

## History of Neurosurgery

# Influence of Aging on Blood-Brain Barrier Permeability and Free Radical Formation Following Experimental Brain Cold Injury

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## Summary

The aim of this study is to investigate the effects of experimental cold brain injury on blood-brain barrier integrity, on brain oedema formation, and on lipid peroxidation and to compare the results between the aged and young rats. Cold brain injury was used to create a standard model of brain trauma in old and young rats. Disruption of the blood-brain barrier was analyzed by Evans blue method. The values of cerebral water content were calculated by using the fresh and dry weights of the cerebral hemispheres. Lipid peroxidation was assessed by measuring the tissue content of malonyl dialdehyde.

Blood-brain barrier was destroyed significantly in young and old rats, but it was more severe in old rats. Accordingly, cerebral water content was increased in both groups, however this increase was significantly more prominent in old rats. No significant difference was found on malonyl dialdehyde levels between young and old rats.

The blood-brain barrier was more easily disrupted in old rats, and this was supposed to be the basic event causing more secondary damage.

**Keywords:** Aging; brain oedema; blood-brain barrier; cold injury; lipid peroxidation.

## Introduction

Compared with their younger counterparts, elderly patients are thought to have lower resistance to central nervous system (CNS) insults. This idea is certainly prevalent in the literature on head injury; however, the pathogenesis is not well understood [1, 8, 9, 16]. It has been frequently stated that the increased mortality rate in older brain-injured patients is a result of more frequent systemic complications [4, 13]. But, Baxt and Moody [3] have clearly demonstrated that, for multiple-injured patients, the severity of brain injury is the major determinant of mortality. The unfavorable outcome in elderly patients seems to be based upon an alteration in the pathophysiological response of

the aging CNS to severe trauma, rather than on an increased incidence of age-related non-neurological complications [25]. The adverse effect of age in cerebral injury is not restricted to trauma, but also holds true for ischaemia and subarachnoid haemorrhage [27]. The cardinal effects of any insults to the CNS is the disruption of blood-brain barrier (BBB) integrity and resulting brain oedema accompanied by secondary damage which is mainly caused by free radicals.

The aim of this study was to investigate the effects of cold brain injury on BBB integrity, on brain oedema formation, and on lipid peroxidation in the brain of young and old rats.

## Materials and Methods

Forty-seven 3 month old and forty-seven 36 month old male Wistar albino rats, bred at Istanbul University Centre for Experimental Medical Research and Application (DETAM), were used in the study. DETAM maintains a barrier-bred colony of rats of different ages of Wistar albino strain. In the life span of the Wistar albino rat, a 3 month old rat represents a fully mature, young adult, and a 36-month-old rat represents an elderly animal (i.e., approximately a 70-year-old human). Young rats weighed between 200–230 g and aged ones weighed between 400–420 g. The rats were divided into 10 groups (Table 1) – 4 control and 6 trauma – and anaesthetized with intraperitoneal ketamine (50 mg/kg). All animals were free to access water and food before and after the experiments.

### *Craniectomy and Performance of Cold Injury*

This stage of procedure was accomplished as described by Tomi-naga and Ohnishi [22]. Briefly, anaesthetized rats were stabilized in a smooth plane in a prone position. Following a vertical midline skin incision, sagittal and left coronal sutures were identified. Periosteum of left parietal region was peeled away from the midline and temporal muscle was dissected to uncover the temporal bone from its at-

Table 1. *Experiments and Control Groups of the Procedure (' Indicate Aged Animals)*

|   |  |  |
|---|--|--|
| Y | Blood-brain barrier groups (BBB)       | blood-brain barrier trauma (BBBTr) (N = 11)    |
| U |  | blood-brain barrier control (BBBCtrl) (N = 7)  |
| G | Hemispheric water content group (HWC)  | hemispheric water content (HWC) (N = 11)       |
|   | Lipid peroxidation groups (LP)         | lipid peroxidation trauma (LPTr) (N = 11)      |
|   |  | lipid peroxidation control (LPCtrl) (N = 7)    |
| A | Blood-brain barrier groups (BBB')      | blood-brain barrier trauma (BBBTr') (N = 11)   |
| E |  | blood-brain barrier control (BBBCtrl') (N = 7) |
| D | Hemispheric water content group (HWC') | hemispheric water content (HWC') (N = 11)      |
|   | Lipid peroxidation groups (LP')        | lipid peroxidation trauma (LPTr') (N = 11)     |
|   |  | lipid peroxidation control (LPCtrl') (N = 7)   |

