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Original Research paper

Title: Effects of acute exposure to aluminum on blood-brain barrier and the protection of zinc

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Abstract

Aluminum and zinc are two important trace elements in an organism. Although several studies have demonstrated their impacts on the intelligence, very little was known about their effects on the integrity of blood-brain barrier (BBB). To study the effects of aluminum and zinc on the permeability of BBB, different doses of aluminum and appropriate zinc were administered to rats. Evans blue was detected in brain to determine the permeability of BBB. The ultrastructure of BBB was observed under the transmission electron microscope. Immunohistochemistry and Western blot method were used to detect the expression of skeleton protein F-actin and tight junction protein occludin in brain capillary endothelium. The data indicated that compared with the control group, Evans blue in brains increased (P<0.01), the ultrastructure of BBB changed and the expression of F-actin and occludin decreased (P<0.01) in the aluminum-toxic group. Compared with the aluminum-toxic groups, the permeability of BBB to Evans blue decreased (P<0.01), the damage of the BBB ultrastructure was attenuated and the expression of F-actin and occludin increased (P<0.05) in the aluminum-zinc group. Our present studies suggest that aluminum increase the permeability of BBB by changing its ultrastructure and the expression of occludin and F-actin. Zinc can protect the integrity of BBB in juvenile rats that are exposed to aluminum and inhibit the decrease of tight junction protein occludin and F-actin expression in BBB.

Key words: aluminum; zinc; blood-brain barrier; F-actin; occludin

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Recent investigation demonstrates that the primary lesion in Alzheimer's disease, dialysis dementia and Parkinson's disease has been postulated to be an impaired blood-brain barrier (BBB) permeability that allows aluminum (Al) to reach the central nervous system [1, 5, 8, 10, 19, 25]. BBB separates the brain microenvironment from the systemic circulation and keeps the CNS in stable circumstances. As Al itself, it can accumulate in brain, with Al-citrate and Al-transferrin complexes crossing the BBB by carrier [18, 26]. At the same time, a high level of Al leads to an additional increase in BBB permeability [11].

Zinc (Zn) is an important microelement in the body of human and animal. Some studies suggested that Zn played the potential role in keeping the integrity of the BBB [3, 12]. In the present study, we investigated the effects of Al and Zn on the permeability of BBB, while focusing on the ultrastructure of capillary endothelial cells and the expression of F-actin and occludin. The goal of the current study was to evaluate the effectiveness of Zn for the BBB-injury induced by the acute exposure of Al as a novel protective approach for this specific type of Al-toxic pathology.

Male juvenile Sprague-Dawley rats weighing approximately 80g were obtained from the animal facility of China Medical University. Animals were maintained at 21-23ºC under a 12:12-h light-dark cycle, and assigned randomly to 6 groups (n = 50 per group) to provide saline, low or high Al, with and without Zn. Al (as chloride) dissolved in saline was administered for 8 cycles in terms of a three-day continuous intraperitoneal supply with one-day interval for intramuscular injection of mycillin to avoid infection. In the control and Zn group, the same volume of saline as that in

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low-Al group (5mg/kg) and high-Al group (10mg/kg) was administered intraperitoneally. Zn (as sulfate) dissolved in deionized water at 5mg/kg was orally infused once a day for 32 days. All animals were treated humanely and with regard for alleviation of suffering.

Evans blue (EB, 2ml/kg; Sigma) was injected by the femoral vein of rats for the assessment of BBB permeability. The chest was opened thirty minutes later and the brain was transcardially perfused with 300 ml of saline until the colorless perfusion fluid was obtained. The brain was removed and weighed. Then, the samples were placed in formamide solution and incubated for 24 hours at 60 . The absorbance of the solution was measured using a spectrophotometer (Milton Roy, USA) at 620 nm and EB content was expressed as μg/g (brain weight).

The cortex of bilateral parietal lobes was cut into 1 mm^3 blocks after rapid head-breaking in low temperature. The blocks were fixed into glutaral for 2 hours. Then the cubic brain tissues were immersed in 1% osmic acid for further 30 min followed by stepwise dehydration with alcohol. Ultrathin tissue sections (70-nm thick) were made with an ultramicrotome (Reichert-Jung, Germany) and stained with uranyl acetate and lead citrate, before being examined under a transmission electron microscope (JEM-1200, Japan) to observe the ultrastructure of BBB.

