



Original article

Digoxin mitigates diethylnitrosamine-induced acute liver injury in mice via limiting production of inflammatory mediators

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ABSTRACT

The cardiotoxic digoxin has been recently shown to possess an anti-inflammatory potential in numerous metabolic and inflammatory disorders. However, data about digoxin's impact in the setting of acute liver injury and sterile inflammation are still limited. Here, we investigated the potential effect of digoxin pretreatments (0.25 and 0.5 mg/kg, oral) on the severity of acute hepatotoxicity in mice challenged with a single dose of diethylnitrosamine (DN; 150 mg/kg, intraperitoneal) for 24 h. Our results indicated that digoxin pretreatments dose-dependently mitigated DN-induced rise of hepatocellular injury parameters and necroinflammation scores. Digoxin, particularly at dose of 0.5 mg/kg, boosted the number of PCNA positive hepatocytes, leading to improvement of the reparative potential in hepatocytes of DN-intoxicated livers. Digoxin's ameliorative effect on DN-hepatotoxicity coincided with (i) lowering the increased hepatic production and release of the proinflammatory mediators IL-17A, IL-1 β and TNF- α , and (ii) impeding the attraction and infiltration of monocytes to the liver, as denoted by decreasing serum MCP-1 and F4/80 immunohistochemical expression. These effects were attributed to reducing DN-induced activation of NF- κ B and overexpression of CD98 in the liver. Meanwhile, DN elicited a decline in the hepatic production and release of the anti-inflammatory cytokines IL-22 and IL-6, which was intensified by digoxin, especially at a dose 0.5 mg/kg. In conclusion, digoxin conferred liver protection against DN-insult by impairing the overproduction of proinflammatory cytokines and infiltration of inflammatory cells to the liver.

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1. Introduction

Digoxin is used as a cardiotoxic for the management of congestive heart failure and several arrhythmic disorders (Virgadamo et al., 2015). The cardiotoxic effect of digoxin is substantially linked to inhibition of the sodium–potassium pump, leading to accumulation of calcium ions *via* collateral decrease of sodium–calcium exchange pump activity (Whayne, 2018). Regardless of its cardiotoxic effect, digoxin has been reported to be an inverse

agonist of retinoid acid receptor-related orphan nuclear receptor gamma (ROR γ t) that stabilizes C-terminal helix 12 (H12) in a conformation that is optimal for binding of corepressor rather than coactivator, preventing transcription of dependent genes (Huh et al., 2011; Meijer et al., 2020).

The transcription factor ROR γ is present as ROR γ 1 and ROR γ 2 (ROR γ t) isoforms that are stimulated by oxysterols derived from cholesterol catabolism rather than retinoic acid (Rutz et al., 2016). The former isoform is present in several tissues like the liver, kidney, muscle and adipose, where it controls lipid/glucose homeostasis and circadian rhythm. However, the latter isoform is expressed in lymphoid cell types like CD4⁺/CD8⁺ thymocytes and TH-17 cells, where it orchestrates the adaptive and innate immune responses (Jetten et al., 2018). Hence, ROR γ t is an attractive nuclear receptor for pharmacological targeting to hinder the action of TH-17 cells and the dependent inflammation.

There is a growing research interest about the outcomes of the unconventional inhibitory effect of digoxin on ROR γ t. For instance,

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digoxin reduced the severity of experimental multiple sclerosis *via* inhibiting ROR γ t and the dependent induction of IL-17A transcription and differentiation of TH-17 cells (Huh et al., 2011). Moreover, digoxin alleviated arthritis *via* reducing the expression of proinflammatory mediators and the *in vitro* TH-17 differentiation and IgG production (Lee et al., 2015; Saeed et al., 2020). Otherwise, digoxin was reported to abate the rise of plasma lipid levels and atherosclerotic lesion formation in apolipoprotein E-deficient mice through antagonizing ROR γ t and the ensued IL-17A- inflammatory responses (Shi et al., 2016). Digoxin also prolonged cardiac allograft survival by inhibiting IL-6-mediated conversion of Tregs to TH-17 cells (Wu et al., 2013). Most recently, digoxin was beneficial in curbing inflammation at border regions of the brain and during COVID 19 infection (Cho et al., 2020; Jansson et al., 2021; Siniorakis et al., 2021).

Most nitrosamines are well-known of being potent carcinogens that can drive tumorigenesis in several body organs like the stomach, esophagus, lung, nasal sinus, brain, kidney, bladder and liver (Verna et al., 1996). It's no wonder that human exposure to diethylnitrosamine (DN) and other nitrosamines occurs on a daily basis through consuming considerable amounts of nitrite-cured meat, processed and fried foodstuffs, alcoholic beverages and cigarettes (Yalcin and de la Monte, 2016; Crowe et al., 2019; Li et al., 2021). Because of that, we sought to investigate whether digoxin can dampen acute liver damage and sterile inflammation-induced by DN in mice, and if so, what will be the underlying mechanism.

2. Materials and methods

2.1. Mice

Male BALB/c mice weighing 30 ± 3 g were supplied with food-pellets and water throughout the acclimatization and experimental periods. The experimental animal caring and procedures were concordant with the criteria of the National Institutes of Health and the Research Ethics Committee for Care of Laboratory Animals at the institutions.

