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Protective effects of luteolin on the venous endothelium

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Abstract
Luteolin is a flavonoid with antioxidant properties already demonstrated in studies related to inflammation, tumor, and cardiovascular processes; however, there are no available information regarding its antioxidant effects at the venous endothelial site. We investigated the effects of luteolin (10, 20, and 50 μmol/L) in cultures of rat venous endothelial cells. Nitric oxide (NO) and reactive oxygen species (ROS) were analyzed by fluorimetry; 3-nitrotyrosine (3-NT) residues were evaluated by immunofluorescence, and prostacyclin (PGI_2) release was investigated by colorimetry. Intracellular NO levels were significantly enhanced after 10 min of luteolin incubation, with a parallel decrease in ROS generation. These results were accompanied by a significant reduction in the expression of 3-NT residues and enhanced PGI_2 rates. Therefore, luteolin is effective in reducing ROS thereby improving NO availability in venous endothelial cells. Besides, luteolin-induced decrease in 3-NT residues may correlate with the enhancement in endothelial PGI_2 bioavailability. These findings suggest the future application of this flavonoid as a protective agent by improving endothelial function in several circulatory disorders related to venous insufficiency.

Keywords Luteolin · Venous endothelium · Nitric oxide · Superoxide · 3-NT · Prostacyclin

Introduction

Luteolin (3,4,5,7-tetrahydroxyl-flavone) is a flavonoid present in numerous herbs, fruits, and vegetables such as chamomile, apple, pomegranate, cress, parsley, mint, basil, celery, and artichoke leaves. Its use as a food supplement is widespread, and its antioxidant properties have been widely demonstrated in a series of experimental studies related to inflammation, tumor, and cardiovascular processes [1–3].

One of the main proposed mechanism to explain the benefits of flavonoids in the control of various pathological conditions and specifically in cardiovascular diseases is precisely their antioxidant action and prevention of endothelial nitric oxide (NO) degradation [4, 5]. Interestingly, however, the vascular effects of these molecules can be paradoxical. Flavonoids can undergo self-oxidation, produce superoxide anion ($O_2^{\bullet-}$), and destroy NO [6]. The final results and consequences of this ambiguity of effects exerted by flavonoids on the vasculature are not yet fully elucidated. In addition, specialized articles have reported that more precise information regarding flavonoids actions at the endothelial site is still lacking [2, 5].

The endothelial cell is strategically located in the blood vessel, being an interface between blood and the vascular smooth muscle. Several mechanical and biochemical signals can stimulate the production and release of endothelial-derived vasoactive substances such as prostacyclin (PGI_2), NO, and $O_2^{\bullet-}$, which possess endocrine, paracrine, and autocrine functions. It is well accepted that the major influence of endothelium in regulating vascular functions is directly related to its ability in maintaining balanced levels of these signaling molecules, therefore modulating vascular wall tone and growth, besides interfering with blood coagulation and inflammatory responses [7–9].

PGI_2 was the first endothelial vasorelaxant mediator identified and is the most abundant product of arachidonic acid produced by vascular tissues. It is formed by PGI_2 synthase, an enzyme highly expressed in endothelial cells. Once

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synthesized and released, PGI_2 exerts its effects locally, and is quickly converted to an inactive metabolite ($PGF_1\alpha$) through a non-enzymatic process. Among its several properties, PGI_2 causes vasodilation and inhibits platelet aggregation by activating specific G-protein coupled receptors. Generally, its effects involve activation of adenylyl cyclase and the subsequent intracellular cAMP enhancement [8, 10].

NO is one of the main mediators produced and secreted by the vascular endothelium and it is generated in different cell types through the NO synthase (NOS) enzyme. NOS catalyzes the oxidation of the L-arginine amino acid into L-citrulline and NO, involving a complex reaction that requires the presence of the cofactors tetrahydrobiopterin (BH_4), flavin mononucleotide, flavin adenine dinucleotide, NADPH, and molecular oxygen consumption. Once synthesized, it diffuses into the smooth muscle layer of the vessel and stimulates soluble guanylyl cyclase, increasing the cytosolic concentration of cGMP and causing vascular relaxation. In addition to vasodilation, endothelial NO exerts several other effects, such as inhibition of smooth muscle cell proliferation, synthesis of extracellular matrix proteins, inhibition of platelet and leukocyte adhesion, and facilitation of platelet aggregates dissolution [9, 11].

Like the other factors produced by the endothelial cell, $O_2^{\bullet-}$ and other reactive oxygen species (ROS) are produced in a controlled manner and at low concentrations. These signaling molecules participate in the maintenance of endothelial function, and play an important role in intracellular processes such as induction of protein expression involved in the inflammatory process and cell growth. Baseline $O_2^{\bullet-}$ levels are regulated by the balance between the rates of its generation and metabolism, regulated in part by the activity of the Superoxide Dismutase (SOD) enzyme. $O_2^{\bullet-}$ reacts directly with NO, reducing its bioavailability and generating peroxynitrite ($ONOO^-$), a particularly harmful reactive intermediate. $ONOO^-$ is capable of decaying into the hydroxyl radical ($OH^{\bullet}$), and there are evidences that it can induce a number of cellular modifications including nitration of tyrosine residues which are modified in the 3-position of the phenolic ring through the addition of a nitro group (NO_2). 3-nitrotyrosine (3-NT) therefore can be considered a biomarker for $ONOO^-$ action in a variety of disease states and in conditions of cellular damage and oxidative/nitrosative stress [12, 13].

