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Excesive consumption of unsaturated fatty acids leads to oxidative and inflammatory instability in *Wistar* rats

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ABSTRACT

Lifestyle modifications such as increase in high-fat food consumption importantly increases the risks for cardiovascular disease. The principal objective of this study is to analyze effects of different high fat diet (HFD) sources on haemodynamic parameters, lipid and oxidative profile, myeloperoxidase activity, and markers of inflammation (IL-6/pentraxin-3). HFD containing 20% of fat, provided by lard (saturated) or soybean oil (unsaturated), as well as control diet were administering to three groups (L, SO and C). Food efficiency ratio and plasma lipids were significantly elevated in both HFD groups. However, only SO group showed an increase in systolic arterial pressure, oxidative stress index, myeloperoxidase activity, liver lipids as well as markers of inflammation: IL-6 and pentraxin-3 (PTX3). In summary, these results indicate inflammogenic potential of excessive soybean oil consumption in triggering liver damage.

1. Introduction

Cardiovascular diseases (CVD) are a major cause of death and disability worldwide [1], and they are a great extent result of unbalanced diets and physical inactivity. Numerous studies in the 1950s and 1960s showed positive correlation among dietary saturated fatty acids intake (SFAs) and the risk of cardiovascular disease [2], thus SFAs deemed unhealthy. SFAs are often found in high-fat types of meats and dairy products, such as fatty beef, lard, sausage, cream, butter, and cheese. These types of fats are nominated as "bad" kind of fat that raise cholesterol levels in the blood and increase the risk of cardiovascular diseases [3,4].

Soybean oil, as a source of unsaturated fatty acids (UFAs), is the second largest source of vegetable oil, commonly used for making mayonnaise, salad dressing, margarine, and nondairy coffee creamers [5]. It is also a mainstay ingredient in many processed foods throughout the baking, bread-making, cooking, and supermarket supply industries, or for extensive use in the restaurants, hotels, cafeterias, and multiple fast food industry. Soybean oil is derived from soybean seed and has 50% linoleic acid, polyunsaturated omega-6 fatty acid (n-6 PUFAs), 20% oleic acid, monounsaturated omega-9 fatty acid (n-9 MUFAs) [6], and 8% linolenic acids. Dietary oils are one of main sources of essential fatty acids in the human body, actually, linoleic acid is one of two essential fatty acids for humans, who must obtain it through ingestion. Linoleic and linolenic acids are also precursors of hormones that regulate smooth muscle contraction, arterial blood pressure, and the growth of healthy cells [7]. [1]

Fatty acids, the building blocks of the fat/oil, are bioactive

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[1] Abbreviations: CVD, cardiovascular diseases; SFAs saturated fatty acids; UFAs unsaturated fatty acids; L, lard; SO, soybean oil; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; CO, cardiac output; TPVR, total peripheral vascular resistance; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; AIP, atherogenic index of plasma; AOPP, advanced oxidation protein products; TBARS, thiobarbituric acid reactive substances; PON1, paraoxonase 1; ALTL, alanine aminotransferase; ASTL, aspartate transaminase; LDHI, lactate dehydrogenase; UA, uric acid; TAC, total antioxidant capacity; TOS, total oxidant status; OSI, oxidative stress index; O_2^-, superoxide anion radical; NO_2, nitrites; PAB, prooxidant-antioxidant balance; MPO, myeloperoxidase enzyme activity; CAT, catalase; SOD, superoxide dismutase; IL-6, interleukin 6; PTX-3, pentraxin-3; ROS, reactive oxygen species.

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compounds involved in numerous homeostatic processes including metabolism. However, excessive consumption of some fatty acids, primarily SFAs, results in health hazards. Randomized controlled trials and prospective studies provide strong evidence that replacing SFAs by UFAs reduced serum cholesterol and LDL-C [8,9]. According to these investigations, nutritional guidelines encouraged people to reduce their intake of SFAs, typically found in foods from animal origin and otherwise, increase intake of UFAs found in plant oils [10], such as those found in soybean oil [7]. This recommendation has begun to apply in the food industry, and in the last two decade the soybean oil has been becoming the main oil in food industry. The relation between risk of CVD and high intake of UFAs, like linoleic and oleic acid, the main components of soybean oil, has been less extensively studied. The results of the previous study showed that only 14 days of 20% soybean oil supplemented diet could help in reducing the risk of hyperlipidemia and atherosclerosis [7]. Therefore, the question is could the long-term use of high doses of soybean oil possibly be unsafe.

One of the main factor that favor nutritional oxidative stress is an excessive consumption of fat. By literature data, it is well known that in conditions of continuous high-fat intake degree of faty acids saturation might have a crucial role in the development of hyperlipidemia and nutritional oxidative stress, trough reactive oxygen species formation in vascular cells [11]. A high fat diet also reduced activity of liver Nrf2, a controller of the antioxidant enzymes activity, which is related to the high production of the free radicals and consequent lipid and protein oxidation in the liver [12]. Accumulation of lipid metabolites generates harmful intracellular substrates which in molecular pathway induce vascular hypertrophy, interstitial fibrosis, and apoptosis [13]. Thus, during lipid peroxidation produced free radicals induce oxidative damages of proteins and DNA and cause cellular damage, hypertrophy, and remodeling [14]. Advanced oxidation protein products (AOPP) are capable to induce releasing of inflammatory mediators and thus represent a new class of inflammatory mediators and markers of oxidative stress [15]. According to the literature, increasing in HDL-C is associated with a CVD protection [16], but when anti-oxidant and anti-inflammatory functions of HDL-C are overwhelmed by pathological processes, such as inflammation, HDL-C is converted into a 'dysfunctional' pro-inflammatory particle [17].

Additional dates conforming that myeloperoxidase (MPO), heme-containing enzyme that catalyzes the hydrogen peroxidase-mediated oxidation of halide ions to hypochlorous acid, stimulates the proinflammatory response by releasing cytokines such as IL-1 and IL-6 [18]. The role of IL-6 in the liver pathology is very complex. It activates several type of cells, such as immune cells, hepatocytes, hematopoietic stem cells, etc. Furthermore, it is one of the mediators that contributes to non-alcoholic fatty liver disease [19], and one of the most common causes of abnormal liver function. Finally, these processes result in chronic liver injury that is characterized by the accumulation of large triglyceride droplets within hepatocytes and massive steatosis of the liver [20]. In addition, IL-6 was known to induce the liver to synthesize a group of proteins called acute phase proteins [19], among which is pentraxin-3 (PTX3), a member of the humoral immune molecules released locally at the site of injury [21]. To date, PTX3 levels were usually investigated by the research studies in assessment of CVD [22,23]. However, the level and role of PTX3 in liver injury and in response to inflammation are not well understood.

Therefore, this work addresses the hypothesis that fats from different sources (animals/plants) cause similar degree of oxidative damage in both cellular proteins and lipids that correlates with similarly alteration in haemodynamic and inflammatory responses, with special reference to link between pentraxin-3 and myeloperoxidase activity in normotensive *Wistar* rats.

