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Modulatory Effects of Oleanolic Acid on Cardiac Anti-Oxidant Status and Inflammatory Response in High Fructose-Fed Neonatal Sprague-Dawley Rats

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ABSTRACT

This present study investigated the antioxidant and inflammatory properties of oleanolic acid (OA) on neonatal rats administered with high fructose diet (HFD). Neonatal rats (24) were assigned at random to four (4) groups namely: Group A (control) which had distilled water only; Group B was administered with OA only; Group C was administered with HFD; Group D received HFD and OA. Animals were administered orally using orogastric gavage at a dosage of 10 ml/kg for 7 days (postnatal day 7-14. The antioxidant status of the hearts such as TEAC, Ferric Reducing Anti-oxidant Power, FRAP, Trolox Equivalence Antioxidant Capacity and oxidative stress biomarkers (MDA, Malondialdehyde and GSH, Glutathione) were evaluated using standard procedures. The levels of inflammatory cytokines in the hearts were determined using magnetic bead-based assays procedure. **T**he TEAC values were significantly decreased in HFD+OA treatment ($p < 0.05$) in comparison with HFD group. Glutathione concentration in the HFD group had significant increase ($p < 0.05$) following treatment with oleanolic acid. FRAP values and MDA level were significantly ($p < 0.01$) elevated post exposure to HFD and treatment with oleanolic acid insignificantly decreased MDA level when compared with HFD group. The pro-inflammatory cytokines (IL-1β, IL-6, IL-12, IFN-γ, TNF-a and MCP-1) were significantly ($p < 0.05$) increased HFD group when compared to the control. Oleanolic acid administration significantly reduced inflammation in postexposure to HFD. Neonatal intake of oleanolic acid may help to prevent inflammation and oxidative damage in the progression of cardiovascular related diseases.

Keywords: High fructose diet (HFD), Oleanolic acid (OA), Inflammation, Oxidative stress .

Introduction

Fructose is a simple sugar which is generally known as fruit sugar and a major constituent of sweeteners such as honey, table sugar and high fructose corn syrup.[1] Fructose is a common ingredient of the Westernized food because it is less expensive as well as production efficiencies and growing toddlers population is being fed with high fructose diet in the early days of postnatal life.[2] Excessively consuming fructose by neonates results in the development of cardiometabolic dysfunctions via oxidative stress and inflammation amongst other mechanisms.[3] Oxidative stress develops from an imbalance between buildup of oxidants in a biological system and capacity of the living system to antagonize same oxidants.[4]

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Lipid peroxidation, protein oxidation, DNA damage, and ROS interference with signal transduction pathways are consequences of the redox imbalance.[5] Inflammation is a biological process which makes immune cells to release various chemicals called cytokines which mobilize other immune cells to the site of attack by the oxidant.[6] Inflammation become chronic when the condition is there continuous inflammatory reaction which leads to destroy the tissues.[7] The pro-inflammatory cytokines are released multifaceted disease that causes heart failure.[8] The cytokines are also involved in the modulation of cardiovascular function by supporting the remodeling of the left ventricle, initiation of the dysfunction of contractile properties and uncoupling of myocardial beta-adrenergic receptors.[9] There is visceral fat deposit in fructose-mediated neonatal programming both in single hit and a double hit models,[3] which can be an early sign of cardiac complications. Medicinal plants use in treatment and prevention of diseases are gaining more attention due to health benefits that are provided by their constituent phytochemicals. Oleanolic acid, abbreviated as OA, is a bioactive phytochemical that is rich in Oleaceae family plants. Oleanolic acid is structurally made up of pentacyclic triterpenoid which is synthesized through the acetic acid-mevalonic acid reaction pathway and cyclization.[10] Oleanolic acid possesses biological properties some of which are anti-oxidant, anti-tumour, anti-microbial, anti-diabetic properties and anti-inflammatory properties.[11] Administration of oleanolic acid during developmental period has been shown to ameliorate oxidative stress without adversely affecting health

