

Adenosine deaminase and xanthine oxidase inhibitory effect of valproic acid abates glucose dysregulation and liver triglyceride accumulation independent of corticosteroids in estrogen/progestin-treated female rats

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Running title: Inhibition of XO by VPA abates disrupted glucoregulation and elevated liver TG

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ABSTRACT

Elevated circulating uric acid has been postulated to play an important pathophysiological role in estrogen-progestin combined oral contraceptive (COC)-induced hypertension and endothelial dysfunction. We therefore also hypothesized that disruption of glucoregulation and liver triglyceride (TG) accumulation induced by COC use would be abated by valproic acid (VPA) treatment through suppression of adenosine deaminase (ADA) and xanthine oxidase (XO) activities. Female Wistar rats aged 9-10 weeks old were treated with a combination of estrogen-progestin COC steroids (1.0 µg ethinylestradiol and 5.0 µg levonorgestrel; *po*) with or without VPA (100.0 mg/kg; *po*) daily for 6 weeks. The result shows that the disrupted glucose regulation, elevated hepatic ADA activity, UA, plasma and hepatic XO activity, TG/HDL-cholesterol, total cholesterol, malondialdehyde, induced by COC treatment were attenuated by VPA treatment. However, VPA did not have any effect on plasma aldosterone, corticosterone, UA, ADA, circulating and hepatic free fatty acid. Our results demonstrate that suppression of plasma and hepatic XO activities, along with hepatic ADA activity and uric acid by VPA treatment protects against disrupted glucose regulation and increased liver TG by COC independent of elevated corticosteroids. The findings imply that VPA would provide protection against the development of cardiometabolic disorder via inhibition of ADA/XO/UA-mediated pathway.

Keywords: Cardiometabolic syndrome, Corticosteroids, Hepatic lipid accumulation, Hormonal contraceptive, Hypeuricaemia

Introduction

Insulin resistance (IR), defined as inhibition of insulin stimulation of several metabolic pathways including glucose transport, glycogen synthesis and antilipolysis, is of considerable clinical relevance because it is pathophysiologically linked to several serious medical problems including type 2 diabetes, hypertension, atherogenic dyslipidemia, abnormalities of fibrinolysis and non-alcoholic fatty liver disease (Bray 2004). IR is a crucial component of cardiometabolic syndrome, which is rapidly becoming one of the major public health challenges, signifying an enormous economic burden in men and women globally (Wang et al. 2010). Interestingly, women with IR develop clinically significant CVD not only readily or more frequently but also more severely than men with IR or diabetes. Hence, the disproportionate occurrence of CVD in women with IR (DeFronzo 2000; Lee et al. 2000; Park et al. 2010). Recent studies have demonstrated the important role of glucocorticoids (Fransson et al. 2013) and mineralocorticoids in the pathogenesis of IR and cardiometabolic risk factors associated with increased mortality (Sherajee et al. 2012). Also, the incidence and prevalence of type 2 diabetes is increasing rapidly worldwide, and is expected to affect 439 million adults by 2030 (Shaw et al. 2010; Park et al. 2013). To overcome the burden of this epidemic, measures for early identification of individuals with IR and appropriate intervention might reduce the incidence of diabetes and associated CVD. Elevated fasting circulating glucose, triglyceride, free-fatty acids (Bergman and Ader 2000; Beshara et al. 2016), 1-hour postload glucose (Abdul-Ghani et al. 2008) and lactate (Matsushita et al. 2013) levels have been shown to be independent risk factors for developing type 2 diabetes mellitus, even when individuals are within the normoglycemic range in various populations (Wilson et al. 2007; Tirosh et al. 2008).

101 The liver plays a central regulatory role in whole body glucose and lipid homeostasis, by uptake
102 of glucose in the postprandial state and conversion to glycogen and triglyceride (TG) and also by
103 production of glucose in the postabsorptive state by glycogenolysis and gluconeogenesis
104 (Wasserman 2009; Agius 2010). Defects in the mechanisms by which insulin regulates hepatic
105 glycogen metabolism disrupt blood glucose homeostasis and are highly associated with
106 metabolic disorders such as type 2 diabetes (Shulman 2000), non-alcoholic fatty liver disease
107 (Aroor et al. 2015) and glycogen storage disease (Roach et al. 2012). The TG content of the liver
108 has important physiological and metabolic functions. The liver takes up free-fatty acids (FFAs)
109 from the circulation, re-esterify them into TGs, and then secrete them back into the circulation as
110 very low-density lipoprotein triglycerides (Postic and Girard 2008; Yki-Järvinen 2010).
111 Excessive accumulation of TG in the liver is a characteristic of several metabolic diseases
112 including IR and non-alcoholic fatty liver disease (NAFLD). NAFLD is the most common
113 chronic liver disease in western countries, affecting 17-46% of adults (Vernon et al. 2011). The
114 presence of the hepatic IR with increased liver TG content has been reported to accelerate the
115 development of type 2 diabetes, hypertension and sequelae atherothrombotic cardiovascular
116 disease (CVD) (Després et al. 2008; Sanal 2008). When accumulation of liver TG is not
117 contained, it might promote progression to chronic liver diseases such as hepatic steatosis,
118 cirrhosis and hepatic carcinoma. Hence, in recent years, there has been great interest in
119 identifying factors that affect liver TG content as a possible prelude to the development of
120 preventive and/or therapeutic strategies to reduce liver TG accumulation (Sanal 2008).

121
122 Diet interferes with whole-body metabolism and its regulation through actions on hormones,
123 transcription factors, and lipid metabolic pathways are considered to play a central role in

124 NAFLD. Over nutrition or inappropriate diet are considered to lead to chronically increased
125 glucose, insulin, and FFAs concentrations in the blood. Both excessive carbohydrate intake and
126 excessive fat intake could play a role in increasing blood glucose, FFAs, and insulin
127 concentrations, independently or together and contribute to excessive accumulation of neutral
128 lipids such as TG and ceramides in the liver. These dietary conditions contribute to resistance to
129 insulin-stimulated glucose uptake at the level of the adipose tissue and skeletal muscle as well as
130 resistance to the insulin mediated suppression of TG hydrolysis in adipose tissue and liver
131 (Bergman and Ader 2000; Beshara et al. 2016). The uptake of glucose in the liver is not insulin
132 dependent, and hyperglycemia leads to increased glucose uptake by the liver. Insulin mediated
133 stimulation of *de novo* lipogenesis leads to an increased conversion of glucose to fatty acids
134 resulting in fatty liver (Bergman and Ader 2000; Beshara et al. 2016). Regardless of the notion
135 that weight loss, dietary and lifestyle changes are suggested as primary treatment for fatty liver,
136 no specific plan exists pertaining to diet (Finelli and Tarantino 2012).

137
138
139 Uric acid, a by-product of oxidation of purines that is filtered by glomeruli in the kidney and
140 reabsorbed by the proximal tubule, may promote IR by inhibiting endothelial cell function
141 (Tsouli et al. 2006; Feig et al. 2008). Indeed, some studies found that higher plasma uric acid is
142 associated with incident diabetes, independent of other known risk factors (Bhole et al. 2010;
143 Juraschek et al. 2014).

144
145 Adenosine deaminase (ADA) is an enzyme involved in purine metabolism. It converts adenosine
146 into inosine through an irreversible deamination reaction (Brady 1942). It is present in virtually
147 all the mammalian cells and its primary function in human is the development and maintenance

of the immune system. However, studies have reported highest ADA activity in the liver, skeletal muscle, fatty tissues and lymphoid (Niedzwicki and Abernethy 1991). Likewise, an increase in ADA activity in type 2 diabetic patients has been reported (Kurtul et al. 2004). Xanthine oxidase (XO) catalyzed the last two steps of purine metabolism, modulating hypoxanthine to xanthine, and then uric acid (Mandal and Mount 2015). Expression of XO is highest in the liver and intestine (Hanison 2002). Enhanced XO activity might be responsible for the pro-oxidant property of uric acid and its negative impact on cardiometabolic disorders (Kelley et al. 2010). XO uses molecular oxygen as an electron acceptor, thus generating superoxide anion and other reactive oxygen species (ROS) as by-product and this is responsible for the pathological conditions implicated in hyperuricemia (Kelley et al. 2010). Moreover, accumulated ROS is a key player in IR progression and establishment of NAFLD (Utzschneider and Kahn 2006; Gusdon et al. 2014).

Oral estrogen-progestin combined oral contraceptive (COC) has been shown to be associated with glucose deregulation and increased circulating triglyceride levels in humans (Krauss and Burkman 1992) and female rats (Michael and Olatunji 2017). We have also recently reported that treating female rats with oral estrogen/progestin steroids induces hypertension that is accompanied by elevated circulating and renal uric acid (Olatunji et al. 2016). Previously studies have demonstrated that valproic acid (VPA) has beneficial effects, such as, mineralocorticoid receptor antagonism, antihypertensive, antifibrotic, anti-inflammatory, antihypertrophic and improved glucose regulatory properties via the histone deacetylase (HDAC) inhibitory action in rats (Zhao et al. 2012; Khan and Jena 2016; Amirzargar et al. 2017).

171 However, the inhibitory effect on ADA/XO-mediated pathway has not been explored for its
172 plausible protective activity against glucose dysregulation and liver disorders. Hence, the present
173 study was designed to test the hypothesis that VPA improves glucose dysregulation and hepatic
174 TG accumulation through the involvement of ADA/XO-mediated pathway. We also sought to
175 determine whether the role of ADA and XO activities would be glucocorticoid-or
176 mineralocorticoid-dependent.

177
178 **Materials and methods**

179 **Animals**

180 Animal care and experimental protocols were executed according to the approved guidelines set
181 by the University of Ilorin Ethical Review Committee, which is in agreement with the Guide for
182 the Care and Use of Laboratory Animals' prepared by the National Institute of Health and
183 Canadian Council on Animal Care and every effort was made to minimize both the number of
184 animals used and their suffering. Eight to ten weeks old female Wistar rats, weighing 120 to
185 125g were used for the study. Rats had unrestricted access to standard rat chow and tap water.
186 After 1 week of acclimatization, the animals were randomly assigned to four groups (n=6/group).
187 Rats were maintained under standard environmental conditions of temperature, relative humidity
188 and dark/light cycle. Body weight, food and water intakes were monitored weekly.

189
190 **Treatment**

191 Control group received distilled water (vehicle; *p.o.*) daily. COC-treated group received (*p.o.*) a
192 combination of 1.0 mg ethinylestradiol and 5.0 mg levonorgestrel (Wyeth–Ayerst, Inc.,
193 Montreal, Canada) daily. VPA-treated group received (*p.o.*) 100 mg valproic acid (Sigma-Aldrich,

194 St. Louis, MO) daily. COC+VPA-treated group received (*p.o.*) a combination of COC steroids
195 plus VPA daily. The treatment lasted for 8 weeks.

