AA-861 appears to suppress leukocyte infiltration induced by traumatic brain injury in rats

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ABSTRACT

الأهداف: دراسة أثر 5،3،2 ترمثايل 1–6، (12هيدروكسي5 ، 10دوديكادين1)–1، 4–بينزوكوينون، على عنصر (AA– 861) على (ICAM–1) وظهور (P–selectin)، ومستوى ليكوترين بي 4 (LTB4)، ونشاط مايلوبيركسيديس (MPO) لمدة 24 ساعة بعد التعرض للإصابة في الدماغ (TBI).

الطريقة: أجريت هذه الدراسة بمختبر قسم الصيدلة السريرية بجامعة عثمان غازي – تركيا في عام 2006م. تم التحريض بإصابة الدماغ (TBI) لمجموعتين من الحيوانات (الجرذان) باستعمال طريقة فينيس لسقوط الوزن. تم استعمال المجموعة الأولى لدراسة ظهور (ICAM–1)، و(P–selectin)، و(CD11a) والخلايا المحببة باستعمال الجسم المستضد (HIS48). تم استعمال المجموعة الثانية لتغيير النسيج في مستوى (LTB4) ونشاط (MPO). تم التضحية بالجرذان عند 0.5، 4، 24، 48، 72 ساعة بعد الإصابة.

النتائج: كانت ذروة ICAM–1–p=0.000001 و P–selectin p=0.00002 عند 24 ساعة، وبقيت مرتفعة عند 48 ساعة (p=0.00012) و (p=0.00002)، وعند 72 ساعة (ICAM–1–p=0.000008) (P–selectin–p=0.00002) ارتفعت نسبة (HIS48) عند 24–72 ساعة (p=0.022)، بينما كانت شدة (CD11a) ملحوظة فقط عند 72 ساعة (p=0.040). ازداد نشاط (MPO) بشكل ملحوظ عند 24 ساعة (p=0.00077)، وبلغ ذروته عند 48 ساعة (p=0.00001). ازداد (LTB4) بشكل ملحوظ عند 4 ساعات (p=0.000004)، وبلغ ذروته عند 24 ساعة (p=0.000001). خمدت ما قبل المعالجة بعنصر (AA–861) ظهور ICAM–1–p=0.0053، و P–selectin–p=0.0018 على بطانة الأوعية الدقيقة و انخفض نشاط MPO–p=0.0007 ومستوى LTB4–p=0.008 عند 24 ساعة.

خاتمة: إن عنصر (AA–861) قد يكون وسيط رئيسي في علاج إصابات الدماغ (TBI).

Objective: To study the effect of 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA-861) on intercellular adhesion molecule 1 (ICAM-1) and P-selectin expression, leukotriene B4 (LTB4) level, and myeloperoxidase (MPO) activity 24 hours after traumatic brain injury (TBI).

Methods: This study was carried out in the laboratory of the Department of Clinical Pharmacology, Osmangazi University, Eskisehir, Turkey in 2006. Traumatic brain injury was induced in 2 sets of animals using Feeney's weight-drop method. The first set was used to study the expression of ICAM-1, P-selectin, CD11a, and mouse anti-rat granulocyte monoclonal antibody (HIS48). The second was used to study tissue changes in LTB4 level, and MPO activity. The rats were sacrificed at 0.5, 4, 24, 48, and 72 hours post-injury.

Results: Intercellular adhesion molecule (p=0.000001) and P-selectin expression (p=0.00002) peaked at 24 hours, remained high at 48 hours (p=0.00012 for ICAM-1, and p=0.00002 for P-selectin), and 72 hours (p=0.000008 for ICAM-1, p=0.0011 for P-selectin). The HIS48 intensity was significantly increased at 24-72 hours (p=0.022), while the intensity of CD11a became significant only at 72 hours (p=0.040). Myeloperoxidase activity increased notably at 24 hours (p=0.00077), and peaked at 48 hours (p=0.00001). The LTB4 increased markedly at 4 hours (p=0.000004), and peaked at 24 hours (p=0.000001). Pretreatment with AA-861 considerably suppressed the expression of ICAM-1 (p=0.0053), and P-selectin (p=0.0018) on microvascular endothelium, and lowered MPO activity (p=0.0007), and LTB4 level (p=0.008) at 24 hours.

Conclusion: The present results suggest that AA-861 might be a potential mediator in the treatment of brain inflammation in TBI.

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Accumulation of polymorphonuclear leukocytes (PMLs) and monocytes in traumatic brain injury (TBI) is identified to contribute to the pathogenesis of secondary brain damage. Inflammatory leukocytes migrating from blood vessels following TBI can mediate, at least in part, neuronal injury, cerebral edema, elevated intracranial pressure, and hyperemia.[1-3] An influx of neutrophils at sites of lesion occurs 24-48 hours after TBI, and requires the interaction of circulating inflammatory cells with vascular endothelium.[4-6] Blood-borne inflammatory cells in TBI initially bind to vascular endothelium by low-affinity adhesive forces that are sensitive to shear stress. This low affinity binding, results in a rolling phenomenon of circulating inflammatory cells, a process mediated by the selectin family of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1). Activation in the expression of CD11/CD18 glycoprotein on leukocytes, and up-regulation of endothelial ICAM-1 and P-selectin, strengthens inflammatory cell-vascular endothelium interaction, thereby, resulting in firm adhesion that is resistant to shear stress.[6-8] Myeloperoxidase (MPO), a lysosomal enzyme secreted by activated PML, is released in phagolysosomes during phagocytosis.[9] Myeloperoxidase activity is used as an indicator of leukocyte accumulation in the injured brain.[10] Moreover, production of leukotrienes from arachidonic acid through 5-lipoxygesase (5-LO) pathway, is shown to be involved in the pathogenesis of inflammatory immune reactions, blood-brain barrier damage, edema formation, cerebral blood flow disturbances, and ultimately neuronal death.[11-13] Leukotriene B4 (LTB4), and 5-hydroxyeicosatetraenoic acid (5-HETE) are prominent chemo attractants factors for PMLs in inflammation.[14] The control of PMLs influx following TBI is shown to attenuate secondary brain damage in a rat model.[2] In addition to blocking binding of leukocytes to vascular endothelium using antibodies,[15,16] several compounds that inhibit PMLs influx were used.[11,17] As metabolites of 5-LO pathway contribute to inflammation in injured brain,[11] we postulated that 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA-861), a specific 5-LO inhibitor, can be used to restrain PMLs influx. The AA-861 is demonstrated to inhibit the formation of 5-HETE, and LTB4 synthesized from endogenous arachidonic acid from stimulated rat macrophages.[18] Although the effects of AA-861 on blood brain barrier permeability and edema was examined in ischemia and experimental brain tumors,[12,19] the effect of AA-861 on PMLs accumulation in post traumatic brain was not studied. In this study, we aimed to evaluate the therapeutic value of AA-861 by examining ICAM-1, and P-selectin expression on microvascular vessels, MPO activity, and LTB4 production in a TBI rat model.

