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4. Resveratrol: A polyphenol with multiple effects

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Abstract. *trans*-Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a natural polyphenol that occurs in grapes, berries, peanuts, and several traditional medicines. A number of studies have demonstrated that this polyphenol holds promise against numerous age-associated diseases including cancer, diabetes, Alzheimer, cardiovascular and pulmonary diseases. In view of these studies, resveratrol's prospects for use in the clinics are rapidly accelerating. This review summarizes our work on the mechanisms involved in the intestinal absorption and its population pharmacokinetics. Finally, various targets of resveratrol and its therapeutic potential are described.

Introduction

trans-Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenolic compound naturally occurring in plants and found in dietary products. Along the past

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decade our research was devoted to study in depth the mechanism involved in its intestinal absorption as the first step to explain the bioavailability of this compound. The present review also discusses the processes implicated on its distribution and elimination as well as its beneficial effects in colon cancer and spermatogenesis.

1. Chemistry of *trans*-resveratrol

trans-Resveratrol (3,5,4'-trihydroxistilbene, Fig. 1) is a polyphenol that belongs to the stilbene family which is characterized by an essential structural skeleton of two aromatic rings joined by an ethylene bridge.

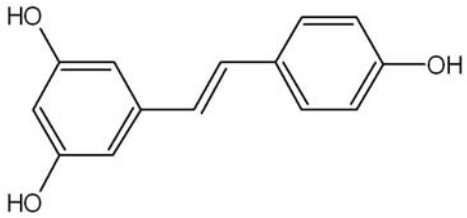

Figure 1. Chemical structure of *trans*-resveratrol.

trans-Resveratrol is formed via a condensation reaction between three molecules of malonyl-CoA and one molecule of 4-coumaroyl CoA catalyzed by *trans*-resveratrol synthase. The synthesis of *trans*-resveratrol in plants is triggered in response to exogenous stress factors, such as injury, ultraviolet radiation and fungal infection. For this reason, this secondary metabolite of plants is defined as a phytoalexin [1]. The concentration of *trans*-resveratrol peaks approximately 42-72 h after stress exposure, and declines after 42-72 h as a result of activation of stilbene oxidase [1].

This polyphenol exists in both *cis*- and *trans*-isomeric forms, the *trans*-isomer being the most commonly found and extensively studied. In plants, *trans*-resveratrol is also found as 3-O-β-D-glucosides, a compound also known as piceid or polydatin, which is more stable and less susceptible to oxidative degradation than the parent compound. Other analogs of *trans*-resveratrol such as pterostilbene (3,5-trimethoxy-4'-hydroxystibene) or piceatannol (3,4,3',5'-*trans*-tetrahydroxystilbene) have also been reported in plants [1].

2. Sources of *trans*-resveratrol

trans-Resveratrol was first isolated in 1940 as a component of the roots of the white hellebore (*Veratrum grandiflorum*). The term resveratrol comes

from this plant, being "res" a latin prefix that means "which comes from" and "ol" a suffix indicating the presence of an alcohol moiety in its structure. In 1963, it was identified as an active constituent of the dried roots of *Polygonum cuspidatum* (0.524 mg/g), also called *kojo-kon* and used in traditional Asian medicine against suppurative dermatitis, gonorrhea and hyperlipemia [2]. In *Vitis vinifera* it was detected in 1976 where this polyphenol is exclusively synthesized in leaf epidermis and in grape skins, but not in the flesh [3], and in wine in 1992 [4]. Afterward this polyphenol has been described in more than 70 plant species [5].

Table 1. Content of *trans*-resveratrol in dietary sources.

Source	*trans*-Resveratrol concentration
Red wine	0.1 – 14.3 mg/L
White wines	0.1 – 2.1 mg/L
Grapes	50 – 100 μg/g
Peanuts	0.02 – 1.92 μg/g
Pistachios	0.09 – 1.67 μg/g
Blueberries	~ 32 ng/g
Bilberries	~ 16 ng/g

In grapes the concentrations of *trans*-resveratrol ranged from 50 to 100 μg/g fresh weight. During mashing, in the winemaking process, part of *trans*-resveratrol present in the skin is dissolved in the must. Thus, the concentrations of this polyphenol are higher in red wines since the contact between berry skin and must is more prolonged, than in white wines, where the must is immediately separated from the grape residues. Content of *trans*-resveratrol in red wine ranges from non-detectable concentrations to 14.3 mg/L (62.7 μM). The average concentrations of *trans*-piceid in a red wine could be as much as 29.2 mg/L (128.1 μM), i.e., three times that of *trans*-resveratrol [6].

In addition to grapes and wine (Table 1), *trans*-resveratrol is found in several edible natural products such as peanuts (*Arachis hypogaea*), berries (blueberries, cranberries, blueberries, bilberries, from the *Vaccinium* family), rhubarb (*Rheum rhabarbarum*), pistachio (*Pistacia vera*) and hop (*Humulus lupulus*) [1,5].

The occurrence of *trans*-resveratrol has also been documented in a number of trees, such as eucalyptus and spruce, as well as the tropical deciduous tree *Bauhinia racemosa*. It has also been found in a few flowering plants [1].