The venous compartment is crucial for the functioning of the circulatory system, since venous circulation can store about 60–80% of the total blood volume in mammals at rest [14]. For this reason, changes in venous tone (promoted by neurotransmitters, hormones, or drugs) induce physiological consequences similar to those promoted by acute changes in blood volume and, thus, can significantly alter cardiac output and all blood circulation. Furthermore, venous endothelium is the first site of inflammatory processes by increasing permeability and expressing several proteins involved in leukocyte behavior [15]. Nevertheless, studies concerning venous endothelial function and endothelial-derived factors from veins are still very scarce. To date, very little is known about the ability of the venous endothelium in the management of redox state.

From these observations, the present study investigated the effects of the flavonoid luteolin on the venous endothelium by using immortalized cultures of endothelial cells previously obtained from rat vena cava. After luteolin treatment, NO and ROS production was determined by fluorimetric methodologies. In parallel, the presence of 3-NT residues was evaluated by immunofluorescence, and the release of PGI_2 was analyzed by colorimetry.

Methods

Cell culture and viability

Venous endothelium cultures were generated in our previous work where endothelial primary cultures were obtained from rat vena cava explants [16]. In this preceding study, we had demonstrated that primary cultures of vena cava endothelium achieved the highest number of viable cells at the 6th passage. From this moment, cultures entered in senescence, which lasted until 8th passage. After this, cellular multiplication was recovered, and growth rates were markedly increased between the 9th and 10th passages. Then, cultures were propagated (15th to 20th passages) and spontaneously immortalized. In the present study, these endothelial cells were cultivated in Dulbecco's Modified Eagle's Medium low glucose supplemented with Fetal Bovine Serum (FBS, 10%) and penicillin (50 U/mL)/streptomycin (50 μg/mL), pH 7.4, in a 5% CO_2 incubator (Sheldon Mfg. Inc. USA), at 37 °C. Cells grew to confluence and propagated in 1:2 ratio using TrypLE™ Express Enzyme (Thermo Fisher Scientific, USA). Functional assays were performed during semi-confluent stages. All procedures were approved and performed in accordance with the guidelines of the Ethics Committee of UNIFESP (Protocol no. 2689270319).

In order to evaluate the cell viability after luteolin incubation, cells were seeded ($n = 5$/group) in 24-well plates and left overnight. Cells were washed in PBS and luteolin (previously diluted in culture medium) was added at concentrations of 10, 20, or 50 μmol/L. Negative and positive control groups were performed by incubating cells with culture medium and dimethyl sulfoxide (DMSO, 5%), respectively. After 24 h, cells were washed and trypsinized. Then, each sample was tested by an automated cell counter (LUNA-FL™ Logos Biosystems, KOR) equipped with dual fluorescence optics, allowing simultaneous quantification of alive and dead cells by propidium iodide and acridine orange

staining, respectively. Results from this viability test were expressed as the percentage of alive cells in each sample.

In another set of experiments, cellular viability was also tested by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Thermo Fisher Scientifc, USA), a colorimetric assay. On the previous day, endothelial cells (10^4) were seeded in 96-well plates ($n = 3$/group) in phenol red-free culture medium and then incubated for 24 h with 10, 20, and 50 μmol/L luteolin. The control group (baseline) was incubated with the culture medium only. After incubation period, cultures were washed in PBS and incubated with MTT reagent (10 mmol/L/well) for 4 h at 37 °C. Then, a HCl [0.01 mol/L]/SDS [0.3 mol/L] solution was added and incubated for another 4 h at 37 °C. Samples were homogenized and absorbance was read on a spectrophotometer (Epoch-Biotek Instruments, USA), at 570 nm. Data were analyzed and plotted according to the manufacturer's protocol. Results were expressed in arbitrary units.

Confocal imaging studies for NO and ROS detections

NO production was determined by using the fluorescent cell permeable dye 4,5-diaminofluorescein diacetate (DAF-2DA) (Ex: 485 nm; Em: 538 nm) [17]. ROS production was determined in endothelial cells by using the fluorescent cell permeable dye dihydroethidine (DHE) (Ex: 490 nm; Em: 590 nm) [18].

Cells (6×10^4) were seeded (in duplicate) on sterile glass coverslips (pre-treated with 1% gelatin) and incubated with culture medium for 24 h. On the day of experiment, cells were washed with PBS and incubated with 10 μmol/L DAF-2DA or 10 μmol/L DHE during 30 min, at 37 °C. Cells were washed again and incubated with 10, 20, or 50 μmol/L luteolin for 10 min, at 37 °C. Basal control groups were performed by incubating cells with culture medium. Then, cells were washed in PBS and fixed in paraformaldehyde (4% PFA) at room temperature for 30 min. Coverslips were mounted with Fluoromount™ Aqueous Mounting Medium and observed in a confocal microscope (TCS SP2—Leica Microsystems, GER). Images were analyzed by densitometry using the ImageJ Image Processing and Analysis™ in Java software. Specific software tools were employed in order to recognize and demarcate cells individually. Fluorescence intensity quantification was determined by the sum of the pixels in relation to the area of each cell. Detection of NO and ROS production was determined, at least, in six fields of each slice, and fluorescence intensity was expressed in arbitrary units.

