq, Cu_{FD} and Sa, Cu_{FD} and Sq, and between Zn_{FD} and Ssk (Table 2). Furthermore, Fe_{FD} with Sa, Sq and with Sz as well as Na_{FD} with Sa and Sq exhibited tendencies toward positive correlations (Table 2).

Significant positive and negative correlations as well as trends toward them were also observed between the V_{FD} level and some indices of the Fe status, the content of Fe in the internal organs of rats and some haematological parameters, between the Fe_{FD} level and some indices of Fe status, the content of Fe in the rat's internal organs and some haematological indices as well as between the content of V in FD and the content of this metal in the liver, kidney, spleen, and cerebral hemisphere (CH) (Table 2, Fig. 8).

3.5 XPS narrow scans

C 1s (59.9%), O 1s (21.6 %), N 1s (11.9 %), Ca 2p3 (4.2 %), P 2p (1.9 %), Si 2p (0.3 %), and Mg 2s (0.2 %) were identified and illustrated in Fig. 9A-F. The V 2p3 line was below 0.1 % (below the detection limit). The C 1s peak (Fig. 9A) showed asymmetry and it was fitted using the peak deconvolution approach. It was decomposed into components which corresponded to the C-C and C-H bonds (284.70 eV), the C-N bonds (285.66 eV), the C-OH bonds (286.20 eV), as well as the carbonyl group (287.89 eV) and the carboxyl group (288.90 eV). The O 1s (Fig. 9B) was deconvoluted to two peaks. The main component at 531.20 eV corresponded to the O linked only with a phosphorous atom in PO_4^{3-} ions. The other one, with a higher energy (532.63 eV), was combined with the Si-O peak. In turn, the N 1s peak showed good symmetry, which implies that the binding energy is stable. It was referenced at 399.76 eV (Fig. 9C). The N 1s peak at 399.76 eV was assigned to the N-C=0 and N-H chemical bindings.[73] The N-H bond corresponded to an amine group that is a part of collagen fibres. This group is combined with the carbon chain through a C-N bond that was recognized at 286.20 eV. Further, the Ca 2p3 spectrum showed a doublet at 347.10 eV and 350.62 eV (Fig. 9D). The main component at 347.10 eV (red line) – Ca2p3 – corresponds to hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$]. The other one, with higher energy 350.62 eV (blue line) – Ca2p1 - corresponds to Ca or Ca-O. The P 2p also showed a doublet at 132.88 eV and 133.72 eV (Fig. 9E). The P 2p peak at 132.88 eV corresponded to the P-C and P-O bonds. Variation in the binding energy for P 2p (132.4 eV to 135.8 eV) depending of the O environment was found by Textor et al..[74] The P-O bond is a part of PO_4^{3-} ions which are partly replaced by silicate SiO_4^{4-} ions (Si 2p peak at 102.22 eV) (Fig. 9F). Substitution of PO_4^{3-} by SiO_4^{4-} has been indicated by Balas et al.,[75] who investigated the Si-containing HA (Si-HA) which can be described by the following formula: $Ca_{10}(PO_4)_{6-y}(SiO_4)_y(OH)_{2-y}$. In our case, the concentration of P and Si taken from deconvoluted peaks were equaled to 2.4 and 0.4 % by weight, respectively, which corresponded to a P/Si molar ratio 5.41. The remaining 0.41 mole of P is bonded to the organic phase of bone (collagen). The characteristic peaks for HA were recognized at 531.20 and 532.63 eV (O 1s), 347.10 and 350.62 eV (Ca 2p3) as well as at 132.88 and 133.72 eV (P 2p).

4 Discussion

The current paper is the next report that contains a subsequent part of the results, originating from the *in vivo* studies on outbred male Wistar rats, referring to the interaction between V, which has an occupational and environmental toxicological impact,[76] and Mg, which has been reported to have beneficial effects against poisoning with some metals.[77-79] To the best of our knowledge, this is the first article focused on providing more insight into the effect of the 12-week separate or combined administration of both the above-mentioned elements (as SMV and MS, respectively) on the rat

femoral content/concentration of minerals required for metabolic processes directly or indirectly related to bone and on femoral bone micromorphology tested by a non-contact optical profilometric method. It clearly shows that V and Mg are able to modulate the response in an interactive manner to produce a specific effect that is distinct from that observed during the separate administration of both agents. It also contains preliminary data about the chemical composition of the inorganic ingredient of bone of the SMV-exposed rats and sheds new light on the relationships between some examined indices providing better understanding of the changes in the investigated parameters in the chosen experimental conditions. On the other hand, the present stage of the studies did not allow us to interpret the results provided in this report more precisely. Therefore, further examinations are needed to explain the action of V and Mg on the bone tissue more accurately.

Our earlier papers from the research field concerning the V-Mg interaction presented the effects of the 12-week administration of V and Mg (as SMV and MS) separately or in combination a) on the activity/level of selected antioxidants in the blood of rats,[52] b) on the level of some oxidative stress (OS) markers in selected rat's internal organs,[51,53] c) on some rat's haematological indices, the blood biochemical parameters, and the level of selected bioelements in the rat's blood/organs,[54] and d) on the total content/concentration of some macro- and microelements in the liver, kidney, spleen, and cerebral hemisphere (CH) simultaneously drawing attention to the probable mechanisms underlying the SMV-induced disorders in homeostasis of Fe in the rat's selected tissues.[56] The influence of the 18-week administration of V and Mg (as SMV and MS) separately or together on lipid peroxidation (LPO) in the rat hepatic tissue has also been published.[55] In all the above-mentioned reports the effects of the mutual interaction between V and Mg were presented.

The lack of data about the influence of V and Mg administered in combination on the femoral bone mineral status and on the femoral bone surface morphology at the microscale (presented in the current report) made the comparison of our results with those described by other researchers practically impossible. Therefore, the discussion section was mainly limited to our findings. Moreover, changes in such basic parameters as fluid and food intake and body weight in rats after the 12-week SMV and/or MS administration together with a short comment concerning the consumption of the V and Mg doses by the rats have already been provided by us previously.[56] Similarly, changes in the fluid and food intake and in the body weight of the SMV-exposed rats were also discussed in our earlier paper.[80] Therefore, these aspects have been passed over in the present report.

As demonstrated, the 12-week exposure to SMV (0.125 mg V/ml) separately or in combination with MS led to accumulation of V in bone, the inorganic part of which (in the SMV-exposed rats) consisted of the Si-HA phase: $Ca_{10}(PO_4)_5(SiO_4)(OH)$ that was cross-linked with an

organic – collagen phase. Since V was below the detection limit the depth-profiling secondary ion mass spectrometry (SIMS) analysis of bones of the SMV-exposed rats and those receiving MS alone and MS in combination with SMV will be performed in the nearest future, as it will allow us to provide more details about the chemical composition of the inorganic component of the femoral bone of rats treated with the above-mentioned compounds. Mravcová et al.[15] found that epiphyseal cartilage and the subperiosteous zone of the diaphysis accumulate V and that deposition of this metal is greatest in areas of recent bone formation and mineralization. It has also been reported that vanadate (VO_4^{3-}) is incorporated into the HA lattice [$Ca_{10}(PO_4)_6(OH)_2$] replacing phosphate anions (PO_4^{3-})[10,81] and forming calcium vanadate apatite [$Ca_{10}(VO_4)_6(OH)_2$],[82] whereas, oxidovanadium(IV) (VO^{2+}) [vanadyl] is strongly adsorbed on the apatite lattice surface.[8] The incorporation of small quantities of VO_4^{3-} in the PO_4^{3-} sites has been highlighted to produce only slight distortions in the macroscopic and microscopic structure of the mineral phase of bones.[8]

Taking into account the V_{FD}, Mg_{FD}, and Ca_{FD} contents in the rats receiving SMV alone (Group II) or in combination with MS (Group IV) (Fig. 2A-2C) and the 2-way ANOVA results referring to the above-mentioned elements (Table 1) we may conclude that V as SMV, in the used concentration, may lead to demineralization of the femur. The negative correlations between the V_{FD} and Mg_{FD} or Ca_{FD} contents (Table 2) may confirm the connection between the V_{FD} accumulation and the Mg_{FD} and Ca_{FD} imbalance. The higher doses of V have been reported to lead to inhibition of bone mineralization.[2] However, some authors found that V did not affect the content of Mg and Ca in bone which may result from different experimental conditions used.[16]

Mg can be adsorbed on the surface of HA crystals (serving as a reservoir for maintaining a normal extracellular level) or incorporated inside the crystal structure and released during bone resorption.[33,83-85] Tsuboi et al.[86] demonstrated that higher concentrations of Mg are maintained in the periosteal (outer) and endosteal (inner) bone surfaces, whereas Laurencin et al.[87] provided direct evidence of the preferential substitution of Mg in one of the two crystallographic Ca sites of apatites, referred to as Ca(II), and suggested that incorporation of Mg in the Ca(II) position has a direct impact on the properties and reactivity of the Mg-substituted HA (Mg-HA).[87]

Our findings also provided clear evidence that the 12-week administration of SMV and MS individually (at the selected level) may significantly reduce the concentration of P_{FD} (Fig. 2D), which is critical to maintaining healthy bone,[88] and that supplementation with MS as well as the combined application of SMV with MS (in the used concentrations) may have a negative impact on the femoral bone Cu status (Fig. 2H). Simultaneously, our results allowed us to state that exposure to SMV (0.125 mg V/ml) may induce disturbances in the K_{FD} and Zn_{FD} concentrations (Fig. 2F and 2I). On the other hand, we revealed that antagonistic interaction between V and Mg (Fig. 3), the exact mechanism of which is currently unknown and requires additional analyses, might limit to

some degree the drop in the P_{FD} and K_{FD} levels and normalize the Zn_{FD} concentration in the rats supplemented with MS during the SMV exposure. Taking into account our present (this report) and previous[64] results we may not exclude that the SMV-induced depletion of K from the rat's femur might partly lead to elevation of the level of K in the plasma of those animals.[64]

