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Extracellular Ferritin Contributes to Neuronal Injury in an In Vitro Model of Ischemic Stroke

--Manuscript Draft--

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Abstract:	Previous clinical and experimental studies have shown that neurological decline and poor functional outcome after acute ischemic stroke in humans are associated with high ferritin levels in serum and cerebrospinal fluid (CSF) within 24 hours of ischemic stroke onset. The aim of the present study was to find out if and how high extracellular ferritin concentrations can increase the excitotoxicity effect in a neuronal cortical culture model of stroke. Extracellular ferritin (100 ng/ml) significantly increased the excitotoxic effect caused by excessive exogenous glutamate (50 μM and 100 μM) by leading to an increase in lipid peroxidation, a reduction in mitochondrial membrane potential and a decrease in neuron viability. Extracellular apoferritin (100 ng/ml), the iron-free form of the protein, does not increase the excitotoxicity of glutamate, which proves that iron was responsible for the neurotoxic effect of the exogenous ferritin. We present evidence that extracellular ferritin iron exacerbate the neurotoxic effect induced by glutamate excitotoxicity and that the effect of ferritin iron is dependent of glutamate excitotoxicity. Our results support the idea that body iron overload is involved in the severity of the brain damage caused by stroke and reveal the need to control systemic iron homeostasis.
Response to Reviewers:	The authors would like to thank the editor and two anonymous referees who kindly reviewed the earlier version of this manuscript and provided valuable suggestions and comments. We present our response to the comments and suggestions from the reviewer #2 Reviewer #2: Figure 2A shows a statistically significant dose-dependant effect of glutamate. But in

figure 2B, the authors show no statistically significance of any effect, so they can not conclude anything from those experiments. Of course, this might be just due to a mistake (missing signs). But anyway, a look at the plot might support the claim that the effect is greater when adding ferritin, but it also may suggest that apoferritin is "protective", a result that the authors should equally discuss.

Answer:

We apologize for the missing signs on Figure 2B, now added. According to your remarks, the results have been analyzed with the post-hoc Tukey's multiple comparisons test and the significant differences vs the group with apoferritin are shown.

Until now, research on ferritin has focused on the function of maintaining iron homeostasis, storing and releasing iron according to the needs of the cell, tissues and organism, and on protection against oxidative iron damage. Other iron-independent functions of ferritin are poorly understood. Recently published evidence (references 16, 18) shows that ferritin is a multifunctional protein and therefore suggest that new functions of ferritin in non-iron dependent processes could be described in the near future.

We have added these comments in the results section. New references have also been incorporated into the discussion. The changes are shown in the revised version of the manuscript as underlined text.

Re: JPBY-D-19-00193_R2

Dear Editor,

We are pleased to submit the revised manuscript **“Extracellular Ferritin Contributes to Neuronal Injury in an In Vitro Model of Ischemic Stroke”** for consideration in Journal of Physiology and Biochemistry.

The authors would like to thank the editor and two anonymous referees who kindly reviewed the earlier version of this manuscript and provided valuable suggestions and comments.

The reviewer # 2 pointed out that in Figure 2B, the results could support the claim that the effect of glutamate is greater when ferritin is added, but could also suggest that apoferritin is "protective", a result that the reviewer asked us to discuss.

Until now, research on ferritin has focused on the function of maintaining iron homeostasis, storing and releasing iron according to the needs of the cell, tissues and organism, and on protection against oxidative iron damage. Other iron-independent functions of ferritin are poorly understood. Recently published evidence (references 16, 18) shows that ferritin is a multifunctional protein and therefore suggest that new functions of ferritin in non-iron dependent processes could be described in the near future.

We have added these comments in the results section, and they have been incorporated into the discussion. The changes suggested by the reviewer # 2 are shown in the revised version of the manuscript as underlined text. A detailed response is also shown in the “Response to Reviewers” file.

We hope the revised manuscript is now suitable for publication.