196 **Oral glucose tolerance test (OGTT) and insulin resistance (IR)**

197 Glucose challenge test was performed, one week, prior to the sacrifice of the rats. The rats had
198 12-h overnight fast. Glucose (2g/kg *b.w.*) was given (*p.o.*). Blood was obtained from the tail
199 before glucose load and then sequentially after 30, 60, 90 and 120 min. Terminal fasting blood
200 glucose (FBG) was determined after the rats had 12-h overnight fast on the day of sacrifice.
201 Blood glucose levels were determined with a glucometer (ONETOUCH®-LifeScan, Inc.,
202 Milpitas, CA, USA). Glucose tolerance was expressed as a function of the area under the OGTT
203 curve (AUC) as previously described (Olatunji et al. 2016). IR and β -cell function were
204 determined using the homeostasis model assessment for IR (HOMA-IR= fasting glucose
205 (mmol/l)*fasting insulin (μ U/l)/22.5) and β -cell function (HOMA- β = (20*fasting insulin)/
206 (fasting glucose-3.5) respectively while quantitative insulin sensitivity check index; QUICKI =
207 $1/[\text{Log (fasting insulin)} + \text{Log (fasting glucose)}]$. Triglyceride-glucose index on the hand is
208 expressed as TyG = $\text{Ln (TG (mg/dl)} \times \text{FPG (mg/dl)/2)}$ (Simental-Mendía et al. 2008).

209 **Sample preparation**

210 At the end of treatment, the rats were anesthetized with pentobarbital sodium (50 mg/kg, *ip*).
211 Blood sample was collected by cardiac puncture into heparinized bottle and was centrifuged at
212 3000 rpm for 5 min. Plasma was stored frozen until needed for biochemical assay. The liver was
213 excised, cleared of adhering connective tissues, blotted and weighed immediately. After
214 weighing, 100 mg of liver tissue was carefully removed and homogenized with a glass
215 homogenizer in cold phosphate-buffered solution.

216 **Visceral adiposity**

Following blood sample collection, all intra-abdominal fat depots including mesenteric, urogenital and retroperitoneal were dissected out and weighed immediately with an electronic weighing scale after dissection to avoid evaporative weight loss.

Biochemical assays

Plasma insulin was determined using ELISA kit from Ray Biotech, Inc. (Georgia, USA). Uric acid level, ADA and XO activities was measured by standardized enzymatic colorimetric method using assay kit obtained from Fortress diagnostics (Antrim, UK). Plasma and liver nitric oxide was measured by quantification of nitrite using Griess Reagent and malondialdehyde (MDA), as marker of oxidative stress was measured by standardized enzymatic colorimetric methods using assay kits from Oxford Biomedical Research, Inc. (Oxford, MI, USA). Plasma and liver free fatty acids (FFA), lactate, aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), fasting triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) were measured by standardized enzymatic colorimetric methods using reagents obtained from Randox Laboratory Ltd. (Antrim, UK). TG/HDL-C ratio was estimated as index of atherogenic dyslipidemia.

Statistical analysis

All data were expressed as means $\pm$ SEM. Statistical group analysis was performed with SPSS statistical software (Chicago, IL). One way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups. Bonferroni's test was used to identify the significance of pair wise comparison of mean values among the groups. Statistically significant differences were accepted at $p < 0.05$.

Results

VPA did not affect body weight and liver weight but reduced visceral adiposity in COC exposure.

COC treatment led to decreased body weight change and increased visceral adiposity. In addition, liver weight was not affected by the treatment when compared with control (Figure 1). Food intake was increased in VPA-treated rats whereas COC treatment led to increased water intake when compared with control (Table 1). However, VPA treatment did not affect body weight and liver weight but led to decreased visceral adiposity in COC-treated rats when compared to COC-treated group.

VPA treatment resolves COC-induced glucose deregulation.

COC treatment led to significant impairment in glucose regulation and insulin sensitivity indicated by elevated fasting glycaemia, insulinemia, 1-hr post load glycaemia, AUC of OGTT and HOMA-IR. This impairment is accompanied by evidence of pancreatic β -cell dysfunction (Figure 2; Table 1). However, the glucose deregulation observed during COC treatment was attenuated by VPA treatment including improvement in pancreatic β -cell dysfunction when compared with COC-treated group (Figure 2; Table 1).

VPA treatment attenuates COC-induced dyslipidemia and hepatic lipid accumulation

COC treatment led to elevated plasma and hepatic FFA, TG, and TG/HDL-C when compared with control (Figure 3). COC treatment also led to increased plasma TC but hepatic TC was not affected. Plasma HDL-C was decreased across the experimental groups whereas VPA treatment led to reduced hepatic HDL-C when compared with control (Table 1). However, VPA treatment significantly attenuated plasma and hepatic FFA, TG and TG/HDL-C and plasma TC and unaltered hepatic TC level thereby resolving the lipotoxicity observed in COC-treated rats when compared with COC-treated group.

VPA treatment attenuates hepatic LDH levels in COC-treated rats

COC treatment led to elevated plasma and hepatic LDH (Figure 4 b & e), a marker of oxidative stress and inflammation. But this treatment did not affect hepatic oxidative capacity indicated by stable plasma and hepatic lactate and hepatic glycogen levels when compared with control. However, VPA treatment led to decreased plasma and hepatic LDH whereas plasma and hepatic

268 lactate were not affected in COC-treated rats when compared with COC-treated group (Figure
269 4e).

270 **VPA treatment did not affect the liver injury enzyme markers in COC-treated rats**

271 Liver injury enzymes such as AST, ALT and GGT were not affected by the treatment across the
272 experimental groups (Figure 5).

273 **VPA treatment attenuated COC-induced lipid peroxidation and nitric oxide suppression**

274 COC treatment led to increase in plasma and hepatic lipid peroxidation maker, MDA and NO
275 suppression when compared with control group. However, VPA treatment led to decreased
276 plasma and hepatic MDA levels and increased NO levels when compared with COC-treated
277 group (Figure 6a-d).

278 **VPA treatment attenuated COC-induced elevated ADA/XO/UA activities**

279 COC treatment led to increased plasma XO activity and UA level when compared with control
280 whereas VPA treatment attenuated plasma XO activity and decreased plasma UA level when
281 compared with COC-treated group. On the other hand, plasma ADA activity was not affected by
282 the treatment when compared with control (Figure 7a-c). Also, COC-treatment led to elevated
283 hepatic ADA, XO activities and UA level and this effect was attenuated by VPA treatment in
284 COC-treated rats when compared with COC-treated group (Figure 7d-f).

285 **VPA treatment did not affect the G6PD-dependent glutathione antioxidant capacity in** 286 **COC-treated rats**

287 Plasma and hepatic G6PD activity, NADPH, GSH/GSSG ratio were comparable among the
288 experimental groups (Figure 8a-e).

289 **VPA treatment increases circulating corticosteroids in COC-treated rats**

290 Circulating corticosteroids (corticosterone and aldosterone) were increased in all the treatment
291 groups when compared with control (Figure 9a&b).

292

DISCUSSION

The current study demonstrates that COC-induced glucose deregulation and insulin insensitivity is accompanied by elevated visceral adiposity, plasma and hepatic FFA, TG accumulation, TG/HDL-C ratio, MDA, LDH and plasma corticosteroids. The study further extend that the COC-induced dysmetabolic effect is linked to increased activity of ADA/XO/UA-dependent pathway. However, COC-induced glucose deregulation and hepatic TG accumulation is not accompanied by altered body weight gain, lactate production, liver injury enzymes and plasma and hepatic G6PD-dependent antioxidant capacity. The results from the present study also provide evidence that VPA suppresses circulating XO activity and prevents hepatic glucolipotoxicity via the attenuation of ADA/XO/UA pathway (Figure 10 & 11).

Estrogen-progestin therapy has been shown to cause cardiometabolic syndrome that is characterized by IR, glucose deregulation and endothelial dysfunction together with activation of hypothalamus-pituitary-adrenal axis (HPA) causing a rise in circulating corticosteroid (Olatunji et al. 2017). The COC-induced glucose deregulation shown in this study is characterized by elevated fasting glycaemia, IR or impaired insulin sensitivity indicated by elevated HOMA-IR, AUC of OGTT and 1-h postload glycaemia (Figure 2). Increase body weight is a ubiquitous risk factor for metabolic diseases of which glucose deregulation is the hallmark. COC-induced glucose deregulation in the present study is however not associated with increased body weight gain, indicating that metabolic disturbances may be independent of changes in body weight. Insulin insensitivity or IR is a major component of cardiometabolic syndrome linking the various metabolic risk factors and it is indicative of T2DM and NAFLD (Williams et al. 2013). This study also shows that COC induces elevated plasma and hepatic FFA (Figure 3) and this is in consonance with previous study (Lee and Goldberg 2008). Circulating FFAs are usually sourced

316 from lipolytic process in adipocytes. They enter the liver to participate in divergent processes
317 depending on the systemic metabolic status except there is a local hepatic derangement which
318 may exaggerate fat accumulation. Elevated plasma FFA on its own can negatively impact
319 glucoregulation by activating destructive processes in the pancreatic β -cell. Also, FFA when
320 used up in the liver can promote oxidative stress through lipid peroxidation by production of
321 MDA, leading to cellular damage and inflammation. This is evident by the result of the present
322 study which shows impairment in pancreatic β -cell function (Figure 2) and elevated MDA
323 (Figure 7). Cellular oxidative incapacity, another known indicator of glucose deregulation, IR
324 and T2DM has been shown to be indicated by elevated resting plasma lactate (Crawford et al.
325 2010) whereas lactate dehydrogenase, the enzyme that catalyzes conversion of pyruvate to
326 lactate releasing NAD^+ is also an indicator of oxidative stress (Khan et al. 2013) . In the present
327 study, COC-induced glucose deregulation was accompanied by elevated hepatic LDH but neither
328 plasma nor hepatic lactate was elevated, which shows that oxidative insufficiency is not involved
329 in COC deleterious metabolic effects and hepatic LDH increase along with maintained lactate
330 concentration might support pyruvate production for oxidative mitochondrial process.

331 Interestingly, COC treatment in the present study induced hepatic TG accumulation which is
332 accompanied by elevated hepatic FFA compared with control group. This finding supports the
333 fact that there is hepatic de novo lipogenesis owing to increased FFA hepatic delivery. Studies
334 have shown that IR promotes hepatic de novo lipogenesis, which in turn augment hepatic lipid
335 accumulation (Bergman and Ader 2000; Postic and Girard 2008; Yki-Järvinen 2010) and
336 predicts NAFLD. The present study further reiterates that COC-induced IR is accompanied by
337 dyslipidemia (increased plasma and hepatic FFA, hepatic TG/HDL-C and resultant elevated
338 hepatic TG), elevated MDA and reduced NO, without changes in liver injury enzyme markers

(Figure 5). Hepatic lipid content is regulated by the interplay between the delivery of lipids to the liver from extra hepatic source (adipocytes) and the hepatic uptake, synthesis, oxidation and secretion of lipids. Alterations in the equilibrium of one or more of these processes can promote hepatic steatosis. Taken together, despite the marked COC-induced hepatic lipotoxicity found in this study, liver injury enzymes were still not increased in this study. This is corroborated in a previous report which showed that a liver can remain fatty without disturbing liver function (McCullough 2004). Likewise, another study also documented that in NAFLD, liver function test can be normal and the degree of abnormalities does not correlate with the degree of steatosis (Noguchi et al. 1995).

