Oral ethinylestradiol-levonorgestrel normalizes fructose-induced hepatic lipid accumulation and glycogen depletion in female rats
Oral ethinylestradiol-levonorgestrel normalizes fructose-induced hepatic lipid accumulation and glycogen depletion in female rats

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Running title: EEL normalizes hepatic lipid accumulation and glycogen depletion

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ABSTRACT

The present study investigated effects of oral ethinylestradiol-levonorgestrel (EEL) on hepatic lipid and glycogen contents during high fructose (HF) intake and determined whether pyruvate dehydrogenase kinase-4 (PDK-4) and Glucose-6-phosphate dehydrogenase (G6PD) activity were involved in HF and/or EEL-induced hepatic dysmetabolism. Female Wistar rats weighing between 140-160g were divided into groups. Control, EEL, HF and EEL+HF groups received water (vehicle, p.o), 1.0µg ethinylestradiol plus 5.0µg levonorgestrel (p.o), fructose (10%; w/v) and EEL plus HF respectively, daily for 8 weeks. Results revealed that EEL or HF led to insulin resistance, hyperinsulinemia, increased hepatic uric acid production, triglyceride (0.80 ± 0.06 or 1.21 ± 0.08 versus control; 0.41 ± 0.05mg/100mg tissue; \(p<0.05\)), reduced glycogen content (0.59 ± 0.11 or 0.61 ± 0.10 versus control; 1.15 ± 0.09ug/100mg tissue; \(p<0.05\)), plasma or hepatic glutathione- and G6PD-dependent antioxidants. HF but not EEL also increased fasting glucose and hepatic PDK-4. Nonetheless, these alterations were attenuated by EEL in HF-treated rats. Our results demonstrate that hepatic lipid accumulation and glycogen depletion induced by HF is accompanied by increased PDK-4 and defective G6PD activity. The findings also suggest that EEL would attenuate hepatic lipid accumulation and glycogen depletion by suppression of PDK-4 and enhancement of G6PD-dependent antioxidant barrier.

Keywords: Glycogen synthesis, Insulin resistance, Lipid accumulation, PDK-4, Estrogen-progestin therapy, G6PD.
Introduction

Generally, insulin resistance (IR) is a multifaceted disruption of the communication between insulin and its target cells in such that there is reduced tissue sensitivity to physiological action of insulin, resulting in metabolic defects including obesity, type-2 diabetes (T2DM) and cardiometabolic syndrome (Sears and Perry 2015; Kitade et al. 2017). IR-driven hepatic lipid accumulation and glycogen depletion is a known risk factor for T2DM and cardiovascular disease (Marchesini et al. 2003; Fattahi et al. 2016). It is also the metabolic pathway to severe liver disease. Interestingly, IR-mediated metabolic dysfunction has been reported as an independent predictor of non alcoholic fatty liver disease (NAFLD), which is the hepatic expression of cardiometabolic disorders (Kusunoki et al. 2002; Marchesini et al. 2003; Kotronen et al. 2007; Kitade et al. 2017). However, NAFLD is well recognized as a component of epidemic metabolic syndrome worldwide (Postic and Girard 2008; Cheng et al. 2017). Hepatic lipid accumulation is the hallmark of NAFLD and reports have it that over 30 % of Americans suffer from hepatic triglyceride (TG) accumulation-related metabolic disorders and over 90 % of obese T2DM develop NAFLD (Tolman et al. 2007, Fattahi et al. 2016; Cheng et al. 2017; Kitade et al. 2017; Sawar et al. 2018). Cheng et al. 2017; Kitade et al. 2017; Sawar et al. 2018

The pathogenesis of NAFLD has been markedly associated with TG accumulation which promotes disrupted antioxidant barrier leading to hepatic lipotoxicity or injury (Lieber 2004; Tolman et al. 2007; Mantena et al. 2008). Liver is not programmed to store excess fat unlike adipose tissue, hence the foremost metabolic defect of IR is the accumulation of lipids in the liver, which possibly progress to NAFLD (Araya et al. 2004; Tolman et al. 2007; Rector et al. 2013). In addition, liver is crucial in glucose and lipid regulation for homeostatic control and
glycogen and TG production are central hepatic homeostatic events either in the postprandial or post absorptive state respectively (Wasserman 2009; Agius 2011). Hepatic glycogen content is crucial in counter regulation of hypoglycemia in individuals with T2DM and/or IR increasing lipogenesis which possibly results to hepatic TG accumulation and consequently fatty liver (Roach et al. 2012; Stapleton et al. 2013; Winnick et al. 2016; Olaniyi and Olatunji 2019).

