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Effects of the relationship between ^{65}Zn and blood cells. A dynamic and morphological study

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Summary. We have studied the dynamic pathway of ^{65}Zn and its autoradiographic location in blood cells, even at the ultra-structural level. We have found evidence that tends to confirm the old biochemical postulates about the capacity of this isotope to displace iron in the haemoglobin molecule.

Recently, the bibliography has demonstrated that ^{57}Co is also able to perform this displacement, but unlike ^{65}Zn it does not invalidate the Redox function of the molecule.

In the case of ^{65}Zn, the mentioned displacement invalidates this function because the radionuclide can only use valence 2. We have also contributed evidence of erythrocytes destruction by the spleen after the incorporation of ^{65}Zn, as well as the clearly marked degradation of haematic pigments inside the spleen.

Key words: Zinc, Blood cells, ^{65}Zn pathway, Autoradiography

Introduction

More than half a century ago, Tupper and collaborators (1951) published the first evidence of marking erythrocytes and leukocytes with ^{65}Zn. Moreover, the studies of Warburg and Christian (1942), which were endorsed by Vallee and Gibson (1948) and interpreted by Oelshlegel et al. (1973a,b), showed that ^{65}Zn did not displace its stable homonym from its covalent bond in the carbon anhydrase molecule. This achievement added extra interest to the use of ^{65}Zn as a marker, especially when studying the distribution of Zn in tissues, as it enables us to rule out carbon anhydrase activity.

As a consequence of the contamination of the Columbia River due to the accident of the 5-Mile Island nuclear power plant, many studies on the effects of ^{65}Zn incorporation into living beings were published in 1965. Out of these studies, we highlight those describing the effects of the incorporation of ^{65}Zn into blood cells (Beloborodova and Red'kina, 1965; Red'kina, 1965; Volkova, 1965).

Much more recently, Chesters and Will (1978) highlight the importance of the uptake rate of ^{65}Zn in blood as information data on the health status of rats.

Lykken (1983) studies the absorption of ^{59}Fe and ^{65}Zn in humans by using ultra-low doses, which permits the long-term monitoring of the incorporation and permanence of the marking.

Tandon et al. (2005) and Malhotra and Dhawan (2008) provide data of lithium interference in biokinetics and uptake of ^{65}Zn. Also, they evaluate the palliative effect to the unfavourable reactions of treatments with lithium when administering Zn.

Simons (1995) places emphasis on the different bivalent metals -including Zinc- in terms of replacing iron in the haemoglobin molecule.

Finally, Simonsen et al. (2011) have observed how ^{57}Co is uptaken by erythrocytes. By means of electrophoresis, ^{57}Co is identified when emigrating with the haemoglobin peak and, therefore, it is considered to be incorporated into the globin. This uptake is maintained for the 120 days' life of the erythrocyte, so it is assumed that the ^{57}Co replaces the Fe physically and functionally, thus being able to produce the oxidation

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from Co^{2+} to Co^{3+}. Moreover, when comparing ^{65}Zn to ^{57}Co, Simonsen stated that ^{65}Zn is uptaken exponentially by the erythrocytes whilst the ^{57}Co is uptaken linearly and maintains the fixation rate for longer.

By extending a preliminary study on distribution of Zinc in tissues (Pérez-Castejón, 1977), we develop our tissue uptake and autoradiography studies with ^{65}Zn in blood cells, which aims to explain the behavioural differences between the uptake of ^{65}Zn and that of ^{57}Co.

Material and methods

Sixty young adult Wistar rats, weighing an average of 250g, were injected intraperitoneally with 0.5 ml of a saline solution of $^{65}ZnCl$, named Z.A.D. and supplied by Nuclear Iberica, with calibrated activity of 18,500 MgBq.

Sacrifice of animals was carried out considering the classic criteria by Khristov (1965) about the biological half-period of ^{65}Zn in the rat. Accordingly, groups of four rats were sacrificed daily until day 15 after injection.

Prior to sacrifice, a sample of blood from the great right jugular vein was extracted. In the act of sacrifice, visceral samples, including the spleen, were taken from all animals.

