Ultrastructural Pathology

ISSN: 0191-3123 (Print) 1521-0758 (Online) Journal homepage: https://www.tandfonline.com/loi/iusp20

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To cite this article: Mohamed A. Haidara, Mohammad Dallak, Abbas O. El Karib, Mohamed Abd Ellatif, Refaat A. Eid, El Hassan A. Heidar & Bahjat Al-Ani (2018) Insulin protects against hepatocyte ultrastructural damage induced by type 1 diabetes mellitus in rats, Ultrastructural Pathology, 42:6, 508-515, DOI: 10.1080/01913123.2018.1551258

To link to this article: https://doi.org/10.1080/01913123.2018.1551258

ULTRASTRUCTURAL PATHOLOGY
2018, VOL. 42, NO. 6, 508–515
https://doi.org/10.1080/01913123.2018.1551258

BASIC RESEARCH

Insulin protects against hepatocyte ultrastructural damage induced by type 1 diabetes mellitus in rats

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ABSTRACT
Diabetic complications that affect vital organs such as the heart and liver represent a major public health concern. The potential protective effects of the hormone insulin against hepatocyte ultrastructural alterations induced secondary to type 1 diabetes mellitus (T1DM) in a rat model of the disease have not been investigated before. Therefore, rats were injected once with 65 mg/kg streptozotocin (T1DM group) and the protection group (T1DM+Ins) received a daily injection of insulin 48 h post diabetic induction by streptozotocin and continued until being sacrificed at week 8. The harvested liver tissues were examined using transmission electron microscopy (TEM) and blood samples were assayed for biomarkers of liver injury enzyme, glycemia, lipidemia, inflammation, and oxidative stress. TEM images showed that T1DM induced profound hepatocyte ultrastructural alterations as demonstrated by pyknotic nucleus, condensation of chromatin, irregular nuclear membrane, swollen mitochondria, dilated rough endoplasmic reticulum, damaged intercellular space, and accumulation of few lipid droplets inside the hepatocyte cytoplasm, which were substantially protected with insulin. In addition, the blood chemistry profile complements the TEM data as demonstrated by an increase in serum levels of alanine aminotransferase (ALT), dyslipidemia, C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and malondialdehyde (MDA) by T1DM that were significantly ($p < 0.05$) reduced with insulin injections. Thus, we conclude that insulin effectively protects against T1DM-induced liver injury in rats for a period of 8 weeks, possibly due to the inhibition of inflammation, oxidative stress, and dyslipidemia.

ARTICLE HISTORY
Received 31 October 2018
Accepted 19 November 2018

KEYWORDS
Hepatocyte ultrastructure; T1DM; insulin; inflammation; animal model

Introduction

An estimated 347 million people were reported to have diabetes mellitus worldwide.[1] High fasting blood glucose level is considered as a hallmark feature of diabetes mellitus caused either by an autoimmune disease that destroys the insulin-producing pancreatic β-cells in children (type I diabetes mellitus) or a decline in insulin sensitivity on its target site like muscle and adipose tissues (type II diabetes mellitus; also called adult onset diabetes).[2] High blood sugar leads to multiple organ dysfunction, especially the eyes, kidneys, liver, and heart.[3] Although the pathophysiological basis of type I diabetes mellitus (T1DM) complications remains uncertain, uncontrolled glycemia, oxidative stress, and inflammation appear to play a central role in the progression of the disease and its complications.[4] In addition, several studies have demonstrated a link between T1DM and liver disease,[5,6] and liver alterations have been suggested to be associated with other diabetic complications, such as chronic kidney disease.[5]

Insulin is the main hormone that regulates the utilization of glucose by the body and maintains normal levels of blood glucose by facilitating glucose uptake by cells, regulating the metabolism of carbohydrate, protein and lipid, and promoting cell growth and division via hepatic production of insulin-like growth factors.[7] Several biochemical and morphological defects occur due to glucose and insulin abnormalities such as diabetic

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retinopathy[8], diabetic nephropathy[9], vascular injury[10], and liver injury.[11] Therefore, insulin or insulin stimulating drugs are essential to immediately treat glycemia.

