Association between inflammation and the risk of cardiovascular disorders in atherogenic male rats: Role of virgin and refined olive oil

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Abstract: The aim of the present study was to determine changes in inflammatory markers, lipid profile and vascular wall integrity, (monitored as nitric oxide levels) in the male rats with experimental atherosclerosis. Also, to evaluate the role of two olive oils (virgin and refined) in these changes. Experimental atherosclerosis was induced by feeding rats normolipidemic diet (NLD) supplemented with (4% cholesterol, 1% cholic acid and 0.5% thiouracil, w/w) for three months. Feeding atherogenic diet (AD) exhibited marked elevation in serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL) and triglycerides (TG), along with decreased high density lipoprotein cholesterol (HDL-C). Besides, an elevation in serum level of the two inflammatory markers, tumor necrosis factor-α (TNF-α) and fibrinogen was demonstrated with a lowered nitric oxide (NO) levels in both aorta and cardiac tissues, indicating impaired vessel wall integrity and development of cardiovascular disorders in response to hyperlipidemia and enhanced inflammation. Subsequently, marked elevations in total leucocytes and other inflammatory mediators, including monocytes and lymphocytes have been recorded in the atherogenic diet fed rats. In addition, a significant reduction in erythrocytes count, hemoglobin (Hb) content and other hematologic indices was demonstrated, indicating further signs of inflammation. However, administration of olive oil (OO) [(in particular virgin olive oil (VOO)] to atherogenic rats exhibited improved inflammatory status, lipid profile and NO levels. Therefore, VOO might be a good candidate to replace other fats in the functional food for retarding atherosclerosis and risk of cardiovascular disorders.
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1. Introduction:

Atherosclerosis is a slow and complex disease that represents one of the most prevalent causes of death world wide (Jaffer *et al.,* 2006). It is an arterial disease in which fatty substances, cellular wastes, and other elements build up in the inner lining of the artery. This build is called plaque that can grow large enough to significantly reduce blood flow throw the vessel (Earnest e*t al.,* 2005). Within the arterial wall, many processes act in a seemingly concerted manner to initiate the formation of lesions that result in occlusion of blood flow (Ross 1999). These processes include injury to the endothelium and retention of lipoproteins within the arterial wall, (Brown *et al.,* 2004) which, in turn leads to the formation of foam cells and the development of atherosclerosis (Hansson, 2009).

In broad outline, atherosclerosis can be considered to be a form of chronic inflammation resulting from interaction between lipoproteins, monocyte-derived macrophages, T cells, inflammatory cytokines and the normal cellular elements of the arterial wall. This inflammatory process can ultimately lead to the development of complex lesions, or plaques, that protrude into the arterial lumen (Glass and Witztum, 2001). It is now recognized that atherosclerosis is an inflammatory disease of the arterial wall that underlies many of the common causes of cardiovascular diseases (CVD) (Zhang, 2009).

Dietary, oils may affect vascular wall integrity and consequently the process of atherosclerosis and its related cardiac complications (Sekalska *et al.,* 2007). Among edible oils, olive oil (OO) is the only one produced from a fresh fruit and can be consumed just as obtained from the olive, like fruit juices. It is a pure natural product requiring and is becoming increasingly present in food as the healthiest alternative to other oils (Perona *et al.,* 2005). Olive oil is extensively used by people in the

Mediterranean region that has been associated with reduced incidence of CVD in these people (Nagaraju & Belur, 2008). This may be the result of its high content of monounsaturated fatty acids (MUFAs) mainly oleic acid and minor biologically active components, such as phenolic compounds (PhCs) (Miles *et al.,* 2005).

The present study was carried out to evaluate the effect of prolonged intake of OO on the risk of cardiovascular disorders in male rats with experimental atherosclerosis, in terms of selected inflammatory markers and lipid parameters, as well as NO levels. Two olive oils; refined olive oil (ROO) and VOO (identical in their fatty acids composition, but different in phenolics content) were used to compare their effects.

2. Materials and methods

Experimental animals:

This study was performed on male albino rats of Wistar strain, initially weighing 105±5 g. Rats were obtained from the Institute of Ophthalmic Disease Research, Cairo. They were housed in stainless steel cages at a well ventilated animal house. Rats were permitted adequate standard diet and given water *ad libitum* for one week of adaptation period prior to the experimental work.

