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ESCULETIN EFFECTS ON MICE-IMMORTALIZED MESANGIAL CELLS SUBMITTED TO HIGH-GLUCOSE MEDIA

EFEITOS DA ESCULETINA EM CÉLULAS MESANGIAIS IMORTALIZADAS DE CAMUNDONGOS SUBMETIDAS A MEIO COM ALTA CONCENTRAÇÃO DE GLICOSE

EFECTOS DE LA ESCULETINA EN CÉLULAS MESANGIALES INMORTALIZADAS DE RATÓN SOMETIDAS A MEDIO CON ALTA CONCENTRACIÓN DE GLUCOSA

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ABSTRACT

Introduction: Diabetes mellitus (DM) has been a major research focus due to its high prevalence and mortality in Brazil and worldwide. Characterized by hyperglycemia, DM leads to comorbidities like atherosclerosis, retinopathy, neuropathy, and nephropathy. Its pathophysiology involves disrupted enzyme and receptor functions, inflammation, and increased reactive oxygen species (ROS). Esculetin, a coumarin-derived polyphenol, has shown potent antioxidant activity, improving lipid profiles and reducing proinflammatory cytokine synthesis.

Objective: To study the effect of esculetin on immortalized mouse mesangial cells subjected to high-glucose medium. Methods: Immortalized mesangial cells from mice (MiMCs) were cultured in culture plates with DMEM/F12 supplemented with 5% fetal bovine serum. The cells were subsequently allocated into four groups: NG (control, D-glucose 6.7 mM)); NG+ESCt (control treated with esculetin hydrate 10, 25, 50, 100, or 200 µg/mL); HG (high glucose, D-glucose 30 mM); and HG+ESCt (high glucose treated with esculetin hydrate 10, 25, 50, 100, or 200 µg/mL) for 24, 48, or 72 hours. In these groups, we evaluated the viability, cell proliferation, and antioxidant capacity of esculetin in addition to its effects on oxidative and nitrosative stress. Statistical analyses were performed with GraphPad Prism 9.0 software. The results are presented as the means ± SEs, with $P < 0.05$ indicating statistical significance.

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Results: The DPPH test revealed the antioxidant effect of esculetin. ROS production was greater in the HG group than in the NG group, and TBARS production was lower in the treated groups than in the control groups. There was an increase in the amount of catalase enzyme in the HG group compared with the NG group, a decrease in the treated groups compared with the control groups, and increased activity of SOD and GPx enzymes in the ESCt groups.

Conclusion: Our data suggest that esculetin reduces oxidative stress in this experimental model. Future studies may be promising in identifying esculetin as an effective intervention for the prevention and treatment of DM.

Keywords: Esculetin. Mesangial Cells. Diabetes Mellitus. Oxidative Stress. Nitrosative Stress.

RESUMO

Introdução: O diabetes mellitus (DM) tem sido um importante foco de pesquisa devido à sua alta prevalência e mortalidade no Brasil e no mundo. Caracterizado pela hiperglicemia, o DM leva a comorbidades como aterosclerose, retinopatia, neuropatia e nefropatia. Sua fisiopatologia envolve alterações no funcionamento de enzimas e receptores, inflamação e aumento das espécies reativas de oxigênio (ERO). A esculetina, um polifenol derivado da cumarina, tem demonstrado potente atividade antioxidante, melhorando o perfil lipídico e reduzindo a síntese de citocinas pró-inflamatórias.

Objetivo: Estudar o efeito da esculetina em células mesangiais imortalizadas de camundongos submetidas a meio com alta concentração de glicose.

Métodos: Células mesangiais imortalizadas de camundongos (MiMCs) foram cultivadas em placas de cultura contendo meio DMEM/F12 suplementado com 5% de soro fetal bovino. Posteriormente, as células foram distribuídas em quatro grupos: NG (controle, D-glicose 6,7 mM); NG+ESCt (controle tratado com esculetina hidratada 10, 25, 50, 100 ou 200 µg/mL); HG (alta glicose, D-glicose 30 mM); e HG+ESCt (alta glicose tratada com esculetina hidratada 10, 25, 50, 100 ou 200 µg/mL) por 24, 48 ou 72 horas. Nesses grupos, foram avaliadas a viabilidade celular, a proliferação celular e a capacidade antioxidante da esculetina, além de seus efeitos sobre o estresse oxidativo e nitrosativo. As análises estatísticas foram realizadas com o software GraphPad Prism 9.0. Os resultados são apresentados como média ± erro padrão (EP), sendo $P < 0,05$ considerado estatisticamente significativo.

Resultados: O teste DPPH revelou o efeito antioxidante da esculetina. A produção de ERO foi maior no grupo HG em comparação ao grupo NG, e a produção de TBARS foi menor nos grupos tratados em relação aos grupos controle. Observou-se aumento da enzima catalase no grupo HG em comparação ao grupo NG, redução nos grupos tratados em relação aos controles e aumento da atividade das enzimas SOD e GPx nos grupos ESCt.

Conclusão: Nossos dados sugerem que a esculetina reduz o estresse oxidativo neste modelo experimental. Estudos futuros podem ser promissores para identificar a esculetina como uma intervenção eficaz na prevenção e no tratamento do DM.

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Palavras-chave: Esculetina. Células Mesangiais. Diabetes Mellitus. Estresse Oxidativo. Estresse Nitrosativo.

RESUMEN

Introducción: La diabetes mellitus (DM) ha sido un importante foco de investigación debido a su alta prevalencia y mortalidad en Brasil y a nivel mundial. Caracterizada por la hiperglucemia, la DM conduce a comorbilidades como aterosclerosis, retinopatía, neuropatía y nefropatía. Su fisiopatología implica alteraciones en la función de enzimas y receptores, inflamación y aumento de especies reactivas de oxígeno (ERO). La esculetina, un polifenol derivado de la cumarina, ha demostrado una potente actividad antioxidante, mejorando el perfil lipídico y reduciendo la síntesis de citocinas proinflamatorias.

