Research Square

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Exogenous adiponectin with full Peroxisome proliferator-activated receptor agonists (pioglitazone) abrogates streptozotocin induced oxidative stress and vascular abnormalities in Spontaneously hypertensive rats.

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Research

Keywords: Streptozotocin, PPAR-γ agonists, adiponectin, oxidative stress, arterial stiffness

Posted Date: July 27th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-45024/v1

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Abstract

Background Oxidative stress, associates with metabolic and anthropometric perturbations, leads to reactive oxygen species production and decrease in plasma adiponectin concentration. We investigated pharmacodynamically the pathophysiological role and potential implication of exogenously administered adiponectin with full and partial peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists on modulation of oxidative stress, metabolic dysregulation and antioxidant potential in streptozotocin induced Spontaneously hypertensive rats (SHR).

Methods Group I (WKY) serve as normotensive control, whereas 42 male SHRs were randomized equally into 7 groups *(n = 6)*, group II: SHR control, groups III: SHR diabetic control, group IV, V and VI treated with irbesartan (30 mg/kg), pioglitazone (10 mg/kg) and adiponectin (2.5 µg/kg), whereas, groups VII and VIII received co-treatments as irbesartan + adiponectin), (pioglitazone + adiponectin) respectively. Diabetes was induced using an intra-peritoneal injection of Streptozotocin (40 mg/kg). Plasma adiponectin, lipid contents, arterial stiffness with oxidative stress bio-markers were measured using an in-*vitro* and *in-vivo* analysis.

Results Diabetic SHRs exhibited hyperglycaemia, hypertriglyceridemia, hypercholesterolemia, increased arterial stiffness with reduced plasma adiponectin and antioxidant enzymatic levels ($P < 0.05$). Diabetic SHRs pre-treated with pioglitazone and adiponectin separately exerted improvements in antioxidant enzyme activities, abrogated arterial stiffness, offset the increased production of reactive oxygen species and dyslipidaemic effects of STZ, except for blood pressure values which were more pronounced with irbesartan treated groups (all $P < 0.05$).

Conclusions The combined treatment of exogenously administered adiponectin with full PPAR-γ agonist augmented the improvement in lipid contents and adiponectin concentration, restores arterial stiffness with antioxidant potential effects, indicating degree of synergism between adiponectin and full PPAR-γ agonists (pioglitazone).

Background

Diabetes mellitus (DM) and hypertension (HTN) are being considered as foremost public health and medical issues, therefore, their co-existence have received greater attention because of rising epidemic globally as common chronic diseases, which associate mainly with micro and macro cardiovascular complications (1) and accelerate in the metabolic syndrome. Metabolic syndrome represents a cluster of various risk factors comprising of atherogenic dyslipidaemia, insulin resistance, hypertension, endothelial and inflammatory disorders (2), characterising a bunch of anthropometric disorders (3). Additionally, the concepts of oxidative stress and endothelial dysfunction have gained interest in recent years as contributing factors in the pathogenesis of hypertension and diabetes.

Of note, endothelial dysfunction connects with atherosclerosis progression (4) although, hyperglycaemia and diabetic complications, as metabolic perturbations, influences the endothelial dysfunction in the first

step of vascular changes (5) through complex mechanisms including oxidative stress (OS) and reactive oxygen species (ROS) (6). Oxidative stress and derivatives of reactive oxygen metabolites significantly aggravates in diabetes, essential hypertension (7) and hyperlipidaemic disorders (8), whereas, pathogenesis of metabolic syndrome (MS) relates to the biological antioxidant capacity of the body (9). Additionally, recent studies have shown that oxidative stress negatively regulates the adiponectin gene expression (10), therefore, its concentration in plasma impacts the oxidative stress (11), (12).

Adiponectin, an adipokine in human body (13) serves as a biomarker for the determination of metabolic syndrome status of the body (14). Adiponectin, upon binding to its receptors increases oxidation of fatty acids and glucose uptake via activating peroxisome proliferator activator receptor (PPAR-γ) ligand pathway (15), thus possess potential for the treatment of diabetic complications (16) with vascular disorders (17), whereas, PPAR-γ on activation directly impacts the adiponectin gene transcription (18).

Thiazolidinedione's derivative, pioglitazone, acts as a PPAR-γ agonist (19), therapeutically, improve insulin resistance and promotes adipocyte differentiation (20). In addition, the antihypertensive effect of pioglitazone has been ascribed to a reduction in vascular sensitivity (21) with an increase in plasma adiponectin concentration through PPAR-γ activation (22).

In addition, various antihypertensive agents including β-blockers, calcium channel blockers, ACE inhibitors and AT1-antagonists partially mediate their effects by decreasing oxidative stress (23). There are reports that angiotensin-II receptor blockers (ARBs) also possesses partial agonistic activity for PPAR-γ (24), therefore, irbesartan increase adiponectin production directly by activating PPAR-γ, self-regulating of its AT1R blocker characteristics (25). Moreover, long-term ARBs treatment causes reduction in pulse wave velocity (PWV) (26), thus inhibits arterial stiffness, independently, of their antihypertensive property (27).

Nonetheless, very less is known about the antioxidant potential of adiponectin in metabolic syndrome status in the genetic model of spontaneously hypertensive rats (SHRs). In the light of the above background, we tried to evaluate pharmacodynamically the pathophysiological role of exogenously administered adiponectin with PPAR-γ agonists in attenuating oxidative stress, arterial stiffness and metabolic syndrome factors including blood pressure, glycaemia and hypertriglyceridemia in streptozotocin (STZ) induced SHRs employing both *in-vivo* and *in-vitro* parameters. The hypothesis also explored whether a potentiating potential or synergism exists between adiponectin with either partial or full PPAR-γ agonists, in alleviating oxidative stress caused by STZ in SHRs. Moreover, the relationships between plasma adiponectin and arterial stiffness using pulse wave velocity (PWV) in diabetic SHRs was also investigated.

Material And Methods

Animals grouping and experimental protocol:

Forty two spontaneously hypertensive rats (SHR) and six Wistar Kyoto rats (WKY), averaging 230-255g body weight, divided randomly into eight groups, were kept in stainless, metabolic cages for 3 days for acclimatization purpose and were fed with commercial rat chow (Gold Coin Sdn. Bhd., Penang, Malaysia) with tap water ad-libitum in the animal care facility, Universiti Sains Malaysia, Malaysia, (where *n*=6 in each cage). All procedures and animal handling were carried out in accordance with the guidelines research centre "Animal Research and Service Centre (ARASC), USM (Main Campus)" with ethical approval number: 2012 (75) (352) by the "Animal Ethics Committee, Universiti Sains Malaysia, (AECUSM), Malaysia". Eight groups of rats were studied, (n=6) were treated as follows:

1. Wistar Kyoto rats: treated with vehicle (WKY+CNT)
2. SHR: treated with vehicle (SHR+CNT)
3. SHR treated with streptozotocin (SHR+STZ) served as SHR diabetic model
4. SHR+STZ+Irb: given irbesartan (30mg/kg) by oral gavage for 28 days starting from day 1.
5. SHR+STZ+Pio: given pioglitazone (10mg/kg) orally for 28 days starting from day 1.
6. SHR+STZ+Adp: given adiponectin 2.5µg/kg/day, intra-peritoneal, from day 21 to day 28
7. SHR+STZ+Irb+Adp: given irbesartan (30mg/kg) by oral gavage for 28 days starting from day 1, and adiponectin 2.5µg/kg/day, intra-peritoneal, from day 21 to day 28.
8. SHR+STZ+Pio+Adp: given pioglitazone (10mg/kg) by oral gavage for 28 days starting from day 1, and adiponectin 2.5µg/kg/day, intra-peritoneal, from day 21 to day 28.

We prepared a model of Type 1 diabetic SHRs using a single intra-peritoneal injection (I/P) of (STZ) (Nova Laboratories, Sdn, Bhd, Malaysia), 40 mg/kg body weight, dissolved in citrate buffer (10 mM, pH 4.5) (28), whereas, all the STZ induced SHRs were given glucose (10%) in for the first 48 hours after injection to offset the early hypoglycaemic shock. Blood glucose levels were evaluated using a standard Glucometer (Free Style, Abbott, Malaysia) and rats with glucose levels > 300 mg/dL at the 7th day were selected for the experiment. Physiological and metabolic perturbations including body weight, 24 hrs water intake and urine collection and were performed on day 0, to establish the basal variables, following on day 08, 21 and 28 of the experiment. Systemic hemodynamic parameters including systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) were measured non-invasively (NIBP) using the CODA equipment (Kent Scientific Corporation, Torrington, CT) on similar day pattern as for metabolic and physiological indices. Pulse wave velocity (PWV) was measured on the acute study day *i.e.* day 28. Blood samples (2ml) were obtained on each day of urine collection, and plasma samples were obtained following centrifugation and stored at -30◦C for further biochemical analysis including oxidative and antioxidant bio-markers, plasma levels of cholesterol, triglycerides, and low & high density lipoproteins measured values. The estimation of plasma adiponectin concentration was carried out using a quantitative assay max rat adiponectin Elisa kit (Chemtron, Biotechnology Sdn, Malaysia).

Drugs used in the experimental protocol:

1. Pioglitazone,(±)-5[4[2(5-ethyl-2-pyridyl)ethoxy]benzyl]-thiazolidine-2,4-dione monohydrochloride) (Searle, Pvt, Ltd., Pakistan).
2. Streptozotocin (STZ, Nova Laboratories, Sdn, Bhd., Selangor, Malaysia).
3. Irbesartan (Approvel, Sanofi, Aventis, France).
4. Adiponectin (Chemtron Biotechnology Sdn, Bhd, Malaysia).

A stock solution of pioglitazone (10mg/ml) and irbesartan (30 mg/ml) were prepared by dissolving their tablets in distilled water, whereas, full length recombinant adiponectin was dissolved in 200µl phosphate buffer saline (29).

Measurement of *in-vivo* oxidative stress and antioxidant markers

The collected blood plasma samples before the termination of acute experiment, were subjected to a variety of biochemical analysis in order to access the oxidative and anti-oxidative status of experimental diabetic SHRs. The levels of plasma oxidative stress bio-markers including malondialdehyde (MDA), antioxidant enzymes activities *i.e.* total superoxide dismutase (TSOD, nitric oxide (NO), total anti-oxidative activity (TAOC) and glutathione peroxidase (GSH) in collected plasma samples were measured using the spectrophotometric detection kits following the instruction manual provided by Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China.

Plasma malondialdehyde

In the biological system oxygen free radicals can be generated by enzymatic and non-enzymatic reactions. Oxygen free radicals upon generation react with polyunsaturated fatty acids resulting in lipid peroxidation and generate lipid peroxide such as aldehyde group (malondialdehyde MDA), ketone and hydroxyl group with some oxygen free radicals. MDA, a product of lipid peroxidation reactions, generated as a result of the reaction between free radicals and polyunsaturated fatty acids in the cell membrane (30). Therefore, evaluation of the MDA concentration in the biological samples could reflect the extent of lipid peroxidation and indirectly signify the extent of cell oxidative state.

