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# Insulin protects against type 1 diabetes mellitus-induced aortopathy associated with the inhibition of biomarkers of vascular injury in rats

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**Short title:** Insulin protects aorta structures against diabetes

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**ABSTRACT**

**Background:** We sought to investigate the protective effect of insulin against type 1 diabetes mellitus (T1DM)-induced aortic injury (aortopathy) associated with the inhibition of biomarkers of vascular injury.

**Material and methods:** T1DM was induced in rats by streptozotocin (65 mg/kg), and the protection group started insulin treatment two days post diabetic induction and continued until being sacrificed at week 8.

**Results:** Aortopathy was developed in the diabetic rats as demonstrated by profound alterations to the aorta ultrastructure, which was substantially protected by insulin. In addition, insulin significantly inhibited diabetes-induced soluble sVCAM-1 and sICAM-1, dyslipidemia, oxidative stress and inflammation. However, blood levels of these biomarkers in the insulin-treated group were still significant ($p<0.05$) compared with the control group, whereas insulin treatment returned blood glucose and triglyceride to control levels.

**Conclusions:** We demonstrate effective protection by insulin against T1DM-induced aortopathy in rats, which is associated with the inhibition of vascular injury biomarkers.

**KEYWORDS:** Aortopathy; type 1 diabetes mellitus; insulin; vascular activation; rat model

## Introduction

It was estimated that almost 347 million people have diabetes globally and this disease claims the lives of around 3.4 million every year due to diabetic complications such as cardiovascular, renal diseases and hypertension (Danaei et al., 2011). The loss of insulin production by the β cells of the pancreas is believed to be caused by autoimmune antibodies that destroy these cells leading to type 1 diabetes mellitus (T1DM) in children, commonly between the ages of 7-19 years (Pugliese, 2004, Narendran et al., 2005, Atkinson et al., 2014). Hyperglycaemia stimulates the generation of inflammatory biomarkers and reactive oxygen species (ROS) such as superoxide through both mitochondria and NADPH oxidase and represents a common mechanism underlying the vascular injury observed in diabetes (Shoelson et al., 2006).

Vascular activation that leads to the release of the soluble form of vascular cell adhesion molecules such as VCAM-1, ICAM-1, E-selectin, and P-selectin is induced by diabetes mellitus, and are recognised biomarkers of diabetes-induced vascular injury (McLeod et al., 1995, Khare et al., 2005). In addition, dyslipidemia is regarded as a risk factor for the development of vascular injury(Husain et al., 2015) and progression of hypertension(Dai et al., 2019). 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 (Wilcox, 2005). Several biochemical and morphological defects occur due to glucose and insulin abnormalities such as diabetic retinopathy(Hammes et al., 2011),

diabetic nephropathy(Gross et al., 2005), and vascular injury(Rask-Madsen and King, 2013). Therefore, insulin or insulin-stimulating drugs are essential to immediately treat glycaemia. However, aortic ultrastructural alterations induced by T1DM associated with the modulation of biomarkers of vascular injury in the presence and absence of insulin has not been investigated before. Therefore, the aim of the present study was to investigate the effects of insulin on T1DM-induced aortopathy in rats and to monitor blood levels of the vascular endothelial activation biomarkers sVCAM-1 and sICAM-1, and dyslipidemia, oxidative stress and inflammation in the blood of these rats.

## Material and methods

### *Animals*

Healthy male Wistar rats at 10 weeks age and weighing 150-200 g were used for these studies. The rats were fed with a standard laboratory diet, given water and maintained under laboratory conditions of temperatures ranging 22±3°C, with 12 hour light and 12 hour dark cycles. All experimental procedures involving the handling and treatment of animals were approved by the Ethical Committee of King Khalid University Medical School (Abha, KSA) and were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

### *Experimental design*

After a one week adaptation period, rats were randomly allocated into 3 groups (n= 6) as follows: The non-diabetic control group (*Control*) were injected intraperitoneally (i.p.)

once with citrate buffer (0.1 M, pH 4.5); Insulin-dependent diabetic group (*T1DM*): DM was induced in rats by a single i.p. injection of streptozotocin (STZ) in a dose of 65 mg/kg. Rats that showed glucose levels above 300 mg/dl were considered to be diabetic and included in the study (Haidara et al., 2004); and T1DM and insulin group (*T1DM+ Ins*): T1DM rats were treated 48 hours post diabetic induction with mixtard insulin subcutaneously in a dose of 0.75 IU/100 gm weight in 0.75 ml volume once daily (El Karib et al., 2016) until being sacrificed, end of week 8.