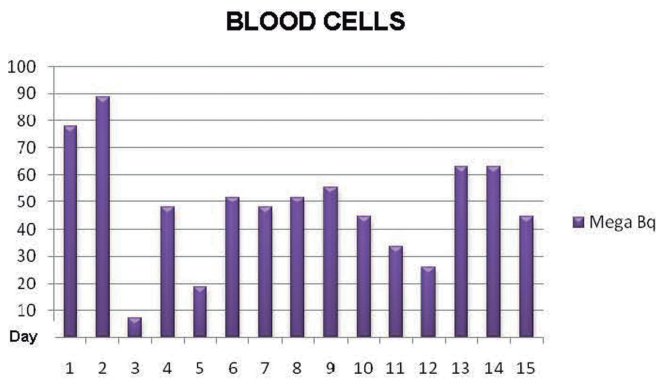
Each visceral sample was divided into 3 pieces; each of them was subjected to different technical studies: the measurement of the radiation doses, ^{65}Zn autoradiography at conventional histological level and ^{65}Zn autoradiography at electron microscope level.

To measure the radiation doses, we placed the samples in Wheaton sterile bottles, performing a double weight to determine the real weight of the sample. 5 ml of nitric acid was added to solid samples in order to homogenize them, while liquid samples –such as blood- were centrifuged in order to separate the cell fraction from the serum fraction. The cell fraction was treated as a solid sample. After, all samples were subjected to radiation measurement in an automatic pit counter CGR Gammamatic-2.

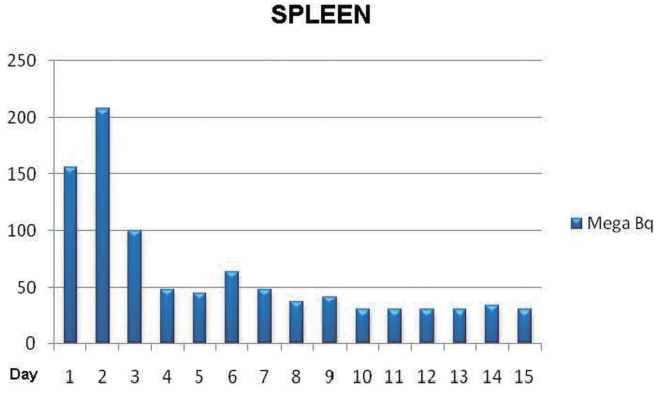
For autoradiography at conventional histological level, the samples were fixed in a Carnoy solution and were embedded in paraffin and sectioned at a thickness of 6 μm. The autoradiographic method was carried out by dipping in 50% Ilford K5 emulsion, dissolved in double-distilled water. Exposure was in black plastic boxes with a drying system, inside a refrigerator at 4°C for 21 days. Kodak D19 was used as developer and Hypam as fixer.

Following the procedure of Parry and Blackett (1973), for autoradiography at electron microscopy level, the samples were subject to double fixation with glutaraldehyde and osmium tetraoxide, embedded in Epon 812 and cut with ultra microtome LKB. The ultrathin sections were transported to conventional glass slides, which had been previously prepared with a bath of formvar and introduced in a high vacuum apparatus for carbon shading. To carry out the dipping autoradiographic procedure, Ilford L4 emulsion mixed with distilled water (1 vol/3 vol respectively) was used. The exposition was three months in a dark plastic box, in a desiccated environment and 4°C of constant temperature. Kodak D19 was used as developer and Hypam as fixer. We detached, elevated and separated the group "formvar/carbon, sample and photographic emulsion" from the glass by using diluted FLH. Then, the group was placed in a grating where it was stained with Reynolds' citrate and uranyl acetate. After, the sample was visualized and photographed in an electron microscopy Philips 301.

Another six animals were used to develop the histochemical technique for location of heavy metals described by Timm (1958), but following the protocol of the Bargmann School. The animals were subjected to thiopental anaesthesia and they were perfused through the heart with sulphide alcohol. After fixation, samples of spleen were extracted, paraffin embedded and sectioned at a thickness of 6 μm. Developing was carried



Graph 1. Uptake profile of ^{65}Zn in blood cells, carried out with the average radiation measurements -expressed in MegaBecquerels- of the samples obtained from the animals, slaughtered daily from the 1st to the 15th day.



