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Original article

Protection against nonalcoholic steatohepatitis through targeting IL-18 and IL-1alpha by luteolin

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ABSTRACT

Background: The management of nonalcoholic steatohepatitis (NASH) is still a crosstalk so the current study was designed to evaluate the effect of different luteolin doses on an experimental model of NASH and to elucidate novel anti-inflammatory pathways underlying its effect.
Methods: Adult male Wistar rats (200–220 g; n = 60) were used. Rats were fed a high carbohydrate/high fat diet (~30% carbohydrate and 42% fat) daily for 12 weeks to induce NASH. Luteolin (10, 25, 50 or 100 mg/kg/day) was administered as a suspension (10% w/v in 0.9% NaCl) using an oral gavage. Histopathological changes (necrosis, inflammation and steatosis) were evaluated. Biomarkers for liver function, lipid peroxidation, extracellular matrix deposition and anti-oxidant activity were measured. Levels of IFN-γ, TNF-α and IL-1α and IL-18 were measured.
Results: Obtained results showed ability of luteolin to reduce activity of ALT and AST and to decrease levels of bilirubin, hyaluronic acid and malondialdehyde significantly ($p < 0.05$). Also, luteolin showed an anti-oxidant activity as indicated by the significant ($p < 0.05$) increase in reduced glutathione. Finally, a significant ($p < 0.05$) decrease in IFN-γ, TNF-α, IL-1α and IL-18 levels was observed most notably in groups that received high doses of luteolin (50 and 100 mg/kg).
Conclusions: Luteolin can protect against non-alcoholic steatohepatitis through targeting the pro-inflammatory IL-1 and Il-18 pathways in addition to an antioxidant effect.

Introduction

Non-alcoholic steatohepatitis (NASH) is becoming increasingly problematic from both the health and economic point of view. Although several drugs with different targets have shown efficacy against NASH in many trials, a therapy to prevent or delay its occurrence has not been approved yet. This limitation in treatment may be owed to unidentified risk factors and underlying cellular and molecular mechanisms in NASH [1]. Steatohepatitis is an inflammatory response with hepatocyte injury that results from accumulation of triglycerides in the hepatocytes [2–4]. Modulation of the underlying inflammatory signalling pathways may represent potential novel therapeutic strategies for the treatment of NASH [5]. Natural flavones with anti-inflammatory anti-oxidant properties represent a bounce hope that may improve disease outcomes in NASH [6].

The dietary flavone, Luteolin, has anti-inflammatory, anticancer, anti-estrogenic, pro-apoptotic and anti-angiogenic effects [7–11]. Several studies investigated luteolin effect in different liver injuries. Zhang et al. [12] demonstrated luteolin ability to attenuate mercuric chloride induced chronic liver injury through regulation of the Nrf2/NF-κB/P53 signalling pathway. It has been proved that luteolin is effective in protecting mice from hepatotoxicity induced by carbon tetrachloride *in vivo* [13]. Furthermore, it has an anti-fibrotic effect as it inhibits stellate cells functions *in vitro* by inhibiting AKT/mTOR/p70S6K and TGFβ/Smad signalling pathways [14].

Since luteolin showed marked improvement in liver injuries including palmitic acid-induced hepatic steatosis [15], the current study was designed to investigate its effect on high carbohydrate high fat diet induced steatohepatitis in rats. In addition, possible underlying mechanisms relevant to inflammatory pathways were explored.

Materials and methods

Experimental design

After 2 weeks of acclimation, rats (adult male Wistar; 200–220 g; n = 60; VACSERA, Egypt) were randomly divided into six groups and received different treatments as illustrated in (Table 1).

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Table 1
Experimental Design.

Group	High carbohydrate/high fat diet*	Luteolin**	Saline***
1#	–	–	+
2	+	–	+
3	+	(10 mg/kg)	–
4	+	(25 mg/kg)	–
5	+	(50 mg/kg)	–
6	+	(100 mg/kg)	–

Rats received standard normal chow; components (g/kg diet): protein 208, carbohydrate 412, fat 236 and others 144 (vitamins, minerals, fibers, and water).
* Daily for 12 weeks; diet components (g/kg diet): beef tallow 300, molasses 200 and standard rat chow 500.
** Daily by oral gavage, suspended in 3 mL saline (0.9%), purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ, USA).
*** Daily by oral gavage, 3 mL, (0.9% w/v).

