

Distinct effects of different doses of kaempferol on D-GalN/LPS-induced ALF depend on the autophagy pathway

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Abstract. Kaempferol, a flavonoid compound, has various biological functions, such as anti-inflammatory and antitumor activities. Acute liver failure (ALF) is a lethal clinical syndrome that occurs due to severe damage of the liver function. In the present study, the mechanisms underlying the therapeutic effects of kaempferol in ALF were evaluated. An ALF mouse model was established using D-galactosamine (D-GalN; 700 mg/kg)/lipopolysaccharide (LPS; 10 μ g/kg). A total of 2 h before the administration of D-GalN/LPS, mice were pretreated with different doses of kaempferol (2.5, 5, 10, 20 and 40 mg/kg), and 6 h after injection of D-GalN/LPS, mice were euthanized. The survival rate, liver function and levels of inflammatory cytokines were assessed. The results demonstrated that kaempferol pretreatment protected hepatocytes from ALF induced by D-GalN/LPS via regulation of the autophagy pathway, both *in vivo* and *in vitro*. Pretreatment with a high dose of kaempferol significantly decreased the survival rates and increased severe liver damage; however, pretreatment with a low dose of kaempferol had the opposite effect. Furthermore, pretreatment with a high dose of kaempferol enhanced the levels of proinflammatory cytokines [TNF- α , IL-6, IL-12p40, IL-1 β , C-X-C motif chemokine ligand (CXCL)-2, CXCL-10] and markers of the MAPK signaling pathway [phosphorylated (p)-JNK, p-ERK, p-p38], whereas pretreatment with a low dose of kaempferol had the opposite effect. Pretreatment with a high dose of kaempferol decreased autophagy, whereas pretreatment with a low dose of kaempferol increased autophagy *in vivo* and *in vitro*. It was also shown that pretreatment with 3-methyladenine or autophagy related 7 small interfering RNA, to inhibit autophagy, partially

abrogated the hepatoprotective effects of pretreatment with 5 mg/kg kaempferol in the ALF mouse model. These results demonstrate that the effects of different doses of kaempferol on D-GalN/LPS-induced ALF varies based on the dose, and that kaempferol exerted its effects via regulation of the autophagy pathway.

Introduction

Acute liver failure (ALF) is a fatal hepatic disease associated with rapid loss of liver function, resulting in multiorgan dysfunction, encephalopathy and coagulopathy in patients. This critical illness has fatal consequences, and the only treatment option is an emergency liver transplant (1). Therefore, an effective drug for treating ALF is urgently required.

Kaempferol is a flavonoid that is primarily extracted from the root of *Kaempferia galanga* L., and it is widely present in various natural plants, fruits, vegetables, beverages and teas (2,3). Kaempferol possesses several pharmacological properties, including cardioprotective, neuroprotective, antioxidative, antidiabetic and anticarcinogenic effects (4). A growing number of studies have shown that kaempferol can reduce the risk of developing various cardiovascular diseases, diabetes and cancer, amongst other diseases (4-6). It has been reported that kaempferol may be associated with the treatment of numerous diseases. For example, in a time- and concentration-dependent manner, kaempferol can decrease HeLa cervical cancer cell viability and induce apoptosis by downregulating the activity of the PI3K/AKT signaling pathway (7). Previous research also revealed that kaempferol distinctly inhibited the proliferation of the EJ bladder cancer cell line by inducing S cell cycle arrest and apoptosis, and increasing the expression levels of phosphorylated (p)-p53 via regulation of the mitochondrial-mediated apoptotic signaling pathways (8).

The potential protective effects of kaempferol on liver injury have been previously demonstrated (9-11), where it has been shown to exhibit effective anti-inflammatory properties in liver cells (12). In addition, kaempferol has been demonstrated to significantly alleviate acute liver injury, inflammation and early hepatocyte apoptosis caused by propacetamol (13). Our previous study revealed that kaempferol pretreatment could alleviate liver damage in D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced mice (14). However, the underlying molecular mechanisms via which kaempferol exerts its effects in ALF remain to be determined.

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Autophagy is an intracellular catabolic signaling pathway, which is a highly conserved evolutionarily process, where biomolecules and organelles can be degraded by the lysosomes. Autophagy is crucial for maintaining the homeostasis of cells and replenishing several types of substances for cell survival under stressful conditions (15). Autophagy is closely associated with liver disease. It has previously been observed that autophagy may suppress the growth of tumors in chronic liver disease, and impaired autophagy can lead to a significant increase in glycolysis in liver cancer cells (16). In addition, autophagy can protect against the accumulation of fat in hepatocytes during nonalcoholic fatty liver disease (17). In our previous study, it was identified that activation of autophagy protected mice from ALF by inhibiting GSK-3 β activity (18). In current therapeutic research, the problem of drug toxicity remains a persistent issue. To the best of our knowledge, there are no previous studies showing the effects of different doses of kaempferol on ALF. Therefore, the toxicity of kaempferol requires further study.

Given the aforementioned information, in the present study, a mouse model of ALF induced by D-GalN/LPS was utilized, as it has been widely used to examine the underlying mechanisms of potential therapeutic drugs for the treatment of ALF (19,20). The functional effects of different doses of kaempferol were determined, the survival rate, liver function and levels of inflammatory cytokines were assessed, and the related regulatory pathways were evaluated in the context of ALF.

Materials and methods

Animals and treatments. A total of 40 male wild-type mice (C57BL/6; age, 8-12 weeks) were purchased from Capital Medical University (CMU; Beijing, China). The mice were housed in a standard environment with a controlled temperature of 22 \pm 2°C and humidity of 55 \pm 5%, under a 12-h light/dark cycle. Animals were provided with *ad libitum* access to water and food. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of CMU (approval no. AEEI-2020-009 on 2019/12/30).

The mice were injected intraperitoneally with D-GalN (700 mg/kg; Sigma-Aldrich; Merck KGaA) and LPS (10 μ g/kg; InvivoGen) to induce ALF, or were injected with an equivalent volume of normal saline. A total of 2 h before the administration of D-GalN/LPS, mice were pretreated with different doses (2.5, 5, 10, 20 or 40 mg/kg) of kaempferol (Sigma-Aldrich; Merck KGaA) via tail vein injection (n=8/group). Survival analysis was conducted based on the number of survivors in each group. 3-methyladenine (3-MA; Sigma-Aldrich; Merck KGaA) can be used to block autophagy, and chloroquine (CQ; Sigma-Aldrich; Merck KGaA) can be used to inhibit the fusion of lysosomes and autophagosomes, and are commonly used to assess autophagic flux (21,22). 3-MA (10 mg/kg) or CQ (60 mg/kg) were administered intraperitoneally 2 h before the administration of D-GalN/LPS. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). Liver tissues were collected for further analysis. Blood samples (~100 μ l) were collected from the eyeballs. Subsequently, mice were sacrificed by intraperitoneal injection of 100 mg/kg sodium pentobarbital after 24 h, and death was confirmed

by observing respiration and by using the corneal reflection method.

