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Research Report

Caspase-dependent cell death involved in brain damage after acute subdural hematoma in rats

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ARTICLE INFO

Article history:
Accepted 27 June 2006

Keywords:
Subdural hemorrhage
Caspase inhibitor
Apoptotic cell death
Neuroprotection
Brain

ABSTRACT

Traumatic brain injury is associated with acute subdural hematoma (ASDH) that worsens outcome. Although early removal of blood can reduce mortality, patients still die or remain disabled after surgery and additional treatments are needed. The blood mass and extravasated blood induce pathomechanisms such as high intracranial pressure (ICP), ischemia, apoptosis and inflammation which lead to acute as well as delayed cell death. Only little is known about the basis of delayed cell death in this type of injury. Thus, the purpose of the study was to investigate to which extent caspase-dependent intracellular processes are involved in the lesion development after ASDH in rats. A volume of 300 μL blood was infused into the subdural space under monitoring of ICP and tissue oxygen concentration. To asses delayed cell death mechanisms, DNA fragmentation was measured 1, 2, 4 and 7 days after ASDH by TUNEL staining, and the effect of the pan-caspase inhibitor zVADfmk on lesion volume was assessed 7 days post-ASDH. A peak of TUNEL-positive cells was found in the injured cortex at day 2 after blood infusion (53.4±11.6 cells/mm^2). zVADfmk (160 ng), applied by intracerebroventricular injection before ASDH, reduced lesion volume significantly by more than 50% (vehicle: 23.79±7.62 mm^3; zVADfmk: 9.06±4.08). The data show for the first time that apoptotic processes are evident following ASDH and that caspase-dependent mechanisms play a crucial role in the lesion development caused by the blood effect on brain tissue.

1. Introduction

Acute subdural hematoma (ASDH) is a common mass lesion following traumatic brain injury (TBI). Investigations revealed that global and local cerebral blood flow (CBF) is reduced after ASDH (Bouma et al., 1992; Inglis et al., 1990; Kuroda and Bullock, 1992; Patel et al., 1999b; Sawauchi et al., 2003). This leads to critical energy supply, ionic imbalance, edema formation and to widespread cell death. However, there is no pharmacological treatment in the clinical routine besides surgical evacuation of blood and reduction of intracranial pressure (ICP) by e.g. mannitol to target cell loss additionally. Although removal of the blood (<4 h after ASDH) reduces mortality (Seelig et al., 1981), many patients do not profit from surgery and still die or remain disabled. Studies indicate that CBF is often not sufficiently restored following surgery (Kuroda and Bullock, 1992) and that decompression leads to reperfusion injury due to free radicals (Kwon et al., 2003).

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doi:10.1016/j.brainres.2006.06.105

Thus, reduced CBF and mediators of secondary injury continue to compromise cell viability.

Following traumatic and ischemic brain injury, cells within the injury core die acutely due to e.g. cessation of energy and oxygen supply, disruption of ionic gradients and the initiation of intracellular death pathways. In the surrounding tissue, delayed cell death is additionally initiated involving, e.g. caspase-dependent apoptotic death signals which cause further maturation of the injury (Conti et al., 1998; Knoblach et al., 2002; Newcomb et al., 1999; Rink et al., 1995; Yakovlev et al., 1997). Targeting apoptotic death signals has become a major focus in brain research in order to find effective drug treatments blocking delayed mechanisms. After ASDH, an acute damage has been shown (Miller et al., 1990; Tsuchida et al., 1999) which further grows over time. Studies investigating intracerebral hemorrhage (ICH) suggest that apoptotic cell death plays an important role in the development of an infarction after bleeding (Matsushita et al., 2000; Qureshi et al., 2001; Rodrigues et al., 2003). Furthermore, the contact of blood with brain tissue may cause inflammatory responses (e.g. activation of cytokines) even days after intracranial blood accumulation (Castillo et al., 2002; Gong et al., 2000; Sercombe et al., 2002; Xue and Del Bigio, 2000a). In both cases, apoptosis as well as inflammation, caspase-dependent pathways are involved in the degradation of brain cells after injury (Kang et al., 2000; Matsushita et al., 2000; Yakovlev et al., 1997). Supporting this idea, the injection of the broad-spectrum caspase (pan-caspase) inhibitor zVADfmk (benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone) that is known to block activation of caspases early in the activation cascade (Sun et al., 1999) reduces apoptotic cell death in a model of collagenase-induced ICH (Matsushita et al., 2000). In acute subdural hematoma, the involvement of caspase-dependent mechanisms on brain damage has not been investigated at all. A subdural hemorrhage produces global ischemia during ICP increase, focal ischemia after restriction of arterial blood flow in the arachnoidea underneath the blood and inflammation due to contact of extravasated blood with brain tissue. Thus, caspase-induced signals might be an ideal target for treatment of ASDH-related cell death. Therefore, we investigated the neuroprotective effect of a pan-caspase inhibitor after acute subdural hematoma in rats.

