Allantoin ameliorated liver fibrosis in a mouse model of non-alcoholic steatohepatitis: role of nuclear factor kappa B/cyclooxygenase 2/prostaglandin E2 pathway

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ABSTRACT

Introduction: Non-alcoholic steatohepatitis (NASH) is considered as current and critical liver disease and liver fibrosis is an initial step to vast NASH injuries. Allantoin is an important and sure composite, which has remark effects on inflammation and apoptosis. This study was done to evaluate the allantoin duty on liver fibrosis and its pathways in mice-induced NASH.

Methods: In the control groups, inbred mice took saline and allantoin. In the NASH group, NASH was provided with a methionine-choline deficient (MCD) diet for eight weeks, and finally, in the NASH-Alla group, allantoin was injected for four weeks in the mice with an MCD diet. For collagen deposition evaluation, trichrome Masson staining and for cellular evaluations, real-time PCR and ELISA assays were performed.

Results: Allantoin treatment improved liver steatosis and fibrosis. Protein expression of nuclear factor kappa B (NFĸB-p65) \( (P<0.05) \) and genes expressions of transforming growth factor-β (TGFβ) \( (P<0.001) \), cyclooxygenase 2 (COX2) \( (P<0.001) \), matrix metalloproteinases 9 (MMP9) \( (P<0.001) \) and alpha-smooth muscle actin (αSMA) \( (P<0.001) \) were also decreased. Moreover, hepatic prostaglandin E2 (PGE2) levels lowered after allantoin treatment \( (P<0.05) \).

Conclusion: Attenuating effects of allantoin on liver fibrosis may be due to the inhibition of NFĸB/TGFβ, NFĸB/MMP9, and NFĸB/Cox2/PGE2 pathways, which decrease αSMA expression and collagen deposition and ameliorate liver fibrosis.

Implication for health policy/practice/research/medical education: Since allantoin has herbal origin and is safe, and has shown antioxidant, anti-steatosis and antiapoptotic effects; after enough and accurate studies, it might be used as a complementary and therapeutic agent in liver diseases, especially liver fibrosis.


Introduction
Non-alcoholic fatty liver disease (NAFLD) is propounded for the extra accumulation of triglycerides in the hepatocytes (steatosis), which may promote to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatic cancer (1). Steatosis, cellular ballooning, and lobar inflammation in the liver provide NASH (2). Today, NAFLD and NASH are increasing and becoming the first liver disease worldwide (3). Increased hepatic fatty acid levels usually increase steatosis neutrophils, resident Kupffer cells, and other innate immune cells activation, which release types of cytokines/chemokines and induce inflammation and then fibrosis (4). Liver fibrosis takes place as a result of the wound-healing response, which arises from hyperactivation of hepatic stellate cells (HSCs) and increase of unwanted deposition of extracellular matrix (ECM) and formation of collagen fibers. Multiple connections between inflammation and fibrosis pathways have also been reported (5,6). Transforming growth factor-β (TGF-β), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and matrix metalloproteinases (MMP) are key factors (7), which cross talk between inflammation and fibrosis. In the NASH disease, hepatocytes and Kupffer cells upregulate NF-κB, as a transcriptional factor, and different types of MMPs lead to liver inflammation and fibrosis (2,7). Specifically, it has been known that MMP9 is upregulated and activates HSCs for the deposition of ECM in cirrhotic patients (8).

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TGF-β, another essential molecule in fibrosis promotion, increases the expression of alpha-smooth muscle actin (αSMA), ECM, and other involved molecules by HSCs activation and causes liver fibrosis (9). Moreover, NF-κB also activates TGF-β and persuades HSCs to hyper-express αSMA and then myofibroblasts to synthesize collagen fibers (10). In another pathway, tissue damage, inflammation, and fibrosis enhance PGE2 production from arachidonic acid (AA) in cyclooxygenase (COX) related pathway. NASH disease, as an inflammatory disease, is associated with increased PGE2 levels (11). Documents show that COX2 and PGE2 levels increase in people with NASH disease (12). It has been reported that NF-κB is able to induce COX2 and increase PGE2 level, too (11,13). PGE2 also enhances TGF-β and αSMA expressions (14). Based on this evidence, various molecular pathways cross link in NASH, providing fibrosis and inflammation.

