

JPET #239087

JPET/2016/239087 2<sup>nd</sup> Revision

# **PDE5 Inhibitor Tadalafil and Hydroxychloroquine Co-Treatment Provides Synergistic Protection against Type 2 Diabetes and Myocardial Infarction in Mice**

Rui Wang, Lei Xi, and Rakesh C. Kukreja

*Pauley Heart Center, Division of Cardiology, Virginia Commonwealth University.*

*Richmond, Virginia (R.W., L.X., R.C.K.)*

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**Running title:** Tadalafil and Hydroxychloroquine Protect Diabetic Heart

**Corresponding author:**

Rakesh C. Kukreja, Ph.D.

Scientific Director, Pauley Heart Center

Professor of Medicine, Physiology, Biochemistry

Division of Cardiology, Box 980204

Virginia Commonwealth University

1101 East Marshall Street, Room 7-020D

Richmond, VA 23298-0204, U.S.A.

Phone: 804-628-5521 Fax: 804-828-8700

E-mail: rakesh.kukreja@vcuhealth.org

**Number of text pages:** 33

**Number of tables:** 0

**Number of figures:** 8

**Number of references:** 53

**Number of words in Abstract:** 256

**Number of words in Introduction:** 904

**Number of words in Discussion:** 1679

**Abbreviations:** AUC, area under the curve; Erk1/2, extracellular signal-regulated kinase 1/2; HCQ, hydroxychloroquine; IGF-1, insulin-like growth factor-1; I/R, ischemia/reperfusion; ITT, insulin tolerance test; K-H, Krebs-Henseleit; mTOR, mammalian target of rapamycin; NEFA, non-esterified fatty acids; NO, nitric oxide; OGTT, oral glucose tolerance test; PDE5, phosphodiesterase 5; T2D, type 2 diabetes; TAD, tadalafil; TTC, 2,3,5 triphenyltetrazolium chloride.

**Recommended section assignment:** Cardiovascular

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## Abstract

Diabetes is associated with high risk of ischemic heart disease. We previously showed that phosphodiesterase 5 inhibitor - tadalafil (TAD) induces cardioprotection against ischemia/reperfusion (I/R) injury in diabetic mice. Hydroxychloroquine (HCQ) is a widely used antimalarial and anti-inflammatory drug, which was reported to reduce hyperglycemia in diabetic patients. Therefore we hypothesized that combination of TAD and HCQ may induce synergistic cardioprotection in diabetes. We also investigated the role of insulin-Akt-mTOR signaling, which regulates protein synthesis and cell survival. Adult male db/db mice were randomized to receive vehicle, TAD (6 mg/kg), HCQ (50 mg/kg), or TAD+HCQ daily by gastric gavage for 7 days. Hearts were isolated and subjected to 30-min global ischemia followed by 1-hour reperfusion in Langendorff mode. Cardiac function and myocardial infarct size were determined. Plasma glucose, insulin and lipid levels and relevant pancreatic and cardiac protein markers were measured. Treatment with TAD+HCQ reduced myocardial infarct size ( $17.4\pm 4.3\%$  vs.  $37.8\pm 4.9\%$  in Control group,  $P<0.05$ ) and enhanced production of ATP. The TAD+HCQ combination treatment also reduced fasting blood glucose, plasma free fatty acids, and triglyceride levels. Furthermore, TAD+HCQ increased plasma insulin levels ( $513\pm 73$  vs.  $232\pm 30$  mU/L,  $P<0.05$ ), with improved insulin sensitivity, larger pancreatic  $\beta$ -cell area and pancreas mass. IGF-1 levels were also elevated by TAD+HCQ ( $343\pm 14$  vs.  $262\pm 22$  ng/mL,  $P<0.05$ ). The increased insulin/IGF-1 resulted in activation of downstream Akt/mTOR cellular survival pathway. These results suggest that combination treatment with TAD and HCQ could be a novel and readily translational pharmacotherapy for reducing cardiovascular risk factors and protecting against myocardial I/R injury in type 2 diabetes.

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## Introduction

Type 2 diabetes (T2D) is one of the major risk factor for developing cardiovascular disease and the resultant devastating morbidity and mortality in the world (Ginter and Simko, 2012;Nichols, et al., 2013). Diabetic patients with myocardial infarction have worse prognosis than non-diabetic patients with myocardial infarction (Miettinen, et al., 1998). In diabetic hearts, the reduced glucose uptake and increased circulating free fatty acids lead to shift of energy substrate from carbohydrates to fatty acids, resulting in less ATP production with more oxygen consumption, which makes cells more susceptible to myocardial ischemia (Barsotti, et al., 2009). Recently it has been suggested that type 2 diabetic hearts retain metabolic flexibility to adapt to hypoxia and become more dependent on oxidative metabolism following hypoxia with 30% lower glycolytic rates and 36% higher fatty acid oxidation than non-diabetic controls, which led to functional deficit in response to ischemic stress (Mansor, et al., 2016). Thus, normalization of circulating glucose and lipid levels is a critical target of therapeutic intervention in the management of diabetic patients (Gilbert and Krum, 2015;Fukushima, et al., 2015;Nichols, et al., 2013).

Phosphodiesterase 5 (PDE5) inhibitors are a class of drugs widely used to treat erectile dysfunction. Two of the PDE5 inhibitors sildenafil and tadalafil (TAD) are also approved by FDA for the treatment of pulmonary arterial hypertension (Hemnes and Champion, 2006;Galie, et al., 2009). In addition, several studies from our laboratory have shown that PDE5 inhibitors protect against myocardial ischemia/reperfusion (I/R) injury in both healthy and diabetic animals (reviewed in Das, et al., 2015). The underlying molecular mechanism involves the induction of nitric oxide (NO) synthase (Das, et al., 2005;Salloum, et al., 2003), which leads to subsequent NO-cGMP-protein kinase G signaling cascade with promotion of hydrogen sulfide production

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(Salloum, et al., 2009), activation of extracellular signal-regulated kinase 1/2 (Erk1/2) pathway (Das, et al., 2009) and opening of mitochondrial  $K_{ATP}$  channels (Ockaili, et al., 2002). Our previous studies showed that 4-week chronic treatment of db/db mice with long-acting PDE5 inhibitor – TAD resulted in reduction of blood glucose and triglyceride levels, and smaller myocardial infarct size (Varma, et al., 2012), indicating beneficial effects in maintaining homeostasis of whole body metabolism and protecting against I/R injury under T2D conditions.

Hydroxychloroquine (HCQ) is a first-line anti-malarial and anti-inflammation drug, which has been widely used to treat malaria, systemic lupus erythematosus and rheumatoid arthritis (Anderson, 1995;Morand, et al., 1992). Chloroquine is reported to improve insulin sensitivity through the activation of Akt, resulting in increased glucose uptake and glycogen synthesis in L6 muscle cell lines (Halaby, et al., 2013). Also, clinical case reports indicated that both chloroquine and HCQ improved glycemic control in type 1 diabetes patients, partially through the inhibition of insulin degradation (Blazar, et al., 1984;Hage, et al., 2014). A 3-day oral chloroquine treatment improved the serum lipid profile of T2D patients, with increased fasting insulin level (Powrie, et al., 1993). HCQ users among rheumatoid arthritis patients had lower risk of developing diabetes (Bili, et al., 2011) suggesting a protective effect of HCQ against insulin resistance. However, whether this anti-diabetic effect of HCQ could further lead to cardioprotection against I/R injury, the most common cause of death in T2D, is unknown. Interestingly, a recent study reported that oral administration of 200 mg/kg HCQ for 3 days prior to I/R injury significantly decreased myocardial infarct size in non-diabetic rats (Bourke, et al., 2015).