VPA, a short branched chain fatty acid, has been widely explored for its protective role in neurodegenerative diseases. It also possesses HDAC inhibitory activity and recently, accumulated evidences show the effect of VPA on glucoregulation and its protective role in CMD and CVD (Zhao et al. 2012; Khan and Jena 2016; Olatunji et al. 2016; Amirzargar et al. 2017). Despite the usual controversies trailing the repositioning of VPA, results from the present study indicates that VPA improved glucose regulation in COC-treated female rats (Figure 2a-f) in consonance with reports from our laboratory (Olatunji et al. 2016) and earlier clinical (Ebbesen et al. 2000) and animal (Khan and Jena 2016; Akindele et al. 2015) studies, implying that VPA may impact beneficial effect on glucose regulation during COC use.

More so, our finding that COC-induced IR is associated with elevated plasma and hepatic FFA is line with report that dysregulation of fat metabolism play a foremost contributory role in development of IR (Bergman and Ader 2000; DeFronzo 2000). Furthermore, insulin-resistant individuals manifest an elevated level of plasma FFA concentration and increased fat content in insulin-responsive tissues, skeletal muscle, and liver (Després et al. 2008; Postic and Girard

2008; Tirosh et al. 2008; Yki-Järvinen 2010; Beshara et al. 2016). The main mechanism underlying the linkage between elevated plasma FFA and IR has not been clearly elucidated. However, FFA-oxidative stress-IR pathway has been proposed as a possible mechanism that FFAs increases free radicals and ROS via increase in NADPH oxidase, which may deregulate proinflammatory cytokines (Han et al. 2012). The present study also corroborates suppression of oxidative stress as a contributory mechanism by which VPA ameliorates COC-induced dysmetabolism through reduction of MDA (Figure 7), a product of lipid peroxidation and LDH. VPA-mediated enhanced antioxidant and anti-inflammatory activities have been confirmed in previous reports to protect the multiple organ damage in numerous pathological conditions (Akindele et al. 2015; Amirzargar et al. 2017). It is noteworthy that VPA treatment suppressed plasma and hepatic FFA in COC-induced insulin resistant rats which is in line with the report that reduction of plasma FFA concentration by acipimox treatment led to improvement of insulin-mediated glucose disposal in obese non-diabetic and in T2DM individuals (Santomauro et al. 1999) and study by Akindele and coworkers (Akindele et al. 2015). VPA-mediated suppression of plasma and hepatic FFA is associated with unaltered lactate production, an index of mitochondrial function in COC-treated female rats. This is in agreement with report by Daniele and colleagues where chronic reduction of plasma FFA improves mitochondrial function and whole-body insulin sensitivity in obese and type 2 diabetic individuals (Daniele et al. 2014).

Hepatic lipogenesis has been linked to the activation of the mammalian target of rapamycin (mTOR) signaling pathway. mTOR signaling is responsible for regulating lipid biosynthesis, reducing insulin signaling and promoting hepatic IR. mTOR signaling is also high in the liver of obese rodents. mTOR signaling therefore seemed to be an interesting pathway for hepatic TG accumulation or steatosis and clearly has an essential role in regulating cell growth and

metabolism such as lipogenesis, protein translation, and autophagy. Hence, inhibition of mTOR signaling pathway is promising as it had been suggested to play a protective role in coronary artery disease and NAFLD (Sarbasov et al. 2006; Wang et al. 2010; Tarantino and Capone 2013). Contrary to our finding that VPA ameliorates hepatic TG accumulation and glucose dysregulation, report exist that VPA promotes hypertrophy and inhibits apoptosis by activating mTOR signaling pathway (Gurpur et al. 2009). Furthermore a metabolomics profiling of steatosis in HepaRG® cells revealed a contrasting report that VPA causes upregulation of neutral lipids such as TG and ceramides in combination with a downregulation of carnitine through inhibition of betaoxidation thereby increasing free fatty acid pool (CuyKx et al. 2018).

The finding that COC treatment activates HPA resulting in elevated corticosteroids which might be responsible for glucose deregulation via genomic and non-genomic pathways causing insulin insensitivity, depleted NO, endothelial dysfunction and T2DM (Olatunji et al. 2017) is in consonance with the report that increased reactive oxygen species (ROS) generation is associated with chronic corticosteroid-mediated IR (Sherajee et al. 2012; Fransson et al. 2013; Suwanjang et al. 2013). The findings of this study that COC-induced fatty liver and visceral adiposity is accompanied by elevated circulating corticosteroids (Fig. 9) is noteworthy and in line with previous reports in rodents and human where hypercortisolism was implicated in the pathogenesis of metabolic disorders characterized by elevated visceral adiposity and NAFLD (Cooper and Stewart 2009; Tarantino and Finelli 2013). Progressively elevated visceral adiposity has been associated with increased production of cortisol by the action of 11beta-hydroxysteroid dehydrogenase type 1(11 β -HSD1) causing hepatic hypercortisolism (Cooper and Stewart 2009).

Despite, the ameliorative effects of VPA on COC-induced dysmetabolic impacts in the present study, we found that circulating corticosteroids remain elevated, indicating that the effects of

VPA is independent of circulating corticosteroids. Therefore, our findings suggests that VPA can be well positioned as a corrective measure with circulating corticosteroid sparing property in metabolic disorders involving ROS generation, inflammatory response and elevated FFA.

The present study is the first to elucidate the effect of COC on G6PD-mediated cellular antioxidant capacity or the glutathione antioxidant system and we showed that hepatic G6PD, GSH/GSSG and NADPH were not affected by COC-treatment (Figure 8a-e). G6PD is the rate limiting enzyme in the oxidative pentose phosphate pathway that produces NADPH for lipid synthesis and GSH production, replenishing the glutathione antioxidant system. Studies have shown that alterations in the level of G6PD (deficiency or overexpression) can induce deleterious effects (Cappellini and Fiorelli 2008). Strikingly, regardless of marked dysmetabolism, COC treatment in this study maintained hepatic G6PD activity, NADPH levels and its associated pathways as well as the GSH/GSSG redox status. This outcome shows that the COC-induced hepatic lipogenesis, elevated TG and depressed NO are independent of G6PD-derived NADPH. Also, the LDH/lactate outcome discussed above alongside our novel finding that COC treatment did not affect glycogen level or glycogen synthase (GS) activity (Figure 4c&f), confirmed that COC treatment sustained mitochondrial function since lactate and glycogen levels were maintained. Taken together, COC treatment results in dysmetabolism derived from neither defective cellular antioxidant status nor oxidative insufficiency. Oxidative insufficiency has been shown to be a selective or partial predictor of IR (Han et al. 2012). Interestingly, the results of the present study therefore provide evidence that VPA may be therapeutic in metabolic derangements where mitochondrial function is preserved.

431 ADA activity is involved in enzymatic inflammation signaling and consequent decreased
432 oxidative capacity probably through the subsequent reactions it fuels. Type 2 diabetes mellitus
433 patients have been shown to have elevated ADA activity with hepatic dysfunction (Niedzwicki
434 and Abernethy 1991, Kurtul et al. 2004) and ADA is also suggested to modulate insulin
435 bioactivity. In chronic injury, adenosine is detrimental due to the promotion of excessive tissue
436 repair and fibrotic responses. The present study revealed that COC treatment led to elevated
437 plasma and hepatic ADA activity suggesting that ADA might partly contribute to COC-induced
438 dysmetabolism by promoting ROS production and lipogenesis through provision of substrate for
439 XO to produce UA. Contrary to our finding of COC-induced elevated plasma and hepatic ADA
440 activity, a study in LDLR/ApoE mice with developing atherosclerosis resulted in lower activity
441 of ADA in liver (Rybakowska et al. 2018).

442 XO catalyzes the oxidation of hypoxanthine to xanthine and furthers the oxidation of xanthine to
443 UA with the production of superoxide radicals and hydrogen peroxide (Kelley et al. 2010). In the
444 liver, Kupffer cells and endothelial cells, as well as hepatocytes, contain xanthine dehydrogenase
445 that is readily converted into xanthine oxidase (Saksela et al. 1991) especially in hypoxic
446 conditions. COC treatment is shown in this study to elevate both plasma and hepatic XO activity
447 revealing that XO may be involved in COC derived derangements. In addition, plasma and
448 hepatic uric acid in the present study were non-surprisingly elevated being the end point of
449 ADA/XO/UA pathway (Figure 6) and has also been linked to IR, impaired NO bioavailability
450 and fructose induced fatty liver (Nakagawa et al. 2006). Taken together, COC-induced
451 dysmetabolism in this study may therefore be through ADA/XO/UA-dependent pathway.
452 Interestingly, VPA treatment ameliorated COC-induced glucose deregulation and hepatic TG
453 accumulation through suppression of plasma XO and hepatic ADA/XO/UA activities. Therefore,

it is noteworthy that VPA treatment prevented the development of fatty liver in COC-treated female rat through ADA/XO/UA-dependent pathway.

VPA is a known human teratogen and exposure in pregnancy is associated with approximately three-fold increase in the rate of major anomalies, such as spinal bifida, cardiac, craniofacial, skeletal and limb defects (Ornoy 2009). In addition, VPA due to its structural resemblance with fatty acids, it competes with the endogenous lipid metabolism, provoking a vulnerability to induce NAFLD or steatosis (CuyKx et al. 2018).

In conclusion, suppression of plasma and hepatic XO activities, along with hepatic ADA activity and UA by VPA treatment protects against COC-induced glucose deregulation and increased hepatic TG independent of elevated corticosteroids (Figure 10 & 11). The findings imply that VPA treatment would provide protection against the development of cardiometabolic disorder via inhibition of ADA/XO/UA-mediated pathway.

Disclosure statement

The authors have no conflicts of interest to declare.

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Table 1: Effect of valproic acid treatment on body weight, food and water intake, fasting blood glucose and insulin in COC-treated rats

	CTR	VPA	COC	COC+VPA
Final body weight (g)	159.0 ± 14.4	151.8 ± 7.4	138.9 ± 9.4*	149.8 ± 17.0 [#]
Food intake (g/kg <i>bw</i>)	65.2 ± 6.0	85.3 ± 11.4*	65.5 ± 11.8	58.0 ± 4.8
Water intake (ml/kg <i>bw</i>)	103.4 ± 3.2	110.9 ± 5.4	86.5 ± 12.6*	103.6 ± 6.0 [#]
FBG (mmol/l)	3.7 ± 0.4	3.8 ± 0.8	4.1 ± 0.6	4.0 ± 0.2
Insulin (μU/l)	15.8 ± 2.4	16.0 ± 6.4	50.4 ± 12.4*	14.7 ± 1.8 [#]
Plasma HDL (mg/dl)	48.9 ± 2.2	28.8 ± 2.0*	15.1 ± 3.2*	25.1 ± 3.6*
Liver HDL (mg/100mg tissue)	83.4 ± 7.4	64.5 ± 3.4*	82.7 ± 2.7	88.9 ± 2.3

Data are expressed as mean ± SD of 6 rats per group. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test (*p<0.05 vs CTR; [#]p<0.05 vs COC).

