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Pioglitazone ameliorates high fat diet-induced hypertension and induces catechol o-methyl transferase expression in rats

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ABSTRACT

Our study aimed to investigate the effect of pioglitazone (PIO) on the obesity-associated metabolic effects and whether this effect is associated with modulation of catechol O-methyl transferase (COMT) expression in the high fat diet (HFD) induced obese rats. Male Wistar rats fed HFD were used to evaluate the effect of PIO on obesity-associated hypertension and the expression of COMT. The HFD-induced obesity was confirmed by the change in body weights, the fasting serum insulin (FSI) which assessed by ELISA, homeostasis model assessment - insulin resistance (HOMA-IR), fasting blood glucose (FBG), oral glucose tolerance test (OGTT) and lipid profile which were determined by colorimetric methods. Plasma epinephrine (EP) and norepinephrine (NE) were determined by ELISA and the systolic blood pressure (SBP) was recorded using the tail-cuff method. COMT expression was assessed by quantitative real time-polymerase chain reaction (qRT-PCR), and western blotting. The HFD-induced obesity was associated with glucose intolerance, derangement of the lipid profile, increased SBP, reduced COMT expression with a concomitant increase in plasma catecholamines. Most importantly, treatment with PIO ameliorated the HFD-induced metabolic changes, improved the lipid profile, reduced SBP, increased COMT expression, and reduced plasma catecholamines. Treatment with PIO reversed HFD-induced glucose intolerance and the associated metabolic derangement. In addition, these effects of PIO were associated with up-regulating COMT expression with a subsequent reduction in plasma catecholamines levels.

1. Introduction

Obesity and its associated co-morbidities including insulin resistance, diabetes, and hypertension are highly prevalent and represent a major health problem (de Almeida et al., 2014). Obesity is an established risk factor for development of hypertension (Modan et al., 1985). Indeed, epidemiological studies revealed that obesity-induced hypertension represents about two-thirds of the hypertension incidences (Booth et al., 2016).

Moreover, the risk of developing hypertension is enhanced when obesity is associated with insulin resistance (Ormazabal et al., 2018). However, the molecular mechanisms underlying the association of obesity, insulin resistance and hypertension remain incompletely understood. Experimental and clinical evidence indicate that obesity-induced hypertension is complex and is associated with insulin resistance and hyperinsulinemia (Hall et al., 2015); over activation of the sympathetic nervous system (SNS) (Thorp and Schlaich, 2015), and overproduction of inflammatory mediators (Ormazabal et al., 2018).

Indeed, obesity-associated insulin resistance and hyperinsulinemia may contribute to the obesity-associated hypertension through several mechanisms. Catecholamines excess, reflecting an adrenergic overdrive of the SNS has been proposed to be linked to hyperinsulinemia in obesity and may contribute to the development of obesity-associated hypertension (Canale et al., 2013). Previous study demonstrated that basal plasma norepinephrine (NE), the principal catecholamine as an index of SNS activity, was persistently elevated and highly correlated with insulin resistance and hyperinsulinemia (Murabayashi et al., 2020).

The inactivation of catecholamines from both adrenal medulla and

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nerve endings results from the reuptake into specific granules in the sympathetic nerve endings as well as catecholamine-O-methyl transferase (COMT)-mediated metabolism in the liver and the target organs (Goldstein, 2010).

A recent study revealed that COMT expression is significantly reduced in obese mice fed high fat diet (Kanasaki et al., 2017). Furthermore, the reduction of COMT expression is associated with impaired glucose tolerance (Salih et al., 2008), hypertension, and heart failure (Houston, 2007). Indeed, knockout of the COMT gene in mice is associated with hypertension (Kanasaki et al., 2008; Stanley et al., 2012). Similarly, prior studies have demonstrated a significant reduction in COMT activity and protein expression in the liver (Tsunoda et al., 2002), brain (Masuda et al., 2006), and renal cortex (Ooshima et al., 2009) of spontaneously hypertensive rats.

Previous study from our groups and others had extensively investigated the regulation of COMT expression at the transcriptional levels and demonstrated that some inflammatory mediators, including the obesity-associated mediators, regulate COMT expression (Kamel et al., 2012; Salama et al., 2009; Tchivileva et al., 2009).

To this end, the molecular mechanisms linking obesity, insulin resistance, and hypertension is not fully understood. Thus, the objective of the current study is to test the hypothesis that insulin resistance in obese rats causes a reduction in COMT expression, which in turn causes hypertension. We also hypothesize that; insulin sensitizers such as Pioglitazone (PIO) may ameliorate obesity-associated hypertension with associated up-regulating COMT expression.

2. Materials and methods

2.1. Chemicals and reagents

Pioglitazone hydrochloride (PIO) was kindly provided by Medical Union Pharmaceuticals (Ismailia, Egypt). The mouse monoclonal antibody COMTD1 (C-5) (Cat# SC-515490) obtained from (Santa Cruz, CA, USA). Insulin ELISA kit (Cat# ERINS-21704) obtained from Thermo Fisher Scientific (Pierce™, Inc. USA). Colorimetric kit for blood glucose assay (Cat#GAGO-20) obtained from Sigma-Aldrich (St. Louis, MI, USA). Colorimetric kits for assessment of serum cholesterol (Cat# CH-1220), triglycerides (TG) (Cat# TG- 2030), and high-density lipoprotein (HDL) (Cat# CH-1230) were obtained from (Biodiagnostics, Egypt). Plasma epinephrine (EP)/NE ELISA kit (Cat# KA1877) was obtained from Abnova (Taipei, Taiwan). Other chemicals were of the highest analytical grade available commercially.

2.2. The computational analysis of the promoter region of COMT

The in-silico analysis of the promoter regions of COMT (COMT promoters 1 and 2) was carried out using MatInspector (www.genomatix.de) software, for detection of the presence of the cognate response element of insulin.

2.3. Experimental animals and treatment protocol

All animal procedures and the experimental protocols were following the ethical standards and approved by the Ethics Committee on the Use and Care of Animals of Faculty of Medicine, Al-Azhar University, Cairo with the following reference number: bio._8med. research_0000008.

Forty male Wistar rats (4-6-week-old, weighing 100–130 g) were obtained from the Nile Company for Pharmaceuticals & Chemical Industries in Cairo, Egypt. Rats were housed in the animal house of Faculty of Pharmacy, Al-Azhar University. They were randomly grouped and housed in polyacrylic cages its side lengths were 70 cm, with not more than five animals per cage under controlled conditions (25 ± 1 °C constant temperature, 55% relative humidity, 12-h lighting cycle). The animals were kept under laboratory conditions for two weeks before the experiment for acclimation and received a normal standard diet (NSD) and water *ad libitum*. Then, the animals were fed either NSD that consisted of 10% fat, 30% protein, and 60% carbohydrate or high fat diet (HFD) that consisted of 59%fat, 11% protein, and 30% carbohydrate (Parrott, 2014). The diets were supplied by El-Nasr Company.

Rats were randomly allocated into 4 groups (10 rats each) as follows:

1 Group #1: Rats on NSD.
2 Group # 2: Rats on NSD and treated with PIO (20 mg/kg/day; P.O daily suspended in distilled water and given by oral tube).
3 Group # 3: Rats on HFD diet.
4 Group # 4: Rats on HFD diet and treated with PIO (20 mg/kg/day; P. O daily suspended in distilled water and given by oral tube).

Group 1 and group 2 fed NSD while group 3 and group 4 fed HFD for 24 consecutive weeks. At the beginning of the 13th week, animals from groups 2 and 4 were treated with PIO and continue until the end of the experiment. PIO was prepared freshly as a suspension in distilled water every time before administration. The dose of PIO and the duration of treatment were guided by several previous publications (DING et al., 2005) as well as a preliminary experiment in our laboratory.

