Vol. 15(3), pp. 49-59, October-December 2021
DOI: 10.5897/AJBR2021.1128
Article Number: D67B06968067
ISSN 1996-0778

http://www.academicjournals.org/AJBR

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African Journal of Biochemistry Research

Full Length Research Paper

Study of isoforms of nicotinamide adenine dinucleotide phosphate oxidase of the heart in a model of rats fed on several vegetable oils

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Received 14 August, 2021; Accepted 18 October, 2021

Palm oil has long been incriminated in obesity, and this obesity would be responsible for the development of cardiac fibrosis, several authors have evoked the role of free radicals in the pathophysiological mechanism of the fibrotic response linked to obesity; the aim of this study was therefore to evaluate the profile of NOX2 in the development of cardiac fibrosis in rats subjected to a diet rich in the fat of several vegetable oils, in this case palm oil. A total of forty young male Wistar rats were subjected to several diets (soybean, red and branched palm oil, olive and lard). After twelve weeks of experimentation, the rats were sacrificed after anaesthesia, and the parameters of oxidative stress, inflammation and the level of interstitial fibrosis of the heart were assessed. Our study showed that red palm oil consumption did not lead to overexpression of oxidative stress parameters and inflammatory RNA markers. The expression of myocardial nicotine adenine dinucleotide phosphate oxidase did not change in rats consuming red palm oil compared to the control diet. However, consumption of palm olein, olive and lard resulted in a significant change in myocardial nicotine adenine dinucleotide phosphate oxidase activity. This study seems to show that red palm oil, because of its richness in antioxidants, would be less deleterious for the heart.

Key words: Oxidative stress-inflammation-palm oil-cardiac fibrosis.

INTRODUCTION

In recent years, obesity has become a matter of concern and is reportedly associated with metabolic disorders and Cardiovascular disease (CVD) (Stepien et al, 2012, 2014). Obesity leads to cardiac pressure overload and hypervolaemia (Kaltman and Goldring, 1976). Both factors lead to ventricular hypertrophy associated with

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increased collagen deposition (Xia et al., 2009; Ulasova et al., 2011) resulting in the development of cardiac fibrosis. Several molecular processes have been implicated in the regulation of the fibrotic response to obesity. These include activation of the renin-angiotensin-aldosterone system, oxidative stress, inflammation and leptin-induced actions (adipokines), (Eschalier et al., 2014). The mechanism induced by oxidative stress is not yet well understood. Reactive oxygen species (ROS) play an important role in the development of cardiovascular disease. In the cardiovascular system, several enzyme systems contribute to the formation of ROS. These include nicotinamide adenine dinucleotide phosphate oxidases (NOX), nitric oxide synthase, respiratory chain enzymes, cytochrome P450 monoxygenases and xanthine oxidase. Although all these systems are important in various disease states, NOX appears to play a central role in the dysfunction of these enzymes. The initial generation of ROS by NOX triggers the release of other sources of radical species (Landmesser et al., 2003). There are seven isoforms of NADPH oxidases expressed in mammals, but the most important for the cardiovascular system are NOX2, NOX1 and NOX4 (Lassegue and Clempus, 2003). NOX2 is the most widely expressed isoform. It is expressed in vascular smooth muscle cells, fibroblasts, endothelial cells and perivascular adipocytes (Van Buul et al., 2005; Infanger et al., 2006; Paravicini and Touyz, 2008). The expression profile of NOX varies in different disease states and their enzymatic activities can be increased in response to stimuli such as cytokines (De Keulenaer et al., 1998) and growth factors (Brandes et al., 2001). Palm oil (PA) has long been implicated as an important risk factor in the development of obesity and cardiovascular disease due to its high saturated fatty acid composition (Ellie Brown, 2005; Kabagambe et al., 2005). A diet containing myristic acid is thought to induce cell hypertrophy in the heart of C57BL / 6J mice (Russo et al., 2012).

According to some authors, palmitate induces apoptosis, activation of protein kinases associated with oxidative stress in ventricular cardiomyocytes (Miller et al., 2005). Studies on dietary fat composition remain one of the conflicting areas of biology due to the complexity of the structure and diversity of functions of FAs (Hamilton et al., 2001). The aim of this study was therefore to assess the profile of NOX2 in the development of cardiac fibrosis in rats fed a high-fat diet of several vegetable oils, in particular palm oil.

