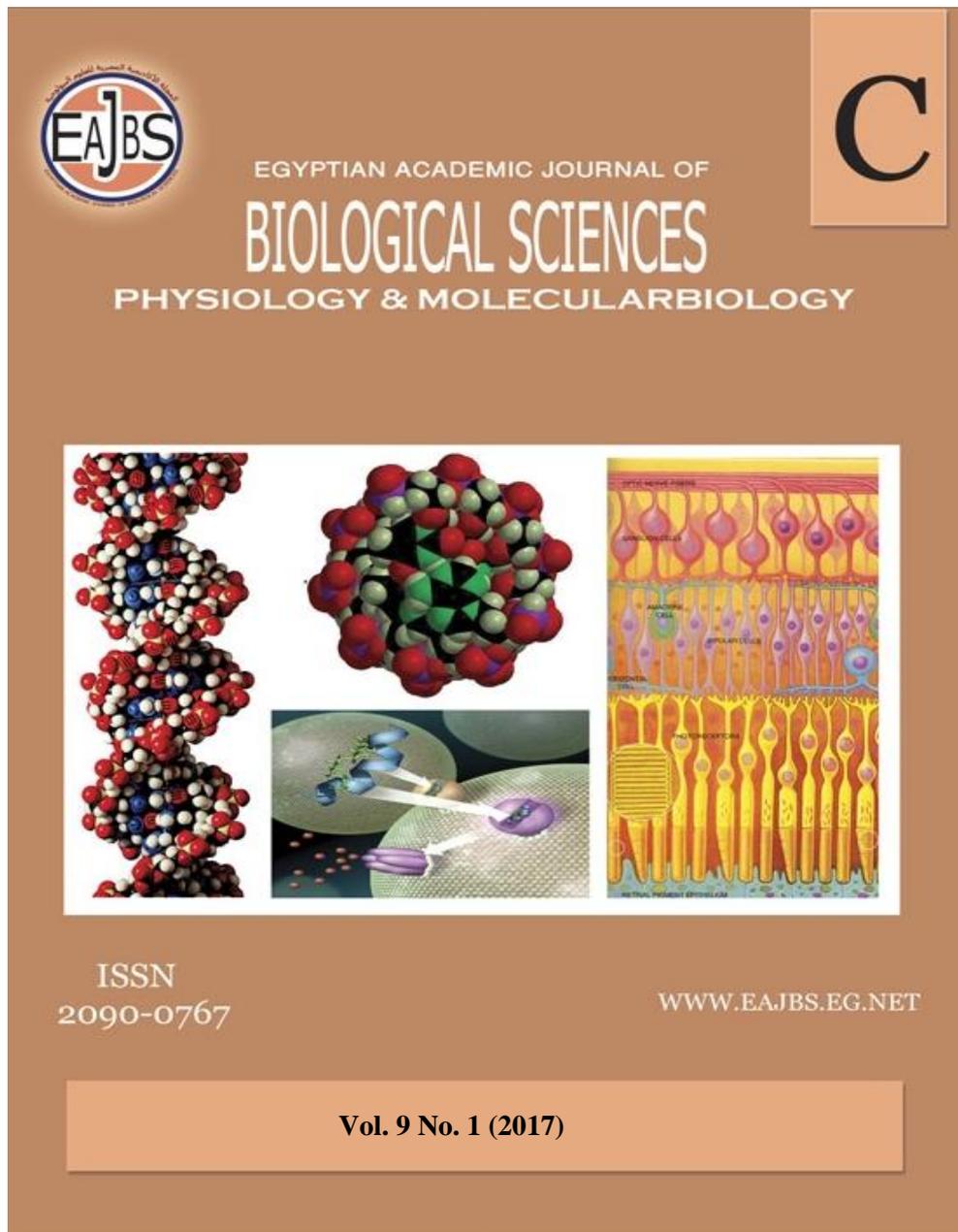


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Cyto-protective Role of Autophagy Against High Fat Diet Induced Hepatocellular injury

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ABSTRACT

Autophagy is catabolic machinery that regulates cellular homeostasis in normal and stress conditions. There is a contradiction concerning the role of autophagy in liver injury. It has been reported that autophagy may act as profibrogenic or anti-fibrogenic cellular machinery. Here we investigated the role of autophagy in high fat diet induced liver injury in mice. Mice were divided into four groups: normal diet feeding as control, high fat diet (HFD) feeding treated or not with chloroquine or rapamycin for autophagy modulation. The expression of active caspase 3, LC3 and P62 was evaluated in liver by western blot for testing the progressing of apoptosis and autophagy, respectively. Levels of mRNA of inflammation markers (Ccl2 and Ccl5) and fibrogenic markers (COL1a1 and PAI1) were quantified by QRT-PCR in liver of all animal groups, besides histological and biochemical means of general liver injury markers. The level of apoptosis, inflammation and fibrosis markers were increased in HFD mice and massively increased in chloroquine treated group whereas, rapamycin reduced all of them. Histopathological and biochemical observations coincided with the above mentioned results concerning liver injury under autophagy modulation. Autophagy acts as protective cellular machinery that attenuates the signs of liver injury including apoptosis, inflammation and fibrosis.

