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Irisin attenuates SARS-CoV-2 entry into cells and cell damage in 2D and 3D cultures of human subcutaneous adipocytes

Maria Teresa De Sibio^{1,*}, Ester Mariane Vieira^{1,*}, Paula Barreto Da Rocha¹, Miriane De Oliveira¹, Regiane Marques Castro Olímpio¹, Vinícius Vigliuzzi Peghinelli¹, Lucas Solla Mathias¹, Helena Paim Tilli¹, Bianca Mariani Gonçalves¹, Igor Deprá¹, Maria Beatriz Bravin¹, Mariana Menezes Lourenço¹, Giovanna Bonatto Luca¹, Matheus de Souza Marino¹, Pedro Henrique Soares Kossooski¹, Camila Renata Corrêa², Marna Eliana Sakalem³, Cormarie Fernández Pulido¹ and Célia Regina Nogueira¹

¹Department of Internal Medicine, Botucatu Medical School – University of Sao Paulo State (UNESP), Botucatu, SP, Brazil

²Department of Pathology, Botucatu Medical School – University of Sao Paulo State (UNESP), Botucatu, SP, Brazil

³Department of Anatomy, State University of Londrina (UEL), Londrina, PR, Brazil

Correspondence should be addressed to M T De Sibio: maria.sibio@unesp.br

*(M T De Sibio and E M Vieira contributed equally to this work)

Abstract

Introduction: COVID-19 is associated with an inflammatory pathophysiology and, when associated with chronic diseases, can trigger severe infection and increase death risk. Irisin, a hormone produced by skeletal muscle during physical activity, has demonstrated therapeutic effects against metabolic disorders and exhibits anti-inflammatory and antioxidant effects. There is great interest in investigating irisin's influence on the interaction between SARS-CoV-2 and host cells. The aim of the present study is to investigate the role of irisin in viral infection in monolayers (2D) or three-dimensional (3D) cell cultures of human subcutaneous adipocytes infected with a SARS-CoV-2 pseudovirus (PV).

Materials and methods: Preadipocytes were cultured to maturity in 2D or 3D conditions and divided into four groups: Group 1: adipocytes with no treatment; Group 2: adipocytes optimized for angiotensin-converting enzyme 2 (ACE2) expression; Group 3: adipocytes optimized for ACE2 expression, and then exposed to SARS-CoV-2 pseudovirus (ACE2+PV); and Group 4: adipocytes treated with irisin 20 nM for 24 h, optimized for ACE2 expression and exposed to PV (ACE2+I+PV). Fluorescence levels of SARS-CoV-2 PV and ACE2 were measured to investigate cell infection; lactate dehydrogenase (LDH) activity to investigate cytotoxicity; and malondialdehyde (MDA) and protein carbonylation to assess oxidative stress levels.

Results and discussion: Irisin significantly reduced viral particle (PV) capture in 2D and 3D conditions. In addition, irisin decreased LDH release, MDA, and protein carbonylation levels, both in 2D and 3D conditions.

Conclusion: The results indicate irisin as a promising therapeutic target against COVID-19 pathophysiology by reducing viral entry into adipose cells as well as reducing cytotoxicity and oxidative stress indicators.

Keywords: irisin; SARS-COV-2; 2D and 3D cell culture; human adipocytes

Introduction

Obesity is a metabolic disease whose prevalence has progressively increased over the past years (1). It is defined by the World Health Organization (WHO) as an excess of body fat accumulation, which triggers direct impacts on health (2, 3). Obesity possesses a multifactorial character, resulting in a chronic positive energetic imbalance, leading to physiological imbalances and functional deficiencies. It also presents a direct correlation to premature mortality (4).

Adipose tissue (AT) of mammals can be subdivided into white adipose tissue (WAT) and brown adipose tissue (BAT). The main cell type of either tissue is adipocytes; however, WAT presents lipid droplets that store energy as triacylglycerol (TGL). In addition, mature adipocytes in WAT increase in size and cell number through the differentiation of preadipocytes, *in vivo* or *in vitro*, in a process called adipogenesis (5).

BAT also presents a larger number of mitochondria in comparison with WAT, while presenting fewer lipids. The same overall genes are expressed in both types of tissue, but the mitochondrial uncoupling protein-1 (UCP-1) is a transmembrane protein located in the inner membrane of the mitochondria (6). UCP-1 is primarily a BAT marker, and when this protein is activated, it generates an increase in temperature without generating adenosine triphosphate (ATP). Recent studies have investigated the possibility of converting WAT into BAT, a process known as browning, which is followed by an increase in energy expenditure and thermogenesis, which facilitates metabolism (5).

Irisin is a hormone arising from the cleavage of the fibronectin type III domain-containing protein 5 (FNDC5) and is released mostly by skeletal muscle tissue after physical activity. It presents thermogenic activity and has been linked to WAT browning (7). FNDC5 is stimulated by the peroxisome proliferator-activated receptor γ (PPAR γ) and coactivator 1 α (PGC1- α) after exercise (8) and increases the expression of UCP-1. Once UCP-1 is activated inside the mitochondria, it will not cause ATP synthesis because it is an uncoupling protein; instead, it leads to the release of heat (9).