MATERIALS AND METHODS

Animal model and diets

A total of forty young male Wistar rats (Charles River, L'Arbresle, France) aged 6 weeks were used in the present study. The rats were housed, two per cage, under constant conditions of temperature (20-22°C), humidity (45-50%) and a standard dark cycle (20.00-08.00 h). Rats were randomly divided into five groups of eight animals and fed one of the following semi-purified diets for 12 weeks: (a) control diet containing 5% fat in the form of soybean oil (11% energy from fat) (Control), (b) high fat diets (55% energy from fat) rich in crude palm oil (cPO) (with 2.5% soybean oil and 30% cPO), (c) refined palm oil (rPO) diets (with 2.5% soybean oil and 30% rPO), (d) olive oil (OO) diets (with 2.5% soybean oil and 30% OO, (e) lard oil (LARD) diets (with 2.5% soybean oil and 30% LARD. The cPO and rPO were supplied by the company SANIA (Ivory Coast), the OO was purchased in a supermarket and the lard oil was supplied by the company CELYS, body fat food (ALVA, Rezé, France). The detailed composition of these experimental diets is given in Table 1. Rats were given free access to food and water throughout the experiment and body growth was determined weekly.

Sacrifice of rats and collection of samples

After twelve weeks of experimentation, the rats were sacrificed after anaesthesia by intraperitoneal injection of sodium pentobarbital (CevaSantéAnimale, Libourne, France). All animals were fasted the day before sacrifice. The blood, taken from the abdominal aorta, was divided into a heparinised tube and a dry tube. After centrifugation at 3000 g for 15 min at 4°C, the plasma and serum obtained were stored at -80°C. The red blood cells in the heparinised tube were rinsed twice with saline and stored at -80°C for SOD determination. For the isoprostane assay, plasma was frozen at -80°C with 0.005% BHT (3,5-di-tert-butyl-4-hydroxytoluene). The heart, after being rinsed with saline, was cut into two pieces. One piece for molecular biology stored at -80°C and one piece for histology in a 15 ml tube containing 10% formalin (Sigma, France) making five times the volume of the sample.

Oxidative stress parameters in blood

In plasma, apart from 15-F2t-isoprotane, oxidative stress parameters were determined spectrophotometrically. TBARS were determined by the method of Sunderman et al. (1985). Protein oxidation was assessed by measuring thiol groups (Faure and Lafond, 1995). Superoxide dismutase (SOD) activity was measured according to the method of Marklund (1976). The more specific parameter of lipid peroxidation, 15-F2t-isoprotane, was determined by mass spectrometry as described by Mas et al. (2008). Briefly, aliquots of plasma samples were spiked with 15-F2t-isoprostane D4 as an internal standard prior to extraction using an Agilent Bond Elut Certify II cartridge. Washes were performed with 50% methanol and ethyl acetate/hexane (1/3 v/v) and elution was performed with ethyl acetate/methanol (9/1 v/v). After esterification, the samples were analysed on a ThermoFinnigan Trace DSQ II instrument interfaced with a Trace GC Ultra 2000 gas chromatograph, equipped with an AS 3000 autosampler (ThermoFinnigan).

Expression of core mRNA

Total heart RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Cergy Pontoise, France) according to the method of Chomczynski and Sacchi (1987) using a FastPrep-24 homogeniser (MP biomedicals, France). Reverse transcription reactions were performed on 500 ng of total RNA using a Takara reverse transcription kit (Takara Bio Europe, France) and RT-qPCR was performed using the LightCycler® 480 SYBR Green I Master (Roche Applied Science, France). Results were normalised to the RPLP0 gene. The genes studied were:

Superoxide dismutase 1 and 2 (SOD1 and SOD2); transglutaminase 2 (TGM2); Toll-like cell receptor (TLR2); soluble

Table 1. Composition of the study regimes.

Food (g/kg)	Control	red palm	Palm olein	Olive	Lard
Casein	**165**	**200**	**200**	**200**	**200**
Corn starch	442.5	233.8	233.8	233.8	233.8
Maltodextrin	144	80	80	80	80
Sucrose	100	53	53	53	53
Soybean oil	50	25	25	25	25
Oil red palm	0	300	**0**	**0**	**0**
Palm olein	**0**	**0**	300	**0**	**0**
Olive oil	**0**	**0**	**0**	300	**0**
Lard	**0**	**0**	**0**	**0**	300
Cellulose	50	50	50	50	50
Minerals (AIN-93M)	**35**	**42**	**42**	**42**	**42**
Vitamins (AIN-93M)*	**10**	**12**	**12**	**12**	**12**
L-Cystine	2	2.4	2.4	2.4	2.4
Choline chloride	1.5	1.8	1.8	1.8	1.8

interleukin 33 receptor (ST2); growth differentiation factor 15 (GDF15): transforming growth factor beta (TGFβ); interleukin 6 (IL6); metalloproteinase 2 (MMP2); NADPH Oxidase (NOX); collagen I (Col I) Supplementary Table 1.

