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Cerebral Hemorrhage

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Preface

These are the proceedings of the 2nd International Symposium on Cerebral Hemorrhage, which was held on November 10–11th, 2007 in Shanghai, China. This symposium followed the successful first symposium held in Ann Arbor, Michigan, USA, in 2005. The aim of the conference was to bring together experts on cerebral hemorrhage from throughout the world to present and discuss data on this understudied form of stroke. The conference covered both clinical and basic science studies on cerebral hemorrhage and particularly intracerebral hemorrhage. Papers from the conference in these proceedings cover the full gamut, from molecular biology to clinical trials and epidemiology. They show that our knowledge of cerebral hemorrhage has greatly expanded in recent years and there is hope that this will be translated into new therapies for the clinic.

The symposium in Shanghai was a joint collaboration between Fudan University and the University of Michigan. It was to have been co-chaired by Professors Liang-Fu Zhou and Julian T. Hoff. Unfortunately, Professor Hoff died prior to the meeting and his presence was sorely missed by all. This volume includes a paper on Professor Hoff and his contributions to the field of cerebral hemorrhage research by Dr. Karin Muraszko, who succeeded Dr. Hoff as Chair of Neurosurgery at the University of Michigan.

With the success of the 1st and 2nd symposia, there was a unanimous desire for these meetings to continue. The 3rd International Symposium on Cerebral Hemorrhage will be held in Palm Springs, USA, under the direction of Professors John Zhang and Austin Colohan of Loma Linda University. Symposium attendees look forward to a successful meeting in that city in November 2009. Plans are also underway to host a 4th meeting in Newcastle, England, in 2011.

The editors wish to thank Ms. Holly Wagner and the staff of Springer-Verlag for the commitment and editorial skills necessary to prepare these proceedings.

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Feng-Ping Huang, Ya Hua, Yi-Cheng Lu,
Karin M. Muraszko

Acta Neurochir Suppl (2008) 105: VI

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Contents

Human intracerebral hemorrhage

Experimental intracranial hemorrhage (non-intracerebral hemorrhage)

Human intracranial hemorrhage (non-intracerebral hemorrhage)

Listed in Current Contents

Acta Neurochir Suppl (2008) 105: XI–XII

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Julian Theodore (Buz) Hoff 1936–2007

K. Muraszko

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On April 16, 2007 the world of neurosurgery lost one of its great leaders, Julian T. (Buz) Hoff. He passed away after a 7-month battle with acute leukemia and died peacefully at home with the comfort of his loving family. Although his life seemed to be cut short, it was a life well-lived and he will be missed.

Dr. Hoff was a graduate of Caldwell High School in Caldwell, Idaho. He received his A.B. degree from Stanford University and he attended Cornell Medical College, graduating in 1962. He completed his neurosurgical training at New York Hospital in 1970 and went on to the University of California at San Francisco where he quickly rose to the rank of professor. Dr. Hoff left San Francisco in 1981 to assume the position as head of the neurosurgery section here at the University of Michigan. He was appointed Richard C. Schneider Professor in 1992 and the section became the Department of Neurosurgery in 2001. Dr. Hoff served as the first chairman of the Department of Neurosurgery at the University of Michigan Health System from 2001 until 2005.

Buz had a distinguished career in neurosurgery, having served on the editorial boards of the major neurosurgical journals and as co-chair of the Editorial Board for the Journal of Neurosurgery from 1997 to 1999. He maintained continuous NIH funding from 1972 until the present day. His most recent R01 focused on mechanisms of brain edema after intracerebral hemorrhage. He received the prestigious Jacob Javitz Award for Neuroscience Research twice, and was a member of the Institute of Medicine National Academy of Sciences since 1999. He received the Cushing Medal from the American Association of Neurological Surgeons, the Grass Prize from the Society of Neurological Surgeons, and was the honored guest of the Congress of Neurological Surgeons. He received the Distinguished Alumni Award from Caldwell High School in Caldwell, Idaho. He also received the Distinguished Service Award from the Society of Neurological Surgeons. He was the author of numerous papers, book chapters, plus the editor of several books.

Buz's research career was a distinguished one. His first publication was on cerebral hemangioblastomas in a patient with Von Hippel–Lindau disease. As he progressed through his residency he continued to publish. In his early career he became very interested in cerebral edema, and specifically, intracerebral hemorrhage. In the 1970s he published a series of papers looking at the effects of alpha-adrenergic blockade on cerebral circulation as well as the effect of hypoxia on the Cushing reflex in intracranial pressure. He looked at various mechanisms to abate injury secondary to cerebral edema. His research interests focused on intracranial pressure and the effects of hematoma, both intraparenchymal as well as subdural, in patients with head injury. As a result of his outstanding research, he was awarded the Grass Award by the Society of Neurological Surgeons in 2002. The selection committee particularly commented on his sustained interest in cerebral edema and the important contributions he and his laboratory made to our understanding of cerebral edema under a variety of circumstances.

