Chemoprevention of metaplasia initiation and carcinogenic progression to esophageal adenocarcinoma by resveratrol supplementation

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Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol found in the skin of the grape and red wine, has been found to have chemopreventitive effects in some carcinogenic models. The effects of resveratrol on the initiation of Barrett's metaplasia and the carcinogenic progression to esophageal adenocarcinoma have not been evaluated. The aim of this study was to evaluate the effects of resveratrol on the transition from reflux esophagitis to Barrett's metaplasia to dysplasia to esophageal adenocarcinoma in an established rat model. Male Sprague–Dawley rats underwent esophagoduodenal anastomosis as per institutional approved protocol. They were then treated twice weekly with intraperitoneal injection of 7 mg/kg of resveratrol or saline. Additional nonoperated rats served as controls. The rats in each group were assigned to 1, 3, or 5-month subgroups. The distal esophagus was preserved for blinded histopathological examination at the time of harvest. Thirty-one animals in the 5-month resveratrol group showed a decreased severity of esophagitis ($P<0.0001$), incidence of intestinal metaplasia ($P=0.3567$), and incidence of carcinoma ($P=0.4590$) as compared with both the saline and nonoperated control groups. In conclusion, morphological characteristics consistent with decreased esophagitis and incidences of metaplasia and esophageal adenocarcinoma were seen on histopathology in the resveratrol group. Resveratrol resulted in a small diminution of the carcinogenic effects and progression to metaplasia, and further human studies are designed to explore the potential anticarcinogenic mechanism.

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Introduction

In the United States population, esophageal cancer occurs as one of two histiotypes: squamous cell carcinoma (SCC) or adenocarcinoma (AC), historically with a preponderance of SCC. Recently, however, that distribution has begun to change, with the morbid obesity epidemic and associated gastroesophageal reflux disease, and now AC is as common if not more common [1]. Other Western countries, such as France and Italy, have reported rates of esophageal adenocarcinoma (EAC) that are lower than those seen in the United States [2]. Although Western, these countries traditionally have different lifestyles and social customs. Specifically, there is not only an increased rate of wine production in each country as compared with the United States, but also, and more importantly, an increased rate of wine consumption per capita.

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol found in the skin of the grape and red wine, has been found to have chemopreventitive effects in some carcinogenic models. It seemed that reseveratrol most likely exerts these effects through modulation of oxidative stress [3]. Studies have examined the role of resveratrol as a chemopreventative agent for several types of cancers [4] as well as heart disease [5] and have noted beneficial effects in terms of decreased oxidative stress and decreased initiation or progression of disease.

The accepted pathway for the transition from normal esophageal epithelium to invasive AC begins with an inciting event, most commonly gastroesophageal reflux disease. Subsequent to this, it is thought that there is an orderly progression to intestinal metaplasia (Barrett's esophagus), dysplasia, in-situ carcinoma, and finally invasive carcinoma [6]. These neoplastic changes seem to be mediated by oxidative stress and inflammation. Therefore, a substance that prevents this inflammation may ultimately inhibit carcinogenesis. Given the observed differences in the rates of esophageal AC between countries with very different rates of wine consumption, a surrogate for resveratrol intake, it is postulated that resveratrol may exert chemopreventative effects for the initiation of pathways leading to EAC. However, the effects of resveratrol on the initiation of Barrett's metaplasia and the carcinogenic progression to EAC have not been evaluated. The aim of this study was to evaluate the effects of resveratrol on the transition from reflux

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esophagitis to Barrett's metaplasia to dysplasia to EAC in an established rat model.