FIGURE LEGENDS

Figure 1. Effect of valproic acid treatment on body weight (A), liver weight (B) and visceral adiposity (C) in COC-treated rats. COC treatment led to decrease body weight gain whereas COC+VPA treatment did not affect body weight gain. Liver weight was comparable in all experimental groups. Visceral adiposity was increased by COC treatment compared with control group. However, there was a significant decrease in visceral adiposity by COC+VPA treatment compared with COC-treated group. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 6 rats per group (* p <0.05 vs CTR; # p <0.05 vs COC).

Figure 2. Effect of valproic acid treatment on oral glucose tolerance test (OGTT; A), area under curve (AUC) of OGTT (B), HOMA-IR (C), HOMA- β (D), TyG (E) and QUICKI (F) in COC-treated rats. COC treatment led to increased 1-hr postload glucose, AUC, HOMA-IR, HOMA- β and TyG values respectively compared with control. The increase in 1-hr postload glucose, AUC, HOMA-IR, HOMA- β and TyG were attenuated in COC+VPA-treated rats compared to COC-treated rats. On the other hand, QUICKI was decreased by COC treatment but was increased by COC+VPA treatment. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 6 rats per group (* p <0.05 vs CTR; # p <0.05 vs COC).

Figure 3. Effect of valproic acid treatment on plasma and liver FFA, TG, TC and TG/HDL-C in COC-treated rats. Plasma and liver TG, TC, TG/HDL-C ratio and FFA were increased by COC treatment. However, COC+VPA treatment attenuated plasma and liver TG, TC, TG/HDL-C and FFA levels compared with COC-treated rats. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 6 rats per group (* p <0.05 vs CTR; # p <0.05 vs COC).

Figure 4. Effect of valproic acid treatment on plasma and liver lactate, lactate dehydrogenase (LDH), liver glycogen and glycogen synthase in COC-treated rats. Plasma and hepatic lactate, hepatic glycogen and glycogen synthase were not altered by the treatment. However, COC treatment led to increased plasma and liver LDH activity which was attenuated by COC+VPA treatment. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 6 rats per group (* p <0.05 vs CTR; # p <0.05 vs COC).

Figure 5. Effect of valproic acid treatment on liver aspartate transaminase (AST; A), alkaline phosphatase (ALP; B) and γ -glutamyl transferase (GGT; C) activities in COC-treated rats. Liver AST, ALP and GGT activities were not affected by the treatment compared with control. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 6 rats per group.

Figure 6. Effect of valproic acid treatment on plasma and liver malondialdehyde (MDA; A) and nitric oxide (NO; B) levels in COC-treated rats. COC treatment led to increased plasma and liver MDA levels whereas plasma and liver NO level was decreased by COC treatment compared with control. However, COC+VPA treatment led to decreased plasma and liver MDA whereas plasma and liver NO levels was increased by COC+VPA treatment compared with COC-treated rats.

775 Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are
776 expressed as mean $\pm$ SD of 6 rats per group (* p <0.05 vs CTR; # p <0.05 vs COC).

777 **Figure7.** Effect of valproic acid treatment on plasma and liver uric acid level, xanthine oxidase
778 (XO) and adenosine deaminase (ADA) activities in COC-treated rats. COC treatment led to
779 increases in plasma and liver uric acid level and XO. Plasma ADA activity was not affected by
780 the treatment compared control. However, COC+VPA treatment led to decreases in plasma and
781 liver uric acid, XO and liver ADA activities compared with COC treatment. Data were analyzed
782 by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD
783 of 6 rats per group (* p <0.05 vs CTR; # p <0.05 vs COC).

784 **Figure 8.** Effect of valproic acid treatment on plasma and liver glucose-6-phosphate
785 dehydrogenase (G6PD), reduced glutathione (GSH)/oxidized glutathione (GSSG) and liver
786 nicotinamide adenine dinucleotide phosphate (NADPH) activities in COC-treated rats. Plasma
787 and liver G6PD, GSH/GSSG and liver NADPH activities were comparable across the
788 experimental groups compared with control group. Data were analyzed by one-way ANOVA
789 followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 6 rats per group.

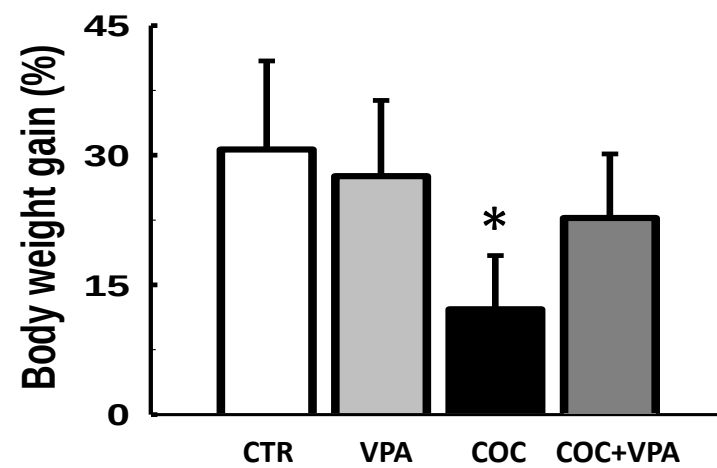
790 **Figure 9.** Effect of valproic acid treatment on circulating corticosteroids (corticosterone and
791 aldosterone) in COC-treated rats. Circulating corticosteroids (corticosterone and aldosterone)
792 were increased in all the treatment groups when compared with control group. Data were
793 analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as
794 mean $\pm$ SD of 6 rats per group. (* p <0.05 vs CTR; # p <0.05 vs COC).

795 **Figure 10.** Schematic diagram of the possible intra-hepatic mechanism by which VPA improves
796 hepatic TG accumulation (fatty liver) in COC-induced insulin resistant female rats. ADA;
797 adenosine deaminase, COC; combined oral contraceptive, FFA; free fatty acid, IR; insulin
798 resistance, TG; triglyceride, UA; uric acid, XO; xanthine oxidase, VPA; valproic acid.

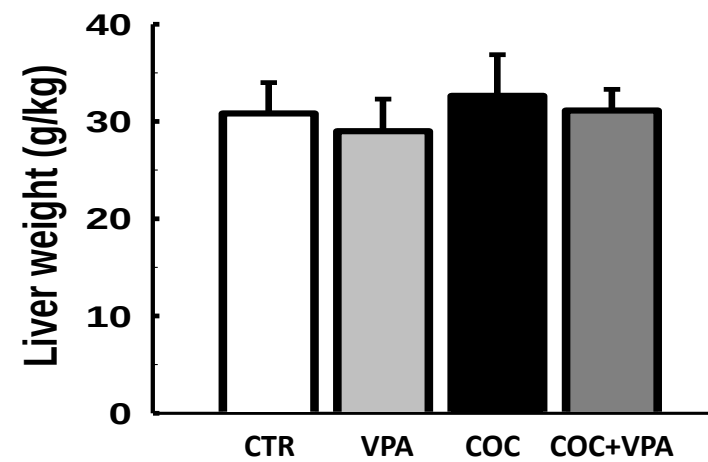
799 **Figure 11.** Schematic diagram of the pathways involved in COC-induced impaired
800 glucoregulation and hepatic TG accumulation. ADA; adenosine deaminase, COC; combined oral
801 contraceptive, CMD; cardiometabolic disorders, FFA; free fatty acid, IR; insulin resistance, TG;
802 triglyceride, T2DM; type 2 diabetes mellitus, UA; uric acid, XO; xanthine oxidase, VPA;
803 valproic acid.