The mechanism of increased deposition of Zn_{FD} found in the SMV-intoxicated rats is also unknown. At this stage of our studies, we may only state that the difference in the accumulation of Zn_{FD}, which plays a physiological role in the regulation of bone metabolism,[89] did not simply reflect the plasma Zn concentration, because the level of Zn in the plasma of the SMV-exposed rats remained unchanged in comparison with the control.[64] Moreover, analysis conducted in order to determine whether the exposure to SMV alone (at the selected level) may produce differences in the activity of ALP in bone, sufficient to account for differences in the Zn_{FD} concentration, did not show any significant changes in the activity of ALP_{FD} in the SMV-exposed rats (Fig. 7). Therefore, further investigations will be necessary to explain the mechanism of the Zn_{FD} deposition in the conditions of SMV exposure. The positive correlation revealed between the level of V_{FD} and Zn_{FD} (Table 2) may confirm the connection between bone V storage and bone Zn accumulation. A high level of Zn, which may incorporate into the Ca(II) site of HA,[90] in bone has been reported to attenuate bone mineralization.[91]

The results obtained also allowed us to suggest that the synergistic interaction between V and Mg (Fig. 3) could account for the rise in the Na_{FD} level in the SMV-MS co-supplied rats (Fig. 2E), and that the exposure to SMV (0.125 mg V/ml) may induce Fe_{FD} deposition (Fig. 2G). Our findings also clearly showed that accumulation of V and Fe in bone may affect TIBC, TS, the faecal Fe excretion as well as the hepatic, renal, splenic, and cerebral Fe level (Fig. 8), the results of which were presented and discussed by us previously.[54,56] Additionally, correlations of V_{FD} and Fe_{FD} with such parameters as haematocrit (Ht), haemoglobin (Hb), mean corpuscular volume (MCV), and/or red blood cell distribution width (RDW) (Fig. 8), the results of which were also discussed by us previously,[54] point to the clear connection between the above haematological indices and the bone V or Fe accumulation. It has been reported that Fe overload, the signs of which were observed by us in the rats during the SMV exposure (previous [54,56] and present report), may have deleterious effects on bone. Experiments with male mice have demonstrated that Fe overload results in increased bone resorption and OS, leading to changes in bone micro-architecture and material properties and thus bone loss.[92] The fact that OS may be involved in the pathogenesis of metabolic bone diseases such as osteoporosis, as demonstrated by analysis of bone metabolism of Fe-overloaded rats, has been reported as well.[93] *In vitro* data, in turn, have confirmed that Fe, which plays a significant role in bone formation, acting as a cofactor in enzymes involved in collagen bone matrix synthesis,[3]

decreases mineralization.[94] Therefore, the issue associated with the accumulation of Fe in the FD of the rats exposed to SMV will be further examined.

An optical profilometric study of the rat femoral bone surface roughness (FBSR), in which some surface roughness parameters were selected in order to perform a detailed analysis of the femoral bone surface at the microscale, provided completely new data. We have demonstrated, for the first time, that V, the action of which in improving the quality of bone structure and morphology is not exactly known,[81] administered alone as SMV at the selected level did not significantly affect the femoral bone roughness (Fig. 6B). The non-significantly altered value of Sq (which is slightly more sensitive to high peaks and low valleys than Sa)[95] obtained for the femoral bone surface of the SMV-exposed rats (Group II), compared with the control (Fig. 5B) may confirm this conclusion. We have also found that the content of V in FD was correlated with roughness of the femoral bone surface (Table 2) and provided, for the first time, clear evidence that supplementation with Mg (as MS), in the chosen concentration, may cause a reduction in roughness of the femoral bone surface (Fig. 6C). The lowest surface roughness value (Sq) obtained for the femoral bone surface of the MS-supplemented rats (Group III) (Fig. 5B) confirms this statement. Additionally, the negative correlations between the content of Mg in FD and Sa or Sq (Table 2) can suggest that the decrease in the content of Mg in bone may elevate the bone surface roughness. The next novel and important information is the fact that V and Mg administered in combination (Group IV) may act synergistically (Fig. 3) in increasing the surface roughness of the rat femoral bone (Fig. 5A, 5B and 6D). In implantology, which focuses on improvement of the physico-chemical properties of implants/bone-substitute biomaterials, it is confirmed that roughness of a surface and its chemical composition are one of the deciding factors in evaluation of progress of the active surface of an implant.[95] Taken together, we may state that Mg itself and a combination of V with Mg markedly affect the internal structure of the rat femur. Consequently, the surface roughness of the femoral bone of those rats decreased and increased, respectively. The explanation of the mechanisms of the above-mentioned alterations will be one of the interesting problems for our subsequent research on a rat model. In turn, the correlations between Sa/Sq and the Ca_{FD} content and between Sa/Sq and the Cu_{FD} concentration as well as the trends towards them between Sa/Sq and the Fe_{FD} and Na_{FD} levels (Table 2) clearly pointed to relationships between the level of the above-mentioned minerals in the femur and possible changes in roughness of the femoral bone surface.

We also showed that the synergistic interaction between V and Mg (Fig. 3), applied in combination, was mainly responsible for the rise in the activity of ALP_{FD} (Fig. 7). However, it is no clear how V and Mg, administered simultaneously, increase ALP_{FD} while the individual treatment with these elements did not affect the activity of the mentioned enzyme. As a result, an alternative method (polymerase chain reaction - PCR or immunohistochemical determination) is planed to be

perform in the future. At this stage of our study the results obtained allowed us to suggest that the diversified ALP_{FD} activity might partly be linked with the diversified degree of FBSR. This statement was confirmed by the following facts: the highest activity of ALP_{FD} corresponded to the highest roughness of the femoral bone surface (reflected by the highest Sq value in the SMV-MS co-administered rats), the lowest activity of ALP_{FD} corresponded to the lowest roughness of the femoral bone surface (reflected by the lowest Sq value in the MS-supplemented rats), and the slightly lowered ALP_{FD} activity corresponded to the slightly reduced Sq value in the SMV-intoxicated rats. Consequently, the above-mentioned data clearly pointed to the following relationship between the activity of ALP_{FD} and Sq: ALP_{FD} [SMV-MS>Control>SMV>MS] <=> Sq [SMV-MS>Control>SMV>MS]. However, it is not easy to explain how a morphological parameter could affect the ALP_{FD} activity.

Some literature data given below, which cannot be compared with our *in vivo* study, show that the activity of ALP may be affected by surface roughness. Cells cultured on a rougher surface have been observed to show the elevated activity of the above-mentioned enzyme.[96] Surface roughness has also been found to alter osteoblast proliferation, differentiation, and matrix production *in vitro*.[97] The studies of Hatano et al.[98] demonstrated that the surface roughness itself causes an increase in osteoblastic proliferation and differentiation in cell cultures, whereas the studies of Boyan et al.[99] showed that osteoblasts are sensitive to subtle differences in both surface roughness and surface chemistry. An increase in ALP expression in response to increased in roughness was also observed.[98] The Authors indicated that some sort of signal might have been provided from the cell surface through contact with the peaks and valleys because the roughness itself, as they state, seems to cause the response. It has also been shown that cells on a rougher surface released higher levels of prostaglandin F_2 (PGF_2) and transforming growth factor beta 1 (TGF-β1), known to be involved in regulation of bone formation.[100] Investigators emphasize that this effect indicates that surface roughness has a direct effect on cell activity and may have an indirect effect mediated through local factor production.[101] In addition, the study of Schwartz et al.[102] has demonstrated that surface roughness also affects chondrocyte proliferation, differentiation, and matrix synthesis, and this regulation is cell maturation dependent. Taking into account our results, it would be interesting to extract osteoblasts from the bone of the tested rats and compare the ability of cells to express osteoblastic markers in relation with the bone composition. Examinations in this direction will be undertaken in the future.

Other facts related to the activity of ALP have drawn our attention. We found that the activity of ALP_{FD} in the SMV (0.125 mg V/ml)-exposed rats did not alter significantly, compared with the control (Fig. 7), whereas the activity of ALP in the plasma, urine and kidney of the SMV (0.125 mg V/ml)-intoxicated rats behaved in a different manner - it was markedly reduced.[64]

Cortizo et al.,[103] who examined the activity of ALP in the soluble and particulate fraction of rat osteosarcoma cell line, found that the activity of this enzyme was inhibited by vanadate. They also demonstrated that different forms of V were direct inhibitors of ALP activity, and highlighted that this effect was dependent on the enzymatic activity and on the origin of ALP.

On the basis of the results obtained for Ssk (skewness) and Sku (kurtosis) investigated in order to complete the information about the femoral bone surface micromorphology, we can state that the femoral bone surface of all the tested animal groups had a preponderance of valleys because Ssk in those groups of rats had only negative values (Fig. 5C). The obtained Ssk values also allowed us to suggest that the surface of the femur of the SMV-MS co-administered and the MS-supplemented rats had the greatest and the lowest number of valleys, respectively. Since all roughness parameters are determined by surface micromorphology and its strong correlation with porosity of bones, the highest Ssk value reflects the highest porosity. In the optical profilometric measurements, such a surface points to the highest number of holes. It was interesting that neither Sa nor Sq were correlated with Ssk and Sku (Table 2) which might result from the fact that Sa and Sq, in contrast with Ssk and Sku, do not provide any information about the shape of irregularities of a surface.

Further, the roughest femoral bone surface had the highest value of Sq which corresponded to the highest negative value of Ssk. This situation was observed in the SMV-MS co-administered rats, the femoral bone surface of which exhibited the highest number of valleys. On the contrary, the smoothest femoral bone surface had the lowest value of Sq which corresponded to the lowest negative value of Ssk. This situation was observed in the MS-supplemented rats, the femoral bone surface of which exhibited the lowest number of valleys. Thus, our results clearly pointed to the following relation in which the absolute values for Ssk have been taken into account: Sq [MS<SMV<Control<SMV-MS] ⇔ Ssk [SMV-MS>Control>SMV>MS] (Fig. 5B and 5C).

The present findings also allowed us to conclude that supplementation with Mg, in the used concentration (0.06 mg Mg/ml), may have a positive influence on creating a regular shape of a surface, which was manifested by a lowered (by 16%) Sz value, compared with the control, and that neither V alone (0.125 mg V/ml) nor the V-Mg combination affected the local irregularities of the rat femoral bone surface which, in turn, was confirmed by significantly unaltered value of Sz, in comparison with the control (Fig. 5E). However, the positive correlation between V_{FD} and Sz clearly points to relationship between both parameters (Table 2).