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Buz's contributions within the laboratory were also notable for his significant ability to mentor young faculty as well as residents in the pursuit of academic careers. At present, there are 5 chairs of neurosurgical departments who trained under Buz and who received mentoring and tutelage from him with respect to their academic careers. He sponsored numerous residents in the laboratory and consistently provided protected time for laboratory work during residency training. Numerous residents went on to win awards by the Congress of Neurological Surgeons as well as the American Association of Neurological Surgeons. Many also received funding from the Neurosurgery Research and Education Foundation (NREF).

Buz served in executive positions in every major neurosurgical society including the American Academy of Neurological Surgeons, as president of the American Association of Neurological Surgeons, and as vice-president of the Congress of Neurological Surgeons. He was a member of the Residency Review Committee for neurosurgery from 1987 to 1993 and continued to serve on the appeals panel for neurosurgery. Within the University of Michigan he served on a variety of committees. Whenever a complex job needed to be done, Buz was always selected to perform that job, and he did so with wisdom and an affable personality.

An Eagle Scout in his early life, Dr. Hoff was particularly beloved for his strong leadership abilities that were displayed in a collegial and kind fashion. In choosing residents, he often indicated that he employed the lessons learned in his early life with the Boy Scouts. A strong advocate of resident education, he sought to create a collegial environment in which even the most complex political and scientific issues could be discussed in a manner of warmth and openness.

Dr. Hoff was particularly pleased that in 2006, the Department of Neurosurgery completed an endowed chair honoring him. Dr. Karin Muraszko serves as the first Julian T. Hoff Professor of Neurosurgery and Chair of the Department of Neurosurgery. The Department has also established a Resident Education and Research Fund in Dr. Hoff's name to continue his outstanding legacy of leadership in academic neurosurgery and his longstanding support of residency education. A Hoff Lectureship has now been established in the Department of Neurosurgery and is associated with a Hoff Alumni Day. Dr. John McGillicuddy will give the first Hoff Lectureship this summer.

Dr. Hoff is survived by his wife of 45 years, Diane (Shanks) Hoff, and by 3 children, Paul Hoff, MD (Donna Hoff, MD), Allison Hoff, MA, and Julia Haughey, MSW (Michael Haughey). He leaves 5 grandchildren, Lauren Hoff, Kiersten Hoff, Kathryn Haughey, Kelly Haughey, and Charles Haughey.

A memorial service at the First Presbyterian Church in Ann Arbor, Michigan on May 19, 2007 celebrated Dr. Hoff's outstanding life. It was a time in which friends from around the world gathered in memory of a truly outstanding leader and great friend to neurosurgery. All who have had the privilege of knowing Dr. Hoff will remember him for his wonderful smile, his bright eyes, and his unfailingly pleasant personality. Behind all this was a blazing intellect that has left behind a legacy of outstanding scholarship, strong national leadership, and a personal ethical and moral compass to which we all aspire. Dr. Hoff's legacy goes beyond a summary of his many accomplishments and is reflected by a generation of scientists, residents, colleagues, patients, and friends who felt honored and privileged to know him, and who lead lives that will forever be influenced by his presence.

Acta Neurochir Suppl (2008) 105: XIII

Printed in Austria

Hoff Fellowships

To honor the memory of Dr. Julian T. Hoff and his contributions to the field of intracerebral hemorrhage research, a number of fellowships were established to allow young scientists and clinicians to attend and present at the 2nd International Symposium on Cerebral Hemorrhage in Shanghai. The awardees are to be applauded for their research. They were as follows:

Xuhui Bao, Department of Neurosurgery, Fudan University, China

Tim Lekic, Department of Physiology, Loma Linda University, USA

Timothy Morgan, Department of Neurology, Johns Hopkins University, USA

Molly E. Ogle, Department of Pathology, Laboratory Medicine, Medical University of South Carolina, USA

Qing Xie, Department of Neurosurgery, Fudan University, China

Feng Zhou, Department of Neurosurgery, 2nd Affiliated Hospital, Zhejiang University, China

Experimental intracerebral hemorrhage – iron, hemoglobin and free radicals

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Deferoxamine therapy for intracerebral hemorrhage

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Summary

Intracerebral hemorrhage (ICH) is a subtype of stroke with very high mortality. Experiments have indicated that clot lysis and iron play an important role in ICH-induced brain injury. Iron overload occurs in the brain after ICH in rats. Intracerebral infusion of iron causes brain edema and neuronal death.

Deferoxamine, an iron chelator, is an FDA-approved drug for the treatment of acute iron intoxication and chronic iron overload due to transfusion-dependent anemia. Deferoxamine can rapidly penetrate the blood–brain barrier and accumulate in the brain tissue in significant concentration after systemic administration. We have demonstrated that deferoxamine reduces ICH-induced brain edema, neuronal death, brain atrophy, and neurological deficits. Iron chelation with deferoxamine could be a new therapy for ICH.

Keywords: Brain edema; cerebral hemorrhage; deferoxamine; iron; neuronal death.

Introduction

Spontaneous intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. If the patient survives the ictus, the resulting hematoma within brain parenchyma triggers a series of events leading to secondary insults and severe neurological deficits [23, 39]. Although the hematoma in humans gradually resolves, the neurological deficits in ICH patients are usually permanent and disabling.

