

The treatment with all-trans retinoic acid causes apoptosis without chromosomal instability in adipose-derived stem cells and might act starting the browning process

O tratamento com ácido all-trans retinóico causa apoptose sem instabilidade cromossômica em células-tronco derivadas do tecido adiposo e pode atuar iniciando o processo de escurecimento

El tratamiento con ácido retinoico todo trans provoca apoptosis sin inestabilidad cromosómica en células madre derivadas de tejido adiposo y puede actuar iniciando el proceso de oscurecimiento

Received: 02/09/2022 | Reviewed: 02/16/2022 | Accept: 02/25/2022 | Published: 03/07/2022

Layna de Carvalho Schweich-Adami

ORCID: <https://orcid.org/0000-0002-9838-7340>

Universidade Federal de Mato Grosso do Sul, Brasil

E-mail: laynnaschweich@gmail.com

Andréia Conceição Milan Brochado Antonioli-Silva

ORCID: <https://orcid.org/0000-0002-2525-7019>

Universidade Federal de Mato Grosso do Sul, Brasil

E-mail: andrea@corporesanosau.de.com.br

Rodrigo Juliano Oliveira

ORCID: <https://orcid.org/0000-0003-3514-3346>

Universidade Federal de Mato Grosso do Sul, Brasil

Universidade Estadual de Londrina, Brasil

E-mail: rodrigo.oliveira@ufms.br

Abstract

Introduction: The knowledge about the biology of obesity and apoptosis induction of adipose-derived stem cells (ADSCs) may support in the development of new therapies. **Objective:** The present research aimed to investigate the toxicogenic effect of all-trans retinoic acid (ATRA) as well as its influence in the adipogenic differentiation and expression of genes related to DNA damage, cell cycle and thermogenesis. **Methodology:** Cell cultures of human adipose-derived stem cells were treated for 12 hours with ATRA (20.75 μ M) and were performed the adipogenic differentiation, comet assay, micronucleous, cell death, cell cycle and qPCR. **Results:** The ATRA treatment decreased the capacity of adipogenic differentiation of ADSCs. The comet assay demonstrated increase in nucleoid frequency with genomic damage and scoring. However, these damages were not fixed at the chromosome level, since the tail moment was not significant. The treatment with ATRA increased cytotoxicity and increased cell death by apoptosis. The relative expression of CHEK-1, CHEK-2, CDC25A, CDC25C, ATM and ATR decreased and only UCPI increased significantly. **Conclusion:** The results of the present study demonstrate that the use of ATRA induces genomic damage in ADSCs, although it was eliminated in the apoptosis process. Therefore, administration of ATRA in ADSCs did not cause chromosomal instability, which suggests toxicogenic safety. Also it was considered that ATRA might active the browning effect. Thus, this compound is considered a promising candidate for the development of novel therapies for the treatment of obesity and / or localized fat by the use of mesotherapy.

Keywords: Mesotherapy; Cytotoxicity; Adipose-derived stem cells; ATRA; Browning.

Resumo

Introdução: O conhecimento sobre a biologia da obesidade e indução de apoptose de células-tronco derivadas do tecido adiposo humano pode auxiliar no desenvolvimento de novas terapias. **Objetivo:** A presente pesquisa teve como objetivo investigar o efeito toxicogênico do ácido all-trans retinóico (ATRA), bem como sua influência na diferenciação adipogênica e expressão de genes relacionados a danos no DNA, ciclo celular e termogênese. **Metodologia:** Culturas celulares de células-tronco foram tratadas por 12 horas com ATRA (20,75 μ M) e foram realizadas a diferenciação adipogênica, ensaio cometa, micronúcleo, morte celular, ciclo celular e qPCR. **Resultados:** O tratamento com ATRA diminuiu a capacidade de diferenciação adipogênica das células-tronco. O ensaio do cometa demonstrou aumento na frequência de nucleóides com dano genômico. No entanto, esses danos não foram fixados no nível cromossômico, uma vez que o momento de cauda não foi significativo. O tratamento com ATRA aumentou a citotoxicidade e aumentou a morte celular por apoptose. A expressão relativa de CHEK-1, CHEK-2, CDC25A, CDC25C, ATM e ATR diminuiu e apenas UCPI aumentou significativamente. **Conclusão:** Os resultados do presente estudo demonstram que o uso de ATRA induz danos genômicos em células-tronco, porém são eliminados com o processo de apoptose. A administração de ATRA não causou instabilidade cromossômica, o que sugere segurança toxicogênica. Também foi considerado que o ATRA pode ativar o efeito de escurecimento dessas células. Assim,

este composto é considerado um candidato promissor para o desenvolvimento de novas terapias para o tratamento da obesidade e/ou gordura localizada pelo uso da mesoterapia.

Palavras-chave: Mesoterapia; Citotoxicidade; Células-tronco; ATRA; Escurecimento.

