

Edaravone inhibits apoptosis caused by ischemia/reperfusion injury in a porcine hepatectomy model

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Author contributions: Kubota K made substantial contributions to conception and design, drafting the article and revising it critically for important intellectual content; Iwasaki Y analyzed the data; all authors approved the version to be published.

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Received: April 16, 2011 Revised: September 9, 2011

Accepted: May 12, 2012

Published online: July 21, 2012

Abstract

AIM: To investigate the effect of E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) on hepatic ischemia-reperfusion (I/R) injury and liver regeneration in a porcine hepatectomy model.

METHODS: One hour ischemia was induced by occluding the vessels and the bile duct of the right and median lobes. A 40% left hepatectomy was performed after reperfusion. Six animals received Edr (3 mg/kg per hour) intravenously and six control animals received saline just before reperfusion. Remnant liver volume, hemodynamics, aspartate aminotransferase (AST), alanine aminotransferase, lactate dehydrogenase and lactic acid, were compared between the groups. The expression of transforming growth factor- β (TGF- β 1) and toll-like receptor (TRL) mRNA in hepatic tissues was examined using reverse transcription polymerase chain reaction. Apoptosis was demonstrated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, respectively.

RESULTS: Serum AST ($P = 0.029$), and toll like receptor 4 level ($P = 0.043$) were significantly lower after 3 h

in animals receiving Edr. In addition, TUNEL staining in Edr-treated pigs showed significantly fewer hepatocytes undergoing apoptosis compared with control pigs. After 1 mo, all factors were non-significantly different between the two groups.

CONCLUSION: Edr is considered to reduce hepatic injury in the early stage of I/R injury in a porcine model.

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Key words: Edaravone; Ischemia-reperfusion injury; Liver resection; Transforming growth factor- β ; Toll like receptor 4

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Shimoda M, Iwasaki Y, Okada T, Kubota K. Edaravone inhibits apoptosis caused by ischemia/reperfusion injury in a porcine hepatectomy model. *World J Gastroenterol* 2012; 18(27): 3520-3526 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i27/3520.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i27.3520>

INTRODUCTION

A potent free radical scavenger, E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) has been shown to protect cardiomyocytes and brain against ischemia/reperfusion (I/R) injury^[1,2]. However, this beneficial protection after I/R injury in the liver, and the effect on liver regeneration is still unknown^[3].

Compression of the hepatoduodenal ligament is routinely used during partial hepatectomy for control of bleeding, but this can lead to postoperative I/R injury that may adversely affect outcome^[4]. Hepatic I/R damage is caused by multiple factors, including ischemia-induced

hypothermia, cytokines, coagulopathy and increased levels of cell adhesion molecules. Expression of transforming growth factor- β (TGF- β) has been demonstrated in a wide variety of diseases and in normal cells and tissues, and is known to be most abundant in platelets and bone^[5]. TGF- β 1 has inhibitory effects on cell growth, and acts in a stimulatory manner in fibrosis and certain mesenchymal cells^[6]. It is particularly germane in the liver, as TGF- β 1 is a potent inducer of apoptosis in hepatocytes, and inhibits liver cell proliferation *in vitro*^[6]. It also has a crucial role in terminating liver regeneration after partial hepatectomy in rats^[7].

Furthermore, Kupffer cells play a prominent role in I/R injury in the liver as this type of injury induces the release of high mobility group box 1 (HMGB1) from damaged liver cells, which then stimulate nonparenchymal cells, such as Kupffer cells through toll like receptor 4 (TLR4)^[8]. In addition, TLR4 triggers the secretion of tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 by Kupffer cells, resulting in liver regeneration^[8].

To explore the effect of Edr in liver I/R injury and to extend the application of Edr clinically, experiments using large animal models are essential. We have attempted to define the effects of Edr after I/R injury in a porcine liver resection model.

In this study, we investigated the effect of Edr on hepatic I/R injury and liver regeneration, and found that Edr significantly inhibits apoptosis in liver.

