

Ubiquitin Reduces Contusion Volume after Controlled Cortical Impact Injury in Rats

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ABSTRACT

Recent data suggest that ubiquitin has anti-inflammatory properties and therapeutic potential after severe trauma and brain injuries. However, direct evidence for its neuroprotective effects has not yet been provided. We hypothesized that ubiquitin treatment is neuroprotective, and thus reduces brain edema formation and cortical contusion volume after closed traumatic brain injuries. To test this hypothesis, a focal cortical contusion was induced using a controlled cortical impact (CCI) model in Sprague-Dawley rats. Animals ($n = 27$) were randomized to either 1.5 mg/kg ubiquitin or vehicle (placebo) intravenously within 5 min after CCI. Blood pressure, arterial blood gases (ABG) and intracranial pressure (ICP) were monitored. Ubiquitin serum and cerebrospinal fluid levels were measured by ELISA. Brain water content was quantified gravimetrically after 24 h and cerebral contusion volume was determined in triphenyltetrazolium-chloride stained brains after 7 days. All animals recovered to normal activity. ICP and cerebral perfusion pressures were normal at the end of the observation period. Ubiquitin serum and CSF levels at 24 h and 7 days after CCI were similar in both groups. With ubiquitin brain water content of the injured hemisphere was slightly lower ($n = 6/\text{group}$; $79.97 \pm 0.29\%$ vs. $81.11 \pm 0.52\%$; $p = 0.08$). Cortical contusion volume was significantly lower with ubiquitin ($n = 7\text{--}8/\text{group}$; $32.88 \pm 2.1 \text{ mm}^3$ vs. $43.96 \pm 4.56 \text{ mm}^3$; $p = 0.025$). This study shows that ubiquitin treatment after brain injury has direct neuroprotective effects, as demonstrated by improved brain morphology 7 days after brain injury. In connection with its beneficial effects in our previous studies, these data suggest ubiquitin as a promising candidate protein therapeutic for the treatment of brain injuries.

Key words: brain edema, controlled cortical impact, contusion, neuroprotection, ubiquitin

INTRODUCTION

UBIQUITIN is a small (8.5 kDa), heat stable and highly conserved protein that fulfills essential intracellular functions in all eukaryotic cells (Goldstein et al., 1975;

Özkaynak et al., 1984; Hershko and Ciechanover, 1998). In contrast to its intracellular role, little attention has been paid to its possible role outside the cell. Ubiquitin is a normal constituent of serum, plasma and cerebrospinal fluid (CSF) (Manaka et al., 1992; Okada et al., 1993; As-

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seman et al., 1994). Elevated ubiquitin concentrations in serum, plasma and CSF have been described in several pathological conditions (Asseman et al., 1994; Okada 1993; Kudo et al., 1994; Kurimura et al., 1997; Takagi et al., 1999; Akarsu et al., 2001), including significantly increased serum concentrations in severely injured blunt trauma patients and increased CSF concentrations in patients with severe traumatic brain injuries (Majetschak et al., 2003, 2005).

Previous observations suggested pleiotropic effects of ubiquitin on immune functions and host defense mechanisms *in vitro*, such as cytokine responses, alloreactivity, apoptosis, growth regulation and microbial defense (Daino et al., 2000; Majetschak et al., 2003; Kieffer et al., 2003; Earle et al., 2006; Patel et al., 2006). In a recent series of *in vivo* studies, we provided evidence for ubiquitin's therapeutic potential in models of infectious and non-infectious inflammation and showed that it has anti-inflammatory and immuno-suppressive actions (Majetschak et al., 2004a,b; Earle et al., 2005, 2006). In models of severe extremity trauma and traumatic brain injury combined with hemorrhagic shock, ubiquitin significantly reduced fluid shifts into the tissue and profoundly reduced intracranial pressure (ICP) in a porcine model of fluid percussion brain injury (Majetschak et al., 2004b; Earle et al., 2005). Although these data suggested indirectly that it reduces brain edema formation after traumatic brain injury, direct evidence for possible neuroprotective effects of exogenous ubiquitin has not been provided. Therefore, we hypothesized that ubiquitin treatment is neuroprotective, and thus reduces brain edema formation and cortical contusion volume after closed traumatic brain injuries.