NO and ROS detection by spectrofluorimetry

Cells (1.5×10^4) were seeded in 96-well microplates and left overnight ($n = 7$–8). Wells were washed in PBS and incubated for 10 min (for NO assay) or 24 h (for ROS assay) with 10, 20, or 50 μmol/L luteolin at 37 °C, 5% CO_2. Basal groups received culture medium, and 600 μmol/L Tempol, a SOD mimetic, was used as an antioxidant control (for ROS assay). Then, cells were treated with 10 μmol/L DAF-2DA or 10 μmol/L DHE for 30 min at 37 °C. At the end of incubation periods, cells were washed and 50 μL of PBS were added to each well before reading. Fluorescence was determined in a microplate reader (Synergy HT/BioTek, USA), as previously described [19]. Fluorescence intensity was expressed in arbitrary units.

3-NT detection by immunofluorescence

Cells (10^5) were seeded (in duplicate) on sterile glass coverslips (13 mm) pre-treated with 1% gelatin distributed in 12-well microplates and left overnight. Then, cells were washed in PBS and incubated with 10, 20, or 50 μmol/L luteolin for 10 min, at 37 °C. Coverslips were washed in PBS and fixed with 4% PFA at room temperature. Cells were then permeabilized with 1% Nonidet P40 and blocked with 1% bovine serum albumin (BSA) in PBS, for 30 min. Samples were incubated overnight at 4 °C with primary rabbit antibody anti-nitrotyrosine (#A-21285, Invitrogen), 1:50 dilution. Protein staining was detected using secondary antibody bovine anti-rabbit IgG-Texas Red conjugated (sc-2365, Santa Cruz Biotech), during 60 min, at room temperature, 1:100 dilution. Control assays were performed in coverslips incubated only with 1% BSA followed with secondary antibody. The cell nucleus was counterstained with 4′,6-diamidino-2-phenylindole (DAPI) at 1:400 dilution for 5 min at 37 °C. Coverslips were mounted with Fluoromount™ Aqueous Mounting Medium and observed in a confocal microscope (TCS SP2—Leica Microsystems, GER) by using appropriate wavelengths for Texas Red stain detection. Obtained images were analyzed by densitometry using the ImageJ Image Processing and Analysis in Java™ software. Images were acquired in at least five to six fields, covering an average of 120 cells evaluated in each group. Fluorescence intensity was expressed in arbitrary units.

Determination of PGI_2 levels by enzyme immunoassay

The levels of PGI_2 (measured as its metabolite 6-keto-$PGF_{1\alpha}$) were determined by enzyme immunoassay kits (Cayman Chemical Co., USA). Cells (7×10^4) were seeded in 48-well plates containing culture medium, placed in the CO_2 incubator, and left overnight ($n = 5$). Cultures were washed in PBS and incubated with phenol red-free culture medium in the presence or absence of 10, 20, or 50 μmol/L luteolin for 24 h, 37 °C, 5% CO_2. Medium was collected and dilutions of supernatants were incubated

with the conjugated eicosanoid-acetylcholinesterase, as well as with the specific antiserum in 96-well plates precoated with anti-rabbit IgG antibodies. After overnight incubation at 4 °C, plates were washed and the enzyme substrate (Ellman's reagent) was added for 60–120 min at 25 °C. The optical density of the samples was determined at 560 nm in a microplate reader (Epoch-Biotek Instruments Inc., USA). PGI_2 concentration was calculated from the standard curve, as described by the manufacture's protocol. Data were normalized by basal values and expressed as arbitrary units.

Statistical analysis

Results were presented as the means ± standard error mean (SEM). Data were analyzed by ANOVA followed by Tukey–Kramer test. *P* values <0.05 were considered to be statistically significant.

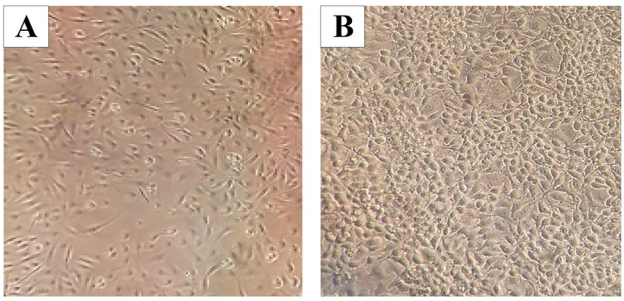

Fig. 1 Representative images of venous endothelial cells in cultures at semiconfluence (**a**) and confluence (**b**) stages, observed by optical microscopy (100×)

Drugs and reagents

Luteolin was purchased from Cayman Chemical; DAPI, DMSO, Fluoromount Aqueous Mounting Medium from Sigma-Aldrich, DAF-2DA and Tempol from Enzo Life Sciences and DHE from Polyscience. FBS (South American) was provided by Thermo Fisher Sci. Materials and solutions for cellular cultivation and all other reagents were from Thermo Fisher Sci.

Results

Cell culture and viability

Immortalized cultures of rat venous endothelial cells presented a "cobblestone" morphology pattern at confluence (Fig. 1). When exposed for 24 h to several concentrations of luteolin these cells presented high viability in all tested groups. By using an automated cell counter, no differences in the percentage number of alive cells were detected, with values (%) of 90.3 ± 3.9 for basal (untreated) group, 89.4 ± 2.4 for 10 μmol/L luteolin, 84.9 ± 4.3 for 20 μmol/L luteolin, and 84.8 ± 4.6 for 50 μmol/L luteolin (Fig. 2a). These results were confirmed by MTT assay, were no significant alterations in cell viability, and were found among tested groups (Fig. 2b). Positive control with DMSO 5% decreased cellular viability in about 40% (not shown).

