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Effects of Curcumin on Autophagy and Nrf2 signaling pathway in a Rat Model of High fructose diet induced Steatohepatitis

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Abstract

Increased fructose consumption predisposes to nonalcoholic steatohepatitis (NASH).There no effective treatment to be used as the second line when lifestyle modification is insufficient .So, the aim of this study was to evaluate the effect of Curcumin on some biochemical markers in fructose induced NASH . Sixty male albino rats were divided randomly and equally into three groups. Group I (control group). Group II (NASH group), received 70% fructose for 5 weeks .Group III (curcumin treated NASH group), fed as group II followed by daily administration of curcumin at a dose of 50 mg/kg orally for 6 weeks. Blood samples were collected to measure lipid profile, and liver enzymes activities . Liver tissues samples were collected for estimation of mRNA expression levels of nuclear factor E2-related factor-2 (Nrf2) using quantitative real-time PCR ,levels of Beclin 1, AMPK, IL6 and Nrf2 DNA-binding activity by ELISA method, and oxidative stress markers malondialdehyde (MDA), superoxide dismutase (SOD) ,GSH spectrophotometrically. NASH was confirmed by histopathology as well as increased activity of liver enzymes and marked dyslipidemia .Curcumin ameliorated biochemical disturbance in NASH by upregulating autophagy process through increasing Beclin 1, increasing AMPK and hampered IL6 levels in the liver . It also restored redox balance as evident by suppressing MDA formation and preservation of intracellular antioxidants status via increasing GSH, activating SOD and Nrf2 signaling pathway. Results suggested that curcumin has an effective role in counteracting both abnormalities in autophagy process and redox balance, supporting the potential utility of this natural product in effective treatment of NASH.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide with a prevalence rate ranging from 6 to 35 % [1]. NAFLD represents a wide spectrum of liver diseases from simple steatosis to a more severe and treatment-resistant clinical entity characterized by the appearance of inflammation, termed nonalcoholic steatohepatitis (NASH), which may in turn progress to cirrhosis and hepatocellular carcinoma [2]. NASH is the most common chronic liver disease in the Western world, it is primarily a disease of the obese and the prevalence of simple steatosis in obese patient reaches 60%, among those, 20–25% will develop NASH [2].

The pathogenesis of NASH is still not fully understood, the disease is characterized by fatty accumulation in the liver due to an imbalance among hepatic lipid intake, synthesis, degradation and secretion. Patients with simple steatosis progress to NASH according to the "two-hit" theory; liver steatosis sensitizes hepatocytes to the second hits, which may be increased in oxidative stress, mitochondrial dysfunction, and cytokine/adipokine imbalance leading to hepatocyte damage, inflammation and fibrosis [3, 4].

Among the causes of hyperlipidemia and steatosis is excessive intake of fructose because the metabolic intermediates of fructose breakdown, glyceraldehyde and dihydroxyacetone phosphate enter the glycolytic pathway bypassing the regulatory mechanisms imposed on phosphofructokinase-1 , with unlimited synthesis of pyruvate and acetyl coA which is forwarded to lipogenesis and excessive formation of triacylglycerol leading to hyperlipidemia and fatty liver [5].

Autophagy (self-eating process) is evolutionarily conserved and genetically regulated critical pathway for the degradation of intracellular components by lysosomes it is a multistep process including initiation, elongation, enclosure, maturation and degradation. Three main types of cellular autophagy have been identified: macroautophagy, chaperone-mediated autophagy, and macroautophagy [6]

Established functions for both macroautophagy and chaperone-mediated autophagy in hepatic lipid metabolism, insulin sensitivity and cellular injury suggest a number of potential mechanistic roles for autophagy in NASH. [7]. It is widely acknowledged that about 30 mammalian homologs of yeast autophagy-related proteins (Atg) have been identified which are involved in initiation and elongation of the isolation membrane, as beclin 1[8].

Beclin 1, the mammalian orthologue of yeast Atg6, is a 60-kDa coiled-coil protein that was discovered as a direct interactor of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein and was therefore given the name Bcl-2-interacting myosin like coiled-coil protein (Beclin 1). Beclin 1 is responsible for the nucleation process of autophagy and is downregulated by the mammalian target of rapamycin complex1 (mTORC1) .It plays a critical role in modulating the magnitude of the autophagic response involved in several physiological and pathological processes inside the mammalian cell [8].

AMP activated protein kinase (AMPK) is a critical regulator of energy homeostasis in eukaryotic cells. It is a heterotrimeric serine-threonine protein kinase composed of a catalytic α-subunit and non-catalytic β and γ subunits. Activation of this enzyme triggers phosphorylation of many regulatory proteins including mTORC1 that leads to inhibition of mTORC1 and liberation of beclin 1 that initiates autophagy [9].

Oxidative stress defined as an imbalance between prooxidant antioxidant status plays a crucial role in the pathogenesis of many diseases including NASH. The relationship between oxidative stress and autophagy has not been fully elucidated [10].

