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Increasing expression of H- or L-ferritin protects cortical astrocytes from hemin toxicity

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Abstract
Iron toxicity may contribute to oxidative injury in cells surrounding an intracerebral haematoma. Cells detoxify iron by sequestering it in ferritin, a 24-mer heteropolymer constructed of H and L subunits. The relative antioxidant efficacy of H- and L-ferritin has not been defined and was tested in this study using an established model of hemin toxicity. Consistent with prior observations, cultures treated with 30 μM hemin sustained loss of approximately half of the cells by 6 h, as measured by LDH and MTT assays, and a 14-fold increase in protein carbonyls. Increasing expression of either ferritin by adenoviral gene transfer prior to hemin treatment had a similar protective effect. Quenching of calcein fluorescence, a marker of the labile iron pool, in hemin-treated cultures was also equally reduced by either subunit. These results suggest that over-expression of either H- or L-ferritin protects astrocytes from hemin and may be beneficial after CNS haemorrhage.

Keywords: *Cell culture, free radical, haemoglobin toxicity, intracerebral haemorrhage, iron chelation, oxidative stress, stroke*

Introduction

Iron toxicity may contribute to cell injury in tissue surrounding an intracerebral haemorrhage (ICH) [1]. The putative source of this iron is extravascular haemoglobin, which tends to release its heme moieties after undergoing auto-oxidation [2]. Their subsequent breakdown by the heme oxygenase enzymes releases equimolar iron and likely accounts for the increase in non-heme iron observed in adjacent neurons and glial cells [3]. Non-heme iron is increased within 1–3 days of experimental ICH produced by direct blood injection in rat and rabbit models and persists for at least 3 months in the former [1,4]. Some of this iron may be sequestered in ferritin, which is rapidly induced in peri-haematomal tissue [5]. However, it is unclear if this ferritin provides cells with any protection from heme toxicity. At best, it appears to be either insufficient or too late, since redox-active iron produces oxidative injury after experimental ICH, despite upregulation of ferritin expression [6,7].

Mammalian ferritin is a 24-mer heteropolymer constructed of H and L subunits, with considerable variability in subunit composition in different cell populations. The antioxidant efficacy of H-rich and L-rich ferritin heteropolymers has been directly compared only in HeLa cells to date. Cozzi et al. [8] reported that iron availability was negatively regulated by increasing expression of H-ferritin by gene transfer, but that L-ferritin had no effect *per se*. The vulnerability of these cells to hydrogen peroxide (H_2O_2), which is an iron-dependent injury [9], was also inversely related to H-ferritin but not L-ferritin levels. In contrast, Orino et al. [10] reported that over-expression of either H- or L-ferritin reduced oxidative stress equally in the same cell line after H_2O_2 treatment.

Differences in the antioxidant efficacy of H- and L-ferritin have been attributed to the ferroxidase activity of the former, which is essential for rapid iron uptake by the heteropolymer and is lacking in the L-subunit [11]. Consistent with a critical role of H-ferritin expression in cellular iron homeostasis, homozygous

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H-ferritin knockout mouse embryos die at 3–9 days of development [12]. However, *in vitro* evidence suggests that increasing expression of L-ferritin may offer certain advantages to cells subjected to iron-loading conditions, due to the greater iron storage capacity, solubility and stability of ferritins containing over 70–80% L-subunits [13]. These characteristics may account for the predominance of L-rich ferritin in cells that store iron, such as hepatocytes. The relative efficacy of H- and L-ferritin in protecting CNS cells from injury produced by supraphysiologic iron concentrations has not yet been defined.

Although iron chelators are protective in some experimental ICH models [6,7], therapy with currently-available chelators may be limited by myriad toxic effects in humans, particularly if administered in the absence of systemic iron overload or at the high doses required for benefit in rodents [14–16]. An alternate or perhaps complementary approach is to increase the iron-sequestering capacity of cells adjacent to a haematoma via gene transfer, using vectors administered by stereotactic injection. Toward that end, we have constructed adenoviruses encoding murine H- and L-ferritin genes driven by the human CMV promoter. In the present study, we compared the effect of increasing H- or L-ferritin expression in an established astrocyte model of hemin toxicity.

Materials and methods

Primary astrocyte cultures

All astrocyte cultures were prepared from 1–3 day postnatal C57BL/6 X 129/Sv mice that were bred in our animal facility. Mice were euthanized for culture preparation by deep isoflurane anaesthesia followed by decapitation, via a protocol approved by the local Institutional Animal Care and Use Committee. Cortices were dissected free and incubated for 30 min in 0.09% trypsin. Tissue was dissociated by trituration through a Pasteur pipette with a narrowed (flame-polished) tip. The cell suspension was plated at a density of 1 hemisphere per 24-well plate, in medium consisting of minimal essential medium (MEM, Gibco/Invitrogen, Grand Island, NY) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT), 10% equine serum (Hyclone), 10 ng/mL mouse epidermal growth factor (Sigma, St. Louis, MO), 23 mM glucose and 2 mM glutamine. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Approximately two-thirds of the culture medium was replaced at 5 days *in vitro* and then twice weekly, using growth medium containing MEM, 23 mM glucose, 2 mM glutamine and 10% equine serum.

H-ferritin heterozygous knockout (FTH+/−) cultures were prepared using a similar procedure, except that cortices from each mouse were dissociated and plated separately. FTH+/− mice were then distinguished from wild-type littermates by PCR-based genotyping, using genomic DNA extracted from residual brain tissue and the following primer pair specific for the knockout gene:

5′-ATA CTT TCT CGG CAG GAG CA-3′
5′-TCA GAG CCA CAT CAT CTC GGT C-3′

Adenovirus preparation and astrocyte transfection

The open reading frames of the murine H-ferritin and L-ferritin genes were excised by digestion with Eco RI on the 5′ end and NotI on the 3′ end from pCMVSPORT6-mFTH1 (ATCC, GeneBank #BC0 12314) or pCMVSPORT6-LFER (ATCC, GeneBank# BC106146). The 0.9 Kb (H-ferritin) and ~1 Kb (L-ferritin) fragments were introduced to adenoviral shuttle vector pDUAL-CCM (Vector Biolabs, Philadelphia, PA) creating pDUAL-CMV-mFTH1 and pDUAL-CMV-mLFer. These constructs were sequenced and transfected to primary cultured wild type astrocytes (5 days *in vitro*, 1 μg DNA per well) using Lipofectamine plus reagent in serum-free medium (OptiMEM, Invitrogen) for expression evaluation via immunoblotting. Sequence analysis demonstrated promoter–insert orientation and insert identity to mouse ferritin heavy chain or light chain sequences, using the Chromas sequences analysing software and NCBI–The Basic Local Alignment Search Tool (BLAST).

