Ischemia reperfusion injury of the marginal liver:

Identification and evaluation of novel therapeutic strategies

Dissertation
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Table of contents

Abbreviations

atg4b: autophagy-related protein 4b

ATP: Adenosine Triphosphate

BMI: Body Mass Index

eNOS: endothelial nitric oxide synthase

IRI: Ischemia Reperfusion Injury

JNK: c-Jun N-terminal kinase-1

LC3: light chain 3

NAFLD: Nonalcoholic Fatty Liver Disease

NO: nitric oxide

PKC: protein kinase C

TNFα: tumor necrosis factor alpha

UNOS: United Network for Organ Sharing

WHO: World Health Organization

Zusammenfassung

Hintergrund:

Der Bedarf an Lebertransplantaten ist in den letzten Jahrzehnten kontinuierlich gestiegen. Die Diskrepanz zwischen verfügbaren Spenderorganen und dem exponentiell ansteigendem Bedarf wird immer deutlicher. Eine Strategie das Problem anzugehen und den Spenderpool zu erweitern, ist die Verwendung marginaler Organe, wie z.B. steatotische Lebern oder Lebern älterer Spender. Es ist jedoch bekannt, dass marginale Lebern anfälliger für den Ischämie/Reperfusions-Schaden (IRI), eines der größten Risiken für post-operative Komplikationen und Morbidität, sind. Jüngste Studien legen nahe, dass einige dieser Mechanismen sowohl an der Entwicklung von Steatose und Seneszenz als auch dem IRI beteiligt sind. Wir stellten die Hypothese auf, dass therapeutische Ansätze, die auf diese Mechanismen abzielen, besonders nützlich sein könnten, um marginale Lebern vor IRI zu schützen.

Ziel:

Wir legten besonderes Augenmerk auf die Erforschung der gemeinsamen Mechanismen der Prozesse, die zu Steatose und Seneszenz sowie dem IRI beitragen. Uns interessierte die Identifikation und Evaluation einer therapeutischen Strategie, um steatotische Lebern vor IRI zu schützen. Weiterhin diskutierten wir die gemeinsamen Mechanismen und schlugen neuartige therapeutische Ansätze vor, die auf die gemeinsamen Mechanismen aller drei Prozesse - Steatose, Seneszenz und IRI - abzielen.

Ergebnisse und Diskussion:

Wir konnten zeigen, dass der intrazelluläre Energiestoffwechsel, die Entzündungsantwort und die Autophagie kritische Mechanismen sowohl in Steatose als auch in IRI sind. Wir stellten die Hypothese auf, dass therapeutische Strategien, die auf diese Mechanismen abzielen, besonders effektiv zum Schutz der steatotischen Lebern vor IRI eingesetzt werden könnten. Wir zeigten, dass die Behandlung mit Lithiumchlorid durch die Modulation der GSK3b and ERK1/2-Signalwege autophagieinduzierend wirkt und außergewöhnlich protektive Effekte beim IRI in steatotischen Lebern zeigte. Wir schlussfolgerten daher, dass Strategien, die auf gemeinsame Mechanismen, wie zB die Autophagie, abzielen, besonders nützlich sein könnten, um steatotische Lebern vor IRI zu schützen.

Wir legten in einen systematischen Review dar, dass der intrazelluläre Energiestoffwechsel, die Entzündungsantwort und die Autophagie sowohl in Seneszenz als auch in den IRI involviert sind. Wir haben festgestellt, dass therapeutische Strategien, die auf diese Mechanismen abzielen, ebenfalls besonders nützlich sind, um ältere Lebern vor IRI zu schützen. Erste Ergebnisse beim Einsatz von Lithiumchlorid legen dessen potentiellen Nutzen auch in diesem Zusammenhang nahe. Wir beobachteten, dass eine Behandlung mit Lithiumchlorid die Werte der Leberenzyme senkte und die nekrotischen Areale in älteren Lebern nach IRI reduzierte.

Weiterhin diskutierten wir gemeinsame Mechanismen in den Steatose-, Seneszenz- und IRI-Prozessen. Wir konnten zeigen, dass Steatose und Seneszenz auf unterschiedliche Weise den intrazellulären Energiestoffwechsel beeinträchtigen. Therapeutische Ansätze, die allein entweder den Glukose- oder den Fettstoffwechsel anvisieren, schützen ältere steatotische Lebern wahrscheinlich nicht vor IRI. Kombinierte Therapien oder die maschinelle ex-vivo Perfusionstechnik sollten als vielversprechende therapeutische Strategien in Erwägung gezogen werden. Die Entzündungsantwort ist der kritischste Prozess in sowohl Steatose als auch bei der Seneszenz. Therapeutische Ansätze, die die Entzündungsantwort adressieren, wie beispielsweise die Verwendung von Melatonin, haben nachweislich steatotische sowie auch seneszente Lebern vor IRI geschützt. Daher könnte Melatonin ein guter Kandidat sein, alte und steatotische Lebern vor IRI zu schützen. Autophagie ist ein weiterer Mechanismus, der sowohl in Steatose als auch Seneszenz eine Rolle spielt. In unserer Studie konnten wir zeigen, dass die Autophagieinduktion durch die Gabe von Lithiumchlorid steatotische Lebern vor IRI zu schützen vermochte. Da Autophagie in steatotischen und älteren Lebern beeinträchtigt ist, sollte die Induktion der Autophagie ebenfalls als eine der vielversprechenden Strategien angesehen werden, alte und steatotische Lebern vor IRI zu schützen.

Schlussfolgerungen:

Anhand einer Literaturauswertung konnten wir darlegen, dass intrazellulärer Energiestoffwechsel, Entzündungsantwort und Autophagie drei kritische Mechanismen darstellen, die den Steatose-, Seneszenz- und IRI-Prozessen gemein sind. Wir konnten zeigen, dass die Behandlung mit Lithiumchlorid die Regulation von Autophagie beeinflusst und erstaunliche protektive Effekte bei IRI in steatotischen Lebern zeigt. Weiterhin konnten wir zeigen, dass therapeutische Strategien, die auf die gemeinsamen Mechanismen in Steatose und Seneszenz abzielen, besonders nützlich sein könnten marginale Lebern, insbesondere alte und steatotische Lebern vor IRI zu schützen.

Summary

Background:

The need for liver grafts has constantly increased in the past decades. The discrepancy between the declining availability of donor organs and the exponentially growing demand has becoming more and more obvious. To expand the donor organ pool by accepting marginal organs such as steatotic livers and aged livers is one of the strategies to solve this problem. However it is well accepted that marginal livers are more vulnerable to ischemia reperfusion injury (IRI), which is considered as one of the major risks of post-operative morbidity and mortality. Recent studies suggested that there were common mechanisms involved in both steatosis respectively senescence and the IRI processes. We hypothesized that therapeutic strategies targeting these common mechanisms might be especially useful in protecting marginal livers from IRI.

Aim:

We put our emphasis on exploring the common mechanisms in senescence/steatosis process and IRI process. We also aimed to identify and to evaluate one of these therapeutic strategies targeting the common mechanisms to protect steatotic livers from IRI. Furthermore, we discuss common mechanisms and suggest novel therapeutic strategies targeting common mechanisms in all three, steatosis, senescence and IRI processes.

Results and Discussions:

We demonstrated in a literature work up that intracellular energy metabolism, inflammatory response and autophagy are critical common mechanisms involved in both steatosis and IRI processes. We hypothesized that therapeutic strategies targeting these mechanisms might be particularly effective in protecting steatotic livers from IRI. We demonstrated that lithium chloride treatment induced autophagy and exhibited outstanding hepatoprotective effects during IRI in steatotic livers via a modulation of GSK3b and ERK1/2 pathways. We concluded that strategies targeting common mechanisms such as autophagy might be especially useful to protect steatotic livers from IRI.

We concluded in a systematic review that intracellular energy metabolism, inflammatory response and autophagy are also involved in both senescence and IRI processes. We have

determined that the therapeutic strategies targeting these mechanisms are also particularly useful for protecting aged livers from IRI. Preliminary results using lithium chloride suggest its potential suitability also in this setting. We observed that treatment with lithium decreased the level of liver enzymes and reduced the area of confluent necrosis in aged livers after IRI.

We further discussed common mechanisms in all three processes: steatosis, senescence and IRI. We demonstrated that steatosis and senescence processes are associated with different type of impairments of intracellular energy metabolism. Therapeutic strategies targeting only either glucose or lipid metabolism might be not expected to protect aged steatotic livers from IRI. Combination therapies or ex-vivo machine perfusion technique should be considered as promising therapeutic strategies. The inflammatory response is the most critical process in both steatosis and senescence processes. Therapeutic strategies targeting the inflammatory response, such as the use of melatonin, have been proven to protect both steatotic livers and aged livers from IRI. Therefore, melatonin could be a drug candidate to protect aged steatotic livers from IRI. Autophagy is another common mechanism in both steatosis and senescence processes. We demonstrated in our study that induction of autophagy by lithium chloride treatment protected steatotic livers from IRI. Since autophagy is inhibited in both steatotic and aged livers, induction of autophagy could also be considered as one of promising strategies to protect aged steatotic livers from IRI.

Conclusions:

We concluded that intracellular energy metabolism, inflammation and autophagy are three critical common mechanisms in steatosis, senescence and IRI processes. We concluded that lithium chloride treatment targets the regulation of autophagy and exhibits outstanding hepatoprotective effects during IRI in steatotic livers. Furthermore, we demonstrated that therapeutic strategies targeting common mechanisms in steatosis and senescence processes may be particularly helpful in protecting marginal livers such as aged steatotic livers from IRI.

Introduction

The need for liver grafts is rising constantly. Since the first case was performed by Thomas Starzl in 1963, human liver transplant is considered as the ultimate strategy for the patients with end-stage liver diseases, acute liver failure or hepatic malignancies (Zarrinpar und Busuttil 2013). Over the past decades, the number of liver transplant cases has constantly increased. According to the report from United Network for Organ Sharing (UNOS), 7841 cases of liver transplantation were performed in the United States in 2016. However, the number of patients still on the waiting list has increased even faster. Based on UNOS data as of July 15, 2017, due to the lack of adequate donors over 20 patients die in average every day while waiting for transplantation in the United States. To ease the discrepancy between the substantially growing demand and the constant insufficient supply of donors, extending the donor pool is a matter of urgency.

Organs from extended criteria donors, so called “marginal organs”, include steatotic livers and aged livers (Busuttil und Tanaka 2003). To expand the donor organ pool by widely accepting marginal organs is the most practical strategy to relieve the problem of donor organs shortage.

However, the use of marginal livers is identified as an obvious risk of post-operative morbidity and mortality (Busuttil und Tanaka 2003). The increased risk is partly related to an increased IRI. It is well accepted that steatotic livers are more vulnerable to IRI (Chu et al. 2013). It also has been demonstrated already in 1994, that old livers are more sensitive to hypoxia/reoxygenation stress (Le Couteur et al. 1994).

The key mechanisms of hepatic IRI have been well explored since 1990s (Jaeschke et al. 1990, Jaeschke 1991, Jaeschke und Farhood 1991). It is well accepted that reperfusion injury occurs when blood supply returns to livers after a period of ischemia. There are two phases of reperfusion, which are defined as initial phase and later phase. During ischemia, the intracellular energy content of hepatocytes is depleted. Damaged mitochondria produce less adenosine triphosphate (ATP) but more free radicals, which leads to a direct hepatic damage (Fondevila et al. 2003). Inflammation plays the most critical role in both, initial and later phases in IRI (Jaeschke und Farhood 1991, Serracino-Inglott et al. 2001). The activation of Kupffer cells is considered as the central event of initial phase. The activation of neutrophils is the most critical event of later phase. The aggravated inflammatory response leads to both

programed and non-programed cell death, also known as apoptosis and necrosis. Recent studies reported that autophagy primarily affects the later phase of reperfusion (Cursio et al. 2015). Autophagy protects hepatocytes during IRI by degrading dysfunctional mitochondria and increasing the intracellular energy content.

Recent studies suggested that there are common mechanisms in both steatosis/senescence and IRI processes. Intracellular energy metabolism, inflammatory response and autophagy are considered to be involved in these processes. These findings support the hypothesis that therapeutic strategies targeting these central mechanisms might be especially useful in protecting marginal livers from IRI.

In this study, we want to investigate common mechanisms in steatosis, senescence and IRI processes, to identify potential therapeutic strategies targeting these mechanisms and to evaluate one of these therapeutic strategies to protect steatotic and aged livers from IRI in a rat model.

Hypotheses

Based on the described considerations we raise the following hypothesis:

- Steatosis, senescence and IRI share common mechanisms.
- Therapeutic strategies targeting the common mechanism of steatosis, senescence and IRI processes might be especially useful in protecting marginal livers from IRI.

Aims

We set three aims for this study:

- To identify common mechanisms and to identify potential therapeutic strategies targeting common mechanisms in steatosis and IRI processes as well as to evaluate one of these therapeutic strategies to protect steatotic livers from IRI.
- To identify common mechanisms and to identify potential therapeutic strategies targeting common mechanisms in senescence and IRI processes.
- To discuss common mechanisms and to suggest novel therapeutic strategies targeting common mechanisms in all three, steatosis, senescence and IRI processes.

Study Design

In order to reach the aims we designed a study composed of three corresponding steps:

- Generate a literature work up to identify the common pathophysiological mechanisms in steatosis and IRI processes and to identify promising therapeutic strategies to protect steatotic livers from IRI. Select and evaluate one of the strategies to protect steatotic livers from IRI in a rat model.
- Generate a systematic review to identify the pathophysiological mechanisms rendering aged livers highly susceptible to IRI and identify promising therapeutic strategies to protect aged livers from IRI.
- Discuss common mechanisms in steatosis, senescence and IRI processes to generate novel hypothesis for further testing of promising therapeutic strategies.

Results and Discussion Part 1:

IRI in steatotic livers and the protective effect of the autophagy inducing drug lithium chloride

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1a. Pathophysiology of IRI in hepatic steatosis and potential therapeutic strategies.

In 21th century, the obesity rate of the human population is constantly rising. A fact sheet from World Health Organization (WHO) in 2014 noted that more that 13% of adults, equal to 600 million people, were obese (Body mass index (BMI) ⩾30). Nearly 2 billion adults were overweight with a BMI ⩾ 25. High obesity rate is associated with a high incidence rate of nonalcoholic fatty liver disease (NAFLD) (Rinella 2015). Due to the fact that NAFLD is normally a silent disease, the knowledge of the exact incidence of the world is still limited. According to studies of different countries, NAFLD affects 10% to 24% of general population or 57.5% to 74% of obese population (Obika und Noguchi 2012).

Accepting steatotic livers is a promising strategy to expand the donor organ pool. However previous studies reported that using steatotic liver leads to an increased risk for postoperative morbidity and mortality after liver transplantation due to an increased IRI (Reiniers et al. 2014).

From the early description of NAFLD, the pathogenesis of this disease is still not fully understood. Nevertheless, intracellular energy metabolism, inflammation and autophagy are considered as three key mechanisms involved in the development of NAFLD (Rinella 2015, Byrne und Targher 2015, Lavallard und Gual 2014).

It is well known that the intracellular energy metabolism disorder, especially the lipid disorder, plays a central role in the development of NAFLD. As a result of the energy metabolism disorder, fat accumulates in hepatocytes. The aggravated accumulation of fat in hepatocytes ultimately leads to a NAFLD (Rinella 2015).

The activation of the inflammatory response is considered to play a critical role in the development of NAFLD. Mixed inflammatory cell infiltration is one of the most typical biopsy features in livers with steatohepatitis. The activation of inflammatory response is also considered as the major reason leading to hepatic fibrosis and ultimately even to hepatic cirrhosis, the alter stages of NAFLD (Rinella 2015).

Recent studies have also shown that autophagy plays a key role in the development of steatosis. It is reported that hepatic steatosis is associated with a reduced autophagic flux (Lavallard et al. 2012). Autophagy involves the recycling of dysfunctional organelles, and is essential to the maintenance of hepatic metabolism. As a self-protective response, autophagy protects hepatocytes from different types of stress (Yorimitsu und Klionsky 2005, Codogno und Meijer 2005). The impairment of autophagy weakens the ability of hepatocytes to resist steatohepatitis. Liu et al. demonstrated in a comprehensive review that steatosis impaired autophagy via a modulation of insulin-mTOR signaling pathway in mice (Liu et al. 2009). Furthermore, a number of studies suggested that activation of autophagy ameliorates hepatic steatosis (Sinha et al. 2014, Lin et al. 2013, Singh et al. 2009). The accumulation of fatty acids is one of the main pathological features in the development of NAFLD. Since autophagy maintains intracellular energy levels via a recycling of cellular components including the lipid droplets, induction of autophagy to reduce hepatic triglyceride levels is a promising strategy to ameliorate hepatic steatosis.