It is very important to stimulate antioxidant/detoxification enzymes for defending against ROS toxicity, hepatocytes are equipped with many cytoprotective enzymes ensure protection against the toxic effects of endogenous and exogenous oxidants , they are encoded by genes containing antioxidant response elements (AREs) in their promoter regions. [11]. AREs are cis-acting enhancer sequence that mediates transcriptional activation of genes in response to changes in the cellular redox status, Transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that regulates transcriptional induction of ARE-containing genes encoding antioxidant enzymes, electrophile-conjugating enzymes, ubiquitin/ proteasomes, and chaperone and heat-shock proteins in response to cellular stresses including ROS . [12]. In normal physiological conditions, Nrf2 is expressed at low level and resides mainly in cell cytoplasm, where it is promoted to degradation via ubiquitination by a repressor protein, kelch-like ECH-associated protein-1 (Keap1)1 in the cytoplasm [13]. Under oxidative stress or through Nrf2 activators, Nrf2 dissociates from Keap1, translocate to the nucleus where heterodimer with Maf is formed, and then expression of genes with antioxidant response element (ARE) is activated [14].

Curcumin is a phenolic compound extracted from popular Indian spice turmeric, derived from the rhizome of Curcuma longa [15]. Curcumin regulates lipid metabolism; has anti-inflammatory, antioxidant and anti-cancer effects [16]. Despite the large number of studies demonstrating the hepatoprotective effects of curcumin, there are few reports on the effects of this polyphenolic compound on the NASH model induced by a high-fructose diet under in vivo conditions, and the possible molecular underlying mechanisms.

Therefore, the aim of this study was to evaluate whether curcumin could attenuate biochemical disturbance associated with high-fructose diet-induced NASH in a rat model to provide a novel mechanism for the regulatory role of curcumin in hepatic lipid metabolism, making it a good candidate for the attenuation of NASH. Also, to clarify the role of autophagy, as monitored by Beclin 1 levels, in the pathogenesis of steatohepatitis, and correlates Beclin 1 levels with levels of AMP activated protein kinase, and parameters of oxidative stress.

Materials and methods

1. Chemicals:

D-fructose (CAS no. 57-48-7, purity ≥99 %), Curcumin (CAS no. 458-37-7) and most other

chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2. Animals

2.1. *Experimental animals and diets*

This study was conducted on 60 male albino rats, (weight, 190±20 g) obtained from the animal breeding laboratory, Faculty of science, Tanta University, Egypt. Animals were kept in wire mesh cages in our animal facility, Faculty of medicine; Tanta University at 25 ± 2 °C and a relative humidity of 40–45% with alternative day and night cycles of 12 h each. Animals were allowed free access to food and water ad libitum for a week. Animal care and experiments were conducted in accordance with the protocols approved by the Ethics Research Committee, Faculty of medicine, Tanta University, following the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.85-23, revised in 1996).

2.2. *Experimental design and animal grouping:*

After an initial acclimatization for a period of one week, the rats were randomly divided into three equal groups as follows: **Group I (control group)** fed standard caloric diet (consisting of 59.7% carbohydrates, 10.6% fat, and 27.3% protein) and ad libitum plain water. **Group II (NASH group)** fed standard caloric diet and 70% fructose-sweetened water (w/v) for 5 weeks to induce NASH [17]. **Group III** (**curcumin treated NASH group)** fed standard caloric diet and 70% fructose-sweetened water (w/v) for 5 weeks as group 2, followed by daily administration of curcumin by oral gavage at a dose of 50 mg/kg suspended in 0.5% carboxymethyl cellulose (CMC) for 6 weeks. Concomitantly, equal volumes of (CMC) were intragastrically administered to the control group for 6 weeks [18].

The dosage was adjusted every week according to any change in body weight to maintain similar dose per kg body weight of rat over the entire period of study.

3. Blood and tissue sampling:

3.1. Blood sampling: At the end point of the experiment, all animals were fasted overnight and sacrificed by decapitation under ketamine (0.2 mL/100 g) anesthesia. Blood was collected in a dry ,sterile tube to obtain serum and then centrifuged at 3000 x g for 20 minutes at 4°C and stored at -70 º C until the time of analysis.

3.2. Tissue sampling: The liver was excised, perfused in situ with ice-cold 0.9% (w/v) NaCl solution, blotted dry on a filter paper, and divided into 2 parts. One part was preserved in 10% buffered paraformaldehyde for histopathological examination .The other part was stored at -70 º C till used for gene expression analysis and preparation of tissue homogenate.

Preparation of Liver tissue homogenate:

One piece of each specimen was weighed and homogenized in ice-cold 10 mM potassium phosphate containing 1 mM EDTA, pH 7.4 in a ratio of 1/5 w/v using a Potter–Elvenhjem tissue homogenizer. Homogenates were centrifuged at 12, 000 x g for 30 minutes at 4º C and the resultant supernatant was frozen at -70 °C till used for further analysis.

- **Preparation of liver nuclear extracts:** A nuclear extract of liver cells was prepared using the

Nuclear/Cytosol Fractionation Kit (Cat #K266-25, BioVision, Inc., CA, USA) according to the protocol of the manufacturer

4. Biochemical analysis

4.1. Lipid profile assessment: Total cholesterol (TC) and triacylglycerols (TAGs) were measured by enzymatic-colorimetric methods (Biodiagnostic., Egypt).

4.2. Serum ALT and AST activities were measured using Randox kits according to manufacturer's instructions.

4.3. Total proteins assay: Concentrations of total proteins in the samples were determined according to the method of Bradford [19] with bovine serum albumin as a standard (#Cat no.500–0006, Bio\\Rad Protein Assay) .

