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Caffeic acid reduces lipid accumulation and reactive oxygen species production in adipocytes

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Caffeic acid (CA) is a hydroxycinnamic acid, a group of natural organic phenolic compounds used in folk medicine and in foods for the prevention and treatment of obesity. CA is present in a variety of plants, as it is an intermediate in secondary metabolism. It is found in coffee, wine and olive oil. The aim of this work was to study the influence of caffeic acid (CA) on the reduction of intracellular lipid accumulation, intracellular reactive oxygen species (ROS) formation and in mitochondrial transmembrane potential alterations in differentiated 3T3-L1 cells. The pre-adipocyte cell line 3T3-L1 was differentiated using a differentiation cocktail. Cells were treated with CA, and the MTT assay was performed to assess the effect of CA on pre-adipocytes. The quantification of lipids accumulated within the mature cells was performed using Oil Red O dye. Flow cytometry was used to evaluate the production of ROS through 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation and changes in mitochondrial transmembrane potential by Rhodamine 123 dye (Rho123). CA did not exhibit significant toxicity on the 3T3-L1 cell line at the studied concentrations. Caffeic acid causes significant reduction of lipid content in the cells submitted to the post- and co-treatment, being more effective in co-treatment. Caffeic acid results in an absolute decrease in the formation of intracytoplasmic reactive oxygen species. The treatment with caffeic acid protects against oxidative stress caused in the mitochondria by the adipocyte differentiation process. Thus, CA acts on adipogenesis, reducing intracellular lipid accumulation in the 3T3-L1 cells. It reduces intracellular ROS formation and mitochondrial transmembrane potential alterations in differentiated cells.

Key words: Caffeic acid, adipocytes, reactive oxygen species, oxidative stress.

INTRODUCTION

Obesity is described as a chronic inflammatory state associated with oxidative stress, leading to severe cardiovascular damage. The disease has a worldwide distribution, resulting in increased costs for health

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systems, especially in industrialized countries and is associated with several other comorbidities, such as metabolic syndrome, hypertension, type-2 diabetes, dyslipidemia and cardiovascular risk, with significant morbidity and mortality (Bocco et al., 2016; Omech et al., 2016). Worldwide, at least 2.8 million people die each year as a result of being overweight or obese (Organization, 2016). In Brazil, it is estimated that 50% of the adult population is overweight and 15% are obese (Alves and Faerstein, 2015).

Natural plant products are a source of compounds that could be potentially active against obesity and overweight. Hydroxycinnamic acids, such as caffeic acid (CA), are natural organic phenolic compounds used in folk medicine and in foods for the prevention and treatment of obesity (Rondanelli et al., 2014). CA is present in a variety of plants as intermediary in secondary metabolism. It is found in coffee, wine and olive oil; worldwide, popular antiobesity uses have been traditionally attributed to this polyphenolic compound (Wider et al., 2013; Rondanelli et al., 2014; Crevar-Sakac et al., 2016; Rangboo et al., 2016).

Previous studies have demonstrated that esters containing caffeic acid have antioxidant activity and decreasing obesity in models *in vivo* and *in vitro* (Farmer, 2006; Juman et al., 2012). Thus, this short communication aims to study the influence of isolated CA on the reduction of intracellular lipid accumulation in differentiated 3T3-L1 pre-adipocytes cells and to evaluate oxidative stress through intracellular reactive oxygen species (ROS) formation, as well as in mitochondrial transmembrane potential alterations (ΔΨm).

