Biomedicine & Pharmacotherapy 131 (2020) 110659

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

SIRT1-dependent effects of resveratrol and grape juice in an *in vitro* model of preeclampsia

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ARTICLE INFO

Keywords:
SIRT1
Preeclampsia
Resveratrol
Grape juice
Endothelium
Nitric oxide
Oxidative stress

ABSTRACT

Preeclampsia (PE) is a multifactorial hypertensive disorder of pregnancy that is partly responsible for both maternal and fetal morbidity and mortality levels worldwide. It has been recently discovered that sirtuin-1 (SIRT1) is reduced in the circulation and in an *in vitro* model of PE. Therefore, in this study, we investigated the effects of trans-resveratrol, a potent antioxidant and activator of SIRT1, on oxidative stress and nitric oxide (NO) production in an *in vitro* model of PE compared to gestational hypertensive (GH) and healthy pregnant (HP) women. Furthermore, we also evaluated the effects of an acute intake of grape juice on women with PE to assess whether it could mimic *in vitro* trans-resveratrol supplementation.

(1) In the GH group, resveratrol decreased intracellular reactive oxygen species (ROS) and increased their antioxidant capacity, while inhibiting SIRT1 reestablished previous levels. (2) In PE, inhibition of SIRT1 increased antioxidant activity. (3) Intracellular NO and supernatant nitrite levels were increased by inhibiting SIRT1 in the PE group. (4) Grape juice intake increased intracellular NO levels *versus* before grape juice intake control; however, the inhibition of SIRT1 before grape juice intake initially increased NO, but decreased it 1 h after grape juice intake.

In conclusion, activating SIRT1 by using resveratrol alone may not be beneficial to women with PE, and GH and PE seem to have different responsive mechanisms to this molecule. Furthermore, grape juice intake seems to have different effects compared to resveratrol supplementation alone in this *in vitro* model of PE, demonstrating the potential of the combination of other biologically active molecules from grape juice over the SIRT1-eNOS-NO in PE treatment.

- GH and PE groups respond differently to resveratrol *in vitro*.
- Grape juice intake has different effects compared to resveratrol in PE.
- Other molecules from grape juice may have potential to reverse eNOS uncoupling.

1. Introduction

Preeclampsia (PE) is a multifactorial gestational hypertensive disease characterized by the onset of hypertension after 20 weeks of gestation associated with proteinuria or other end-organ damage [1]. It is one of the major causes of maternal and fetal morbidity and mortality worldwide, affecting 3%–8% of all pregnancies [2,3]. The pathophysiology of PE is thought to occur in two stages, the first being the poor vascularization of the placenta leading to placental ischemia and the consequent release of several factors in the bloodstream of these pregnant women. This promotes the second stage of the disease, or maternal syndrome, which includes systemic endothelial dysfunction, characterized by an imbalance between the endothelium-derived vasodilators and vasoconstrictors. [4]. The incubation of plasma and serum from women with PE for the assessment of endothelial cells is well-established as an *in vitro* model and facilitates research for a better understanding of

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https://doi.org/10.1016/j.biopha.2020.110659
Received 16 June 2020; Received in revised form 10 August 2020; Accepted 17 August 2020
Available online 28 August 2020
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endothelial dysfunction in PE [5–8].

Ischemic placental microparticles, released into the maternal circulation, contribute to the elevated levels of reactive oxygen species (ROS) [9,10]. These have been pointed out as one of the causes of vascular endothelial dysfunction [11,12]. Although clinical trials using antioxidants, such as vitamins C and E, have been unsuccessful in reducing the risk of PE [13,14], some studies have reported the effect of resveratrol, a natural antioxidant, in PE [15–17]. Resveratrol, one of the polyphenol stilbenes found in grapevines and a variety of other plant species [18], is known for its protective cardiovascular effects associated with wine consumption ("French Paradox") [19,59]. Furthermore, this molecule has shown beneficial effects in cancer, neurological diseases, energy homeostasis, and diabetes [20–23]. Indeed, a recent studie using this molecule have shown that resveratrol supplementation in patients with type 2 diabetes mellitus and coronary heart disease had beneficial effects on glycemic control, HDL-cholesterol levels, the total HDL-cholesterol ratio, total antioxidant capacity, and malondialdehyde levels [24]. Moreover, the supplementation of an allopathic treatment with resveratrol had a beneficial effect on blood pressure, body mass index as well as all parameters of the lipid profile, and glucose (in nondiabetic patients) in patients who had a stroke compared to the control group [25]. However, the molecular and cellular mechanisms of resveratrol are subjects of much debate within the literature and is suggested to involve estrogen receptors, protein kinases, phosphodiesterases, heat shock proteins, regulators of cellular bioenergetics, and protein deacetylases such as sirtuins [26].

