Resveratrol acts as an antioxidant in leukocytes of patients with diabetes mellitus through the AMPK signaling pathway

O Resveratrol atua como antioxidante em leucócitos de pacientes com diabetes mellitus por meio da via de sinalização AMPK

El Resveratrol actúa como antioxidante en leucocitos de pacientes con diabetes mellitus a través de la vía de señalización AMPK

ABSTRACT
Diabetes is characterized by disturbances in serum glucose levels caused by insulin resistance, with consequent high blood glucose levels. Permanently increased blood glucose tends to lead to an increase in reactive oxygen species (ROS). Therefore, one of the mechanisms to contain this process is to consider antioxidant treatment. Within this context, resveratrol (RSV) stands out. Among the pathways of action of this compound, AMPK plays a fundamental role in regulating its antioxidant activity. The objective of the work aims to verify the antioxidant profile of RSV, through the AMPK pathway, in leukocytes of patients with type 2 diabetes mellitus (DM2) between 60 and 80 years old. 12 individuals were selected, divided into two groups: control group (n=6) and experimental group (n=6). After blood collection and leukocyte separation, the luminol-dependent chemiluminescence assay was performed. To analyze the
involvement of the AMPK pathway, the inhibitor compound C was used. The result obtained showed that there was an antioxidant behavior of AMPK in cells stimulated with peroxide and treated with RSV, this in turn decreased the production of ROS in leukocytes of all samples, but this effect was less significant in DM2 patients. This oxidation, which is more pronounced in diabetics, can lead to target organ damage. As RSV played an important role in reducing ROS, we concluded that it is an important compound in preventing target organ damage in patients with this comorbidity, acting through the AMPK pathway.

**Keywords:** Resveratrol, AMPK, diabetes mellitus 2.

**RESUMO**
A diabetes é caracterizada por perturbações nos níveis séricos de glucose causadas pela resistência à insulina, com consequentes níveis elevados de glucose no sangue. O aumento permanente da glicose no sangue tende a levar a um aumento das espécies reactivas de oxigénio (ROS). Por isso, um dos mecanismos para conter esse processo é considerar o tratamento com antioxidantes. Dentro desse contexto, destaca-se o resveratrol (RSV). Dentre as vias de ação deste composto, a AMPK desempenha um papel fundamental na regulação de sua atividade antioxidante. O objetivo deste estudo foi verificar o perfil antioxidante do RSV, através da via da AMPK, em leucócitos de pacientes com diabetes mellitus tipo 2 (DM2) com idade entre 60 e 80 anos. Foram selecionados 12 indivíduos, divididos em dois grupos: um grupo de controlo (n=6) e um grupo experimental (n=6). Após a coleta de sangue e separação dos leucócitos, foi realizado o ensaio de quimioluminescência dependente de luminol. Para analisar o envolvimento da via da AMPK, foi utilizado o inibidor composto C. O resultado obtido mostrou que houve comportamento antioxidante da AMPK nas células estimuladas com peróxido e tratadas com RSV, que por sua vez diminuiu a produção de ROS nos leucócitos de todas as amostras, mas esse efeito foi menos significativo nos pacientes com DM2. Esta oxidação, que é mais acentuada nos diabéticos, pode causar danos nos órgãos-alvo. Como o RSV desempenhou um papel importante na redução das ERO, concluímos que é um composto importante na prevenção de danos a órgãos-alvo em pacientes com essa comorbidade, atuando pela via da AMPK.