At the end of the study, rats were anesthetized with thiopental sodium (50 mg/kg, intraperitoneal injection). Blood samples were collected through a cardiac puncture, allowed to clot then centrifuged at 3000 rpm for 15 min to separate serum. Liver was isolated, cut into sections and fixed in 8% (w/w) neutral buffered formalin solution in order to be processed for histopathological examination. Liver tissue homogenate (10% w/v) was prepared manually using an ice cooled glass homogenizer in a phosphate buffer saline (pH 7.4–7.5). The obtained homogenate was divided into aliquots to avoid freeze-thaw cycles and stored at -80°C.

All procedures performed in studies involving animals were in accordance with the ethical standards of the Scientific Research Ethics Committee in Faculty of Pharmacy, Mansoura University (approval code:2018-102).

Measurement of liver function biomarkers

Serum level of total bilirubin and activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as liver function biomarkers were measured using ready to use colorimetric kits (Spectrum Diagnostics, Cairo, Egypt) as directed by the manufacturer. Briefly, ALT or AST in samples transferred 2-oxoglutarate in presence of alanine or L-aspartate respectively into a colored hydrazone after addition of

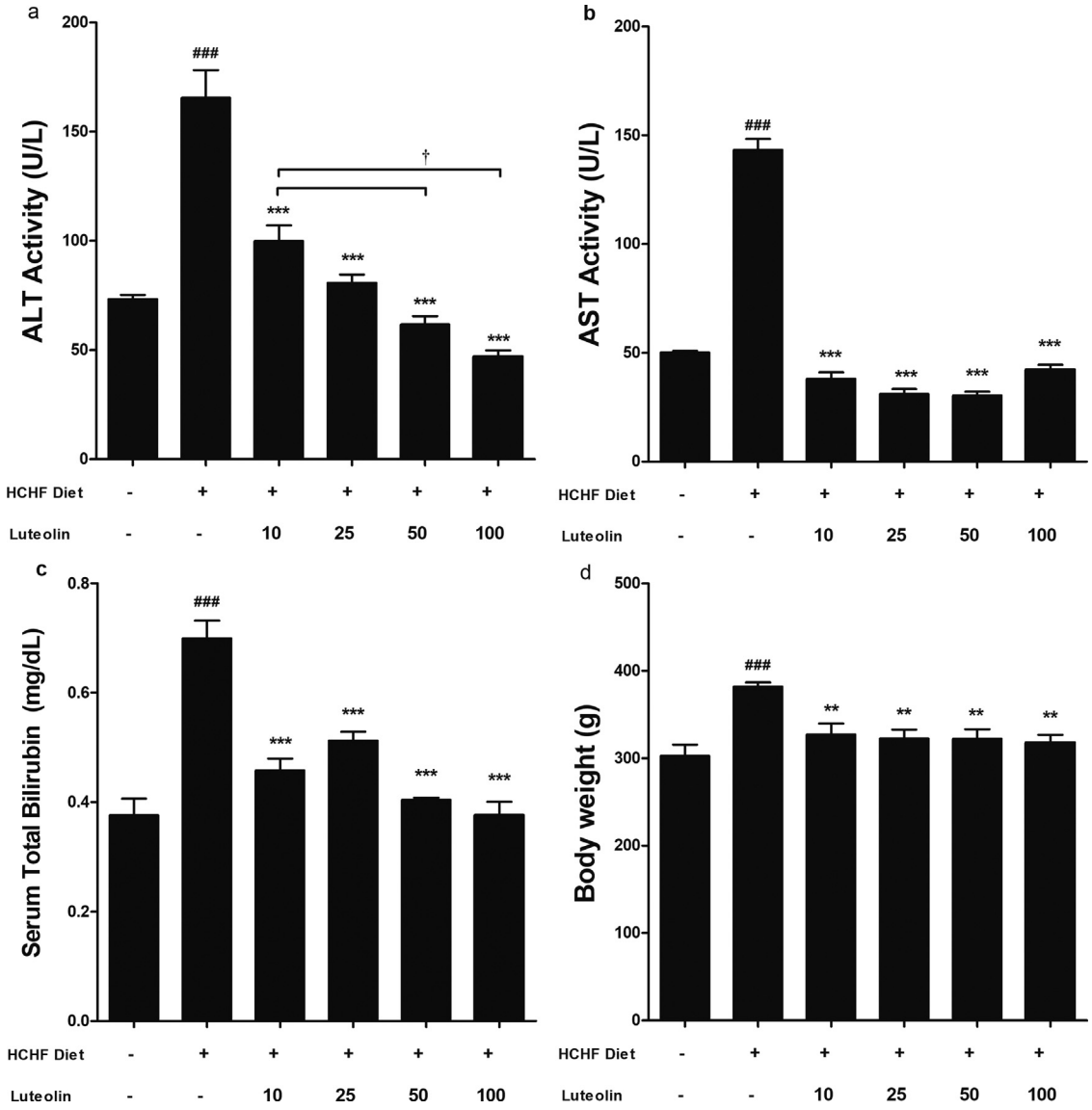

Fig. 1. Effects of luteolin (10, 25, 50, 100 mg/kg) on liver function biomarkers a) ALT b) AST c) total bilirubin and d) animals' body weight in non-alcoholic steatohepatitis induced by high carbohydrate/high fat (HCHF) diet. ### $p < 0.001$ compared with standard chow received group, **, *** $p < 0.01, 0.001$ compared with HCHF diet received group, † $p < 0.05$.

Table 2
Effect of luteolin on necro-inflammation score and steatosis percentage in high carbohydrate/high fat diet induced non-alcoholic steatohepatitis.

Group	Necro-inflammation score	Steatohepatitis (%)
High carbohydrate/high fat diet	2.6 ± 0.16	61.2 ± 1.39
Diet + Luteolin (10 mg/kg)	2.4 ± 0.16	53.9 ± 1.99
Diet + Luteolin (25 mg/kg)	1.8 ± 0.13	36.7 ± 2.50*
Diet + Luteolin (50 mg/kg)	1.3 ± 0.15***	27.8 ± 1.10***
Diet + Luteolin (100 mg/kg)	0.8 ± 0.13***	08.3 ± 1.59***

*,*** $p <$ 0.05, 0.001 (respectively) compared with high carbohydrate/high fat diet group.

