Recent research on changes in genomic regulation and protein expression in intracerebral haemorrhage

Jason R. Cannon[1]*, Guohua Xi[2], and Richard F. Keep[2,3]

Abstract Intracerebral haemorrhage (ICH) is a devastating form of stroke that accounts for roughly 10% of all strokes and the effects on those that survive are often debilitating. To date, no suitable therapy exists. Recent work has examined alterations in gene and protein expression after ICH. The focus of this review is to outline the current knowledge of changes in genetic and protein expression after ICH and how those changes may affect the course of brain injury. Both animal and human data are reviewed.

Introduction

Spontaneous intracerebral haemorrhage (ICH) is a devastating form of stroke that results in roughly 30–50% mortality. Those that survive often suffer debilitating permanent neurological deficits. Currently, no satisfactory treatment exists (1, 2). A major clinical trial failed to show a benefit for surgical haematoma evacuation, although it is possible that a subset of patients may benefit (3). Similarly, although initially promising (Phase II clinical trial), the use of recombinant activated factor VII (rFVIIa) to prevent haematoma expansion failed to produce long-term benefits (Phase III clinical trial) (4, 5). Thus, there is a continual search for effective therapeutics.

The mechanisms of injury in ICH are becoming better understood and have been recently reviewed (2). While much of the neurological effects of ICH can be likely attributed to the initial mass effect of blood in the brain, several pathogenic events occur after the initial haemorrhage including haema-

toma expansion, midline shift, oedema formation, and blood–brain barrier disruption. Much of the injury that occurs after an ICH appears to be due to clot-derived factors and, therefore, the mechanisms of injury differ from cerebral ischaemia. Identifying changes in genomic regulation and protein expression after ICH may provide additional details of the pathological processes that occur in ICH and aid in the development of new and more effective therapeutics. Indeed, with the advent of microarray technology, identifying genetic changes has become more practical. Recent studies have identified numerous genes that undergo altered regulation after experimental ICH, although it is still much less studied than cerebral ischaemia. Here, the current knowledge of changes in genetic expression and protein alterations that result from clinical and experimental ICH is reviewed. Additionally, the role these discoveries may have in the development of new therapeutics is also discussed.

Genetic expression and protein changes in animal models of ICH

The use of DNA micro-array technology has allowed for the expression of thousands of genes to be tested under experimental conditions for each sample. This technology has recently been applied to a prominent experimental ICH model. Lu *et al.* (6) provided strong evidence that widespread changes in genetic expression are evident after infusion of autologous blood into the rat striatum. Therefore, important regulatory events likely take place in the region of the haematoma, surrounding tissue, and overlying cortex. They found 369 genes were to be regulated by ICH, either in the striatum or in the surrounding cortex (6). The findings in this report prompt the need for further understanding of alterations in genomic expression after ICH. Indeed, only 59 (16%) of the 369 regulated transcripts had been previously reported in ICH studies (Fig. 1a). Many of the regulated genes in the stress response, enzymes/inhibitors, and extracellular matrix categories were somewhat expected and have been reported before. However, many categories of genes were reported for the first