Ever since its discovery in 2012 (8), the role of irisin, a myokine, has been investigated in multiple systems and different pathologies. In Parkinson's disease, irisin has proven to present a neuroprotective effect, reducing apoptosis and oxidative stress, restricting mitochondrial fragmentation, and promoting respiration and biogenesis (10). Irisin has also shown beneficial effects in osteogenesis, reducing fracture incidence and skeletal defects (11). In the cardiovascular system, irisin has reduced vascular senescence and stiffness (12), along with a significant improvement in myocardial performance after hemorrhagic shock (13).

Coronavirus disease-19 (COVID-19) is a pathology developed after a viral infection caused by the highly pathogenic SARS-CoV-2 virus. This virus demands different cell factors for successful cell entry and replication (14). Infectivity and SARS-CoV-2 cell entry in human cell hosts are mediated by the action of the viral membrane protein spike (S), which propagates on the viral surface and can bind to receptors located in the host cell membrane (15). SARS-CoV-2 uses angiotensin-converting enzyme II (ACE2) as a membrane receptor for cell infection due to its high binding capacity and affinity (16). Human transmembrane serine protease II (TMPRSS2) on the cell surface has also been known to allow the activation of S proteins linked to ACE2 in the host cell and the direct entry of the virus into human cells (17). Both ACE2 and TMPRSS2 are present in high concentrations in obese patients, which have been previously linked as a possible explanation for the more prominent negative effect of the virus in these patients. In addition, the increase in inflammatory cytokines produced and released by the adipose tissue can significantly contribute to the increased morbidity risk related to the infection in this population (18). Given the need to further elucidate the behavior of SARS-CoV-2 and which interventions might minimize its negative effects on the organism, the evaluation of the specific tissue response – in this case, of the adipose tissue – is important to identify the ability of irisin to act on SARS-CoV-2 and, consequently, on COVID-19. The present study aims to investigate irisin's role on oxidative stress markers and its cytotoxicity in 2D and 3D cell cultures of human subcutaneous adipocytes infected with a SARS-CoV-2 pseudovirus.

Materials and methods

Cultivation of human subcutaneous preadipocytes

The multiuser laboratories of the Experimental Research Unit (UNIPLEX) at the Botucatu Medicine Faculty were used to conduct all experimental procedures. Human subcutaneous preadipocytes (Catalog #: HPAd-802s-05A) were obtained from Cell Applications, Inc. (USA) through Sigma-Aldrich (USA). The remaining materials employed will be showed according to the experimental procedure, as follows.

In vitro investigations

Human subcutaneous preadipocytes were kept in culture until they achieved a confluence of approximately 80%, at a temperature of 37°C and an atmosphere of 5% CO₂, in Human Preadipocyte Growth Medium (Sigma, 811-500) and 1% antibiotic-antimycotic (Sigma, A5955). After achieving confluence, cells were washed with phosphate-buffered saline solution (PBS) from Dulbecco

(# 17-512F) and detached from the flasks with trypsin (CC-3232 Trypsin/EDTA Solution). The resulting cell suspension was centrifuged at 300 *g* for 10 min to obtain a cell pellet for further plating at a concentration of 10,000 cells/well in a 96-well cell culture plate.

Differentiation of preadipocytes into mature adipocytes: two-dimensional (2D) and three-dimensional (3D) cultures

After centrifugation, preadipocytes were seeded in 96-well cell culture plates with growth media and no bedding for 2D cell cultivation, or with growth media and the addition of 3D matrix CellFate® (Biocelltis Biotecnologia, Brazil) for 3D cell cultivation. Cells were maintained at 37°C and 5% CO₂ until a confluence of approximately 90% was achieved. Then, preadipocytes were induced to differentiate into mature subcutaneous adipocytes. The differentiation process was initiated with Human Adipocyte Differentiation Medium (Sigma-Aldrich, 811D-250) supplemented with 1% antibiotic–antimycotic (Sigma, A5955), and cells were maintained with differentiation medium for 16 days. The evaluation of the differentiation process was conducted by microscopic observation using an Inverted Research Microscope ECLIPSE Ti (Nikon, Japan); lipid vacuoles in induced cells were identified and used as a parameter for differentiation. Intracellular lipid vacuoles were first observed after 4 days of induction and in the following days showed increased number and size for 16 consecutive days.

After cellular differentiation, mature adipocytes were hormone-depleted in the culture media by using Human Adipocyte Starvation Medium (Sigma, 811S-250) for 24 h (19). After the completion, cultures were divided into groups, and a SARS-CoV-2 pseudovirus (Montana Molecular Fluorescent Biosensors for Live Cell Discovery) was employed for the experimental groups. The experimental design was:

Group 1 (Control): adipocytes without treatment; Group 2 (ACE2): adipocytes treated with ACE2 – used to control SARS-CoV-2 transduction; Group 3 (ACE2 + pseudovirus): adipocytes treated with ACE2 and then treated with SARS-CoV-2 pseudovirus; Group 4 (ACE2 + Irisin + pseudovirus): adipocytes treated with ACE2, followed by 20 nM irisin treatment, and then SARS-CoV-2 pseudovirus.

The irisin dose (20 nM) and 24 h treatment period were selected based on prior studies demonstrating that this concentration replicates post-exercise physiological levels and is sufficient to elicit metabolic effects in adipocytes (8, 20, 21, 22).

