Study of Heparin in Intestinal Ischemia and Reperfusion in Rats: Morphologic and Functional Evaluation

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ABSTRACT

To study whether treatment with heparin (HEP) attenuates intestinal dysfunction caused by ischemia (I) and reperfusion (R), rats were treated with HEP (100 U/kg intravenously) or saline solution (SS) before I (60 min), which was produced by occlusion of the superior mesenteric artery, and R (120 min). After I or I/R, we mounted 2-cm jejunal segment in an organ bath to study neurogenic contractions stimulated by electrical pulses or KCl, using a digital recording system. Thin jejunal slices were stained with hematoxylin and eosin for optical microscopy. Compared with the sham group, jejunal contractions were similar in the I + HEP and the I/R + HEP groups, but reduced in the I + SS and the I/R + SS groups. The jejunal enteric nerves were damaged in the I + SS and the I/R + SS, but not in the I + HEP and the I/R + HEP cohorts. These results suggested that HEP attenuated intestinal dysfunction caused by I and I/R.

Intestinal ischemia (I) and reperfusion (R) injuries occur in a variety of surgical practices, such as transplantation, and critical illnesses, such as neonatal necrotizing enterocolitis.[1] The I and R injuries can lead to hypovolemia, hypotension, hypoxia, and sepsis.[1–3] They dramatically compromise motor and secretory functions of the intestine owing to cellular lesions caused by deprivation of oxygen and nutrients.[1,2] In addition, vessels that suffer R after I can experience many cellular lesions and death mainly owing to lipid peroxidation of cell membranes caused by accumulation of free oxygen radicals and other cytotoxic substances.[2,3] In response to ischemia, cells undergo changes in enzyme activities, mitochondrial functions, cytoskeletal structures, membrane transport, and antioxidant defenses, which collectively predispose to reoxygenation injury.[2,3] The morphologic and functional injuries after I and R remain a major challenge for patients undergoing transplantation or major surgery.

The motor functions of mammalian intestines are regulated by several excitatory and inhibitory transmitters which are released from enteric nerves, including acetylcholine, 5′-triphosphate-adenosine (ATP), nitric oxide, neuropeptides, and other substances.[4] The actions of these transmitters are highly dependent on the integrity of the enteric nerves.[5] However, intestinal motor activity is severely compromised in I/R mainly owing to a loss of structural and functional integrity of the enteric nerves.[5,6]

Several drugs have been proposed to attenuate or prevent the structural and functional lesions caused by I and R.[7–12] Compounds such as heparin (HEP) and its derivates may alter the activity of superoxide smutase (SOD), the content of malondialdehyde (MDA), and the intracellular Ca^{2+} concentration. Some studies have suggested that HEP and its derivatives, that are currently used as anticoagulant and antithrombotic agents, act as free radical scavengers and inhibitors of apoptosis in several cell types, protecting them from I/R lesions.[17–22] Oxygen-derived free radicals may be among the most important factors involved in the I/R process.[13,14] Because these radicals are highly reactive, they immediately affect cellular membranes, which consist of polyunsaturated fatty acids, leading to lipid peroxidation and generating potentially cytotoxic products.[13,14] Various pharmacologic agents, including allopuri-

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nol ascorbic acid, have been evaluated to reduce free radical levels in the ischemic process.[15,16]

Therefore, in the present work we investigated the effects of HEP on the motility and histology of rat jejunal segments undergoing intestinal I and R in rats.

MATERIALS AND METHODS

Male Wistar EPM-1 rats (270–300 g) were anesthetized with ketamine (60 mg/kg) and xylazine (40 mg/kg), intravenously before occlusion of the superior mesenteric artery with a metallic clip for 60 minutes of ischemia (I) followed by blood recirculation (R) for 120 minutes after removal of the clip. Twelve rats underwent only I (I group) and 12 I and R (I/R group) in addition to a sham group (n = 6).

Among the I group, 6 rats were treated with saline solution 0.9% (SS) and 6 with HEP (100 mg/kgm) injected via the femoral vein 5 minutes before I. In the I/R group, 6 rats were treated with SS and 6 with HEP (100 mg/kg) injected via the femoral vein 5 minute before 1 and 5 minutes before R as well as 55 minutes after R.

