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Effect of a new dietary supplement formula on the activity and expression of pro-inflammatory genes in vitro

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PURPOSE

Wet Age-related Macular Degeneration and Diabetic Macular Edema are characterized by chronic inflammation and high levels of Vascular Endothelial Growth Factor (VEGF). Since anti-VEGF agents do not possess a direct effect on inflammation, we set out to investigate the potential anti-inflammatory activity of a nutritional supplement formula codenamed AVS (SIFI S.p.A.) as a candidate support to anti-VEGF therapies. To this end, we exposed stimulated murine macrophage (J774.2) and human lung adenocarcinoma epithelial (A549) cells to AVS in order to evaluate the effect exerted upon expression and/or activity of interleukine-1beta (IL-1β) and inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2), respectively.

METHODS

Cell culture and treatment

J774.2 and A549 cell lines, obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C.), were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200mM L-glutamine, 100 units/ml of penicillin/streptomycin and incubated at 37°C in a humidified CO_2 incubator. These cells were plated at a density of $2.5x10^4$ cells per well in a 96-well plate until sub-confluence. Briefly, J774.2 and A549 sub-confluent cells were pre-treated (2 h) with AVS (S.I.F.I. S.p.A.) (1.9 mg·ml^{-1}) and then stimulated with LPS (1 µg·ml^{-1}) or IL-1β (10 ng·ml^{-1}), respectively. Following overnight incubation, the medium was collected and nitrites or prostaglandin E_2 (PGE_2) production were determined by Griess Reagent or Enzyme Immuno Assay (EIA) test, respectively. Moreover, cells were harvested in 1 ml Trizol reagent and total RNA was extracted following the manufacturer's instruction in order to assess the expression of IL-1β, iNos and COX-2 mRNAs by Real Time RT-PCR.

Nitrite determination by Griess assay

The nitrite concentration in the culture medium of J774.2 cells was measured as an indicator of nitric oxide (NO) production using the Griess reaction. Supernatant from each well (20 µl) was transferred to a fresh 96-well plate and 50 µl of 1% sulfanilamide plus 50 µl of 0.1% napthyethylene-diamine were added. When combined, these compounds (known as Griess reagent), form a violet colour in the presence of nitrite. After 10 min incubation at room temperature, the absorbance of each well was measured at 550 nm (SPECTRAFluor Plus, Tecan). Nitrite concentration was calculated with reference to a standard curve of sodium nitrite generated by known concentrations, ranging from 0 to 100 µM.

PGE_2 EIA assay

PGE_2 concentration in the culture medium of A549 cells was assessed with the PGE_2 EIA kit (Caymal Chemicals). Briefly, supernatant from each well was transferred to a fresh 96-well plate, properly diluted and incubated with 50 µl of PGE_2 monoclonal antibody and 50 µl of PGE_2 AChE tracer for 18 h at 4°C. Standard curve was generated with serial dilution of PGE_2 EIA standard in EIA buffer prepared according to Cayman's protocol. After incubation, plate was washed 5 times with Wash Buffer and than 200 µl of Ellman's Reagent was added into each sample/standard well for development. The plate was placed on a shaker for 60 min and then read at a wavelength of 412 nm (SPECTRAFluor Plus, Tecan).

Quantitative Real Time RT-PCR

Total RNA was extracted from J774.2 and A549 cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. Total RNA (1 µg) was treated with DNase I Amplification Grade (Invitrogen) to eliminate possible genomic DNA contamination and reverse-transcribed into cDNA with SuperScript II Reverse Transcriptase (Invitrogen) and Random Hexanucleotide mix (Roches). Quantitative real-time RT-PCR was performed using AbiPrism 7000 thermal cycler (Life Technologies) and TaqMan Gene Expression Assay (Life Technologies) to evaluate the expression levels of the IL-1β, iNOS and COX-2 genes. The fold change in gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method (Ct, cycle threshold) using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as endogenous reference gene and the average ΔCt of negative control (CTRL-) samples as calibrator.

Statistical analysis

Results are expressed as mean ± SEM of 8 replicates from 4 different experiments. Statistical analysis was carried out using unpaired t-test (GraphPad Prism Software Inc., San Diego, CA). P values ≤0.05 were considered statistically significant.

