Secondary Damage after Traumatic Brain Injury: Epidemiology, Pathophysiology and Therapy

Sedondaire schade na traumatisch schedelhersenletsel:
epidemiologie, pathofysiologie en therapie

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Zweckberger K, Erös C, Zimmermann R, Kim SW, Engel DC, Plesnila N. Effect of Early and Delayed Decompression Craniotomy on Secondary Brain Damage after Controlled Cortical Impact in Mice. *J Neurotrauma*. 2006 Jul;23(7):1083-93.

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CHAPTER 1. GENERAL INTRODUCTION

Traumatic brain injury

Traumatic brain injury (TBI) is defined as a microscopic or macroscopic injury to the brain caused by external physical forces. Road traffic accidents, falls, sports injuries (i.e. boxing), recreational accidents (i.e. parachute jumping), the use of firearms, assault, child abuse, and several rare causes e.g. the use of nail guns or lawn mowers have all been described as causes of TBI. The pathology of TBI can be classified by mechanism (closed versus penetrating); clinical severity (Glasgow Coma Scale) and structural damage (imaging e.g. CT-examination). In most cases TBI is graded according to injury severity assessing the level of consciousness of the patient by, most frequently, the Glasgow Coma Scale (GCS). The GCS scores patients based on their ability to open their eyes, perform limb movements and respond adequately to simple questions (Teasdale and Jennett 1974) (see table 1). Mild TBI, e.g. a light concussion, is defined as a patient with a GCS of 13-15 possibly suffering from short-term memory and concentration deficits (Rimel et al., 1981; Mosenthal et al., 2004). Moderate TBI is scored by a GCS of 9-12, e.g. a lethargic and stuporous patient. A comatose patient, unable to open eyes or follow commands has been severely injured and has a GCS of 3-8.

Table 1. Glasgow Coma Scale (GCS) Score

EYE OPENING	*MOTOR RESPONSE*	*VERBAL RESPONSE*
	6. Obeys Commands	*5. Oriented*
	5. Localizes Pain	*4. Confused*
4. Spontaneous	*4. Withdraws (Pain)*	*3. Inappropiate Words*
3. To Voice	*3. Flexion (Pain)*	*2. Incomprehensive Words*
2. To Pain	*2. Extension (Pain)*	*1. None*
1. None	*1. None*	*t Intubated*

Epidemiology

Many articles and books concerning TBI start out with the following, or a similar, sentence: TBI is a leading cause of death and disability in young people in Western industrialised societies. In Europe the one year prevalence of TBI was estimated to be 708,954 (Andlin-Sobocki et al., 2005). A systematic review published in 2006 by Tagliaferri et al. showed that epidemiological studies on TBI in Europe differ greatly in study design, which makes it difficult to compare. An incidence of about 235 per 100,000 and a mortality rate of about 15 per 100,000 were derived. These facts implicate that TBI represents a highly relevant medical and socioeconomic burden for modern societies (Murray et al., 1997, Ghajar 2000). Another approach to assessing incidence and mortality of TBI was used in Cologne between the years 1990 and 2000 (Bouillon et al., 1999; see chapter 2A). The total prevalence of brain disorders in Europe is 127 ,012,482 (Andlin-Sobocki et al., 2005). Thus less than 0.5% of all brain disorders (including depression and stroke, Alzheimer's disease etc.) is caused by TBI. Polytrauma is ranked 4th in death rates in ICD-9 chapters, after circulatory, neoplastic, and respiratory diseases. Data on the prevalence of different injuries in polytraumatised patients showed that in 34% of all patients TBI was present (Gennarelli et al., 1994). Polytraumatised patients suffering from additional TBI, however, account for over 50% of all polytrauma-related mortality. This indicates that TBI is one of the most important prognostic factors for polytraumatised

individuals (Caldwell and McGovern 1993, Acosta et al., 1998, Dereeper et al., 1998, Jennett et al., 1998, Hodgson et al., 2000). Patients that survive TBI suffer from short or long term minor to major disabilities (Sosin et al., 1996, Thornhill et al., 2000, Bruns et al., 2003). Both moderate and severe TBI are associated with a higher lifetime risk to develop Alzheimer's disease (2.3 and 4.5 times respectively; Langlois et al., 2006).
Besides the severity of the primary impact other factors have been identified to influence outcome. These factors can provide predictive information because they influence the vicious circle (described below). The predictive information can provide assistance in decision making during treatment. The influence of predictive factors is measured by outcome parameters. A common outcome score is the Glasgow Outcome Scale (GOS) (Jennett et al., 1981) (see table 2).

Table 2. Glasgow Outcome Scale (GOS) Score

1	*Good recovery*	*normal social life and ability to return to work.*
2	*Moderate disability*	*indepent, but disabled: ability to look after themselves, but no return to pre-injury level of daily life.*
3	*Severe disability*	*conscious, but dependent: need of another person for some – all activities during the day.*
4	*Vegetative state*	*non-sentient survival (Jennett and Plum 1972), no communication possible and even simple commands cannot be obeyed.*

Many possible predictive factors have been investigated prospectively and retrospectively in reasonably small datasets in the past few years (Tiret et al., 1990; Chesnut et al., 1993; Asikainen et al., 1998; Ono et al., 2001; Hoofien et al., 2002; Servadei et al., 2002; Jeremitsky et al., 2003). Some factors, i.e. gender, race and hypothermia were proven to not correlate with better or poorer outcome. For other factors, i.e. age and hypotension, different consequences of presence or absence of the factors have been published in small datasets (Chesnut 1993; Manley et al., 2001; Walia and Sutcliffe, 2002; Hukkelhoven et al., 2003). This led to disagreement amongst researchers. The IMPACT study investigated these factors in a pooled set of individual data of 9205 patients (see chapters 2B and C). Again, these factors are important for treatment decision-making.

Pathophysiology
Traumatic brain injury can be focal, multifocal or diffuse. In this thesis mainly focal/multifocal injury will be discussed. This kind of injury leads to a contusion, or an epidural or subdural haemorrhage caused by mechanical impact of trauma. The direct physical impact can injure neurons, glia and blood vessels throughout the brain in one or several regions. The pathophysiology of contusions is complex. Several processes after the primary brain damage lead to secondary brain damage, causing the contusion to expand over time. The more braintissue is lost, the larger the chance of function loss. Figure 1 illustrates processes leading to secondary damage in detail. A number of factors are thought to contribute to contusion expansion, i.e. brain oedema formation leading to capillary compression and hypoperfusion (Bullock et al., 1991, Katayama et al., 1998), microthrombosis (Lafuente et al., 1999, Schwarzmaier et al., 2007), progressive bleeding causing microvasospasm (Bullock et al., 1992), glutamate excitotoxicity (Nilsson et al., 1990, Tanaka et al., 1994), or activation of intracellular cell

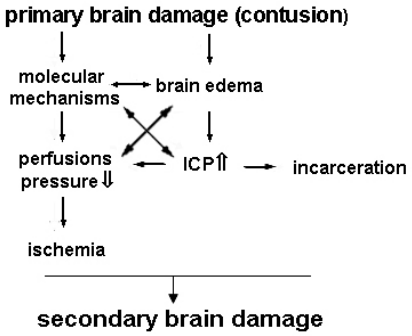

Figure 1. Pathophysiological mechanisms of secondary brain injury in severe head injury.
The primary damage leads to mechanical damage, that causes molecular mechanisms to start off a vicious circle in which all factors interact with and aggrevates the other factors directly or indirectly.

death pathways (Colicos et al., 1996, Newcomb et al., 1999, Smith et al., 2000).
The reduction of cerebral blood flow (CBF) in the peri-contusional area (penumbra) was one of the first factors to be thought of (Rosomoff et al., 1996). This has been studied the past forty years by the use of i.e. Xe-CT in TBI patients (Fieschi et al., 1974), brain tissue of deceased TBI patients (Graham et al., 1978) and different experimental techniques in animals (Martins et al., 1977, DeWitt et al., 1986, Cherian et al., 1994, Lundblad et al., 2004). Most studies show that post-traumatic cerebral blood flow is reduced following focal and diffuse brain damage (Bouma et al., 1991, Alexander et al., 1994, Kochanek et al., 1995, Liu et al., 2002, Lundblad et al., 2004). A severe reduction of CBF, >80% of normal value, is in most cases associated with cortical contusions, and is found in these contusions (Bouma et al., 1991; Schroder et al., 1995; Garnett et al., 2001). Only few studies tried to correlate reduction of CBF and tissue loss in the penumbra. Schroder et al. (1995) and von Oettingen et al. (2002) showed a clear positive correlation between the level of CBF reduction and the distance from the core of the contusion. It suggested that progressive penumbral cell death might be caused by ischemia. Bryan et al. (1995) compared the volume of ischemic tissue 30 minutes and 4 hours after controlled cortical impact injury (see *Technical approaches* in this chapter) in the rat and reported a trend towards an increased volume of ischemic tissue over time. This suggests that progressive penumbral ischemia may be a major cause for peri-contusional cell death.
Other factors, besides systemic and demographic factors, affecting contusion expansion include progressive bleeding, oedema and various pathophysiologic conditions such as glutamate excitotoxicity (Bullock et al., 1991, Maas et al., 2000, Weber, 2004). These processes lead to cumulative damage resulting in brain oedema, consecutively increased intracranial pressure (ICP) leading to more cell death and in worst case the patients' death (Unterberg 2004); a vicious circle has developed (figure 1). Figure 2 illustrates pathophysiological events on molecular level.
Brain oedema is categorised in vasogenic, cytotoxic, and osmotic oedema. Cytotoxic oedema is not directly reliant on the blood brain barrier (BBB), and involves cell swelling of mostly glia because of changed cell membrane permeability (Unterberg et al., 2004). When neurons and glia disrupt, calcium, glutamate and potassium ions are involuntarily released in excess into the extracellular space. These substances are detrimental to neurons. The elevated concentration over stimulates receptors on healthy astrocytes and to lesser extent neurons, leading to excessive influx of calcium and potassium, followed passively by sodium, chloride

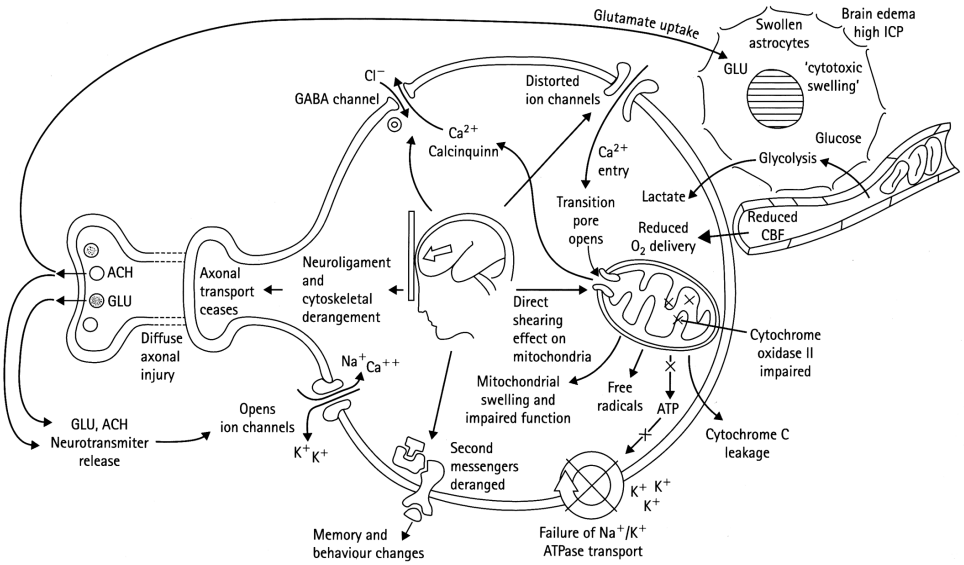

Figure 2. Molecular events in TBI.
Hypothetical scheme to depict post-traumatic events, with opening of ion-channels and uptake of potassium by astrocytes which jeopardizes the microcirculation. From: Head injury, editors: Reilly and Bullock (2005).

and water (Weber 1999). As a result of this osmotic load water will accumulate intracellular. Simultaneously degradation enzymes causing organelle swelling, plasma membrane swelling (Choi et al., 1995), necrosis (Wyllie et al., 1980) and apoptosis (Zipfel et al., 2000). The elevated intracellular calcium also leads to changes in the mitochondria (Muizelaar 1989, Sutton et al., 1994, Katayama et al., 1998), leading to release of free radicals. Free radicals are always present in organisms as a normal by-product of oxidative metabolism within mitochondria (Kontos, 1985, Siesjo, 1992a (II)). Yet, following trauma free radical activity is increased (Hall and Braughler, 1989, Siesjo, 1992 a,b), leading to cellular damage by peroxidation of proteins, DNA and lipid membranes and thereby necrosis and apoptosis (Kermer et al., 1999, Leker and Shohami, 2002). Thereby several reactive oxygen species (ROS) are generated excessively, such as superoxide and hydroxyl radicals (Leker and Shohami, 2002). Nitric oxide (NO) appears to be increased as well, which is generated by the enzyme nitric oxide synthase (NOS) (Samdani et al., 1997). NO contributes to several important physiological processes, such as cerebral vasodilation and neurotransmission (Dawson and Dawson, 1996). However, excess NO can interact with the ROS superoxide radical, resulting in the generation of peroxynitrite. Peroxynitrite is postulated to be one of the most damaging free radical species (Dawson, 1999, Liu et al., 2002). The idea exists that the application of a NOS-inhibitor protects cells from peroxidation.

Osmotic oedema develops almost immediately after injury by high osmolarity in the necrotic tissue caused by increased substances in the extracellular space leading to increased intercellular water (Katayama and Kawamata, 2003). Vasogenic oedema results from disruption of the BBB, an increased permeability of the capillary endothelial cells for proteins caused by

ischemia or trauma itself, causing increased extracellular fluid as well (Betz et al., 1989). Katayama et al. (1998) emphasize the importance of the osmotic gradients caused by the processes described above within contusions, causing oedema, and thereby increased ICP. ICP raises the most within the first 24 hours after injury in human (Bouma et al., 1991,1992) and animal studies (Zweckberger et al., 2003). In these 24 hours the brain is most vulnerable and susceptible to secondary injury insults by i.e. hypoperfusion, leading to progressive cell death (Jenkins, 1989). Ischemic changes have been demonstrated in over 90% of patients who died from head injury (Graham et al., 1978, Ross et al., 1993). As mentioned earlier decreased CBF is related to worse outcome (Graham et al., 1978, Jaggi et al., 1991, Garnett et al., 2001, Hlatky et al., 2004). The exact role of CBF, hypoperfusion and ischemia for secondary brain damage following mechanical injury is not clear. In different experimental settings the effect of CBF and ischemia was investigated in relation to cell death or tissue loss (see Chapter 3).

Current therapeutic options

Prevention is the best cure. In the past 30 years preventive measures have resulted in a great reduction of TBI in western societies. The major profit was made in road traffic TBI by several changes; i.e. road construction (Evans, 1991, Kraus, 1993), speed control (Brindle, 1992), motor vehicle design, airbags (Zador and Ciccone, 1991, Stewart et al., 2003), seat belts (Orsay 1990, Bradbury and Robertson 1993) motorcycle helmets (Sosin et al., 1990, McSwain and Belles, 1990, Kraus et al., 1994, Chiu et al., 2007), and, especially in the younger victims, bicycle helmets (Rivara et al., 1994, Sosin et al., 1996, Shafi et al., 1998, Kopjar et al., 2000, Thompson et al., 2000, Wesson et al., 2000). Occupational hazards have been prevented by strict regulations on the use of tools and wearing helmets at construction sites. Unfortunately, the prevention of falling of elderly from stairs or in the shower has not been accomplished yet.

Often, TBI is accompanied by injuries of other parts of the body, leading to hypovolemic shock and multiple organ failure. The Advanced Trauma Life Support (ATLS) (Vestrup et al., 1988, Bell et al., 1999) has standardised the care of trauma patients as much as possible. The implementation of ATLS and other changes within emergency medical systems (i.e. implementation of helicopters) have improved treatment (Klemen and Grmec, 2006, Chiu et al., 2007). However, injured patients in rural areas have worse outcome than patients closer to advanced medical care (Wald et al., 1993, Chiu et al., 2007, Tiesman et al., 2007). These patients cannot always reach optimal care within reasonable time. The Brain Trauma Foundation manages guidelines for the acute management of TBI patients from accident site to hospital discharge. First priority is to assess, stabilise and treat according to the basic resuscitation protocols, starting airway, breathing and circulation. Thereafter consciousness is assessed by the GCS. Patients with moderate to severe TBI (9-13 and 3-8, resp.) need to be transferred to a trauma centre. The appointed trauma centre should have:

- 24-hour CT scanning availability
- 24-hour available operating room and neurosurgical care.
- ability to measure and treat intracranial pressure (ICP) promptly.

Pre-hospital the patient needs to be re-assessed every 5 minutes, maintaining an O_2 saturation at >90% or PaO_2 60 mmHg, systolic blood pressure > 90mmHg. These values should be maintained both pre-hospital and in-hospital.

In-hospital ICP needs to be assessed, when GCS is 3-8, and an abnormal CT-scan (haematoma, contusions, swelling, herniations, or compressed basal cisterns). In patients aged over 40 years, uni- or bilateral motor posturing, or hypotension ICP measuring should be considered. The most accurate and cost effective way of ICP measuring is intraventricular, though other methods are available and secure as well. When ICP raises above 20-25 mmHg treatment should be started. To assess the presence of ischemia the cerebral perfusion pressure (CPP) is calculated (mean arterial pressure (MAP) – ICP). The threshold for CPP lays around 50-60 mmHg. When increased ICP is most likely to be caused by an intracranial haematoma or contusion operative treatment should be considered. When increased ICP is caused by other, non-operable, reasons, barbiturates or mannitol (hyperosmolar therapy) should be considered. These options should only be used for severe increased ICP (signs of transtentorial herniation), special care is needed to maintain systolic blood pressure > 90 mmHg.
Unfortunately the above-summarised guidelines often fail in treating raised ICP. Decompressive craniectomy (DC) is an old treatment that has been revived the past couple of years. DC can be considered when ICP is raised and is uncontrollable with conservative therapy. By opening the skull ICP reduces almost immediately; pathophysiological pathways leading to ischemia, apoptosis and necrosis are discontinued. Cerebral blood flow (CBF) is likely to be altered by DC after TBI. DC after TBI was first described already in 1901 by Kocher and was investigated in the 1960s&70s (Kjellberg and Prieto, 1971, Ransohoff, 1971, Venes and Collins 1975, Cooper 1976, Britt and Hamilton, 1978). The suggestion that DC would worsen brain swelling and induce herniation through the DC opening, made many neurosurgeons abandon this option of therapy. In the late 1980s new criteria for when and how to perform DC were introduced among other therapeutic strategies to reduce raised ICP (Alexander et al., 1987, Gaab et al., 1990, Marshall, 2000, Naredi et al., 1998,2001, Grande et al., 2002). Experimental and clinical data show promising beneficial effects (Polin et al., 1997, 2003, Guerra et al., 1999, Yoo et al., 1999, Taylor et al., 2001, Whitfield et al., 2001, Hejazi et al., 2002, Kontopoulos et al., 2002, Schneider et al., 2002, Simma et al., 2002, Albanese et al., 2003, Figaji et al., 2003, Zweckberger et al., 2003, Messing-Junger et al., 2003). DC, when performed early could be effective (Skoglund et al., 2006). In the paediatric population DC has been accepted as valid ‘second tier’ therapy for raised intracranial pressure (Rutigliano et al., 2006, Jagannathan et al., 2007). This is not (yet) the case in adult TBI patients. Clinics throughout Germany and the Netherlands seem to use different protocols and criteria (personal communication). In stroke early decompressive surgery has been proven to be beneficial in a pooled analysis of three randomised controlled trials (RCTs) (Vahedi et al., 2007). Rosenfeld (2006) advocates early large, generous, craniectomy in war victims of cranial blast injuries. Prospective RCTs of early DC after TBI in adults injured outside war zones are currently running worldwide (RESCUEicp and DECRA) hopefully giving a last conclusive answer on clinical outcome. Why exactly DC works, besides taking off the pressure of the rigid box, is unknown. The hypothesis rose that CBF is improved after DC and thereby affect different pathophysiological pathways leading to secondary brain damage (see Chapter 5).

Technical Approaches

Great effort has been made the past decades to investigate pathophysiological pathways and to test possible treatment strategies. As TBI itself and subsequent (patho)physiological processes occur rapidly after each other, it is difficult to study these processes in human beings, let alone on a test dummy. The development of imaging by computer tomography (CT) and

magnetic resonance imaging (MRI) must be mentioned in the development of knowledge in science in general, but certainly in TBI research. CT enables a glance into the brain quickly after trauma and thereby the ability to diagnose rapidly and non-invasively (Marshall et al., 1992). Thereafter a more targeted treatment can be started. Images of both post-mortem and CT/MRI investigations have shown that there is great heterogeneity between patients and mechanisms of injury. This has led to different treatment protocols for i.e. diffuse injury versus brain contusions (Brain Trauma Foundation 2007 updated guidelines). However, not everything can be investigated that way. Animal models are able to model responses of body and brain after TBI. Therefore many studies have been and still are continued in the laboratory *in vitro* (in cell cultures) and *in vivo* (laboratory animals) to study mechanisms of reactions to TBI from RNA level to systemic responses alongside post-mortem and clinical investigations. Several methods are used to image brains (incl. pathophysiological mechanisms), brain structures, cells, and even the inside of cells by pathologic anatomy approaches (i.e. immunohistochemistry, immunocytochemistry), CT and MRI. Autoradiography, Laser Doppler and fMRI are methods used to measure blood flow or metabolism of glucose indirectly. Metabolism and extracellular conditions can (in)directly be measured by microdialysis.

In vitro

In *in vitro* studies cultured cells are utilized. For this thesis primary (embryonic) mouse cultures were grown on a silastic membrane that, when a fluent layer has been formed after 9-12 days *in vitro*, can be mechanically injured by the *in vitro* 94A Cell Injury Controller (CIC) (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA, USA; see figure 3). Ellis et al. first described this device in 1995 (Ellis et al. 1995). Several cells in the fluent layer are disrupted directly by the mechanical impact of stretching (necrosis). Some of the remaining cells go into apoptosis because of extracellular changes; the human situation of primary and secondary damage alike. The amount of cell damage depends on the amount of stretch. This model is thought to represent the same mechanical impact on the brain when the brain is hit by physical impact or de-/acceleration, according to a mathematical approach (Ljung et al., 1975; Schreiber et al., 1995). Cells can be dyed or stained after injury and looked at morphologically on the silastic membrane; i.e. by the use of propidium iodide and Hoechst, or Microtubulin Associated Protein 2 (MAP2) and 4',6-Diamidine-2'-phenylindoldihydrochloride (DAPI) respectively. Substances released in the cell media can be determined by several techniques.

In vivo

Several *in vivo* models were designed to mimic a more realistic setting. In the 1960s researchers started to develop experimental *in vivo* head injury models (Terao 1963, Unterharnscheidt 1963, Ommaya et al., 1964). A precursor of an experimental TBI model that is still being used today, though with some adjustments, was first described in 1976 by Sullivan et al.: the Fluid Percussion Injury (FPI) model (Sullivan et al., 1976). This model mimics diffuse injury together with intraparenchymal haemorrhage or subarachnoid haemorrhage. It was used on opened or closed skulls. The use of the FPI model on closed skulls had various practical problems. On the other hand, the Closed Head Injury model of Marmarou et al. was able to induce more severe diffuse injury than the FPI model (Marmarou et al., 1994). Chen et al. (1996) made it possible to use a model alike in mice. As mice are to date the only rodents that can be genetically enhanced the enhancement of this model enabled a new field

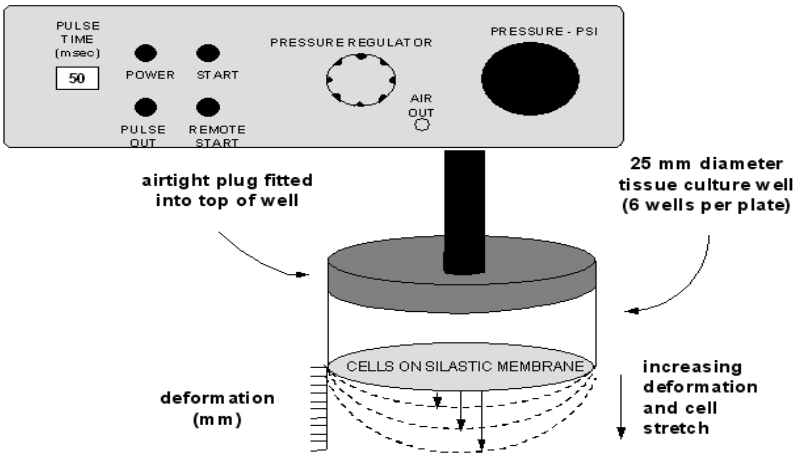

Figure 3. Stretch injury model: 94A cell injury controller.
Cells are cultured on the silastic membrane in the well. On top of the cells is a layer of media, which contains nutrients, oxygen and cellular waste products. These nutrients and oxygen are needed to maintain the confluent layer of the living cells. To apply trauma the airtight plug is fitted on the well. Through the airtight plug an impuls of air is given during an upfront set amount of pressure and duration, after pushing the 'start' button. The silastic membrane, and the cells, will be deformed several millimeters for the set duration of time, and thereby be injured. Pressure and duration can be altered in order to modify the severity of injury.

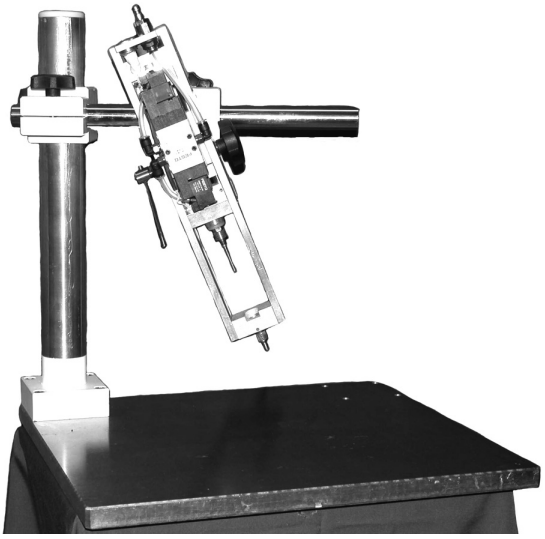

Figure 4. Control cortical impact model.
The exact depth and speed of impact are set before placement of the mouse. The head of the anaesthetised mouse is fixed in a stereotactic frame. A craniotomy is made located as shown in figure 5. A pneumatic pressure causes the impactor to move with the set speed directly onto the dura, a contusion is made. The severity can be regulated by alteration of depth and/or speed.

of studies in closed skull experimental TBI as well as in open models. Lighthall et al. of the General Motors Research Laboratories introduced the Controlled Cortical Impact model in 1988 (Lighthall et al., 1988). The CCI model is the model used in this thesis (figure 4). Mice have to be craniectomised as drawn in figure 5. Thereafter mice receive mainly focal injury applied by a pneumatic impact tip (impactor), leading to a contusion and/or haemorrhage. Regulating depth, width and/or speed of the impactor can vary the injury severity. Acutely the most important aspect is the expansion of the primary contusion by several factors within the first 24 hours. ICP, CBF, BBB, oedema and other parameters can be measured at different time points after injury. The location of ICP and CBF by Laser Doppler measurements is also shown in figure 5. After the application of injury the skull can be closed again by tissue glue, or left open (craniectomy instead of craniotomy) to investigate decompressive effects.

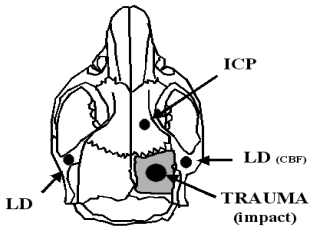

Figure 5. Schematic drawing of a mouse skull.
Placement of the following devices are depicted:
- craniectomy (light grey),
- trauma impact,
- intracranial pressure (ICP) (through the skull),
- Laser Doppler (LD) (on the skull).

Scope of thesis

The overall aim of this thesis is to investigate the importance of ischemia in traumatic brain injury from different perspectives, ie. epidemiology, pathophysiology and therapeutic options, hypothesizing that the role of ischemia is essential in secondary damage after TBI. Epidemiology, pathophysiology and therapeutic strategies will be discussed in the following chapters.

In order to be able to examine an issue in depth, the issue should be clear. Epidemiology is the start of every medical query when searching for pathophysiological mechanisms and possible therapeutic strategies. Many factors might influence (patho)physiological conditions and outcome. Some might be treatable, some might not. Chapter 2 describes the role of hypotension and hypoxia, which are both thought to influence CBF. It also describes incidence, outcome and other potential predictive factors of TBI.

Pathophysiology of TBI has been investigated to large extent the past decades, but certainly not everything is clear. Ischemia is one issue that is under constant investigation. Every year more pieces of the puzzle are put on the right spot. Chapter 3 considers the role of ischemia *in vitro* and *in vivo*, as to whether ischemia, as supposed, kills more cells when superimposed after mechanical injury *in vitro*. The possible protective role of free radical scavengers in reducing secondary damage *in vitro* is evaluated as well *in vitro*. *In vivo* CBF is measured by autoradiography in mice, leading to a good comparison of CBF in and around the contusion.

And lastly, by understanding further pathophysiological conditions and pathways future therapy is the final goal. As described above, many therapeutic options have been investigated, but not found effective. Decompressive craniectomy (DC) is a possible treatment currently under investigation. DC and correlations with pathophysiological factors, e.g. CBF, were investigated experimentally and shown and described in chapter 4.

CHAPTER 2. EPIDEMIOLOGY.

A) Incidence and Outcome of Traumatic Brain injury in an Urban Area in Western Europe over 10 years

Abstract

Introduction: Valid epidemiological data on incidence and outcome of traumatic brain injury (TBI) show great variability. A study on incidence, severity and outcome of TBI was conducted in an urban area of one million inhabitants. *Materials and Methods*: 130,000 prehospital emergencies were screened for TBI. Inclusion criteria: Glasgow Coma Scale (GCS) score ≤ 8 and/or Abbreviated Injury Scale for head injuries (AIS_{head}) score ≥ 2 with confirmed TBI via appropriate diagnostics. *Results*: Annual incidence was 7.3/100,000. Overall mortality rate was 45.8%: 182 (28%) were prehospital deaths, 116 (17.8%) patients died in hospital. Two hundred and fourteen of 352 (60.8%) surviving patients were sufficiently rehabilitated at discharge [Glasgow Outcome Scale (GOS) score = 1], but 138 patients (39.2%) survived with persisting deficits. GOS was associated with initial GCS and AIS_{head}. *Conclusion*: The incidence of TBI was lower compared to the literature. The overall mortality was high, especially prehospital and early in-hospital mortality rates.

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability world-wide. In the United States an estimated number of 1.6 million people sustain TBI each year accounting for 52,000 deaths and 80,000 patients suffering from permanent neurological impairment (Sosin et al., 1996, Bruns and Hauser, 2003). TBI represents a highly relevant medical and socioeconomic burden for modern societies (Murray and Lopez, 1997, Ghajar, 2000).

Despite its clinical relevance, valid epidemiological data on incidence, severity and outcome of TBI from Europe, in particular from Germany, are scarce and show great variation (Hartwig et al., 1993, Jennett, 1996; Lehr et al., 1997). A major problem with epidemiological studies on TBI is still associated with inconsistencies in TBI definition and classification of injury severity (Jennett, 1996, Engberg, 1995, Kraus and McArthur, 1996, Bouillon et al., 1997, Fearnside and Simpson, 1997, Ingebrigtsen et al., 1998). Subsequently, the true impact of this injury with respect to its magnitude as well as the effectiveness and efficiency of therapeutic concepts remains difficult to estimate (Ghajar et al., 1995; Prough, and Lang, 1997; Tolias and Bullock, 2004).

The value of a functioning emergency medical system for TBI patients has frequently been emphasized (Roy, 1987, Colohan et al., 1989, Sampalis et al., 1995, Mullins et al., 1996, Marshall, 2000). Major improvements in outcome and mortality can only be achieved by preventive strategies, early recognition of complications and stabilization of patients (for review see guidelines of the Brain Trauma Task Force and the Brain Trauma Foundation (Brain Trauma Foundation, 2000). However, optimal care of TBI patients requires sufficient resources with respect to logistics, management, organization and finances. Valid data on incidence, severity, and outcome are essential prerequisites to adequately consider and allocate these resources, and thus to improve emergency medical care (Jennett, 1996).

We therefore conducted a 10 year epidemiological study to provide representative data on the significance of TBI on the incidence, injury severity, and outcome in a western European urban center of one million inhabitants, namely Cologne (Germany), using strictly defined stan-

dardized scoring systems for inclusion, injury severity and outcome at hospital discharge.

Materials and Methods

The design of the present study has been previously published by Bouillon and colleagues (Bouillon et al., 1999). Briefly, certain conditions encouraged us to initiate the present epidemiological study in Cologne (Germany). First of all, the city of Cologne has a population of approximately one million inhabitants which meets the requirements of an urban area. Subsequently, Colognes pre-hospital emergency medical care system has a central organization in which all severely injured patients are seen by experienced physicians at the injury scene. Since 1987, all emergencies are prospectively documented by the physician involved on a standardized pre-hospital record (Bouillon et al., 1990). The central organization as well as the prospective documentation of all relevant data using standardized documentation sheets allowed the identification of potentially brain injured patients at the scene and ensured a reliable collection of all the necessary data for the study.

