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The colon epithelium as a target for the intracellular antioxidant activity of hydroxytyrosol: A study on rat colon explants

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ABSTRACT

The study aimed at investigating the intracellular antioxidant activity of hydroxytyrosol (HT), one of the most potent natural antioxidant phenolic compounds, directly on the colon epithelium, under basal physiological and pro-oxidant conditions. *In situ* confocal microscopy on rat colon explants loaded with the ROS sensitive fluorescent probe CM-H_2DCFDA was applied. HT exerted a dose-dependent decrease of the basal ROS production of superficial colonocytes. Also, it induced a dose-dependent antioxidant action on the colon mucosa exposed to an H_2O_2 challenge. The effect of 100 μM HT was comparable to that of 10 μM Trolox. The HT effect was also tested against tert-butyl peroxide, another pro-oxidant. The results showed that HT can directly contribute to the redox balance of colonic epithelium by reducing ROS in both basal and pro-oxidant conditions, and support the potential of HT as a functional food ingredient with applications in protecting the intestinal mucosa against oxidative stress.

1. Introduction

The gastrointestinal (GI) tract is exposed to several pro-oxidant conditions *via* endogenous and exogenous routes (Circu & Aw, 2012). Some reactive species are present in food, such as lipid peroxides, aldehydes, hydrogen peroxide, heme proteins, or some food contaminating pollutants (Halliwell, 2007). Other reactive species arise from chemical reactions of dietary components in the stomach, where lipid peroxidation, generation of free radicals, secondary lipid peroxidation products and co-oxidation of vitamins easily occur at low pH with the formation of aldehydes, ketones, and epoxides (Sies, Stahl, & Sevanian, 2005). ROS production can also be increased by the activation of immune cells naturally present in the GI tract by diet-derived bacteria and toxins (Strus et al., 2009). Besides, the GI can produce reactive species itself, such as those released by the activity of some enzymes highly expressed in the intestine, such as NADPH oxidase and xanthine oxidase (Tian, Wang, & Zhang, 2017).

Considering the complex functions of the intestinal epithelium as an interface between the external and the internal environment of the organism and the wide variety of oxidative exposures, the maintenance of the redox balance in the GI tract is of pivotal importance. As outlined by Pérez and coll. (Pérez, Taléns-Visconti, Rius-Pérez, Finamor, & Sastre, 2017), redox signaling is important for the physiological function of the intestinal epithelium, contributing to the regulation of proliferation, progression, and differentiation during epithelium self-renewal. However, the excess of reactive species under anomalous conditions and the following loss of redox homeostasis can contribute to the progress of GI pathologies, including Barrett's esophagus, esophageal adenocarcinoma, peptic ulcer, gastric cancer, ischemic intestinal injury, inflammatory bowel disease and colorectal cancer (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014; Kim, Kim, & Hahm, 2012; Klaunig, Kamendulis, & Hocevar, 2010; Pérez et al., 2017; Perše, 2013). Along the GI tract, the colon is one of the segments more exposed to pro-oxidant conditions (Sanders et al., 2004). Here, the prolonged transit time of bolus causes longer exposure to oxidized food particles, toxins, and redox-active minerals. In addition, bacterial microflora generates a consistent quantity of reactive metabolites (Stone, Krishnan, Campbell, & Palau, 2014). Colon showed a less effective response to oxidative stress compared to other GI segments, and this could contribute to an increased cancer incidence at this site (Sanders et al., 2004).

Maintenance of the correct GI redox balance, through the involvement of endogenous and exogenous antioxidant defenses (mainly deriving from the diet), is an important aspect for human health (Circu & Aw, 2012; He et al., 2017). Plant polyphenols represent the most abundant antioxidants in the diet (D'Archivio et al., 2007), contributing to the exogenous antioxidant defenses of the organisms. Particularly, phenolic compounds typically included in the Mediterranean diet arose

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great interest for their health benefits after their consumption, use as dietary supplements, and use as functional ingredients.

One of the most potent natural antioxidant phenolic compounds is hydroxytyrosol (HT) (3,4-dihydroxyphenylethanol) (Echeverría, Ortiz, Valenzuela, & Videla, 2017). It naturally occurs in olive leaves and pulp and it is significantly present in olive oil, which is the most diffused fat source in Mediterranean countries (Sánchez-Villegas & Sánchez-Tainta, 2018). HT is also present in red grape and, in turn, in aged red wine and white wine (Fernandez-Marin, Mateos, Garcia-Parrilla, Puertas, & Cantos-Villar, 2012). The antioxidant properties of HT are related to its high hydrogen-atom donation capacity and to its ability to increase radical stability by generating an intramolecular hydrogen bond between the free hydrogens of their hydroxyl groups and their phenoxyl radicals (Visioli, Poli, & Galli, 2002). HT has been recognized to protect low-density lipoproteins (LDL) from oxidative damage by the European Food Safety Authority (EFSA Panel on Dietetic Products and Nutrition and Allergies (NDA), 2011), in relation to its ability to reduce the expression of adhesion proteins in endothelial cells, thus preventing LDL oxidation (Cheng, Schmelz, Liu, & Hulver, 2014). Moreover, it has been demonstrated to protect cells against H_2O_2-induced DNA damage and apoptosis by mitigation of intracellular labile iron level (Kitsati, Mantzaris, & Galaris, 2016; Warleta et al., 2011). Besides, several other beneficial effects, including anti-inflammatory activity and anticancer effects have been reported (Echeverría et al., 2017).

