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Research Article

Bolus Administration of Polyamines Boosts Effects on Hepatic Ischemia-Reperfusion Injury and Regeneration in Rats

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Keywords
Bolus administration · Hepatic ischemia-reperfusion injury · Hepatic regeneration · Polyamine · Rat

Abstract
Background: It was demonstrated that polyamines ameliorate ischemia-reperfusion injury (IRI) and promote regeneration in the liver. An optimal protocol of polyamine treatment remains unknown in the clinical setting. We examined 2 types of administration methods using rat models. ***Methods:*** Experiment 1: evaluation of pharmacokinetics of polyamines. Experiment 2: for 3 days preoperatively and 5 days postoperatively, polyamines were given to male Lewis rats in the following three groups: the control group, no polyamine administration; the chow group, 0.05% polyamines mixed in chow; the bolus group, polyamines (200 μmol/kg) given by gastric tube once a day. All rats received 70% hepatectomy after 40 min of warm IRI. Postoperatively, IRI and regeneration were evaluated with assessment of serum levels of hepatic enzymes, histology and immunohistochemistry of liver tissue, and measurement of remnant liver weight. ***Results:*** The blood concentrations of polyamines in the portal vein increased at 1 h of bolus administration, while they did not increase without the bolus. The bolus group was significantly associated with lower serum levels of aspartate/alanine aminotransferases ($p < 0.05$), decreased hepatocyte congestion, vacuolization and necrosis in histopathological scoring ($p < 0.05$), a lower number of TUNEL-positive hepatocytes ($p < 0.05$), higher remnant liver weight at

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24, 48, and 168 h ($p < 0.05$), and a higher Ki-67 labeling index (24 h, $p < 0.01$) compared with the chow group. ***Conclusion:*** The bolus administration of polyamines was more effective in ameliorating IRI and promoting regeneration than chow administration. Perioperative bolus administration of polyamines might be an optimal treatment, when clinically applied.

Introduction

Polyamine is a generic term for amine compounds, putrescine (PUT), spermidine (SPD), and spermine (SPM). Polyamine is an important component in cell proliferation, growth, and differentiation in a wide range of organisms, from prokaryotes to eukaryotes, modulating transcription and translation [1, 2]. In eukaryotic cells, polyamine protects against oxidative stress by scavenging reactive oxygen species or modulating mitochondrial permeability. Moreover, polyamines initiate and accelerate cell proliferation via their involvement in the cell cycle and their activation of the eukaryotic translation initiation factor [3, 4].

Previous reports demonstrated that polyamines ameliorate ischemia-reperfusion injury (IRI) and promote regeneration [5–7]. We also reported that oral polyamine administration by gastric tube alleviates IRI and promotes regeneration in inbred rats [8]. This showed the exogenous polyamines' potential to contribute to improving the outcome of liver surgery, including liver transplantation. However, there is no previous report specifically describing the optimal methodology of polyamine administration in clinical liver surgery, despite its detailed importance. Therefore, in this study, we focused on the influences of administration methods on IRI and regeneration. Previous reports have shown that bolus administration of polyamines significantly attenuates injury or promotes regeneration in rat models [7, 9–12]. We hypothesized that bolus administration of polyamines by gastric tube in a perioperative period will have a greater positive effect on hepatic IRI and regeneration compared with administration by mixed chow. The purpose of this study is to assess the effects of administrations of polyamines mixed in chow and bolus administration on hepatic IRI and regeneration. We used a rat model of hepatectomy accompanied by hepatic warm IRI.

Methods

Experiment 1: Measurement of Blood Levels of Polyamines in the Portal Vein

To assess the pharmacokinetics of the polyamines, rats were randomly divided into three groups ($n = 3$/group): the control group, the chow group, and the bolus group. In the control group and the chow group, rats

