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Exploring the health benefits of high amylose wheat phenolic extract in human endothelial cell model: Inhibitory effects on endothelial activation

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ABSTRACT

Newly developed high amylose durum wheat genotypes, characterized by a high content of resistant starch, is gaining interest as a healthy ingredient for functional food. The objective of the present study was to investigate the effects of Svevo high amylose phenolic extracts (HAPE) on endothelial activation, the first obligatory step of the atherosclerotic process, investigating the underlying mechanisms of action. To this aim, human microvascular endothelial cells were treated with HAPE (1–10 μg/mL) and then challenged with the pro-inflammatory and pro-atherogenic cytokine TNF-α. Endothelium-leukocyte adhesion, the expression of endothelial inflammatory mediators, intracellular reactive oxygen species (ROS) levels and the activation of nuclear factor (NF)-κB were evaluated by multiple assays. The results showed that, HAPE, already at 5 μg/mL, suppressed the TNF-α stimulated expression of endothelial adhesion molecules as well as the adhesion of leukocytes to endothelial cells by more than 50% in comparison with TNF-α alone. These effects were associated with a significant ($p < 0.05$) reduction of intracellular ROS levels and NF-κB activation. In summary, our findings underscore the potential of Svevo high amylose as a functional food able to blunt endothelial activation, by inhibiting the concerted expression of endothelial markers involved in leukocyte recruitment and early atherogenesis.

1. Introduction

Atherosclerotic cardiovascular disease continues to be the leading cause of death not only in Western countries, but also worldwide due to the increased prevalence of cardiovascular risk factors such as diabetes and obesity (Roth et al., 2020). Atherosclerotic disease begins in childhood, progresses silently through a long preclinical stage, and eventually manifests clinically, usually from middle age. Over the last years, it has become clear that the initiation and progression of disease depends on deep changes in vascular biology (Libby, 2024). Endothelium has emerged as the key regulator of vascular homeostasis, as it has not merely a barrier function but also acts as an active signal transducer for circulating triggers. Alteration in endothelial function precedes the development of atherosclerosis and can also contribute to atherosclerotic lesion progression. Qualitatively and quantitatively abnormal stimuli associated with atherosclerotic risk factors, likely through the induction of heightened oxidative stress, alter the normal homeostatic function of the vascular endothelium. A complex of functional phenotypic changes makes the endothelium adhesive to circulating leukocytes, more permeable to solutes, and capable of the enhanced synthesis of mitogenic, chemoattractant, and inflammatory mediators. This concerted transcriptional activation of genes involved in leukocyte recruitment into the intima is termed 'endothelial activation', which is a subset of a more complex perturbation of normal functional properties

Abbreviations: HA, high amylose; HAPE, Svevo high amylose phenolic extracts; HMEC-1, human microvascular endothelial cells-1; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; GAE, gallic acid equivalents; d.m., dry matter.

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of the endothelium known as 'endothelial dysfunction' (Gimbrone and Garcia-Cardena, 2016).

The leukocyte recruitment to the activated endothelium is coordinated by the expression of endothelial cells adhesion proteins such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), which facilitate leukocyte rolling and extravasation into the intimal layer, as well as the cytokine interleukin 6 (IL-6) and the chemotactic factor monocyte chemoattractant protein-1 (MCP-1), which attracts circulating monocytes (Botts et al., 2021). Appreciation of the central role of the endothelium throughout the atherosclerotic process has led to the development of strategies to prevent or counteract atherosclerotic diseases by affecting endothelial function.

The dietary approach based on Mediterranean diets is recognized as an effective strategy for the prevention of atherosclerotic cardiovascular diseases (Martinez-Gonzalez et al., 2019). Cereals, including wheat, are considered basic products of the Mediterranean diet. The interest in improving the health benefits of cereal foods is continuously increasing. This is essentially due to the high frequency of their consumption worldwide, and to the chance of using them to vehicle health promoting components in the diet. Improving the nutritional quality of basic foods such as cereals offers a promising strategy to counteract atherosclerotic vascular disease (Guo et al., 2022). In this context, wholegrain wheat is a particularly important strategy for its well-established health promotion potential. Notably, wholegrain cereals are good sources of complex dietary carbohydrates (starch and dietary fiber), minerals, B vitamins and phytochemicals (Hernandez-Espinosa et al., 2020). Wheat flour is mainly composed of readily digestible carbohydrates, in the form of starch which is structured as amylose and amylopectin. In wheat, starch constitutes 65%–75% of the grain dry weight, with amylose contributing to 20%–30% of the total starch and amylopectin to the remaining 70%–80% (Zhang et al., 2017). Resistant starch, which is the fraction of dietary fiber that escapes digestion and absorption in the upper gastrointestinal tract and flows to the large intestine, serving as a substrate for resident bacteria, has the potential to improve dietary fiber in foods. Resistant starch has been found to have many benefits in foods such as lowering glycaemic index, promoting satiety, prebiotic properties (Di Rosa et al., 2023; Hallstrom et al., 2011; Sissons et al., 2020). Possible health benefits from consumption of slowly digested carbohydrates and resistant starch include improved metabolic control and decreased risk of cardiovascular disease, obesity, and type 1 and 2 diabetes (Maiya et al., 2023).

In addition to fibres, phenolic acids are important components found in wholegrains that are largely responsible for their health advantages. Phenolic acids as free, conjugated and bound forms exhibit significantly benefits in the prevention from chronic diseases such as cardiovascular disease and diabetes (Calinoiu and Vodnar, 2018).

In the present study, we characterized the Svevo High Amylose (SvevoHA) phenolic extract (HAPE) for phenolic acid profile and antioxidant capacity and investigated its potential anti-inflammatory nutraceutical properties. To this aim, we used a well-known model of vascular inflammation and atherogenesis represented by cultured human endothelial cells stimulated with tumour necrosis factor-α (TNF-α), an inflammatory cytokine produced by macrophages/monocytes during inflammatory and atherosclerotic process. We monitored the nutraceutical effects of HAPE, by evaluating the leukocyte adhesion to inflamed endothelial cells as well as the related endothelial expression of adhesion molecules. Moreover, the gene expression of inflammatory mediators, including chemokines and pro-inflammatory cytokines was analyzed.