MATERIALS AND METHODS

Mouse embryo fibroblast pre-adipocyte cell line 3T3-L1 was obtained from the cell bank of Federal University of Rio de Janeiro and maintained in DMEM culture medium supplemented with 2.5 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L $NaHCO_3$ and 10% foetal bovine serum (Gibco, Grand Island, NY, USA), 100 units/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin (Sigma), in atmosphere with 5% CO_2 in culture plates until they reached 80% confluence. Before the experiments, the culture medium was removed, and cells were displaced using trypsin-EDTA solution for 5 min at 37ºC. For studies in mature adipocytes, cells were differentiated two days after confluence with DMEM-IDI (isobutylmethylxanthine -IBMX 0.5 mM; dexamethasone 0.25 mM, and insulin 10 µg/ml - Sigma) (day 0). Two days after differentiation initiation, DMEM-IDI was substituted by DMEM. After 8 days, approximately 80% of the cells were differentiated (Lin et al., 2005). An MTT assay was performed in order to assess the range of caffeic acid cytotoxicity on pre-adipocyte (Mosmann, 1983). 3T3-L1 cells (10^5 cell/mL) were treated with different concentrations (1000, 500, 250, 125, 62.5, 31.25 and 15.1 mM) of caffeic acid (Sigma-Aldrich, St. Louis, MO, USA, purity of 99.5%) diluted in saline solution and incubated for 24 h at 37°C and 5% CO_2. Sterile PBS, pH 7.4 was used as a negative control. DMSO 0.5% was used as vehicle control and it did not cause any effect.

To observe the effects of caffeic acid on the inhibition activity of lipids droplets, treatments were performed after the last day of differentiation for 24 h (post-treatment) or daily from day 0 of differentiation (co-treatment) at concentrations of 62.5 and 31.25 µM, which were considered the lowest non-toxic in the MTT assay.

In order to assess the differentiation grade of preadipocytes into adipocytes and the quantification of lipids accumulated within the mature cells, the Oil Red O dye coloration was performed (Simons et al., 2005). After differentiation, cells were fixed with p-formaldehyde (4%) and stained with Oil Red O solution at 0.3% (w/v) in isopropanol: Distilled water (3: 2). After 4 washes with distilled water and drying of the plate at room temperature, cells were visualized and photographed microscopically (Nikon 50i, Nikon, Japan) at a magnification of 400×.

Subsequently, in order to quantify the Oil Red O staining, the dye was dissolved in 100 uL of 100% isopropanol and the absorbance was measured at 492 nm using a micro plate reader. Results of spectrophotometric analysis were expressed as relative percentage of adiposity compared between the groups. The percentage of control absorbance was scored as 100% and the other groups obtained proportional values.

In order to investigate the potential of caffeic acid to protect the cells from the redox imbalance caused by adipogenesis, flow cytometry (FACSCalibur - Becton Dickinson, São Paulo, SP) assays were performed, using differentiated pre-adipocytes in post- and co-treatment with caffeic acid at 62.5 and 31.25 µM. The results were expressed as relative geometric mean of fluorescence intensity compared to the control group, which was normalized as relative fluorescence intensity equal to one.

ROS production was assessed through 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation (Zhou et al., 2016). The cells were labeled with DCFH-DA (20 µM), washed and submitted to fluorescence analysis (excitation at 488 nm and measurement by FL1 detector at 515-545 nm). Changes in mitochondrial transmembrane potential (ΔΨm) were determined by Rhodamine 123 (Rho123) (Sigma-Aldrich, St. Louis, MO, USA, 10 µg/mL) accumulation (Johnson et al., 1980). Cells were excited at 488 nm and measured by FL2 detector (563-606 nm).

All data were expressed as mean ± standard error of the mean (SEM). For statistical comparisons between groups, one-way ANOVA was used followed by Bonferroni test. Significance was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism5.0 (USA).

RESULTS

In this work, it was evaluated the ability of co- and post-treatment with CA to reduce lipid accumulation in 3T3-L1 cells. Moreover, analysis of redox balance by flow cytometry was performed using DCFH-DA and Rhodamine123. Co-treatment was able to reduce lipid accumulation on cytoplasmic droplets, decrease ROS formation analyzed through DCFH oxidation and improved mitochondrial transmembrane potential. All the results are better that the post-treatment.