On the basis of the results obtained for other roughness parameters we may also state that both V and Mg, administered separately or in combination in the chosen concentration, were not able to significantly affect corrugation and development of the femoral bone surface. This statement was confirmed by the lack of marked alterations in the values of Sdq and Sdr, in comparison with

the control (Fig. 5F and 5G, respectively). Moreover, the values of Sa and Sq and the values of Sdq and Sdr were the lowest in the MS-supplemented rats, whereas in the SMV-MS co-administered rats the first two values were the highest but the latter were kept within the range of the values represented by the MS-supplemented animals. Additionally, Sdq was not correlated with Sq. This was mainly caused by the fact that Sq is strongly dependent on a lateral resolution, which in our case was 0.47 μm. The above-mentioned facts may indicate that the shape of protrusions and valleys of the femoral bone surface of the MS-supplemented and SMV-MS co-administered rats was the same. The SMV-MS co-supplied rats had only the higher Sq value.

Our results also demonstrated that the femoral bone surface of the MS-supplemented and SMV-MS co-administered rats was characterized by the lowest curvature of peaks, as reflected in the lower Ssc values (Fig. 5H). The lowest curvature of peaks might be associated with some mechanical properties of bones. It cannot also be excluded that such specific shapes of peaks of surface irregularities could be an effect of bone cutting. However, the similar level of the values of Ssc (which is known to be associated with flexibility of the sample and its reaction to the applied force that derives, for example, from the saw blade) found in the rats receiving MS alone or MS in combination with SMV may suggest that the mechanical feature of the femur and the influence of the saw on the femoral bone samples obtained from those groups of rats were similar. Thus, differences in the level of the Sq values found between the MS-supplemented rats and those receiving SMV in combination with MS were not determined by the effect evoked by the cutting itself, but they probably originated from the bone internal structure, first of all, from distribution and the hole size.

5 Conclusions

The findings illustrated in the current report are completely novel. They expand the knowledge about the V-Mg interaction and provide more insight into the consequences of the action of both the above-mentioned elements on the bone tissue. In our *in vivo* study on a rodent model, we showed for the first time the possible changes in the rat femoral bone mineral status and in the rat femoral bone micromorphology during the administration of V and Mg, the functions of which have not been fully recognized yet, (as SMV and MS, respectively) separately or in combination, in the chosen concentrations, and indicated in which way the examined parameters were modified under the SMV-MS co-administration. Simultaneously, we revealed the character of the V-Mg interaction (synergistic/antagonistic) with regard to indices investigated in the rat bone tissue and proposed the preliminary chemical composition of the inorganic ingredient of bone of the SMV-exposed rats: $Ca_{10}(PO_4)_5(SiO_4)(OH)$.

Conflict of interest statement

There is no conflict of interest of authors.

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Legends:

Figure 1. Schematic illustration showing the preparation of the rats' femurs obtained from all the four tested animal groups (Control, SMV, MS and SMV-MS) for profilometric evaluation. PFE: proximal femoral epiphysis; DFE: distal femoral epiphysis; FD: femoral diaphysis; FCS: femoral cross-section.

Figure 2. The content of V (A), Mg (B), and Ca (C) as well as the concentration of P (D), Na (E), K (F), Fe (G), Cu (H), and Zn (I) in the FD of the tested animal groups (Control, SMV, MS and SMV-MS). Differences are indicated by: [a]*vs*. control, [b]*vs*. SMV-intoxicated, [c]*vs*. MS-supplemented, and [d]*vs*. SMV-MS co-administered rats ([1] Tukey's test and [2] T3 Dunnett's test). Numerical values on the bars indicate how many times the V content increased (↑), compared with the control (italic bold underline) and the MS-supplemented (normal bold) rats. Other values show the percentage of the decrease (↓) in the content of V, Mg, and Ca and in the concentration of P, K, Fe, and Cu compared with the control (italic alone), the SMV-intoxicated (bold underline), the MS-supplemented (italic underline), and the SMV-MS co-administered (normal underline) rats, as well as the percentage of the increase (↑) in the content of Mg and in the concentration of Na, Fe, and Zn, compared with the control (italic bold), the SMV-intoxicated (normal bold alone), the MS-supplemented (normal alone), and the SMV-MS co-supplied (bold underline) animals. Data are presented as the mean ± SEM (n = 11 rats/group). [*]$P < 0.05$; [†]$P < 0.01$; [‡]$P < 0.001$; [»]$P = 0.08$; [«]$P = 0.09$; [•]$P = 0.12$; [ƒ]$P = 0.14$; [§]$P = 0.17$.

Figure 3. Antagonistic (A), synergistic (B) and no interactive (C) effects between V and Mg visualized for the SMV-MS co-administered male Wistar rats with respect to all the examined parameters.

- red solid lines: the V-Mg antagonistic interaction
- blue solid lines: the V-Mg synergistic interaction
- green dashed lines: lack of interactive effect between V and Mg

The effect of V and Mg in combination < or > sum of the effects of V and Mg alone (antagonistic or synergistic interaction, respectively). FD: femoral diaphysis. FCS: femoral cross-section. V_{FD}, Mg_{FD}, and Ca_{FD}: the content of vanadium (μg), magnesium (mg), and calcium (mg) in FD, respectively. P_{FD}, Na_{FD}, K_{FD}, Fe_{FD}, Cu_{FD}, and Zn_{FD}: the concentration of phosphorus (μM/g tissue), sodium (mg/g tissue), potassium (mg/g tissue), iron (μg/g tissue), copper (μg/g tissue), and zinc (μg/g tissue) in FD, respectively. Sa: the mean roughness (nm); Sq: the root mean square roughness (nm); Ssk: skewness; Sku: kurtosis; Sz: the ten point height (nm); Sdq: the root mean square surface

slope (rad); Sdr: the developed interfacial area ratio (%); Ssc: the mean summit curvature (1/nm). ALP_{FD}: alkaline phosphatase in FD (U/g of tissue).

Figure 4. The graphs of the interaction between V and Mg referring to the concentration of Zn (A), Na (B), K (C), and P (D) in FD, Sa (E), Sq (F), and Sz (G), as well as the activity of ALP (H) in FD. V+, V-, Mg+, Mg-, presence (+) or lack (-) of V or Mg. Differences between the means are indicated by the *t*-test.

Figure 5. Amplitude (A, B, C, D, E) and hybrid (F, G, H) parameters of the femoral bone cross-section surface at the microscale for Control, SMV, MS, and SMV-MS animal groups. Differences are indicated by: [a]*vs*. control, [b]*vs*. SMV-intoxicated, [c]*vs*. MS-supplemented, and [d]*vs*. SMV-MS co-administered rats ([1] Tukey's test and [2] T3 Dunnett's test). Numerical values on the bars indicate the percentage of the decrease (↓) in the measured indices, compared with the control (italic alone), the SMV-intoxicated (underline bold) and the SMV-MS co-administered (underline normal) rats, as well as the percentage of the increase (↑), compared with the SMV-intoxicated (bold alone) and the MS-supplemented (normal alone) animals. Data are presented as the mean ± SEM (n = 11 rats/group). $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$; $^{\#}P = 0.06$; $^{**}P = 0.09$; $^{\dagger\dagger}P = 0.11$; $^{\ddagger\ddagger}P = 0.13$; $^{\S}P = 0.17$.

Figure 6. OP micrographs of the femoral bone cross-section surface. Each micrograph is the most representative sample selected from each of the four experimental groups of rats: Control (A), SMV-intoxicated (B), MS-supplemented (C), and SMV-MS co-administered (D).

Figure 7. The activity of ALP_{FD} in the tested animal groups (Control, SMV, MS, and SMV-MS). Differences are indicated by: [a]*vs*. control, [b]*vs*. SMV-intoxicated, and [c]*vs*. MS-supplemented rats (Tukey's test). Numerical values on the bars indicate the percentage of the increase, compared with the control (italic bold), the SMV-intoxicated (bold alone), and the MS-supplemented (normal alone) animals. Data are presented as the mean ± SEM (n = 11 rats/group). $^{\dagger}P < 0.01$; $^{*}P < 0.05$; « $P = 0.09$.

Figure 8. Relationships between some measured parameters.

- red: relationships between Fe_{FD} and Fe in the examined organs and between Fe_{FD} and some other investigated indices
- green: relationships between V_{FD} and some other parameters examined
- blue: relationships between V_{FD} and Fe_{FD} and between V_{FD} and Fe in the examined organs

(-): negative correlation, (+): positive correlation. $T_{(-)}$: trend toward the negative correlation. $T_{(+)}$: trend toward the positive correlation. [FD, TIBC, TS, F, Ht, Hb, MCV, RDW] Femoral diaphysis, total iron binding capacity, transferrin saturation, faeces, haematocrit, haemoglobin, mean corpuscular volume, and red blood cell distribution width, respectively. Correlation analysis was performed for rats from all the tested groups. Changes (increase/decrease) in the investigated parameters illustrated in this scheme were presented for the SMV-exposed rats. [√] Previous reports.[54,56] [*] Present report .

Figure 9. Binding energies (eV) of C 1s (A), O 1s (B), N 1s (C), Ca 2p3 (D), P 2p (E), Si 2p (F), and Mg 2s (F) in bone sample of the SMV-exposed rats. Dashed lines indicate location of the top of peaks in XPS spectra's; a. u. – arbitrary units.

