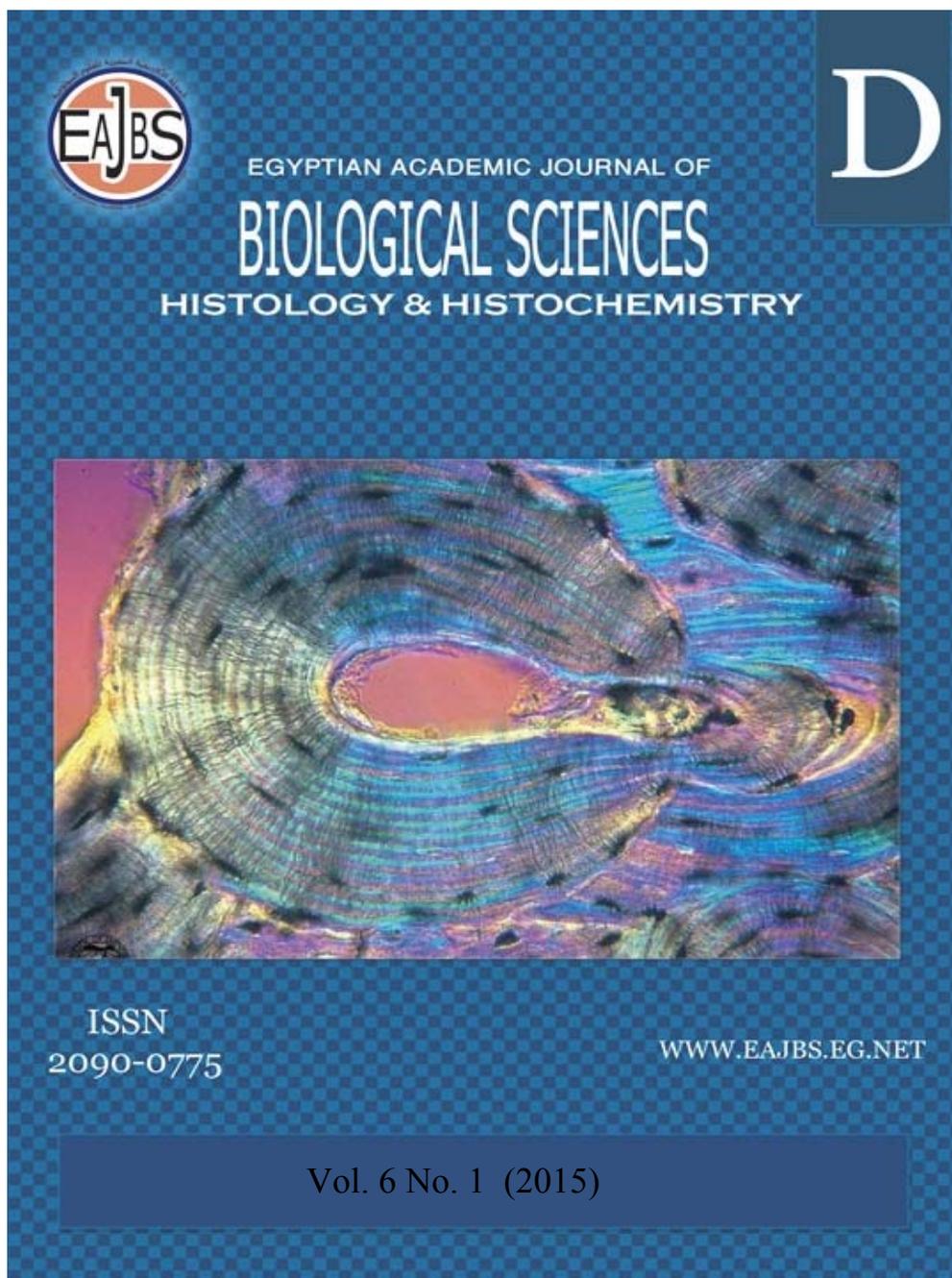


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Hyperglycemia and hyperinsulinemia induced hepatocellular autophagy in male mice

