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Original Article

Protective effects of edaravone and UW perfusion on ischemia reperfusion injury of amputated swine extremities

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Abstract: To reduce tissue ischemic damage and explore the best preservation for amputated extremities, 30 hind limps were randomly divided into five groups: the control, saline, UW, saline and edaravone, and the UW and edaravone group. At 2, 4, and 6 hours after amputation, the tissue homogenate were collected to detect the concentration of ALT, ALP, and GLU, and the activity of SOD and GSHPx. Muscle tissues were also collected for immunohistochemical staining, and histopathological observation by light and electron microscopy. It was indicated from the biochemical indexes, the activity of SOD and GSHPx, and IOD values of BOD, Bcl-2 protein and Bcl-2/BaxIOD ratio that the necrosis of tissue changed constantly with the increasing of time, and the frequency of changed values in the UW and edaravone group was significantly different from other groups ($P<0.05$). The UW and edaravone group was observed to have less edema, less fracture, and less cell dissolution in the muscle fibers. The results demonstrate that limb preservation using UW liquid and edaravone has a better impact on preventing I/R-induced tissue injury.

Keyword: Edaravone, UW solution, ischemia reperfusion injury

Introduction

With the progress of science and technology, the number of severe traumas, especially high-energy traumas has gradually increased, and people are increasingly subjected to tissue defects, amputated extremities, and other injuries in the modern society. Although replantation of extremities has become a well-established surgical procedure, the salvage of extremities after prolonged ischemia remains a great challenge in the clinical practice. Amputated extremities provoke a series of pathophysiological changes including metabolic and toxic substance accumulation, cytoplasmic vacuolization, mitochondrial edema, decreased function, decreased lysosomal stability, cell degeneration, and tissue necrosis. Because of ischemia, hypoxia, and the disturbances of normal metabolic process, can be generally referred to as ischemia and reperfusion injury [1]. It cannot only affect the replanted or transplanted extremities, but also harm distant organs like heart, lung, kidney, and other distant organs [2]. Reaction time for different tissue parts of body varies during the ischemic condition, such as for muscles, nerves, adipose, skin and bone tissues is up to 4, 8, 13, 24, and 4 hours respectively [3]. It has been suggested that skeletal muscles are highly vulnerable to ischemia. Nevertheless, the replantation time of amputated extremities is related to the amputated extremities' off plane and environmental factors. In general, the replantation time of limbs should not be extended beyond 6 hours, otherwise it will jeopardize the surgical success [1, 2]. However, it is not always practical to minimize ischemia time before replantation when patients are accompanied by fractures, craniocerebral trauma, and other complex injuries needed to undergo life-saving surgeries first, or are subject to the conditions

of treatment and the patients have to be transferred to other hospitals which adversely prolong the limb ischemia time. Therefore, preservation of amputated extremities is the key to the success before the replantation or the reconstruction of blood circulation to reduce ischemic damage, delay degeneration and tissue necrosis, and subsequently ensure survival and functional outcomes.

The study of the preservation of limbs has made great achievements in recent years. In the past decade, it is common to preserve amputated extremities using low temperature and dry methods [4, 5]. But resistance to ischemia and hypoxia in muscle cells is weak and therefore many reperfusion injury and complications occur after replantation surgery. Additionally there are other methods for storage, such as organ preservative solution, hyperbaric oxygen, perfusion, and ectopic foster methods, etc [6-9]. Perfusion preservation not only provides oxygen and metabolic substrate, but also protects endothelial cell, prohibit the inflammatory response, and scavenges free radicals which are the key substances causing irreversible damage in tissues [10]. According to the different components organ preservation solution can be divided into imitation cell liquid type (such as E-C liquid), imitation of extracellular liquid (such as U-W liquid), plasma solution, oxygen storage solution, and non-liquid type preservation solution. E-C liquid and U-W liquid are most studied [6]. A consensus was reached to use UW solution which was first developed by the University of Wisconsin to preserve kidney before the transplantation [5]. It can provide the components with hypotonicity of 320 MOSM/L and PH 7.4, such as impermeant, an effective buffering system and the substrates to regenerate high-energy phosphates, which can reduce the onset of action potential during ischemia and conserves energy for the cells. 11 Furthermore, it provides ATP to tissues using ADP as a direct energy substrate, and contains antioxidants, like allopurinol and glutathione, can improve the organ tissue survival while inhibiting the generation of oxygen free radicals, thereby inhibiting apoptosis and reducing reperfusion injury [11-13]. It was confirmed in clinical trials that UW played a good protective effect on the preservation of kidney by inhibiting low temperature edema and cell damage, significantly prolonging the tolerance of tissues and organs to hypothermic ischemia and decreasing organ damage [14].

Edaravone was approved for patients with cerebral infarction in 2001 in Japan. The efficacy has been proved to be significant in clinical trials for many years. Many experimental studies have shown that it can spread to many organs, such as lung, liver, myocardium, intestine, kidney, pancreas etc., and reduce the reperfusion injury of target organs through multiple ways [15-17]. The recent report demonstrated that edaravone can scavenge free radicals, reduce lipid peroxidation and subsequent infiltration of inflammatory cells, ensure the integrity of mitochondrial structure and function, inhibit mRNA expression of Bax gene relating with apoptosis, thereby improving skeletal muscle contractility [18-22]. Therefore, edaravone can play a protective effect to reperfusion injury from all aspects.

Although cryopreservation is economical and easy to operate, a variety of organ preservation solution and perfusion fluid also have a good extension of the replantation time, temporary ectopic foster and then re-implanted can save many amputated extremities, and hyperbaric oxygen (HBO) preservation of the limb is still in the experimental stage, but has also achieved a certain effect. Application of a single method may have more or less limitations, so possible combined usage of various methods or putting forward more innovative techniques for prevention and treatment of ischemia-reperfusion injury, will be the key to the success replantation and in urgent need of exploration.