The association between type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD), which describes a wide spectrum of liver disorders from steatosis to cirrhosis, has been thoroughly investigated.[12,13] However, little is known about the evolution of liver injury in patients and animals with T1DM, and animal models of the disease using chemical induction by alloxan (ALX) or streptozotocin (STZ) provided valuable information about T1DM-induced liver injury.[14,15] Therefore, the aim of the present study was to investigate the protective effect of insulin on hepatocyte ultrastructural alterations induced secondary to T1DM in rats.

Methods

Animals

All experimental procedures were approved by the medical research ethical committee at King Khalid University and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. (NIH publication No. 85–23, revised 1996). Sprague Dawley rats (n = 18) weighing 170–200 g were used in this study. All rats were bred and housed in the research center of King Khalid University, college of medicine (Abha, Saudi Arabia), at temperature of 23 ± 1°C and a 12-h light: 12-h dark cycle. Rats had free access to tap water and fed standard laboratory chow during the acclimatization period.

Experimental design

After a 1-week adaptation, the rats were randomly divided into three groups (n = 6 rats each). Animals in group 1 (Control) were used as the control group and fed with standard laboratory chow for 8 weeks. Animals in group 2 (T1DM group) were injected once with 65 mg/kg streptozotocin (STZ) and fed a standard laboratory chow for 8 weeks. Diabetes was confirmed by fasting blood glucose levels >300 mg/dl measured using a Randox reagent kit (Sigma-Aldrich, Randox Laboratories, Antrim, UK). The third group, protection group (T1DM+Ins) received a daily injection of insulin (0.75 IU/100 gm weight in 0.75 ml volume) 48 h post diabetic induction by STZ and continued until being sacrificed at week 8.

Biochemical measurements

Blood samples

At the end of experimental period, blood samples were collected by cardiac puncture under anesthesia (sodium thiopentone at 40 mg/kg body weight) after an overnight fast of 12 h. These blood samples were collected without anticoagulant, left for 10 min, then centrifuged for 10 min at 4,000 r/min to obtain serum, which was stored at minus 20°C until further biochemical analysis for determination of serum glucose, ALT, Lipids, oxidative stress, and inflammatory biomarkers.

Determination of serum levels of ALT, glucose, hs-CRP, TNF-α, IL-6, MDA, TG, CHOL, LDL, and HDL

After 8 weeks, animals were sacrificed and liver function was evaluated by assessing serum liver injury enzyme (ALT) levels using an enzymatic kit (Randox Laboratories, Crumlin, UK) according to the manufacturer's instructions. Serum glucose was determined colorimetrically using a Randox reagent kit (Sigma-Aldrich). Serum levels of C-reactive protein (hs-CRP, ELISA kit Cat. No. ERC1021-1; ASSAYPRO, USA), TNF-α (ELISA kit BIOTANG INC, Cat. No. R6365, MA, USA), and IL-6 (ELISA kit BIOTANG INC, Cat. No. RB1829, MA, USA) were used as recommended by the manufacturer. MDA (measured as TBARS) using Thiobarbituric Acid Reactive Substances, TBARS Assay kit, Cayman Chemical Item Number 10009055 according to the manufacturer's instructions. The concentrations of serum total cholesterol (CHOL), triglyceride (TG), low-density lipoprotein–cholesterol (LDL), high-density lipoprotein–cholesterol (HDL-Ch) were measured using the corresponding commercial enzyme kits (HUMAN Diagnostics, Wiesbaden, Germany).

Transmission electron microscopy (TEM)

Small pieces of liver tissues were removed and immediately fixed in 2.5% glutaraldehyde for 24 h and washed with phosphate buffer (0.1 M, PH 7.4). Post fixation was made in 1% osmium tetroxide buffered to PH 7.4 with 0.1 M phosphate buffer at 4°C for 1–2 h. The samples washed in phosphate buffer to remove excess fixative, dehydrated through ascending grades of ethanol followed by clearing in propylene oxide. The specimens were embedded in Araldite 502, to form gelatin capsules. Polymerization was obtained by placing the capsules at 60°C. Semi-thin sections (~1 mm thick) were stained with toluidine blue for orientation and observation. Ultra-thin sections (100 nm) were prepared using ultra-microtome and picked up on uncoated copper grids. Following double staining with uranylacetate and lead citrate, three to five random micrographs for each section were examined and photographed using a JEM-1011-JEOL transmission electron microscope, Japan, at 80 Kv.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Data were processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). One-way ANOVA was done followed by Tukey's post hoc test. Pearson correlation statistical analysis was done for detection of a probable significance between two different parameters. Results were considered significant if $p \leq 0.05$.