INTRODUCTION

Autophagy is catabolic cellular machinery for degradation of the excess and damaged cytosolic proteins and organelles in order to recycling their macromolecular components (Yorimitsu and Klionsky, 2005). Autophagy is initiated in many biological activities and diseases (Ravikumar *et al.*, 2011; Mizushima *et al.*, 2008). There are three types of autophagy (i.e, macroautophagy, microautophagy and chaperone-mediated autophagy) depending on cellular function and pathway of cargo-lysosomal delivery (Mizushima *et al.*, 2008; Boya and Codogno, 2012). The well-characterized type in eukaryotic cells is the macroautophagy (hereafter referred to as autophagy). Autophagy starts by the sequestration of the cytoplasmic material into a double membrane vesicle known as autophagosome.

The latter originates from a membrane elongation from endoplasmic reticulum called phagophore. Finally the autophagosome fuses with lysosome to form the autophagolysosome where the cargo material is degraded by lysosomal enzymes (Mizushima, 2007). Autophagy initiation is controlled by many diverse signals including amino acids, glucose and growth factors (Jewell and Guan, 2013).

Under normal physiological condition autophagy is an important regulator of liver homeostasis and also in various liver diseases including non-alcoholic fatty liver, viral hepatitis and hepatocellular carcinoma (Codogno and Meijer, 2013; Czaja *et al.*, 2013; Rautou *et al.*, 2011). During liver injury, hepatic fibrogenic cells accumulate at the site of injury in response to paracrine and autocrine signal sources (Mallat and Lotersztajn, 2013; Lotersztajn *et al.*, 2005). The main source of hepatic fibrogenic cells is the hepatic stellate cells (HSC). Liver injury induces signals that in turn promote the transformation of quiescent HSC to active form with proinflammatory and fibrogenic characters (Schuppan and Kim, 2013; Iredale, 2013; Hernandez-Gea and Friedman, 2011). The active HSC has phagocytic activity towards apoptotic hepatocytes which provides resistance of HSC to apoptotic cell death and in turn increases its fibrogenic power (Jiang *et al.*, 2010). It has been reported that autophagy attenuates liver fibrosis through hepatocytes protection against apoptosis and inflammation (Amir *et al.*, 2013; Lodder *et al.*, 2013). In contrast, other studies reported that autophagy contributes to fibrosis through activation of HSC (Hernandez-Gea *et al.*, 2012; Thoen *et al.*, 2011). In the present study, we investigated the role of autophagy in liver cells apoptosis, inflammation and fibrosis induced by high fat diet feeding. The obtained results indicate that pharmacological modulation of autophagy works as Cyto-protective machinery against liver injury.

MATERIALS AND METHODS.

Materials.

Chloroquine (phosphate salt), rapamycin, Tritonx-100, Tween-20, Sodium dodecylsulphate, polyacrylamide gel electrophoresis

(SDS-PAGE) chemicals, Nitrocellulose membrane, rabbit anti-LC3 IgG, Rabbit anti P62IgG antibodies and protease inhibitor cocktail were purchased from Sigma (USA). Super Signal West Pico chemiluminescent substrate was from Pierce Biotechnology (USA). Mouse anti-caspase 3 IgG, goat anti-mouse IgG-HRP, goat anti- β actin IgG, mouse anti-goat IgG-HRP and mouse anti-rabbit IgG HRP-conjugated were from Santa Cruz Biotechnology (USA). RNase inhibitor was from Promega (USA) and RNeasy Mini Kit from Invitrogen (USA). Protein Assay Kit was from Bio-Rad (Austria). All other chemicals of high grade –if not mentioned- were purchased from local Suppliers.

Animals and experimental design.