Sirtuin-1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD^+)-dependent class III histone deacetylase that can be activated by resveratrol directly, by binding to its N-terminal domain, or indirectly by activating AMPK and consequently augmenting NAD^+ bioavailability [27,28]. It regulates many physiological functions, including cell senescence, gene transcription, energy balance, inflammatory processes, and oxidative stress [29–33]. Moreover, SIRT1 can increase nitric oxide (NO) synthesis by deacetylation of endothelial nitric oxide synthase (eNOS) in the vascular endothelium [29]. Previous studies have shown that this protein might play an important role in PE defective placentation [34,35]. More so, another study proposed that SIRT1 is responsible for protecting the PE endothelium by inhibiting the liberation of heat-shock protein 70 (HSP70) and high mobility group box-1 (HMGB1). Furthermore, a previous study from our group showed that SIRT1 was reduced in the circulation of women with PE and endothelial cells incubated with plasma from these women, compared with gestational hypertensive (GH) and healthy pregnant (HP) women [36].

2. Materials and methods

2.1. Patient included in the in vitro studies using trans-resveratrol

The study was approved by the Research Ethics Committee of the Faculty of Medicine of Ribeirao Preto (HCFMRP-USP), Brazil (process number 4682/2006, approved on June 20th, 2006), following the principles of the Declaration of Helsinki. Diagnostic criteria for PE and GH were defined by the American College of Obstetricians and Gynecologists (ACOG) [1]. Exclusion criteria were: non-white women, smokers, >34 years old, twin pregnancies, chronic hypertension, hemostatic abnormalities, diabetes mellitus, fetal abnormalities, cancer, and cardiovascular, autoimmune, renal, and liver diseases. All volunteers received detailed information about the project and signed the informed consent form. From these, 15 mL of venous blood was collected in standard heparin Vacutainer tubes (Becton-Dickinson). Then, the tubes were centrifuged at room temperature for 10 min at 3200 *g* and plasma aliquots were separated and stored together at -80 °C until use. For each pool of plasma, we selected 10 plasma samples from HP, 10 plasma samples from GH, and 10 plasma samples from PE women collected at the Clinics Hospital of Ribeirao Preto.

2.2. Pilot clinical study using grape juice

For this pilot study, four PE patients, diagnosed with PE according to ACOG guidelines [1], were recruited at HCFMRP-USP. They were offered a 200 mL shot of commercial organic whole grape juice (Carraro, RS, Brazil). This study was approved by the Institutional Review Board at Ribeirao Preto Medical School, Brazil (process number 2.602.100/2018, approved on April 16, 2018), following the principles of the Declaration of Helsinki and all subjects gave written informed consent. Exclusion criteria included smokers, chronic alcohol consumption, pre-gestational obesity (BMI > 30), gestational diabetes and diabetes mellitus, dyslipidemia, cardiovascular diseases, inflammatory diseases, and cancer. These women arrived at the hospital after 12 h fasting and had their blood was drawn before grape juice intake (basal) and after 1 h (1 h). The blood samples were collected in tubes containing heparin, which was rapidly centrifuged for 10 min at 1000 *g* at room temperature. Plasma samples were stored at −80 °C for the *in vitro* assays.

We chose to collect the blood after 1 h of grape juice intake based on a previous study that showed that this period had the highest absorption of polyphenols [37].

2.3. In vitro assays

Human umbilical vein endothelial cells (HUVECs), strain CRL 2873 from ATCC (American Type Culture Collection ATCC, Manassas, Virginia, USA), were cultured until reaching 70–80 % confluence, at 37 °C in 5% CO2 in DMEM medium (Gibco, CA, USA) supplemented with 10 % (v/v) fetal bovine serum (Gibco CA, USA), 50 μg/mL penicillin, 100 μg/mL streptomycin and 0.5 μg/mL amphotericin B (Gibco, CA, USA). Then, we replanted the Human Umbilical Vein Endothelial Cells (HUVECs) in 96 well plates (Jet Biofil) and after reaching the necessary confluence, the culture medium was removed and the HUVECs were washed with saline solution (PBS) to remove traces of fetal bovine serum. Then, we incubated the cells with a 10 % (v/v) plasma pool from HP, GH, and PE groups for 24 h at 37 °C in 5% CO_2. Besides, the same procedures were performed in the presence of 10 μM of trans-resveratrol (Cayman Chemical®, MI, USA) for 24 h in the presence or absence of 30 μM of EX-527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide), a potent and specific SIRT1 inhibitor that inhibits SIRT1 approximately 100-fold more potently than SIRT2 and SIRT3 and has no effect on SIRT5 deacetylation activity [38], (Sigma Aldrich, Saint Louis, MO, USA).

For trans-resveratrol intervention *in vitro*, the concentration of 10 μM was chosen based on previous studies that evaluated the response of the endothelial cells to trans-resveratrol *in vitro*. These studies showed that, at this concentration, resveratrol augmented eNOS miRNA levels and eNOS promoter activity [39], besides upregulating SIRT1 protein expression and enzymatic activity that was associated to increased mRNA and protein expression of eNOS, which was prevented by knockdown of SIRT1 [40].