Resumen

Introducción: El conocimiento sobre la biología de la obesidad y la inducción de apoptosis de células madre derivadas del tejido adiposo humano puede ayudar en el desarrollo de nuevas terapias. **Objetivo:** La presente investigación tuvo como objetivo investigar el efecto toxicogenético del ácido transretinoico (ATRA), así como su influencia en la diferenciación adipogénica y la expresión de genes relacionados con el daño del ADN, el ciclo celular y la termogénesis. **Metodología:** Los cultivos celulares de células madre se trataron durante 12 horas con ATRA (20.75 μ M) y se realizó diferenciación adipogénica, ensayo cometa, micronúcleo, muerte celular, ciclo celular y qPCR. **Resultados:** El tratamiento con ATRA disminuyó la capacidad de diferenciación adipogénica de las células madre. El ensayo del cometa demostró un aumento en la frecuencia de nucleoides con daño genómico. Sin embargo, estos daños no se fijaron a nivel cromosómico, ya que el momento de la cola no fue significativo. El tratamiento con ATRA aumentó la citotoxicidad y aumentó la muerte celular por apoptosis. La expresión relativa de CHEK-1, CHEK-2, CDC25A, CDC25C, ATM y ATR disminuyó y solo UCP1 aumentó significativamente. **Conclusión:** Los resultados del presente estudio demuestran que el uso de ATRA induce daño genómico en células madre, pero se elimina con el proceso de apoptosis. La administración de ATRA no causó inestabilidad cromosómica, lo que sugiere seguridad toxicogenética. También se consideró que ATRA puede activar el efecto de oscurecimiento de estas células. Así, este compuesto se considera un candidato prometedor para el desarrollo de nuevas terapias para el tratamiento de la obesidad y/o grasa localizada mediante el uso de mesoterapia.

Palabras clave: Mesoterapia; Citotoxicidad; Células madre; ATRA; Oscureciendo.

1. Introduction

The all-trans retinoic acid (ATRA) is the most active form of vitamin A in the body (Noy, 2010) and it is known that the adipose tissue is a target organ for ATRA (Bonet et al., 2003). In spite of this many studies have been made to discover how this substance really act in the adipogenesis process (Lobo et al., 2010; Moon et al., 2007; Morikawa et al., 2013; Ribot et al., 2001). To understand this complex pathway of differentiation, is necessary know from where this cells came. Studies start to explore researches in 3T3-L (Lobo et al., 2010; Moon et al., 2007; Morikawa et al., 2013), which is immortalized preadipocytes lineage, during the differentiation, but this still needs to be more accurate. So the attention was turned to the research with adipose-derived stem cells (ADSCs) (Takeda et al., 2016).

The focus of this research is the understanding of the way that ATRA acts on adipose tissue, for the development of an aiding treatment for overweight and obesity. For this analysis, therefore, we start from the knowledge that exist three types of adipocyte (White, Beige and Brown) with distinct functions very well described (Giralt & Villarroya, 2013), but the target tissue of this treatment will be white adipose tissue (WAT). That is because, during the weight gain process, the adipocytes are developed by hyperplasia and/or hypertrophy. Also have the ability to completely deregulate the organism by synthesis and secretion of cytokines and hormones, during a framework of systemic inflammation caused by obesity (Fruhbeck et al., 2001).

Our group already performed a previous study with ADSCs and evaluated the influence of ATRA during the proliferation, differentiation and induction of apoptosis. This treatment was effective in inhibiting the early stages of adipogenesis. So the local mesenchymal stem cells of adipose tissue are more sensitive to ATRA than the pre-adipocytes and adipocytes. ATRA also activates the Bcl-2, CASP-3, BAK, BAX genes, directing the cells to apoptosis by mitochondrial pathways. Thus, this treatment could prevent adipose tissue hyperplasia in the region received the application (de Carvalho Schweich et al., 2017).

In order to increase the knowledge and to continue to understand about the mechanisms, that promote this apoptosis and how this treatment can interfere in the cell cycle was choose in the present study the genes: ATM and ATR that are a key regulator of multiple signaling cascades which respond to DNA strand breaks induced by damaging agents or by normal processes; CHEK-1 and CHEK-2 that are involved in a cell cycle checkpoint that plays an important role in the DNA feeding pathway; CDC25A and CDC25C that are responsible for cell division events (von Stechow et al., 2014); and UCP1 that are

involved in mitochondrial activation and thermogenic effect (Giralt & Villarroya, 2013). Thus, the present study investigated the toxicogenic effect of ATRA in ADSCs and during their adipogenic differentiation .

2. Methodology

Isolation and culture of primary Human ADSCs

The biological material used in this research came from 3 female patients aged between 20-30 years old, who would already be submitted to the liposuction procedure with a private Plastic Surgeon. This material was obtained and manipulated after approval of Ethics Committee for Research on Human Beings of Federal University of Mato Grosso do Sul (Universidade Federal de Mato Grosso do Sul– UFMS) (No. 1.464.098).

The liposuction surgical procedure was performed in infra-abdominal region and was made as described in Pesarini, *et al.* (2017) (Pesarini et al., 2017). To processing the adipose tissue was used Type 1 collagenase (Gibco® 280U/mg) as already described (de Carvalho Schweich et al., 2017; Markarian et al., 2014). For each 1mL of adipose tissue, 0,87mg of collagenase was diluted in 3mL of Phosphate Buffer Solution (PBS). The medium of culture used was Dulbecco's Modified Eagle's Medium (DMEM Sigma®) – low glucose with HEPES 10 mM, 10% of foetal bovine serum (Sigma-Aldrich®) and 1% antibiotic (Penicilin/Estreptomycin/Amphotericin, Sigma-Aldrich ®). ADSCs among 2nd and 5th passage were used in the experiments.

