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Blood–Brain Barrier Breakdown and Edema Formation following Frontal Cortical Contusion: Does Hormonal Status Play a Role?

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ABSTRACT

The present experiment was designed to evaluate and correlate the time course of blood–brain barrier (BBB) integrity and cerebral edema in adult male rats given medial frontal cortex contusions. The effect of sex hormones on BBB integrity in the same injury model was also examined, because previous work has shown that progesterone can reduce cerebral edema (Roof et al., 1993). BBB breakdown was assessed by Evans blue extravasation and albumin immunostaining while edema formation was measured by the wet weight dry weight technique. These processes were examined beginning 2 h and continuing up to 10 days after injury. Our findings show that medial frontal contusion in rats produces changes in cerebral water content and opening of the BBB that endures at least 7 days postinjury. Although pseudopregnancy has been shown to reduce cerebral edema at day 1 postinjury, we did not find any evidence that this hormonal state is associated with BBB repair.

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) results in a series of delayed or secondary pathophysiological events including hemorrhage, intracranial hypertension, blood–brain barrier (BBB) breakdown, and cerebral edema (Miller, 1993). Cerebral edema is considered one of the most serious complications following TBI (Lobato et al., 1988; Luerssen et al., 1988). As described by Betz et al. (1989), "the presence or absence of brain edema may be the single most important determinant of outcome in patients with a variety of neurological injuries and disorders." Despite the importance of this problem, there is currently no completely effective clinical treatment that can eliminate brain swelling. In order to find appropriate pharmacological agents to treat edema, it is important to establish an animal model of head injury in which the course of edema formation and related pathological events can be characterized.

Formation of cerebral edema has been reported in various models of TBI, mainly after a closed head injury (Persson and Hansson, 1976; Mitchell et al., 1979; Tornheim et al., 1984; Cortez et al., 1989; Soares et al., 1992; Tanno et al., 1992; Shapira et al., 1993). Cerebral edema can be defined as the excess accumulation of water in the intra- and/or extracellular spaces of the brain. There are two major types of cerebral edema: vasogenic and cytotoxic. Vasogenic edema is the most common form of cerebral edema and

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is initiated by loss of the integrity of the BBB. The disruption of the BBB enables substances from the blood to enter the brain such as metals, kinins, prostaglandins, blood-borne cells, and proteins (Betz et al., 1989; Cortez et al., 1989; Greenwood, 1991). Plasma proteins, which are normally excluded from the brain, enter through the open BBB into the brain extracellular space and induce a water influx due to osmotic forces. This plasma protein extravasation results in formation of edema fluid (Klatzo, 1987). Vasogenic edema is the one most frequently seen in a clinical setting in response to trauma, primary and metastatic tumors, focal inflammation, and the later stages of cerebral ischemia (Pollay, 1985).

Cytotoxic edema is due primarily to derangement of cellular metabolism, which results in inadequate functioning of the sodium and potassium pump in the glial cell memebrane. The failure of the sodium and potassium pump allows Na^+, and therefore water, to accumulate within cells. Cytotoxic edema implies intracellular swelling of neurons, glia, and endothelial cells, with concomitant reduction of brain extracellular space. This type of cerebral edema is seen in various intoxications, Reye's syndrome, severe hypothermia, and the early stages of ischemia (Pollay, 1985).

Resolution of edema may occur by one or both of the following mechanisms: (1) edemic fluid flows from its source to the ventricular system, driven by a hydrostatic pressure gradient between the lesion itself and the lower pressure cerebrospinal fluid (CSF) pool; edemic fluid is then cleared from the ventricles (Betz et al., 1989); (2) the extravasated proteins in edematous tissue are taken up from the extracellular space and degraded by neurons and glia. This uptake reduces the extracellular osmotic force that has created edema (Klatzo et al., 1980; Betz et al., 1989). These processes are coupled with the repair of the damaged BBB, thus creating a favorable osmotic gradient, for water to move from the extracellular space of the brain into the blood.

In order to measure the BBB integrity after brain insult, the abnormal presence of blood proteins in brain tissue, such as albumin, can be used as an endogenous marker (Todd and Graham, 1990). Evans blue (EB) dye, which does not normally cross into the intact brain in appreciable amounts, is also a reliable indicator of BBB compromise after injury. Both of these methods were used in the present study to assess the effect of cerebral contusion on BBB disruption and repair.