MATERIALS AND METHODS

Experimental groups

The study was performed using male pigs, weighing 23-26 kg (SEASCO, Saitama, Japan), in accordance with the Guidelines for the Care and Use of Laboratory Animals, Dokkyo Medical University. Two groups of animals were prepared: Edr group ($n = 6$) in which Edr (supplied by Mitsubishi-Tanabe Pharma Co., Ltd., Osaka, Japan) was administered intravenously at a dose of 3 mg/kg per hour from the commencement of clamping for 30 min, and a control group ($n = 6$) which received physiological saline administered intravenously for the same period. The Edr dose was the same as that used in the treatment of human brain infarction^[9] (Figure 1).

Surgical procedure

A chevron incision was made under general anesthesia, and each branch of the portal vein, hepatic artery and bile duct were carefully isolated and taped. Hemi-hepatic (approximately 60%) liver ischemia was induced by clamping the right and middle hepatic vessels and bile duct using vessel tapes and maintained for 60 min; the left portal branch was kept patent to avoid bowel congestion, and the left lobe played a role in the bypass. After declamping, the left portal vein and artery were ligated, and a left hemi-hepatectomy was performed (approximately 40%). Liver transection was achieved by the crush-clamping method using Pean forceps. During the liver transection, the exposed Glisson's vessels were ligated

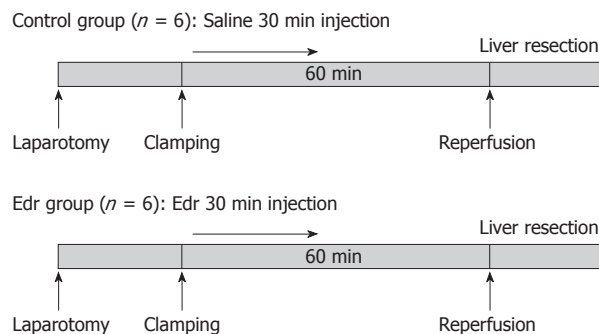


Figure 1 Two groups of animals were prepared. One group received saline and the other received E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) which was administered intravenously at a dose of 3 mg/kg per hour from commencement of clamping to completion of the liver resection.

with 2-0 or 3-0 silk. The hepatic vein was closed by continuous sutures using 4-0 proline. The influence of I/R injury was assumed to be on the right lobe only. During this procedure, hemodynamic parameters (systolic and diastolic arterial pressure) were monitored by a femoral arterial line. All pigs received Ringer's solution during the procedure. After the operation, the pigs were transferred to the Dokkyo Medical University animal center and unlimited oral intake was allowed after the first postoperative day. No antibiotics were administered either orally or intravenously after surgery.

Measurements and sampling protocol levels

Blood samples were obtained from the arterial line immediately after laparotomy, 5 and 180 min after reperfusion, and after 1 mo. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and lactic acid (LA) were evaluated. Hepatic tissues were obtained from the right lobe at laparotomy, after reperfusion, and after 1 mo, and were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and TLR4 and TGF- β 1 mRNA were determined. Serum AST, ALT, LDH and LA were measured using standard clinical methods for automated analysis (Model 7170, Hitachi, Inc., Tokyo, Japan).

Experimental pigs were sacrificed 1 mo after operation and remnant liver was obtained. Total liver weight was calculated based on 30 pigs. We calculated the average percentage of total liver weight to body weight (2.64%), estimated the total liver weight from pre-operative body weight, and calculated the estimated remnant liver weight 1 mo after operation by subtracting the resected liver weight at the time of operation from the estimated total liver weight. Liver weight increasing ratio (%) was calculated as follows; estimated remnant liver weight at resection/remnant liver weight after 1 wk \times 100.