Histological examinations

Sections of 5 µm were taken with a microtome (Leïca RM 2145, Microsystems Nussloch GmbH, Germany). Sirius red staining on 5 µm heart section slides was used to objectify areas of fibrosis on each category of rats using a microscope. Fibrosis was evaluated as the percentage of red stained pixels (collagenous tissue) in relation to the sum of green and red pixels (total tissue area) × 100% using Image J software. Immunostaining was performed using CD68 antibody (Bio-Rad, France) followed by infrared microscopy.

Statistical analyzes

The values were expressed as mean ± standard deviation. Statistical analysis is based on a two-way ANOVA, followed by Tukey Kramer's multiple comparison test. Statistical analyzes of the data were performed with StatView software (SAS Institute, Cary, NC, USA). The differences observed were considered significant for a p value <0.05. The Bravais-Pearson correlation test was used to evaluate linear regressions; the closer the values are to 1 (in absolute value), the stronger the relationship.

Ethical considerations

The research protocol for this study and all experimental procedures were approved by the local ethics committee in Montpellier, France (Reference CEEA-LR-12002).

RESULTS

Weight evolution kinetics of the animals

Figure 1 shows the kinetics of weight change of the rats fed the different diets. They were weighed weekly. The different diets resulted in a significant increase in the weight of the rats at the end of the 12 weeks compared to the rats fed the control diet.

Study of interstitial fibrosis and cardiomyocyte size

Figure 2A shows the micrographs of interstitial fibrosis lesions induced by the different oil-based diets in each category of rats on the heart (magnification x 200). Figure 2B shows the cardiomyocyte size in μm^2 and the proportion of interstitial fibrosis expressed as a percentage in animals fed the different diets. Cardiomyocyte size and the proportion of interstitial fibrosis were significantly increased in rats fed lard oil compared to the control diet. In contrast, they did not vary significantly in rats fed the red palm oil, palm olein diets compared to the control diet.

Oxidative stress, inflammatory and cardiac cytokine parameters

We determined oxidative stress parameters in the left ventricle of the hearts of rats fed the different diets by RTqPCR. RNA expression of the three NOX isoforms did not vary in the red palm oil fed rats compared to the control diet. The expression of NOX2 was significantly increased in rats fed lard oil (Figure 3). Furthermore, the RTqPCR study of SOD in the myocardium showed no variation between the different groups of rats (Figure 3). At the systemic level, no significant variation was observed in the oxidative stress parameters, regardless of the diet (Table 2). The expression of pro-inflammatory parameters (TGM2, TLR2 and ST2) did not vary in the red palm oil fed rats, but did vary in the other diets

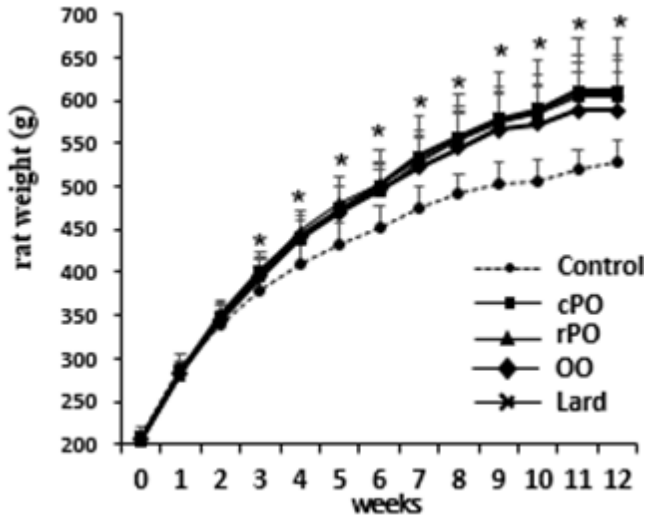

Figure 1. Kinetics of rat weights.