Methods. The experiment was carried out in the laboratory of the Department of Clinical Pharmacology, of Osmangazi University Medical School, Eskisehir, Turkey in 2006. In this study, a total of 97 adult male Sprague-Dawley rats weighing 250-350 grams were used. Animal care was in compliance with the guidelines of the Medical Faculty of Eskisehir Osmangazi University Research Council Criteria. The animals were kept at room temperature, and had free access to food and water. Animals were anesthetized with a combination of ketamine hydrochloride (60 mg/kg), and xylazine (12 mg/kg) administered intraperitoneally. Atrophin sulfate (10 mg/kg) was also given to prevent excessive pulmonary secretion. The trachea was intubated with a 14 gauge angiocatheter, and lungs were mechanically ventilated using a small rodent ventilator (Ugo Basile Biological Research Apparatus, Comerio, Italy). Ventilation setting was adjusted for maintaining an arterial carbon dioxide partial pressure of 30-40 Torr, and a pH 7.3-7.5. There was no significant difference between groups related to pH, and blood gases. A femoral catheter was inserted into the left femoral artery for continuous monitoring of blood pressure and arterial blood sampling (Nihon-Coherder System, Tokyo, Japan). The experiment was performed under normotensive conditions, and a rectal probe was also inserted for continuous monitoring of body temperature, which was maintained at 37±0.5°C by means of an automated heat lamp. Traumatic brain injury was induced using a modification of Feeney's weight-drop method. We designed a new device to semi-automate the experimental trauma method of Feeney.[20] A craniotomy was made over the right parietal cortex with a dental drill. The coronal and interparietal sutures were used as landmarks. Cerebral injury was introduced using an electrical circuit switch, which caused a 10 g brass rod (3.0 mm diameter, 15.2 cm length) to drop from a distance of 5 cm through a Plexiglas guide tube, onto exposed dura over the right parietal cortex. After the scalp trauma incision was closed, the rats were allowed to ventilate mechanically at least 0.5 hours until they recovered. Then, the rats were returned to their cages. The experiment was performed in 2 sets of animals. The first set was used to study the expression of ICAM-1 (CD54), P-selectin, CD11a (the alfa-chain of lymphocyte function-associated antigen-1), and a mouse anti-rat granulocyte monoclonal antibody (HIS48). A group of animals (n=3) undergoing craniotomy alone was used as control. The rats subjected to cerebral injuries were randomly divided in 5 groups (n=4 for each group) as: surviving 0.5, 4, 24, 48, and 72 hours post-injury. The rats were reanesthetized and perfused with 100 ml saline, via left cardiac ventricle, and killed by decapitation. The brains were removed and sectioned coronally through the center of the contusion. Three

coronal sections passing through the contusion taken from each rat were analyzed. Freshly prepared slides were used to study the expression of ICAM-1 (CD54), P-selectin, CD11a, and HIS48. Both anterior and posterior segments of brain were placed in mounting media. Alternate coronal cryostat sections (7 µm) were placed on poly-L-lysine coated glass slides, fixed in acetone (5 minutes, at 25°C), and stored at -20°C. Subsequently, the sections were washed for 2 minutes in phosphate-buffered saline (PBS) at pH 7.4, incubated for 3 minutes in 3% H_2O_2 to quench endogenous peroxidase activity, then washed in PBS containing 2% normal goat serum. The sections were incubated with primary monoclonal antibodies including anti-rat granulocyte (HIS48, used at 1:50 dilution, Pharmingen, San Diego, CA, USA), CD11a integrin α chain (LFA-1, used at 1:100 dilution, Pharmingen), P-selectin (CD62P, used at 1:100 dilution, Pharmingen), and purified anti-rat CD54 (ICAM I, used at 1:500 dilution, Pharmingen). Antibody staining was optimized using different dilutions until obtaining a clear signal with minimal background staining. The sections were first incubated with primary monoclonal antibodies in a humidified chamber for one hour. After thorough washing in PBS for 10 minutes, the sections were reacted with biotinylated goat-anti-mouse IgG (at 1:100 dilutions) for one hour. After washing the sections in PBS, the reaction was visualized using ABC reagent for 30 minutes. The slides were counterstained with hematoxylin, dehydrated, and mounted before the microscopic evaluation. An observer blinded to the treatment group obtained all immunohistochemical data. To quantitate the expression of ICAM-1 and P-selectin, the mean number of immuno-labeled vascular profiles in 10 microscopic fields (x400) of slides produced from the injured and non-injured hemispheres was counted. The microscopic fields were randomly selected from different regions of the slides. The intensity of immunoreactivity was evaluated as minimal, moderate, and intensive. The expression of CD11a and HIS48 was evaluated semi-quantitatively using an Olympus microscope at x400 and scored on a 3-point scale (0: no staining, 1: faint or moderate staining, and 2: intense staining). The second set was used to determine the time dependent tissue changes in LTB4 level and MPO activity. Sham-operated rats (n=7) that underwent craniotomy alone were used as a control. The rats were randomly divided into 5 groups (n=9 for each group) and allowed to survive 0.5, 4, 24, 48, and 72 hours post-injury. The rats were re-anesthetized and perfused with 100 ml saline via left cardiac ventricle. The brains were removed quickly and frozen in liquid nitrogen and stored at -80°C to determine LTB4 level and MPO activity. Myeloperoxidase activity and LTB4 were examined on the brain cortical and subcortical tissue taken from the contusion site including 3-4 mm in diameter neighboring area. Myeloperoxidase activity was assayed according to the method of Suzuki et al[21] and expressed as U/g wet weight. Briefly, the samples were homogenized initially in 50 mmol/lt potassium phosphate buffer and were centrifuged at 1500g for 10 minutes. A total of 500 ml homogenate was then centrifuged at 40,000g for 15 minutes at 4°C. The supernatant was used to determine MPO activity. Analysis of LTB4 was carried out using an enzyme immunoassay (EIA) commercial kit as instructed by the provider (Amersham Life Science, UK) and expressed as pg/g wet weight. AA-861 (Wako Chemicals GmbH, Germany) was dissolved in dimethylsulfoxide (DMSO) and 0.1-0.125 ml of this preparation was used for each injection as described by Iyobe and coworkers.[22] Three animals in the first set and 9 animals in the second set were treated with AA-861 at 60 mg/kg dose intraperitoneally, 0.5 hour before the TBI. The same volume of DMSO was used as vehicle. The AA-861-treated animals in the first set were used to examine the effect of AA-861 on ICAM-1 and P-selectin expression on microvascular endothelium. The AA-861-treated animals in the second set were used to assess the effect of AA-861 on LTB4 level and MPO activity. The vehicle (0.1-0.125 ml of DMSO) was given into 3 rats in the first set and 7 rats in the second set of animals. Both AA-861-and vehicle treated rats were killed at 24 hours post-injury

Statistical evaluations. The data were presented as means ± SEM and analyzed using SPSS for windows 15.0. If data showed normal distribution and did not have homogeneity of variances, the significance of the differences between groups was determined by Tamhane test after calculating one-way analyses of variance (ANOVA). If the data had a normal distribution and homogeneity of variance, Turkey's tests were performed. Semiquantitatively scored data for granulocyte and CD11a were analyzed using the rank sum test to determine significant difference from sham control. Values smaller than $p<0.05$ were accepted as statistically significant.