Initially, cell viability assay showed no significant toxicity of caffeic acid on the 3T3-L1 cell line at the studied concentrations (Table 1). Regarding caffeic acid influence on pre-adipocyte differentiation through staining with Oil Red O, a significant reduction of lipid content was observed in the cells, which was greater in those submitted to co-treatment, when compared with the ones submitted to post-treatment. Cells treated with 31.25 µM CA presented relative adiposity 68.5 ± 0.27% at co-treatment and 85.2 ± 0.15% at the post-treatment; in the 62.5 µM CA co-treatment the found values were 52.6 ±

Table 1. Cell viability by MTT assay of 3T3-L1 cells treated with caffeic acid (CA).

Group	Cell viability	± SEM
Control	100	0.56
CA 1000 µM	98.8	0.82
CA 500 µM	97.9	0.55
CA 250 µM	96.5	0.49
CA 125 µM	95.9	0.96
CA 62.5 µM	99.1	0.19
CA 31.25 µM	98.5	0.62

The data are expressed as percentage of viability ± SEM. * p <0.05 vs. group control. In Bonferroni's comparison test was not found significant.

0.12%, in the post-treatment were 88.2 ± 0.19%, as shown in Figure 1. These findings were corroborated by the photomicrography that shows Oil Red O accumulation on cytoplasmic droplets.

Figure 2 shows the flow cytometry assay results. It was observed that co-treatment with caffeic acid causes an absolute decrease in the formation of intracytoplasmic ROS associated with adipogenesis on both concentrations, post-treatment did not reduced significantly the fluorescence (Figure 2A). Additionally, analysis of mitochondrial transmembrane potential (Figure 2D) showed that treatment with caffeic acid protects against the stress caused in the mitochondria by the adipocyte differentiation process; both used concentrations, at co- and post-treatment improved ΔΨm. Numerical data are expressed as relative fluorescence intensity, showing more effective activity in the co-treatment in all analyses. These results are represented in histograms, ratifying the cell labeling profile; Figures 2B and C representing DCFH-DA labeling and Figures 2E and F representing Rho123 labeling.

DISCUSSION

Hydroxycinnamic acids, such as CA and the derivative esters are able to inhibit mRNA expression of PPARγ and other adipogenic and lipogenic genes, such as C/EBPα and fatty acid synthase (FAS) in rats submitted to a high-fat diet induced body weight gain (Shin et al., 2014). In this study, the ability of CA to reduce the accumulation of lipids related to the differentiation of 3T3-L1 pre-adipocytes was evaluated.

The *in vitro* model better characterized for the study of adipogenesis is the use of 3T3-L1 preadipocyte cell lines, derived from Swiss mouse embryos. The cocktail used to differentiate the cells acts by activating the gamma receptor activated by peroxisome proliferator – PPARγ. Differentiation occurs through the activation of glucocorticoid receptors by dexamethasone, IGF-1 receptor by insulin and inhibition of phosphodiesterase by IBMX, modulating the cAMP signaling pathway (Lin et al., 2005).

Previous studies have shown that caffeic acid-derived esters significantly inhibits differentiation into adipocytes in 3T3-L1 cells and inhibit triglyceride deposition through the analysis of oil red O accumulation (Yasui et al., 2013; Shin et al., 2014). Additionally, a recent work shows that the unbalance of peroxiredoxins, cysteine-dependent peroxidase enzymes, lead to the generation of ROS in 3T3-L1 cells, increasing DCFH-DA fluorescence and resulting in adipogenesis, corroborating with the study's results (Kim et al., 2018). In this study, isolated CA showed inhibition of adipocyte differentiation in the same cell lineage and reduced lipid deposition with additional reduction on oxidative stress.

