RESEARCH ARTICLE

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Effects of Grape Juice in Superoxide Dismutase and Catalase in Colorectal Cancer Carcinogenesis Induced by Azoxymethane

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Abstract

Background: The intestinal mucosa is commonly exposed to oxidant nutrients and carcinogens, which can lead to the generation of free radicals. The antioxidants present in the diet assume great importance as possible protective agents, reducing the oxidative damage. In this way, we evaluated the antioxidant action of grape juice on preneoplastic lesions induced by azoxymethane (AOM) in Wistar rats. **Methods:** The colorectal carcinogenesis was induced by two intraperitoneal injections of 15mg/kg of AOM in Wistar rats. The animals were divided in 7 groups and treated with 1 and 2% concentrations of grape juice before and after carcinogen administration. After euthanasia, the expression of antioxidant enzymes catalase (CAT), copper-zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) CAT, SOD1 and SOD2 were evaluated by immunohistochemistry. **Results:** AOM decreased the expression of CAT and Mn-SOD enzymes, but not for Cu/Zn-SOD. We observed an increase expression of CAT and Mn-SOD after grape juice administration in some concentrations according to the time of administration of the grape juice before the carcinogen or just after the carcinogen. **Conclusion:** Our results suggest an independent action of each enzyme and a possible antioxidant action of the grape juice components in the diet being able to balance the body to neutralize the superoxide radicals and not leave them in the cell-damaging form.

Keywords: Colorectal cancer- aberrant crypt foci- grape- antioxidants- free radicals

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Introduction

Colorectal cancer (CRC) is one of the main causes of cancer in the world and in Brazil. According to the National Institute of Cancer (INCA) for 2018 was estimated 36.360 new cases in Brazil (18.980 in women and 17.380 in men) (INCA, 2018). Several acquired and hereditary risk factors have been involved in cancer development. Important role is attributed to chronic exposure of the intestinal mucosa to carcinogens, causing lesions that may progress to cancer (INCA, 2018). It has been well established that the carcinogenesis of CRC is mediated by a sequence of mutations in the cell cycle control genes implicated on proliferation, differentiation, adhesion and apoptosis. Recent studies have shown that hypermethylation in the promoter region of some genes and oxidative damage to DNA (oxidative stress) represents the main mechanisms related to the initial stages of CRC carcinogenesis (Ribeiro et al., 2007).

Maintaining the balance between the production of free radicals and antioxidant defenses (enzymes and non-enzymatic molecules) is an essential condition for the normal functioning of the organism. Oxidative stress is a condition of imbalance, which can damage cellular lipids and DNA. Free radicals are produced during normal cell functioning, mostly in the form of reactive oxygen species (ROS) and nitrogen (RNs) (Roessner et al., 2008). Recent evidence suggests that the generation of ROS can play an important role in all stages of carcinogenesis (Fukumura et al., 2006).

The intestinal mucosa is commonly exposed to oxidant nutrients and carcinogens, which can lead to the generation of free radicals. The production of these species over a long period results in cells and tissues lesions and, consequently, a persistent inflammation. This inflammatory and oxidative environment favors an increase in the production of hydroperoxides, which can damage healthy cells. After a long time, this mechanism may lead to stimulate carcinogenesis (Valko et al., 2007; Guz et al., 2008).

The antioxidant defense system is made up of numerous molecules that work together and are classified in three forms. The primary superoxides: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx),

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glutathione reductase (GR). The secondary: vitamin E, vitamin C, uric acid, beta-carotene, bilirubin and albumin and the tertiary: biomolecules damaged by free radicals, cell defense elements (Fukumura et al., 2006).

In humans, SOD is known to be in at least 2 forms: the copper-zinc superoxide dismutase (Cu/Zn-SOD), present in the cellular cytoplasm and the manganese superoxide dismutase (Mn-SOD), present in the mitochondrial matrix (Holley et al., 2011; Pisoschi and Pop, 2015). Catalase, which is present in peroxisomes, is an enzyme that plays an important role in controlling the concentration of hydrogen peroxide in human cells, converting H_2O_2 into H_2O and O to protect cells and prevent oxidative damage. Both molecules limit the oxidizing effect on tissues and are activated during cell injury. They act together to eliminate ROS.

