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## Vascular Dysfunction in Brain Hemorrhage: Translational Pathways to Developing New Treatments from Old Targets

Paul A. Lapchak<sup>1,\*</sup> and Qiang Wu<sup>2</sup>

<sup>1</sup>Director of Translational Research, Cedars-Sinai Medical Center, Department of Neurology, Davis Research Building, D- 2091, 110 N, George Burns Road, Los Angeles, CA 90048 USA

<sup>2</sup>Project Scientist, Cedars-Sinai Medical Center, Department of Neurology, Davis Research Building, D-2094E, 110 N. George Burns Road, Los Angeles, CA 90048 USA

### Abstract

Hemorrhagic stroke which is a form of stroke that affects 20% of all stroke patients is a devastating condition for which new treatments must be developed. Current treatment methods are quite insufficient to reduce long term morbidity and high mortality rate, up to 50%, associated with bleeding into critical brain structures, into ventricular spaces and within the subarachnoid space. During the last 10–15 years, significant advances in the understanding of important mechanisms that contribute to cell death and clinical deficits have been made. The most important observations revolve around a key set of basic mechanisms that are altered in brain bleeding models, including activation of membrane metalloproteinases, oxidative stress and both inflammatory and coagulation pathways. Moreover, it is now becoming apparent that brain hemorrhage can activate the ischemic stroke cascade in neurons, glial cells and the vascular compartment. The activation of multiple pathways allows comes the opportunity to intervene pharmacologically using monotherapy or combination therapy. Ultimately, combination therapy or pleiotropic compounds with multi-target activities should prove to be more efficacious than any single therapy alone. This article provides a comprehensive look at possible targets for small molecule intervention as well as some new approaches that result in metabolic down-regulation or inhibition of multiple pathways simultaneously.

### Keywords

Antioxidant; Matrix membrane metalloproteinase; Factor VIIa; Hemostasis; Subarachnoid hemorrhage; Normobaric oxygen; Hyperbaric oxygen; Hypothermia; Hydrogen gas

### Brain Hemorrhage: Incidence and Pathophysiology

Brain hemorrhage stroke occurs in approximately 20% of all patients with a stroke [1–3]. There are two main categories of hemorrhagic stroke, intracranial hemorrhage including epidural hematoma, subdural hematoma and subarachnoid hemorrhage (SAH), and intracerebral hemorrhage (ICH) including intraparenchymal hemorrhage (IPH) and

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\*Corresponding author: Dr. Paul A. Lapchak Ph.D,FAHA, Director of Translational Research, Cedars-Sinai Medical Center, Department of Neurology, Davis Research Building, D- 2091, 110 N, George Burns Road, Los Angeles, CA 90048 USA, Tel: 310-248-8188, Fax: 310-248-7568, Paul.Lapchak@cshs.org.

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intraventricular hemorrhage (IVH). The terms ICH and hemorrhagic stroke are often used interchangeably, but they can be distinguished from hemorrhagic transformations normally associated with ischemic stroke.

ICH is associated with a rapid and higher mortality rate than ischemic stroke and substantial brain damage occurs through multiple mechanisms. The 30-day mortality rate for ischemic stroke is 8–12%, whereas hemorrhagic stroke is estimated to be around 50% [4–7]. Moreover, despite presenting with similar focal neurologic deficits, patients with hemorrhagic stroke tend to be more severe than patients with ischemic stroke and less than 20% of hemorrhagic stroke patients regain functional independence. There are still no acceptable specific treatments for most types of ICH [7,8]. Patients with ICH may require neurosurgical evaluation to detect and repair the source of bleeding, and remove the clot to prevent expansion and secondary repercussions such as ischemic damage.

There are many causes for ICH, which generally occurs in small arteries or arterioles and is commonly due to hypertension, intracranial vascular malformations or cerebral amyloid angiopathy [9]. The pathophysiology of ICH is triggered by bleeding directly into the brain parenchyma, often as a result of leakage from small intracerebral arteries that have been compromised. Vascular damage due to cerebral amyloidosis primarily affects the elderly and represents up to 10% of hemorrhagic strokes. In addition, commonly used therapeutics such as anticoagulants, platelet inhibitors [10,11] and thrombolytics (tissue plasminogen activator; tPA, Alteplase™) typically used to treat acute myocardial infarction (AMI) or acute ischemic stroke (AIS), in particular, can lead to iatrogenic hemorrhagic stroke and a prior history of stroke, per se, also represents an important risk factor for ICH [12].