Research design:

Rats were randomly divided into six groups of five animals each. The first one was the control group received NLD without any supplementation (The Nutrient Requirements of Laboratory Animals, 1995). The second and third groups were fed NLD supplemented with 10% ROO or VOO, respectively (El-Seweidy *et al.,* 2005). The fourth group received AD (NLD supplemented with 4% cholesterol, 1% cholic acid and 0.5% thiouracil, w/w) (Parmer and Kar, 2007), while the fifth and sixth groups received AD supplemented with ROO (10%) or VOO (10%), as described in 2nd and 3rd groups. All animal groups were maintained on their respective diets for duration of three months.

Samples collection:

At the end of the study period, overnight fasted rats were sacrificed under ether anesthesia. From each rat, two blood samples were collected. The first blood sample was taken on EDTA as anticoagulant for determination of hematological parameters. The second blood sample was collected with no additives to obtain serum by centrifugation at 3000 r.p.m for 10 minutes for biochemical analysis. Immediately after collecting blood, the heart and aorta from each rat were removed, and placed into clean and dry tubes. The tissues were then homogenized using a homogenizer surrounded with an ice jacket and the homogenate was kept frozen at $-20C^0$ until being analyzed.

Biochemical analysis:

Serum concentrations of glucose, TC, HDL, and TG were determined calorimetrically using Spectrophotometer with Kits from Spinreact Co., according to the manufacture instructions, as described by McCleary and Codd (1991); Zoppi and Fellini (1976); Wahlefeld (1974); Naito (1984), respectively. LDL-C and VLDL:-C, as well as atherogenic index (AI) was calculated according the following equation:

LDL-C = TC – HDL-C – TG/5 (Ahmedi *et al;* 2008). VLDL-C = TG/5 (Satheesh and Pari, 2008).

AI = (VLDL-C + LDL-C) / HDL-C (Pandya *et al.,* 2006).

The concentration of nitric oxide (NO) was measured by determination of total nitrate and nitrite concentrations according to the method of Green *et al.* (1982).

Inflammatory and hematologic analysis:

Serum TNF-α was measured, using ELIZA technique, that is a solid phase enzyme amplified sensitivity immunoassay, according to Aggarwal (1985). Quantitative determination of fibrinogen in serum was carried out using Multifibren – U – Kit, as described by Cooper and Douglas (1991).

Red blood cells (RBCs), platelets (Plts) and total white blood cells (WBCs) count was measured by hemocytometer neubauer slide, while differential count of WBCs was determined by Leishman's stained blood film (Harvey, 2001). Whole blood Hb was measured colorimetrically by Randox kits according to Haemat (1967). Hematocrit (Hct) value was determined using hematocrit capillary tubes. The percent of Hct was determined by the use of microscopically reader (Alwan *et al.,* 2009). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated using the formulae mentioned by Dacie and Lewis (2001).

Statistical analysis:

All data are represented as means ± SE. One-way analysis of variance ANOVA was used to analyze the results. The level of statistical significance at $P < 0.05$ was performed by Least Significant Difference (LSD) test (SPSS Inc. 1999).

3. Results

The present study investigates the impact of two types of olive oil (OO) [(refined olive oil (ROO)

and virgin olive oil (VOO)] on biochemical, inflammatory and hematological changes developed in male rats with experimentally induced atherosclerosis. The statistical evaluation of obtained data were calculated for the atherogenic group according to the normolipidemic diet (NLD) group and for OO+AD treated groups according to both the atherogenic and the NLD groups (no comparison between the two groups OO+AD and ROO, VOO groups were carried out).

In the present study, administration of ROO or VOO to normal rats did not produce significant changes in all measured parameters if compared to NLD group (Table 1, 2, 3, 4). On the other hand, administration of atherogenic diet exhibited elevated serum lipids (TC, TG and LDL-C), along with decreased HDL-C concentrations. Besides, serum glucose level was increased in the atherogenic animals, if compared to NLD group (Table 1). Meanwhile, the levels of NO in both aorta and cardiac tissues showed significant reduction (Table 2), along with marked elevation in the serum levels of the two inflammatory markers, TNF-α and fibrinogen (Table 3). Additionally, Table 4 showed elevations of Plts count, as well as WBCs, monocytes and lymphocytes, concomitantly with a reduction in RBCs count, Hb content and other indices (Hct%, MCV, MCH and MCHC) in the atherogenic rats, in comparison with NLD group. On the other hand, feeding rats an atherogenic diet supplemented with ROO or VOO caused an ameliorative effect on all these parameters, as evidenced by restoration of the changed lipid profile, inflammatory markers, NO level and hematologic indices near to normal values. However, the greatest effect was recorded with VOO administration.