Consumption of virgin olive oil in the Mediterranean countries is around 30–50 g/day (Peyrol, Riva, & Amiot, 2017) corresponding to an intake of about 4–9 mg/day of olive oil phenols, mainly represented by the HT precursors oleuropein and ligstroside and to a lesser extent by hydroxytirosol and tyrosol. During its transit along the GI tract, ingested HT maintains its stability in gastric juice and duodenal fluid (Corona et al., 2006; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002). The HT that arrives in the small intestine may be considerably increased compared to the quantity present in the ingested olive oil because of the oleuropein and ligstroside hydrolysis at the acidic conditions of the stomach.

Hydroxytyrosol is well absorbed by the small intestinal epithelium (Tuck & Hayball, 2002; Visioli et al., 2000; Vissers et al., 2002), which is able to absorb from 40% to 95% of ingested HT, depending on the association with other aliments in the diet (Peyrol et al., 2017). Manna and coll. demonstrated HT transport *via* passive diffusion in CaCo-2 cell monolayers (Manna et al., 2000).

At the colon level, HT not absorbed in the previous GI tracts is accumulated, and this amount is increased by the activity of the intestinal microflora, which releases HT from oleuropein, which is conversely poorly absorbed at the intestinal level (López de las Hazas et al., 2016). The concentration of HT in the colon is stable concerning the intestinal microflora action, as recently demonstrated *in vitro* with human fecal microflora (Mosele et al., 2014), and it has been estimated in the high micromolar range (Corona et al., 2006). If we consider that the HT concentration measured in the plasma is in the nanomolar range (Miro-Casas et al., 2003), it is evident that the intestinal epithelium represents the tissue of the body exposed to the highest HT concentration with respect to all the other body tissues (De la Torre, 2008; Peyrol et al., 2017).

In spite of the increasing number of studies on the biological properties of HT and on its potential application as functional ingredient and supplement (EFSA Panel on Dietetic Products and Nutrition and Allergies (NDA), 2017; González-Santiago, Fonollá, & López-Huertas, 2010), no studies are available in the literature about the biological effect of HT directly on the intestinal mucosa.

Therefore, the present work aimed to assess whether or not HT can exert any intracellular antioxidant activities directly in the colon epithelium, and whether or not it can contribute to the protection of the intestinal epithelium from pro-oxidant conditions. The study was carried out by using confocal *in situ* detection on freshly isolated rat colon explants using the ROS-sensitive cell-permeant fluorescent probe CM-H_2DCFDA. This experimental model with intact tight junctions closely resembles the functional and morphological characteristics of the absorptive GI epithelium cells *in vivo*, as it maintains the complex cellular community and architecture of the intestinal epithelium, thus providing more realistic information on the intracellular antioxidant activity of HT compared to bi-dimensional intestinal cell cultures.

2. Methods

2.1. Materials

Male Wistar rats (70–100 g) were purchased from Harlan (Carezzana, Italy) and housed individually in animal cages (experimental conditions: temperature 22 ± 1 °C; 12:12 h light–dark cycle, 30–40% humidity, *ad libitum* access to food and water). This study was carried out in strict conformity with the EU Directive 2010/63/EU for animal experiments.

All chemicals were reagent grade. Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of analytical grade.

The cell-permeant probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluoresceindiacetate (CM-H_2DCFDA) was purchased from Life Technologies-Molecular Probes.

2.2. Effect of HT on colon transepithelial electrophysiological parameter Rt, Vt, and Isc

Small colon segments were opened and vertically oriented as a flat sheet in a modified Ussing chamber (CHM6, World Precision Instruments) were they were perfused on both sides by Krebs solution (containing, in mM: NaCl 124, KH_2PO_4 1.25, $MgCl_2$ 1.8, KCl 1.75, $CaCl_2$ 1.6, $NaHCO_3$ 25 and glucose 10) bubbled with a mixture of 5% CO_2 95% O_2 to yield a pH of 7.4. The chamber opening exposed 0.6 cm^2 of serosal surface area to 10 ml of circulating oxygenated Krebs at 37 °C. Tissues were connected to an automatic short-circuit current device (WPI's DVC-1000) by four Ag/AgCl electrodes in contact with the bathing solution through Agar-Krebs filled cartridges. Vt was measured with respect to the mucosal bath (grounded); Isc was measured by the passage of sufficient current to reduce Vt to 0 automatically (the resistance of the chamber fluid was subtracted automatically); Rt was determined at an applied current of 33 μA cm^{-2} (500 ms).

After 30 min equilibration period to achieve steady-state conditions, HT (100 μM) was added to the apical or basolateral compartment of the chamber and Vt, Isc, and Rt were monitored for 1 h.