For fluorescent staining of F-actin, rats were deeply anesthetized with pentobarbital (70mg/kg) and perfused with 300 ml of phosphate buffered saline (PBS) followed by 300 ml of ice-cold 4% paraformaldehyde. After the perfusion fixation, the animals were kept in ice for 30 min, and then perfused with 1000 ml of ice-cold

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15% sucrose. The brain was cut into 12μm-thick sections in a cryostat. After washing with PBS containing 0.1% Triton X-100 for 3 min, sections were blocked by 5% bovine serum albumin for 30min and incubated with rhodamine- phalloidin (1:200; Sigma) for 45~60min at 37 . Washed coverslips were mounted in glycerol and examined by fluorescence microscope (Olympus BX60, Japan).

Occludin in brain capillary endothelium was examined by immunohistochemistry and Western blot separately. For immunohistochemical staining, sections (12μm-thickness) were incubated overnight at 4 with rabbit anti-occludin (H279) polyclonal antibody (1:400; Santa Cruz Biotechnology), followed by goat anti-rabbit antibody conjugated with horseradish peroxidase (1:200; Wuhan Boster Biotechnology). The rabbit anti-occludin (H279) polyclonal antibody used in this study was raised against amino acids 132-411 mapping within an internal region of occludin of human origin, which cross-reacted with the rat protein as indicated by the manufacturer. Immunostaining was performed with streptoavidin–biotin complex (SABC; Wuhan Boster Biotechnology) and a chromogenic agent, 3, 3'-diaminobenzidine (DAB; Wuhan Boster Biotechnology). Five sections from bilateral parietal lobes of each rat were visualized and three fields at ×200 magnification of each section were photographed. The images were processed with Motic Images Advanced 3.2 image analysis software (Motic Instruments). For Western blot, 0.1g parietal cortex homogenate was centrifuged at 1700g for 1h. Samples were separated by electrophoresis on 4 and 7.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After being blocked in PBS containing

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0.1% Tween 20 and 4% skimmed milk for 4 h, the membrane was incubated with rabbit antibodies against occludin (1:1000; Santa Cruz Biotechnology) for 2h, followed by goat anti-rabbit antibody (1:2000; Santa Cruz Biotechnology) for 2 h at room temperature. Detection was carried out by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). The bands were scanned and analyzed as described [7, 15]. Differences between groups were analyzed by *t*-test (SPSS 13.0). $P<0.05$ was considered statistically significant.

Fig. 1 showed that an administration of Al was coupled with the breakdown of BBB integrity. EB extravasation increased in both low-Al group and high-Al group compared with control group ($P<0.01$). Although Zn had no significant effect on the permeability of BBB ($P>0.05$) compared with the control group, it protected the integrity of BBB in groups that coupled with Al administration. Compared with the corresponding Al-toxic group, the EB content in brains of low-Al/Zn group and high-Al/Zn group was significantly decreased ($P<0.01$) (Fig. 1).

Observed under the transmission electron microscope, the ultrastructure of BBB in control and Zn groups was normal (Fig. 2A, B). Al treatment disrupted the integrity of BBB. A cleavage on the capillary endothelium and an enhanced electron density on the tight junction (Fig. 2C arrows) were seen in low-Al group. The heterochromatin aggregation, mitochondria vacuolization, and shrunk capillary lumen (Fig. 2E arrows) were seen in high-Al group. In Al-Zn groups, the condition was much better. The integrity of capillary endothelium was still almost normal with a regular capillary lumen and a clear base membrane (Fig. 2D, F).

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Fluorescent staining showed that F-actin (red), which was considered as the skeleton protein specially associated with the endothelial permeability [23], localized along the capillary endothelial cells. For control and Zn groups, the expression of F-actin was bright and clear. The brain capillary vessels were intact (Fig. 3A, B). For Al-toxic groups, the expression of F-actin was thinned, blurred and unconnected (Fig. 3C arrows). The lumens of BBB were even shrunk in high-Al group (Fig. 3E arrowheads). For Al-Zn groups, the capillary lumens were much more regular. The red fluorescence was almost continuous. (Fig. 3D, F).

