

Pilot study for the evaluation of safety profile of a potential inhibitor of SARS-CoV-2 endocytosis

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Abstract. *Background and aim of the work:* Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the current pandemics of coronavirus disease. This virus is able to attack the cells of the airway epithelium by binding to the transmembrane angiotensin I converting enzyme 2 (ACE2). We developed an oral spray that could inhibit the SARS-CoV-2 endocytosis. The spray contains hydroxytyrosol for its anti-viral, anti-inflammatory and anti-oxidant properties, and α -cyclodextrin for its ability to deplete sphingolipids, that form the lipid rafts where ACE2 localizes. The aim of the present pilot multi-centric open non-controlled observational study was to evaluate the safety profile of the “Endovir Stop” spray. *Methods:* An MTT test was performed to evaluate cytotoxicity of the spray in two human cell lines. An oxygen radical absorbance capacity assay was performed to evaluate the antioxidant capacity of the spray. The spray was also tested on 87 healthy subjects on a voluntary basis. *Results:* The MTT test revealed that the spray is not cytotoxic. The ORAC assay showed a good antioxidant capacity for the spray. Endovir Stop tested on healthy volunteers showed the total absence of side effects and drug interactions during the treatment. *Conclusions:* We demonstrated that Endovir Stop spray is safe. The next step would be the administration of the efficacy of the spray by testing it to a wider range of people and see whether there is a reduced infection rate of SARS-CoV-2 in the treated subjects than in the non-treated individuals. (www.actabiomedica.it)

Key words: SARS-CoV-2, COVID-19, ACE2, hydroxytyrosol, α -cyclodextrin

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the current pandemics of coronavirus disease known as COVID-19. This virus is able to attack the cells of the airway epithelium by binding its spike (S) protein to the transmembrane angiotensin I converting enzyme 2 (ACE2)

with the help of the transmembrane serine protease 2 (TMPRSS2). Both proteins, ACE2 and TMPRSS2, are localized in the cholesterol-rich lipid rafts of the cell membrane (1,2). Two main mechanisms may influence the SARS-CoV-2 pathogenesis: the entry into the cells via endocytosis, and the triggering of an exaggerated inflammatory response (3). With regard to the literature in the current knowledge, we devel-

oped an oral spray containing compounds that could inhibit both the endocytosis and the inflammatory response with the consequent oxidative damage as a preventive measure to the SARS-CoV-2 infection or to help in reducing the negative effects of virus on the body once it starts spreading. The spray contains hydroxytyrosol extracted from olive leaves and fruits for its anti-viral, anti-inflammatory and anti-oxidant properties (4), and α -cyclodextrin for its ability to deplete sphingolipids, that, together with cholesterol, form the lipid rafts where ACE2 localizes (5). Hydroxytyrosol has a broad-spectrum antiviral activity, especially against enveloped viruses like influenza virus, HIV or coronaviruses. For instance, it is able to reduce HIV replication (6), and fusion *in vitro* (4,7,8). HT is also able to induce morphological changes that reduce influenza virus infectivity (9). It also enhances anti-inflammatory effects by decreasing the levels of pro-inflammatory cytokines IL-6 and TNF- α , as observed in animal models (10). Interestingly, those two cytokines rapidly increase in the most severe cases of COVID-19 (11). Whereas, α -cyclodextrin is able to deplete sphingolipids from the lipid rafts where the ACE2 receptor, specific for SARS-CoV-2, localizes (5), and to reduce serum phospholipids, which are necessary for the SARS-CoV-2 endocytosis into cells, replication, transcription, and assembly of novel virus particles (12).

The aim of the present pilot multi-centric open non-controlled observational study was to evaluate the safety profile of the “Endovir Stop” spray in a SARS Cov-2 free population.

Materials and methods

Materials

Eagle’s Minimum Essential Medium (EMEM), Fetal bovine serum (FBS), penicillin–streptomycin, Dulbecco’s phosphate buffered saline pH 7.4 (PBS) were from Thermo Fisher Scientific (Waltham, MA, USA). L-glutamine, trypsin, and tetrasodium salt (EDTA) were from Microtech Srl (Pozzuoli, NA, Italy). Dimethyl sulfoxide (DMSO) was purchased from Carlo Erba Reagents Srl (Milan, Italy).

Ethanol 96% (EtOH), 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazoliumbromide (MTT) and 2,20-azobis (2-methylpropionamide) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (Ravenna, Italy).