**Palavras-chave:** Resveratrol, AMPK, diabetes mellitus 2.

**RESUMEN**
La diabetes se caracteriza por alteraciones de los niveles séricos de glucosa causadas por la resistencia a la insulina, con los consiguientes niveles elevados de glucosa en sangre. El aumento permanente de la glucemia tiende a provocar un aumento de las especies reactivas del oxígeno (ROS). Por ello, uno de los mecanismos para contener este proceso es considerar el tratamiento con antioxidantes. En este contexto, destaca el resveratrol (RSV). Entre las vías de acción de este compuesto, la AMPK juega un papel clave en la regulación de su actividad antioxidante. El objetivo de este estudio fue verificar el perfil antioxidante del RSV, a través de la vía AMPK, en leucocitos de pacientes con diabetes mellitus tipo 2 (DM2) con edades comprendidas entre 60 y 80 años. Se seleccionaron 12 individuos y se dividieron en dos grupos: un grupo de control (n=6) y un grupo experimental (n=6). Tras la extracción de sangre y la separación de los leucocitos, se realizó el ensayo de quimioluminiscencia dependiente del luminol. Se utilizó el inhibidor compuesto C para analizar la implicación de la vía AMPK. Los resultados mostraron que existía un comportamiento antioxidante de la AMPK en las células estimuladas con peróxido y tratadas con RSV, lo que a su vez reducía la producción de ROS en los leucocitos de todas las muestras, pero este efecto era menos significativo en los pacientes con DM2. Esta oxidación, que es más pronunciada en los diabéticos, puede causar daños en los
órganos diana. Dado que el RSV desempeñó un papel importante en la reducción de ROS, concluimos que es un compuesto importante en la prevención de daños en órganos diana en pacientes con esta comorbilidad, actuando a través de la vía AMPK.

**Palabras clave:** Resveratrol, AMPK, diabetes mellitus 2.

### 1 INTRODUCTION

With increasing rates of obesity, poor diet, lack of physical activity, among other factors, there is a concomitant increase in adults with diabetes. This increase becomes extremely relevant for the health system, thus requiring improvements in early diagnosis, increased access to quality care for diabetes control and the development of strategies to promote healthy lifestyles and nutrition (Cardoso et al. 2024). Permanently increased glycemia in diabetes tends to worsen this imbalance between the production of pro-oxidant and antioxidant species, the latter being the most impaired, which, in the long term, leads to an increase in reactive oxygen species (ROS) (Poblete et al. 2018).

New strategies to improve the quality of life of these patients who already live with the disease are always studied and many can be really effective. In this context, the use of antioxidant compounds for diabetic patients has been widely disseminated, with promising results for improving the redox status of these patients (Dos Santos et al. 2023; Ferreira-Fiochi et al. 2023; Huang et al. 2020).

Studies involving diabetic individuals demonstrate that resveratrol induces effects that can contribute to the protection of β cells, in addition to inhibiting the action of cytokines and attenuating oxidative damage to pancreatic tissue (Huang et al. 2020). Furthermore, one of the targets in the treatment of diabetes is the AMPK (Adenosine Monophosphate-Activated Protein Kinase) signaling pathway, since this pathway is compromised in diabetes. Some medications used to treat DM2 are known to activate the AMPK signaling pathway, such as metformin. Some plant-derived products are also known to activate the AMPK pathway, such as resveratrol (Joshi et al. 2018).

The pharmacological activation of AMPK in insulin-resistant rodents led to an improvement in lipid profile, blood glucose homeostasis and blood pressure. Consequently, AMPK may be a new target that can be explored in the treatment of DM2 (Saltiel & Kahn, 2001). At a cellular level, high glucose levels negatively affect the function of several cell populations, including leukocytes (Burke et al. 2004). Diabetic cardiac injury is characterized by increased leukocyte mobilization, secretion of pro-inflammatory cytokines and oxidative...
stress (Yu et al. 2011). Thus, disturbances in metabolic and inflammatory signaling pathways during the progression of diabetes are associated with changes in leukocyte activation and increased chronic inflammation (Hernandez et al. 2013).

Given this, studies are still needed to understand the effect of RSV on the leukocytes of diabetic patients, in order to clarify whether the AMPK signaling pathway can be a target in treatment with a consequent reduction in ROS production in these cells, as is the case with objective of this study.