2,4-dinitrophenylhydrazine. The enzymes activity was detected by monitoring the concentration of the formed hydrazone in proportion to the color intensity measured at 546 nm.

Total bilirubin level was measured using Jendrassik Grof method. Briefly, the concentration was determined in presence of caffeine by the reaction of bilirubin in samples with diazotized sulphanilic acid which produced a diazo dye with color intensity (measured at 580 nm) proportional to the concentration of total bilirubin.

Measurement of oxidative stress and lipid peroxidation

Levels of malondialdehyde (MDA) and reduced glutathione (GSH) were measured within one week of sample collection in liver homogenate spectrophotometrically (Bio-Diagnostic, Cairo, Egypt) as directed by the manufacturer. For MDA, thiobarbituric acid was added to samples in acidic medium (at 95 °C for 30 min) then the absorbance of the reactive colored product was measured at 534 nm. Concentration of GSH was directly proportional to the chromogen color intensity produced after addition of 5,5`dithiobis (2-nitrobenzoic acid) to the samples. Absorbance was measured at 405 nm.

Evaluation of necroinflammation and steatosis

Necrosis and inflammation were scored using the Histological Activity Index modified by [16] and calculated in hematoxylin-eosin stained sections (5 μm) as described by [17] as the sum of: 0–4 portal inflammation, 0–4 periportal or periseptal interface hepatitis, 0–6 confluent necrosis, 0–4 focal necrosis, apoptosis and focal inflammation. Hepatic steatosis was graded as the percentage of affected hepatocytes: grade 0 (0%), grade 1 (<30%), grade 2 (30–60%) and grade 3 (>60%) [18].

Measurement of inflammatory cytokines

Tissue levels of the pro-inflammatory cytokines, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Platinum ELISA, eBioscience, San Diego, CA, USA), were measured in liver homogenate (10% w/v). Levels of the IL-1 family members, IL-1α (eBioscience, San Diego, CA, USA) and IL-18 (Sandwich ELISA, BosterBio Technology Co., Ltd., Fremont, CA, USA), were measured as well. Additionally, the extracellular matrix component hyaluronic acid (HA) was measured (BlueGene Biotech CO., Shanghai, China). All measurements were carried out as directed by the manufacturer's instructions at 460 nm.