Table (1): Effect of refined or virgin olive oil on serum lipid profile and glucose level in atherogenic diet fed rats.

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
TC (mg/dl)	189.5±3.62	189.1±3.00	182.2±2.51	382.1±9.57^{a}	247.0±5.74^{ab}	204.6±2.96^{abc}
TG (mg/dl)	125±0.43	121.6±2.25	118.9±1.41	183.6±3.37^{a}	163.2±3.47^{ab}	128.5±1.08^{bc}
LDL-C(mg/dl)	96.9±2.22	96.5±1.12	89.5±2.01	316.3±8.14^{a}	179.6±4.59^{ab}	121.1±2.08^{abc}
VLDL-C(mg/dl)	25.00±0.09	24.31±0.45	23.78±0.23	36.72±0.67^{a}	32.64±0.69^{ab}	25.70±0.21^{bc}
AI	1.80±0.03	1.77±0.07	1.65±0.04	12.12^{a}±0.29	6.10±0.31^{ab}	2.54±0.02^{abc}
HDL-C (mg/dl)	67. 7±1.54	68.3±2.70	68.9±1.71	29.1±1.10^{a}	34. 8±1.68^{ab}	57.7±0.98^{abc}
Glucose (mg/dl)	106.7±1.88	105±3.08	104.3±1.09	125.3±3.28^{a}	121.0±1.09^{a}	111.3±1.18^{bc}
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

Table 2: Effect of refined or virgin olive oil on aorta and cardiac nitric oxide (NO) level in atherogenic diet fed rats

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
Aorta NO (µmol/g)	1.6±0.02	1.6±0.04	1.7±0.02	0.9±0.07^{a}	1.0±0.06^{a}	1.5±0.04^{bc}
Heart NO (µmol/g)	1.1±0.16	1.2±0.18	1.3±0.19	0.5±0.10^{a}	0.9±0.08^{ab}	1.0±0.10^{b}
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

Table 3: Effect of refined or virgin olive oil on serum level of tumor necrosis factor- α (TNF-α and fibrinogen in atherogenic diet fed rats

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
TNF-α (pg/mg)	7.4±0.13	7.2±0.12	6.7 ±0.17	11.0 ±0.45[a]	9.6±0.20[ab]	7.6±0.15[bc]
Fibrinogen (mg/dL)	223.6±0.93	223.2 ±1.96	222.2±1.36	389.0 ±5.00[a]	333.0 ±3.48[ab]	234.6±2.27[abc]
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

Table 4: Effect of refined or virgin olive oil on different hematologic parameters in atherogenic diet fed rats

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
WBCs ($X10^3/\mu L$)	5.2±0.17	5.1±0.16	5.0±0.34	6.5±0.34[a]	6.1±0.62	5.2±0.40[bc]
Lymphocytes%	42.2±1.10	43±2.10	40±0.60	55.6±1.96[a]	47.4±1.12[ab]	43.2±1.77[bc]
Monocytes %	5.60±0.37	5.60±0.25	5.40±0.25	6.8±0.58[a]	6.0±0.45	5.8±0.58[b]
PLts ($X10^3/\mu L$)	228.6±5.09	229.6±6.55	227.0±6.61	387.8±15.92[a]	268.8±8.72[ab]	231.0±9.56[bc]
RBCs ($X10^6/\mu L$)	5.5±0.09	5.8±0.26	5.9±0.22	4.2±0.27[a]	4.2±0.07[ab]	5.1±0.20[bc]
Hb(g/dL)	13.3±0.66	12.7±0.24	13.4±1.00	9.0±0.40[a]	10.3±0.37[a]	10.5±0.17[ab]
Hct%	41.8±1.59	40.7±0.57	41.2±1.30	27.2±1.65[a]	27.9±0.79[a]	39.8±1.70[bc]
MCV (*f*L)	76.6±4.23	75.7±2.67	76.6±1.45	57.9±1.66[a]	70.8±2.56[b]	76.0±1.36[b]
MCH (pg/ml)	22.4±1.14	21.3±0.66	21.8±0.25	19.7±0.51[a]	20.5±0.78[a]	20.8±0.59[b]
MCHC%	31.2±0.99	31.0±0.32	32.4±1.36	26.3±2.29[a]	28.5±1.48[a]	30.8±1.11[b]
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