outcomes related with metabolic syndrome or precocious gastrointestinal tract development in neonatal rats.[12] We have also recently shown the impact of oleanolic acid in controlling glucose homeostasis in fructose-induced derangements through peroxisome proliferator-activated receptor gamma (PPARγ) expression.[13] Oleanolic acid has also been shown to have beneficial cardiovascular benefits. These cardiovascular beneficial properties included cardioprotection during Ischaemia,[14] improved antioxidant status of the heart in streptozotocin-induced diabetic rats[15] and amelioration of cardiac risk factors in pore-diabetic rats.[16] Previous experimental works on the health promoting effects of OA have been conducted in adult animal models. Given the impact of high dietary fructose in the perinatal period on later health outcomes, it is important to investigate the potential benefits of strategic intake of Oleanolic acid during the early postnatal period on Cardio-metabolic health. The study investigated the anti-oxidant and anti-inflammatory effects of OA in the hearts of neonatal rats that were administered with HFD.

Materials and Methods

Ethical consideration
Ethical approval was granted by the University of the Witwatersrand Animal Ethics Screening Committee, South Africa with clearance ID: 2014/47/D.

Research animals
Sprague Dawley breed of *Rattus norvegicus* were used in the study. Each rat dam had between 8 and 12 pups as provided by the University of the Witwatersrand Research Animal Facility. Perspex cage with wooden shavings as bedding was used to house each of the rat dam and its pups. The room temperature of 25±2°C was maintained. Light and dark cycle of 12-h was followed with lights on by 7 am. Throughout the experiment, the dams were fed regular rat food (Epol®, Johannesburg, South Africa) with water *ad-libitum*.

Study design
Seven-days old rats (pups) where used in this interventional, randomized prospective study. Each nursing dam's litter was allocated randomly to one of the four experimental groups, with each treatment group consisting of six rat pups. To avoid dam-effect bias, littermates were added to one of the four separate groups at random. The rat pups were distinguished using a color-coded numbering method which involved painting their tails with non-toxic ink of permanent markers in various colors. The rat pups were weighed regularly to assess their health and to ensure a consistent dosage per body weight. The different groups included;

Group A (Control): Rats were received with distilled water.

Group B (Oleanolic acid - OA): Rats were administered with OA (60 mg/kg).[3]

Group C (HFD): Rats were administered 25% HFD only.

Group D (OA+HFD): Rats received OA (60 mg/kg) and HFD (25% w/v).

The fluids were administered at 10 ml/kg body weight once a day. Pups were given the dietary treatments (60 mg/kg OA) prophylactically for 7 days. On postnatal day 14 the pups, were euthanized. The blood was obtained via cardiac puncture into heparin-coated microtubes, centrifuged and the plasma separated, then stored in -20ºC. The hearts were also removed, made frozen in liquid nitrogen, and preserved in cryovial tubes at -80ºC freezer for later analysis.

Evaluation of anti-oxidant capacity
Ferric reducing anti-oxidant power assay
This assay was conducted with the method of Benzie and Strain.[17] Briefly, 100 µl cardiac homogenate was added to FRAP reagent (200 µl). The reagent was prepared by adding buffered acetate (30 ml, pH 3.4), tripyridyl triazine (TPTZ, 3 ml), $FeCl_3$ (3ml) and dH_2O (6.6 ml) together as a mixture. The sample was then incubated for 30 min at room temperature and absorbance measured at 539 nm. The standard was ascorbic acid (AA), and the results were given in µmole ascorbic acid equivalents/g sample

Trolox equivalent anti-oxidant capacity assay
This assay was conducted based on 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging principle as described previously.[18] The ABTS reagent was prepared by adding $K_2S_2O_8$ (88 µl) to ABTS (5 ml) and was allowed to stay for 24 hr before it was used. The heart homogenate (25 µl) was added to ABTS (200 µl) solution and incubation was done for 30 min at room temperature. The absorbance of the mixture was measured at 734 nm. The standard solution used was Trolox and the results were rexpressed as µmol Trolox equivalent/g.