Mice were maintained in Assiut University Joint Animal Breeding Unit, College of medicine. All experimental procedures were conducted in strict compliance with the guide of National Institute of Health for the Care and Use of Laboratory Animals as well as the protocol approved by Assiut University of experimental Animal usage. Nine-to-ten-week old mice (*Mus musculus* Linnaeus, 1758) males (n=40) weighing 25-29 g were reared at $23\pm 2^{\circ}\text{C}$ on a 12/12 h light/dark cycle, relative humidity of 57 ± 9 with free access to food and water. Animals were categorized into four groups, ten mice in each group. Animals were fed for 12 weeks on normal diet (ND), normal mice chow (8% energy from fat), high fat diet (HFD), high fat mice chow (46% energy from fat). At the week number 6 of HFD feeding, modulation of autophagy was achieved Pharmacologically by IP administration of chloroquine (60 mg/kg) for inhibition of autophagy (HFD+CQ) or rapamycin (2 mg/kg) for enhancement of autophagy (HFD+Rap) every 3 days, for another 6 week with continued HFD feeding.

Western blot analysis.

Tissues were lysed by hand held homogenizer at 4°C in 500 µl of RIPA lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. Insolubilized tissue debris was removed by centrifugation at 10000 x g for 10 min at 4°C. Supernatant was collected and protein concentration was determined. Aliquots containing 30 µg proteins were subjected to 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using Bio-Rad dry electro-transfer. The membranes were blocked with 5% skim milk in TBS with 0.05% Tween 20 and incubated with primary antibodies (overnight, 4°C) and HRP-conjugated secondary antibodies (1 h, room temperature) in blocking solution. Immunoreactive bands were visualized by chemiluminescent substrate kit. Anti-β actin goat polyclonal antibody was used for equal loading confirmation. Estimation of each band optical density was carried out using Image J software referenced to the corresponding β actin band.

Histology.

After dissection, immediately the liver was cut into small pieces, fixed in 10% formalin. After paraffin embedding, 5µm sections were mounted on glass slides. Sections were deparaffinized twice in xylol for 30 minutes and hydration with ethanol series then stained routinely with Hematoxylin & Eosin and Masson's trichrome.

Quantification of hepatic injury and fibrosis.

Liver injury was estimated by a pathologist who was blind to experimental treatments according to Heijnen's method (Heijnen *et al.*, 2003). Five liver injury parameters were considered in at least six sections of different animals of each animal group. These parameters are tissue

vacuolization, cytoplasmic color fading, nuclear condensation, nuclear fragmentation and erythrocyte stasis. Collagen content in liver sections stained with Masson's trichrome was assessed by morphometric analysis as described (Wang *et al.*, 2007; Bataller *et al.*, 2003). Briefly, the Masson's trichrome positive areas were measured using image J software in entire liver section of at least six sections of different animals of each animal group. The percentage of fibrosis was calculated as follow

$$\% \text{ of fibrosis} = \frac{\text{Masson's trichrome positive area}}{\text{Total section area} - \text{vascular lumen area}} \times 100$$
Quantification of alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH) activities in blood plasma were determined using kits (Boehringer Mannheim, Mannheim, Germany) according to manufacturer procedures.

Quantitative Real time-PCR (qRT-PCR).

Liver tissue was frozen in liquid nitrogen immediately after dissection. Total RNA was extracted from tissue samples using RNeasy Mini Kit (Invitrogen) according to the manufacturer protocol. Reverse transcription was done using (SMART_PCR cDNA synthesis kit, Clontech Inc., Palo Alto, CA), no internal control template was used. Quantitative RT-PCR was performed in duplicate in total volume of 25 µl mixture containing 1 µl template cDNA, SYBR green PCR Master mix (Applied Biosystems), 10 pmol forward and reverse Primers (listed in Table 1). Reactions were run in (iCycler iQ, Bio-Rad). The level of each cDNA amplicon was normalized to the corresponding glyceraldehyde-3 phosphate dehydrogenase (GAPDH) cDNA amplicon level.

Table1:List of qRT-PCR primers

Genes	forward primers	reverse primers
Ccl2	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCTTCTTG
Ccl5	TGCCCCAGTCAAGGATATTT	TTCTCTGGGTTGGCACACAT
Col1a1	AAGAGGCGAGAGAGGTTTCC	AGAACCATCAGCACCTTTGG
Pal1	AGGGCTTCATGCCCCACTTCTTCA	AGTAGAGGGCATTACCAGCACCA
GAPDH	AACTTTGGCATTGTGGAAGG	GTCTTCTGGGTGGCAGTGAT

Statistical analysis.

Data are presented as mean \pm SD and were evaluated using post hoc tests after ANOVA. Statistics were performed using GraphPad Prism 3 software.

RESULTS

High fat diet induces autophagy and apoptosis in liver.

