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Effect of (R)-(−)-Linalool on endothelial damage: Sex differences

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ABSTRACT

Oxidative stress and inflammation are responsible for endothelial damage displaying many sex differences. Lipopolysaccharide (LPS) is a pathogenic stimulus that can trigger inflammation, contributing to endothelial dysfunction. Given the scientific evidence on the effectiveness of herbal extracts in managing endothelial dysfunction, we considered the (R)-(−)-Linalool (LIN), an aromatic monoterpene alcohol, as a bioactive phytochemical compound that could prevent and improve endothelial injury. In this study, we evaluated the effect of the LIN on LPS-induced damage in female and male human umbilical vein endothelial cells (FHUVECs and MHUVECs), measuring cell viability, cytokines release (IL-6 and TNF-α), malondialdehyde (MDA), and nitrites.

LPS significantly reduced viability both in MHUVECs and FHUVECs. Moreover, LPS increased the IL-6, TNF-α, and MDA level only in FHUVECs if compared to basal value; despite that, LPS reduced nitrites only in MHUVECs. LIN alone did not affect the parameters measured except for an increase in nitrites in FHUVECs. Nevertheless, LIN reduced damage and restored endothelium viability reduced by LPS without a clear sex difference. Under LPS, LIN inhibited IL-6 release and reduced MDA levels only in FHUVECs.

The present data confirm the existence of sex differences in the behavior of HUVECs under LPS conditions. The administration of LIN seems to have a more evident effect on FHUVECs after damage induced by LPS. These LIN effects are important to conduct further well-designed studies on the sex-specific use of this compound on vascular endothelial injury.

1. Introduction

The endothelium plays a crucial role in cardiovascular physiology and pathophysiology by regulating factors such as vascular tone, inflammation, coagulation, fluid and solute exchange, and angiogenesis. It produces vasodilators like nitric oxide and vasoconstrictors like endothelin. An imbalance in the production of these vasoactive substances leads to a loss of endothelial function, known as endothelial dysfunction [1]. A greater production of reactive oxygen species (ROS) is involved in the progression of inflammatory disorders that can causes endothelial dysfunction and tissue injury [2]. Endothelial dysfunction and inflammation are implicated in the pathogenesis of many disease states and is regulated by numerous factors such as lifestyle, behavioural factors, intestinal microbiota, autonomic nervous system, immune activation and sex [3]. Furthermore, endothelial dysfunctions display many sex differences regarding many cellular processes, including the cellular redox balance and inflammation [4], but the specific mechanisms underlying these differences are not yet fully understood.

Lipopolysaccharide (LPS) is a highly proinflammatory molecule that affects many cellular processes, including those related to endothelial dysfunction, such as upregulation of cytokines, adhesion molecules and tissue factors and endothelial cell death [5–7]. Additionally, numerous studies have shown that LPS is a potent activator of neuroinflammation [8].

Several studies reported that endothelial damage can be prevented with natural anti-inflammatory [9,10] and antioxidant agents [11,12].

Linalool (2,6-dimethyl-2,7-octadien-6-ol) is a naturally occurring aromatic monoterpene alcohol, widely found in essential oils of plants,

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including lavender, basil, coriander, rosemary, jasmine, and some cannabis plants [13,14]. The properties of linalool have been extensively investigated *in vivo* and *in vitro*. It has been shown that linalool, in its racemic form, exhibits anti-inflammatory and antioxidant properties [15–17] as well as neuroprotective effects in different cerebral ischemia models [18]. Interestingly, the enantiomer (R)-(−)-Linalool (LIN) displays neuroprotective effects and scavenges peroxyl radicals [19,20] in various models of intracellular oxidative stress. LIN anti-inflammatory properties has been studied *in vivo*, showing effects both in acute [16] and in chronic inflammation as well as in neuropathic hypersensitivity [21]. However, molecular mechanisms of LIN remain still largely unknown.

The study aimed to expand understanding of the effects of LIN and investigate its potential sex-specific impact on endothelial damage induced by LPS in both female and male human umbilical vein endothelial cells (FHUVECs and MHUVECs). Consequently, the following assays were performed: a) cell viability assay; b) cytokines release (IL-6 and TNF-a); c) malondialdehyde (MDA), and d) nitrites determination.