Our laboratory has developed and evaluated an impact contusion injury model of cortical damage in the adult rat brain (Fülöp et al., 1992; Roof et al., 1992, 1993; Sutton et al., 1993; Hoffman et al., 1994). Lesions are produced by using a pneumatic piston device that contuses the exposed brain while controlling for the parameters of velocity and depth of impact. In our recent studies we chose the medial frontal cortex (MFC) as the injury site (Fülöp et al., 1992; Roof et al., 1992, 1993; Hoffman et al., 1994). The site of injury is relevant because frontal impact is one of the most common causes of TBI in humans following automobile accidents (Lighthall et al., 1989; Mattson and Levin, 1990). Rats with cortical contusions show profound and long-lasting behavioral deficits, as well as the development of cerebral edema (Roof et al., 1992, 1993), tissue necrosis, cavity formation (Hoffman et al., 1994), and reactive gliosis (Fülöp et al., 1992).

We have also observed a significant influence of sex hormones on edema resolution following this type of head injury (Roof et al., 1993). In our experiment we found that following MFC contusions, cerebral edema was much lower in pseudopregnant females (PPF) compared to normally cycling females (NCF) and males. By ovariectomizing females and then replacing their hormones, we were able to demonstrate that progesterone is responsible for this beneficial effect. Although the mechanisms by which progesterone relieves edema are not yet known, one possibility lies in the repair of the BBB. There is literature to suggest that certain steroids may act to repair the BBB (Reid et al., 1983; Gamache and Ellis, 1986; Hedley-Whyte and Hsu, 1986; Ziylan et al., 1988; Guerin et al., 1992). For example, dexamethasone was shown to produce a decreased vascular permeability to α-aminobutyric acid and sucrose in normal rats (Ziylan et al., 1988) and to modify the permeability of cerebral blood vessels to horseradish peroxidase in normal mice (Hedley-Whyte and Hsu, 1986). In a recent study, Guerin et al. (1992) showed that dexamethasone treatment resulted in reduction of vascular permeability to EB in a rat brain–glioma model. Pretreatment with U-74006F, a 21-aminosteroid, normalized capillary permeability after subarachnoid injection of blood (Zuccarello and Anderson, 1989). The role of steroids in BBB function and repair has been examined in various experimental models, but not following TBI and not specifically with progesterone. Based on this information, the present study was designed to compare BBB injury-involved changes in males, NCF, and PPF.

METHODS

Subjects

Eighty-two male and 20 female Sprague–Dawley rats, approximately 90 days of age at the start of the experiment, served as subjects. Rats were housed individually in suspended rack-mounted cages, with a 12 h:12 h light:dark reversed light cycle. Food and water were provided *ad libitum*.

Surgery

Seventy males and 12 females were anesthetized with sodium pentobarbital V-pento™ (50 mg/kg, IP) and mounted in a Kopf™ stereotaxic device. A midline incision was made in the scalp and the fascia were retracted to expose the cranium. A dental drill was used to create a 6-mm-diameter bilateral craniotomy immediately anterior to bregma. The contusion was created with a pneumatic piston impactor device attached to the stereotaxic carrier. The device consists of a small (9/16 in.), dual stroke, air cylinder containing a 3-mm-diameter piston. A circular (5-mm-diameter) stainless steel tip was attached to the bottom of the piston and activated with compressed air (Sutton et al., 1993).

To create the injury, the tip of the piston was first placed gently on the dura in order to determine the extent of piston penetration into the cortex, and then retracted back to the original position above the exposed dura. The height of the tip was recorded and then lowered 2 mm. The impact was then made with a force of 20 psi, at a velocity of 2.25 m/sec, and the tip remained in contact with the brain for 0.5 sec. After the impact, cortical surface hemorrhaging was stopped by placing Gelfoam™ sponge on the injury site and then the fascia and the scalp were sutured.

The contusions were produced on the sixth day of pseudopregnancy or on the day of proestrus in the case of NCF. To determine the day of proestrus, estrous cycle was monitored by the lavage method for two full cycles; that is for approximately 10 days (Asdell, 1946). Pseudopregnancy was induced by pressing the rubber plunger of a 1-mL syringe against the cervix for 1 min, using 300 *g* of force.

An additional 12 males and 8 females (4 NCF and 4 PPF) served as sham-operated controls, receiving anesthesia and scalp incision, but no cortical injury. These controls did not receive craniotomy because this procedure may produce edema and the object was to compare rats with injury to those without it.

