



N-Acetylcysteine Inhibits Lipids Production in Mature Adipocytes through the Inhibition of Peroxisome Proliferator-Activated Receptor γ

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Authors' contributions

This work was carried out in collaboration among all authors. Author DS performed cellular incubation experiments and western blotting. Author CM performed molecular biology experiments and managed literature searches. Author EF performed cellular incubation, viability and measurement of intracellular thiol experiments. Author LM performed biochemical determinations and statistical analysis. Author MCV performed molecular biology experiments analysis. Author JCC managed literature searches. Author LNG performed experimental design and implementation; experiments and statistical analysis; wrote the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Reports regarding the effects of antioxidants in obesity have been contradictory. Antioxidant N-acetylcysteine is usually considered a nutritional supplement. Our aim is to evaluate bioactivity of N-acetylcysteine (NAC) on mature adipocytes, which is a close model to in vivo condition.

Study Design: *In vitro* study.

Place and Duration of Study: Department of Basic Science (Universidad Nacional de Lujan), Department of Chemical Biology (Universidad de Buenos Aires), CONICET – INEDES and CONICET – IQUIBICEN, between March 2017 and June 2019.

Methodology: We evaluated the bioactivity of different concentrations of NAC for 5 days (0.01 mM to 5 mM) on fully differentiated 3T3-L1 cells (mature adipocytes).

Results: We demonstrated that NAC treatment was not toxic to mature adipocytes. Only 5mM NAC inhibited reactive oxygen species production. 5 mM NAC treatment resulted in a 60% decrease in cellular triglycerides content and inhibited 70% cholesterol accumulation. We also determined the mRNA and protein expression levels of Peroxisome Proliferator-Activated Receptor γ as well as, mRNA levels of lipid protein Perilipin in NAC treated adipocytes; we observed that 5mM NAC treatment caused nearly 30% decrease in the expression of these parameters.

Conclusion: These results suggest that NAC could avoid lipid accumulation in mature adipocytes; the antioxidant NAC could be beneficial in obesity treatment.

Keywords: Antioxidants; obesity; peroxisome proliferator-activated receptor gamma.

1. INTRODUCTION

Obesity is a disorder involving an excessive accumulation of adipose tissue [1]. An increase in the number and size of adipocytes contributes to the etiology of obesity, characterized by excessive accumulation of lipids in white adipose tissue. White adipose tissue is widely distributed in the body, mainly as dermal, subcutaneous and mesenteric fat tissue. Oxidative stress occurs in this tissue, associated with insulin resistance, lead to systemic alterations such as metabolic syndrome [2]. Mature adipocytes account for about sixty percent of adipose tissue; also, blood cells, endothelial cells and adipocyte precursors with different degrees of cell differentiation are present in this tissue.

3T3-L1 preadipocyte cell line has been isolated from cells derived from Swiss 3T3-8 mice embryos [3]. This cell line could differentiate into mature adipocytes and it is a useful model to test biological effect of antioxidant N-acetylcysteine (NAC) during adipogenesis [4-7]. 3T3-L1 adipocyte differentiation occurs in response to insulin and dexamethasone, 70% - 90% of cells increase lipid droplets and their cellular triglyceride content after seven days of beginning cellular differentiation process [4]. Once the cells went through commitment checkpoint, they reach the complete restriction and differentiate in a terminal way [8]. Terminal adipocyte differentiated state (mature adipocytes) is maintaining by expression of some master

regulators as adipocyte transcription factors such as Peroxisome Proliferator-Activated Receptor γ (PPAR γ) that coordinate expression of the responsible genes for developing and maintenance of adipocyte phenotype [9]. We distinguish mature adipocytes based on their ability to accumulate triglycerides and express fat specific proteins such as ap2 and a protein surrounding lipid vacuoles named perilipin. Activation of PPAR γ is crucial for adipocyte differentiation [5], because this transcription factor recognize a specific site in the promoter of terminal differentiation protein ap2 gene to induce its expression in adipocytes [6]; PPAR γ is also responsible for perilipin expression in mature adipocytes [10].

Treatment of 3T3-L1 with insulin, during differentiation protocol, rapidly generates a burst of hydrogen peroxide, which could have a strong impact on insulin signal transduction [11], thus reactive oxygen species (ROS) could be a component of intracellular redox signaling cascades [12,13]. In fact, ROS could facilitate preadipocyte differentiation [14]. It has been shown that adipogenesis is sensitive to extracellular redox environment [15]. We have previously showed that a pro-oxidant state occurred during preadipocyte to adipocyte differentiation pathway; addition of 0.01 mM NAC to cells increased the total intracellular GSH content by 25% after 1 day of treatment and inhibited fat accumulation in the cells [5].