3. Intestinal absorption of *trans*-resveratrol

The processes that take place in the intestine during the absorption of *trans*-resveratrol have been studied *in vivo* in rats using a perfusion method [7]. A nutritionally relevant concentration of 25 μmol/L of *trans*-resveratrol was used to evaluate the absorption across the jejunum as a function of time. Nearly 72% of the luminally perfused polyphenol disappeared from the buffer after 30 min, indicating that there was an efficient uptake of this polyphenol. At the same time, the result showed that *trans*-resveratrol was efficiently conjugated inside the enterocyte with glucuronic acid by UDP-glucuronosyltransferase or with sulfate by sulfotransferase, and 42% and 12%, respectively, were subsequently pumped back to the luminal side.

The kinetic study of *trans*-resveratrol was performed in jejunal and ileal loops perfused with increasing concentrations of this compound. The transport rates of the unconjugated polyphenol were directly proportional to the initially applied *trans*-resveratrol, indicating that the uptake occurs by simple diffusion, without the participation of a mediated transport, as indicated with perfusion using 2,4-dinitrophenol. The apparent diffusion constant (Kd) normalized to segment dry weight was 8.1 ± 0.3 μL/5 min·mg dry weight in the jejunum and 10.7 ± 0.2 μL/5 min·mg dry weight in the ileum [7]. These results are in accordance with findings for Caco-2 cells, where this compound crosses the cells by passive diffusion [8].

The efflux of the intracellulary formed *trans*-resveratrol glucuronide and sulfate towards the intestinal lumen was higher in the jejunum than in the ileum, indicating a region-dependent metabolism of *trans*-resveratrol in the small intestine according to the regional distribution of UDP-glucuronosyltransferase and sulfotransferase in the intestine [9]. In both segments, the kinetic analysis of *trans*-resveratrol transformation indicated that in the enterocyte, glucuronidation was favored over sulfation, with similar rates of reaction. Glucuronidation seems to be the predominant conjugation pathway in the rat, and *trans*-resveratrol glucuronide was excreted form the enterocyte to the intestinal lumen with an average concentration 4.5 times higher than that of the sulfate [7].

The secretion of the conjugates out of the enterocyte was thought to be mediated through the ATP-binding cassette (ABC) transporters which are efflux proteins that act as “gatekeepers” in the intestine thus controlling the oral availability of many substances [10]. This ABC superfamily contains membrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, sterols, and drugs. Of the numerous members of the ABC transporter family, three have been described in the apical membrane of the enterocyte: the P-glycoprotein

(Pgp; ABCB1), the multidrug resistance-associated protein (MRP2; ABCC2), and the breast cancer resistance protein (BCRP; ABCG2) [10,11]. Pgp recognizes a wide range of structurally and pharmacologically unrelated neutral and positively charged hydrophobic compounds [10]. MRP2 has a relatively hydrophobic substrate spectrum, including glucuronide, glutathione and sulfate conjugates of endogenous and exogenous [12]. BCRP is the most recently discovered of these efflux transporters and recognizes mainly hydrophilic anticancer agents as well as negatively charged drug conjugates[13].

The role of intestinal ABC transporters in the absorption of *trans*-resveratrol was investigated with the use of specific inhibitors (Fig. 2). Consequently, P-glycoprotein was evaluated by using verapamil and cyclosporine [10]. These inhibitors ruled out the possibility that either *trans*-resveratrol or its conjugates constituted a substrate of this protein. Intestinal perfusions of *trans*-resveratrol with the MRP2 inhibitors probenecid [14] and MK571 [15] did not exhibit any effect on *trans*-resveratrol. However, secretion of glucuronide and sulfate conjugates decreased, thus implicating MRP2 in their efflux. These results are in agreement with findings for isolated perfused livers, which have indicated that MRP2 exclusively mediates the biliary excretion of resveratrol glucuronides and only partly mediates that of sulfates [16]. The role of BCRP was analyzed in the presence of Ko143, a high affinity and specific BCRP inhibitor [17]. Perfusions with Ko143 significantly decreased the secretion of *trans*-resveratrol glucuronide and sulfate conjugates without affecting the absorption of the parent compound. The role of BCRP was confirmed with the use of BCRP1$^{-/-}$ mice that were orally administered with 60 mg/kg of *trans*-resveratrol [18]. *trans*-Resveratrol and its metabolites were measured in intestinal content at 30 min after administration and showed a decrease of 71% and 97% of resveratrol glucuronide and sulfate respectively, compared to the wild-type mice, thus indicating a lower efflux from the enterocytes [18].

trans-Resveratrol conversion to a mono-glucuronide and a mono-sulfate was the only observed transformation in rat intestine under our experimental conditions with no cytochrome P450-mediated phase I metabolic transformation or methylation [7]. The latter possibility did not take place due to the lack of catechol function in the *trans*-resveratrol molecule, which is a requirement for catechol-O-methyltransferase (COMT). In addition to the conjugates, another metabolite of *trans*-resveratrol has been described in the intestine [19]. This polyphenol is quantitatively transformed into dihydroresveratrol, which is a metabolite that may be produced by the intestinal microflora since its formation involves the hydrogenation of the aliphatic double bond of the parent compound. Dihydroresveratrol was found

