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Post-traumatic brain hypothermia reduces histopathological damage following concussive brain injury in the rat

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Abstract The purposes of this study were (1) to document the histopathological consequences of moderate traumatic brain injury (TBI) in anesthetized Sprague-Dawley rats, and (2) to determine whether post-traumatic brain hypothermia (30 °C) would protect histopathologically. Twenty-four hours prior to TBI, the fluid percussion interface was positioned over the right cerebral cortex. On the 2nd day, fasted rats were anesthetized with 70 % nitrous oxide, 1 % halothane, and 30 % oxygen. Under controlled physiological conditions and normothermic brain temperature (37.5 °C), rats were injured with a fluid percussion pulse ranging from 1.7 to 2.2 atmospheres. In one group, brain temperature was maintained at normothermic levels for 3 h after injury. In a second group, brain temperature was reduced to 30 °C at 5 min post-trauma and maintained for 3 h. Three days after TBI, brains were perfusion-fixed for routine histopathological analysis. In the normothermic group, damage at the site of impact was seen in only one of nine rats. In contrast, all normothermic animals displayed necrotic neurons within ipsilateral cortical regions lateral and remote from the impact site. Intracerebral hemorrhagic contusions were present in all rats at the gray-white interface underlying the injured cortical areas. Selective neuronal necrosis was also present within the CA3 and CA4 hippocampal subsectors and thalamus. Post-traumatic brain hypothermia significantly reduced the overall sum of necrotic cortical neurons (519 ± 122 vs 952 ± 130, mean ± SE, P =0.03, Kruskal-Wallis test) as well as contusion volume (0.50 ± 0.14 vs 2.14 ± 0.71 mm^3, P = 0.004). These data document a consistent pattern of histopathological vulnerability following normothermic TBI and demonstrate hypothermic protection in the post-traumatic setting.

Key words Traumatic brain injury · Hypothermia
Histopathology · Fluid percussion · Rat · Contusion

Introduction

Experimental studies of traumatic brain injury (TBI) are an attempt to duplicate the pathological conditions of human closed head injury [20]. Previous studies using animal models of brain trauma have documented changes in energy metabolism, cerebrovascular dysfunction, and parenchymal histopathology following TBI (for review see [26]). Recently, rodent models of TBI have been developed which facilitate the systematic examination of traumatic consequences and the testing of potential pharmacotherapeutic agents [17, 36, 45, 60). The rat model of fluid percussion injury represents a reliable model of mild-to-moderate diffuse brain injury with minimal or no focal tissue damage at the site of impact [17]. This model is felt to mimic the clinical situation in which patients experience a concussion-like injury characterized by brief neurological and systematic physiological alterations without severe structural damage [17].

Although rat models of brain injury are advantageous for many reasons, recent data in the setting of cerebral ischemia have demonstrated that small variations in brain temperature can significantly affect injury outcome and influence model reproducibility (for reviews see [12, 21]). For example, it is known that a 2 °C difference in intra-ischemic brain temperature significantly affects the neuronal and microvascular consequences of global cerebral ischemia [5, 13, 14]. While mild hypothermia (33 °C) protects the ischemic brain, mild hyperthermia (39 °C) significantly worsens histopathological and behavioral outcome. In more recent studies, intra-ischemic hypothermia (30 °C) has also

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been shown to reduce the behavioral deficits associated with transient global ischemia [23]. The importance of head and brain temperature in rodent models of TBI has also been demonstrated in studies where both pre- and post-traumatic brain hypothermia improved behavioral recovery [10]. More recently, Jiang et al. [35] have demonstrated that moderate hypothermia prior to injury reduced blood-brain barrier (BBB) disruption following traumatic brain injury in the rat.

Experiments in which intra-ischemic or pre-traumatic brain injury are manipulated provide a powerful tool with which to investigate injury pathomechanisms. However, of greater relevance to the clinical setting are *post-injury* temperature modifications which may be of potential utility. In models of global ischemia, the beneficial affects of post-ischemic hypothermia have been restricted to relatively short ischemic periods with early post-ischemic cooling [6, 7, 9, 16, 27]. For example, Busto and colleagues [6] demonstrated protection of the CA1 hippocampus when a 3-h post-ischemic hypothermic (30 °C) period was initiated 5 min, but not 30 min, into the post-ischemic recirculation period. The ability of post-injury cooling to protect the injured brain could depend on several variables including injury type (ischemia vs trauma), injury severity (selective neuronal necrosis vs pan-necrosis), as well as the degree and duration of the hypothermic period.