Characterization of ADSCs

The cell surface markers of the extracted cells were examined using a flow cytometer (FACScalibur - Becton Dickinson, San Diego, CA). To this 2.5×10⁵ ADSCs in 5th passage were dissociated in trypsin, centrifuged and incubated during 30 min at 4 °C using the following antibody cell markers: CD105, CD90, MHC II and CD45 (Pharmingen BD, San DIEGO, CA. USA). The cells were analyzed in 10⁴ events using the 488nm laser FACScalibur (Becton Dickinson, San Diego, CA) in the CellQuest software. The MDI 2.8 software was used to generate the histograms.

Test substance: All-trans-retinoic acid

All-trans-retinoic acid (ATRA-Fagron®, CAS: 302-79-4, Lot: 15073822) was diluted in DMSO and stored as a stock solution. At the time of the experiment, the diluted ATRA was added to the DMEM. The concentration used was the IC₅₀ (dose that inhibits 50% of the studied biological event - cell viability) determined by MTT assay as previously published (de Carvalho Schweich et al., 2017). First, four ATRA concentrations (0.5, 5, 50 and 100 µM) were assessed at 24, 48 and 72h and after the results the concentration of 20.75 µM was determined. Normal culture received the same quantity of DMSO in the DMEM, this medium was used as negative control. All experiments were performed in triplicate.

Adipogenic differentiation assay

For this 1x10⁵ cells / well (3rd passage) were seeded in 6-well plates. After cell adhesion (24 hours), 3 wells with DMEM was discarded and received ATRA treatment. After 12h the medium culture of all 6 plates were discarded. All the wells received in this moment the specific medium, containing 0.714 µL of insulin (Insunorm R1, Aspen Pharma/Aspen Brazil, 0.35 mg/mL), 100 µM indomethacin (0.0447 g indomethacin, Sigma® catalogue number I7378, in 5 mL of dimethyl sulfoxide (DMSO), 3.5 µM rosiglitazone (0.0089 g of rosiglitazone, Sigma®, in 5 mL of DMSO) and 10⁻⁵M dexamethasone. To 1 mL of DMEM was supplemented 20% foetal bovine serum (de Carvalho Schweich et al., 2017; Hermeto et al., 2016; Hermeto et al., 2015). At 14th day the accumulation of intracellular lipids was visualized by *Oil Red O* (Sigma®), and analyzed under bright

field microscope (NIKON® Eclipse E200) at 40X magnification. The quantity of lipid vacuole was evaluated using the Fiji program, using the Feret method (Schweich-Adami et al., 2021). The assay was made and analyzed, in triplicate.

Comet assay

This assay was made according to Oliveira et al. (2007) (Oliveira et al., 2007) with modifications. To this ADSCs (3rd passage) were seeded in a flask with 25cm³ (3 x 10⁵ cells/flask), maintained for 24 hours in the incubator with 5% CO₂, 37° C to cell adhesion. The ATRA treatment happened for 12 hours in 3 flasks, and other 3 flasks maintained the normal culture medium. After the treatment all ADSCs were collected and the assay was performed according Schweich-Adami, et al. (2021). After the trypsinization the supernatant was discarded, an aliquot (40µL) was resuspended in 120µL of low melting point agarose (0.75%) at 37°C, and distributed on slides previously coated with 1.5% normal melting point agarose, covered with coverslips and immediately placed in the refrigerator. to solidification of the agarose for 20 min. After this the coverslips were removed carefully and the slides received lysis solution (2.5M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA) titriplex, 10 mM Tris (pH 10), Triton X-100 at 1 % and 10% dimethylsulfoxide (DMSO), being totally covered also in refrigerator for 2 hours. Then the slides remained in an electrophoresis vat, with alkaline buffer (pH 12), for 20 min (10N NaOH, 200mM EDTA-titriplex) for DNA denaturation. Electrophoresis (300 mA and 25 V) was performed with the same buffer for another 20 min. Subsequently, the slides were neutralized with 0.4 M Tris, pH 7.5, for 15 min (3x for 5 min each), fixed in absolute ethanol for 10 min. The material was stained with 100 µL of ethidium bromide. The nucleoids were photographed under a fluorescence microscope (Leica, DMI8) with a magnification of 200X. Subsequently, more than 200 nucleoids were analyzed per repetition using the CometScore 2.0.0.38 TriTek program, the parameters of the Tail Moment were evaluated (% DNA in the tail multiplied by the length of the tail).(Schweich-Adami et al., 2021).

