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Dietary freeze dried black raspberry's effect on cellular antioxidant status during reflux-induced esophagitis in rats

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Abstract

Introduction—Esophageal cancer consists of two distinct types –esophageal adenocarcinoma (EAC) and squamous cell carcinoma (SCC), both of which differ significantly in their etiology. Freeze dried black raspberry (BRB) has been consistent in its ability to modulate the biomarkers and reduce the incidence of carcinogen-induced SCC in rats. In our previous studies in the esophagoduodenal anastomosis (EDA) model, we have shown that the early modulation of Manganese Superoxide dismutase (MnSOD) significantly correlates with the development of reflux-induced EAC in rats. In this study we looked at the short-term effects of a BRB supplemented diet on the modulation of antioxidant enzymes in reflux-induced esophagitis.

Methods—Male SD rats (8 wo; n=3–5) were randomized into 3 groups- sham-operated, fed control AIN-93M diet (SH-CD), EDA operated and fed either control diet (EDA-CD) or 2.5% (w/w) BRB diet (EDA-BRB). The effect of both reflux and dietary supplementation was analyzed 2 and 4 weeks after EDA surgery.

Results—Animals in EDA groups had significantly lower weight gain and diet intake compared to SH-CD ($p<0.05$). The sham operated animals, received an average esophagitis score of 0.1 ± 0.1, this increased significantly in EDA –CD animals to 1.8 ± 0.14 ($p< 0.001$ vs SH-CD) and in EDA-BRB group to 1.7 ± 0.06 ($p< 0.001$ vs SH-CD), with BE changes also present. However, dietary supplementation of BRB did not alter or ameliorate the grade of esophagitis or the induction of BE. BRB diet caused a 43% increase in MnSOD levels compared to EDA-CD (0.73 ± 0.16; $p=0.09$), however, this effect was not statistically significant and at 4 weeks, EDA-CD (0.58 ±0.12) showed an increase in MnSOD expression compared to SH-CD (0.34 ± 0.01).

Conclusions—In conclusion, our data suggests that dietary BRB does not increase the levels of cellular antioxidant enzymes or reduce the levels of lipid peroxidation compared to a control diet, in a short-term study of gastroesophageal reflux induction in the EDA animal model. However, it remains to be tested whether this is indicative of its ineffectiveness to inhibit reflux-induced EAC incidence over long-term.

Keywords

Black raspberry; reflux-esophagitis; barrett's esophagus; esophagoduodenal anastomosis; esophageal adenocarcinoma; superoxide dismutase; cellular antioxidant enzymes

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Introduction

Esophageal cancer incidence increased at a rate >300% in the past 3 decades, from 3.8 to 23.3 cases per million[33]. In the year 2000, esophageal cancer contributed to 87,000 and 59,000 person years of life lost (PYLL) due to cancer mortality in men aged under and over 65, respectively. This translated to a total of $22 billion in value of life lost[5,55]. It is projected that this would increase by 59% in the year 2020, the third highest increase among all cancers, to cost the American economy $35 billion in value[54]. These statistics are important since, esophageal cancer has a higher cost for younger males due to its rapid advancement, late detection and poor prognosis[34,36]. These grim statistics underscore the importance of extensive and urgent research on mechanisms, prevention, detection and therapy of esophageal cancer.

There are 2 distinct types of esophageal cancer. The esophageal squamous cell carcinoma (SCC) has been decreasing in incidence, whereas esophageal adenocarcinoma (EAC) has increased steadily over the last 3 decades[32]. The etiologies of these cancers differ significantly as well. Typically, SCC has been associated with several exogenous factors including smoking, alcohol and other carcinogen exposures, malnourishment along with low body weight, diet lacking in fruits and vegetable and a low socioeconomic status[28]. The strongest risk factor for EAC has been postulated to be gastro esophageal reflux disease (GERD) and its progression to the premalignant lesion- Barrett's esophagus (BE)[2,4]. Unlike for SCC, quitting tobacco use does not reduce the risk for EAC and alcohol is not strongly associated with EAC risk[6]. Further EAC incidence is also associated with obesity, high meat take and industrialized culture[13,27]. Although, these facts suggests that the two main types of esophageal cancers may differ considerably in their etiology, the low intake of fruits and vegetables is the only risk factor consistently associated with both SCC and EAC[12,29].