2.2. Digoxin treatment and diethylnitrosamine (DN) model of acute liver injury

Digoxin was supplied as sterile vial containing 0.5 mg/2 ml (Alexandria Pharmaceuticals, Egypt), while DN was supplied as a liquid vial containing 99%/10 ml (Sigma, USA; density 0.95 g/mL; Cat. no. 73861). Both doses of digoxin (0.25 and 0.5 mg/kg/10 ml) and DN (150 mg/kg/10 ml) were diluted in sterile normal saline. Two groups of mice received digoxin doses (0.25 and 0.5 mg/kg, oral) 2 h prior to intoxication with DN dose (150 mg/kg, intraperitoneal) for 24 h. Another group of mice administered the normal saline vehicle orally devoid of digoxin 2 h prior to the intraperitoneal DN-intoxication and served as DN-untreated mice. The last group were normal control mice that were administered the normal saline vehicle twice (orally and intraperitoneally) in a period of 2 h in between. After 24 h, mice were anesthetized by a dose of 100 mg/kg/10 ml of thiopental (Sigma Pharmaceuticals, Egypt), and blood samples were collected *via* cardiac puncture. Thereafter, blood samples were centrifuged at 2500 g for 12 min at room temperature for separating serum, which was kept at -80 °C for further analysis. Portions of liver tissue were also kept at -80 °C for immunological assays. Some portions of liver were also stored in plastic cassettes and immersed in a solution of 4% (v/v) neutral-buffered formalin for fixation, followed by applying the procedures of histopathology and immunohistochemistry.

2.3. Serum biochemical parameters of liver injury

The extent of liver injury in mice was evaluated by measuring alanine aminotransferase (ALT; cat. no. 265 001), aspartate aminotransferase (AST; Cat. no. 261 001) and lactate dehydrogenase (LDH; cat. no. 279 001) levels in prediluted serum samples by kinetic kits (Spectrum-diagnostics, Egypt).

2.4. Histopathological and immunohistochemical inspections in the liver

The fixed liver tissues in the plastic cassettes were first dehydrated by graded concentrations of ethanol in water (70, 80, 95 and 100% v/v), followed by immersing in ethanol:xylene (1:1) solution and finally, pure xylene solution to remove traces of ethanol. Thereafter, liver tissues were embedded into paraffin blocks, cut by a microtome as 4 μ m thickness sections that were picked up by glass slides. The sections were then subjected to dewaxing by immersing 2 times in xylene solution, followed by rehydration in ethanol solutions in the reverse order of dehydration (100, 95, 80 and 70% v/v) and staining with hematoxylin and eosin (HE). The applied scores for hepatic necrosis were 0 (lack of necrosis), 1 (spotty necrotic hepatocytes), 2 (confluent necrotic hepatocytes) and 3 (bridging necrosis) (González-Pérez et al., 2006). The scores assigned for hepatic inflammation were 0 (lack of inflammatory cells), 1 (scattered inflammatory cells), 2 (inflammatory foci) and 3 (diffuse inflammatory cells) (Veteläinen et al., 2006). In immunohistochemical evaluation, liver sections (4 μ m paraffin-embedded) were placed on coated glass slides for detecting the target proteins under investigation. Slides were then subjected to protocols for retrieving antigens and abolishing endogenous peroxidases alongside non-specific protein binding. Thereafter, slide sections were sequentially incubated with primary antibodies, secondary antibodies, and substrate/chromogen. The used primary antibodies were for nuclear factor-kappa B (NF- κ B) (BioLegend, USA; Cat. no. 622601), F4/80 (BioLegend, USA; Cat. no. 123101), proliferating cell nuclear antigen (PCNA) (BioLegend, USA; Cat. no. 307902) and CD98 (Santa Cruz, USA; Cat. no. 9160). The pictures of immunohistochemistry were quantified by the ImageJ software (NIH, USA).

2.5. Enzyme-linked immunosorbent assay (ELISA) of hepatic and serum cytokines

By using ELISA MAX[™] Deluxe kits (BioLegend, USA), mouse tumor necrosis factor- α (TNF- α ; Cat. no. 430904) and interleukins (ILs)-1 β (Cat. no. 432604), 6 (Cat. no. 431304), 17A (Cat. no. 432504) and 22 (Cat. no. 436304) were quantitated in both the lysed liver and serum samples, while monocyte chemoattractant protein (MCP-1; Cat. no. 432704) was estimated only in serum samples. Liver lysate was prepared by mincing 0.1 g of liver in 0.9 ml of cooled lysis buffer (150 mM NaCl, 0.5% v/v Triton X-100, 10 mM Tris pH 7.4) supplied with a protease inhibitor (Roche, Germany; Cat. no. 11836170001), followed by centrifuging the tubes at 5000 g for 10 min at 4 °C. The isolated supernatants of liver lysis and serum samples (prediluted with phosphate buffered saline in a ratio 2:1) were loaded on the precoated ELISA plates with the appropriate capture antibody, and the manufacturer protocol was followed. The protein concentration was also estimated in the lysed liver samples as previously described (Bradford, 1976).

2.6. Quantification of hepatic malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)

The total concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) was quantified spectrophotometrically at a wavelength of 586 nm as formerly mentioned (Gérard-

Monnier et al., 1998). In brief, liver homogenate samples were prepared as previously described (Shaker et al., 2016), followed by mixing 0.4 ml of sample with 0.65 ml of the reagent mixture [10.3 mM 1-methyl-2-phenylindole (Sigma-Aldrich, USA; Cat. no. 404888) dissolved in acetonitrile and diluted with 32 μ M FeCl₃ dissolved in methanol in ratio 3:1] and 0.15 ml of 15.4 M methanesulfonic acid (Merck, Germany; Cat. no. 8060220250). The sample tubes were subsequently kept at 45 °C for a period of 40 min, cooled on ice and centrifuged at 4000 g for 10 min. The collected supernatants were measured spectrophotometrically against sample blanks. A standard calibration curve of tetramethoxypropane (Sigma-Aldrich, USA; Cat. no. 108383) was conducted to estimate the concentration of total MDA + 4-HNE.