Adipogenic differentiation verification

To document adipocyte differentiation and to determine lipid accumulation, Oil Red O (Sigma-Aldrich, O1391)

staining was conducted. Initially, cells were washed twice with PBS and fixed with 4% formaldehyde for 30 min at room temperature. After fixation, cells were again washed twice with PBS. For staining, 200 µL Oil Red O was diluted in Milli-Q water (3:2) (Milli-Q® Direct Water Purification System, Milli-Q Type Ultrapure Water, Sigma-Aldrich), and cells were incubated with the solution for 2 h at 37°C. Stained cells were rinsed three times with Milli-Q water and kept in a greenhouse (Thermo Scientific™ Series 8000 Water-Jacketed CO₂ Incubator, 184L, catalog number: 3429) at 37°C for 30 min. After preparation, cells were observed under the microscope to verify differentiation – noted as red stains on lipid droplets.

SARS-CoV-2 pseudovirus (PV) infection and ACE2 expression

To evaluate the presence of ACE2 and PV in cells, immunofluorescent markers were employed. Angiotensin-Converting Enzyme 2 (ACE2) Red Reporter Kit (catalog number #C1100R) and Pseudo SARS-CoV-2 Green Reporter (Catalog number #C1110G) were acquired from Montana Molecular. The ACE2-Red assay was used to mark protein expression with previous optimization of protein expression in adipocytes, and the PV Green Reporter assay was used to mark the presence of the PV in cell culture. These effects were measured by fluorescence reading using a plate reader and by manual inspection of triplicate fluorescence images.

SARS-CoV-2 pseudovirus (PV) capture and ACE2 levels

PV and ACE2 capture were used to quantify expression in cell culture. Cells were incubated for the transduction reaction using the ACE2-Red Reporter (C1100R) assay to optimize ACE2 expression. For the solution, 5 µL of fluorescent ACE2 BacMam was added to 0.6 µL of sodium butyrate (SB) 500 mM and 44.4 µL of complete adipocyte culture medium, for a total volume of 50 µL. Then, the transduction reaction solution was added to the wells with the cells and 100 µL of culture medium and incubated for five minutes at room temperature. Cells were then incubated for 24 h under regular cellular growth conditions. After this period, cells were treated with 20 nM irisin (SRP8039-10UG IRISIN – Sigma-Aldrich) for 24 h, and then a solution containing 100 µL of medium with 8×10^7 viral genes of SARS-CoV-2 pseudovirus (C1110G). Cells were incubated for 12 h in the darkness under regular cellular growth conditions. Before fluorescence reading, cells were rinsed with PBS. The capture of fluorescence levels was conducted using a plate reader with the range 506/517 (Ex/Em) for the SARS-CoV-2 pseudovirus (Green Reporter assay) and 558/603 (Ex/Em) for ACE2 (Red Reporter assay).

Confocal microscopy

The same procedures for fluorescence aspects were used for confocal microscopy analyses. The images were obtained using a confocal microscope, Leica TCS SP8 (LSCM, Leica Microsystems, Germany), equipped with LAS X software, a 20× objective, and a 0.75× zoom. All images were captured using the 552 nm laser and a detection window of 583–649 (red channel) and the 488 nm laser and a detection window of 510–536 nm.

LDH liberation analysis

Extracellular LDH levels were also investigated. LDH is a cytosolic enzyme that is released into the cell culture after damage to the plasmatic membrane (23). The levels of LDH were determined using the CyQUANT™ LDH Cytotoxicity Assay (catalog numbers C20300 – No. MAN0018500), and the procedures were conducted as instructed by the manufacturer. Triplicate samples were used. Absorbance was evaluated using 490 and 680 nm readers and using wavelength as a reference. The percentage calculation of cytotoxicity employed was as described by the manufacturer (Fig. 1).

Evaluation of oxidative stress and protein carbonylation biomarkers

Carbonylation was quantified as described by Mesquita *et al.* (25), with minor modifications. Briefly, 100 µL of adipocyte culture supernatant was used for 2,4-dinitrophenylhydrazine (DNPH) (10 mM in 2 M HCl). Samples were conducted in quintuplets, incubated for ten minutes at room temperature, then 50 µL NaOH (6 M) was added and incubated for ten minutes at room temperature. Reading was conducted with a microplate reader, Spectra Max 190 (Molecular Devices®, USA), at 450 nm, and the result was obtained from the absorbance of samples and the molar extinction coefficient (22,000 M⁻¹ cm⁻¹). The final result is expressed as nmol/mg of proteins.

Malondialdehyde (MDA)

For MDA quantification, 250 µL of adipocyte cell culture supernatant and 750 µL of 10% trichloroacetic acid were used for protein precipitation. Samples, in quintuplicates, were centrifuged at 900 *g* for five minutes using an

Eppendorf® Centrifuge 5804-R (Germany). After centrifugation, the supernatant was removed and added to 0.67% thiobarbituric acid (TBA), in a 1:1 proportion. Then, samples were warmed for 45 min in a water bath at 100°C. MDA reacted with TBA in a 1:2 proportion (MDA: TBA), and after cooling, reading was conducted in a microplate reader (Spectra Max 190, Molecular Devices®, USA) at 535 nm. MDA concentration was obtained by using the molar extinction coefficient (1.56 × 10⁵ M⁻¹ cm⁻¹) and sample absorbances, and the final result is expressed as nmol/g of protein (26).

Statistical analysis

Data are expressed as mean ± standard deviation. After normality verification using the Shapiro–Wilk test, all treatments within groups were analyzed using two-way variance analysis (ANOVA) followed by multiple Tukey's test comparisons. A value of *P* < 0.05 was considered statistically significant.

