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Individual components of a nutraceutical formula synergistically contribute to the effect on COX-2 and iNos activity and expression

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PURPOSE

Anti-VEGF agents currently available for the treatment of Diabetic Macular Edema (DME) and Wet Age-Related Macular Degeneration (Wet AMD) do not possess a direct effect on inflammation. Here we set out to investigate whether a nutritional supplement may aid the activity of anti-VEGF drugs by providing anti-inflammatory support. To this end stimulated murine J774.2 macrophages were exposed to a nutritional supplement formula codenamed AVS (S.I.F.I. S.p.A.) and its individual components (astaxanthin, piperine, boswellic acid, bromelain, coenzyme Q10, copper, curcumin, lutein, anthocyanin, resveratrol, safranal, salicin, zeaxanthin and zinc), in order to evaluate the effect exerted upon activity and/or expression of inducible nitric oxide synthase (iNos) or cyclooxygenase-2 (COX-2).

METHODS

Cell culture and treatment

J774.2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovin serum (FBS) conteining 200mM L-glutamine and 100 units/ml of penicillin/streptomycin and maintained at 37°C in a humidified CO_2 incubator. Cells were then plated in a 96-well plate at a density of $5x10^4$ cells/well until sub-confluence. In particular, sub-confluent cells were pretreated (2 h) with AVS (1.9 mg·ml^{-1}) and its individual components: astaxanthin (2.4 μg·ml^{-1}), piperine (12 μg·ml^{-1}), boswellic acid (480 μg·ml^{-1}), bromelain (360 μg·ml^{-1}), coenzyme Q10 (24 μg·ml^{-1}), copper (2.4 μg·ml^{-1}), curcumin (240 μg·ml^{-1}), lutein (24 μg·ml^{-1}), Oxi-fend® blackcurrant extract (anthocyanin, 240 μg·ml^{-1}), resveratrol (72 μg·ml^{-1}), safranal (48 μg·ml^{-1}), salicin (360 μg·ml^{-1}), zeaxanthin (2.4 μg·ml^{-1}), zinc (30 μg·ml^{-1}) or mix J (mix of components inactive when tested individually: astaxanthin, piperine, boswellic acid, coenzyme Q10, copper, curcumin, lutein, resveratrol and zeaxanthin). Cells were than stimulated with lipopolysaccharide (LPS) (1 μg·ml^{-1}) and 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 RNA was isolated following the manufacturer's instruction. Total RNA was extracted to assess the expression of 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 J774.2 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).

Real Time RT-PCR

Total RNA was extracted from J774.2 cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. Total RNA (1 μg) was treated with DNase I Amplification Grade and reverse-transcribed into cDNA with SuperScript II Reverse Transcriptase and Random Hexanucleotide mix. Quantitative Real-Time RT-PCR was performed using AbiPrism 7000 thermal cycler and TaqMan Gene Expression Assay to evaluate the expression levels of the iNos and COX-2 genes. The PCR thermal cycling conditions were as follows: initial denaturation of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. 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 6 replicates from 3 different experiments. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's post-hoc test (GraphPad Prism Software Inc., San Diego, CA). P values ≤0.05 were considered statistically significant.

RESULTS

We found that LPS induced the accumulation of 24.3±2.8 μmol·l^{-1} nitrites and 21.4±5.3 ng·ml^{-1} PGE_2 in J774.2 cells. Interestingly, treatment with AVS, bromelain, zinc, salicin, anthocyanin or mix J effectively inhibited the accumulation of nitrites by 63-90% (Fig. 1). Similarly AVS, bromelain, anthocyanin or mix J significantly inhibited PGE_2 accumulation by 38-99% (Fig. 2). Surprisingly, components inactive when tested individually showed a synergistic effect on inhibition of nitrites and PGE_2 accumulation when mixed together (mix J). Moreover, the inhibition of nitrites and PGE_2 accumulation was paralleled by a significant inhibition of iNos (~95%) and COX-2 (60-85%) expression, in cells treated with AVS, bromelain, zinc or anthocyanin. Treatment with mix J significantly inhibited iNos expression (94%) while COX-2 inhibition (55%) failed to reach statistical significance (Fig. 3).