2.3. In situ detection of HT intracellular antioxidant activity in colon mucosa

2.3.1. Colon explants preparation

In situ detection of intracellular antioxidant activity in superficial colonocytes was assessed according to Giordano and coll. (Giordano et al., 2016). Briefly, small explants (about 0.36 cm^2 in size) were excised from the distal colon of adult rats sacrificed under dietylether anesthesia. Each explant was washed with Hepes-Tris buffer containing in mM: NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Hepes 10, glucose 10, pH 7.4. Then, the explants were placed in 60 mm plastic tissue-culture dishes with the epithelium facing the gas-medium interface. The Trypan blue exclusion test was used to assess cell viability. The cell viability was about 100%.

The explants were immediately incubated with the ROS sensitive probe CM-H_2DCFDA dissolved in Tris-buffer (5 μM, final concentration) for 30 min (at 37 °C). CM-H_2DCFDA is hydrolyzed in the cell to the DCFH carboxylate anion, retained intracellularly, which produces the fluorescent probe dichlorofluorescein (DCF) after two-electron oxidation. CM-H_2DCFDA reacts mainly with peroxides and to a lesser extent with other ROS (Arriazu, Pérez de Obanos, López-Zabalza, Herraiz, &

Iraburu, 2010).

In HT treated explants, tissues were pre-incubated with the polyphenol for 1 h; then they were washed and treated with the fluorescent probe.

The probe was removed from the extracellular medium by repeated washing with Tris-buffer solution. Then, the explants were incubated with 400 μM H_2O_2 or tert-butyl hydroperoxide for 15 min to induce intracellular pro-oxidant conditions. Four hundred μM H_2O_2 was able to cause apoptosis induced by oxidative stress in rat colon explants after 24 h exposure as previously demonstrated (Antico, Lionetto, Giordano, Caricato, & Schettino, 2013). In the present study, it was utilized for a shorter time period (15 min) according to Giordano and coll. (Giordano et al., 2016).

2.3.2. Confocal visualization

Colon explants (0.09 cm^2), charged with the probe, were visualized using a confocal laser scanning microscope Nikon C1 (Nikon Instruments, Florence, Italy), mounted on an inverted optical microscope Nikon Eclipse TE 300 (objective: Plan-Apochromat 63×/1.40 immersion-oil DIC objective). The software EZC1 (Coord, Amsterdam, NL) was used for image acquisition, storage, and analysis. The probe was excited at 488 nm with an Argon laser. At least 10 fields randomly chosen in each explant were considered. Fluorescence intensity was measured on 50–70 superficial colonocytes per field. Ten fields per explant were acquired.

Unlabeled preparations did not exhibit any fluorescence under the conditions used.

The intracellular antioxidant activity of HT on rat colon mucosa was assessed on explants pre-incubated with the phenolic compound for 1 h and then washed three times with the Hepes-Tris buffer solution before CM-H_2DCFDA loading. On the other hand, the effect of HT against an exogenous oxidative challenge was assessed on explants pre-incubated with HT and then exposed to a pro-oxidant agent (H_2O_2 or terbutyl peroxide) before charging with the probe. The negative control was represented by explants incubated for 1 h with only Tris buffer prior to H_2O_2 treatment. In this case, H_2O_2-induced fluorescence did not show any significant changes. The positive control was represented by explants incubated for 1 h with Trolox (at concentrations ranging between 2 and 8 μM), a synthetic analog of vitamin E, known to be a scavenger of H_2O_2-derived ROS (Distelmaier et al., 2012).

The fluorescence intensity per cell was measured in 50–70 superficial colonocytes per field. Ten fields per explants were acquired.

2.4. Statistical analysis

Data were expressed as mean ± S.E.M. The Student's *t* test or one-way ANOVA (as indicated in the captions to figures) were applied to assess the significance of differences of the means. The IC_{50} values were reported at ± 95% confidence intervals (± 95% CI) and were calculated by GraphPad software (Prism 5.0, San Diego, CA).

3. Results

3.1. Effect of HT on Vt, Isc, and Rt in rat colon mucosa

Small colon segments were mounted vertically in a modified Ussing chamber to first evaluate the effect of HT on the transepithelial electrophysiological parameters of colon mucosa (transepithelial potential Vt, short circuit current Isc, and transepithelial resistance Rt), which are the expression of the functional transepithelial ion transport activity of the epithelium (Lionetto et al., 2008).

The effect of HT on Vt, Isc and Rt on intestinal mucosa was calculated as the percentage of the initial value, measured before the incubation of the epithelium with HT. As reported in Table 1, the exposure of rat colon to HT (100 μM) did not significantly affect Vt, Isc, and Rt, when compared with the control (colon segments only perfused with Krebs solution). These results suggest that HT does not alter the permeability of the epithelium or its ion transport properties. In particular, Rt values are strong indicators of the integrity of the epithelial barrier.