We have previously evaluated bioactivity of different non-toxic doses of antioxidant NAC on adipogenic differentiation pathways of 3T3-L1 preadipocytes [5-7] and mouse embryonic fibroblasts [16]. We observed that NAC inhibited phosphorylation of mitogen-activated protein kinases in cell cultures [7,16] on the first day of differentiation protocol; this finding was consistent with another study using a known antioxidant such as epigallocatechin gallate [17]. We also demonstrated that NAC induced a decrease in PPAR γ protein expression on the fourth day of differentiation process [5], similar to other groups, which confirmed that NAC blocks lipid accumulation by reducing cellular PPAR γ protein expression during adipocyte differentiation [18-20]. However, the effects of NAC related with PPAR γ in mature adipocytes have not yet been studied, which it is essential because adipose tissue is mainly composed of mature adipocytes and in a low percentage by differentiating precursor cells. Therefore, new experiments are necessary to evaluate NAC effect on cells when differentiation took place. We have previously evaluated antioxidants effectivity for treating several pathologies [21-24]. Here, our aim is to evaluate the effect of NAC on 3T3-L1 mature adipocytes, which represent a better model for obesity than those previously used.

2. MATERIALS AND METHODS

2.1 Cell Cultures and Drugs

The 3T3-L1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured at 37°C with 5% CO $_2$ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 25 mM glucose and 10% fetal bovine serum. N-Acetylcysteine was purchased from Sigma- Aldrich Inc., USA.

2.2 3T3-L1 Adipocyte Differentiation

The 3T3-L1 preadipocytes were first cultured in MDI medium (0.5 mM 3-isobutyl-1-methyl xanthine, 0.1 μ M dexamethasone, and 2 μ M insulin) for 48 hours. Then they were transferred to fresh DMEM (with 25 mM glucose and 10% FBS) supplemented with 2 μ M insulin and incubated for three days. After three days, cells were then cultured in fresh DMEM (with 25 mM glucose and 10% FBS) for the remainder of the experiment. Day 0 of differentiation protocol was defined as the time at which the cells were first cultured in MDI medium. At day 7, the cellular

triglyceride (Tg) content dramatically increased by 70 – 80%, thereby generating refractive droplets that were clearly observed by microscopy. These cells (at day 7) were considered as mature adipocytes (AC). On day 7 of differentiation protocol, various concentrations of NAC were added to the cells and maintained throughout the remainder of the experiment; these cells were considered as NAC-treated mature adipocytes (ACN). DMEM (with 25 mM glucose and 10% FBS) was used as the vehicle and vehicle-treated cells were considered as control cells (CC) as previously described [5].

2.3 Measurement of Intracellular Free – SH (thiol: -SH)

To evaluate cellular free thiols, we performed assays on mature adipocytes (AC) using different concentrations of NAC including 0.01 mM, 0.1 mM NAC, 1 mM, 5 mM and 10 mM. On day 7 of differentiation protocol, NAC was added to the cells, and the treatment continued for 5 days. At day 12 of differentiation protocol, the cells were washed with PBS at RT and then 0.2 - 1 mL of water was added. The cells were then scraped and evaluated for cellular free thiols and proteins for each treatment. Free thiols content were assessed using Raggi method [25]. Briefly, the technique is based on an oxide-reduction reaction. The oxidation of thiols (present in the molecules to be evaluated) in the presence of Fe (III) and phenanthroline, gives a stable compound 1,10 phenanthroline - Fe (II) which could be determined at 515nm. Proteins were quantified by the Bradford method using crystalline bovine serum albumin as the standard. Results of experiments were normalized by protein content. The absorbance of mature adipocytes without NAC treatment (0) was evaluated at day 12 and set to 1, the results are presented as fold increase of them [5].

2.4 MTT Assay

To evaluate the toxicity of NAC treatment, on mature adipocytes (AC), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay was performed [26]. The technique is based on the presence of mitochondrial enzymes in viable cells that reduce the MTT dye and produce a purple color. We followed the previous published protocol [16]. To evaluate toxicity, cell viability assays were performed using different concentrations of NAC including 0.01 mM, 0.1 mM, 1 mM and 5 mM. On day 7 of differentiation protocol, NAC was added

to the cells, and the treatment continued for 5 days. Cellular viability was evaluated at day 12 of differentiation protocol for each treatment. The absorbance of mature adipocytes without NAC treatment (0) was evaluated at day 12 and set to 1, the results are presented as fold increase of them [16].

2.5 Measurement of Intracellular ROS Generation

To evaluate ROS in mature adipocytes, the cells were washed with 0.01 M phosphate buffered saline at room temperature and, then incubated in the dark for 1 hour at 37°C with a cell-permeable probe CM-H2DCFDA that becomes fluorescent under oxidation and emits green fluorescence. Fluorescence emission was detected at 520 nm, with an excitation wavelength at 505 nm. ROS production was determined from a standard curve using H₂O₂ (0.1–5 µM), the fluorescence intensities of H₂O₂ solutions were plotted vs H₂O₂ nmoles. Results of experiments were normalized by protein content [5]. Mature adipocytes without NAC treatment (AC) and NAC treated adipocytes (ACN - 0.01: 0.01 mM NAC; ACN - 0.1: 0.1 mM NAC; ACN - 1: 1 mM NAC; ACN - 5: 5 mM NAC) were evaluated, as well as, non-differentiated 3T3-L1 control cells (CC). Briefly, on day 7 of differentiation protocol, NAC was added to mature adipocytes, and the treatment continued for 5 days. Fluorescence was evaluated at day 12 of differentiation protocol for each treatment. The absorbance of control cells without NAC treatment (CC) was evaluated at day 12 and set to 1, the results are presented as fold increase of them [5].