Results

Characterization of differentiation

To verify cellular differentiation, human subcutaneous preadipocytes were plated and stained using Oil Red, and the results can be observed in Fig. 2.

ACE2 expression and viral infection confirmation

ACE2 expression and viral infection using the pseudovirus were confirmed using immunofluorescence staining for both 2D and 3D cell cultures. The viral infection and ACE2 expression were marked using a green reporter assay and a red reporter assay, respectively. In both types of culture, human subcutaneous adipocytes showed high expression of ACE2 (Fig. 3B). In addition, the pseudovirus was able to successfully infect the cultivated cells (Fig. 3C). Finally, the double staining (Fig. 3D) proved that cells containing a higher expression of ACE2 were more infected with the pseudovirus, both in 2D and 3D conditions.

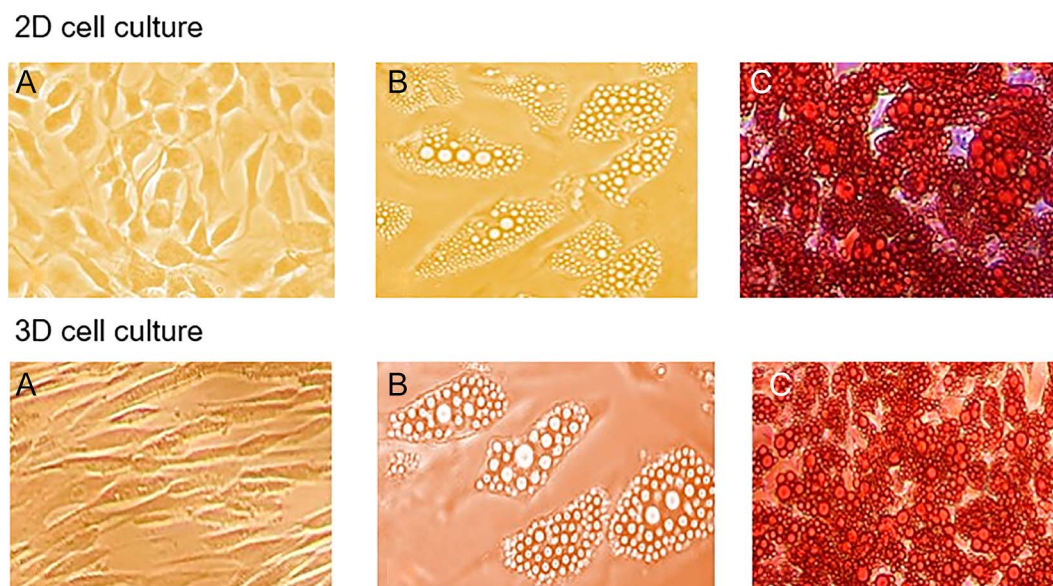
Capture of ACE2 levels

Cells optimized for ACE2 expression showed increased protein expression in comparison with the control group. Irisin treatment was effective in reducing ACE2 capture levels in cells exposed to the pseudovirus in 2D (ACE2+PV, 38,378 ± 53 vs ACE2+PV+I, 36,779 ± 311) and 3D (ACE2+PV, 40,368 ± 97 vs ACE2+PV+I, 38,805 ± 129). The results can be observed in Fig. 4.

$$\% \text{ Cytotoxicity} = \left[\frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right] \times 100$$

Figure 1

Formula to indicate cytotoxicity percent, as indicated by the manufacturer (Elkholly *et al.* 2024 (24)).

**Figure 2**

Cellular cultivation of human subcutaneous preadipocytes and differentiation into mature adipocytes in monolayer under 2D (top row) and 3D (bottom row) conditions. (A) Plating in monolayer; (B) differentiation; (C) Oil Red O staining.

Capture of SARS-CoV-2 pseudovirus levels

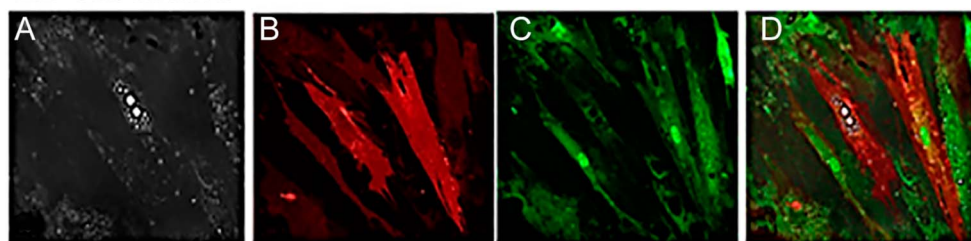
There was no difference between the control group and the group with optimization of ACE2 expression (C vs ACE2). However, groups that received irisin treatment showed a reduction of PV capture levels in 2D conditions (ACE2+PV, $48,553 \pm 62$ vs ACE2+PV+I, $44,745 \pm 207$) and 3D conditions

(ACE2+PV, $50,436 \pm 41$ vs ACE2+PV+I, $45,433 \pm 158$). The capture levels for all groups can be observed in Fig. 5.