2.7. Statistical analysis

Parametric data (means \pm SE) were analyzed by one way ANOVA, ensued by the post test of Tukey-Kramer. The non-parametric scores of hepatic necrosis and inflammation were compared by the non-parametric Kruskal-Wallis test, ensued by the post test of Dunn's multiple comparison. Statistical analysis of all data was conducted by the GraphPad Prism 7 software (USA).

3. Results

3.1. Digoxin mitigates DN-induced hepatocellular injury and necroinflammation

Administration of DN significantly ($P < 0.001$) increased serum ALT, AST and LDH activities in mice, relative to the normal control group (Fig. 1). DN also elicited a significant ($P < 0.001$) elevation of hepatic necrosis and inflammation scores in comparison to the normal control group (Fig. 2). Intriguingly, these hepatic abnormalities induced by DN-administration were dose-dependently mitigated by digoxin pretreatments (0.25 and 0.5 mg/kg).

3.2. Digoxin reverses DN-induced alteration of proinflammatory cytokines

To examine whether the ameliorative effect of digoxin on DN-hepatotoxicity is related to inhibition of the inflammation cascade, we evaluated the changes of the proinflammatory cytokines TNF- α , IL-1 β and IL-17A in both serum and liver. The results indicated that administration of DN to mice markedly increased the serum concentrations of these proinflammatory cytokines (Fig. 3A–C). Digoxin pretreatments (0.25 and 0.5 mg/kg) to DN-intoxicated mice elicited a dose dependent inhibition of the increase in the serum concentrations of these proinflammatory cytokines. However, DN-intoxication insignificantly raised TNF- α and lowered IL-1 β and IL-17A in the liver, compared to control mice (Fig. 3D–F). At the dose level of 0.5 mg/kg, digoxin reversed DN-elicited abnormalities in hepatic concentrations of TNF- α and IL-1 β , but not IL-17A.

3.3. Digoxin abates DN-induced nuclear accumulation of NF- κ B in the liver

To identify the underlying mechanism for digoxin in limiting DN-mediated release of proinflammatory cytokines in the blood circulation, hepatic NF- κ B expression was evaluated. DN-intoxication caused a significant rise of hepatic NF- κ B immunohistochemical expression in the nuclei of hepatocytes and non-parenchymal cells, compared to the control group (Fig. 4A and C). However, these abnormalities were significantly

hindered by 0.25 and 0.5 mg/kg of digoxin pretreatments at $P < 0.05$ and $P < 0.01$, respectively.

3.4. Digoxin attenuates DN-induced attraction and infiltration of monocytes to the liver

Next, we investigated whether digoxin's hepatoprotective effect on DN-intoxication was associated with interference of attraction and infiltration of monocytes into the liver. Accordingly, alterations of MCP-1 and the monocyte/macrophage parameter F4/80 were assessed in the blood circulation and liver, respectively. Mice intoxicated with DN without digoxin pretreatment were having higher serum MCP-1 concentration and hepatic F4/80 immunohistochemical expression (Fig. 4B, D and E). Meanwhile, pretreating DN-intoxicated mice with digoxin significantly limited the rise of these parameters in both serum and liver, especially at a dose of 0.5 mg/kg

3.5. Digoxin improves the reparative potential in hepatocytes of DN-intoxicated livers

After hepatocellular injury, nuclear repair mechanisms are initiated to spare hepatocytes from damage. Accordingly, we evaluated the alterations of PCNA immunohistochemical expression in hepatocytes of DN-intoxicated mice with and without digoxin pretreatments. The immunohistochemical assessment indicated that the nuclear PCNA expression was insignificantly elevated with DN alone (Fig. 5A and C). Meanwhile, PCNA expression was elevated by pretreating DN-mice with digoxin, pronouncedly at a dose of 0.5 mg/kg ($P < 0.001$ versus control group and $P < 0.01$ versus DN group).

3.6. Digoxin mitigates DN-induced overexpression of CD98 in the liver

CD98 regulates multiple cellular functions like amino acid transportation, proliferation, diffusion and adhesion. We next sought to examine whether digoxin acts through impairing CD98 abnormalities induced by DN-intoxication. DN-intoxicated mice were having higher hepatocellular CD98 expression in comparison to normal control mice, as evidenced by immunohistochemistry assessment and quantification (Fig. 5B and D). Interestingly, both digoxin pretreatments (0.25 and 0.5 mg/kg) abrogated DN-induced overexpression of CD98 in the liver hepatocytes.

3.7. Digoxin does not impair DN-induced lipid peroxidation in the liver

DN is known to partially mediate its liver injury by increasing the generation of lipid peroxidation products like MDA and 4-HNE. Thereby, we quantitated the hepatic concentration of these lipid peroxidation products to ascertain whether the hepatoprotective effects of digoxin can be linked to preventing DN-induced lipid peroxidation. Unexpectedly, DN-induced elevation of hepatic MDA and 4-HNE concentration was not lowered by pretreating the mice with either 0.25 or 0.5 mg/kg of digoxin (Fig. 5E).

3.8. Digoxin intensifies DN-induced decline in production and release of IL-22 and IL-6

IL-22 and IL-6 are known to have anti-inflammatory and reparative roles in vast liver injury situations. Next, we assessed the effect of digoxin on DN-induced alterations in serum and hepatic concentrations of IL-22 and IL-6 in mice. Acute DN-intoxication led to decline of serum IL-22 and IL-6 concentrations, compared to the control group (Fig. 6). In the liver, DN also caused reduction of IL-22, but no effect on IL-6. However, digoxin (0.5 mg/kg)