2.6 Measurement of Triglyceride, Cholesterol and Protein Levels

Adipocytes (AC) and 5 mM NAC treated adipocytes (ACN - 5) were evaluated, as well as, non-differentiated 3T3-L1 control cells (CC). Briefly, on day 7 of differentiation protocol, NAC was added to mature adipocytes, and the treatment continued for 5 days. Cell were washed with PBS at RT and then 0.2 - 1 mL of water was added. The cells were then scraped and evaluated for triglycerides, cholesterol and proteins. Triglyceride and cholesterol accumulation were assessed using the TG color GPO/PAP AA kit (Wiener Laboratory, Rosario, Argentina) and Colestat AA kit (Wiener Laboratory, Rosario, Argentina), respectively. Proteins were quantified by the Bradford method using crystalline bovine serum albumin as the

standard. Results of experiments were normalized by protein content. The absorbance of control cells without NAC treatment (CC) was set to 100, the results are presented as percentage of them [4].

2.7 Western Blotting

Adipocytes (AC), NAC treated adipocytes (ACN - 0.01: 0.01 mM NAC; ACN - 0.1: 0.1 mM NAC; ACN - 1: 1 mM NAC; ACN - 5: 5 mM NAC) and non-differentiated 3T3-L1 control cells (CC) samples were obtained on day 12 of the differentiation protocol. NAC was added on day 7 of differentiation protocol to mature adipocytes, and the treatment continued for 5 days. We followed the previous published protocol [16]. The primary antibodies included anti-PPAR γ and GAPDH (PPAR γ (E-8) and GAPDH, Santa Cruz Biotechnology, USA). The membranes were then probed with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) and protein signal was detected using the enhanced chemiluminescence (ECL) substrate kit (Amersham ECL Plus Western Blotting Detection System, GE Healthcare, USA). PPAR γ expression was normalized to GAPDH expression. Control cells (CC) without NAC treatment was evaluated at day 12 and set to 1, the results are presented as fold increase of them. The results are expressed as arbitrary units.

2.8 RNA Extraction and RT-qPCR Analysis

Adipocytes (AC) and 5 mM NAC treated adipocytes (ACN - 5) were obtained on day 9 of differentiation protocol. Briefly, on day 7 of differentiation protocol, NAC was added to mature adipocytes, and the treatment continued for 2 days. Total RNA extraction from 3T3-L1 cells was performed using the TRI Reagent (Molecular Research Center, USA). The relative mRNA expression was determined by quantitative real-time PCR using a Bio-Rad MyIQ2 thermal cycler. Each PCR reaction was performed using 5 pmol of each primer. The following cycling conditions were used: 94°C for 5 minutes, followed by 40 cycles of 94°C for 20 seconds, 58°C for 30 seconds and 72°C for 40 seconds. Target gene mRNA expression was normalized to acidic ribosomal protein (Rplp0) as a reference gene. Perilipin 1 (Plin1) primers were designed using Primer-3 (v.-0.4.0) software. Rplp0 and PPAR γ primer sequences were

obtained from published reports. Mature adipocytes without treatment (AC) was evaluated at day 9 and set to 1, the results are presented as fold increase of them. The results are expressed as arbitrary units [27].

The sequences of primers are shown below:

Plin1-Fwd: 5'TGAAGGGTGTACGGATAACG3';
 Plin1-Rev: 5'TGAAGGGTTATCGATGTCTCG3';
 PPARγ-Fwd:5'CCAGAGCATGGTGCCTTCGC
 T3';PPARγ-Rev: 5'CAGCAACCATTGGGTCAG
 CTC3'; Rplp0-Fwd: 5'GAGGAATCAGATGAGG
 ATATGGGA3';Rplp0-Rev:5'AAGCAGGCTGAC
 TTGGTTGC3'.

2.9 Statistics

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance followed by post hoc analysis [28]. The results represent the average of four independent experiments (mean ± SD), **P* = .001 vs mature adipocytes without NAC treatment was considered statistically significant.

3. RESULTS

We induced differentiation of 3T3-L1 preadipocytes and at day 7, we observed fully differentiated cells; they showed refractive lipid droplets as detected by microscopy and increased their triglyceride content compared to 3T3-L1 non-differentiated preadipocytes (non-differentiated control cells). Therefore, we considered these fully differentiated cells as mature adipocytes (AC). On day 7, triglyceride (Tg) content in AC was almost four times higher than Tg content observed in non-differentiated control cells (CC) (0.83 ± 0.15 g Tg/g protein [AC] vs. 0.23 ± 0.1 g Tg/g protein [CC], *P* = .000).

