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Oil emulsion from *Plukenetia huayllabambana* (Sacha inchi) modifies nitric oxide and leptin in the liver and antioxidant and inflammation markers in the adipose tissue in obese rats

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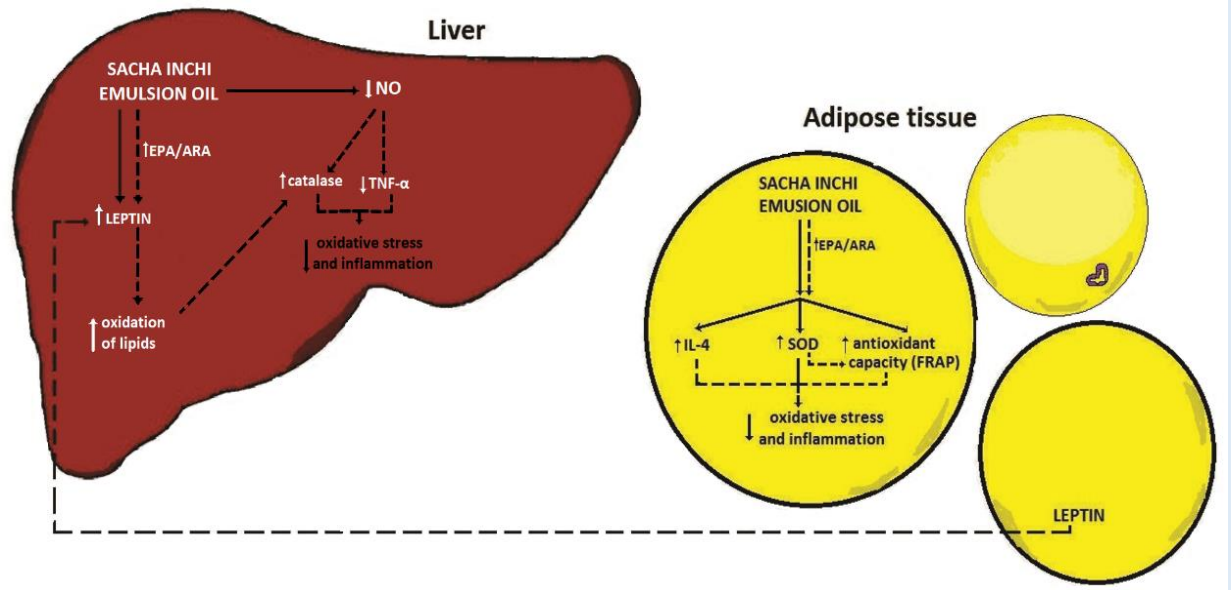
ABSTRACT

Background: Obesity is characterized by excessive accumulation of adipose tissue and is associated with higher risk of metabolic diseases and other comorbidities. Efficacious strategies including a diet high in "functional foods" are promising. *Plukenetia huayllabambana* known as Sacha Inchi (SI), is a legume which seeds are rich in proteins, tocopherols, and fatty acids such as omega-3 (ω-3). The latter has emerged as a potential protective nutrient against the cardiometabolic risks associated with obesity. Omega-3 changes the membrane lipid profile of hepatic and adipose cells triggering the expression of antioxidant and anti-inflammatory genes. However, there are few reports in relation to the effect of these oils in inflammatory and stress response related to obesity. In this sense, the present study evaluated the effect of SI oil emulsion on nitric oxide and leptin levels in the liver and some markers of oxidative stress and inflammation in adipose tissue from the rodent obesity model.



Methods: Six groups were formed: Not obese control group (Noc), obese control (Oc), two groups treated with the emulsion of SI oil (Os1:0.25g ω-3/day; Os2:0.5g ω-3/day), one obese group treated with atorvastatin (Oa) and one group treated with atorvastatin plus the emulsion of SI oil (Oas2).

Results: Os1 and Os2 lowered nitric oxide and increased liver leptin levels. In the adipose tissue, the superoxide dismutase and reducing antioxidant power increased significantly in Os1 and Os2 groups. The anti-inflammatory marker IL-4 was also increased in Os2, Oa and Oas2 compared to the Oc and IL-10 increased in Oas2 group.

Conclusion: Our study suggests that the emulsion of SI oil can modify the inflammatory and stress responses associated with obesity and it can be incorporated as a promising functional food.

Keywords: Inflammation, leptin, obesity, nitric oxide, oxidative stress, SI oil emulsion.