Serum aminotransferase activity. Blood samples were collected from the mice 6 h after D-GalN/LPS administration. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are markers of hepatic damage, were measured using a multiparametric analyzer (AU 5400; Olympus Corporation) according to the manufacturer's protocol (23).

Histopathological analysis. Liver samples were fixed in 4% formaldehyde solution for 12 h at 4°C and embedded in paraffin wax, after which, they were sectioned (4 μ m) and stained using H&E, according to routine procedure for histopathological evaluation; sections were stained with hematoxylin for 10 min and with eosin for 10 sec at room temperature. Tissues were observed using a light microscope (magnification, x200).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from liver tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT of RNA (2.5 μ g) into cDNA was performed using a SuperScript III first-strand synthesis system according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). A DNA Engine with a Chromo 4 detector (MJ Research, Inc.; Bio-Rad Laboratories, Inc.) was used for qPCR. The final reaction volume was 20 μ l and consisted of 1X super mix (Platinum SYBR-Green qPCR kit; Invitrogen; Thermo Fisher Scientific, Inc.), 2 μ l cDNA and 0.5 μ l each primer. The amplification conditions were as follows: Initial denaturation at 50°C (2 min), 95°C (5 min), followed by 50 cycles of 95°C (15 sec) and 60°C (30 sec). The mRNA expression levels were calculated using the 2^{- $\Delta\Delta C_q$} method (24). The sequences of the PCR primers used are listed in Table I and were produced by Sangon Biotech, Co., Ltd.

Western blotting. Liver tissue samples or cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with phosphatase and protease inhibitors, and the concentration was estimated using a Bradford Protein Assay kit (Beyotime Institute of Biotechnology). A total of 20 μ g protein per sample was loaded onto a 12% SDS gel, resolved using SDS-PAGE at 80 V for 30 min and 120 V for 1 h, and then transferred to a PVDF membrane using a Bio-Rad blotting transfer system (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% skimmed milk at room temperature for 1 h, and incubated overnight at 4°C with the following primary antibodies: β -actin (1:1,000; cat. no. 8457S; Cell Signaling Technology, Inc.), p-JNK (1:1,000; cat. no. 4668S; Cell Signaling Technology, Inc.), p-ERK (1:1,000; cat. no. 4348S; Cell Signaling Technology, Inc.), p-p38 (1:1,000; cat. no. 9215S; Cell Signaling Technology, Inc.), LC3B (1:1,000; cat. no. 3868S; Cell Signaling Technology, Inc.), autophagy related 7 (Atg7; 1:1,000; cat. no. 8558S; Cell Signaling Technology, Inc.) and p62 (1:1,000; cat. no. 23214S; Cell Signaling Technology, Inc.). After washing, the membranes with TBS-0.5% Tween-20 (TBS-T) and the corresponding HRP-conjugated secondary antibody (1:2,000; cat. no. 7074S; Cell Signaling Technology, Inc.) in 10 ml blocking buffer was

Table I. Sequences of the primers used for reverse transcription-quantitative PCR.

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
TNF- α	GCCTCTTCTCATTCCTGCTTGT	TTGAGATCCATGCCGTTG
CXCL-10	AAGTGCTGCCGTCATTTTCT	GTGGCAATGATCTCAACACG
CXCL-2	AGTGAAGTGCCTGTCAATG	TTCAGGGTCAAGGCAAACCTT
IL-6	GCTACCAAAGTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
IL-1 β	TTGACGGACCCCAAAAGAT	GATGATCTGAGTGTGAGGGTCTG
IL-12p40	CAGCTTCTTCATCAGGGACAT	CTTGAGGGGAGAAGTAGGAATGG
IL-10	ACTGCACCCACTTCCCAGT	TGTCCAGCTGGTCCTTTGTT
LC3	AGCAGCATCCAACCAAAATC	CTGTGTCCGTTACCAACAG
Atg7	ACCCAGAAGAAGCTGAACGA	CTCATTTGCTGCTTGTTCAC
HPRT	TCAACGGGGGACATAAAAGT	TGCATTGTTTTACCAGTGTCAA

CXCL-10, C-X-C motif chemokine ligand-10; CXCL-2, C-X-C motif chemokine ligand-2; Atg7, recombinant autophagy related protein 7; HPRT, hypoxanthine guanine phosphoribosyl transferase.

added and incubated for 1 h at room temperature. Next, the membranes were washed three times with TBS-T for 30 min, and signals were visualized using an ECL kit (Thermo Fisher Scientific, Inc.). Restore Western Blot Stripping Buffer was used to re-probe new target proteins in a same membrane (Thermo Fisher Scientific, Inc.). The blot was placed in Restore Western Blot Stripping Buffer and incubated for 5-15 min at 37°C. Then, the blot was removed from the Restore Western Blot Stripping Buffer and washed in PBS for 15-20 min. Next, as mentioned previously, the blot was incubated with new primary and secondary antibodies. Densitometry analysis was performed using ImageJ (version 1.49p; National Institutes of Health).

Atg7 small interfering (si)RNA treatment in vivo. siRNA (3 mg/kg; Shanghai Genepharma Co., Ltd.) and an Entranster™ *in vivo* transfection reagent (Engreen Biosystem Co.) were used to knock down Atg7 expression via the administration of a hydrodynamic tail vein injection in mice. After transfection for 24 h, subsequent experimentation was performed. The sequence of Atg7 siRNA was 5'-GCAUCAUCUUCGAAGUGAATT-3'. Scrambled siRNA (3 mg/kg) was used as a control and the sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. These steps were performed in accordance with the manufacturer's protocol.

Isolation of primary mouse hepatocytes. Hanks' solution containing collagenase was used to perfuse mouse livers when the mice were 7 weeks old, and, as described previously, live hepatocytes were separated using Percoll isocratic centrifugation at 4°C for 10 min at 10,000 \times g (25). The number of mice used was 3, and they were sacrificed by intraperitoneal injection of 100 mg/kg sodium pentobarbital after 24 h; death was confirmed by observing respiration and by using the corneal reflection method.

Starvation-induced autophagy in vitro. The most robust method of inducing autophagy is starvation of cells (26). Primary hepatocytes were transfected with the GFP-LC3 plasmid (1 μ g/ml; Shanghai Genepharma Co., Ltd.) for 12 h

at 37°C, and were treated with serum-free medium for 4 h at 37°C. The percentage of cells was calculated based on the number of GFP-LC3 puncta in the different treatment groups. GFP-positive cells were regarded as cells that exhibited bright, punctate staining. Per condition, ~50 cells were counted, and the experiment was repeated at least three times. Cells were observed using a fluorescence microscope (magnification, \times 200).