Allantoin is a compound in many plants, including N. nucifera rhizome, yam, sugar beet, and leguminous (15-17). Previously, the positive effects of allantoin on tissue regeneration and wound healing have been demonstrated (17,18). Newly, the effects of allantoin on lipid and glucose lowering have been distinguished (19). It has been reported that allantoin incremented the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), sirtuin 1 (SIRT1), and nuclear factor erythroid 2-related factor 1 (Nrf-1) in the skeletal muscle tissue (17). In a previous study, we showed that allantoin ameliorated NASH by downregulation of steatosis-dependent genes and attenuation of apoptosis pathways in MCD diet-induced animals (20). Allantoin also improves oxidative stress and inflammation (20,21). We recently also reported that the antioxidant effects of allantoin are mediated through SIRT1/Nrf2 pathway in mice with NASH (22). In this study, our aim is to evaluate the effects of allantoin on the attenuation of liver fibrosis and its possible pathways in a mouse model of NASH.

Material and Methods

Materials
Allantoin was purchased from Sigma-Aldrich (Purity: 98%, Germany) and the prostaglandin E2 kit was purchased from ZellBio GmbH, Germany (ZB-10504C-R9648).

Experimental procedures
C57/BL6 male mice (25-27 g) were taken at a temperature of 22-25°C and 12:12 light/dark cycle with free access to food and water. The protocols of this experiment were planned in accordance with the Guidelines for Animal Care and Use at the Qom University of Medical Sciences (IR.MUQ.REC.1400.055). Animals were randomly distributed into four equal groups (n=6).
1– Control group: free access to standard diet for eight weeks with a daily injection of saline (ip) from 4th week during four weeks.
2– Allantoin group: free access to standard diet for eight weeks with a daily injection of allantoin (5 mg/kg, ip) from the fourth week for four weeks (20).
3– NASH group: free access to methionine-choline deficient (MCD) diet for eight weeks to cause NASH (24).
4– NASH-Alla group: free access to MCD diet for eight weeks to induce NASH and injection of allantoin (5 mg/kg, ip) from the fourth week during four weeks daily.

At the end experiment, the animals were anesthetized with sodium pentobarbital (25) and the abdomen was exposed. The liver was immediately taken out and washed with saline.

Histological study
One lobe of the liver was cut and taken in 10% formalin solution. The paraffin-embedded sections from liver tissue were then obtained, and trichrome-Masson staining was performed to find collagen fibers.

Western Blot
NF-κB-p65 (the active form of NF-κB) expression in the liver tissue was determined by Western immunoblotting. Briefly, the protein concentration was determined by the Bradford assay kit (Sigma Aldrich, USA). The proteins were transferred to PVDF membranes and explored with primary antibodies versus NF-κB-p65 (ab16502) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab181602). The HRP-conjugated secondary antibody (1:7000, Cell Signaling) was attached to the membranes. The membranes were enhanced with chemiluminescence (ECL, Amersham) reagents in a dark room and exposed to an X-ray film and visualization of the chemiluminescence of the binding by means of a visualizing machine. The diameter of the bands was measured by ImageJ software (II 1.46r version, NIH, USA) and normalized by the GAPDH as a housekeeping protein.

Real-time RT-PCR
Using the RNX-plus kit from frozen tissue samples, total RNA was extracted (Cinnagen, Tehran, Iran), and the quantity and purity of the RNA samples were measured by a Nanodrop spectrophotometer (Thermo Scientific, USA). By the cDNA synthesis kit, complementary DNAs (cDNA) were produced (Yektatajhiz, Iran) and for real-time assay, 2× SYBR Green master mix (Biofact, Korea: 1670-5695), template, forward and reverse specific primers, and DEPC of treated water were mixed and incubated. GAPDH was used as an internal control. The analysis of data was performed by the comparative 2-ΔΔCT method. Primers for the genes studied were designed by primer3 software (v. 0.4.0) (http://primer3.ut.ee). Primer sequence similarity and specificity were established with BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as follows:
α-SMA: (F) CCCAGACATCAGGGAGTAATGG (R) TCTATCGGATACTTCAGCGTCA
TGF-β: (F) CTTCAATACGTCAGACATTCGGG (R) GTAACGCCAGGAATTGTTGCTA
COX2: (F) CTGGTCTGATGATGTATGCC (R) TCTATGAGTATGAGTCTGCTG
MMP9: (F) CACTTTCCCTTCACCTCC (R) TTAGCCTGCTTATCGTAG
GAPDH: (F) TGGCCTTCCGTGTTCCTAC (R) GAGTTGCTGTGGAAGTCGCA

PGE2 measurement
A part of the hepatic texture was weighted, mixed with phosphate-buffered saline (PBS) buffer (100 mM, PH 7.4, 1 ml/100 mg tissue with anti-protease mixture), homogenized by an electrical homogenizer (IKA-3420000, Germany), and centrifuged (4000-6000 rpm for 10 minutes). The prepared supernatants were then inquired for PGE2 levels through an ELISA kit, according to the manufacturer's instructions, and read by an ELISA reader at 450 nm.