4. Discussion

Atherosclerosis is generally assumed to develop following prolonged exposure of the vascular wall to elevated levels of cholesterol and glucose and also to several inflammatory and hematological changes. However, due to special anatomical position of the vascular endothelium (between circulation and vascular wall) it is a primary target for these risk factors, which in turn causes endothelial dysfunction. Dysfunction of vascular endothelium appears to be a key event in initiating, progression and complications of atherosclerosis (Vallance & Chan, 2001). One of the principal mechanisms underlying endothelial dysfunction is through increased generation of reactive oxygen species (ROS) with consequent oxidative stress (OS) (Hassanabad *et al.,* 2010). Lipid metabolic abnormalities are considered the main cause for increased OS under atherogenic state (Rizzo *et al.,* 2009). However, hyperglycemia (occurred as a result of insulin resistance) associated with lipid abnormalities seemed to be also responsible (Ferroni *et al.,* 2006). In this regard, the present study has shown that feeding rats an atherogenic diet induced elevations in serum levels of glucose and lipids (TC, LDL-C and TG), which may reflect increased generation of ROS that contribute to atherosclerosis development.

Increased ROS is suggested to play a role in atherosclerosis by inducing endothelial dysfunction characterized by impaired production of NO (Balakumar *et al.,* 2009). ROS including O_2^- can react with NO to form peroxynitrite ($OONO^-$), thus inactivating NO and directly decreased endothelium synthesis of NO (Perona *et al.,* 2006). Consistent with this, it was reported that patients with developing atherosclerosis have reduced NO bioavailability in both coronary and peripheral vasculature (Barbato & Tzeng, 2004). Additionally, functional NO has been demonstrated to decrease in plasma and tissues of experimental atherogenic conditions (Deepa and Varalakshmi, 2004). In the

present study, observed finding of decreased aorta and cardiac NO levels following atherogenic diet feeding could therefore indicate development of atherosclerosis with increased risk of CVD.

Nitric oxide normally functions to maintain vascular homeostasis through a number of physiologic processes. One prevalent action involves the activation of soluble guanyl cyclase, which then produces cyclic guanisine monophosphate (cGMP), being responsible for vasorelaxation (Dhir and Kulkarni, 2007). Nitric oxide can also act directly on calcium dependent potassium channels, leading to relaxation of vascular smooth muscles (SMCs) (Costa & Assreuy, 2005). Moreover, the vasoprotective effect of NO includes promotion of endothelial proliferation and protection of endothelial cells (ECs) from apoptosis and adherence of inflammatory cells (Hengartner, 2000) thus serves to limit endothelial inflammation. Based on this, the reduction in NO, as in case of atherosclerosis has suggested to promote pro-inflammatory endothelial response (Napoli *et al.,* 2006). It involves enhanced vulnerability of vascular wall to circulating leukocytes and other inflammatory mediators, leading to atherosclerotic vascular injury (Naseem, 2005). It is now clear that inflammatory mediators are intimately involved in atherogenic process (Libby, 2002), thereby demonstrating atherosclerosis as an inflammatory disease (Burger-Kentischer *et al.,* 2006).