Increasing fructose consumption has been associated with IR and the risk of development of T2DM with or without hepatic complications in humans and experimental animals (Lee et al. 2009; Wu et al. 2014). Female animals have been shown to be more susceptible to deleterious hepatic impacts of fructose than the male counterparts by reducing hepatic insulin signaling (Vilà et al. 2011). Hepatic IR has been shown to cause elevated TG (Kostogrys et al. 2010; Huang et al. 2010). Likewise the interaction between the liver and vascular wall possibly accounts for the strong associations between liver steatosis and increased atherosclerosis. Reports show that severe hepatic steatosis was associated with increased atherosclerosis in diabetic patients by increasing plasma lipids particularly TG and total cholesterol (Toledo et al. 2006; Huang et al. 2010). Other studies also revealed that increased fructose intake causes unregulated conversion into triose phosphates which promotes excessive lipogenic and gluconeogenic fluxes that accounts for elevated TG and hyperglycemia respectively (Tappy and Lê 2010; Caton et al. 2011; Samuel 2011). Nonetheless, the pathophysiology of lipid accumulation and glycogen depletion induced by high fructose intake is not completely understood. A recent study has linked these metabolic effects of high fructose with increased lipid production and pyruvate kinase (Chambers et al. 2011; Rebollo et al. 2014), hence unraveling the possible involvement of
hepatic pyruvate dehydrogenase kinase-4 (PDK-4) and Glucose-6-phosphate dehydrogenase (G6PD) activity will contribute to the pool of knowledge.

Gluconeogenic process increases in hepatic IR at the step regulated by PDKs especially PDK-4, which suppresses the activity of pyruvate dehydrogenase (PD) to regulate pyruvate dehydrogenase complex (PDC). PDC controls the entry of carbohydrate-derived pyruvate into the TCA, which is suppressed by PDK-4 promoting hepatic glucose production from pyruvate or lactate (Kumashiro et al. 2011; Rector et al. 2013). Increased activity of hepatic PDK-4 and its deficiency have been documented in IR-related steatosis (Sunny et al. 2011; Longato 2013; Rebollo et al. 2014), and increased lactate during starvation (Jeoung et al. 2006) respectively, suggesting the crucial role of PDK-4 in glucoregulation. More so, as a key player, the role of reactive oxygen species cannot be undermined in IR-linked NAFLD (Rains and Jain 2011). Oxidative stress results from an imbalance between reactive oxygen species and cellular antioxidant species. The cellular antioxidant capacity depends primarily on G6PD activity. G6PD is a house-keeping enzyme which controls the alteration of glutathione redox potential (Dentin et al. 2012). Decreased G6PD activity has been implicated in IR and T2DM (Niazi 1991; Stanton 2012). Besides causing metabolic derangements, G6PD deficiency decreases antioxidant capacity and increases cell death including pancreatic β–cell (Jain et al. 2003).

The estrogen-progestin oral contraceptive particularly ethinylestradiol-levonorgestrel (EEL) is the first extended-cycle and the most widely used combined oral contraceptive (Wilson and Kudis 2005; Panicker et al. 2014) even by adolescent age group for several reasons such as contraception, dysmenorrhea, polycystic ovarian syndrome, uterine bleeding and ovarian
insufficiency especially in premenopausal women. In addition, sex hormones particularly estrogen-progestin have been shown to modulate fructose-induced metabolic defects (Vatner et al. 2015; Bundalo et al. 2016; 2017), but the effect of EEL on hepatic lipid accumulation and glycogen depletion induced by fructose intake is not known. Therefore, this study attempted to investigate the effect of EEL on hepatic lipid accumulation and glycogen depletion and evaluate the possible roles of PDK-4 and G6PD activity in high fructose and/or EEL-induced hepatic dysmetabolism.

**Materials and methods**

**Animals and treatment**

The study protocol was approved by University of Ilorin Ethical Review Board, and the investigation was conducted in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory animals. Female Wistar rats weighing between 140-160g were procured from the animal house of the College of Health Sciences, University of Ilorin, Nigeria. The animals were given unrestricted access to standard rat chow and tap water, and randomly divided into 4 groups (n=6/group) after one week of acclimatization: Control, EEL, HF and EEL+HF groups. Animals were house under standard environmental conditions of temperature (22-26°C), relative humidity (50-60%), and 12-hour dark/light cycle.

Control, EEL, HF and EEL+HF groups received water (vehicle, *p.o*), 1.0µg ethinylestradiol plus 5.0µg levonorgestrel (*p.o*; Wyeth-Ayerst, Inc., Montreal), fructose (10%; *w/v*; Merck KGaA, Darmstadt, Germany) and EEL plus HF respectively, daily for 8 weeks. Initial and final body weights were determined.
**Sample preparation**

The animals were anesthetized with sodium pentobarbital (50 mg/kg, *ip*) at the end of the treatment and blood was collected by cardiac puncture into heparinized bottle. Blood was centrifuged at 3000 rpm for 5 min at room temperature and plasma was decanted and stored frozen until required for biochemical analysis.

**Hepatic tissue homogenate**

The liver was isolated, blotted and weighed. After weighing, 100 mg of tissue was carefully removed and homogenized with a glass homogenizer, centrifuged at 10000 rpm for 10 min at 4°C and supernatant was collected for analysis of glycogen content, glycogen synthase activity and PDK-4 immediately. The remaining sample was stored frozen until required for other biochemical analysis. The liver weights were normalized to tibial lengths to eliminate variabilities.

