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Neuroprotective Effect of Post Ischemic Treatment of Acetylsalicylic Acid on CA1 Hippocampus Neuron and Spatial Learning in Transient MCA Occlusion in Rat

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The present study was designed to examine the neuroprotective effects of post-ischemic treatment with ASA on pyramidal neurons of the hippocampal CA1 sector and spatial learning and memory. Animals were allocated to ASA (20, 40 and 80 mg kg^{-1}) and vehicle groups. Ischemia was induced by 20 min middle cerebral artery occlusion. ASA solution was administered intraperitoneal at 30 min, 6 and 24 h after induction of ischemia. Four days after ischemia, animals were subjected to 5 days of training in the Morris water maze (MWM); 4 days with the invisible platform to test spatial learning and the 5th day without platform to test spatial memory. At the end of behavioral test rats were sacrificed and pyramidal neurons in the CA1 sector were stained with Hematoxylin and Eosin. We demonstrated that repeated injections of 20 mg kg^{-1} ASA ($p<0.05$) but not 40 and 80 mg kg^{-1} ASA ($p = 0.989$) increased percentage of spent time in the target zone across the four days post stroke test period significantly in the MWM as compared to vehicle group. But repeated injections of 20, 40 and 80 mg kg^{-1} of ASA after ischemia not reduced the prolongation of the escape latency significantly as compared to vehicle group ($p = 0.556$). In treated groups there was no significant effect on spatial memory ($p = 0.987$) as compared to vehicle group. Histological verification of CA1 area showed that repeated injections of 20, 40 and 80 mg kg^{-1} ASA at 30 min, 6 and 24 h after ischemia reduced the number of dead hippocampal neuronal cells significantly ($p<0.001$). Study findings show that repeated ASA injections at 30 min, 6 and 24 h after stroke onset decreases neuronal injuries following ischemia and attenuates cerebral ischemia-induced learning dysfunctions.

Key words: Brain ischemia, hippocampus, morris water maze, ASA, spatial learning

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INTRODUCTION

The Central Nervous System (CNS) is solely dependent on the glucose and oxygen delivered by the blood. It is highly vulnerable to brain injury and inadequate blood supply to the brain, such as that produced by cerebral ischemia, can easily trigger neuronal death (Lee *et al*., 1999; Siesjö *et al*., 1976; Wang *et al*., 1997). Stroke is one of the leading causes of death in the world. In the USA, estimated 700,000 suffer from stroke; 30% of these stroke-afflicted patients die, while 20-30% become severely and permanently disabled (Pulsinelli *et al*., 1997).

Of the neighboring regions and different cell types that undergo delayed cell death in response to ischemia, the neurons that are most vulnerable are found in the hippocampus-a key region in the learning and memory processes. The order of vulnerability of the regions within the hippocampus is CA1, hilar>CA2>CA3>dentate gyrus DG (Butler *et al*., 2002). Hippocampal CA1 neurons are critically involved in spatial learning and memory and degeneration of these neurons results in deficiencies in such functions (Morris *et al*., 1982). During brain ischemia, extra cellular glutamate concentration increases, reaching levels that activate the NMDA type of glutamate receptor, thereby, causing neuronal death (Choi and Rothman, 1990; Castillo *et al*., 2003). Recent studies on pharmacological treatment of acute ischemic stroke have been directed to improving blood supply to the ischemic zone margins and/or interrupting the neurochemical cascade initiated by ischemia leading to cell death. Accordingly, acetylsalicylic acid (ASA) is suggested as a therapy (The International Stroke Trial, 1997), besides its use in primary or secondary prophylaxis of ischemia stroke (Patrono, 1994). Testing ASA as a therapy in acute ischemic stroke is consistent with experimental evidence that platelet activation occurs repeatedly during the first 48 h after the onset of ischemic stroke symptoms (Van Kooten *et al*., 1994) and ASA or salicylic acid significantly reduces glutamate excitotoxicity *in vitro* (Grilli *et al*., 1996). ASA neuroprotective effects were observed in experimental focal cerebral ischemia (Khayyam *et al*., 1999). Glutamate, the most abundant excitatory neurotransmitter in the mammalian CNS that is necessary for learning and memory function (Bliss and Collingridge, 1993; Menschik and Finkel, 1998). In the other hand a very modest treatment regimen with ASA in aged rats that were not subjected to ischemia is sufficient to induce obvious improvements in the spatial learning (Smith *et al*., 2002). Therefore, the current study was conducted to investigate the neuroprotective effect of post-ischemic treatment of ASA on CA1 hippocampal neurons and spatial Learning and Memory in rat model of transient focal cerebral ischemia.