Measurements of Edema

Edema was measured in 20 contused male rats at four time points after surgery: Two hours ($n = 4$), 1 day ($n = 6$), 3 days ($n = 6$), and 7 days ($n = 4$). Edema was measured also in 4 additional sham-operated males. Data from females have been collected in an earlier study (Roof et al., 1993) and are presented in the discussion. The edema measurement was based on the method of Karpiak and Mahadik (1984). Briefly, rats were decapitated under general (V-pento™, 75 mg/kg, IP) anesthesia and the brain surface was exposed within 90 sec. Four, 2-mm core punches were taken from areas bordering the lesion (no more than 1 mm from lesion). Four additional punches were taken from the occipital/posterior parietal areas, distal to the lesion area. Each tissue punch was quickly placed in a preweighed container, which was then capped immediately to prevent evaporation. The tissue and the container were then weighed together. Weights were recorded to the nearest 0.1 mg. The caps were then removed and the containers placed in a vacuum oven and dried at 60°C, 0.3 atm pressure, for 24 h. After drying, the containers were removed from the oven, immediately recapped, and reweighed. All weighing was done on the same balance. The percent water was calculated by the following equation:

$$\% \text{ water} = \frac{(\text{wet sample weight} - \text{dry sample weight}) \times 100}{(\text{wet sample weight})}$$

Measurements of Vascular Permeability to Evan's Blue

Vascular permeability to EB was evaluated on 28 contused male rats at 2 h ($n = 4$), 1 day ($n = 10$), 3 days ($n = 5$), 7 days ($n = 5$), and 10 days ($n = 4$) days after injury. At 1 day postinjury, vascular permeability to EB was also measured in PPF ($n = 6$) and NCF ($n = 6$). An additional 4 rats per hormone group

served as sham-operated controls. Thirty minutes prior to perfusion, deeply anesthetized animals (Pentobarbital, V-pento™; 50 mg/kg, IP) were intravenously infused (via the femoral vein) with EB solution (1% EB in 0.9% saline, 2 mL per rat) for 5 min. The dose used in this study exhibits total binding to serum albumin (Cole et al., 1991). Thirty minutes after EB injection, the rats were given an overdose of pentobarbital (V-pento™; up to 75 mg/kg, IP) followed by intracardiac vascular perfusion with cacodylate buffer (8.6 g NaCl and 1 g sodium cacodylate in 1000 mL of dH_2O adjusted to pH 7.5) and then by fixative (200 mL of 20% paraformaldehyde, 5.9 g $CaCl \cdot 2H_2O$, 3.6 g NaCl, and 1 g sodium cacodylate in 800 mL of H_2O adjusted to pH 7.5) (Gallyas, 1970). The brains were removed, left in the fixative overnight, and then for 3 days in fixative containing 30% sucrose. The brains were frozen in dry ice and cut at 40 μm on a sliding microtome. EB was visualized using fluorescent light (nm = 550). The borders of EB extravasation were drawn on 4 representative sections of each brain, at distances of 3.7, 2.7, 1.7, and 0.7 mm rostral from bregma (Paxinos and Watson, 1982) using a camera lucida. The drawings were then assessed using a Bioquant™ Image Analyzer to delineate and quantify the area of EB extravasation.

Albumin Immunohistochemistry

The presence of albumin was studied in 22, contused male rats at 1 day (n = 10), 3 days (n = 4), 7 days (n = 4), and 10 days (n = 4) after injury. At 1 day postinjury, albumin presence was also measured in PPF (n = 6) and NCF (n = 6). An additional 4 rats per hormone group served as sham controls. The presence of albumin in brain parenchyma was detected by antialbumin immunohistochemistry on paraformaldehyde-fixed brain sections. Forty-micron-thick floating brain sections were stained immunohistochemically for albumin using the peroxidase–antiperoxidase (PAP) method (Sternberger et al., 1970). Sections were pretreated with 0.3% H_2O_2 for 30 min, and preincubated for 30 min in diluent (3% goat serum, 1% bovine serum albumin, 0.1% Triton, and 0.1% glycine in PBS, pH 7.4) to block nonspecific binding. Sections were then incubated with antialbumin antibody (Biogenex Labs), diluted 1:5000 in the same diluent, and labeled with Multi-link™ and Label™ reagents (Biogenex Labs) in dilutions of 1:100 for 20 min each, and stained with 3,3′-diaminobenzidine (Sigma). The entire procedure was performed at room temperature. Four stained sections from each brain, at distances of 3.7, 2.7, 1.7, and 0.7 mm from bregma (Paxinos and Watson, 1982), were assessed under a light microscope attached to the Bioquant Image Analyzer to quantify the area of albumin staining.