2. Materials and methods

2.1. Phenolic acids extraction and analyses

Svevo-HA plant material was grown under conventional farming at the experimental field affiliated with the Experimental Farm "Nello Lupori" (University of Tuscia), located in Viterbo (Italy, lat. 42°26′ N, long. 12°04' E, altit. 310 m a.s.l.), in the 2021–2022 growing season. The semolina sample was produced by Grandi Mulini Italiani S.r.l. (Rovigo, Italy) using an industrial miller. The extraction of phenolic acids was performed according to Laddomada et al. (2015) to extract the soluble free, soluble conjugated and insoluble bound fractions. To evaluate the composition of individual phenolic acids each fraction was analyzed using an Agilent 1100 high-performance liquid chromatography (HPLC) equipped with a photodiode array detector (DAD) (Agilent Technologies, Waldbronn, Germany) following the conditions used in Bonanno et al., (2019); Bonanno et al. (2019). The individual phenolic acids were quantified via rationing to the internal standard (3, 5-dichloro-4-hydroxybenzoic acid) added to each sample and using calibration curves of phenolic acid standards having undergone the same extraction procedure. The three phenolic acid fractions were combined into one sample (Svevo HAPE) which was analyzed using a rapid method to measure the total phenols content. Briefly, HAPE (50 μL) at different dilutions were mixed with the Folin–Ciocalteu reagent (1:5, v/v, 50 μL) and sodium hydroxide solution (0.35 mol/L, 100 μL) was added to each well. The absorbance value at 760 nm was recorded after 5 min incubation. Gallic acid was used to obtain a calibration curve in the range from 2.5 to 40.0 mg/L ($R \geq 0.9997$). Gallic acid equivalents (GAE) were used to express the total phenol content of samples. Then, HAPE was characterized for total antioxidant capacity by ABTS according to Romano et al. (2022).

2.2. Cell culture and treatment

The human microvascular endothelial cell line (HMEC-1), obtained from Dr. Thomas J. Lawley, was cultured in MCBD 131 added with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin, and grown at 37 °C in a humidity-controlled 5% CO2 cell culture incubator, as previously described (Ades et al., 1992; Calabriso et al., 2018). Human monocytoid THP-1 cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium added with 10% FBS, 2 mmol/L glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO2 humidified atmosphere at 37 °C.

To evaluate the anti-inflammatory activity of HAPE, sub-confluent endothelial cells were shifted to medium supplemented with 5% FBS and subsequently treated with increasing concentrations of high amylose wheat phenolic extract (HAPE, 1, 5, 10 and 25 μg/mL GAE) for 2 h before stimulation with TNF-α (10 ng/mL) for 0–24 h. As vehicle control, endothelial cells were incubated with an appropriate amount of solvent (<0.025% v/v).

2.3. Cell viability

Endothelial cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This is a method based on the ability of viable cells to convert MTT, a soluble tetrazolium salt, into an insoluble precipitate of formazan, which is then quantified spectrophotometrically (Mosmann, 1983). Briefly, HMEC-1 cells (2×10^4 cells/well) were seeded onto 96-well cell culture plates and treated with increasing concentrations of HAPE (1, 5, 10, and 25 μg/mL) for 24 h. Subsequently, the cells were incubated with MTT (0.5 mg/mL) for 4 h, and then the formazan products were dissolved in isopropanol, and the absorbance was measured at 540 nm using a microplate reader. Results were expressed as a percentage of untreated cells.

2.4. Leukocyte adhesion assay

HMEC-1 cells (2×10^5 cells/well) were grown to confluence in 6-well cell culture plates, incubated in the absence (vehicle) or presence

of HAPE (1, 5, 10 μg/mL) for 2 h, and stimulated with TNF-α (10 ng/mL) for another 16 h. THP-1 cells were labeled with 1 μmol/L calcein AM (Molecular Probes, a brand of Thermo Fisher Scientific, Waltham, MA, USA) for 30 min, seeded at $5x10^5$ cell density onto endothelial monolayers, and incubated under rotating conditions (63 rpm) at 21 °C (Calabriso et al., 2016). After gently washing twice with PBS, the adherent THP-1 cells were observed under an inverted phase contrast and fluorescence microscope by obtaining five photomicrographs from each well. Furthermore, the fluorescence intensity of each well was measured in a microplate reader with an excitation/emission wavelength of 485/530 nm.

2.5. Detection of endothelial cell surface molecules

HMEC-1 cells (2×10^4 cells/well) were grown to confluence in 96-well cell culture plates, incubated in the absence (vehicle) or presence of HAPE (1, 5, and 10 μg/mL) for 2 h, before stimulation with TNF-α (10 ng/mL) for additional 16 h. The detection of endothelial cell surface molecules VCAM-1 and ICAM-1 was carried out by cell surface enzyme immunoassays (EIA), using primary mouse anti-human monoclonal antibodies against VCAM-1, ICAM-1 (HU5/3), or the monoclonal antibody against the non-cytokine-inducible and constitutive endothelial cell antigen E1/1, as previously described (Calabriso et al., 2016).

2.6. Measure of secreted inflammation markers

HMEC-1 cells (1×10^5 cells/well) were grown to confluence in 12-well cell culture plates, incubated in the absence (vehicle) or presence of HAPE (5 and 10 μg/mL) for 2 h, before stimulation with TNF-α (10 ng/mL) for additional 16 h. The conditioned media were collected, and the levels of secreted MCP-1 and IL-6 were determined using the corresponding enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions. Specifically, MCP-1 levels were determined with ELISA kit by Invitrogen (a brand of Thermo Fisher Scientific, Waltham, MA, USA) with a sensitivity 2.3 pg/mL and an assay range of 15.6–1000 pg/mL and IL-6 levels were determined with ELISA kit by Cayman Chemicals (Ann Arbor, MI, USA) with a sensitivity 7.8 pg/mL and an assay range of 7.8–250 pg/mL.

2.7. RNA isolation and real-time quantitative PCR

HMEC-1 cells (2×10^5 cells/well) were grown in 6-well cell culture plates, incubated in the absence (vehicle) or presence of HAPE (5 and 10 μg/mL) for 2 h, before stimulation with TNF-α (10 ng/mL) for additional 4 h. Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and quantified spectrophotometrically. Total RNA (1 μg) was converted into first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy). Quantitative RT-PCR was performed, with 16 ng cDNA, in CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy) using a SYBR Green PCR Master Mix (Bio-Rad Laboratories, Milan, Italy) and the synthesized primers (Thermo Fisher Scientific, Waltham, MA, USA) listed in Table 1. All reactions were assessed in triplicate. The relative changes in gene expression were calculated by comparative critical threshold method ($2^{-\Delta\Delta CT}$). As housekeeping gene was used GAPDH was quantified for each sample, and the data were normalized accordingly. Results are expressed as fold increase relative to unstimulated control (=1).

2.8. Preparation of nuclear protein extracts and NF-κB activation assay

HMEC-1 cells (2×10^5 cells/well) were grown in 6-well cell culture plates and treated with HAPE (5 and 10 μg/mL) for 2 h and stimulated with TNF-α (10 ng/mL) for an additional 2 h, after which nuclear proteins were purified by using a kit from Active Motif (Carlsbad, CA, USA). Briefly, HMEC-1 cells were collected in ice-cold PBS containing phosphatase inhibitors and centrifuged at 300×*g* for 5 min. Pellets were resuspended in a hypotonic buffer and centrifuged at 14,000×*g* for 30 s. Then, nuclear proteins were solubilized in lysis buffer containing proteasome inhibitors, and protein concentrations were quantified by Bio-Rad protein assay (Bio-Rad Laboratories, Milan, Italy). The DNA-binding activity of NF-κB (p65 subunit) was evaluated using the Active Motif's ELISA-based "TransAM kit" following the manufacturer's instructions.