The histopathological consequences of fluid percussion TBI in the rat can vary according to the level of injury and the location of impact [11, 17, 36, 45]. The major aim of this study was to establish a rodent model of fluid percussion brain injury under controlled physiological conditions that resulted in consistent histopathological damage. In addition, the consequences of post-traumatic brain hypothermia on histopathological outcome were assessed since it is not known whether post-traumatic brain hypothermia would provide histopathological protection from fluid percussion TBI.

Materials and methods

Surgical preparation

These experiments were conducted on 24 fasted male Sprague-Dawley rats weighing between 250 and 300 g. Rats were initially anesthetized with 3 % halothane, 30 % oxygen and a balance of nitrous oxide. Tracheal intubation was performed and rats were placed in a stereotaxic frame. As previously described [10, 17], a 4.8-mm craniotomy was made overlying the right parietal cortex 3.8 mm posterior to bregma and 2.5 mm lateral to the midline [64]. A plastic injury tube was next placed over the exposed dura and bonded by adhesive. Dental acrylic was then poured around the injury tube. After the acrylic had hardened, the injury tube was plugged with a gelfoam sponge, the scalp sutured closed and the animal returned to its home cage and allowed to recover overnight.

Traumatic brain injury

A fluid percussion device was used to produce experimental TBI [10, 17]. This device, as previously discussed, consisted of a saline-filled Plexiglas cylindrical reservoir bent at one end with a rubber-covered piston and with the opposite end fitted with a transducer housing and injury screw adapted for the rat's skull. The metal screw was firmly connected to the plastic injury tube of the intubated anesthetized rat (70 % nitrous oxide, 1.5 % halothane and 30 % oxygen) and the injury induced by the descent of a metal pendulum which struck the piston. In this study, rats underwent mild-to-moderate head injury ranging from 1.7 to 2.1 atmospheres. Brain temperature was indirectly monitored with a thermistor probe inserted into the temporalis muscle as previously described [5]. Temporalis muscle temperature has recently been shown to be a good indicator of brain temperature following traumatic brain injury in rodents [34]. Readings were taken 30 min prior to trauma and held at 37 °C prior to TBI. Rectal temperature was also measured and maintained at 37 °C prior to and throughout the 3-h post-traumatic monitoring period. In animals which post-traumatic brain temperature was lowered, cold air was blown directly onto the skull. In these studies, post-traumatic brain temperature was artificially reduced starting 3 min following the traumatic insult and continued for 3 h. Moderate post-traumatic hypothermia was achieved in all rats within 5 min post-trauma.

Histopathological analysis

Three days following traumatic brain injury, rats were perfused-fixed for histopathological and morphometric analysis. Animals were anesthetized and perfused with isotonic saline delivered under a constant pressure of 100–120 mm Hg for 15 s followed by fixative (FAM, a mixture of 40 % formaldehyde, glacial acetic acid and absolute ethanol; 1:1:8 by volume). Following perfusion for 20 min, animals were decapitated and the heads immersed in FAM at 4 °C for 24 h. The brains were then carefully dissected from the skull and reimmersed in FAM for an additional 24 h. Next, the brains were blocked and embedded in paraffin; 10-μm-thick sections were obtained at 100-μm intervals throughout the neuraxis. These were stained with hematoxylin and eosin. Assessment of histopathological damage was carried out using several methods. To assess quantitatively the numbers of necrotic neurons within the entire cerebral cortex, numbers of eosinophilic neurons per high microscopic field (100X) were counted in the lateral cortex by an investigator (WDD) who was blinded to the experimental groups. Each microscopic field corresponded to approximately 1.65 mm^2. For this purpose, seven coronal sections spanning the antero-posterior extent of the cortical injury were selected (0.8, 1.8, 3.3, 4.3, 5.8, 6.8 and 7.3 mm posterior to bregma) [64]. To determine contusion volume, areas to tissue necrosis in coronal sections spanning the entire antero-posterior extent of the injury were first traced using a camera lucida microscope attachment. Each drawing was then retraced onto a digitizing tablet interfaced to a computer which calculated contusion areas. Contusion volume was calculated by numeric integration of sequential areas. Finally, the presence or absence of selective neuronal necrosis was also determined within subcortical brain regions.