Table 1
Effect of the incubation of rat colon explants with 100 μM HT (1 h) on the transepithelial electrophysiological parameters. Data are expressed as percentage of Vt, Isc and Rt value measured after 1 h incubation with HT with respect to the initial value measured before the addition of HT in the luminal chamber. Control is represented by tissues not exposed to HT. Data are expressed as mean ± SEM.

	% Vt	% Isc	% Rt
Control	96.5 ± 4.5	95.2 ± 3.1	94.2 ± 3.5
+HT	94.1 ± 3.6	94.2 ± 2.9	95.1 ± 4.1

3.2. Effect of HT on basal ROS production

Under basal conditions, the cells showed a slight fluorescence (Fig. 2A), indicative of the basal ROS production of the cells during normal oxidative metabolism, according to Giordano and coll. (Giordano et al., 2016).

As reported in Fig. 1, pre-incubation of the tissue with HT in the concentration range 10–200 μM induced a marked dose-dependent decrease of the basal fluorescence of superficial colonocytes charged with CM-H_2DCFDA. The HT concentration able to decrease the fluorescence of the probe by 50% (IC_{50}) was 94.1 ± 9.2 μM, as assessed by interpolation on the curve.

3.3. Effect of HT against an exogenous oxidative challenge induced by H_2O_2 exposure

When colon explants were exposed to an oxidative challenge of 400 mM H_2O_2 for 15 min (Fig. 2B), the fluorescence of the superficial colonocytes charged with the probe increased compared to the untreated tissues (Fig. 2A). Absorptive cells, which represent the main cell type of colon mucosa, showed a homogeneous cytoplasmatic green fluorescence. The choice of 15 min incubation comes from a previous time-dependence analysis of H_2O_2-induced oxidative challenge in the isolated intestinal mucosa (Giordano et al., 2016).

The pre-incubation with HT (100 μM) before H_2O_2 exposure (Fig. 2C) was able to exert a marked inhibition of the intracellular

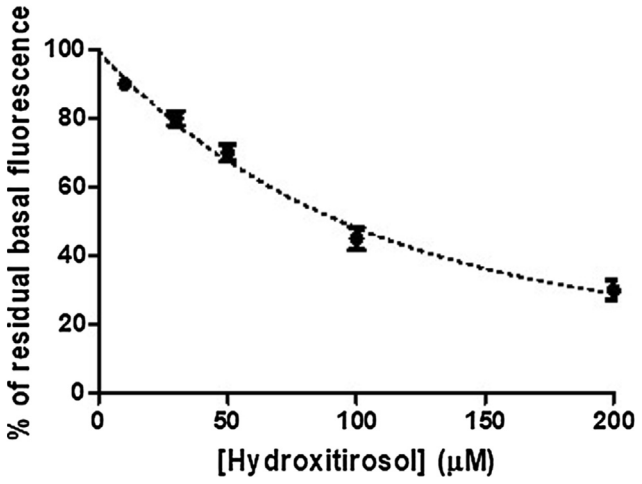

Fig. 1. Effect of increasing HT concentrations on the basal fluorescence of colonocytes charged with the CM-H_2DCFDA probe. Data are expressed as percentage residual fluorescence with respect to negative control (explants not pre-incubated with HT).

