# Iron Overload Exacerbates the Risk of Hemorrhagic Transformation After tPA (Tissue-Type Plasminogen Activator) Administration in Thromboembolic Stroke Mice

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***Background and Purpose***—Recanalization with tPA (tissue-type plasminogen activator) is the only pharmacological therapy available for patients with ischemic stroke. However, the percentage of patients who may receive this therapy is limited by the risk of hemorrhagic transformation (HT)—the main complication of ischemic stroke. Our aim is to establish whether iron overload affects HT risk, to identify mechanisms that could help to select patients and to prevent this devastating complication.

***Methods***—Mice fed with control or high-iron diet were subjected to thromboembolic stroke, with or without tPA therapy at different times after occlusion. Blood samples were collected for determination of malondialdehyde, matrix metalloproteinases, and fibronectin. Brain samples were collected 24 hours after occlusion to determine brain infarct and edema size, hemorrhage extension, IgG extravasation, and inflammatory and oxidative markers (neutrophil infiltration, 4-hydroxynonenal, and matrix metalloproteinase-9 staining).

***Results***—Despite an increased rate of recanalization, iron-overload mice showed less neuroprotection after tPA administration. Importantly, iron overload exacerbated the risk of HT after early tPA administration, accelerated ischemia-induced serum matrix metalloproteinase-9 increase, and enhanced basal serum lipid peroxidation. High iron increased brain lipid peroxidation at most times and neutrophil infiltration at the latest time studied.

***Conclusions***—Our data showing that iron overload increases the death of the compromised tissues, accelerates the time of tPA-induced reperfusion, and exacerbates the risk of HT may have relevant clinical implications for a safer thrombolysis. Patients with stroke with iron overload might be at high risk of HT after fibrinolysis, and, therefore, clinical studies must be performed to confirm our results.

***Visual Overview***—An online visual overview is available for this article. **(*Stroke*. 2018;49:2163-2172. DOI: 10.1161/STROKEAHA.118.021540.)**

**Key Words:** blood-brain barrier ■ fibrinolysis ■ hemorrhage ■ humans ■ iron



Stroke is a leading cause not only of death but also of long-term disability and dementia in developed countries. Recanalization with tPA (tissue-type plasminogen activator) is the only pharmacological therapy available for patients with ischemic stroke.[1,2] However, to limit hemorrhagic transformation (HT)—the main complication of intravenous thrombolysis, this drug is used only under restrictive conditions.[3] The percentage of patients with stroke who receive intravenous tPA remains between 5% and 10%, and successful recanalization is achieved approximately in <40% of the treated patients—another factor that limits the benefits of the therapy. Endovascular thrombectomy with new-generation devices improves clinical outcome in patients with large anterior cerebral arterial occlusions who have contraindications or

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have failed intravenous tPA revascularization.[4] Because of the mechanic retrieval of the clot by thrombectomy, effective recanalization is achieved in >80% of the patients, extending the beneficial effect of the thrombolysis. The meta-analysis of clinical trials has shown similar proportion of symptomatic intracranial hemorrhage in the thrombectomy and control groups, most of them treated with intravenous tPA,[4] so intracerebral bleeding remains the main complication of revascularization therapies.

During an ischemic episode, the blood-brain barrier (BBB) is damaged and undergoes structural alterations that contribute to brain injury. Several studies have focused on elucidating the mechanisms by which the vasculature is altered during stroke. Among others, oxidative stress, activation of proteases, and infiltration of circulating white cells seem to play an important role in short-term BBB damage and HT, in particular after tPA-induced recanalization.[5]

Because of its double redox nature, iron is an essential element that catalyzes processes such as mitochondrial respiration, oxygen transport in blood,[6,7] and neurotransmitter synthesis in the brain. Paradoxically, this bivalence also makes iron potentially toxic because by undergoing Haber-Weiss reactions, it can generate reactive oxygen species, which damage cellular substrates. Iron toxicity is generally avoided by highly regulated homeostatic mechanisms that keep iron under its less reactive, ferric form ($Fe^{3+}$), and chelated by specific binding proteins. These controlling mechanisms are especially important in brain, which maintains iron levels constant by the regulation of its uptake through the BBB. Even in disorders causing systemic iron accumulation, such as hemochromatosis, brain iron levels remain unaltered.[8] However, it is widely accepted that iron homeostasis is impaired early after cerebral ischemia, being one of the first mediators of damage in the ischemic cascade.[9] Importantly, iron overload in patients has been described to be associated to poor outcome after stroke.[10–13] Experimental studies confirm this detrimental effect[14,15] and suggest that iron overload could accelerate the damage of the compromised tissue during acute ischemia.[16] BBB could be also more vulnerable in patients with iron overload—a fact supported by some clinical studies that found high levels of iron associated with a higher rate of HT after intravenous tPA therapy.[10,17]

The aim of this study is to establish experimental evidence of iron overload on the HT risk, to identify the mechanisms involved in this effect and to find some related prognostic markers that could help to avoid this feared complication.

## Methods

Details of materials and experimental procedures are available in the online-only Data Supplement. The data that support the findings of this study are available from the corresponding author on reasonable request.

### Animals and Diet

Male Swiss mice (Harlan Laboratories, Barcelona, Spain) were used for this study, following the guidelines of the Animal Welfare Committee of the Universidad Complutense (EU directives 86/609/CEE and 2003/65/CE). Animals were fed with 1 of 2 different diets: a standard rodent maintenance diet for the control group or a diet supplemented with an additional 2.5% of carbonyl iron for the iron-overload group.[16] After a feeding period of at least 9 weeks, mice weighing 35 to 45 g were used for the experiments. We have previously shown that this iron overload induces an almost 4-fold increase in serum ferritin (100±11% in control group versus 380±20% in iron-overload group; $P<0.05$; basal level in control group was 170±19 ng/mL).[16] Further details are provided in the online-only Data Supplement.