METHODS

To test this hypothesis, we used a well described controlled cortical impact (CCI) injury model in rats. The study protocol was approved by the state animal care and use committee, Berlin, Germany. All animal procedures were performed according to National Institutes of Health Guidelines for Use of Laboratory Animals. Male Sprague Dawley rats ($n = 27$; 300–350 g body weight) were anesthetized with isoflurane (1.6–2.2 vol%) in N₂O (0.6 L/min) and O₂ (0.3 L/min) and allowed to breathe spontaneously via a mask. During anesthesia, rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ using a homeothermic heating pad. The right femoral artery and vein were instrumented with catheters for measurement of mean arterial blood pressure (MABP), blood sampling, and intravenous drug administration. Intracranial pressure (ICP) was measured by positioning an ICP micro sensor

(Codman, Johnson & Johnson, Norderstedt, Germany) in the right frontal hemisphere (relative to bregma: 0 to –4 mm), as described (Stover et al., 2003). A left parietotemporal craniotomy (7×7 mm) was performed along the sagittal, lambdoid and coronal sutures and the zygomatic arch. Animals were then subjected to a CCI injury, as described in detail previously (Kroppenstedt et al., 2003; Stover et al., 2003; Thomale et al., 2004). The cortical contusion was induced by a pneumatically accelerated bolt (diameter 5 mm; velocity 7 m/sec; penetration depth 1.5 mm; contact time 300 msec; pressure 5.2 bar). Bolt position was at a 45 degree angle perpendicular to the surface of the cerebral convexity approximately 3 mm lateral to the sagittal suture. The dura remained intact in all animals. Within 5 min after CCI, rats were randomized to receive either physiological saline solution (0.9% NaCl, 1 mL/kg body weight; placebo) or the same volume containing 1.5 mg/kg body weight ubiquitin (Boston Biochem, Cambridge, MA) in 0.9% NaCl intravenously via the femoral vein. Blood samples for arterial blood gas analyses (ABG) were obtained and MABP and ICP recorded at baseline, after CCI and 10 min after ubiquitin/saline injection. The bone flap was then reattached with bone cement and scalps were sutured. Catheters were removed and the animals returned to their cages. At 24 h after CCI, six animals of each group were re-anesthetized as described. The femoral artery was cannulated for MABP measurements and blood sampling for ABG and ubiquitin plasma levels. Animals were then sacrificed by exsanguination, brains were harvested and cerebrospinal fluid collected. Hemispheric water content was determined gravimetrically. Dissected hemispheres were weighed before and after drying at 100°C for 24 h to assess wet (WW) and dry weight (DW). Water content was calculated as follows:

$$\text{Water content}_{L/R} [\%] = (\text{WW}_{L/R} - \text{DW}_{L/R}) / \text{WW}_{L/R} * 100.$$

The remaining animals were re-anesthetized 7 days after CCI, and then sacrificed by exsanguination after measurement of MABP, ICP and ABG. CSF was collected and brains were harvested to determine cortical contusion volume. Directly before brain harvest bone flaps were removed for inspection of the contusion in all animals. A rigid fixation of the bone flap could be verified in all animals. Brains were cut in 1.2-mm slices beginning at the occipital pole using a commercially available matrix for rat brain (Brain Blocker, AgnTho's AB, Lidköping, Sweden). After incubation in 2% triphenyltetrazolium-chloride (TTC; Sigma) solution at 37°C for 20 min images of brain sections were recorded photographically. Off-line calculation of the contusion area was carried out using a computerized image analysis system

(ImageJ 1.34s, National Institutes of Health, USA) by multiplying the average area from the front and back of each slice by its thickness and adding all slices as previously described (Stover et al., 2000). Plasma and CSF ubiquitin concentrations were quantified as described in detail previously (Majetschak et al., 2004a,b; Earle et al., 2005), using an indirect competitive ELISA employing ubiquitin as a standard. The correlation coefficients for each standard curve were 0.95–1. Plasma from uninjured animals ($n = 8$) served as control. The lower detection limit was 0.9 ng ubiquitin/mL. The recovery of ubiquitin in spiked serum and CSF was 94–105%. Data are expressed as mean \pm SEM.