Fig. 1

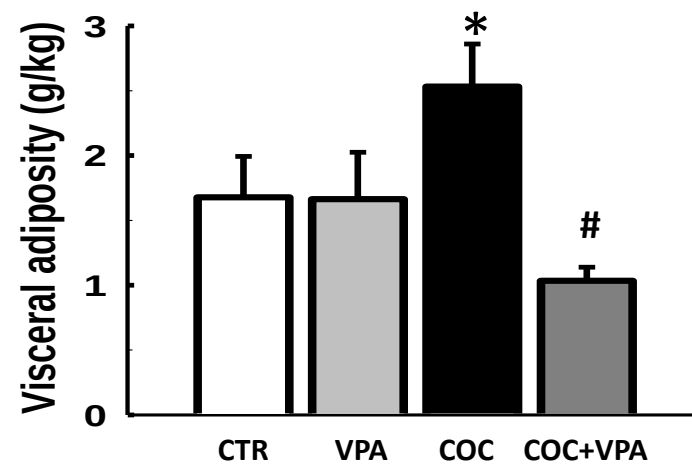
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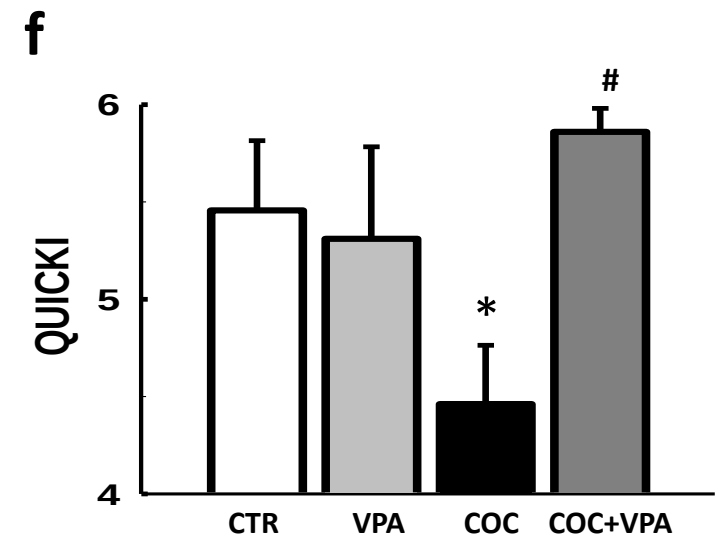
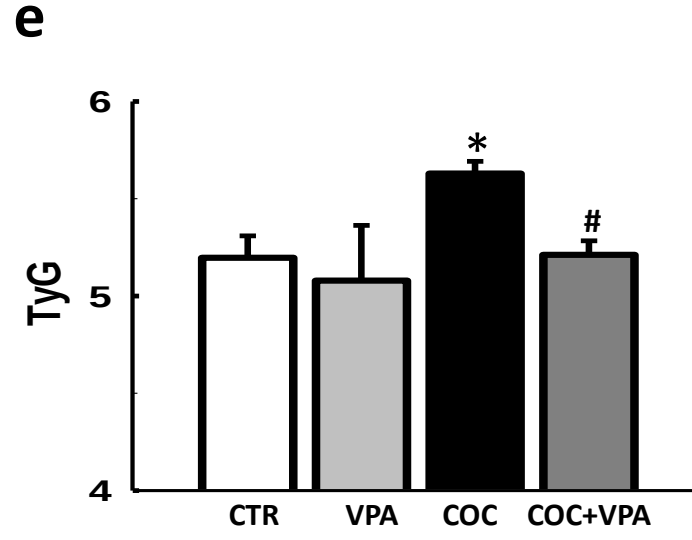
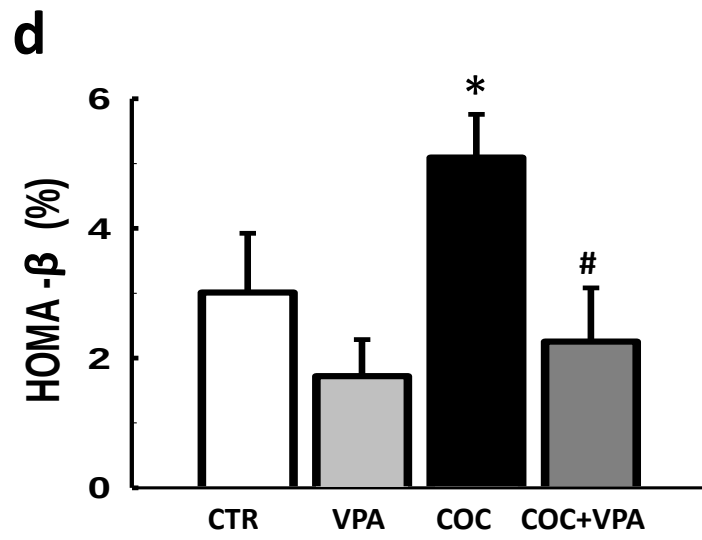
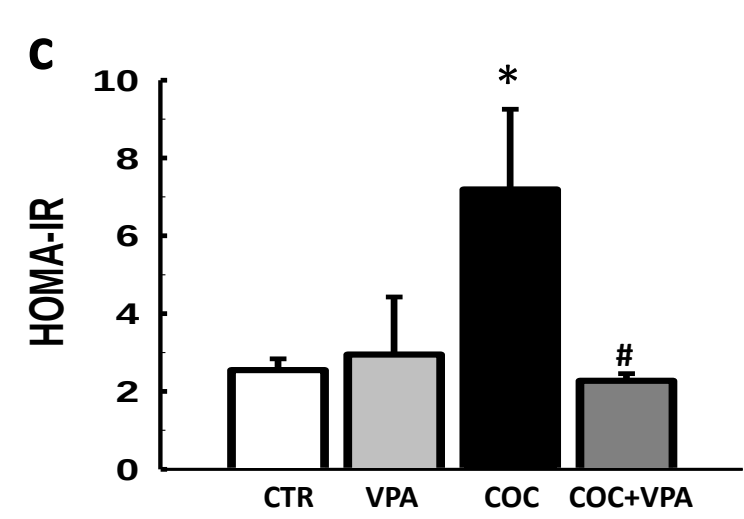
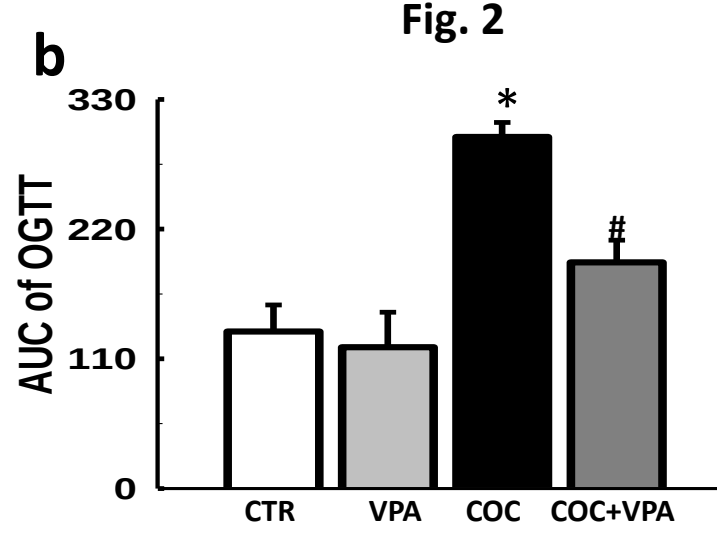
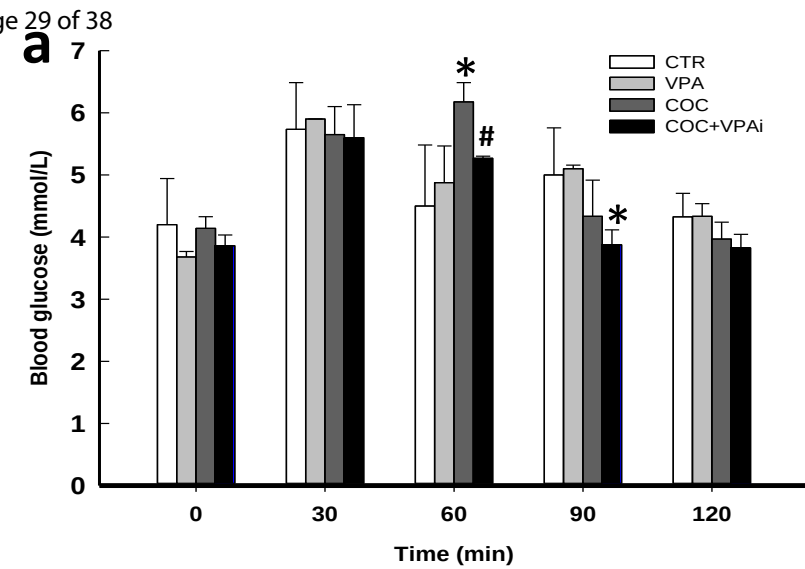
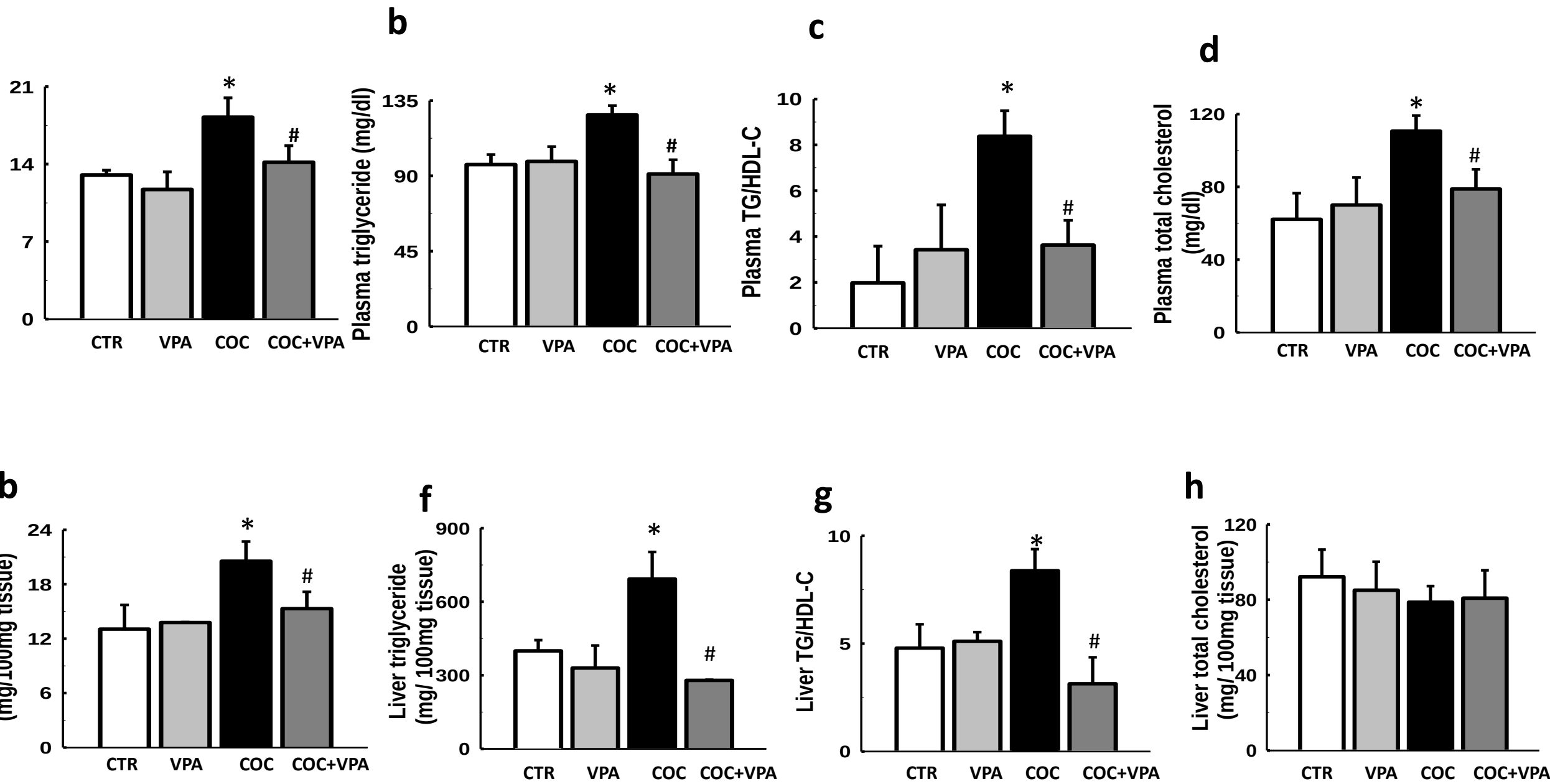