Statistical analysis

Histopathological scoring was analyzed using the non-parametric Kruskal-Wallis test by rank followed by Dunn's *post hoc* tests. Other results were analyzed using one way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test as a *post hoc* test. GraphPad Prism program V5.01 (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistics and design graphs using mean ± SE.

Results

The present study was conducted to elucidate the hepatoprotective effect of luteolin in an experimental model of NASH. Wistar rats were fed high carbohydrate-high fat diet for 12 weeks to induce steatohepatitis. Luteolin (10, 25, 50 and 100 mg/kg) was administered by oral gavage concurrently with diet. Stained liver sections were examined for histopathological

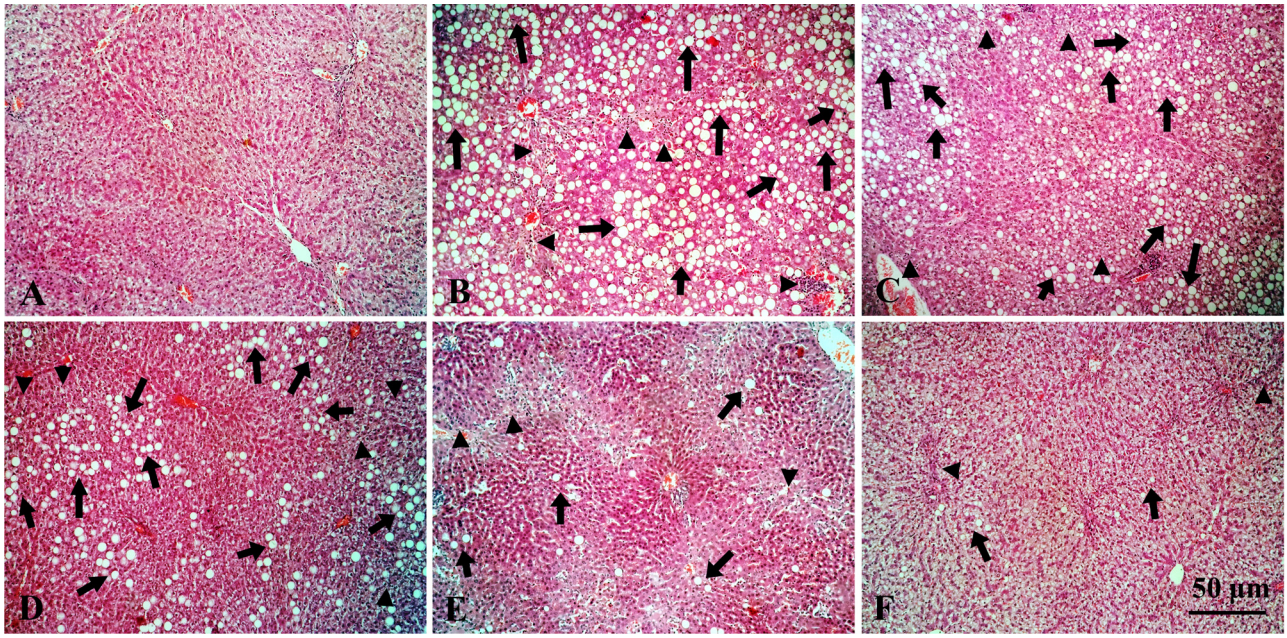

Fig. 2. Representative photographs for hematoxylin-eosin stained liver sections isolated from A) standard show B) high carbohydrate/high fat diet C) luteolin 10 mg/kg D) 25 mg/kg E) 50 mg/kg F) 100 mg/kg received groups. Arrow heads indicate mixed inflammatory cells infiltration (macrophages and lymphocytes) while arrows indicate steatosis.