Sincerely yours,

Teresa Carbonell and Ramon Rama

Title Page

Extracellular Ferritin Contributes to Neuronal Injury in an *In Vitro* Model of Ischemic Stroke

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Short Title: Extracellular Ferritin Increases Excitotoxicity

Keywords: Apoferritin; Excitotoxicity; Ferritin; Glutamate; Iron; Stroke

Abstract

Previous clinical and experimental studies have shown that neurological decline and poor functional outcome after acute ischemic stroke in humans are associated with high ferritin levels in serum and cerebrospinal fluid (CSF) within 24 hours of ischemic stroke onset. The aim of the present study was to find out if and how high extracellular ferritin concentrations can increase the excitotoxicity effect in a neuronal cortical culture model of stroke.

Extracellular ferritin (100 ng/ml) significantly increased the excitotoxic effect caused by excessive exogenous glutamate (50 μ M and 100 μ M) by leading to an increase in lipid peroxidation, a reduction in mitochondrial membrane potential and a decrease in neuron viability. Extracellular apoferritin (100 ng/ml), the iron-free form of the protein, does not increase the excitotoxicity of glutamate, which proves that iron was responsible for the neurotoxic effect of the exogenous ferritin.

We present evidence that extracellular ferritin iron exacerbate the neurotoxic effect induced by glutamate excitotoxicity and that the effect of ferritin iron is dependent of glutamate excitotoxicity. Our results support the idea that body iron overload is involved in the severity of the brain damage caused by stroke and reveal the need to control systemic iron homeostasis.

Key Points

Extracellular ferritin increases glutamate excitotoxicity in neuronal cultures.

Patients at risk of cerebral stroke should take care of systemic iron level.

Introduction

Stroke is the second most common cause of death and the leading cause of acquired disability in adults' worldwide [13]. There are two major types of stroke: ischemic and hemorrhagic. Ischemic strokes caused by the interruption of the blood flow (ischemia) due to thrombosis or embolism account for the majority of strokes. Less frequently (<15%), strokes are caused by hemorrhage or cardiac arrest. The pathophysiological mechanisms involved in cerebral ischemia are multifactorial and complex [6, 21, 25]. Among the mechanisms involved in stroke, excitotoxicity is considered a primary intracellular event that induces neuronal death [21]. Neuronal excitotoxicity generally refers to the injury and death of neurons that arise from prolonged exposure to glutamate, as occurs in a stroke [21]. Overactivation of glutamate receptors impairs cellular calcium homeostasis, and activates oxidative stress and programmed cell death [9].

Deregulation of brain iron homeostasis may act as a mediator of neuronal damage in important neurodegenerative diseases including stroke [4, 20]. Ferritin is an iron binding protein whose main function is the sequestration of excess iron to prevent the presence of iron in free form and the increase in the production of reactive oxygen species (ROS)[7]. Despite this, ferritin also has the ability to release iron from its core and, under certain conditions, can act as a major source of free iron [22, 23].

In previous clinical study, we found that high plasma and CSF ferritin concentrations within the first 24 h from the onset of ischemic stroke were associated with early neurologic deterioration [11]. Subsequent experimental studies in rats fed with an iron-rich diet, which lead to increase the body iron stores and the levels of serum ferritin, confirm that increased body iron stores contribute to stroke progression by

enhancing the cytotoxic mechanisms in cerebral ischemia [8]. In this study, the progression of stroke was not associated with a greater amount of iron in brain tissue. These findings suggest that ferritin iron, involved in the acceleration of neurological deterioration in acute cerebral infarction, would be of systemic origin. Research on the role of iron in ischemic diseases has focused on the relationship between raised ferritin stores, atherosclerosis and risk of ischemic stroke [1, 28].

The aim of the present study is to analyze whether extracellular ferritin-iron can contribute to increasing the excitotoxic effect that occurs in strokes. We evaluated the effects of extracellular ferritin on lipid peroxidation, mitochondrial dysfunction and neuronal viability by exposing primary neuronal cortical cultures to excessive exogenous glutamate to model ischemic stroke.