Quantitative real-time polymerase chain reaction

At laparotomy, 30 mg of surgical tissue was stored in liquid nitrogen and kept at -80°C until extraction of total RNA using a Nucleospin II kit (Macherey-Nagel, Germany). Reverse transcription reactions were performed using a SuperScript III First-strand Synthesis System for t-poly-

merase chain reaction (PCR) (Invitrogen, Carlsbad, CA, United States). Briefly, 1 µg of total RNA, oligo dT primer, and dNTPs were incubated at 65 °C for 5 min, and then 10 µL of cDNA synthesis mixture was added and incubated at 50 °C for 50 min. The reaction was terminated by adding 1 µL of RNase H and incubated at 37 °C for 20 min. Real-time PCR was performed using a ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, United Kingdom). The PCR reaction was carried out in a final volume of 1 µL cDNA, 2 µL 10 × SYBR Green (Applied Biosystems), using 40 cycles at 95 °C for 30 s and 60 °C for 30 s. The specific primers were designed using Primer 3 software (http://frodo.wi.edu/cgi-bin/primer3/primer3/primer3_www.cgi) and synthesized by Sigma Genosys (Hokkaido, Japan). The sequences of each primer were as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5'-CCACCCAGAAGACTGTGGAT-3', anti-sense 5'-TTCAGCTCAGGGATGACCTT-3'; TLR4 and TGF-β1: sense 5'-CCCCTGTCCATCCCTTATT-3', anti-sense 5'-AAGCCCCAGTTCCTAATCTT-3'. For each PCR run a standard curve was constructed from serial dilutions of cDNA from the PANCI cell line. The level of expression of TLR4 and TGF-β1 were calculated using the formula: relative expression (t) = [(copy number of TLR4 + TGF-β1 number/copy number of GAPDH)] × 1000. For non-template reactions and standard cDNA dilutions from PANCI cells, liver samples were assayed in triplicate. The average and standard deviation were calculated and the *t*-value was determined from the averages.

Histological examination

Tissue samples were obtained at the time of laparotomy, the remnant liver just after hepatectomy, 3 h after declamping of the right and middle hepatic arteries and portal vein, and after 1 mo, and were fixed with 10% formalin for 24 h and embedded in paraffin. Sections 3-µm thick were stained by the *in situ* terminal TUNEL method using an apoptosis *in situ* detection kit (Wako Pure Chemical, Inc., Osaka, Japan) according to the manufacturer's instructions. The mean numbers of apoptotic cells per 10 random high-power fields were calculated and compared between the two groups.

Statistical analysis

All values are expressed as mean ± SD. Parameters were evaluated using the Student *t* test and Mann Whitney *U* test. Differences between the two groups were evaluated using analysis of variance with *P* < 0.05 considered to be significant.

RESULTS

There were no significant differences in weight, amount of intraoperative hemorrhage, liver resection time, and weight of resected liver (Table 1). There was no significant difference in liver weight increasing ratio at 1 mo after operation between the two groups (*P* = 0.228, Figure 2). There were no significant differences in systolic and diastolic blood pressures between the two groups (Figure 3).

Table 1 Weight, amount of intraoperative hemorrhage, liver resection time, and weight of resected liver in the two groups (mean ± SD)

	Control	Edr	<i>P</i> value
Body weight	24.6 ± 1.1	24.7 ± 0.5	0.99
Body weight (after 1 mo)	29.4 ± 3.3	29.4 ± 3.1	0.97
Bleeding (g)	59.5 ± 44.7	57.4 ± 46.8	0.82
Resection time (min)	24.2 ± 1.6	24.7 ± 0.4	0.49
Resected liver volume (g)	295.7 ± 25.7	271 ± 41.0	0.32
Liver volume (after 1 mo)	606.3 ± 92.7	579.3 ± 60.0	0.53

Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one; RxLV: Resected liver volume.

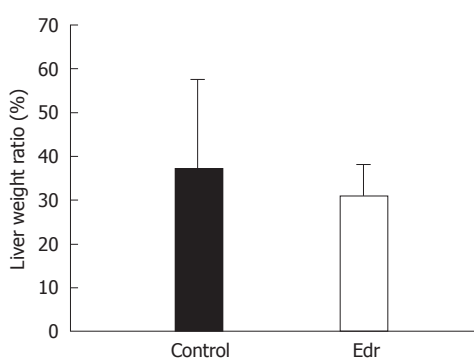


Figure 2 After 1 mo, increasing remnant liver weight ratio showed no difference between the two groups (*P* = 0.228). Liver weight increasing ratio (%) = estimated remnant liver weight at resection/remnant liver weight after 1 mo × 100. Total liver weight was calculated based on previous experimental data of 30 pigs. We calculated the average percentage of total liver weight to body weight (2.64%). Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one.

