Effects of Caffeine Intake on Oxido/Inflammatory Axis in Rat Model of Non-Alcoholic Fatty Liver Disease

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Abstract

The precise mechanism of caffeine modulating effects on NAFLD is completely unknown. So, the aim of this study was to evaluate the modulating effects of caffeine on some biochemical markers in obesity induced NAFLD. Forty male albino rats were divided into four groups of 10 rats each. The rats of group 1 served as normal control. Group 2 received control diet and caffeine in a concentration of 1g/L orally. Group 3 received a high fat diet (HFD). Group 4 received a high fat diet and was given caffeine just like group 2. After 20 weeks, serum and liver tissues were obtained from the sacrificed rats. Total lipid profile, blood glucose levels, liver triacylglycerols (TAGs) and serum activity of alanine aminotransferase (ALT) were estimated. Tissue 4-hydroxy-2-nonenal (4-HNE) as a marker for oxidative stress, calgranulin S100 A8, receptor for advanced glycation end products (RAGE) and toll like receptor-4 (TLR4) were immunoassayed. Plasma caffeine levels were estimated by ultra performance liquid chromatography. NAFLD was confirmed by histopathological results as well as increased serum activity of ALT, marked dyslipidemia and high blood glucose levels. NAFLD led to elevations in liver TAGs, 4–HNE, calgranulin S100 A8, RAGE and TLR4. Improvement in all assessed parameters except TLR4, was observed in NAFLD rat group treated with caffeine. Caffeine had a modulatory action on NAFLD which may be through improving the antioxidant status in hepatic tissue and/or suppressing some inflammatory cascades. It is hoped that these findings would assist in development of NAFLD management strategies that may use caffeine as a potential drug for treatment.

Keywords

- Obesity
- Non-alcoholic fatty liver disease (NAFLD);
- Caffeine
- 4-hydroxy-2-nonenal (4-HNE)
- Calgranulin S100 A8
- Receptor for advanced glycation end products (RAGE)
- Toll like receptor-4 (TLR4)

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Introduction

One of the most common causes of chronic liver diseases worldwide is non-alcoholic fatty liver disease (NAFLD). NAFLD is ranged from non-progressive simple steatosis to non-alcoholic steatohepatitis (NASH) with ballooning degeneration, fibrosis and inflammation which may ends after decade years with hepatocellular carcinoma. Also, hypertension and dyslipidemia with increased lipids accumulation, mainly triacylglycerols and cholesterol are associated with NAFLD (1). These features may suggest the role of NAFLD in metabolic syndrome. Inflammatory processes and oxidative stress are induced by lipotoxicity of hepatic fat which trigger progressive liver damage. Necroinflammation of the liver and reactive oxygen species (ROS) with subsequent induction of hepatic steatosis and fibrosis appear to be a result of oxidative stress which is considered a main contributor of simple fatty liver progression to NASH (2). ROS can initiate lipid peroxidation by attacking polyunsaturated fatty acids (PUFA) and cause the formation of 4-hydroxy-2-nonenal (4-HNE) or malondialdehyde (MDA) which are aldehyde by-products (3). ROS has shorter half-lives than 4-HNE and MDA, which can diffuse into other sites, spreading oxidative stress. 4-hydroxynonenal is a biomarker for oxidative stress, as well as, an important mediator of a number of signaling pathways (4). The family of S100 calcium-binding proteins, is composed of subgroups, which are S100A8 (calgranulin A, referred to as MRP8), S100A9 (calgranulin B, MRP14) and S100A12 (calgranulin C). Their hallmark is at the sites of chronic inflammation, where they are accumulated (5). In neutrophils, the binding of polyunsaturated fatty acids like arachidonic acid to S100A8/A9 is the main contributor of their uptake and release which is regulated by zinc (6). Internalization of arachidonic acid-calprotectin complex to form eicosanoids occurs by nearby cells and is responsible for inflammatory responses initiation and regulation. Also, S100A8/A9 increases ROS generation by facilitating the activation of NADPH oxidase and arachidonic acid binding (7). Acute and chronic inflammation was mediated by the important roles of both receptor for advanced glycation end products (RAGE) and toll like receptors (8). RAGE was firstly activated by binding to bovine S100/calgranulins which mediate a pro-inflammatory response in macrophages and in cultured endothelial cells (9). Increased secretion of the inflammatory markers, IL-6, TNFα and IL1β was mediated by the cell surface receptor RAGE (9). In addition, S100/calgranulins mediated amplification of inflammation, autoimmunity, infection and cancer occurred by binding to its toll like receptor 4 (TLR-4) binding partner (10). Caffeine (1, 3, 7-trimethylxanthine) is widely consumed, for its central nervous system stimulating effect and is present in several foods and beverages like coffee and tea (11). Caffeine has been widely used in athletic competitions due its ergogenic effects on skeletal muscle metabolism. Recent studies showed a beneficial role of caffeine on liver injury in animals and in vitro studies. The precise mechanism is completely unknown. Previous studies did not investigate the modulating effects of caffeine on the oxidative stress marker, 4-HNE and the inflammatory marker, calgranulin
S100A8 and its mediators, RAGE and TLR-4 in an obesity induced NAFLD rat model. In view of these considerations, we investigated caffeine effect on the previous biochemical markers, hoping to provide new mechanistic insights of caffeine action on these parameters.