The Cologne Emergency Medical Care System

The treatment of trauma patients including TBI begins at the site of injury. The emergency call reaches the central Cologne Emergency Dispatch Center, that coordinates medical emergencies. Emergency physicians are dispatched if the patient's vital functions are possibly impaired or endangered. Following stabilization at the scene by the emergency physician patients are transferred to the most appropriate hospital that meets the diagnostic and therapeutic needs according to suspected injuries. In case of suspicion of TBI the patient is routinely admitted to one of the two designated trauma centres of the region with 24h availability of computed tomography (CT) and neurosurgical care. These two centres are level I trauma centers according to the criteria of the American College of Surgeons (ACS). During the 10 year study period (January 1, 1990 – December 31, 1999), more than 100 emergency physicians (70% surgeons, 20% anesthesiologists, 10% others) and 1000 paramedics served this emergency care. One helicopter, five physician-staffed emergency vehicles (NEF), and 23 paramedic-only-staffed vehicles (RTW) cover a core area of 405 km^2 with an average population of approximately one million people. The five NEFs and the helicopter served approximately 13,000 and 1,000 emergency missions per year, respectively. The Cologne Emergency Medical Care System works 24/7 a week all year round on a rendez-vous-system basis. The responsibility for further treatment was taken by the staff on duty in each trauma center.

Documentation

Based on preliminary studies, a standardized procedure has been implemented to precisely document the prehospital course of each patient beginning at the site of injury. Since January 1, 1987, a standardized documentation sheet is mandatory for each emergency physician on duty; meanwhile, it has reached a high level of acceptance with a completion rate of > 90%. This documentation sheet is similar to the Germany-wide well-accepted DIVI emergency documentation sheet (Moecke et al., 1994) and includes two scoring systems: (1) the Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974) to assess the neurological state, and (2) the Trauma Score (TS) (Champion et al., 1981; 1989) to estimate the physiological response to severe trauma. It contains information on the patient's age, gender, mechanism of injury, initial vital status, suspected diagnosis, prehospital monitoring and care, triage, and

early time course after the injury (Bouillon et al., 1997). It further provides space to document specific measures in case of complications. This procedure ensures that data collection begins as early as possible. One prehospital documentation sheet was added to the patient's files while a copy was archived at the emergency vehicle's base. The latter was used in this study for the identification of TBI cases. Information about the hospital course, including diagnostics, interventions, length of intubation, length of stay and outcome at discharge [mortality, Glasgow Outcome Scale (GOS)] (Jennett and Bond, 1975), was extracted from hospital records. All prehospital and hospital records were reviewed by two independent research assistants.

Assessment of relevant traumatic brain injury

TBI was assessed and graded using the GCS and the Abbreviated Injury Scale for head injuries (AIS_{head}). A GCS score of 13–15 is considered as mild TBI, which means in most cases a concussion with full neurological recovery, although many patients present with short-term memory and concentration deficits (Rimel et al., 1981). Patients with a GCS score of 9–12, moderate TBI, are lethargic and stuporous. Comatose (GCS score ≤ 8) patients, unable to open their eyes or to follow specific commands, are regarded as having severe TBI (Teasdale and Jennett, 1975). The AIS_{head} is another commonly used instrument for the categorization of injury severity based on anatomical features using a 6-grade scale system (Walder et al., 1995). AIS_{head} scores ≥ 2 are considered moderate while scores ≥ 3 are severe to life-threatening. The AIS is further used to calculate other scoring systems, for example, the Injury Severity Score (Osler et al., 1997). In the present study, relevant TBI was assumed when the initial GCS score was ≤ 8 or the AIS_{head} score was ≥ 2. Relevant (AIS score ≥ 2) and severe (AIS

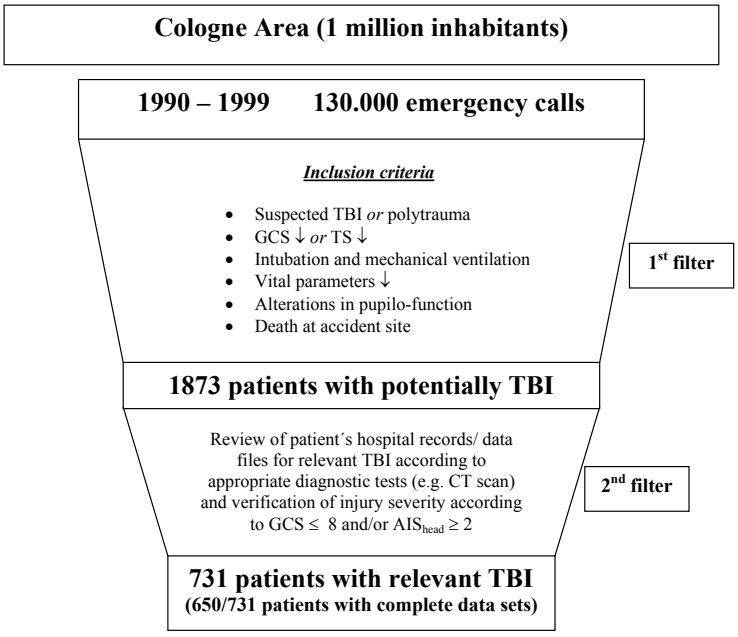

Figure 1. Filter criteria to identify patients with relevant TBI.

score ≥ 3) concomitant injuries of the extremities, abdomen, and thorax were graded accordingly. Pre- and in-hospital treatment strategies of TBI patients included in the present study principally followed the guidelines for the management of severe head injury as published by the Brain Trauma Foundation, the American Association of Neurological Surgeons, and the Joint Section on Neurotrauma and Critical Care, published in the Journal of Neurotrauma in 1996, with an update in 2000 (Brain Trauma Foundation 1996, 2000). These guidelines are endorsed by the World Health Organization's Committee on Neurotraumatology.

Data Collection

All prehospital records completed by the on-the-scene emergency physician between January 1, 1990, and December 31, 1999, were reviewed for eligibility. Data collection was performed both retrospectively (January 1, 1990, to June 30, 1996) and prospectively (July 1, 1996, to December 31, 1999) as part of a region wide joint research project funded by the German Ministry for Research and Education (BMBF-Verbundstudie 'Neurotrauma NRW'). As the corresponding procedures and instruments for data collection were applied during both phases, data quality was constant. To distinguish TBI from the high number of patients having sustained other emergencies, two filter systems were applied (fig. 1). At first, broad initial screening criteria were set as follows: (1) suspected TBI or polytrauma, (2) GCS score ≤ 12, (3) TS ≤ 14 (Teasdale and Jennett, 1974; Champion et al., 1981, 1989), (4) prehospital intubation and mechanical ventilation, (5) marked reduction in vital parameters and/or at pupil assessment, and (6) death at the scene. In the presence of at least one criterion, the patient was considered for detailed evaluation. These initial screening criteria were intentionally wide-ranging in order not to miss any patients with relevant TBI. In the second filter, hospital records of potentially eligible patients were reviewed. Only those patients in whom a relevant TBI was confirmed via appropriate diagnostic tests (e.g., CT) and who fulfilled the criteria of a prehospital GCS score ≤ 8 or an AIS_{head} score ≥ 2 were finally included.

Data Analysis

After verification, the information was recorded in a database and analyzed anonymously. As we conducted an area-based analysis, only those patients whose accident occurred within the Cologne city limits were eligible. A univariate analysis of demographic data was performed. Incidence rates and occurrence of events were calculated. Data were analyzed using SPSS 11.0 statistical software.

Results

Incidence of relevant TBI

Between January 1, 1990, and December 31, 1999, approximately 130,000 emergency missions were documented by the Cologne Emergency Medical Care System, leaving the same number of prehospital records to be reviewed. 1,873 patients passed the first filter (fig. 1), and 1,101 patients passed the second filter. Seven hundred and thirty-one patients sustained their injury within the city limits of Cologne. Based on these data, the annual incidence for relevant TBI (GCS score ≤ 8 or AIS_{head} score ≥ 2) in Cologne was estimated to be 7.3/100,000. Complete prehospital and in-hospital data sets were available from 650 patients (89%); these data were selected for further analysis.

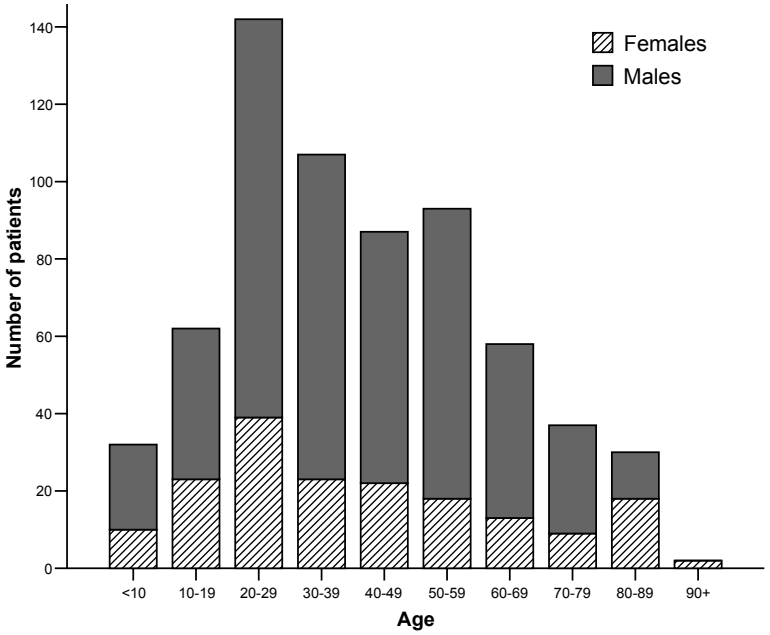

Figure 2. Distribution of age and gender among the study population (n = 650).

Patient Characteristics

The majority of patients who sustained TBI was male (73%, n = 473) and the average age was 40.3 years. A detailed distribution of age is shown in figure 2 . The mean age in the second half of the study (1995–1999) was 2 years higher ($p < 0.001$; t test). The percentage of patients over 60 years of age was 16% during the first year of the study versus 28% during the last year of the study.

Mechanism of Injury

In 617 cases (94.9%), the type of injury was blunt, while 33 cases (5.1%) sustained a penetrating trauma. Eighty cases (12.3%) resulted from suicide attempts. The predominant cause of injury was traffic-related (55.3%), followed by high (> 3 m, 22.8%) and low falls (< 3 m, 12.2%). Among the traffic victims (358/650 = 100%), 142 (39.6%) were pedestrians, 115 (32.1%) car or truck drivers, 63 (17.6%) cyclists, and 38 (10.7%) motorcyclists. There was no change with respect to mechanism of injury over the 10-year study period.

Scores

TBI patients transfered to a hospital had a mean GCS score of 8 ± 4; those who died at the scene (n = 182; 28%) had a GCS score of 4 ± 1. Among the 444 patients sustaining a TBI graded $AIS_{head} \geq 2$, 104 (23.5%) patients were graded $AIS_{head} = 2$. The remaining 340 (76.5%) patients sustained a severe TBI as graded $AIS_{head} \geq 3$.

Concomitant Injuries

Among patients in whom diagnostics could sufficiently be completed upon hospital admission (n = 451, preclinical deaths excluded), a significant number sustained concomitant in-

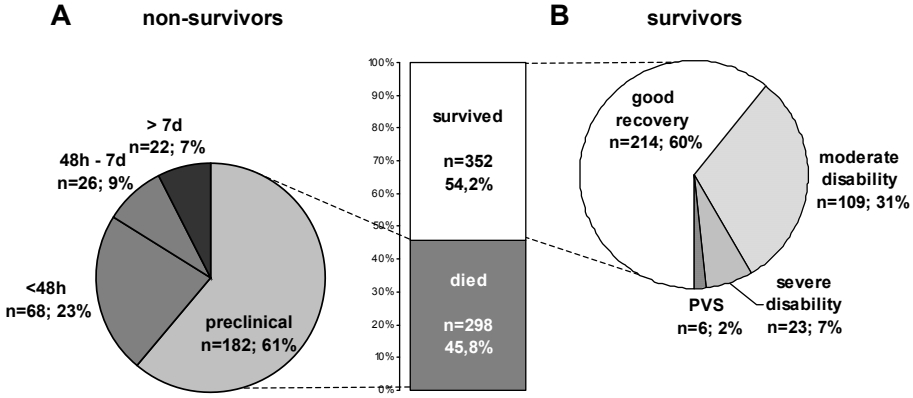

Figure 3. Mortality and outcome from TBI (n = 650). (**A**) Nonsurvivors are classified according to time until death. (**B**) Survivors are presented according to the GOS. PVS = Persistent vegetative state.

juries. As expected, concomitant injuries were observed most frequently in the extremities (37%; $AIS_{ext.}$ score ≥ 2); 20% of these injuries were considered severe ($AIS_{ext.}$ score ≥ 3). Twenty-four percent sustained relevant thoracic injuries, of which 20% were severe. Relevant concomitant injuries of the abdomen were observed in 11%, of which 8% were severe. Overall, in more than half of the patients (52%), at least one relevant concomitant injury was observed. Thirty-four percent sustained at least one concomitant injury graded as severe (AISscore ≥ 3).

Length of Intubation and Intensive Care Unit/Hospital Stay

TBI patients who were admitted to a hospital remained intubated and mechanically ventilated for 5.8 ± 10 days. They stayed in the intensive care unit for 8.9 ± 13 days, and the overall length of stay in the hospital was 18.2 ± 22 days.

Mortality and Outcome

The overall mortality rate for the study population was 45.8% (n = 298). Prehospital deaths occurred in 182 patients (61%), whereas 116 patients (39%) died in hospital. This corresponds to an overall in-hospital mortality rate of 24.8%. A significant number of patients died within 48 h upon admission (n = 68; 23% of all deaths), while 26 (9%) died within days 3–7 after admission. The remaining 22 nonsurvivors (7%) died later (fig. 3). Among the 352 surviving patients, 214 (60%) were sufficiently rehabilitated according to the GOS score at discharge, while 138 patients (40%) survived but with persisting deficits of various degrees (fig. 3).

Discussion

We present data over a 10-year study period from an urban area of approximately one million inhabitants in western Europe on the incidence, injury severity, and outcome of patients sustaining relevant TBI. 130,000 prehospital emergency protocols from the Cologne Emergency Medical Care System (Germany) were screened.

The number of patients we included into the present study per year was consistent over the

entire study period. Fifty-one percent of cases occurred during the first 5 years of the study (1990–1994). The annual incidence of relevant TBI for the Cologne area, a typical urban area in western Europe of one million inhabitants, was found to be 7.3/100,000 inhabitants. This rate is low compared to other reports. In the literature, the annual incidence rates for TBI range between 13.6 and 300/100,000 (Annegers et al., 1980, MacKenzie et al., 1989, Tiret et al., 1990, Annoni et al., 1991, Kraus, 1992, Hartwig et al., 1993, Lehr et al., 1997, Tate et al., 1998, Bruns and Hauser 2003). Besides a general decrease in incidence, one reason for these discrepancies may have been a problem of TBI definition (Engberg, 1995, Fearnside and Simpson, 1995, Jennett, 1996, Kraus and McArthur, 1996, Bouillon et al., 1997, Ingebrigtsen et al., 1998). Lack of consensus on injury classification led to considerable variability in defining inclusion criteria and subsequently to difficulties in comparing study results. Nevertheless, it appears that according to our definition (GCS score ≤ 8 and/or AIS_{head} score ≥ 2), the incidence of relevant TBI is lower than expected. For further studies and better comparability of results, the use of common criteria and scoring systems to precisely define TBI is highly recommended. Tagliaferri et al. (2006) noted critical differences in methods employed in the past 20 years across several reports on the epidemiology of TBI in Europe resulting in considerable difficulties to reach a consensus on all epidemiological findings across the 23 published European studies to date. In conclusion, these authors highly recommended the development of research guidelines to standardize definitional, case finding, and data reporting parameters to help establish a more precise and hence more useful description of the epidemiology of TBI in Europe.

Another point of discussion with respect to the lowincidence reported here is related to the fact that the present study only included relevant TBI that occurred inside the 405 km^2 Cologne area. It might be possible that a relatively large number of Cologne citizens sustained a relevant TBI outside the area and thus may have been missed in the present study. TBI sustained outside the urban area will probably represent the majority of high-velocity injuries. Conversely, noninhabitants of this area if injured within the Cologne city limits were included. Therefore, it needs to be emphasized that the authors of the present study did not follow the urban population of one million inhabitants but the incidence of relevant TBI within a typical western European urban area of one million inhabitants.

TBI is predominantly the injury of young males. Our data confirm previous reports regarding gender and age (Farin et al., 2003). This can be mainly attributed to a higher level of risk tolerance. Considerable differences in patient characteristics and case management between various geographical regions, reflecting variations in social, cultural, and organizational aspects have been reported (Hukkelhoven et al., 2002). Considering the distribution of age within our study population, a second peak was noted for the group between 50 and 59 years of age, but far less pronounced as compared to the younger age groups. In most other epidemiological studies on TBI, a second peak in the elderly, mostly due to falls, is observed (Sundstrom et al., 2007). As our approach within the given study was to analyze relevant TBI based upon prehospital emergency system documentation, minor to moderate falls in the elderly may have been missed.

With respect to cause of injury, it was striking that only 55% of TBI were related to traffic accidents, whereas 45% had other causes. Percent ranges of 48–57% for traffic-related TBI have been published previously (Kraus et al., 1984, Sosin et al., 1989). This is of great significance as TBI to date has mostly been considered and thus investigated in the context of traffic victims. It is likely that only about half of the affected population has thus been recognized

and studied in other investigations.

The overall mortality rate of 45.8% reported in our study appears to be higher as compared to the literature (Foulkes et al., 1991, Chesnut et al., 1993, Winchell and Hoyt, 1997), although there are reports of similar (Tiret et al., 1990) or even higher (Annegers et al., 1980; Kraus 1992) overall mortality rates associated with TBI. One aspect may be related to our very thorough and strict data inclusion from the scene of the accident until the in-hospital phase. An important difference to many other studies is the inclusion of all prehospital deaths. The possibility exists that some prehospital deaths might have involved brain injury but that TBI was not the primary cause of death. In our study, the occurrence of relevant concomitant injuries was 52%. This is in accordance with previous findings (Bouillon et al., 1992; Genarelli et al., 1994; Meixensberger and Roosen, 1998). In-hospital mortality ranges between 20 and 30% according to the literature (MacKenzie et al., 1989, Tiret et al., 1990, Annoni et al., 1991, Tate et al., 1998). In the present study, a similar in-hospital mortality rate was noted (24.8%). In view of prehospital versus in-hospital mortality, we found a similar ratio to Tiret et al. (1990) who applied a comparable approach. In both studies, the prehospital mortality rate was higher than the in-hospital rate.

Early mortality is an important matter since the majority of all deaths occur within 48 h following the accident (Tiret et al., 1990, Lieberman et al., 2003). A well-functioning emergency system, in which the time course of prehospital care is limited to < 45 min, was accountable for the fact that a substantial number of severely brain-injured patients reached the hospital alive. An organized emergency medical services system has been shown to substantially improve outcome following TBI (Colohan et al., 1987, Roy, 1987, Sampalis et al., 1995, Mullins et al., 1996, Watts et al., 2004, Klemen et al., 2006). We have to emphasize that the present study was undertaken in an urban area in western Europe with a highly developed emergency medicine system; in rural areas, the situation may be different due to a longer prehospital period.

Combining the annual incidence of severe TBI (7.3/100,000) with the overall mortality rate of 45.8% from severe TBI reported here, an annual mortality rate of 3.3/100,000 inhabitants can be calculated. This rate is low compared to other reports. For example, Kraus et al. (1984) reported a mortality rate of 13.5/100,000 from the San Diego area (United States) while Sundstrom et al. (2007) reported median TBI death rates for the four Scandinavian countries to range between 9.5 and 21.2/100,000 per year. More recently, Tagliaferri et al. (2006) systematically reviewed brain injury epidemiology from 23 European studies and reported an average mortality rate of approximately 15 per 100,000, but there were also considerable difficulties to reach consensus on all epidemiological findings across these studies due to critical differences in methods and classifications employed. The low annual mortality from relevant TBI reported here may be attributed to our strict inclusion criteria as well as to the inclusion of brain injuries that had occurred inside the 405 km^2 Cologne area only.

Among surviving patients, the average length of intensive care unit and total in-hospital stay was 8.9 and 18.1 days, respectively. These data correspond to previous reports (Vitaz et al., 2003). More than half of the survivors were successfully rehabilitated at discharge, whereas one third remained disabled, while only a few patients remained in a persistent vegetative state (1.4%). Similar observations have previously been reported (Hoffmann et al., 2002, Pfenninger and Santi, 2002, Rudehill et al., 2002, Vitaz et al., 2003).

Apart from age, mechanism of injury, and pathologies evolving from the impact (Gennarelli et al., 1982, Changaris et al., 1987, Mazaux et al., 1997, Asikainen et al., 1998, 1999a,b,

McCleary et al., 1998, Satz et al., 1998a,b, Geraldina et al., 2003, Mazzini et al., 2003, Chamelian et al., 2004, Demetriades et al., 2004a,b, Levin et al., 2004), the initial GCS score on admission is a strong predictor for mortality and functional outcome (Changaris et al., 1987, Levin et al., 1991, Asikainen et al., 1996, 1999a, Hoffmann et al., 2002; Demetriades 2004a,b). For example, a GCS score of 3 due to head trauma is associated with a high mortality rate (70%) (Liebermann et al., 2003); in our study, this was 92.5%. Comparable data exist for the AIS head (Walder et al., 1995; Demetriades et al., 2004a,b). Similarly, in the present study, both scores, the initial GCS and the AIS head scores, were associated with the GOS. Surprisingly, there is no good correlation between GCS and AIS head themselves (Demetriades et al., 2004a), indicating that both measures will be useful in future studies conducted on therapeutic strategies and their outcome.

The present study is the first of its kind to provide substantial data on the incidence, magnitude and outcome of TBI from an urban area of approximately one million inhabitants in western Europe over a 10-year period. The study was conducted in an environment with an organized emergency medical services system including designated trauma facilities, 24-hour availability of CT, full neurosurgical care including intracranial pressure monitoring, and experienced critical care management. Our data are similar to previous findings in other areas of the western industrialized society, except for the prehospital mortality rates, which is probably due to the inclusion criteria. The authors believe that these inclusion criteria demonstrate a more complete view on the epidemiology of TBI in urban areas. Still, optimizing pre- and intrahospital care and management remains the major challenge in preventing secondary brain damage which affects outcome and prognosis.

CHAPTER 2.

B) The Prognostic Value of Demographic Characteristics in Traumatic Brain Injury: Results from the IMPACT* Study

***International Mission on Prognosis and Analysis of Clinical Trials in TBI**

Abstract

Outcome following traumatic brain injury (TBI) is not only dependent on the nature and severity of injury and subsequent treatment, but also on constituent characteristics of injured individuals. We aimed to describe and quantify the relationship between demographic characteristics and six month outcome assessed by the Glasgow Outcome Scale (GOS) after TBI. Individual patient data on age (N=8719), gender (N=8720), race (N=5320) and education (N=2201) were extracted from eight therapeutic Phase III randomized clinical trials and three surveys in moderate or severe TBI, contained in the IMPACT database. The strength of prognostic effects was analyzed with binary and proportional odds regression analysis and expressed as an odds ratio. Age was analyzed as a continuous variable with spline functions, and the odds ratio calculated over the difference between the 75th and 25th percentiles. Associations with other predictors were explored. Increasing age was strongly related to poorer outcome (OR 2.14; 95% CI 2.00-2.28) in a continuous fashion that could be approximated by a linear function. No gender differences in outcome were found (OR: 1.01; CI 0.92-1.11), and exploratory analysis failed to show any gender/age interaction. The studies included predominantly Caucasians (83%); outcome in black patients was poorer relative to this group (OR 1.30; CI 1.09-1.56). This relationship was sustained on adjusted analyses, and requires further study into mediating factors. Higher levels of education were weakly related to a better outcome (OR: 0.70; CI 0.52-0.94). On multivariable analysis adjusting for age, motor score and pupils the prognostic effect of race and education were sustained. We conclude that outcome following TBI is dependent on age, race, to a lesser extent on education but not on gender.

Introduction

Outcome following traumatic brain injury (TBI) is dependent on many factors including demographic characteristics. Access to the IMPACT database (Marmarou et al., 2006) permitted us to analyze the prognostic value of age, gender, race and education. Age is one of the strongest and best documented predictors in TBI, with a large body of evidence demonstrating that outcome is poorer with increasing age. Uncertainty exists about the nature and shape of the association between age and outcome. Most studies categorize age or report on threshold values. (Hukkelhoven et al., 2003) investigated the relationship between age and outcome in more detail in a meta-analysis of the literature and of individual patient data. They found a continuous association and argued that reported threshold values might have been artefactual due to the typically skewed age distribution in TBI. Considerable preclinical and clinical interest exists in gender issues related to TBI, particularly in relation to hormonal influences and possibilities for hormonal treatment. This interest is in part socially motivated (adequate representation, equal rights) and in part scientific. Anatomical dimorphism and hormonal influences have been implicated as factors involved in possible gender-related differences in outcome. Whether gender differences in outcome actually exist remains debat-

able. (Groswasser et al., 1998) describe better overall responses to rehabilitation therapy in females when controlling for age and severity. Other authors (Klauber et al., 1981, Kraus and Nourjah, 1988) describe a higher mortality in females. A meta-analysis (Farace and Alves, 2000) found on average poorer outcome in females on primarily subjective outcome measures, and concluded that adjusted outcome analysis with gender as a covariate should be conducted in clinical trials in TBI. This statement may be considered strong in relation to the uncertainties which exist on the effect of gender on outcome after TBI.

Even less is known about associations between race and outcome after TBI. Within the USA, African Americans reportedly have a 35% higher incidence of TBI than Caucasians (Jager et al., 2000), but to our knowledge no studies have addressed the relationship between race and outcome assessed with the GOS, following severe and moderate TBI. Racial differences in outcome deserve consideration and are particularly relevant to clinical trials in traumatic brain injury, as metabolic pathways and consequently responses to injury and drug pharmacokinetics may differ due to biological differences.

A demographic factor that, unlike gender and race, is not intrinsic to the individual is the level of education. Educational level may be related to outcome after TBI due to interactions with cause of injury and differences in socio-cultural and socio-economical status influencing access to health care facilities.

The aim of our study was to describe and quantify the relationship between demographic characteristics and six month outcome after TBI. Specific objectives were to determine whether the use of threshold values for describing the relationship between age and outcome is appropriate, and to investigate whether any gender effects at different ages could be identified.

Methods

Patients and data collection

Individual patient data on demographic characteristics were included in the eight randomized controlled trials and three unselected prospective surveys assembled in the IMPACT* data base (Marmarou et al., 2006). We selected patients with complete data on outcome (N=8721). In six studies the actual date of birth was recorded, permitting calculation of age. In four studies (UK4, EBIC, HIT I, HIT II) age was recorded in years. For the purposes of analysis we calculated age in years, rounded to whole numbers for all studies. Gender was recorded in all studies as a binary variable. Data on race were available in five RCTs (TIUS, TINT, PEG, SKB, SAP) and in one of the prospective series (TCDB). All studies recorded the main differentiation: Caucasian/ Black/Asian/other. Some studies included differentiation of the category "other", for instance into American Indian or Alaskan. This differentiation was not included in our analysis. Data on education were available in three studies (TIUS, TINT and TCDB). The codings for education differed between the Tirilazad trials and the TCDB. We recoded education into the categories: zero to 8 years, 9 to 12 years, 13 years or more. The primary endpoint for prognostic analysis was the Glasgow Outcome Scale (GOS) as recorded in the studies. If the 6 month GOS was missing we imputed the three month GOS (N=1613, including 1510 patients from the PEGSOD trial).

Statistical analysis

Descriptive analyses included the calculation of the median and interquartile ranges for age, and of the male/female ratio per study. The prevalence of racial distribution and educational

categories was derived per study. The relationship between demographic characteristics and outcome was first analyzed with cross tabulation. The shape of the relationship of age to outcome was further examined with restricted spline functions. These functions are smooth and flexible, allowing for an adequate description of non linear relationships.
Logistic regression models were applied to quantify the predictive strength of the demographic characteristics. In these regression models the GOS was dichotomized in four different ways: good recovery vs. less than good recovery, favorable outcome vs unfavorable outcome, conscious survival vs. death/vegetative state and survival vs. death. We also considered an ordinal analysis with proportional odds methodology which reflects prognostic effects across the various GOS categories (McHugh et al, 2006). Data from all of the studies were pooled using a random effects model. The strength of the associations was expressed using odds ratios with 95% confidence intervals.
Exploratory analyses of clinically meaningful associations with other predictors were performed, and included adjusted analysis for the association between race and outcome.
Finally, multivariate analysis was performed, adjusting for the three main clinical predictors: age, motor score and pupillary reactivity. This analysis was restricted to patients > 14 years of age.

Results

Descriptive analysis

The availability and distribution of demographic characteristics within and across studies is summarized in Tables 1 and 2. The median age between studies varied from 26 to 37 and was in general higher in the European studies compared to the primarily North American studies.

Table 1. Descriptive statistics for age and gender

		Age (N=8719)			Gender (N=8720)	
Study	**N**	**N (%) available**	**Median**	**IQR**	**N (%) Available**	**% Male**
TCDB	604	604 (100)	26	(21-40)	604 (100)	77
UK4*	986	986 (100)	29	(17-51)	986 (100)	75
HIT I	350	350 (100)	34	(21-47)	350 (100)	84
HIT II	819	819 (100)	33	(22-49)	819 (100)	77
TIUS	1042	1041 (100)	30	(23-41)	1041 (100)	78
TINT	1121	1121 (100)	30	(21-45)	1121 (100)	76
PEG	1510	1510 (100)	27	(20-38)	1510 (100)	77
EBIC	835	834 (100)	37	(23-58)	834 (100)	75
SLIN	409	409 (100)	28	(21-43)	409 (100)	78
SKB	126	126 (100)	27	(20-39)	126 (100)	76
SAP	919	919 (100)	32	(23-47)	919 (100)	80
Overall^	8721	100	30	(21-45)	100	77

a) This study included 162 children; on exclusion of patients <14 years of age, the median age is 36 (IQR: 22–55), b) the summary data presented as overall are simple totals rather than any more sophisticated pooled estimate. IQR, Interquartile range.

We found a clear male excess in the overall population (average 77% male; range 75-84%). However, this excess was mainly due to the contribution of younger patients and the male/female ratio decreased to one above age 65 (Figure 1). Six studies reported the distribution of race, and within these studies data were available for virtually 100% of patients.
Most patients were classified as Caucasian (66-98%), with higher percentages being observed in the primarily European studies (TINT/SAP: 96-98%). Data on education were available for a total of 2201 patients over three datasets (TCDB, TIUS, TINT), but were missing in a considerable proportion of these patients (16-37%).

Table 2. Descriptive statistics for Race and Education

		Race (N=5320)					Education in years (N=2201)			
Study	N	N (%) available	Caucasian N (%)	Black N (%)	Asian N (%)	Other N (%)	N (%) available	<9	9-12	>12
TCDB	604	604 (100)	507 (84)	81 (13)	13 (2)	3 (<1)	381 (63)	19 (5)	271 (71)	91 (24)
TIUS	1042	1040 (100)	687 (66)	149 (14)	35 (3)	169 (16)	872 (84)	59 (7)	525 (60)	288 (33)
TINT	1121	1121 (100)	1071 (96)	12 (1)	29 (3)	9 (<1)	948 (85)	249 (26)	423 (45)	276 (29)
PEG	1510	1510 (100)	1170 (77)	179 (12)	29 (2)	132 (9)	NA	-	-	-
SKB	126	126 (100)	93 (74)	17 (13)	0 (0)	16 (13)	NA	-	-	-
SAP	919	919 (100)	903 (98)	6 (<1)	10 (1)	0 (0)	NA	-	-	-

Demographic characteristics and outcome
The relationship between demographic characteristics and outcome is summarized in Figures 2 and 3. Figure 2 shows that the relationship between age and outcome is continuous for different points of dichotomization of the GOS. The spline functions can be well approximated by a linear function, but indicate a possible change point for the splits for mortality and death/vegetative at around age 35.
Figure 3 demonstrates a similar distribution of GOS categories for each gender. We further explored a possible gender/age interaction with outcome. Figure 4 demonstrates the spline function for the probability of outcome differentiated by age for males and females separately. This graph shows a very weak trend towards more favorable outcome in female patients at older ages but the differences were not statistically significant, with widely overlapping confidence intervals. We conclude that no clear evidence was found for a gender effect on outcome in TBI.
Table 3 summarizes the odds ratios calculated in binary and proportional odds analyses across the studies. We found a strong prognostic effect of age (odds ratio 2.14; 95% CI 2.00-2.28), a clear prognostic effect of race (odds ratio for black patients relative to caucasians: 1.30; 95% CI (1.09-1.56) and a weak effect of education (odds ratio for >12 years relative to 0 to 8 years 0.70; 95% CI 0.52-0.94).
No gender effect was detected. The results were consistent across studies (Figure 5). On multivariate analysis, adjusting for age, motor score and pupillary reactivity, the prognostic effects observed in univariate analysis were sustained (Table 3).
Significant differences were observed in the outcome distribution differentiated for race: out-

come was poorer in black patients with increased morality and a lower percentage of favorable outcome (Fig 3; p=0.03). The number of Asian patients included in the various studies was relatively low (N=116) and no differences in outcome compared to Caucasians were found.