SwaI endonuclease was used to release the insert from shuttle pDUAL-CMV-mFTH1 or pDUAL-CMV-mLFer and ligate the insert directly into the viral plasmid vector (pAd-VEC). For viral packaging in HEK293 cells, pAd-VEC-CMV-mFTH1 and pAd-VEC-CMV-mLFer were transfected in linear form (digested with Pac-I), to produce Ad-HF and Ad-LF. After propagation and harvesting, titer was quantified by cytopathic effect assay.

Adenoviral infection of astrocytes was accomplished in medium similar to feeding medium, except that it contained 3.3% equine serum. In a prior study using this culture system, treatment with 100 MOI (multiplicity of infection) of adenovirus serotype 5 in this medium resulted in transfection of ~80% of astrocytes [17].

Immunoblotting

Culture medium was aspirated and cultures were then washed once with 1 ml MEM. After medium aspiration, 100 μl ice-cold lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1% sodium dodecyl sulphate, 0.1% Triton X-100) was added. Cells were collected, sonicated on ice and centrifuged. Protein concentration of the supernatant was quantified by the BCA method (Pierce Biotechnology, Rockford, IL). Samples (20 μg in 30 μl) were diluted with 10 μl loading buffer (Tris-Cl 240 mmol/L, β-mercaptoethanol 20%, sodium dodecyl sulphate

8%, glycerol 40% and bromophenol blue 0.2%) and heated to 95°C for 5 min. Proteins were separated on 12% SDS-PAGE gels (Ready Gel, Bio-Rad, Hercules, CA) and were then transferred to a polyvinylidene difluoride (PVDF) membrane (Imobilon-P, Millipore, Billerica, MA). After washing, non-specific sites were blocked with 5% non-fat dry milk in a buffer containing 20 mM Tris, 500 mM NaCl and 0.1% Tween 20 (pH 7.5) for 1 h at room temperature. For experiments depicted in Figure 1, membranes were incubated at 4°C overnight with goat anti-H-ferritin or goat anti-L-ferritin (Santa Cruz Biotechnology, Santa Cruz, CA, Product# SC-14416 and C-14420, 1:200 dilution). For experiments using FTH+/− and paired wild-type cultures (Figure 7), rabbit anti-H-ferritin was a gift from Dr James Connor (Pennsylvania State University, Hershey, PA, 1:10,000 dilution) and anti-L-ferritin was a gift from Dr Paolo Arosio (Università di Brescia, Brescia, Italy, 1:2000). After washing, membranes were treated with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce, Rockford, IL) and Kodak Gel Logic 2200.

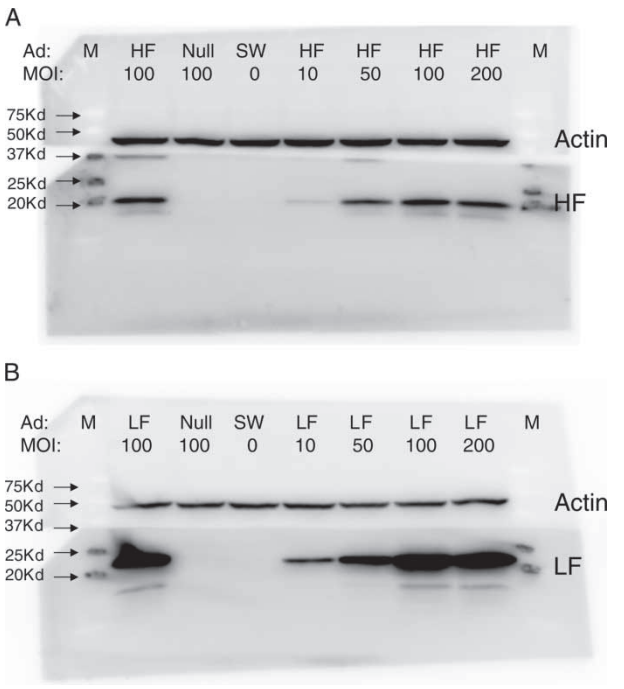

Figure 1. Dose–response effect of adenoviruses encoding murine H (Ad-HF) or L-ferritin (Ad-LF) in primary astrocyte cultures. Representative immunoblots from astrocyte cultures treated with 10–200 MOI (multiplicity of infection) Ad-HF (A), Ad-LF (B) or control empty adenovirus (Null) for 24 h, stained with goat antibody specific for H- or L-ferritin (Santa Cruz Biotechnology). Control cultures were subjected to medium exchange (sham-wash, SW) only. M denotes protein molecular weight marker. Membranes were also stained with rabbit anti-β-actin (1:1250, Sigma) as a gel loading control. Entire membranes are shown, which were cut to allow separate incubation with different secondary antibodies.

Cytotoxicity experiments

Experiments were conducted after 21 days *in vitro*. Confluent cultures were washed free of growth medium and were then exposed to hemin in serum-free medium consisting of MEM with 10 mM glucose (MEM10). In all experiments, cell injury was estimated at the end of the exposure period by visualizing cultures using phase contrast microscopy. Cell viability was then quantified using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and lactate dehydrogenase (LDH) release assays, as previously described in detail [18].

Cell protein oxidation was quantified by derivatized carbonyl assay, using the Oxyblot™ kit (Chemicon, Inc., Temecula, CA) and following the manufacturer's instructions. Briefly, proteins in cell lysates were protected from *in vitro* oxidation by adding 2-mercaptoethanol to a final concentration of 1%. 2,4-dinitrophenylhydrazine was then added to convert carbonyl groups to 2,4-dintrophenylhydrazone (DNP) derivatives. Proteins were separated on 12% polyacrylamide gels and were transferred to a PVDF membrane filter. After incubation with rabbit anti-DNP primary antibody (1:150) followed by goat anti-rabbit HRP-conjugated secondary antibody (1:300), immunoreactive proteins were detected as described above. Oxidized protein bands densities were analyzed using Kodak 1D software.