Correspondence: Jason Cannon*, Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, 3501 5th Avenue, 7th Floor BST3-7045/13B, Pittsburgh, PA 15260, USA. Tel: (412) 648-9154; Fax: (412) 648-7029; e-mail: cannonjr@upmc.edu
[1]Pittsburgh Institute for Neurodegenerative Diseases, Department of Neurology, University of Pittsburgh, Pittsburgh, PA 15260, USA
[2]Department of Neurosurgery, University of Michigan, Ann Arbor, MI 48109, USA
[3]Department of Physiology, University of Michigan, Ann Arbor, MI 48109, USA
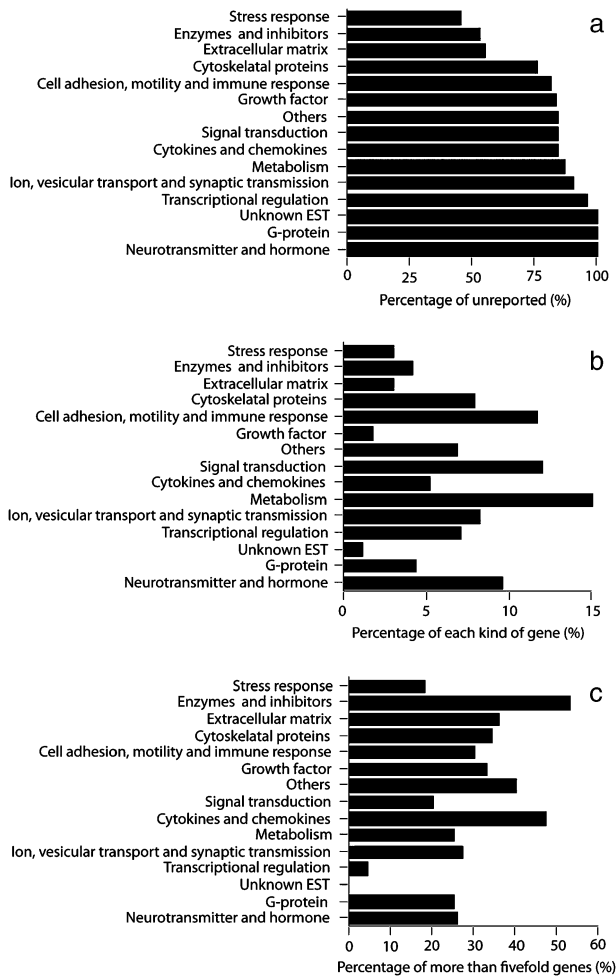
Fig. 1 Trends in genomic regulation after intracerebral haemorrhage (ICH) [summarized from a report by Lu *et al.* (6)]. (a) Percentage of previously unreported genes regulated after ICH by functional category. (b) Percentage genes within each functional category relative to the total number of genes regulated by ICH. (c) Percentage of genes within each category to undergo more than fivefold regulation.

time and contain a significant percentage of total regulated genes (Fig. 1b). Additionally, in many of the categories more than one-third of the regulated genes underwent a greater than fivefold regulation (Fig. 1c). In Lu *et al.* (6), the authors place the genes in functional categories. While genes often fall into multiple categories, it is important to categorize genes into functional categories to understand the broad regulatory changes that occur as a result of ICH. Here, it is useful to discuss what is currently known about changes in expression in genetic functional categories from both this report and previous work. The goal of this review is not to discuss every reported change to mRNA or protein levels that have been reported in experimental or clinical ICH, but, rather to highlight important changes in expression that are likely to have functional effects. To that end, those expression changes that have been corroborated by observed changes in protein levels are given the greatest consideration.

Stress and immune response

This category of genes is the best studied in terms of altered expression after ICH. Perhaps the most prominent of stress proteins studied in ICH are the heat shock proteins (HSPs). These proteins in general are wide ranging in their expression profile, with some being constitutively expressed and others, which are inducible, are expressed at low levels under normal conditions. Induction of HSPs occurs under a wide variety of biological conditions and in considering the effects of induction in ICH, it is important to explore the time-course, cell type, and spectrum of HSPs present. Several of the HSPs have indeed been found to be up-regulated in response to experimental ICH.

HSP70 induction in the brain occurs after a wide variety of insults and serves as an indicator of cell injury (7–10). HSP70 mRNA is increased (∼3-fold) after the infusion of lysed autologous blood 24 h after the lesion (6). Additionally, HSP70 tissue protein levels are increased in both neurons and glia in that model, as observed by immunohistochemistry (11). Lysed blood was required, as whole blood did not exert this effect. Neurons, both adjacent to and spatially remote to the haematoma, exhibited an induction of HSP70. Indeed, HSP70 induction under these circumstances is unsurprising, as it is induced under a wide variety of cellular insults. In humans, plasma levels of HSP70 have been correlated with mortality (12). HSPs are often thought to be a protective response to stress. Thus, the induction of HSP70 before ICH has been found to be protective in a collegenase model of ICH (13).

The inducible haeme oxygenase-1 (HO-1), also referred to as HSP32, converts haeme to biliverdin, carbon monoxide, and iron. HSP32 protein has been found to be upregulated in a rat model ICH in neurons (lysed blood only) and glia (both whole and lysed blood) (11) through immunohistochemistry. The induction of HSP32 protein is also detectable in striatal tissue after experimental ICH by Western blotting (14, 15). In aged rats, which exhibit increased brain oedema and neurological deficits following experimental ICH (autologous blood infusion), enhanced HSP32 induction relative to young rats has been observed (14). Additionally, HSP32 is detectable in white matter of the pig after the infusion of either whole or lysed blood (16).