Methods

Cell culture

A primary culture of cortical neurons was used in this study. Cortical neurons were chosen because the cortical area is one of the areas affected by a stroke. Moreover, the neurons in this area contain a large number of glutamate receptors, so it is prone to excitotoxicity. Primary neurons were isolated from Wistar rats (Harlan, Spain) on embryonic day 17. All experimental procedures were carried out following the guidelines of the Committee for the Care of Research Animals of the University of Barcelona, in accordance with the directive of the Council of the European Community (2010/63 y 86/609/EEC) on animal experimentation. The experimental protocol was approved by the local University Committee (CEE-UB, Comitè Ètic d'Experimentació Animal de la Universitat de

Barcelona) and by the Catalan Government (Generalitat de Catalunya, Departament de Territori i Sostenibilitat) with the approval number #9431.

The cells were cultured for 9 days in growth medium for Neurobasal[®] neurons containing Penicillin/Streptomycin, enriched with GlutaMAX[®] and B27[®] (Antioxidants) and supplemented with 1/5 of Neurobasal Medium plus GlutaMAX[®] and B27[®] previously conditioned in astrocyte cultures, in culture incubator at 37°C in a humidified atmosphere containing 5% CO₂/95% air (pH 7.2). To halt the proliferation of non-neuronal cells, 3 days after plating, half the culture medium was replaced with medium containing cytosine arabinoside (AraC) (final concentration: 10 µM). On day 5 after plating, half of the medium was replaced with Neurobasal[®] medium containing 2 mM glutamine and 2% B27 (AO+), and the neurons were analyzed at day 9. Approximately 98% of the cells were Map2-positive.

On the twelve day in culture, the medium was replaced with conditioned Neurobasal medium containing 2 mM glutamine and 2% B-27.

To test the neurotoxic effect of glutamate, doses of 50 and 100 µM of glutamate were applied and cells incubated for 1 hour at 37°C. After this incubation, the medium containing glutamate was removed and replaced by Neurobasal medium. Cells were further incubated for 3, 24 and 48 hours at 37°C. Ferritin (100 ng / ml) and apoferritin (100 ng / ml), were added when indicated, and maintained for 3, 24 and 48 hours at 37°C in a CO₂ incubator, as is summarized in Fig. 1. Neuron viability was analyzed over time of incubation.

Assay of glutamate and ferritin neurotoxicity

Cytotoxicity was quantified by measuring the activity of the lactate dehydrogenase (LDH, Sigma St Louis, MO, USA) released, using the method described by Dringen et al [14]. The data presented are the means $\pm$ S.E.M. for five independent experiments.

Lipid peroxidation

Lipid peroxidation was evaluated by the formation of thiobarbituric acid reactive substances (TBARS), in accordance with the method described by Uchiyama and Mihara [26], with minor modifications substances [10]. The cells were incubated in Neurobasal medium with glutamate and ferritin at the indicated concentrations. At 48 hours, cells were collected and sonicated over ice. An aliquot of the cell lysate was added to the reaction mixture and heated at 95°C for 60 min. The formation of TBARS was spectrophotometrically measured at 540 nm and the protein concentration of cell lysates was determined using a protein assay (Bio-Rad, Hercules, CA, USA). Lipid peroxidation was calculated as nmols of TBARS per milligram of protein and converted to relative units of the control. Data represent mean $\pm$ SEM of three wells derived from five independently prepared neuronal cultures.

Mitochondrial permeability transition potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was performed using 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzamidozyl-carboncyanine (JC-1, Invitrogen, Carlsbad, CA, USA). JC-1 is a cationic dye whose mitochondrial uptake is directly related to the magnitude of the $\Delta\Psi_m$. In healthy cells, the dye stains the mitochondria bright red. Cationic fluorescent probes (JC-1) are an invaluable tool

for monitoring mitochondrial function in intact cells during excitotoxic injury [27]. The negative charge established by the intact $\Delta\Psi_m$ allows the lipophilic dye to enter the mitochondrial matrix, where it accumulates. In apoptotic cells, the $\Delta\Psi_m$ collapses and the JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytoplasm in a green fluorescent monomeric form. The greater the mitochondrial uptake, the greater the concentration of JC-1 aggregate forms that have a red fluorescent emission signal, as opposed to the JC-1 monomer that emits green fluorescence [24]. Several studies have demonstrated a linear relationship between the red and green ratio of JC-1 fluorescence and membrane potential over a physiological range.