Statistical Analysis

Changes over time (ICP, MABP, ABG) were evaluated for statistical significance by analysis of variance (ANOVA). Statistical comparisons of brain edema and cortical contusion volume between the placebo and ubiquitin group were performed using the Student's *t*-test. Analyses were calculated with the Sigma Stat 3.0 program (Jandel Scientific, Erkrath, Germany). Differences were considered significant on a two-tailed $p < 0.05$ level.

RESULTS

MABP decreased immediately after CCI to 70.1 ± 14 mm Hg and returned to baseline values (89.1 ± 9 mm Hg) within 3 min. Except for this short time period MABP was stable and CPP above 65 mm Hg in all animals. ABG analyses of the spontaneously breathing animals were within the normal range at all time points (data not shown). Animals of both groups recovered uneventfully and returned to normal activity at 24 h and 7 days post-CCI. The body weight at 24 h and 7 days after CCI was comparable in both groups (with placebo: $-4.7 \pm 0.5\%$ at 24 h; $-5.0 \pm 2\%$ at 7 days; with ubiquitin: $-4.8 \pm 0.4\%$ at 24 h; $-3.1 \pm 1.2\%$ at 7 days; $p > 0.05$ for all comparisons), suggesting that ubiquitin treatment was well tolerated after CCI. We showed previously that peak ubiquitin serum concentrations after i.v. bolus injection of 1.5 mg/kg reached 11–15 $\mu\text{g/mL}$ and that the half-life of ubiquitin is approximately 60 min in serum, and 1.4 h in CSF (Majetschak et al., 2004b, 2005; Earle et al., 2005). Thus, the finding that ubiquitin plasma levels were unchanged between the placebo and ubiquitin group 24 h after i.v. injection was expected (placebo: 1945 ± 202 ng/mL; ubiquitin: 2024 ± 184 ng/mL). However, both treatment groups showed significantly elevated ubiquitin plasma levels, when compared with control animals (916 ± 97 ng/mL; $p < 0.05$). We interpret this increase to reflect the systemic response to TBI and

surgical trauma, as described in patients and animals (Majetschak et al., 2003, 2004b).

In agreement with our previous finding that hemolysis is one source of CSF ubiquitin *in vivo* (Majetschak et al., 2005), we also detected in the present study that CSF ubiquitin levels were 5–7-fold higher when hemolysis was present (not shown). Nevertheless, clear CSF samples were obtained in three to five animals per group. These CSF ubiquitin concentrations were 327 ± 166 ng/mL in the placebo group ($n = 4$) and 297 ± 108 ng/mL in the ubiquitin group ($n = 3$) at 24 h after CCI and decreased to 103 ± 63 ng/mL ($n = 3$) and 70 ± 24 ng/mL ($n = 5$) 7 days after CCI, respectively. The CSF ubiquitin levels 7 days after CCI are in line with baseline CSF concentrations detected previously in swine (Earle et al., 2005; Majetschak et al., 2005). Therefore, these data confirm increased ubiquitin release into CSF after TBI and suggest its clearance within 7 days. ICP was not significantly altered at any time point post CCI (Fig. 1A). In connection with stable hemodynamic parameters and an uneventful recovery of normal activity in all animals, these data show that the CCI is a model of moderate brain injury. At 24 h after CCI water content of the uninjured hemispheres were comparable in both groups ($78.37 \pm 0.28\%$ with ubiquitin vs. $78.45 \pm 0.50\%$ with placebo; $p > 0.05$) and lower than water content of the contused hemispheres ($p < 0.001$ for ubiquitin and placebo groups). These data are consistent with our previous findings in the same CCI model (Thomale et al., 2004, 2006). Although water content of the traumatized hemispheres showed a tendency towards decreased values with ubiquitin treatment, differences did not reach statistical significance ($79.97 \pm 0.29\%$ with ubiquitin vs. $81.11 \pm 0.52\%$ with placebo; $p = 0.08$; Fig. 1B). Despite the possibility that ubiquitin treatment does not affect brain edema formation 24 h after injury in rats, it should be considered that the statistically significant increase in brain water content of the injured hemisphere by 1.6–2.6% was overall modest and might not have been high enough to discriminate effects of ubiquitin with the given sample size.