Assessment of the labile iron pool

Changes in cell chelatable iron were detected by quantifying calcein fluorescence quenching in hemin-treated cultures [19]. After medium exchange to remove extracellular hemin, cultures were loaded with calcein by incubation for 15 min with 0.1 μM calcein-AM, its cell-permeable ester. Cultures were then examined using an inverted microscope with epifluorescence attachment and an FITC filter. Digital 100× images in the center of each well (1 field/culture) were rapidly captured and fluorescence intensity was quantified using Scanalytics IPLab software. Membrane integrity, essential for calcein retention, was verified by staining with propidium iodide, which is excluded by intact cells. For these experiments, hemin exposure was conducted in 3.3% equine serum, which attenuates the toxicity of hemin, and was terminated by medium exchange at 3 or 5 h. Fluorescence of hemin-treated cultures was expressed as a percentage of that in sister cultures from the same plating treated with the same adenovirus, but not with hemin.

Data analyses

All data were analysed using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test, using Graphpad (San Diego, CA) Prism.

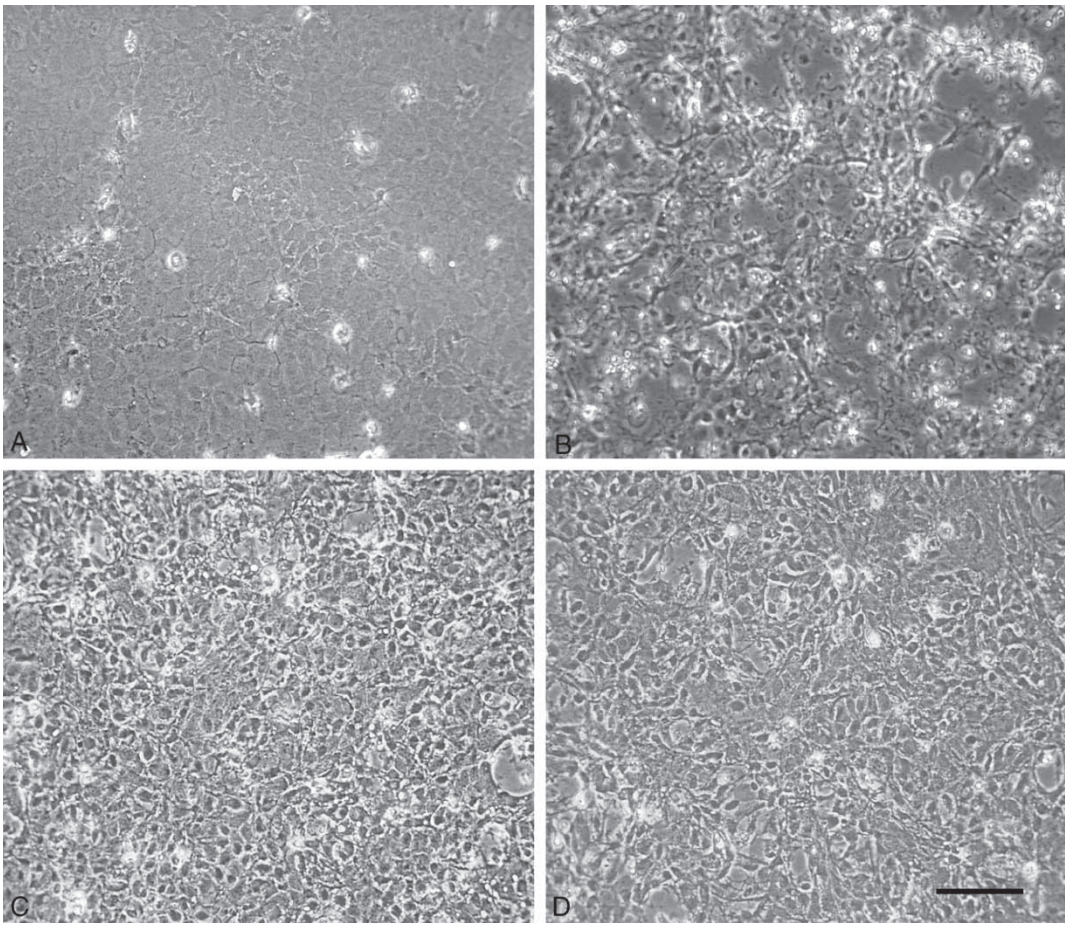

Figure 2. Ad-HF and Ad-LF protect astrocytes from hemin. Phase-contrast photomicrographs of astrocyte cultures, 6 h after: (A) medium change only; (B) exposure to 30 μM hemin after 24 h incubation with 100 MOI Ad-Null; (C) exposure to 30 μM hemin after 24 h incubation with 100 MOI Ad-HF; (D) exposure to 30 μM hemin after 24 h incubation with Ad-LF. Scale bar = 100 μm.

Results

Treatment with Ad-HF and Ad-LF increases ferritin expression

We have previously demonstrated that transgene expression using adenovirus serotype 5 in this culture system is optimized at a dose of 100 MOI for 24 h [17]. These observations were confirmed using ad-HF and ad-LF (Figure 1). In cultures subjected to medium exchange only, baseline H and L-ferritin levels were minimal when membranes were probed with isoform-specific antibodies purchased from Santa Cruz Biotechnology. Over a range of 10–200 MOI, maximal ferritin expression was observed at 100 MOI (Figure 1) for each construct. Treatment with an empty control adenovirus (Ad-Null) at this dose did not increase endogenous H or L-ferritin expression.

Protective effect of H or L-ferritin overexpression

Consistent with prior observations in this system [17], cultures pre-treated with 100 MOI Ad-Null for 24 h and then treated with 30 μM hemin sustained widespread cell injury, which was morphologically apparent within 6 h (Figure 2). At this time point, $44.0 \pm 1.5\%$ of culture LDH was released into the culture medium (Figure 3A). In cultures pre-treated with either Ad-HF or Ad-LF, less injury was detected, with release of $22.0 \pm 1.7\%$ and $23.3 \pm 2.0\%$ of culture LDH, respectively. A similar but somewhat weaker effect was observed when injury was assessed by the ability of cells to reduce MTT to formazan (Figure 3B).