Total superoxide dismutase

SOD plays an important role in cellular environments in the prevention of diseases linked to oxidative stress. SOD scavenges the superoxide anion free radicals and protects the cells from being injured from oxidative stress in a biological system. We investigated SOD measurement in blood plasma samples using the method as described by Oyanagui (31).

Nitric oxide

The universal inter and intracellular molecule, nitric oxide (NO), is involved in regulating pathophysiology of CVS. Its biological activity is recognized as EDRF responsible for vasodilatation. It is a gaseous free biological molecule with a half-life of few seconds or less *in vivo*, whereas, it's altered levels are

associated with several pathological conditions like hypertension, hypoxia and diabetes mellitus. The NO detection kit utilizes the nitrate reductase method and provides an accurate and convenient method for the measurement of total nitrate/nitrite concentration in the biological sample.

Total anti-oxidant capacity

The antioxidant defence consists of enzymatic and non-enzymatic components. Defence system protects the biological system from oxidation through three pathways. Firstly, it eliminates activated oxygen and free radicals, secondly decomposes superoxide to block the oxidation chain and lastly, gets rid of catalytic metal ions (32). All different antioxidants yields greater protection against attack by nitrogen radicals and reactive oxygen. Hence, total anti-oxidant capacity (T-AOC) provides more concise biological information about anti-oxidant status of an organism compared to that obtained by the measurement of individual components.

Plasma glutathione

Glutathione is naturally occurring tri-peptide and is a significant component of antioxidant system and offers protection against oxidative damage and in the detoxification processes in the cell. Glutathione is mostly present in its reduced form (GSH) than in the oxidized form (GSSG). GSH is a cofactor for antioxidant enzymes participating in detoxication mechanisms, e.g. glutathione peroxidase, glutathione transferase, dehydroascorbate and reductase. GSH scavenges hydroxyl radicals ($HO^{\bullet -}$) radical and singlet oxygen ($^1O_2$) directly, whereas, the cells redox state can be determined by using the ratio between GSSG/GSH (33).

Measurement of plasma cholesterol, triglycerides and lipoproteins (LDL, HDL) levels

Triglycerides (esters derivative from fatty acids and glycerol) are transported in plasma by lipoproteins, whereas, the excess quantity of carbohydrates converts into triglycerides and deposits in the adipose tissue (34). We employ phosphate oxidase/peroxidase method using biochemical analyzer (ChemWell®, Awareness Technology, Inc., FL, U.S.A) for the measurement of plasma triglyceride in collected plasma samples.

Surgical intervention for pulse wave velocity (PWV) measurement

All animals were fasted overnight (12-14 hours) prior to the surgical interventions used for acute surgery. All cannulae and the transducer were filled with heparinized saline (20.0 units/ml). All animals were anaesthetized with an intra-peritoneal injection of 60mg/kg sodium pentobarbitone (Nembutal^{Ò}, CEVA Sante Animale, Libourne, France). The trachea was cannulated with a PP240 tube to get a clear airway passage to facilitate respiration. The left jugular vein was cannulated with a PP50 cannula, to which a 50 ml syringe on an infusion pump (Perfusor secura FT 50 ml, B. Braun) that delivered normal saline throughout the experiment was attached. The left carotid artery was catheterized with PP50 tube for the

direct measurement of arterial B.P via pressure transducer (P23 ID Gould, Statham Instruments, USA) coupled to computerized data acquisition system (PowerlabÒ, ADInstruments, Australia). A midline abdominal incision was carried out to expose the left kidney and the whole dissection process was done using electrical cautery knife and the abdominal contents were moved with great care to the right to get the clear exposure of the left kidney. The left kidney was exposed via a ventral mid-line incision and a laser Doppler probe (OxyFlo® Probe, Oxford Ltd., UK) attached to the Powerlab® system was placed on the dorsal surface of kidney for the direct observation of renal cortical blood perfusion (RCBP) values throughout the experiment. Additionally, the left iliac artery was catheterized with a PP50 cannula and was advanced through abdominal aorta lay at the entrance of renal artery, whereas PP50 cannula was kept patent via infusing saline @ 6 ml/hr. A time period of 60 minutes were allowed to stabilize the animals after the completion of surgical protocol. Blood pressure waves from the two pressure transducers were simultaneously imported and displayed on a data acquisition system at a sampling rate of 400/s for 30 min. The measurement of PWV was done as per our lab technique methodology, described by Swarup *et al.,* (35), and was calculated by dividing the propagation distance (d) by propagation time (t) and expressed as meters per second.

Propagation distance and time

After euthenisation of animal, the full length of aorta was exposed and tip of the two cannulae from the carotid and iliac arteries were identified and marked. The distance between these two points was determined by using a cotton thread. After that the thread was laid straight for the measurement of distance and used to calculate PWV. At the end of recording, the animal was euthanized with sodium pentobarbitone (200 mg/kg) and the full length of aorta was exposed. A damp silk thread was placed along contour of aorta and marked at the tips of the two cannulae. The thread was then removed, laid straight and the distance between the two marks was measured. This pulse wave propagation distance was used to calculate the PWV. The propagation time was determined using a manual ‘foot to foot' technique. The time consumed by the pulse wave (t) to move from the aortic arch to abdominal aorta was measured manually by the time delay between the upstrokes (foot) of each pressure wave front. The manual foot to foot method has been widely used and is considered a reliable method for determining PWV (35) (26). The time for the propagation of the pulse wave from the aortic arch to the abdominal aorta was measured by the time delay between the upstrokes (foot) of each pressure wave front. The average of 10 normal consecutive cardiac cycles was used to calculate the propagation time. Any abnormal waveform within the cycles measured was rejected and next viable waveform was measured. All the animals used in the experiment were sacrificed with an overdose of sodium pentobarbitone, Nembutal®, CEVA, France), at the termination of the study and were disposed off in accordance with guidelines of Animal Ethics Committee of Universiti Sains Malaysia, Malaysia.

Statistical analysis

The statistical analysis was performed using GraphPad Prisim® version 5.00 for Windows (GraphPad Software, San Diego California U.S.A). Metabolic parameters including body weight, blood glucose level and plasma adiponectin concentration, the haemodynamics parameters during the treatment period were analyzed using repeated measures one-way ANOVA followed by Bonferroni *post hoc* test. Data expressed as mean±S.E.M and differences between the means were considered significant at the 5% level.

Results

Biochemical and metabolic indices

The mean values for metabolic indices including body weight, fluid intake, urine output and blood glucose concentration of all eight experimental groups were measured on four occasions during the study period, i.e. on day 0, day 8, day 21, and day 28 and are given in Table 1. The initial body weight at day 0 was not significantly different among all the groups including WKY and SHR control groups (WKY+CNT, SHR+CNT) on all four days of observation ($P>0.05$), whereas, the respective body weights of these groups increased on day 8,21 and 28 were significantly higher as compared to day 0, ($P<0.05$). As study progressed, the body weight of SHR diabetic control (SHR+STZ) and SHR diabetic treated groups *i.e.* SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Adp+Irb, SHR+STZ+Adp+Pio follow decreasing body weight pattern significantly with duration of diabetes, irrespective of various treatments as compared to day 0 and control groups on day 8, 21 and 28 of the protocol ($P<0.05$), (Table 1).

There was no significant difference of fluid intake in WKY+CNT group ($P>0.05$) but was significantly lower in the SHR+CNT group as compared to the WKY control group on all 4 days of observation ($P<0.05$). However, in the STZ induced diabetic model, the SHR+STZ group showed higher water intake on day 8, 21 and 28 as compared to day 0. Similarly SHR diabetic treated groups including SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Adp+Irb and SHR+STZ+Adp+Pio exhibited polydipsia compared to SHR+CNT group on day 8, 21 and 28 ($P<0.05$). No significant difference was observed in separate and combined treatment of adiponectin with either irbesartan or pioglitazone on respective days as compared to SHR+STZ group ($P>0.05$), (Table 1).

Similarly mean values of urine flow rate of all experimental groups were observed as per experimental protocol and was significantly lower in the SHR+CNT as compared to the WKY+CNT group on all 4 days of observation, whereas, contrary to SHR+CNT group, SHR+STZ treated rats showed polyuria on day 8, 21 & 28 ($P<0.05$). However, SHR+STZ+Irb and SHR+STZ+Pio treated groups did not show significant difference on day 8, 21 & 28 ($P>0.05$), but significantly increased in SHR+STZ+Adp, SHR+STZ+Irb+Adp and SHR+STZ+Pio+Adp groups on day 28 only as compared to SHR+STZ group and statistically with greater values in SHR+STZ+Pio+Adp group as compared to SHR+STZ+ADP and SHR+STZ+Irb+Adp groups ($P<0.05$), (Table 1).

All STZ administered animals developed diabetes resulting in a significant rise in blood glucose levels of SHR+STZ versus SHR+CNT group ($P<0.05$), whereas, no significant difference was observed between WKY and SHR control groups on all four days ($P>0.05$). Similarly, the SHR+STZ and SHR+STZ treated

groups showed greater values of blood glucose on day 8, 21 & 28 as compared to SHR+CNT group (P<0.05), whereas, statistically there was no significant effect on the blood glucose levels with any set of treatment during the experiment (P>0.05), (Table 1).

Systemic haemodynamics

As per study protocol, baseline values and the changes in the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) of eight groups of experimental rats were measured by the tail cuff method on day 0, day 8 after induction of diabetes, day 21 and finally on day 28 of the study, as shown in table 2. We observed that SBP and MAP were significantly higher in SHR+CNT as compared to WKY+CNT on all 4 days of observation (P<0.05), whereas, SHR+STZ groups exhibited higher SBP and MAP values as compared to SHR+CNT on day 21 and 28 (P>0.05). The SHR+STZ+Irb and SHR+STZ+Pio groups exhibited significant decrease in SBP and MAP values on day 21 and 28, and in SHR+STZ+Adp (day 28 only) as compared to SHR+STZ group (P<0.05). Interestingly, SHR+STZ+Irb+Adp group expressed greater significant reduction in the values of SBP and MAP on day 28 versus other sets of treatments used in the study (P<0.05), and the values obtained were comparable to WKY+CNT group, (Table 2).

In addition, after induction of diabetes, the mean values of DBP was significantly higher in SHR+CNT as compared to WKY+CNT (P<0.05), but no significant difference was observed in SHR+CNT and SHR+STZ (P>0.05) groups on all days of observation, whereas, SHR+STZ+Irb and SHR+STZ+Pio groups exhibited decreased DBP as compared to SHR+STZ+CNT group on day 21 (P<0.05). The DBP of SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp & SHR+STZ+Pio+Adp groups significantly decreased on day 28 (P<0.05), with a greater extent in SHR+STZ+Irb+Adp group in comparison to SHR+STZ+Irb, SHR+STZ+Adp and SHR+STZ+Pio+Adp groups (P<0.05), (Table 2).