At 7 days post-CCI, cortical contusion volume was significantly lower in the ubiquitin group than in the placebo group (32.88 ± 2.1 mm³ with ubiquitin vs. 43.96 ± 4.56 mm³ with placebo, $p = 0.025$; Fig. 1C). These findings provide initial evidence for its direct neuroprotective properties and further affirm ubiquitin's therapeutic potential.

DISCUSSION

Obvious limitations of the present study are the small sample size, a timing of treatment that is not feasible in

the clinical situation and the lack of sensorimotor and cognitive outcome measures. However, this study was designed to obtain proof of principle for direct neuroprotective effects of ubiquitin, which have been demonstrated for its effects on cerebral contusion volume. Another limitation is that we cannot provide new information on ubiquitin's possible mechanism of action. We showed previously that ubiquitin inhibits pro-inflammatory responses *in vitro* (Majetschak et al., 2003; Earle et al., 2006, Patel et al., 2006) and *in vivo* (Majetschak et al., 2004a,b). Recent data also suggest that it may enhance the anti-inflammatory response *in vivo* (Garcia-Covarrubias et al., 2007). Thus, its beneficial effects in the current and the four preceding animal studies could be mediated by its immune modulatory actions. On the other hand, we presented the concept that extracellular ubiquitin may target the intracellular ubiquitin proteasome pathway through a receptor mediated uptake mechanism (Majetschak et al., 2006). Thus, another pos-

sible explanation for its therapeutic effects *in vivo* could be that it protects the cell through modulation of the targeted cells intracellular ubiquitin proteasome pathway. Since the ubiquitin proteasome pathway regulates a variety of essential intracellular functions, such as protein metabolism or cell cycle control (Hershko and Ciechanover, 1998), its clinically relevant actions could also be independent of its effects on immune function.

Taken together, this pilot study shows for the first time that ubiquitin treatment after brain injury has direct neuroprotective effects, as demonstrated by improved brain morphology 7 days after brain injury. Since ubiquitin is an endogenous protein in the extracellular space and its administration was well tolerated in the current and all previous animal trials with dosing regimen for up to 2 weeks without noticeable side effects, its toxicity profile appears to be low (Earle et al., 2005, 2006; Majetschak et al., 2004a,b, 2005). The results of the present study in connection with its beneficial effects in a previous

