Concanavalin-A as a Model for Induction of Murine Autoimmune Hepatitis: Role of TNF-α and NF-κβ During The Acute Phase

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Autoimmune hepatitis (AIH) is a heterogeneous immune-mediated chronic liver disease affecting children and adults. It is important to rely on a specific animal model to study the hepatic changes and to evaluate the roles played by pro-inflammatory cytokines such as tumor necrosis factor alpha "TNF-α" and transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells "NF-κβ" in the pathogenesis and outcome of the disease. This will help to identify specific targets for treatment of AIH. This study aimed at evaluating Concanavalin-A (Con A) as a model for induction of AIH and assessing splenocytes' TNF-α and hepatocytes' NF-κβ levels at comparable durations after induction of hepatitis with Con A to evaluate the relationship between both factors. Materials and methods: A total of 130 outbreed CD1 mice were divided into group (1) which included 100 mice with induced AIH and group (2) included 30 normal mice as negative controls. Intra-peritoneal injection of Concanavalin-A was used to induce hepatitis. Hepatic injury was evaluated by the levels of liver enzymes, histopathological evidence for hepatic inflammatory infiltrate and/or apoptosis. Splenocytes and hepatocytes were cultured for assessment of TNF-α and NF-κβ levels, respectively. Results: Con A injection caused a significant elevation in ALT and AST levels, portal inflammatory infiltrate, remarkable hepatocytes degeneration and marked increase of TNF-α levels, particularly within 24 hours, but all returned to normal within 1 week. Administration of another dose of Con A resulted in sharp significant elevation of liver enzymes, inflammatory infiltrate and hepatocyte apoptosis after 24 hours and sustained till the end of the study. There was a significant increase in NF-κβ throughout most of the study duration following Con A injection as compared to that of normal mice. In conclusions, intra-peritoneal administration of Con A, particularly two doses, represents an efficient approach for induction of immune-mediated hepatitis. T-cells play a major role in AIH through release of TNF-α. Coincidently, hepatitis seems to be associated with elevation of NF-κβ to protect hepatocytes. Thus TNF-α and NF-κβ can represent targets for treatment of AIH either through inhibition or augmentation, respectively.

Autoimmune hepatitis (AIH) is a heterogeneous immune-mediated chronic liver disease characterized by interface hepatitis, hyper-gammaglobulinaemia, circulating auto-antibodies, remarkable necroinflammatory changes of hepatocytes, impaired immune regulation and dramatic response to immunosuppressive therapy [1, 2, 3]. It can affect children and adults but it is more frequently seen in young women [4].

The marked heterogeneity of AIH; regarding its variable presenting features, spectrum of disease severity, type of characteristic autoantibodies and the recommended therapy, has led to classification of the disease into three main types; AIH type-1 (predominant form) affects adolescent and adult females and is

characterized by elevated liver enzymes, circulating antibodies to nuclei (ANA) and smooth muscle (SMA) [5]; AIH type-II which is characterized by antibodies to a particular epitope on cytochrome P450 (IID6) enzyme located in liver and kidney microsomes (anti-LKM), and AIH Type-III which is accompanied by the presence of autoantibodies to Soluble Liver Antigen/Liver Pancreas (anti-SLA/anti-LP). Titers of these autoantibodies correlate with indices of disease severity [6].

Concanavalin-A (Con A) is a type of lectin that is extracted from *Canavalia brasiliensis*. Con A experimental model is a well-established model for investigating T-cell dependent hepatic injury in mice because it mimics the pathogenic mechanisms and pathological changes of patients [7].

There is accumulating evidences suggesting that hepatotoxins induce hepatocellular stress and lead to activation of antigen presenting cells as well as liver resident macrophages (Kupffer cells) that act as effector cells in the destruction of hepatocytes by producing harmful soluble mediators. Chemokines released by kupffer cells are responsible for chemo-attraction of cytotoxic and regulatory T-cells as well as induction of gene expression of cell adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule -1(VCAM-1) on the portal and/or sinusoidal endothelial cells. These molecules allow the recruitment and sinusoidal transmigration of inflammatory cells toward hepatocyte causing liver injury [8, 9].

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that has an indispensable role in the development of AIH through promoting a strong inflammation, up regulating hepatic CCL20 expression, recruiting deregulated splenic T-cells to the liver and inducing c-Jun N-terminal kinases (JNK) and caspase pathways resulting in hepatocyte apoptosis [10, 11].