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ABSTRACT

The aim of the present study is to investigate the role of hyperglycemia and hyperinsulinemia in autophagy induction in the liver of male mice. Autophagy is a catabolic cellular process that recycles the aged or damaged cellular organelles and inclusions under certain circumstances. Hyperglycemia is induced by a single dose of alloxan IP injection (180 mg/kg) and hyperinsulinemia is induced by high fat diet together with glucose feeding for short period (2 weeks) and long period (3 months). Hyperglycemia and hyperinsulinemia were estimated by measuring blood glucose level by glucometer and insulin level by specific ELISA kit, respectively. Autophagy induction was investigated morphologically by electron microscopy examination and biochemically by immunodetection of microtubular associated light chain protein 3 (LC3) conversion from LC3I to LC3II form and by immunodetection of the phosphorylated and non-phosphorylated forms of mammalian target of Rapamycin (mTOR). Our results revealed that hyperglycemia and hyperinsulinemia independently induced hepatocellular autophagy as indicated by the accumulation of autophagosomes and autolysosomes in EM examination and by the increase of the level of LC3II and decrease of the phosphorylated form of mTOR in western blot analysis. This study throw the light on the autophagy of hepatocytes as a cellular mechanism induced under diabetic conditions which may contribute in better understanding of our knowledge concerning nutrients metabolic disorders.

INTRODUCTION

Two major cellular strategies for protein degradation are carried out; the ubiquitin-proteasome system for disposal of short-lived proteins and the lysosomal system for degradation of intracellular and extracellular proteins. Autophagy is a part of the lysosomal system, it is an evolutionarily conserved cellular strategy to engulf and degrade long-lived cytosolic proteins and organelles. It provides substrates for energy metabolism and to recycle amino acids, fatty acids and nucleotides for the biosynthetic needs of the cell.

It also may occur in the face of nutrient deprivation, growth factor withdrawal or other stressors (Lum *et al*, 2005; Heymann, 2006; Keith and John, 2008). Three major forms of autophagy have been described in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy (Kim and Klionsky, 2000). In mammals, regulation of autophagy appears to be highly complicated. In the first step of autophagosome formation cytoplasmic constituents, including organelles, are sequestered by a unique membrane called the phagophore which is a very flat organelle like a Golgi cisterna. Complete sequestration by the elongating phagophore results in formation of the autophagosome, which is typically a double-membrane organelle. This step is a simple sequestration and no degradation occurs. Since autophagosomes are generated, it is called the "preautophagosomal structure (PAS)" (Kim *et al.*, 2001; Suzuki *et al.*, 2001; Suzuki and Ohsumi, 2007). In the last step the autophagosome fuses with lysosome to form the autophagolysosome or autolysosome where the included material is degraded by lysosomal enzymes (Mizushima, 2007). Autophagy is initiated by many diverse signals including amino acids, glucose and growth factors (Jewell and Guan, 2013). It is now believed that the endocrine system, particularly insulin, manages autophagy regulation *in vivo*, as example, it was found that liver autophagy is suppressed by insulin and enhanced by glucagon (Mortimore and Pösö, 1987). Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects of insulin secretion and/or increased cellular resistance to insulin. Chronic hyperglycemia and other metabolic disturbances of DM lead to long-term tissue and organ damage as well as dysfunction involving many organs and

systems. Type 1 diabetes used to be called juvenile diabetes and insulin-dependent diabetes mellitus. It is an autoimmune disease in which the immune system mistakenly destroys the insulin-making β -cells of the pancreas. Non-insulin-dependent diabetes mellitus also referred to as type II diabetes is the most common of all metabolic disorders. Type II diabetes currently affects about 6–7% of the US population, with a cumulative risk of 17% by age 80 (Warram *et al*, 1995). The association between liver disease and DM is well known; DM itself may be a cause of liver disease via non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), cirrhosis and ultimately hepatocellular carcinoma. It was found that Post-transplantation DM is a major cause of morbidity and mortality in subjects following liver transplantation (Simona *et al*, 2007). Autophagy is important for proper β -cells function and viability. Originally, autophagy was reported to be activated in β -cells upon stress induction as a protective mechanism (Las and Shirihai, 2010). Autophagic cell death contributes to loss of pancreatic β -cells mass in diabetes. Beta cells apoptosis plays a major role in reducing their mass in diabetes, autophagic cell death can also contribute to loss of β -cell mass (Ze-fang *et al*, 2011). Here we investigated the induction of autophagy in the liver of two different cases of metabolic disorders. In alloxan induced type I diabetes characterized by hyperglycemia and hypoinsulinemia and in high fat diet feeding characterized by normoglycemia and hyperinsulinemia.

