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# Cholestyramine Modulates Non-Alcoholic Steatohepatitis Induced by Methionine and Choline Deficiency in Rats: Cross-linking AGEs, MIP2, EMR1 and Adiponectin

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**Abstract**

The pathophysiological mechanisms underlying NASH mainly involve lipid accumulation and build-up in the hepatic tissues. Cholestyramine is a bile acid sequestrant commonly used for the treatment of hypercholesterolemia. Forty juvenile male albino Wistar rats were divided into four groups (n=10). The first group received a normal pellet diet for 2 months and served as the normal group. The remaining 3 groups received a Methionine and Choline-deficient diet (MCD) for 2 months to induce NASH. Group 2 was administered 0.5 ml distilled water and served as the NASH group. Groups 3 and 4 were administered oral cholestyramine (10 & 20 mg/kg; p.o.) for 2 months. MCD resulted in NASH as was manifested by significant alteration of hepatic histopathological pictures along with elevation of serum AST, ALT activities, serum TC, TG & LDL-cholesterol and a reduction in HDL-cholesterol levels. The liver weight and the liver index were also significantly elevated. Hepatic levels of AGEs, NF-kβ, TNF-α, MMP-9, MIP-2 & PPAR-γ along with iNOS & EMR1 gene expression were elevated while adiponectin level was reduced versus the normal group. Cholestyramine treatment (10 & 20 mg/kg) amended all the aforementioned parameters as compared to the NASH group. The current study is the first to identify cholestyramine's beneficial actions in NASH induced in rats by MCD. Furthermore, the study navigates the possible molecular mechanisms beyond cholestyramine's actions. cholestyramine treatment reduces hepatic fat accumulation as well as hepatic tissue inflammation.

Cholestyramine; NASH; MIP2, EMR1; Adiponectin; Rats.

## 1. Introduction

In mammals, the liver is the primary site that controls key lipid metabolic pathways, including fatty acid oxidation. Non-alcoholic fatty liver disease (NAFLD) is characterized by the buildup of fat in the hepatic tissues of patients who do not drink alcohol excessively [1]. NAFLD can arise from decreased hepatic lipid droplet turnover. Simple steatosis is the initial symptom, and over time, nonalcoholic steatohepatitis (NASH) and even hepatocellular cancers may develop [2]. NASH usually develops when the rate of the manufacture and/or importation of fatty acids exceeds the rate of catabolism and /or removal by the hepatocytes [3]. The pathophysiological mechanisms underlying NASH mainly involve lipid accumulation and build-up in the hepatic tissues where long-chain fatty acids; either originating from adipose tissues and/or increased dietary consumption; are excessively transported into the hepatocytes. Additionally, elevation in the indigenous hepatic triglycerides and long-chain fatty acids production occurs accompanied by the reduced synthesis of the very low-density lipoproteins along with reduced mitochondrial oxidation eventually leading to failure in the elimination of long-chain fatty acids and further build-up in the hepatocytes [4]. NASH is then spontaneously followed by a solid inflammatory response characterized by elevated pro-inflammatory cytokine release, accompanied by irregular hepatic free fatty acids and cholesterol piling, oxidative stress, apoptosis, hyperinsulinemia and eventually hepatotoxicity [5]. Moreover, elevated oxidative stress forms a positive feedback loop with the pro-inflammatory cytokines thus augmenting the inflammatory response to further increase the oxidative stress in NASH [1].

Patients with liver disorders, particularly NASH, frequently experience malnutrition and vitamin

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deficiencies. Methionine and choline deficiency (MCD) is a robust frequently-used animal model used for the induction of NASH in experimental animals. MCD causes significant beta-oxidation in the hepatic tissues, leading to increased synthesis of pro-inflammatory cytokines, inflammation, oxidative stress along with fibrogenesis [3].

The hepatic conversion of cholesterol into bile acids represents a significant mechanism for the elimination and catabolism of cholesterol. Bile acids also act as signaling molecules with systemic actions. Recently, alterations in bile acid-activated signaling pathways have emerged as a promising therapeutic target for the management of hypercholesterolemia [6].