Table 1 Main and interactive effects of V and Mg on the measured variables in male Wistar rats after 12-week administration of both elements as SMV and MS in combination

Variables[a]	Two-way analysis of variance (2-way ANOVA)[b]		
	Main effect of V	Main effect of Mg	Interactive effect (V x Mg)
V_{FD}	$F = 696.900$, $P = 0.000$	NS	NS
Mg_{FD}	$F = 50.924$, $P = 0.000$	NS	NS
Ca_{FD}	$F = 5.517$, $P = 0.024$	NS	NS
P_{FD}	$F = 8.082$, $P = 0.007$	$F = 3.273$, $P = 0.078$	$F = 11.790$, $P = 0.001$
Na_{FD}	NS	NS	$F = 3.815$, $P = 0.058$
K_{FD}	NS	NS	$F = 4.014$, $P = 0.052$
Fe_{FD}	$F = 11.876$, $P = 0.001$	NS	NS
Cu_{FD}	NS	$F = 10.162$, $P = 0.003$	NS
Zn_{FD}	$F = 4.305$, $P = 0.044$	NS	$F = 3.722$, $P = 0.061$
Sa	$F = 9.551$, $P = 0.004$	NS	$F = 23.677$, $P = 0.000$
Sq	$F = 10.401$, $P = 0.003$	NS	$F = 25.129$, $P = 0.000$
Ssk	NS	NS	NS
Sku	NS	NS	NS
Sz	$F = 4.365$, $P = 0.043$	$F = 3.897$, $P = 0.056$	$F = 2.349$, $P = 0.134$
Sdq	NS	NS	NS
Sdr	NS	NS	NS
Ssc	NS	$F = 7.817$, $P = 0.008$	NS
ALP_{FD}	$F = 3.968$, $P = 0.053$	NS	$F = 9.902$, $P = 0.003$

[a] FD: femoral diaphysis. V_{FD}, Mg_{FD}, and Ca_{FD}: the content of vanadium (μg), magnesium (mg), and calcium (mg) in FD, respectively. P_{FD}, Na_{FD}, K_{FD}, Fe_{FD}, Cu_{FD}, and Zn_{FD}: the concentration of phosphorus (μM/g tissue), sodium (mg/g tissue), potassium (mg/g tissue), iron (μg/g tissue), copper (μg/g tissue), and zinc (μg/g tissue) in FD, respectively. Sa: the mean roughness (nm); Sq: the root mean square roughness (nm); Ssk: skewness; Sku: kurtosis; Sz: the ten point height (nm); Sdq: the root mean square surface slope (rad); Sdr: the developed interfacial area ratio (%); Ssc: the mean summit curvature (1/nm). ALP_{FD}: alkaline phosphatase in FD (U/g of tissue). [b] Data are presented as *F* values and the levels of significance (*P*). NS: no significant effect.

Table 2 Correlation coefficients for the compared variables

Variables	Elements								
	Ca_{FD}	V_{FD}	Mg_{FD}	Cu_{FD}	Fe_{FD}	Zn_{FD}	Na_{FD}	K_{FD}	P_{FD}
Ca_{FD}	1								
V_{FD}	**-0.296** ††	1							
Mg_{FD}	**0.776** ‡	**-0.681** ‡	1						
Cu_{FD}	0.114	-0.141 §	0.054 §	1					
Fe_{FD}	*-0.251* #d	**0.472** †	**-0.407** †	0.042 §	1				
Zn_{FD}	0.028 §	**0.360** *	*-0.212* #h	-0.149 §	0.005 §	1			
Na_{FD}	-0.034 §	0.117 §	-0.066 §	-0.038 §	0.043 §	-0.126 §	1		
K_{FD}	-0.114 §	*-0.258* #c	-0.009 §	*0.264* #b	0.124 §	**-0.296** ††	**0.381** *	1	
P_{FD}	0.071 §	**-0.406** †	**0.291** ††	*0.225* #f	-0.184 §	-0.161 §	**0.314** *	**0.408** †	1
Variables	Amplitude parameters						Hybrid parameters		
	Sa	Sq	Ssk	Sku	Sz		Sdq	Sdr	Ssc
Sa	1								
Sq	**0.998** †	1							
Ssk	-0.065 §	-0.063 §	1						
Sku	-0.144 §	-0.108 §	-0.153 §	1					
Sz	**0.422** †	**0.446** †	**-0.343** *	**0.494** †	1				
Sdq	0.021 §	0.033 §	**-0.303** *	0.112 §	**0.628** †		1		
Sdr	-0.003 §	0.009 §	**-0.299** *	0.126 §	**0.616** †		**0.994** †	1	
Ssc	0.007 §	0.015 §	*-0.223* #f	*0.233* #e	**0.578** †		**0.608** †	**0.621** †	1
Variables	Elements								
	Ca_{FD}	V_{FD}	Mg_{FD}	Cu_{FD}	Fe_{FD}	Zn_{FD}	Na_{FD}	K_{FD}	P_{FD}
Sa	**-0.307** *	**0.378** *	**-0.356** *	**-0.345** *	*0.269* #b	0.022 §	*0.239* #e	-0.020 §	0.068 §
Sq	**-0.323** *	**0.398** *	**-0.368** *	**-0.337** *	*0.275* #a	0.003 §	*0.230* #f	-0.020 §	0.070 §
Ssk	-0.088 §	-0.170 §	-0.022 §	0.154 §	0.126 §	**-0.359** *	0.204 §	0.204 §	0.074 §
Sku	-0.127 §	0.050 §	0.043 §	0.040 §	0.206 §	-0.066 §	-0.166 §	0.013 §	0.074 §
Sz	-0.038 §	**0.374** *	-0.144 §	-0.036 §	*0.266* #c	0.062 §	-0.220 §	-0.118 §	-0.157 §
Sdq	0.043 §	0.215 §	-0.138 §	0.101 §	0.103 §	-0.019 §	-0.088 §	-0.019 §	-0.284 §
Sdr	0.052 §	0.184 §	-0.098 §	0.100 §	0.094 §	-0.036 §	-0.110 §	-0.012 §	0.240 §
Ssc	0.120 §	0.047 §	-0.012 §	0.136 §	0.150 §	0.084 §	-0.094 §	0.066 §	0.148 §

Variables	Fe_L √	Fe_K √	Fe_S √	Fe_{CH} √	Fe_P √	Fe_F √	TIBC √	TS √
Fe_{FD}	*0.330* #b	**-0.376** *	**0.460** *	**-0.479** †	0.198 §	*-0.291* #c	*-0.266* #c	**0.407** †
V_{FD}	**0.502** †	**-0.397** *	**0.545** †	**-0.654** ‡	-0.082 §	**-0.523** †	**-0.566** ‡	**0.501** †

Variables	V_L √	V_K √	V_S √	V_{CH} √
V_{FD}	**0.974** ‡	**0.946** ‡	**0.955** ‡	**0.827** ‡

Variables	RBC √	Ht √	Hb √	MCV √	RDW √
Fe_{FD}	*0.264* #b	*-0.218* #g	*-0.265* #c	**-0.467** †	0.082 §
V_{FD}	0.156 §	**-0.609** ‡	*-0.233* #f	**-0.658** ‡	**0.528** ‡

FD, L, K, S, CH, P, F Femoral diaphysis, liver, kidney, spleen, cerebral hemisphere, plasma, and faeces, respectively. TIBC, TS, RBC, Ht, Hb, MCV, and RDW: total iron binding capacity, transferrin saturation, red blood cell count, haematocrit index, haemoglobin level, mean corpuscular volume, and red blood cell distribution width. Data are presented as the correlation coefficients (r) and the levels of statistical significance (P). The significant correlations and tendencies toward them are highlighted as bold text and italics, respectively. § Lack of a linear relationship. ‡ $P < 0.001$; † $P < 0.01$; * $P < 0.05$; †† $P = 0.05$; # $P > 0.05$; #a $P = 0.07$; #b $P = 0.08$; #c $P = 0.09$; #d $P = 0.10$; #e $P = 0.12$; #f $P = 0.14$; #g $P = 0.15$; #h $P = 0.16$. √ Previous reports.[54,56]

Supplementary file

$$S_q = \sqrt{\frac{1}{MN}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}\left[z(x_k, y_l)\right]^2}$$

where x and y are the coordinates, z is the perpendicular deviation from the ideally smooth surface, M is the number of points in the x direction and N is the number of points in the y direction

$$S_{dq} = \sqrt{\frac{1}{(M-1)(N-1)}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}\left(\frac{z(x_k,y_l)-z(x_{k-1},y_l)}{\delta x}\right)^2+\left(\frac{z(x_k,y_l)-z(x_k,y_{l-1})}{\delta y}\right)^2}$$

$$S_a = \frac{1}{MN}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}|z(x_k, y_l)|$$

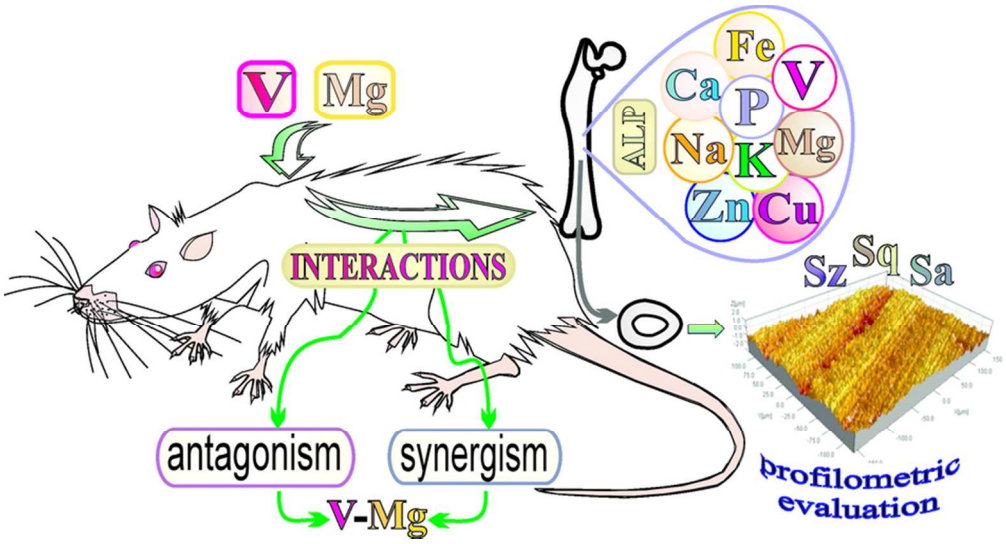
$$S_{sc} = \frac{-1}{2n}\sum_{i=1}^{n}\left(\frac{\delta^2 z(x,y)}{\delta x^2}\right)+\left(\frac{\delta^2 z(x,y)}{\delta y^2}\right)$$

$$S_{ku} = \frac{1}{MNS_q^4}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}\left[z(x_k, y_l)\right]^4$$

$$S_{sk} = \frac{1}{MNS_q^3}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}\left[(z(x_k, y_l)\right]^3$$

$$S_z = S_t = S_y = z_{\max} - z_{\min}$$

$$S_{dr} = \frac{\left(\sum_{k=0}^{M-2}\sum_{l=0}^{N-2}A_{kl}\right)-(M-1)(N-1)\delta x\delta y}{(M-1)(N-1)\delta x\delta y}100\%$$