***Preparation of blood and tissues for analysis***

After 8 weeks, fasting blood samples were collected under anaesthesia using 40 mg Kg-1 sodium thiopentone, i.p., and animals were then culled. Aortic tissues were collected and fixed in 2.5% glutaraldehyde for scanning electron microscopy examinations. Sera were separated and stored at -80°C for subsequent measurements of biochemical parameters.

***Transmission electron microscopy (TEM)***

As we previously reported (Dallak et al., 2018), small pieces of aortic tissue were removed and immediately fixed in 2.5% glutaraldehyde for 24 hours 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 $4^{o}$C for 1-2 hours. 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$^{o}$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. The effect of insulin was determined by assessing the integrity of aortic endothelium and smooth muscle cells in 50 fields scored as % positivity.

***Serum measurements of glucose, triglyceride, cholesterol, LDL-C, HDL-C, MDA, SOD, TNF-α, and hs-CRP***

Animals were sacrificed 8 weeks post-diabetic induction, and serum glucose was determined colorimetrically using a Randox reagent kit (Sigma-Aldrich). Triglycerides (TG), cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using commercial kits supplied by SPINREACT, Spain, according to the manufacturer's instructions. Malondialdehyde (MDA) measured as Thiobarbituric Acid Reactive Substances, TBARS Assay kit, Cayman Chemical Item Number 10009055 and Superoxide dismutase assay kit (SOD), rat, Cayman Chemical, Cat. No. 706002 was used as recommended by the manufacturer. Rat tumour necrosis factor alpha, TNF-α, (ELISA kit BIOTANF INC, Cat. No. R6365) was used as recommended by the manufacturer. Serum levels of high

sensitive C-reactive protein were measured using ELISA kit (hs-CRP, Cat. No. ERC1021-1) from ASSAYPRO, USA.

### *Serum measurements of soluble VCAM-1 and ICAM-1*

The level of vascular cell adhesion protein 1 (sVCAM-1) and intercellular adhesion molecule-1 (sICAM-1) was determined in the serum of all animal groups 8 weeks after the induction of T1DM using rat ELISA Assay kits purchased from MyBioSource (San Diego, USA) as recommended by the manufacturer.

### *Statistical analysis*

The data were expressed as the mean ± standard deviation (SD). Data were processed and analyzed using Graph Pad Prism software (version 5). One-way ANOVA was done followed by Tukey's post hoc test. Pearson correlation statistical analysis was done for the detection of a probable significance between two different parameters. Results were considered significant if $p \leq 0.05$.

## Results

### *Insulin protects against T1DM-induced aortopathy*

To evaluate the effect of insulin treatment on protection against T1DM-induced aortopathy using the TEM approach, one group of diabetic rats was treated for 8 weeks with insulin, and aortic tissues were prepared for transmission electron micrographs