Statistical analysis

Histopathological data were expressed as mean values ± standard error (SEM). Ischemic cells counts and contusion areas were compared in normothermic versus hypothermic rats by repeated-measures analysis of variance. Data at individual coronal levels were compared by the Kruskal-Wallis one-way analysis of variance by ranks. Statistical analysis was performed using SAS routines (SAS Institute, Inc., Cary, NC). Differences were regarded as statistically significant at $P < 0.05$. Linear regression analysis was also used to compare the relationship between the number of necrotic neurons and contusion volume.

Results

Physiological data

Physiological data from normothermic and post-traumatic hypothermic rats are shown in Table 1. Arterial blood gases and pH prior to and 1 and 3 h after TBI were generally within the normal range and were similar in the normothermic and post-traumatic hypothermic groups. In all TBI rats, arterial blood pressure increased immediately following trauma and remained elevated for approximately 1 min. Peak blood pressure increases averaged 223 ± 34 mm Hg (mean ± SEM).

Histopathological findings

Normothermic rats

In rats that underwent sham operations ($n = 6$), histopathological injury was not seen either beneath the injury screw or elsewhere. In contrast, widespread histopathological damage was detected throughout the injured hemisphere of normothermic TBI rats (Figs. 1, 2). In the cerebral cortex *underlying* the central injury screw, a focal area of tissue necrosis was seen in only one of nine rats. However, in cortical areas *lateral* to the injury site, necrotic neurons with eosinophilic cytoplasm and pynotic nuclei were present in all rats. Coronal levels that commonly contained necrotic neurons included levels extending between 1.8 to 7.3 mm posterior to bregma (Fig. 3). Cortical areas affected thus included the lateral portion of the primary somatosensory parietal cortex (Par1), the supplementary somatosensory cortex (Par2) as well as the gustatory cortex. In more posterior coronal sections, portions of the affected temporal cortex included areas 1, 2 and 3 of the auditory cortex. Although necrotic neurons were seen in all cortical layers, damaged cells were most numerous within the deeper cortical layers (i.e., 5 and 6). Necrotic neurons were sometimes grouped in columns and associated with penetrating cortical vessels. Astrocytic cell bodies also appeared mildly swollen at sites of cortical neuronal injury. In one rat, selective neuron necrosis was replaced by pan-necrosis involving cortical layers 2 through 6.

Underlying the damaged cortical areas, focal contusion was commonly seen at the gray-white interface (Fig. 1). The contused site demonstrated different degrees of hemorrhage. In some cases, large amounts of extravasated blood were present within the subcortical white matter, while in other cases, individual erythrocytes and small petechiae were evident. In all cases, the contused regions contained swollen white matter tracts and macrophages (Fig. 1). The antero-posterior extent of the contusion was detected at coronal levels extending 1.8 to 7.3 mm posterior to bregma (Fig. 4).

In addition to the injury to the subcortical white matter, other white matter tracts were also affected. For example, focal hemorrhage or necrosis of the fimbria of the hippocampus was seen in two rats, and the internal capsule was disrupted in one rat. Blood was detected in subarachnoid spaces of six of nine TBI rats (Fig. 2A). Specific regions that most commmonly contained blood included the pial surface of the traumatized hemisphere, the longitudinal cerebral fissue and other cerebrospinal fluid-containing spaces. Luminal leukocytes were occasionally detected within pial and cortical vessels within the traumatized hemisphere (Fig. 2B). In addition, clusters of perivascular leukocytes were also seen within damaged cortical areas (Fig. 2C).

Necrotic neurons were also detected in subcortical areas. For example, within the hippocampus, eosinophilic neurons were detected in nine out of nine normothermic TBI rats (Table 2). Abnormal cells were present in the CA3 and CA4 subsectors as well as the dentate hilus (Fig. 2D, E). In thalamus, necrotic neurons were seen in all TBI rats and were most numerous in the lateral dorsal and geniculate nuclei. In contrast, the reticular and substantia nigra nuclei appeared relatively normal as did the cerebellum and brain stem. However, evidence for central chromatolysis was seen in brain stem neurons.