MATERIALS AND METHODS

Materials

Alloxan, Glucose, Tritonx-100, Tween-20, Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) chemicals, EDTA (ethylene

diamine Tetra-acetic acid), EGTA (ethylene glycol Tetra-acetic acid), Bromophenol Blue, Nitrocellulose membrane, TMED, PMSF, 2-Mercaptoethanol, Goat Anti Rabbit IgG HRP conjugated, Rabbit Anti mouse IgG HRP conjugated and protease inhibitor cocktail were from (Sigma Co.). Protein assay kit was purchased from Bio Rad, USA. Rb anti LC3 Ab, Mo Anti mTOR and Mo anti pmTOR antibodies were purchased from (Affinity BioReagent, Denver, CO, USA). Goat anti- β actin IgG and mouse anti-goat IgG-HRP were from Santa Cruz Biotechnology. SuperSignal West Pico Chemiluminescent Substrate was from Pierce Biotechnology (USA). All other chemicals of high grade were purchased from local Suppliers.

Animals and experimental design

Thirty five adult male Swiss albino mice weighing 25-30 g. were used in the present work. They were purchased from and maintained in Assiut University Joint Animal Breeding Unit. Suitable temperature of almost 23 ± 2 °C and 12 hours of light /dark cycle were also into consideration. All animals were given free access to standard chow and tap water. All experimental procedures were conducted in strict compliance with the guide of National Institute of Health for the Care and Use of Laboratory Animals. Mice were categorized into four groups. Control group (cnt) fed with normal rodent chow (8% energy from fat), allox group injected intraperitoneally with freshly prepared single dose of alloxan monohydrate (180 mg/kg) to become diabetic, short period high fat diet feeding group (shfd) fed with high fat diet rodent chow (46% energy from fat) together with orally administration of glucose (0.5 ml of 25% glucose every 6 hours) for 2 weeks and long period high fat diet group (lhfd) fed as shfd group but for 3 month.

Histological and histochemical preparations

For histological preparation of pancreas and histochemical examination of general proteins, immediately after sacrifice, pieces of organs were fixed in 10% of neutral buffered formalin pH 7.2, dehydrated in ascending series of alcohols, cleared in cedar wood oil and embedded in paraffin wax. Paraffin sections of 5 micrometer in thickness were prepared and then stained routinely with Harris haematoxylin and eosin stain. For histochemical examination of general proteins, liver sections were stained with bromophenol blue as described (Mazia *et al*, 1953). The colour intensity was estimated by Image J software and expressed as arbitrary units. Morphometric analysis for the area of islets of Langerhans was done using Image J software. For each section, 5 fields were examined; at least 10 different sections for each treatment were calculated.

Electron microscopy

For electron microscopy, small slices of liver were fixed in 2.5 % glutaraldehyde in cacodylate buffer. The specimens were washed in cacodylate buffer (0.1 M, PH 7.2) for 1-3 hours and then post fixed in 1 % osmium tetroxide for 2 hours. The specimens were placed in propylene oxide for 1 hour, then in pure epon 812 and incubated in a special polymerization incubator (one day at 37 °C, second day at 45 °C and then three days at 60°C). Ultrathin section (50 nm) were mounted in copper grids and stained with uranyl acetate, lead citrate and examined by "Jeol TEM" in the electron microscopic unit, Assiut University.

Fasting Blood glucose and insulin levels measurements

Mice were fasted for almost 8 hours and then glucose levels were measured by hand-held glucose test monitor (Lifescan, Johnson and Johnson) from whole tail vein blood and expressed as mg/dl. Serum insulin was quantified using mice

insulin ELISA kit (Crystal Chem, USA) according to manufacturer protocol.

Western blot analysis

Liver tissues were lysed in 500 μ l of RIPA lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail and homogenized slowly by hand held homogenizer at 4°C. Tissue debris was removed by centrifugation at 10000 x g for 5 min at 4°C. Supernatant was collected and protein concentration was determined. Aliquots containing 40 μ g proteins were subjected to 12% SDS-PAGE, and then transferred to nitrocellulose membranes. Blocking of active sites was carried out 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. Target proteins 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 and neutralized to the corresponding β actin band.

Statistical analysis

Data were presented as mean \pm SD. Statistical analyses were performed using ANOVA. P value less than 0.05 was considered significant.