Antioxidants can be found in foods, especially in nutrients with phenolic compounds and flavonoids (Federico et al., 2007). In this perspective, the antioxidants present in the diet (seeds, cereals, vegetables, fruits, leaves, roots, spices and herbs) assume great importance as possible protective agents, reducing the oxidative damage and may be involved in reducing the risk of disease, such as cancer (Martinet al., 2007; Gerhauser 2008; Peng et al., 2014).

Grapes (*Vittis sp.*) are considered one of the major sources of phenolic compounds when compared to other fruits and vegetables (Ogawa et al., 2012). The phenolic compounds of the grapes are classified in flavonoids and non-flavonoids. In the first group are flavanols (catechin, epicatechin and epigallocatechin), flavonoids (caempferol, quercetin and myricetin) and anthocyanins. The non-flavonoids group is represented by the phenolic acids, hydroxybenzoic and hydroxycinnamic acids (Padidar et al., 2012).

The varieties of red grapes (*Vitis labrusca L.*) are widely cultivated in North and South America, and in Brazil, represent more than 80% of processed grapes, mainly destined for grape juice production, mostly of the Concord variety (Nixdorf and Hermosín-Gutiérrez, 2010; Aguiar et al., 2011, Toaldo et al., 2013). Generally, the main phenolic compounds presented in grape juices are anthocyanins, flavan-3-ols, flavonols, phenolic acids and resveratrol (Natividade et al., 2013) These compounds are associated with modulation of important physiological parameters, such as antioxidant protection, inhibition of platelet function and reduction of inflammatory biomarkers (Vitseva et al., 2005; Sharma et al., 2007; Dani et al., 2009, Nichols and Katiyar, 2010).

In a previous study, the supplementation with concentrate grape juice reduced the number of the aberrant crypt foci (ACF) and the mRNA expression of cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-kB) after injection of the carcinogen azoxymethane (AOM). COX-2, a glycoprotein that catalyzes the conversion of arachidonic acid to pro-inflammatory substances, such as prostaglandins, stimulates the growth of tumor cells and suppresses immune surveillance. COX-2 also activates carcinogens for more reactive substances that cause genetic damage as tumor necrosis factor alpha (TNF-α), NF-kB and inducible nitric oxide synthasae (iNOS). These results suggest that grape juice may be a preventive food in the process of carcinogenesis (Campanholoet al., 2015; Silva et al., 2015).

In this context, to complement the findings and to better understand the mechanism of action of concentrate juice, the present research proposes to evaluate the effectiveness of grape juice as a possible antioxidant, studying the immunoexpression of the enzymes CAT, Cu/Zn-SOD and Mn-SOD after the injection of AOM.

Materials and Methods

Ethic aspects

All procedures were in accordance with guidelines on animal experimentation and the Ethics Committee of UNIFESP-EPM approved the study (CEUA Nº 4187280114).

Experimental design

Forty male Wistar rats aged 5-6 weeks, provided by the Center for the Development of Experimental Models in Medicine and Biology (CEDEME), Universidade Federal de São Paulo - Escola Paulista de Medicina (UNIFESP/EPM), were divided in 7 groups (5-7 animals per group) as a follow: G1) Negative control group: treated with a single intraperitoneal injection of saline solution; G2) Positive control group: treated with two intraperitoneal injections of 15 mg/kg of AOM at an interval of one week; G3) AOM treated with 1% grape juice for 15 days before and after AOM injection; G4) AOM treated with 2% grape juice for 15 days before and after AOM injection; G5) AOM treated with 1% grape juice after 4 weeks of AOM injection; G6) AOM treated with 2% grape juice after 4 weeks of AOM injection and G7) only treatment with 2% grape juice.