2.9. Detection of intracellular ROS production

Cellular ROS levels were assessed using a carboxy-2,7-dichlorofluorescein diacetate (CM-H2DCFDA) probe, as described previously (Calabriso et al., 2018). CM-H2DCFDA is hydrolysed in the cytosol to form the DCFH carboxylate anion. Oxidation results in the formation of fluorescent DCF, which is maximally excited at 495 nm and emits at 520 nm. HMEC-1 cells (5×10^4 cells/well) were grown in 24-well cell culture plates and incubated in the absence (vehicle) or presence of HAPE (5 and 10 μg/mL) for 2 h and stimulated with TNF-α for an additional 2 h, and, after that, loaded with the probe CM-H2DCFDA (10 μmol/L) for 45 min at 37 °C in the dark. After gently washing the endothelial cell monolayers twice with PBS, phenol red-free medium was added, and fluorescence was monitored by spectrofluorimetric and microscopic analysis.

2.10. Determination of endothelial nitric oxide production

Production of nitric oxide (NO) by endothelial cells was measured via its stable oxidation product, nitrite, using a commercial kit by Cayman. Nitrite accumulation in culture supernatants during 24 h of incubation with TNF-α, in the absence or presence of HAPE was determined using the diazotization reaction (Griess assay), as described previously (Suschek et al., 1993). The nitrite concentrations in the cell culture supernatant were normalized against the number of live cells as assessed by neutral red staining.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences between two groups were determined by unpaired Student's t-test. Multiple comparisons were performed by one-way analysis of variance (ANOVA), and individual differences were then tested by the Fisher's protected least-significant difference test after the demonstration of significant inter-group differences by ANOVA. A p value < 0.05 was considered to be statistically significant.

Table 1
Oligonucleotides used for quantitative real-time PCR analysis.

Gene name	Accession number	Forward Primer	Reverse Primer	Size (bp)
IL6	NM_000600.3	5′-AGGAGACTTGCCTGGTGAAA-3′	5′-CAGGGGTGGTTATTGCATCT-3′	180
CCL2/MCP1	NM_002982.3	5′-CCCCAGTCACCTGCTGTTAT-3′	5′-TCCTGAACCCACTTCTGCTT-3′	166
VCAM1	NM_001078.3	5′-CATGGAATTCGAACCCAAAC-3′	5′-CCTGGCTCAAGCATGTCATA-3′	140
ICAM1	NM_000201.2	5′-AGACATAGCCCCACCATGAG-3′	5′-CAAGGGTTGGGGTCAGTAGA-3′	190
GAPDH	NM_002046.3	5′-ATCACTGCCACCCAGAAGAC-3′	5′-TTCTAGACGGCAGGTCAGGT-3′	210

3. Results and discussion

This research activity was part of the MEDWHEALTH, a project funded by PRIMA Foundation (Horizon, 2020), whose objective is to improve the health value of traditional durum wheat-based foods from the Mediterranean area, using innovative materials such as the Svevo-HA, a durum wheat variety characterized by high content of amylose (Romano et al., 2022; Sestili et al., 2015). Previous evidence showed that Svevo HA wholemeal flour extract is characterized by a higher phenolic acids content, and a higher antioxidant activity when compared to Svevo (control) and the other MEDWHEALTH genetic materials (Romano et al., 2022). Therefore, in this work we decided to further investigate the anti-inflammatory activity of Svevo HA phenolic acids extracts. The aim of the present research was to analyse the antioxidant and anti-inflammatory properties of a durum wheat phenolic extract isolated from high amylose semolina in an *in vitro* model of inflammation and atherosclerosis (inflamed endothelial cells) and to evaluate the underlying mechanisms of action.

3.1. Characterization of phenolic acid profile and antioxidant capacity of Svevo-HA phenolic extract

Svevo-HA phenolic extract (HAPE) was characterized for its content and composition in individual free, conjugated, and bound phenolic acids (Table 2). The results showed that the bound forms of phenolic acids were the most abundant (337.97 μg/g d.m.), followed by the conjugated (66.68 μg/g d.m.) and the free forms (2.99 μg/g d.m.). Overall, ferulic acid was the most abundant compound, particularly in the bound insoluble form (305.98 ± 3.10 μg/g d.m.), representing about 74.8% of total phenolic acids (Table 2). Sinapic acid was second for abundancy both in the conjugated (35.70 μg/g d.m.) and bound (24.84 μg/g d.m.) forms, whereas other compounds, namely *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid, and syringic acid, were less represented (Table 2). Overall, the bound phenolic acids of HAPE accounted for about 83% of total phenolic acids, whereas the conjugated and free forms contributed for about 16% and less than 1%, respectively. These results suggest that Svevo-HA semolina has similar nutraceutical value to that of wholegrain flours, due to the elevated content of phenolic compounds (Romano et al., 2022). These results may have relevant implications on the daily uptake of wheat polyphenols since the consumption of whole wheat grain foods is still modest compared to more refined products made using semolina (Laddomada et al., 2015; Romano et al., 2022). The total antioxidant capacity of Svevo-HAPE was 26.7 mmol TE/kg which was higher than that of Svevo (control) and other MEDWHEALTH materials (data not shown).

3.2. High amylose wheat phenolic extract prevents endothelial activation

Our previous evidence showed that Svevo HA semolina extract was characterized by a higher phenolic acids content, and a higher antioxidant activity when compared to Svevo (control) (Romano et al., 2022). Based on that, in this work we decided to investigate the effect of increasing concentrations of HAPE on endothelial activation in order to evaluate the concerted expression of gene products involved in leukocyte recruitment and early atherogenesis (Carluccio et al., 1999, 2003; Pober and Cotran, 1990).

To this aim, the HAPE biological properties were analyzed in an *in vitro* model represented by human cultured microvascular endothelial cells, HMEC-1, challenged with tumour necrosis factor (TNF)-α, a classical, pleiotropic pro-inflammatory cytokine, able to trigger endothelial activation and dysfunction (Pober, 2002).