Cholestyramine is a bile acid sequestrant that blocks the intestinal reabsorption of bile acids and decreases the trans-hepatic bile acids flux enhancing the conversion of cholesterol into bile acids [7]. Bile acid sequestrants are positively-charged undigestible resins that form an insoluble complex that is excreted in the feces upon binding to bile acids in the intestine [8]. Cholestyramine is commonly used for the treatment of hypercholesterolemia as a monotherapy or combined with other lipid-lowering drugs [9]. Recent studies report that cholestyramine is effective in the treatment of type 2 diabetes where it improves both lipid and carbohydrate metabolism. However, the mechanisms underlying these effects remain unclear [10, 11].

Therefore, the present study aims to pinpoint the potential modulatory effects of cholestyramine against NASH induced by methionine and choline deficiency in rats. This study also looks into the central molecular mechanisms underlying cholestyramine effects in NASH. To the authors' knowledge, this is the first study to address the effects of cholestyramine against NASH induced by MCD in rats.

## 2. Experimental

### *2.1. Animals*

Forty juvenile male albino Wistar rats weighing 60-70 g were used in the current study. Animals were obtained from the animal house colony of the National Research Center (NRC, Egypt) and kept under standard conditions all over the study. The study was conducted following the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and following the National Research Centre–Medical Research Ethics Committee for the use of animal subjects (MREC), Approval number: 44112012023.

### *2.2. Drugs and chemicals*

Cholestyramine (Questran®, 4 g oral powder sachets; Bristol-Myers Squibb, USA) was administered orally throughout the study. All other chemicals were of the highest analytical grade available.

### *2.3. Experimental design and treatment protocol*

Animals were weighed to obtain the initial body weight and then randomly allocated into four groups (10 rats each). The first group (Normal group) received 0.5 ml oral distilled water plus a normal pellet diet for 2 months. The remaining 3 groups received a Methionine and Choline-deficient diet (MCD; Dyets, Bethlehem, Pa., USA) for 2 months to induce NASH [3]. Group 2 was administered 0.5 ml distilled water orally for 2 months and served as the NASH group. Groups 3 and 4 were administered oral cholestyramine (10 and 20 mg/kg body weight; respectively) for 2 months [12]. At the end of the experiment, the final body weight of all animals was recorded.

### 2.4. Methods

#### *2.4.1. Serum biochemical analysis*

At the end of the experiment, animals were sacrificed under sodium thiopental anesthesia (20 mg/kg; i.p.) and blood samples were withdrawn from the retro-orbital venous plexus. Collected blood samples were allowed to stand for 10 min at room temperature then centrifuged at 4ºC using a cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000 r.p.m for 10 min and sera were separated for the assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) using commercially available kits according to the manufacturer's instructions (Biodiagnostic®, Egypt) [13].

#### *2.4.2. Tissue biochemical analysis*

Immediately after blood sampling, animals were sacrificed by cervical dislocation under anesthesia. The livers were harvested from each rat, dissected out and rinsed with PBS to remove excess blood. The livers were then weighed to calculate the liver index of each rat. The liver index of each rat is calculated by the following equation: Liver index= (Liver Weight / Total Body Weight) *100 [2].

Parts from liver tissues were homogenized (MPW-120 homogenizer, Med instruments, Poland) to obtain 20% homogenate that was stored overnight at –20°C. The homogenates were centrifuged for 5 minutes at 5000 x g using a cooling centrifuge (Sigma and laborzentrifugen, 2k15, Germany) [14].

Liver tissue contents of tumor necrosis factor-alpha (TNF-α: Sunlong Biotech Co., Ltd; UK), nuclear factor kappa light chain enhancer of activated B cells (NF-kβ: Sunlong Biotech Co., Ltd; UK), Advanced glycation end products (AGEs: Sunlong Biotech Co., Ltd; UK), Matrix metalloproteinases 9 (MMP-9: SinoGeneClon Biotech Co., Ltd; China), Macrophage

inflammatory protein-2 (MIP-2: Sunlong Biotech Co., Ltd; UK), Peroxisome proliferator-activated receptor gamma (PPAR-γ: Sunlong Biotech Co., Ltd; UK) and Adiponectin (Elabscience, Biotech, Co., Ltd, China) were assessed using commercially available rat ELISA kits according to the manufacturer's instructions.

**2.4.3. Real-time polymerase chain reaction (PCR) quantification of inducible Nitric Oxide Synthase (iNOS) and Epidermal growth factor (EGF) module-containing mucin-like receptor 1 ($EMR_1$)**

Total RNA was extracted from homogenized tissues of all different groups with Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP. USA) and then quantity and quality were assessed by Beckman dual spectrophotometer (USA).