Based on this background information, we hypothesized that TAD in combination with HCQ could synergistically protect T2D db/db mouse heart from I/R injury through mechanisms

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involving insulin-Akt/mTOR pathway and modulation of circulatory energy substrate levels. We also investigated the effects of TAD and HCQ alone and in combination on the islets of Langerhans in pancreas.

## Materials and Methods

**Animals.** All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, Revised 1996). The animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Adult male db/db mice (B6.BKS(D)-*Lepr*<sup>db</sup>/J) were obtained from Jackson laboratory (Bar Harbor, ME). Mice with a fasting glucose level between 200 and 400 mg/dL were included in the experiments.

**Drugs and Treatment Protocols.** Seventy six male db/db mice were assigned into 4 groups (n=19/group) and received the following treatments via daily oral gavage for 7 days: *Group 1*: Control vehicle (CTRL, 0.5 % hydroxypropyl methy cellulose and 1 % Tween 80 in sterile water); *Group 2*: Tadalafil (TAD, Lilly USA, Indianapolis, IN) 6 mg/kg/day mixed in vehicle solvent; *Group 3*: Hydroxychloroquine sulfate (HCQ, Plaquenil, Sanofi-Aventis U.S., Bridgewater, NJ) 50 mg/kg/day mixed in vehicle solvent; and *Group 4*: TAD (6 mg/kg/day) + HCQ (50 mg/kg/day) mixed in vehicle solvent. The oral dose of HCQ was chosen based on a recent study (Long, et al., 2013), which used 50 mg/kg/day in adult rats administered daily by intraperitoneal injection for 3 weeks. Chronic treatment with this dose of HCQ provided significant beneficial effects against pulmonary hypertension. In addition, this experimental dose of HCQ is compatible with the clinically prescribed dose i.e., 250 mg per day (~3.8 mg/kg for an

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adult with 65 kg body weight) for the treatment of rheumatoid arthritis and systemic lupus erythematosus. According to the Food and Drug Administration, the human HCQ dose (250 mg/kg/day) can be converted into mouse dose (i.e. human dose multiple by 12.5 = 50 mg/kg/day). The final numbers of animals used for data analysis are n=19 for CTRL, TAD, and HCQ group respectively, n=17 for TAD+HCQ group (2 TAD+HCQ-treated mice died apparently due to physical injury during the oral gavage procedure). Body weight was recorded and fasting blood glucose levels were measured using Bayer™ Contour™ blood glucose monitoring system.

After completion of the 1-week treatment, animals were anesthetized with intraperitoneal injection of pentobarbital sodium (100 mg/kg) and euthanized, their hearts and pancreata were surgically collected. Besides a subset of hearts were subjected to I/R in Langendorff model (n=5 for TAD, HCQ, TAD+HCQ groups and n=6 for CTRL group), the tissue samples from other animals were snap frozen in liquid nitrogen and stored at -80°C. Meanwhile, blood samples were collected from all animals into heparinized tubes and plasma was separated by centrifugation at 4 °C, 3000 rpm for 30 min.

**Ischemia/Reperfusion Studies.** I/R studies were performed in isolated perfused hearts using Langendorff model as described previously (Xi, et al., 1999). In brief, following anesthetization, the hearts were isolated and immediately immersed into 95% oxygen/5% CO<sub>2</sub> saturated ice cold Krebs-Henseleit (K-H) buffer, containing (in mM) 118 NaCl, 24 NaHCO<sub>3</sub>, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 11 D-(+)-Glucose, 0.5 EDTA, 2.5 CaCl<sub>2</sub>. Within 3 min, the aorta was cannulated and mounted onto a Langendorff perfusion system and the hearts were retrogradely perfused with K-H buffer maintained at 37 °C under a constant pressure of 55 mmHg. The heart apex was attached to a Grass FT03 force-displacement transducer with

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surgical thread and a rigid metal hook for measuring ventricular contractile force. The beat-by-beat cardiac contractile signals were continuously recorded with a PowerLab/8sp system. After 30 min of equilibration, hearts were subjected to 30 min of normothermic no-flow global ischemia (37 °C), followed by 1 hour of reperfusion. Coronary flow rate was measured via timed collection of coronary effluents at 15 min before ischemia and 15 min after reperfusion. At the end of experiment, hearts were collected, weighed and stored at -20°C for further analysis.

**Infarct Size Measurement.** The day after Langendorff experiment, frozen hearts were transversely cut into 6 or 7 sections, 1 mm in thickness and placed in Petri dish. The heart sections were stained with 10% 2,3,5 triphenyltetrazolium chloride (TTC, Sigma Aldrich, St. Louis, MO) for 30 min, and then transferred into 10% neutral buffered formalin for fixation (Sigma Aldrich, St. Louis, MO). After taking pictures of each sections, infarct size was quantified by computer morphometry using ImageJ software. The infarct size was presented as percentage of risk area (i.e. the total ventricular area minus cavities). A portion of the heart tissue was used for preparation of protein extracts.

**Insulin, Insulin-like Growth Factor and Lipid Assays.** Plasma insulin levels were determined using insulin ELISA kit (Crystal Chem INC., Downers Grove, IL). Insulin-like growth factor-1 (IGF-1) levels were measured in plasma samples using mouse/rat IGF-1 Quantikine® ELISA (R&D systems, Minneapolis, MN). Non-esterified fatty acids (NEFA), triglycerides and cholesterol levels were assayed using NEFA, L-Type Triglyceride M and Cholesterol E assay kits (Wako Pure Chemical Industries, Japan). The cardiac levels of triglycerides and total cholesterol were normalized with total protein content.

**Glucose and Insulin Tolerance Test.** The glucose tolerance test was performed in mice after overnight fasting. Following measurement of body weight, each animal received glucose (2



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mg/kg body weight) via oral gavage (n=5/group). Blood samples were drawn from the tail vein at baseline, 15, 30, 60 and 120 min after glucose administration. The plasma glucose and insulin levels were measured by Glucose Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) and insulin ELISA Kit (Crystal Chem INC., Downers Grove, IL). For insulin tolerance test, the animals (n=5/group) were fasted for 6 hours. After measuring body weight, regular human insulin (Novolin<sup>®</sup>, Novo Nordisk, Princeton, NJ) was administered intraperitoneally (0.9 IU/kg). Blood glucose were taken at baseline, 15, 30, 60, 90 and 120 min after insulin injection, glucose levels were measured using Bayer<sup>™</sup> Contour<sup>™</sup> blood glucose monitor.

**Immunofluorescence and Hematoxylin and Eosin Histology.** Mouse pancreata were weighed and ratio of pancreas weight vs. body weight were calculated (n=5/group). Immunohistology was performed according to a previous published method with modifications (Kim, et al., 2011). Briefly, the mouse pancreata were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue was sectioned to a thickness of 5  $\mu$ m. Multiple sections (200  $\mu$ m apart) from each animal were chosen and stained with goat anti-insulin (Santa Cruz biotechnology), followed by secondary antibody conjugated with FITC (Life technology). Images of insulin positive  $\beta$ -cells were taken under microscope, and cross-sectional areas of insulin positive  $\beta$ -cells as well as relative area of pancreas were determined. The insulin positive  $\beta$ -cell area/pancreas area was calculated. Also, sections of pancreas were stained with hematoxylin and eosin (n=8 for TAD and n=9 for CTRL, HCQ, and TAD+HCQ). Whole sections were scanned with NanoZoomer 2.0-HT Digital slide scanner (Hamamatsu, Japan) and cross-sectional islet numbers were counted. The number of islets per mm<sup>2</sup> tissue area were calculated.