As described before, IRI process is dominated by three critical mechanisms: intracellular energy metabolism, inflammatory response and autophagy. Intracellular energy metabolism plays a critical role in ischemia phase and early phase of reperfusion. Inflammatory response is the most pivotal mechanism in both early and later phases of reperfusion. Autophagy mainly affects the later phase of reperfusion.

Since intracellular energy metabolism, inflammatory response and autophagy are involved in both steatosis and IRI processes, therapeutic strategies targeting these mechanisms might be particularly effective in protecting steatotic livers from IRI. Studies investigating therapeutic strategies to protect steatotic liver grafts against IRI in are listed in following Table 1.

Therapeutic strategies targeting intracellular energy metabolism are very efficient to protect steatotic livers from IRI. As early as 2009, Evans et al. demonstrated that vitamin E succinate treatment protects fatty livers from warm IRI by enhancing the hepatic energy status. Zaoualí et al. demonstrated that melatonin protected fatty livers from cold IRI by relieving intracellular energy disorder. It is well accepted that ex-vivo machine perfusion technique significantly increases the level of intracellular energy contents and reduces oxygen free radicals release. Bruinsma et al. reported that subnormothermic machine perfusion is particularly useful for protecting steatotic livers from IRI.

Therapeutic strategies targeting the inflammatory response are also well explored in steatotic livers. A series of drugs have been reported to prevent hepatic IRI by addressing the inflammatory response. Zaoualí et al. demonstrated that melatonin protected steatotic livers from IRI by modulation the nitric oxide (NO) pathway. Other inflammatory regulating drugs such as ankaflavin (inhibition of the activity of Kupffer cells) and theaflavin (regulation of TNFα pathway) are also reported as promising therapeutic strategies to protect steatotic liver grafts against IRI.

Current experimental evidence regarding the efficacy of targeting autophagy to protect steatotic livers from IRI is still limited. However, in 2011 a clinical study suggested that ischemic preconditioning protects fatty livers from IRI by inducing autophagy (Degli Esposti et al. 2011).

Therefore, we hypothesize that a therapeutic strategy inducing autophagy is especially effective to reduce IRI in steatotic livers. A number of drugs such as rapamycin (Pan et al. 2009), carbamazepine (Hidvegi et al. 2010), verapamil (Kania et al. 2017) as well as lithium chloride (Motoi et al. 2014) were proven to induce autophagy. Liu et al. demonstrated that lithium chloride treatment protects normal livers from IRI via a modulation of autophagy (Liu et al. 2013). Since autophagy is involved into both steatosis and IRI processes, induction of autophagy by lithium chloride treatment is also a promising to protect steatotic livers from IRI.

Table 1: Therapeutic strategies against hepatic IRI in steatotic livers

Authors and Year	Animal model	Steatosis model	IRI model	Key statement
(Evans et al. 2009) [#]	C57BL/6 mice	Leptin -/- knockout	Total liver warm IRI 15 min	Vitamin E succinate protects fatty livers from IRI by enhancing hepatic energy status and preventing oxidative damage.
(Andraus et al. 2010)	Wistar rats	protein-free diet for 3 weeks	70% liver warm IRI 60 min	S-Nitroso-N-Acetylcysteine protects against IRI in fatty livers but not in normal livers.
(Zaouali et al. 2011) [#,$]	Homozygous Zucker rats		Cold IRI for 24 hours	Melatonin protects normal and fatty livers from IRI by addressing intracellular energy disorder and inflammatory response.
(Luo et al. 2012) [$]	C57BL/6 mice	Methionine- and choline-deficient high fat diet for 2 weeks	Total liver warm IRI 15 min	Theaflavin protects fatty livers from IRI by addressing oxidant stress, inflammatory response and apoptosis.
(Yang et al. 2014)	Sprague-Dawley rats	Methionine- and choline-deficient diet for 12 weeks/ High fat diet for 8 weeks	70% liver warm IRI 60 min	Losartan protects fatty livers from IRI.
(Bejaoui et al. 2015a)	Homozygous Zucker rats		Cold IRI for 24 hours	Acetazolamide protects fatty livers from IRI.
(Bejaoui et al. 2015b)	Homozygous Zucker rats		Cold IRI for 24 hours	Carbonic anhydrases II protects fatty livers from IRI.
(Matsuda et al. 2015) [$]	C57BL/6 mice	High fat diet for 9 weeks	70% liver warm IRI 60 min	Activated protein C protects normal and fatty livers from IRI by addressing inflammatory response.
(Cahova et al. 2015) [$]	Wistar rats	High fat diet for 10 weeks	Total liver warm IRI 20 min	Metformin protects fatty livers from IRI by addressing reactive oxygen species formation and inflammatory response.
(Yang et al. 2015) [$]	C57BL/6 mice	High fat diet for 4-8 weeks	70% liver warm IRI 60 min	Ankaflavin protects fatty livers from IRI by modulating the function of Kupffer cells.

Therapeutic strategies targeting energy metabolism
$ Therapeutic strategies targeting inflammatory response

1b. Induction of autophagy reduces ischemia/reperfusion injury in steatotic rat livers

Introduction:

To investigate whether inducing autophagy by treatment with lithium chloride could protect fatty livers from IRI, a rat model of hepatic steatosis was used in this study. After induction of moderate hepatic steatosis, rats were pretreated with lithium chloride and then subjected to 70% selective warm ischemia for 60 minutes. In this experiment, autophagy, inflammation, apoptosis and potential related signal transduction pathways were investigated.

Summary:

In the first part of this study, we demonstrated that therapeutic strategies targeting intracellular energy metabolism, inflammatory response and autophagy are promising to protect steatotic livers from IRI. Since autophagy is a common mechanism of both steatosis and IRI processes, it was of great interest to extend this setting to steatotic livers. Therefore, we validated the hepatoprotective effect of autophagy in a rodent model of warm ischemia of the steatotic liver.

We confirmed in this study, that steatotic livers are more sensitive to IRI than normal livers. Comparing our results with the results of Liu et al., at 6 h after reperfusion, the plasma ALT level in steatotic livers was 20% higher than the level in normal livers.

After lithium chloride treatment, liver enzymes were reduced by half at 6 h after reperfusion. The inflammatory response, apoptosis and necrosis were also reduced in the lithium chloride treatment groups. The expression levels of phospho-GSK3b and phospho-ERK1/2 were higher in the treatment group compared to the control group. We demonstrated that treatment with lithium modulated both GSK3b and ERK1/2 pathways, induced autophagy and protected steatotic livers from IRI.

Conclusion:

In conclusion, lithium chloride treatment exhibited outstanding hepatoprotective effects during IRI in steatotic livers via a regulation of autophagy. Furthermore, we demonstrated that simultaneous modulation of GSK3b and ERK1/2 pathways is an interesting strategy to reduce IRI. This strategy might be especially useful in steatotic livers with an underlying impairment of autophagy.

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Manuscripts

Manuscript I

Induction of autophagy reduces ischemia/reperfusion injury in steatotic rat livers

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Authorship

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C. Kan, A. Liu., H. Fang., O. Dirsch and U. Dahmen contributed to conception and design, as well as for critical revision of the article;

C. Kan contributed for data collection, analysis, and interpretation;

M. Boettcher contributed for measurement of lithium chloride plasma levels;

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Induction of autophagy reduces ischemia/reperfusion injury in steatotic rat livers

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ABSTRACT

Background: Steatotic livers are particularly vulnerable to ischemia/reperfusion injury (IRI). One of the reasons is an underlying impairment of autophagy. Autophagy is regulated by glycogen synthase kinase 3b (GSK3b) and extracellular signal-regulated kinases (ERK1/2) pathways. Both of them are target proteins of a cell-protective drug, lithium chloride. Lithium chloride treatment reduces IRI in many organs including liver. Therefore, we aimed to investigate the effect of lithium chloride treatment on autophagy induction in steatotic rat livers. We also wanted to evaluate the related cell-protective effects on the enhanced hepatic IRI.

Materials and methods: After inducing hepatic steatosis, rats were injected with lithium chloride or normal saline for 3 d before being subjected to 70% selective warm ischemia for 60 min. After reperfusion, rats were observed for 30 min, 6, 24, and 48 h.

Results: Lithium chloride appeared to protect hepatocytes from IRI via its ability to induce autophagy by modulation of both GSK3b and ERK1/2 pathways. Hepatic damage was significantly decreased in the treatment group as indicated by a reduced inflammatory response, less apoptosis, less necrosis, and lower liver enzyme levels.

Conclusions: Simultaneous modulation of GSK3b and ERK1/2 pathways might be an interesting strategy to reduce IRI in steatotic livers with an impairment of autophagy.

Introduction

The lack of adequate organs for transplantation to meet the existing demand has resulted in a major organ shortage crisis. One of the strategies to solve this problem is to expand the donor organ pool by accepting marginal organs such as steatotic livers.[1]

Hepatic steatosis is associated with an increased risk for postoperative morbidity and mortality after major liver surgery. Evidence is accumulating that steatotic livers are particularly vulnerable to ischemia/reperfusion injury (IRI). A growing number of studies suggest that compared with normal livers, steatotic livers show a reduced autophagy level.

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The reduction of autophagy contributes to the aggravation of hepatic damage after IRI.[2,3]

Autophagy is a cellular response to various types of cell damage, also including ischemia. Cells are protected from death via maintaining intracellular energy levels by disassembling nonessential cellular components and by degrading impaired organelles such as mitochondria.[4,5] It increases the chance of cells to survive under extenuating circumstances such as starvation, hypoxia, and IRI.[5]

IRI is a multifactorial process that affects liver function after major hepatic surgery such as extended hepatectomy with Pringle manoeuver or liver transplantation.[6] During IRI, the activation of glycogen synthase kinase 3b (GSK3b)[7] and extracellular signal-regulated kinases (ERK1/2)[8] pathways are two of the major events modulating autophagy independently. On one hand, GSK3b pathway modulates autophagy indirectly by downregulating the activity of mTOR, which is well known as an inhibitor of autophagy.[7] On the other hand, phosphorylation of ERK1/2 leads to an activation of the beclin 1 pathway, which is directly involved in the autophagy progress.[9]

On the basis of these studies, a drug which induces autophagy via a modulation of both GSK3b and ERK1/2 pathways may represent a novel strategy for protecting steatotic livers from hepatic IRI.

Lithium chloride as a neuroprotective drug has been used in bipolar disorder treatment for over 100 y.[10] It is involved in a wide range of cellular functions, including cell cycle, death, and carcinogenesis, by inhibiting GSK3b pathway directly and indirectly.[11,12] In addition, lithium also acts on stress and survival pathways such as the ERK1/2 pathway[13] to protect neurons against neurodegenerative diseases.[14,15] In previous studies, treatment with lithium chloride regulated autophagy positively.[12,16,17]

Growing evidence suggests that lithium chloride treatment may reduce IRI in many organs such as brain, kidney, heart, and liver.[18-24] However, the potential of this compound to reduce IRI in steatotic liver with the known underlying impairment of autophagy is still unclear.

In this study, we want to investigate the hypothesis that enhancing autophagy with lithium chloride treatment could protect steatotic liver from ischemia/reperfusion injury.

Materials and methods

Experimental design

Rats were given a methionine/choline deficient with high fat-content (MCD + HF, Ssniff Spezialdiaeten GmbH, Soest, Germany; key components listed in Supplementary table) diet for 2 wk to induce moderate hepatic steatosis.[25,26] During the last 3 d of feeding the diet, rats in the treatment group were pretreated with lithium chloride. Animals in the control group were subjected to treatment with an equal volume of normal saline instead of lithium chloride. Thereafter, they were subjected to 70% selective warm ischemia for 60 min. Rats were sacrificed 0.5, 6, 24, and 48 h after reperfusion ($n = 6$/group). The following parameters were investigated: light chain 3 II/I (LC3) and p62 levels to assess autophagy; hepatic high-mobility group box (HMGB1) translocation and expression, neutrophil infiltration, as well as serum interleukin 6 and interleukin 10 levels to assess inflammation; cleavage of caspase 3 for apoptosis; phosphorylation level of GSK3b, ERK1/2, and JNK; and mechanistic target of rapamycin (mTOR) to investigate the underlying mechanism. Furthermore, we investigated the extent of hepatic necrosis and serum liver transaminase levels to quantify hepatic damage.

Animals

Male inbred Lewis rats (Charles River Laboratories, Sulzfeld, Germany) with a body weight from 250-320g at the start of the experiment were employed in the present study. Animals were housed under standard care conditions (humidity: 45%-70%, temperature: 21 ± 03°C, 12-hour light/dark cycle). Tap water and the respective diet were offered to the rats ad libitum. All procedures were carried out according to the German Animal Welfare Legislation (Thuringia State office for consumer protection, Germany, protocol number: 02-038/14).

Drug administration

0.9% saline solution was used to dissolve lithium chloride (Sigma-Aldrich Chemie GmbH, Munich, Germany). The concentration of lithium chloride solution was 2 mmol/mL. The solution was diluted immediately before injection. In the treatment group, rats were subjected to lithium chloride treatment (2 mmol/kg subcutaneously once daily) at 72, 48, and 24 h before operation, which was continued for 24 or 48 h according to the respective observation time.[20,24,27] Rats in the control group received an equal volume of saline solution.

Partial hepatic warm ischemia/reperfusion

Rats were placed in an anesthesia induction chamber using a concentration of 3% isoflurane (Piramal Healthcare Ltd, Northumberland, United Kingdom) in a vaporizer (Penlon Limited, Abingdon, United Kingdom) and oxygen flow of 0.5 L/min. After placing a transversal incision on the abdomen, the liver was exposed and the interlobular ligaments were dissected. The left portion of the hepatoduodenal ligament (including portal vein, bile duct, and hepatic artery to median and left lateral lobes) was clamped with a microvascular clamp for 60 min to induce 70% selective warm ischemia.[28] Animals were sacrificed 0.5, 6, 24, and 48 h after reperfusion. Left lateral lobe and right superior lobe were collected for histological analysis. Right median lobe and serum were collected for further protein analysis, snap frozen, and stored in liquid nitrogen until use.

Serum aminotransferases analysis

Serum alanine and aspartate aminotransferases were analyzed by using the AEROSET System (Abbott Laboratories GmbH, Hannover, Germany) according to the instructions of the manufacturers. The levels were compared to the results of serum alanine and aspartate aminotransferases levels in nonsteatotic livers, taken from our previously published study[19] (presented in Fig. 1B).

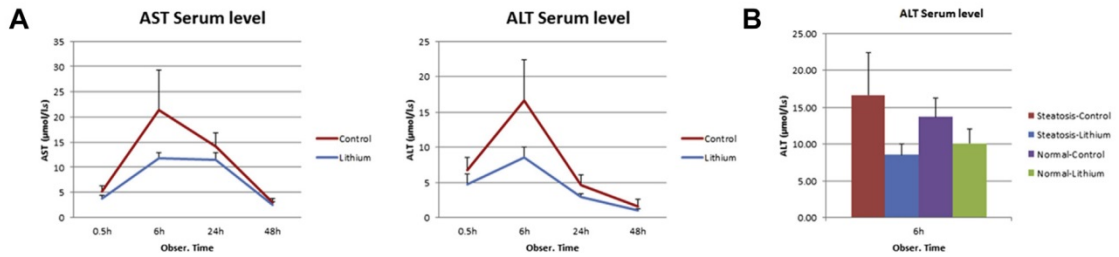

Fig. 1 – Induction of autophagy reduced hepatic injury induced by IRI. (A) Serum levels of AST and ALT after IRI. AST and ALT levels were obtained as indicators of hepatocellular injury. The serum levels of ALT after reperfusion were decreased by half from 16.65 to 8.52 (μmol/l.s) ($P = 0.01$) at 6 h postreperfusion. Similar results were obtained for AST. (B) Serum levels of ALT at 6 h after reperfusion in steatotic and normal liver (Liu, 2013). The effect of Lithium was more pronounced in steatotic livers than that in normal livers. Data are shown as means and standard deviations ($n = 6$ per group). (Color version of figure is available online.)