Objetivo: Estudiar el efecto de la esculetina en células mesangiales inmortalizadas de ratón sometidas a medio con alta concentración de glucosa.

Métodos: Células mesangiales inmortalizadas de ratón (MiMCs) fueron cultivadas en placas con medio DMEM/F12 suplementado con 5% de suero fetal bovino. Posteriormente, las células se distribuyeron en cuatro grupos: NG (control, D-glucosa 6,7 mM); NG+ESCt (control tratado con esculetina hidratada 10, 25, 50, 100 o 200 µg/mL); HG (alta glucosa, D-glucosa 30 mM); y HG+ESCt (alta glucosa tratada con esculetina hidratada 10, 25, 50, 100 o 200 µg/mL) durante 24, 48 o 72 horas. En estos grupos se evaluaron la viabilidad celular, la proliferación celular y la capacidad antioxidante de la esculetina, además de sus efectos sobre el estrés oxidativo y nitrosativo. Los análisis estadísticos se realizaron con el software GraphPad Prism 9.0. Los resultados se presentan como media ± error estándar (EE), considerándose significativo $P < 0,05$.

Resultados: La prueba DPPH reveló el efecto antioxidante de la esculetina. La producción de ERO fue mayor en el grupo HG en comparación con el grupo NG, y la producción de TBARS fue menor en los grupos tratados respecto a los grupos control. Se observó un aumento de la enzima catalasa en el grupo HG en comparación con el grupo NG, una disminución en los grupos tratados respecto a los controles y un aumento en la actividad de las enzimas SOD y GPx en los grupos ESCt.

Conclusión: Nuestros datos sugieren que la esculetina reduce el estrés oxidativo en este modelo experimental. Estudios futuros pueden ser prometedores para identificar la esculetina como una intervención eficaz en la prevención y el tratamiento de la DM.

Palabras clave: Esculetina. Células Mesangiales. Diabetes Mellitus. Estrés Oxidativo. Estrés Nitrosativo.

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1 BACKGROUND

Diabetes mellitus (DM) is a chronic condition characterized by hyperglycemia. The worldwide prevalence of the disease in 2021 was 537 million people, a large increase compared with the 108 million in 1980 (1). In 2019, there were two million deaths from diabetes worldwide (2). DM patients cannot efficiently capture blood glucose and remain in a state of metabolic fasting, with great catabolic activity to synthesize ATP (3). This disorder, classified into two different types, represents a functional defect of the hormone insulin: either it is not synthesized (Type 1 Diabetes; DM1), or its action is impaired in the body, and consequently, glucose is not internalized in the tissues (Type 2 Diabetes; DM2) (4).

Hyperglycemia, if maintained for a long period, induces damage in many body systems, especially in nerves and blood vessels (5). The endothelial dysfunction present in DM results in the formation of peroxynitrite ($ONOO^-$), an oxidizing agent that causes lipoperoxidation, tyrosine nitration, reduction of cellular antioxidants, induction of apoptosis by breaking down nucleic acids and deactivation of the enzyme nitric oxide synthase (NOS) by the oxidation of tetrahydrobiopterin (6). $ONOO^-$ activates some enzymes with repair mechanisms, such as poly (ADP–ribose) polymerase-1 (PARP), which functions in DNA repair. On the other hand, it inactivates glyceraldehyde phosphate dehydrogenase (GAPDH), leading glucose to alternative pathways, increasing the levels of polyol and hexosamine and activating protein kinase C (PKC) (7).

Glucose oxidation by such alternative pathways gives rise to many procoagulants and pro-inflammatories, particularly reactive oxygen species (ROS), in which the superoxide anion (O2•-) is obtained via the electron transport chain from mitochondria as a byproduct of oxidative phosphorylation (8). The increased production of ROS due to hyperglycemia results in the nonenzymatic glycation of lipids and proteins, which results in the formation of end products of advanced glycosylation (AGEs) (9). These products interact with specific receptors (RAGEs) that contribute, in part, to the micro- and macrovascular pathophysiology of DM by reducing the production of nitric oxide (NO) and increasing the synthesis of ROS and proinflammatory enzymes (10). In other words, when the production of these ROS exceeds the antioxidant defense, oxidative stress can irreversibly modify some macromolecules, causing complications for the patient (10). A consequence of these complications is the loss of renal function in the phase known as end-stage renal disease (ESRD), the main cause of which is diabetic nephropathy (DN)(11). However, hyperglycemia alone is not enough to cause nephropathy (12), as genetic susceptibility and environmental

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agents are important factors for the onset and progression of this complication (13). Thus, the generation of ROS leads to the occurrence of oxidative stress, which can also be observed in the oxidation of the lipid layer of the cell membrane, resulting in increased levels of thiobarbituric acid reactive substances (TBARS) (11, 14). In the context of oxidative damage, the presence of the Nrf2 factor (nuclear factor erythroid 2-related factor) is responsible for the transcription of genes that encode antioxidant enzymes, in addition to acting in inflammatory modulation responses (15). Therefore, drugs that act on the activation of this gene may be promising in the treatment of DM, such as esculetin (ESCt) (16).

ESCt is a coumarin derivative with the chemical formula $C_{15}H_{16}O_9$; therefore, once it has five hydroxyls and an aromatic group in its composition (17), it is a polyphenol, which is considered a natural antioxidant and has therapeutic properties (18). When administered orally, it is metabolized by the microbiota to esculin (ESC) through a deglycosylation process (17). Both compounds, ESCt and ESC, have biological activity and can be found in northern hemisphere plants of the *Oleaceae* family, commonly from the genus *Fraxinus* (19), but can also occur in other more well-known southern hemisphere species, such as mugwort (*Artemisia capillaris*) and lemon (*Citrus limon*) (20). These derivatives may be potential future therapies for DM, since they can promote glycemic homeostasis through increasing insulin secretion (21) or through the uptake of circulating glucose (22). In addition, these compounds can help combat the development of comorbidities related to DM by improving the lipid profile (23), reducing the levels of proinflammatory cytokines (24) and exerting their antioxidant effects. There is also evidence that this substance reduces the accumulation of AGEs in the kidneys and decreases tubular damage (22, 23).