Methods

Animals and treatment

This study was approved by the Institutional Animal Care and Use Committee at the University of Louisville. Eight-week old Sprague–Dawley rats (Harlan, Indianapolis, Indiana, USA) were housed three per cage, given commercial rat chow and tap water, and maintained on a 12-h light/dark cycle. They were allowed to acclimatize for 2 weeks before surgery. Solid food was withdrawn 1 day before and 1 day after surgery. Esophagoduodenal anastomosis (EDA) is an established model for the initiation of EAC pathways. EDA was performed on rats according to the operating procedure described previously [7]. In brief, the animals were anesthetized with 60 mg/kg sodium pentobarbital, using the pedal reflex to monitor the depth of anesthesia. The gastroesophageal junction was ligated flush with stomach and the distal esophagus transected proximal to the ligature. A duodenal enterotomy (2 mm) was made 1 cm distal to the pylorus on the antimesenteric border. The distal esophagus was then anastomosed to the duodenal enterotomy with accurate mucosal to mucosal opposition using a two-layer technique. Postoperatively, the rats were given water after 2 h and rat chow the following day.

Following a postoperative recuperative period of 1 week, the rates were dosed with 7 mg/kg of resveratrol (Sigma-Aldrich, St. Louis, Missouri, USA) weekly divided into two doses. The resveratrol was dissolved in 100% ethanol, and the solution was administered through intraperitoneal (i.p.) injection. Rats in the control arm of the study underwent EDA and were subsequently dosed twice weekly with an identical volume of saline. Additional nonoperative rats were maintained as controls for baseline histology and were not dosed. The animals were weighed weekly. Rats were euthanized after 1, 3, and 5 months of treatment as per our previous publication showing the optimal timing for esophagitis, Barrett's, and EAC [7,8]. The entire esophagi were collected and examined for macroscopic and microscopic changes of esophagitis, intestinal metaplasia, or EAC, as well as markers of oxidative stress.

Histopathology

The entire esophagus was removed and opened longitudinally to examine for evidence of gross abnormalities. Samples of esophageal tissue (0.5 cm in length) were taken from the distal part of the esophagus (at the level of the EDA) and fixed in 10% buffered formalin for 24 h and transferred into 80% ethanol. The formalin-fixed esophagus was embedded in paraffin. Serial sections of 5-μm were mounted onto glass slides for histopathological and immunohistochemical analyses. Hematoxylin and eosin-stained slides were obtained for each rat. The slides were reviewed by an independent pathologist blinded to the individual animal's grouping. Evidence for reflux esophagitis was identified in the esophageal epithelium, such as the infiltration of inflammatory cells, basal cell hyperproliferation, papillae hypertrophy, dilation of venules, ingrowth of the capillaries, epithelial sloughing, and ulceration [9]. Esophagitis was graded using the Hetzel grading system as follows: grade 0, normal appearing mucosa; grade 1, mucosa edema hyperemia and/or friability; grade 2, superficial erosions involving less than 10% of mucosal surface; grade 3, superficial erosions or ulcerations involving 10–50% of the esophageal squamous mucosa; grade 4, deep peptic ulceration anywhere in the esophagus or confluent erosion of greater than 50% of the esophageal squamous mucosa. Histiological identification of intestinal metaplasia, dysplasia, or EAC was also noted where appropriate.

Measures of oxidative stress

TUNEL assay

ApopTag in-situ apoptosis detection kit (Intergen Company, Purchase, New York, USA) was used to detect the apoptotic cells according to a procedure reported previously [10,11]. Briefly, after endogenous peroxidase was blocked with H_2O_2 in methanol for 20 min, the sections underwent proteinase K digestion for 15 min. DNA fragments were tailed using digoxigenin–deoxyuridine triphosphate along with anti-digoxigenin antibody conjugation with horseradish peroxidase along with the substrate (diaminobenzidine–H_2O_2) to develop a brown color. The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive epithelial cells were counted against negative cells under a light microscope at a magnification of ×40, and six visual epithelium fields were chosen on each slide, and all sections from each animal were examined. An apoptotic index (the number of epithelial nuclei labeled by the TUNEL method/the number of total epithelial nuclei) was calculated.

