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ERYTHROPOIETIN NEUROPROTECTION IS ENHANCED BY DIRECT CORTICAL APPLICATION FOLLOWING SUBDURAL BLOOD EVACUATION IN A RAT MODEL OF ACUTE SUBDURAL HEMATOMA

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Abstract—Recombinant human erythropoietin (EPO) has been successfully tested as neuroprotectant in brain injury models. The first large clinical trial with stroke patients, however, revealed negative results. Reasons are manifold and may include side-effects such as thrombotic complications or interactions with other medication, EPO concentration, penetration of the blood–brain-barrier and/or route of application. The latter is restricted to systemic application. Here we hypothesize that EPO is neuroprotective in a rat model of acute subdural hemorrhage (ASDH) and that direct cortical application is a feasible route of application in this injury type. The subdural hematoma was surgically evacuated and EPO was applied directly onto the surface of the brain. We injected NaCl, 200, 2000 or 20,000 IU EPO per rat i.v. at 15 min post-ASDH (400 µl autologous venous blood) or NaCl, 0.02, 0.2 or 2 IU per rat onto the cortical surface after removal of the subdurally infused blood t at 70 min post-ASDH. Arterial blood pressure (MAP), blood chemistry, intracranial pressure (ICP), cerebral blood flow (CBF) and brain tissue oxygen ($ptiO_2$) were assessed during the first hour and lesion volume at 2 days after ASDH. EPO 20,000 IU/rat (i.v.) elevated ICP significantly. EPO at 200 and 2000 IU reduced lesion volume from 38.2 ± 0.6 mm^3 (NaCl-treated group) to 28.5 ± 0.9 and 22.2 ± 1.3 mm^3 (all $p < 0.05$ vs. NaCl). Cortical application of 0.02 IU EPO after ASDH evacuation reduced injury from 36.0 ± 5.2 to 11.2 ± 2.1 mm^3 ($p = 0.007$), whereas 0.2 IU had no effect (38.0 ± 9.0 mm^3). The highest dose of both application routes (i.v. 20,000 IU; cortical 2 IU) enlarged the ASDH-induced damage significantly to 46.5 ± 1.7 and 67.9 ± 10.4 mm^3 (all $p < 0.05$ vs. NaCl). In order to test whether Tween-20, a solvent of EPO formulation 'NeoRecomon®' was responsible for adverse effects two groups were treated with NaCl or Tween-20 after the evacuation of ASDH, but no difference in lesion volume was detected. In conclusion, EPO is neuroprotective in a model of ASDH in rats and was most efficacious at a very low dose in combination with subdural blood removal. High systemic and topically applied concentrations caused adverse effects on lesion size which were partially due to increased ICP. Thus, patients with traumatic ASDH could be treated with cortically applied EPO but with caution concerning concentration.

Key words: acute subdural hemorrhage, clot evacuation, recombinant erythropoietin, neurotoxicity, rat.

INTRODUCTION

Acute subdural hemorrhage (ASDH) is still a devastating consequence of traumatic brain injury (TBI) and worsens mortality and disability of severely head-injured patients. Despite decades of clinical and experimental research ASDH is mainly treated by the surgical removal of extravasated blood volume (e.g. Bullock et al., 2006). This can reduce mortality significantly but does not stop pathophysiological processes which have already been initiated by trauma and hemorrhage (Hlatky et al., 2007). Until now neuroprotective drug effects in pre-clinical studies failed to translate into a successful treatment of TBI patients. This is partially due to the fact that the treatment approach includes a single neuropathological mechanism. Targeting multiple neuropathological processes which contribute to injury development may help to reduce neuronal cell death, and improve repair and functional recovery following TBI. ASDH contributes to this process by adding a pronounced ischemia and contact of blood-derived factors or mediators from extravasated blood with brain tissue. We and others could show that blood-derived factors or mediators play a major role for lesion development following hemorrhage (Kuroda et al., 1992; Dreier et al., 2000). Furthermore, apoptosis, free radicals and inflammation are part of the pathophysiological cascade that is initiated (Kwon et al., 2003; Alessandri et al., 2006; Wang and Dore, 2007).

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Abbreviations: ASDH, acute subdural hemorrhage; BBB, blood–brain-barrier; CBF, cerebral blood flow; EPO, erythropoietin; EPOR, erythropoietin receptor; ICP, intracranial pressure; LDU, Laser-Doppler units; PI3-K, phosphatidylinositol-3-kinase; $ptiO_2$, brain tissue oxygen; rtPA, recombinant tissue plasminogen activator; TBI, traumatic brain injury.

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Erythropoietin (EPO) is an endogenous cytokine that is essential for erythropoiesis. EPO and its receptors (EPOR) are produced and expressed in endothelial cells, neurons and astrocytes (Hasselblatt et al., 2006). Erythropoietin exerts tissue protection by anti-apoptotic, anti-inflammatory, anti-oxidative, angiogenic and neurotrophic mechanisms (Morishita et al., 1997; Siren et al., 2001, 2009; Wakida et al., 2007; Hartley et al., 2008; Velly et al., 2010), factors which are also important for lesion development after traumatic ASDH. Recombinant human erythropoietin shows neuroprotection in various models of human disease such as stroke (Sakanaka et al., 1998; Kilic et al., 2005; Kawata et al., 2006; Wang et al., 2007; Gonzalez et al., 2009), subarachnoid hemorrhage (Alafaci et al., 2000; Grasso, 2001), spinal cord injury (Celik et al., 2002; Gorio et al., 2002; Grasso et al., 2006), concussive brain injury (Brines et al., 2000; Yatsiv et al., 2005; Chen et al., 2007; Zhang et al., 2009) and intracerebral hemorrhage (Lee et al., 2006).