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) is a transcription factor, first discovered in 1986 that reported to have an important role in protecting hepatocytes from apoptosis induced by TNF-α and other death-inducing signals through the expression and activation of critical genes encoding anti-apoptotic proteins [11]. Moreover, NF-κB signaling can be involved in the initiation and progression of autoimmunity at different stages, including: breakdown of central and peripheral tolerance, development of initial autoimmune inflammatory response as well as its persistent maintenance. Thus it is important to focus on the proposed mechanisms that have been formulated currently, especially in the context of TNFα-mediated liver injury which is an important aspect in the pathogenesis of autoimmune hepatitis. Precisely, we will focus on the mechanisms behind the crosstalk between NF-κB-mediated inhibitions of TNFα-mediated liver injury.

Thus this study aimed at evaluating Con A as a model for induction of autoimmune hepatitis. We also aimed to elucidate the role of TNF-α in disease development through determination of its level in splenocytes (effector cells) at various time durations after induction of hepatitis. Additionally, levels of NF-κβ were assessed at comparable durations to evaluate the relation between both factors.

Materials and Methods

Study Design

The present study was approved by the Animal Care and Use Committee of Medical Research Institute,

Alexandria University, and performed according to the National Animal Health Guidelines.

The study was carried out on a total of 130 outbreed CD1 male mice (6–8 weeks old, 30 ± 2 g) divided into three groups; group (1) included 50 mice injected with single dose Con A to induce AIH, group (2) included 50 mice injected with two doses of Con A, and group (3) included 30 normal mice as negative controls. All animals were accommodated for 1 week in a 12-h day/night rhythm, at a temperature of 25 ± 2°C, 55% relative humidity, with free access to food and water. Con A was dissolved in normal saline solution at a concentration of 20 mg/kg body weight [12, 13].

At the start of 2nd week, hepatitis was induced in group (1) and (2) by intra-peritoneal injection of 600 µl Con A dissolved in saline. Negative control group was injected with saline at the same time. Ten mice from group (1) and 3 mice from group (3, control) were sacrificed by cervical dislocation at intervals of 24h, 48h, 72h, 1 week and 2 weeks following the primary Con A injection. At the start of 3rd week, a second dose of Con A was given to group (2) (secondary injection). Negative control group was injected with saline at the same time. Following the second dose, ten mice from group (2) and 3 mice from group (3, negative control) were sacrificed after 24 h, 48h, 72h, 1 week and 2 weeks.

Sampling

Mice were quickly dissected to obtain blood directly from the heart; in addition, livers and spleens were dissected. Blood samples were collected in plain tubes and centrifuged to prepare sera which are used for determination of the main liver enzymes ALT and AST. Spleens were isolated and used to prepare sufficient amounts of splenocytes that were employed in short term tissue cultures for assessment of NF-κβ and TNF-α levels.

Finally, excised livers were divided into 2 pieces; one employed in short term tissue cultures for assessment of NF-κβ and the other was fixed immediately in 10% buffered formalin for histopathologic evaluation of hepatitis, inflammatory cell infiltrates and necrosis.

Liver functions tests

Aspartate transaminase (AST) and alanine transaminase (ALT) were photometrically measured in all mice serum sample and expressed as µ/l [14].

Histopathological examination of liver:

Livers were excised from normal and Con A-injected mice, immediately fixed in a 10% formalin for 24 h, paraffin blocks were prepared, then 3 µm thick liver sections were cut and stored at room temperature. Paraffin sections were stained with hematoxylin and eosin (H&E) to observe the level of inflammation, tissue damage and apoptosis by light microscopy [15].

All sections were examined blindly by a Pathologist using light microscope and scored according to the following criteria: 0: none; 1: individual cell necrosis; 2: ≤30% lobular necrosis; 3: ≤60% lobular necrosis; and 4: >60% lobular necrosis [13].

Preparation of splenocyte suspension and cell culture

Excised spleens were collected separately into sterile plastic petri-dishes containing 50 mM phosphate buffer saline (PBS) pH 7.2 to extract splenocytes that were washed twice with 50 mM PBS and cultured with supplemented RPMI-1640 tissue culture media (Sigma Aldrich, USA) at 37°C in humidified CO_2 incubator (5% CO_2 and 95% O_2) for 2 days [16].

Preparation of primary hepatocytes and cell culture

Hepatocytes were isolated by in-situ collagenase perfusion of liver samples according to the method described by Tawfik S., et al., (2015) [17]. Cell viability was consistently more that 85% as determined by trypan blue dye exclusion technique, based on impermeability of viable cells to trypan blue. Hepatocytes (1,50,000 cells/cm^2) were cultured in medium containing 5% fetal calf serum for 4 h. Afterwards, the medium was removed and replaced by fresh culture medium without fetal bovine serum. Each sample was cultured in triplicate.