Additionally, all the analyses showed that co-treatment was more effective than the post-treatment. These data corroborate with previous study that demonstrated the anti-inflammatory and antiadipogenic action of supplementation of β-carotene, an antioxidant substance, in 3T3-L1 adipocytes culture are related to suppression of TNF-α and modulation of NF-κB pathway (Cho et al., 2018). These findings suggest the use of CA to prevent and treat the medium- and long-term consequences of obesity.

It has been shown that caffeic acid esters suppress oxidative stress associated with adipocyte differentiation, by acting on intracellular enzymatic antioxidant systems (Yasui et al., 2013). Oxidative stress disrupts electron transport chains and causes mitochondrial depolarization, which triggers the apoptotic pathway, chronic inflammation and increased cardiovascular risk (Lin et al., 2005; Farmer, 2006). This study showed a reduction in intracellular ROS formation and alterations in mitochondrial transmembrane potential in differentiated cells treated with caffeic acid. Also, this study confirmed data in the literature that showed that caffeic acid is an antioxidant agent and inhibits apoptosis in adipocytes (Lin et al., 2005; Yasui et al., 2013; Zhou et al., 2016).

Several authors have described the influence of mitochondria on adipocyte differentiation and obesity development. Mitochondria-mediated autophagy is associated to triglycerides accumulation and lipid droplets formation, since this organelle plays a key role on fatty acid oxidation (Zeng et al., 2018). Also, it has been