SuperScript IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) was utilized for reverse transcription of extracted RNA followed by PCR. 96-well plate StepOne instrument (Applied Biosystem, USA) was used in a thermal profile as follows: 10 minutes at 45 ºC for reverse transcription, 2 minutes at 98 ºC for RT inactivation and initial denaturation by 40 cycles of 10 seconds at 98ºC, 10 seconds at 55 ºC and 30 seconds at 72 ºC for the amplification step. After the RT-PCR run, the data were expressed in Cycle threshold (Ct) for the target genes and housekeeping gene. Normalization for variation in the expression of each target gene; iNOS &$EMR_1$; was performed by referring to the mean critical threshold (CT) expression value of the GAPDH housekeeping gene by the ΔΔCt method. The relative quantitation (RQ) of each target gene is quantified according to the calculation of the 2-ΔΔCt method. Gene bank accession number for the iNOS gene is (NM-AJ230480.1), for the $EMR_1$ gene is (NM-001007557.2) and for the GAPDH housekeeping gene is (NM_001394060.2). The primer sequences used for the RT-PCR are presented in table 1.

***2.5. Statistical analysis***

All the values are presented as means ± standard error of the means (SEM). Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) followed by *Tukey's* multiple comparison post hoc test. Difference was considered significant when $p < 0.05$. GraphPad prism® software (version 8 for Windows, San Diego, California, USA) was used to carry out these statistical tests.

**Table 1: The primer sequences used for RT-PCR quantification of inducible Nitric Oxide Synthase (iNOS) and Epidermal growth factor (EGF) module-containing mucin-like receptor 1 ($EMR_1$)**

| | Forward gene | Reverse gene |
|---|---|---|
| **iNOS** | 5′-CAGCATCCACGCAAGAA -3′ | 5′-CAGGTGTTCCCCAGGTAGGTAG -3’ |
| **$EMR_1$** | 5′-TCAAGGATACGAGGTTGCTGA -3′ | 5′-CTGAAGGCTGTTGATAGTGGTGA -3’ |
| **GAPDH Housekeeping** | 5'-TGTGTCCGTCGTGGATCTGA-3' | 5'-TTGATGTTGAAGTCGCAGGAG-3'. |

***2.4.5. Histopathological examination of liver tissues***

Different sections from the livers of all groups were cut and fixed in 10% neutral buffered formalin. After routine processing, the tissues were cut into 5µm thick sections, stained with H&E and examined through a light electric microscope [15].

**3. Results**

**3.1. Effects of cholestyramine on body & liver weights and liver index in NASH in rats.**

Feeding the rats for 8 weeks with the MCD diet resulted in induction of NASH as manifested by a significant reduction in body weight to ≈87% associated with a significant elevation in the liver weight and liver index to ≈129% and 149% versus the normal group; respectively.

Cholestyramine treatment (10 mg/kg/day; p.o.) elevated the reduced body weight to ≈109%, while it reduced the elevated liver weight and liver index to ≈77% and 70% versus the NASH group; respectively.

Cholestyramine treatment (20 mg/kg/day; p.o.) elevated the reduced body weight to ≈111%, while it reduced the elevated liver weight and liver index to ≈77% and 69% versus the NASH group; respectively (Table 2).

**Table 2: Effects of cholestyramine on body & liver weights and liver index in NASH in rats.**

| | Normal | NASH | NASH+ Cholestyramine (10 mg/kg) | NASH+ Cholestyramine (20 mg/kg) |
|---|---|---|---|---|
| **Initial Body weight (g)** | **65.80 ± 1.24** | **68.60 ± 0.89** | **68.40 ± 0.47** | **68.60 ± 0.68** |
| **Final Body weight (g)** | **196.20 ± 5.51** | **170.00a ± 4.79** | **185.40 ± 2.53** | **188.00b ± 1.83** |
| **Liver weight (g)** | **5.06 ± 0.29** | **6.55a ± 0.23** | **5.04b ± 0.36** | **5.05b ± 0.10** |
| **Liver Index (%)** | **2.60 ± 0.002** | **3.87a ± 0.002** | **2.71b ± 0.002** | **2.69b ± 0.001** |

Data is presented as mean ± SEM (n=10). Data is analyzed by one-way ANOVA followed by *Tukey's* post hoc test, [a] Significantly different *vs* normal group at $p < 0.05$ and [b] Significantly different *vs* NASH group at $p < 0.05$.