2. Material and methods

2.1. Cell culture

Umbilical cords were collected within 24 h after natural birth at the Obstetrics and Gynecology Clinic at the University of Sassari. Donors were healthy, no-smoking and non-obese mothers, as demonstrated by body mass index calculated at the beginning of pregnancy (Table 1). Furthermore, HUVECs were obtained only from the umbilical cords of normal-weight female and male neonates (FHUVECs and MHUVECs, respectively), according to Bertino and co-workers (2012) [47] (Table 1).

Informed consent was obtained from the mothers of all subjects donating umbilical cords following the Declaration of Helsinki.

HUVECs were isolated using collagenase treatment (Sigma-Aldrich, Italy), as previously described [22].

2.2. Treatments

The concentrations of LIN and LPS were chosen based on data available in the literature [7,23–26] and obtained from our pilot experiments. HUVECs were used at P3 and all experiments were conducted in duplicate.

HUVECs were incubated at 37 °C in a 5 % CO_2 humidified incubator with.

a) M199 only for 24 h,
b) 10 μg/ml LPS (Sigma-Aldrich, Italy) for 5 h,
c) LIN 10 μM (Sigma-Aldrich, Italy) for 24 h,
d) LIN 10 μM for 24 h, after that the medium was replaced with 10 μg/ml LPS for a further 5 h.

2.3. Cell viability

Cell viability was detected by the crystal violet assay following Elengoe and Hamdan [27] and as previously reported by Campesi and co-workers (2022). The absorbance was detected at 540 nm and the cell viability was calculated as the percentage of basal cells for the cells treated with LPS and LIN, for which a viability percentage of 100 % was assumed and in relation to cells treated with LPS for those who have been pre-treated.

Table 1
Physical data of donors.

	Male (n = 13)	Female (n = 9)	p-value
Age of mothers (Years)	33.8 ± 4.4	34.8 ± 5.7	0.65
Body Weight of Mothers (Kg)	69.2 ± 8.9	74.1 ± 10.7	0.26
Body mass index (kg/m2)	25.7 ± 3.3	24.4 ± 9.7	0.66
Body Weight of Neonates (Kg)	3.4 ± 0.4	3.5 ± 0.5	0.61
Gestational age (weeks)	39.1 ± 1.5	39.4 ± 1.01	0.61

Values are reported as the mean ± standard deviation (SD).

2.4. Cytokines detection

The expression levels of IL-6 and TNF-α were evaluated with a commercial ELISA kit (DuoSet ELISA kit, R&D Systems, Italy). The assay allows both quantitative and qualitative detection by antigen-antibody-specific binding.

2.5. MDA determination

The levels of lipid peroxidation were evaluated on 50 μl of supernatant (medium conditioned by the cells based on the treatment) as previously described by Campesi and colleagues (2021). A standard curve of MDA was used for the construction of the calibration curve and samples concentration calculation after spectrophotometric absorption at 535 nm.

2.6. Nitrites determination

Nitrites are the final product of nitric oxide metabolism and were measured in 50 μL of supernatant using the Griess reaction (sulfanilamide 2 % in H_3PO_4; and 1-naphthyl ethylenediamine dihydrochloride 0,2 % in MQ water (Sigma-Aldrich, Italy), as already described by Campesi and colleagues (2023). The absorbance was detected at 535 nm, and nitrite concentrations were calculated on a standard curve of sodium nitrite ranging from 50 to 1 μM.

2.7. Statistics

Data were reported as the mean ± SD. Statistical analysis was performed using a Pairwise Multiple Comparison *t*-test after Bonferroni's correction using Sigma-Stat 3.1 software (Systat Software, Germany). The distribution of samples was assessed via the Kolmogorov–Smirnov and Shapiro tests. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of LIN on cell viability

The treatment of HUVECs with LPS significantly reduced viability both in FHUVECs and in MHUVECs. LIN administration did not result in any change in the viability both in FHUVECs and in MHUVECs. Interestingly, pre-treatment with LIN reduced LPS damage and restored viability to basal value, without a clear sex difference (Fig. 1).

3.2. Effects of LIN on cytokines release

LPS significantly increases the release of both IL-6 (Fig. 2, panel A) and TNF-α (Fig. 2, panel B) in comparison with basal conditions, only in FHUVECs. This effect on FHUVEC was stronger than that on MHUVECs ($p < 0.05$). LIN administration did not affect the release of the analyzed cytokines both in FHUVECs and MHUVECS (Fig. 2, panels A and B). Finally, the pre-treatment with LIN significantly inhibited the release of IL-6 (induced by LPS) only in FHUVECs (Fig. 2, panel A). This effect on FHUVECs was greater than that observed in MHUVECs ($p < 0.05$) (Fig. 2, panel A). LPS induced the release of TNF-α only in FHUVECs and LIN pretreatment did not alter this damage (Fig. 2, panel B).

