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Neuroradiological findings and molecular markers as predictors for secondary brain injury and outcome after intracerebral hemorrhage (ICH)

Thesis

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Contrast Extravasation on CT Angiography Predicts Clinical Outcome in Primary Intracerebral Hemorrhage: A Prospective Study with 139 cases.

Contents

General introduction

Stroke is a serious medical emergency that frequently causes permanent neurological damage, complications, and death. It is the leading cause of adult disability and the second leading cause of death worldwide. Stroke occurs when blood flow to the brain is impaired by either the blockage or the rupture of a supplying artery, resulting in brain cell death and abnormal brain function. According to the World Health Organization data, 15 million people worldwide suffer a stroke each year. Of these, 5 million die and another 5 million are permanently disabled. Spontaneous intracerebral hemorrhage (ICH) is the second most common form of stroke accounting for 10–30% of first-ever strokes, but represents the most devastating subtype. ICH has a worse prognosis than ischemic stroke considering the grade of post-stroke disability and the mortality rate. It was reported by a systematic review and meta-analysis that the overall incidence of ICH was 24.6 per100 000 person-years, the case fatality at 1 month was around 40%, and independency rates between 12% and 39% (VAN ASCH et al. 2010). Incidence of ICH per 100 000 person-years was 24.2 in white people, 2.9 in black people, 19.6 in Hispanic people, and 51.8 (38.8–69.3) in the Asian population (VAN ASCH et al. 2010). In the past two decades, the incidence and mortality rate of stroke did not decrease. This holds true especially for the high-morbidity and high-mortality associated subtype of ICH (VAN ASCH et al. 2010). With the aging of the population stroke incidence can be expected to further increase and to induce higher costs, overwhelming the already overburdened health-care systems and challenging an already terrible situation.

It is well known that the key factor that affects ICH outcome is the initial hematoma volume (QURESHI et al. 2009). However the complications post-ICH also considerably contribute to mortality and poor outcome. Those complications include early hematoma expansion (HE), perihematomal edema (PHE), intraventricular extension of hemorrhage with hydrocephalus, seizures, venous thromboembolic events, hyperglycemia, increased blood pressure, fever, and infections (BALAMI u. BUCHAN 2012). As the major secondary brain injuries, HE and PHE play a crucial role in clinical outcome, particularly in patients with small to medium hematomas (QURESHI et al. 2009). HE has been identified as one of the most important determinants of early neurological deterioration, mortality and poor clinical outcome in primary ICH (FUJII et al. 1994; BROTT et al. 1997; KAZUI et al. 1997; FUJII et al. 1998; LEIRA et al. 2004; S. M. DAVIS et al. 2006; DELCOURT et al. 2012). In contrast, the significance of PHE is still controversial.PHE including vasogenic edema (VE) and cytotoxic edema (CE) are detected in the perihematomal region (XI et al. 2006). It is important to understand the development of and the pathophysiological mechanisms behind the secondary brain injury, and to search for predictive molecular or neuroradiological findings which allow the identification of patients at high risk of secondary brain injuries, and more important of poor clinical outcome. Thereby, early stratification and optimized therapeutic approaches could be expected for future clinical trials undertaken in those patients. Bearing this in mind we conducted 3 prospective studies trying to examine the predictive value of some neuroradiological and molecular markers for the development and extent of secondary brain injury and clinical outcome after ICH, aiming at further elucidation of these

unclear situations.

Part I: Hematoma expansion (HE)

Historically, ICH was considered to be a monophasic event that stopped within minutes of onset as a result of clotting and tamponade by surrounding brain tissue (CMJ. 1986). Further studies indicated that ICH is a dynamic and active process in the first couple of hours. Brott et al. showed in a prospective observational study for the first time the occurrence of hematoma expansion (HE) during the first 24 hours after symptom onset (BROTT et al. 1997). The definition of HE is heterogenous in different studies varying from a relative value of hematoma volume increase of 33% to 50%, to absolute values of 6, 12.5, 20 ml, or their combination (KAZUI et al. 1996; BROTT et al. 1997; KAZUI et al. 1997; FUJII et al. 1998; BRODERICK et al. 2007; WADA et al. 2007; ANDERSON et al. 2008; DELGADO ALMANDOZ et al. 2009; DOWLATSHAHI et al. 2011b). The highest incidence rate of HE has been observed within the first 6 hours after symptom onset, but from 6 to 24 hours a high risk to develop HE remains (MAYER 2003). The incidence of early HE ranges from 14-38% depending on the time from symptom onset to initial imaging and different definitions of HE in different studies. In absence of acceptable animal models, the precise mechanism of HE during the acute phase is poorly understood. HE is speculated to represent ongoing bleeding and re-bleeding from single or multiple ruptured arteries or arterioles (WARTENBERG u. MAYER 2007). The proposed mechanism is a heterogeneous process including: i) mechanical injury resulting from increased intravascular hydrostatic pressure, increased local

tissue pressure and shear forces; ii) dysregulation of hemostasis via inflammatory cascade activation and matrix metalloproteinase (MMPs) overexpression; iii) breakdown of the blood–brain barrier (BBB) in the perihematomal tissue. Hemostasis dysregulation and BBB disruption are elicited by thrombin, hemoglobin degradation products, and plasmin released from the hematoma (MAYER et al. 2005).

A number of clinical studies tried to sort out predisposing factors of HE. The initial hematoma volume was regarded as an important one. Patients with a larger hematoma size were prone to develop HE, in contrast to those with a small one (KAZUI et al. 1997; FUJII et al. 1998; DOWLATSHAHI et al. 2011b). It was reported that HE is more likely to occur in patients with short admission time after symptom onset, alcohol abuse, irregularly shaped hematoma, low Glasgow Coma Scale (GCS) score, low level of fibrinogen, or high systolic and diastolic blood pressure (FUJII et al. 1998; MAYER 2002; LIM et al. 2008). Prior warfarin medication is associated with a higher risk of HE (FLIBOTTE et al. 2004; KUWASHIRO et al. 2010), but contradictory results are found for prior use of antiplatelet therapy (TOYODA et al. 2005; SANSING et al. 2009; TOYODA et al. 2009; DUCRUET et al. 2010; MOUSSOUTTAS et al. 2010; DE GEA-GARCIA et al. 2012). Lower serum LDL-C level is shown to be independently predictive for HE (RODRIGUEZ-LUNA et al. 2011). However, none of these predictors could be used as a tool for a reliable identification of patients at high risk of HE. In contrast, advanced imaging technique might provide this possibility. Recently, several studies showed that contrast extravasation (so-called spot sign) in the hematoma on CT angiography (CTA) provided the visual evidence of progressive

bleeding several hours after ICH onset. The contrast extravasation has been regarded as an independent predictor of HE with high specificity and might serve as a promising surrogate for identification of early HE (GOLDSTEIN et al. 2007; WADA et al. 2007; KIM et al. 2008; DELGADO ALMANDOZ et al. 2009).

The clinical hemostatic trials targeting HE in the ultra-early stage of ICH by the use of recombinant factor VII (rFVIIa) have shown that rFVIIa successfully restricts the extent of HE in both coagulopathic and non-coagulopathic ICH patients, via promoting local hemostasis at sites of vascular injury (MAYER et al. 2005). However, there is no clear clinical benefit neither on functional outcome nor on mortality in overall ICH patients (MAYER et al. 2008). Unfortunately, current clinical HE studies in ICH are not comparable due to the heterogeneous definition of HE. It is suggested to use an absolute growth criterion since absolute growth seems more clinically relevant than relative growth, in particular for more severe outcomes (DOWLATSHAHI et al. 2011a). However, the discussion of an appropriate clinically meaningful cutoff value of HE is ongoing (HANLEY 2010; DOWLATSHAHI et al. 2011a). Therefore a reliable predictor which could identify not only distinct HE but also high risk of poor outcome is critically needed. Contrast extravasation on CTA could be a promising marker. Several retrospective studies suggested that contrast extravasation could independently predict mortality and poor outcome in ICH patients (BECKER et al. 1999; KIM et al. 2008; DELGADO ALMANDOZ et al. 2010). The first prospective study of 39 cases performing CTA in the hyperacute stage of primary ICH, however, failed to prove any association between contrast extravasation and outcome,

possibly due to the small number of cases (WADA et al. 2007). Therefore, we aimed to prospectively determine whether contrast extravasation on multidetector CTA (MDCTA) in the hyperacute stage of primary ICH is an effective predictor in identifying patients at high risk of poor clinical outcome (LI et al. 2011a) (Publication I).

Part II: Perihematomal edema (PHE)

Mechanisms of PHE development

Perihematomal edema (PHE) is the most common secondary brain injury induced by the hematoma after ICH. Compared to the knowledge about the pathology behind edema development in ischemic stroke data regarding the development of perihematomal edema are sparse. It was suggested that there are 2 origins of PHE including vasogenic (extracellular) and cytotoxic (intracellular) edema, and 3 phases of edema formation after ICH (XI et al. 2006; QURESHI et al. 2009). In the very early phase within the first few hours after ICH onset vasogenic edema (VE) develops resulting from hydrostatic pressure and clot retraction with movement of serum from the clot into the surrounding tissue. In contrast, cytotoxic edema (CE) formation in the very early phase involves mechanical disruption of the neurons and glia, followed by oligemia, neurotransmitter release (such as glutamate), mitochondrial dysfunction, membrane depolarization by calcium influx and sodium accumulation, and mechanical deformation (mass effect) (QURESHI et al. 2009). The results of injury range from temporary metabolic suppression (hibernation phase) to cellular swelling and necrosis

depending on the severity of mitochondrial dysfunction. The second phase (first 24 hours till 2 days) of PHE is related to the coagulation cascade and thrombin production, since high concentration of thrombin surrounding the hematoma induces significant neurotoxicity. The third phase of PHE is related to erythrocyte lysis, hemoglobin and iron toxicity, which is predominantly responsible for this delayed edema. Thrombin and lysed erythrocytes released from the hematoma are major factors causing BBB disruption, VE and CE (HUA et al. 2007).

Thrombin is an essential component in the coagulation cascade. Its production in the brain parenchyma is immediately upregulated after ICH, since the concentration of prothrombin in the plasma is high enough to produce a substantial amount of thrombin(HUA et al. 2007). It has been demonstrated that thrombin at high concentrations (>5 U/ml) induces significant BBB disruption, brain edema, neuron and astrocyte injury and death (K. R. LEE et al. 1997; XI et al. 2003). Delayed and systemic administration of thrombin inhibitors such as argatroban could reduce ICH-induced edema in a rat model (KITAOKA et al. 2002). In contrast, low doses of thrombin (1-2 U/ml) have beneficial effects in ICH, attributable to hemostasis and prevention of hematoma expansion via the effect of cleavage of fibrinogen to fibrin. Notably, thrombin preconditioning could induce a tolerance to ICH and focal cerebral ischemia in animal models (XI et al. 2003). This effect is thought to be mediated by protease-activated receptors (PAR-1, PAR-3 and PAR-4), which are found in neurons and astrocytes (VAUGHAN et al. 1995).

Lysis of erythrocytes results either from depletion of intracellular energy reserves or activation of the complement system, or both (XI et al. 2006; HUA et al. 2007). It is

associated with clot resolution and delayed brain damage in both animal models and clinical studies (WAGNER et al. 2003; G. WU et al. 2006). Animal models suggest that erythrocyte lysis can take place as early as 24 hours after hemorrhage and peaks at 2 days (MARLET u. BARRETO FONSECA JDE 1982; J. WU et al. 2003). It has been demonstrated in experimental studies that hemoglobin, or its degradation products such as hemin and iron which are released from lysed erythrocytes result in pronounced brain edema formation, BBB disruption and DNA injury within 24 hours (HUANG et al. 2002; J. WU et al. 2002; F. ZHAO et al. 2011). Treatment with minocyclineas iron chelators and nonspecific inhibitor of MMPs can inhibit these effects (C. Z. LEE et al. 2007; F. ZHAO et al. 2011). A clinical MRI study found a correlation between iron content in the hematoma estimated by the signal intensity on T2-weighted images and the relative PHE volume on day 3 after ICH (LOU et al. 2009).

The interplay between inflammation and oxidative and nitrosative stress induced by thrombin and lytic products from erythrocytes aggravates the brain injury after ICH. Evidence from experimental and clinical studies has shown that the pathological mechanisms involve a family of zinc-dependent enzymes - matrix metalloproteinases (MMPs) (XUE u. YONG 2008), which degrade the components of the extracellular matrix and cleave intracellular substrates (YONG et al. 2001; CAUWE u. OPDENAKKER 2010), and mediators of oxidative and nitrosative stress such as asymmetric dimethylarginine (ADMA) which can influence nitric oxide synthase (NOS) and nitric oxide (NO) (LI et al. 2011b). It has been shown in experimental studies that thrombin and hemin activate microglia and astrocytes, and thereby induce overexpression of pro-inflammatory cytokines, proteolytic enzymes and

mediators of oxidative stress (TNF-alpha, IL-1, IL-6, iNOS and MMPs) (RYU et al. 2000; POWER et al. 2003; HUA et al. 2006; R. L. DAVIS et al. 2008; LAIRD et al. 2008; J. WU et al. 2008). Additionally, perihematomal hypoperfusion can induce overexpression of neuronal NOS (nNOS) (BAUSER-HEATON u. BOHLEN 2007). Thereby a substantial amount of NO is produced by iNOS and nNOS after ICH. Animal models demonstrate that overexpression of NOS and NO (LI et al. 2011b), as well as MMPs such as MMP-3 and MMP-9 (XUE u. YONG 2008) play an important role in central nervous system injury, including neuronal cell injury, BBB disruption, and PHE formation in the perihematomal region after ICH. Of note, nNOS and iNOS up-regulate MMP-9 activity in neurons and macrophages (MANABE et al. 2005; LEE CZ 2009;), and high concentration of s-nitrosylated MMP-9 metabolized by NO and MMP-9 causes neuronal death (GU et al. 2002). These findings address the complex interaction of inflammatory response, oxidative and nitrosative stress. Therefore modulating these involved molecules might establish novel therapeutic strategies for ICH.

