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EXPERIMENTAL STUDY

Could Ambroxol reduce cytokines in hepatic ischemia--reperfusion injury in rats?

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ABSTRACT
OBJECTIVES: The aim of the study is to examine the effect of Ambroxol on TNF-α and IL-1β released after liver ischemia-reperfusion injury.
BACKGROUND: Many drugs are being tried to reduce ischemia-reperfusion injury, which is life threating problem after many liver surgeries. In this study, it was investigated whether Ambroxol reduces the release of pro-inflammatory cytokines released after liver ischemia-reperfusion injury.
METHODS: Twenty-four Wistar albino rats were divided into 3 groups as Control (CTR; n=8), hepatic ischemia reperfusion (H-IR; n=8) and hepatic ischemia reperfusion+Ambroxol (H-IR+AMB; n=8). In H-IR+AMB group, Ambroxol (30 mg/kg) was administered orally 30 minutes before ischemia period. In H-IR and H-IR+AMB groups underwent 45 minutes of hepatic ischemia followed by a 60-minute reperfusion period. After reperfusion period, tissue and blood samples were collected from euthanised animals. ALT, AST, ALP, LDH, TNF-α, IL-1β concentrations and liver tissues were evaluated.
RESULTS: Serum ALT, ALP, AST, LDH, TNF-α and IL-1β values were lower in the H-IR+AMB group compared to the H-IR group. In the histopathological examination, hepatocyte degeneration and congestion in the H-IR group were higher than in the H-IR+AMB group.
CONCLUSION: It was determined that Ambroxol treatment suppressed the production of pro-inflammatory cytokines TNF-α and IL-1β in rats undergoing hepatic ischemia reperfusion *(Tab. 1, Fig. 2, Ref. 28)*. Text in PDF *www.elis.sk*
KEY WORDS: Ambroxol, hepatic ischemia reperfusion, pro-inflammatory cytokines, rat.

Introduction

Hepatic ischemia/reperfusion (H-IR) injury can occur due to variable conditions such as trauma, shock, sepsis, heart failure, liver transplantation, and hepatectomy operations (1). Kupffer cells, livers' stellate macrophages, produce cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) stimulating T lymphocytes (2, 3). These cytokines, one of the main damaging factors for hepatic cells, resulting in increased microvascular permeability and interstitial oedema, disrupt vasoregulation, inflammatory cell infiltration and parenchymal cell degeneration (4–6).

Ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl]benzylamine) is a drug widely used for the treatment of respiratory tract diseases due to its mucolytic and secretory properties (7). Besides, Ambroxol is endowed with the potent capacity to exert anti-inflammatory action (8, 9). Ambroxol reduces lipopolysaccharide (LPS)-induced cytokine synthesis in macrophages. It has been reported that the anti-inflammatory effect of Ambroxol is to reduce TNF-α and neutrophil infiltration in rat lungs (10). Besides, Ambroxol also reduced the release or production of some cytokines such as IL-1β (11).

Rodents are often used in experiments based on animal study models of tissue injury because of similarities to humans (12, 13). Subsequently, rat, a genus of rodents, was selected as the model animal for this study. This study aimed (i) to answer whether Ambroxol administration protects against hepatic damage induced by the ischemia-reperfusion injury model in rats.

Materials and methods

Ethical statement

The local animal ethics committee of Near East University approved the study protocol (Approval no: 2019-96). The laboratory staff was blinded to the groups and the administration protocols allocated to the rats.

Animals

Wistar albino rats, a total of twenty-four 200–250 g of both sexes, were assigned to study and housed in humidity (60 %) and temperature-controlled room (22±2 °C), with a 12-hour light/

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dark cycle that used fluorescent lighting. Outbred Wistar albino rats were housed in a conventional rectangular, solid plastic cages 20 cm high with a wire mesh lid. The rats had access to pelleted food and water *ad libitum*.