### Experimental Groups

Animals were randomly subdivided into 6 experimental groups: middle cerebral artery occlusion (MCAO), in which the middle cerebral artery was permanently occluded using the in situ thromboembolic model; tPA 20 minutes, tPA 1 hour, and tPA 3 hours, in which artery recanalization was achieved administering tPA at 20 minutes, 1 or 3 hours, respectively, after MCAO; and Sham, group with the middle cerebral artery exposed but not occluded and treated with saline or tPA. The in situ thromboembolic model was performed as described previously.[18] Further details are provided in the online-only Data Supplement.

### Blood Sample Collection and Determination

Blood samples were collected from the femoral vein the day before (t=0) and 3, 6, and 24 hours after surgery to obtain serum. Malondialdehyde (MDA), cellular fibronectin, and matrix metalloproteinase (MMP)-2 and 9 were measured by ELISA kits. Details for determination of immunohistochemical studies, IgG extravasation, 4-hydroxynonenal ($HNE^+$) cells, MMP-9 expression, neutrophil quantification, and serum zymography are provided in the online-only Data Supplement.

### Infarct Outcome Determination and Quantification of Hemorrhages

Infarct outcome was assessed by Nissl staining 24 hours after MCAO. Hemorrhages were studied by staining it with diaminobenzidine.[18] Hemorrhages were classified by their extension in 5 groups following previously established criteria. Further details are provided in the online-only Data Supplement.

### Statistics

Results were expressed as mean±SEM. Normality was assessed by Kolmogorov-Smirnov, performing parametric or nonparametric test according to the result. For all comparisons, $P<0.05$ was considered significant. Further details are provided in the online-only Data Supplement.

## Results

### Iron Overload Increases the Rate of Successful Recanalization After Thrombolytic Treatment

To study cerebral blood flow recovery, recanalization rates by spontaneous clot autolysis and after tPA infusion were measured (Figure 1A through 1C). A low percentage of animals experienced spontaneous recanalization after thrombin injection (2% in control and 9% in iron overloaded animals; Figure 1B) and were excluded of further analysis. Mice with normal diet (control group) displayed significantly lower recanalization rates than iron overloaded animals when administering the tPA 20 minutes, 1 hour, and 3 hours after ischemia (Figure 1). As a whole, all the animals with iron overload had a higher rate of reperfusion than controls ($P<0.05$; Figure 1A and 1B). Among the animals that reperfused, the time point at which laser Doppler flowmetry overtook 50% of the original value after tPA treatment (reperfusion time) was longer in control groups than in the iron overloaded groups, being significant in tPA 1-hour and tPA 3-hour groups (Figure 1C). Again, when evaluating all the groups as a whole, control mice