**Materials and methods**

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

**Animals**

All animals received appropriate care in compliance with the Public Health Service Policy on Use of Laboratory Animals published by the National Institutes of Health and were approved by the Ethical Committee of the Faculty of Medicine, Tanta University, Egypt. All the procedures described below were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments.

**Experimental design:**

The study included 40 male albino wistar rats, each weighing 210-245 grams. The rats were housed at a constant temperature of 28±4°C, and 12 h light /dark cycle. The animals were fed a control diet and given tap water ad libitum. After an acclimation period of one week, the 40 rats were divided randomly into four groups (10 rats in each group). The rats of the first group (group 1) served as control group and kept on normal feeding without any treatment. The rats of the second group (group 2), received control diet and caffeine in a concentration of 1g/L that was administered in drinking water during the period of the experiment, (group 3) received a high fat diet (HFD) and was given tap water ad libitum. The fourth group (group 4) received also high fat diet and was given caffeine just like group 2. The compositions of the control diet and HFD were as follows: in each 100 grams of control diet there were 60 grams carbohydrates, 4 grams fat, 20 grams protein and of 3.56 calories. Also, in each 100 grams of HFD there were 20 grams carbohydrates, 41 grams fat, 22 grams protein and of 5.73 calories.

**Blood sampling**

After 20 weeks, rats were fasted overnight with allowance for free access to caffeine solutions or water. Afterwards, the animals were killed by cardiac puncture after ether anesthesia. Blood was collected by cardiac puncture and 1.5 ml of the blood sample was added to heparinized polypropylene tubes and centrifuged for 10 min at 3500 rpm under a controlled temperature of 20°C. The plasma supernatant was carefully collected in polypropylene tubes and frozen at −80°C until analyzed for measurement of caffeine levels. Serum samples were obtained by centrifuging the remaining blood at 3000 rpm at 4°C for 10 minutes and the supernatants were transferred into tubes for separate biochemical assay and maintained at −80°C.

**Preparation of liver tissue homogenates**

The liver pieces were washed in ice cold isotonic saline and blotted individually on ash-free filter paper, the tissues were divided into 3 pieces, one piece was homogenized separately in cold 5% phosphate buffer saline (0.1 M, pH 7.4), then stored overnight at -20°C. Then two freeze-thaw
cycles were performed to break the cell membranes; the homogenates were centrifuged for 15 minutes at 5000 x g at 2 - 8°C. The clear supernatants were stored at -80 °C until use. The second piece of liver tissue was fixed in 10% buffered formalin, processed and embedded in paraffin for hematoxylin-eosin (H&E) staining and histolopathological examination, and the third one was stored at -80°C for estimation of triacylglycerols content. Tissue protein levels were measured according to the method used by Lowry et al (12).