2. Results

2.1. *Evaluation of pathophysiological changes by 300 μL ASDH*

The assessment of our model using 300μL blood volume showed that mean arterial blood pressure was 73.8±1.8mm Hg before ASDH and 81.2±5.5mm Hg at the end of the monitoring period after ASDH. Baseline ICP was 7.3±0.7mm Hg and increased to a peak value of 47.0±4.7mm Hg at 7min after starting the infusion. Within 25min, ICP stabilized at about 12mm Hg (Fig. 1). $ptiO_2$ decreased immediately after infusion start from a baseline value of 32.6±4.3mm Hg to 11.4±0.7mm Hg within 6min, but increased thereafter over time slightly.

Since zVADfmk was injected before ASDH in the main study (see below), possible effects of zVADfmk on physiological parameters were tested by application of zVADfmk in sham-operated rats. The result showed that ICP increased shortly by 1.5mm Hg due to the intraventricularly injected volume and that measured local CBF was also not affected by zVADfmk. CBF was 31.1±0.5 LDU during baseline and 31.9±0.1 LDU post-injection of zVADfmk. In addition, the drug had no effect on mean arterial blood pressure in these two sham animals (before injection: 79.5±0.5mm Hg; 20min after injection: 78.5±0.5mm Hg).

2.2. *Time-course of apoptotic cell death (TUNEL staining)*

No blood pressure was recorded in the TUNEL staining series. Endtidal CO_2 and inspiratory O_2 values for all animals were 41.5±4.0 and 132±4.1mm Hg before, and of 42.6±0.6 and 114.4±4.3mm Hg after ASDH.

TUNEL-positive cells appeared mostly in a cluster of several cells in the cortex underneath the bleeding (Fig. 2). The number of counted cells peaked out at day 2 with 53.4±11.6 cells/mm^2 (Fig. 3). At all time points, no TUNEL-positive cells were observed in the hippocampus.

2.3. *Effect of caspase inhibition by zVADfmk on lesion volume*

Mean arterial blood pressure was 82.6±0.5 (zVADfmk group) and 82.1±0.3mm Hg (vehicle group) before (n.s.) and 95.4±0.4 and 89.5±0.5mm Hg after ASDH ($p<0.001$). The injection of venous blood induced a clearly demarcated lesion area with a lesion volume in the vehicle group of 23.79±7.62mm^3 when