Fig. 1. Experimental protocols. **a** In the control and chow groups, rats were administered 0% polyamine chow and 0.05% polyamine chow through the experimental methods, respectively. In the bolus group, in addition to being fed 0% polyamine chow, the rats were administered polyamines (200 μmol/kg) by gastric tube once a day, 4 times for 3 days. For measurement of blood polyamine levels, blood was sampled from the PV at the time points of 0, 1, 2, and 6 h. **b** In the control and chow groups, rats were administered 0% polyamine chow and 0.05% polyamine chow, respectively, from 3 days prior to surgery through the experiments. In the bolus group, in addition to feeding 0% polyamine chow, rats were administered polyamines (200 μmol/kg) by gastric tube once a day from 3 days to 2 h prior to surgery. After the surgery, this treatment was continued from postoperative day 1–7. The polyamines were a mixture of SPD trihydrochloride (160 μmol/kg) and SPM tetrahydrochloride (40 μmol/kg). **c** Our rat model of 70% hepatectomy with IRI (40 min of warm ischemia). The left portal pedicle was clamped for 40 min, then 70% hepatectomy was performed. The remnant liver was in the right portion of the median lobe, which was subject to ischemia-reperfusion injury.

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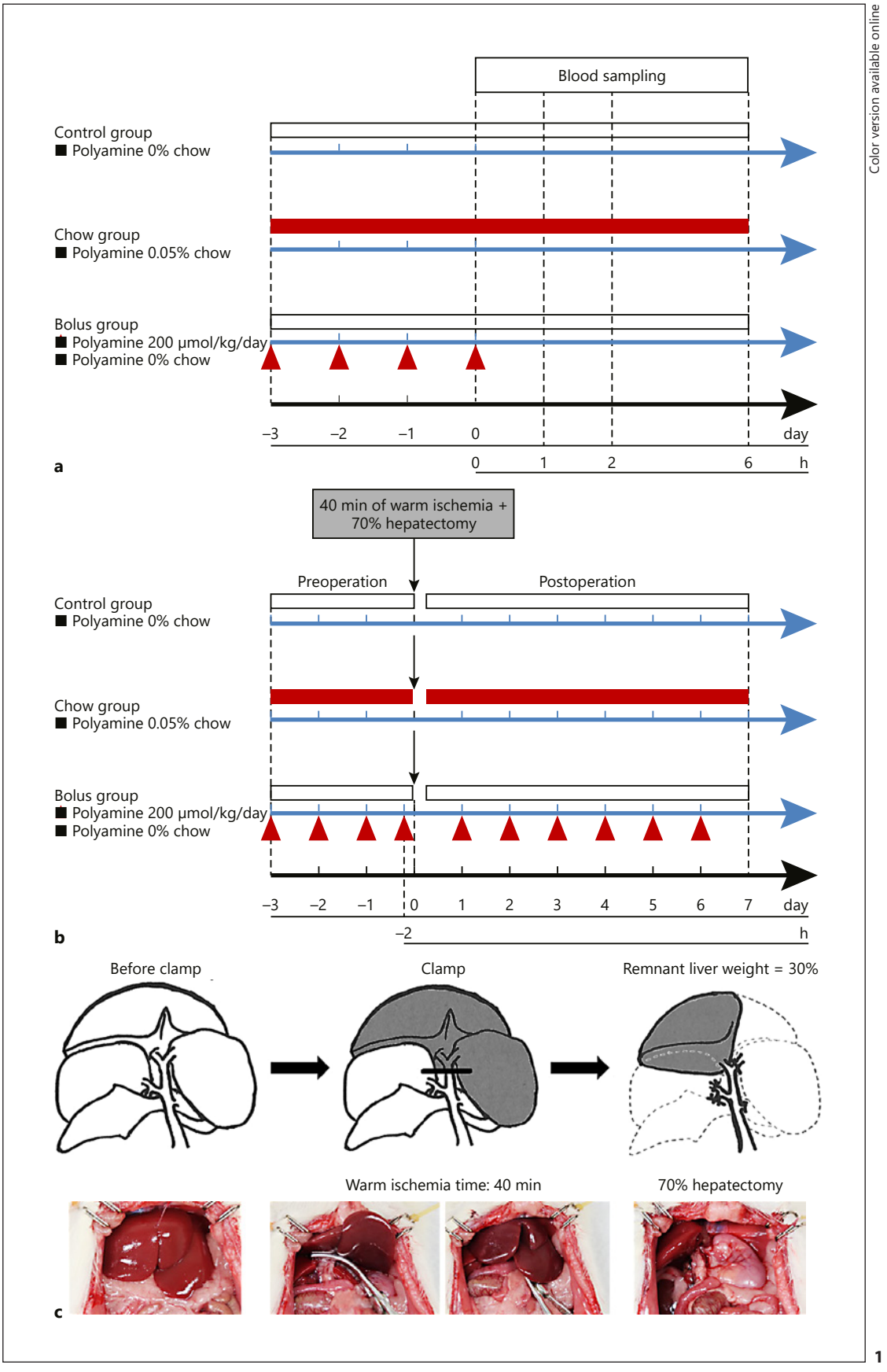