Hear rate of all groups were observed on the same pattern of days *i.e.,* day 0, 8, 21 and 28. The heart rate of SHR+CNT group remained significantly higher as compared to WKY+CNT on all four points of observation, whereas, heart rate of SHR+STZ+CNT group was higher as compared to SHR+CNT on day 21 and 28 (P<0.05). However, treating diabetic SHRs significantly reduced the heart rate in SHR+STZ+Irb, SHR+STZ+Pio and SHR+STZ+Adp as compared to SHR diabetic control group on day 28 only, (P<0.05), whereas, the values obtained in SHR+STZ+Adp were of greater extent as compared to SHR+STZ+Irb and SHR+STZ+Pio groups. No significant effect was observed in case of combined treatment of adiponectin with either irbesartan or pioglitazone, (P>0.05), (Table 2).

Plasma adiponectin and Lipid profile determination

Plasma adiponectin concentration and lipid profile were measured on day 28 only in SHR and SHR diabetic pre-treated groups. A significant increases in plasma adiponectin concentration was observed in WKY and SHR control groups as compared to SHR+STZ group (P<0.05). The diabetic SHRs treated with irbesartan (30mg/kg/day), pioglitazone (10mg/kg/day) and adiponectin (2.5µg/kg/day), expressed significant increase in plasma adiponectin concentration as compared to SHR+STZ+CNT group (P<0.05).

Moreover, the combined treatment of adiponectin in SHR+STZ+Irb+Adp and SHR+STZ+Pio+Adp significantly increased plasma concentration of adiponectin as compared to their separate treatments, (P<0.05), however, the extent of increase in plasma concentration of adiponectin in SHR+STZ+Pio+Adp group was greater as compared to the combination of adiponectin with irbesartan in SHR+STZ+Irb+Adp group (P<0.05), but didn't reach to the level of WKY control group (Figure 1).

As far as lipid profile of SHR diabetic treated groups are concerned, SHR+STZ showed a significant increase in triglycerides, low density lipoproteins, total serum cholesterol and decrease in high density lipoproteins as compared to SHR+CNT group as shown in table 3 (P<0.05). Interestingly, adiponectin treatment caused a significant improvement in all these parameters (P<0.05), whereas, combination of adiponectin with pioglitazone caused more significant decrease in triglycerides, low density lipoproteins, total serum cholesterol, and increases in high density lipoproteins as compared to separate treatment of adiponectin and combination of adiponectin with irbesartan (P<0.05), thus improved the lipid contents of diabetic treated SHRs (Table 3).

Pulse wave velocity and renal cortical blood perfusion

Recordings for the pulse wave velocity (PWV) and renal cortical blood perfusion (RCBP) for groups including SHR control, STZ induced diabetic SHRS and SHR diabetic treated groups were determined during the acute surgical intervention *i.e* day 28. The renal cortical blood perfusion (RCBP) in SHR diabetic group was lower as compared to WKY and SHR control groups (133±12 vs. 247 ±11, 167±9)bpu respectively (P<0.05). In SHR diabetic treated groups (SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp), renal cortical blood perfusion was significantly higher as compared to SHR+STZ group, *i.e.* 163±9, 166±12, 187±9 vs. 133±12 bpu) respectively (P<0.05). Moreover, RCBP in SHR+STZ+Adp group was significantly higher as compared to irbesartan and pioglitazone separate treatments, but still remained significantly lower as compared to WKY control group. The combined treatment in SHR+STZ+Pio+Adp further increased RCBP (209±12)bpu, and this increase was statistically higher as compared to SHR+STZ+Irb+Adp group (194±6) bpu, (P<0.05), (Figure 2).

Moreover, it was observed that the pulse wave velocity (PWV) of SHR control group was significantly higher as compared to WKY control group, whereas after induction of diabetes the velocity of SHR diabetic group was significantly higher compared to SHR group. This increase in PWV was blunted in SHR+STZ+Irb (6.17±0.17)m/s, SHR+STZ+Pio+Adp (6.14±0.21)m/s, and SHR+STZ+Adp (5.49±0.22)m/s, however, the tendency to decrease PWV in adiponectin group was more as compared to separate irbesartan and pioglitazone groups. Adiponectin with pioglitazone in SHR+STZ+Pio+Adp group further reduce PWV and reach to the level of WKY group (5.27±0.31) m/s, (P<0.05), (Figure 3).

Antioxidant Biomarkers

Plasma total superoxide dismutase and malondialdehyde

The plasma total superoxide dismutase (T-SOD) of eight experimental groups including diabetic control SHRs and diabetic treated SHRs was measured as shown in figure 4. We observed that plasma T-SOD level of SHR groups of rats was significantly lower as compared to WKY control *i.e.* (108.75±3.9 vs. 145.50±3.87) U/mL (P<0.05), whereas STZ induced diabetic SHRs expressed significantly lower values as compared to SHR control group, (100.58±4.77 vs. 108.75±3.9)U/mL (P>0.05), which significantly increased in SHR+STZ+Irb, SHR+STZ+Pio and SHR+STZ+Adp groups after separate treatments SHR+STZ group, (119.14±2.68, 125.52±4.51 and 138.56±3.97 vs. 100.58±4.77) U/ML respectively (P<0.05). The SHR+STZ+Adp group exhibited higher T-SOD level as compared to SHR+STZ+Irb and SHR+STZ+Pio groups. Combination treatment in SHR+STZ+Pio+Adp further increase T-SOD values and reach to the level of WKY control (146.27±5.01 vs. 145.50±3.87) U/mL, (P>0.05), as compared to SHR+STZ+Irb+Adp group which did not show difference as compared to SHR+STZ+Adp group (143.25±3.81) U/mL).

We also obtained the plasma malondialdehyde (MDA) levels of these groups, which were significantly higher in SHR control as compared to WKY control group *i.e.* 5.91±0.22 vs. 2.85±0.19 nmol/mL (P< 0.05), whereas, in STZ induced rats the plasma MDA levels were significantly higher as compared to SHR control group (6.61±0.25 vs. 5.91±0.23)nmol/mL. The separate treatments with either irbesartan or pioglitazone significantly decrease the MDA concentrations as compared to SHR diabetic control group (5.25±0.25, 4.99±0.21 vs. 6.61±0.25) nmol/mL respectively, (P<0.05). Furthermore, treatment with adiponectin alone (SHR+STZ+Adp) and combination with irbesartan (WKY+STZ+Irb+Adp), or pioglitazone (WKY+STZ+Pio+Adp) groups further significantly decreased the plasma MDA concentration (3.01±0.17, 2.99±0.11, 2.95±0.01) nmol/mL respectively. No significant difference was observed between SHR+STZ+Adp group as compared to the combined treatment with either irbesartan or pioglitazone groups (P>0.05), (Figure 5).

The plasma nitric oxide and total antioxidant capacity

The plasma nitric oxide (NOx) levels were estimated by measuring the total nitrate/nitrite concentrations in plasma. We observed that plasma NO level of SHR group was significantly lower as compared to WKY control .(22.54±0.77 vs. 33.12±0.97) µmol/L, whereas, the plasma NO level of SHR diabetic group was significantly lower as compared to SHR control group (20.51±0.86 vs. 22.54±0.77) µmol/L respectively (P<0.05). Interestingly, pioglitazone and adiponectin singly treatments significantly increased plasma NO levels in SHR+STZ+Pio and SHR+STZ+Adp groups as compared to SHR+STZ and SHR+STZ+Irb groups (23.56±0.65, 28.52±0.39 vs. 20.51±0.86 and 21.70±0.71)µmol/L respectively with more values in SHR+STZ+Adp group (P<0.05). Combined treatment of adiponectin with pioglitazone in SHR+STZ+Pio+Adp groups further increased plasma NO level (32.77±0.88) µmol/L (P<0.05) which was comparable to WKY control group (Figure 6).

Our observations also recorded a significant decreased values for total antioxidant capacity (TAC) in SHR as compared to WKY control group (1.37±0.09 vs. 1.99±0.05) U/mL (P<0.05), whereas, after induction of diabetes the plasma TAC values in SHR diabetic control group further reduced significantly as compared

to SHR control group (1.12±0.07 vs. 1.37±0.09) U/mL, (P<0.05). However, the treated SHRs with either irbesartan (SHR+STZ+Irb) or pioglitazone (SHR+STZ+Pio) caused significant increase in the TAC levels in plasma as compared to SHR+STZ control group (1.33±0.08, 1.39±0.05 vs. 1.12±0.07) U/mL respectively (P<0.05), whereas, SHR+STZ+Adp, SHR+STZ+Irb+Adp and SHR+STZ+Pio+Adp groups further significantly increased the TAC levels in blood plasma (1.70±0.09, 1.85±0.11, 2.01±0.07) U/mL respectively, (P<0.05), with the values obtained in SHR+STZ+Pio+Adp (2.01±0.07) U/mL) comparable to WKY control group (1.99±0.05) U/mL), (Figure 7).

Plasma glutathione

In the last we also measured the plasma glutathione (GSH) values in diabetic SHR pre-treated groups, which presents a significant lower values in SHR control group as compared to WKY control group (120.19±3.85 vs. 160.08±4.10) µmol/L. However, treating with STZ, diabetic SHR group significantly reduced GSH values as compared to SHR control (110.23±3.77 *vs.* 120.19±3.85) µmol/L, (P<0.05). The respective treatments with irbesartan, pioglitazone and adiponectin separately significantly increased GSH values in SHR+STZ+Irb, SHR+STZ+Pio and SHR+STZ+Adp as compared to SHR+STZ control group (133.49±3.77, 139.22±3.66 and 145.49±5.13 *vs.* 110.21±3.77) µmol/L respectively (P<0.05). However, co-treatment of adiponectin with pioglitazone in SHR+STZ+Pio+Adp group further increased GSH values (156.27±3.77) as compared to SHR+STZ+Irb+Adp (150.25±4.77) µmol/L, group respectively, but was not comparable to WKY control group (160.08±4.10) µmol/L, (Figure 8).

Discussion

To the best of our knowledge, this study is amongst few investigating the pathophysiological role and impact of exogenously administered adiponectin with PPAR-γ agonists in Streptozotocin (STZ) induced Spontaneously hypertensive rats (SHRs) by measuring *in-vivo* and *in-vitro* antioxidant potential, plasma lipid contents, glycaemic and endogenous adiponectin levels with systemic and renal blood pressure measurements. The present study also assessed the relationship between pulse wave velocity (PWV) and oxidative stress makers. Our study indicates that STZ administration leads to a complex mechanism of diabetes and hypertension development, possibly due to the enhanced oxidative stress, indicated by increase MDA and decreased plasma SOD, NOx and T-AOC levels. The 28 days study period, pharmacodynamically, revealed that adiponectin, as a biomarker, in combination with full PPAR-γ agonist, pioglitazone abrogates oxidative stress including PWV, ameliorates lipid profile, systemic and renal blood pressure without effecting glycemic levels, signifying the synergistic antioxidant potential and vasodilator action in pretreated diabetic SHRs.