2. Material and methods

2.1. Animals

The experimental protocol was in accordance with the National Law of AnimalWelfare ("Sl.gl.RS" 41/09 and 39/10) and the Directive 2010/63/EU. The study protocol was approved by the Ethic Committee of the Institute for Medical Research (IMR), University of Belgrade, Serbia and Veterinary Directorate, Ministry of Agriculture and Environmental Protection, Republic of Serbia (No. 323-07-06069/2015-05). Protocols were performed on 5-month-old male *Wistar* rats (200–250 g), bred at IMR. Experimental animals were housed in groups of four in plastic cages (Macrolon® cage type 4, Bioscape, Germany) with sawdust bedding (Versele-Laga, Belgium) certificated as having contaminant levels below toxic concentrations. Environmental conditions were controlled and monitored by a central computer assisted system with a temperature of 22 ± 2 °C, relative humidity of 55 ± 15%, 15–20 air changes/h, and artificial lighting of approximately 220 V (12 h light/dark cycle). Experimental animals had free access to tap water from municipal mains, filtered through 1.0 μm filter (Skala Green, Serbia).

The animals were randomly divided into three groups (n = 8 rats in each group) and were feading for 8 weeks. Control group (C) received normal-fat diet consisted of commercial chow for laboratory rats ("Veterinarski zavod" Subotica, Serbia). HFD groups received standard chow for laboratory rats enriched with lard (L) or soyabean oil (SO) at 20% of the total mass chow (Institute of food tehnology, University of Novi Sad, Serbia) (Table 1).

Diets were prepared weekly to minimize external oxidation of lipids. Body weight and food intake were monitored once per week. At the end of the experiment, the body weight impact of diverse diets was evaluated by calculating the cumulative weight gain and food efficiency ratio of the rats, food intake and calorie intake, as described by Machaba et al. [24].

2.2. Haemodynamic measurements

Haemodynamic measurements were performed at the end of the diets. Systolic (SAP) and diastolic arterial pressure (DAP) were measured directly in anaesthetized rats (sodium pentobarbital 35 mg/kg i.p.), through a femoral artery catheter (PE-50, Clay-Adams Parsippany, NJ,

Table 1
Diet ingredients and composition.

	NFD	HFD	
		L	SO
Ingredients (%)			
Protein	20	20	20
Cellulose	5	5	5
Fat	4	4	4
Calcium	1	1	1
Lysine	0.90	0.90	0.90
Methionine+cystine	0.75	0.75	0.75
Phosphor	0.50	0.50	0.50
Sodium	0.20	0.20	0.20
Lard	0	20	0
Soybean oil	0	0	20
Composition (mg/kg)			
Zinc	100	100	100
Iron	100	100	100
Manganese	30	30	30
Copper	20	20	20
Iodine	0.5	0.5	0.5
Selen	0.1	0.1	0.1
Vitamin A (IJ/kg)	10,000	10,000	10,000
Vitamin D (IJ/kg)	1600	1600	1600
Vitamin E (mg/kg)	0.5	0.5	0.5
Vitamin B_{12} (mg/kg)	0.02	0.02	0.02

NFD, normal (standard) diet; HFD, high-fat diet; L, lard; SO, soybean oil.

USA) connected to physiological data acquisition system (9800TCR Cardiomax III-TCR Thermodilution Cardiac Output, Columbus Instruments', Columbus, OH, USA). A jugular vein was cannulated with polyethylene tubing PE-50 for the injection of cold saline. Further, the left carotid artery was cannulated with PE-50 tubing and attached to a Cardiomax III thermo sensor for the determination of cardiac output (CO). The second Cardiomax III thermocouple sensor was placed in cold saline. Following 20 min for stabilization after surgery, cold saline (0.2 ml) was injected through the jugular vein and mean arterial pressure (MAP) and CO were recorded. Total peripheral vascular resistance (TPVR) was calculated by dividing MAP with CO normalized for body weight and expressed as mmHg x min x kg/ml.

2.3. Biochemical measurements

Biochemical parameters were determined after haemodynamic measurements and blood sample collection. Blood was collected by punctation of the abdominal aorta, poured in a tube containing lithium heparin (Li-heparin, Sigma, USA), and centrifuged at 4000 rpm at 4 °C, 20 min. After blood sample collection all animals were sacrificed by pentobarbital overdose injection. Plasma was collected and stored at − 20 °C until assaying. Livers were harvested, weighted, and stored at − 70 °C for later analysis. Alanine aminotransferase (ALTL), aspartate transaminase (ASTL), lactate dehydrogenase (LDHI), uric acid (UA) plasma levels and total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglyceride (TG), were determined in plasma and liver samples using commercial kits for automatic analyzer Cobas Integra 400 (Hoffmann-La Roche, Germany). Triglyceride concentration in the liver is presented as mg of triglyceride/g of the liver tissue. Non-HDL (atherogenic cholesterol) was calculated according to the following equation: non-HDL=LDL-C+VLDL-C [25]. The atherogenic index of plasma (AIP) was calculated according to the following equation: AIP= (LDL-C+VLDL-C)/HDL-C, with units for LDL-C and HDL-C in mmol/L [26].

2.4. Evaluation of systemic and liver redox state

Plasma total antioxidant capacity (TAC) method is based on the bleaching of characteristic color of a more stable ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation by antioxidant sample constituents [27]. Total peroxide concentration (TOS) of plasma is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in the plasma samples, in the presence of xylenol orange which produces a colored ferric-xylenol orange complex whose absorbance can be measured spectrophotometrically, [28]. Ratio of total peroxide to TAC levels was accepted as oxidative stress index (OSI) and expressed in arbitrary units.

In order to determine the level of lipid peroxidation, we measured plasma and liver thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. [29] using 4,6-Dihydroxy-2-mercaptopyrimidine. Plasma and liver advanced oxidation protein products (AOPP) were measured in acidic condition in the presence of potassium iodide [30]. Concentration of nitrites (NO_2^-) was measured in plasma by the Griess reagent method as previously described [31]. The concentration of superoxide anion radical (O_2^-) in plasma was measured at 530 nm, after the reaction of nitro blue tetrazolium in TRIS buffer [32].

Paraxonase activity (PON1) in plasma was evaluated by phenyl acetate [33]. Determination of prooxidant-antioxidant balance (PAB) in liver tissue was performed using 3,3′, 5,5′-tetramethylbenzidine as a chromogen [34]. The activities of antioxidant enzymes: superoxide dismutase (SOD) and catalase (CAT) were measured in liver homogenates by the spectrophotometric method, as previously described [35,36]. Myeloperoxidase enzyme activity in plasma and liver samples was determined by o-dianisidine-H_2O_2 method as described by Kothari et al. [18].

2.5. Inflammatory markers of liver tissue

Quantitative determinations of IL-6 and PTX3 in liver homogenate were performed by rat-specific ELISA kits (IL-6 and PTX3: Elabscience Biotechnology inc., Houston, USA). Previously frozen liver tissue were processed in accordance with the manufacturer's recommendations. Briefly, liver tissue were dissolved with phosphate buffer, and stored at − 20 °C for 30 min. After three freeze-thaw cycles to break up the cell membranes, lysates were centrifuged at 10,000 rpm, 4 °C for 5 min. The supernatant was further assayed, and values were expressed as ng/ml. All samples were measured in triplicate in a single assay according to the manufacturers' instructions. For IL-6 and PTX3 detection, the sensitivity of the system was 7.5 pg/ml and 0.4 ng/ml, and the intra- and interassay coefficients of variation of 3 samples with low, mid range and high level were 5.9, 4.83, 5.03% and 5.09, 5, 4.9%, respectively.