At the end of 12th, the rats were fasted for overnight and blood samples from the tail were obtained, and at the end of the experiment, the rats were fasted for overnight and anesthetized with diethyl ether, and then rats were killed by decapitation, blood samples were collected by aortic puncture for measurement of blood chemistry. Blood samples collected into centrifuge tubes and left to stand at room temperature for 20 min, then centrifuged at 1559 g for 10 min to separate serum then the serum was stored at −80 °C till analysis achievement. Other part of blood sample was collected into fluoride and EDTA tubes for fasting blood glucose (FBG) and plasma epinephrine (EP) and norepinephrine (NE) assay respectively, and the rats were perfused with cold isotonic saline solution through the abdominal aorta. Both kidneys and liver were removed, and pieces of tissue were frozen and conserved at −80 °C for real time PCR and western blotting (Fig. 1).

2.4. Assessment of body weight gain

Before the commencement of treatment, the individual body weight of rats in each group was measured. The measurements obtained were used as baseline data. Thereafter, the body weight was weighed twice weekly and the change in body weight was reported.

2.5. Measurement of glucose and insulin resistance

At the end of the 12th week, the metabolic phenotype of the rats was evaluated by the assessment of body weight changes; insulin resistance; and FBG levels. Blood glucose level was determined using a commercial kit from Sigma-Aldrich (St. Louis, MI, USA) according to the manufacturer's instruction. Fast serum insulin (FSI) levels were assayed using Rat Insulin ELISA Kit from Thermo Fisher Scientific (Frederick, MD, USA); Homeostasis Model Assessment - Insulin Resistance (HOMA-IR) and determination of Oral Glucose Tolerance Test (OGTT) were performed after overnight fasting. HOMA-IR was determined according to the following formula: ***FSI (μIU/mL) X FBG (mg/dL)/405*** (Matthews et al., 1985).

2.6. Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT) was performed on overnight fasting rats. In brief, rats have fasted overnight before the administration of a single intraperitoneal injection of a loading dose of a glucose load (2 g/kg). Blood samples were drawn from the tail vein of each rat at 0 (the baseline fasting level), 30, 60, 90, and 120, min after the administration of glucose. Blood glucose level was determined calorimetrically using a commercial kit from Sigma-Aldrich (St. Louis, MI, USA) according to the

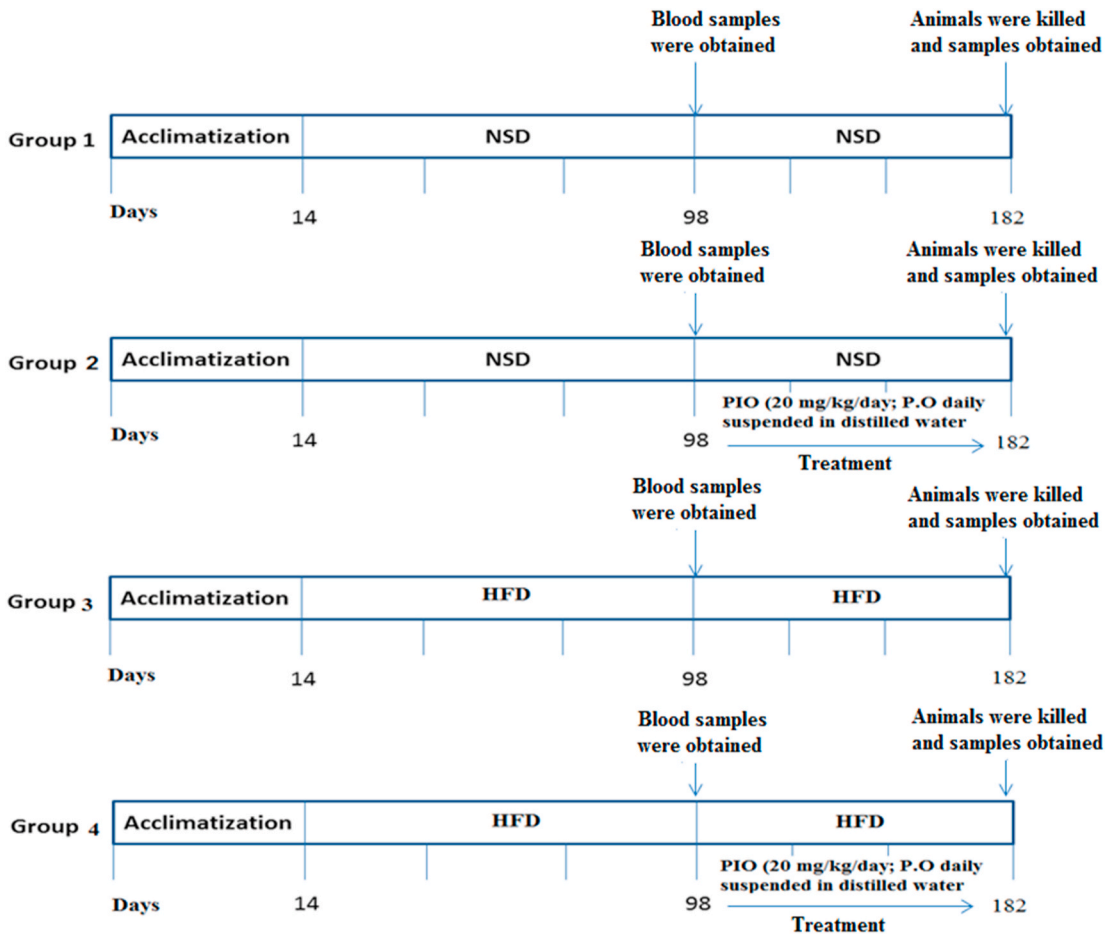

Fig 1. Schematic diagram for experimental scale design and procedure in rats fed high fat diet (HFD) or normal standard diet (NSD) and treated with or without pioglitazone (PIO)., n = 10 rats (per each treatment group and the body weight was weighed twice weekly.

manufacturer's instruction. The area-under-the-curve (AUC-OGTT) value of glucose was determined using the total AUC from the sampling period from 0 to 120 min (Antunes et al., 2016).

2.7. Measurement of a lipid profile

Serum cholesterol, TG and HDL were estimated by colorimetric procedure using the commercially available kit from Biodiagnostics (Cairo, Egypt). Low density lipoprotein (LDL) was calculated using Friedewald formula ***LDL(mg/dL) = TC (mg/dL) – [HDL(mg/dL) – TG (mg/dL)/5]*** (Knopfholz et al., 2014).

2.8. Measurement of plasma catecholamines

For biochemical analysis of plasma catecholamines (EP and NE), blood was collected after decapitation by an aortic puncture. Plasma was separated from the blood and used for the assessment of EP and NE concentrations using an EP/NE ELISA kit (Abnova, Taipei, Taiwan) according to the manufacturer's instructions (Westermann et al., 2002).

2.9. Measurement of SBP

The systolic blood pressure (SBP) was measured in conscious rats from different groups using a non-invasive Blood Pressure (BP) recorder (Power Lab, NIBP controller, AD instruments, serial number 221–1400, Australia) after 20 min rest in calm dark conditions (Gangwar et al., 2014).

2.10. Quantitative real-time RT-PCR

2.10.1. RNA isolation

Total RNA from liver or kidney tissues was isolated using TRIzol reagent from Invitrogen (Thermo Fisher Scientific, Waltham, USA); according to the manufacturer's protocol. After completion of the isolation procedures, the RNA pellet was stored in DEPC- treated water. To digest the potential DNA residues, the pellets of isolated RNA were treated with RNAse-free DNAse kit (Invitrogen, Germany). RNA aliquots were stored at −20 °C or utilized immediately for reverse transcription (Salem et al., 2018).