Histopathology

Formalin-fixed samples from left lateral lobe were processed, dehydrated, and embedded using standard techniques. Sections (4 μm) were cut and used for Hematoxylin-Eosin (HE), HMGB1 immunohistochemistry, and naphthol-AS-D-chloroacetate esterase (ASDCL) staining. A slide scanner (Hamamatsu Electronic Press Co, Ltd, Iwata, Japan) was used to digitize slides and to evaluate inflammation and tissue necrosis after staining.

Semiquantitative evaluation of hepatic necrosis

For semiquantitative analysis of hepatic damage, 10 high-power field (HPF; 400×) were selected randomly. Necrosis was expressed as the percentage of necrotic tissue for each HPF: 0, no necrosis; 1, less than 25%; 2, 25%-50%; 3, 50%-75%; and 4, at least 75% necrosis.[29] Necrosis score was shown as the sum of results for all 10 HPF. All histologic analyses were performed in a blinded fashion with respect to the experimental groups.

Quantitative evaluation of hepatic steatosis

Computer-based fully automatic software, Histokat (Fraunhofer MEVIS, Bremen, Germany) was used to quantify the severity of hepatic steatosis.[30] The severity of steatosis was determined as the relative area occupied by fat vacuoles in respect to total area. The steatosis quantification mode of the software application divided the whole slide scans into small tiles with a size of 128 pixels and 0.25 μm/pixel resolution at ×400 magnification. In the first training round, 30 tiles representing the pattern of the morphologic structure in question were marked using a specific overlay color. Each morphologic structure was assigned to a separate category (background, liver tissue, and lipid droplets). Only droplets with a diameter of more than 5 μm are recognized by this software. In the following training rounds, the tiles marked falsely by the software were reassigned to the desired category by the observer. This procedure was repeated until the software marked all tiles with the given pattern correctly. The percentage of surface covered by droplets (>5 μm) *versus* the total surface of tissue was calculated by the software.

HMGB1 immunohistochemistry

After deparaffinization and rehydration with standard techniques, antigen retrieval was performed using citrate-EDTA buffer (10-mM citric acid, 2-mM EDTA, 0.05% Tween 20, and pH 6.2) for 20 min at 100°C. Then, sections were incubated with an HMGB1 antibody (Abcam, Cambridge, United Kingdom; 1:500) for 1 h at room temperature, followed by detection using PowerVision System (ImmunoLogic, Duiven, Netherlands) and Fast Red (Dako, Glostrup, Denmark) as a substrate. After digitizing the section, the translocation rate of HMGB1 was analyzed by Histokat in nuclear positivity analysis mode.[30] In the first training round, 30 HMGB1-positive nuclei (brown staining) and 30 HMGB1 negative nuclei (blue staining) were marked by the observer. In the following training rounds, the erroneously marked nuclei could be deleted by the observer until the software marked all nuclei correctly using a specific overlay color. HMGB1 translocation rate was determined by calculating the ratio of total number of negative nuclei to total number of nuclei.

LC3 immunohistochemistry

After deparaffinization, rehydration, and antigen retrieval with standard techniques, sections were incubated with an LC3 antibody (Abcam; 1:500) for 1 h at room temperature, followed by detection using PowerVision System (ImmunoLogic). A slide scanner (Hamamatsu Electronic Press Co, Ltd, Iwata, Japan) was used to evaluate the positive signals.

ASDCL staining

Rehydrated sections were stained by using the naphthol AS-D chloroacetate esterase technique (Sigma-Aldrich) to investigate the neutrophil infiltration in the liver after reperfusion, as described previously.[31] After scanning, ASDCL positive neutrophils were counted manually in 10 randomly selected high-

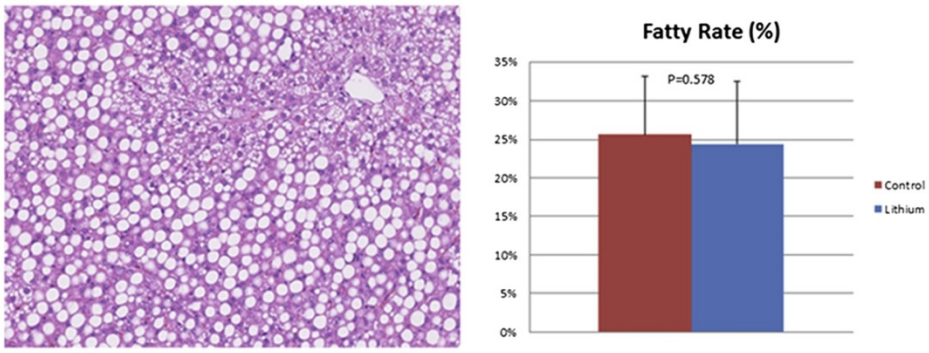

Fig. 2 – Feeding with MCD + HF diet induced hepatic steatosis. Tissues were obtained from right median lobe of liver of the rat fed with MCD + HF diet for 2 wk (original magnification ×200). Computer-assisted quantification of hepatic steatosis (HistoCAD) revealed a similar severity in treatment and control group as indicated by the relative surface of lipid droplets: 24.37 ± 8.15% for the lithium-injected rats *versus* 25.64 ± 7.50% for the saline-injected rats, $P = 0.58$ ($n = 6$ per group). (Color version of figure is available online.)

power fields using 400× magnifications. The result was shown as the mean number of positively stained neutrophils per HPF.

Gel electrophoresis and Western blotting

After isolation and quantification, equal amounts of protein from all animals of the same group were pooled. Protease and phosphatase inhibitor cocktail (Santa Cruz, Dallas, TX) were added to the protein extract. Western blotting was performed as described previously.[19] Rabbit anti GSK3b, phospho-GSK3b (Ser^{9}), caspase 3, ERK1/2, phospho-ERK1/2 ($THR^{202/204}$), JNK, phospho-JNK ($THR^{183/185}$), mTOR and phospho-mTOR (Ser^{2448}; 1:1000, Cell Signaling Technology, Danvers, MA), rabbit anti LC3 (1:1000, Abcam), rabbit anti p62 (1:1000, Sigma-Aldrich), and rabbit anti GAPDH (1:10,000, Sigma-Aldrich) were used as primary antibodies. Goat polyclonal antibody to rabbit IgG (1:5000, Abcam) was used as secondary antibody. The membranes were exposed and digitalized with an automated Western blot imaging system Fusion FX7 (Labtech International Ltd, Heathfield, United Kingdom). After normalization with GAPDH, all results were quantified with the Gel Analyzer Module provided by ImageJ (National Institutes of Health, Bethesda, MD).

Enzyme-linked immunosorbent assay

Serum IL6 and IL10 levels were investigated with commercial enzyme-linked immunosorbent assays (ELISA; R&D Systems, Minneapolis, MN) according to the instructions of the manufacturers.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical differences between groups were analyzed using the two-tailed paired sample Student's *t*-test. All tests were performed using SigmaStat, v3.5 (Systat-Software, Erkrath, Germany). A *P* value below 0.05 was considered statistically significant.

Results

In the first step, we examined whether treatment with lithium chloride affected the severity of hepatic steatosis. Compared with normal rats, HE-stained liver sections from rats fed with MCD + HF diet showed fatty changes in both lithium chloride–injected and saline-injected rats of similar severity. The steatosis occurred predominantly as macrosteatosis. (Percentage of the relative surface of lipid droplets: 24.37 ± 8.15% for the lithium chloride–injected rats *versus* 25.64 ± 7.50% for the saline-injected rats, $P = 0.58$, Fig. 2)

In the second step, we explored the effect of lithium chloride on the autophagic process in livers subjected to IRI. We investigated the autophagy related proteins LC3 and p62, as well as the cellular signal pathways related proteins GSK3b, mTOR, ERK1/2, and JNK.

The protein levels of LC3-II were substantially higher at all observation time points in liver samples from lithium chloride–treated rats during IRI. After 0.5 h reperfusion, the ratio between LC3-II and LC3-I in treatment group was also twice as high compared to the control group (ratio between LC3-II and LC3-I at 0.5 h after reperfusion: 12.81 for the lithium chloride-injected rats *versus* 5.89 for the saline-injected rats). In contrast, hepatic protein levels of p62, which correlate inversely with autophagic flux were substantially lower (Fig. 3A). To confirm this result, we explored the expression of LC3 with immunohistochemistry. Positive signals which may correspond to autophagosomes were also observed in the lithium chloride–treated rats at 6 h after reperfusion (Fig. 3B).

The expression level of phospho-GSK3b did rapidly increase, starting at 0.5 h and reaching a peak at 24 h after reperfusion. Compared to the levels in the saline-treated rats, the expression levels of phospho-GSK3b were 3-fold

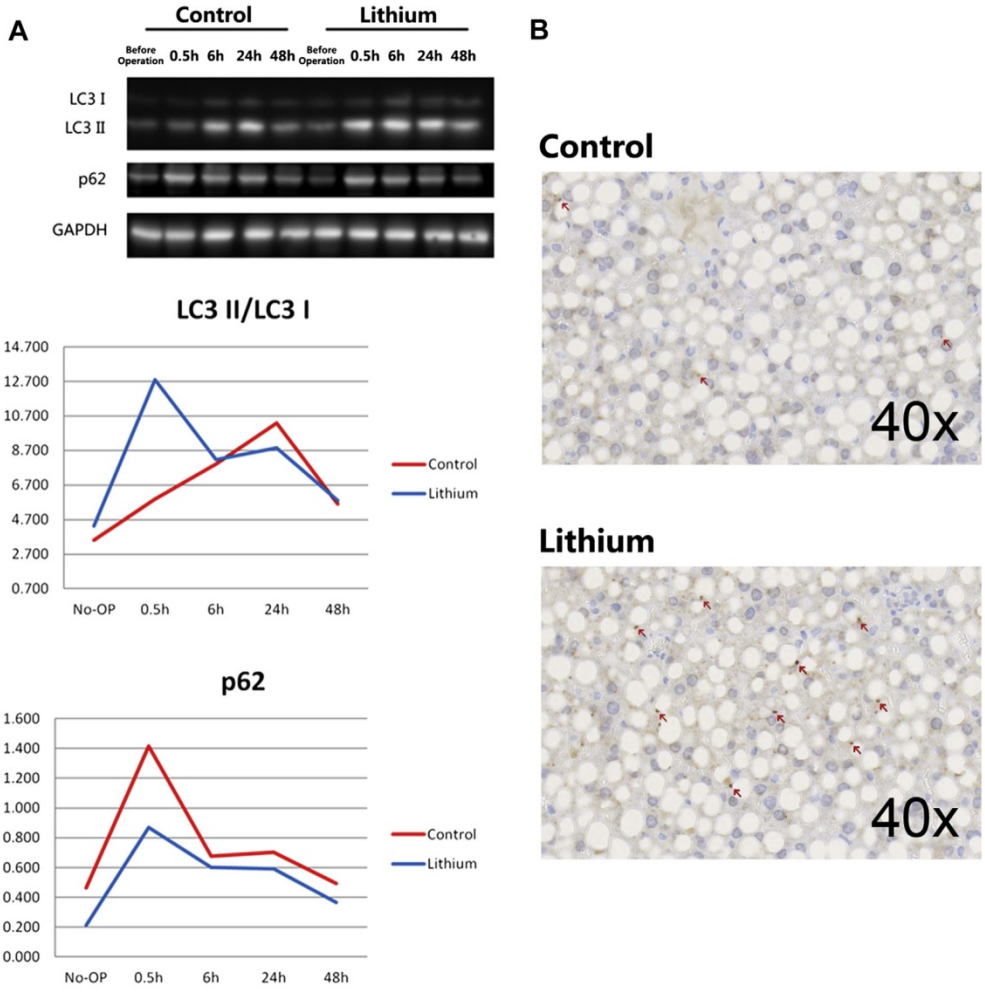

Fig. 3 – Lithium chloride enhanced hepatic autophagy. (A) LC3 and p62 protein expression in the ischemic lobes was examined with Western blot analysis at various time points after reperfusion. Lithium treatment increased the expression level of LC3-II and decreased the expression level of p62 protein after I/R injury. (B) Immunohistochemical staining for LC3 was performed on left lateral lobe sections from rats subjected to 1 h of ischemia followed by 6 h of reperfusion (original magnification ×400). (Color version of figure is available online.)

higher in the treatment group at 6 h after reperfusion. However, the total cellular levels of GSK3b were not affected by lithium chloride treatment. Furthermore, the phosphorylation at Ser^{2448} of mTOR was greater in the control group compared to the lithium chloride–treated rats (Fig. 4).

After reperfusion, the phosphorylation levels of ERK1/2 and JNK were increased in both, control and treatment groups. However, the expression of phosphorylated ERK1/2 was more pronounced in the treatment group compared to the control group. In contrast, phosphorylated JNK, indicating activation was lower in the lithium chloride–treated rats. However, lithium chloride treatment did not affect the expression levels of total ERK1/2 and JNK (Fig. 5).

In the third step, we looked at the inflammatory response, apoptosis, necrosis, and liver enzyme levels to determinate the cell-protective effect of lithium chloride.

Hepatocytes nuclear HMGB1 staining was substantially reduced in saline-injected rats as early as 30 min after reperfusion. In contrast, lithium chloride treatment reduced translocation of HMGB1 (HMGB1 negative nuclei rate: 3.52 ± 1.04% for the lithium chloride–injected rats *versus* 5.38 ± 1.00% for the saline-injected rats, $P = 0.01$, Fig. 6A). Compared with the control group, the IL10 serum level was decreased by more than half from 158.7 ± 43.98 to 63.9 ± 20.15 (μg/mL) ($P < 0.001$) at 0.5 h postreperfusion in treatment group. A similar result was observed for IL6 (201.36 ± 58.38-116.33 ± 16.89; μg/mL; $P = 0.006$; Fig. 6B). Intensity of

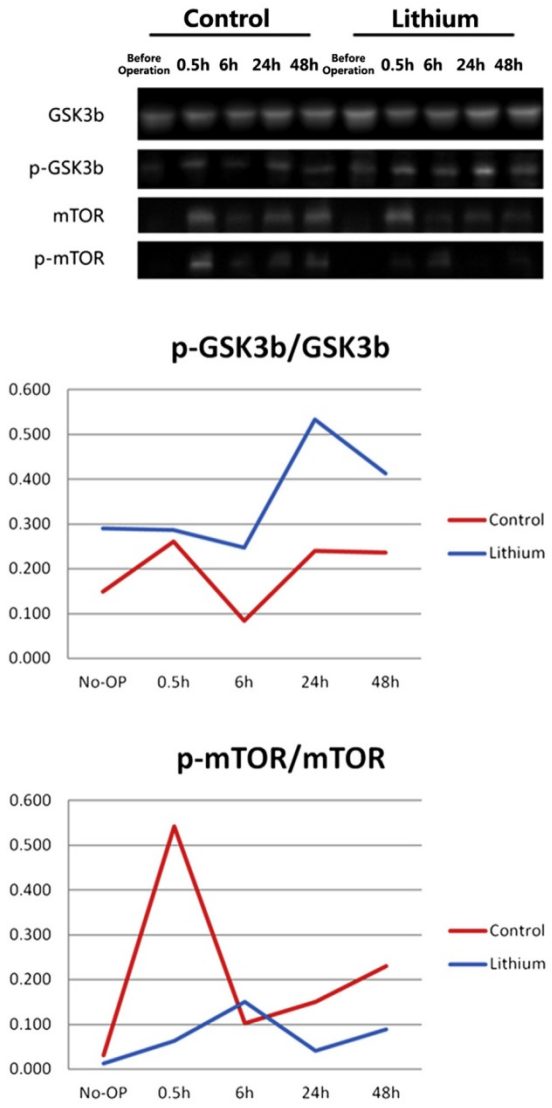

Fig. 4 – Lithium chloride modulated GSK3b-mTOR pathway. Phosphorylated and total of both GSK3b and mTOR expression levels in the ischemic lobes were visualized with Western blots. (Color version of figure is available online.)