Results. To determine the ICAM-1 expression at selected times, purified anti-rat CD54 (ICAM-1) was used. Representative sections illustrating typical labeling patterns for anti-rat CD54 are presented in **Figure 1**. The ICAM-1 positive vessels were few in number in sham-operated rats. In traumatized animals the number of ICAM-1 positive vessels started to increase at 0.5 hours, peaked at 24 hours ($p=0.000001$), and remained high at 48 hours ($p=0.00012$) and 72 hours ($p=0.000008$) post-injury when compared to sham control (**Figure 2**). The number of ICAM-1 positive vessels in non-traumatized

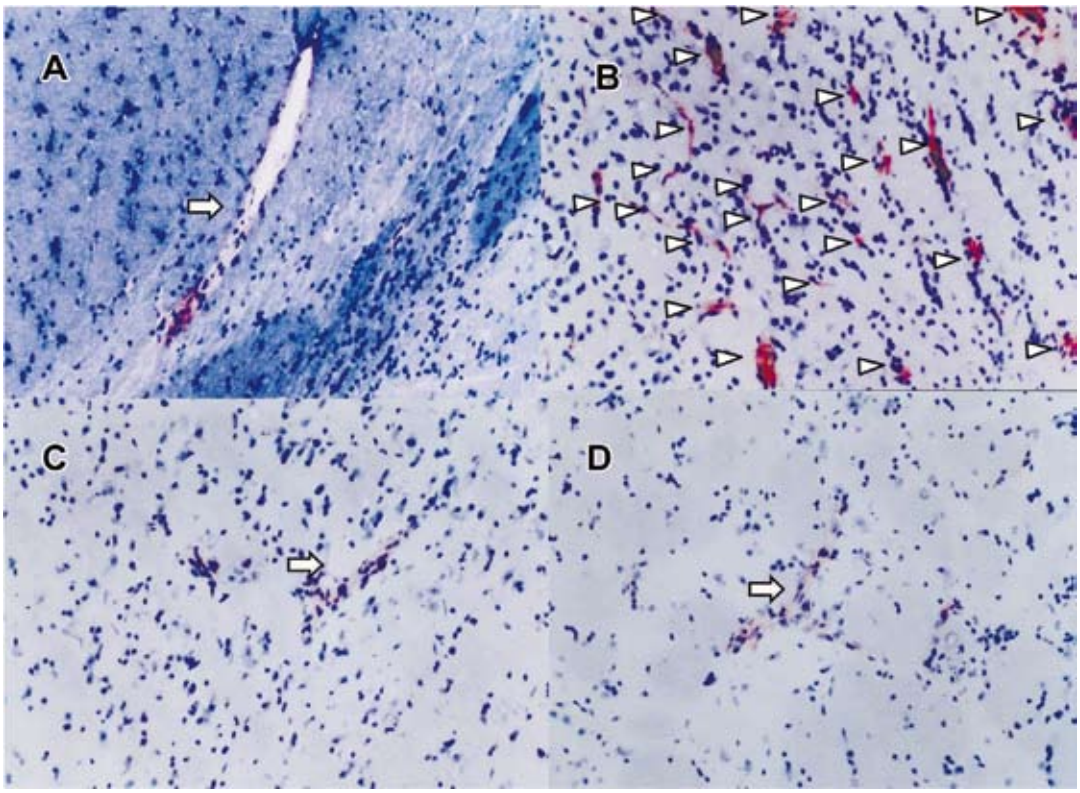

Figure 1 - Adhesion molecule 1 (ICAM-1) expression on cerebrovascular endothelium at selected times. Panels **A**, **B**, and **C** show ICAM-1 immunoreactivity at 0.5 (arrow), 24 (arrow heads), and 72 hours (arrow), after traumatic brain injury in traumatized hemispheres. Panel **D** shows the effect of AA-861 on ICAM-1 expression at 24 hours post trauma. The ICAM-1 immunoreactivity is weak at 0.5 and 72 hours (panels **A** & **C**), intense at 24 hours (panel **B**). The AA-861 pretreatment significantly lowered the ICAM-1 intensity (panel **D**, arrow). Note that the number of ICAM-positive cells was numerous at 24 hours after injury. Original magnification, x200. AA-861 - 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone

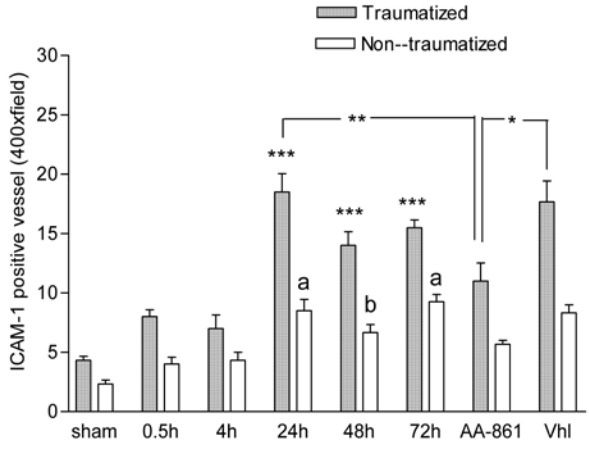

Figure 2 - The number of adhesion molecule 1 (ICAM-1) positive vessels in traumatized and non-traumatized hemispheres after traumatic brain injury. AA-861 administration notably reduced the number of ICAM-1 positive vessels at 24 hours compared to 24 hours of traumatized and vehicle-treated (Vhl) groups. Data are expressed as means±SEM. *Significant at $p<0.05$, **Significant at $p<0.01$, and ***Significant at $p<0.001$. Letter 'a' denotes statistical significance at $p<0.001$ and 'b' at $p<0.01$ in non-traumatized hemispheres compared to non-traumatized cortex in sham-operated rats.

hemispheres also increased by 24 hours (p=0.000078) and stayed similar at 48 hours (p=0.0077) and 72 hours (p=0.000017). Pretreatment with AA-861 significantly suppressed the number of ICAM-1 positive vessels at 24 hours in comparison to 24 hours of traumatized (p=0.0022) and vehicle-treated (p=0.0053) groups. P-selectin regulation was studied at selected times with anti-rat CD62P reactivity (**Figure 3**). Sham-operated animals showed few numbers of vessels having weak anti-rat CD62P reactivity. In traumatized animals, the number of P-selectin positive vessels began to increase at 0.5 hours, and reached a maximum at 24 hours (p=0.00002), then while slightly reduced, it remained high at the 48 (p=0.0002), and 72 hours (p=0.0011) (**Figure 4**). The number of P-selectin positive vessels in non-traumatized hemispheres was increased notably at 24 hours (p=0.021), and 72 hours (p=0.0063), compared to that of non-traumatized hemispheres of sham-operated animals. Similar to ICAM-1 findings, AA-861 administration considerably reduced the number of P-selectin positive vessels at 24 hours (p=0.0018). No significant effect of vehicle treatment was observed on the number of P-selectin positive vessels at 24 hours,