RESULTS

Fasting blood glucose and insulin levels

The fasting blood glucose levels (fbgl) significantly increased in alloxan treated animals compared with control (Fig. 1 A). The fbgl slightly decreased in shfd and lhfd groups compared with control. These decreases were statistically non-significant (Fig. 1 A). Because shfd and lhfd groups did not show any significant increase of fbgl, so they may have a mechanism for reducing blood glucose. Accordingly, we measured serum insulin level in different

experimental groups (Fig.1 B). Serum insulin was reduced in allox group compared with control group. High insulin level was detected in high fat diet feeding groups with the highest level recorded in shfd group. We decided to examine Langerhans islets looking for any change in their structure which may indicate the change of insulin level. It was found that the islet area significantly decreased in alloxan treated group compared with control (Fig. 1 C and D). The mean values were ($79\pm 3.2\mu\text{m}^2$, $140.1\pm 9.5\mu\text{m}^2$) for allox and cnt groups, respectively. Inversely, the islet area significantly increased in shfd and lhfd groups compared with control. The mean values were ($3592\pm 151.3\mu\text{m}^2$, $392.5\pm 35.5\mu\text{m}^2$) respectively. It is clear that high fat diet for short period enhanced the enlargement of islets and then the islet area reduced with continuous high fat feeding for long time. This may indicate the compensatory hyperinsulinemia upon high fat and glucose feeding.

Induction of autophagy by hyperglycemia or hyperinsulinemia

The conversion of the lipidated LC3I form to the cytosolic LC3II form significantly increased in allox, shfd and lhfd groups as indicated by LC3II/LC3I ratio (Fig. 2 A and B). This result indicates the elevated level of autophagy intensity in hyperglycemia or by short or long period of high fat diet feeding. To confirm the autophagy induction in hyperglycemia or hyperinsulinemia another autophagic marker was used; mTOR and its phosphorylated form pmTOR. Immuno-detection of mTOR and pmTOR revealed that level of pmTOR decreased in allox, shfd and lhfd groups compared with control (Fig. 2 A). The pmTOR/mTOR ratio showed low levels in above mentioned groups compared with control (Fig. 2 C).

Autophagy morphology by EM

Electron microscopic examination of normal hepatocyte revealed normal

appearance of organelles including rounded nucleus with normal distribution of euchromatin. In addition, the hepatocyte contains round and elongate mitochondria which were found intermingled with cisternae of rough endoplasmic reticulum. Lysosomes and abundant glycogen are also observed (Fig. 3, upper left panel). Ultrastructural examination of allox, shfd and lhfd groups showed the presence of autophagy process. Numerous autophagosomes of varying sizes bounded by double membranes could be observed in the majority of the examined hepatocytes of these groups. Some of autophagosomes contain glycogen granules and assortment of organelles of variable electron density (mitochondria, endoplasmic reticulum and ribosomes) of various stage of degradation (Fig. 3, upper right panel and lower panels). In addition, the cisternae of rough endoplasmic reticulum especially around the nucleus were separated by numerous areas of rarified cytoplasm. In shfd group large numbers of rarefied areas in cytoplasm containing electron dense myelinated figures which are end products of autophagy process were observed (Fig. 3, lower left panel). These myelinated figures appeared abundantly in shfd and rarely in lhfd, which indicates the high intensity of autophagy process in these groups.

General protein depletion in hyperglycemia and hyperinsulinemia induced autophagy.

In control liver tissue, the hepatocytes showed an intensive reaction for total proteins as indicated by a dense blue colour (Fig. 4 A). In allox, shfd and lhfd groups, marked and significant depletion in protein content was observed in hepatocytes as indicated from the optical density measurement (Fig. 4 B). In shfd group, Protein inclusions of the liver were the most reduced one compared with other groups (Fig. 4 B); this may indicate the intensity of

autophagy in shfd group compared with allox and lhfd groups.