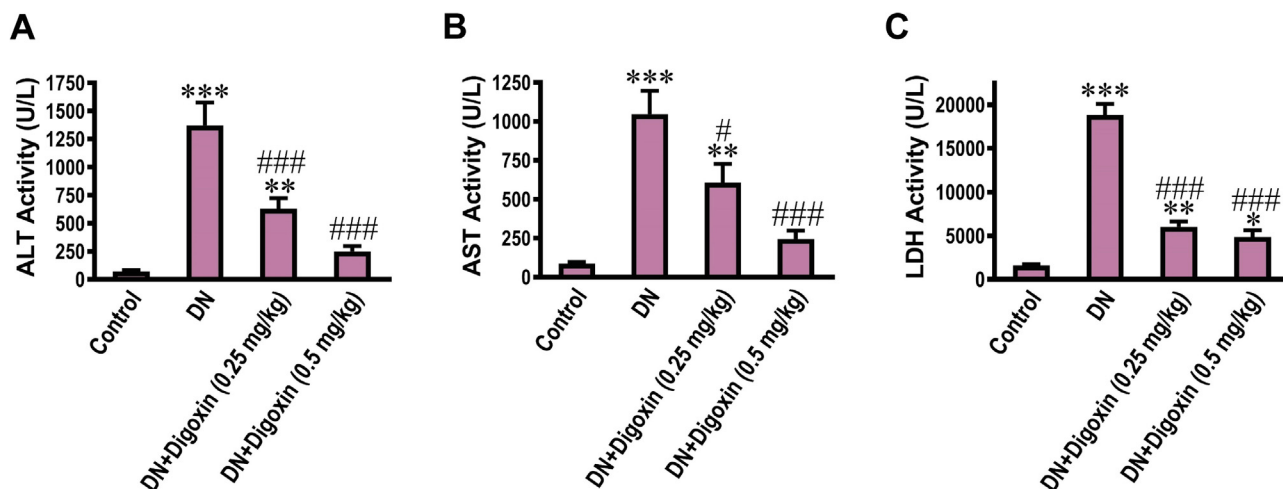


Fig. 1. Impact of digoxin pretreatments (0.25 and 0.5 mg/kg) on diethylnitrosamine (DN)-instigated variations in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities in mice ($n = 6/\text{group}$). Levels of statistical differences against the control group were $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, while those against the DN group were $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$.

intensified DN-induced decline in these parameters, rather than reversing them.

4. Discussion

Digoxin-therapeutic usefulness in acute hepatotoxicity is still not known to date. To fully explore this hypothesis, the potential of digoxin to limit liver injury and sterile inflammation was investigated in acutely DN-intoxicated mice. The results showed that digoxin dose-dependently curbed DN-induced elevation of hepatocellular injury parameters (ALT, AST, LDH) and necroinflammation scores. The protective effect of digoxin was further confirmed at a dose level of 0.5 mg/kg by reducing DN-chemoattraction (MCP-1) and infiltration of monocytes/macrophages (F4/80) to the liver alongside intensifying the hepatocellular proliferation (PCNA).

In liver, IL-17A is produced by multiple inflammatory cells other than CD4⁺/CD8⁺ T cells like Kupffer cells, macrophages, neutrophils and others (Chackelevicius et al., 2016). In cholestatic and CCl₄-induced fibrosis, genetic deletion of IL-17 receptor A or IL-17A in mice led to less production of IL-6, IL-1 β and TNF- α by hepatic Kupffer and T cells and lowered collagen production from activated hepatic stellate cells in comparison to the wild type counterparts (Yan et al., 2012). In acute liver injury induced by ischemia/reperfusion or concanavalin A, IL-17A and IL-17RA of Kupffer cell were shown to increase the severity of liver injury and production of other proinflammatory mediators, including IL-1 β , IL-6, TNF- α and MCP-1 (Kono et al., 2011; Yan et al., 2012), while interfering with the IL-17A action by digoxin may lessen inflammation in the liver.

Challenging mice with DN generated a hepatic sterile inflammatory response that was ensued by marked release of the proinflammatory factors IL-1 β , TNF- α and IL-17A in the systemic circulation, but these abnormalities were limited by pretreating these mice with digoxin. Digoxin and other ROR γ t inverse agonists were reported to counter NLRP3-inflammasome processing for IL-1 β through inhibition of hypoxia-inducible factor (HIF)-1 α -induced pro-IL-1 β gene transcription in LPS/galactosamine-challenged livers and LPS/ATP-stimulated macrophages (Ouyang et al., 2018; Zhao et al., 2019). In the same context, digoxin reduced high fat diet-induced hepatic inflammation via binding to pyruvate kinase M2 and impairing dependent HIF-1 α transactivation at doses lower those needed for a cardiotoxic action (Ouyang et al., 2018). These findings were also corroborated by showing that

macrophages isolated from mice lacking ROR- γ t exhibited lower expression of the NLRP3-inflammasome and pro-IL-1 β alongside lesser secretion of the cleaved IL-1 β (Billon et al., 2019). Based on these findings, digoxin limited DN-induced activation of IL-1 β mediated by the NLRP3-inflammasome via upstream impairment of ROR γ t and HIF-1 α .

The data indicated that digoxin pretreatments also reduced DN-induced activation and nuclear translocation of NF- κ B in the liver cells. NF- κ B is linked to the inhibitory proteins I κ B in the cytoplasm, which is phosphorylated by IKK and degraded by the proteasome subsequent to stimulation of TLR4 or certain cytokines receptors (Iwai 2014). This eventually drives nuclear NF- κ B translocation and transcription of multiple NF- κ B-dependent target genes like TNF- α , IL-6 and Pro-IL-1 β (Liu et al., 2017). LPS-stimulated human peripheral blood mononuclear cells exhibited less production of TNF- α and IL-6 by digoxin pretreatment that interfered with the NF- κ B activation (Ihenetu et al., 2008). Moreover, digoxin-related cardiac glycosides suppressed TNF- α mediated activation for NF- κ B and JNK signaling pathways by interfering with the upstream recruitment of TRADD to TNF-receptor 1 (Yang et al., 2005). Otherwise, TNF- α inhibitors like etanercept and adalimumab were shown to downregulate ROR γ t expression and production of IL-17A and IL-22 in human TH-17-polarized T cells by impairing recruitment of NF- κ B-associated acetyltransferases and acetylation of histones H3 and H4 in the ROR γ t promoter area (Lin et al., 2017). Taken these findings together, digoxin impairment of NF- κ B and ROR γ t activation may be the underlying mechanism for lessening the inflammation cascade.