INTRODUCTION

Obesity is a multifactorial disease whose prevalence has increased in the last years reaching a pandemic level. It represents a risk for human health since it is associated with a higher probability of Type 2 diabetes mellitus, fatty liver, hypertension, myocardial infarction, stroke, mental illness, osteoarthritis, obstructive sleep apnea and various types of cancer, diminishing the quality and life expectancy [1–3]. Obesity has also been linked to be a unifying risk factor for severe COVID-19 infection because it reduces protective cardiorespiratory reserve as well as potentiates the immune dysregulation. This latter appears, at least in part, to mediate the progression to critical illness and organ failure in a proportion of patients with COVID-19 [4,5].

The most common cause of this illness is an imbalance between energy intake and expenditure, which causes an increase in fat storage related to an inflammatory state. It stimulates the infiltration of monocytes and macrophages in hepatic and adipose

tissue increasing the production of pro-inflammatory cytokines such as TNF-α, leptin, and IL-6 and decreasing adiponectin, IL-10, IL-4 [6–8]. Increased leptin levels in serum cause the blockage of their anorexigenic effects and benefit the inflammatory process. However, leptin has also lipolytic functions that can lower tissue triglycerides [9,10].

Nitric oxide (NO) has been increased in obesity models contributing to insulin resistance and vascular effects [11]. Under normal physiological conditions NO can act as a second messenger and antioxidant, though in pathological cases like sepsis, inflammation, ischemia or obesity, could lead to cytotoxicity by the production of reactive nitrogen species (RNS) [12]. Oxidative stress (OS) is another biological process disrupted in obesity which induces the expression of malondialdehyde (MDA), a marker of lipid oxidation, diminishing its antioxidant capacity [13,14]. Increasing OS, especially in epididymal adipose tissue, could be reduced by the catalase (CAT) and superoxide dismutase (SOD) activity as antioxidant enzymes [15–17]. SODs convert superoxide radical into hydrogen peroxide and molecular oxygen, while the catalase converts hydrogen peroxide into water [18].

Sacha inchi (*Plukenetia huayllabambana*) also named Inca inchi, is a native plant to the Peruvian Amazon and is recognized as a sustainable crop with commercial applications. The oil has been investigated due to its composition in proteins and omega 3 fatty acid (ω-3) [19,20]. This latter has emerged as a potential protective nutrient against the cardiometabolic risks associated with obesity [21,22]. Sacha Inchi (SI) oil was previously reported by our group to have an antilipemic effect in serum and liver and it was investigated due to its composition and potential health benefits [19,23]. However, it is necessary to continue evaluating its effects in liver and in adipose tissue since it regulates the lipid metabolism by the adipokines release. Consequently, the aim of this study was to evaluate the effect of Sacha Inchi (SI) oil emulsion on NO and hepatic leptin levels, and in some oxidative stress and inflammation markers of adipose tissue in a high-fat diet-induced obesity rat model.

MATERIALS AND METHODS

Animals and diet: One hundred male rats of the Sprague Dawley strain, approximately 6 weeks old and 180g of weight, were used. The animals obtained from the animal facility of the *Universidad Peruana Cayetano Heredia* (UPCH) were acclimatized for one week under 12 hours of light and 12 hours of darkness. They received a diet high in lipids of 45% Kcal (HFD; D12451, 45kcal% of lipids, Research Inc. USA) for a period of 16 weeks. Approximately 40% of them were classified as obese by their body weight, Lee and Roher index, total cholesterol, and triglycerides [24–26]. These animals were grouped for treatment: obese control (Oc, n = 6), obese 2.5 ml dose of the SI emulsion 1 (Os1, 0.25g ω-3/day, n = 7), obese 2.5 ml dose of the SI emulsion 2 (Os2, 0.5g ω-3/day, n = 7), obese with 10mg/Kg atorvastatin dose (Oa, n = 6) and obese with 10mg/Kg atorvastatin (10mg/Kg) plus 2.5ml SI emulsion 2 (Oas2, n = 6). Another group received a standard diet with 10% Kcal of lipids (LFD, D12450B, 10kcal% of lipids, Research Diets, Inc. USA) to conform the non-obese control group (Noc, n = 6). After an eleven-week orogastric treatment period, they were sacrificed on an empty stomach with pentobarbital to extract blood by cardiac puncture. Then, the blood was centrifuged to obtain the serum and stored it at -20°C for subsequent laboratory

analysis [27,28]. The liver and adipose tissue were also removed and frozen with liquid nitrogen to be stored at -80°C.