It has been reported that autophagy play a role in liver injury induced by high fat diet (Papackova *et al.*, 2012). Therefore, it is important firstly to test whether autophagy and apoptosis initiated under high fat diet condition. We conducted immunodetection of

autophagy markers LC3 and P62 and apoptosis marker caspase3 in HFD and ND fed mice. The results showed that both autophagic markers are increased in HFD compared with ND (Fig.1A and B). The ratio of lipidated membrane-bound LC3II to the cytosolic form LC3I indicates the autophagy intensity. This ratio was elevated in HFD mice almost two folds higher than ND mice (Fig.1 C). Also, level of active caspase 3 is markedly elevated under the condition of HFD (fig.1 A and B). These results indicate that HFD can promote both autophagy and apoptosis in hepatocytes.

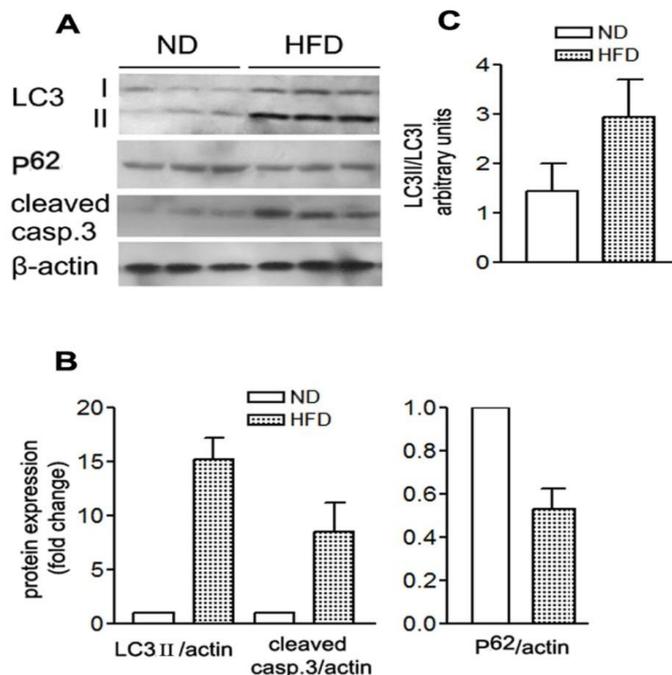


Fig. 1: HFD induced both autophagy and apoptosis: liver tissues from ND and HFD group were lysed and protein extraction was subjected to SDS electrophoresis. Transferred onto nitrocellulose membrane then immune-detection of cleaved caspase 3, LC3 and P62 were performed as described in methods, actin level for equal loading confirmation (A). The level of protein expression was quantified and expressed as fold change of protein/actin ratio after normalization to the control ND (B) or LC3II/LC3I ratio (C). Data are presented as mean \pm SD from at least three independent experiments.

Modulation of autophagy alters the apoptosis of liver cells in HFD mice.

To test the effect of autophagy modulation on the apoptosis of hepatocytes, HFD mice were administrated chloroquine for autophagosome degradation blocking or rapamycin for enhancement of autophagy. LC3II and P62 were significantly elevated after chloroquine administration compared to HFD (Fig. 2 A and B). In contrast, rapamycin treatment markedly downregulated LC3II and p62 compared with HFD. The

LC3II/LC3I ratio indicates the higher autophagy intensity after chloroquine and its down regulation after rapamycin compared with HFD (Fig. 2 C). Autophagy modulation in HFD mice affected the apoptosis state of hepatocytes, inhibition of autophagy leads to increase of active caspase 3 level (Fig. 2 A and B) whereas autophagy enhancement leads to reduction of its level. Accordingly, autophagy enhancement is critical for attenuation of hepatocytes apoptosis induced by HFD.