2.6. Statistical analysis

Results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was applied as appropriate. Fisher LSD test was performed as a post hoc multiple comparison test (Statistica 8). The Pearson correlation between the examined parameters was also determined. P-value < 0.05 was considered significant.

3. Results

3.1. Body weight and food intake

The body weight increased significantly during the experimental period in all groups ($p < 0.001$), but there were no significant differences in mean of weights after the intervention period between the groups (Fig. 1A). Food intake in the groups of rats treated with HFD (L or SO) was significantly lower compared to standard food (Fig. 1B). Concerning the food efficiency ratio, L and SO groups had a significant increase compared to control (Fig. 1C; $p < 0.001$). It was followed by significantly increased calorie intake in both HFD groups (Fig. 1D).

3.2. Haemodynamic results

Soybean oil feeding caused a significant increase in SAP compared to the standard diet (Fig. 2A). DAP was significantly increased after 8 week consumption in both lard ($p < 0.05$) and soybean oil ($p < 0.01$) groups compared to control group (Fig. 2B). On the other side, both HFD did not significantly change cardiac output and total vascular resistance (Fig. 2C.D).

3.3. Biochemical results in plasma

Plasma total cholesterol was significantly higher in the groups receiving L or SO compared to C group (Fig. 3A.; $p < 0.001$; $p < 0.01$; respectively), while there were no significant changes in triglyceride levels (data no show). In similar way, LDL-C, non-HDL (Fig. 3B. D.; $p < 0.001$), as well as the atherogenic index were increased (Fig. 3E.; $p < 0.05$). In relation to HDL-C, groups that were on lard or soybean oil diets showed higher values than group on standard diet (Fig. 3C.; $p < 0.001$; $p < 0.01$; respectively). Plasma ALTL was significantly increased in both high-fat diet groups (Fig. 3G. $p < 0.05$). On the other side, ASTL and LDHI2 were significantly higher only in SO group compared to control group (Fig. 3H.I; $p < 0.05$).

Plasma TBARS was significantly increased in L and SO groups, compared to control group (Fig. 4A. $p < 0.01$; $p < 0.05$; respectively), followed by significantly higher plasma AOPP in both high-fat diet groups (Fig. 4B.; $p < 0.001$). More interesting was that 8 weeks consumation of enriched SO diet was statistically decreased TAC (Fig. 4E., $p < 0.01$) while TOS and OSI levels were significantly higher compared to standard diet (Fig. 4D.F; $p < 0.05$; respectively). The highest level of

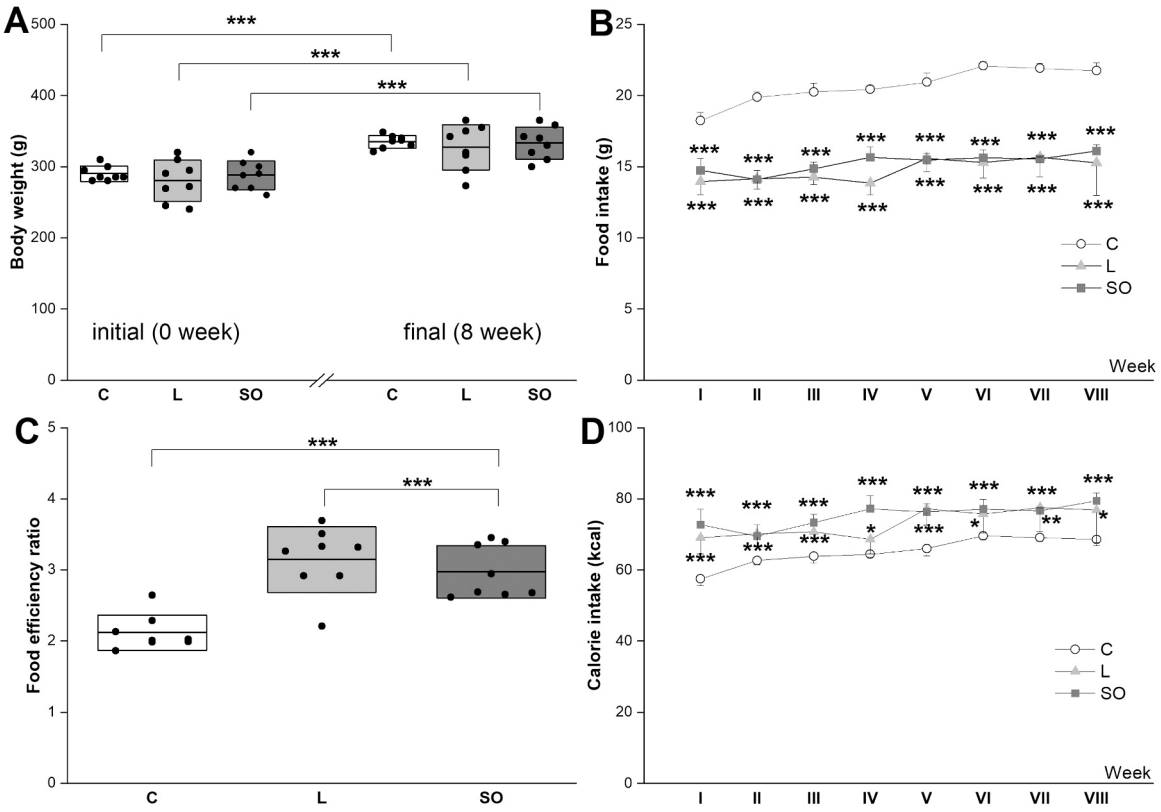

Fig. 1. Body weight (A), food intake (B), food efficiency ratio (C), and calorie intake (D) of rats assigned to different diet. C-control, L-lard, SO-soybean oil group. Values are means ± SD; * **, * *, * indicate $p < 0.001$, 0.01, 0.05.