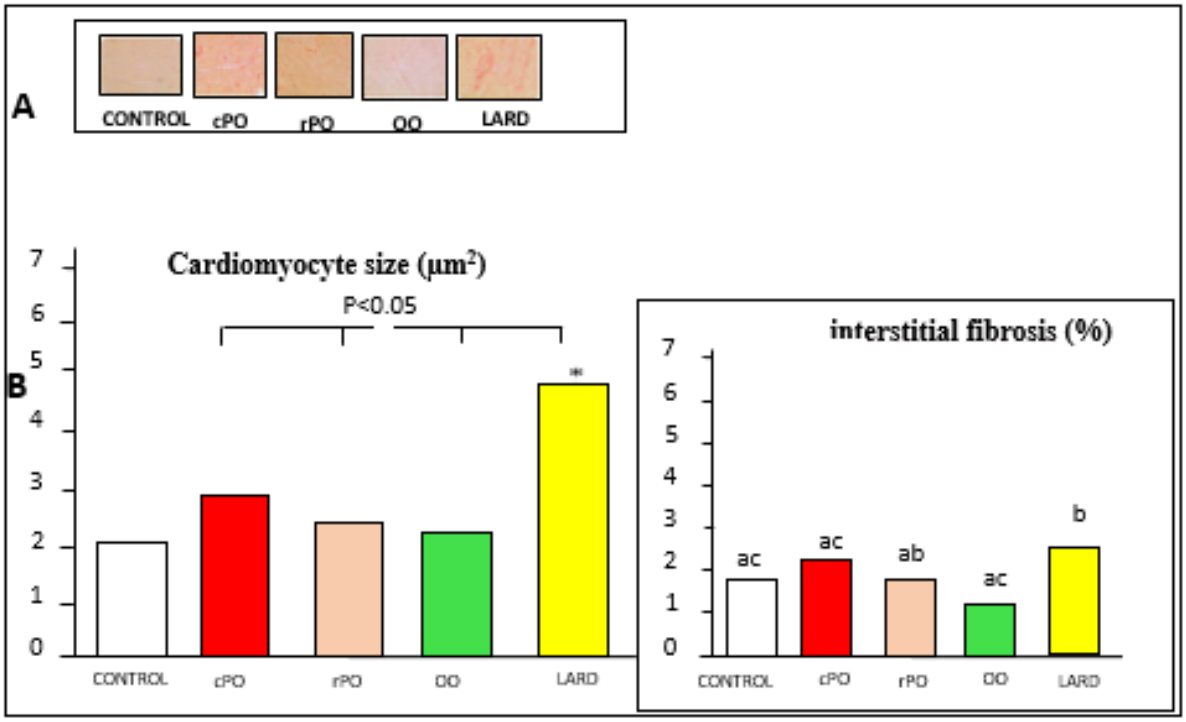

Figure 2. Effect of different regimens on cardiomyocyte size and proportion of interstitial fibrosis. The different letters (a, b, c) mean that the comparison between the different groups is statistically significant. The star * indicates the significant difference between the different groups compared to the control. Ctrl Diet = Control, Crude Palm Oil Diet = cPO, Refined Palm Oil Diet = rPO, Olive Oil Diet = OO, Lard Oil Diet = Lard.

(Figure 4).

Figure 5A shows micrographs of histological sections of the left ventricle (n = 10-20/rat) showing macrophage infiltration after labelling with primary CD68 antibody (magnification × 200). They show the effect of consumption of several vegetable oils on macrophage infiltration in the left ventricle. The results were expressed as a percentage of the tissue area infiltrated by macrophages. Results were expressed as mean values ± SD, n = 7-8 animals per group (Figure 5B). Our results show that macrophage

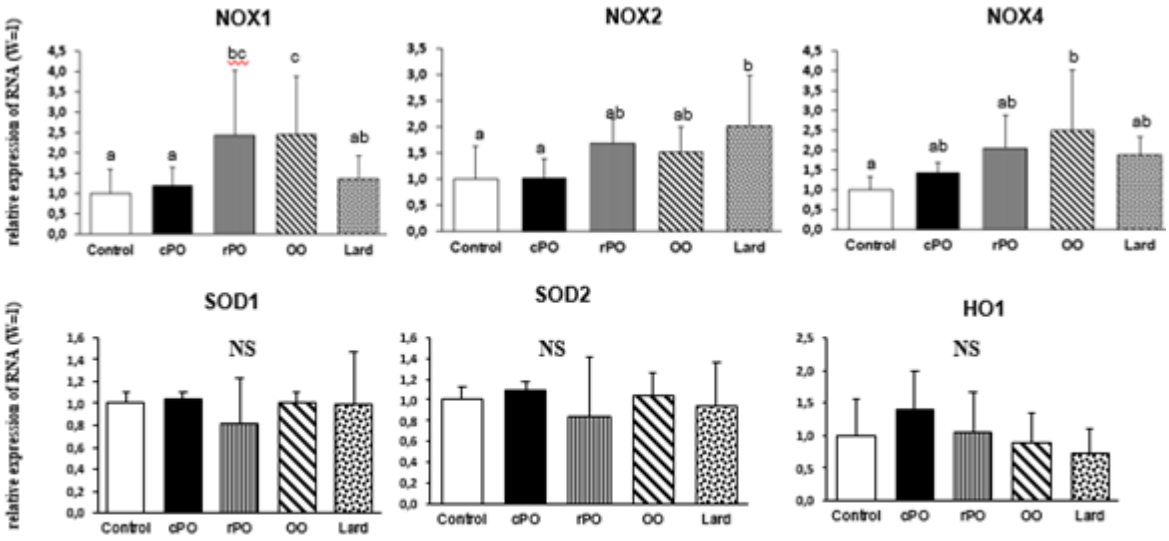

Figure 3. Study of markers of oxidative stress in the myocardium.