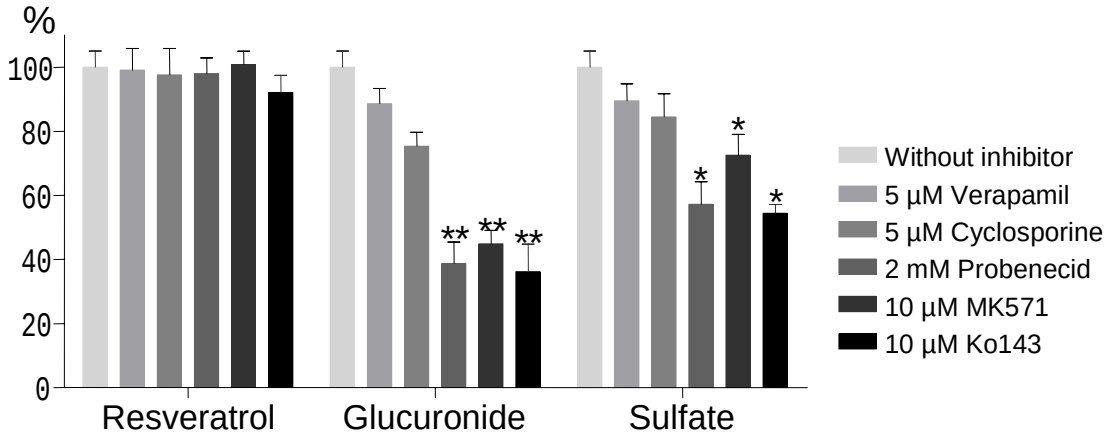

Figure 2. Effects of inhibitors of Pgp (verapamil, cyclosporine), MRP2 (probenecid, MK571) and BCRP (Ko143) on the absorption of *trans*-resveratrol and the secretion of intracellulary formed glucuronide and sulfate conjugates. Results are expressed as percentage of *trans*-resveratrol absorbed or glucuronide and sulfate secreted in the absence (100%) or presence of the inhibitor under study. Values are expressed as means + SE (n = 4-6). * $P < 0.05$, significantly different from perfusion with *trans*-resveratrol alone; ** $P < 0.001$.

to be the most abundant metabolite in the colon after the oral administration of 60 mg/kg of *trans*-resveratrol for 49 days, and 24 hours after the last administration, with concentrations of 303.0 ± 34.7 nmol/g, 446-fold that of the parent compound [19].

4. Population pharmacokinetics of *trans*-resveratrol

The bioavailability and metabolism of *trans*-resveratrol have been widely studied in rats and humans given that its efficacy depends on its absorption and metabolism [20]. However, it is difficult to provide an adequate pharmacokinetic description of the intricate processes that determine the bioavailability of this polyphenol. A preliminary evaluation of the plasmatic pharmacokinetics of *trans*-resveratrol was carried out after the oral administration of 2 mg/kg to overnight fasted rats [21]. Blood samples were extracted at different time points over an hour, and showed low plasmatic concentrations of unchanged *trans*-resveratrol with peak concentration of 550 ng/mL at 10 minutes [21]. The low bioavailability for *trans*-resveratrol indicates that the small intestine comes out as the first bottleneck to the entry of this compound to the organism [18,21]. In addition, the metabolism in the liver cannot be underestimated [22,23] before the distribution to tissues where ABC proteins are also present, given that all these processes influence the

distribution and subsequent elimination from the organism [18]. As a result of this complex interplay between enzyme activities and efflux transporters, the concentrations of *trans*-resveratrol in plasma have been reported to be low [20,21].

Given the important metabolism observed for *trans*-resveratrol, an integrated pharmacokinetic model that could describe the parameters of both the parent compound and its conjugated metabolites was investigated after i.v. and p.o. administration of 2, 10 and 20 mg/kg of *trans*-resveratrol in Sprague-Dawley rats [24]. Analyses of the plasmatic data through the population pharmacokinetic approach provided important advantages such as overcoming the limitations of blood sampling in studies using experimental animals, and at the same time preserving animal individuality. In addition, the population approach allows the estimation of the typical pharmacokinetic parameters as well as the inter-animal variability. To this end, all the data (from parent compound and conjugates) obtained after the i.v. administrations of three doses of *trans*-resveratrol were simultaneously analyzed. Once the best intravenous model was found, the disposition parameters were fixed and the oral data of the three compounds were added to estimate the absorption parameters (absorption rate constants and bioavailability).

