Journal of Functional Foods 86 (2021) 104731

Contents lists available at ScienceDirect

Journal of Functional Foods

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

Citrus flavonoids effects on human umbilical vein

Christian Pinna [a], Angelo Sala [a,b,*]

[a] Department of Pharmaceutical Sciences, University of Milan, via Balzaretti 9, 20133 Milano, Italy
[b] IRIB, National Research Council, Via Ugo la Malfa 153, 90146 Palermo, Italy

ARTICLE INFO

Keywords:
Human umbilical vein
Vasorelaxation
Flavonoids
Gender-related activity
Endothelium

ABSTRACT

Flavonoids intake is associated to beneficial effects on the cardiovascular system. Aim of this study was to investigate the biological activities of hesperidin and diosmin on the vascular tone of human umbilical vein (HUV). Umbilical cords obtained at delivery from healthy women were set up for recording of isometric tension; vasorelaxing activity of flavonoids was assessed in serotonin-precontracted HUV rings. Hesperidin evoked a more profound vasorelaxation than diosmin, and inhibition of NO-synthase significantly impaired relaxant responses to hesperidin but completely abolished those to diosmin. The residual vasorelaxation to hesperidin was prevented by glybenclamide (a K_{ATP} channel blocker). Endothelium-dependent responses to hesperidin were larger in female- when compared to male-derived HUV. β-estradiol and a direct NO-donor compound caused a marked vasorelaxation irrespective of gender of the HUV. Diosmin and, in particular, hesperidin may exert a beneficial effect on the vascular and endothelial function of HUV, and help preventing fetal vascular complications.

1. Introduction

Natural polyphenols are the most common dietary antioxidants, abundantly present in many fruits, vegetables and seeds; they exert a wide range of beneficial effects related to the prevention or the modulation of chronic diseases including gestational diabetes (Pham, Do, & Lee, 2019) and cardiovascular disease (Habauzit & Morand, 2012). Polyphenols prevent endothelial dysfunction improving the release of NO (Monsalve, Concha-Meyer, Palomo, & Fuentes, 2017; Oak et al., 2018), and protect cell constituents against oxidative damage (Billingsley & Carbone, 2018). Preeclampsia occurs more frequently among women who rarely consumed daily serving of fruit and vegetables during pregnancy (Pistollato et al., 2015), and an appropriate antioxidant capacity has been reported to prevent or attenuate its severity (Ly et al., 2015) as well as that of other pregnancy disorders such as intrauterine growth restriction (Parlapani et al., 2019).

Diosmin and hesperidin are the predominant flavonoid found in many *Citrus* species, and their titrated formulation is widely used in venous insufficiency, where significant evidence of efficacy are available (Ramelet, 2001; Ulloa, 2019). Diosmin has also been reported as antihypertensive and vasoprotective, including the ability to reverse nitric oxide alterations in hypertensive rats (Silambarasan & Raja, 2012); hesperidin is presumed to contribute to the vascular protective effects of several *Citrus* products and beverages in humans (Constans et al., 2015; Morand et al., 2011), and a recent clinical trial reported that 4-week consumption of orange juice (500 ml/day) as well as of control drink plus hesperidin resulted in a significantly decrease of diastolic blood pressure in healthy volunteers (Habauzit & Morand, 2012). However, scant evidence is available in support of their use during gestation (Achamrah & Ditisheim, 2018; Dodd, O'Brien, & Grivell, 2014; Pistollato et al., 2015) and only little is known about flavonoid-induced activity on human umbilical vein (HUV) (Protić et al., 2014). Indeed, not all flavonoids have shown biological activity on endothelial cells (Nicholson, Tucker, & Brameld, 2010) or are able to cross the placental barrier. In addition, there are significant anatomical and physiological differences between HUV and other venous vessels, such as it carries oxygenated blood from placenta to the fetus and its vascular tone is not under control of the nervous system (Lachenmayer, 1971; Sastry, 1997). Additionally, acetylcholine as well as histamine cause vasoconstriction rather than vasorelaxation, this latter being the response observed in almost all kinds of intact blood vessels (Chen et al., 2015).

Given their reported vasoprotective activities and their ability to affect endothelial-derived nitric oxide production, the present study was designed to evaluate the effects of *Citrus*-derived flavonoids hesperidin and diosmin on HUV.

* Corresponding author at: Department of Pharmaceutical Sciences, University of Milan, via Balzaretti 9, 20133 Milano, Italy.
E-mail addresses: Christian.pinna@unimi.it (C. Pinna), asala@fulbrightmail.org (A. Sala).

https://doi.org/10.1016/j.jff.2021.104731
Received 12 April 2021; Received in revised form 29 August 2021; Accepted 4 September 2021
Available online 9 September 2021

2. Materials and methods

Human umbilical cords were collected by the local Hospital Macedonio Melloni (20129, Milan). Informed consent was provided according to the Declaration of Helsinki.