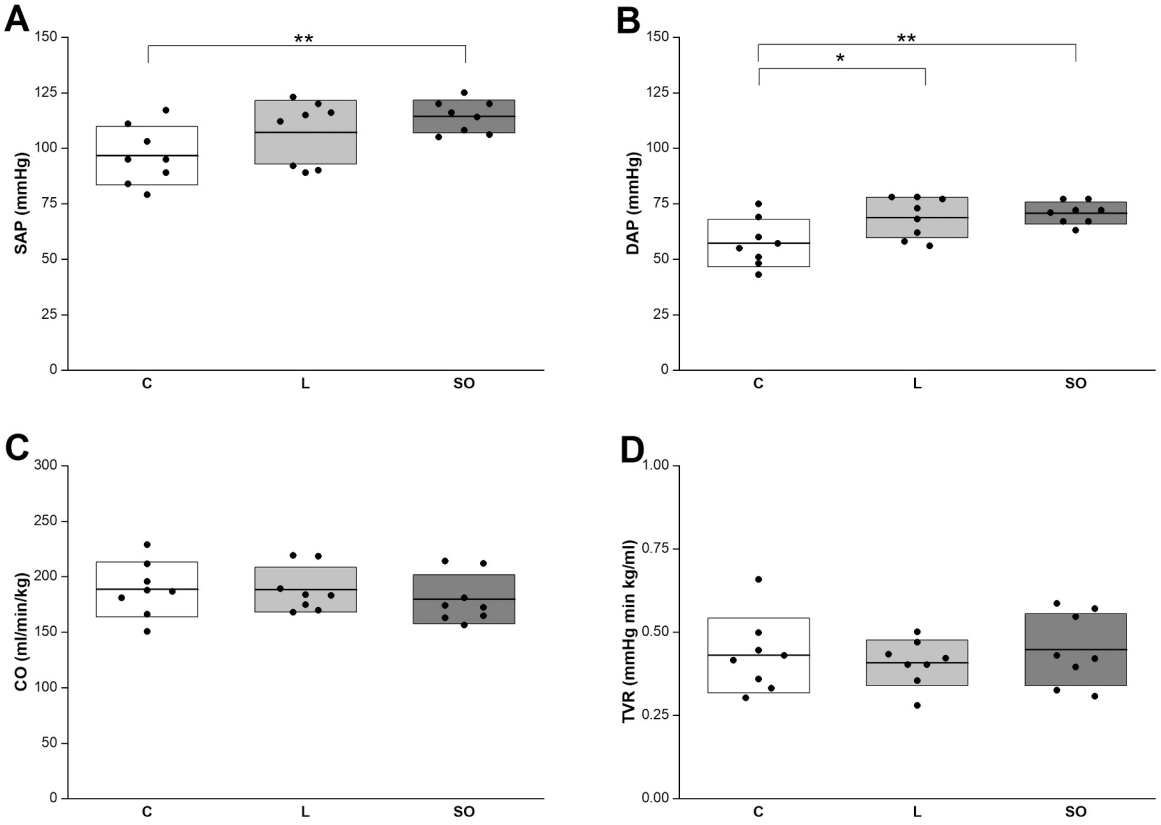

Fig. 2. Systolic arterial pressure (A), diastolic arterial pressure (B), cardiac output (C) and total vascular resistance (D) in experimental groups. C-control, L-lard, SO-soybean oil group.Values are means ± SD; * *, * indicate $p < 0.01$, 0.05.

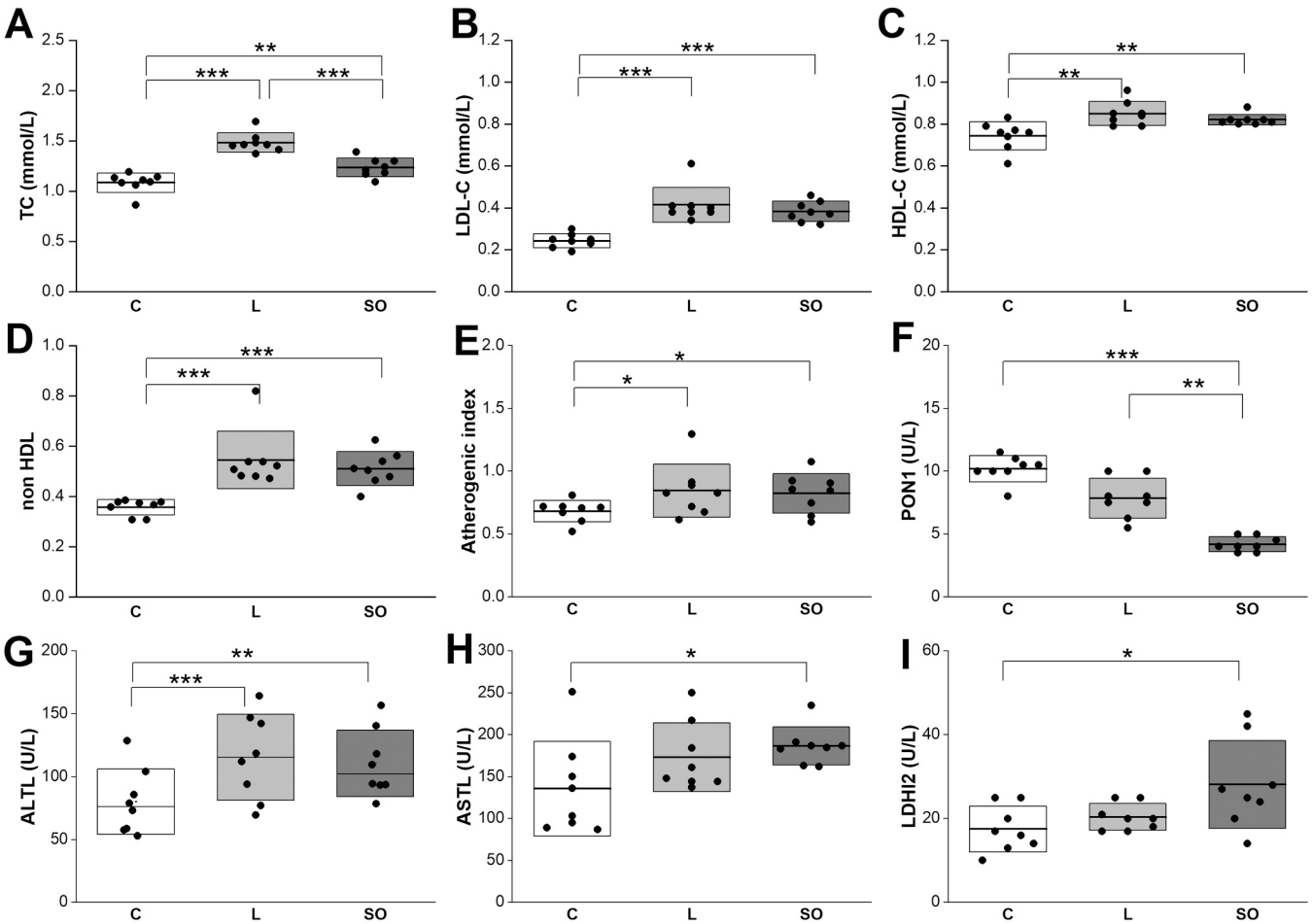

Fig. 3. Biochemical results: TC/total cholesterol (A); LDL-C/low density lipopropein cholesterol (B), HDL-C/high density lipoprotein cholesterol (C), non-HDL cholesterol (D), atherogenic index (E), PON1/paraoxonase 1 (F), ALTL/alanine aminotransferase (G), ASTL/aspartate transaminase (H), LDHI/lactate dehydrogenase (I) in experimental groups. C-control, L-lard, SO-soybean oil group.Values are means ± SD; * ** , * *, * indicate $p < 0.001$, 0.01, 0.05.

O_2^- and NO_2^- were noticed in SO group (Fig. 4G.H. $p < 0.001$; $p < 0.05$; respectively). Level of UA was significant 1.7-fold increased, as well as the activity of MPO which was even four times increased, only in SO group compared with C group (Fig. 4C. I. $p < 0.001$; $p < 0.01$; respectively).

3.4. Lipid status in the liver tissue

Compared with the control diet, the SO diet significantly increased liver lipids: total cholesterol, triglyceride, LDL-C, and non-HDL (Fig. 5A. B.D.E. $p < 0.01$; $p < 0.001$; $p < 0.05$; $p < 0.01$; respectively) that implies serious steatosis of the liver. Liver triglycerides content ranging from 6.57% to 21.81% among groups.