Occludin is an integral membrane protein specifically associated with tight junctions which contributes to the barrier function of this intercellular seal [2]. The expression of occludin can be determined by its average optical density (AOD, integrated optical density/area) [6, 13] as well as the ratio of integrated density value (IDV) [7, 13] of occludin to that of β-actin. These values are related to the level of occludin expression. The AOD and the IDV were obtained by immunohistochemistry analysis (Fig. 4) and Western blot analysis (Fig. 5) respectively. There was no significant difference in the expression of occludin between Zn group and control group ($P>0.05$). Compared with control group, the expression of occludin decreased in Al-toxic groups ($P>0.01$) and compared with the corresponding Al-toxic groups, the expression of occludin increased in Al-Zn groups to some degree ($P>0.05$).

In the present study, EB extravasation increased in brains of Al-toxic groups. The ultrastructure of BBB showed that Al induced the damage of cell membrane, organs, and the tight junction of brain capillary endothelium. The results of immunochemistry

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and Western blot analysis showed that Al induced a decrease in the expression of F-actin and occludin. All these results suggested that Al toxicity might be related to the change of the permeability and the integrity of BBB. This may be the original damage of CNS by Al, which may induce neuronal cell injury [21], demented brain [9, 20] or other pathological changes in a long stage [27].

Zn is an essential nutrient involved in many aspects of cell function. It is a very important microelement for the brain to retain normal learning and memory ability [14, 16, 22]. At the same time, it also acts as a favorable factor in keeping the integrity of BBB [3, 12]. Although Di Cello demonstrated that human brain microvascular endothelium cells, which constituted the BBB, resisted Zn depletion and responded by enhancing their barrier function [4]. However, Noseworthy demonstrated that Zn deficiency exacerbated loss of BBB integrity induced by hyperoxia measured by dynamic magnetic resonance imaging. Zn deficiency resulted in a significant increase in both BBB permeability and tissue interstitial leakage space, indicating a severely disturbed BBB [12]. Our study showed that Zn could protect the integrity of BBB and increased the expression of F-actin and occludin which was decreased by Al.

Although an appropriate dose of Zn was favorable for keeping the integrity of BBB and antagonizing cognitive impairment [3, 24, 28], Zn levels in brain tissue itself were significantly elevated (more than twofold) in the most severely demented cases [17]. Brain Zn accumulation was even a prominent feature of advanced Alzheimer's disease and was biochemically linked to brain amyloid beta-peptide

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accumulation and dementia severity [17]. Hence, the mechanism of interaction of metal ions in central nervous system is not clear. Further study is needed to learn about Al toxicity on BBB and the potential role of Zn to develop new methods of protecting and treating the metal toxicity on juvenile animals.

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Figure 1. EB content in different rat brains. Data are presented as mean±S.E.M. (n=10/group). $^{*}P<0.01$ compared with control group; $^{\#}P<0.01$ compared with low-Al group; $^{\blacktriangle}P<0.01$ compared with high-Al group.

Figure 2. Ultrastructure of BBB for all treatment groups. A: control group; B: Zn group; C: low-Al group; D: low-Al/Zn group; E: high-Al group; F: high-Al/Zn group. Scale bar, 10μm.

Figure 3. Fluorescent staining of F-actin (red) in brain capillary endothelium for all treatment groups. A: control group; B: Zn group; C: low-Al group; D: low-Al/Zn group; E: high-Al group; F: high-Al/Zn group. Scale bar, 50μm.

Figure 4. Immunohistochemistry analysis of occludin for all treatment groups. (A)Expression of occludin in brain capillary endothelium by immunohistochemistry staining. Occludin located on the capillary cell membrane as spotty or linear brown stain. a: control group; b: Zn group; c: low-Al group; d: low-Al/Zn group; e: high-Al group; f: high-Al/Zn group. Scale bar, 50μm. (B) AOD value of occludin. Data are presented as mean±S.E.M. (n=10/group). $^{*}P<0.01$ compared with control group; $^{\#}P<0.05$ compared with low-Al group; $^{\blacktriangle}P<0.05$ compared with high-Al group.

Figure 5. Western blot analysis of occludin for all treatment groups. (A) Expression of occludin and β-actin by Western blot. a: control group; b: Zn group; c: low-Al group; d: low-Al/Zn group; e: high-Al group; f: high-Al/Zn group. (B) The ratio of occludin IDV to β-actin IDV. Data are presented as mean±S.E.M. (n=10/group). $^{*}P<0.01$ compared with control group; $^{\#}P<0.05$ compared with low-Al group; $^{\blacktriangle}P<0.05$ compared with high-Al group.