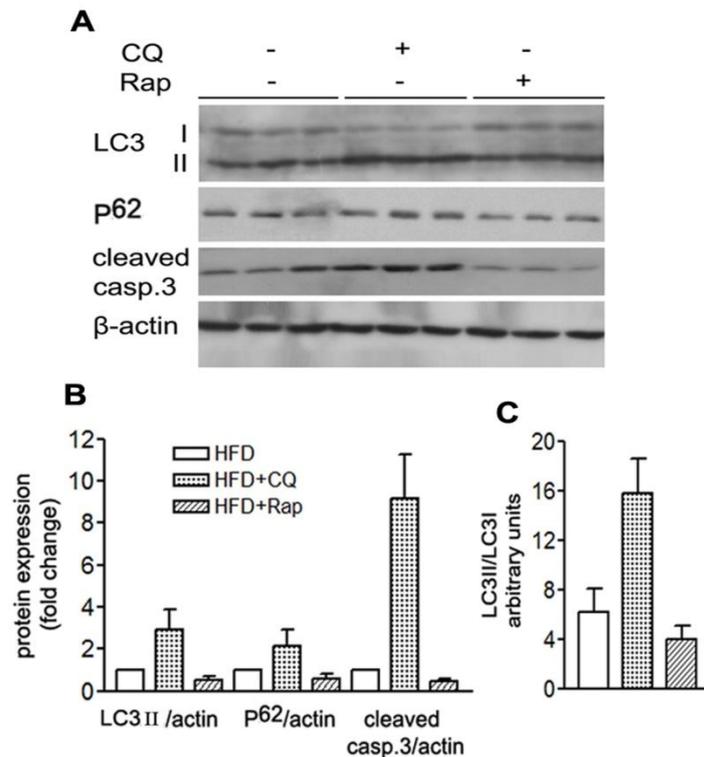


Fig. 2: Autophagy protects liver cells from apoptosis: Pharmacological modulation of autophagy was carried out by i.p administration of chloroquine for autophagy flux blockade or rapamycin for autophagy upregulation. Immunodetection of cleaved caspase 3, LC3 and P62 was performed (A). The fold changes in protein/actin ratio (B) and LC3II/LC3I ratio(C) as described in fig. 1. Data are presented as mean ± SD from at least three independent experiments.

Liver histopathology in HFD mice under autophagy modulation.

After 12 weeks of HFD feeding, liver tissue appears to have marked histological changes as vacuolation and necrobiotic changes compared with the normal liver histology of the control ND

mice (Fig. 3). After prevention of autophagosome degradation by chloroquine administration more severe tissue damage was observed including extensive vacuolation and necrobiotic changes. Inversely, autophagy enhancement by rapamycin

administration greatly restores the normal histology of the liver compared to the HFD mice. Accordingly, Heijnen's score recorded highest degree in HFD+CQ mice (Fig. 4), it is almost two folds higher than HFD mice and the latter is almost two folds higher than the control ND mice. In contrast, HFD+Rap mice show reduced Heijnen's score more or less like that of control ND mice (Fig.4).

Liver fibrosis and morphometric analysis of collagen deposition is demonstrated by Masson's trichrome staining performed on liver sections. In HFD mice collagen deposition is almost two folds higher than ND mice which show normal appearance of collagen fibers (Figs. 3 and 4). Chloroquine administration markedly raises collagen deposition whereas, rapamycin reduces it.

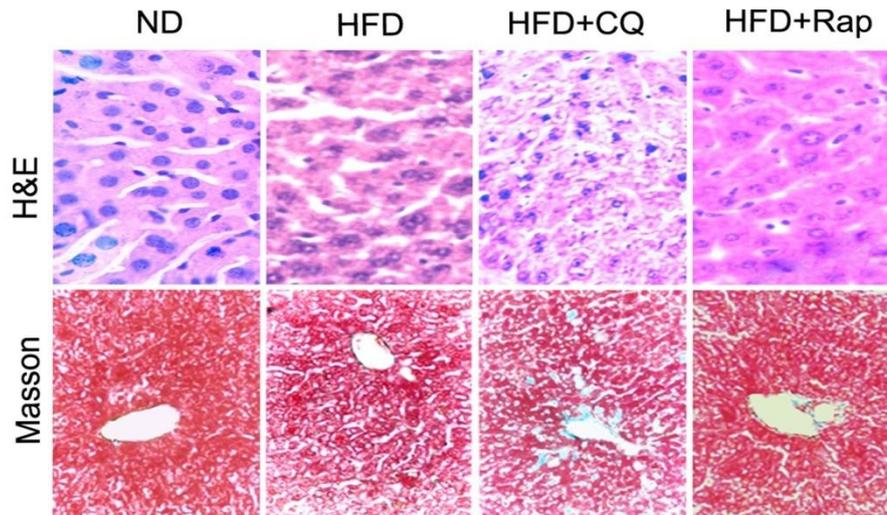


Fig. 3: Histopathology of liver tissue after HFS feeding and autophagy modulation: liver tissue from ND, HFD, HFD+CQ and HFD+Rap groups were processed for hematoxylin and eosin staining, magnification (x 400) or Masson's trichrome staining, magnification (x 100).