Protein carbonylation is a sensitive marker of hemin-mediated oxidative injury in this culture system [18]. Protein carbonyl levels were increased by 14-fold over baseline in cultures pre-treated with 100 MOI Ad-Null for 24 h followed by 30 μM hemin for 6 h. Carbonyls were reduced by $\sim 40\%$ in cultures pre-treated with either Ad-HF or Ad-LF (Figure 4).

Effect of ferritin over-expression on the labile iron pool

In cultures pre-treated with Ad-Null, hemin treatment for 3–5 h reduced calcein fluorescence by over 90% (Figures 5 and 6). Most of this quenching was prevented in cultures pre-treated with either Ad-HF or Ad-LF. Fluorescence differences in cultures

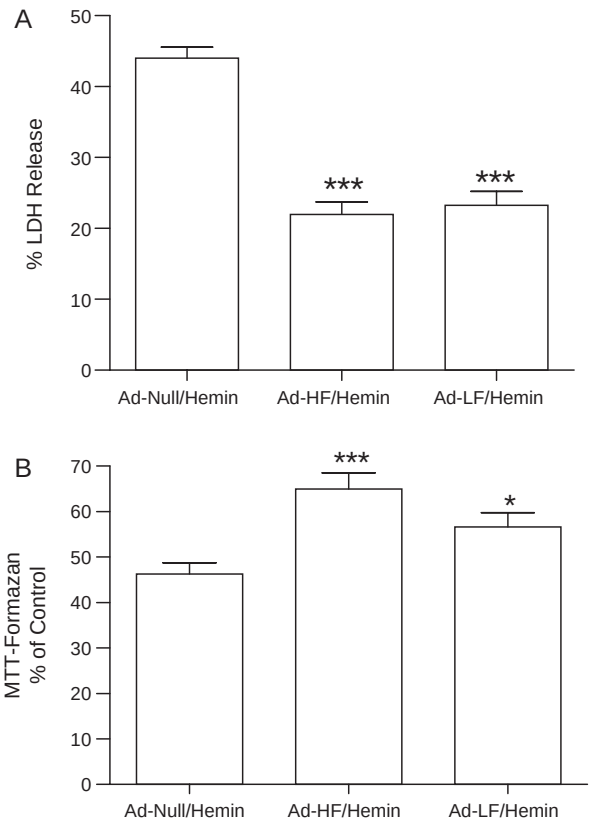

Figure 3. Quantification of the protective effect of Ad-HF and Ad-LF. (A) Mean culture percentage LDH release (±SEM), 6 h after treatment with 30 μM hemin, preceded by 24 h incubation with 100 MOI Ad-Null, Ad-HF or Ad-LF. Values are scaled to those in control sister cultures treated with 0.1% Triton X-100 (=100), which releases 100% of culture LDH. The LDH values in sister cultures subjected to sham-wash only were subtracted from all values. (B) Quantification of cell viability using the MTT assay at 3 h. Values are scaled to those in control sister cultures subjected to sham-wash only (=100). ***$p<0.001$, *$p<0.05$ vs mean value in cultures pre-treated with Ad-Null, $n=14$–24/condition, Bonferroni multiple comparisons test.

pre-treated with Ad-HF and Ad-LF were not statistically significant. Quenching was completely reversed by treating cultures with the cell-permeable iron chelator 1,10-phenanthroline (100 μM) for 15 min. The fluorescence intensity in cultures treated with hemin for 5 h followed by 1,10-phenanthroline for 15 min exceeded that of control cultures treated with 1,10-phenanthroline after exposure to the experimental medium (MEM10) only.

Effect of decreased H:L ferritin ratio on cell vulnerability to hemin

Homozygous H-ferritin gene knockout is lethal in early gestation, but heterozygotes (FTH+/−) survive and are fertile [20]. These mice express less H-ferritin in the CNS but compensate by increasing L-ferritin expression. In order to investigate the effect of altering the H:L ferritin ratio on astrocyte vulnerability to hemin, cultures were prepared from FTH+/− mice and their wild-type littermates.

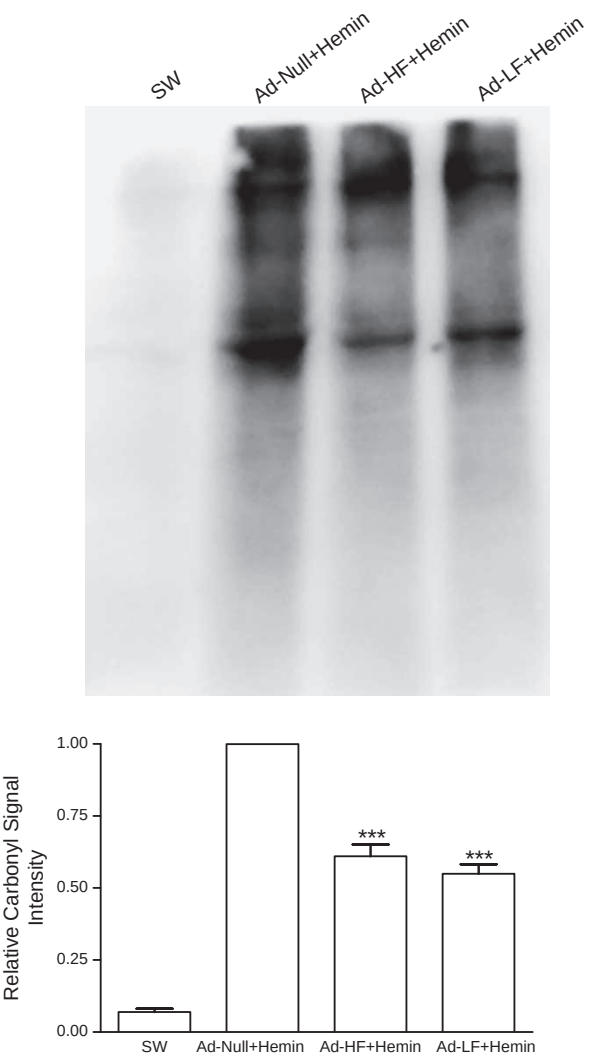

Figure 4. Astrocyte protein oxidation after hemin treatment is reduced by pre-treatment with Ad-HF or Ad-LF. Upper figure is a representative immunoblot of lysates from astrocyte cultures treated with sham wash (SW) only or with 30 μM hemin for 6 h, preceded by 24 h incubation with 100 MOI Ad-Null, Ad-HF or Ad-LF. Bars represent mean carbonyl signal intensities (±SEM, $n=5$/condition). ***$p<0.001$ vs mean value in cultures pre-treated with Ad-Null, Bonferroni multiple comparisons test.