Increases in HSP32 may serve to increase the metabolism of haeme introduced from lysed blood cells. HSP32 is involved in the clearance of haeme from the brain and, thus, plays a role in haematoma resolution. However, because of the potential for haeme degradation products (e.g. iron) to be neurotoxic, HSP32 activity may induce brain injury. In a pig model of ICH, inhibition of HSP32 activity reduces the haematoma size and oedema volume (17). In the rat, inhibition of HSP32 is protective against haemoglobin-induced brain oedema (18). The effect of HSP32 on ICH-induced brain injury likely depends on the degree of induction and its timing. Induction before the haemorrhage is associated with protection against

oedema formation and neurological deficits resultant from high-dose thrombin or ICH (19–21).

HSP27 is also induced in ICH with tissue mRNA increasing in both the ipsilateral striatum ($\sim$6-fold) and the overlying cortex ($\sim$13-fold) (6). Additionally, HSP27 protein levels are also increased in ICH in both aged and young rats. As with HSP32, induction of HSP27 is increased in aged rats in association with increased neurological deficits and perihaematomal brain swelling (14). However, upregulation of HSP27 protein before administration of high-dose thrombin (5 U) was found to be protective against brain oedema formation (21). Therefore, the effects of HSP induction vary depending on the experimental conditions, particularly the time of induction.

The leakage of blood into the parenchyma elicits an immune response. Part of this response includes the release of cytokines. Indeed numerous genes that are targets of NF-κB activation are increased in ICH (6). The immune response, including an influx of neutrophils and monocytes into the haemorrhage, is a part of the mechanism involved in clot resolution. Indeed, augmentation of microglial-mediated phagocytosis is protective (22). However, as with HSP32, the immune response may also participate in brain injury. Previous reports have indicated that immune system inhibition in experimental ICH is protective (23, 24).

Oxidative stress and oxidative damage occurs in experimental ICH (25, 26). Indeed both mRNA and protein levels of enzymes involved in oxidative stress are increased. Expression of the metal-binding proteins, metallothionein-1, -2 mRNA is increased after ICH (6). NADPH oxidase, a superoxide-producing enzyme, also undergoes increased expression and knockouts are less susceptible to the deleterious effects of ICH (6, 27).

Enzymes and metabolism

After ICH, mRNA of many matrix metalloproteinase (MMP)-related genes are up-regulated (6). Induction of these zinc-dependent endopeptidases is thought to contribute to oedema formation as they degrade the extracellular matrix affecting blood–brain barrier function. In a mouse model of ICH, MMP-9 protein was found to be colocalized with astrocytes surrounding the haematoma region and oedema was reduced in MMP-9 knockouts (28). Interestingly an inhibitor of MMP, tissue inhibitor of metalloproteinase-1 (TIMP-1) also undergoes induction (6), perhaps as a form of compensation. Indeed in clinical ICH baseline serum MMP-9 activity is correlated with perihaematomal volume, while TIMP levels are negatively correlated (29–31). Additionally, increases of both MMPs and their inhibitors tend to occur in the acute phase of injury (30). The authors also found MMP-3 levels to be a predictor of death. The regulation of TIMPs in ICH is also interesting. In humans, TIMP-1 levels increase rapidly, while TIMP-2 levels are decreased compared to controls (30). Indeed, TIMP-2 may provide protection against blood-brain-barrier disruption

(32). In a collegenase model of ICH, mRNA from several MMPs is increased, with the macrophage metalloelastase, MMP-12, being most prominently regulated ($\sim$80-fold) (33).

Several enzymes related to glycolysis undergo regulation following experimental ICH, as evidenced by changes in mRNA (6). The authors report decreases in mRNA expression of rate-limiting enzymes in the haematoma region (phosphofructokinase-M), but increases (type II hexokinase) in the overlying cortex. This corresponds to previously observed decreases in 2-deoxyglucose uptake in the haematoma region, in conjunction with increased uptake in the perihaematomal region (34).