Experimental model

Twenty-four rats were divided into three groups, each containing eight animals as follows: control (CTR) group (rats operated without ischemia/reperfusion), hepatic ischemia-reperfusion group (H-IR), and hepatic ischemia/reperfusion + Ambroxol treatment group (H-IR+AMB). No randomization method was determined in the study. Exclusion criteria were determined as rats dying during ischemia and/or reperfusion period. There were no rats excluded from the study. Ambroxol was given in a dose of 30 mg/kg orally in H-IR+AMB groups 30 minutes before the ischemia (14). In all groups rats were anaesthetised by intraperitoneal injection with a mixture of Ketamine (10 % Ketamine, Dutchfarm, 100 mg/kg) and Xylazine (2 % Vetaxyl 20 mg, Vetagro, 10 mg/kg) and fixed in a supine position. Access to the abdominal cavity was performed via a ventral midline incision in all groups. In CTR group, abdominal cavities were closed without any manipulation. In the H-IR and H-IR+AMB groups to induce ischemia, the hepatoportal vein and hepatic artery were ligated for 45 minutes, and the colour change in the liver was observed. The liver was re-perfused for 1 hour by opening the ligature. The rats were euthanised with overdose of Xylazine+Ketamine anaesthesia at the end of the reperfusion period. The livers were excised, and blood samples were collected (3). All surgical procedures and sample collections were performed by the same veterinary surgeon in the experimental work section under the housing unit conditions.

Biochemical analysis

Blood samples were collected from rats into serum separator tubes (SST). Samples had stood for 30 minutes for complete blood clot formation, and then sera were separated at 1500 g x 10 minutes. The samples were kept at a temperature of –20 °C until analysis.

Alanine aminotransferase (ALT, in U/L), aspartate aminotransferase (AST, in U/L), alkaline phosphatase (ALP, in U/L), lactate dehydrogenase (LDH, in U/L) activities from sera were measured to assess the hepatocellular injury. Based on IFCC methods, commercially available assay kits were run using an automated analyser (BS120, Mindray, Shenzhen, China).

Serum TNF-α (pg/mL) and IL-1β (pg/mL) concentrations were determined using rat-specific enzyme immunoassay kits following the manufacturers' directions. (Rat TNF-α ELISA Kit Catalog No: E-EL-R0019; Rat IL-1β Catalog No: E-EL-R001, Elabscience Biotechnology Inc., TX, USA).

Histopathological analysis

Removed livers were fixed in 10 % neutral-buffered formalin solution. Liver tissues were then dehydrated with a series of varying alcohol concentrations, and embedded in paraffin blocks. After blocking process, sections with a thickness of 5 – 6 µm were stained using hematoxylin and eosin (H&E). Histopathological analyzes were performed under a light microscope (Zeiss-Axio Scope A1, Carl Zeiss, Göttingen, Germany).

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA). All results were expressed as the means ± SD. A one-way analysis of variance (ANOVA) was used to compare the measured parameters. Tukey's test was used for further analysis of binary comparisons. p values below 0.05 were regarded as statistically significant.

Results

ALT, AST, ALP, LDH values are used to determine the effects of ischemia-reperfusion injury on liver cells. After the ischemia-reperfusion injury, a significant elevation in ALT, AST, ALP and LDH activities in the H-IR group was detected compared to the CTR group ($p < 0.001$). However, in the Ambroxol administered group (H-IR+AMB), ALT, AST, ALP and LDH activities significantly decreased compared to H-IR group, $p < 0.001$, $p < 0.01$, $p < 0.001$, $p < 0.0001$c, respectively (Tab. 1).

The pro-inflammatory cytokines TNF-α and IL-1β levels were determined to evaluate inflammatory response after the ischemia-reperfusion injury and Ambroxol administration. Rats in H-IR

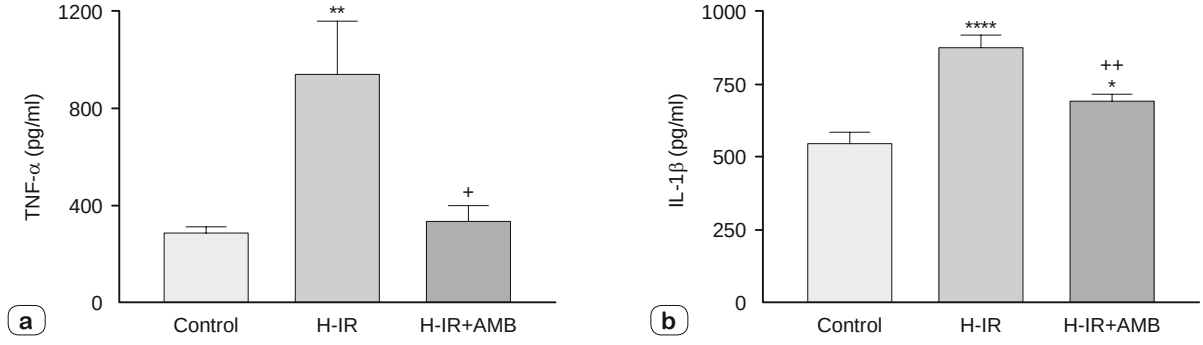

Fig. 1. TNF- α (a) and IL-1 β (b) levels in the serum samples of groups CTR (control), H-IR (Hepatic ischemia/reperfusion) and H-IR+AMB (Hepatic ischemia/reperfusion+ Ambroxol). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$ compared with the control (CTR) group, + $p < 0.05$, ++ $p < 0.01$ compared with the H-IR group.