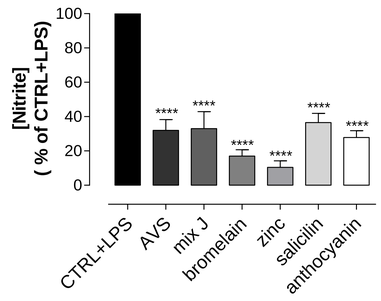

Fig. 1 Effect of AVS, mix J or single components on nitrites accumulation in LPS-induced J774.2 cells. ****p≤0.0001 vs. CTRL+LPS (One-way ANOVA + Dunnett's post-hoc test). Data represent the mean ± SEM of 3 different experiments.

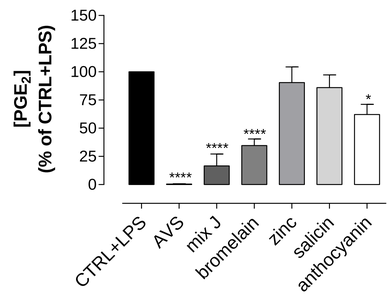

Fig. 2 Effect of AVS, mix J, bromelain or anthocyanin on PGE_2 accumulation in LPS-induced J774.2 cells. *p≤0.05; ****p≤0.0001 vs. CTRL+LPS (One-way ANOVA + Dunnett's post-hoc test). Data represent the mean ± SEM of 3 different experiments.

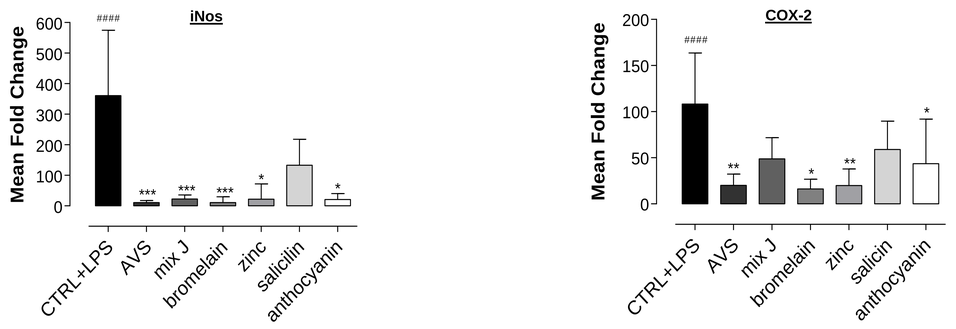

Fig. 3 iNos and COX-2 mRNA expression in response to AVS, mix J or single components treatment in LPS-induced J774.2 cells. ####p≤0.0001 vs. CTRL-. *p≤0.05; **p≤0.01; ***p≤0.001 vs. CTRL+LPS (One-way ANOVA + Dunnett's post-hoc test). Data represent the mean ± SEM of 3 different experiments.

CONCLUSIONS

These data demonstrate that the AVS formula was effective in inhibiting nitrites and PGE_2 accumulation in murine J774.2 macrophages. These activities appear to rely upon inhibition of iNos and COX-2 expression. Interestingly, components found to be inactive when tested individually were found to act synergistically when tested as a mix. Therefore, the AVS nutritional formula appears to be endowed with anti-inflammatory properties that may well prove useful to support anti-VEGF therapy currently available.

Commercial Relationships Disclosure Maria Grazia Mazzone: Commercial Relationship(s);S.I.F.I. S.p.A.:Code E (Employment) | Cristina Zappulla: Commercial Relationship(s);S.I.F.I. S.p.A.:Code E (Employment) | Luca La Rosa: Commercial Relationship(s);S.I.F.I. S.p.A.:Code E (Employment) | Manuela Santonocito: Commercial Relationship(s);S.I.F.I. S.p.A.:Code E (Employment) | Santa Viola: Commercial Relationship(s);S.I.F.I. S.p.A.:Code E (Employment) | Francesco Giuliano: Commercial Relationship(s);S.I.F.I. S.p.A. :Code E (Employment)