Neurotransmitters and synaptic transmission

The effects of ICH on neurotransmitter systems have received little attention. Interestingly, Lu *et al.* (6) found that in the rat striatal blood injection model, genes associated with multiple neurotransmitters systems were down regulated. Prominent examples included a 15-fold down regulation in an AMPA receptor subunit (Glu-R-C) in the striatum, a 25-fold decrease in dopamine receptor 2 in the cortex, and a 25-fold decrease in the muscarinic cholinergic receptor 1 in the striatum. Neurochemical alterations in this model have also been observed. Ipsilateral and contralateral dopamine levels have been found to be increased in experimental ICH as compared with modest increases in GABA, which may elicit the decrease in receptor expression (35). These changes in glutamate, GABA, and monoamine systems are an exciting finding and may be amenable to therapeutic interventions but much work will be needed to characterize the neuro chemical phenotype that occurs in ICH.

Ion channels and transporters

In their genomic analysis of rat ICH, Lu *et al.* (6) reported a number of changes in ion channel expression. Here, 90% of the genes found to be regulated were previously unreported (Fig. 1a). Most of these genes were down regulated and related to voltage-gated potassium channels. Kcnab2, a gene associated with the voltage-gated potassium channel beta subunit, was found to be down regulated in both micro-array and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Additionally, some other sodium and chloride channels were also down regulated. For these newly reported changes in ion channel mRNA, the functional importance still needs to be determined and Western blotting and immunohistochemistry will be needed to assess the actual impact of these changes. However, ion channel blockers could potentially be employed in an experimental protective approach, when changes are more fully characterized.

Iron-transport-related proteins have been extensively studied in ICH and the role of iron and iron-transport proteins in ICH has been previously reviewed (36, 37). The breakdown of

haeme releases iron, which results in the iron accumulation in the brain. Iron chelation can reduce ICH-induced brain injury in animal models of ICH (38), suggesting that changes in endogenous iron-handling proteins may also affect brain damage. ICH is associated with increases in the iron transport protein transferrin (Tf) and its receptor (TfR), and there are marked increases in ferritin, a naturally occurring iron chelator (15). Increased Tf and TfR before ICH are associated with protection (21).

Additional categories

The genomics study by Lu *et al.* (6) identifies numerous changes in mRNA of additional functional categories after ICH. Changes in signaling proteins, for example, affect a range of processes from growth to inflammation. Again, most of these changes have not been previously reported and will require much further work to assess their involvement in the pathogenesis of ICH (39).

Needs for future research

The regulated genes discussed here present several potential opportunities for therapeutic intervention. For example, receptor agonists or reuptake inhibitors for affected neurotransmitter systems could be tested for impact on behavioral deficits after ICH. Additionally, over-expression of outward-conducting potassium channels has been found to be neuroprotective against excitotoxic lesions (40). Here, it may be that the down regulation of potassium channels contributes to excitoxicity.

The study by Lu *et al.* (6) provides an important step in understanding genomic changes after ICH. Ultimately, new therapeutic pathways may be discovered as a result of these findings. However, much intermediate research will first need to be conducted:

(1) Validation of the micro-array findings of relevant genes will need to be performed.

(2) Analysis of protein levels or conformation changes would provide additional convincing support. It is widely known that changes in genetic expression do not always translate to changes in protein expression. Therefore, to understand the functional changes that occur after ICH it is important to identify changes in protein levels. Here, we have attempted to integrate current knowledge of mRNA and protein changes but much work is still required to determine the extent to which changes in gene expression are reflected in actual changes in protein levels or activity.

(3) The cell types (e.g. neurons, glia, endothelial cells) involved will also give important information on the potential role of these changes. Laser microdissection would allow such an examination.

(4) There is also much work to be done examining the temporal patterns of gene and protein expression. The genomic study of Lu *et al.* (6) looked at a single time point (24 h) after ICH. However, much of the injury that occurs early after an ICH and whether changes in endogenous protein levels affect that injury are still uncertain. Similarly, there may be late changes that affect neuronal plasticity, neurogenesis, and behavioral recovery after an ICH.

