Biological Trace Element Research
https://doi.org/10.1007/s12011-019-01909-x

Could biological tissue preservation methods change chemical elements proportion measured by energy dispersive X-ray spectroscopy?

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Received: 5 August 2019 / Accepted: 16 September 2019

Abstract
Energy dispersive X-ray spectroscopy (EDS) is a powerful technical tool used in the biomedical field to investigate the proportion of chemical elements of interest in research, such as heavy metal bioaccumulation and the enzymatic cofactors and nanoparticle therapy in various pathologies. However, the correct evaluation of the proportion of the elements is subject to some factors, including the method of sample preservation. In this study, we seek to investigate the effect of biological tissue preservation methods on the proportion of chemical elements obtained by the EDS methodology. For such, we used EDS to measure the proportion of chemical elements with biomedical interest in preserved livers, using three common methods for preserving biological tissues: (a) freezing, (b) paraformaldehyde fixative solution, and (c) Karnovsky solution. We found an increased level of sodium and reduced contents of potassium and copper in samples fixed in fixative solutions, when compared to frozen samples ($p < 0.05$). Our data indicate that preservation methods can change the proportion of chemical elements in biological samples, when measured by EDS. Frozen preservation should be preferred to retain the actual chemical content of samples and allow a correct assessment of the proportion of their elements.

Keywords Energy dispersive X-ray spectroscopy · Fixative methods · Chemical element proportion

Introduction

The energy dispersive X-ray spectroscopy (EDS) also known as electron probe X-ray microanalysis (EDX) is a powerful technical tool that uses the generation and incidence of X-rays associated to electron microscopy (EM) in atoms of different types of specimens [1, 2]. This technique is used to reveal and/or characterize different chemical elements present over the tested sample surfaces [3]. In the biomedical field, this tool has been applied to investigate or detect the bioaccumulation of minerals and/or pollutants in organs and tissues [4, 5]; residues of heavy metals in skin [6] or in cancerous cells [7]; morphological/ultrastructural damage associated with oxidative stress in different tissues due to bioaccumulation of heavy metals [8–10]; and nanoparticles associated to the improvement and therapeutic performance of some chemotherapeutic agents [3].

Considering the substantial applicability inherent to the microanalysis of chemical elements by this technique, it is fundamental to search for results with a considerable degree of analytical sensitivity [1]. From this perspective, the biological sample preparation aiming at the maximum chemical preservation of microelements must be prioritized [1, 3]. Since EDS is linked to the EM technique [1], a single specimen can be usually prepared for simultaneous use, including for morphological microanalysis and the investigation of the chemical profile. However, it should be considered that, while the purpose of sample preparation for EM is to prevent tissue deformation by using fixation and dehydration so as to ensure structure preservation [1], in the EDS, methods capable of preventing movement or loss of chemical elements should be prioritized to guarantee the reliability of the data obtained [1, 3].

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Published online: 25 October 2019

Although analytical studies have shown an isolated relationship between aldehyde fixative solutions (e.g. paraformaldehyde and Karnovsky) and the diffusion loss of low weight elements or molecules [1, 11, 12], to date, no study has shown the relationship between these fixatives and the semi-qualitative or semi-quantitative analysis of the chemical elements by EDS [2]. Therefore, this work aims to investigate the effect of biological tissue preservation methods on the proportion of the chemical elements obtained by the EDS methodology.

