

## Original Research Article

# Tetrazole exerts anti-hepatitis effect in mice via activation of PI3K/Akt pathway, inhibition of cell autophagy and suppression of inflammatory cytokine expressions

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### Abstract

**Purpose:** To investigate the effect of tetrazole on concanavalin A (Con A)-induced hepatitis in mice, and the underlying mechanism(s).

**Methods:** Thirty 5-week-old, male BALB/c mice (mean weight,  $30.5 \pm 1.04$  g) were used for this study. They were randomly assigned to six groups of five mice each: control group, hepatitis group and four treatment groups. With the exception of control group, hepatitis was induced in all mice with Con A (20 mg/kg) via their tail veins. The treatment groups received varied doses of tetrazole (1.0 - 6.0 mg/kg) within 1 h after hepatitis induction, while mice in the control group received an equivalent volume of normal saline in place of tetrazole. Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined while expressions of interleukin-2 (IL-2), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and interferon gamma (IFN- $\gamma$ ) were evaluated by enzyme-linked immunosorbent assay (ELISA) kits. Expressions of protein kinase B (Akt), phosphoinositide 3-kinase (PI3K), nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B), and autophagy-related genes were determined by real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting.

**Results:** Con A-induced hepatitis significantly increased the activities of serum ALT and AST in the mice. However, after treatment with tetrazole, the activities of these enzymes were significantly and dose-dependently reduced in the treatment groups, relative to hepatitis group ( $p < 0.05$ ). The levels of IL-2, IFN- $\gamma$  and TNF- $\alpha$  were significantly increased in hepatitis group when compared with the control group ( $p < 0.05$ ). However, treatment with tetrazole significantly inhibited the expressions of these parameters. There were no significant differences in the levels of expressions of Akt mRNAs among the treatment groups ( $p > 0.05$ ). The levels of expressions of LC3II and Beclin 1 were also significantly upregulated in hepatitis group, when compared with control group ( $p < 0.05$ ). However, expression levels of LC3II and Beclin 1 were significantly and dose-dependently reduced by tetrazole treatment.

**Conclusion:** Tetrazole is effective in the treatment of hepatitis via mechanisms involving the activation of PI3K/Akt pathway, inhibition of cell autophagy and suppression of inflammatory cytokines expressions.

**Keywords:** Hepatitis, Concanavalin A, Tetrazole, Inflammatory cytokines, Expression, Autophagy

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## INTRODUCTION

Hepatitis is a liver disease caused by viral infection, exposure to toxins, excessive alcohol consumption and immunological disturbance [1,2]. Its pathogenesis is complex and involves several pathways and molecules [3-5]. The disease progression involves activation of T cells which in turn stimulate the secretion of inflammatory cytokines and enzymes in the blood [6,7]. The activities of ALT and AST, and levels of inflammatory cytokines are usually elevated in the blood following the activation of T cells [8,9].

Concanavalin A (Con A), a plant lectin, activates T lymphocytes [10]. Liver damage in hepatitis is caused by aggregation and infiltration of T-lymphocytes [11,12]. In addition, the expressions of IL-2, TNF- $\alpha$  and IFN- $\gamma$  are involved in the pathogenesis of hepatitis [13]. Nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) regulates the expressions of inflammatory cytokines in hepatitis [13]. However, the activity of NF- $\kappa$ B is regulated by I $\kappa$ B $\alpha$  with the involvement of PI3K/Akt pathway [13]. Autophagy, a type of programmed cell death acts by engulfing cellular organelles in the form of autophagosomes and transferring them to lysosomes for degradation [14,15]. The autophagic process is regulated by activation of several pathways such as c-Jun-N and AMPK. The formation of an autophagosome involves Beclin 1 and mTOR pathways which are regulated by the PI3K/Akt pathway [16,17]. The four nitrogen atoms in tetrazole ring are responsible for the biological activity of the compound. Tetrazole-bearing compounds possess chemotherapeutic effects such as anti-inflammatory, antimicrobial, anti-nociceptive and anticonvulsant activities [18]. There is a need for the development of new and effective chemotherapeutic agents that can effectively ameliorate the symptoms and complications of hepatitis. The aim of this study was to investigate the effect of tetrazole on Con A-induced hepatitis in mice, and the underlying mechanism(s).

## EXPERIMENTAL

### Materials

The BALB/c mice were purchased from Beijing HFK Bioscience Co., Ltd., while ALT and AST automated biochemical analyser was a product of Olympus AU1000 (Japan). ELISA kits were purchased from Santa Cruz Biotechnology Inc. (USA), while Kinematica tissue pulverizer was obtained from Shanghai Xin Yu Biotech Co., Ltd. RNeasy Mini kit was purchased from Qiagen, Inc. (USA) and NanoDrop 1000 spectrophotometer was obtained from Thermo

Fisher Scientific Inc. (USA). Real time polymerase chain reaction (RT-PCR, 7900HT model) was a product of ABI (USA), while SYBR Premix EX Taq was obtained from Takara Biotechnology Inc. (Japan).

