Original Article

Anthocyanin-Rich Diet in Chemically Induced Colitis in Mice

(flavonoids / protein oxidation / inflammatory bowel disease / oxidative stress)

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Abstract. The aetiology of inflammatory bowel diseases is unclear, but oxidative stress plays a key role in the pathogenesis. Anthocyanins – plant polyphenols – were shown to have antioxidant and anti-inflammatory properties. The aim of this study was to investigate the potential protective effects of anthocyanins on the oxidative status in mice with chemically induced colitis. Adult male mice were randomly divided into a control group drinking tap water and a colitis group drinking 1% dextran sulphate sodium solution. Animals had *ad libitum* access to a control wheat-based diet or food based on wheat producing anthocyanins. Bodyweight and stool consistency were monitored daily for 14 days. At the end of the experiment, colon length was measured and tissue samples were collected for the assessment of histology and oxidative status. Mice with colitis had lower body weight, higher stool score and shorter colon than control mice. Anthocyanins had neither an effect on stool consistency, nor on bodyweight loss and colon length. In the colon, liver and plasma, analysis of oxidative stress markers and antioxidant status revealed no significant differences between the groups. Food made from wheat producing anthocyanins did not protect mice from the consequences of chemically induced colitis. The measured biomarkers do not confirm the role of oxidative stress in this model of colitis. Further optimization of the anthocyanin-rich food might be needed before further experiments are conducted.

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, represent chronic, gradually progressive and incurable diseases of the intestine (Angelberger et al., 2009). Intestinal tissue damage occurs after interaction of intestinal microflora with intestinal mucosa, which causes the immune response and inflammation (Thompson-Chagoyan et al., 2005). Chronic inflammation is associated with production of reactive oxygen species (Tüzün et al., 2002). Oxidative stress is defined as an imbalance between the production of free radicals and the activity of antioxidant mechanisms (Omata et al., 2009; Tothova et al., 2013).

Several studies deal with oxidative stress and antioxidant status markers in patients with IBD. Rana et al. (2014) observed increased lipid peroxidation in the plasma of patients with ulcerative colitis compared to healthy controls. On the other hand, Tüzün et al. (2002) observed no differences in plasma malondialdehyde (MDA) between patients with IBD and the control group. Higher concentrations of nitric oxide were observed in exhaled air of patients with ulcerative colitis compared to controls (Koek et al., 2002). Parmar et al. (2014) observed increased amounts of MDA in the co-

lon tissue of mice with DSS-induced colitis (5 %) compared to control mice, suggesting a rationale for the antioxidant treatment of colitis.

Polyphenolic compounds have antioxidant properties (Bi et al., 2014; Liang et al., 2014) and beneficial effects in oxidative stress-related diseases (Witaicenis et al., 2014). They can be found in fruits (blackcurrants, apples, etc.) and vegetables (e.g. red onion) (Norberto et al., 2013). One group of polyphenols are anthocyanins – plant pigments (Sancho and Pastore, 2012). Anthocyanins display anti-tumour, antimicrobial and anti-inflammatory effects (Bi et al., 2014; Chan et al., 2014). Philippe et al. (2012) found that pre-treatment of mice with anthocyanin-rich Lacto-Wolfberry formulation before and after inducing colitis increased TAC concentration in the plasma compared to mice without Lacto-Wolfberry administration. Another study observed that oral intake of anthocyanin-rich extract from blueberries decreased the level of nitric oxide in the colon of mice with colitis compared to mice without the extract intake (Wu et al., 2011). These studies showed that administration of anthocyanins has a beneficial effect on the oxidative stress status in the animal model of colitis.

Several studies have previously evaluated antioxidant and anti-inflammatory effects of various types of not only anthocyanins but also polyphenols in different animal models of colitis (Mandalari et al., 2011; Bruckner et al., 2012; Philippe et al., 2012), but none of them investigated the effect of *ad libitum* daily intake of anthocyanins incorporated into common food (except for the above-mentioned study with Lacto-Wolfberry). The aim of the present study was to analyse the effects of anthocyanin intake on the colitis and markers of oxidative and antioxidant status in a mouse model of IBD.

Material and Methods

Animals and diet

Twenty male 129sv4 adult mice (Anlab, Prague, Czech Republic) were randomly divided into the following groups (5 mice per group): control groups (CTRL, CTRL+A) receiving water, and colitis groups (1% DSS, 1% DSS+A) receiving 1% dextran sulphate sodium (DSS) solution in water. Mice from each group had *ad libitum* access to control wheat food or anthocyanin-rich wheat food (A). Mice in each group were housed separately in plastic cages with a 12 h light/dark cycle. The experimental protocol was approved by the ethics committee of the Institute of Molecular Biomedicine.