2.1. Isolated human umbilical vein rings

The umbilical cords were obtained after delivery from anonymized healthy pregnant women (N = 90, 37–41 weeks, not affected by pathologies or presenting clinical biochemistry alterations) and were kept at 5 °C until the experiment was carried out the same day of delivery, taking note of the sex of the newborn. No statistically significant difference in the age of male and female newborn mother's was observed. A rapid degradation of the endothelium associated to an increased response to serotonin (5-HT) and to the absence of vasorelaxant responses was observed in preparations maintained overnight and prepared the following day.

Medial segments of umbilical vein were carefully isolated from adherent connective tissue and cut into 4–5 mm long rings. From each vein, up to six vascular rings were prepared, and care was taken in order to avoid damage of the endothelial cell layer during the preparations of the HUV rings. The rings were mounted in 5 ml organ baths and perfused with Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5.5 mM glucose, pH 7.4) at 37 °C, continuously bubbled with 95% O_2 and 5% CO_2. Tension was recorded using a PowerLab/8SP (ADInstruments, UK) connected to isometric force transducers. Tissues were equilibrated for 1 h under a resting tension of 2.0 g. In preliminary experiments, concentration–response curves were obtained by cumulative addition of U46619 (thromboxane mimetic) (10^{-9}–10^{-6} mol/l), histamine (10^{-8}–10^{-4} mol/l), 5-HT (10^{-9}–10^{-4} mol/l), ATP (10^{-7}–10^{-3} mol/l), adrenaline (10^{-8}–10^{-4} mol/l), angiotensin-II (10^{-10}–10^{-6} mol/l). Upon verification that 5-HT at 1 μM (approximate EC_{80} concentration) induced a stable and long-lasting contraction of the vascular preparations, concentration–response curves to vasorelaxing adenosine (10^{-9}–10^{-5} mol/l), sodium nitroprusside (10^{-9}–10^{-6} mol/l), different flavonoids (10^{-8} to 3×10^{-4} mol/l), and β-estradiol (10^{-9} to 3×10^{-5} mol/l), were obtained in endothelium-intact preparations of the HUV. Each preparation was challenged with one agonist only, and time controls were carried out to ensure that responses were reproducible. Concentration-response curves to β-estradiol in the absence and presence of a competitive ERα-/ERβ-antagonist (ICI 182780) were also carried-out in male- and female-derived HUV. The affinity of ICI 182780 for estrogen receptors was estimated by the pA_2 value, calculated using the Schild regression analysis on GraphPad Prism 5.0.

Contractile responses to agonists were measured as the increase in tone above baseline (mN/mg tissue), whereas vasorelaxing responses were expressed as percentage of relaxation of 5-HT- precontracted tissues.

A separate set of experiments was carried out using preparations devoid of endothelium. The removal of the endothelium was obtained by gentle scraping the lumen of umbilical vein ring with a needle (size: 1 mm), and its absence was confirmed by an increased vasoconstricting response to 5-HT and, eventually, by the lack of vasorelaxation to hesperidin (1 μM).

2.2. Drugs and chemicals

Acetylcholine hydrochloride, adrenaline hydrochloride, adenosine, 17β-estradiol, glybenclamide, histamine, ICI 182780, Nitro-L-arginine methyl ester hydrochloride (L-NAME), sodium nitroprusside (SNP), 5-hydroxytryptamine (5-HT), 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α (U46619), hesperidin, and diosmin, were purchased from Sigma-Aldrich. All compounds were freshly dissolved in distilled H_2O, except for flavonoids which were dissolved in DMSO.

2.3. Statistical analysis

All data were expressed as mean ± S.E.M. of eight experiments and represent unpaired data. Concentration-response curves were fitted and compared by analysis of variance (ANOVA) using GraphPad Prism 5.0. If P values were <0.05 the treatment affected the response over the tested range of concentration (Ludbrook, 1994). Maximal responses and pD_2 values (−log EC_{50}) for each agonist were estimated using GraphPad InStat, and comparison were carried out by one-way ANOVA followed by Tukey's *post hoc* test.

3. Results

3.1. Vasorelaxant effects of exogenous sodium nitroprusside and adenosine

The NO donor sodium nitroprusside (SNP), when added cumulatively (10^{-9}–10^{-5} mol/l) to the HUV rings precontracted with 5-HT, produced endothelium-independent vasorelaxations that were not gender specific (Fig. 1). Tissue sensitivity to SNP, expressed as pD_2 was comparable in female- (6.54 ± 0.10) and in male-derived tissues (6.86 ± 0.28).

Adenosine (ADO, 1 nM – 10 μM) also induced concentration-dependent relaxations of HUV rings precontracted with 5-HT (Fig. 1), with a pD_2 of 6.54 ± 0.10. On the contrary, acetylcholine (ACh, 10^{-7}–10^{-4} mol/l) was ineffective on 5-HT-precontracted preparations.