Statistical analysis
Data were reported as mean ± standard error of the mean (SEM). The Kolmogorov–Smirnov test determined normality of the data. Comparison between groups was made by one-way analysis of variances (ANOVA) and Tukey's post hoc test using SPSS. \( P < 0.05 \) was considered statistically significant.

Results
Allantoin attenuated liver injuries in the mice with NASH
Our findings from trichrome-Masson staining showed much lipid-droplet accumulation (empty spaces), inflammation, and collagen fibers deposition in the surround of the central and portal veins in MCD induced mice, whereas, steatosis, inflammation, and fibrosis were improved in the NASH-Alla group (Figure 1).

Allantoin decreased liver NFκB-p65 expression in the mice with NASH
Western blot results revealed a marked increase in protein expression of NFκB-p65 in the NASH group compared with the control group (\( P < 0.01 \)). However, allantoin administration significantly reduced the expression of NFκB-p65 (\( P < 0.05 \)) (Figure 2).

Allantoin decreased TGF-β and α-SMA expressions in the mice with NASH
Our data in Figure 3 showed that the gene expressions of TGF-β and α-SMA significantly upregulated in the NASH group compared with the control group (\( P < 0.001 \)), meanwhile allantoin markedly decreased them in the NASH-Alla group compared with the NASH group (\( P < 0.001 \)).

Allantoin decreased MMP9 expression in the mice with NASH
The obtained results displayed a marked increment in MMP9 gene expression in the NASH group compared with the control group (\( P < 0.001 \)), and allantoin could significantly decrease MMP9 expression in the NASH-Alla group compared with the NASH group (\( P < 0.001 \)) (Figure 4).

Allantoin decreased COX2 expression and PGE2 level in the mice with NASH
As presented in Figure 5, hepatic COX2 expression and PGE2 level were significantly increased in the NASH group.
compared with the control group (P<0.01 and P<0.05, respectively), although in the NASH-Alla group, allantoin markedly lowered COX2 and PGE2 level compared with NASH group (P<0.001 and P<0.05, respectively).

Discussion
Allantoin could downregulate NF-κB, TGF-β, COX2, MMP9, and αSMA expressions, decrease PGE2 levels, and finally improve liver fibrosis. This is the first report in which the effects of allantoin have been evaluated on the NASH-induced liver fibrosis by cross talking between inflammation and fibrosis pathways.

Allantoin is a synthetic compound but is plenty found in yam, N. nucifera rhizome, sugar beet, leguminous and other herbs (15). Allantoin has been used as a cosmetic and wound healing agent but now its other effects are being revealed (18,25). Recent studies have shown the metabolic, anti-inflammatory, and anti-oxidative effects of allantoin in animal models (21,26,27). We previously reported that allantoin attenuated steatosis and liver injury in the animals with NASH, through the improvement of lipids metabolism and decrement of inflammation, oxidative stress, and apoptosis (20,22).

Liver fibrosis results from chronic and vast liver damage that activate HSCs and enforce to hypersecretion of ECM and formation of fibrosis tissues (29). In the prolonged NASH disease, steatotic and inflammatory processes lead to HSC cell activation and differentiation into myofibroblasts and collagen fibers formation (1,29,30).