A first clinical trial to study the efficacy and safety of three injections of 33,000 IU EPO in stroke patients revealed benefits on outcome parameters and that the agent is well tolerated (Ehrenreich et al., 2002). A later large scale trial showed, however, no beneficial outcome and even higher mortality in stroke patients treated with 40,000 IU EPO (Ehrenreich et al., 2009). In this trial recombinant tissue plasminogen activator (rtPA) was allowed and analysis of data raised safety concerns especially in rtPA + EPO-treated patients. An adverse effect of rtPA on EPO treatment could later be confirmed in animals (Jia et al., 2010). Other reasons for a negative result could be the injury-dependent degree of the blood–brain-barrier (BBB) opening that may result in a wide range of cerebral EPO concentrations or the used dosage which is generally higher in animal models. In a case report by Nirula et al. (2010) treatment of severe TBI patients with a single dose of 40,000 IU EPO had no clear beneficial effect on outcome parameters. Another study treating patients after subarachnoid hemorrhage (SAH, NCT00626574) has been terminated due to the risk of increased mortality in the EPO-treated group. Nevertheless, several clinical trials using EPO for the treatment of TBI patients are on-going (www.clinicaltrials.gov: NCT00987454, Australia/New Zealand; NCT00313716, USA). The problem of EPO dose, dosing interval, number of doses required and route of application to improve patient outcome following brain injury seems not to be resolved. Similarly, neuroprotection by EPO treatment has not been fully surveyed in pre-clinical trials after TBI and not at all after ASDH. Since only small amounts of EPO penetrate the blood–brain-barrier (Banks et al., 2004; Xenocostas et al., 2005) the route of application becomes of interest especially in patients with ASDH. In these cases the brain tissue is exposed in order to remove the subdural blood volume and EPO could be applied topically, thus bypassing the blood–brain-barrier. In order to examine neuroprotective effects of EPO following ASDH we treated rats with post-ASDH injections of various doses and compared results of i.v. injection with the application on the cortical surface after surgical removal of the subdural blood volume.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (Charles River, Germany) were used for all experiments. They had free access to food and water and were housed at a 12:12-h light:dark cycle and 50% humidity. Experiments were approved by the local ethics committee and performed according to guidelines for use and care of laboratory animals.

Anesthesia and surgical preparation

Rats were anesthetized with chloral hydrate (36 mg/ml; Dept. of Pharmacy, University Medical Center, Mainz, Germany). First, they received an i.p. bolus injection of 1 ml/100 g body weight. Thereafter, approx. 1 ml chloral hydrate was injected hourly through an intraperitoneal catheter. Atropin (1 mg) was injected s.c. Body temperature was kept at 37 ± 0.5 °C with a rectal temperature probe connected to a heating blanket control unit (Harvard Instruments, USA). Before surgical preparation animals were intubated and mechanically ventilated with a mixture of room air/O_2.

Tail artery and jugular vein were cannulated (0.8–0.96 mm o.d. PE tubing; Portex, UK) for mean arterial blood pressure (MAPB) and blood gas analysis and for the withdrawal of autologous venous blood. In order to prepare for neuromonitoring and subdural blood infusion rats were fixed in a stereotaxic frame (TSE, Germany). After a skin incision the exposed skull was cleaned and disinfected using 3% H_2O_2. A craniotomy was performed by drilling posterior to the Bregma suture (diam. 3 mm). The dura mater was penetrated using a G26 needle and an L-shaped, blunted needle (G23, B + Braun, Germany) was carefully inserted underneath the dura and fixed in place by Histoacryl® (Bbraun, Germany) and dental cement (Palavit®55VS). Anterior to the Bregma suture an area of 2 × 2 mm was thinned out with a high speed drill for cerebral blood flow (CBF) monitoring (Vasamedics Laserflo® BPM2, St. Paul, USA). Contralateral to the subdural needle a small burr hole was drilled and an intracranial pressure (ICP) catheter (NeuroventP 3F; Raumedic, Germany) was inserted into the cortical tissue. A Licox oxygen sensor (1 mm^2 sensing area; Integra) was placed close to the ICP catheter and brain tissue oxygen tension ($ptiO_2$) was measured continuously in study 1 only.

Monitoring and acute subdural hematoma

Implanted subdural needle, ICP catheter, $ptiO_2$ probe and LD-probe were left in place until stable ICP and CBF values were reached. Thereafter, a 15-min baseline monitoring period started and values were recorded every minute throughout the entire experiment. At the end of baseline, venous blood from the jugular vein was withdrawn and 400 µl was subdurally infused at a rate of 50 µl/min. The subdural needle was left in place until the end of monitoring, then clipped off as close to the skull as possible and completely sealed off by Histoacryl® in Experiment 2. Post-ASDH monitoring continued in this experiment for 60 min (Fig. 1). In Experiments 3 and 4 the needle was carefully removed at 60 min post-ASDH, the dura mater cut off and all visible blood evacuated. The craniotomy was finished by replacing and sealing off the conserved bone flap. In these series animals were monitored for another 15 min (Fig. 1). All animals were allowed to survive for 48 h.