from all the groups. TEM images of the aortic wall layers, tunica intima (Figure 1A, 1C, 1E) and tunica media (Figure 1B, 1D, 1F) are shown here. Representative TEM image of the tunica intima obtained from the control animal group (Figure 1A ) showing the regular architecture of endothelium as demonstrated by the appearance of a normal endothelial cell (En) with its nucleus (N) displaying peripheral heterochromatin, resting on clear continuous basement membrane (arrow), lining the vessel's lumen (Lu). The image of tunica media shown in Figure 1B obtained from the control animal group showed regularly arranged spindle-shaped smooth muscle cells (SMC) and intact mitochondria (m). In addition, fine moderately electron-dense collagen fibres (arrows) in extracellular matrix are revealed. TEM images represent aortic sections of T1DM rats (Figure 1C and 1D) show, in the tunica intima layer (Figure 1C) markedly distorted vacuolated endothelial cell (En) with an irregular heterochromatic nucleus (N) and detached from the underlying basement membrane (arrow). Thick electron dense elastic lamina (e) and irregular distorted smooth muscle cells (SMC) are also seen. Whereas, the aortic wall layer tunica media (Figure 1D) is showing distorted smooth muscle cells (SMC), swollen mitochondria (m) with disrupted cristae, cytoplasmic vacuolations (v), and lipid droplet (L). Also, fragmented electron-dense extracellular matrix (arrow) and the irregular electron-dense elastic laminae (e) were also present. Approximately 85% of the scanned aortic endothelial cells and 50% of the vascular SMC had poor morphology.

Treatment of the T1DM rats with insulin provided substantial protection against the development T1DM-induced aortopathy, as demonstrated by normal endothelial cells (En) with euchromatic nuclei (N) resting on a clear intact basement membrane

(black arrow), lining the vessel's lumen (Lu). Regularly-arranged spindle-shaped smooth muscle cells (SMC) with euchromatic nuclei (N) and intact mitochondria (m) are seen with slightly disrupted electron-dense extracellular matrix (white arrow) in between (Figure 1E and 1F). Approximately 85% of the scanned aortic endothelial cells and vascular SMC had normal morphology due to insulin treatment.

***Insulin inhibits T1DM-induced inflammation and endothelial activation***

The link between inflammation, endothelial activation, and vascular injury is well established (Mu et al., 2015). To investigate whether the observed protection of aortopathy by insulin was also associated with the inhibition of biomarkers of inflammation and endothelial activation, we measured TNF-$\alpha$, hs-CRP, soluble VCAM-1 and ICAM-1 in serum samples collected from the insulin-treated (T1DM+Ins), T1DM and control groups 8 weeks after the induction of diabetes (Figure 2). Insulin treatment significantly ($p<0.05$) reduced diabetes-induced TNF-$\alpha$ (Figure 2A), hs-CRP (Figure 2B), circulating sVCAM-1 (Figure 2C), and sICAM-1 (Figure 2D). However, the level of these parameters in the T1DM+Ins group was significantly elevated compared with the control group of rats.

***Insulin inhibits T1DM-induced hyperglycemia and oxidative stress***

To investigate whether the observed protection to aortic tissues in the diabetic rats treated with insulin shown above was also associated with glycemic control, reduction in

the blood levels of oxidative stress, and augmentation of anti-oxidant biomarkers, we measured blood glucose, MDA, and SOD in the insulin-treated group (T1DM+Ins) and compared it to the T1DM and control groups, 8 weeks after the induction of diabetes (Figure 3). Compared to the diabetic group (T1DM), insulin treatment (T1DM+Ins) significantly ($p<0.05$) reduced blood glucose (Figure 3A) and MDA (Figure 3B), and increased SOD (Figure 3C) to levels comparable to the control group in (A), but still significant to the control group in (B) and (C).

***Insulin inhibits T1DM-induced modulation of blood lipids***

The link between dyslipidemia and vascular injury is well-known(Flores et al., 2017). To investigate whether the observed protection of aortopathy by insulin was also associated with the inhibition of dyslipidemia, we measured blood levels of TG (Figure 4A), CHOL (Figure 4B), LDL-C (Figure 4C), and HDL-C (Figure 4D) in all animal groups 8 weeks after the induction of diabetes. Insulin treatment significantly ($p<0.05$) modulated diabetes-induced TG, CHOL, LDL-C, and HDL-C. However, the level of these parameters in the T1DM+Ins group was significantly elevated compared with the control group of rats except TG.