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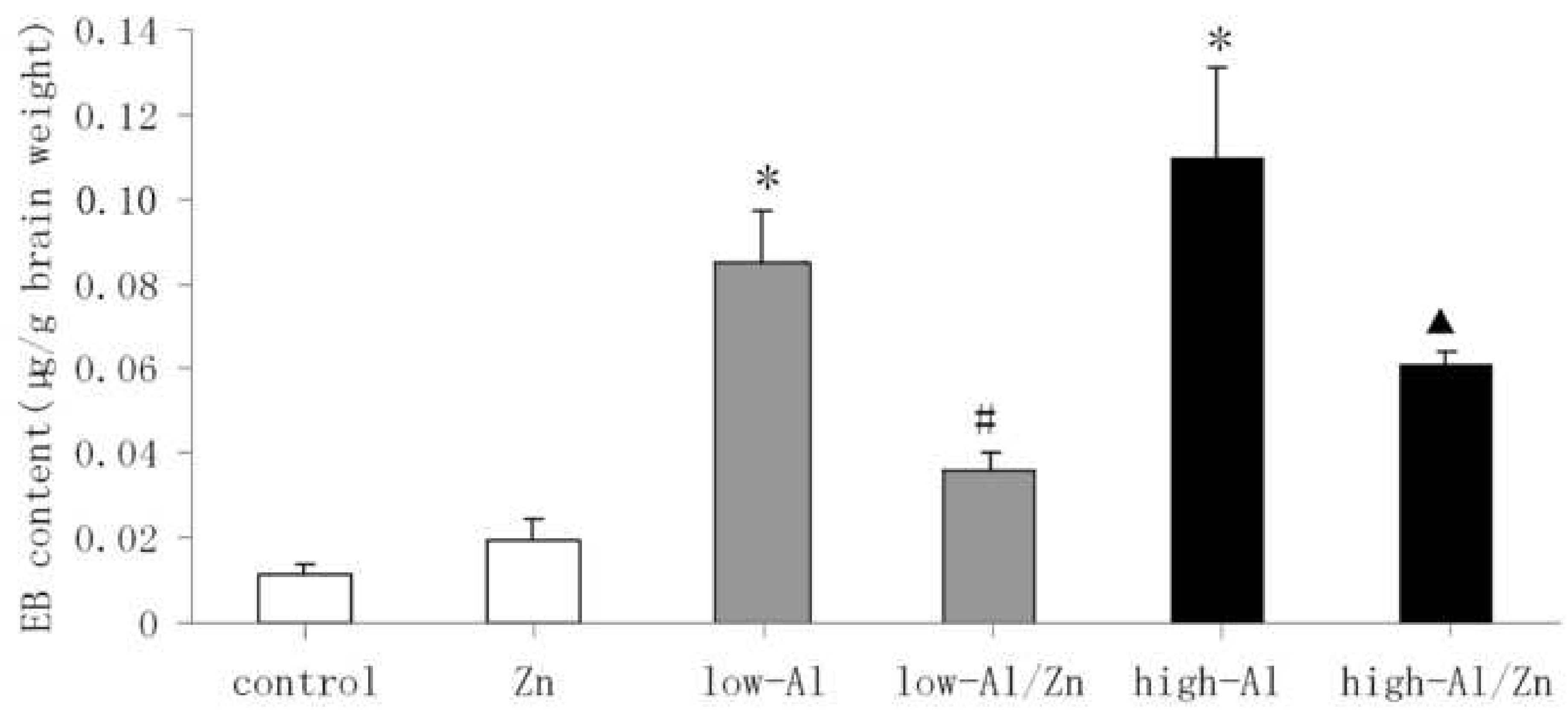
new Figure 1