We explored various factors to investigate whether they might explain the poorer outcome in black patients and found that assaults were more common as the cause of injury (20%) compared to Caucasians (3%) and that more black patients had a motor score of 1 on admission (23% vs 15%). The prognostic effect of race remained on adjustment for cause of injury (OR 1.31;CI 1.08-1.57) and for age, motor score and pupils (Table 3).On further multivariable analysis, adjusting for seven clinical predictors the strength of the association increased (OR 1.45; 1.07-1.96) (Murray et al., 2006).

A weak relationship was found between education and outcome: patients with higher levels of education showed better outcome (Figure 3). Although weak, this relationship was sustained on adjusted analysis (Table 3).

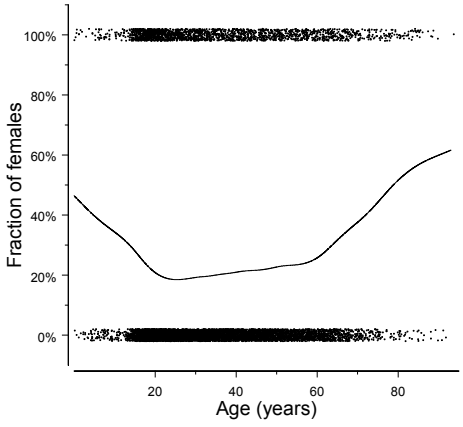

Figure 1. Gender ratio in relation to age. The horizontal bars show the population density for different ages (top, females; bottom, males).

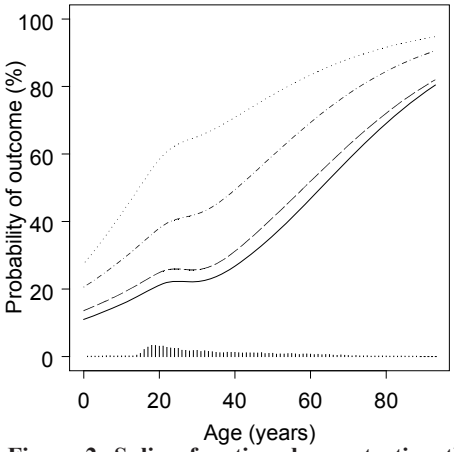

Figure 2. Spline function demonstrating the continuous relationship between age and outcome. _____, probability of mortality; - - -, probablity of death/vegetative; -.-, probability of unfavorable outcome; . . . ., probability of less than good recovery.

Secondary analysis

Exploratory analysis of clinically meaningful associations between demographic characteristics and other predictors was performed. Results are reported in more detail in subsequent manuscripts (Butcher et al., 2006; Maas et al., 2006). In brief, the age distribution differed per cause of injury. Falls and work-related injuries occurred predominantly in older patients.

Assaults as cause of injury were more common in blacks, road traffic accidents occurred more in patients with higher levels of education and falls were more frequent in patients with lower levels of education.

Increasing age was also associated with more mass lesions, particularly the occurrence of

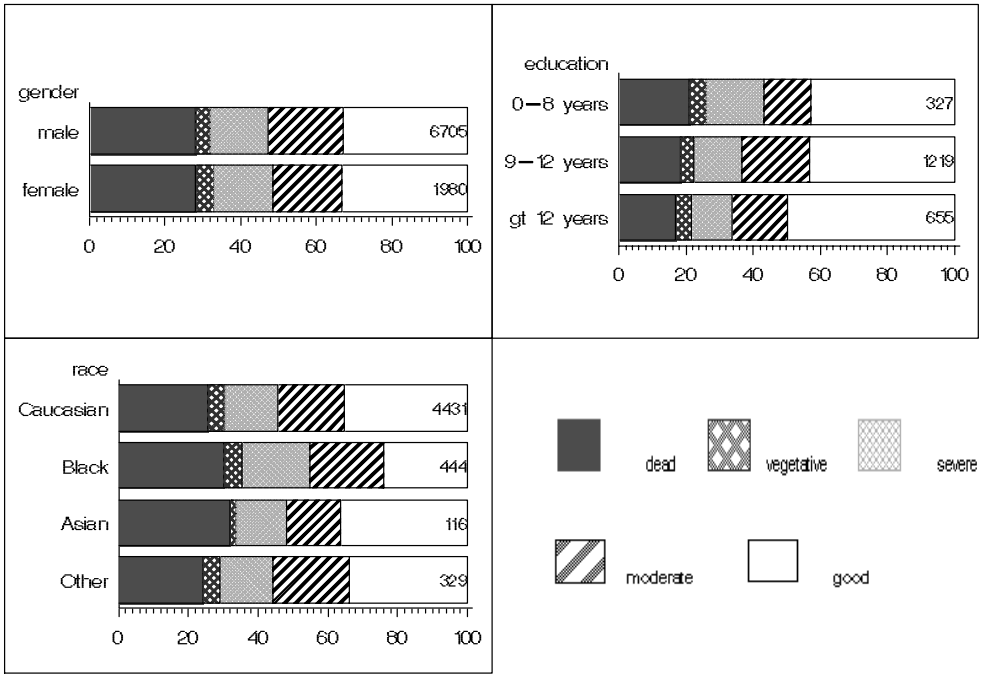

Figure 3. Bar charts illustrating the distribution of the 6-month Glasgow Outcome Scale (GOS) differentiated for gender, race, and education.

acute subdural hematomas. No age-related differences were found for patients with an epidural hematoma or a contusion on the CT scan.

Discussion

We found that age, race and education, but not gender are associated with outcome after moderate and severe TBI. These findings emphasize the importance of demographic characteristics in predicting outcome and underscore the opinion that each patient's personal characteristics at the time of injury need to be taken into account when making statements about outcome (Wagner et al., 2000). Consideration of these variables is particularly relevant to trials in the field of TBI as gender, race and age may also influence drug metabolism.

Age

Our study investigating the relationship between age and outcome in the largest database of TBI patients with severe and moderate head injury assembled to date confirms that this relationship is continuous, in agreement with previous studies (Balestreri et al., 2004, Combes et al., 1996, Ellenberg et al., 1996, Gomez et al., 2000, Hukkelhoven et al., 2003, Lannoo et al., 2000). The strong relationship between age and outcome in TBI has been demonstrated in many prognostic studies. Most of these studies have documented threshold values varying from 30 to 60 years of age, whilst others described a stepwise categorical relationship (Table 4). We found that the continuous relationship holds across the different points of dichotomization for the GOS and is further confirmed by the proportional odds analysis. The relationship can be well approximated by a linear function, which we consider more appropriate and

informative than the stepwise categorical approach adopted in previous studies. The spline function analysis indicated a change point at approximately 30 years of age for the relationship between age and mortality, and change point analysis showed a marginally better performance than a linear fit. This contradicts a study by Signorini (Signorini et al., 1999) which reported a change point at age 50. The difference in change point can possibly be explained by the higher mean age (42 with a SD of 21) in the study reported by Signorini et al.
The strength of the relationship between age and outcome found in our current studies (odds ratio 2.14) is greater than in the previous study from (Hukkelhoven et al., 2003) (odds ratio 1.49, 95% CI 1.43-1.56), although this study was based on four of the individual patient series included in the IMPACT database. This difference can be explained by different approaches to analysis. In our study the odds ratio was calculated for the shift in outcome between the 75th and 25th percentile age ranges (age 45/21) and in the study by Hukkelhoven per age decade. Consequently the age difference over which the odds ratio was calculated was larger in the present study, resulting in a higher odds ratio. A limitation of our study is that relatively few children are included in the IMPACT database, thus precluding strong statements on the association between age and outcome in the pediatric TBI population. For this reason also, we restricted the adjusted analyses to patients > 14 years of age.
The strong association between age and mass lesions, in particular acute subdural hematomas, has been described previously (Gan et al., 2004, Gomez et al., 2000, Mosenthal et al., 2002, Munro et al., 2002, Ono et al., 2001) and confirmed in our study. This observation has direct consequences for healthcare planning, particularly in relation to the increasing age of the population and the increasing incidence of TBI in the elderly population (Kannus et al., 2001, Luukinen et al., 1999).

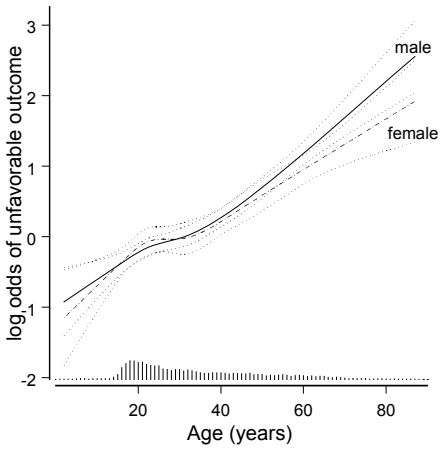

Figure 4. Spline function analysis demonstrating the relationship between age and unfavorable outcome differentiated for gender.

Gender
Our studies show gender-related differences in the incidence of TBI, but not in outcome. Many studies have reported on the increased risk of males to sustain TBI (Bayir et al., 2004,

Table 3. Strength of the associationos of demographic characteristics with Glasgow Outcome Scale (GOS) at 6 months after Traumatic Brain Injury

	Age (n=8719)	Gender (n=8720)	Race (n=5320)			Education in years (n=2201)	
		Female	Black	Asian	Other	9-12	> 12
Dichotomous OR	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Less than good recovery	2.15 (1.94-2.39)	0.98 (0.88-1.09)	1.41 (0.91-2.17)	1.01 (0.67-1.51)	1.12 (0.87-1.44)	0.88 (0.67-1.15)	0.72 (0.53-0.99)
Unfavorable vs favorable outcome	2.15 (2.00-2.31)	1.04 (0.93-1.16)	1.32 1.00-1.73)	1.15 (0.79-1.69)	1.03 (0.81-1.31	0.62 (0.37-1.03)	0.59 (0.39-0.91)
Death/vegetative Vs conscious survival	2.06 (1.92-2.21)	1.03 (0.93-1.15)	1.20 (0.97-1.49)	1.20 (0.75-1.91)	1.08 (0.83-1.40)	0.79 (0.58-1.07)	0.81 (0.58-1.12)
Death vs alive	2.08 (1.91-2.26)	1.01 (0.90-1.13)	1.24 (0.98-1.55)	1.42 (0.94-2.15)	1.15 (0.77-1.71)	0.92 (0.56-1.54)	0.87 (0.57 – 1.34)
Proportional OR							
Unadjusted analysis	2.14 (2.00-2.28)	1.01 (0.92-1.11)	1.30 (1.09-1.56)	1.09 (0.78-1.51)	1.08 (0.88-1.34)	0.78 (0.57-1.07)	0.70 (0.52-0.94)
Adjusted for age/ motor score/pupils*	-	0.94 (0.85-1.04)	1.44 (1.08-1.93)	1.22 (0.84-1.78)`	1.11 (0.89-1.40)	0.87 (0.67-1.13)	0.74 (0.56-0.97)

[a]Adjusted analysis restricted to patients ≥ 14 years of age.
Odds ratios are calculated for the GOS dichotomized as less than good recovery (D/V/SD/MD/ vs. GR), unfavorable outcome (D/V/SD vs. MD/GR), death/vegetative state versus conscious survival (D/V vs. SD/MD/GR) and death versus survival, as well aswith a proportional odds model, with their 95% confidence intervals. Reference categories: gender (male), race (Caucasian), education (< 9 years). The odds ratio for age was calculated on the shift in outcome between the 75th and 25th percentiles.

Groswasser et al., 1998, Hukkelhoven et al., 2003, Kirkness et al., 2004, Kraus and Nourjah, 1988, Pentland et al., 1986). Our studies indicate that young adult males are particularly at risk for TBI but that the male/female ratio declines with increasing age, reaching an approximate one to one ratio at ages over 65. Similar findings have been reported (Pentland et al., 1986) . The recent surge in interest in sex difference research has been stimulated by the realization that women had sometimes been excluded from drug studies because of fear that novel treatments could negatively affect their fecundity and because fluctuations in hormonal status could have undesirable influences on drug pharmaco-kinetics (Stein, 2005). In our current study we found a slight difference in the percentage of females included in the observational studies (24%) compared to the RCTs (22%), but this difference was not significant and can be explained by chance and by exclusion from trials for reasons of suspected pregnancy. Reports on gender-related differences in outcome after TBI have raised interest in hormonal

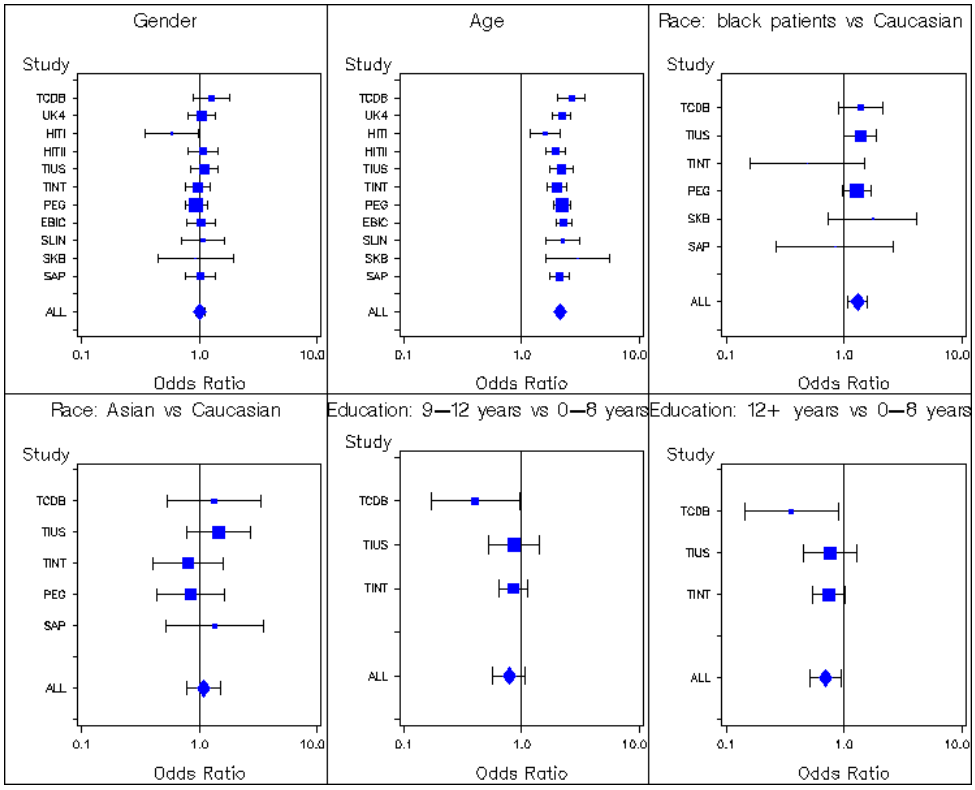

Figure 5. Forest plots demonstrating the consistency of the associations between demographic characteristics and outcome across studies.

influences and generated research into neuroprotective effects of estrogen and progesterone. Higher levels of estrogen relative to progesterone were considered as a possible explanation for the poorer outcome observed by Farin (Farin et al., 2003) in females under 50 years of age. The sample size in the study was very small however, and the conclusions therefore open to criticism. We did not find any evidence for a gender/age interaction on outcome. Only a few authors report better outcome in females (Groswasser et al., 1998). Most studies indicate poorer outcome in females (Bayir et al., 2004, Farace and Alves, 2000, Gan et al., 2004, Kirkness et al., 2004, Wagner et al., 2000). Our studies definitively show that no gender-related differences in outcome as assessed by the GOS exist in TBI. A limitation of our studies is that we could not analyze a possible relationship between the period of the menstrual cycle or use of oral anticonception in females on outcome, but the presence of such an influence is considered unlikely given the large number of patients included in our studies.

Race

A surprising finding in our study was the higher risk for poorer outcome in black patients with TBI compared to Caucasians. This significant effect even increased in adjusted analyses. Two previous studies have similarly shown poorer outcome in black patients (Jorge et al., 1994, Wagner et al., 2000). Other studies however (Burnett et al., 2003, Schreiber et al., 2002) and

(Johnstone et al., 2003) did not find any differences in outcome related to race for a variety of outcome measures. Hart et al., 2005 (2005) found a significant lower social integration in African Americans following TBI, compared to Caucasians, but no differences in other outcome measures. The reason for poorer outcome in black patients observed in our study can only be speculated upon. Ethnic minorities have been associated with lower educational level (Harrison-Felix et al., 1998, Jorge et al., 1994), lower pre-morbid employment (Burnett et al., 2003, Wagner et al., 2000) and to increased drug abuse pre-injury (Wagner et al., 2000). Such associations are insufficient explanation for our findings. Differences may exist between lacks and whites in health perception and in methods for coping with negative life events (Yeates et al., 2002). These differences however, are more relevant to Health-Related Quality of Life measures than to the general functional outcome after TBI as determined by the GOS. The response to injury may be different in black patients due to biological differences, and access to acute (Bazarian et al., 2003) and postacute (Burnett et al., 2003) care may be more limited. We consider the poorer outcome observed in blacks an important area for future research, which should include disparities in care, and in particular any difference in acute care.

Education
Our study indicates a weak association between educational level and outcome. Higher educated patients (> 12 years) more often have a favorable outcome than lower educated patients. Similar results have been reported by (Wagner et al., 2000), noting that patients with less education than high school are 4.2 times less likely to achieve their best possible levels of functional recovery assessed by the disability rating scale. (Burnett et al., 2003) however did not find an influence of education on outcome after TBI when measured by discharge disposition and post injury employment.

Conclusions
Demographic characteristics are important in predicting outcome after traumatic brain injury. Age is strongly related to outcome in a continuous relationship that can be well approximated by a linear function. Threshold values do not exist. Clear gender differences exist in the incidence of TBI in younger patients, but these differences disappear on increasing age. Gender is not related to outcome as determined by GOS. Outcome is poorer in black patients, which cannot readily be explained by associations with other predictors. A weak association exists between educational level and outcome.

CHAPTER 2.

C) The Prognostic Value of Secondary Insults in TBI: Results from the IMPACT* Study

***International Mission on Prognosis and Analysis of Clinical Trials in TBI**

Abstract

We determined the relationship between secondary insults (hypoxia, hypotension and hypothermia) occurring prior - to or on admission to hospital and six month outcome after traumatic brain injury (TBI). A meta analysis of individual patient data, from seven Phase III randomized clinical trials (RCT) in moderate or severe TBI and three TBI population-based series, was performed to model outcome as measured by the Glasgow Outcome Scale (GOS). Proportional odds modeling was used to relate the probability of a poor outcome to hypoxia (N=5661), hypotension (N=6629) and hypothermia (N=4195) separately. We additionally analyzed the combined effects of hypoxia and hypotension and performed exploratory analysis of associations with CT classification and month of injury. Having a pre-enrollment insult of hypoxia, hypotension or hypothermia is strongly associated with a poorer outcome (odds ratios of 2.1 95%CI (1.7-2.6), 2.7 95%CI (2.1-3.4) and 2.2 95%CI (1.6-3.2) respectively). Patients with both hypoxia and hypotension had poorer outcomes than those with either insult alone. Radiological signs of raised intracranial pressure (CT class III or IV) were more frequent in patients who had sustained hypoxia or hypotension. A significant association was observed between month of injury and hypothermia. The occurrence of secondary insults prior-to or on admission to hospital in TBI patients is strongly related to poorer outcome and should therefore be a priority for emergency department personnel.

Introduction

Following an initial trauma the injured brain is vulnerable to secondary damage which may be exacerbated by secondary insults. For more than 20 years it has been reported that hypoxia and hypotension are associated with adverse outcome following traumatic brain injury (TBI) (Miller et al., 1978). Later reports have confirmed this finding (Manley et al., 2001, Walia and Sutcliffe, 2002), and Chesnut (1993a) reported the combined effect of hypoxia and hypotension as being substantially greater than the sum of their two effects. Hypothermia has also been shown to be associated with adverse outcome following TBI (Strachan et al., 1989).

Many of the published reports of the impact of secondary insults on outcome have concentrated on insults which develop while the patient is in the Intensive Care Unit. Indeed, one of the key principles of the modern management of severely brain injured patients is to reduce the risk of such insults occurring (EBIC/ABIC guidelines). The main focus of the IMPACT project (Maas et al., 2006b) is to improve the methodology for clinical trials in TBI, and so we have focused on insults which are present on the patient's admission to 'neuro' care. Such insults are still of great clinical relevance, and if they are confirmed to be related to adverse outcome then there is the potential to improve outcome by intervening to correct the abnormality and/or to interrupt the processes which lead from secondary insult to secondary brain damage.

We had the unique opportunity to perform analyses on data from 9205 patients in order to investigate the associations between hypoxia, hypotension and hypothermia and outcome measured on the Glasgow Outcome Scale (GOS) (Jennett and Bond, 1975).

Methods

Data on at least one of the secondary insults were available for 10 of the 11 studies in the IMPACT database (Marmarou et al., 2006). PEGSOD (Young et al., 1996) alone had no relevant data captured in the record forms. In general the insults were recorded as being absent, suspect or definite at or before admission. The classification of 'suspect' was based on a clinical impression, whereas the classification of 'definite' was based on an objective measurement of oxygenation (hypoxia), blood pressure (hypotension) or temperature (hypothermia). The definition for "definite" insults was consistent across studies: hypoxia was defined as an arterial pO2 < 60 mmHg, hypotension as a systolic blood pressure < 90 mmHg and hypothermia as < 35 °C. For all of the formal analyses the categories of suspect and definite were pooled, so that each insult was reduced to a binary absent/present categorization. The outcome examined in these analyses was the Glasgow Outcome Scale at six months. Where this was missing or systematically not recorded, the GOS at three months was substituted instead (102 cases). The strength of the association between each separate insult and outcome was analyzed univariately using binary and proportional odds regression models. For the binary models all four possible dichotomies were considered: death versus survival, death or vegetative state versus better, death, vegetative state or severe disability (conventionally termed 'unfavorable outcome') versus better and worse than good recovery versus good recovery. For the proportional odds analysis the GOS was modeled as a four point scale with death and vegetative state categories combined. Results were expressed as odds ratios for an adverse

Table 1. Availability and prevalence of Hypoxia, Hypotension and Hypothermia at admission for each study

		Hypoxia		Hypotension		Hypothermia	
Study	**N**	**Available N (% of total)**	**Prevalence N (% of available)**	**Available N (% of total)**	**Prevalence N (% of available)**	**Available N (% of total)**	**Prevalence N (% of available)**
TCDB	604	604(100)	109 (18.0)	604 (100)	143 (23.7)	NR	-
UK4	986	968 (98.2)	227 (23.5)	974 (98.8)	241 (24.7)	NR	-
HIT I	350	NR	-	342 (97.7)	17 (5.0)	NR	-
HIT II	819	NR	-	815 (99.5)	81 (9.9)	552 (67.4)	50 (9.1)
TIUS	1042	923 (88.6)	266 (28.8)	1013 (97.2)	224 (22.1)	941 (90.3)	157 (16.7)
TINT	1121	989 (88.2)	149 (15.1)	1091 (97.3)	155 (14.2)	1018 (90.8)	39 (3.8)
EBIC	835	830 (99.4)	236 (28.4)	829 (99.3)	201 (24.2)	827 (99.0)	51 (6.2)
SLIN	409	409 (100)	24 (5.9)	NR	-	NR	-
SKB	126	84 (66.7)	29 (34.5)	104 (82.5)	21 (20.2)	NR	-
SAP	919	854 (92.9)	110 (12.9)	857 (93.3)	128 (14.9)	857 (93.3)	111 (13.0)
All *	8721	5661	1150 (20.3)	6629	1211 (18.3)	4195	408 (9.7)

[a]The summary data presented as "all" are simple totals rather than any more sophisticated pooled estimates. NR, not recorded in study.

outcome when the insult was present relative to the insult being absent, with corresponding 95% confidence intervals. Odds ratios of greater than one indicate that an insult being present is associated with an increased risk of an adverse outcome. All analyses were performed for the individual trials/studies and an overall summary measure derived using random effects pooling (Taylor et al., 2006). Tests of heterogeneity were performed to assess consistency of effects across studies.

Given the suggestion in the literature that hypoxia and hypotension have a synergistic adverse impact on outcome, an additional analysis was performed where the two insults were combined into a single four category variable: neither hypoxia nor hypotension; hypoxia alone; hypotension alone; hypoxia and hypotension. To test for evidence of synergism a proportional odds model was fitted with main effects for hypoxia and hypotension together with their interaction.

Results

Descriptive Analysis

Table 1 shows the number and percentage of subjects with the three secondary insults recorded for each of the studies separately. Both the availability and prevalence in each study are shown. The presence of hypoxia was recorded in eight studies, and with the exception of SKB the data were relatively complete for each of these studies. The prevalence of hypoxia varied widely between the studies ranging from 6% in SLIN to 35% in SKB. Hypotension was recorded in nine studies. Again the data were virtually complete for these studies apart from SKB. The prevalence of hypotension ranged from 5% in HIT I to 25% in the UK4 centres study. Hypothermia was only recorded in five studies with the data being at least 90% complete for four of these five studies. One third of the data were missing for HIT II. The prevalence of hypothermia varied between four and seventeen percent.

Figure 1 shows the distribution of GOS with and without hypoxia, hypotension and hypothermia. It can be seen that many more subjects die and fewer have a good outcome when any of he secondary insults has occurred.

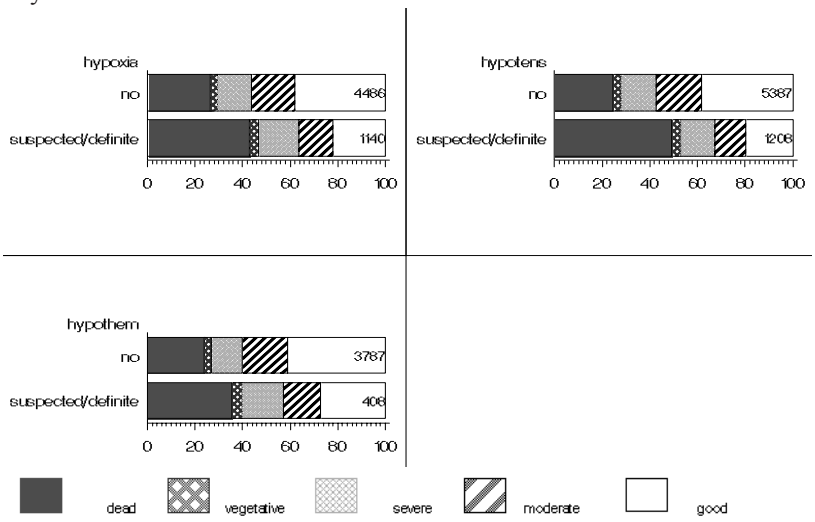

Figure 1. Bar charts showing the distribution of Glasgow Outcome Scale (GOS) with and without hypoxia, hypothermia, and hypotension.

Table 2. Summary of pooled univariate analysis of hypoxia, hypotension, and hypothermia

Model	Hypoxia	Hypotension	Hypothermia
	OR 95%CI	OR 95%CI	OR 95%CI
Dichotomous OR			
GOS D,V,S,M vs. G	2.13 (1.70,2.67)	2.43 (2.00,2.95)	2.09 (1.43,3.06)
GOS D,V,S vs. M,G	2.14 (1.72,2.67)	2.67 (2.18,3.28)	2.27 (1.66,3.11)
GOS D,V vs. S,M,G	2.08 (1.71,2.54)	2.68 (2.02,3.54)	2.13 (1.45,3.14)
GOS D vs. V,S,M,G	2.02 (1.61,2.55)	2.62 (1.99,3.47)	2.11 (1.35,3.30)
Proportional odds Unadjusted	2.08 (1.69,2.56)	2.67 (2.09,3.41)	2.21 (1.56,3.15)
Adjusted for age/ motor score/ pupils	1.65 (1.37,2.00)	2.06 (1.64, 2.69)	1.63 (1.11,2.40)

aAdjusted analysis restricted to age ≥ 14.
Odds ratio (OR) is calculated for the GOS dichotomized as less than good recovery (D/V/SD/MD vs. GR), unfavorable outcome (D/V/SD vs. MD/GR), death/vegetative state versus conscious survival (D/V vs. SD/MD/GR), and death versus survival, as well as with a proportional odds model, with their 95% confidence intervals.

Primary Analysis

Table 2 shows the odds ratios for each secondary insult and each possible dichotomy of the GOS, the unadjusted proportional odds model and the proportional odds model adjusted for age, motor and pupil scores. The dichotomous odds ratios are remarkably consistent however the GOS is grouped. This implies that the proportional odds model gives an excellent summary of the association between each secondary insult and the GOS (Taylor et al., 2006).

Hypoxia and hypothermia are both associated with the odds of an adverse outcome being approximately doubled. The impact of hypotension is even greater, with a common odds ratio from the unadjusted proportional odds model of 2.67. The odds ratios from the adjusted proportional odds model show that hypoxia, hypotension and hypothermia are independent predictors of an adverse outcome even when age, motor, and pupil scores are taken into account. Forest plots of the three secondary insults are shown in Figure 2. This shows excellent consistency in the estimated common odds ratios between the studies for hypoxia and hypothermia. There is more variation for hypotension but the variation only relates to the magnitude of the odds ratio. There is a very consistent pattern of the direction of the effect, namely that hypotension is associated with an increased risk of an adverse outcome.

Secondary analyses

The relationship between hypoxia and hypotension combined and outcome was studied as was the relationship between CT class and hypoxia and hypotension. The hypothesis that there is seasonal variation in the prevalence of hypothermia was also examined.

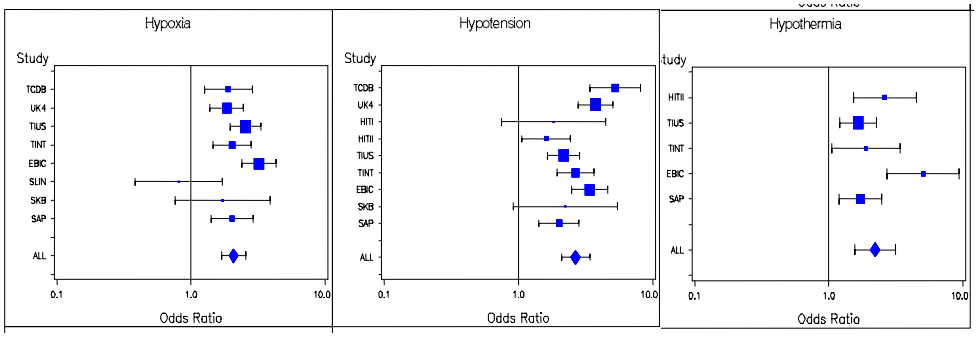

Figure 2. Forest plots of hypoxia, hypotension, and hypothermia.

Hypoxia and Hypotension. In those studies which measured both hypoxia and hypothermia subjects were categorized into four mutually exclusive groups: neither hypoxia nor hypotension; hypoxia and not hypotension; hypotension and not hypoxia and both hypoxia and hypotension. Table 3 shows the distribution of GOS for each of these groups. There is a clear gradation with outcome deteriorating as one moves from neither insult to hypoxia alone to hypotension alone to both insults together. However, the deterioration in outcome going from hypotension alone to hypotension plus hypoxia is less than the deterioration going from neither insult to hypoxia alone. The formal statistical modeling resulted in a borderline significant interaction between hypoxia and hypotension and GOS ($p=0.034$). The direction of the interaction was that the effect of the two insults together was sub-additive. That is to say the combined effect was less than the sum of the two effects.

CT characteristics, hypoxia and hypotension. The relationship between CT classification and hypoxia and hypotension was examined. Hypoxia and hypotension were both most prevalent in those with swelling/shift as shown in Table 4. However, multivariable regression showed that both hypoxia and hypotension remain powerful independent predictors of GOS even after adjustment for age, GCS motor score, pupil reaction and CT class (Murray et al., 2006).

Month of injury and hypothermia. Table 5 shows the anticipated strong seasonal effect for hypothermia, with a substantial increase in prevalence from October through to February. An association was also observed between the type of referral and the prevalence. The prevalence was 11% for primary referrals and 8% for secondary referrals (Chi-squared = 10.9, d.f. 1, $p<0.001$). No such association was observed for hypoxia or hypotension.

Discussion

Our results demonstrate that hypoxia, hypotension and hypothermia are all relatively common on admission, with an observed overall prevalence of 20%, 18% and 10% respectively. There was substantial variability from study to study in these prevalence figures. The variability will reflect differences in definitions and cutoffs and also, most likely, systematic differences in how rigorously investigators sought evidence of these insults having occurred. This latter point is supported too by the reduced prevalence of hypothermic insults recorded when patients were secondary referrals rather than direct admissions to the study hospital, reflecting quite possibly the loss of information between the receiving hospital and the study hospital.

The past 10 to 15 years have seen considerable advances in emergency care services and a decrease in incidence of secondary insults on admission might be expected. We did not observe

Table 3. Hypoxia and Hypotension (mutually exclusive categories) by derived Glasgow Outcome Scale (GOS)

		GOS									
Variable	Total	Dead		Vegetative State		Severe Disability		Moderate Disability		Good Recovery	
		N	%	N	%	N	%	N	%	N	%
Neither Hypoxia nor Hypotension	3506	803	22.9	107	3.1	502	14.3	651	18.6	1443	41.2
Hypoxia only	632	224	35.4	25	4.0	116	18.4	101	16.0	166	26.3
Hypotension only	581	282	48.5	18	3.1	77	13.3	80	13.8	124	21.3
Both Hypoxia and Hypotension	465	254	54.6	20	4.3	68	14.6	55	11.8	68	14.6

this. It may be argued that the most recent dataset in the IMPACT database is nearly 10 years old. However, in the Pharmos dexanabinol study, conducted from 2001 to 2004, hypotension was observed in 15% of patients and hypoxia in 25% (Maas et al., 2006a).