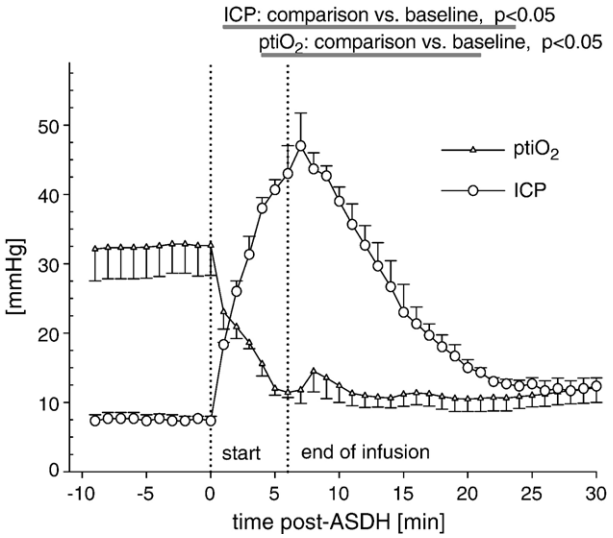

Fig. 1 – Effect of infusion of 300 μL venous blood into the subdural space on intracranial pressure (ICP) and brain tissue oxygen ($ptiO_2$). Dotted lines indicate start and end of infusion. Probes were allowed to equilibrate for at least 20min. Thereafter, ICP and $ptiO_2$ were monitored for 10min before and for 30min after ASDH induction (start: at time point 0). Values are given as mean±SEM, and the gray bar indicates a significant difference to baseline.

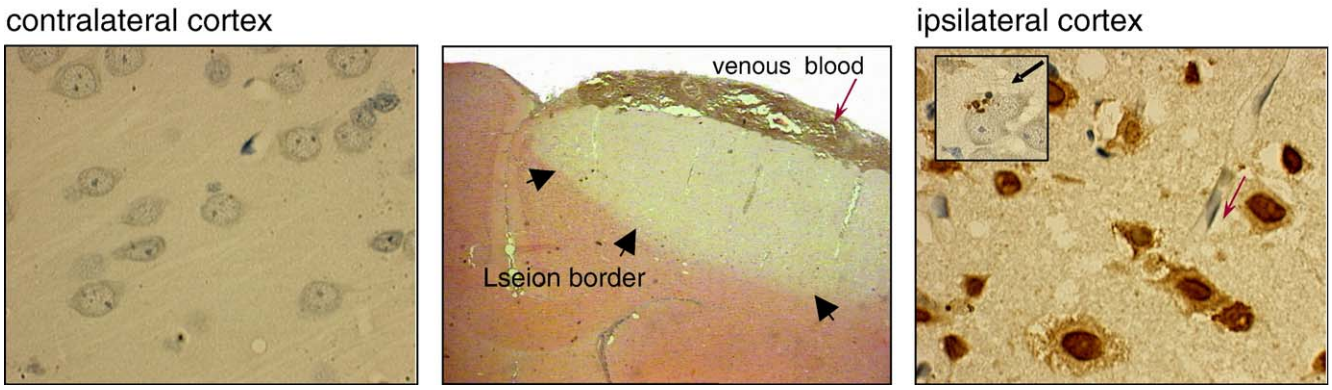

Fig. 2 – Example of TUNEL-positive neuronal cells in the contra- (left panel) and ipsilateral cortex (right panel) at day 2 after ASDH. Sections were counterstained with hematoxylin. There were no positive cells found at any time point in the contralateral hemisphere. The arrow in the inlet of the right panel indicates cell with apoptotic bodies. The middle panel shows expansion of the cortical lesion below the subdural blood clot (H&E staining).

measured 7 days after ASDH (Fig. 4). The application of the caspase-inhibitor zVADfmk reduced this volume significantly to 9.06 ± 4.08 mm^3 (Mann–Whitney *U* test).

3. Discussion

Intracranial bleeding is a frequent complication in human traumatic brain injury (TBI) and especially acute subdural hemorrhages (ASDH) worsen outcome of patients dramatically. Early removal of the blood mass only is able to reduce mortality effectively, but many patients do not recover or still die from their injury (Seelig et al., 1981). Since ASDH unleashes a plethora of degenerative processes, understanding the nature of the injury is important for treatment selection. We could now demonstrate that caspase-dependent cell death plays a major role for the lesion development in this type of TBI. For the first time shown after ASDH, the application of the pan-caspase inhibitor zVADfmk could reduce lesion volume by more than 50% when assessed 7 days after injury. Thus, injection of pan-caspase inhibitors after traumatic brain hemorrhage might be a future option for treating patients.

