Original Article

Preoperative short-term fasting protects liver injury in patients undergoing hepatectomy

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Background: Our previous study demonstrated that preoperative short-term fasting attenuates mice hepatic ischemia/reperfusion injury (IRI), which greatly piqued our interest in verifying if fasting produces similar protective effects in patients undergoing hepatectomy.

Methods: Eighty patients with liver tumors were randomized into control (Ctrl, n=40, preoperative fasting for 6 h) or fasting group (Fasting, n=40, preoperative fasting for 24 h). Serum was collected at pre-operation (Pre-Op), post-operation 1 day (POD-1), post-operation 3 days (POD-3), and post-operation 7 days (POD-7). Liver tissue was removed from the resected specimen.

Results: Sixty-three patients were eventually enrolled, with 33 in Ctrl and 30 in Fasting group. Our data showed that 24 h fasting effectively attenuated elevated sALT and sAST levels after operation ($P<0.05$), but serum total bilirubin was significantly lower at only POD-3 ($P<0.05$); and serum albumin was not markedly different in either of the groups. Interestingly, 24 h fasting partially attenuates expression of pro-inflammatory cytokine (TNF-α) and improves oxidative stress (MDA and SOD). Our data further showed short-term fasting triggered Nrf2 signaling pathway.

Conclusions: This study demonstrates preoperative short-term fasting effectively improves clinical outcomes and markedly attenuates inflammatory responses and oxidative stress in patients undergoing hepatectomy, and Nrf2 signaling pathway may play a key role in fasting against inflammatory responses and oxidant stress.

Keywords: Short-term fasting; hepatectomy; inflammatory cytokines; oxidative stress; nuclear factor erythroid-derived 2-related factor 2 signaling (Nrf2 signaling)

Submitted Jun 05, 2018. Accepted for publication Oct 16, 2018.

doi: 10.21037/atm.2018.10.64

View this article at: http://dx.doi.org/10.21037/atm.2018.10.64

Introduction

The temporary occlusion of the hepatic inflow (pringle maneuver) or the hepatic inflow and outflow (total hepatic vascular occlusion) is a common method used during liver resection or liver transplantation. The prolonged interruption results in liver ischemia/reperfusion injury (IRI) caused by progressive hepatocellular injury and death after reperfusion, which is considered to be a risk factor for recent and distant damage to residual liver or transplant liver (1-3). The development of a protective strategy against IRI is warranted to alleviate the consequences of hepatic IRI. Calorie restriction (CR) and fasting have

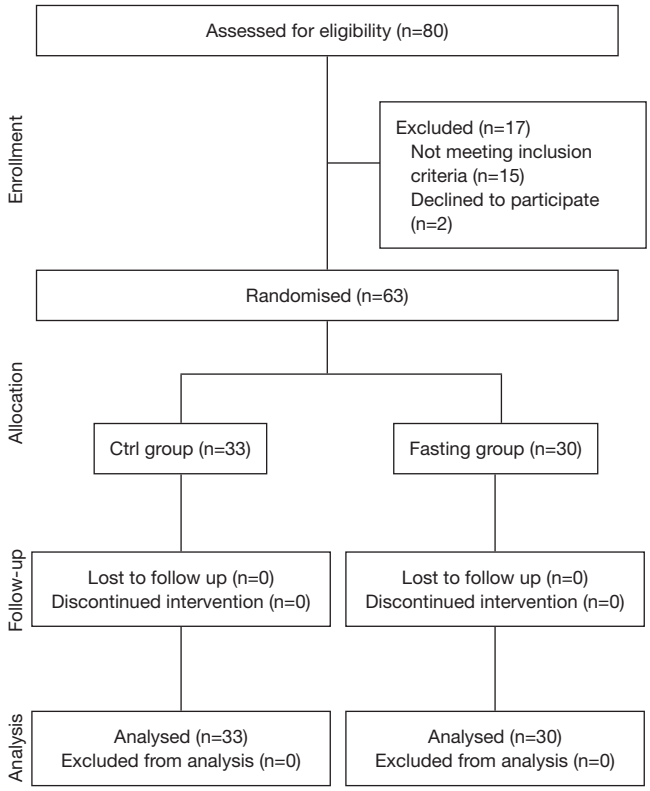

Figure 1 Flowchart of patient enrollment, allocation follow-up and analysis.