Digoxin (0.5 mg/kg) pretreatment to DN-intoxicated mice also suppressed the dependent-attraction and infiltration of monocytes to the liver, as evidenced by digoxin-mediated decline of DN-rise of serum MCP-1 and number of F4/80 positive cells in the liver. Furthermore, digoxin dose-dependently receded DN-overexpression of CD98 in the liver. Targeting CD98 has been shown to abate the severity hepatic injury, inflammation and steatosis in mice (Canup et al., 2017; Canup et al., 2020). CD98 can increase the ligand-affinity for integrin- β 1, which can be liberated from hepatocytes during stress into the circulation for attracting monocyte-derived macrophages to the sinusoidal endothelial cells in the setting of hepatic inflammation (Cai et al., 2005; Guo et al., 2019). Furthermore, switching off integrin- β 1-signaling prevented the mice inflammatory response driven by

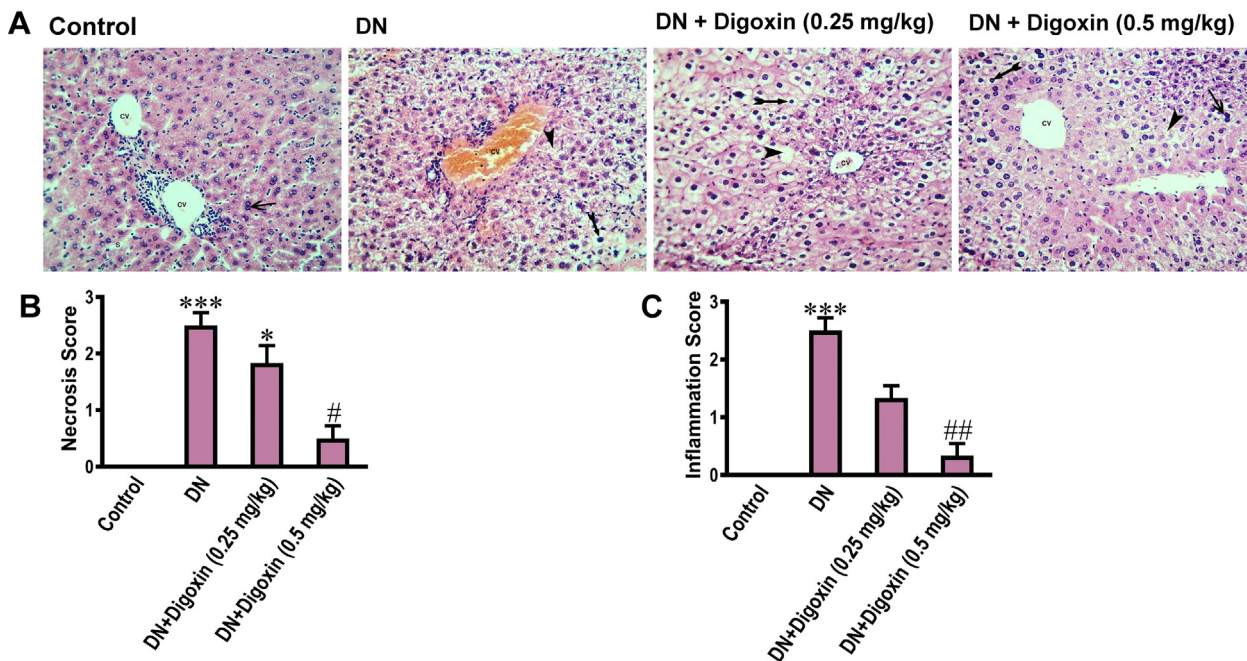


Fig. 2. Impact of digoxin pretreatments (0.25 and 0.5 mg/kg) on diethylnitrosamine (DN)-instigated variations in hepatic histopathology (200×, A) and scores of necrosis (B) and inflammation (C) in mice (n = 6/group). Levels of statistical differences against the control group were **P* < 0.05 and ****P* < 0.001, while those against the DN group were #*P* < 0.05 and ##*P* < 0.01. The liver section of the control mice revealed normal arrangement of the hepatocytes around the central veins (CV) with intervening sinusoids (S). DN-intoxicated mice showed extensive liver changes in the form of congestion of the CV, pyknosis of the hepatocytes' nuclei (tailed arrow) and vacuolated cytoplasm (arrow head). DN-mice pretreated with digoxin (0.25 mg/kg) exhibited moderate liver affection in the form of minimal congestion of the CV, less frequent pyknosis of the hepatocytes' nuclei (tailed arrow) and vacuolated cytoplasm (arrow head). DN-mice pretreated with digoxin (0.5 mg/kg) showed mild liver affection in the form scanty pyknosis of the hepatocytes' nuclei (tailed arrow) and vacuolated cytoplasm (arrow head).