We preliminarily examined the effects of HAPE on endothelial cell viability using the MTT assay. For this purpose, HMEC-1 cells were treated with increasing concentrations (1, 5, 10 and 25 μg/mL) of HAPE for 24 h (Fig. 1A). The MTT assay results showed that HAPE at 25 μg/mL started to be toxic for HMEC-1 cells (>20% toxicity) compared with the control vehicle. Therefore, in the following experiments we used lower HAPE concentrations, which were ≤10 μg/mL. Then, we investigated the effects of HAPE on the expression of endothelium-leukocyte adhesion, a crucial step in inflammatory and atherosclerotic process. HMEC-1 cells were pre-exposed to increasing concentrations (1, 5 and 10 μg/mL) of HAPE or ethanol vehicle control (CTR) for 2 h, before stimulation with TNF-α (10 ng/mL) for 16 h. Subsequently, stimulated endothelial monolayers treated with or without HAPE were exposed to a monocyte suspension under rotating conditions to reproduce the monocyte adhesion step in atherogenesis and evaluate the functional role of HAPE in the endothelial-monocyte interaction. The number of leukocytes adhering to control unstimulated HMEC-1 cells (CTR) was very low, whereas it increased strongly when HMEC-1 cells were stimulated with TNF-α (Fig. 1). As shown in the bar graph (Fig. 1B) and representative images (Fig. 1C), HAPE treatment significantly reduced leukocyte adhesion to TNF-α-stimulated HMEC-1 monolayers in a concentration-dependent manner, with a significant inhibitory effect already evident at 5 μg/mL (Fig. 1B and C). HAPE significantly lowered TNF-α-stimulated leukocyte-endothelium adhesion by 59% and 68% at concentrations of 5 and 10 μg/mL, respectively (Fig. 1B).

An extract obtained from Svevo (control) was also used preliminarily as a comparison. Analyzing the biological effects at the same phenolic concentration, no differences were noticed (data not shown). This can be explained by the similar composition in phenolic acids between Svevo HA and Svevo control.

Since leukocyte-endothelial cell adhesion is mediated by increased expression of endothelial adhesion molecules, we investigated the effects of HAPE on TNF-α-induced expression of VCAM-1 and ICAM-1, by cell surface EIA. Both endothelial adhesion molecules were expressed at low levels in unstimulated control HMEC-1 cells and their expression was strongly upregulated by TNF-α stimulation (Fig. 2). HAPE significantly inhibited the TNF-α-stimulated expression of both endothelial adhesion molecules, with a marked effect on VCAM-1. The expression of VCAM-1 and ICAM-1 was reduced in a concentration-dependent manner with significant effects already evident at 5 μg/mL, reaching a reduction of approximately 56% for VCAM-1 and 30% at 10 μg/mL for ICAM-1 (Fig. 2). The inhibitory effect of HAPE occurred only under pro-inflammatory conditions mimicked by TNF-α stimulation, without affecting the expression of the endothelial surface constitutive antigen E1/1 (data not shown).

To understand the potential mechanism of action underlying the inhibitory effect of HAPE on TNF-α-induced endothelial cell surface expression of adhesion molecules, we evaluated the mRNA levels of VCAM-1 and ICAM-1, using quantitative RT-PCR. The results show that

Table 2
Phenolic acids composition and content of individual free, conjugated and bound phenolic acids (μg/g dry matter) in HAPE.

Phenolic acid fraction	*p*-Hydroxybenzoic acid	Syringic acid	Vanillic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Total sum
Free	0.07 ± 0.01 ^{c}	0.19 ± 0.01 ^{c}	0.37 ± 0.07 ^{c}	n.d.	1.77 ± 0.07 ^{c}	0.60 ± 0.02 ^{c}	2.99 ± 0.18 ^{c}
Conjugated	2.49 ± 0.07 ^{a}	1.43 ± 0.02 ^{a}	4.65 ± 0.02 ^{a}	0.22 ± 0.03 ^{b}	22.18 ± 1.71 ^{b}	35.70 ± 1.47 ^{a}	66.68 ± 3.31 ^{b}
Bound	0.55 ± 0.03 ^{b}	1.20 ± 0.02 b	0.79 ± 0.01 ^{b}	4.61 ± 0.22 ^{a}	305.98 ± 3.10 ^{a}	24.84 ± 0.24 ^{b}	337.97 ± 3.62 ^{a}
Total							**407.6 ± 7.09**

Values are mean ± SD of three determinations (n = 3). One-way ANOVA followed by Bonferroni's post-hoc comparisons tests was performed to establish significant differences among phenolic acids means ($p < 0.05$). Values with the different superscript letters within each column indicate significant differences ($p < 0.05$).