2.10.2. Reverse transcription reaction

The synthesis of the First Strand cDNA from the isolated RNA was performed using RevertAid First Strand cDNA Synthesis Kit (Catalog number: K1621) from Thermo Fisher Scientific (Waltham, MA, USA) as per the manufacturer's protocol. Briefly, 1 μg of RNA was mixed with H_2O and oligo (dT) primers, pre-heated for 5 min at 25 °C, and finally incubated at 43 °C for 1 h in the presence of DNTPs, reverse transcriptase, and reaction buffer containing $MgCl_2$ (5 mM) (Khalil et al., 2018).

2.10.3. Quantitative real time RT-PCR

SYBR® Premix Ex TaqTM kit (TaKaRa, Biotech. Co. Ltd.) was used to perform the qRT-PCR analyses using the synthesized cDNA copies from liver and kidney tissues. For each reaction, a melting curve profile was conducted. The quantitative values of the COMT were normalized on the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (Table 1). The $2^{-\Delta\Delta CT}$ method was

employed to determine relative gene level differences with GAPDH qPCR products used as a control.

2.11. Western blotting

Western blots were performed using liver and kidney tissue lysates prepared by homogenizing the liver and kidneys with a tissue mixer (Ultra-Turrax T8, IKA-Werwe; IKA, Staufen, Germany) at 4 °C in homogenization RIPA lysis buffer (Sigma-Aldrich, Milan, Italy) (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% NP-40 or 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing appropriate phosphatase and protease inhibitors.

The tissue lysates were sonicated and centrifuged at 14031 g for 20 min. The supernatants were collected, divided into aliquots, and stored at – 80 °C until used for immunoblotting. Protein concentration was determined using the Biuret method (Gornall et al., 1949; Wang et al., 1996). Proteins in each homogenate were denatured at 95 °C for 5 min in 2 × Laemmli buffer containing 5% β-mercaptoethanol. SDS–PAGE and western blot were achieved according to the method described by (Burnette, 1981).

2.12. Statistical analysis

Numerical data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test for the comparison among the multiple groups. Mann-Whitney test was used for the comparison between two groups; GraphPad Prism software-6 was used. A P-value of less than 0.05 was considered to show a significant difference between groups. Data expressed as Mean ± S.D.

3. Results

3.1. Analysis of COMT promoter regions for insulin response elements

We began with the in-silico analysis of the promoter regions (promoter 1 and 2) of the *COMT* gene by MatInspector (www.genomatix.de) for the presence of the cognate response elements of insulin. Our results demonstrated the presence of several classical recognition motifs for insulin including the activator protein 1(AP- 1)(Sutherland et al., 2007), carbohydrate response element-binding protein (ChREBP) (Vijayakumar et al., 2017), serum response factor (SRF) (Sarkar et al., 2011), sterol-response-element-binding protein (SREBP) (Mounier and Posner, 2006) in COMTP1 and COMTP2 which are known response elements for insulin (Fig. 2).

3.2. Effect of HFD on rats SBP and biochemical parameters

We first establish the rat model of HFD-induced obesity and insulin resistance. As indicated in (Fig. 3A and B), feeding the rats with HFD resulted in a significant and progressive increase in the rat's body weights as compared to NSD-fed rats.

In HFD-fed rats, the change in the body weights (gm) from the initial weight was (53.04 ± 11.52; 91.33 ± 19.15; and 97.80 ± 22.89) at 4th, 8th and 12th weeks respectively, while in the NSD-fed rats, the change in the body weights was (36.09 ± 10.85; 35.17 ± 11.13; and 30.74 ± 9.22) at 4th, 8th and 12th weeks respectively. Thus, the AUC for the change in the body weight increased significantly in HFD-fed rats as compared to NSD-fed rats (17055 ± 509.90 and 11180 ± 531), respectively.

Then we investigated the effect of HFD on insulin resistance. As demonstrated in (Fig. 4), feeding the rats with HFD for 12 weeks resulted in a significant increase in levels of FSI (μIU/mL) as compared to NSD-fed rats (0.33 ± 0.79 and 0.16 ± 0.48), respectively (Fig. 4A). Similarly, the FBG levels (mg/dL) were (133.20 ± 12.15 and 85.25 ± 8.86), respectively (Fig. 4B).

To investigate whether the increase in FBG and FSI levels observed in HFD-fed rats is indicative for insulin resistance, we perform OGTT and determine the HOMA-IR for HFD-fed rats and NSD-fed rats. In HFD-fed rats, the AUC of OGTT was significantly higher as compared to NSD-fed rats (14215 ± 826.40 and 11470 ± 701.40), respectively (Fig. 4C and D). Also, the HOMA-IR in HFD-fed rats was significantly higher as compared to NSD-fed rats (0.13 ± 0.03 and 0.04 ± 0.01), respectively (Fig. 4E).

Interestingly our data also revealed that HFD-induced metabolic changes were associated with a significant increase in SBP after 12 weeks of HFD regimen in rats. Thus, the mean SBP (mmHg) in HFD-fed rats was significantly higher in HFD-fed rats as compared to NSD-fed rats (144.30 ± 5.95 and 101.80 ± 2.44), respectively (Fig. 4F).

Table 1
Primers sequence used for *RT-qPCR*.

Gene	Forward	NCBI Reference
GAPDH	F: AGACAGCCGCATCTTCTTGT R: CTTGCCGTGGGTAGAGTCAT	NM_017008.4
COMT	F: ATCTTCACGGGGTTTCAGTG R: AGATGTGGTGTGAGCTGCTG	NM_012531.2

GAPDH; glyceraldehyde 3-phosphate dehydrogenase and COMT; catechol o-methyl transferase.

3.3. Effect of PIO on HFD-induced metabolic and SBP changes in rats

As depicted in (Fig. 5A and B), the treatment with PIO did not cause significant changes in the body weight either in HFD-fed rats or NSD-fed rats.

Treatment of HFD-fed rats with PIO resulted in improving the insulin sensitivity that was manifested as a significant reduction in the FSI levels (μIU/mL) as compared to untreated HFD-fed rats (3.53 ± 0.52 and 7.10 ± 1.10), respectively. Indeed, the FSI levels in HFD-fed rats treated with PIO was not significantly different from NSD-fed rats (3.53 ± 0.52 and 2.70 ± 0.57), respectively. On the other hand, treatment NSD-fed rats with PIO did not cause any significant changes in FSI levels as compared with untreated NSD-fed rats (2.43 ± 0.61 and 2.70 ± 0.57), respectively (Fig. 6A).

Similarly, as anticipated, treatment with PIO significantly reduced the FBG levels (mg/dL) in HFD-fed rats as compared to untreated HFD-fed rats (98.60 ± 3.37 and 132.60 ± 3.85), respectively. While, FBG level was not significantly different in NSD-fed rats treated with PIO as compared to untreated NSD-fed rats (81.50 ± 11.04 and 87.20 ± 16.28), respectively. Accordingly, the FBG level in the HFD-fed rats treated with PIO was comparable to the corresponding value in NSD-fed rats treated with or without PIO (98.60 ± 3.37; 81.50 ± 11.04 and 87.20 ± 16.28), respectively (Fig. 6B).

Concerning the glucose tolerance, the AUC of OGTT in HFD-fed rats treated with PIO was significantly lower as compared to untreated HFD-fed rats (10860 ± 474.30 and 13895 ± 474.30), respectively. While, AUC of OGTT in NSD-fed rats treated with PIO was not significantly different as compared to untreated NSD-fed rats (9505 ± 443.80 and 10543 ± 474.60), respectively. Thus, in HFD-fed rats treated with PIO, the AUC of OGTT was comparable with that for NSD-fed rats whether treated with PIO or not (10860 ± 474.30; 9505 ± 443.80; and 10543 ± 474.60), respectively (Fig. 6C and D).