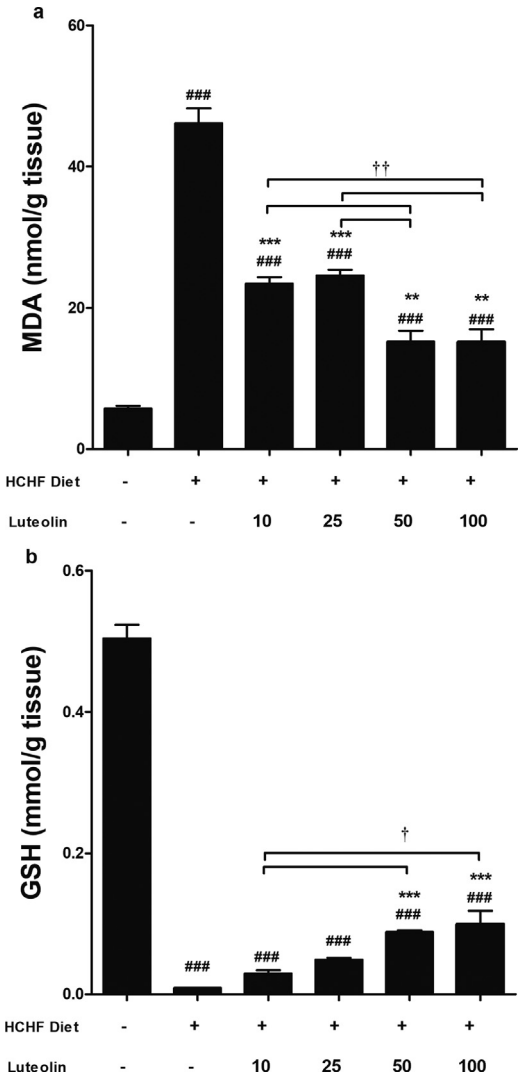

Fig. 3. Effects of luteolin (10, 25, 50, 100 mg/kg) on hepatic tissue levels of a) malondialdehyde (MDA) b) reduced glutathione (GSH) in non-alcoholic steatohepatitis induced by high carbohydrate/high fat (HCHF) diet. ### $p < 0.001$ compared with standard chow received group, **, *** $p < 0.01$, 0.001 respectively compared with HCHF diet received group, †, †† $p < 0.05$, 0.01 respectively.

changes as necro-inflammation and steatosis. Biological markers for liver function (ALT, AST, total bilirubin) were assessed in serum. Hyaluronic acid as a biomarker for NASH and the lipid peroxidation biomarker MDA were also measured. To identify possible mechanisms underlying luteolin effect, IFN-γ, TNF-α, IL-1α, IL-18 and the anti-oxidant biomarker (GSH) were measured in liver homogenate.

Activity of ALT, AST, and total bilirubin were significantly ($p < 0.001$) elevated by diet compared with negative control group. Luteolin significantly ($p < 0.01$) decreased these biomarkers in a dose dependent manner (Fig. 1) when administered concurrently with diet. A significant increase in rats' body weight was observed in the group that received the HCHF diet ($p < 0.001$). All groups receiving luteolin showed a significant ($p < 0.01$) decrease in body weights compared to diet receiving group (Fig. 1d).

In line with the results of liver function biomarkers, the examined liver sections stained with hematoxylin-eosin showed a significant ($p < 0.05$) increase in necro-inflammation scores after feeding rats with the diet (Table 2). High doses of luteolin (50 and 100 mg/kg) significantly decreased inflammation compared to the group fed diet alone ($p < 0.05$) (Fig. 2). In addition, percentage of steatosis elevated by diet was significantly ($p < 0.01$) reduced by luteolin (50 and 100 mg/kg).

Malondialdehyde level was significantly increased by diet ($p < 0.001$) indicating lipid peroxidation (Fig. 3a). Groups that received luteolin showed a decrease in MDA level ($p < 0.001$) with higher decrease in luteolin 50 and 100 mg/kg received groups compared to those received 10 and 25 mg/kg ($p < 0.01$).

The antioxidant GSH level was significantly ($p < 0.001$) elevated by 50 and 100 mg/kg luteolin administration when compared to its level in groups that received diet alone or with low luteolin doses (Fig. 3b). However, the level of GSH did not reach to its normal level of negative control group in all groups that received luteolin.

Furthermore, HA was significantly ($p < 0.001$) elevated by diet compared to negative control. Tested doses of luteolin significantly decreased HA ($p < 0.001$) (Fig. 4a). The pro-inflammatory cytokine, TNF-α, was significantly reduced by luteolin (Fig. 4b) especially in the dose of 50 mg/kg ($p < 0.001$). Level of IFN-γ was reduced in all groups that received luteolin to a level comparable to its level in control group (Fig. 4c).

Liver tissue level of IL-1α was significantly ($p < 0.001$) increased by high carbohydrate high fat diet. All tested doses of luteolin decreased IL-1α level significantly ($p < 0.001$) but its level in luteolin (10 and 25 mg/kg) received groups remained significantly higher than in negative control group (Fig. 5a).