Clinical significance of PHE

Cytotoxic edema (CE) is characterized by cellular swelling mostly due to failure of ATP-dependent ion transport possibly involving several different membrane bound channel molecules. It can be detected by diffusion-weighted imaging (DWI) as decreased apparent diffusion coefficient (ADC) in the corresponding region (LIANG et al. 2007). CE has been detected in the perihematomal region in part of the ICH patients in a limited number of studies. It has been reported that CE occurs in the hyper-acute stage (<6 hours) until day 6

post-ICH (CARHUAPOMA et al. 2000; KIDWELL et al. 2001; SCHELLINGER et al. 2003; OLIVOT et al. 2010; TSAI et al. 2011). Kidwell et al. visualized a rim of perihematomal decreased ADC values within 6 hours in 3 among 12 patients with ICH[69]. Schellinger et al. observed a decreased ADC value within 6 hours in 7 among 32 patients within 6 hours after ICH onset (SCHELLINGER et al. 2003). Patients with the presence of CE surrounding the hematoma in the ultra-early stage of ICH were prone to develop poor clinical outcome (KIDWELL et al. 2001; SCHELLINGER et al. 2003). Recently Olivot et al. examined 23 ICH patients within 3 days after symptom onset. They found that 2/3 of the patients exhibited patchy regions with increased diffusivity mixed with reduced diffusion in the perihematomal region (OLIVOT et al. 2010). But they did not investigate the association of CE with outcome. Tsai et al. demonstrated that CE within 24 hours was associated with poor outcome at 6-months based on 46 cases using voxel-based analysis of ADC (TSAI et al. 2011). However, other DWI studies detected that ADC values increased globally in the perihematomal region (CARHUAPOMA et al. 2002; BUTCHER et al. 2004). Therefore more work is needed in this field to elucidate the causes and the development of CE over time and their meaning for clinical outcome.

There are limited studies involving the progression of PHE volume, and the association between PHE volume and clinical outcome. These studies demonstrate that PHE forms in the hyper-acute stage of ICH and develops rapidly over the first 3 days. In a CT study of 142 ICH patients, it showed that PHE occurred as early as within 3 hours after symptom onset and had an increase in volume of approximately 75% during the first 24 hours after ICH (GEBEL et al.

2002a). PHE volume was highly correlated with hematoma volume. ICH patients with lower baseline relative PHE (rPHE) volume were likely to increase PHE volume during the first 24 hours. rPHE was defined as the absolute PHE volume divided by hematoma volume. A sub-study from the INTERACT trial including 270 patients performed 3 sequential CT scans: within 6 hours of symptom onset, at 24 and 72 hours after the initial CT (ARIMA et al. 2009). It showed that the PHE volume increased over the whole time interval, and that hematoma volume was related to both, absolute PHE and rPHE volume. Chronological changes of PHE were investigated by several CT studies but showed controversial results. Zazulia et al. found that PHE progression occurred in the late stage of ICH (from 9 to 21 days), and was related to mass effect (ZAZULIA et al. 1999). In contrast, Inaji et al. showed in 14 cases that PHE increased rapidly over the first 3 days after ICH, slowly increased until day 14, and decreased thereafter (INAJI et al. 2003). This is consistent with 2 other studies (G. WU et al. 2006; X. ZHAO et al. 2006). Recently a MRI study investigated the natural history of PHE measured on FLAIR images in 27 ICH patients (VENKATASUBRAMANIAN et al. 2011). MRIs were done at 48±12 hours, 7±1 days, 14±2 days, and 21±3 days. PHE volume increased rapidly in the first 2 days, and peaked towards the end of the second week (12 days). This finding is in line with the majority of previous CT studies. Notably, PHE volume mainly consists of VE since CE commonly restricts to a rim of the perihematomal region. In this regard, DWI has the advantage in distinguishing VE and CE over CT scans.

Conflicting results have been reported with respect to the association of PHE volume with clinical outcomes. Inaji et al. showed that the rapid development of PHE within 3 days

after ICH led to subsequent clinical deterioration (INAJI et al. 2003). Gebel et al. showed that rPHE within 3 hours strongly predicted favorable functional outcome in patients with hyperacute spontaneous ICH without intraventricular extension, assessed by modified Rankin Scale (mRS≤2) and Barthel Index (BI<80) at 3 months, whereas absolute PHE volume did not (GEBEL et al. 2002b). This association was independent of other previously reported predictors of outcome in ICH, such as hematoma volume, Glasgow Coma Scale score, hematoma location, age, mass effect, hydrocephalus, and time from ICH onset to CT scan. However, in the study by Arima et al. who also found a significant association between the initial degree and expansion of PHE and rPHE and 90-day clinical outcome, this association got lost after adjustment for hematoma volume (ARIMA et al. 2009).

We therefore investigated the temporal pattern of the development of CE and PHE volume in a serial MRI study of primary ICH patients, and the relation between CE and PHE development and clinical outcome (Manuscript I).

Part III: Predictive value of molecular markers for HE, PHE and clinical outcome

The number of clinical studies which linked circulating levels of molecular biomarkers and their changes over time to clinical outcome is rather limited. Experimental studies have indicated a possible role of MMPs in ICH via degradation of the extracellular matrix components and intracellular substrates (YONG et al. 2001; CAUWE u. OPDENAKKER 2010), which contributes to neurotoxicity, BBB disruption and brain edema (XUE u. YONG

2008). Clinical studies tried to elucidate the role of MMPs in humans. Alvarez-Sabin et al. investigated the temporal profile of MMPs and their inhibitors with regard to PHE development and mortality after ICH (ALVAREZ-SABIN et al. 2004). Their study is the first study upon the time course of MMPs after ICH. Blood samples were collected on admission (<12 hours after symptom onset), at 24 hours, 48 hours, 7 days and 3 months in 21 ICH patients. Highest levels of MMP-2 and tissue inhibitor of metalloproteinases-2 (TIMP-2) were found at baseline, for MMP-9 and TIMP-1 at 24 hours, and for MMP-3 at 24-48 hours. Baseline MMP-9 was related to PHE, and MMP-3 was related to mortality at 3 months. Both MMP-3 and MMP-9 were related to 3-month residual cavity volume. Due to the small number of cases, no multivariate analysis was performed in this study. Silva et al reported that significantly increased MMP-9levels were found in patients who developed early hematoma expansion (HE) (SILVA et al. 2005). However levels of interleukin-6 (IL-6) which represents inflammatory activation and cellular fibronectin (c-Fn) which indicates the degradation of BBB basal membrane components by MMP-9 were independently predictive for HE, whereas MMP-9 was not. Abilieira et al showed that increased MMP-9 but not MMP-3 levels were associated with PHE and neurological deterioration in patients with deep ICH (ABILLEIRA et al. 2003). In a further study from the same group significantly higher levels of MMP-9 were detected in glial cells in human brain tissue surrounding the hematoma (ROSELL et al. 2006). These clinical studies aimed to shed light on the association of MMP-3 and MMP-9 with PHE, HE, and mortality after ICH. However data upon the implications of MMPs in secondary brain injury and clinical outcome after ICH in humans are still scarce. A better understanding of the molecular mechanisms involved in secondary brain injury of ICH and their predictive value with regard to clinical outcome might help to identify new therapeutic options in the acute phase. We therefore prospectively investigated the relationship between

MMP-3 and MMP-9 levels, PHE and clinical outcome in a cohort of 59 ICH patients. In contrast to former studies related to alterations of MMPs and PHE diffusion weighted MRI was used to assess PHE volume and CE presence (Manuscript II).

The results of the currently limited number of clinical studies on the role of NO in ICH are contradictory (RASHID et al. 2003; CHIANG et al. 2006). One study showed that increased NO levels in cerebrospinal fluid collected 3-14 days after ICH were associated with poor outcome at 6 months (CHIANG et al. 2006). In contrast, another study showed that lower NOx (nitrite and nitrate) plasma levels collected within 3 days were associated with poor clinical outcome at discharge (RASHID et al. 2003). Asymmetric dimethylarginine (ADMA), the endogenous inhibitor of NOS, is regarded as not only a risk marker of endothelial dysfunction, but also a mediator of oxidative stress due to the inhibition and uncoupling of NOS (SYDOW u. MUNZEL 2003). It has been shown in many clinical and experimental studies that over-expressed ADMA is harmful to the vascular and nervous systems in many diseases including ischemic stroke (LI et al. 2011b). However the role of ADMA in ICH is still unclear. Therefore, the current knowledge of ADMA and the NO-NOS-ADMA pathway in ICH has been described in (Publication II).

Publication I

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Contrast Extravasation on CT Angiography Predicts Clinical Outcome in Primary Intracerebral Hemorrhage: A Prospective Study of 139 cases

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Abstract

Background and Purpose: Several retrospective studies suggested that contrast extravasation on CT angiography (CTA) predicts hematoma expansion, poor outcome and mortality in primary intracerebral hemorrhage (ICH). We aimed to determine the predictive value of contrast extravasation on multi-detector CTA (MDCTA) for clinical outcome in a prospective study.

Methods: In 160 consecutive spontaneous ICH patients admitted within 6 hours of symptom onset, noncontrast CT (NCCT) and MDCTA were performed on admission. A follow-up NCCT was done at 24 hours. MDCTA images were analyzed to identify the presence of contrast extravasation. Clinical outcome was assessed by modified Rankin Scale (mRS) on discharge and at 90 days.

Results: A total of 139 primary ICH patients were included in the final analysis. Contrast extravasation occurred in 30 (21.6%) patients. The presence of contrast extravasation was associated with increased hematoma expansion (P<0.0001), in-hospital mortality (P=0.008), prolonged hospital stay (P=0.006), poor outcome on discharge (P=0.025), increased 3-month mortality (P=0.009) and poor clinical outcome (P<0.0001). In multivariate analysis, contrast extravasation was a promising independent predictor (OR=10.5, 95%CI 3.2-34.7, P<0.0001) for 90-day poor clinical outcome, followed by the presence of intraventricular hemorrhage (OR 3.4, 95%CI 1.5-7.7, P=0.003) and initial hematoma volume (OR 1.0, 95%CI 1.0-1.1, P=0.013).

Conclusions: The presence of contrast extravasation on MDCTA in hyper-acute stage

ICH-patients is an independent and strong factor associated with poor outcome. Any ICH patient with such sign on MDCTA should be monitored intensely and treated accordingly.

Key Words: Contrast extravasation, CT angiography, intracerebral hemorrhage, hematoma expansion, outcome, spot sign

Manuscript I

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Temporal pattern of cytotoxic edema in the perihematomal region after intracerebral hemorrhage: a serial MRI study

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Abstract

Background and Purpose

Perihematomal edema (PHE) is considered a major contributor to secondary brain injury in intracerebral hemorrhage (ICH). Knowledge on PHE and in particular on cytotoxic edema (CE) in ICH patients, however, is still limited. We aimed to characterize the temporal profile and composition of PHE in the acute stage of ICH.

Methods

Twenty-one patients with primary ICH were prospectively studied with sequential multimodal magnetic resonance imaging (MRI) at day 1, 3 and 7 after symptom onset. Hematoma and PHE volumes were measured on fluid-attenuated inversion recovery images. CE was identified using diffusion-weighted imaging. National Institutes of Health Stroke Scale score was assessed at admission and with each MRI. Clinical outcome was assessed by modified Rankin Scale at 90 days.

Results

PHE appeared in all patients and CE in half of the patients within the first 24 hours. CE peaked on day 3 and was self-limited by day 7 represented by an increase of ADC values towards normal (P=0.032). PHE volume increased over the first week, with predominance on day 1. Larger 3-day PHE volume (P=0.02) and presence of CE on day 3 (P=0.07) was associated with poor clinical outcome.

Conclusion

Considering CE as an indicator of ongoing neuronal injury during the first days after ICH and

its association with poor clinical outcome, further attempts should be made to understand the pathology behind CE development to be able to elaborate new therapeutic strategies.

Introduction

Intracerebral hemorrhage (ICH) is the most severe stroke subtype. It is associated with higher mortality and worse clinical outcome than ischemic stroke (IS), and currently no effective therapy is available[1]. Subsequent to the initial physical trauma and mass effect of the bleeding, secondary brain injury such as perihematomal edema (PHE) develops during the first few days after ICH onset[2]. In contrast to edema after IS, the pathophysiological mechanism of PHE formation is poorly understood. Experimental studies suggest that PHE within the first few hours after the bleeding results from hydrostatic pressure and clot retraction. The second and third phase of PHE development is related to the interaction of thrombin and hemoglobin toxicity and inflammation, which contribute to vasogenic (extracellular) and cytotoxic (intracellular) edema as the consequences of blood brain barrier disruption and neurotoxicity in the perihematomal area[2]. In clinical studies, vasogenic edema (VE) is commonly described in the perihematomal regions, whereas the existence of cytotoxic edema (CE) is controversially discussed[3-9], different from IS[10]. Some previous diffusion-weighted imaging (DWI) studies showed an increase of the apparent diffusion coefficient (ADC) in the perihematomal region[8, 9]. Other studies observed a decrease of ADC in the perihematomal area in part of the patients in the hyper-acute stage (<6 hours) till day 6 post-ictus and suggested a possible presence of CE in ICH[3-7]. One study showed patchy regions of increased

diffusivity mixed with reduced diffusion in two thirds of the patients who were included within 3 days after symptom onset[7]. The temporal development and the impact of PHE, and CE in particular, on clinical outcome in patients with primary ICH remain uncertain.

Therefore, we aimed to investigate prospectively the temporal profile of PHE and pattern of CE within the first week after ICH and their impact upon clinical outcome.