Iron plays an important role in brain injury after ICH [31]. Our recent studies suggest that iron overload occurs in the brain following ICH, which results in brain damage [18, 35]. In particular, we have found that an iron chelator, deferoxamine, can reduce ICH-induced brain edema, neuronal death and neurological deficits in rats [5, 7, 18, 19].

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Erythrocyte lysis and brain edema

Brain edema around the hematoma is commonly observed during the acute and subacute stages following ICH, contributing to poor outcomes [25, 41]. Although the mechanisms of edema formation following ICH are not fully resolved, several mechanisms are responsible for edema development. These include hydrostatic pressure during hematoma formation and clot retraction, coagulation cascade activation and thrombin production, hemoglobin and iron toxicity, complement activation, mass effect, secondary perihematomal ischemia, reperfusion brain injury, and blood–brain barrier (BBB) disruption [6, 13, 15, 16, 30, 36–38]. Various animal models of ICH have allowed detailed study of these mechanisms and their roles in the pathophysiological events that occur in brain tissue after ICH.

Edema around a hematoma reaches its peak several days after the ictus [3, 17, 29]. In rats, the edema peak occurs on the third or the fourth day after experimental ICH [36, 40]. In contrast, thrombin-induced brain edema peaks within 48 h [36]. This difference in time course led us to examine whether there might be a third phase (after 3 days) of injury involving red blood cell (RBC) lysis and hemoglobin-induced neuronal toxicity. Infusion of packed erythrocytes caused edema after about 3 days, suggesting that RBCs are associated with delayed edema formation [36]. A clinical study of edema and ICH indicated that delayed brain edema is related to significant midline shift after ICH in humans [41]. This delayed brain edema formation (in the second or third weeks

after onset in human) is probably due to RBC lysis and hemoglobin degradation products.

Hemoglobin, hemoglobin degradation products, and brain injury

Hemoglobin causes brain damage through its degradation products [39]. Heme from hemoglobin is degraded by heme oxygenase in the brain into iron, carbon monoxide, and biliverdin. Biliverdin is then converted to bilirubin by biliverdin reductase [12]. Our previous studies demonstrated that an intracerebral infusion of hemoglobin and its degradation products, hemin, iron, and bilirubin, cause the formation of brain edema within 24 h. Hemoglobin itself induces heme oxygenase-1 (HO-1) upregulation in the brain and heme oxygenase inhibition by tin-protoporphyrin (SnPP) reduces hemoglobin-induced brain edema. In addition, an intraperitoneal injection of a large dose of deferoxamine (an iron chelator) attenuates brain edema induced by hemoglobin. These results indicate that hemoglobin causes brain injury by itself and through its degradation products, particularly iron [9].

Iron toxicity and neuronal death

Iron is essential for normal brain function, but iron overload may have devastating effects [1]. Iron overload contributes to many kinds of brain injury including hemorrhagic and ischemic stroke [8]. Clot lysis and iron release from heme occurs within the first week after ICH in animal studies, and this appears to contribute to acute brain edema formation [36]. However, brain atrophy occurs several weeks later, suggesting that iron exposure causes cell damage that results in delayed cell death. For example, iron may cause sufficient damage that the cell cannot repair itself. If such death occurs in cells that are storing iron released from the hematoma, the new iron release may affect nearby cells leading to amplification of the lesion. Alternately, the iron stored in cells after resolution of the clot may be naturally released for clearance across the BBB or through cerebrospinal fluid, but with potential for causing further tissue damage.

After erythrocyte lysis, iron concentrations in the brain can reach very high levels. Our recent data showed a 3-fold increase in brain non-heme iron content after ICH in rats, and it remains high for at least 28 days [35]. Intracerebral infusion of iron causes brain edema and an iron chelator reduces hematoma- and hemoglobin-induced edema, suggesting that iron plays an important role in edema formation after ICH [9, 18].

Iron-induced brain damage may result from oxidative stress. Cortical iron injections cause focal epileptiform paroxysmal discharges [4, 33]. Iron and lipid peroxidation also have an important role in hemoglobin-induced brain injury. For example, a subpial injection of $FeCl_2$ induces brain edema and lipid peroxidation in the brain [34]. Iron can also stimulate the formation of free radicals leading to neuronal damage. It is known that ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron react with lipid hydroperoxides to produce free radicals [27]. Antioxidants block neuronal toxicity induced by hemoglobin and iron [24, 32].

Iron transport and storage proteins in the brain

A number of proteins, including transferrin (Tf), transferrin receptor (TfR), and ferritin are involved in maintaining brain iron homeostasis. We found that brain Tf, TfR, and ferritin levels are increased after ICH [35]. Tf, an 80-kDa protein, is a major iron distributor in the brain. Cellular uptake of Tf-bound iron is achieved by binding to the TfR. In normal brain, TfR is expressed at very high levels at the BBB, where it is involved in iron uptake into brain. However, a recent report indicates that there is rapid efflux of Tf from brain to blood across the BBB [42], suggesting that Tf could contribute to iron clearance when there is brain iron overload. TfR is normally only expressed on brain parenchymal cells at low levels but it can be increased under pathophysiological conditions. The brain can produce ferritin, the iron storage protein. Ferritin has 2 subunits, heavy-ferritin (21 kDa) and light-ferritin (19 kDa) [2]. Ferritin is found mainly in glial cells but not neurons.