Excessive inflammation leads to patho-logical situation that including leucocytes migration into the inflammatory site. During vascular atherosclerosis, leucocytes and ECs are the major cellular players of the inflammatory reactions, while numerous mediators are involved in augmenting the inflammatory process (Zakynthinos & Pappa, 2009). It was explained that ECs coordinate the recruitment of inflammatory leucocytes to sites of vascular injury that consequently result in production and release of inflammatory cytokines, which are large family of small chemoattractant proteins (Reape & Groot, 1999). Among these, TNF-α is recognized to have a major role in inflammation, apoptosis, cell proliferation and stimulating synthesis of other cytokines, which further augment expression of cell adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Hennig & Toborek, 2001). Circulating leucocytes (in particular monocytes) are attracted by these molecules and adhered to endothelium, from which they migrate to the subendothelial space. Once within the vascular wall, monocytes differentiate into macrophages, which express scavenger receptors, allowing them to scavenge oxidized LDL-C (Ox-LDL-C) (Fuhrman *et al.,* 2008). LDL-C can be oxidized by ECs, SMCs and macrophages present in the arterial wall and the formed Ox-LDL-C may directly damage the endothelium and contribute to atheroma plaque formation, through increased adherence and migration of monocytes and lymphocytes (predominantly T-lymphocytes) into the vascular wall (Quehenberger, 2005). T lymphocytes recognize specially antigens that are produced in plaques including Ox-LDL-C (McLaren and Ramji, 2008). In this regard, the study of Kentischer *et al.* (2002) demonstrated that monocytes, macrophages and T-lymphocytes are critical cells characterizing all stages of atherosclerosis. Once stimulated they increase production of several pro-inflammatory cytokines, such as TNF-α, which are important in initiating the expression of variety of genes that in turn promote cell adhesion and other processes required for progression of atherosclerosis (Zhang *et al.,* 2007). Further evidence that atherosclerosis is linked to inflammation is provided by the present observation that feeding rats on atherogenic diet induced marked elevation in several inflammatory markers, including total leucocytes, monocytes and lymphocytes, as well as serum level of the pro-inflammatory and pro-atherogenic TNF-α. In other studies, the interactions between these inflammatory mediators promote SMCs migration and proliferation which in turn leads to atheroma plaque rupture with subsequent adherence and aggregation of platelets, leading to progression of atherosclerotic injury (Chon *et al.,* 2006).

Platelets are circulating cells that play a key role in hemostasis system (Ni & Freedman, 2003). By adhering to damaged blood vessels PLts become activated, which in turn release PLts derived growth factor that promote plaque growth contributing to progression of atherosclerotic disease (Crowther, 2005). Besides, activated PLts play a role in atherosclerosis through providing other hemostatic factors, including fibrinogen (Ni and Freedman, 2003). This latter effect has already been demonstrated in the present study, as evidenced from the raised fibrinogen levels in parallel to the increased PLts count following atherogenic diet feeding. Raised plasma fibrinogen is associated with development of atherosclerosis and is considered a risk factor for CVD (Sato *et al.,* 2000). Several reasons for the association between fibrinogen and the risk of CVD have been suggested. They include the effect on plasma viscosity, coagulation, fibrinolysis (Junker *et al.,* 1998), promotion of vascular SMCs proliferation (Rauch *et al.,* 2007) and also the induction of PLts aggregation (Willoughby *et al.,* 2002). In addition, recent studies indicated that fibrinogen accumulation in the vessel wall may

contribute to the early vascular inflammation (Heffron *et al.,* 2009). Other studies showed that fibrinogen induced production of the systemic inflammatory marker C-reactive protein (CRP) in SMCs, both in messenger ribonucleic acid (m-RNA) and protein levels (Guo *et al.,* 2009), suggesting that fibrinogen possesses a pro-inflammatory properties which is related to atherosclerosis development. In support, Guo *et al.,* (2009) reported that macrophages accumulation in the arterial wall may be associated with increased plasma concentrations of both fibrinogen and CRP, two markers of inflammation thought to be early signs of atherosclerosis and CVD. Therefore, increased fibrinogen level as detected in this study may indicate enhanced inflammatory status under present atherogenic condition.

Apart from the role of the two hemostatic factors, fibrinogen and PLts in atherosclerosis there is evidence that RBCs may also serve as a potential partner in this process, through activation of the vascular endothelium leading to vascular inflammation and atherosclerosis (Blum, 2009). A number of changes can affect RBCs and thereby lead to development and progression of atherosclerosis. Researchers have indicated that RBCs are particularly exposed to oxidative hazards because of their specific role as oxygen carriers (Manna *et al.,* 1999). Under normal physiologic conditions, there is a steady state balance between the production of ROS and their destruction by the endogenous antioxidant defense system (Takenaka *et al.,* 1991). However, if ROS are over produced, as in atherosclerosis, this can damage both plasma membrane and cytosolic components, leading to oxidative hemolysis of RBCs and decreased survival of oxidized RBCs in circulation (Manna *et al.,* 1999). Therefore, the possibility of RBCs oxidative modification appears to be an explanation for the reduction in RBCs count and other hematologic indices (Hb, Hct, MCV, MCH and MCHC) observed under present atherogenic condition.