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**Western Blot Analysis for Protein Expression.** The heart tissue was crushed in liquid nitrogen, transferred into RIPA buffer (Thermo Scientific™ Pierce™ #PI-89900) and further homogenized. Samples were then centrifuged at 4°C with 12,000 rpm for 10 min and supernatant was collected. Protein concentration was determined using BCA reagent (Thermo Scientific #23227). Samples were then mixed with 2X sample buffer (Bio-Rad #1610737) and boiled for 5 min prior to loading on 4-15% Criterion TGX Gel system (30 µg/sample) for electrophoresis. A wet transfer was performed using Bio-Rad Criterion™ blotter system. Membranes were then incubated in 5% non-fat milk for 1 hour at room temperature, followed by incubation with primary antibodies (1:1000 dilution) at 4°C overnight. The membranes were further incubated with secondary antibodies, and then developed using 20X LumiGLO Reagent and 20X Peroxide (Cell Signaling, #7003). We chose β-actin as the internal reference. Antibodies used were rabbit anti-p-mTOR #5536, rabbit anti-mTOR #2938, rabbit anti-p-S6 #4858, mouse anti-S6 #2317, rabbit anti-pAkt<sup>Thr308</sup> #4056, rabbit anti-p-Akt<sup>Ser473</sup> #4060, rabbit anti-Akt #4691, rabbit anti-Raptor #2280, rabbit anti-Rictor #2114 (Cell Signaling); mouse anti-β-actin sc-58679 (Santa Cruz Biotechnology).

**ATP Assay.** Frozen myocardial tissue samples (10 mg from each animal, n=5/group) were used to quantify ATP levels in the tissue using ATP Assay Kit (Abcam) according to the manufacturer's instructions.

**Statistics.** Data were analyzed using GraphPad Prism 6 software. One-way ANOVA and Dunnett's post-hoc test were performed for global and pair-wise comparisons of the multiple groups. Data were presented as Mean±SEM and p<0.05 was considered statistically significant.

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## Results

**Treatment with Tadalafil and Hydroxychloroquine Reduces Myocardial Infarct Size Following Ischemia/Reperfusion Injury.** The experimental protocol is shown in Figure 1. Mice treated with the combination of TAD and HCQ had a significantly smaller myocardial infarct size as shown by TTC staining (Figure 2). After 7 days of the drug treatment, no difference was observed in cardiac contractile function based on ventricular developed force (Figure 3A) and rate-force product (Figure 3B), prior to ischemia and at the end of reperfusion. Heart rate (Figure 3C) and coronary flow rate (Figure 3D) were also not different among the treatment groups.

**Effect of Tadalafil and Hydroxychloroquine Treatment on Blood Glucose, Insulin, IGF-1 and Lipids.** Baseline levels of fasting glucose were measured before any treatment. The plasma level of insulin was increased following 7 days treatment with HCQ alone or in combination with TAD as compared to the control. (Figure 4A,  $p < 0.05$ ). The same trend was observed in TAD treated group as well. IGF-1 levels increased only in the TAD+HCQ combination treatment group (Figure 4B). The blood glucose level increased significantly from the baseline level in the control group. However no such increase in glucose levels was observed in TAD, HCQ or especially TAD+HCQ group, which had a significantly lower glucose levels as compared to the control group following 1 week of treatment (Figure 4C). Lipid profile was also altered after TAD+HCQ treatment, with a significant decrease in the plasma levels of free fatty acids and triglycerides in the TAD+HCQ group (Figure 4D and 4E). There was a trend towards decreased cholesterol levels in all 3 drug-treated groups although the differences did not reach statistical significance, (Figure 4F). Interestingly, cardiac levels of triglycerides increased in

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HCQ-treated group (Figure 4G), whereas cholesterol levels in the combination treated hearts were decreased (Figure 4H).

**Beneficial Effects of the Combination Therapy with Tadalafil and Hydroxychloroquine on Insulin Sensitivity.** We performed oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) to assess the effect of combination treatment of TAD and HCQ on insulin sensitivity in the db/db mice. The results showed no significant difference in glucose uptake or insulin release between the control and TAD+HCQ groups during OGTT (Fig. 5A to 5D). However, TAD+HCQ treatment group showed a larger decline in blood glucose levels in response to insulin injection (Fig. 5 E to 5G), as indicated by approximately 20% drop in the area under the curve (AUC) (Fig. 5F), suggesting improved insulin sensitivity.

**Tadalafil and Hydroxychloroquine Improved Pancreatic  $\beta$ -cell Area.** We next asked the question whether the observed increase in fasting insulin levels (Figure 4A) was due to a protective effect of the drug treatment on the pancreatic  $\beta$ -cells. Immunofluorescence staining showed that the total insulin positive  $\beta$ -cell area was markedly increased following treatment with either TAD or HCQ alone and in combination (Figure 6A and 6C). There was significant increase in the islet number per mm<sup>2</sup> in the pancreas (Figure 6B and 6D). In addition, TAD, HCQ, and TAD+HCQ treated animals had a clear trend towards increased pancreas mass/body weight with statistical significant increase in the HCQ and TAD+HCQ groups (Figure 6E).

**Effect of Tadalafil and Hydroxychloroquine on Cardiac Insulin/IGF-1-Akt-mTOR Pathway.** Insulin/IGF-1 trigger downstream Akt-mTOR pathway in response to food and energy intake to stimulate glucose uptake and glycogen and protein synthesis. We observed increased Akt phosphorylation at site Thr<sup>308</sup> following I/R in mice treated with TAD, HCQ, and TAD+HCQ combination (Fig. 7A and 7B). As a result, the downstream mammalian target of

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rapamycin (mTOR) was activated. Phosphor-mTOR was increased by the combination treatment (Fig. 7A and 7C). We also investigated if both of mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) were activated by drug treatments. The expression of mTORC1 protein Raptor was increased in all three drug treated groups following I/R (Figure 7A and 7D), which was associated with enhanced phosphorylation of S6, the mTORC1 downstream ribosomal protein (Figure 7A and 7E). The total S6 protein expression was also increased in TAD, HCQ and TAD+HCQ treated groups (Figure 7A and 7E). The mTORC2 protein Rictor expression was also upregulated in TAD, HCQ and TAD+HCQ treated hearts (Figure 7A and 7F). Consequently, phosphor-Akt<sup>Ser473</sup>, the downstream target of mTORC2 was dramatically increased in the HCQ and TAD+HCQ treatment groups (Figure 7A and 7G).

**TAD+HCQ Combination Treatment Improved ATP Levels in Heart.** To determine whether the beneficial effects of TAD and HCQ on cardiac cell survival, circulating insulin/IGF-1 or the blood profile of lipids are correlated with the improvement in the final product of cardiac energy metabolism, we also measured ATP level in the heart. Our results showed that both HCQ and TAD+HCQ groups had significantly increased levels of cardiac ATP production after 7-day treatment as compared to TAD or HCQ treated groups ( $p < 0.01$ , Figure 8).

## Discussion

The main features of T2D are hyperglycemia, hyperlipidemia, insulin resistance and reduced insulin production in the late stage. It has been well recognized that diabetic individuals are resistant to most of the cardioprotective drugs that would be beneficial only in the non-diabetic people. The pathology of myocardial I/R injury involves multiple pathways, which

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include calcium overload, pH paradox, generation of reactive oxygen species, inflammation, endothelial dysfunction, and altered myocardial metabolism (Yellon and Hausenloy, 2007). Numerous therapeutic strategies targeting single mechanistic pathway have limited effect on human and animal models, indicating that myocardial I/R injury is a complex confluence of divergent biological signaling.

