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Histopathological evaluation of the role of negative electrical charge on renal ischemia/reperfusion injuries on brain and heart tissues in rat

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Abstract

This study was performed to evaluate the role of electroacupuncture on kidney 1 (Ki1) acupoint to prevent the heart and brain injury following ischemia/reperfusion of both kidneys. 24 Sprague Dawley rats were randomly assigned into four equal groups. In the treatment 1 group, following anesthesia, acupuncture needles were inserted on Ki1 on the palm of both hindlimbs and connected to electroacupuncture unit for a 3.00 Hz direct current, 1 hr before surgery until the end of surgery. In treatment two groups, the electroacupuncture was also performed 48 and 24 hr before the operation, with the same protocol as treatment 1. Control 1 and control 2 groups had the same procedures like the treatment ones, except for acupuncture. Immediately after reperfusion, the samples of brains and hearts were taken and prepared for microscopic examination. Histopathological study of the heart in the control and treatment groups showed the breakage of myofibrils, hyaline necrosis, edema and disorganization of myocytes. The severity of cardiac lesions was decreased in both treatment groups in comparison with the controls. Brain in control and treatment groups showed ischemic necrosis, disorganization of the neurons in the hippocampus, and edema. The severity of lesions was reduced in the treatment groups and showed a significant difference between the control and treatment 1. It could be concluded that electroacupuncture on the Ki1 point could reduce the severity of damages induced by renal ischemia/reperfusion in the remote organs of the heart and brain.

Introduction

Ischemia is defined as an insufficient supply of the nutrient and oxygen to the organs. During ischemia, the level of glutathione, phosphocreatine, and ATP are reduced and hypoxanthine level gets elevated. Altered ion distribution i.e. increase in intracellular Ca^{2+}/Na^{2+}, cellular swelling, cytoskeletal disorganization and acidosis of cells occur. During reperfusion, blood flow supply returns to the cardiac muscle (tissue) that causes cellular edema, excess release of intracellular Ca^{2+} and damage to the cell membrane. Cellular damage after reperfusion of previously viable ischemic tissues is known as ischemia/reperfusion (I/R) injury.In clinical studies and in experimental models, ischemia followed by reperfusion resulted in the release of free radicals into bloodstream which in turn damaged the remote vital organs.[1]

Ischemia/reperfusion injury (IRI) results in a local and systemic inflammatory response characterized by production of oxidative stress, leukocyte endothelial cell adhesion, transendothelial leukocyte migration, increased microvascular permeability and decreased endothelium-dependent relaxation. Timely reperfusion of the ischemic area at risk remains the cornerstone of clinical practice.[1]

Acupuncture is an energy-based system of healing that is several thousand years old and addresses and activates the self-healing powers of the body. The influence of acupuncture stimulates causes the flow of energy within

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the body along the channels and hence in the entire organism. According to Traditional Chinese Medicine (TCM), it removes blockages along the acupuncture channels, moves a stagnation and supplies mental or emotional disorder with new energy. As a result, we can reduce pain and revive disturbed organ functions. Successful acupuncture restores the natural balance of the organism. It is not able, however, to heal permanently destroyed tissue. As one important aspect of this healing process, acupuncture teaches the body to restore its balance on its own. The acupuncturist hereby has the role of showing the diseased organism the path to heal by means of the needle.[2]

Very few studies are available for reducing I/R injury with electroacupuncture. Electroacupuncture has been used to inhibit apoptosis and provide neuroprotection.[3] It also enhances endogenous brain-derived neurotrophic factor (BDNF) mRNA expression, which may improve the survival environment for intracerebral neurons and inhibit the apoptosis of hippocampal cells.[4] It has been used to treat the polycystic ovary syndrome (PCOS), and to improve obesity-related indexes, insulin sensitivity, and adiponectin (APN) level.[5] It has also been reported that electroacupuncture treatment of cerebral I/R regulates Atp2a2, Cacna1e, Camk2a, Gnas, Grm1, Rapgef3 gene in the calcium signaling pathway.[6]

In this study, we evaluated the effect of negative electrical charges on damages induced from renal I/R on the remote organs of the heart and brain.