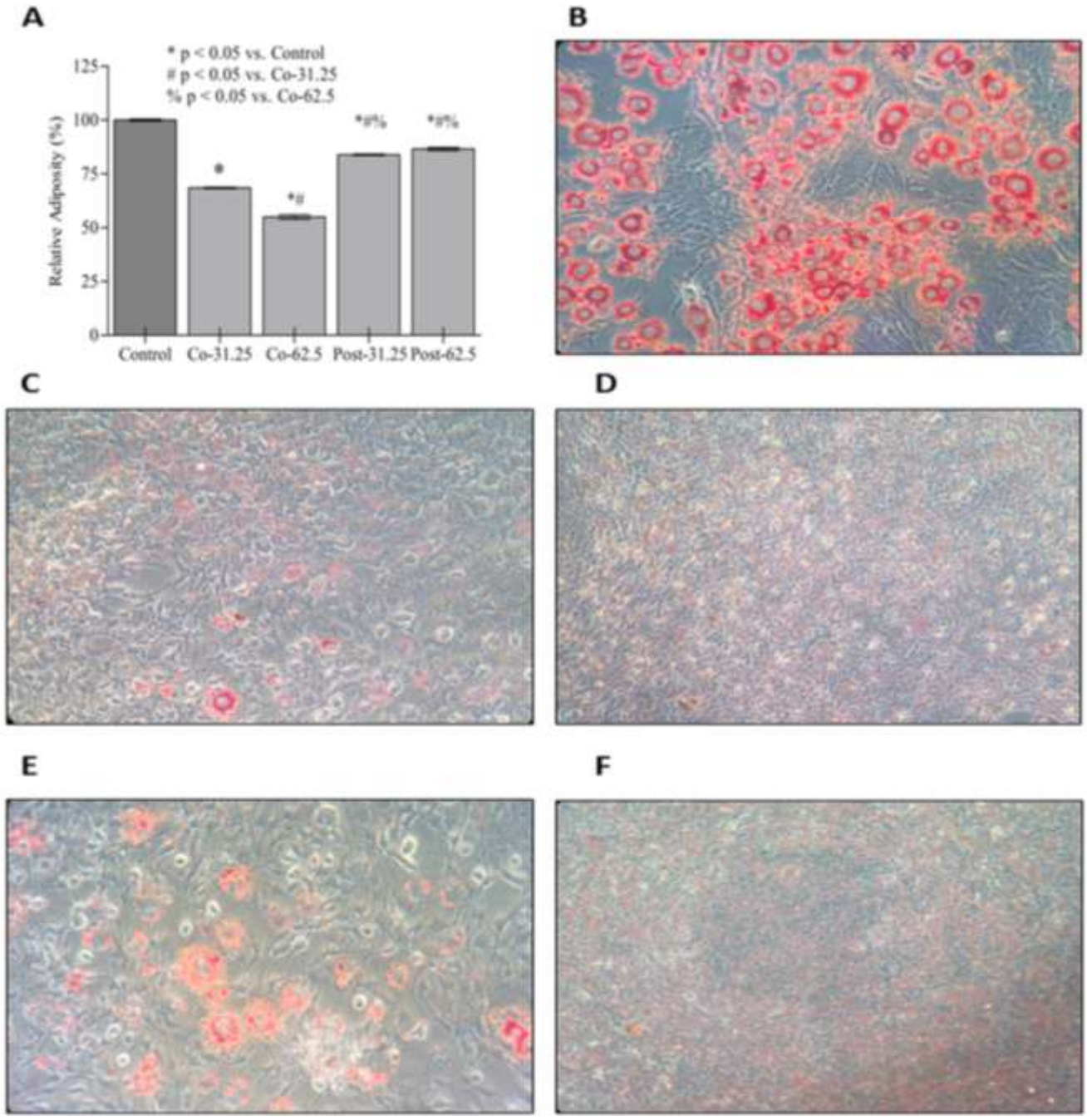

Figure 1. Assessment of lipid accumulation using Oil Red O assay. (A) Relative adiposity compared between the groups. The result is expressed in percentage related to the absorbance, and results are show as mean ± SE. *p < 0.05 compared to the vehicle control group. Photomicrography of the well containing differentiated 3T3-L1 cells: (B) Control group, (C) Co-treatment with caffeic acid 31.25 µM, (D) Co-treatment with caffeic acid 62.5 µM, (E) Post-treatment with caffeic acid 31.25 µM, (F) Post-treatment with caffeic acid 62.5 µM. Olympus™ microscope, 400× of magnification.

demonstrated that mitochondria-generated hydrogen peroxide is an important mediator on adipogenesis, mainly through induction of mitochondrial complex III (Tormos et al., 2011; Kim et al., 2018).

Recently, some works show that caffeic acid protected the cell death from oxidative stress condition in macrophages and microglia cells, increasing mRNA and protein expression levels of phase I antioxidant enzymes (Bajpai et al., 2018). Additionally, these data corroborate with other findings that shows that caffeic acid phenethyl ester ameliorates metabolic syndrome by activating PPAR-γ in adipose tissue, reducing inflammation,