All three insults were confirmed as powerful markers of adverse outcome, with each insult being associated with at least a doubling of the odds of adverse outcome. The strongest prognostic effects were noted for hypotension. The importance of hypoxia and hypotension as systemic secondary insults and the association between these insults and poorer outcome has been well documented (Chesnut et al., 1993b). We observed that hypoxia does add to hypotension although the effects are sub-additive rather than synergistic. Limitations of our analysis are that we were restricted by the definitions given by the individual studies as to

Table 4. Computed Tomography (CT) class, and hypoxia and hypotension

CT Class	Hypoxia		Hypotension	
	N	Prevalence	N	Prevalence
NVP	317	18.6%	402	15.9%
Diffuse injury	1540	16.9%	1736	16.1%
Swelling/shift	888	24.9%	938	23.3%
Mass	1573	19.8%	1864	14.4%

NVP, no visible pathology

what constituted hypotension as a secondary insult and no information was available on the duration of insults. On analysis of the relation between the actual levels of blood pressure on admission and outcome we found a smooth U shaped relationship without any evidence of an abrupt threshold effect (Butcher et al., 2006) . The relatively strict definition for hypotension (SBP < 90 mmHg) used during data collection may have been too restrictive and the actual incidence of hypotensive insults much greater.

The strength of the prognostic effect of hypothermia is very similar to that of hypoxia. Few

Table 5. Month of injury and hypothermia

	Hypothermia	
	N	Prevalence
January	258	13.6%
February	570	10.7%
March	589	8.0%
April	565	7.6%
May	374	9.1%
June	358	6.4%
July	338	8.3%
August	322	5.9%
September	292	8.2%
October	296	10.8%
November	274	17.1%
December	251	12.7%

previous studies have investigated the relationship between hypothermia on admission and final outcome and results are conflicting. Zauner (Zauner et al., 1998) in a relatively small study of 60 patients with severe TBI describe an association between spontaneous hypothermia and poorer outcome but this association was not statistically significant. Jeremitski (Jeremitsky et al., 2003) report a significant increase in mortality in patients who were hypothermic on admission. However, in this series hypothermia was defined as a brain temperature below 35ºC, and it may be argued that the low brain temperature might have resulted from low cerebral blood flow and may not have been representative of core body temperature. Steinemann (Steinemann et al., 1990) found no association between early post traumatic hypothermia and outcome in patients with multi trauma including head injury. The strong evidence of an adverse effect of hypothermia as a secondary insult seems paradoxical in the light of the strong experimental evidence that hypothermia is neuroprotective. This perhaps points to the mechanism of the adverse effect of hypothermia as a secondary insult being related to its management and in particular to an adverse effect of rewarming. This would be consistent with a secondary analysis of the pivotal Phase III trial of hypothermia in TBI (Clifton et al., 2001). Here patients who arrived hypothermic, were rewarmed and then subsequently randomized to the hypothermia treatment group had particularly poor outcomes.

In summary, all three secondary insults examined have shown to be strongly associated with the risk of an adverse outcome. Although these results are purely observational, they do point to the need for great vigilance in their early identification, and the potential for therapeutic research into their optimal management.

CHAPTER 3. PATHOPHYSIOLOGY: ISCHEMIC FACTORS

A) Combined Effects of Mechanical and Ischemic Injury to Cortical Cells: Secondary Ischemia Increases Damage and Decreases Effect of Neuroprotective Agents

Abstract

Traumatic brain injury (TBI) involves direct mechanical damage, which may be aggravated by secondary insults such as ischemia. We utilized an *in vitro* model of stretch-induced injury to investigate the effects of mechanical and combined mechanical/ischemic insults to cultured mouse cortical cells. Stretch injury alone caused significant neuronal loss and increased uptake of the dye, propidium iodide, suggesting cellular membrane damage to both glia and neurons. Exposure of cultures to ischemic conditions for 24 h, or a combination of stretch and 24 h of ischemia, caused greater neuronal loss compared to stretch injury alone. Next, we tested the neuroprotective effects of superoxide dismutase (SOD), and the nitric oxide (NO) synthase inhibitors 7-nitroindazole (7-NINA) and lubeluzole. In general, these agents decreased neuronal loss following stretch injury alone, but were relatively ineffective against the combined injury paradigm. A combination of SOD with 7-NINA or lubeluzole offered no additional protection than single drug treatment against stretch alone or combined injury. These results suggest that the effects of primary mechanical damage and secondary ischemia to cortical neurons are cumulative, and drugs that scavenge superoxide or reduce NO production may not be effective for treating the secondary ischemia that often accompanies TBI.

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability in Western industrialized societies (Jennett, 1996). Many factors contribute to the outcome of TBI besides the direct physical damage caused to neurons and glia by a mechanical insult. Secondary insults, such as glutamate excitotoxicity, calcium-mediated toxicity, and ischemia, play a large role in brain damage (Maas et al., 2000; Weber, 2004). For example, decreases in cerebral blood flow (CBF), and thus a corresponding decrease of brain tissue oxygen pressure ($PbrO_2$) as measured in both rats and humans (Clark et al., 1997, van Santbrink et al., 2002, van den Brink et al., 2000), can also aggravate cellular damage. In clinical studies, patients with decreased $PbrO_2$, measured within the first 48 h after injury, have a less favourable Glasgow Outcome Scale score than patients without a decrease in $PbrO_2$ (van Santbrink et al., 2003). This would implicate that a combination of the primary mechanical insult and secondary mechanisms, such as ischemia, contribute to cell damage and death following TBI. Therefore, experimental models that mimic both primary and secondary injury processes may provide a more realistic assessment of trauma at the cellular level.

Our first goal was to establish a model of combined mechanical and ischemic injury *in vitro* using cultured mouse cortical cells. We utilized a well-described model of stretch-induced mechanical injury (Ellis et al., 1995), which has previously been characterized in rat cortical neurons and glia (Ellis et al., 1995, McKinney et al., 1996, Weber et al., 1999), rat septo-hippocampal cultures (Pike et al., 2000), and mouse hippocampal (Slemmer et al., 2002) and

cerebellar cultures (Slemmer et al., 2004). We analyzed the effects of mechanical injury, ischemic injury, and a combination of these insults by measuring cortical cell damage and neuronal loss.

Ischemia is accompanied by hypoxia, as well as by hypercapnia and acidosis (Hoffman et al., 1996; Raghupathi and McIntosh, 1998). In addition, during brain ischemia, several reactive oxygen species (ROS) are generated, such as superoxide and hydroxyl radicals (Leker and Shohami, 2002). There also appears to be an increase in nitric oxide (NO), which is generated by the enzyme nitric oxide synthase (NOS) (Samdani et al., 1997). Three major isoforms of NOS have been identified in the brain: the neuronal form (nNOS), the endothelial form (eNOS), and an inducible form (iNOS) (Dawson, 1999, Wada et al., 1999). NO contributes to several important physiological processes, such as cerebral vasodilation and neurotransmission (Dawson and Dawson, 1996). However, excess NO can interact with the superoxide radical (O_2), resulting in the generation of peroxynitrite ($ONOO^-$), postulated to be one of the most damaging free radical species (Dawson, 1999, Liu et al., 2002). Free radicals can cause cellular damage by peroxidation of proteins, DNA and lipid membranes (Kermer et al., 1999; Leker and Shohami, 2002). For this reason, decreasing the amount of NO and superoxide radicals, and thereby blocking the production of peroxynitrite, appears to be a possible protective mechanism against cell death after mechanical and combined mechanical/ischemic injury. Hence, we evaluated the neuroprotective potential of superoxide dismutase (SOD) and inhibitors of NOS (7-nitroindazole and lubeluzole) separately, and in combination, in our *in vitro* model of combined injury.

Methods

Animal welfare

All experiments were conducted in accordance with the European Communities Council Directive and were approved by the animal welfare committee of Erasmus Medical Center Rotterdam. Efforts were made throughout these studies to minimize animal suffering and the number of animals used.

Cell culture

Primary cortical cell cultures (approximately 10% neurons and 90% glia) were prepared from E17/18 wildtype FVB/N mouse embryos similar to previously described methods for hippocampal cell cultures (Slemmer et al., 2002). Briefly, dissociated cortices were diluted in serum-containing media [Basal Medium Eagles (GIBCO, Grand Island, NY) containing 10% horse serum (GIBCO), 10 µg/ml gentamycin (Sigma, St. Louis, MO), 0.5% glucose (Sigma), 1 mM sodium pyruvate (GIBCO) and 1% N2 supplements (GIBCO)] to a concentration of 500,000 cells per ml; cells were then plated in 1 ml aliquots onto collagen-coated sixwell FlexPlates (FlexCell, Hillsborough, NC) coated overnight with poly-L-ornithine (500 µg/ml; Sigma). All cultures were maintained in a humidified incubator (5% CO_2, 37˚C). Neuronally enhanced cultures were obtained by replacing half of the media 2 days after plating, and then twice per week, with serum-free media containing 2% B27 supplements (GIBCO). Glia formed a confluent monolayer that adhered to the membrane substrate while neurons adhered to the underlying glia. Cells were used for experiments within 9-12 days *in vitro* (DIV).

Cell injury

Primary mouse cortical cultures were stretch-injured using a model 94A Cell Injury Control-

ler (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA, USA) as described previously (Ellis et al., 1995; Slemmer et al., 2002). In brief, the Silastic membrane of the FlexPlate well is rapidly and transiently deformed by a 50-ms pulse of compressed nitrogen, which deforms the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure. The extent of cell injury - produced by deforming the Silastic membrane on which the cells are grown - is dependent on the degree of deformation, or stretch. Based on previous work (Ellis et al., 1995), two levels of cells injury were chosen (5.5 and 6.5 mm deformations) and defined as mild and moderate, respectively. These degrees of membrane deformation result in a biaxial strain or stretch of 31 and 38%, respectively. This range of cell stretch has been shown to be relevant to what would occur in humans after rotational acceleration/deceleration injury (Schreiber et al., 1995). Uninjured control wells were contained in the same FlexPlates as injured wells, and thus underwent the same manipulations, except that they did not receive rapid deformation of the Silastic membrane. To simulate ischemia due to low CBF, FlexPlates of primary mouse cortical cultures were placed in a humidified incubator set at 20% CO_2 and 37˚C. Incubation of cells for up to 6 h in ischemic conditions caused no measurable cell damage or death; however, 24 h of ischemia resulted in cell damage. The cells were incubated for 24 h and then fixed and stained, or returned to the normal incubator for an additional 24 h before being fixed and stained (described below). Incubation of cells for 24 h in the ischemic incubator resulted in a pH change from ~7.4 to ~7.0. For the combined mechanical and ischemic injury paradigm, cells were injured at 5.5 mm deformation and immediately placed in the ischemic chamber for 24h.

Cell viability

The cells were fixed and stained at 15 min, 24 h or 48 h after mechanical injury, or at 24 h or 48 h after combined mechanical and ischemic injury. Cell injury was assessed in cultured cells using the dyes propidium iodide (PrI; Sigma) and Hoechst (Sigma), similar to previously described methods (Slemmer et al., 2002; Zhao et al., 2000). Hoechst labels the nuclei of all cells, whereas PrI is normally excluded from intact cell membranes. If membranes are damaged, PrI will enter cells and stain the nucleus, resulting in a bright red fluorescence. Cultures were treated with PrI and Hoechst for 20 min at 4˚C, then fixed and stained with 4% paraformaldehyde for 20 min at room temperature. Images were captured using Texas Red and UV filters (for PrI and Hoechst, respectively) on a Leica DMRBE fluorescence microscope, equipped with a Hamamatsu C4880 CCD camera. PrI and Hoechst images were taken in five contiguous 100X or 160X images, and were counted and averaged per well. All images were taken from the center portion of the well, as this region was previously shown to receive equal impact from the cell injury controller (Ellis et al., 1995). All PrI and Hoechst cell counting were performed by one of the authors blinded to experimental conditions. Data are expressed as the ratio of PrI positive cells to total cell number (as determined by Hoechst staining of cell nuclei). In control cultures, PrI staining was low, accounting for less than 5% of total cell number at all time points, consistent with previous reports (Slemmer et al., 2002; Zhao et al., 2000). Changes in total cell number are expressed as percentage of control values. All experiments were completed using at least two separate culture preparations at 9-12 DIV.

Immunohistochemistry and neuronal cell counts

Cultured cortical cells received either a mild (5.5 mm) or a moderate (6.5 mm) injury, or

remained uninjured; at either 15 min, 24 h or 48 h post-injury, the cultures were fixed for 20 min with 4% paraformaldehyde as previously described (Slemmer et al., 2004). For the ischemia and combined injury experiments, the cells were incubated for 24 h in the ischemic chamber and then fixed and stained, or returned to the normal incubator for an additional 24 h before being fixed and stained. In brief, cultures were incubated in primary antibody [anti-microtubule-associated protein 2 (MAP2, 1:500; Sigma)] for 1 h, followed by incubation with secondary antibody (Alexa-594-conjugated goat-antimouse, 1:300; Molecular Probes, Eugene, OR) for 1 h. Cultures were dehydrated with ethanol and mounted with 4',6-diamidino-2-phenylindole (DAPI). MAP2 specifically labels the dendrites and soma of neurons, while DAPI labels the nuclei of all cells, similar to Hoechst. Images were captured using a Leica DMRBE microscope (see Section 2.4). MAP2-positive neurons were counted in five contiguous fields at a magnification of 100X or 160X.

Drug treatment

The drugs used for treatment were 7-nitroindazole monosodium salt (7-NINA, Tocris Cookson, U.K.); lubeluzole (supplied by Janssen Pharmaceuticals, Belgium); and superoxide dismutase (SOD; Sigma). 7-NINA and lubeluzole were initially dissolved in dimethylsulfoxide (DMSO) and subsequently diluted in dH_2O. The maximal final concentration of DMSO that was added to the culture wells was 0.1%. SOD was dissolved in dH_2O and added to culture wells in a final amount of 100 units/ml. The concentration ranges of 7-NINA (1 μM and 10 μM) and lubeluzole (10 nM and 100 nM), which were used in the current study, have been previously shown to protect cultured neurons against hypoxia (Huang et al., 2002), and glutamatemediated toxicity (Culmsee et al., 1998), respectively. An amount of 100 units/ml of SOD has been demonstrated to reduce oxygen free radical production *in vitro* (Saito et al., 1997, Shanker and Aschner, 2003). Also, this amount of polyethylene glycol-conjugated SOD was utilized in a previous study using the same stretch-induced injury model (McKinney et al., 1996). The addition of the drugs alone to uninjured control wells (for 24 h) caused no significant difference in PrI uptake, total cell number, or number of MAP2-positive neurons compared to untreated wells at the final concentrations used in this study. Higher levels of 7-NINA (100 μM) or lubeluzole (1 μM) increased PrI uptake in uninjured controls, suggesting some toxicity at these concentrations. The drugs were either added to the media 15 min prior to mechanical injury, as pre-treatment, or 15 min after mechanical injury, as post-treatment. Unlike 7-NINA and lubeluzole, SOD is sensitive to proteolytic and oxidative inactivation. Therefore, in one set of experiments, SOD was added to injured cultures at two timepoints, 15 min and 12 h after injury. For ischemic injury only, or for combined injury, the plates were placed in the ischemic incubator immediately after the drugs were added.

Data analysis

The data were analyzed using the statistical program GB Stat (Dynamic Microsystems, Silver Spring, MD). Data were computed as meansGstandard error (SE) values. Statistical significance was established by oneway analysis of variance (ANOVA) followed by Fisher's protected least significance difference test. Data are considered significant at $p<0.05$.

Results

Stretch injury increases PrI uptake in cortical cells

In uninjured control wells, there was little PrI staining (Fig. 1A and C, *see page 129*), indicat-

ing that most cells were healthy and viable, with intact cell membranes. Fifteen minutes after injury, however, cultures demonstrated an increased amount of PrI staining, the majority of which occurred in the underlying glial layer (Fig. 1B and C), similar to our previous findings in hippocampal cultures (Slemmer et al., 2002). PrI uptake decreased with time post-injury, but remained significantly elevated through 24 h after 5.5 mm deformation (mild injury) and 48 h after 6.5 mm deformation (moderate injury). Mild injury also caused a drop in cell number 24 h after injury (determined by the amount of Hoechst-positive nuclei), which returned to control levels at 48 h (Fig. 1D). Moderate injury caused a more dramatic effect on total cell number, which was decreased vs. control at 15 min and 24 h post-injury, and then elevated at 48 h post-injury, suggesting glial proliferation. We also observed an increased amount of condensed nuclei (see Fig. 1B) in cell cultures following both levels of injury, which is generally an indication of delayed or apoptotic cell death (Toescu, 1998).

Stretch injury causes a loss of neurons in cortical cell cultures

To evaluate more closely the effects of mechanical injury on neuronal cells, we counted the number of MAP2-positive neurons in injured cultures as a percentage of neurons in control wells (Fig. 2, *see page 130*). Uninjured neurons exhibited an intact soma and smooth neurites (shown in Fig. 2A). Injured neurons often displayed beaded neurites and condensed nuclei (shown in Fig. 2B). Counterstaining with DAPI further supported the finding of condensed nuclei in both neuronal and glial cells, as previously observed with Hoechst staining. Mild injury caused a slight, but non-significant decrease in the number of neurons to 86.4 ± 5.2% of control at 15 min, while moderate injury caused an even greater decrease to 40.6 ± 8.1% of control. The amount of MAP2 staining did not change significantly over time after moderate injury, therefore the decrease in MAP2-positive neurons is an early event. However, after mild injury there was a significant decrease in neurons at 24 h and 48 h, suggesting a delayed neuronal loss at this injury level.

Effects of ischemic and combined mechanical/ischemic insults

To evaluate the effects of secondary ischemic damage to cortical cells, we compared the effects of stretch injury alone to ischemic injury, and to a combined mechanical and ischemic injury paradigm (as described in Section 2). Ischemic injury alone and combined injury caused an increase in PrI at 24 h, similar to that caused by stretch injury alone (Fig. 3A, also compare to Fig. 1C). PrI uptake remained elevated at 48 h after ischemic and combined injury, unlike stretch injury alone. However, there was no difference in PrI uptake between ischemia alone and combined injury at 48 h. Neither ischemia alone nor the combined injury paradigm caused a significant change in total cell number at either time point (data not shown). Ischemia alone caused a reduction in MAP2-positive neurons to 48.0 ± 9.5% of control at 24 h (Fig. 3B). The combined insult paradigm caused an even greater loss of neuronal cells to 32.9 ± 4.0% of control. At 48 h, neuronal cell counts were 47.9 ± 6.5% of control following ischemia alone and 25.5 ± 4.4% of control following the combination injury. These results suggest an additive effect of mechanical and ischemic damage to cortical neurons, and a delayed loss of neurons during ischemic conditions.

Evaluation of neuroprotective agents against mechanical and ischemic insults

We evaluated the neuroprotective potential of several compounds using our *in vitro* model of combined injury: 7-NINA, an inhibitor of neuronal NOS (Huang et al., 2002, Wada et al.,

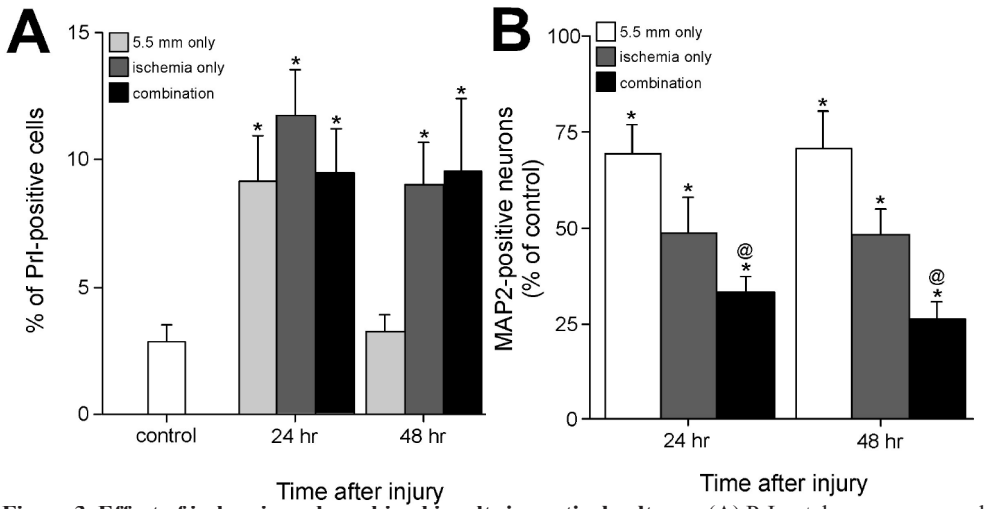

Figure 3. Effect of ischemia and combined insults in cortical cultures. (**A**) PrI uptake was measured following 5.5 mm stretch only, ischemia only, or a combination of stretch and ischemia. Data are shown at 24 h and 48 h after stretch injury or the onset of ischemia (means ±SE values; n= 5-13). Uptake of PrI was increased at 24 h in all three conditions and remained elevated at 48 h in the ischemia and combination injury conditions. *p<0.05 vs. control. (**B**) Effect of ischemia and combined insults on the number of MAP2-positive neurons in cortical cultures. Data are shown at 24 h and 48 h after stretch injury or the onset of ischemia and is expressed as percent of control values (means ±SE values; n=6-8). All three conditions caused a decrease in the amount of MAP2-positive neurons. *p<0.01 vs. control, @p< 0.05 vs. 5.5 mm injury only at same time point.

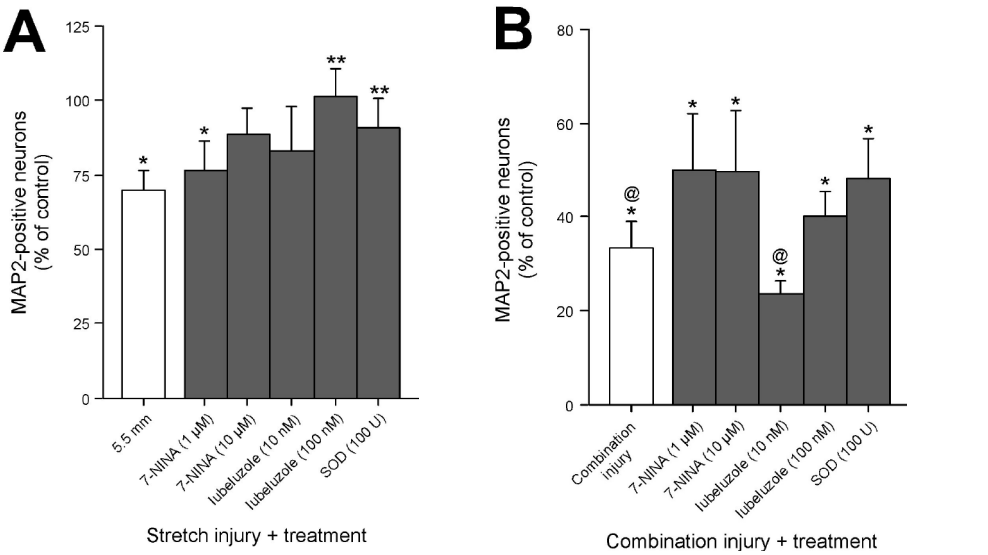

Figure 4. Effect of drug pre-treatment on injury. (**A**) The number of MAP2-positive neurons (% of control; means ±SE values) is shown at 24 h after stretch injury (n= 11-12). Drugs were administered 15 min prior to stretch. *p<0.01 vs. control (uninjured), **p< 0.05 vs. 5.5 mm injury. (**B**) The number of MAP2-positive neurons 24 h after combined injury (means ±SE values; n= 6-8). Drugs were administered 15 min prior to stretchConset of ischemia. *p<0.01 vs. control (uninjured), @p<0.01 vs. 5.5 mm injury only.

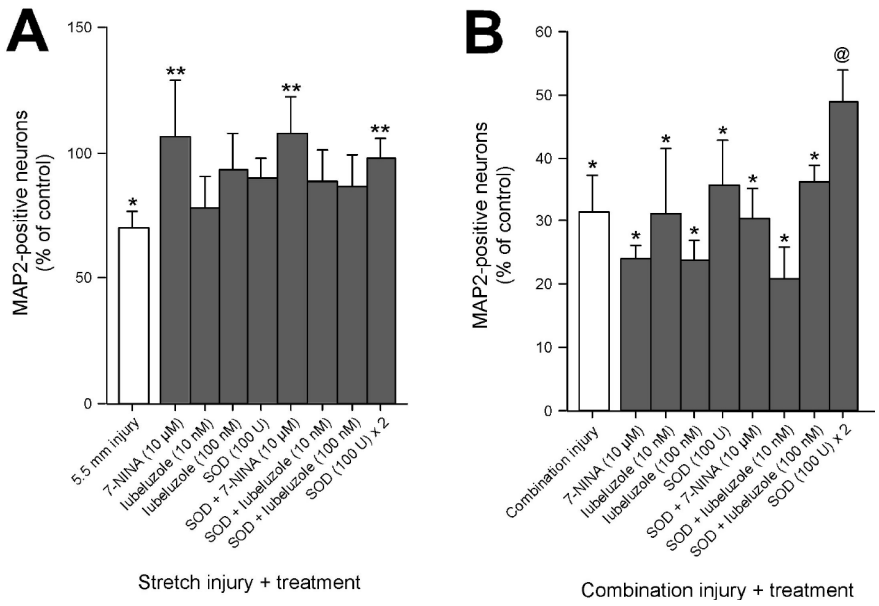

Figure 5. Effect of drug post-treatment on injury. (**A**) The number of MAP2-positive neurons (% of control; means ±SE values) is shown at 24 h after stretch injury (n=6-10). Drugs were administered 15 min after stretch. *p! 0.01 vs. control (uninjured), **p<0.05 vs. 5.5 mm injury. (**B**) The number of MAP2-positive neurons 24 h after combined injury (means ±SE values; n=5-9). Drugs were administered 15 min after stretchConset of ischemia. *p<0.01 vs. control (uninjured), @p<0.05 vs. combination injury.

1999); lubeluzole, a NOS inhibitor, which in addition decreases glutamate release and blocks sodium and calcium channels (Maiese et al., 1997; Mueller et al., 2003); and SOD (Ikeda et al., 1994; Kamii et al., 1996; McKinney et al., 1996). Since the combination injury paradigm did not produce a profound effect on PrI uptake in cultures as compared to the dramatic neuronal loss, we decided to investigate the effects of these compounds only against MAP2 staining. The number of MAP2-positive neurons measured at 24 h after ischemic and combination injuries was not significantly different from the number of neurons measured following these conditions at 48 h, suggesting no further loss of neurons after 24 h (Fig. 3B). We therefore chose to evaluate the effects of the neuroprotective agents at the 24 h timepoint.

We evaluated the neuroprotective effects of both pretreatment (Fig. 4) and post-treatment (Fig. 5) with 7-NINA (1 μM, 10 μM), lubeluzole (10 nM, 100 nM) and SOD (100 U/ml). As shown in Fig. 4A, cultures pretreated with 10 μM 7-NINA or 10 nM lubeluzole no longer exhibited a significant reduction in neurons 24 h after 5.5 mm injury alone, suggesting a protective trend of these compounds vs. stretch-induced mechanical injury. Treatment with 100 nM lubeluzole or 100 U/ml of SOD showed significant protection against neuronal loss induced by stretch injury, whereas 1 μM 7-NINA exhibited no measurable protective effect. In the combined injury paradigm, cultures treated with 1 μM or 10 μM 7-NINA, 100 nM lubeluzole, or 100 U/ml SOD, no longer showed a significant reduction of MAP2-positive neurons in comparison to stretch injury alone. Cultures treated with 10 nM lubeluzole, however, did not exhibit a trend towards protection. For the post-treatment studies, we evaluated 7-NINA

(10 μM), lubeluzole (10 and 100 nM), single and repeated (15 min and 12 h) administration of SOD (100 U/ml), and combinations of these compounds for protection against stretch injury alone and combined stretch/ischemia. With stretch injury alone, 10 μM 7-NINA, the combination of 10 μM 7-NINA and 100 U/ml SOD, and the double addition of 100 U/ml SOD showed a significant decrease in reduction of MAP2-positive neurons (Fig. 5A). The other compounds, and combinations of compounds, demonstrated a protective trend against stretch injury alone, as the number of neurons was not significantly decreased at 24 h after injury compared to controls in these conditions. After combined stretch and ischemic injury, only the repeated addition of 100 U/ml SOD demonstrated a significant protection as measured by the number of MAP2-positive neurons (Fig. 5B). The other compounds and combinations showed no trend towards protection against combined injury.

Discussion

Evidence from both the clinic and experiments *in vivo* demonstrates that TBI is often accompanied by a reduction in CBF in the early post-traumatic period (Clark et al., 1997, van Santbrink et al., 2002, van den Brink et al., 2000). This low CBF often produces ischemia, which is accompanied by hypoxia, hypercapnia and acidosis (Hoffman et al., 1996; Raghupathi and McIntosh, 1998). Therefore, experimental models that mimic both mechanical injury and secondary insults, such as ischemia, should prove useful for analyzing the mechanisms of cell damage and death following TBI. In the current study we utilized an *in vitro* model of mechanical injury, which generates a highly reproducible amount of stretch to cultured cells. We also analyzed the effects of ischemic insults superimposed on stretch-induced injury in cultured mouse cortical cells.

Stretch injury on its own increased uptake of PrI in cortical cells. This effect was dependent on the level of stretch and time post-injury and was similar to previous findings using the same injury model in cultured cortical cells from rat (McKinney et al., 1996, Weber et al., 1999). In addition, the total cell count was also affected following stretch injury alone. This was especially true after moderate injury where a decrease in cell number was evident at 15 min and 24 h after injury, and an elevated number of cells was observed at 48 h postinjury, suggesting that glial cells had proliferated. This finding is similar to the astrogliosis that is often reported following TBI *in vivo* (McGraw et al., 2001). We also specifically analyzed the effects of stretch on neuronal viability. There were fewer MAP2-positive neurons at 24 h and 48 h after mild injury compared to control or 15 min post-injury, demonstrating some delayed neuronal death at this injury level. After moderate injury there was a dramatic neuronal loss by 15 min, which did not change over time. This finding suggests that the neuronal loss following moderate injury occurs within the initial injury phase, and that cells die by necrotic means or that they lift from the membrane substrate, as no additional delayed cell death is apparent.

Our *in vitro* model of combined insults is similar to the model previously described by Glass et al. (2002). They also used the same stretch injury model originally described by Ellis et al. (1995), and superimposed hypoxic insults of various duration (1 h-24 h) following stretch injury of cultured rat cortical cells. We superimposed ischemic insults after stretch injury, which encompasses a drop in O_2 levels, an increase in CO_2, and a decrease in pH. Although we did not precisely control the level of O_2 in our study, we believe that the hypercapnia and resulting acidosis that was induced may more closely mimic the overall effects of a global reduction in CBF, rather than specific regions of hypoxia occurring after TBI. In addition,

unlike Glass et al. (2002) who measured trypan blue exclusion and lactate dehydrogenase release from injured cultures, we analyzed the effects of combined insults more specifically on neurons.

PrI uptake was similar following stretch alone, 24 h of ischemia, or both of these insults when measured at 24 h. Although PrI uptake returned to control levels at 48 h following stretch alone, it remained elevated following ischemia and combined injury. This suggested sustained damage to cells, but we were unable to discern whether this was due to glial or neuronal damage. When we investigated MAP2-positive neurons specifically, we found a profound difference between the three injury paradigms. Ischemia alone caused greater neuronal loss than stretch injury alone, while combined ischemia and stretch caused an even greater reduction in neurons. As with the findings of Glass et al. (2002), the effects of ischemia and stretch appear to be additive in our *in vitro* model. When cultures were placed back into a normal incubator for 24 h following 24 h of ischemia, there was no additional neuronal loss compared to measurements directly after 24 h of ischemia. Therefore, restoring normal levels of O_2 and CO_2 inhibited further neuronal death in our culture system. This finding also suggested that the first 24 h after the initiation of combined insults was most appropriate as a target window for drug treatment.

Many free radical scavengers have been evaluated in experiments related to TBI. With our model, injuries are easily reproducible, providing an adequate means to screen possible therapeutic strategies. We decided to evaluate the protective effects of SOD and NOS inhibitors using our model, as cell damage due to TBI and ischemia are believed to involve excess production of both superoxide and NO (Hall et al., 2004, Ikeda et al., 1994, Kermer et al., 1999, Samdani et al., 1997, Wada et al., 1998). For protection against NO-mediated toxicity, we utilized the NOS inhibitors 7-NINA and lubeluzole. 7-NINA has demonstrated neuroprotection *in vitro* (Huang et al., 2002) and *in vivo* (Wada et al., 1999). Lubeluzole has shown beneficial effects against excitotoxicity and NO *in vitro* (Culmsee et al., 1998, Maiese et al., 1997) as well as *in vivo* ischemia (Culmsee et al., 1998, Mueller et al., 2003).