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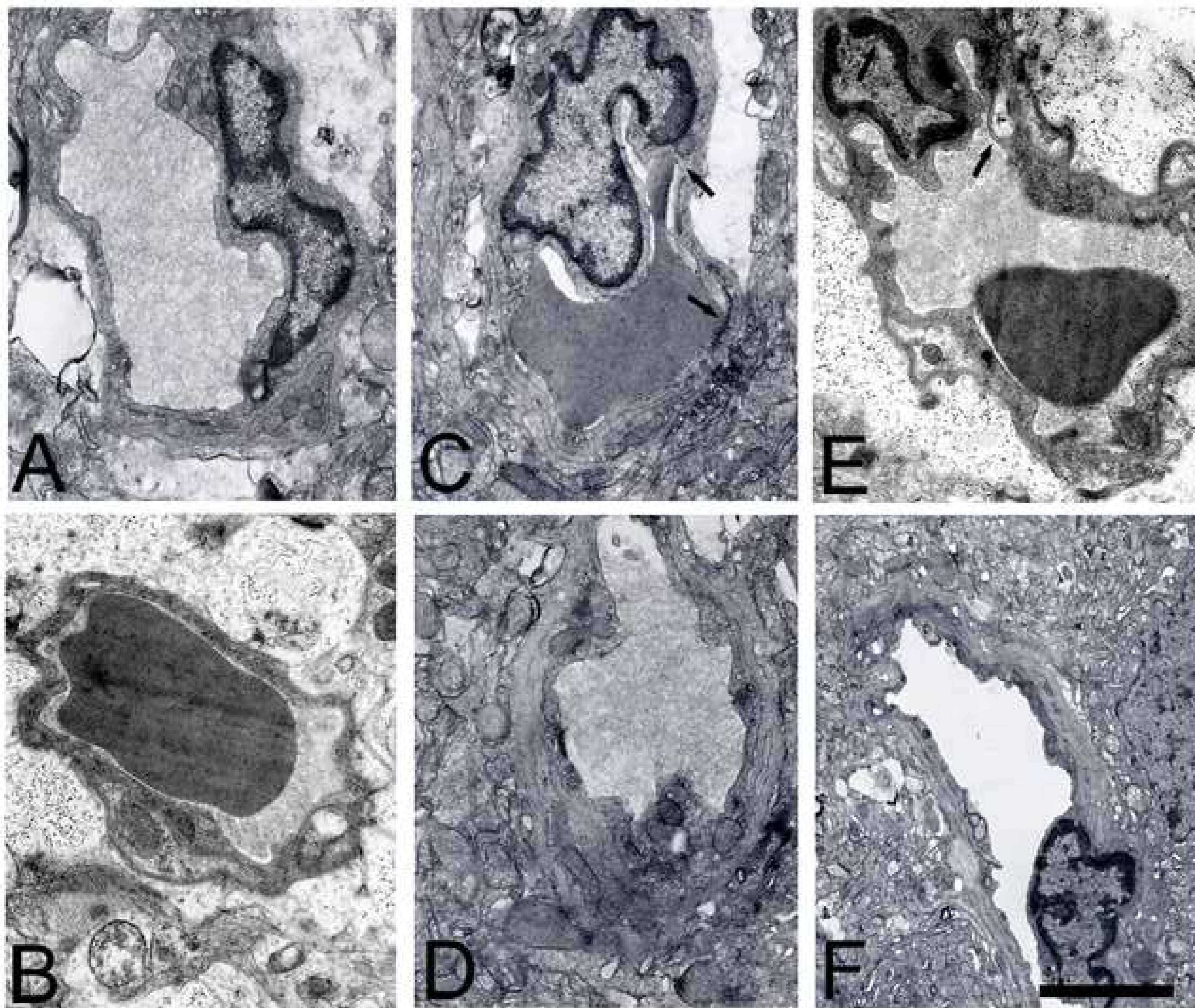