## Preclinical Models and Translational Studies

An extensive amount of funding has been dedicated to preclinical research studies to identify mechanisms involved in ICH and to subsequently test strategies in animal models, not only for ICH reduction, but also for clinical improvement. Three of the most commonly used animal models are briefly reviewed here: collagenase injection model, whole-blood model and embolization-induced hemorrhage. There is significant scientific value in using a combination of 2 or more of the models to develop mechanism-based treatments strategies to be applied to stroke patients.

The collagenase model has been used by many leading hemorrhage groups including Lyden and colleagues [13–15] and Rosenberg et al. [16–18]. Briefly, the injection of the membrane protease, collagenase into the striatum results in dissolution of the membrane matrix that supports cells resulting in the formation of a hematoma. It appears that Type IV collagen in the basal lamina is the substrate for the bacterial collagenase used in most studies [19,20]. This model has been used by many research laboratories as a model of ICH or SAH [13–16,18,19,21–26]. Many investigators target cortical substructures such as the caudate putamen in this model and doing so allows for a behavioral function measures due to a lesion in the nigrostriatal pathway. A lesion in the pathway produces neuronal degeneration and behavioral asymmetry that can be detected in rodents as rotational behavior. The advantages allow investigator to study treatments that can limit the collagenase-induced bleeding response and hematoma expansion. The model is particularly relevant to the clinical condition because continued bleeding occurs in 14–20% of all ICH patients and lasts for over 6 h in 17% of cases [27,28]. However, the drawbacks are that there is no underlying vascular pathology, and bleeding results from the rupture of many vessels, whereas rupture of a small, deep-penetrating artery in humans.

In the whole-blood or blood product injection model, an animal's own blood (autologous) or donor blood (non-autologous) is injected directly into the brain tissues [13–16,18,21–24,29–

37], also using the caudate putamen in many cases. Zhang and colleagues have successfully used the model to identify target mechanisms for treatment [38–41]. The advantage of the whole-blood model is that only blood is introduced into the model system. The drawbacks are the lack of underlying vascular pathology and vessel rupture, which is normally seen in the human stroke population.

An embolism-based model has also been used to study interventions that can reduce ICH. The rabbit large clot embolism model (RLCEM) has been used extensively to decipher mechanisms involved in hemorrhage and for the preclinical development of pharmacological strategies that may reduce embolism- and tPA-induced hemorrhage [5,42–47]. The favorable characteristics of the RLCEM include the study of 3 types of ICH: hemorrhagic infarction, petechial hemorrhage and parenchymal hemorrhage (PH) [45] regardless of whether or not thrombolytics are administered. The symptomatic PH defined as large collections of blood in the parenchyma without intervention into brain tissue, originally is thought to occur consequent to reperfusion into a devitalized artery [48]. In the model, no specific structure can be targeted because the clot is injected through an indwelling carotid catheter and becomes lodged in one or more vessels in brain, depending on whether the clot remain intact or fragments. Most often, the result is blockage of the middle cerebral artery (MCA), which causes a large reduction of cerebral blood flow to one brain hemisphere. In essence, the RLCEM is an embolic stroke model with a significant hemorrhage component. This model has been used by many research laboratories as a model of ICH and tPA-induced ICH [44–47,49,50].

## Inflammatory Reactions and Vascular Damage Coagulation factors

As a consequence of brain hemorrhage, enzymes involved in blood clotting and clot lysis are produced [47,51–53]; however, many of them have potential toxicities. Primary hemostasis is initiated when vascular injury triggers adherence of platelets to proteins within in the vascular endothelium using glycoprotein (GP) Ia/IIa receptors. This is followed by platelet aggregation by means of docking proteins or sites to form the primary hemostatic clot. The sequence of platelet aggregation is mediated by von Willebrand factor, which forms links between the platelet GP Ib/IX/V and collagen [54–56]. Once they bind and are thereby activated, platelets undergo structural and conformational changes and begin platelet-platelet aggregation via the GP IIb/IIIa receptors. Secondary hemostasis, which is comprised of two specific pathways, the “intrinsic” or “contact activation” pathway and the “extrinsic” or “tissue factor” pathway, results in local activation of plasma coagulation factors and the generation of a fibrin clot that reinforces the platelet aggregate. The classical model of blood coagulation involves a complex “cascade” of zymogen activation reactions involving coagulation factors, 6 in the intrinsic pathway (factors VIII, IX, XI, XII, prekallikrein, and high-molecular weight kininogen), 1 in the extrinsic pathway (factor VII), and 4 in a common pathway (factors II, V, X, and fibrinogen). The main role of the extrinsic pathway is to generate thrombin to convert soluble fibrinogen into insoluble strands of fibrin which is the backbone of a clot. Following damage to the blood vessel, Endothelium Tissue Factor (ETF) is released, forming a complex with Factor VIIa, which then activates Factor IX and X. Factor VII is a coagulation factor in the extrinsic coagulation pathway and is part of a series of hemostatic defense mechanisms [55,56]. Factor VII itself can also be activated by thrombin leading to a cyclic complex including the activation of prothrombin to thrombin. Factor VIIa is present in excess of any other activated coagulation factor could be a potential target for ICH therapy. However, clinical results in hemorrhage trials with Novoseven have been quite disappointing [57], primarily because there was an increased incidence of thromboembolism causing ischemic damage and clinical deficits.