Thiobarbituric acid reactive substance assay
This assay measures the level of malondialdehyde (MDA) which is a marker of oxidative stress. It was conducted according to method previously reported.[19]. MDA levels were determined using prepared solution (4 µM MDA) as diluted from 4 mM MDA stock. The heart homogenate (50 µl) was added to the of prepared MDA solution (40 µM), this was followed by addition of 15 % (w/v) trichroloacetic acid (TCA, 100 µl) and thiobarbituric acid (TBA, 0.375%). The sample was subjected to heating at boiling temperature for 15 min, and was allowed to cooled, for it to be centrifuged at 3000 g for 5 min. The absorbance of the mixture was read at 535 nm, and the results were reported as nmolMDA/ng protein.

Glutathione assay
Determination of the antioxidant glutathione (GSH) was conducted using the standard method. [20] Glutathione standard solution of 0.001 M GSH in 0.2 M EDTA solution was prepared. The EDTA dilutions were ranged between 0 - 250 µl. Reaction mixture consist of phosphate buffer (50 µl), sodium azide (NaN_3) (10 µl), GSH (20 µl), H_2O_2 (1 µl), heart homogenate (50 µl) with distilled water. Incubation of sample was done at 37ºC for 3 min, followed by the addition of TCA (50 µl). The final mixture was centrifuged at 3000 × *g* at 4ºC for 5 min. Supernatant (50 µl) was used to determine GSH as it was being added to 100 µl of phosphate buffer and 50 µl of DTNB. The wavelength was read at 412 nm. GSH concentration was extrapolated from a standard curve and results expressed as µM/mg protein.

Measurement of inflammatory biomarkers
Using the Luminex platform, the plasma levels of the interleukins (IL-1β, IL-6 and IL-12), monocyte interferon- γ (IFN-γ), tumor necrosis factor-α (TNF-α) and chemoattractant protein-1 (MCP-1) in the heart samples were assessed as previously described.[31] Following optimization experiments, undiluted samples were blindly assessed. In this assay, Bio-Plex Pro kits b (Bio-Rad Laboratories, Hercules, CA, USA) were used according to the manufacturer's instructions. For all analytes, the standard curve varied from 3 to 12000 pg/mL. For bead capture and analysis, the Bio-Plex Manager software, version 6.1, was employed.

Statistical analysis
Data analysis was done using one-way analysis of variance and reported as mean ± SEM. Student t-test and Tukey post-hoc test was performed using Microsoft Excel 2010 version, and SPPS 16.0 version. Results were considered significant at $^{**}p < 0.01$ and $^{*}p < 0.05$.

Results and Discussion

Oleanolic acid on anti-oxidant capacity

Diets rich in fats and refined sugars have widely been part of the contributing factors and even the main drivers of the increased cardiovascular diseases.[21] The management of cardiovascular diseases places a heavy burden on health care globally. Cardiovascular disease is associated with inflammation and oxidative stress.[22] and they are both closely connected with both the progression of cardiovascular

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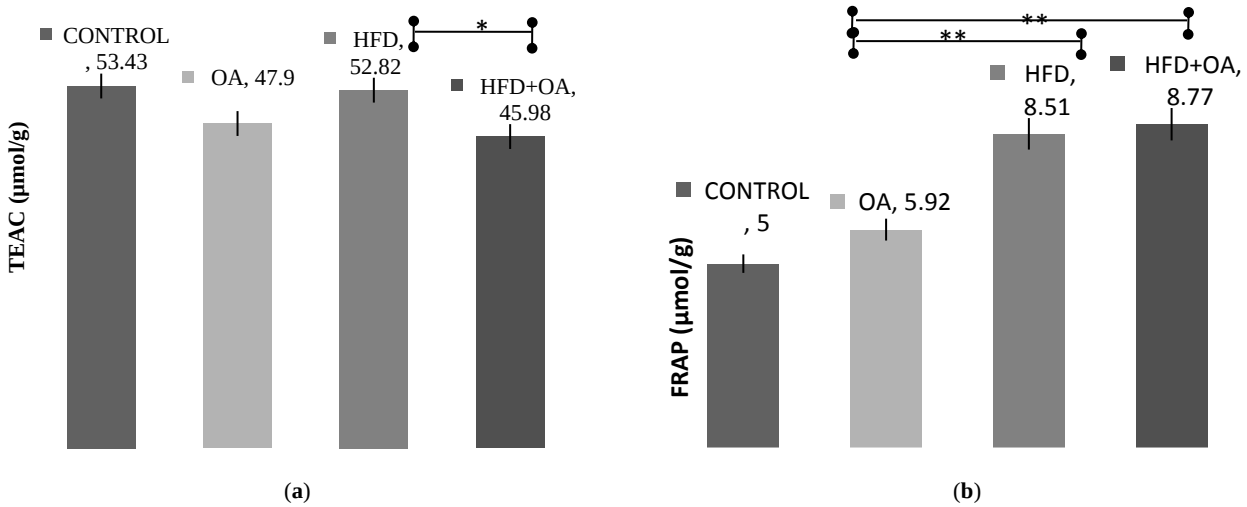
derangements and acute coronary syndromes.[8] Oleanolic acid from plants source and the synthetic derivatives have been shown to have a variety of biological properties in a variety of disease models via various action mechanisms..[11] The anti-oxidative and anti-inflammatory potentials of OA are supported by our findings from our study. TEAC, FRAP and ABTS assays are based on the capacity of antioxidants in samples to scavenge radical cations.[26] They assesses anti-oxidant's ability to neutralize reactive species produced in organic and aqueous environments.[23] The capability of a material to chemically reduce the vivid blue ferric tripyri-dyltriazine complex to its ferrous form is also measured by the FRAP assay.[17] However, in this study, TEAC values were decreased following treatment of HFD group with OA while there were significant increase in FRAP in both HFD and HFD+OA ($p < 0.01$) as against the control group (Figure 1).