Consequently, the HOMA-IR level was significantly declined in HFD-fed rats treated with PIO as compared to untreated HFD-fed rats (2.80 ± 0.88 and 6.12 ± 0.99), respectively. While, the HOMA-IR level was not significantly different in NSD-fed rats treated with PIO as compared to untreated NSD-fed rats (1.40 ± 0.47 and 1.60 ± 0.67), respectively. But the HOMA-IR level in HFD-fed rats treated with PIO was higher as compared to NSD-fed rats treated with and without PIO (2.80 ± 0.88; 1.40 ± 0.47; and 1.60 ± 0.67), respectively (Fig. 6E).

Interestingly, the treatment of HFD-fed rats with PIO resulted in a significant reduction in the SBP level (mmHg) as compared to HFD-fed rats (107 ± 5.23 and 141.00 ± 6.96), respectively. On the other hand,

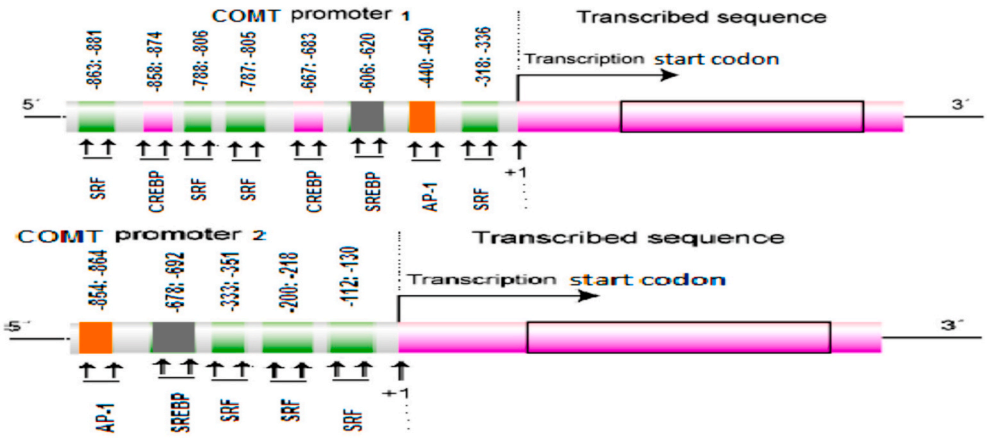

Fig 2. Schematic representation of *COMT* gene promoter regions (Genbank accession numbers Z26491 and Z26490) showing some consensus sequence of SREBP, ChREBP, SRF, and AP-1. COMTP1 and COMTP2 sequence were located by MatInspector (www.genomatix.de). COMT; catechol o-methyl transferase, AP- 1; the activator protein 1, ChREBP; carbohydrate response element-binding protein, SRF; Serum response factor, SREBP; sterol-response-element-binding protein, COMTP1; catechol o-methyl transferase promoter 1 and COMTP2; catechol o-methyl transferase promoter 2.

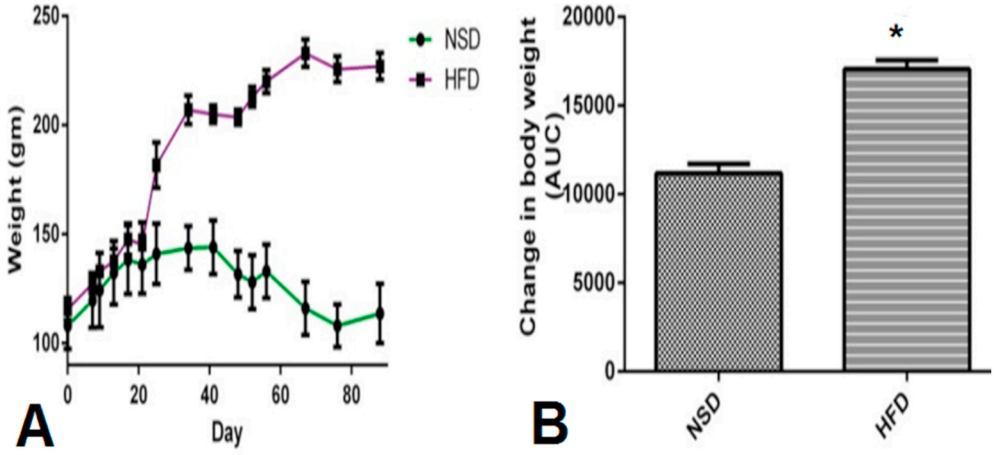

Fig 3. Effect of the diet on the change in body weights. (A) and (B) effect of HFD on the change in body weights and AUC of change in body weights for NSD and HFD groups, data expressed as Mean ± S.D., n = 10. HFD: high fat diet, AUC: area under the curve and NSD: normal standard diet.
*: Significant from NSD group at $P < 0.05$.

treatment of NSD-fed rats with PIO did not cause any significant changes in SBP levels as compared with untreated NSD-fed rats (99.63 ± 3.29 and 106.10 ± 6.46), respectively. Thus, in HFD-fed rats treated with PIO, the SBP was comparable with that for NSD-fed rats whether treated with PIO or not (107 ± 5.23; 99.63 ± 3.29; and 106.10 ± 6.46), respectively (Fig. 6F).

3.4. Effects of HFD and/or PIO on lipid profile

Feeding the rats with HFD resulted in a significant increase in fast serum cholesterol (mg/dL) as compared to NSD-fed rats (102.20 ± 6.68 and 85.32 ± 7.06), respectively. Treatment with PIO significantly ameliorated HFD-induced increase in fast serum cholesterol. Thus, fast serum cholesterol level in HFD-fed rats treated with PIO was comparable with NSD-fed rats whether treated with or without PIO (81.43 ± 18.03; 78.55 ± 11.83; and 85.32 ± 7.06), respectively (Table 2).

Similarly, the LDL levels (mg/dL) were increased significantly in HFD-fed rats as compared to NSD-fed rats (73.89 ± 16.56 and 33.12 ± 19.38), respectively. Treatment the HFD-fed rats with PIO resulted in a significant reduction in the LDL levels as compared to HFD-fed rats (30.22 ± 17.86 and 73.89 ± 16.56), respectively. Thus, the LDL levels in HFD-fed rats treated with PIO were comparable with NSD-fed rats whether treated with or without PIO (30.22 ± 17.86; 17.57 ± 11.56; and 33.12 ± 19.38), respectively (Table 2).

Also, the HFD resulted in a significant increase in TG levels (mg/dL) in HFD-fed rats as compared to NSD-fed rats (77.65 ± 7.10 and 53.5 ± 15.01), respectively. Treatment with PIO significantly ameliorated HFD-induced increase in the TG levels as a significant reduction in the TG levels as compared to untreated HFD-fed rats (50.37 ± 6.91 and 77.65 ± 7.10). Thus, the TG levels in HFD-fed rats treated with PIO were comparable with NSD-fed rats whether treated with or without PIO (50.37 ± 6.91; 49.63 ± 8.59; and 53.50 ± 15.01), respectively (Table 2).

On the contrary, the HDL levels (mg/dL) were declined significantly in HFD-fed rats as compared to NSD-fed rats (26.88 ± 7.04 and 44.50 ± 6.43), respectively. Treatment with PIO significantly improved HFD-induced decline in the HDL levels as a significant increase in the HDL levels in treated HFD-fed rats as compared to untreated HFD-fed rats (42.50 ± 4.24 and 26.88 ± 7.04). Thus, the HDL levels in HFD-fed rats treated with PIO were comparable with NSD-fed rats whether treated with or without PIO (42.50 ± 4.24; 50.60 ± 1.07; and 44.50 ± 6.43), respectively (Table 2).