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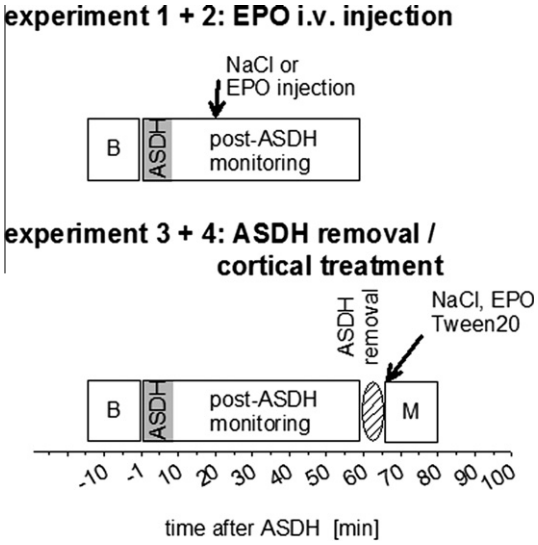

Fig. 1. Experimental design of Experiments 1 + 2 consisted of a 15-min baseline, infusion of 400 µl autologous venous blood (8 min) and a 60-min monitoring period. NaCl (0.9%) or EPO (200, 2000 or 20,000 IU/animal) was injected i.v. 15 min after the end of ASDH (gray bar). At the end of 60-min post-ASDH monitoring animals were either euthanized for CSF and removal of brains (Experiment 1) or allowed to survive for 2 days (Experiment 2). Experimental design for Experiments 3 + 4 consisted of a 15-min baseline, 8-min subdural blood infusion, a 60-min post-ASDH monitoring period and a 15-min monitoring period after the evacuation of subdural blood. 150 µl of NaCl, EPO (0.02, 0.2 or 2 IU; Experiment 3) or Tween-20 (Experiment 4) was applied directly on the cortical surface after removal of the subdural hematoma. Rats were allowed to survive for 48 h. Note that blood evacuation was finished within 5 min. B = 15-min baseline; M = 15 min monitoring after subdural blood evacuation.

Assessment of lesion volume

Rats were transcardially perfused by 4% buffered paraformaldehyde, and their brains were removed and post-fixed in paraformaldehyde for 24 h. Brains were embedded in paraffin and coronal sections spaced 250 µm apart were taken throughout the lesion. Sections were then stained with hematoxylin and eosin to delineate the injury. The damaged area on each section was photographed with a CCD camera (SSC-C370P, Sony) which was connected to a light microscope (Zeiss, Oberkochen, Germany) and was outlined using an image-analyzing software (Optimas 6.51, VSG, UK). Lesion volume consisted of the sum of all measured injured areas multiplied by 250 µm.

Experimental series, groups and treatment

The study consisted of four separate experiments.

Experiment 1. Pharmacokinetics of EPO in blood, cerebrospinal fluid (CSF) and brain tissue was studied in the ASDH model. Rats received an i.v. injection of either NaCl (n = 3), EPO 200 IU (n = 4), EPO 2000 IU (n = 3) or EPO 20,000 IU (n = 4). Blood samples were withdrawn before and 15, 30, 40 and 60 min after ASDH. CSF was collected from the cisterna magna at about 75 min post-ASDH (approx. 60 min after EPO injection). Thereafter, brains were carefully removed (80 min post-ASDH), and split into hemispheres. Blood plasma, CSF and brain tissue were frozen immediately and analyzed for EPO content by ELISA. (R + D Systems).

Experiment 2. Experiment 2 examined the neuroprotective effect of i.v. injected EPO at 200 (n = 11), 2000 (n = 9) and 20,000 IU/animal (n = 8). EPO (NeoRecomon®, Roche, Switzerland) was injected 15 min post-ASDH. The control group (n = 9) received the same volume of NaCl 0.9%. Sham animals (n = 9) received 0.9% NaCl, but no ASDH. Histological analysis of brain damage was performed 48 h after ASDH or sham-operation.

Experiment 3. Experiment 3 explored the neuroprotective effect of cortically applied EPO after evacuation of subdurally infused blood at 60 min post-ASDH. After removal of the infused blood volume the bone flap was reinserted. Through a small burr hole in this bone flap 150 µl EPO or NaCl was applied onto the cortical surface with minimal fluid loss at around 70 min post-ASDH. Animals received 0.02, 0.2 or 2 IU (n = 9/group). Vehicle-treated animals (n = 9) received NaCl 0.9% and sham animals (n = 9) were treated with either NaCl or EPO. Brains were removed for histological analysis of lesion volume at 48 h after ASDH or sham-operation.

Experiment 4. An additional experimental series was started to test whether Tween-20 (Sigma), a component of NeoRecomon® (0.1 mg/0.3 ml), was responsible for adverse effects of cortically applied EPO. The identical experimental protocol as for Experiment 3 was used with the exception that 100 µl EPO was replaced by 0.033% Tween-20 solution. Control animals received NaCl 0.9%. No sham group was included. Brains were removed for histological analysis at 48 h post-ASDH.

Statistical analysis

Statistical analysis was performed by the statistical program SigmaStat 3.10 (Systat). Groups were compared by one-way analysis of variance (ANOVA) with Student–Newman–Keuls post hoc test for individual differences. If normality of data was not reached, a one-way ANOVA on ranks was performed. All values are displayed as mean ± SEM.

RESULTS

Physiological data

All parameters were within physiological ranges and EPO had no effect on MABP, blood gases and hematocrit (Table 1). ASDH did not affect any parameters significantly. A slight 'Cushing reflex' i.e. physiological blood pressure response to increased ICP, was observed that lasted from the 4th to 20th-min after the start of blood infusion.