**Glucoregulatory parameters**

Oral glucose tolerance test (OGTT) was carried out 48 h before the termination of experiment. After subjecting the rats to 12-hour overnight fast, the fasting blood glucose levels were determined from the tail capillary droplet of blood and glucose (2 g/kg *b.w*) was given (*p.o.*) to each rat. After glucose load, blood glucose levels were determined sequentially after 30, 60, 90 and 120 min. Blood glucose levels were determined with a glucometer (ONETOUCH®-LifeScan, Inc., Milpitas, CA, USA), and glucose tolerance curve was prepared. IR was estimated using the homeostasis model assessment for IR (HOMA-IR), which is expressed as fasting glucose (mmol/l) * fasting insulin (μIU/l)/22.5 (Hsing et al. 2003). Whereas triglyceride-glucose (TyG) index was estimated as the product of fasting TG and glucose expressed as TyG index; Ln
[TG (mg/dL)*FPG (mg/dL)/2] (Guerrero-Romero et al. 2010). TG/HDLc and TC/HDLc ratios were also estimated.

**Biochemical analysis**

Hepatic PDK-4 and plasma insulin were determined using ELISA kits procured from Korain Biotech Co., Ltd (Shanghai, China) and Ray Biotech, Inc. (Georgia, USA) respectively. Plasma and hepatic TG, total cholesterol (TC), high density lipoprotein cholesterol (HDLc), uric acid were determined by standardized non-enzymatic colorimetric methods using assay kits obtained from Fortress Diagnostics Ltd. (Antrim, UK).

Malondialdehyde (MDA), reduced glutathione (GSH), free fatty acid (FFA), oxidized glutathione (GSSG), lactate and glycogen contents were measured by standardized colorimetric methods using assay kits from Oxford Biomedical Research, Inc. (Oxford, MI, USA).

Adenosine deaminase (ADA), G6PD; Gamma-Glutamyl transferase (GGT), lactate dehydrogenase (LDH), glycogen synthase and xanthine oxidase (XO) activities were also estimated by standard spectrophotometric method using assay kits procured from Randox Laboratory Ltd. (Co. Antrim, UK) and Oxford Biomedical Research, Inc. (Oxford, MI, USA) respectively.

**Histopathological studies**

For hematoxylin and eosin (H & E) stains, a section of the right lobe of liver was fixed in 10% formolsaline overnight and thereafter dehydrated, embedded in paraffin and sectioned at 5-μm thickness. In brief, the sections were deparaffinised by flaming the slide in burner and dipping in xylene. The tissue sections were hydrated by passing through decreasing concentration of alcohol baths and water and stained in hematoxylin for 3-5 minutes, washed in running tap water
until sections stained blue for 5 minutes or less. Then differentiated in 1% acid alcohol for 5mins and washed in running tap water until sections are again blue by dipping in alkaline solution and washed again in running tap water. It was thereafter stained in 1% eosin for 10minutes, washed in tap water for 1-5minutes, dehydrated in different concentrations of alcohol, cleared in xylene and mounted in mounting media. The slides were observed under light microscope.

**Statistical analysis**

Data from the present study were expressed as means ± SEM. Group analysis was performed with SPSS software and one-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups while repeated measure of ANOVA was used to compare means of OGTT. Bonferroni’s test was used to identify the significance of pair wise comparisons of mean values among the groups. Significant differences were considered at p less than 0.05.

**Results**

*High fructose intake with or without EEL did not alter body and liver weights in female rats*

Fructose intake with or without EEL treatment did not significantly alter the body weight (187.7 ± 13.2 or 197.5 ± 10.4g) and liver weight (27.8 ± 2.4 or 28.2 ± 2.4gm/mm) when compared with control group (190.5 ± 3.5g and 25.3 ± 1.3mg/mm respectively; Table 1).

*EEL attenuated hyperglycemia, glucose intolerance, hyperinsulinemia and IR during high fructose intake in female rats*

EEL treatment did not affect the fasting blood glucose (4.03 ± 0.06mmol/L), whereas fructose intake significantly increased fasting blood glucose (5.13 ± 0.09mmol/L) when compared with control (3.78 ± 0.14mmol/L). However, treatment with EEL attenuated fructose-induced hyperglycemia (3.72 ± 0.13mmol/L; Figure 1A). EEL treatment or fructose intake caused
impaired glucose tolerance and significantly increased fasting plasma insulin (48.78 ± 3.40 or 40.93 ± 2.60µIU/mL), HOMA-IR (8.65 ± 1.31 or 9.35 ± 1.11) and TyG index (8.50 ± 0.21 or 8.75 ± 0.06) compared to the control group (22.40 ± 0.71µIU/mL, 3.77 ± 0.21 and 7.62 ± 0.20 respectively), which were attenuated in fructose intake with EEL treatment (32.88 ± 1.67µIU/mL, 6.38 ± 0.59 and 7.88 ± 0.19 respectively; Figure 1B, C, D, E).