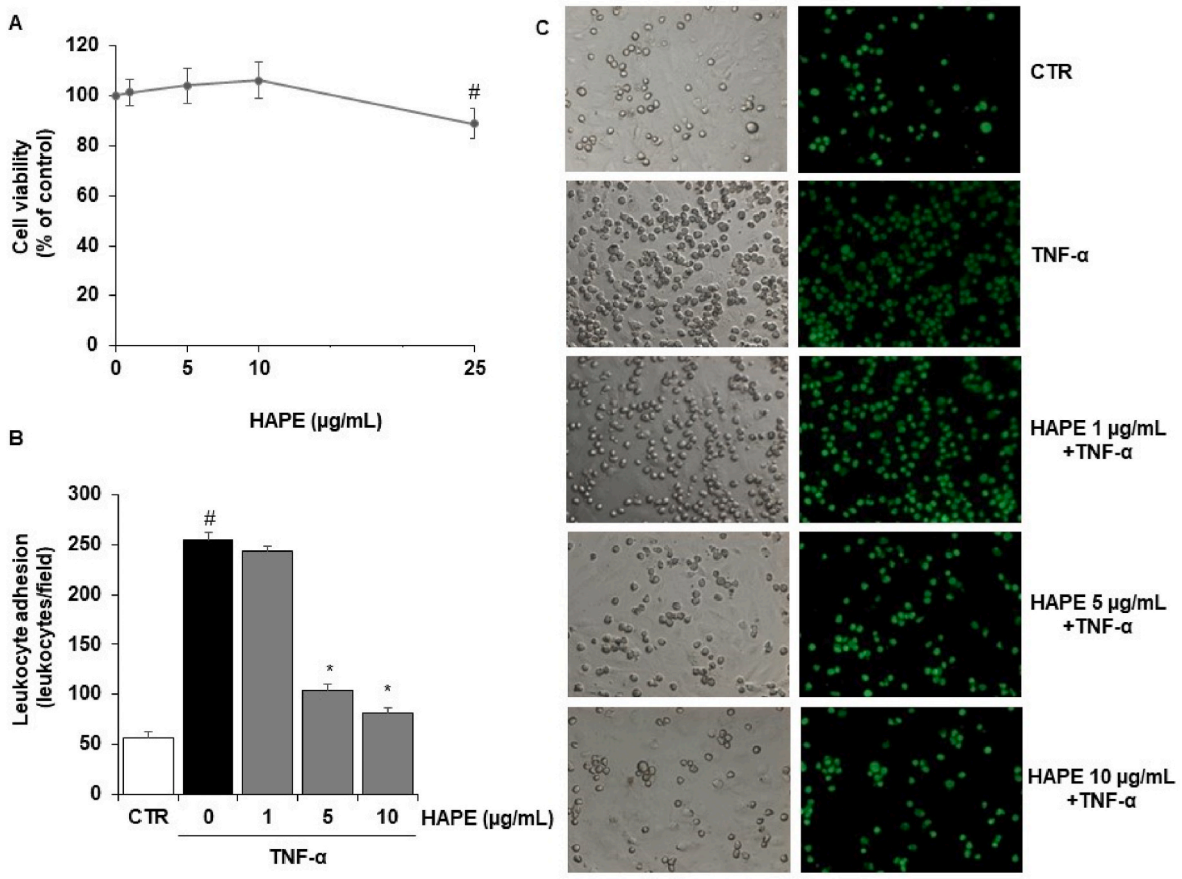

Fig. 1. Effects of HAPE on endothelial cell viability and TNF-α-stimulated endothelium-leukocyte adhesion. HMEC-1 cells were exposed to increasing concentrations of HAPE (1, 5, 10 and 25 μg/mL) for 24 h and cell viability was analyzed by MTT assay (A). HMEC-1 cells were pre-treated with HAPE (1, 5, and 10 μg/mL) for 2 h, followed by incubation with TNF-α (10 ng/mL) for 16h and then co-cultured with labeled monocytic THP-1 cells for 1 h (B and C). The number of adherent THP-1 cells was measured by the fluorescence plate reader (B) or monitored by fluorescence microscope (C). Each experiment was performed in triplicate (n = 3). #$p < 0.01$ versus control; *$p < 0.05$ versus TNF-α alone.

HAPE reduced the mRNA levels of VCAM-1 and ICAM-1 in a concentration-dependent manner in TNF-α-stimulated endothelial cells (Fig. 2), thus suggesting that the inhibitory action of HAPE occurred at a pre-translation level. These results confirmed the effect of HAPE in counteracting endothelium-leukocyte adhesion through reduced expression of the adhesion molecules VCAM-1 and ICAM-1.

These results showed for the first time that phenolic extract of Svevo-HA semolina possesses endothelial protective properties by inhibiting endothelial monocyte adhesion and endothelial adhesion molecule expression (VCAM-1 and ICAM-1), so blunting the stimulated endothelial activation.

These results agree with our previous data which showed that that biofortified bread, obtained by adding different durum wheat milling by-products rich in phenolic compounds, exhibited multiple anti-inflammatory and anti-atherosclerotic properties (Calabriso et al., 2020).

Here, biofortified bread extracts inhibited monocyte adhesion to LPS-stimulated endothelial cells, in a concentration-dependent manner by reducing mainly endothelial VCAM-1 expression. Phenolic acid extracts contained in 10 mg biofortified bread downregulated the LPS-induced expression of chemokines MCP-1, M-CSF, and CXCL-10 as well as pro-inflammatory cytokines TNF-α and IL-1β, in endothelial cells and monocytes, with CXCL-10 as the most reduced inflammatory mediator. Among biofortified bread phenolic acids, ferulic, sinapic, and *p*-coumaric acids significantly inhibited the LPS-stimulated CXCL-10 expression in vascular cells. The reduced pro-inflammatory response was related to a slightly but significant reduction of intracellular oxidative stress. In addition, in the present, as in a previous study, the dampening of the monocyte adhesion to inflamed endothelium and the associated expression of endothelial adhesion molecules occurred at phenolic acid concentrations of about 5 and 10 μg/mL.

Our data are consistent with other research studies reporting an improvement in endothelial function by wheat phenolic extracts as a whole, as well as by pure phenolic acids or fermented products which are able to counteract the harmful effect on the endothelium induced by inflammatory stimuli (Fuentes and Palomo, 2014; Lucchesi et al., 2014).

In vitro studies with fermented powder obtained from organic whole grains (Triticum aestivum) also showed protective effects in human microvascular endothelial cells exposed to oxidized-LDL (Lucchesi et al., 2014). Similar endothelial beneficial effects were observed in human endothelial progenitor cells in response to oxidative stress (Giusti et al., 2017).

These healthful properties of wheat phenolic extracts could be biologically relevant as were obtained at concentrations achievable in vivo after intake of wheat-based foods (Di Lorenzo et al., 2021; Manach et al., 2004). In animal models, the plasma concentration of phenolic acids has been reported to enhance in a dose-dependent manner, reaching maximum values of 110 μmol/L. Concordantly, human nutritional studies revealed phenolic acids in plasma after a single wholegrain breakfast and showed threefold increase after regular wholegrain consumption (Calinoiu and Vodnar, 2018). The reduced endothelial activation after conditioning of the endothelial monolayer with HAPE extract could be due by the presence in the extract of various cinnamic and benzoic derivative, and particularly ferulic acid (both in free and