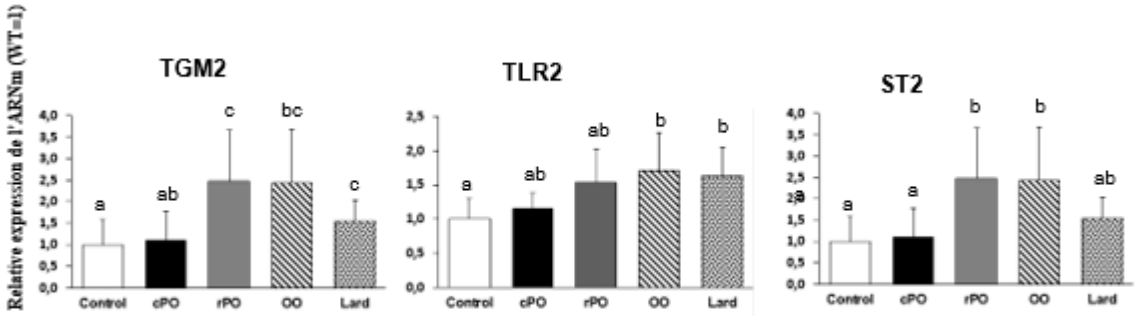

Figure 4. Studies of the parameters of inflammation and macrophage infiltration.

infiltration was significantly higher in the group of rats fed with lard oil. These macrophage infiltrations did not vary with red palm oil and the other diets. Also, the cardiac cytokines studied in the heart did not vary with the different diets (Table 3). On the other hand, a correlation between NOX2, NOX4 and the membrane receptor for interleukin 33 was observed in Figure 6. Results were expressed as mean ± standard deviation, n = 7–8 animals per group. Ctrl Diet = Control, Crude Palm Oil Diet = cPO, Refined Palm Oil Diet = rPO, Olive Oil Diet = OO, Lard Oil Diet = Lard.

DISCUSSION

We investigated the role of oxidative stress markers in the development of cardiac fibrosis in rats fed several high-fat diets, including crude palm oil, palm olein, olive and lard. Consumption of crude palm oil did not lead to overexpression of oxidative stress and inflammation parameters. Crude palm, palm olein and olive oil did not significantly increase the proportion of interstitial collagen and cardiomyocyte size compared to the control (lower calorie) diet.

Lard consumption resulted in a significant increase in cardiomyocyte size and proportion of interstitial fibrosis in rats at the end of 12 weeks compared to the control diet. Our study was in agreement with Kubant et al. (2015) who showed that lard fat consumption led to weight gain, visceral obesity, ventricular hypertrophy and cardiac fibrosis. High-fat diets induce obesity by altering carbohydrate metabolism (Lima-Leopoldo et al., 2011; White et al., 2013; Oliveira-Junior et al., 2014). The relative expression of NOX 1, NOX2 and NOX4 did not change in rats fed red palm oil compared to the control diet considered to be lower in calories. Consumption of red palm oil did not induce overexpression of NOX. Red palm oil appears to decrease free radical production through low NOX expression.