After I or I/R, the rats were killed to obtain jejunal segments (2 cm) that were isolated, washed, cleared of surrounding tissues, and mounted under 1 g tension at 37°C in an organ bath containing 10 mL aerated nutrient solution of composition (mmol/L) NaCl 138, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15, and dextrose 5.5 (pH 7.4). We studied neurogenic contractions induced by electrical field stimulation (EFS) or by the depolarizing agent KCl (70 mmol/L) with the use of a digital recording system.[6–8] EFS (5 and 30 Hz, 1 ms duration, 60 V) was performed by means of platinum electrodes connected to an S88 electrical stimulator (Grass, USA).[6–8] Responses to EFS and KCl were recorded by force-displacement transducers connected via a bridge amplifier to an analog/digital recording system (AD Instruments, USA). Data on contractile responses were subjected to statistical analysis using 1-way analysis of variance and Student *t* test.[7,8]

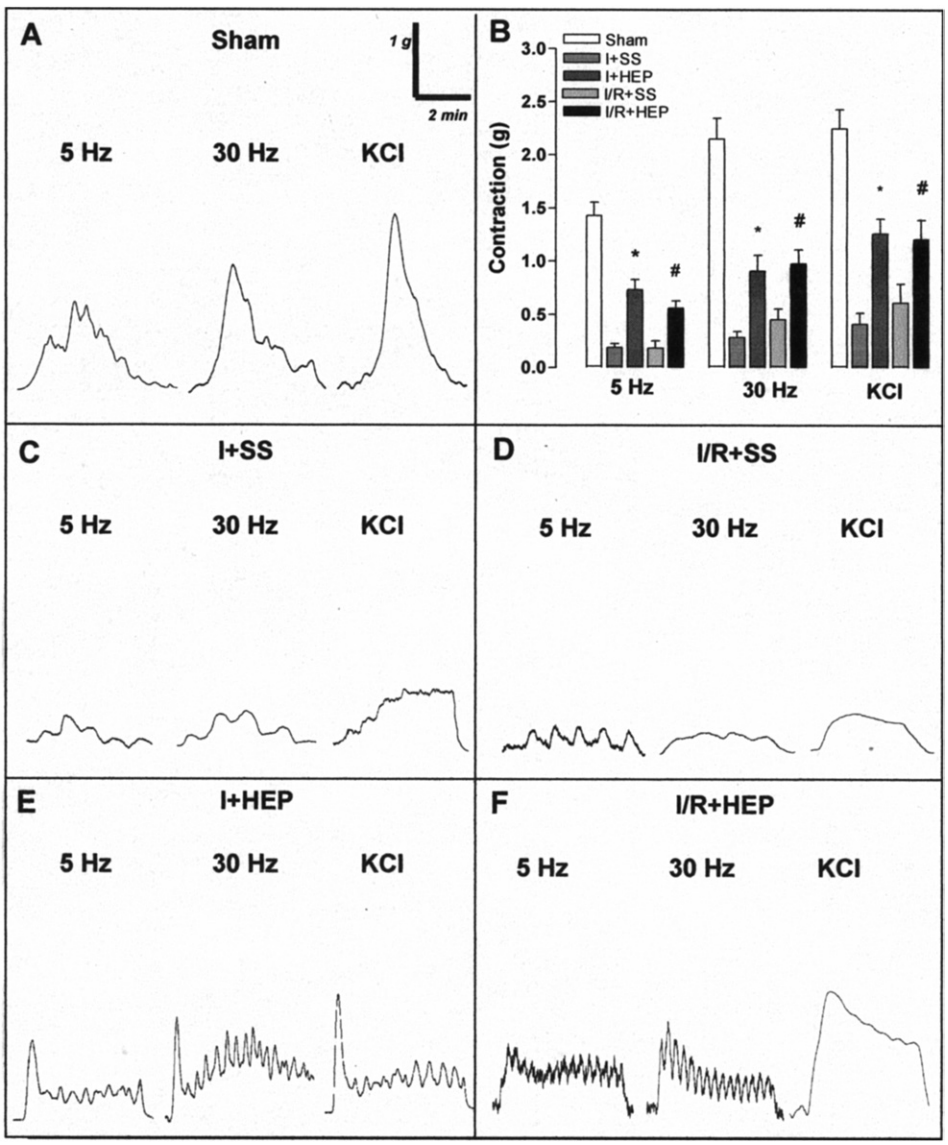

Fig 1. Typical records of neurogenic contractions induced by electrical field stimulation (5 and 30 Hz) or KCl (70 mmol/L) in jejunal segments of rats treated with HEP or SS and submitted to I or I/R. *Statistically different from I + SS ($P < .05$; $n = 6$); #Statistically different from I/R + SS ($P < .05$; $n = 6$). HEP, heparin; SS, saline solution; I, ischemia; R, reperfusion.

Table 1. Values of Amplitude of Neurogenic Contractions (expressed in grams of tension) Induced by EFS (5 and 30 Hz) or KCl (70 mmol/L) in Jejuna of Rats Treated with HEP or SS and Submitted to Intestinal I or I/R

Groups	5 Hz	30 Hz	KCl
Sham	1.43 ± 0.12	2.15 ± 0.19	2.25 ± 0.18
I + SS	0.18 ± 0.04$^{\Delta}$	0.28 ± 0.05$^{\Delta}$	0.41 ± 0.10$^{\Delta}$
I + HEP	0.73 ± 0.10*	0.91 ± 0.15*	1.26 ± 0.14*
I/R + SS	0.18 ± 0.06	0.45 ± 0.10	0.61 ± 0.17
I/R + HEP	0.56 ± 0.07$^{\#}$	0.98 ± 0.13$^{\#}$	1.21 ± 0.18$^{\#}$

Data presented as mean ± SEM (n = 6).
$^{\Delta}$Statistically different from sham ($P < .05$).
*Statistically different from I + SS ($P < .05$).
$^{\#}$Statistically different from I/R + SS ($P < .05$).