Effects of ASDH in patients and animals are related to the volume of extravasated blood in the cranium. Infarct size in rats reaches from around 25 mm^3 using 200 μL (Eijkenboom et al., 2000, 1999) to above 100 mm^3 using 400 μL subdural blood volume (Alessandri et al., 1999b; Kwon et al., 2003). As one immediate effect of many devastating factors, blood volume increases ICP (Fig. 1) to above 70 mm Hg using up to 400 μL (Mauler et al., 2004; Miller et al., 1990; Sawauchi et al., 2003). In the rat model, ICP drops quickly to values around 15 mm Hg but remains elevated for at least 24 h (Mauler et al., 2004). An

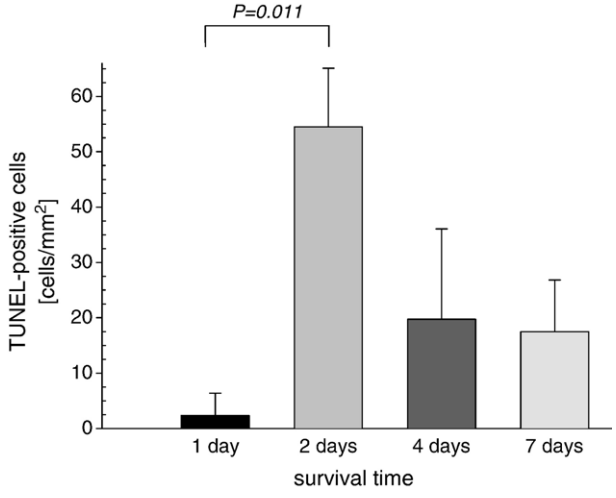

Fig. 3 – Number of TUNEL-positive cells per mm^2 (mean ± SEM) in the cortical tissue underneath the subdural hematoma. A peak of positive cells was found at day 2 after induction of blood into the subdural space. The number of positive cells increased significantly from survival day 1 to day 2 (*t* test: $p = 0.011$).

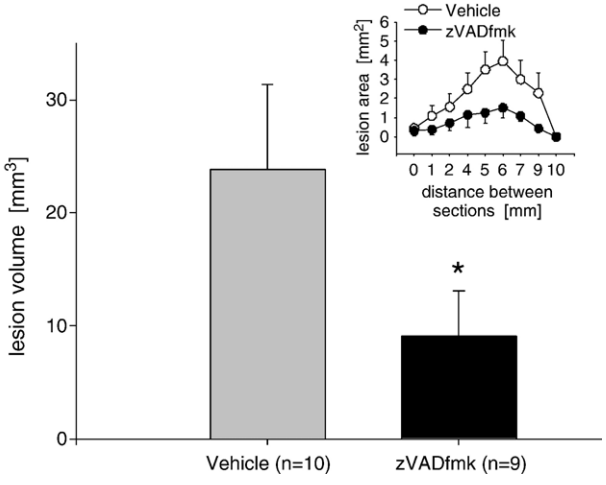

Fig. 4 – Effect of the pan-caspase inhibitor zVADfmk (160 ng) on lesion volume (mm^3, mean ± SEM), assessed 7 days after acute subdural hematoma. Inlet shows the distribution of the lesion area (mm^2, mean ± SEM) of both groups. Asterisk (*) indicates significant difference between groups (Mann–Whitney *U* test, $p < 0.05$).

ischemic cerebral perfusion pressure (CPP) develops mainly in the acute phase, and local pressure on the arachonidea causes focal ischemic CBF. The injury cascade involves increased energy metabolism, massive glutamate release and reduced tissue oxygen level (Fig. 1) (Inglis et al., 1990; Patel et al., 1999b; Sawauchi et al., 2003). Such acute changes lead to acute/necrotic cell death that is indicated by a large infarct size already at a few hours after ASDH (Miller et al., 1990; Tsuchida et al., 1999). However, a significant (40%) secondary lesion growth from 50 mm^3 at 4 h to 70 mm^3 at 24 h following a 400 μL ASDH is observed, despite a rapid decrease of ICP and normalization of CPP in most rat models (Miller et al., 1990) that suggest delayed cell death. Partially, this late cell death can still be explained by a secondary ischemic effect of ICP which might be elevated for more than 24 h after ASDH due to massive swelling and therefore causes prolonged CBF reduction (Patel et al., 1999a,b).