Micronucleus assay

For this, the ADSCs were seeded 3 x 10⁵ cells/flask with 25cm³, maintained for 24 hours in the incubator, with 5% CO₂, 37° C. The ATRA treatment happened for 12 hours in 3 flasks, and other 3 flasks maintained the normal culture medium. After this the all the flasks were washed twice with PBS, and then incubated again with DMEM medium containing cytochalasin-B (Cyt-B, Sigma) (3 µg/mL) for 36 h. Cells were trypsinized and after was neutralized with the previously saved medium with one drop of 40% formaldehyde. The suspension was centrifuged and after the supernatant was discarded and then rapidly hypotonized with 1.5 mL of sodium citrate (1%). The pellet was suspended and centrifuged again under the same conditions as described above. The supernatant was discarded and 5 mL of methanol-acetic acid (3:1) were added to fix the cells. After this, the cells were transferred to slides and stained with 5% Giemsa. (Oliveira et al., 2006) The assay was made in triplicate and the slides were analyzed (Oliveira et al., 2007) with modifications, were examined both mononuclear and binucleate cells in a total 2000 cells/ repetition with an bright field microscope (NIKON® Eclipse E200) at 40X magnification.

Cytological and molecular cell death assay

The technique to determine the indexes for cell viability, apoptosis and necrosis was differential staining with acridine orange and ethidium bromide, using fluorescence microscopy. For this the flasks with 25cm³ were prepared using 3x10⁵ cells, after the adhesion the ATRA treatment happened for 12 hours in 3 flasks, and other 3 flasks maintained the normal culture medium. After this all the flasks were trypsinized, centrifuged and the supernatant was discarded, the pellet was resuspended in 1mL of PBS. An aliquot of 20 µL was take off and stained with 2 µL of the solution contained 100 µg/mL acridine orange and 100µg/mL ethidium bromide, both diluted in PBS, and then deposited in a slide and covered by a coverslip

to continue the reading. In the examination cells, a total of 100 cells per repetition were examined with a fluorescence microscope (NIKON® Eclipse E200), using a 60x objective (Oliveira et al., 2007).

After checking the results, the ADSCs was also evaluated by flow cytometry with the Annexin V Apoptosis Detection Kit (BD Pharmingen™) and Tali™ Cell Cycle Kit (Invitrogen™) according to the manufacturer's instructions. The acquisitions happened with 10^4 events were acquired (Cytoflex – Beckman Coulter).

qPCR

The qPCR was made to determine changes in gene expression patterns. For this, 2×10^5 ADSCs after 12 hours of exposure to the ATRA treatment have their RNA extracted using kit SV Total RNA Isolation System (cat. no. Z3100, Promega) according to the manufacturers's specifications, the quantification was made using a NanoVue™ spectrophotometer (GE Healthcare – Life Sciences®). The cDNA synthesis was performed using 250 ng of total RNA in a final reaction volume of 20 μ L. The cDNA was quantified and diluted to a concentration of 150 ng/ μ L for use in real-time PCR. The real-time reaction was performed using a Real-Time PCR Rotor Gene® (Qiagen). The GoTaq® qPCR Master Mix Kit (cat. no. A6002, Promega) was used at a volume of 10 μ L with the oligonucleotide primers (5 pmol each), cDNA template (500 ng) and free water ribonuclease q.s. 20 μ l. Real-time PCR was performed under the following conditions: 95°C for 10 min. and 40 cycles of 95°C/15 seconds and 60°C/60 seconds. At the end of reaction, the melting curve was used to evaluate the specificity of each oligonucleotide. Beta-actin (ACTB) was used housekeeping gene (de Carvalho Schweich et al., 2017). The primers CHECK 1, CHECK 2, CD25A, CD25C, ATM, ATR and UCP1 were analyzed.

Statistical analysis

The results were expressed as mean \pm standard deviation mean. Statistical analysis was performed by ANOVA / Bonferroni. Tukey's method was used to analyze the cell diameter data in particular. All differences were considered significant when $p \leq 0.05$ (GraphPad InStat 5 software). The qPCR was analyzed through the REST program (Pfaffl et al., 2002), was considered significant difference, when the level of relative expression was $p \leq 0.5$ or higher than 2 (Biazi et al., 2017; Navarro et al., 2018; Rabacow et al., 2018).