### 3.2. Effects of cholestyramine on serum liver function parameters and lipid profile in NASH in rats.

NASH induced in rats fed on the MCD diet for 8 weeks was manifested by a significant elevation in AST, ALT, total cholesterol (TC), triglycerides (TG) and low-density lipoprotein-cholesterol (LDL-C) to ≈ 287%, 234%, 200%, 240% and 282% respectively, while the high-density lipoprotein-cholesterol (HDL-C) was reduced to ≈ 45% versus the normal group.

Cholestyramine treatment (10 mg/kg/day; p.o.) significantly reduced the elevated AST, ALT, TC, TG and LDL-C to ≈ 84%, 80%, 72%, 75% and 74% respectively, while HDL-C was elevated to ≈184% versus the NASH group. Similarly, cholestyramine treatment (20 mg/kg/day; p.o.) significantly reduced the elevated AST, ALT, TC, TG and LDL-C to ≈ 53%, 68%, 58%, 52% and 54% respectively, while HDL-C was elevated to ≈173% versus the NASH group (Table 3).

**Table 3: Effects of cholestyramine on serum liver function parameters and lipid profile in NASH in rats.**

| | Normal | NASH | NASH+ Cholestyramine (10 mg/kg) | NASH+ Cholestyramine (20 mg/kg) |
|---|---|---|---|---|
| AST (U/L) | 61.40 ± 4.79 | 176.30a ± 0.89 | 148.50a,b ± 5.96 | 93.20a,b ± 8.94 |
| ALT (U/L) | 26.38 ± 0.49 | 61.75a ± 0.61 | 49.68a,b ± 2.94 | 42.00a,b ± 2.44 |
| TC (mg/dl) | 92.61 ± 3.13 | 185.38a ± 3.88 | 132.61a,b ± 5.14 | 107.22b ± 2.93 |
| TG (mg/dl) | 76.80 ± 3.45 | 184.32a ± 5.54 | 138.83a,b ± 2.78 | 96.46a,b ± 2.27 |
| HDL-C (mg/dl) | 47.62 ± 3.63 | 21.39a ± 1.08 | 31.58a,b ± 1.74 | 39.98a,b ± 1.70 |
| LDL-C (mg/dl) | 46.17 ± 0.57 | 130.37a ± 3.67 | 96.57a,b ± 2.11 | 70.46a,b ± 1.63 |

Data is presented as mean ± SEM (n=10). Data is analyzed by one-way ANOVA followed by *Tukey's* post hoc test, [a] Significantly different *vs* normal group at $p < 0.05$ and [b] Significantly different *vs* NASH group at $p < 0.05$. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol and LDL-C: Low-density lipoprotein-cholesterol.

### 3.3. Effects of cholestyramine on liver tissue contents of AGEs, TNF-α and NF-kβ in NASH in rats.

NASH induced in rats fed on the MCD diet for 8 weeks revealed a significant elevation in liver tissue contents of AGEs, TNF-α and NF-kβ to ≈ 207%, 310% and 245% respectively versus the normal group.

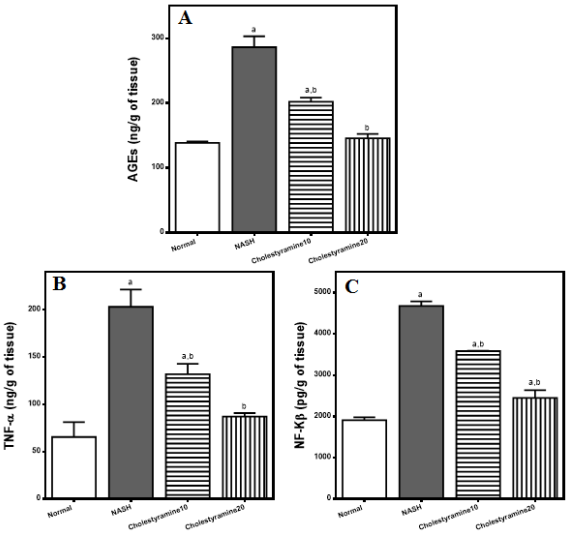
Cholestyramine treatment (10 mg/kg/day; p.o.) significantly decreased the raised liver tissue contents of AGEs, TNF-α and NF-kβ to ≈ 71%, 65% and 77% respectively versus the NASH group. Alternatively, cholestyramine treatment (20 mg/kg/day; p.o.) significantly decreased the raised liver tissue contents of AGEs, TNF-α and NF-kβ to ≈ 51%, 65% and 52% respectively versus the NASH group (Figure 1).