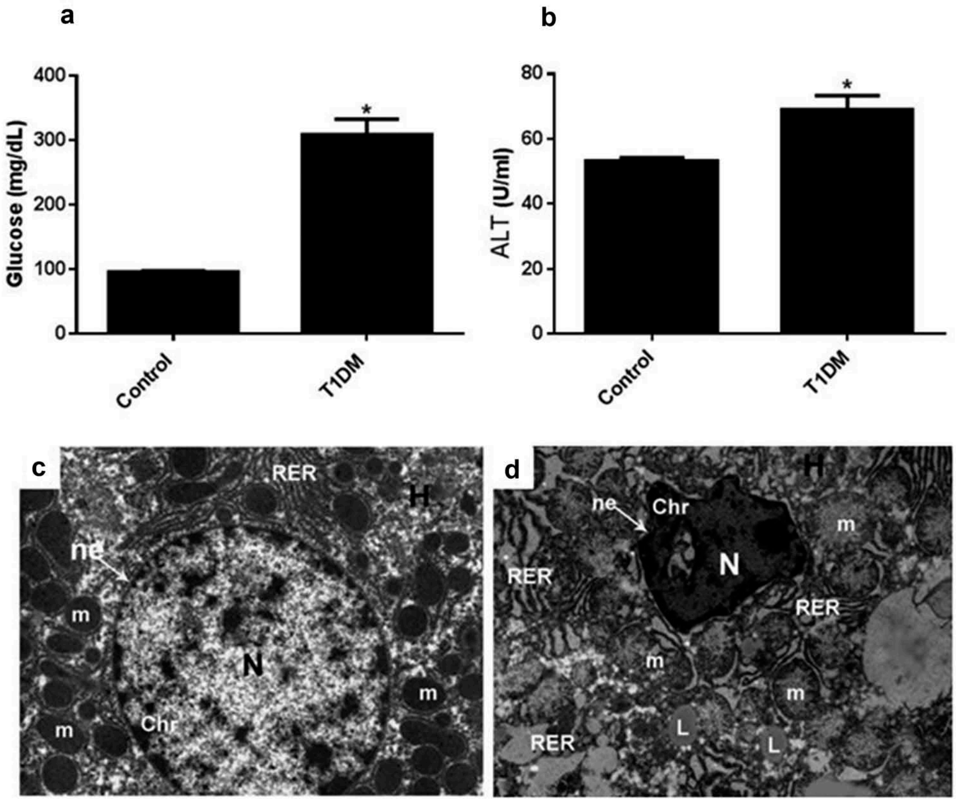

Figure 1. Induction of T1DM and liver injury in rats.

Blood levels of glucose (**a**) and ALT (**b**) were measured in the model group (T1DM) compared to the control group of rats ($n = 6$ for each group). Results represent the mean (±SD); and experiments were performed in triplicate. *$p < 0.05$ versus control. TEM images (12,000×) of harvested tissues obtained from the liver of model group (**d**) compared to control group (**c**) rats are visualized using transmission electron microscopy. Note that arrows point to nuclear membrane. Abbreviations: H, hepatocyte; N, nucleus; L, lipid droplets; m, mitochondria; RER, rough endoplasmic reticulum; ne, nuclear envelop; chr, chromatin.