tachment. A small hole using a dental drill was opened on the central part of the left parietal bone which was constantly irrigated with isotonic solution to cool off throughout this process. The hole was enlarged using a mini-curved haemostat. Dura remained intact. A craniectomy of 10 × 15 mm was performed beginning from the left of midsagittal plane and extending to the temporoparietal region. Extreme care was taken not to damage the sagittal sinus. Bonewax (Ethicon) and Surgicel (Ethicon) were used to stop bleeding if required. Following the exposure, a copper probe of 4 × 10 mm was used to create a standard focal freeze injury using liquid nitrogen at -70 °C which was kept in contact with bare dura for 45 seconds. Monolayer suturing during closure was performed.

#### Methods for Evaluation

**Blood-brain barrier integrity:** Animals were anaesthetized again 24 hours after the cold injury and were placed supine. Left femoral vein was identified under the operating microscope and 1 mL of Evans blue was injected intravenously with the aid of a 29 G sterile needle. Five minutes later, when blue appearance was observed on the sclera of the rats, the thorax was dissected and inserting a needle into the left ventricle, the rats were perfused with isotonic saline solution. Perfusion was continued for at least twenty minutes till clear isotonic fluid was drawn from dissected jugular veins. Normal appearance of the sclera of the rats was routinely observed at the end of this procedure. The dead rats were decapitated and the brains removed in a period shorter than three minutes trying to avoid any additional damage. The brains were divided in the mid-callosal plan. The hemispheres were weighed on preweighed aluminium foils, homogenized with 50% trichloroacetic acid, centrifuged 15 000 rpm for 20 minutes and absorption was measured at 615 nm [2]. The results were expressed in terms of Evans blue (µg)/tissue (g). The same procedure was applied to the rats in the control groups without performing cold brain injury.

**Hemispheric water content:** Twenty four hours after the cold injury, the animals were sacrificed and the brains were quickly removed with no additional insults. The cerebral hemispheres were separated and were taken to numbered aluminum foils with predetermined weight. The fresh weight of each specimen was noted. The samples

were heated at 105 °C and kept in a constant temperature for 48 hours. The values were calculated using the fresh and dry weights of the hemispheres [22].

**Lipid peroxidation:** Lipid peroxidation was assessed by measuring the tissue content of malonyl dialdehyde (MDA), one of the end products of lipid peroxidation. Twenty-four hours after the cold injury, the rats were decapitated and the brains were removed and divided in the mid-callosal plane to obtain only cold injury left hemispheres and stored at -70 °C until the homogenization procedure. Tissue samples were homogenized in ice-cold trichloroacetic acid (TCA)(1 g. tissue plus 1 ml 10%, wt/vol, TCA plus 8 ml 5%, wt/vol, TCA, or equivalent amounts) in an ultra Turrax tissue homogenizer. After centrifugation, a volume of the supernatant was added to give an equal volume of 0.67% (wt/vol) thiobarbituric acid, and the mixture was heated at 100 °C for 10 minutes. The absorption spectrum was then recorded over 480–600 nm. The spectrum was quite similar to that obtained with an MDA standard produced by the acid hydrolysis of 1,1,3,3-tetra-ethoxypropane and run under the same conditions. The MDA concentration was calculated from the absorption at 532 nm. (absorption maximum) of the difference spectrum, with the use of a molar extinction coefficient of  $1.56 \times 100\,000$ , as reported by others and also recalculated from our standards. The same procedure was applied to the rats in the control groups without performing cold injury to the brain.

#### Statistical Analysis

All values are expressed as means ± SD. The results were analyzed by using Student's t-test. A p-value less than 0.05 ( $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ ) was considered statistically significant.