Pharmacokinetics of i.v. injected EPO after ASDH

EPO concentration in blood plasma was not detectable in baseline samples and 15 min after ASDH. Venous injection of 200, 2000 or 20,000 IU EPO per animal at 15 min post-ASDH led to an immediate increase of values to 0.88 ± 0.25, 134.6 ± 27.8 and 1484.7 ± 299.5 IU/ml, respectively. Thereafter, EPO concentration in blood samples decreased continuously (Fig. 2). In CSF samples EPO reached values of 458.1 ± 70.2, 3406.8 ± 693.5 and 100,499.0 ± 52,517.1 mIU/ml at around 75 min post-ASDH. EPO of NaCl-treated rats was undetectable in CSF samples (Fig. 2). In brain tissue EPO was dose-dependently increased at around 80 min after ASDH. Ipsilateral concentration was

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Table 1. Physiological data before and after ASDH of Experiment 2 given as mean ± SEM. MAP = mean arterial blood pressure; pCO_2/pO_2 = arterial carbon dioxide and oxygen pressure; Hb = hemoglobin; Hct = hematocrit

Groups	MAP [mmHg]	pH	pCO_2 [mmHg]	pO_2 [mmHg]	Hb	Hct [%]	Glucose [mg/dl]	Lactate [mmol/L]
Baseline								
Sham	75.3 ± 2.4	7.42 ± 0.01	38.2 ± 2.82	129.0 ± 4.98	15.3 ± 0.37	46.5 ± 1.55	177.4 ± 29.52	1.08 ± 0.14
NaCl	74.6 ± 2.65	7.42 ± 0.02	39.6 ± 3.56	117.7 ± 6.49	15.0 ± 0.95	45.9 ± 1.48	176.0 ± 8.65	0.98 ± 0.16
EPO 200 IU	73.8 ± 2.2	7.39 ± 0.02	41.9 ± 3.94	119.7 ± 7.22	15.4 ± 0.55	44.6 ± 1.73	176.4 ± 27.89	0.93 ± 0.14
EPO 2000 IU	75.7 ± 2.93	7.40 ± 0.02	41.3 ± 1.46	122.8 ± 7.17	14.9 ± 0.29	45.7 ± 0.94	202.7 ± 14.38	0.97 ± 0.15
EPO 20.000 IU	74.3 ± 2.17	7.39 ± 0.01	39.4 ± 1.64	126.6 ± 4.73	14.8 ± 0.42	43.3 ± 1.25	186.0 ± 15.3	0.93 ± 0.11
End of monitoring								
Sham	72.1 ± 1.52	7.40 ± 0.01	38 ± 1.23	122.5 ± 3.77	14.8 ± 0.2	44.6 ± 1.41	176.8 ± 14.78	1.02 ± 0.08
Vehicle	81.6 ± 2.87	7.38 ± 0.02	39.4 ± 1.98	120.0 ± 3.61	14.7 ± 0.86	44.7 ± 1.07	196.0 ± 9.94	1.04 ± 0.14
EPO 200 IU	79.1 ± 2.56	7.39 ± 0.02	42.8 ± 1.53	123.5 ± 3.95	15.3 ± 0.5	43.8 ± 1.23	204.7 ± 20.17	0.98 ± 0.13
EPO 2.000 IU	77.5 ± 2.93	7.41 ± 0.01	40.0 ± 1.33	127.3 ± 1.55	15.0 ± 0.32	45.8 ± 0.98	196.3 ± 8.75	1.00 ± 0.08
EPO 20.000 IU	79.0 ± 1.87	7.37 ± 0.01	40.9 ± 1.53	122.9 ± 5.34	15.1 ± 0.36	44.2 ± 1.31	200.8 ± 7.16	0.95 ± 0.15

24.1 ± 10.7, 89.8 ± 54.3 and 1794.3 ± 1074.7 µIU/ml brain homogenate in groups receiving 200, 2000 or 20,000 IU EPO, respectively. EPO has been as well detected, but at much lower levels in the contralateral hemisphere (EPO 200: 3.5 ± 0.8; EPO 2000: 45.3 ± 27.4; EPO 20,000: 86.8 ± 214.2 µIU/ml) (Fig. 2).

Monitoring of ICP, CBF and $ptiO_2$

In Experiment 2 ICP of all groups was within a narrow range of 7.1 ± 1.5 (EPO 200) and 7.9 ± 1.0 mmHg) (EPO 20,000) (n.s. between all groups). ASDH temporarily increased ICP to 84.7 ± 5.9 (NaCl), 79.4 ± 1.9 (EPO 200), 83.6 ± 7.0 (EPO 2000) and 87.1 ± 3.4 mmHg (EPO 20,000) (n.s. between injured groups). At time point of EPO injection, ICP had decreased to 18.0 ± 3.0, 18.2 ± 1.8, 18.5 ± 2.4 and 20.0 ± 1.0 mmHg (sham: 7.3 ± 0.9 mmHg). Thereafter, ICP stabilized and values were 18.1 ± 2.5 (NaCl), 17.3 ± 1.4 (EPO 200), 17.1 ± 2.2 mmHg (EPO 2000); (sham: 7.2 ± 0.9 mmHg) at 60 min post-ASDH. Only ICP of EPO 20,000 increased after EPO injection and was significantly higher than in all other groups (22.0 ± 2.3 mmHg; $p < 0.05$ vs. all groups).

Baseline ipsilateral CBF was 33.7 ± 0.5 (NaCl), 33.5 ± 0.4 (EPO 200), 33.1 ± 0.4 (EPO 2000) and 33.0 ± 0.4 Laser-Doppler units (LDU; EPO 20,000). As depicted in Fig. 3 blood flow dropped to 15.0 ± 2.1%, 18.3 ± 3.7%, 13.6 ± 1.3% and 12.2 ± 0.9% of baseline LDU. Thereafter, CBF slowly increased to 47.8 ± 3.4%, 42.7 ± 4.8%, 49.2 ± 4.6% and 43.3 ± 2.4% of baseline LDU at the end of monitoring 60 min post-ASDH. Sham animals showed a stable CBF throughout the experiment (baseline: 33.0 ± 0.5 LDU; 60 min post-ASDH 32.8 ± 0.2 LDU).