Spray composition

One dose of the solution (4 sprays = 0.5 ml, density = 1.1 g/ml) contains the following ingredients: water (52.57%), active compounds: hydroxytyrosol (3.80%), α -cyclodextrin (0.20%), co-emulsifier: glycerin (3.80%), flavoring: lemon flavor (0.98%), acidifier: citric acid (0.30%), preservatives: sodium benzoate (0.10%), potassium sorbate (0.10%), viscosity control: xanthan gum (0.05%), sweeteners: fructose (38.06%), steviol glycosides (0.02%), sucralose (0.02%).

In vitro experiments

Cell lines

The human HepG2 cell line (ATCC HB 8065) was derived from a human hepatoblastoma (13,14) and was obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna “Bruno Ubertini” (Brescia, Italy). All experiments were performed on HepG2 cells at passages between 101 and 113.

The human Caco2 cell line were purchased by ATCC and derive from a tumor of a male (Caucasian ethnicity) patient suffering from colorectal adenocarcinoma (15). All experiments were performed on HepG2 cells at passages between 61 and 75.

Both cells were grown as monolayer cultures in Eagle’s Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂.

In vitro cytotoxicity assay

Cell lines are seeded onto a 96-well plate at a density of 1×10^4 cells/well with complete medium. After 24 h fresh complete medium is replaced for treatment

with different concentrations of Endovir Stop Spray solution. in completed medium for 24 h and 48 h. Then, MTT reagent is dissolved in PBS 1X and added to the culture at 0.5 mg/ml final concentration. After 3 h of incubation at 37°C, the supernatant is carefully removed, and formazan salt crystals are dissolved in 200 µl DMSO (16,17). After 30 min the absorbance (OD) values are measured spectrophotometrically at 540 nm using an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). As a positive control DMSO in three different percentages (1%, 2% and 4%) were used. Each experiment is performed two times in triplicate. Cell viability is expressed as a percentage relative to that of the control cells as described previously (18,19).

Oxygen Radical Absorbance Capacity (ORAC) assay

The antioxidant capacity of Endovir Stop Spray is determined using the ORAC method (20). The ORAC assays were carried out on a FLUOstar OPTIMA microplate fluorescence reader (BMG LABTECH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The procedure is based on the method of Zulueta et al. (21) with slight modifications. Briefly, 2,20-azobis (2-methylpropionamide) dihydrochloride (AAPH) is used as a peroxy radical generator, Trolox is used as a reference antioxidant standard, and fluorescein is used as a fluorescent probe. The data are expressed as micromoles of Trolox equivalents (TE) per ml of sample (µmolTE/ml).

Subject selection

If the null hypothesis value is 0% (given that the component are considered suitable for human consumption as food), and a proportion of at least 2% of side effects to be significantly different from the expected, with an error α of 5% and an error β of 10%, then the required sample size was at least 81. Therefore, 87 subjects enrolled in this study on a voluntary basis. They all signed the informed consent and the study was conducted according to the ethical principles of the "Declaration of Helsinki".

Results

In vitro assays

The *in vitro* cytotoxicity assay revealed that the spray is not cytotoxic up to a concentration of 1,4 µg/mL, corresponding to 120 µl of the product in both HepG2 (Figure 1) and Caco2 (Figure 2) cell lines. In fact, the obtained results highlighted that the cell viability was always > 80%. In particular, with concentrations less than 230 ng/mL the cell viability is higher than negative control. Moreover, considering that four sprays of Endovir Stop correspond to 0,5 mL and the density is equal to 1.1 g/mL we can conclude that the antioxidant capacity of the spray is 561,15 µmol TE/g. In comparison we previously published a paper on *Ly-cium barbarum* berries and the extract exhibits an antioxidant activity value of 225.07 ± 1.40 µmolTE/g (16).

Experimentation on healthy volunteers

The clinical data of the enrolled subjects are included in Table 1. Endovir Stop was used on a population (87 individuals) heterogeneous in age, sex, comorbidity and drug use without significant differences from the general population. All subjects used Endovir Stop twice a day for 7 days with a total absence of side effects (we did not expect any specific side effects because the active components of Endovir Stop are considered novel foods (22,23)) and drug interactions was evidenced. In addition, none of the subjects withdrew from the study.

Discussion

The experimentation that we performed revealed that the Endovir Stop spray was not cytotoxic up to a concentration of 1,4 µg/mL, corresponding to 120 µl of the product in both HepG2 and Caco2 cell lines. Based on the MTT results it is possible to conclude that the Endovir Stop spray is safe in the physiologic concentration range.