Experimental design

The experiment lasted 14 days and the design of the study is described in Fig. 1. Tap water was given to each group for the first three days. Colitis groups received 1% DSS solution *ad libitum* instead of tap water for the following seven days. All groups received tap water for the last four days of the experiment. Weight and stool consistency were monitored daily. The scores for the stool
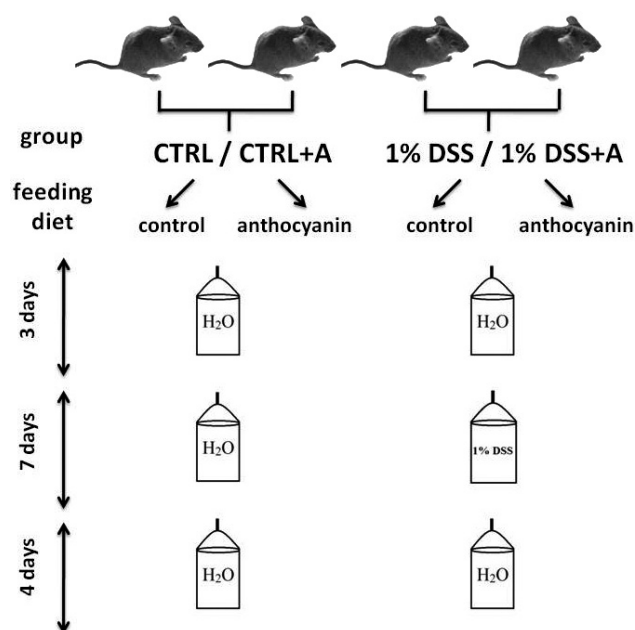

Fig. 1. Experimental design and groups: CTRL and CTRL+A, 1% DSS and 1% DSS+A had *ad libitum* access to water/1% DSS and control/anthocyanin (A) food during the entire experiment; all groups received water for the first three days of the experiment; the next seven days, 1% DSS and 1% DSS+A groups received 1% DSS solution and control groups received water; the last four days, all groups had free access to water.

consistency were as follows: 0 – normal stool, 1 – soft stool, 2 – diarrhoea, 3 – blood in stool (Gardlik et al., 2012). After 14 days all animals were sacrificed under isofluran anaesthesia. Blood was collected by heart puncture into Microvette EDTA tubes (Sarstedt, Nümbrecht, Germany), centrifuged immediately at 2000 *g* for 7 min at room temperature and plasma samples were stored at -80 °C. The colon length was measured and samples of colon and liver were excised and homogenized in phosphate-buffered saline (PBS, pH 7.2) to prepare 10% homogenates. The homogenates were centrifuged for 10 minutes at 2000 *g* at 4 °C. The supernatants were collected and stored at -80 °C for further measurements.

Histopathological examination

Colonic tissue sections were fixed in 4% formaldehyde and dehydrated with graded ethanol and xylene. Paraffin-embedded tissues were cut at 5 μm. Haematoxylin and eosin stain was performed according to the standard protocol. Representative photomicrographs were taken using a digital camera attached to a Leica DM2000 light microscope (Leica Microsystems, Wetzlar, Germany).

Analysis of oxidative and antioxidant status

Markers of oxidative stress and antioxidant status were measured in plasma, colon and liver homogenates. The measurement of advanced oxidation protein products (AOPP) was described by Witko-Sarsat et al. (1996). Briefly, chloramin T with potassium iodide was used to
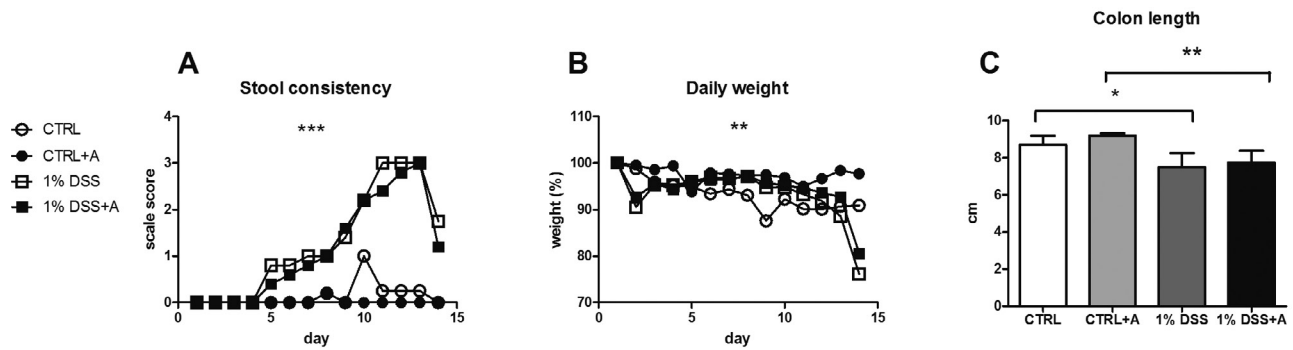
Fig. 2. Analysis of **A**) stool consistency and **B**) body weight monitored daily during the experiment, **C**) colon length analysed immediately after termination of the experiment
* denotes P < 0.05, ** denotes P < 0.01, data are presented as mean + SD.