Materials and methods

Four male Swiss mice (80 days old; 37.24 ± 2.48 g) were housed individually in polypropylene cages, under controlled conditions of temperature (22 ± 2 °C), humidity (60–70%), and light-dark cycles (12/12 h). The use of animals was approved by the Ethics Committee on the Use of Animals at the Federal University of Viçosa (CEUA/UFV—protocol number 07/2018). All animals received standard rodent diet and water ad libitum for 7 days. Then, the animals were euthanized by deep anesthesia (ketamine 150 mg/kg and xylazine 10 mg/kg i.p.) followed by cardiac puncture [10]. Their livers were removed, weighed, and divided into three fragments (i.e., three test groups) for treatment with different preservation methods, as follows: (1) frozen liver (frozen), (2) liver fixed in Karnovsky (Karnovsky), and (3) liver fixed in paraformaldehyde (paraform). The first fragment was put in a plastic microtube and frozen in liquid nitrogen and immediately stored at − 80 °C; the second one was immersed in Karnovsky fixative solution (4% paraformaldehyde and 5% glutaraldehyde) in 0.1 M sodium phosphate buffer, pH 7.2 [10]; and the third fragment was immersed in 4% paraformaldehyde fixative solution in 0.1 M sodium phosphate buffer, pH 7.2 [5]. The fragments remained at the fixative solution for 24 h and were subsequently transferred to a 70% ethanol solution, in which they remained for 24 h. Then, the next step was conducted.

The presence of the chemical elements sodium (Na), potassium (K), calcium (Ca), manganese (Mn), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), and selenium (Se) in the liver was evaluated by the EDS methodology [5]. These elements were selected for being commonly analyzed in medical research, as they are cofactors of antioxidant enzymes and determinant in the operation of ion pumps, often measured to evaluate cellular homeostasis [5, 7, 9, 10]. Briefly, the liver fragments, which were frozen, fixed in Karnovsky solution or fixed in paraformaldehyde, were dried at 60 °C for 96 h in a glass plate, coated with carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK), and analyzed in a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with a X-ray detector system (Tracor TN5502, Middleton, WI, USA). Oven drying was chosen to allow the analysis of the frozen fragment as increasing serial dehydration of alcohol would not be possible by disintegrating the unfixed sample. Carbon coating is a prerequisite step in EDS analysis, which was performed for all samples. The experiment was designed to ensure that the only source of variation was the fixative treatment without interference of the analysis method. The analysis was performed at × 150 magnification, using an accelerating voltage of 20 kV and a working distance of 10 mm. The data were normalized using the carbon (C), nitrogen (N), oxygen (O), phosphorus (P), and sulfur (S) measurements. The results were expressed as a mean value.

Prior to statistical analysis, the data were transformed by angular transformation, since the data were provided as percentages. All the results were submitted to the Shapiro-Wilk test for normality assessment. The data were analyzed by one-way analysis of variance (one-way ANOVA) followed by the Holm-Sidak post hoc test for multiple comparisons. Statistical significance was established at $p \leq 0.05$. All tests and graphics were performed using the GraphPad Prism 6.0 statistical software system (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as mean ± standard deviation (mean ± SD).

Results

Figure 1 presents the proportion of chemical elements, highlighting Na, K, Ca, Mn, Mg, Fe, Cu, Zn, and Se, present in liver fragments treated with three different preservation techniques (i.e., freezing, Karnovsky, and paraformaldehyde fixative).

The K and Cu percentage levels decreased considerably ($p < 0.05$) in the samples fixed with Karnovsky and paraformaldehyde, compared to the frozen control. Conversely, the proportion of Na increased significantly in the same groups. However, the proportions of the relative values of other investigated elements (Ca, Mn, Mg, Fe, Zn, Se) presented no significant changes.

Discussion

The EDS method has been widely used for the study of chemical elements of interest in medical research [3–10, 13]. Therefore, the selection of an appropriate preservation method is fundamental to ensure the chemical integrity of the samples [1, 3].

As Fernandez-Segura et al. [1] had already pointed out, since the loss or acquisition of elements during sample preparation is prevented, the freezing method is safer, compared