**Biochemical assays**

Serum levels of glucose, triacylglycerols (TAG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and very low density lipoprotein cholesterol (VLDL-C), as well as alanine transaminase (ALT) activity were estimated by the use of colorimetric diagnostic kits supplied by Spinreact, Germany. They were measured by spectrophotometer (CA, USA). The LDL-cholesterol was estimated according to the following formula, LDL-C = TC – HDL-C - TG/5 (13).

**Liver triacylglycerols** content were measured by a commercial kit (Catalog# ab65336, triglyceride quantification assay kit, Abcam). Frozen liver tissues were taken and washed in cold PBS then re-suspended and homogenized in 1 mL of 5%NP-40/ddH2O solution (4-Nonylphenyl poly ethylene glycol). Then the samples were slowly heated to 80 – 100°C in a water bath for 2 -5 minutes and until the NP-40 became cloudy, then cooled down to room temperature. Heating was repeated one more time to solubilize the triacylglycerols. Then the samples were centrifuged for 2 minutes at top speed using a microcentrifuge to remove any insoluble material. Finally, the samples were diluted 10-fold with double distilled water (ddH2O) before proceeding with the assay. In this assay, triacylglycerols were converted to free fatty acids and glycerol. The glycerol was then oxidized to generate a product which reacts with the probe to generate colour. Colour intensity was measured spectrophotometrically at 570 nm.

**Liver 4-HNE, calgranulin S100 A8, RAGE and TLR4** were measured in tissue homogenates using ELISA kits: Catalog #AMS.E02H 0203, Rat 4 Hydroxynonenal, Amsbio; Catalog# MBS924882, Rat / S100A8, MyBiosource; Catalog# ab100780, rat RAGE, Abcam and Catalog# SEA753Ra, rat TLR4, Cloud Clone Corp , respectively, according to the manufacturer's instructions. The intensity of colours was measured spectrophotometrically at 450 nm in a microplate reader.

**Caffeine levels in plasma were measured using ultra performance liquid chromatography (UPLC):** One hundred microliters of 30% (v/v) perchloric acid (40 µg) (i.e 30 % solution of perchloric acid (70% w/v) that contains 40 µg perchloric acid in each 100 µl) were added to 250 µl of plasma samples and calibration standards in a 1.5 ml microcentrifuge tubes. The tubes were vortexed for 20 seconds then centrifuged at 12000 rpm for 10 min. The supernatants clear layer were collected and injected into the chromatographic system using an autosampler. Chromatography was performed on a Waters Acquity UPLC (Waters Associates, Inc., Milford, MA) consisting
of a quaternary pump, autosampler, column thermostat, and photodiode array detector. ARP Atlantis C18 (4.6×150mm, 5-µm) column and a guard pak pre-column module with a Bondapak C18, 4-µm insert were used for the separation. The data were collected with a Pentium IV computer using Empower Chromatography Manager Software. The mobile phase consisted of 15 mM potassium phosphate (pH 3.50±0.05, adjusted with phosphoric acid) and acetonitrile (83:17, v/v). The analysis was carried out under isocratic conditions using a flow rate of 600 µl/min. Chromatograms were recorded at 274 nm with a runtime of 5 min.

**Histopathological study:**

The liver tissue was kept in 10% formalin solution and embedded in paraffin. Five-µm slices were stained with hematoxylin & eosin (H&E) and photomicrographs were taken at 200 x.

**Statistical methods:**

The data were statistically treated using unpaired Student’s t test. A p-value of 0.05 was considered significant. The statistical analyses were carried out with Prism version 4 (GraphPad, San Diego, USA). Data are shown as mean ± standard deviation.