Serum chemistry showed that the AST level at 3 h after reperfusion was significantly lower in the Edr-treated group than in the control group (*P* = 0.029). There were no significant differences in ALT, LDH and LA levels at any of the observation points between the two groups (Figure 4). Figure 5 shows the expression of TGF-β1-mRNA in liver tissues. Expression of TGF-β1-mRNA in the Edr-treated group tended to be lower than in the control group at 3 h (*P* = 0.285) and 1 h (*P* = 0.172) after operation. Expression of TLR4 mRNA was significantly lower in the Edr-treated group than in the control group at 3 h after operation (*P* = 0.043), however, there was no significant difference between the two groups 1 mo after operation (Figure 6). Figure 7A shows TUNEL staining. There were more TUNEL-positive cells in Control pigs than in Edr-treated pigs 5 min after hepatectomy and 3 h after declamping. Actual numbers of apoptotic cells in the Edr-treated and control groups were 22.16 ± 8.97 and 36.85 ± 1.83 at 5 min after hepatectomy (*P* = 0.015), and 24.66 ± 3.11 and 42.25 ± 3.43 at 3 h after declamping, respectively (*P* = 0.0001) (Figure 7B).

DISCUSSION

The Pringle method is usually performed to reduce the amount of hemorrhage during hepatectomy^[4]. However, this method inevitably causes I/R injury and has a risk

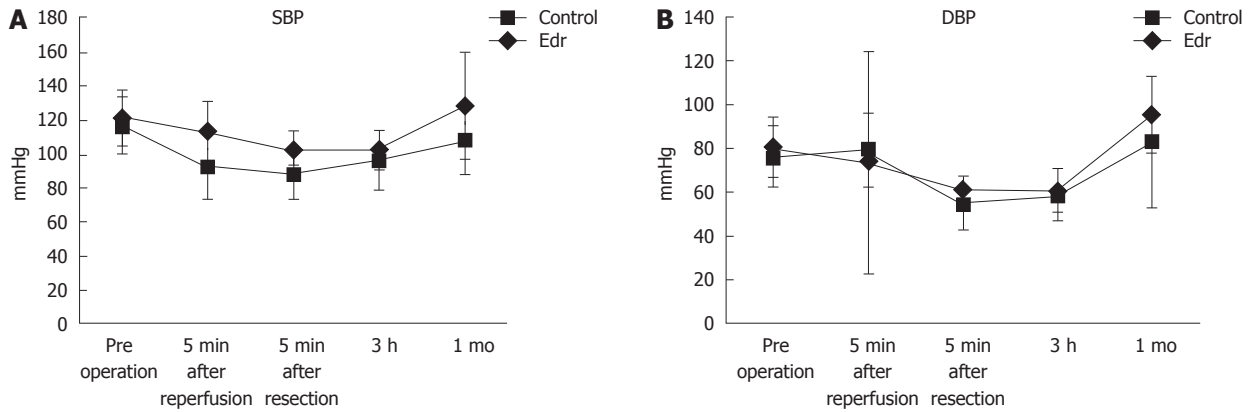


Figure 3 Systolic blood pressure and diastolic blood pressure were not different between the two groups. A: Systolic blood pressure (SBP); B: Diastolic blood pressure (DBP); Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one.