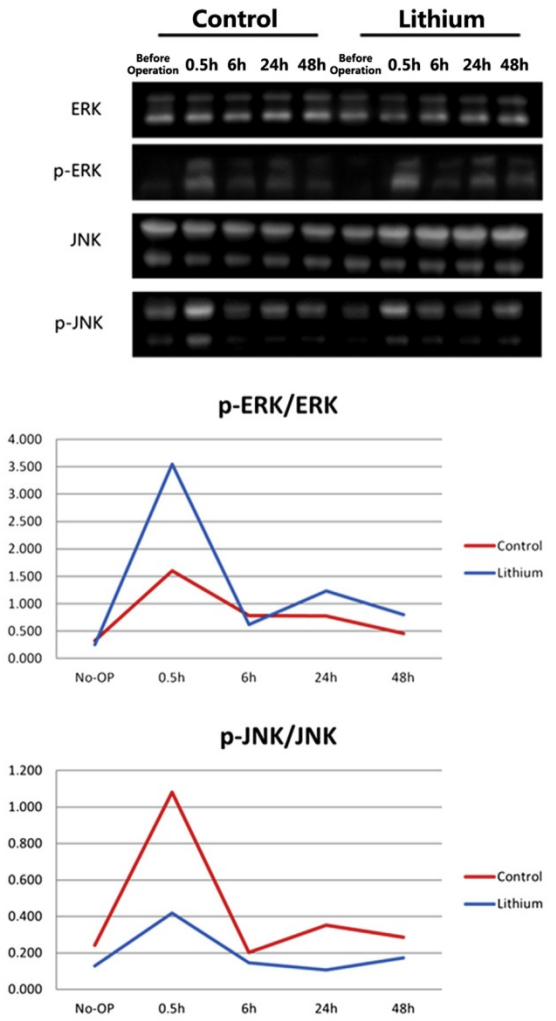

Fig. 5 – Lithium chloride modulated MAPK pathways. Phosphorylated and total ERK1/2 and JNK expression levels in the ischemic lobes were investigated using Western blots. (Color version of figure is available online.)

neutrophil infiltration, indicated by the number of ASDCL positive cells in the hepatic parenchyma,[31] was significantly higher in the saline-injected rats compared to lithium chloride–treated rats at 24 h after reperfusion (ASDCL positive nuclei: 7.77 ± 5.87/HPF for the lithium chloride–injected rats *versus* 20.48 ± 4.93/HPF for the saline-injected rats, $P < 0.001$, Fig. 6C).

Large areas of confluent necrosis were observed in the liver tissue from saline-treated rats. In contrast, the livers from rats with lithium chloride pretreatment did not show any relevant necrotic areas. (necrosis score at 24 h: 0.17 ± 0.41 for the lithium chloride–injected rats *versus* 1.83 ± 1.72 for the saline-injected rats, $P = 0.04$; necrosis score at 48 h: 0.33 ± 0.26 for the lithium chloride–injected rats *versus* 3.67 ± 3.01 for the saline-injected rats, $P = 0.03$, Fig. 7).

Lithium chloride treatment inhibited the caspase 3 cleavage induced by IRI. Total amount of caspase 3 was not affected by lithium chloride treatment (Fig. 8).

After 1 h of warm ischemia, the increase of serum aminotransferase became obvious at 0.5 h postreperfusion, reached a highest point at 6 h, and then started to decrease. Treatment with lithium chloride did dramatically reduce IRI, as showed by the lower serum levels of both liver enzymes. The ALT serum levels after reperfusion were decreased by half

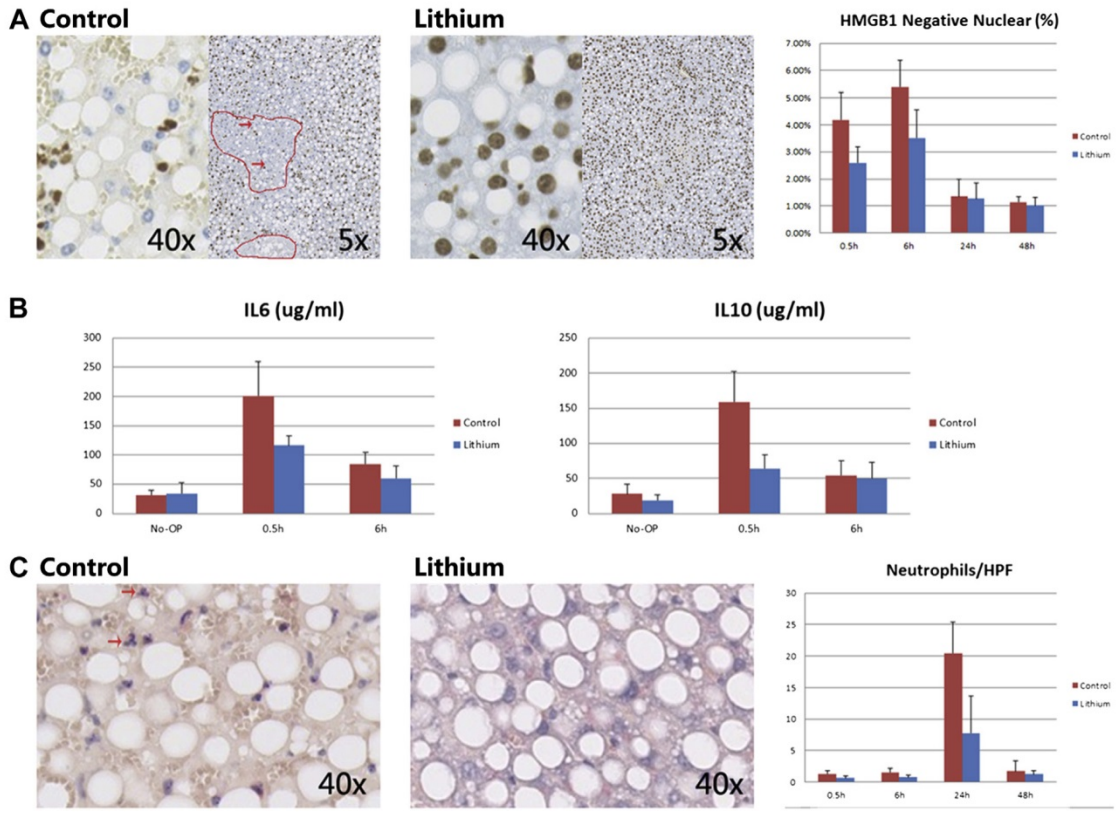

Fig. 6 – Induction of autophagy decreased inflammatory response. Immunohistochemical staining for HMGB1 and ASDCL staining, respectively, was performed on left lateral lobe sections from rats subjected to 1 h of ischemia followed by 0.5 h of reperfusion (original magnification ×400) (A) HMGB1 negative nuclei rate 3.52 ± 1.04% for the lithium-injected rats *versus* 5.38 ± 1.00% for the saline-injected rats, $P = 0.01$. (B) IL10 serum level: 158.7 ± 43.98 μg/mL for the lithium-injected rats *versus* 63.9 ± 20.15 μg/mL for the saline-injected rats at 0.5 h after reperfusion, $P < 0.001$. IL6 serum level: 201.36 ± 58.38 μg/mL for the lithium-injected rats *versus* 116.33 ± 16.89 μg/mL for the saline-injected rats at 0.5 h after reperfusion, $P = 0.006$. (C) Rate of ASDCL positive nuclei: 7.77 ± 5.87/HPF for the lithium-injected rats *versus* 20.48 ± 4.93/HPF for the saline-injected rats, $P < 0.001$. Data were shown as means and standard deviations ($n = 6$ per group). (Color version of figure is available online.)

from 16.65 to 8.52 (μmol/l.s; $P = 0.01$) at 6 h postreperfusion. A similar result was observed for AST (Fig. 1A).

Discussion

A model of nonalcoholic fatty liver disease (NAFLD) was used in this study. Dietary induced accumulation of lipids in the liver is the most common etiology of hepatic steatosis followed by alcoholic fatty liver disease.[32] Gender is not considered to be the most important factor affecting IRI.[33] However to eliminate a possibly gender-related effect, only male rats were used in this study.

Steatosis was induced by feeding Lew rats for 2 wk with MCD + HF diet.[25] This protocol resulted in a predominantly moderate macrosteatosis in periportal zone and mid-zone, a pattern frequently found in patients with NAFLD.[32] Macrosteatosis is considered to be the more severe threat in hepatic surgery than microsteatosis.[34] However, in some animals, small areas of microsteatosis were also observed.

In this study, a 70% partial hepatic warm IRI rat model was used. The setting of 60-min ischemic time was based on a pilot exploring the tolerance of rats with steatotic liver (data not shown). The dose and route of lithium chloride application were selected in accordance with previous studies.[19,20,27] In these studies, lithium chloride was used to protect brain, kidneys, heart, and liver from IRI. Li *et al.* reported that rats injected with a dose of 2-mmol/kg lithium chloride subcutaneously presented with drug levels in the therapeutic range between 0.5 and 1.0 mmol/L plasma lithium levels at 12 h.[24] In 2013, Liu *et al.* demonstrated that pretreating rats with lithium chloride following this schedule did reduce liver IRI in normal nonsteatotic rats.[19] In our study, the plasma levels of lithium were measured at the end of the observation period in each

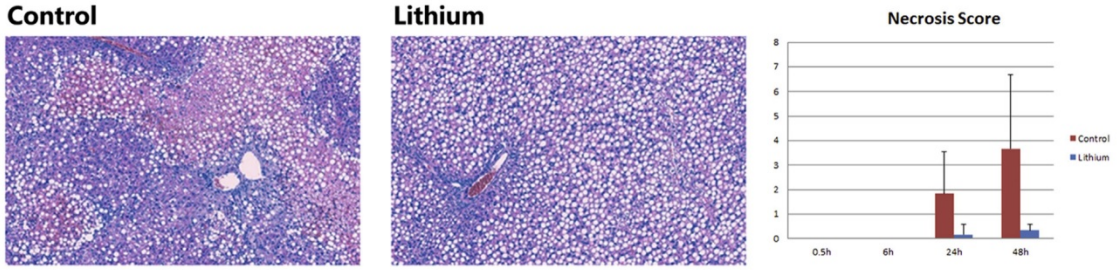

Fig. 7 – Induction of autophagy reduced necrosis. Tissues were obtained from left lateral lobe sections from rats subjected to 1 h of ischemia followed by 24 h of reperfusion (original magnification ×100). Necrosis score at 24 h: 0.17 ± 0.41 for the lithium chloride–injected rats *versus* 1.83 ± 1.72 for the saline-injected rats, $P = 0.04$; necrosis score at 48 h: 0.33 ± 0.26 for the lithium chloride–injected rats *versus* 3.67 ± 3.01 for the saline-injected rats, $P = 0.03$. Data were shown as means and standard deviations ($n = 6$ per group). (Color version of figure is available online.)

group. The values were dependent on the time between last drug injection and sacrifice in different groups and ranged between 0.13-2.66 mmol/L (median: 0.252 mmol/L).

Summarizing our results, lithium chloride treatment protected steatotic livers from IRI. The effect was associated with a reduced inflammatory response, less apoptosis, less necrosis, and lower liver enzyme levels. We demonstrated that pretreatment with lithium chloride did reduce hepatic IRI in steatotic rats by upregulating autophagy via modulation of both, the GSK3b and ERK1/2 pathways.

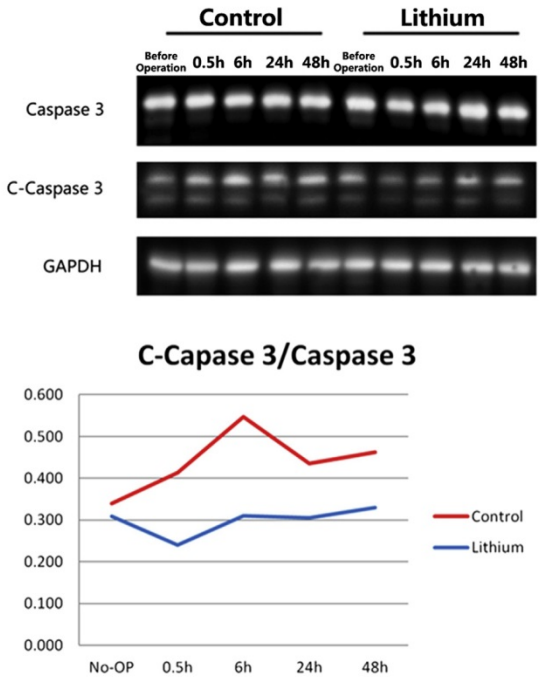

Fig. 8 – Induction of autophagy decreased hepatocytes apoptosis. Caspase 3 cleavage in the ischemic lobes was assessed using Western blots. (Color version of figure is available online.)

Autophagy is a highly conserved cellular pathway that targets cell constituents to the lysosome for degradation.[4,5] The breakdown of cellular components promotes cellular survival during IRI by maintaining cellular energy levels.[2] During IRI, targeted cytoplasmic constituents are isolated from the rest of the cell within an autophagosome. This autophagosome then fuses with a lysosome, and its cargo is degraded and recycled.[4] A growing number of studies claimed that lithium chloride treatment upregulated autophagy in brain, kidney as well as liver.[17,19,35] LC3, also known as mammalian orthologue of atg8 in yeast, has been modified from its initial form LC-I into phosphatidylethanolamine conjugated form LC-II during the constitution of autophagosomes.[36] The ratio between LC3-II and LC3-I is the most widely accepted indicator to evaluate autophagic flux.[37] As an adaptor protein of ubiquitin system, p62 participates in the aggregation of ubiquitinylated proteins, which play a critical role in the fusion of autophagosomes with lysosomes.[38] The intracellular p62 level correlates with autophagic flux inversely, due to the degradation of autophagosomes.[37] In our study, we demonstrated that pretreatment with lithium chloride significantly increased autophagy as indicated by the ratio between LC3-II and LC3-I and p62 level after IRI in the steatotic livers. To better visualize autophagy, an immunohistochemistry of LC3 was implemented in both control and lithium group. At 6 h after reperfusion, we observed in lithium chloride–treated animals some positive signals which may correspond to autophagosomes. However, according to the recently published consensus article,[37] autophagy-related proteins including LC3 can be located in organelles other than in autophagosomes. Therefore, LC3 Western blotting is the recommended method to quantify autophagy.

Lithium chloride is considered a direct and indirect inhibitor of GSK3b, which plays an important role in hepatic IRI.[11,12,39] GSK3, which is a serine/threonine protein kinase, mediates the addition of phosphate molecules onto serine and threonine amino acid residues.[40,41] There are two isoforms of GSK3: GSK3a and GSK3b. Recent studies reported that GSK3b may act as a multifunctional enzyme playing a critical role in cell proliferation, division, differentiation, survival and

apoptosis, as well as autophagy.[42,43] GSK3b is considered as an augmenter of mTOR, which is a well-known inhibitor of autophagy.[7] On one hand, treatment with lithium chloride inhibits GSK3b activity directly by binding to the magnesium-sensitive active site of the enzyme. On the other hand, it also upregulates the phosphorylation at Ser^9 by other kinases to modulate the GSK3b pathway indirectly.[11,12] The reduced GSK3b activation results in the inhibition of mTOR activity, which also induces autophagy indirectly.[7,17] A number of studies suggested that GSK3b plays a critical role in liver IRI, whereas its inhibition protects hepatocytes from damage. Besides modulating autophagy, GSK3b inhibition ameliorates liver IRI also by reducing local inflammation, downregulating apoptosis, and promoting hepatocyte proliferation after reperfusion.[40,42,43] Our results showed that the expression level of phosphorylated GSK3b was significantly higher in the treatment group after reperfusion. These data suggest that lithium chloride treatment is involved in hepatoprotection by enhancing autophagy via an inhibition of GSK3b-mTOR pathway in steatotic liver.