## Discussion

The present report examines the development of aortopathy secondary to T1DM in a rat model of the disease using transmission electron microscopy approach that demonstrates effective protection by insulin, which substantially slows down the progression of the disease. In addition, this approach links the pathophysiology of aortopathy with the known causes, oxidative stress, inflammation, endothelial activation, and dyslipidemia. These conclusions are supported by the data indicating that induction of diabetes using a high dose of STZ caused profound damage to the aortic wall layers, tunica intima and tunica media, which was prevented following daily administration of insulin for 8 weeks (Figure 1). Furthermore, insulin significantly reduced circulating biomarkers of inflammation (TNF-$\alpha$ and hs-CRP), vascular injury (sVCAM-1 and sICAM-1), oxidative stress (MDA), and hyperlipidemia (Figures 2 - 4) that are known to be elevated in cardiovascular disease secondary to diabetes (Dai et al., 2016, Berk et al., 2016, Rodriguez-Castaneda et al., 2018) these references maybe mentioned before]. However, only blood sugar and triglyceride returned to control levels following treatment with insulin. These observations may point to a possible decline in the degree of protection provided by insulin as time goes by.

The aorta is a known target of T1DM in humans and animals (Hagensen et al., 2017, McCulloch et al., 2015, Turkbey et al., 2013), and our transmission electron micrographs (Figure 1) confirm the development of aortopathy as one of the diabetic complications occurring 8 weeks after the induction of diabetes in rats, are in agreement with part of a study that investigated the ultrastructural changes of aorta in T1DM rats

over a span of 150 days (Searls et al., 2012). Though insulin is the only drug of choice to treat T1DM, patients with T1DM still develop cardiovascular complications even with insulin treatment (Chillaron et al., 2014). Our data indicate good glycemic control with insulin but only partial reduction of pro-inflammatory, oxidative stress, and vascular activation biomarkers. This is in broad agreement with a recent report (Al Hariri et al., 2017) demonstrating a significant increase in post-translational protein modification in the aorta and kidney of T1DM rats that was partially reversed by insulin treatment despite blood glucose levels returning to control levels. It also supports work that showed incomplete protection by insulin alone against the development of osteoarthritis secondary to T1DM in rats until we co-administered vanadium with insulin, which provided much greater protection (El Karib et al., 2016). Indeed, a meta-analysis study on 4351of diabetic patients monitored over five years showed that hypoglycaemic agents failed to prevent the development of diabetic nephropathy (Lachin et al., 2011).

The link between aortopathy and elevated levels of sVCAM-1 and sICAM-1 is well established in the aortic tissues of atherosclerotic patients (Mu et al., 2015), patients undergoing open heart surgery (Andresen et al., 2002), and non-rheumatic aortic valve disease (Ghaisas et al., 2000). These are in agreement with our TEM and biochemical data that clearly draw the link between these parameters, and insulin was able to substantially “break“ the link between these points; oxidative stress, inflammation, endothelial activation, and aortopathy.

In conclusion, using transmission electron microscopy and blood chemistry, we demonstrate the development of aortopathy as a secondary complication to type 1

diabetes mellitus in a rat model, which is significantly protected by insulin for a period of 8 weeks. We also demonstrate an association between aortopathy and biomarkers of vascular endothelial activation, dyslipidemia, oxidative stress and inflammation. Future investigations will determine whether the protective effect of insulin against the development of aortopathy can be extended over a longer period of time.

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**Disclosures**: We declare no competing financial interests.