2 OBJECTIVE

The aim of this project was to study the effects of ESCt on oxidative and nitrosative stress in mesangial cells from mice subjected to high-glucose media.

3 METHODS

3.1 CELL CULTURE

We used immortalized mouse mesangial cells (MiMCs) acquired from the American Type Culture Collection (ATCC) and provided by the Nephrology Division, UNIFESP/EPM, which were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin (50 U/ml)/streptomycin (50 µg/ml) supplemented

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with F12 nutrients at a ratio of 3:1. All products were obtained from Gibco (Thermo Fisher; USA). The cells were kept in an incubator at 37°C with 5% CO2, and their growth was monitored every 48 hours when the culture medium was changed. When the cell monolayer became semiconfluent for lineage perpetuation, subculture was performed via a trypsin solution with 0.25% EDTA, followed by two washes with sterile phosphate-buffered saline (PBS). The cells were centrifuged at 1200 RPM for 5 minutes and then resuspended in 3 ml of DMEM supplemented as described and subcultured in plates. This process was repeated until the time of the experiments, when they were placed in culture plates of adequate size for each analysis.

3.2 DEFINITION OF GROUPS AND DOSE–RESPONSE TIMES

For the experiments, the cells were placed in culture plates and used when they reached a semiconfluency of 60–70%, when the culture medium was replaced with DMEM + 0.5% SBF, and treated according to the following groups:

- NG, control, cultured in normal concentrations of D-glucose (6.7 mM);
- NG+ESCs were cultured in normal concentrations of D-glucose (6.7 mM) or esculetin hydrate (Sigma Aldrich, Germany) at different concentrations (10, 25, 50, 100 or 200 µg/mL) for 24 h;
- HG, high glucose, cultured with a high concentration of D-glucose (30 mM, *i.e.*, 6.7 mM of medium plus 23.3 mM of D-glucose);
- HG+ESCs were cultured with high concentrations of D-glucose and esculetin hydrate (Sigma Aldrich, Germany) at different doses (10, 25, 50, 100 or 200 µg/mL) for 24 h.

The ESCt dose, as well as the cell treatment time, was established on the basis of the dose and time of response. The viability, cell proliferation and antioxidant capacity of the abovementioned groups were evaluated.

3.3 CELL VIABILITY AND PROLIFERATION

For these experiments, MiMCs were cultivated in 12-well cell culture plates at a concentration of $5x10^4$ cells per well, and after they reached 50% confluence, they were incubated with DMEM/F12 for a period of 24 hours for cell cycle synchronization in a humid atmosphere at 37°C and then treated according to the mentioned groups and time points and with the different concentrations of ESCt described. Then, the cells were trypsinized,

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centrifuged and resuspended in 1 mL of fresh medium, and viability was analyzed via a hemocytometer (Countess™ Automated Cell Counter; Invitrogen; USA) with 0.4% trypan blue dye. This dye penetrates the membrane of ruptured cells, giving them a purplish color. Viable cells do not acquire this stain. Analysis was performed by direct counting and pipetting 10 µL of trypan blue into the cell counter chamber.

Another method used to assess cell proliferation and viability is the methyl thiazole tetrazolium assay (MTT; Sigma–Aldrich; Germany), a colorimetric assay used to assess cellular metabolic activity. Under defined conditions, NADPH-dependent cellular oxidoreductases can reflect the number of viable cells present. These enzymes can reduce MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble purple form. After treatment, the cells were trypsinized and centrifuged; the supernatant was discarded, and the cells were resuspended in 1 mL of PBS and analyzed by MTT. The optical density (OD) was obtained at a wavelength of 570 nm in a microplate reader. The OD of the cells in the control group (NG) was assigned a value relative to 100, and the results were obtained from averages of three independent experiments performed in octuplicate. MTT assays were performed in the dark, as the reagents are sensitive to light.

3.4 ESCULETIN ANTIOXIDANT ACTIVITY

The ESCt antioxidant activity at the different concentrations described was determined via the 2,2-diphenyl-1-picryl-hydrazyl (DPPH)33 assay, which evaluates the ability of a substance to scavenge the DPPH free radical through the IC50 parameter, which represents the concentration of the material in question necessary to inhibit 50% of the DPPH radicals. It was read at 515 nm and converted to the percentage of antioxidant activity.

3.5 EVALUATION OF THE EFFECT OF ESCULETIN ON OXIDATIVE AND NITROSATIVE STRESS IN CELLS TREATED WITH HIGH GLUCOSE

Some components involved in oxidative and nitrosative stress were analyzed in MiMC distributed according to the groups described: NG, NG+ESCt, HG and HG+ESCt. With respect to the time of treatment and the dose of ESCt used, we analyzed the best dose and time according to the data obtained from the determination of its antioxidant capacity, as well as through the analysis of cell viability and proliferation.

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3.6 TBARS METHOD (THIOBARBITURIC ACID REACTIVE SUBSTANCES)

Malondialdehyde (MDA) is a natural lipid peroxidation product, a well-established mechanism of cell injury in plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxidation can be estimated via the TBARS method. Briefly, 250 µl of the cell suspension was combined with 500 µl of 30% trichloracetic acid, vortexed and centrifuged at 5000 × g for 15 min. Then, 500 µl of the acid-soluble supernatant was added to an equal volume of 1% thiobarbituric acid in 0.05 M NaOH, and the mixture was heated in a water bath at 95°C for 10 min. The absorbance of the developed pink chromophore was determined at 532 nm, and the values were corrected by the protein content measured in the cells (25).