**Results**

The weight gain in grams was calculated as the difference between the body weight at the end and at the beginning of the 20 weeks. As seen in fig.1, The differences between group 1 and 2 was not significant, while there was a statistically significant increase in weight gain in group 3 compared to other groups, also group 4 showed significant increase when compared to groups 1 and 2 and a significant decrease when compared to group 3. As shown in Table 1, rats fed a HFD alone displayed higher circulating levels of TC, TAG, LDL, and VLDL levels than did groups 1 and 2 of rats. After 20 weeks, the serum TAG levels in the HFD group increased approximately two-fold compared to the control group, whereas the TC concentration only increased by 62.8% in the HFD group; no significant difference was observed between group 2 and Control groups with respect to circulating TC, and TAG levels. Also, there was no significant difference between the circulating levels of TC, HDL, and LDL in groups 3 and 4. High fat diet caused significant reduction in the HDL levels and this reduction was not corrected by caffeine intake. However; caffeine caused statistically significant reduction in TAG, and VLDL levels in HFD fed animals. Also, HFD caused increased fasting blood glucose level, however; caffeine caused statistically significant reduction in fasting blood glucose in both normal diet and HFD fed groups comparing to the other two groups. Also, HFD caused raising of the serum levels of ALT to about double their normal values, and this increase was not observed in animals fed HFD and received caffeine orally. In table 2, liver tissue showed higher content of triacylglycerol in HFD fed animals, whether they received caffeine or not, however; caffeine caused some reduction in the liver tissue triglyceride content in HFD fed animals, but it still higher than the normal diet fed animals. As regard to 4-NHE tissue content, HFD caused marked elevation in its level, caffeine treatment caused normalization of its level in HFD fed group. In groups that
received normal diet, caffeine caused also reduction in 4-NHE levels. Liver tissue content of calgranulin A8S100 was highest in HFD fed group, caffeine reduced its level but it was still higher than that in the normal diet fed group. However, this effect of caffeine was not manifest in normal diet fed animals. As well, the liver tissue levels of RAGE were significantly increased in animals consuming HFD, caffeine treatment did not change the elevated levels of TLR in HFD fed animal groups. Fig. 2, showed chromatogram of caffeine standard. As expected, by UPLC, plasma caffeine was undetectable in groups 1 and 3, while its levels were 2.84 ±1.8 µg/ml in group 2, and 3.12+1.04 µg/ml in group 4 with no statistically significant difference between the two groups.

Table 1. Serum lipid profile, fasting blood glucose levels and serum ALT activity in the studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum total cholesterol (mg/dl)</th>
<th>Serum TAG (mg/dl)</th>
<th>Serum HDL (mg/dl)</th>
<th>Serum LDL (mg/dl)</th>
<th>Serum VLDL (mg/dl)</th>
<th>Serum glucose (mg/dl)</th>
<th>Serum ALT activity (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n=10)</td>
<td>93.1±2.1 c,d</td>
<td>98.21±3.3 c,d</td>
<td>26.6±1.38 b,c,d</td>
<td>31.11±1.12 b,c,d</td>
<td>15.24±1.04 b,c,d</td>
<td>126±13.66 b,c,d</td>
<td>47.73±3.17 c</td>
</tr>
<tr>
<td>Group 2 (n=10)</td>
<td>90.31±3.4 c,d</td>
<td>95.4±2.6 c,d</td>
<td>24.7±2.1 a,c,d</td>
<td>29.4±2.01 a,c,d</td>
<td>13.14±0.97 a,c,d</td>
<td>119±12.43 a,c,d</td>
<td>44.97±2.11 c</td>
</tr>
<tr>
<td>Group 3 (n=10)</td>
<td>148.22±5.1 a,b,c,d</td>
<td>188.77±3.2 a,b,c</td>
<td>19.2±1.4 a,b</td>
<td>48.32±4.30 a,b</td>
<td>27.61±1.81 a,b</td>
<td>220±14.82 a,b</td>
<td>88.52±4.08 a,c,d</td>
</tr>
<tr>
<td>Group 4 (n=10)</td>
<td>151±4.1 a,b,c</td>
<td>164.2±6.3 a,b,c</td>
<td>20.32±2.2 a,b</td>
<td>45.61±5.23 a,b</td>
<td>23.3±2.24 a,b</td>
<td>195±15.21 a,b,c</td>
<td>46.81±5.87 a,b,c</td>
</tr>
</tbody>
</table>

Statistically significant differences when compared to the group number shown using unpaired Student’s t test. Data are presented as mean ±SD. P≤0.05 was considered significant. Group 1 (control), group 2 (control caffeine), group 3 (high fat diet), group 4 (high fat diet treated with caffeine).TAG: triacylglycerols; HDL: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; VLDL: very low density lipoprotein cholesterol; ALT: alanine amino transferase.