### Mice

A total of thirty 5-week-old BALB/c male mice weighing 28.2 to 32.8 g (mean weight = 30.5  $\pm$  1.04 g) were used for this study. They were housed in plastic cages under standard conditions of animal care and had free access to standard feed and water. The mice were exposed to 12 h light/dark cycles and maintained at 25 °C and 48 % humidity. The study protocol was approved by the Laboratory Animal Committee of China Medical University (approval no. CMU/17/187), and the study procedures were carried out according to the guidelines of National Institutes of Health [19].

### Treatment

The mice were randomly assigned to six groups of five mice each: control group, hepatitis group and four treatment groups. With the exception of control group, hepatitis was induced in the mice with Con A (20 mg/kg) through their tails veins. The treatment groups received varied doses of tetrazole (1.0 - 6.0 mg/kg bwt) within 1 h after induction of hepatitis, while mice in the control group received equivalent volumes of normal saline.

### Determination of activities of ALT and AST, and serum levels of inflammatory cytokines

After 12 h of treatment, the mice were sacrificed under isoflurane anaesthesia and blood samples were collected through cardiac puncture. The blood was centrifuged at 3000 rpm for 30 min at room temperature to obtain serum which was used for biochemical analysis. Serum activities of ALT and AST were determined using automated biochemical analyser, while the expressions of IL-2, TNF- $\alpha$ , and IFN- $\gamma$  were determined using appropriate ELISA kits.

### Western blotting

Liver tissues collected from the mice were stored in liquid nitrogen at -80 °C and sliced into thin sections (5  $\mu$ m) using refrigerated microtome, and homogenized using Kinematica tissue pulveriser. The resultant tissue homogenate was washed twice with phosphate-buffered saline (PBS) and centrifuged at 13,000 g for 25 min at 4 °C. The protein concentration of the supernatant was determined using BCA assay kit. A portion

of total tissue protein (20 - 30 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min.

	GGTGC	TTCTTGGG
PI3K	ACACCACGGTTTGG	GGCTACAGTAGTG
	ACTATGG	GGCTTGG
Akt	ATGAACGACGTAGC	TTGTAGCCAAATAAA
	CATTGTG	GGTGCCAT
β-actin	GGCTGTATTCCCCT	CCAGTTGGTAACAA
	CCATCG	TGCCATGT

Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of IL-2, TNF-α, IFN-γ, AKT, p-AKT, PI3K, p-P13K, LC3II, Beclin 1 and β-actin at a dilution of 1 to 500. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software (4.6.2). Respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

#### Quantitative polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from portions of liver homogenate using RNeasy Mini kit and determined spectrophotometrically. The RNAs were reverse-transcribed to cDNAs, using random primers at 45 °C for 2 h. The samples were heated at 95 °C for 10 min. The PCR amplification of the reverse-transcribed reaction mixture was carried out using 20 µl reaction mixture and equal volume of SYBR Premix Ex Taq™ II. The PCR conditions were: pre-denaturation at 95 °C for 30 sec, denaturation at 95 °C for 3 sec, annealing at 60 °C for 34 sec, and 50 cycles. The procedure was performed in triplicate. Relative expression was quantified using Stratagene Mx3000P software, and β-actin gene was used as internal reference. The primers sequences used for qRT-PCR are shown in Table 1.

**Table 1:** Primers sequences used for qRT-PCR

Gene	Primer sequence	
	Forward	Backward
Interleukin-2	TGAGCAGGATGGA	GTCCAAGTTCATCT
TNF-α	GAATTACAGG	TCTAGGCAC
	CAGGCGGTGCCTAT	CGATCACCCCGAA
	GTCTC	GTTCAGTAG
Interferon-γ	ATGAACGCTACACA	CCATCCTTTTGCCA
Beclin 1	CTGCATC	GTTCCCTC
	ATGGAGGGGTCTAA	TGGGCTGTGGTAA
	GCCGTC	GTAATGGA
LC3II	GACCGCTGTAAGGA	AGAAGCCGAAGGT

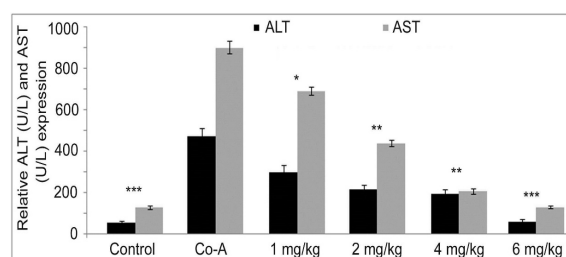
#### Statistical analysis

Data are expressed as mean ± SD, and the statistical analysis was performed using SPSS (11.5). Groups were compared using Student's *t*-test. *P* < 0.05 was considered statistically significant.