Tab. 1. Serum ALT, AST, ALP, LDH, activities in the control (CTR), hepatic ischemia/reperfusion (H-IR) and hepatic ischemia/reperfusion+Ambroxol (H-IR+AMB) groups.

	CTR	H-IR	H-IR+AMB
ALT (U/L)	65.28±15.90	2228±229****	869.5±209.8$^{*,+++}$
AST (U/L)	118.7±17.8	1303±160****	619.2±112.3$^{*,++}$
ALP (U/L)	108.3±9.2	182.2±7.74****	119.6±6.1^{+++}
LDH (U/L)	3778±498	13060±804****	4694±986^{++++}

$^{*}p < 0.05$; $^{****}p < 0.001$, compared with the control (CTR) group. $^{++}p < 0.01$; $^{+++}p < 0.001$, compared with the H-IR group

group secreted significantly higher levels of TNF-α ($p < 0.01$) and IL-1β ($p < 0.001$) than rats in CTR group. Nevertheless, TNF-α and IL-1β levels declined in H-IR+AMB group compared to H-IR group, $p < 0.05$ and $p < 0.01$, respectively (Fig. 1).

Histopathological findings demonstrated that the CTR group had a regular appearance with sinusoids and hepatocytes (Fig. 2a). In contrast, the H-IR and H-IR+AMB groups had dense congestion throughout the tissue and massive degeneration in the cytoplasm of the hepatocytes (Fig. 2b). It was also observed that the disseminated congestion and regression of degeneration of the hepatocytes decreased in the H-IR+AMB group (Fig. 2c).

Discussion

Ischemia-reperfusion injury is a series of reactions, mainly including infiltration of inflammatory cells to ischemic tissues, thus promoting cytokine secretion and necrosis, which develop due to interruption of blood flow in tissues and organs (15). Hepatic ischemia-reperfusion may be caused by transplantation and operations, pathophysiological processes that develop due to several organ failures or trauma. Although the development and stages of ischemia-reperfusion injury are still being investigated, many therapeutic approaches have been tried in experimental studies to prevent damage (16, 17). Therefore, we investigated the possible protective effect of Ambroxol on hepatic damage caused by ischemia-reperfusion injury through evaluating pro-inflammatory cytokines, as well as hepatic leakage enzymes.

The liver is a crucial organ with many functions such as metabolic homeostasis, detoxification, and immunity and is often referred to as the metabolic chief of the body. The liver is subject to various conditions that can cause liver dysfunction (18). After liver diseases, ALT, AST, ALP or LDH enzyme activities are measured as a non-invasive method to determine and evaluate the liver's parenchymal and cellular damage and healing, IR injury is a phenomenon that is characterised by the disruption of functional and anatomical integrity in the organ or tissue, even if blood flow is provided in the ischemic organ (19, 20). Our study's histopathological examination results have demonstrated that hepatocyte damage and congestion in the H-IR group were more prominent than the damage in the H-IR+AMB group. Consistent with the histopathological results, an increase was determined in ALT, AST, ALP and LDH activities, addressing hepatocellular damage after IR injury. However, the increase in enzyme activities significantly declined in H-IR+AMB group. Thus, administration of Ambroxol prior to ischemia-reperfusion injury may be associated with regression of ischemia-reperfusion injury in liver cells. Besides, Jiang et al. (21) showed that Ambroxol regulates increased ALT and AST levels in a liver injury model. However, they did not evaluate the role of pro-inflammatory cytokines TNF-α and IL-1β and ALP and LDH activities. Although the findings of our study in terms of ALT and AST are parallel to Jiang's study, our study differs in terms of increased LDH and cytokines, emphasizing the importance of LDH activation and cytokines. This may also demonstrate the importance of LDH as a general indicator of tissue damage and an inflammatory marker (22). Besides, LDH is also considered as a prognostic factor (23).