**EEL attenuated lipid accumulation induced by high fructose intake in female rats**

Fructose intake or EEL treatment significantly increased plasma and hepatic lipids with correspondent increase in TG, TC, TG/HDLc and TC/HDLc respectively (plasma TG; 125.50 ± 10.94 or 138.23 ± 17.00mg/dL and hepatic TG; 1.21 ± 0.08 or 0.80 ± 0.06mg/100mg tissue) without altering the plasma and hepatic FFA (plasma; 391.00 ± 31.90 or 410.00 ± 55.06µmol/L and hepatic; 0.50 ± 0.04 or 0.58 ± 0.09µmol/100mg tissue respectively) when compared with control group (TG; 69.63 ± 13.47mg/dL or 0.41 ± 0.05mg/100mg tissue and FFA; 375.39 ± 40.83µmol/L or 0.49 ± 0.03µmol/100mg tissue respectively). Nevertheless, EEL administration in high fructose intake attenuated increased plasma and hepatic lipids respectively (TG; 89.64 ± 8.00mg/dL or 0.61 ± 0.13mg/100mg tissue and FFA; 356.45 ± 10.41µmol/L or 0.58 ± 0.11µmol/100mg tissue; Table 2, figure 2).

**EEL protected against hepatic glycogen depletion during high fructose intake in female rats**

Fructose intake or EEL treatment significantly decreased hepatic glycogen contents (0.61 ± 0.10 or 0.59 ± 0.11ug/100mg tissue) and glycogen synthase activities (15.53 ± 1.02 or 16.91 ± 1.26u/g protein) when compared to the control group (1.15 ± 0.09ug/100mg tissue and 23.83 ± 1.92u/g protein respectively). However, EEL treatment attenuated fructose-induced glycogen depletion (0.94 ± 0.08ug/100mg tissue and 20.33 ± 0.95u/g protein; Figure 3A, B).

**EEL protected against lipid peroxidation during high fructose intake in female rats**
Fructose intake or EEL treatment increased lipid peroxidation (Plasma; 6.74 ± 0.20 or 7.63 ± 0.65µM and hepatic; 0.22 ± 0.02 or 0.18 ± 0.03µM/100mg tissue respectively) when compared to the control group (4.70 ± 0.32µM and 0.11 ± 0.01µM/100mg tissue respectively), which was attenuated when fructose intake was combined with EEL treatment (5.30 ± 0.30µM and 0.15 ± 0.01µM/100mg tissue respectively; Figure 4).

**EEL reduced hepatic uric acid production during high fructose intake in female rats**

Fructose intake or EEL treatment significantly increased hepatic uric acid production (0.82 ± 0.07 or 0.67 ± 0.07U/100mg tissue) by increasing ADA (14.86 ± 1.39 or 12.70 ± 1.20U/g protein), XO (7.58 ± 0.75 or 6.59 ± 0.76U/g protein) and decreasing adenosine (0.15 ± 0.004 or 0.17 ± 0.008mg/100mg tissue); Figure 5A-D) when compared to the control group (0.34 ± 0.08U/100mg tissue, 0.63 ± 1.10U/g protein, 4.18 ± 0.50U/g protein and 0.22 ± 0.006mg/100mg tissue respectively), which were attenuated in EEL treatment with high fructose intake (0.58 ± 0.02U/100mg tissue, 10.25 ± 0.90U/g protein, 5.18 ± 0.34U/g protein and 0.21 ± 0.008mg/100mg tissue respectively). However, fructose intake but not EEL significantly increased the activity of plasma ADA (7.24 ± 0.29U/mL) and decreased plasma adenosine (2.76 ± 0.39U/dL) whereas EEL administration or fructose intake increased the plasma level of xanthine oxidase and uric acid concentration (3.04 ± 0.09 or 3.89 ± 0.08 and 3.54 ± 0.25 or 3.76 ± 0.28U/mL) respectively compared to the control group (6.04 ± 0.50U/mL, 5.00 ± 1.20U/dL, 1.83 ± 0.10U/mL and 2.74 ± 0.20U/mL; Table 3).

**EEL reduced lactate synthesis and liver function enzyme markers during high fructose intake in female rats**

Fructose intake or EEL treatment increased plasma lactate content (16.74 ± 0.94 or 13.30 ± 0.90mg/dL), plasma and hepatic LDH (plasma; 633.43 ± 31.70 or 642.51 ± 29.00U/L and
hepatic; 0.26 ± 0.01 or 0.27 ± 0.02U/g protein respectively) compared to the control group (10.18 ± 0.70mg/dL, 550.91 ± 20.06U/L and 0.21 ± 0.02U/g protein respectively), which were attenuated in fructose with EEL treatment (11.09 ± 0.87mg/dL, 443.03 ± 35.84U/L and 0.20 ± 0.01U/g protein respectively; Figure 6A, C, D). EEL treatment did not affect the hepatic level of lactate, whereas fructose intake increased hepatic lactate content (0.091 ± 0.01mg/100mg tissue) compared to the control group (0.043 ± 0.002mg/100mg tissue). However, EEL treatment attenuated increased hepatic lactate induced by fructose intake (0.051 ± 0.003mg/100mg tissue; Figure 6B). In addition, fructose but not EEL treatment increased plasma and hepatic GGT (16.36 ± 1.43U/L and 25.31 ± 2.24U/g protein) when compared to the control group (9.04 ± 1.62U/L and 10.58 ± 4.60U/g protein). However, EEL treatment attenuated HF-induced elevated plasma GGT and hepatic GGT activity (11.20 ± 0.66U/L and 17.97 ± 1.03U/g protein; Figure 6E).