## RESULTS

#### Serum ALT and AST

Con A-induced hepatitis significantly increased the activities of serum ALT and AST in the mice. However, after treatment with tetrazole, the activities of these enzymes were significantly and dose-dependently decreased in the treatment groups, relative to hepatitis group (*p* < 0.05). Their activities in hepatitis mice treated with 6 mg/kg bwt tetrazole were 62 ± 19 U/L and 131 ± 32 U/L, respectively (Figure 1).



**Figure 1:** Effect of tetrazole treatment on the activities of ALT and AST; \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001, when compared to hepatitis group

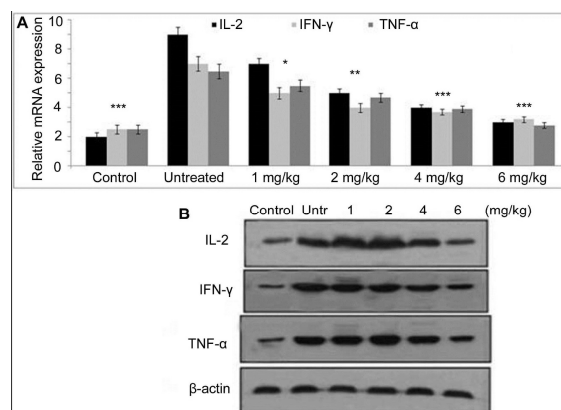
#### Inflammatory cytokines

The expression levels of IL-2, IFN-γ and TNF-α were significantly increased in hepatitis group, when compared with the control group (*p* < 0.05). However, treatment with 6 mg/kg tetrazole producing the most significant inhibition (*p* < 0.05). These results are shown in Figure 2 A and B.

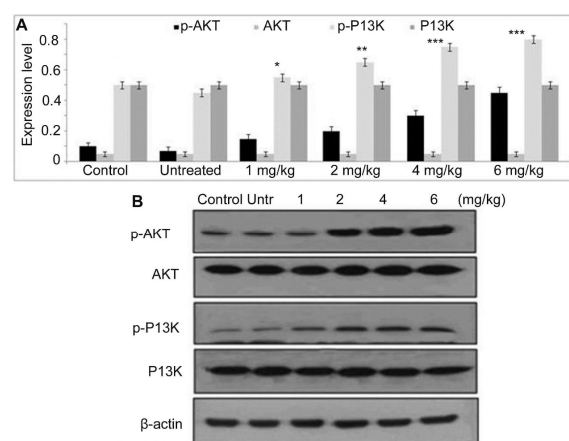
#### Expressions of Akt and PI3K

As shown in Figures 3 A and B, the expressions of Akt and PI3K were significantly and dose-dependently enhanced in treatment groups, when compared with the control and hepatitis groups (*p* < 0.05). However, there were no significant differences in the levels of

expressions of Akt mRNAs among the treatment groups ( $p > 0.05$ ). The expressions of p-Akt and p-PI3K were also significantly higher in the treatment groups than in the hepatitis group ( $p < 0.05$ ).



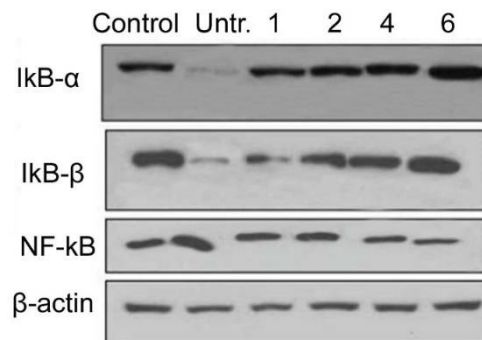
**Figure 2:** Effect of tetrazole on the levels of inflammatory cytokines in hepatitis mice; **A:** Expressions of inflammatory cytokines as determined using qRT-PCR; **B:** Expressions of inflammatory cytokines as determined using Western blotting. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , when compared to hepatitis group



**Figure 3:** Effect of tetrazole on the level of expressions of Akt and PI3K in hepatitis mice; **A:** expressions of Akt and PI3K as determined using qRT-PCR; **B:** expressions of Akt and P13K as determined using Western blotting; \* $p < 0.05$ , \*\* $p < 0.01$  \*\*\* $p < 0.001$ , when compared to control group