Fig. 3



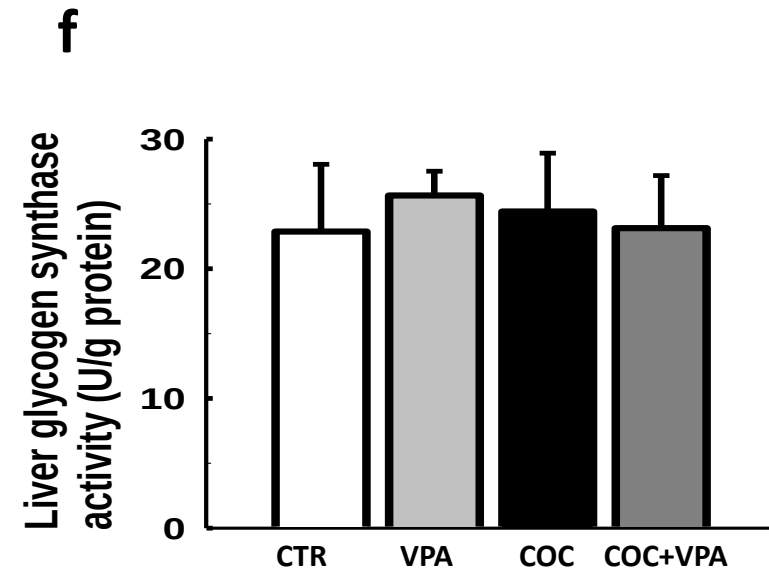
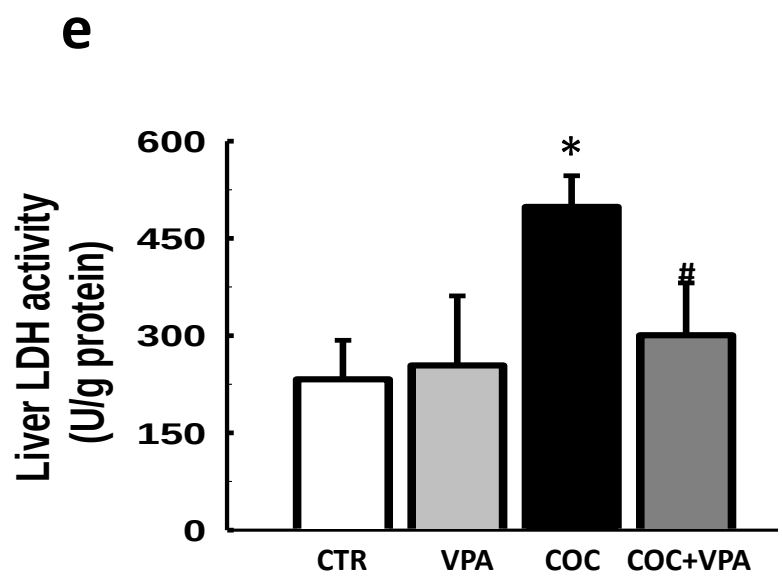
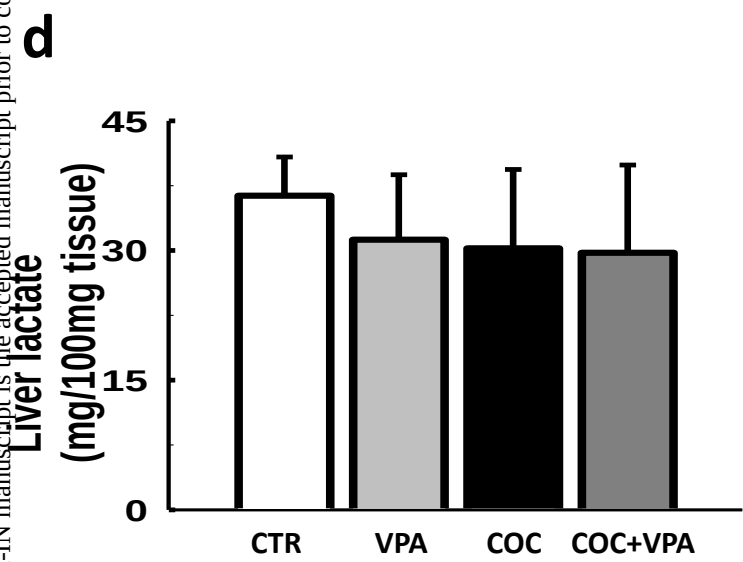
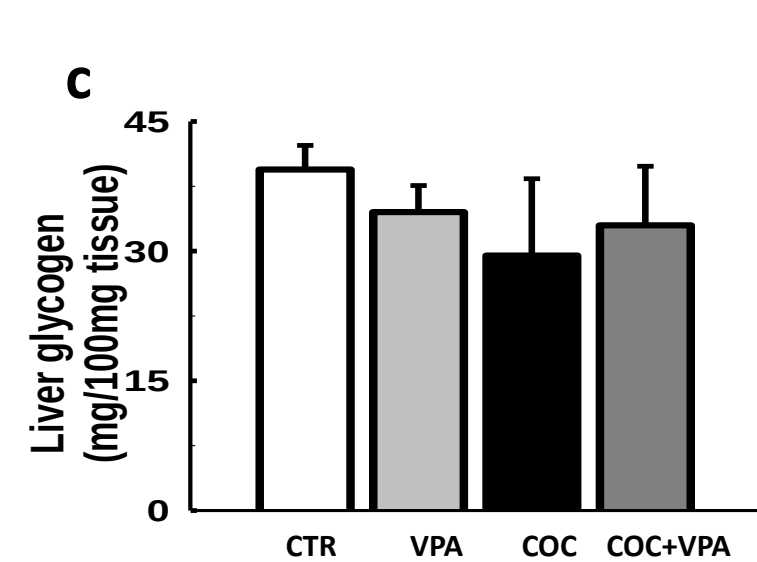
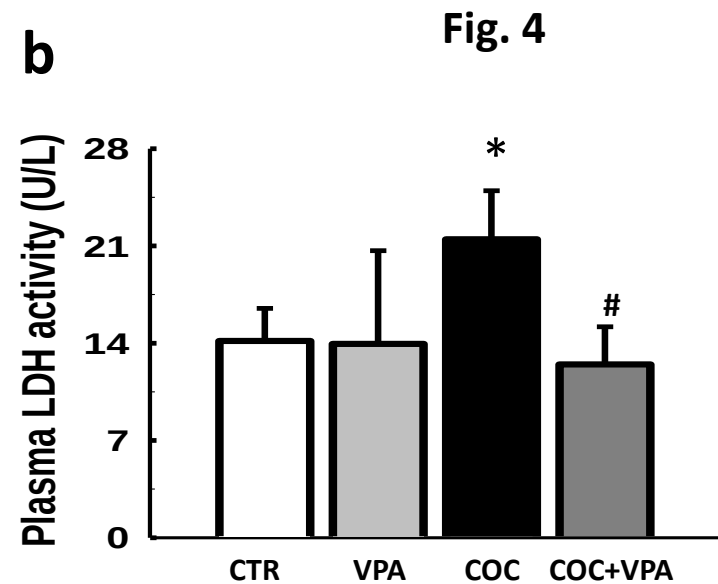
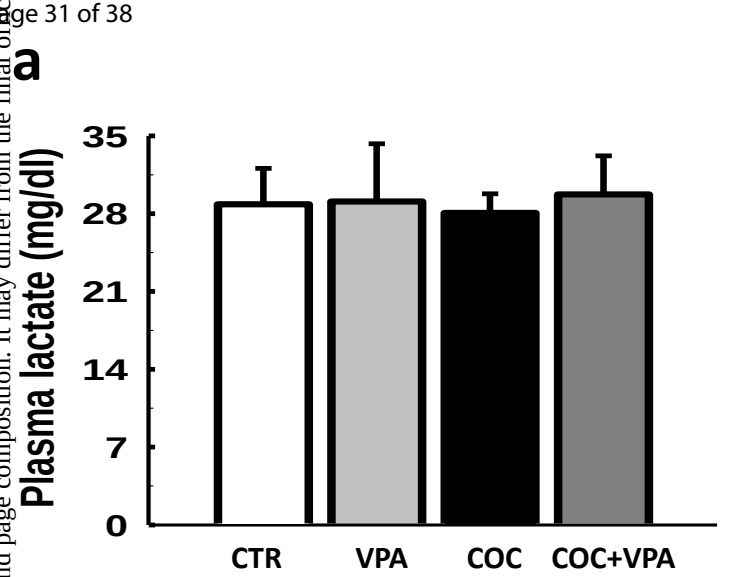