NO production

Confocal microscopy experiments showed a consistent basal NO production in cells pre-treated with 10 μmol/L DAF-2DA (Fig. 3a, basal group). After incubation with 10, 20,

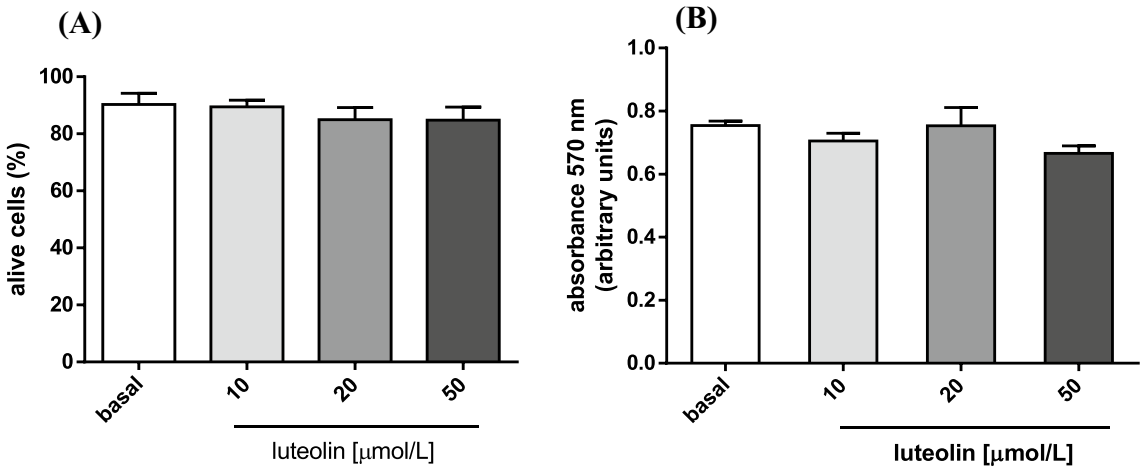

Fig. 2 Cellular viability after 24 h of incubation with luteolin. Data were obtained by an automated cell counter ($n = 5$) (**a**), and MTT method ($n = 3$) (**b**). Values represent mean ± SEM

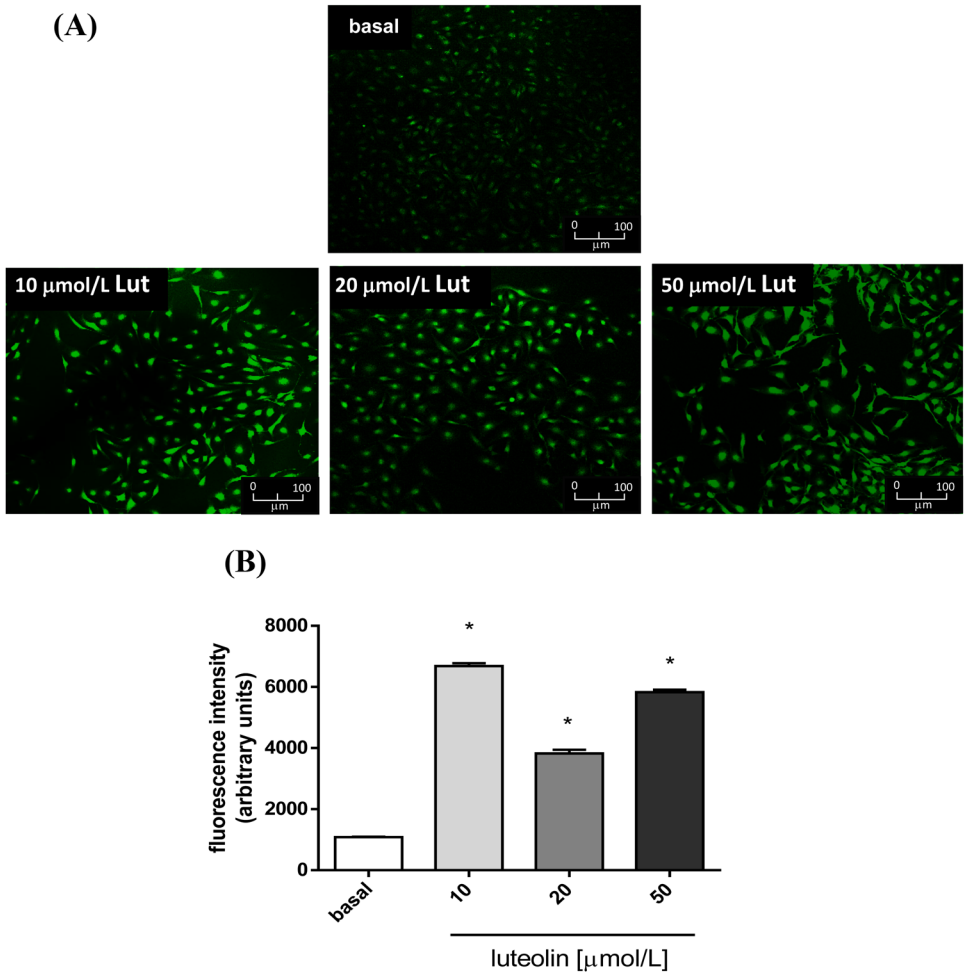

Fig. 3 NO detection by confocal microscopy in venous endothelial cells pre-treated with 10 μmol/L DAF-2DA and incubated with luteolin (Lut) for 10 min. (**a**) Representative images (200×) and (**b**) fluorescence intensity determined by densitometry. Values represent mean ± SEM ($n = 6$) *$P < 0.05$ vs basal

and 50 μmol/L luteolin, a significant enhancement of NO release was observed, with no differences among luteolin treated groups. Representative images and fluorescence intensity are demonstrated in Fig. 3.