3.3. Effects of LIN on MDA

An additional experiment was conducted to test the hypothesis that

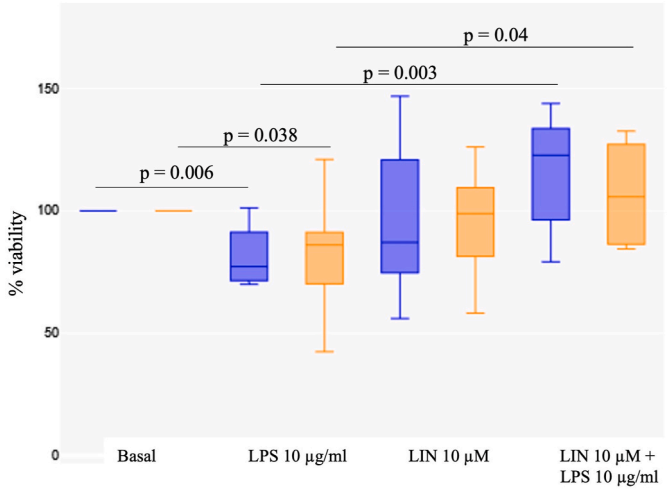

Fig. 1. Effect of LPS (10 μg/ml) and LIN (10 μM) on FHUVECs (blue bars) and MHUVECs (orange bars) viability (n = 6–7 for each sex and treatment). Data are reported as the means ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

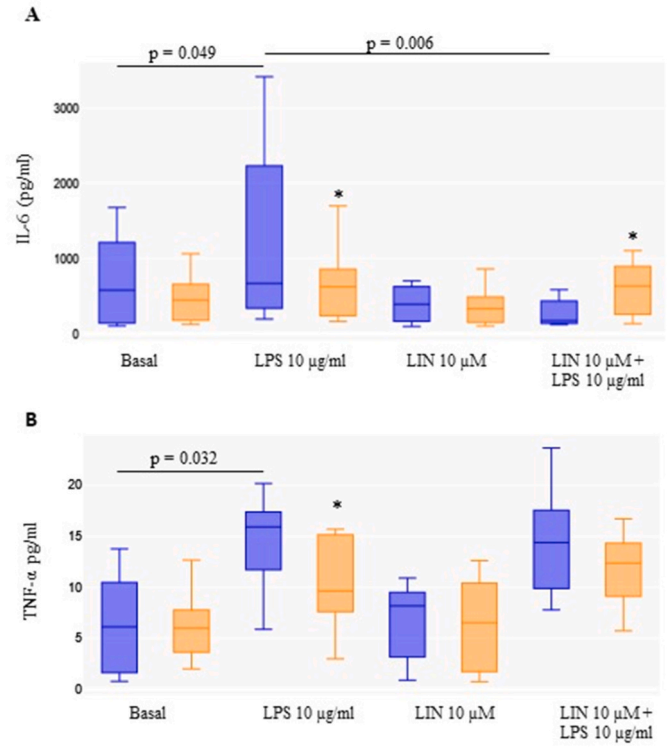

Fig. 2. Effect of LPS (10 μg/ml) and LIN (10 μM) on IL-6 (panel A) and TNF-α (panel B) release on FHUVECs (blue bars) and MHUVECs (orange bars). Data are reported as the means ± SD of at least 8–9 samples for each sex and treatment. *p < 0.05 versus the corresponding FHUVECs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

LIN protects from oxidative stress. MDA levels, a lipid peroxidation index, were assayed in the supernatants of FHUVECs and MHUVECs. LPS significantly increased MDA levels only in FHUVECs, while LIN alone did not modify basal conditions and without a sex-specific effect (Fig. 3, panel A). The pre-treatment with LIN resulted in a significant reduction ($p < 0.05$) of LPS-induced MDA increase only in FHUVECs with respect to the LPS group (Fig. 3, panel A).