In Experiment 3, ICP was between 6.0 ± 0.7 mmHg (EPO 0.02) and 8.1 ± 0.5 mmHg (sham) (n.s. between all groups). ICP increased to a maximum of 67.0 ± 7.8 (NaCl), 73.0 ± 4.1 (EPO 2), 68.8 ± 2.7 (EPO 0.2) and 73.7 ± 3.8 mmHg (EPO 0.02; n.s. between injured groups). Immediately before the evacuation of subdural blood ICP reached 20.7 ± 2.7, 15.0 ± 0.7, 14.3 ± 1.1 and 16.2 ± 1.1 mmHg. Beginning of craniotomy and blood evacuation reduced ICP to 12.6 ± 1.4, 12.0 ± 0.6, 12.3 ± 1.0 and 12.8 ± 0.7 mmHg ($p < 0.007$ all injured groups vs. sham). End of craniotomy and injection of NaCl or EPO elevated ICP again to 22.4 ± 1.2, 18.2 ± 1.5, 19.4 ± 1.5 and 22.5 ± 2.3 mmHg ($p < 0.001$ all injury groups vs. sham).

Baseline CBF was 30.5 ± 0.2 (sham), 31.0 ± 0.4 (NaCl), 31.2 ± 0.5 (EPO 2), 30.4 ± 0.3 (EPO 0.2) and 30.0 ± 0.4 LDU (EPO 0.02). CBF of sham-operated animals remained stable and was 31.4 ± 0.7 LDU at the end of the experiment. ASDH reduced ipsilateral CBF to 10.3 ± 1.3% (NaCl), 9.5 ± 1.1% (EPO 2), 10.8 ± 2.1% (EPO 0.2) and 11.6 ± 1.5% (EPO 0.02) of baseline LDU. CBF recovered slowly to 30% and increased further after the beginning of craniotomy and the evacuation of subdural blood (NaCl: 35.5 ± 2.7%; EPO 2: 41.6 ± 4.0%; EPO 0.2: 43.3 ± 4.5%; EPO 0.02: 45.7 ± 4.9%). End of craniotomy and the injection of 100 µl NaCl or EPO decreased CBF again, ranging from 18.1 ± 1.0 (NaCl) and 28.2 ± 4.8% (EPO 0.2). At any time point, injured groups did not differ significantly from each other (see Fig. 3C, D).

Neuroprotection by EPO

As depicted in Fig. 4A lesion volume at 48 h after ASDH and i.v. injection of NaCl or EPO was 38.2 ± 0.6 mm^3 (NaCl), 28.5 ± 0.9 mm^3 (EPO 200), 22.2 ± 1.3 mm^3 (EPO 2000) and 46.5 ± 1.7 mm^3 (EPO 20,000).

Evacuation of subdurally infused venous blood produced a lesion volume of 36.0 ± 5.2 mm^3 at 48 h post-ASDH (Fig. 4B). Cortical application with 0.02 IU reduced damage to 11.2 ± 2.1 mm^3 ($p = 0.007$), whereas 0.2 IU had no effect on lesion size (38.0 ± 9.0 mm^3) and 2 IU increased injury massively to 67.9 ± 10.4 mm^3 ($p = 0.006$).

In Experiment 4, treatment with NaCl or Tween-20 led to a lesion volume of 49.1 ± 8.4 mm^3 and 42.5 ± 3.6 mm^3 at 48 h following ASDH ($p = 0.490$).

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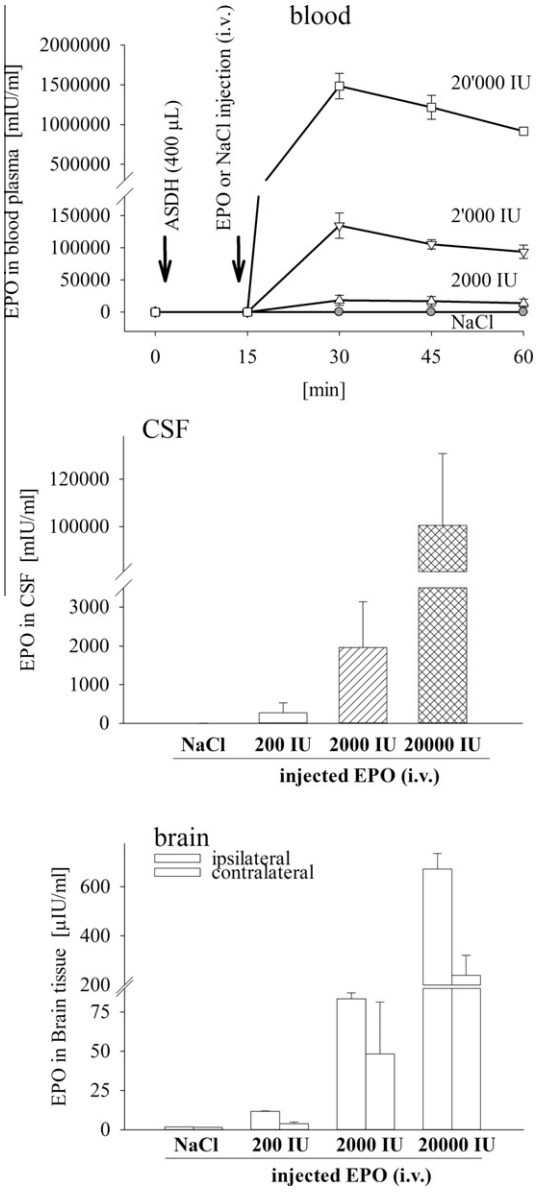

Fig. 2. EPO concentrations in blood plasma, cerebral spinal fluid (CSF) and brain tissue. CSF and tissue samples were harvested about 75 min and 80 min after ASDH, respectively. Values are given as mean ± SEM.

DISCUSSION

Two large on-going clinical trials (NCT00987454; NCT00313716) explore the hypothesis that a recombinant human erythropoietin is neuroprotective in traumatic brain injury as it has been proved in many pre-clinical trials (Brines et al., 2000; Yatsiv et al., 2005; Lee et al., 2006; Chen et al., 2007; Cherian et al., 2007; Zhang et al., 2009) The present pre-clinical study could demonstrate that EPO is also neuroprotective in a model of acute subdural hematoma and that direct cortical application after evacuation of the subdurally infused blood is the most effective route of application in this injury type. In addition, EPO caused adverse effects on lesion volume independent of systemic or direct cortical application at the highest tested concentration, revealing a devastating side-effect of EPO.