In this study, porcine limbs were used as an animal model with different organ perfusion fluid at low temperature to observe the pathogenesis of the limbs' skin, fat, and muscle at different time points. The best technique for successful replantation was decided through pathological observation, immunohistochemical staining (IHC) and ultrastructure examination, detection of biochemical markers, and superoxide dismutase in the above-mentioned composite tissues and exploration of the best preservation of the limbs for prolonged retention time.

Methods

Ethics statement

All experimental animals in current study were conducted in compliance with the Guide for the Care and Use of Laboratory Animals of People's Liberation Army General Hospital. The study

was approved by the local animal experimentation committee of People's Liberation Army General Hospital.

Grouping criteria

Fifteen healthy Bama miniature pigs (25±5.5 kg) having same birth date and weight from the same source and irrespective of gender were included in this study. Any animal which had recently suffered from zoonotic diseases or swine infectious diseases or had abnormal vascular development were excluded from the study. Hind limbs of 30 healthy Bama miniature was randomly divided into five groups based on the methods of extracorporeal perfusion. The five groups were categorized as follows: Low temperature and drying group as control group (cut-off limbs wrapped by the wet cloth, and kept at 4°C for 6 hours); Saline perfusion group (1000 mL saline solution added to perfusion system); UW solution perfusion group (1000 mL UW solution added to perfusion system); Edaravone and saline perfusion group (30 mg edaravone and 1000 ml saline added into the perfusion system); UW solution and edaravone perfusion group (30 mg edaravone mixed with 1000 ml UW solution added to the perfusion system).

Surgical procedure

All animals underwent premedication and anesthesia with ketamine (20 mg/kg) and xylazine (2 mg/kg) by intramuscular injection, followed by intravenous midazolam (0.5 mg/kg) and atropine (0.05 mg/kg) under mechanical ventilation (O_2/air 1:3, isoflurane 1-1.5 volume %). Through a semicircular incision at bilateral groin, subcutaneous tissues and the neurovascular bundle were laid open and then the femoral artery and vein were separated and cannulated with 10 French cannulas. The average of 400 ml arterial blood was collected into transfusion bags to which 10000 IU of heparin were added and stored at 4°C. Both limbs were then amputated, and the animals were subsequently sacrificed by intravenous injection of 10 mL 20% potassium chloride. The amputated limbs were then weighed and assigned to different groups. The control group was not perfused and was wrapped by the wet cloth to reduce the exposed area and avoid excessive evaporation. These limbs were kept at 4°C for 6 hours and then directly kept at 4°C.

Perfusion protocol

At first 250 mL of synthetic colloidal hydroxyethyl starch solution (HAES; Voluven, Fresenius, Bad Homburg, Germany) was primed into the extracorporeal circuit and perfused into the amputated limb during the first 5 minutes via the cannulated artery in order to wash out metabolic products. Thereafter, HAES was replaced by the heparinized autologous blood accumulated in the extremity. The perfusion was performed in a standardized manner with a low flow rate of 100-150 mL/min (MEDOS DataStream blood pump, model DP2; Medos Medizintechnik AG, Stolberg, Germany), perfusion pressures of 18 cm H_2O, temperature at 4°C (Heater-Cooler Unit HCU30; Maquet GmbH & Co KG, Rastatt, Germany) and O_2 at 21% by membrane oxygenation (MEDOS Hilite 800 LT; Medos Medizintechnik AG, Stolberg, Germany). Capillary blood running freely from the open wound surface was collected into a sterile reservoir bag and then drained back into the perfusion system, complementally reflowed via the cannulated veins. Then 1000 ml saline, 1000 ml UW Solution, 1000 ml saline mixed with 30 mg edaravone, and 1000 ml UW solution mixed with 30 mg edaravone were added to perfusion system for group 2, 3, 4 and 5 respectively.

Detection method

A small amount of muscle tissues (around 200 mg) were collected at 3 cm above the knee joints at 2, 4, and 6 hours after the start of perfusion both in perfusion group and control group. First, muscle tissues were washed with saline and homogenized with 5 ml saline by Tissue Lyzer (Qiagen GmbH, Hilden, Germany) then centrifuged at 3500 rpm for 1 minute. Supernatant was used for the following detection.

Biochemical indexes including alkaline phosphatase (ALP), alanine aminotransferase (ALT), and glucose (GLU) were detected by automatic biochemical analyzer (Chemistry analyzer 7170; Hitachi, Japan). Superoxide dismutase (SOD) was detected by detection kit (SOD detection kit, A001-2; Nanjing Jianchen, China) and spectrophotometer (Ultraviolet-visible spectrophotometer UV2600/2700; Shimadzu, Japan. Glutathione peroxidase (GSH-Px) was detected with detection kit (GSH-Px detection kit, A005; Nanjing Jianchen, China) and spectrophotome-

Table 1. Content of indicators in effluent collected at different time points after amputation ($\bar{X}$±SD)

Indicator	Collection time	Control	4°C + drying	4°C+ UW	4°C + edaravone	4°C + UW + edaravone
ALP (U/L)	2 h	3.50±1.03	3.04±1.39	2.35±0.92	3.22±1.40	2.41±0.57
	4 h	4.07±0.73	3.73±1.27	2.50±1.12	3.53±1.26	2.64±0.84
	6 h	4.42±1.21	4.62±1.8	3.24±0.63	4.30±1.35	3.19±0.98
ALT (U/L)	2 h	382.38±45.69	375.30±32.79	384.54±38.34	370.68±47.18	337.25±19.56
	4 h	402.15±67.53	392.28±34.15	355.00±34.51	409.82±35.67	360.06±47.63
	6 h	461.27±29.01	424.78±23.79	391.30±23.28	430.34±18.55	373.79±28.91
GLU (mmol/L)	2 h	0.30±0.06	0.35±0.06	0.36±0.11	0.33±0.05	0.37±0.08
	4 h	0.25±0.05	0.24±0.03	0.30±0.12	0.26±0.04	0.33±0.07
	6 h	0.22±0.03	0.20±0.07	0.26±0.08	0.21±0.12	0.25±0.04

ter Ultraviolet-visible spectrophotometer UV-2600/2700; Shimadzu, Japan).