Spontaneously hypertensive rats (SHRs) are more susceptible to diabetogenic effect of Streptozotocin, most frequently used for the induction of diabetes (36) and causes increased production of reactive oxygen species (ROS) with activation of poly adenosine diphosphate ribosylation and nitric oxide release in SHRs (37). Consequently, we attempted to develop a well-known rat model of combined state of essential hypertension with diabetes.

Physiological and metabolic indices were kept into consideration to assess the experimental diabetes in SHRs, including body weight, which was significantly reduced as one of the pronounced effect of STZ on β -cells (38). Polyuria and polydipsia were also observed as significant metabolic perturbations of diabetic SHRs and found to be in accordance with the observations of Khan (39).

Hyperglycemia with elevated B.P cause damage to the vascular endothelial cells with increased oxidative stress & vascular reactivity (40) and are considered as vital phenomenon of metabolic syndrome (2). In our study, glycemic level was not influenced after either irbesartan, adiponectin or pioglitazone either singly or combination treatment protocol. This probably corresponds with the type of the diabetic model using STZ similar to human type 1 diabetic model. It is well known that endothelial dysfunction, occurs in diabetic complications (6), associates with atherosclerotic progression (4) and elevates in oxidative stress. In diabetic SHRs, variable observation in terms of increased or decreased SBP and MAP are reported (41), (42), whereas, in our study findings, the MAP and SBP values of diabetic SHRs were considerably higher, which could be due to the rapid destruction of nitric oxide (NO) in STZ induced SHRs (43), although, diminished NO bioactivity and bioavailability are key characteristics for arterial hypertension and endothelial disorders (44).

Moreover, we also observed that hyperglycemic SHRs exhibited decreased RCBP which supports previous observations of our laboratory findings in diabetic model of rats (41), (39), which probably due to stimulation of local ANG-II & intra-renal RAAS (45). In our findings, three weeks irbesartan (partial PPAR-γ agonist) in combination with adiponectin significantly reduced RCBP, SBP and MAP values to a larger extent as compared to adiponectin and pioglitazone (full PPAR-γ agonist) either singly or combination pre-treatments, which could be possibly due to up regulation of PPAR-γ receptors besides an increase in production of nitric oxide (NO) (46), (47). The significance of NO in the kidney vasculature cannot be ruled out which performs various pivotal role including renal hemodynamics regulation, modulation of renal sympathetic neural activity and inhibition of tubular sodium reabsorptive mechanism (48). We observed that irbesartan @ (30 mg/kg/day) caused maximal dose for blockade of RAAS whilst its partial PPAR-γ agonistic activity also contributed in its B.P reduction and renoprotective characteristics in non-diabetic SHRs as observed previously in our findings (49).

Nonetheless, regulation of MAP and vascular tone depends upon NO, which acts as endothelium-derived molecule (50), whereas, plasma adiponectin stimulates production of NO with reduction in sensitivity to Ang-II (51). Adiponectin receptors (Adipo R1 and Adipo R2) in endothelial cells mediate adiponectin-induced phosphorylation of AMPK and eNOS which together lead to an increase in NO production (52). In our findings activation of PPAR-γ with partial and full agonists (irbesartan and pioglitazone) respectively up-regulates plasma adiponectin levels probably by stimulating the expression of proteins involved in adiponectin assembly, for instance endoplasmic reticulum oxidoreductin-1 protein (Erol-Lα) and adiponectin secretion such as disulfide-bond A oxidoreductase-like protein (DsbA-L), (53), however, we did not measure these proteins in our experimental protocol.

Moreover, in our findings, the heart rate of STZ induced SHRs remained higher which could be due to the SNS over-activation (54), whereas, hypertensive state correlate with SNS activity, which, therefore, intricately involved with the initiation and progression of hypertension causing an increases in the heart rate (55) and supports our values obtained in diabetic SHRs. Previous findings confirms the adiponectin existence in the cerebrospinal fluid (56), thus controls and reduces the sympathetic nerve activity and heart rate (57), indicating adiponectin merely responsible for the reduction in heart rate of diabetic SHRs treated groups.

Adiponectin concentration in plasma and lipid profile

Diabetes induced by high-dose STZ is similar to human type 1 diabetic model (58), thus reduction of plasma adiponectin concentration with STZ administration would contribute to the diabetic condition of SHRs, and is in agreement with findings of Thule (59). Interestingly, in our experimental protocol STZ induced SHRs treated with pioglitazone for 3 weeks in combination with exogenous adiponectin significantly increased adiponectin levels as compared to the other sets of treatment. It is also evident that pioglitazone while acting as an agonist for PPAR-γ, improve endothelial function (60), with B.P reduction and lipid metabolism (61) via stimulating the production of plasma adiponectin (22) and reduction in vascular sensitivity in diabetic SHRs (21).

In addition, we also measured lipid contents of experimental diabetic and genetic model of hypertensive pre-treated rats. Plasma triglyceride concentrations were higher in control SHRs as compared to control WKY during treatment period, whereas, STZ treatment aggravate the condition in genetic model of hypertensive rats, leading to a further significant increase in plasma triglyceride, LDL, total serum cholesterol, with a decrease in adiponectin and HDL plasma concentrations, indicating anthropometric and physiological disorders. Previous studies reveals that full-length adiponectin activates AMP-activated protein kinase (AMPK) phosphorylation, (62) stimulates fatty-acid oxidation and glucose utilization by activating AMP-activated protein kinase, thus suppressing gluconeogenesis in liver (15). However, phosphorylation of AMPK regulates enzymes responsible for the synthesis of triglycerides and fatty acids with their transcription factors, thus constrains basal and oxidized low-density lipoproteins through NADPH oxidase inhibition in endothelial cells (63), eventually leading to decrease in adipose tissue mass through activation of adiponectin receptors present mainly in lateral hypothalamic nuclei (64). Therefore, PPAR-γ agonists used in our study probably influenced the gene expression responsible for lipid and carbohydrate metabolism without affecting glycaemic levels in diabetic SHRs. To support our observations previous observation proved that pioglitazone attenuated dyslipidemia in cyclosporine induced hypertensive rats (65), whereas, in another study, Hussain proved a much greater beneficial effect of a combination of rosiglitazone and telmisartan offered more improvement in serum TGs and adiponectin (66). Interestingly, treating diabetic rats with exogenous adiponectin and pioglitazone as full PPAR-γ agonist, produced significant attenuation of metabolic dysfunctions, as evidenced by the significant decrease in TC, TGs, LDL, but an increase in HDL and adiponectin plasma concentrations as similar conclusion were drawn for plasma adiponectin concentration.

Pulse wave velocity and antioxidant changes

Oxidative stress defines an imbalance between production of free radicals, its reactive metabolites and so-called oxidants or reactive oxygen species (ROS), whereas, their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism (67), however, oxidative stress and reactive oxygen derivatives further aggravates in diabetes and hypertension (7). In SHR, oxidative stress appears to be the cause of hypertension development on a larger scale and major effect of PPAR-γ activation is the reduction of oxidative stress levels (68). Recent epidemiological studies together with human diabetic models have suggested an association between adiponectin concentration and oxidative stress, thus, decreased circulating adiponectin levels predominates in increased oxidative stress, which are closely linked with metabolic syndrome (69), (12), and a the key feature of increased production of ROS and pro-inflammatory pathways (11). Production of ROS reduces bioavailability of NO due to uncoupling of eNOS, with enhanced levels of superoxide anions leading to formation of peroxynitrite, thus aggravates the impairment of eNOS activity and reduces NO production (70). In our experiment, an imbalance between antioxidants and oxidative stress was observed in diabetic SHRs, which can be confirmed from the increased plasma levels of free radical mediated products of lipid peroxidation (MDA), decreased plasma concentration of enzymatic antioxidant SOD and non-enzymatic antioxidant GSH. A decrease in TAC further confirms this imbalance indicates free radicals production with weak antioxidant defence system in diabetic SHRs, signifying the importance of OS as common denominator in all these pathways.

Arterial stiffness is linked with endothelial dysfunction, whereas, the pulse wave velocity (PWV) is considered as surrogate marker (a well-established index for arterial stiffness) (26) and vascular diseases. The stiffer artery would lead to increase in the PWV due to the persistent hyperglycaemia leads to depletion of antioxidant defence mechanism, generating free radicals (71) resulting in endothelial dysfunction and reduced vascular elasticity. Therefore, pulse wave velocity of diabetic SHRs was significantly higher as compared to control rats indicating the marked decrease in the extensibility of blood vessels in diabetic condition leading to increased arterial stiffness.

However, we observed that exogenously administered adiponectin attenuated the arterial stiffness (PWV) of diabetic SHRs along decrease in SBP and MAP, which could be at least partially mediated through its potent antioxidant characteristics and was attenuated by blocking endothelial derived nitric oxide synthase activity, suggesting that relaxant effect was possibly mediated by nitric oxide. However, the combination with pioglitazone resulted in significantly greater decrease in PWV as compared with combined treatment of adiponectin with irbesartan and separate treatments. Previous clinical studies have demonstrated that partial PPAR-γ agonist (ARBs), protects vascular endothelium via an increase of endothelial NO synthesis (72) and plasma adiponectin concentration (47) thus prevents endothelial dysfunction more effectively as compared to non PPARγ-agonists ARBs (73). Likewise, full PPAR-γ agonist, pioglitazone, stimulates the production of NO and moderates oxidative stress through activation of signaling cascades, such as cAMP–PKA and AMPK–eNOS component (60), and by increasing glutathione levels, thus supports the fact that AMPK serves as a major downstream molecule for

adiponectin production (55). In this study, combined treatment of exogenous adiponectin with full PPAR-γ agonist (pioglitazone) significantly attenuates the oxidative status to a larger extent as compared to co-treatment of adiponectin with irbesartan in experimentally induced diabetic SHRs. There was a marked increase in NO, SOD and T-AOC plasma levels that indicates improvement in arterial stiffness with decreased oxidative stress in diabetic SHRs. Similarly the reduced lipid peroxidation (MDA) values denotes decrease in free radical production, thus substantiates our findings and supports our hypothesis tested. Increased antioxidant levels (SOD and GSH) imply better defense against ROS. These antioxidants protect the cells from oxidative damage, thereby decreasing the oxidative stress mediated vascular complications through antioxidant-mediated pathways.