3.5. Redox status in the liver tissue

Fig. 6. shows the redox state in liver tissue. AOPP levels were significantly higher in both high-fat diet groups, while TBARS was significantly higher only in SO group (Fig. 6A.B. $p < 0.001$; $p < 0.01$; respectively). On the other hand, the most significant differences in PAB, as well as MPO activity were observed only in SO group (Fig. 6C.D. $p < 0.001$; $p < 0.001$ respectively). The similar trend was maintained in both superoxide dismutase (SOD) and catalase (CAT) activity (Fig. 6E.F. $p < 0.01$; $p < 0.001$; respectively).

3.6. Markers of inflammation in the liver tissue

Cytokines implicated in inflammatory pathways, IL-6 and PTX3 in the liver tissue, were significantly increased in SO group in comparison to both C and L groups (Fig. 7A.B). In SO group, IL-6 levels remained significant with approximately 1.7-fold and 2-fold difference compared to C and L group, ($p < 0.01$; $p < 0.001$ respectively). In the same group, the level of PTX3 increase 6.5-fold compared to C group and 4.9-fold compared to L group ($p < 0.001$).

3.7. Correlation

We found significant correlations between HDL-C function (characterized by PON1 and MPO activities), PAB, and the levels of inflammatory biomarkers IL-6 and PTX3 measured in liver. Namely, plasma PON activity was in high negative correlation with both plasma and liver MPO activities, as well as with the expressions of IL-6 and PTX3, while MPO activity either in plasma or liver tissue markedly and positively correlate with the levels of IL-6 and PTX3 (Table 2).

4. Discussion

The present study examined whether diets varying in faty acids composition could differentially affect the rate of body weight gain, lipid and oxidative status, as well as the inflammatory response. We demonstrated that both HFD, despite the reduced food intake, increased calorie intake and compromised plasma lipid profile. The lower food

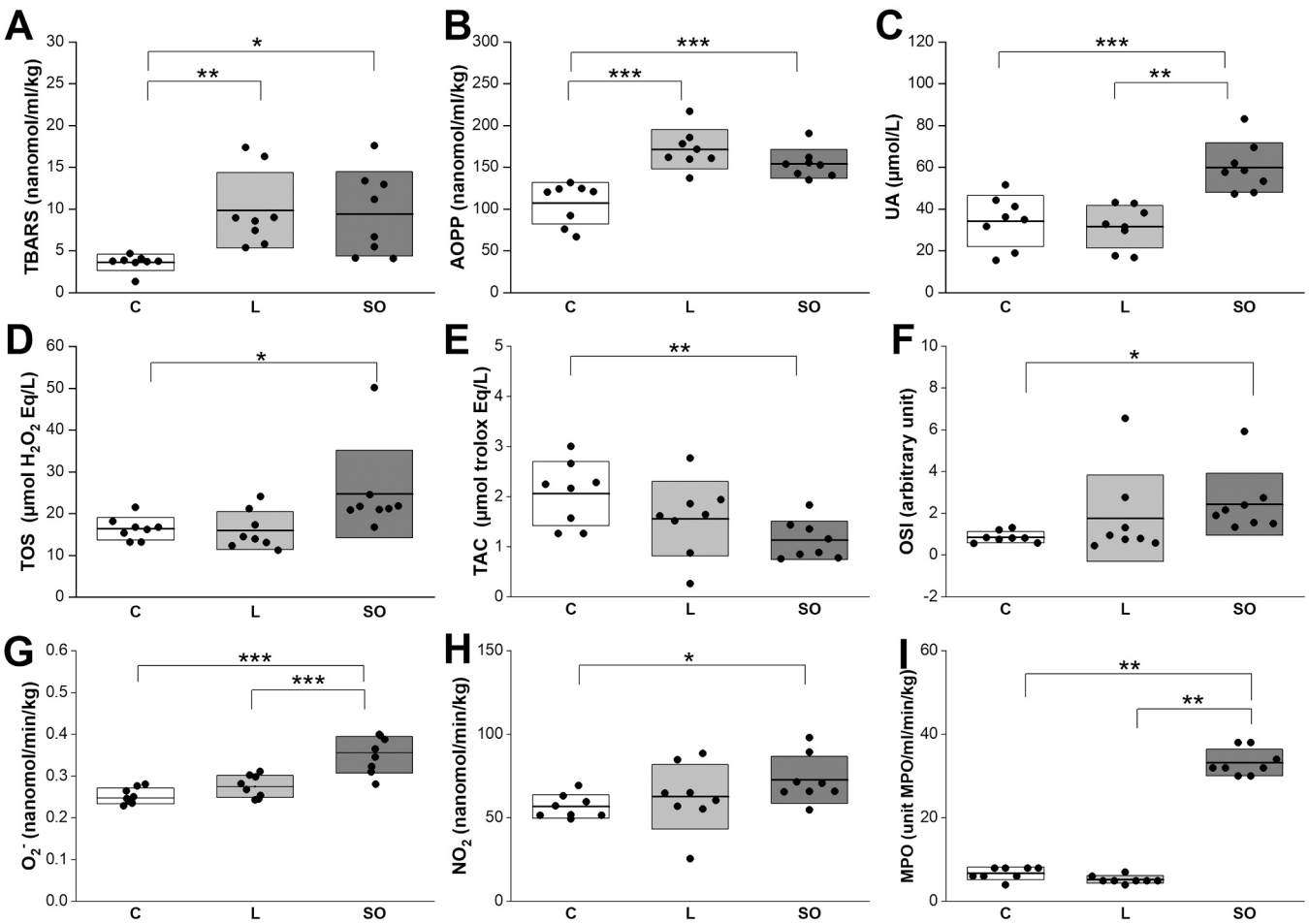

Fig. 4. TBARS/thiobarbituric acid reactive substances (A), AOPP/advanced oxidation protein products (B), UA/uric acid (C), TOS/total oxidant status (D), TAC/total antioxidant capacity (E), OSI-oxidative stress index (F), O_2^-/ superoxide anion radical (G), NO_2^-/ nitrites (H) and MPO/myeloperoxidase enzyme activity (F) of plasma in experimental groups. C-control, L-lard, SO-soybean oil group.Values are means ± SD; * ** , * *, * indicate $p < 0.001$, 0.01, 0.05.

consumption in both high-fat diet (vs. controls) equalled out caloric intake between all groups, which resulted in similar final body weights. These data agree with previous experiments that high calorie intake was enough to cause a smaller food consumption [37]. Besides, our results show that, in normotensive *Wistar* rats, only consumption of food enriched with soyabean oil increased systolic blood pressure, disrupted plasma/liver oxidative stability which was closely related to enhanced plasma/liver MPO activity, and liver inflammation confirmed by elevated IL-6 and PTX3 expressions.