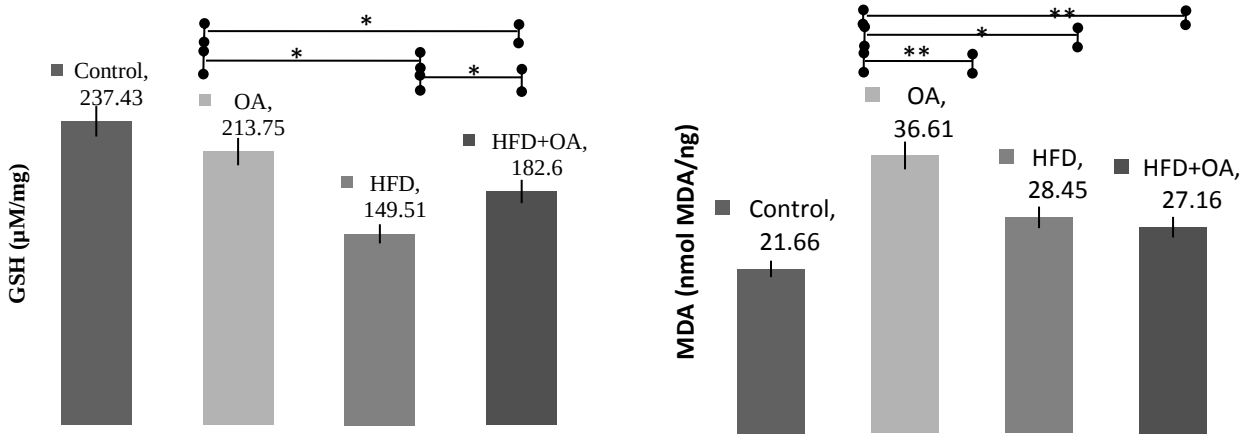
Oleanolic acid on oxidative stress biomarkers

Glutathione (GSH), a tripeptide which act by scavenging the lone pair of electron from free radicals either from the peroxidized lipids or from other sources, thus becoming oxidized glutathione (GSSG). The peroxidized lipid has its end product as MDA which is commonly quantified to assess the level of damage to the lipid biomembrane of the cells. Our findings demonstrated that neonatally administering of HFD to rat pups increased MDA concentration significantly which shows that HFD provokes oxidative overload. Treatment with OA did not significantly increase the level of MDA in rats that were fed with HFD (Figure 2b). However, it has been reported that OA extracted from Ligustrum lucidum exhibited an anti-oxidative role by reducing the MDA concentration in alloxan induced-diabetic rats.[25] OA has also been shown to have beneficial antioxidant effects on red blood cells in streptozotcin-induced diabetic rats.[27] The differences in these studies compared to the current one can possibly be attributed to different experimental models used to investigate the effect of OA on the heart. We used a high fructose diet while the previous studies used chemically induced methods. Furthermore, it is well known that there are differences in the metabolism and substrate preference of hearts of neonates compared to those of adults[28] and this may also have had an impact on the findings. A significant reduction of GSH in the HFD-administered ratswas reported in comparison with the control group (Figure 2a). Treatment with OA significantly increased the concentration of GSH in neonatal rats that were fed with HFD. This result is similar to Wang, Ye[24] who reported an increased GSH production and expression of key antioxidant enzymes as a result of OA treatment.