## Thrombin

Thrombin is an essential component of the coagulation cascade and it is produced in the brain immediately after ICH. Recent research has suggested that thrombin is a pleiotropic molecule that causes vascular damage, an inflammatory response, oxidative stress, and also has direct cellular toxicity, which is mediated in part by protease activated receptors (PARs) [58–60]. Some studies have noted that thrombin at high concentrations can activate potentially harmful pathways [51,61,62]. Direct infusion of large doses of thrombin into the brains causes inflammatory-cell infiltration and brain edema formation partly due to the direct opening of the blood brain barrier (BBB). A recent key study [63] showed that thrombin mediates endothelial permeability and the introduction of thrombin in brain causes vascular damage, a result that can be blocked by the small molecule direct thrombin antagonist argatroban. Moreover, thrombin stimulates PARs expressed on microglia/macrophages to activate these cells via recruitment of mitogen-activated protein kinases (MAPKs), and produces several inflammatory mediators, which contribute to edema formation through disruption of the BBB [61]. Similarly, another recent study has suggested that thrombin induced edema is mediated by stimulating PARs to activate src family kinases, which are a family of protooncogene tyrosine kinases [64]. Src family kinase members mediate BBB permeability changes and brain edema by phosphorylating membrane metalloproteinases (MMP's), tight junction proteins and other BBB-related proteins [65]. Thus, these kinases may be appropriate targets for further development to attenuate the downstream detrimental effects of thrombin.

## Cytokines

The major inflammatory cells that are activated and accumulate within the brain after ICH are blood-derived leukocytes, macrophages, and resident microglia [66,67]. Microglia is believed to be the first non-neuronal cells to react following CNS injury. After brain injury, microglia are activated to undergo morphologic including upregulation of pro-inflammatory cytokines, migration, proliferation and phagocytic behavior [68]. Microglia, “brain macrophage”, which when activated release a variety of cytokines [69–71], reactive oxygen species (ROS) [72–74], and other potentially toxic factors, suggesting that activated microglia/macrophages might contribute to hemorrhage-induced early brain injury [26,75]. Infiltrating leukocytes are also believed to play a role in ICH-induced brain injury. Neutrophils are the earliest leukocyte subtype to infiltrate into the hemorrhagic brain, and these may damage brain tissue directly also by producing ROS, releasing proinflammatory proteases [76], and modulating BBB permeability [77]. Substantial evidences have suggested that leukocytes/macrophages and activated microglia are major CNS sources of cytokines, chemokines, prostaglandins, proteases, and other immunoactive molecules after ICH [66,78–81].

Many investigators have studied cytokine changes after ICH and have shown that two primary molecules, TNF- $\alpha$  and IL-1 $\beta$  are elevated in various experimental models of brain injury. TNF- $\alpha$  is a pleiotropic cytokine that received a lot of attention because it has multiple biologic activities that are temporally organized including the stimulation of acute phase protein secretion, vascular permeability and post-ICH brain edema formation [82,83]. TNF- $\alpha$  expression is increased in response to ICH when presented with either autologous blood or thrombin [83,84,85]. TNF also mediates hemorrhage in brain following embolic strokes [86]. TNF and its family of receptors represent possible interventional targets to reduce hemorrhage damage or hemorrhage-induced ischemic damage [87,88]. Like TNF, there is a persistent increase in IL-1 $\beta$  observed in brain following autologous blood injection [80,89]. Similarly, expression of the TNF receptor and IL-1 $\beta$  were both upregulated following intrastriatal blood infusion [90]. TNF and its family of receptors and IL-1  $\beta$  represent

possible interventional targets to reduce hemorrhage damage or hemorrhage-induced ischemic damage [87,88].