7-NINA, lubeluzole and SOD displayed some neuroprotective effects against stretch injury alone when administered either before or after injury. A differene between the NOS inhibitors was manifest with pretreatment of stretch injury.Whereas 7-NINA showed no significant protective effect, 100 nM lubeluzole did show such an effect when administered before stretch injury. This effect appeared to be reversed with post-treatment. A possible explanation for this could be found in the characteristics of the compounds. 7-NINA is reported to specifically decrease NO production, whereas lubeluzole also decreases glutamate release and blocks sodium and calcium channels (Maiese et al., 1997, Mueller et al., 2003). Thus, it is possible that solely decreasing NO production before injury is not beneficial to cells, and a drug with more broad effects, such as lubeluzole, leads to cell protection. However, lubeluzole showed no significant protective effects when used in posttreatment. Similarly, some studies have shown that lubeluzole is not effective against TBI *in vivo* (Kroppenstedt et al., 1999, O'Dell et al., 2000). Also, lubeluzole failed to demonstrate neurological improvement in recent clinical trials for stroke (Diener et al., 2000), which can be compared to our superimposed secondary ischemia, where this compound was also ineffective.

McKinney et al. (1996) had previously demonstrated that polyethylene glycol-conjugated SOD did not decrease PrI uptake after stretch injury of mixed cultures of neurons and glia. However, the effect of SOD pretreatment specifically on neurons was not evaluated. In our hands, SOD demonstrated significant neuroprotective effects against mechanical injury with

both pre- and post-treatment. The difficulty with SOD, however, is that it is metabolized more quickly than the NOS inhibitors. Thus, post-treatment against stretch injury required a more continuous higher concentration in order to obtain a substantial protective effect. Therefore, the repeated addition of SOD did have a significant effect, whereas the single administration did not. This repeated administration of SOD also proved efficacious against the combined injury paradigm, suggesting that scavenging of superoxide is pertinent for several hours, and not just in the initial time period after injury.

The data with NOS inhibitors and SOD added alone to the cultures suggest that there is more damage occurring in combined mechanical and ischemic injury than these compounds can prevent. However, the trends towards protection with these drugs with pre-treatment against mechanical/ischemic injury, and against mechanical injury alone, do suggest that the pathways they are inhibiting are involved in the cell damaging processes. Consequently, it is likely that a combination of ischemia on top of the primary mechanical injury produces an increase in both superoxide and peroxynitrite, against which a single drug is not efficacious. Therefore, we treated cells with a combination of SOD and either 7-NINA or lubeluzole, aimed at reducing superoxide and NO production (and thus excess peroxynitrite). A combination of SOD and 10 μM 7-NINA post-injury was significantly protective against mechanical injury only. However, post-treatment with 10 μM 7-NINA only was equally as protective as the combination of SOD and 10 μM 7-NINA, suggesting that 7-NINA alone was responsible for this protective effect. These drug combinations also did not provide protection against neuronal loss in the combined injury paradigm.

Overall, these results suggest that, although mechanical injury alone may be amenable to treatment with drugs that scavenge superoxide and reduce NO, this therapy may not be effective in the presence of secondary ischemia, which often accompanies TBI in the clinic. It is possible that other mechanisms, such as glutamate or calcium-mediated toxicity, may be playing a more prominent role in cell death when ischemia is superimposed on mechanical injury. Also, because NO is a vasodilator (Dawson and Dawson, 1996), it may be important to block certain sources of NO and not others. For example, a recent study has shown that iNOS-derived NO is necessary for the phenomenon of ischemic preconditioning in the brain (Cho et al., 2005). In addition, although blockade of nNOS can protect neurons from ischemia-induced damage (Huang et al., 2002, Samdani et al., 1997), blockade of iNOS has been demonstrated to worsen CBF following *in vivo* TBI (Steiner et al., 2004). Our current findings further emphasize the importance of preventing secondary ischemic insults in the clinical situation, and indicate that the likelihood of demonstrating beneficial effects of neuroprotective agents in TBI may be significantly decreased in the presence of secondary ischemic insults.

CHAPTER 3.

B) Changes of Cerebral Blood Flow during Secondary Expansion of a Cortical Contusion Assessed by Non-invasive ^{14}C-iodoantipyrene Autoradiography in Mice

Abstract

Although changes of cerebral blood flow (CBF) in and around traumatic contusions are well documented, the role of CBF for the delayed death of neuronal cells in the traumatic penumbra ultimately resulting in secondary contusion expansion remains unclear. The aim of the current study was therefore to investigate the relationship between changes of CBF and progressive peri-contusional cell death following traumatic brain injury (TBI). CBF and contusion size were measured in C57/Bl6 mice under continuous on-line monitoring of $ETpCO_2$ prior to trauma and 15 min or 24 hrs following controlled cortical impact by ^{14}C-iodoantipyrine autoradiography (IAP-AR; n=5-6 per group) and by Nissl staining, respectively. Contused and ischemic (CBF < 10%) tissue volumes were calculated based on eleven adjacent section pairs collected at equal distances throughout the contusion and compared over time. All physiological parameters were stable throughout the experiment. Cortical CBF in non injured mice varied between 69-93 ml/100mg/min depending on the anatomical location. Fifteen minutes after trauma CBF decreased in the whole brain by ~50% ($p<0.05$), except in contused tissue where it fell by more than 90% ($p<0.001$). Within 24 hours after TBI CBF recovered to normal values in all brain areas with the exception of the contusion where it remained reduced by more than 90% ($p<0.001$). Contusion volume expanded from 24.9 to 35.5 mm^3 ($p<0.01$) from 15 minutes to 24 hours after trauma (+43%) whereas the area of severe ischemia (CBF<10%) showed only a minimal (+13%) and not significant increase (22.3 to 25.1 mm^3). The current data therefore suggest that the delayed secondary expansion of a cortical contusion following traumatic brain injury is not caused by a reduction of cerebral blood flow alone.

Introduction

Contusions are the most frequent focal abnormalities on CT examination in head injured patients and are associated with high mortality and unfavorable functional outcome (Leitgeb et al., 2007, Maas et al., 2007). The pathophysiology of contusions is complex and difficult to investigate since many contusions tend to increase in size over time, a finding termed secondary contusion expansion and recently characterized in detail experimentally (Zweckberger et al., 2006). Several factors have been proposed to contribute to contusion expansion, e.g. brain edema formation leading to capillary compression and hypoperfusion (Bullock et al., 1991, Katayama et al., 1998), microthrombosis (Lafuente et al., 1999, Schwarzmaier et al., 2007), progressive bleeding causing microvasospasm (Bullock et al., 1992), glutamate excitotoxicity (Nilsson et al., 1990, Tanaka et al., 1994), or activation of intracellular cell death pathways (Colicos et al., 1996, Newcomb et al., 1999, Smith et al., 2000). One of the first factors, however, postulated to be responsible for the expansion of cerebral contusions was the reduction of cerebral blood flow (CBF) in the peri-contusional area (see Rosomoff et al. (1996) for review).

Starting with the pioneering work of Fieschi et al. using Xe-CT in TBI patients, Graham et al. using specimens of human TBI victims, and Martins et al. in experimental animals (Fieschi et al., 1974, Martins et al., 1977, Graham et al., 1978) changes of post-traumatic CBF have been studied extensively both in patients and animals during the past three decades. Most studies show that post-traumatic CBF is reduced following focal and diffuse brain damage (see (Verweij et al., 2007, Werner et al., 2007) for review), however, there is general understanding that in man (Tenjin et al., 1990, Bouma et al., 1991, Bullock et al., 1992, Alexander et al., 1994, Garnett et al., 2001, von Oettingen et al., 2002) as well as in experimental animals (Nilsson et al., 1977, Pollay et al., 1980, Zhuang et al., 1992, Cherian et al., 1994, Bryan, Jr. et al., 1995, Kochanek et al., 1995, Maeda et al., 1997, Hendrich et al., 1999, Liu et al., 2002, Lundblad et al., 2004, Zweckberger et al., 2006) a severe reduction of CBF, i.e. cerebral ischemia, is in most cases associated with cortical contusions. The most severe CBF reductions (>80% of normal CBF) are found in the contused tissue itself (Bouma et al., 1991, Bullock et al., 1992, Alexander et al., 1994, Bryan, Jr. et al., 1995, Kochanek et al., 1995, Schroder et al., 1995, Maeda et al., 1997, Hendrich et al., 1999, Garnett et al., 2001, von Oettingen et al., 2002, Zweckberger et al., 2006) and are observed almost immediately after TBI (Zweckberger et al., 2006). However, due to the dynamic nature of metabolic and structural changes in the penumbra and its relatively small size at any given time point, knowledge on blood flow changes in this crucial region is much less profound. In this crucial region delayed neuronal cell death results in gradual contusion expansion. Depending on the time of measurement, reductions (Bouma et al., 1991, Bryan Jr. et al., 1995, Kochanek et al., 1995, Schroder et al., 1995, Maeda et al., 1997, von Oettingen et al., 2002, Liu et al., 2002) as well as increases (Bullock et al., 1992, Garnett et al., 2001) of penumbral blood flow were reported. Only few studies tried to correlate reductions of CBF and tissue loss in the traumatic penumbra or to trace penumbral blood flow changes over time. Schroder et al. (1995) and von Oettingen et al. (2002) showed a clear positive correlation between the level of CBF reduction and the distance from the core of the contusion thereby suggesting that progressive penumbral cell death may be caused by ischemia (Schroder et al., 1995, von Oettingen et al., 2002). Bryan and colleagues compared the volume of ischemic tissue 30 minutes and 4 hours after controlled cortical impact injury (CCII) in the rat and reported a trend towards an increased volume of ischemic tissue over time (Bryan, Jr. et al., 1995). The latter suggests that progressive penumbral ischemia may be the cause for pericontusional cell death.

Despite these findings it still remains unclear whether tissue ischemia is the result of the primary lesion and remains therefore confined to the region of initially necrotic tissue, or if CBF drops to ischemic levels in the traumatic penumbra over time and is responsible for pericontusional cell death. Therefore the aim of the current study was to measure the volumes of ischemic and non-viable tissue volumes immediately after experimental TBI and 24 hours later. Thus verifying the hypothesis that contusion volume will grow in parallel with the volume of ischemic peri-contusional tissue.

Materials and Methods

Animals

Male C57/Bl6 mice (25 – 28 g, Charles River, Kisslegg, Germany) were used for this study. All efforts were made to minimize suffering and the number of animals according to the guidelines of the German animal protection law and derived guidelines on the ethical use of animals. Animals were kept under controlled light and environmental conditions (12 h

dark/light cycle, 23 ± 1ºC, 55 ± 5% relative humidity) and had free access to food (Altromin, Germany) and water at all times before and after the experiments.

Anesthesia

For trauma application anesthesia was initialized in a halothane chamber (4%) and maintained with a face mask using 30% O_2, 69% N_2O, and 1.2% halothane.

For measurements of absolute cerebral blood flow anesthesia was carried out by an i.p. injection of medetomidine (0.5 mg/kg b.w., Domitor®, Dr. E. Graeub AG, Basel, Switzerland), fentanyl (0.05 mg/kg b.w, Janssen-Cilag, Neuss, Germany), and midazolam (5 mg/kg b.w., Dormicum®, Roche, Basel, Switzerland), a protocol well known not to influence cerebral blood flow. Mice were then intubated with an oro-tracheal tube and mechanically ventilated using 30% O_2 in room air (MiniVent 845, Hugo Sachs Elektronik, March-Hungstetten, Germany). Physiological monitoring (blood pressure and pCO_2) was performed non-invasively as previously reported in order to prevent disturbances of cardiovascular function by arterial or venous catheterization and to minimize anesthesia time (Thal et al., 2007). Briefly, end-tidal pCO_2, which closely correlates with pO_2, was measured with a microcapnometer (Cl240, Columbus Instruments, Columbus, USA) and adjusted to 35-40 mmHg by respective ventilation. Blood pressure was measured at the tail using a non-invasive blood pressure monitoring system (RTBP 2000, Kent, USA). A thermostatically regulated, feedback-controlled heating

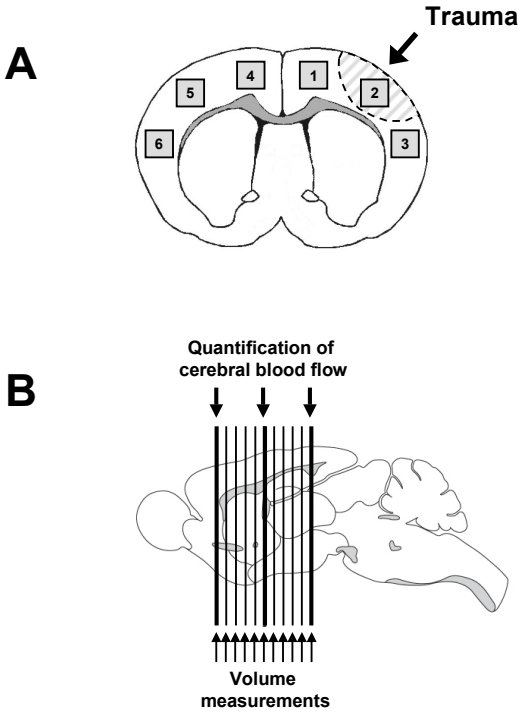

Figure 1
A) Localization of regions of interest (ROI) used for measurement of cerebral blood flow and,
B) coronal plains from where coronal brain sections were taken for quantification of cerebral blood flow and volume of ischemic and contused brain tissue.

pad was used to maintain body temperature at 37°C (FHC, Bowdoinham, USA).

Controlled cortical impact (CCI)
Traumatic brain injury was performed as described previously (Zweckberger et al., 2003b, Zweckberger et al., 2006, Plesnila et al., 2007). Briefly, after induction of anesthesia the head was fixed in a stereotactic frame and a large craniectomy was performed above the right parietal cortex using a high speed drill. Special attention was paid to leave the dura mater intact. CCI was performed perpendicular to the surface of the brain (diameter of the impactor tip: 3 mm, impact velocity: 8 m/s, impact duration: 150 ms, indentation: 1 mm). Thereafter, the craniectomy was closed with the initially removed bone flap using conventional tissue glue (Histoacryl®, Braun-Melsungen, Melsungen, Germany). The skin over the craniotomy was carefully closed and animals were transferred to an incubator heated to 35°C until recovery of spontaneous motor activity.

Quantification of cerebral blood flow by ^{14}C-iodoantipyrine autoradiography
Absolute cerebral blood flow was measured by ^{14}C-iodoantipyrine autoradiography 15 min and 24 hr after injury as described previously (Maeda et al., 2000, Zweckberger et al., 2006). Briefly, after induction of anesthesia and stabilization of mean arterial blood pressure and end tidal pCO_2 (35-40 mmHg) mice received 20 µCi ^{14}C-iodoantipyrine by intraperitoneal injection. Thirty seconds afterwards, animals were sacrificed by whole body snap freezing, and stored at - 80 ºC until further use. Coronal sections (20 µm) were collected every 500 µm from the brains on a cryostat (CryoStar HM 560, Microm, Walldorf, Germany) and exposed to autoradiography film (Biomax MR, Kodak, Germany) for 9-11 days with respective radioactive standards (RPA504, Amersham, Piscataway, NJ, USA). A blood sample was taken from the frozen heart and the amount of ^{14}C-radioactivity was measured by ß-counting as an internal standard. The values for blood ^{14}C-radioactivity and the autoradiograms were processed as described previously by Maeda et al. (2000). Briefly, autoradiograms were digitized with a CCD camera connected to an image processing system (ImageMG, NIH, Bethesda, MD, USA). Absolute CBF was quantified in six regions of interest (ROIs) in the cortex of the injured (ROI 1-3) and non-injured hemisphere (ROI 4-6; Fig. 1A) on three coronal brain section obtained from the rostral to the occipital brain (bregma 1.7, -0.90, and -2.7 mm; Fig. 1B).

Quantification of cerebral blood flow laser Doppler fluxmetry (LDF)
LDF measurements were performed as previously described (Plesnila et al., 2004, Groger et al., 2005, Trabold et al., 2006). Briefly, a flexible laser Doppler probe was glued onto the exposed left parietal skull over the territory of the middle cerebral artery (MCA) for continuous monitoring of regional cerebral blood flow (rCBF; Perimed 4001 Master, Perimed, Järfälla, Sweden). Data were digitized (Dasylab 5.0, measX, Germany) and analyzed off-line (Flexpro 6.0, Weisang, Germany). LDF values are expressed as % pre-traumatic recordings (baseline).

Quantification of contusion and ischemic tissue volumes
Contused and severely ischemic (CBF < 10%) tissue volumes were calculated based on eleven adjacent section pairs collected at equal distances throughout the contusion (Fig. 1B). Contused brain tissue was identified by cresyl violet staining as previously described

(Zweckberger et al., 2003, Zweckberger et al., 2006, Plesnila et al., 2007). The area of severe ischemia (CBF < 10%) was visualized on autoradiograms by allocating color codes only to CBF values from 10-90% of normal blood flow thereby leaving areas with CBF < 10% blank (Fig. 3A). Areas of contused brain and CBF < 10% were quantified with a standard image analysis software (Optimate 6.52, Media Cybernetics, Silver Spring, MD, USA) by an investigator blinded to the treatment of the animals. Contusion and ischemic tissue volumes (Vi) were calculated based on the contused/ischemic areas (A) obtained from 11 sections by the equation: Vi = 0.5 x (A1+ A2+…+A11).

Statistical analysis

Statistical analysis was performed using the SigmaStat 3.0 statistical software package (SPSS inc., Richmond, CA). Parametrical data were analyzed with independent-samples T test or one-way ANOVA, non-parametrical data with ANOVA on ranks procedures. Differences were considered significant at $p<0.05$. Results are presented as means ± SEM if not indicated otherwise.

Results

All animals used for the measurement of CBF had physiological blood pressure (90-130 mmHg systolic), endtidal pCO_2 (35-40 mmHg), and body temperature (36.5-37.5 °C) during the whole duration of the experiment (Table 1). Due to the rapid set-up of the non-invasive monitoring protocol used in the current study CBF measurements were performed in each animal in less than 30 min after induction of anesthesia.

CBF in control, non-injured, mice showed the highest values in the basal ganglia, while the lowest values were observed in the corpus callosum, i.e. in the white matter (Fig. 2A, *see page 131*). In the cerebral cortex blood flow was at intermediate levels and varied between 69-93 ml/100mg/min (Fig. 2B-D; n=5-6). In all examined sections there was a trend towards higher CBF values in the lateral cortex and towards lower levels near the interhemispheric cleft (Fig. 2B-D). All measurements of cerebral blood flow showed a satisfactory small inter-individual variability (Fig. 2, 4, and 5).

Fifteen minutes following traumatic brain injury by controlled cortical impact CBF decreased in the not impacted tissue of the traumatized hemisphere and in the contralateral hemisphere by 40-60% (Fig. 3A and 4, $p<0.01$, *see page 132, 133*). Global post-traumatic reduction of CBF by 40-50% lasted about 30 min as observed by laser Doppler fluxmetry, a method which does not measure absolute CBF as ^{14}C-iodoantipyrene autoradiography but detects relative CBF changes with high temporal resolution (Fig. 3B; n=11; $p<0.001$). In the following 30 min after brain injury CBF recovered slowly and reached 80-90% of baseline CBF 60 min after trauma. Directly at the impact site, i.e. the medio-lateral right cortex and in areas frontal

Table 1

	sham	***15 min***	***24 hours***
T (°C)	*37.3 ± 0.0*	*37.1 ± 0.4*	*37.2 ± 0.1*
SBP (mmHg)	*103.6 ± 22.1*	*88.6 ± 13.2*	*112.1 ± 22.0*
pCO_2 (mmHg)	*36.5 ± 0.8*	*36.8 ± 0.6*	*36.2 ± 0.6*

Body temperature, systolic blood pressure, and endtidal pCO_2 15 min and 24 hours after TBI immediately before assessment of cerebral blood flow by ^{14}C-iodoantipyrine autoradiography

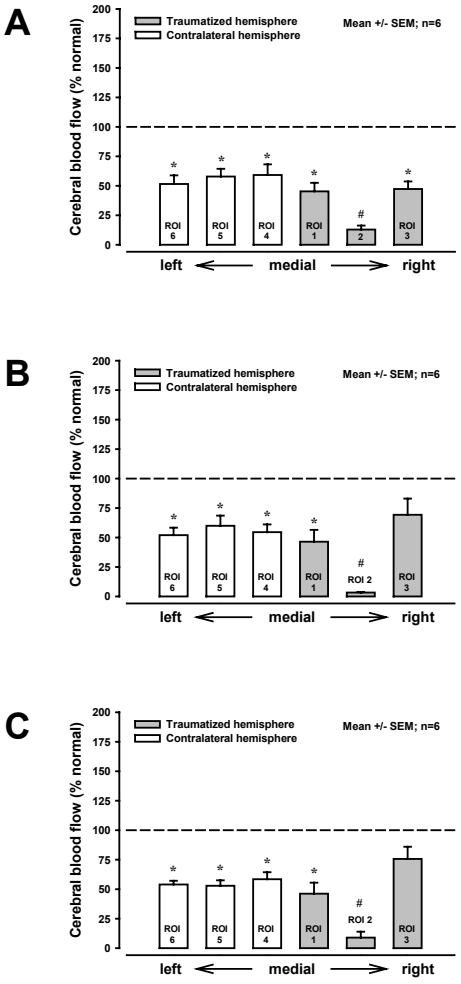

Figure 4. Cerebral blood flow expressed as % of CBF in normal brain (dotted line)15 minutes after experimental TBI in mice (n=5-6) in rostral (**A**), striatal (**B**), and occipital (**C**) coronal brain sections. CBF was quantified in six regions of interest (ROI) arranged according to their anatomical location, i.e. from the left lateral to the right lateral cortex. 15 min after trauma CBF decreased significantly in all regions of the brain (A-C; *p<0.01), most dramatically at the site of the impact (#p<0.001).

and occipital to the impact site CBF decreased by 96%, 87%, and 91%, respectively (Fig. 3A and 4, ROI 2). No recovery was observed up to 24 hours after TBI (Fig. 3A and 5A-C, ROI2). In contrast to the impact site CBF recovered in most other brain regions to normal or high-normal values within 24 hours (Fig. 3A and 5A-C, ROI 1 and 3-6).

To test the main hypothesis of the current study, i.e. that the expansion of a traumatic contusion over time is accompanied or preceded by tissue ischemia, we compared the temporal changes of contused and severely ischemic tissue volumes by morphometrical analysis. As reported previously, a cortical contusion expands after trauma and reaches its maximal volume 24 hours thereafter (Zweckberger et al., 2006), a finding well reproduced in the current study (Fig. 6B, D, and E, white columns). While contusion volume was 28.9 mm^3 15 min after injury, it expanded significantly to 39.3 mm^3 within 24 hours ($p<0.01$). In contrast to the expansion of contusion volume the zone of severely ischemic brain parenchyma did not increase from 15 min to 24 hours after TBI (20.6 mm^3 vs. 25.1 mm^3). The volume of contused

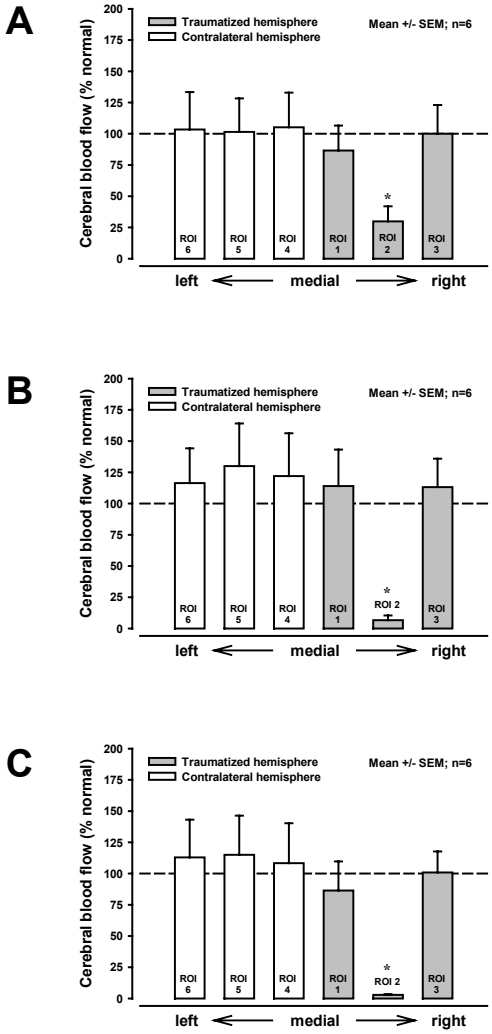

Figure 5. Cerebral blood flow expressed as % of CBF in normal brain (dotted line) 24 hours after experimental TBI (n=5-6) in rostral (**A**), striatal (**B**), and occipital (**C**) coronal brain sections. CBF was quantified in six regions of interest (ROI) arranged according to their anatomical location, i.e. from the left lateral to the right lateral cortex. 24 hours after trauma CBF recovered significantly in all regions of the brain (A-C), except at the site of injury (*p<0.001).

brain parenchyma and the volume of ischemic brain tissue were not significantly different 15 minutes after injury. Twenty-four hours after injury contusion volume was significantly larger than the volume of ischemic tissue ($p<0.01$).

Discussion

In the present study we confirmed our previous findings that the volume of a cortical contusion expands significantly over time, i.e. during the first 24 hours after experimental TBI (Zweckberger et al., 2003) and addressed the hypothesis that ischemia in pericontusional cerebral tissue, i.e. the traumatic penumbra, is responsible for secondary contusion expansion. However, measurements of absolute CBF by ^{14}C-iodoantipyrine autoradiography revealed that the volume of ischemic tissue does not expand significantly after TBI indicating that progressive penumbral ischemia may play only a minor or bystander role for delayed pericontusional cell death and hence contusion expansion.

Experimental Set-up

As discussed previously (Zweckberger et al., 2003, Zweckberger et al., 2006, Plesnila et al., 2007), the current model used for the induction of cortical contusions, controlled cortical impact (CCI), was specifically adapted to mice thereby reducing inter- and intraindividual data scattering to a minimum. The craniectomy necessary for the induction of CCI was carefully resealed after trauma allowing the subsequently evolving brain edema to cause pathologically elevated intracranial pressure (Zweckberger et al., 2003). Therefore this model resembles many features of contusions found in patients after TBI and seems to have a higher relevance for the clinical condition of TBI as compared to previous experimental TBI models lacking a kinetic component or significant increases in intracranial pressure (Martins et al., 1978, Pollay et al., 1980, Zhuang et al., 1992, Bryan Jr. et al., 1995). Special care was taken to avoid any experimental interventions which may have influenced animal physiology, e.g. systemic blood pressure, arterial pCO_2, and CBF. Therefore we used an anesthesia protocol well known not to influence CBF, i.e. a combination of fentanyl, midazolam, and medetomidine, and a completely non-invasive set-up for the measurement of blood pressure and arterial pCO_2 (Thal et al., 2007). Non-invasive animal monitoring has the great advantage to make the time consuming placement of arterial lines unnecessary and in case of the measurement of pCO_2 by end-tidal microcapnometry to monitor the most important determinant of CBF on-line and continuously. Accordingly, CBF was measured in less than 30 min after induction of anesthesia under conditions reducing any influences on animal physiology to a minimum. This is especially relevant for mice which tend to have an unstable cardiovascular baseline during extended period of anesthesia, i.e. longer than 45-60 min.

To further minimize any investigation-related influences on animal physiology and hence CBF we used a method for the measurement of CBF developed by Mies and colleagues which doesn't need placement of venous or arterial lines (Maeda et al., 2000). The radioactive tracer is injected intraperitoneally from where it enters the systemic circulation at a constant rate. Accordingly, plasma levels can be calculated from a single arterial sample obtained from the heart post-mortally. Thereby intravital blood sampling is not necessary resulting in the advantages discussed above, i.e. short anesthesia time, non-invasiveness and no blood volume loss. Another crucial advantage of the currently used method for the measurement of CBF is the termination of the experiment by whole body snap freezing thereby receiving snapshot CBF measurements under perfect physiological conditions, i.e. without the usual delay caused by decapitation of the animal and removal of the brain. By combining non-invasive in vivo monitoring of blood pressure and pCO_2 together with non-invasive systemic application and determination of ^{14}C-iodoantipyrine we are confident that the CBF values obtained in the current study were only minimally influenced by the experimental conditions and represent physiological values.

Cortical Blood Flow

The current values found for murine cortical blood flow were between 69-93 ml/100mg/min. Interestingly, there was a clear trend towards higher CBF values in the lateral and towards lower levels in the medial cortex. Since the cytoarchitecture, i.e. the density of neurons, does not differ significantly between these cortical regions and thereby no reason to assume metabolic need of these cortical regions may be different, differences in CBF are most likely caused by decreasing perfusion pressure occurring from the proximal to the distal end of the arteries. These perfuse the lateral and the medial cortex, i.e. the middle cerebral and the

anterior cerebral artery, respectively.
CBF in the cortex of not traumatized control mice is significantly lower than previously published by other groups (Jay et al., 1988, Maeda et al., 2000, Wellons, III et al., 2000, Lundblad et al., 2004) (66-93 ml/mg/min vs 144-220 ml/mg/min). These differences are probably due to different experimental set-ups. As already pointed out above, the animals in the current study were intubated, ventilated, and anesthetized with medetomidin, fentanyl, and midazolam. These drugs do not influence CBF. Thereby animals were kept under strict non-invasive control of key physiologic parameters until the moment of CBF measurement. Additionally, mice were sacrificed by whole body snap freezing at –80 ºC, avoiding all possible post-mortem artifacts on CBF. The only other laboratory which measured CBF after TBI in mice used isoflurane anesthesia and froze the brains with some delay after sacrifice (Lundblad et al. 2004, 2007). Since isoflurane is well known to increase CBF significantly (Holmstrom et al., 2005) and post-mortem diffusion of ^{14}C-iodoantipyrine from the intravascular space into the brain parenchyma is one of the most prominent sources for overestimating CBF using the^{14}C-iodoantipyrine method (Lumblad 2004), it is conceivable that by avoiding these two sources of possible artifact we may have obtained lower, though more physiological cortical blood flow measurements than previously reported.

Cortical Blood Flow following Traumatic Brain Injury
Changes of CBF following experimental TBI were reported in non human primates (Martins et al., 1977, Martins et al., 1978), pigs (Madsen, 1990, Zhuang et al., 1992), cats (Lewelt et al., 1980, Wei et al., 1980, Dewitt et al., 1986, Tornheim et al., 1990, Dewitt et al., 1992), rats (Nilsson et al., 1977, Pollay et al., 1980, Ishige et al., 1987, Yuan et al., 1988), and mice (Liu et al., 2002, Lundblad et al., 2004, Zweckberger et al., 2006) by hydrogen clearance (Martins et al., 1977, Martins et al., 1978, Zhuang et al., 1992), ^{14}C-iodoantipyrine autoradiography (Nilsson et al., 1977, Pollay et al., 1980, Ishige et al., 1987, McIntosh et al., 1987), labeled microbeads (Yuan et al., 1988, Yamakami et al., 1989, Madsen, 1990, Tornheim et al., 1990, Dewitt et al., 1992), laser-Doppler fluxmetry (Muir et al., 1992, Cherian et al., 1994, Nilsson et al., 1996, Maeda et al., 1997, Liu et al., 2002) and MRI (Hendrich et al., 1999). Despite this wealth of information on changes of CBF after TBI, only few clinical (Bouma et al., 1991, Bullock et al., 1992, Schroder et al., 1995, Garnett et al., 2001, von Oettingen et al., 2002) and experimental studies were designed to investigate changes of CBF in and around cortical contusions (Sutton et al., 1994, Bryan, Jr. et al., 1995, Kochanek et al., 1995, Hendrich et al., 1999, Lundblad et al., 2004, Zweckberger et al., 2006).
One major early finding after contusional TBI is global reduction of cerebral blood flow by ~ 50% of baseline in the whole brain, i.e. not only at or near the area of impact, but also in remote areas of the ipsilateral and the contralateral hemisphere (Cherian et al., 1994, Bryan, Jr. et al., 1995, Kochanek et al., 1995, Hendrich et al., 1999, Liu et al., 2002). The global CBF reduction after TBI depends on the severity of injury (Cherian et al., 1994), lasts more than 3-8 hours (Cherian et al., 1994, Liu et al., 2002), and is restored to baseline values 24 hours after trauma (Bryan, Jr. et al., 1995, Kochanek et al., 1995, Hendrich et al., 1999). Our current data in mice are well in agreement with these findings. We detected also a 50% reduction of CBF in the whole brain immediately after trauma, which resolved 24 hours later. These changes seem also to be dependent on injury severity in the currently used animal species, i.e. in mice, since a recent study using a milder murine CCI model did not detect global CBF changes (Lundblad et al., 2004). Hence, this is the first study to demonstrate that mice show

the same qualitative and quantitative CBF changes after TBI as previously reported in rats thereby demonstrating that mice are valid organisms for this kind of investigation.
Also changes of cerebral blood flow inside cortical contusions seem to strongly depend on injury severity. If the impact is strong enough to irreversibly damage the affected tissue immediately or within the first few minutes after the injury, then low CBF values inside the contusion represent tissue necrosis rather than evolving tissue ischemia as recently demonstrated experimentally (Zweckberger et al., 2006) and previously suggested by results obtained in TBI patients (Schroder et al., 1995, von Oettingen et al., 2002). If the initial impact is not strong enough, contused tissue may well survive as a whole or in part and may therefore show normal or even elevated blood flow values as shown in experimental models of diffuse (Ginsberg et al., 1997, Dietrich et al., 1998) and mild contusional TBI (Lundblad et al., 2004) or in patients with milder trauma (von Oettingen et al., 2002). Accordingly, CBF inside cortical contusions may well be a good marker to differentiate between moderate and severe contusional TBI and to identify patients at risk for secondary insults.
Following severe experimental contusional TBI, i.e. with rapid loss of perfusion inside the impacted tissue like in the current study, the initially healthy tissue in the traumatic penumbra around the contusion will die over time resulting in an increased total contusion volume. The enlargement of a cortical contusion over time is a well known clinical observation and was recently quantified to account for ~30% of the final contusion volume peaking 24 hours after experimental TBI (Zweckberger et al., 2006). Since the expansion of a traumatic contusion can be prevented by decompression craniectomy (Zweckberger et al., 2003b, Zweckberger et al., 2006), by inhibition of cell death signaling, and by activation of cell survival pathways (Plesnila et al., 2007), we termed this process “secondary contusion expansion” (SCE). Despite the mechanistic insight into the pathophysiology of SCE from our and other previous studies, the role of CBF for pericontusional cell death is still unclear. Several factors may account for this fact. Experimental studies using non-invasive imaging techniques to monitor CBF and tissue damage, e.g. by MRI, lack spatial and temporal resolution to track the changes occurring in the rather small traumatic penumbra (Hendrich et al., 1999). Clinical studies addressing the same issue often suffer from high patient heterogeneity, low patient numbers, unavailability of histological data due to the nature of the studies, and/or, most importantly, lack of imaging opportunities early after TBI,when SCE occurs (Bouma et al., 1991, Bullock et al., 1992, Schroder et al., 1995, Garnett et al., 2001, Steiner et al., 2003). Going back to the remaining experimental literature on blood flow changes after contusional TBI there is only the study by Bryan and colleagues left which tries to evaluate if the volume of ischemic contusional tissue is stable after trauma or if it increases over time (Bryan Jr. et al., 1995). The severity of injury in this study was high enough to produce a non-perfused, i.e. necrotic contusion core, although not high enough to induce a global reduction of CBF. Quantification of the volume of ischemic CBF revealed that when data 4 hours after TBI were compared to 30 min after TBI the area of ischemic tissue showed a trend towards increase, but due to a small sample size and large variability of the measurements this increase was not statistically significant (Bryan, Jr. et al., 1995). Our current results are well in agreement with these previous results. Also our ischemic tissue volume data show only a trend to increase from 15 min to 24 hours after TBI (+13%).
Most importantly we assessed the volume of necrotic tissue by cresyl violet staining on sections adjacent to those used for CBF measurements as well and demonstrate that the area of necrotic tissue is larger from the beginning and increases to a much higher degree as com-

pared to the area of severely ischemic tissue (+43%). This finding suggests two important points: 1. that already the primary contusion is not caused by tissue ischemia alone, but most likely also by or together with different mechanisms, e.g. on the cellular/molecular level, and 2. that pericontusional ischemia is most likely not the only responsible factor for secondary contusion expansion as previously anticipated by us and others (Bouma et al., 1991, Bullock et al., 1992, Bryan Jr. et al., 1995, Schroder et al., 1995, von Oettingen et al., 2002, Zweckberger et al., 2006). Accordingly, ischemia seems to be a necessary but not sufficient factor for secondary contusion expansion following TBI.