3.7 INTRACELLULAR EVALUATION OF ROS (H2O2)

This was carried out via the use of the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma–Aldrich; Germany), which reacts with H2O2 and is converted into DCF. After treatment, the MiMCs were washed with PBS free of calcium and magnesium and incubated with DCFH-DA (9 µM) at 37°C for 30 min. The medium was removed, and the cells were washed 2x with PBS. The fluorescence was measured via a microplate reader (Synergy HT, Biotek, USA); the excitation was read at 480 nm, and the emission was detected at 520 nm. The relative production of ROS was expressed as the mean fluorescence intensity (26).

3.8 DETERMINATION OF SUPEROXIDE ANION (O2•-)

The level of superoxide anion was detected via nitroblue tetrazolium reagent (NBT, AMRESCO, Biosystems, USA); the anion reduces NBT to formazan at pH 7.4 at room temperature, and formazan generation is followed by spectrophotometry by changing the pale yellow color of NBT to a purple color of formazan at an optical density of 560 nm (27).

3.9 DETERMINATION OF ANTIOXIDANTS: CATALASE, GLUTATHIONE, AND SUPEROXIDE DISMUTASE (SOD)

For catalase determination, we used the EnzyChromTM Catalase Assay Kit (ECAT-100) from BioAssay Systems (Hayward, USA). For glutathione, we used a Glutathione Fluorometric Assay Kit (#K251-100; BioVision Inc., Boulevard, USA). For SOD, we used the Superoxide Dismutase Activity Assay Kit (ab65354; Abcam, UK).

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3.10 DETERMINATION OF NITRIC OXIDE (NO)

The cells were treated according to the groups, and 10 µL of culture medium was collected from each plate for determination of NO by the chemiluminescence method, which is considered the gold standard in the determination of this molecule, using a nitric oxide analyzer device (NOATM 280, Sievers Instruments, Inc., Boulder, USA)37. After the culture medium was collected, the cells were lysed with RIPA buffer in the presence of a protease inhibitor for the measurement of intracellular proteins via a BCA protein kit (ab102536, Abcam, United Kingdom) (modified Lowry method) for normalization of the NO values (28).

3.11 STATISTICAL ANALYSIS

The results are expressed as the mean ± standard deviation (SD). For parametric data, statistical analysis was conducted via ordinary one-way ANOVA followed by Tukey's multiple comparisons test. Nonparametric data were analyzed via the Kruskal–Wallis test with Dunn's multiple comparisons. All the statistical analyses were performed via GraphPad Prism 9.0 (GraphPad Software Inc., USA). An F test was conducted in Python 3.8 via the 'scipy.stats' package to compare the residual variances of the dose–response curves. A p value of < 0.05 was considered statistically significant for all analyses.

4 RESULTS

4.1 DPPH

The antioxidant activity of esculetin was measured via the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The antioxidant activity was determined by the concentration required to reduce 50% of the DPPH free radical (IC_{50}), and the results were fitted via nonlinear regression models, resulting in dose–response curves for each compound. The initial concentrations of the standard solutions of esculetin and its control, gallic acid, were 1 mg/mL, with diluted standards prepared at 0.1 mg/mL and 0.01 mg/mL for both.

An F test was used to compare the dose–response curves obtained for gallic acid and esculetin, considering both the IC_{50} values and the slopes of the curves. The analysis of the F test results revealed an F value of 0.402 and a P value of 0.0035, indicating a significant difference between the dose–response curves of the two compounds (Figure 1).

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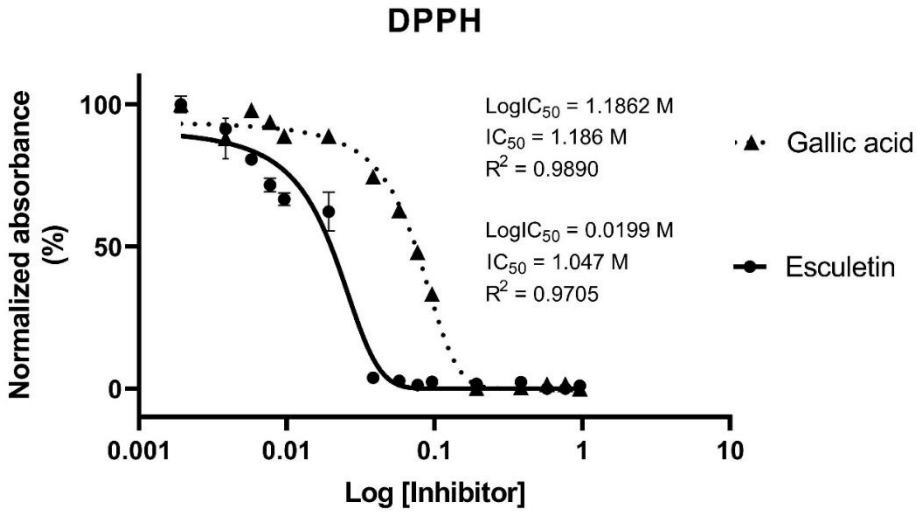
Figure 1

DPPH assay to analyze dose-responsedose–response curves for esculetin (test) and its control, gallic acid. F- value: 0.042. P- value: 0.0035



4.2 CELL VIABILITY

An assay with different concentrations of DMSO (1.25%, 2.5%, and 5%) revealed that the cell viability in the 1.25% DMSO group was the closest to that in the control group (without DMSO). Therefore, we chose 1.25% DMSO for the treatments, as it minimally reduces cell viability while efficiently diluting esculetin. In the assays with different concentrations of esculetin (100 µg/mL, 250 µg/mL, and 500 µg/mL), the group with 100 µg/mL esculetin had the greatest cell viability compared with the control. Therefore, we chose a concentration of 100 µg/mL esculetin for the treatments.