Grape juice concentrate intake

The administered dose of grape juice was calculated based in an amount of polyphenols presents in four glasses (200 mL each) adjusted to the animal metabolism (twice as fasts as humans) (Golluckeet al., 2008). In this way, the animals from the groups 3-7 received, daily, by oral gavage 222 mg/ day (1% concentration) or 444 mg/day (2% concentration) of concentrate grape juice (G8000®). Supplied by Gold juice™ (Farroupilha, RS, Brazil), this product was obtained by nanofiltration of the juice from red grapes (Vitis labrusca, mostly of the Concord variety), with subsequent concentration at 68º Brix by evaporation. The chemical characterization of grape juice was previously published (Aguiar et al., 2011).

The animals were euthanized after 22 weeks. After euthanasia, the colon was removed and processed for histological and molecular analysis. The preliminary results have already been published (Campanholo et al., 2015; Silva et al., 2015).

Immunohistochemical expression

Paraffin blocks were used to analyze the immunoexpression of antioxidant enzymes. Sections of 3 μm were placed on silanized slides and conserved at 56-60 ºC for one night and then submitted to dewaxing

in xylol for 30 min. Antigen retrieval was performed in a steamer as a follow: in TrilogyTM (pH 9.0) buffer (Cell Marque, Sigma Aldrich) for catalase (20 min), in citrate buffer (pH 6.0) for Cu/Zn-SOD (40 min) and in EnVision FLEX HIGH (pH 9.0) buffer (DAKO, Glostrup, Denmark) for Mn-SOD (30 min). Endogenous peroxidase was blocked by the application of 3% hydrogen peroxide followed by washing in water and incubation in phosphate buffer (PBS) pH 7.4 for 30 min. The slides were incubated with rabbit polyclonal anti-catalase (Santa Cruz Biotechnology, Ltd, Santa Cruz, CA); rabbit polyclonal anti-Cu/Zn-SOD (Santa Cruz Biotechnology, Ltd, Santa Cruz, CA) and rabbit monoclonal anti-Mn-SOD (Santa Cruz Biotechnology, Ltd, Santa Cruz, CA) at 4ºC overnight. Subsequently, the slides were incubated with the secondary antibody post-primer (NovoLinkTM, Leica Biosystems, Newcastle Ltd, UK) for 30 min. After washing with PBS, the slides were incubated with the polymer from the same kit (30 min). The reaction was developed with 3,3'-diaminobenzidine (DAB Liquid-DAKO, Glostrup, Denmark) at room temperature. The sections were washed in distilled water, counterstained with Harry´s Hematoxylin and covered with Entellan resin.

The reactions were evaluated according to a previously described scoring system with staining intensity graded as negative (0), weak (1), moderate (2) and strong (3) , and positivity stained area as < 10% (0), 10-40% (1), 40-70% (2) and >70% (3). Total scores for grade and area classified as 3 or more were defined as positive and less than 3 as negative (Paiotti et al., 2007).

Statistical analyses

Values were expressed qualitatively as positive and negative and semiquantitatively as mean ± standard deviation. The One-Way analysis of variance (ANOVA), followed by Tukey´s test was used for CAT, Cu/Zn-SOD and Mn-SOD immunoexpression. Analysis was performed using the Graph Pad Prism software version 4.0. Differences were considered significant when the p value were less than 0.05.

Table 1. Percent of Colon Tissues with Positive Imunoexpression According to the Groups

Group	N	Catalase	Cu/Zn-SOD	Mn-SOD
		N (%)	N (%)	N (%)
G1	5	3 (60)	4 (80)	2 (40)
G2	4	3 (75)	4 (100)	2 (50)
G3	5	4 (80)	2 (40)	4 (80)
G4	5	0 (0)	5 (100)	5 (100)
G5	5	0 (0)	4 (100)	4 (80)
G6	5	3 (60)	4 (80)	5 (100)
G7	5	2 (40)	2 (40)	5 (100)

G1 – negative control group, G2 - positive control group, G3 - grape juice1% before and after azoximethane, G4 - grape juice 2% before and after azoximethane, G5 - grape juice 1% after azoximethane, G6 - grape juice 2% after azoximethane and G7 grape juice 2% control group.