Graph 2. Uptake profile of ^{65}Zn in the spleen homogenisation, carried out with the average radiation measurements -expressed in MegaBecquerels- of the samples obtained from the animals, slaughtered daily from the 1st to the 15th day.

out in a laboratory with an average temperature of 21°C and soft illumination. Developing was interrupted when the reaction changed into a silver reflectance and control inspection showed the expected reaction in those tissues. Then, the normal process of washing in hot water was performed.

Controls of both histochemical and autoradiographical techniques were implemented. Regarding the latter, fading and blackening controls were carried out in order to discard chemography. The background index was also controlled.

Results

The average uptake profile in blood cells (Graph 1) shows an increasing maximum peak in the first 48 hours. This is followed by a sudden drop to the minimum value observed in the 15 days of experience, appearing as if the majority of the blood cells marked disappeared from the bloodstream. A rise in the uptake is observed towards the 4th day and then drops once again on the 5th day. A re-uptake occurs from day 6 -the biological half-period according to Khristov, 1965- and remains quite steady and less sudden until the end of the experience. Significant downward oscillations may be observed from day 6 and on.

Timm's histochemical technique shows positivity in the erythrocytes that are present in the blood vessels (Fig. 1). Likewise, the autoradiographic marking of ^{65}Zn

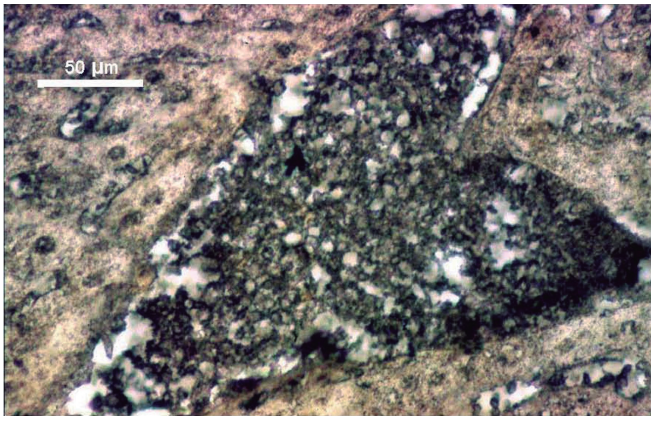


Fig. 1. Preparation showing a hepatic vessel and its blood content stained using Timm's histochemical technique, showing the abundant histochemical positivity in the blood cells.

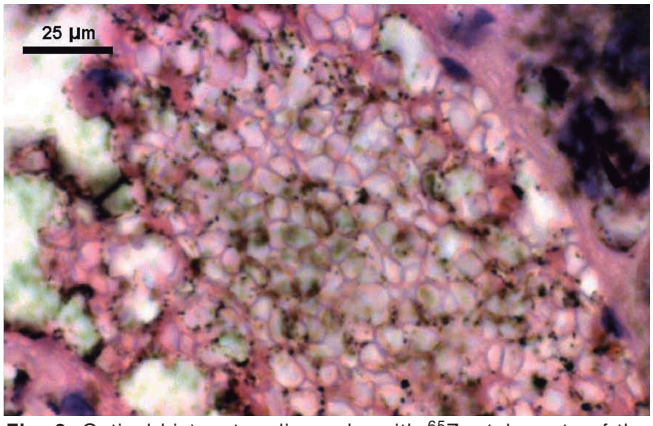


Fig. 2. Optical histoautoradiography with ^{65}Zn; take note of the abundant erythrocytes marked by the radioisotope. Eosin haematoxylin background stain.

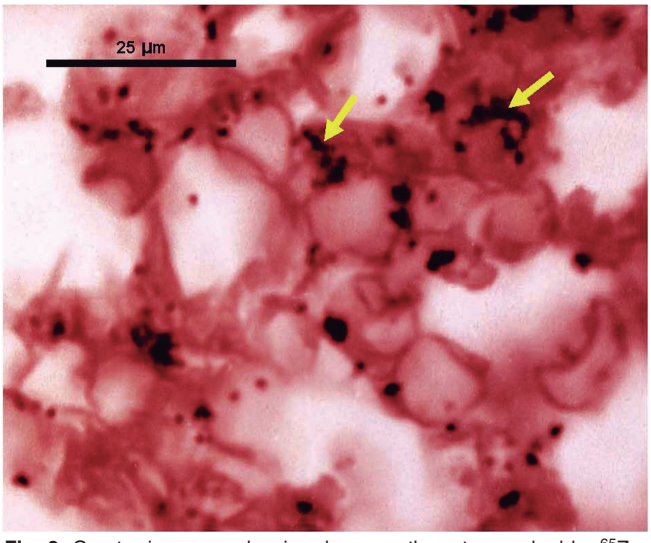


Fig. 3. Greater increase, showing dense erythrocytes marked by ^{65}Zn. Indicated by arrows. Eosin haematoxylin background stain.

