SHORT COMMUNICATION

Effects of flavonoid derivatives on human microvascular endothelial cells

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\textbf{ABSTRACT}

Some natural compounds, including flavonoids, are active in vasculature re-growth during hair follicle disruption, but their effects have not been yet evaluated directly on microvascular endothelial cells. Skin vascularisation regulates the physiological blood supply required for hair growth and its dysregulation is the basis of several human diseases. Follicle-derived vascular endothelial growth factor (VEGF) release from follicular keratinocytes promotes perifollicular vascularisation and increases follicle and hair size, while blockade of VEGF-mediated angiogenesis leads to impaired hair growth. Here, we tested three flavonoids, namely visnadin (VSD), hesperidin (HSP) and baicalin (BC), on cultured human microvascular endothelial cells (HMEC), comparing their effects with minoxidil (MXD), a synthetic drug broadly used in the treatment of androgenetic alopecia. The response to these compounds was assayed in terms of endothelial survival, proliferation, tubulogenesis and proangiogenic signalling. We show that BC promotes HMEC proliferation, while both VSD and MXD enhance tubulogenesis. Interestingly, only HSP increases VEGFR-2 phosphorylation.

\textbf{KEYWORDS}

Natural products; flavonoids; endothelial cells; angiogenesis; hair follicle

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1. Introduction

Flavonoids are a large family of compounds synthesised by plants that have a common chemical structure. Although it was initially hypothesised that the biological effects of flavonoids would be related to their renowned antioxidant properties, many of their biological effects are related to their ability to modulate cell-signalling pathways. Many flavonoids are suggested to be helpful for coronary heart disease prevention and anticancer activity (Yao et al. 2004; Takır et al. 2014).

Different flavonoids inhibit 5α-reductase activity, differently targeting type-1 or type-2 isoforms (Hiipakka et al. 2002). Since some of these compounds are part of the normal diet or in supplements, they may be useful for the prevention or treatment of metabolic and androgen-dependent disorders. Among the latter, androgenetic alopecia (AGA) is related to impaired 5α-reductase activity, and furthermore, it is associated with an extended alteration of perifollicular vascularisation and some flavonoids regulate endothelial growth and hair follicle (HF) microvasculature (Lachgar et al. 1998).

The biological importance of vascularisation in HF growth is broadly recognised, and the molecular mechanisms underlying this process are under investigation. Pronounced angiogenesis occurs during murine HF cycling and an increasing amount of experimental evidences strongly supports the involvement of proangiogenic VEGF-related signalling in the regulation of HF growth (Yano et al. 2001; Semalty et al. 2011).

Beside the recently approved minoxidil (MXD) and finasteride (Lachgar et al. 1998; Gubelin Harcha et al. 2014) a variety of natural compounds, including flavonoids, have been suggested as HF growth regulators in diseases related to HF growth, including AGA (Kim et al. 2014).

Interestingly, the flavonoids, visnadin (VSD), hesperidin (HSP) and baicalin (BC), are active in vasculature re-growth during HF disruption (Hiipakka et al. 2002). VSD is a vasodilator extracted from the fruit of *Ammi Visnaga* (Durate et al. 1997). HSP is an anti-inflammatory flavonoid obtained from *Citrus* (Assini et al. 2013). In mouse models, *Citrus*-derived flavonoids suppress atherogenesis through improved metabolic parameters and also through direct impact on the vessel wall. In bovine aortic endothelial cells, HSP acutely stimulates phosphorylation of Src, Akt, AMP kinase, as well as NO release, all signalling mediators commonly involved in vascular modifications like vasodilation and increased permeability (Rizza et al. 2011). In a clinical study, HSP treatment increased flow-mediated dilation and reduced concentrations of circulating inflammatory biomarkers (Rizza et al. 2011). BC is the major component found in *Scutellaria baicalensis* root, a widely used herb in traditional Chinese medicine. Although it has been employed for thousands of years to treat stroke, the mechanisms of action of BC have not been fully elucidated. It acts as an anti-inflammatory and antioxidant agent, and regulates angiogenesis (Zhang et al. 2011). BC inhibits androgen activation signalling and promotes human dermal papilla cell proliferation, suggesting that they could be used as active ingredients for treating androgen-associated disorders, such as AGA (Kim et al. 2014).

In this work, we tested the effects of the aforementioned flavonoids on survival, proliferation, tubulogenesis and signalling of human microvascular endothelial cells (HMEC). MXD, topically applied for AGA treatment, was used as a non-flavonoid comparative compound.
2. Results and discussion

In order to set a suitable pattern of compound concentrations to be tested, we performed a preliminary dose–response curve of their cytotoxicity on HMEC at 24 h of treatment. As indicated by the graphs, all the flavonoid derivatives and MXD-induced cytotoxicity only at high concentrations. No significant variation of cell viability was observed for the lower doses (Figure S1(a)–(d)).

Then, we measured the mitogenic activity of different compounds on HMECs at 48 h of treatment. Only BC exerted a small but significant proliferative effect compared to the negative control (DMEM 2%), while HSP, VSD and MXD did not result effective at any of the concentrations tested (Figure S1(e)–(h)). The lack of pro-mitogenic effects by HSP and VSD on endothelium supports their anticancer properties observed in prostate cancer or in colon carcinogenesis (Sambantham et al. 2013).

In order to evaluate the proangiogenic potential of the compounds in vitro, we measured the formation of endothelial tubule networks in three-dimensional matrigel structure (in vitro tubulogenesis assay, see Methods). VSD enhanced the formation of pseudocapillary structures in a dose-dependent manner. Similar results were observed after the treatment with MXD. This evidence is in nice agreement with MDX ability to trigger vascular endothelial growth factor (VEGF) release. On the other hand, BC and HSP did not affect HMEC tubule formation (Figure S2(a)–(d)).

Finally, we asked whether any of the flavonoids used in this work could interfere with endothelial signalling induced by VEGF, one of the most powerful endogenous proangiogenic factors. Interestingly, we found that VEGFR2 (KDR) phosphorylation was significantly increased only by stimulation with HSP, while no other compounds exert any detectable effect (Figure S2(e)). It is worth noting that the ability of HSP to enhance VEGFR2 phosphorylation is not sufficient to promote tubulogenesis in HMEC, as shown in Figure S2(c). It could interfere on vascular effects regulated by VEGF (permeability, vasodilation) but not on the others (endothelial migration and tubule formation). Accordingly, HSP is reported to stimulate phosphorylation of Src, Akt, AMP kinase, and NO release, all mediators involved in vasodilation and increased permeability (Sambantham et al. 2013).

3. Conclusion

The beneficial properties of flavonoids have been established in both various cell lines and different animal models. Studies in cell lines have also demonstrated that these compounds can affect a range of signalling and metabolic pathways resulting in improving various symptoms including endothelial dysfunction (Wu et al. 2014). In conclusion, this work supports in vitro a selective role of some flavonoids in the control of vascular-mediated HF growth.

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Eleonora Bassino analysed the data and wrote the paper. Franco Gasparri contributed essential reagents or tools and designed the research study. Susanna Antoniotti performed experiments and analysed the data. Luca Munaron designed the research study, analysed the data, wrote the paper and contributed essential reagents or tools.
Disclosure statement
No potential conflict of interest was reported by the authors.

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