Vanadium and magnesium effects on bone mineral status and micromorphology were showed in in vivio experimental model
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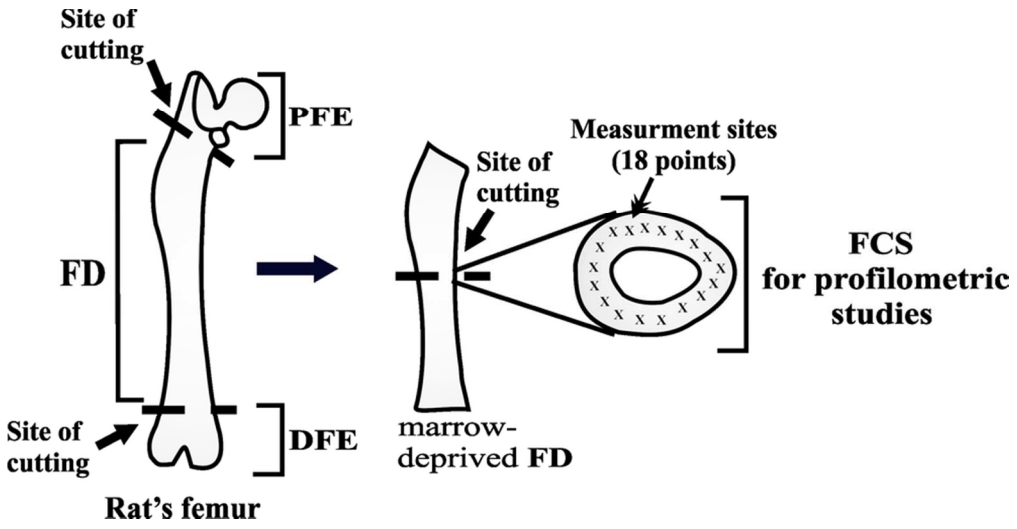

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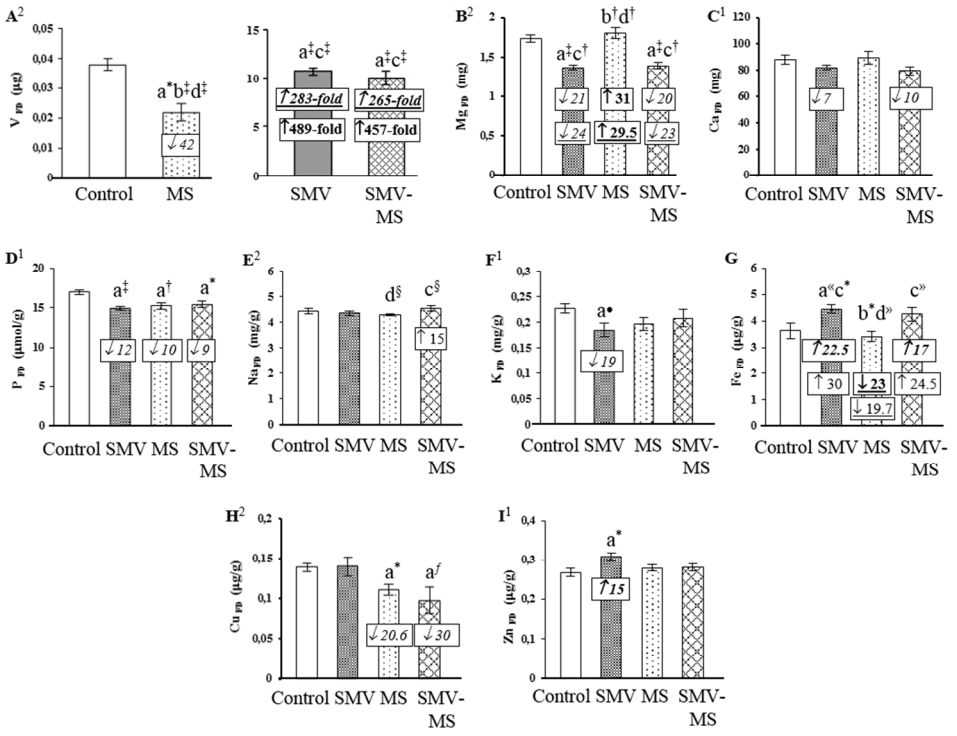

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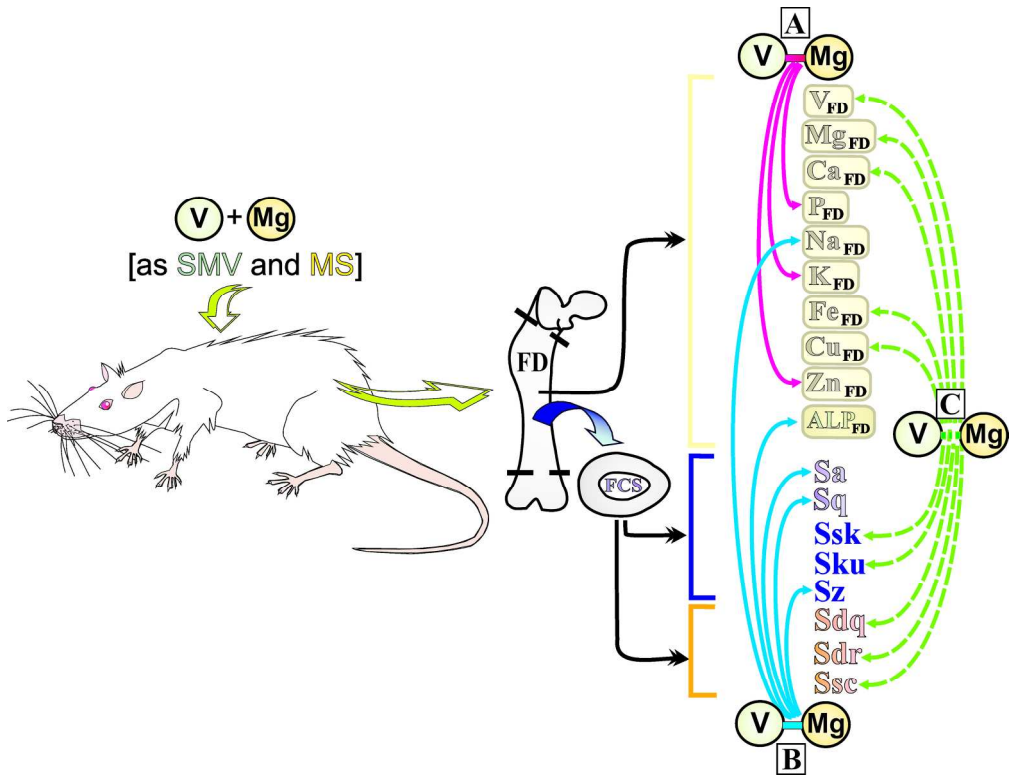

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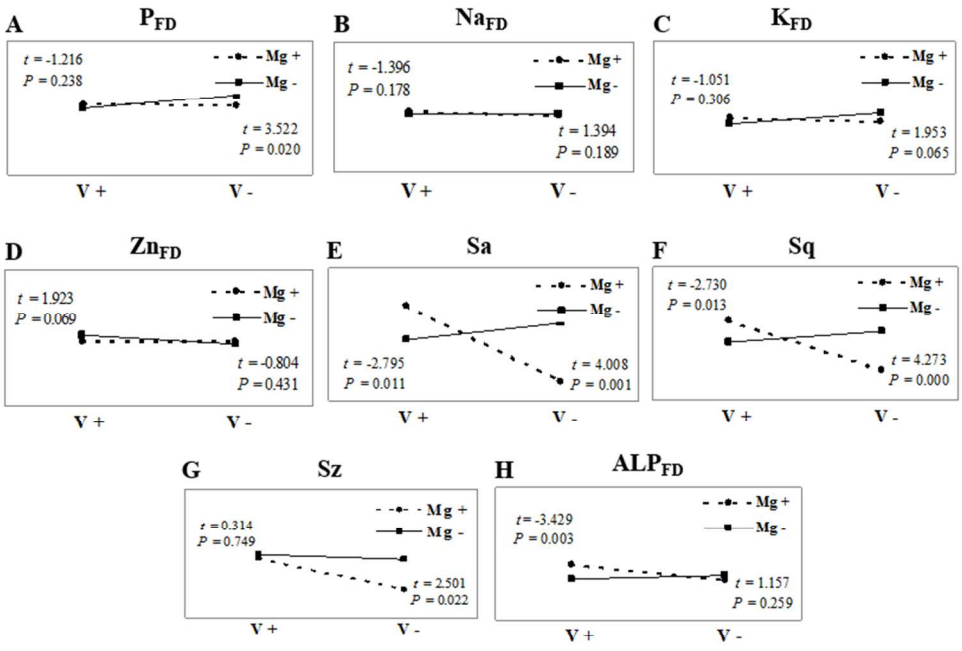

V- and Mg- (Control); V+ and Mg- (SMV-intoxicated)
V- and Mg+ (MS-supplemented); V+ and Mg+ (SMV-MS co-administered)

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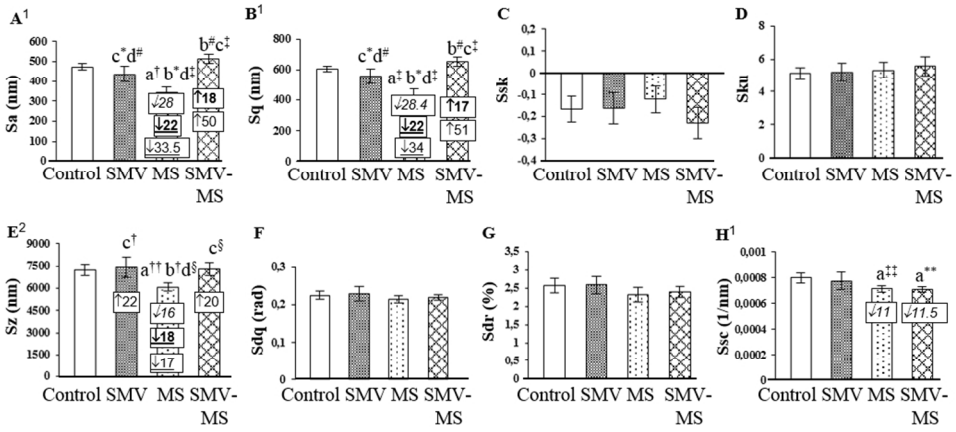