Table 1 Physiological variables. Mean ± SEM (*MAP*, mean arterial pressure)

	Normothermia ($n = 9$)	Hypothermia ($n = 9$)
Pre-trauma		
PCO_2	39.3 ± 0.93	38.2 ± 0.69
PO_2	150.4 ± 10.3	138.9 ± 5.44
pH	7.420 ± 0.015	7.439 ± 0.019
MAP	120.5 ± 2.50	126.3 ± 1.95
Post-trauma (1 h)		
PCO_2	39.7 ± 1.32	40.5 ± 7.12
PO_2	146.5 ± 7.44	137.6 ± 7.12
pH	7.417 ± 0.022	7.395 ± 0.018
MAP	131.5 ± 6.58	130.0 ± 4.52
Post-trauma (3 h)		
PCO_2	36.9 ± 0.57	37.9 ± 0.84
PO_2	145.0 ± 6.65	147.9 ± 6.98
pH	7.421 ± 0.017	7.392 ± 0.025
MAP	124.4 ± 6.98	125.0 ± 3.22

Post-traumatic hypothermia

Post-traumatic hypothermia reduced the extent of histopathological damage when compared to normothermic TBI rats. The frequency of necrotic cortical neurons was significantly reduced at the three coronal levels showing the most severe damage (Fig. 3). Repeated-measures ANOVA revealed a significant between-subjects effect of temperature group ($F(1, 16) = 5.91$, $P = 0.027$); and the overall sum of necrotic cortical neurons across levels was significantly lower in