Table 2. Liver tissue content of TAG, 4NHE, calgranulin A8S100, RAGE and TLR4 in the studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver triacylglycerols (nmol/mg protein)</th>
<th>Liver 4NHE (µg/mg protein)</th>
<th>Liver calgranulin A8S100 (ng/mg protein)</th>
<th>Liver RAGE (ng/mg protein)</th>
<th>Liver TLR4 (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n=10)</td>
<td>98±5.53 a,b,c,d</td>
<td>1.7±0.10 b,c</td>
<td>112±14.2 2 a,b</td>
<td>17.3±2.1 c,d</td>
<td>0.8±0.13 c,d</td>
</tr>
<tr>
<td>Group 2 (n=10)</td>
<td>92±6.27 a,b,c,d</td>
<td>1.3±0.08 a,c,d</td>
<td>124±11.8 1 c,d</td>
<td>18.1±3.2 c,d</td>
<td>0.9±0.10 c,d</td>
</tr>
<tr>
<td>Group 3 (n=10)</td>
<td>416±11.46 a,b,c,d</td>
<td>2.5±0.35 a,b,d</td>
<td>760±42.7 1 b,d</td>
<td>32.4±6.21 b,d</td>
<td>1.82±0.30 a,b</td>
</tr>
<tr>
<td>Group 4 (n=10)</td>
<td>262±7.16 a,b,c,d</td>
<td>1.8±0.14 b,c</td>
<td>320±21.6 1 b,c</td>
<td>23.1±4.41 b,c</td>
<td>1.91±0.11 a,b</td>
</tr>
</tbody>
</table>

Statistically significant differences when compared to the group number shown using unpaired Student’s t test. Data are presented as mean ±SD. P≤0.05 was considered significant. Group 1 (control), group 2 (control caffeine), group 3 (high fat diet), group 4 (high fat diet treated with caffeine).4NHE: 4-hydroxy-2-nonenal; RAGE: receptor for advanced glycated end product; TLR4: toll like receptor-4; ALT: alanine amino transferase.
Modulating effects of caffeine on non-alcoholic fatty liver disease

Figure 1. Body weight gained by the rats in different groups in grams at the end of the experiment. Group 1 (control), group 2 (control caffeine), group 3 (high fat diet), group 4 (high fat diet treated with caffeine).

Figure 2. Chromatogram of caffeine standard

Histopathological examination:
Normal lobular architecture exhibiting normal hepatocytes arranged in cords around central vein was seen in sections of control group 1 (fig.3). Also, sections of group 2 showed normal liver architecture and pattern (fig.4). Sections of group 3 showed diffuse severe fatty degeneration, local inflammatory cellular infiltration associated with foci of cellular necrosis (fig.5). Liver sections of group 4 showed few inflammatory cellular infiltrate associated with normal liver architecture and normal hepatocytes (fig. 6).