3.5. Expression of COMT in rat's liver and kidney

Then, we assessed whether the expression of COMT in the kidney and liver tissues is modulated by HFD and/or PIO treatment.

To verify whether the COMT expression is modulated at the transcriptional level by HFD and/or treatment with PIO, the expression of COMT mRNA was analyzed in liver and kidney tissues from rats fed HFD or NSD and treated with or without PIO by real-time RT-PCR. The real

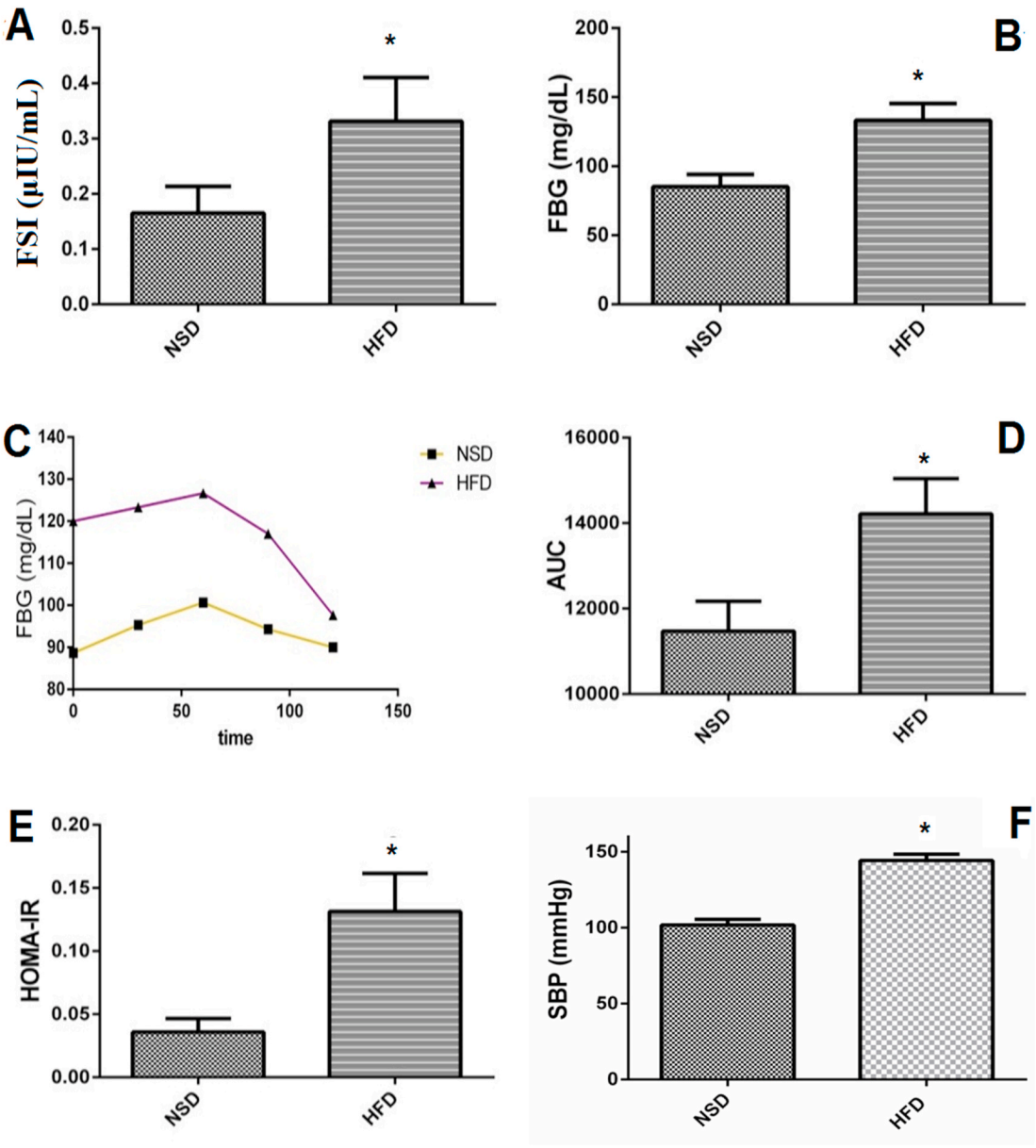

Fig 4. Effect of HFD on metabolic parameters. (A) effect of HFD on FSI (µIU/mL) for NSD and HFD groups (B) effect of HFD on FBG (mg/dL) for NSD and HFD groups (C) and (D) effect of HFD on AUC of OGTT for NSD and HFD groups (E) effect of HFD on HOMA-IR for NSD and HFD groups (F) effect of HFD on SBP for NSD and HFD groups, data expressed as Mean ± S.D., n = 10. HFD: high fat diet, FSI: fasting serum insulin, FBG: fasting blood glucose, AUC: area under the curve, OGTT: oral glucose tolerance test, HOMA-IR: Homeostasis Model Assessment - Insulin Resistance, SBP: systolic blood pressure and NSD: normal standard diet.
*: Significant from NSD group at $P < 0.05$

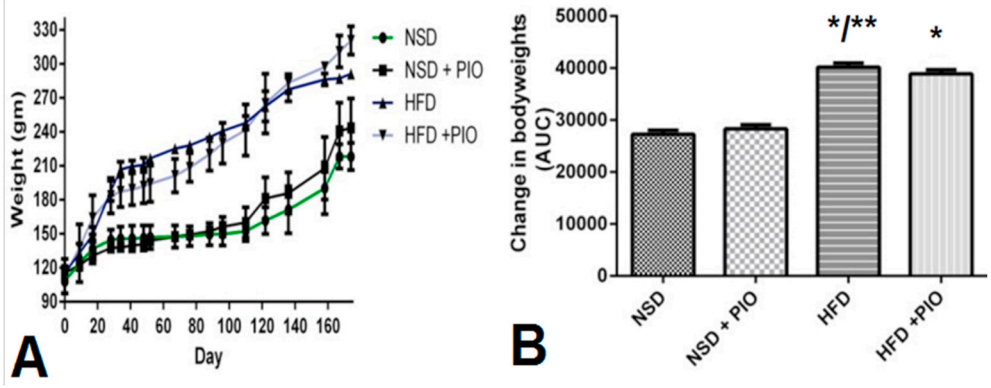

Fig 5. Effect of the diet and/or PIO on the change in body weights. (A) and (B) effect of HFD and/or PIO on the change in body weights and AUC of change in body weights for NSD, NSD + PIO, HFD, and HFD + PIO groups data expressed as Mean ± S.D., n = 10. HFD: high fat diet, PIO: pioglitazone, AUC: area under the curve and NSD: normal standard diet.
*: Significant from NSD group at $P < 0.05$
**: Significant from NSD + PIO group at $P < 0.05$.