### Free radicals and vascular damage

Free radicals have been a focal point in the development of treatments for stroke. They are reactive molecules that have one or more unpaired electrons. These reactive species often are divided into two groups, ROS and reactive nitrogen species (RNS). ROS usually refers to superoxide, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical, singlet oxygen, whereas an RNS can include nitric oxide (NO) and peroxynitrite. Free radicals and their related non-free radical reactive species have been implicated in stroke pathophysiology as an important contributor to cell and tissue injury [91–93]. Increased levels of free radicals can cause damage to virtually all cellular components, including DNA, lipids, and proteins, which then leads to injury of neurons, glial cells, blood vessels and the vasculature. Free radicals can exert effects directly on cells and also interact with various cellular molecular pathways which contributes to the development of brain edema and cell death [66].

ICH is also associated with robust induction of heme oxygenase (HO) in microglia/macrophages, and the enzyme catalyzes degradation of heme into iron, carbon monoxide (CO), and biliverdin, which is then converted to bilirubin by biliverdin reductase [94]. ICH causes iron accumulation in the brain and non-heme iron has been shown to increase threefold after ICH in rats [95]. Furthermore, intracerebral infusion of iron causes brain injury and deferoxamine reduces ICH-induced brain damage, suggesting that iron plays an important part in brain injury after ICH [96–98]. Iron and other products contribute to pathological changes such as increase in oxidative stress, formation of edema, infiltration of neutrophils, and induction of neuron death [61]. Moreover, Iron and iron-related products catalyze hydroxyl radical production and lipid peroxidation [99,100], which expose the brain cells to increased levels of oxidative stress. The production of ROS is an inevitable consequence of normal oxidative metabolism, but high ROS levels can be lethal [101], [102,103] and must be regulated because they are involved in a series of processes such as contributing to brain edema by triggering the induction and activation of MMP family members both directly and indirectly [92]. The direct process may involve the oxidation or nitrosylation of MMP, resulting in MMP activation [104]. The indirect process may involve redox-sensitive elements of transcription factors such as nuclear factor kappa-light-chain enhancer of activated B cells (NF- $\kappa$ B) and activator protein 1 (AP-1), which is known to be an integral part of the binding sites for MMP transcription [105,106]. Excessive hydrogen peroxide production has been suggested to induce MMP-1 mRNA expression in fibroblasts, and sublethal exposure to hydrogen peroxide has been found to increase the expression and activation of MMP-2 in human endothelial cells [107]. The treatment of fibroblasts with xanthine/xanthine oxides results in the induction of MMP-2 and MMP-9; furthermore, superoxide-stimulated extracellular signal-regulated kinase activation has been shown to mediate MMP-9 induction in vascular smooth muscle cells [108]. Studies using genetically manipulated mice have suggested that superoxide and/or hydrogen peroxide are involved in the induction and activation of MMPs [109]. These studies have strongly implied that superoxide and/or hydrogen peroxide might mediate BBB disruption through the activation of MMPs. Studies have also shown that excessive production of superoxide radicals result in increased water and sodium content in the brain and the extravasation of Evans blue, suggesting the development of vasogenic edema [110]. Moreover, the superoxide radical has been identified as the primary radical involved in increased vascular permeability and edema development in various disease models [111]. Based on the amount of evidence, it is clear that free radicals, and the resulting oxidative stress, are involved in BBB disruption and brain injury after stroke. The challenging task in the future is to how to attenuate free radical damage at the cellular and molecular level.



## Membrane metalloproteinases in vascular damage: a potential drug target

This section will continue with the theme of MMPs, which are a group of important proteolytic enzymes whose catalytic mechanism involves a metal. MMPs are zinc- and calcium-dependent endopeptidases which are known to degrade many components of the extracellular matrix (ECM) including fibronectin, laminin, proteoglycans and type IV collagen [112,113]. They have been categorized into four groups of enzymes based on protein structure: collagenases, stromelysins, gelatinases, and membrane-type MMPs [114]. These enzymes are secreted as a latent form, but once they become activated they regulate many physiological and pathological processes. Cytokines, such as TNF $\alpha$  and IL-1 induce the transcription of MMP3 and MMP9 which are important in both acute and chronic neuroinflammation. In addition, several activation mechanisms have been suggested including other proteases and free radicals [104,115]. The activity of MMPs might be controlled by free radicals, either through activation of the latent forms or by induction of mRNA through signaling via the nuclear factor-kappaB site [116].