2 METHODOLOGY
2.1 DONOR SELECTION

The study is part of a project by the Laboratory of Biochemistry of Aging and Related Diseases, which seeks to understand the antioxidant mechanisms of resveratrol. Due to this, it has already been approved by the UFMG Research Ethics Committee (CAAE: 33842420.4.0000.5149). 12 individuals aged between 40 and 60 were selected, men and women with type 2 diabetes. The samples were provided by our partner, the laboratory In Vitro Cells Pesquisa Toxicológica Ltda. After collection, the blood was taken to the Biochemistry of Aging and Related Diseases laboratory at the Federal University of Minas Gerais (UFMG), at the Institute of Biological Sciences (ICB), the institution responsible for the custody, confidentiality of results and manipulation.

The individuals were divided into 2 groups: Control group (n=6) in which they did not have a diagnosis of Type 2 Diabetes Mellitus and an experimental group (n=6) in which they had a diagnosis of Type 2 Diabetes Mellitus. Regarding the inclusion criteria for the control group were accepted: individuals of both sexes and aged 60 to 80 years, within the normal range of body mass index (18.5 - 24.99 kg/m2), normal blood glucose (below 99 mg/dL), normal triglycerides (<150 mg/dL), normal serum creatine (0.5 - 1.5 mg/dL for men and 0.6 - 1.2 mg/dL for women) and no cardiovascular complications. Regarding the inclusion criteria for the experimental group (DM 2), there are: individuals of both sexes and aged 60 to 80, previously diagnosed with DM 2 by the responsible doctor. Those who are able to participate signed a Free and Informed Consent Form (TCLE).

2.2 OBTAINING HUMAN LEUKOCYTES

In short, 4ml of heparinized blood was added to 3ml of Leucopaque ® gradient in glass tubes. After centrifugation at 2200 rpm for 15 minutes, two distinct phases separated by two interphase rings were obtained. The plasma, corresponding to the first phase formed after
centrifugation, was discarded. The leukocytes were placed in a siliconized tube, which had its volume filled with PBS (pH 7.3) for two washing sessions at 1500 rpm for 10 minutes each. After two washes, the cells were resuspended in 1.0 mL of RPMI culture medium. The final volume will be adjusted to 1x10^7 cells/mL (Franco et al. 2023). Furthermore, viability during the experiments was monitored by the Trypan Blue assay.

2.3 QUANTITATIVE ROS ANALYSIS

For the quantitative evaluation of Reactive Oxygen Species and the antioxidant effect of resveratrol, the luminol-dependent Chemiluminescence assay was used. For this, 1x10^6 cells were added to a tube along with 500μL of luminol and 100μL of PBS buffer or resveratrol (5μM). The groups were: (1) Control, (2) Resveratrol only, (3) 150 μM hydrogen peroxide – H₂O₂ and (4) H₂O₂ + RSV. The reading was performed immediately in 10-minute runs with Relative Light Units/minute (RLU/min) values.

2.4 ASSESSMENT OF THE AMPK ANTIOXIDANT PATHWAY

The AMPK signaling pathway inhibitor, Compound C, was used at the concentration previously established in the laboratory (20 μM) using a dose-response curve (Caldeira et al. 2021; Franco et al. 2023). The assay used was also luminol-dependent chemiluminescence. The same four groups described previously were preincubated with the inhibitor for 30 min. Then, the samples were centrifuged at 1500 rpm for 10 min and treatments were carried out followed by readings on the equipment.

2.5 STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation. All data were analyzed using the GraphPad Prism 7.0 software using the ANOVA test followed by the Dunnett test. With *p<0.05 considered significant.