3.2. Effect of hesperidin

Fig. 2A shows the concentration–response curves to hesperidin (10 nM – 0.3 mM) in 5-HT-precontracted preparations. Female-derived HUV exhibited significantly greater vasorelaxations in response to hesperidin ($P < 0.01$) than male-derived tissues. Maximal responses were (54.0 ± 4%) and (35.0 ± 5%) in female- and male-derived tissues, respectively. The effect observed at highest concentration tested in female tissues was comparable to the maximal vasorelaxation obtained with SNP (Fig. 1), and higher that that observed with adenosine.

Pre-treatment with the NO-synthase inhibitor, nitro-L-arginine-methyl ester (L-NAME, 10 μM, 30′), significantly impaired hesperidin-mediated vasorelaxation ($P < 0.01$) both in male- and female-derived rings. The NO component of the hesperidin-mediated vasorelaxation

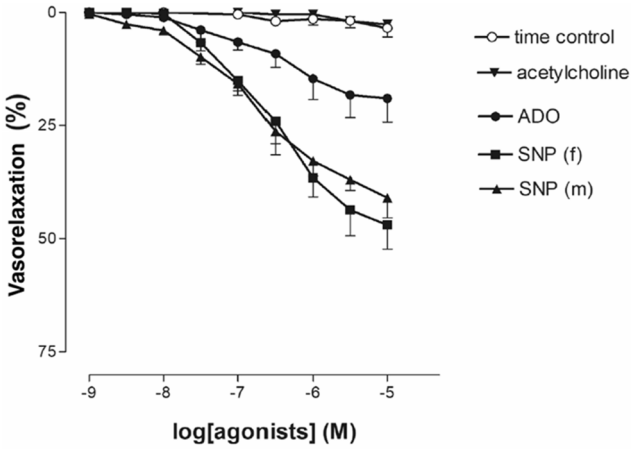

Fig. 1. Cumulative concentration–response curves in 5-HT-precontracted rings to acetylcholine (ACh), adenosine (ADO) and sodium nitroprusside (SNP, in female- and male-derived HUV). Vasorelaxation was expressed as percent inhibition of the 5-HT-induced (EC_{80}) contraction. Points show mean ± S.E.M. of 8 experiments, unless occluded by symbol. No significant difference was observed for SNP-induced responses in the female- and male-derived preparations.

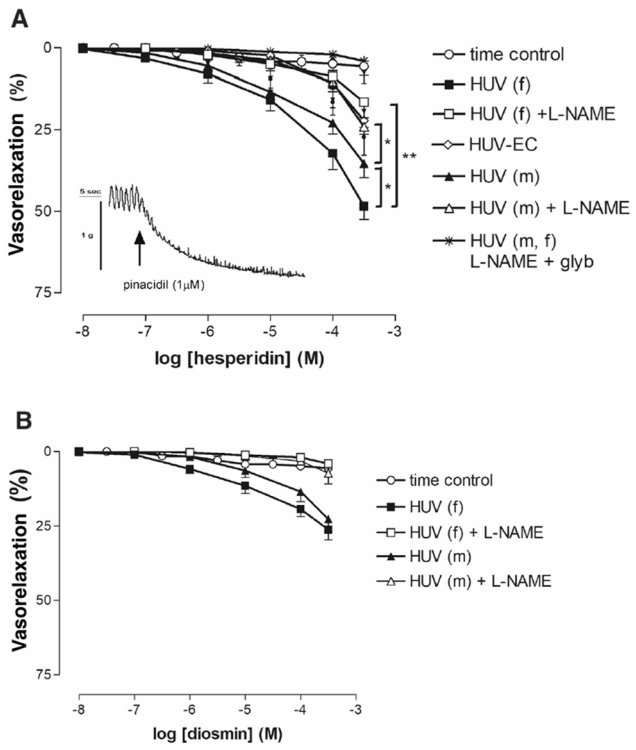

Fig. 2. Panel A. Cumulative concentration–response curves to hesperidin in rings from female- and male-derived human umbilical vein (HUV). Vasorelaxation was expressed as percent inhibition of the 5-HT-induced (EC_{80}) contraction. The curve HUV-EC represent preparations where the endothelial cells were removed as described in Materials and methods. Data are expressed as mean ± S.E.M. of 8 experiments. Statistical significance for curves $^{*}P < 0.05$: female-derived vs. male-derived and male-derived vs. male-derived + L-NAME; $^{**}P < 0.005$: female-derived vs. female-derived + L-NAME. The ATP-sensitive K^+ channel inhibitor, glybenclamide (glyb, 1 μM), completely blocked hesperidin-induced vasorelaxation in the presence of L-NAME. Typical tracing of the effect of pinacidil (1 μM) in 5-HT-precontracted HUV rings (inset). **Panel B.** Cumulative concentration–response curves to diosmin in rings from female- and male-derived HUV. Vasorelaxation was expressed as percent inhibition of the 5-HT-induced (EC_{80}) contraction. Data are expressed as mean ± S.E.M. of 8 experiments, unless occluded by symbol. Statistical significance for curves $^{*}P < 0.05$: female-derived vs. female-derived + L-NAME and male-derived vs. male-derived + L-NAME.

evaluated as percentage of inhibition induced by L-NAME was more evident in female- than in male-derived tissues. Human umbilical vein rings devoid of endothelium exhibited a markedly decreased, but not abolished, vasorelaxation in response to hesperidin, comparable to the results obtained in tissues pretreated with L-NAME.