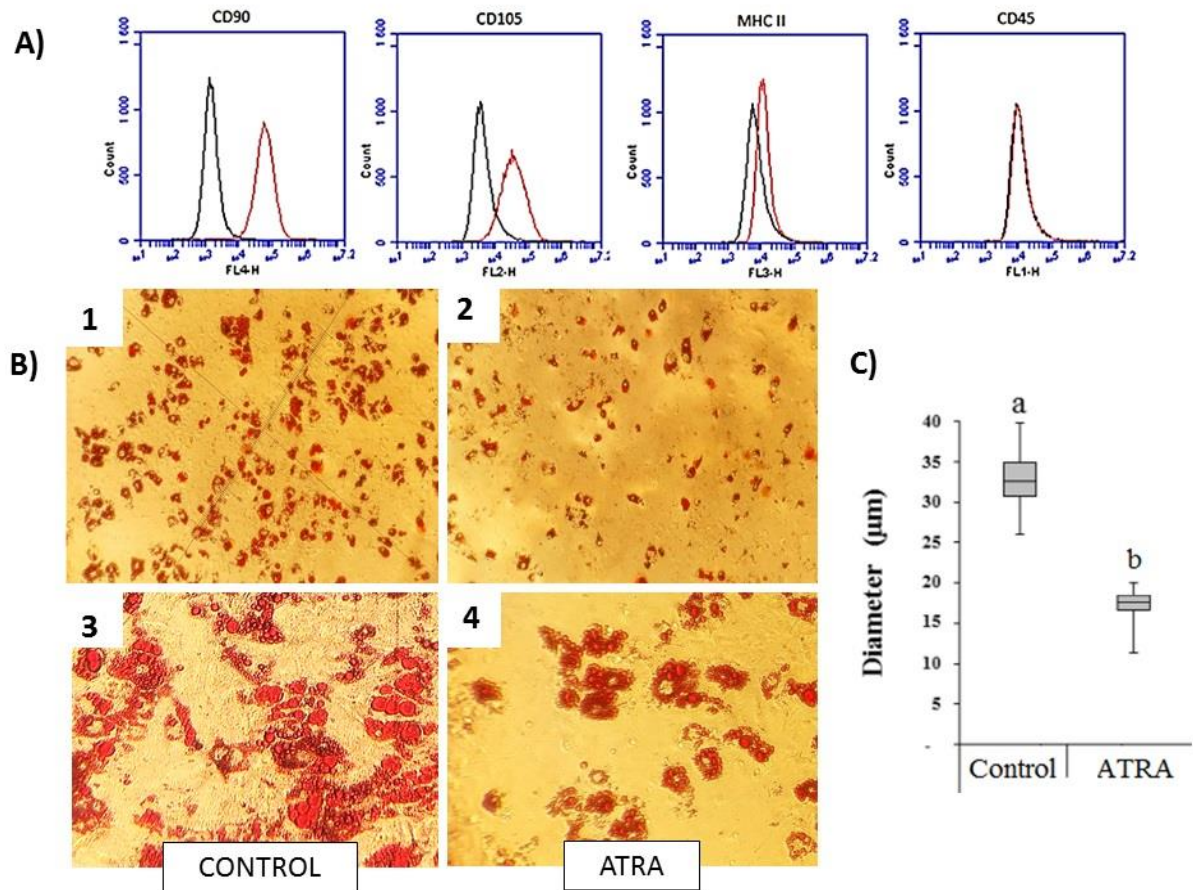
3. Results

Characterization and differentiation of ADSCs

The ADSCs used in this study have the ability to adhere to the flask and express in the immunophenotyping assay the CD105 and CD90 markers and do not express MHC II and CD45 (Figure 1A). These properties confirms that the cells used are mesenchymal stem cells derived from adipose tissue.

The treatment with ATRA for 12h before exchanging the specific medium of differentiation decrease the adipogenic differentiation capacity of ADSCs. Was demonstrated a significant reduction ($p < 0.05$) of 49% in the quantitie of lipid vacuoles when compared with the control culture. Also was observed that ATRA treatment made the ADSCs decrease significantly ($p < 0.05$) the ability to form large vacuoles, because the control culture showed mean of 34.17 ± 3.44 of size and the ATRA culture 16.61 ± 1.43 (Figure 1B).

Figure 1- Characterization and differentiation of ADSCs. A) Immunophenotypic profile of mesenchymal stem cells expressed the markers for CD105 and CD90 and did not express MHC class II and CD45; B) adipogenic differentiation with presence of lipid vacuoles stained with Oil Red (1 – control (ADSCs after adipogenic differentiation) and 2 – ATRA (ADSCs in adipogenic differentiation after treated with ATRA), 10X magnification) (3- control (ADSCs after adipogenic differentiation) and 4- ATRA (ADSCs in adipogenic differentiation after treated with ATRA), 40X magnification); C) Upper limit, 1st quartile, median, 3rd quartile and lower limit on the size of lipid vacuoles (μm).