Deferoxamine therapy

Deferoxamine, an iron chelator, is a Food and Drug Administration (FDA)-approved drug for the treatment of acute iron intoxication and of chronic iron overload due to transfusion-dependent anemias. Its molecular weight is 657. Deferoxamine can rapidly penetrate the BBB and accumulate in brain tissue in significant concentration after systemic administration [11, 20]. The terminal half-life of deferoxamine after intravenous infusion is 3.05 h [21]. Deferoxamine chelates iron by forming a stable complex that prevents the iron from

entering into further chemical reactions. It readily chelates iron from ferritin and hemosiderin but not readily from transferrin. *In vivo*, deferoxamine can reduce hematoma- and hemoglobin-induced brain edema [9, 18]. Our recent studies show that deferoxamine reduces ICH-induced neuronal death, brain atrophy, and neurological deficits [5, 7, 28].

Deferoxamine binds ferric iron and prevents the formation of hydroxyl radical via the Fenton/Haber-Weiss reaction. Deferoxamine reduces hemoglobin-induced brain Na^+/K^+ ATPase inhibition and neuronal toxicity [24, 26]. Favorable effects of iron chelator therapy have been reported in various cerebral ischemia models [10, 14].

Although deferoxamine is an iron chelator, it can have other effects. Thus, it can act as a direct free radical scavenger [10, 14] and it can induce ischemic tolerance in the brain [22]. The latter has been demonstrated *in vivo* and *in vitro*, and it may be related to a deferoxamine induction of hypoxia-inducible transcription factor-1 binding to DNA [22].

Deferoxamine, however, may cause hypersensitivity reactions, systemic allergic reactions, cardiovascular, hematologic, and neurological adverse reactions. Serious adverse reactions include significant hypotension and marked body weight loss. In addition, deferoxamine may cause cytotoxicity because deferoxamine inhibits DNA synthesis *in vitro*.

Remarks

At present, there is no effective treatment that attenuates brain edema and improves long-term outcome in ICH. Our previous studies have shown that iron plays an important role in edema formation, neuronal death, brain atrophy, and behavioral deficits. Iron chelation with deferoxamine could be a new therapy for ICH. Such a therapy would be of great benefit, not only to the patients, but also to their caregivers and to society in general by reducing the cost of care for hemorrhagic stroke patients.

Deferoxamine has just entered Phase I clinical trial in humans.

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Bilirubin oxidation products, oxidative stress, and intracerebral hemorrhage

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Summary

Hematoma and perihematomal regions after intracerebral hemorrhage (ICH) are biochemically active environments known to undergo potent oxidizing reactions. We report facile production of bilirubin oxidation products (BOXes) *via* hemoglobin/Fenton reaction under conditions approximating putative *in vivo* conditions seen following ICH.

Using a mixture of human hemoglobin, physiological buffers, unconjugated solubilized bilirubin, and molecular oxygen and/or hydrogen peroxide, we generated BOXes, confirmed by spectral signature consistent with known BOXes mixtures produced by independent chemical synthesis, as well as HPLC-MS of BOX A and BOX B. Kinetics are straightforward and uncomplicated, having initial rates around 0.002 μM bilirubin per μM hemoglobin per second under normal experimental conditions. In hematomas from porcine ICH model, we observed significant production of BOXes, malondialdehyde, and superoxide dismutase, indicating a potent oxidizing environment. BOX concentrations increased from 0.084 ± 0.01 in fresh blood to 22.24 ± 4.28 in hematoma at 72 h, and were 11.22 ± 1.90 in adjacent white matter (nmol/g). Similar chemical and analytical results are seen in ICH *in vivo*, indicating the hematoma is undergoing similar potent oxidations.

This is the first report of BOXes production using a well-defined biological reaction and *in vivo* model of same. Following ICH, amounts of unconjugated bilirubin in hematoma can be substantial, as can levels of iron and hemoglobin. Oxidation of unconjugated bilirubin to yield bioactive molecules, such as BOXes, is an important discovery, expanding the role of bilirubin in pathological processes seen after ICH.

Keywords: Bilirubin oxidation products; edema; reactive oxygen species; pathology; stroke.

Introduction

Radical processes are well-known in biochemistry and are proposed to occur in living organisms, creating many known disease states. Oxidation of common metabolic products by oxygen, hydrogen peroxide, free iron, and other simple compounds have been widely studied and reported [16, 21–23]. The nature of these oxidation products is not well understood, even though they may have significant physiological impact and lead to subsequent tissue damage. There is growing interest in the oxidations that occur in intracerebral hematoma after intracerebral hemorrhage (ICH), because there appears to be a compromised region around the hematoma that is refractory to current therapies or even hematoma evacuation [6, 9, 20, 22, 26].