Materials and Methods

Animals. The study was performed on 24 female Sprague Dawley rats weighing about 250-300 g. All animals were housed in an air-conditioned room (12 hr light/ dark cycles with a temperature of 21.00 ˚C and relative humidity of 50.00%). The animals were fed with standard laboratory rodent pellet diet, (Javaneh Khorasan Co., Mashhad, Iran) and tap water *ad libitum*. All animals received human care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). The study was approved by the local ethics (624, 94. 4. 20) committee of Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

Experimental groups. The animals were randomly assigned into four groups (n = 6 rats in each) as follows:
Control 1 group) Following general anesthesia using an intraperitoneal injection of 60.00 mg kg^{-1} ketamine (Alfasan, Woerden, Holland) and 10.00 mg kg^{-1} xylzine (Alfasan), 40 min both kidneys ischemia followed by 30 min reperfusion was done. Treatment 1 group) Following general anesthesia, acupuncture was administered, acupuncture needles were inserted on Ki1 on the palm of both hindlimbs and connected to electroacupuncture unit for a 3.00 Hz direct current from 1 hr before surgery until the end of the surgery, with 40 min both kidneys ischemia followed by 30 min reperfusion. Control 2 group) General anesthesia was performed 48 hr and 24 hr before surgery and on the day of surgery. Ischemia for 40 min both on kidneys was performed, followed by 30 min reperfusion was done. Treatment 2 group) General anesthesia was performed 48 hr and 24 hr before and on the day of surgery. Acupuncture was administered, the electroacupuncture was performed 24 and 48 hr before the operation, with the same protocol as group B, with 40 min both kidneys ischemia, followed by 30 min reperfusion.

Surgical procedures. Following surgical preparations and general anesthesia, both kidneys were approached from the ventral midline and each renal pedicle was occluded by a Rumel tourniquet for 40 min. Then, the tourniquets were released and 30 min reperfusion was administered, followed by the sacrificing of the animals for sampling.

Sampling. The anesthetized rats were decapitated, and the entire brains and hearts were removed and kept in 10.00% buffered formalin for seven days. Following the completion of the fixation period, 6.00 µm sections were prepared and stained with Hematoxylin and Eosin (H & E) for light microscopy (Eclipse 50i; Nikon, Tokyo, Japan).

Histopathology evaluation. All sections were systematically examined at 200× total magnification, and all regions with ischemic, necrotic, neurons were noted. Necrotic neurons in affected neuronal populations, bilaterally, were manually counted by a veterinary pathologist and put into five ranked categories as follow: 0 necrotic neurons = 0; 1-15 = (+1); 16-30 = (+2); 31-50 = (+3); and > 50 = (+4).

Ranked category assignments for all regions were summed to obtain the overall brain histologic damage score. Karyorrhectic nuclei were counted by visually reconstructing each nucleus from the fragments.[7] The severity of lesions in the cardiac muscles was evaluated according to the severity of muscular edema and signs of myocyte necrosis. The procedure was performed blindly under 100 × magnifications, according to the following scores: Absent =0, rare =1, moderate =2, and severe =3.

The signs of myocyte necrosis included: cellular vacuolization, band contraction and/or cellular destruction.[8]

Statistical Analysis. All statistical analyses were carried out using SPSS statistical software (version 16.0; IBM Corp. Armonk, USA). The one-way ANOVA analysis of variance and post hoc Tukey HSD test were performed on the data of the histopathologic lesions to examine differences between the groups. A $p < 0.05$ was considered significant.

Results

Histopathological findings in the heart. In the normal control group, muscle fibers were arranged regularly, with vesicular nuclei located in the center of the fibers. The sarcolemma was intact and did not show any defect including no break, degenerative changes, and necrosis in the cardiac fibers (Fig. 1A). In control 1, the order of muscle fibers was changed, interstitial edema and increased interfibrillar space could be seen, and sarcolemma was damaged and broken down. In some areas, cardiac fibers showed Zenker's necrosis as hyaline change. The severity of lesions in the majority of samples was moderate (2) to severe (3) with mean = 2.16 (Figs. 1B and 1C). In treatment 1, the structure of cardiac muscle fibers was disrupted, and hyaline necrosis and intercellular edema occurred.

The severity of injuries was lower than control 1 and varied from mild (1) to moderate (2). The mean necrosis severity was mean = 1.83 (Fig. 1D). In control 2, coagulation necrosis, breaking in myofibrils and increasing the space between the cells were observed (mean = 1.66), (Fig. 1E). In treatment 2, pyknotic nuclei, hyalinization of the cytoplasm, coagulation necrosis, breaking in myofibrils and increase in space between cardiac fibers were visible (mean = 1.33), (Fig. 1F).