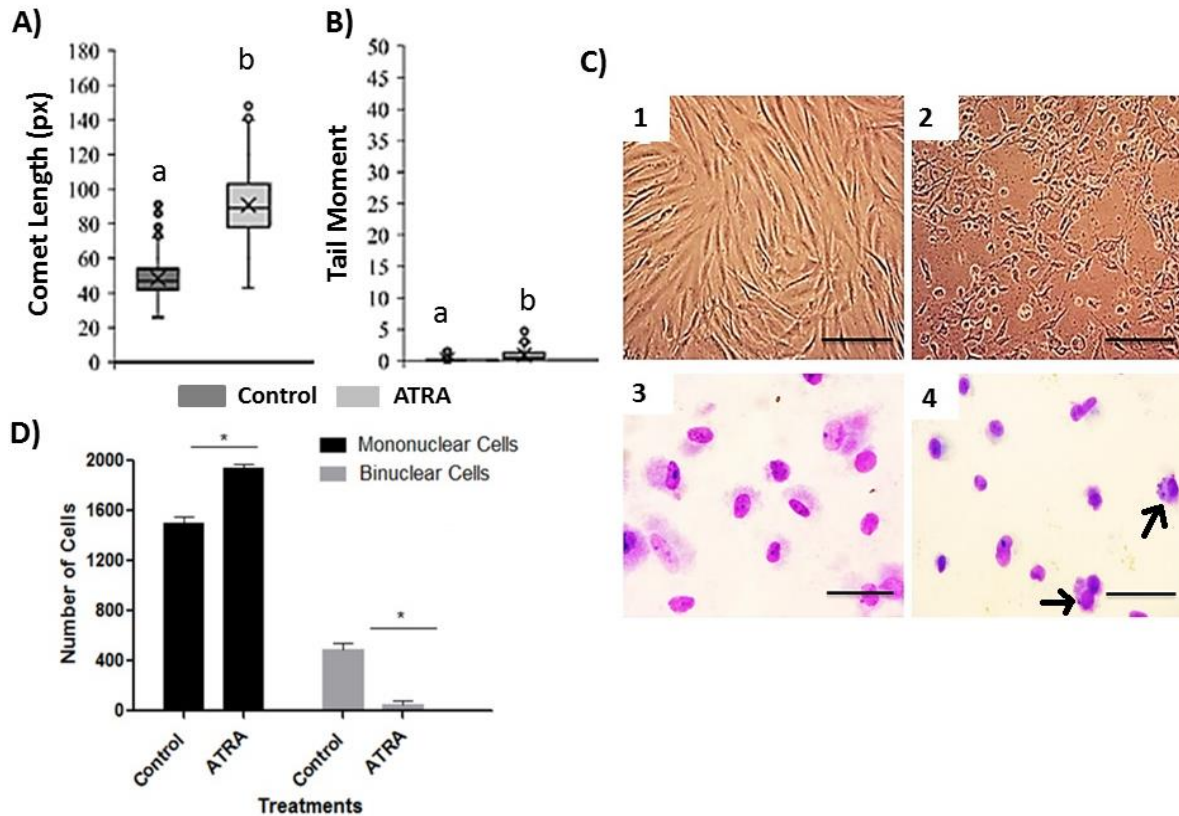


Source: Authors.

Comet assay

The ATRA induced genomes in the ADSCs and the increases ($p < 0.05$) in the nucleoid frequency with genomic damage (comets) (Figure 2A), but did not have significance ($p > 0.05$) in the increased of the tail moment, indicating little amount of DNA fragment in the existing tails (Figure 2B).

Figure 2 - Comet and Micronucleous assay. A – B) Upper limit, 1st quartile, median, 3rd quartile and lower limit, frequency of DNA damage (Comet Length and Tail Moment); C) Photomicrographs (1–control and 2– ATRA) of the cultures of ADSCs and stained by Giemsa (3 - control; 4 - ATRA). In 2 ATRA-treated cells showed cytoplasmic retraction, reduced nucleolus frequency and morphology change when still adhered to the culture flask as well as after trypsinization and giemsa staining, and in 4 presence of micronucleus in mononuclear cells (black arrow) are observed; D) Frequency of mono and binucleate cells. Bar represents 50 μ m. * Indicate statistically significant differences (Statistical Test: ANOVA / Bonferroni, $p < 0.05$).



Source: Authors

Micronucleous assay

The analysis of the micronucleus assay demonstrated that at 36h in the presence of cytochalasin B less than 1/4 of the ADSCs completed the cell cycle, since the mean frequency of binucleated cells was 493.44 ± 44.24 in 2×10^3 cells analyzed. Even though there was a low frequency of binucleated cells in the untreated cultures, a reduction ($p < 0.05$) of 9.86x was observed when the cultures were treated with ATRA. In addition, no micronucleus were found in binucleate cells and the frequency of micronucleus in mononuclear cells did not differ between untreated cells and those treated with ATRA (Figure 2D).

