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Iron overload, measured as serum ferritin, increases brain damage induced by focal ischemia and early reperfusion

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ABSTRACT

High levels of iron, measured as serum ferritin, are associated to a worse outcome after stroke. However, it is not known whether ischemic damage might increase ferritin levels as an acute phase protein or whether iron overload affects stroke outcome. The objectives are to study the effect of stroke on serum ferritin and the contribution of iron overload to ischemic damage.

Swiss mice were fed with a standard diet or with a diet supplemented with 2.5% carbonyl iron to produce iron overload. Mice were submitted to permanent (by ligature and by *in situ* thromboembolic models) or transient focal ischemia (by ligature for 1 or 3 h).

Treatment with iron diet produced an increase in the basal levels of ferritin in all the groups. However, serum ferritin did not change after ischemia. Animals submitted to permanent ischemia had the same infarct volume in the groups studied. However, in mice submitted to transient ischemia followed by early (1 h) but not late reperfusion (3 h), iron overload increased ischemic damage and haemorrhagic transformation.

Iron worsens ischemic damage induced by transient ischemia and early reperfusion. In addition, ferritin is a good indicator of body iron levels but not an acute phase protein after ischemia.

1. Introduction

Iron is an essential element for life that catalyses many biological reactions due its dual redox nature. However, iron is also able to produce free radicals and oxidative stress. In order to avoid this harmful effect, the organism has developed a set of mechanisms that keep free iron under toxic levels (Eisenstein, 2000; Moos et al., 2007; MacKenzie et al., 2008). Among them, ferritin is one of these mechanisms, being responsible for storing excess iron inside the cells, avoiding free radicals production from free iron. Indeed, serum ferritin is a reliable index of iron stores under healthy conditions (Wang et al., 2010) but it is also a well-known protein of acute phase, synthesized in the liver as a part of the systemic response to infection (Beard et al., 2006).

In this context, brain has one of the highest rates of metabolic activity and for that reason it has the second greatest amount of iron after the liver. It has been suggested that the impairment of iron homeostasis and its consequent accumulation could be a very important factor as a trigger or mediator in many neurodegenerative disorders (for revision see Berg and Youdim, 2006; Jomova et al., 2010). Despite the fact that the specific mechanisms have to be elucidated, it seems clear that cerebral ischemia also impairs iron homeostasis, leading to iron release in the ischemic tissue and therefore to an increased oxidative damage (Ishimaru et al., 1996).

Some clinical studies have found that high levels of iron stores, measured by serum ferritin, are associated with poor outcome after ischemic stroke (Davalos et al., 1994, 2000; Erdemoglu and Ozbakir, 2002; Millan et al., 2007, 2008) and intracerebral haemorrhage (Mehdiratta et al., 2008; Perez de la Ossa et al., 2010). However, direct evidences of iron toxicity after experimental stroke are controversial. Some groups have shown that iron overload increases brain damage after ischemia (Castellanos et al., 2002; Mehta et al., 2004), whereas others failed to show this detrimental effect (Christensen et al., 2002; Millerot et al., 2005).

Therefore, the role of iron in stroke is unclear and needs to be clarified. The main two goals of the present study are: (a) to describe the kinetic of serum ferritin after stroke to clarify whether ferritin is an acute phase protein after stroke or is a reliable marker of iron stores and (b) to study the consequences of iron overload

Abbreviations: IST, *in situ* thromboembolic model; mNSS, modified neurologic score severity; pMCAO, permanent middle cerebral artery occlusion; ROS, reactive oxygen species.

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after cerebral ischemia in mice, attending primarily to the lesion size and haemorrhagic transformation.

2. Material and methods

2.1. Animals and diet

Male Swiss mice (Harlan laboratories, Barcelona, Spain) were used for this study according to the guidelines of the Animal Welfare Committee of the Universidad Complutense (following EU directives 86/609/EEC and 2003/65/EC). One-month-old animals, weighing 15 g, were assigned by randomization to two groups fed with different diets for nine weeks. Control group received a standard diet for rodent maintenance (2014, Harlan laboratories), containing 50 mg of Fe per kg. Iron overload group was fed with a diet supplemented with an additional 2.5% of carbonyl iron (TD 08704, Harlan) (Castellanos et al., 2002). Finally, after the feeding period, 3–5-month-old mice weighing 35–45 g were used for this experimental study. During all the procedures, animals were kept under standard conditions of humidity, ventilation and temperature, with a 12 h dark/light cycle (on at 8:00 h) and with free access to food and water.

2.2. Experimental groups

Animals were assigned by randomization to five further different groups: IST-pMCAO, in which the MCA was permanently occluded using the *in situ* thromboembolic (IST) model (Orset et al., 2007; Garcia-Yebenes et al., 2011); Lig-pMCAO, Lig-1hMCAO, Lig-3hMCAO where the ischemia was achieved by permanent or transient ligature (Lig) (Chen et al., 1986; Zarruk et al., 2012) of the MCA for one and three hours respectively, and Sham group in which the MCA was just exposed but not occluded.