We added NAC to the media on day 7 of differentiation protocol and evaluated its effect on 3T3-L1 mature adipocytes after 5 days. We used doses from 0.01 to 10 mM to determine cellular uptake of NAC, we observed that only the highest doses, such as 5 mM and 10 mM, significantly increased free total thiols in a saturable way in NAC treated mature adipocytes (Fig. 1).

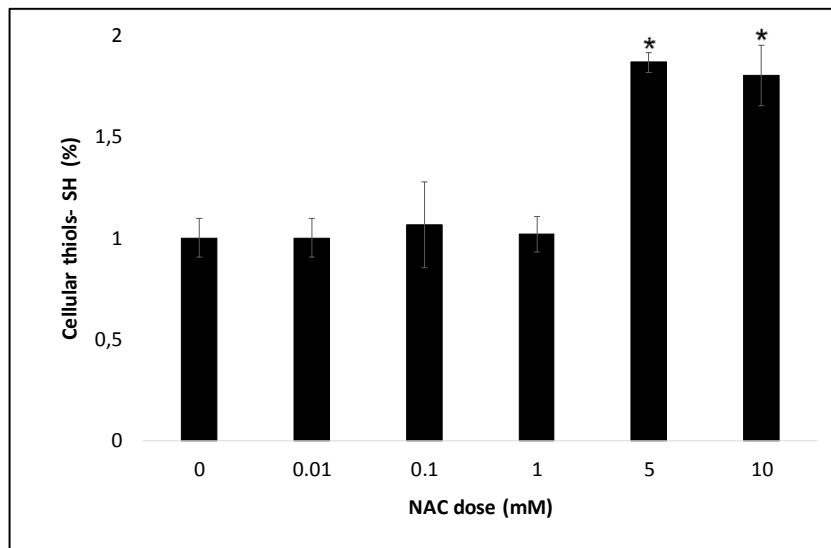


Fig. 1. Effect of NAC on intracellular free-SH in 3T3-L1 mature adipocytes: 3T3-L1 mature adipocytes were treated on day 7 of differentiation protocol with 0.01mM NAC (0.01), 0.1 mM NAC (0.1), 1 mM NAC (1), 5 mM NAC (5) or 10mM NAC (10). 3T3-L1 mature adipocytes without NAC treatment were also evaluated (0). Cells were harvested on day 12 and intracellular free thiols level was determined. The absorbance of mature adipocytes without NAC treatment (0) was evaluated and set to 1, the results are presented as fold increase of them. The results are the average of four different experiments (mean ± SD). * This indicates *P* = .001 : statistically significant when compared to mature adipocytes without NAC treatment (0)

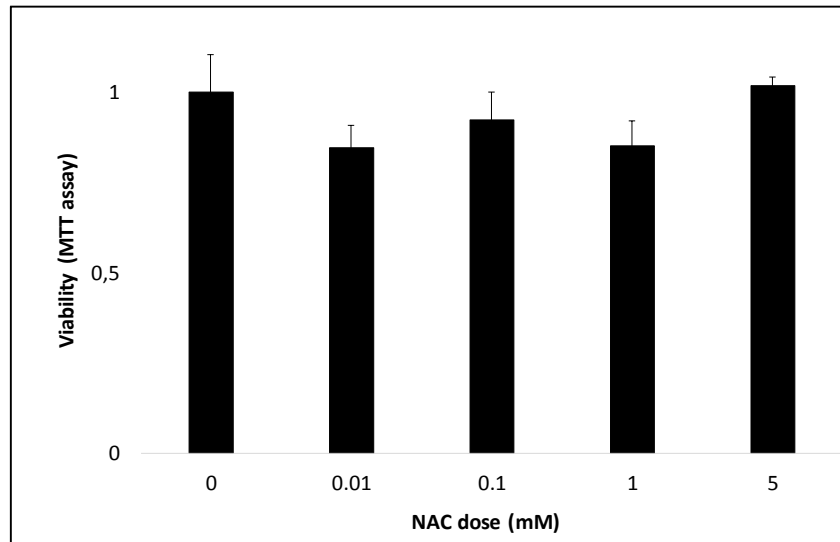


Fig. 2. Effect of NAC on cell viability in 3T3-L1 mature adipocytes: 3T3-L1 adipocytes were treated on day 7 of differentiation protocol with 0.01 mM NAC (0.01), 0.1 mM NAC (0.1), 1 mM NAC (1) or 5 mM NAC (5). 3T3-L1 mature adipocytes without NAC treatment were also evaluated (0). Cells were harvested on day 12 and MTT assay was performed. The absorbance of mature adipocytes without treatment (0) was evaluated and set to 1, the results are represented as a fold increase of them. The results are the average of four different experiments (mean \pm SD)