Statistical Analysis

The data were statistically evaluated using analysis of variance (ANOVA) and then Student's t test between each pair of time points, using Supernova™ and Statview™.

RESULTS

Edema formation, measured as an increase in brain tissue water content, was found only in regions immediately around the injury area (medial frontal cortex), while in distal areas (noninjured tissue) the percentage of water content (78.8% ± 1.2) was similar to that found in intact brains (79.8% ± 1.4). Analysis of variance of water content of brain areas around the injury revealed significant changes [$F(3,16) = 10.87$; $p < 0.001$] over time after surgery. The increase in cerebral water content in injured tissue relative to distal, noninjured tissue at four time points after injury is shown in Figure 1. Edema was detected as early as 2 h after injury. By 1 day after injury, the tissue water content had significantly increased [$t(9) = 2.77$; $p < 0.05$; 2 h vs 1 day], decreased on the third day, and significantly dropped by 7 days postinjury [$t(9) = 3.63$; $p < 0.01$; 3 days vs 7 days].

Analysis of variance of the area of EB extravasation revealed a significant effect [$F(4,17) = 12.36$; $p < 0.0001$] of time after surgery on BBB integrity. EB was detected in the injured region as early as 2 h and had significantly increased by 3 days [$t(8) = 4.43$; $p < 0.001$; day 1 vs day 3], and disappeared by day 10. The time course of the changes in area of EB extravasation is shown in Figure 2.

Albumin immunostaining also changed significantly with time after surgery [$F(3,12) = 13.51$, $p < 0.001$] and showed a similar temporal patterning to that observed for EB extravasation. The immunostaining was

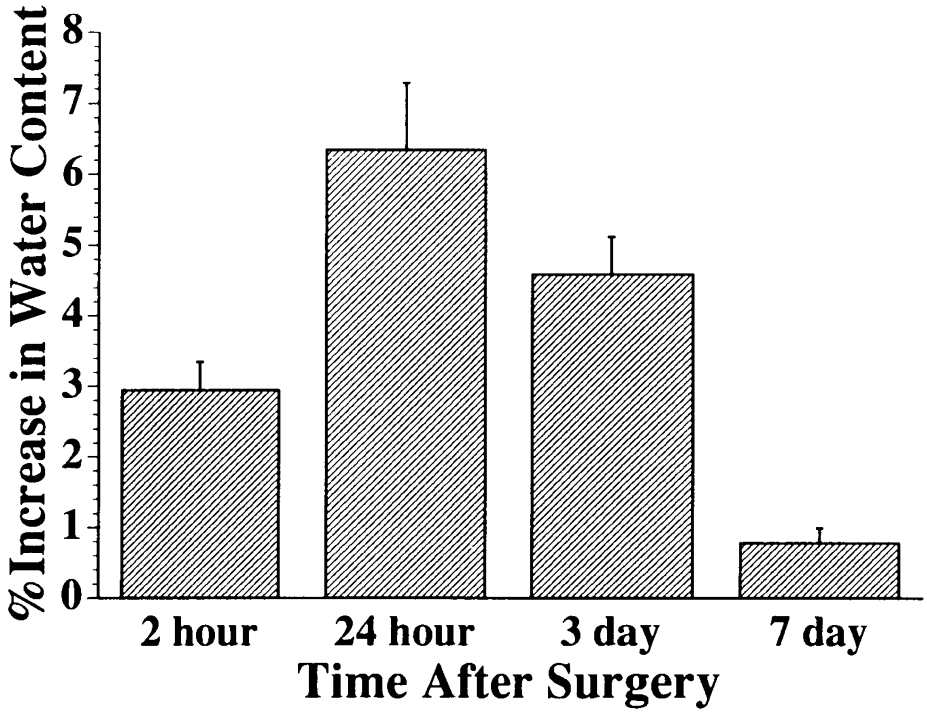

FIG. 1. Changes in percentage increase of cerebral water content in injured brain tissue of male rats relative to non-injured tissue, as a function of time after surgery. The data are represented as mean ± standard error (SE).