(5) Mechanistic research on the implications of altered gene/protein regulation is necessary to identify the role of these changes in pathogenesis and/or the potential for therapeutic modulation. It is often unclear whether some of the changes in gene/protein expression that occur after ICH are beneficial or detrimental. This is exemplified by up-regulation in HSP32 and inflammatory mediators which may be necessary for clot resolution but may also enhance brain injury. Indeed, the effects of such up-regulation may depend on the nature (size/site) of the initial haemorrhage. Thus, inhibitor studies and/or gene knockdown experiments are needed to help define the role of changes in gene/protein regulation.

References

1 Qureshi AI, Tuhrim S, Broderick JP, Batjer HH, Hondo H, Hanley DF: Spontaneous intracerebral hemorrhage. *N Engl J Med* 2001; **344:** 1450–60.

2 Xi G, Keep RF, Hoff JT: Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol* 2006; **5:**53–63.

3 Mendelow AD, Gregson BA, Fernandes HM *et al.* Early surgery versus initial conservative treatment in patients with spontaneous supratentorial intracerebral haematomas in the International Surgical Trial in Intracerebral Haemorrhage (STICH): a randomised trial. *Lancet* 2005; **365:**387–97.

4 Mayer SA: Recombinant activated factor VII for acute intracerebral hemorrhage. *Stroke* 2007; **38:**763–7.

5 Mayer SA, Brun NC, Begtrup K *et al.* Recombinant activated factor VII for acute intracerebral hemorrhage. *N Engl J Med* 2005; **352:** 777–85.

6 Lu A, Tang Y, Ran R, Ardizzone TL, Wagner KR, Sharp FR: Brain genomics of intracerebral hemorrhage. *J Cereb Blood Flow Metab* 2006; **26:**230–52.

7 Ananthan J, Goldberg AL, Voellmy R: Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 1986; **232:**522–4.

8 Gonzalez MF, Shiraishi K, Hisanaga K, Sagar SM, Mandabach M, Sharp FR: Heat shock proteins as markers of neural injury. *Brain Res Mol Brain Res* 1989; **6:**93–100.

9 Li Y, Chopp M, Garcia JH, Yoshida Y, Zhang ZG, Levine SR: Distribution of the 72-kd heat-shock protein as a function of transient focal cerebral ischemia in rats. *Stroke* 1992; **23:**1292–8.

10 Massa SM, Swanson RA, Sharp FR: The stress gene response in brain. *Cereb Brain Metab Rev* 1996; **8:**95–158.

11 Matz PG, Weinstein PR, Sharp FR: Heme oxygenase-1 and heat shock protein 70 induction in glia and neurons throughout rat brain after experimental intracerebral hemorrhage. *Neurosurgery* 1997; **40:** 152–60; discussion 60–2.

12 Fang HY, Ko WJ, Lin CY: Inducible heat shock protein 70, interleukin-18, and tumor necrosis factor alpha correlate with outcomes in spontaneous intracerebral hemorrhage. *J Clin Neurosci* 2007; **14:** 435–41.

13 Sinn DI, Chu K, Lee ST *et al.* Pharmacological induction of heat shock protein exerts neuroprotective effects in experimental intracerebral hemorrhage. *Brain Res* 2007; **1135:**167–76.

14 Gong Y, Hua Y, Keep RF, Hoff JT, Xi G: Intracerebral hemorrhage: effects of aging on brain edema and neurological deficits. *Stroke* 2004; **35:**2571–5.

15 Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G: Iron and iron-handling proteins in the brain after intracerebral hemorrhage. *Stroke* 2003; **34:**2964–9.

16 Wagner KR, Packard BA, Hall CL *et al.* Protein oxidation and heme oxygenase-1 induction in porcine white matter following intracerebral infusions of whole blood or plasma. *Dev Neurosci* 2002; **24:**154–60.

17 Wagner KR, Hua Y, de Courten-Myers GM *et al.* Tin-mesoporphyrin, a potent heme oxygenase inhibitor, for treatment of intracerebral hemorrhage: in vivo and in vitro studies. *Cell Mol Biol (Noisy-le-Grand, France)* 2000; **46:**597–608.

18 Huang FP, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT: Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products. *J Neurosurg* 2002; **96:**287–93.

19 Xi G, Hua Y, Keep RF, Hoff JT: Induction of colligin may attenuate brain edema following intracerebral hemorrhage. *Acta Neurochir* 2000; **76**(Suppl)**:** 501–5.