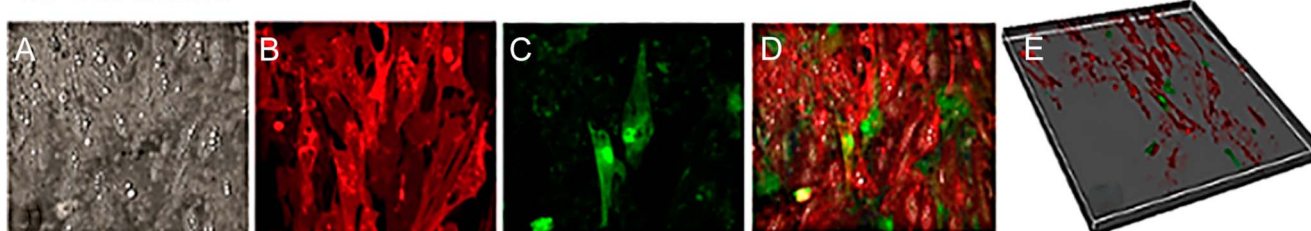
Cytotoxicity assay

Cytotoxicity effects were investigated in all groups, both under 2D and 3D cell culture conditions. The evaluation was

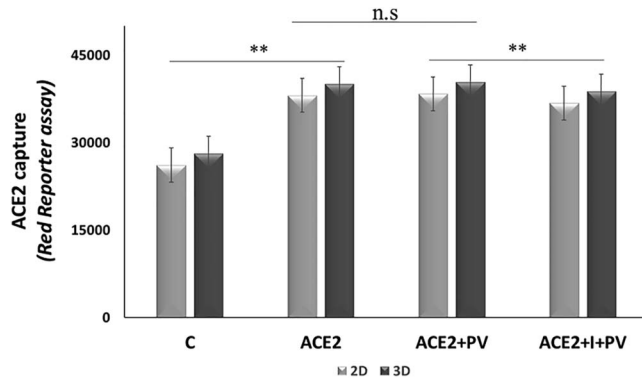
2D cell culture



3D cell culture

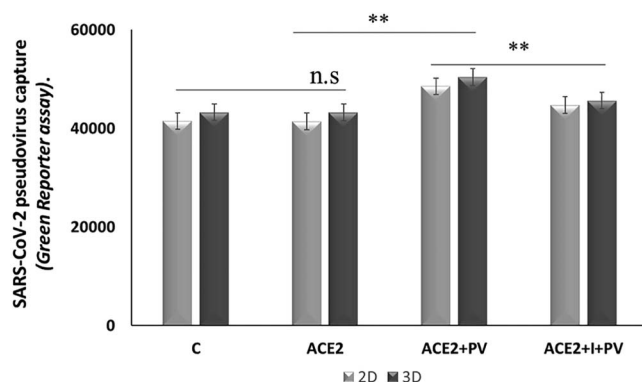
**Figure 3**

Confocal microscopy to verify ACE2 expression and presence of SARS-CoV-2 pseudovirus; cell cultivation in monolayer under 2D (top row) and 3D (bottom row) conditions of human subcutaneous adipocytes infected with SARS-CoV-2 pseudovirus. (A) Cells without treatment (Control group); (B) ACE2 (Red Reporter assay); (C) SARS-CoV-2 pseudovirus (Green Reporter assay) (PV); (D) ACE2 (Red Reporter assay) + SARS-CoV-2 pseudovirus (Green Reporter assay), double staining (ACE2+PV); (E) three-dimensional resource of cell culture to confirm cell adhesion and depth under 3D conditions.

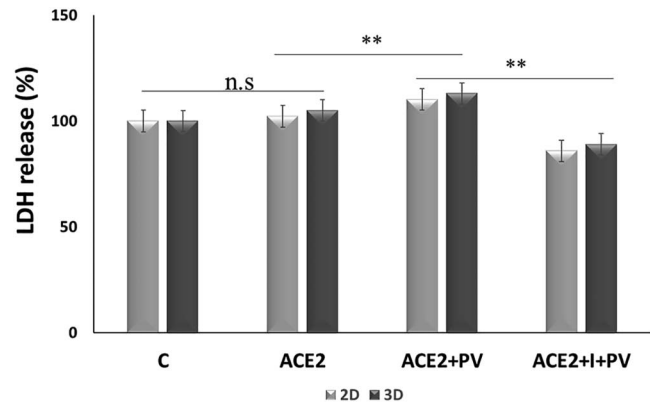
**Figure 4**

Capture of ACE2 levels. ACE2 capture levels, observed by fluorescence read in a microplate reader at excitation of 540 nm and emission of 580 nm (Red Reporter assay), in 2D and 3D cell cultures of human subcutaneous adipocytes (C: Control group, cells with no treatment; ACE2: cells with ACE2 expression optimized; ACE2+PV: ACE2 cells with ACE2 expression optimized and exposed to SARS-CoV-2 pseudovirus (PV); and ACE2+I+PV: ACE2 cells with ACE2 expression optimized, treated with 20 nM irisin for 24 h, and exposed to SARS-CoV-2 pseudovirus (PV)). All groups were analyzed in triplicated samples and tested with ANOVA, followed by Tukey's test. $**P < 0.01$; n.s., not significant.

conducted using a microplate reader for lactate dehydrogenase enzyme (LDH), using an absorbance range of 490–680 nm. A reduction in the cytotoxic levels of human subcutaneous preadipocytes was observed after irisin treatment in 2D (ACE2+PV, 110 ± 3.9 vs ACE2+PV+I, 86 ± 2.2) and in 3D (ACE2+PV, 113 ± 1.6 vs ACE2+PV+I, 89 ± 0.9). All data can be seen in Fig. 6.