HUVEC were also incubated with 10 % (v/v) from PE basal plasma (before grape juice intake) and PE plasma from after 1 h of grape juice intake for 24 h.

2.4. PrestoBlue viability assay

Cell viability was assessed using PrestoBlue Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific, California, USA), which in the presence of metabolically active cells is converted into the reduced form (resorufin) by mitochondrial enzymes, resulting in changes in fluorescence [41]. To perform the assay, we seeded the HUVECs in a 96-well microplate with a dark side (Synergy 4, BioTek, VT, USA) ($\sim 1 \times 10^4$ / well) and after 24 h of incubation with the treatments, the supernatant was discarded and the wells were washed once with PBS. Then, we added 90 μL of medium and 10 μL of the PrestoBlue® Cell Viability

reagent (Invitrogen, Thermo Fisher Scientific, California, USA). Fluorescence was obtained from reading on a spectrophotometer (Synergy 4, BioTek, VT, USA) at the excitation and emission of 560 nm and 590 nm, respectively.

2.5. Intracellular reactive oxygen species

The quantification of intracellular ROS was performed by determining the fluorescence of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Cayman Chemical®), an excellent tool to verify the release of hydroxyl radical, peroxyl, alkoxyl radical, peroxynitrite, as well as hydrogen peroxide [42]. To perform this assay, after incubation for 24 h in a 96-well microplate with a dark side (Synergy 4, BioTek, VT, USA) (~ 1×10^4/well) with the treatments, the supernatants were collected and the HUVEC were incubated with 25 μM of DCFH-DA diluted in PBS for 30 min. Then the wells were washed with PBS once and filled with 100 μL of PBS. Fluorescence was determined using wavelengths of 502 nm excitation and 523 nm emission in a multifunctional plate reader (Synergy 4, BioTek, VT, USA).

2.6. Ferric reducing ability of supernatant (Frap)

Total Antioxidant Capacity from supernatant was assessed using Ferric Reducing Antioxidant Power (FRAP) assay [43]. FRAP reagent was prepared by mixing 50 mL of 23 mM acetate buffer (pH = 3.6), 5 mL of 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution and 5 mL 20 mM $FeCl_3.6H_2O$. We added 10 μL of supernatant sample to a 96 well microplate, followed by 290 μL of FRAP reagent and incubated the microplate at 37 °C for 4 min. Absorbance was read at a 593 nm wavelength. The standard solution was prepared using $Fe_2SO_4.7H_2O$ and the unit used was mM equivalent Fe2+.

2.7. Intracellular nitric oxide quantification

A total of 5×10^4 HUVEC were seeded per well in a 96-well microplate with a dark side (Synergy 4, BioTek, VT, USA). After 24 h of incubation with plasmas and treatments, cells were washed, and we added 95 μL of PBS and incubated for 30 min at 37 °C. Then, we added 5 μL of DAF-FM ™ (5 μM) (Cayman Chemical®, MI, USA). Once inside the cell, this compound is deacetylated by intracellular esterases forming highly fluorescent DAF-FM. The reading was performed using wavelengths of 485 nm excitation and 538 nm emission in a multifunctional plate reader (Synergy 4, BioTek, VT, USA).

2.8. Measurement of nitrite

Nitrite concentration was evaluated in the cell supernatants using a *Griess Reagent kit* (Invitrogen, Thermo Fisher Scientific, California, EUA). After 24 h of HUVECs incubation with plasmas and treatments, the supernatants were collected and used as recommended by the manufacturer. The plate was read at 540 nm in a spectrophotometer (Synergy 4, BioTek, VT, USA).

2.9. Statistical analysis

Replicates of 5 per group combined with treatments were performed in each experiment. We used Kolmogorov–Smirnov normality test to determine if the following test should be parametric or not. When two groups were compared, we used a *t*-test (parametric) or Mann-Whitney test (non-parametric). When comparing three or more groups we used 1-way analysis of variance test (parametric) or Kruskal-Wallis test (non-parametric). Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and statistical significance was defined as $P < 0.05$.

3. Theory

To our knowledge, no study has investigated resveratrol's SIRT1-related activity in an *in vitro* model of PE. Therefore, in this study, we further investigated the effect of trans-resveratrol on the SIRT1-mediated signal pathways, antioxidant capacity, intracellular ROS, NO production, and nitrite levels in endothelial cells incubated with plasma from PE, GH, or HP. Moreover, we have also analyzed these parameters in endothelial cells incubated with plasma from women with PE who acutely ingested grape juice.