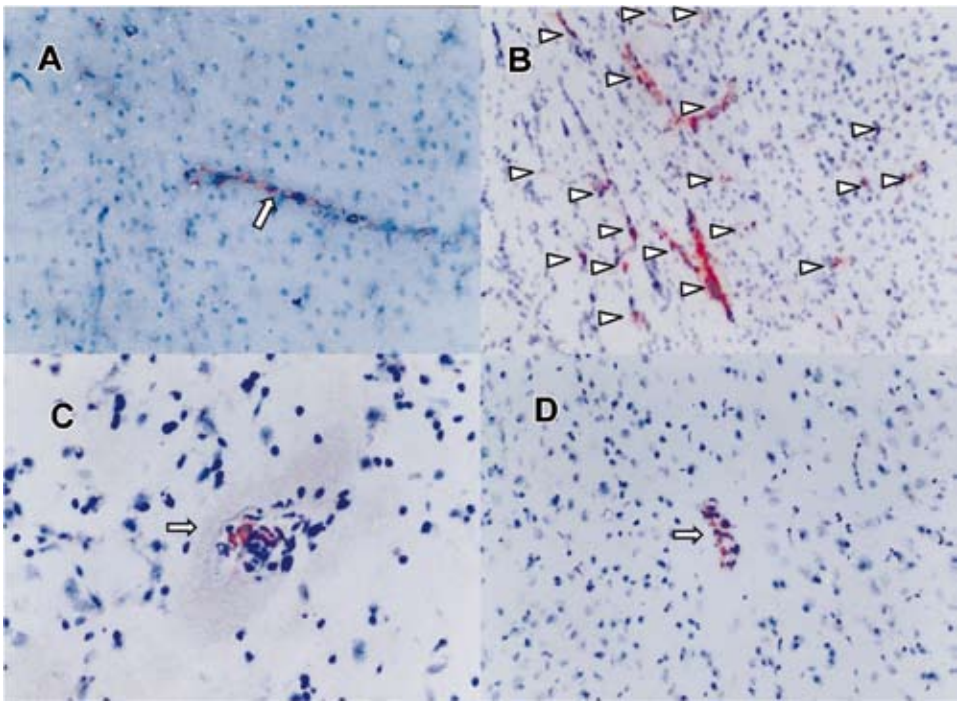

Figure 3 - P-selectin expression on cerebrovascular endothelium at selected times. Panels **A**, **B**, and **C** show P-selectin immunoreactivity at 0.5 (arrow), 24 (arrow heads), and 72 hours (arrow), after traumatic brain injury in traumatized hemispheres. Panel **D** shows the effect of AA-861 on P-selectin expression. P-selectin immunoreactivity was weak at 0.5 and 72 hours (panel **A** & **C**); intense at 24 hours (panel **B**). The AA-861 pretreatment also significantly lowered the P-selectin intensity (panel **D**, arrow). Note that there are several P-selectin-positive cells at 24 hours after injury. Original magnification was x200 for panel **A, B, D** and x 400 for panel **C**. AA-861 - 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone

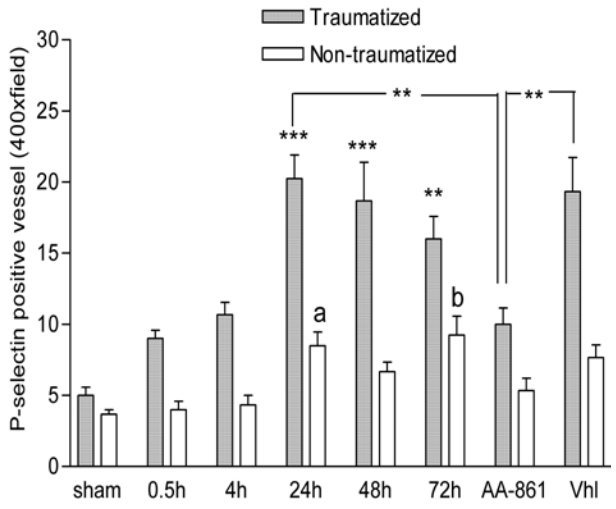

Figure 4 - The number of P-selectin positive vessels in traumatized-and non-traumatized hemispheres after traumatic brain injury. In addition to adhesion molecule 1 (ICAM-1), AA-861 pretreatment considerably lowered the number of P-selectin positive vessels at 24 hours. Vhl: Vehicle-treated group. Data are expressed as means±SEM. **Significant at $p<0.01$, and ***Significant at $p<0.001$. Letter 'a' denotes statistical significance at $p<0.05$ and 'b' at $p<0.01$ in non-traumatized hemispheres compared to non-traumatized cortex in sham-operated rats.

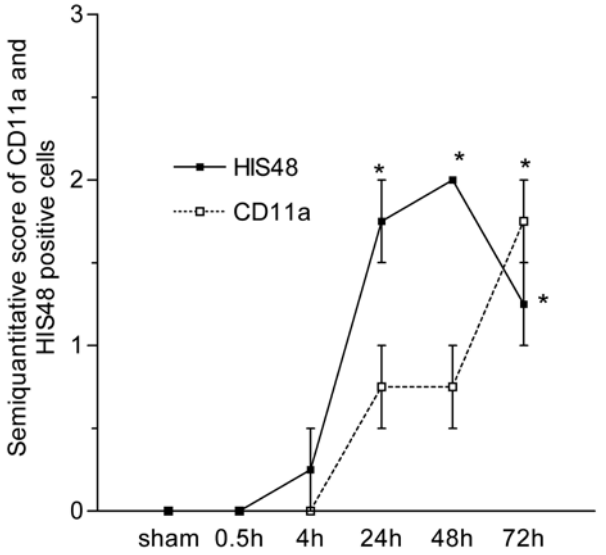

Figure 5 - Semiquantitative scores of CD11a and HIS48 positive cell in the injured cortex at selected times. The HIS48 positive cell number was most intense at 48 hours. Values represent the means semi-quantitative immunohistochemical intensity score±SEM. Mann-Whitney (Rank sum test) test was used to determine significant differences from sham control. *Significant at $p<0.05$.