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Table 1. Complete ingredients and nutrition contents of AIN-93G and the two test chows used in the experiments

Ingredients	AIN-93G	0% polyamine chow	0.05% polyamine chow
Casein	20.00	20.00	20.00
L-Cystin	0.30	0.30	0.30
Corn starch	39.7486	39.7486	39.7486
Pregelatinized corn starch	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00
Refined soybean oil	7.00	–	–
Corn oil	–	7.00	7.00
Cellulose	5.00	5.00	5.00
AIN93G mineral mix	3.50	3.50	3.50
AIN93 vitamin mix	1.00	1.00	1.00
Choline bitartrate	0.25	0.25	0.25
Tertiary butylhydroquinone	0.0014	0.0014	0.0014
SPD trihydrochloride	–	–	0.073 (0.04)
SPM tetrahydrochloride	–	–	0.018 (0.01)

Values in parentheses are the compounding ratio (w/w, %).

were fed on 0% polyamine chow and 0.05% polyamine chow, respectively. In the bolus group, the rats were fed on 0% polyamine chow and 200 μmol/kg polyamine (160 μmol/kg SPD trihydrochloride and 40 μmol/kg SPM tetrahydrochloride) was administered by gastric tube once daily. The time point of the 4th administration of polyamines in the bolus group was considered to be 0 h. Experiment 1 lasted from day –3 to 6 h (Fig. 1a). Whole blood was collected from the portal vein (PV) at the time points of 0, 1, 2, and 6 h. Concentrations of PUT, SPD, and SPM were measured using high-performance liquid chromatography as previously described [8] (Fig. 1a).

Experiment 2: Hepatectomy with IRI

Rats were randomly divided into three groups: the control group, the chow group, and the bolus group. In the control group and the chow group, rats were fed on 0% polyamine chow and 0.05% polyamine chow, respectively, from 3 days prior to surgery throughout the experiments. In the bolus group, the rats were fed on 0% polyamine chow and the same 200 μmol/kg polyamine mixture was administered by gastric tube once daily from 3 days prior to surgery until the end of the experiment. On the day of surgery, 200 μmol/kg polyamine was administered in the same manner 2 h before surgery (Fig. 1b).

Animals

Male inbred Lewis rats (8–12 weeks old, weighing 240–340 g; Charles River, Kanagawa, Japan) were used. Rats were handled and cared for according to the Institutional Guidelines for Animal Welfare. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University (protocol ID: Med Kyo 18177). All animals were housed in a specific pathogen-free animal facility at Kyoto University under the following conditions: 50 ± 10% relative humidity, 12/12 h light-dark cycle, and a temperature of 24 ± 2 °C.

Rat Chow

Two types of test chow were prepared for the animal experiments, one that was free of polyamine (0.00% polyamine chow), and another with 0.05% polyamine content (0.05% polyamine chow). Fortified rodent chow for growth (AIN-93G; Oriental Bio Service, Kyoto, Japan) was modified into 0% polyamine or 0.05% polyamine chow [13]. The soybean oil contained in AIN-93G was replaced with corn oil as a source of fat, because soybeans and soybean products contain high concentrations of polyamine. For polyamine 0.05% chow, SPD trihydrochloride and SPM tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were mixed so that the net concentrations of SPD and SPM were 0.04% and 0.01% (w/w), respectively (Table 1).