Conclusion

The present study demonstrates that by using a completely non-invasive protocol absolute values of CBF can be measured by ^{14}C-iodoantipyrine autoradiography reliably and with high precision under physiological conditions and following traumatic brain injury in mice.
Immediately following TBI cerebral blood flow decreases in the whole brain by ~50%. In the contusion proper CBF is reduced by over 90%. While blood flow outside the contusion recovers within 60 min, contusional blood flow does not recover significantly.
The volume of a cortical contusion expands over 24 hours by over 40%, while the volume of severely ischemic cortical tissue remains almost unchanged. These findings suggest that pericontusional ischemia alone is not sufficient for secondary contusion expansion (SCE).

CHAPTER 4. THERAPY: DECOMPRESSIVE CRANIECTOMY

A) Effect of Early and Delayed Decompressive Craniectomy on Secondary Brain Damage after Controlled Cortical Impact in Mice.

Abstract

The timing of decompressive craniectomy for the treatment of increased intracranial pressure (ICP) after traumatic brain injury (TBI) is a widely discussed clinical issue. Although we showed recently that early decompression is beneficial following experimental TBI, it remains unclear to what degree decompression craniectomy reduces secondary brain damage and if craniectomy is still beneficial when it is delayed by several hours as often inevitable during daily clinical practice. The aim of the current study was therefore to investigate the influence of craniectomy on secondary contusion expansion and brain edema formation and to determine the therapeutic window of craniectomy. Male C57/Bl6 mice were subjected to controlled cortical impact injury. Contusion volume, brain edema formation, and opening of the blood–brain barrier were investigated 2, 6, 12, and 24h and 7 days after trauma. The effect of decompression craniectomy on secondary brain damage was studied in control mice (closed skull) and in animals craniotomized immediately or with a delay of 1, 3, or 8 h after trauma. Twenty-four hours after trauma, the time point of maximal lesion expansion (+60% vs. 15 min after trauma) and brain edema formation (+3.0% water content vs. sham), contusion volume in craniotomized mice did not show any secondary expansion; that is, contusion volume was similar to that observed in mice sacrificed immediately after trauma (18.3±5.3 vs. 22.2±1.4 mm^3). Furthermore, brain edema formation was reduced by 52% in craniotomized animals. The beneficial effect of craniectomy was still present even when treatment was delayed by up to 3 h after trauma ($p<0.05$). The current study clearly demonstrates that early craniectomy prevents secondary brain damage and significantly reduces brain edema formation after experimental TBI. Evaluation of early craniectomy as a therapeutic option after TBI in humans may therefore be indicated.

Introduction

Traumatic brain injury (TBI) is the leading cause of death among young adults (<45 years) and one of the major causes of disability in the overall population (Jennett, 1998). The deformation of the brain in the moment of the mechanical impact leads to parenchymal damage, which due to its immediate character is not amenable to therapy. This primary injury is followed by a multitude of delayed secondary events on the cellular and molecular level which continue to cause neuronal damage and ultimately lead to secondary expansion of the primary lesion (Gennarelli, 1993).

Among the mechanisms responsible for secondary brain damage following TBI, brain edema formation resulting in a refractory increase of intracranial pressure (ICP) remains the most common cause for unfavorable outcome (Murray et al., 1999). In addition to treatment strategies recommended on the basis of scientific evidence, for example, hyperosmotic therapy or mild hyperventilation (Maas et al., 1997), decompressive craniectomy has been used intuitively for over a century for the treatment of malignant post-traumatic brain edema (Kocher,

1901, Ogawa et al., 1974, Jennett et al., 1980, Wilberger et al., 1991). Although a recent randomized clinical trial suggests that functional outcome and quality of life may be better in early craniotomised children (Taylor et al., 2001) and data in adults point into the same direction (Polin et al., 1997; Albanese et al., 2003; Meier et al., 2003), the lack of relevant class I evidence still does not allow to draw a definite conclusion about the efficacy of decompressive craniectomy for the treatment of TBI (Winter et al., 2005).

The discussion about the use of decompressive craniectomy after TBI is also fueled by the discrepancy of experimental data (Moody et al., 1968, Cooper et al., 1979, Gaab et al., 1979, Burkert et al., 1989, Rinaldi et al., 1990, Zweckberger et al., 2003). Most studies agree that craniectomy lowers pathological ICP (Moody et al., 1968, Gaab et al., 1979, Rinaldi et al., 1990, Zweckberger et al., 2003); however, the results on brain edema formation and histopathological damage are heterogeneous. For example, Cooper et al. (1979) reported a sevenfold increase in Evans Blue stained brain tissue following cold lesion in craniotomized dogs. Gaab et al. (1979) showed no interruption of edema evolution after craniectomy using the same model in cats, and decompression had neither a positive nor a negative effect on brain edema formation following cold lesion in rabbits (Rinaldi et al., 1990). A recent study from our laboratory using a kinetic TBI model, controlled cortical impact (CCI), demonstrated that morphological brain damage and functional outcome were significantly improved when decompressive craniectomy was performed immediately after trauma (Zweckberger et al., 2003). It remained unclear, however, to what degree craniectomy reduced secondary brain damage, if decompression had an effect on brain edema formation, and if there is a beneficial effect of craniectomy when decompression is delayed for several hours, a condition that often cannot be avoided during daily clinical practice.

Since the development of secondary brain damage following CCI has not been quantified yet, the first aim of the current study was to quantify lesion volume, brain edema development, and blood–brain barrier disruption in this model. In a second line of experiments we investigated the effect of decompressive craniectomy on secondary brain damage and brain edema formation, and determined the therapeutic window of this procedure following experimental TBI.

Methods

Anesthesia and Trauma Application

Traumatic brain injury using a CCI device was induced as previously described (Zweckberger et al., 2003). Briefly, male C57/Bl6 mice (25–28 g b.w., Charles River, Kisslegg, Germany) were anaesthetized in a halothane chamber (4%), and anesthesia was maintained with a face mask using 1.2% halothane, 30% O_2, and 69% N_2O during surgery (<15 min). In animals used for measurements of absolute cerebral blood flow, anesthesia was performed by i.p. injections of medetomidine (0.5 mg/kg b.w., Domitor®, Dr. E. Graeub AG, Basel, Switzerland), fentanyl (0.05 mg/kg b.w, Janssen-Cilag, Neuss, Germany), and midazolam (5 mg/kg b.w., Dormicum®, Roche, Basel, Switzerland). Mice were intubated with an orotracheal tube, and mechanically ventilated using 30% O_2 (MiniVent 845, Hugo Sachs Elektronik, March-Hungstetten, Germany). End-tidal pCO_2, which represents arterial pCO_2, was measured with a microcapnometer (Cl240, Columbus Instruments, Columbus, OH) and adjusted to 35 mmHg by respective ventilation. Blood pressure was measured every minute at the tail using a noninvasive blood pressure monitoring system (RTBP 2000, Kent, OH). A thermostatically regulated, feedback-controlled heating pad was used to maintain rectal

temperature at 37°C (FHC, Bowdoinham, ME). After induction of anesthesia, the head was fixed in a stereotactic frame and a large craniectomy was performed above the right parietal cortex (Fig. 1). Special attention was paid to leave the dura mater intact. CCI (Mouse-Katjuscha 2000, L. Kopacz, University of Mainz, Germany) was performed perpendicular to the surface of the brain (diameter of the impactor tip: 3 mm, impact velocity: 8 m/sec, impact duration: 150 msec, brain displacement: 1 mm). The craniectomy was closed immediately after CCI with the initially removed bone flap using conventional tissue glue (Histoacryl®, Braun-Melsungen, Melsungen, Germany). In animals receiving decompression the craniectomy was left open immediately or was reopened 1, 3, or 8 h after trauma. The skin over the craniectomy was carefully closed, and animals were transferred to an incubator heated to 32°C until recovery of spontaneous motor activity. As indicated previously, craniectomy did not alter brain temperature (Zweckberger et al., 2003).

Experimental Groups

A total of seven experimental groups were investigated. In Group 1, contusion volume was measured by histomorphometry 15 min, 2 h, 6 h, 12 h, 24 h, 4 days, and 7 days after trauma (n = 7 per group) in order to quantify secondary lesion growth. In Group 2, brain water content was quantified 6, 12, 24, and 48 h after trauma to assess the maximum of brain edema formation after CCI injury in mice (n = 6 per group). In Group 3, Evans Blue extravasation was quantified in brain lysates by spectrophotometry in order to assess blood–brain barrier dysfunction (n = 4 per group). In Group 4, we assessed the effect of decompression craniectomy performed immediately after trauma on secondary contusion expansion (n = 6 per group). In Group 5, we measured the effect of decompression craniectomy performed immediately after trauma on brain edema formation (n = 6 per group). In Group 6, we determined the therapeutic window of decompression craniectomy following TBI (n = 7 per group) by performing a delayed craniectomy and assessing the contusion volume 24 h after CCI. In Group 7, cerebral blood flow was measured by ^{14}C-Iodoantipyrine autoradiography 15 min after trauma (n = 5).

Histology and Determination of Contusion Volume

At the end of the observation period, animals were sacrificed by cervical dislocation in deep halothane anesthesia. The brains were carefully removed, frozen in powdered dry ice, and

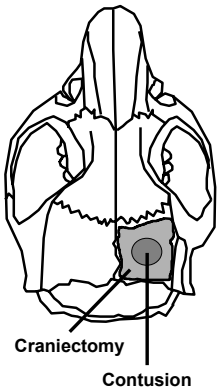

Figure 1. Drawing of a mouse skull indicating the position of craniectomy and brain trauma.

stored at -20°C until further use. Ten µm coronal sections were prepared every 500 µm on a cryostat (CryoStar HM 560, Microm, Walldorf, Germany) and stained with cresyl violet. Sections were photographed with a digital camera system. The contusion area, the area of the non-traumatized tissue of the ipsilateral hemisphere, and the area of the contralateral hemisphere were determined on the digitized images with a standard image analysis software (Optimate 6.51, Media Cybernetics, Silver Spring, MD) by an investigator blinded to the treatment of the animals. In order to correct for the swelling of contused tissue, the contusion area was calculated by subtracting the area of the nontraumatized tissue of the ipsilateral hemisphere from the area of the contralateral hemisphere. Contusion volume was calculated based on the contusion areas corrected for brain edema obtained from 15 sections as following: 0.5 x (A1 + A2 + . . . + A15).

Measurement of Absolute Cerebral Blood Flow

Absolute cerebral blood flow was measured by ^{14}C-iodoantipyrine autoradiography (Maeda et al., 2000). Briefly, after induction of anesthesia and stabilization of mean arterial blood pressure and end tidal pCO_2 mice received 20 µCi ^{14}C-iodoantipyrine by i.p. injection. Thirty seconds later, animals were sacrificed by whole body snap freezing. Thereafter the heart and the brain were removed for quantification of radioactivity in blood and tissue by beta-counting and autoradiography, respectively. Brains were sectioned (20 µm) and exposed to film (Biomax MR, Kodak, Germany) for 8 days with respective radioactive standards (RPA504, Amersham, Piscataway, NJ). Autoradiograms were digitized, and absolute blood flow values were calculated as previously described (Maeda et al., 2000).

Measurement of Brain Water Content

Brain water content was determined as previously described (Vakili et al., 2005). Briefly, 24 h after trauma, mice were killed and brains were removed as described above. Pons and olfactory bulb were removed, and brains were weighed in order to obtain their wet weight (ww). Thereafter brains were dried at 110°C for 24 h for determination of their dry weight (dw). Brain water content was calculated by using the following formula: (ww – dw)/ww x 100.

Evaluation of Blood–Brain Barrier Permeability

Blood–brain barrier (BBB) permeability was evaluated by measuring Evans Blue (EB) extravasation, as previously described (Vakili et al., 2005). Briefly, mice received 4 mL/kg of a 2% EB solution by tail vein injection 30 min after trauma. Twenty-three hours after reperfusion, animals were perfused transcardially with 100 mL of saline solution to wash out intravascular EB. Thereafter, brains were removed, and the hemispheres were separated and weighed. For extraction of EB from brain tissue hemispheres were placed in 2 mL of 50% trichloroacetic acid (wt/vol) and homogenized by sonication. The homogenate was centrifuged at 10,000 rpm for 20 min, and the supernatant was diluted 1:12 with ethanol. EB was measured at an excitation wavelength of 620 nm and an emission wavelength of 680 nm using a fluorescence spectrophotometer (Model 650-10S, Perkin-Elmer, Norwalk, CT). EB concentrations were calculated and expressed as µg/g brain tissue by using a standard curve (100–500 ng/mL).

Statistical Analysis

All data are given as means ± standard error of the mean (SEM) if not indicated otherwise.

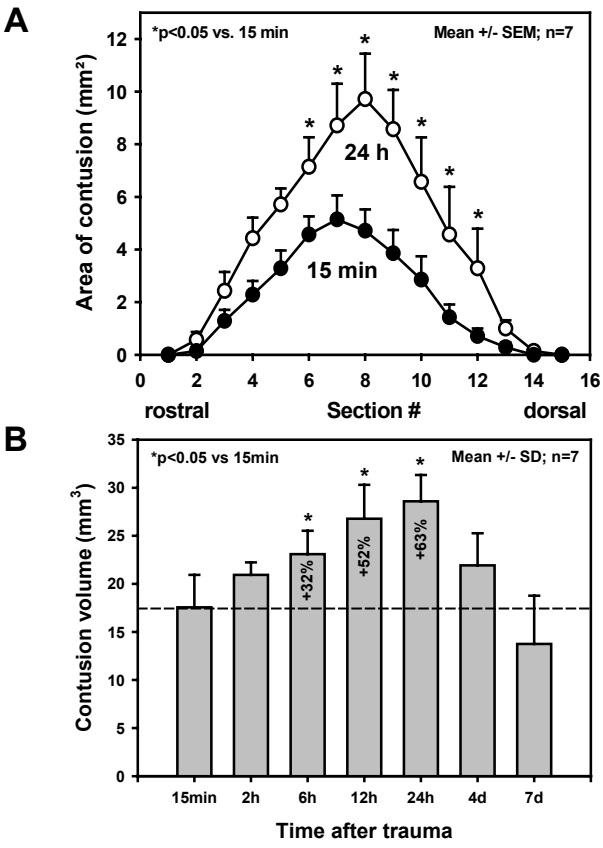

Figure 3. **Contusion area and volume.**
A. Contusion areas from 15 coronal sections prepared throughout the injured brain obtained from animals sacrificed 15 min (black) or 24 h (white) after trauma (n=7 per group). B. Contusion volume of mice sacrificed at different time points after controlled cortical impact (n=7 per group). Contusion volume increases over time and reaches a maximum 24 h after trauma as compared to animals sacrificed immediately after trauma. Removal of dead tissue starting later than 24 h after trauma results in an apparent shrinkage of the contusion on days 4 and 7.

For statistical comparisons, the between-groups Student t-test was used for normally distributed and the Mann-Whitney Rank Sum test for not normally distributed data. Measurements over time (body weight and beam walk) were tested versus baseline with Friedman Repeated Measures analysis of variance (ANOVA) on Ranks followed by Dunnett's All Pairwise Multiple Comparison Procedure as post hoc test. Calculations were performed with a standard statistical software package (SigmaStat 2.0, Jandel Scientific, Erkrath, Germany).

Results

Except for three animals which died from traumatic subarachnoid hemorrhage within 24 h after trauma, no mortality was observed in the present study. Anesthesia and craniectomy did not have any influence on brain histology, functional outcome, or body weight as observed in sham operated animals (anesthesia and craniectomy but no trauma; data not shown).
Mice sacrificed for evaluation of primary brain damage 15 min after trauma showed a large cortical contusion with massive hemorrhage. Visual inspection of freshly prepared coronal brain sections demonstrated widespread hemorrhage within the contused brain tissue (data not shown). Histological staining of coronal brain sections confirmed that the cortical contusion reached down to the medial part of the corpus callosum (Fig. 2A, *see page 134*). Higher magnification photomicrographs demonstrated disruption of almost all cellular elements in

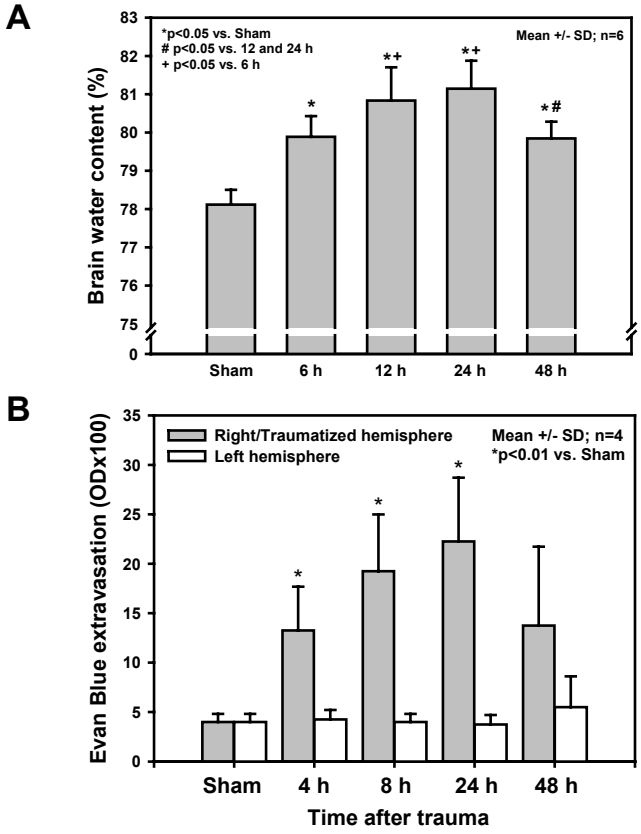

Figure 4. Brain edema formation after controlled cortical injury in mice without craniectomy.
Brain water content (A) as well as Evans Blue extravasation, an indicator of blood–brain barrier disruption (B), show their maximal increase within the first 12 h after trauma. Maximal values for brain water content and Evans Blue extravasation are observed after 24 h (n=6 and n=4 per group, respectively).

the core of the contusion (Fig. 2D) as compared to uninjured cortex (Fig. 2C). Most cells had an irregular shape and pyknotic nuclei indicating cell death (Fig. 2D). Measurement of cerebral blood flow by ^{14}C-iodoantipyrine revealed that the primary contusion did not show any perfusion already at this early time point after trauma (CBF = 0 ± 0 mL/100/mg/min; $p < 0.001$ vs. contralateral cortical blood flow; n = 5; Fig. 2F).

Mice sacrificed 24 h after trauma showed significantly larger contusion volumes as compared to those sacrificed 15 min after trauma (Fig. 2B). The contusion expanded mainly into the lateral cortex, a region of the brain not affected by cell death immediately after trauma. Cells in the lateral cortex were shrunken and showed nuclear condensation although no signs of bleeding or mechanical tissue disruption were observed (Fig. 2E). The expansion of the necrotic contusion into healthy brain was also demonstrated by quantitative analysis (n = 7 per group; Fig. 3A). The contusion area on sections obtained from the center of the contusion more than doubled during the first 24 h after trauma. During the same period of time the traumatized hemisphere swelled by less than 10% (data not shown).

Analysis of contusion volume over time revealed that the area of necrotic tissue expanded continuously during the first 24 h after trauma by 62% reaching a maximum of 163% of the volume of the primary contusion (Fig. 3B). During the next 6 days the contusion volume gradually decreased reaching 78% of the volume of the primary contusion.

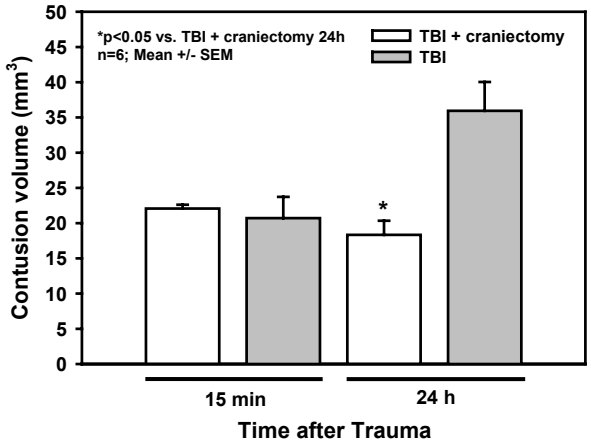

Figure 5. Contusion volume of craniectomized (open bar; n=6) and not craniectomised mice (filled bar: n=6) 15 min and 24 h after controlled cortical injury. Craniectomized animals do not show secondary brain damage.

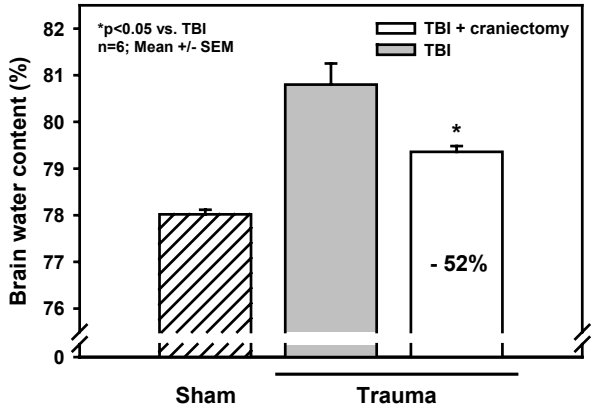

Figure 6. Brain water content of sham-operated (sham; n=6), non-craniectomized (filled bar; n=6) and craniectomized mice (open bar; n=6) 24 h after trauma. Brain edema formation is reduced by 52% in craniectomized animals.

Brain edema, quantified by the measurement of brain water content, developed most quickly during the first 6 h after trauma and reached its maximum at 12–24 h postinjury (Fig. 4A). Forty-eight hours after trauma, brain water content decreased indicating resolution of brain edema formation, that is, net efflux of brain edema fluid from the injured tissue. This finding is also supported by the quantification of blood–brain barrier disruption using Evans Blue (Fig. 4B). More than 80% of Evans Blue extravasated into the brain parenchyma within the first 8 h after trauma. Thereafter, little extravasation was observed. Forty-eight hours after trauma tissue Evans Blue decreased thereby indicating closure of the blood–brain barrier.

After determining the time course of lesion expansion and brain edema formation, i.e., the development of secondary brain damage following CCI, we quantified the effect of decom-

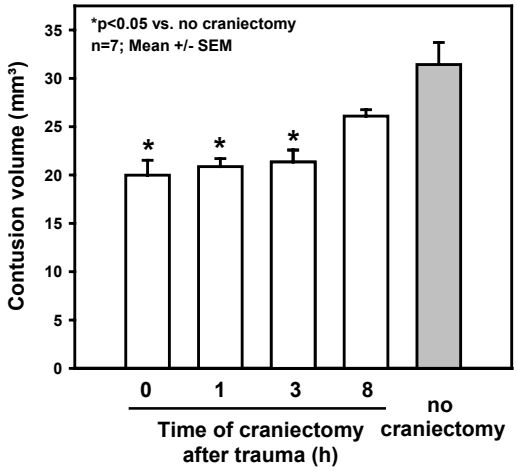

Figure 7. Therapeutic window of decompressive craniectomy after controlled cortical injury. Animals of the control group (closed bar; n=7) were not craniectomized, while the animals from the other groups (open bars; n=7 each) were craniectomized immediately, and 1, 3, or 8 h after trauma. Only animals decompressed 1–3 h after trauma showed a significant prevention of secondary brain damage.

pression craniectomy on lesion expansion (Fig. 5). Primary lesion volume assessed 15 min after trauma was not different between craniectomized (open bars) and non-craniectomized (closed bars) mice; however, while lesion volume showed an expansion of 89% in non-craniotomized animals, no lesion expansion was observed in craniectomized mice within the first 24 h after trauma (Fig. 5).

The significant reduction of secondary brain damage was also supported by the decrease of brain edema formation in the craniectomized animals. Brain water content 24 h after trauma, the time point of maximal brain swelling, was reduced by more than 50% in the craniectomy group (Fig. 6).

In the last series of experiments, we aimed to determine the therapeutic window of decompression craniectomy. For this purpose, we used non-craniectomized animals as controls (Fig. 7, closed bar) and compared their contusion volume 24 h after trauma to mice craniectomized immediately 1, 3, or 8 h after controlled CCI (Fig. 7, open bars). Craniectomy performed within the first 3 h after trauma significantly prevented lesion expansion. The contusion volume assessed in the noncraniectomized group was about 50% larger as compared to the contusion volumes of the animals subjected to early craniectomy, indicating that most of secondary lesion expansion was prevented by early craniectomy. If performed too late (i.e., 8 h after trauma), contusion expansion progressed already so far (cf. Fig. 2) that decompression could not prevent secondary brain damage.

Discussion

In the present study, we demonstrate that decompressive craniectomy (DC) reduces brain edema formation by more than 50% and completely prevents secondary brain damage as morphologically defined in this study when performed early enough (i.e., during the first 3 h after trauma).

Together with our previous report (Zweckberger et al., 2003), we clearly demonstrate that early craniectomy significantly reduces brain edema formation, abolishes ICP increase, prevents secondary brain damage, and improves functional outcome following TBI in a kinetic, that is, clinically relevant, animal model.

As discussed previously (Zweckberger et al., 2003), the currently used CCI device was specifically developed for mice thereby reducing inter- and intraindividual data scattering to a minimum (cf. Figs. 3, 5, and 7). Together with the randomized and blinded experimental procedures, the fact that DC does not affect systemic parameters and does not, as intuitively expected, cause hypothermia (Zweckberger et al., 2003) we believe that the current experiments were carried out with the most possible care to avoid all relevant sources of experimental artifacts which may confound the interpretation of our data.

One aim of the current study was to assess the effect of decompression craniectomy on secondary brain damage. We recognized that although the concept and mechanisms of secondary brain damage are discussed among the scientific community for decades (Baethmann et al., 1988; Gentleman, 1990), its clear morphological definition and experimental quantification following experimental TBI was missing yet. In the current study we defined secondary brain damage as the difference between the morphological damage observed immediately after trauma, i.e. the primary damage, and the maximal damage observed thereafter. Using this definition we determined that the primary damage in a mouse subjected to controlled cortical impact injury has a volume of about 20 mm^3 (Figs. 3 and 5). The injury is characterized by massive hemorrhage on the brain surface and in the parenchyma, as also observed following human TBI (Ribas et al., 1992), and pan-necrosis of the affected tissue (Fig. 2). The fact that these changes were present already 15 min after trauma suggest that hemorrhage and cell death were caused by disruption of blood vessels and cell membranes as a direct result of the mechanical impact. The assumption that the primary lesion/contusion consists of non vital tissue is also supported by the fact that no cerebral blood flow was detected in this region of the brain already 15 min after trauma (Fig. 2F) as also observed by others in a similar time window (Kochanek et al., 1995; Bryan, Jr. et al., 1995).

According to the above mentioned definition, secondary brain damage is the portion of the damage which develops over time on top of the primary insult. Our experiments show that the expansion of the contused brain area could be observed in every single investigated brain section obtained 15 min and 24 h after trauma and resulted in an almost doubling of the contusion area (Fig. 3A). Since opening of the blood-brain barrier and brain edema formation, which developed during the same period of time (Fig. 4), resulted in a hemispheric swelling of less than 10%, the observed expansion of the contusion by 100-200% (Fig. 3A) can by no means be explained by mere swelling of the affected hemisphere. The assumption that quantification of contusion expansion is not affected by brain swelling is also supported by the fact that contusion expansion can be completely prevented by craniectomy while brain edema is still present (Figs. 5 and 6) and by the finding that the expanded contusions do not only increase in size but also affect anatomical areas of the brain not affected by the primary lesion, e.g. the lateral cortex and the hippocampus (Fig. 2A,B). These results are also supported by the calculation of lesion volume which revealed that cortical contusions following CCI in mice expanded from ~20 mm^3 immediately after trauma to a maximum of ~30 mm^3 24 h later (Figs. 3 and 5) indicating that 35-40% of the final lesion develop secondarily and are therefore amenable to therapeutic interventions. Interestingly, our previous experiments on the therapeutic efficacy of decompression craniectomy showed that decompression re-

duced lesion volume 24 h after trauma by 39.5% (from 30 to 18 mm^3) (Zweckberger et al., 2003) indicating that craniectomy may almost completely reduce secondary brain injury. The present results substantiate these findings by demonstrating that decompression craniectomy indeed completely prevents contusion expansion (Fig. 5).

The complete prevention of contusion expansion, i.e. secondary brain damage, by decompression stresses the utmost importance of increased ICP for the pathophysiology of secondary brain damage following TBI and sheds new light on the mechanisms how a cerebral contusion may expand over time. Although many studies stressed the role of molecular mechanisms for post-traumatic cell death, e.g. apoptosis or free radical formation (Raghupathi et al., 2000; Lewen et al., 2000; Fiskum, 2000; Eldadah et al., 2000), it remained unclear how these events were related to the daily observations made by clinicians during the treatment of TBI patients. Our results clearly indicate that the formation of brain edema originating from the primary contusion is the first step in a cascade of events ultimately leading to delayed neuronal cell death and secondary brain injury. The evolving ICP increase (Zweckberger et al., 2003) reduces perfusion pressure thereby causing a reduction of cerebral blood flow. This may not be relevant for healthy brain tissue, however in the tissue at risk, i.e. the still viable tissue surrounding the primary lesion, where blood flow is affected by the primary insult (Bullock et al., 1992), a slight reduction of blood flow may already have detrimental sequels, e.g. further reduction of blood flow by astrocytic swelling (Bullock et al., 1991), the induction of apoptosis, and the formation of free radicals (Raghupathi et al., 2000; Lewen et al., 2000; Fiskum, 2000; Eldadah et al., 2000). The addition of further damage to the primary lesion increases the area of tissue and vessel damage and initiates a vicious circle by further bloodbrain barrier disruption and brain edema formation. This pathophysiological concept is clearly supported by our previous (Zweckberger et al., 2003) and current data showing that the pathological ICP increase is the cause of secondary lesion expansion and by demonstrating that brain edema formation is reduced by ~50% if secondary brain damage is prevented. Accordingly, these data show that in the current trauma model half of the edema originates from the primary lesion, i.e. it is not preventable by decompression craniectomy, while the other half develops due to secondary mechanisms and can be prevented by decompression.

The proposed pathophysiology of secondary lesion expansion, i.e. a slow starting phase ultimately followed by a self amplifying final phase, is further supported by the fact that decompression during the first 1-3 hours after TBI blunts secondary lesion expansion to a high proportion, while later craniectomy is not or only marginally effective (Fig. 7).