Pathological observation

Muscle tissues with size of 2 cm*2 cm*0.2 cm were collected at 3 cm above the knee joints for observing pathological changes at 2, 4, and 6 hours after initiation of ECC (Extracorporeal circulation). Tissues fixed in 4% buffered formaldehyde solution were used for paraffin embedding and histologic evaluation (hematoxylin-eosin staining). The light microscope (DM100, LEICA, Germany) was used to observe histopathological changes.

Immunohistochemical staining

In each group of 2 paraffin sections were performed the immunohistochemically staining of Bax and bcl-2 proteins. First, paraffin sections were placed in 58°C oven until the tissue wax melted, then put slides into xylene for 10 minutes for three times, absolute ethanol for 5 minutes for two times, then 95% ethanol, 90% ethanol, 80% ethanol, 70% ethanol each time for 5 minutes, and washed with distilled water for three mins for two times. Antigen retrieval process was performed in citrate buffer solution (pH:6.0) two times first 6 minutes, later 4 minutes boiled in microwave oven at 700 W. They were allowed to cool to room temperature and washed in PBS (phosphate buffer saline; pH-7.2) for 5 minutes three times. Endogenous peroxidase was removed in 5% Hydrogen peroxide for 20 minutes and the slides were washed with PBS for 5 mins three times. Non-immunized goat serum was applied for 10 minutes prior to the application of primary antibody. Rabbit anti-porcine polyclonal Bax or Bcl-2 antibody (ABCAM, US) was diluted with PBS to be 1:50, and incubated under 37°C for 1 hour. Horseradish enzyme-labeled goat anti-rabbit secondary antibody (Solarbio, China) was applied for 30 minutes under room temperature. Slides then were added with Diaminobenzidine (DAB, Invitrogen, Carlsbad, CA) as a chromogen for 5 minutes and then washed with distilled water. Control slides were also prepared as mentioned above except the primary antibodies. After counterstaining with Hematoxylin, washing in tap water for 5-10 minutes and in distilled water for 2 x 4 minutes, the slides were mounted. Each slice was observed in the 200 × microscope (DM100, LEICA, Germany) and five fields with the same brightness were randomly selected to take pictures. Image-pro Plus 6.0 image analysis software was used to analyze the photos.

Ultrastructural observation

Gastrocnemius muscle tissues with same distance (3 cm) from the knee joint were collected at 6 hours after the starting of the perfusion. After washing with saline solution repeatedly, tissues were fixed by 2.5% glutaraldehyde solution at 4°C for 3 hours and were washed three times with PBS solution for 45 minutes each. Afterwards 1% osmium acid solution were used for fixing, tissues were dehydrated with acetone and ethanol by gradually increasing their concentration and concluded by embedding in epoxy resin. After staining with saturated uranium acetate and lead citrate, ultra-thin slices were prepared and observed for ultrastructural changes under electron microscopy (H-9500, Hitachi, Japan).

Statistical Analysis

The biochemical indexes were analyzed using Repeated Measures Analysis of Variance (Gr-

