

Original Article

Antiapoptotic Effects of Aspirin on CA1 Pyramidal Neurons in Adult Rats

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ABSTRACT

Background and Objective: As one of the widely used drugs, aspirin (acetyl-salicylic acid, ASA) plays an important role in stroke treatment and prevention. In a previous study, we demonstrated ASA injection at 30 min after ischemia onset is neuroprotective. To determine whether the neurons protected by ASA had a normal ultrastructure, hippocampal CA1 pyramidal neurons were examined by Transmission Electron Microscope (TEM).

Material and Methods: Adult male wistar rats were divided into three different groups (6 animals/group): Sham-operated, control (48 MCAO+vehicle) and aspirin (48 MCAO + ASA). ASA (30 mg/kg) was injected 30 min after ischemia onset. The animals were killed 2 days after ischemia induction and their brain removed, processed, and examined under a TEM.

Results: Apoptotic changes were observed in rats not treated with ASA. In contrast, pyramidal neuron ultrastructure appeared normal in rats that exhibited neuroprotection (defined at the light microscope level) by ASA when studied two days after ischemia.

Conclusion: We conclude that administration of ASA after permanent focal cerebral ischemia remains a considerable therapeutic strategy.

Keywords: Aspirin, Ischemia, Apoptosis

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Introduction

Stroke is a major leading cause of death in the world and at the first place of disability, so effective therapy, or prevention of this event is of great importance (1). Aspirin (acetyl-salicylic acid, ASA) has become standard treatment for acute ischemic stroke. It produces some benefit in long-term outcome and survival if given within 14 days of stroke onset (2). Depending on its dosage, ASA has a wide spectrum of pharmacological activities and multiple sites of action (3). It reduces the risk of stroke in patients with cerebrovascular disease due to its antiplatelet effect through the inhibition of cyclooxygenase (COX) (4). Studies also showed that higher doses of ASA inhibit prostacyclin production and have a significant anti-inflammatory effect, which may reduce vascular events in humans (5).

Few studies reported a primary neuroprotective effect of ASA in doses ranging between 15 and 80 mg/kg when administered once shortly prior to permanent middle cerebral artery occlusion (MCAO), but not when given as a single bolus after the onset of cerebral ischemia (6). Previously, we demonstrated ASA (30 mg/kg) injection at 30 min after ischemia (permanent MCAO) onset is neuroprotective (7). We designed an experimental setting with permanent MCAO model using Tamura's method (1981) (8).

The objective of this study was to test whether the neurons protected by ASA had a normal ultrastructure, so hippocampal CA1 pyramidal neurons were examined by Transmission Electron Microscope (TEM).

Materials and Methods

Animal protocols were approved by Ethical Committee of Kerman Neuroscience Research Center. Male NMRI rats (n=18) weighing 250-350 g were used in this study. The animals were divided into 3 different groups (6 animals/group): Sham-operated group, Vehicle group (48 h MCAO + Distilled Water) and ASA

(Sigma; St. Louis, USA) treated group (48 h MCAO+ASA injection after 30 min of ischemia onset).

Induction of focal cerebral ischemia

Under chloral hydrate (Sigma; St. Louis, USA) anesthesia, the left MCA was exposed using the method of Tamura *et al.* (1981) (8). The ipsilateral Common Carotid Artery (CCA) was also exposed. Ischemia resulted when ligation of the CCA immediately followed cautery of the MCA, which is coagulated from its junction with the olfactory tract to the inferior cerebral vein. The arterial occlusions were maintained for 48 h. Rectal temperature was monitored continuously and maintained between $37\pm 0.5^{\circ}\text{C}$ throughout the anesthesia. Neurological evaluation (9) was performed for being assured of MCAO after full recovery and the animals without clinical signs were excluded.

Electron Microscopy Preparation

After two days, the animals were anesthetized (chloral hydrate, 400mg/kg) and perfused through a cannula inserted through the left ventricle into the ascending aorta with a short rinse of 0.5 M phosphate buffer (PB)(150 ml) followed by fixation fluid (250 ml of 4% paraformaldehyde, pH 7.4 at room temperature). The brains were removed and immersed in the same fixative solution overnight. A 400- μm block of area CA1 was dissected and fixed in buffered 2.5% glutaraldehyde for an additional 48h. The sections were then washed in PB solution and postfixed in 1% OSO_4 for 2h at room temperature. After dehydration in ascending graded ethanol, were embedded in Epon 812 resin. After that, they were put onto slices with resin and polymerized for 48 h at 60°C . Semithin sections (0.3 μm) were stained with toluidine blue to identify the region of interest. Subsequently, 70 nm sections were cut and stained with 1% uranyl acetate and 2% lead citrate. Sections were examined with a Philips EM300 transmission electron microscope.