Measurement of NF-κβ and TNF-α level

NF-κβ and TNF-α levels were determined in the supernatant of splenocytes and hepatocytes cultures, respectively using commercially available ELISA kits; according to the manufacturer's instructions. Results were expressed as optical density (O.D) [13].

Statistical Analysis

Data were analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using mean, standard deviation and median.

Comparison between groups was tested using mixed model analysis of variance (ANOVA) and Kruskal–Wallis test. Significance of the obtained results was judged at the 0.05 level [18].

Results

Effects of Con A injection on liver enzymes

Aspartate transaminase (AST) and alanine transaminase (ALT) were photo-metrically measured in all serum sample, expressed as μ/l. AST levels in group 1 (single dose of Con A) showed marked increase after 24 hours then gradually declined by week 1 and remained at low levels. However, in group 2 (two doses of Con A) AST reached maximum levels after 48 hours and maintained at high levels till the end of study (figure 1, Table 1).

Concerning the effect of Con A injection on serum level of ALT; group 1 (single dose of Con A) showed marked increase after 24 hours then rapid normalization. However, in group 2, (two doses of Con A) ALT levels were maintained at high level till the end of study (figure 2, table 2).

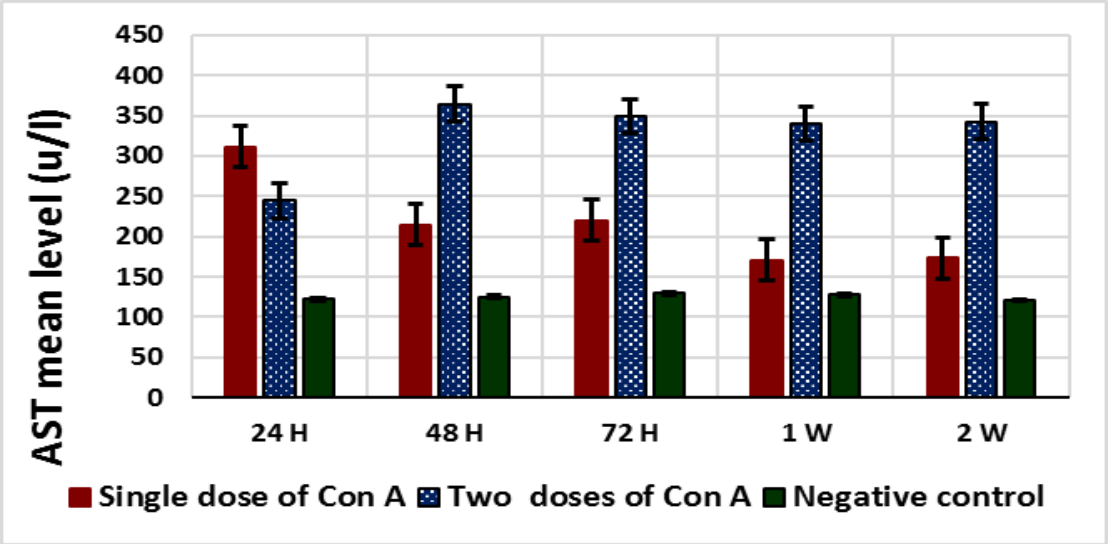

Figure 1. AST levels at different times among Con A injected mice and negative controls

Table 1. Mean levels of serum AST among the studied groups

Groups	AST mean levels (u/l)					*P* Value
	24 H	48 H	72 H	1 W	2 W	
Single dose of Con A	311.7	214.3	219.7	170.63	173	*P*1= 0.003*
Two doses of Con A	244.3	364.57	348.9	339.8	342.76	*P*2<0.0001*
Negative control	122	125.1	129.24	127.1	120.49	*P*3= 0.005*

*P*1 comparison between negative control and single dose of Con A groups at different times
*P*2 comparison between negative control and two doses of Con A groups at different times
*P*3 comparison between single dose of Con A and two doses of Con A groups at different times
P<0.05 is significant