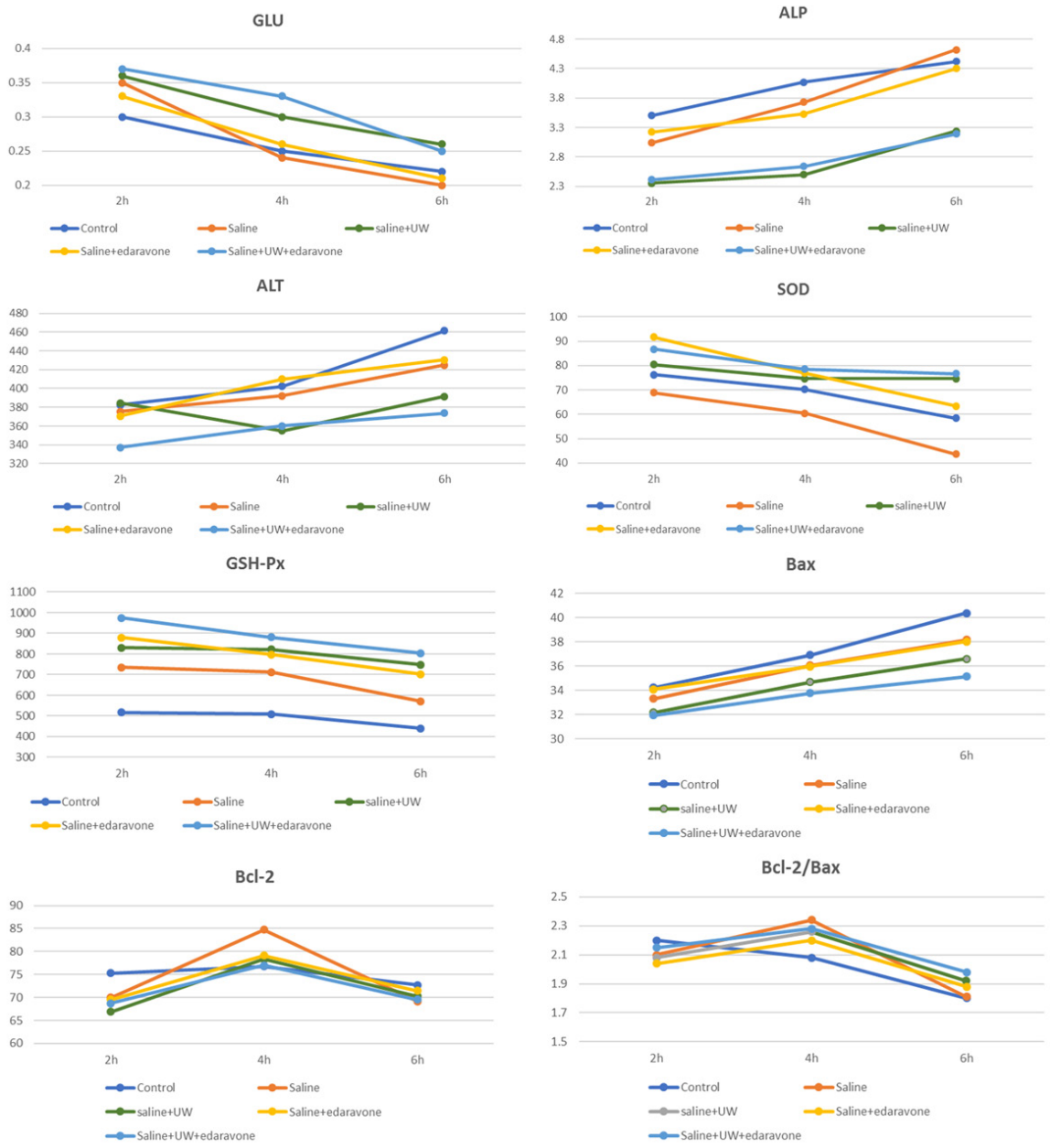

Figure 1. Trends of different indicators in effluent collected at different time points after amputation.

eenhouse-Geisser test), followed by Mauchly's W testing. *P* values<0.05 were considered significant. The analysis was performed using SPSS 17.0 software.

Results

Detection results

According to the statistical analysis, the content of ALT and ALU in UW perfusion group and UW mixed with edaravone perfusion group were lower than other three groups (P<0.05), while GLU were significantly higher in these two groups than other three groups (P<0.05). The content of ALT, ALU, and GLU in control groups were significantly different from other groups (P<0.05). But there was no statistically significant difference in the content of three biochemical indexes between saline and edaravone perfusion group, UW perfusion group as well as UW and edaravone perfusion group (**Table 1**). According to the **Figure 1**, ALT and ALP increased after amputation among all groups, while the content of GLU decreased in

Table 2. The Enzyme activity test results at different time points after amputation ($\bar{X}$±SD)

Indicator	Collection time	Control	4°C + drying	4°C + UW	4°C + edaravone	4°C + UW + edaravone
SOD (U/mgprot)	2 h	76.22±9.25	68.91±14.80	80.47±16.70	91.72±7.56	86.78±9.32
	4 h	70.25±13.18	60.49±9.65	74.65±26.13	76.97±18.90	78.60±15.70
	6 h	58.42±15.37	43.70±8.58	74.60±14.60	63.43±18.85	76.64±14.71
GSH-Px (U/mgprot)	2 h	516.89±155.60	734.30±83.61	830.06±201.02	880.17±246.50	975.34±223.93
	4 h	509.47±213.19	711.54±212.46	821.68±211.61	797.79±222.71	880.35±227.11
	6 h	440.55±137.69	570.62±182.13	748.39±234.52	702.26±215.03	804.00±164.53

Table 3. The IOD staining results of immunohistochemistry at different time points after amputation ($\bar{X}$±SD)

Indicator	Collection time	Control	4°C + drying	4°C + UW	4°C + edaravone	4°C + UW + edaravone
Bax	2 h	34.22±11.72	33.31±5.12	32.17±3.21	34.09±5.27	31.96±6.20
	4 h	36.91±16.20	36.07±4.39	34.67±6.25	35.98±7.33	33.77±7.92
	6 h	40.38±13.42	38.19±11.72	36.60±13.35	38.02±9.90	35.13±9.34
Bcl-2	2 h	75.28±8.24	69.96±5.88	66.91±1.36	69.55±4.36	68.71±2.76
	4 h	76.77±12.26	84.76±6.45	78.35±5.42	79.16±1.53	77.00±9.56
	6 h	72.68±9.05	69.19±4.58	70.27±2.17	71.48±7.56	69.56±5.74
Bcl-2/Bax	2 h	2.20±0.12	2.10±0.27	2.08±0.07	2.04±0.18	2.15±0.47
	4 h	2.08±0.24	2.34±0.24	2.26±0.25	2.20±0.11	2.28±0.13
	6 h	1.80±0.492	1.81±0.37	1.92±0.45	1.88±0.36	1.98±0.07

each group with the passing time after amputation.

The activity of SOD was higher in edaravone perfusion group, UW and edaravone perfusion group compared with control group having low temperature with drying group ($P<0.05$). The activity of GSHPx was lower in the control group than other groups ($P<0.05$) (**Table 2**). The activity of SOD and GSHPx in each group decreased with increasing time after amputation (**Figure 1**).

Immunohistochemical staining results

With the passing time after amputation, IOD value of BOD protein in each group gradually increased, while IOD value of Bcl-2 protein and Bcl-2/BaxIOD ratio first increased and then decreased (**Figure 1**). IOD value of Bax protein were higher than UW perfusion group, and UW mixed with edaravone group ($P<0.05$). IOD value of Bcl-2 protein were significantly different in saline group compared with other groups. Bcl-2/BaxIOD ratio were also significantly different between the control group and other groups, the saline group and other groups (**Table 3**, Supplement Figure 1).

Histopathological observation by the light microscope

After 2 hours of amputation, muscle fibers were clear in all groups except slightly vague in control group. All groups had no obvious edema and the muscle space were slightly widen and nucleus were uniform and no significant sarcoplasmic coagulation (**Figure 2**).

After 4 hours of amputation, muscle fibers had developed mild edema and the arrangement were uneven and muscle space were slightly widened in all groups. Additionally, the stripes of muscle cells appeared to be dissolved slightly, and small muscle fibers fractured except the UW perfusion as well as UW and edaravone group (**Figure 2**).

At 6 hours after amputation, the muscle fibers had obvious edema, and more muscle fibers fractured, and the arrangement of fibers were obviously disordered. However, in UW perfusion and UW mixed with edaravone group, the muscle fibers in UW and edaravone group were less injured only with slightly muscle fibers swollen, no dissolved muscle cells and no sarcoplasm solidification (**Figure 2**).