Fig. 5

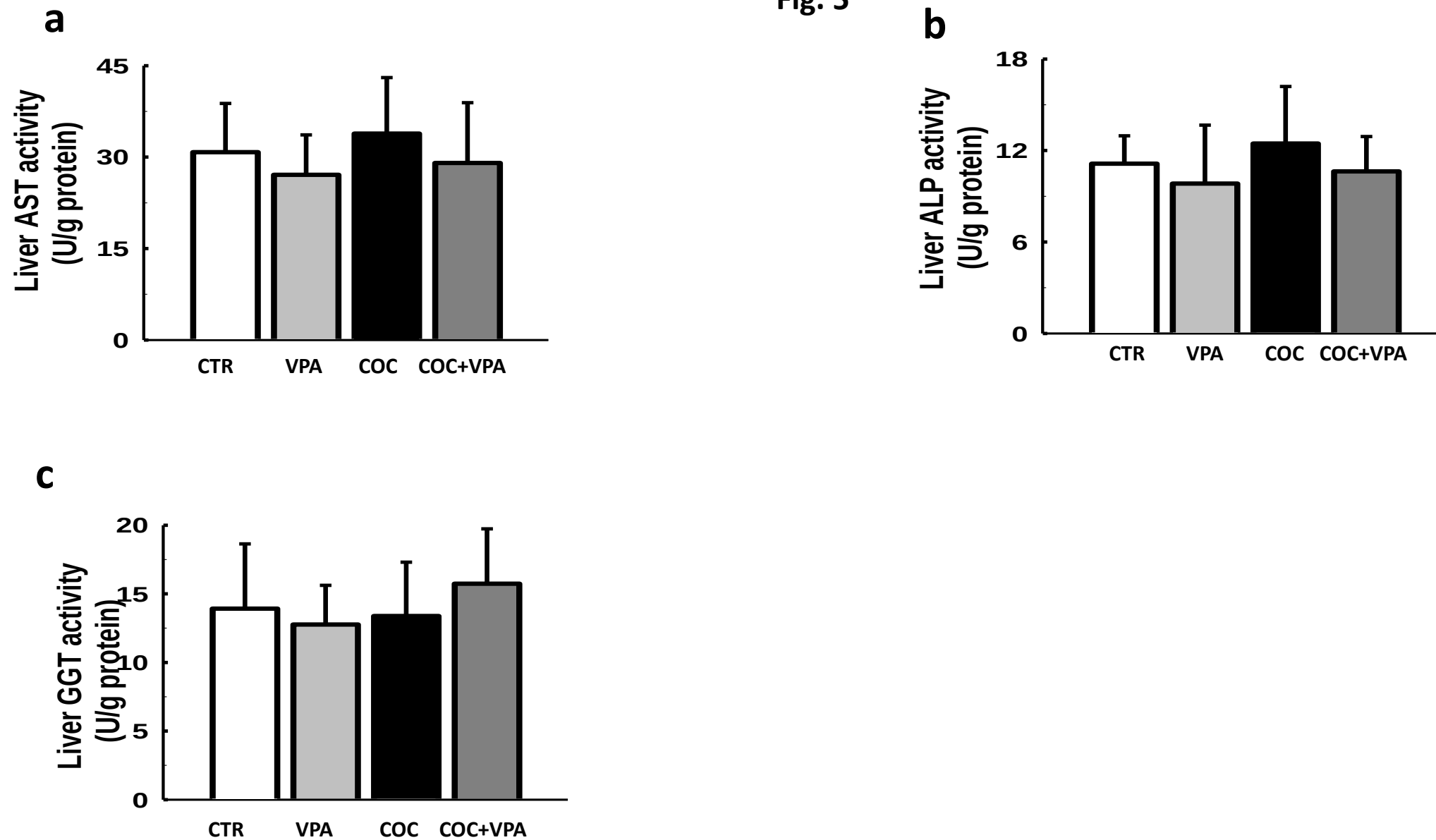


Fig. 6

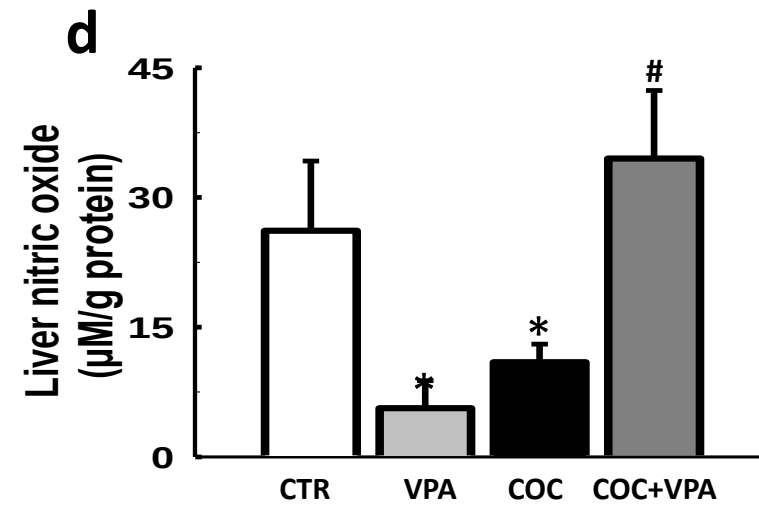
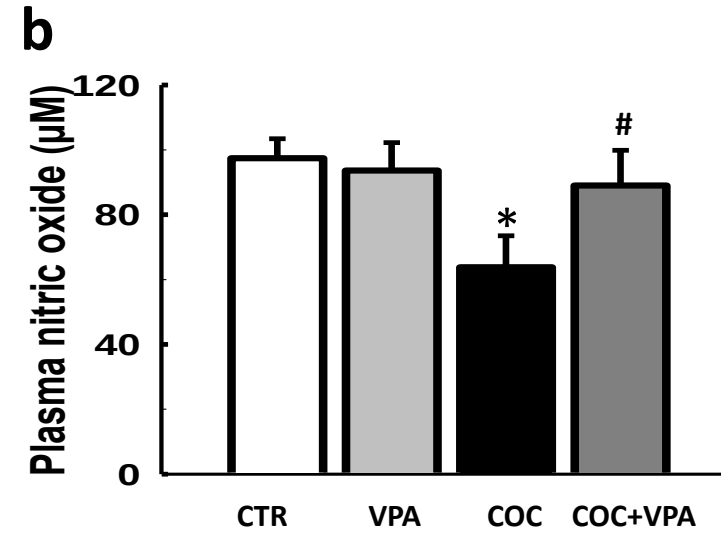
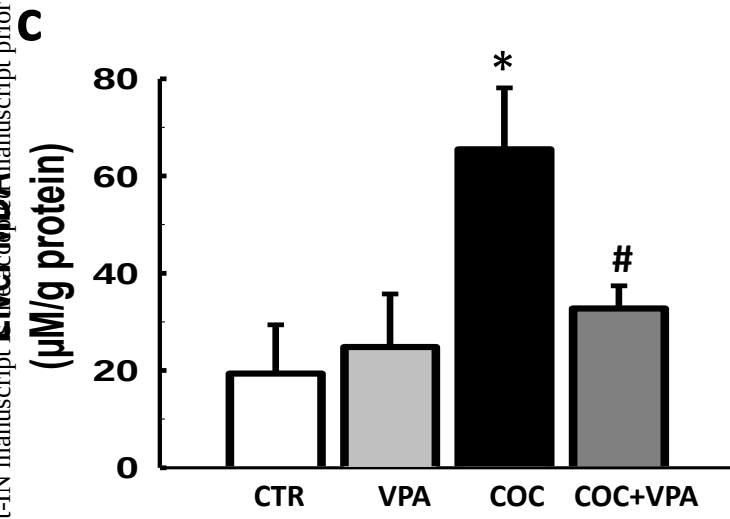
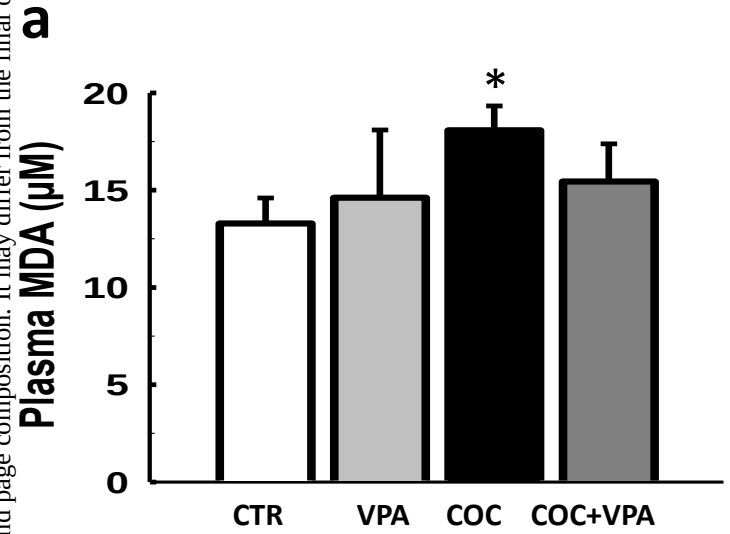


Fig. 7

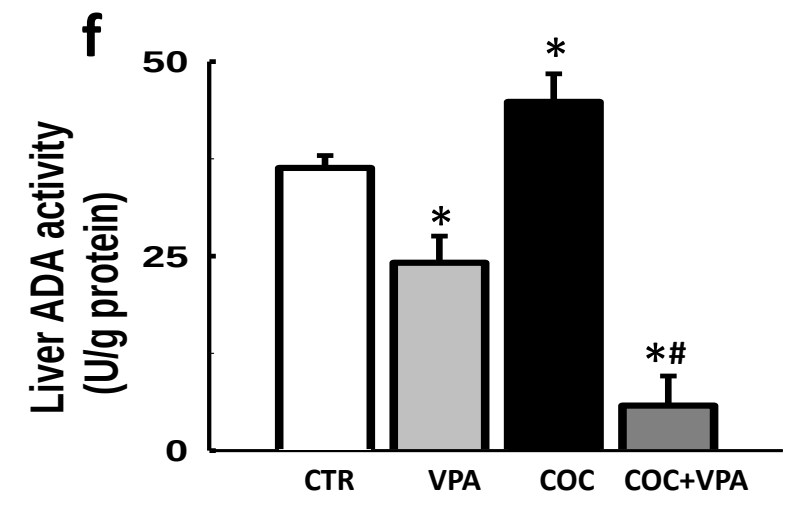
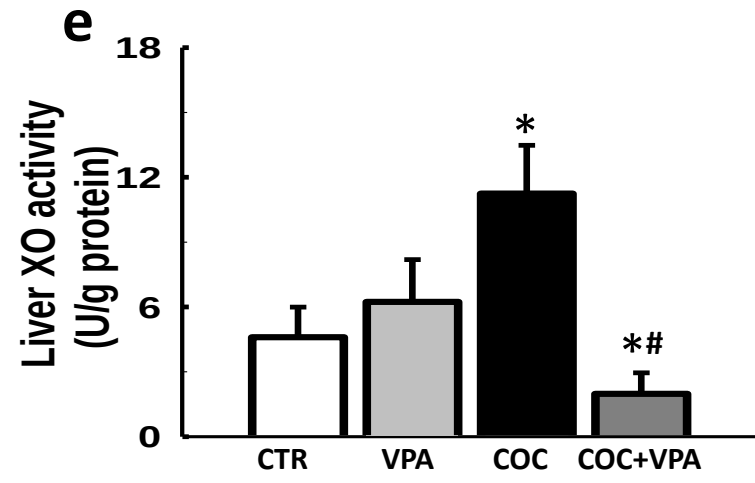
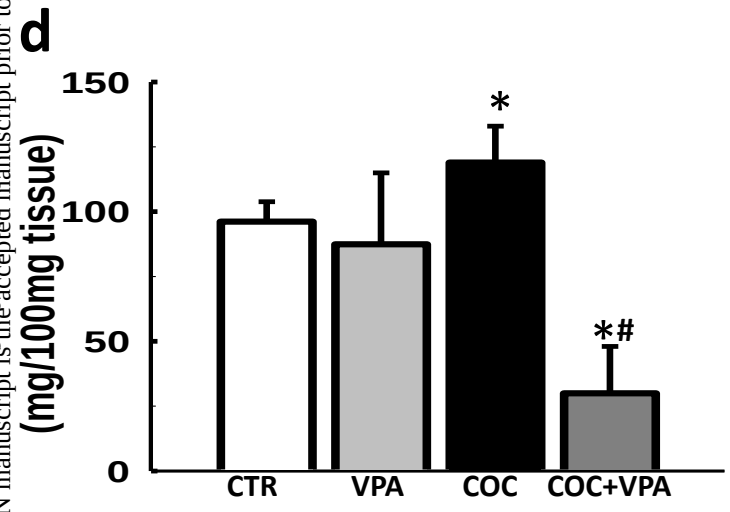
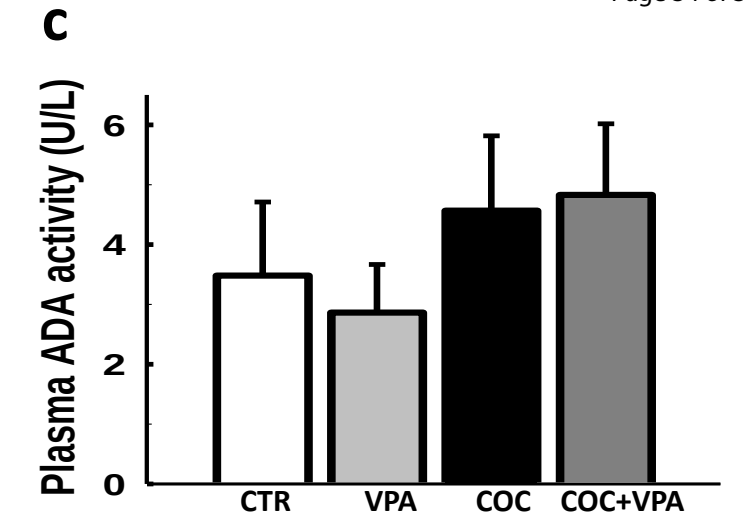
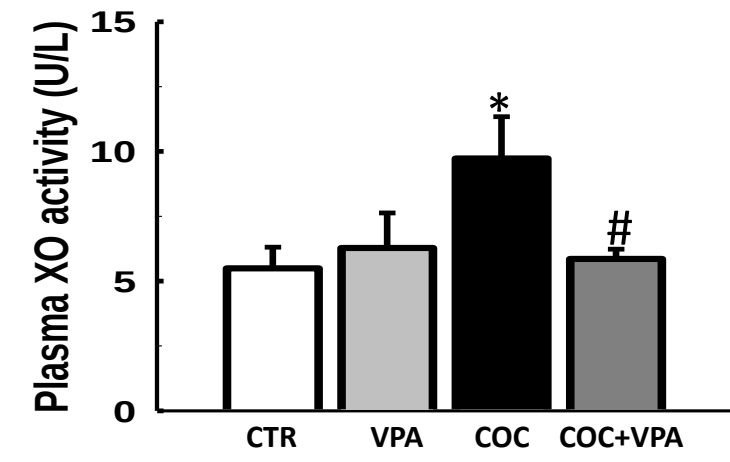
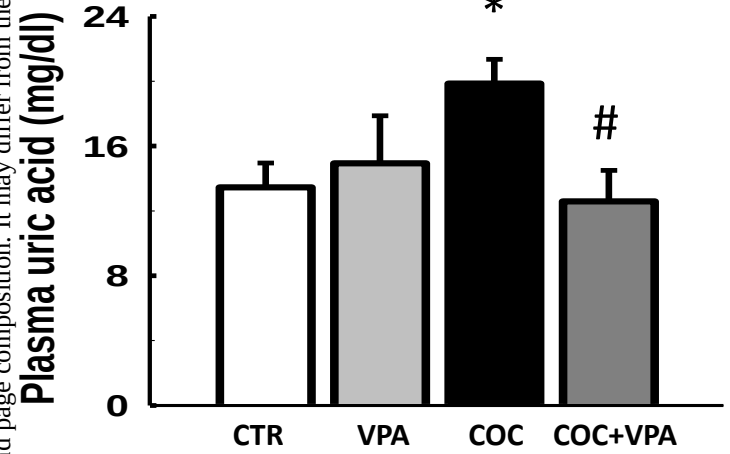