RESULTS

Our results show that LPS treatment induced the accumulation of 69.9±10.5 µmol·l^{-1} nitrites in the J774.2 cell culture medium (Fig. 1). Treatment with AVS inhibited the accumulation of nitrites by 89% compared to control (p≤0.001) (Fig. 1). Consistently, AVS produced a significant (p≤0.0001) inhibition of iNOS and IL-1β mRNA expression by 670-fold (99%) and 1100-fold (96%), respectively (Fig. 2). In addition, AVS effectively inhibited PGE_2 accumulation by 99% compared to control (p≤0.001) (Fig. 3), while COX-2 mRNA expression was not affected by AVS treatment in IL-1β-stimulated A549 cells (Fig. 4).

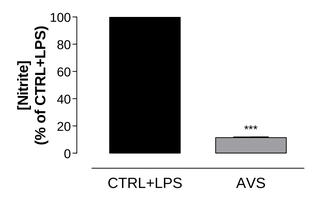

Fig. 1 Treatment with AVS inhibits nitrite accumulation in LPS-induced J774.2 cells. ***p≤0.001 vs CTRL+LPS (Unpaired t-test). Data represent the mean ± SEM of 4 different experiments.

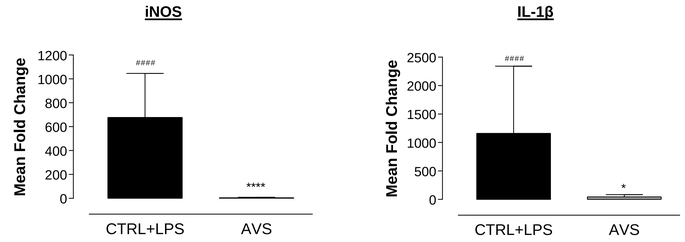

Fig. 2 iNOS and IL-1β mRNA expression levels are significantly inhibited by AVS treatment in LPS-induced J774.2 cells. ####p≤0.0001 vs CTRL-. ****p≤0.0001; *p≤0.05 vs CTRL+LPS (Unpaired t-test). Data represent the mean ± SEM of 4 different experiments.

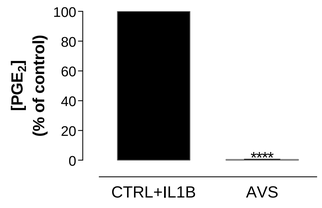

Fig. 3 Treatment with AVS inhibits PGE_2 accumulation in IL-1β-stimulated A549 cells. ****p≤0.0001 vs CTRL+LPS (Unpaired t-test). Data represent the mean ± SEM of 4 different experiments.

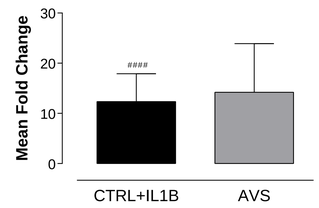

Fig. 4 mRNA expression levels of COX-2 in response to AVS treatment in IL-1β-stimulated A549 cells. ####p≤0.0001 vs CTRL- (Unpaired t-test). Data represent the mean ± SEM of 4 different experiments.

CONCLUSIONS

The specific composition of AVS was shown to be endowed with anti-inflammatory properties. Indeed, AVS was effective in inhibiting nitrites accumulation in J774.2 cultures, most likely by inhibiting the expression of iNOS and IL-1β genes. Interestingly, AVS effectively inhibited the synthesis of PGE_2 in A549 cells while unable to inhibit COX-2 expression. These findings suggest that AVS is able to act at multiple levels modulating the expression and/or the activity of important pro-inflammatory genes. Therefore, the AVS formula may well prove a useful anti-inflammatory aid in patients undergoing standard anti-VEGF therapy.

Commercial Relationships Disclosure: Giuliano Francesco (Commercial Relationship: S.I.F.I. S.p.A.; code: E); Santonocito Manuela (Commercial Relationship: S.I.F.I. S.p.A.; code: E); La Rosa Luca (Commercial Relationship: S.I.F.I. S.p.A.; code: E); Zappulla Cristina (Commercial Relationship: S.I.F.I. S.p.A.; code: E); Viola Santa (Commercial Relationship: S.I.F.I. S.p.A.; code: E) and Santocono Marcello (Commercial Relationship: S.I.F.I. S.p.A.; code: C)