Finally, feeding rats with tested diet increased ($p < 0.001$) IL-18 level in liver homogenate (Fig. 5b). Luteolin reduced the cytokine level significantly ($p < 0.001$). Luteolin 50 mg/kg decreased IL-18 level significantly ($p < 0.001$) when compared to 10 and 25 mg/kg.

Discussion

A precise understanding of the mechanisms underlying the pathogenesis of NASH is still relatively distant. Hepatocellular injury, immune cell mediated inflammation and progressive liver fibrosis represent the main characteristics of NASH [19].

In our study, high carbohydrate high fat diet was used to induce NASH in rats. Diet intake for 12 weeks resulted in marked steatosis and infiltration of inflammatory cells in examined liver sections. Liver function biomarkers and HA were elevated as previously demonstrated by Panchal et al [20]. Hyaluronic acid has been investigated as a non-invasive marker for fibrosis in patients with NASH. Its level was found to be higher in patients with NASH even without fibrosis compared with healthy individuals and also strongly correlates to fibrosis grades [21,22]. Luteolin administration attenuated structural and functional changes induced by HCHF diet in liver. It reduced steatosis and inflammation and restored normal activities of ALT, AST and total bilirubin level. Similar hepatoprotective effect of luteolin was previously studied in an acetaminophen induced liver failure [23]. The effect was due to inhibition of i-NOS, NF-κB hepatic expression and an anti-endoplasmic reticulum stress property. The decrease in HA acid level by luteolin may halt progression of NASH and fibrosis development.

Oxidative stress has been recognized as an important mechanism in the pathogenesis of NASH [24]. It is considered a key step for the second hit leading to hepatocellular injury and disease progression [25,26].

Our results showed a rise in MDA and a reduction in the endogenous anti-oxidant biomarker GSH after feeding rats the high carbohydrate high fat diet which subsequently would lead to oxidative burden. Administration of luteolin significantly improved the balance in the favor of anti-oxidant mechanism as indicated by the increase in GSH and the decrease in MDA tissue levels (most notably with 50 and 100 mg/kg luteolin). The anti-oxidant effect of luteolin may account for its hepatoprotective effect and its beneficial effects on injuries of other organs as lung,