4. Results

The general clinical parameters of HP, GH, and women with PE whose plasma samples were collected and pooled to perform *in vitro* studies are shown in Table 1. No differences were observed in maternal age, percentage of primigravids, body mass index before pregnancy (BMI), newborn weight, and gestational age (GA) at sampling. GA at delivery was lower in the PE group than in the HP and GH groups, systolic blood pressure (SBP) was higher in GH and PE, and diastolic blood pressure (DBP) was increased in the PE group compared to the HP group. Moreover, hemoglobin and hematocrit levels were increased in GH compared with HP.

No differences were observed in cell viability between the HP, GH, and PE groups (data not shown). Furthermore, adding resveratrol alone or in combination with EX-527 (SIRT1 inhibitor) did not alter the viability in any of the experimental groups (all $p > 0.05$, data not shown).

To investigate the oxidative stress, we evaluated the intracellular ROS production in HUVECs incubated with the experimental group pool of plasma before and after adding 10 μM resveratrol and EX-527 treatments. In the HP group (Fig. 1A), resveratrol did not alter ROS levels, but adding EX-527 to the treatment increased its value significantly ($p < 0.01$). Furthermore, resveratrol reduced the intracellular ROS production in the GH group (Fig. 1B), and by adding EX-527, this reduction was reversed. Finally, the generation of ROS in PE increased after incubation with EX-527 when compared with resveratrol alone (Fig. 1C).

We further investigated the effect of the treatments on the

Table 1
Clinical characteristics of HP, GH, and PE pregnant women enrolled in this study.

Parameters	HP(n = 10)	GH(n = 10)	PE(n = 10)
Maternal Age (years)	23 ± 5	20 ± 3	23 ± 3
Primigravida (%)	70	80	70
BMI before pregnancy (kg/m^2)	25 ± 8	32 ± 6	27 ± 4
SBP at sampling (mmHg)	106 ± 12	127 ± 11**	135 ± 18***
DBP at sampling (mmHg)	68 ± 9	79 ± 7	82 ± 11*
GA at sampling (weeks)	37 ± 1	36 ± 5	36 ± 2
Hb (g/dl)	10 ± 0.72	13 ± 2.5**	12 ± 1
Hct (%)	30 ± 2.5	37 ± 3*	36 ± 2
GA at delivery (weeks)	40 ± 2	39 ± 2	37 ± 4*
Newborn weight (g)	3320 ± 382	3237 ± 394	2825 ± 738
Antihypertensive drugs at blood collection[1]			
α-Methyldopa (%)	NA	80	90
Nifedipine (%)	NA	0	20
Hydralazine (%)	NA	0	10

Values are the means ± S.D. or percentage. HP, Healthy Pregnant; GH, gestational hypertension; PE, preeclampsia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; Hb, hemoglobin; Hct, hematocrit; NA, not applicable. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* healthy pregnant.

[1] Some GH and PE patients were under antihypertensive drug treatment. More than one drug might have been combined.

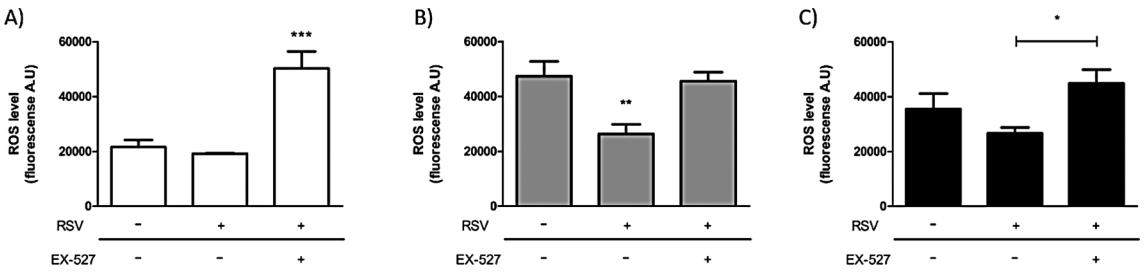

Fig. 1. ***Intracellular ROS production after 24 h of incubation.*** **(A)** the combination of resveratrol (RSV) and EX-527 increased intracellular ROS levels in the HP group compared to the treatment with only plasma and the treatment with just plasma and RSV ($p < 0.01$). **(B)** RSV decreased ROS production in GH and adding EX-527 to the treatment reversed this decrease ($p < 0.05$). **(C)** in PE group only RSV treatment did not alter ROS concentration but adding EX-527 increased these levels when compared to RSV treatment ($p < 0.05$) HUVECs were incubated with 10 % (v/v) plasma samples from HP, GH and PE ($n = 10$ per group) for 24 h in the absence (−RSV) or presence of trans-resveratrol (+RSV) at 10 μM and without (−EX-527) or combined with EX-527 (+EX-527). DMSO was used as a vehicle for RSV (10 μM) and EX-527 (30 μM) (DMSO < 0.12 %). Data presented as mean ± *S.E.M.* (**(A)** Oneway ANOVA $p = 0.0008$; **(B)** Oneway ANOVA $p = 0.0137$; **(C)** Oneway ANOVA $p = 0.0336$). * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

antioxidant capacity in the cell culture supernatant. No differences were observed with the treatments in the HP group (Fig. 2A). In the GH group, we found that the resveratrol treatment increased the antioxidant capacity of HUVECs ($p < 0.05$) and the addition of EX-527 reversed this increase ($p < 0.05$) (Fig. 2B). Interestingly, in the PE group, resveratrol alone did not affect the antioxidant capacity of the cells, but the combination of resveratrol and EX-527 increased this activity ($p < 0.05$) (Fig. 2C).