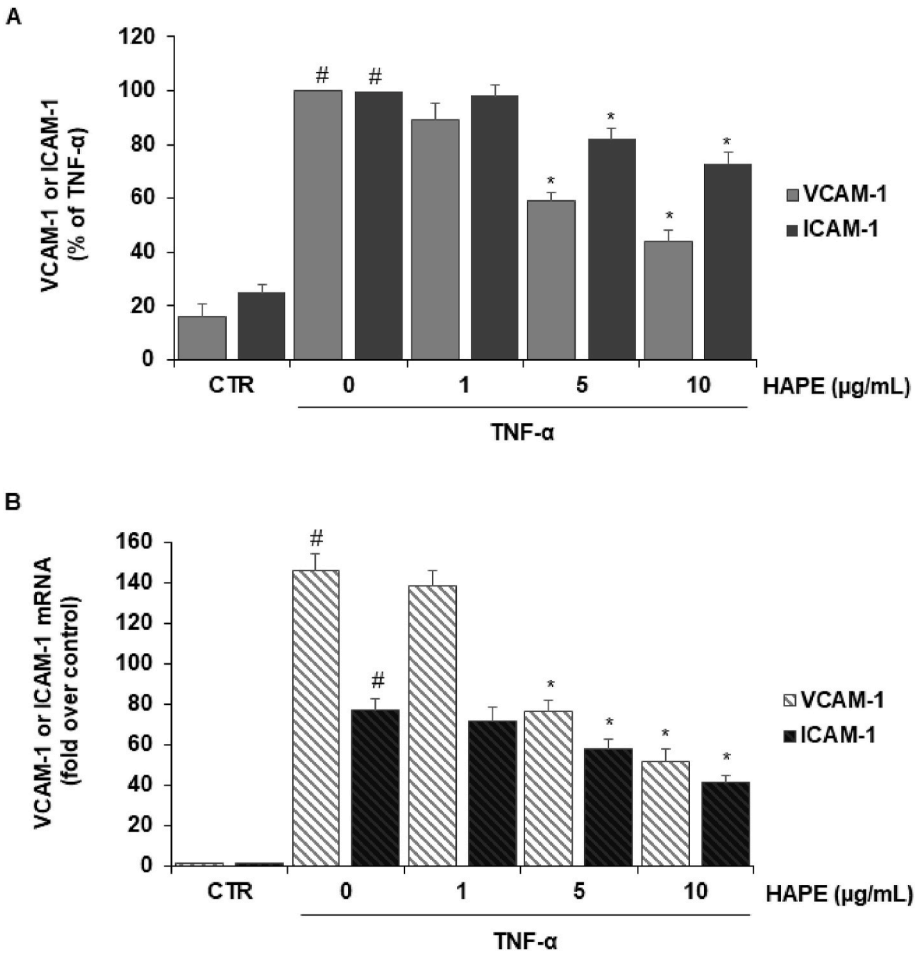

Fig. 2. Inhibitory effects of HAPE on the TNF-α-stimulated expression of endothelial adhesion molecules. HMEC-1 cells were pre-treated with increasing concentrations of HAPE (1, 5, and 10 μg/mL) for 2 h, followed by incubation with TNF-α (10 ng/mL) for 4 h (B) or 16h (A). Endothelial cell surface expression of VCAM-1 and ICAM-1 was assessed by cell surface EIA and expressed as percentage of TNF-α-stimulated endothelial cells (A). VCAM-1 and ICAM-1 mRNA levels were analyzed by quantitative RT-PCR and expressed as fold over unstimulated control (mean ± SD, n = 3) (B). Each experiment was performed in triplicate. #$p < 0.01$ versus control; *$p < 0.05$ versus TNF-α alone.

bound form).

It has been recognized that the content of cinnamic and benzoic derivative present in several food, including wheat, contribute to endothelial protection (attenuated oxidative stress, improved nitric oxide bioavailability and decreased E-selectin, ICAM-1 and VCAM-1 expression, among others) (Fuentes and Palomo, 2014).

In addition, previous studies found that phenolic acids significantly inhibited events connected with endothelial activation, along with the stimulated expression of adhesion molecules, such as VCAM-1 and ICAM-1 (Calabriso et al., 2020). This effect was accompanied by a functional counterpart, i.e., reduced monocyte adhesion to cytokine-activated endothelium (Calabriso et al., 2020; Del Bo et al., 2016; Liu et al., 2018; Ma et al., 2010; Wang et al., 2005).

Liu et al. (2018) showed that ferulic acid inhibited the formation of advanced glycation end products (AGEs) and mitigates the AGEs-induced inflammatory response in human umbilical vein endothelial cells (HUVEC) by suppressing the activation of NF-κB and p38 MAPK signaling pathway.

Moreover, ferulic acid attenuated inflammation and oxidative stress in a rat model of LPS-induced acute respiratory distress syndrome (Zhang et al., 2018). Other phenolic acids, such as p-coumaric acid, sinapic acid and vanillic acid, which are present in HAPE, may contribute to the HAPE vasculo-protective action (Amin et al., 2015; Calabriso et al., 2020; Krga et al., 2018).

Regarding the in vivo relevance of selected concentrations, we recognize that this is an *in vitro* study, the results of which should be verified in vivo. However, to the best of our knowledge, the vascular anti-inflammatory effects observed in this study could be biologically relevant, occurring at concentrations of phenolic acids achievable in vivo. Indeed, in rat plasma, the concentration of ferulic acid, the major phenolic acid of our extract, has been reported increase in a dose-dependent manner, reaching maximum values of 110 μmol/L (Zhao and Moghadasian, 2008). Intervention studies in humans confirmed the presence of ferulic acid in plasma after a single consumption of whole grain breakfast cereals (Kern et al., 2003) and showed a threefold increase in plasma of fasting subjects after regular whole grain consumption (Costabile et al., 2008), indicating the potential nutraceutical relevance of phenolic acids used under our experimental conditions.

3.3. *High amylose wheat phenolic extract inhibited TNF-α-stimulated expression of proinflammatory mediators in endothelial cells*

To further investigate the vascular anti-inflammatory properties of the phenolic component of high-amylose wheat, we evaluated HAPE effects on gene expression and protein release of key endothelial inflammatory mediators such as proinflammatory cytokines and chemokines, playing a crucial role in the triggering and maintaining the inflammatory environment that fuels endothelial activation. To this aim, HMEC-1 cells were challenged with the pro-inflammatory cytokine TNF-α, which significantly increased the release of the chemokine monocyte

chemoattractant protein-1 (MCP-1) and the cytokine interleukin-6 (IL-6) (Fig. 3A and B). Pre-treatment with increasing concentrations (1, 5 and 10 μg/mL) of HAPE reduced, in a concentration-dependent manner, the release of MCP-1 and IL-6 in HMEC-1 (Fig. 3A and B). The inhibitory effect of HAPE was confirmed at the transcription level, as reported by the reduced mRNA levels of both inflammatory mediators (Fig. 3C and D).

These results help to explain the vasculo-protective and anti-inflammatory action of the phenolic components of wheat. Indeed, HAPE reduced the TNF-α-induced expression of inflammatory mediators such as MCP-1 and IL-6 involved in the recruitment of leukocyte cells to the sites of inflammation and atherogenesis.

An increased production of pro-inflammatory cytokines is related to disease progression and promotes atherosclerosis (Ait-Oufella et al., 2011; Fatkhullina et al., 2016). Cytokine-induced activation of endothelial cells can cause endothelium dysfunction accompanied by upregulation of adhesion molecules and chemokines, which promotes migration of immune cells (monocytes, neutrophils, lymphocytes) into atherosclerosis site (Szmitko et al., 2003). Moreover, IL-6 promotes endothelial cell activation and induces expression of cell adhesion molecules such as ICAM-1, VCAM-1 and E-selectin on endothelial cells (Wung et al., 2005)

The results of the present study were in agreement with previous findings showing that phenolic extracts from biofortified wholegrain bread blunted the endothelial expression on MCP-1 and IL-6 (Calabriso et al., 2020), thus suggesting a beneficial role of the phenolic acids present in wheat extract.