Subjects and Methods

Patients

Patients >18 years with primary supratentorial ICH who presented within 24 hours of symptom onset at Hannover Medical School (MHH) were prospectively included. Exclusion criteria were secondary ICH (hemorrhage due to aneurysm, vascular malformation, hemorrhagic infarction, tumor, or impaired coagulation), contraindication to perform MRI, undergoing a surgical procedure or refusal of participation. Demographic and clinical data of patients were collected on admission. The variables included gender, age, body mass index, alcohol and tobacco use, a detailed history of vascular risk factors and concomitant medications, body temperature, systolic and diastolic blood pressure, and laboratory tests. Stroke severity was evaluated by National Institutes of Health Stroke Scale (NIHSS) score at admission, 3 and 7 days. Clinical outcome was assessed by modified Rankin Scale (mRS) on 90 days. Informed consent was obtained from patients or relatives. The study has been approved by the local ethics committee.

Imaging protocol

Noncontrast computed tomography (CT) scans were done on admission, contiguous images with 2.5mm slice thickness were reconstructed (Light speed VFX, GE, Milwaukee, USA). Sequential MRI was performed using a 1.5 Tesla scanner (Magnetom Avanto, Siemens, Erlangen, Germany) within 24 hours, 72±12 hours, and 7±1 days after symptom onset. MRI included the following sequences: conventional gradient-echo T2*imaging (repetition time/echo time=760ms/23ms, 24 slices, 256×256 matrix, field of view=24 cm, 5.5mm/0.55mm slice thickness / gap), 3D - fluid attenuated inversion recovery (FLAIR) images (3D TSE sequence with slab selective variable excitation pulse, repetition time/echo time= 6000ms/335ms, 176 contiguous slices, 256×218 matrix, field of view=25.6 cm, slice thickness 1mm), triplanar DWI using two levels of diffusion sensitization (repetition time/echo time=3700ms/89ms, 192×192 acquisition matrix, field of view=24 cm, 5.5mm/0.55mm slice thickness / gap, 24 sections; x, y, and z axes averaged; b=0 and 1000 seconds/mm^2), and ADC maps calculated from DWI images by the image analysis system.

Image analysis

Image analysis was done by an experienced neuroradiologist who was blinded to clinical information. Hematoma location and the presence of intraventricular hemorrhage (IVH) were recorded. Hematoma volume was measured on the admission CT and FLAIR sequence of the sequential MRIs using a free image analysis software (ITK-SNAP) based on manually outlined hematoma boundaries[11]. PHE volume was measured on FLAIR images for all sequential time points using the same method, except for those with distortion due to motion

of patients. Relative PHE (rPHE) volume was defined as absolute PHE volume divided by baseline hematoma volume on FLAIR images. ADC values were calculated on ADC maps from DWI using the Stejskal-Tanner equation. Restricted diffusion lesions were qualitatively identified as a hyperintense signal on DWI (b=1000) with corresponding hypointense signal on ADC maps, which had to be located outside the boundary of the hematoma on T2*-weighted images. The region of interest (ROI) of restricted diffusion lesions was outlined manually. The cytotoxic edema was finally confirmed by the definition as relative ADC (rADC) ratios ≤0.9, which was calculated by using the mean ADC value of the lesion ROI divided by the mean ADC value of the individual patient's mirror ROI.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package Version 11.5. Categorical variables are shown as numbers and percentages. Continuous variables are presented as mean±SD, or median values [interquartile range (IQR)] as appropriate. Tests performed were the Fisher exact test for categorical variables, and the Student *t* test or the Mann–Whitney U test for continuous variables between groups as appropriate. Within group comparisons of the hematoma and PHE volumes, and ADC and rADC values at different time points were analyzed by repeated ANOVA. Spearman correlation analysis was used to study correlations between continuous variables. A value of $P<0.05$ was considered significant.

Results

Twenty-one patients were prospectively enrolled. The demographic and clinical data are shown in Table 1. Twenty patients had MRI on day 1 (15±9 hours), 19 patients had MRI on day 3 (2.9±0.5 days), and 18 patients had MRI on day 7 (7.0±0.9 days). There was no significant difference between ICH volume measured on admission CT and different time points of MRI (P=0.468). A strong correlation was found between ICH volume measured on the CT at admission and MRI on day 3, followed by MRI on day 1 and 7 (r=0.954, r=0.949, r=0.879, respectively; all P<0.001). ICH size measured on 1-day MRI was used as baseline hematoma volume (n=19), whereas ICH volume on 3-day MRI was used in patients who had no or distorted MRI on day 1 (n=2). Median hematoma volume was 11.4 ml [IQR 3.1-19.7] on admission CT, and 9.4ml [IQR 3.1-24.1] on baseline MRI, and remained stable throughout the study.

Table 1. Demographic and clinical characteristics of patients (n=21)

Demographic and medical history data	
Male gender	9 (42.9)
Age (years)	73±13
BMI	26.1±4.5
History of hypertension	15 (71.4)
History of diabetes	1 (4.8)
History of coronary artery disease	1 (4.8)
Tobacco use	1 (4.8)
Alcohol use	2 (9.5)
Antiplatelet medication	4 (19.0)
Clinical data	
Admission systolic blood pressure (mmHg)	185±29
Admission diastolic blood pressure (mmHg)	97±15
Admission GCS	15 [13-15]
Admission NIHSS	8 [6-15]
NIHSS on day 3	6 [4-14]
NIHSS on day 7	6 [3-14]
Imaging data	
Basal ganglion location of ICH	12 (57.1)

Thalamus location of ICH	4 (19.0)
Lobar location of ICH	5 (23.8)
Intraventricular hemorrhage extension	2 (9.5)
Hematoma volume on admission CT (ml)	11.4 [3.1-19.7]
Hematoma volume on MRI (ml)	9.4 [3.1-24.1]
PHE volume on day 1 (ml)	12.0 [6.5-27.9]
PHE volume on day 3 (ml)	15.4 [11.1-54.7]
PHE volume on day 7 (ml)	21.2 [10.1-60.6]
CE on day 1 (n=20)	9 (45.0)
CE on day 3 (n=19)	9 (47.4)
CE on day 7 (n=18)	6 (33.3)

PHE indicates perihematomal edema; CE: cytotoxic edema; BMI: body mass index. PHE volume is presented for patients who had 3 MRIs (n=13). Numbers represent number of patients and percentage if not otherwise indicated.

Temporal profile of CE

Among the 20 patients who had MRI on day 1, CE was detected in 9 (45%) cases (Figure 1). One of the 9 cases was lost to follow-up for MRIs on day 3 and 7. CE was present on day 3 in all remaining cases who had CE on day 1, but disappeared in 2 cases on day 7. Of note, in 1 case an additional CE lesion was detected on day 3 but had disappeared on day 7. In 1 case, CE was apparent on day 3 for the first time and remained until day 7 (Figure 2). CE was mostly located in a position medial, above or occipital to the hematoma (Table 2). The mean

ADC value of CE was decreased by about 33% ($529\pm91\times10^{-6}$ mm^2/s) relative to the mirror ROI on day 1, by about 37% ($504\pm96\times10^{-6}$ mm^2/s) on day 3, and 24% ($596\pm105\times10^{-6}$ mm^2/s) on day 7. Both ADC and rADC values of CE decreased from day 1 to day 3, then significantly reversed towards normal values on day 7 (ADC: F=4.447, P=0.032; rADC: F=5.586, P=0.016; respectively. Figure 3).

Patients showing CE on day-1 MRI had a significantly higher NIHSS score on admission and day 3 than those without (day 1: 9 [7-18] vs 6 [5-9], P=0.031; day 3: 10 [5-16] vs 4 [3-7], P=0.020; respectively). On day 7 NIHSS score did not significantly differ (P=0.112). Patients with CE on day 1 had a significantly larger PHE volume than those without (25.8 [10.3-51.1] vs 8.8 [4.7-16.1] ml, P=0.036). This did not apply for days 3 and 7. Patients who developed CE tended to have a larger hematoma volume than those without (23.5 ml [6.0-27.3] vs 3.4 ml [3.0-11.7], P=0.068) (Table 3). No correlation of ADC and rADC values of CE with ICH or PHE volume was found.

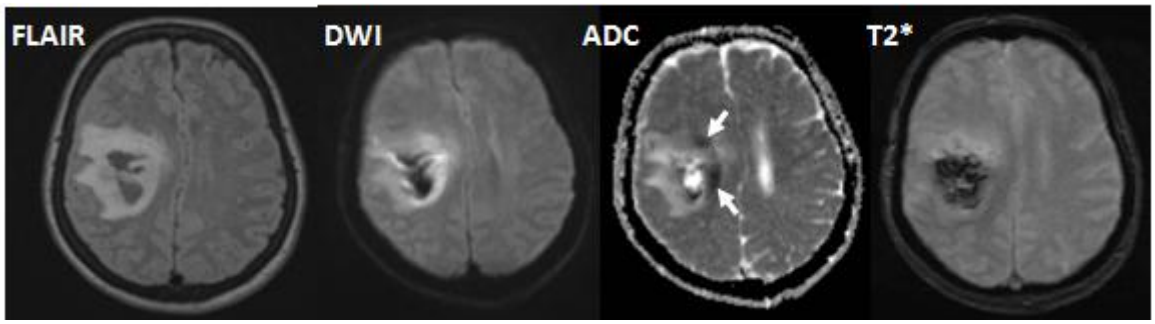

Figure 1. Example of CE on MRI. White arrow indicates CE on the ADC image.

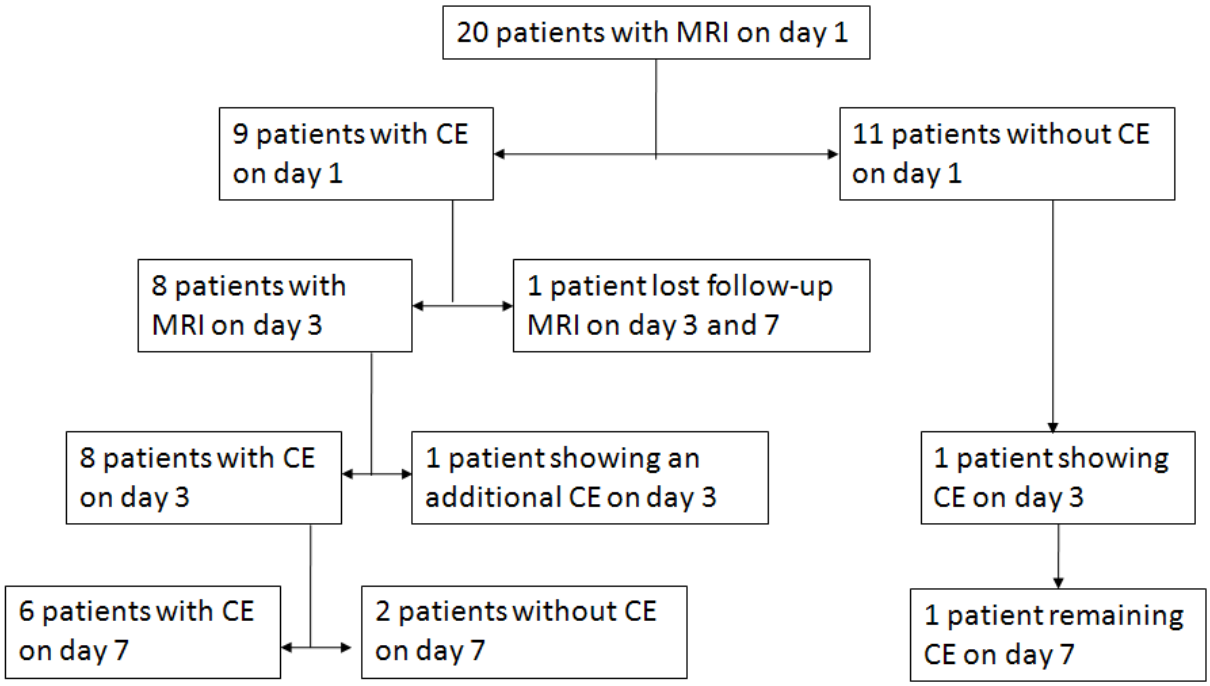

Figure 2. Temporal pattern of CE in the acute stage of primary ICH.

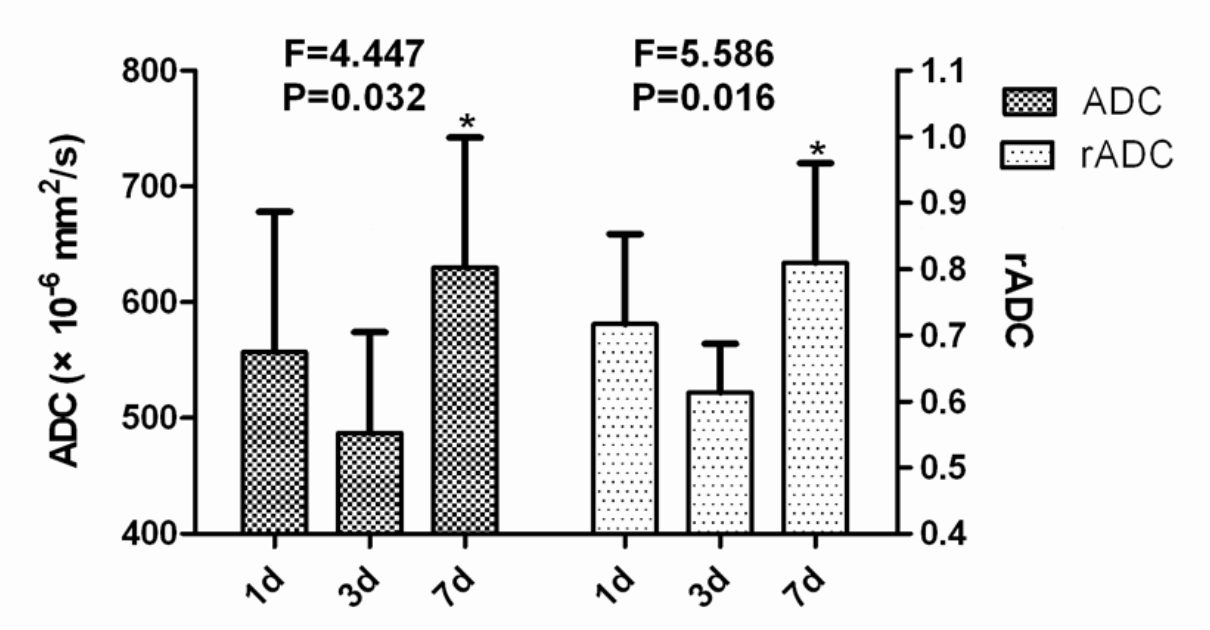

Figure 3. Temporal profile of ADC and rADC values in the acute stage of primary ICH.