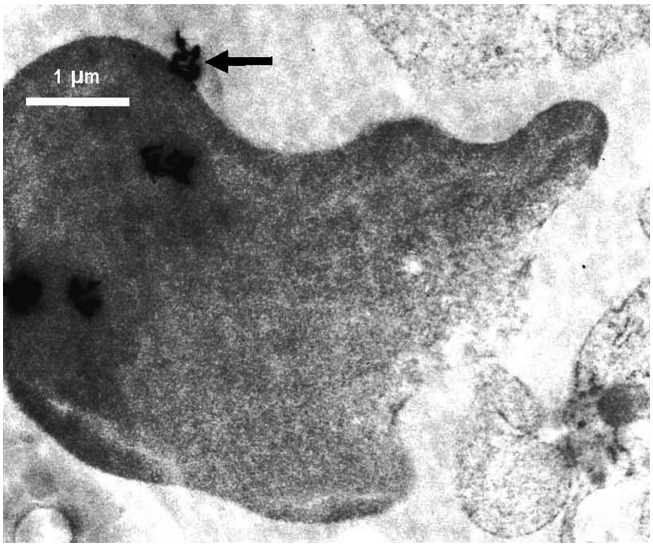


Fig. 4. Autoradiography at electronic microscopic level showing an erythrocyte densely marked with ^{65}Zn. The arrow marks a clear impact on the cellular membrane.

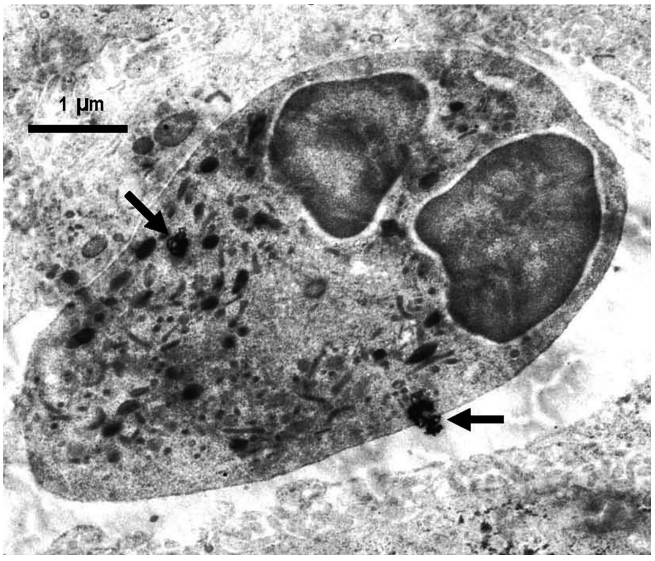


Fig. 5. Autoradiography at electronic microscopic level, showing a polymorphonuclear that shows uptake of ^{65}Zn, in connection with its granular structure. The arrows indicate the location of the impacts.

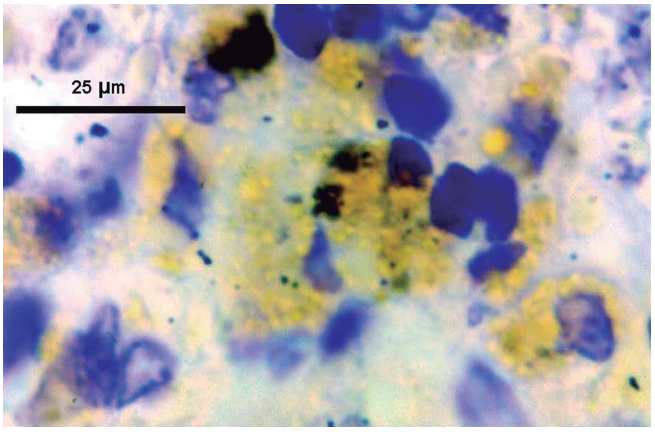


Fig. 7. Optical histoautoradiography with ^{65}Zn of the spleen. Take note of the uptake of radioisotope in the haemolytic remains of the destroyed erythrocytes. Martin's trichromic background stain.

in the intravascular erythrocytes is evident in optical microscopy (Figs. 2, 3).