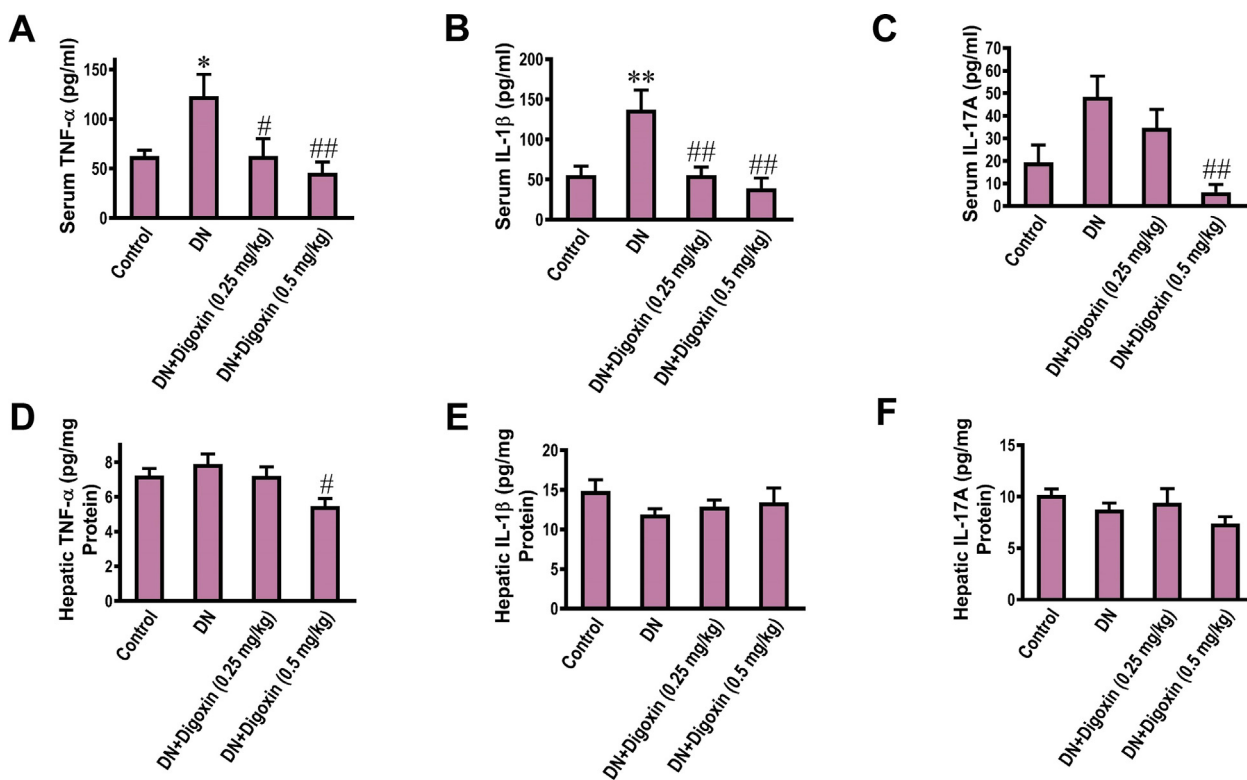


Fig. 3. Impact of digoxin pretreatments (0.25 and 0.5 mg/kg) on diethylnitrosamine (DN)-instigated variations in serum (A-C) and hepatic (D-F) concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-17A in mice (n = 6/group). Levels of statistical differences against the control group were **P* < 0.05 and ***P* < 0.01, while those against the DN group were #*P* < 0.05 and ##*P* < 0.01.