**Figure 5**

SARS-CoV-2 pseudovirus capture levels, observed by fluorescence levels read in a microplate reader at excitation of 506 nm and emission of 517 nm (Green Reporter assay), in 2D and 3D cell cultures of human subcutaneous preadipocytes (C: Control group, cells with no treatment; ACE2: cells with ACE2 expression optimized; ACE2+PV: ACE2 cells with ACE2 expression optimized and exposed to SARS-CoV-2 pseudovirus (PV); and ACE2+I+PV: ACE2 cells with ACE2 expression optimized, treated with 20 nM irisin for 24 h, and exposed to SARS-CoV-2 pseudovirus (PV)). All groups were analyzed in triplicated samples and tested with ANOVA, followed by Tukey's test. $**P < 0.01$; n.s., not significant.

**Figure 6**

Cytotoxicity assay – investigation of lactate dehydrogenase enzyme (LDH) after SARS-CoV-2 pseudovirus infection, with and without irisin treatment. (C: Control group, cells with no treatment; ACE2: cells with ACE2 expression optimized; ACE2+PV: ACE2 cells with ACE2 expression optimized and exposed to SARS-CoV-2 pseudovirus (PV); and ACE2+I+PV: ACE2 cells with ACE2 expression optimized, treated with 20 nM irisin for 24 h, and exposed to SARS-CoV-2 pseudovirus (PV)). All groups were analyzed in triplicated samples and tested with ANOVA, followed by Tukey's test. $**P < 0.01$; n.s., not significant.

Evaluation of oxidative stress and protein carbonylation markers

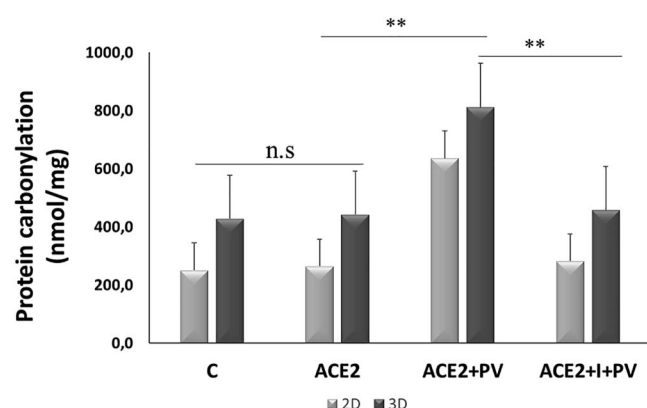
Protein carbonylation is a result of the direct action of reactive oxygen species (ROS) and leads to oxidative modifications of released proteins. In the present investigation, there was a significant increase in ROS in human subcutaneous preadipocytes after SARS-CoV-2 pseudovirus infection, in 2D and 3D conditions. In addition, the group that had been treated with irisin before pseudovirus exposure showed less ROS in comparison with the non-treated group (2D culture: ACE2+PV, 637 ± 14 vs ACE2+PV+I, 282 ± 11 ; 3D culture: ACE2+PV, 813 ± 15 vs ACE2+PV+I, 459 ± 15). Data are shown in Fig. 7.

Malondialdehyde (MDA)

Malondialdehyde (MDA) is an important biomarker used to investigate oxidative stress. In the present investigation, a significant increase was observed in MDA in groups infected with PV in comparison with non-treated groups, in both 2D and 3D conditions. In addition, a reduction in MDA was seen in the group pre-treated with irisin before PV exposure, also in both 2D and 3D conditions (2D: ACE2+PV, $22,307 \pm 297$ vs ACE2+PV+I, $16,832 \pm 99$; 3D: ACE2+PV, $25,675 \pm 134$ vs ACE2+PV+I, $20,199 \pm 149$). Data are shown in Fig. 8.

Discussion

Obesity is a vastly investigated condition, recognized as one of the major concerns in public health worldwide.

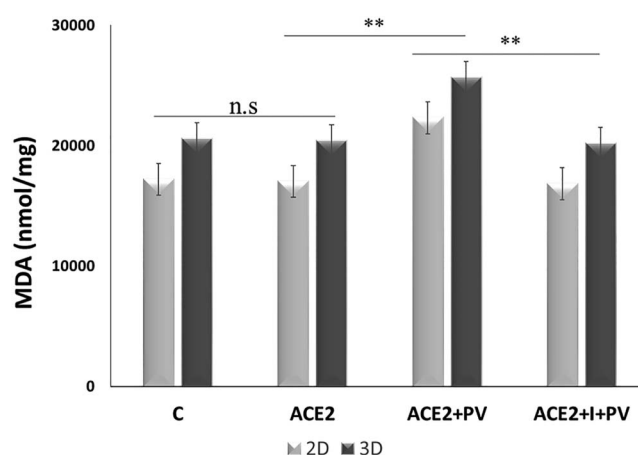
**Figure 7**

Protein carbonylation. (C: Control group, cells with no treatment; ACE2: cells with ACE2 expression optimized; ACE2+PV: ACE2 cells with ACE2 expression optimized and exposed to SARS-CoV-2 pseudovirus (PV); and ACE2+I+PV: ACE2 cells with ACE2 expression optimized, treated with 20 nM irisin for 24 h, and exposed to SARS-CoV-2 pseudovirus (PV)). All groups were analyzed in triplicated samples and tested by ANOVA, followed by Tukey's test. $^{**}P < 0.01$; n.s., not significant.