Statistical analysis. The results from three independent experiments are presented as the mean \pm SD. Survival analysis was conducted using a Kaplan-Meier plot with the log-rank test, and the P-value was corrected by Bonferroni's test. Statistical differences were determined using one-way ANOVA followed by Bonferroni's post hoc analysis in GraphPad Prism version 7 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of kaempferol on D-GalN/LPS-induced ALF. First, the effects of different doses of kaempferol on ALF induced by D-GalN/LPS in mice were examined. Different doses of kaempferol (2.5, 5, 10, 20 or 40 mg/kg) were intraperitoneally administered. As shown in the survival analysis (Fig. 1A), the survival rate of the 5 and 10 mg/kg kaempferol group was significantly increased compared with that in the D-GalN/LPS-treated group, and the 5 mg/kg kaempferol group showed the highest survival rate. This result suggested that 5 mg/kg kaempferol treatment effectively reduced D-GalN/LPS-induced liver injury.

Pretreatment with and 40 mg/kg kaempferol resulted in higher serum ALT (sALT) and serum AST (sAST) levels compared with the D-GalN/LPS induced group; whereas the 2.5, 5 and 10 mg/kg-treated mice exhibited lower levels of sALT and sAST, particularly the 5 mg/kg kaempferol group (Fig. 1C). Consistent with the ALT and AST activities, the liver histopathology in the 20 mg/kg kaempferol group showed increased hepatocyte injury, similar to that of the D-GalN/LPS group, but mice in the groups treated with

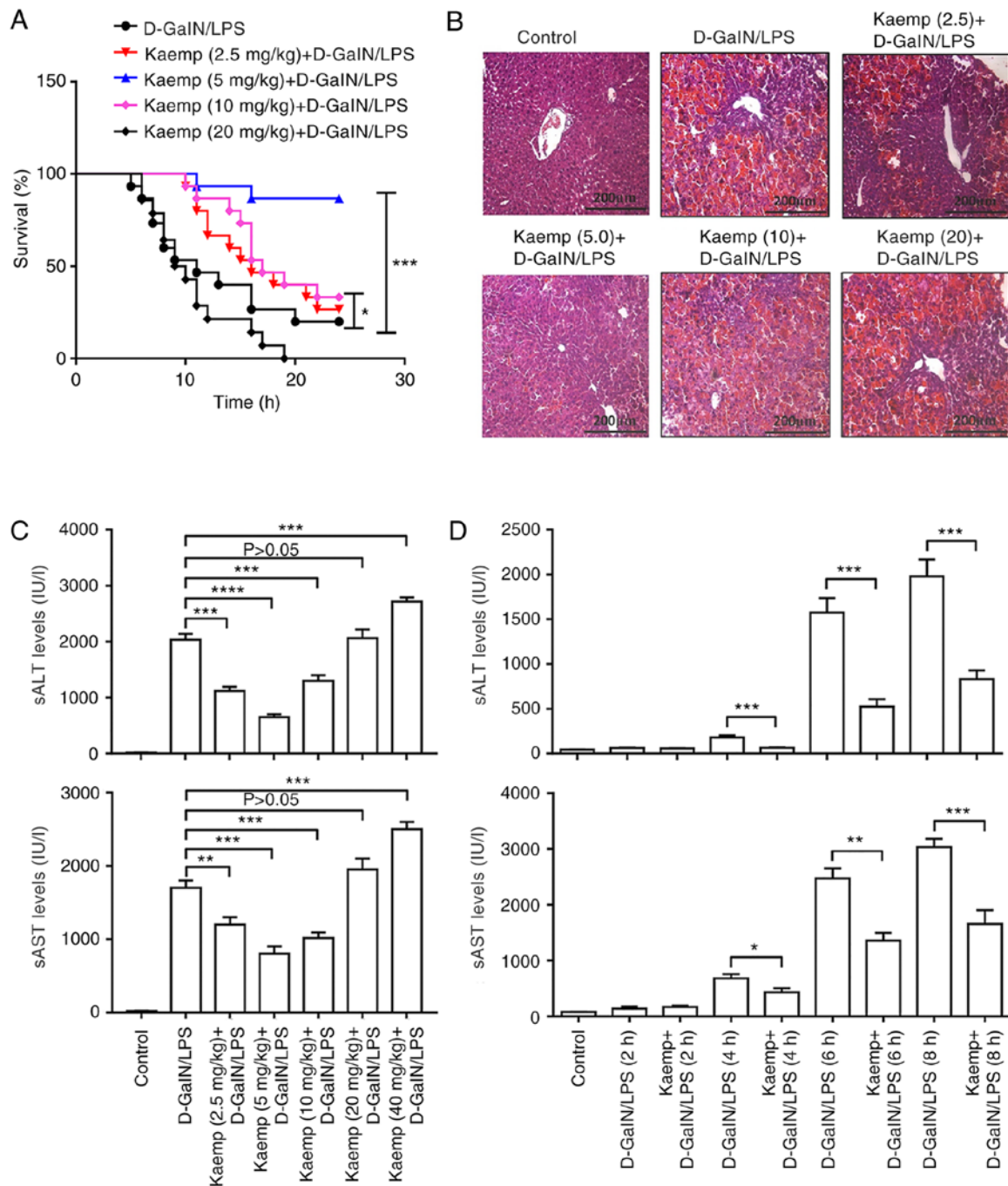


Figure 1. Effects of different doses of kaempferol on D-GalN/LPS-induced ALF. Mice treated with kaempferol + D-GalN/LPS were administered kaempferol (2.5, 5, 10, 20 or 40 mg/kg; intravenously) prior to or after D-GalN/LPS injection (n=20). Control mice were pretreated with saline before D-GalN/LPS injection (n=20). (A) Survival rates of ALF-induced mice treated with different doses of kaempferol and the PBS + D-GalN/LPS-treated group. * $P < 0.05$, *** $P < 0.001$ vs. D-GalN/LPS treated group. (B) H&E-stained liver sections of ALF induced model mice treated with different doses of kaempferol and the D-GalN/LPS-treated mice. Scale bar, 200 μ m. (C) sALT and sAST enzyme levels in the different groups. (D) sALT and sAST enzyme levels in the different groups, including mice pretreated with 5 mg/kg kaempferol, were measured to evaluate liver injury in mice after 2, 4, 6 and 8 h of D-GalN/LPS administration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ALF, acute liver failure; sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; D-GalN/LPS, D-galactosamine/lipopolysaccharide; kaempferol, kaempferol.

2.5 and 5 mg/kg kaempferol showed decreased hepatocyte injury, and the 5 mg/kg kaempferol group exhibited the lowest degree of injury (Fig. 1B).

Next, the protective effects of kaempferol (5 mg/kg) pretreatment on liver injury in mice 2, 4, 6 and 8 h after D-GalN/LPS administration was determined. The results demonstrated that the D-GalN/LPS-induced increases in sALT and sAST levels were significantly decreased by 5 mg/kg

kaempferol after 4, 6 and 8 h (Fig. 1D). These results suggested that high doses of kaempferol can induce more severe injury, whereas pretreatment with low doses of kaempferol significantly increased the survival rates of mice and protected against ALF induced by D-GalN/LPS.