Fig. 8

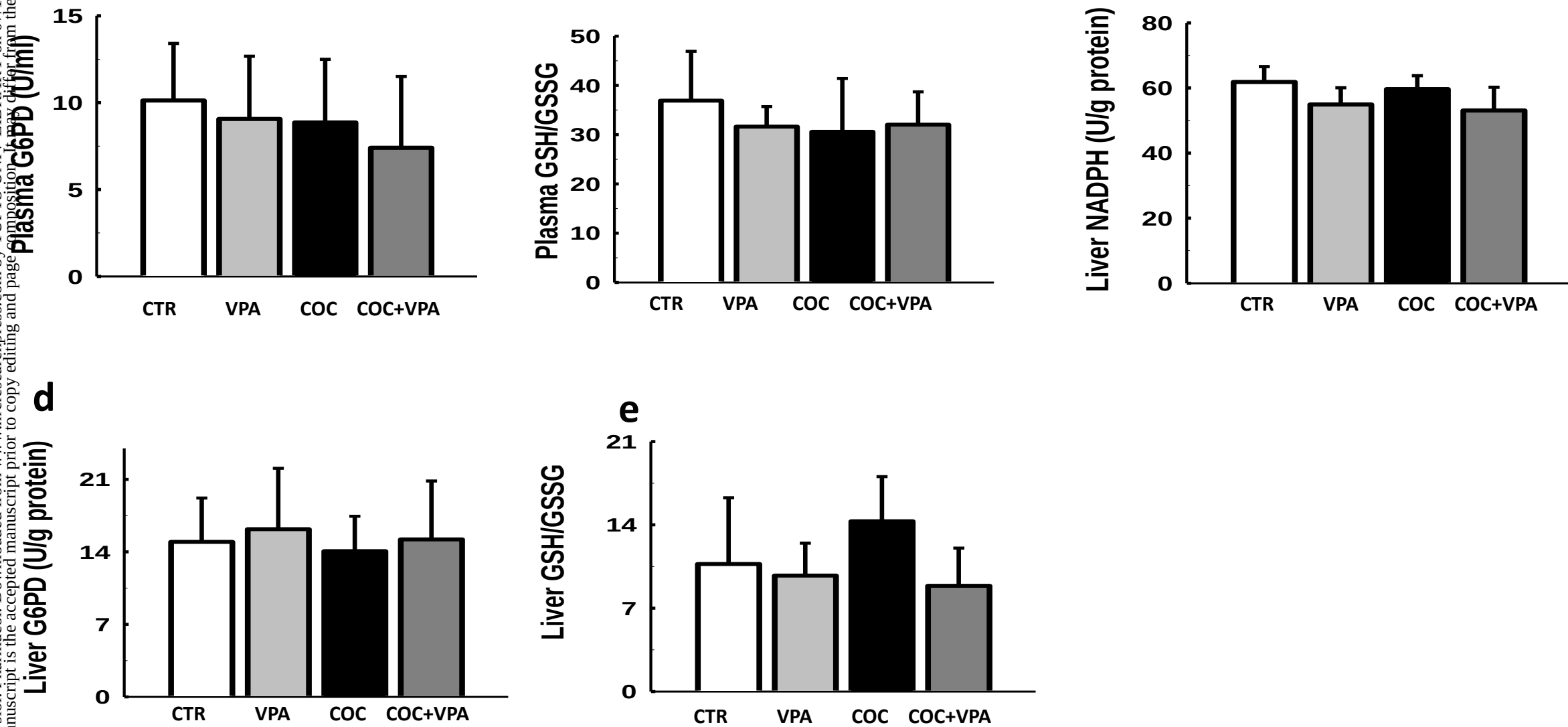


Fig. 9

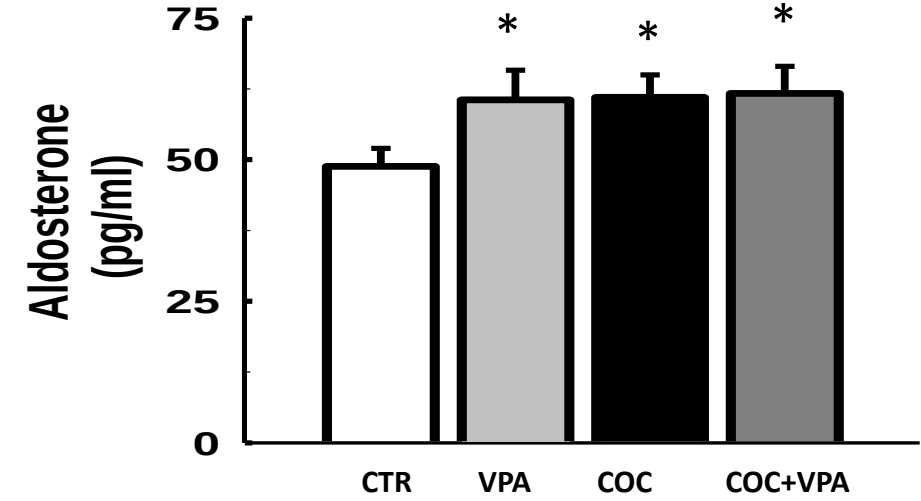
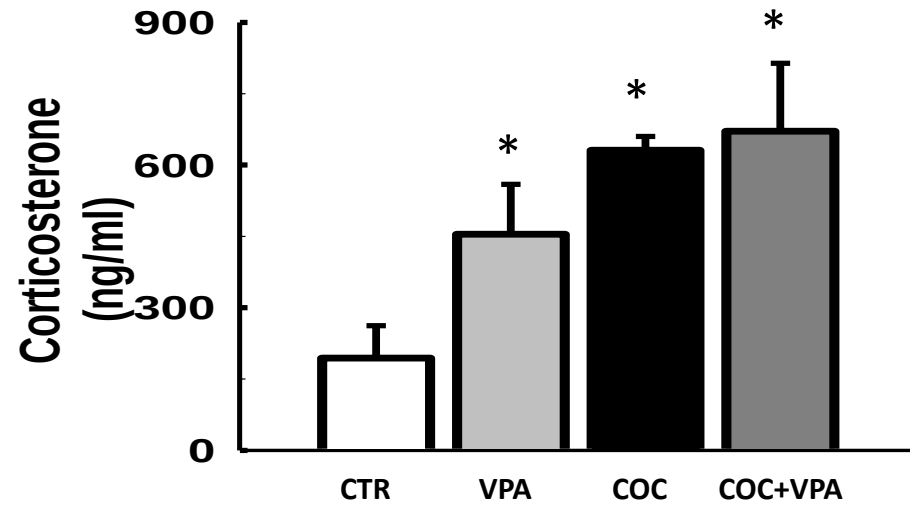


Fig. 10

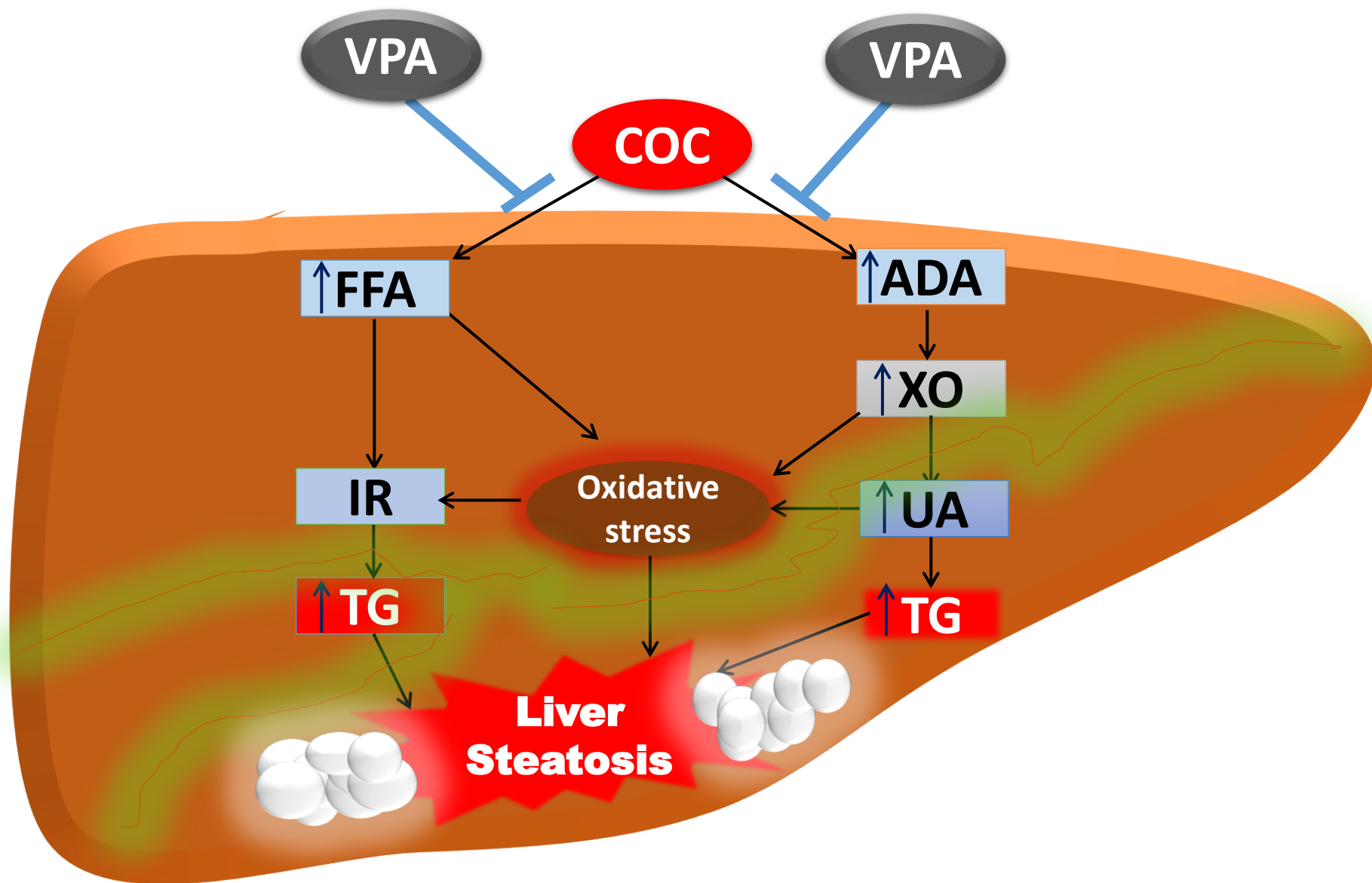


Fig. 11