Many investigators have emphasized the role MMP-2, 3, 9, and 12 in ICH [18,22,25,75,90,117]. Firstly, a study has demonstrated that activation of MMP-2 and MMP-9 was increased after collagenase-induced ICH in rats [18]. Subsequently, another study has indicated that brain MMP2, 3, 7, 9 and 12 mRNA levels were increased in collagenase-induced hemorrhage rat model [75]. Furthermore, a dramatic increase of MMP-9 activity was observed by gel and *in situ* zymography in collagenase-induced hemorrhage rat model [22]. Moreover, early increases in MMP-9 mRNA and activity have been confirmed in other animal ICH models including mouse [25] and pig [117]. The findings are consistent and show that MMP's were regulated by hemorrhage and also played a crucial role in vascular damage. Similarly, several clinical studies have reported an evaluation of MMP-9 levels in blood of patients with ICH [118–120]. Taken together, these data support the view that some of the MMPs, especially MMP-3, 9, and 12, might play an important role in the pathophysiology of ICH.

Cellular components of the BBB include endothelium, pericytes/ microglia and the perivascular astrocytic processes. Previous studies have shown that astrocytes, neurons, oligodendroglia, endothelial cells, pericytes, and microglia produce MMPs [121,122]. Mixed microglia and astrocytes in culture produce an active form of MMP-9, whereas astrocytes alone in culture induces proMMP-9 but failed to produce the active form of MMP-9. Moreover, immunostaining of mixed glial cultures showed that MMP-3 was expressed by microglia, but not by astrocytes. This finding suggested that the microglia-derived MMP-3 was critical for activation of the MMP-9 during the inflammatory response. Activation of the proMMPs is a critical step in the proteolytic process. In cell cultures, microglia interact with the pericytes, endothelial cells and astrocytes to activate MMPs. Therefore, microglia may be necessary for the activation of the proMMP-9 which could be done by MMP-3.

As mentioned above, inflammatory mediators, such as TNF- $\alpha$  and IL-1 $\beta$ , induce the production of the MMPs. In addition, activation processes of MMPs involve proteases and free radicals. Therefore, microglia and tissue macrophages play a critical role in the inflammatory response both by releasing the MMPs and by forming molecules that activate them. Previous studies have shown that TNF- $\alpha$  stimulates cells to produce active MMPs, which facilitate leukocyte extravasation and brain edema by degradation of extracellular matrix components and the opening of the BBB that could be blocked by the use of the MMP inhibitor [123]. Astrocytic end feet and tight-junctioned endothelial cells act in concert with the basal lamina and the pericytes/microglia to form BBB as a neurovascular unit. The tight junctions of the endothelial cells comprise the first line of defense. Basal lamina forms a layer around the endothelial cells to provide a charged barrier and may

impede diffusion of larger molecules. MMPs affect the function of the neurovascular structures by degrading the components of the basal lamina around the cerebral vessels to increase the permeability of the BBB, thereby contributing to brain edema and hemorrhagic brain injury [18,92]. Overall, MMPs cause increase in permeability of the BBB by targeting the matrix proteins, resulting in the final common pathway downstream of acute neuroinflammatory damage to induce the vasogenic edema. Research evidence suggests that the development and use of specific MMP inhibitors may reduce hemorrhage expansion, vascular damage and ischemic damage.

### Potential targets to treat brain hemorrhage

Some important, but very preliminary steps toward therapeutics for hemorrhage have been made using preclinical animal models. Therapies aimed at reducing the cascade of injuries surrounding a hemorrhage may reduce the deleterious effects of hemorrhage including edema, apoptotic and necrotic cell death. As describe above, there are many key processes involved in hemorrhage and the deficits that occur subsequent to hemorrhage. Inflammatory reactions, free radicals, BBB damage and edema are primary targets for useful hemorrhage treatments. This section will identify a few targets that should be further pursued.