DISCUSSION:
NAFLD is the most common chronic liver disease and is associated with metabolic problems (1). A high fat diet induced NAFLD in rat model was utilized in the current work to study the modulatory effects of caffeine on this disease. Many animal models for NAFLD have been developed, these models do not replicate the exact full picture of the disease in humans, however; they can be utilized to verify different hypotheses on the pathogenesis and development of NAFLD, as well as performing interventional studies. The HFD model reported by Lieber et al. (14)
constituted of 71% of energy from fat, 18% from proteins and 11% from carbohydrates was used with some modifications in this study to induce NAFLD in rats. Using of this HFD formula for 20 weeks induced a picture resembling NAFLD in our rats, as shown by increased liver size in the gross picture, marked pan lobular steatosis in histopathology, duplication of the TAG content of hepatic tissue and ALT levels in blood as shown by the biochemical analysis. These changes were in consistence with those found by Ito et al. (15) regarding the body weight of the animals in the different groups in this study, HFD feeding caused significant increase in body weight, however, caffeine consumption partially ameliorated this effect. Weight gain induction by HFD was also observed by Chen et al. (16) and Matsuzawa-Nagataa et al.(17). On the other hand, other studies showed that HFD feeding did not induce weight gain, and rats received HFD consumed less feed. A possible explanation for these contradictory findings is the discrepancy in
the time of exposure of the animals for the HFD; it is well known that HFD induces a positive fat balance on a short-term basis because of the imbalance between fat consumption and oxidation. In the long term, fat accumulate and lead to weight gain, so, the longer the duration of exposure to HFD, the greater the gain in body weight (18). Another factor that may affect the weight gain of the rats is the type of lipid used in the HFD formula, there is absence of standardization of HFD used in different studies. In this study caffeine reduced weight gain, and this result is in agreement with the results of other studies. It has been shown that caffeine causes weight loss by increasing the sympathetic tone of adipose tissue (19). Studies on rats demonstrated that caffeine can cause release of great amounts of norepinepherine (NE) by stimulation of sympathetic post-synaptic nerves onto the richly innervated adipose tissue. This effect results in increasing sympathetic stimulation throughout the body, thus, indirectly stimulates thermo-genesis. Also, caffeine can increase thermo-genesis and elevation of cytoplasmic cAMP by inhibiting its destruction by phosphodiesterase (PDE) enzyme. As well, caffeine is an adenosine receptor antagonist that can increase cAMP levels in adipose cells by preventing the inhibition of NE release by extracellular adenosine (19). Moreover, in an in vitro study, it was suggested that caffeine can inhibit adipocyte differentiation and development through inhibition of adipogenic related factors (20). These roles of caffeine may explain the reduction of body weight after caffeine treatment. Regarding fasting blood glucose levels and the lipid profile, HFD feeding caused significant hyperglycaemia and dyslipidemia. Both of them were more or less improved by caffeine consumption. This was in agreement with the findings of Xie et al. (21) who found that HFD caused hyperglycaemia and hypercholesterolemia in rats. Also, other studies found that caffeine consumption caused reduction in TC and LDL levels in both animals and humans. On the other hand, Akagiri et al. (22) did not detect any difference in the fasting blood glucose levels between control and HFD fed groups, which may be due to exposure of the animals in this study for a shorter duration to HFD. It has been found that caffeine increases glucose transporter type (GLUT-4) mRNA in skeletal muscles and increase insulin independent glucose transport by adenosine monophosphate activated protein kinase (AMPK) (23). Caffeine also activates the cyclic AMP dependent protein kinase alpha-1 isoform, and increases AMP kinase-1 that promotes glucose transport (24). Another mechanism could be the action of caffeine as an antagonist of adenosine A1 receptor, which improves glucose tolerance in rats. An imbalance between formation and scavenging of ROS and an impaired antioxidant defence is observed in cases of NAFLD, this explains the elevated tissue levels of 4-NHE in the livers of HFD fed rats with NAFLD (25). The present study results were in line with other studies which showed that caffeine reduces liver tissue oxidative stress that may be through increasing the expression of antioxidant and stress sensor proteins (25). Also, it may be due to β-oxidation by an auto phago-lysosomal pathway stimulation by caffeine. Another study done by Kamat et al. (26) showed that caffeine inhibited lipid peroxidation by reducing lipid peroxides in rat liver microsomes. These findings explain the