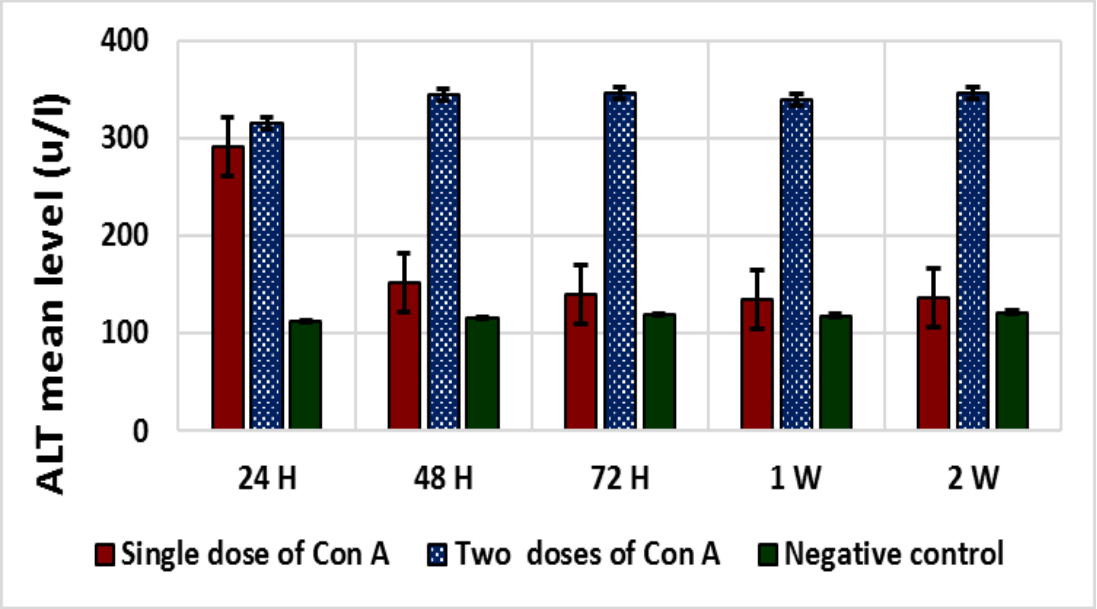

Figure 2. ALT levels at different times among Con A injected mice and negative controls

Table 2. Mean levels of serum ALT among the studied groups

Groups	ALT mean levels (u/l)					*P* Value
	24 H	48 H	72 H	1 W	2 W	
Single dose of Con A	290.5	152	139.8	133.75	136	*P*1= 0.057*
Two doses of Con A	315.36	344.5	346.75	340	347	*P*2<0.0001*
Negative control	112.1	115.6	119	117.4	121.3	*P*3= 0.0002*

*P*1 comparison between negative control and single dose of Con A groups at different times
*P*2 comparison between negative control and two doses of Con A groups at different times
*P*3 comparison between single dose of Con A and two doses of Con A groups at different times
P<0.05 is significant

Liver Histopathological alterations induced by concanavalin-A

Microscopic examination of hepatic sections from mice injected with Con A focused on the presence of inflammatory cell infiltration and morphological evidence of apoptosis (figures 3a-f).

Liver sections in group 1 (single dose of Con A), after 24 hours, revealed inflammatory infiltrates in 60% of mice, apoptosis in 30% while normal livers constituted 10%. After 48 hours, percentage of normal livers increased to 40% compared to 30% inflamed livers and 30% with apoptotic changes. After one week, 70% of examined mice showed normal livers corresponding to 15% with inflammatory changes and 15% each with apoptotic cells.

Liver sections in group 2 (two doses of Con A), after 24 hours, showed marked hepatitis in 60% of examined mice, 20% with apoptotic changes and 20% normal livers. After 48 hours, neither of the examined mice showed normal livers while 50% of them had inflammatory infiltrates versus 50% with apoptotic changes. One week later, there were inflammatory

infiltrates and apoptotic changes in 42% and 38% of examined liver sections, respectively. Finally, 2 weeks post second injection, 70% of examined mice showed normal livers, 15% showed inflammatory infiltrates while 15% had apoptotic changes.

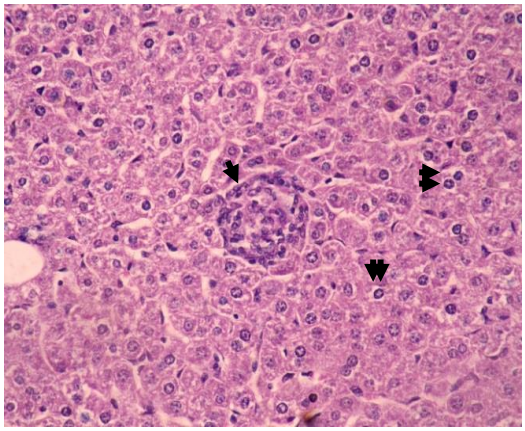

Figure 3a. Liver section show mild inflammatory lobular infiltrate (arrow) and dispersed apoptotic cells (double arrow) (score 1) (H&E, X400)