We also performed histologic analyses using optical microscopy of jejunal pieces embedded in paraffin, cut into thin slices, and stained with hematoxylin and eosin.

RESULTS

Figure 1 shows that EFS (5 and 30 Hz) and KCl (70 mmol/L) produced contractile responses in all jejunal segments: sham, I + SS, I + HEP, I/R + SS, I/R + HEP. However, the amplitude of these contractions was similar in I + HEP and IR + HEP groups but reduced in I + SS and I/R + SS groups compared with the sham group (Fig 1; Table 1).

Histologic analysis showed a loss of structural integrity of enteric nerves in the jejunal segments of I/R + SS group (Fig 2B), but not in I/R + HEP (Fig 2C).

DISCUSSION

The complex mechanisms involving cellular death after I/R insults are not fully understood. Significant increases in tissues exposed to I/R have been observed in the content of reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions, and hydrogen peroxide, associated with decreased antioxidant enzyme activities. ROS play a major role in the pathophysiology of the ischemic injury via oxidative damage to membrane lipids and proteins.[15,16]

SOD represents the first line of defense against oxidative stress, by catalyzing the dismutation reaction of superoxide anion to hydrogen peroxide.[17] MDA, one of the major products of lipid peroxidation, has been extensively measured as an index of lipid peroxidation.[18] Calcium is an important second messenger in signal transduction and neurotransmitter release. A sustained increase of Ca^{2+} destabilizes the neuronal cytoarchitecture, resulting in cell damage and eventual death. Ca^{2+} enters neurons via NMDA- and AMPA-receptor–operated channels, voltage-gated Ca^{2+} channels, store-operated channels, and the reverse operation of the Na^{+}/Ca^{2+} exchanger. Additionally, release from the organelles, such as endoplasmic reticulum, mitochondria, and synaptic vesicles, as well as from Ca^{2+}-binding proteins further increase intracellular Ca^{2+} levels. Thrombin also induces Ca^{2+} transients and subsequent nitric oxide (NO) production in vascular endothelial cells. Thrombin cleaves protease-activated receptors resulting in activation of intracellular signals.[29]