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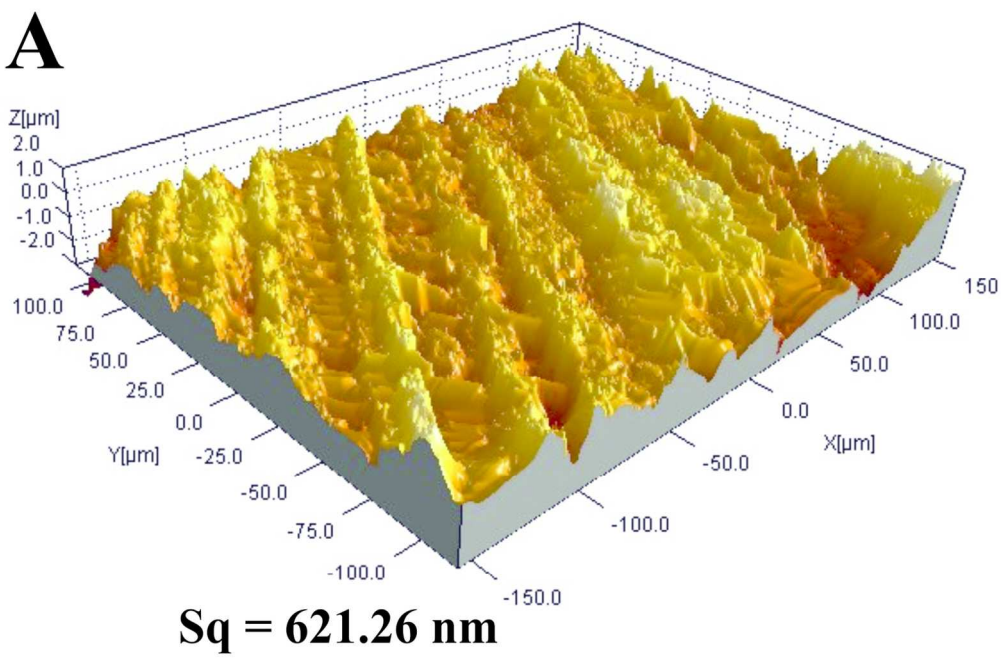

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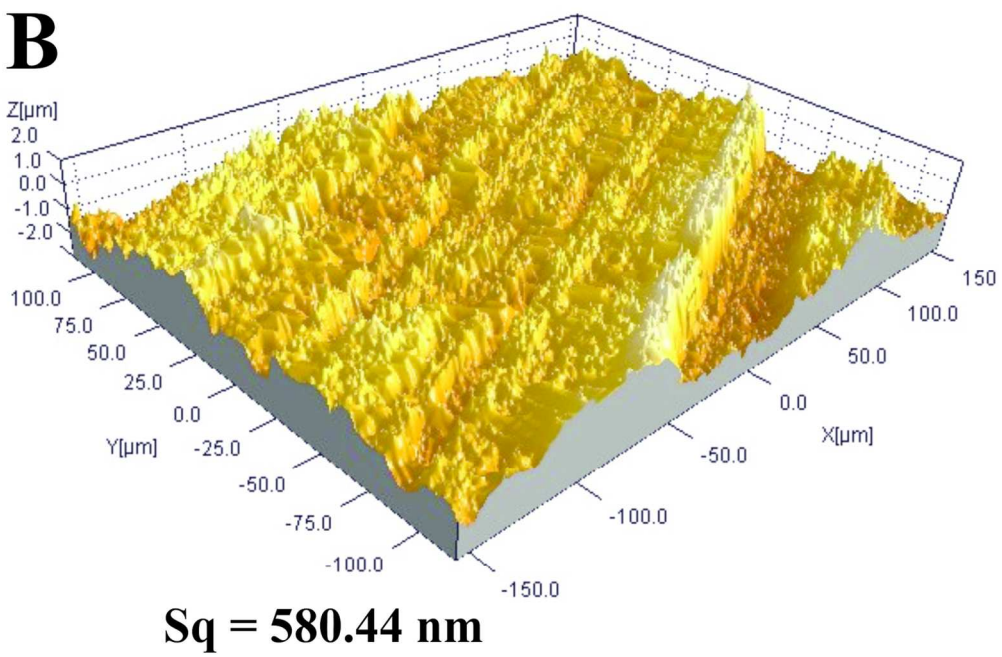

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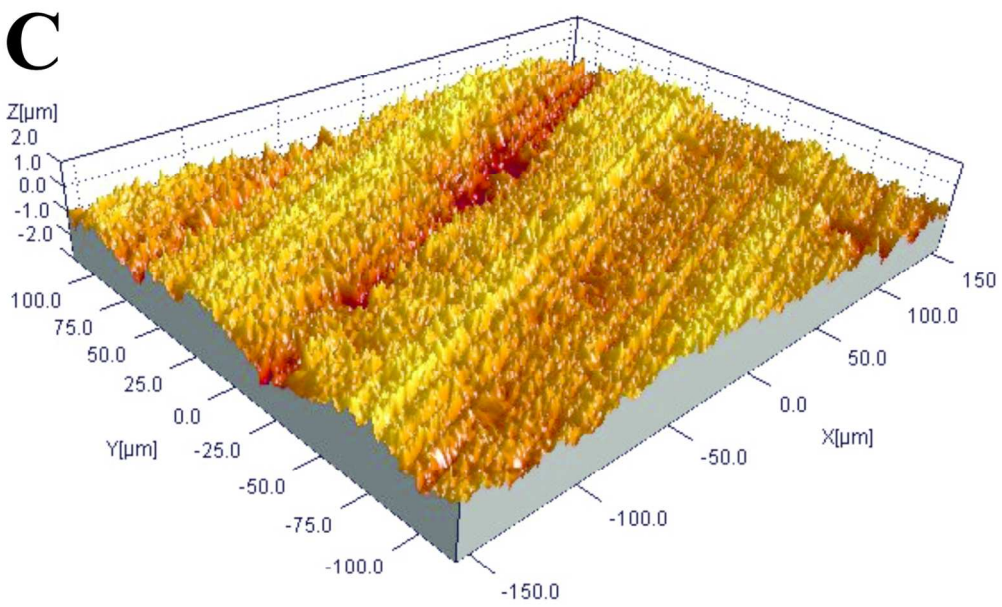

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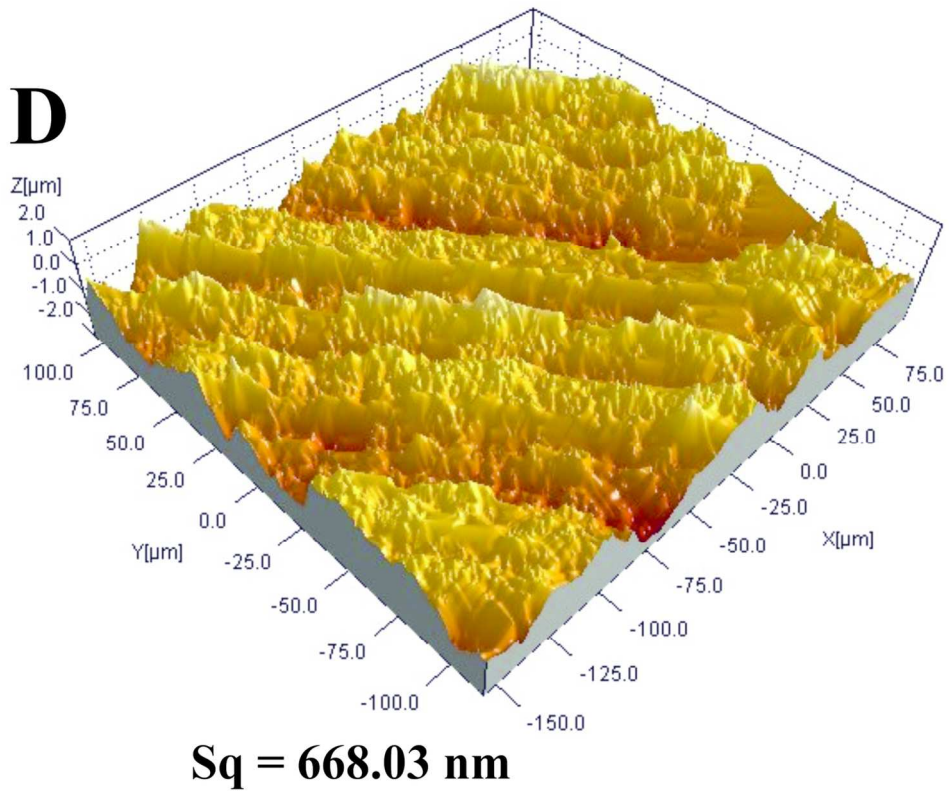

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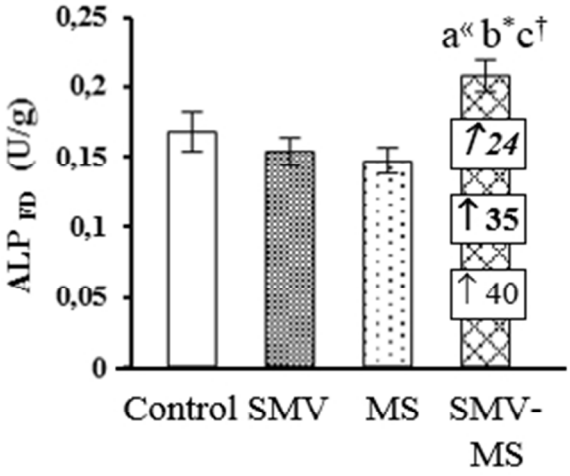