Operative Procedures

The surgical operation performed in the present study was a 70% partial hepatectomy (PH) after 40 min of warm ischemia as previously described (Fig. 1c) [8, 14]. All surgical procedures were performed under inhalation anesthesia using 1.5% isoflurane. The abdomen was opened via a midline incision. Clamping the left portal pedicle with a vessel clip induced 70% partial hepatic ischemia. The left portal pedicle included the PV, the hepatic artery, and the bile duct for the median and the left lateral lobes of the liver. The vessel clip was released to initiate hepatic reperfusion at 40 min after clamping. Then, the nonischemic lobes (the superior right lateral, the inferior right lateral, the anterior caudate, and the posterior caudate lobes) and the ischemic lobes (the left lateral lobe and the left portion of the medial lobe) were excised with only the ischemic right portion of the medial lobe left behind.

Assessment of Liver Functions

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed as previously prescribed (n = 6/group) [14].

Histological Analysis

Conventional morphological evaluation with hematoxylin and eosin (HE) staining was performed using the Suzuki score, which measures three parameters of hepatic IRI: sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and parenchymal necrosis (n = 6/group) [8, 14, 15].

Immunohistochemical Detection of Apoptosis of Hepatocytes

Apoptosis in liver sections was determined by in situ detection of deoxyribonucleic acid fragmentation using a TUNEL (terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling) assay (Trevigen Inc., Gaithersburg, MD, USA). Quantification of TUNEL-positive hepatocyte nuclei was performed by counting the number of TUNEL-positive hepatocytes in 10 random 200× fields per animal (n = 4–6/group).

Liver Regeneration Rate

The liver regeneration rate was calculated and assessed to examine restoration of the remnant liver (n = 5–8/group). The resected liver was weighed at the time of PH, while the remnant liver was excised and weighed following euthanasia. The liver regeneration rate was expressed as the percentage of remnant liver weight to the whole liver weight. The original whole liver weight at the point of PH was extrapolated by dividing the resected liver weight by 0.691, based on results of a pilot study showing that the resected liver weight in our model was equal to 69.1% of the original whole liver weight (69.1 ± 2.7%, in inbred young male Lewis rats, n = 9).

Immunohistochemical Detection of Active Proliferation of Hepatocytes

The proliferative activity of hepatocytes in liver tissue was evaluated by immunochemical detection of the nuclear non-histone protein Ki-67. Ki-67 staining was performed using rabbit monoclonal SP6 antibody with 3,3′-diaminobenzidine chromogen (Lab Vision Corporation, Thermo Fischer Scientific, Fremont, CA, USA). The mean percentage of Ki-67-positive hepatocytes among total hepatocytes in 10 random 200× fields per animal was used for the Ki-67 labeling index (n = 6/group).

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis was conducted using a Student's t test and a non-repeated measurement analysis of variance (ANOVA) followed by a Bonferroni post hoc comparison test. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Experiment 1

Peak Levels of Polyamines of the PV in the Bolus Group

In the bolus group, the blood levels of SPD in the PV increased at 1 h, and dropped to the baseline level at 2 h. The blood levels of SPD in the PV in the bolus group at 1 h was

significantly higher compared with those in the control and chow groups (n = 3/group; Fig. 2a). There was no significant difference in the blood levels of SPM among the control, the chow, and the bolus groups (Fig. 2b). PUT levels were below the level of detection in all rats.