When intracellular NO levels were measured a microplate reader, a significant increase was observed in cells incubated with 50 μmol/L luteolin, with no detectable effect for the lower concentrations tested. Results are shown in Fig. 4.

ROS production

Confocal microscopy experiments were performed in cells pre-treated with the fluorescent dye 10 μmol/L DHE. The original image was cropped and the color pattern was changed so that it could be recognized by the ImageJ software. After being processed, the pattern recognition tool was applied and each cell was selected individually for later fluorescence quantification. Quantitative analysis was based in the fluorescence intensity determined by the sum of the pixels in relation to the area of each cell (Fig. 5a). At resting conditions, the venous endothelium exhibited significant fluorescence intensity, as represented in Fig. 5b (basal

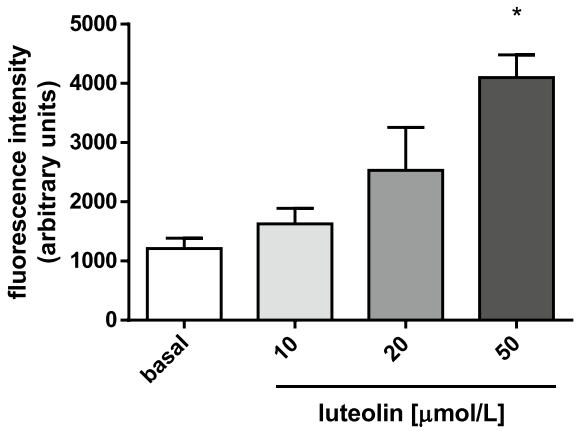

Fig. 4 NO detection by spectrofluorimetry in alive venous endothelial cells pre-treated with 10 μmol/L DAF-2DA and incubated with luteolin for 10 min. Values represent mean ± SEM ($n = 7$) *$P < 0.05$ vs basal

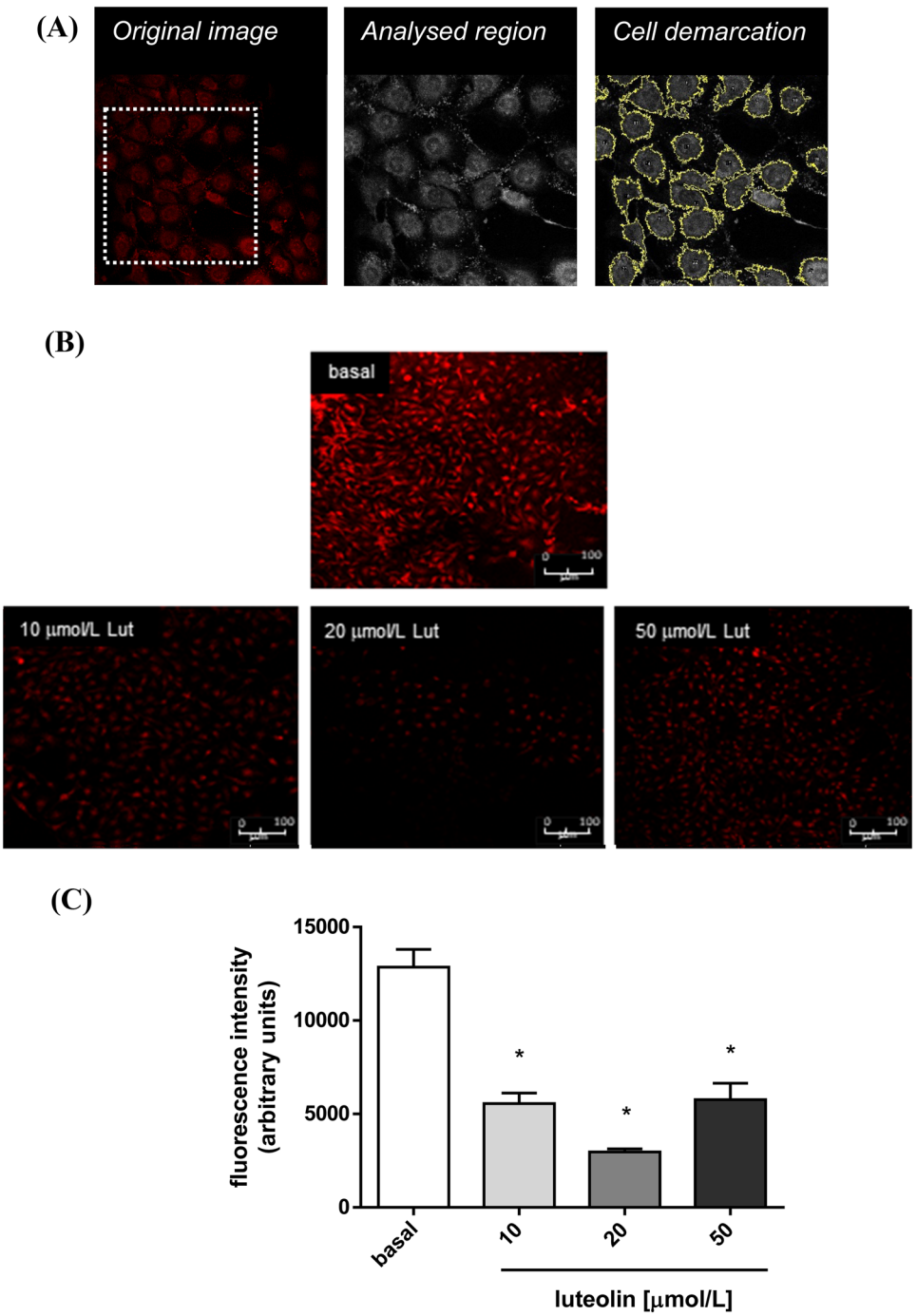

Fig. 5 ROS detection by confocal microscopy in venous endothelial cells pre-treated with 10 μmol/L DHE and incubated with luteolin (Lut) for 10 min. (**a**) Image processing for analysis (400×); (**b**) representative images of basal and treated groups (200×), and (**c**) fluorescence intensity determined by densitometry. Values represent mean ± SEM ($n = 6$) *$P < 0.05$ vs basal

group). Luteolin incubation for 10 min was highly effective in reducing ROS production. This effect was observed for all luteolin-tested concentrations, with no differences among exposed groups (Fig. 5c).