displayed some beneficial effects on the prolongation of life and increased resistance to stress. In rodents, CR increases resistance to paraquat toxicity (4). Further, CR is also associated with improved resistance against multiple stressors, such as IRI to the kidney, liver, brain, and heart (5-7). Dietary restriction (DR), which is defined as reduced food intake without malnutrition, is a commonly used CR method, but it is not easily implemented in the clinical setting due to the length of time required (8-11). Interestingly, fasting can rapidly produce similar benefits to long-term DR in terms of gene expression, physiology, and stress resistance. It has recently been reported that fasting can rapidly induce DR-like effects on hepatic IRI. In fact, fasting for 3 days has been shown to be as effective as a month of DR in reducing IRI, which implies a new and promising noninvasive strategy for protecting the liver against the detrimental effects of hepatic IRI (12). Our previous study demonstrated that short-term starvation effectively attenuated liver IRI by inhibiting hepatocellular apoptosis in the mouse liver IRI model (1). In the present study, we aimed to verify whether preoperative short-term fasting could induce similar protective effects in patients undergoing hepatectomy.

Methods

Subjects and clinical design

This is a prospective, single-blinded, randomized study. The Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, in Nanjing, China (Ref: 2014/185) granted permission for the study protocol, and the trial was registered at the beginning of the study (Ref: ChiCTR-TRC-11001530, September 2014). The research was performed from November 2015 to May 2016 in the First Affiliated Hospital of Nanjing Medical University. Patients with liver tumors were enrolled in this study when they were scheduled for a hepatectomy with inflow occlusion and informed consent was signed by each participant. We used the following inclusion criteria: (I) Child-Pugh classification for A, and (II) hepatectomy with Pringle maneuver, and the exclusion criteria: (i) <18 and >75 years old; having any of the following conditions: being completely blind and deaf; unconsciousness; Alzheimer's disease; anorexia nervosa; alcoholic insanity; epilepsy; schizophrenia; paralytic disease; serious chronic infection; extensive metastasis tumor; AIDS; gastric ulcer with hemorrhage; pregnancy and breastfeeding; weakness; malnutrition; diabetes type 1; diabetes type 2 requiring continuous insulin injections; cardiac failure; arrhythmia; uremia; or secondary hypertension (13-16); and (ii) intraoperative additional implementation of hepatic artery ligation. All patients were randomly divided into the control group (Ctrl group, traditional fasting) or the fasting group (Fasting group, preoperative fasting for 24 h). The randomization sequence was computer generated, and patients were distributed in a table of simple random numbers, which was showed in a CONSORT Figure (*Figure 1*).

Surgery

All of the operations were conducted by the same medical team to minimize bias. The operation was classified into 2 grades based on the extent of liver resection: Grade I was defined as local resection of the tumor, while Grade II was defined as a combined liver segmentectomy, including hemihepatectomy. After mobilization of the liver, inflow occlusion was performed with a 4-mm Mersilene

Table 1 Comparison of the demographic data between the Fasting group and the Ctrl group

Characteristic	Fasting group (n=30)	Ctrl group (n=33)	P value
Male/female (n)	27/3	26/7	0.51
Maximum tumor diameter (cm)	6.12±3.19	6.18±3.16	0.93
Multiple tumors N/P (n)	24/6	28/5	0.61
BCLC stage A/B	27/3	30/3	0.90
JIS score 0/1/2/3(n)	5/19/6/0	7/22/4/0	0.67
Platelets (104/aL)	10.53±4.17	10.15±3.89	0.39
Prothrombin time (%)	79.40±11.59	76.39±10.28	0.28
AFP (ng/dL)	187.30±286.83	284.36±330.71	0.22
ICG-15, %	6.97±1.92	4.39±2.00	0.25
Tumor types HCC/others (n)	22/8	29/4	0.16
Ischemia time (min)	21.53±4.71	19.67±6.90	0.22
Total operation time (min)	100.83±13.00	103.43±14.48	0.42
Blood loss (mL)	157.50±87.56	144.24±94.23	0.56
IBT N/P (n)	29/1	32/1	0.95
Grade I/II resection (n)	23/7	28/5	0.4