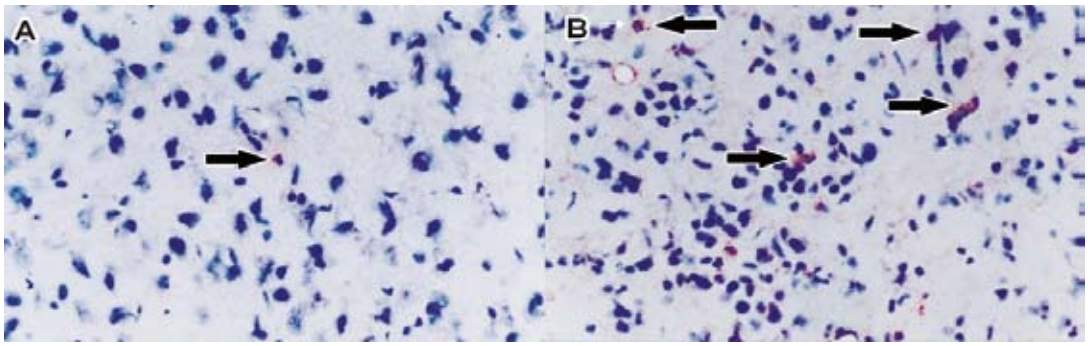

Figure 6 - CD11a expression in traumatized brain parenchyma: A few CD11a positive leucocytes were observed at 24 hours (panel **A**, arrow) and further increased at 72 hours at the injury site (panel **B**, arrows). Magnification x 400.

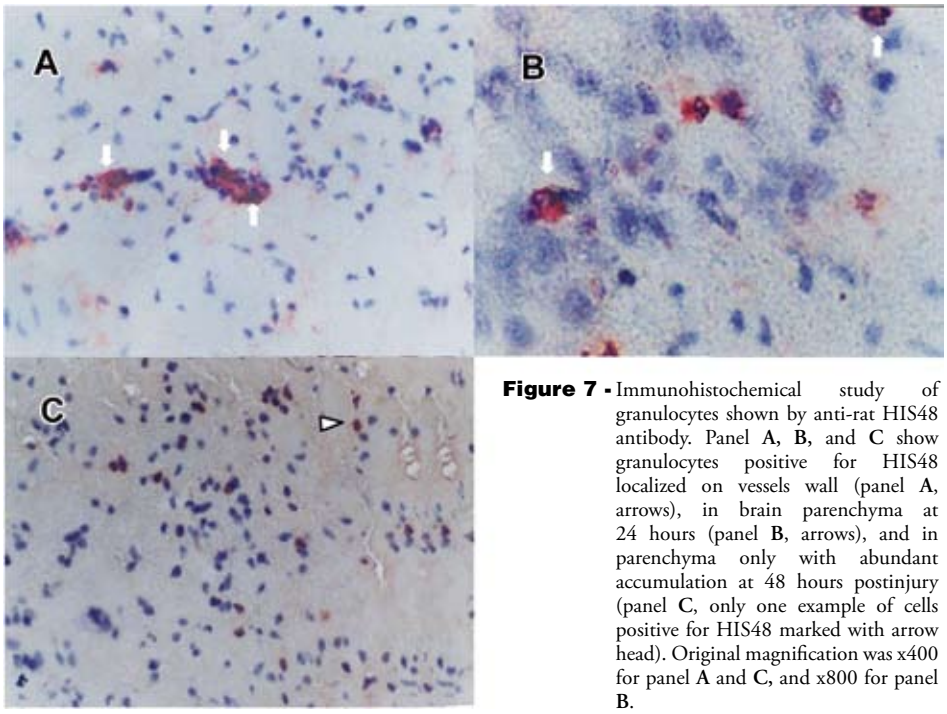

Figure 7 - Immunohistochemical study of granulocytes shown by anti-rat HIS48 antibody. Panel **A, B**, and **C** show granulocytes positive for HIS48 localized on vessels wall (panel **A**, arrows), in brain parenchyma at 24 hours (panel **B**, arrows), and in parenchyma only with abundant accumulation at 48 hours postinjury (panel **C**, only one example of cells positive for HIS48 marked with arrow head). Original magnification was x400 for panel **A** and **C**, and x800 for panel **B**.

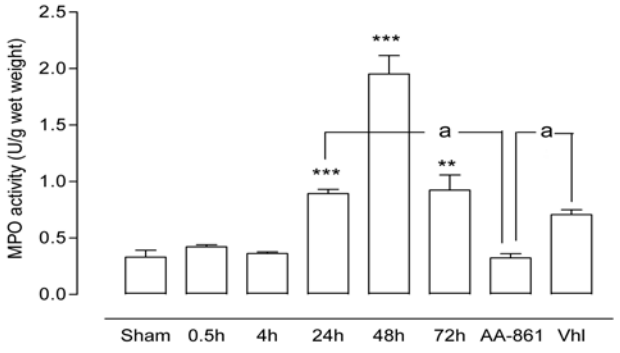

Figure 8 - Myeloperoxidase (MPO) activity in the traumatized hemispheres. The AA-861 significantly lowered MPO activity at 24 hours when compared to trauma and vehicle-treated (Vhl) groups. Data are expressed as means±SEM. Tamhane post hoc test was used following ANOVA. **Significant at $p<0.01$, ***Significant at $p<0.001$. Letter 'a' denotes statistical significance at $p<0.001$.

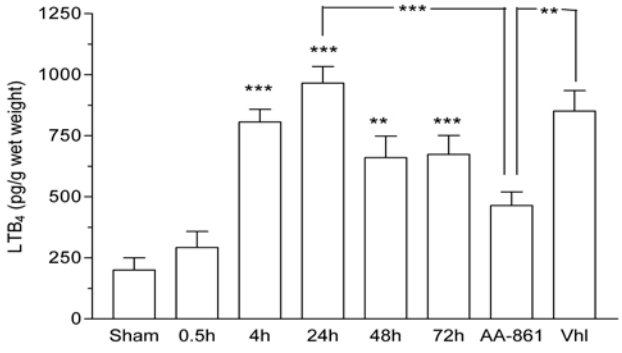

Figure 9 - Leukotriene B4 (LTB4) level in the contused tissue after traumatic brain injury. The AA-861 injection obviously reduced the LTB4 level at 24 hours when compared to traumatized and vehicle treated (Vhl) groups at 24 hours. Data are expressed as means±SEM. **Significant at $p<0.01$, and ***Significant at $p<0.001$.

and the inhibitory capacity of AA-861 was evident with regard to vehicle.