The antioxidant capacity of Endovir Stop spray was really high compare with other foods, probably due to the presence of hydroxytyrosol molecule (3.80%). The ORAC assay showed an antioxidant capacity of

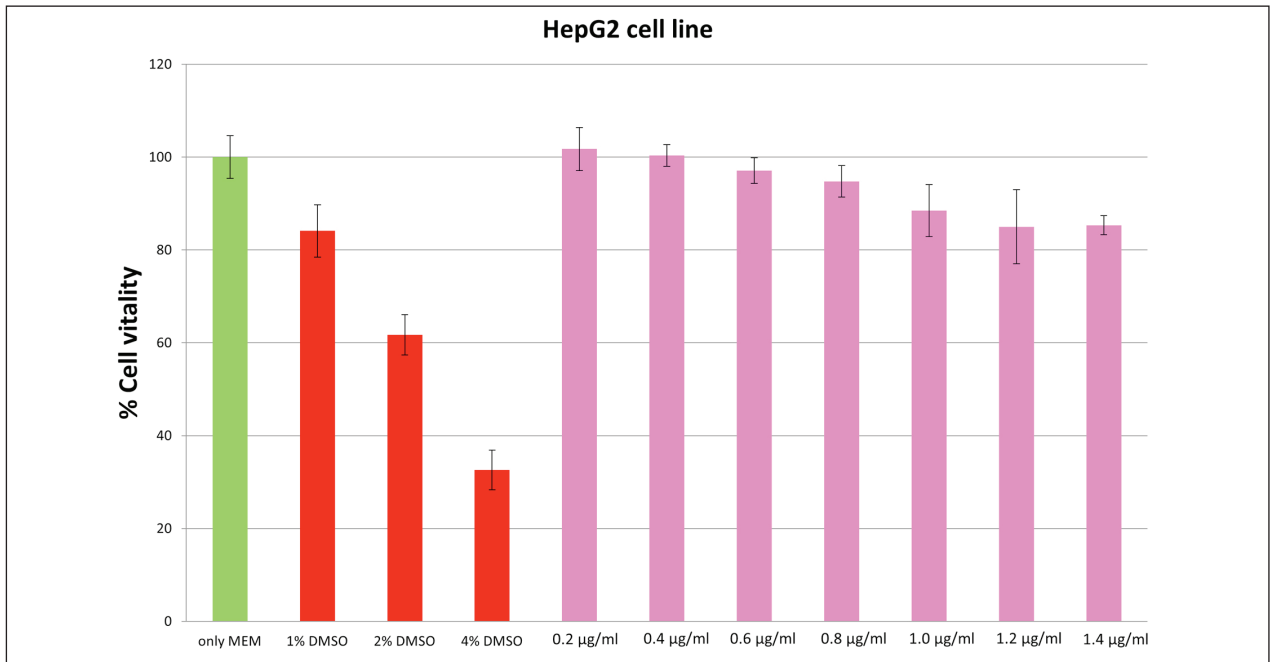


Figure 1. Histogram representing cell vitality after MTT test at increasing Endovir Stop spray concentrations in HepG2 cells. The treated cells were compared with the negative control and with cells grown in a medium containing the cytotoxic compound DMSO (see Materials and Methods section for details).