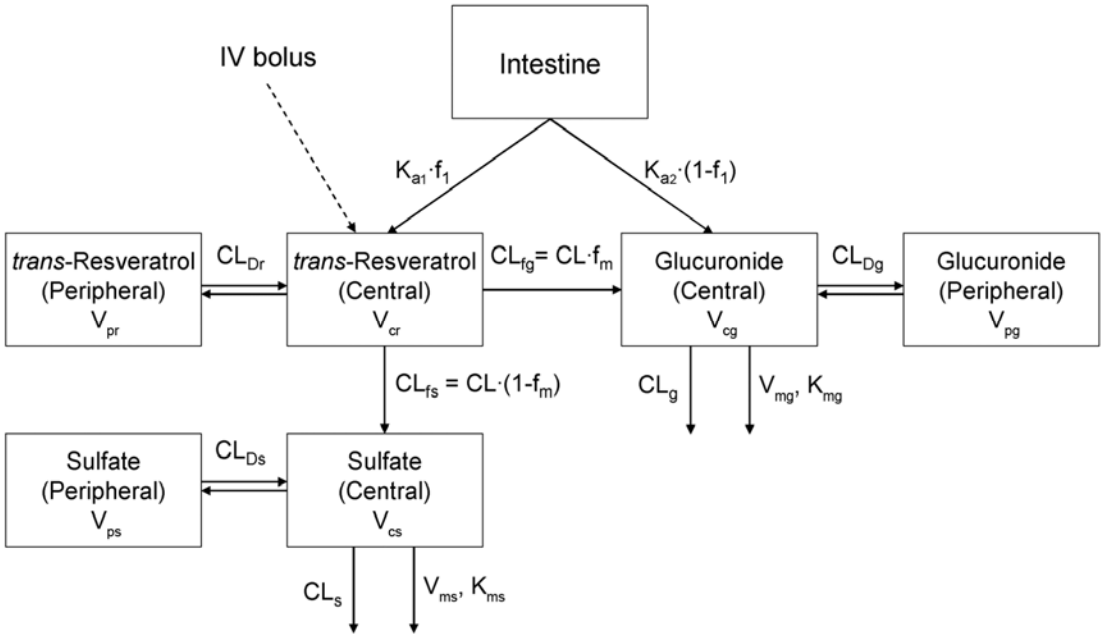

Figure 3. Schematic representation of the pharmacokinetic model to simultaneously describe the data of *trans*-resveratrol and its glucuronide and sulfate conjugates after i.v. and p.o. administration.

The pharmacokinetics of *trans*-resveratrol and its conjugates that best described our results was a three-linked two compartment model (Fig. 3). Elimination of *trans*-resveratrol by conversion to its glucuronide and sulfate took place by a first-order kinetic process. It is noteworthy that the transformation of the parent compound to its conjugates was not saturable even at the plasmatic concentrations achieved after the highest dose assayed (20 $mg \cdot kg^{-1}$). Meanwhile, clearance of *trans*-resveratrol glucuronide and sulfate was best described by parallel first-order and Michaelis-Menten kinetics. These results are in agreement with existing knowledge about elimination mechanisms of glucuronide and sulfate in tubular cells and hepatocytes [18,25]. The total clearance value of *trans*-resveratrol was slightly higher than ¾ times the hepatic blood flow in the rat (0.90 L/h for a body weight of 0.25 kg). According to the f_m value obtained (0.540) approximately the same percentage of both metabolites was formed. Clearances of formation of the glucuronide and sulfate were 0.67 and 0.57 $L \cdot h^{-1}$, respectively. Distribution volume of *trans*-resveratrol (total distribution volume = 3.05 L) exceeded the total body water in the rat (0.15 $L \cdot kg^{-1}$ for a body weight of 0.25 kg) suggesting extensive distribution into tissues. A short half-life value was estimated for *trans*-resveratrol (0.55 h) that was in agreement with its clearance and distribution volumes values. By contrast smaller distribution volumes were found for the metabolites (total distribution volumes = 0.38 and 0.14 L for the glucuronide and sulfate, respectively).

In order to model the oral data, a depot compartment (intestinal compartment) was added to the intravenous model where *trans*-resveratrol was administered. When going into the gastrointestinal tract, *trans*-resveratrol was supposed to be subject to first-pass metabolism and to reach the systemic circulation intact or as its glucuronide according to a simple diffusion process. The rate limiting step is most likely the absorption process of *trans*-resveratrol and its glucuronide rather than the metabolism. The first-order absorption rate constants (K_{a1} = 0.442 h^{-1}, absorption half life = 1.57 h, K_{a2} = 0.256 h^{-1}, absorption/metabolism half life = 2.71 h) confirmed rapid absorption/metabolism kinetics for both compounds. The data did not support the inclusion of first-pass metabolism of *trans*-resveratrol to sulfate, so the model was simplified to absorption/presystemic metabolism of *trans*-resveratrol to its glucuronide and absorption of the formed glucuronide. The inclusion of a fraction of the parent compound never absorbed, due to metabolism to the conjugates and efflux of them back into intestine, did not improve the fit. One of the reasons why *trans*-resveratrol sulfate could not be included in the presystemic metabolism of *trans*-resveratrol might be the low concentrations of this metabolite that reached the bloodstream. These low concentrations could be attributed to a lower sulfation compared to the

glucuronidation of *trans*-resveratrol in the rat intestine [26,27]. Moreover, the higher affinity and capacity of BCRP for *trans*-resveratrol sulfate compared to the one observed for the glucuronide could account for a higher efficiency in the secretion of the sulfate [25]. Andlauer *et al.* [26] appointed that only 0.3% of the absorbed resveratrol reaches the blood as sulfate. Altogether, these processes could explain why the model did not support the inclusion of presystemic metabolism of *trans*-resveratrol to its sulfate conjugate.

The proportions of absorbed intact *trans*-resveratrol and its glucuronide were estimated as f_1 and $(1-f_1)$, respectively. The results showed that when the dose administered increased a lower fraction of *trans*-resveratrol remained unchanged (f_1 = 0.420, 0.207 and 0.060 for the doses of 2, 10 and 20 $mg \cdot kg^{-1}$, respectively, and a body weight of 0.25 kg). By contrast, the relative fraction of glucuronide formed/absorbed from the intestine increased with the dose with values of 0.580, 0.793 and 0.94 for the doses of 2, 10 and 20 $mg \cdot kg^{-1}$ and a body weight of 0.25 kg, respectively. This suggested a non linear pharmacokinetic behaviour of *trans*-resveratrol, after p.o. administration from 2 to 20 $mg \cdot kg^{-1}$.