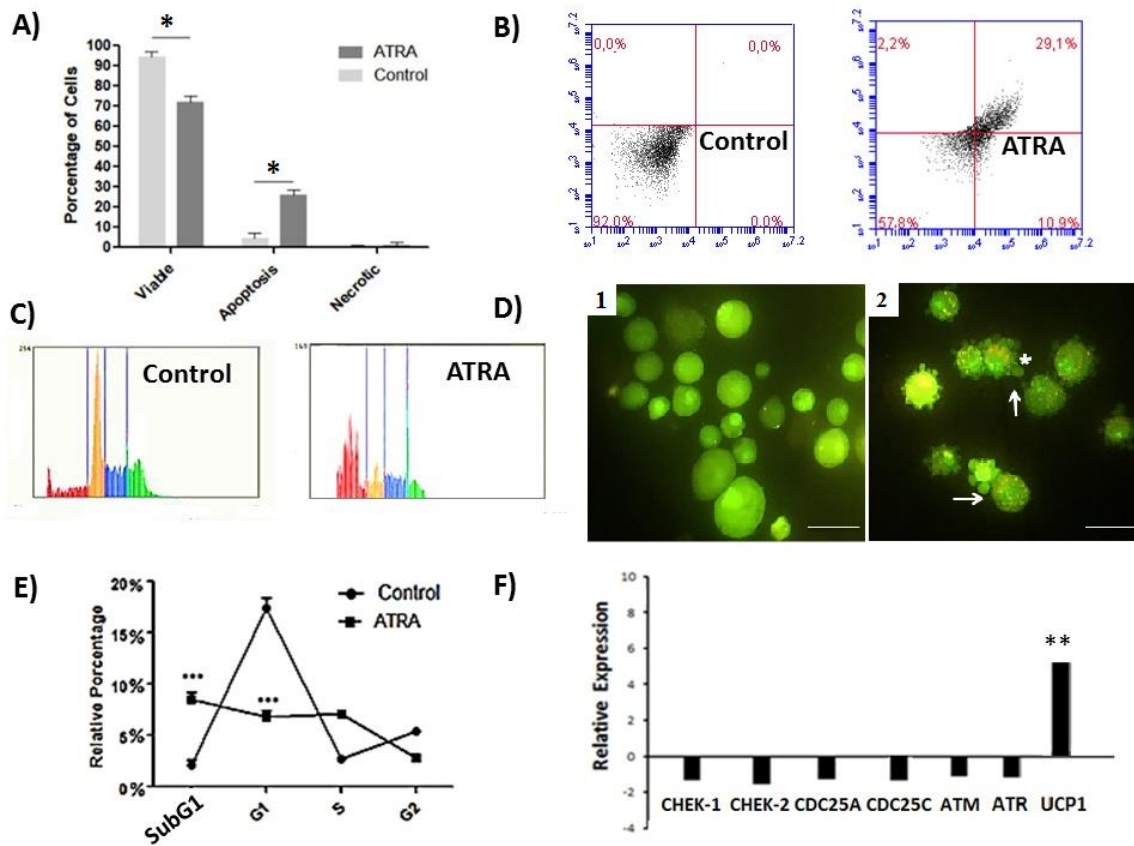
Cell death assay

ATRA induces cell death in ADSCs (Figure 3A). There was reduction ($p < 0.05$) of 1.31x in cell viability and increase ($p < 0.05$) 5.64x in the frequency of apoptosis. The frequency of necrosis in the culture of untreated and treated cells did not show significant differences and ranged from 0.67 ± 0.58 to 1.33 ± 1.15 (Figure 3A).

This events was confirmed by the Annexin V assay. Was demonstrated that in the ATRA treatment the most part of the percentage of events analyzed, even with a significant decreased of cells were viable (59.06 ± 3.98), and the ADSCs that were

significantly affected was in early apoptosis (27.09 ± 3.98), late apoptosis (12.06 ± 0.87) and the minority in necrosis (1.79 ± 0.40) (Figure 3B).

Figure 3 – Cell death assays, cell cycle and qPCR. A) Frequency of viable, apoptosis and necrotic cells; B) Example of the results in Annexin V assay by flow cytometry; C) Example of the results in cell cycle V assay by flow cytometry; D) Photomicrographs of ADSCs stained with ethidium bromide and acridine orange (1) Control; (2) ATRA with morphological characteristics of apoptosis (arrow with *) (nuclear fragmentation, marginalization of chromatin, formation of blebs and apoptotic bodies); E) Relative percentage of cell cycle phases; F) Relative expression of CHEK-1, CHEK-2, CDC25A, CDC25C, ATM, ATR and UCP1, over housekeeping Beta-actin (ACTB) in ADSCs treated with ATRA. * They indicate statistically significant differences (Statistical Test: ANOVA / Bonferroni; $p < 0.05$). Bar represents 50 μm .



Source: Authors.

Cell cycle

Cell cycle analysis demonstrated that the treatment with ATRA increased ($p < 0.05$) the frequency of events in SubG1 at 5.78x and reduced ($p < 0.05$) and the amount of cells in the G1 phase at 2.47x. The amount of cells in S and G2 / M phases did not differ between control and treatment with ATRA (Figure 3C e E).

Relative expression of genes

The treatment with ATRA reduced the relative expression of CHEK-1 in -1.31x, CHEK-2 in -1.54x, CDC25A in -1.27x, CDC25C in 1.34x, ATM in -1.14x and ATR in -1.11x, but this decrease was not considered significant. The UCP1 increased significantly in 5.4x (Figure 3F).

4. Discussion

In a previous study our research group demonstrated that ATRA is cytotoxic to ADSCs and that this cytotoxicity reduces as the cells progress in the process of adipogenic differentiation (de Carvalho Schweich et al., 2017). Therefore the use of ATRA in mesotherapy would be indicated to reduce and / or prevent the hyperplasia of the cells that originate the adipocytes. In this case, the ideal is the cell death, that does not cause inflammation, since the subcutaneous adipose tissue is the target tissue in this treatment (Takeda et al., 2016) and also that the process of apoptosis happens through the mitochondrial pathway in ADSCs, a fact that was proven by us with the use of ATRA (de Carvalho Schweich et al., 2017). However, in order to reinforce the indication of ATRA in mesotherapy, as a possible innovative therapy for the treatment of obesity and/ or localized fat, it was necessary to demonstrate the toxicogenic safety of this treatment, so it was the motivation to continue this study. Studies report that ATRA induces genomic damage (Di Francesco et al., 2015; Tokarz et al., 2016). Therefore, was expected that ATRA causes extensive and irreversible genomic lesions in ADSCs, would increase expression of GADD45 and p53, and also the cells undergo apoptosis due to DNA damage. However, we already verified that the treatment of ADSCs with ATRA caused increased of GADD45 expression and reduced p53 expression and, even so, part of ADSCs underwent to apoptosis. Therefore, apoptosis is thought to have occurred via the mitochondrial pathway and not due to genomic damage (de Carvalho Schweich et al., 2017). In addition, in the present study was verified that there was no stopping of cell cycle, by the inhibition of CHECK-1 and CHECK-2 that are essential components to delay cell cycle progression. This observation reinforces the absence of DNA damage. Thus, these data indicate toxicogenic safety, but still is insufficient, more assays are necessary for this statement.