### 3.4. Effects of cholestyramine on liver tissue contents of MMP-9, MIP-2, PPAR-γ and Adiponectin in NASH in rats.

NASH induced in rats fed on the MCD diet for 8 weeks revealed a significant rise in liver tissue contents of MMP-9, MIP-2 and PPAR-γ to ≈ 194%, 371% and 281% respectively versus the normal group. Hepatic adiponectin was significantly lowered in the NASH group to ≈ 80% of the normal group.

Cholestyramine treatment (10 mg/kg/day; p.o.) significantly decreased the raised liver tissue contents of MMP-9, MIP-2 and PPAR-γ to ≈ 71%, 67% and 76% respectively versus the NASH group. Hepatic adiponectin was significantly elevated following cholestyramine (10 mg/kg) treatment to reach ≈ 162% of NASH rats.

**Figure 1: Effects of cholestyramine on liver tissue contents of (A) AGEs, (B) TNF-α (C) NF-kβ in NASH in rats.**

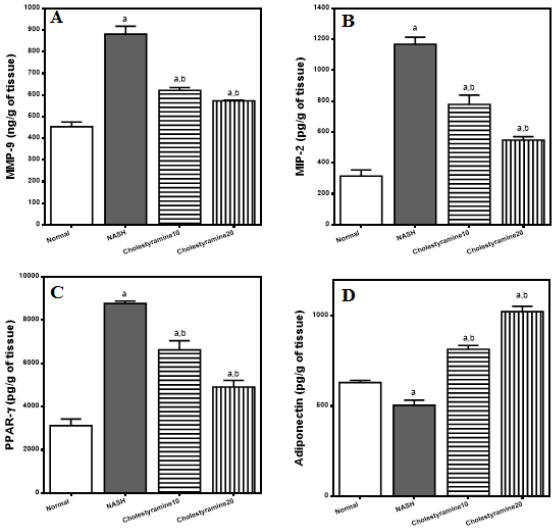



Data is presented as mean ± SEM (n=10). Data is analyzed by one-way ANOVA followed by *Tukey's* post hoc test, [a] Significantly different *vs* normal group at $p < 0.05$ and [b] Significantly different *vs* NASH group at $p < 0.05$. AGEs: Advanced glycation end products; TNF-α: Tumor necrosis factor-alpha and NF-kβ: Nuclear factor kappa light chain enhancer of activated B cells.

In addition, cholestyramine treatment (20 mg/kg/day; p.o.) significantly decreased the raised liver tissue contents of MMP-9, MIP-2 and PPAR-γ to ≈ 65%, 47% and 59% respectively versus the NASH group. Hepatic adiponectin was significantly

elevated following cholestyramine (20 mg/kg) treatment to reach ≈ 203% of NASH rats (Figure 2).

**Figure 2: Effects of cholestyramine on liver tissue contents of (A) MMP-9, (B) MIP-2, (C) PPAR-γ and (D) Adiponectin in NASH in rats.**




Data is presented as mean ± SEM (n=10). Data is analyzed by one-way ANOVA followed by *Tukey's* post hoc test, [a] Significantly different *vs* normal group at $p < 0.05$ and [b] Significantly different *vs* NASH group at $p < 0.05$. MMP-9: Matrix metalloproteinases 9; MIP-2: Macrophage inflammatory protein-2, and PPAR-γ: Peroxisome proliferator-activated receptor gamma.

### 3.5. Effects of cholestyramine on liver tissue relative iNOS and $EMR_1$ Quantitative Real Time-PCR gene expression in NASH in rats.

Liver tissue iNOS and $EMR_1$ gene expression were expressively elevated in NASH-control rats relative to the normal rats. Cholestyramine treatment (10 and 20 mg/kg/day; p.o.) lead to a significant reduction in liver tissue iNOS and $EMR_1$ gene expression in comparison to the NASH-control group (Figure 3).