20 Xi G, Keep RF, Hua Y, Hoff JT: Thrombin preconditioning, heat shock proteins and thrombin-induced brain edema. *Acta Neurochir* 2000; **76**(Suppl)**:** 511–5.

21 Xi G, Keep RF, Hua Y, Xiang J, Hoff JT: Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke* 1999; **30:**1247–55.

22 Zhao X, Sun G, Zhang J *et al.* Hematoma resolution as a target for intracerebral hemorrhage treatment: role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. *Ann Neurol* 2007; **61:**352–62.

23 Peeling J, Yan HJ, Corbett D, Xue M, Del Bigio MR: Effect of FK-506 on inflammation and behavioral outcome following intracerebral hemorrhage in rat. *Exp Neurol* 2001; **167:**341–7.

24 Xi G, Hua Y, Keep RF, Younger JG, Hoff JT: Systemic complement depletion diminishes perihematomal brain edema in rats. *Stroke* 2001; **32:**162–7.

25 Hall NC, Packard BA, Hall CL, de Courten-Myers G, Wagner KR: Protein oxidation and enzyme susceptibility in white and gray matter with in vitro oxidative stress: relevance to brain injury from intracerebral hemorrhage. *Cell Mol Biol (Noisy-le-Grand, France)* 2000; **46:** 673–83.

26 Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G: Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. *Brain Res* 2002; **953:**45–52.

27 Tang J, Liu J, Zhou C *et al.* Role of NADPH oxidase in the brain injury of intracerebral hemorrhage. *J Neurochem* 2005; **94:**1342–50.

28 Tejima E, Zhao BQ, Tsuji K *et al.* Astrocytic induction of matrix metalloproteinase-9 and edema in brain hemorrhage. *J Cereb Blood Flow Metab* 2007; **27:**460–8.

29 Abilleira S, Montaner J, Molina CA, Monasterio J, Castillo J, Alvarez-Sabin J: Matrix metalloproteinase-9 concentration after spontaneous intracerebral hemorrhage. *J Neurosurg* 2003; **99:**65–70.

30 Alvarez-Sabin J, Delgado P, Abilleira S *et al.* Temporal profile of matrix metalloproteinases and their inhibitors after spontaneous intracerebral hemorrhage: relationship to clinical and radiological outcome. *Stroke* 2004; **35:**1316–22.

31 Silva Y, Leira R, Tejada J, Lainez JM, Castillo J, Davalos A: Molecular signatures of vascular injury are associated with early growth of intracerebral hemorrhage. *Stroke* 2005; **36:**86–91.

32 Rosenberg GA, Kornfeld M, Estrada E, Kelley RO, Liotta LA, Stetler-Stevenson WG: TIMP-2 reduces proteolytic opening of blood-brain barrier by type IV collagenase. *Brain Res* 1992; **576:**203–7.

33 Power C, Henry S, Del Bigio MR *et al.* Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. *Ann Neurol* 2003; **53:**731–42.

34 Ardizzone TD, Lu A, Wagner KR, Tang Y, Ran R, Sharp FR: Glutamate receptor blockade attenuates glucose hypermetabolism in perihematomal brain after experimental intracerebral hemorrhage in rat. *Stroke* 2004; **35:**2587–91.

35 Cannon JR, Nakamura T, Keep RF, Richardson RJ, Hua Y, Xi G: Dopamine changes in a rat model of intracerebral hemorrhage. *Acta Neurochir* 2006; **96**(Suppl)**:** 222–6.

36 Hua Y, Keep RF, Hoff JT, Xi G: Brain injury after intracerebral hemorrhage: the role of thrombin and iron. *Stroke* 2007; **38**(2 Suppl)**:** 759–62.

37 Wagner KR, Sharp FR, Ardizzone TD, Lu A, Clark JF: Heme and iron metabolism: role in cerebral hemorrhage. *J Cereb Blood Flow Metab* 2003; **23:**629–52.

38 Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, Xi G: Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *J Neurosurg* 2004; **100:**672–8.

39 Wang J, Dore S: Inflammation after intracerebral hemorrhage. *J Cereb Blood Flow Metab* 2007; **27:**894–908.

40 Lee AL, Dumas TC, Tarapore PE *et al.* Potassium channel gene therapy can prevent neuron death resulting from necrotic and apoptotic insults. *J Neurochem* 2003; **86:**1079–88.