Much research around the world has shown that the replacement of saturated fats with reasonable amounts of polyunsaturated fats, such as those found in soybean oil, is recommended [8,9]. Also, Uhegbu et al. showed that soybean oil diet 10%, 20% and 30%, respectively for 14 days can help in reducing the risk of hyperlipidemia and atherosclerosis [7]. Our results clearly show that both 20% of L and SO diets after 28 days supplementation significantly increased total plasma cholesterol and LDL-C levels, as well as the atherogenic index, compared to the regular diet. Hence, an essential question in our study is why the SO diet, rich in UFAs, causes a disorder of the lipid profile, especially when epidemiological and basic research suggest otherwise. Namely, Shen et al. [38] showed that in human population 12 week treatment with soybean capsule, which contains 910 mg of soybean oil, inhibited cholesterol absorption and lowered LDL-C levels. Soybean oil contains linolenic, linoleic, and oleic acid, which have different cholesterol and LDL-C lowering effects [7]. Also, some studies have documented that higher intake of linoleic acid reduces LDL-C [39] and reduces the risk of hypertension [40]. On the other hand, it was shown that diet with increasing doses of unsaturated linoleic acids (110–1100 mg/kg/day) significantly increase total cholesterol in rats after 11 week treatment [41]. Also, Syrian hamsters fed 6 weeks with the HFD containing 14% soybean oil showed significantly higher TG, TC, LDL-C, and HDL-C in serum compared with animals on low-fat basal diet [42]. Such contradictory results might be explained by the fact that increase in the intake of linoleic acid increases the linoleic acid content in very-low-density lipoproteins (VLDL), which increase their susceptibility to oxidize. Indeed, linoleic acid is the most common oxidized fatty acid in LDL particules. Once linoleic acid becomes oxidized in LDL, aldehydes and ketones covalently bind apolipoprotein B, creating unrecognizable LDL for the LDL receptors in the liver, but is now recognized by scavenger receptors on macrophages leading to the classic foam cell formation and atherosclerosis [43].

A fat enriched diet and excessive calorie intake among the environmental factors are known to increase incidence of metabolic disorders such as hyperlipidemia, diabetes, hypertension, obesity, and cancer [44]. In our study, the excessive consumption of L or SO resulted in extensive lipid and protein oxidations (significant increase in plasma TBARS and AOPP levels) that could be associated with the occurrence of vascular remodeling, as indicated by an elevated atherogenic index. These findings are in accordance with recent evidences in rodents showing induced elevation of lipid peroxidation [45] and AOPP accumulation [46] with controversial results regarding the level of NO metabolites in different organs or tissues [45].

In the present study, both HFD, L, and SO increase HDL-C compared to the regular rat food. However, there is a question about HDL-C

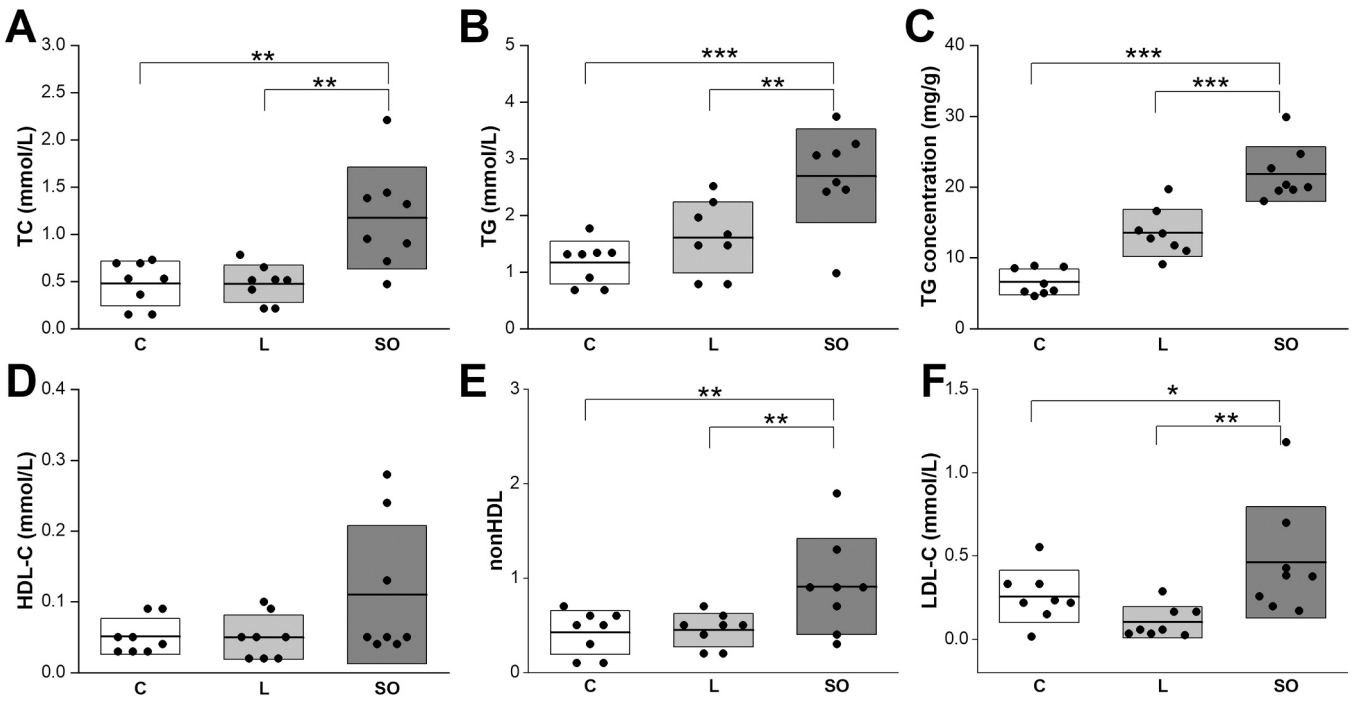

Fig. 5. Liver lipid status: total cholesterol (A), triglyceride (B), triglyceride concentration (C), HDL-C/high density lipoprotein cholesterol (D), non-HDL (E), LDL-C/ low density lipopropein cholesterol (F) in experimental groups. C-control, L-lard, SO-soybean oil group.Values are means ± SD; * ** , * *, * indicate $p < 0.001$, 0.01, 0.05.