The antibodies used in these experiments were more sensitive than the commercially-available antibodies used in experiments depicted in Figure 1 and detected H- and L-ferritin expression in control cultures subjected to medium exchange only. In confluent FTH+/− cultures, H-ferritin expression was reduced to almost half of that in wild-type cultures at baseline or after ferritin induction by haemoglobin (Figure 7), which slowly delivers heme to astrocytes and is non-toxic to this cell population at low micromolar concentrations [21]. L-ferritin expression was increased by 2.2-fold at baseline and remained significantly increased with induction. The vulnerability of FTH+/− and wild-type cells to hemin was not significantly different despite the decreased H:L ferritin ratio of the former (Figure 7).

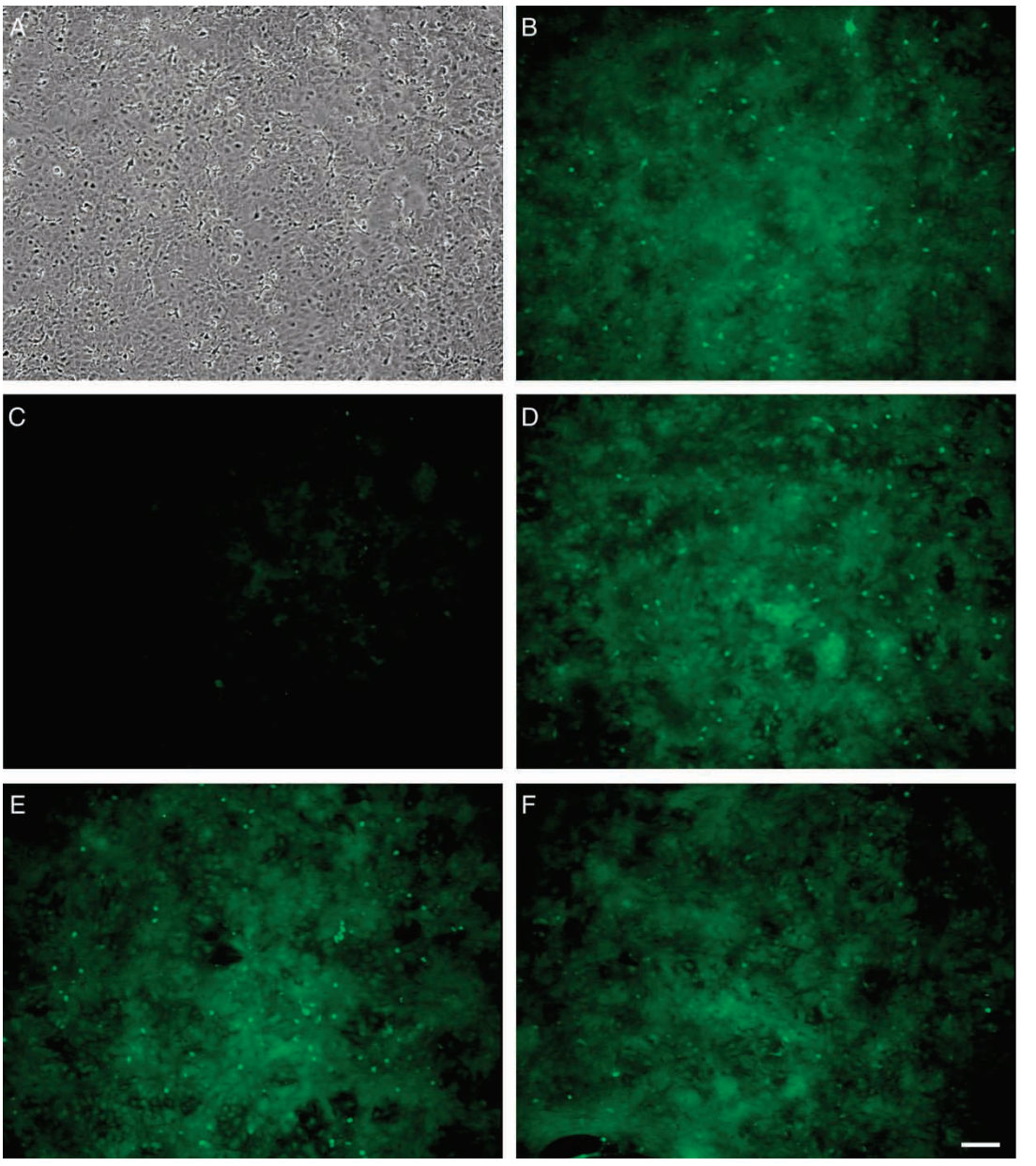

Figure 5. Calcein fluorescence quenching after hemin treatment is attenuated by pre-treatment with Ad-HF or Ad-LF. (A) Phase contrast photomicrograph of culture treated with 100 MOI Ad-Null for 24 h; (B) Same culture as (A), demonstrating baseline calcein fluorescence without hemin treatment; (C) Sister culture, treated with 100 MOI Ad-Null for 24 h followed by 30 μM hemin for 3 h; fluorescence intensity is markedly diminished; (D) Same culture as C, 15 min after treatment with 100 μM 1,10-phenanthroline, demonstrating reversal of fluorescence quenching with a cell-permeable iron chelator; (E, F) Cultures pre-treated with 100 MOI Ad-HF (E) or Ad-LF (F) for 24 h prior to 3 h hemin treatment; fluorescence quenching is reduced compared with culture pre-treated with Ad-Null.