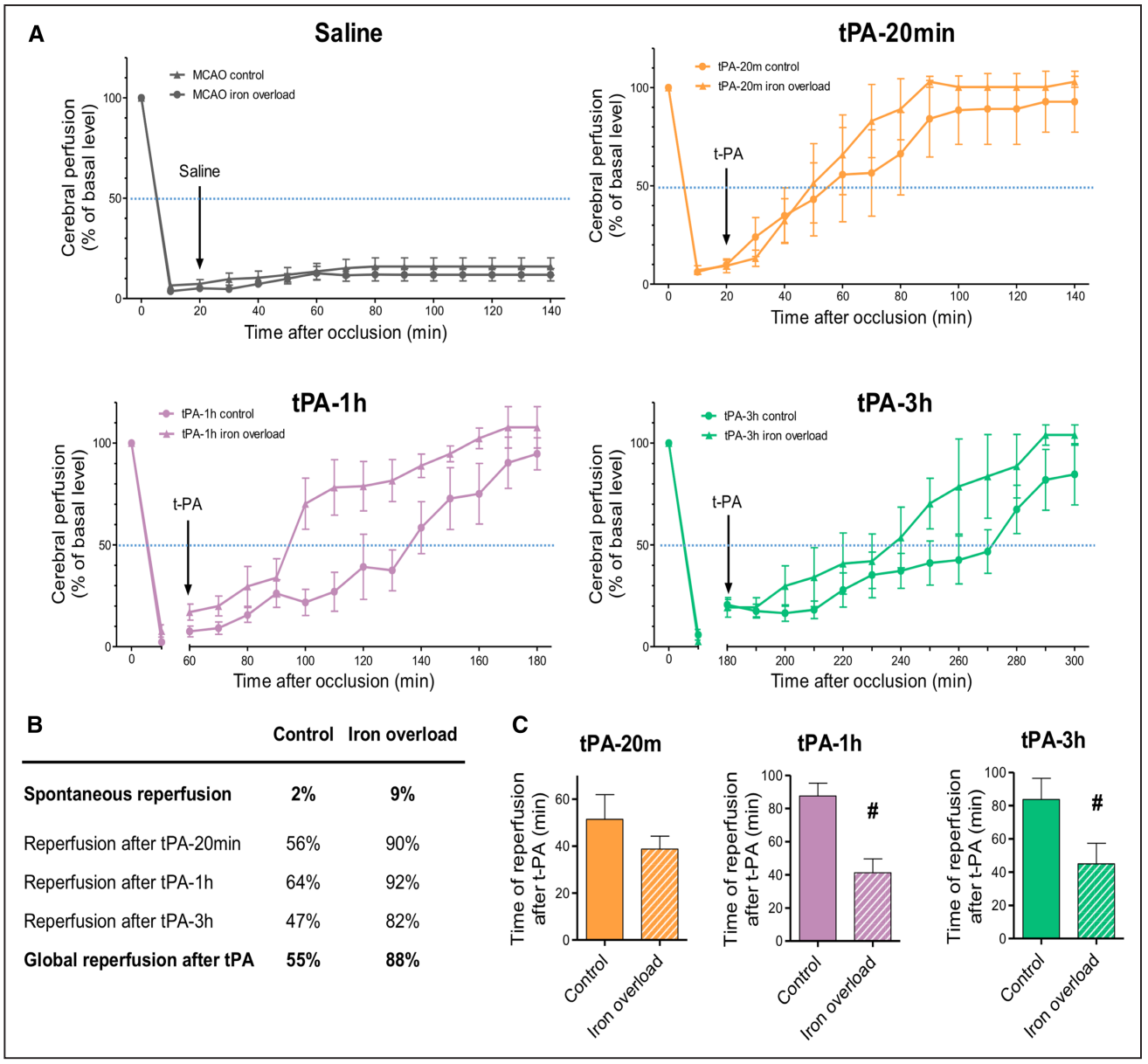


**Figure 1.** Study of the cerebral perfusion by laser Doppler flowmetry. **A**, Recordings of perfusion in saline and tPA (tissue-type plasminogen activator)-treated groups (20 minutes, 1 hour, and 3 hours, respectively) showing a recovery beyond 50% of the initial values. **B** and **C**, Percentage (**B**) and time of recanalization (**C**) after tPA treatment in all groups or in each individual group. Data are expressed as mean±SEM and analyzed by Mann-Whitney *U* test for time of reperfusion and Fisher exact for contingency tables (n=8). #*P*<0.05 vs control diet.

reperfused significantly later than animals with iron overload (*P*<0.05; Figure 1C).

## Iron Overload Reduces the Viable Brain Tissue After Early Recanalization

To assess the effect of iron overload on the size of the lesion, infarct volume was measured 24 hours after permanent ischemia induced by the in situ thromboembolic model. Both control and iron overloaded mice presented similar infarct volumes (Figure 2A). Late recanalization of the artery after MCAO (tPA 3-hour groups) did not modify the size of the lesion either in control or in iron overloaded animals (*P*>0.05 versus MCAO; Figure 2A). As expected, early reperfusion at 20 minutes or 1 hour after the ischemia resulted in smaller infarcts in control animals (*P*<0.05 versus control MCAO; Figure 2A). Interestingly, in iron overloaded mice, early reperfusion by thrombolysis resulted in smaller infarct volumes only when tPA was administered at 20 minutes (*P*<0.05 versus iron-overload MCAO; Figure 2A) but not at 1 hour (*P*>0.05 versus iron-overload MCAO; Figure 2A). Besides, iron overload increased the size of the lesion comparing with control mice in both tPA 20 minutes and tPA 1 hour groups (*P*<0.05 versus control tPA 20 minutes and tPA 1 hour; Figure 2A).

## Iron Overload Exacerbates the Risk of tPA-Induced HT

With the purpose of quantifying the bleeding, a classification of the type of hemorrhage (Figure 2B, top) and a measurement