### Anti-inflammatory drugs

When ICH occurs, blood components including erythrocytes, leukocytes, macrophages, and plasma proteins (thrombin and plasmin) immediately enter the brain. Therefore, brain tissue injury occurs after an inflammatory reaction which comprises both cellular and molecular components. Anti-inflammatory strategies have been tested in previous studies. As discussed above, there are detrimental effects of microglial activation in ICH-induced brain injury [124]. Therefore, it seems that there exists the therapeutic potential of treatments based on the inhibition of microglial activation shortly after the onset of ICH. In previous studies, tuftsin fragment 1–3 macrophage/microglial inhibitory factor (MIF) inhibited microglial activation and macrophage infiltration following collagenase-induced ICH [26,74]. The treatment also reduced stroke injury volume and improved behavior. These findings further support that microglial activation promotes inflammatory reactions after ICH and MIF could be a valuable neuroprotective agent for the treatment of ICH. Furthermore, another study using a rodent ICH model showed that antileukocyte intervention reduced neutrophil infiltration, behavioral deficits and neuronal damage [125]. Therefore, the strategies targeting leukocytes and microglial activation may merit further evaluation either as alternative or adjunctive therapeutic approach to ICH.

### TNF and MMP's

Activation of cytokines is an important component in the cascade of events that lead to damage following a stroke. Mature pro-TNF $\alpha$  can be cleaved to biologically active TNF $\alpha$  by several membrane MMP's, one which is known as TNF-alpha-converting enzyme (TACE) [126– 130]. While MMP's are involved in the processing of pro-TNF $\alpha$ , mature TNF $\alpha$  can also induce MMP-9 in the CNS [16,18,21,131,132] which perpetuates the cycle of TNF $\alpha$  production that can result in membrane damage [122]. Synthesis of TNF $\alpha$  from pro-TNF $\alpha$  is blocked by MMP inhibitors like BB-2284 [133], BB-94 [134–136] as well as specific TACE inhibitors such as Ro32–7315[137] and DPH-067517[138]. A previous study showed that ICH can be reduced by administration of a non-specific MMP inhibitor BB-94 in a rat collagenase model [18]. In the RLCEM, the MMP inhibitor BB-94 also lowered the rate of tPA-induced hemorrhage, while not affecting significantly hemorrhage rate in the absence of tPA administration [45]. It is interesting to note that BB-94 could reduce TNF $\alpha$  levels in rabbit brain even though BB-94 is a non-selective MMP inhibitor [139]. Also, other groups have reported that BB-94 can reduce tissue levels of TNF $\alpha$  [134–136]. The reduction in hemorrhage rate consequent to BB-94 administration is consistent with the previously

described role of MMP's as important factors in the BBB vasculature function and extracellular matrix remodeling following a stroke [121] but may also expand the roles of processing TNF $\alpha$ . Of particular promise are MMP-9 inhibitors that have recently been shown to reduce brain injury and apoptosis following SAH [140,141].

There is some evidence in ischemic stroke and hemorrhage patients that MMP-2 and MMP-9 may be involved either BBB breakdown or remodeling following the injury. In stroke patients, serum MMP-2 and MMP-9 levels increased during the course of ischemia [142,143]. In SAH patients serum MMP-2 levels from SAH patients MMP-2 levels are significantly decreased while MMP-9 levels are increased relative to controls. MMP-2 levels remain decreased 12 days post SAH, but MMP-9 levels appeared to recover [142,143]. There is also evidence linking MMP-2 to SAH from intracranial aneurysm rupture[144], which substantiates the hypothesis that MMP-2 and/or MMP-9 may be directly involved in the progression of stroke and hemorrhage. In a post-mortem study, there was increased endothelial expression of both MMP-2 and MMP-9 suggesting that endothelial expression of MMPs may affect vascular matrix stability and contribute to hemorrhage [145]. The results from preclinical studies suggest that MMP's and possibly TNF $\alpha$  are directly involved in blood barrier breakdown and hemorrhage in brain following a variety of insults. MMP-2, MMP-9 and TACE are all valid targets for the development of small molecules to reduce spontaneous ICH, SAH or hemorrhage resulting from the administration of thrombolytics.

Interleukin-1 has gained much more attention as a therapeutic target for stroke because extensive evidences support the direct involvement of interleukin-1 in the neuronal injury that occurs in acute neurodegeneration [146]. Extensive evidences have shown that inhibiting IL-1 release or activity markedly reduces ischemic cerebral and cerebral hemorrhagic damage [147]. Moreover, previous studies have reported that over expression of IL-1ra attenuated brain edema formation and thrombin-induced intracerebral inflammation in a rat autologous blood injection model of ICH [148,149]. Therefore, these studies suggested that IL-1ra could be considered as a potential therapeutic agent for patients with ICH and could be the focus of additional preclinical and clinical research.