Immunohistochemical assay

Immunohistochemical assays were performed to detect proliferating cell nuclear antigen (PCNA). Immunohistochemical staining was carried out on the paraffin-embedded material using the DAKO EnVision + System Kit (DAKO Corporation, Carpinteria, California, USA). In brief, peroxidase blocking was performed for 5 min, and primary antibodies were applied for 30 min (Santa Cruz Biotechnology Inc., CA). Incubation was performed in the Dako Envision-labeled polymer for 30 min, and the substrate-chromogen solution (diaminobenzidine) was added as a visualization reagent. The PCNA-positive epithelial cells were counted against negative cells under a light microscope at a magnification of ×40. As with the apoptotic index, six visual fields were chosen on each slide, and a proliferation index is calculated as a ratio of

the number of PCNA-positive epithelial nuclei and the number of total epithelial nuclei.

Thiobarbituric acid reactive substances assay

Lipid peroxidation was quantified by an OXItek TBARS Assay Kit (ZeptoMetrix Corporation, Buffalo, New York, USA) measuring the malondialdehyde concentrations as described in the provided instruction. Briefly, the tissue homogenate was processed for thiobarbituric acid reaction following the procedure described. Then, the reaction mixture was covered and incubated at 95°C for 60 min, and then cooled to room temperature in an ice bath for 10 min. Samples were centrifuged at 3000 rpm for 15 min, and the absorbance was measured with a microplate reader reading at 532 nm.

Western blot analysis of MnSOD expression

Western blot was performed to determine the MnSOD protein expression in the esophageal mucosal layer and muscle layer. In brief, total protein was isolated from fresh tissue samples by homogenization in ice cold buffer containing 20 mmol/l HEPES (pH 7.5), 1.5 mmol/l $MgCl_2$, 0.1 mmol/l dithiothreitol, 0.4 mol/l NaCl, 20% glycerol, 0.5 mmol/l phenylmethylsulfonyl fluoride, and 0.5 mmol/l leupeptin at 4°C. The insoluble cellular material was removed by microcentrifugation at 16 000*g* for 5 min, and total protein was determined spectrophotometrically. The protein samples were separated by SDS/polyacrylamide gel electrophoresis and subsequently transferred to the nitrocellulose membrane for western blot as described earlier [7].