Results

Light and electron microscopy observation in the present study showed morphological changes in the rat hippocampal CA1 region after 48 h ischemia.

Light microscopy findings

In sham-operated animals, semithin sections of CA1 region showed a layer of cell bodies of pyramidal neurons with large nuclei and prominent nucleoli (Fig. 1A). In control group, the neurons showed apoptotic changes characterized by formation of apoptotic bodies and loss of inflammatory cells (Fig. 1B). When ASA was injected 30 min following ischemia, neuronal death was partially prevented at two days of survival (Fig. 1C).

Electron microscopy findings

Pyramidal cells were examined with a transmission electron microscope two days after MCA Occlusion (MCAO). In sham-operated animals, CA1 pyramidal neurons had oval nuclei with evenly dispersed chromatin and clear nucleoli. Cell and nuclear membranes were intact. The cytoplasm contains mitochondria of various sizes, well developed Rough Endoplasmic Reticulum (RER) and polyribosomes formed characteristic rosettes (Fig.2 A). Their neuropil were normal too (Fig. 2B).

Two days after permanent MCAO, ultrastructure changes were observed in control group. The changes were visible within the cytoplasm and nucleus as shown in (Fig. 3A). Ultrastructure observation showed chromatin aggregation and formation of apoptotic bodies (Fig. 3B & C). Dendritic pathology was also evident with numerous electron dense dendritic shafts and empty spaces in the neuropil (Fig. 3D).

Neuronal ultrastructure of treated rats with ASA examined two days after ischemia too as

shown in Fig. 4.A. The ultrastructure of most pyramidal neurons was preserved. Apoptotic bodies were not found in this group. The dendritic fields were unremarkable, except for relative dendritic shafts swelling (Fig. 4B).

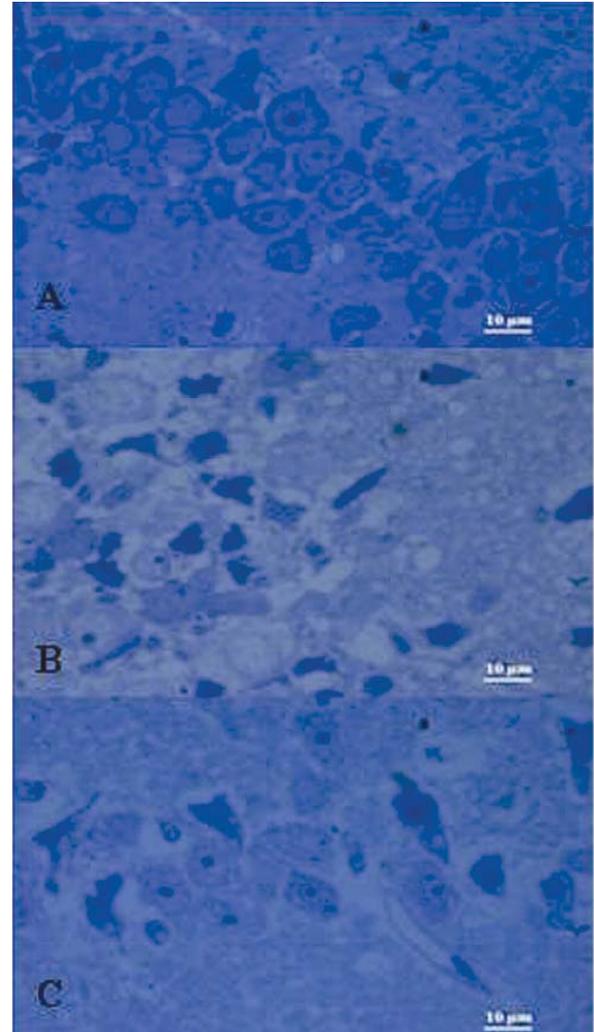


Fig. 1- Light micrographs of semithin sections of hippocampus CA1 region of male rats stained with toluidine blue on day 2 after ischemia in the different groups. (A) sham-operated group, (B) control group and (C) aspirin-treated group; (A) Micrograph Shows a layer of pyramidal neurons with large and prominent nuclei, (B) Micrograph showing apoptotic neurons. Apoptotic body is considerable; (C) Micrograph showing protected neurons in aspirin-treated group. Bar: 10µm.