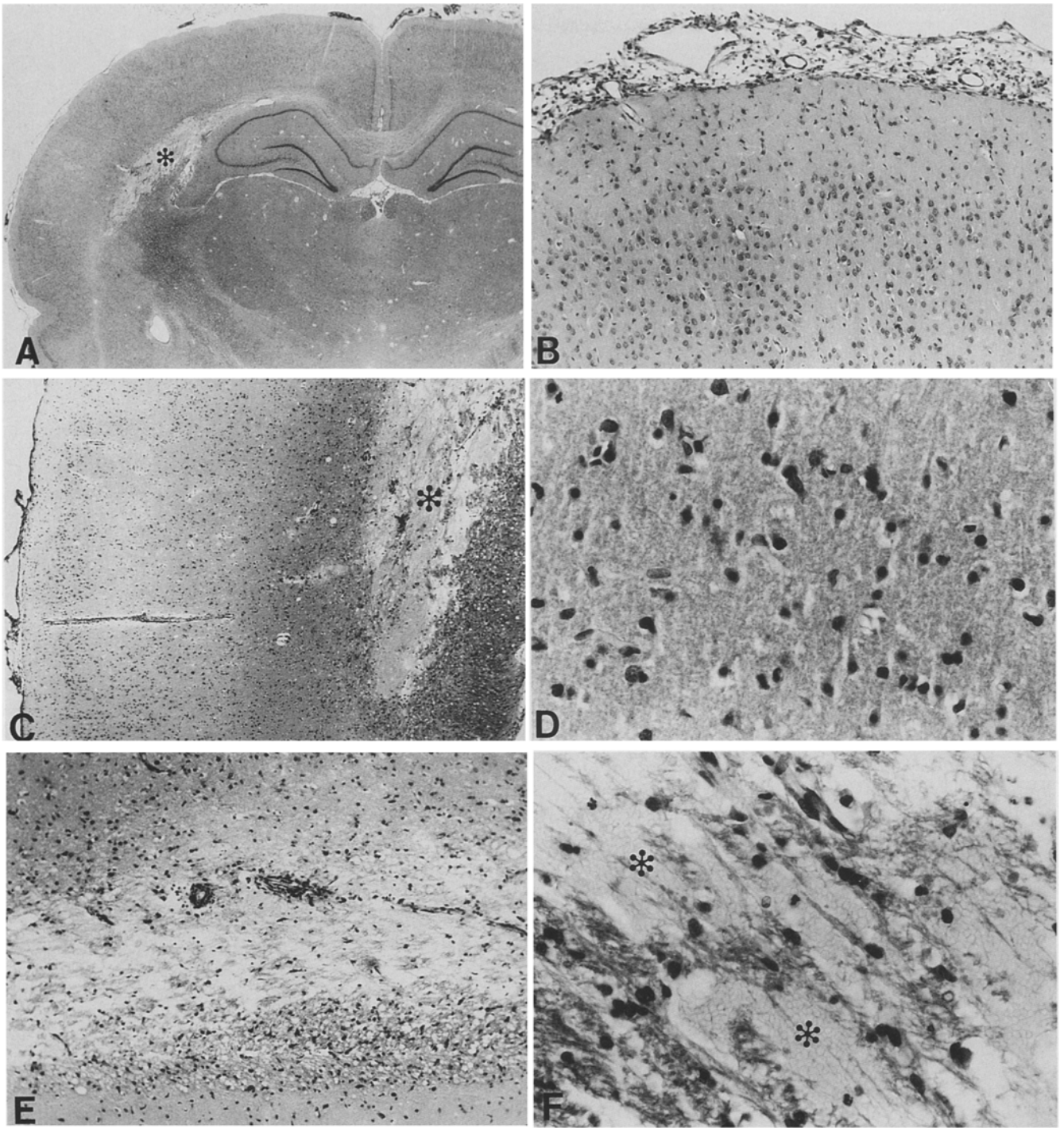

Fig. 1. A–F Paraffin-embedded sections of rat brain stained with hematoxylin and eosin 3 days after fluid percussion brain injury. **A** Focal contusion (*asterisk*) is present at the gray-white interface lateral to hippocampus. A focal region of cortical pallor is present overlying the contusion. **B** Cortical region at site of impact appears unremarkable compared to sham-operated controls. **C** Cortical pallor with selective neuronal necrosis overlying area of contusion (*asterisk*). **D** Necrotic neurons within damaged cerebral cortex. **E** Contused area contains severely swollen white matter tracts. **F** Hemorrhagic contusion containing extravasated eythrocytes (*asteriks*). **A** ×27; **B, E** ×270; **C** ×110; **D, F** ×1070

the hypothermic than in the normothermic group (519 ± 122 vs. 952 ± 130 per field, respectively; $P = 0.03$, Kruskal-Wallis test).

Similarly, when contusion areas were analyzed across coronal levels by repeated-measures ANOVA, there was a significant between-subjects effect for temperature group, denoting protection by hypothermia compared to normothermia ($F(1, 16) = 5.86$, $P = 0.028$). Contusion areas were significantly reduced at three of the six coronal levels investigated (Fig. 4). In addition, the overall contusion volume was significantly reduced