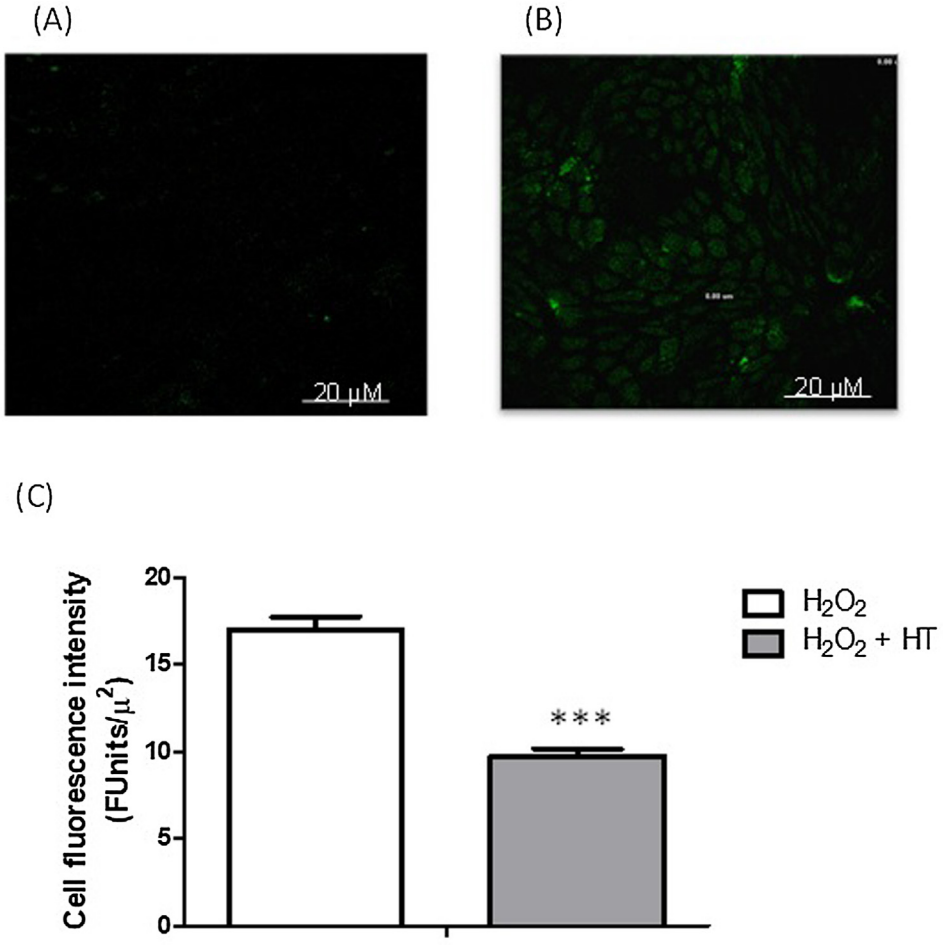

Fig. 2. Representative confocal image of the rat colon surface of explants charged with the CM-H_2DCFDA probe and exposed (B) or not (A) to H_2O_2 for 15 min. The epithelial surface was visualized using confocal laser scanning microscopy (see Section 2) at 100× objective. (C) Fluorescence emitted by superficial colonocytes pre-incubated with HT (100 μm) or PBS for 1 h, loaded with CM-H2DCFDA, and then exposed to H_2O_2 for 15 min respectively. ***p < 0001 (Student's *t* test).

fluorescence intensity.

As reported in Fig. 3, the HT inhibition on the H_2O_2-induced fluorescence was dose-dependent with a significant effect already detectable at 5 μM. The HT concentration able to decrease the fluorescence of the probe by 50% (IC_{50}) was 53.6 ± 12.1 μM.

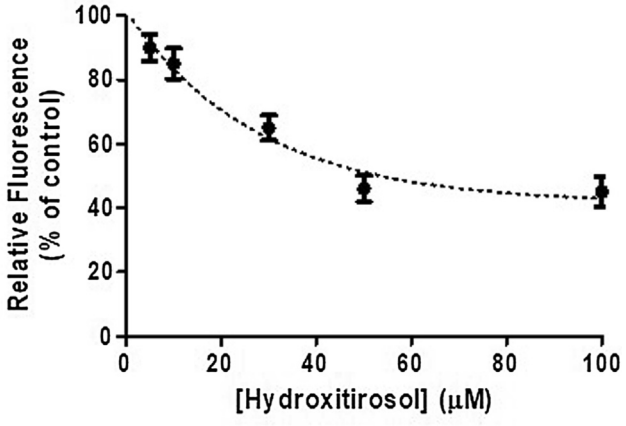

Fig. 3. Effect of increasing HT concentrations on H_2O_2 induced fluorescence. The graph shows the percentage residual fluorescence (*vs.* control) recorded from superficial colonocytes of explants pre-incubated with increasing HT concentrations for 1 h, loaded with CM-H_2DCFDA and then treated with 400 μM H_2O_2 for 15 min. Control cells were from explants not pre-incubated with HT.

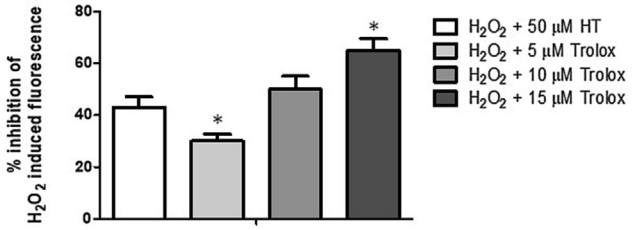

Fig. 4. Comparison between the antioxidant effect of HT and the effect of Trolox on rat colonocytes. Percentage inhibition exerted by 100 μM HT on superficial rat colonocytes compared with the effect exerted by increasing concentrations of Trolox. *p < 0.05 (One Way ANOVA and Dunnett's multiple comparison test).

The antioxidant effect of HT (50 μM) on superficial colonocytes was compared to the effect exerted on the same experimental model by increasing concentrations of Trolox, a vitamin E synthetic analog, widely utilized as antioxidant standard (Fig. 4). The inhibitory effect of HT on the CM-H_2DCFDA fluorescence induced by H_2O_2 was comparable to the effect exerted by 10 μM Trolox (Fig. 4).