On the other hand, inflammation and apoptosis following ischemia and TBI are major contributors to delayed cell death (Hengartner, 2001; Liou et al., 2003; Schmidt et al., 2005) and, thus, to secondary lesion growth. ASDH resembles many pathophysiological mechanisms of ischemia and TBI and induces most likely inflammation- and apoptosis-related cell death. In both cases, caspase-dependent signaling pathways are involved in the degradation of brain cells (Liou et al., 2003; Schmidt et al., 2005). Caspase-dependent mechanisms have been investigated in brain injury and play a crucial role in delayed cell death (Chen et al., 1998; Didenko et al., 2002; Franz et al., 2002; Gong et al., 2001; Knoblach et al., 2002; Matsushita et al., 2000; Tenneti and Lipton, 2000; Yakovlev et al., 1997). Therefore, the goal of the present study was to demonstrate that apoptotic cell death is evident in this type of injury and that caspase-dependent processes are key players in the genesis of the damage. The application of a pan-caspase inhibitor promised to be the most effective protective strategy since many of the abovementioned pathomechanisms following ischemia and TBI which lead to caspase activation are involved in the injury development following ASDH.

Effector caspases such as caspase-3 can be activated either through the release of cytochrome *c* from mitochondria by, e.g. free radicals or through membrane-bound death receptors (e.g. CD95 = Apo-1/Fas; TNF = tumor necrosis factor) which mobilize upstream caspases such as caspase-8 (Hengartner, 2001). The final result of this cascade is controlled DNA breakdown/fragmentation, generally visualized by DNA laddering or by labeling of DNA breaks (TUNEL staining (Matsushita et al., 2000)). TUNEL-positive cells with apoptotic morphology are considered as rough marker of apoptosis and peak out often 1 day after ischemia and TBI (Linnik et al., 1995; Newcomb et al., 1999; Rink et al., 1995; Sasaki et al., 2000). Most positive cells were found at day 2 after ASDH, whereas only a few were observed at day 1 in our model. A similar time-course is found following ICH with only a few TUNEL-positive cells around the blood clot at day 1, but with a peak at day 3 (Gong et al., 2001; Matsushita et al., 2000; Qureshi et al., 2001). This is a first indication that apoptosis is initiated in the ASDH model. A possible explanation for the delayed appearance of TUNEL-positive cells could be that extravasated whole blood causes, at a later time point, additional and long-lasting inflammatory responses (Peeling et al., 2001; Xue and Del Bigio, 2000b) which lead to delayed release of death signals (e.g. TNFalpha) (Mayne et al., 2001). This notion is supported by the fact that the appearance of TUNEL-positive cells can be diminished further by a second injection of zVADfmk before and 24 h after ICH (Matsushita et al., 2000). That TUNEL-positive cells are partially related to caspase activation has been shown by a co-localization of staining for TUNEL and caspase-3 (Gong et al., 2001; Namura et al., 1998; Sasaki et al., 2000). Furthermore, blockage of caspase-activation can reduce TUNEL staining as well as improve histological and functional outcome after brain injury (Chen et al., 1998; Fink et al., 1999; Knoblach et al., 2002; Matsushita et al., 2000; Mayne et al., 2001; Yakovlev et al., 1997). This indicates that caspase activation contributes to lesion development. In a mouse contusion model, Fink et al. (1999) showed a neuroprotective effect of zVADfmk pre-treatment by more than 50% which is comparable to the findings in the present study. Post-injury treatment led to a reduction of only 20%. This suggests that caspase-related processes are started already early after brain injury. Recent data also indicate that caspase-3 is not only involved in apoptotic, but also necrotic cell death through a calpain-related death pathway which is activated early after TBI (Knoblach et al., 2004). In addition, a rapid translocation of the apoptosis-inducing factor (AIF) from mitochondria into cell nucleus occurs after injury and leads to already early cell death. Although the AIF pathway is considered to be caspase-independent, blocking of caspases was found to protect against AIF-induced cell loss, partially due to better mitochondrial protection (Cao et al., 2003; Chiari et al., 2000). However, these mechanisms may reduce the neuroprotective efficacy of caspase inhibitors if applied after injury as reported by Fink et al. (1999).