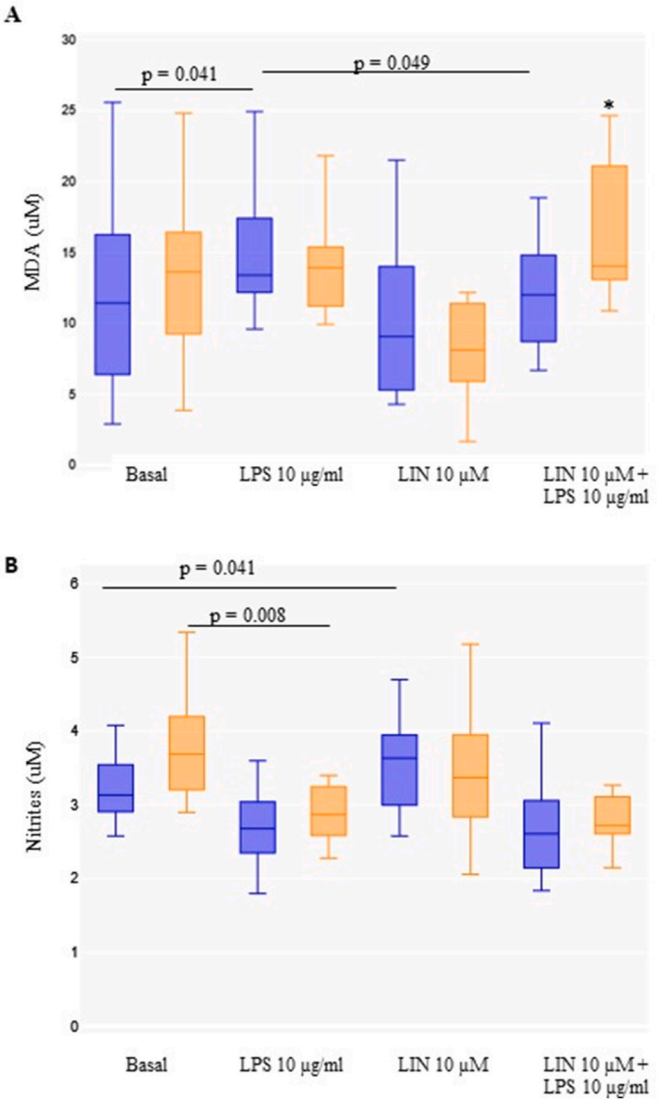

Fig. 3. Effect of LPS (10 μg/ml) and LIN (10 μM) on MDA (panel A) and nitrites (panel B) levels on FHUVECs (blue bars) and MHUVECs (orange bars). Data are reported as the means ± SD of at least 8–9 samples for each sex and treatment. *p < 0.05 versus the corresponding FHUVECs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. *Effects of LIN on nitrites*

Nitrites levels, as products of nitric oxide metabolism were assayed in the supernatants of FHUVECs and MHUVECs. LPS-treated MHUVECs displayed a significantly lower concentration if compared to the basal group (Fig. 3, panel B). Under LIN condition, there is an increase of nitrites in FHUVECs but not in MHUVECs. Under LPS condition, LIN pretreatment did not modify nitrites levels both in FHUVECs and in MHUVECs (Fig. 3, panel B).

4. Discussion

Endothelial dysfunction caused by inflammation and oxidative stress may contribute to several disorders and is a sex-dependent process [3,4, 28–31].

By activating transcriptional proteins, LPS initiates the production of proinflammatory mediators [49] [24], leading to sustained inflammation and contributing to inflammatory diseases such as vascular

dysfunction [32].

Theoretically, natural anti-inflammatory and antioxidant molecules can be useful in preventing endothelial dysfunction [33,34], but only a few reports describe a sex-specific action [12,35].

As previously describe, the racemic form of linalool as well as its active enantiomer (LIN) have anti-inflammatory properties [15–17,21] and neuroprotective effects as well as scavenging peroxyl radicals [19, 36].

Our results show that HUVECs under LPS experienced a reduction of viability that was abolished under LIN treatment, in both FHUVECs and MHUVECs without a clear sex difference in accordance with the findings of Hou and co-workers (2024) [48]. Furthermore, the treatment with LIN alone did not alter the parameters studied except for an increase in nitrites in FHUVECs and restored endothelium viability reduced by LPS without a clear sex difference.