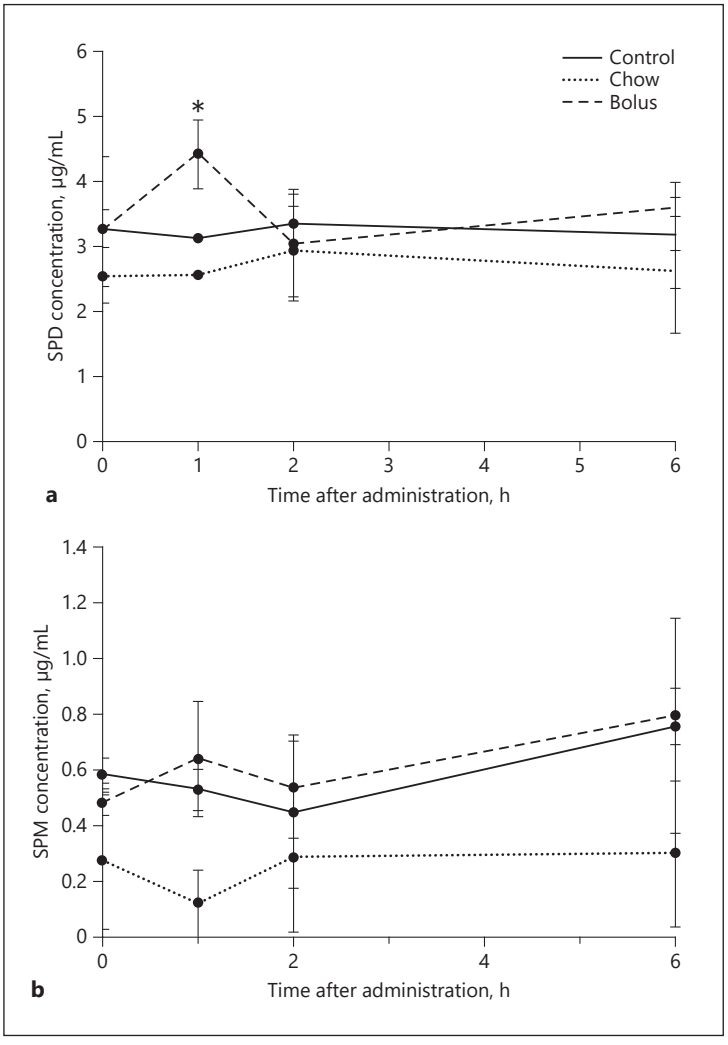

Fig. 2. Kinetics of SPD and SPM. **a** Concentrations of SPD in the PV increased until 1 h after bolus administration of polyamines, and dropped to the baseline level at 2 h (* p < 0.01, ANOVA, n = 3/group). **b** Concentrations of SPM in the PV did not increase after bolus administration (n = 3/group). SPD, spermidine; SPM, spermine.

Fig. 3. IRI. **a** Serum levels of AST and ALT at 6 and 24 h of reperfusion. Serum levels of AST and ALT in the bolus group were significantly lower than those in the chow group (* p < 0.01, ** p < 0.05, ANOVA, n = 6/group). **b** Representative HE-stained sections at 6 and 24 h of reperfusion. Original magnification ×400. Scale bars, 200 μm. **c** Assessment of HE-stained sections by Suzuki scoring. At 6 or 24 h, congestion, vacuolization, necrosis, and Suzuki scores in the bolus group were significantly lower than those in the chow group (* p < 0.01, ** p < 0.05, ANOVA, n = 6/group). **d** Representative TUNEL-stained sections at 6 h of reperfusion. Arrows indicate TUNEL-positive nuclei of hepatocytes. Original magnification ×200. Scale bars, 100 μm. **e** Cell count of TUNEL-positive hepatocytes. The number of TUNEL-positive hepatocytes counted at 6 h of reperfusion in the bolus group was significantly lower than that in the chow group (* p < 0.01, ** p < 0.05, Student's t test, n = 4–6/group).