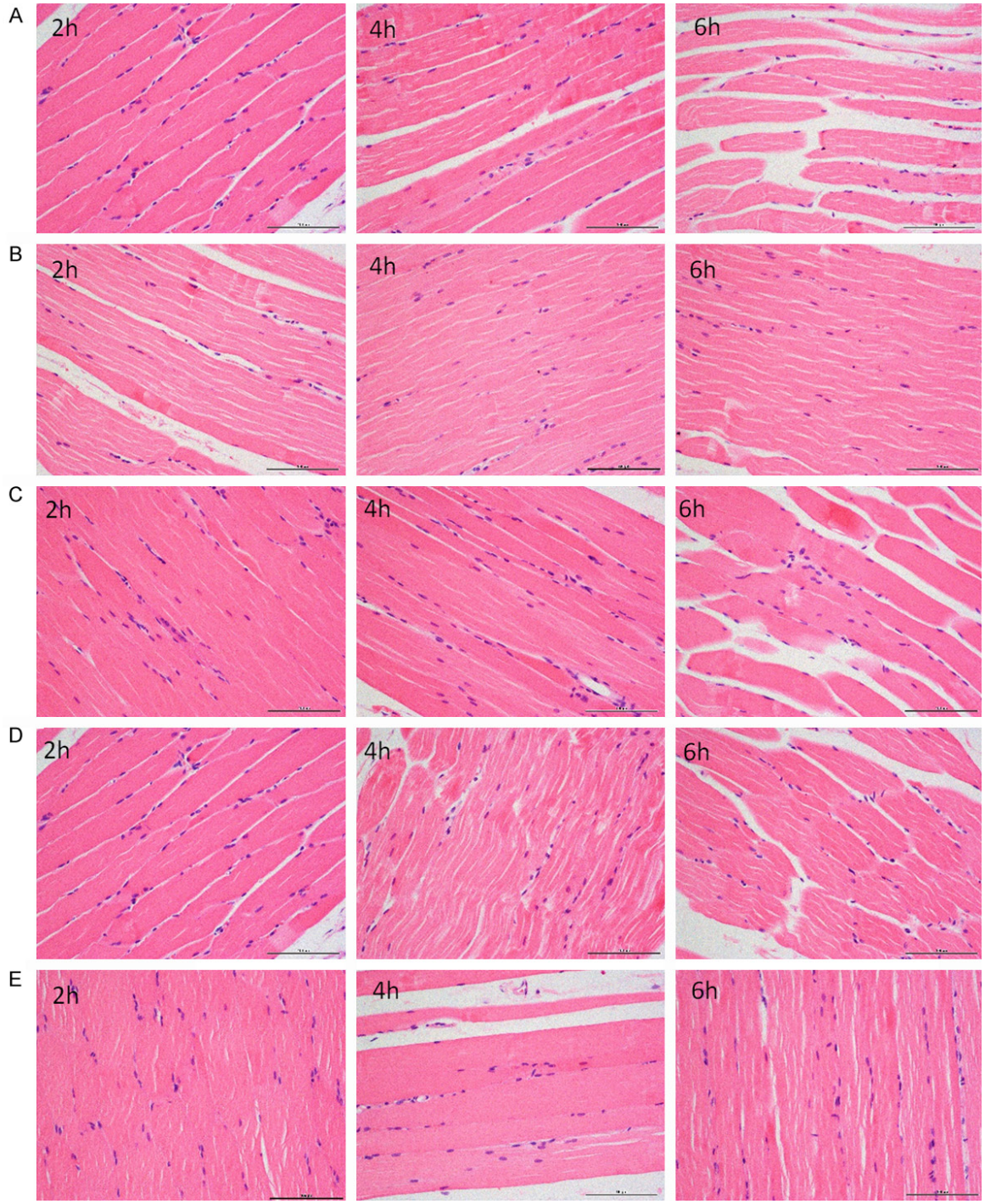

Figure 2. Histological observation (× 200) by the light microscope at different time points after amputation. (A) Control; (B) Saline; (C) saline + UW; (D) Saline + edaravone; (E) Saline + UW + edaravone.

Ultrastructural observation by electron microscopy

In the UW and edaravone perfusion group, muscle fibers observed in **Figure 3** arranged more normally, with no obvious muscle fiber breakage, less mitochondria swelling, less vacuolization and more glycogen granules than other groups. The group added with edaravone had a more complete mitochondrial structure and less vacuolization than only the UW perfusion group. Saline group as well as saline and