MATERIALS AND METHODS

Subjects: Male albino Wistar rats (280-330 g) were used in the study. Animals were housed individually in standard cages in a temperature and light controlled (12 h light: 12 h dark) environment. All of the animals had free access to food and water throughout the experiment. Experiments were performed in conformity with the Kerman university research council guidelines ethics for conducting animal studies. All process was done in labs of the Neuroscience Research Center of Kerman University of Medical Sciences in 2006-2007.

Experimental protocols

Rats were divided randomly into four groups: Vehicle and ischemia+ASA (20, 40 and 80 mg kg^{-1}). Transient unilateral occlusion of right middle cerebral artery was induced for 20 min and followed by reperfusion (Memezawa *et al*., 1992). ASA was used in pure substance (Sigma). It was dissolved in distilled water and injected via the i.p route. Ischemia+ASA subjects received different doses of ASA (20, 40, 80 mg kg^{-1}) at 30 min, 6 and 24 h after ischemia induction. Vehicle subjects received injections of saline at all same time points.

Induction of focal ischemia: The rats were anesthetized with chloral hydrate (400 mg kg^{-1} i.p.). Under an operation microscope, right Common Carotid Artery (CCA) and External Carotid Artery (ECA) were exposed. The Internal Carotid Artery (ICA) was also dissected to the level of petrygopalatine artery. The CCA and ECA were occluded permanently and ICA temporarily using a micro vascular clip. Then, a small incision was made on CCA and a nylon thread (3-0) was inserted through. While, holding the thread around ECA tightly to prevent bleeding, the micro vascular clip on ICA was removed and the nylon thread was carefully and slowly pushed forward through CCA until a light resistance was felt. Such resistance was indication that tip of nylon thread was wedged at the beginning of anterior cerebral artery (20-22 mm from CCA bifurcation), resulting in occlusion of the middle cerebral artery (Vakili *et al*., 2006). At 20 min after induction ischemia, the filament was slowly removed. Animals were then recovered from anesthesia and kept in single cages. Rectal temperature was measured by a thermometer and maintained at 37±0.1°C, throughout the experiment using an electrical blanket.

Learning and memory: Spatial learning and memory were evaluated in Morris water maze (Morris *et al.*, 1982), which consisted of a circular pool, 180 cm in diameter. The animals were monitored by a video tracking system (Radyab soft ware, Home made).

Animals were trained for four consecutive days with four trials/day from any of the four pseudo-randomly selected starting points. The platform was submerged 1 cm below the water (22±1°C) surface in a fixed position. Several prominent extra-maze cues were present and kept constant throughout the experiment. A trial was terminated as soon as the rat had climbed on the escape platform or when 90 sec had elapsed. A rat was allowed to stay on the platform for 20 sec. Then it was taken from the platform and the next trial was started. Rats that did not find the platform within 90 sec were put on the plat form by the experimenter and were allowed to stay there for 20 sec. After completion of the 4th trial, rats were gently dried with a towel, kept warm for an hour and returned to their home cage.

Escape latency (time to reach the platform), path length the rat swam to find the platform and percentage of target zone entries as well as the swim speed was automatically recorded and analyzed. At the 5th day, a 60 sec probe trial without the platform was used to assess how well rats remembered the location of the platform, Time spent and traveled distance in the target zone of the pool was analyzed (Majlessi *et al.*, 2002).

Histology: At the completion of the memory test, animals were deeply anesthetized and perfused with normal saline (10 min) followed by a mixture of 40% formaldehyde, glacial acetic acid and methanol (FAM, 1:1:8 by volume) and brain blocks were embedded in paraffin. Five- micron-thick sections from hippocampus were cut and stained with Hematoxylin and Eosin. In the hippocampus, numbers of normal pyramidal neurons were quantitated of five fields of CA1 region by an observer blinded to the experimental groups. Data were expressed as numbers of normal pyramidal neurons per high-power (40x) microscopic field.

Statistical analysis: The data of first 4 training days with hidden platform were initially subjected to a two-way ANOVA followed, by Tukey post-hoc analysis. Data of the 5th day and part of histology were analyzed by one-way ANOVA. In all comparison, $p<0.05$ was used as statistical significance.