**EEL preserved hepatic tissue during high fructose intake in female rats**

The histology of liver showed hepatic tissue with preserved architecture comprising cords of hepatocytes, central veins and portal triad with no degenerative change in control group. EEL group showed hepatic tissue with foci of moderate periportal and sinusoidal lymphocytic inflammation. Fructose group showed hepatic tissue with foci of severe periportal and sinusoidal infiltration by lymphocytes while EEL plus fructose group showed hepatic tissue with preserved hepatic architecture, there are foci of mild periportal lymphocytic inflammation (Figure 7).

**EEL improved G6PD- and glutathione-dependent antioxidant barriers during high fructose intake in female rats**

Fructose intake or EEL reduced GSH/GSSG (0.087 ± 0.009 or 0.096 ± 0.008) and G6PD activity (8.77 ± 1.19 or 10.48 ± 1.25U/L) when compared to the control group (0.130 ± 0.006 and 14.86 ±
0.88U/L), which were attenuated when fructose intake was combined with EEL treatment (0.114 ± 0.008 and 12.54 ± 1.29U/L; Figure 8).

**EEL normalized hepatic PDK-4 during high fructose intake in female rats**

The level of hepatic PDK-4 (6.32 ± 0.46ng/100mg tissue) was significantly increased in rats treated with fructose but not EEL when compared to the control group (3.70 ± 0.40ng/100mg tissue), which was attenuated when fructose was concomitantly treated with EEL (4.34 ± 0.68ng/100mg tissue; Figure 9).

**Discussion**

The main finding of the present study is that EEL ameliorates hepatic IR, lipid accumulation, glycogen depletion and their correlates during high fructose intake in rats, which was associated with decreased PDK-4 and enhanced G6PD-dependent antioxidant barrier. Our data showed that EEL or high fructose induced IR, which triggered hepatic lipid accumulation and glycogen depletion that was accompanied by increased uric acid synthesis, oxidative stress, lipid peroxidation, blood lactate and decreased G6PD activity. In addition, high fructose intake but not EEL induced hyperglycemia, hepatic lactate accumulation, altered liver function enzyme markers and increased PDK-4. Histology of the liver also revealed that high fructose intake led to disrupted hepatic tissue architecture with foci of severe periportal and sinusoidal infiltration by lymphocytes, EEL treatment caused foci of moderate periportal and sinusoidal lymphocytic inflammation, whereas, EEL with high fructose intake resulted in preserved hepatic tissue architecture with foci of mild periportal lymphocytic inflammation but no significant degenerative changes. However, treatment with EEL protected against fructose-induced
metabolic defects which was accompanied by decreased PDK-4 and enhanced G6PD-dependent antioxidant barrier (Figure 10).

Accumulating evidence suggests IR that is associated with hyperinsulinemia as a strong predictor of NAFLD even without obesity or T2DM (Marchesini et al. 2003; Kotronen et al. 2007). This study revealed that high fructose or EEL administration caused IR/hyperinsulinemia that was accompanied by hepatic lipid accumulation. Lipid accumulation such as TG, TC and atherogenic lipids precedes the development of NAFLD which is a predictor of severe liver disease and integral feature of metabolic disorders, including T2DM, obesity, arterial hypertension and atherosclerosis (Tolman et al. 2007; Postic and Girard 2008; Rector et al. 2013). In addition, IR induced by high fructose or EEL treatment is clearly reiterated with elevated TyG index which serves as a simple surrogate marker of IR (Lee et al. 2009; Bundalo et al. 2017). In addition, the present study showed that IR/hyperinsulinemia and hepatic lipid accumulation observed during high fructose but not EEL treatment was associated with hyperglycemia, which implies that high fructose intake clearly increases the risk of T2DM (Lee et al. 2009; Tran et al. 2009) aside from inducing IR-driven hepatic lipid accumulation. This finding is inconsonance with previous findings in humans and experimental animals (Tran et al. 2009; Wu et al. 2014).

Earlier studies demonstrate the direct relationship between IR and hepatic lipid accumulation with corresponding crosstalk between metabolic deregulation and liver diseases (Kotronen et al. 2007). The present study showed as an extension that high fructose or EEL-induced IR is not only accompanied by hepatic TG accumulation but also by glycogen depletion. Glycogen stores and releases glucose for metabolic and synthetic requirements of the cell (Philip et al. 2012), and...
this process is controlled by a combination of substrate availability and regulation of catalytic activities, especially glycogen synthase. The present study revealed a significant reduction in hepatic glycogen content with correspondent decrease in glycogen synthase activities during fructose intake or EEL treatments. However, liver has been shown to play a crucial role in maintaining energy balance in postprandial and postabsorptive states by stimulating glycogenic or lipogenic enzymes activities, and disruption of this metabolic process results in glucose/lipid dysmetabolism, a clear indicator of metabolic dysfunction, which has been observed in endemic T2DM, obesity and cardiometabolic syndrome (Postic and Girard 2008; Tran et al. 2009). This finding indicates that high fructose or EEL treatment disrupts glucose homeostasis as earlier reported (Tappy and Lê 2010; Caton et al. 2011; Samuel 2011; Vatner et al. 2015) leading to IR-mediated impaired hepatic glycogen synthesis and increased hepatic triglyceride production that correspond with hepatic glycogen depletion and lipid accumulation respectively regardless of FFA levels, suggesting that lipid accumulation is not due to de novo lipogenesis but excessive influx of TG into the hepatocytes during high fructose or EEL treatment.