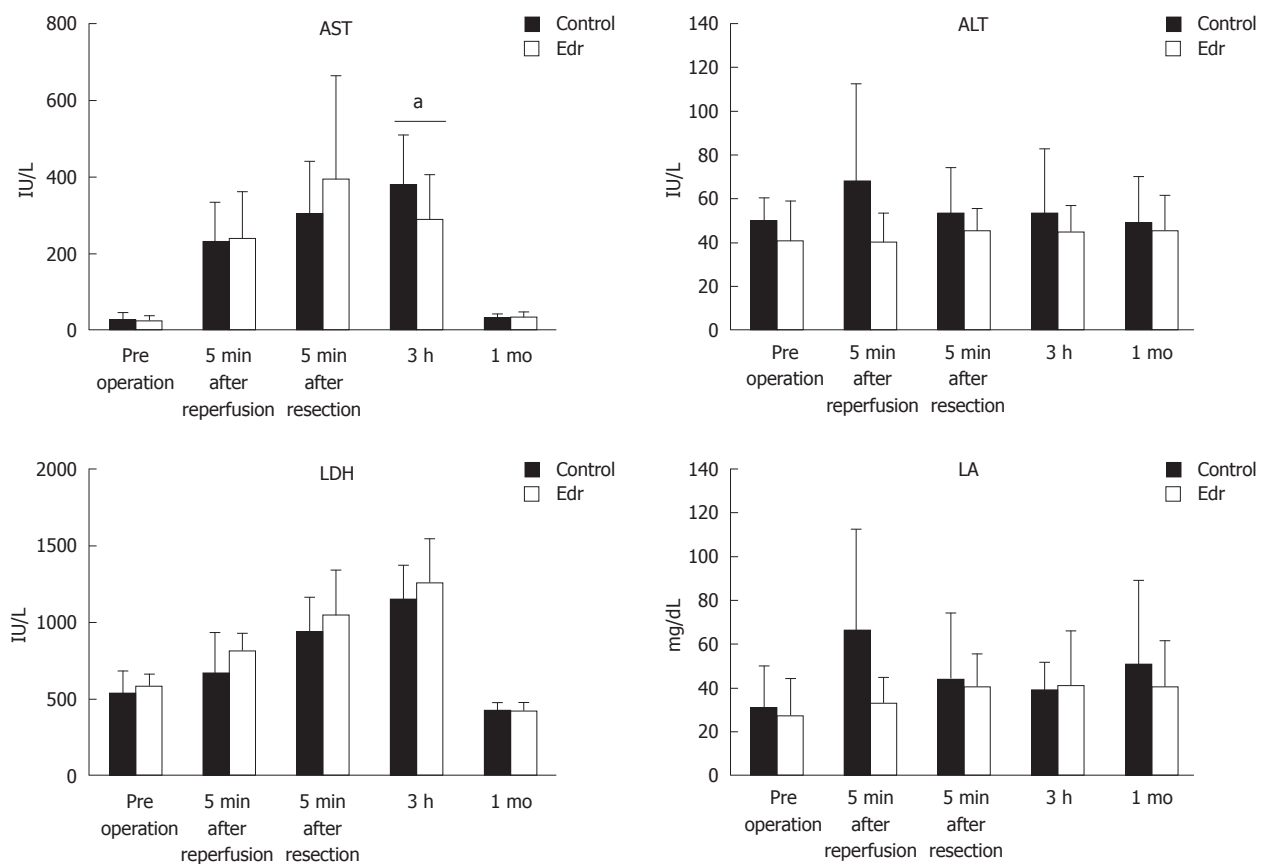


Figure 4 Aspartate aminotransferase was significantly lower 3 h after reperfusion compared to the control ($^*P = 0.029$). Alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and lactic acid (LA) were not different after reperfusion in the E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr)-treated group compared to the control group. AST: Aspartate aminotransferase.

of inducing abnormally high hepatic enzyme levels, icterus, hyperammonemia, lacticidemia, and an intractable accumulation of pleural and peritoneal effusion postoperatively. I/R injury associated with the Pringle maneuver may affect outcome when the ischemic time is long, or during hepatectomy for diseased liver, such as in cases of hepatic cirrhosis and fatty liver, despite the reduction in intraoperative hemorrhage achieved using the Pringle method^[10].