After an ischemia-reperfusion injury, swelling of endothelial cells, vasoconstriction, platelet aggregation in sinusoids, and deterioration in microcirculation occurs. Swelling of endothelial and Kupffer cells results from disruption of active transmembrane transport due to ischemia and formation of intracellular oedema (24). Kupffer cells, activated in the early stages of reperfusion, synthesize and release cytokines such as TNF-α and IL-1β, involved in hepatocellular and endothelial damage in hepatic ischemia-reperfusion injury. TNF-α directly contributes to liver injury by activating or triggering important inflammatory factors and increasing neutrophil infiltration and parenchymal damage. Similarly, IL-1β causes liver cell damage with some effects, such as activating NF-kB, increasing leukocyte aggregation, and upregulating TNF-α production by Kupffer cells (17, 25, 26). Some

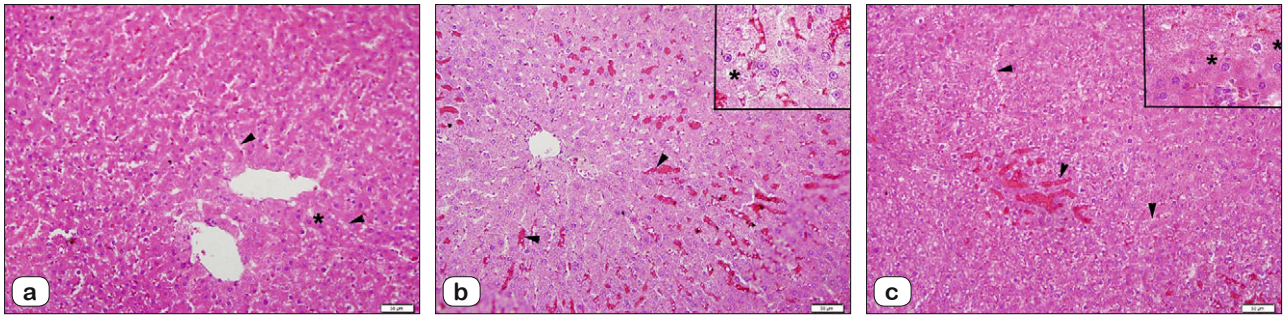

Fig. 2. a) Control group, regular structure with sinusoids (arrowheads) and hepatocytes (*), b) H-IR group, disseminated congestion in sinusoids (arrowheads) and severe cytoplasmic degeneration of hepatocytes (*), c) H-IR+AMB group, reduced sinusoidal congestion (arrowheads) and amelioration in hepatocyte cytoplasm (*). HE staining, inset (20 µm)

studies have reported that Ambroxol reduces the production and release of cytokines (10, 11). In this study, histopathological findings showed that ischemia-reperfusion injury caused congestion and degeneration in the cytoplasm of the hepatocytes, but these effects diminished in the H-IR+AMB group compared to the H-IR group. In addition, increases in TNF-α and IL-1β values were detected after IR, and levels of cytokines in H-IR were higher than in the H-IR+AMB group. The decline in hepatocellular and parenchymal damage in the H-IR+AMB group was consistent with the decrease in serum TNF-α and IL-1β levels. Ambroxol has been shown to reduce serum TNF and IL-1β levels with its anti-inflammatory effects in clinical and experimental studies (27, 28). Thus, the results of our study support the anti-inflammatory effect of Ambroxol; thus, ambroxol treatment reduced ischemia-reperfusion injury in the liver by decreasing TNF-α and IL-1β levels.

Hepatic ischemia-reperfusion injury is a set of cellular and inflammatory reactions that affect many organs and systems, especially after transplantation and organ surgeries. Many studies have established methods for determining the various tissue damage mechanisms and repairing or reducing the damage. Among the preventive approaches that can be used primarily to minimise the damage, some drugs' anti-inflammatory and cell-protective effects are being tested. For this purpose, Ambroxol, which has shown some protective and anti-inflammatory effects in various models, has been the subject of our study. Our results suggested that Ambroxol is among the options that can benefit from its protective effects before operations that may cause hepatic ischemia-reperfusion injury through its possible mechanism of activity by reducing the TNF-α and IL-1β levels, which were the subject of the study. Thus, more extensive experimental research is essential to clarify the mechanism of action.

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