Briefly, 0.5 ml of cell suspension were transferred into a sterile centrifuge tube and centrifuged at 400 *g* for 5 minutes at room temperature. The supernatant was removed and the cells were resuspended in JC-1 reagent. After 15 minutes of incubation at 37°C in a 5% CO₂ incubator, the pellet was removed, resuspended in 2 ml assay buffer, centrifuged and the supernatant removed. The remaining pellet was resuspended in 0.3 ml of assay buffer and immediately observed in a fluorescence microscope using a dual band-pass filter to detect fluorescein. In live non-apoptotic cells, mitochondria appear red following aggregation of the JC-1 reagent. Loss of mitochondrial membrane potential is indicated by a decrease in the 590/530 fluorescence intensity ratio. The data presented are means $\pm$ S.E.M for five independent experiments.

Statistics

Results were expressed as mean $\pm$ S.E.M. Data were evaluated by analysis of variance (ANOVA) followed by a post-hoc Tukey's multiple comparisons test

using GraphPad InStat statistical software. Differences were considered significant when $P \leq 0.05$.

Results

LDH release due to glutamate-induced cell death increased with extracellular ferritin

Incubation with glutamate (50 μ M and 100 μ M) caused the release of LDH in a dose- and time-dependent manner (Fig. 2A). As shown in Fig. 2B, the neurotoxic effect of glutamate (50 μ M) is greater when ferritin (100 ng/ml) but not apoferritin (100 ng / ml) are present in the extracellular medium, suggesting the neurotoxic effect of ferritin is due to iron and further showing that apoferritin inhibits the neurotoxic effect of glutamate. In previous studies, (data not shown) we found that neuron cells incubated with 100 ng ferritin-iron/ml for 3 h, 24 h or 48 h released LDH at similar rates to control neurons indicating that the neurotoxic effects described do not derive simply from extracellular ferritin-iron.

Extracellular ferritin increased the lipid peroxidation and the loss of mitochondrial membrane potential caused by excitotoxicity

Excitotoxicity is known to cause an increase in the formation of ROS that can damage the structural molecules of membrane cells. Here we assessed the formation of lipid peroxidation. The results show that exogenous glutamate significantly increase lipid peroxidation. When extracellular ferritin was added to the glutamate group, the oxidation of lipids was significantly enhanced (Fig. 3A). We also assessed the mitochondrial membrane potential ($\Delta\Psi_m$) as an indicator of possible mitochondrial function impairment using the membrane-permeant JC-1

dye. The results show that glutamate (50 μ M) in the extracellular medium leads to a reduction in the red/green ratio (Fig. 3B), which indicates a loss of $\Delta\Psi_m$. This reduction in $\Delta\Psi_m$ was greater in the presence of extracellular ferritin (100 ng/ml). The loss of $\Delta\Psi_m$ is an indicator of mitochondrial dysfunction and a hallmark of apoptosis [17].

Discussion

In this study, we provide the first evidence that neuronal damage caused by the presence of excess extracellular glutamate (excitotoxicity) in cultured cortical neurons is aggravated by the presence of ferritin in the extracellular medium. We provide evidence that this neurotoxic effect of ferritin is attributable to the presence of stored iron in the ferritin molecule, since apoferritin, the iron-free form of the protein, does not increase the excitotoxicity of glutamate. It is striking that apoferritin not only does not contribute to increasing the excitotoxic effect of glutamate but also eliminates it, showing a protective action. After more than 80 years of research, ferritin is known for its role in iron homeostasis as a molecule that stores iron in a safe and bioavailable form and in protecting against the oxidative damage of iron [2]. Recent data shows that ferritin is a multifunctional protein [16] and we can expect to find new functions of ferritin in non-iron dependent processes in the near future [3, 18].