It is known that *trans*-resveratrol and its conjugates accumulate significantly in the liver [18,28] and are further excreted into the bile, leading to enterohepatic recirculation [16,29]. The contribution of the biliar pathways to the elimination of the conjugated metabolites could not be captured by the present model although a light rebound was observed after 6 h of the i.v. administration, supporting the occurrence of the enterohepatic cycle. Previously, Marier et al. [29] have reported that the enterohepatic recirculation using a linked-rat model induced significant increase of plasma concentrations of resveratrol and its glucuronide in bile-recipient rats at 4 to 8 h. The population pharmacokinetic model built adequately described the plasmatic concentrations of *trans*-resveratrol and its major metabolites in the rat after i.v. and p.o. administrations, as it was verified with internal validation procedures [24]. Its predictive capacity was also evaluated using an external data set obtained after the i.v. administration of 15 $mg \cdot kg^{-1}$. The results showed a close agreement between the observed data and the predicted concentrations for the dose and administration route assayed, supporting the hypothesis of the robustness of the model. In summary, the population PK model built allowed to increase the knowledge to better understand the PK of this polyphenol but also may be applicable to many other compounds which have similar PK properties. In addition the model may also be useful for planning future PK–PD studies to establish the relative contribution of the conjugates to the overall efficacy.

In humans, Walle et al. showed that the majority of a dose of 25 mg administered p.o and i.v. was converted to *trans*-resveratrol sulfate within 30 min, and the serum peak concentrations of the parent compound were inferior to

22 nM [30]. Five metabolites were identified in urine, *trans*-resveratrol monosulphate, two isomeric monoglucuronides, dihydroresveratrol monosulphate and dihydroresveratrol monoglucuronide. The total sulfate and glucuronide conjugates accounted for approximately 37 and 19%, respectively of the metabolites in urine, and there were only trace amounts of free *trans*-resveratrol [30]. A study of the pharmacokinetics of oral *trans*-resveratrol was performed after the administration of a single dose of 0.5, 1, 2.5 or 5 g in healthy volunteers [31]. An extensive metabolism after the oral administration was also reported since six metabolites were recovered from plasma and urine. Peak plasma concentration of *trans*-resveratrol was 2.4 μM at 1.5 hours after the oral administration of 5 g. Peak levels of two monoglucuronides and resveratrol 3-sulphate were 3- to 8-fold higher. The area under the plasma concentration curve values for *trans*-resveratrol-3-sulphate and *trans*-resveratrol monoglucuronides were 23 times greater than those of *trans*-resveratrol. Urinary excretion of *trans*-resveratrol and its metabolites was rapid, with 77% of all urinary agent derived species excreted within 4 h after the lower dose (0.5 g).

5. Toxicology

Several studies have evaluated the toxicity in mice and rats in short-term and subchronic administration of *trans*-resveratrol [32-34]. The repeated dosage of 20 mg/kg of *trans*-resveratrol during 28 days, which corresponds to 1000 times the amount that may be consumed by a person drinking one glass of red wine a day, did not induce any adverse effect [32]. Body weight, food and water consumption were not altered in rats exposed to *trans*-resveratrol, when compared to the control group. Hematologic and biochemical variables were not affected by the treatment. An increased relative brain weight and testicular weight normalized to body weight was observed in the group administered with *trans*-resveratrol whereas no histopatological changes in these organs were detected. In addition, *trans*-resveratrol did not alter any vital organ weight or induce histopathologic changes [32].

The effect of higher doses was evaluated by the Crowell et al. [33]. Male and female Sprague-Dawley rats were administered by gavage 300, 1000 and 3000 mg/kg of *trans*-resveratrol for 4 weeks. The results showed that no adverse effects were observed in male and female rats exposed to 300 mg/kg. Minor effects were reported for the dose of 1000 mg/kg, such as reduced body weight in females and elevated leukocyte counts in males. The most remarkable adverse events took place at the highest dose of 3000 mg/kg, when a reduced body weight and food consumption, elevated kidney weight,

and increased incidence of kidney lesions were observed in male and female rats.

Those results were supported by the studies in which *trans*-resveratrol was incorporated in the diet at 50, 150 and 500 mg/kg during 28 days [34]. The dietary exposure did not induce any treatment-related effects on body weight clinical signs, hematological, clinical chemistry, histopatology or urinary parameters at any dose.

The reproductive toxicity of *trans*-resveratrol has been comprehensively evaluated. The administration of *trans*-resveratrol in the diet at 750 mg/kg during 90 days did not negatively affect sperm parameters or estrous cycle in the rat or any histopathological effect on reproductive organs [34]. *trans*-Resveratrol has been administered at 0, 120, 300 and 750 mg/kg to pregnant rats, showing no adverse effects on the number of implantations, resorptions, live young, or pre- and post-implantation losses. In addition no negative findings were observed in the placental, litter and fetal weight; or incidence of major or minor fetal abnormalities at any of the doses tested [34].