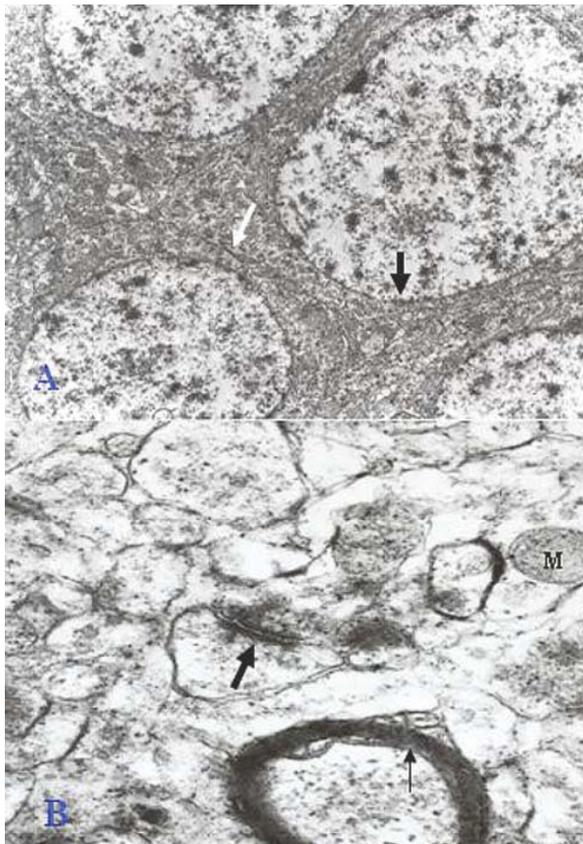


Fig. 2- Electron micrograph of typical CA1 pyramidal neurons from sham-operated rat group (A). (A) Intact cytoplasm within the somata and normal cell membrane (white arrow). Nuclear membrane (black arrow) is smooth and the chromatin material is dispersed throughout the nucleus. (B) Electron micrograph with higher magnification shows neuropil (stratum radiatum) filed of pyramidal neurons; normal synapse (thick arrow), normal myelin sheath (thin arrow), and mitochondrion (M) are considerable. Magnification in (A): $\times 5200$, in (B): $\times 39000$

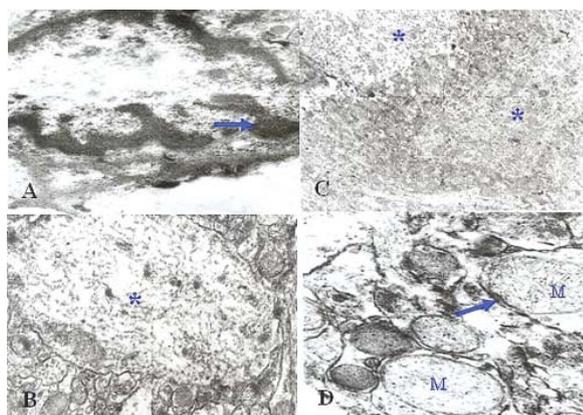


Fig. 3- Electron micrograph of the CA1 pyramidal neurons on day 2 after permanent ischemia. (A) Pyramidal neuron showing degenerative changes. Note nuclear deformity and chromatin aggregation (arrow). (B) and (C) Micrographs of lower magnification showing apoptotic neurons (asterisks). Micrograph showing typical apoptotic bodies. (D) Within the neuropil, degenerating dendrites show increased electron density and synapses are irregular too (arrow). Swelling mitochondria (M) are noticeable. Magnifications: (A) and (D): $\times 39000$ (B), (C): $\times 6195$