Semiquantitative scores of CD11a, and HIS48 labelling are shown in **Figure 5**. Expression of LFA-1 (CD11a), ligand for ICAM-1, was assessed using an antibody against it. The HIS48, an anti-rat granulocyte monoclonal antibody, was used to indicate leukocyte accumulation following TBI. Based on our 3-point scale system, no CD11a and HIS48 positive cells were found in the sham-operated animals. There was no CD11a stained cells at 0.5 hours, and at 4 hours in the traumatized groups. The CD11a positive cells started appearing at the 24 hours (**Figure 6A**), and reached a peak at 72 hours (p=0.040, **Figure 6B**). Similarly, HIS48 stained cells were absent at 0.5 and 4 hours, except for one animal that showed positive staining at 4 hours. The HIS48 positive granulocytes were abundant at 24 hours (p=0.022), and further increased by 48 hours (p=0.025), and began decreasing at 72 hours (p=0.0022). Granulocytes resided on the vascular wall, or in brain parenchyma at 24 hours (**Figure 7A & 7B**), whereas, by 48 hours, most of the granulocytes were localized exclusively in brain parenchyma (**Figure 7C**). A comparison of MPO activity in the injured hemisphere was made among sham-operated animals, traumatized animals, traumatized animals pretreated with AA-861, and traumatized animals pretreated with vehicle. Profiles of MPO activity in the injured brain tissue homogenate are illustrated in **Figure 8**. There was weak MPO activity in controls. Post traumatic brain PMLs accumulation, as measured by MPO activity, in the traumatized group was not significantly different from the sham-injured group at 0.5 and 4 hours after TBI. Polymorphonuclear leukocytes influx into injured cortical and subcortical regions was significantly increased at 24 hours (p=0.00077), peaked at 48 hours (p=0.00001), and then began reducing at 72 hours (p=0.003) after trauma. The AA-861 administration significantly reduced PMLs influx into injured tissue by 64% compared to traumatized animals (p=0.00021), and by 54% compared to vehicle treated animals (p=0.0007) 24 hours after trauma. Leukotrienes are potent chemotactic substances that increase vascular permeability, and exacerbate the cellular damage upon their release. Therefore, in this study, we measured LTB4 as a cerebral injury marker in traumatized animals, and examined the AA-861 effect on its production. Leukotriene B4 levels in the contused brain tissue are illustrated in **Figure 9**. Leukotriene B4 levels in traumatized animals were maintained at the sham-injured control level at 0.5 hours after TBI. Leukotriene B4 level in the traumatized group was markedly elevated at 4 hours (p=0.000004), and peaked at 24 hours (p=0.000001), and maintained its elevated levels at 48 hours (p=0.0077) and 72 hours (p=0.0005). The AA-861 pretreatment markedly reduced the LTB4 level at the 24 hours when compared to traumatized (p=0.00005) and vehicle treated (p=0.008) groups at 24 hours.

Discussion. Mild focal traumatic cerebral injury was chosen in this study, to assess the mechanisms of inflammation after TBI, as previously applied.[4,15,23] Inflammation-derived secondary injuries in brain are frequent sequelae of ischemic, and traumatic injury in the clinical settings. Consistent with earlier studies,[15,24-26] the present study showed an increased number of ICAM-1 and P-selectin-immunostained microvessels, preferentially capillaries at the site of the injury. By comparing the results with sham-operated animals, we observed an up-regulation of ICAM-1 and P-selectin in the endothelial cells of microvessels at the site of the lesion, and this event lasted for at least 72 hours after injury. The kinetics of P-selectin expression was similar to that of ICAM-1, indicating a simultaneous regulation of these molecules. The up-regulation of ICAM-1 and P-selectin, therefore, coincides in time with the period at which many secondary injuries are produced in the traumatized or ischemic rat brain.[25-29] Furthermore, tumor necrosis factor-α (TNF-α) and IL-1β stimulation are shown to increase P-selectin and ICAM-1 expression in the cultured murine brain microvascular endothelial cells.[30,31] A similar study[32] shows that stimulation of cultured brain endothelial cells by TNF-α increases P-selectin expression, and leucocytes adhesion. Similar to our results, in previous studies endothelial adhesion molecules in ischemic brain injury are shown to remain up-regulated at least for 7 days.[27,28] Persistent up-regulation of ICAM-1 and P-selectin expression might be explained by the stimulation of cytokines secreted by cells at the site of inflammation. Moreover, expression of these molecules on vessels in the contralateral, non-traumatized hemisphere was reduced, and delayed (meaningful at 24, 48, and 72 hours for ICAM-1, and only 72 hours for P-selectin). This observation suggests that up-regulation, and persistent expression of adhesion molecules on vasculature endothelium remote from the injury site, may be due to regional release of cytokines that are shown to be elevated following TBI.[33]

While PMLs play a beneficial role in fighting against bacterial infection,[34] they have been shown to be detrimental in the response to ischemia, reperfusion or trauma, through production of toxic oxidative metabolites, and hydrolytic enzymes.[35-37] In order for PMLs to migrate and accumulate at the injury site, they must first adhere to local microvasculature endothelial

cells. One of the essential molecules in this process includes CD11a/CD18 (LFA-1) in leukocytes, its principal endothelial ligand is ICAM-1 or ICAM-2.[7] The CD18 expression in stroke patients seems to appear later in blood, and stay for several days. Kim et al[38] showed that CD11a expression significantly increases within 72 hours, and remains up-regulated for 5-7 days while CD18 expression becomes weak at later days. Another study[28] reports similar results, in which several CD11a positive leucocytes are seen at day 3, and sustain remarkably for 7 days after MCA occlusion/reperfusion injury in rats. This finding suggests that CD11a-mediated leukocyte interaction with endothelial ICAM-1 appears to take place at the late phases of brain injury.

When the present data are considered along with previously published data,[3,15,25,28,39] demonstrating a deleterious role for ICAM-1 and P-selectin expression in stroke and trauma, it becomes increasingly apparent that there are multiple means for recruiting PMLs to post traumatic cortex and that blockade of each, represents a potential strategy to improve trauma outcome in patients. Monoclonal antibodies directed against CD11a, ICAM-1, and P-selectin epitopes or knocking out the genes that encode these molecules, have been shown to reduce PMLs accumulation, edema, and infarct volume and improve motor test scores, and spatial memory as well.[3,15,16,29,32,40,41] In this study, granulocytes were at contact with endothelial cells on vessel wall at 24 hours. At 48 hours post-injury, granulocytes were more abundant, and MPO activity was at its peak level, suggesting that much more PMLs accumulation occurs in 48 hours. Carlos et al[15] obtained similar results in a weight drop trauma model in which the peak PMLs infiltration occurred at 48 hours after TBI. Moreover, our results are further supported by the study of Clark et al.[4] In their study, they showed that PML infiltration as indicated by MPO activity was widespread at 24-48 hours in both controlled cortical impact, and weight drop models. In contrast to earlier studies showing that PMLs accumulation starts to increase 4-6 hours post trauma,[5,23] such regulation of PML was not observed in this study. The reason for this conflict may be explained by the severity of the trauma generated during the experiment.