Results

Induction of liver injury secondary to T1DM

We first sought to characterize the animal model of liver injury induced secondary to T1DM. T1DM was induced in male Sprague Dawley rats by injecting these rats once with 65 mg/kg STZ. High blood levels of glucose (exceeding 300 mg/dl; Figure 1(a)) and ALT (a 25% increase; Figure 1(b)), and TEM images of liver sections (Figure 1(c and d)) confirmed liver injury and abnormal changes to hepatocytes secondary to diabetes. Liver section from the diabetic group (Figure 1(d)) displays degenerate hepatocytes with pyknotic nucleus surrounded by irregular nuclear envelop and the condensation of chromatin masses. In addition, cytoplasm displays swollen mitochondria, dilated endoplasmic reticulum, and accumulation of few lipid droplets (steatosis). However, a TEM image of another hepatocyte at similar magnification prepared from liver sections of the control rats (Figure 1(b)) shows an unremarkable hepatocyte (H), with centrally placed nuclei (N) and crowded cytoplasm with organelles, particularly rough endoplasmic reticulum (RER) and mitochondria (m).

Insulin protects hepatocyte ultrastructural damage induced by T1DM

To test the hypothesis that insulin can protect the ultrastructure of hepatocytes against damage induced secondary to T1DM, we assessed the effect of daily injection of insulin for 8 weeks given 48 h post diabetic induction using TEM. Representative TEM images of liver sections obtained from the control animal group (Figure 2 (a and b)) show normal architecture of hepatocytes, which are surrounded by an intact intercellular space (thick arrows). The nuclei are rounding in shape with one or more nucleoli, and the cytoplasm display many organelles, particularly rough endoplasmic reticulum and mitochondria. TEM images represent liver sections of T1DM rats (Figure 2(c and d)) display blebbing of membranes, nuclear disaggregaton of granular elements, mitochondrial swelling, dilation of endoplasmic reticulum, accumulations of lipid droplets and vacuoles. Also, damaged intercellular space and abnormal bile canaliculus (Bc) with lost microvilli can be seen. Treatment of diabetic rats with insulin (Figure 2(e and f)) 2 days after the induction of diabetes completely protected the hepatocellular architecture for 8 weeks against alterations induced secondary to T1DM. This is demonstrated by TEM images showing intact hepatocytes with normal nuclear and cytoplasmic compartments comparable to the control group.

Insulin inhibits T1DM-induced lipidemia

There is a strong correlation between type 2 diabetes mellitus and non-alcoholic fatty liver disease (NAFLD), and little is known about the association between NAFLD and T1DM.[16] Therefore, we assessed blood levels of triglycerides (TG), cholesterol (CHOL), low-density lipoprotein (LDL-Ch), and high-density lipoprotein (HDL-Ch) in all rats at week 8. Figure 3 shows that T1DM caused a significant ($p < 0.05$) increase in TG (Figure 3 (a)), CHOL (Figure 3(b)), and LDL-Ch (Figure 3 (c)), which were significantly ($p < 0.05$) but not completely decreased by insulin, whereas induction of T1DM caused a significant ($p < 0.05$) decrease in HDL-Ch levels (Figure 3(d)) that were partially increased with insulin.

Insulin inhibits T1DM-induced oxidative stress and inflammation

To determine the effects of insulin in our model on suppressing the release of the oxidative stress and inflammatory biomarkers, MDA, hs-CRP, TNF-α, and IL-6, which are known to be involved in the pathology of hepatic damage, we measured the blood levels of these cytokines in all animal groups at week 8. Serum levels of MDA (Figure 4 (a)), hs-CRP (Figure 4(b)), TNF-α (Figure 4(c)), and IL-6 (Figure 4(d)) were significantly ($p < 0.05$) higher in the model group (T1DM group) compared with the control group. Daily injection of insulin 48 h post diabetic induction caused a significant ($p < 0.05$) decrease in these cytokines in the treated group (T1DM+Ins) compared to T1DM group.