Animal models are very helpful in discovering mechanisms of and interventions for a disease. Rat models of SCC typically require carcinogen administration[53], whereas those of EAC require reflux-induction with or without co-carcinogen administration[20]. The research on prevention of EAC using animal models is still nascent. Recently, Hao et al., have shown the efficacy of α-tocopherol and N-acetyl cysteine in the prevention of reflux-induced EAC[11]. In our laboratory we have successfully used the esophagoduodenal anastomosis (EDA) model to induce reflux in male rats and we have consistently shown that EDA surgery produces EAC in 40% of animals within 6 months[25]. A relevant early biomarker for esophageal carcinogenesis is Manganese Superoxide Dismutase (MnSOD), whose levels and activity are significantly reduced 4-weeks after EDA surgery[26], replacement of which results in reduced EAC incidence[30].

Gary Stoner and colleagues have done extensive studies on the effect of freeze dried black raspberries (BRB) in the prevention of carcinogen-induced SCC[47]. It has been consistently and unequivocally shown that dietary BRB at various doses prevents the formation of SCC and reverses the biomarkers of SCC development[37,46]. However, the effects of these berries in a reflux-induced EAC animal model have not been studied. Since, the mechanisms of SCC and EAC development vary we evaluated the effect of dietary BRB on the short-term biomarkers of reflux-induced EAC such as esophagitis, cellular antioxidant enzyme levels and activities, as well as lipid peroxidation markers.

Materials and Methods

Animals and treatment

Male Sprague-Dawley rats (8 weeks old) were purchased from Harlan, Inc. (Indianapolis, IN) and acclimated for a week before surgery. Food and water were provided ad libitum. Animals were randomized into 3 groups and 2 time points as shown in Table 1. The EDA-operation was performed on those that weighed over 300 g as described[24]. Animals were weighed weekly, before and after surgery. At the end of respective treatment periods (2 or 4 weeks) animals were euthanized and the esophagus was collected for analysis. One half of the EDA junction was fixed in 10% buffered formalin for 24 hours, transferred to 80% ethanol and paraffin embedded. The distal part of the esophagus, proximal to the EDA-junction was collected, the mucosal and smooth muscle layers were separated and flash frozen in liquid nitrogen. Only the mucosal layer was used for immunoblots, enzyme activity analysis and lipid peroxidation assays.

Esophagitis was scored based on the Hetzel-Dent system (Grade 0- Normal appearing mucosa; grade 1- basal cell hyperplasia, mucosal edema, hyperemia, mucosal friability; grade 2- superficial erosion involving <10% of mucosal surface of the last 5 cm of the esophageal squamous mucosa; grade 3- superficial erosions or ulcerations involving 10–50% of the mucosal surface and grade 4- deep peptic ulceration and confluent erosion of >50% of the esophageal squamous mucosa) on 5 random regions for each sample and the scores averaged.

Diet preparation and Feed intake

Freeze dried black raspberry for this study was provided kindly by Dr. Ramesh Gupta (Department of Pharmacology and Toxicology, University of Louisville, KY) and sourced from Van Drunen Farms (Mommence, IL). The AIN-93M diet was supplemented with 2.5% (w/w) BRB as described[1]. The cornstarch and fiber components of the AIN-93M diet were replaced for the berry diet, based on the nutritional information available for black raspberry (http://www.nal.usda.gov/fnic/foodcomp). Previous proximate analysis of the 2.5% BRB diet has shown it to be isocaloric to regular AIN-93M diet[1]. The feed intake was measured for each cage over a period of 5 days and the feed intake/rat/day was calculated as described[1]. This procedure was repeated every week to evaluate diet intake. Berry diet was started 2 days before the EDA surgery, which mimics simultaneous feeding rather than pre- or post-feeding of chemopreventive agent.