The SI emulsion was synthesized from seeds of *Plukenetia huayllabambana* (LMH-E1410) by HERSIL S.A. (Lima Peru). The emulsion was mixed with excipients at a ratio of 1: 1.74. The SI oil was composed of 54.79% linolenic acid, 26.69% linoleic acid, 9.71% oleic acid, 5.2% palmitic acid and 1.9% stearic acid measured by High Performance Liquid Chromatography. All the procedures were approved by the IRB from UPCH.

Determination of antioxidant stress and anti-inflammatory markers in epididymal adipose tissues: MDA was measured by thiobarbituric acid reactive species (TBARS, SIGMA) concentration at a wavelength of 532 nm. It was added to 100 ul of beta hidroxitoluene (BHT, SIGMA), 100 ul of $FeCl_3$ $6H_2O$, 1.5ml of acetate buffer and 1.5ml of thiobarbituric acid (TBA, SIGMA) to 100 ul of the sample (1:10). Then, it was mixed vigorously and incubated for one hour at 95-100ºC, 2.5 ml of butanol and 0.5 ml of water were added and blended for one minute to be centrifuged after at 4000 rpm for 10 minutes. The concentration of MDA in adipose tissue (nmol/g of protein) was estimated using a calibration curve with 1,3,3-tetraethoxypropane (SIGMA) [29,30].

Two methods were used to determine the antioxidant capacity in adipose tissue, the ferric reducing antioxidant power (FRAP) and $ABTS^+$ tests [31]. 0.5g of adipose tissue was homogenized in 2 ml phosphate buffer 10 mM at 4ºC, then centrifuged at 1500 rpm for 10 minutes. The resulting supernatant was diluted 1:2 and used for an antioxidant capacity test [32]. For the standard curves, ferrous sulphate at concentrations of 62.5-2000 µM and trolox (Calbiochem) at 1-5 mM were used for the FRAP and $ABTS^+$ assays at 593nm and 660 nm, respectively [33,34].

The enzymatic activity of SOD and CAT in adipose tissue were determined by the capacity to inhibit the autoxidation of pyrogallol and the formation of chromic acetate. Adipose tissue was homogenized (25% weight/volume) at 4°C with tris-cacodylic acid buffer containing 1mM EDTA, pH 8.2. Then, it was centrifuged at 6000 g at 4°C for 10 minutes. The supernatant was used to measure the activity of SOD at 420 nm [35,36]. For the catalase activity, the epididymal adipose tissue was homogenized (25% weight/volume) in phosphate-buffered saline pH 7 at 4°C and centrifuged at 10000 rpm at 4 °C for 15 minutes. The supernatant at a dilution de 1/1000 in phosphate buffer with 0.5% BSA was used to evaluate the activity of catalase at 570nm [37,38].

To evaluate IL-4, IL-10 and TNF-α in adipose tissue, 1g was homogenized with 4ml of RIPA buffer and protease inhibitor, then centrifuged at 13000 rpm for 10 min at 4°C. Supernatants diluted 1:4 were used to determinate cytokines according to DB OptEIA commercial kit procedures (BD Biosciences). The samples were read at 550 nm in a microplate reader VERSAmax, CA 94089, Molecular Devices.

Determination of NO and leptin in the liver: 0.5 g of liver tissue was homogenized with 2 ml of RIPA buffer (SIGMA-ALDRICH) and protease inhibitor at 4ºC [39]. It was centrifuged 10000 g at 4ºC for 60 minutes in plates with filters (10-30 kDa) and the supernatants were used to determine nitric oxide according to Nitrate/Nitrite Assay Kit user manual (CAYMAN CHEM). To determine liver leptin, the tissue was homogenized at a weight-volume ratio of 1:5 with RIPA buffer and protease inhibitor at 4°C

[39]. After that, the homogenized tissues were incubated at 4°C for 60 min and then centrifuged at 16000 g at 4°C for 1 hour. The supernatants were used to measure liver leptin according to kit method Millipore ELISA (catalog number: EZRADP-62K). The samples of NO were read at 550 nm in a microplate reader VERSAmax, CA 94089, Molecular Devices.

Determination of protein levels: To normalize the protein concentration in liver and adipose tissue, 25 ul of homogenate was used at a dilution of 1:20 and 1:10, respectively. Then, Pierce® BCA commercial kit (THERMO SCIENTIFIC) procedures were performed.