Understanding the physiological mechanisms involved in body weight regulation is mandatory and has been the focus of our research group for the past years. During the COVID-19 pandemic, researchers have devoted time and effort toward understanding the association between COVID-19 and obesity (27, 28, 29), and there was an increased number of investigations regarding adipose tissue to better elucidate the relation to the virus (30). Similar to cardiovascular and respiratory tissues, subcutaneous adipose tissue also presents an elevated amount of ACE2 receptor on the cell membrane, which implicates virus entry into the cell and consequent viral replication (31).

COVID-19 possesses a pro-inflammatory profile and is known to increase oxidative stress, as well as tissue damage (32, 33). Irisin was first discovered as a molecule with beneficial potential for the prevention and treatment of obesity, but it also presents potent anti-inflammatory effects and participates in adipose tissue browning, thus supporting multiple tissues through thermogenesis (34, 35, 36).

In a recent study evaluating the genetic expression in human subcutaneous adipocytes, our research group demonstrated by global transcriptome analysis that the cells cultivated with or without irisin in the medium showed a total of 14,857 expressed genes. From those, irisin reduced the expression of genes associated with viral entry (TLR3, HAT1, KDM5B, SIRT1, RAB1A, FURIN, and ADAM10) and increased the expression of the ones that prevent viral entry into cells (HDAC2 and TRIB3) (30). These results indicate that irisin presents a beneficial effect on the physiopathology of COVID-19 by hindering the entrance of SARS-CoV-2 into adipocytes (30). Further investigations were necessary to fully comprehend how

**Figure 8**

Evaluation of oxidative stress by malondialdehyde (MDA) verification. (C: Control group, cells with no treatment; ACE2: cells with ACE2 expression optimized; ACE2+PV: ACE2 cells with ACE2 expression optimized and exposed to SARS-CoV-2 pseudovirus (PV); and ACE2+I+PV: ACE2 cells with ACE2 expression optimized, treated with 20 nM irisin for 24 h, and exposed to SARS-CoV-2 pseudovirus (PV)). All groups were analyzed in triplicated samples and tested by ANOVA, followed by Tukey's test. $^{**}P < 0.01$; n.s., not significant.

irisin affects COVID-19 and relates to the adipose tissue. The present study focused on investigating the role of irisin in viral infection, oxidative stress markers, and cytotoxicity.

Preadipocytes were successfully matured into adipocytes after 15 days, presenting significant morphological changes. Cells exhibited a rounded aspect with an accumulation of lipid droplets in the cytoplasm, stained red by Oil Red O staining (Fig. 2). The exposure to the SARS-CoV-2 pseudovirus (PV) was effective in enabling viral entry into cultured cells, confirmed by immunofluorescence staining (Green Reporter assay). These results indicate the efficacy of the used model and are in agreement with other investigations using the same cells and the PV (Fig. 3) (37). More importantly, irisin treatment before PV exposure caused a decrease in the PV capture levels (Fig. 5). Previous investigations have confirmed that irisin presents a significant effect on the expression of genes that are linked with SARS-CoV-2 entry into cells (30, 37, 38). Nevertheless, to our best knowledge, this is the first report demonstrating reduced viral entry into cells after irisin treatment. Further experiments were conducted to try to elucidate potential mechanisms for this effect.

SARS-CoV-2 utilizes the ACE2 receptor as a gateway to enter the cell by endocytosis and proceeds to undergo viral replication inside the host cell (39). The PV used in this study does not possess the ability to replicate intracellularly or alter genetic material, but it succeeds in mimicking the viral infection effectively.

Irisin treatment before PV exposure was able to drastically reduce cell entry by SARS-CoV-2 PV (Fig. 5), while also reducing ACE2 expression (Fig. 4). Considering that ACE2 expression plays a key role in reducing viral entry, one of the mechanisms by which irisin reduced viral entry is mediated by ACE2 reduction. Previous investigations aiming to understand how irisin acts as a protective agent against severe COVID-19 have demonstrated that, in addition to reducing the expression of genes associated with viral entry and improving the expression of protective genes (30), irisin also acts on other tissues, mostly acting to regulate inflammation and reduce the cytokine storm, but also improving metabolic regulation (40).

In addition to the effect on virus entry into cells, the present investigation aimed to understand the impact on cell death of irisin treatment and virus exposure. The liberation of LDH has been widely used to verify the potential of cytotoxicity in *in vitro* models, and it is considered a marker for poor prognosis in diverse diseases, including COVID-19 (41). Increased levels of LDH in the blood indicate acute cellular death in patients with the disease (42). Similarly, in *in vitro* investigations, elevated LDH demonstrates increased cellular death. The group exposed to the PV (ACE2+PV) showed an increase in LDH levels in comparison with the control group, as well as in comparison with the group with ACE2 optimization. In addition, this increase was significantly reverted once cells were treated with irisin, suggesting a protective effect of this myokine against the cytotoxic effects of SARS-CoV-2 (Fig. 6). These results demonstrate the cytotoxic potential of the virus in comparison with normal conditions. More importantly, they also indicate the beneficial effect of irisin in preventing cell death, even in the presence of the viral agent. Thus, in addition to reducing the entry of the PV into cultured cells, irisin treatment was effective in refraining from exacerbated cellular death post-infection.