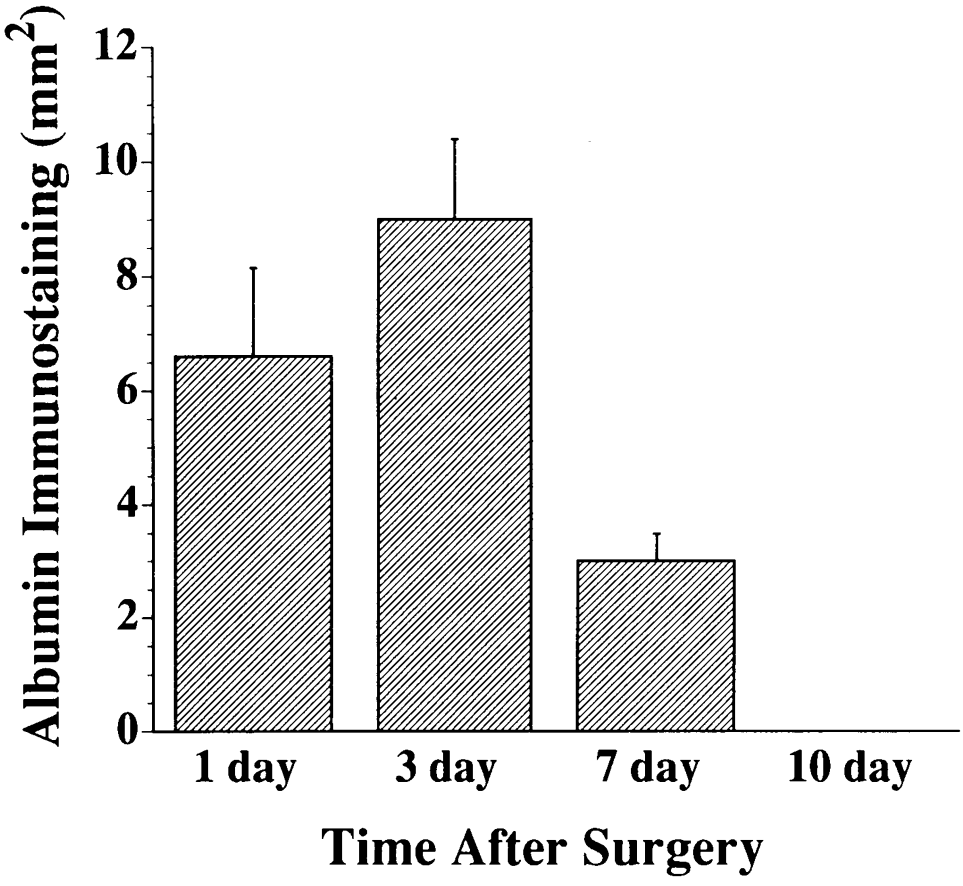

FIG. 2. Changes in the area of albumin immunostaining in contused male rat brains as a function of time after surgery. The data are represented as mean ± SE.