BCLC, Barcelona-Cl Liver Cancer; N/P, negative/positive; IBT, intraoperative blood transfusion; JIS, The Japan Integrate Staging.

tape around the hepatic pedicle. Time of continuous inflow occlusion was determined according to the required operation. We excluded patients with additional intraoperative implementation of hepatic artery ligation and other peculiar vascular clamping manners, which are considered to have great influence on postoperative outcomes (17). All of the complications occurring during and after surgery were recorded, such as blood loss, bile leaks, infections, and so on. All of the patients received similar treatment after surgery in the same medical unit, and were followed for their entire hospitalization.

General data and hepatic function

General data of patients and intraoperative and postoperative complications were collected in an electronic medical record system (SimLink Information System Co., Ltd., China) as shown in the study flow chart (*Table 1*, *Figure 1*). The elevation of serum aminotransferase occurs when liver cells are subjected to acute damage; hepatic IRI is an acute injury, so we chose hepatic function to reflect the real extent of IRI directly (18). Hepatic function was determined before and after the operations by Automatic Biochemistry Analyzer (Beckman Kurt Co., Ltd., USA).

Enzyme-linked immunosorbent assay (ELISA)

Serum TNF-α, IL-6, and IL-10 secretions were measured by ELISA kits (eBioscience Affymetrix Inc., USA) according to the manufacturer's standard protocols, which principally involved the mechanism of inflammation of hepatic IRI. Absorbance was read on a Multiscan FC plate reader and analyzed with SkanIt for Multiscan FC software (Thermo Scientific, Schwerte, Germany).

Quantitative real-time PCR

Liver tissue was harvested from the tissue adjacent to the tumor. Total RNA was extracted from liver tissue samples that had been frozen at –80 ℃ using a TRIzol kit following the manufacturer's protocol (Invitrogen, Shanghai, China), and RNA concentration was measured by a spectrophotometer (Thermo Scientific, Schwerte, Germany). cDNA was synthesized according to the manufacturer's instructions using HiScriptII Q RT SuperMix (+gDNA wiper) (Vazyme, USA). Polymerase chain reaction (PCR) was performed with SYBR Green Master Mix (High ROX Premixed) (Vazyme, USA). using the following primers: TNF-α (5'-CCT CTC TCT AAT

Table 2 Comparison of the postoperative complications between the Fasting group and the Ctrl group

Characteristic	Fasting group (n=30)	Ctrl group (n=33)	P value
Hemorrhage (n)	0	1	0.34
Biliary leakage (n)	1	0	0.30
Ascites (n)	2	3	0.72
Pleural effusion (n)	0	0	–
Incision infection (n)	1	0	0.30

CAG CCC TCT G-3' and 5'-GAG GAC CTG GGA GTA GAT GAG-3'); IL-6 (5'-ACT CAC CTC TTC AGA ACG AAT TG-3' and 5'-CCA TCT TTG GAA GGT TCA GGT TG-3'); IL-10 (5'-GAC TTT AAG GGT TAC CTG GGT TG-3' and 5'-TCA CAT GCG CCT TGA TGT CTG-3'); and β-actin: 5'-AGC GAG CAT CCC CCA AAG TT-3' and 5'-GGG CAC GAA GGC TCA TCA TT-3'). β-actin was used as an endogenous control.

Serum MDA and SOD assay

MDA and SOD are commonly used as markers to estimate oxidative stress (19). Serum MDA was measured using kits and performed according to the manufacturer's directions (Jiangcheng Biotechnology, Nanjing, China). The results are expressed in nmol MDA per liter of serum. SOD activity was measured by an indirect competition assay between SOD and the indicator compound, nitroblue tetrazolium (NBT), for the superoxide produced by xanthine/xanthine oxidase according to the method of Spitz and Oberley. The data are expressed as μmol/L.