Leukotriene B4 was shown to be involved in the pathogenesis of ischemic brain edema and PML infiltration.[11,42,43] Brain tissue LTB4 level in the current study increased significantly 4 hours after TBI, and remained elevated throughout the experiment. Eicosanoid production in early stages of trauma, and infiltrated leukocyte can be counted for elevated LTB4 level. The lipoxygenase pathway involved in the production of LTB4 from PMLs in response to microorganism-derived products, and in autoimmune encephalomyelitis.[37,44] Furthermore, concentrations of LTB4 in the cerebro-spinal fluid of rats after TBI are shown to increase significantly at 4 hours, and peak at 24 hours, and changes in LTB4 concentration are directly correlated with that of leukocyte infiltration, indicating that increased LTB4 concentration induces initiation and accumulation of PML infiltration.[45] In addition, LTB4 has potent chemotactic activity for PMLs.[14] Binding of LTB4 to its receptor has been reported to be highly correlated with MPO activity in a timely manner in cerebral focal stroke in rats.[46] Although alterations in the expression of endothelial molecules in animal models of trauma, ischemia and reperfusion have been studied, in this study, we define the effect of AA-861 pretreatment on the expression of these molecules following TBI. As ICAM-1 and P-selectin expression, LTB4 level, and MPO activity notably increase at 24 hours after TBI, we examined the effect of AA-861 pretreatment 24 hours after TBI on these molecules. The results of this study confirm that AA-861 pretreatment exerts a protective effect in trauma by lowering ICAM-1 and P-selectin expression, thereby reducing PMLs accumulation as indicated by MPO activity. In the early studies, AA-861 is shown to inhibit elevation of LTC4/LTB4 ischemia/reperfusion injury in rat, and 5-lipoxgenase pathway in guinea pig peritoneal PMLs.[47,48] Furthermore, the administration of AA-861 is shown to reduce LTB4 concentration, and PML numbers in lung lavages of rats exposed to hyperoxia for 60 minutes.[49] This study further supports our present findings.

In conclusion, similar to previous studies, in this study both ICAM-1 and P-selectin up-regulations on microvessels are shown to have a crucial role in the PMLs rolling into injury sites after TBI. Although the inflammatory process in response to traumatic injury is a complex phenomenon, the current study indicates that the 5-lipooxigeanase pathway of the arachidonic acid metabolism can be suppressed using AA-861, to prevent LTB4-induced inflammation after TBI. The present results suggest that AA-861 has the potential to inhibit inflammation and deserves to be studied further in detail.

References

1. Zhuang J, Shackford SR, Schmoker JD, Anderson M. The association of leukocytes with secondary brain injury. ***J Trauma*** 1993; 35: 415-422.
2. Uhl MW, Biagas KV, Grundl PD, Barmada MA, Schiding JK, Nemoto EM, et al. Effects of neutropenia on edema, histology, and cerebral blood flow after traumatic brain injury in rats. ***J Neurotrauma*** 1994; 11: 303-315.
3. Matsuo Y, Onodera H, Shiga Y, Shozuhara H, Ninomiya M, Kihara T, et al. Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. ***Brain Res*** 1994; 656: 344-352.