Results

About the immunoexpression of antioxidant enzymes we noted heterogeneous results between the groups. As expected the immunoreactivity of CAT was lower in the positive control group (G2) compared to the negative control group (G1) or group treated only with 2% grape juice (G7), however these differences were not significant. On the other hand, a strong cytoplasmic immunoreactivity was verified in the animals treated with grape juice in the concentration of 1% before and after AOM injection (G3) when compared with the positive control group (G2) (p=0.039). In the same way, the G3 group also had a higher expression when compared to the other groups (G4 or G5, p=0.003) (Figure 1).Cu/Zn-SOD immunoreactivity was increased in the colon of the animals treated with 2% grape juice before and after AOM injection (G4) when compared to the others groups (p=0.012). However, the positive control group (G2) not demonstrated decrease in this expression (Figure 2).

Relative to Mn-SOD immunoreactivity, we observed significant difference between the groups. The positive control group had a less expression compared to the G7 (p=0.001), G4 (p=0.002), G6 (p=0.001), respectively. G3

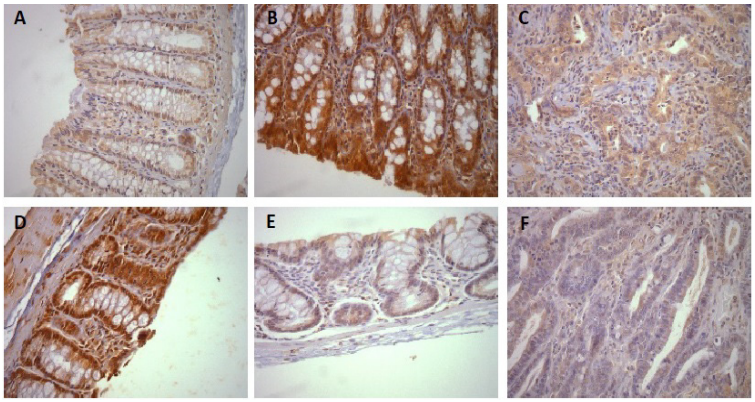

Figure 1. Cytoplasmic Immunostaining of Catalase (CAT). A) Negative control group (G1) - moderate immunostaining; B) Grape juice 2% control group (G7) -strong immunostaining; C) Positive control group (G2) – shown weak immunostaining in dysplasic area; D) Grape juice 1% before and after azoximethane (G3) - strong immunostaining; E) Grape juice 2% before and after azoximethane (G4) and F) Grape juice 2% after azoximethane (G6) - weak immunostaining in dysplasic area. 400X magnification.

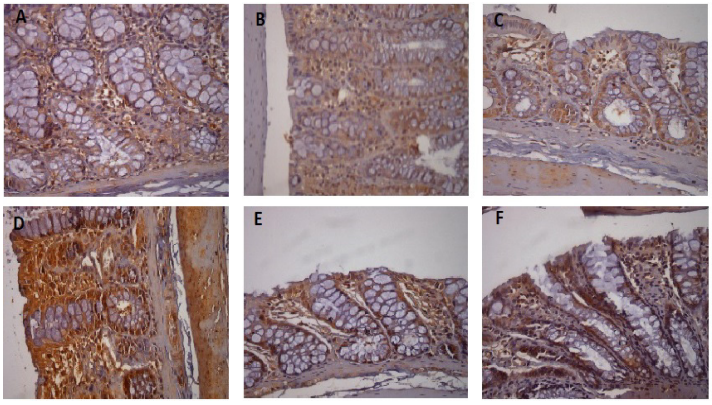

Figure 2. Cytoplasmic Immunostaining of Copper-zinc Superoxide Dismutase (Cu/Zn-SOD). A) Grape juice 2% control group (G7), B) Positive control group (G2) and C) Grape juice 1% before and after azoximethane (G3) - shown moderate immunostaining; D) Grape juice 2% before and after azoximethane (G4, E) Grape juice 1% after azoximethane (G5) and F) Grape juice 2% after azoximethane (G6) - strong immunostaining. 400X magnification.