LPS can enhance cytokines release (IL-6 and TNF-α) [37,38] only in FHUVECs. This is in accordance with the fact that females have, in general, higher inflammatory responses and develop stronger immune response [39] although the mechanisms remain elusive and unpredictable. In our study, FHUVECs under LPS conditions exhibited significantly increased IL-6, TNF-α and MDA levels if compared to basal conditions and to MHUVECs. Likewise, LIN pretreatment inhibited the release of IL-6 and reduced MDA levels only in FHUVECs. Nevertheless, LIN pretreatment, under LPS condition, inhibited the release of IL-6 and reduced MDA levels only in FHUVECs.

Furthermore, LPS treatment produces a decrease in nitrites levels only in MHUVECs. Nitric oxide plays a crucial role as vasodilator molecule and is involved in inflammatory responses by damaging cells and modulating the release of inflammatory mediators. However, its effects can be either pro- or anti-inflammatory, depending on its location and concentration [40,41]. Moreover, nitric oxide, acts as an adaptive molecule limiting inflammatory signaling in various cell types and tissues [42], and it also acts as a potent inhibitor of lipid peroxidation by scavenging peroxyl lipid radicals [43]. This could explain the reduction in nitrites after LPS treatment observed in MHUVECs which also display lower cytokines levels. Likewise, previous research showed that the treatment with LIN before the exposure of LPS-stimulated J774. A1 macrophages inhibited nitrites accumulation through an inhibitory interaction with the iNOS enzyme [44].

Taken together, our findings suggest that LPS induces greater deleterious effects in FHUVECs well in agreement with the observations of Cignarella and colleagues, 2023.

This report confirms previously observed sex differences in inflammatory responses both *in vivo* as well as *in vitro* [5,45,46].

Consistent with these findings, other authors have described similar effects of LPS in HUVECs, even if without stratification of data by donors' sex [4]. Overall, these data reinforce the existence of sex differences in the behaviour of HUVECs [4,12,22] validating their use as a model for the study of sex differences in endothelial functions.

Mostly, these results highlight a sex-specific protective effect of LIN only in conditions of LPS-induced endothelial damage, and this effect is more evident in FHUVECs. The antioxidant and radical scavenger properties of LIN could justify these results as already demonstrated by Franconi and colleagues 2023. The protective effects of LIN against LPS-induced endothelial damage in HUVECs could be supported by various mechanisms described in the literature. For example, our recent study showed that LIN prevents the reduction of cell viability induced from H_2O_2 in PC12 cells highlighting overall a neuroprotective effect [19]. Likewise, Sabogal-Guáqueta and co-workers (2019) reported that linalool counteracts cell death mediated by glutamate-induced mitochondrial oxidative stress in immortalized neuronal HT-22 cells. Additionally, a study demonstrated antioxidant properties of the racemic linalool by showing its ability to inhibit oxidation in unsaturated fatty acids from guinea pig brains, which were exposed to H_2O_2 to induce oxidative stress [15].

These findings highlight the potential of LIN as a neuroprotective, antioxidant, and anti-inflammatory agent, suggesting its possible use as a useful drug in the clinical management of diseases in which endothelial dysfunction plays a major role.

5. Conclusions

Overall, our results provide novel valuable information that the enantiomer LIN could be an efficacy remedy able to prevent endothelial damage, especially in female cells.

Further experiments are required to explore the mechanisms underlying the protective effect of LIN on endothelial damage in greater detail. Specifically, certain molecular aspects related to the regulation of inflammation, such as apoptosis, autophagy, and interactions with regulatory factors like miRNAs and cell migration, will be investigated more thoroughly, as previously done in a similar experimental model [4, 12]. Despite that, our study provides new insight into the sex-specific action of antioxidant molecules.

6. Limitations

The current study includes some limitations. First, only one pathogenic stimulus (LPS) was measured, and, thus, a complete picture of vascular injury was not fully captured. However, as an agent widely used *in vitro*, including in models of endothelial dysfunction, it still provides a good basis for studying sex differences. Moreover, due to the observed sex differences and the divergences within the measured parameters, the effects of sex and LIN likely complement each other, necessitating further research.

CRediT authorship contribution statement

Laura Doro: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Alessandra T. Peana:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization. **Rossana Migheli:** Writing – review & editing, Writing – original draft. **Giampiero Capobianco:** Writing – review & editing, Writing – original draft. **Massimo Criscione:** Methodology. **Andrea Montella:** Writing – review & editing, Writing – original draft. **Ilaria Campesi:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflict(s) of interest to declare.

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Data availability

Data will be made available on request.

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