Excitotoxicity is considered a primary intracellular event that induces neuronal death in stroke [21]. It is well known that excitotoxic mechanisms primarily cause free radical generation and mitochondrial dysfunction [19]. We found that excessive amounts of extracellular glutamate caused an increase in lipid peroxidation levels, mitochondrial membrane potential loss and a reduction in neuronal viability. Our results are consistent with those obtained in other studies on the neurotoxic effect of excessive

glutamate levels in cortical neuronal culture [27]. Both excessive free radicals and mitochondrial dysfunction have been described as events involved in the activation of the apoptotic cascade that occurs in the ischemic penumbra. [5].

Several lines of research indicate that oxidative stress is a main mediator of neurological injury after cerebral ischemia [9]. In particular, clinical [11], experimental [6] and epidemiological [1] studies show that poor outcomes after stroke are associated with increased ferritin content in plasma and CSF. Iron release from ferritin is facilitated by the presence of reactive oxygen and nitrogen species [22, 23] all of which increased in the ischemic region during a stroke [1, 7].

Initially, it was thought that increased brain injury associated with iron content after stroke was due to increased iron levels in the brain because of alterations in brain iron homeostasis [12]. CSF ferritin levels were interpreted to indicate the iron content of brain tissue and, similarly, plasma ferritin levels were thought to be directly proportional to the body's iron stores and could be used to assess systemic iron overload in the absence of inflammation, cancer and infectious diseases [29]. However, experimental studies using rats fed an iron-enriched diet to induce systemic iron overload confirm that a worsened outcome after stroke was associated with the ferritin level in serum and not with iron in the brain tissue [8]. Although iron accumulation in the brain has been associated with many neurodegenerative disorders [30], it should be noted that the increased neurotoxic effect in stroke is due to an iron overload in systemic tissue [8].

These results raise the question of how an iron overload situation in the systemic tissue can cause higher levels of brain damage in stroke. When a stroke occurs, the integrity of the blood-brain barrier (BBB) is lost in the infarct area [15]. This facilitates the entrance to the ischemic brain area of the accumulated ferritin in the microvasculature and in the plasma. Once ferritin is in the ischemic zone, factors caused by excessive

glutamate in the extracellular medium, such as ROS and acidic pH, can release iron from ferritin [15]. This free iron may increase oxidative damage in the area of the ischemic penumbra, contributing to the neurological decline and, finally, causing a greater neurological injury in stroke than would occur without alterations in iron metabolism.

Possible mechanisms of ferritin action were summarized in Fig. 4. The results suggest that blood-barrier-brain disruption in the ischemic penumbra enables plasma ferritin to enter the interstitial fluid, and factors caused by excessive glutamate in the extracellular medium, such as superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$) and acidic pH, can release iron from ferritin. Under these conditions, ferritin can release and increase the amount of free iron. This free iron can increase free radical production in the ischemic penumbra area, which causes greater oxidative stress, contributes to neurological decline in ischemic stroke, and finally, causes greater neurological injury in stroke than in normal body iron metabolism.

Conclusions

We have demonstrated that extracellular ferritin increases the excitotoxicity of glutamate in a primary culture of cortical neurons. Our results support that body iron overload is involved in the severity of brain damage caused by stroke and point to take care of systemic iron levels in patients at risk of cerebral stroke.

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Compliance with Ethical Standards

Authors Contributions: The authors declare that all data were generated in-house and

that no paper mill was used.

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Informed consent: All experimental procedures (approval number #9431) were carried out following the guidelines of the Committee for the Care of Research Animals of the University of Barcelona, in accordance with European directive (2010/63 and 86/609/EEC).