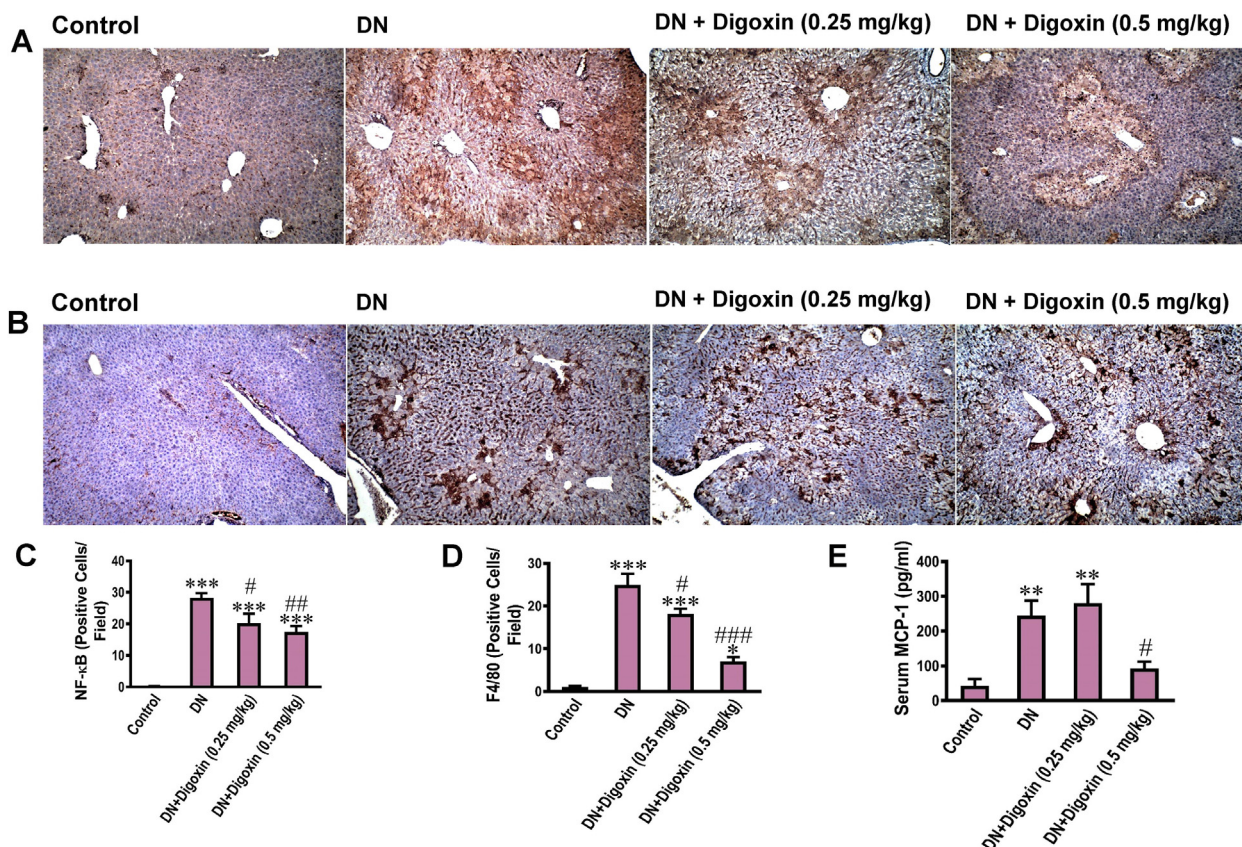


Fig. 4. Impact of digoxin pretreatments (0.25 and 0.5 mg/kg) on diethylnitrosamine (DN)-instigated variations in the hepatic immunohistochemical expression and quantification of nuclear factor-kappa B (NF-κB; A and C; 100×), F4/80 (B and D; 100×) and serum monocyte chemoattractant protein (MCP-1; E) concentration in mice (n = 6/group). Levels of statistical differences against the control group were * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, while those against the DN group were # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$.

TLR4-stimulation with LPS (Hakanpaa et al., 2018). Accordingly, digoxin reduction for DN-increased expression of CD98 is an additional mechanism for reducing monocyte infiltration and the inflammation cascade beside digoxin-mediated impairment of NF-κB and RORγt activation.

The data indicated that mice administered digoxin (0.5 mg/kg) alongside DN pronouncedly had lower concentrations of IL-22 in the systemic circulation and liver to an extent greater than those administered DN only. DN-insult also caused a discrepancy between IL-17A and IL-22 concentrations, raising the possibility of involvement of immune cells other than TH-17T cells. Unlike IL-17A, IL-22 is involved in protection and repair of hepatocytes, and mice lacking IL-22 were having aggravated liver fibrosis (Zenewicz et al., 2007). It has been recently shown that NK-22 cells-double positive for NKp46 and RORγt are the main liver-infiltrating lymphocytes that produce IL-22 and provide protection against hepatic ischemia-reperfusion injury, and IL-22-production by these cells was found to be regulated by RORγt (Eggenhofer et al., 2016). NK cells were also involved alongside T and NKT cells in driving concanavalin-A-induced liver injury in mice lacking glycine N-methyltransferase (Gomez-Santos et al., 2012). Accordingly, DN seems to interfere with the action of NK-22 and deplete their capacity for IL-22 production, which was more severe upon inhibition of RORγt by digoxin. Beyond NK-22 cells, TH-22 cells are another producer for IL-22 that are infiltrated in the context of liver injury and regulated by IL-6 and TNF-α, but not RORγt (Lai et al., 2015). Thus, digoxin-mediated reduction of serum IL-6 concentration will subsequently lower TH-22-mediated secretion of IL-22.

Like IL-22, serum and hepatic concentration of IL-6 was declined by DN administration alone or with digoxin, particularly at a dose of 0.5 mg/kg. Induction of IL-6 is well known to rise in acute DN-challenge via different signaling cascades. In hepatic immune cells like Kupffer cells, debris of necrotic hepatocytes and HMGB1 stimulates TLR4, leading to induction of IL-6 mediated by MyD88/ NF-κB axis (Naugler et al., 2007; Gaskell et al., 2018). The IL-6 released can induce activation of STAT3 in hepatocytes for proliferation and repair in a DN-acute liver injury model, but this response is negatively regulated by and SOCS3 and RORα (Böhm et al., 2010; Kim et al., 2019). Thus, our data regarding DN-mediated decrease of IL-6 after 24 h of intoxication in mice disagreed with these previous studies.

Hepatocellular toxicity of DN originates from the metabolic conversion by cytochrome P450 into α-hydroxynitrosamine, which is then N-dealkylated into acetaldehyde and ethyl diazohydroxide (Chowdhury et al., 2010). The ethyl diazohydroxide is a highly reactive intermediate that can trigger DNA-alkylation, formation of N7-ethyl-guanine adducts, impairment of base pairing, mutation and tumorigenesis of hepatocytes (Boysen et al., 2009). DN-metabolism also generates the reactive hydroxyl radical (*OH) that leads to oxidative DNA damage and excessive production of the lipid peroxidation aldehydes (MDA + 4-HNE). According to hepatic MDA and 4-HNE concentrations, digoxin pretreatments did not lower DN-induced oxidative stress in the stage of early liver injury. Thus, digoxin mediates its hepatoprotection via interfering with the inflammation cascade, rather than oxidative stress. Noteworthy, the intense rise of hepatocellular proliferation in DN-mice