Statistical analysis

Data are presented as mean ± SD. SPSS software (Chicago, IL, USA) was used to calculate the statistical significance by performing one-way analysis of variance. The χ^2 test was used, when appropriate, for analysis of categorical data. All of the P values were two-sided, and P<0.05 was considered to be statistically significant.

Results

Demographic data from the two groups

We randomized 80 patients, of whom 17 were later excluded; occlusion of hepatic blood flow or hepatic resection was not performed in 11 patients for poor liver reserve function (7 in the Fasting group and 4 in the Ctrl group), the hepatic artery was ligated in 4 patients (1 in the Fasting group and 3 in the Ctrl group), and another 2 patients did not continue to fast due to nurses misleading messages (2 in the Fasting group). The final number of patients eligible for analysis was 63 (30 in the Fasting group and 33 in the Ctrl group). No differences were observed in clinical and demographic data, including operative time, intraoperative blood loss, tumor types and sizes, time of clamping portal hepatis and the extent of liver resection BCLC stage, JIS score, platelets, prothrombin time, AFP, ICG-15, % (*Table 1*). Complications occurring throughout the entire hospitalization did not differ between the groups. Details of postoperative complications are reported in *Table 2*.

Short-term fasting improves hepatic function in patients undergoing hepatectomy

We first determined the effects of short-term fasting on hepatic function in patients undergoing hepatectomy. Transaminase (ALT and AST), albumin, and total bilirubin (TBIL) were collected to assess the liver injury pre-operation and at 1, 3, and 7 days post-operation. As shown in *Figure 2A,B*, sALT and sAST levels were significantly increased at POD-1, POD-3, and POD-7 compared with the pre-operation levels, especially at 1- and 3-day post-operation (P<0.05). Interestingly, preoperative 24 h fasting effectively attenuated an increase of sALT and sAST after hepatectomy, especially at 1- and 3-day post-operation (P<0.05). In addition, albumin and total bilirubin were analyzed, and there was no significant difference between the Ctrl group and the Fasting group (*Figure 2C,D*); but total bilirubin more rapidly declined in the Fasting group compared with the Ctrl group at 3 days post-operation (P<0.05; *Figure 2D*). These data indicate that preoperative short-term fasting effectively attenuates surgery-related liver injury.

Short-term fasting reduces inflammatory response in patients undergoing hepatectomy

Next, we estimated the effects of short-term fasting on the inflammatory response in patients undergoing hepatectomy. Gene expression of pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokines (IL-10) were examined in ischemic liver tissues.