* indicates a significant difference of ADC and rADC on day 7 compared to day 3 (P<0.05).

Table 2: Characteristics of ICH and CE

Patient	Age	Sex	ICH location	ICH volume (ml)	IVH	CE on D1	Mean rADC	CE on D3	Mean rADC	Ce on D7	Mean rADC	Ce location
1	80	F	BG	3.6	No	No	0.980	Yes	0.556	Yes	0.792	M,T
2	77	M	lobe	3.0	No	No	-	/	/	/	/	-
3	49	M	BG	9.4	No	Yes	0.751	/	/	/	/	M,T
4	44	M	BG	1.4	No	Yes	0.591	Yes	0.631	Yes	0.882	M,T,O
5	87	M	lobe	60.5	No	Yes	0.505	Yes	0.771	/	/	I
6	61	F	BG	26.7	No	Yes	0.567	Yes	0.506	Yes	0.524	M,T,O
7	80	F	BG	14.2	Yes	/	/	No	-	No	-	-
8	71	F	BG	6.5	Yes	No	-	No	-	No	-	-
9	55	M	BG	27.9	No	Yes	0.676	Yes	0.563	No	0.939	M,I,O
10	76	M	thalamus	3.1	No	No	-	No	-	No	-	-
11	84	F	thalamus	2.2	No	No	-	No	-	No	-	-
12	84	M	thalamus	2.6	No	Yes	0.716	Yes	0.725	Yes	0.879	T, F
13	71	M	BG	3.0	No	No	-	No	-	No	-	-
14	82	F	BG	11.7	No	No	-	No	-	No	-	-
15	86	F	thalamus	1.2	No	No	-	No	-	No	-	-
16	79	M	BG	21.9	No	Yes	0.719	Yes	0.700	Yes	0.782	M,O

17	63	F	lobe	26.7	No	No	-	No	-	No	-	-
18	87	F	lobe	17.3	No	No	-	No	-	No	-	-
19	83	F	lobe	24.5	No	Yes	0.646	Yes new	0.620 0.756	No	0.955	M,O
20	70	F	BG	3.40	No	No	-	No	-	No	-	-
21	62	F	BG	23.5	No	Yes	0.840	Yes	0.611	Yes	0.653	M,T

BG indicates basal ganglia;CE: cytotoxic edema; ICH: intracerebral hemorrhage; IVH, intraventricular hemorrhage; M, T, O, or I, the location of cytotoxic edema is medial, top occipital, or inferior to the hematoma; rADC: relative ADC value of cytotoxic edema; / means no MRI was performed at this time point due to the clinical condition; new means an additional CE lesion was detected.

Table 3: Demographic and clinical characteristics by the presence of CE on day 1

	Patients without cytotoxic edema (n=11)	Patients with cytotoxic edema (n=9)	P value
Demographic data			
Male gender	3 (27.3)	6 (66.7)	0.175
Age (years)	77±8	67±16	0.124
BMI	24.9±3.8	28.3±4.7	0.108
Clinical data			
History of hypertension	8 (72.7)	7 (77.8)	1.000

Anticoagulant medication	0	1 (11.1)	0.450
Antiplatelet medication	3 (27.3)	1 (11.1)	0.591
Admission systolic blood pressure (mmHg)	179±20	192±39	0.360
Admission diastolic blood pressure (mmHg)	93±11	101±19	0.412
Admission GCS	15 [14-15]	14 [11-15]	0.201
Admission NIHSS	6 [5-9]	9 [7-18]	0.031*
NIHSS on day 3	4 [3-7]	10 [5-16]	0.020*
NIHSS on day 7	4 [3-8]	8 [4-16]	0.112
Imaging data			
Deep location of ICH	8 (81.8)	7 (77.8)	1.000
Intraventricular hemorrhage extension	1 (9.1)	0	1.000
Hematoma volume on FLAIR (ml)	3.4 [3.0-11.7]	23.5 [6.0-27.3]	0.067
PHE volume on day 1 (ml)	8.8 [4.6-16.1]	25.8 [10.3-51.1]	0.036*
PHE volume on day 3 (ml)	14.1 [9.0-25.3]	54.7 [18.7-81.6]	0.059
PHE volume on day 7 (ml)	14.3 [9.6-22.0]	58.6 [19.5-79.0]	0.161
rPHE volume on day 1	1.7 [1.2-2.5]	1.5 [1.0-3.0]	1.000
rPHE volume on day 3	3.5 [1.8-4.1]	2.8 [1.7-4.3]	0.888
rPHE volume on day 7	3.8 [1.5-4.7]	2.5 [2.2-3.6]	1.000
mRS score at 3-months	2 [1-4]	4 [3-4]	0.175

Data are expressed as n (%), mean±SD, or median [IQR] as appropriate.

*indicates $P<0.05$

Temporal profile of PHE

PHE was visible as a region of hyperintense signal on FLAIR images in all patients. Thirteen patients had FLAIR images at all three time points (8 had distorted or incomplete series of FLAIR images). Median PHE volume measured on these FLAIR images was 12.0 ml [IQR 6.5-27.9] on day 1, 15.4 ml [IQR 11.1-54.7] on day 3, and 21.2 ml [IQR 10.1-60.6] on day 7. PHE volume gradually increased during the acute stage of ICH (F=11.067, P=0.004; Figure 4A). Absolute PHE growth was fastest in the first 24 hours, presuming a PHE volume of 0 ml at ICH onset (Figure 4B). Median relative PHE volume was 1.75 [IQR 1.05-2.79] on day 1, 3.63 [IQR1.79-4.32] on day 3, and 3.45 [IQR 2.16-4.97] on day 7. Larger hematoma volume at baseline was accompanied by larger absolute PHE volume on day 1, 3 and 7, respectively (r=0.86, r=0.80, r=0.80, respectively; all P<0.001). However, larger hematoma was accompanied by less rPHE on day 1, 3 and 7, respectively (r=-0.58, P=0.019; r=-0.64, P=0.004; r=-0.50, P=0.033; respectively).

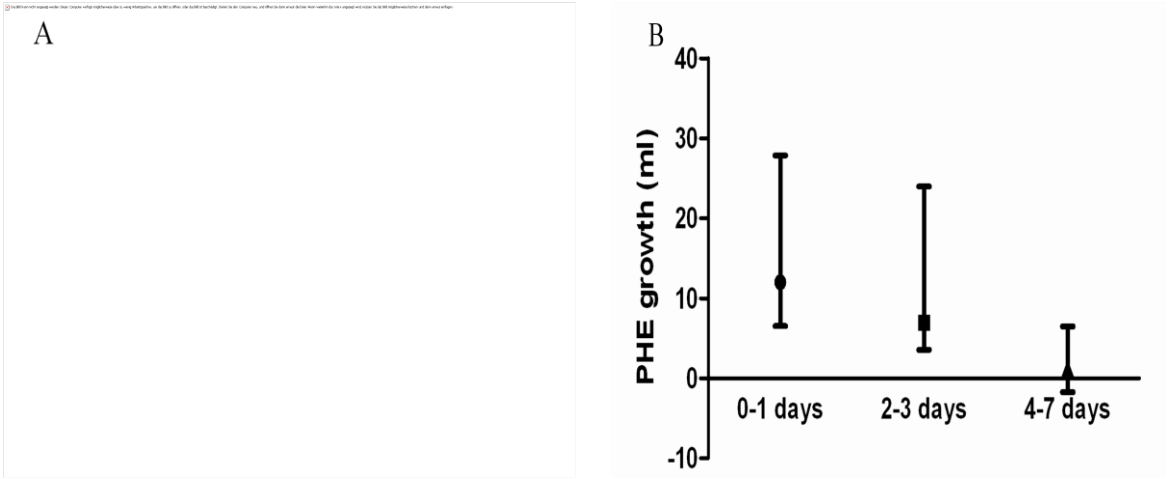

Figure 4. Temporal profile of PHE volume (A) and PHE volume growth (B) in the acute stage of primary ICH.

* indicates a significant difference of PHE volume on days 3 and 7 compared to day 1($P<0.05$).

Association of PHE and CE with clinical outcome

At 90-day follow-up 12 patients showed favorable (mRS 0-3) and 9 unfavorable outcome (mRS 4-6). Larger absolute PHE volume on day 3 was observed in patients with unfavorable clinical outcome in comparison to those with favorable outcome (54.8 ml [37.0-82.3] vs. 14.1 ml [9.1-35.2], P=0.020). No similar association was found between rPHE volume and clinical outcome. Patients with the presence of CE on day 3 tended to develop unfavorable outcome (P=0.07). Baseline hematoma volume was not associated with outcome (P=0.213).

Discussion

The main findings of the present study are that (i) CE occurs in nearly half of the patients within the first 24 hours after spontaneous ICH, and is pronounced on day 3 but tends to be reversible towards day 7; and that (ii) PHE is present in all patients and in contrast to CE progressive in volume during the first week after ICH.

To the best of our knowledge, this is the first prospective study to investigate the temporal profile of CE after ICH. CE is restricted diffusion due to cellular swelling mostly referred to failure of ATP-dependent ion transport possibly involving several different membrane bound channel molecules, which could be demonstrated by DWI as decreased ADC in the corresponding region[10]. In a few clinical ICH studies, CE was detected in the perihematomal region in part of ICH patients within the first week[3-7]. It was shown that

patients with the presence of this cellular impairment within 6 hours of symptom onset were prone to develop unfavorable clinical outcome[3, 4]. Therefore it was put into question if there is a salvageable perihematomal ischemic penumbra similar to that in IS[4, 12]. One study using DWI and perfusion-weighted images (PWI) within 6 hours of symptom onset proposed that there was no ischemic penumbra in ICH patients since only hypoperfusion but no ischemic damage was found in the perihematomal region[4]. This conclusion was based on the finding that in average increased ADC values were detected in the 1cm broad swath of tissue surrounding the hematoma, rather than decreased ADC values when considering all examined patients. However, Warach argued in an editorial to this study that a subset of patients in this study who presented a decreased ADC and developed poor clinical outcome, might have had undetected perihematomal tissue changes since no follow-up DWI was performed.[12]. Our study demonstrated that CE in ICH was pronounced 3 days after ICH and was self-limited since the ADC values improved spontaneously until day 7 (Figure 3). This is in line with the concept that CE is still a reversible step in any cellular dysfunctional process if compensatory mechanisms such as ionic channel or ATP pump activity are still effective, whereas CE turns into oncotic cell death when the compensatory mechanisms fail[13]. While the hypothesis that an ischemic penumbra exists in ICH was disapproved from perfusion computed tomography and PWI data[12, 14, 15], more evidence suggested the existence of a non-ischemic metabolic crisis surrounding the hematoma[16-20]. Analysis of brain tissue samples of ICH patients who had been operated on within 72 hours after ICH onset demonstrated mitochondrial dysfunction in the perihematomal region[16], which was thought to contribute to a reduction in oxidative metabolism and oxygen utilization in this region[17]. A transient focal increase in perihematomal glucose metabolism was observed in ICH patients 2-4 days post-ictus, and resolved on day 7[18]. A similar change of glucose utilization was seen in traumatic ICH

patients[21]. These observed metabolic changes over time are in line with the CE changes in our study.

The presence of CE was associated with stroke severity on admission represented by higher NIHSS score, larger hematoma and PHE volumes. These findings are consistent with previous DWI studies[3, 5, 7]. We found that PHE is progressive during the first week after ICH, while the fastest growth occurs within the first 24 hours (Figure 4). This is in accordance with data about the natural history of PHE from a recent MRI study and a large CT study[22, 23]. In our study, poor 90-day clinical outcome was significantly associated with larger absolute PHE volume on day 3 and tended to be associated with pronounced CE on day 3, but not with baseline hematoma volume. This finding implies that secondary brain injury might play a role as important as initial hematoma size in ICH patients with small to medium hematomas. This assumption is supported by studies showing that ICH patients with appearance of CE within 6 -24 hours are prone to develop unfavorable clinical outcome[3-5]. Experimental and clinical studies suggested that local compression, diaschisis and locally mediated toxic clot components such as thrombin and hemoglobin degradation compounds, particularly iron, are likely to be responsible for perihemorrhagic tissue damage[2, 24,25]. Such ongoing neuronal injury and its relationship with poor clinical outcome may therefore represent an important therapeutic target.

Our findings cannot be generalized to patients with coma on admission and those non-eligible for MRI performance. Particularly in patients with large hematoma pronounced CE and larger PHE volume can be expected. This could not be proven in this study since it is difficult to examine these patients sequentially by MRI due to adverse clinical conditions.

Secondly, we examined a relatively small number of cases. This might influence the strength of the association between CE and poor outcome.

Conclusion

Our current prospective data in acute ICH patients show that CE is pronounced on day 3 but tends to be reversible after 1 week and is unrelated to PHE growth during the first week. The temporal pattern of CE complies with the metabolic change in the perihematomal region and might link metabolic crisis and ongoing neuronal injury after ICH. Further studies are warranted to investigate the pathology behind CE development before it might be considered as new treatment target.

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Manuscript II

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Association of molecular markers with perihematomal edema and clinical outcome in intracerebral hemorrhage

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Abstract

Background and Purpose

Perihematomal edema (PHE) contributes to secondary brain injury in intracerebral hemorrhage (ICH). Increase of matrix metalloproteinases (MMPs) and growth factors (GFs) is considerably involved in blood-brain barrier disruption and neuronal cell death in ICH models. We therefore hypothesized that increased levels of these molecular markers are associated with PHE and clinical outcome in ICH patients.