## Results

### Blood-Brain Barrier Permeability

The results of extravasated Evans blue were evaluated separately for each hemispheres of the four BBB groups and are shown in the Table 2. Table 2 contains also the p values of various comparisons between these groups. As could be expected, the degree of BBB disruption as indicated by extravasation of Evans blue was significantly greater in the left cerebral hemisphere exposed to cold injury than in the undamaged left

Table 2. *Mean ± SD Values [Evans Blue (µg)]/Tissue (g)] for Blood-Brain Barrier (BBB) Permeability of the Right (R) and Left (L) Hemispheres of the Four BBB Groups and the Statistical Comparison Between Various Groups (' Indicate Aged Animals)*

| Groups    | Mean ± SD    | Compared groups     | P      |
|-----------|--------------|---------------------|--------|
| BBBTrL    | 0.60 ± 0.026 | BBBCtrlL-BBBCtrlR   | NS*    |
| BBBTrR    | 0.57 ± 0.059 | BBBCtrlL'-BBBCtrlR' | NS     |
| BBBCtrlL  | 0.55 ± 0.052 | BBBTrL-BBBCtrlL     | <0.05  |
| BBBCtrlR  | 0.57 ± 0.052 | BBBTrL'-BBBCtrlL'   | <0.001 |
| BBBTrL'   | 0.72 ± 0.041 | BBBCtrlL-BBBCtrlL'  | NS     |
| BBBTrR'   | 0.59 ± 0.087 | BBBCtrlR-BBBCtrlR'  | NS     |
| BBBCtrlL' | 0.52 ± 0.061 | BBBTrL-BBBTrR       | NS     |
| BBBCtrlR' | 0.56 ± 0.048 | BBBTrL'-BBBTrR'     | <0.001 |
|           |              | BBBTrL-BBBTrL'      | <0.001 |

\* Not significant.

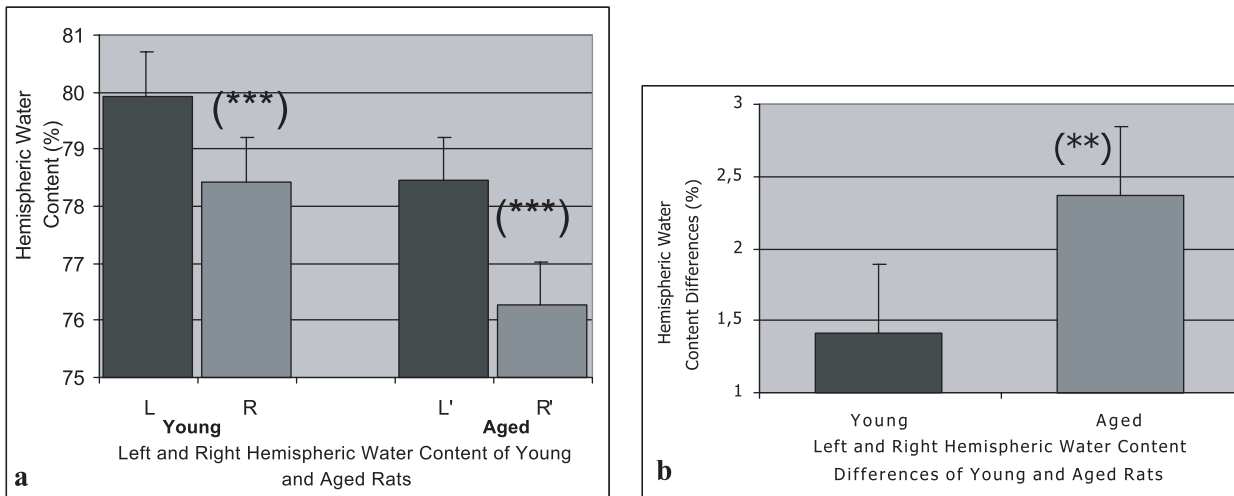


Fig. 1. (a) Left and right hemispheric water content of young and aged rats. (b) Left and right hemispheric water content differences of young and aged rats

hemisphere of the control groups both in young and old rats. However, the degree of disruption was significantly greater in old animals as compared with the young ones.