Considering the above, the present study also evaluated the genotoxic damages caused in the ATRA treatment by means of the binucleate cell and micronucleus assays. The results demonstrated as expected (Di Francesco et al., 2015; Tokarz et al., 2016), increased frequency of genomic damage (increased numbers of lesioned cells and comet assay score). However, these lesions were not fixed at the chromosome level since they did not increase the frequency of micronucleus. Moreover, the micronucleus assay demonstrated that ATRA reduces the amount of binucleated cells in the culture. This fact suggests, in a first analysis, that DNA damage, caused by the ATRA and observed in the comet assay, could perhaps block the cell cycle. However, the cell cycle assay, by cytometry, no showed that stopped neither in G1 nor in G2 / M but presented significant increase of events in SubG1. This fact suggest again that this cells are undergoing apoptosis, corresponding to the increase of events in SubG1 (de Oliveira et al., 2018) or for might cell adaptation, it was also demonstrated by the cytological assay of cell death without cell cycle arrest and fixation of chromosomal damage. This hypothesis again was confirmed by the inhibition of the genes evaluated (CHEK-1, CHEK-2, CDC25A, ATM and ATR).

The WAT has important functions for the regulation of human metabolism, such as lipid metabolism, glucose metabolism, insulin sensitivity, endocrine regulation, release of inflammatory cytokines, thermogenesis, among others (Ailhaud & Hauner, 2004). The local stem cells have the role of regulating the function of this tissue and, in turn, being a reserve source for possible differentiations, such as in myofibroblasts or pre-adipocytes (Fraser et al., 2007). Among the WAT of this physiological microenvironment also exist beige adipose cells that works in the regulation of this tissue, their amount and functioning capacity, is given through the individual's lifestyle (Giralt & Villarroya, 2013). Differentiation from WAT to BAT is called "browning", so the brown adipocytes that appear in the WAT are often called "inducible, beige or brite"(Kuryłowicz & Puzianowska-Kuźnicka, 2020). In terms of differential function, although there has not yet been a precise bioenergetic analysis of the beige cells, they indicate that they have all the morphological and molecular characteristics of the classic brown adipocytes present in BAT deposits (multilocularity, UCP1 expression, and increased mitochondrial pathways) and therefore likely have intrinsically similar functions(Giralt & Villarroya, 2013). Considering the informations above, together with the significant increase in the UCP1 gene that was demonstrated after treatment with ATRA, the arrest of the cell

cycle in SubG1, and the continuity of adipogenic differentiation even after treatment (even with less amount and presence only of multilocular lipid vacuoles). We raise the hypothesis that ATRA acts as a modulator regarding the browning effect in this ADSCs, that do not suffer apoptosis with the treatment here proposed. But this effect would add a greater thermogenic capacity to the adipose tissue treated, being very important in helping the type of treatment that this research considered. However more assays are necessary to understand better this mechanism.

The set analysis of these data, and what the literature presents, reinforces the hypothesis that the apoptosis of ADSCs treated with ATRA occurs via mitochondrial pathway and further demonstrates (de Carvalho Schweich et al., 2017), first of all, that the DNA-mediated pathway leads the cell to apoptosis directly, and that damage in the DNA are not fixed. Therefore, genomic lesions are eliminated, reducing the possibility of a side effect such as the development of a cancer, which is directly related to the maintenance of mutations (Sancar et al., 2004), especially in cells that have undergone the attempt and / or repair of DNA damage. This fact indirectly suggests toxicogenic safety for the use of ATRA in mesotherapy and in the development of alternative therapies for the treatment of obesity and / or localized fat.

5. Conclusion

The results of the present study demonstrate that the use of ATRA induces genomic damage in ADSCs, although it was eliminated in the apoptosis process. Therefore, administration of ATRA in ADSCs did not cause chromosomal instability, which suggests toxicogenic safety. Also it was considered that ATRA might active the browning effect. Thus, this compound is considered a promising candidate for the development of novel therapies for the treatment of obesity and / or localized fat by the use of mesotherapy. We encourage the performance of in vivo research, with animals with a higher % of body fat, such as rabbits and pigs, to investigate this interaction of ATRA with adipose tissue in a better way and analyzed this possible modification a living organism.

Acknowledgments

The present work was carried out with support from the Federal University of Mato Grosso do Sul - UFMS / MEC - Brazil. This study received the financial supported from FUNDECT, CNPq and (CAPES) institutions, thanks for this.

Disclosure of Interest

The authors report no conflict of interest.

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