### Effect of tetrazole treatment on NF-κB pathway

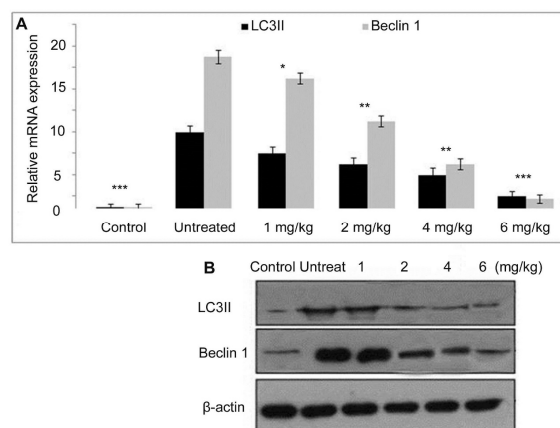
The expression of NF-κB was significantly higher in hepatitis group than in control group, but was significantly and dose-dependently reduced after treatment with tetrazole ( $p < 0.05$ ). However, the expressions of 1κBα and 1κBβ were significantly upregulated in control and treatment groups, relative to hepatitis group ( $p < 0.05$ ; Figure 4).



**Figure 4:** Effect of tetrazole treatment on NF-κB expression

### Expressions of autophagy-related genes

The expressions levels of LC3II and Beclin 1 were significantly upregulated in hepatitis group, when compared with control group ( $p < 0.05$ ). However, LC3II and Beclin 1 were significantly and dose-dependently downregulated by tetrazole treatment, with 6 mg/kg bwt tetrazole producing maximum inhibition (Figures 5 A and B).



**Figure 5:** Beclin 1 and LC3II expressions in the mice. **A:** Expressions of LC3II and Beclin 1 as determined using qRT-PCR; **B:** Expressions of LC3II and Beclin 1 as determined using Western blotting; \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , compared to control group

## DISCUSSION

Hepatitis is a serious health condition caused by viral infection, exposure to toxins, excessive alcohol consumption and immunological disturbance [1]. At present, there are no effective therapeutic agents for hepatitis [19]. The present study investigated the effect of tetrazole on Con A-induced hepatitis in mice, and the underlying mechanism (s). Induction of hepatitis leads to release of inflammatory cytokines such as TNF-α, IFN-γ, IL-2 and IL-6 [20]. In this study, the levels of IL-2, IFN-γ and TNF-α were significantly increased in hepatitis group when compared with

the control group. However, treatment with tetrazole significantly inhibited the expressions of these parameters, with 6 mg/kg bwt tetrazole producing the most significant inhibition. These results are in agreement with those reported in previous studies [20]. It is possible that tetrazole regulated the secretion of these inflammatory cytokines in Con A- induced hepatitis mice.

Increased activities of serum ALT and AST are associated with liver damage [12]. In this study, Con A-induced hepatitis significantly increased the activities of serum ALT and AST in the mice. However, after treatment with tetrazole, the activities of these enzymes were significantly and dose-dependently reduced in the treatment groups, relative to hepatitis group. These results suggest that tetrazole may prevent liver damage in hepatitis by inhibiting the release of inflammatory cytokines. It is likely that the upregulation of inflammatory cytokine expressions enhances the activities of ALT and AST in serum of hepatitis mice.

Expressions of genes associated with the secretion of inflammatory cytokines are regulated by NF- $\kappa$ B [21]. Nuclear transcription factor-  $\kappa$ B (NF- $\kappa$ B) plays a key role in the expression of pro-inflammatory genes and the development of hepatitis [22,23]. The results of Western blotting showed that the expression of NF- $\kappa$ B was significantly higher in hepatitis group than in control group, but was significantly and dose-dependently reduced after treatment with tetrazole. However, the levels of expressions of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  were significantly upregulated in control and treatment groups, relative to hepatitis group. These results suggest that the pathogenesis of hepatitis may involve the degradation of NF- $\kappa$ B, and that tetrazole might prevent I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$  degradation. It is likely that treatment with tetrazole suppressed the translocation of NF- $\kappa$ B to the nucleus of hepatocytes in hepatitis mice. It has been reported that inhibition of I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$  degradation plays a central role in down-regulation of the expressions of inflammatory factors [24]. Induction of cell autophagy is regulated by several factors, the most common of which are PI3K and Akt [14].

In the present study, the expressions of Akt and PI3K were significantly and dose-dependently increased in treatment groups, when compared with the control and hepatitis groups. However, there were no significant differences in the levels of expressions of Akt mRNAs among the treatment groups. The levels of expressions of p-Akt and p-PI3K were also significantly higher in the treatment groups than in hepatitis group.

These results suggest that tetrazole exerts anti-hepatitic effects via the activation of PI3K/Akt pathway. In this study, treatment with tetrazole significantly down-regulated the expressions of Beclin 1 and LC3II, an indication that tetrazole may exert anti-hepatitis effect via the inhibition of cell autophagy.

## CONCLUSION

Tetrazole is effective in the treatment of hepatitis and its anti-hepatitis effect is exerted via mechanisms involving the activation of PI3K/Akt pathway, inhibition of cell autophagy and suppression of inflammatory cytokine expressions.

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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