When pre-treated with both L-NAME (10 μM, 30′) and the ATP-sensitive K^+ channel inhibitor glybenclamide (1 μM, 30′) HUV rings showed a complete lack of response to hesperidin. The presence of ATP-sensitive K^+ channel with vasorelaxant activity in HUV was confirmed by the ability of pinacidil (1 μM), an ATP-sensitive K^+ channel opener, to induce substantial vasorelaxation in 5-HT-precontracted rings (Fig. 2A, inset).

3.3. Effect of diosmin

Concentration-dependent relaxations to diosmin (10 nM-0.3 mM) on 5-HT-precontracted HUV rings were not gender-specific and the two concentration–response curves obtained from male- and female-derived preparations did not differ significantly (Fig. 2B). The responses obtained at the highest concentration tested of diosmin in female- (24.2 ± 3%) and male-derived rings (20.2 ± 4%) were also significantly smaller than those evoked by hesperidin ($P < 0.05$) or nitroprusside, and comparable to the maximal effect of adenosine. Additionally, diosmin-induced vasorelaxations were completely suppressed by pre-incubation with L-NAME (10 μM, 30′) in both groups.

3.4. Effect of β-estradiol

17β-estradiol (10^{-9} to 3×10^{-5} mol/l) caused vasorelaxation in pre-contracted HUV at submicromolar concentrations, but no difference was observed between male- and female-derived vascular tissues (Fig. 3). Pretreatment with the ER-antagonist ICI 182780 (10^{-7} M for 30′), caused a significant shift to the right of the concentration–response curves to 17β-estradiol both male- and female-derived HUV. The estimated tissue sensitivity of ICI 182780 to estrogen receptors, expressed as the pA_2 value was comparable in both groups being (6.87 ± 0.03) and (6.67 ± 0.09) in male- and female-derived HUV, respectively.

4. Discussion

In the present study we aimed at assessing the potential vasoprotective activity of citrus-derived flavonoids hesperidin and diosmin, evaluating their effects on vasoconstriction elicited by 5-HT in HUV. The results obtained showed unanticipated differences between hesperidin and diosmin, both in terms of efficacy in eliciting vasorelaxation, and of sensitivity of male- vs female-derived tissues to the vasorelaxant activity. Hesperidin, in fact, resulted more effective than diosmin in reverting the vasoconstriction elicited by 5-HT, and unlike diosmin, showed a greater vasorelaxation in female-derived HUV when compared to HUV obtained from male newborn. While it is possible that the observed differences could be related to differences in potencies between hesperidin and diosmin, and higher concentrations of the latter could have led to unmasking gender specific differences, it must be noted that at a vasorelaxation of 25% the male vs female curves with hesperidin were already diverging, albeit still not statistically different, while in the case of diosmin the two curves actually appeared to converge.

The difference between male- and female-derived response to hesperidin was the result of a much larger endothelial cell/NO-dependent vasorelaxation in female tissues; this effect was not associated to an increased tissue sensitivity to NO, since relaxation to sodium nitroprusside, a direct NO-donor, was not gender-specific. Our observations is therefore in agreement with the results of Cattaneo and collaborators (Cattaneo et al., 2017) who found sex-specific differences in expression, activity, and function of eNOS in HUVEC, and suggest a specific role of eNOS and NO in the vasorelaxation induced by hesperidin. Similar differences in reactivity to vasoconstrictor and vasorelaxation stimuli, and sex-specific eNOS expression and NO release have also been observed in other vascular beds (Kane, Anselm, Rattmann, Auger, & Schini-Kerth, 2009; Kunert, Dwinell, Drenjancevic Peric, & Lombard, 2008).

Flavonoids are well known for their antioxidant effect arising from the high reactivity of their hydroxyl group (Korkina & Afanasev, 1996), but a number of biological activities have been reported for this important class of natural molecules, including modulation of enzyme functions such as acetylcholine esterase, cyclooxygenases, xanthine oxidase and phosphodiesterases (Panche, Diwan, & Chandra, 2016), as well as ionic channels (Goutman, Waxemberg, Doñate-Oliver, Pomata, & Calvo, 2003)(Naylor et al., 2016)

The mechanism by which bioactive polyphenols initiate their signaling in endothelial cells is still debated, but evidence have been presented that a bilitranslocase-mediated membrane transport may substantially contribute to their endothelium-dependent vasorelaxing effect (Ziberna, Kim, Auger, Passamonti, & Schini-Kerth, 2013), while the increased NO production appears to be the result of a redox-sensitive Src- and Akt-dependent activation of eNOS (Anselm, Chataigneau, Ndiaye, Chataigneau, & Schini-Kerth, 2007); indeed, previous studies provided evidence that hesperidin induce rapid phosphorylation of eNOS, enhancing its activity and NO production in HUVEC (Chiou et al.,