4. 4.Parameters of oxidative stress:

a. Liver tissue malondialdehyde (MDA) Levels were determined using a method depends on the formation of MDA as an end product of lipid peroxidation which reacts with thiobarbituric acid producing thiobarbituric acid reactive substance (TBARS), a pink chromogen, which can be measured spectrophotometrically at 532 nm[20].

b. The activity of superoxide dismutase (SOD) in the liver tissue was assayed according to the method of [21] .Addition of SOD inhibits reduction of nitroblue tetrazolium (NBT) mediated by hydroxylamine hydrochloride. The extent of inhibition is taken as a measure of enzyme activity. Reaction mixture consisted of 0.1 mM Ethylene diamine tetra acetic acid, 50 mM sodiumcarbonate and 96 mM of NBT. In the cuvette, 2 ml of above mixture was taken and 0.05 ml of the supernatant and 0.05 ml of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added to it. The auto-oxidation of hydroxylamine (development of blue colored complex) was observed by measuring the change in optical density at 560 nm for 2 min at 30/60 s intervals. The activity of enzyme was expressed as units/mg protein where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

c) Reduced glutathione levels (GSH) were determined in the liver tissue homogenate spectrophotometrically using a commercially available kit (#Cat: GR 2511, Bio Diagnostic, Egypt) The method is based on the reduction of 5,5′dithiobis (2-nitrobenzoic acid) with GSH to produce a yellow compound, its absorbance is measured at 405 nm. Results were expressed as mg/g liver tissue [22].

4.5. Phosphorylated active form of Adenosine Monophosphate Protein Kinase (AMPK) levels: were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit supplied by (Glory Science Co, USA) according to the manufacturer's instructions.

4.6. Liver tissue Beclin1 levels were assayed by ELISA kit supplied by (Glory Science Co, USA) according to the manufacturer's instructions.

4.7. Liver tissue IL-6 levels were assayed by ELISA kit (Cat# E02I0006, Blue Gene, Shanghai,

China).According to the manufacturer's instructions.

4.8. Quantification of DNA-binding activity of liver nuclear factor erythroid 2-related factor 2 (Nrf-2) was carried out in liver nuclear extracts using ELISA kit (Cat # 600590, Cayman Chemical, Michigan,USA) according to the manufacturer's protocol.

4.9. Quantitative measurement of nuclear factor-erythroid 2-related factor-2 (Nrf2) mRNA by quantitative real-time reverse transcription PCR (RT-PCR):

▪ **RNA extraction:** Total cellular RNA was isolated from sample liver, using Trizol Reagents(Invitrogen Life Technologies, Carlsbad, Calif, United States) according to the manufacturer's instructions. RNA quality was ensured through gel visualization and spectrophotometric analysis (OD260/280). Concentration of RNA was measured with the use of the OD260. On a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA), RNA was then stored at -80°C.

▪ **cDNA synthesis :** Total RNA samples were reverse-transcribed using the RevertAid H Minus First Strand cDNA Synthesis kit (Cat#K1632, Thermo Scientific Fermentas, St. Leon-Ro, Germany) according to the manufacturer's instructions. Briefly, 10 µL of random hexamer primers (Roche, Mannheim, Germany) were added to 21 µL of RNA which was denatured for 5 min in the thermal cycler (Biometra, USA) ,then cooled to 4 °C. The cDNA master mix was prepared (5 µL of first strand buffer, 10 mM of dNTPs,1 µL of RNase inhibitor, 1 µL of reverse transcriptase SuperscriptTM II-RT enzyme and 10 µL of DEPC treated water) according to the kit protocol and was added to each sample. The total volume of the cDNA master mix was 19 µL for each sample ,added to 31 µL RNA-primer mixture resulting in a reaction total volume of 50 µL, which was then incubated in the programmed thermal cycler for one hour at 42 °C, followed by inactivation of enzymes at 95 °C for 5 min, and finally cooled at 4 °C. The first strand cDNA was verified by running on 2% agarose gel for confirmation of the amplified band, then cDNA stored at -20°C until used for PCR.

▪ **Real-time quantitative PCR:**

1 µL of the reverse transcriptase reaction mixture was added to a 20 µL reaction mixture of the QuantiTect SYBR-Green PCR kit (Qiagen) and 0.5 µM from the specific primer pair for rat Nrf2 (GenBank accession no. NM_031789). Samples of cDNA were assayed on the step one Real Time PCR System (Applied Biosystems). Initial denaturation at 95˚C for 5 min was followed by 30 cycles with denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 20 sec. A negative control containing the complete Master Mix without a DNA template was included to detect genomic DNA contamination Primer sequences specific for rat Nrf2 were as follow [23]. : (forward) 5′-CTCTCTGGAGACGGCCATGACT-3′, and (reverse):5′CTGGGCTGGGGACAGTGGTAGT-3′. Primers for b-actin were included as an internal control (forward):5′-CCTCTATGCCAACACAGTGC-3′ and, (reverse) 5′CATCGTACTCCTGCTTGCTG-3′. (GenBank accession no. NM_0311442). The fluorescence intensity of SYBR-Green, specifically

Table 1: Effects of treatment with curcumin on Liver tissue IL-6 and some metabolic parameters