4.3 EVALUATION OF ROS

The results indicated that, compared with the NG group, the HG group presented greater ROS production (1199 ± 355.3 *vs.* 863.8 ± 323.6; $p < 0.05$), suggesting a parallel relationship with *diabetes mellitus*. However, the groups treated with esculetin showed a significant reduction in the production of these reactive oxygen species compared with their respective controls (NGE 133.5 ± 195.7 *vs.* 863.8 ± 323.6; $p < 0.0001$ and HGE 117.9 ± 234.5 *vs.* 1199 ± 355.3 $p < 0.0001$), demonstrating the antioxidant power of the substance under study (Figure 2).

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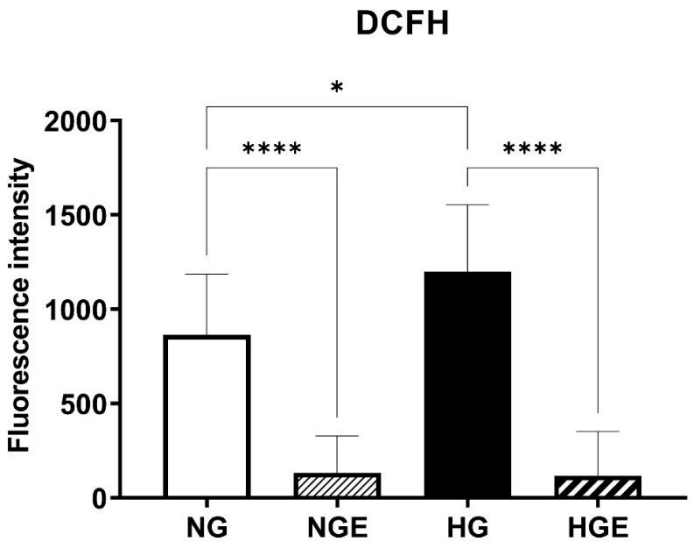
Figure 2

*DCFH assay to analyze ROS production. NG = normal glucose (6.7 mM); NGE = normal glucose (6.7 mM) + esculetin (100 µg/mL); HG = high glucose (30 mM); HGE = high glucose (30 mM) + esculetin (100 µg/mL). n = 12-14. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. * p < 0.05; **** p < 0.0001*



4.4 TBARS

The test revealed greater TBARS production in the HG group than in the NG control group, indicating increased lipid peroxidation due to oxidative stress in the high-glucose group (129.6 ± 46.05 *vs.* 41.93 ± 22.70; $p < 0.05$). Additionally, high glucose combined with esculetin significantly decreased the TBARS levels (56.77 ± 17.03 *vs.* 129.6 ± 46.05; $p < 0.05$), indicating lower MDA production in the treated groups (Figure 3).

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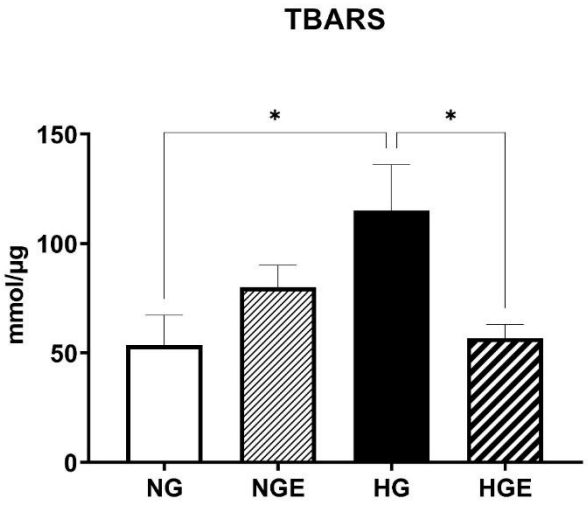
Figure 3

*TBARS assay to analyze lipid peroxidation. NG = normal glucose (6.7 mM); NGE = normal glucose (6.7 mM) + esculetin (100 µg/mL); HG = high glucose (30 mM); HGE = high glucose (30 mM) + esculetin (100 µg/mL). n = 7--8. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. * p < 0.05*



4.5 DETERMINATION OF ANTIOXIDANTS

a. Catalase

Compared with those in the normal glucose group, catalase levels were increased in the high glucose group (6.70 ± 1.59 *vs.* 4.57 ± 0.51; *p* = 0.0013). On the other hand, groups that were treated with esculetin presented decreased levels of these enzymes compared with their respective controls (NGE 2.38 ± 0.73 *vs.* 4.57 0.51; *p* = 0.0005 and HGE 1.84 ± 0.72 *vs.* 6.70 ± 1.59; *p* < 0.0001) (Figure 4).

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Figure 3

*Catalase levels. NG = normal glucose (6.7 mM); NGE = normal glucose (6.7 mM) + esculetin (100 µg/mL); HG = high glucose (30 mM); HGE = high glucose (30 mM) + esculetin (100 µg/mL). n = 7--9. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. ** p = 0.0013; *** p = 0.0005; **** p < 0.0001*