DISCUSSION

In the current study, autophagy was initiated in hyperglycemia induced by alloxan and in normoglycemic hyperinsulinemic mice fed with high fat and glucose diet for short and long periods. Autophagy is not one way process, but it is a complex process initiated in many cellular conditions. In the following, we try to throw the light on the mechanism responsible for autophagy initiation under the effect of hyperglycemia and hyperinsulinemia. The fasting blood glucose levels increased in alloxan treated group and decreased non-significantly in shfd and lhfd groups when compared with cnt group. Alloxan, a β -cytotoxin, is known to induce chemical diabetes in a wide variety of animal species by damaging the insulin secreting cells resulting in increased plasma levels of glucose and a fall in liver glycogen (Rajathi and Daisy, 2011 and Adeyi *et al.*, 2012). Recently Ankur and Shahjad, (2012) reported that DNA fragmentation took place in the β -cells exposed to alloxan which stimulates poly ADP-ribosylation, a process participating in DNA repair. The non-significant change happened in fasting blood glucose levels in shfd and lhfd groups versus that of control group may be resulted from the compensatory increase of islet of Langerhans area "mainly β -cells" to increase insulin secretion and return the blood glucose within normal levels. In present study, measurements of the area of islets of Langerhans in pancreatic tissue showed that the islet area decreased in allox group when compared with cnt group. Whereas, islet area was highly increased in shfd and lhfd groups when compared with cnt group. Nermeen *et al.*, (2010) observed apparent reduction of size and number of islets in pancreas of diabetic mice treated by alloxan. Islets hyperplasia

was observed in mice infused continuously with glucose (Kinash and Haist, 1954) or high carbohydrate diet (Barberà *et al.*, 2003) and in male wistar rats fed on high carbohydrate and high fat diet (Panchal *et al.*, 2011). James *et al.*, (2001) showed that Neonatal rats fed a high carbohydrate formula by gastrostomy were hyperinsulinemic but normoglycemic. They cited that the rapid increase in islet cell mass that occurs in late fetal and neonatal life in the rat may explain why pancreatic morphology is so sensitive to nutritional insult at this time. Li *et al.*, (2006) explained that the expansion of β -cell mass in response to insulin resistance is found in many animal models, and obese humans have an increased islet mass compared with lean individuals. The signal for expansion of islet mass is not clear but likely involves a response to increased glucose flux and may be dependent on intact insulin signaling pathways within the β -cell. In allox, shfd and lhfd groups marked conversion of cytosolic LC3- I into autophagosome specific isoform LC3- II were observed which indicate the presence of autophagy process in the liver. The autophagy induction by hyperglycemia was reported in streptozotocin- induced diabetes in rats (Satoru *et al.*, 2012). The induction of autophagy in hyperglycemia may be related to the increased plasma glucagon level and/or the decreased insulin which is corrected by insulin therapy (Amherdt *et al.*, 1974). Recently, another explanation for autophagy induction in hyperglycemia was introduced by Claudio *et al.*, (2011). They reported that there are diverging upstream pro-apoptotic signals in both types of diabetes, as a result of mitochondrial dysfunction and production of reactive oxygen species (ROS). Elevation in ROS is essential for autophagy to proceed because their presence may control the activity of Atg4, a gene that is necessary for autophagosome formation. In the

current study, hyperinsulinemia also induced autophagy. However, hyperinsulinemia is accompanied by normoglycemia, so it is clear that autophagy is initiated by different mechanism in this case. The high fat and glucose feeding may cause temporary hyperglycemia, which in turn induced autophagy induction regardless of hyperinsulinemia. Our suggestion is enforced by the work of Nirmala *et al.*, (2011); they observed the upregulation of autophagy is reversed when diabetic animals are treated with insulin. Brinda *et al.*, (2010) cleared that several signaling pathways seem to regulate autophagy in mammalian cells. Similar to yeast, the classical pathway involves the mammalian target of rapamycin (mTOR). It is the mammalian ortholog of the yeast protein kinase TOR that negatively regulates autophagy. In this study, Immunoblot detection of mTOR and pmTOR in the liver of allox, shfd and lhfd groups showed low level of pmTOR in comparison to mTOR. The pmTOR/mTOR ratio decreased compared with that in control group which indicate the presence of autophagy process. This result agree with the explanation of Dos *et al.*, (2005) who cleared that mTOR pathway is a key regulator of cell growth and proliferation and increasing evidence suggests that its deregulation is associated with human diseases, including cancer and diabetes. Nakatsu *et al.*, (2010) cited that mTOR is a multifunctional serine/threonine kinase that regulates cell growth and survival. When nutrients are abundant, mTOR is phosphorylated and promotes protein synthesis. On the other hand, in energy depletion, autophagy is induced by dephosphorylation of mTOR. Proud, (2006) reported that insulin stimulates protein synthesis and cell growth by activation of the protein kinase B and mTOR. General protein staining in the current study revealed that hyperglycemia is accompanied by depletion of protein