#### Brain Oedema

The increase of hemispheric water content was significant in both young and old rats but the increase was significantly more pronounced in the group of old rats (Fig. 1a and b). In the old animals, the right and left hemispheric water content difference was 2.37%, whereas it was 1.4% in young animals (Fig. 1b). In general, hemispheric water content was significantly lower in old rats, e.g. 78.44% in the right control hemispheres of young, and 76.26% in the right control hemispheres of old animals (Fig. 1a).

#### Lipid Peroxidation

The MDA content is listed in Table 3 by group. The comparison of the results were achieved a statistical

Table 3. Mean  $\pm$  SD Values of Malonyl Dialdehyde (MDA) (Micromoles MDA per Gram of Wet Weight) in the Experimental Groups and the Statistical Comparison of the Groups (' Indicate Aged Animals)

| Groups  | Mean $\pm$ SD    | Compared groups | p     |
|---------|------------------|-----------------|-------|
| LPTr    | 51.55 $\pm$ 6.49 | LPTr-LpCtrl     | <0.05 |
| LPCtrl  | 44.14 $\pm$ 2.27 | LpTr-LpTr'      | NS*   |
| LPTr'   | 58.73 $\pm$ 9.88 | LpTr'-LPCtrl'   | <0.05 |
| LPCtrl' | 47.86 $\pm$ 3.93 | LPCtrl-LPCtrl'  | NS    |

\* Not significant.

significance in only between the trauma applied animals and their control ones both in young and old rats (Table 3).

#### Discussion

Although many clinical studies exists investigating the effects of age on outcome from head injury, there have been relatively few studies of traumatic brain injury in young as compared to old animals [23]. Using the fluid-percussion brain injury model in rats, Hamm *et al.* [8, 9] investigated in two different studies the effects of aging on outcome after brain injury in rats. Their data demonstrated that aging was associated with an increased mortality rate and increased motor and cognitive deficits. Also, except a decrease in heart rate, no meaningful observations were noted in systemic physiological responses to head injury. They concluded their study by affirming that aged brains were more vulnerable to excitatory neurotransmitters release after traumatic brain injury and they called this the excitotoxic hypothesis [8, 9]. In another experimental study, oedema generation following a standardized cryogenic lesion was found to be markedly enhanced in old versus young rats [23]. Truly, because of associated increased intracranial pressure, altered cerebral blood flow, and release of substances that are toxic to brain, cerebral oedema, which results from disruption of the BBB, is one of the important prognostic factors in various disorder of the brain. Oxygen radical mediated lipid peroxidation has been suggested to be another important factor in neuronal degenera-

tion especially after head injury [7]. In this study, we addressed the age question on the pathogenesis of cerebral oedema or on the deleterious effects of the free radicals on an experimental model to produce cerebral oedema. We investigated the effects of aging on BBB integrity, on cerebral oedema formation and on lipid peroxidation in order to clarify the tenets that predict that young patients are more resilient and recover better than the elderly after sustaining a similar insult.

#### *Trauma Model*

To create a standard cerebral oedema in experimental animals, we adopted a cold brain injury model [22]. The model was effective to disrupt the BBB as seen in the statistical comparisons of the damaged left cerebral hemispheres of both young and old groups with their controls.

#### *Blood-Brain Barrier Disruption and Brain Oedema*

Quantitative analysis of the disruption of the BBB was carried out by measuring the amount of extravasated Evans blue injected from the femoral vein [2]. The comparison of the results between left and right hemispheres of the young and old control groups were not significant, confirming the validation of our method for measuring BBB integrity.