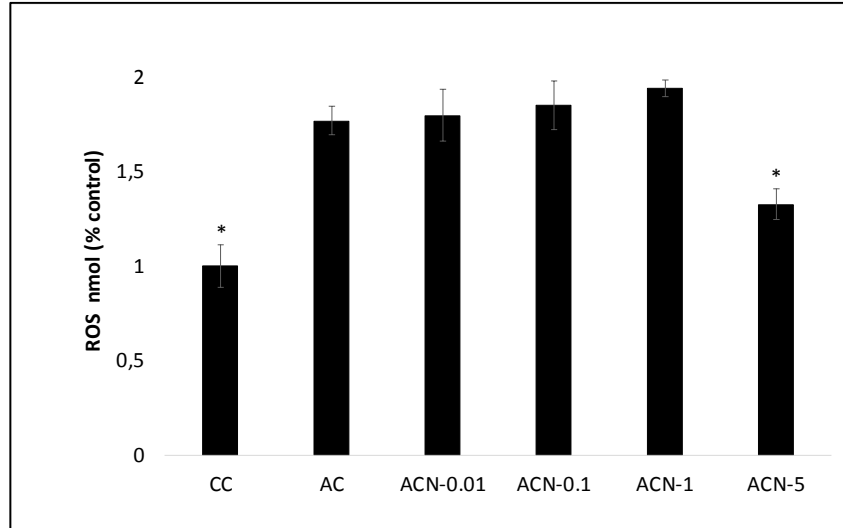


Fig. 3. Effect of NAC on ROS level in 3T3-L1 mature adipocytes: 3T3-L1 mature adipocytes were treated on day 7 of differentiation protocol with 0.01 mM NAC (ACN-0.01), 0.1 mM NAC (ACN-0.1), 1 mM NAC (ACN-1) or 5 mM NAC (ACN-5). 3T3-L1 mature adipocytes without NAC treatment (AC) and 3T3-L1 non-differentiated control cells without NAC treatment (CC) were also evaluated. Cells were harvested on day 12 and intracellular ROS level was determined. The absorbance of control cells (CC) was evaluated and set to 1, the results are presented as fold increase of CC. The results are the average of four different experiments (mean \pm SD). * This indicates P = .000 : statistically significant when compared to mature adipocytes without NAC treatment (AC)

We found that 0.01 – 5 mM NAC doses were not toxic to mature adipocytes as shown in Fig. 2; we set non-treated mature adipocytes viability to 1 (1 ± 0.12 [0 mM NAC] vs 1.05 ± 0.02 [5 mM NAC], $P = 0.85$, no significant difference was observed).

We observed an increase in reactive oxygen species (ROS) level two-fold higher in mature adipocytes than in non-differentiated control cells and, assayed different doses of NAC on mature adipocytes for 5 days. We demonstrated that only 5 mM NAC could significant decrease intracellular ROS level in NAC treated mature adipocytes almost 30% (Fig. 3).

After 5-days treatment with 5 mM NAC in mature adipocytes, at day 12, we observed a significant decrease in cellular Tg content in NAC treated adipocytes compared to non-treated adipocytes (Fig. 4). Is relevant to point out that a 60% decrease in triglyceride accumulation was

observed with the treatment (1.22 ± 0.09 g Tg/g protein [AC] vs 0.49 ± 0.03 g Tg/g protein [ACN-5], $P = .000$). Whereas, on day 12, non-differentiated control cells (CC) had four times lower Tg content than AC (1.22 ± 0.09 g Tg/g protein [AC] vs 0.3 ± 0.05 g Tg/g protein [CC], $P = .000$). 5 mM NAC treatment also decreased nearly 70% cellular cholesterol accumulation in ACN-5 at day 12 compared to AC (Fig. 4).

After 5 days NAC treatment, on day 12 of differentiation protocol, we evaluated PPAR γ protein expression. As shown in Fig. 5, only 5 mM NAC treatment induced a 30% decrease in PPAR γ protein level (PPAR γ /GAPDH: 1.54 ± 0.12 [AC] vs 1.08 ± 0.08 [ACN-5] AU; $P = .005$). After addition of 5 mM NAC to the media for 2 days (on day 9 of differentiation protocol), we evaluated mRNA levels of PPAR γ by RT-qPCR (Fig. 6), and observed a similar decrease in both PPAR γ protein and mRNA levels (PPAR γ / Rplp0: 1 ± 0.07 [AC] vs 0.74 ± 0.14 [ACN-5] AU, $P = .026$).