In addition to the studies performed in rats, the toxicity has also been evaluated in p53 knockout mice, which is a model accepted for oncogenicity bioassays [35]. The animals were administered *trans*-resveratrol by gavage at 1000, 2000 or 4000 mg/kg during six months. No effects were observed on body weight, food consumption, or clinical signs in any dose group. However, a dose-related increase in liver weight and serum cholesterol concentrations were reported in both sexes. Histopathology identified the kidney (hydronephrosis) and urinary bladder (epithelial hyperplasia) as target tissues for resveratrol toxicity. The incidences of both benign and malignant tumors in mice exposed to *trans*-resveratrol were comparable to those in vehicle controls. When administered to p53 knockout mice at its maximum tolerated dose, *trans*-resveratrol demonstrates no evidence of oncogenicity [35]. This study performed in p53 knockout mice address a key regulatory requirement for the entry of resveratrol into clinical trials for cancer prevention.

A phase I study of the oral administration of single doses of *trans*-resveratrol of 0.5, 1, 2.5 or 5 g conducted in 10 healthy volunteers per dose levels did not cause serious adverse events [31]. A safety profile of *trans*-resveratrol was carried out in healthy volunteers that received 25, 50, 100 or 150 mg, six times/day, for two days. This polyphenol was well tolerated and only mild adverse events were reported [36].

Taken as a whole, the absence of symptoms and the normal appearance of the vital organs in rats and mice suggested that *trans*-resveratrol is barely toxic even under the conditions that have been described.

6. Health benefits of *trans*-resveratrol

6.1. Colon cancer: *In vivo* and *in vitro*

Colorectal cancer is one of the leading causes of death in both men and women in Western countries, being usually lethal when diagnosed at later stages of progression [37]. Genetic predisposition as well as environmental aspects are thought to be involved in colon carcinogenesis, among which, dietary habits play a pivotal role. Mediterranean countries have lower rates of colorectal cancer compared with other Western countries [38]. It has been suggested that the environmental factors are mainly dietary and up to 80% of sporadic colorectal cancers are therefore potentially preventable [39].

trans-Resveratrol is gaining acceptance as a potential antitumor agent because of its multiple effects described in different experimental models of carcinogenesis [40,41]. This bioactive compound was shown to inhibit the growth of tumor cell lines derived from various human cancers, an effect that was associated with its ability to arrest cell cycle progression and to induce programmed cell death [40,41]. These antitumoral activities accompanied with the lack of harmful effects [32,34] makes *trans*-resveratrol an attractive chemotherapy and chemopreventive drug for cancer treatment [40]. The low oral bioavailability established for *trans*-resveratrol both in experimental animals and humans, where the intestine has been pointed out as a bottleneck to its absorption, have promoted the large intestine as a potential target site thus favoring its potential chemopreventive activity in colon cancer.

The chemopreventive activity of *trans*-resveratrol against colon cancer was evaluated *in vitro* on the human colorectal carcinoma HT-29 by assessing its anti-proliferative and pro-apoptotic activities. Defects in the regulation of cell cycle progression constitute an important characteristic of transformed cells. Exposure of HT-29 cells to 10-300 μM of *trans*-resveratrol reduced cell growth, with half-maximal effects of around 80 μM [42]. Noteworthy, this polyphenol did not induce necrosis even at concentrations that caused full inhibition of cell growth. The antiproliferative activity of *trans*-resveratrol may be attributed to cell cycle arrest at G2 phase as was previously indicated for HT-29 cells [43] and Caco-2 cells [44,45].

Alterations in programmed cell death mechanisms play important roles in tumor pathogenesis, allowing neoplastic cells to survive beyond their normally intended lifespan, subverting the need for exogenous survival factors, providing protection from hypoxia and oxidative stress as tumor mass expands, and allowing time for accumulative genetic alterations that deregulate cell proliferation, interfere with differentiation, promote

angiogenesis, and increase cell motility and invasiveness during tumor progression [46]. Consequently, compounds that can eliminate aberrant cell clones by the induction of apoptosis may have a chemopreventive or even a therapeutic potential. The pro-apoptotic activities of *trans*-resveratrol were evaluated on HT-29 using a series of *in vitro* assays that allowed the evaluation of different stages of programmed cell death [42]. The activation of the effector caspase-3 was determined since it represents the converging point of different caspase-dependent apoptosis pathways [47]. *trans*-Resveratrol increased the activity of caspase-3 in a time- and a dose-dependent manner. The lowest concentration of *trans*-resveratrol that induced the activation of this protease was 100 μM with an incremented of 200% that of control cells, and the highest activation was achieved at 250 μM, with an increase of 7-fold [42]. Execution of apoptosis beyond activation of caspase-3 by *trans*-resveratrol led to the characteristic hallmarks of programmed cell death, such as disintegration of the plasma membrane that was characterized by cell-staining with Hoechst 33342 dye. Exposure of HT-29 cells with 150 μM of *trans*-resveratrol accumulated Hoechst dye over time, with an increase of 17.0 ± 2.7% and 33.0 ± 3.7% at 20 and 24 h over the control cells [42]. Full execution of apoptosis with increased fragmentation of DNA and chromatin condensation was evidenced by Hoechst 33258 staining. The presence of apoptotic bodies was evidenced in a 14.9 ± 1.4% of cells after 24 h of exposure to 150 μM of *trans*-resveratrol.