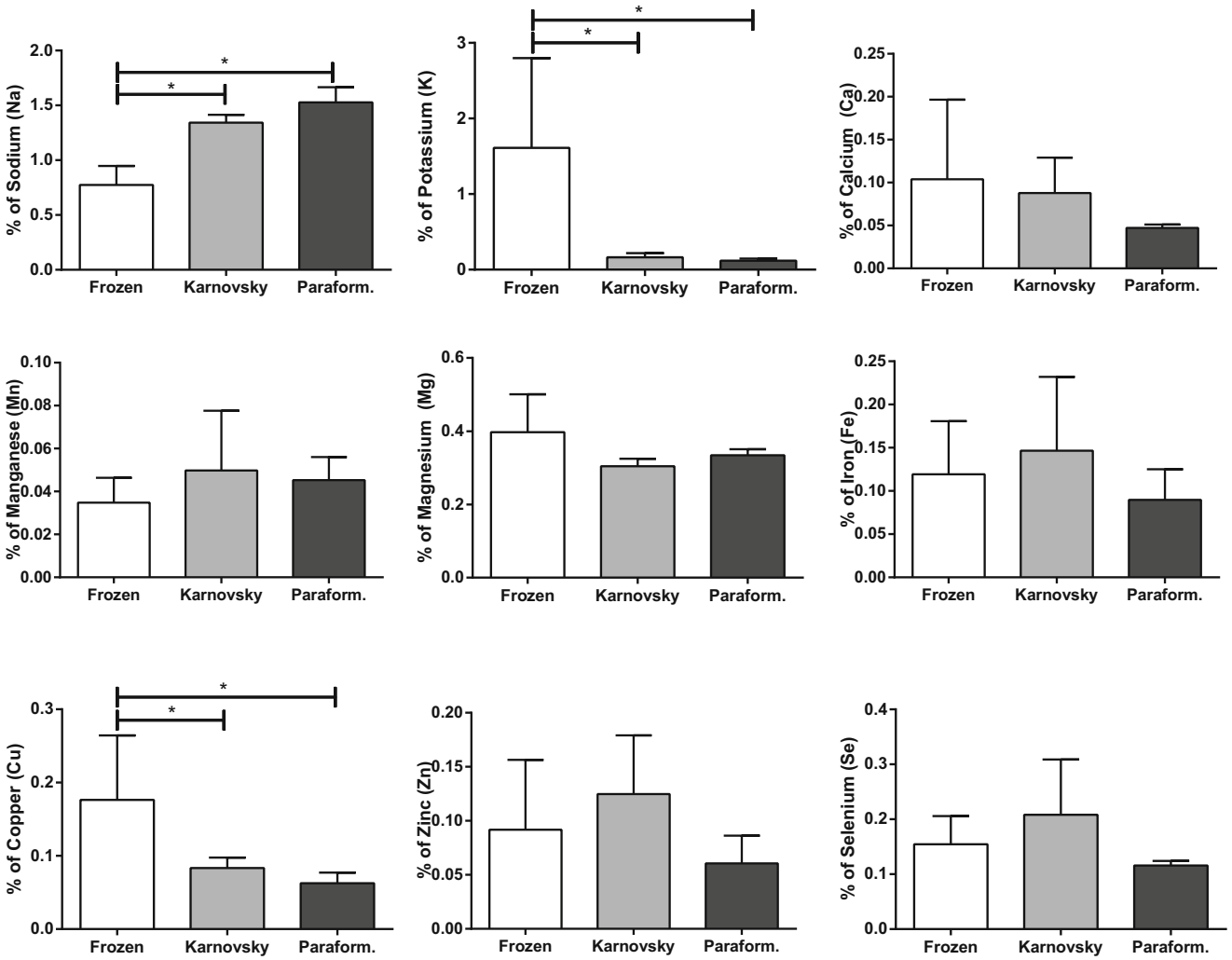

Fig. 1 Percentage of mean values of Sodium (Na), Potassium (K), Calcium (Ca), Manganese (Mn), Magnesium (Mg), Iron (Fe), Copper (Cu), Zinc (Zn), and Selenium (Se) in the liver of the experimental animals. The tissue fragments were preserved by freezing (frozen), fixed in Karnovsky (Karnovsky), or 4% paraformaldehyde (paraform). The graphics present the X-ray emission spectrum for the elements analyzed. The bar refers to the mean, with the standard deviation indicated above. The asterisk (*) denotes different means ($p \leq 0.05$) between the groups by "one way" ANOVA, followed by the Holm-Sidak test