3.4. Effect of HT against an exogenous oxidative challenge induced by terbutyl hydroperoxide exposure

With the aim to deepen the study on the intracellular antioxidant activity of HT on superficial colonocytes, we tested the effect of HT

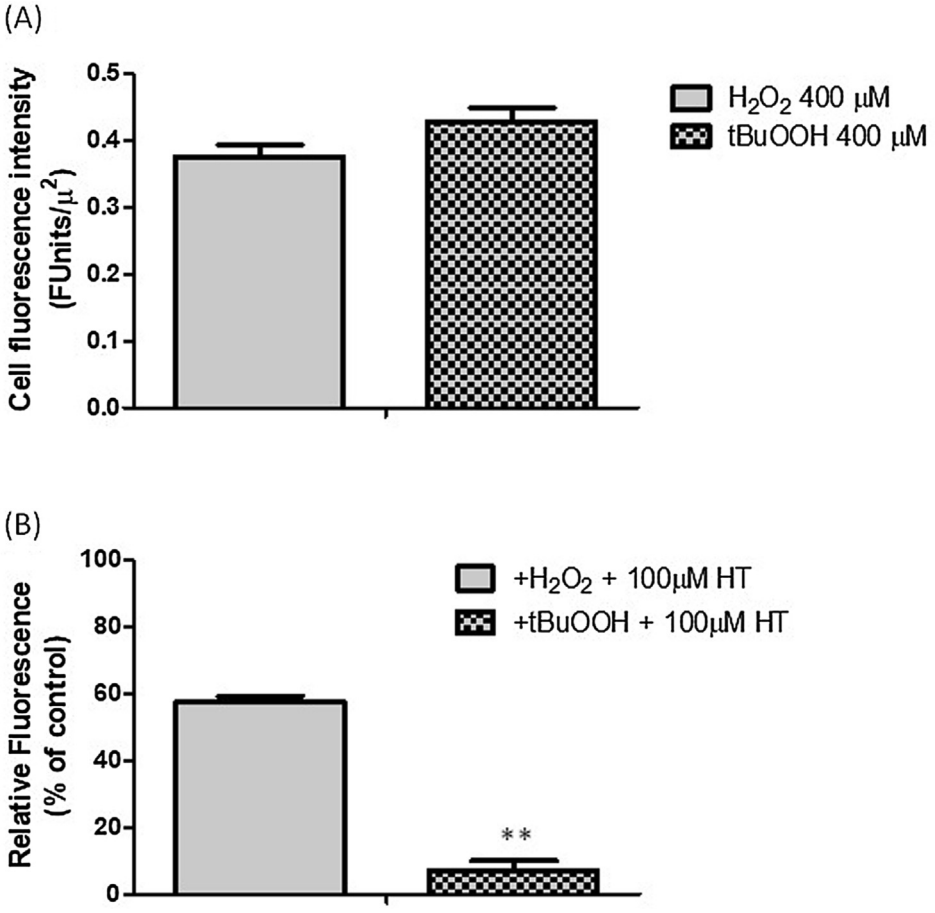

Fig. 5. Effect of HT on H_2O_2 or tert-butyl peroxide induced fluorescence. (A) Fluorescence emitted by superficial colonocytes charged with CM-H_2DCFDA, and then exposed to 400 μM H_2O_2 or tert-butyl hydroperoxide for 15 min respectively. (B) The graph shows the percentage residual fluorescence (*vs.* control) recorded from superficial colonocytes of explants pre-incubated with increasing HT (100 μM) for 1 h, loaded with CM-H_2DCFDA and then treated with 400 μM H_2O_2 or 400 μM tert-butyl hydroperoxide for 15 min. Control cells were from explants not pre-incubated with HT. Data are reported as the mean ± S.E.M. ***$P < 0.001$ (Student's *t*-test).

against an oxidative challenge induced by another pro-oxidant agent, tert-butyl peroxide, a synthetic analog of lipid peroxides. As observed in Fig. 5A, 400 μM tert-butyl peroxide for 15 min was able to evoke an increase in the intracellular fluorescence slightly higher (although not significant) compared to the increase induced by the exposure to H_2O_2 for 15 min.

Fig. 5B reports the inhibition exerted by100 μM HT pre-incubation on the tert-butyl peroxide-induced challenge compared to the effect against an H_2O_2 challenge at the same concentration. While 100 μM HT was able to reduce the H_2O_2-induced oxidative stress by about 50% compared to not exposed explants, the same HT concentration was able to reduce more dramatically the tert-butyl peroxide-induced oxidative stress by about 90%.

4. Discussion

Oxidative stress is one of the main factors involved in many disorders of the GI tract. In recent years, there is a growing interest in the capacity of dietary antioxidants to protect the intestinal epithelium against oxidative stress conditions thanks to the direct interaction of food with the intestinal mucosa.

The present work aimed to study the direct intracellular antioxidant effect of HT, one of the most potent natural antioxidant phenolic compound, on the colon epithelium, either under basal physiological conditions or under pro-oxidant conditions, by using *in situ* confocal microscopy, in order to gain information about possible protective effects of this phenolic compound directly on the intact tissue. Compared to the cellular antioxidant activity assay (CAA), developed to measure the antioxidant activity of several compounds in cell cultures (Wolfe & Liu, 2007), the experimental approach used in the present study allows to obtain information about antioxidant activity and protective effects directly on the intact epithelium, with a preserved architecture of the intact mucosa, and thus offering a more realistic information about the biological properties of HT.