The current experiments clearly demonstrate that decompressive craniectomy completely prevents secondary brain damage when performed within the first few hours after experimental TBI. Although care should be taken when extrapolating experimental results in rodents to the situation in humans our current results suggest that early decompressive craniectomy may have a clinical potential.

Our results further demonstrate that timing seems to be of utmost importance and that decompression has to be performed as early as possible in order to fully exert its therapeutic potential.

CHAPTER 4.
B) Effect of Decompressive Craniectomy on Cerebral Blood Flow after Controlled Cortical Impact in Mice

Abstract
Objectives: Previous studies have shown positive effects of decompression craniotomy (DC) on secondary contusion growth after traumatic brain injury (TBI). Here the effect of DC on cerebral blood flow (CBF) and hippocampal function is examined. *Materials/Methods*: CBF was measured in one group C57Bl6 mice 3 hrs following controlled cortical impact by ^{14}C-iodoantipyrine autoradiography. Contusion volumes were quantified on parallel sections stained by Nissl and compared to areas with CBF < 10%. Intra- and peri-contusional CBF was compared to the corresponding areas of the contralateral hemisphere. A second group of mice was treated similarly and tested with the Object Recognition Test (ORT) 7 days after injury. *Results*: Physiological parameters were stable throughout the experiment. Decompressed mice had significant smaller contusion volumes 3 hours after injury (20 mm^3 vs 36 mm^3). The volume of CBF < 0.10 ml/min/g was significantly smaller in craniectomized mice (8 mm^3 vs 20 mm^3). CBF was significantly decreased (30% of control) ipsilateral to injury in non-decompressed animals. Both closed and craniectomized animals had significantly reduced CBF ipsilaterally, of which the closed group showed significantly lower values. Craniectomized mice performed worse on the memory test (object recognition test). *Conclusion*: Early DC improves CBF in both cortex and hippocampus, but not memory.

Introduction
Traumatic brain injury (TBI) is a major health and socio-economic problem throughout the world. In western industrialized society TBI is the leading cause of death and disability among young people (Jennett, 1996). A recently revived and by some clinics never abandoned surgical therapy is decompressive craniectomy (DC). DC as therapy for uncontrollable raised intracranial pressure (ICP) after TBI has been investigated in the 1960s&70s (Kjellberg and Prieto 1971; Ransohoff 1971; Britt and Hamilton, 1978; Cooper 1976, Venes and Collins 1975). The suggestion that brain swelling could worsen and induce herniation through the DC opening made many neurosurgeons abandone this option for therapy. In the late 1980s new criteria for when and how to perform a DC were introduced among other therapeutic strategies to reduce raised ICP (Alexander 1987, Gaab 1990, Grande 2002, Marshall 2000, Naredi 1998, 2001).
The exact role of DC within TBI treatment has been unclear. Pre-clinical and clinical studies carried out in the past 10 years have shown DC possibly useful in the treatment of severe, focal, TBI (Zweckberger 2003, 2006, Taylor 2001, Albanese 2003, Figaji 2003, Guerra 1999, Hejazi 2002, Kontopoulos 2002, Messing-Junger 2003, Polin 1997, 2003, Schneider 2002, Whitfield 2001). In the pediatric population DC has already been accepted as valid 'second tier' therapy for raised ICP after TBI (Taylor 2001, Jagannathan et al., 2007; Rutigliano et al., 2006). In adult TBI Rosenfeld (2006) advocates early generous DC in war victims of cranial blast injuries. However, circumstances during war do differ from peaceful, optimized and maximized, hospital conditions. In stroke, early decompressive surgery has been proven to be beneficial in a pooled analysis of three randomised controlled trials (RCTs) (Vahedi et al.,

2007). This is not yet the case in adult TBI patients. Early DC, when performed according to current, developing, criteria, could be a more effective treatment because ICP is reduced earlier after injury (Skoglund et al., 2006). The use of different protocols by several clinics has been made it impossible to investigate the effects on outcome. Prospective RCTs of DC in TBI are currently running worldwide (RESCUEicp and DECRA) hopefully giving an answer on the effect of DC on clinical outcome.
Two recent preclinical studies using a kinetic contusion TBI model, controlled cortical impact (CCI), demonstrated that early DC reduces secondary damage and edema formation and improves functional outcome significantly when performed early after TBI (up to three hours) (Zweckberger et al., 2003, 2006). By opening the skull ICP reduces almost immediately; pathophysiological pathways leading to ischemia, apoptosis and necrosis are discontinued. Cerebral blood flow (CBF) is likely to be altered by DC after TBI. How CBF is affected exactly has scarcely been investigated (Yamakami and Yamaura 1993a,b; Lundblad et al., 2004, 2007). Therefore we investigated CBF in the cortex three hours after CCI injury in craniectomized mice using ^{14}C-autoradiography. Hippocampal CBF was measured specifically and hippocampal function was tested accordingly with the Object Recognition Test.

Methods and Materials

Study design and animal care

Experimental head injury was induced in C57/Bl6 mice according to the controlled cortical impact model. All animals were randomized to treatment by decompressive craniectomy (craniectomy), supportive care (closed) or non injured (control). Two sets of experiments were performed: in the first (N=6-7) animals were sacrifised three hours after injury for CBF determination by autoradiography and histologic analysis of contusion volumes. In the second set (N=6-7) animals were allowed to survive and hippocampal function measured on day 7 with the object recognition test. All quantifications were conducted in a blinded fashion. All efforts were made to minimize suffering and the number of animals according to the guidelines of the German animal protection law and derived guidelines on the ethical use of animals. Animals were kept under controlled light and environmental conditions (12 h dark/light cycle, 23 ± 1ºC, 55 ± 5% relative humidity) and had free access to food (Altromin, Germany) and water.

Anesthesia

For induction of experimental head injury male C57/Bl6 mice (25 – 28 g, Charles River, Kisslegg, Germany) were anaesthetized in a halothane chamber (4%), whereafter anesthesia was maintained with a face mask using 1.2% halothane, in a mixture of 30% O_2 and 70% N_2O.
Mice used for measurements of absolute cerebral blood flow by autoradiography were anesthetized by i.p. injection of medetomidine (0.5 mg/kg b.w., Domitor®, Dr. E. Graeub AG, Basel, Switzerland), fentanyl (0.05 mg/kg b.w, Janssen-Cilag, Neuss, Germany), and midazolam (5 mg/kg b.w., Dormicum®, Roche, Basel, Switzerland). Mice were then intubated with an oro-tracheal tube, and mechanically ventilated using 30% O_2 in room air (MiniVent 845, Hugo Sachs Elektronik, March-Hungstetten, Germany). End-tidal pCO_2 was measured with a microcapnometer (Cl240, Columbus Instruments, Columbus, USA) and adjusted to 35-40 mmHg by respective ventilation. Blood pressure was measured at the tail using a non-invasive blood pressure monitoring system (RTBP 2000, Kent, USA). A thermostatically

regulated, feedback-controlled heating pad was used to maintain rectal temperature at 37°C (FHC, Bowdoinham, USA).

Controlled cortical impact (CCI)
Traumatic brain injury was performed as described previously by Zweckberger et al. (2003). Briefly, after induction of anesthesia the head was fixed in a stereotactic frame and a large craniectomy was performed above the right parietal cortex using a high speed drill. Special attention was paid to leave the dura mater intact. CCI (MouseKatjuscha 2000, L. Kopacz, University of Mainz, Germany) was performed perpendicular to the surface of the brain (diameter of the impactor tip: 3 mm, impact peak velocity: 8 m/s, impact duration: 150 ms, indentation: 1 mm). Thereafter, the craniectomy was either closed with the initially removed bone flap using conventional tissue glue (Histoacryl®, Braun-Melsungen, Melsungen, Germany), or the bone flap was left out following randomisation. The skin over the craniotomy was carefully closed and animals were transferred to an incubator heated to 35°C until recovery of spontaneous motor activity (within minutes).

Quantification of cerebral blood flow by autoradiography
Absolute cerebral blood flow was measured by ^{14}C-iodoantipyrine autoradiography 3 hours after injury (Maeda et al., 2000). Briefly, after induction of anesthesia and stabilization of mean arterial blood pressure and end tidal pCO_2 (35-40 mmHg) mice received 20 µCi ^{14}C-iodoantipyrine by intraperitoneal injection. Thirty seconds afterwards, animals were sacrificed by whole body snap freezing, and stored at - 80ºC untill further use. Coronal sections (20 µm) were collected every 500 µm from the brains on a cryostat (CryoStar HM 560, Microm, Walldorf, Germany) and exposed to autoradiography film (Biomax MR, Kodak, Germany) for 9-11 days with respective radioactive standards (RPA504, Amersham, Piscataway, NJ, USA). A bloodsample was taken from the frozen heart and the amount of [^{14}C]-radioactivity was measured by ß-counting as an internal standard. Autoradiograms were processed as well as previously described by Maeda et al. (2000). Briefly, autoradiograms were digitized with a CCD camera connected to an image processing system (ImageMG, NIH, Bethesda, MD, USA). Absolute CBF was quantified in six regions of interest (ROIs) in and around ipsilateral contused (ROI 4-6) and noncontused contralateral (ROI 1-3) brain parenchyma (fig. 1) (Maeda et al., 2000).

Quantification of brain contusion
Parallel radioactive sections were stained by cresyl violet (Zweckberger et al., 2003). The contusion area of each section was measured on digitized images with standard image analysis software (Optimate 6.52, Media Cybernetics, Silver Spring, MD, USA). The volume of necrosis (Vi) was calculated based on the contusion areas (A) obtained from 15 sections by the equation: Vi = 0.5 x (A1+ A2+…+A15).

Quantification of hippocampal function: the Object Recognition Test
Memory function was evaluated by the Object Recognition Test (ORT) as described previously in a second group 7 days after injury (Ennaceur and Delacour, 1988). Briefly, mice were placed in the test-cage for 1 hr on day 6 after injury for acclimatization. On day 7 the mice were replaced in the same cage for 5 min. The cage contained then 2 similar objects, and time of exploration was recorded for each object (right versus left). Three hours later

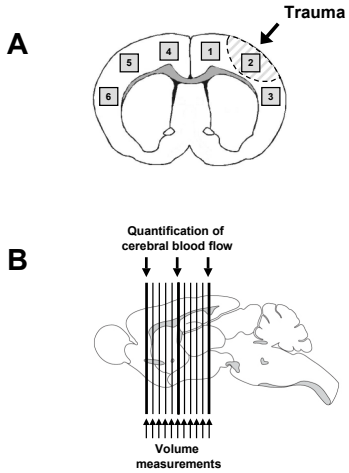

Figure 1 (A) Localization of regions of interest (ROI) used for measurement of cerebral blood flow and (B) coronal plains from where coronal brain sections were taken for quantification of cerebral blood flow and volume of ischemic and contused brain tissue. (*for larger display see page 59*)

mice were placed in the testcage again. This time though, the left object was exchanged for a 'new' object. Again, time of exploration of both objects was recorded during 5 min. Mice will spend an equal amount of time at both objects when not recognizing an object, as all are supposed to in the first baseline test (expected time distribution 50%-50%). During the second test, however, healthy mice recognize the 'old' object, and therefore spend more time at the 'new' object (25%-75%). Non-traumatized healthy mice were tested as controls.

Statistical analysis

Analysis was performed with the SigmaStat 2.0 software package (SPSS inc., Richmond, CA). Data are presented as means +/- SEM or as indicated in the legends. Values were compared by independent-samples T test. Statistical differences are presented at probability levels of $p<0.05$, $p<0.01$, and $p<0.001$.

Results

The contusion volume was significantly smaller in the craniectomy group than in the closed group (fig. 2, *see page 135*, and 3). The average contusion volume, quantified in Nissl-stained sections, was 30.5 $mm^3 \pm 2.70$ (SEM) in closed animals and 17.04 $mm^3 \pm 1.43$ (SEM) (fig. 3). The contusion of the craniectomy group do not differ significantly from (historical) data of the primary contusion volume of the same injury severity and depth (15 minutes after injury) no significant contusion expansion is detected (data not shown). This concurs with our earlier study on DC.

In both groups the volume of non-perfused tissue (CBF < 10%) was significantly smaller than the contusion volume in both groups (closed: 20.29 $mm^3 \pm 2.41$ (SEM), craniectomy: 7.98 $mm^3 \pm 1.21$ (SEM); fig. 3). Figure 2 illustrates a visible discrepancy between non-perfused brain and contused/necrotic brain tissue area in closed animals 3 hours after injury. After DC part of the contusion is pressed out of the skull and is thereby passively relieved. Thus CBF was present in the penumbra, since volume of non-perfused tissue was significantly smaller in both groups when compared with contusion volume.

Figure 4 (*see page 136*) shows quantification of absolute CBF in the cortex 3 hours after injury in the six ROIs. In the contusion region, closed and craniectomized animals showed a significant reduction of CBF of >90–50%. In the occipital region though, CBF was less

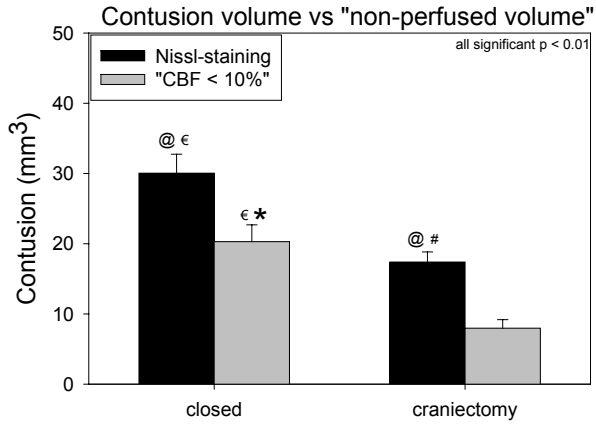

Figure 3. Contused versus non-perfused parenchyma.
Volume of contused (black) versus volume of brain parenchyma with CBF < 10% of baseline (grey) 3 hr after TBI in closed (left) and craniectomized (right) animals (n= 5-10).

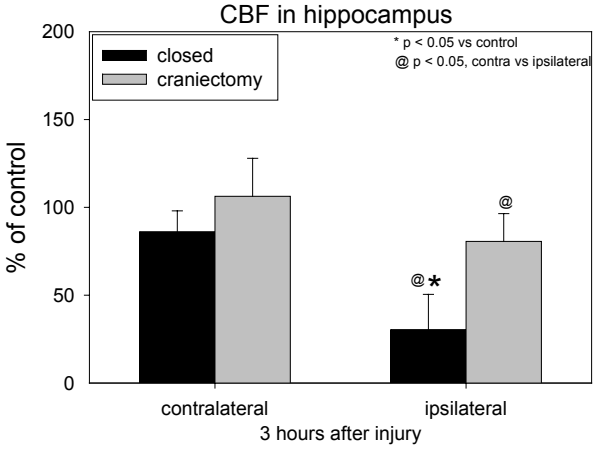

Figure 5. Quantification of absolute CBF in the hippocampus.
CBF in contralateral (left) and ipsilateral (right) to injury in closed (black) and craniectomized (grey) animals. (*/@ $p < 0.05$, n=6-7).

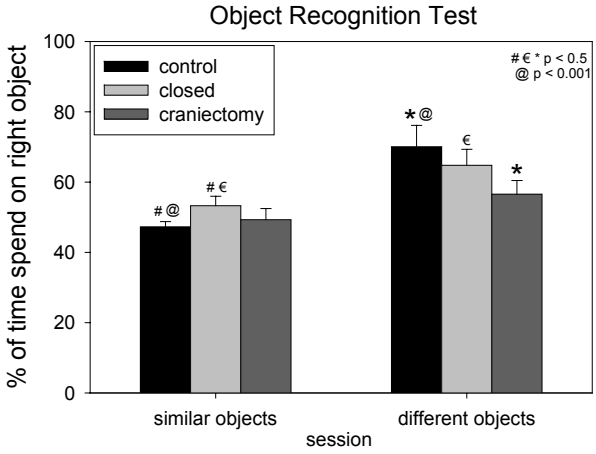

Figure 6. Object Recognition Test.
Percentage of time spend on the right object in control (black), closed (light grey) and craniectomized (dark grey) animals in respect of exploration of 2 similar objects (left) and 2 different (1 old, 1 new) objects (right). (#/€/* $p < 0.05$, @$p < 0.001$, n=6-7)

reduced in the craniectomized group (1.2 % vs 21.0 %). (fig. 4D). In the ipsilateral cortex, in the medial frontal part (ROI 4), closed animals showed a significant reduction when craniectomized did not (fig 4B). This would concur with the contusion expansion suggested in figure 2. Lateral to the contusion (ROI 6) both groups showed a trend towards a reduced CBF frontal, parietal and occipital, though few significant. The closed group showed a significant reduction parietal. The craniectomized group, on the other hand, showed a significant reduction in the occipital ROI (fig. 4F). In the contralateral cortex no significant difference was measured between the closed and craniectomized group (fig. 4A,C,E), nor in comparison to control values (data not shown).
CBF in the hippocampus on the ipsilateral, injured, hemisphere was in both groups significantly decreased. In the craniectomy group CBF in the hippocampus of the ipsilateral hemisphere was significantly higher than in the closed group (80.60% ± 15.81 (SEM) vs. 30.41% ± 20.07 (SEM), resp.; fig. 5). Contralateral, the closed group showed a trend towards reduction of CBF (86.16% ± 11.81 (SEM)).
Hippocampal function was analyzed by the object recognition test. No beneficial effects of DC were found (fig. 6). The closed group recognized the old object significantly better than the craniectomy group. However, this implies that the test was impure. Usually injured animals are not capable of recognizing the old object. Reasons for this discrepancy are unclear.
Unexpected mortality was 1 of 14 mice in both experiments (3 hours and 7 days).

Discussion

Two recent studies from our laboratory using CCI injury, demonstrated that DC reduces contusion expansion and brain edema formation, and improves functional outcome significantly when performed up to three hours after TBI (Zweckberger 2003, 2006). The way DC affects pathophysiological mechanisms besides reducing ICP itself is scarcely known. A vicious circle of known pathophysiological pathways leading to ischemia, apoptosis and necrosis is discontinued when ICP is reduced. Therefore, CBF was likely to be altered and improvement on neurological functioning expected.
CBF was measured using ^{14}C-iodoantipyrine autoradiography (Engel et al., 2007 (chapter 3b); Maeda et al., 2000). This method is from a practical point complicated, but is possible to perform in mice. Autoradiography still is the golden standard for CBF measurements. This method was therefore first choice for this study. It was previously performed in mice after CCI by Lundblad et al. (2004 and 2007). These investigators had used the same injury device and the same sort of mice, but different injury parameters. They used mild injury that didn't cause a real primary contusion. Therefore our present data aren't comparable with their previous ones. Neither did they use whole body snap freezing, but decapitated mice simultaneously with the last blood sample. The authors opinion is that whole body snap freezing is the best, and most simultaneous, way of harvesting heart blood samples and brains of the same timepoint, with loosing least radioactivity.
CBF and contusion volume are measured only at 3 hours after injury/DC. Reason for this is that past this timepoint edema will develop in closed animals (Zweckberger et al., 2006), which distorts a pure comparison of CBF between closed and craniectomized animals. Because DC is a permanent open system ICP will not raise after that time in mice (Zweckberger et al., 2006).
Contusions in craniectomized animals 3 hours after injury didn't expand during previous experiments (Zweckberger 2003, 2006). Nor do our current data show expansion of this volume

at this timepoint in craniectomized animals. Besides contusion volume we have measured CBF after CCI injury previously which showed no expansion of non-perfused brain volume over time, between 15 min and 24 hours (Engel et al., 2007). In the present study 3 hours after injury non-perfused brain volume was significantly smaller in craniectomized animals than in closed ones. This means that perfusion around the contusion was significantly better after early DC.

In our previous study CBF was reduced 15 minutes after injury throughout the whole brain (Engel et al 2007). A reduction of approximately 50% was shown contralateral. Twenty-four hours after injury CBF recovered to control values. Our current data show that the time window for recovery is smaller, already within 3 hours after injury.

In the contusion, that was significantly smaller in the DC group, both groups showed a reduction of CBF of >90 – 50%. In the occipital region though, CBF was reduced less in the DC group. This can be explained by the fact that in closed animals contusions expand into the occipital cortex, and emphasizes thereby that contusions do not expand after early DC.

In our animal model the contusion is evacuated spontaneously by leaving the DC open. Only skin closes on top of it. This automatically leaves space for the hippocampus and subcortical nuclei, which are otherwhise compressed by the expanding hemorrhagic contusion. We hypothesized that this leads to an improved function of these brain regions. As expected, CBF measured in the hippocampus on the injured side was reduced in the closed group, whereas in craniectomized animals no significant reduction was measured. Surprisingly, hippocampal function was not better in craniectomized animals, measured by ORT. The object recognition test has been proven an effecitve method in another injury mouse model (closed head injury) (Shein et al., 2005; Beni et al., 2006; Yatsiv et al., 2005). Unfortunately ORT has failed in our investigation, because control traumatized animals seemed to have either not enough hippocampal damage, or have recovered quicker as expected. By no means a beneficial effect on hippocampal function in combination with early DC was shown.

Clinically, improved outcome expressed in Glasgow Outcome Scale (GOS) has been published previously (Aarabi et al., 2006, Taylor et al., 2001, Bayir et al., 2003, Yamakami and Yamaura, 1993). Jaeger (2005) and Stiefel (2004) have shown that brain tissue oxygen ($PbrO_2$) improved immediately in the contusion area. Translation of preclinical data into the clinic is always complicated. Our present results differ from the few clinical data avaible on CBF and DC (Yamakami 1993). Yamakami et al. (1993) report on CBF measurements in craniectomized patients after TBI. CBF increased immediately underneath the area of decompression. This differs from our rodent model, where an apparent focal contusion is made and CBF isn't detectable. Partially this can be explained by the fact that contusions in human beings are more heterogeneous than the standardized contusion in mice, in which vasculature is damaged acutely in the primary contusion.

In a pediatric population early DC has been accepted as a legitimate treatment for uncontrollable raised ICP (Taylor et al., 2001). Taylor and collaegues performed the first RCT to investigate DC in a pediatric population. Although the sample size was small (n=27 in seven years) a better outcome was seen after DC. Subsequently two multicentre RCTs have been commenced in Australia (DECRA) and other countries (RESCUEicp). These RCTs will inform us in a few months to years whether early DC indeed will grow up to be a legitimate therapy instead of a "second tier" therapy. Our preclinical data show that besides other pathophysiological parameters CBF is improved after TBI in mice, and the authors would like to advocate a more mature position of early DC in the treatment of uncontrollable raised ICP

CHAPTER 5. GENERAL DISCUSSION

The overall aim of this thesis was to investigate the importance of ischemia in traumatic brain injury from different perspectives, i.e. epidemiology, pathophysiology and therapeutic options, hypothesizing that the role of ischemia is essential in secondary brain damage after TBI. Although many preventive strategies such as motor cycle helmets and seat belts have reduced the incidence of severe TBI in the past, incidence has remained stable in an urban area between 1990-2000 (7.3 per 100.000) (see chapter 2A). The start of every medical query is epidemiology: incidence, outcome and potential prognostic factors of TBI, for many factors might influence (patho)physiological conditions and outcome. A problem needs to exist before we can explore, investigate its processes and attempt to solve it. Outside the city boundaries incidence might have been higher because the majority of high-velocity injuries occur here. Young men are most affected by TBI. Despite the preventive strategies mentioned previously road traffic accidents (RTA) have always been and nowadays still are the major cause of injury. Our urban data though, give the impression this number has decreased. Other studies, not performed within city limits show higher rates of RTAs (Butcher et al., 2007). Conversely, the amount of falls will increase. The population is ageing; therefore more elderly TBI patients can be expected in the future (Sundstrom et al., 2007). A significant increase of elderly patients was found between 1990 and 2000 (chapter 2A). This 'second age peak' of age related incidence will develop into a serious impediment to society, leading to both care and financial difficulties. Fall as cause of injury will increase as well with this trend (Butcher et al., 2007). Overall mortality is higher in the present study: 45.8%. Lower numbers had been expected because optimal care was possible within 45 min after injury. However, other studies differ from our findings because more pre-hospital deaths were included by systematic pre-hospital registration in chapter 2A. The higher mortality among elderly and the growing incidence of TBI in the present study will be responsible for a small part of higher mortality. Of the patients that survived more than half was discharged successfully, living a life outside health care facilities with none to minor disabilities. One third remained disabled and only 1.4% was discharged in a persistent vegetative state. These numbers hardly differ from previously published studies (Maas et al., 1997, Marshall, 2000, Thornhill et al., 2001).
Prognostic factors in chapter 2A were age, cause of injury, initial GCS and AIS_{head}. These prognostic factors have been studied before in smaller study samples and showed to more or lesser extent the same effect (Hukkelhoven et al., 2003, Chesnut et al., 1995, Manley et al., 2001). In chapters 2B and 2C several factors were investigated in more detail. High age is associated with worse outcome (OR 2.14), with a change point at 30 years. African-American people had a higher risk for poorer outcome than Caucasians. Several reasons can be taken into consideration; lower social integration (Hart et al., 2005), lower educational level (Harrison-Felix et al., 1998, Jorge et al., 1994), lower pre-morbid employment (Burnett et al., 2003, Wagner et al., 2000), higher drug abuse rates pre-injury (Wagner et al., 2000) and worse access to optimal acute care and rehabilitation (Bazarian et al., 2003, Burnett et al., 2003). None of these factors have been investigated thoroughly yet. A weak association exists between lower educational level and worse outcome, hypothesizing that less educated people come from societies with lower socio-economic status and thereby have less ability to access the correct care and are psychologically less able to fully benefit rehabilitation treatment (Johnstone et al., 2003). After years of discussion gender is no prognostic factor in the present, larger, study. Even when corrected for age, and thereby eliminating the difference between

pre- and postmenopausal women, no significant difference was found. Hypotension, hypoxia and hypothermia are different prognostic factors. An insult of either factor is associated with poorer outcome (ORs 2.7, 2.1, 2.2 respectively). Several insults of one or a combination of hypotension and hypoxia lead to worse outcome than one insult alone (Chapter 2C, Manley et al., 2001). They merge into an evil triad, causing cumulative secondary brain damage. These factors differ from e.g. age in that these directly change physiological conditions in the brain into e.g. decreased CBF. Hypothetically hypoxia, hypotension and hypothermia can be prevented or treated. Unfortunately comorbidity overrules prevention or treatment chronically through i.e. cardiovascular disease. Acute comorbidity is caused by polytrauma, e.g. (excessive) extracranial bleeding, or drugs needed for intubation leading to hypotension. The current studies proved stronger as well as previous smaller studies the detrimental effects of these prognostic factors (Chapter 2A,B,C, Hukkelhoven et al., 2003, Chesnut et al., 1993, Manley et al., 2001, Wagner et al., 2000). These facts will assist physicians with decision-making in the future.

The pathophysiology of TBI has been investigated to a large extent during the past decades, but certainly not everything is clear. Secondary brain damage caused by ischemia was investigated in chapter 3. Ischemia itself leads to cell death *in vitro* also when superimposed by increased CO2 after mechanical injury (chapter 3A). Reactive oxygen species are generated after injury (Leker and Shohami, 2002, Samdani et al., 1997). Superoxide, hydroxyl radicals and nitric oxide, which is generated by nitric oxide synthase, can interact with each other and form one of the most damaging free radical species, peroxynitrite (Dawson and Dawson, 1996). The protective role of free radical scavengers was tested (chapter 3A). Only one of them, 7-NINA, had a small significant effect when administered pre-injury. Combining two free radical scavengers, with a broader range of effects, neither had the expected effect.
According to data shown in chapter 3B ischemia is actually not the main reason for secondary contusion expansion in CCI injury in mice. The volume of a cortical contusion expands significantly over time during the first 24 hours after experimental TBI (Zweckberger et al., 2003, Chapter 3B). Measurements of absolute CBF by ^{14}C-iodoantipyrine autoradiography revealed that the volume of ischemic tissue does not expand significantly after TBI. This indicates that progressive penumbral ischemia may play only a minor role in delayed pericontusional cell death and hence contusion expansion. The ischemic threshold (20 ml/mg/min) was only found within the primary contusion. Hypoperfusion was seen throughout the whole brain, thus in the penumbra as well. Nevertheless, contusions expand and a reduction of CBF is strongly related to an adverse outcome after TBI (Hlatky et al., 2004, Soustiel et al., 2005). Other factors must be more accountable. The vasculature is altered after injury. The blood brain barrier (BBB) becomes permeable to proteins early after injury (Kawamata et al., 2000, Maeda et al., 2003). This results in vasogenic oedema, and therefore causes secondary cell death by fuelling cytotoxic oedema. Osmolality in the penumbra increases, which aggravates further cell death by switching on apoptotic pathways and increasing excitotoxicity (Katayama et al., 1998). Our findings indicate that ischemia is not present either 15 min nor 24 hours after injury, and that mechanisms such as vasogenic and cytotoxic oedema leading to increased intracranial pressure (ICP) fuel apoptosis and excitotoxicity, may be more relevant to contusion expansion. TBI has broad pathophysiological effects and a small and early treatment window, whereas the first twenty-four to forty-eight hours are often the most important. In conclusion, ischemia does kill cells *in vitro*, but free radical scavengers alone

can hardly reduce this cell death. Hence, ischemia is disproved to be the most important factor to SCE after CCI in mice in chapter 3B. With the understanding of several pathophysiological pathways, many therapeutic options have gone through trial and error. Decompressive craniectomy (DC) is one of these therapeutic options that is currently living its third life during modern history. Current research methods combined with old methods explored the pathophysiology and effects of DC in more depth. DC cracks the Monroe-Kellie doctrine of the rigid case in which pressure can only build up. DC releases pressure from a medical uncontrollable raised ICP (chapter 4A). Several parameters have been measured in chapter 4. ICP raise and oedema formation were significantly less in craniectomised mice, though time of performing DC was of utmost importance. In mice, already 8 hours after trauma, DC showed no beneficial effects anymore. CBF measured by Laser Doppler (LD) and autoradiography differed on few parts in the brain only. The combination of LD and autoradiography is of great value, because LD measures CBF through time, but shallow, and autoradiography only at one time point, though with better resolution. In this thesis the non-perfused volume was significantly smaller than contusion volume in mice. The perfusion deficit was even smaller in DC animals; therefore ischemia cannot be the main reason for SCE in this type of injury. However, this does not mean that ischemia is not harmful. As discussed in chapter 3A, ischemia does kill cells, but in order to do so ischemia must be present. It is likely that ischemia is present in other types of injury, such as diffuse (vascular) injury. The presence of ischemia in the penumbra in human studies with multicontusional injury was actually shown (Bouma et al., 1991, Hlatky et al., 2004, Liu et al., 2002).

Future

The expression “treatable” in the context of TBI should be interpreted broadly in the context of prevention, pre/in-hospital medical care and rehabilitation. Prevention is a hot topic in today’s medical world. Is it possible to prevent TBI in the future with further preventive strategies? Can cars become safer for driver, pedestrian and cyclist, although the amount of patients suffering from whiplash injury has increased since the use of seatbelts (Montfoort et al., 2006)? Lower social integration, lower educational level, lower pre-morbid employment, higher drug abuse pre-injury rates, less access to optimal acute care/rehabilitation are all factors that are shown to have a negative effect on outcome. None of these factors have been investigated thoroughly enough yet. A strenuous effort should be made towards the investigation of these parameters.

Although no beneficial effects were seen specifically on CBF after early DC, early DC does show beneficial effects on outcome of mice. More information is needed on mechanisms of DC, in order to improve the identification of patients who possibly benefit from DC. In several chapters the BBB was discussed in the role of oedema formation, and thereby ICP increase. As we know now from CBF, oedema formation and ICP data is that oedema and raised ICP itself, and not a low CBF might be responsible for SCE. In our model oedema could be caused by a disrupted BBB during the first few hours after injury (Zweckberger et al., 2003). Whether and how early DC affects the BBB is unknown.

The outcome of TBI patients after DC will be revealed through the earlier mentioned trials DECRA and RESCUEicp. These studies are very important in future determination of guidelines for the treatment of severe TBI. A great challenge will be standardizing possible changes in favour of performing early DC. In a running study large differences are seen when data between cities and even countries are compared. For example, The Vrije Universiteit

Medical Centre in Amsterdam has performed many more DCs than University Medical Centre Groningen in the past three years (approximately 20% and 4% respectively of all severe TBI patients). Average amount of DCs in the Netherlands (± 9%) in comparison to Germany and Austria (both ± 16%) differ as well. Data on outcome seems promising (data not shown, personal communication), but of course the randomised controlled trials will provide a final answer.
Another field of interest within TBI treatment is, as discussed in chapter 3A, pharmacological agents. An excellent review was written by Schouten (2007), but with disappointing conclusions; no pharmacological agents have been associated with better outcome yet. A combination of pharmacological agents might be useful, though in chapter 3A the use of several reactive oxygen species-inhibitors did not show the expected beneficial effect. Targeting more parts of pathophysiological pathways at once might be the future. Combination of therapeutic options is valuable in e.g. the field of oncology. However, pharmacological agents used in the acute phase can also aggravate damage by causing hypotension. This might be the case with the use of rapid sequence intubation (pre-)hospital.