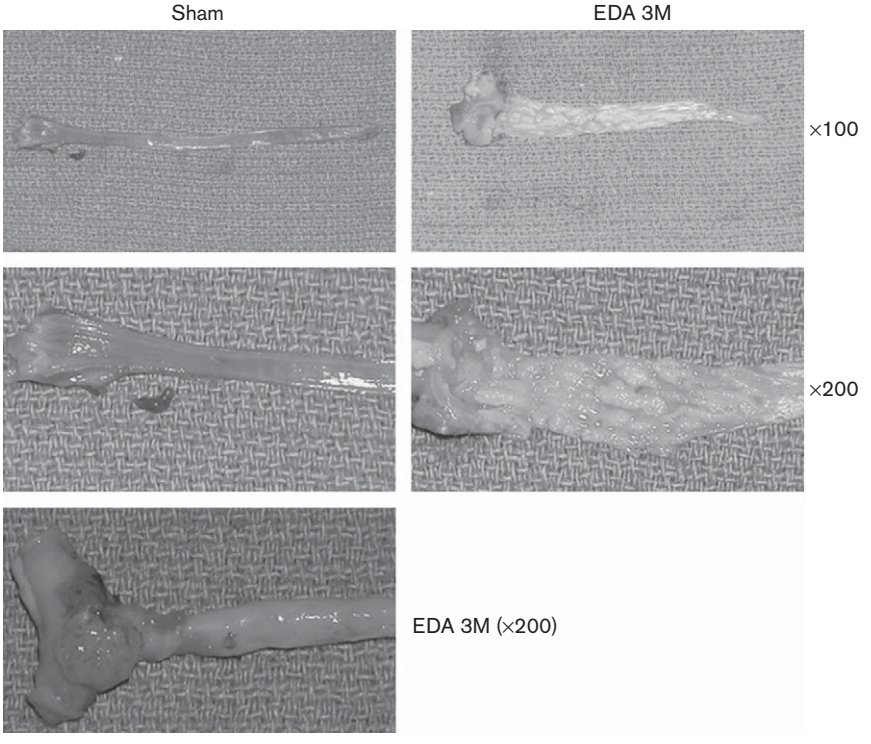
Glutathione assay

Glutathione (GSH) was determined by a Cayman's GSH assay kit (Cayman Chemical, Ann Arbor, Michigan, USA) utilizing an enzymatic recycling method. In brief, using the sulfhydryl group of GSH reacts with DTNB (5,5′-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by GSH reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 412 nm provides an accurate estimation of GSH in the sample. Owing to the use of GSH reductase in this assay, both GSH and glutathione disulfide are measured and the assay reflects total GSH.

Catalase assay

Catalase was determined by a Cayman's GSH assay kit (Cayman Chemical) using the peroxidatic function of

Fig. 1



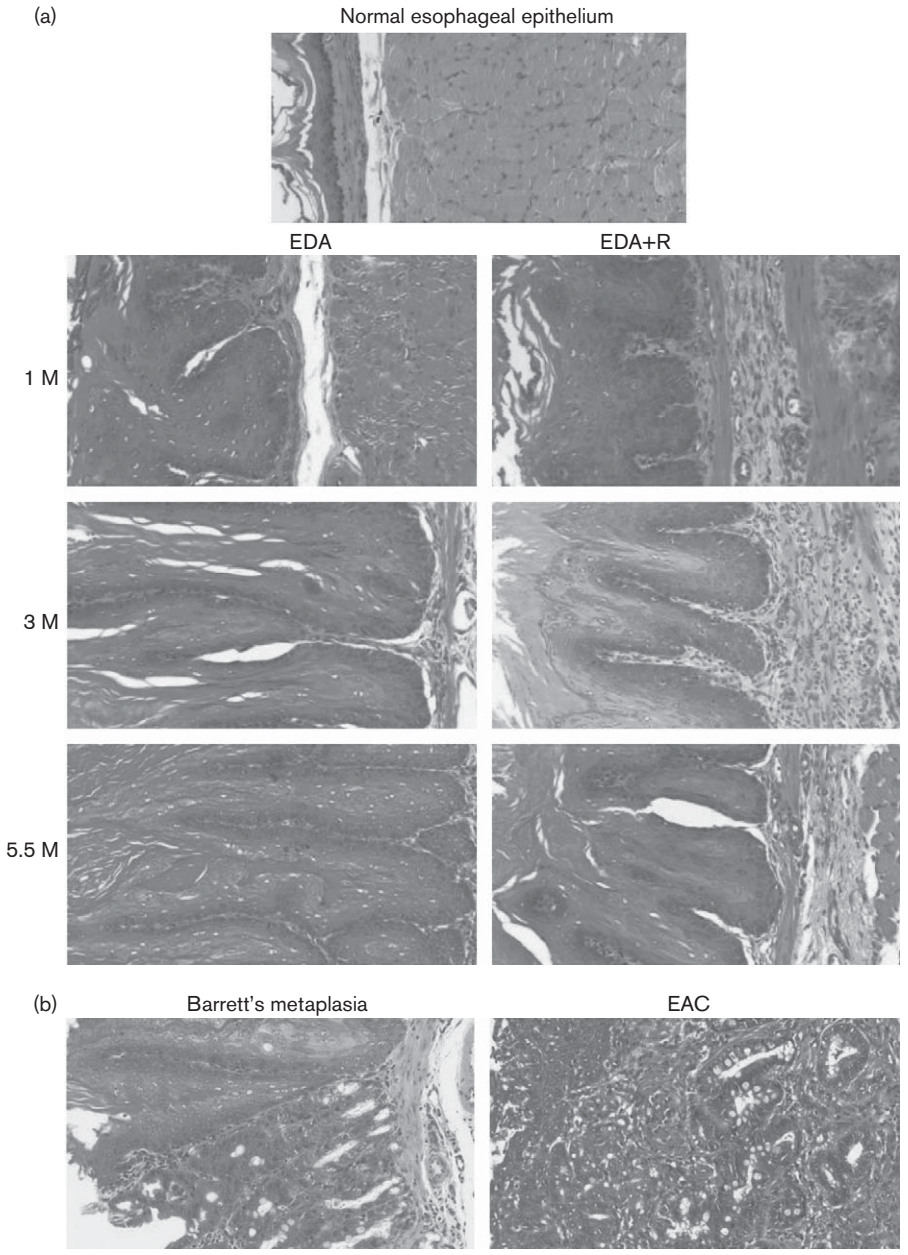
Gross magnified view of esophageal specimens for both the sham and physiological saline-treated rats. EDA, esophagoduodenal anastomosis. M, month.