3 RESULTS

The first stage of the study was to better characterize the profile of donors with DM2, such as blood glucose levels at the time of collection, and whether the patient was taking any medication. The results are described in Table 1. In short, the patients were over 60 years old and are routinely monitored by health professionals, with some using some type of hypoglycemic medication. It is worth noting that in the control group, fasting blood glucose was less than 100 mg/dL and the patients did not have DM2 or any other disease.
Table 1. Profile of the experimental group, patients with DM2. Glycated hemoglobin had been measured up to three months before blood collection.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years old)</th>
<th>Blood glucose at collection (mg/dL)</th>
<th>Glycated Hemoglobin (%)</th>
<th>Year of diagnosis</th>
<th>Insulin use</th>
<th>Insulin dosage (Units)</th>
<th>Use of another medication</th>
<th>Other chronic disease</th>
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<tr>
<td>1</td>
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<td>6.5</td>
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<tr>
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<td>119</td>
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<tr>
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<td>2003</td>
<td>No</td>
<td>-</td>
<td>Metformin 500mg</td>
<td>No</td>
</tr>
</tbody>
</table>

Source: Elaborated by the authors.

The cell viability under treatments was assessed using trypan blue dye. Overall, Figure 1 shows that neither treatment affected cell viability. Leukocytes remained with viability values above 75% for all groups evaluated. According to ISO 10993-5, a reduction in cell viability by more than 30% (i.e. viability below 70%) is considered a cytotoxic effect. Therefore, the treatment conditions are in accordance with international standards for study (International organization for standardization, 2009).

Once the viability of the cells was proven, the antioxidant profile of RSV was then evaluated using the chemiluminescence assay. The data in Figure 2 shows that in both groups there was a significant increase in ROS production in cells exposed to H$_2$O$_2$ (C+) compared to baseline (C-). This was expected, since H$_2$O$_2$ induces an oxidizing environment. H$_2$O$_2$+RSV (RSV+) treatment was able to reduce ROS levels in both groups, when compared to leukocytes stimulated with H$_2$O$_2$ (C+). It is worth noting that in the diabetic group, despite the significant difference, ROS values are 10x higher in RSV+ compared to the control group. In other words, resveratrol had a lower antioxidant capacity in the leukocytes of DM2 patients.
Figure 1. Viability of leukocytes using the trypan blue assay. Leukocytes (5x10^3/well) were added to 96-well plates and incubated for 1h with RSV (5µM) and/or H_2O_2 (150µM). The results were expressed as the mean percentage of viable cells ±SD. The experiments were carried out in triplicate/well (n=6/group). The cells maintained viability > 70% and there was no significant difference between the groups.

Source: Elaborated by the authors.

Figure 2. Evaluation of the effect of resveratrol on ROS production in human leukocytes with DM2. ROS generation was expressed as RLU/min in an average reading of 10 minutes. Being *(p<0.05) and ***(p<0.001). The results were significant using the Kolmogorov–Smirnov normality test, one-way ANOVA and Dunnett's post-test (n=6/group).

Source: Elaborated by the authors.

Once it was demonstrated that resveratrol’s antioxidant effect was reduced in leukocytes from DM2 patients, our next step was to evaluate one of the main signaling pathways that seem to modulate the antioxidant action of this polyphenol - AMPK. The result is shown in Figure 3. When we added the inhibitors and H_2O_2 (C+I), there was an increase in ROS production when we compared only leukocytes stimulated with H_2O_2 (C+). This shows that there is an antioxidant role for this pathway in both experimental groups.
When adding inhibitors $+ \text{H}_2\text{O}_2+\text{RSV}$ (RSV+) and comparing with $\text{H}_2\text{O}_2+\text{RSV}$ (RSV+), a different profile is observed in each group. In the control group, there was no difference between treatments. In other words, RSV does not act through this route. While in the DM2 group, a difference is observed between these last two bars. This indicates that the polyphenol acts through the AMPK cellular signaling pathway, being responsible for its antioxidant effect still observed in this group.

Figure 3. Assessment of AMPK on the effect of resveratrol on ROS production in human leukocytes with DM2. ROS generation was expressed as RLU/min at an average reading of 10 minutes. Being ***(p<0.001) and ****(p<0.0001). The results were significant by the Kolmogorov-Smirnov normality test, one-way ANOVA and Dunnett’s post-test (n=6/group).