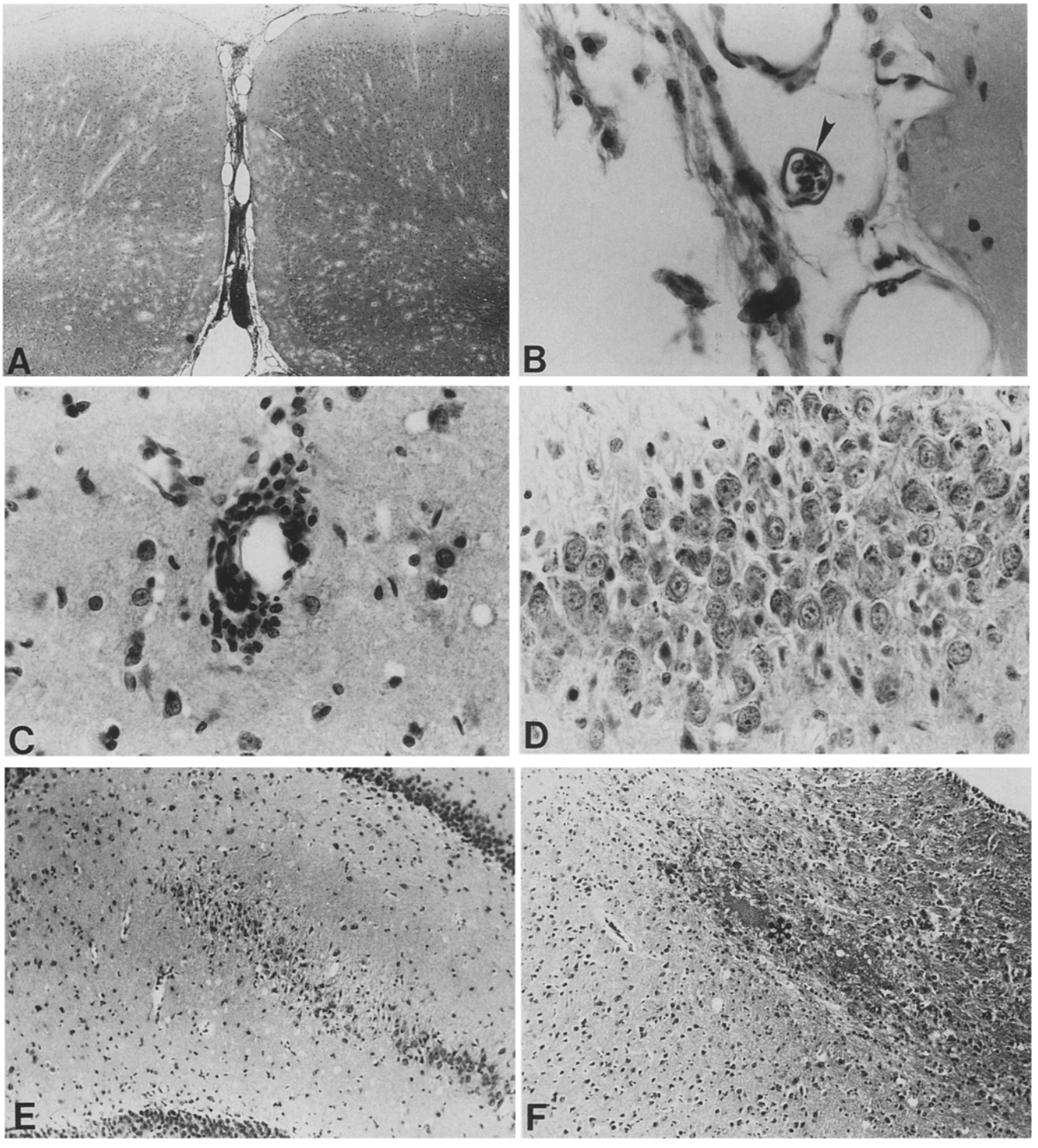

Fig. 2 **A** Blood is present within the longitudinal cerebral fissure of a traumatized rat. **B** Polymorphonuclear white blood cells within pial vessel overlying injured cortical area. **C** Perivascular leukocytes within cerebral cortex. **D** Hippocampal CA3 necrotic neurons. **E** Hippocampal CA4 necrotic neurons. **F** Small hemorrhagic contusion is present at gray-white interface of rat that underwent 3 h of post-traumatic brain hypothermia (30 °C). **A** ×110; **B–D** ×1070; **E, F** ×270

in the hypothermic group compared to normothermic rats (0.50 ± 0.14 vs. 2.14 ± 0.71 mm^3, respectively; $P = 0.004$).

Hypothermic protection was also seen within vulnerable subcortical structures. As shown in Table 2, post-traumatic hypothermia significantly reduced the incidence of selective neuronal necrosis within the hippocampus and thalamus.

Table 2 Selective neuronal injury and contusion in subcortical areas (+, neuronal injury and focal contusion –, damage not seen)

Animals	Brain regions				White matter contusion
	CA1	CA3	CA4	Thalamus	
Normothermia:					
1	–	+	+	+	+
2	–	+	+	+	+
3	–	+	+	+	+
4	–	+	+	+	+
5	–	+	+	+	+
6	–	+	+	+	+
7	–	+	+	+	+
8	–	+	+	+	+
9	–	+	+	+	+
Hypothermia:					
10	–	+	–	–	+
11	–	+	+	–	+
12	–	+	+	–	–
13	–	–	–	–	–
14	–	–	–	–	+
15	–	–	–	–	–
16	–	+	–	–	+
17	–	–	–	–	+
18	–	–	+	–	+
*P =	NS	0.01	0.005	0.00002	NS

* Statistically significant difference between normothermic and hypothermic animals by Fisher exact probability test

Relationship between cortical injury and contusion

In individual animals, each contusion volume was plotted against the respective total number of necrotic neurons. These data are illustrated in Fig. 5 that excludes one outlying data point. Linear regression analysis revealed a positive correlation between contusion volume and frequency of neuronal necrosis for the pooled data set (normothermic + hypothermic rats, $r = 0.81$, $P = 0.0001$) (Fig. 5), and for the normothermic data set alone ($r = 0.86$, $P = 0.006$), but not for the hypothermic data set alone.

Discussion

Cerebral contusion is an important consequence of head injury [1, 2, 4, 49]. In the present study, neuronal necrosis and intracerebral hemorrhagic contusion were seen at sites remote from the impact. According to Holbourn [28], two major types of forces are responsible for brain injury after head injury: one localized at the site of injury and a second characterized mainly by rotational forces that deform brain tissue and through mechanical stress, disrupt blood vessels. Gliding contusions are the result of relative movements either between the cortical surface and skull, or between the cortical and subcortical structures, resulting in a mechanical stress on vessels and intracerebral hematomas [40, 53]. In the present study, shearing strains at the