Several studies have indicated that reactive oxygen species (ROS) production in mitochondria play a role in the initiation and execution of apoptosis [48]. HT-29 cells exposed to 150 μM of *trans*-resveratrol showed markedly increased levels of superoxide anion radicals in mitochondria after 4 hours of incubation. Consequently, the apoptotic effect of this polyphenol was mediated, in part, by the intrinsic pathway, through the production of superoxide anions in mitochondria prior to the initiation of the caspase pathway [42]. These results were in agreement with previous studies that pointed out superoxide anions as secondary messengers in apoptosis provoked by anticancer agents, such as paclitaxel and cisplatin [49,50]. In addition, *trans*-resveratrol was also reported to generate reactive oxygen species prior to the release of mitochondrial proteins to the cytosol, activation of effector caspase-3 and caspase-9, and induction of apoptosis in prostate cancer cells [51]. Reactive oxygen species production occurs in an early phase suggesting that the compound triggers a rapid release of cytochrome c from mitochondria into the cytosol that in turn activates procaspase-9 and the downstream effectors, including the pro-caspases -3, -6, and -7, followed finally by the cleavage of proteins and DNA that characterize the final phase of apoptosis.

Two principal pathways of apoptosis have been described, such as mitochondria-mediated intrinsic pathway and death receptor-induced extrinsic pathway [47]. However, it has been suggested that lysosomes and the endoplasmic reticulum also play important roles in programmed cell death [52]. In HT-29 cells, *trans*-resveratrol also triggers apoptosis through lysosome and demonstrates a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which lysosomal cathepsin D acts upstream of caspase activation [53]. Furthermore, this polyphenol has been reported to promote apoptosis through the endosplasmic reticulum (ER) [54] and the induction of CHOP/GADD153 gene expression, which has been recognized as a proapoptosis gene [55].

The potential cancer chemopreventive activity of *trans*-resveratrol *in vivo* was evaluated in rats that received an intraperitoneal injection of 1,2-dimethylhydrazine (DMH) [19]. The development of carcinogenesis in the DMH rat model takes place through a multistep process as it does in humans, and allowed the measurement of aberrant crypt foci (ACF) and mucin-depleted foci (MDF) as valid early preneoplastic markers. ACF have been identified in humans at high risk and are widely used as a surrogate marker of colon cancer [56]. Prior to the performance of the study the experimental conditions were validated. Three subcutaneous injections of DMH (20 mg/kg, one week apart) followed by an observation period of four weeks proved to be appropriate for the screening of potentially chemopreventive agents [19]. *trans*-Resveratrol was administered at a dose 60 mg/kg since it was regarded as a potential nutraceutical and not in any case as an effect encountered after a long period of consumption of this polyphenol in the diet. *trans*-Resveratrol reduced the number of preneoplastic lesions, since ACF were inhibited by 52%, and the total number of aberrant crypts (AC) by 50% [19]. In addition, MDF were described in carcinogen-treated rodents [57] and in humans at high risk of colon cancer [56]. MDF are characterized by harboring mutations that show Wnt signaling activation like in colon tumors, suggesting that these lesions are precancerous [56]. *trans*-Resveratrol oral administration for 49 days reduced the number of MDF by 50%, thus remarking the protecting activity exerted by this compound in the colon mucosa. Given that *trans*-resveratrol was administered one week prior to the first exposure to DMH, the results demonstrated that *trans*-resveratrol acts as an efficient agent inhibiting cancer initiation [19].

The potential cancer chemopreventive activity of *trans*-resveratrol *in vivo* was examined previously in long-term studies [45,58-61]. The effect of *trans*-resveratrol on azoxymethane-induced colon carcinogenesis was assessed in F344 rats. This phytochemical was administered in drinking water at a dose of 200 μg/kg for 100 day, beginning 10 days before administration of the

carcinogen [59]. *trans*-Resveratrol reduced the growth of colorectal ACF modulating the expression of bax and p21, both involved in the regulation of cell proliferation and apoptosis [59]. Sengottuvelan et al. also assessed the anticarcinogenic activity of 8 mg/kg of *trans*-resveratrol in a model of colon carcinogenesis induced by DMH, but these were long term experiments that lasted 30 weeks [58,60]. Their results showed that in rats, *trans*-resveratrol markedly reduced the number of 1,2-dimethylhydrazine–induced aberrant crypt foci and incidence and size of tumors, possibly through the modulation of antioxidant defense status and activities of carcinogen-detoxifying enzymes [58,60]. In ApcMin mice, which are a model of human familial adenomatous polyposis, *trans*-resveratrol administered in drinking water at a dose of 15 mg/kg for 7 weeks prevented the formation of colon tumors and reduced the formation of small intestinal tumor by 70% by down-regulating genes that are directly involved in cell cycle progression such as cyclin D_1, D_2 and DP-1, and in the inhibition of the carcinogenic process and tumor expansion [45]. In the same animal model, *trans*-resveratrol (0.2% in the diet) decreased the number of adenomas, which was associated with inhibition of COX enzymes and interference with prostaglandin E_2 (PGE_2) generation [62]. In contrast, *trans*-resveratrol administered in the diet at 0, 4, 20, or 90 mg/kg body weight for 7 weeks did not affect intestinal tumorigenesis or COX-2 expression in ApcMin/+ mice [61].