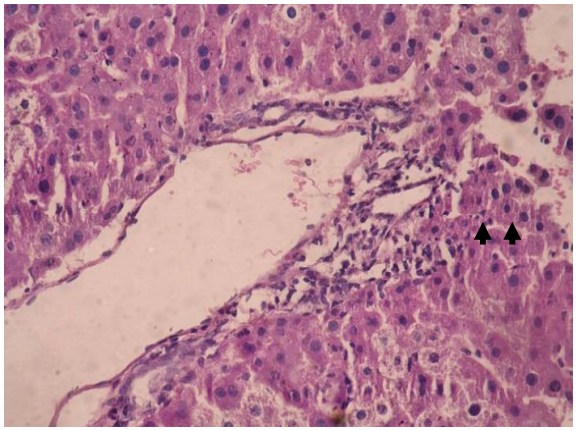

Figure 3b. Liver section show mild portal inflammatory infiltrate with degenerated hepatocytes apoptosis (score 1) (arrow) (H&E, X 400).

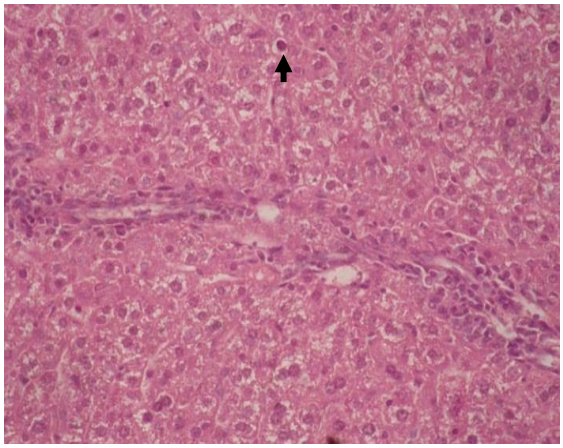

Figure 3c. Section in liver show mild inflammatory infiltrate and apoptosis (score 2) (arrow) (X400).

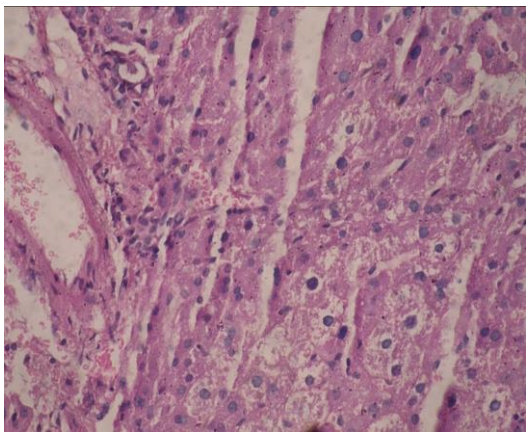

Figure 3d. Section in liver show mild portal inflammatory infiltrate (score 2) (H&E, X400).

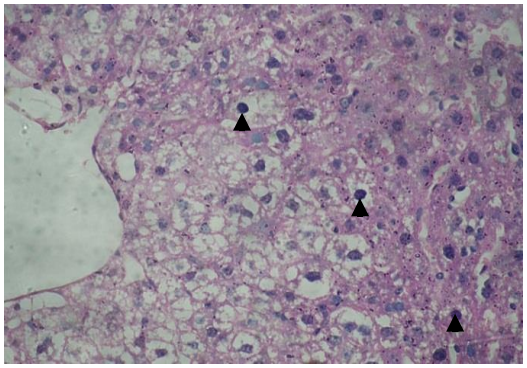

Figure 3e. Moderate hepatic staetosis and apoptosis (arrow) (score 3) (H&E, X400).

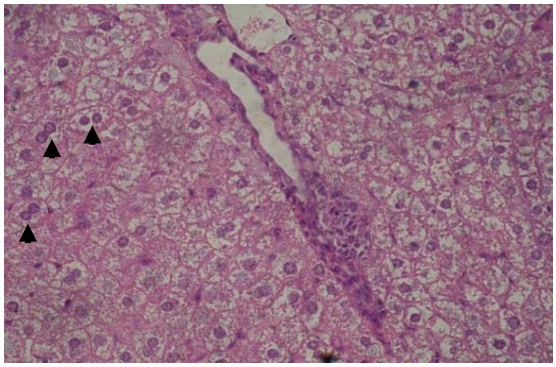

Figure 3f Inflammatory infiltrate, macro-steatotic hepatocytes (bi-nucleated cells, score 4) (X400).

Effect of Con A on splenocyte production of TNF-α

Following either primary or secondary injections with Con A, splenocytes were isolated and maintained in a short-term culture in order to determine the effect of *in-vivo* exposure to the polyclonal T-cell mitogen on production of TNF-α. There was a statistically significant increase in splenocyte TNF-α throughout the study following Con A injection either once or twice as compared to that of normal mice (figure 4, table 3).