The nutritional benefits of palm oil in animals have

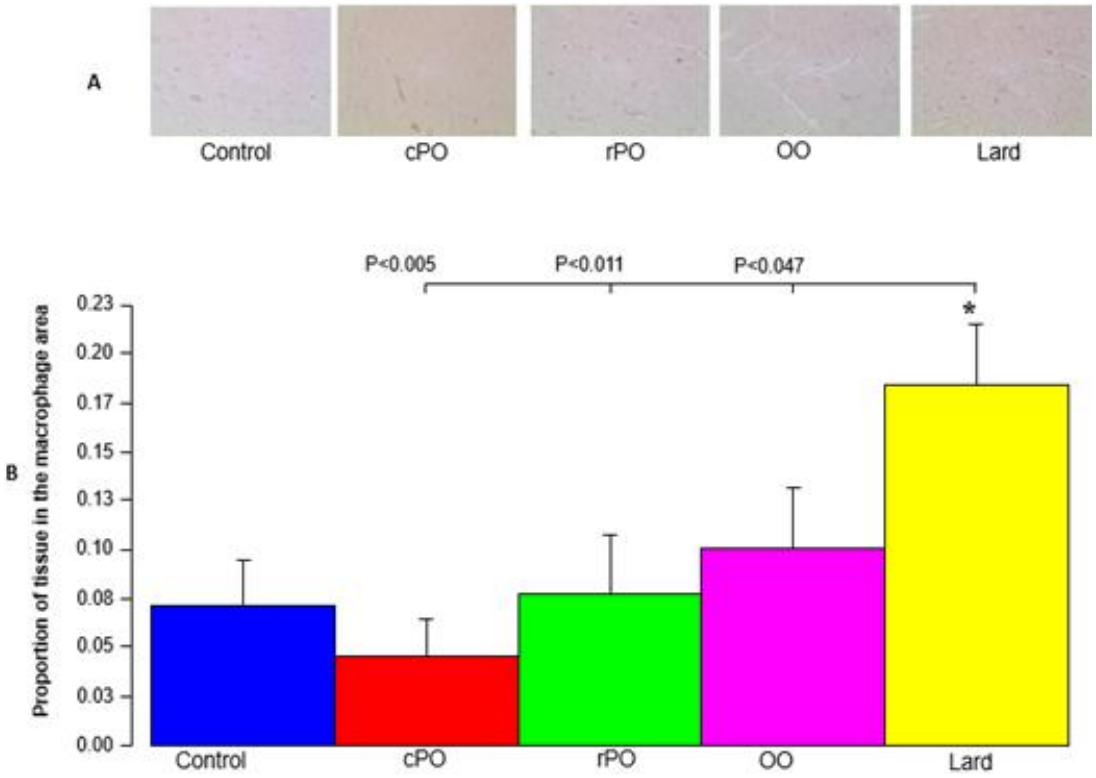

Figure 5. Effect of diets on macrophage infiltration.

Table 2: Study of plasma biomarkers of oxidative stress

Antioxidant system	Control	cPO	rPO	OO	Lard	P
SOD	324 ± 5,3	315 ± 8,7	296 ± 8,8	326 ± 6,6	311 ± 12	NS
GPx	14487±1170	16147±609	15945±1649	13927±478	13972±613	
Thiols (µmol/mL)	0,112±0,018	0,126±0,013	0,124±0,018	0,111±0,016	0,121±0,017	NS
TBARS(nmol/mL)	5,12 ±0,31	5,13 ±0,21	5,26 ±0,39	4,79 ±0,31	4,91 ±0,21	NS
15 -F_2t isoprostane (UA)	0,053±0,006	0,049±0,006	0,044±0,002	0,037±0,006	0,041±0,004	NS

The values of the parameters of the antioxidant system and of the products of lipid peroxidation are expressed as a mean ± SD (n = 7-8). SOD: Superoxide dismutase, GPx: Glutathione peroxidase, TBARS: Reactive substances of tiobarbituric acid. UA: arbitrary units, NOX: NADPH Oxidase, OH: Heme oxygenase, NS: means not significant. Ctrl Diet = Control, Crude Palm Oil Diet = cPO, Refined Palm Oil Diet = rPO, Olive Oil Diet = OO, Lard Oil Diet = Lard.

Table 3. Cardiac cytokine studies.

cytokines	Control	cPO	rPO	OO	LARD	P1(Hf/Cont)	P^2(Fat/Fat)
GDF 15	1,00±0.55	0.86±0.24	1.41±0.84	1.15±0.48	1.03±0.37	NS	NS
TGFβ	1,00±0.12	1.07±0.13	0.94±0.17	0.91±0.12	0.70±0.35	NS	NS
IL6	1,00±1.14	0.72±0.85	0.57±0.40	0.24±0.23	0.24±0.16	NS	NS
IL33	1,00±0.42	1.28±0.17	1.10±0.23	1.45±0.43	1.35±0.30	NS	NS
MCP1	1,00±0.23	0.98±0.44	1.78±1.53	1.09±0.73	1.09±0.61	NS	NS
COL1	1,00±0,25	1.08±0.27	1.17±0.17	0.93±0.35	1.00±0.28	NS	NS

TGM2: Transglutaminase2; TLR2: TOLL type cellular receptor; ST2: Iterleukin33 membrane receptor; GDF15: Growth differentiation factor-15. MMP2: Metalloproteinase 2; IL33: Interleukin 33; IL6: Interleukin 6; ColA1: Collagen A1: TGFβ: Transforming growth factor- β; NS: not significant, Ctrl Diet = Control, Crude Palm Oil Diet = cPO, Refined Palm Oil Diet = rPO, Olive Oil Diet = OO, Lard Oil Diet = Lard.