In addition to image studies, ROS production was also investigated by spectrofluorimetry. In this case, cells were incubated with luteolin for 24 h, and the fluorescent dye DHE was applied 30 min before fluorescence measure. As demonstrated in Fig. 6, ROS production was significantly reduced by 10, 20, and 50 μmol/L luteolin. Values found for luteolin were similar to those observed for the SOD mimetic Tempol. Results are represented in Fig. 6.

Tyrosine residues nitration

The presence of 3-NT was studied by immunofluorescence. As demonstrated in Fig. 7, incubation of venous endothelial cells with 10, 20, or 50 μmol/L luteolin for 10 min significantly decreased the fluorescence staining in comparison to the basal values.

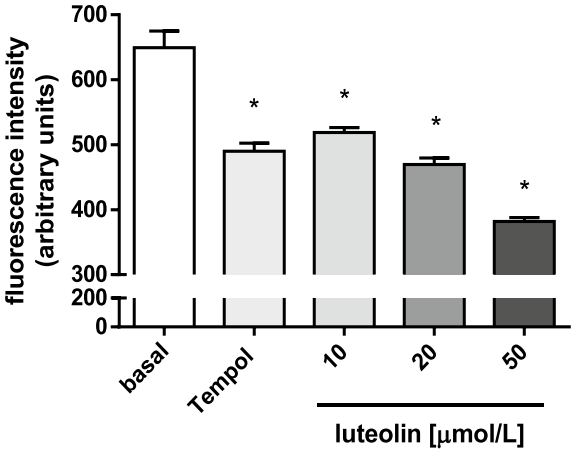

Fig. 6 ROS detection by spectrofluorimetry in alive venous endothelial cells pre-treated with 10 μmol/L DHE and with luteolin for 24 h. Values represent mean ± SEM ($n = 8$) *$P < 0.05$ vs basal

PGI_2 levels

After 24 h of incubation, 50 μmol/L luteolin induced a significant increase in PGI_2 levels detected in the supernatants of venous endothelial cells. The same effect was not observed for lower concentrations of the flavonoid. Results are shown in Fig. 8.

Discussion

Endothelial cells possess an extensive and complex biochemical network involved in both pro- and antioxidant species generation. Unbalanced cellular redox state accounts for important and deleterious consequences in the whole circulatory system, altering blood pressure, cell proliferation, and inflammatory responses, among other effects.

Various plant polyphenols have been recognized as redox active molecules [5]. Luteolin is a natural antioxidant with less pro-oxidant potential than the flavonol quercetin, the best studied flavonoid, but apparently with a better safety profile. It displays excellent radical scavenging and cytoprotective properties, especially when tested in complex biological systems [20]. It also possesses specific effects in the cardiovascular system, such as blood pressure decrease, cardiac remodeling reduction, and proliferation and migration inhibition in vascular smooth muscle cells [21, 22]. In