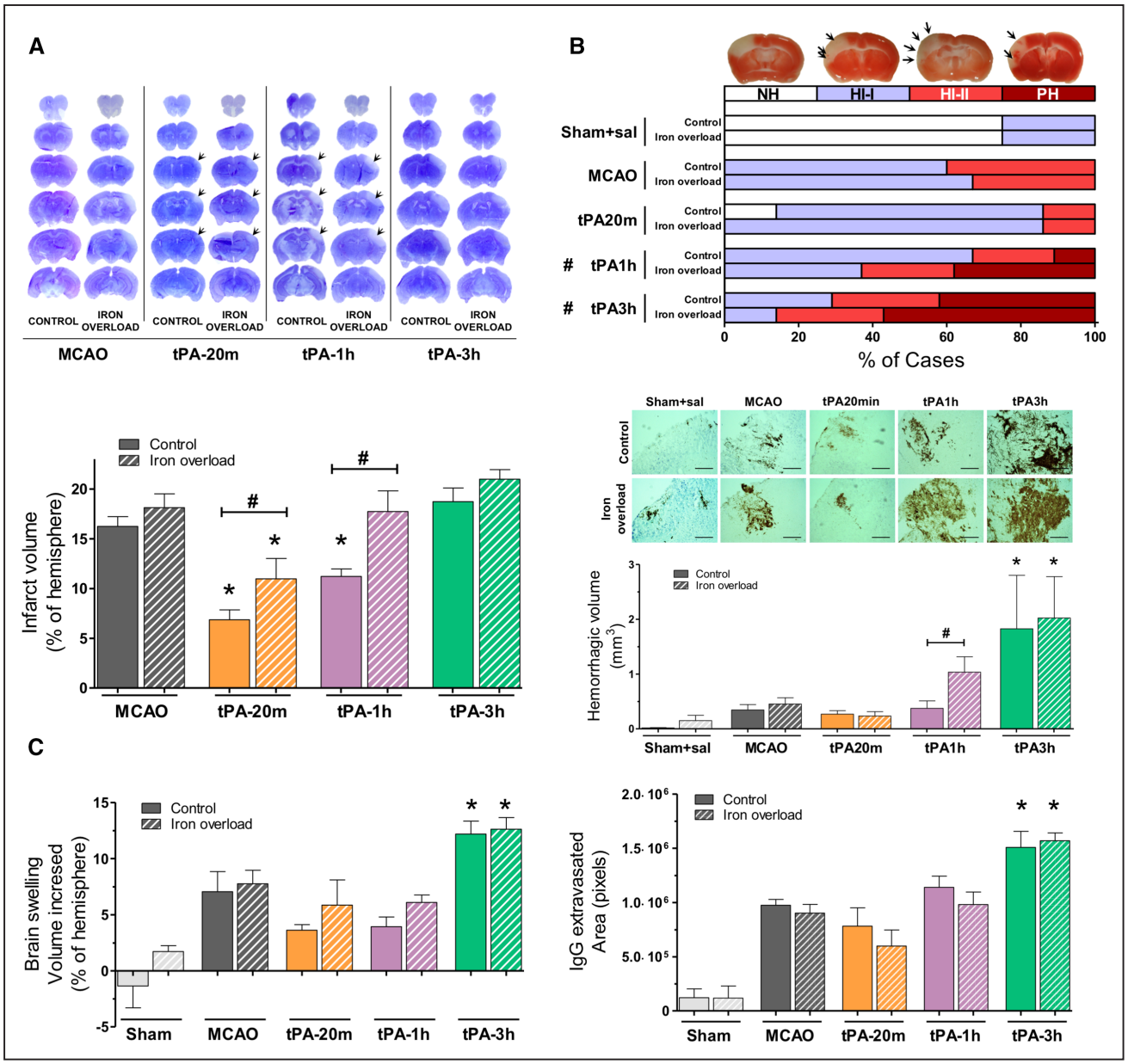


**Figure 2.** Effect of iron overload and recanalization on infarct size, hemorrhagic transformation, and blood-brain barrier (BBB) damage. **A**, Representative slices after Nissl staining showing the area affected by the ischemia (**top**). Infarct volume as a percentage of the hemisphere 24 hours after the surgery (**bottom**). **B**, Macroscopic classification of the hemorrhage by type and extension being the animals classified according to the most severe bleeding in no hemorrhage (NH), hemorrhagic infarction type 1, type 2 (hemorrhagic infarction type I [HI-I] and hemorrhagic infarction type II [HI-II]), and parenchymal hemorrhage (PH), analyzed by $\chi^2$ followed by Fisher exact (**top**). Representative images of the bleeding in each group after red cell staining by endogenous peroxidase detection (scale bar=250 μm) and stereological quantification of extravasated red cells (**bottom**). **C**, BBB damage indicators: brain swelling 24 hours after the infarction (**left**) and area of IgG extravasation (**right**). Data are expressed as mean±SEM and analyzed by 2-way ANOVA followed by Bonferroni test as a post hoc (n=4–8). tPA indicates tissue-type plasminogen activator. *$P$<0.05 vs MCAO, #$P$<0.05 vs control diet.

of its area were performed (Figure 2B, bottom). Twenty-four hours after permanent MCAO, animals showed mild signs of HT and a small bleeding area, which was not affected by high-iron diet ($P$>0.05). Late recanalization by tPA administration, 3 hours after the occlusion, resulted in an increased rate of the most severe hemorrhages, hemorrhagic infarction type II and parenchymal hemorrhage (Figure 2B, top), and in a larger area of extravasated red blood cells in both control and iron overloaded mice (Figure 2B, bottom). In normal-diet mice, early reperfusion at 20 minutes and 1 hour in the control group showed hemorrhage severity and area similar to the MCAO control group ($P$>0.05 versus control MCAO; Figure 2B). Importantly, iron overload resulted in worse hemorrhages and in an increased bleeding area in the tPA 1-hour ($P$<0.05 versus control diet) but not in the tPA 20-minute group ($P$>0.05 versus control; Figure 2B).

As BBB damage indicators, brain swelling and IgG extravasation (Figure 2C; Figure I in the online-only Data Supplement) were measured. Data showed that animals with early reperfusion (tPA 20 minutes and tPA 1 hour) had values similar to those found in mice subjected to permanent ischemia, in both control and iron overloaded groups, despite

the fact that infarct size was smaller. However, independent of iron levels, late reperfusion significantly increased brain swelling and extravasated IgG area in both groups ($P<0.05$ versus MCAO; Figure 2C).

## Iron Overload Increases Some Mediators of HT in the Ischemic Tissue

It has been proposed that oxidative stress, MMPs, especially MMP-9, and infiltrated neutrophils are involved in HT. Therefore, 24 hours after the occlusion, immunoreactivity of these markers was determined in the ischemic tissue.

First, HNE—indicator of lipid peroxidation—was chosen as a marker of oxidative stress (Figure 3). The immunofluorescence analysis showed that lipid peroxidation was distributed in layers and mainly in neuron-like cells (Figure 3C), as well as in some vessels, being the staining especially patent along the boundaries of the lesion. Whereas all the groups showed a similar total number of marked cells, iron overload significantly increased HNE immunoreactivity in MCAO, tPA 20-minute, and tPA 3-hour groups (Figure 3B and 3D).

Second, MMP-9 expression—a widely accepted HT mediator—was studied at 24 hours in the ischemic tissue by immunofluorescence (Figure 4). The results showed that only the tPA 3-hour groups, both control and iron overloaded animals, had a significant increase in the expression of this protein when compared with the Sham groups ($P<0.05$ versus Sham; Figure 4B) or with the respective contralateral hemispheres (data not shown) but not between them ($P>0.05$ control versus iron overloaded; Figure 4B). The expression appeared mainly vascular; in fact, in all groups excluding Sham, some large vessels presented a light MMP-9 staining that was especially intense in tPA 3-hour animals (Figure 4C). To check whether MMP-9 was present in the infiltrated neutrophils or endothelium, a triple immunofluorescence was performed, showing that MMP-9 expression in the tPA 3-hour group colocalized with the marker of brain endothelium glucose transporter-1 but not with neutrophil antibody (NIMP-R14), marker of neutrophils (Figure 4D).

Finally, neutrophil infiltration was also studied to check the importance of these cells in HT. The maps traced showed that the infiltration occurred mainly in the central part of the infarcted tissue and from the cortex surface (Figure 5A through 5C); important accumulations of neutrophils were also observed along the vessel's wall (Figure 5B). The stereological quantification showed that tPA 20 minutes decreased the total number of infiltrated neutrophils compared with MCAO ($P<0.05$ for iron-overload groups; Figure 5D) and that iron overload increased neutrophil infiltration in tPA 3-hour group ($P<0.05$ versus control diet; Figure 5D). Besides specific locations of the neutrophils, vascular- and hemorrhage-associated cells were studied (Figure IIC and IID in the online-only Data Supplement). The results showed that MCAO and tPA 3-hour groups displayed an elevated number of vascular-associated NIMP-R14$^+$ cells, and importantly, a large number of neutrophils were located next to hemorrhages in those groups with larger bleedings and iron overload (Figure IIA and IIB in the online-only Data Supplement).