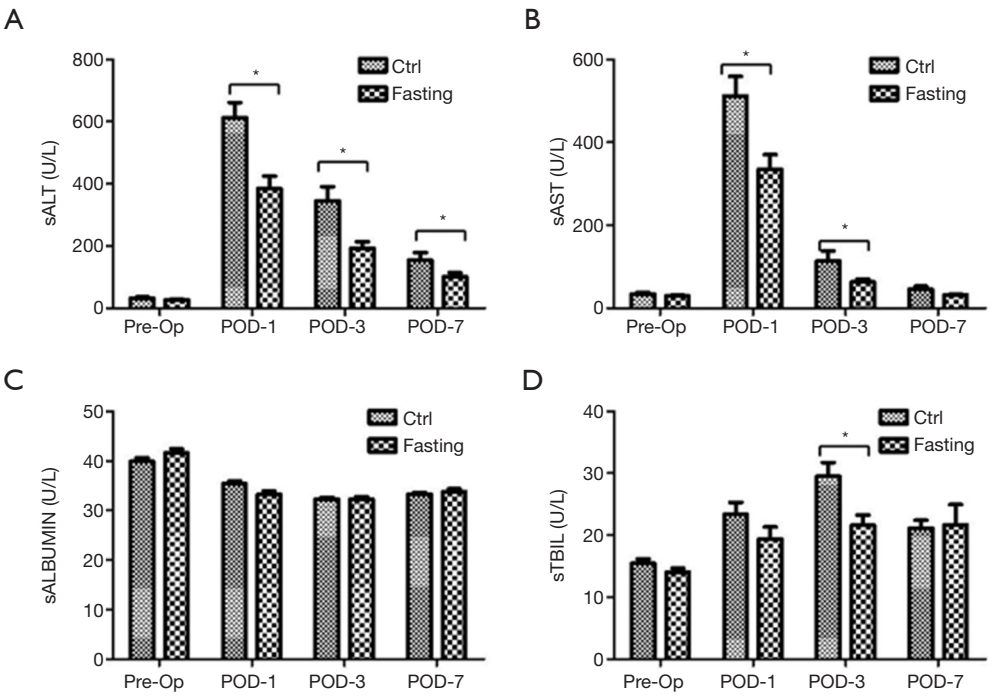

Figure 2 Twenty-four hours fasting improves hepatic function in patients undergoing hepatectomy. All patients were subjected to hepatectomy. Some patients were fasted 24 h prior to surgery. sALT (A), sAST (B), serum album (C), and total bilirubin (D) were collected to assess liver injury at pre-operation, and 1, 3 and 7 days after operation. (mean ± SD, *, P<0.05 *vs.* Ctrl group).

Figure 3A shows that fasting significantly inhibited the surgery-triggered expression of TNF-α (23.39±7.42 and 2.96±0.78, respectively; P<0.05), but there was no difference in IL-6 and IL-10 at the gene level between the Ctrl group and the Fasting group (*Figure 3A*). We further analyzed the expression of these cytokines in serum using ELISA and they were consistent with the gene expressions of the liver tissues. *Figure 3B* showed that fasting markedly attenuated the surgery-triggered expression of TNF-α in serum compared with the Ctrl group (P<0.05); however, the expressions of IL-6 and IL-10 were not significantly different at the protein levels in serum of both of the groups (*Figure 3C,D*). Generally, the above data reflect that preoperative short-term fasting effectively attenuates the inflammatory response, especially TNF-α expression.

Short-term fasting attenuates oxidative stress in patients undergoing hepatectomy

Oxidative stress plays a prominently causative role in liver injury following hepatectomy (20). Oxidative stress can trigger the cascade of cell damage, necrosis/apoptosis, and subsequent pro-inflammatory responses. MDA, an index of lipid peroxidation that indicates the levels of the oxygen free radicals, is significantly increased after liver resection (21). MDA was significantly decreased in the Fasting group compared with the Ctrl group at POD-3 and POD-7, but not at POD-1 (*Figure 4A*). In addition, antioxidant SOD was measured in serum, which showed that SOD was significantly decreased after surgery. Fortunately, SOD activity gradually recovered as time goes by. Interestingly, pre-operation fasting accelerated the recovery process compared with the Ctrl group (*Figure 4B*). These data indicate pre-operation short-term fasting attenuates oxidative stress after hepatectomy.

Short-term fasting activates nuclear factor erythroid-derived 2-related factor 2 (Nrf2) signaling pathway in liver tissue

The Nrf2, a member of the Cap'n'Collar subfamily of bZIP transcription factor family, plays a pivotal role in the defense system against inflammatory responses and oxidative stress. To further investigate relative mechanisms of protective