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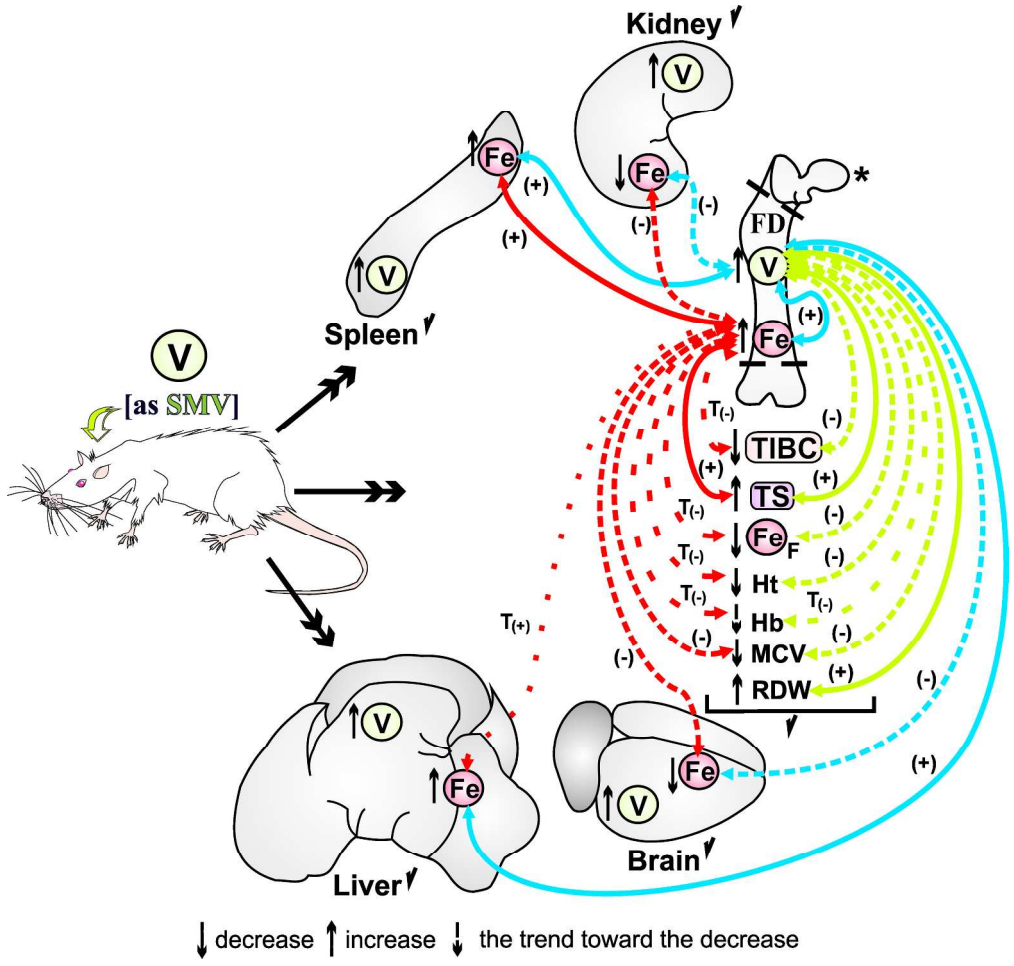

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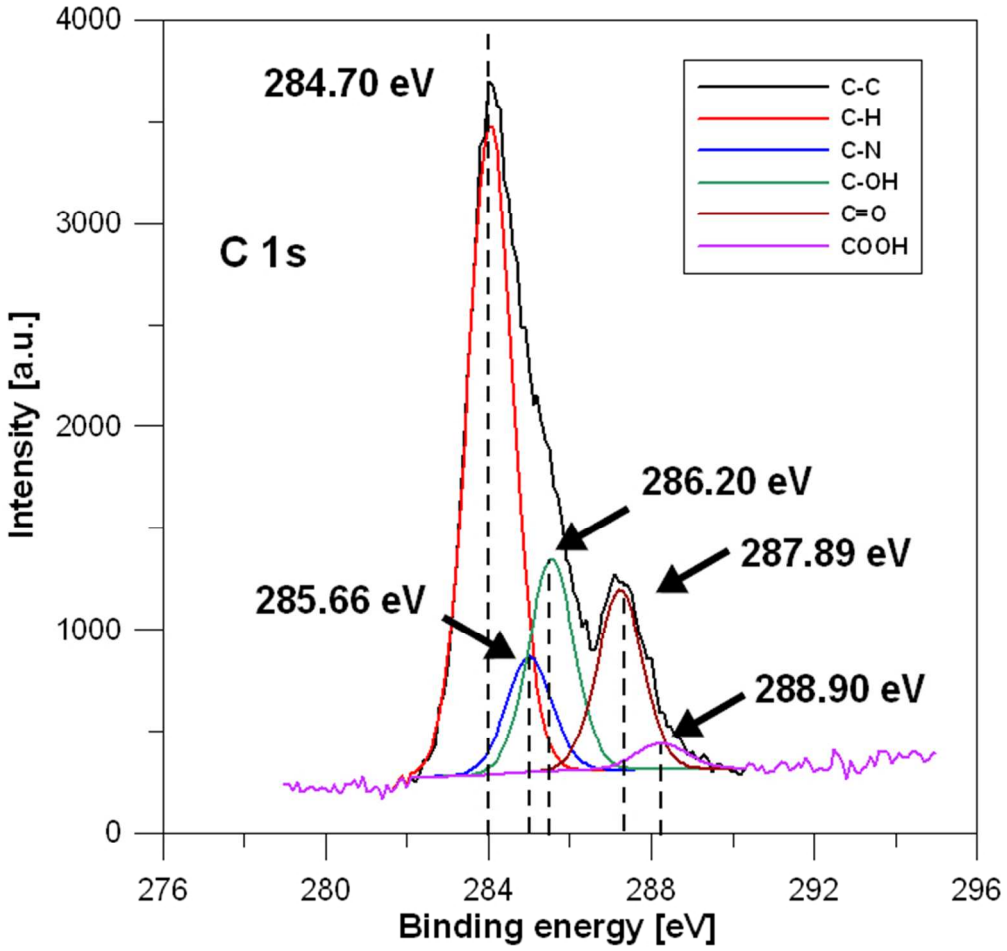

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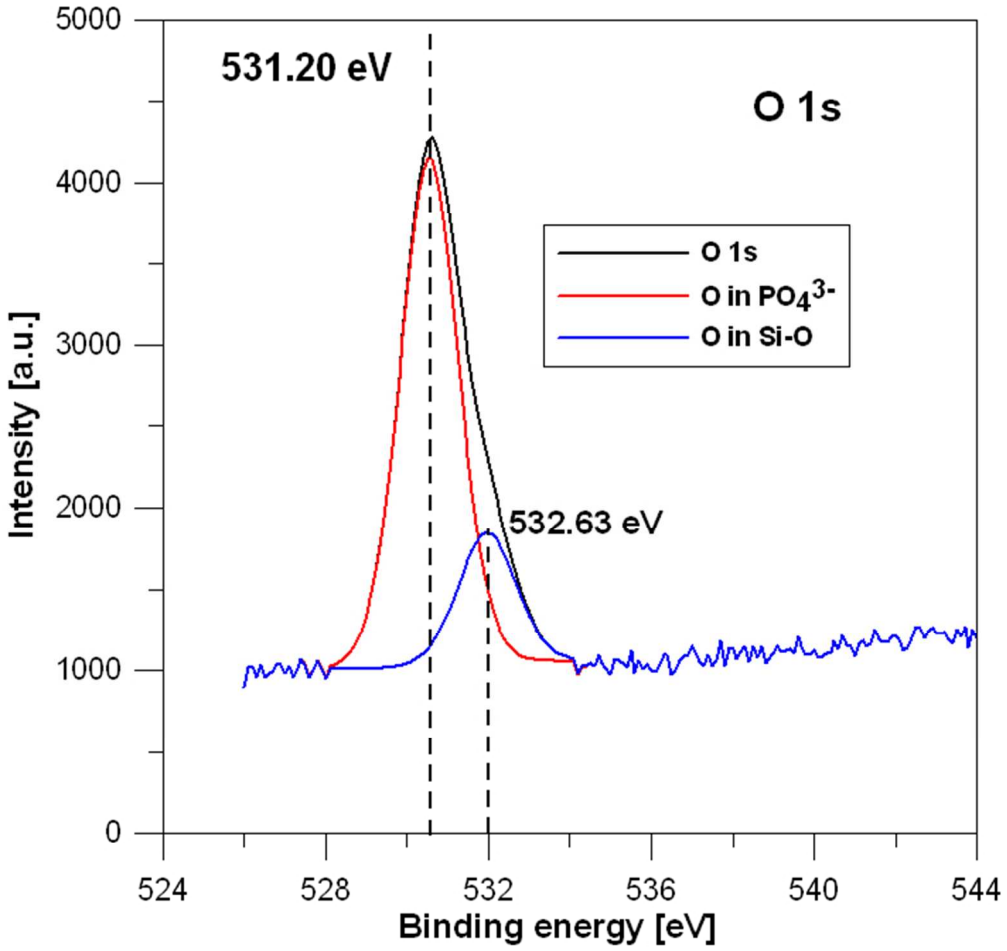

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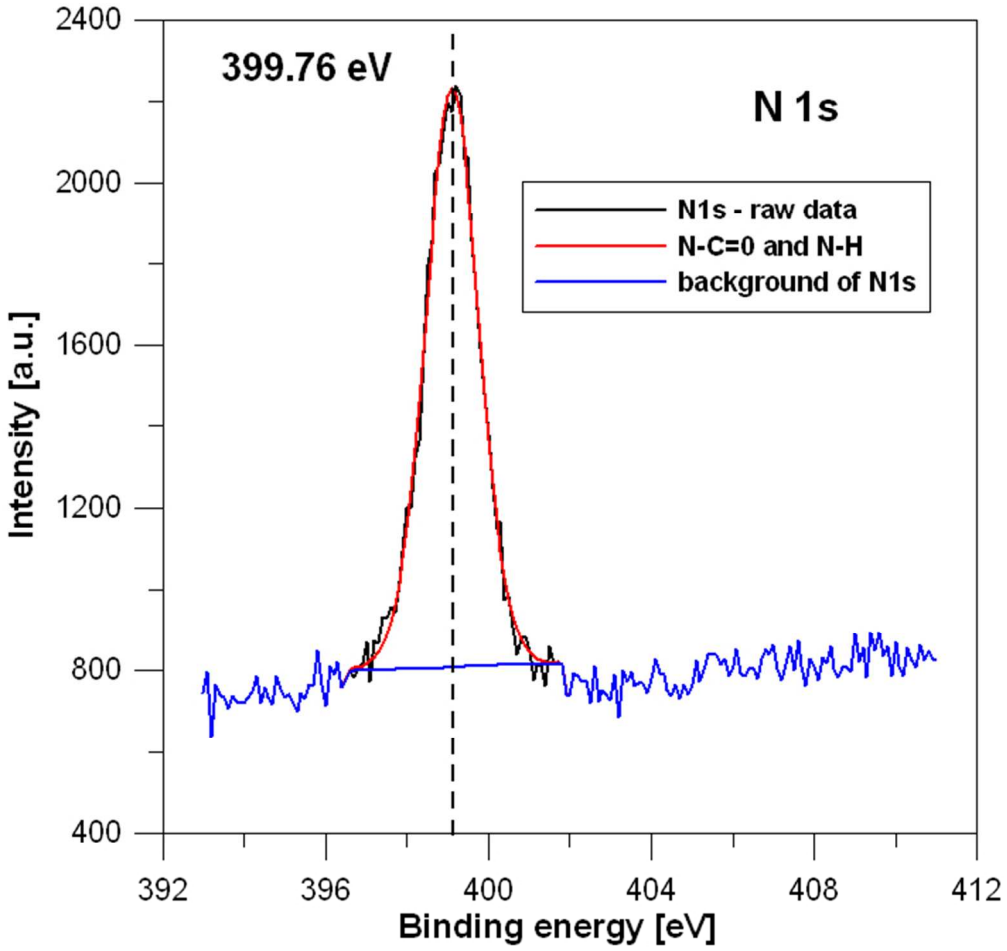