The autoradiography at electronic microscopic level shows the multiple impacts caused by ^{65}Zn radiation in an erythrocyte (Fig. 4) and the visible impacts in a nuclear polymorph (Fig. 5).

Seeking a coherent explanation to the uptake drops of ^{65}Zn during the 3rd and the 5th day, we pose the hypothesis that the marked erythrocytes were quickly eliminated by the haemocatheretic system of the spleen.

A mere routine observation of our spleen samples showed the presence of a marked haemocatheresis (Fig.

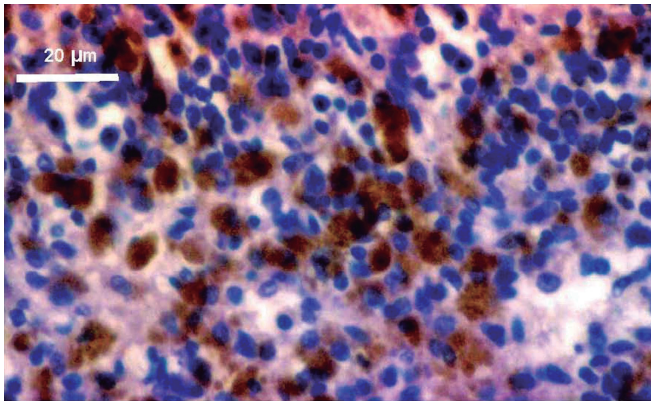


Fig. 6. Image of traditional histology in spleen; see the abundance of haemolytic remains of the destroyed erythrocytes. Eosin haematoxylin stain.

6). The autoradiography at optical microscopic level showed significant uptake of ^{65}Zn in the remains of haemocatheresis of a red series present in the spleen sinusoids (Fig. 7).

The uptake profile of the spleen (Graph 2) bears a certain similarity with that of the blood, although the radiation measured in the spleen is always greater. More specifically, the values of the spleen are double the values measured in the circulating blood cells.

Discussion

Two questions must be discussed now: Why are the erythrocytes marked with ^{65}Zn destroyed so quickly and massively? And, what equals the uptake profiles in measured radioactivity, both in circulating blood cells and in spleen, from the 5th day onwards?.

The only feasible answer to the first question is the confirmation of the proposal made by Oelshlegel et al. in 1973 about the displacement and replacement of Fe by Zn in the haemoglobin molecule; and also, the incapacity of Zn^{2+} to vary its ionic condition, unlike Cobalt and other bivalent metals, which are capable of oxidation such as Co^{2+} to Co^{3+}.

This may explain why the uptake of ^{57}Co is slower but more long-lasting than that of ^{65}Zn. Indeed, ^{57}Co enables the normal longevity of the erythrocyte, something that does not occur in the case of ^{65}Zn, which is uptaken very quickly (Simonsen et al. 2011) but blocks the Redox activity disabling the erythrocyte for its exchange function of O_2 and CO_2 and leading it to its early destruction.

With regards to the second question, we must note that the values drop below 50 Mega Bq., which means maintenance values of residual radioactivity from the functional point of view.

Undoubtedly, slight uptake destruction rises in the erythrocytes will occur, but these are absolutely non-significant when compared to the spectacular

manifestations of the first few days.

Another point to be considered is that in blood, ^{65}Zn is also uptaken by the polymorphonuclear leukocytes as described and shown in Fig. 5 of this study. This uptake was already described some time ago by the authors mentioned in the introduction (Tupper et al., 1951) and its functional meaning was related to the immunity phenomena (Beloborodova and Red'kina, 1965; Beloborodova et al., 1965; Red'kina, 1965; Volkova, 1965)

We believe that this is the explanation for the measured and observed uptake peaks and the final maintenance values of ^{65}Zn in blood cells.

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