Histopathological findings in the brain. Different parts of the brain were investigated and severity lesions in the hippocampus were graded which is shown in Table 1**.** In the normal control group, brain structure was normal and no cell damage, degenerative changes, and necrosis were observed. The neurons in the hippocampus showed a round, bright, and vesicular nucleus (Fig. 2A). In control 1, neuronal necrosis, edema, and status spongiosis in the cerebral cortex and also disorganization of neurons was observed. Astrogliosis was observed in different areas of the cerebrum. In the hippocampus, various degrees of neuronal damage have occurred. The neurons were shrunken and appeared as triangular and hyperchromatic forms in CA1, CA2 and CA3 regions. By the statistical method, the mean severity of neuronal necrosis was calculated as 135.75 ± 14.23. Necrosis of the neurons by following I/R was widespread and extensive in the cortex (Figs. 2B and 2C). In treatment 1, ischemic necrosis in neurons of gray matter, pyknotic nucleus and eosinophilic change of cytoplasm were seen. There were also hyperemia and cerebral edema in white matter.

In the hippocampus, neuronal necrosis has occurred in varying degrees of severity and disorganization of the cellular order. Grading the severity of neuronal necrosis in the hippocampus was more severe and statistically significant differences were observed in comparison with control 1 ($p < 0.05$), (Fig. 2D). In control 2, the neurons were wrinkled, their nuclei were hyperchromic and dark, unspecified, with eosinophilic cytoplasm.

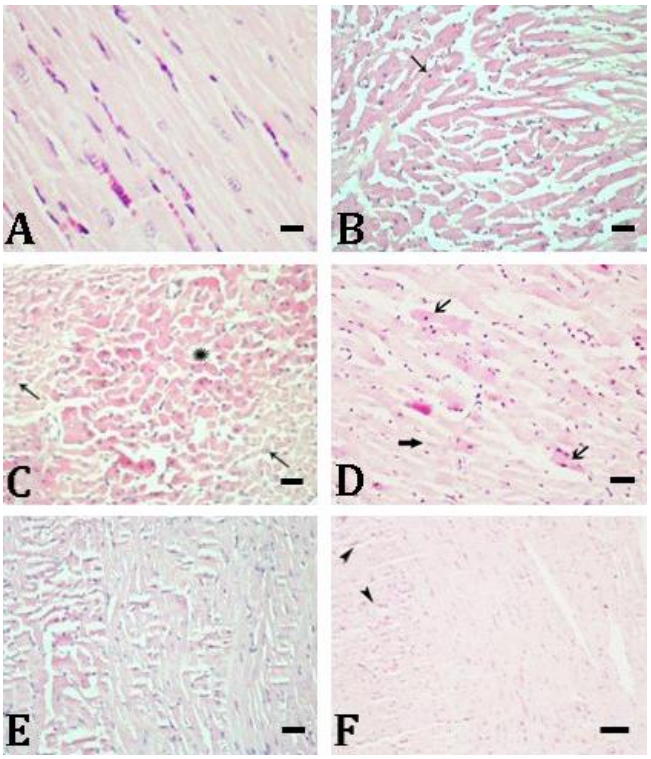

Fig. 1. A) Normal group: Regular arrangement of cardiomuscular fibers with vesicular nuclei in their center. **B)** Control 1: Disorganization of cardiac fibers. Interstitial edema and increase in space between the cardiomyocytes, breaking in myofibers, hyaline necrosis (pyknotic nuclei and eosinophilic cytoplasm) (arrow). **C)** Control 1: Severe hyaline necrosis of cardiac fibers (infarction) (asterisk), normal myocytes with bright pink cytoplasm and vesicular nuclei (arrows). **D)** Treatment 1: Hyaline necrosis of cells (thin arrows), intercellular edema, and the gap among the myofibers, normal myocytes with clear pink cytoplasm and vesicular nuclei (bold arrows) **E)** Control 2: Hyaline necrosis of myofibrils on the left side of the figure. **F)** Treatment 2: Mild necrosis of myocytes, hyalinization of damaged cells (arrows), intercellular edema, and fracture of myofibers (H & E; Figs. 1A, 1B, 1C, 1D, and 1E: Scale bar = 10.00 µm, Fig. 1F: Scale bar = 100 µm).

Table 1. Evaluation of the average of lesions in different areas of the hippocampus. Data are presented as mean ± SE.

Groups	The lesion in the hippocampus*			Total mean lesion*	*p*-value
	CA_1	CA_2	CA_3		
Control 1	135.75 ± 14.23*	59.00 ± 21.69	64.50 ± 7.23*	259.25 ± 30.59	0.03*
Treatment 1	73.33 ± 18.37*	37.00 ± 18.33	20.16 ± 11.58*	130.50 ± 38.71	
Control 2	119.33 ± 55.47	14.00 ± 9.50	76.00 ± 30.43	209.33 ± 85.69	0.29
Treatment 2	66.40 ± 29.96	10.40 ± 5.67	46.80 ± 12.59	123.60 ± 31.33	

* Asterisk indicates a significant difference at $p \leq 0.05$.