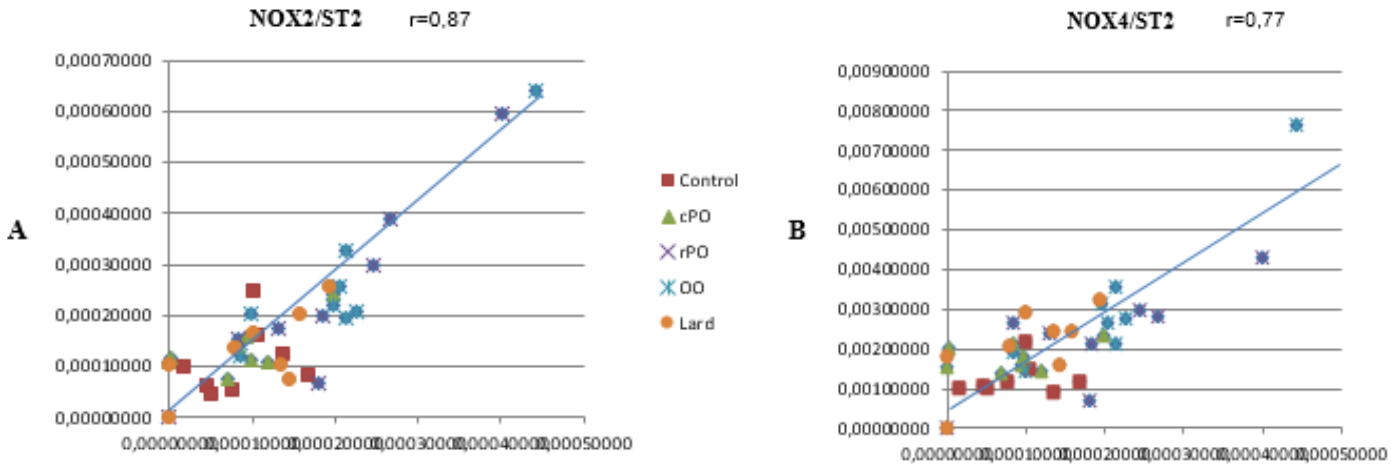

Figure 6. Correlation graph between NOX and ST2.NOX: NADPH Oxidase. ST2: Soluble interleukin 33 receptor, r: Bravais-Pearson correlation coefficient.

been elucidated by numerous studies (Suarna et al., 1993; Azlina et al., 2005) which have demonstrated the antioxidant effects of tocotrienols in palm oil. Coenzyme Q10 (ubiquinone), which is a natural coenzyme in palm oil, is a powerful free radical scavenger (Niklowitz et al., 2007). It has ten times the antioxidant power of carotenoids and vitamin E (Rosenfeldt et al., 2007). NOX2 is the most frequent isoform in the cardiovascular system (Infanger et al., 2006; Paravicini and Touyz, 2008). NOX2 was most expressed in rats consuming lard oil. This could explain the high proportion of interstitial fibrosis found in this group (Figure 2A and B).

Furthermore, NOX2 is involved in the recruitment of macrophages, which is an essential step in the formation of cardiac fibrosis (Van Buul et al., 2005). This observation is in agreement with the results of immunostaining with the CD68+ antibody, which shows a significant difference in macrophage infiltration in the cardiac cells of the lard-fed group of rats (Figure 5A and B). To further elucidate interstitial fibrosis, we analysed collagen I expression from myocardial tissue by RTqPCR. However, the collagen I RNA study did not vary significantly between oils.

Calligaris et al. (2013) confirmed fibrosis by overexpression of collagen types I and III in cardiac mRNA. In their model, cardiac remodelling was associated with thickening of cardiac fibres and the left ventricular wall, resulting in cardiac hypertrophy. NOX1 activity was increased in the palm olein and olive diets and NOX4 activity was higher in the olive group of rats. NOX1 and NOX4 although sharing 60 and 39% amino acid identity with NOX2 respectively (Guzik et al., 2004; Guzik et al., 2006) may have antagonistic functions (Schroder et al., 2012). Their physiological roles are poorly defined but they seem to play a central role in cell signalling (Arbiser et al., 2002; Cifuentes et al., 2006; Nauseef, 2008).

Nox4 and NOX1 mediate transforming growth factor β (TGF-β)-induced differentiation (Sturrock et al., 2006) In addition, cytokines have also been shown to regulate vascular NADPH oxidases, which associate inflammation with oxidative stress. In particular, tumour necrosis factor α (TNF-α) stimulates the expression and activation of Nox1, Nox2 and Nox4 in various vascular cells (Anilkumar et al., 2008; Basuroy et al., 2009; Moe et al., 2011). In our model, the different cytokines studied did not vary between regimes. Quantitative analysis of mRNA levels of molecules related to pro- or anti-inflammatory signals showed that TGFβ mRNA levels did not vary significantly between oils, contrary to data in the literature. (Okada et al., 2005; Lucas et al., 2010). To date, transforming growth factor beta (TGF-β) is the most potent and ubiquitous profibrogenic cytokine in fibrosis formation. It plays a central role in the development of fibrosis involving almost all organ systems (Lenz et al., 1996; Manouryet al., 2005; Rottoli et al., 2005). The factor growth differentiation factor-15 (GDF-15 is a member of the TGF-β superfamily, (Baan et al., 2015; Oshima et al.,2009).