Our previous studies reported that after 4 or 8 weeks of treatment, TAD provided myocardial protection in db/db mice by improving mitochondrial function, increasing NO bioavailability and inhibiting inflammation (Koka, et al., 2014;Varma, et al., 2012). In addition, both *in vivo*, *in vitro*, and cohort-based reports have shown that HCQ has multiple other properties that include beneficial effects on metabolism function, vascular compliance and endothelial function (Bili, et al., 2011;Blazar, et al., 1984;Hage, et al., 2014;Halaby, et al., 2013;Powrie, et al., 1993;Siso, et al., 2008). In the present study, we report a novel and potentially very important combination use of TAD and HCQ in protection against myocardial I/R injury and reduction of cardiovascular risk factors (e.g. hyperglycemia and hyperlipidemia) in T2D. This novel strategy of combining both TAD and HCQ provided superior beneficial effects than a monotherapy in T2D. The short-term treatment for 7 days with TAD and HCQ resulted in improved blood glucose, free fatty acids, triglyceride levels and improved insulin signaling, which ultimately contributed to significant reduction of infarct size. However, the improvement in cardiac function was not evident after the drug treatment (Figure 3). Such dissociation between the infarct size reduction and improvement of contractile function is not unusual because previously published studies by our group and others have also observed that ischemic preconditioning (Xi, et al., 1998;Jenkins, et al., 1995) or stem cell therapy (Moelker, et al., 2006) cause significant reduction in infarct size without concomitant improvement in

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ventricular function. A possible explanation for this dissociation is that there may be two separate mechanisms controlling cardiac cell survival and contractility respectively. A protective mechanism on cardiomyocyte viability may not necessarily translate into a better contractility, or vice versa. The phenomenon of myocardial stunning is another example, in which cardiomyocytes are viable, but their contractility is severely depressed. The experimental and clinical studies have also shown that myocardium reperfused after reversible ischemia exhibits prolonged depression of contractile function (Bolli, et al., 1991). Furthermore, the viable but stunned myocardium can gradually regain its contractility, but infarcted myocardium would likely to have sustained contractile dysfunction. Therefore, the infarct size reduction by TAD+HCQ therapy may eventually lead to a better cardiac function, which needs to be examined in a larger in vivo translational model of T2D following prolonged reperfusion.

In diabetic patients, increased free fatty acids are released from adipose tissue through triglyceride lipolysis (Stich and Berlan, 2004), leading to higher circulating free fatty acid levels that can further result in increased  $\beta$ -oxidation and reduction of glycolysis in cardiomyocytes. The combination treatment of TAD and HCQ reduced plasma free fatty acids and blood glucose levels as well as cardiac cholesterol content, suggesting a myocardial protective effect through regulation of both circulating energy substrates and cardiac lipids (Figure 4C-4H). Previous study showed that higher cardiac cholesterol content in the hypercholesterolemic rats was associated with the loss of sevoflurane-induced cardioprotection against I/R injury via alteration of the survival kinase signaling pathway (Xu, et al., 2013). In addition, hesperidin-induced protection against isoproterenol-induced cardiotoxicity was associated with a reduction in cardiac cholesterol levels (Selvaraj and Pugalendi, 2012). Accordingly we postulate that the

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observed decrease in cardiac cholesterol content in the TAD+HCQ treated diabetic mice (Figure 4H) may also play a cardioprotective role against I/R injury.

In the present study, we observed increased baseline insulin levels in animals treated with combination of TAD and HCQ. Also, a better insulin sensitivity was observed, with improved insulin response 30 min after insulin injection (Figure 5). Furthermore, the mass of pancreas was larger in HCQ as well as TAD and HCQ treated groups, with increased insulin positive  $\beta$ -cell area in the latter group (Figure 6). The pancreas weight /body weight was higher in HCQ and the combination treated groups suggesting a possible protective effect on  $\beta$ -cells as well. These results support previous clinical studies showing improvement in  $\beta$ -cell function with HCQ or TAD in human subjects (Hill, et al., 2009;Wasko, et al., 2015). Interestingly, there was no significant difference in the insulin release during OGTT test, which suggests that the combination treatment is effective only in improving baseline (fasting) insulin secretion.

We also observed higher plasma IGF-1 levels in the combination treatment group which possibly leads to activation of PI3K/Akt/mTOR pathway in  $\beta$ -cells. It has been shown that IGF-1 promotes pancreatic  $\beta$ -cell line INS-1 cell proliferation in vitro within the physiologically relevant glucose concentration (6–18 mM). This synergistic effect induces more than 50-fold cell proliferation (Hugl, et al., 1998). However other studies have reported that pancreatic specific IGF-1 knockout mice had 2.3 fold increase in pancreatic cell mass, suggesting that locally produced IGF-I within the pancreas inhibits islet cell growth (Lu, et al., 2004). In addition,  $\beta$ -cell specific IGF-1 receptor knockout mice showed normal growth and development of  $\beta$  cells but had a defective glucose-stimulated insulin secretion with impaired glucose tolerance (Kulkarni, et al., 2002). Thus the function of IGF-1 in  $\beta$ -cell proliferation remains inconclusive.



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Insulin and IGF-1 are well known to control blood glucose levels (Guler, et al., 1987). The increased levels of insulin and IGF-1 play an important role in improving hyperglycemia. Also, both insulin and IGF-1 bind to insulin receptors and IGF-1 receptors with different affinity, regulating cell survival through activation of the PI3K-Akt pathway (Buerke, et al., 1995; Jonassen, et al., 2001). Our results show that treatment with TAD, HCQ or their combination significantly increased Akt phosphorylation at Thr308 (Figure 7A & 7B), suggesting enhanced cell survival signaling. Insulin has been reported to protect against I/R injury via facilitating glucose transport (Oates, et al., 2009), inhibition of apoptosis and inflammation (Sack and Yellon, 2003), and suppression of reactive oxidative species (Ji, et al., 2010). In the present study, we observed increased ATP production (Figure 8), which is indicative of better fuel supply or improved mitochondrial biogenesis in the TAD and HCQ combination treated hearts. In fact, a previous study showed that insulin and IGF-1 improved mitochondrial function through PI3K/Akt pathway in Huntington's disease knock-in striatal cells (Ribeiro, et al., 2014). The present data supports similar observations in the diabetic mouse heart. Future studies are needed to elucidate the exact roles of mitochondrial respiratory chain and biogenesis in the TAD+HCQ induced enhancement of cardiac ATP production.

It has been shown that preconditioning with IGF-1 protects against I/R injury (Buerke, et al., 1995). In addition, IGF-1 exerts its indirect cardioprotective effect by increasing insulin sensitivity in the peripheral system (Abbas, et al., 2008). Moreover, the cardioprotective effect of insulin and IGF-1 involves activation of biogenesis. The insulin/IGF-1- Akt-mTORC1 pathway regulates protein synthesis through activation of p70S6K and its target S6. There are two mTOR complexes, Raptor binding mTORC1, which is sensitive to nutrient availability and regulates cell cycle and proliferation and Rictor binding mTORC2, which is rapamycin insensitive and

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regulates cell survival in response to growth factors (Lum, et al., 2005). In the present study, combination treatment with TAD and HCQ activated (phosphorylated) mTOR, Akt, and S6 after I/R, indicating an increased biogenesis in response to the energy restoration.

We also observed activation of mTORC2 in mice treated either with HCQ or the combination of TAD and HCQ. mTORC2 is insensitive to acute rapamycin treatment, however it responds to growth factor such as insulin signaling to regulate cell growth and survival (Lamming, et al., 2012;Laplante and Sabatini, 2012). So far the function of mTORC2 has not been fully uncovered. The most studied function of this complex is the phosphorylation of Akt at Ser473, leading to the full activation of the kinase. Our results show that Akt was highly phosphorylated at Ser473 following treatment with either HCQ or the combination of TAD and HCQ after I/R, indicating an increased activity of mTORC2 pathway.

Nevertheless, the present study has several limitations. First, we did not measure cardiac ATP levels during and/or after the global I/R protocol due to the limited availability of post-I/R heart tissues that were used for infarct size measurement and molecular studies. Therefore, we speculate that possible changes in cardiac glucose and fatty acid metabolism following TAD+HCQ treatment may contribute to its infarct-limiting cardioprotective effects observed in the current study. The energy metabolism hypothesis needs to be tested in the in vivo I/R model of under diabetic conditions. Second, both physiological and molecular studies were not performed in the non-ischemic isolated hearts receiving time-matched normoxic buffer-perfusion as the model-specific controls. In this case, any confounding effect due to heart isolation and buffer-perfusion procedures cannot be ruled out.