We previously reported that bilirubin is oxidized in a hemorrhagic medium and *in vivo* [4, 8, 12, 17]. Bilirubin oxidation products (BOXes), 1-(1,5-dihydropyrrole-2-ylidene) acetamide and related compounds, are small vasoactive molecules with demonstrated clinical importance [17]. They are found in the cerebrospinal fluid of patients who have had hemorrhagic strokes such as subarachnoid and intracerebral hemorrhage. These compounds exhibit biological activity *in vivo* and *in vitro* and have been postulated to be a major contributor to cerebral vasospasm, a pathological constriction of arteries. BOXes have been synthesized *in vitro* by the oxidation of unconjugated bilirubin at room temperature with a large excess of hydrogen peroxide. The conversion of bilirubin to BOXes is associated with a biochemical state that may cause or contribute to pathological sequelae after ICH. In this report, we outline the conversion of unconjugated (free) bilirubin and other relevant compounds to compounds well-known to have physiological and pathological effects. This was achieved using an *in vitro* system for modeling chemical oxidations, and *in vivo* using a porcine ICH model. Our results suggest that potent oxidations occur in the hematoma, and that the oxidized products penetrate into the perihematomal region with possible detrimental effects.

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Experimental methods

Reagents and chemicals

All reagents were American Chemical Society grade or better. Chemicals and biomaterials were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise stated. BOXes were prepared as previously described [4, 8, 12]. Gasses used were technical grade or better. Optical spectra were obtained on a micro-Quant microwell plate reader using ultraviolet transparent 96-well plates. All optical densities (OD) are reported as uncorrected for plate. Aliquots of the reaction mixture were taken directly and read for OD within a short period of time from sampling and analyzed for kinetics data. Reaction mixture consisted of 100 μM solubilized bilirubin, 1 g sodium carbonate, 200 mL deionized water, 1 mg human hemoglobin, μM oxygen, and/or 100 μL of 30% hydrogen peroxide. Figure 1 is a schematic of how the in vitro system was designed. Figures 2A and B show data collected using this reaction system.

Pig surgery and tissue sampling

Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Methods to induce ICH have been previously described [18–20]. Briefly, 3 mL of autologous blood was infused into frontal hemispheric white matter of pentobarbital-anesthetized pigs. Brains were frozen *in situ* with liquid nitrogen at various time points up to 72 h. Approximately 20 mg of perihematomal, edematous white matter was sampled and homogenized in 200 μL of homogenization buffer, as previously described [11].

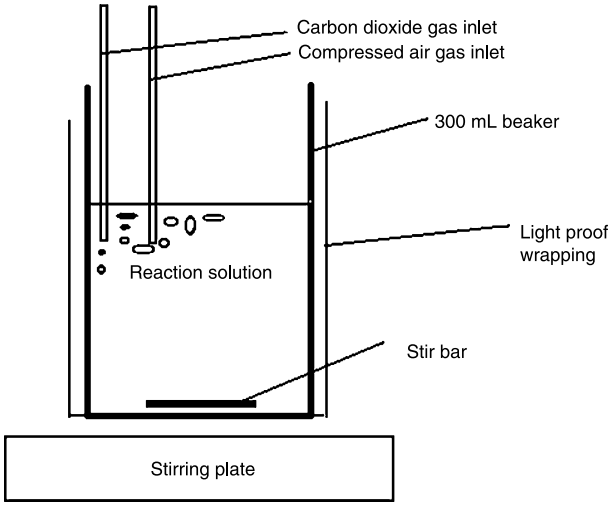

Fig. 1. Simple oxidization reaction vessel showing *in vitro* system for modeling the oxidizing environment in porcine hematoma

Bilirubin determination

Total bilirubin was assayed using a method based on those developed by Michaelsson *et al.* [14] and adapted for use in a microtiter plate, as previously described [17]. Briefly, bilirubin in the sample is first treated with a caffeine solution to release all bilirubin moieties, followed by a diazo reagent to yield a colored product. This is then alkalized until the color is in a range that is not overlapping the absorption range of hemoglobin, and the absorption is recorded at 600 nm. Sample absorbance is compared to a concomitantly run standard curve constructed from commercially available bilirubin standard solutions (Wako Chemicals USA, Inc., Richmond, VA) to determine total bilirubin concentrations.

BOXes determination

BOXes were quantified by spectroscopic analysis at 320 nm, as previously described [17]. Chemically prepared BOXes were used to construct a standard curve. Samples were subjected to a chloroform extraction and evaporation, and re-suspended in 0.9% saline for analysis.

Hemoglobin determination

The sample was exposed to Drabkin's reagent ($NaHCO_3$:$K_3Fe(CN)_6$:KCN, 100:20:5) in order to convert all hemoglobin moieties into cyanomethemoglobin [5]. A surfactant (Brij-35) was added to prevent protein- or lipid-induced turbidity. Concentration of cyanomethemoglobin is determined by comparison to a standard curve constructed with commercially available lyophilized hemoglobin (Sigma-Aldrich), treated with Drabkin's reagent, and the absorbance read at 540 nm.

Malondialdehyde (MDA) determination

MDA was assessed using a commercially available assay kit from Calbiochem (San Diego, CA). Briefly, MDA reacts with N-methyl-2-phenylindole in acetonitrile and ferric ions to form a colored molecule that has maximum absorbance at 568 nm [5].