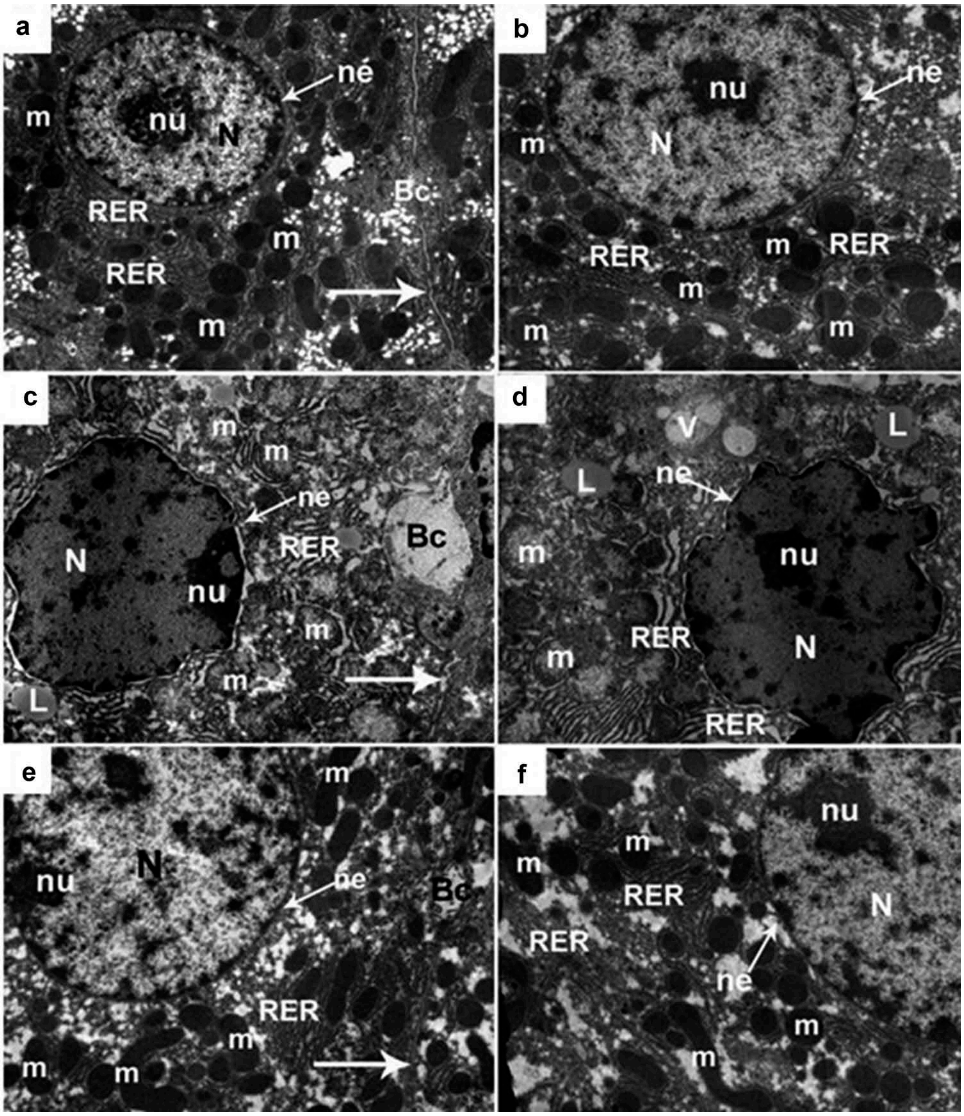

Figure 2. Insulin protects against T1DM-induced hepatocyte ultrastructural damage in rats.

TEM images (10,000×) of the liver obtained 8 weeks post diabetic induction. (**a** and **b**), Control group. (**c** and **d**), T1DM group. (**e** and **f**), T1DM group treated with insulin. Note that large arrows point to intercellular spaces, and small arrows point to nuclear membrane (ne). Abbreviations: H, hepatocyte; N, nucleus; L, lipid droplets; nu, nucleolus; m, mitochondria; RER, rough endoplasmic reticulum; BC, bile canaliculus.

Discussion

The main objective of our study was to investigate the potential protective effect of insulin on hepatocyte ultrastructural alterations induced by T1DM in a rat model of the disease using TEM. Therefore, we induced liver injury in rats after 8 weeks as a secondary complication to T1DM using a high dose of STZ (Figure 1). One group of diabetic rats was treated with insulin for 8 weeks, and the histological and biochemical parameters were monitored and confirmed the beneficial effects of insulin (Figures 2–4).