A

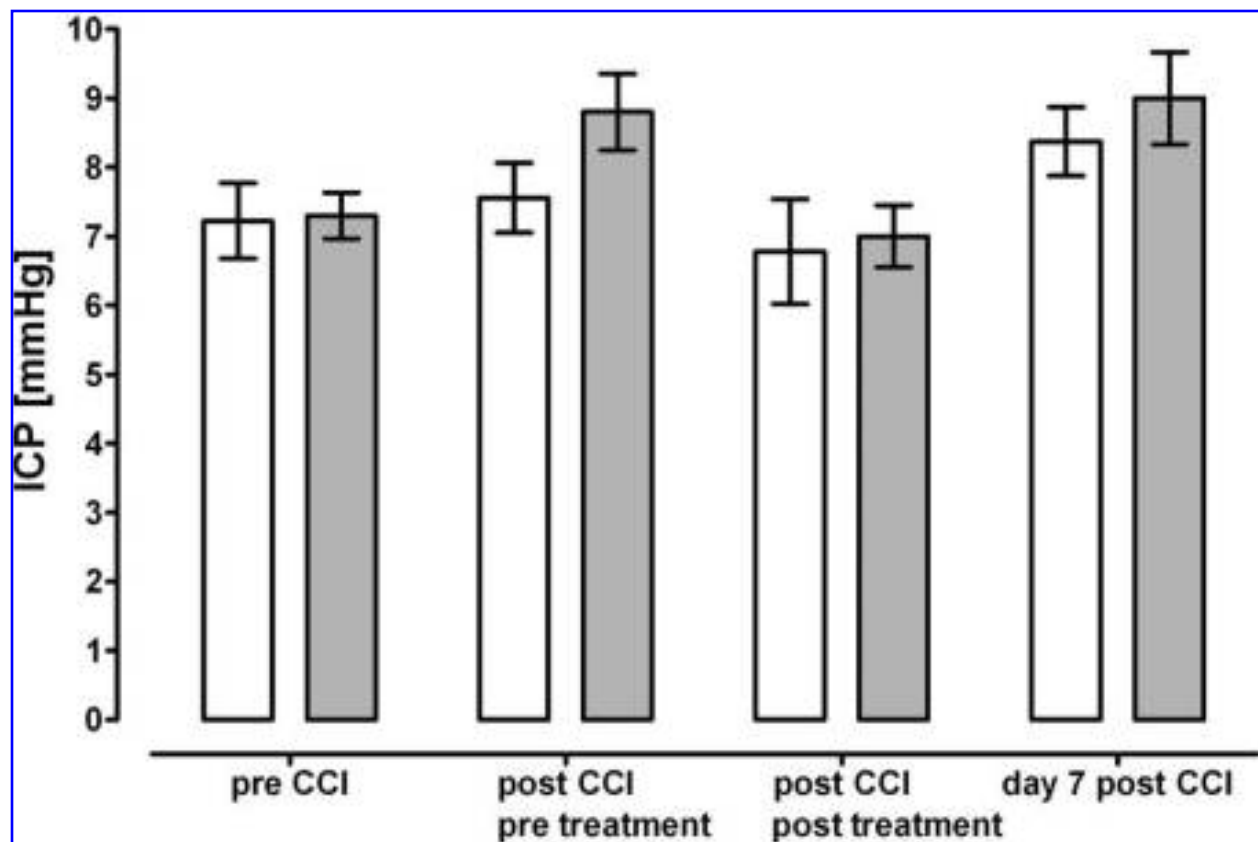
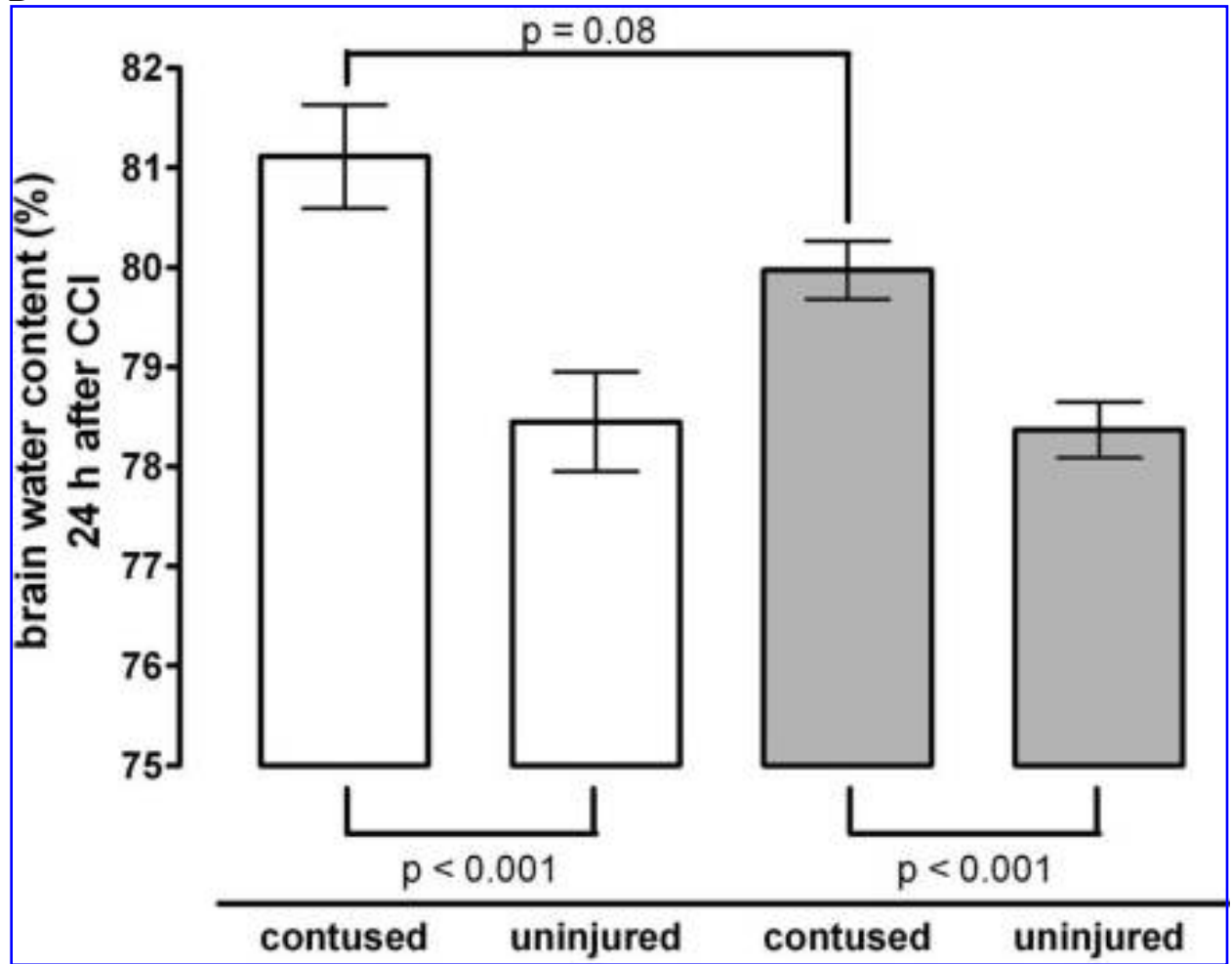
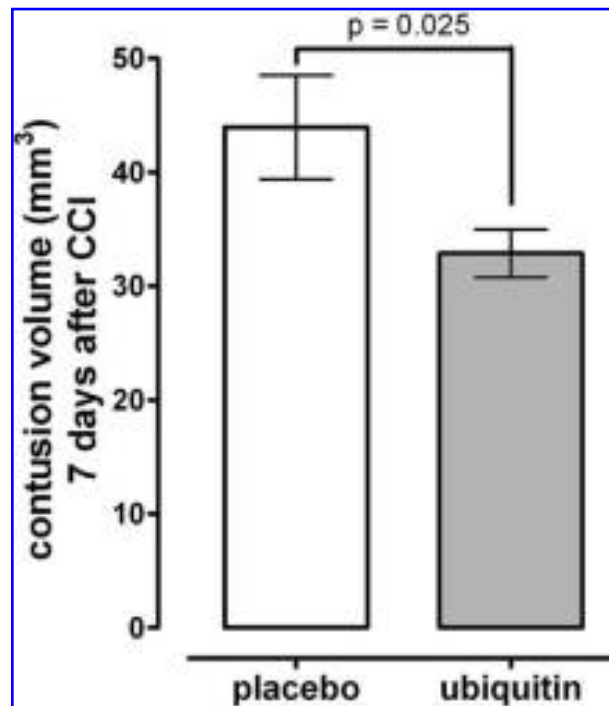


FIG. 1. (A) Intracranial pressure (ICP, mm Hg). CCI, controlled cortical impact; white bars, placebo group ($n = 6-13$); gray bars, ubiquitin group ($n = 7-13$). $p > 0.05$ for all comparisons. (B) Water content (%) of the contused and uninjured hemispheres 24 h after CCI. White bars, placebo group ($n = 6$); gray bars, ubiquitin group ($n = 6$). The level of statistical significance for intra- and inter-group comparisons is shown. (C) Quantification of the cortical contusion volume (mm^3) in triphenyltetrazolium-chloride (TTC)-stained brain sections at 7 days after CCI. White bar, placebo group ($n = 8$); gray bar, ubiquitin group ($n = 7$).

B



C



porcine fluid percussion brain injury study suggest that ubiquitin is as a promising protein therapeutic for the treatment of brain injuries. Based on these data, evaluation of its effects on behavioral outcomes and further characterization of its therapeutic window, dose-effect relationship and side-effect profile appear well justified.

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EFFECTS OF UBIQUITIN AFTER BRAIN INJURY

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