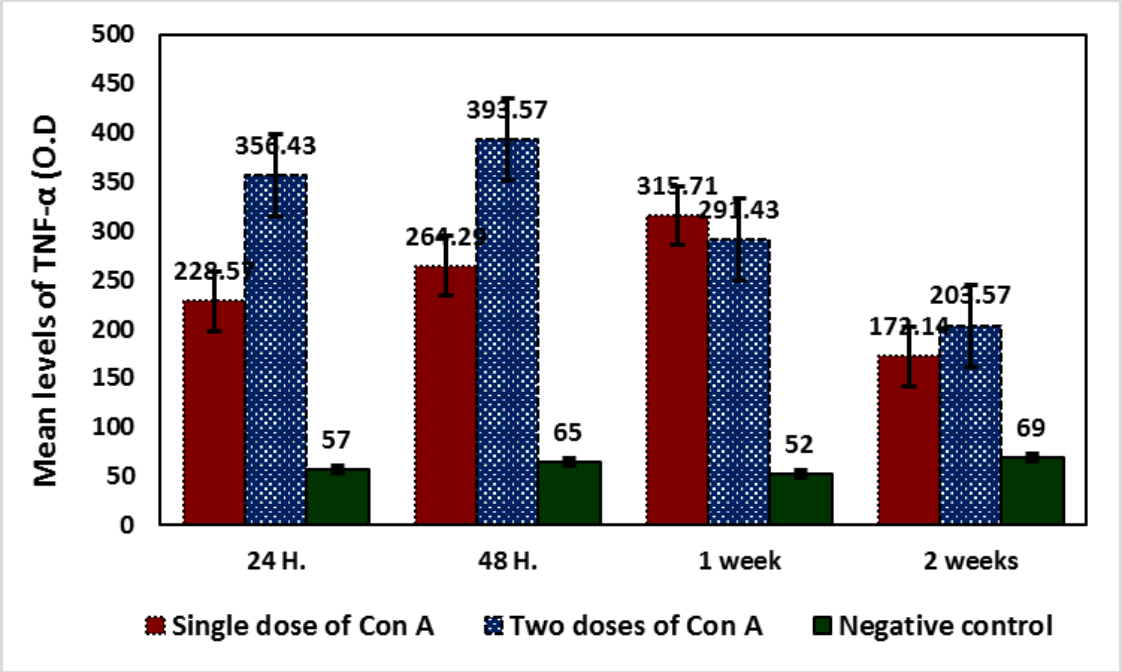

Figure 4. TNF-α mean levels at different times among Con A injected mice and negative controls.

Table 3. Mean levels of Splenocytes' TNF-α among the studied groups

Groups	TNF-α mean levels				*P* value
	24 H.	48 H.	1 week	2 weeks	
Single dose of Con A	228.57	264.29	315.71	172.14	*P*1= 0.0004*
Two doses of Con A	356.43	393.57	291.43	203.57	*P*2= 0.0004*
Negative control	57	65	52	69	*P*3= NS

*P*1 comparison between negative control and single dose of Con A groups at different times
*P*2 comparison between negative control and two doses of Con A groups at different times
*P*3 comparison between single dose of Con A and two doses of Con A groups at different times
P>0.05 is not significant (NS).

Effect of Con A on hepatocytes production of NF-κβ

Following either single or two injections with Con A, hepatocytes were isolated and maintained in a short-term culture in order to determine the effect of *in-vivo* exposure to Con A on production of NF-κβ. There was a statistically significant increase in NF-κβ throughout most of the study duration following Con A injection, particularly with 2 doses, as compared to that of normal mice (figure 5, table 4).