Effects of kaempferol on liver inflammation in mice with D-GalN/LPS-induced ALF. Our previous study revealed

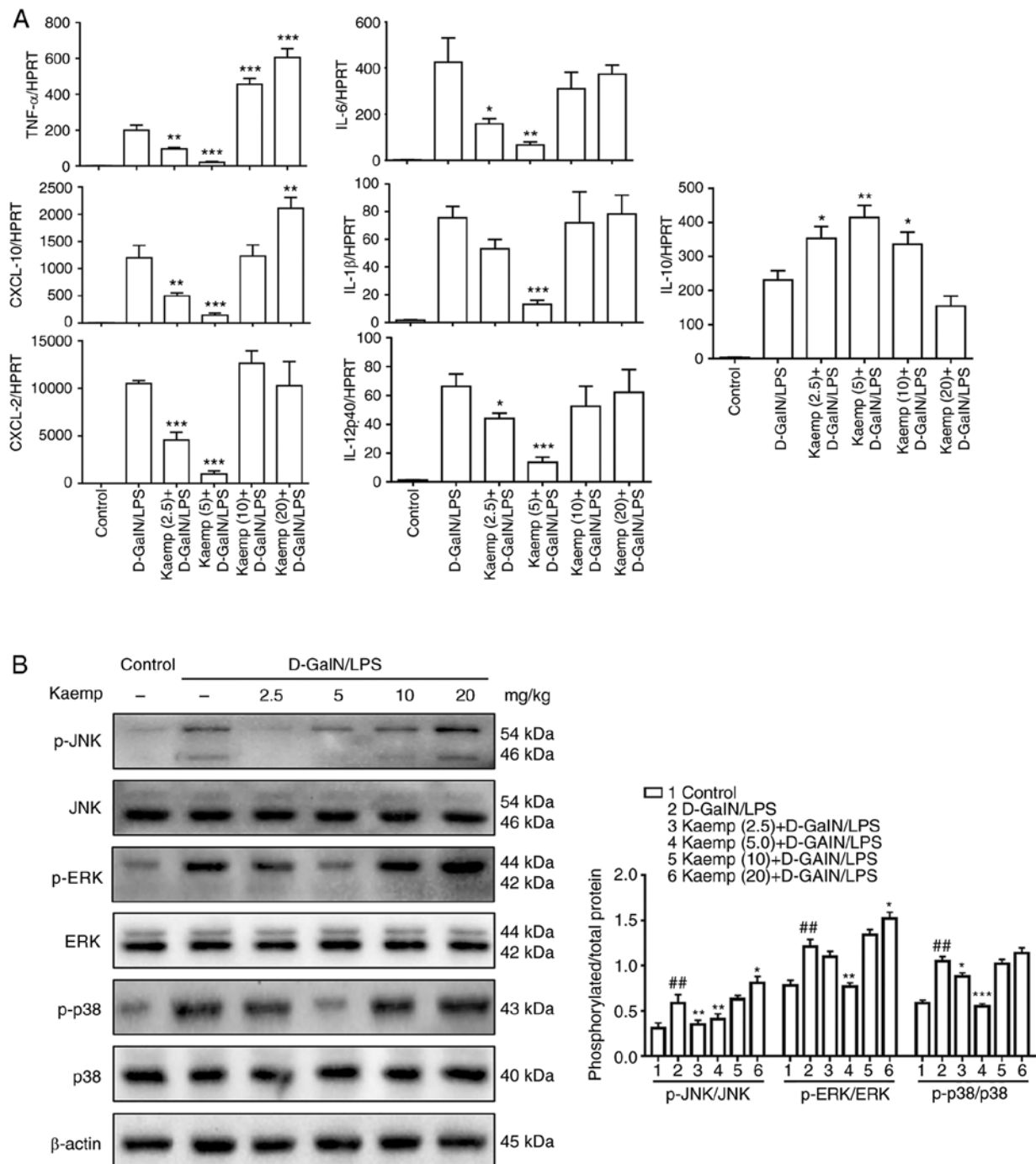


Figure 2. Effects of different doses of kaemp on liver inflammation in D-GalN/LPS-induced ALF. (A) Reverse transcription-quantitative PCR analysis was performed to determine the gene expression levels of cytokines and chemokines, including TNF- α , IL-6, IL-12p40, IL-10, IL-1 β , CXCL-10 and CXCL-2, in the livers of mice in the different groups. (B) Western blot analysis to determine hepatic protein expression levels of p-JNK, JNK, p-ERK, ERK, p-p38, p38 and β -actin. Data are presented as the mean \pm SD. ## P <0.01 vs. control group; * P <0.05, ** P <0.01, *** P <0.001 vs. D-GalN/LPS group. D-GalN/LPS, D-galactosamine/lipopolysaccharide; kaemp, kaempferol; CXCL, C-X-C motif chemokine ligand; p-, phosphorylated.

that the inflammatory response served an important role in ALF (27). Therefore, whether liver inflammation was induced by the different doses of kaempferol in the D-GalN/LPS-induced ALF mice was next determined. As shown in Fig. 2A, compared with the D-GalN/LPS group, the mice pretreated with 2.5 and 5 mg/kg kaempferol exhibited lower gene expression levels of TNF- α , IL-6, IL-1 β , IL-12p40, C-X-C motif chemokine ligand (CXCL)-10, CXCL-2 in the livers, and the 5 mg/kg kaempferol group exhibited the lowest

levels. By contrast, pretreatment with 10 and 20 mg/kg kaempferol resulted in notably increased gene expression levels of these cytokines. Additionally, pretreatment with 2.5, 5 or 10 mg/kg kaempferol resulted in increased gene expression levels of IL-10 when compared with the D-GalN/LPS-induced mice, and the 5 mg/kg kaempferol group exhibited the highest levels.

Next, whether the MAPK signaling pathway was affected by different doses of kaempferol was assessed. The protein

expression levels of p-JNK, JNK, p-ERK, ERK, p-p38, p38 were determined using western blotting. Mice treated with 2.5 or 5 mg/kg kaempferol exhibited reduced levels of these proteins, with the 5 mg/kg kaempferol group exhibiting the lowest levels. By contrast, pretreatment with 10 and 20 mg/kg kaempferol resulted in markedly increased expression levels of these proteins (Fig. 2B). Therefore, these results indicated that pretreatment with high doses of kaempferol can increase the hepatic inflammatory response, whereas pretreatment with a low dose of kaempferol can significantly decrease it in D-GalN/LPS-induced ALF.

