Alcohol hinders the Anti-Carcinogenic effect of Resveratrol in the Colon of rats

O álcool impede o efeito Anticancerígeno do Resveratrol no Côlon de ratos

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ABSTRACT
Resveratrol (RESV), a polyphenol present in wine and other natural sources, has potent anti-oxidant and chemopreventive effect against various types of cancer by multiple mechanisms of action. On the other hand, ethanol, which is also one important component of wine, is associated with increased oxidative stress and risk of cancer in general, including the colonic cancer. It is unknown whether the anti-oxidant and anti-carcinogenic effects of RESV could be counterbalanced by alcohol in vivo. Thus, the present study evaluated the anti-neoplastic action of RESV, pure or associated with ethanol on the colon carcinogenesis induced by N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) in rats. It was performed a detailed analysis of aberrant crypt foci (ACF) formation and the systemic oxidative stress through malondialdehyde (MDA) dosage. The inflammatory process, which is associated to the colonic carcinogenesis, was estimated by the immune-expression of cyclooxygenase-2 (COX-2). Our experiments were performed in 32 wistar rats, divided into four groups: the group C was the control, the group M received MNNG (5mg/ml), the group MR received MNNG and RESV (1mg/kg/day)
and the group MRA received RESV and alcohol (0.377g/kg/day). As a result, RESV decreased the number of ACF and COX-2 expression, but these effects were abolished by the alcohol. RESV reduced the production of MDA in all treated groups, evidencing that its systemic antioxidant effect was also present in the animals treated with alcohol. It was concluded that alcohol hindered the anti-carcinogenic effects of RESV. This effect of alcohol should be attributed to its pro-inflammatory effects, which have avoided the anti-inflammatory properties of RESV.

Keywords: Colon Carcinogenesis, colon cancer, resveratrol, alcohol.

1 INTRODUCTION

Wine is largely consumed around the world and there is a wide spread concept that it may be beneficial to human health (Soleas et al., 1997). Most of the eventual healthy effects of wine have been attributed to the resveratrol (3,4,5-tri-hidroxi-trans-estilben), which is an aromatic fitoalexin found mainly in the skin of grapes and is present in high concentrations in red wine (Delmas et al., 2003). It has been observed that RESV potently inhibits the carcinogenesis process in
general (Athar et al., 2007; Sharmila et al., 2007; Cai et al, 2015) and in the colon in experimental animals (Schneider et al., 2001). However, alcohol, which is one of the most important components of wine, is associated to increased oxidative stress and risk of cancer in general (Brown, 2005; Sarich et al., 2021), including the experimental colonic cancer (Hayashi et al., 2007). The association between moderate or high alcohol intake and colonic cancer has been clearly established, both in humans (Longnecker et al., 1990; Cho et al., 2004; O'Sullivan et al., 2022) and in experimental animals (Cardoso et al., 2011).

Various mechanisms may influence both the pro-carcinogenic effect of alcohol and the protective effects of RESV, what makes it necessary to evaluate in vivo the combination of this two major components of wine.

Our objective was to verify in vivo the effects of RESV, combined or not with alcohol, on the well-known carcinogenesis marker: the formation of aberrant crypt foci (ACF) induced by a chemical carcinogen. The N-methyl-N-nitro-N-nitrosoguanidine (MNNG) was chosen for this aim, because it is a direct carcinogen (Che et al., 2010) and is not metabolized in liver, which would be a bias, since the liver function is affected by the alcohol (Perse & Cerar, 2007). The colonic carcinogenesis process was estimated by the counting of aberrant crypt foci (ACF) in the colonic mucosa. In sequence, we evaluated whether RESV was able to counterbalance the pro-oxidant and the pro-inflammatory effects of alcohol by evaluating, respectively, the peroxidation marker malondialdehyde (MDA) (Marnett, 1999) and the inflammatory enzyme cyclo-oxygenase 2 (COX-2), as previously reported (Cardoso et al., 2011).

2 MATERIAL AND METHODS

Thirty-two male Wistar rats from the central bioterrorium of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP) were used in the experiment. The animals were 7 weeks old, weighed approximately 225g at the beginning of the experiment and were randomly divided into 4 groups (n=8). The animals were fed with a standard Purine® diet for rats, with approximate composition (g/100g): protein 21.0; fat 3.5; carbohydrates 60.0; exit 5.3; Vitamin 20 and ash (data provided by the producer), which provides approximately 3.5 kcal/g (data provided by the producer). Water was offered ad libitum.
Apart from the control group (C), the other groups received 4 successive intrarectal deposits of the carcinogen MNNG (Sigma-Aldrich, Steinheim, Germany), 2 times/week for 2 weeks. Each injection consisted of 0.5 ml of MNNG (5 mg/ml) diluted in distilled water, applied 7 cm from the anal margin by using a bulb-tip needle, accordingly to the established protocol (Cardoso et al 2011). In the control group, it was applied distilled water in equal volume and repetitions using the same procedure. The group MR also received resveratrol (1mg/kg) and the group MRA received MNNG, RESV and alcohol (0,377g/kg/day). The control animals received saline in the same volume. The treatments were administered by gastric gavage, once a day for four weeks, starting four days after the last application of the carcinogen. The treatments were carried out in the late afternoon so as not to disturb the sleep of the rodents, which have nocturnal habits. Rats were housed in a number of four per box. All animals were weighed at the beginning and at the end of the project. All rats were euthanized at 13 weeks (91 days) of life, 4 weeks after the start of treatment, a period sufficient for the appearance of aberrant crypts in the animals treated with MNNG (Cardoso et al 2011).