Pathophysiological mechanisms which play an important role for lesion development after ASDH in animals are increased ICP (Miller et al., 1990; Sawauchi et al., 2004; Jussen et al., 2008), wide-spread ischemia and CBF reduction underneath the hemorrhage (Kuroda and Bullock, 1992a,b; Tsuchida et al., 1999), inflammation (Fahrig et al., 2005) apoptotic cell death (Alessandri et al., 2006) and blood constituents from extravasated blood (Kuroda et al., 1992; Baechli et al., 2010). Elimination of one or more of these pathomechanisms will reduce brain tissue damage following ASDH (Sawauchi et al., 2004; Alessandri et al., 2006; Jussen et al., 2008). Thus, a combination of surgical intervention as well as the use of a multi-functional drug as proposed by Vink and Nimmo (2009)) could be a potent treatment strategy. Recombinant human erythropoietin is such a multi-functional drug and EPO receptors are found on endothelial cells, neurons, astrocytes, oligodendrocytes and microglia (e.g. Hasselblatt et al., 2006). EPO induces neuroprotection after brain injury through various mechanisms (for review see: Hasselblatt et al., 2006; Xiong et al., 2009; Velly et al., 2010) of which many are involved in ASDH-induced injury development.

Systemically injected EPO concentration increased dose-dependently in blood, CSF and brain tissue with a more pronounced increase ipsilaterally due to BBB breakdown (Fig. 2). Only 0.05–0.1% of i.v.-injected EPO is crossing the intact BBB (Banks et al., 2004) and EPO reduces BBB breakdown after ischemia (Chi et al., 2008). Both factors may influence dose selection and effective treatment with EPO. A dose of 5000 IU/kg has been found most efficacious in models of ischemia, TBI and intracerebral hemorrhage (Lee et al., 2006; Chen et al., 2007; Cherian et al., 2007; Wang et al., 2007; Hartley et al., 2008; Xiong et al., 2010). For the first time we could show that 200 (620 IU/kg) and 2000 IU/ animals (6200 IU/kg) were neuroprotective after ASDH and reduced lesion volumes by 25% and 42%, respectively. This enormous protective effect of 2000 IU is in line with reduced lesion volume or atrophy by i.v. 5000 IU/kg EPO following focal ischemia (49% Li et al., 2007, ±17% Jia et al., 2010), traumatic brain injury (>50%: Cherian et al., 2007, 35%: Hartley et al., 2008, ±37%: Xiong et al., 2010) or intracerebral hemorrhage (40%: Lee et al., 2006).

In order to avoid the BBB as confounding factor we applied EPO directly onto the cortical surface after subdural blood removal in study 2. Early evacuation generates decreased mortality in patients (e.g. Bullock et al., 2006) which is partially due to reduced ICP, improved microcirculation and effects of removed blood constituents (see Fig. 3C, D, Kuroda and Bullock, 1992b; Kinoshita et al., 1994; Verweij et al., 2001; Sawauchi et al., 2004; Jussen et al., 2008; Baechli et al., 2010). A combination of removal of a subdural hematoma and pharmacological treatment has been seldom performed. Most experimental studies left the subdural volume in place and targeted a single

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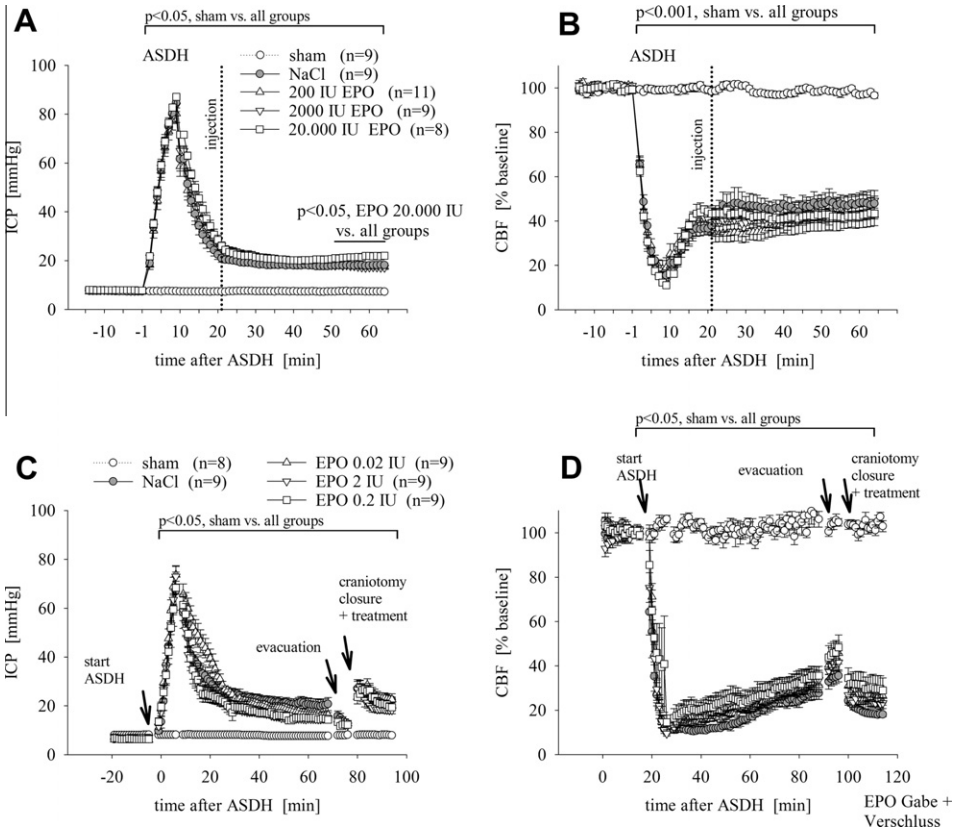