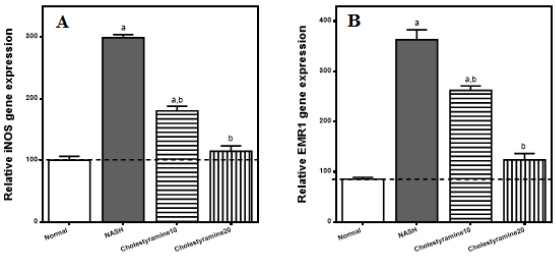



**Figure 3: Effects of cholestyramine on liver tissue relative (A) iNOS and (B) $EMR_1$ qRT-PCR gene expression in NASH in rats.**

Data is presented as mean ± SEM (n=10). Data is analyzed by one-way ANOVA followed by *Tukey's* post hoc test, [a] Significantly different *vs* normal group at $p < 0.05$ and [b] Significantly different *vs* NASH-control group at $p < 0.05$. iNOS: inducible nitric oxide synthase and $EMR_1$: Epidermal growth factor module-containing mucin-like receptor 1.

### 3.6. Histopathological examination of liver tissues

Livers of normal rats showed no histopathological alteration and a normal histological structure of the central vein and surrounding hepatocytes in the lobules of the parenchyma (Fig.4A). On the other hand, livers of the NASH group showed severe congestion in both portal and central veins associated with fatty changes in the hepatocytes of most lobules (Fig.4B&C). Cholestyramine treatment (10 mg/kg) showed a mild improvement of the histopathological picture as manifested by moderate degeneration of the hepatocytes of some lobules (Fig.4D&E). Alternatively, cholestyramine treatment (20 mg/kg) showed a huge improvement in the hepatocytes of most lobules, whereas the parenchyma showed only little degenerative changes (Fig.4F&G).

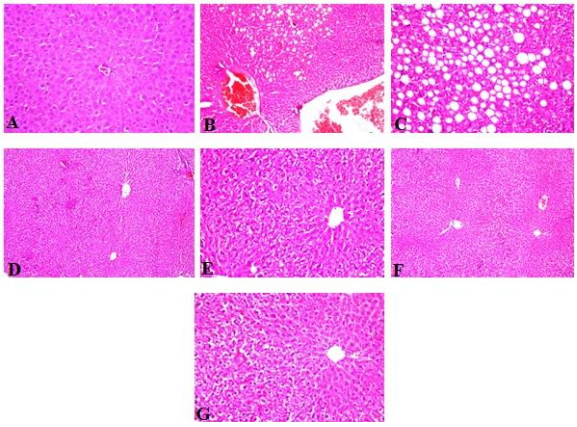



**Figure 4: Histopathological examination of liver tissue.**

Liver of (**A**) normal rats showing normal histological structure of the central vein and the surrounding hepatocytes in parenchyma (Stain:H&E, X40), (**B**) NASH-control rats showing marked fatty changes in the hepatocytes of some lobules with congestion of the central and portal veins(Stain:H&E, X16), (**C**) A magnification of the section B of NASH-control rats to identify the fatty changes in the hepatocytes (Stain:H&E, X40), (**D**) NASH+Cholestyramine (10 mg/kg) group showing moderate degeneration in the hepatocytes of some lobules (Stain:H&E, X16), (**E**) A magnification of the section of NASH+Cholestyramine (10 mg/kg) group to identify the moderate degenation in the same hepatocytes (Stain:H&E, X40), (**F**) NASH+Cholestyramine (20 mg/kg) group showing huge improvement with little degenerative changes in the hepatocytes in some lobules (Stain:H&E, X16), and (**G**) A magnification of the section of NASH+Cholestyramine (20 mg/kg) group to identify the little degenative changes in the hepatocytes (Stain:H&E, X40).

## 4. Discussion

The current study mainly identifies the potential modulatory actions of cholestyramine (10 &20 mg/kg, p.o) in non-alcoholic steatohepatitis (NASH) induced experimentally in rats via methionine and choline deficiency (MCD). To the authors' knowledge, the current study is the first to identify cholestyramine's beneficial actions in NASH and to navigate the possible molecular mechanisms beyond these actions.