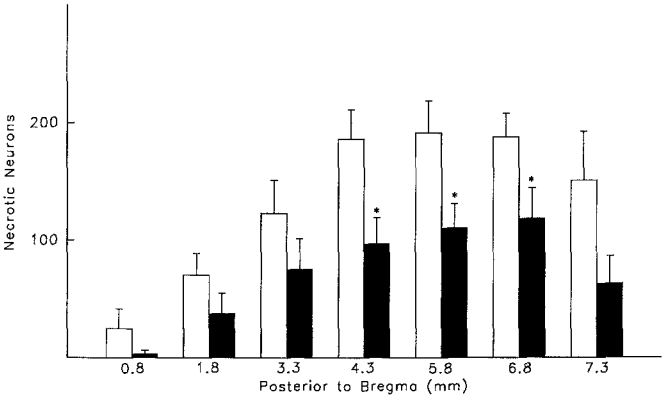

Fig. 3 Bar graph of mean ± SEM number of cortical necrotic neurons per microscopic field (1.65 mm^2) at seven coronal levels. Data taken from both normothermic (*clear bars*) and post-traumatic hypothermic (*black bars*) rats (*, significantly reduced compared to normothermia by the Kruskal-Wallis one-way analysis by ranks)

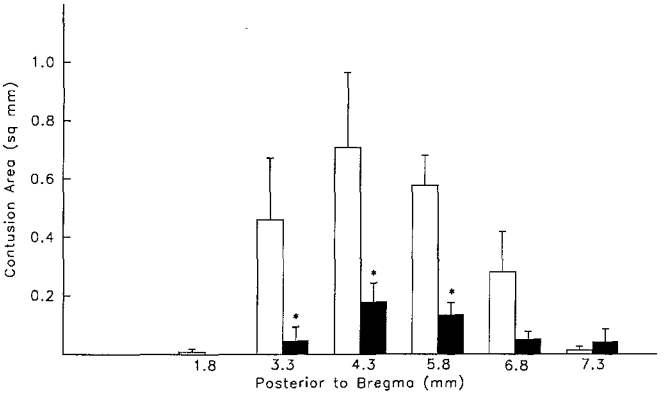

Fig. 4 Bar graph of mean ± SEM contusion area from normothermic (*clear bars*) and post-traumatic hypothermic (*black bars*) rats at 6 coronal levels (*, significantly reduced compared to normothermia by the Kruskal-Wallis one-way analysis by ranks)

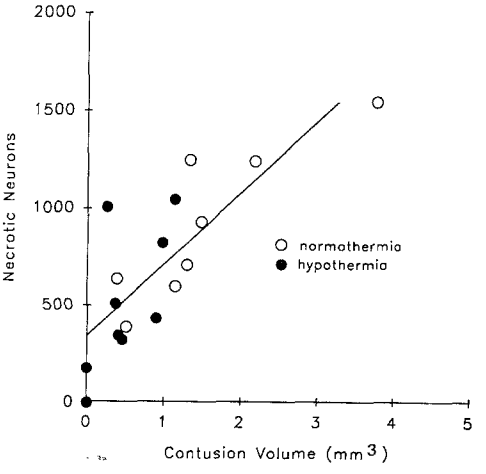

Fig. 5 Relationship between total numbers of cortical necrotic neurons and contusion volume. Linear regression annalysis revealed a positive correaltion between contusion volume and number of necrotic neurons for the entire pooled data set (P = 0.0001)

gray-white interface of the lateral cortex caused by tissue displacement at the impact site may have led to contusion formation.

A spatial relationship between contusion location and selective neuronal necrosis within overlying cortical areas was demonstrated in this study. In addition, animals having the largest contusions also contained the largest numbers of cortical necrotic neurons. These relationships might suggest that the pathologies of these two types of tissue injury are interrelated. For example, in this injury model, impact-induced structural displacement at the gray-white interface may have led to the acute rupture of small vessels causing intracerebral hemorrhage. Subsequent BBB disruption and edema formation with brain swelling might then secondarily reduce blood flow within the overlying cerebral cortex. BBB breakdown accompanying acute brain trauma is commonly described [11, 52, 56]. In the rat, reduced levels of cerebral blood flow (CBF) also have been documented following fluid-percussion brain injury [30, 59, 63]. Edematous fluid and extravasated blood-borne factors include vasoactive and neurotoxic substances, [8, 15, 18, 41, 51]. Thus, cortical pathology after TBI may be a secondary consequence of acute microvascular damage within the contused region. In this regard, a spatial relationship between ischemia-induced BBB breakdown and acute neuronal damage has been recently documented following temperature-controlled global forebrain ischemia [15].