3.4. High amylose wheat phenolic extract prevented the TNF-α-stimulated NF-κB activation and intracellular ROS levels in endothelial cells

The expression of inflammatory genes is mainly regulated by the activation of the transcription factor NF-κB. Generally, NF-κB is relegated to the cytoplasm of resting cells in an inactive form; inflammatory stimuli activate NF-κB, which translocates to the nucleus, where it binds to the promoters of target genes, such as those that encode proinflammatory proteins. In endothelial cells, we evaluated the effects of HAPE on TNF-α-stimulated NF-κB activation by an ELISA-based method, which measures the DNA binding activity of the p65 subunit of NF-κB. Fig. 4 shows that TNF-α induced NF-κB activation, as evidenced by increased levels of the p65 subunit in endothelial cell nuclear extracts. Pretreatment with HAPE prevented, in a concentration-dependent manner, TNF-α-induced nuclear translocation of p65 subunit (Fig. 4A), demonstrating the ability of the wheat phenolic extract to modulate NF-κB activation in response to inflammatory stimuli.

Since NF-κB contribute to regulate the expression of genes that have NF-κB consensus binding sites in their promoter, the reduced activation of NF-κB by HAPE could explain the reduced expression of inflammatory genes regulated by NF-κB, including VCAM-1, ICAM-1, MCP-1 and IL-6. Pertinently to a potential anti-oxidant effect by HAPE, NF-κB activation can be induced by an alteration of the cellular redox balance, between oxidants and antioxidants, which can be inhibited by treatment with antioxidants, such as phenolic compounds (Chaudhary et al., 2023).

Excessive ROS production in endothelial cells can result in a condition of oxidative stress which can lead to vascular damage by targeting several homeostatic processes, including vasodilation by reducing the levels of the vasodilator nitric oxide (NO), which is produced by nitric

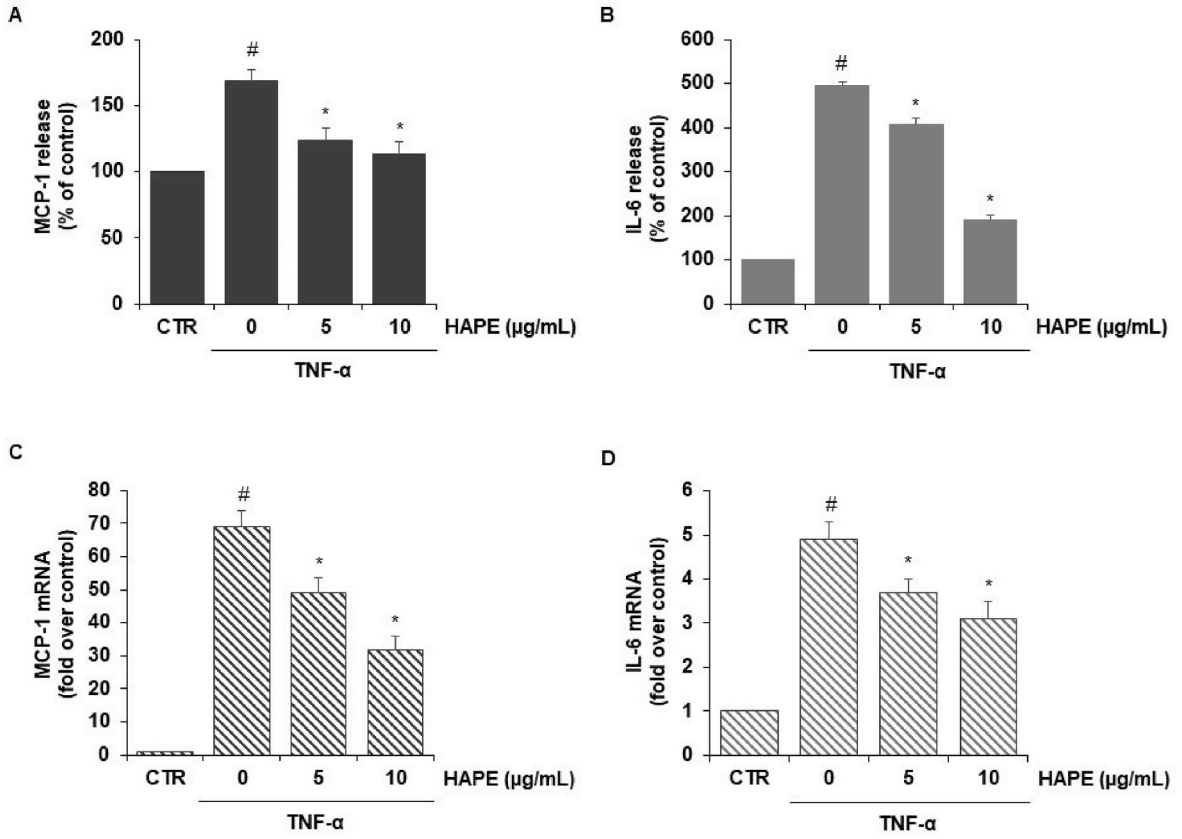

Fig. 3. Inhibitory effects of HAPE on MCP-1- and IL-6 release and expression in TNF-α stimulated endothelial cells. HMEC-1 cells were pre-treated with HAPE (5 and 10 μg/mL) for 2 h, followed by incubation with TNF-α (10 ng/mL) for 4 h (C, D) or 24 h (A, B). IL-6 and MCP-1 release in culture medium was analyzed by ELISA assay (A, B). Results are shown as percentage of unstimulated control (mean ± SD, n = 3). IL-6 and MCP-1 mRNA levels were determined by quantitative RT-PCR (C, D) and are expressed as fold over unstimulated control (mean ± SD). Each experiment was performed in triplicate. #$p < 0.01$ versus control; *$p < 0.05$ versus TNF-α alone.