4. Clark RS, Schiding JK, Kaczorowski SL, Marion DW, Kochanek PM. Neutrophil accumulation after traumatic brain injury in rats: comparison of weight drop and controlled cortical impact models. ***J Neurotrauma*** 1994; 11: 499-506.
5. Royo NC, Wahl F, Stutzmann JM. Kinetics of polymorphonuclear neutrophil infiltration after a traumatic brain injury in rat. ***Neuroreport*** 1999; 10: 1363-1367.
6. Smith CW. Endothelial adhesion molecules and their role in inflammation. ***Can J Physiol Pharmacol*** 1993; 71: 76-87.
7. Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. ***Blood*** 1990; 75: 1037-1050.
8. Kim MB, Sarelius IH. Role of shear forces and adhesion molecule distribution on P-selectin-mediated leukocyte rolling in postcapillary venules. ***Am J Physiol Heart Circ Physiol*** 2004; 287: H2705-H2711.
9. Bretz U, Baggiolini M. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. ***J Cell Biol*** 1974; 63: 251-269.
10. Biagas KV, Uhl MW, Schiding JK, Nemoto EM, Kochanek PM. Assessment of posttraumatic polymorphonuclear leukocyte accumulation in rat brain using tissue myeloperoxidase assay and vinblastine treatment. ***J Neurotrauma*** 1992; 9: 363-371.
11. Chu LS, Fang SH, Zhou Y, Yu GL, Wang ML, Zhang WP, et al. Minocycline inhibits 5-lipoxygenase activation and brain inflammation after focal cerebral ischemia in rats. ***Acta Pharmacol Sin*** 2007; 28: 763-772.
12. Baskaya MK, Hu Y, Donaldson D, Maley M, Rao AM, Prasad MR, et al. Protective effect of the 5-lipoxygenase inhibitor AA-861 on cerebral edema after transient ischemia. ***J Neurosurg*** 1996; 85: 112-116.
13. Rao AM, Hatcher JF, Kindy MS, Dempsey RJ. Arachidonic acid and leukotriene C4: role in transient cerebral ischemia of gerbils. ***Neurochem Res*** 1999; 24: 1225-1232.
14. Brain SD, Williams TJ. Leukotrienes and inflammation. ***Pharmac Ther*** 1990; 46: 57-66.
15. Carlos TM, Clark RSB, Franicola-Higgins D, Schiding JK, Kochanek PM. Expression of endothelial adhesion molecules and recruitment of neutrophils after traumatic brain injury rats. ***J Leukocyte Biol*** 1997; 61: 279-285.
16. Grady MS, Cody RF Jr, Maris DO, McCall TD, Seckin H, Sharar SR, et al. P-selectin blockade following fluid-percussion injury: behavioral and immunochemical sequelae. ***J Neurotrauma*** 1999; 16: 13-25.
17. Weaver M, Leshley K, Sands H, Gritman KR, Legos JJ, Tuma R. FLEX032, a novel recombinant serpin, protects the brain after transient focal ischemia. ***Microvasc Res*** 2002; 63: 327-334.
18. Ashida Y, Saljo H, Kuriki H, Makino H, Terao S, Maki Y. Pharmacological profile of AA-861, a 5-lipoxygenase inhibitor. ***Prostaglandins*** 1983; 26; 955-972.
19. Baba T, Chio CC, Black KL. The effect of 5-lipoxygenase inhibition on blood-brain barrier permeability in experimental brain tumors. ***J Neurosurg*** 1992; 77: 403-406.
20. Durmaz R, Kanbak G, Akyuz F, Isiksoy S, Yucel F, Inal M, et al. Lazaroid attenuates edema by stabilizing ATPase in the traumatized rat brain. ***Can J Neurol Sci*** 2003; 30: 143-149.
21. Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. ***Anal Biochem*** 1983; 132: 345-352.
22. Iyobe T, Izumi R, Shimizu K. The immunosuppressive effect of 5-lipoxygenase inhibitor on liver allotransplantation in rats. ***Transplantation*** 1993; 56: 518-523.
23. Schoettle RJ, Kochanek PM, Magargee MJ, Uhl MW, Nemoto EM. Early polymorphonuclear leukocyte accumulation correlates with the development of posttraumatic cerebral edema in rats. ***J Neurotrauma*** 1990; 7; 207-217.
24. Isaksson J, Farooque M, Holtz A, Hillered L, Olsson Y. Expression of ICAM-1 and CD11b after experimental spinal cord injury in rats. ***J Neurotrauma*** 1999; 16: 165-173.
25. Hang CH, Shi JX, Li JS, Wu W, Yin HX. Concomitant upregulation of nuclear factor-kB activity, proinflammatory cytokines and ICAM-1 in the injured brain after cortical contusion trauma in a rat model. ***Neurol India*** 2005; 53: 312-317.
26. Zhang RJ, You C, Cai BW, He M, Yang YB, Liu Z, et al. Changes in P-selectin expression after brain injury in rats (in Chinese). ***Nan Fang Yi Ke Da Xue Xue Bao*** 2006; 26: 348-351.
27. Suzuki H, Abe K, Tojo S, Morooka S, Kimura K, Mizugaki M, et al. Expressions of P-selectin- and HSP72-like immunoreactivities in rat brain after transient middle cerebral artery occlusion. ***Brain Res*** 1997; 759: 321-329.
28. Kato H, Kogure K, Liu XH, Araki T, Itoyama Y. Progressive expression of immunomolecules on activated microglia and invading leukocytes following focal cerebral ischemia in the rat. ***Brain Res*** 1996; 734: 203-212.
29. Connolly ES Jr, Winfree CJ, Prestigiacomo CJ, Kim SC, Choudhri TF, Hoh BL, et al. Exacerbation of cerebral injury in mice that express the P-selectin gene: identification of P-selectin blockade as a new target for the treatment of stroke. ***Circ Res*** 1997; 81: 304-310.
30. Barkalow FJ, Goodman MJ, Gerritsen ME, Mayadas TN. Brain endothelium lack one of two pathways of P-selectin-mediated neutrophil adhesion. ***Blood*** 1996; 88: 4585-4593.
31. Barkalow FJ, Goodman MJ, Mayadas TN. Cultured murine cerebral microvascular endothelial cells contain von Willebrand factor-positive Weibel-Palade bodies and support rapid cytokine-induced neutrophil adhesion. ***Microcirculation*** 1996; 3: 19-28.
32. Carvalho-Tavares J, Hickey MJ, Hutchison J, Michaud J, Sutcliffe IT, Kubes P. A role for platelets and endothelial selectins in tumor necrosis factor-alpha-induced leukocyte recruitment in the brain microvasculature. ***Circ Res*** 2000; 87: 1141-1148.
33. Schmidt OI, Morganti-Kossmann MC, Heyde CE, Perez D, Yatsiv I, Shohami E, et al. Tumor necrosis factor-mediated inhibition of interleukin-18 in the brain: a clinical and experimental study in head-injured patients and in a murine model of closed head injury. ***J Neuroinflammation*** 2004; 28; 1: 13.
34. Moslen MT. Reactive oxygen species in normal physiology, cell injury and phagocytosis In: Armstrong D, editor. Free radicals in diagnostic medicine. New York (NY): Plenum Press; 1994. p. 17-27.
35. Matsuo Y, Kihara T, Ikeda M, Ninomiya M, Onodera H, Kogure K. Role of neutrophils in radical production during ischemia and reperfusion of the rat brain: effect of neutrophil depletion on extracellular ascorbyl radical formation. ***J Cereb Blood Flow*** 1995; 15: 941-947.
36. Geffner JR, Giordano M, Palermo MS, Prat A, Serebrinsky GP, Isturiz MA. Neutrophil-mediated cytotoxicity triggered by immune complexes: the role of reactive oxygen metabolites. ***Clin Exp Immunol*** 1987; 69: 668-675.
37. Valera I, Vigo AG, Alonso S, Barbolla L, Crespo MS, Fernandez N. Peptidoglycan and mannose-based molecular patterns trigger the arachidonic acid cascade in human polymorphonuclear leukocytes. ***J Leukoc Biol*** 2007; 81: 925-933.
38. Kim JS, Chopp M, Chen H, Levine SR, Carey JL, Welch KM. Adhesive glycoproteins CD11a and CD18 are upregulated in the leukocytes from patients with ischemic stroke and transient ischemic attacks. ***J Neurol Sci*** 1995; 128: 45-50.
39. Isaksson J, Lewen A, Hillered L, Olsson Y. Up-regulation of intercellular adhesion molecule-1 in cerebral microvessels after cortical contusion trauma in a rat model. ***Acta Neuropathol*** 1997; 94: 16-20.

40. Connolly ES Jr, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, et al. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. ***J Clin Invest*** 1996; 97: 209-216.
41. Knoblach SM, Faden AI. Administration of either anti-intercellular adhesion molecule-1 or a nonspecific control antibody improves recovery after traumatic brain injury in the rat. ***J Neurotrauma*** 2002; 19: 1039-1050.
42. Di-Gennaro A, Carnini C, Buccellati C, Ballerio R, Zarini S, Fumagalli F, et al. Cysteinyl-leukotrienes receptor activation in brain inflammatory reactions and cerebral edema formation: a role for transcellular biosynthesis of cysteinyl-leukotrienes. ***FASEB J*** 2004; 18: 842-844.
43. Ford-Hutchinson AW, Bray MA, Smith MJ. Lipoxygenase products and the polymorphonuclear leucocyte. ***Agents Actions*** 1980; 10: 548-550.
44. Neu IS, Wildfeuer A, Mallinger J, Mehlber L. Suppression of experimental autoimmune encephalomyelitis by a new specific inhibitor of leukotriene biosynthesis. ***Arzneimittelforschung*** 1992; 42: 1083-1086.
45. Schuhmann MU, Mokhtarzadeh M, Stichtenoth DO, Skardelly M, Klinge PM, Gutzki FM, et al. Temporal profiles of cerebrospinal fluid leukotrienes, brain edema and inflammatory response following experimental brain injury. ***Neurol Res*** 2003; 25: 481-491.
46. Barone FC, Hillegass LM, Tzimas MN, Schmidt DB, Foley JJ, White RF, et al. Time-related changes in myeloperoxidase activity and leukotriene B4 receptor binding reflect leukocyte influx in cerebral focal stroke. ***Mol Chem Neuropathol*** 1995; 24: 13-30.
47. Namura Y, Shio H, Kimura J. LTC4/LTB4 alterations in rat forebrain ischemia and reperfusion and effects of AA-861, CV-3988. ***Acta Neurochir Suppl (Wien)*** 1994; 60: 296-299.
48. Yoshimoto T, Yokoyama C, Ochi K, Yamamoto S, Maki Y, Ashida Y, et al. 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861), a selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis. ***Biochim Biophys Acta*** 1982; 713: 470-473.
49. Taniguchi H, Taki F, Takagi K, Satake T, Sugiyama S, Ozawa T. The role of leukotriene B4 in the genesis of oxygen toxicity in the lung. ***Am Rev Respir Dis*** 1986; 133: 805-808.

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