content in liver tissue. Also, in shfd and lhfd groups are accompanied by depletion of protein content. So it is suggested that insulin is not the main agent which controls protein synthesis as previously reported by (Proud, 2006). It is suggested that autophagy is the most responsible process for protein depletion in allox, shfd and lhfd groups of the current study. Here we introduced evidences for the induction of autophagy in two different cases of metabolic disorders, hyperglycemia and hyperinsulinemia. Our findings may throw the light on the mechanism and importance of autophagy in diabetes and its complications.

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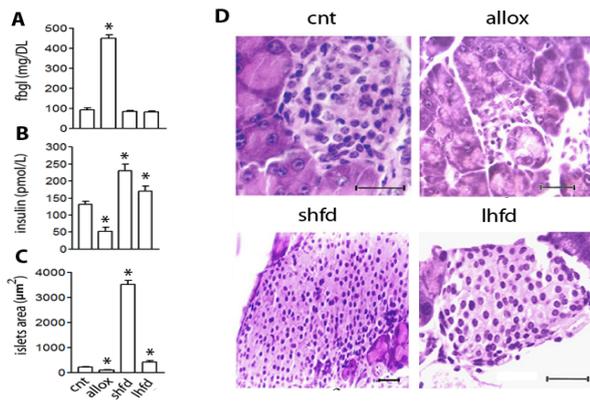


Fig. 1: Fasting blood glucose and insulin levels in different treatments: fasting blood glucose level (fbgl) and serum insulin were estimated as described in method section (A and B). Measurement of Langerhans islets area (C). Representative photomicrographs of pancreas sections stained with H & E of different experimental groups showing Langerhans islets (D), Bar equals 50μm. Data representing the mean ± SD, * P < 0.05 compared with control.

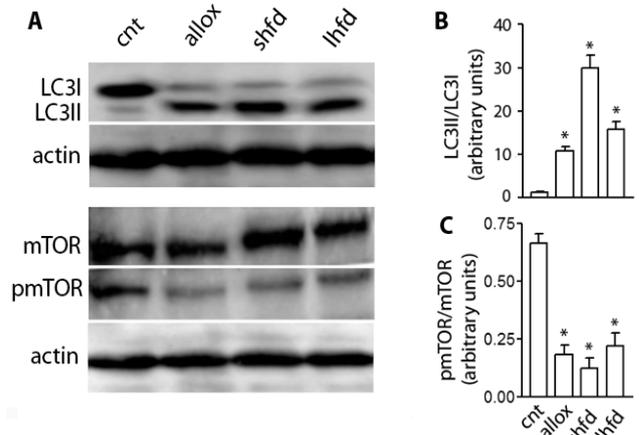


Fig. 2: immunodetection of LC3 and mTOR: LC3I & II and mTOR & pmTOR were detected by western blot in liver tissues of different treatments, representative figures out of at least three independent experiments are shown (A). Estimation of band optical density was carried out by Image J software and normalized to the corresponding actin band and expressed as arbitrary units (B and C). Data representing the mean ± SD, * P < 0.05 compared with control.

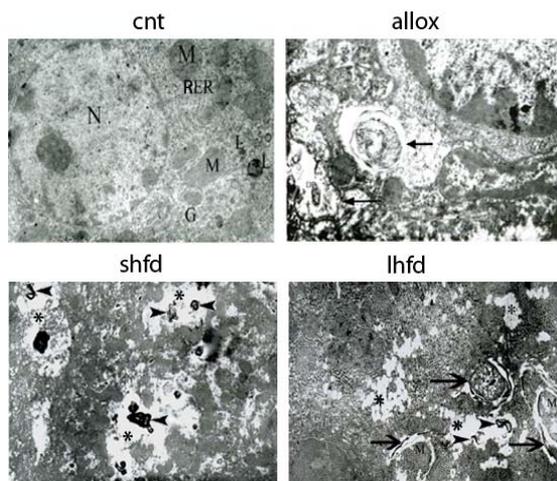


Fig. 3: Electron micrographs of hepatocytes of different experimental groups. Ultra-thin sections (50 nm) were prepared for TEM examination as described in method section. Electron micrographs (X 6700) showing autophagosomes (arrows), nucleus (N), mitochondria (M), rough endoplasmic reticulum (RER), lysosomes (L) and glycogen granules (G). In shfd and lhfd groups there are many electron dense myelinated figures (arrowhead) representing residual bodies of late lysosomes indicating extensive autophagy process in rarified areas in the cytoplasm (asterisk).