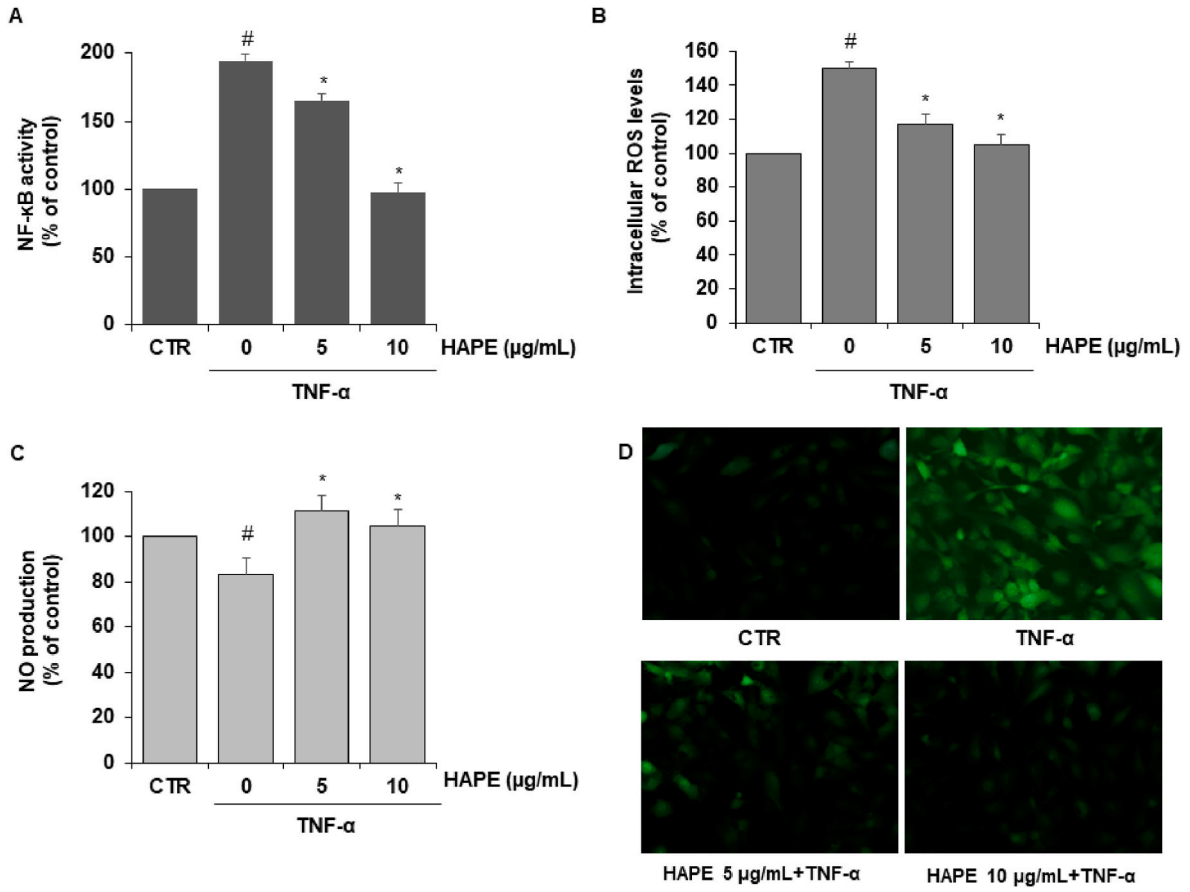

Fig. 4. Inhibitory effects of HAPE on TNF-α-stimulated NF-κB activation and oxidative stress in endothelial cells. HMEC-1 cells were pre-treated with HAPE (5 and 10 μg/mL) for 2 h, followed by incubation with TNF-α (10 ng/mL) for 2 h. NF-κB activation was assessed in nuclear proteins by an ELISA-based method measuring the DNA-binding activity of p65 NF-κB subunit (A). Intracellular ROS were analyzed by using carboxy-H2DCFDA staining by fluorescence plate reader (B) and fluorescence microscope (D). (A–C) Data are reported as unstimulated control percentage (mean ± SD, n = 3). Each experiment was performed in triplicate. #$p < 0.01$ versus control; *$p < 0.05$ versus TNF-α alone.

oxide synthase.

Indeed, ROS such as superoxide anion react with NO forming peroxynitrite (OONO−), thereby reducing NO bioavailability. In addition, increased ROS causes uncoupling of endothelial NO synthase (eNOS), which favors the production of O2– over NO, further increasing ROS in endothelial cells and thus exacerbating inflammation and endothelial activation/dysfunction (Endemann and Schiffrin, 2004).

Therefore, we evaluated the effects of HAPE on TNF-α-induced intracellular ROS levels, using the ROS-sensitive carboxy-H2DCFDA fluorescent probe, as well as the NO production by Griess method (Suschek et al., 1993).

Our findings showed that the stimulation with TNF-α produced a significant increase in intracellular ROS levels and decreased NO levels in cultured endothelial cells. These effects were attenuated by pre-treatment with HAPE (Fig. 4B–D).

These results highlight significant antioxidant action of HAPE which can explain its effects on NF-kB activation and the related regulation of the concerted expression of endothelial inflammatory mediators.

Altogether, the present study demonstrates that phenolic extracts from HA wheat exhibited antioxidant effects in TNF-α challenged vascular endothelial cells confirming their antioxidant properties showed in cell-free systems (Romano et al., 2022). Furthermore, these data are consistent with previous results that showing an attenuation of intracellular oxidative stress, associated with a reduction of lipid peroxidation by whole grain phenolic extracts in endothelial cells (Calabriso et al., 2020). The extract antioxidant properties were associated with the content of phenolic acids including ferulic, sinapic and p-coumaric acids (Calabriso et al., 2016; Sakai et al., 2012; Yun et al., 2008). The antioxidant activity may also explain the nutrigenomic properties of HAPE which, by reducing the activation of NF-κB, can dampen the expression of inflammatory genes and endothelial activation leading to improved vascular function.

4. Conclusions

As part of the MEDWHEALTH project, Svevo-HA may be proposed as a novel wheat-based food with high amylose content and resistant starch able to counteract the problem of non-communicable diseases, especially atherosclerosis, the leading causes of mortality globally.

Atherosclerotic vascular diseases are increasing in their rate due to the changing lifestyles and eating habits also in the Mediterranean regions. Therefore, exploring the anti-atherosclerotic properties of new wheat genetic materials is of extreme interest. In an *in vitro* model of early atherogenesis based on cultured endothelial cells challenged with inflammatory stimuli, we observed that the phenolic extract of the recently developed durum wheat Svevo-HA, rich in amylose, is able to reduce the stimulated surface expression of endothelial leukocyte adhesion molecules, such as VCAM-1 and ICAM-1, in a concentration dependent fashion, also decreasing the stimulated production of endothelial chemoattractants, such as MCP-1 and IL-6. The effect appears to be a generalized 'quenching' of endothelial activation and is accompanied by a functional counterpart in terms of inhibition of monocytoid cell adhesion to the endothelium. Furthermore, the vascular protective effects of the phenolic component of high-amylose wheat counteract the

excessive production of ROS and inhibit the activation of redox-sensitive transcription factors.

These findings suggest that Svevo-HA could be used as a functional food ingredient with multiple anti-inflammatory properties associated to its phenolic profile, which was able to reduce endothelial activation and counteract or prevent atherosclerotic process. To validate these properties in vivo, clinical trials are presently under investigation within the MEDWHEALTH project frame.

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CRediT authorship contribution statement

Nadia Calabriso: Writing – review & editing, Writing – original draft, Validation, Investigation, Conceptualization. **Marika Massaro:** Writing – review & editing, Resources, Methodology. **Egeria Scoditti:** Writing – review & editing, Methodology. **Stefano Quarta:** Investigation. **Barbara Laddomada:** Writing – review & editing, Writing – original draft, Resources, Investigation. **Aurelia Scarano:** Investigation. **Samuela Palombieri:** Resources. **Francesco Sestili:** Resources, Funding acquisition. **Maria Annunziata Carluccio:** Writing – review & editing, Writing – original draft, Validation, Resources, Conceptualization.

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.

Data availability

Data will be made available on request.
CNR (Original data) ()

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2024.104028.

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