Methods

Fifty-nine patients with spontaneous ICH admitted within 24 hours of symptom onset were prospectively investigated. Noncontrast CT was performed on admission for diagnosis of ICH and quantification of initial hematoma volume. MRI was performed on day 3 in order to evaluate PHE. Concentrations of MMP-3, MMP-9, as well as vascular endothelial growth factor (VEGF) and Angiopoietin-1(Ang-1) on admission were determined by enzyme-linked immunosorbent assays. Clinical outcome was assessed by modified Rankin Scale (mRS) at 90days.

Results

Increased MMP-3 levels were independently associated with PHE volume ($P<0.05$). Cytotoxic edema (CE) surrounding the hematoma was seen in 36 (61%) cases on 3-day MRI. CE did not correlate with the level of any of the biomarkers studied. Levels of MMP-3 ≥12.4 ng/ml and MMP-9 ≥192.4 ng/ml but not VEGF and Ang-1 predicted poor clinical outcome at 90 days (mRS>3) independent of stroke severity and hematoma volume at baseline (OR 25.3,

P=0.035; OR 68.9, P=0.023; respectively).

Conclusion

Metalloproteinases 3 and 9 seem to be significantly involved in secondary brain injury and outcome after primary ICH in humans and thus should be further evaluated as targets for therapeutic strategies in this devastating disorder.

Introduction

Intracerebral hemorrhage (ICH) accounts for about10–15% of strokes in Western countries and up to 20–30% in Asian countries[1]. ICH is associated with higher mortality and worse clinical outcome than ischemic stroke, and no effective therapy is available[1, 2]. Subsequent tothe initial physical trauma and mass effect of the bleeding, secondary brain injury such as perihematomal edema (PHE) including vasogenic (extracellular) and cytotoxic (intracellular) edema develops[3]. Both types of PHE have been shown to be associated with poor outcome[4-7]. Matrix metalloproteinase (MMP)-3 and MMP-9[8-10],as well as growth factors (GFs) such as vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1)[11], are expressed in abnormally high concentrations in brain and peripheral blood in ICH patients. In ICH animal models elevated MMP-9 and MMP-3 contribute to blood–brain barrier (BBB) disruption[12, 13], brain edema[14, 15] and neuronal cell death[16, 17]. Other animal models of ICH and brain injury showed that alteration of VEGF and Ang-1 was related to increased BBB permeability and brain edema[18-22].

Data upon the implications of MMPs and GFs in secondary brain injury after ICH in humans are still scarce. A better understanding of these molecular pathophysiological mechanisms involved in secondary brain injury of ICH might help to develop new therapeutic options. Therefore we aimed to investigate the relationship between the molecular markers MMP-3, MMP-9, VEGF and Ang-1and PHE as well as clinical outcome in patients with primary ICH.

Subjects and Methods

Study Population

All patients with spontaneous ICH admitted to the Neurological Department of Beijing Tiantan Hospital from January 2011 to December 2011 were screened for this study. Inclusion criteria were time from symptom onset to admission <24 hours, age <80 years and absence of coma. Exclusion criteria were secondary ICH (hemorrhage due to aneurysm, vascular malformation, hemorrhagic infarction, tumor, or impaired coagulation), history of acute or chronic infection, malignant diseases and immunosuppressive treatment, contraindication for MRI, undergoing a surgical procedure or refusal of participation. A total of 59 patients were prospectively included after informed consent from patients or their relatives. The study has been approved by the local ethics committee.

Clinical data of patients were collected on admission. The variables included gender, age, body mass index, alcohol and tobacco use, a detailed history of vascular risk factors and

concomitant medications, body temperature, systolic and diastolic blood pressure, and laboratory tests. Stroke severity was evaluated by Glasgow Coma Scale (GCS) and National Institutes of Health Stroke Scale (NIHSS) at admission. Poor clinical outcome was defined as modified Rankin Scale (mRS) >3 assessed at 90-day follow-up.

Radiological Protocol

Noncontrast cerebral computed tomography (NCCT) scans were performed on admission, according to the protocol of the neuroradiological department with an image matrix of 512x512, 4.5-mm-wide slices for posterior fossa and 9-mm-wide slices for medium and anterior fossae. Cerebral magnetic resonance imaging (MRI) was performed by 3.0 Tesla scanners (Trio-Tim, Siemens, Erlangen, Germany) on 3±1 days. MRI included the following sequences: conventional T1-weighted and T2-weighted images, gradient-recalled echo imaging (T2*), diffusion-weighted images (DWI) using two levels of diffusion sensitization (b=0 and 1000 s/mm^2), and apparent diffusion coefficient (ADC) maps created from DWI images by the image analysis system.

Investigators who analyzed the images were blinded to clinical and biomarker information. Hematoma location (supratentorial deep location versus others including lobar and infratentorial locations), and the presence of intraventricular hemorrhage (IVH) were recorded. Hematoma volume on baseline NCCT was calculated by the summation-of-area method on each slice multiplied by the interslice thickness[23]. PHE was evaluated on 3-day MRI. MRI measures were performed jointly by two investigators using an open consensus

method. The boundaries of the hematoma and PHE were outlined manually on T2-weighted images. The absolute PHE volume was measured by subtracting hematoma volume from total lesion area, according to the same volumetric method mentioned. Relative PHE volume was defined as absolute PHE volume divided by hematoma volume, yielding a dimensionless ratio variable. Cytotoxic edema (CE) was defined as relative ADC (rADC) ratios ≤0.9 outside the boundary of the hematoma, which was calculated by using the ADC of the individual patient's mirror segmentation in the healthy hemisphere as the denominator. To ensure that the perihematomal regions with low ADC values were outside of the hematoma, only region of interest (ROI) residing outside of hematoma projection on T2* images were considered (Figure 1) .

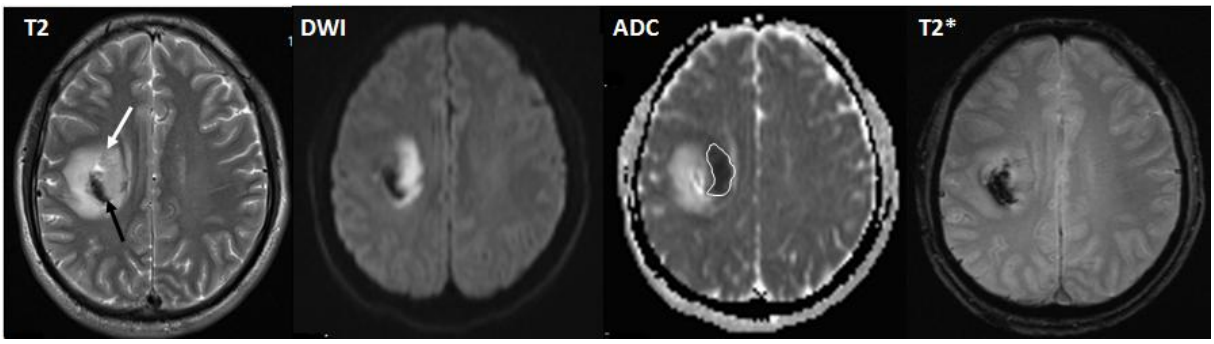

Figure 1: Example of edema surrounding the hematoma on 3-day MRI. Black and white arrows indicate hematoma and PHE on the T2-weighted image, respectively. ROI region of CE is outlined on the ADC image.

Immunoassay Methods

For the measurement of markers, blood samples were drawn using EDTA and serum tubes

from each patient on admission. Plasma was immediately separated by centrifugation at 3000 rpm for 15 minutes and stored at -80°C. The levels of MMP-3, MMP-9, VEGF and Ang-1 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems). MMP-9 was measured in EDTA plasma, MMP-3, VEGF and Ang-1 in serum.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package Version 11.5. Categorical variables are shown as numbers and percentages. Continuous variables are presented as mean±SD, or median values [interquartile range] as appropriate. Tests performed were the χ^2 or Fisher exact tests for categorical variables, and the Student *t* test or the Mann–Whitney U test for continuous variables as appropriate. Spearman correlation analysis was used to study correlations between continuous variables. Cutoff points of studied markers were determined using the receiver operating characteristic (ROC) curve and Youden Index. Multivariable analysis using a multiple linear regression model was performed to assess the relationship between the molecular markers and the PHE volume at day 3. A stepwise logistic regression analysis was performed to determine factors that could be considered as independent predictors of unfavorable clinical outcome at 3 months. Variables were retained in the logistic regression model for $P \leq 0.10$. A value of $P < 0.05$ was considered significant.

Results

Baseline clinical, neuroimaging and laboratory findings

Baseline variables of the 59 included patients are shown in Table 1. Median time from symptom onset to baseline NCCT was 3.5[1.6-7.5] hours. Median time to follow-up MRI was 76 [69-88] hours. MMP-3, MMP-9, VEGF and Ang-1 levels did not correlate with hematoma volume at baseline.

Table 1: Baseline clinical, radiological and laboratory characteristics of all patients and patients grouped by 90-day outcome

	All (n=59)	Clinical outcome at 90 days		
		Favorable outcome (n=50)	Unfavorable outcome (n=9)	P value
Male gender	41 (69.5)	35 (70.0)	6 (66.7)	1.000
Age (years)	56±11	56±11	60±14	0.338
BMI	25.3±3.5	25.4±3.5	24.7±3.8	0.601
History of hypertension	52 (88.1)	44 (88.0)	8 (88.9)	1.000
History of diabetes	11 (18.6)	9 (18.0)	2 (22.2)	0.670
History of stroke	13 (22.0)	12 (24.0)	1 (11.1)	0.673
Tobacco use	24 (40.7)	19 (38.0)	5 (55.6)	0.619
Alcohol use	26 (44.1)	22 (44.0)	4 (44.4)	1.000
Antihypertensive medication	26 (44.1)	21 (42.0)	5 (55.6)	0.697
Antiplatelet medication	8 (13.6)	6 (12.0)	2 (22.2)	0.494
GCS	15 [14-15]	15 [14-15]	14 [13-15]	0.009*
NIHSS	6 [3-10]	5 [2-9]	13 [10-15]	<0.001*

Systolic blood pressure (mmHg)	168±25	165±24	180±25	0.108
Diastolic blood pressure (mmHg)	97±15	98±15	91±15	0.163
Hematoma volume	10.0 [5.2-23.9]	9.5 [4.1-20.1]	25.2 [16.5-38.1]	0.003*
PHE volume	6.5 [3.0-11.9]	5.7 [2.5-11.7]	10.0 [6.7-22.1]	0.062
Deep location	49 (83.1)	40 (80.0)	9 (100)	0.322
Intraventricular hemorrhage extension	13 (22.0)	12 (24.0)	1 (11.1)	0.673
Leukocyte ($10E^9$/L)	9.0±2.7	8.9±2.7	9.9±3.1	0.313
Platelet ($10E^9$/L)	220.7±47.3	218.5±49.1	232.9±34.9	0.406
Glucose (mmol/l)	7.0±2.1	7.2±2.2	6.0±0.8	0.144
Creatinine (µmol/l)	64.4±18.4	64.2±19.6	65.6±10.2	0.844
INR	0.99±0.06	0.99±0.06	0.99±0.06	0.928
Fibrinogen (g/L)	2.6±0.9	2.5±0.8	3.0±0.8	0.118
MMP-3 (ng/ml)	10.7 [7.5-21.0]	9.9 [6.9-18.7]	17.0 [10.3-28.1]	0.124
MMP-9 (ng/ml)	140.2 [102.4-217.0]	135.2 [100.4-192.2]	232.7 [97.7-306.8]	0.124
VEGF (pg/ml)	324.9 [186.7-516.2]	331.7 [197.2-523.2]	270.6 [164.6-404.9]	0.411
Ang-1 (ng/ml)	28.2 [21.2-34.8]	26.1 [20.9-34.4]	28.8 [24.7-41.4]	0.273

* P<0.05 was considered significant. Data are expressed as n (%), mean±SD, or median [IQR] as appropriate. Chi-square test was used for dichotomizing variables, the Student's t test or Mann-Whitney U test was used for continuous variables.

Association of PHE volume with hematoma volume and molecular markers

PHE was present in all patients on 3-day T2-weighted images. A high correlation was found between absolute PHE and hematoma volume at baseline and day 3 (r=0.922 and r=0.959,

respectively; both P<0.001) (Figure 2). Absolute PHE volume was positively correlated with MMP-3 (r=0.311, P=0.017), but not with MMP-9, VEGF and Ang-1. The linear stepwise regression model revealed that MMP-3 was independently associated with absolute PHE volume (Beta=0.370, P=0.004), irrespective of age and gender. MMP-3 remained independently associated with absolute PHE volume when baseline hematoma volume was also considered (Beta=0.138, P=0.043).

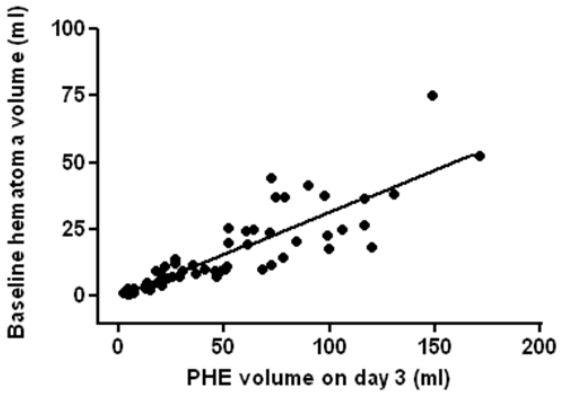

Figure 2: Relation between baseline hematoma and 3-day PHE volumes after ICH. Baseline hematoma volume vs 3-day PHE volume: r=0.922, *P*<0.001.

Association of CE with hematoma volume and molecular markers

Among the 59 included patients, CE was detected in 36 (61%) on 3-day MRI. Mean absolute and relative ADC value of CE were 527.5±83.4 $x10^{-6}mm^2/s$ and 0.665±0.116, respectively. Absolute and relative ADC value were negatively correlated with baseline hematoma volume (r=-0.337, *P*=0.044; r=-0.352, *P*=0.035; respectively). Patients with CE had a significantly larger hematoma volume at baseline and day 3 than those without (baseline: 14.7 [7.7-25.1]

vs 6.9 [2.8-12.5] ml, *P*=0.011; day 3: 21.1 [10.0-41.3] vs 7.6 [2.9-17.5] ml, *P*=0.001; respectively) (Figure 3). CE and ADC values did not correlate with MMP-3, MMP-9, VEGF or Ang-1 levels. Radiological data and levels of measured markers grouped according to the status of CE are provided in Table 2.