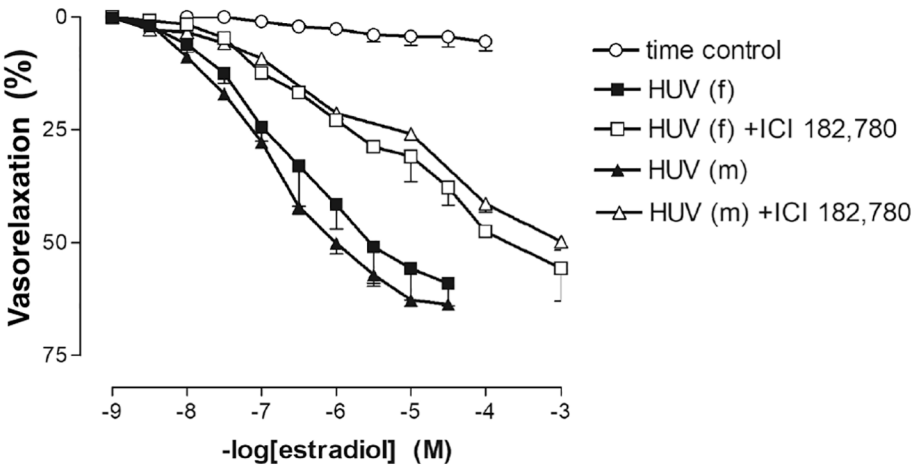

Fig. 3. Cumulative concentration–response curves to 17β-estradiol in 5-HT-precontracted rings from female- and male-derived human umbilical vein (HUV), before and after incubation with the estrogen receptor antagonist ICI 182780 (10^{-7} M). No significant difference is present between concentration–response curves to 17β-estradiol in female- and male-derived groups either before or after pretreatment with ICI 182780. Statistical significance for curves $^{*}P < 0.01$: female-derived vs. female-derived + ICI 182780 and male-derived vs. male-derived + ICI 182780.

2008), supporting a potential role of NO in the observed differences between male and female vasorelaxation in response to hesperidin.

The observed differences between diosmin and hesperidin also showed the presence of an endothelial cell/NO-independent vasorelaxation in response to hesperidin only, an effect that was abolished by pretreatment with glybenclamide, suggesting the contribution of a potential activation of ATP-sensitive K^+ channel to the effects of hesperidin. ATP-sensitive K^+ channels are thought to play an important functional role in vascular responses of several vascular beds (Brayden, 2002), including HUV (Protić et al., 2015), and this was confirmed by the potent vasorelaxing effect resulting from their activation in response to pinacidil. Flavonoids have been reported to cause vasorelaxation through the activation of ATP-sensitive K^+ channels (Ng, Poh, Lam, & Hoe, 2013) and our results are well in agreement with the endothelium-dependent and -independent vasorelaxant actions induced by total flavonoids of Elsholtzia splendens (Wang et al., 2014), confirming that different flavonoids, but not all of them, may share this activity.

Estrogen receptors (ER) beside their classical function of regulators of gene expression, have been associated to numerous non-genomic functions (Banerjee, Chambliss, Mineo, & Shaul, 2014), including the regulatory function of eNOS activity and direct vasorelaxation (Kim, Moriarty, & Bender, 2008; Mineo & Shaul, 2012). In consideration of the sex-related differences we observed in the vascular response to hesperidin, we tested 17β-estradiol in our HUV preparations, under the hypothesis that hesperidin activity might reflect a potential estrogenic effect. In agreement with results obtained in different vascular beds, 17β-estradiol was able to cause a potent vasorelaxation of HUV at submicromolar concentrations, resulting from the activation of membrane ER as shown by the significant rightshift of the concentration–response curves caused by the specific ER antagonist ICI 182780. Unfortunately, no significant difference could be observed between male- and female-derived tissues, ruling out the hypothesis that the increased e-NOS activity in female-derived HUV in response to hesperidin could involve the activation of the estrogen receptor. Indeed, the chemical structures of the aglycone of hesperidin and, in particular, of diosmin are very similar to that of the phytoestrogen genistein, but it is known that the estrogenic effect is lost upon glucuronidation (Balakrishnan, Thorstensen, Ponnampalam, & Mitchell, 2010), making it unlikely that the glycosides could have been able to interact with the ER.