Effects of kaempferol on liver autophagy in mice with D-GalN/LPS-induced ALF. Given that our previous study observed that autophagy serves a significant role in D-GalN/LPS-induced ALF (18), whether different doses of kaempferol affected liver autophagy in the D-GalN/LPS-induced ALF was next assessed. The results of the RT-qPCR analysis revealed that pretreatment with 2.5, 5, 10 or 20 mg/kg kaempferol significantly increased the gene expression levels of LC3 compared with the untreated D-GalN/LPS-induced ALF in mice (Fig. 3A). Moreover, pretreatment with 2.5, 5 and 20 mg/kg kaempferol increased the gene expression levels of Atg7, and the 5 mg/kg kaempferol group exhibited the highest expression levels of these genes. The western blot analysis produced consistent results. Pretreatment with 2.5, 5, 10 or 20 mg/kg kaempferol resulted in increased LC3 protein expression, and the 5 mg/kg kaempferol group exhibited the highest expression levels. In addition, pretreatment with 2.5 and 5 mg/kg kaempferol significantly decreased the protein expression levels of p62, whereas 10 and 20 mg/kg kaempferol significantly increased its protein expression. Furthermore, pretreatment with 2.5, 5, 10 and 20 mg/kg kaempferol significantly increased the protein expression levels of Atg7, and the 5 mg/kg kaempferol group exhibited the highest expression levels (Fig. 3B).

To further demonstrate the effect of kaempferol (5 mg/kg) on autophagy flux, the fusion of lysosomes with autophagosomes was inhibited using CQ pretreatment. It was found that CQ pretreatment did not further increase LC3II conversion and did not further decrease p62 levels compared with the 5 mg/kg kaempferol pretreated D-GalN/LPS induced ALF mice (Fig. 3C). These data suggest that kaempferol pretreatment may facilitate autophagosome function and inhibit autophagic flux in D-GalN/LPS-induced ALF.

Effects of kaempferol on starvation-induced autophagy in vitro. To further support the results of the *in vivo* experiments, the effects of kaempferol on starved primary hepatocytes were assessed *in vitro*. To observe the formation of autophagosomes, the GFP-LC3 plasmid was transfected into hepatocytes. As shown in Fig. 4A, compared with that of the control group, the GFP-LC3 signal was weak in the starved group and high-dose kaempferol-treated groups, but was bright, with a lower number of puncta, in the low-dose kaempferol-treated groups.

Next, the protein expression levels of LC3, p62 and Atg7 were measured via western blotting (Fig. 4B). The starved and low-dose kaempferol-treated groups exhibited higher LC3 protein expression levels, and the high-dose kaempferol group

exhibited lower expression levels compared with the control group. Furthermore, the low-dose kaempferol groups showed reduced protein expression levels of p62, and the high-dose groups also appeared to show decreased p62 expression compared with the control. Additionally, low-dose kaempferol groups showed reduced protein expression levels of Atg7, and the high-dose kaempferol groups exhibited lower expression levels compared with the control group. Collectively, the presence of autophagic flux was demonstrated by increased expression of LC3 and decreased expression of p62. Thus, the results suggest that pretreatment with low doses of kaempferol promotes the induction of autophagic flux, but that the pretreatment with high doses of kaempferol restrains the induction of autophagic flux in starvation-induced hepatocytes *in vitro*.

Kaempferol ameliorates injury in the livers of ALF model mice through autophagic mechanisms. The aforementioned experiments demonstrated that 5 mg/kg kaempferol significantly reduced ALF induced by D-GalN/LPS. To further determine whether pretreatment with 5 mg/kg kaempferol contributed to the induction of autophagy to protect against liver injury, Atg7 knockdown using siRNA was performed *in vivo*. The results indicated that 3-MA or Atg7 siRNA partially reversed kaempferol-mediated hepatoprotection in the ALF model mice, as shown by the increased levels of sALT and sAST (Fig. 5A), and the histological analysis showing a relatively less well-preserved liver architecture (Fig. 5B). Additionally, the protein expression levels of LC3, p62 and Atg7 were measured using western blotting (Fig. 5C). The Atg7 siRNA group had lower protein expression levels of LC3II and Atg7, and higher protein expression levels of p62. Furthermore, the expression levels of LC3II, Atg7 and p62 were assessed following pretreatment with 3-MA, and the expression levels of Atg7 were reduced with pretreatment of 3-MA. The results demonstrated that, compared with mice treated with kaempferol (5 mg/kg) and D-GalN/LPS, 3-MA pretreatment decreased conversion of LC3II and increased the degradation of p62 (Fig. 3C). These data indicated that pretreatment with 5 mg/kg kaempferol ameliorated liver injury by regulating autophagy in ALF model mice.

Discussion

Kaempferol is an ingredient in traditional Chinese herbs, and is found in various vegetables and fruits, including tomatoes, citrus fruits, grapefruit, onion, broccoli, cabbage and apples. Kaempferol is a flavonoid that has been shown to possess a broad range of pharmacological activities, such as antidiabetic, antioxidative, cardioprotective, angiogenic and anticancer properties (28,29). A previous study revealed that kaempferol can suppress liver gluconeogenesis by reducing the activity of pyruvate carboxylase and glucose-6 phosphatase, thereby increasing hepatic glucose metabolism and insulin resistance in diet-induced obese mice (30). In the present study, it was demonstrated that pretreatment with kaempferol at various doses had different functional effects on D-GalN/LPS-induced ALF. The results indicated that 5 mg/kg kaempferol pretreatment significantly protected against liver injury induced by D-GalN/LPS in mice, whereas a high dose of kaempferol decreased the survival rate of