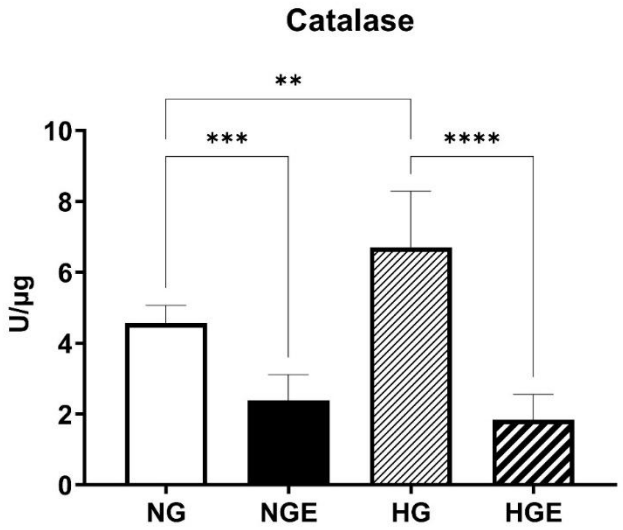
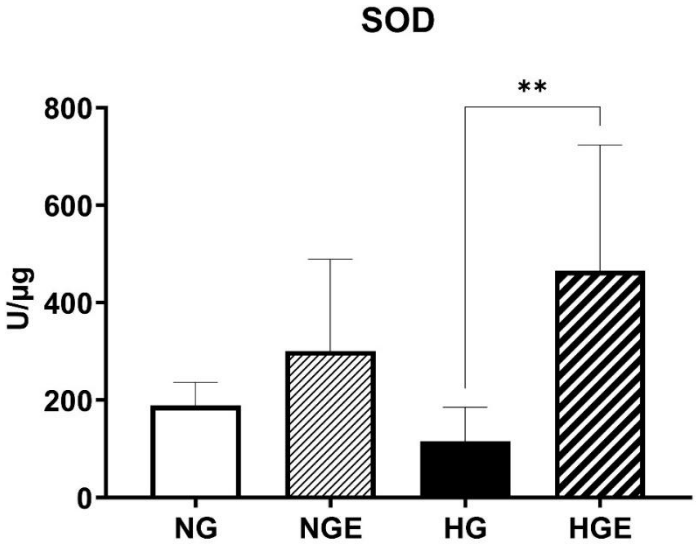


Figure 4

*Superoxide dismutase levels. NG = normal glucose (6.7 mM); NGE = normal glucose (6.7 mM) + esculetin (100 µg/mL); HG = high glucose (30 mM); HGE = high glucose (30 mM) + esculetin (100 µg/mL). n = 5-7. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. ** p = 0.0033*



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b. Superoxide dismutase (SOD)

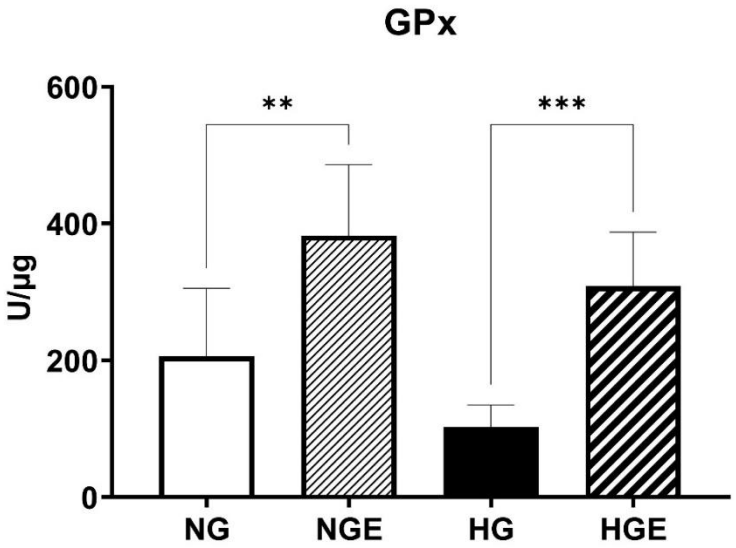
SOD levels were decreased but not significantly decreased in the high-glucose groups. Compared with the control, esculetin significantly increased SOD levels in normal and high-glucose media (465.8 ± 258.1 *vs.* 115.4 ± 69.88; $p = 0.0033$) (Figure 5).

c. Glutathione peroxidase (GPx)

An increase in GPx levels was observed in the esculetin-treated groups compared with the corresponding control groups (NGE 382.4 ± 103.8 *vs.* 206 ± 99.61; $p = 0.0015$ and HGE 308.3 ± 79.29 *vs.* 102.8 ± 32.11; $p = 0.0006$) (Figure 6).

Figure 5

*Glutathione peroxidase levels. NG = normal glucose (6.7 mM); NGE = normal glucose (6.7 mM) + esculetin (100 µg/mL); HG = high glucose (30 mM); HGE = high glucose (30 mM) + esculetin (100 µg/mL). n = 6--8. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. ** p = 0.0015; ***p = 0.0006*



4.6 NITRIC OXIDE (NO) DETERMINATION

In the present study, we observed greater production of nitric oxide in the HG group than in the NG group (17.62 ± 3.73 *vs.* 3.91 ± 0.49; $p < 0.0001$). Treatment with esculetin in the HG group appeared to reduce NO levels, but we did not observe significant differences in these data (Figure 7).

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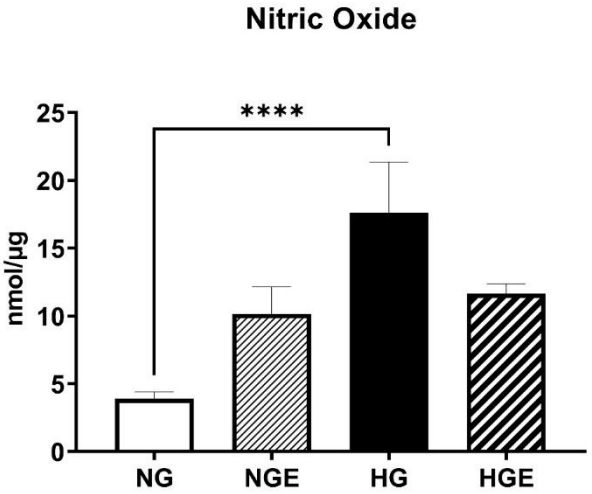
Figure 6

*Nitric oxide levels. NG = normal glucose (6.7 mM); NGE = normal glucose (6.7 mM) + esculetin (100 µg/mL); HG = high glucose (30 mM); HGE = high glucose (30 mM) + esculetin (100 µg/mL). n = 8--9. The Kruskal–Wallis test was followed by Dunn's multiple comparisons test. ** **p < 0.0001*



6 DISCUSSION

Diabetes mellitus (DM) is a multifactorial disease, the prevalence of which is increasing worldwide, with an estimated 783 million people in 2045 (1). One of its complications, diabetic nephropathy (DN), is currently considered the leading cause of chronic renal disease, resulting in renal replacement therapy, such as dialysis and kidney transplantation (29, 30).