In the IST-pMCAO group, ischemia was carried out as previously described (Orset et al., 2007; Garcia-Yebenes et al., 2011). Mouse alpha-thrombin (2 UI) was injected into the MCA to induce a clot. A clot was defined as stable when laser Doppler flowmetry displayed a drastic fall of brain perfusion (mean reduction of 70–80%) that remained stable during 60 min. Animals with spontaneous reperfusion (20%) were excluded of further analysis being finally $n = 5$ per group.

In the Lig-MCAO groups, the MCA and the ipsilateral common carotid were tied either permanently (Lig-pMCAO) or for one (Lig-1hMCAO) or three hours (Lig-3hMCAO) (Chen et al., 1986; Zarruk et al., 2012). Considering the distal ligature of the MCA as a cortical model, animals with striatal lesion were excluded of further analysis (7%), being $n = 6–7$ in Lig-pMCAO and Lig-3hMCAO groups, and $n = 11$ in Lig-1hMCAO groups.

Animals in which both carotid and middle cerebral arteries were exposed but not occluded were considered as Sham group. Before all the surgical procedures mice were anaesthetised in a chamber ventilated with 2.5% and then maintained at 1.5–2% isoflurane in a 30/70% mixture of O_2/air. Body temperature was maintained at 36.5–37 °C using a feedback-controlled heating blanket. No spontaneous mortality was found after MCAO and this was unaffected by the different experimental treatments.

Physiological parameters (pH, pCO_2, pO_2, haematocrit, haemoglobin and mean arterial blood pressure) were measured in a different group of mice, $n = 4$ and were not significantly different between iron overload and control animals, at both times studied (basal and 15 min after ischemia; Table 1).

2.3. Blood samples collection and measurement of serum ferritin

Blood tail samples were collected the day before ($t = 0$), 3 and 24 h after the surgery and kept at room temperature for 1 h and at 4 °C overnight, allowing coagulation. Then, samples were centrifuged at 1500*g* and 4 °C, and finally serum was extracted and kept at −80 °C until its use. Serum ferritin was measured by a blinded investigator, using an ELISA kit (E-90F, Immunology Consultants Laboratory, Inc., Newberg, OR, USA) and expressed as normalised values by basal control levels ($n = 4$ per group).

2.4. Neurological deficit evaluation

Functional outcome was assessed 24 h after ischemia by a blinded observer using a modified neurologic score severity (mNSS) (Li et al., 2000; Zarruk et al., 2011). Neurologic score severity comprises motor, sensory and reflex tests.

2.5. Determination of infarct size, brain swelling and haemorrhagic areas

Twenty-four hours after MCAO, mice were sacrificed by an overdose of sodium pentobarbital to assess infarct outcome and were transcardially perfused with 0.1 M phosphate buffer (pH 7.4) to eliminate intravascular blood for further analysis. Then, brain was removed, cut into 1-mm thick coronal slices and stained with 2,3,5-triphenyltetrazolium chloride (1% TTC in 0.2 M phosphate buffer). Photographs of each coronal section were made with a digital camera and analysed using Image J 1.44 k (NIH, Bethesda, Washington). All the determinations were measured by a blinded investigator.

Infarct volume was measured as previously reported (Garcia-Yebenes et al., 2011). Infarct area was delineated and determined (in mm^2) by counting the number of pixels within the outline. Infarct volume (in mm^3) was calculated as the sum of the orthogonal projections of each damaged area over the section thickness. In order to exclude the brain edema effects, infarct area was corrected by the ratio of the entire area of the ipsilateral hemisphere to that of the contralateral one.

In addition, brain swelling was calculated according to the following formula: (ipsilateral hemispheric volume – contralateral hemispheric volume)/contralateral hemispheric volume × 100 (Maier et al., 1998).

All noticeable haemorrhages, both petechial and parenchymal ones, were quantified on all the images of TTC-stained sections as previously described (Zhang et al., 1999; Garcia-Yebenes et al., 2011). The boundary of each bleeding was drawn using Image J 1.44 k. The haemorrhage area (mm^2) was calculated by a summation of the area of every bleeding.

2.6. Statistics

Results are expressed as mean ± standard error of the mean. Two way analysis of variance (two way ANOVA) with Boferroni's test as *post hoc* was performed to analyse differences between serum ferritin values and the rostro-caudal distribution of the infarction. ANOVA with Bonferroni's test as *post hoc* (or Kruskal–Wallis with Dunn's test as *post hoc* when data were not normally distributed) was used to analyse differences between groups. Mann–Whitney test was used for comparison of two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of iron overload and ischemic stroke on serum ferritin levels

Iron overload produced a significant increase on the basal levels of serum ferritin in all the groups studied (100 ± 11% in control group *vs.* 380 ± 20% in iron overload group, $p < 0.05$; basal level