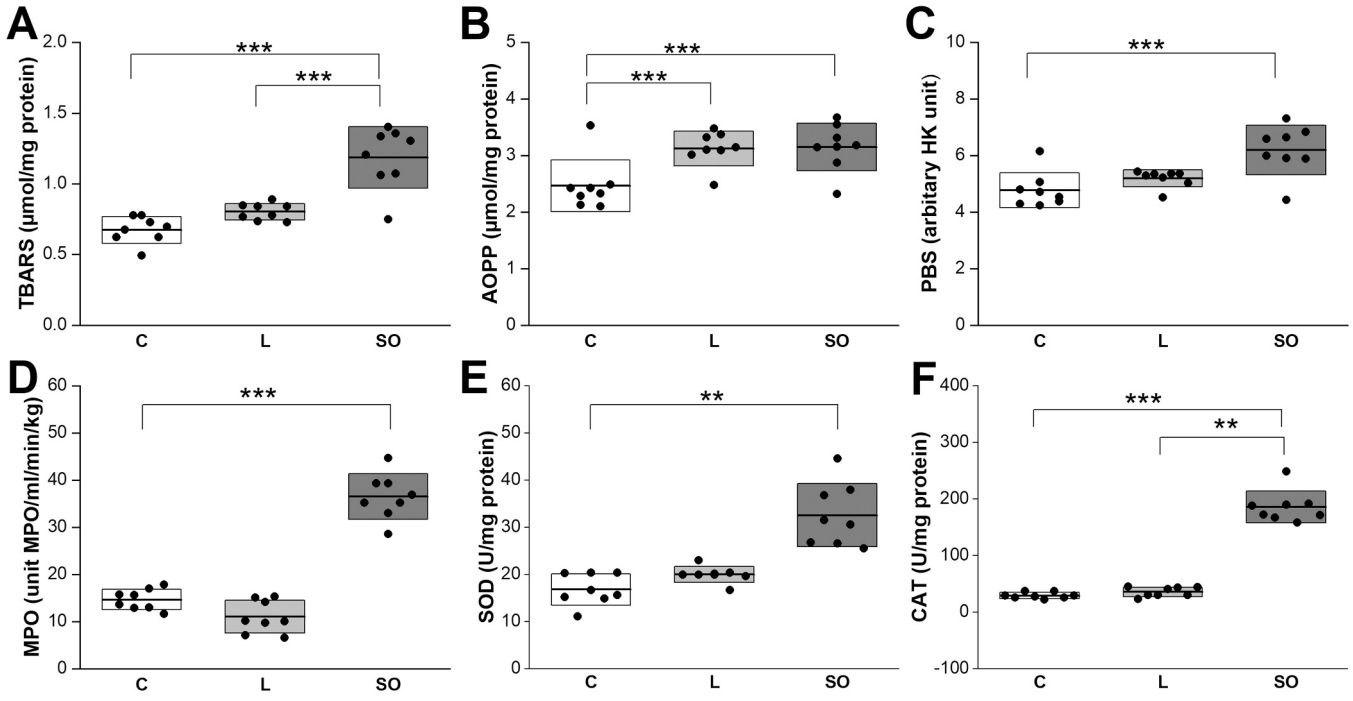

Fig. 6. Redox status in liver tissue: TBARS/thiobarbituric acid reactive substances (A), AOPP/advanced oxidation protein products (B), PBS/ prooxidant-antioxidant balance (C), MPO/myeloperoxidase enzyme activity (D), SOD/superoxide dismutase (E) and CAT/catalase (F) in experimental groups. C-control, L-lard, SO-soybean oil group.Values are means ± SD; * ** , * * indicate $p < 0.001$, 0.01.

functionality. Functional HDL-C have potent antioxidant properties, as well as anti-inflammatory, antithrombotic, cytoprotective, vasodilatory activity. This wide spectrum of biological activities likely reflects the heterogeneity of HDL-C particles, and proteomic analysis showed about 48 plasma circulate proteins, including: lecithin-cholesterol acyl-transferase, phospholipid transfer protein, paraoxonase, and acetylhydrolase which could modulate HDL-C and convert it to HDL-C ester [47,48]. This can induce varying degrees of HDL-C dysfunction reflected in impaired reverse cholesterol transport and resulting in high plasma levels of a dysfunctional free cholesterol-rich HDL-C. Finally, such increasing of plasma cholesterol bioavailability produces whole-body hypercholesterolemia [49]. Someone studies indicate that

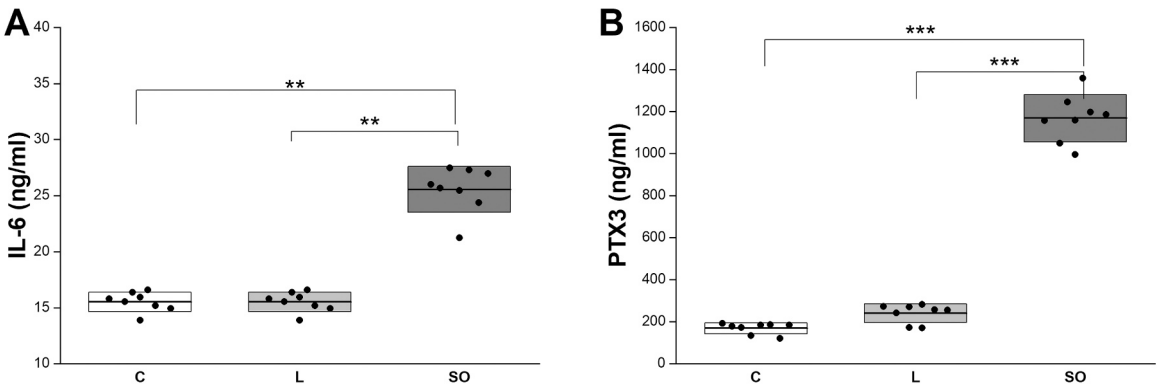

Fig. 7. Liver marker of inflammation: IL-6/interleukin-6 (A) and PTX3/pentraxin-3.
(B) level in experimental groups. C-control, L-lard, SO-soybean oil group. Values are means ± SD; * ** , * * indicate $p < 0.001$, 0.01.

Table 2
Correlations between HDL-C function (characterized by PON1 and MPO activities), prooxidant-antioxidant balance (PAB), and levels of inflammatory biomarkers in the liver: IL-6 and PTX3. Marked correlations are significant at p < ,05000. N = 24.

	Plasma MPO	Plasma PON	Liver PAB	Liver MPO	Liver IL6	Liver PTX3
Plasma MPO	1,0000	-,5980	,6215	,6494	,4648	,5819
	p = —	p = ,002	p = ,001	p = ,001	p = ,022	p = ,003
Plasma PON	-,5980	1,0000	-,5895	-,5558	-,5270	-,6252
	p = ,002	p = —	p = ,002	p = ,005	p = ,008	p = ,001
Liver PAB	,6215	-,5895	1,0000	,5146	,6220	,6346
	p = ,001	p = ,002	p = —	p = ,010	p = ,001	p = ,001
Liver MPO	,6494	-,5558	,5146	1,0000	,4610	,7745
	p = ,001	p = ,005	p = ,010	p = —	p = ,023	p = ,000
Liver IL6	,4648	-,5270	,6220	,4610	1,0000	,4670
	p = ,022	p = ,008	p = ,001	p = ,023	p = —	p = ,021
Liver PTX3	,5819	-,6252	,6346	,7745	,4670	1,0000
	p = ,003	p = ,001	p = ,001	p = ,000	p = ,021	p = —

the HDL-C from patients with CVD does not have a protective action, but does stimulate inflammation and free radical synthesis [17,48]. Also, prospective studies in human population showed that very high plasma HDL-C levels, as well as very large HDL-C particles were associated with higher risk for CVD [50]. This data suggests that HDL-C, commonly recognized as protective in some circumstances, becomes pro-atherogenic and dysfunctional. Dysfunctional HDL-C is followed by decreased level and activity of anti-inflammatory/anti-oxidative PON1, an essential collaborator of HDL-C protection against of LDL-C [51]. On the other hand, *in vitro* studies have demonstrated that oxidized lipoproteins, like LDL-C, contribute to HDL-C dysfunctionality through decreasing of PON1 activity [52]. Considering literature data in human population, the plasma PON1 activity markedly decreases in response to a diet rich in trans fatty acids [53], as well as that high-cholesterol diet in rabbits reduced PON1 activity [54]. Results of our study also demonstrated reduced PON1 activity in rats fed with a SO diet.