The pathophysiology of DM involves the formation of many procoagulants and proinflammatory substances, resulting in reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as enzymes that participate in the defense against these substances, *i.e.*, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (31-33). An imbalance between ROS/RNS and these antioxidants results in oxidative/nitrosative stress (OS/NS), leading to apoptosis and tissue damage (31, 32).

The toxic effects related to oxygen have been known since the XIX century, but the identification of free radicals responsible for this toxicity was possible only approximately 70 years ago by Denham Harman, who proposed a relationship between these radicals and mutagenesis, cancer and aging (34, 35). These studies were enhanced with the discovery of SOD in 1968 by McCord and Fridovich (36). According to Philips et al., OS-induced

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complications from DM include coronary artery disease, neuropathy, nephropathy (DN), and retinopathy (37).

In our laboratory, many studies in experimental models of diabetes, both in vitro and in vivo, revealed the participation of OS in this disease. Rodrigues et al. reported that streptozotocin-induced diabetic rats presented high levels of urinary albumin (an early marker of DN) and TBARS (a marker of lipoperoxidation, *i.e.,* OS) (38). Our group subsequently demonstrated that N-acetylcysteine, an antioxidant that enhances muscle cysteine and glutathione availability (39), protected rats against DN through the control of OS/NS in this same model of DM (40). We also showed that kefir, a probiotic fermented milk known as an antioxidant, reduced TBARS and superoxide anion levels in the renal cortex of diabetic rats (41). Additionally, cupuacu (*Theobroma grandiflorum*), a fruit abundant in the Amazon rainforest that contains phenols, catechin and quercetin, also has a protective effect on the OS of mouse-immortalized mesangial cells (42). Research conducted elsewhere also supports the role of hyperglycemia in the generation of OS, resulting in endothelial dysfunction in diabetic patients (43). Furthermore, increased levels of glucose and insulin, as well as dyslipidemia, result in microangiopathies and OS, leading to atherosclerosis (44).

In the present study, we utilized mouse-immortalized mesangial cells (MiMCs) cultured in high-glucose medium (HG), which is a widely used and accepted model of in vitro DM (45-48). We studied the effects of esculetin (ESCt) on OS/NS in these cells. ESCt is a coumarin derivative commonly found in trees of the genus *Fraxinus* (49). Its dried bark, also known as *Cortex Fraxini*, is frequently used in traditional Chinese medicine under the name Qinpi (49, 50). ESCt and its glycoside form, esculin (ESC), are the most studied active ingredients in this product (50).

There is evidence that ESCt and ESC have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antibacterial, antitumor, antidiabetic, antiatherosclerotic, and immunomodulatory effects (51-53). The antioxidant effects of ESCt and ESC are associated with the activation of the nuclear factor erythroid-derived factor 2-related factor 2 (Nrf2) signaling pathway; Nrf2 can regulate the expression of several endogenous antioxidant proteins, such as SOD, GPx and glutathione (GSH) (54, 55).

In our study, we first determined the scavenging effect of ESCt on DPPH (Figure 1). The DPPH method is a widely used technique for assessing the antioxidant activity of compounds, especially in plant extracts. DPPH is a stable free radical that can be reduced in the presence of an antioxidant agent, resulting in DPPH-H, a more stable molecule that

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does not exhibit the reactive properties of the original radical (56, 57). Gallic acid is commonly used as a control for antioxidant activity in DPPH assays because of its high capacity to reduce this radical to a less reactive form (56, 57). In our experiments, we demonstrated that ESCt has greater antioxidant potential than gallic acid does, as a lower concentration of this molecule is required to inhibit at least 50% of the DPPH present in the medium.

The antioxidant activity of ESCt observed via the DPPH assay was confirmed via the DCFH-DA assay. In this method, DCFH-DA is taken up by the cells and cleaved by a cellular esterase, resulting in DCFH, which, in the presence of ROS, is oxidized and converted to DCF, emitting fluorescence that can be quantified (26). As expected, cells cultured in HG medium produced more ROS than those cultured in NG medium did. Notably, ESCt demonstrated a strong antioxidant role, successfully reducing ROS production in both the NG and the HG (Figure 2). ESCt can initially play an antioxidant role in a direct manner because of its catechol structure, which consists of two hydroxyl groups at positions 6 and 7 of its benzopyrone ring (17). These hydroxyl groups are key to their ability to neutralize ROS by donating electrons or hydrogen atoms, effectively scavenging free radicals such as hydroxyl radicals and superoxide anions (16, 17, 58).

Once produced in a dysregulated manner, ROS can exacerbate the progression of DM by promoting nonenzymatic protein glycation, glucose autoxidation, activation of the polyol pathway, and degradation of lipid membranes through lipid peroxidation (59, 60). Malondialdehyde (MDA) is one of the most studied byproducts of lipid peroxidation and can be quantified through the TBARS method (25). This molecule also amplifies the deleterious effects present in DM by inducing a vicious cycle of interaction with biomolecules (*e.g.*, nucleic acid bases, creating interstrand cross-links in DNA and histones) and activating proinflammatory genes that subsequently produce more ROS and more lipid peroxidation products (61-63). Recent studies have shown that MDA plays a significant role in the development of atherogenesis and thickening of cardiovascular tissue (63, 64), as well as in other diseases (61, 65-67). As expected, we observed elevated TBARS levels in cells cultured in a medium that mimics a hyperglycemic environment and that treatment with ESCt reduced TBARS levels under the same conditions (Figure 3). This effect can be attributed to the antioxidant properties of ESCt, which reduce ROS and, consequently, the degree and extent of lipid peroxidation.