6.2. Spermatogenesis

The mammalian testis fulfills two main functions, namely, the synthesis of steroid hormones and the production of spermatozoa, which are controlled by gonadotrophins and testosterone together with locally produced factors [63]. The repeated oral administration of 20 mg/kg of *trans*-resveratrol during 90 days enhanced both functions. Sperm counts were significantly greater in the resveratrol treated rats ($24.8 \pm 3.30 \cdot 10^7$) than in the control group ($14.1 \pm 0.80 \cdot 10^7$) [64]. The experimental data showed that the repeated oral administration of *trans*-resveratrol induced a 71% decrease in the mean diameter of the seminiferous tubules with a 100% increase in the testicular tubular density. Taken together, these changes result in an overall increase in the size of the spermatogenic tissue. Therefore, this enlargement would be in the basis of the observed increase of sperm production. It is noteworthy that sperm collected from the epididymis showed a correct maturation since the morphological examination evidenced the same percentage of abnormalities between groups. As a conclusion, these results suggest that the *trans*-resveratrol-induced increment in total sperm content would be due more to an enlargement in the overall tissue that produces mature sperm cells than to an

increase in the rhythm of sperm production, which would remain unchanged with respect to the control animals [64].

Spermatogenesis depends on the delicate balance that constitutes the hypothalamic-pituitary-gonadal axis. The endocrine regulation of this axis in the male is intricate with a role for estradiol and testosterone, making obvious the complexity of the steroid-feedback mechanisms [65]. The sites for these feedback effects include cells in the hypothalamus, that are in close proximity to gonadotrophin-releasing hormone (GnRH) neurons, and gonadotrophins in the pituitary, that may respond directly to androgens due to the expression of the androgen receptor (AR), whereas the presence of aromatase and the estrogen receptor (ER) allows the conversion of androgens into estrogens, and the subsequent activation of ER signaling pathways [65]. The oral administration of 20 mg/kg of *trans*-resveratrol for 90 days also exerted a stimulatory effect on the secretion of gonadotrophins that are the major endocrine regulators of spermatogenesis [64]. The concentration of FSH, which acts within the tubules to stimulate spermatogenesis, and LH, that signals the production of testosterone in Leydig cells were both elevated in the resveratrol group with respect to the control rats. Testosterone, which is essential for promoting spermatogenesis, was also enhanced. These results seem to indicate that the effect of *trans*-resveratrol on sperm count was caused by the hypophisary stimulation of the testicular function.

A possible explanation to the described effect could be that *trans*-resveratrol binds ER as a mixed weak agonist/antagonist, without estrogenic properties [40]. The fact that *trans*-resveratrol lacks estrogenic activity could be confirmed by the absence of deleterious effects on testes, in opposition to the toxicity described for diethylstilbestrol (DES) [66]. Rats treated with DES, which is a structural analog of *trans*-resveratrol, and a potent estrogen agonist, showed reduction in testicular weight, impaired seminiferous tubular morphology and reduced testosterone concentration [66]. The distinct activity shown by two structurally similar compounds can be explained by subtle differences in both molecules. Compared to *trans*-resveratrol, DES lacks the 3-OH and 5-OH groups, but on the contrary possesses a 4-OH group and two additional ethyl groups. These features provide differential binding characteristics to ER [67]. DES holds similar affinity as estradiol to ER acting as a full potent agonist, thus accounting for the harmful effects described.

In addition, the daily oral administration of 20 mg/kg of *trans*-resveratrol for 90 days did not induce any differences in body weight, food and water consumption in the treated group with respect to the control rats [64]. Given that growth inhibition is a sensitive indicator of estrogenic effects [68], this lack of reduction in body weight in the treated rats substantiates that

trans-resveratrol does not act as an estrogen agonist, in agreement with other *in vivo* studies [69,70].

Consequently, *trans*-resveratrol could interact with ER, thus increasing the secretion of gonadotrophins which leads in turn to an increment in testosterone and sperm output. Furthermore, the effects described above could have been reinforced through androgen antagonism, since *trans*-resveratrol has also been revealed to antagonize androgen action in prostate cancer cells by inhibiting AR activity as well as by suppressing AR expression [71,72]. Besides the effect of *trans*-resveratrol on the hypothalamic-pituitary-gonadal axis, its antioxidant and anti-inflammatory activity also warrants attention. Taken as a whole, these actions could also account for the increase on sperm output observed in healthy rats. In Western society, infertility is a growing problem, the causes are diverse and considerable effort is being made to offer effective therapy. In the search for solutions, in the case of male infertility, antioxidants, anti-inflammatories, androgens and antiestrogens are among the treatments used. However, truly effective treatment has still to be found [73]. Therefore, the effect of *trans*-resveratrol on spermatogenesis merits further research since this compound may be useful in the treatment of male infertility.

7. Conclusions

In the present review we summarize the current evidence about the role of ABC transporters both in the absorption and distribution of *trans*-resveratrol that contribute to the low bioavailability of this compound. Moreover, we provide evidence that *trans*-resveratrol, as a pharmacological agent, has an important effect in the prevention of colon cancer as well as in masculine infertility. The wide spectrum of this compound may reflect its simultaneous action on multiple molecular targets.

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