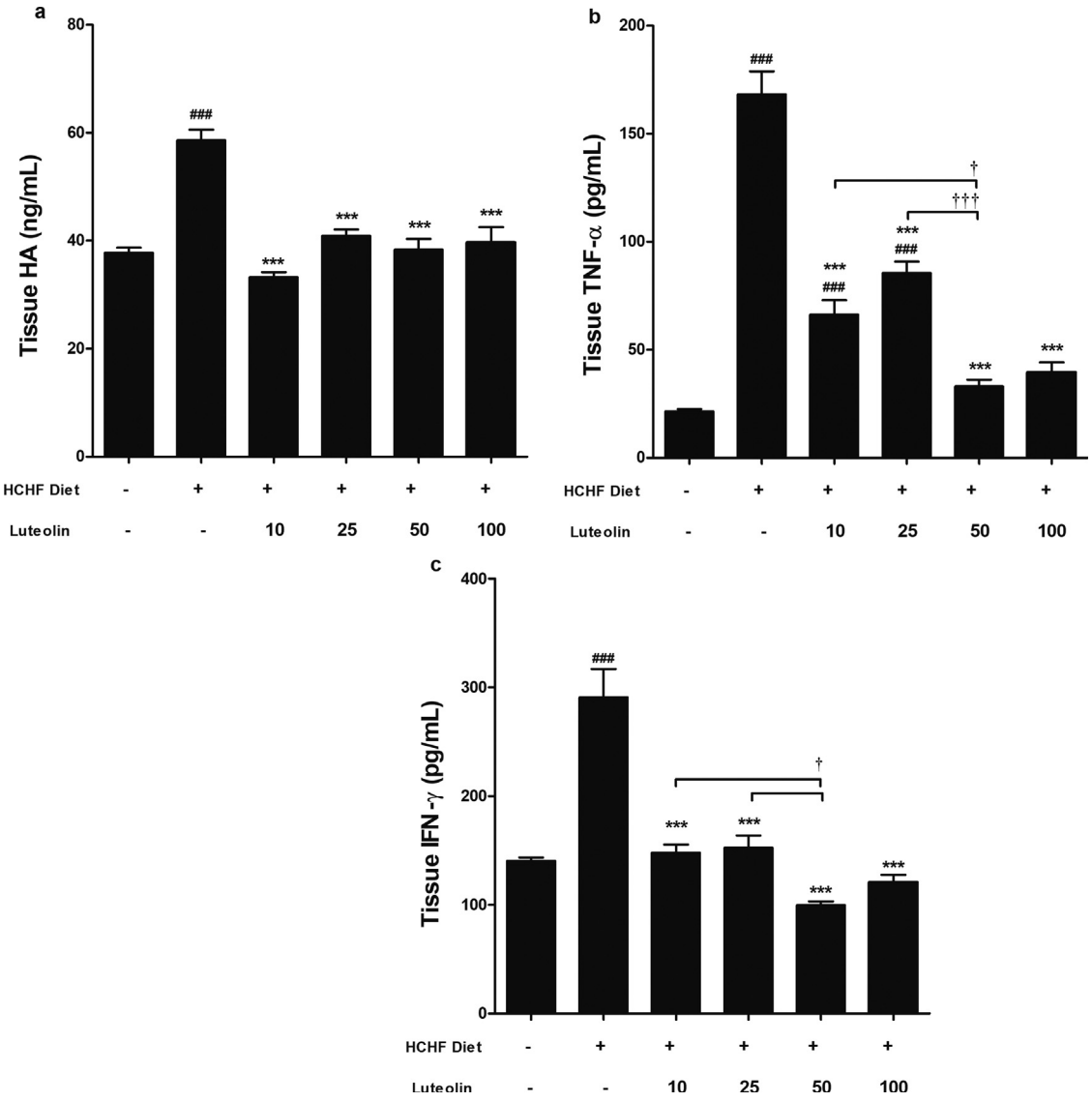

Fig. 4. Effects of luteolin (10, 25, 50, 100 mg/kg) on hepatic tissue levels of a) hyaluronic acid (HA), b) tumor necrosis factor (TNF)-α, c) interferon (IFN)-γ in non-alcoholic steatohepatitis induced by high carbohydrate/high fat (HCHF) diet. ### $p < 0.001$ compared with standard chow received group, *** $p < 0.001$ compared with HCHF diet received group, †, ††† $p < 0.05, 0.001$.

kidney and heart as well [27–29]. Interestingly, groups received small doses of luteolin, 10 mg/kg and 25 mg/kg did not show a clear anti-oxidant effect as indicated by their effects on MDA and GSH tissue levels if compared to the effects of larger doses. This difference in effect between doses on oxidative stress biomarkers was also obtained by Domitrović et al [13]. Luteolin is suggested to possess both anti-oxidant and pro-oxidant properties (free radical generation) thus shows versatile effects as anti-apoptotic in normal cells and pro-apoptotic in cancer cells. The outcome of luteolin-induced effects on cellular redox status may relay on the condition and microenvironment of the cell [30]. We also suggest that the balance between the pro-oxidant and anti-oxidant effects of luteolin may be a matter of dose i.e in larger dose the anti-oxidant effect become more prominent.

Many studies elucidated that inflammatory cytokines, chemokines, and other inflammatory mediators are key regulators in the pathogenesis of NASH [31,32]. From these mediators the cytokines of interlukin-1 family that are considered inducers for inflammation and tissue injury in many body tissues as liver and lung [33] in addition they have a pivotal role in diabetes [34]. Our results revealed a significant increase in IL-1α and IL-18 levels in liver tissue in the group that received HCHF diet for 12 weeks indicating a possible central role of these two members in the liver immune-response to such type of injury.

Normal hepatocytes have a precursor for IL-1α which has a major role in various liver diseases/injuries [35,36]. Activation of this precursor by injury initiates an inflammatory cytokine cascade including TNF-α and IFN-γ [37]. A reduction in TNF-α level was observed in groups treated with luteolin indicating an anti-inflammatory effect. An important study made by Kamari et al [38] reported that mice deficient in IL-1α and IL-1β were protected from inflammation after diet-induced steatosis revealing their role in the immune-response of the liver towards HCHF induced injury. Our study may add an additional support to the role of IL-1α in sterile liver inflammation and suggests that luteolin can protect against steatohepatitis through interfering with IL-1α mediated inflammation.