Conclusion

In a nut shell, exogenous adiponectin administration attenuated the vascular abnormalities, fluctuating from endothelial dysfunction to ROS production, through nitric oxide and antioxidant enzymatic properties with abrogation of arterial stiffness. Nonetheless, owing to the full PPAR-γ agonist activity of pioglitazone, co-treatment with adiponectin significantly augmented to a larger extent with improvement in oxidative status, serum triglycerides, restoration of atrial stiffness (in-vivo biomarker) with antioxidant enzymatic potential indicating a degree of synergism existence between adiponectin and pioglitazone.

Abbreviations

DM
Diabetes mellitus
HTN
Hypertension
MS
Metabolic syndrome
OS
Oxidative stress
ROS
Reactive oxygen species
PPAR-γ
Peroxisome proliferator activated gamma receptor
TZD
Thiazolidinedione
ARBs
angiotensin receptor blockers
PWV
Pulse wave velocity
STZ

Streptozotocin

SHR

Spontaneously hypertensive rat

WKY

Wistar Kyoto

CNT

Control

Pio

Pioglitazone

Irb

Irbesartan

Adp

Adiponectin

SBP

Systolic blood pressure

DBP

Diastolic blood pressure

MAP

Mean arterial pressure

HR

Heart rate

NIBP

Non-invasive blood pressure

MDA

Malondialdehyde

SOD

Superoxide dismutase

NO

Nitric oxide

GSH

Glutathione

TC

Total cholesterol

TG

Total triglycerides

LDL

Low density lipoproteins

HDL

High density lipoproteins

TAOC

Total antioxidant capacity
GSH
Glutathione peroxidase
RCBP
Renal cortical blood perfusion

Declarations

Ethics approval and consent to participate:

All procedures and animal handling were carried out in accordance with the guidelines research centre “Animal Research and Service Centre (ARASC), USM (Main Campus)” with ethical approval number: 2012 (75) (352) by the “Animal Ethics Committee, Universiti Sains Malaysia, (AECUSM), Malaysia”.

Consent for publication:

All authors are responsible for the reported research, and have equally participated in the concept, design, analysis and interpretation of data, drafting or revising of the manuscript, and have approved the manuscript to be submitted for publication.

Availability of data and materials:

The analyzed data have been incorporated in the tables and figures of the manuscript, whereas, the values for these analysis of the data have been provided in the supplementary files submitted with the manuscript.

Competing interests:

All authors have no competing or conflict of interest for this study.

Funding:
The authors fully acknowledge the Universiti Sains Malaysia, Research grant # 1001/PFARMASI/815078, provided by Universiti Sains Malaysia for this work.

Authors' contributions:
Dr. Sheryar Afzal conceptualize the study, perform the experiments, collect and analyze experimental data and draft manuscript.

Prof. Dr. Munavvar supervised the whole project, provide lab facilities, verify data analysis and edit manuscript for submission.

Prof. Edward Johns helped in designing the study protocol and experiment groups, verify data analysis and finalize the manuscript.

Prof. Dr. Olorunfemi Eseyin helped in the statistical analysis of the study and data collection of the experimental groups.

Acknowledgements:
Sheryar Afzal is a recipient of USM Fellowship from the Institute of Post-Graduate Studies (IPS), Universiti Sains Malaysia (USM), Penang, Malaysia, and this support is gratefully acknowledged.

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Tables

Table1: Metabolic parameters of WKY, SHR control and SHR diabetic treated groups with irbesartan, pioglitazone, adiponectin, and a combination of adiponectin with irbesartan or pioglitazone.

Parameters	Groups	Days of Observation			
		Day 0	Day 8	Day 21	Day 28
Body Weight (g)	WKY+CNT	245±5	250±7	275±4*	289±8*
	SHR+CNT	242±3	248±6^	267±8*	284±9*
	SHR+STZ	245±3	200±5*^	208±7*^	209±10*
	SHR+STZ+Irb	250±5	215±3*	211±8*	212±6*
	SHR+STZ+Pio	254±6	205±4*	207±7*	217±5* ж
	SHR+STZ+Adp	252±4	213±7*	215±9*	206±4*
	SHR+STZ+Irb+Adp	251±7	208±3*	206±8*	204±6*
	SHR+STZ+Pio+Adp	247±5	201±9*	200±10*	209±5*
Water intake (ml/d)	WKY+CNT	43±1	44±2	45±3	44±2
	SHR+CNT	32±2^	34±2!	34±3!	37±4!
	SHR+STZ	33±2	48±3^*	48±2^*	59±3^*
	SHR+STZ+Irb	34±2	48±3*	50±2*	50±3*
	SHR+STZ+Pio	35±2	47±2*	51±2*	60±3*
	SHR+STZ+Adp	36±2	46±3*	47±2*	57±3*
	SHR+STZ+Irb+Adp	35±2	47±2*	49±2*	56±3*
	SHR+STZ+Pio+Adp	34±2	45±3*	49±2*	55±3*
UFR (mL/min/100 g)	WKY+CNT	3.84±0.44	3.63±0.21	3.92±0.21	3.95±0.21
	SHR+CNT	3.10±0.05 !	2.98±0.09 !	2.94±0.54 !	2.93±0.43!
	SHR+STZ	3.09±0.04	12.35±0.52^	12.79±0.62^	13.18±0.04^
	SHR+STZ+Irb	3.06±0.02	12.57±0.25*	12.57±0.37^*	12.58±0.22*#
	SHR+STZ+Pio	3.07±0.03	13.58±0.55*	13.57±0.27*	13.58±0.30*

			δ	δ	δ ж
	SHR+STZ+Adp	3.06±0.02	13.28±0.49* δ	13.35±0.15* δ	16.25±0.13* δ
	SHR+STZ+Irb+Adp	3.08±0.02	13.57±0.39* δ	13.35±0.25* δ	17.79±0.15* δ Φ
	SHR+STZ+Pio+Adp	3.09±0.03	13.57±0.29* δ	13.58±0.24* δ	20.28±0.29* δ ζ
Blood glucose (mg/dl)	WKY+CNT	89±3	88±2	86±2	88±3
	SHR+CNT	91±3	90±2	88±3	89±3
	SHR+STZ	90±3	460±18*^	471±14*^	489±25*^
	SHR+STZ+Irb	89±4	458±11*	462±17*	470±16*
	SHR+STZ+Pio	90±5	471±21*	477±19*	474±15*
	SHR+STZ+Adp	88±2	465±19*	462±18*	484±27*
	SHR+STZ+Irb+Adp	89±2	488±10*	484±16*	479±19*
	SHR+STZ+Pio+Adp	86±3	479±21*	486±18*	480±22*

Notes: The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by repeated measure one-way ANOVA followed by *Bonferroni post hoc* test. Values with (P<0.05) were statistically significant during and at the end of treatment.

! indicates significant difference (P<0.05) between SHR and WKY control groups.

^ indicates significant difference (P<0.05) between WKY and SHR control groups in comparison to SHR diabetic control group.

*indicates significant difference (P<0.05) in comparison to day 0 of the respective group.

δ indicates significant difference (P<0.05) of diabetic Irb, Pio, Adp, Irb+Adp, Pio+Adp groups in comparison to SHR diabetic control group.

# indicates significant difference (P<0.05 between diabetic Irb and Adp groups.

ж indicates significant difference (P<0.05) between diabetic Pio and Adp groups.

Φ indicates significant difference (P<0.05) of diabetic Adp group in comparison to diabetic Irb+Adp group at day 21 and 28.

ζ indicates significant difference (P<0.05) of diabetic Adp group in comparison to diabetic Pio+Adp group at day 21 and 28.

Table 2: Systemic haemodynamics parameters of WKY, SHR control and SHR diabetic treated groups with irbesartan, pioglitazone, adiponectin, and a combination of adiponectin with irbesartan or pioglitazone.

Parameters	Groups	Observation Days of			
		Day 0	Day 8	Day 21	Day 28
Systolic blood pressure (mmHg)	WKY+CNT	118±5	117±2	117±2	120±3
	SHR+CNT	159±4 !	164±6 !	162±3 !	157±8 !
	SHR+STZ	161±5^	173±3^*	177±4^*	175±3^*
	SHR+STZ+Irb	163±4	177±5*	147±4* δ	135±3* δ
	SHR+STZ+Pio	165±6	179±4*	155±3* δ ж	148±6* δ ж
	SHR+STZ+Adp	162±4	175±2*	174±3*#	138±4* δ
	SHR+STZ+Irb+Adp	164±3	176±3*	148±4* δ	118±3 δ Ф
	SHR+STZ+Pio+Adp	163±5	177±4*	154±4* δ	134±3* δ
Diastolic blood pressure (mmHg)	WKY+CNT	79±3	84±4	80±3	86±5
	SHR+CNT	119±6 !	117±7 !	108±7 !	120±6 !
	SHR+STZ	117±3	119±2	120±3	118±4
	SHR+STZ+Irb	118±2	121±3	101±2* δ	92±1* δ
	SHR+STZ+Pio	116±3	119±3	109±5* δ	106±4*
	SHR+STZ+Adp	117±4	120±4	118±4# ж	96±2* δ# ж
	SHR+STZ+Irb+Adp	115±4	118±3	103±3* δ	88±2* δ Ф
	SHR+STZ+Pio+Adp	118±3	120±2	111±3* δ	98±2* δ
Mean arterial pressure	WKY+CNT	92±8	97±4	92±4	97±5

	SHR+CNT	132±5 !	133±4 !	126±5 !	132±6 !
	SHR+STZ	132±7	137±5^*	143±4^*	144±5^*
	SHR+STZ+Irb	133±5	140±6*	118±3* δ	106±4* δ
	SHR+STZ+Pio	132±4	139±4*	124±4* δ	120±5* δ ж
	SHR+STZ+Adp	132±3	138±7*	137±6*	110±8* δ
	SHR+STZ+Irb+Adp	131±4	137±5*	118±4* δ	98±5 δ Φ
	SHR+STZ+Pio+Adp	133±5	139±5*	125±3* δ	105±6* δ
Heart rate (BPM)	WKY+CNT	312±10	310±4	309±11	303±8
	SHR+CNT	386±9 !	390±10 !	392±14 !	389±11 !
	SHR+STZ	387±5 ! ^	394±7^	402±4^*	407±5^*
	SHR+STZ+Irb	385±4	396±4*	393±4* δ#	386±3 δ#
	SHR+STZ+Pio	388±6	400±3*	380±3* δ	367±4* δ ж
	SHR+STZ+Adp	383±3	398±5*	395±4* ж	356±3* δ
	SHR+STZ+Irb+Adp	386±5	401±4*	396±3* δ	360±4* δ
	SHR+STZ+Pio+Adp	387±7	403±5*	377±6* δ	351±5* δ

Notes: The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by repeated measure one-way ANOVA followed by *Bonferroni post hoc* test. Values with (P<0.05) were considered statistically significant.

! indicates significant difference (P<0.05) between SHR and WKY control groups during and at the end of treatment.

*indicates significant difference (P<0.05) in comparison to day 0 of the respective group.