Immunoblot analysis

The antibodies for MnSOD and gluatathione peroxidase (GPX) were purchased from Millipore (Temecula, CA) and the antibodies for catalase (CAT) and β-actin was purchased from Sigma Corp. (St Louis, MO). Fresh tissue homogenate of esophageal mucosa was prepared in ice-cold lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 400 mM Nacl and 20% glycerol; pH 7.5) containing protease inhibitors as described[23]. Insoluble cellular material was removed and the total protein levels were determined using a spectrophotometer. Thirty μg of protein was subjected to SDS/PAGE using a 10% gel, followed by transfer to a PVDF membrane. The membrane was blocked with 5% milk, incubated with the primary antibody at a dilution of 1: 2000, 1 h at room temperature and detected with chemiluminescent detection using appropriate secondary antibodies (1:5,000–10,000 dilution depending on previous standardization). The specific protein bands were detected based on their molecular weight with reference to pre-labeled markers and quantitated using UnScanIt software (Silk Scientific Inc., Orem, UT). The average pixel of each protein band was divided by that of the β-actin band so that the expression levels for all proteins were normalized using respective β-actin levels.

Lipid peroxidation assay (Thiobarbituric acid reactive substances-TBARS assay)

TBARS was quantified using an OXItek TBARS assay kit (Zeptometrix Corporation, Buffalo, NY) according to manufacturer's instruction. The insoluble cellular material that primarily consists of cell membrane components obtained during the protein isolation was used for these analyses and equivalent amount of protein was loaded for each sample. Malondialdehyde (MDA) levels were measured using a spectrophotometer and the MDA concentration of each sample was derived using values from a standard curve.

SOD activity assays

SOD activity was determined using a SOD assay kit-WST (Dojindo Molecular Technologies, Inc., Gaithesburg, MD) as described[22]. This kit uses xanthine-oxidase to generate superoxide radicals which can then react with either the total SOD present in the samples or a water-soluble tetrazolium salt, which then forms a formazan dye that can be detected spectrophotometrically. The total SOD was quantitated based on a standard curve generated concurrently. To selectively measure the MnSOD activity, the Cu/ZnSOd activity was inhibited using KCN (10 μM) and measured simultaneously.

Statistics

All data are represented as mean ± SEM. The weight gain data was compared using a Two-way ANOVA. For the other analyses, the mean value was compared using a one way ANOVA followed by a Tukey's post hoc test and a p-value < 0.05 was considered significant. All analyses were done using the Graphpad Prizm software (San Diego, CA).

Results

Effect of EDA surgery on short-term weight loss and feed intake

There were no significant differences in initial weight between any groups of animals. Animals that underwent EDA surgery, regardless of the diet, had significant weight loss 1 week following surgery (Figure 1A) and also had significantly lower feed intake (Figure 1B). However, feed intake recovered 2 weeks after surgery (Figure 1B). Although both groups of EDA animals began to gain weight starting at 2 weeks, this weight gain did not match that of the sham surgery group and continued to remain significantly different until 4 weeks (Figure 1A).

Effect of BRB supplementation on histopathological changes in the esophagus after EDA surgery

Esophagitis was observed in all EDA animals, regardless of diet, both 2 and 4 weeks after surgery (Figure 2). BE was assessed based on the presence of columnar lined esophagus consisting of absorptive and goblet cells. The sham operated animals, received an average esophagitis score of 0.1 ± 0.1. This increased significantly in EDA –CD animals to 1.8 ± 0.14 ($p< 0.001$ vs SH-CD) and in EDA-BRB group to 1.7 ± 0.06 ($p< 0.001$ vs SH-CD). Early BE changes were also present in certain samples (Figure 2B and C; blunt arrows). Dietary supplementation of BRB did not alter or ameliorate the grade of esophagitis or the induction of BE.