Groups	Group 1	Group 2	Group 3	One way ANOVA	
Variables	n= 20	n= 20	n= 20	F value	p-value
Final body weight in gram	256.25±15.15	320.75±10.6 [a]	264.5±12.5 [b]	148.4	<0.0001*
Serum triacylglycerol levels (mg/dl)	77.3±4.5	210.82± 15.93 [a]	95.53.± 35.0 [a, b]	209.8	<0.0001*
Serum total cholesterol levels (mg/dl)	90.5±6.2	186.05± 11.56 [a]	97.12.± 7.1 [a, b]	767.8	<0.0001*
Serum AST(U/L)	27.4±0.8	90.85± 8.65 [a]	40.1± 2.4 [a, b]	832.6	<0.0001*
Serum ALT(U/L)	22.7±0.3	52.45± 7.42 [a]	28.2 ±1.5 [a, b]	261.9	<0.0001*
Liver tissue triacylglycerol levels (mg TAG/mg protein/ml)	0.23± 0.062	0.45± 0.094 [a]	0.29± 0.052 [a, b]	55.51	<0.0001*
Liver tissue IL-6 levels(pg/mL)	77.9 ± 17.2	133.1 ± 49.9 [a]	86.9 ± 15.5 [b]	17.39	<0.0001*

Values are expressed as mean ± SD. *P was considered significant at <0.05; a Significance vs control group , b Significance vs NASH induced group using One way ANOVA followed by Tukey's post hoc test for multiple comparison. ALT alanine aminotransferase, AST aspartate aminotransferase, IL-6 interleukin-6

Table 2: Effects of treatment with curcumin on liver tissue AMPK , and oxidative stress markers.

Groups	Group 1	Group 2	Group 3	One way ANOVA	
Variables	**n= 20**	**n= 20**	**n= 20**	**F value**	**p-value**
Liver tissue MDA (nmol/mg protein/ml)	61.32± 18.68	185.97± 66.38[a]	77.5± 19.5[b]	53.68	<0.0001*
Liver tissue SOD Activity units/mg protein	436.06± 76.17	254.6± 60.42 [a]	449.6± 79.4 [b]	45.15	<0.0001*
Liver tissue GSH (mg/ g liver tissue)	1.2 ± 0.1	.8 ± 0.2 [a]	1.1 ± 0.2[b]	28.89	<0.0001*
DNA-binding activity of liver Nrf2	0.45 ± 0.02	.53 ± 0.01 [a]	2.70 ± 0.02 [a, b]	108642	<0.0001*
Liver tissue phosphorylated active form of AMPK level (ng/mg protein /ml)	1.927± 0.364	1.436 ± 0.323 [a]	3.248± 0.692 [a,b]	73.63	<0.0001*

Values are expressed as mean ± SD. *P was considered significant at <0.05; a Significance vs control group , b Significance vs NASH induced group using One way ANOVA followed by Tukey's post hoc test for multiple comparison. MDA malondialdehyde, GSH glutathione, SOD superoxide dismutase, Nrf2 nuclear factor-erythroid 2-related factor

incorporated in the double-stranded DNA amplicon reflecting the amount of formed PCR product, was read after each extension step at72˚C. RNA amounts were determined with the Applied Biosystems software using the comparative cycle threshold (ΔΔCt) method in mode relative to reference gene b-actin, which was not altered by the experimental conditions

5. Histopathological study: After fixation of liver tissue samples in neutral 10% buffered formalin (pH 7.2) at room temperature, tissues were dehydrated through graded alcohol solutions, and embedded in paraffin. Sections were then stained with hematoxylin and eosin for histopathological analysis.

Statistical analysis: The results were expressed as the mean ± S.D. in each group. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. The data were analyzed with Graph prism statistical software (version 6), San Diego, CA, USA. A statistical probability of $P < 0.05$ is considered to be significant.

Results

Effect of curcumin on body weight:

The weight gain in grams was calculated as the difference between the body weight at the end and at the beginning of the 12 weeks as shown in table (1) Significant differences were observed between model and control groups regarding body weight ($p<0.0001$) . In the treatment group, a remarkable reduction was observed in body weight as compared to model group ($p<0.0001$), and non-significant differences compared to control group.

Effect of curcumin on liver parameters, and lipid parameters:

Rats fed on the high fructose diet (model group) showed an increase ($p<$**0.0001**), in all lipid parameters (i.e. serum TAGs, liver tissue TACs, and TC) and in serum liver enzymes activity (ALT,AST) when compared to those of rats fed the standard diet. Curcumin treatment resulted in significant reduction ($p<$**0.0001**) of these lipid and liver parameters but still higher than control group (Table 1).

Effect of curcumin on the inflammatory cytokine IL-6 levels in liver tissue:

Rats in the model group had higher liver contents of IL-6 than those in the normal group ($p<$**0.0001**) .The administration of curcumin resulted in a remarkable reduction of this inflammatory cytokine ($p<$**0.0001**) when compared to model group and non-significant difference compared with control group. (Table 1)

Effects of curcumin on oxidative stress markers, liver tissue AMPK and autophagy protein, beclin 1:

Administration of a high fructose diet to rats increased the liver protein MDA significantly versus the model group, ($p<$**0.0001**) whereas curcumin treatment decreased the MDA content of the liver to basal levels. Concerning the hepatic contents of GSH and activity of SOD, the model group displayed the lowest values, significantly different ($p<$**0.0001**) from the normal and treatment groups. Regarding Nrf-2 DNA-binding activity, it showed significant increase in NASH group compared to control group ,while treatment group revealed, the highest values , significantly

different (p<**0.0001**) from the normal and model groups. (Table 2).