Cerebral edema and dilatation of space around the vessels and neurons and white matter sponge were visible. The hippocampus was also affected by the neuronal order, including shrinkage of the cell, hyperchromatic nuclei, and dark basophilic cells. The severity of the injury in this area was calculated as 209.33 ± 85.69, (Fig. 2E). In treatment 2, neuronal necrosis, cerebral edema and hyperemia could be seen. The severity of neuronal necrosis was 123.60 ± 31.33, which was lower than that of control 2, however, there was no significant difference ($p > 0.05$), (Fig. 2F).

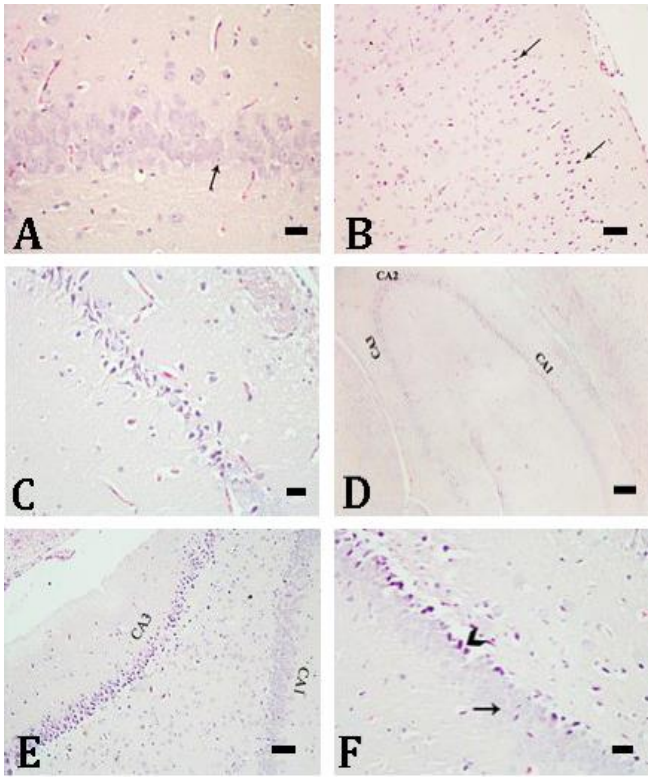

Fig. 2. A) Normal group: The neurons were regular in the hippocampus and had a round, bright, and vesicular nucleus. **B)** Control 1: Cerebral cortex. Neuronal necrosis with a pyknotic nucleus, and enhanced eosinophilic cytoplasm (arrows). **C)** Control 1: Severe necrosis of the neurons in the hippocampus and increase the distance between the cells. **D)** Treatment 1: Neuronal shrinkage with a pyknotic nucleus in the CA1, CA2 and CA3 regions of the hippocampus. **E)** Control 2: Neuron necrosis in the CA3 region of the hippocampus. **F)** Treatment 2: Mild necrosis of neurons with pyknotic nuclei and eosinophilic cytoplasm (arrowhead), normal neurons with bright, vesicular nuclei (arrow), (H&E, Figs. 2A, 2C and 2F Scale bar = 10.00 μm, Figs. 2B, 2D and 2E Scale bar = 100μm).

Discussion

The occurrence of injuries following I/R in different tissues is depended on the duration of ischemia.[9] Releasing of cytokines, reactive oxygen species (ROS) and chemo-kines from activated endothelium and mast cells in the blood are main responses to I/R. However, differences in various organs influence the severity, extent, and reversibility of damage.[10] with irreversible damage already detectable at less than 20 min of ischemia,[11] brain is the most sensitive organ to reduce in its blood supply.[12]

In the present study, the effect of electroacupuncture was investigated on the heart and brain following renal I/R. On histopathologic examination, cardiac lesions included coagulation necrosis was characterized by pyknotic nuclei, cytoplasm hyalinization, and also breaking of the myofibrils and increase in distance between cells. In the brain, lesions were observed as neuronal necrosis, edema, astrogliosis, status spongiosis in the cerebral cortex and also various degrees of neuronal damages in the hippocampus. We have demonstrated that electroacupuncture could decrease I/R damages in the heart and brain in both treatment groups in comparison with the controls.