Furthermore, the present data also revealed that hepatic lactate production increased during high fructose intake with correspondent elevated circulating and hepatic lactate content, whereas EEL treatment increased plasma lactate and LDH without affecting the hepatic lactate content. However, elevated lactate content has been reported as an indication of mitochondrial impairment in oxidative phosphorylation (Iossa et al. 2004) and largely associated with IR, T2DM and the incident NAFLD (Crawford et al. 2010). Therefore, elevated plasma lactate may target metabolic tissues to reduce glucose utility and flux, resulting in systemic IR that may be associated with hyperglycemia through increased gluconeogenic activity. Hence, fructose-driven
lactate production is another event causing systemic and/or local insulin resistance, which is consistent with previous observation (Leite et al. 2011).

It is noteworthy that high fructose or EEL-induced hepatic lipid accumulation and glycogen depletion is associated with elevated uric acid synthesis with corresponding increase in ADA and XO activities, although the plasma ADA was not affected by EEL treatment. ADA deaminates adenosine to inosine (Brady 1942) and its increased activity has been documented as a marker of inflammatory response in T2DM and hepatic injury (Rabinovic et al. 2001) in spite of its primary function as immune defense (Rodrigues et al. 2012). Likewise the increased activity of XO with corresponding increase in uric acid content is a reflection that inflammatory response is triggered in the pathogenesis of high fructose or EEL-induced hepatic metabolic defects, which is in agreement with earlier studies (Grieve and Shah 2003; Paravicini and Touyz 2008; Olatunji et al. 2016; Mandal et al. 2017). Histological studies also revealed that the hepatic tissue derangement observed during high fructose intake is inflammatory driven.

Moreso, oxidative stress and lipid peroxidation is a key player in the pathogenesis of hepatic injury, T2DM and atherosclerosis (Dentin et al. 2012). Elevated levels of LDH and MDA have been considered as markers of oxidative stress and lipid peroxidation respectively. The present data also showed that high fructose intake or EEL treatment significantly increased LDH and MDA. These findings are in agreement with previous studies including study from our laboratory animals (Springer et al. 2013; Olatunji et al. 2016). In addition, the present result indicates that fructose or EEL-induced hepatic metabolic defects is accompanied by increased oxidative stress and lipid peroxidation, which promotes liver injury in high fructose intake but not EEL-treated
rats as also indicated by increased plasma or hepatic GGT, whereas hepatic GGT, ALT and AST did not change in EEL-treated rats.

Furthermore, G6PD, a house-keeping, rate limiting and non-oxidative enzyme that provides NADPH for glutathione system (Niazi 1991; Stanton 2012) was found to be significantly reduced in high fructose or EEL-treated rats, with correspondent decrease in GSH-GSSG ratio. Reduced activity of G6PD has earlier been demonstrated to decrease cellular resistance to oxidative stress resulting in cell death including hepatocytes in insulin resistant conditions such as T2DM, cardiometabolic disorders among others (Jain et al. 2003). Therefore, this present findings imply that high fructose or EEL-induced IR-driven lipid accumulation and glycogen depletion is also accompanied by decreased G6PD-dependent antioxidant barrier.

Importantly, PDK4 has been demonstrated to increase gluconeogenic process by inhibiting PD activity thereby preventing the entry of glycolytic products into TCA in starved or diabetic mammals and humans (Kumashiro et al. 2011; Rector et al. 2013). In this present study, high fructose but not EEL treatment significantly increased hepatic PDK-4. It is earlier reported that insulin suppresses PDK-4 expression in metabolic tissues including hepatic (Longato 2013; Rebollo et al. 2014), which is consistent with the present finding that IR induced by high fructose intake is associated with increased hepatic PDK-4, leading to reduction in glucose oxidation, thus promoting increased gluconeogenic and lipogenic processes. The finding that high fructose but not EEL induces hepatic metabolic defects with correspondent increased hepatic PDK-4 suggests for the first time that fructose-induced hepatic lipid accumulation and glycogen depletion is possibly driven by increased PDK-4, hence suppression of PDK-4 would be a
potential therapy for predisposing conditions to NAFLD particularly lipid accumulation and glycogen depletion.

Interestingly, rats treated with fructose and EEL showed no hepatic lipid accumulation and glycogen depletion, implying that EEL treatment attenuates fructose-induced hepatic lipid accumulation, glycogen depletion and their correlates which is accompanied by decreased PDK-4 and enhanced G6PD reflecting the hepatoprotective effects of EEL during fructose intake. To the best of our knowledge, this is the first study to report the ameliorative effect of EEL on hepatic lipid accumulation and glycogen depletion induced by high fructose intake. Recent studies demonstrated ameliorative effects of estrogen deficiency through menopause and ovariectomy in insulin-resistant humans and animals (Boldo and White 2011; Ko et al. 2013; Adeyanju et al. 2018). In addition, administration of EEL during non-smoking nicotine exposure has also been reported to improve insulin sensitivity, endothelial dysfunction mediators such as inflammation and thrombosis (Ko et al. 2013; Michael and Olatunji 2017). However, the present findings suggest that the use of EEL during high fructose intake may have hepatic metabolic beneficial effects by suppression of PDK-4 and enhancement of G6PD-dependent antioxidant barrier, thereby preventing lipid accumulation and glycogen depletion.