Discussion

At low micromolar concentrations, hemin produces an iron-dependent oxidative injury in cultured cells [22,23]. The protective effect of ferritin over-expression is consistent with prior observations that pre-treatment with horse spleen ferritin, which is taken up by endocytosis, attenuated hemin toxicity in endothelial cells and astrocytes [22,24]. However, data comparing the efficacy of H- and L-ferritin against heme-mediated oxidative injury have not been previously reported. The present results demonstrate that in primary cultured astrocytes, increasing expression of either subunit has a comparable effect on the labile iron pool and cell injury after hemin treatment. The hypothesis that both H- and L-subunits are protective in this model is further supported by observations that altering the H:L ferritin ratio by heterozygous H-ferritin gene knockout, which is associated with a compensatory increase in L-ferritin, had no effect on astrocyte vulnerability to hemin.

The present results are consistent with those of Orino et al. [10], who reported that over-expression of either H- or L-ferritin attenuated cellular reactive oxygen species levels after H_2O_2 treatment. However,

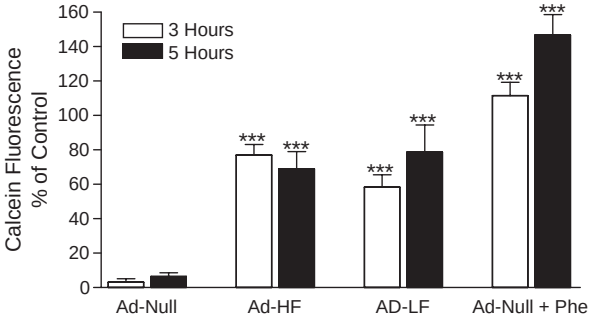

Figure 6. Quantification of calcein fluorescence quenching by hemin after treatment with viral vectors. Cultures were treated with 100 MOI Ad-Null, Ad-HF or Ad-LF for 24 h. Half were then treated with 30 μM hemin for 3 or 5 h. Mean calcein fluorescence (±SEM, $n = 5$–8/condition) is expressed as a percentage of that in control cultures not treated with hemin. Cultures pre-treated with Ad-Null were subsequently treated with 100 μM 1,10-phenanthroline (Phe) to reverse quenching. ***$p < 0.001$ vs corresponding value in cultures pre-treated with Ad-Null.

they disagree with data of Cozzi et al. [8], suggesting that cell iron availability and vulnerability to oxidant challenge were primarily regulated by H-ferritin levels only. These disparate results are likely reconciled by differences in endogenous H-ferritin expression in these models. Iron uptake by ferritin heteropolymers is optimal when the H-ferritin content is 20–30%, suggesting that additional ferroxidase activity provides no benefit [13,25]. Furthermore, L-rich heteropolymers are more soluble and incorporate up to 4-fold more iron than H-homopolymers [13]. Astrocytes in this culture system rapidly upregulate H-ferritin after exposure to heme, in contrast with the very weak expression observed in neurons [26]. Since hemin breakdown by the heme oxygenases releases ferrous iron [27] and since ferritin heteropolymers incorporate only ferric iron in their mineral core, ferroxidase activity is likely critical for rapid iron uptake. The present results suggest that adequate ferroxidase activity is provided by endogenous H-ferritin in cultured astrocytes, even in heterozygous knockouts.

The effect of ferritin gene transfer on cell viability as measured by the MTT assay was less than that observed with the LDH release assay. Reduction of MTT to its formazan product is catalysed in part by mitochondrial enzymes [28]. Heteropolymers constructed of H- and L-ferritin are the primary cytoplasmic iron regulators. However, a unique ferritin encoded by a separate nuclear gene predominates in mitochondria [29] and was not directly altered in the current series of experiments, although indirect alterations as a consequence of the experimental conditions cannot be excluded. The effect of specifically increasing expression of mitochondrial ferritin on heme-mediated astrocyte injury seems a worthy topic for future investigation. It is noteworthy that when over-expressed in HeLa cells, it reduced the levels of reactive oxygen species after treatment with H_2O_2 or antimycin A [30].

The protection provided by H- or L-ferritin gene transfer was also less than predicted from its effect on the labile iron pool, as measured by calcein fluorescence quenching. Two factors may contribute to this discrepancy. First, hemin is lipid soluble and accumulates in membranes, where it may catalyze free radical reactions in a cellular compartment that is inaccessible to hydrophilic ferritin and calcein [31]. Second, the calcein method may underestimate the labile iron pool. Tenopoulou et al. [32] have recently reported that calcein does not measure lysosomal iron, due to its limited ability to penetrate lysosomal membranes and chelate iron in an acidic environment. Lysosomal iron mediates most of the cell death produced by H_2O_2 *in vitro* [33]. Although its role in heme-mediated injury has not been defined, any contribution may not be detected by changes in calcein fluorescence.

Ferritin expression is minimal at baseline in this culture system, but it is induced within 6 h by heme treatment [26]. While that induction likely contributes to the resistance of astrocytes to the more-slowly developing toxicity of haemoglobin, it apparently occurs too late to prevent widespread cell death *per se* in this hemin toxicity model. This observation is consistent with the hypothesis that endogenous ferritin levels may be suboptimal in cells adjacent to an intracerebral haematoma, which has a hemin content in the high micromolar range [34], and that therapies aiming to upregulate its expression prior to a heme-mediated oxidative insult may be beneficial. In support of this hypothesis, we have recently reported that neurons lacking iron regulatory protein-2 (IRP2), which represses H- and L-ferritin translation by binding to an iron responsive element in the 5′ regions of their mRNA, are highly resistant to iron-dependent oxidative injury *in vitro* and to experimental intracerebral haemorrhage *in vivo* [26,35]. It remains to be determined if any treatment administered after the onset of haemorrhage would alter ferritin levels quickly enough to increase cell resistance to hemin. It is noteworthy, however, that iron-mediated injury is delayed until ~2–3 days after experimental ICH, which likely reflects the time required for erythrocyte lysis and haemoglobin oxidation [36]. The therapeutic window for the component of injury produced by heme breakdown may therefore be sufficient for delivery of viral vectors to astrocytes adjacent to a haematoma.