Effect of BRB supplementation on antioxidant enzyme levels in the esophagus

The protein levels of 3 cellular antioxidant enzymes- MnSOD, CAT and GPX were measured using immunoblot analysis. At 2 weeks after surgery, compared to SH-CD (0.46 ± 0.16) group, MnSOD levels were unaltered in EDA-CD (0.51 ± 0.04; Figure 3A). BRB diet caused a 43% increase in MnSOD levels compared to EDA-CD (0.73 ± 0.16; Figure 3A) ($p=0.09$), however, this effect was not statistically significant. At 4 weeks, EDA-CD (0.58

±0.12) showed an increase in MnSOD expression compared to SH-CD (0.34 ± 0.01; Figure 3A). This increase was higher than in EDA-BRB (0.48 ± 0.2), but not statistically significant ($p = 0.09$). EDA surgery did not affect the levels of the other antioxidant enzymes (CAT and GPX) measured at either time points (Figure 3B and C).

Effect of BRB supplementation on SOD activity

At 2 weeks after EDA-surgery, the total SOD activity was similar among the 3 groups. However, the MnSOD activity mirrored the expression levels and increased with the presence of EDA–surgery. It was the highest in EDA-BRB group and significantly different from SH-CD group ($p = 0.02$). On the other hand, the reverse trend was seen for Cu/ZnSOD, with EDA-BRB having the lowest activity (Figure 4A). At 4 weeks, there was an insignificant reduction in total SOD activity for the EDA-BRB group (Figure 4B). Once again, the MnSOD activity mimicked the expression level and there was a slight reduction in MnSOD activity for EDA-BRB groups compared to the other 2 (Figure 4B). CuZnSOD activity was the same for all groups at 4 weeks.

Effect of BRB supplementation on lipid peroxidation

In these studies, reflux induction did not increase lipid peroxidation either at 2 or 4 weeks after surgery. In fact, 4 weeks after EDA-surgery, EDA-PD group had a lower lipid peroxidation level than SH-PD. BRB supplementation significantly increased the measured MDA concentration compared to EDA-PD at 2 weeks ($p < 0.05$), but had no effect at 4 weeks (Figure 5).

Discussion

Extensive studies by Stoner and colleagues have shown repeatedly that freeze-dried dietary black raspberry is highly effective in the prevention of N′-nitrosomethyl benzylamine (NMBA)-induced esophageal SCC[1,38,45]. However, the effect of these berries in reflux-induced EAC has not been studied. This is the first study to report the effects of dietary BRB in an animal model of reflux-induced esophagitis. Compared to the control diet, BRB diet did not show any effect on the development of esophagitis. BRB did demonstrate an increased in the level and activity of MnSOD as well as TBARS at 2 weeks, but had no effect on the other antioxidant enzymes (CAT and GPX), with this effect lost at 4 weeks.

BRB diet increased the levels of both MnSOD and TBARS at 2 weeks. There is evidence to suggest that the SOD/CAT ratio plays a role in the ultimate levels of cellular oxidative stress[8] and this oxidative stress may be a precursor to other cellular effects such as apoptosis[15]. Since MnSOD generates the substrate for CAT- H_2O_2, an oxidant, an increase in MnSOD without a concurrent increase in CAT levels can lead to increased oxidative stress and lipid peroxidation as seen in our results. Nevertheless, this effect is not sustained until 4 weeks, suggesting that this is an early modulatory response to BRB diet. Several reports have shown the effects of various black raspberry phytochemicals such as ellagic acid and anthocyanins on antioxidant enzymes[7,10,19,50]. However, it is difficult to compare our results to these data since studies were done in cell types other than esophageal epithelium, used phytochemicals in black raspberry but extracted from a different food source and exposed to different treatment conditions or methodologies. There are at least 2 human short-term studies that show lack of antioxidant effect in peripheral blood cells when berry juices were provided orally[9,31].

Studies done by Stoner and colleagues are the most relevant to our work. However, these investigators have primarily looked at a dietary BRB dose of 5 and 10% in NMBA-induced squamous cell carcinoma[17,40,44]. Given the decided different animal model and

completely different histology, comparisons to Stoners work is limited at best. Also, they have focused primarily on xenobiotic metabolism, signal transduction and cell regulation[18,41,51]. In their recent report analyzing the early molecular changes induced by BRB diet in the esophageal epithelium, in response to NMBA treatment, they do not report any changes in the expression of the antioxidant enzymes SOD, CAT and GPX[42].