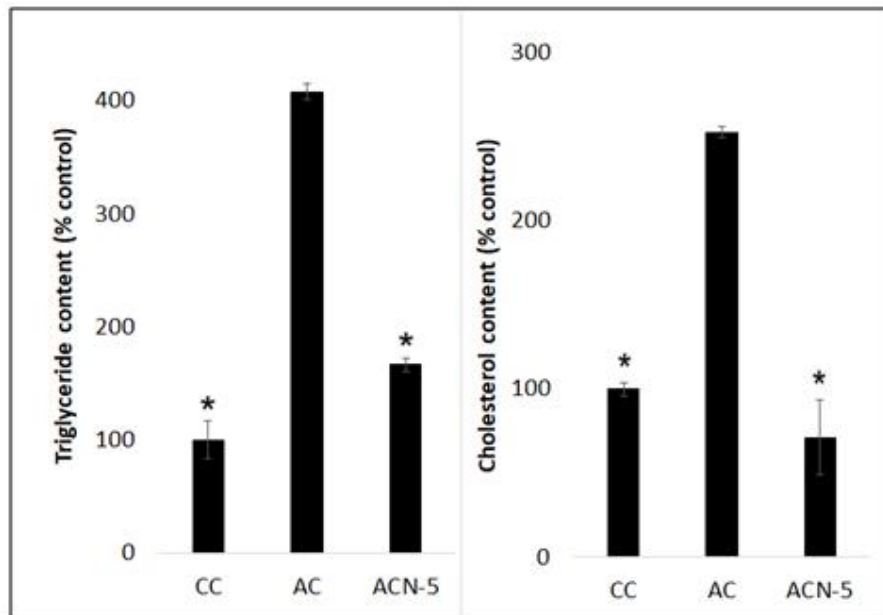


Fig. 4. Effect of NAC on lipid accumulation in 3T3-L1 mature adipocytes: 3T3-L1 mature adipocytes were treated on day 7 of differentiation protocol with 5mM NAC (ACN-5). 3T3-L1 mature adipocytes without NAC treatment (AC) and 3T3-L1 non-differentiated control cells without NAC treatment (CC) were also evaluated. Cells were harvested on day 12 and intracellular triglyceride or cholesterol content was determined. The absorbance of control cells (CC) was evaluated and set to 100, the results are presented as percentage of CC. The results are the average of four different experiments (mean \pm SD). * This indicates $P = .000$: statistically significant when compared to mature adipocytes without NAC treatment (AC). Triglyceride content: * $P = .000$ [ACN-5 vs AC]; * $P = .000$ [CC vs AC]. Cholesterol content: * $P = .000$ [ACN-5 vs AC]; * $P = .000$ [CC vs AC]

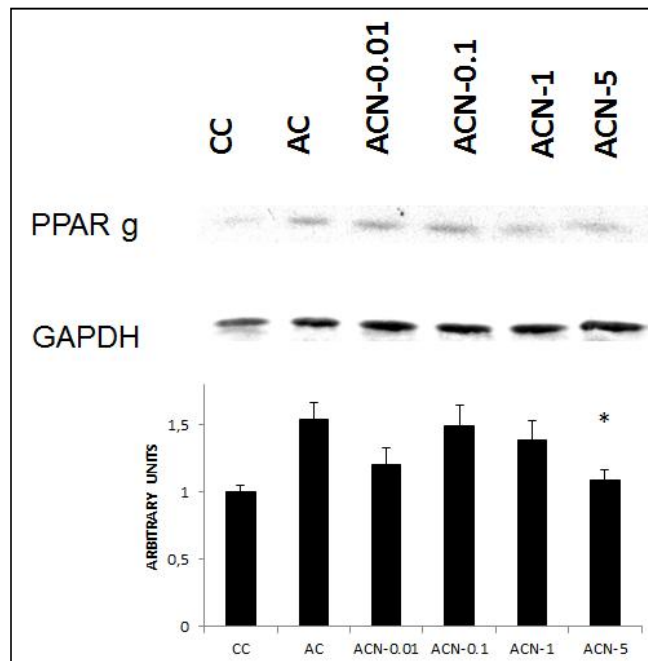


Fig. 5. Effect of NAC on PPAR γ (PPAR g) in 3T3-L1 mature adipocytes: 3T3-L1 mature adipocytes were treated on day 7 of differentiation protocol with 0.01mM NAC (ACN-0.01), 0.1mM NAC (ACN-0.1), 1mM NAC (ACN-1) or 5mM NAC (ACN-5). 3T3-L1 mature adipocytes without NAC treatment (AC) and 3T3-L1 non-differentiated control cells without NAC treatment (CC) were also evaluated. Cells were harvested on day 12 and western blot assay was performed. PPAR γ protein expression was normalized to GAPDH expression. The values represent fold increase in the expression of adipogenic transcription factor versus the expression of control cells (CC) harvested on day 12. Representative results from one of three independent western blot experiments with similar results are shown. The results are represented as arbitrary units and they are the average of three different experiments (mean \pm SD). *P = .005 vs mature adipocytes without NAC treatment (AC). PPAR γ (PPARg): peroxisomal proliferator activated receptor gamma; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

We also quantified mRNA levels of perilipin, after treating mature adipocytes with 5 mM NAC for 2 days (Fig. 6). We could not detect mRNA perilipin in non-differentiated 3T3-L1 control cells; therefore, we set AC content mRNAs to 1. A significant decrease was observed in ACN-5 compared to AC (1 ± 0.09 AU [AC] vs 0.72 ± 0.07 AU [ACN-5], $P = .013$).

4. DISCUSSION

3T3-L1 preadipocyte cell line has been widely used for the study of adipogenic differentiation process [9,29]. As a result, it is possible to observe in mature adipocytes the phenotypical changes occurring during adipogenesis; at day 7 of differentiation protocol lipid droplets are visible at optical microscope and a drastic cellular morphology of the cells is also observed [6, 7].