Fig. 3. Time-course of ICP (mmHg) and CBF (% baseline). Following a baseline period 400 µl venous blood was infused at a rate of 50 µl/min (gray bar, arrow). NaCl and EPO have been given by i.v. injection 15 min after the infusion of subdural blood (panel A, B) or by direct cortical application after evacuation of infused blood (panel C, D). All values are given as mean ± SEM. ASDH = acute subdural hemorrhage. $^{*}p < 0.05$ vs. NaCl-treated ASDH group.

neuroprotective mechanism such as excitotoxicity or hydroxyl radicals (Kuroda et al., 1994; Tsuchida et al., 1996; Alessandri et al., 1999, 2006; Uchida et al., 2001; Reinert et al., 2002; Kwon et al., 2003; Mauler et al., 2003, 2004; Fahrig et al., 2005). Jussen et al. (2008) combined surgery at 1 h post-ASDH with hyperoncotic/hypertonic solution treatment and achieved more than 50% lesion reduction and significant recovery of motor function. Unpublished results from our laboratory indicate that ASDH-induced tissue damage doubles from 1 to 6 h and again from 6 to 24 h post-ASDH. This time-course implicates that surgery in combination with a multi-functional drug such as EPO at 60 min post-ASDH could have the potential to attenuate lesion growth and behavioral deficits in rats massively. Lesion volume at 48 h post-ASDH decreased by 68% from 36.0 ± 5.2 mm^3 to 11.2 ± 2.1 mm^3 using 0.02 IU in combination with hematoma evacuation. Similar to Q7 results from combining surgery and HHT by Jussen et al. (2008) behavioral improvement can also be expected with EPO treatment. Short-time monitoring of ICP and CBF indicate that these parameters were not responsible for EPO-induced effects. In addition to injury reduction by hematoma removal (Jussen et al., 2008) EPO contributed to the strong neuroprotection of the combined treatment by its broad function. This could include mechanisms such as anti-apoptosis, anti-inflammation, anti-oxidation, protection from glutamate excitotoxicity and preserved energy metabolism in the brain which all play a well-known role in lesion development after experimental ASDH (Kwon et al., 2003; Fahrig et al., 2005; Alessandri et al., 2006; Baechli et al., 2010). Evacuation of subdurally extravasated blood and thus, exposing the underlying injured brain tissue enables the circumvention of the BBB. As a major consequence we could reduce the amount of EPO necessary for neuroprotection from 200 IU/animal down to 0.02 IU/animal. In the clinical setup this would add up to an at least 10,000 times lower EPO load for patients suffering from traumatic ASDH, thereby reducing systemic side-effects as well as treatment costs.

No protective effect could be found with topical application of 0.2 IU and even an adverse effect by 2 IU (67.9 ± 10.4 mm^3) and by i.v. injection of 20,000 IU (equals 62,000 IU/kg; 46.5 ± 1.7 mm^3). A bell-shaped dose–response curve has already been reported *in vitro* and *in vivo* (e.g. Weishaupt et al., 2004). However, multiple i.p. or s.c. injection of 30,000 IU/kg did not reveal an adverse but on the contrary a neuroprotective effect on ischemic damage in adult mice (Wakida et al., 2007) and newborn rats (Kellert et al., 2007). Even a dose of as high as 50,000 IU/kg prevented isoflurane-induced neurodegeneration in the developing brain

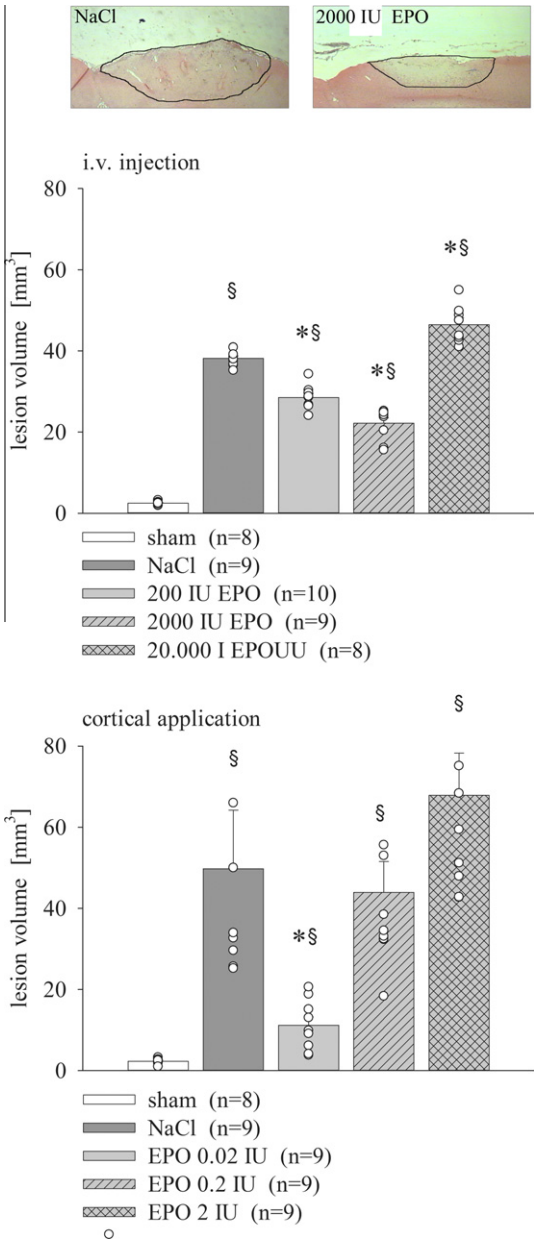

Fig. 4. Lesion volume at 48 h after ASDH. Animals were treated with NaCl or EPO either 15 min after the end of subdural blood infusion by i.v. injection (upper graphic) or 70 min after ASDH by direct cortical application following ASDH evacuation (lower graphic). All values are given as mean ± SEM. * indicates significant difference to NaCl-treated animals; § indicates significant difference to sham-operated animals (all $p < 0.05$; one-way ANOVA with Student–Newman–Keuls post hoc comparison). The two histological sections show a cortical lesion after i.v. NaCl- (left) and 2000 IU EPO-treatment (right). Sections were stained with hematoxylin–eosin.