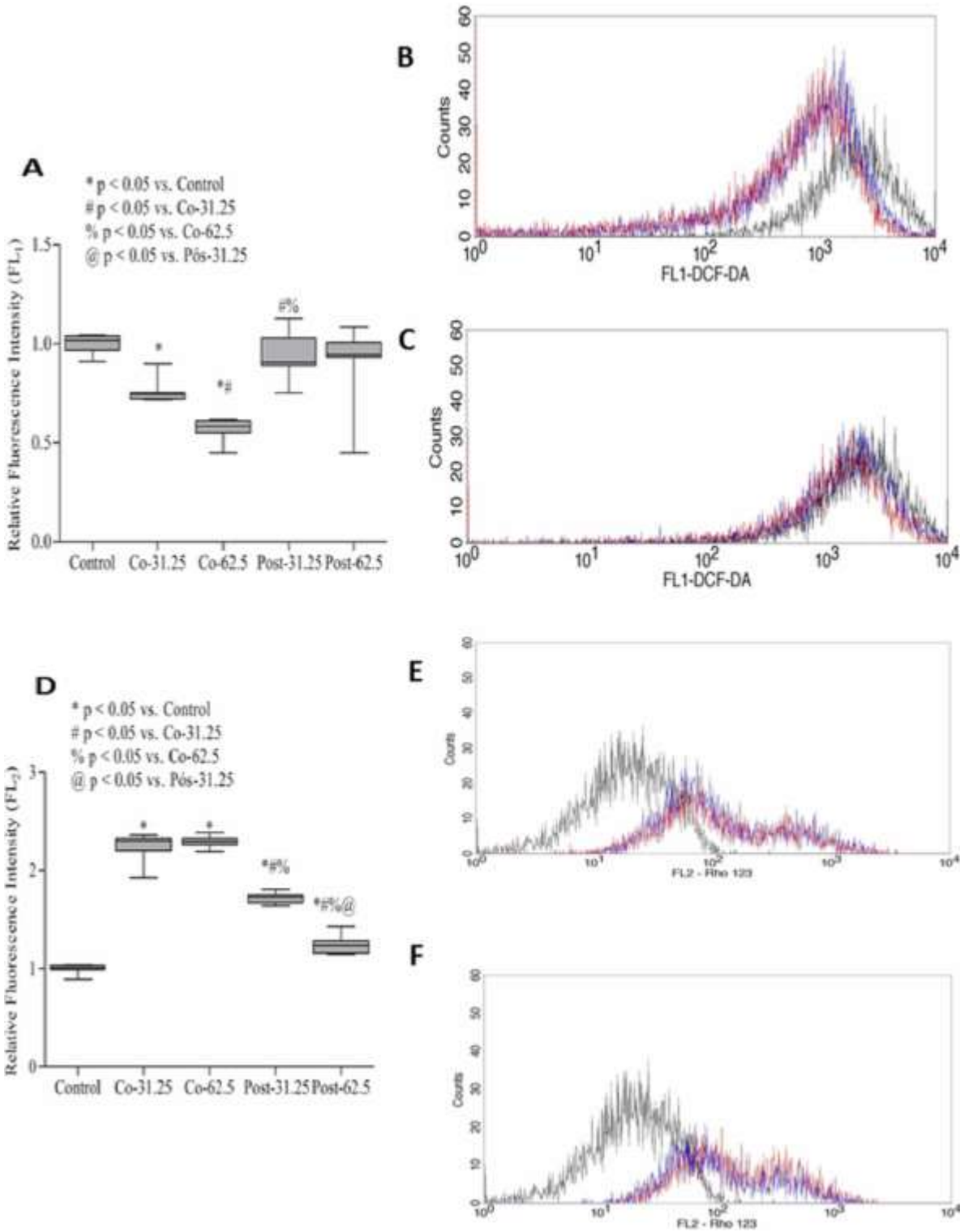

Figure 2. Flow cytometry assays. (A) Ratio of relative fluorescence intensity of DCFH-DA assay, showing that caffeic acid reduces ROS production on 3T3-L1 cells; (B) histogram representation of ROS production in co-treatment; the cells were treated with caffeic acid at 31.25 µM (blue line) and 62.5 µM (red line); (C) histogram representation of ROS production in post-treatment; the cells were treated with caffeic acid at 31.25 µM (blue line) and 62.5 µM (red line); (D) Ratio of relative fluorescence intensity of Rhodamine 123 assay; (E) histogram representation of mitochondrial transmembrane potential in co-treatment; the cells were treated with caffeic acid at 31.25 µM (blue line) and 62.5 µM (red line); (F) histogram representation of mitochondrial transmembrane potential in post-treatment; the cells were treated with caffeic acid at 31.25 µM (blue line) and 62.5 µM (red line). The data are expressed as fluorescence ratio relative to control ± SEM. * p <0.05 vs. group control.

modulating transcription of pro-angiogenic proteins, reducing obesity, hyperlipidemia and hyperglycemia (Kim et al., 2018).

Altogether, results suggest that caffeic acid acts on adipogenesis, reducing intracellular lipid accumulation in the 3T3-L1 cells. The main finding is that CA was able to reduce lipid accumulation related to the differentiation of 3T3-L1 pre-adipocytes, reducing ROS and altering transmembrane mitochondrial potential, mainly in the co-treatment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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