The long-term consequences of iron accumulation in the ferritin of astrocytes after haemorrhagic injury is undefined and may best be addressed *in vivo*, due to the shorter lifespan of cells in primary cultures. Studies using genetically modified mice suggest that sustained upregulation of ferritin *per se* may be associated with toxicity, although precise mechanisms have yet to be delineated. In ageing ceruloplasmin knockout mice, ferritin-bound iron accumulation in

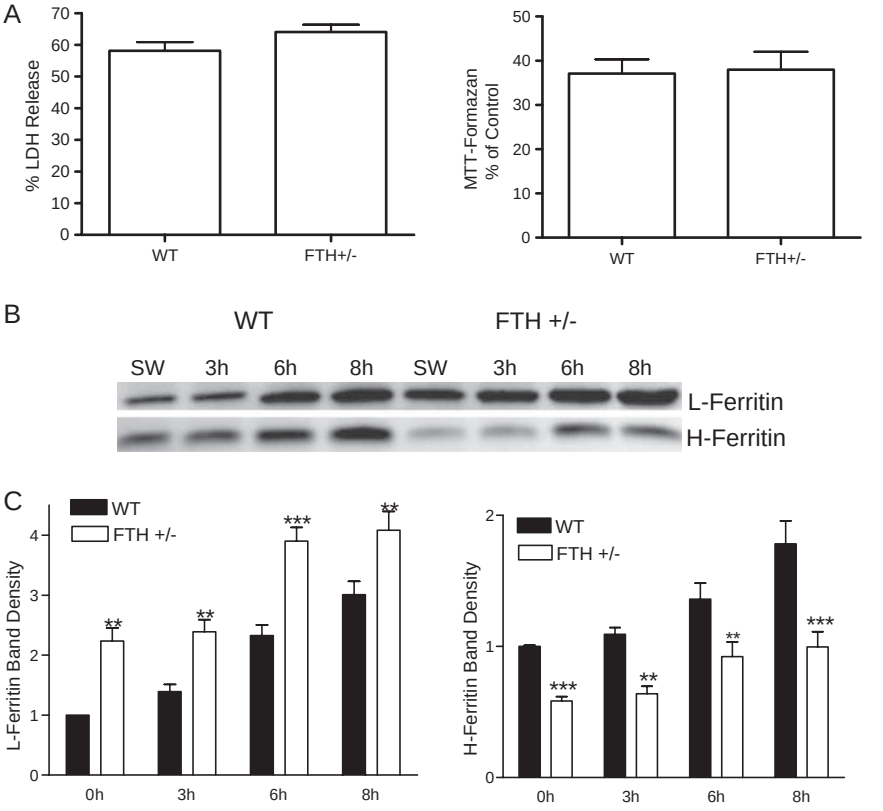

Figure 7. Heterozygous H-ferritin gene deletion has no effect on astrocyte vulnerability to hemin. (A) Cell injury, as assessed by LDH release and MTT assays, in wild-type and H-ferritin heterozygous knockout (FTH+/−) cultures produced by exposure to 30 μM hemin, as in Figure 3. Mean culture LDH values (±SEM, $n=28$–32/condition) are scaled to those in sister cultures treated with 0.1% Triton X-100 (=100), after subtraction of mean values in sham-washed (SW) controls, to yield the value associated with hemin toxicity. Values for MTT reduction to formazan ($n=24$/condition) are scaled to those in sister cultures subjected to sham-wash only (=100). (B) Expression of H-ferritin and L-ferritin in astrocyte cultures prepared from wild-type (WT) and FTH+/− mice, at indicated intervals after treatment with 10 μM haemoglobin, using non-commercial antibodies described in Methods. (C) Bars represent mean ferritin band densities (±SEM, $n=4$ condition). $^{**}p<0.01$, $^{***}p<0.001$ vs corresponding wild-type condition, Bonferroni multiple comparisons test.

astrocytes is associated with reduced immunoreactivity to the astrocyte markers glial fibrillary acidic protein and S100β, consistent with cell loss [37]. IRP2 knockout mice may develop a late-onset movement disorder, in which iron deposition in white matter tracts and nuclei precede neurodegeneration [38]. The toxicity of sustained ferritin over-expression may be mitigated by the use of adenoviral vectors, which tend to produce transient gene expression [39]. However, any benefit may then be negated by a direct cytotoxic or inflammatory response [40]. Further investigation is needed to determine if ferritin gene transfer is feasible *in vivo* and if it also protects neurons and other vulnerable cell populations.

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References

[1] Hua Y, Nakamura T, Keep RF, Wu J, Schallert T, Hoff JT, Xi G. Long-term effects of experimental intracerebral hemorrhage: the role of iron. J Neurosurg 2006;104:305–312.
[2] Bunn HF, Jandl JH. Exchange of heme along hemoglobins and between hemoglobin and albumin. J Biol Chem 1967;243:465–475.
[3] Koeppen AH, Dickson AC, Smith J. Heme oxygenase in experimental intracerebral hemorrhage: the benefit of tin-mesoporphyrin. J Neuropathol Exp Neurol 2004;63:587–597.
[4] Koeppen AH, Dickson AC, McEvoy JA. The cellular reactions to experimental intracerebral hemorrhage. J Neurol Sci 1995;134(Suppl):102–112.
[5] Wu J, Hua Y, Keep RF, Nakemura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003;34:2964–2969.
[6] Wan S, Hua Y, Keep RF, Hoff JT, Xi G. Deferoxamine reduces CSF free iron levels following intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:199–202.
[7] Masuda T, Hida H, Kanda Y, Aihara N, Ohta K, Yamada K, Nishino H. Oral administration of metal chelator ameliorates