In relation to liver tissue AMPK,(table 2)and beclin 1(figure 1) , there was significant decrease in model group compared to control group ,meanwhile the treatment group showed the highest values, significantly different (p<0.0001) from the normal and model groups .

Effects of curcumin on alterations in liver gene expression of antioxidant marker (Nrf-2) in rats

As shown in Fig. 2; high fructose diet rats were found to have Significant increase in mRNA expression of Nrf-2 as compared to control group group (p<0.0001). Meanwhile the treatment group showed the highest values, significantly different (p<0.0001) from the normal and model groups.

Histopathology

As illustrated in Fig.(3,4,5)

control group displayed normal hepatocytes with a well-preserved cytoplasm, and well defined nuclei, with no steatosis or lymphocytic infiltration while, NASH-induced group showed small fat vacuoles filling hepatocytes cytoplasm (marked microvesicular steatosis), with nuclei pushed to the periphery, with thickened portal tracts, and mild to moderate inflammatory lymphocytic cellular infiltration the intensity of hepatic steatosis and inflammation were significantly alleviated in curcumin treatment group.

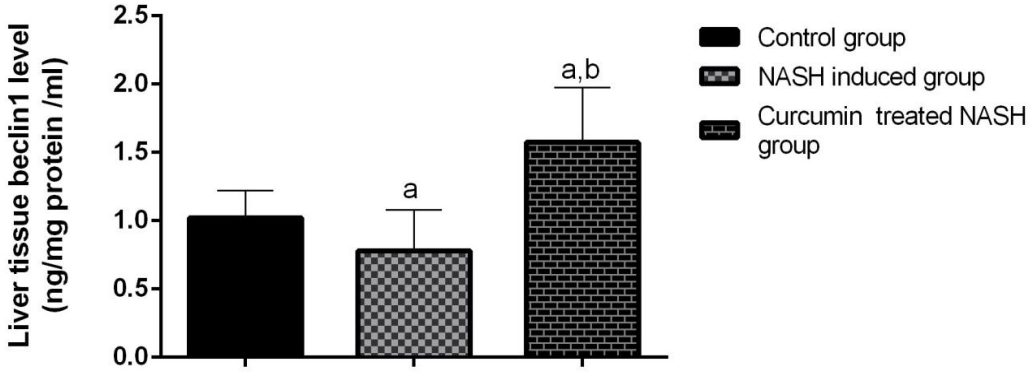

Fig. 1. Liver tissue beclin1 levels. Values are expressed as mean ± SD. *P was considered significant at <0.05; a significant as compared to the control group, b significant as compared to NASH group using One way ANOVA followed by Tukey's post hoc test for multiple comparison.

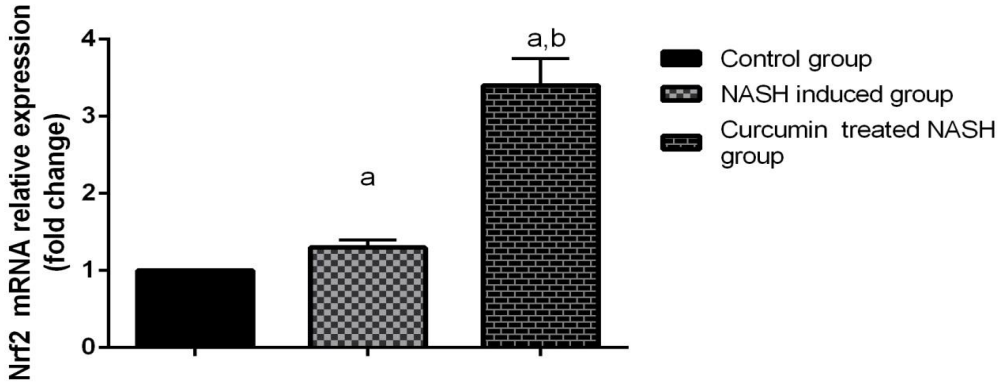

Fig. 2. Nrf2 mRNA relative expression. Values are expressed as mean ± SD. *P was considered significant at <0.05; a significant as compared to the control group, b significant as compared to NASH group using One way ANOVA followed by Tukey's post hoc test for multiple comparison

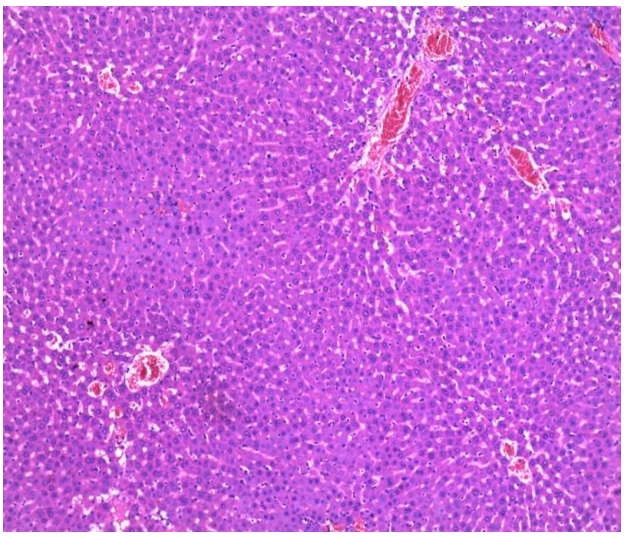

Fig 3. Micrograph from group 1 (control group) showing normal hepatocytes with a well-preserved cytoplasm, and well defined nuclei(H&E 400X).