Hesperidin is known to be contributing, more than other *Citrus*-derived flavonoids, to the blood pressure lowering effects of orange products consumption (Constans et al., 2015), and a deficiency of hesperidin has been associated with capillary leakiness (Garg, Garg, Zaneveld, & Singla, 2001). Recently, a clinical randomized control trial confirmed that both orange juice and hesperidin-enriched orange juice decrease blood pressure in mild hypertensive subjects (Valls et al., 2020), but not all *Citrus*-derived flavonoids are equally effective in inducing eNOS activity: for instance, naringenin (the aglycone form of naringin after hydrolysis of phenolic glycoside) exhibits lower activity than hesperetin (the aglycone form of hesperidin) in HUVEC cells (Liu, Xu, & Cheng, 2008). Indeed, it is interesting to note how minor differences in the structures of diosmin and hesperidin (the latter is a flavanone, while the former is a flavone) may result is such different biological activities. While different studies would be necessary to define the structure–activity relationship for this activity it must be noted that the additional double-bond present in the flavonic molecule of diosmin is generating a planar core structure to which the 3-hydroxy-4methoxyphenil group bind on the same planar surface, while this group is heading on a different plane from the core structure in the non-planar, flavanonic molecule of hesperidin, therefore generating a rather different spatial configuration. This non-coplanar configuration may result critical for the interaction with the ATP-sensitive K^+ channels that appear to be responsible for the L-NAME-resistant vasorelaxing effect of hesperidin only.

In conclusion, our functional data suggest that *Citrus*-derived hesperidin and diosmin may exert beneficial effects on placental circulation through different mechanisms. Hesperidin has been found to be more active then diosmin, especially in female-derived HUV, but additional studies are warranted in order to define the specific mechanism responsible for these differences. *Citrus* flavonoids may contribute to maintain critically important endothelial function in the HUV, that may help reducing the risk of fetal vascular complications such as pre-eclampsia and intrauterine growth restriction.

5. Ethics statement

Informed consent for umbilical cord collection from anonymized healthy pregnant women was obtained according to the Declaration of Helsinki.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors and was sponsored by institutional funds of the Department of Pharmaceutical Sciences, University of Milan.

CRediT authorship contribution statement

Christian Pinna: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Angelo Sala:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to the medical personnel of Macedonio Melloni Hospital for their assistance in the umbilical cord collection. We acknowledge support from the Department od Pharmaceutical Sciences for partial coverage of Open Access publication costs.

References

Achamrah, N., & Ditisheim, A. (2018). Nutritional approach to preeclampsia prevention. *Current Opinion in Clinical Nutrition and Metabolic Care, 21*(3), 168–173. https://doi.org/10.1097/MCO.0000000000000462.

Anselm, E., Chataigneau, M., Ndiaye, M., Chataigneau, T., & Schini-Kerth, V. B. (2007). Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS. *Cardiovascular Research, 73*(2), 404–413. https://doi.org/10.1016/j.cardiores.2006.08.004.

Balakrishnan, B., Thorstensen, E. B., Ponnampalam, A. P., & Mitchell, M. D. (2010). Transplacental transfer and biotransformation of genistein in human placenta. *Placenta, 31*(6), 506–511. https://doi.org/10.1016/j.placenta.2010.03.007.

Banerjee, S., Chambliss, K. L., Mineo, C., & Shaul, P. W. (2014). Recent insights into non-nuclear actions of estrogen receptor alpha. *Steroids, 81*, 64–69. https://doi.org/10.1016/j.steroids.2013.11.002.

Billingsley, H. E., & Carbone, S. (2018). The antioxidant potential of the Mediterranean diet in patients at high cardiovascular risk: An in-depth review of the PREDIMED. *Nutrition & Diabetes, 8*(1), 13. https://doi.org/10.1038/s41387-018-0025-1.

Brayden, J. E. (2002). Functional roles Of KATP channels in vascular smooth muscle. *Clinical and Experimental Pharmacology and Physiology, 29*(4), 312–316. https://doi.org/10.1046/j.1440-1681.2002.03650.x.

Cattaneo, M. G., Vanetti, C., Decimo, I., Di Chio, M., Martano, G., Garrone, G., … Vicentini, L. M. (2017). Sex-specific eNOS activity and function in human endothelial cells. *Scientific Reports, 7*(1), 9612. https://doi.org/10.1038/s41598-017-10139-x.

Chen, N., Lv, J., Bo, L., Li, N., Wu, C., Yin, X., … Xu, Z. (2015). Muscarinic-mediated vasoconstriction in human, rat and sheep umbilical cords and related vasoconstriction mechanisms. *BJOG: An International Journal of Obstetrics & Gynaecology, 122*(12), 1630–1639. https://doi.org/10.1111/bjo.2015.122.issue-1210.1111/1471-0528.13144.

Chiou, C.-S., Lin, J.-W., Kao, P.-F., Liu, J.-C., Cheng, T.-H., & Chan, P. (2008). Effects of hesperidin on cyclic strain-induced endothelin-1 release in human umbilical vein endothelial cells. *Clinical and Experimental Pharmacology and Physiology, 35*(8), 938–943. https://doi.org/10.1111/j.1440-1681.2008.04939.x.