motor dysfunction after a small hemorrhage near the internal capsule in rat. J Neurosci Res 2007;85:213–222.
[8] Cozzi A, Corsi B, Levi S, Santambrogio P, Biasiotto G, Arosio P. Analysis of the biologic functions of H- and L-ferritins in HeLa cells by transfection with siRNAs and cDNAs: evidence for a proliferative role of L-ferritin. Blood 2004;103: 2377–2383.
[9] Starke PE, Farber JL. Ferric iron and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide. Evidence for the participation of hydroxyl radicals formed by an iron-catalyzed Haber-Weiss reaction. J Biol Chem 1985;260:10099–10104.
[10] Orino K, Lehman L, Tsuji Y, Ayaki H, Torti SV, Torti FM. Ferritin and the response to oxidative stress. Biochem J 2001;357:241–247.
[11] Arosio P, Levi S. Ferritin, iron homeostasis, and oxidative damage. Free Radic Biol Med 2002;33:457–463.
[12] Ferreira C, Bucchini D, Martin ME, Levi S, Arosio P, Grandchamp B, Beaumont C. Early embryonic lethality of H ferritin gene deletion in mice. J Biol Chem 2000;275: 3021–3024.
[13] Levi S, Santambrogio P, Cozzi A, Rovida E, Corsi B, Tamborini E, Spada S, Albertini A, Arosio P. The role of the L-chain in ferritin iron incorporation. Studies in homo and heteropolymers. J Mol Biol 1994;238:649–654.
[14] Porter JB, Huehns ER. The toxic effects of desferrioxamine. Baillieres Clin Haematol 1989;2:459–474.
[15] Kushner JP, Porter JP, Olivieri NF. Secondary iron overload. Hematology 2001;2001:47–61.
[16] Chaston TB, Richardson DR. Iron chelators for the treatment of iron overload disease: relationship between structure, redox activity, and toxicity. Am J Hematol 2003;73:200–210.
[17] Teng ZP, Chen J, Chau LY, Galunic N, Regan RF. Adenoviral transfer of the heme oxygenase-1 gene protects cortical astrocytes from heme-mediated oxidative injury. Neurobiol Dis 2004;17:179–187.
[18] Chen J, Regan RF. Increasing expression of heme oxygenase-1 by proteasome inhibition protects astrocytes from heme-mediated oxidative injury. Curr Neurovasc Res 2005;2: 189–196.
[19] Kakhlon O, Cabantchik ZI. The labile iron pool: characterization, measurement, and participation in cellular processes(1). Free Radic Biol Med 2002;33:1037–1046.
[20] Thompson K, Menzies S, Muckenthaler M, Torti FM, Wood T, Torti SV, Hentze MW, Beard J, Connor J. Mouse brains deficient in H-ferritin have normal iron concentration but a protein profile of iron deficiency and increased evidence of oxidative stress. J Neurosci Res 2003;71:46–63.
[21] Chen-Roetling J, Regan RF. Effect of heme oxygenase-1 on the vulnerability of astrocytes and neurons to hemoglobin. Biochem Biophys Res Commun 2006;350:233–237.
[22] Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: a cytoprotective strategem of endothelium. J Biol Chem 1992;267:18148–18153.
[23] Goldstein L, Teng ZP, Zeserson E, Patel M, Regan RF. Hemin induces an iron-dependent, oxidative injury on human neuron-like cells. J Neurosci Res 2003;73:113–121.
[24] Regan RF, Kumar N, Gao F, Guo YP. Ferritin induction protects cortical astrocytes from heme-mediated oxidative injury. Neuroscience 2002;113:985–994.
[25] Santambrogio P, Levi S, Cozzi A, Rovida E, Albertini A, Arosio P. Production and characterization of recombinant heteropolymers of human ferritin H and L chains. J Biol Chem 1993;268:12744–12748.
[26] Regan RF, Chen M, Li Z, Zhang X, Benvenisti-Zarom L, Chen-Roetling J. Neurons lacking iron regulatory protein-2 are highly resistant to the toxicity of hemoglobin. Neurobiol Dis 2008;31:242–249.
[27] Yoshida T, Migita CT. Mechanism of heme degradation by heme oxygenase. J Inorg Biochem 2000;82:33–41.
[28] Bernas T, Dobrucki J. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 2002;47:236–242.
[29] Santambrogio P, Biasiotto G, Sanvito F, Olivieri S, Arosio P, Levi S. Mitochondrial Ferritin expression in adult mouse tissues. J Histochem Cytochem 2007;55:1129–1137.
[30] Campanella A, Rovelli E, Santambrogio P, Cozzi A, Taroni F, Levi S. Mitochondrial ferritin limits oxidative damage regulating mitochondrial iron availability: hypothesis for a protective role in Friedreich ataxia. Hum Mol Genet 2009;18:1–11.
[31] Hebbel RP, Eaton JW. Pathobiology of heme interaction with the erythrocyte membrane. Sem Hematol 1989;26:136–149.
[32] Tenopoulou M, Kurz T, Doulias PT, Galaris D, Brunk UT. Does the calcein-AM method assay the total cellular 'labile iron pool' or only a fraction of it? Biochem J 2007;403: 261–266.
[33] Starke PE, Gilbertson JD, Farber JL. Lysosomal origin of the ferric iron required for cell killing by hydrogen peroxide. Biochem Biophys Res Commun 1985;133:371–379.
[34] Letarte PB, Lieberman K, Nagatani K, Haworth RA, Odell GB, Duff TA. Hemin: levels in experimental subarachnoid hematoma and effects on dissociated vascular smooth muscle cells. J Neurosurg 1993;79:252–255.
[35] Chen M, Chen-Roetling J, Regan RF. Reduced oxidative injury after experimental intracerebral hemorrhage in iron regulatory protein-2 knockout mice. Neuroscience Meeting Planner, Washington, DC: Society for Neuroscience, Online; 2008;757.12.
[36] Xi GH, Keep RF, Hoff JT. Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. J Neurosurg 1998;89:991–996.
[37] Jeong SY, David S. Age-related changes in iron homeostasis and cell death in the cerebellum of ceruloplasmin-deficient mice. J Neurosci 2006;26:9810–9819.
[38] LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, Drake SK, Miller G, Abu-Asab M, Tsokos M, Switzer R 3rd, Grinberg A, Love P, Tresser N, Rouault TA. Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. Nat Genet 2001;27: 209–214.
[39] Gonzalez SC, McMenamin MM, Charlton HM, Goodman J, Lantos T, Simpson C, Wood MJ. Readministration of adenoviral gene delivery to dopamine neurons. Neuroreport 2007;18:1609–1614.
[40] McMenamin MM, Lantos T, Carter EE, Hamilton L, Charlton HM, Gonzalez SC, Wood MJ. Neuropathological consequences of delivering an adenoviral vector in the rat brain. J Gene Med 2004;6:740–750.

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