RESULTS

Morris water maze: The two way ANOVA of the escape latency during the 4 days with invisible platform was no significant difference ($F_{9, 96} = 0.144$, $p = 0.998$) between groups over days of training (Table 1). The differences in escape latency ($F_{3, 96} = 12.269$, $p<0.001$) between days of training were significant. The percentage of time spent by each of them in the target zone had significant difference between groups ($F_{3, 96} = 5.472$, $p<0.05$). Tukey post-hoc analysis indicated a significant differences between ASA 20 mg kg^{-1} group and vehicle ($p<0.05$) (Fig. 1). A two-way

Table 1: Mean escapes latencies in seconds per groups over 4 days in the Morris water maze. On days 1-4, rats learned to reach a hidden platform

Groups/days	The 1st day	The 2nd day	The 3rd day	The 4th day
Vehicle	55.67±6.12	36.25±5.86	28.53±11.31	25.32±8.26
ASA (20 mg kg^{-1})	46.03±8.56	26.71±7.73	20.64±5.78	19.46±4.07
ASA (40 mg kg^{-1})	51.32±8.49	39.75±7.21	22.25±8.30	26.03±9.48
ASA (80 mg kg^{-1})	71.64±6.63	48.32±7.07	37.21±6.77	42.85±7.78

Four groups (n = 7) were tested. Data have shown as Means±SEM

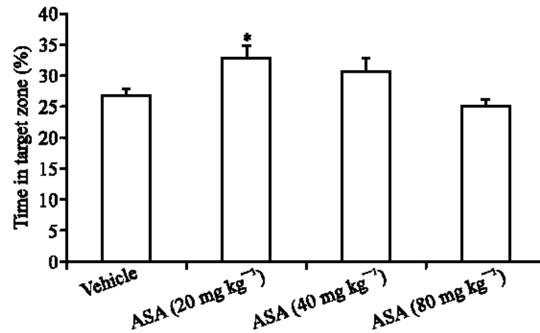

Fig. 1: Mean percentage of time spent in the target zone±SEM during the 4 days of training in a water maze with the invisible platform (data were averaged to obtain a mean performance for each animal). Four groups (n = 7) were tested: vehicle, ASA20 mg kg^{-1}, ASA40 mg kg^{-1} and ASA80 mg kg^{-1}. *$p<0.05$ vs. Vehicle

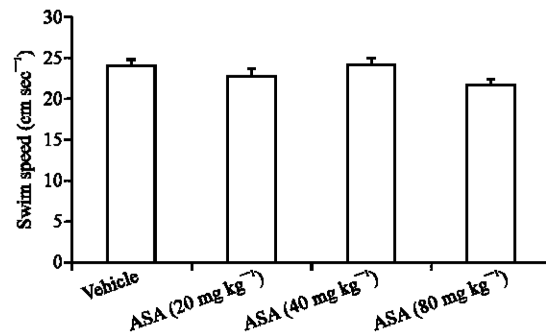

Fig. 2: Mean swim speeds ±SEM during the 4 days of training in a Water maze with the invisible platform (data were averaged to obtain a mean performance for each animal). Four groups (n = 7) were tested: vehicle, ASA20 mg kg^{-1}, ASA40 mg kg^{-1} and ASA80 mg kg^{-1}

ANOVA showed no significant difference between the swim speed of the four groups ($F_{3, 96} = 2.312$, $p = 0.081$) over days of training ($F_{3, 96} = 2.271$, $p = 0.085$) (Fig. 2). The interaction between group and day was not significant ($F_{9, 96} = 0.405$, $p = 0.930$). In the memory test the percentage of spent time in the target quadrant at 5th day was not statistically significant between groups ($F_{3, 27} = 0.055$, $p = 0.983$) (Fig. 3).

Histology: Photomicrographs represent the hippocampus CA1 sector from each experimental group (Fig. 4). One way ANOVA showed significant differences in number of normal CA1 hippocampal pyramidal neurons in five field among four groups of ASA20 mg kg^{-1}, ASA40 mg kg^{-1}, ASA80 mg kg^{-1} and vehicle ($p<0.05$). Post hoc analysis indicated a significant differences between three groups of different doses of ASA (20, 40 and 80 mg kg^{-1}) and vehicle ($p<0.05$, Fig. 5).