**Conclusion**

This study demonstrates that NAFLD-predisposed factors particularly lipid accumulation and glycogen depletion induced by high fructose treatment is associated with increased PDK-4 and defective G6PD-dependent antioxidant barrier. Thus the study suggests that EEL has a protective role in fructose-induced hepatic lipid accumulation and glycogen depletion by attenuating lipid
accumulation, glycogen depletion, uric acid synthesis and lactate production, which is accompanied by decreased PDK-4 and enhanced G6PD-dependent antioxidant barrier.

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FIGURE LEGENDS

**Fig.1.** Effect of oral ethinylestradiol-levonorgestrel (EEL) on fasting blood glucose (a), oral glucose tolerance test (b) plasma insulin (c) HOMA-IR (d) and TyG index (e) during high fructose (HF) intake in female rats. HF increased fasting blood glucose and EEL or HF caused impaired glucose tolerance and increased plasma insulin and HOMA-IR, which were attenuated in HF with EEL treatment. HF and EEL also increased TyG, which was attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA except Oral glucose tolerance that was analysed by repeated measure ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; #p<0.05 vs. HF).

**Fig.2.** Effect of oral ethinylestradiol-levonorgestrel (EEL) on hepatic triglyceride (a), total cholesterol (b) TG/HDL-cholesterol (c) TC/HDL-cholesterol (d) and free fatty acids (e) during high fructose (HF) intake in female rats. HF or EEL treatment increased hepatic TG, TC, TG/HDL-cholesterol and TC/HDL-cholesterol without affecting FFA. These alterations were attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control;
Fig.3. Effect of oral ethinylestradiol-levonorgestrel (EEL) on hepatic glycogen (a) and glycogen synthase activity (b) during high fructose (HF) intake in female rats. HF or EEL treatment decreased hepatic glycogen and glycogen synthase, which were attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; #p<0.05 vs. HF).

Fig.4. Effect of oral ethinylestradiol-levonorgestrel (EEL) on plasma (a) and hepatic (b) malondialdehyde (MDA) during high fructose (HF) intake in female rats. HF increased plasma and hepatic MDA, which were attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; #p<0.05 vs. HF).

Fig.5. Effect of oral ethinylestradiol-levonorgestrel (EEL) on hepatic adenosine (a), adenosine deaminase (b) xanthine oxidase (c) and uric acid (d) during high fructose (HF) intake in female rats. HF or EEL treatment increased hepatic ADA, XO and uric acid decreased adenosine, which were all attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; #p<0.05 vs. HF).

Fig.6. Effect of oral ethinylestradiol-levonorgestrel (EEL) on plasma and hepatic lactate (a, b), lactate dehydrogenase (c, d) and Gamma-Glutamyl transferase (e, f) during high fructose (HF) intake in female rats. HF increased lactate, LDH and GGT which were attenuated in HF with EEL treatment. EEL treatment alone increased plasma lactate, plasma and hepatic LDH. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; #p<0.05 vs. HF).

Fig.7. Photomicrographs of the liver from female Wistar rats. Control group shows hepatic tissue with preserved architecture comprising cords of hepatocytes, central veins and portal triad. There moderate periportal and sinusoidal lymphocytic inflammation (b), Fructose group shows hepatic
tissue with foci of severe periportal and sinusoidal infiltration by lymphocytes (c) and EEL plus fructose group shows hepatic tissue with preserved hepatic architecture, there are foci of mild periportal lymphocytic inflammation (d) (H & E paraffin stain; transverse section). CV; central vein and P; portal triad or foci of periportal vein.

**Fig. 8.** Effect of oral ethinylestradiol-levonorgestrel (EEL) on plasma or hepatic glutathione (a, b) and G6PD (c, d) activities during high fructose (HF) intake in female rats. HF or EEL treatment decreased plasma or hepatic glutathione and G6PD activities which were attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; # p<0.05 vs. HF).

**Fig. 9.** Effect of oral ethinylestradiol-levonorgestrel (EEL) on hepatic PDK4 during high fructose (HF) intake in female rats. HF increased hepatic PDK-4, which was attenuated in HF with EEL treatment. Data are expressed as mean±S.E.M. n=6. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. control; # p<0.05 vs. HF).