### Free radical scavengers

Free radicals have been proposed to mediate an array of injuries following a stroke [150–156]. Reactive oxygen species cause brain injury via many different pathways. Compounds that can counteract or reverse the effects of free radicals have received a great deal of attention in recent years. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent lipid soluble hydroxyl and peroxy radical scavenger used clinically for treatment of ischemic stroke in Japan. Previous study has shown that edaravone attenuated ICH-induced brain edema, neurologic deficits, and oxidative injury and also reduced iron- and thrombin-induced brain injury, suggesting that edaravone is a potential therapeutic agent for ICH [157]. Moreover, the edaravone clinical trial [158] reported that there was a significant improvement in functional outcome as evaluated using the modified Rankin Scale, when treatment was started within 72 hr of onset. Furthermore, another study reported preliminary findings of a clinical trial showing that patients treated with edaravone prior to administration of intravenous tPA had a reduced incidence of ICH compared with placebo-treated tPA-treated patients [159]. Thus, edaravone, or other lipophilic free radical scavengers may be useful to treat either acute ischemic stroke or hemorrhagic stroke

Also, other studies have attempted to target pro-oxidant heme or iron to recue a potential source of ROS production during hemorrhage. It is postulated that the regulation of HO might decrease ICH-induced toxicity because the enzyme metabolizes heme to release iron [160]. Several studies have also shown that nonselective inhibitors of HO (tin-mesoporphyrin IX, tin-protoporphyrin, and zinc protoporphyrin) decreased ICH-induced



brain edema and neurologic deficits [30,97,161,162]. Moreover, a ferric iron chelator (Deferoxamine) was shown to have a similar neuroprotective effect after ICH [96,163], suggesting that ROS could be a potential target for ICH therapy. Furthermore, the effect of melatonin, a potent antioxidant and freeradical scavenger, on outcomes was investigated in rat collagenase-induced ICH model. The results showed that brain edema and neurological function at 24 h were unchanged in spite of oxidative stress reductions. However, repeated treatment with the lower dose of melatonin (5 mg/kg) given at 1 h and every 24 h thereafter for 3 days after ICH, led to normalization of striatal function, norNew treatment possibilitiesmalized memory tasks and reduced brain atrophy, suggesting that melatonin is safe for use after ICH and is protective [164].

### **New treatment possibilities**

The following section will review new pharmacological agents still in early stages of development. However, since some of the most important recent advances have been non-pharmaceutical in nature, they will be highlighted below. All of the novel treatments have the potential to reduce hemorrhage and may be the hemorrhage treatment of the future.

### **Brain Hypothermia (BH)**

The secondary consequences of ICH including inflammation, edema, and oxidative damage all contribute to cell death after ICH. Preclinically, brain hypothermia (BH) has been used as an effective neuroprotective treatment in experimental brain ischemia and traumatic brain injury [165–168]. Before reviewing the intriguing results, BH must be defined. BH is now classified by the depth of cooling below normal body temperature (i.e. 37–38°C). Mild hypothermia reduces body temperature by 3–6°C, whereas deep hypothermia reduces body temperature by 10°C [169]. Although there is no consensus on an optimal temperature, the largest neuroprotective benefit is obtained with 34°C [170]. Neuroprotection by mild hypothermia is associated with mitochondrial preservation and suppression of apoptosis. Pathophysiological mechanisms involved in the beneficial effects of hypothermia are being elucidated [171]. Evidence suggests that significant metabolic downregulation including attenuation of the inflammatory response and reduced reactive oxygen species may be the basis for neuroprotection [171,172]. Suppression of both can reduce the activity of MMP's to prevent BBB damage and edema [173,174]. Mild BH significantly reduces the brain edema formation after ICH and several neuroprotective mechanisms including reduced BBB disruption, inflammation and oxidative damage are suggested in this study [175]. Because preclinical studies are promising, the recommendation by Groysman [176], should be followed. Clearly, trials are warranted to define the specific operating conditions for effective hypothermia including the therapeutic window and temperature regimen [169]. Clinical trials of hypothermia for stroke (ICTuS-L trial) have had mixed results. A recent study showed the feasibility of hypothermia in stroke patients, but there were significant side effects related to the treatment. Pneumonia occurred in 50% of hypothermic patients compared to 10% of normothermic patients ( $p=0.001$ ); however, there was an almost equal number of deaths in both groups ( $p>0.05$ ) [177]. The results raise the following question: Is BH a cool method of neuroprotection or will the side effects limit its use? Only additional studies will be able to provide an answer.