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On the other hand, nitric oxide (NO) may also be dysregulated due to hyperglycemia (68). This molecule is known to be a potent vasodilator and plays a role in inhibiting platelet aggregation and leukocyte adhesion—processes closely linked to atherogenesis and the aforementioned thickening of cardiovascular tissue—making it a good marker of endothelial health (69, 70). The elevated ROS produced as a result of hyperglycemia can interact directly with NO, forming peroxynitrite, which leads to the uncoupling of the enzyme responsible for NO synthesis, nitric oxide synthase (NOS), and reduces the cofactors necessary for the proper functioning of all isoforms of this enzyme (68, 70, 71). Once uncoupled, NOS begins to produce superoxide anions instead of NO, exacerbating oxidative stress (71). In our study, we observed an increase in NO production in cells cultured in high-glucose media. When treated with ESCt, there was a considerable reduction in NO production (~ 34%), although the values did not reach statistical significance (Figure 7). This increase in NO production has also been observed in other studies under similar conditions, and it was attributed to a higher protein content of the inducible NOS (iNOS) isoform, which is responsible for the activation of proinflammatory pathways (45, 72).

In addition to the direct antioxidant effects promoted by ESCt, such as the neutralization of ROS, its role in modulating certain antioxidant enzymes is notable. In living organisms, there are three main antioxidant defense pathways (73). The first pathway is enzymatic, comprising enzymes that dismute the superoxide radical (such as SODs) and those that remove hydrogen peroxide (glutathione and catalase) (73, 74). The second antioxidant defense pathway is more nutritious in nature and involves vitamins, flavonoids, and carotenoids (73-75). The third and final pathway involves various complex mechanisms, including repair processes (e.g., of DNA, oxidized proteins, and oxidized lipids) and recycling mechanisms for cellular components damaged by oxidation (73, 76).

We determined that the glutathione peroxidase (GPx) levels of cells cultured in HG medium were depleted to approximately half the original level. However, treatment with ESCt significantly increased GPx levels in cells cultured in both normoglycemic and hyperglycemic environments (Figure 6). The same profile was observed for superoxide dismutase (SOD) levels, which slightly differed, suggesting impaired antioxidant defense in HG-cultured cells (Figure 5). The scavenging effect of ESCt over free radicals was demonstrated previously by Wang et al. (77) and is also in agreement with other models of toxicity, such as ethanol-induced liver injury in human HepG2 cells, where the authors showed that ESCt can restore depleted GSH levels and inhibit MDA by activating the Nrf2

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signaling pathway (78). In animal models of OS caused by myocardial ischemia–reperfusion (79) and in models of myocardial toxicity (80), ESCt can improve OS by restoring the activity of enzymes involved in the first-line antioxidant defense.

Curiously, in this study, an analysis of the activity of catalase (CAT) revealed an interesting response to treatment with ESCt (Figure 4). While the amount of this enzyme was greater in the HG group than in the NG group, the ESCt-treated groups presented a decrease in the CAT content. This observation may suggest that CAT was activated in the HG environment in an attempt to control OS and that increasing GPx and SOD levels in ESCt made CAT 'dispensable' under these conditions.

7 CONCLUSION

Thus, our study provides convincing evidence that esculetin has antioxidant properties and can attenuate oxidative stress in an experimental model, which is an important aspect of the pathophysiology of DM. Future studies may further elucidate the therapeutic potential of esculetin in the prevention and treatment of disorders associated with oxidative stress, such as diabetes mellitus.

8 LIST OF ABBREVIATIONS

AGEs - Advanced Glycation End Products
ANOVA - Analysis of Variance
ATCC - American Type Culture Collection
CAT - Catalase
DCFH-DA - 2',7'-dichlorofluorescein diacetate
DCF - Dichlorofluorescein
DM - Diabetes Mellitus
DM1 - Type 1 Diabetes Mellitus
DM2 - Type 2 Diabetes Mellitus
DMEM - Dulbecco's Modified Eagle's Medium
DN - Diabetic Nephropathy
DPPH - 2,2-diphenyl-1-picrylhydrazyl
ESC - Esculin
ESCt - Esculetin
ESRD - End-Stage Renal Disease

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FBS - Fetal Bovine Serum
GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase
GPx - Glutathione Peroxidase
HG - High Glucose
HGE - High Glucose + Esculetin
IC50 - Inhibitory Concentration 50%
MiMCs - Mouse Immortalized Mesangial Cells
MDA - Malondialdehyde
MTT - Methyl Thiazole Tetrazolium
NBT - Nitroblue Tetrazolium
NG - Normal Glucose
NGE - Normal Glucose + Esculetin
NO - Nitric Oxide
NOS - Nitric Oxide Synthase
Nrf2 - Nuclear Factor Erythroid 2-related Factor 2
OD - Optical Density
$ONOO^-$ - Peroxynitrite
OS - Oxidative Stress
PARP - Poly (ADP-ribose) Polymerase
PBS - Phosphate-Buffered Saline
PKC - Protein Kinase C
RAGEs - Receptors for Advanced Glycation End Products
RNS - Reactive Nitrogen Species
ROS - Reactive Oxygen Species
SD - Standard Deviation
SOD - Superoxide Dismutase
TBARS - Thiobarbituric Acid Reactive Substances
UNIFESP/EPM - Universidade Federal de São Paulo/Escola Paulista de Medicina

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

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Consent for publication

Not applicable.

Availability of data and materials

Data and materials can be accessed in https://drive.google.com/drive/folders/1iTa1MkmEHtv6SEGSAdPcORwGc7ylZz8Z?usp=sharing

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

The authors contributed equally to the development of this work.

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Authors' information (optional)

Nothing to declare.

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