Constans, J., Bennetau-Pelissero, C., Martin, J.-F., Rock, E., Mazur, A., Bedel, A., … Bérard, A. M. (2015). Marked antioxidant effect of orange juice intake and its phytomicronutrients in a preliminary randomized cross-over trial on mild hypercholesterolemic men. *Clinical Nutrition, 34*(6), 1093–1100. https://doi.org/10.1016/j.clnu.2014.12.016.

Dodd, J. M., O'Brien, C., & Grivell, R. M. (2014). Preventing pre-eclampsia - are dietary factors the key? *BMC Medicine, 12*(1), 176. https://doi.org/10.1186/s12916-014-0176-4.

Garg, A., Garg, S., Zaneveld, L. J. D., & Singla, A. K. (2001). Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytotherapy Research, 15*(8), 655–669. https://doi.org/10.1002/ptr.1074.

Goutman, J. D., Waxemberg, M. D., Doñate-Oliver, F., Pomata, P. E., & Calvo, D. J. (2003). Flavonoid modulation of ionic currents mediated by GABAA and GABAC receptors. *European Journal of Pharmacology, 461*(2–3), 79–87. https://doi.org/10.1016/S0014-2999(03)01309-8.

Habauzit, V., & Morand, C. (2012). Evidence for a protective effect of polyphenols-containing foods on cardiovascular health: An update for clinicians. *Therapeutic Advances in Chronic Disease, 3*(2), 87–106. https://doi.org/10.1177/2040622311430006.

Kane, M. O., Anselm, E., Rattmann, Y. D., Auger, C., & Schini-Kerth, V. B. (2009). Role of gender and estrogen receptors in the rat aorta endothelium-dependent relaxation to red wine polyphenols. *Vascular Pharmacology, 51*(2–3), 140–146. https://doi.org/10.1016/j.vph.2009.05.002.

Kim, K. H., Moriarty, K., & Bender, J. R. (2008). Vascular cell signaling by membrane estrogen receptors. *Steroids, 73*(9–10), 864–869. https://doi.org/10.1016/j.steroids.2008.01.008.

Korkina, L. G., & Afanasev, I. B. (1996). Antioxidant and chelating properties of flavonoids. *Advances in Pharmacology, 38*, 151–163. https://doi.org/10.1016/S1054-3589(08)60983-7.

Kunert, M. P., Dwinell, M. R., Drenjancevic Peric, I., & Lombard, J. H. (2008). Sex-specific differences in chromosome-dependent regulation of vascular reactivity in female consomic rat strains from a SS × BN cross. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 295*(2), R516–R527. https://doi.org/10.1152/ajpregu.00038.2008.

Lachenmayer, L. (1971). Adrenergic innervation of the umbilical vessels. *Zeitschrift Fur Zellforschung Und Mikroskopische Anatomie, 120*(1), 120–136. https://doi.org/10.1007/BF00331246.

Liu, L., Xu, D., & Cheng, Y. (2008). Distinct effects of naringenin and hesperetin on nitric oxide production from endothelial cells. *Journal of Agricultural and Food Chemistry, 56*(3), 824–829. https://doi.org/10.1021/jf0723007.

Ludbrook, J. (1994). Repeated measurements and multiple comparisons in cardiovascular research. *Cardiovascular Research, 28*(3), 303–311. https://doi.org/10.1093/cvr/28.3.303.

Ly, C., Yockell-Lelièvre, J., Ferraro, Z. M., Arnason, J. T., Ferrier, J., & Gruslin, A. (2015). The effects of dietary polyphenols on reproductive health and early development. *Human Reproduction Update*. https://doi.org/10.1093/humupd/dmu058.

Mineo, C., & Shaul, P. W. (2012). Regulation of eNOS in Caveolae. In Advances in experimental medicine and biology (pp. 51–62). https://doi.org/10.1007/978-1-4614-1222-9_4.

Monsalve, B., Concha-Meyer, A., Palomo, I., & Fuentes, E. (2017). Mechanisms of endothelial protection by natural bioactive compounds from fruit and vegetables. *Anais Da Academia Brasileira de Ciências, 89*(1 suppl), 615–633. https://doi.org/10.1590/0001-3765201720160509.

Morand, C., Dubray, C., Milenkovic, D., Lioger, D., Martin, J. F., Scalbert, A., & Mazur, A. (2011). Hesperidin contributes to the vascular protective effects of orange juice: A randomized crossover study in healthy volunteers. *American Journal of Clinical Nutrition, 93*(1), 73–80. https://doi.org/10.3945/ajcn.110.004945.

Naylor, J., Minard, A., Gaunt, H. J., Amer, M. S., Wilson, L. A., Migliore, M., … Bon, R. S. (2016). Natural and synthetic flavonoid modulation of TRPC5 channels. *British Journal of Pharmacology, 173*(3), 562–574. https://doi.org/10.1111/bph.13387.

Ng, H.-K., Poh, T.-F., Lam, S.-K., & Hoe, S.-Z. (2013). Potassium channel openers and prostacyclin play a crucial role in mediating the vasorelaxant activity of Gynura procumbens. *BMC Complementary and Alternative Medicine, 13*(1), 188. https://doi.org/10.1186/1472-6882-13-188.