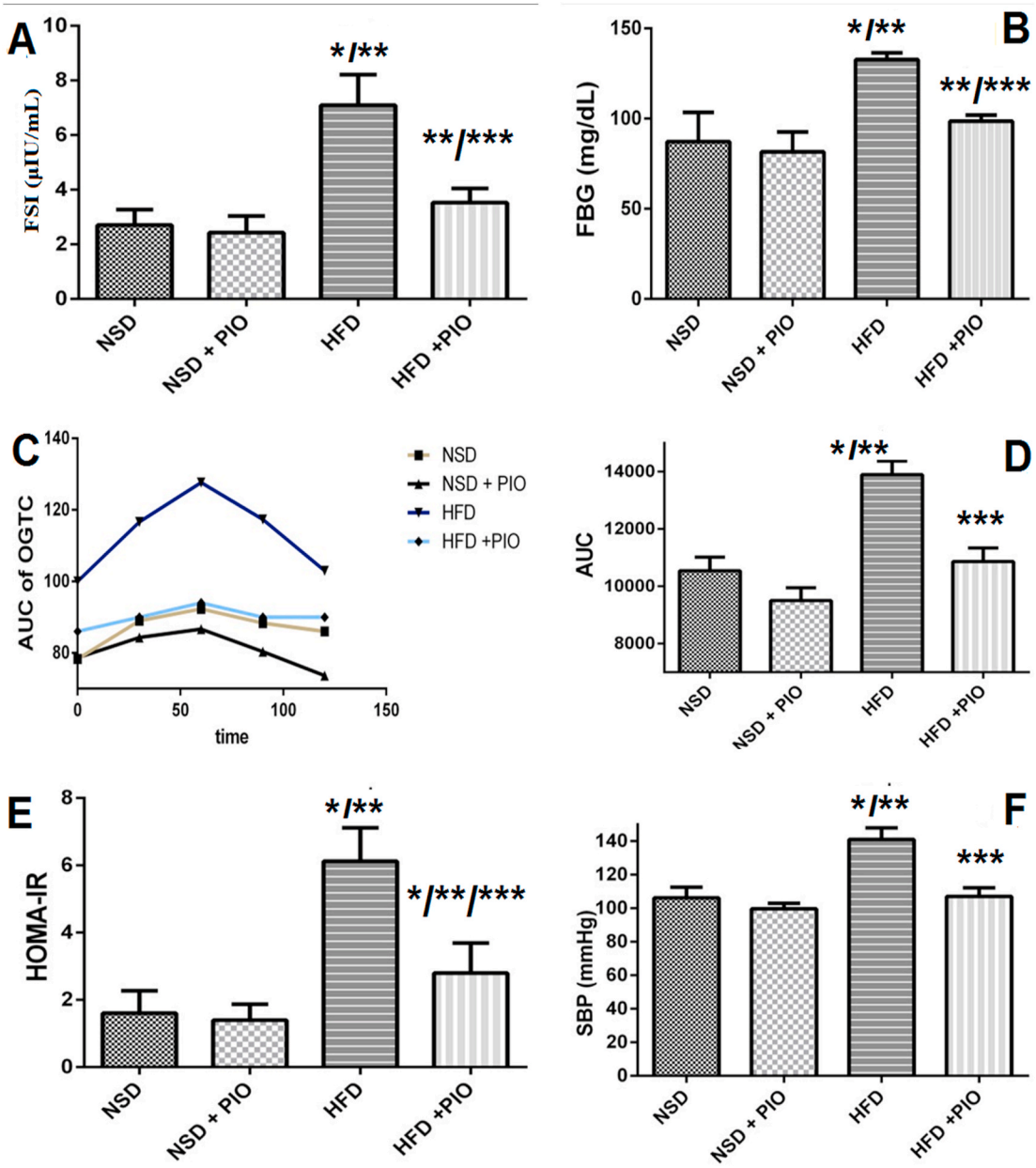

Fig 6. Effect of HFD and/or PIO on metabolic parameters. (A) effect of HFD and/or PIO on FSI (µIU/mL) for NSD, NSD + PIO, HFD, HFD + PIO groups (B) effect of HFD and/or PIO on FBG (mg/dL) for NSD, NSD + PIO, HFD, HFD + PIO groups (C) and (D) effect of HFD and/or PIO on AUC of OGTT for NSD, NSD + PIO, HFD, HFD + PIO groups (E) effect of HFD and/or PIO on HOMA-IR for NSD, NSD + PIO, HFD, HFD + PIO groups (F) effect of HFD and/or PIO on SBP (mmHg) for NSD, NSD + PIO, HFD, HFD + PIO groups, data expressed as Mean ± S.D., n = 10. HFD: high fat diet, PIO: pioglitazone, FSI: fasting serum insulin, FBG: fasting blood glucose, AUC: area under the curve, OGTT: oral glucose tolerance test, HOMA-IR: Homeostasis Model Assessment-Insulin Resistance, SBP: systolic blood pressure and NSD: normal standard diet.
*: Significant from NSD group at $P < 0.05$
**: Significant from NSD + PIO group at $P < 0.05$
***: Significant from HFD group at $P < 0.05$.

time RT-PCR assay indicated that COMT mRNA expression in both liver and kidney tissues is significantly down-regulated in HFD-fed rats as compared to NSD-fed rats. Treatment with PIO resulted in a significant increase in COMT mRNA expression in the kidney (Fig. 7A) and liver tissues (Fig. 7B) as compared to NSD-fed rats. In HFD-fed rats, treatment with PIO caused a substantial increase in COMT mRNA expression in the kidney (Fig. 7A) and liver tissues (Fig. 7B) as compared to untreated HFD-fed rats.

Similarly, western blotting demonstrated that the expression of COMT is significantly lower in the kidney and liver tissues from HFD-fed rats as compared to NSD-fed rats. Most significantly, treatment with PIO resulted in an increase in the expression of COMT in the kidney and liver tissues in either HFD-fed rats or NSD-fed rats as compared to the corresponding untreated rats. Indeed, the effect of PIO was more significantly pronounced in HFD-fed rats as compared to NSD-fed rats (Fig. 8A, B, C, D, E, and F).

3.6. *Effects of HFD and/or PIO on plasma catecholamines*

On the light of the finding that PIO ameliorated HFD-induced down regulation of COMT expression and increased SBP, we investigated whether these effects were associated with changes in plasma

Table 2
Data of lipid profile in all groups.

	Cholesterol (mg/dL)	LDL (mg/dL)	TG (mg/dL)	HDL (mg/dL)
NSD	85.32 ± 7.06	33.12 ± 19.38	53.50 ± 15.01	44.50 ± 6.43
NSD + PIO	78.55 ± 11.83	17.57 ± 11.56	49.63 ± 8.59	50.60 ± 1.07
HFD	102.20 ± 6.68[a, b]	73.89 ± 16.56 [a, b]	77.65 ± 7.10 [a, b]	26.88 ± 7.04 [a, b]
HFD + PIO	81.43 ± 18.03 [c]	30.22 ± 17.86 [c]	50.37 ± 6.91 [c]	42.50 ± 4.24 [c]

Data expressed as Mean ± S.D. LDL; low-density lipoprotein, TG; triglycerides, HDL; high-density lipoprotein, NSD; normal standard diet, PIO; pioglitazone and HFD; high fat diet.
[a] Significant from NSD group at $P < 0.05$.
[b] Significant from NSD + PIO group at $P < 0.05$.
[c] Significant from HFD group at $P < 0.05$.

catecholamines levels.

Indeed, our results demonstrated that EP level (pg/mL) was not significantly different in NSD-fed rats treated with or without PIO (91.96 ± 17.62 and 109.20 ± 12.70), respectively. More significantly, in HFD-fed rats, the EP level was significantly higher as compared to NSD-fed rats (178.30 ± 11.99 and 109.20 ± 12.70), respectively. Interestingly, treatment of HFD-fed rats with PIO was associated with a marked reduction in EP levels as compared to untreated rats (115.40 ± 11.49 and 178.30 ± 11.99), respectively (Fig. 9A).

A similar pattern of NE was observed. Thus, the level of NE (pg/mL) in NSD-fed rats, NSD-fed rats treated with PIO, HFD-fed rats and HFD-fed rats treated with PIO were (151.60 ± 12.86; 133 ± 6.84; 376.10 ± 31.06; and 212.50 ± 13.35) respectively (Fig. 9B).