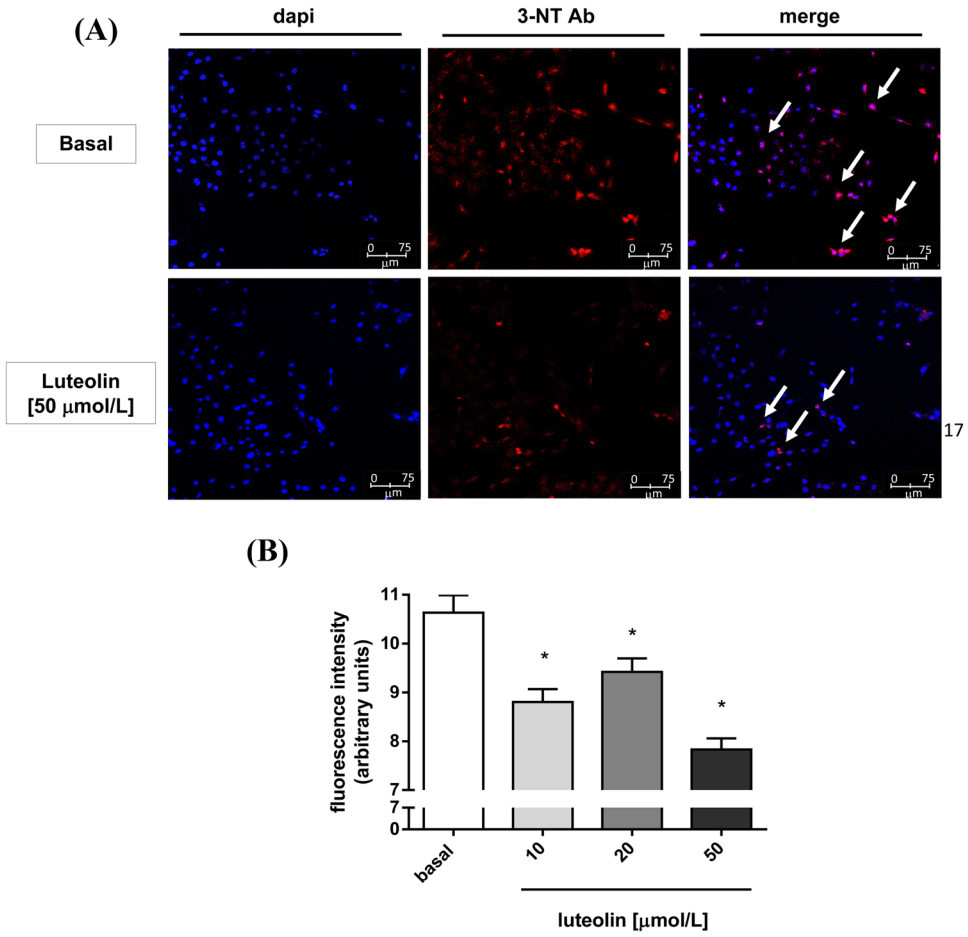

Fig. 7 Expression of 3-NT in venous endothelial cells detected by immunofluorescence. Representative images of cells at basal state and after incubation with 50 μmol/L luteolin, observed by confocal microscopy (200×); 3-NT residues were stained in red (Texas Red) and nucleus counterstained in blue (DAPI); arrows indicate positive staining (**a**). Fluorescence intensity was determined by densitometry (**b**). Values represent mean ± SEM ($n = 5$–7 fields) *$P < 0.05$ vs basal. (Color figure online)

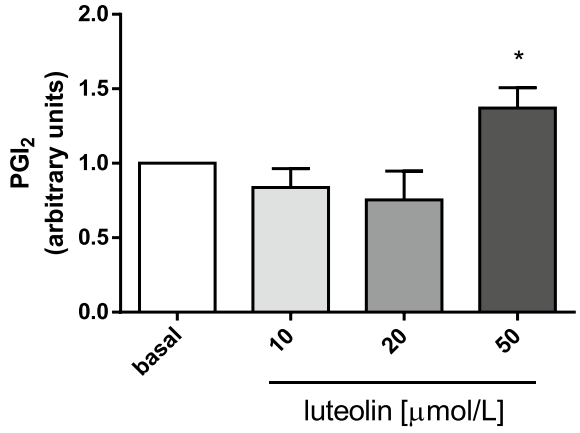

Fig. 8 PGI_2 levels measurements in the supernatant of cultured venous endothelial cells determined by enzyme immunoassay. Values represent mean ± SEM ($n = 5$) *$P < 0.05$ vs basal

arterial beds preparations, luteolin evokes dose-dependent vasorelaxation, but the dependence of the endothelium in this effect is still debatable [23–25]. Despites this uncertainty, luteolin appears to increase the production of NO and prevent ROS generation [25–27]. It can also improve endothelial function in cultured cells submitted to high glucose levels [28], attenuate diabetes-induced impairment in endothelial-dependent relaxation [29], and prevent systemic metabolic and vascular alterations associated with obesity [30]. Taken together, these data point to important effects of luteolin in the whole circulatory system by preventing oxidative stress and vascular injury. In order to provide a better understanding about luteolin actions at the endothelial site, the present study investigated its ability in modulating venous endothelial redox state by using immortalized cultures of rat venous endothelial cells.

Firstly, we analyzed luteolin cytotoxicity by two different methodologies. In both, exposure of venous endothelial cell cultures to 10, 20, and 50 μmol/L of luteolin for 24 h had no effect on cell viability; therefore, these concentrations were chosen for all further experiments. These results are in agreement with the previous findings in human endothelial cell lines [4, 31], however, luteolin safety levels at endothelium are still highly controversial. Similar concentrations of luteolin exhibited significant cytotoxicity in human umbilical vein endothelial cells after 48 h of incubation [28] with a LC_{50} value of 57 μmol/L [32]. These conflicting results reveal that luteolin cytotoxicity might be further elucidated, thereby making it possible to determine the appropriate therapeutic dosages.

In our experiments, NO production was assessed by DAF-2DA, a cell permeable fluorescent dye specific for NO detection. Once inside the cells, DAF-2DA is hydrolyzed by cytosolic esterases thus releasing DAF-2. The reaction between DAF-2 and NO yields the corresponding stable bright green-fluorescent 4,5-diaminofluorescein triazole (DAF-2T). Incubation of resting venous endothelial cells with micromolar concentrations of luteolin resulted in significant enhancement of intracellular fluorescence, which correlates with NO production. This result corroborates previous findings obtained indirectly by measuring the NO metabolites nitrite/nitrate in human endothelial cell lines pre-treated with luteolin [25, 28, 33]; however, to our knowledge, this is the first direct evidence of the intracellular endothelial NO production after luteolin treatment.

In a second step of our study, the probe DHE was used to verify the presumed antioxidant effect of luteolin in the venous endothelium. Endothelial cells are permeable to DHE, which is converted to the fluorescent products 2-hydroxyethidium (2-E^+OH) and ethidium (E^+). The first one is produced solely in the presence of $O_2^{\bullet-}$, so that 2-E^+OH detection is a marker of $O_2^{\bullet-}$ among many other ROS. In parallel, E^+ identification comprehends the total oxidant generation, including $OH^\bullet$ and $ONOO^-$ [34, 35]. Since both compounds (2-E^+OH and E^+) are fluorescent, the methodologies employed by our group (confocal microscopy and fluorometric detection) had limitations because they did not distinguish the effect of luteolin over amounts of each component of pro-oxidant signaling cascade ($O_2^{\bullet-}$, $OH^\bullet$, and $ONOO^-$). Nonetheless, these results clearly indicate that micromolar concentrations of luteolin are competent to promote both early (10 min) and late (24 h) antioxidant effects in resting venous endothelial cells.