2.1 TISSUE COLLECTION AND PROCESSING

Blood and plasma were collected immediately after the animals were euthanized with a high dose of anesthesia. Blood was withdrawn by cardiac puncture, collected through 5.0 ml yellow BD Vacutainer SST II Advance® tubes. The material was centrifuged at 7000 rpm for 10 minutes. Serum was collected and stored in Eppendorfs® tubes and frozen in a freezer at -70°C for a posteriori analysis. The liver was removed and a 200mg sample was separated into 2ml Axygen® tubes for analysis of the biochemical parameters of oxidative stress. The colons were carefully isolated and removed, washed in saline and opened longitudinally along the mesenteric border. Approximately 10 centimeters of the distal colon were spread on cardboard plates with the mucosa facing up, protected with tissue paper and submerged in 10% buffered formalin for a minimum period of 24 hours. After this period, the tissues were removed from the formalin, cut and processed.
2.2 HISTOPATHOLOGICAL PROCEDURES

After staining with Hematoxylin and Eosin, an analysis of the mucosa of the distal portion of the colon was performed by microscopy using a 40X objective magnifying lens, where the AFC's were identified, qualified and the frequency per unit area in cm² was calculated.

Once cut, the pieces underwent a dehydration process by immersion in alcohols of increasing concentrations (70%, 80%, 90%, 100%) for a total period of approximately 5 hours. Subsequently, for diaphanization of the material, they were submerged in 100% xylene baths for approximately 1 hour and 20 minutes. The samples were submerged in liquid paraffin baths at a temperature of 60°C for a period of 4 hours, followed by inclusion in blocks, where part of the sample was included horizontally with the mucosa down, and vertically to obtain longitudinal and transverse sections respectively. The blocks were cut in a microtome to obtain cuts with 5μm thickness; later they were mounted on slides. Slides were stained with Harris Hematoxylin for 3 minutes and counterstained with alcoholic Eosin Phloxin solution for 10 seconds. For subsequent dehydration, the slide was submerged in increasing concentrations of alcohols and then diaphanized in xylene for mounting the slides. Immunohistochemistry for detection of the expression of COX-2 was performed as described elsewhere (Cardoso et al., 2011).

2.3 BIOCHEMICAL ANALYSIS FOR MDA DOSAGES

An amount of 0.5 ml of serum was used to analyze the concentration of MDA, to which 1 ml of the TBA-TCA-HCl solution was added and heated for 15 minutes in a boiling water bath. After cooling, the samples were centrifuged for 10 minutes at 3000 rpm at room temperature. The supernatant was collected and used for reading in a spectrophotometer at 535 nm.

2.4 STATISTICAL ANALYSIS

The results were submitted to statistical analysis by using the Graph Pad Prism 5.0 software (San Diego, California). To verify the differences between the means of the groups for each dependent variable, Analysis of Variance was used in one way (One-way) - ANOVA. We adopted a significance level of 0.05; and the
mean was given +/- standard error of the mean. When there was a difference between the means (p < 0.05), Tukey’s (One-way) post-test was used.

3 RESULTS

No animal presented signs of disease or died and there was no statistically significant difference in the weight gain among the groups during the experimental period.

3.1 ANALYSIS OF THE CARCINOGENESIS PARAMETERS

The histopathological analysis showed the presence of ACF (as shown in Figure 1) in the colonic mucosa of animals treated with MNNG. The treatment with RESV markedly reduced the number of ACF. However, the animals simultaneous treated with alcohol had a similar number of ACF when compared to the animals treated only with MNNG (Figure 2).

Figure 1 – H&E histology of one aberrant crypt. (Black arrow)
Figure 2 – Graphic of ACF quantification - shows the mean counts of ACF's in the groups exposed to the carcinogen. There was a significant reduction in the total number of ACF's in the group treated with resveratrol (MR) when compared to the group exposed to the carcinogen MNNG without treatment (M). The group treated with MNNG + resveratrol + alcohol (MRA) did not show a statistically difference when compared to the M group. One-way ANOVA P<0.0001. Tukey's post-test. ****p <0,0001 “M vs MR”, “M vs MRA”; **p=0,0029”.