## Iron Overload Increases Basal Lipid Peroxidation and Accelerates Ischemia-Induced Serum MMP-9 Increase

Serum MMP-9 levels showed differences over time in some groups. Permanent MCAO did not change the MMP-9 basal values, whereas tPA 1 hour after ischemia transiently increased this protein and its active form in serum (at 3 hours, $P<0.05$ versus t=0 and $P<0.05$ versus MCAO; Figure 6A and 6B). Moreover, iron overload accelerated MMP-9 activation because animals treated with tPA 20 minutes showed higher values of active MMP-9, than control animals ($P<0.05$ versus control; Figure 6B). Finally, thrombolytic treatment 3 hours

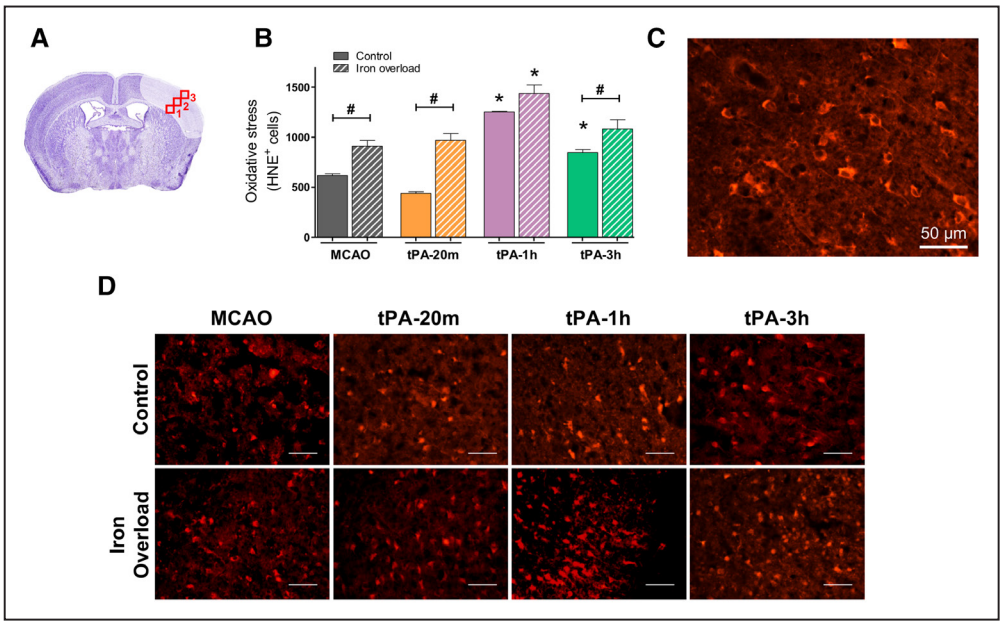


**Figure 3.** Effect of iron overload on the oxidative stress after cerebral ischemia. **A**, Explanatory figure of the image capture for the quantification of HNE+ cells. **B**, Total HNE+ cells counted as a measurement of lipid peroxidation after ischemia. **C**, Image showing the typical staining mostly in neuronal-like cells. **D**, Representative images of each group after HNE staining (scale bar=50 µm). Data are expressed as mean±SEM and analyzed by 2-way ANOVA followed by Bonferroni test as a post hoc (n=6). tPA indicates tissue-type plasminogen activator. *$P<0.05$ vs MCAO, #$P<0.05$ vs control.