There is a close relationship between obesity and oxidative stress [30], with increasing evidence suggesting a role for oxidative stress and free radicals in promoting adipogenesis [14, 31]. A pro-oxidant state occurs during adipogenic differentiation in 3T3-L1 cell line, in the absence of an appropriate compensatory response from the endogenous antioxidant network, the system could become overwhelmed. Therefore, to avoid accumulation of ROS and their potentially deleterious effects, several cellular enzymes such as SOD and GPx detoxify them [5]. Most of the studies evaluate antioxidants effects on this cell line during 3T3-L1 cellular adipocyte differentiation and, external agents are adding together with inducing adipogenic agents to the cells. Nevertheless, mature adipocytes could not response to external agents. Since more than fifty percent of the cells in adipose tissue are

mature adipocytes, new studies could be necessary to evaluate external antioxidants effects on them.

Several reports, summarized in a recent review, demonstrated that NAC could inhibit ROS production as well as lipid accumulation during differentiation of 3T3-L1 preadipocytes [32]; in fact, we had confirmed that 0.01mM NAC assayed on preadipocytes, was effective in decreasing ROS and lipid accumulation during cellular adipocyte differentiation [5]. Here, we used NAC on terminal adipocyte differentiated cells to study a possible lipid accumulation. We added NAC to the culture medium at day 7 of differentiation protocol; at pH medium (nearly 7 - 8), NAC should be negatively charged [33]. Therefore, NAC could enter in mature adipocytes via anion exchange protein transport [34]. We conducted experiments to determine NAC adipocytes uptake, by testing cellular free-thiol content, because the presence of thiol group in NAC. We observed that dose of 5 mM NAC allowed the greatest uptake compared to lower doses. It is important to point out, that cells contain different kind of molecules with thiol groups, such as glutathione, which it is high in mature adipocytes [5]; an increase in free-thiols level in NAC treated mature adipocytes above

basal content could suggest that cells incorporated NAC. We showed saturable NAC incorporation, 10 mM dose did not induce a higher free-thiols cellular uptake than 5 mM dose.

Dose from 0.01 mM to 5 mM NAC were not toxic to cells during the treatments. We observed an increase on ROS level in mature adipocytes, but only 5 mM NAC was effective in decreasing cellular oxidant species, according to NAC uptake. Other authors used similar doses of NAC in mature adipocytes to inhibit ROS production [35,36]. Therefore, we decided to evaluate this dose on lipid accumulation in 3T3-L1 mature adipocytes.

5 mM NAC treatment was significantly effective in inhibiting Tg and Cholesterol accumulation in 3T3-L1 mature adipocytes. Triglyceride content significantly decreased from day 7 to day 12 in NAC treated mature adipocytes; we could suggest that NAC treatment was effective in avoiding triglyceride accumulation in these cells. Tg accumulation in adipocytes correlates with an increase in cellular ROS level [5,37], meanwhile NAC is a ROS scavenger and precursor to GSH, one of the main cellular antioxidant molecule. Since an increase in free radicals occurs during

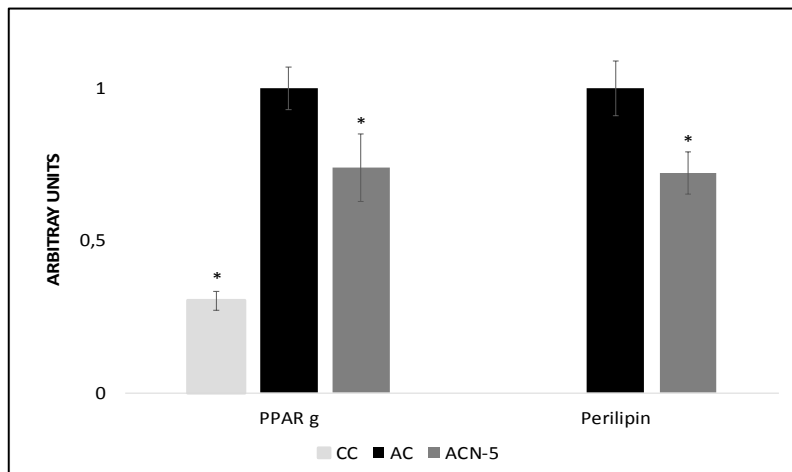


Fig. 6. Effect of NAC on mRNA PPAR γ (PPAR g) or mRNA perilipin in 3T3-L1 mature adipocytes: 3T3-L1 mature adipocytes were treated on day 7 with 5mM NAC (ACN-5). 3T3-L1 mature adipocytes without NAC treatment (AC) and 3T3-L1 non-differentiated control cells without NAC treatment (CC) were also evaluated. Cells were harvested on day 9 and qPCR assay was performed. Relative expression of mRNA PPAR γ or mRNA perilipin were normalized to acidic ribosomal protein (Rplp0) as a reference gene. The values represent fold increase in mRNA level versus the level in mature adipocytes without NAC treatment (AC) harvested on day 9. None mRNA perilipin was detected in CC. The results are represented as arbitrary units and they are the average of three different experiments (mean \pm SD). PPAR γ : *P = .026 [ACN-5 vs AC]; *P = .000 [CC vs AC]. Perilipin: *P = .013 [ACN-5 vs AC]. PPAR γ (PPARg): peroxisomal proliferator activated receptor gamma