Preliminarily, we assessed that the experimental exposure of rat colon mucosa to HT did not alter the ion transport properties of the intact epithelium, as emerged by the electrophysiological measurements of Vt and Isc on Ussing chamber-mounted colon explants exposed to 100 μM HT, one of the highest concentrations tested in our experimental conditions or its permeability characteristics. Moreover, the measurement of Rt on colon explants exposed to HT allowed excluding any effect of the polyphenolic compound on the tight junction permeability, which is fundamental for the barrier function of the epithelium and its para-cellular transports (Shimizu, 2010; Turner, 2009). The obtained results are in agreement with previous data on cell lines and animal models that excluded any cytotoxic effect of HT and contribute to improve the knowledge in this field with data on the intact epithelium which are lacking in literature (Auñon-Calles, Canut, & Visioli, 2013; Auñon-Calles, Giordano, Bohnenberger, & Visioli, 2013; D'Angelo et al., 2001).

HT is known to be absorbed in a dose-dependent manner both in the small intestine and colon (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010). In rat colon mucosa, we demonstrated that HT can decrease the basal intracellular ROS production of superficial colonocytes dose-dependently following 1 h exposure to increasing physiological concentrations (Corona et al., 2006). In intestinal cells, like in other cell types, a large proportion of basal ROS generation comes from the mitochondrial electron transport chain. Besides, a significant contribution arises from the activity of multiple enzymes highly expressed in the large intestine (including peroxidases, NADPH oxidase, xanthine oxidase). Moreover, the availability of transition metal ions and the depletion of reducing compounds

(Bhattacharyya et al., 2014) can contribute to ROS production. HT is known to act as a free radical scavenger and a radical chain breaker thanks to its catechol structure, and, in addition, it is known to exert metal chelation (Bernini, Merendino, Romani, & Velotti, 2013).

Our results show that after 1 h incubation HT reaches intracellular concentrations suitable to exert significant dose-dependent reduction of basal ROS generation in colonocytes, which suggests a possible preventive role in oxidative stress-associated conditions.

The direct intracellular antioxidant effect of HT on colon mucosa is clear on rat colon explants following a strong exogenous oxidative challenge simulating what can happen in the GI tract under abnormal conditions where reactive species overproduction contributes to the development of various physio-pathological processes, from inflammatory processes to multistage carcinogenesis (Bhattacharyya et al., 2014; Kim et al., 2012; Klaunig et al., 2010; Pérez et al., 2017).

In our work, we have chosen H_2O_2 as a pro-oxidant agent. It is permeable through the plasma membrane due to e.g. aquaporins (Miller, Dickinson, & Chang, 2010). Peroxide radicals are the major oxygen free radicals generated as a result of oxygen activation. They are commonly released by bacteria and phagocytic cells during inflammation and represent a physiological oxidative stress actor in colonic epithelial cells (Nathan, 2002; Ocaña, de Ruiz, Hogado, & Nader Macías, 1999; Strus et al., 2009; Uchiyama et al., 2011). They are stable, if compared with other oxygen free radicals and, therefore, they show the ability to diffuse to remote cellular locations including nucleus and DNA, participating in reactions that are relevant to tumor initiation and promotion (Lisanti et al., 2011; Marnett, 1987). Moreover, as outlined by Barrett and McCole (2016), hydrogen peroxide overproduction is of relevance for inflammatory bowel disease where the secretory activity of the numerous activated phagocytes can produce millimolar concentration of H_2O_2 in close proximity of colonic cells. Therefore, the choice of peroxide radical removal as a tool for detecting antioxidant activity aroused form the fact that it represents a biological relevant reaction from a physiological and pathophysiological point of view.

The H_2O_2 concentration applied in our experiments (400 μM) is included in the range of H_2O_2 concentrations that can be found in the colon under chronic inflammatory conditions (Becker, Soukup, & Gallagher, 2002; Giordano et al., 2016). In addition, another pro-oxidant agent, i.e. tert-butyl hydroperoxide, was chosen. It is an organic cell-permeable synthetic analog of lipid peroxides, widely utilized for investigating lipid peroxidation damage (Chamulitrat, 1998). It can generate t-butoxyl radicals *via* the Fenton reaction, resulting in lipid peroxidation and depletion of intracellular glutathione (GSH), one of the main intracellular redox systems (Domanski, Lapshina, & Zavodnik, 2005).

According to Giordano and coll. (Giordano et al., 2016), the incubation time with the pro-oxidant agent in rat colon explants loaded with CM-H_2DCFDA was 15 min, which corresponds in our experimental model to a temporal threshold for the activation of intracellular peroxidative reactions induced by H_2O_2 entry into the cell.