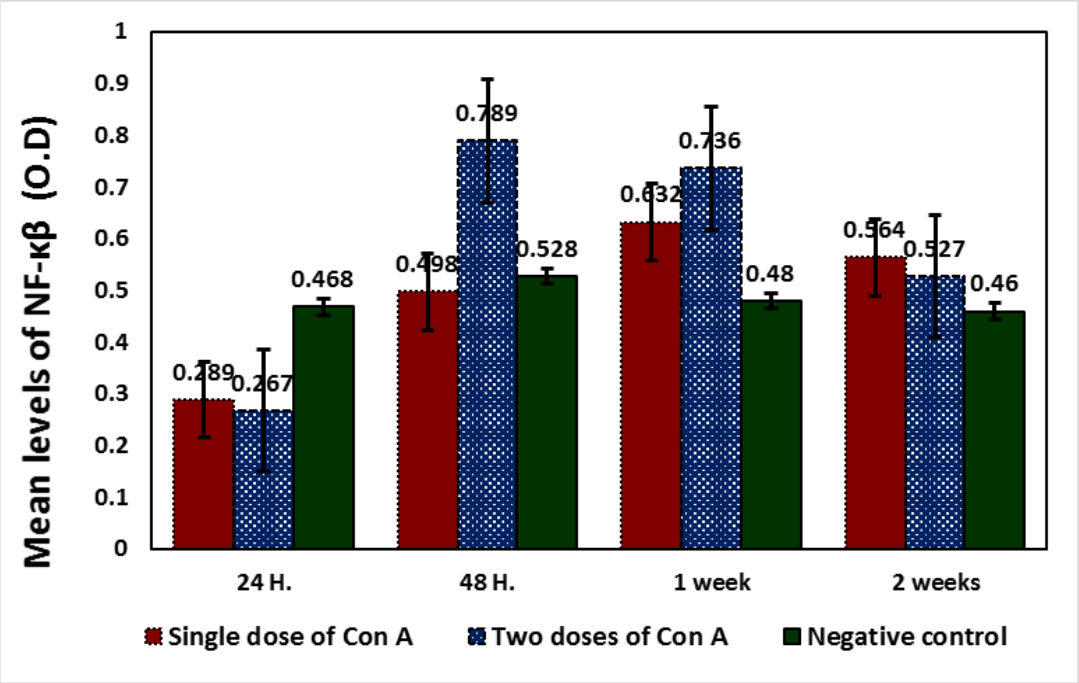

Figure 5. Mean levels of NF-κβ at different times among Con A injected mice and negative controls

Table 4. Mean levels of hepatocytes' NF-κβ among the studied groups

Groups	NF-κβ mean levels				*P* value
	24 H.	48 H.	1 week	2 weeks	
Single dose of Con A	0.289	0.498	0.632	0.564	*P*1= NS
Two doses of Con A	0.267	0.789	0.736	0.527	*P*2= NS
Negative control	0.468	0.528	0.48	0.46	*P*3=NS

*P*1 comparison between negative control and single dose of Con A groups at different times
*P*2 comparison between negative control and two doses of Con A groups at different times
*P*3 comparison between single dose of Con A and two doses of Con A groups at different times
P>0.05 is not significant (NS).

Discussion

Autoimmune hepatitis (AIH) is an un-resolving progressive liver disease that can affect any age or sex. However, its prevalence and manifestations seem to vary according to race and ethnicity [19]. Diagnosis of AIH remains difficult due to the absence of specific and definitive serological markers for the disease and heterogeneity of its clinical, laboratory and histological features. Con. A is a mitogenic plant lectin widely used for *in-vitro* T- cells activation studies. It has been recognized as a powerful *in-vivo* inducer of T-cell and macrophage dependent inflammation causing hepatic injury in mice, mimicking the findings seen in AIH patients [20].

It was stated that TNF-α is essential in the induction of autoimmune hepatitis in mice through up-regulation of hepatic CCL20 expression [10]. On the other hand, the transcription factor NF-κβ is suggested to promote hepatocyte survival by opposing the JNK-induced hepatocyte death. Thus identification of the interplay between TNF-α and NF-κβ is required to identify novel targets for the treatment of autoimmune hepatitis.

In our study, we used Con A at a concentration of 20 mg/kg to induce AIH among outbreed CD1 mice then the extent of hepatitis was assessed by liver enzymes (ALT and AST) and histopathological evidences for inflammatory infiltrate and/or apoptosis as essential markers for hepatic injury. Results revealed significant earlier and more remarkable elevation in ALT and AST levels among Con A-injected mice. Furthermore, there was an increase in percentage of mice with portal inflammatory infiltrate and degenerated hepatocytes particularly 24 hours after Con A injection. Upon administration of 2nd dose of Con A, the elevation of liver enzymes was much more pronounced, and sustained at such high levels until the end of the study. Also, there was an increase in the relative percentages of mice with inflammatory cell infiltrates that peaked at 48 hours. These histopathological changes were consistent with levels of AST and ALT.

Our results were in agreement with many researchers; Wan, *et al.*, [21] have reported that IV administration of Con A in Female BALB/c mice resulted in a significant increase in serum ALT and AST and massive infiltration of inflammatory cells in liver, 4 and 24 h after Con A administration. Wang C, et al., [22] have demonstrated that mice with Con A- induced hepatitis showed high serum levels of ALT and AST concomitant with hepatic injury. Also, Ye, *et al.*, [13] stated similar results with the use of Con A. All these results agree with the use of Con A-induced liver injury in mice as an appropriate model of immune hepatitis.

There is strong evidence for the major role of T-cells in AIH because it was found that T-cell reactivity to liver antigens dominates the histological and pathological disease [13]. Goto, *et al.*, [23] suggested that NKT cells are known to contribute to the pathogenesis of Con A-induced hepatitis by producing cytokines such as TNF-α and inducing FasL-mediated hepatocyte injury.