As a frequent outcome of SARS-CoV-2 infection, there is a significant increase in oxidative stress (32, 33). The imbalance between free radicals and antioxidants induces cellular and tissue damage, and it has been previously shown that SARS-CoV-2 infection triggers the production of these harmful molecules, not only due to the infection per se but also because of the body's immune response to the virus. The direct consequences observed in patients were shortness of breath and respiratory issues, fatigue and muscle weakness, and increased risk of heart, kidney, and liver damage (33, 43). In the present investigation, the group of cells with ACE2 optimization and PV exposure (ACE2+PV) showed a significant increase in the oxidative stress marker used in comparison with the control group with no viral exposure (Fig. 7). This marker reflects the oxidative damage caused by reactive oxygen species (ROS). Elevated carbonylated proteins are associated with increased oxidative stress in multiple diseases (44).

On the other hand, it has been indicated that irisin can modulate chronic inflammation associated with obesity (45), and once again, our results are in accordance with the literature, since irisin treatment before PV exposure reduced oxidative stress levels, demonstrating the protective effect of the molecule (Fig. 8). Malondialdehyde (MDA) is a well-established biomarker of oxidative stress, representing the final product of lipid peroxidation and reflecting cellular damage induced by free radicals. Investigations with animal models have also demonstrated a significant reduction of oxidative stress in obese animals after irisin treatment (46). In addition, investigations using pancreatic cell cultures also proved irisin's protective effect on oxidative stress (47). These findings reinforce the importance of irisin as a molecule capable of impairing viral infection and evoking a positive effect on cytotoxicity and oxidative stress parameters.

Cytotoxicity and oxidative stress are relevant parameters in COVID-19 since oxidative stress leads to cellular and tissue damage, causing a negative progression of the disease and the escalation of symptoms. In addition, chronic inflammation and oxidative stress are related to a hyperactive immunological response, which can trigger COVID-19 complications (47). Therefore, if irisin is able to reduce oxidative stress in the context of cells exposed to SARS-CoV-2, this finding can help decrease the severity of symptoms and improve the immune response to the virus. These results corroborate the proposition of more effective therapeutic models to fight severe respiratory infectious diseases, including COVID-19 and other viral pathologies. Epidemiological studies investigating this effect of irisin have demonstrated that serum irisin levels of patients with severe COVID-19 were significantly lower compared to those with milder symptoms; additionally, there was an inverse correlation between serum irisin concentrations and multiple clinical characteristics, and the severity score. Moreover, patients with lower serum irisin at admission had an increased risk of mortality within 1 month, suggesting that irisin may serve as a prognostic biomarker in COVID-19, particularly in populations with cardiometabolic comorbidities (48).

The results from the present investigation indicate that irisin evoked a significant decrease in viral entry into human subcutaneous adipocytes and reverted deleterious effects caused by viral infection, including cytotoxicity and oxidative stress markers. Although the pseudovirus used for the experiments mimics cell entry, it does not present the pathogenic effects of a viral infection, and investigations using the actual virus should be conducted to confirm the findings. On the positive side, the PV succeeded in mimicking cell entry and presents a very low risk to the researchers. Nevertheless, as a limitation, it is important to mention that it is possible that parameters such as cytotoxicity assays might be different in comparison with a viral infection by SARS-CoV-2 due to the consequent effects

of the infection per se in the cells. The present findings open precedent to new investigations focusing on viral and/or infectious pathologies, which can be conducted with pseudoviruses of different viral agents, resulting in rapid and assertive results.

As a limitation, although irisin plays an important role in inflammation modulation, the present study aimed at investigating irisin's relation to SARS-CoV-2 pseudovirus cell entry and its effects on oxidative stress and cytotoxicity. Future studies should incorporate inflammatory cytokine analyses to determine whether irisin's protective effects are mediated through adipose tissue inflammation reduction. As future perspectives, it is mandatory to better understand how irisin affects other infectious diseases, considering that investigations before the appearance of SARS-CoV-2 enabled a more rapid and assertive action once the pandemic began. This also directs us towards the investigation of the beneficial effects of constant physical exercise and the consequent higher production of irisin, and how such measures can impact the clinical outcome of infections. In addition, pre- and post-viral infection treatments with irisin should contribute to revealing if irisin is active in both scenarios and if it interferes with viral replication besides cell entry. The production of an irisin analog is a reality that must be considered in the will to possess more options against the aggravation of COVID-19, and potentially against other pathologies.

Conclusion

The use of the SARS-CoV-2 pseudovirus was effective as a model to mimic viral infection *in vitro*, and irisin treatment before exposure to the PV could significantly decrease cell entry in human subcutaneous adipocytes. Irisin treatment was also effective in reverting deleterious effects evoked by viral infection, such as cytotoxicity and oxidative stress. Together, these results indicate irisin as a target against COVID-19 and the aggravation of symptoms, and raise the hypothesis of investigating irisin against other viral pathologies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work reported.

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Author contribution statement

MTS was responsible for data curation, investigation, methodology, project administration, writing the original draft, and reviewing and editing. EMV contributed to data curation, investigation, methodology, writing the original draft, and reviewing and editing. MO, RMCO, and VVP were involved in data

curation, investigation, and reviewing and editing. LSM, HPT, BMGM, ID, PBR, MBB, MML, GBL, MSM, PHS, CRC, and CFP contributed to data curation and reviewing and editing. MES handled data curation, validation, and reviewing and editing. CRN was responsible for funding acquisition, methodology, supervision, validation, and reviewing and editing.

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