reduction or 4-NEH observed in liver tissue of animals received caffeine orally. Oxidative stress can provoke neutrophil inflammatory response, and it has been well established as a trigger for inflammation and the pathogenesis of NAFLD (26). S100A8 are actively secreted by phagocytes during inflammation, and are considered as a marker for the inflammatory process. (5) In our study, liver tissue of HFD fed animals showed significantly higher levels of S100A8, while HFD fed animals receiving caffeine did not show such high levels, this indicates that caffeine has a role is suppressing the inflammatory process. These finding are in consistence with Hosoi et al. (27) who suggested that caffeine improves the endoplasmic reticulum stress, and also reduces the level of the inflammatory cytokines. Endoplasmic reticulum (ER) is an organelle that performs modification, folding and trafficking of membrane and secretory proteins to the Golgi compartment. In case of disruption, ER generates the unfolded protein response (UPR), which is an adaptive signalling pathway to maintain its homeostasis. However, failure to restore this homeostasis, results in ER death signaling pathways initiation (28, 29). There have been numerous evidences contributing NAFLD to ER stress and activation of the inflammatory signaling pathways (30). Expression of genes associated with ER stress were found to be markedly reduced by caffeine including, inositol-requiring transmembrane kinase/endonuclease 1 (IRE1), pancreatic ER kinase (PERK) activating transcription factor 6 (ATF6) and CCAAT/enhancer-binding protein homologous protein (CHOP), indicating that caffeine may improve ER stress to protect liver cell (30). S100A8 released by activated neutrophils is suggested to exert its extracellular pro-inflammatory effects via interaction with other receptors such RAGE and TLRs (9, 10). RAGE was first described as a receptor for advanced glycation end products (AGEs) which are formed as a result of hyperglycaemia, oxidative stress, and inflammation. It has been found that in addition to AGEs, RAGE has other natural ligands such as the calgranulin s100A8 (31).In our study, RAGE levels were higher in HFD fed rats, which was in consistence with other studies that suggested that increased fatty acid and lipid peroxidation in the liver may promote the induction of NAFLD, and enhance RAGE expression (32).On the other hand caffeine consumption caused a significant reduction in RAGE liver tissue levels. A study done by Yeh et al. (33) showed that fructose fed animals treated with caffeine displayed significantly reduced levels of RAGEs expression. This could be also helped by the improved glycaemia in caffeine treated animals that led to lower levels of AGEs formation, as well as S100A8 reduction. Both factors could cause down-regulation of RAGE expression, as it is known that RAGE ligands upregulate RAGE expression in the liver. Regarding the levels of TLR4, HFD fed animals showed higher levels compared to control, regardless of caffeine treatment. This was in agreement with other studies and was related to many factors like exposure of the hepatocyte to high levels of TLR ligands derived from gut bacterial products caused by altered gut permeability noticed in cases of NAFLD, or high levels of free fatty acids and denatured DNA in such cases (34).On the other hand, a study done on cord blood monocytes exposed to high concentrations of caffeine showed
that caffeine can induce TLR4 gene expression (35). However, this study used very high concentrations of caffeine. Moreover, caffeine may inhibit the inflammatory process through the inhibition of TLR mediated inflammatory cascades by suppressing calcium mobilization (36). In addition, a study of Chen et al. (37) showed that S100A8 induced modest levels of TLR4-mediated cytokine production from human peripheral blood mononuclear cells.

CONCLUSION:
Caffeine could be considered as a potential drug for improving NAFLD due to its modulatory action through improving the antioxidant status in hepatic tissue, evidenced by 4-NHE levels reduction and suppressing some inflammatory cascades through inhibition of calgranulin S100A8 formation and RAGE levels.

Limitations of the study:
There were certain limitations to the study. As this was an animal study, it would be useful to repeat the study on other experimental models of NAFLD, to verify the results. Further research is required to determine the possible secondary effects of caffeine on both animals, and humans and to induce NAFLD prior to administering caffeine, to observe whether this effect can be modified in existing disease.

Acknowledgments:
We would like to acknowledge dr.Yomna Zamzam, Lecturer of Pathology, Faculty of Medicine, Tanta University, for the histopathology results of this work.

Funding:
This research did not receive any specific grant or funding.

Conflict of interests:
The authors declare that they have no conflict of interests in relation to this work.

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