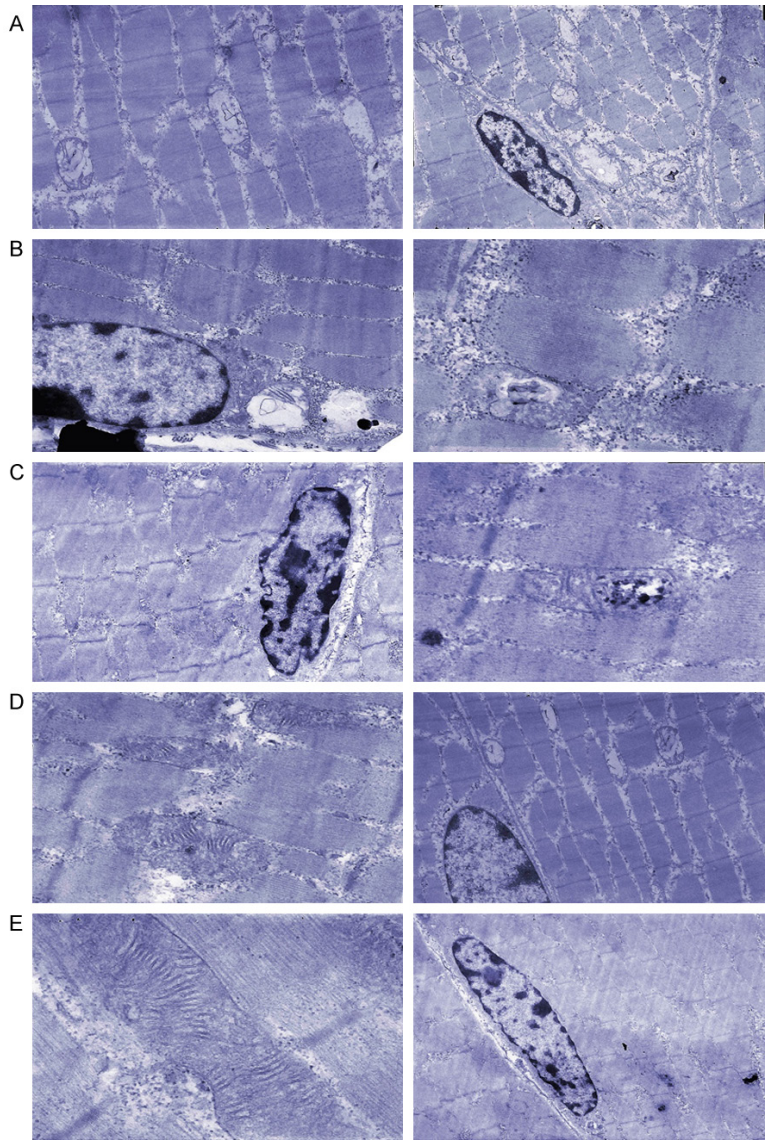

Figure 3. Ultrastructural observation (× 1000) by electron microscopy at 6 h after amputation. (A) Control; (B) Saline; (C) saline + UW; (D) Saline + edaravone; (E) Saline + UW + edaravone.

edaravone group perfusion group have less injury than the control group.

Discussion

In this study, the porcine model was used as the experimental object, which had more resemblance with human limb size, physiology, and anatomy, allowing for an appropriate clinical comparison of the investigated variables with the ones found in humans. Furthermore, using the full disengaged limbs as model, the obtained experimental result is more reliable than the previous research using aortic clipping method. As shown in the results, the biochemical indexes indicated the necrosis of tissue changed constantly with the increasing of time, and the frequency of changed values in the UW and edaravone perfusion group was significantly different from other groups. It was evident from the study of histopathological characteristics of tissue under light microscope, IHC, and EM that, UW and edaravone perfusion group were less severely damaged when compared to other groups.

With the increasing of *in vitro* time, a large number of bioactive enzymes were released into the blood stream because skeletal ischemia caused damage to muscle cells and cell membrane rupture, therefore ALT and ALU had shown an increase in their value. The content of ALT is mainly high in muscle cells and ALU is mainly distribute in the basement membrane of capillary endothelial cells [23]. At the early stage of ischemic injury, the level of ALT and ALP increases sharply for a certain period and then falls gradually after reaching the peak [23]. So the extent of ischemic injury in muscle cells of isolated limb can be determined by measuring the level of ALT and ALP. GLU is an important factor of carbohydrate metabolism and energy supply. When the lower limbs began to develop ischemic injury, the normal oxidative pathway gets hindered, and the yield of ATP for direct energy supply declines [24]. Large amounts of creatine phosphate converses to supply energy, and the glycolytic pathway unilaterally strengthens, resulting in declining of GLU significantly [24]. The detection results of biochemical indexes showed that ALT and ALP represented of the muscle cell damage gradually increased, while GLU, which represents energy metabolism, was gradually declining as time went by. In the control group

ALT and ALP were significantly higher than other groups and GLU decreased to the lowest level among all groups, which revealed that cell damage and energy loss in this group were most serious, and had high rate of anaerobic metabolism. ALP and ALT in UW perfusion group and UW mixed with the edaravone perfusion group were significantly lower than other three groups, while GLU was higher than other groups. However, there are no significantly different between UW perfusion and UW mixed with the edaravone perfusion group, as well as the saline perfusion group and saline mixed with the edaravone perfusion group. The possible reason was that edaravone, as a free radical scavenger, has a less protective effect on cell direct injury caused by ischemia and hypoxia.

SOD (superoxide dismutase) is widely present in the cytoplasm, nucleus and lysosomes of mammalian cells. Under normal conditions, there is a dynamic equilibrium formed between its formation and elimination with good enzyme activity. When the body is damaged or oxidative stress occurs, the activity of SOD increase, which catalyzes the disproportionation of O_2- (apoptosis of skeletal muscle cells by oxidized low-density lipoprotein) to H_2O_2 and O_2, and protects the skeletal muscle tissue [25]. However, when the limbs are amputated and lack of blood supply, free radicals are accumulated and cannot be cleared on time, then SOD are self-consumed and its activity decrease [25]. Therefore, the activity of SOD can reflect the amount of oxygen free radical generated by the body and whether SOD is directly self-damaged or consumed because of hypoxia or hypoxia [26]. GSH-Px (glutathione peroxidase) is a metalloenzyme antioxidant enzyme, which main function is acted as catalyst to prompt the reduction reaction of glutathione hydrogen and oxidation, block lipid peroxidation chain-lock reaction, and remove a variety of biological macromolecular peroxides, including almost all organic hydroperoxides, especially lipoperoxides. This enzyme plays an important role in protecting the membrane structure of cells and various biological macromolecules [27] 1 mg. Thus, GSH-Px, SOD can catalyze superoxide radicals to induce disproportionation reaction, protecting the cell membrane from damage by free radicals' attack, and their activity can reflect the body's antioxidant capacity of the enzyme system [28]. In the present study, the activity of SOD and GSH-Px gradually decreased as time passed, and the UW and edaravone perfusion group showed the highest activity of SOD and GSH-Px in all groups. It was indicated that edaravone took roles in protecting and enhancing SOD activity, inhibiting lipid peroxidation and generating free radicals, and providing electrons to directly scavenge hydroxyl radicals.