Regarding intracellular NO production, we found that neither resveratrol nor the combination of resveratrol and EX-527 changed NO concentrations in the HP and GH groups (Fig. 3A and **3**B, respectively). In contrast, the combination of resveratrol and EX-527 increased the NO concentration in the PE group (Fig. 3C).

We further investigated the NO results by measuring the nitrite concentration in the supernatant of the treated HUVECs. Similarly, we found no alterations in the nitrite levels in the HP group after resveratrol and EX-527 treatments (Fig. 3D). In addition, there were no differences in the GH group (Fig. 3E). In PE, the results were similar to those observed in intracellular NO levels, and only the combination of resveratrol and EX-527 increased nitrite levels (Fig. 3F).

A comparison between the experimental groups results (HP, GH, and PE) can be found in the supplementary materials .

We also investigated intracellular ROS and NO levels in PE after acute grape juice intake, to verify how similar they were to the resveratrol treatment and their SIRT1 relation. Even though there was no difference in ROS concentrations before and after grape juice intake, EX-527 increased ROS concentrations in both basal and after 1 h (Fig. 4A). Furthermore, after 1 h, grape juice increased NO levels compared to basal control, and adding EX-527 decreased NO production after 1 h of grape juice intake, but increased its basal production (Fig. 4B).

5. Discussion

To our knowledge, our study is the first group to demonstrate the findings that follow. First, in the GH group the resveratrol treatment reduced ROS levels and increased the antioxidant capacity. Moreover, inhibiting SIRT1 increased ROS levels in HP and PE, and increased antioxidant capacity in PE. Second, inhibiting SIRT1 in this PE *in vitro* model increases intracellular NO and supernatant nitrite. Lastly, the effects of grape juice intake by women with PE were also assessed *in vitro*. Under basal conditions, the inhibition of SIRT1 resulted in an increase in ROS and a decrease in NO. After acute grape juice intake, inhibiting SIRT1 increased ROS and intracellular NO levels.

An increase in oxidative stress is considered a normal phenomenon in normal pregnancy. However, in PE, circulating oxidative stress levels are exaggerated, even though these women have been shown to present an increased antioxidant capacity [44,48]. Previous studies proposed that the increased products of oxidative stress might be an underlying mechanism of maternal endothelial dysfunction [9,46]. For this reason, the search for antioxidant molecules has been a major focus for possible treatments of this condition. Indeed, studies that focused on the use of vitamins C and E were not successful in reducing the risk of developing PE [13,14]. Likewise, our results showed that resveratrol did not reduce intracellular ROS levels in the PE group, and this result is similar to that observed by our group in a previous study using 1 μM resveratrol in pregnant women who subsequently developed PE [47]. Interestingly, the same concentration of resveratrol was able to decrease the intracellular ROS levels observed in the GH group. Furthermore, inhibiting