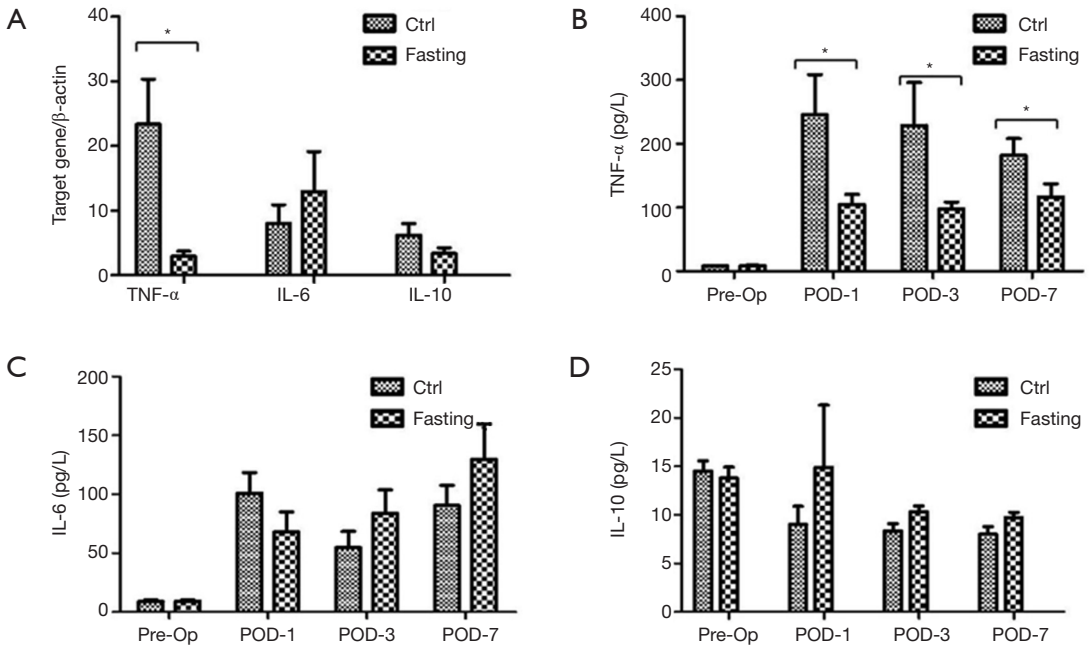

Figure 3 Twenty-four hours fasting reduces inflammatory response in patients undergoing hepatectomy. All patients were subjected to hepatectomy. Some patients were fasted 24 h prior to surgery. Expression of TNF-α, IL-6 and IL-10 (A) was analyzed by real-time PCR in liver tissue. Serum TNF-α (B), IL-6 (C) and IL-10 (D) were measured to assess inflammatory responses by ELISA at pre-operation, and 1 day, 3 and 7 days after operation. (mean ± SD, *, P<0.05 *vs.* Ctrl group).

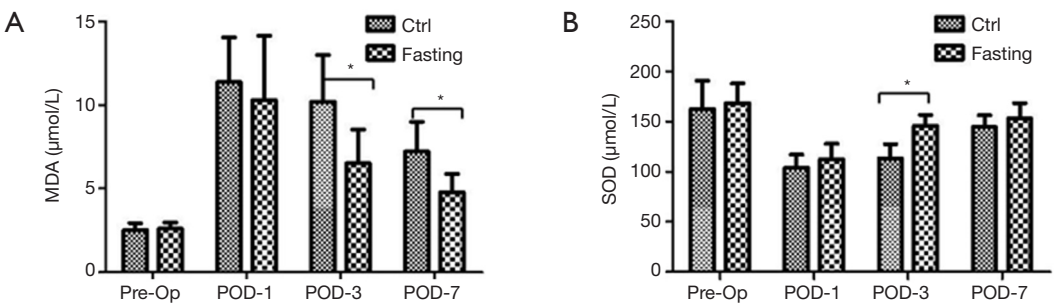

Figure 4 Twenty-four hours fasting attenuates oxidative stress in patients undergoing hepatectomy. All patients were subjected to hepatectomy. Some patients were fasted 24 h prior to surgery. Serum MDA (A) and SOD (B) were measured to assess oxidative stress by kits at pre-operation, and 1, 3 and 7 days after operation. (mean ± SD, *, P<0.05 *vs.* Ctrl group).

effects of short-term fasting on hepatectomy, Nrf2 signaling pathway was analyzed in liver specimens. As shown in *Figure 5A*, Nrf2 mRNA expression was significantly increased in fasting group compared with ctrl group. In addition, Nrf2 related genes, HO-1 and Nqo1, were further analyzed, which showed mRNA expression of HO-1 and Nqo1 were also markedly increased in fasting group (*Figure 5A*). Next, Nrf2 protein expression further determined by Western blot, which supported above results (*Figure 5B*). These data indicate short-term fasting activates Nrf2 signaling pathway.