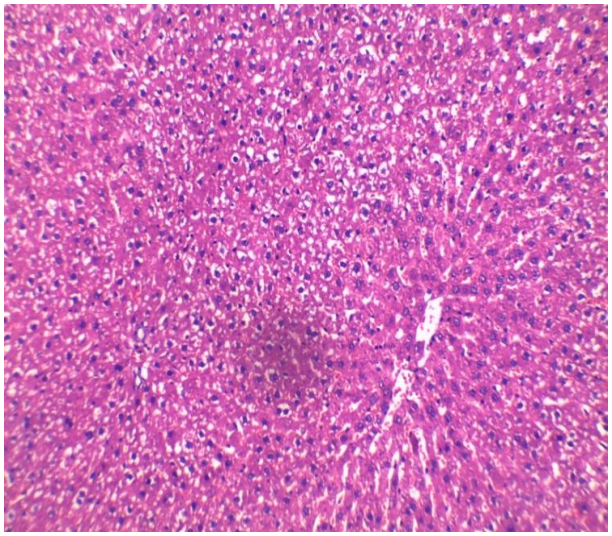

Fig. 4. Micrograph from group 2 (NASH-induced group) showing small fat vacuoles filling hepatocytes cytoplasm (marked microvesicular steatosis), with nuclei pushed to the periphery, with thickened portal tracts, and mild to moderate inflammatory lymphocytic cellular infiltration (H&E200X).

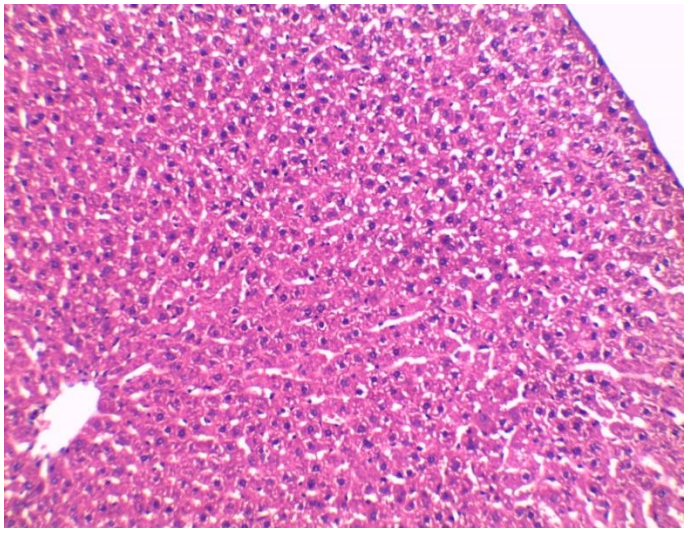

Fig. 5. Micrograph from group 3(curcumin treatment group) showing few inflammatory cellular infiltrate associated with normal liver architecture and normal hepatocytes(H&E200X).

Discussion:

Non-alcoholic fatty liver disease (NAFLD) is the most common hepatic alteration in both affluent and developing countries [24]. The most severe form of this disease is NASH, the role of dietary fructose in inducing NASH has become a focus of attention [25].

Autophagy, oxidative damage, signaling and inflammatory changes have been implicated in the pathogenesis of NASH .So; we used a fructose induced rat model of NASH to understand the potential mechanisms underlying curcumin beneficial effects on these NASH associated changes.

In this current study, NASH has been induced in 20 male albino rats, fructose-induced obesity has been recorded by significant increase in body weight in all rats compared to control group ,concomitant with histopathological alteration of liver specimens and high levels of ALT and AST indicating hepatocellular damage . Hypertriglyceridemia, hypercholesterolemia and significant increase in TAGs content of the liver, were also reported in NASH-induced group. These results came In accordance with previous researches they attributed increased accumulation of fat in the liver to increased hepatic de novo lipogenesis, inhibition of fatty acid beta oxidation, impaired TG clearance and reduced VLD export [26-29].

In fact an increasing body of evidence indicates that fructose in the diet causes obesity and fatty liver disease that commonly progress to NASH , mimicking metabolic disorders in humans [30]. fructose metabolism in the liver is an important player in hepatic steatosis , the major pathway of fructose metabolism in the liver involves the formation of fructose-1-P, under the effect of fructokinase, which is splitted by Aldolase-B to glyceraldehyde and dihydroxyacetone phosphate , both are converted to glyceraldehyde-3-P that is ultimately converted to pyruvate and then acetyl-CoA, bypassing the regulatory mechanisms imposed on phosphofructokinase-1. Excess acetylcoA will be forwarded for de novo synthesis of fatty acids and cholesterol biosynthesis [31]. De novo lipogenesis explained also on the basis that fructose administration induces the carbohydrate response element binding protein and acts synergistically with sterol response element binding protein where both proteins increase the expression of lipogenic genes including those encoding acetyl-coA carboxylase, fatty acid synthase and stearoyl-coA desaturatase enzymes[32]. Meanwhile, it has been reported that hepatic acyl-CoA diacylglycerol acyltransferase enzyme that catalyzes the final step in TG synthesis is often activated in states of energy excess such as fructose-induced obesity[33].