This thesis shows that TBI is still a harmful disease that is not likely to be diminished easily. Many factors play a role; though the hypothesis that ischemia plays a main role must be falsified for contusional injury. This thesis though verifies that pathophysiological pathways start very early and treatment must be initiated as early as possible. There is no time to sit and wait, and think about what can be done in a few hours, when ICP has reached medical uncontrollable heights. DC must be considered early in the decisive therapeutic process i.e. within hours of rising ICP. This necessitates ongoing optimal cooperation among (neuro)surgeons, intensivists and anaesthesiologists.

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Samenvatting:
Secundaire Schade na Traumatisch Schedelhersenletsel: Epidemiologie, Pathofysiologie en Behandeling

Traumatisch schedelhersenletsel is een van de meest voorkomende doodsoorzaken van jonge mannen in de westerse samenleving, maar mensen van alle leeftijden hebben hiermee te maken. De oorzaak van traumatisch schedelhersenletsel is vaak een verkeersongeluk of een val van bepaalde hoogte, maar ook veroorzaakt tijdens sport of vuurwapengevecht en anderszins geweld. Eenieder kan er dus mee in aanraking komen. De incidentie op Europees niveau is ongeveer 235 per 100 000 inwoners. Zowel mild, middelmatig en ernstig traumatisch schedelhersenletsel worden hiertoe gerekend. Vijftien per 100 000 inwoners overlijdt per jaar hieraan. De overige patiënten zijn echter niet gevrijwaard van late gevolgen. Zo'n 70 van de 235 patiënten blijven gehandicapt en hebben de rest van hun leven hulp nodig bij de algemene dagelijkse levensbehoeften. Ongeveer een zelfde hoeveelheid patiënten zal een lichte handicap overhouden en niet meer op het niveau van voor het letsel kunnen functioneren. Dit brengt niet alleen veel teweeg bij de patiënten zelf, maar evenzo bij familie en vrienden. Ook de kans op de ziekte van Alzheimer is groter na een traumatisch schedelhersenletsel. De epidemiologische kenmerken zijn in dit proefschrift nogmaals bevestigd in het 2e hoofdstuk. Daarbij kwam als nieuwe informatie naar voren dat naarmate de bevolking verouderd ook het aantal traumatisch schedelhersenletsel patiënten van hogere leeftijd aanzienlijk stijgt, met als zeer waarschijnlijk gevolg een verhoging van de daarbij behorende (zorg)kosten. Verdere maatregelen ter preventie zijn hier geïndiceerd. Enkele omstreden prognostische factoren werden geverifieerd. Uit de meta-analyse is gebleken dat het geslacht van de patiënt weliswaar op epidemiologisch niveau een verschil maakt in de hoeveelheid mannen versus vrouwen, maar hun afzonderlijke uitkomst na trauma wordt er niet door bepaald. Afro-amerikaanse afkomst en een lager opleidingsniveau toonde wel een slechtere prognose. Gezien het feit dat deze factoren vaak samen voorkomen moeten deze factoren nog in meer detail uitgezocht worden. Het voorkomen van hypotensie, hypoxie en hypothermie tijdens de eerste 24 uur na trauma heeft ook een slechtere prognose. De prognose verslechtert nog meer als deze hypo's samen voorkomen.

Het pathofysiologische deel van dit proefschrift is in een in vitro en een in vivo model uitgevoerd. De hoofdgedachte was dat in focaal trauma een lage doorbloeding van de hersenen tot ischemie leidt, en daarmee meer hersenweefsel verloren gaat. Uitkomsten van het in vitro onderzoek toonden dat ischemie een cumulatief schadelijk effect heeft in combinatie met mechanisch letsel. Uit andere onderzoeken is reeds bekend dat vrije radicalen een grote rol spelen bij ischemische schade. In het in vitro model is vervolgens met enkele farmaceutische middelen (Lubeluzole, 7-NINA, SOD-dismutase) geprobeerd de schade tegen te gaan, dan wel te verminderen. Het effect van de ischemische schade tezamen met de mechanische is echter zo overweldigend dat slechts een klein effect werd gezien. In het vivo model is gekeken naar de doorbloeding (perfusie) in het brein na trauma op verschillende tijdsmomenten. Er werd wel een hypoperfusie gezien, maar geen duidelijke ischemie, terwijl in het gebied om het trauma heen (de penumbra) het weefselverval wel toenam. Ook hieruit blijkt dat er andere factoren zijn, zoals een verhoogde intracraniële druk en verschillende moleculaire processen (apoptose pathways), die belangrijker zijn. De reeds beschreven hypoperfusie en de anderszins onbehandelbare verhoogde intracraniële druk zijn vervolgens getracht te verbeteren

in hetzelfde in vivo model door middel van een craniëctomie. Een craniëctomie betekent het weghalen van een deel van de schedel, waardoor de gezwollen hersenen meer ruimte krijgen. De hypoperfusie, weefselverval en de intracraniële druk verbeterden inderdaad. De craniëctomie is reeds in 1901 beschreven door Kocher en is daardoor zeker geen nieuwe therapie, maar, door nieuwe meettechnieken krijgt deze oude therapie een nieuwe kans. Door dit onderzoek, en het onderzoek van enkele andere groepen over de wereld, wordt steeds duidelijker wanneer het zinvol is een craniëctomie uit te voeren en welke patiënt hier baat bij kunnen hebben. Voor de keuze van patiënten wordt ook gebruik gemaakt van prognostische factoren, waarover hierboven reeds is geschreven. Het belangrijkste is dat de craniëctomie vroeg na het trauma, of na het begin van het bestaan van een verhoogde intracraniële druk, die niet reageert op andere (non-invasieve) technieken, wordt uitgevoerd. Dit is een groot discussiepunt in de kliniek tussen neurologen, neurochirurgen en intensivisten.
Dit proefschrift draagt bij aan de actuele kennis van de epidemiologie van traumatisch schedelhersenletsel: de samenstelling van de bevolking verandert, de incidentie van dit ziektebeeld echter ook. Het zal hoogstwaarschijnlijk leiden tot zorgproblemen op zowel praktisch als financieel niveau. Het signaleren ervan biedt de mogelijkheid vroeg te anticiperen, dan wel verdere preventie te ontwikkelen. Door de resultaten over de doorbloeding van de hersenen na unifocaal letsel is duidelijk geworden dat de doorbloeding weliswaar belangrijk is voor secundaire schade, maar niet het belangrijkste. Het belangrijkste lijkt vooralsnog de verhoogde intracraniële druk, die door middel van een wel overwogen, doch spoedige, craniëctomie in de toekomst meer patiënten beter behandeld kunnen worden.

List of Publications

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Curriculum Vitae

I, Doortje Caroline Engel, was born the 27th of September 1983 in Rotterdam. I went to the 'van Oldenbarnevelt' primary school, and thereafter to the 'Marnix Gymnasium'. I started medical school at the Erasmus University Rotterdam in 2001. During medical school I did an orientation project at the department of Neurosurgery with Sanjay Harhangi, who was one of the senior residents. From then on my interest in neuroscience started to grow. I joined the Master of Neuroscience Programme of the Erasmus MC in 2002 and started research in Neurotrauma in the group of dr. John Weber, in cooperation with dr. Andrew Maas of the department of Neurosurgery. To learn about patients, patient care & the clinical part of neuroscience as well I worked as a nurse-assistant at the department of Neurosurgery for two years.

After obtaining the 'Doctoraal' of medical school and the Master of Science's degree in February 2005 I left Rotterdam to live and work in Munich for over a year. I worked in 'the neuroteam' of prof. Nikolaus Plesnila in the Institute for Surgical Research. During that time I flew to Jerusalem to learn about different experiments from Esther Shohami and I went to Cologne to finalize other experiments with professor Gunter Mies at the Max Plank Institute for Neurological Research. I also worked for the IMPACT study of dr. Andrew Maas. During the past few years I was given the opportunity to travel around the world (Switzerland, France, USA, Australia, Germany, Italy & Israel); of course it was entirely my pleasure to take these opportunities.

In June 2006 I returned to Rotterdam to start my internships ('Co-schappen'), write articles and this thesis. I missed being abroad so I left for my surgical internships and a bit of research to Cologne (Germany) and Melbourne (Australia). I will finish medical school on June 27th of this year, and what the future will hold isn't clear when this thesis was being printed. I do know I would like to continue combining hospital and science within the surgical field. The question is, however, where? And next to all this study & work there was also some play: hockey, cello and repairing house/cars/bikes etc. Some of these hobbies hope to get a bit more of my time after June 27th 2008…

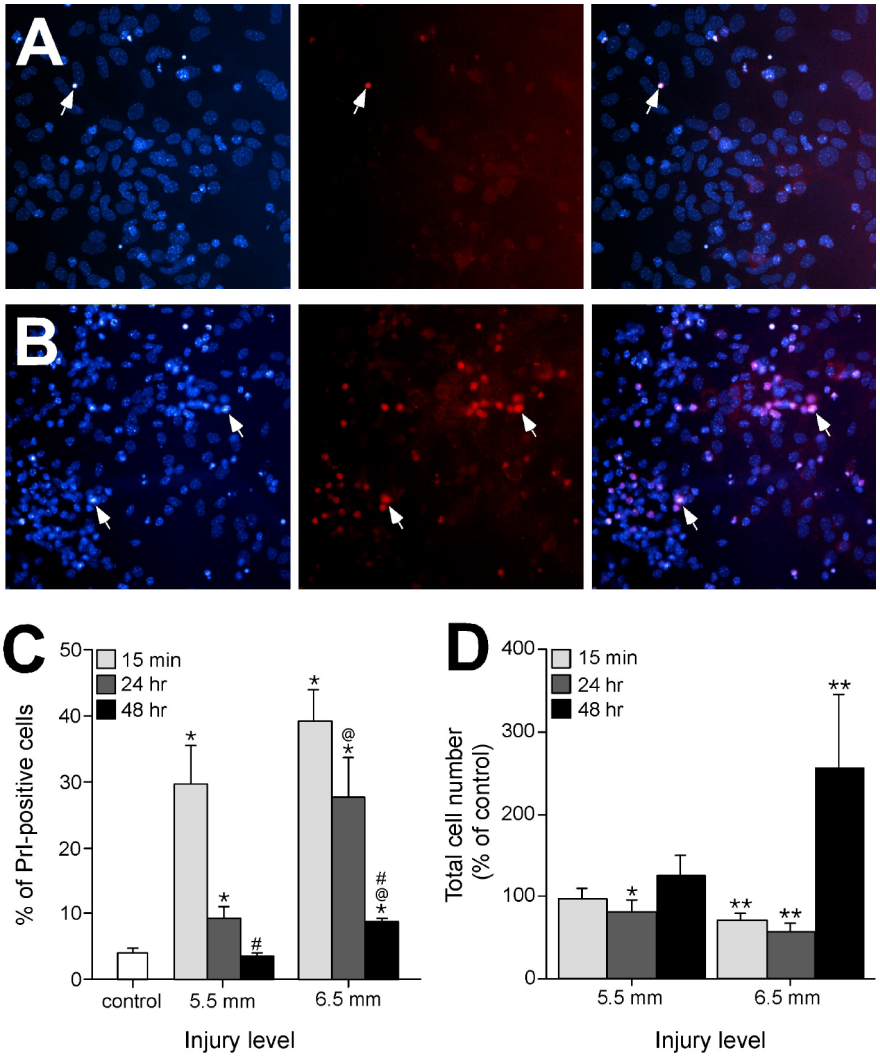


Figure 1. Effect of stretch injury on cortical cultures.
(**A**) Representative images from an uninjured control well stained with Hoechst (blue, left panel) and counterstained with propidium iodide (PrI; red, middle panel). The right panel shows an overlay of both pictures. Note that the underlying glial monolayer is intact, as indicated by Hoechst nuclear staining. Note also the very low PrI uptake (example shown by arrow), which indicates very few cell membranes were damaged. The scale bar in the left panel is 160 mm and applies to all images. (**B**) Representative images from an injured culture well after 24 h (5.5 mm deformation). In the left panel the number of Hoechst condensed nuclei is markedly increased (examples shown by arrows). The middle panel shows PrI uptake, which is noticeably increased. The right panel represents an overlay of PrI and Hoechst. (**C**) PrI uptake is positively correlated to the degree of stretch. Data are shown at 15 min, 24 h and 48 h post-injury (means ±SE values). Uptake of PrI increased with mild and moderate levels of stretch at 15 min post-injury vs. control. At 24 h, PrI levels remained elevated at both levels of injury, and at 48 h after a moderate injury. *p<0.01 vs. control, @p<0.05 vs. 5.5 mm injury at same time point, #p<0.05 vs. 24 h at same injury level (n= 4-7). (**D**) Total cell number in injured cortical cultures. Data are derived from the same cultures shown in (**C**), and is expressed as percent of control values (means ±SE values). *p!0.05 vs. control, **p !0.01 vs. control.

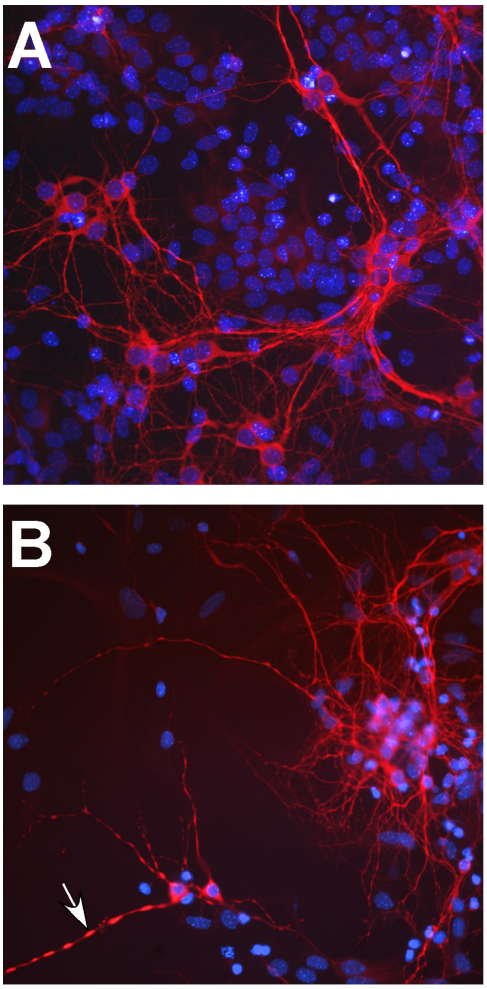


Figure 2. Stretch injury causes a reduction in MAP2-positive neurons. (**A**) Representative image from an uninjured control well stained against MAP2 (red) and counterstained with DAPI (blue). Uninjured neurons have an intact soma and smooth neurites. Note that the underlying glial monolayer is intact, as indicated by DAPI nuclear staining. The scale bar is 160 mm and applies to both images. (**B**) Representative image from an injured culture well (6.5 mm deformation). Injury caused beaded neurites (indicated by the arrow) as well as a disruption of the glial monolayer, indicated by DAPIstained nuclei. (**C**) Summary of the number of MAP2-positive neurons at 15 min, 24 h and 48 h after injury. Data are expressed as percent of control values (meansGSE values; n=6-8). *p < 0.01 vs. control, @p < 0.05 vs. 5.5 mm injury at same time point.

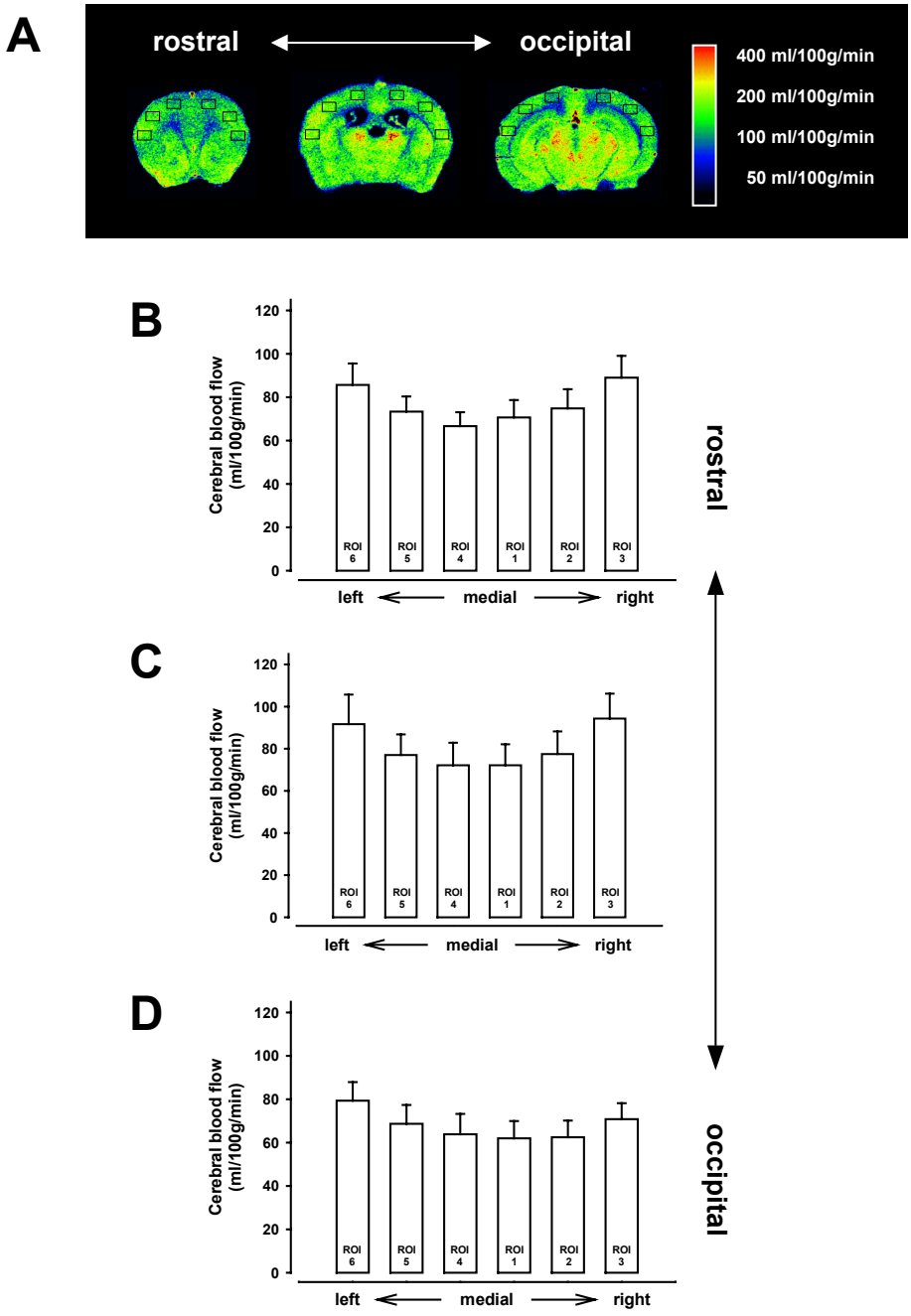
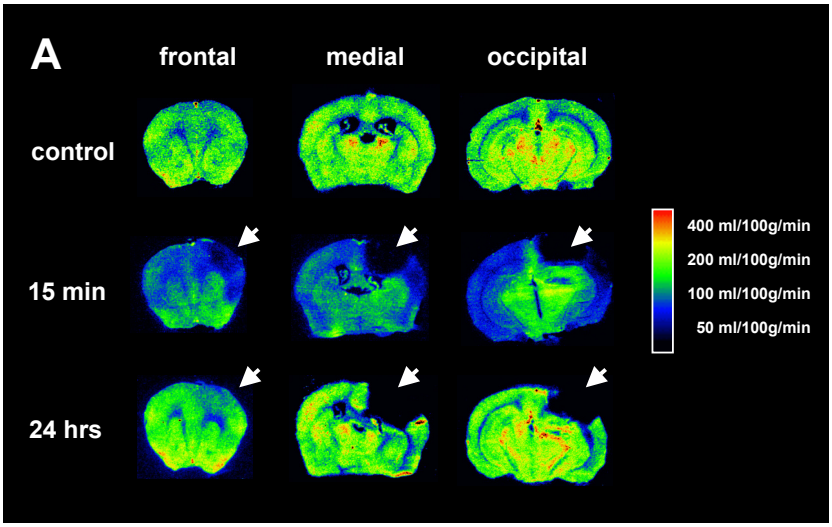


Figure 2. (**A**) Representative 14C-IAP autoradiograms of non-injured control animals. (**B-D**) Quantification of cerebral blood flow (ml/100g tissue/min) in the regions of interest (ROI) defined in figure 1. Data are arranged according to the anatomical location of the ROIs, i.e. from the left lateral to right lateral cortex (n=5). Panel B shows the data from the most rostrally, panel D the data from the most occipitally located brain section.



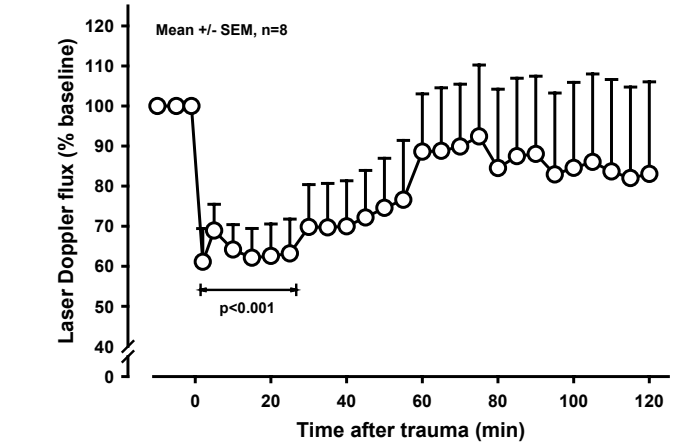


Figure 3. (A) Representative 14C-IAP autoradiograms of mice subjected to experimental traumatic brain injury by controlled cortical impact to the right hemisphere (arrows) and scarified 15 minutes or 24 hours after TBI. As compared to non-injured controls cerebral blood flow decreased in the whole brain including the hemisphere contralateral to the impact 15 min after TBI. No blood flow was detectable at the location of the contusion. Twenty-four hours after TBI cerebral blood flow recovered in the contralateral hemisphere while still no blood flow could be detected at the contusion site. (**B**) Temporal changes of local cerebral blood flow measured by laser Doppler fluxmetry over the contralateral hemisphere after controlled cortical impact in mice.

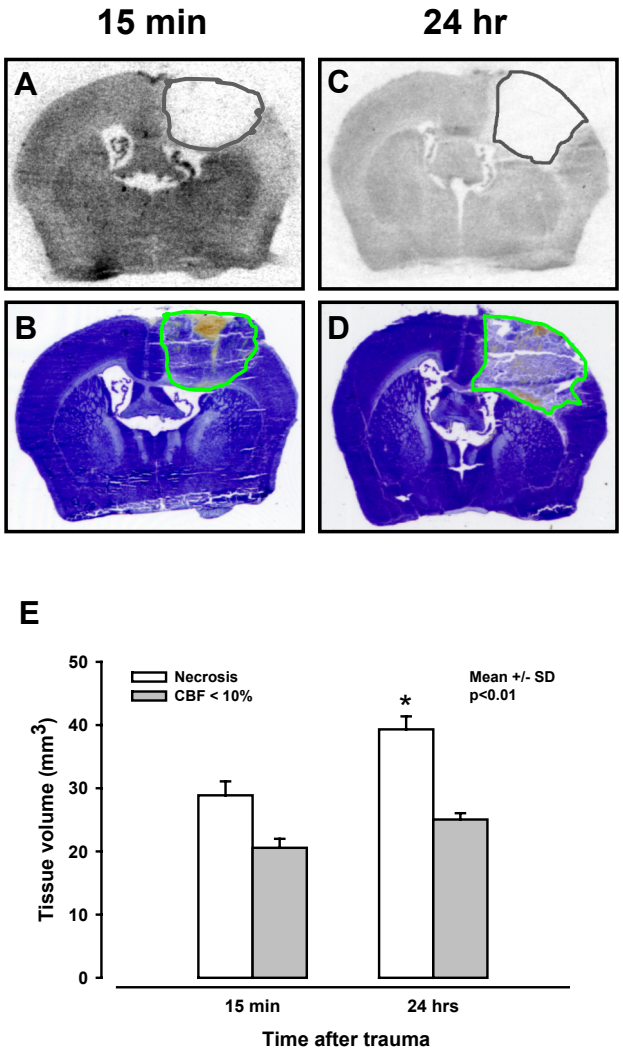


Figure 6. Two pairs of adjacent coronal brain sections taken at equal distances throughout the contused brain were used for the determination of ischemic and necrotic brain tissue volume by 14C-IAP autoradiography and Nissl staining, respectively (see Fig. 1B).
A-D. Representative autoradiograms (**A & C**) and histological sections (**B & D**) obtained 15 min and 24 hours after TBI showing the areas of ischemic and contused brain tissue, respectively. E. The volume of contused (white bars) as compared to the volume of ischemic/not perfused brain parenchyma (CBF < 10% of baseline; grey bars) 15 min and 24 h after TBI. While the volume of contused brain tissue expands significantly over time (+43%; *p<0.01), the volume of non-perfused tissue shows only a moderate, not significant change over time (n=5-7).

Colour figures chapter 4A

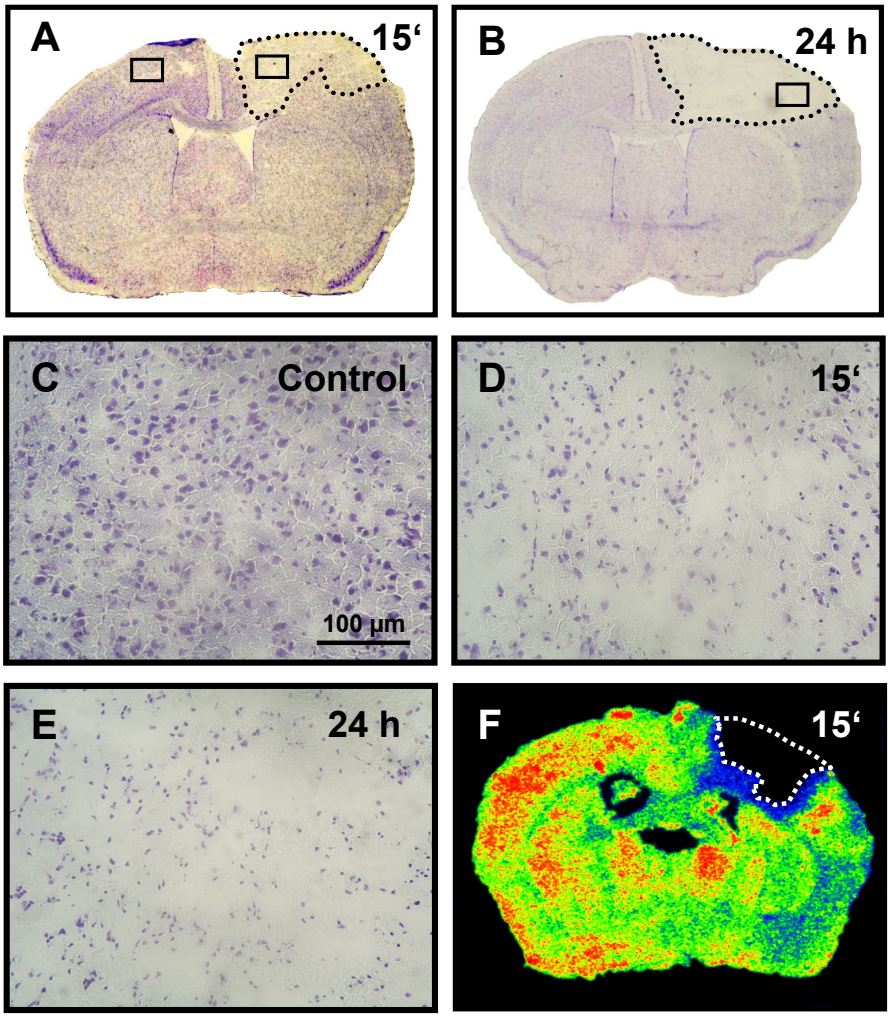


Figure 2. Nissl-stained coronal brain section showing a cortical contusion 15 min (**A**) and 24 h (**B**) after controlled cortical injury. Higher magnifications (200) from the areas indicated in A (**C,D**) and B (**E**). Autoradiography showing blood flow values in the brain of a mouse sacrificed 15 min after trauma (**F**). No blood flow (black) is detectable within the primary contusion.

Colour figures chapter 4B

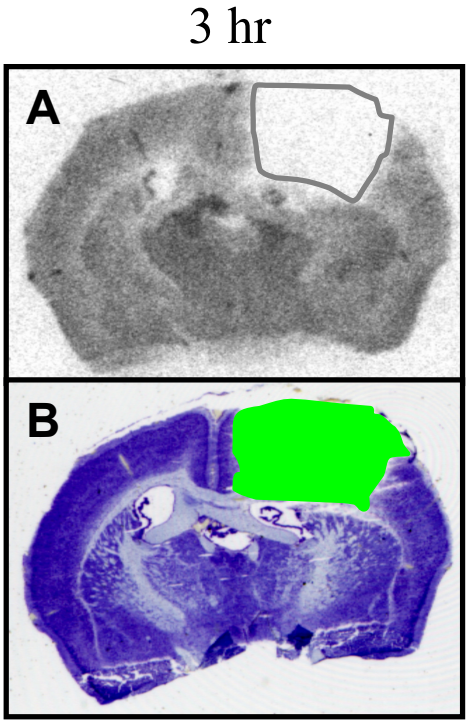


Figure 2. Representative adjacent brain sections.
Autoradiogram (**A**) and Nissl-stained (**B**). Note the difference between the contusion/non-perfused area.

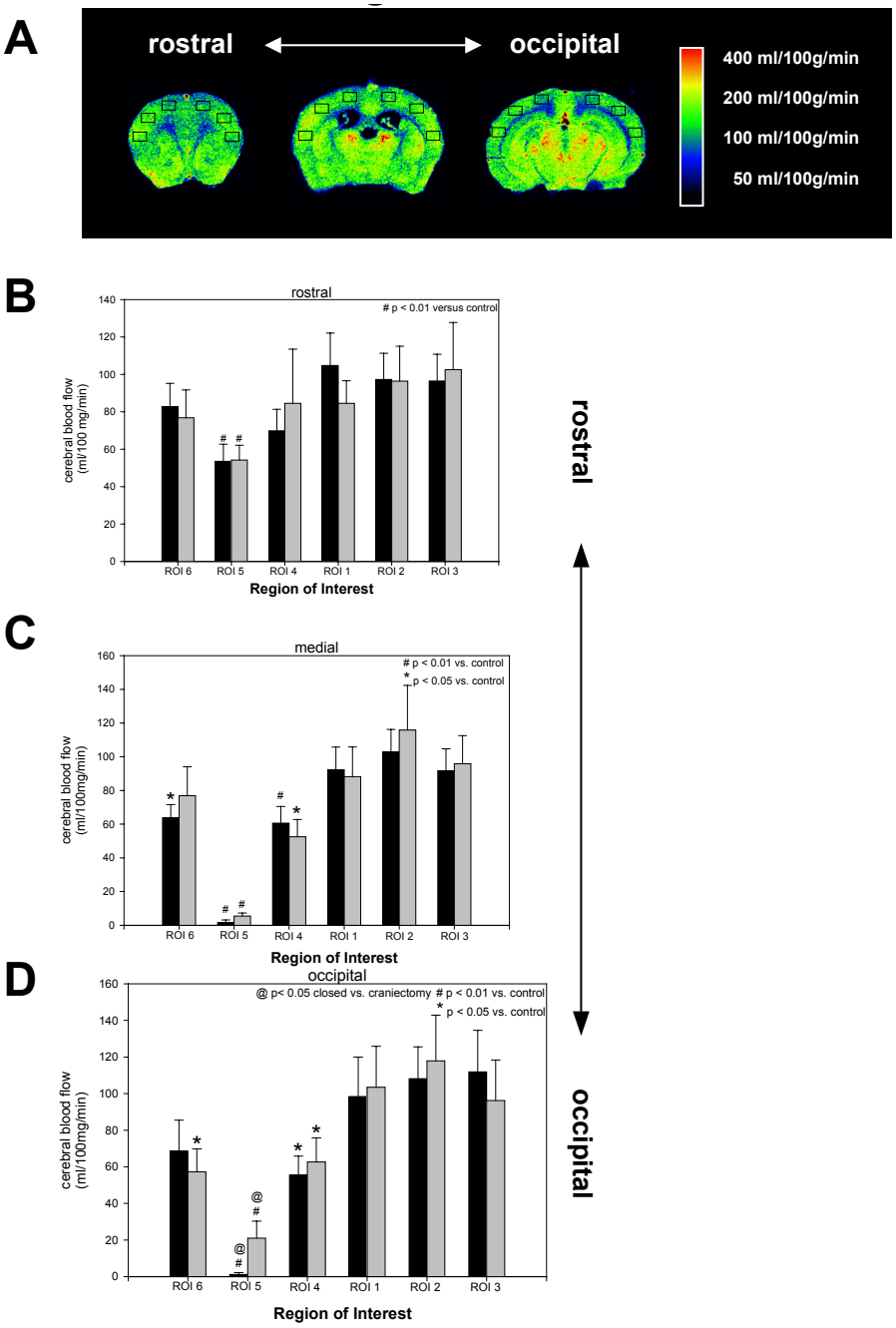


Figure 4. Quantification of absolute CBF.
CBF in 6 ROIs (**A-F**) of closed (left) and craniectomized (right) animals in frontal (black), parietal (light grey) and occipital (dark grey) sections. (* $p < 0.05$, #$p < 0.05$, n=6-7).