(Tsuchimoto et al., 2011). Negative effects of exogenous EPO are mainly reported in cancer research (for review see: Hadland and Longmore, 2009). Since tumor cells express few EPO receptors adverse effects are attributed rather to thrombovascular events than to a direct EPO effect on tumor cells. EPO treatment induces higher hemoglobin levels, platelet activation and neovascularization by the activation of endothelial cells. Similarly, an increased concentration of erythrocytes is a potentially harmful effect for the ischemic and traumatized brain and should therefore be avoided. Hematocrit increases continuously to almost 60% after multiple injections of 5000 IU/kg in rats within 4 days (Klemm et al., 2008; Zhang et al., 2009). Five injections of 10,000 IU/kg EPO increased hematocrit only by 10% on day 4 and by 20% on day 7 in a mouse anemia model (Kabaya et al., 1995). Neuroprotection in our study was assessed at day 2 post-ASDH, i.e. before elevated hematocrit levels of 55–60% are reached. Therefore, EPO-induced thrombovascular events seem not to be responsible for the acute adverse effect of EPO. Results that transgenic mice overproducing EPO only in brain cells have no elevated hematocrit but are protected against brain ischemia (Frietsch et al., 2007) present a similar aspect. Already concentrations as low as 2 (Fig. 4B) and 200 IU (data not shown) which could not have influenced hematocrit significantly had detrimental effects on lesion volume in the present study. Thus, other factors are responsible for this adverse effect. Overexpressing brain EPO in these transgenic mice increase the CBF by 11% and metabolic rate of glucose by 22% (Frietsch et al., 2007). It is unclear whether an EPO-induced CBF effect could still occur in the penumbral region where vessels are already dilated due to increased energy demand. On the other hand, EPO-induced elevated glucose metabolism might push already compromised cells after ASDH (Kuroda and Bullock, 1992b) over the edge, thereby expanding brain injury. As seen in Fig. 3B 20,000 IU per animal caused a significant CBF drop but did not influence the CBF time-course. Simultaneously an increase of ICP indicated an adverse effect for this systemically applied concentration. Since CBF (Fig. 3B) and $ptiO_2$ did not show a parallel reaction the effect of EPO on ICP might be an epiphenomenon. However, delayed effects of high systemically or topically applied EPO concentrations on lesion development through ICP, CBF or glucose metabolism cannot be ruled out. We tested also the idea that Tween-20, a detergent and potentially harmful ingredient of the used NeoreRecomon® might have played a role in injury development after treatment with high EPO concentrations. Direct application of a Tween-20 solution onto the cortical surface did not augment lesion volume which rules out Tween-20 as harmful ingredient.

The fact that cortically applied 2 IU also expanded lesion volume extremely point at a direct effect of EPO through its receptor and activated pathways. EPO activates two types of EPO receptors, a high-affinity homodimer and low-affinity heteromeric receptor which belong to the cytokine type-1 family receptors (Brines, 2010). The low-affinity receptor with its neuroprotective properties is expressed immediately after TBI that is followed by the stimulation of high-affinity EPO receptor at 24 h post-injury and then by astrocytic and neuronal EPO production (e.g. Bernaudin et al., 1999). The consequences of the simultaneous activation of both receptors or over-stimulation of the low-affinity receptor on signaling cascades have not been thoroughly

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examined in respect to detrimental processes. The EPO receptor has no intrinsic kinase activity but ligand binding causes dimerization that enables autophosphorylation and phosphorylation of Janus-tyrosine kinase-2 (JAK-2). Significant activated signaling pathways for the neuronal functions seem to be the activation of phosphatidylinositol-3-kinase (PI3-K)-Akt/protein kinase B pathway, the Ras-mitogen-activated protein kinases, signal transducers and activators of transcription-5 (STAT-5) and necrosis factor-kB-dependent transcription (Bernaudin et al., 1999; Kilic et al., 2005; Hasselblatt et al., 2006; Zhang et al., 2006; Valable et al., 2010). Disturbance of one or more pathways might lead for example to altered endothelial NO production via PI3 kinases or bcl-2 production via AKT signaling pathways and thereby to blocking/reversing pivotal protective mechanisms. Since i.v. injection of more than 60,000 IU/kg is unlikely for the treatment of head-injured patients our reported neurotoxic side-effects of EPO are of theoretical interest. In the case of topical application for which a concentration as low as 2 IU per rat was devastating, our reported adverse effect is important. Comparable to our topical application is the intracerebroventricular (i.c.v.) injection of EPO (Bernaudin et al., 1999; Calapai et al., 2000; Zhang et al., 2006). Post-ischemic i.c.v. injection of 5–50 IU was neuroprotective, i.e. at dosages with which we already observed detrimental effects. Factors which could explain this discrepancy could be dilution and different distribution after i.c.v. injection or interactions with anesthesia-induced processes (e.g. Hockel et al., 2012). In order to perform EPO treatment of traumatic ASDH it will be necessary to address such factors in further pre-clinical studies beforehand.

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