Lithium chloride is also known for its ability to enhance ERK1/2 activation,[13] which induces autophagy by regulating beclin 1 pathway.[44] The activation of ERK1/2-beclin 1 pathway after redox stress is protective in cells.[8] The neuroprotective effect of lithium chloride to protect against bipolar disorder is partly dependent on the modulation of ERK1/2 pathway.[45] It has also been reported that treatment with lithium chloride reduces IRI by an activation of ERK1/2 in brain.[13] Our results confirmed that lithium chloride pretreatment of steatotic rats substantially increased the phosphorylation level of hepatic ERK1/2 after reperfusion. Phosphorylated ERK1/2 regulates autophagy positively by activating beclin 1.[44] Beclin 1 as the first identified autophagy related gene in mammals has been well studied since 1998.[46] As a mammalian orthologue of atg6 in yeast, beclin 1 induces autophagy by binding to the PI3KcIII complex, which is essential for the initiation of autophagosome creation.[47] Furthermore, ERK1/2 is also known as an important inhibitor of the inflammatory response, which determines the evolution of damage after IRI.[8] Our results confirmed that the phosphorylated ERK1/2 level is significantly increased in the treatment group after reperfusion. It suggests that lithium chloride treatment may also regulate autophagy positively by activating the ERK1/2-beclin 1 pathway in steatotic livers.

To determinate the benefit of autophagy modulation on IRI, we investigated IRI-related inflammatory response, which is considered to be one of the most important mechanisms of liver damage.[6,48,49] Three parameters were selected to represent different phases of the inflammatory cascade: HMGB1 for the early initiating phase, cytokine release and leukocyte infiltration for the late effector phase. HMGB1 as a nuclear chromatin protein is perceived as an important mediator during the initial phase of the inflammatory response.[50,51] Nuclear HMGB1 may translocate to the cytoplasm of hepatocytes as well as into the extracellular space and trigger the release of inflammatory cytokines in the early phase after reperfusion.[52,53] Quantification of serum interleukins expression and neutrophil infiltration are frequently used and represent well-accepted methods to investigate the late inflammatory response in IRI.[48] Here, we confirmed that modulation of autophagy by lithium chloride treatment reduced inflammatory response after IRI in steatotic livers as indicated by less HMGB1 translocation, lower serum IL6 and IL10 level as well as less neutrophil infiltration.

Necrosis also has a prominent role in the evolution of organ damage after liver IRI.[48] Due to diminished sinusoidal spaces and reduced microcirculatory blood flow, there is an increased risk of necrosis in the steatotic liver undergoing IRI compared to the normal liver.[54] We and others observed necrosis areas in steatotic livers from 24 h after reperfusion.[55] It is well known that modulation of autophagy maintains the energy level of cells and protects cells from necrosis.[5] In agreement with our previous study, we demonstrated that pretreatment with lithium chloride decreased necrosis significantly after IRI in steatotic livers by enhancing autophagy.

Programmed cell death, also known as apoptosis, is considered a critical indicator for evaluation of IRI-related organ damage.[55] Same as autophagy, apoptosis is also a process activated in stressed or damaged cells.[56] Furthermore, apoptosis and autophagy are sharing a number of same pathways such as mitogen-activated protein kinases pathways.[57] But different from autophagy, which is generally considered as a prosurvival process, the activation of apoptosis results always in cell death.[56] During IRI, the early molecular events of apoptosis such as caspase 3 protein cleavage appear around 6 h after reperfusion.[48,49] It has been reported that lithium chloride treatment prevents both IRI-induced and non-IRI-induced apoptotic cell death.[39,45,58] These reports were consistent to our data showing a lower level of cleaved caspase 3 protein in the reperfused steatotic liver.

To study how lithium chloride induced autophagy but reduced apoptosis, the JNK activity was investigated. Although JNK and ERK1/2 both are members of mitogen-activated protein kinases family, however different from ERK1/2, JNK activation increases apoptosis after IRI and is deleterious for hepatocytes.[28] Activated JNK translocates to nucleus and mitochondria, and induces apoptosis independently. In nucleus, JNK induces phosphorylation of c-Jun, which is involved in apoptosis by modulation of the level of distinct apoptotic-related proteins such as p53. In mitochondria, JNK induces apoptosis via promoting the formation of apoptosomes consisting of cytochrome C, caspase 9, and Apaf-1.[59] Previous findings demonstrated that lithium chloride treatment protected neurons from apoptosis by inhibition of JNK activity during ischemia.[15] In agreement with these findings, our results demonstrated that lithium chloride treatment inhibited JNK activation after IRI also in steatotic livers. Based on these results, we speculated that lithium chloride treatment induced autophagy by activating ERK1/2 and reduced apoptosis by inhibiting JNK.

In 1990s, the mechanism of hepatic IRI has been well explored by Jaeschke *et al.* in a series of studies.[60-64] According to his studies, hepatic ischemia is followed by the two phases of ischemia/reperfusion injury: the initial and the later phase.[63] During ischemia, hepatic parenchymal cells suffer from the lack of oxygen leading to a reduction of the hepatocyte energy level and to an increase in oxidative stress.[61] The first hour after reperfusion are considered as the initial phase, also known as biochemical phase.[63] During this phase, HMGB1 translocates from nucleus to cytoplasm and extracellular

space, where it activates endothelial cells and Kupffer cells promptly after reperfusion.[52] These cells produce more reactive oxygen species leading to acute, mild hepatic damage. Translocated HMGB1 also triggers the release of inflammatory cytokines, leading to the aggravation of hepatic damage in terms of elevation of liver enzymes and subsequently to the recruitment of neutrophils in the later phase.[65] During the later phase (after 6 h of reperfusion), neutrophils transmigrate from vessels and sinusoids into the hepatic parenchyma as a response to the activation of inflammatory mediators. Numerous oxidants and proteases which are released by infiltrating neutrophils cause direct injury to hepatocytes and endothelial cells leading to the observed aggravation of hepatic damage, now manifesting in the extent of confluent necrosis.[63]

Our results demonstrated that lithium chloride treatment enhanced autophagy via a modulation of both GSK3b-mTOR and ERK1/2-beclin 1 pathways. Enhanced autophagic flux maintains energy level and reduces oxidative stress by degrading dysfunctional mitochondria and impaired organelles during ischemia. Furthermore, lithium chloride treatment prevents translocation of HMGB1 during the initial phase, thereby leading to a reduction of hepatic damage as well as a reduction of neutrophils infiltration during the late phase. Lithium chloride treatment was associated with reduced hepatic damage in terms of enzyme release (6 h) and confluent necrosis (24 h) which may well be related to its enhancing effect on autophagy.

Comparing our results to the previous study of Liu, the protective effect was more pronounced in steatotic livers than in normal livers with the same dose of lithium chloride treatment.[19] Steatotic livers are especially vulnerable to IRI as indicated by the serum ALT levels. At 6 h after reperfusion, the serum ALT levels of untreated steatotic rats were higher than of untreated nonsteatotic normal rats. However, ALT serum levels of treated steatotic rats were even lower than the ALT levels of treated nonsteatotic normal rats (Fig. 1B). Recent studies have implicated that autophagy is impaired in nonalcoholic fatty liver disease.[2,66] The inhibition was attributed to the increase of mTOR complex.[2] Our study suggested that lithium chloride treatment of steatotic animals might augment the hepatoprotective effects and result in a more pronounced enhancement of autophagy via modulation of both GSK3b and ERK1/2 pathways. Our data suggested that modulation of autophagy reduced IRI in steatotic livers even to a larger extent than in normal livers.

Study limitations

This study was based on a selective hepatic warm IRI model. Selective hepatic warm IRI model in rats is a well-accepted experimental model for a variety of ischemia settings in hepatic surgery, for example, extended hepatectomy, but also for transplantation. However, we have to notice the fact that the warm IRI experimental scenario does not fully reflect the pathophysiology of cold ischemia as present in transplantation. Cold IRI is considered to be the more severe threat in liver transplantation than warm IRI.[1]

Our result demonstrated that modulation of autophagy by lithium chloride treatment showed an outstanding hepatoprotective effect in both normal and steatotic livers undergoing IRI. Therefore, it is also great interest to extend this setting to a transplantation model in future.

Conclusions

Taken together, we concluded that an induction of autophagy via modulating both GSK3b and ERK1/2 pathways by treatment with lithium chloride protects steatotic livers from IRI. These results further support the hypothesis that simultaneous modulation of GSK3b and ERK1/2 pathways might be an interesting strategy to reduce IRI. This strategy might be especially useful in steatotic livers with an underlying impairment of autophagy.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jss.2017.04.012.

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Results and Discussion Part 2:

IRI in aged livers – the energy metabolism, inflammatory response and autophagy

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Introduction:

Over the past century, the life expectancy of mankind has been greatly improved. According to the WHO, the life expectancy at birth in the United States did increase from 76.8 in 2000 to 79.3 in 2015. The United Nations' World Population Ageing Report 2015 noted that the global population aged 60 or over has increased from 607.1 million in 2000 to 900.9 million in 2015 and is projected to rise further to 1402.4 million in 2030 and 2092.0 million in 2050. The population aged 80 or over are expected to increase by 6-fold, from 71.0 million in 2000 to 434.4 million in 2050.

Since 2000s, the physiological changes of liver in senescence process were explored by a series of studies (Hoare et al. 2010, Kim et al. 2015). The impaired intracellular energy metabolism, the enhanced inflammatory response and the reduced autophagic flux were considered as the most important physiological processes in aging. Impaired mitochondria in aged livers produce less ATP but generate more free radicals. The accumulation of free radicals was considered one of the major forces of senescence process (Harman 1956). It is also reported that compared to normal livers, aged livers are more vulnerable to external stress. The increased vulnerability of aged livers is associated with an increased inflammatory response (Matsutani et al. 2007) and a decreased autophagy (Rubinsztein et al. 2011).

To better understand how senescence affects liver IRI, a literature work up spanning over the existing studies was generated.

Summary:

In the second part of this study, we systematically reviewed the pathophysiological mechanisms of IRI in aged livers. We identified therapeutic strategies targeting intracellular energy metabolism, inflammation or autophagy which might be also especially useful to protect aged livers from IRI.

Similar to steatotic livers, aged livers are also more sensitive to IRI. Current experimental evidence for therapeutic strategies targeting intracellular energy metabolism, inflammation or autophagy protecting aged livers from IRI are promising, but still limited. Selzner et al.

demonstrated that treatment with d-glucose, which increases the hepatocellular intracellular energy content, reversed the reduced effect of ischemic preconditioning in aged livers. Selzner et al. and Kireev et al. presented in their studies that treatment with pentoxifylline and melatonin, which are considered as inflammatory inhibitors, significantly reduced IRI related hepatic damage in aged livers.

We generated own preliminary but promising experimental evidence that using lithium chloride as a drug targeting inflammatory response and autophagy alleviates the IRI in aged livers. Therefore, it is also of great interest to explore other therapeutic strategies targeting autophagy in future.

Conclusion:

In conclusion, the intracellular energy metabolism, inflammatory response and autophagy are three critical common mechanisms in both senescence process and IRI process. It is expected that therapeutic strategies targeting these three processes are particularly useful for aged livers with an increased risk of IRI after primary liver surgery.

A comprehensive review entitled “Ischemia-Reperfusion Injury in Aged Livers - The Energy Metabolism, Inflammatory Response, and Autophagy” was published by Transplantation (IF 3.678) and can be accessed via https://doi.org/10.1097/TP.0000000000001999.

Manuscripts

Manuscript II

Ischemia reperfusion injury in aged livers - the energy metabolism, inflammatory response and autophagy

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Authorship

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Authors' Contribution

Kan C., Dirsch O. and Dahmen U. contributed to conception and design;

Kan C. participated in writing the article;

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Dirsch O. and Dahmen U. participated in the critical revision of the article and contributed to obtain funding.

Review

Ischemia-Reperfusion Injury in Aged Livers—The Energy Metabolism, Inflammatory Response, and Autophagy

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Abstract: Because of the lack of adequate organs, the number of patients with end-stage liver diseases, acute liver failure or hepatic malignancies waiting for liver transplantation is constantly increasing. Accepting aged liver grafts is one of the strategies expanding the donor pool to ease the discrepancy between the growing demand and the limited supply of donor organs. However, recipients of organs from old donors may show an increased posttransplantation morbidity and mortality due to enhanced ischemia-reperfusion injury. Energy metabolism, inflammatory response, and autophagy are 3 critical processes which are involved in the aging progress as well as in hepatic ischemia-reperfusion injury. Compared with young liver grafts, impairment of energy metabolism in aged liver grafts leads to lower adenosine triphosphate production and an enhanced generation of free radicals, both aggravating the inflammatory response. The aggravated inflammatory response determines the extent of hepatic ischemia-reperfusion injury and augments the liver damage. Autophagy protects cells by removal of damaged organelles, including dysfunctional mitochondria, a process impaired in aging and involved in ischemia-reperfusion–related apoptotic cell death. Furthermore, autophagic degradation of cellular compounds relieves intracellular adenosine triphosphate level for the energy depressed cells. Strategies targeting the mechanisms involved in energy metabolism, inflammatory response, and autophagy might be especially useful to prevent the increased risk for ischemia-reperfusion injury in aged livers after major hepatic surgery.

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The number of patients awaiting liver transplantation is constantly increasing. The development of liver transplantation has significantly increased the survival rate of patients with end-stage liver diseases, acute liver failure and hepatic malignancies. According to the report from United Network for Organ Sharing, 7841 cases of liver transplantation were performed in patients with end-stage liver diseases, acute liver failure, or hepatic malignancies in the United States in 2016. However, more patients are still dying on the waiting list because of the lack of adequate organs. Based on United Network for Organ Sharing data as of July 13, 2017, 14 302 patients in the United States are waiting anxiously for a liver donor. The discrepancy between the exponentially growing demand and the constant or even decreasing supply of donor is becoming more and more severe.[1]

To solve this problem, one strategy to expand the donor pool is to accept older donors. In these years, the percentage of liver grafts obtained from aged donors older than 70 years is constantly raising.[2] The number of cases of liver transplantation in the United States using donors older than 50 years has accounted for over 30% of the total cases and has increased from 2273 to 2640 over the past 5 years (see Figure 1).

CLINICAL PROBLEM

However, the use of aged liver grafts can lead to severe problems. During the first decade in 21st century, old donor age was identified as an important cause of posttransplantation morbidity and mortality by a series of clinical studies. In Busquets et al[3] and Markmann et al's[4] studies, using grafts from aged donors was associated with lower graft and

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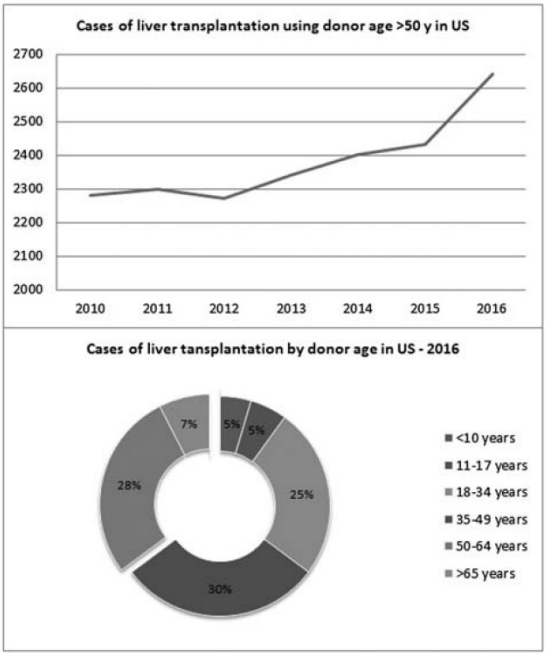

FIGURE 1. Increasing number of aged donors. The number of liver donors aged 50 years or older has accounted for over 30% of the total donors and has increased from less than 2300 in 2010 to more than 2600 in 2016.

recipient survival. In 2007, Johnson et al[5] described in a clinical study covering more than 10 000 cases of liver transplantation that the risk of graft loss and death was twice as high when using organs from donors older than 60 years compared with donors younger than 40 years. Similar observations were obtained in other clinical studies as presented in Table 1.

In contrast, Dasari et al[12] demonstrated in a comprehensive review that part of the risk of graft loss and death is related to the higher rate of further impairments such as fibrosis and steatosis in aged liver grafts. Alterations of the hepatic vascular system in aged liver also increase the risk of posttransplant vascular complications. Dasari et al concluded that this risk can be reduced by a careful selection of suitable aged donors and low-risk recipients. Selection can be improved by thorough investigation of the donor condition including a liver biopsy for better assessment of graft quality. A similar recommendation was given by Jimenez-Romero et al[13] in 2014. He proposed to restrict the use of aged liver grafts to those with normal liver function, without atherosclerosis in the hepatic artery, without histological alteration in the biopsy, intensive care unit stay less than 72 hours, warm ischemia time less than 1 hour, cold ischemia time less than 8 hours, macrosteatosis less than 30%, and with good hemodynamic and preharvesting conditions. Bertuzzo et al[2] confirmed in 2017 that performing a routine liver graft biopsy and discarding the grafts with moderate/severe macrosteatosis, fibrosis, necrosis, and even more important, limiting the cold ischemia time to 8 hours, the recipients of aged liver graft could achieve a similar long-term survival rate.