MCD in rats resulted in a significant induction of NASH, characterized by steatosis, oxidative stress and inflammation of the hepatocytes. This is attributable to the accumulation of lipids - mainly triglycerides- in the hepatocytes. This results from a discrepancy in the metabolic pathways involving fatty acids synthesis and oxidation where steatosis is brought on by lipolysis, which raises the levels of free fatty acids in the blood and also the hepatocyte fatty acid absorption [16]. Therefore, NASH, in the current work, was associated with a significant alteration in liver function tests as manifested by elevations in serum AST and ALT activities. Moreover, serum TC and TG, LDL-cholesterol were significantly elevated and HDL-cholesterol was significantly lowered. The liver weight and the liver index were also significantly elevated as compared to the normal rats; which can be explained by fat accumulation in the hepatic tissues of the NASH group. Likewise, the histopathological examination of liver tissues of NASH rats showed severe alterations characterized by significant fatty changes in the hepatocytes of most lobules. Many studies reported similar data following the induction of NASH [1-3].

In the current work, hepatic levels of AGEs, NF-kβ, TNF-α, MMP-9 and MIP-2 along with iNOS and $EMR_1$ gene expression were elevated in NASH induced experimentally in rats via MCD. NASH progression promotes oxidative and inflammatory changes in the hepatocytes accompanied by mitochondrial dysfunctionalities and adipokines release from the adipose tissue [16]. The development of the advanced glycation endproducts (AGEs) is stimulated by oxidative stress [17]. AGEs are heterogeneous moieties created *in vivo* through the interactions between sugars, protein, and lipid adducts [18]. The liver is primarily responsible for AGEs metabolism and elimination. Interestingly, the hepatocytes suffer oxidative as well as inflammatory reactions in response to elevated levels of AGEs in a positive feedback loop. Where elevated AGEs impair the liver performance, consequently reducing their own metabolism and elimination eventually resulting in AGEs buildup in the blood and the hepatocytes [14]. Elevated levels of AGEs trigger inflammatory signaling pathways in the hepatocytes and adipose tissue through the activation of nuclear factor-kappa beta (NF-kβ) and its downstream target; tumor necrosis factor (TNF-α) [19]. Moreover, the overexpression of NF-kβ in the hepatic tissues causes activation of NF-kβ sites in the inducible nitric oxide synthase (iNOS) promoter, thus stimulating iNOS up-regulation [20]. Significant iNOS gene expression upregulation in the hepatocytes is documented following induction of NASH [21]. Hepatic NF-kβ levels were elevated in rats fed on a high-fat diet leading to overexpression of TNF-α and thus moving stepwise from steatosis to NASH [22]. Additionally, accumulated data revealed that elevated hepatic levels of NF-kβ trigger the expression of matrix metalloproteinases (MMP) in response to inflammatory cytokines like TNF-α [23]. MMPs are well known for their ability to degrade the extracellular matrix thus causing hepatic tissue injury and loss of the normal hepatocyte architecture. MMP-9 is recently used for the prediction of hepatic inflammation and damage [24]. Macrophage inflammatory protein-2 (MIP-2), also known as chemokine CXC ligand (CXCL2), is released by various cell types in response to inflammation. Macrophages produce MIP-2 in response to inflammatory stimuli. Activated Kupffer cells are potent effectors of MIP-2 in response to liver injury [25]. Moreover, Sun et al. (2018) reported an elevation of MMP-9 and MIP-2 in the hepatic tissues of mice following $CCl_4$ administration [24]. Interestingly, it is well established that lipid accumulation in the liver and adipose tissue cause severe inflammation accompanied by activation of chemokines as the epidermal growth factor module-containing mucin-like receptor 1 ($EMR_1$). Furthermore, inflammatory cytokines and growth factors initiate $EMR_1$ expression in hepatic tissues. The hepatic expression of the $EMR_1$ is very high as compared with other cell types since it plays a pivotal signaling pathway role in acute and chronic liver injury [26].