Furthermore, in the present study excesive consumption of soybean oil reduced TAC in plasma as consequence of elevated oxidative stress and high values of TOS. Hydrogen peroxide is one of the main components of TOS and it oxidizes the enzyme MPO to a higher oxidation state, which further builds specific adducts in the cell with certain molecules. Actually, in the presence of nitrite and H_2O_2 myeloperoxidase is also able to form reactive nitrogen species, which can lead to further oxidative instability. Early study has demonstrated that MPO, PON1, and HDL-C form a functional complex in which PON1 partially inhibits the MPO activity, while MPO also partially inactivates the PON1 [53]. Also, serum MPO/PON1 ratio could be potencial indicator of HDL-C dysfunctionality [55]. On the other hand, there is no data about the effects of fat rich diet on MPO/PON1 relation contemporary. Our results clearly show that the highest increase in plasma MPO activity was observed only in SO group, evidenced with a drop in plasma PON 1 activity. Therefore, the strong negative corelation between MPO and PON 1 indicates that the HDL-associated protein PON1 may be targeted for oxidative modification and functional inactivation by MPO. This data reveals the effects of different dietary compounds on MPO/PON1 ratio and oxidative stability. This implies compromised antioxidative defense after unsaturated fat rich diet.

The reductive reaction carried out by xanthine oxidase reduces oxygen into a superoxide anion and produces uric acid. Hence, in our study, in the SO group, significantly increased level of uric acid is accompanied by an increase superoxide anion. Superoxide anion is a one of the major limitator of NO biosynthesis and bioavailability and can thus modify endothelial function. In this research, after SO diet, there is a disbalanced release of relaxing and contracting factors, NO_2 and O_2^-, which can be accounted to increase systolic blood pressure, thereby contributing to further progression of vascular and organ damage. In that sense obtained increase in the ALTL, ASTL and LDHI2 after the SO diet appears to be logical. High levels of LDH indicate some form of tissue damage, whereas levels of ALT and AST are indicator of hepatocellular damage. Actually these enzymes are poor prognostic markers of the severity of liver injury [56].

Nutritional stress, such as high fat intake could lead to hepatic lipid accumulation [57]. However, if more than 10% percent of the liver's weight is fat, then it is called a fatty liver (or liver in steatosis) [20]. Considering that is in normal conditions liver does not store triglyceride, recent studies suggest that normal liver may contain up to 5.5% triglyceride and that a triglyceride content greater than 8% may be a reliable sign of pathologically fatty liver [58]. In our study, livers of soybean oil group and lard group contain 21.81% and 13.51%, respectively, triglycerides that reveal strong hepatic steatosis. There are two concepts of steatosis confirmation: biochemical and histopathological. Keeping in mind apparentness of pathophysiology, biochemical concept has some advantages. Namely, some authors suggest that histopathological analysis of steatosis is not an adequate method due to a

two-dimensional semi-quantitative nature susceptible to inter-individual visual estimation [59], therefore they recommend direct measurement of lipid in the liver as most reliable. In this regard, we noted that in our study soybean oil ingestion developed steatosis as evidenced with 218% and 230% increases in liver cholesterol and triglyceride levels vs control, that is similarly as in the report of Echeverría et al. [57]. Another study has shown that mice fed with HFD had reduction in hepatic n-3 long-chain polyunsaturated fatty acids which induced deactivation of transcription factor peroxisome proliferator-activated receptor α (PPAR-α) and upregulated sterol regulatory element-binding protein- 1c (SREBP-1c). This alteration trigger FA oxidation and favouring de novo lipogenesis which can contribute to storage of triglyceride and liver steatosis [60]. Steatosis is associated with the onset of oxidative stress in the liver, as evidenced by significant changes in protein oxidation responses and 1.8-fold increase of lipid peroxidation. Increased level of both liver lipids and activity of antioxidant enzymes (SOD and CAT) may also contribute to the increased free oxygen radicals, leading to disturbed PAB and significantly increased activity of the MPO in the liver. Reactive oxygen species (ROS) overproduction is also an important factor of liver mitochondrial dysfunction. This dysfunction diminishes expression of the nuclear respiratory factor 1 (NRF-1) which deranges the expression of nuclear DNA and mtDNA encoded oxidative phosphorylation genes, thus decreases mitochondrial biogenesis [61] and reduces oxidative stability. The precise mechanism by which soybean oil intake produces liver injury is unknown, but it is believed that oxidative stress modulate this process. Most ingested lipids are incorporated in membrane phospholipids making them more susceptible to free radical attacks. This mainly occurs with UFAs which have unstable molecular bounds and therefore are more susceptible to oxidative stress and inflammation. This hypothesis is supported by our results, where SO diet induced significantly increase of hepatic MPO activity. Actually, in SO group, in states of increased inflammation via activity of MPO, lipid peroxidation product levels is also altered. MPO activity is linked to lipid peroxidation, which is a prominent characteristic of fatty livers, promoting activation of stellate cells and attraction of inflammatory cells [62,63].

MPO/HDL-C ratio in the liver was significantly elevated in SO group. Higher MPO/HDL-C level in this group was associated with increased level of inflammation factors: IL-6 and PTX3. These results suggest that dyslipidemia and nutritional oxidative stress deepenes inflammation in the liver after chronic ingestion of UFAs. On the other side, the metabolites of linoleic acid can mediate inflammation. Linoleic acid (n-6 PUFAs), the main component of soybean oil, is metabolized to arachidonic acid which metabolites, eicosanoids, in large quantities can contribute to the formation of thrombus and atheroma, and induce pathological proliferation of cells [64]. Thus, a diet rich in n-6 PUFAs shifts the physiological state to one that is proinflammatory.

Searching for the possible regulatory factors of inflammation in conditions of continuous high-fat intake, we can say that liver damage could be mediated by IL-6 and PTX3 after excessive UFAs intake. Liver PTX-3 was found significantly higher in the SO group than in C and L groups. Short components of this family are mainly produced by hepatocytes in response to inflammation. In normal liver tissue hepatocytes are negative for PTX3 expression [65]. In the context of liver diseases levels of PTX3 were found to be elevated in chronic liver diseases: nonalcoholic steatohepatitis, nonalcoholic fatty liver disease, liver cirrhosis, and chronic viral hepatitis [66–68]. Today, it is well known that increased caloric intake and reduced physical activity undoubtedly contribute to the prevalence of non-alcoholic fatty liver disease. It is an intra-hepatocellular accumulation of lipids (mainly triglycerides) that affects almost 30% of the Western world population. Several diagnostic panels have been developed to predict liver injuries. Although, liver biopsy and imaging studies demonstrate limited sensitivity while serum markers (like transaminase), platelet count, and high-sensitivity C-reactive protein has not been standardized for evaluating the severity of liver injuries. However, Yoneda et al. reported that PTX3 levels are strongly correlated with the severity of liver disease [69]. In accordance with this finding, our results of high positive correlation between PAB and MPO, but IL-6 and PTX3 on the other, indicates a close connection between the molecular mechanisms that regulate nutritional stress and inflammation in this experimental design. Because of this we think that PTX-3 could be a useful inflammatory marker of liver damage after chronic excessive high-fat intake.

In conclusion, contrary to expectation, our study shows that excessive consumption of the soybean oil is more inflammogenic than lard. By correlation analysis we confirmed the existence of association between nutritional oxidative stress-induced HDL-C dysfunction and inflammation after prolonged soybean oil consumption. These results give a new insight on the negative impact of excessive soybean oil diet on cardiovascular system by compromising oxidative stability and inducing inflammation. Therefore, we suggested the use of different diet compositions and different fatty acids as the goals of the future studies in order to clarify safety and healthy nutrition as well as creative a food environment where healthy foods are accessible, affordable, and desirable as the main precursor of reduction of cardiovascular disease.

Declaration of Interest statement

The authors have no competing interests to declare. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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