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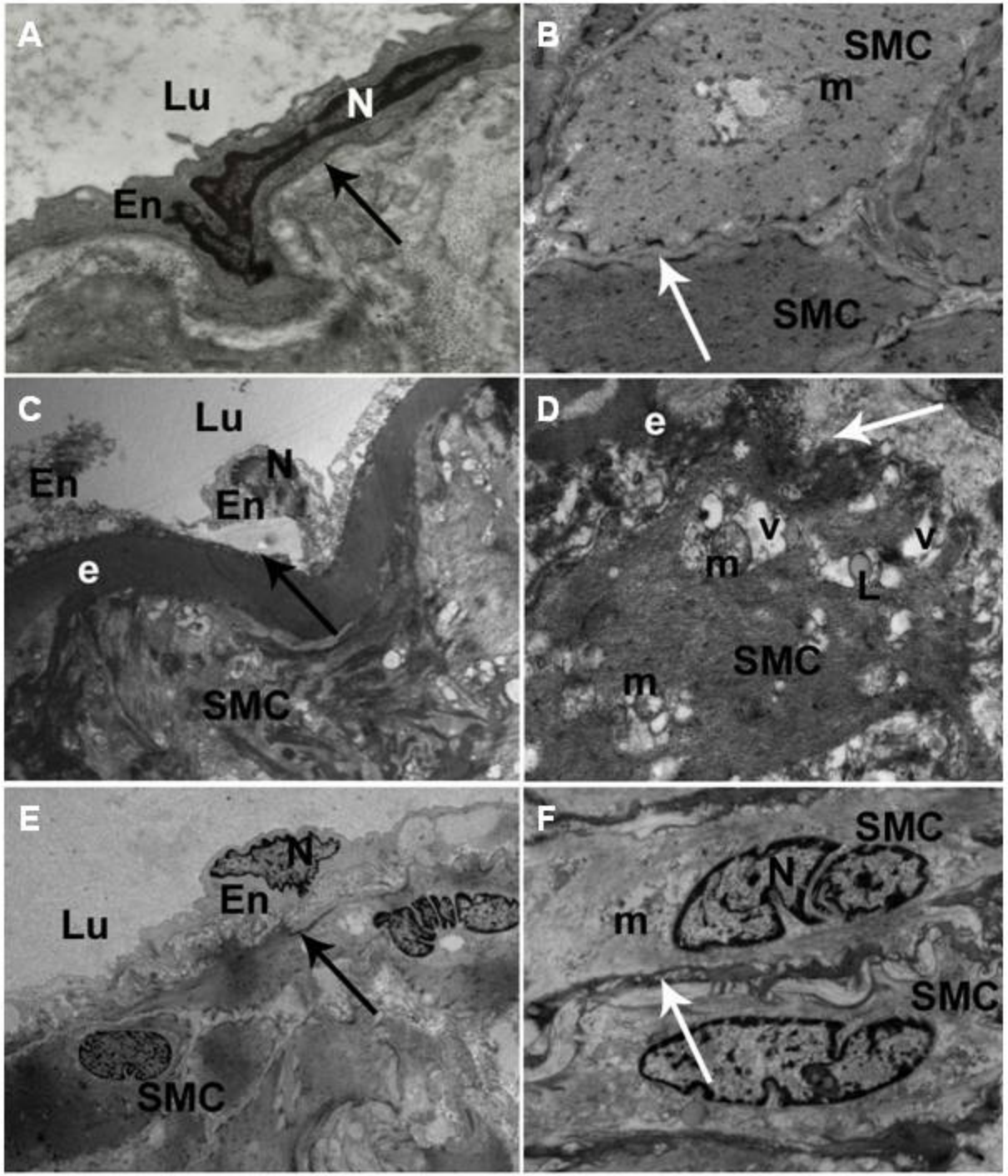
## Figure legends

**Figure 1. Insulin protects against aortopathy induced secondary to T1DM in rats.** TEM images (8000x) of the aortic wall layer tunica intima (**A, C, E**) and aortic tunica media (**B, D, F**) obtained 8 weeks post diabetic induction. (**A** and **B)** Control group. (**C** and **D**) Diabetic group.(**E** and **F**). Diabetic group treated with insulin. Abbreviations: N, nucleus; SMC, smooth muscle cells; m, mitochondria; v, vacuoles; En, endothelial cell; Lu, lumen; e, elastic lamina; and L, lipid droplet.