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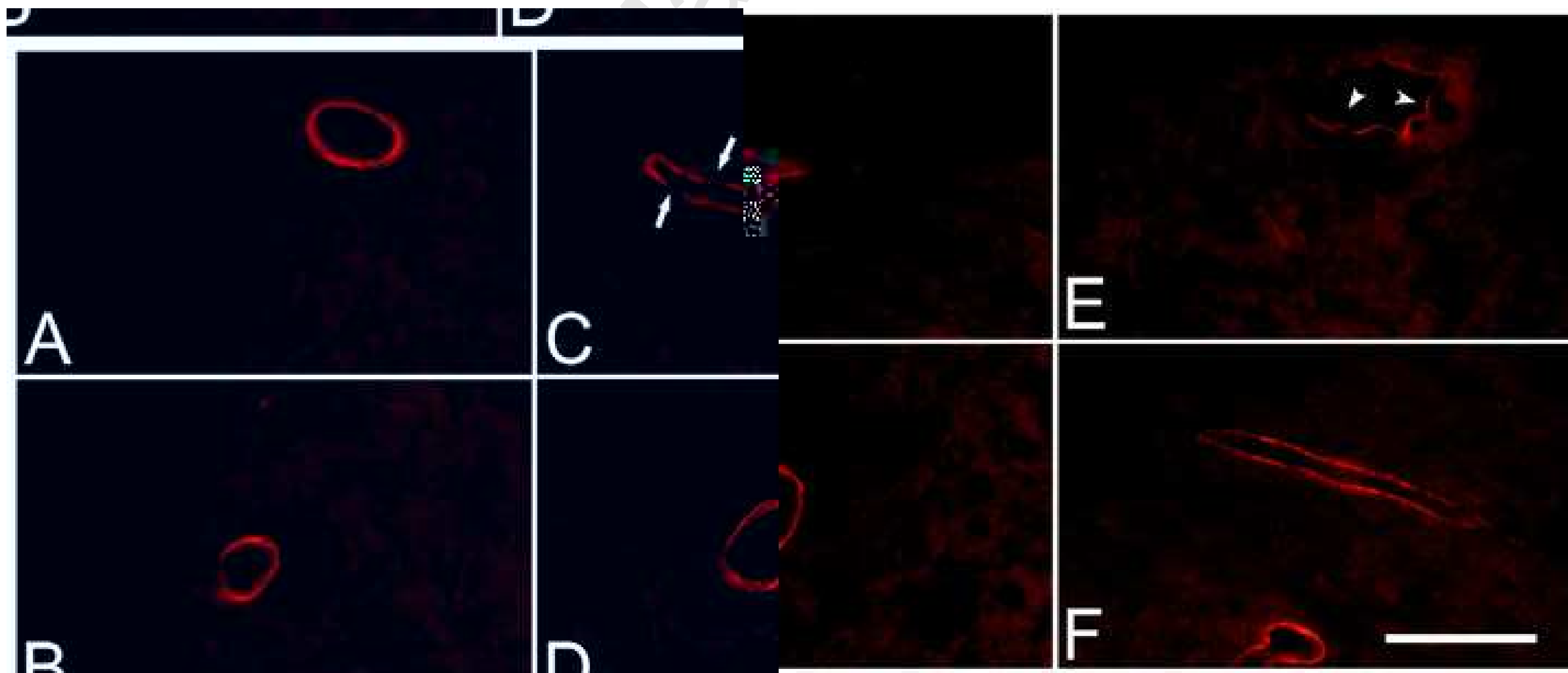

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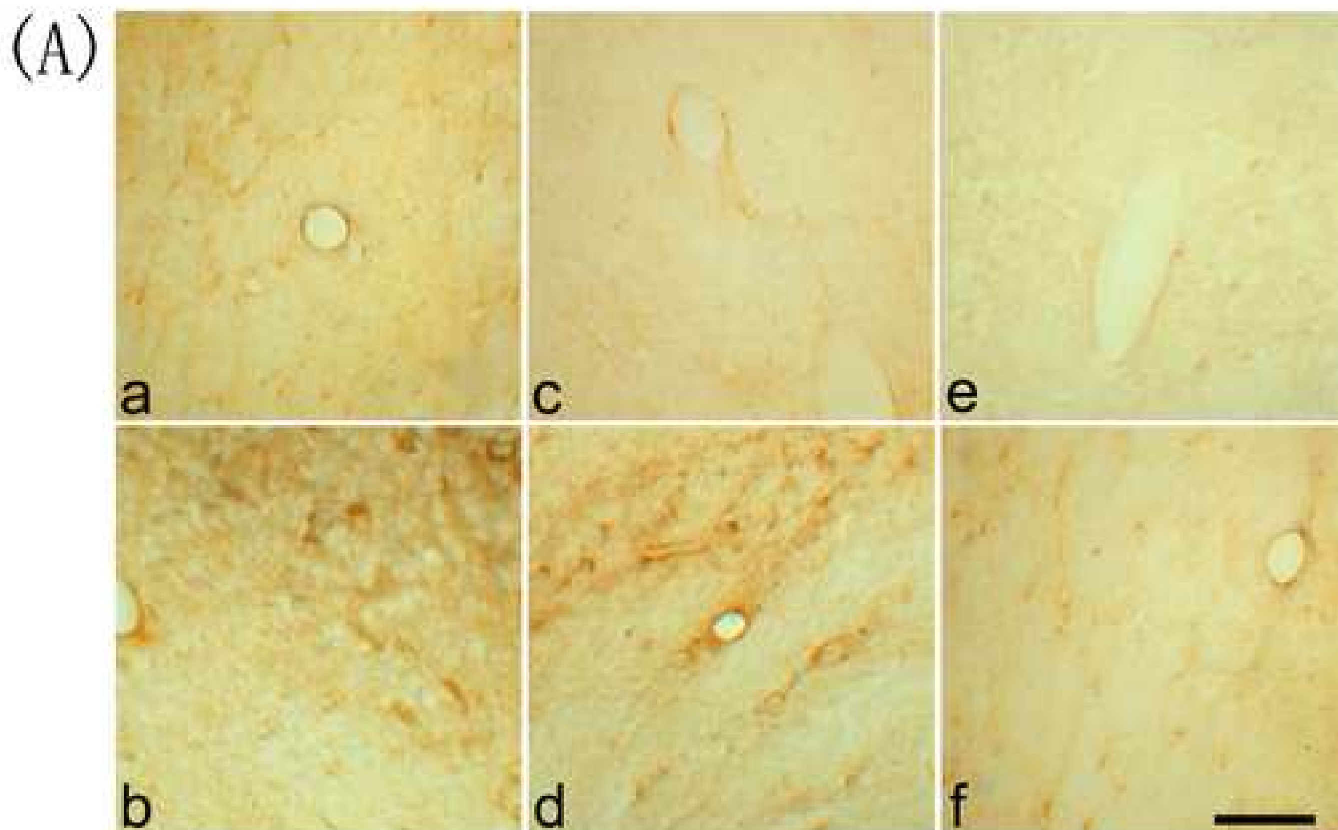

