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MILD HYPOTHERMIA DOES NOT AFFECT LIVER REGENERATION AFTER PARTIAL HEPATECTOMY IN MICE

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Abstract

BACKGROUND—The use of mild Hypothermia has been suggested to be therapeutically useful in the setting of acute liver failure. It is not known if hypothermia influences liver regeneration.

AIM—To assess the effect of hypothermia on liver regeneration in mice.

METHODS—After partial (70%) hepatectomy (PHx) C57BL6/J mice were randomly assigned to either a hypothermic group or normothermic group. Controlled mild-hypothermia was maintained up to three hours after surgery. In addition, assessment of liver mass restitution, was examined studying the induction of key cell cycle proteins (cyclin A, D1 and E) and hepatocyte proliferation [assessment of proliferating cell nuclear antigen (PCNA) protein expression] by western blotting and DNA synthesis by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation by immunohistochemical techniques 45 hours after PHx.

RESULTS—PHx induced a vigorous proliferative response in the remnant livers of both groups of mice (normothermic and hypothermic group), as evidenced by the induction of key cyclins, PCNA and incorporation of BrdU after PHx. The liver/body weight ratio and both cyclin and PCNA protein expression as well as BrdU incorporation did not differ between the regenerating livers of hypothermic and normothermic groups.

CONCLUSION—Mild hypothermia does not influence liver regeneration in mice.

Keywords

liver transplantation; acute liver failure; cell cycle

Acute liver failure (ALF) is a clinical syndrome characterized by sudden hepatic synthetic dysfunction associated with hepatic encephalopathy and coagulopathy (1,2). ALF represents a major challenge for hepatologists, because it can rapidly lead to multiorgan failure and death (3). Major complications of acute liver failure that require active intervention include metabolic disorders, coagulopathy, cerebral edema, renal failure, and infection. The focus of management of ALF is to provide comprehensive supportive care in an intensive care unit and assess the need for liver transplantation (4,5). Intracranial hypertension due to cerebral edema leading to brain herniation is among the most serious complications of ALF and is associated with a poor prognosis (6). Management protocols for intracranial hypertension

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include several standard therapeutic measures such as head elevation, hyperventilation, mannitol administration and barbiturate infusion (7,8). The use of intracranial pressure monitoring is controversial but could be useful to guide therapy (9–12). Recently, the use of hypothermia as a treatment modality for increased intracranial pressure has been proposed based in the ability of this measure to reduce cerebral hyperemia and other cerebro-protective mechanisms (13–15). By reducing intracranial pressure mild hypothermia may, improve neurological end points which, if confirmed in well-designed clinical trials, can be viewed as an important clinical advance in this area (16).

A potentially troubling aspect of the use of hypothermia in patients with ALF is its hypothetical effects on hepatic regeneration (17). ALF is characterized by active regeneration which actually leads to some patients to full recovery due to the vigorous capacity of the liver parenchyma to regenerate (18,19). In fact, a significant proportion of patients with ALF survive with intensive medical therapy alone (8,20). Hypothetically, if hypothermia impairs hepatic regeneration some patients might have to undergo liver transplantation which could have been otherwise avoided under normothermic conditions(17). To date, no studies have specifically addressed this issue and no preclinical studies assessing the effect of hypothermia on hepatic regeneration have been conducted. Thus the aim of the present study is to directly explore the effect of hypothermia on liver regeneration in a mouse model.