Kresty *et al.*, have shown that lyophilized black raspberry reduces the level of urinary 8-epi-prostaglandin-F2α in the BE patients, they also report that BRB had no effect on the length of BE. Further, they show that there is a high variability in the reduction of urinary 8-hydroxyguanosine and do not report changes in the biomarkers of the actual BE mucosa[16]. Dr. Gary Stoner in his most recent review has suggested that this lack of effect may be due to the rapid transit of the berry slurry through the esophagus[39]. The effect of BRB phytochemicals in a preclinical model of mixed gastroesophageal reflux has not yet been shown. Reflux is the operative word, since reports on the effect of ellagic acid and BRB diet on phase I and II enzymes and other antioxidant biomarkers in the esophageal mucosa in vivo are published[41,43,52].

Additional molecular markers, specifically P53 have begun to be evaluated as a potential molecular predictive marker in BE carcinogenesis. P53 expression has been demonstrated as early as 2004, when Segal et al demonstrated pos P53 staining in 39% of cases with IM and 60% with BE, with the degree of dysplasia not presented[35]. This was one of the single highest reported expression of P53 in BE without dysplasia, since more recent reports have demonstrated positive staining in 0% of BE[14], 10% of BE[49], and 62.5% of BE[3]. Thus the true predictive valuate of P53 in BE without dysplasia remains to be proven and was one of the reasons this molecular marker was not studied in this experiment. Similarly the effects of BRB on P53 expression remain to be evaluated.

It is possible that dietary BRB is ineffective in this study due to the following reasons. First, there is ineffective digestion of BRB due to lack of stomach and the rapid transit of food through the EDA junction. Due to the lack of a functional stomach, the EDA junction in this animal model expands to accommodate food storage. It is known that anthocyanins are partially absorbed in the stomach of rats[48]. Thus this lack of stomach may lead to both reduced digestion and absorption of protective phytonutrients, assuming that systemically absorbed phytonutrients provide protective effect on esophageal mucosa. Second, BRB constituents are known to affect enzymes of carcinogen metabolism[50]. Hence the high effectiveness of BRB in the SCC animal model may arise out of its effect on carcinogen metabolism as opposed to its effect on bile/acid reflux. Third, the dose of BRB supplemented may be too low to be effective. Finally, even though BRB has no effect on the antioxidant enzymes, it may be effective through other mechanisms such as inhibition of cell proliferation and induction of apoptosis etc. These biomarkers are yet to be analyzed in our study. Currently, a pilot clinical trial to study the effects of oral BRB on esophageal dysplasia is ongoing (34). The results from this study may contextualize our data regarding the effects of BRB on the esophageal dysplasia or vice versa.

The lack of effectiveness of BRB further underscores the differences in the mechanisms of SCC and EAC development[21]. However, these results are from a short-term study and it remains to be seen whether BRB when fed over longer term is effective in reducing reflux-induced EAC incidence. Also, data on other molecular markers such as apoptosis and cell proliferation, which are altered by BRB, must also be gathered. Currently we are conducting long-term esophageal tumorigenesis studies and analysis of other potential biomarkers to evaluate this.

In conclusion, our data suggests that dietary BRB does not increase the levels of cellular antioxidant enzymes or reduce the levels of lipid peroxidation compared to a control diet, in a short-term study of gastroesophageal reflux induction in the EDA animal model. However, it remains to be tested whether this is indicative of its ineffectiveness to inhibit reflux-induced EAC incidence over long-term.