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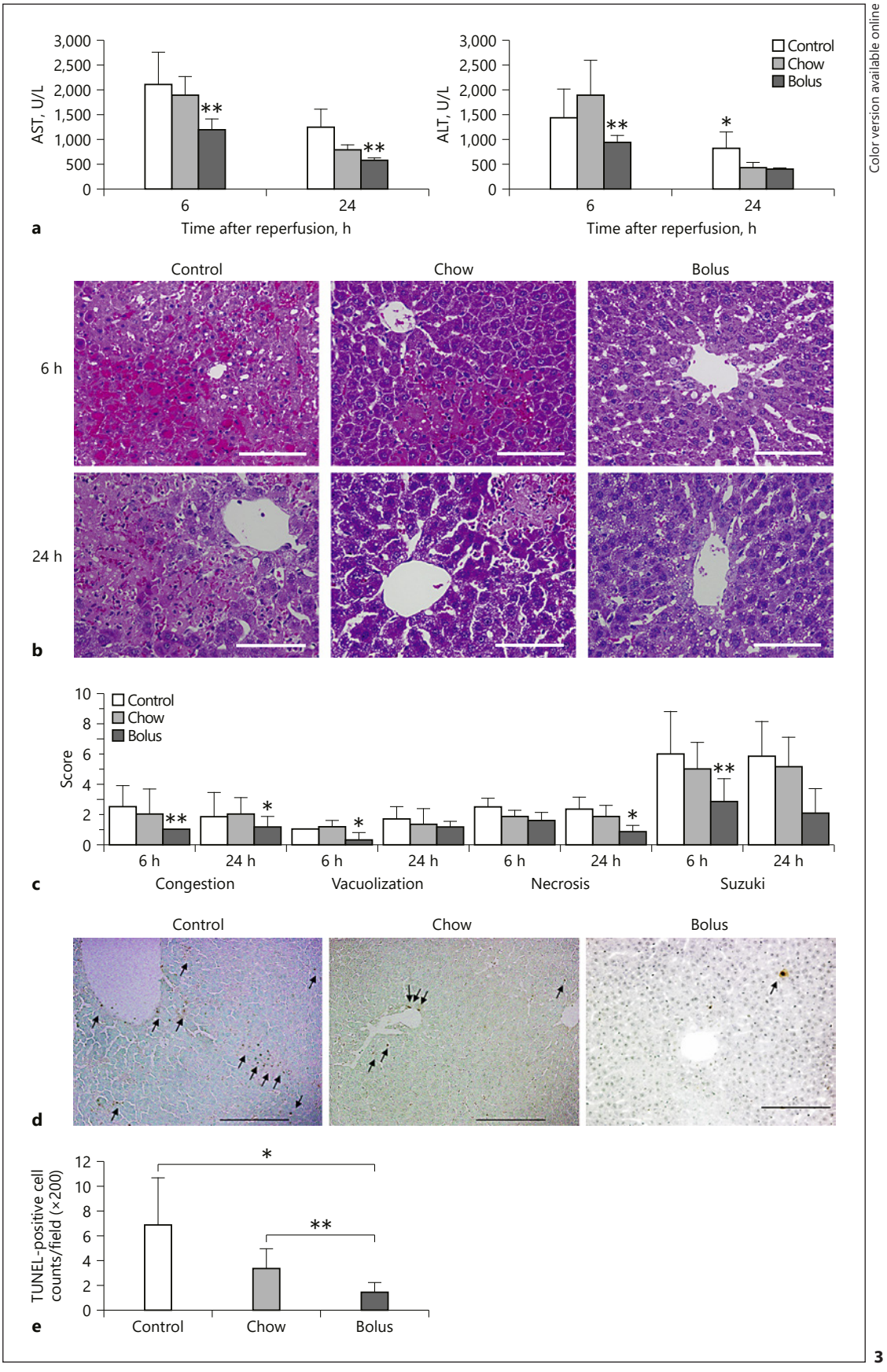

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Experiment 2

Serum Levels of AST and ALT

At 6 and 24 h of reperfusion, serum levels of AST and ALT were significantly lower in the bolus group compared with the chow group ($p < 0.05$, $n = 6$/group; Fig. 3a).

HE and TUNEL Staining

At 6 and 24 h of reperfusion, representative HE-stained sections are shown in Figure 3b. Histological scoring assessment on IRI revealed that congestion, vacuolization, necrosis, and Suzuki scores were lower in the bolus group compared with the chow group ($p < 0.05$, $n = 6$/group; Fig. 3c).

Representative TUNEL-stained sections are shown in Figure 3d. The number of TUNEL-positive hepatocytes counted at 6 h of reperfusion was significantly lower in the bolus group compared with the chow group ($p < 0.05$, $n = 4–6$/group; Fig. 3e).

Liver Regeneration Rate and Ki-67 Staining

The liver regeneration rates at 24, 48, and 168 h of reperfusion in the bolus group were significantly higher than in the chow group (24 h: $p < 0.05$; 48 h: $p < 0.01$; 168 h: $p < 0.05$, $n = 5–8$/group; Fig. 4a). At 24 h, the number of Ki67-positive hepatocytes was significantly greater in the bolus group compared with the chow group ($p < 0.01$, $n = 6$/group; Fig. 4b, c).