As a consequence of oxidative modification, drastic changes in RBCs morphology occur, causing RBCs activation thereby, microvesicles of RBCs are formed (Leopold & Loscalzo, 2009). Formation of membrane vesicles is associated with loss of plasma membrane asymmetry, consequently, RBCs shed microvesicles from their main body, and circulating levels of microvesicles are augmented, as in case of most CVD (Blum, 2009). Microvesicles activate ECs, attract leucocytes and enhance their adherence to ECs, as well as induce production of pro-inflammatory cytokines and upregulation of ICAM-1 involved in the recruitment of monocytes into the arterial wall, thereby promoting the whole cascade of inflammatory events, leading to evolution of atherosclerosis with increased risk of CVD (Kaperonis *et al.,* 2006).

In contrast to these multiple atherogenic events, there is compelling evidence that nutrition can affect genesis of atherosclerosis by modulating functional properties of vascular ECs. In particular the lipid environment of the vascular endothelium may profoundly influence the inflammatory response during atherosclerosis and thereby confer an overall beneficial effect (Ringseis & Eder, 2010). With the discovery of the relationship between dietary fat and atherosclerosis; olive oil rich diets have been acclaimed for its protective effects on several of changes associated with atherosclerosis and heart disease (Acın *et al.,* 2007). This was in accordance with the present observations, where feeding rats on atherogenic diet supplemented with VOO or ROO has shown lowered atherogenic hazards. This effect was observed mainly in terms of modulating a number of inflammatory markers. Besides, a reduction in the hyperlipidemic status, concomitant with increased NO levels were recorded. The present data showed also that VOO was more effective than refined ROO, where the maximal protection was observed with VOO administration.

Evidence from other studies indicated that each of VOO or ROO consists mainly of monounsaturated fatty acids (MUFAs), with minor constituents, like phenolic compounds (PhCs) that contribute to the stability of the oil and exhibit wide range of biological activities (Ruano *et al.* 2005). From previous studies, it was indicated that PhCs are influenced by procedures of OO extraction and that PhCs are lost when OO is refined (Mir´o-Casas *et al.,* 2001). Therefore, VOO obtained by physical procedures has high amounts of PhCs. Actually, both VOO and ROO contain similar proportions of MUFAs; however the total PhCs concentration is greater in VOO, which may contribute to the more beneficial properties of VOO compared to ROO, as observed in this study.

In some studies the healthy effects of OO on cardiovascular risk factors have been attributed to its high content of MUFAs, such as oleic acid (Massimo *et al.,* 2009). In fact, MUFAs are suggested to be effective in improving serum lipid profile, through a decrease in TC, LDL-C and TGs and increase in HDL-C (Moreno & Mitjavila, 2003). Besides, OO has been shown to lower blood glucose levels (Tahvonen *et al.,* 2005), which together with improved blood lipids (as shown in this study) may reflect some protection against development of atherosclerosis.

Other studies suggested that nutrients, including MUFAs may modulate atherosclerosis by

affecting vascular endothelium (Covas, 2007), through increasing the amount of oleic acid in the arterial wall and displacing saturated fatty acids (SFAs), while leaving polyunsaturated fatty acid (PUFAs). This change in vessel wall composition is favorable for three reasons: (1) Saturated fatty acids are atherogenic and favor platelet aggregation through decreasing prostacyclin and increasing thromboxan production. They can thus be considered prothrombotic substances. (2) Polyunsaturated fatty acids reduce platelet activity and prothrombotic capacity of the arterial wall. (3) The accumulation of MUFAs in the arterial wall decreases the expression of several endothelial adhesion molecules involved in the selective monocytes recruitment in the arterial wall (Perona *et al.,* 2006). Thus, oleic acid may contribute to prevention of atherosclerosis, mainly through a protective effect against atherogenic vascular wall lesions.