to other fixative methods. Small molecules and diffusible elements are quickly lost during immersive fixation and dehydration process. On the other hand, freezing methods achieve fixation and secure that the elements are maintained at their intracellular sites of action [1]. In fact, our results show an increased Na proportion and reduced K and Cu in paraformaldehyde and Karnovsky solution-fixed liver samples. Although changes were not detected in any other elements, we consider that the freezing method may be more appropriate for the EDS analysis, as it ensures the chemical integrity of the sample. Otherwise, tissue preservation with fixative solutions can be a great problem during X-ray detection.

In this sense, Scimeca et al. [3] indicated that one of the most relevant limitations during elemental analysis is the inter-element reactions, which can be exemplified by the chemical interactions between the sample and the fixative solution for tissue preservation. So, how do fixative solutions change the proportion of the elements in a sample?

Studies indicate that aldehyde fixative solutions, such as paraformaldehyde and Karnovsky, favor the cross-linking between proteins in the tissue [11, 12]. The cross-linkages cause proteins to bind to each other. Membrane proteins can bind in the intra and extracellular environment [11] and change the permeability of the membranes, consequently altering the chemical micro environment around them [11, 12, 14]. The cross-links may disrupt the cellular membrane, thus exposing the cytoplasmic content to the extracellular environment and favoring the chemical exchange between the sample and the fixative solution [11]. Our results present the outcome of such exchanges, in which significant changes ($p < 0.05$), especially in Na and K levels, were found in samples fixed with Karnovsky and paraformaldehyde, when compared to those preserved by freezing. It indicates

that this process is directly linked to the movement of elements of the cellular microenvironment and the solution in which the sample is immersed in both directions.

In this context, Shepherd et al. [11] also reinforce that the activity of the sodium-potassium ATPases are impaired by aldehyde fixatives. Such carriers exhibit abnormal activity and work to the complete failure of the energy in cells, which contributes to the modification of the gradient of the chemical elements. In addition, these fixatives change the electrochemical charges in the cellular membrane, which increases membrane pore sizes [11] and affects the Cu carrier [15].

In hepatocytes, Cu is bound to proteins and is not detected in a free form as a mechanism to avoid toxicity, which hinders chemical exchanges by gradient movement [16, 17]. However, the interaction between the metal and the binding sites of proteins is highly affected by variations in pH and temperature [18, 19]. This is corroborated by a study with cultured hepatocytes, which demonstrated that washing the samples with phosphate buffer at pH 7.0 can reduce the Cu content up to 20% [18]. Although we have not evaluated pH and temperature, this proposition may explain the reduced levels of copper found in the liver fragments treated with fixative solutions, when compared to the frozen sample, which is further evidence to support the findings of this study.

The mechanisms discussed may work similarly in other organs, given the effect of fixative solutions on biological samples. Tissue-to-tissue variations can be found, as each organ differs from the others in chemical content, for several reasons, and some of them may contain higher levels of elements such as Na and K [5, 9] than other tissues.

Taken together, our results indicate that paraformaldehyde and Karnovsky fixative solutions can alter the proportion of chemical elements measured by EDS. Because they are proportions, changing one element alters the quantification of all others, which further increases the importance of choosing the proper preservation method. In this sense, frozen preservation should be preferred over fixative solutions in order to preserve the actual chemical content of the samples and allow a correct assessment of the proportion between them. This is the only alternative to guarantee the correct output of EDS results for biological samples and it should be the preferred preservation method for this purpose.

Acknowledgements The authors are thankful to the "Núcleo de Microscopia e Microanálise - NMM" of the Federal University of Viçosa (UFV); Almeida, E. L. M. for the insights in the discussion; Sacramento, E. for English language editing; "Programa de Pós-Graduação em Biologia Celular e Estrutural" of UFV for the resources to perform the analyses; and "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) for the L. C. M. Ladeira Ph.D. scholarship provided (process nº 88882.436984/2019-01).

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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