Interestingly, our data depicted that treatment with curcumin resulted in marked reduction of serum ALT and AST ,TC,TG levels ,liver TG content and alleviated hepatic steatosis and inflammation. These data agree with that reported by Li et al [34] who showed that Curcumin administration led to lower degrees of hepatic steatosis and inflammation , lower levels of serum aminotransferases, lipids, homeostasis model assessment of insulin resistance as well as lower serum and hepatic cytokines level. It is assumed that curcumin reduces both hepatic and non-hepatic lipids by lowering the fatty acid synthesis: oxidation ratio ,through activation of a key fatty acid oxidizing enzyme, acyl-CoA oxidase [35].Also it may exert its beneficial effects via reducing insulin and leptin resistance, and attenuating inflammatory cytokine expression [36].

While our data and some reported data showed the hypolipidemic effect of curcumin [37], others indicated that plasma lipid levels are not affected by curcumin supplementation [38]. This discrepancy may be due to diet composition, method of supplementation, duration of treatment and concentration of Curcumin.

According to the two-hit theory , the inflammatory progression to NASH occurs with two sequential hits, the first being hepatic steatosis, and the second being hepatic inflammation caused by oxidative stress associated with lipid peroxidation, cytokine

activation, ROS and endogenous toxins of high fructose intake. [39]

In this study, rats with induced NASH were evidently suffering from both ,oxidative stress, which was manifested as elevated levels of MDA, in face of a concomitant decreased activity of SOD and GSH in the liver and from hepatic inflammation manifested by significant increase in hepatic IL-6 levels. This finding is supported by related study that gave an evidence of increased levels of MDA and reduced activity of SOD in the sera of patients with NASH, and stated that treatment approaches that affect the antioxidant enzymes may be beneficial in patients with NASH. [40] These findings may point to the causal relationship between fat accumulation and emergence of both cytokine activation and oxidative stress in NASH. [41,42] Accumulation of free fatty acids and cholesterol in mitochondria of hepatocytes leads to TNF-α and IL-6 mediated liver injury and reactive oxygen species formation. Endogenous toxins produced secondary to excess dietary fructose intake leads to ROS formation their accumulation in the portal vein can contribute to the development of NASH [42]. It has been reported also, that when antioxidant reserves are depleted, by activating hepatic microsomal oxidases involved in fatty acid oxidation, more oxidative stress and cellular injury occur [44] .

Curcumin treatment resulted in amelioration of oxidative stress, as emphasized by a significant decrease in MDA levels in the liver and boosting antioxidant status by increasing hepatic GSH levels and SOD activity. Curcumin treatment also effects significant decrease in hepatic IL6 levels. Thus confirming the antioxidant effect and the anti-inflammatory role of Curcumin in fructose induced NASH model. This finding matches with that of Inzaugarat ME,et al [53] they have demonstrated the remarkable pro-inflammatory and pro-oxidant influence of curcumin in human and mouse NAFLD/ NASH and showed that Curcumin prevented the development of immunological alterations in this disease.

Given that Nrf2, as a critical transcription factor, regulates cellular lipid metabolism and antioxidant response, the current study detected the gene expression and DNA-binding activity of Nrf-2 in liver tissues. Our data reported an increase in Nrf-2 DNA binding activity and gene expression in liver of NASH group compared to control group, this is explained on the basis of Nrf2 action as a prooxidant stressors sensor so, it is activated in compensation to diminish the harmful effects of ROS [45] . Furthermore, our results revealed that Curcumin treatment group exhibited the highest values, significantly different from the control and model groups . our finding is supported by matched study[34] emphasized the beneficial effect of curcumin in rat model with NASH through activation and modulation of the Nrf2–Keap1 signaling pathway and verifying the central role of Nrf2 in mediating a cytoprotective response against a wide variety of stress and toxic insults.

In fact, there are several stages within this disease progression at which activation of Nrf2 by oxidative or electrophilic stress may exert a potential therapeutic effect. The first is in the initial stages of the disease, when lipids are accumulating in the hepatocytes Nrf2 activation has been shown to effectively decrease gene expression of fatty acid synthesis enzymes [45,47] The second is through

prevention of inflammation by increasing gene expression of Heme oxygenase 1 (Ho-1) and NAD(P)H quinone oxidoreductase 1 (Nqo1),which in turn have inhibitory effects on inflammation through repression of nuclear factor-κB, an important cell-signaling molecule for the inflammatory response. [47].Another mechanism is through increasing expression of Nrf2-regulated gene products including GSH, glutathione peroxidase, HO-1, NAD(P)H quinine oxidoreductase1, glutamate cysteine ligase and many other factors [48]. Finally,Nrf2 could contributes to alleviation of fibrosis in NASH by regulating transforming growth factor-β (TGF-β), a profibrotic signaling factor in plasma[49]. Besides its role in regulating cellular anti-oxidative defense, Nrf2 has also been shown to attenuate insulin resistance and has anti-obesity effect. [50]. The above findings suggest that Nrf2 associates with NASH and may be a novel therapeutic target for the prevention and treatment of fatty liver disease.