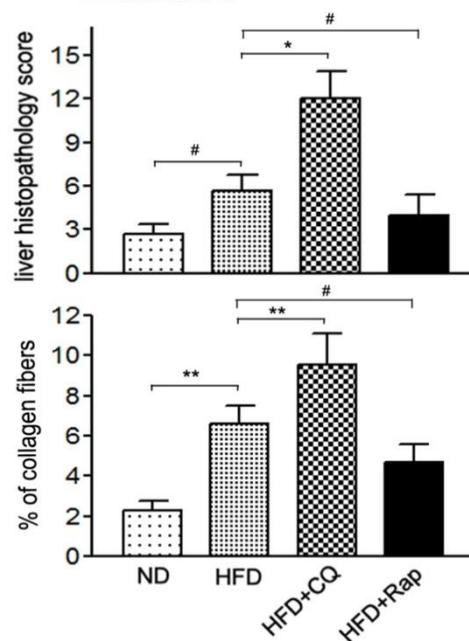


Fig. 4: Heijnen's and fibrosis scores of liver: Liver histopathology score was assessed by Heijnen's scores and percentage of liver fibrosis was calculated as described in method. Data are expressed as mean \pm SD, * $p < 0.001$, ** $p < 0.01$ and # $p < 0.05$.

Morphometric analysis of collagen deposition demonstrated 35-47% increase in hepatic fibrosis after chloroquine administration compared with HFD mice, but rapamycin causes 27-36% reduction in collagen content compared with HFD mice (Fig. 4). This result indicates that autophagy is important for attenuation of liver injury and fibrosis induced by HFD.

Effect of autophagy modulation on liver function in HFD mice.

Feeding on HFD for 12 weeks significantly increases the levels of ALT,

AST and LDH compared to the control ND mice (Fig. 5). Chloroquine administration increases the level of these enzymes by 180%, 54% and 91%, respectively than HFD mice. In contrast, rapamycin reduces enzymes levels by 25%, 30% and 33%, respectively compared with HFD mice. Liver injury enzyme markers are greatly reduced after autophagy augmentation by rapamycin which enforce the role of autophagy in liver injury attenuation in HFD mice.

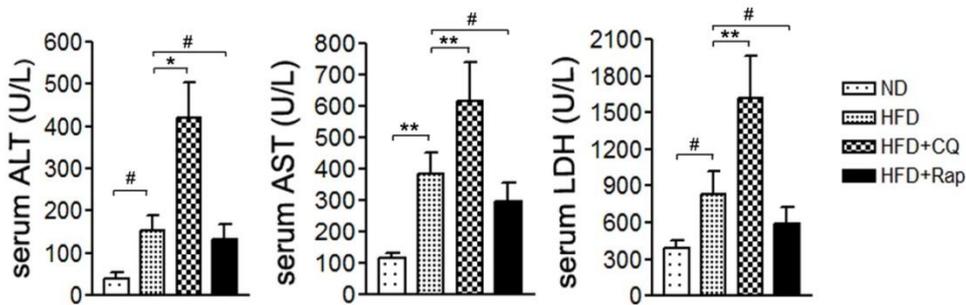


Fig. 5: Liver function enzymes after HFD and autophagy modulation: The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in the four animal groups. Data are expressed as mean ± SD, *p < 0.001, **p < 0.01 and #p < 0.05.

Effect of autophagy modulation on liver fibrosis and inflammatory genetic markers in HFD mice.

The mRNA of fibrogenic markers Collagen type 1 alpha 1 (COL1a1) and Plasminogen activator inhibitor 1 (PAI1) and inflammatory markers Chemokine (C-C motif) ligand 2 (Ccl2) and Chemokine (C-C motif) ligand 5 (Ccl5) were quantified by qRT-PCR in control ND, HFD group and after modulation of autophagy by chloroquine and rapamycin. HFD feeding resulted in significant elevation of both (COL1a1

and PAI1) and (Ccl2 and Ccl5) mRNA compared to control ND feeding group (Fig. 6). Furthermore, exclusive elevation of both fibrogenic and inflammatory markers mRNA level was observed after chloroquine administration compared to HFD group. Inversely, rapamycin administration resulted in significant attenuation of mRNA of these markers compared to HFD group. Collectively, these results confirm the importance of autophagy as a suppressive process for the progress of HFD induces liver fibrosis and inflammation.