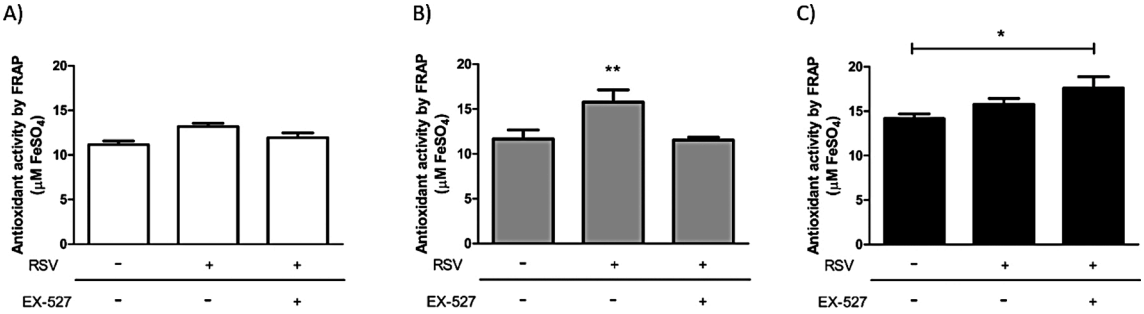

Fig. 2. ***Supernatant antioxidant activity by FRAP.*** **(A)** Neither resveratrol alone nor combined to EX-527 altered antioxidant activity in the HP group. **(B)** RSV increased antioxidant activity in GH ($p < 0.05$). **(C)** in PE group only RSV treatment did not alter antioxidant capacity but adding EX-527 increased these levels ($p < 0.05$). HUVECs were incubated with 10 % (v/v) plasma samples from HP, GH and PE ($n = 10$ per group) for 24 h in the absence (−RSV) or presence of trans-resveratrol (+RSV) at 10 μM and without (−EX-527) or combined with EX-527 (+EX-527). DMSO was used as a vehicle for RSV (10 μM) and EX-527 (30 μM) (DMSO < 0.12 %). Data presented as mean ± *S.E.M.* **(A)** Oneway ANOVA $p = 0.0261$; **(B)** Kruskal-Wallis test $p = 0.0182$; **(C)** Kruskal-Wallis test $p = 0.0410$). * $p < 0.05$, **$p < 0.01$.

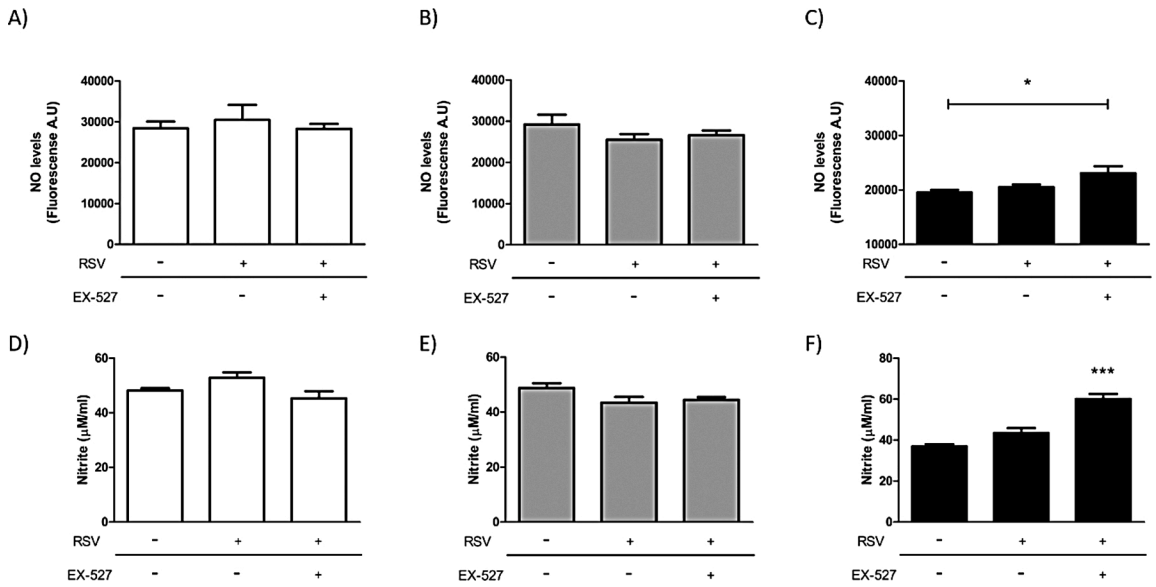

Fig. 3. ***Intracellular NO levels and supernatant nitrite levels after 24 h of incubation.*** **(A)** Neither resveratrol (RSV) nor combined RSV and EX-527 altered NO levels in the HP group. **(B)** Neither RSV nor RSV combined to EX-527 altered NO production in the GH group ($p < 0.05$). **(C)** In PE group only RSV treatment did not alter NO concentration but adding EX-527 increased these levels ($p < 0.05$). **(D)** Neither RSV nor RSV combined to EX-527 altered nitrite levels in the HP group. **(E)** Neither RSV nor RSV combined EX-527 altered nitrite levels in the GH group. **(F)** In PE group RSV treatment did not alter nitrite concentration but adding EX-527 combined to RSV increased these levels ($p < 0.05$). HUVECs were incubated with 10 % (v/v) plasma samples from HP, GH and PE ($n = 10$ per group) for 24 h in the absence (−RSV) or presence of trans-resveratrol (+RSV) at 10 μM and without (−EX-527) or combinated with EX-527 (+EX-527).). DMSO was used as a vehicle for RSV (10 μM) and EX-527(30 μM) (DMSO < 0.12 %). Data presented as mean ± *S.E.M.*. **(A)** Oneway ANOVA $p = 0.7809$; **(B)** Oneway ANOVA $p = 0.3252$; **(C)** Oneway ANOVA $p = 0.0377$; **(D)** Kruskal-Wallis test $p = 0.1042$; **(E)** Oneway ANOVA $p = 0.0945$; **(F)** Oneway ANOVA $p < 0.0001$). * $p < 0.05$, *** $P < 0.001$.