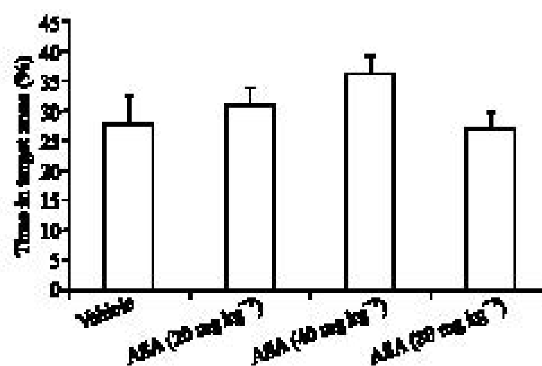

Fig. 3: Mean percentage of time spent in the target zone ±SEM during the 5th day of training in a Water maze without platform. Four groups (n = 7) were tested: vehicle, ASA20 mg kg^{-1}, ASA40 mg kg^{-1} and ASA80 mg kg^{-1}

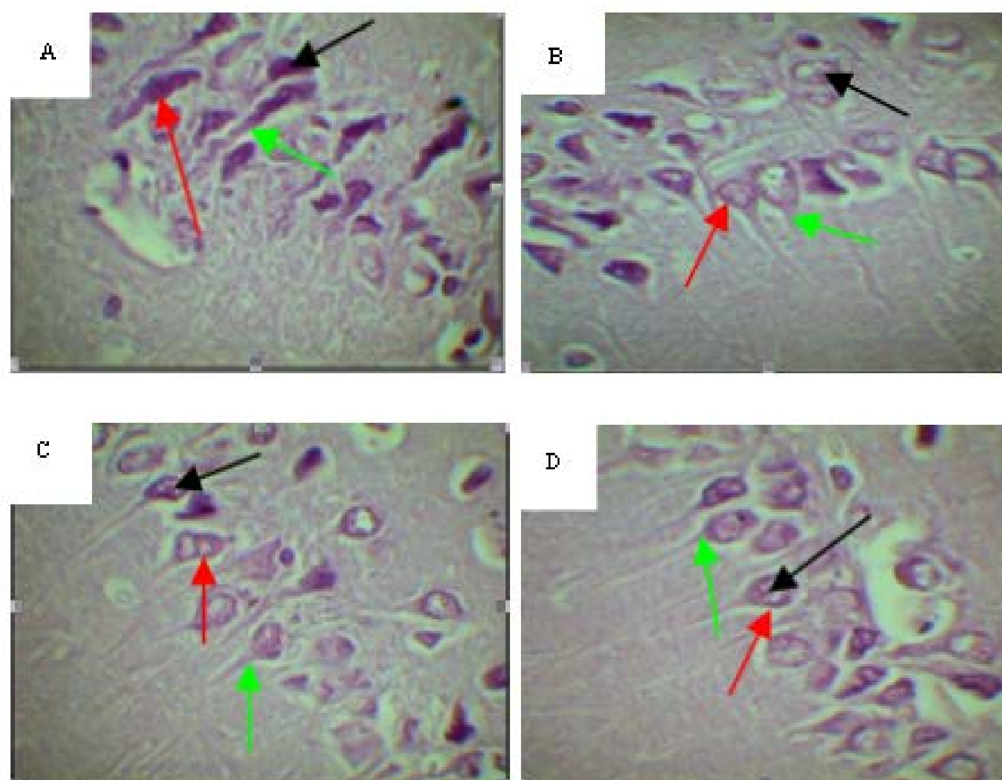

Fig. 4: Protection effect of ASA against ischemia-mediated cell loss in the CA1 hippocampus area after transient focal cerebral ischemia in rat. Photomicrographs illustrate neurons within the CA1 region of the hippocampus stained with hematoxylin and eosin at a magnification of 40X after ischemia. A: there is an almost complete neuronal necrosis of the CA1 sector in rats subjected to 20 min transient focal cerebral ischemia. In contrast, administration of different doses of ASA (20, 40 and 80 mg kg^{-1} i.p) at 30 min, 6 and 24 h after ischemia conferred neuroprotection by markedly reduced number of damaged pyramidal cells in the CA1 subfield (respectively BCD). Black arrow show nucleus, red arrow show nucleus, green arrow show cytoplasm

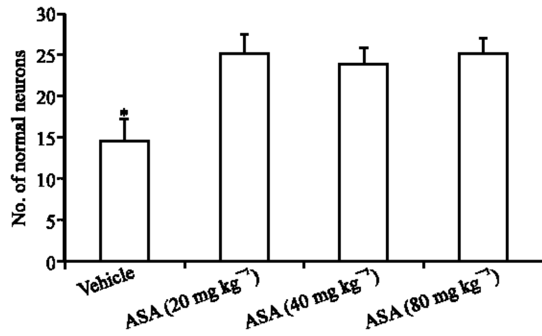

Fig. 5: Bar graph of normal neuron counts in the hippocampal CA1 sector. ASA (20, 40 and 80 mg kg^{-1}) results in a significant protection against ischemic neuronal death, when administrated at 30 min, 6 and 24 h after ischemia. Values are means±SEM. *Show significant differences between ASA (20, 40 and 80 mg kg^{-1}) and vehicle, $p<0.05$