Statistical analysis: The statistical analyses were performed using the STATA12 program. The results were expressed as mean ± standard error (SE). The data was evaluated using a one-way ANOVA test. The Bonferroni multiple comparison tests were calculated when the p-value in the ANOVA was significant ($p<0.05$). The Kruskal-Wallis test and Wilcoxon test of sum and ranges was performed when the distribution was not normal, or equality of variances were not equal.

RESULTS

Effect of the emulsion of SI oil emulsion on antioxidant stress and anti-inflammatory markers from adipose tissue: FRAP marker significantly decreased in the Oc group compared to Noc but increased significantly in group Os2 (42%) compared with the Oc group ($p<0.05$). The SOD activity significantly decreased in Oc group (58%) compared to Noc. The groups Os1 and Os2 treated with the SI oil emulsion have 4.4 to 2.5 times more SOD activity than the Oc group, respectively ($p<0.05$). Anti-inflammatory markers such as IL-4 and IL-10 showed differences in their concentrations($p<0.05$). IL-4 increased in Os2, Oa and Oas2 groups compared to Oc ($p<0.01$). Although Os1 group showed an increase of more than 60% in IL-4 concentration, it was not significant. Additionally, it increased their concentration in Noc group respect to Oc. IL-10 increased their concentration in the Oas2 compared to the Oc group. There were not significant differences between Noc and Oc groups and within treated SI oil emulsion group in relation to the TNF-α, CAT, MDA and ABTS concentration (Table 1).

Table 1. Effect of the emulsion of Sacha Inchi (*Plukenetia huayllabambana*) oil on antioxidant stress: malonaldehyde (MDA), total antioxidant capacity (FRAP, ABTS), superoxide dismutase (SOD) and catalase (CAT) activities in adipose tissue of obese rats induced with a high lipid diet.

	Noc	Oc	Os1	Os2	Oa	Oas2
MDA (nmol/mg protein)	27.8±2.9	22.7±1.7	29± 3.6	35.5±2.3	29.5± 4.2	19.4±3.3
FRAP (µmol/mg protein)	265.7±8.2**	211.3±16.6	206.7± 15.9	300.1± 17.4**	278.3±34.6	261±38.8
ABTS (mmol/mg protein)	1.21± 0.05	1.08±0.16	0.91± 0.1	1.17± 0.05	1.16± 0.18	1.01±0.03
SOD (U/mg protein)	5.9±0.8*	2.5±0.5	11±2.8**	6.2±0.8**	6.8± 2.3	3.3±0.7
CAT (µmol H_2O_2/mg protein)	1474±125.2	1333±120.3	1256±77.8	1370± 60.7	1431±104.5	1246±59.8
TNF-α (pg/mg protein)	90±7.5	58±7.2	69.8±10.1	84±12.1	81.6±7	69±4.1
IL-4 (pg/mg protein)	8.4±0.9**	4.8±0.4	6.2±0.8	7.7±0.9*	7.4±0.8*	6.4±0.2*
IL-10 (pg/mg protein)	13.9±0.7	9.4±2.7	16.2±3.1	15.2±1.1	12.9±4.1	26.5±3**

Noc: non-obese control group, Oc: obese control group, Os1: obese group with emulsion of SI oil 1, Os2: obese group with emulsion of SI oil 2, Oa: obese group with atorvastatin, Oas2: obese group with emulsion of SI oil 2 plus atorvastatin. *p<0.05 Compared with Oc. **p<0.01 Compared with Oc.

Effect of SI oil emulsion on NO and leptin in the liver: Hepatic NO level was 5.2 times higher in Oc group than Noc group; however, it diminished in 35% and 32% in Os1 and Os2 group in contrast with Oc group. Leptin hepatic levels did not change significantly between Oc and Noc groups, but they were increased in Os1 and Os2 groups in 36 and 64%, respectively in comparison with Oc group ($p<0.05$).

Table 2. Effect of the emulsion of Sacha Inchi (*Plukenetia huayllabambana*) oil on nitric oxide and leptin of liver tissue, and adipose tissue cytokines (TNF-α, IL-4 and IL-10) in obesity-induced with a high lipid diet.