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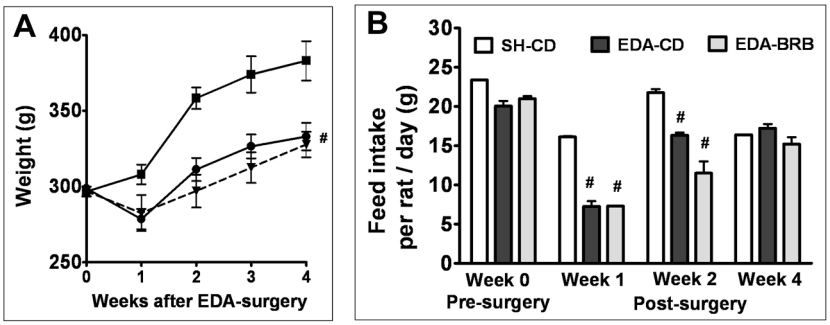

Figure 1. Comparison of weight gain (A) and diet intake (B) in rats that underwent EDA surgery

EDA surgery to induce mixed-reflux was performed in 8 weeks-old male SD rats as described in Materials and Methods. (A) Weight gain and (B) diet intake were measured weekly after surgery and then compared using either two-way ANOVA (weight gain) or one-way ANOVA (diet intake).

#- significantly different from SH-CD ($p<0.05$).

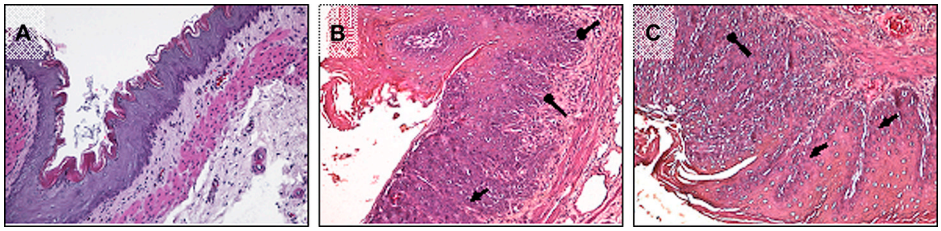

Figure 2. Histopathological changes observed in the esophageal epithelium 4 weeks after reflux-induction

Representative H&E stained sections of esophageal mucosa (200X magnification) from (A) SH-CD –normal mucosa (B) EDA-PD and (C) EDA-BRB. Esophagitis (denoted by triangular arrowhead) and Barrett's esophagus (blunt arrow head) can be visualized in both EDA-operated groups (B and C).

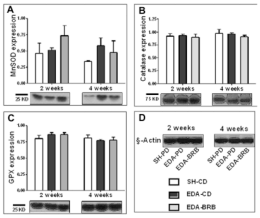

Figure 3. Effect of dietary BRB on antioxidant enzyme expression levels in the esophageal mucosa in the presence of reflux

The enzymes (A) Manganese superodxide dismutase (MnSOD), (B) Catalase and (C) Glutathione peroxidase (GPX) in the esophageal mucosa 2 and 4 weeks after EDA surgery were measured using immunoblot and normalized to tissue β-actin levels (D). SH-CD - sham operated on control diet; EDA-CD – EDA-operated on control diet and EDA-BRB – EDA-operated on BRB diet.