I/R injury may be a causal factor in the above symp-

oms, and various countermeasures have been proposed. These include modified surgical methods, such as an intermittent Pringle method, partial hepatic pedicle clamping, and ischemic pre-conditioning, as well as drug therapy with steroids, prostaglandin E1, Sivelestat or Erythropoietin^[10-19]. However, assessment of the effectiveness of these approaches has been limited, and this may be clarified by studies in animals of similar size to humans. In general, the process of I/R injury consists of multiple steps. Hypoxia due to cessation of blood supply

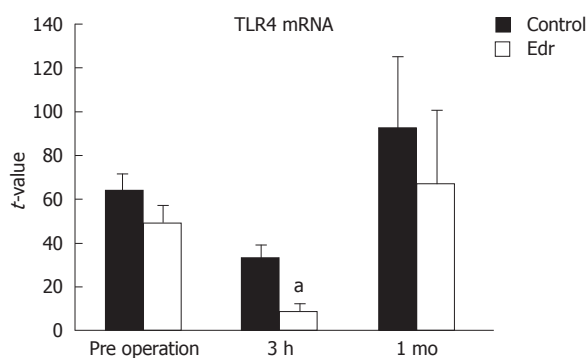
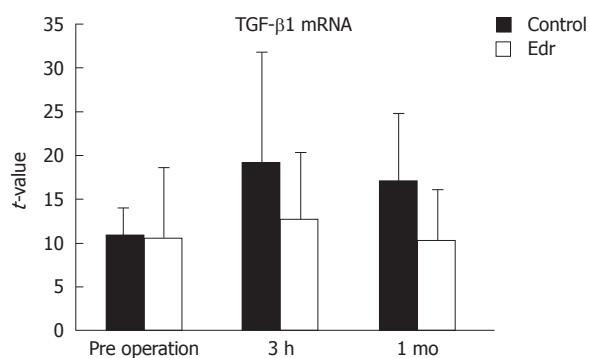


Figure 5 Transforming growth factor-β1 mRNA expression in liver tissue. Expression of transforming growth factor-β1 (TGF-β1) mRNA in the E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr)-treated group tended to decrease 3 h and 1 mo after reperfusion, however, no significant difference between the two groups was observed ($P = 0.285$, $P = 0.172$).

Figure 6 Toll like receptor 4 mRNA level. Toll like receptor 4 (TLR4) mRNA level significantly decreased in the E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr)-treated group at 3 h after surgery ($^aP = 0.004$). At 1 mo after operation, Toll like receptor 4 level showed no differences between the two groups.

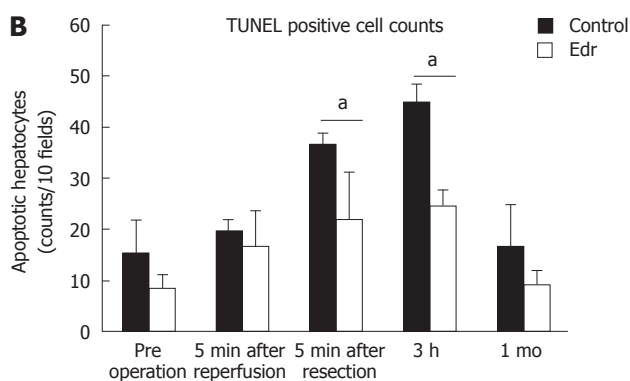
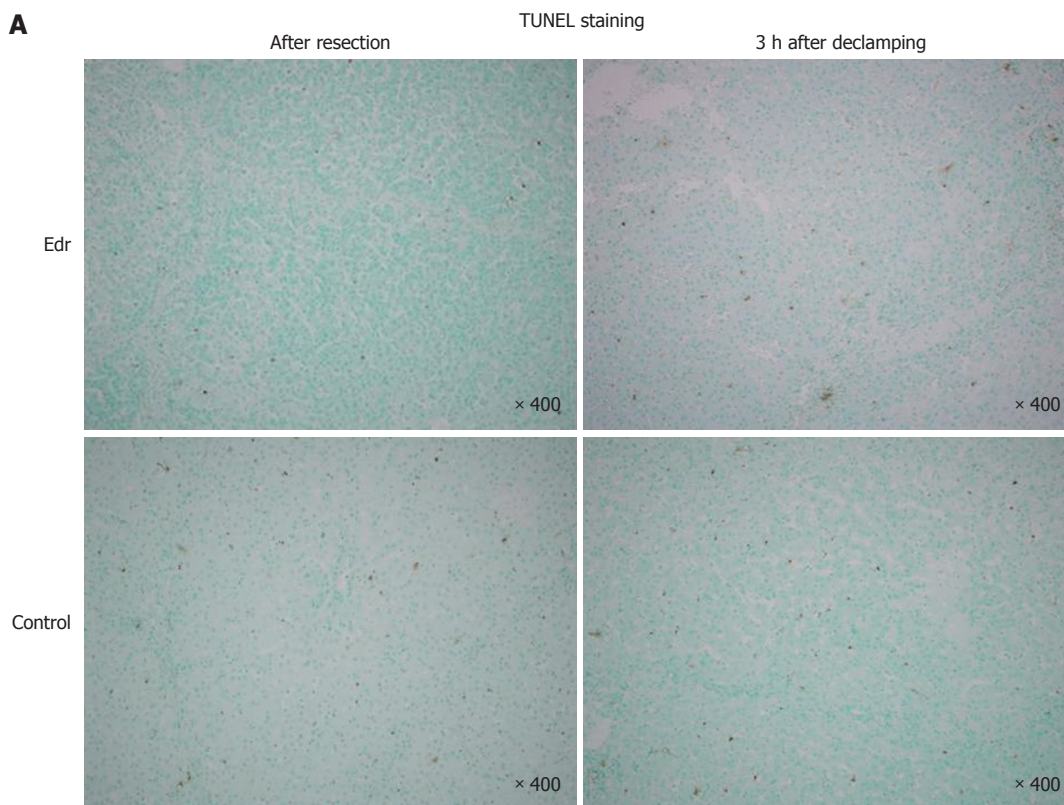