MATERIALS AND METHODS

Products and Reagents

Unless otherwise stated all chemicals and reagents were obtained from Sigma (St. Louis, MO) and Bio-Rad Laboratories (Hercules, CA). Mouse monoclonal antibodies against 5-bromo-2-deoxyuridine (BrdU) were bought from Roche Diagnostics (Indianapolis, IN). Antibodies against proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin A, cyclin E and the goat-anti-mouse HRP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals, operations and experimental designs

Twelve-week-old C57BL6/J male mice (23–26 g body weight) were used for this study. Animals were maintained on a standard diet and kept on a daily 12-h light/12-h dark cycle with free access to food and water. Partial hepatectomy (PHx) (70%) was performed in mice by the individual ligation and resection of the left lateral and median lobes based on a conventional surgical technique (21,22) under isoflurane anesthesia. Gall bladder was intact after the resection of liver lobes. All experimental groups underwent hepatectomy at the same time of the day [between 1.00–2.30 pm] to avoid any confounding influences of circadian variation (23). The resected liver served as non-proliferating control tissue (0 h). Mice were randomly assigned to two experimental groups: the normothermic (NT) group (n=5) and the hypothermic (HT) group (n=9). Mice in the NT group were actively warmed with heating pads and heating lamps to maintain a body temperature at 36°C. Mice subjected hypothermia were allowed to undergo spontaneous reduction of the temperature by the withdrawal of active heating, achieving an average temperature of 32°C; this condition was maintained for 3 hours. Body temperature was measured continuously using a rectal thermometer. Animals were then allowed to recover and the remnant livers were harvested under anesthesia at 45h after PHx. This time point was chosen to assess regeneration because DNA synthesis closer to its peak, and to avoid confounding effects from circadian variation (36 h harvest involves hepatectomy in the morning and harvest in the evening approximately 12 hrs apart in the time of the day). Livers were weighed and preserved for subsequent analysis of proteins, histology and immunohistochemistry. The liver weight/body weight

ratio was calculated. All animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and the Ethics Committee of the Pontificia Universidad Catolica de Chile.

Western Blot Analysis of nuclear proteins

Nuclear proteins were isolated from the remnant livers after PHx as described by Hwang et al. (24). In brief, liver tissue was homogenized in a protease inhibitor cocktail and nuclear extracts were separated in 12% SDS-polyacrylamide gels. Protein concentrations of the homogenates were assessed using the BCA Protein Assay with bovine serum albumin as the reference and 20 µg of protein were loaded for each sample (Pierce Chemical Co, Rockford, IL). Gels were transferred to nitrocellulose membranes (Bio-Rad Transblot 0.2-µm membrane; Hercules, CA) by electroblotting. Membranes were blocked for 1 hour in Tris-Buffered Saline Tween-20 (TBST) (Tris-HCl 0.05 mol/L, NaCl 0.3 mol/L, Tween 20 at 0.1%, pH 7.6) plus 5% nonfat-dry milk. Membranes were then incubated overnight at 4°C with different antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions ranging from 1:500 to 1:2000 in TBST + 5% nonfat-dry milk and then washed before a secondary incubation with goat anti-mouse/rabbit antibody at a 1:2,000 dilution in TBST + 5% nonfat-dry milk for 1 h. Protein signal was revealed using a standard enhanced chemiluminescence method following the manufacturer's protocol (Perkin Elmer Life Sciences, Boston, MA). Bands were quantified densitometrically using the ImageQuant™ software. Density values for each sample were expressed as a percentage of the control from the same blot.

BrdU Immunohistochemistry

To assess the effect of hypothermia on liver DNA synthesis, mice subjected to PHx were injected with BrdU (50 mg/kg intraperitoneally) 2 h prior to the harvest of liver tissue. Liver sections were then immunostained for BrdU incorporation. In brief, paraffin-embedded liver sections were dewaxed by heating at 60°C and rehydrated. Slides were incubated in 0.1 N HCl with pepsin (EC 3.4.23.1; 0.05 mg/mL) for 4 minutes at 37°C. After rinsing, samples were incubated at 37°C in 2 N HCl for 20 minutes, and twice in 0.1 mol/L borate buffer (pH 8.5) for 5 minutes. Endogenous peroxidase activity was quenched by using 0.3% H₂O₂ in PBS. Nonspecific binding sites were blocked with PBS, 10% goat serum, and 0.5% Triton X-100 for 1 hour. Slides were incubated with an anti-BrdU mouse monoclonal antibody (1:200) for 1 hour in PBS, 2% goat serum. After washing, sections were exposed for 1 hour to biotinylated goat anti-mouse immunoglobulin G antibody (1:200) in PBS, 1.5% goat serum, followed by incubations with Vectastain ABC reagent, and 3,3'-diaminobenzidine containing urea-H₂O₂. Randomly selected fields (n=5) of H&E and BrdU immunostained liver sections were analyzed by light microscopy (40X) for BrdU incorporation hallmark of liver regeneration in response to PHx. The average number of BrdU-positive hepatocytes in each animal was used for subsequent analysis.