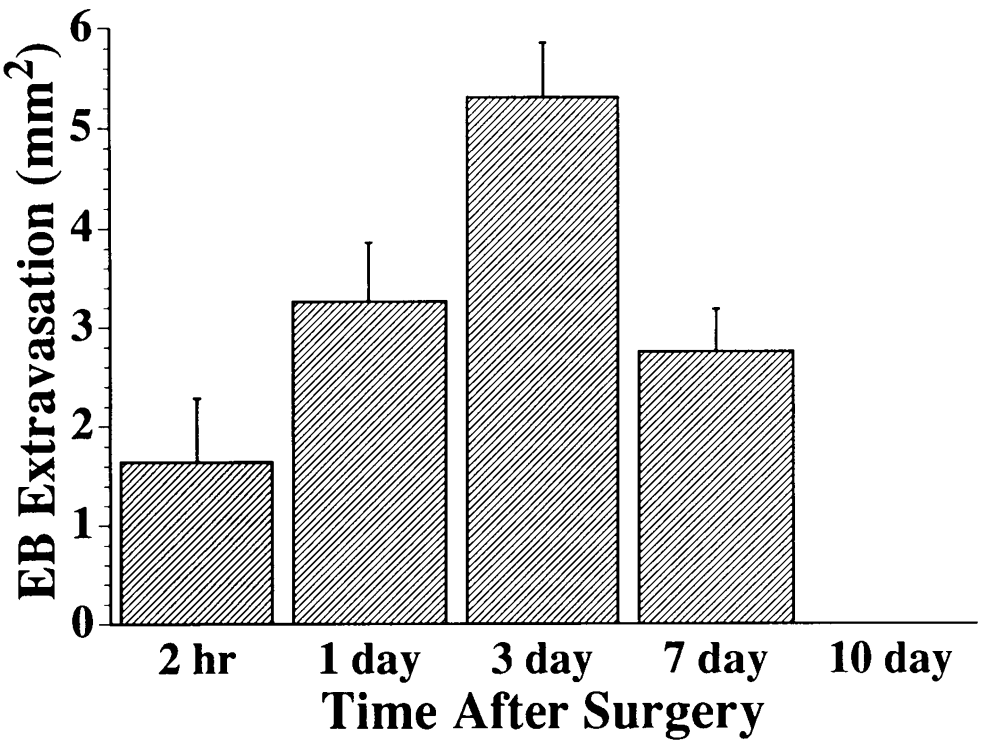

FIG. 3. Changes in the area of EB extravasation in contused male rat brains as a function of time after surgery. The data are represented as mean ± SE.

detected in injured brains at 1 day, decreased significantly at 7 days [$t(7) = 2.34$; $p < 0.05$; 3 days vs 7 days] after contusion, and disappeared by 10 days. The time course of the changes in area of albumin immunostaining is shown in Figure 3. No EB extravasation or albumin immunostaining was observed in intact brains. Schematic diagrams of rat brain coronal sections [modified from original drawings (Paxinos and Watson, 1982)] summarizing the distribution of albumin immunostaining and EB on representative brain sections from 1 day to 7 days after injury are shown in Figure 4. Minimal and maximal extent of albumin immunostaining or EB extravasation was illustrated according to the areas measured. This figure also shows the cavity that was formed by the contusion injury and visible beginning at 3 days after injury. Note that the areas of EB extravasation or albumin immunostaining could not be measured wherever tissue was missing.

Analysis of hormonal status showed that males, NCF, and PPF did not differ in either EB extravasation (Fig. 5A) or albumin immunostaining (Fig. 5B). No albumin staining or EB extravasation was evident in sham-operated males, NCF, or PPF.

DISCUSSION

Changes of BBB Permeability and Cerebral Edema

One of the aims of this study was to examine the time course of edema formation and BBB disruption following bilateral MFC contusions. Early postinjury we observed both a significant accumulation of water in brain tissue and opening of the BBB. Cerebral edema and disruption of the BBB endured up to 7 days postinjury. Then, by 7 days postinjury, the cerebral edema disappeared, while the BBB remained open for up to 10 days, even after normalization of water content in the brain tissue.

A similar pattern of BBB disruption and repair was reported following cerebral cold injury (Bodsch et al., 1982). The authors suggested that since edema can be resolved by two mechanisms, its disappearance may occur even while the BBB remains open. Only one of the mechanisms for resolution of edema in-