Figure 7 Morphometric evaluation of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive hepatocytes in terminal deoxynucleotidyl transferase dUTP nick end labeling-stained tissue samples after resection and after 1 mo (A, B). The differences between the two groups were statistically significant 5 min after hepatectomy and 3 h after declamping (values are expressed as mean \pm SD, $n = 6$ in both groups). $^aP = 0.001$. Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

impairs oxidative phosphorylation in the mitochondria, leading to profound cellular damage^[20]. Furthermore, reperfusion exacerbates cellular damage by producing reactive oxygen species, activating pro-inflammatory cytokines such as IL-10 and TNF- α ^[21], and up-regulating cell adhesion molecules such as P-, E-, and L-selectins^[22,23], resulting in tissue injury.

Edr has potent hydroxyl radical scavenging activity^[24]. In various experimental models, Edr has been reported to protect several organs such as brain^[25], heart^[26] and liver^[27-29] from free radical-mediated injuries.

Several previous studies have investigated the effect of Edr on I/R injury in the liver. Edr improves portal flow and decreases the level of hepatic enzyme^[30]. Edr also decreases oxidative damage to mitochondria in liver^[27]. However, these findings only indicate that Edr is effective in the acute phase of I/R injury. In the present study, we investigated the effect of Edr at both very early time points and at a late point (one mo after hepatectomy). No improvements in the markers of liver function including AST, ALT, LDH and LA, except AST at 3 h after hepatectomy, were noted at any of the observation periods. However, Edr successfully reduced apoptotic cells in the liver at 5 min and 3 h after hepatectomy. The anti-apoptotic effect of Edr was not observed at one mo after hepatectomy. Thus, the results suggest that Edr has a protective effect against I/R injury in liver by inhibiting apoptosis in the early phase after injury, which was reflected in the decrease in markers. However, prevention of I/R injury, particularly in the early phase, did not affect liver regeneration.

TGF- β is a key mediator involved in the progression of liver disease. TGF- β exerts cytotoxic effects, such as induction of apoptosis and aggravation of microcirculation disorders, due to induction of the expression of cell adhesion molecules^[31]. TGF- β had antiproliferative action after partial liver resection in a rat model^[7]. When administered at the time of liver resection, TGF- β clearly delayed hepatocyte proliferation^[7]. In the present study, we observed that Edr tended to suppress TGF- β 1-mRNA expression in liver tissue at 3 h and 1 mo after hepatectomy.