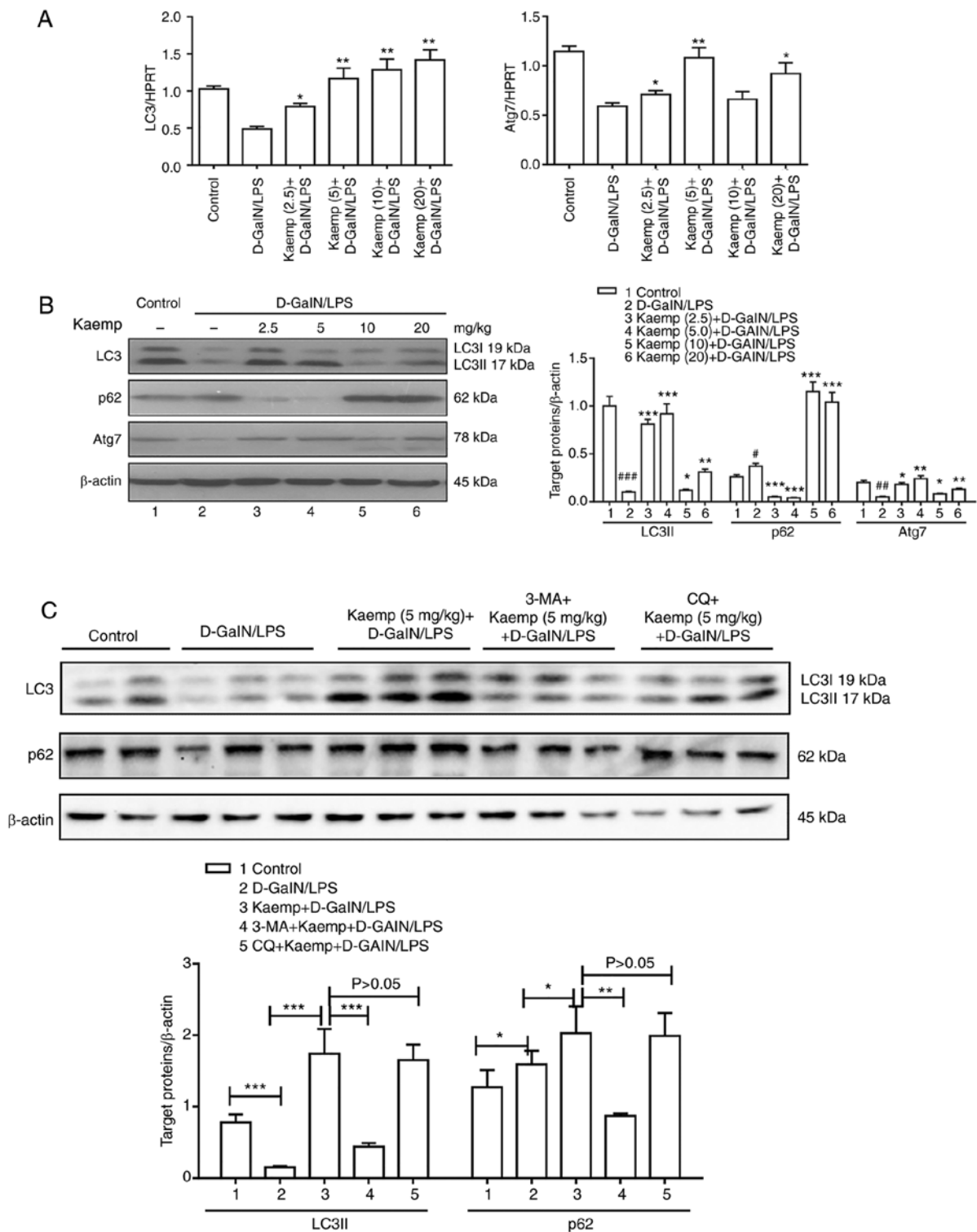


Figure 3. Effects of different doses of kaemp on liver autophagy in D-GalN/LPS-induced ALF. (A) Gene expression levels of LC3 and Atg7 in livers from mice in the different groups. HPRT was used as the reference gene. * $P < 0.05$, ** $P < 0.01$ vs. D-GalN/LPS group. (B) Western blotting was performed to determine hepatic protein expression levels of LC3, p62 and Atg7, with β -actin as the loading control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. D-GalN/LPS group. (C) Mice were pretreated for 2 h with or without kaemp (5 mg/kg, intraperitoneally), and then stimulated with D-GalN/LPS for 6 h (n=12). Mice treated with 3-MA + kaemp + D-GalN/LPS were co-administered 3-MA (10 mg/kg) and kaemp 2 h before D-GalN/LPS injection (n=12). Mice treated with CQ + kaemp + D-GalN/LPS were co-administered CQ (60 mg/kg) and kaemp 2 h before D-GalN/LPS injection (n=12). A total of 2 h after vehicle injection, control mice were pretreated with PBS (n=12). Western blot analysis was performed to determine hepatic protein expression levels of LC3, p62 and β -actin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as the mean \pm SD. D-GalN/LPS, D-galactosamine/lipopolysaccharide; 3-MA, 3-Methyladenine; CQ, chloroquine; kaemp, kaempferol; Atg7, autophagy related 7; HPRT, hypoxanthine guanine phosphoribosyl transferase.

mice and resulted in more severe injury. Thus, when using kaempferol to treat ALF, it is important to pay attention to

the differential effects caused by different doses, and choose the appropriate dose for treatment.