Amputated limb, the skeletal muscle will occur degeneration and necrosis over time, if the replantation too late, a large number of the toxin material will enter the blood circulation, affecting the patient's body and other organs and tissues. Many clinical trials have confirmed that the release of a large amount of myoglobin after rhabdomyolysis will block the renal tubules, resulting in acute renal failure or even uremia [29]. Myocyte necrosis will release toxic substances into the bloodstream and can cause systemic infections and even shock [29]. Therefore, the degree of muscle cell apoptosis, degeneration, and necrosis are important indications of the replantation. It was believed that apoptosis is a major form of skeletal muscle ischemia and hypoxia injury and ischemia-reperfusion injury [29]. Apoptosis has a very complex regulatory mechanism and is a programmed process with many genes involved, among which the Bcl-2 gene family is currently recognized to be closely related. Bcl-2 gene, called "anti-apoptotic gene" as a member of the family, promote cell survival and prolong cell life [30]. The Bax gene is a pro-apoptotic gene in the Bcl-2 gene family. It was recently reported that Bax plays a role by forming a dimer with Bcl-2 protein. When Bax is overexpressed, Bax/Bax homodimers will be formed to promote cell apoptosis. When Bcl-2 is overexpressed, Bcl-2/Bax will be generated and then Bax pro-apoptotic effects will be diminished or even disappeared, and apoptosis was inhibited. Therefore, the ratio of Bcl-2 to Bax is a key determinant of apoptosis [30]. In current study, with the extension of the amputation time, the IOD value of Bax protein gradually increased, while the IOD value of Bcl-2 protein and IOD ratio of Bcl-2/Bax first increased and then decreased. The control group had the highest IOD value of Bax protein while the UW mixed with edaravone perfusion group had the lowest. Additionally, for Bcl-2, saline perfusion showed highest value. It has been shown that

apoptosis increased gradually with prolongation of time regardless of the method of preservation, and the number of apoptotic cells in the control group was always the highest at all time points. Both UW solution and edaravone inhibited to a certain extent apoptosis. Saline infusion, may be due to the fast cell swelling, stimulated the expression of Bcl-2 apoptotic gene which can inhibit apoptosis. Moreover, we conclude that the IOD value of Bcl-2 and the IOD ratio of Bcl-2/Bax first increased and then decreased, because many factors that induced apoptosis in the early stage of ischemia, the Bcl-2 gene were over-expressed of in to prevent the cells from apoptosis. However, with prolongation of the ischemic time, more and more free radicals were constantly produced, the cell injury was aggravated, and eventually the expression of Bcl-2 was inhibited.

According to the H&E staining results, the histological changes in both UW perfusion group and UW mixed with edaravone group were the least changed, while the control group changed most. According to the observation of ultramicrostructure at the end of storage, the tissue damage was the lightest in UW perfusion group and UW mixed with edaravone group, and the damage in saline group and saline and edaravone group was lighter than control group. We conclude that because of the high osmotic pressure of UW solution and its strong viscidity, its ability to maintain the cell morphology was stronger than saline. However, the wider interstitial space of muscle fiber than saline group during late period may be related to the cell swelling in saline group.

Based on the findings of the above experimental results, it can be concluded that the protective effect of amputated limbs preservation at 4°C was obviously better than the traditional low temperature drying method. Furthermore, UW solution had better preservation effect than that of saline solution. Edaravone can further reduce the occurrence of apoptosis and ischemia-reperfusion injury, protect the activity of antioxidant enzymes, and better maintain the functional integrity of replanted limbs.

The preservation method through perfusion in porcine model in present study needs to be further validated by the success of subsequent replantation through the detection of circulating indexes after the replantation, observation of pathological changes, and neurophysiological tests. In addition, combined usage with some other perfusion solution needed to be further explored to determine whether they can improve the conditions for the perfused limb and extend the time of limb preservation.