3.2 OXIDATIVE STRESS

The RESV developed a potent systemic antioxidant effect, evaluated by the analysis of the MDA levels in the liver of the rats. This anti-oxidant effect was also present in the animals treated with alcohol (Figure 3).

Figure 3 – Graphic of Oxidative Stress – RESV was able to reduce oxidative stress in both groups treated with MNNG and resveratrol only (MR) or resveratrol associated with alcohol (MRA), demonstrating its antioxidant effect. One-Way ANOVA p<0,0001. Tukey's post test; ”M vs MR” ** p=0,0016; “M vs MRA” ** p=0,0030; “MR vs MRA” p=0,8058.
3.2 CYCLO-OXYGENASE 2

As shown in figure 4, the MNNG induced a marked increase in the COX-2 expression, both in epithelial and in stromal cells of the colonic crypts. The treatment with RESV avoided this increase in COX-2 expression, but not in the animals also treated with alcohol (Figures 5 and 6).

Figure 4 – Immunohistochemistry for detection of cyclo-oxygenase 2 (COX-2). A) Colon of control animals with no treatment. B) Rat colon with marked expression of COX-2, both in the stromal (white arrow) and in epithelial cells (black arrow), at the base of a colonic crypt. Magnification: 400X
Figure 5 – Graphic of COX-2 expression in colonic epithelial Cells – shows the mean of percentage of cells stained in epithelial cells by crypt. There was a significant reduction in the production of COX-2 in the group treated with resveratrol alone in comparison to the groups M and MRA. One-way ANOVA p<0.0001. Tukey’s post-test **** p<0.0001 “M vs MR” and “MR vs MRA”; p=0.3703 “M vs MRA”.

![COX2 Epithelial Graph](image1)

Figure 6 – Graphic of COX-2 expression in colonic stromal cells – shows the mean of percentage of cells stained with COX-2 in stromal cells by crypt. There was a significant reduction in the production of COX-2 in the group treated with resveratrol alone in comparison to the groups M and MRA. Also, alcohol seems to enhance inflammation as showed in the group MRA. One-way ANOVA p<0.0001. Tukey’s post-test **** p<0.0001 “C vs M”, “C vs MRA”, “M vs MR” and “MR vs MRA”; **p=0.0015 “M vs MRA”; p=0.2355 “C vs MR”.

![COX2 - Stromal Graph](image2)
4 DISCUSSION

The present study was designed specifically to verify whether the well-described beneficial effects of RESV against colonic carcinogenesis could counterbalance the pro-carcinogenic effects of alcohol in experimental settings. First, we have confirmed that the RESV treatment reduced the incidence of ACF induced by the carcinogen MNNG, corroborating the literature data regarding to its anti-carcinogenic properties (Jang et al., 1997; Schneider et al., 2001). In fact, there is a large body of experimental evidence that RESV possesses chemopreventive effects against the cancer development, including inhibition of chemically-induced colonic carcinogenesis (Tessitore et al., 2000). On the other hand, the literature clearly shows that alcohol stimulates human (Park et al., 2009) and experimental colonic carcinogenesis (Niwa et al., 1991; Hayashi et al., 2007). Unprecedentedly, here we have observed that RESV was not able to reduce the formation of ACF in the animals that also received alcohol. In order to gain insights on the mechanisms involved in this finding, we have evaluated two major modulators of carcinogenesis that are related both to RESV and to the alcohol consumption: the oxidative and the inflammatory damages (Reuter et al., 2010).

We have observed a marked fall in the liver MDA levels, confirming the previously described systemic anti-oxidant properties of RESV (Izzo et al., 2021). Interestingly, this effect was present even when alcohol was also administrated to the rats, as shown in figure 3. This means that the anti-oxidant properties of RESV surpassed the pro-oxidant ones from alcohol in our experimental model, indicating that oxidative stress may not explain the lack of protection against carcinogenesis in the animals that received alcohol in the experimental conditions of this study.

We have observed a marked increase of COX-2 expression in the animals treated with MNNG. Inflammation and COX-2 expression have been directly linked to the ACF formation and colonic carcinogenesis (Mariani et al., 2009). Furthermore, we found that RESV inhibited the increase of the COX-2 expression, induced by MNNG. This finding could be expected because it had been previously reported that RESV suppresses the human colorectal cancer through inhibition of COX-2 expression (Gong et al., 2017). However, differently from the findings regarding the anti-oxidant properties of RESV, we have observed that RESV did not reduce the COX-2 expression in the MNNG-treated animals that received...
alcohol. This indicates that the anti-inflammatory effect of RESV was not able to counterbalance the pro-inflammatory effects of alcohol.

In summary, we have found that alcohol hindered the anti-carcinogenic effects of RESV in the colon and that possibly this was due to the pro-inflammatory effects of alcohol, which have avoided the anti-inflammatory properties of RESV accessed by the evaluation of COX-2 expression. These findings may be useful for the design of further studies regarding the balance between anti and pro-carcinogenic components in the human diet.
REFERENCES