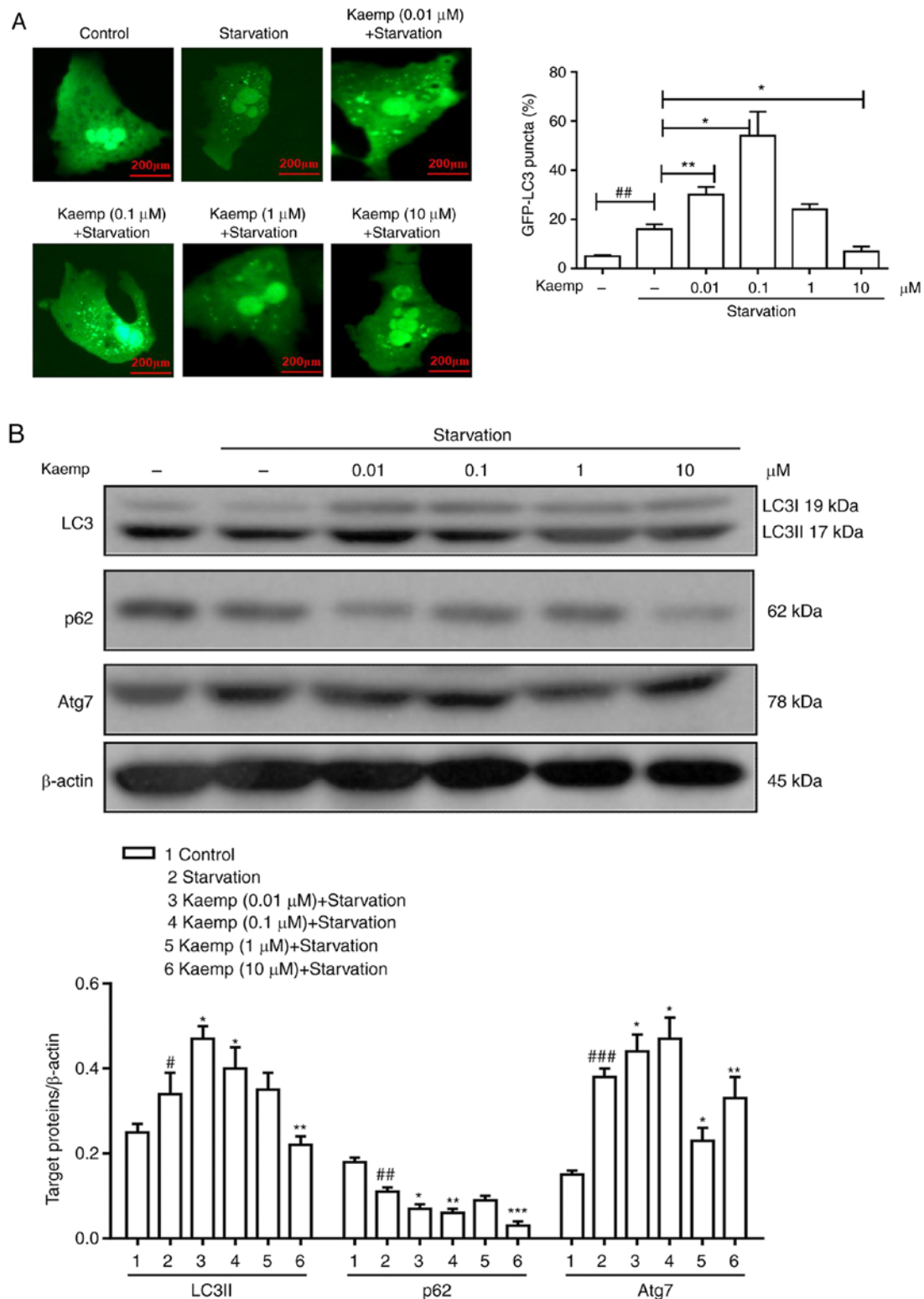


Figure 4. Effects of different doses of kaemp on starvation-induced autophagy *in vitro*. (A) Primary hepatocytes were transfected with GFP-LC3 plasmids for 12 h and preincubated with kaemp (0.01, 0.1, 1 or 10 μ M) for 12 h, after which, the formation of autophagosomes was observed. (B) Western blotting was performed to determine the protein expression levels of autophagy-related proteins, including LC3, p62 and Atg7, in primary hepatocytes under conditions of starvation. Data are presented as the mean \pm SD. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. control group; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. starvation group. kaemp, kaempferol; Atg7, autophagy related 7.

ALF is associated with high mortality rates; the clinical symptoms include coagulopathy, hepatic dysfunction and abnormal liver biochemical parameters. In addition, ALF is closely associated with the inflammatory response, and is

an injury process associated with inflammation-mediated hepatocellular carcinoma (31). There are currently no effective treatments for ALF. Our previous study showed that endoplasmic reticulum stress can reduce inflammation by regulating the

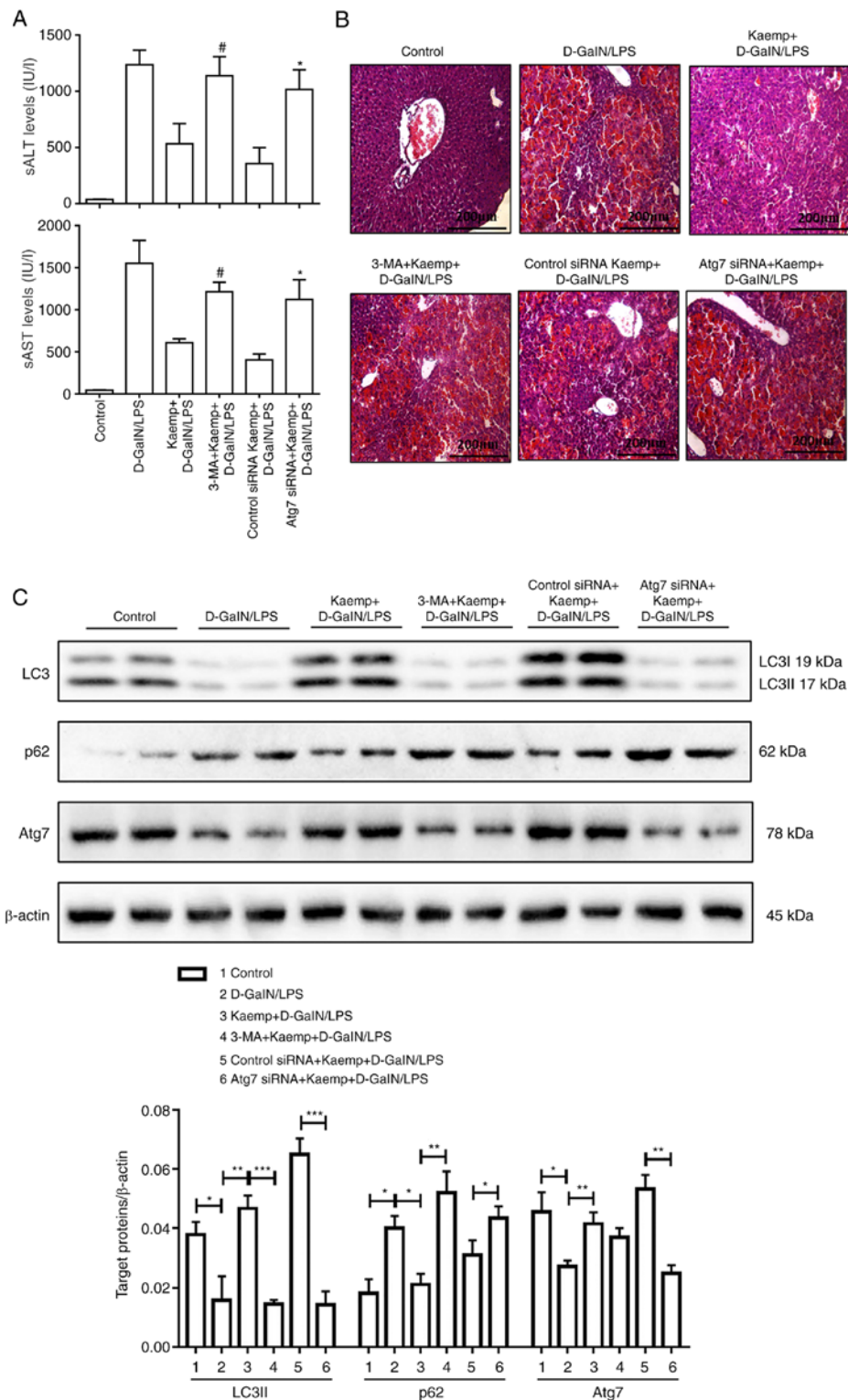


Figure 5. Kaempferol protects mice against ALF by regulating the autophagy pathway in the liver. Mice were pretreated for 2 h with or without kaempferol (5 mg/kg, intraperitoneally), and then stimulated with D-GalN/LPS for 6 h (n=20). Mice treated with 3-MA + kaempferol + D-GalN/LPS were administered 3-MA (10 mg/kg) and kaempferol 2 h before D-GalN/LPS injection (n=20). Mice treated with control siRNA or Atg7 siRNA + kaempferol + D-GalN/LPS were injected via tail vein for 48 h with control or Atg7 siRNA (3 mg/kg), and were administered kaempferol 2 h before D-GalN/LPS injection (n=20). A total of 2 h after vehicle injection, control mice were pretreated with PBS (n=20). (A) sALT and sAST enzyme levels in the different groups. *P<0.05 vs. kaempferol + D-GalN/LPS group; **P<0.05 vs. kaempferol + D-GalN/LPS + control siRNA group. (B) Representative H&E-stained liver sections from different groups. (C) Western blot analysis was performed to determine hepatic protein expression levels of LC3, p62, Atg7 and β -actin in the different groups. *P<0.05, **P<0.01, ***P<0.001. Data are presented as the mean \pm SD. ALF, acute liver failure; sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; D-GalN/LPS, D-galactosamine/lipopolysaccharide; Atg7, autophagy related 7; 3-MA, 3-Methyladenine; kaempferol, kaempferol; siRNA, small interfering RNA.