adipogenesis, the antioxidant environment generated by NAC treatment had an inhibitory effect on lipid accumulation. We demonstrated that NAC, despite its low cellular uptake, could be effective as antioxidant. Our results suggest that reduction of oxidative state, due NAC addition, could cause inhibition in Tg and cholesterol accumulation in mature adipocytes. Other authors that have used NAC during adipocyte differentiation, reported similar results [18,20,38,39]. Therefore, we could speculate about a possible use of this molecule as a new drug or as an adjuvant to decrease lipid content in human adipose tissue during obesity development.

Early studies have demonstrated that PPAR γ expression increase during adipogenesis; it is necessary and sufficient for adipocytes differentiation, thereby establishing PPAR γ as a master regulator of adipogenesis [40]. Some authors have shown that elevation of ROS is required in activation of PPAR γ in mesenchymal stem cells, during adipocyte differentiation [19], while antioxidants are effective inhibiting adipogenesis *in vitro* [41]. We have also reported a correlation between ROS and PPAR γ expression during differentiation of 3T3-L1 preadipocytes, while NAC was effective in inhibiting PPAR γ expression during cellular differentiation [5]. Therefore, we decided to evaluate the effect of this antioxidant on this adipogenic transcription factor in mature adipocytes. Here, we showed that PPAR γ mRNA and protein levels were higher in mature adipocytes than in non-differentiated preadipocytes. We observed that 5 mM NAC treatment in mature adipocytes caused a reduction in both PPAR γ mRNA level and protein expression. These results were in contradiction with those reported by Liu et al. [42]; but they evaluated NAC treatment on tamoxifen-pretreated 3T3-L1 adipocytes, and informed that NAC increased PPAR γ protein level in these cells. However, they showed that PPAR γ protein expression was lower in NAC tamoxifen-pretreated adipocytes than in mature adipocytes. Since PPAR γ is a key factor in adipogenesis and its expression is enough to maintain mature adipocyte phenotype, we suggest that NAC could mediate its effect by inhibiting this transcription factor expression in these cells, and therefore lipid accumulation effectively decreased in mature adipocytes.

The decrease of PPAR γ , suggest that adipocytes could lose their storage capacity of lipids, the

main function of mature adipocyte. In preadipocytes small lipid droplets are coated with an adipocyte differentiation related protein named ADRP, once cells differentiated into mature adipocytes, these cells produce much larger fat droplets, that could see at microscope, and perilipin protein displaces ADRP from droplets surface. Perilipin is a protein found exclusively on the outer surface of lipid vacuoles in mature adipocytes, which protects lipids from de action of lipases [43]. Therefore, we determined perilipin in NAC treated mature adipocytes, and showed that its mRNA level significantly decreased compared to none-treated NAC adipocytes. This fall in perilipin could partly explained since its expression is upregulated by PPAR γ [10]. We could speculate a possible lipolysis process, because perilipin could modulate lipolysis by controlling the access of lipases within lipid droplets [44].

We have previously showed an inhibitory effect of NAC on MAPKs signaling pathway during adipocyte differentiation in both 3T3-L1 preadipocytes [7] and mouse embryonic fibroblasts [16]. We observed that a minimum dose of NAC, such as 0.01 mM, could inhibit mitotic clonal expansion [7] and, terminal lipid protein ap2 expression [6] during 3T3-L1 cellular differentiation. However, this is the first time to assay NAC on mature adipocytes, after complete 3T3-L1 cellular differentiation, and we observed that higher dose of NAC was active in mature adipocytes than that previously used. NAC is usually included in food supplements; our results suggest that NAC could prevent lipid accumulation in mature adipocytes. It is unclear to what extent these new results from this *in vitro* study might extrapolate to *in vivo* condition, but it is clear that NAC could potentially have a regulatory effect on lipid accumulation in mature adipocytes. Some authors reported that treatment of obese patients with 2 mM NAC administered daily decreased body fat mass, producing an increase of 25% of total plasma thiol in these patients [45]. Another report revealed that antioxidant rich diet is inversely associated with adiposity in healthy young adults [46].

5. CONCLUSION

This is the first report evaluating the effect of NAC on 3T3-L1 mature adipocytes. Our results demonstrated that a dose of 5 mM NAC prevents cellular cholesterol and triglyceride accumulation in mature adipocytes through a mechanism

involving the inhibition of PPAR γ expression in the cells. More studies are necessary to investigate the potential impact of NAC on obesity treatment; but this approach is closer to that could happen in adipose tissue during obesity, since mature adipocytes are the principal cells of this tissue.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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