Nicholson, S. K., Tucker, G. A., & Brameld, J. M. (2010). Physiological concentrations of dietary polyphenols regulate vascular endothelial cell expression of genes important in cardiovascular health. *British Journal of Nutrition, 103*(10), 1398–1403. https://doi.org/10.1017/S0007114509993485.

Oak, M.-H., Auger, C., Belcastro, E., Park, S.-H., Lee, H.-H., & Schini-Kerth, V. B. (2018). Potential mechanisms underlying cardiovascular protection by polyphenols: Role of the endothelium. *Free Radical Biology and Medicine, 122*, 161–170. https://doi.org/10.1016/j.freeradbiomed.2018.03.018.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science, 5*, Article e47. https://doi.org/10.1017/jns.2016.41.

Parlapani, E., Agakidis, C., Karagiozoglou-Lampoudi, T., Sarafidis, K., Agakidou, E., Athanasiadis, A., & Diamanti, E. (2019). The Mediterranean diet adherence by pregnant women delivering prematurely: Association with size at birth and complications of prematurity. *The Journal of Maternal-Fetal & Neonatal Medicine, 32*(7), 1084–1091. https://doi.org/10.1080/14767058.2017.1399120.

Pham, N. M., Do, V. V., & Lee, A. H. (2019). Polyphenol-rich foods and risk of gestational diabetes: A systematic review and meta-analysis. *European Journal of Clinical Nutrition, 73*(5), 647–656. https://doi.org/10.1038/s41430-018-0218-7.

Pistollato, F., Sumalla Cano, S., Elio, I., Masias Vergara, M., Giampieri, F., & Battino, M. (2015). Plant-based and plant-rich diet patterns during gestation: Beneficial effects and possible shortcomings. *Advances in Nutrition, 6*(5), 581–591. https://doi.org/10.3945/an.115.009126.

Protić, D., Beleslin-Čokić, B., Spremović-Rađenović, S., Radunović, N., Heinle, H., Šćepanović, R., & Gojković Bukarica, L. (2014). The different effects of resveratrol and naringenin on isolated human umbilical vein: The role of ATP-sensitive K + channels. *Phytotherapy Research, 28*(9), 1412–1418. https://doi.org/10.1002/ptr.5145.

Protić, D., Radunović, N., Spremović-Rađenović, S., Živanović, V., Heinle, H., Petrović, A., & Gojković-Bukarica, L. (2015). The role of potassium channels in the vasodilatation induced by resveratrol and naringenin in isolated human umbilical vein. *Drug Development Research, 76*(1), 17–23. https://doi.org/10.1002/ddr.v76.110.1002/ddr.21236.

Ramelet, A. A. (2001). Clinical benefits of Daflon 500 mg in the most severe stages of chronic venous insufficiency. *Angiology, 52*(1_suppl), S49–S56. https://doi.org/10.1177/000331970105200 1S07.

Sastry, B. V. R. (1997). Human placental cholinergic system. *Biochemical Pharmacology, 53*(11), 1577–1586. https://doi.org/10.1016/s0006-2952(97)00017-8.

Silambarasan, T., & Raja, B. (2012). Diosmin, a bioflavonoid reverses alterations in blood pressure, nitric oxide, lipid peroxides and antioxidant status in DOCA-salt induced hypertensive rats. *European Journal of Pharmacology, 679*(1–3), 81–89. https://doi.org/10.1016/j.ejphar.2011.12.040.

Ulloa, J. H. (2019). Micronized purified flavonoid fraction (MPFF) for patients suffering from chronic venous disease: a review of new evidence. *Advances in Therapy*. https://doi.org/10.1007/s12325-019-0884-4.

Valls, R. M., Pedret, A., Calderón-Pérez, L., Llauradó, E., Pla-Pagà, L., Companys, J., … Solà, R. (2020). Effects of hesperidin in orange juice on blood and pulse pressures in

mildly hypertensive individuals: A randomized controlled trial (Citrus study). *European Journal of Nutrition, 1–12*. https://doi.org/10.1007/s00394-020-02279-0.

Wang, H.-P., Lu, J.-F., Zhang, G.-L., Li, X.-Y., Peng, H.-Y., Lu, Y., … Qian, L.-B. (2014). Endothelium-dependent and -independent vasorelaxant actions and mechanisms induced by total flavonoids of Elsholtzia splendens in rat aortas. *Environmental Toxicology and Pharmacology, 38*(2), 453–459. https://doi.org/10.1016/j.etap.2014.07.019.

Ziberna, L., Kim, J.-H., Auger, C., Passamonti, S., & Schini-Kerth, V. (2013). Role of endothelial cell membrane transport in red wine polyphenols-induced coronary vasorelaxation: Involvement of bilitranslocase. *Food & Function, 4*(10), 1452. https://doi.org/10.1039/c3fo60160a.