In general, these studies suggested that when performing a strict donor management to identify healthy donors with a biological age below their calendarical age, the overall outcome using old livers or transplantation may be acceptable in terms of survival.

Despite observing comparable survival rates when careful selecting and matching the aged donor graft with a suitable recipient, these positive observations do not contradict the fact that aged livers are more sensitive to external stress. Age itself and age-related geriatric diseases, such as atheromatous disease, aortic valve stenosis, and systolic hypertension, are still considered as major risk factors potentially leading to an increased posttransplantation morbidity and mortality.[12]

For a better overview, currently available clinical studies evaluating the effect of senescence on clinical outcome in liver transplantation are presented in Table 1.

The increased risk of posttransplantation morbidity and mortality is partly due to the enhanced ischemia-reperfusion injury (IRI). Compared with normal grafts, grafts from aged donors show increased sensitivity to IRI.[14] The increased IRI enhances necrosis and apoptosis, as well as reduced hepatocyte

TABLE 1.

Clinical studies assessing the effect of age on clinical outcome in liver transplantation

Year	Authors	Total number of cases	Definition of "old"	Key statement
Donor age affects outcome				
2001	Markmann et al	1148	>65 y	Donor age is associated with lower graft and recipient survival.[4]
2001	Busquets et al	348	>70 y	Donor age is associated with lower graft and recipient survival.[3]
2003	Clavien et al	100	>60 y	The protective effect of ischemic preconditioning is lost in old patients.[6]
2005	Lake et al	11 670	>40 y	Donor age is an important predictor of outcome in patients with hepatitis C.[7]
2007	Johnson et al	10 545	>40 y	Donor age is associated with lower graft and recipient survival.[5]
2011	Lai et al	188	>70 y	Donor age is associated with lower graft and recipient survival.[8]
Donor age may result in comparable outcome				
2004	Nardo et al	90	>80 y	When performing an appropriate donor management, the use of old donors in liver transplantation can achieve a normal early functional recovery.[9]
2008	Cescon et al	553	>50/60/70/80 y	Routine graft biopsy and short ischemia time may improves the outcome of liver transplantation with old donors are acceptable.[10]
2009	Ravaioli et al	287	>60 y	When performing an appropriate donor management, the outcome of liver transplantation with old donors are acceptable.[11]
2017	Bertuzzo et al	1083	>70 y	When performing an appropriate donor management, the outcome of liver transplantation with old donors are acceptable.[2]

regeneration, and ultimately leads to a higher rate of primary liver failure and long-term graft dysfunction after transplantation.[5,8,15]

Age is not only considered an additional risk in liver transplantation but is also of importance for transplantation of other organs. Lim et al[16] demonstrated in a large clinical study comprising over 6317 patients that the graft outcomes for recipients of kidney grafts from donors older than 60 years were inferior to those obtained with kidney grafts from younger donors. A recently published risk index suggested that donor older than 50 years should be identified as a risk for graft loss after kidney transplantation due to an aggravated IRI.[17]

This review aims to provide an overview of the most relevant pathophysiological mechanisms rendering the aged liver highly susceptible to IRI. Furthermore, we want to identify strategies to prevent IRI in aged donors.

HEPATIC IRI

Since the 1990s, the mechanism of hepatic IRI has been explored by a series of experimental and clinical studies.[18-22] It is widely accepted that ischemia is followed by 2 distinct phases of reperfusion injury, the initial and the later phase.

Recent studies revealed that energy metabolism, inflammatory response, and autophagy are 3 critical processes involved in hepatic IRI. Energy metabolism is mostly affected during ischemia and the initial reperfusion phase.[23] Inflammation is the most pivotal mechanism in both the initial and the later phases of reperfusion.[24] Autophagy plays its role predominantly in the later phase.[25]

Intracellular energy metabolism is considered the key mechanism in the ischemia phase. During ischemia, hepatocytes suffer from the lack of oxygen and nutrients, leading to a reduction of the intracellular energy content level and to an increase in oxidative stress.[23,24] Free radicals and oxidant chemicals impair mitochondria function and lead to an increased permeability of the inner membrane. The inner membrane becomes freely permeable to molecules with a molecular weight less than 1500 Da.[26-28] This phenomenon is called mitochondrial permeability transition (MPT) and is followed by a loss of cell activity.[29]

The first hour after reperfusion is considered as the initial phase, also known as biochemical phase.[30] MPT plays a critical role in this phase. High concentration of Ca^{2+} after reperfusion aggravates ischemia-related MPT.[31] The release of cytochrome c from damaged mitochondria is reported to be a critical event of apoptosis via a regulation of caspase activation.[32] Besides, impaired mitochondria generate more oxidative radicals. The release of oxidative radicals results in an early inflammatory response. Endothelial cells and Kupffer cells are activated after reperfusion. Upon activation, these cells produce less nitric oxide (NO) and release more inflammatory mediators, including tumor necrosis factor alpha (TNFα), interleukin (IL) 1, and leukotriene, leading to acute hepatic damage.[24] Translocation of high-mobility group box 1 (HMGB1) from the cell nucleus to the cytoplasm and the extracellular space in endothelial cells, Kupffer cells as well as hepatocytes plays a critical role in this process.[33]

HMGB1 is a short-chain protein which is located in the nuclei of hepatocytes. Upon activation, HMGB1 translocates into cytoplasm and releases into the extracellular space.[33] A study of Liu et al[34] suggested that activated HMGB1 translocates from nuclei to cytoplasm during the early phase of hepatic IRI. Translocated and released HMGB1 leads to the activation of redox-sensitive transcription factors including nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). The activation of redox-sensitive transcription factors modulates the expression of inflammatory mediators such as TNFα and IL-12, leads to the later phase of liver injury.[35]

The later phase, called inflammation phase, starts around 6 hours and lasts up to 48 hours after reperfusion.[30] Activation of the inflammatory response, especially the transmigration and activation of neutrophils is the central event in the later phase of IRI.[24] During this phase, neutrophils transmigrate from vessels and sinusoids into the hepatic parenchyma as a response to the release of inflammatory mediators, including TNFα, IL-1, IL-12, and macrophage inflammatory proteins (MIPs). Numerous oxidants and proteases, which are released by infiltrating neutrophils, cause direct injury to hepatocytes and endothelial cells.[36] The excessive inflammatory response ultimately induces apoptosis and necrosis but also autophagy in hepatocytes via the modulation of multiple signal pathway molecules, such as extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and glycogen synthase kinase 3 beta (GSK3b).[26,37-40] Apoptosis and necrosis induced by JNK pathway progresses to cell death.[37] However, autophagy is considered as a prosurvival response.[25,41,42]

Autophagy is a self-digesting process that disassembles dysfunctional or nonessential components when cells are under stress.[43] Two controversial opinions regarding the role of autophagy in IRI are currently discussed. One of the views suggests that autophagy is another form of programmed cell death pathway besides apoptosis. The other view, described in most studies, considers autophagy as a self-protective response during IRI.[42] Ischemia-reperfusion–related autophagy is induced by the activation of ERK and GSK3b pathways.[44,45] The degradation of damaged organelles especially the damaged mitochondria reduces the generation of free radicals and provides urgently needed energy to hepatocytes.[46] Autophagy relieves the stress and improves the intercellular energy metabolism during hepatic IRI[42] (see Figure 2).

AGING PROCESS IN LIVER

Almost 3000 years ago, the ancient Greeks had already realized that liver has an outstanding ability of regeneration. According to Greek mythology, Prometheus' liver was eaten by an eagle during daytime and regenerated during night as his eternal punishment for stealing fire from Mount Olympus and giving it to mankind. Comparing the liver from adolescents and young adults, the blood flow and total liver volume decreases slightly in livers from elderlies.[47,48] Until recently, it was considered that under normal conditions, this reduction does not lead a noticeable impairment of hepatic function.[14] However, growing evidence suggests that the opinion of the ancient Greeks may not be perfectly correct. Similar to other organs, the liver also undergoes an aging progress as an inevitable natural phenomenon.[49]

There are evidences that the aging process impairs the hepatic stress response.[50-55] The impairment of the hepatic stress response occurs irrespective of the underlying etiology, including the responses not only to infection and intoxication but also to IRI.[14] In the last 10 years, a series of experimental studies aimed to investigate the IRI relevant mechanism in

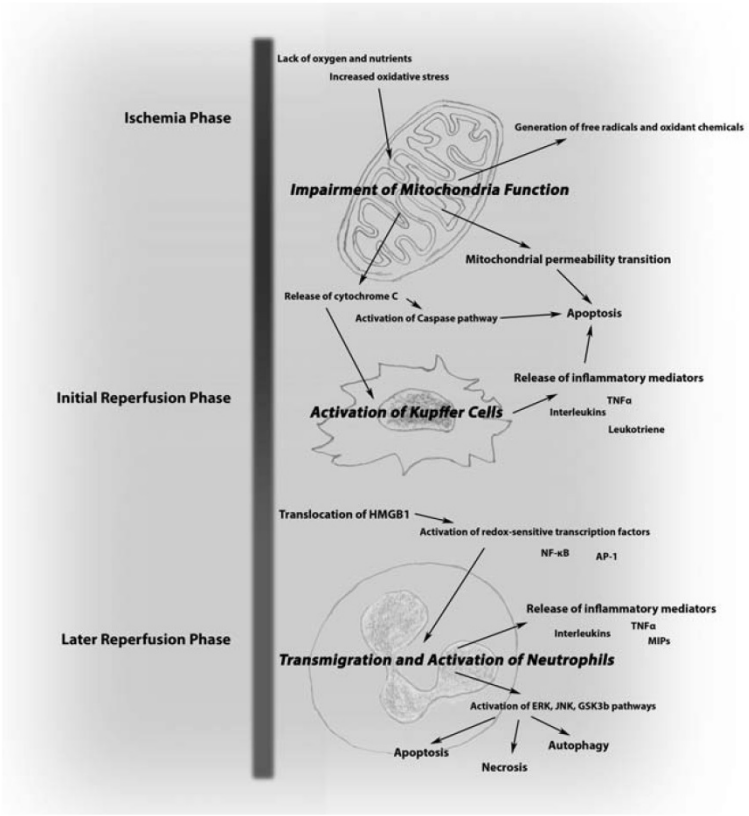

FIGURE 2. Scheme of key processes in the time course of hepatic IRI. IRI leads to a depletion of hepatic energy reserves which causes an increased oxidative stress and ultimately causes an impairment of mitochondria function. The impairment of mitochondria function leads to a mitochondrial permeability transition and is followed by a loss of cell activity. In the early phase of reperfusion, injury activation of the inflammatory response is initiated with the activation of Kupffer cells. Activated Kupffer cells produce less NO, release more inflammatory mediators which leads to acute hepatic damage. The late phase of reperfusion is dominated by the transmigration and activation of neutrophils leading to the aggravated inflammatory response. The release of inflammatory mediators regulates multiple signal pathway molecules including ERK, JNK, and GSK3b and ultimately induces apoptosis, necrosis, and autophagy in hepatocytes. The activation of apoptosis and necrosis progresses to cell death. The activation of autophagy relieves the oxidative stress and improves the intercellular energy metabolism during the later phase of hepatic IRI.

aged liver (see Table 2). All of these studies using observational und interventional experimental designs supported the view that IRI is enhanced in aged liver and examined different aspects of the underlying mechanism.

Experimental studies in aging are mainly based on rodent models using animals aged 12 to 24 months, which is considered to be comparable to humans aged 40 to 80 years.[68] In 2005 and 2007, 2 independent studies using different rodent models demonstrated that hepatic IRI is more severe in aged liver. Okaya et al[56] reported that compared with 8 to 12 weeks old mice, 12 to 13 months old ones presented an increased hepatic damage and an increased inflammatory response after 70% warm ischemia for 90 minutes followed by 8 hours reperfusion. Park et al[57] reported a similar result in 9 months old rats (compared with 3 months old ones) subjected to 70% warm ischemia for 75 minutes followed by 8 hours reperfusion. The studies from Okaya and Park strongly insinuated that the aging process undermined hepatic function and especially impaired the hepatic stress response despite showing only minimal histological changes before the insult.

A growing number of experimental studies suggest that the aging process of the liver is dominated by the growing impairment of 3 processes, playing also an important role in hepatic IRI: impairment of intracellular energy metabolism, enhancement of inflammatory response and alteration of autophagy.[56-64] In the following 3 chapters, we describe the underlying pathophysiology in age and in IRI as well as the resulting considerations for a therapeutic strategy.

ENERGY METABOLISM

The impairment of intracellular energy metabolism because of the dysfunction of mitochondria is one of the major biologic changes of the aging process in a number of organs. Since the early reports from 1956, the mitochondrial-free radical theory of aging is well accepted.[69] According to this theory, the functional degradation of the mitochondria is considered as one of the major forces of the aging process. Study from Lesnefsky et al[70] demonstrated that the cytochrome C binding site in mitochondria is impaired in old rats, which was associated with an aggravated cardiac IRI. Growing evidence supports that mitochondria in aged organs including livers are producing less adenosine triphosphate (ATP) but more free radicals.[71,72] Selzner et al[58] reported that

TABLE 2.

Experimental studies investigating the influence of age on hepatic IRI

Year	Authors	Animal model	Age	IRI model	Key observation	Potential therapy strategy
2005	Okaya et al	C57BL/6 mice	12-13 mo	70% liver WIR 90 min	IRI is more severe in aged livers in mice.[56]	—
2007	Park et al	Lewis rat	9 mo	70% liver WIR 75 min	IRI is more severe in aged livers in rat.[57]	—
2007	Selzner et al	C57BL/6 mice	60 wk	70% liver WIR 60 min	The decreased ability to produce mitochondrial ATP in aged livers aggravated IRI. Ischemic preconditioning did not protect against hepatic IRI in aged livers.	Enhancing intercellular ATP level by glucose administration preserves the lost effects of ischemic preconditioning in aged liver.[58]
2009	Selzner et al	C57BL/6 mice	60 wk	70% liver WIR 60 min	Enhanced induction of TNFα -dependent apoptosis aggravated IRI in aged livers.	Inhibition of apoptosis pathways protects against IRI in aged livers.[59]
2009	Schiesser et al	Sprague-Dawley rats	20-24 mo	70% liver WIR 45 min	—	Intermittent ischemia is effective in restoring bile flow after IRI in aged livers.[60]
2009	Huber et al	C57BL/6 mice	12-14 mo	70% liver WIR 30/60/ 90 min	Ubiquitin-proteasome pathway is significant downregulated in aged livers. Decreased expression of PSMD4 in aged livers leads to a defect in hepatic NF-κB signaling during IRI.[61]	—
2011	Wang et al	C57BL/6 mice	26 mo	Total liver WIR 20 min	Loss of atg4b in aged livers increases their sensitivity to IRI.	Induction of autophagy may suppress age-dependent hepatic IRI.[62]
2012	Kireev et al	Wistar rat	14 mo	70% liver WIR 35 min	IRI stimulated expression of p roinflammatory cytokines is increased in aged rats.	Melatonin treatment reduced the expression of IRI stimulated proinflammation genes in aged livers.[63]
2014	Trocha et al	Wistar rat	12-14 mo	70% liver WIR 60 min	The influence of IRI on ADMA-DDAH-NO pathway in rat livers is dependent on age.[64]	—
2014	Liu et al	Wistar rat	16-18 mo	Liver transplantation	—	Induction of exogenous hTERT gene by adenovirus protects IRI in aged livers.[65]
2014	Huang et al	Sprague-Dawley rats	20 mo	70% liver WIR 90 min	—	Downregulation of miR-34a gene expression enhance the protective effect of H_2S on hepatic IRI in aged livers.[66]
2016	Limani et al	C57BL/6 mice	20-24 mo	70% liver WIR 60 min	—	Instead of ischemic preconditioning and intermittent clamping, remote ischemic preconditioning prevents IRI in aged livers.[67]