Vasogenic brain oedema is the type of oedema most frequently seen in a clinical setting in response to trauma, primary and metastatic tumors, focal inflammation, and the later stages of cerebral ischaemia. Disruption of the cerebral capillary provides the underlying mechanism for the development of vasogenic oedema. On the other hand, age-related changes in the cerebral microvasculature include changes in the cross-sectional area of the capillary wall, reduced numbers of endothelial cells, and mitochondria in the endothelial cells, gliofibrillar proliferation, and increased basement thickness [17, 21]. Aging was also associated with significant arteriovenous shunting in the cerebral microvessels [18]. However, a number of reports have shown that there is no significant alteration in BBB permeability with senescence per se in the absence of neurological disease [19, 20, 26]. Accordingly, the results of our study showed that in undamaged rats, BBB permeability was not affected by aging as seen in comparison with the old and young control groups. But, it was also documented that conditions com-

monly associated with aging, such as hypertension [11, 14] and transient cerebral ischaemia [15], have an important effect on the barrier function of the cerebral vasculature. In agreement with this, our study showed a significant disruption of the BBB in the cold injury hemispheres of the old rats, but not in young ones, and a more remarkable result, which confirmed a marked disruption of the BBB in old rats, was obtained by comparison of the trauma applied to hemispheres of the young and old rats.

On the other hand, confirming the results of other studies concerning the effects of experimental cold induced brain injury on brain oedema [12, 23], hemispheric water content was found significantly higher in the left hemispheres of young and old rats. But, the hemispheric water content differences between young and old rats showed that oedema generation following a standardized cryogenic lesion is markedly enhanced in old versus young rats. This remark was previously made by Unterberg *et al.* [23]. We attribute the cause of more brain oedema in old animals to the severity of the BBB disruption in old animals.

#### *Lipid Peroxidation*

The free radical theory proposed by Harman [10] postulates that free radical damage contributes to the aging phenomenon. Oxidative stress has also been implicated in the pathogenesis of neurodegenerative disease [6]. However, the results of studies investigating cerebral lipid peroxidation in old animals are still controversial [5].

In the injured brain, free radicals formed around the oedematous areas of the brain can cause lipid peroxidation of the cellular membrane, followed by calcium influx into the cell through calcium channels. These secondary insults may aggravate vasogenic brain edema. It has been reported that the lipid peroxidation level in the injured brain tissue reached its maximum 2 hours after the trauma and progressively slowed down afterwards [24]. We evaluated the effects of cold injury on lipid peroxidation by assessing MDA formation in the brain at 2 hours after the cold injury. A statistical comparison of the trauma groups with their control ones showed a significant difference in the MDA level confirming previous observations. But, the same observation could not be seen when we compared brain tissue MDA levels in young and old rats subjected to cold trauma. Also, we have found no statistical significance between the young and old control groups. We

concluded that our trauma model was effective in causing oxidative stress in animals, but free radicals had no more deleterious effects in old animals.

## Conclusion

In conclusion, it has been observed that BBB was destroyed considerably by focal cold injury in both young and old rats, however the destruction was more severe in the older animals. Similarly, hemispheric water content increased in both traumatized groups but more so in older ones. Nevertheless, lipid peroxidation level did not demonstrate any significant difference between young and old rats.

The severity of the BBB dysfunction is supposed to be the basic event for explaining the more favorable outcome in young patient after sustaining a similar insult to the CNS. The reason for this may be understood better after further ultrastructural studies.

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## Comments

The aim of this study was to investigate the effects of age on brain edema formation, blood brain barrier integrity and lipid peroxidation, following experimental cold injury. The authors conclude that blood brain barrier disruption and edema formation are more pronounced in older animals. No differences were found on lipid per-

oxidation as assessed by measuring the tissue content of malonyl dialdehyde. The objectives of the study are clearly stated, the methodology and results concisely described. One may debate the validity of extrapolating results from a model in cold injury to the clinical situation of patients with traumatic brain injury. The remarks of the authors that vasogenic edema is the type of edema most frequently seen in a clinical setting in response to for instance trauma has been challenged by other investigators. Nevertheless, this study is of good scientific quality and addresses the important issue of the effect of age on outcome.

*A. Maas*

The objective of this study is interesting i.e., to investigate the mechanisms responsible for the poor response of the aging brain to different types of injury, which remains still largely unexplained. The conclusions of the study are that the brain of aged rats exposed to a cold injury develops greater BBB disruption, and consequently more marked edema than the brains of young animals. These findings are expected and have been previously reported by other investigators.

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