In our study, GDF 15 expression did not vary significantly with diet. Our data are different from those of Tran et al. (2018) who objected that GDF15 deficiency promoted high-fat diet-induced obesity in knockout mice. On the other hand, pro-inflammatory parameters such as transglutaminase 2 (TGM2), Toll-like receptor (TLR2) and interleukin-33 membrane receptor (ST2) were significantly varied in the different diets, except in rats fed red palm oil (Figure 4A).There was a strong correlation between NOX4, NOX1 and soluble IL33 receptor. This observation could be explained by the fact that inflammation and oxidation are two fundamental processes underlying the pathogenesis of most human disease states.

Furthermore, it is now accepted that these two distinct mechanisms are in constant interaction, which is

particularly evident in the vessel wall (Lichtman et al., 2013; Miller et al., 2011; Takac et al., 2012). Vascular oxidative stress regulates the development of vascular inflammation which has recently been implicated in the pathogenesis of atherosclerosis (Harrison et al., 2011). Analysis of other parameters of the antioxidant system showed no significant difference between the different regimes, both in plasma and in the left ventricle.

In our study, the expression of superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) RNAs did not differ between the different diets. In contrast, in animal models, obesity was found to decrease the mRNA expression of antioxidant enzymes such as SOD, catalase (CAT) and GPx in white adipose tissue. (Furukawa et al., 2004). Several recent studies have shown that the expression of extracellular SOD or (SOD3) is decreased in the failing heart, and this has been associated with evidence of increased myocardial oxidative stress and endothelial dysfunction (Landmesser et al., 2003; Chen et al., 2005).

Noelia (2010) demonstrated that the absence of Gpx1 promotes angiotensin II-induced left ventricular hypertrophy and left heart dysfunction. The products of lipoperoxidation did not vary with the different diets, although all diets resulted in a significant increase in rat body weight compared to the control diet. Furthermore, F2 -IsoPs levels did not vary significantly between these diets, although multiple studies have clearly shown that F2 -IsoPs levels, measured in plasma, increase in adult obese patients (Basu, 2008; Kaikkonen et al., 2013). In the study by Furukawa et al. (2004), which involved several animal models of obesity, dietary fat intake caused an increase in lipid peroxidation (Furukawa et al., 2004).

Conclusion

Our study showed that red palm oil consumption did not result in overexpression of RNA parameters of oxidative stress and inflammatory markers. Myocardial NADPH oxidase expression did not change in rats consuming red palm oil compared to the control diet. However, consumption of palm olein, olive and lard resulted in a significant change in myocardial NADPH oxidase activity. This study seems to show that red palm oil, because of its high antioxidant content, is less harmful to the heart.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The authors extend their gratitude to all the volunteers who accepted to participate in this study.

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SUPPLEMENTARY MATERIALS

Table 1. The gene sequences used in the study.

Primer sequence	Forward	Reverse
EMR1	GCCATAGCCACCTTCCTGTT	ATAGCGCAAGCTGTCTGGTT
GDF15	TGTTCCTGCTGCTCTTGCTG	TCGCACCTCTGGACTGAGTATC
COL A I	GACTGTCCCAACCCCCAAAA	TGGGTCCCTCGACTCCTATG
TGFβ	GACCGCAACAACGCAATCT	GACAGCCACTCAGGCGTATC
IL33	CCCTGAGCACATACAACGACC	CACCATCAGCTTCTTCCCATC
ST2	ATGATTGGCAAATGGAGAAT	TTCTAGACCCCAGGATGTTT
MCP1	TGTCTCAGCCAGATGCAGTT	CAGCCGACTCATTGGGATCA
SOD1	AGA GAG GCA TGT TGG AGA CCT G	ACG GCC AAT GAT GGA ATG CTC
SOD2	TCT GAA CGT CAC CGA GGA GAA G	AGT GCA GGC TGA AGA GCA AC
NOX1	CCAAACGTGACAGTGATGTATGC	AGCTGAAGTTACCATGAGAACCAA
NOX2	CGTATTGTGGGAGACTGGACTGA	AGGGCCCATCAACTGCTATCT
NOX4	GCCTAGGATTGTGTTTGAGCAGA	GCGAAGGTAAGCCAGGACTGT
TLR2	GAGGTCTCCAGGTCAAATCTCAG	ACACACCAGCAGCATCACAT
TGM2	CACTGTCAGCTACAACGG	CGCACCTTGATGAGGTTT
Rplpo	CACTGGCTGAAAAGGTCAAGG	GACTTGGTGTGAGGGGCTTA