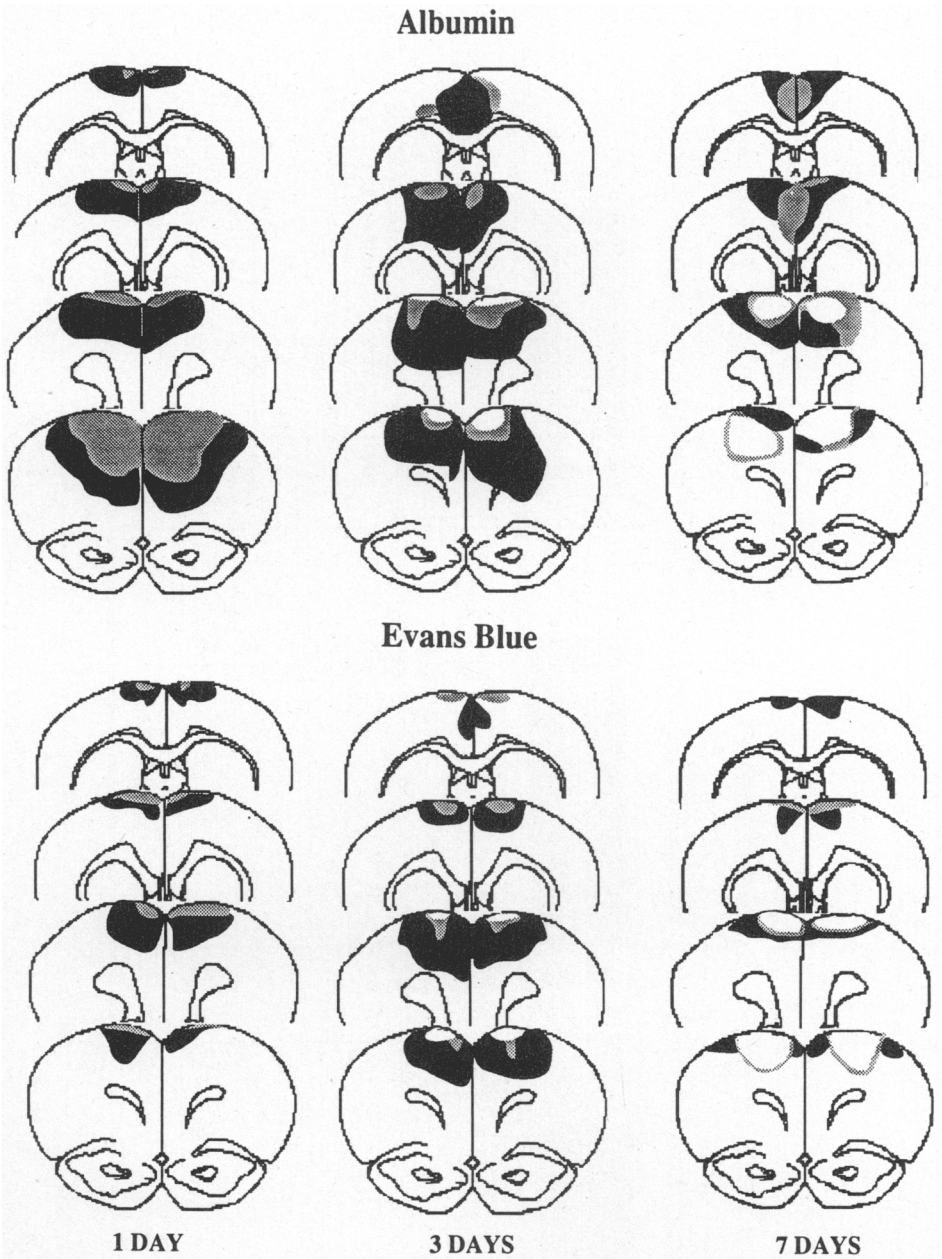

FIG. 4. A schematic diagram showing the distribution of EB extravasation and albumin immunostaining in contused male rat brains at 1 day, 3 days, and 7 days after surgery. The darkened areas correspond to maximal distribution area, and the lightened areas correspond to minimal distribution area. At 3 days and 7 days white areas correspond to area of cavitation.

volves intracellular degradation of extravasated proteins that have entered through the open BBB (Klatzo et al., 1980; Betz et al., 1989), while the second mechanism involves bulk flow of edemic fluid into the ventricles. These two mechanisms work in parallel and the end result might be that edemic fluid is resolved through the ventricles, although edema-related proteins remain in the cells.

The contusion injury in our model includes severe physical damage to the BBB. It can be expected that repair of the BBB, which has a complex biology (i.e., interaction of astrocytes, pericytes, and endothelial cells in formation of continuous tight junctions), will take longer than restoration of the brain water con-