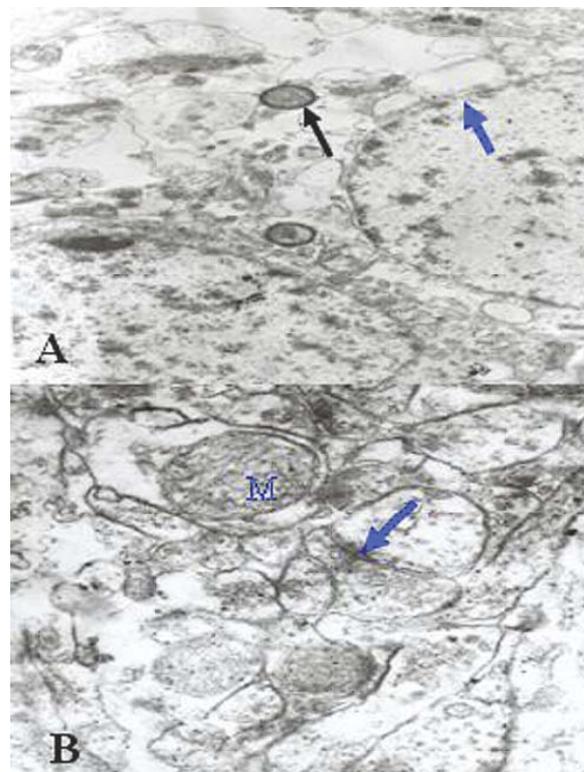


Fig. 4- Effect of aspirin on area CA1 Ultrastructure two days after permanent ischemia. (A) In this pyramidal cell, the nuclear membrane is intact and relatively smooth (black thick arrow), but increased density occurs within the nucleoplasm along with chromatin aggregation. Myelin sheath (black thin arrow) is normal. (B) Normal appearance of synapses (arrow) and mitochondria in stratum radiatum. Magnification in (A): $\times 5200$, in (B): $\times 39000$

Discussion

In the present study, we show that ASA, administered soon after cerebral ischemia, prevent cell death of CA1 hippocampal pyramidal cells. We found that ASA could preserve the normal ultrastructure of CA1 pyramidal cells for at least 2 days after ischemia. Light microscopy evaluation showed the apoptotic changes of CA1 hippocampal neurons following permanent MCAO. These changes were including formation of apoptotic bodies and loss of inflammatory cells.

Electron microscopy evaluation showed chromatin aggregation and formation of apoptotic bodies, while neither plasma nor mitochondrial membranes were disrupted. Our findings are agreement with that reported evidence of apoptosis (10-13).

Previous studies compared the different doses of ASA applied before and after the ischemia induction. Khayyam *et al.*(14) reported that ASA in different doses (between 15 and 80 mg/kg) reduced infarct size only when administered 2 h or 30 min before permanent MCAO induction, but not when it was administered 8 or 24 h before or 30 min after ischemia onset. Treatment of rats with 20 mg/kg ASA 1 to 6 h before slice preparation decreased the decline of ATP content of hippocampal slices during hypoxia (15). A dose of 30 mg/kg ASA injected 2 h prior to permanent MCAO attenuated glutamate release in the infarct core (16). These studies showed that a neuroprotective effect of ASA when given before the onset of ischemia. Post-ischemic administration of a single ASA bolus failed to reduce infarct size in one single report (17) as it did in our research.

Obviously, the type of ischemia – if permanent or temporary – accounts for the effectiveness of ASA as neuroprotective agent. At the time of filament removal in the

transient ischemia model, striatal glutamate concentration rapidly decreased reaching almost preischemic values after 5 h. On the contrary, permanent ischemia led to a sustained release of glutamate though its concentration also gradually decreased after 2 h (18).

Reduction of glutamate release it seems that is one of the mechanisms reflecting neuroprotection by ASA at least in transient ischemia though it may not be causative. In permanent ischemia, glutamate release in the infarct core could be reduced when ASA (30 mg/kg) was administered 2 h prior to stroke onset (6). In the present study, ASA was injected 30 min after ischemia induction and this intervention showed some evidence regarding neuroprotective effect of it.

As ASA may act at different stages of ischemia via multiple pharmacological mechanisms (6), so the exact protective mechanism of ASA is not clear, but multiple neuroprotective mechanisms of ASA could explain the observed effect.

We conclude that administration of ASA after permanent focal cerebral ischemia may remain a considerable therapeutic strategy.

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