catalase for the determination of enzyme activity. In brief, the method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. Measurement of the absorbance at 540 nm provides an accurate estimation of catalase enzymatic activity in the sample.

Statistical analysis

JMP software (SAS Institute, Cary, North Carolina, USA) was utilized. Student's *t*-tests with an assumption of unequal variances were carried out to compare means between groups. The results are expressed as mean values ± standard deviation. The χ^2 analyses were used

Fig. 2



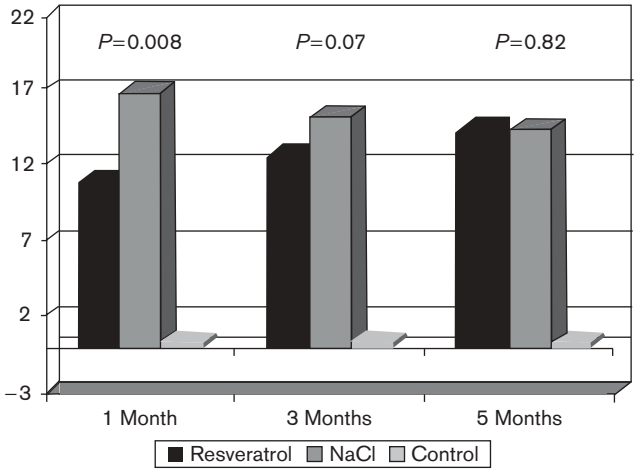
(a) Histopathological presentation of both the saline and resveratrol-treated rats (R), showing severe esophagitis at 1 and 3 months in the saline-treated rats. (b) Barrett's (left slide) and esophageal adenocarcinoma (EAC) (right slide) in the saline-treated rats esophagus. EDA, esophagoduodenal anastomosis. M, month.

Table 1 Histopathological results in the control, saline, and resveratrol-treated animals

Histopathology	Control 1 month	Control 3 months	Control 5 months	Saline 1 month	Saline 3 months	Saline 5 months	Resveratrol 1 month	Resveratrol 3 months	Resveratrol 5 months	*P* value
n	1	1	2	2	3	3	3	6	9	–
Esophagitis[a]	–	–	–	1.3 ± 0.5	2.2 ± 0.7	3.1 ± 0.5	1.1 ± 0.7	1.0 ± 0.9	1.9 ± 0.6	<0.0001
Intestinal metaplasia, number (incidence)	–	–	–	–	1 (33%)	2 (66%)	–	1 (16%)	2 (22%)	0.4895
Esophageal adenocarcinoma, number (incidence)	–	–	–	–	–	1 (33%)	–	–	–	0.7629