WIR, warm ischemia/reperfusion; ADMA-DDAH-NO, asymmetric dimethylarginine-dimethylarginine dimethylaminohydrolase-nitric oxide;hTERT, human telomerase reverse transcriptase.

the hepatic energy content in 60-week-old mouse livers was decreased by half compared with 6-week-old mouse livers. The generation of reactive oxygen species by mitochondria during aerobic respiration increases with age and causes cellular damage.[71]

This reactive oxygen species theory suggests that the aged organs are more vulnerable to oxidative stress such as ischemia or hypoxia due to dysfunction of mitochondria. On one hand, less ATP generated by aged mitochondria leads to a lower intracellular energy content which aggravates ischemic stress. On the other hand, generation of higher levels of free radicals leads to more direct cell damage after reperfusion.[73]

The disorder of intracellular energy metabolism as one of the major biologic phenomenon in IRI is more severe in aged animals compared with younger ones. The intracellular energy level is under fierce fluctuations during hepatic IRI.[15,23] Mitochondria play a critical role in this process.[22] As we described before, decreased mitochondrial functions due to aging process have been observed in aged liver. As early as 1994, Le Couteur et al[14] described their observation about "old" Wistar rats (24-28 months) with or without 24 hours fasting representing different responses to hypoxia/reoxygenation injury compared to young animals. In 2007, Selzner et al[58] documented a decreased ATP level after IRI in mice with 60 weeks of age. This study suggested a decreased ability to produce mitochondrial ATP in aged livers from C57/BL6 mice subjected to 70% warm ischemia for 60 minutes. Decreased ATP levels induced cell death processes, such as apoptosis or necrosis, after IRI in aged livers and led to more hepatic damage. Furthermore, the aging process-induced

mitochondria dysfunction resulted in a more severe IRI-related MPT, which is aggravated after inflammatory response.[26]

Interestingly, well-known therapeutic concepts to alleviate hepatic IRI apparently do not yield the expected beneficial results in aged livers. In previous clinical trials, some strategies only protected young livers but not aged livers against ischemia-reperfusion. Ischemic preconditioning is widely used in surgery since first reported by Murry et al[74] in 1986. It has been proven that ischemic preconditioning is a simple and stable strategy to protect organs from IRI by regulating a number of stress- and survival-related molecules, including NF-κB, signal transducer, and activator of transcription 3, p38, JNK, and cyclin D1.[22] However, a clinical study documented that ischemic preconditioning did not protect against hepatic IRI in patients older than 65 years.[6] This might be related to the reduced intracellular energy content. As we described before, a potential explanation is that the reduced ATP levels in aged livers could impair the interplay of intracellular death and survival mediators. According to this explanation, reduced ATP levels decrease the tolerance of livers to ischemia, which may even result in damage to the organ after brief periods of ischemic preconditioning.[58]

Based on this hypothesis, a simple strategy to preserve the lost effects of ischemic preconditioning in aged liver is to enhance intercellular ATP level by glucose administration. Selzner et al[58] reported that the protective effect of ischemic preconditioning could be restored by 0.2 mL of 10% D-glucose injection before ischemia and reperfusion in senescent rats aged 60 weeks. He also demonstrated that pretreatment with D-glucose and ischemic preconditioning before ischemia prevented the development of necrosis in old mice. This result suggested that the reduced intracellular energy content because of the impairment of mitochondrial function in aged livers is one of the major biologic changes which aggravate IRI. According to this result, glucose administration is one of the most simple and economic strategies to protect aged liver from IRI.

In summary, the aging process leads to mitochondria dysfunction resulting in reduced intracellular energy levels, which is aggravated upon IRI. Simply replenishing energy substrates by glucose administration did reduce the augmented IRI in a rodent model.

Over the past decade, machine perfusion technique was introduced in transplantation as one of the most promising strategies to protect donor grafts from cold IRI.[75] A number of studies demonstrated that the use of machine perfusion not only significantly improves the posttransplant graft function but also provides an opportunity for surgeons to monitor the viability of the graft.[75-81] Furthermore, the use of machine perfusion may improve the quality of marginal grafts by offering the chance to deliver therapeutic interventions during transport.[82] In general, the application of machine perfusion improves the intracellular energy metabolism of grafts by increasing the energy contents and reducing the generation of oxygen-free radicals.[75,83] The ex vivo machine perfusion may be static or pulsatile and may be hypothermic, subnormothermic, or normothermic.[83] Schlegel et al[78] demonstrated that a short period (1-2 hours) of hypothermic oxygenated perfusion after cold storage improved the viability of the liver grafts, especially for liver grafts donated after circulatory death (DCD) by regulating mitochondrial respiration rate. Bruinsma et al[77] reported that the use of subnormothermic (21°C) machine perfusion protected DCD liver grafts during cold ischemia by improving the activity of energetic cofactors and redox shifts. Minor et al[79] reported that persufflation with pulsatile oxygen flow improved the early liver grafts recovery upon reperfusion. It is also reported that the use of normothermic machine perfusion improves liver graft function after transplantation using DCD liver grafts.[76]

However, the evidence regarding the effect of machine perfusion on aged liver donors is still limited. Pezzati et al[84] reported a case recently that the viability of a graft from an 83-year-old brain-dead donor was successful preserved by normothermic machine perfusion. It also remains unclear which strategy of machine perfusion should be performed on marginal donors for maximal protective effect. Nevertheless, because machine perfusion greatly improved the intracellular energy levels of grafts during cold ischemia, it is also a promising therapeutic strategy to protect aged liver donors from IRI. Besides, it is worth mentioning that an ongoing clinical trial sponsored by Universitaria Pisana (NCT02940600) is specifically investigating the potential benefit of normothermic machine perfusion in very old liver grafts (70 years or older). The primary result of this clinical trial is expected to be available in April 2018.

INFLAMMATION

Growing evidences suggested that using aged grafts leads to a more severe postoperative inflammatory response than the use of young and middle-aged grafts.[85] As an aerobic organ, liver is highly vulnerable to hypoxic stress.[86]

An age-dependent protein, heat shock protein 70 (HSP70) plays a critical role in the inflammatory response after hepatic ischemic damage.[56] In normal liver, the expression of HSP70 is increased promptly after damage occurs. As a protective response, enhanced HSP70 level reduces liver injury by inhibiting the inflammatory response. High expression of HSP70 in the liver reduces hepatic NF-κB activation as well as high levels of HSP70 reduce serum MIPs and TNFα generation.[87] NF-κB and MIPs are well accepted as important proinflammatory mediators, which affect the inflammatory response via a modulation of inflammatory cytokines expression.[88,89] Okaya et al[56] reported in his study that after liver damage, aged livers produced less HSP70 than young livers. Matsutani et al[90] demonstrated that the expression of inflammatory mediators, such as TNFα and IL6, was significantly enhanced in aged mice compared with younger ones.

The inflammatory response plays a critical role in both initial and later phases of hepatic IRI in young and even more in old animals.[24,39] Activation of Kupffer cells is a central event in the initial phase of hepatic I/R injury, so is the activation of neutrophils in the later phase.[19] A series of studies suggested that the activation of a ubiquitous transcription factor, NF-κB, is considered a major checkpoint of inflammatory response induced by IRI.[88,91,92] The activity of both Kupffer cells and neutrophils is regulated by NF-κB. It has been reported that the activity of NF-κB is age-dependent. Compared with mice aged 8 to 12 weeks, 12 to 13 months old ones show an increased activation of liver NF-κB during hepatic IRI.[56] NF-κB regulates inflammatory processes by modulating the expression and release of a number of proinflammatory cytokines, such as TNFα, MIPs, inducible NO synthases (iNOS), and intercellular adhesion

molecule 1 after IRI.[91] Kireev et al[63] reported a similar result in a rat model of 70% warm ischemia for 35 minutes followed by 36 hours reperfusion. The activation of Kupffer cells in the initial phase of hepatic I/R injury regulates NO production, which is involved in the inflammatory response by modulating the generation of reactive oxygen species.[24] A recent study demonstrated that asymmetric dimethylarginine dimethylaminohydrolase pathway, which is age related, regulates NO activity after hepatic I/R injury. Trocha et al[64] reported that 12 to 14 months old rats present with significantly higher aminotransferase levels as well as significantly lower activity of iNOS compared with 2 to 4 months old rats. As an important proinflammatory mediator, iNOS regulates the ischemia-reperfusion–related inflammatory response via modulating the metabolism of reactive oxygen species.[93] The aggravated inflammatory response in aged livers ultimately leads to large areas of hepatic necrosis.[37] The inflammatory response also activates other cell death and survival processes, such as apoptosis and autophagy, by regulating the phosphorylation rate of signaling molecules, such as JNK, ERK, and GSK3b pathways.[40]

Because the inflammatory response plays a critical role in hepatic IRI, suppression of the augmented inflammatory response is a promising strategy to treat IRI in aged animals. A number of studies selected inflammation-related strategies to protect aged livers from IRI. As one of most important downstream mediators of NF-κB, the activation of TNFα is considered as a critical event of the inflammatory response followed IRI.[91] To inhibit the activation of TNFα is a promising strategy to protect hepatocytes from IRI. Selzner et al[59] presented that inhibition of the reperfusion-related TNFα activation via different strategies resulted in stronger protection to IRI in 60-week-old mice compared to 6-week-old ones. Two independent methods targeting the reperfusion-related increase of TNFα were used in this study: TNF receptor 1 knockout mice and treatment with pentoxifylline, which is an inhibitor of TNFα synthesis. The authors demonstrated that the postoperative AST levels were decreased by more than half after pentoxifylline treatment. Furthermore, a lower postreperfusion apoptosis and necrosis rate were observed in the treatment group. However, the protective effect of pentoxifylline was only observed in aged animals but not observed in young ones. Melatonin is considered a regulator of inflammation by modulation of NO formation and activation.[94,95] In 2012, Kireev et al[63] demonstrated that intraperitoneal injection of 10 mg/kg melatonin significantly reduced warm ischemia-reperfusion–related hepatic damage in Wistar rats aged 14 months.

In brief, aging leads to an aggravation of the IRI-related inflammatory response. Promising first results to reduce the age-augmented aggravation of the inflammatory response were obtained with using pentoxifylline to inhibit the activation of TNFα and by using melatonin to regulate NO formation.

AUTOPHAGY

Hepatic autophagy is also affected by the aging progress.[96] Autophagy is considered as a prosurvival response to protect hepatocytes against stress.[25] Mitochondria as well as other organelles can be damaged during ischemia. Dysfunctional mitochondria generate less ATP but more free radicals, which aggravates the injury.[27]

Autophagy is a self-digesting process to promote cell survival by degrading dysfunctional organelles generated when cells are under stress. By autophagy, damaged organelles, such as mitochondria, are degraded to maintain intracellular energy level and to reduce free radicals generation. Reduced autophagic flux impairs the ability of cells to withstand oxygen damage, such as ischemia or hypoxia.[46] Wang et al[62] reported that the expression level of autophagy-related protein 4b (atg4b), which is considered as one of the most important autophagy-related proteins, is reduced in aged liver. The reduced expression of atg4b inhibits autophagy by regulating light chain 3 activity. It is also confirmed by other studies that livers lose their autophagy activity with aging process.[96,97]

The reduced autophagic flux in aged liver leads to an aggravated IRI. The growing number of studies suggested that autophagy protects hepatocytes from death during and after IRI.[41,42,98] A study in 26-month-old mice demonstrated that the loss of atg4b in aged livers led to the reduction of light chain 3 activity, which resulted in a reduced autophagic flux and a more severe IRI. In this study, isolated hepatocytes were incubated in Krebs-Ringer hydroxyethylpiperazine-N-2 buffer at pH 6.2 for 2 hours to simulate the ischemia phase and were incubated in Dulbecco modified eagle medium buffer for 12 hours to simulate the reperfusion phase. Compared with those from young mice, hepatocytes from old mice presented a highly reduced autophagy response after IRI simulation. This result was considered as a consequence of atg4b loss in hepatocytes from aged liver. An overexpression of atg4b induced by adenoviral delivery reversed the reduction of autophagy, as well as reversed the enhancement of IRI in aged hepatocytes. This finding was validated on a rodent model using 20 minutes total liver ischemia and 40 minutes reperfusion. Compared with wild type mice of similar age, 26-month-old mice with atg4b overexpression presented with a significantly reduced IRI.[62]

According to these findings, modulation of autophagy might be another promising strategy to reduce IRI-related necrosis. As we discussed before, autophagy is a prosurvival response which is highly reduced in aged liver. Upregulation of autophagy protects cells from death during starvation, hypoxia, and ischemia.[42] There are a number of drugs proven to promote hepatic autophagy.[99] Some of them, such as carbamazepine, may either affect hepatic metabolism or increase the risk of surgery. These drugs should be avoided in elderly patients especially under ischemia-reperfusion condition. However, lithium treatment is considered a simple, safe, and reliable strategy to induce hepatic autophagy via a modulation of both mechanistic targets of rapamycin–dependent and independent pathways.[41] In our previous studies, we demonstrated that induction of autophagy by lithium treatment prevented warm IRI in normal[41] and steatotic[100] livers. We extended this setting to a model of 70% warm IRI in rats with age of 2 years. We observed in a preliminary experiment that treatment with lithium also protected aged liver from IRI in terms of lower release of liver enzymes and less confluent necrosis (unpublished observations).

In short, aging leads to an impairment of autophagy, which contributes to the reduced tolerance of aged livers to IRI. First limited observations suggested that modulation of autophagy by treatment with autophagy inducers, such as

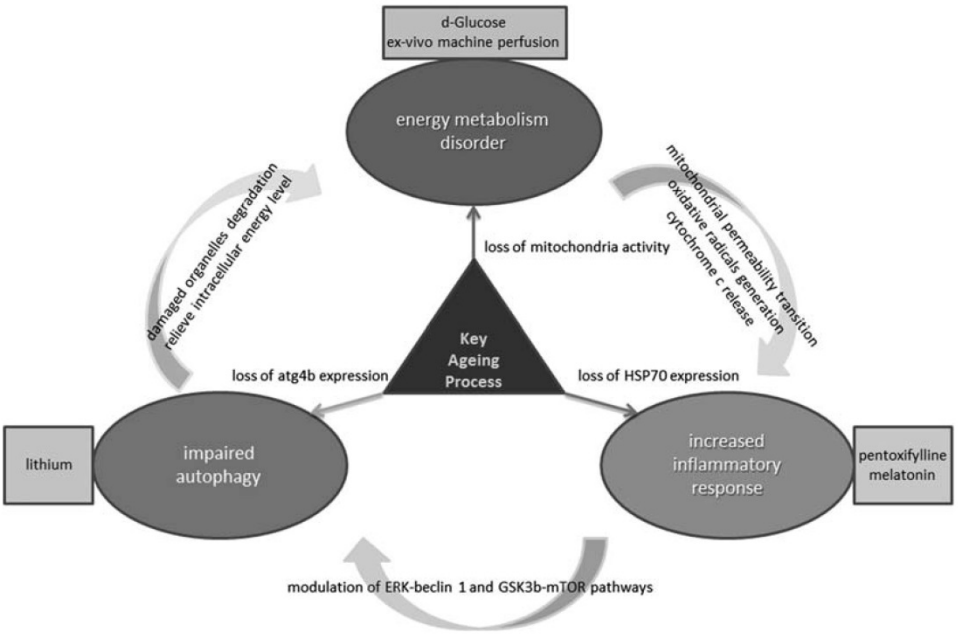

FIGURE 3. Scheme of key mechanisms of IRI in aged livers and promising therapeutic strategies. The aging progress of liver donors results in an energy metabolism disorder, an increased inflammatory response and an impaired autophagy. These biological changes aggravate IRI and ultimately lead to an increased risk of posttransplantation morbidity and mortality. Therapeutic strategies targeting in these mechanisms such as ex-vivo machine perfusion and treatment with D-glucose, pentoxifylline, melatonin, or lithium may be particularly useful for protecting aged liver donors from IRI.

lithium, could be a promising strategy to restore the reduced tolerance to hepatic IRI.

LIMITATION OF EXPERIMENTAL MODELS

The use of experimental studies based on rodent models has limitations, when considering the translation into the clinical setting.