### **Hyperbaric Oxygen (HBO)**

Hyperbaric oxygen (HBO), 100% O<sub>2</sub> at 3 bar or ATA [178] is a promising non-invasive method to decrease BBB damage, reduce hemorrhagic transformation and reduce hemoglobin extravasation in ischemic zones following embolic stroke [178]. Moreover, HBO has been found to reduce thrombolytic-induced hemorrhage [178]. Investigators have found that HBO has diverse effects [179,180] including reduced non-thrombin mediated edema, and edema reduction mediated by activation of ribosomal protein S6 kinases (p70 S6

K), which are important to protein synthesis [180]. Furthermore, Ostrowski et al. [181,182], who extensively studied mechanisms of HBO in models of SAH, showed that HBO-induced reduced mortality and behavioral improvement was somewhat correlated with reduced lipid peroxidation measured by detecting malondialdehyde, the degradation product of polyunsaturated lipids. HBO treatment also reduced the expression superoxide producing enzyme, NADPH oxidase (NOX) [182]. The study suggests that HBO decreases oxidative stress via an early inhibition of NOX. Matchett et al. [183] reviewed the use of HBO and suggested that the treatment has mainly positive effects in animal models, since HBO reduces BBB breakdown, decreases inflammation and oxidative stress, reduces edema and suppresses apoptosis [184].

### **Normobaric oxygen (NBO)**

Normobaric oxygen (NBO) therapy has been studied by Lo and colleagues to treat both stroke and hemorrhage [185–187]. Like HBO, NBO, 100% O<sub>2</sub> has some neuroprotective effects. Most work has been done in embolic stroke, rather than hemorrhagic stroke models. Lo and colleagues [186] showed that NBO could be safely administered with tPA in a rat embolic stroke model, leading the way for additional studies and clinically relevant measures. A follow-up study by Sun et al. [178] using a thrombin-induced hemorrhage model showed that NBO decreases infarct size and tPA-induced hemorrhage. In the same study, in parallel rats, they found that HBO reduced hemoglobin extravasation in the ischemic brain. Moreover, both NBO and HBO treatment decreased BBB damage and the incidence of hemorrhagic transformation. In a hemorrhage model, NBO did not affect collagenase-induced blood volume or edema and was ineffective at reducing neurological outcome [185]. Interpretation of a negative study using a single treatment regimen and is quite difficult, especially given the fact that the study was uncontrolled (i.e. no positive control). It is premature to definitively conclude that NBO treatment is ineffective to treat hemorrhagic stroke until various treatment regimens are tested in parallel in multiple hemorrhage models with proper positive controls in each study [188].

### **Hydrogen gas (HG)**

A novel and quite interesting observation was recently made by Chen et al. [189] and Zhang et al. [190]. The discovery seems to tie together many of the key mechanisms described above including free radicals, oxidative stress and MMP's. The authors showed that hydrogen gas by inhalation can exert neuroprotective effects and reduce hemorrhagic transformation following MCA occlusion. It is hypothesized that H<sub>2</sub> gas decreases oxidative stress and reduces MMP-9 activation and/or activity.

### **Conclusion**

ICH will continue to be an important problem as the population ages in the United States and worldwide. As can be gleaned from this article, there are many suitable targets for drug development including inhibition of specific MMP's, TNF $\alpha$ , thrombin, inflammation and the coagulation pathway. Many preliminary attempts at monotherapy have not been effective. This is most likely due to simultaneous activation of a "hemorrhage cascade" and a "stroke cascade" with some overlapping key mediators [51,66,93,191,192]. There is a growing consensus that the treatment of ischemic stroke will require pleiotropic drugs or combination therapy [93,193–200]. This may be even more crucial when one considers the effect of brain blood on tissue plus the fact that brain bleeds will cause tissue ischemia and edema. For hemorrhage treatment, the following drug classes hold promise for an eventual treatment: anti-inflammatory drugs, anti-oxidants, thrombin, and HO inhibitors. Surprisingly, some of the most effective preclinical treatments are noninvasive therapies such as hyperthermia, HBO, NBO and HG. Although it is early in their development and investigation, they are all

pleiotropic therapies that have multiple physiological consequences. Continued mechanism-based research and translational research will provide a better understanding of the mechanisms involved in hemorrhagic stroke so that preclinical knowledge can be translated into a clinically useful therapeutic.

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