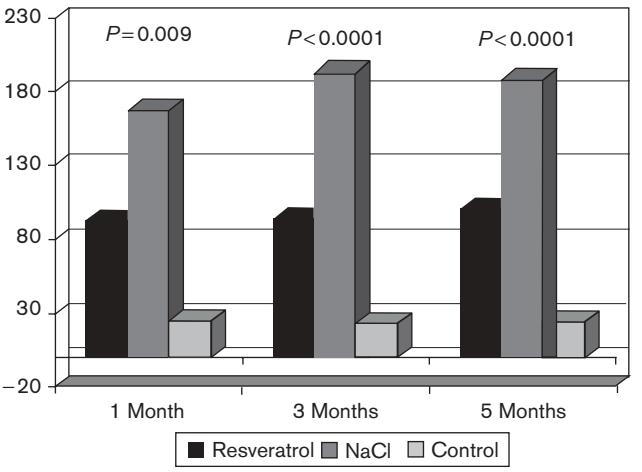
The Hetzel grading system is as follows: grade 0, normal appearing mucosa; grade 1, mucosa edema hyperemia and/or friability of mucosa; grade 2, superficial erosions involving <10% of mucosal surface of the esophageal squamous mucosa; grade 3, superficial erosions/ulcerations involving 10–50% of the esophageal squamous mucosa; grade 4, deep peptic ulceration anywhere in the esophagus or confluent erosion of more than 50% of the esophageal squamous mucosa.
[a]The data represent means ± SD of all animals with esophagitis.

Fig. 3



Percentage of apoptotic cells in the nonoperative, saline, and resveratrol-treated rats.

Fig. 4



Degree of proliferating cell nuclear antigen (PCNA) staining in the nonoperative, saline, and resveratrol-treated rats. Values representative are a proliferation index calculated as a ratio of the number of PCNA-positive epithelial nuclei and the number of total epithelial nuclei.

to compare the incidences of intestinal metaplasia and AC between groups. A P value of less than 0.05 was considered statistically significant for all tests.