Relative to the clinical situation, rodent models represent an ideal condition. In terms of donors, aged human organ donors often suffer from further impairments, such as fibrosis and steatosis, a phenomenon which is normally not observed in rodent models. In terms of recipients, in clinical reality, most recipients waiting for a liver transplantation are patients with end-stage liver disease, or hepatic malignancies. Few patients are healthy individuals suffering from acute liver failure. In contrast, preclinical experimental studies in rodents are performed with healthy animals not suffering from multimorbidity. Patients, donors, as well as recipients, suffer in general from additional complicating factors, such as intensive care unit stay time or surgical respective infectious complications, which may affect the clinical course of IRI as well.[12] Therefore, rodent models will never reflect the wide range of complexity as seen in the clinical reality.

As outlined above, therapeutic strategies targeting the described common mechanisms are particularly effective for protecting marginal livers from IRI. Therefore, it is and will be of great interest to evaluate successful experimental strategies in clinical trials in the future.

PROSPECT

Overall, intracellular energy metabolism, inflammatory response, and autophagy are 3 critical mechanisms involved in both age and IRI processes not only in liver but also in kidney[101,102] and heart.[103,104] Therapeutic strategies targeting these 3 mechanisms may be particularly useful for protecting aged liver, but potentially also other aged organs from IRI. In particular, machine perfusion is one of the most promising therapeutic strategies to protect old liver grafts from IRI via improvement of the intracellular energy levels of grafts during cold ischemia. It is of great interest to further explore the use of machine perfusion in other types of marginal liver grafts even more in other marginal organs in the future.

SUMMARY

To summarize, 3 critical processes are governing the aging progress of liver: the intracellular energy metabolism, inflammatory response, and autophagy. These 3 processes are also considered to determine the severity of hepatic IRI, especially in aged liver.

The increased risk of posttransplantation morbidity and mortality associated with the use of liver grafts obtained from aged donors is partly due to the underlying impairment of the intracellular energy metabolism of hepatocytes, to the enhancement of the inflammatory response, and to the alteration of autophagy in the aged donors.

We demonstrated in this review that the strategies targeting 1 of these 3 processes might be especially useful in aged donors with an increased risk for IRI after transplantation (see Figure 3). It remains to be demonstrated that combination strategies are even more useful to alleviate the augmented IRI in aged donors and render them a reliable additional source of organ grafts.

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Results and Discussion Part 3:

Discussion: Common mechanisms in steatosis, senescence and IRI processes and resulting hypothesis for the design of promising therapeutic strategies

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We demonstrated in part 1 that intracellular energy metabolism, inflammation and autophagy are common mechanisms shared by steatosis and IRI processes. Therapeutic strategies targeting these mechanisms such as induction of autophagy by lithium chloride treatment can reduce IRI in steatotic livers. We have demonstrated in part 2 that these mechanisms are also shared by senescence and IRI processes. Therapeutic strategies targeting these mechanisms are also especially useful to protect senescent livers from IRI.

However, marginal livers of the future are not only steatotic livers or aged livers but also livers suffering from both, age and steatosis, at the same time. The overwhelming changes in life style leading to the growing prevalence of obesity and the demographic changes leading to the increased number of senescent donors will result in a higher number of donor organs affected by both, steatosis and age. In our study, we identified and evaluated therapeutic strategies targeting intracellular energy metabolism, inflammation and autophagy that are promising to protect steatotic or aged livers from IRI. Since steatosis process and senescence process sharing common mechanisms, therapeutic strategies targeting these mechanisms may also be promising to protect aged and steatotic livers from IRI. On the basis of these two papers and previous studies of common mechanisms in steatosis, senescence and IRI processes, we want to further generate a novel hypothesis for reducing IRI in the aged steatotic liver.

Intracellular energy metabolism

Intracellular energy metabolism disorder is an important factor of both steatosis and senescence processes. It has been shown that therapeutic strategies targeting intracellular energy metabolism can protect either steatotic livers or aged livers from IRI. Nevertheless, the mechanisms of intracellular energy metabolism disorder in steatosis and senescence processes are different.

The fat accumulation resulting from lipid disorder plays a critical role in the development of NAFLD. Samuel et al. demonstrated in a series of studies that feeding rats a high fat diet resulted in an activation of the protein kinase C (PKC) and c-Jun N-terminal kinase-1 (JNK).

The activation of PKC and JNK leads to a reduced tyrosine phosphorylation level of insulin receptor substrate by regulating the activity of insulin receptor kinase and leads to a hepatic insulin resistance. According to the studies of Samuel, hepatic insulin resistance results in an impairment of insulin-mediated suppression of lipolysis and does ultimately cause the fat accumulation in hepatocytes (Samuel et al. 2010, Samuel et al. 2007, Samuel et al. 2004).

However, in aged livers, the intracellular energy metabolism disorder is mainly associated with dysfunctional mitochondria and reduced intracellular energy content. The impairment of the mitochondria function is one of the major forces of the senescence process. On one hand, mitochondria in aged livers are producing less ATP resulting in a reduced intracellular energy level. On the other hand, more free radicals generated by mitochondria in aged livers lead to a higher level of oxidative stress (Harman 1956, Cadenas und Davies 2000, Navarro und Boveris 2007).

As we described before, the development of steatosis is mainly related to a disturbance in the lipid metabolism leading to a lipid accumulation in the hepatocytes whereas and the aging is a result of impairment in the function of mitochondria. Therefore intracellular energy metabolism is not affected in the same way and therefore cannot be considered as a common mechanism in both processes. Therapeutic strategies targeting only the glucose or lipid metabolism might be not suitable to protect aged steatotic livers from IRI. Combination therapies such as Vitamin E succinate and glucose targeting both lipid and glucose metabolisms could be more promising therapeutic strategies in aged steatotic livers.

In addition, recently published studies suggested that ex-vivo machine perfusion is a promising therapeutic strategy that can significantly increase intracellular energy levels and reduce the generation of oxygen free radicals during graft transportation (Marecki et al. 2017). Depending on the temperature of the perfusate, the machine perfusion may be hypothermic, subnormothermic or normothermic (Marecki et al. 2017). Bessems et al. demonstrated that preservation of steatotic livers by hypothermic machine perfusion reduces hepatic damage during cold ischemia (Bessems et al. 2007). Due to the lipid accumulation, the hepatocyte size increases substantially in steatotic livers, resulting in a reduced sinusoidal space and ultimately leading to a reduced hepatic flow (Rinella 2015). The observation that machine perfusion is particularly effective in steatotic livers may be associated with the mechanical pressure improving the hepatic flow (Bessems et al. 2007). However, up to now, no systematic study dedicated to explore the effect of machine perfusion on the age-related augmentation of hepatic IRI was yet performed. A case was reported recently of using

normothermic machine perfusion successful preserving the viability of a graft from an 83 year old brain dead donor (Pezzati et al. 2017). Since ex-vivo machine perfusion protects steatotic liver donors and other type of marginal liver donors from IRI, it is of great interest to also explore the effect of machine perfusion on aged livers or even aged steatotic livers in the future.

Inflammatory response

The inflammatory response is considered to be the most pivotal mechanism in both steatosis development and in aging. On one hand, it is well accepted that the inflammatory response plays a critical role in the development of NAFLD. To a certain extent, NAFLD itself is considered as a chronic inflammatory disease. The inflammatory response is considered as the major reason leading to hepatic fibrosis and cirrhosis (Rinella 2015). On the other hand, aged livers are also more vulnerable to IRI-induced inflammatory response. As described in detail before, heat shock protein 70 (HSP70) plays a critical role in the inflammatory response following hepatic ischemic damage. Okaya et al. demonstrated that aged livers produced less HSP70 than young livers. The activation of HSP70 protected the aged livers from the aggravated inflammatory response after IRI by reducing liver nuclear factor-κB activation and by reducing serum macrophage inflammatory proteins and tumor necrosis factor alpha (TNFα) generation (Okaya et al. 2005). Besides, the expressions of other inflammatory cytokines such as IL6 were also significantly increased in aged mice compared to younger ones (Matsutani et al. 2007).

Therapeutic strategies targeting the inflammatory response have been well studied in marginal livers. Melatonin (N-acetyl-5-methoxytryptophan) is mainly produced by pineal gland in humans. The major biological function of melatonin is to regulate the circadian rhythm. Moreover, it was reported recently that melatonin also presented a strong activity to protect against oxidative stress (Claustrat und Leston 2015). In 2011 and 2012, two independent studies employing different rodent models demonstrated that melatonin therapy protected both steatotic livers and aged livers from IRI by regulating the inflammatory response (Zaouali et al. 2011, Kireev et al. 2012).

Previous studies demonstrated that melatonin treatment exhibited hepatoprotective effects during IRI in marginal livers via a regulation of NO signaling pathway. Zaoualí et al. reported in 2011 that melatonin protected against hepatic IRI by inducing the generation of NO in homozygous Zucker rats (Zaouali et al. 2011). NO produced by endothelial NO synthase

(eNOS) maintained the hepatic microcirculation, inhibited leukocyte adhesion and migration as well as inhibited platelet aggregation (Fleming 2010). The activation of NO protected against hepatic IRI by relieving the oxidative stress and reducing the inflammatory response (Thomas et al. 2008). Melatonin induced NO formation and activation via an overexpression of heme oxygenase-1 (Li et al. 2014). Compared with normal livers, the activity of NO signal pathway was inhibited in both steatotic and aged livers during IRI. Persico et al. demonstrated recently in a clinical study that a marked eNOS dysfunction has been observed in the liver from NAFLD patients. This study suggested that the eNOS dysfunction is a peculiar and essential mechanism in the occurrence of NAFLD (Persico et al. 2017). Knott et al. demonstrated in a comprehensive review that NO signaling is also impaired with aging. The impaired NO signaling was considered to be involved in the development of a variety of geriatric diseases such as atheromatous disease, aortic valve stenosis and systolic hypertension (Knott und Bossy-Wetzel 2010).

Overall, the NO signaling pathway was inhibited in both steatotic livers and aged livers. Due to the fact that treatment with melatonin reduced hepatic IRI by inducing the activation of NO signaling pathway, we can raise the hypothesis that melatonin treatment can prevent IRI also in aged steatotic livers. Therefore, it is of great interest to further explore the protective effect of melatonin in aged steatotic livers.

Besides melatonin, a number of other drugs also protect marginal livers from IRI. Luo et al. demonstrated that theaflavin treatment attenuates IRI in steatotic livers via a regulation of TNFα pathway (Luo et al. 2012). Yang et al. reported that ankaflavin protects steatotic livers from IRI by inhibiting the function of Kupffer cells (Yang et al. 2015). In addition, metformin (Cahova et al. 2015) and pentoxifylline (Selzner et al. 2009) are proven to reduce IRI in marginal livers by targeting inflammatory response. Since both steatosis process and senescence process aggravates inflammatory response, it is of great interest to explore the protective effect of these drugs in other type of marginal livers and even in aged and steatotic livers.

Autophagy

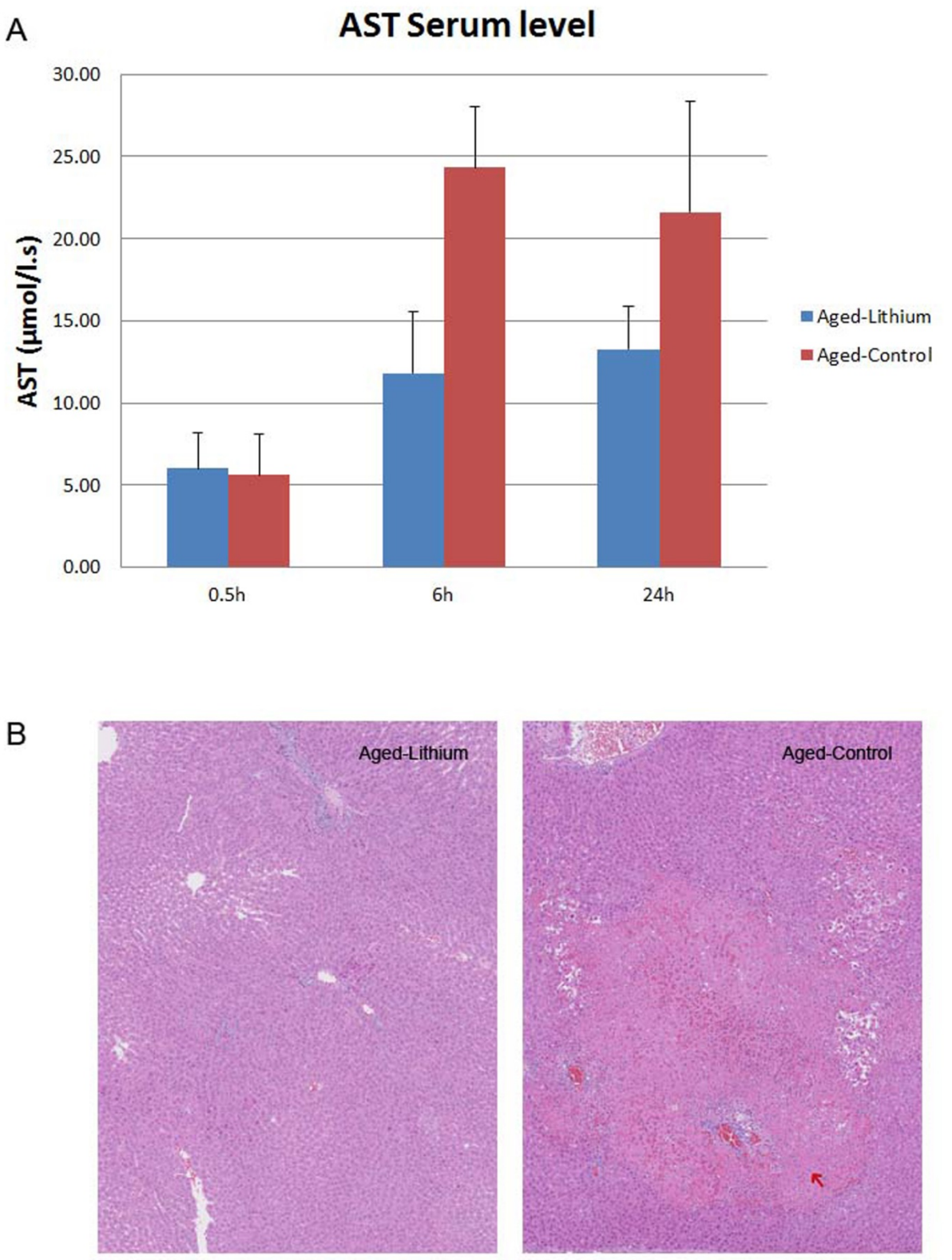
An impairment of autophagy was also observed in both, steatotic livers and aged livers. In 2009, Liu et al. reported that steatosis development affects insulin-mTOR signaling pathway and inhibits autophagy (Liu et al. 2009). In 2011, Wang et al. demonstrated that the expression level of autophagy-related protein 4b (atg4b) is reduced in aged liver. The reduced

expression of atg4b regulates light chain 3 (LC3) activity and inhibits autophagy (Wang et al. 2011).

Since autophagy is reduced in both steatotic and aged livers, it is also of great interest to explore the protective effect of induction of autophagy in aged and steatotic livers undergoing hepatic IRI. Current experimental evidence for therapeutic strategies targeting autophagy in hepatic IRI in aged livers is still limited. In this study, we generated own preliminary data that using lithium chloride as a drug targeting autophagy protected against IRI in aged livers. This result was indicated by lower serum liver enzyme levels and less necrotic areas (Figure 1).

Since induction of autophagy by lithium chloride treatment reduced IRI in both normal and steatotic livers and we already generated preliminary evidence regarding the effect in aged livers, it is also of great interest to further explore the protective effect of autophagy in the setting of aged steatotic livers in IRI.

Figure 1: Treatment with lithium chloride reduces (A) serum liver enzyme levels and reduces (B) necrotic areas after IRI in aged livers



Conclusion

Taken together, we concluded that intracellular energy metabolism, inflammation and autophagy are three critical mechanisms which are involved in steatosis, senescence and IRI processes.

Previous studies and our experiments demonstrated that therapeutic strategies targeting one or more of these three mechanisms are especially useful to protect steatotic and aged livers or even “aged steatotic livers” from IRI.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Anding Liu, Haoshu Fang, Michael Böttcher, Luisa Ungelenk, Amelie Lupp, Olaf Dirsch und Uta Dahmen,

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