4. Discussion

Obesity is closely associated with various metabolic syndromes, including insulin resistance; glucose intolerance, dyslipidemia, and hypertension. The prime feature of obesity is the accumulation of the excessive amount of adipose tissues which plays a crucial role in the development of obesity-associated complications (Jung and Choi, 2014).

In the current study, we attempted to investigate how obesity can cause the development of hypertension. We hypothesized that IR associated with obesity may be associated with down regulation of the COMT gene which plays a central role in catecholamines metabolism. It is documented that catecholamines can regulate vital physiological processes including insulin, glucose, vascular tone, and heart rate (Hall et al., 2016). Furthermore, catecholamines also play a fundamental role in regulating insulin secretion and thus glucose intolerance (Hall et al., 2016).

Following these notions, several reports demonstrated that low-activity COMT allele is associated with abdominal obesity, glucose intolerance, and increased level of HbA1c (Bozek et al., 2017). However, the molecular link between obesity; glucose intolerance; insulin resistance; and hypertension is still unclear. Thus, we postulated that COMT expression might represent the molecular link between obesity, development glucose intolerance, and insulin resistance.

It is well-documented that insulin/insulin receptor can regulate the expression of genes harboring insulin response elements (IRE), the activator protein 1(AP- 1) (Sutherland et al., 2007), carbohydrate response element-binding protein (ChREBP) (Vijayakumar et al., 2017), Serum response factor (SRF) (Sarkar et al., 2011), sterol response element-binding protein (SREBP) (Mounier and Posner, 2006) in their promoters. Indeed, in the current study, our computational analysis of the COMT promoter regions, COMTP1 and COMTP2, harbors multiple consensus sequences of SREBP, ChREBP, SRF and, AP1. These findings suggest that insulin may regulate COMT expression. Accordingly, our current report confirms our previous study which demonstrated that COMT expression is down-regulated in insulin resistance (Salih et al., 2008).

The role of insulin in regulating COMT expression provides a plausible explanation for our finding that COMT expression is significantly reduced in the kidney from HFD-fed rats which are associated with insulin resistance by 51.16% (0.60 ± 0.08 as compared to 1.21 ± 0.06 in NSD-fed rats; $P < 0.05$). Similarly, in liver tissues from HFD-fed rats, COMT expression is significantly reduced by 41.97% (0.47 ± 0.01 as compared to 0.81 ± 0.01 in NSD-fed rats; $P < 0.05$). Furthermore, it worth mentioning that, in obesity which is considered as a chronic inflammatory state, a plethora of cytokines such as interleukins and adipokines are released (Khan and Joseph, 2014). Many of these factors can also regulate COMT expression. For instance, previous studies reported that TNF-α and leptin down-regulate COMT expression (Shouman et al., 2016).

COMT plays an indispensable role in the metabolism of endogenous catecholamines (adrenaline, noradrenaline, and dopamine) which in turn play a critical role in regulating blood pressure (Paravati and Warrington, 2019). The role of COMT expression in the development of hypertension is substantiated by the finding that COMT knockout mice are hypertensive (Kanasaki et al., 2017).

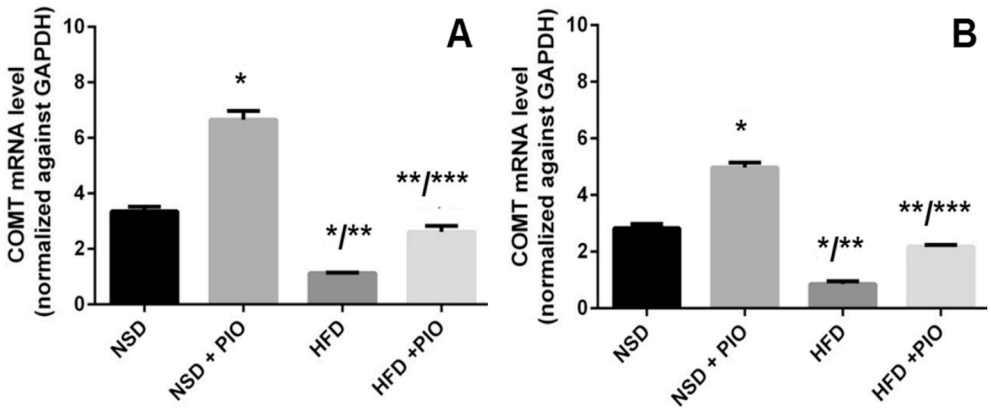

Fig 7. Quantitative analysis of COMT mRNA expression levels with real time RT-PCR. Tissue samples from randomly selected 5 animals from each group. Expression levels in the kidney (A) and liver tissues (B) are displayed as the mean ratio of target-to-reference gene ± S.D. Data was normalized to GAPDH. COMT; catechol o-methyl transferase, RT-PCR; quantitative real time-polymerase chain reaction and GAPDH; glyceraldehyde 3-phosphate dehydrogenase
*: Significant from NSD group at $P < 0.05$
**: Significant from NSD + PIO group at $P < 0.05$
***: Significant from HFD group at $P < 0.05$.