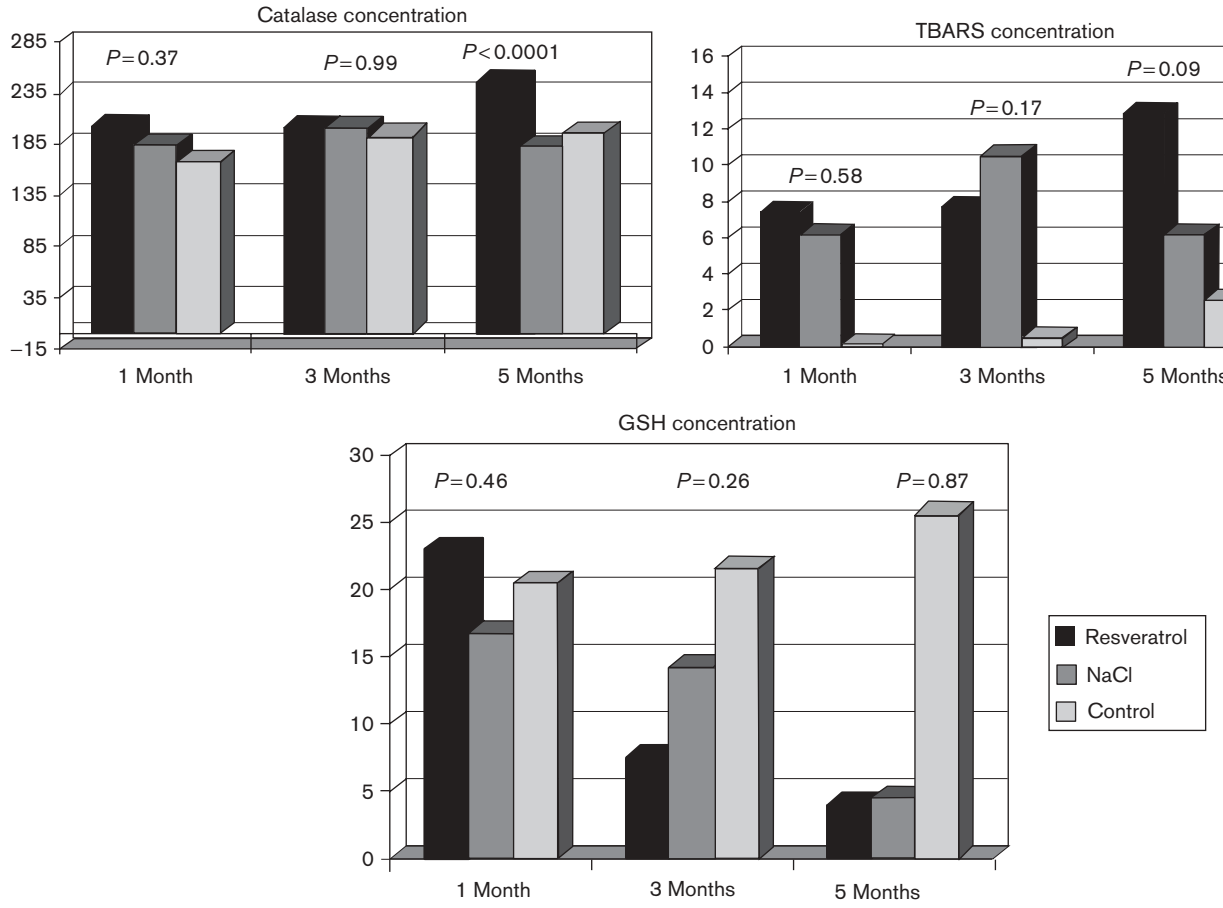
Results

Twenty-eight animals underwent EDA performed by the same surgeon using an identical technique as outlined above. One rat died in the immediate perioperative period and was never treated. An additional four animals were not operated on and served as nonoperative controls throughout the experiment. In the nonoperated group, one rat was assigned to the 1-month group, one to the 3-month group, and two to the 5-month group. In the EDA group treated with saline only, there were two rats in the 1-month group and three rats in both the 3 and 5-month groups. Finally, in the treatment group (EDA group treated with resveratrol), there were three rats in the 1-month group, six in the 3-month group, and nine in the 5-month group.

Animals in the nonoperated control group continued to gain weight normally throughout the experiment. However, a plateau was reached after approximately 2 months in all of the EDA rats, after which they failed to gain additional body mass and most began to slowly lose weight. This was observed in both the NaCl-treated group as well as the resveratrol-treated group. The difference was significant between the two groups with the NaCl weighing more for weeks 5 through 10 of the study ($P < 0.05$), but there was no significant difference in the weeks 1–4 or after 10 weeks of the study.

Esophagitis was present in all rats that underwent EDA. Initially, in the 1-month group, there was no difference between those administered with NaCl or those treated with resveratrol in the grade of esophagitis present (Fig. 1). However, in both the 3 and 5-month groups, a higher grade of severity was noted in the animals receiving NaCl ($P < 0.0001$). Intestinal metaplasia was noted in one (33%) of the rats treated with NaCl at 3 months and two (66%) at 5 months (Figs 2a and b). This compared with one (16%) of the 3-month resveratrol

Fig. 5



Antioxidant effects of resveratrol compared with nonoperative controls and saline-treated rats. GSH, glutathione; TBARS, thiobarbituric acid reactive substances.

group and two (*22%*) of the 5-month group. These differences were not statistically significant ($P = \text{NS}$). A single case of AC was identified, in an animal from the 5-month NaCl group ($P = \text{NS}$) (Table 1).

The percentage of apoptotic cells in the mucosa and submucosa of the esophagus was increased over time in the resveratrol group; conversely, it decreased in the Nacl group and remained the same in the control animals. These differences were significant in the 1-month ($P = 0.008$) group, but not in the 3-month ($P = 0.07$) or 5-month($P = 0.82$)groups. PCNA was significantly less in the resveratrol group at all three time points.