immune mechanism in ALF (32). The present study suggested that 10 and 20 mg/kg kaempferol pretreatment increased the

expression of proinflammatory cytokines, and the expression levels of proinflammatory cytokines were significantly decreased

in the ALF mice pretreated with 2.5 or 5 mg/kg kaempferol. Therefore, these data suggest that a high-dose of kaempferol pretreatment can promote the hepatic inflammatory response, whereas low-dose kaempferol pretreatment can significantly suppress it in D-GalN/LPS-induced ALF. The levels of proinflammatory cytokines were downregulated by the low dose of kaempferol, which may be the result of the increased production of anti-inflammatory cytokines, such as IL-13, which reduce TNF- α production *in vivo* (33). A previous study revealed that IL-13 significantly reduced the lethal effects of LPS in a neonatal mouse model of endotoxin shock (34). In a similar manner to IL-10, a high dose of kaempferol may also decrease the production of IL-13 (35).

Autophagy is a process of self-digestion that attempts to maintain cell homeostasis, supply a variety of substrates for cellular energy generation and ensures cell survival under stressful conditions to a certain degree. Autophagy is an important physiological process, and is tightly associated with regulation of cell death in specific tissues, such as the liver and brain (36). Autophagy and inflammation are also closely related (37). Our previous study reported that peroxisome proliferator activated receptor (PPAR) α activation alleviated the inflammatory response by promoting autophagy in ALF model mice induced by D-GalN/LPS (38). Moreover, it was confirmed that inhibition of GSK-3 β activity increased PPAR α expression and decreased the inflammatory response by further increasing autophagy (32). The present study demonstrated that different doses of kaempferol had differential effects on the induction of autophagy and autophagosome formation *in vivo* and *in vitro*. The presence of autophagic flux was identified based on the increased LC3 expression combined with decreased p62 expression (39). These current results indicated that a low-dose of kaempferol upregulated the expression levels of genes associated with autophagy, and increased LC3II conversion and p62 degradation, whereas a high-dose of kaempferol decreased LC3II conversion and p62 degradation, and increased autophagosome formation. Moreover, pretreatment with CQ did not significantly alter the effects of 5 mg/kg kaempferol on the expression levels of LC3 and p62 in the ALF mouse model. It was thus concluded that the inflammatory response was attenuated by pretreatment with a low dose of kaempferol, and that this low dose upregulated autophagic activity. However, it is possible that the effects of kaempferol on the autophagic flux at different concentrations is a highly complicated physiological phenomenon, and therefore requires further study in the context of ALF.

Previous studies have shown that the excessive activation of mTOR can result in disordered apoptosis and autophagy, which leads to the occurrence of human immunodeficiency virus (HIV)-related diseases (40). Hence, it could be hypothesized that kaempferol may also be useful for conditions characterized by dysregulated autophagy, such as HIV-associated neurodegenerative diseases. Additionally, it also can be assumed that kaempferol may be effective against diseases characterized by abnormal activation of the PIK3/Akt/mTOR pathway that benefit from mTOR inhibitors, including infectious diseases, such as HIV and severe acute respiratory syndrome coronavirus 2 (41-43), and autoimmune diseases, such as multiple sclerosis and systemic lupus erythematosus (44-46), as well as cancer (47-49).

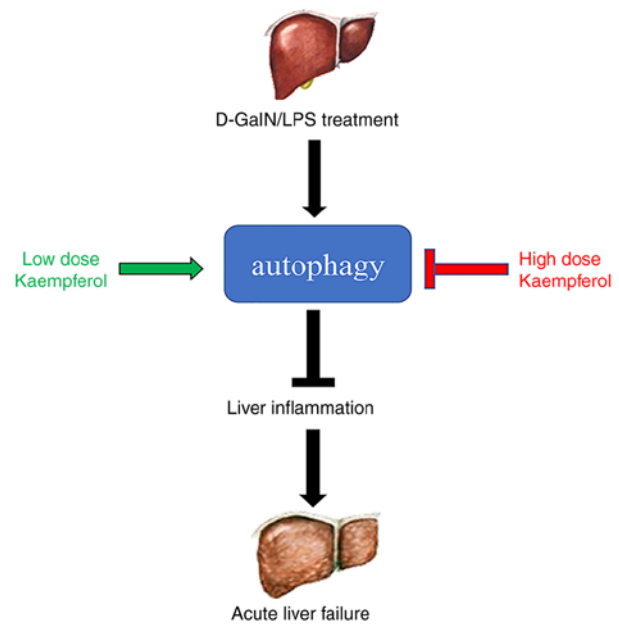


Figure 6. Schematic representation of the study findings. The effects of kaempferol on ALF at various doses had different functional outcomes, which was mediated by differential regulation of the autophagy pathway. ALF, acute liver failure; D-GalN/LPS, D-galactosamine/lipopolysaccharide.

In reviewing the literature, kaempferol has been shown to antagonize Toll-like receptor (TLR) signaling, and in particular TLR4 signaling (50). It has previously been observed that autophagy can be regulated by the TLR4 signaling pathway (51,52). TLR4 inhibitors, including VGX-1027 and monoclonal antibodies, have been reported to possess multiple functions in models of immune-inflammation and autoimmunity, such as anti-inflammatory and anti-injury effects, and they are being developed for use in clinical settings (53-56). Therefore, kaempferol may be used as an emerging class of TLR4 inhibitor to treat immune inflammation and autoimmune diseases, which highlights the significant pharmacological potential of kaempferol.

Although this study revealed the different effects of different doses of kaempferol on D-GalN/LPS-induced ALF via regulation of the autophagy pathway, the specific molecular pathway through which autophagy is regulated remains unclear. Further research is required to explore how autophagy is regulated.

In conclusion, the results of the present study suggested that the effects of kaempferol on ALF at various doses had different functional outcomes, and this was mediated by differential regulation of the autophagy pathway. It was found that a low dose of kaempferol can significantly protect mice from liver injury in ALF. A schematic diagram of the potential mechanisms identified by the present study is presented in Fig. 6. Overall, the optimal dose of kaempferol should be further assessed, and this may serve as an effective strategy for treatment of ALF. For further preclinical studies of autophagy agonists, it is necessary to develop clinically applicable therapeutic strategies for ALF.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YT and FR wrote the manuscript and performed the experiments. YT and LX collected and analyzed the data. FR and XZ designed the experiments. FR and XZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were reviewed and approved by Institutional Animal Care and Use Committee of Capital Medical University (approval no. AEEI-2020-009 on 2019/12/30).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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