In summary, our results clearly demonstrate that short-term treatment with the combination of TAD and HCQ significantly reduced myocardial infarct size following I/R in

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diabetic mice. Fasting glucose as well as circulating levels of plasma free fatty acids and triglycerides were decreased following combination treatment. Moreover, TAD and HCQ treatment resulted in better insulin sensitivity, higher baseline insulin levels which were associated with larger pancreatic  $\beta$ -cell area and pancreas mass. These results suggest that combination treatment with TAD and HCQ could potentially be a novel therapy for both reducing cardiovascular risk factors and protecting against myocardial I/R injury in T2D. We believe that treatment with TAD and HCQ could potentially lead to a novel line of therapy in diabetes clinics to manage cardiovascular risk factors and to improve clinical outcomes of diabetic patients suffering from acute myocardial infarction or possibly other ischemic injuries. Uniquely, this drug combination may also be beneficial in alleviating other common diabetic comorbidities such as erectile dysfunction and nerve/joint pain through the current FDA approved drug indications for TAD and HCQ.

## **Acknowledgements**

We thank Dr. Jorge A. Almenara from Department of Pathology, VCU for helping with imaging the pancreas H&E stained whole slides picture. Preliminary results of this study were presented at the American Heart Association Scientific Sessions in November, 2015 (Orlando, FL).

## **Authorship Contributions**

*Participated in research design:* Wang, Xi, Kukreja.

*Conducted experiments:* Wang, Xi.

*Performed data analysis:* Wang, Xi, Kukreja.

*Wrote or contributed to the writing of the manuscript:* Wang, Xi, Kukreja.

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## **Footnotes**

This study was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute [Grants R01HL118808 and R37HL51045] to R.C.K.

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## Legends for Figures

### Figure 1

**Experimental design and protocol.** The schematic diagram shows the sequence and timing of various experimental procedures including the drug treatment, oral glucose tolerance test (OGTT), insulin tolerance test (ITT), harvest of tissue, global ischemia/reperfusion in Langendorff mode, histology analysis as well as plasma and cardiac tissue biochemical measurements.

### Figure 2

**Effect of tadalafil, hydroxychloroquine and combination treatment on post-ischemic infarct size in db/db mice.** Top: Representative images of transverse sections of 2,3,5-triphenyltetrazolium chloride (TTC)-stained hearts collected after 1-week respective drug treatments and ex vivo global ischemia-reperfusion. Bottom: Bar diagram showing myocardial infarct size presented as % of risk area (Mean±SEM, n=6 for CTRL and n=5 for TAD, HCQ, and TAD+HCQ). \* indicates  $p < 0.05$  versus Control group. Abbreviations: Control (CTRL), Tadalafil (TAD), hydroxychloroquine (HCQ).

### Figure 3

**Effect of tadalafil, hydroxychloroquine and combination treatment on cardiac function.** Post-I/R ventricular developed force (A), rate-force product (B), heart rate (C), and coronary flow rate (D) are presented as % of pre-ischemia baseline (Mean±SEM; n=6 for CTRL and n=5 for TAD, HCQ, and TAD+HCQ). No statistical significance was observed among the 4

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treatment groups in any of the cardiac function indices. Abbreviations: Control (CTRL), Tadalafil (TAD), hydroxychloroquine (HCQ).

#### **Figure 4**

**Changes in blood glucose, plasma insulin, IGF-1 levels, and lipid profile following treatment with tadalafil and hydroxychloroquine.** Graph A and B: Plasma insulin and IGF-1 levels measured with ELISAs. Graph C Blood glucose levels measured before and after the 1-week drug treatment respectively (Mean±SEM; n=13 for CTRL and HCQ, n=15 for TAD, and n=12 for TAD+HCQ); Graph D to H: Plasma and cardiac levels of free fatty acids, triglycerides and total cholesterol measured using enzymatic assays (Mean±SEM, n=8 for TAD, n=9 for HCQ, and n=12 for CTRL and TAD+HCQ). \* indicates  $p<0.05$  and \*\*\* indicates  $p<0.001$  versus Control group. Abbreviations: Control (CTRL), Tadalafil (TAD), hydroxychloroquine (HCQ).

#### **Figure 5**

**Insulin and glucose tolerance tests following treatment with TAD, HCQ and combination of TAD and HCQ.** Animals were fasted overnight before oral glucose tolerance test. Glucose (2 mg/kg) was administered via oral gavage and blood samples were taken from tail. (A) Glucose levels; (B). Area under the curve; (C) Insulin levels and (D) Area under the curve. Insulin tolerance test was performed after the animals were fasted for 6 hours. Insulin (0.9 IU/kg regular human) was given intraperitoneally. (E) Blood glucose levels; (F) Area under the curve; (G) Insulin response curve presented as percentage to baseline glucose. Data are Mean±SEM (n=5/group.), \* indicates  $p<0.05$  versus Control group. Abbreviations: Control (CTRL), Tadalafil + hydroxychloroquine (TAD+HCQ or T+H).

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## Figure 6

### Effect of tadalafil, hydroxychloroquine and combination treatment on pancreatic islets. (A):

Representative pictures of immunofluorescent stained paraffin sections of pancreata. Goat anti-insulin antibody was used to detect insulin inside islets, and a secondary antibody conjugated with FITC was used. (B): Representative pictures of hematoxylin and eosin stained pancreas paraffin-fixed sections with further magnified representative images of the pancreatic islets at lower-right corners. (C): Insulin positive  $\beta$ -cell area vs pancreas area (n=5/group). (D): Bar diagram showing pancreatic islet number per mm<sup>2</sup> pancreas area in all treatment groups (n=8 for TAD and n=9 for CTRL, HCQ, and TAD+HCQ). (E): Bar diagram showing percentage of pancreas mass vs. body weight (n=5/group). Data are Mean $\pm$ SEM, \* indicates p<0.05 and \*\* indicates p<0.01 versus Control group. Abbreviations: Control (CTRL), Tadalafil (TAD), hydroxychloroquine (HCQ).

## Figure 7

Effect of tadalafil, hydroxychloroquine or combination treatment on mTOR activation following ischemia/reperfusion (I/R). (A) Representative Western blot images; (B) Bar diagram showing quantitative analysis of cardiac p-Akt<sup>Thr308</sup>/Akt; (C) p-mTOR/mTOR; (D) Expression of Raptor; (E) p-S6/S6; (F) Rictor and (G) p-Akt<sup>Ser473</sup>/Akt following I/R. Data are presented as Mean $\pm$ SEM (n=4/group), \* indicates p<0.05, \*\* indicates p<0.01, \*\*\* indicates p<0.001, \*\*\*\* indicates p<0.0001 versus Control group. Abbreviations: Control (CTRL), Tadalafil (TAD), hydroxychloroquine (HCQ).