Source: Elaborated by the authors.

4 DISCUSSION

There are several complications described regarding diabetes mellitus, such as hypoxia, increased C-Reactive Protein (CRP) inducing inflammation, increased intracellular formation of advanced glycation end products (AGE), in addition to the association with other chronic diseases, such as hypertension. All of these phenomena result from a single process induced by
hyperglycemia: the production of reactive species. The most described in the case of DM are the overproduction of superoxide by the mitochondrial electron transport chain. Superoxide is the initial oxygen free radical formed by mitochondria, which is then converted into other more reactive species that can damage cells in various ways (Giacco et al. 2010).

Korshunov et al. (1997) had already demonstrated that in endothelial cells in culture, intracellular hyperglycemia increased the voltage across the mitochondrial membrane, increasing the production of ROS. Yu et al. (2006) also observed that mitochondria exposed to a high concentration of glucose also produced a high amount of these reactive species.

Studies of resveratrol in diabetes are mainly in vitro studies and animal model experiments, and it can clearly be seen that resveratrol has multiple protective effects against diabetes. Baur et al. (2006) observed that resveratrol treatment can improve insulin sensitivity in diabetic mice. Insulin resistance was monitored using a homeostasis assessment model, and the results further confirmed that resveratrol can improve insulin resistance through activation of the AMPK signaling pathway.

The increase in glucose uptake induced by resveratrol also depends mainly on the stimulation of GLUT4 expression and translocation (Tan et al. 2012). Studies have shown that insulin-resistant mice (T2DM) fed resveratrol significantly increased glucose uptake by increasing the level of GLUT4 (Do et al. 2012). Resveratrol combined with insulin has been reported to improve GLUT4 translocation and thus glucose uptake in diabetic rats more than resveratrol or insulin treatment alone (Ruderman et al. 2004). Resveratrol also increases AMPK phosphorylation by activating or binding to an estrogen receptor, further increasing GLUT4 expression and translocation, thereby affecting glucose uptake by skeletal muscle cells (Rogers et al. 2009).

Numerous studies have demonstrated that resveratrol can activate AMPK, and AMPK participates in the antioxidant response to stress by interacting with sirtuin protein 1 (SIRT1) or jointly regulating numerous downstream effector molecules. AMPK alone or in conjunction with SIRT1 stimulates downstream PGC-1α activation and alleviates damage to endothelial function and other T2D-related complications (Ahmad et al 2020). In our study, we observed that in DM2, resveratrol was able to activate this pathway, having an antioxidant effect on leukocytes in both experimental groups.

Furthermore, it is described that AMPK activates the transcription factor FOXOs through phosphorylation with SIRT1, triggering the expression of antioxidant enzymes to restore glycemic homeostasis (Canto et al. 2009). Resveratrol also indirectly increases PPARα activity by activating AMPK, SIRT1, and PGC-1α, thereby inhibiting NF-κB (an important
inflammatory pathway) and attenuating oxidative stress and inflammation (Guellich et al. 2007).

These literature data indicate that resveratrol can, in fact, activate multiple pathways in addition to AMPK, culminating in a more effective response. For example, there is crosstalk between the AMPK-SIRT1-PGC-1α, AMPK-SIRT1-FOXOs and AMPK-SIRT1-PPARα pathways to reduce the oxidative stress present in DM2 (Poynter et al. 1998; Hayashida et al. 2010).

5 CONCLUSION

Resveratrol was shown to be an effective antioxidant in both groups to reduce oxidative stress. However, it is more effective in elderly people who do not have DM2. Since one of the consequences of this disease is an exacerbated increase in the production of ROS. The AMPK cellular signaling pathway is activated by resveratrol, being responsible for several physiological responses, including the reduction of oxidative stress. In leukocytes of DM2 patients, this pathway was shown to be active in combating the reduction of ROS. With more research into this pathway, AMPK could become a target protein for future therapies and improvement in DM2.
REFERENCES