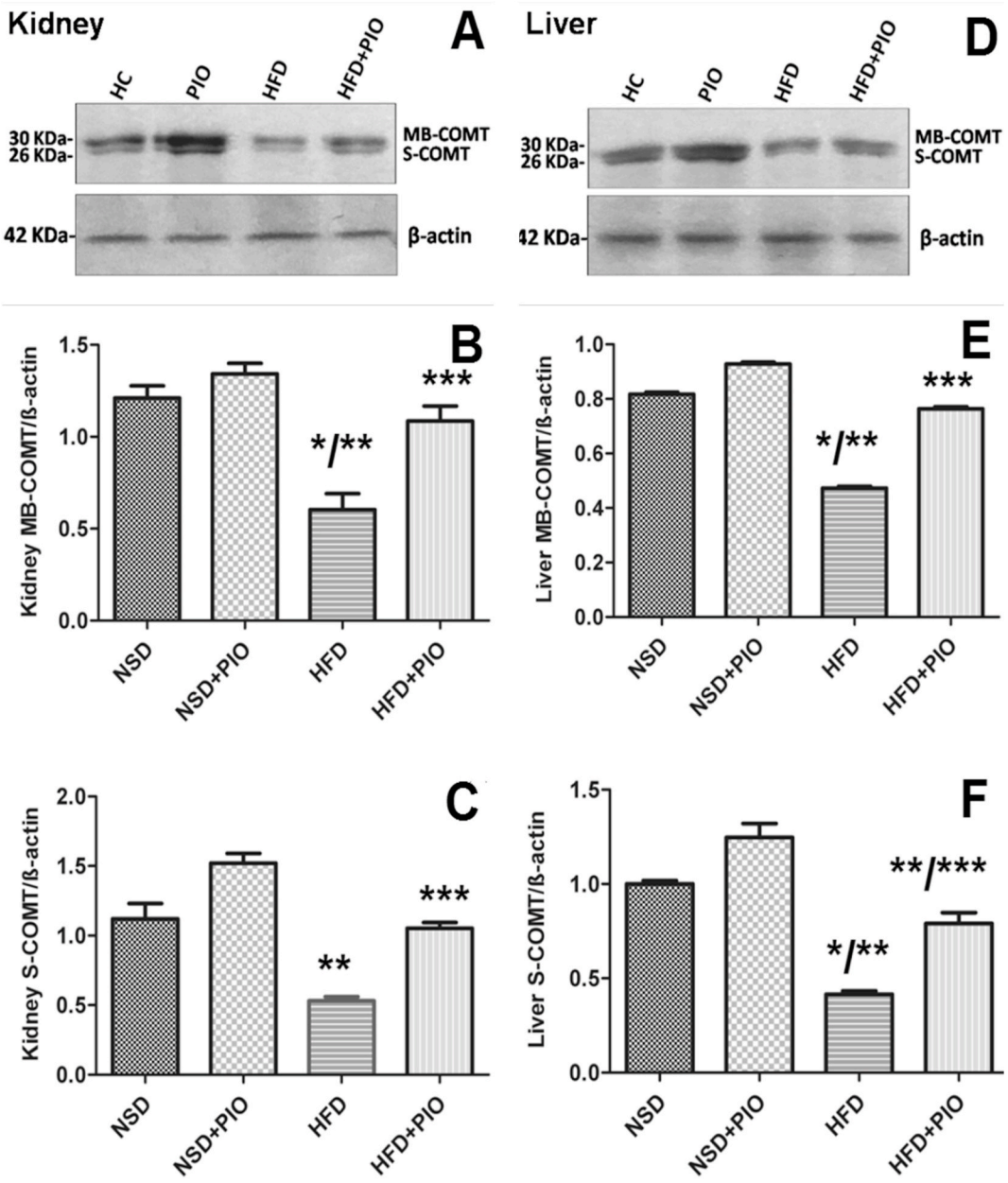

Fig 8. Western blot for COMT. (A), (B) and (C) representative western blot analyses of MB-COMT and S-COMT expressions in kidney tissue homogenates of randomly selected 3 rats from each group and (D),(E) and (F) representative western blot analyses of MB-COMT and S-COMT expressions in liver tissue homogenates of randomly selected 3 rats from each group. β-actin was used in parallel to internal control. The right panels represent the corresponding quantification of each blot measured by Image J software and expressed as the relative band density to β-actin. The levels of significance were accepted with $p < 0.05$ and all relevant results were graphically displayed as Mean ± S.D. MB-COMT: membrane-bound- catechol o-methyl transferase and S-COMT: soluble-catechol o-methyl transferase
*: Significant from NSD group at $P < 0.05$
**: Significant from NSD + PIO group at $P < 0.05$
***: Significant from HFD group at $P < 0.05$.

In light of this information, it is anticipated that insulin sensitizers such as PIO which enhances insulin signaling, including its transcriptional activities (Jiang et al., 2014) may be associated with increased COMT expression.

Furthermore, PIO has been reported to decrease TNF-α and adipokines expression, an effect that may increase COMT expression (Tchivileva et al., 2009). Collectively, these data explain how PIO enhances COMT expression and accordingly amelioration of hypertension associated with HFD-induced obesity. Our finding that PIO ameliorates HFD diet-induced hypertension is consistent with a previous study showing that PIO lowered BP in fructose-fed rats (Buchanan et al., 1995). The observed effect of PIO on COMT expression could explain the reduction of SBP in HFD-fed rats treated with PIO.

Consistent with the effect of HFD and/or PIO on COMT expression, our data revealed that changes in COMT expression were paralleled with circulating levels of catecholamines (EP and NE). The levels of catecholamines (EP and NE) were significantly increased in HFD-fed rats as compared to NSD-fed rats by 64% and 150% respectively, ($P < 0.05$). In addition, treatment with PIO significantly reduced EP and NE levels in HFD-fed rats as compared to NSD-fed rats by 35.39% and 43.61% respectively, ($P < 0.05$). These catecholamines are known substrates for COMT and play a critical role in regulating blood pressure (Bisogni et al.,

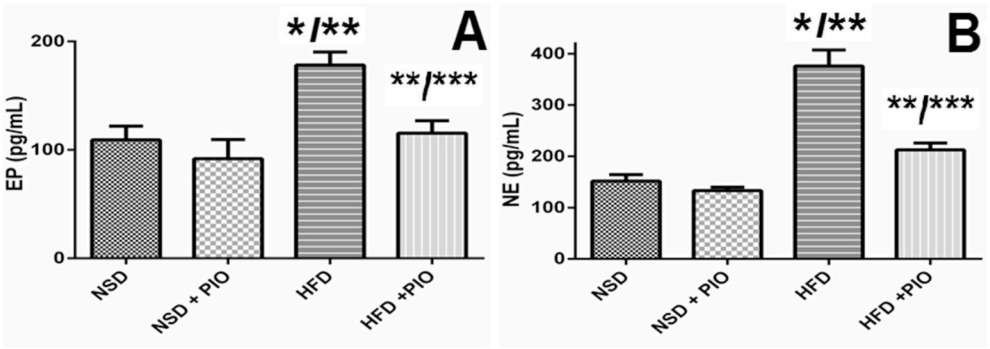

Fig 9. Epinephrine and norepinephrine levels. (A) effect of HFD and/or PIO on the plasma levels of epinephrine (EP) for NSD, NSD + PIO, HFD, HFD + PIO groups and (B) effect of HFD and/or PIO on the plasma levels of norepinephrine (NE) for NSD, NSD + PIO, HFD, HFD + PIO groups, data expressed as Mean ± S.D., n = 10. HFD: high fat diet, PIO: pioglitazone and NSD: normal standard diet.
*: Significant from NSD group at $P < 0.05$
**: Significant from NSD + PIO group at $P < 0.05$
***: Significant from HFD group at $P < 0.05$.

2016). Indeed, prior studies demonstrated that COMT expression was lower in hypertension (Ueki et al., 2017) and plasma levels of catecholamines were significantly higher in kidney tissues from diabetic hypertensive mice (Watson et al., 2019).

Pioglitazone is a well-known agonist for PPAR-γ which is widely expressed in various parts of the body including white adipose tissues, kidney, liver and vascular system which play roles in regulating blood pressure (Lamichane et al., 2018).

To the best of our knowledge, this is the first study to address the potential role of the PPAR-γ agonist PIO in regulating COMT expression and catecholamine levels in HFD-induced obesity in rats. However, it remains unknown whether other isoforms of PPAR receptors or other PPAR agonists can also modulate COMT expression and ameliorate HFD-induced metabolic changes and hypertension.

Nonetheless, there are some pitfalls in our study. For instance, the functional significance of the presence of IRE; AP-1, SRF, SREBP, and ChREBP in the COMT promoters should be thoroughly investigated using advanced molecular techniques such as reporter gene assay, chromatin immune precipitation, and site-directed mutagenesis. From this study we can only make conclusions regarding associations between variables and that causality one way or the other cannot be attributed.

Accordingly, it might be concluded that treatment with PIO reversed HFD-induced glucose intolerance and the associated metabolic defects with increasing the COMT expression with a subsequent reduction in plasma catecholamines levels.

5. Conclusion

COMT expression is down-regulated in HFD-fed rats. Reduced COMT expression may be causally associated with glucose intolerance, insulin resistance, reduced plasma catecholamines, and hypertension. Treatment with insulin sensitizer PIO is associated with increased COMT expression in HFD-fed rats, an effect which may result in enhancement of glucose tolerance and amelioration of catecholamine levels and hypertension. These results suggest that COMT is involved in glucose homeostasis and insulin may regulate the expression of COMT.

Ethical approval

Animal Care and Use Committee of Faculty of Medicine, Al-Azhar University, Cairo approved all our experiments. All procedures performed in studies involving animals were following the ethical standards of the institution or practice at which the studies were conducted.

CRediT authorship contribution statement

Maghawry Hegazy: Conceptualization, Software, Formal analysis, Writing - original draft, Writing - review & editing. **Mostafa El-Shafey:** Supervision. **Ahmed Ibrahim Abulsoud:** Investigation. **Bakheet E.M. Elsadek:** Methodology, Resources. **Adel I. Abd Elaziz:** Methodology. **Salama Abdou Salama:** Conceptualization, Validation, Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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