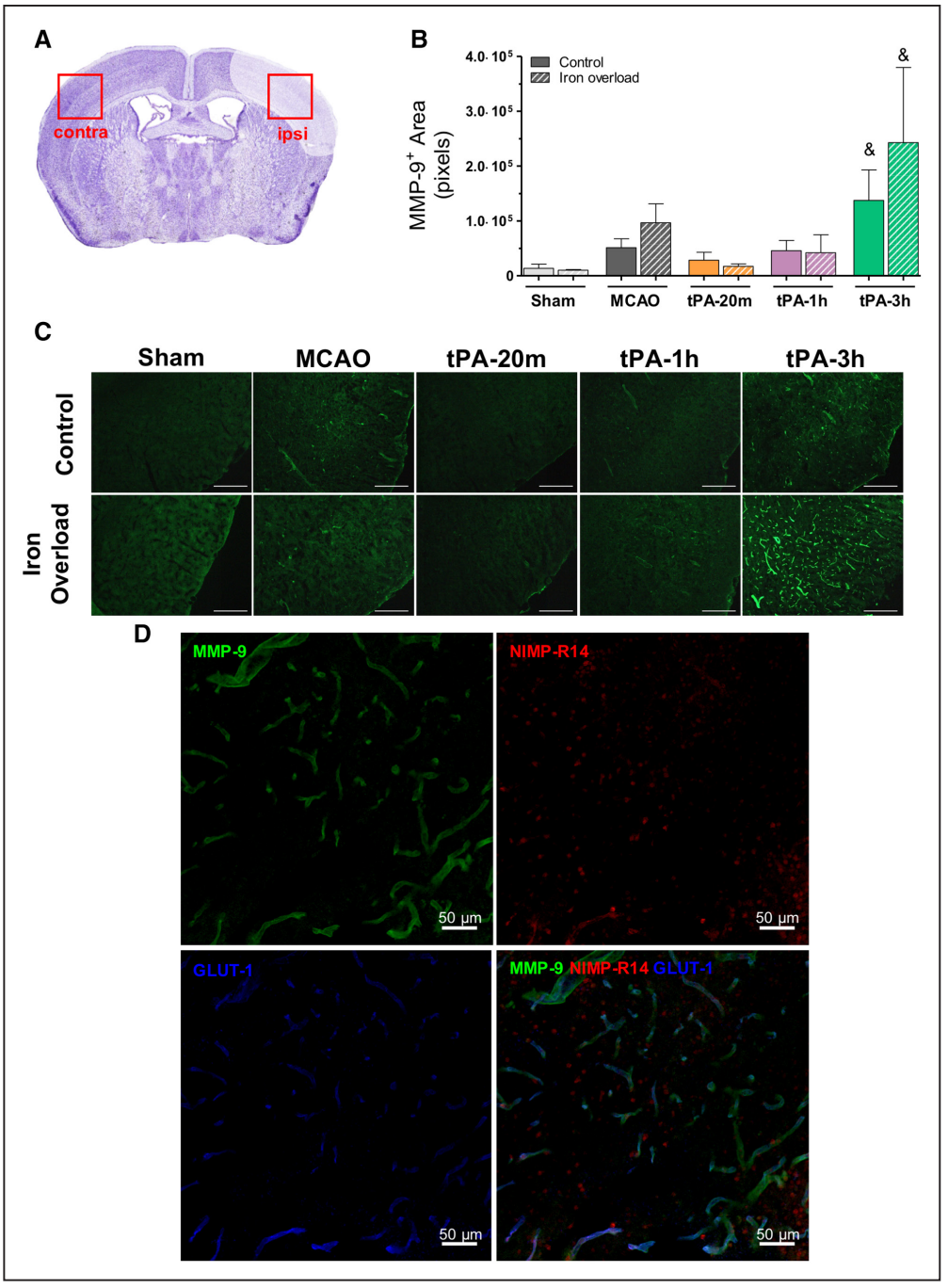


**Figure 4.** Effect of iron overload on the matrix metalloproteinase (MMP)-9 expression in the ischemic brain. **A**, Explanatory diagram of the image capture. **B**, Densitometry of MMP-9 expression in the affected area. **C**, Representative image of the staining in the different groups (scale bar=250 µm). **D**, Triple immunohistochemistry with MMP-9, neutrophil antibody (NIMP-R14), and glucose transporter-1 (GLUT-1) antibodies showing that MMP-9 is mainly expressed in the endothelium of the affected tissue and not in infiltrated neutrophils 24 hours after ischemia. Data are expressed as mean±SEM and analyzed by 2-way ANOVA followed by Bonferroni test as a post hoc (n=6). tPA indicates tissue-type plasminogen activator. &$P$<0.05 vs sham.

after the ischemia produced a delayed and long-lasting elevation of MMP-9 expression and activation in both control and iron overloaded animals (at 24 hours, $P$<0.05 versus t=0 and $P$<0.05 versus MCAO; Figure 6A and 6B). ELISA and zymography were unable to detect a significant increase of serum MMP-9 at any studied time in sham animals treated with this drug (n=8; data not shown).

To analyze the temporal profile of some HT blood markers, serum values of cFn, MDA, MMP-2, and MMP-9 were measured. Levels of activated MMP-9 were also checked by zymography. No significant differences among groups were found for cFn, MDA, or MMP-2, being the values unchanged after ischemia or tPA injection (Figure III in the online-only Data Supplement). In contrast, basal values of MDA were significantly increased in animals with iron overload compared with control mice (20±1 µmol/L versus 16±1 µM; $P$<0.05), indicating a higher basal oxidative stress.