The rapid recovery of elevated liver enzymes and histopathological evidence of hepatitis after primary Con A injection were explained by Biburger *et al.*, [24] who stated that accelerated inflammatory responses induced by Con A challenge might be beyond the ability of affected livers to regenerate/recover. However, they demonstrated that upon Con A re-challenge, treated mice develop a sort of tolerance to immune-mediated liver injury. In fact, the

observed variability in kinetics of liver enzyme release and liver cell/activated T cell apoptosis between our study and others could also be attributed to strain differences of the employed mice.

Santucci, *et al.,* [25] showed that in Con A model of liver injury there are at least 2 partially independent pathways by which activated T-cells cause liver cell death; the first is the release of Th1-like cytokines such as TNF-α and IFN-γ, and the second is the activation of Fas/FasL apoptotic pathway on the hepatocyte cell surface. In the liver, TNF-α directly and indirectly induces cell death of hepatocytes, whereas it can mediate production of inflammatory mediators, hepatocyte proliferation, and liver regeneration [26].

This was in accordance with our study where we employed levels of TNF-α to assess inflammation in culture supernatants of splenocytes. Statistical analysis revealed a significant increase in splenocyte TNF-α throughout most of the study duration following Con A injection either once or twice as compared to that of normal mice.

In agreement with our results, Yamashita, *et al.,* [27] showed that activated T-cells and subsequent production of cytokines play a critical role in the pathogenesis of hepatitis. Upregulation of pro-inflammatory cytokines such as TNF-α and IFN-γ by Con A injection directly induce hepatocellular apoptosis and necrosis. Wang, *et al.,* [22] have clarified that mRNA expression levels of TNF-α in the liver of Con A-treated mice were significantly up-regulated at three time points 6h ,12h and 24hours. All these results supported that these pro-inflammatory cytokines are mainly implicated in the pathogenesis of AIH.

Wullaert A. *et al.,* [28] have clarified that binding of TNF-α to TNF-R1 leads to formation of complex I whose signaling leads to NF-κβ activation. Then NF-κβ translocate to the nucleus to induce the transcription of genes with an NF-κβ consensus site in their promoter region. In addition, signaling from complex I result in recruitment of Fas-associated death domain (FADD) and pro-caspase-8 leading to the formation of the cytosolic complex II, where caspase-8 is activated [29]. Caspase-8 initiates the mitochondrial pathway that causes release of cytochrome C that activates the other caspases, resulting in full-blown caspase activity and subsequent apoptosis [2, 28].

On the other hand, accumulating evidences [11, 22, 29-32] have shown that NF-κβ have cytoprotective effects against TNFα-induced hepatotoxicity. This is consistent with the results of our study where NF-κβ levels increased gradually and reached maximum after 24 hours of Con A injection followed by a gradual decline by week 1 that was maintained till week 2; sharp and highly significant elevation was recorded 24 hours following second dose of Con A that was sustained at high levels till the end of the first week. This can be explained by its combating effect against TNF-α.

Papa, *et al.,* [11] have explained this to be through suppression of c-Jun N-terminal kinase (JNK) activity following TNF-α challenge. Others reported that NF-kB-induces release of some anti-apoptotic proteins, such as the caspase-8 inhibitor c-FLIP(L), the Bcl-2 family members Bcl-xL and A1/Bfl-1, X-linked inhibitor of apoptosis (XIAP), and cellular inhibitor of apoptosis (c-IAP)1 and c-IAP2 that interfere with TNF-induced apoptosis at various levels in the signaling pathways [22, 29-32]. For instance, c-FLIP(L) competes with caspase-8 for binding to the TNF-R1 complex II and hinder the formation of the

Death Inducing Signaling Complex (DISC) [29]. Bcl-xL and A1/Bfl-1 prevent tBid-induced mitochondrial permeabilization, thus reducing the production of ROS and cytochrome C mediated caspase-9 activation [30]. XIAP as well as the c-IAPs directly bind distinct caspases and inhibit their proteolytic activity [31, 32].

In conclusions, intra-peritoneal administration of Con A, either as a single or two doses represents an efficient approach for induction of immune-mediated hepatitis. Single dose of Con A is sufficient to induce acute AIH. T-cells play the major role in AIH through release of TNF-α. Coincidently, hepatitis seems to be associated with activations of NF-κβ to oppose the effect of TNF-α. Thus TNF-α and NF-κβ can represent targets for treatment of AIH either through inhibition of TNF-α or augmentation of NF-κβ, respectively.

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