When the colon explants were incubated with increasing HT concentrations for 1 h, the fluorescence induced by 15 min incubation with H_2O_2 decreased in a dose-dependent manner, suggesting that HT can induce a dose-response protective effect against pro-oxidant conditions directly on the colon epithelium. In doing so, HT contributes to the intracellular antioxidant defenses, helping the peroxide removal activity exerted by the antioxidant enzymatic defenses catalase and glutathione peroxidase, widely expressed in the colon mucosa (Tian et al., 2017). Catalase catalyzes the conversion of H_2O_2 to H_2O and O_2 and its activity has been demonstrated to reduce inflammation in rodent colitis models (Yavuz, Yüksel, Yeğen, & Alican, 1999). Glutathione peroxidase (GPX) reduces H_2O_2 into H_2O or lipid hydroperoxydes into alcohols using the oxidation of GSH. GPX2 is the isoform mainly expressed in the colon mucosa and its activity contributes to the prevention of inflammation (Tian et al., 2017). HT is known to upregulate catalase expression in vascular endothelial cells (Zrelli, Matsuoka, Kitazaki, Zarrouk, & Miyazaki, 2011). However, due to the short incubation time (1 h) of the explants with HT in our experimental model, the hypothesis of a possible effect of HT on the expression of intracellular antioxidant enzymes can be excluded. Therefore, it is reasonable to hypothesize that the HT antioxidant activity, due to the specific molecular characteristics of the phenolic compound, could act in an additive way to the peroxide removal activity exerted by the antioxidant enzymes in our experimental model.

A detectable HT effect on H_2O_2 induced fluorescence was already evident at 5 μM HT and reached its maximal effect at 50 μM. Higher HT concentrations (> 50 μM) kept antioxidant activity constant, and still partial. This saturable activity of HT against an H_2O_2 challenge is similar to the effects observed in other studies on polyphenolic compounds employing the cellular antioxidant activity (CAA) assay, where dose–response curves for a number of polyphenolic compounds exhibited a hyperbolic shape with apparent saturation at higher concentrations (Kellett, Greenspan, & Pegga, 2018; Wolfe & Liu, 2007). This saturation behavior indicates the interaction of HT with specific oxidation sites. Another aspect to be outlined is the lower IC_{50} associated to the HT effect under pro-oxidant condition (H_2O_2 exposure) (53.6 ± 12.1 μM) with respect to the IC_{50} associated to the HT effect under basal condition (94.1 ± 9.2 μM). It is possible that under pro-oxidant conditions, the antioxidant action of HT is more clearly expressed because of the depletion of endogenous intracellular antioxidants, such as GSH (required for the activity of glutathione peroxidase), which has been demonstrated to be oxidized very quickly after application of exogenous H_2O_2 (Dringen & Hamprecht, 1997). On the other hand, under homeostatic condition with well-charged endogenous antioxidant defenses a marked concentration of HT would be required in order to observe a significant effect on the basal ROS.

In our experimental model, the effect of 50 μM HT against an H_2O_2 challenge is comparable to the effect exerted by 10 μM Trolox, widely used as a standard in *in vitro* assays for antioxidant activity determination. Also, tert-butyl peroxide induces a detectable oxidative challenge to colon explants, loaded with the oxidative stress fluorescent probe CM-H_2DCFDA, comparable to the effect exerted by H_2O_2. At the highest concentration tested (100 μM HT), the protective effect of the polyphenol is more pronounced against the tert-butyl peroxide-induced oxidative stress than the H_2O_2. This result is in line with previous observations about the antioxidant activity of the peptide lunasin on CaCo-2 cells exposed to H_2O_2 or tert-butyl peroxide challenge (García-Nebot, Recio, & Hernández-Ledesma, 2014), where an increased effect of the peptide was observed at higher concentrations against tert-butyl-induced oxidative stress than against H_2O_2. This suggests that the oxidative challenge of the two agents can involve different oxidative reactions and sites whose sensitivity to HT scavenging activity might differently act. Moreover, the inhibitory effect exerted by HT incubation against the tert-butyl peroxide induced fluorescence confirms the known preventive action of HT on lipid peroxidation, demonstrated in a number of cell types such as hepatocytes (Ruiz-Gutierrez, De La Puerta, & Catala, 2001), peripheral blood lymphocytes (Gargouri et al., 2011), renal tubular cells (Deiana et al., 2011), and also colonic cells (Deiana et al., 2010).

5. Conclusions

Our results demonstrate that HT decreases the basal intracellular ROS production of superficial colonocytes in an intact rat colonic mucosa dose-dependently and that it exerts a direct antioxidant action on the colon mucosa exposed to oxidizing species. Indirectly, these findings document the successful dose-dependent, intracellular accumulation of HT in the colon mucosa to intracellular levels sufficient to exert an antioxidant protective effect. The antioxidant activity is dose-dependent and exhibits a saturation behavior with respect to H_2O_2-induced oxidative stress. At the highest concentration tested (100 μM),

HT is more effective against a tBuOOH than an H_2O_2 challenge, suggesting that the oxidative challenge exerted by these two agents may involve different oxidative reactions and different sites whose sensitivity to HT scavenging activity is different.

Moreover, our results indicate that HT directly contributes to the redox balance of the colon epithelium by reducing ROS in both basal and pro-oxidant conditions and potentially protects the mucosal epithelium from oxidative stress-related damage.

Overall, our data support the potential of HT as a functional food ingredient with applications in the protection of intestinal mucosa against oxidative damage, and open to further studies involving HT aimed at the prevention and treatment of pathological conditions associated with excessive exposure to oxidative stress in the GI tract.

Ethical statements

All animal experiments were carried out according to the EU Directive 2010/63/EU for animal experiments.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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