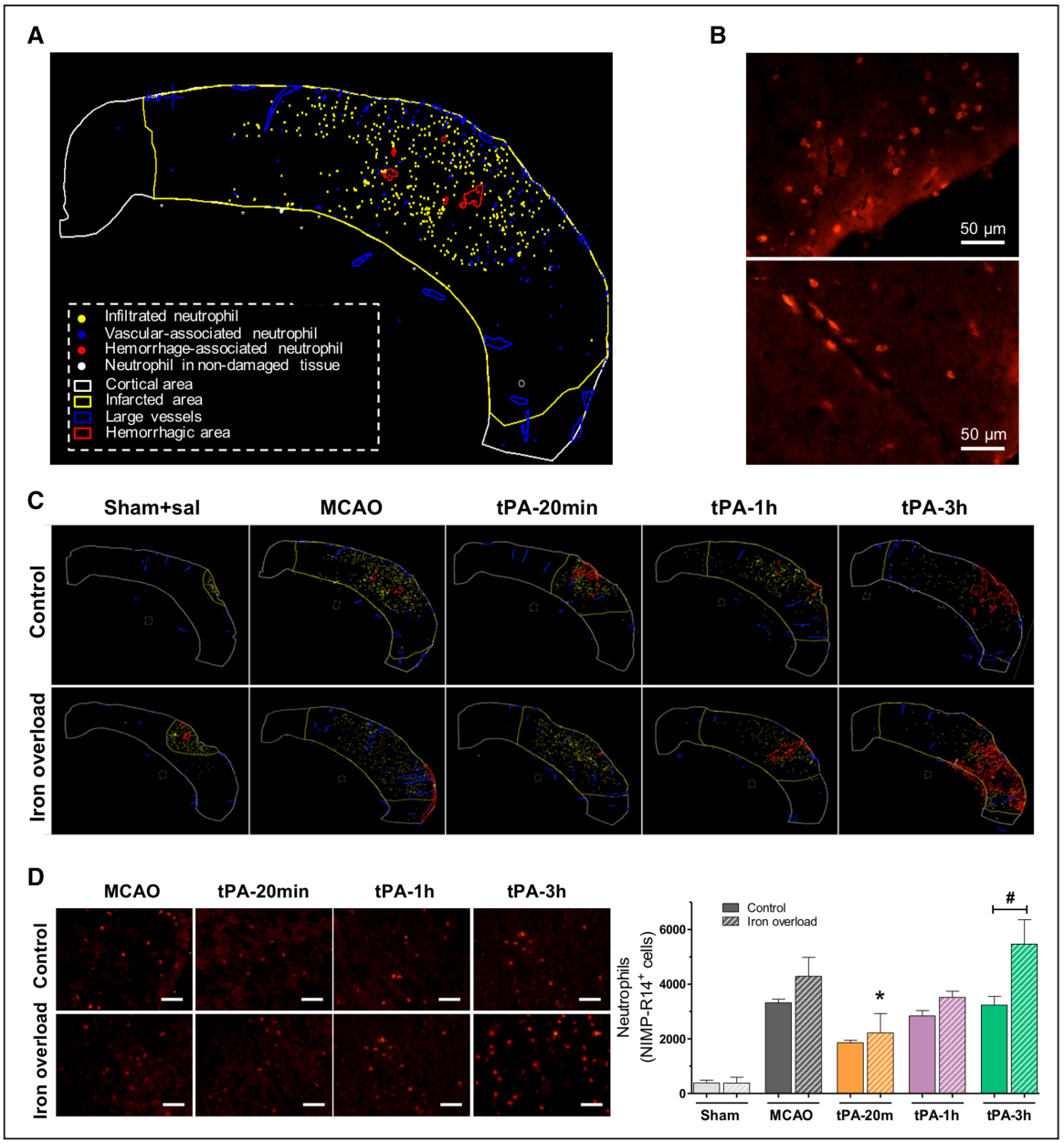


**Figure 5.** Neutrophil infiltration in the ischemic tissue. **A**, Infiltration maps drawn with the software Stereo Investigator (Visiopharm, Denmark) using a unique section per animal (bregma, −0.4±0.4). **B**, Representative images showing periphery-associated (**top**) and vasculature-associated neutrophils (**bottom**). **C**, Representative maps of each group showing that extravasation is especially strong in the central part of the affected region and in the outside border. **D**, Neutrophils in the ipsilateral cortex. Representative images of neutrophil infiltration in each group (scale bars=50 µm; **left**) and total number of neutrophils quantified by stereology (**right**). Data are expressed as mean±SEM and analyzed by 2-way ANOVA followed by Bonferroni test as a post hoc (n=4). tPA indicates tissue-type plasminogen activator. **P*<0.05 vs MCAO, #*P*<0.05 vs control diet.

## Discussion

Recanalization therapies, either pharmacological or mechanical, have proven benefits in patients with stroke. However, both treatment modalities are associated with an increased risk of symptomatic intracerebral hemorrhage—a life-threatening complication. Identification of both the underlying mechanisms and the patients more prone to bleed could improve the risk-benefit of the therapy. Our findings demonstrate that iron overload is a predisposing factor for complications after thrombolytic therapy. Iron overload (1) increases the rate and accelerates the time of the tPA-induced reperfusion, (2) increases the death of the compromised tissue, reducing the beneficial effect of reperfusion, and (3) exacerbates the risk of HT after early tPA administration, by increasing some mediators of this complication in tissue and plasma.

First, our data demonstrate that successful recanalization after tPA infusion is achieved earlier and at higher rates in iron overloaded animals than in controls. To our knowledge, this constitutes the first evidence of a factor making animals more prone to reperfusion, but the precise mechanisms participating are still unknown. Under an anemic situation, both platelet reactivity[19] and thrombocytosis[20] are increased as part of the physiological response to stop bleedings that could be producing the pathology. Although further studies are needed to

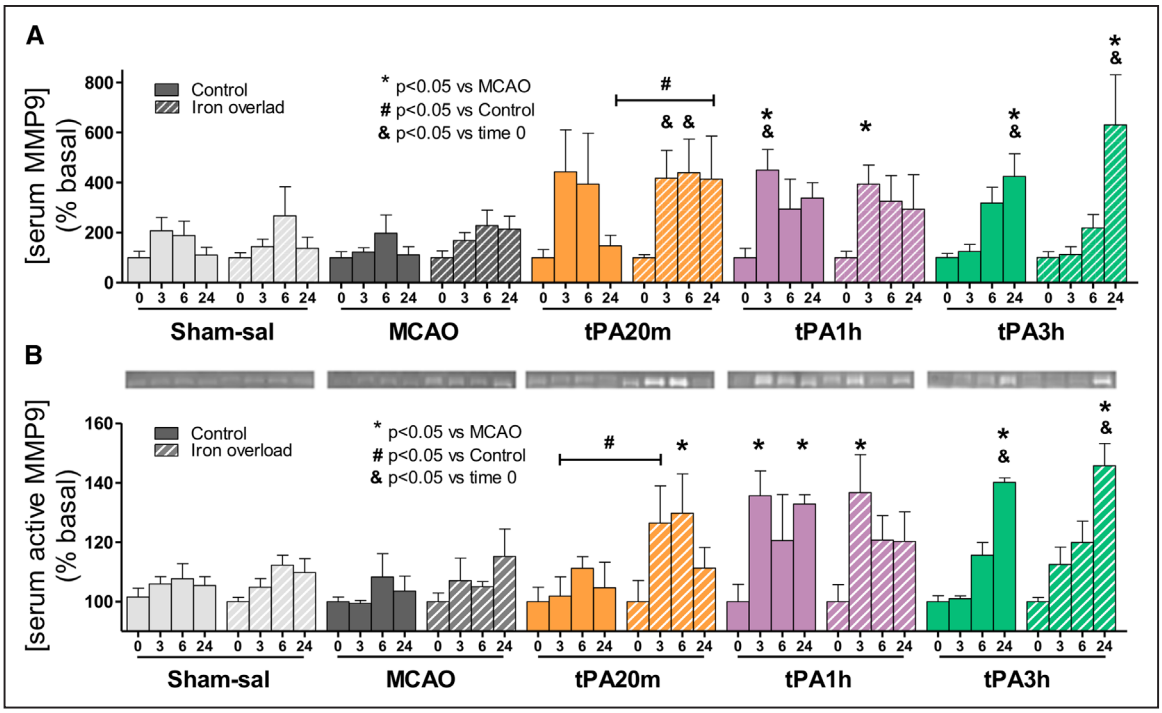


**Figure 6.** Temporal profile of serum matrix metalloproteinase (MMP)-9 during the first 24 hours after ischemia. **A**, MMP-9 expression measured by ELISA. **B**, Active MMP-9 measured by densitometry after zymography. Data were calculated as a percentage of the original values, expressed as mean±SEM and analyzed by Kruskal-Wallis to assess induction along the temporal profile in each group and by 2-way ANOVA followed by Bonferroni test as a post hoc to check differences between groups (n=4–8). tPA indicates tissue-type plasminogen activator. *$P$<0.05 vs MCAO, #$P$<0.05 vs control diet, &$P$<0.05 vs time 0.

elucidate this issue, it might be that elevated iron levels could reduce platelet reactivity or number in blood, affecting coagulation/thrombolysis and causing labile fibrin-enriched clots easier to dissolve after tPA administration.