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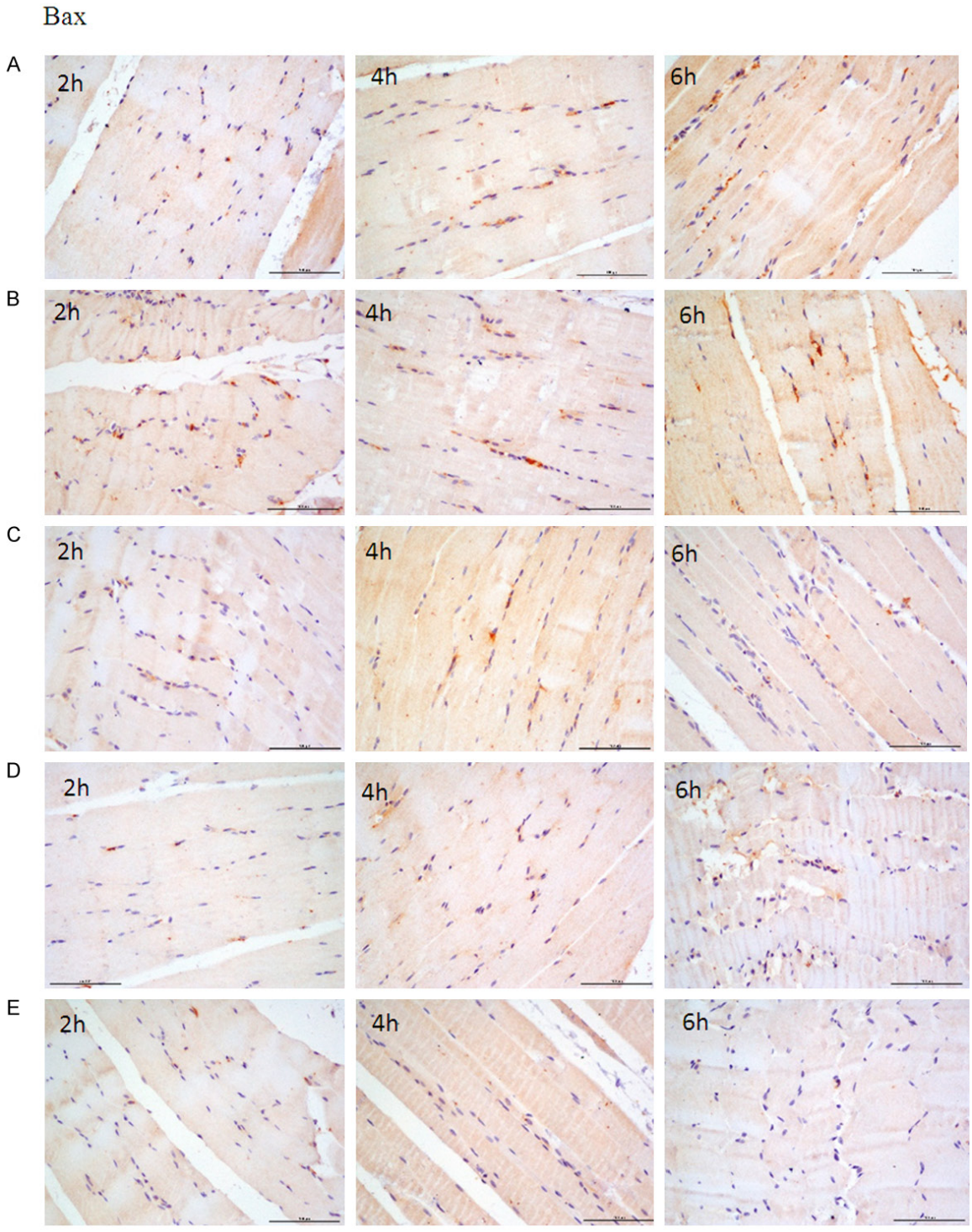
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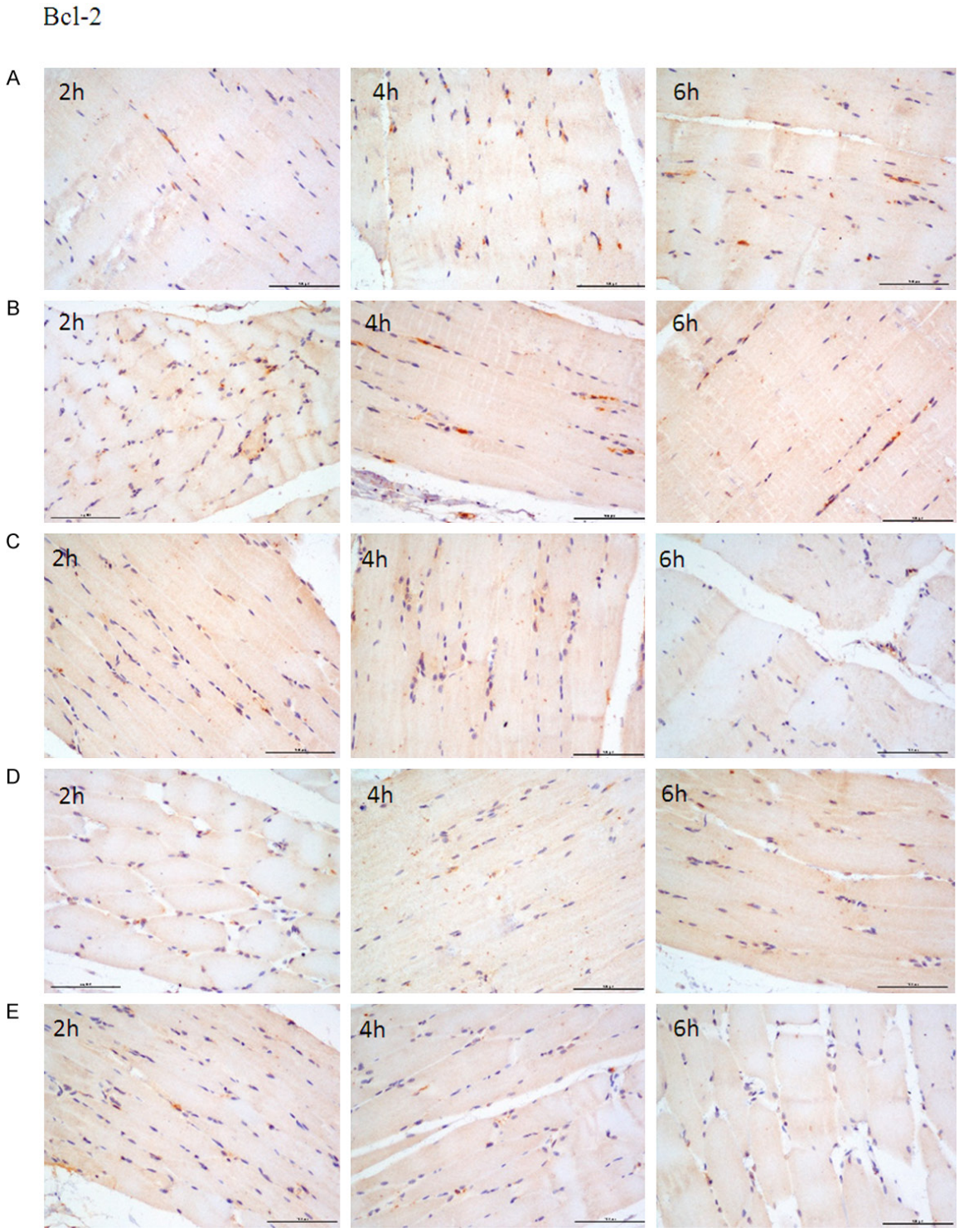

Supplement Figure 1. Immunohistochemical staining (200 ×), nuclei were stained blue, deposited brown particles were set as a positive area. (A) Control; (B) Saline; (C) saline + UW; (D) Saline + edaravone; (E) Saline + UW + edaravone.