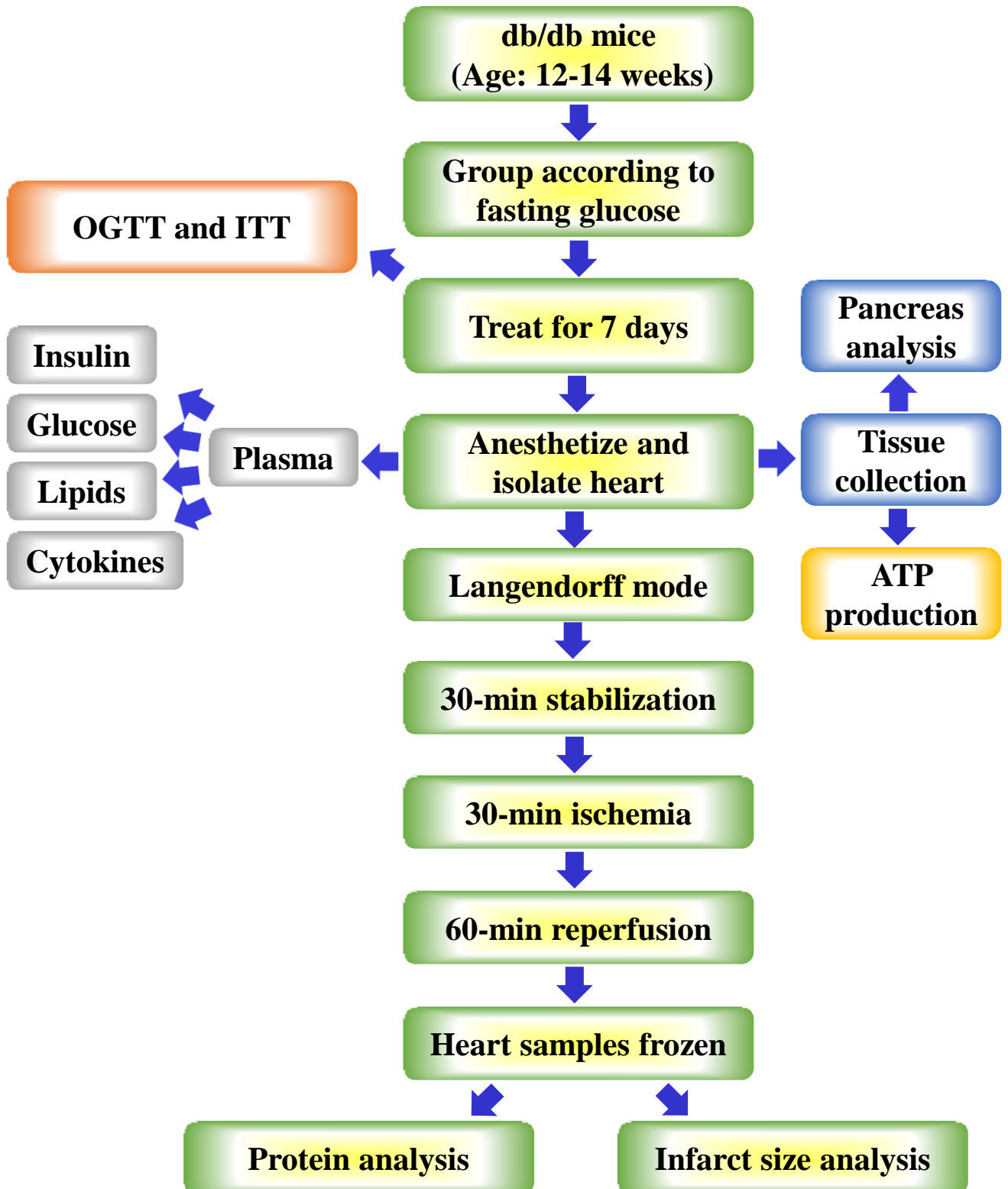


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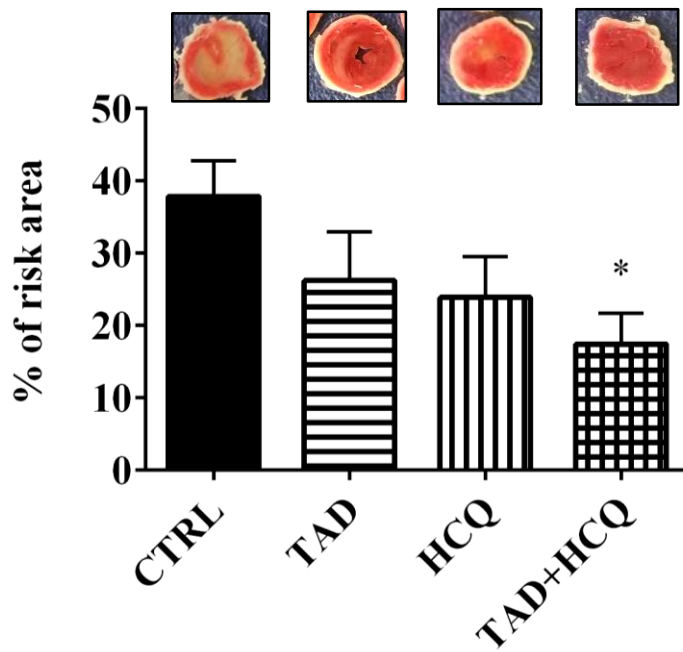
## Figure 8

**Effect of tadalafil, hydroxychloroquine and combination treatment on myocardial ATP production.** ATP levels normalized with respective protein concentration for each sample. Data are presented as Mean±SEM (n=4/group). \*\* indicates p<0.01 versus control group. Abbreviations: Control (CTRL), Tadalafil (TAD), hydroxychloroquine (HCQ).