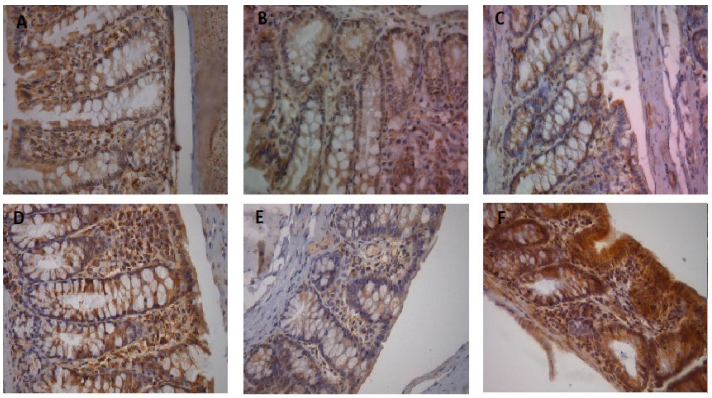

Figure 3. Cytoplasmic Immunostaining of Manganese Superoxide Dismutase (Mn-SOD). A) Negative control group (G1), B) Grape juice 2% control group (G7), C) Grape juice 1% before and after azoximethane (G3), D) Grape juice 2% before and after azoximethane (G4) and E) Grape juice 1% after azoximethane (G5) - shown moderate immunostaining; F) Grape juice 2% after azoximethane (G6) - shown strong immunostaining. 400X magnification.

had less expression than G4 (p=0.017) and G6 (p=0.007). G6 had a strong expression than G3 (p=0.012) and G5 (p=0.001). G6 had a strong expression compared to G5 (p=0.010) (Figure 3). The positivity frequency of each enzyme was summarized in Table 1 and the mean values in Table 2.

Table 2. Mean of the Scores of the Imunoexpression of the Enzymes CAT, Cu/Zn-SOD and Mn-SOD According to the Groups

	CAT	Cu/Zn-SOD	Mn-SOD
G1	5.24±3.56 [a]	6.60±3.91 [h]	4.20±4.38 [o]
G2	3.00±2.00 [c]	7.00±2.45 [j]	2.75±1.50 [q]
G3	7.60±3.13 [d]	2.80±2.28 [k]	3.80±2.49 [r]
G4	1.60±0.54 [e]	9.00±0.00 [l]	7,80±1.64 [s]
G5	1.80±0.05 [f]	8.00±2.23 [m]	2.20±3.89 [t]
G6	3.80±2.95 [g]	5.20±3.80 [n]	8.40±1.34 [u]
G7	4.40±4.27 [b]	2,40±1.67 [i]	8.40±1,34 [p]
P	0.032[b]	0.003	<0.001

c ≠ d (p=0.039), d ≠ e (p=0.003), d ≠ f (p=0.003), p ≠ q (p=0.001), q ≠ s (p=0.002), q ≠ u (p=0.001), r ≠s (p=0.017), r ≠u (p=0.007), t ≠u (p=0.012), t ≠ v (p=0.01) (ANOVA – Tukey´s test). Legends, G1 – negative control groupG2 – positive control group, G3 - grape juice 1% before and after azoximethane; G4 - grape juice 2% before and after azoximethane, G5 - grape juice 1% after azoximethane, G6 - grape juice 2% after azoximethane and G7 – grape juice 2% control group.

Discussion

The worldwide increase in the incidence of chronic diseases such as cancer has aroused interest in bioactive components that can prevent these diseases. Several studies have demonstrated the benefits of rich diet in natural foods. The consumption of fruit flavonoids is associated with a reduction in the incidence of neoplasms, diabetes and cardiovascular diseases (Feskanich et al., 2000; Knektet al., 2000; Knektet al., 2002; Zhou and Raffoul 2012).