Similar histopathological observations were reported following cardiac or brain I/R in different studies. In a study by Han *et al*. the effects of licochalcone B in reducing I/R-induced myocardial structure injury in the rat was investigated.[13] Zhang *et al*. have also investigated the protective effect of tribulosin against cardiac I/R injury and the underlying mechanism in rats. In their study, the heart was exposed to 30 min of ischemia followed by 120 min reperfusion using Langendorff's technique. In the I/R group irregularity, large areas of necrosis, interstitial edema with neutrophil infiltration, sarcoplasm agglomeration and unclear transverse striation in cardiac muscle fibers were observed.[14]

Maxwell and Lip, and Park and Lucchesi have reported that reperfusion can decrease the extension of myocardial necrosis with the magnitude of the sparing directly related to the timing of the intervention. However, the effects of reperfusion were complex and contained some deleterious effects collectively referred to as reperfusion injury.[15, 16]

Our histopathologic results in the brain due to I/R were in agreement with the previous researches. Chandrashekhar *et al*. induced cerebral ischemia in rats by occlusion of bilateral common carotid arteries for 30 min followed by 1 hr reperfusion. They observed congestion, neutrophil infiltration, and neuronal necrosis.[17] Garcia *et al*., and Petito and Babiak have shown that astrocytic swelling was one of the most important and first responses to cerebral ischemia.[18,19] Lukaszevicz *et al*. have reported that after permanent blockage of the middle cerebral artery in mice, fibrous astrocytes located at the brain surface displayed a transient and limited hypertrophy, without conspicuous cell death, while cortical protoplasmic astrocytes were more vulnerable.[20] Pantino *et al*. have reported the existence of selective oligodendrocyte damage in the rat cerebral white matter after middle cerebral artery (MCA) occlusion.[21] For this, oligodendrocyte swelling occurred early in 30 min after

MCA obstruction, and pyknosis of these cells was expanded after 6 hr.[21] In the present study the brain was subjected to indirect result of kidney I/R and the histopathological manifestations were similar to those studies in which the brain underwent direct I/R.

In acupuncture, the main function of kidneys, similar to the allopathic medical knowledge, is to control the body water distribution. The Kidney 1 (Ki1) Yongquan acupuncture point, named "Gushing spring" or "Bubbling well", is the first acupuncture point on the Kidney meridian. It is the only meridian point on the sole of the foot. According to TCM, Ki1 is a major energy vortex that has the ability to revitalize body, mind, and spirit. This point can be activated with acupuncture, acupressure, herbal plasters, exercise and even floral essences.[22]

Acupuncture neutralizes the free radicals. It can decrease the levels of malondialdehyde, as an indicator of oxidative stress. Our results were consistent with the study of Zhao *et al*. that reported electroacupuncture protected the neurons from cerebral IRI in a mouse by reducing neuronal apoptosis.[3]

Many studies have reported that acupuncture could prevent inflammatory reactions during the process of I/R. The results of a study by Yu *et al*. confirmed the beneficial effects of acupuncture on Parkinson's in rats and found a close relationship between Parkinson's disease and reactive oxygen species, which could damage neurons. Evidence suggests that acupuncture has an antioxidant effect, and this can be due to the antioxidant defense system.[23] Zhang *et al*. examined the effect of acupuncture on psychological function, brain blood flow, mitochondrial respiration, and oxidative stress in the brain of rats suffering from several strokes. The results of acupuncture treatment significantly increased the activity of superoxide dismutase.[24] Qi *et al*. have shown that acupuncture treatment could inhibit the release of inflammatory cells and thus reduced immune-related lesions.[25]

Abe *et al*. reported the effect of intracellular nitric-oxide on the mitochondrial energy of the heart during I/R in rat, and concluded that nitric-oxide produced in reperfusion was the main cause of mitochondrial malfunction and ultimately increased contractility in the heart.[26]

Akhlaghi and Bandy have investigated the effect of flavonoids on heart I/R and demonstrated their positive effects through various mechanisms, most important of which interacting with ionic channels.[27] Özbal *et al*. have studied the effect of selenium on the reduction of the destructive effects of restoring blood flow to the brain in rat, and reported positive effects in the treatment of these injuries.[28] Yamauchi *et al*. have investigated the effect of RS9, a novel Nrf2 activator, One of the transcription factors, on I/R in brain in rats. After 2 hr of ischemia and obstruction of the cerebral artery, reperfusion has happened and 0.20 mg kg^{-1} of RS9 was injected into the peritoneum of the rat. Wound healing after ischemia was investigated and decreased oxidative stress and inflammation of the nerves. It should be noted that RS9 plays a protective role against oxidative stress.[29]

It could be concluded that the use of electro-acupuncture at the kidney point 1 could reduce the complications of renal I/R on the heart and brain. To translate the results of this study in clinical practice, further studies should be performed, indeed.

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Conflict of interest

The authors declare no potential competing conflict of interest.

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