Figure 1

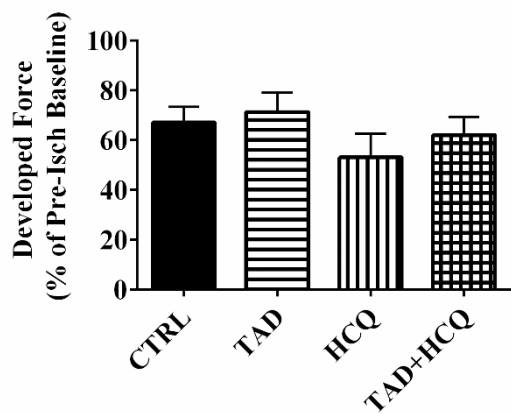


**Figure 2**

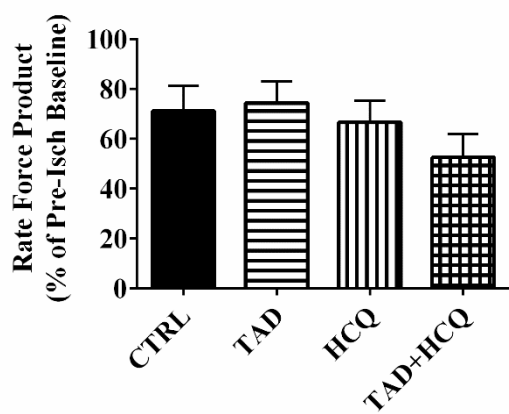


**Figure 3**

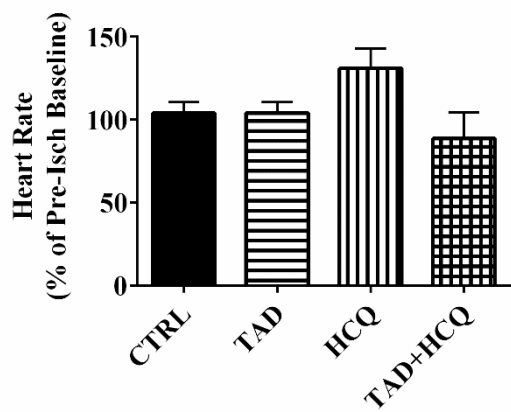
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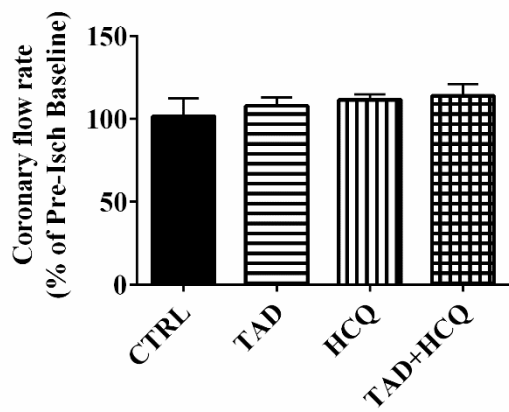
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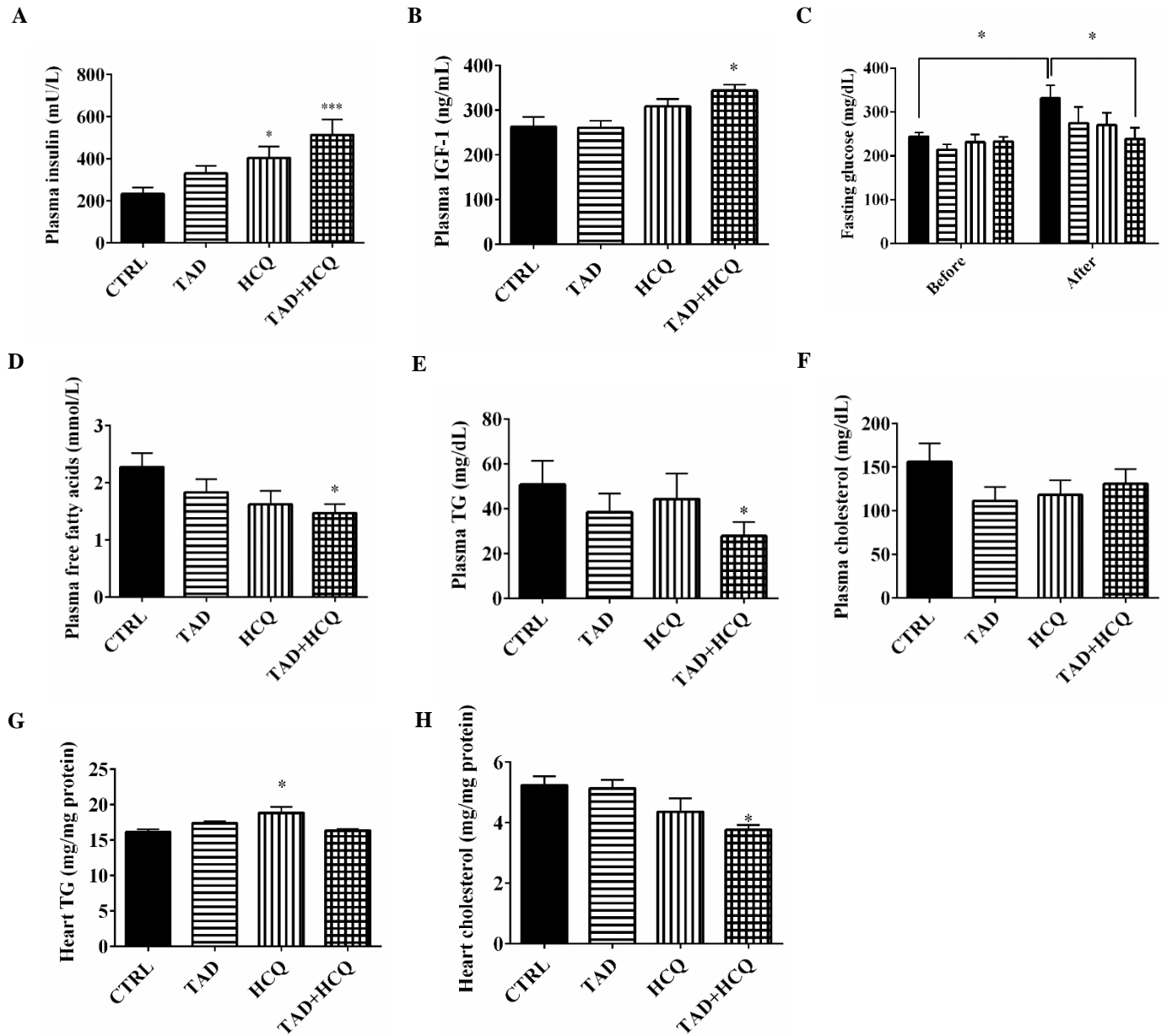
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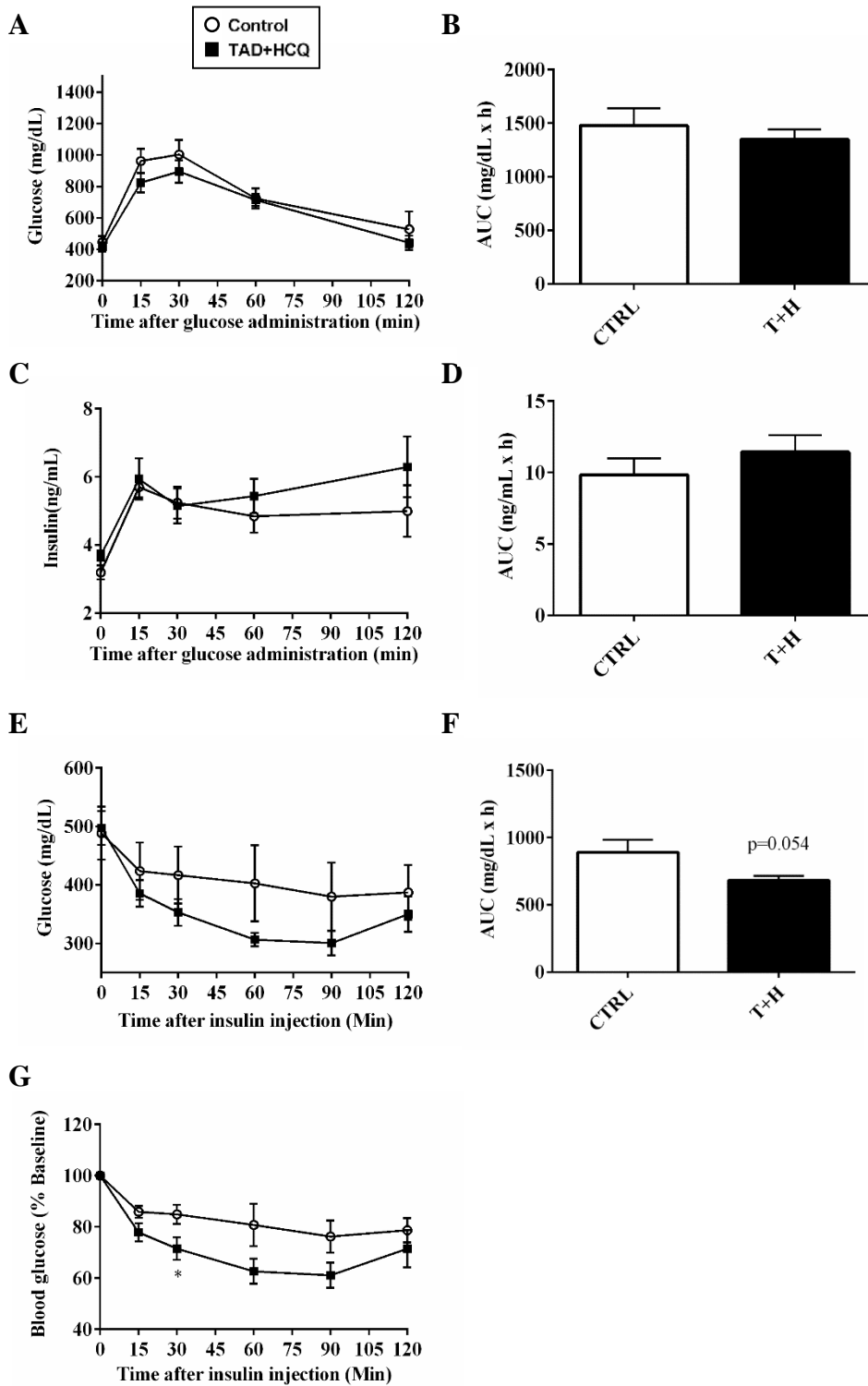
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**Figure 4**