Discussion

In this study, we demonstrated that bolus administration of polyamines by gastric tube was significantly protective against hepatic IRI and also promoted regeneration compared with the administration of polyamines mixed in chow. These results suggested that bolus administration might be one optimal treatment in clinical liver surgery.

In the bolus group of Experiment 1, blood levels of SPD in the PV increased to reach a peak at 1 h and decreased to the baseline at 2 h. This result implied that the absorption process of SPD through the alimentary tract was completed within 2 h. These findings were compatible with a previous report using a rat ex vivo small intestine model [16]. In contrast, SPD blood levels in the PV did not increase at 0, 1, 2, or 6 h in the chow group of Experiment 1. Next, the daily total intake in the chow group was calculated from the chow intake weight in a pilot study (SPD: 190 ± 19.9; SPM: 45 ± 10.3; SPD + SPM: 240 ± 23.5 μmol/kg/day). There was no significant difference in the daily amount of polyamine intake between the chow and the bolus groups. Despite the same amount of polyamine intake, in Experiment 2, IRI was significantly alleviated and regeneration was promoted in the bolus group compared with the chow group. These results implied that the peak level of SPD beyond the baseline level might be needed to obtain significant effects of polyamines on IRI and regeneration. This finding is supported by other publications reporting that bolus administration of polyamines significantly attenuates injury or promotes regeneration [6, 7, 9, 10]. Additionally, it is also supported by pharmacokinetics/pharmacodynamics theory in pharmacy, which describes the time course of effect intensity in response to administration of an antibiotics dose [17].

Based on serum levels of AST and ALT, and HE and TUNEL staining, IRI in the bolus group was alleviated through suppressed congestion, vacuolization, necrosis, and apoptosis of hepatocytes compared with the chow group. These results were consistent with the polyamine's characteristics including protection against hepatic damage [6, 7, 10, 12, 18]. Generally, in the acute phase of IRI, reactive oxygen species are generated, triggering inflammatory cascades with proinflammatory cytokines and chemokines. Subsequently, activated Kupffer cells and T lymphocytes recruit neutrophils into the liver after reperfusion [19, 20].

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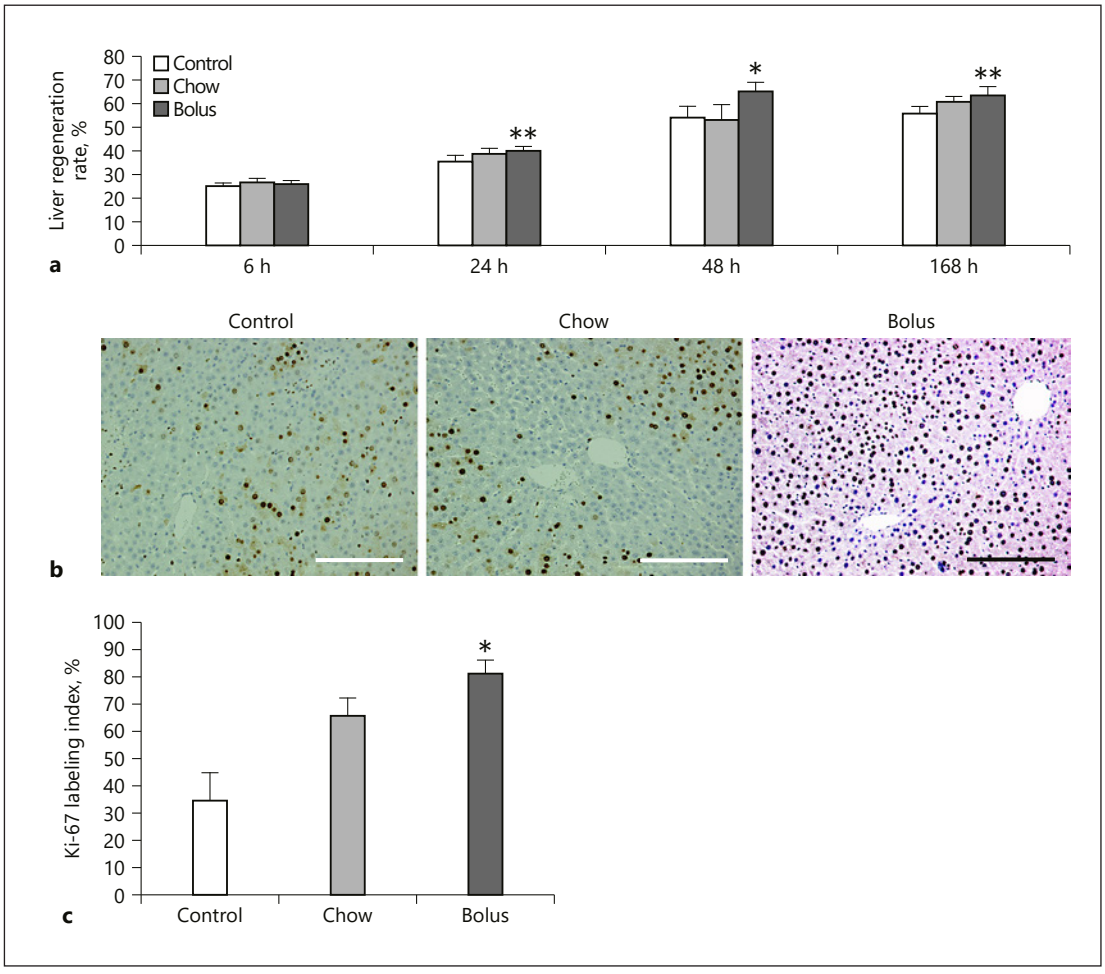