Several lines of evidence, highlighted curcumin as Nrf2 activator because it increases Nrf2 expression at the transcriptional and translational levels [51-53]. Curcumin is electrophile , covalently modify the cysteine sulfhydryl groups of KEAP1, thereby altering its conformation and preventing the KEAP1–NRF2 interaction, enables Nrf2 to translocate to the nucleus , binds to the antioxidant-responsive element and initiates the transcription of genes coding antioxidants against oxidative/nitrative stress and inflammation. [51] Curcumin can also elicit its prostate cancer chemopreventive effect inTRAMP C1 cells, potentially through epigenetic modification of the Nrf2 gene with its subsequent induction of the Nrf2-mediated anti-oxidative stress cellular defense pathway [54].All of these support the protective role curcumin in opposing NASH pathogenic changes by triggering Nrf2 signaling.

Autophagy is a protective self-digestion of intracellular organelles in response to stress to maintain homeostasis. Lipophagy is another pathway for lipid degradation besides lipolysis.

In this study, the autophagy pathway has been monitored by measuring Beclin-1, mediator of the nucleation process of autophagy in liver tissue .It displayed significant decrease in NASH group compared to control group .Our results came in line with several researches [55-58], they found that autophagy is partially suppressed in NAFLD/NASH patients and animal models, and restoring autophagy may slow the progression of NAFLD.

Decreased autophagic function in particular may promote the initial development of hepatic steatosis. Steatosis together with oxidative stress that promote overactivation of the JNK/c-Jun signaling pathway and inflammatory cytokines, will trigger hepatocellular injury and death, leading to progression of steatosis to liver injury and NASH. [59]

In the current study, the use of curcumin markedly improve autophagy checked by elevated Beclin-1. Beclin 1 can intervene at every major step in autophagic pathways, from autophagosome formation, to autophagosome/endosome maturation many of these effects are mediated through the activation of specific Beclin 1-binding proteins .Also It plays a critical role in modulating the

magnitude of the autophagic response by regulating phosphatidyl inositol 3 kinase dependent generation of phosphatidyl inositol 3 phosphate and subsequent recruitment of additional proteins involved in autophagosome formation . [60] .

Autophagy have Many protective mechanisms that lead to cell survival from death stimuli including the removal of damaged organelles or proteins that contribute to cellular dysfunction as abnormal mitochondria, that may lead to oxidative stress or the release of mitochondrial factors that trigger apoptosis [60] .In addition ,autophagy decrease triglyceride and cholesterol accumulation, improve insulin signaling, and prevent cellular injury from oxidative stress by Nrf2 Activation via interaction of the selective autophagy substrate p62 with the Nrf2 binding site on Keap1.Also, it Block TNFα and Fas death receptor-mediated liver injury ,Reduce endoplasmic reticulum stress and the resultant cellular damage and insulin resistance ,and Prevent hepatocellular carcinoma development. [61] However, autophagy is a double-edged sword it protects hepatocytes by inhibiting oxidative stress and inflammation yet, its over-stimulation may result in autophagic cell death that aggravates any existing liver damage[63].

NAFLD is associated with low hepatic ATP levels and increased nucleotide turnover resulting in substantial AMP generation. Surprisingly, despite of reductions in liver ATP contents and in agreement with previous studies [64-66],our results demonstrated significant decrease in liver AMPK levels in NASH group compared to control group, suggesting that additional factors are important for controlling AMPK activity in the liver during NASH. One possibility may be that inflammatory factors known to be elevated with NASH, such as lipopolysaccharide (LPS) tumor necrosis factor-α (TNFα), and IL6 reduce AMPK activity [66] another possibility posit that surges of fructose in the portal vein lead to increased unregulated flux to trioses accompanied by unavoidable methylglyoxal production, sudden flux exerts carbonyl stress on the three arginines on the c subunits AMP binding site of AMPK, irreversibly blocking some of the enzyme molecules to allosteric modulation, this explains why, even when fructose quick phosphorylation increases AMP and should therefore activate AMPK, the effects of fructose are compatible with inactivation of AMPK, which then solves the apparent metabolic paradox. [69].

In harmony with other studies [70-71], our data depicted that treatment with curcumin resulted in marked increase in liver AMPK, that is associated with improvement of liver markers, lipid profile and histopathology and explained as follow: once activated, AMPK-mediated downstream phosphorylation events switch cells from active ATP consumption to active ATP production thus, it increases glucose transport, glycolysis, beta oxidation, and inhibits lipogenesis and cholesterol biosynthesis. AMPK also inhibits mTOR pathways leading to activation of mitochondrial biogenesis and autophagy. [72-73].

In conclusion, our study provided some insights into the pathogenesis of NASH, and highlighted the potential ameliorative role of Curcumin as it modify many serial and parallel pathways as autophagy, inflammatory response, AMPK activity, and redox balance as evident by marked alleviations of the liver MDA and elevating both SOD activity and GSH levels and activating Nrf2

signaling pathway .Since curcumin has a good safety profile, its role in the prevention and treatment of NASH merits further investigation evaluating its effect of on patients with NAFLD.

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