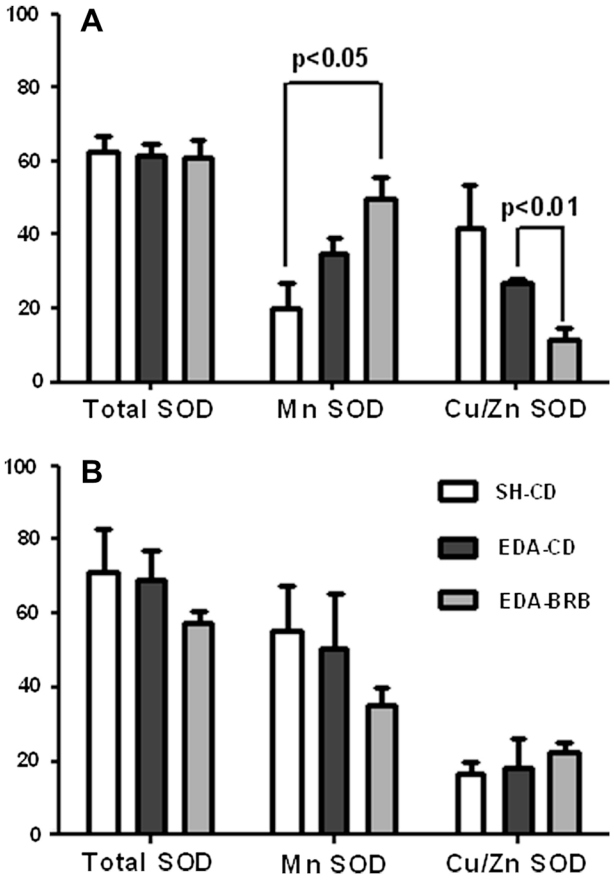

Figure 4. Effect of dietary BRB on SOD enzyme activities in the esophageal mucosa in the presence of reflux

SOD enzyme activities in the esophageal mucosa 2 (A) and 4 weeks (B) after EDA surgery were measured using an SOD assay kit according to attached instructions. Cu/ZnSOD activity was inhibited using KCN (10 μM) and the MnSOD was measured alongside total SOD activity. Cu/ZnSOD activity was then derived by subtracting MnSOD from total SOD activity.

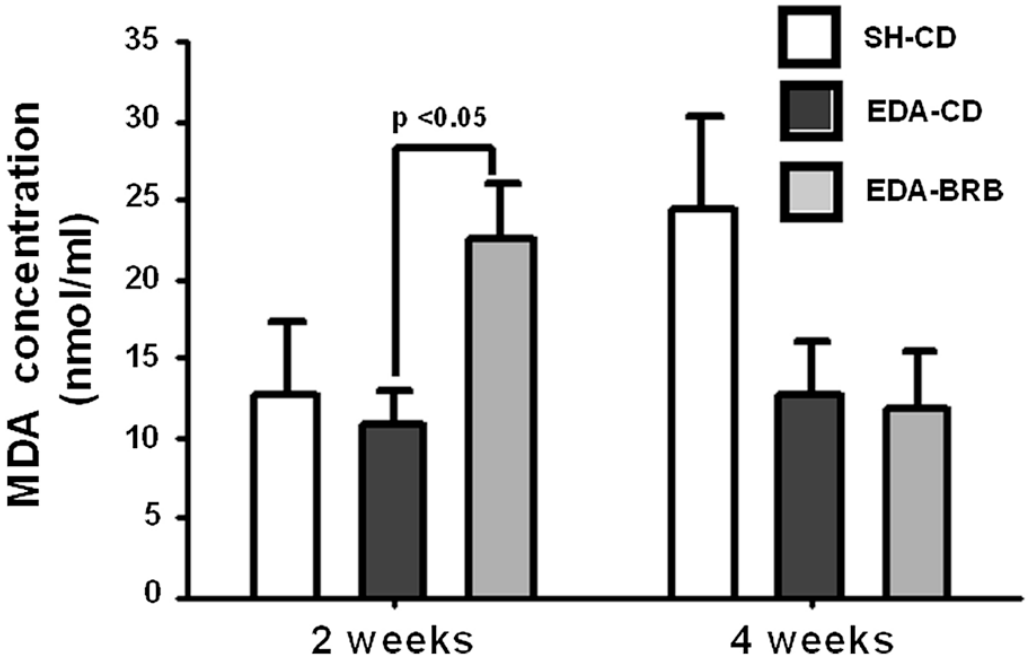

Figure 5. Effect of dietary BRB on lipid peroxidation of the esophageal mucosa in the presence reflux

MDA concentration (nmol/ml) was measured in the esophageal mucosa 2 and 4 weeks after EDA surgery using a commercially available TBARS assay kit as described in Materials and Methods.

Table 1

Experimental Design

Diet	Surgery	Time point	n
CD	Sham	2 4	3 3
CD	EDA	2 4	4 5
BRB	EDA	2 4	4 5

Eight week old male SD rats were acclimated for a week and then randomized into 6 groups. EDA surgery was performed as described in Materials and Methods. BRB supplementation was started 2 days before the EDA surgery. Body weight gain and diet intake were measured weekly through out the study.