	Noc	Oc	Os1	Os2	Oa	Oas2
NO (nmol/mg protein)	3.9±0.59**	20.3±1.32	13.3±1.27**	13.9±0.73*	16.9±1.86	18.6±1.85
Leptin (ng/mg protein)	5.6±0.37	5.65±0.44	7.67±0.80*	9.26±0.89*	8.08±1.52	6.21±0.57

Noc: non-obese control group, Oc: obese control group, Os1: obese group with emulsion of SI oil 1, Os2: obese group with emulsion of SI oil 2, Oa: obese group with atorvastatin, Oas2: obese group with emulsion of SI oil 2 plus atorvastatin. *$p<0.05$ Compared with Oc. **$p<0.01$ Compared with Oc.

DISCUSSION

An increase in oxidative stress has been reported in obesity and models for obesity research due to an imbalance between reactive oxygen and nitrogen species in the body´s antioxidant system [15,40,41]. In the present study, markers of oxidative stress in the epididymal adipose tissue showed positive changes in the ferric reducing antioxidant power (FRAP) and enzymatic activity of SOD. Antioxidants protect us against oxidative cellular damage; for this reason, measuring the antioxidant capacity in biological samples using FRAP and $ABTS^{+}$ methods can be a protective predictor. In our study, the FRAP antioxidant capacity values of epididymal adipose tissue of the obese control (Oc) decreased confirming that obesity decrease the antioxidant capacity [13,14]. The FRAP marker increased in the treated groups due to a higher SOD activity (Table 1), tocopherols, flavonoids and other antioxidants like OH-1 and NQO1; probably regulated by ω-3 [42–44] but further analysis is required. The increase in antioxidant capacity in epididymal adipose tissue and hepatic tissue shows that the emulsion of SI oil has a tissular effect but not extracellular [23].

About SOD activity in adipose tissue, a significant decreased was observed in the Oc group compared to the Noc group, which agrees with findings reported in obese induced rodents [15]. This decrease in SOD activity and the lower antioxidant capacity in the Oc group can increase oxidative stress (Table 1) and lead to deregulation in adipokines production like leptin [15,23]. In treated groups with SI oil emulsion (Os1 and Os2), the enzymatic SOD activity increased compared to the Oc group meaning that a hydrogen peroxide concentration also increased. Hydrogen peroxide is degraded by glutathione peroxidase (GPx) or CAT, however, catalase activity did not show a significant increase in treated group. Furthermore, the measure of GPx activity and other markers such as J3-isoprostanes (a product of EPA and DHA oxidation) that induces the expression of Nrf2, a transcription factor that regulates antioxidant genes expression [45,46], could help to clarify the antioxidants pathway in further studies. Although the levels of MDA in treated groups with SI oil emulsion did not change significantly, they showed a growing trend (Table 1). For this reason, it can be interesting to further study

it because MDA is a marker of lipid peroxidation that increase their concentration during obesity [15,41] and possibly the treatment could have benefited the increasing of ω-3 LCPUFA, as reported in previous studies using chia oil that have a high content of ω-3 [47].

NO is synthesized from the amino acid L-arginine, NADPH, and O_2 employing the inducible nitric oxide synthase (iNOS). This enzyme is different from the other isoforms (eNOS, nNOS) because it can produce more NO and binds with calmodulin to be activated, even with low calcium concentration levels [48]. Hepatic NO level was 5.2 times higher in the Oc group than the Noc group, possibly due to an increase in iNOS activity [49,50]. These findings agree with results of high levels of NO associated with inflammation and hepatic damage in obesity and obesity models [50–52]. The treatment of SI oil emulsion lowered NO levels, as a probable consequence of ω-3 metabolites (EPA and DHA) that can generate peroxidized metabolites that reduce iNOS or can avoid protein and gene expression of NF-κB and iNOS [53–56]. TNF-α, which is capable of increasing iNOS and COX 2, decreased with the treatment and consequently the NO levels [23,57].

Leptin regulates food intake by acting on cell receptors in the arcuate nucleus, but in obesity, the brain does not respond to this hormone, despite the unusually high levels; this leads to "leptin resistance" that produces an inflammation state [9,58]. SI oil emulsion decreased serum leptin levels in the rat groups induced to obesity [23]; nevertheless, the hormone levels increased in hepatic tissue, which would contribute to less storage of triglycerides and fatty acid oxidation since it reduces acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), and promotes the expression of CPT1 [10,59]. This finding reinforced the idea that mitochondrial oxidation and peroxisomal increased in treated rats with oils with high content of ω-3 [60]. The emulsion with a high content of ω-3, may change the lipid composition of the cellular membrane in hepatocytes that stimulates the uptake of leptin by the tissue contributing to lower leptin levels in serum [23,56,61].