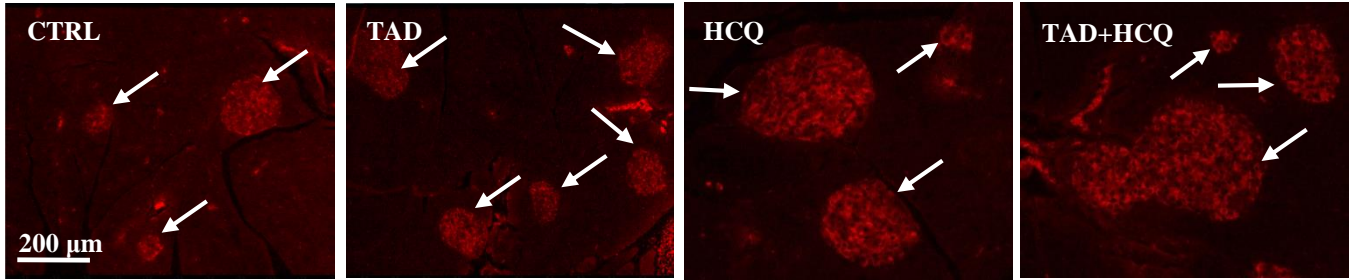


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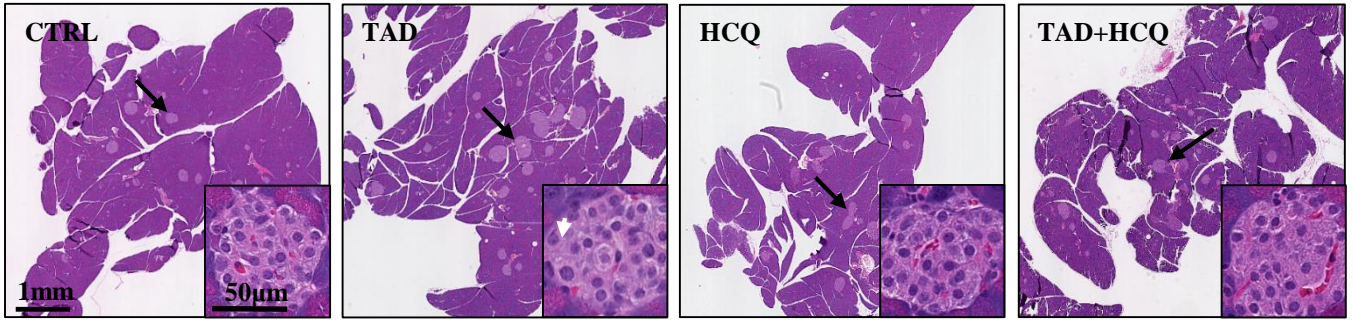


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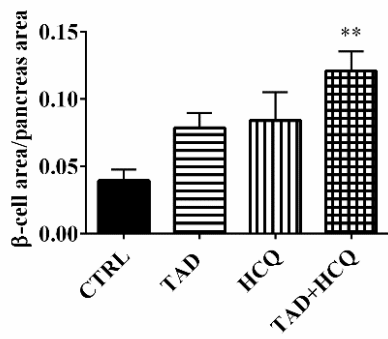
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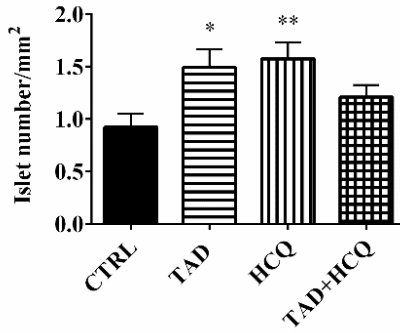
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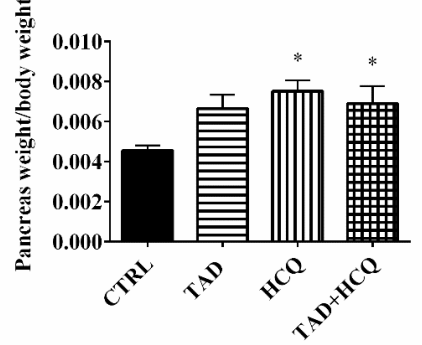
**C**



**D**



**E**



**Figure 7**

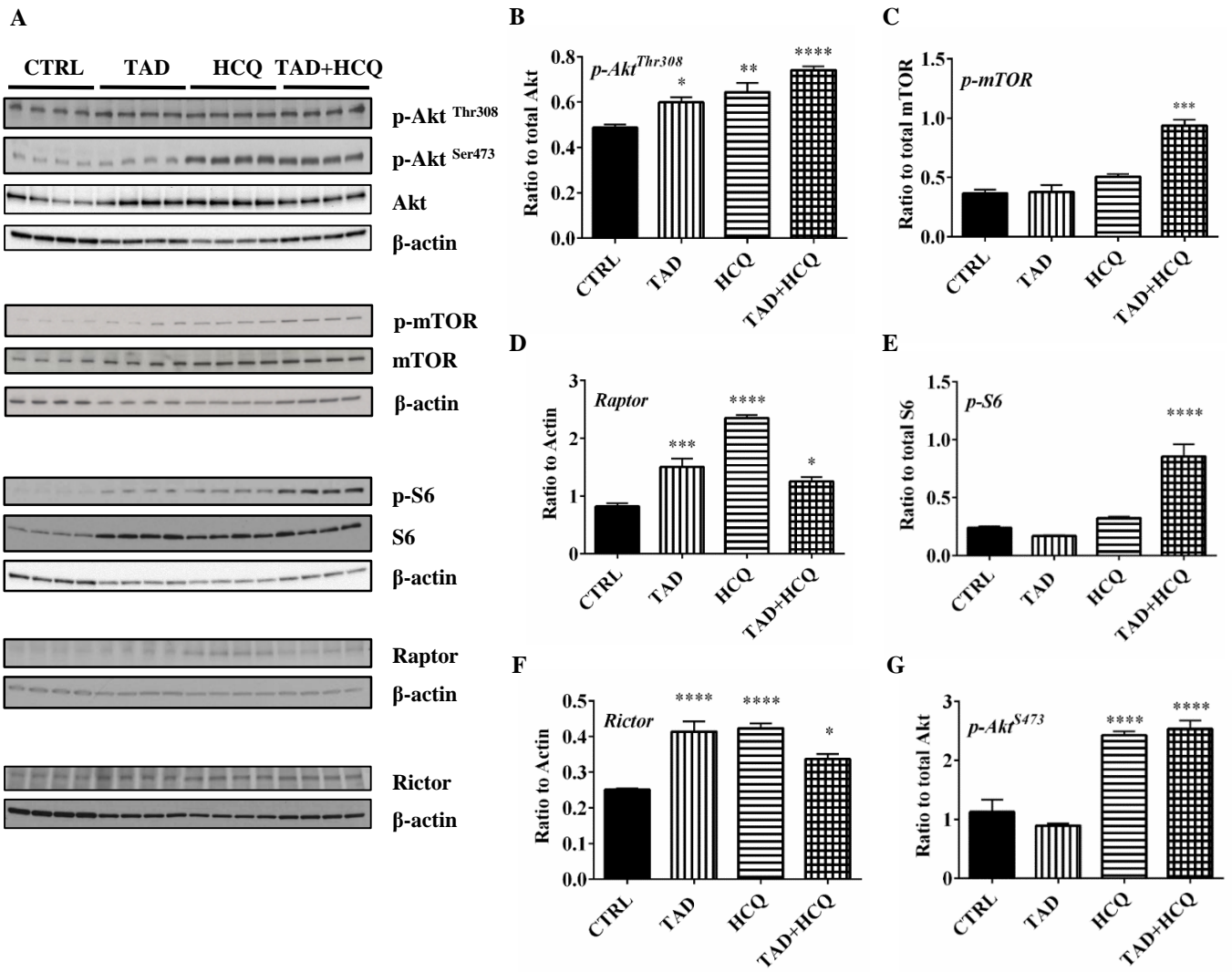




Figure 8