(**a**) (**b**)

Figure 1: The effect of orally administered oleanolic acid on antioxidant capacity; (a) TEAC (µmol Trolox equivalent/g) (b) FRAP (µmol Ascorbic acid equivalent/g) concentrations. Data represented as mean ± SEM; n = 6 per treatment. Control = 10 ml/kg dH_2O; oleanolic acid = 60 mg/kg; HFD = 10 ml/kg 25% w/v fructose; HFD + OA = 10 mL/kg 25% fructose + 60 mg/kg of oleanolic acid. ** $p < 0.01$ and * $p < 0.05$ were considered significant.



(**a**) (**b**)

Figure 2: The effect of orally administered Oleanolic acid on oxidative stress biomakers; (a) GSH (µM/mg) (b) MDA (nmol MDA/ng protein) concentrations. Data represented as mean ± SEM; n = 6 per treatment. Control = 10 ml/kg dH2O; oleanolic acid = 60 mg/kg; HFD = 10 ml/kg 25% w/v fructose; HFD + OA = 10 mL/kg 25% fructose + 60 mg/kg of oleanolic acid. ** $p<0.01$ and * $p<0.05$ were considered as significant.

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Oleanolic acid on Inflammatory biomarkers

Early developmental exposure of both animals and humans to inappropriate diets, such as high fructose diets, affects the expression of anti-inflammatory and pro-inflammatory genes involved in lipid and glucose metabolism.[3,29] In pathological inflammation, the cascade in a complex whole-cellular pathway starts with generation of excess free radicals that often comes from mitochondria.[8] Cytokines are small soluble proteins that give instructions and act as mediators in communication among immune and non-immune cells.[30]

Pro-inflammatory cytokines like interleukin-1 beta (IL-1β), interleukin-1 alpha (IL-1α), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10). tumor necrosis factor (TNF-α) and interferon gamma (INF-γ) have been reported to be involved with heart failure and dilated cardiomyopathy.[9] Findings from this study corroborate this claim, as there were significant increases in the levels IL-1β, IL-6, IFN-γ, TNF-α and MCP-1 in rat pups fed with HFD when compared with control pups. Neonatal treatment with HFD and OA significantly ameliorated increased IL-1β, IL-6, IFN-γ, TNF-α and MCP-1 concentrations as against the HFD fed pups (Figure 3a – f).