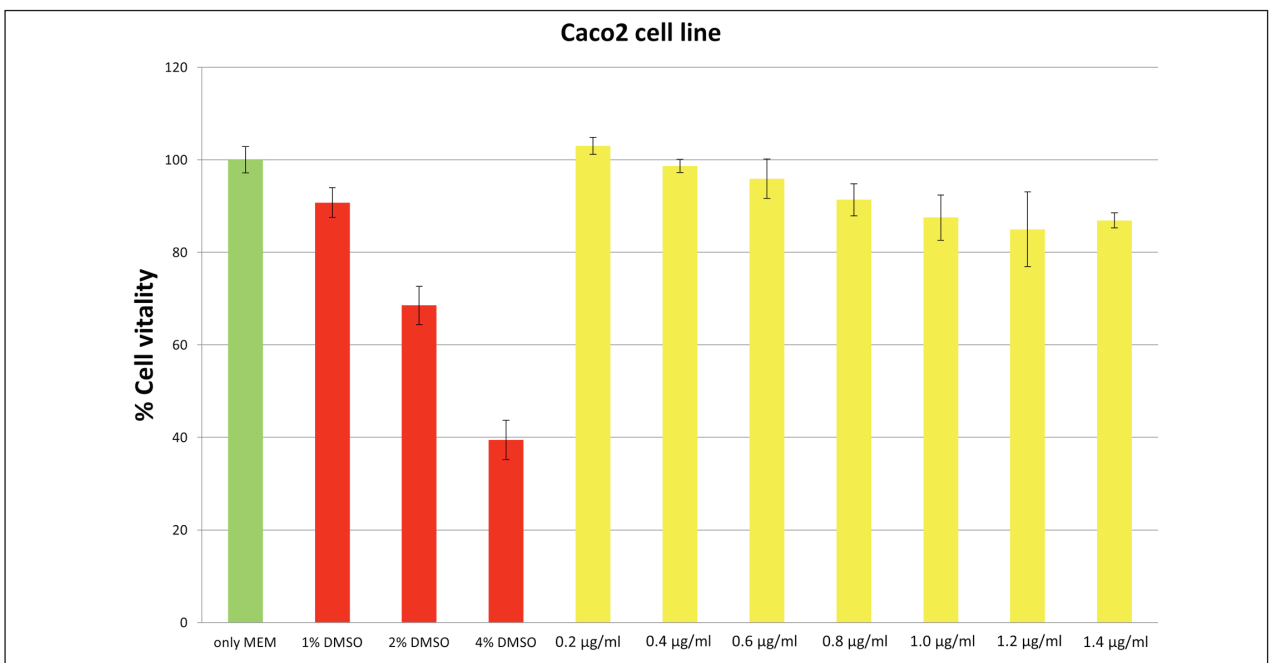


Figure 2. Histogram representing cell vitality after MTT test at increasing Endovir Stop spray concentrations in Caco2 cells. The treated cells were compared with the negative control and with cells grown in a medium containing the cytotoxic compound DMSO (see Materials and Methods section for details).

Table 1. Clinical data of the enrolled subjects

Mean age \pm SD (Min-Max)	52.6 \pm 17.6 (24-86)
Males/Females (%Males)	45/42 (51.7%)
Smoker Yes/No (%Yes)	36/51 (41.4%)
Comorbidity Yes/No (%Yes)	36/51 (41.4%)
Diabetes	12/51 (23.5%)
Obesity	9/51 (17.6%)
Hypercholesterolemia	11/51 (21.6%)
Hypertension	17/51 (33.3%)
Cardiovascular	11/51 (21.6%)
Dyslipidemia	4/51 (7.8%)
Pollen allergy	1/51 (2%)
Anemia	1/51 (2%)
Pharmacological treatment Yes/No (%Yes)	44/43 (50.6%)
ACE inhibitors	13/44 (29.5%)
β -blockers	11/44 (25%)
Antithrombotic agents	9/44 (20.5%)
Hypoglycemic agents	9/44 (20.5%)
Statins	8/44 (18.2%)
Mean weight (Kg) \pm SD (Min-Max)	78.6 \pm 12.6 (55-107)
Mean height (m) \pm SD (Min-Max)	1.73 \pm 0.1 (1.53-1.92)
Mean BMI \pm SD (Min-Max)	26.1 \pm 3.7 (17-37.7)
Type of exposure to virus (continuous/occasional)	36/51 (41.4%)
Place of exposure (home/workplace)	28/58 (32.5%)
Withdrawal from the study	0%
Side effects	0%

the spray of $1247.43 \pm 4.05 \mu\text{mol TE/mL}$. Regarding these results, we recently analyzed the antioxidant capacity of human breast milk in a heterogeneous population of breast feeding mothers with different diets (24). Interestingly, milk samples of mothers fed with Mediterranean diet showed an antioxidant activity value of $584.16 \pm 29.51 \mu\text{mol TE/mL}$ higher than milk samples of mother fed with vegetarian diet (rich in vegetables and fruit and with very low content of meat, fish, eggs and cereals). In the current study, we analyzed the antioxidant potential of four different milk formulas and was really high, due to an enrichment with vitamins and others nutrients.

Furthermore, the analysis on a heterogenous population of COVID-19-negative volunteers confirmed

the absence of side effects and interactions with other medications after one week of administration.

The next step of our experimentation will be the administration of the spray to a wider range of people and see whether there is a reduced infection rate of SARS-CoV-2 in the treated subjects than in the non-treated individuals. The use of cyclodextrins could be a new path to follow for helping at fighting SARS-CoV-2 infections that are spreading worldwide. In fact, cyclodextrins “are listed in the *generally regarded as safe* list of the Food and Drug Administration for use as a food additive (25) and α -cyclodextrins are approved as novel food ingredients by the European Commission”, therefore its approval for use would be easier than for other molecules (22,26). Furthermore, hydroxytyrosol

could aid the activity of α -cyclodextrin in its anti-viral endocytosis given its virucidal, anti-inflammatory and anti-oxidant properties. In addition, hydroxytyrosol is already allowed “for use as an antioxidant and antimicrobial agent in conventional foods such as beverages, fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces at use levels of 5.0 mg/serving” by the Food and Drug Administration in US (27) and as a novel food in the European Union (23). Therefore, it would be easier as well to have its approval for human use.

Conflict of interest: SP, EM, MD, MCE, TS, MF, GF, GMT and MB declare that they are co-inventors of the Endovir Stop spray. They submitted the invention to the Italian Patent Office (date: 13/10/2020 - number: 102020000024118).

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