Despite the beneficial effects attributed to oleic acid, it is very likely that other minor components with antioxidant and anti-inflammatory properties, such as PhCs could be responsible for OO effects observed in this study and in other experimental trials. Oleuropin and its derivative hydroxytyrosol have the strongest radical scavenging properties among all olive oil PhCs (Oram *et al.,* 2004). Massaro *et al.* (2002) hypothesized that these components may exert direct vascular athero-protective effects by inhibiting endothelial dysfunction through quenching ROS and reversing the imbalance between increased OS and impaired antioxidant status. Association between OS and impaired endothelial function, characterized by reduced NO bioavailability have been demonstrated in humans and experimental animal models of atherosclerosis (Moraes *et al.,* 2007). Endothelial dysfunction could be reversed in atherogenic patients by administration of agents capable of scavenging ROS (Jackson *et al.,* 1998), such as OO and its PhCs (Covas, 2007). In this regard, it was reported that oleuropein and hydroxytyrosol are potent scavengers of ROS and O_2^- in neutrophils, which might prevent formation of the powerful oxidant peroxynitite ($OONO^-$) leading to an increase in NO levels, being linked to endothelial improvement and inhibition of atherosclerosis (Briante *et al.,* 2001). In support, the present data showed increased NO levels in both aorta and cardiac tissues in rats fed atherogenic diet supplemented with OO.

Other mechanisms have been suggested to explain beneficial effects of olive oil PhCs. They include: (1) Antioxidant protection of LDL-C from oxidative modification, either directly or with interaction with endogenous antioxidants likes vitamin E (Rajasekhar *et al.,* 2004). (2) Reduction of microphage uptake of Ox-LDL-C (Rosenblat *et al.,* 2008). (3) Increase in size of LDL-C particles that become less pro-atherogenic (Perona *et al.,* 2006). Apart from protecting LDL-C from oxidation, PhCs exhibited also some beneficial effects on PLts (Singh *et al*., 2006), fibrinogen and erythrocytes, as consequence of its antioxidant properties (Covas, 2007). PhCs from olive leaf extract significantly inhibited PLts aggregation *in vitro,* possibly through their H_2O_2 scavenging properties, which may offer a degree of protection from thrombosis and other CVD risk factors (Singh *et al.*, 2006). Moreover, OO with different phenolic contents were found to reduce fibrinogen levels, but increase RBCs count (Huang & Sumpio 2009), as well as Hb concentration, MCHC and Hct (Ashour *et al.,* 2007). Similar observations were also demonstrated with the present study, where feeding rats an atherogenic diet supplemented with OO showed a lowering effect on both PLts and fibrinogen levels, concomitant with elevations in RBCs count and other hematological indices (Hb, Hct, MCV, MCH, and MCHC), thereby suggesting a potent antiatherogenic role of OO.

Other studies in humans and animals evidenced that PhCs from OO have demonstrated anti-inflammatory effects, through inhibiting a number of inflammatory mediators released by endothelial cells (Ros *et al.,* 2004). De La Puerta *et al.* (1999) studied a range of VOO phenolics (oleuropein glycoside, caffiec acid and tyrosol) in rat peritoneal leucocytes, indicating inhibited production of leukotriene B_4 (LTB_4), which is a chemoattractant and activator of neutrophiles. Moreover, Miles *et al.* (2005) found a very strong effect of oleuropein glycocide on production of cytokines, IL-6 and TNF-α. Also, monocyte adhesion to ECs can be modulated by VOO- PhCs, through inhibiting mRNA expression of several adhesion molecules, including VCAM-1 (Carluccio *et al.,* 2003). This occurs as a result of interfering with activation of the most important transcription factor controlling endothelial activation, nuclear factor kB (NFkB) (Perona *et al.,* 2007). In this respect the present study showed marked reduction in total leucocytes, monocytes and lymphocytes, as well as the pro-inflammatory marker TNF-α in rats fed atherogenic diet supplemented with OO. The out come of these effects in all, is indicative of lowered inflammatory tendency in response to OO intake, which in turn may decrease the risk of atherosclerosis and cardiovascular disorders.

In conclusion, the results of this study illustrated the role of inflammatory changes as an etiology of atherosclerosis and further indicated the ability of OO (in particular VOO) to favorably influence these changes. Therefore, VOO may be

useful in designing dietary strategies to minimize development of atherosclerosis and related cardiovascular disorders in humans.

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