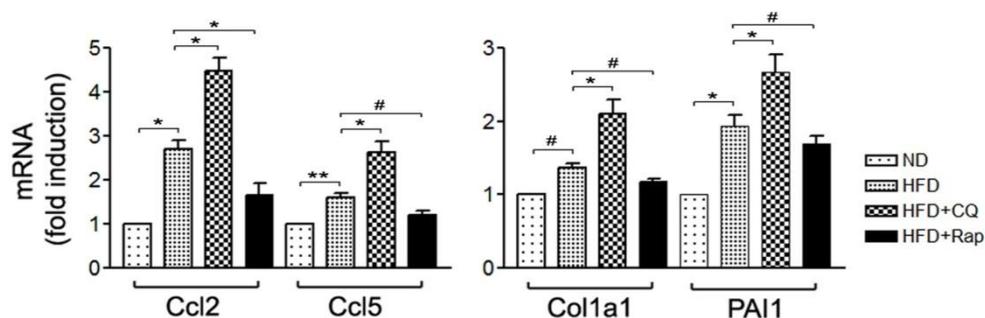


Fig. 6: qRT-PCR analysis of gene expression involved in inflammation and fibrosis: The level of mRNA transcription of inflammation markers (Ccl2 and Ccl5) and fibrogenic markers (COL1a1 and PAI1) were quantified by real time PCR. Values were normalized to the corresponding level of GAPDH. Data represent mean \pm SD, * $p < 0.001$, ** $p < 0.01$ and # $p < 0.05$.

DISCUSSION

Impairment of nutrients metabolism contributes to liver injury which is initiated by liver cell death associated with inflammatory response and activation of fibrogenic cells. Autophagy plays an important role to maintain liver function in basal conditions (Komatsu *et al.*, 2005). It has been established the engagement of autophagy as a protective mechanism in liver diseases, including ethanol-induced steatosis (Lin *et al.*, 2013; Ding *et al.*, 2010). Hepatocellular carcinoma (Shimizu *et al.*, 2012), ischemia and oxidative stress (Wang *et al.*, 2012; Cardinal *et al.*, 2009). In the current study, we investigated the role of autophagy in liver inflammation, apoptosis and fibrosis induced by HFD feeding. After 12 weeks of HFD feeding, autophagic markers P62 and LC3II/LC3I ratio were increased significantly compared to the ND feeding mice. Furthermore, active caspase 3, Liver injury enzymes, inflammatory and fibrosis markers were all also increased. Suppression of autophagy flux by chloroquine administration in HFD mice greatly elevated liver autophagy and injury markers compared to HFD mice. Inversely, autophagy enhancement by rapamycin administration significantly attenuated these markers compared with HFD mice. Unrecoverable liver injury caused by HFD may be the reason for

autophagy initiation. The cytosolic form of the microtubule associate protein light chain 3 (LC3I) is lipidated by phosphatidylethanolamine during the formation of autophagosome forming (LC3II) which associated with autophagosomal membrane and degraded upon lysosome-autophagosome fusing (Rubinsztein *et al.*, 2009). The ratio of LC3II/LC3I is a good indicator for the autophagy intensity. P62 is an ubiquitin-binding scaffold protein that binds to LC3 protein and co-localize together in autophagosomal membrane. It has been shown that autophagy activation leads to P62 degradation and inversely, autophagy inhibition leads to accumulation of P62 (Klionsky *et al.*, 2012). The accumulation of both LC3II and P62 is a good indication for the autophagy flux blockade not as previously considered as a sign for autophagy induction (Ganley *et al.*, 2011). Chloroquine is a lysosomotropic agent that interferes with autophagosomes degradation. The mechanism of action of chloroquine or its hydroxylated form (hydroxychloroquine) on autophagy is due to the lysosomal acidification followed by blocking the autophagosome-lysosome fusion (White, 2012), so in fact it prevents autophagy flux but does not inhibit autophagy process (Liu *et al.*, 2014). In the present study HFD resulted in elevation of autophagy intensity monitored by

LC3II/LC3I ratio and also autophagy flux monitored by LC3II and P62 downregulation compared with ND mice. Blocking of autophagy flux by chloroquine resulted in massive accumulation of LC3II and P62 and higher LC3II/LC3I ratio than HFD mice. HFD may increase the accumulation of lipids, unfolded proteins and damaged organelles which in turn increase the autophagy as a feedback mechanism in an attempt to overcome this problem. However, it seems that autophagy intensity induced by HFD is not sufficient to overcome nutrient overload leading to initiation of liver injury represented in upregulation of inflammation and fibrosis markers (Ccl2 and Ccl5) and (COL1a1 and PAI1), respectively. Accordingly, liver injury enzymes (ALT, AST and LDH) and apoptotic cell death as demonstrated by elevated cleaved caspase 3 level were also upregulated. However, the lipid-autophagy relationship is not investigated in the present study but other investigators proved that autophagy inhibition increased lipid accumulation in hepatocytes and reversely, massive increase in intracellular lipids impaired autophagy (Liu and Czaja, 2013; Singh *et al.*, 2009). This may explain why autophagy was not sufficient to prevent liver injury induced by HFD in the present study. This assumption is enforced by two observations 1- blocking of autophagy flux by chloroquine resulted in massive elevation of these mentioned liver injury signs 2- enhancement of autophagy by rapamycin significantly downregulated these signs compared with those of HFD mice. It has been reported that rapamycin alleviated steatosis, blood levels of triglycerides and liver enzymes in alcoholic and non-alcoholic fatty liver in mice while chloroquine acted inversely (Lin *et al.*, 2013). However, there is no straight line of evidences for a particular role of autophagy in liver injury, controversial