**Fig. 10.** Schematic diagram depicting the metabolic pathways involved in the attenuation of fructose-induced lipid accumulation and glycogen depletion by suppression of PDK-4 and enhancing G6PD-dependent antioxidant barrier by EEL. EEL: ethinylestradiol-levonorgestrel, HF: high fructose intake, PDK-4: pyruvate dehydrogenase kinase-4, G6PD: Glucose-6-phosphate dehydrogenase.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.
Table 1: Effects of EEL on body and liver weights during HF intake in female rats

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>EEL</th>
<th>HF</th>
<th>HF+EEL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>143.0± 2.1</td>
<td>142.7 ± 5.8</td>
<td>143.3 ± 3.6</td>
<td>141.3 ± 2.6</td>
</tr>
<tr>
<td>Final</td>
<td>190.5± 3.5</td>
<td>175.7± 14.7</td>
<td>197.5± 10.4</td>
<td>187.7± 13.2</td>
</tr>
<tr>
<td><strong>Liver weight (mg/mm)</strong></td>
<td>25.3± 1.3</td>
<td>26.5± 2.2</td>
<td>28.2± 2.4</td>
<td>27.8± 2.4</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. n=6. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. CTL (Control), EEL (Ethinylestradiol-levonorgestrel) and HF (High fructose).

Table 2: Effects of EEL on plasma lipids during HF intake in female rats

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>EEL</th>
<th>HF</th>
<th>HF+EEL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free fatty acid (µmol/L)</strong></td>
<td>375.39±40.83</td>
<td>410.00±55.06</td>
<td>391.00±31.90</td>
<td>359.45±10.41</td>
</tr>
<tr>
<td><strong>Other lipids (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride (TG)</td>
<td>69.63± 13.47</td>
<td>133.23±17.00*</td>
<td>125.50±10.94*</td>
<td>89.64±8.00#</td>
</tr>
<tr>
<td>Total cholesterol (TC)</td>
<td>130.24±6.98</td>
<td>153.12±1.75*</td>
<td>168.08±3.56*</td>
<td>146.18±8.14#</td>
</tr>
<tr>
<td>TG/HDL-cholesterol</td>
<td>4.64±0.90</td>
<td>11.01±1.50*</td>
<td>10.95±1.04*</td>
<td>6.12±1.01#</td>
</tr>
<tr>
<td>TC/HDL-cholesterol</td>
<td>8.68±0.41</td>
<td>12.38±0.35*</td>
<td>14.66±0.31*</td>
<td>9.90±0.56#</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. n=6. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. (*p< 0.05 vs. CTL; #p<0.05 vs. HF). CTL (Control), EEL (Ethinylestradiol-levonorgestrel), HF (High fructose) and HDL (High density lipoprotein).
Table 3: Effects of EEL on plasma uric acid synthesis during HF intake in female rats

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>EEL</th>
<th>HF</th>
<th>HF+EEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine (U/mL)</td>
<td>5.00±1.20</td>
<td>3.35±0.59</td>
<td>2.76±0.39*</td>
<td>3.83±0.98</td>
</tr>
<tr>
<td>ADA (U/mL)</td>
<td>6.04±0.50</td>
<td>6.44±0.33</td>
<td>7.24±0.29*</td>
<td>5.79±0.34#</td>
</tr>
<tr>
<td>XO (U/mL)</td>
<td>1.83±0.10</td>
<td>3.04±0.09*</td>
<td>3.86±0.08*</td>
<td>2.33±0.12#</td>
</tr>
<tr>
<td>Uric acid (U/mL)</td>
<td>2.74±0.20</td>
<td>3.54±0.25*</td>
<td>3.76±0.28*</td>
<td>2.31±0.30#</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. n=6. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. (*p< 0.05 vs. CTL; #p<0.05 vs. HF). CTL (Control), EEL (Ethinylestradiol-levonorgestrel), HF (High fructose), ADA (Adenosine deaminase) and XO (xanthine oxidase).
(A) Bar graph showing blood glucose (mmol/L) levels with significance markers.

(B) Graph showing blood glucose level over time (mins) with different conditions.

(C) Bar graph showing plasma insulin (µIU/mL) levels with significance markers.

(D) Bar graph showing HOMA-IR values with significance markers.

(E) Bar graph showing TyG index values with significance markers.
Fig. 3

(A) Glycogen (μg/100mg tissue)

(B) GS (u/g protein)
**Fig. 4**

(A) Plasma MDA (µM) vs. Treatment Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma MDA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF</td>
<td>3.0</td>
</tr>
<tr>
<td>EEL</td>
<td>6.0</td>
</tr>
</tbody>
</table>

(B) Hepatic MDA (µM/100mg tissue) vs. Treatment Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic MDA (µM/100mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF</td>
<td>0.1</td>
</tr>
<tr>
<td>EEL</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Denotes a significant difference from the control group.
#Denotes a significant difference from the EEL group.

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Figure 5

(A) Adenosine (mg/100mg tissue)

(B) ADA (U/g protein)

(C) XO (U/g protein)

(D) Uric acid (U/100mg tissue)
Fig. 8

(A) Plasma GSH/GSSG

(B) Hepatic GSH/GSSG

(C) Plasma G6PD (U/L)

(D) G6PD (U/100mg tissue)
Hepatic PDK-4 (ng/100mg tissue)

HF         -           -            +            +
EEL        -          +             -            +

Fig. 9

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Fig. 10

EEL → HF → Uric acid production → G6PD → Oxidative stress → Lipid peroxidation, Insulin resistance, Triglyceride → NAFLD

- PDK-4
- Glycogen
- Glucose Utility
- Lactate

- HF (Heart Failure)
- G6PD (Glucose 6-phosphate dehydrogenase)
- NAFLD (Non-alcoholic fatty liver disease)