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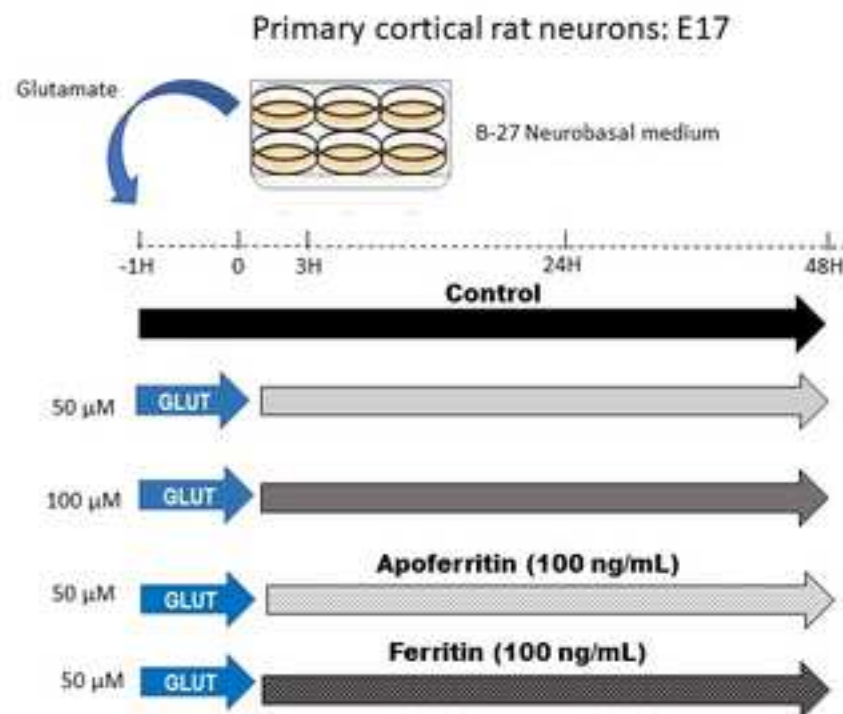
Figure 1: Schematic representation of experimental design. For more details please refer to the material and methods section.

Figure 2: Effect of iron and glutamate on the viability of primary culture rat neurons.

A. Cells were incubated with glutamate at the indicated concentrations for 1h. After this incubation, the medium containing glutamate was removed and replaced by Neurobasal medium **B.** Cells were incubated with glutamate (1h), After this incubation, the medium containing glutamate was removed and replaced by Neurobasal medium. Ferritin (100 ng / ml) and apoferritin (100 ng / ml), were added when indicated, and maintained for 3, 24 and 48 hours at 37°C in a CO₂ incubator. Cell viability was determined by lactate dehydrogenase (LDH) assay at 3, 24 and 48 hours. The data represent mean ± SEM of three wells derived from five independently prepared neuronal cultures. Significant differences from control values: ** $P < 0.01$ and *** $P < 0.001$. Significant differences from Glutamate 50 µM values: + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$. Significant differences from Glutamate 50 µM + Apoferritin values: ### $P < 0.001$.

Figure 3: Extracellular ferritin increases lipid peroxidation and the loss of mitochondrial membrane potential caused by excitotoxicity. **A.** Lipid peroxidation was determined by thiobarbituric acid reaction substances (TBARS) assay at 48 hours. **B.** Mitochondrial membrane depolarization was determined by adding JC-1 at 48 hours. Data represent mean ± SEM of three wells derived from five independently prepared neuronal cultures. Significant differences from control values: *** $P < 0.001$. Significant differences from Glutamate 50 µM values: + $P < 0.05$, +++ $P < 0.001$

Figure 4: Proposed model for the role of body ferritin stores in stroke. When stroke occurs, a disruption of the blood-brain barrier (BBB) can allow serum ferritin to enter the ischemic zone, where reactive oxygen species (ROS) produced by excitotoxicity can release iron from ferritin, exacerbating the cascade of events that lead to neuronal death in the penumbra zone. Purple arrows: Mechanisms activated by Stroke. Arrows in blue: expanded effects due to ferritin in the interstitial fluid.



- Cytotoxicity: LDH
- Lipid peroxidation
- MPT Assay