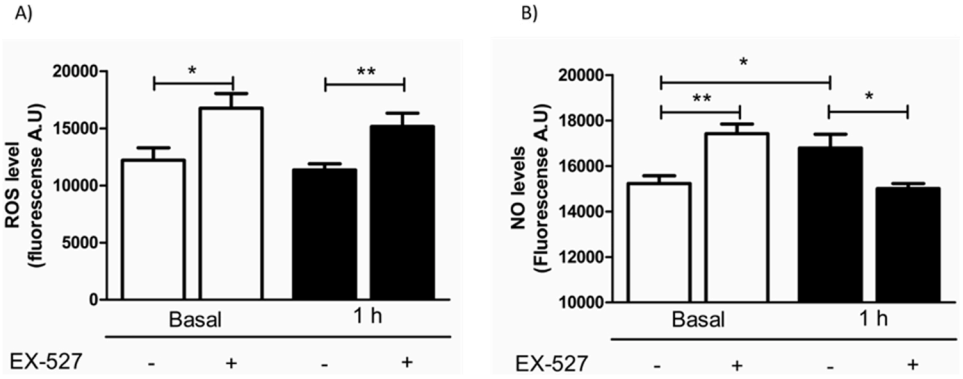

Fig. 4. Intracellular levels of ROS and NO of the grape juice pilot study. **(A)** ROS levels were not altered by the grape juice intake and adding EX-527 to the plasma from PE patients, both before (basal) and after 1 h of grape juice intake, increased intracellular ROS concentration ($p < 0.05$). **(B)** After the 1 h of the grape juice ingestion, NO levels were increased compared to basal levels. Adding EX-527 to as treatment reduced NO levels in HUVEC incubated with the 1 h after grape juice consumption plasma but increased intracellular NO in the basal group ($p < 0.05$). HUVEC were incubated with 10 % (v/v) plasma samples from PE patients separated in before (basal) and after (1 h) of consumption of 200 mL of whole red grape juice with (+ EX-527) or without (−EX-527) EX-527 (30 μM). DMSO was used as a vehicle for EX-527 (30 μM) (DMSO < 0.12 %). Data presented as mean ± *S.E.M.* (**(A. Basal)** Student's *t*-test $p = 0.0359$; **(A. 1 h)** Student's *t*-test $p = 0.0148$; **(A. Basal *vs* 1 h)** Student's *t*-test $p = 0.4728$; (**B. Basal**) Student's *t*-test $p = 0.0159$; **(B. 1 h)** Student's *t*-test $p = 0.0418$; **(B. basal** vs 1 h) Student's *t*-test $p = 0.0317$). * $p < 0.05$, ** $p < 0.01$

SIRT1 by adding EX-527 to the treatment augmented ROS concentrations, to its initial values, in the GH group and augmented ROS level in PE compared to resveratrol treatment alone. Hence, inhibiting SIRT1 in the HP group also increased the intracellular ROS levels by almost 3-fold. These results suggest that the decreased endothelial and plasma levels of SIRT1 in PE, previously observed by our group, might be a possible mechanism related to the increased oxidative stress characteristic of this disease and, for that reason, antioxidant mechanisms may differ in PE when compared to the GH group, which has no alterations in SIRT1 expression [36].

This hypothesis was further improved by our supernatant antioxidant capacity results, in which we found that resveratrol was able to increase the antioxidant activity of the GH group while EX-527 reduced it to the previous level. In contrast, in the PE group, resveratrol alone did not affect the antioxidant capacity until it was combined with EX-527. In addition, we believe that no significant alterations were observed in HP because these women were not exposed to a great amount of oxidative stress. The grape juice pilot study further confirmed our resveratrol treatment results by showing no decrease in ROS intracellular levels after 1 h of grape juice intake, and that inhibiting SIRT1 increased ROS

levels before and after grape juice intake. The increased antioxidant capacity observed in the FRAP assay in PE, in our study and a few others, is still not fully understood [44,48]. It has been suggested that this result might be related to the increased uric acid levels observed in PE. However, no positive correlation between plasma levels of FRAP and serum concentrations of uric acid was found in a previous study [48]. Moreover, another group observed that increased catalase and glutathione peroxidase activities in preeclamptic women could serve as a compensatory mechanism to prevent damage by reactive radicals or other toxins present in the circulation of these women [46]. We believe that the same proposed compensatory mechanisms are responsible for the increase in the total antioxidant capacity observed in the PE group treated with resveratrol and EX-527, since inhibiting SIRT1 increases ROS levels and could consequently increase glutathione peroxidase and catalase activity. This increase in antioxidant capacity may also be related to the observed increase in NO and nitrite levels, which will be further discussed next.