Second, we have also shown that systemic iron overload results in an increased infarct volume when reperfusion therapy is given at 20 minutes or 1 hour after ischemia, showing no effect after permanent ischemia or late administration of tPA at 3 hours. These results strongly suggest that iron overload reduces viable brain volume during the time window of opportunity for reperfusion therapies by accelerating evolution of the ischemic penumbra because of both basal systemic and brain oxidative stress. In this context, some experimental studies demonstrate that low systemic levels of iron result in protection after cerebral ischemia,[21] whereas iron overload exacerbates the ischemic damage[14–16] as we have said before. Additionally, iron-driven macrophage M1 polarization[22] was associated with a worse resolution, and an increased damage has been observed in models of spinal injury[23] and wound healing.[24] In contrast, a clinical[25] and an experimental study[26] failed to prove the detrimental effect of elevated iron storages after permanent occlusion.

It is widely accepted that iron homeostasis is lost early after cerebral ischemia. In these circumstances, iron is rapidly released within the ischemic tissue from its binding proteins to its most reactive form, ferrous ($Fe^{2+}$)—one of the first mediators of the ischemic cascade that contributes to brain damage through reactive oxygen species production.[9,27] In fact, after cerebral ischemia, not only free iron levels are increased (1 hour post-occlusion) but also total brain iron is higher.[28] As a consequence, blood-borne iron, increased in iron overloaded animals, may enter into the ischemic parenchyma contributing to a larger damage. Supporting this fact, it has been found that iron depositions are observed close to vessels, which may be inducing an elevated BBB damage.[15,29]

Finally, we have also found more severe HT and increased hemorrhage-related parameters when reperfusing with tPA as soon as 1 hour after ischemia in animals with iron overload. In this context, it is difficult to discriminate whether the bleeding induced after tPA administration is because of tPA or reperfusion. Among the molecular mechanisms studied and involved in HT, oxidative stress and neutrophil infiltration could be exacerbated by reperfusion per se, whereas MMP-9 has been associated with tPA administration. To our knowledge, this is the first experimental evidence of the detrimental effect of iron overload on tPA-associated HT, which is supported by clinical results showing that higher iron levels are associated to worse outcome and higher HT risk in patients receiving tPA.[10,17]

We analyzed the detrimental effect of iron overload on HT during ischemia and reperfusion by means of determining potential mediators of BBB disruption. Our results demonstrate that animals develop an increased systemic basal oxidative stress as a consequence of iron overload shown by an elevated serum MDA previous to surgery. In baseline condition, however, brain oxidative stress, indicated by $HNE^+$ cells, is not altered as shown previously[15] probably because of an intact BBB that preserves brain iron content unaltered.[8] Remarkable is the fact that all the groups with iron overload showed increased lipid peroxidation (brain $HNE^+$ cells) after ischemia compared with control animals, pointing to a basal systemic oxidative status and to an increased brain oxidative stress, both induced by iron overload itself indicating

the lack of iron homeostasis affecting brain parenchyma that participates in reactive oxygen species generation. Of note, this environment could be both a cause and a consequence of a macrophage M1 polarization of myeloid phenotypes[22] and subsequent proinflammatory status. Nonetheless, oxidative stress does not seem to be the main mediator of bleeding. On the other hand, neutrophil infiltration, which according to the literature takes places mainly in the infarct core extending from the cortical surface along large vessels,[30] was decreased in tPA 20-minute groups and increased in iron overloaded animals when administering tPA 3 hours after ischemia, likely as a consequence, respectively, of reduced infarct size in early reperfusion and increased reactive oxygen species production in iron overloaded animals. Although some publications point to neutrophils as key players in HT,[31,32] our results support just a secondary role. However, because of an increased oxidative damage in animals with high levels of iron, neutrophil N1 polarization[33] could also contribute to vascular damage. BBB disruption after stroke has been classically attributed to protease induction and activation[34]—an effect potentiated by tPA.[35,36] Our results do not show an MMP-9 increase in the ischemic brain but clearly demonstrate an induction in animals with delayed reperfusion after tPA and, therefore, with HT. Besides, the association between this protease and bleeding is also supported by our results proving an endothelial location of this protease—as other authors showed before,[37] contrary to the previously proposed neutrophil origin of MMP-9–mediating BBB damage[31,32,38]—that may be because of the different models of study used and the time points selected for the analysis. Although no significant differences were found according to iron burden, iron-mediated oxidative stress could result in enhanced MMP-9 activation[39] in iron-overload animals, explaining the increased bleeding in this group. Indeed, serum zymograhy showed an elevated activation of this protease in the iron-overload group at least in the tPA 20-minute group. Additionally, serum MMP-9 levels showed a transient increase after tPA administration that can be a consequence of neutrophil degranulation,[40] remaining elevated at 24 hours in tPA 3-hour group and being, therefore, a good marker of HT as clinical evidence suggests.[41–44] Interestingly, neither MMP-2 nor fibronectin (an MMP-9 substrate) levels were modified in serum during 24 hours after ischemia, suggesting that those studies showing increased serum cFn in patients with HT[45] may be indicating an endothelial damage previous to stroke or reflecting larger intracerebral hemorrhage volumes, such as parenchymal hemorrhage type 2. These large hemorrhages were not found in our experimental model.

Our results showing that iron overload increases the death of the compromised tissue, accelerates the time of the tPA-induced reperfusion, and exacerbates the risk of HT, may have relevant clinical implications to make thrombolysis safer. High body iron stores have been associated with poorer clinical outcome of ischemic stroke both in non-tPA–treated patients in whom persistent occlusion or late recanalization can be assumed[11–13,46] and in patients treated with intravenous tPA.[10] Because elevated iron burden is relatively common in the population—being caused by a plethora of diseases like hemochromatosis, one of the most common genetic diseases among white people, thalasemia, frequent among African origin people, or diabetes mellitus—future combined reperfusion and neuroprotective should, among others, target iron-related injury in ischemic stroke.[47]

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## Disclosures

None.

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