**Figure 2. Insulin reduces circulating markers of inflammation and endothelial activation induced by T1DM.** Blood levels of TNF-$\alpha$ (**A**), hs-CRP (**B**), sVCAM-1 (**C**), and sICAM-1(**D**) were measured 8 weeks post diabetic induction in 3 groups of rats; control, diabetic (T1DM), and diabetic plus insulin (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 diabetic group, T1DM.

**Figure 3. Insulin prevents hyperglycemia and modulates circulating levels of oxidative and anti-oxidative stress biomarkers in streptozotocin-induced T1DM.** Blood glucose (**A**), MDA (**B**), and SOD (**C**) were measured 8 weeks post diabetic induction in 3 groups of rats; control, diabetic (T1DM), and diabetic plus insulin (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 diabetic group, T1DM.

**Figure 4. Insulin modulates dyslipidemia in streptozotocin-induced T1DM.** Blood levels of TG (**A**), CHOL (**B**), LDL-C (**C**), and HDL-C (**D**) were measured 8 weeks post diabetic induction in 3 groups of rats; control, diabetic (T1DM), and diabetic plus insulin (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 diabetic group, T1DM.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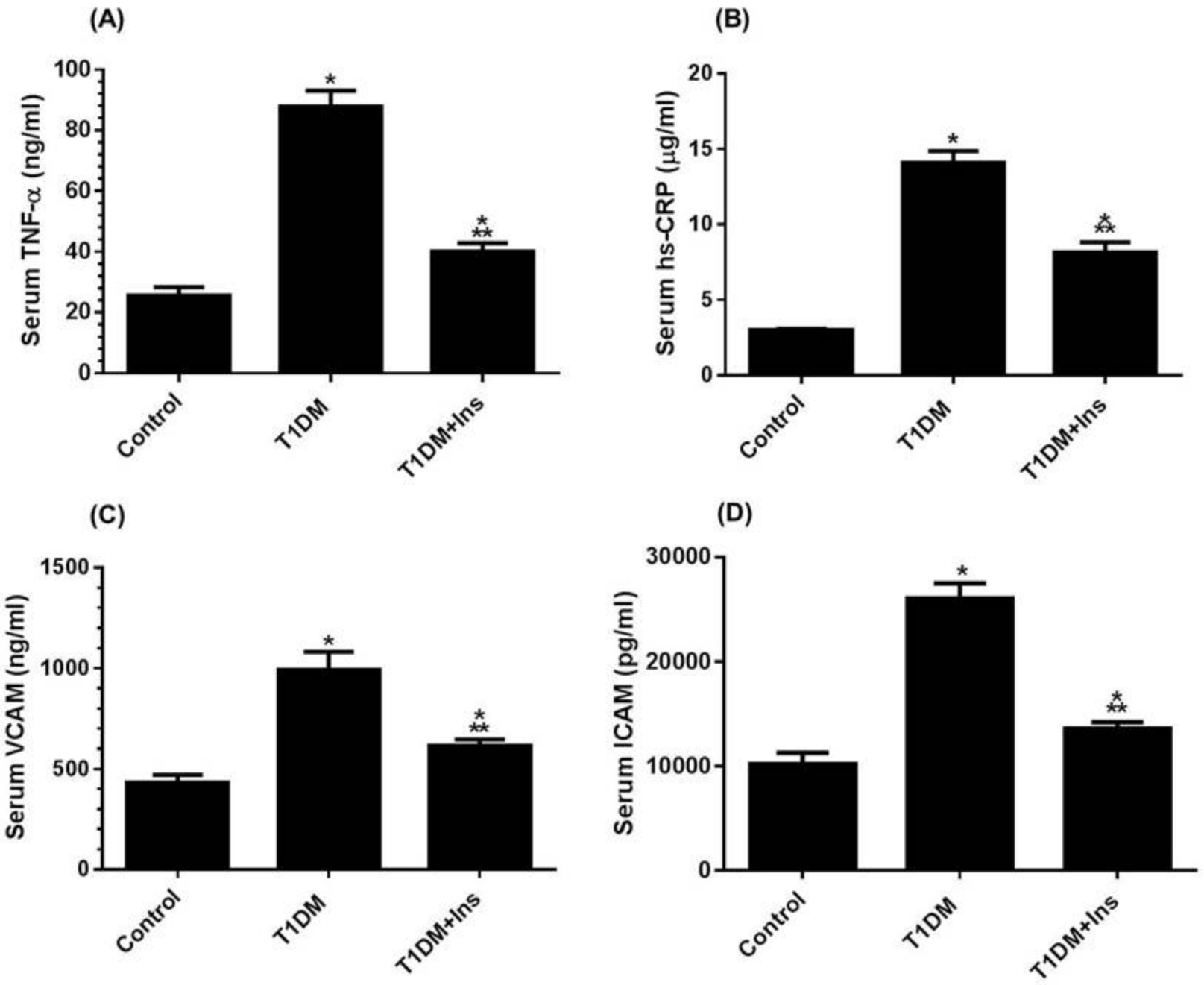
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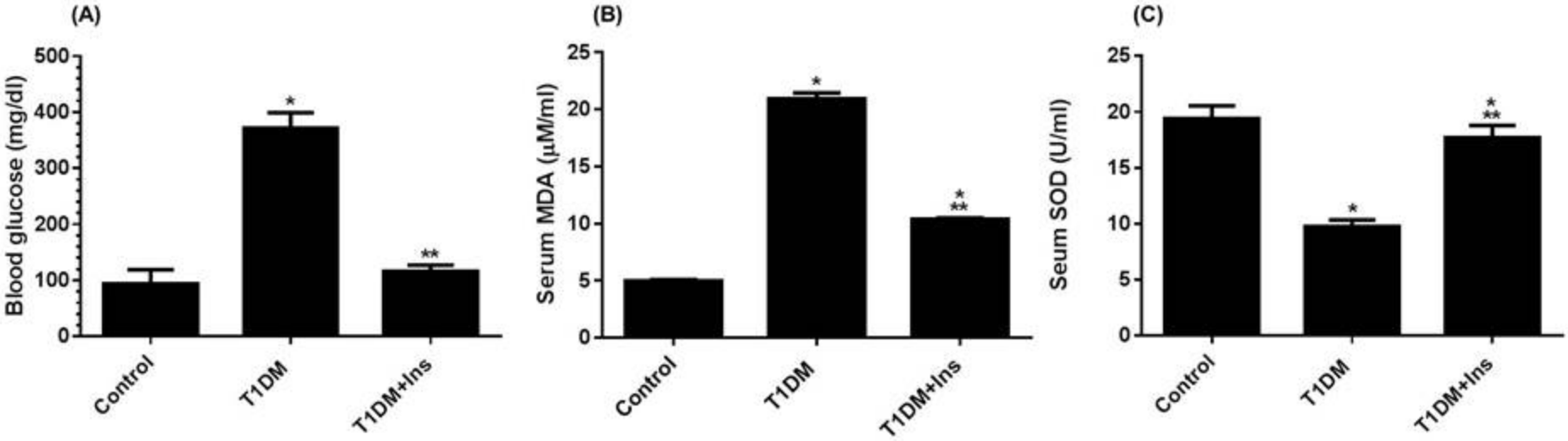
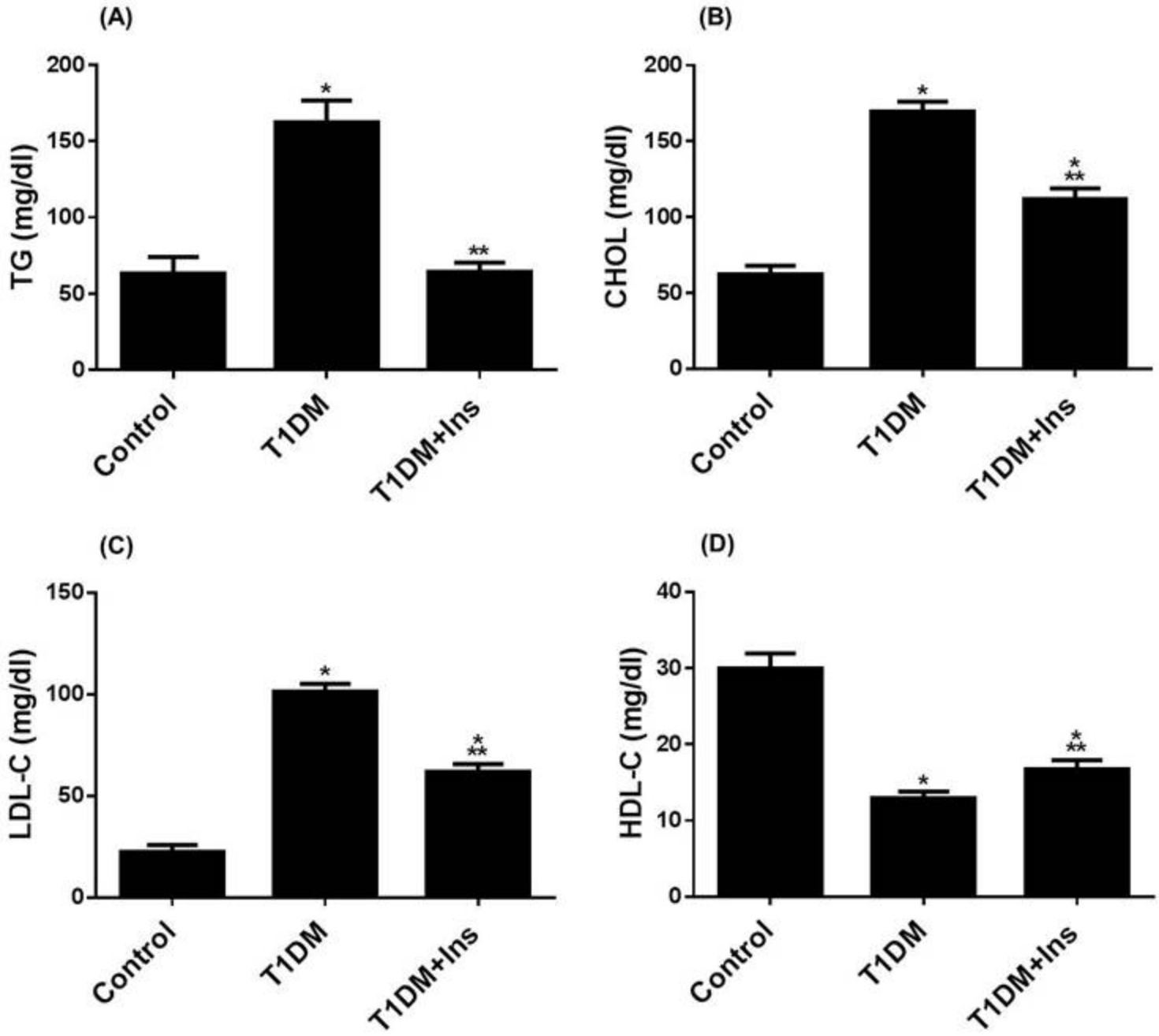

(A)
Blood glucose (mg/dl)
500
400
300
200
100
0
*
**
Control
T1DM
T1DM+Ins
(B)
Serum MDA (μM/ml)
25
20
15
10
5
0
*
*
**
Control
T1DM
T1DM+Ins
(C)
Seum SOD (U/ml)
25
20
15
10
5
0
*
*
**
Control
T1DM
T1DM+Ins


(A)
TG (mg/dl)
200
150
100
50
0
*
**
Control
T1DM
T1DM+Ins
(B)
CHOL (mg/dl)
200
150
100
50
0
*
*
**
Control
T1DM
T1DM+Ins
(C)
LDL-C (mg/dl)
150
100
50
0
*
*
**
Control
T1DM
T1DM+Ins
(D)
HDL-C (mg/dl)
40
30
20
10
0
*
*
**
Control
T1DM
T1DM+Ins