Regarding the intracellular NO and supernatant nitrite levels, we found that resveratrol alone did not alter any of the groups NO and nitrite production but adding EX-527 to the treatment increased these parameters only in the PE group. We suppose that this outcome might be related to the eNOS uncoupling observed in PE. We believe that by adding resveratrol as treatment, we caused an increase in SIRT1 deacetylase activity, leading to a consequent increase in eNOS expression and activity as showed previously in another study [49]; however, specifically in PE, eNOS may be uncoupled, due to an increased L-arginine degradation by arginase II, liberating superoxide ($O_2^{\bullet-}$) instead of NO [50]. Superoxide then sequestrates NO to form peroxynitrite ($ONOO^-$), a highly damaging reactive intermediate capable of forming hydroxyl radicals ($^{\bullet}OH$), which might be related to the insufficient activity of resveratrol in reducing intracellular ROS levels in PE . Furthermore, we suppose that by inhibiting SIRT1 with EX-527, we break the eNOS uncoupling loop by decreasing its SIRT1 dependent expression and activation, leading to a decrease in $O_2^{\bullet-}$ production and a consequent increase in intracellular NO and supernatant nitrite. Moreover, NO and nitrite levels were not altered by any of the treatments in the HP and GH groups, showing that the results observed in the PE group are dependent on the particular mechanisms of this disease.

Additionally, intracellular NO results from the grape juice pilot study showed that, in contrast to resveratrol alone, grape juice intake was able to increase intracellular NO levels. Moreover, the addition of EX-527 before and after grape juice intake showed different results.

Before grape juice intake, the addition of EX-527 increased the intracellular NO levels, probably due to the same mechanism of inhibition of eNOS uncoupling, as discussed previously. After 1 h of grape juice intake, EX-527 decreased NO levels, as expected in a SIRT1 inhibition condition. Collectively, these results suggest that this difference might be explained by the great variety of biologically active compounds present in grape juice, such as anthocyanins, flavanols (or flavan-3-ols or catechins), and proanthocyanidins [51], rather than stilbenes alone, such as resveratrol and its derivatives (pterostilbene, piceid, and viniferins). Interestingly, a previous study showed that even resveratrol-poor red wines are capable of modulating the expression of sirtuins in renal cells [52] and other molecules present in grape juice and wines, such as catechin and epicatechin, have shown beneficial results in reducing arginase and NADPH oxidase activity in HUVECs from women with PE [53]. A previous study showed that organic whole grape juices from the same location (Rio Grande do Sul) and the same variety (bordo) from the juice used in this study have concentrations of 3.73 ± 0.19 mg/L of trans-resveratrol as well as catechin (500.52 ± 12.33) and other molecules of possible interest [54]. Here, we propose that the combination of the grape juice biologically active molecules successfully recovered the eNOS uncoupling and reestablished the physiological NO endothelium balance, enabling SIRT1 proper activity over eNOS.

Although resveratrol concentrations utilized *in vitro* are several-fold higher than those measured *in vivo* following dietary supplementation [55], many factors might affect free polyphenol concentrations and should be considered in interpreting these studies. For example, the presence of serum (both human and bovine) in the culture medium is an important factor that limits comparisons between *in vitro* and *in vivo* concentrations, since resveratrol is highly lipophilic and binds to serum albumin and other proteins. Therefore, free resveratrol concentrations in which HUVECs were initially exposed might have been much lower than the initial 10 μM dose proposed in our study after 24 h [56,57]. Thereupon, we can not completely refuse possible solo resveratrol beneficial effects in PE at higher concentrations and further studies are necessary to assess resveratrol *versus* grape juice intake beneficial effects in PE.

6. Conclusion

Finally, our study suggests that although resveratrol might have shown beneficial effects in cardiovascular diseases, it has no positive effects on our endothelial *in vitro* model of PE at a 10 μM dose. Furthermore, GH and PE groups seem to have different responsive mechanisms to this molecule, emphasizing the differences in the pathophysiology of these diseases. Moreover, SIRT1 seems to play an important role in PE regarding endothelial antioxidant activity and NO bioavailability, denoting the necessity of further investigation on the activity of this protein in PE pathophysiology. Our grape juice study results proposed that the combination of grapevine biologically active compounds might have better results in reestablishing endothelial NO bioavailability in PE. We conclude that activating SIRT1 by using resveratrol alone without controlling the eNOS uncoupling characteristic of PE may not be beneficial to these women. Instead, future studies should investigate the possible combinations of biologically active molecules present in grapevines in the SIRT1-eNOS-NO axis in PE.

Data statement

The data that supports the findings in this study are available from the corresponding author upon reasonable request.

Funding sources

This work was supported by the National Council for Scientific and Technological Development (CNPq-Brazil) [Grant Numbers #2014-5/305587, #301293/2018-0], and by the São Paulo Research Foundation (FAPESP-Brazil) [#2015/20461-8, #2019/06118-0, #2019/07230-8].

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

All authors thank Pamela Camila Furlanetto for assistance in cell culture maintenance and experiments.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110659.

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