Clinical and experimental evidence suggests that T1DM affects the liver in addition to blood vessels, kidneys, retina, and nerves.[17–19] Although those complications are commonly associated with T2DM, increasing evidences show that T1DM is also linked to an increased risk of chronic liver injury.[20] However, the recognition of T1DM as the

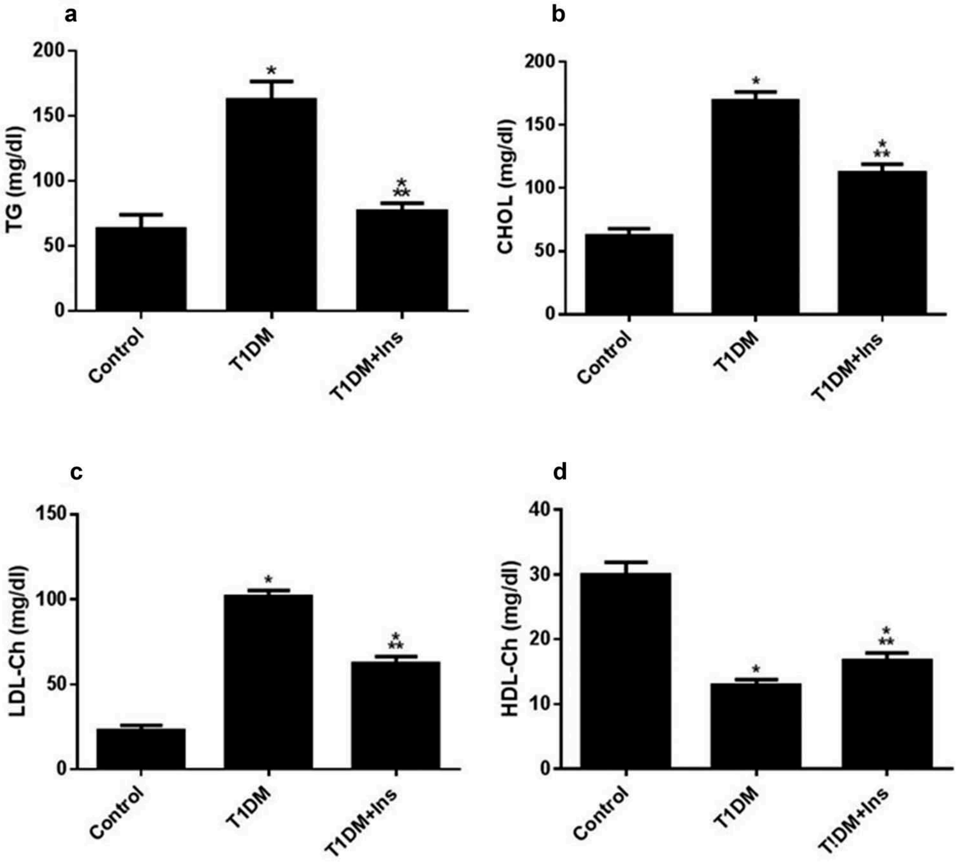

Figure 3. Insulin inhibits dyslipidemia induced by T1DM.
Blood levels of TG (**a**), CHOL (**b**), LDL-Ch (**c**), and HDL-Ch (**d**) were measured at the end of the experiment, after 8 weeks in three groups of rats; Control, the diabetic group (T1DM), and the treated group (T1DM+Ins). Results represent the mean (±SD); $n = 6$ for each group. Experiments were performed in triplicate. *$p < 0.05$ versus control, **$p < 0.05$ versus T1DM.

primary cause of chronic liver disease is neglected in medical practice because of the wide variety of clinical, metabolic and hormonal conditions that can lead NAFLD.[15,21] Our data that point to the induction of both lipidemia and hepatocyte ultrastrcutral alterations due to T1DM in rats (Figures 1–3) together with other previously published work[15]further strengthen the link between T1DM and liver injury and steatosis.

Elevated levels of inflammatory biomarkers like TNF-α and IL-6 are suggestive of active inflammatory diseases such as T1DM[22], T2DM[23], and hepatic injury.[24,25] These reports are in agreement with our findings (Figure 4), and the significant reduction in the levels of these markers upon treatment with insulin (Figure 4) might account for the observed improvement of the liver histology as indicated by a normal architecture of the hepatocytes and bile canaliculus with normal microvilli (Figure 2).