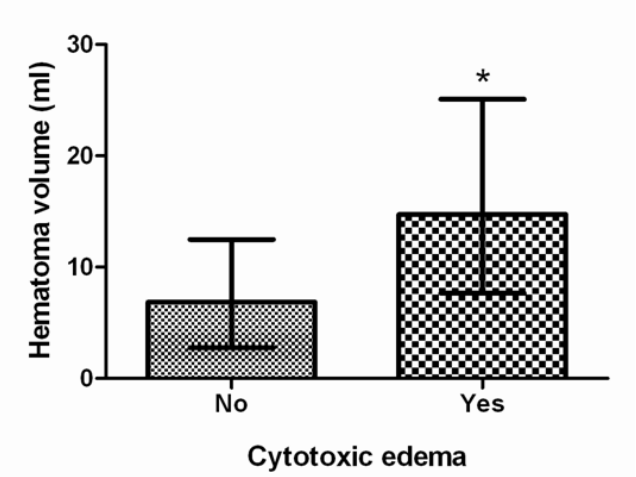

Figure3: Baseline hematoma volume in patients with and without CE.

* indicates P<0.05

Table 2: Radiological characteristics and marker levels grouped by presence of CE

Radiological data	Patients without cytotoxic edema (n=23)	Patients with cytotoxic edema (n=36)	P value
Deep location of hematoma	17 (73.9)	32 (88.9)	0.166
Intraventricular hemorrhage extension	9 (39.1)	4 (11.1)	0.011*
Baseline hematoma volume (ml)	6.9 [2.8-12.5]	14.7 [7.7-25.1]	0.011*
Baseline PHE volume(ml)	4.7 [0.1-9.3]	8.0 [4.7-16.3]	0.010*
Time to follow-up MRI (h)	78.6±13.1	76.6±11.0	0.237
Hematoma volume on day 3(ml)	7.6 [2.9-17.5]	21.1[10.0-41.3]	0.001*
PHE volume on day 3(ml)	20.3 [6.9-40.9]	52.1 [28.7-98.6]	0.001*
Relative PHE on day 3	3.0 [2.4-3.9]	2.7 [2.0-3.5]	0.359
MMP-3 (ng/ml)	9.5 [6.0-21.0]	11.1 [8.0-20.8]	0.484
MMP-9 (ng/ml)	140.2 [102.7-232.7]	139.6 [96.5-196.2]	0.938
VEGF(pg/ml)	341.8 [284.2-517.5]	302.5 [177.9-504.1]	0.446
Ang-1(ng/ml)	28.3 [22.5-34.8]	25.7 [20.5-35.0]	0.524

Data are expressed as n (%), mean±SD, or median [IQR] as appropriate.

Non-contrast CT scan was performed on baseline and MRI on day 3. Studied molecules were measured from blood withdrawals at baseline.

Association of PHE and molecular markers with clinical outcome

At 90-day follow-up 50 patients showed favorable outcome (mRS 0-3) and 9 unfavorable outcome (mRS 4-6). Clinical, radiological and laboratory data according to outcome are presented in Table 1. Larger PHE volume was observed in those patients with unfavorable clinical outcome in comparison to those with favorable outcome (P<0.001). No association was found between outcome and presence or absence of CE. ROC analysis was performed for concentrations of MMP-3, MMP-9, VEGF and Ang-1. A possible cut-off point for the discrimination of dichotomized clinical outcome was calculated for MMP-3 as 12.4 ng/ml, MMP-9 as 192.4 ng/ml, VEGF as 317.6 pg/ml, and Ang-1 as 27.3 ng/ml. Using these cut-off points univariate analysis found that MMP-3 ≥12.4 ng/ml and MMP-9 ≥192.4 ng/ml predicted unfavorable 90-day outcome (*P*=0.029, *P*=0.018, respectively), whereas VEGF and Ang-1 did not (*P*=0.277, *P*=0.252, respectively). In addition to all the significant variables in univariate analysis including categorized MMP-3 and MMP-9, hematoma volume, GCS and NIHSS score on admission, age and gender and were included in a multivariate logistic analysis to determine independent predictors for unfavorable outcome. As a result, MMP-3 ≥12.4 ng/ml (OR 25.3, *P*=0.035), MMP-9 ≥192.4 ng/ml (OR 68.9, *P*=0.023) and NIHSS at baseline (OR=1.7, *P*=0.005) were identified as independent predictors of unfavorable 90-day outcome.

Discussion

The main findings of the present study in acute patients with spontaneous ICH are that (i) the circulating levels of MMP-3 are correlated with absolute PHE volume, (ii) increased levels of MMP-3 and MMP-9 at admission are independent predictors of poor clinical outcome, and (iii) no association of the investigated markers (MMP-3, MMP-9, VEGF and Ang-1) with the presence of CE was detected.

Our finding that baseline MMP-3 and MMP-9 levels predict poor clinical outcome, independently of stroke severity and baseline hematoma volume, can be explained pathophysiologically. After ICH, secondary brain injuries including BBB disruption, brain edema and neuronal cell death, are induced by interaction of the coagulation cascade and thrombin production, erythrocyte lysis and hemoglobin toxicity, together with inflammation[3]. Pro-MMPs can be activated by plasmin, thrombin, hemoglobin products, and reactive oxygen species produced following ICH. Activated MMP-3 can activate MMP-9 which predominantly degrades the components of the extracellular matrix[24]. In ICH animal models, several studies showed that elevated levels of MMP-9 lead to BBB breakdown and brain edema[12-15]. It was recently demonstrated that MMP-9 and MMP-3 jointly contributed to blood-induced lesions and neuronal cell death[16, 17]. In previous clinical studies, MMP-9 levels significantly correlated with PHE volume in the first 24 hours after symptom onset[25, 26]. In our study, MMP-3 independently predicted PHE volume on day 3 after symptom onset. This phase of PHE is thought to result predominantly from BBB disruption and neuronal injury due to the interplay of hemoglobin toxicity and inflammation[3]. Our study showed a

relationship between 3-day PHE volume and clinical outcome. However, it is still controversially discussed if PHE independently influences clinical outcome[4, 5, 27].

Previous studies showed increased MMP-9levels in patients who developed hematoma expansion and neurological deterioration after ICH[25, 28]. Increased MMP-3 levels on admission were reported being associated with mortality in ICH patients, both, MMP-3 and MMP-9, with the residual scar volume at 3 months[26]. Our study confirmed a significant role of the MMPs in the pathology after ICH as both, MMP-3 and MMP-9, were associated with unfavorable 90-day outcome independent of hematoma volume and stroke severity at baseline. Therefore targeting MMPs for preventing unfavorable clinical outcome might be a potential strategy in ICH treatment.

CE is defined as a pre-morbid cellular process, which can either be reversed or develop into necrotic cell death[29]. However, the existence of CE in ICH is controversially discussed[6, 7, 30-33]. Some previous DWI studies showed that ADC values increased globally in the perihematomal region[30, 31]. Other studies observed a decrease of ADC in the perihematomal area in part of the patients in the hyper-acute stage (<6 hours) till day 6 post-ictus and therefore suggested the presence of CE in ICH[6, 7, 32, 33]. CE surrounding the hematoma was detected in the ultra-early stage after ICH in 2 MRI studies[6, 7]. Kidwell et al. visualized a rim of perihematomal decreased ADC values within 6 hours in 3 among 12 patients with ICH[6]. Schellinger et al. detected CE in 7 among 32 patients within 6 hours after ICH onset[7]. Patients with CE tended to develop poor clinical outcome[6, 7]. Carhuapoma et al. found 1 among 9 patients with a decreased ADC on day 6[32]. Recently Olivot et al. examined 23 ICH patients

within 3 days after symptom onset. They found that two-thirds of the patients exhibited patchy regions with increased diffusivity mixed with reduced diffusion in the perihematomal region[33]. This is in line with our data. We detected CE in a similar proportion of patients (61%) on day 3. Instead of an association of CE with the MMP and growth factor levels we found that CE was highly correlated with the hematoma volume. We therefore hypothesize that this secondary brain cell injury is likely a consequence of the primary mass effect, or caused by cytotoxic molecules which are released from the clot or the destroyed brain parenchyma.

VEGF and Ang-1 levels were found to be related to BBB disruption and brain edema in animal models of ICH and brain injury[18-22]. In a clinical ICH study high levels of VEGF and Ang-1 at 72 hours after symptom onset were associated with good functional outcome and reduced lesion volume[11]. In our study neither VEGF nor Ang-1 were associated with PHE or clinical outcome. This could be due to the different time points of GF assessment.

Our study has some limitations. Overall, the sample size is small. However, considering the fact that MRI was used, and thus the best available method to quantify PHE[25, 34], this study includes a relatively high number of cases. Secondly, our findings cannot be generalized to patients with coma on admission and to those non-eligible for MRI. Particularly in patients with large hematoma who were not eligible for this study according to the exclusion criteria, pronounced CE and larger PHE volume can be expected.

In conclusion, our data show that MMP-3 levels are associated with perihematomal edema in acute spontaneous ICH patients. Increased levels of MMP-3 and MMP-9 are

independently associated with poor clinical outcome. Further investigations are needed to explain the mechanisms behind, which might lead to treatment options for the prevention of secondary brain damage and unfavorable outcome.

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Nitric Oxide (NO) and Asymmetric Dimethylarginine (ADMA): Their Pathophysiological Role and Involvement in Intracerebral Hemorrhage

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Abstract

Objective: Nitric oxide (NO) has a variety of functions in physiological systems, particularly in the vasculature and the central nervous system. Currently, the imbalance of the pathway involving nitric oxide, nitric oxide synthase, and asymmetric dimethylarginine (NO–NOS–ADMA) is increasingly discussed in connection with endothelial dysfunction. Knowledge about the role of this pathway in intracerebral hemorrhage (ICH), which represents the most devastating stroke subtype, is increasing but still sparse. This article aims to review the current knowledge about the role and metabolism of NO and ADMA. It will also address the role of the NO–NOS–ADMA pathway in ICH and delineate some questions that should be addressed by future studies.

Methods: A literature search regarding the data about NO, NOS, and ADMA and its role in ICH was conducted in PubMed.

Results: Experimental data from cell culture and animal models indicate that, after the occurrence of ICH, neuronal and inducible nitric oxide synthases (nNOS and iNOS) are both overexpressed and uncoupled through the induction of blood compound metabolites, including thrombin and inflammatory mediators. ADMA, the most potent endogenous inhibitor of NOS, is also overproduced following dysregulation of its metabolizing enzymes. Dysfunction of the NO–NOS–ADMA pathway results in cell death, blood–brain barrier (BBB) disruption, and brain edema via different pathological mechanisms. However, the available data from clinical studies are still rare and partially contradictory.

Conclusion: Experimental data suggest an important role for the NO–NOS–ADMA pathway for secondary injury after ICH. Since the literature shows contradictory results, further studies are needed to address current confusion.

Key words: Asymmetric dimethylarginine, intracerebral hemorrhage, nitric oxide, nitric oxide synthase

General discussion

Although intracerebral hemorrhage (ICH) is the most devastating subtype of stroke, research upon the secondary brain injury after ICH, which has a major impact upon the patients' prognosis, is comparatively sparse. Up to now, no effective therapy exists for these ICH patients. Understanding the mechanisms of secondary brain injury after ICH is the precondition for the development of any therapeutic strategy. Molecular or imaging markers could help to identify patients at high risk of secondary brain injury such as early hematoma expansion (HE) and perihematomal edema (PHE) after ICH and poor clinical outcome. These patients would be most suitable for the evaluation of new treatments.

Hematoma enlargement (HE) is seen in one third of acute ICH patients and has been identified as one of the most important determinants of poor clinical outcome, including early neurological deterioration, higher disability and mortality (LEIRA et al. 2004; S. M. DAVIS et al. 2006; DELCOURT et al. 2012). Recombinant factor VIIa (rFVIIa), a hemostatic drug, has been shown to be effective on reduction of HE but not on improvement of clinical outcome (MAYER et al. 2005, 2008). This discrepancy might be due to the unselected inclusion of acute ICH patients rather than the candidates at high risk of HE. Furthermore, patients with large hematomas will invariably have bad outcomes, with or without subsequent HE. Therefore a reliable marker predictive of HE and poor clinical outcome irrespectively of hematoma volume could aid the selection of patients for optimized treatment, such as hemostatic therapy and intensive blood pressure lowering treatment. By prospectively

studying a cohort of 139 cases (Publication I), we have shown that contrast extravasation present on CTA images could strongly predict HE and poor clinical outcome represented by longer length of hospital stay, increased mortality and disability (mRS>2) on discharge and at 90 days with high specificity and positive predictive value (LI et al. 2011a). Importantly, contrast extravasation could predict 90-day poor clinical outcome independent of hematoma volume and the presence of intraventricular hemorrhage. Currently, the definitions of HE in different studies are inconsistent, and the optimal definition of HE with regard to its power to predict poor clinical outcome is debatable. Our study examined contrast extravasation simultaneously with HE as predictors for clinical outcome, and provided the direct evidence that contrast extravasation is a predictor of 90-day poor outcome, which is independent of HE. Our results suggest that this sign could serve as a promising tool for the identification of patients who are vulnerable to develop HE and unfavorable outcome. Our findings have been recently confirmed by a multicentre prospective observational cohort study –the PREDICT Study (DEMCHUK et al. 2012). Here, considering the accumulated evidence, contrast extravasation has been recommended as an entry criterion for future trials of hemostatic therapy in patients with acute ICH (DEMCHUK et al. 2012).