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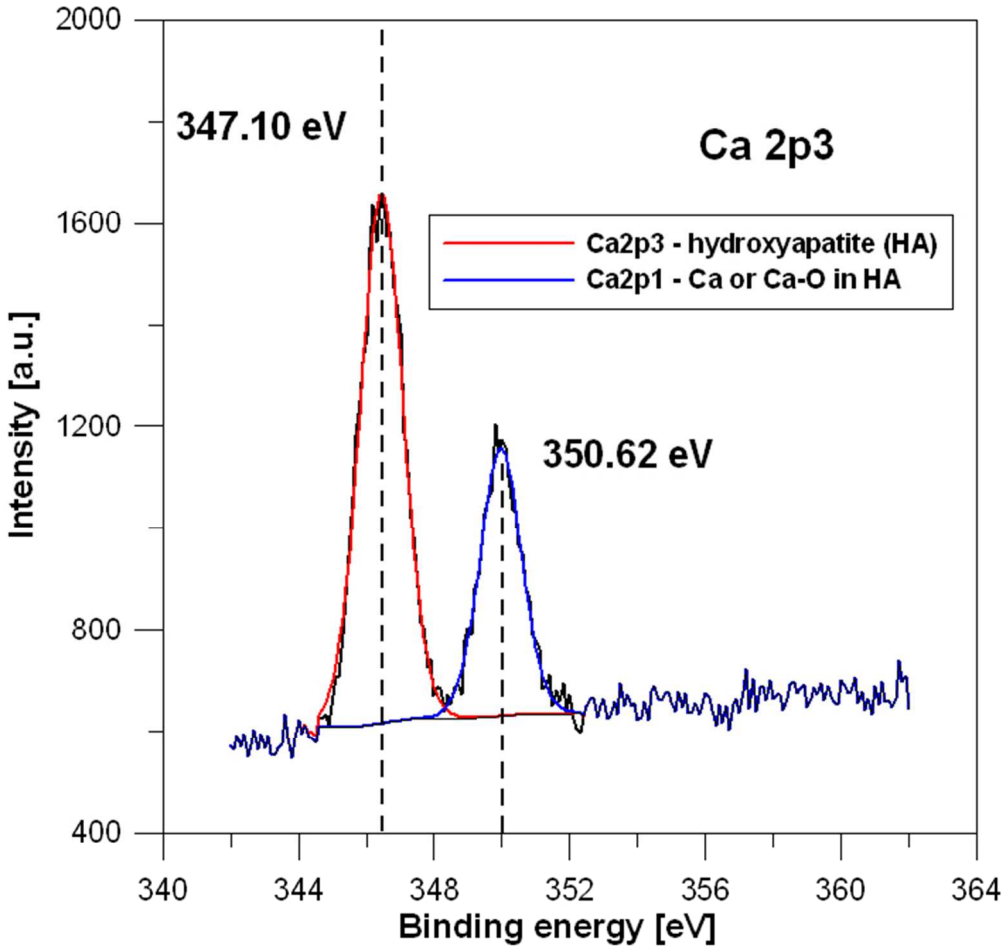

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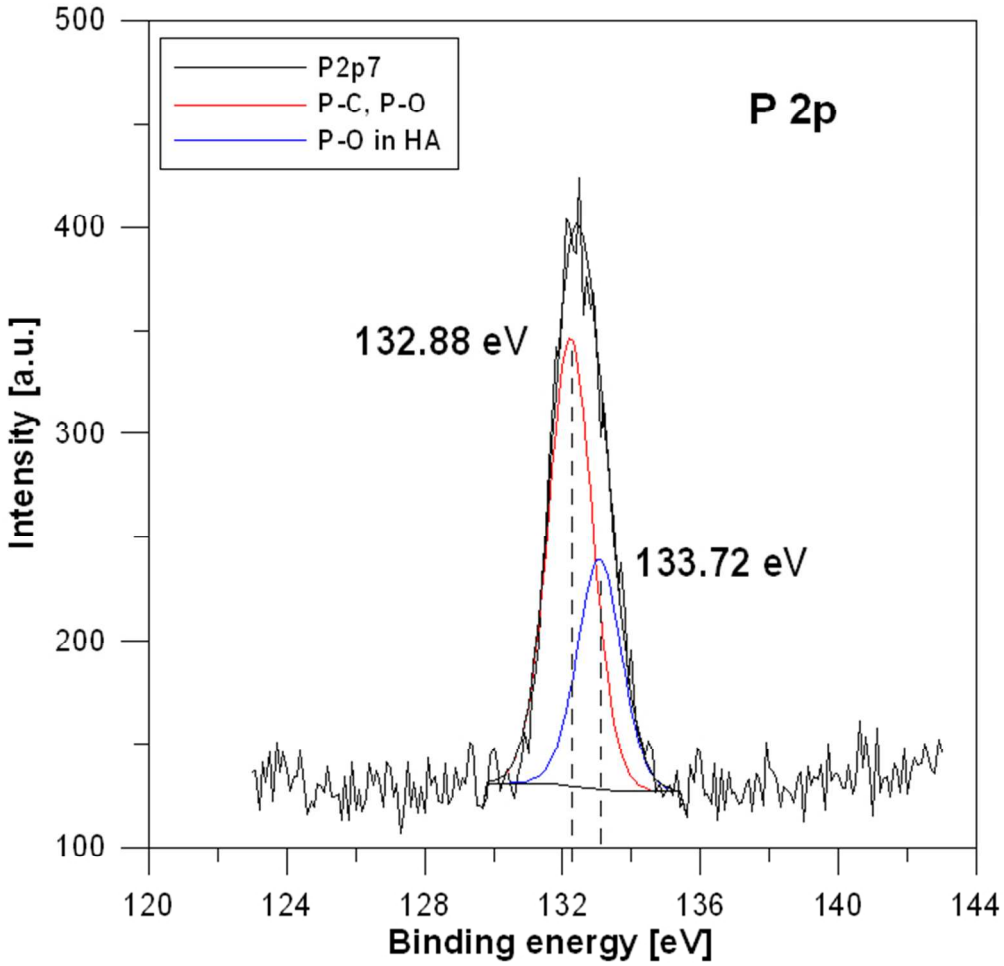

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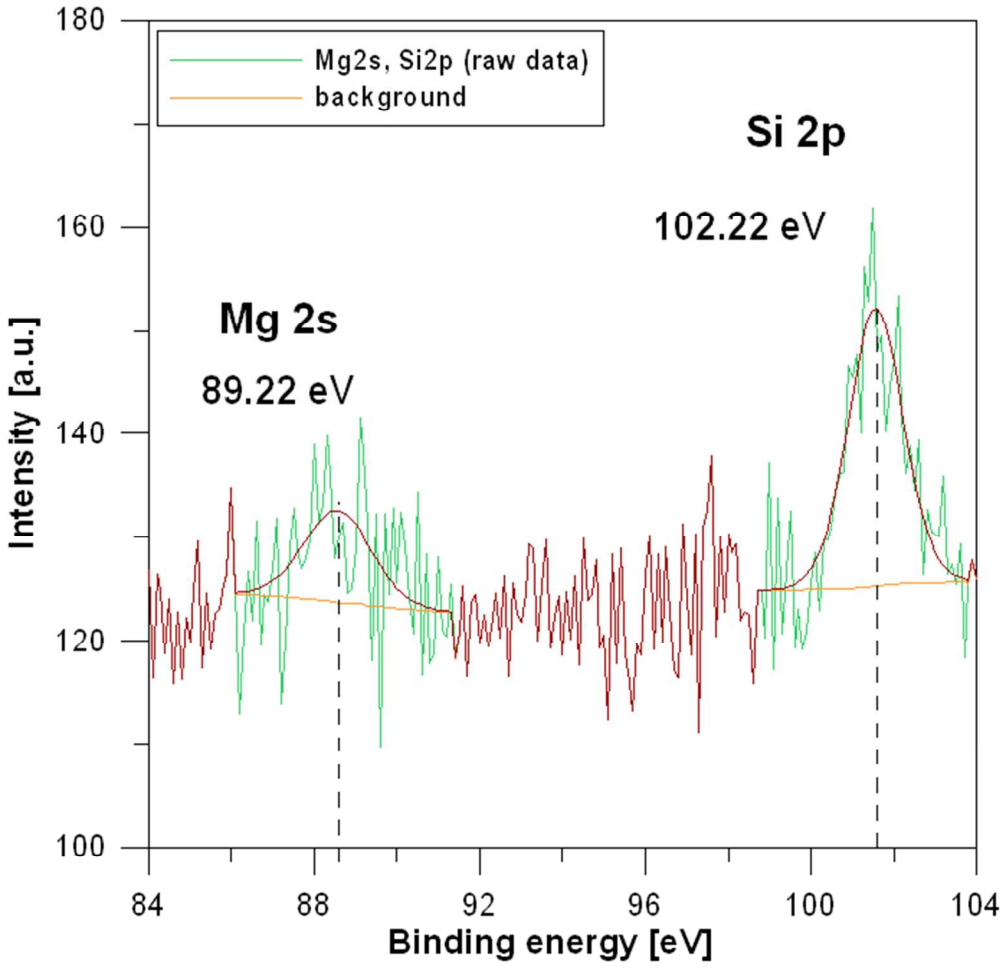

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