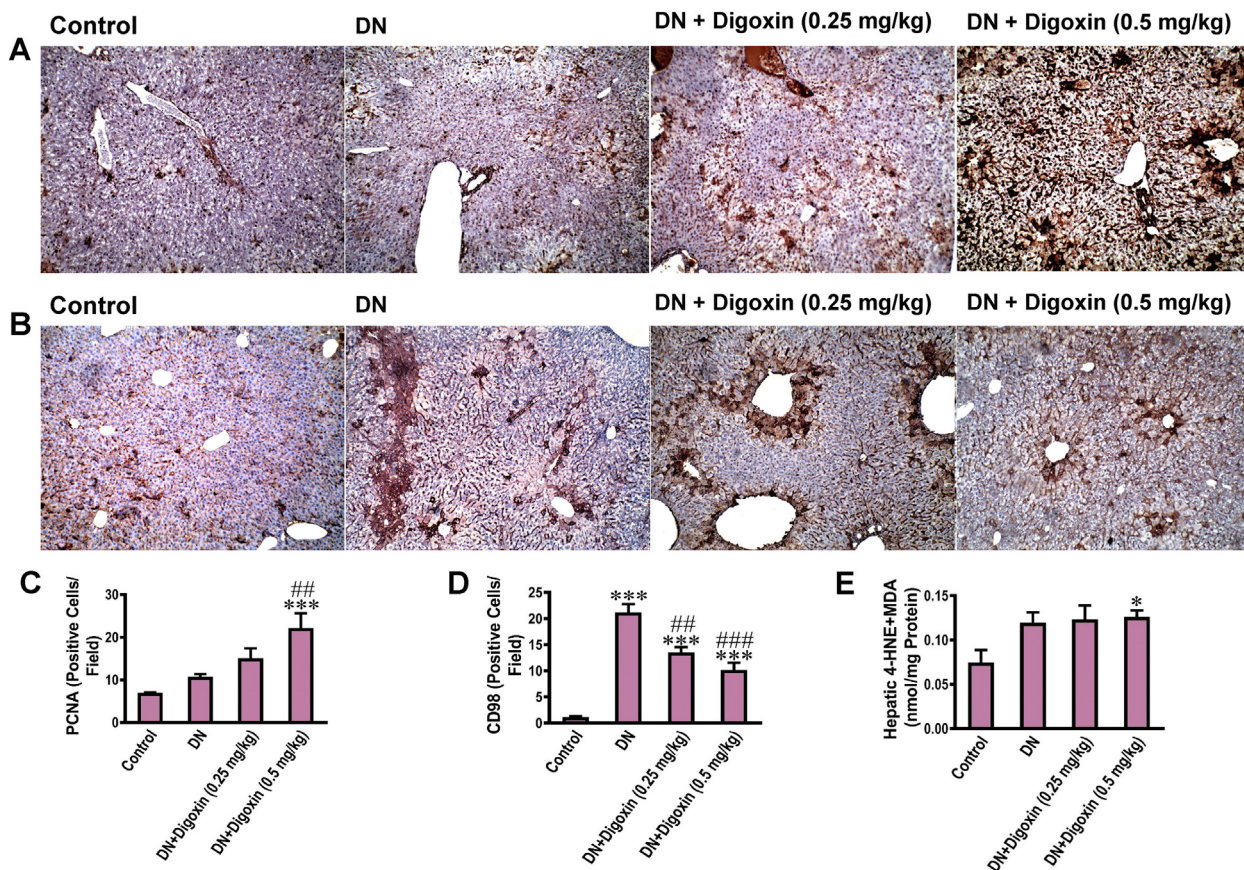


Fig. 5. Impact of digoxin pretreatments (0.25 and 0.5 mg/kg) on diethylnitrosamine (DN)-instigated variations in the hepatic immunohistochemical expression and quantification of proliferating cell nuclear antigen (PCNA; A and C; 100×) and CD98 (B and D; 100×), as well as hepatic lipid peroxidation products (E) in mice (n = 6/group). Levels of statistical differences against the control group were *P < 0.05, **P < 0.01 and ***P < 0.001, while those against the DN group were #P < 0.05, ##P < 0.01 and ###P < 0.001.

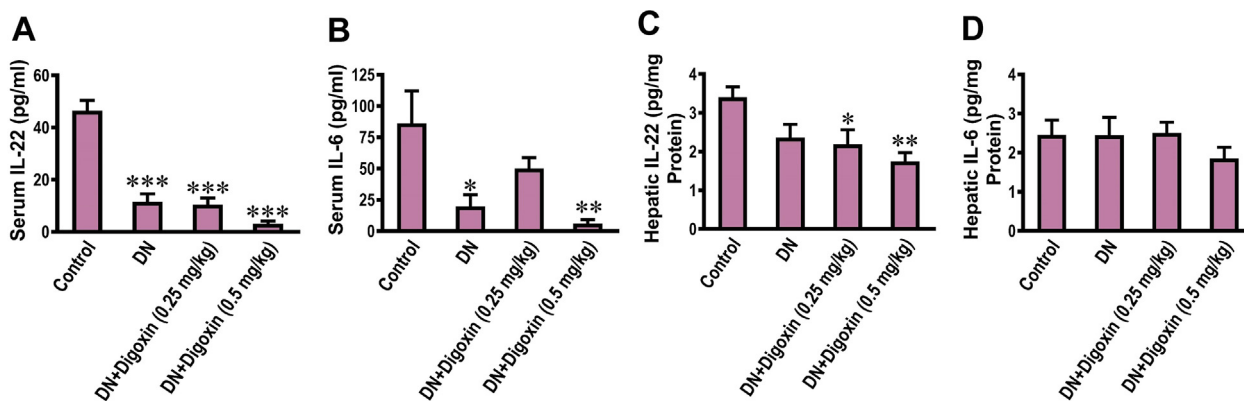


Fig. 6. Impact of digoxin pretreatments (0.25 and 0.5 mg/kg) on diethylnitrosamine (DN)-instigated variations in serum (A, B) and hepatic (C, D) concentrations of interleukin (IL)-22 and IL-6 in mice (n = 6/group). Levels of statistical differences against the control group were *P < 0.05, **P < 0.01 and ***P < 0.001.

pretreated with digoxin (0.5 mg/kg) may be regarded a double edged sword; this can be considered a driver for repair of hepatocytes on the short term or a potent trigger for carcinogenesis on the long term.

In conclusion, digoxin conferred hepatoprotection against acute DN-intoxication via interfering with several inflammation cascades. These include inhibition of the production IL-17A, IL-1β and TNF-α that coincided with attenuation of hepatic NF-κB activation and infiltration of immune inflammatory cells in the liver. Future studies are needed to examine the clinical applicability of

digoxin in the diverse contexts of hepatic inflammatory disorders and elucidate its detailed mechanism as an inflammatory-modulator drug to provide prospective options for therapeutic targeting.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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