results concerning the role of autophagy in liver steatosis and fibrosis were investigated (Kwanten *et al.*, 2014). This debate concerning autophagy is a lipolytic or lipogenic cell machinery may be explained by the degree of intracellular lipid accumulation. In fact, short-term exposure to high fat diet upregulated autophagy while, long-term exposure inhibited it (Papackova *et al.*, 2012). Accordingly, there is a clear evidence that autophagy is a defense cellular machinery against lipid accumulation and liver injury when the latter is recent or in recoverable state for autophagy. But massive lipid accumulation and cellular injury leads to inhibition of autophagy. In the current study we present confirm evidences that autophagy is defense cellular machinery against liver injury induced by HFD. Autophagy markers together with apoptotic, inflammatory and fibrosis markers were significantly upregulated by HFD feeding. Upon autophagy flux blockade by chloroquine all these markers were massively elevated indicating the importance of autophagy in attenuation of liver injury. Moreover, rapamycin administration enhanced autophagy and downregulated liver injury markers. Better understanding of the role of autophagy and its molecular mechanism in liver diseases leads to development of effective and new therapeutic strategies.

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ARABIC SUMMERY

الدور الوقائي الخلوي للالتهام الذاتي ضد إصابة خلايا الكبد المستحث بواسطة نظام غذائي عالي الدهون

منى محمد عطية

معمل بيولوجيا الخلية الجزيئية - قسم علم الحيوان - كلية العلوم - جامعة أسيوط - أسيوط ٦١٥١٧ - جمهورية مصر العربية

إن عملية الالتهام الذاتي الخلوي هي عملية ايض هدمي يعمل على تنظيم التوازن الخلوي في الظروف العادية أو الاجهادية للخلية . هناك آراء متضادة حول دور الالتهام الذاتي في الإصابات الكبدية. فعلى سبيل المثال يوجد أبحاث تؤكد أن الالتهام الذاتي يعمل على زيادة تليف الكبد وعلى النقيض يوجد أبحاث أخرى تبين أن الالتهام الذاتي هو ميكانيكية مضادة لتليف الكبد. لذلك فإن هذا البحث يتناول دور الالتهام الذاتي في إصابات خلايا كبد الفئران الناتجة عن حمية غذائية عالية الدهون. قسمت مجموعة الفئران في التجربة إلى أربع مجموعات, مجموعة ذات نظام غذائي عادي وهي المجموعة الضابطة ومجموعة ذات نظام غذائي عالي الدهون والتي تركت كما هي أو تم تجريعها بالكلوروكوين أو الراباميسين لتحويل عملية الالتهام الذاتي. تم دراسة بروتينات الالتهام الذاتي وهي (LC3 and P62) وبروتين الموت الخلوي المبرمج وهو (caspase 3) بواسطة الكشف المناعي البروتيني كما تم تقدير مستوى النسخ الجيني لدلالات التليف الكبدية وهي الجينات (COL1a1 and PAI1) ودلالات الالتهابات الكبدية وهي الجينات (Ccl2 and Ccl5). هذا بالإضافة إلى الدراسات النسيجية والكيمياء النسيجية والكيموحيوية لكبد الفئران في مختلف المجموعات. أثبتت النتائج أن مستوى الموت الخلوي المبرمج وكذلك دلالات الالتهابات والتليف قد زادت بدرجة ملحوظة في الفئران ذات الحمية عالية الدهون بالمقارنة بالمجموعة الضابطة. بينما التجريع بواسطة عقار الكلوروكوين والذي يوقف الالتهام الذاتي أدى إلى ارتفاع كبير جدا في كل من مستوى الموت الخلوي المبرمج وكذلك دلالات الالتهابات والتليف وعلى النقيض فإن تنشيط الالتهام الذاتي الخلوي في خلايا الكبد بواسطة عقار الراباميسين أدى إلى اختزال كبير في تلك العوامل سابقة الذكر. تم تأكيد هذه النتائج بدراسة التغيرات النسيجية والكيمياء النسيجية والكيموحيوية والتي أكدت أن الالتهام الذاتي الخلوي يحمي خلايا الكبد من الموت الخلوي المبرمج والالتهاب والتليف الناتج عن النظام الغذائي عالي الدهون.