In obesity, there is an accumulation of pro-inflammatory immune cells in visceral adipose tissue such as T cells and macrophages that can lead to chronic inflammation [62–64]. Also, an association between obesity and hyperinsulinemia has been reported, the latter with the decrease of IL-10 [65]. Rats treated with a high-fat diet (58% Kcal) and sucrose for 16 weeks showed hyperinsulinemia and reduced expression of IL-10 mRNA in visceral adipose tissue, spleen and T cells. In the present study, high leptin levels [23] likely increased the infiltration of T cells and monocytes into hepatic and adipose tissue [62,63], but in treated groups, the emulsion of SI oil would be regulating serum insulin levels by higher production of IL-10 in infiltrated T cells. Increased IL-10 in the treated groups with the emulsion would also increase insulin sensitivity and protection against macrophage tissue infiltration [66]. Anti-inflammatory cytokines IL-4 are produced by T-cells and natural killers that are negatively correlated with BMI and insulin resistance [67]. Additionally, it was reported that natural killer cells decrease in obesity and thus the IL-4. The emulsion of SI oil increased the cytokine in epididymal adipose tissue that could benefit an anti-inflammatory and lipolytic effect. In the first, IL-4δ2 inhibits the proliferation of T-cells and expression of COX 2, and in the second effect IL-4 can increase the expression and activity of hormone sensitive lipase through

PPARγ or PKA [68–70]. This improves the release of fatty acids to the serum and oxidation by hepatic acyl-CoA oxidase [60]. The ω-3 of the emulsion in the form of EPA was probably increasing IL-4δ2 since it was tested in an inflammation model using LPS after rats were supplemented with EPA and gamma-linolenic acid [71]. Also, EPA and DHA are increased in adipose tissue of rats treated with SI oil of the species *Plukenetia volubilis* [72]. In our study, the IL-4 increase in adipose tissue and the growing tendency of hepatic IL-4 [23] showed that the emulsion would have benefits in the inhibition of lipid accumulation in adipose and hepatic tissue and decrease the inflammation. These statements should be demonstrated in future studies, especially with different forms of IL-4, since stimulation of lipogenesis and inhibition of lipolysis in an *in vivo* study of the epididymal adipose tissue in non-obese rats was observed [73].

The kinds of acyl groups that are present in SI oil are known to provide protection against cardiovascular disease, rheumatoid arthritis, cancer, and possibly the severity of viral infections. The latter could be considered particularly important in the current context of a pandemic caused by COVID-19, since there is an apparent relationship between obesity and the risk of developing severe COVID-19 illness.

The present study reinforced the proposed idea in our previous study using the same SI oil emulsion [23]. This latter can help to regulate the biochemical alterations of obesity in a rodent model.

CONCLUSIONS

The emulsion of SI oil (*Plukenetia huayllabambana*) influences oxidative stress decreasing NO hepatic marker and increasing superoxide dismutase expression and antioxidant capacity in adipose tissue. It would also contribute to the anti-inflammatory effect by reducing hepatic NO and the increment in IL-4 of epididymal adipose tissue. Finally, the increase in hepatic leptin would be promoting a possible hepatic lipid oxidation mechanism that needs further analysis. All these results would be helping normalize metabolic alterations in the obesity model used.

List of abbreviations: SI: Sacha Inchi, Noc: Not obese control group, Oc: Obese control, Os1: Obese group 1, Os2: Obese group 2, Oa: Obese group treated with atorvastatin, Oas2: Obese group treated with atorvastatin plus the emulsion of SI oil, NO: Nitric oxide, OS: Oxidative stress, MDA: malondialdehyde, CAT: catalase, SOD: superoxide dismutase, TBARS: thiobarbituric acid reactive species, BHT: beta hidroxitoluene, TBA: thiobarbituric acid, FRAP: ferric reducing antioxidant power, IL-4: Interleukin 4, IL-10: Interleukin 10, TNF-?: Tumor necrosis factor, GPx: glutathione peroxidase, iNOS: nitric oxide synthase, ACC: acetyl-CoA carboxylase, FAS: fatty acid synthase

Authors' Contributions: Johnny Ambulay, Percy Rojas, Olga Timoteo, Teresa Barreto, Zayra Vila and Maxy De los Santos performed all clinical study; Johnny Ambulay and Ana Colarossi designed the research; Maria Eguiluz and Johnny Ambulay analyzed the data and prepared the manuscript.

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