Superoxide dismutase (SOD) assay

SOD activity was measured using a commercially available kit (Fluka, St. Louis, MO) according to the manufacturer's instructions. Upon reac-

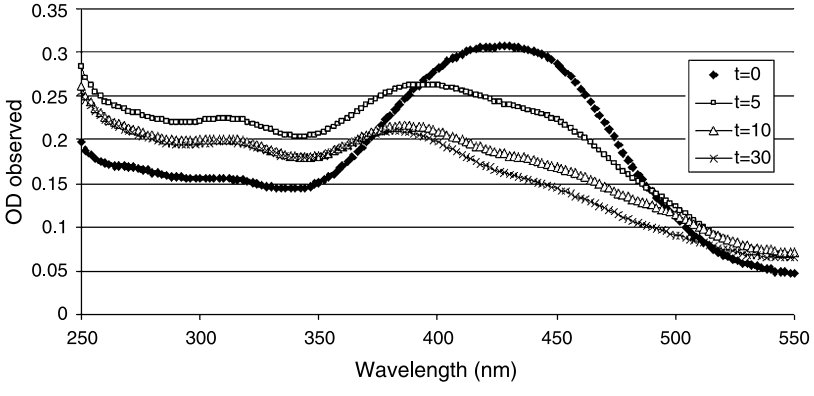

Fig. 2A. A solution of 1 g sodium carbonate, 105 μM solubilized bilirubin, saturating levels of carbon dioxide gas and compressed air, 1 mg human hemoglobin, and 42 mM hydrogen peroxide were allowed to react with constant stirring in an open system. Aliquots of reaction were taken at 0 min (*square*), 5 min (*triangle*), 10 min (*X*), and 30 min (*diamond*). Optical density was followed in the visible region. The pH of this reaction is 6.8

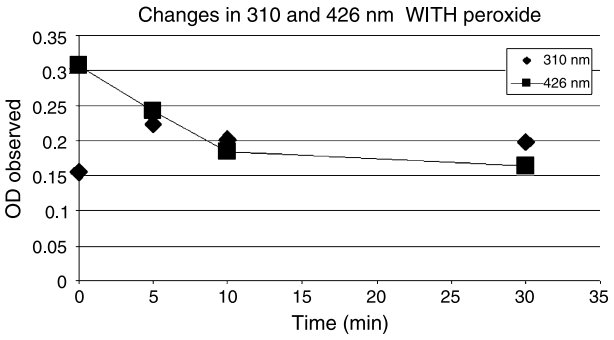

Fig. 2B. The apparent kinetics of bilirubin degradation and BOX production using visible spectroscopy and extinction coefficients for bilirubin and BOXes. The rise in BOXes is apparent within 10 min and plateaus at about 10 min, reaching a steady state. Bilirubin falls at 426 nm, mirroring the production of BOXes

tion with superoxide, Donjino's tetrazolium salt, WST-1, produces a water soluble formazan dye with an absorbance of 450 nm [27, 28]. Samples were treated with Donjino's and xanthine oxidase to produce superoxide. The percent inhibition of formazan formation by SOD was measured.

Total protein determination

Total protein was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Side chains of several amino acids reduce cupric copper to the cuprous oxidation state, and this change is measured at 562 nm. The total protein concentration is interpolated from a standard curve using bovine serum albumin as the standard.

Statistics

Statistical differences were determined using ANOVA or Student *t*-test where indicated. A *p*-value of ≤ 0.05 was considered statistically significant.

Results

Figures 2A and B indicate the facile conversion of bilirubin (loss of OD at 425 nm) and conversion to BOXes (OD at 310 nm). This conversion is rapid and takes only 5 min at room temperature under the experimental conditions described (Fig. 2B). This indicates that the small molecules of BOXes have extreme membrane permeability and high biological activity, and appear to be formed in the model system under pseudo-physiological conditions. Both peroxide and hemoglobin are necessary for the reaction to occur. Carbon monoxide significantly inhibits the reaction; thus, the reaction involves the heme moiety in the hemoglobin (Fig. 3). These observations are indicative of a free radical reaction. Also, deferoxamine has little effect on the reaction, indicating that free iron is not involved in, or essential for, the reaction. Thus, this *in vitro* reaction produces significant amounts of BOXes in about 10 min. Figure 3 shows that there are both activators and inhibitors of this simple reaction.

Table 1 summarizes the production of BOXes as well as related metabolites produced in and around the hematoma after ICH. Of note is that concentrations of BOXes reach substantial levels in hematoma and perihematomal white matter on the ipsilateral side. Bilirubin levels are increased in the hematoma as well as perihematomal region and have not reached a plateau at 72 h. Production of MDA appears to plateau at 24 h and is present in both ipsilateral and contralateral brain. SOD activity was consistently highest in the hematoma brain and was relatively constant following hemorrhage. The