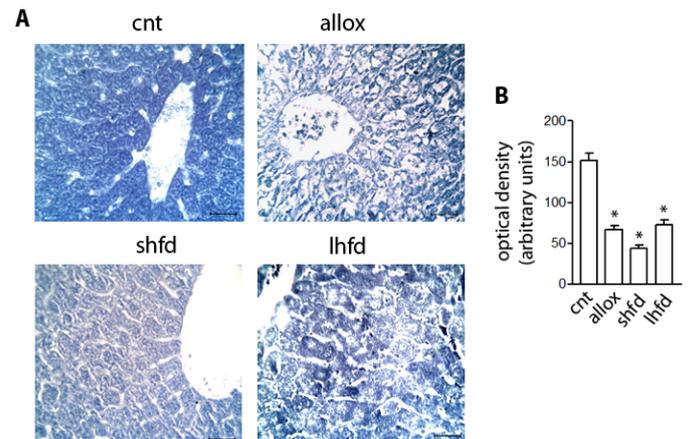


Fig. 4: General protein contents of liver in different treatments. Representative photomicrographs of liver sections stained with bromophenol blue of different experimental groups (A), bar equals 50μm. Estimation of colour optical density was carried out by Image J software and expressed as arbitrary units (B). Data representing the mean ± SD, * P < 0.05 compared with control.

ARABIC SUMMARY

ارتفاع جلوكوز و انسولين الدم يحدثان التهاب ذاتى خلوى فى خلايا كبد ذكور الفئران

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معمل بيولوجيا الخلية الجزيئية- قسم علم الحيوان- كلية العلوم- جامعة اسيوط - اسيوط ٧١٥١٦- جمهورية مصر العربية

الدراسة الحالية تبحث دور ارتفاع سكر الدم او انسولين الدم فى احداث التهاب خلوى ذاتى فى خلايا كبد ذكور الفئران. عملية الالتهام الذاتى الخلوى هى عملية تقوم فيها الخلية باعادة استخدام او تدوير العضيات الخلوية المسنة او التالفة وكذلك محتويات الخلية الغذائية الزائدة وتحللها بواسطة انزيمات الليسوسوم و الاستفادة من نواتج تحللها ويحدث ذلك تحت ظروف خاصة. زيادة مستوى سكر الدم فى الدراسة الحالية تم بواسطة حقن جرعة وحيدة من الالوكسان (١٨٠ ملج/كجم من وزن الجسم) فى التجويف البريتونى وزيادة انسولين الدم تم بواسطة تغذية مجموعة اخرى من الفئران لمدة قصيرة (اسبوعان) او لمدة طويلة (ثلاث اشهر) على نظام غذائى عالى السعرات مع محلول سكر الجلوكوز. تم التأكد من زيادة سكر الدم بواسطة جهاز قياس سكر الدم و انسولين الدم بواسطة مجموعة الكشف المناعى (ELISA). تم دراسة الالتهام الذاتى الخلوى مورفولوجيا عن طريق ملاحظة اجسام الالتهام والتحلل الذاتى فى تحضيرات المجهر الالكترونى النافذ و بيوكمياتيا عن طريق الكشف المناعى البروتينى (western blot) لانتيجينات الالتهام الذاتى و هى (LC3I- LC3II-mTOR- pmTOR). نتائج هذه الدراسة اكدت ان ارتفاع مستوى سكر و انسولين الدم كلا على حده يؤدى الى حث عملية الالتهام الذاتى الخلوى فى خلايا الكبد. هذه النتيجة تلقى الضوء على الالتهام الذاتى الخلوى لخلايا الكبد كآلية خلوية تستحث بواسطة ارتفاع سكر الدم او انسولين الدم والتي تكون السبب فى داء السكرى. هذه النتائج ممكن ان تساعد على تحسين معرفتنا بالاسس الخلوية للاضطرابات الابضية الغذائية عامة ومنها داء السكرى.