In this experiment, allantoin administration could decrease NFκB-p65 (activated form of NF-κB) protein expression. It also declined TGFβ, α-SMA, and MMP9 expressions. NF-κB and TGFβ are two critical nuclear factors in fibrosis, and the activation of NF-κB launches the cascade of inflammatory and fibrotic processes. Activation of TGFβ as a growth factor also promotes liver fibrosis through increasing the expression of α-SMA (4,8,25). In
this context, glycine-based compounds can decrease ALT and AST serum levels, TGFβ1 and NF-κB expressions, and NASH attenuation in mice. El-Sweify and Hassanien have also reported that in cholestatic liver fibrosis, NF-κB and TGFβ levels increased, while montelukast administration, a cysteinyl leukotrienes antagonist, decreased both and improved fibrosis (31). Moreover, MMP9 stimulates and persuades HSCs to overproduce ECM and collagen deposition, too (32). According to available documents, there are multiple interactions among MMP9, TGFβ, and NF-κB, and all of them are upregulated in the NASH disease and able to activate each other (8,31,33). In a study, treatment with coenzyme Q10 attenuated inflammation and fibrosis in patients with pelvic tumors via inhibiting the NF-κB/TGF-β1/MMP-9 pathway and α-SMA expression (8). Inhibition of NF-κB activity also led to the decrease of collagen IV, MMP-9, tissue inhibitors of matrix metalloproteinases expressions, and pulmonary fibrosis improvement (34). On the other hand, allantoin has positive effects on the decrease of inflammation and fibrosis. The alleviating effects of a mixture composed of cepae extract, allantoin, and heparin on epidermal fibrosis have previously been reported at the rat laminecstomy (35). Hydrocolloid film of allantoin also improved diabetic wound healing through the decrease of NF-κB and TGFβ expressions (17). In another report, it has been stated that the application of a patch containing allantoin improves hypertrophic and fibrosis skin scars (36). Our findings are consistent with the previous reports, and probably allantoin, through the suppression of NF-κB, has led to the decrease of TGF-β1 and MMP9 expressions, attenuation of HSCs activity, and then downregulation of α-SMA. Following that, collagen production and deposition have reduced, and liver fibrosis improved.

Chronic inflammation is an important promoter to provide liver fibrosis. One of these inflammatory pathways is COX2/PGE2. COX2 is a key enzyme in transforming arachidonic acid into prostaglandin E2, which is upregulated in the NASH disease (14). PGE2 concentration is increased in NASH in both the liver and the blood levels (11,36,37). NF-κB induces COX2, and PGE2 upregulates NF-κB expression in a vicious cycle (38). PGE2 also can increase TGFβ and α-SMA expressions, activate HSCs to deposit collagen and play a notable role in liver fibrosis (32,39). Our findings showed that allantoin reduced COX2 expression and lowered hepatic PGE2 concentration. Etoricoxib has an antitumorigenic effect via the modulation of NF-κB/COX-2/PGE2 signaling in Hepatocellular carcinoma cells (37). Green tea extract also attenuated NASH-induced liver injury by reducing lipid peroxidation, COX2, and PGE2 expressions (11). An experimental gel containing allantoin could significantly reduce pain, trismus, and inflammation signs after surgery (40). Allantoin also lowered ovalbumin-induced lung inflammation via the inhibition of T-helper-2-type (Th2), interleukin-4 (IL-4), and IL-5 (41). Likewise, we reported that allantoin declined tumor necrosis factor-alpha and IL-6 expressions and ameliorated lobar inflammation in mice with NASH (20). In the light of this evidence, it seems that allantoin could also alleviate hepatic fibrosis through downregulation of NF-κB expression and thereby decrease of COX2 and PGE2 levels and then reduction of TGF and α-SMA expressions. This is another novel pathway, which could support attenuating the effects of allantoin on the NASH-induced liver fibrosis.

The high cost of kits, antibodies, and chemicals prevented more data from being obtained. However, we evaluated some signaling pathways associated with liver fibrosis. This study was the continuation of our previous research, which confirmed the other positive effects of allantoin on NASH disease in mice.

**Conclusion**

This study demonstrated that allantoin had ameliorative effects on NASH-induced liver fibrosis, which resulted from the suppression of NF-κB/TGF-β/α-SMA, NFκB/COX-2/PGE2/α-SMA, and NF-κB/MMP9/α-SMA pathways and thereupon the decrement of collagen deposit in the liver. These findings confirm the notable effects of allantoin in the treatment of NASH injuries.

**Authors’ contributions**

TKM performed the work and AM designed the study, analyzed the data, and wrote the manuscript. All authors read the final version and approved the manuscript.

**Conflict of interests**

The authors declare that they have no conflict of interest.

**Ethical considerations**

The animal care and experimental procedure were approved in accordance with the Guidelines for Animal Care and Use at the Qom University of Medical Sciences (IR.MUQ.REC.1400.055).

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**References**


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