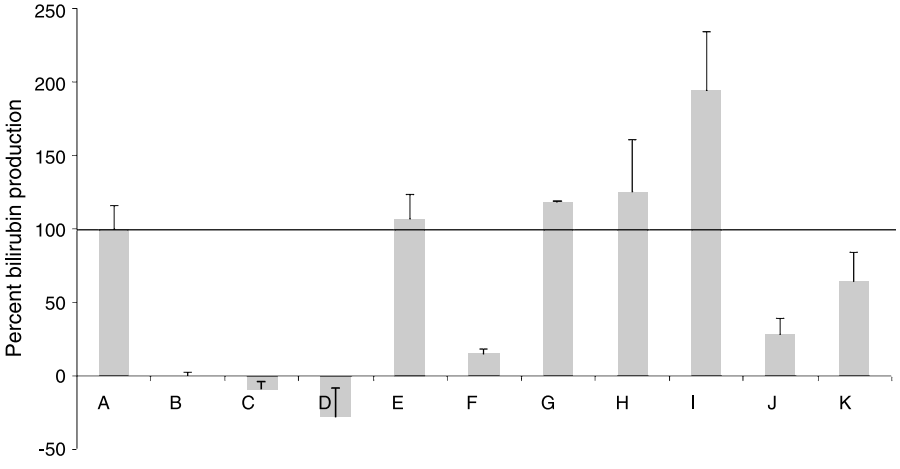

Fig. 3. Relative amounts of BOX production normalized to control condition A. A = hemoglobin (0.64 μM), bilirubin (105 μM), peroxide (383 mM); B = incubation A without hemoglobin; C = incubation without hemoglobin and peroxide; D = incubation A without peroxide; E = incubation A with added deferoxamine (65 mM); F = incubation A with carbon monoxide; G = incubation A with benzoic acid (1.2 mM); H = incubation A with added potassium cyanide (6.5 mM); I = incubation A with added arachidonic acid (4.3 mM); J = incubation A with added oxalic acid (550 mM); K = incubation A with added thiourea (110 mM). Error bars are standard deviation. $N = 3$ or more for all conditions. Reactions were performed in phosphate-buffered saline at pH 7.5

Table 1. *Metabolites present post ICH*

	Malondialdehyde (mM/mg protein)	Superoxide dismutase (% activity/mg/mL protein)	Bilirubin (mg/dL)	Bilirubin oxidation products (nmol/g)
Fresh blood	0.37 ± 0.06		4.18 ± 0.08	0.084 ± 0.01
Sham ipsilateral	0.51 ± 0.041	0.16 ± 0.010		
Sham contralateral	0.54 ± 0.021	0.17 ± 0.011		
1 h hematoma	0.088 ± 0.034	0.029 ± 0.0021		
1 h contralateral	1.15 ± 0.45	0.19 ± 0.041		
1 h ipsilateral	0.86 ± 0.030	0.16 ± 0.040		
8–12 h hematoma	0.56 ± 0.038		13.6 ± 0.98	3.44 ± 0.37
24 h hematoma	0.082 ± 0.036	0.039 ± 0.011	20.31 ± 3.32	
24 h contralateral	0.99 ± 0.33	0.174 ± 0.017	0.71	0.78
24 h ipsilateral	0.47 ± 0.14	0.181 ± 0.012	10.35	3.10
48 h hematoma	0.11 ± 0.097	0.034 ± 0.011		11.22 ± 1.90
48 h ipsilateral	0.39 ± 0.20	0.18 ± 0.025		
48 h contralateral	0.96 ± 0.23	0.16 ± 0.030		
72 h hematoma	0.098 ± 0.011	0.027 ± 0.002	40.72 ± 3.03	22.24 ± 4.28
72 h ipsilateral	0.63 ± 0.07	0.25 ± 0.060		
72 h contralateral	0.88 ± 0.16	0.17 ± 0.002		

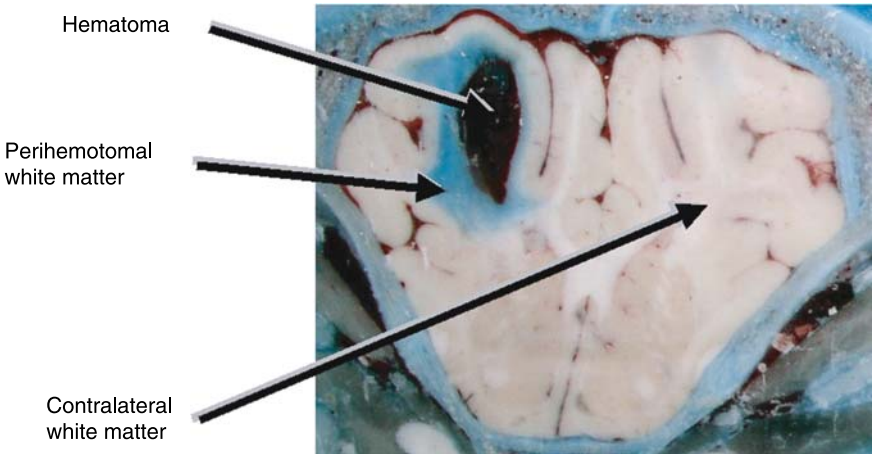

Fig. 4. Representative cross-section of porcine brain 16 h after ICH. Breakdown of blood–brain barrier is indicated by Evans blue perfusion. Arrows indicate relative areas of sampling for use in generating data presented in Table 1

relative location for obtaining samples presented in Table 1 can be seen in Fig. 4, where we show a representative section of pig brain demonstrating the hematoma, perihematomal region, and contralateral white matter. The edema present in the porcine brain after ICH is visible at 1 h and appears to peak at 48 h (Fig. 5).