Liver as an insulin-dependent tissue plays a vital role in glucose and lipid homeostasis.[26] Oxidative stress causing structural and functional alterations in the cellular biomolecules and cell membrane is the result of the development of complications in diabetic individuals.[27] Poor glycemic control leads to NAFLD[16] and also may lead to a decrease in antioxidant defense system that is followed by the damage of cellular organelles and enzymes, increased level of lipid peroxidation in liver tissue and others, and development of insulin resistance.[28,29] Our data that demonstrate an increase in the level of lipid and the oxidative stress biomarker, MDA that measures lipid peroxidation are in agreement with the above findings, and insulin inhibition of MDA and lipids

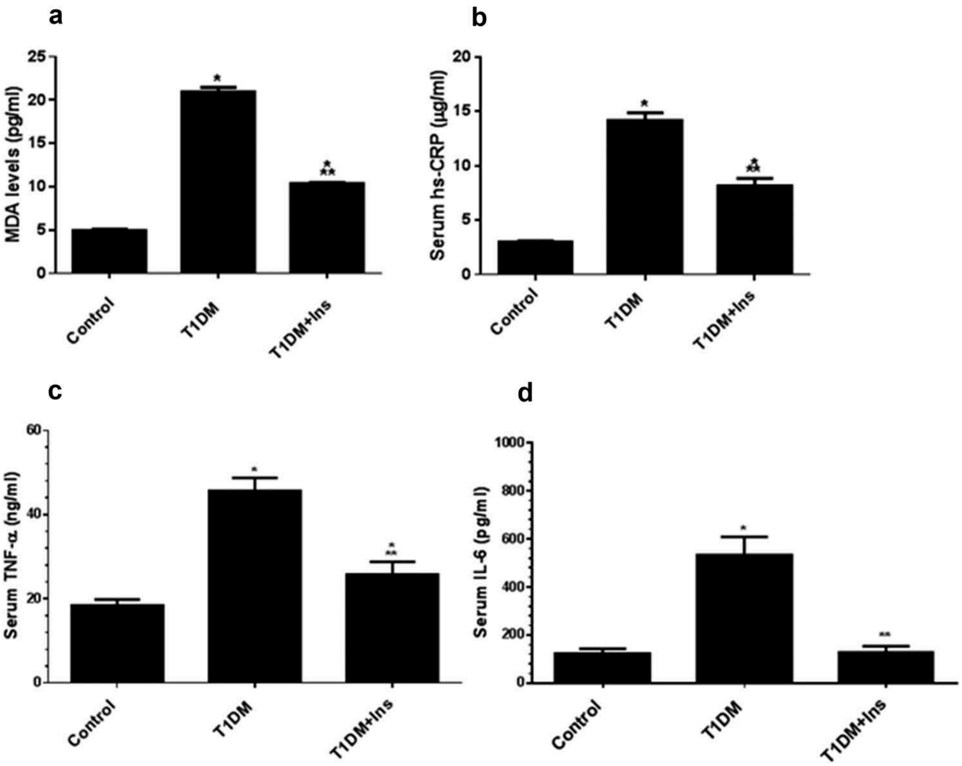

Figure 4. Insulin inhibits the induction of oxidative stress and inflammatory biomarkers caused by T1DM.

Blood levels of MDA (**a**), hs-CRP (**b**), TNF-α (**c**), and IL-6 (**d**) were measured at the end of the experiment, after 8 weeks in three groups of rats; Control, the diabetic group (T1DM), and the treated group (T1DM+Ins). Results represent the mean (±SD); $n = 6$ for each group. Experiments were performed in triplicate. *$p < 0.05$ versus control, **$p < 0.05$ versus T1DM.

could also be a factor in the observed protection of liver cells architecture by insulin (Figure 2).

Collectively, this study demonstrates that induction of T1DM in rats for 8 weeks resulted in hepatocyte ultrastructural alterations, possibly via augmentation of biomarkers of oxidative stress and inflammation, and insulin treatment was very effective in inhibiting liver injury.

Disclosure statement

We declare no competing financial interests.

Funding

This work was supported by King Khalid University grant number KKU-Project No. R.G.P.1/27/38.

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