The infusion of the pan-caspase inhibitor zVADfmk just before ASDH led to a reduction of the hematoma-induced infarct at 7 days after injury. zVADfmk did not affect ICP, blood pressure and brain temperature (Hartmann et al., in preparation) which could influence this neuroprotective effect. Based on the time-course of TUNEL-positive cells with apoptotic morphology, it is likely that zVADfmk reduced caspase-activated 'necrotic' or caspase-independent cell death in the first place as postulated by Knoblach et al. (2004). This is supported by the fact that the half-life time of zVADfmk might be shorter than 24 h (Matsushita et al., 2000), and thus it maybe has inhibited mainly processes induced during the first several hours after ASDH. The peak of TUNEL-positive cells at day 2 after ASDH and data from Matsushita et al. (2000) using an ICH model demonstrate that intracranial hemorrhage induces prolonged caspase-dependent cell death which can be treated by application of a pan-caspase inhibitor at a later time point. Therefore, blocking caspase-dependent pathways by pan-caspase inhibitors such as zVADfmk might be useful in a successful therapeutic strategy to reduce hemorrhage-induced brain damage since not only acute necrotic, but also delayed apoptotic cell death can be prevented.

In conclusion, acute subdural hematoma induced a rapid increase in ICP and decrease in tissue oxygen concentration. These ischemic events caused the appearance of TUNEL-positive cells with apoptotic morphology with a maximum at day 2 after ASDH, indicating delayed cell death mechanisms. The intraventricular application of zVADfmk, a pan-caspase inhibitor, reduced the infarct volume by more than 50%. Thus,

caspase-dependent cell death pathways play a major role in the development of brain damage after ASDH and might be an interesting target for treatment strategies. Further studies will be necessary to elucidate the temporal profile of caspase activation and of caspase-independent mechanisms relation to signaling stimuli (e.g. inflammatory) which are specific for this type of injury.

4. Experimental procedures

4.1. Animals

All experiments were performed with the approval of the institutional Animal Care and Use Committees. A total of 36 male Sprague–Dawley rats, weighing 320–410 g, were used in this study.

4.2. Surgery

All animals were deeply anesthetized (chloral hydrate) and mechanically ventilated (Alessandri et al., 1999b). Femoral artery and vein were cannulated (PE tubing, i.d. 0.58 mm) for blood pressure monitoring and for withdrawal of venous blood for subdural infusion. Animals were fixed in a stereotaxic frame and a small craniotomy (diameter 3 mm) was drilled at a distance of 1.5 mm anterior to the Bregma and lateral to the midline suture. For subdural blood infusion, a J-shaped, blunted needle (G23) was inserted underneath the dura and fixed by cyanoacrylate glue (see Alessandri et al., 1999a,b).

4.3. Acute subdural hematoma (ASDH)

ASDH was induced after withdrawing unheparinized venous blood of which 300 μL was immediately infused at a rate of 50 μL/min. About 30 min after infusion, the needle was removed and the craniotomy sealed off with the bone flap and cyanoacrylate glue.

4.4. Lesion volume

For determination of the lesion volume 7 days after ASDH, 3-μm-thick sections were cut throughout the macroscopically visible infarct. All sections were stained by hematoxylin–eosin. The lesion area on the sections was measured on digitalized images which were scanned by an image analysis system (Leitz Ortho-Plan, Wetzlar, Germany; Sony CCD video camera SSC-C270P; software Optimas 5.1).