Resveratrol supplementation had little effect on the antioxidative enzymes MnSOD, catalase, GSH, and thiobarbituric acid reactive substances (TBARS). All were nonsignificant between the treatment groups, except in the 5-month group when catalase expression was significantly higher in the resveratrol group versus the NaCl group ($P < 0.0001$) (Figs 3–5). GSH and TBARS were both different between the EDA groups compared with the nonoperated controls; this was, however, not observed for catalase. MnSOD expression, as measured by western blot, was also unchanged.

Discussion

This study represents one of the first attempts to examine the chemoprevention of EAC with resveratrol in an animal model. Although it does not show a statistically significant decrease in the incidence of cancer, the animals treated with resveratrol did have a significant decrease in the severity of esophagitis, a known precursor to invasive cancer. The lack of a significant decrease in the incidence of cancer is most likely related to the number of animals that were used in each treatment arm. A well-designed study from Li *et al.* [12] examined the effects of resveratrol on rats administered a carcinogen, *N*-nitrosomethylbenzylamine, and found that it decreased the number and size of SCC tumors in the esophagi in the presence of this agent. There was also a trend toward decreased incidence of

invasive cancer, although this was not statistically significant. This study adds to this information showing that esophagitis-induced AC tomorgenesis may also be prevented using the resveratrol.

The exact mechanisms of this chemoprentative effect are unknown, but are thought to be related to anti-oxidation effects and subsequent decreases in inflammation, a requisite for carcinogensis in this model. Other studies have identified similar results for different types of malignancies, as well as cardiovascular, pulmonary, and renal disease. In this study, the cell turnover, as measured by both apoptosis and PCNA, was certainly less pronounced for animals given resveratrol versus those treated with NaCl, though resveratrol-induced apoptosis was still identified, as reported in other studies [13]. However, we failed to identify statistically significant differences in the expression of other established biomarkers of oxidative stress including GSH, TBARS, and MnSOD; catalase expression was significantly increased in the 5-month group of resveratrol rats. These markers have been variably studied with resveratrol in other disease states and models. The study most relevant to the one at hand examined the use of resveratrol as a chemoprentative agent in colon cancer [14]. In this study, the authors performed TBARs, SOD, catalase, and GSH analyses of colonic epithelium after a 30-week treatment course of resveratrol. As in our study, both catalase and TBARs were increased over the control group; however, expression of SOD and GSH were also increased, results which are contradictory of our findings.

There are limitations to this early feasibility study in that the mode of delivery for the treatment dose was i.p. injection. This likely led to sufficient circulating levels of the study compound, however these were not directly measured. There is no standardized dosing of this agent in the literature; reported dosing schemes are wide ranging from 3 g/kg/day i.p. [15] to 60 mg/day/nasogastric tube [16] to 8 mg/kg orally a day [14] to a study that showed a dose greater than 4 mg/kg delivered i.p. was fatal [12]. Thus, the dose in this study was purposely conservative, not only to protect the animals but also to conceivably be attainable in future in-vivo studies. We chose the intra-peritoneal route as our first step to ensure accurate and similar dosing levels were being acquired in the animals. Our next study will evaluate oral administration in order to be correlate the potential relationship between resveratrol supplementation and decreasing incidence of EAC. Whether the same decrease in esophagitis would be observed in animals given oral resveratrol, perhaps as a dietary additive, is not clearly elucidated by this study. The question of the ability to attain circulating resveratrol levels in humans has been raised by other studies and certainly warrants attention [17,18].

Conclusion

Morphological characteristics consistent with decreased esophagitis and incidences of metaplasia and EAC were seen on histopathology in the resveratrol group. Resveratrol resulted in a small diminution of the carcinogenic effects and progression to metaplasia, and further studies are designed to explore the potential anticarcinogenic mechanism, as it relates to oxidative inflammation.

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