Discussion

Liver IRI remains the major cause of liver dysfunction and failure after hepatectomy and liver transplantation (22,23).

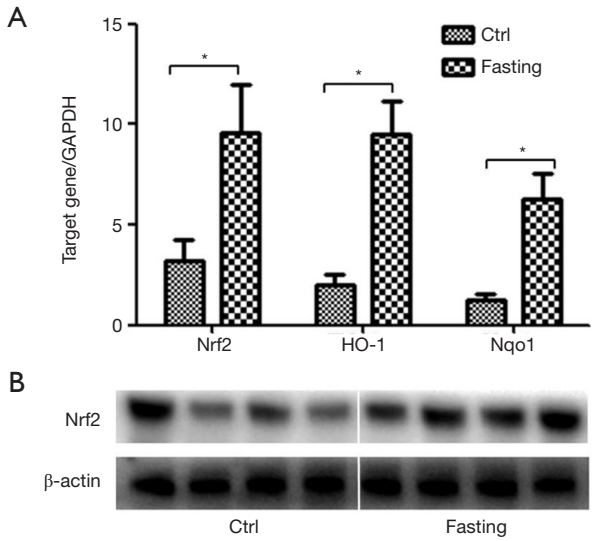

Figure 5 Twenty-four hours fasting activates Nrf2 signaling pathway in liver tissue. All patients were subjected to hepatectomy. Some patients were fasted 24 h prior to surgery. Expression of Nrf2, HO-1 and Nqo1 (A) was analyzed by real-time PCR in liver tissue; (B) expression of Nrf2 was analyzed by Western blot in liver tissue (mean ± SD, *, P<0.05 *vs.* Ctrl group).

CR and fasting have displayed some beneficial effects on the prolongation of life and increased resistance to stress, including liver IRI. Our previous study demonstrated that short-term starvation, including starvation for 1, 2, and 3 days, attenuated liver IRI in the mouse model, which piqued our interest in its clinical application during liver resection (1). Herein, we designed a prospective, single-blinded, randomized study to analyze the effects of preoperative short-term fasting on hepatectomy. One day (24 h), as a period of short-term fasting, is an acceptable and feasible time span for patients. To our knowledge, this is the first study analyzing the effects of short-term fasting on hepatic IRI after hepatectomy in human.

First, we collected the pre- and intra-operative demographic data, including age, gender, tumor size, tumor type, ischemic time, operation time, blood loss, and so on (*Table 1*). After analyzing the data, we noted that there was not a marked difference between the two groups, so comparing post-operative outcomes from both groups was reasonable. Next, we estimated the effects of fasting on liver injury by biochemical outcomes, which showed that 24 h fasting effectively attenuated the increase of sALT and sAST after operation. In addition, 24 h fasting relatively stabilized the effects of surgery on serum TBIL, but fasting did not affect the level of serum albumin. Furthermore, postsurgical complications of both groups were similar during the hospitalization of the patients. Taken together, short-term fasting improves the clinical outcomes to some extent in patients undergoing liver resection. This finding is consistent with our previous research, in which short-term starvation attenuated liver injury-induced by ischemia/reperfusion in the mouse liver IRI model (1). Johnson's group also demonstrated that alternate-day CR improved symptoms and pulmonary function in overweight adults with moderate asthma (24). In addition, CR or fasting improves numerous health indicators, including those associated with risk of cardiovascular disease, type 2 diabetes and cancers, in rodents, monkeys, and humans (25-28).