4.5. TUNEL staining

For the analysis of TUNEL-positive cells, several 5-μm-thick sections were cut through the center of the visible infarct. Three sections per animal were stained using a DNA fragmentation kit (FragEL®, Calbiochem, Cat# QIA33). Apoptotic cells were visualized by DAB (3,3-diaminobenzidine) and counterstained by hematoxylin. Under a light microscope (Nikon Eclipse E400, Nikon Ltd., USA), TUNEL-positive cells with distinct apoptotic characteristics were counted (Rink et al., 1995) in the peripheral region of the lesion.

4.6. Experimental groups

4.6.1. Evaluation of pathophysiological changes by 300 μL ASDH

Since our laboratories mainly used 400 μL as ASDH blood volume, we have evaluated in a first step the effect of a smaller volume (1) on intracranial pressure (ICP) and brain tissue oxygen ($ptiO_2$) and (2) on the effect of zVADfmk (see below for details) on ICP and cerebral blood flow (CBF) in our model.

Therefore, an ICP sensor (size 3F = 1 mm diameter; Raumedic AG, Germany) was inserted into the contralateral hemisphere, and a $ptiO_2$ sensor (active membrane: 1 mm^2; Integra Neuroscience Ltd., UK) was implanted posterior to the blood infusion site. ICP and $ptiO_2$ were monitored for 10 min before and for 30 min after ASDH in three rats each.

In order to exclude possible influence of treatment with the pan-caspase inhibitor (see below) on pathophysiological processes, 8 μL of zVADfmk (n = 2) was applied intraventricularly and MABP, ICP and local cerebral blood flow (CBF, TSI/Vasamedics Laserflo, St. Paul, USA) were monitored. For CBF monitoring, an LD (laser Doppler) probe was placed above a small ipsilateral area frontal to Bregma which was thinned out by a surgical drill and displayed LD units of around 30 (for details, see Soehle et al., 2001).

4.6.2. Time-course of apoptotic cell death (TUNEL staining)

In a second step, we verified the time-course of TUNEL-positive cells with apoptotic features as an indicator of programmed cell death without any intracranial sensors in place or craniectomies (no sensor- or operation-related damage). For this purpose, 12 rats underwent surgical preparation and were assigned to a 1-, 2-, 4- or 7-day survival time point after ASDH (3 rats/group). At the end of each particular survival time, animals were perfusion-fixed (4% paraformaldehyde). Brains were removed, post-fixed for 24 h and embedded in paraffin for analysis of TUNEL-positive/apoptotic cells.

4.6.3. Effect of caspase inhibition by zVADfmk on lesion volume

In a third step, we studied the neuroprotective effect of the caspase inhibitor zVADfmk (Enzyme Systems, USA, #FK-109). Therefore, 19 rats were prepared for ASDH but received a second burr hole over the contralateral hemisphere. A needle (G26) which was connected to a microsyringe was lowered to the ventricle (AP = 1.5 mm, ML = 1 mm from Bregma) and fixed to the skull. Rats received an injection of either vehicle (n = 10) or the pan-caspase inhibitor zVADfmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; n = 9). For injection, 450 μL PBS was mixed with 1 μL DMSO (vehicle) or zVADfmk stock solution (3 mg zVADfmk in 333 μL DMSO). A volume of 8 μL of vehicle zVADfmk solution (160 ng) was injected 5 min before induction of hematoma. After ASDH, animals were returned to the animal room (12:12 h light–dark cycle; >50% relative humidity; 24 °C). One week after trauma, all animals were deeply anesthetized, perfusion-fixed and brains were removed for histological evaluation. The investigator was blinded to treatment until after histological analysis.

4.6.4. *Statistics*

All data are expressed as means ± SEM. Group differences were analyzed by *t* test or Mann–Whitney *U* tests (Sigmastat 2.0.3, SPSS, Chicago, USA). Differences were considered significant at $p < 0.05$.

Acknowledgments

B.A. and O.K. were supported by grants of DFG (KE 338/6-1) and of 'Neurowissenschaft Mainz' (B.A.).

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