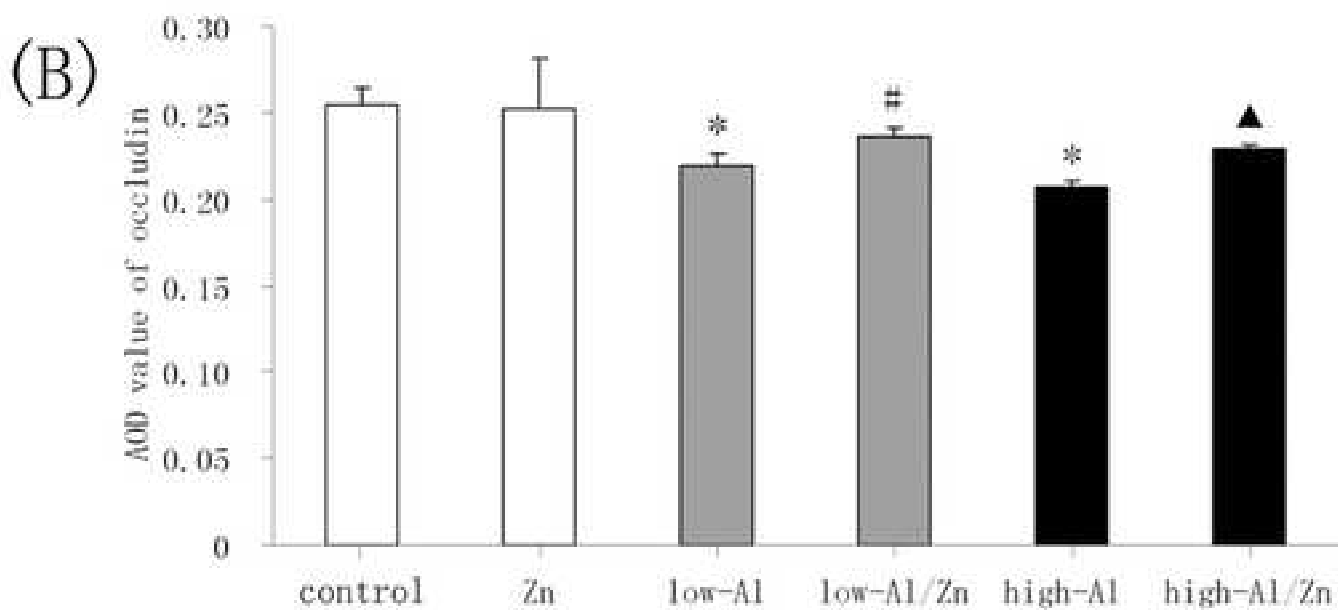

new Figure 5