TLRs consist of 13 mammalian members containing a conserved TIR Toll/IL-1 receptor domain in their intracellular domain and an individual leucine-rich repeat domain in their extracellular domain. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and TLR3, TLR7, TLR8 and TLR9 are expressed on the endosome-lysosome membrane. TLR4 and TLR5 are the receptors for the Gram-negative bacterial cell wall components, lipopolysaccharide, and bacterial flagellin, respectively^[32]. In the liver, TLR4 is an important key factor for liver fibrosis, I/R injury and liver regeneration. Upon I/R injury, the TLR4 ligand, HMGB1, is released from damaged hepatocytes and subsequently stimulates Kupffer cells through TLR4^[33]. Suppression of TLR4 at I/R injury, leads to the control of progress to liver regeneration by controlling liver fibrosis. Unfortunately, in this study, Edr only decreased TLR4 levels in the early phase

(less than 3 h) after liver resection. This result suggested that Edr did not have any effect on liver fibrosis and regeneration.

In conclusion, our results demonstrated that Edr has a protective effect against I/R injury in the liver by inhibiting apoptosis in early period after I/R injury. Although further studies are needed to determine the dose, timing, and duration of Edr treatment to be used in the clinical setting, we have shown, for the first time in a porcine model, that Edr may be a promising agent for ameliorating I/R injury during hepatectomy.

ACKNOWLEDGEMENTS

The authors thank Hisato Hirata and Yoshifumi Machida of the laboratory animal research center at Dokkyo Medical University for their excellent technical assistance.

COMMENTS

Background

Peritoneal adhesions can cause intestinal obstruction and other severe clinical disorders, thus, it is very important to prevent peritoneal adhesions in abdominal surgical operations. However, to date, there are still no ideal methods to prevent peritoneal adhesions in clinical practice. Chitosan is a deacetylated derivative of chitin.

Research frontiers

Chitosan is a natural biological material and has been processed into many forms for medical use. Chitosan has also been used in the prevention of peritoneal adhesions and various researchers are exploring how to modify chitosan using chemical and physical methods to improve its effectiveness in preventing adhesions, and simultaneously reduce its adverse reactions.

Innovations and breakthroughs

In previous reports on the application of chitosan gels to prevent peritoneal adhesions, it was found that the gel was much too fluid and did not remain in the target area for sufficient time, moreover, the gel degraded so fast that it could only maintain effectiveness for a short period. In order to delay degradation and decrease the fluidity of the gel, the authors processed pure chitosan into films, however, the film degraded too slowly and the residual film was encapsulated by surrounding tissue and the peritoneal adhesions worsened. In order to overcome these disadvantages, we mixed chitosan with gelatin and produced blended films. The blended film degraded much faster than the previous pure chitosan film, but it also created a foreign body reaction and formed a severe foreign body granuloma around the blended film. In the present study we chemically modified chitosan during gelatinization to develop a new type of chitosan film, and showed that the film is remarkably effective in preventing peritoneal adhesions induced by wounds, ischemia and infection except foreign body-induced adhesions.

Applications

The study results suggest that the gelatinized-chitosan film is a potential therapeutic material which could be used in preventing peritoneal adhesions induced by wounds, ischemia and infection.

Terminology

Peritoneal adhesion: Peritoneal adhesion is a type of defensive reaction to peritoneal injury mainly wounds, infection, ischemia and foreign bodies, but it can also cause intestinal obstruction and severe clinical disorders; chitosan: Chitosan is a deacetylated derivative of chitin, and chitin is the second most abundant natural biopolymer derived from the exoskeletons of crustaceans and from the cell walls of fungi and insects.

Peer review

This is a good descriptive study in which authors investigate the effect of E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) on hepatic ischemia-reperfusion (I/R) injury and liver regeneration in a porcine hepatectomy model. The results are interesting and suggest that Edr is considered to reduce hepatic injury in the early stage of I/R injury in a porcine model.

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S- Editor Cheng JX L- Editor Webster JR E- Editor Zheng XM