DISCUSSION

Study result in this study showed that repeated administration of 20 mg kg^{-1} ASA at 30 min, 6 and 24 h after MCAO in addition to significant reduction in neuronal cell death in the hippocampal CA1 region caused mild improvement of spatial learning in the water maze test. However repeated injections of higher doses of ASA (40 and 80 mg kg^{-1}) at the same times had no significant effect on spatial learning in the water maze test, but caused significant reduction in neuronal cell death in the hippocampal CA1 region. We also found that administration of different doses of ASA (20, 40 and 80 mg kg^{-1}) at 30 min, 6 and 24 h after MCAO had no effect on spatial memory. These findings indicate that ASA has the potential ability to exert a persistent effect on the learning function of animals with cerebral ischemia. The underling mechanism(s) by which ASA exert neuroprotective effects in CNS is not completely determined yet.

The most important pathological event that contributes to the impairment of brain function is the decrease of glucose and oxygen supply for the brain tissue. Therefore, efficient vascular supply of blood is capable of preventing the progression of ischemic pathogenesis, including cerebral infarction and tissue degeneration and may correlate with functional recovery after cerebral ischemia (Date *et al*., 2004). Treatment with ASA significantly prevented the tissue degeneration. Therefore, ASA may prevent injury to brain vasculature, consequently maintaining glucose and oxygen supply to brain tissue, possibly to the penumbra. Also, study results showed that ASA caused a mild improvement in learning in rat, probably thought excitatory glutamate neurotransmitter.

Glutamate is the most abundant excitatory neurotransmitter in the mammalian Central Nervous System (CNS). It has a principal role in long term potentiation, which is necessary for learning and memory function (Bliss and Collingrige, 1993; Menschik and Finkel, 1998). Thus, a facilitation of glutamate exocytosis may be a potentially important mechanism to strengthen excitatory synapses during learning and memory processes. Wang showed that, in hippocampal nerve terminals, the non selective COX inhibitor, aspirin facilitates 4AP-evoked glutamate release primarily by increasing Ca^{2+} influx through voltage dependent N and P/Q type Ca^{2+} channels (Wang, 2006). A very modest treatment regimen with aspirin in rats is sufficient to induce obvious improvements in the speed of learning; this improvement in cognitive ability was most evident in aged rats tested with Morris water maze (Smith *et al*., 2002). Also Riepe *et al*. (1997) found enhanced recovery of the population spike in ASA-treated slices following hypoxia (Riepe *et al*., 1997).

During brain ischemia, extra cellular glutamate concentration increases, reaching levels that activate the NMDA type of glutamate receptor, thereby causing neuronal death (Choi and Rothman, 1990; Castillo *et al*., 2003). Thus, ASA probably through modulation release of glutamate during ischemia (Cristobal *et al*., 2001) caused neuroprotection and may be increasing spatial learning in the Water maze test.

Whereas acute ischemic phase is characterized by the release of excitotoxic concentrations of glutamate and other exitatory amino acids and decrease of vital ATP levels. The subsequent stage lasting for a few days which cause the induction of inflammatory and immune mechanisms and lead to further neuronal death (Dirnagl *et al*., 1999). These inflammatory reactions are enhanced by reperfusion after tMCAO and consist of a large influx of neutrophils/leukocytes which is driven by specific adhesion molecules and cytokines. The tissue damage potentate by releasing oxygen radicals (Kuhn *et al*., 1995; Lancelot *et al*., 1995) and cytotoxic products (Zhang *et al*., 1994). Therefore, ASA may act at different stages of ischemia via multiple pharmacological mechanisms. During the early stage of cerebral ischemia, ASA may attenuate glutamate-mediated neurotoxicity. ASA inhibited glutamate release in cortical neurons exposed to oxygen-glucose deprivation in rat (Cristobal *et al*., 2003). In human ASA treatment was

associated with significantly lower CSF glutamate concentrations after acute cerebral infarction (Castillo *et al*., 2001). During the following course of the ischemic cascade, its anti-inflammatory properties may be of grater importance.

CONCLUSION

In conclusion, we demonstrated that repeated ASA post treatment has the potential ability to decline learning dysfunction and neuronal degeneration in the CA1 sector of the hippocampus after cerebral ischemia.

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