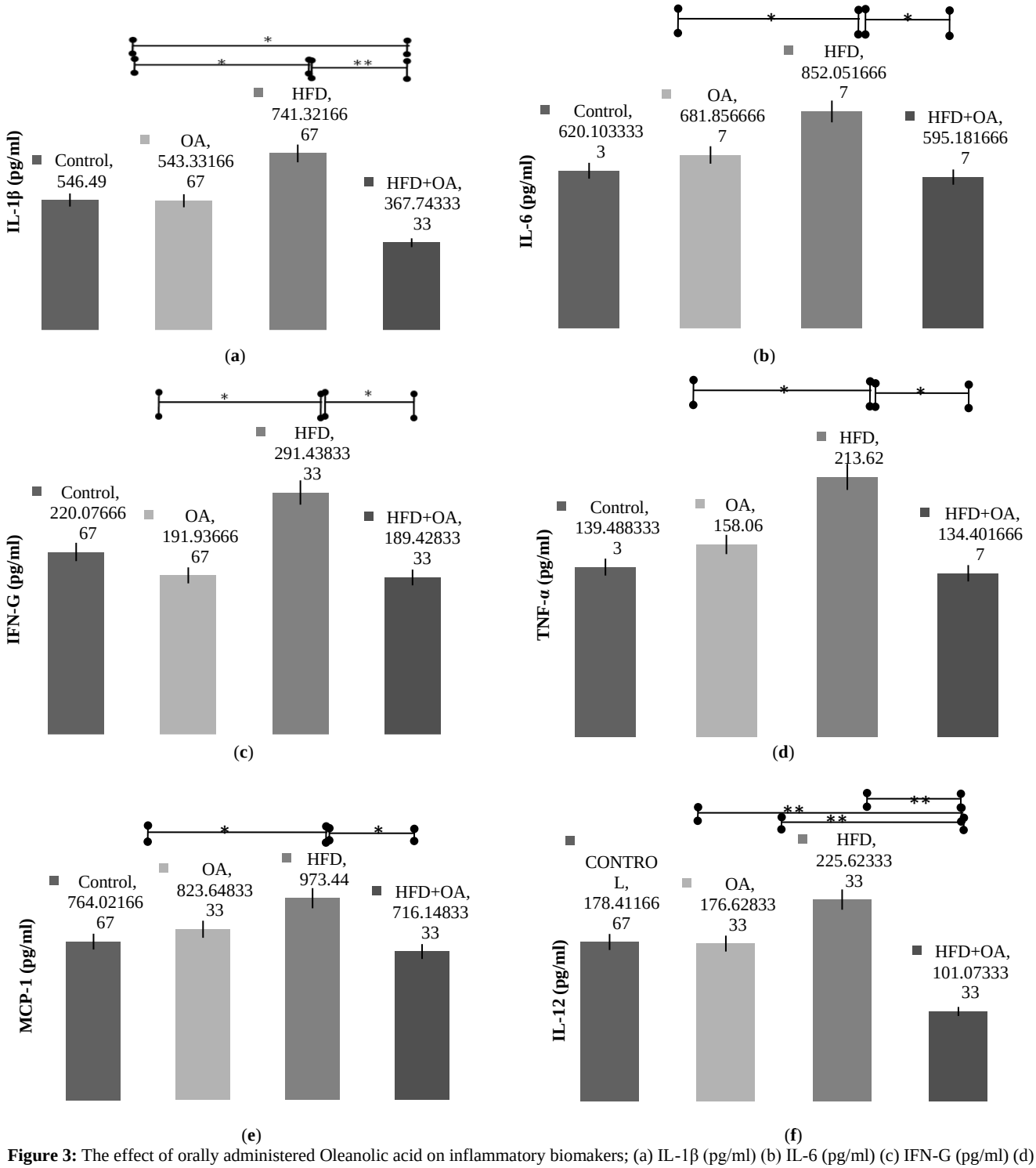

Figure 3: The effect of orally administered Oleanolic acid on inflammatory biomakers; (a) IL-1β (pg/ml) (b) IL-6 (pg/ml) (c) IFN-G (pg/ml) (d) TNF-α (pg/ml) (e) MCP-1 (pg/ml) (f) IL-12 (pg/ml) concentrations. Data represented as mean ± SEM; n = 6 per treatment. Control = 10 ml/kg dH2O; oleanolic acid = 60 mg/kg; HFD = 10 ml/kg 25% w/v fructose; HFD + OA = 10 mL/kg 25% fructose + 60 mg/kg of oleanolic acid. ** $p < 0.01$ and * $p < 0.05$ were considered as significant.

This is supported by Wang, Ye,[24] who reported that upon treating insulin resistant db/db mice with OA, there were evidences of improved inflammatory responses as seen in the concentrations of the inflammatory cytokines assayed in the heart of the pups.

Conclusion

The study revealed the antioxidant and inflammatory properties of oleanolic acid (OA) on neonatal rats fed with HFD. The data revealed that excessive fructose diet in neonates reduces anti-oxidant defense and promote the release pro-inflammatory cytokines. However, neonatal oleanolic acid supplementation has the potential to alleviate the consequences of HFD-induced oxidative stress and inflammation. Future studies are required to investigate whether these early life benefits on cardiac health can have long term cardioprotective properties and consequently reduce the burden of cardiac disease on healthcare provision.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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