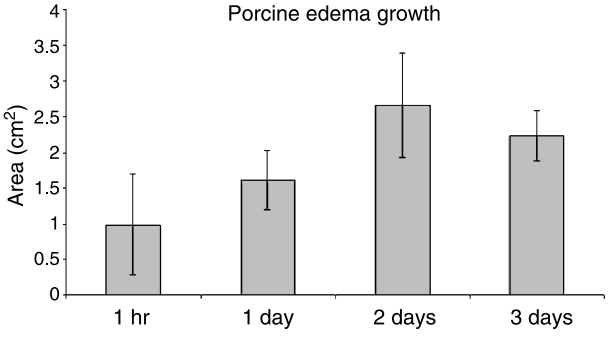

Fig. 5. Edema in the pig brain after ICH is evident in 8 h after hemorrhage and appears to peak at 2 days

Discussion

ICH is a devastating event, with high mortality from the initial hemorrhage. and a tragically high death and disability rate for patients who survive the initial ictus. Complications after ICH include, but are not limited to, increased intracranial pressure, breakdown of blood–brain barrier, clot mass effect, hydrocephalus, necrosis, atrophy, activation of complement, spreading depression, ischemia, and death [2, 7, 9, 10, 15, 24, 25]. Less than 30% of patients who survive an ICH return to normal, and many of the survivors require dependent care [1]. Thus, ICH and complications after ICH are a substantial health burden to the nation and to the fami-

lies of survivors. Better understanding of the mechanisms associated with the complications will be important in treating these patients and providing hope for improved outcomes.

Our findings include: 1) the chemical environment found after ICH produces a potent oxidizing environment; 2) oxidations in the hematoma produce a cocktail of dangerous metabolites; and 3) these metabolites are quickly found in the perihematomal white matter. Taken together, these data suggest that the chemical environment of the hematoma is a substantial cause of the pathological sequelae seen after ICH, and that damage from the hematoma manifests rapidly after ICH. These 3 major findings and how they contribute to the body of evidence concerning the toxic environment produced by the hematoma are discussed below.

The chemical environment produces BOXes and toxic metabolites

In solution, we found that bilirubin is quickly oxidized in minutes, using several single electron reactions. We were able to confirm significant production of BOXes in the presence of the Fenton reaction as well as a carbon dioxide/carbonate 1-electron donor system (Fig. 3). Importantly, but not surprisingly, we found that there was an apparent pH optimum for this reaction of 6.8. This is a pH that is quite plausible after ICH. Reactive oxygen species can also produce an oxidizing environment to produce BOXes and other metabolites. We found that BOXes are produced with a similar time course when hydrogen peroxide is added to the system as compared to a 1-electron donor system. These data suggest that reactive oxygen species, single electron oxidations, and some radical reactions will produce BOXes. Should BOXes and other toxic metabolites be oxidized in this way, we believe that this oxidizing environment may cause or contribute to the pathological sequelae seen after ICH by allowing these compounds to leech into the perihematomal region and have their toxic effects.

Oxidations in the hematoma produce a cocktail of dangerous metabolites

The potent oxidizing environment seen in the hematoma after ICH can produce substantial concentrations of BOXes and other oxidized metabolites relatively quickly after ICH. These concentrations are in the range that has been reported previously [12, 13, 17], and are associated with toxicity responses *in vitro* [13]. We also found that substantial amounts of MDA, SOD, and BOXes are produced in the hematoma at 1 h after ICH. These molecules are associated with oxidative damage and are considered to be indicators of hemorrhagic complications. Some of these are also directly biologically active [3].

Metabolites are found in the perihematomal white matter soon after ictus

If one believes that there are toxic compounds produced in the hematoma and that these toxic compounds cause or contribute to the pathological sequelae seen after ICH, it is necessary to assume that these compounds are diffusing out of the hematoma into the surrounding parenchyma. Therefore, we closely examined the concentration of metabolites and compounds in the perihematomal white matter. Ipsilateral brain consistently had elevated levels of BOXes, MDA, and SOD in our studies, with variations in the time course of these metabolites. It is unclear from our data whether the edema and breakdown of the blood–brain barrier is caused by the diffusion of these compounds out of the hematoma, or if these compounds are contributing to this breakdown. There does, nonetheless, seem to be a positive correlation of oxidative damage, oxidative stress, and BOXes with some of the pathological sequelae seen after ICH.

We also believe it is significant that substantial changes in histopathology as well as chemistry can occur within minutes and are manifested within hours. This might be highly relevant when designing strategies to treat these patients. Our data suggests that damage to the perihematomal white matter is occurring in as little as an hour after ICH, and that simple evacuation strategies might be less than successful if they do not address the harmful metabolites that have already diffused from the hematoma.

Conclusions

In this study, we found that a potent oxidizing environment exists in the hematoma after ICH, and that oxidative stress produces a cadre of compounds, some of which are known to be toxic and biologically active. These compounds diffuse into perihematomal white matter and may cause or contribute to the pathological sequelae seen after ICH. The time course for these chemical changes is also quite rapid (many seen in less than 24 h), suggesting that ultra-early intervention may be needed to prevent damage caused by the hematoma after ICH.