In the present study, necrotic neurons were also observed in subcortical brain regions including the hippocampus and thalamus. In the hippocampus, eosinophilic neurons were present within subsectors CA3 and CA4 and the dendate hilus. Hippocampal injury or evidence of dysfunction are commonly seen in head injured patients as well as in several animals models of brain injury [11, 22, 24, 31, 38]. In a model of lateral fluid percussion brain injury, Cortez et al., [11], reported the development of widespread pathological changes including acidophilic neurons within the CA1, CA2 and CA3 subsectors, dentate gyrus and thalamus. Except for CA1 involvement, this pattern of neuronal necrosis appears to be similar to that described in the present study. Previous studies have suggested a relationship between the degree of hippocampal involvement and traumatic severity [24, 38, 42]. In addition, an apparent directional dependence of traumatic hippocampal neuronal damage has also been described in an acceleration-induced head injury model [38]. In that study, all animals subjected to a lateral injury developed hippocampal lesions while only 43 % developed similar damage when injured in the sagittal plane. Thus, both injury severity as well as impact placement may have contributed to the lack of CA1 hippocampal pathology in our series.

In animal models of recurrent seizures, hippocampal neurons are selectively vulnerable [3, 47, 48, 57]. Although evidence for seizures was not seen in the present series, one explanation for hippocampal damage after TBI is the excessive stimulation of CA3 neurons as a consequence of cortical damage. Excessive neuronal excitation leading to excitotoxic-mediated neuronal injury has previously been implicated in hippocampal vulnerability after head trauma (for review see [26]). Elevations in extracellular glutamate and aspartate have been reported after TBI [19, 36, 38, 50], while NMDA antagonists have provided behavioral protection [19, 25, 32, 46]. Immediately following fluid percussion brain injury, marked elevations in hippocampal glucose metabolism have also been reported [60]. Trauma-induced hypermetabolism within the CA1 subsector is prevented by prior destruction of the CA3 region by kainic acid [61] or by the local administration of excitatory amino acid antagonists [37]. Taken together, these data indicate an important role for glutamate in the pathogenesis of selective neuronal necrosis after TBI. A variety of secondary injury mechanisms have been hypothesized to participate in the pathogeneous of TBI [18, 29, 33, 62]. The fact that post-traumatic brain hypothermia significantly decreased the incidence of selective neuronal necrosis and contusion volume in this study supports such a contention.

Previous clinical and experimental investigations have reported the beneficial effects of whole body hypothermia following TBI [39, 54, 55]. In rodent models of fluid percussion TBI, hypothermic protection has also been documented [10, 35, 43, 44, 58]. Lowering body temperature to 30 °C prior to moderate TBI has been reported to decrease mortality significantly [10]. In that study, beam-balance score deficits and beam-walking latency deficits were also significantly reduced when brain temperature was lowered to 30 °C 5 min after TBI and maintained for 1 h. The present data are the first to provide evidence for histopathological protection with post-traumatic brain hypothermia following moderate fluid percussion TBI.

The mechanism by which brain hypothermia protects the brain after TBI is most likely multifactoral. In models of cerebral ischemia, for example, brain hypothermia has been shown to affect several ischemic processes (for reviews see [12, 21]). In models of TBI, the beneficial effects of hypothermia have also been reported. For example, the degree of BBB disruption following TBI was reduced when brain temperature was selectively lowered to 30 °C prior to and following the injury [35]. Hypothermia induced prior to TBI has also been shown to reduce the elevations in cerebrospinal fluid levels of acetylcholine [43] and attenuate the loss of hippocampal microtubular-associated protein 2 [58]. Interestingly, the therapeutic windows for post-traumatic and post-ischemic hypothermia both appear to be relatively short [7, 43]. Thus, TBI and brain ischemia may share similar temperature-sensitive pathomechanisms that occur early in the post-injury period.

In summary, under the present experimental conditions, fluid percussion brain injury leads to widespread histopathological consequences including contusion and selective neuronal necrosis. This model of TBI

should be advantageous in determining the acute mechanisms of contusion formation and the role of focal contusion in secondary injury. Post-traumatic brain hypothermia induced after trauma significantly reduced the extent of histopathological injury. Structural protection may, therefore, be possible in head-injured patients where early cooling can be initiated.

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Note added in proof Clifton and colleagues have recently reported beneficial effects with moderate hypothermia in a phase II study of severe head-injured patients. J Neurotrauma 10: 263–271, 1993.