PHE is the most common secondary brain injury after ICH. The changes of PHE volume over time have been investigated in several studies. However, studies with respect to cytotoxic edema (CE) are scarce, attributable to the difficulty of studying ICH patients using DWI. In the limited number of DWI studies, CE is detected in the perihematomal region in 1/3 to 2/3 of acute ICH patients in the hyper-acute stage (<6 hours) till day 6 post-ictus

(CARHUAPOMA et al. 2000; KIDWELL et al. 2001; SCHELLINGER et al. 2003; OLIVOT et al. 2010; TSAI et al. 2011). However data showing the development of CE over the first days after ICH was missing. Therefore, we examined 21 acute ICH patients by serial MRI diffusion weighted imaging (DWI) within the first week (on day 1, 3 and 7) after ICH (Manuscript I). We for the first time revealed that CE is more pronounced on day 3 than on days 1 and 7 represented by a lower ADC value, and that it tends to regress till day 7 represented by a spontaneous reversion of ADC towards normalization. Analysis of brain tissue samples of ICH patients demonstrated that mitochondrial dysfunction occurred early from 6 hours till 72 hours post-ICH in the perihematomal region (KIM-HAN et al. 2006), which was thought to contribute to a reduction in oxidative metabolism and oxygen utilization in this region (ZAZULIA et al. 2001). A transient focal increase in perihematomal glucose metabolism was observed in ICH patients 2-4 days post-ictus, and resolved on day 7 (ZAZULIA et al. 2009). A similar change of glucose utilization was seen in traumatic ICH patients (BERGSNEIDER et al. 2000). The temporal profile of CE found in our study complies with the metabolic change surrounding the hematoma observed in previous ICH studies (ZAZULIA et al. 2001; KIM-HAN et al. 2006; ZAZULIA et al. 2009), and thereby suggests a link of CE with the metabolic crisis and ongoing neuronal injury after ICH. Furthermore, our data shows that larger 3-day PHE volumes are associated with poor clinical outcome, and the presence of CE on day 3 has the same tendency. This is consistent with previous observational findings in clinical studies (KIDWELL et al. 2001; SCHELLINGER et al. 2003; TSAI et al. 2011). Two studies showed that patients with ICH who develop CE

within 6 hours after symptom onset are prone to develop unfavorable clinical outcome (KIDWELL et al. 2001; SCHELLINGER et al. 2003). Recently, another study including 46 cases demonstrated that CE development within 24 hours was associated with poor clinical outcome at 6-months (TSAI et al. 2011). These data about ongoing but possibly reversible neuronal injury after the initial event of ICH and its relationship with poor clinical outcome may therefore represent an important therapeutic target.

A huge amount of preclinical studies have demonstrated potential clinical benefits of molecules targeting pathways involved in inflammation or neuroprotection after ICH (HWANG et al. 2011). However bench-to-bedside translation of neuroprotective strategies that aim to improve the clinical outcome through reduction of secondary pathologic processes have not been successful so far. More insight into the pathological mechanisms on preclinical and clinical levels, and more clinical studies linking the molecular markers to secondary brain injury and clinical outcome could be critically helpful in the current situation. Overexpressed MMPs (particularly MMP-3 and MMP-9) play a crucial role in inflammatory reactions which contribute to secondary brain injury including neurotoxicity, BBB disruption and brain edema, and thereby unfavorable outcomes in a variety of central nervous system diseases including ICH. We therefore conducted a prospective study that aimed to investigate the predictive value of MMP-3 and MMP-9 for PHE and poor clinical outcome in patients with ICH (Manuscript II). We showed that both, increased levels of MMP-3 and MMP-9 on admission independently predict poor clinical outcome, whereas MMP-3 is associated with the delayed PHE volume in patients with ICH. In previous clinical studies, increased MMP-3 levels were

associated with mortality in ICH patients, and both, baseline MMP-3 and MMP-9 were related to residual scar volume at 3 months (ABILLEIRA et al. 2003; ALVAREZ-SABIN et al. 2004). Our findings provide more evidence linking these inflammatory markers and clinical outcome, and address the important role of MMPs in humans. We further investigated the time course of MMP-3 and MMP-9 in our prospective MRI cohort with 21 spontaneous ICH patients. Nineteen ICH patients were included in the final analysis since 2 patients developed an infection during their hospital stay and had to be excluded because MMP levels are influenced by infection and inflammation. Plasma MMP-3 and MMP-9 were measured within 6 hours, at 12, 24 hours, as well as 3 and 7 days after stroke onset using enzyme-linked immunosorbent assays (ELISA). MMP-3 levels increased immediately (<6 hours) after ICH with subsequent reduction at 12 hours, then significantly increased again till day 7 (P=0.020). MMP-9 has a similar trend of biphasic increased expression within 7 days (Appendix Figure 1, Table 1). Interestingly, a rapid up-regulation of MMP-9 within 6 hours post-ictus with subsequent decrease was also observed in patients after severe acute ischemic stroke (WORTHMANN et al. 2010). A similar biphasic profile of MMP-9 was observed in a rat ICH model (POWER et al. 2003). This might reflect cellular infiltration and subsequent activation including early neutrophil entry followed by monocyte/macrophage infiltration and activation in response to the bleeding and damaged brain tissue. It is supported by the finding that higher levels of MMP-9 were detected in glial cells in human brain tissue surrounding the hematoma (ROSELL et al. 2006). Nine of 19 patients had a favorable outcome 90 days after stroke onset, 10 patients had an unfavorable outcome (mRS>2). MMP-3 showed a similar profile

irrespective of ICH outcome (Appendix: Figure 2A). In patients with unfavorable outcome MMP-9 levels showed a biphasic increase with peaks at 6 hours and 7 days, whereas levels remained unchanged in patients with favorable outcome (Appendix: Figure 2B). Patients with unfavorable outcome had higher MMP-3 and MMP-9 levels than those with favorable outcome at 6 hours and 7 days after ICH although the difference is not significant. These findings reinforce the hypothesis that MMPs play an important role in secondary brain injury after ICH and suggest therapeutic efforts targeting MMP-3 and MMP-9for prevention of secondary brain damage and unfavorable prognosis.

The nitric oxide (NO), nitric oxide synthase (NOS), and asymmetric dimethylarginine (ADMA) pathway is increasingly discussed in connection with the inflammatory cascade including MMPs in ICH. Therefore we systematically reviewed the pathophysiological role of the NO-NOS-ADMA pathway and their involvement in ICH (Publication II) (LI et al. 2011b). In our published review, we described that overexpressed NO and NOS have been shown after ICH, and that the increased levels of NOS and NO have been shown to be detrimental in animal models of ICH, whereas the role of NO in ICH in humans is still contradictory. Data upon the alteration of ADMA levels after ICH and their association with clinical outcome were missing. Considering the currently available limited data on ADMA in subarachnoid hemorrhage as well as in acute ischemic stroke, we hypothesized that a significant up-regulation of ADMA can be expected within the first week after ICH. We therefore investigated the time course of ADMA and its analogue- symmetric dimethylarginine (SDMA)- in our prospective MRI study in 21 patients with spontaneous ICH compared to 32

age-adjusted healthy controls. Again, only the nineteen patients without concomitant infection were included into the data analysis as described above. Plasma ADMA and SDMA levels were measured within 6 hours, at 12 and 24 hours, as well as 3 and 7 days after stroke onset using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) (MARTENS-LOBENHOFFER u. BODE-BOGER 2006). ADMA levels were significantly higher in the ICH patient group at any time point compared to levels in controls, while SDMA was not (Appendix: Table 2). ADMA levels increased significantly until 7 days after ICH onset (P=0.023), while SDMA levels tended to decrease during the first 3 days but increased at day 7 (P=0.051) (Appendix: Figure 3). This finding is in line with our hypothesis. Two mechanisms –increased production by increased expression of protein arginine methyltransferases (PRMTs), and decreased metabolization by decreased enzymatic activity of dimethylaminohydrolases (DDAHs) – can induce elevated levels of ADMA (SYDOW u. MUNZEL 2003). SDMA is completely excreted through the kidneys, different from ADMA, which is predominantly (>90%) metabolized through DDAHs. Therefore increased SDMA induced by PRMTs could be concealed and SDMA could even show a trend of decrease in case of increased renal excretion until the SDMA production exceeds the renal compensation. In our cohort of 19 ICH patients, 9 patients had a favorable outcome at 90 days after stroke onset, 10 patients had an unfavorable outcome (mRS>2). In patients with a favorable outcome, ADMA levels remained stable during the first 3 days after ICH, increased at day7 but decreased again at 90 days. In patients with an unfavorable outcome, the ADMA levels increased rapidly within the first 7days and decreased again 90 days after ICH onset. At 24

hours the ADMA levels were significantly higher in patients with an unfavorable outcome than those with a favorable outcome (P=0.042, Appendix: Figure 4A). SDMA levels in patients with unfavorable outcome were significantly higher at day 3 (P=0.004) and day 7 (P=0.008) compared to those with favorable outcome (Appendix: Figure 4B). SDMA levels remained unchanged in the patients with favorable outcome during the first 7 days, while they continuously decreased over the first 3 days after ICH onset in those with unfavorable outcome and then increased at day 7. In both groups of patients, SDMA levels converged again until day 90. Our data showed that the temporal profile of plasma ADMA and SDMA alterations was related to ICH outcome. The mechanism behind is not clear but might be attributable to an induction of oxidative and nitrosative stress via uncoupling NOS (endothelial NOS and neuronal NOS), and to an exacerbation of the inflammatory reaction to the hemorrhage via the interplay with inflammatory factors (such as TNF-alpha and interleukins). Thereby the role of SDMA is less clear than that of ADMA. Experimental data suggest that SDMA might enhance reactive oxygen species (ROS) production in monocytes which are recruited to the hemorrhagic lesion (SCHEPERS et al. 2009). Our previous study in patients with acute ischemic stroke has demonstrated that increased ADMA and SDMA levels are linked with inflammation and predict poor clinical outcome in this pathology (WORTHMANN et al. 2011; WORTHMANN H 2011).

Summary

Na Li

Neuroradiological findings and molecular markers as predictors for secondary brain injury and outcome after intracerebral hemorrhage

Intracerebral hemorrhage (ICH) is the most devastating subtype of stroke and carries a high rate of disability and mortality. Substantial efforts need to be made to change the current critical situation and to elaborate effective treatments for acute ICH.

After ICH occurs, secondary brain injury including early hematoma expansion (HE) and perihematomal edema (PHE) develops in most of the patients. HE has been regarded as one of the most important determinants of early neurological deterioration, mortality and poor clinical outcome in primary ICH. In contrast, the significance of PHE is still controversial. It is important to understand the development of these secondary brain injuries, and to elaborate predictive molecular or imaging factors that allow to identify patients at high risk of secondary brain injuries, and more important of poor clinical outcome. These patients would be most suitable for the evaluation of new treatments.

We have shown that the presence of contrast extravasation on CT angiography (CTA) images in the hyperacute stage after ICH onset is a strong predictor of HE and poor clinical outcome, independent of the traditional factors such as hematoma volume and presence of intraventricular hemorrhage. The sign of contrast extravasation has been recommended as an entry criterion for future trials of hemostatic therapy in patients with acute ICH.

PHE - the other type of secondary brain injury - immediately occurs in most of the patients after ICH. However, the chronology of PHE development and the clinical significance of PHE are still unclear. Our study demonstrates that PHE appears in all patients within the first 24 hours. At this time point PHE consists of vasogenic (VE) in all patients and cytotoxic edema (CE) in about half of the patients. PHE volume predominantly increases on day 1, but further increases during the first week after ICH. In patients with small to medium hematomas CE is pronounced on day 3 but tends to regress after 1 week. Of note, the temporal profile of CE is in line with the metabolic change in the perihematomal region and might be linked to ongoing neuronal injury. Furthermore, larger 3-day PHE volume and the presence of CE were associated with poor clinical outcome. Our findings show that PHE might play a role as important as hematoma size in small to medium ICH. In patients with small to medium hematoma volume, more therapeutic efforts should be made to prevent this type of secondary neuronal injury and thereby poor clinical outcome.

Pathologically inflammation and their modulating markers play an important role in secondary brain injury and contribute to poor clinical outcome. Preclinical studies have demonstrated that matrix metalloproteinases (MMPs), a family of proteolytic enzymes, contribute to blood–brain barrier (BBB) disruption, neuronal injury, and brain edema after ICH. Asymmetric dimethylarginine (ADMA), a newly emerged mediator of oxidative and nitrosative stress, interacts with inflammation and might exacerbate the secondary brain injury. Our studies showed a significant association between MMP-3, MMP-9 and ADMA levels and their temporal pattern after acute ICH and clinical outcome. These data suggest these inflammatory markers and mediators as potential targets of ICH therapy at the molecular level.

Zusammenfassung

Na Li

Untersuchungen zum prädiktiven Wert neuroradiologischer Befunde und molekularer Marker für den sekundären Hirnschaden und klinischen Outcome nach intracerebraler Blutung

Die intrazerebrale Blutung (ICB) ist die verheerendste Form des Schlaganfalls und geht mit einer hohen Rate an bleibender Behinderung und Mortalität einher. Es ist dringend notwendig, diesekritische Situation zu ändern und effektive Behandlungen für die akute ICB zu erarbeiten.

Nach Eintritt der ICB treten bei der Mehrzahl der Patienten Komplikationen einer sekundären Hirnschädigung auf, welche insbesondere die frühe Nachblutungund das perihämorrhagische Ödem beinhalten. Die Nachblutung wird bei der primären ICB als eine der wesentlichen Determinanten für frühe neurologische Verschlechterung, schlechtes klinisches Outcome und Mortalität angesehen. Dahingegen wird die Rolle des perihämorrhagischen Ödems für die weitere klinische Entwicklung weiterhin kontrovers diskutiert. Es ist entscheidend, die Entwicklung einer sekundären Hirnschädigung besser zu verstehen und prädiktive molekulare und bildgebende Surrogat parameter zu erarbeiten, welche es ermöglichen, Patienten mit hohem Risiko für Komplikationen, und noch entscheidender für schlechtes klinisches Outcome, bereits im Vorfeld zu identifizieren. An

diesen Patienten könnte die Effizienz neuer Behandlungsstrategien am ehesten beurteilt werden.