Statistical Analysis

Values are expressed as mean + SD. Mann-Whitney U test was used to assess differences between groups. A p value of <0.05 was considered significant. Data was analyzed by using MINITAB software (Version 13.20, Minitab Inc, State College, PA, USA).

RESULTS

Effect of Mild Hypothermia on liver growth

After PHx mice in the NT group were actively warmed and maintained at a significantly higher body temperature than mice of HT group during the 3 h period set in the experimental design. Mice in the HT exhibited a mean body temperature of 32°C (Fig 1). At

the time of harvesting liver weight was similar in both experimental groups [NT group: 0.582 ± 0.06 and HT group: 0.527 ± 0.05 , p : NS]. The body/liver weight ratio at 45 hrs after PHx was also similar in both experimental groups [NT group: 0.0244 ± 0.002 and HT group: 0.0228 ± 0.002 , p : NS] indicating that hypothermia did not affect liver regrowth in response to 70% PHx.

Effect of Mild Hypothermia on Nuclear Protein Expression

Nuclear proteins isolated from the remnant livers at 45 h after PHx were analyzed by western blotting as previously described (22). Protein expression in the remnant lobes of livers after PHx was compared with the protein expression observed in the resected lobe which was considered to be the control or basal value for comparison.

Cyclin D1 protein expression was higher in the remnant livers as compared to the control-resected lobes during partial hepatectomy- (NT group –5.0-fold higher than control, HT group 4.4-fold higher than control; $p < 0.05$), but without a statistically significant difference between NT and Mild-HT group (Fig 2A). Also, no differences were observed in both Cyclin A and PCNA protein expression between the NT and Mild-HT groups. Expression of Cyclin A was higher in NT (7.3-fold higher than control, $p < 0.001$) and HT group (6.6-fold higher than control, $p < 0.05$) while PCNA protein expression was induced by 8.5-fold compared to resected lobes (control) in NT group and 8.4-fold compared to resected lobes (control) in HT group compared to resected liver lobe (control group) (Fig 2B and 2C).

Cyclin E expression did not show a statistically significant difference between both groups or between resected lobes and remnant livers (NT group 1.1-fold of control, HT group 1-fold of control) (data not shown).

Effect of Mild Hypothermia on DNA Synthesis

To evaluate whether hypothermia abrogated hepatocyte proliferation we assessed the degree of DNA synthesis in both groups of remnant livers by BrdU immunohistochemistry.

At 45 hours after PHx a significant number of labeled nuclei was observed in both groups, with no statistical differences between NT and mild-HT mice (Figure 3).

DISCUSSION

Mild hypothermia is associated to several metabolic and hemodynamic effects which may be therapeutically beneficial in patients with intracranial hypertension seen due to ALF (15,25). However, concerns have been raised regarding the clinical use of hypothermia in this setting due to its potential effects on liver regeneration (17). Several mechanisms may be involved in a negative effect of hypothermia on liver regeneration including decrease in cardiac output and mesenteric perfusion and reduction in hepatic blood flow, changes in the expression of several genes associated with hepatic regeneration or relevant down-regulation of biochemical reactions required for hepatic regeneration (17). The present study is the first preclinical study specifically addressing the effects of mild hypothermia on the proliferative process associated with liver tissue regeneration. For that purpose we used the classic experimental model of rodent two-thirds PHx (26) and found no significant differences between NT and HT experimental groups. Both groups exhibited a robust regenerative response consisting of a marked increase in the expression of key cyclins and PCNA as well as an increase in DNA synthesis as shown in BrdU immunohistochemistry analysis. Our data is in agreement with a recent study which, aiming to demonstrate that mild hypothermia is able to attenuate liver injury and improve survival in mice with acetaminophen toxicity, showed that mild hypothermia does not delay or impair hepatocyte DNA synthesis (27).