δ indicates significant difference (P<0.05) of diabetic Irb, Pio, Adp, Irb+Adp, Pio+Adp groups in comparison to SHR diabetic control group.

# indicates significant difference (P<0.05 between diabetic Irb and Adp groups during and at the end of treatment.

ж indicates significant difference (P<0.05) between diabetic Pio and Adp groups during and at the end of treatment.

Φ indicates significant difference (P<0.05) of diabetic Adp group in comparison to diabetic Irb+Adp group at day 21 and 28.

Table 3: Plasma triglycerides and lipoproteins (LDL, HDL, VLDL) level profile of WKY, SHR control and SHR diabetic treated groups with irbesartan, pioglitazone, adiponectin, and a combination of adiponectin with irbesartan or pioglitazone.

Groups	Lipid Profile			
	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
WKY+CNT	50.75 ± 4.09	61.25 ± 2.29	16.33 ± 1.2	36.25 ± 1.8
SHR+CNT	84.80±11.32 !	149.96±17.54 !	67.64±3.97 !	97.08±6.33 !
SHR+STZ	172.4±14.48 *	197.72±12.72 *	42.02±4.63 *	122.49±6.01 *
SHR+STZ+Irb	153.9±10.5 δ#	166.84±15.0 δ#	59.78±4.88 δ#	108.56±7.97 δ#
SHR+STZ+Pio	147.2±10.7δ ж	153.98±11.64 δ ж	72.89±2.85 δ ж	98.96±7.50 δ
SHR+STZ+Adp	93.20±8.29 δ	129.60±12.19 δ	79.22±3.96 δ	96.03±5.20 δ
SHR+STZ+Irb+Adp	85.25±2.35 δ	136.57±5.7 δ	79.25±4.1 δ	95.28±4.5 δ
SHR+STZ+Pio+Adp	73.25±4.5 δ ζ	119.25±6.7 δ ζ	77.28±5.7 δ	91.25±5.4 δ

Notes: The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by *Bonferroni post hoc* test. Values with (P<0.05) were considered statistically significant during and at the end of treatment.

! indicates significant difference (P<0.05) between SHR and WKY control groups.

*indicates significant difference (P<0.05) in comparison to SHR control group.

δ indicates significant difference (P<0.05) of diabetic Irb, Pio, Adp, Irb+Adp, Pio+Adp groups in comparison to SHR diabetic control group.

# indicates significant difference (P<0.05 between diabetic Irb and Adp groups.

ж indicates significant difference (P<0.05) between diabetic Pio and Adp groups.

ζ indicates significant difference (P<0.05) of diabetic Adp group in comparison to diabetic Pio+Adp group at day 21 and 28.

Figures

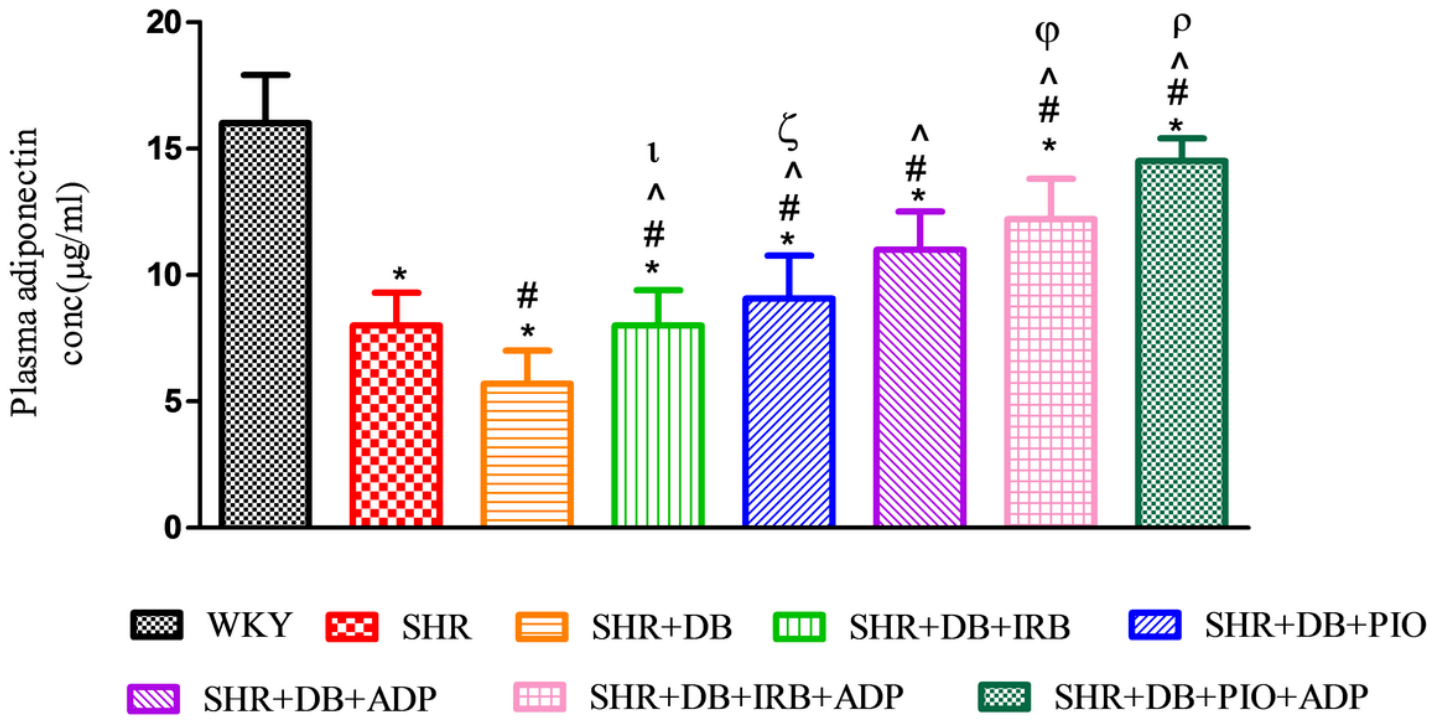

Figure 1

Plasma adiponectin concentration in WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. * (P<0.05) vs. WKY, # (P<0.05) vs. SHR ^ (P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp,

SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp φ indicates significant difference between SHR+STZ+Adp to SHR+STZ+Pio+Adp

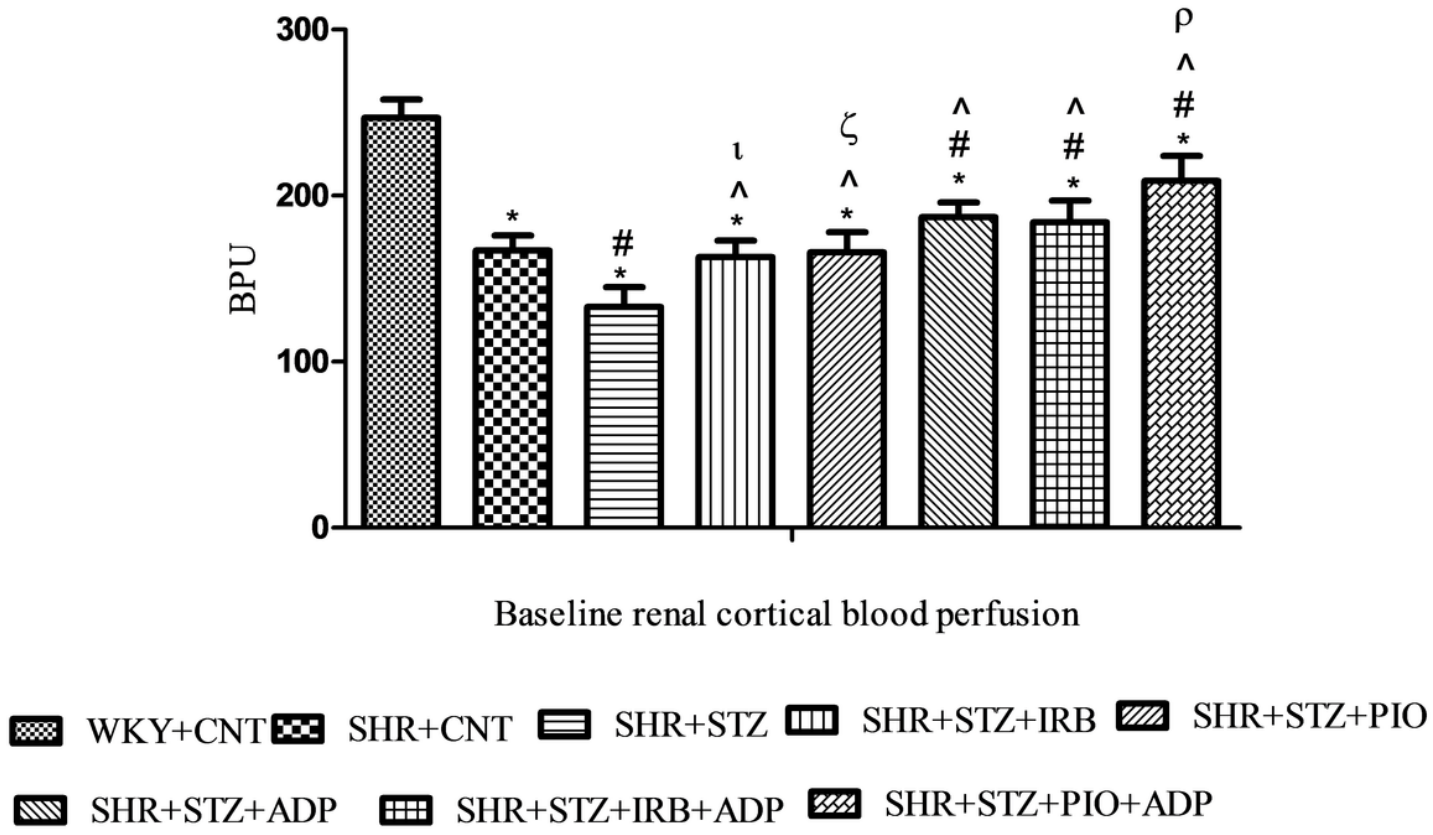

Figure 2

Baseline renal cortical blood perfusion of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. * (P<0.05) versus WKY, # (P<0.05) versus SHR ^ (P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp

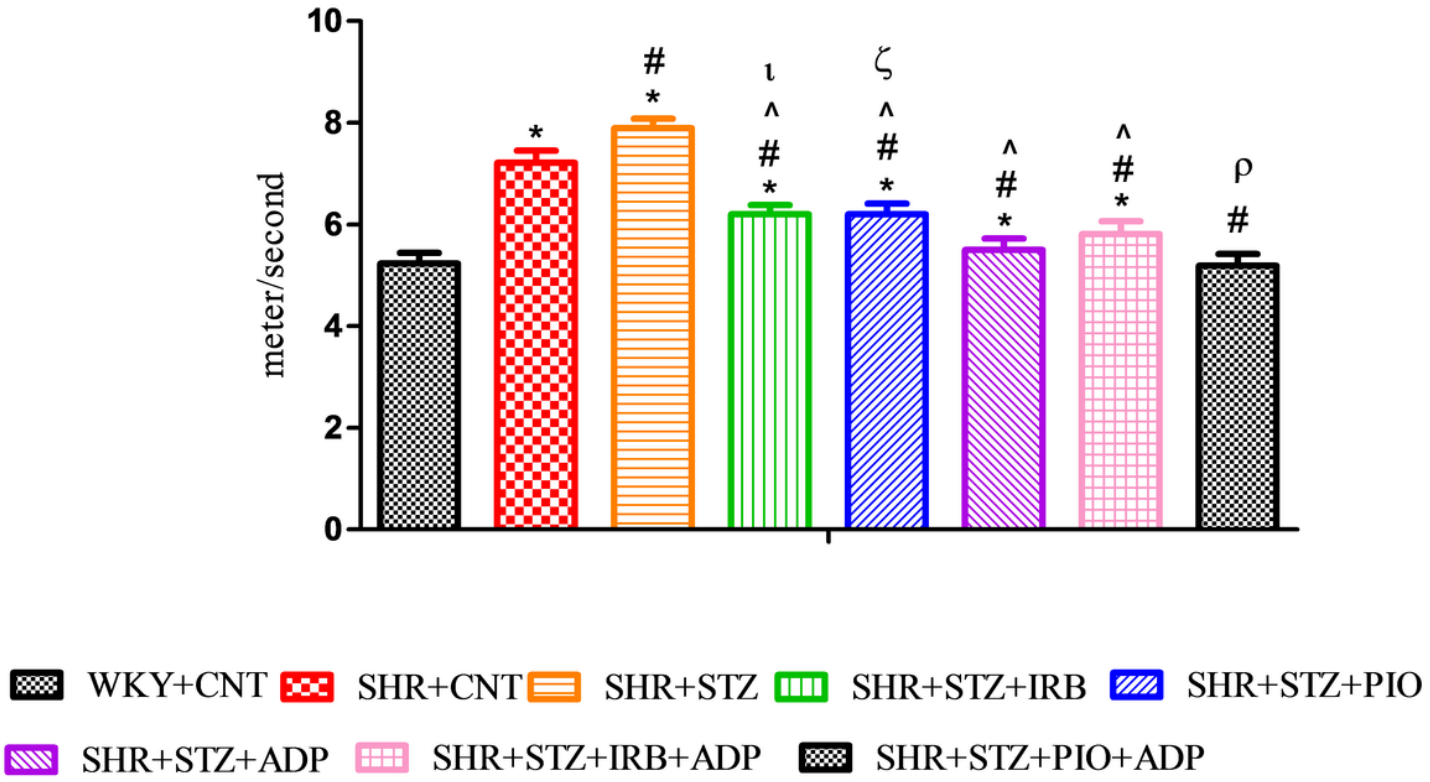

Figure 3

Pulse wave velocity of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. *(P<0.05) versus WKY, # (P<0.05) versus SHR ^ (P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp

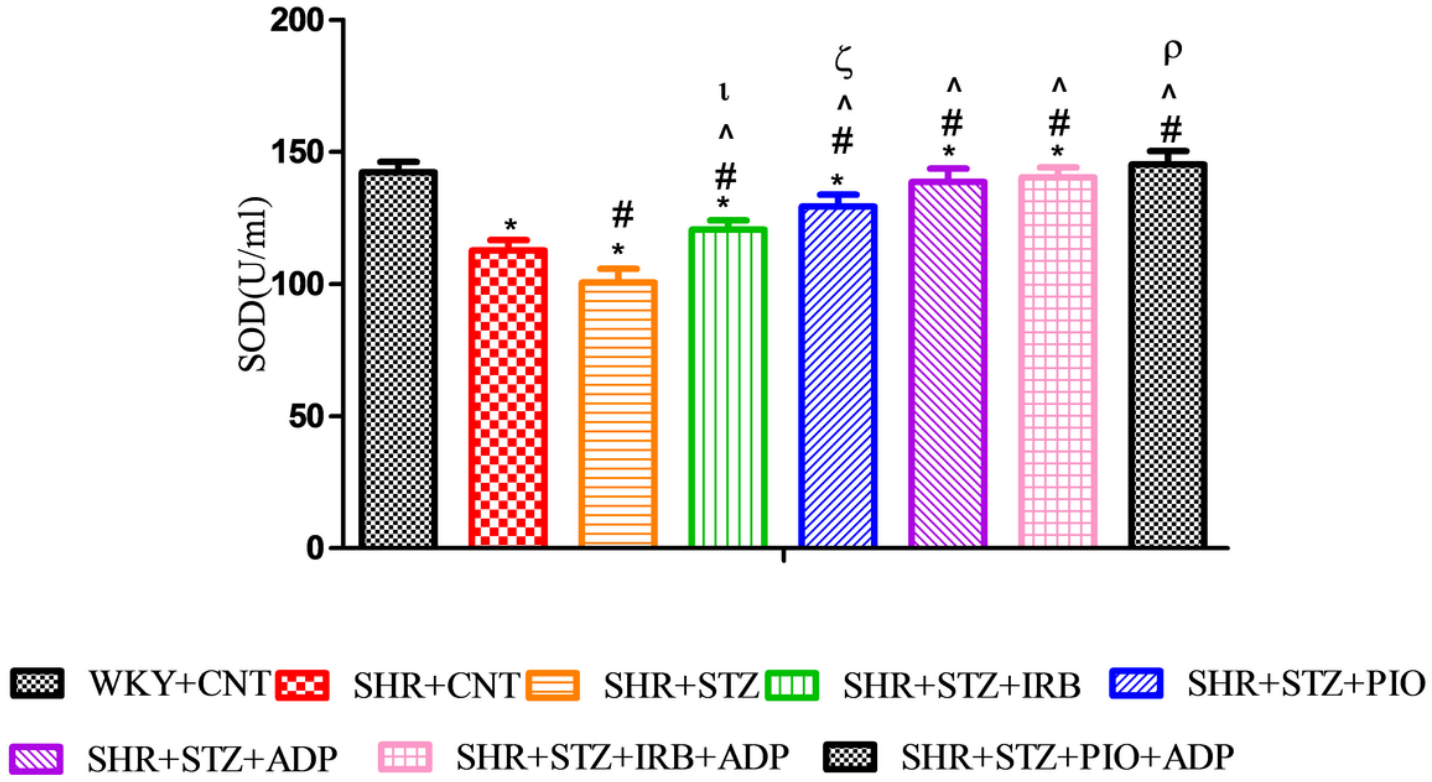

Figure 4

Plasma total superoxide dismutase levels of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. * (P<0.05) versus WKY, # (P<0.05) versus SHR ^(P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp

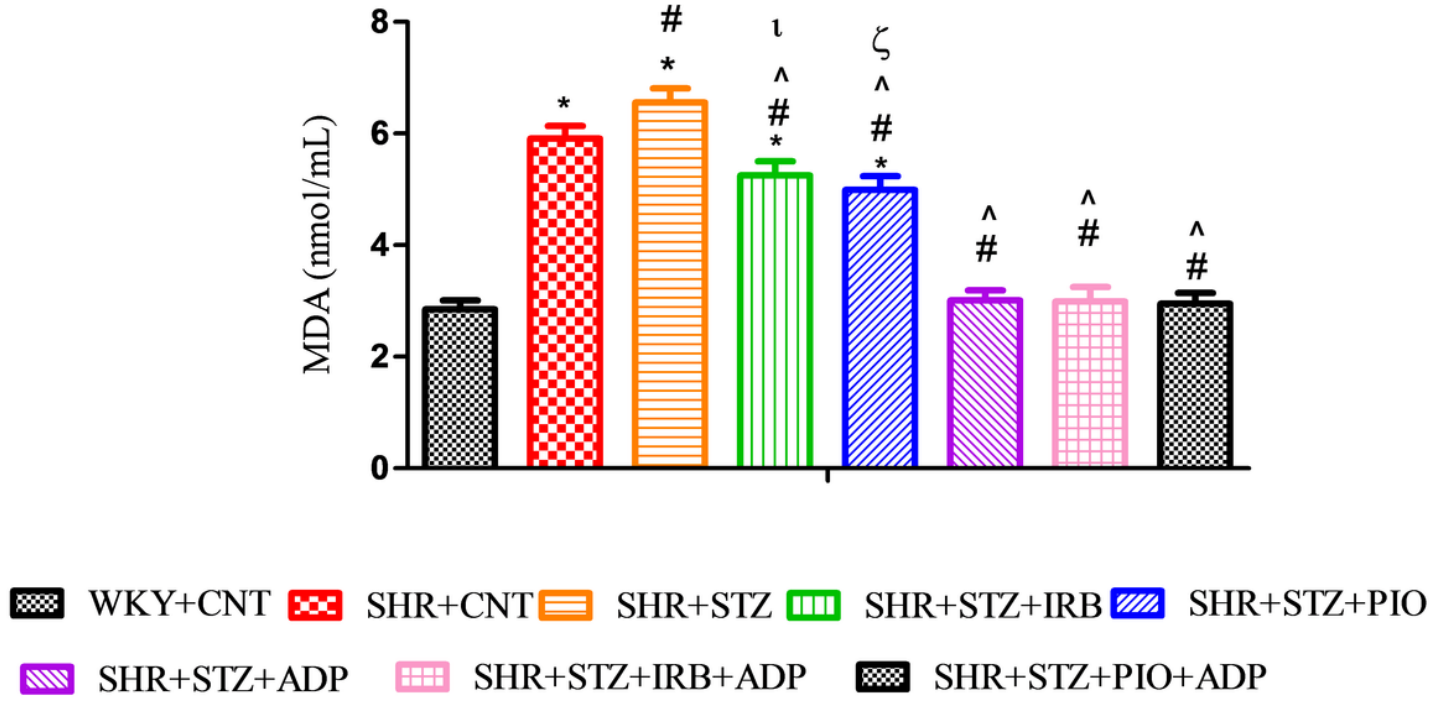

Figure 5

Plasma malondialdehyde levels of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. *(P<0.05) versus WKY, # (P<0.05) versus SHR ^(P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp

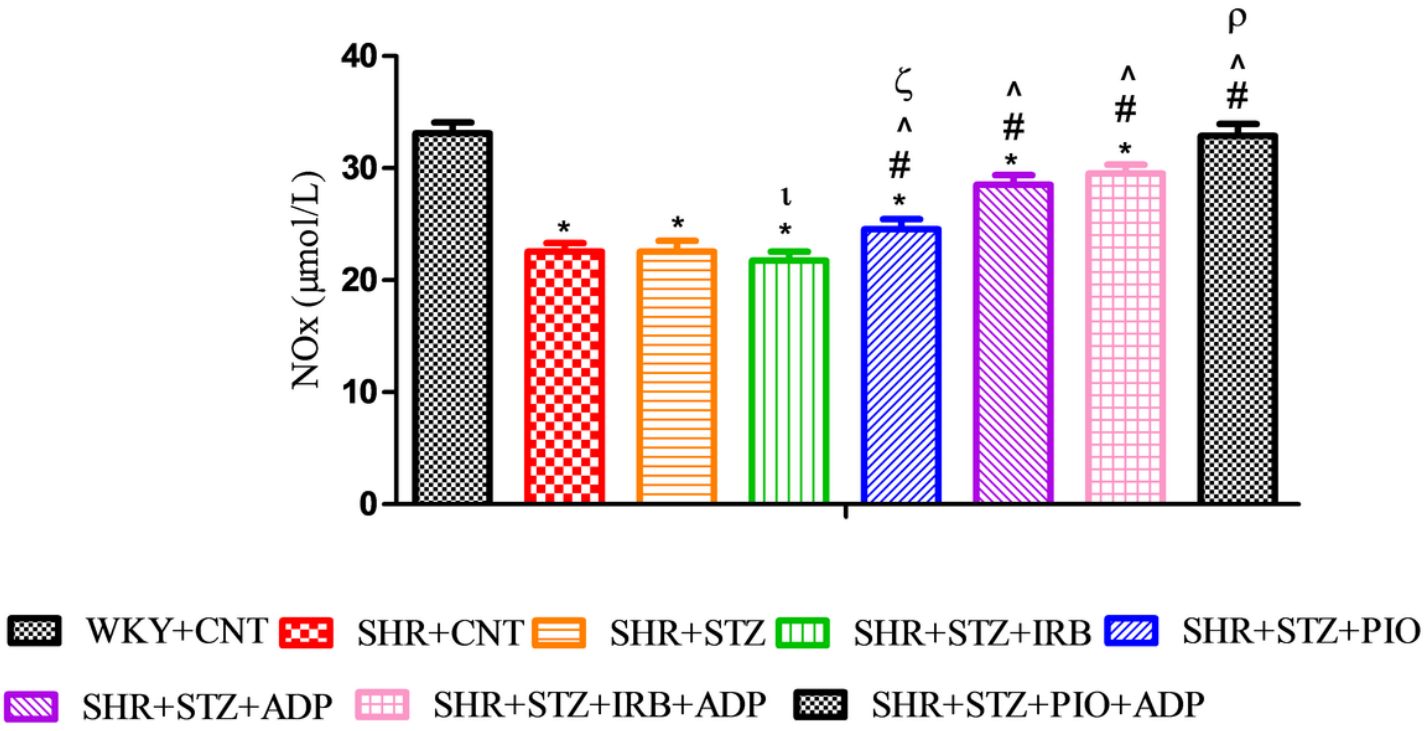

Figure 6

Plasma nitric oxide levels of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. *(P<0.05) versus WKY, # (P<0.05) versus SHR ^ (P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp

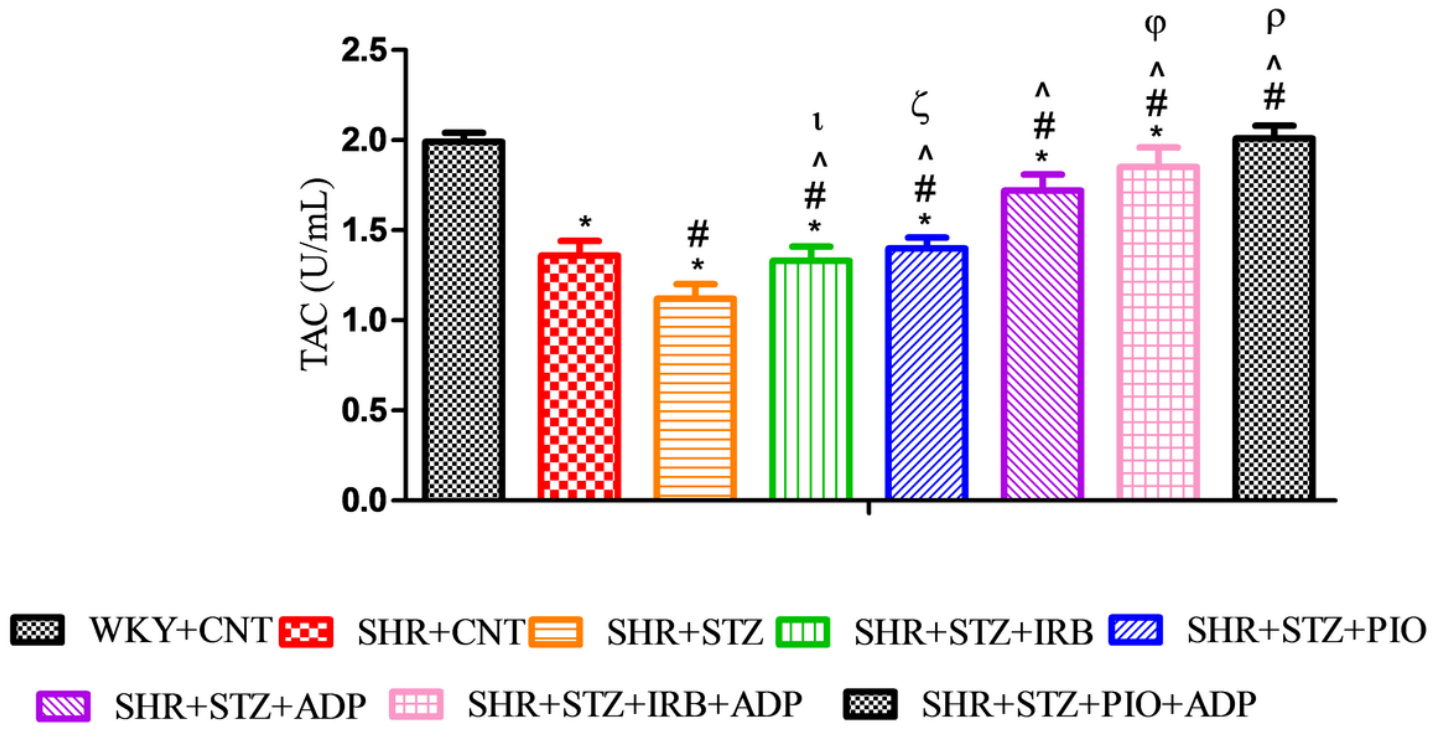

Figure 7

Plasma total antioxidant capacity of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. *P(<0.05) versus WKY, # (P<0.05) versus SHR ^(P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp φ indicates significant difference between SHR+STZ+Adp to SHR+STZ+Pio+Adp

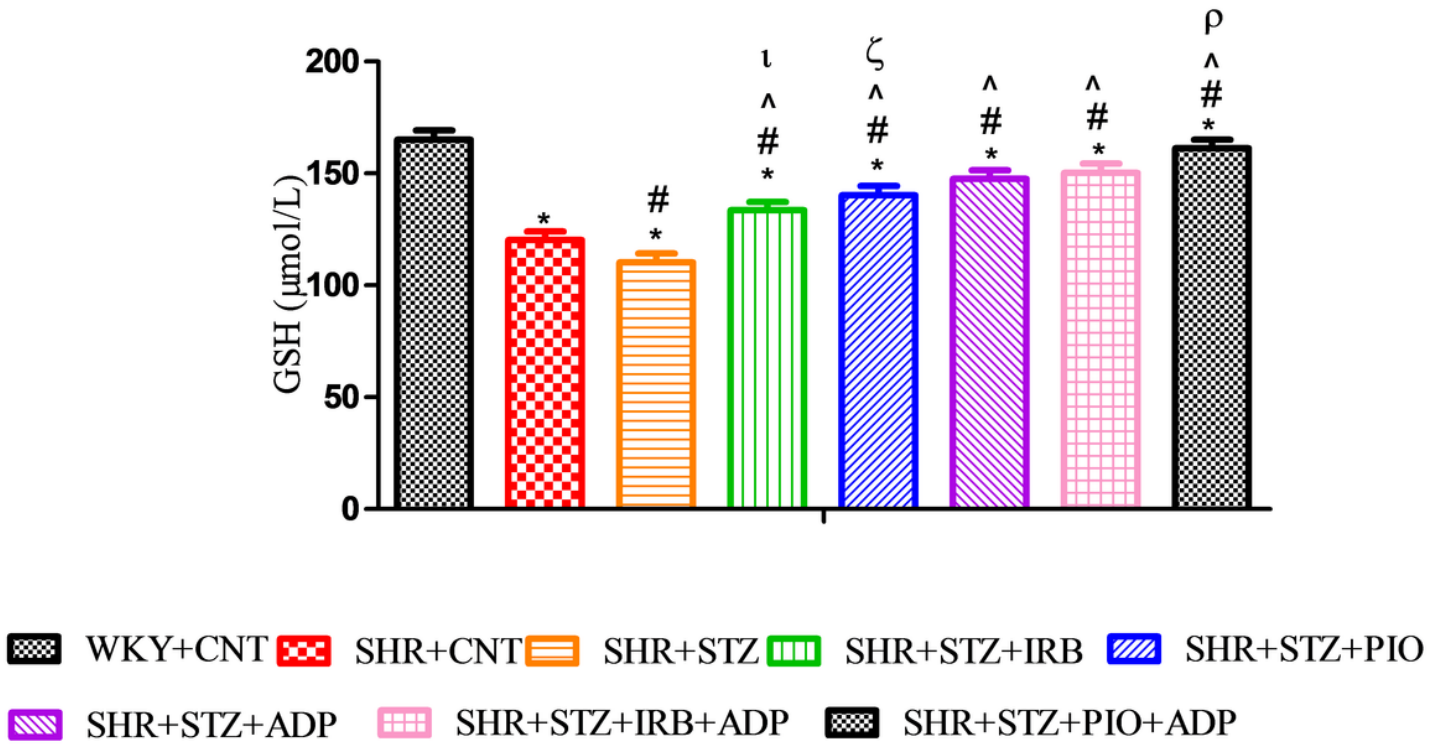

Figure 8

Plasma glutathione level of WKY, SHR diabetic control and SHR diabetic treated rats. The values are presented as mean±S.E.M. (n=6) in each group and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values with (P<0.05) were considered statistically significant. *(P<0.05) versus WKY, # (P<0.05) versus SHR ^(P<0.05) vs. SHR+STZ+Irb, SHR+STZ+Pio, SHR+STZ+Adp, SHR+STZ+Irb+Adp, SHR+STZ+ Pio+Adp groups in comparison to SHR+STZ τ indicates significant difference between SHR+STZ+Irb and SHR+STZ+Adp ξ indicates significant difference between SHR+STZ+Pio and SHR+STZ +Adp ρ indicates significant difference between SHR+STZ+Adp to SHR+STZ+ Pio+Adp.