Wir konnten zeigen, dass das Vorliegen einer „Kontrastmittel extravasation“ in der CT Angiographie in der hyperakuten Phase der ICB ein starker und sogar von traditionellen Faktoren wie Blutungsvolumen oder dem Vorhandensein intraventrikulärer Blutungsanteile unabhängiger Prädiktor für eine Nachblutung und schlechtes klinisches Outcome ist. Das Zeichen der „Kontrastmittel extravasation“ wird mittlerweile als Einschlusskriterium für zukünftige klinische Studien der hämostatischen Therapie bei Patienten mit ICB empfohlen.

Das perihämorrhagische Ödem, als weiterer Bestandteil der sekundären Hirnschädigung, ist bei den meisten Patienten bereits unmittelbar nach Einsetzen der ICB vorhanden. Allerdings ist der zeitliche Ablauf der weiteren Entwicklung des perihämorrhagischen Ödems sowie dessen klinische Signifikanz unzureichend untersucht. Unsere Studie demonstriert, dass es innerhalb der ersten 24 Stunden nach der Blutung bei allen Patienten zum Auftreten eines perihämorrhagischen Ödems kommt. Dei der Hälfte der Patienten finden sich zu diesem Zeitpunkt sowohl ein vasogenes als auch ein cytotoxisches Ödem als Anteile des perihämorrhagischen Ödems. Die stärkste Volumenzunahme des perihämorrhagischen Ödems findet bereits am ersten Tag statt. Allerdings kommt es, wenn auch in geringerer Ausprägung, noch bis zu Tag 7 zu einer weiteren Größenzunahme des perihämorrhagischen Ödems. Bei Patienten mit kleiner bis mittelgroßer ICB ist das cytotoxische Ödem an Tag 3 am ausgeprägtesten und ist bis Tag 7 partiell rückläufig. Diese Zeitkinetik des cytotoxischen Ödems steht im Einklang mit metabolischen Veränderungen in der perihämorrhagischen Region und geht potentiell mit einer fortschreitenden neuronalen Schädigung einher. Entsprechend zeigt sich, dass ein höheres Volumen des perihämorrhagischen Ödems an Tag 3

sowie das Vorhandensein eines cytotoxischen Ödems mit schlechtem klinischen Outcome assoziiert sind. Unsere Ergebnisse zeigen, dass das perihämorrhagische Ödem bei Patienten mit ICB von kleiner bis mittlerer Blutungsgröße eine ebenso entscheidende Rolle wie die Blutungsgröße spielen könnte. Bei diesen Patienten sollte eine Therapieintensivierung unternommen werden, um diese Form der sekundären Hirnschädigung und infolgedessen schlechtes klinisches Outcome zu verhindern.

Pathophysiologisch spielen Inflammation und die sie modulierenden Mediatoreneine entscheidende Rolle für die sekundäre Hirnschädigung und tragen dementsprechend zum schlechten klinischen Outcome bei. Präklinische Studien haben gezeigt, dass Matrix-metalloproteasen (MMPs), eine Familie von proteolytischen Enzymen, durch eine Zunahme der Blut-Hirn-Schranken-Störung wesentlich am perihämorrhagischen Ödem nach ICB beteiligt sind. Asymmetric dimethylarginine (ADMA), ein in den letzten Jahren zunehmend untersuchter Mediator oxidativen und nitrosativen Stresses, interagiert mit Inflammation und könnte zur Exazerbation der sekundären Hirnschädigung führen. Unsere Studien zeigen eine signifikante Assoziation von MMP-3, MMP-9 und ADMA Leveln sowie deren zeitlichem Verlauf nach akuter ICB mit klinischem Outcome. Die Daten legen nahe, dass die untersuchten inflammatorischen Marker und Mediatoren potentielle Angriffspunkte einer Therapie der ICB auf molekularer Ebene darstellen.

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Appendix

Table 1: Levels of matrix metalloprotease (MMPs) in ICH patients

	6h	12h	24h	3d	7d	90d
MMP-3 (ng/ml)	11.49 ± 4.78	7.90 ± 2.83	9.09 ± 3.59	11.48 ± 4.78	12.5 ± 5.73	14.5 ± 7.68
P	0.036	-	<0.001	0.020	0.011	-
MMP-9 (ng/ml)	126.49±160.43	102.92±87.48	74.92±55.49	84.91±65.77	122.5±104.74	91.75±114.39
P	0.696	-	0.172	0. 944	0.283	-

Data are presented as mean±SD. P values indicate differences between 12 hours and other time points in levels of MMP-3 and MMP-9 calculated by repeated measures ANOVA. Levels of MMP-3 and MMP-9 at 12 hours were used as compared since their levels show a rapid decrease after baseline from 6 to 12h and thereafter increase again ($P<0.05$).

Table 2: Levels of Dimethylarginine in ICH patients and controls

	Controls	ICH patients					
		6h	12h	24h	3d	7d	90d
ADMA	0.447	0.496	0.538	0.548	0.502	0.589	0.535
(umol/L)	[0.391-	[0.439-	[0.477-	[0.482-	[0.452-	[0. 506-	[0. 472-
	0.481]	0.548]	0.587]	0.599]	0.581]	0.640]	0.581]
P		0.042	<0.001	<0.001	0.001	<0.001	<0.001
SDMA	0.546	0.580	0.515	0.522	0.490	0.555	0.534
(umol/L)	[0.473-	[0.404-	[0.443-	[0.466-	[0.442-	[0.444-	[0.437-
	0.630]	0.692]	0.658]	0.581]	0.572]	0.691]	0.710]
P		0.849	0.802	0.647	0.092	0.573	0.505

ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine.

Data are presented as median and interquartile range. P values indicate differences between patients and controls in levels of ADMA and SDMA calculated by Student's t test.

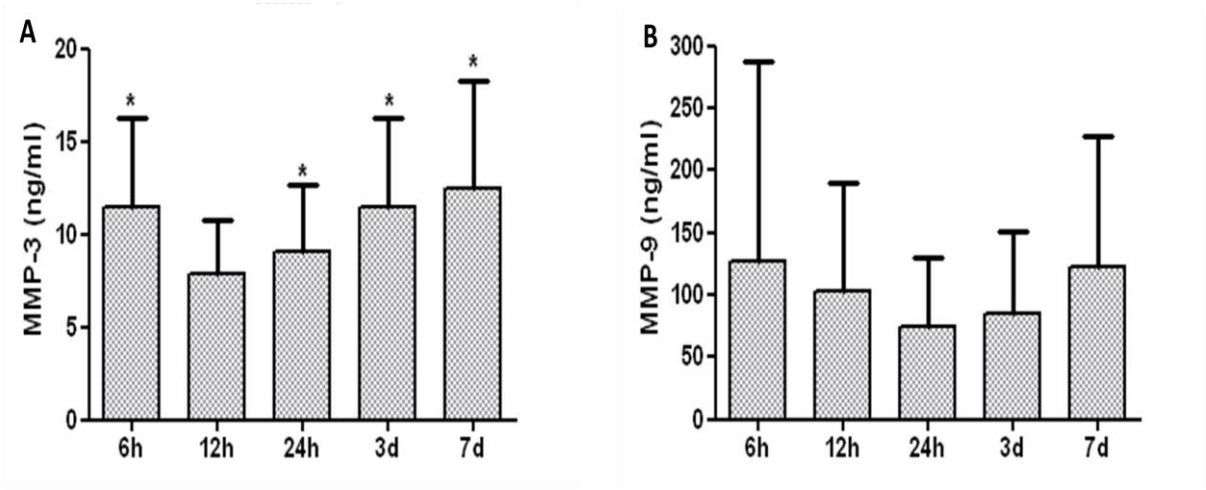

Figure 1. Time courses of MMP-3 (A) and MMP-9(B) after acute ICH onset (mean±SD).

* indicates significant differences between MMP-3 levels at 12 hours after symptom onset and other time points calculated by repeated measures ANOVA (P<0.05).

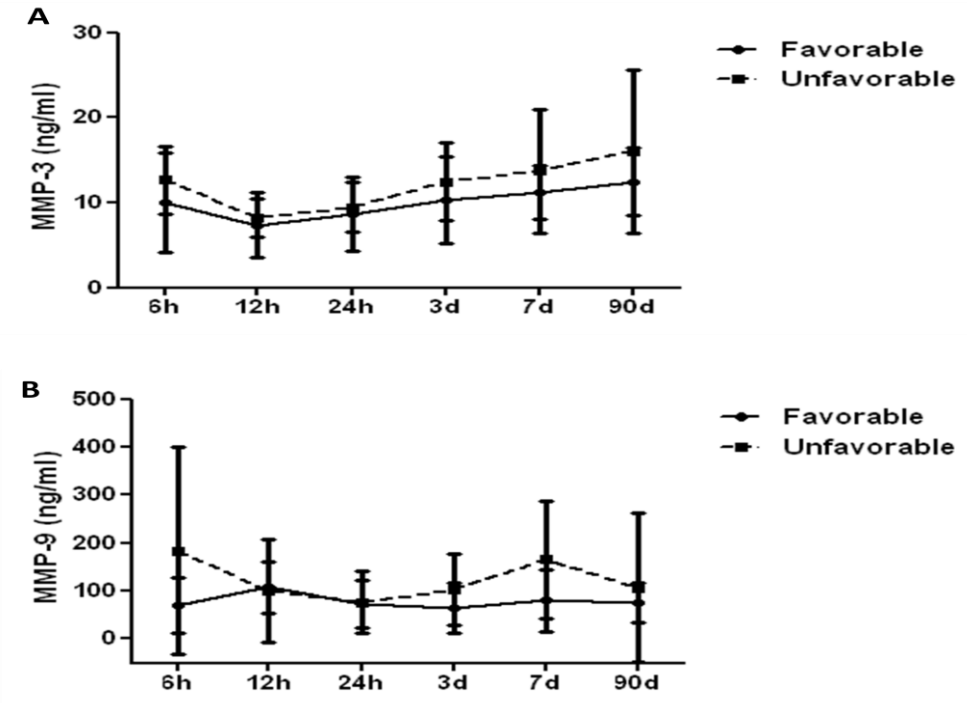

Figure 2. Time courses of MMP-3 (A) and MMP-9 (B) in patients with favorable and unfavorable outcome after acute ICH (mean ± SD). No significant intergroup-difference for MMP-3 andMMP-9 levels was found by Student's t test.

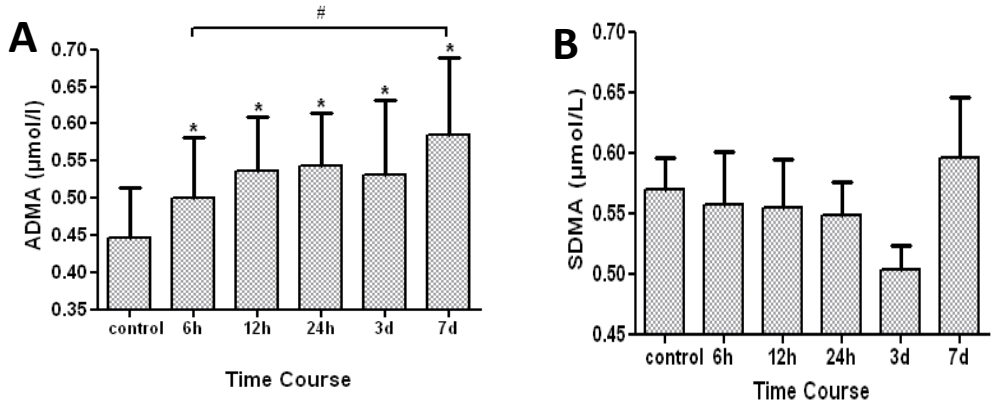

Figure 3. Time courses of dimethylarginine levels after acute ICH onset (mean±SD). A: ADMA; B: SDMA.

* indicates significant differences in ADMA levels for comparison with control calculated by Student's t test,

# indicates significant differences in ADMA levels for comparison of follow-up time points with the 6 hour time point calculated by repeated measures ANOVA; both P<0.05.

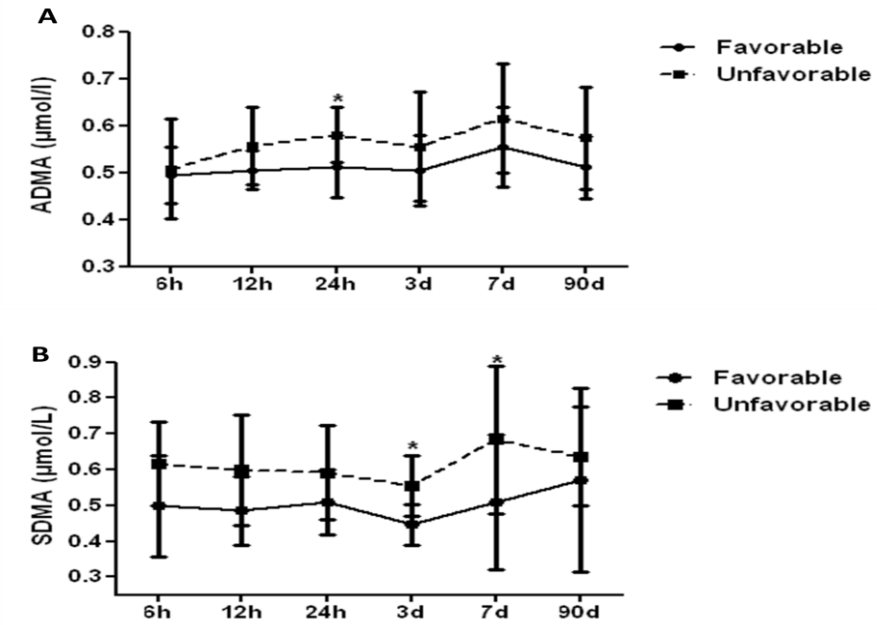

Figure 4.Dimethylargininetime course in patients with favorable and unfavorable outcome (mean ± SD). A: ADMA; B: SDMA.

* indicates significant intergroup differences for ADMA and SDMA levels calculated by Student's t test (P<0.05).