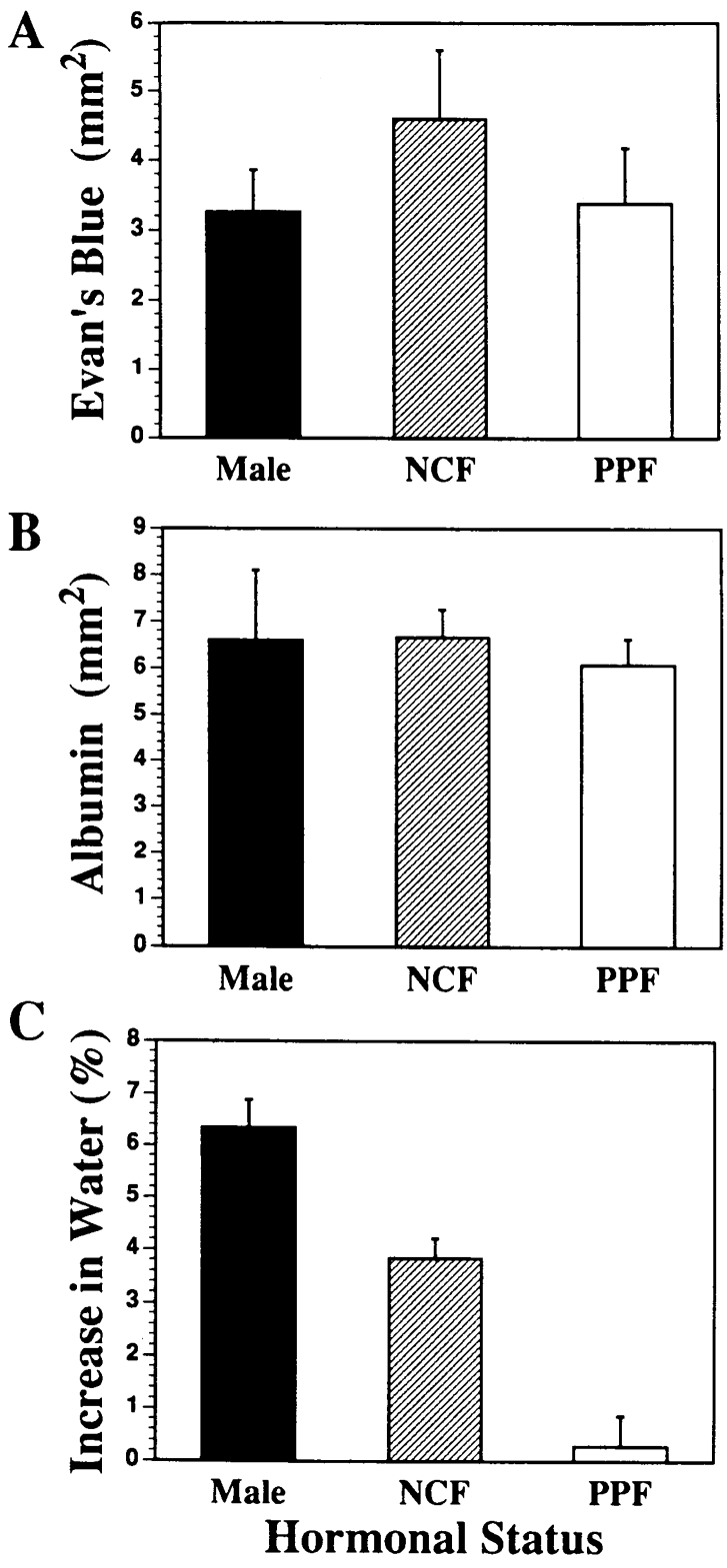

FIG. 5. Changes in the area of EB extravasation (A), area of albumin immunostaining (B), and increase of cerebral water content (C) as a function of hormonal status of contused males and females.

tent to its normal level. The process of regaining homeostasis of brain water content, as described above, might be more rapid than the structural reconstruction.

In our study, we observed prolonged BBB opening and edema. In most experimental studies (Persson and Hansson, 1976; Mitchell et al., 1979; Tornheim et al., 1984; Cortez et al., 1989; Tanno et al., 1992) cerebral edema or opening of the BBB was examined up to 24 h following TBI. For those reports in which later time points were included, prolonged BBB opening and edema were also observed. For example, Soares et al. (1992) showed development of prolonged cerebral edema in a fluid-percussion closed head injury model, which could be detected from 6 h up to 5 days after injury. Shapira et al. (1993) showed that a weight-drop closed-head injury caused BBB opening for as long as 4 days after the insult, while cerebral edema was detected and gradually resolved by 4–7 days.

On the negative side, the prolonged opening of the BBB may have serious implications for brain-impaired individuals, such as the stimulation of inflammatory reactions (caused by penetration of blood-borne cells), leading to additional tissue destruction and behavioral impairments. However, on the positive side, opening of the BBB for an extended period may serve as a "window of opportunity" for systemic treatment by drugs that otherwise would not cross an intact BBB.

The Effect of Hormonal Status on BBB Permeability

In our previous findings (Roof et al., 1993) we found that PPF showed less edema than males or NCF (Fig. 5C). According to this finding and to the known relationship between edema and BBB, we hypothesized that the presence of high levels of progesterone during pseudopregnancy, which significantly reduced postinjury edema within 1 day after injury, would affect BBB breakdown following TBI. Our findings did not support this hypothesis. We found no effect of hormonal status on BBB integrity at 1 day postinjury, the time point in which progesterone produced dramatic reduction in cerebral water content (Roof et al., 1993). One explanation can be related to the existence of two parallel mechanisms for edema resolution, as explained in the first part of the discussion. Accordingly, it seems that in PPF rats edema might have been resolved because edemic fluid was carried off via the ventricles. However, EB and albumin marked some proteins that were still extravasating or proteins that were left behind, indicating that the BBB might still be open. It is possible that even if progesterone contributes the repair of the BBB, it may take longer then 24 h for this effect to be detected.

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