IL-18 is a unique pleotropic pro-inflammatory cytokine involved in liver immune-response to high carbohydrate/high fat diet induced injury, inflammation and liver cell regeneration [39,40]. Its release increases by increasing the stored fats in experimental animals and humans [41]. It has been demonstrated

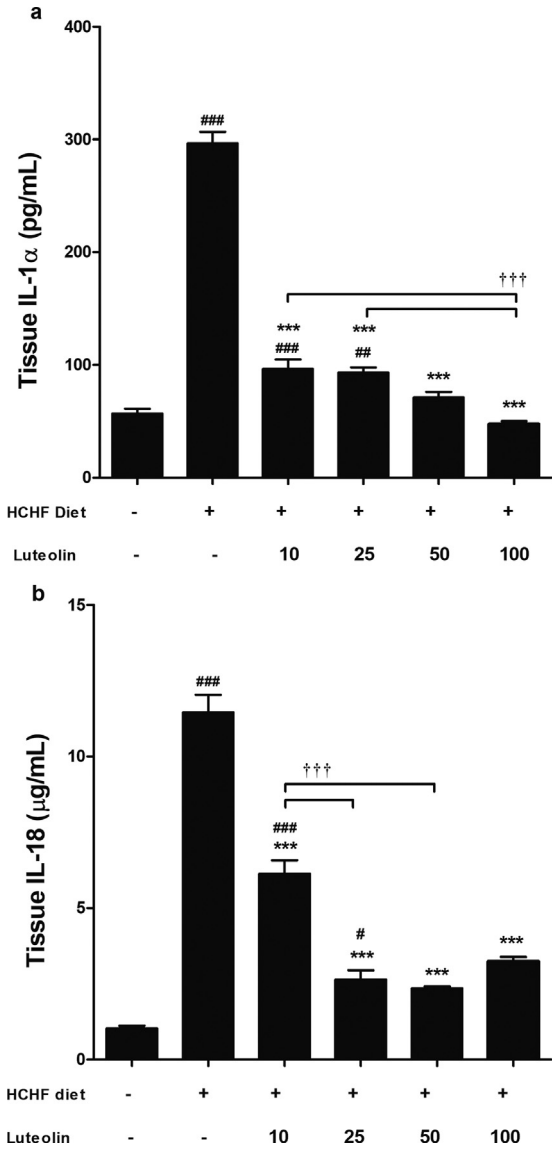

Fig. 5. Effects of luteolin (10, 25, 50, 100 mg/kg) on hepatic tissue levels of a) interleukin (IL)-1α b) IL-18 in non-alcoholic steatohepatitis induced by high carbohydrate/high fat (HCHF) diet. #, ##, ### $p <$ 0.05, 0.01, 0.001 compared with standard chow received group, *** $p <$ 0.001 compared with HCHF diet received group, ††† $p < 0.001$.

by Kaneda et al. [42] that IL-18 in combination with IL-12 can impair microcirculation and mitochondrial function in liver mediating inflammatory steatosis. Activation of inflammasomes by reactive oxygen species allows cleavage and maturation of pro-IL-18 promoting progression of NASH [43]. The observed reduction in IL-18 level may account for the improvement in liver function by luteolin in the current study.

The resident macrophages, Kupffer cells, are the first cells to be activated by liver injury and they produce IL-1α and IL-18 [44]. It is well documented that the uptake of modified low density lipoproteins as oxidized LDL by Kupffer cells results in lysosomal cholesterol accumulation. This triggers an inflammatory response and increases hepatic inflammation during NASH progression [45,46]. In our previous work, luteolin reduced serum cholesterol, triglycerides and low density lipoprotein levels elevated by high carbohydrate high fat diet [47]. Accordingly, it can be said that limitation of lipid intermediates accumulation in hepatocytes by luteolin and the followed decrease in the expected hepatocellular lipotoxicity and eventually hepatocytes death, the main activator of Kupffer cell [19] may be a key mechanism that explain the protective effect of luteolin against HCHF-induced steatohepatitis.

In conclusion, luteolin may be recommended to protect liver against high fat diet induced NASH through an antioxidant anti-inflammatory mechanisms and mainly regulating IL-1α and IL-18 hepatic tissue levels. Further studies should be constructed to test luteolin effect on other models of steatohepatitis.

Author contributions

Nashwa Abu-Elsaad: study design, methodology, data collection and analysis, writing and review.

Amr El-Karef: histopathological examination, data collection and interpretation, writing and review.

Conflict of interest

There are no known conflicts of interest.

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