The compounds present in fruits have a potential antioxidant effect in the organisms and even if present in lower concentrations, have the capacity to decrease or inhibit the action of free radicals (Sies 1993; Maxwell 1995). Thus, the immunoexpression of the CAT, Cu/Zn-SOD and Mn-SOD enzymes in the antioxidant balance x free radicals in preneoplastic lesions induced by AOM was evaluated in the animals treated with concentrate grape juice.

The AOM decreased CAT expression; however this difference was not significant. CAT immunoexpression increased in the group treated with grape juice 1% before and after AOM compared to the other groups supplemented with grape juice before and after at a concentration of 2% or only after AOM at a concentration of 1%. CAT is an important enzyme in response of oxidative stress by catalyzing the hydrolysis reaction of hydrogen peroxide molecules in water and oxygen (Da Costa et al., 2012). In this way, it can be suggest a possible protective role of grape juice treatment.

In a previous study (Campanholo et al., 2015), the grape juice treated groups presented a decrease in the number of aberrant crypts when compared to the AOM positive control group. In addition, the up-regulation of COX-2 was to verified in the groups that received AOM (Silva et al., 2015). It is known that COX-2 can be favors the growth of malignant cells by inhibiting apoptosis and promoting angiogenesis, besides being present in situations of inflammatory nature (Nzeako et al., 2002; Sharmaet al., 2003; Liu et al., 2015).

Curiously, the AOM administration did not decreased the Cu/Zn-SOD immunoexpression in the positive control group. In addition, the findings of previous study conducted demonstrated an increase of NF-kB mRNA in this same group (Campanholo et al., 2015). These events are supported in the literature. Increased stress can trigger events that lead to the import of Cu/Zn-SOD from the cytoplasm and into the nucleus (Zlatković and Filipović, 2011). A majority of inducible Cu/Zn-SOD expression is facilitated by the binding of NF-kB (Miao and St Clair 2009). However, there was a significant difference on the Cu/Zn-SOD immunoexpression in the animals treated with grape juice before and after the administration with AOM.

On the other hand, the Mn-SOD immunoexpression decreased in the positive control group and were again expressed in the groups treated with grape juice 2% before and after AOM or only after AOM. These results suggest that higher concentration of resveratrol, a component of grape juice, acting on free radicals and defending the organism against possible inflammation (Hudsonet al., 2007). Mn-SOD is a mitochondrial antioxidant enzyme encoded in the nucleus, it is essential for the removal of superoxide radicals. Under normal physiological conditions, the superoxide radicals in the mitochondria are rapidly converted by Mn-SOD, which together with CAT and/or GPx (Dhar et al., 2011). It has been shown that oxidative stress is harmful to cells and in particular to DNA in neurodegenerative diseases, diabetes and cancers. Research corroborates the benefits of antioxidants in cancer development. Another study suggest that oncogenes, such as RAS can inactivate Mn-SOD (Papa et al., 2014). Was reported that the activity of SODs tends to decrease in the early stages of the neoplasm and stabilize in the progression phase, in melanoma (Dhar et al., 2011).

It has been reported that the polyphenol content and profile of grape juice is substantially dependent upon the variety from which it is prepared, though environmental factors and methods of cultivation can affect both (Blumberg et al., 2015). In addition, the activity of antioxidants depends on the dose administered, the type of cancer and the time of exposure to the flavonoid or to the carcinogen (Skibola and Smith 2000). Our data, demonstrated an increase of enzymes expression in some concentrations according to the time of grape juice administration before or just after the carcinogen.

In summary, our results suggest an independent action of each enzyme and a possible antioxidant action of the grape juice components in the diet being able to balance the body to neutralize the superoxide radicals and not leave them in the cell-damaging form.

Confict of Interest

The authors declare no conflict of interest.

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