Protocols guiding the use of hypothermia in the clinical setting vary among published reports (13,25). In our experimental setting we followed a widely accepted protocol (27) consisting in a 3h-long single period of hypothermia. It might be possible that longer periods of hypothermia adversely affect regeneration by inducing hemodynamic changes and modifying plasma cytokine levels (28). Thus, when comparing the clinical and experimental setting, the relatively short duration and the one-time use of the hypothermic maneuver could be viewed as a limitation of the present study. Practical limitations of using rodents as experimental model preclude the use of protocols similar to those used in humans. Indeed, further studies are needed to explore if the use of repetitive or continuous hypothermia could abrogate an already triggered regenerative response.

The experimental model used in this study (i.e. two-thirds PHx) is among the most widely used in studying liver regeneration (26). However, one have to take into account that reduction of liver mass is not associated with the extensive tissue damage and necrosis seen when liver injury is caused by toxic or viral agents which should be considered when extrapolating the results of the present study. PHx it is associated to elevated serum aminotransferase levels and presence of apoptotic bodies, scattered foci of swollen hepatocytes variably associated with haemorrhage and small cell infiltrates, indicating the occurrence of some extent of liver injury (29). However, the potential differences among regenerative processes in these two settings have not been studied in detail. Thus, it can be speculated that differences might exists with regard to the occurrence of circulatory abnormalities and changes in plasma cytokine levels as well as in the extent of activation of the progenitor/stem cell compartment (18,30). Differences may also exist with regard to the effects of hypothermia in different experimental models such as PHx, acetaminophen toxicity or ischemia/reperfusion injury. It has been recently recognized that profound hypothermia occurs spontaneously in acetaminophen toxicity and therefore to increase body temperature to 32°C is actually therapeutic in this setting (27). In the case of ischemia/reperfusion injury, the benefit is associated to significant inhibition of the inflammatory response normally seen following hepatic I/R (31). This mechanism could also operate in the PHx model.

In conclusion, the present study shows that mild hypothermia does not have detrimental effects on liver regeneration in mice. Thus, the use of this therapeutic maneuver in the setting of ALF is probably safe in this regard.

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Abbreviations used in this paper

ALF	Acute Liver Failure
PHx	Partial Hepatectomy
BrdU	5-bromo-2-deoxyuridine
HT	Hypothermic
NT	Normothermic
PCNA	Proliferating cell nuclear antigen

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FIGURE 1.

Body temperature of mice subjected to partial hepatectomy. Rectal temperature was continuously registered in both experimental groups. Control mice were actively warmed with heating pads and heating lamps to maintain a body temperature at 36°C while mice subjected hypothermia were put under a spontaneous reduction of the temperature, achieving an average temperature of 32°C.

FIGURE 2.

Representative Western blot analysis and quantitative densitometric analysis of commonly used markers of liver regeneration in mice subjected to partial hepatectomy with and without hypothermia. Protein levels of cyclin D1 (Panel A), cyclin A (Panel B) and proliferating cell nuclear antigen (Panel C) were measured 45 h after partial hepatectomy. Resected livers (0 hrs) served as non-proliferating control tissue. Values are means $\pm$ SD [n= 5 (NT); n= 9 (HT)]

FIGURE 3.

Representative images of BrdU immunohistochemistry in mice subjected to partial hepatectomy with and without hypothermia. Remnant lobes were resected 45 h after hepatectomy and the number of BrdU positive cells per field (40x) was measured. Values are mean + SD. [n= 5 (NT); n= 9 (HT)]