The inflammatory response is an important risk factor of liver injury after hepatectomy, especially innate immune responses involved in inflammatory cytokines (29-31). In our study, pro- and anti-inflammatory cytokines, including TNF-α, IL-6, and IL-10, were analyzed in liver tissues and serum. The reductions in the expression of TNF-α in liver tissues and serum of patients in the Fasting group compared to the Ctrl group were particularly striking; however, IL-6 and IL-10 did not significantly change in either of the groups. TNF-α is mainly responsible for the induction of neutrophil sequestration in the liver and directly mediates tissue injury (32-34), and TNF-α can activate hepatocellular programmed necrosis by the TNFR1 binding Fas-associated death domain (FADD) protein in many liver diseases (35). Thus, the decreased level of TNF-α suggests that 24 h fasting inhibits inflammatory responses and reduces hepatocellular death, which may contribute to the beneficial effects of fasting on clinical outcomes. In fact, Clavien's group and our group have demonstrated that one-day fasting downregulates the circulating levels of HMGB1, reduces inflammatory responses, and attenuates mouse hepatic IRI via the Sirt1-autophagy pathway (36).

Oxidative stress is another risk factor of liver injury in many conditions, including liver IRI, hepatectomy, and so on (37-40). MDA, an end-product of lipid peroxidation, is an important criterion of oxidative stress and reflects the levels of tissue oxidative stress. In this study, levels of MDA were significantly lower in the Fasting group compared with the Ctrl group at POD-3 and POD-7, but not at POD-1, which indicated that 24 h fasting increased the scavenging ability of oxygen free radicals and accelerated the remission of oxidative stress. In addition, SOD, a classical antioxidant enzyme, increases susceptibility of the antioxidant system

during liver IRI. In this study, 24 h fasting only increased the activity of SOD at POD-1 a little, but significantly enhanced the activity of SOD at POD-3, and it had almost the same level as the Ctrl group at POD-7. The above data indicated that fasting accelerated the recovery of SOD activity and then attenuated oxidative stress after hepatectomy, which was consistent with the MDA level. In parallel, fasting and postprandial blood glucose increments contributed to the oxidative stress and inflammation in dyslipidemic type 2 diabetic patients with stable ischemic heart disease (41).

Nrf2 plays a key role in the defense system against inflammatory responses and oxidative stress in various diseases conditions. Under homeostatic conditions, Nrf2 levels are maintained at low levels due to E3 ubiquitin ligase-mediated ubiquitylation and proteasome-dependent degradation of Nrf2 (42). Under stress conditions, Nrf2 degradation was markedly inhibited in the liver, heart, and brain (43-45). Activation of Nrf2 induces the expression of HO-1 and Nqo1, suggesting that Nrf2 is essential for the regulation of HO-1 and Nqo1. Numerous studies have indicated that Nrf2 signaling pathway plays a key role in inflammatory responses and oxidative stress during IRI (46,47). Our data displayed 24 h fasting effectively activates Nrf2 signaling pathway in liver tissues. Thus, Nrf2 signaling pathway may play critical role in short-term fasting against inflammatory responses and oxidant stress.

In summary, our findings show (I) 24 h fasting effectively improves serum hepatic biochemistry in patients undergoing hepatectomy; and (II) the fasting-associated protection feature may be closed related to reduced inflammation and oxidative stress; (III) Nrf2 signaling pathway may play key role short-term fasting against inflammatory responses and oxidant stress. Preoperative short-term fasting is inexpensive, noninvasive, and very attractive to implement in the clinical setting; however, since we do not know the exact fasting duration, including 1, 2, or 3 days, to induce the greatest advantages in patients, further clinical trials will need to be performed.

Acknowledgements

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.
Funding: This study was supported by the Foundation of Jiangsu Collaborative Innovation Center of Biomedical Functional Materials, the Priority Academic Program Development of Jiangsu Higher Education Institutions, the National Natural Science Foundation of China (81871259), Six talent peaks project in Jiangsu Province (2017-WSW-019), Huai'an science and technology planning project (HAB201740).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, in Nanjing, China (Ref: 2014/185) granted permission for the study protocol, and the trial was registered at the beginning of the study (Ref: ChiCTR-TRC-11001530, September 2014).

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Cite this article as: Zhan C, Dai X, Shen G, Lu X, Wang X, Lu L, Qian X, Rao J. Preoperative short-term fasting protects liver injury in patients undergoing hepatectomy. Ann Transl Med 2018;6(23):449. doi: 10.21037/atm.2018.10.64