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Fig. 4. Regeneration. **a** Liver regeneration rate. The liver regeneration rates at 24, 48, and 168 h of reperfusion in the bolus group were significantly higher than those in the control or the chow group (24 h: * $p < 0.01$, ** $p < 0.05$, ANOVA, $n = 5–8$/group). **b** Representative Ki-67-stained sections at 24 h of reperfusion. Original magnification ×200. Scale bars, 100 μm. **c** Ki-67 labeling index at 24 h of reperfusion. The Ki-67 labeling index in the bolus group was significantly higher than that in the chow group (* $p < 0.01$, ANOVA, $n = 6$/group).

Okumura et al. [8] reported that the bolus administration of polyamines suppressed inflammatory cascades in the same rat model as the present study. Therefore, it is suggested that bolus administration of polyamines might have significant protective effects on the acute phase of IRI.

The results of remnant liver weight and the Ki-67 labeling indices in this study showed that polyamines promoted regeneration. These results were consistent with previous reports showing that polyamines promote DNA synthesis of hepatocytes isolated from inbred rats in vitro, and initiate and promote liver regeneration in vivo [11, 21–23]. It was suggested that bolus administration of polyamines might be necessary to promote hepatic regeneration.

The limitations of this study are as follows. First, interpretation of the results is limited by differences in species and a small number of animals. However, we did not increase the number of experimental subjects for animal protection as there was consistency in the results in each experimental group. Second, the postoperative observation period was short. The rat remnant liver after hepatectomy in the control, the chow, and the bolus groups recovered to,

respectively, 55.5 ± 3.3, 60.5 ± 2.4, and 63.6 ± 3.4% of the original liver weight 1 week after surgery. One week is supposed to be appropriate as an observation period of the acute phase after hepatectomy in rats.

In conclusion, exogenously administered polyamines ameliorated hepatic IRI and promoted hepatocyte proliferation. In addition, bolus administration of polyamines was more effective in alleviating hepatic IRI and enhancing regeneration in rats than chow administration. The bolus administration was an optimal method to exert these properties. The results of this study indicate that bolus administration of polyamines by gastric tube might be therapeutic when applied in the clinical setting.

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Statement of Ethics

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body. Animal handling and care met the Institutional Guidelines for Animal Welfare. The Institutional Animal Care and Use Committee of Kyoto University approved the study (protocol ID: Med Kyo 18177).

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.D., T.Te., N.K., and Y.F. designed the research. J.D., T.Te., N.K., M.M., and S.U. conducted the research. J.D., T.Te., and T.Ts. analyzed the data. J.D. wrote the initial draft. J.D., T.Te., T.I., S.Y., and Y.F. reworked further drafts, and T.Te., Y.F., S.Y., and S.U. had primary responsibility for the final content. All authors read and approved the final manuscript.

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