Similar to our work, Chtourou et al. (2015) reported an elevation of hepatic TNF-α, NF-kβ, MMP-2, 9 along with iNOS and $EMR_1$ gene expression in NASH rats [1]. Maeda et al. (2020) demonstrated a significant hepatic expression of TNF-α, MMP-9 and $EMR_1$ in response to liver fibrosis due to NASH in mice [27].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor transcription factor that exerts crucial actions in adipocyte differentiation, glucose metabolism and fat storage. PPARγ is a key player in NAFLD and NASH where its overexpression is linked to hepatic steatosis and adipogenic transformation of hepatocytes [28]. NASH also involves the activation of adipocytes and the subsequent release of adiponectin. Adiponectin is a polypeptide that

decreases fat accumulation in the macrophages, opposes hepatic fatty acid synthesis and increases fatty acid beta-oxidation in the mitochondria. Moreover, it also possesses direct anti-inflammatory effects via the ablation of TNF-α synthesis and release [29]. Recent data demonstrate that NASH induced by high fat diet is associated by up-regulation of PPARγ and down-regulation of hepatic adiponectin levels in mice [30] and in rats [31].

Additionally, the present results also revealed that the hepatic level of PPAR-γ was elevated in NASH induced experimentally in rats via MCD. On the other hand, hepatic adiponectin level was reduced in response to NASH induction in rats as compared to the normal group.

Cholestyramine; commonly used for the treatment of hypercholesterolemia is a bile acid sequestrant that blocks the intestinal reabsorption of bile acids and decreases the trans-hepatic bile acids flux enhancing the conversion of cholesterol into bile acids [7].

In the current work, cholestyramine treatment (10&20 mg/kg; p.o.) modulated NASH induced experimentally in rats via MCD. This was manifested by the reduction of the liver weight & the liver index, serum AST & ALT activities and serum TC, TG & LDL-cholesterol. Serum HDL-cholesterol was significantly elevated as compared to the NASH group. Moreover, cholestyramine significantly reduced the elevated hepatic levels of AGEs, NF-kβ, TNF-α, MMP-9, MIP-2 and PPAR-γ along with iNOS and $EMR_1$ gene expression as compared to NASH rats. Interestingly, hepatic adiponectin levels were significantly elevated in response to cholestyramine treatment. Moreover, cholestyramine treatment in the aforementioned doses managed to improve the histopathological pictures of liver tissues of rats resulting in a huge improvement in the hepatocytes of most lobules.

Mounting evidence states the hypolipidemic effects of cholestyramine treatment. Oliveira et al. (2007) reported a significant reduction in serum TG and LDL-cholesterol accompanied by an elevation in serum HDL-cholesterol after treatment with cholestyramine in rats Oliveira, Ricardo [12]. Another study reported a reduction in serum TG and non-esterified fatty acid accompanied by an elevation of lipid excretion following cholestyramine treatment in mice [6]. Matsumoto and Yokoyama (2010) declared that the exact molecular mechanisms underlying cholestyramine's effects on lipid metabolism and carbohydrate metabolism are still not fully elucidated [9]. Another study reported that cholestyramine treatment has extensive beneficial actions on the components of metabolic syndrome [32]. Moreover, cholestyramine in one study, diminished intestinal ulceration and inflammation in patients with inflammatory bowel diseases [33]. A recent study documented that cholestyramine treatment downregulates the gene expression of cholesterol transporter proteins [34].

## 5. Conclusion

To sum up, the present study is the first to identify cholestyramine's beneficial actions in NASH induced in rats by methionine and choline deficiency. Furthermore, the study navigates the possible molecular mechanisms beyond cholestyramine's actions. MCD resulted in NASH in experimental animals. Cholestyramine treatment amended the elevation in the liver weight and the liver index; signifying a reduction in fat accumulation in the hepatic tissues. Moreover, cholestyramine treatment resulted in a significant improvement in the liver function tests, serum cholesterol & TG levels, hepatic histopathological pictures, hepatic levels of AGEs, NF-kβ, TNF-α, MMP-9, MIP-2 & PPAR-γ along with iNOS & $EMR_1$ gene expression and an elevation in hepatic adiponectin levels; signifying amendment of an on-going inflammation of hepatic tissues. Further studies are warranted to add cholestyramine to the treatment protocol for NASH.

## 6. Conflicts of interest

There are no conflicts to declare.

## 7. Funding

The authors have no relevant financial or non-financial interests to disclose.

## Availability of data and material

All data will be available upon request

## 8. Authors' contributions

All authors contributed to the study's conception and design. Material preparation and data collection were performed by Abeer AA Salama and Mohamed M. Amin. Statistical analysis was performed by Rasha E. Mostafa. The first draft of the manuscript was written by Rasha E. Mostafa and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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