Besides, the present study revealed for the first time the effect of luteolin in reducing protein nitration in endothelial cells. Tyrosine residue nitration is a covalent post-translational modification derived from the reaction of proteins with nitrating agents [12], leading to important alterations and loss of function of enzymes and other signaling proteins. Therefore, tyrosine nitration is a convenient marker of reactive nitrogen-centered oxidants being produced, and it is important to highlight that $ONOO^-$ produced from NO/$O_2^{\bullet-}$ reaction is the most likely source of 3-NT in vivo [36].

Previous studies have shown that, at vasculature, tyrosine nitration of PGI_2 synthase can account for several pathological processes, including endothelial dysfunction and atherosclerosis [37–39]. Our results showed significant effects of luteolin in reducing 3-NT, accompanied by an increase in PGI_2 levels in venous endothelial cells. Accordingly, increases in PGI_2 synthesis have already been reported in cultured human aortic endothelial cells and in plasma of healthy adult subjects after treatment with pomegranate juice, a luteolin-rich beverage [40]. Taken together, these data may indicate a possible correlation between the antioxidant effects of luteolin and the prevention of PGI_2 synthase nitration, enhancing PGI_2 levels in the vasculature.

The importance of endothelium in modulating circulatory functions was firstly evidenced by Furchgott and Zawadzki

in 1980 [41], and represented a major impetus for other discoveries, opening frontiers for a number of studies in the vascular physiology field. More recently, the development of molecular and biochemical tools has allowed a deeper understanding about the role of each endothelial-derived factor as well as the redox state in vascular homeostasis. However, most of these new findings are related to arterial functions with focus on peripheral resistance and blood pressure regulation, while little is known about the venous endothelium. Importantly, in spite of the role of endothelium in modulating venous tonus and blood distribution, venous endothelium-derived mediators also influence the blood physiology that is contained in the venous bed.

In several vascular conditions associated with arterial disease and endothelium dysfunction, the venous endothelium appears to be either altered. For example, in hypertensive subjects, the impairment in endothelium-dependent vasodilation is present in both arterial and venous circuits [42]. Besides, clinical observations have associated the venous thromboembolism with arterial thrombosis, a condition with straight relation to endothelial dysfunction [43].

One of the most commented practical significance of venous endothelium is how it can decisively affect the evolution of vein grafts implanted in situations of coronary artery disease. Vein graft failure in the coronary artery bypass grafting correlates with vascular inflammation, intimal hyperplasia, and accelerated atherosclerosis [44]. The bioavailability of NO and PGI_2 is fundamental to the maintenance of vascular tone of these vein grafts, as well as the regulation of vascular smooth muscle cell proliferation, migration, and contractility. Endothelial cells are highly sensitive to their mechanical environment, for example, the degree of fluid shear stress on their plasma membrane, the stretch due to vessel distension, and the stiffness of the extracellular matrix to which they are attached. Upon acute high shear stress insult following immediate implantation into the arterial circulation, venous endothelial cells activate several inflammatory acute mechanisms. Following, there is activation of a more prolonged (chronic) inflammatory response, including increased ROS generation and expression of adhesion molecules, cytokines, and chemokines. Late stage of graft failure is characterized by the prolonged development of superimposed atherosclerosis on the vessel wall and occurs in approximately 50–60% of grafts within 10 years [45]. There is a consensus that the endothelial dysfunction characterized by reduced NO and PGI_2 availability, accompanied by elevated rates of $O_2^{\bullet-}$ and $ONOO^-$ are responsible for producing a highly atherogenic environment which is directly involved in vascular smooth muscle proliferation and vein graft failure [15].

In addition, altered venous endothelial function appears to be associated with chronic venous disease. Inflammatory responses activated by dysfunctional endothelial cells can contribute to venular degeneration and venous insufficiency, leading to improper blood return from the lower extremities to the heart, and accounting for a range of clinical presentations including tortuous, distended veins in lower extremities, increasing skin pigmentation, and in severe cases, ulceration of the affected skin. Patients with varicose veins exhibit increased synthesis of endothelial-derived pro-inflammatory molecules and reduced vasodilation in response to acetylcholine, an endothelium-dependent mediator [46]. In view of this, authors have suggested that further clinical studies should consider the valid usefulness of antioxidants for the prevention and treatment of venous insufficiency [47].

In summary, the venous endothelium plays a central role in vascular physiology. However, to our knowledge, it is still an unexplored site since few and inconclusive studies have pointed to differences in endothelial NO and ROS production between veins and arteries [42, 48]. Indeed, the capacity of the venous endothelial cell in the management of its redox state might be decisive in several vascular situations. In view of this, the major contribution of our study is the demonstration that luteolin is effective in reducing ROS thereby improving NO availability in cultured venous endothelial cells. Furthermore, these results also suggest that luteolin-induced decrease in 3-NT residues may correlate with the enhancement in endothelial PGI_2 bioavailability. These findings suggest the future application of this flavonoid as a protective agent by improving endothelial function in several circulatory disorders related to venous insufficiency.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were approved and performed in accordance with the guidelines of the Ethics Committee of UNIFESP (Protocol No. 2689270319).

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