Calcium plays a unique role in the pathophysiology of ischemia, because it causes several damaging events by activating a variety of Ca^{2+}-dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, calcium-dependent nitric oxide synthase, and calpain, as well as various proteases and endonucleases. As a result of the formation of cytotoxic products, such as free radicals and leukotrienes, excess intracellular Ca^{2+} triggers irreversible mitochondrial damage, inflammation, and necrotic or programmed cell death.[19]

HEP and its derivatives have long been proposed for stroke treatment. HEP may prevent venous thromboembolic complications, improve neurologic outcomes, reduce mortality, and prevent early recurrence. Unfortunately, only the first objective has been confirmed.[19,20] Low-molecular-weight HEP (LMWH), which is obtained by depolymerization of standard HEP, was designed to reduce the risk of hemorrhage. Compared with the parent compound, it shows substantial advantages owing to greater bioavailability, longer half-life, and reduced interaction with platelets, so it is supplanting

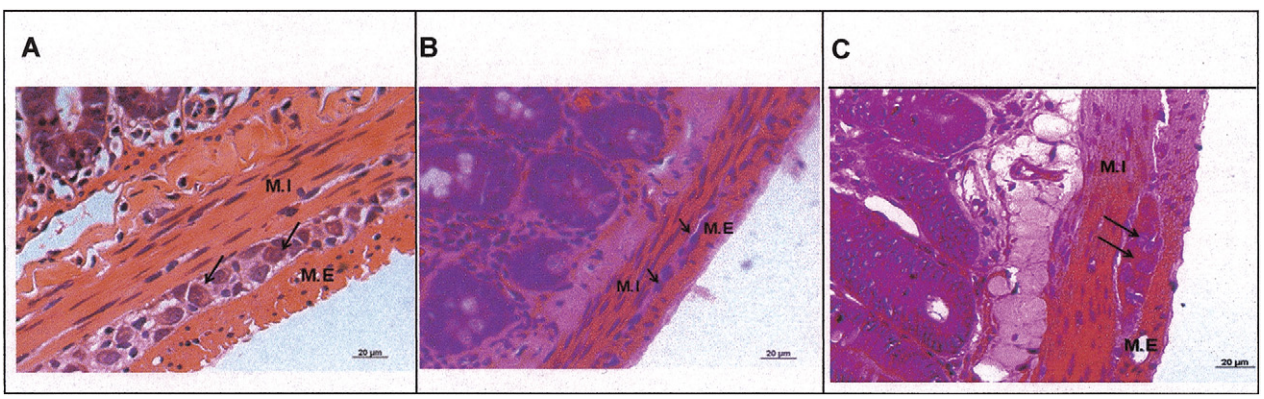

Fig 2. Histologic aspects of jejuna of sham rat. **(A)** without ischemia and/or reperfusion, and treated with **(B)** saline solution or **(C)** heparin andsubmitted to intestinal ischemia and/or reperfusion. The images show the longitudinal muscle (ME), circular muscle (MI), and enteric nerves (*arrows*). (Hematoxylin and eosin stain, ×400).

HEP for various clinical indications. Some LMWH, including nadroparin, danaparoid, certoparin, and tinzaparin, has been tested in the treatment of acute I/R lesions.[21–27] However, follow-up studies have failed to replicate the results. Ultra-LMWH confers a much lower risk of bleeding. It can penetrate the blood-brain barrier, so it may be a safe, effective drug for the treatment of acute ischemic strokes.

In the present study, we investigated the effect of HEP in an animal model of I/R using portal vein and hepatic artery occlusion. HEP attenuated hepatic lesions caused by I/R in rabbits. Studying hepatic I/R, Taha et al. (2009) observed HEP to produce protective effects on rabbits by controlling levels of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase. In addition, histologic analysis showed significant reductions in lesions caused by I/R among livers of HEP-treated rabbits.[28]

Celik et al. (2009) demonstrated that HEP reduced flap necrosis and improved survival owing to radical scavenging, antioxidant effects, and supportive activities on capillary permeability and transudation.[30] More recently, Medeiros et al (2001) showed that heparin also induced intracellular calcium release, activation of phospholipase C and of calcium calmodulin kinase II, and increased NO production,[31] protective factors on vessels during I and R.

In conclusion, HEP produced protection on jejunal segments that had suffered I/R lesions.

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