prepare a calibration curve. Forty hundred μl of the samples were diluted in 160 μl of PBS to final volume 200 μl (pH = 7.2; ratio 1 : 4) and mixed with 20 μl of glacial acetic acid. The absorbance was measured at 340 nm. Total antioxidant capacity (TAC) assay was based on the reducing ability of 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) cations by antioxidants present in the sample. Trolox was used as a standard in the calibration curve. Twenty μl of samples were diluted with acetate buffer (pH = 5.8) and the initial absorbance was measured at 660 nm. The 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) reagent was added and the absorbance was measured after incubation (5 min) at 660 nm (Erel, 2004).

Another marker of antioxidant status, ferric reducing antioxidant power (FRAP), was measured according to the described protocol (Benzie and Strain, 1996). Twenty μl of the fresh, pre-warmed FRAP reagent (acetate buffer, pH 3.6, tripyridyl-*s*-triazine, $FeCl_3 \times 6H_2O$ and distilled water, 37 °C) were added into the microtitre plate and subsequently measured at 593 nm as blank. Twenty μl of samples and standards ($FeSO_4 \times 7H_2O$) were added to the FRAP reagent and the absorbance at 593 nm was measured again (Benzie and Strain, 1996). Protein concentration was analysed using a bicinchoninic acid kit. Bovine serum albumin was used as a standard. All chemicals and the kit used for biochemical analyses were obtained from Sigma-Aldrich. Analyses were performed in a spectrofluorometer Tecan Saphire II (Grödig, Austria).

Statistical analysis

The statistical analysis of daily weight and stool consistency data was performed using repeated measures one-way ANOVA and Tukey post hoc test. One-way ANOVA was used for the analysis of other data (GraphPad Prism5®, La Jolla, CA). P < 0.05 was considered significant. Data are presented as mean + standard deviation (SD).

Results

A significant loss of body weight in 1% DSS and 1% DSS+A groups (23.8 % and 19.42 %, respectively) was observed after colitis induction compared to CTRL and CTRL+A groups (9.11 % and 2.3 %, respectively) (Fig. 2B, P = 0.001). The daily analysis of stool consistency revealed gradually deteriorating stool score in both groups receiving DSS (Fig. 2A, P = 0.0001) from the second day of treatment when compared to control groups. Transiently deteriorated stool consistency was observed in the CTRL group on the 10th day of experiment. No significant differences between 1% DSS and 1% DSS+A groups were found in daily monitored body weight and stool consistency (Fig. 2A and 2B, P = 0.96 and P = 0.91, respectively). The colon length was significantly lower in both groups treated with DSS (7.5 ± 0.76 cm and 7.74 ± 0.64 cm for 1% DSS and 1% DSS+A groups, respectively) compared with both control groups (8.7 ± 0.49 cm and 9.2 ± 0.12 cm for CTRL and CTRL+A groups, respectively) (Fig. 2C, P = 0.0007). However, no significant differences were observed between 1% DSS and 1% DSS+A groups. CTRL and CTRL+A had normal colonic histomorphology (Fig. 3A and 3B). DSS
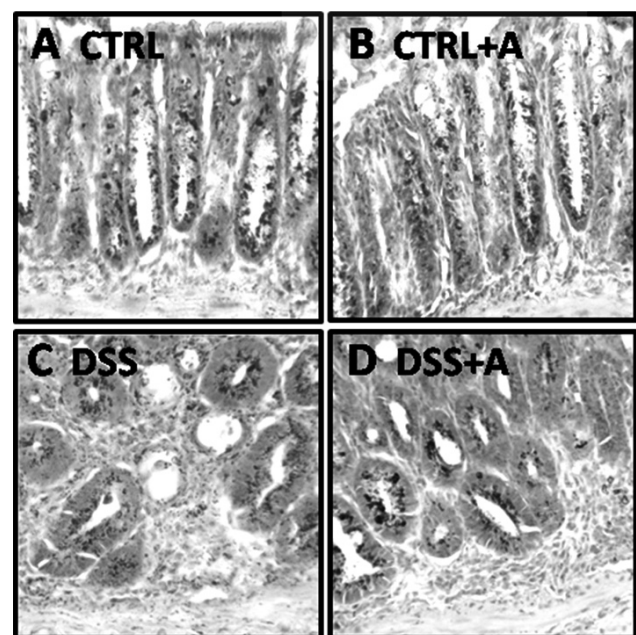

Fig. 3. Histopathological analysis of colonic tissues. **A**) and **B**), normal colonic morphology; **C**) and **D**), areas of crypt damage and inflammation
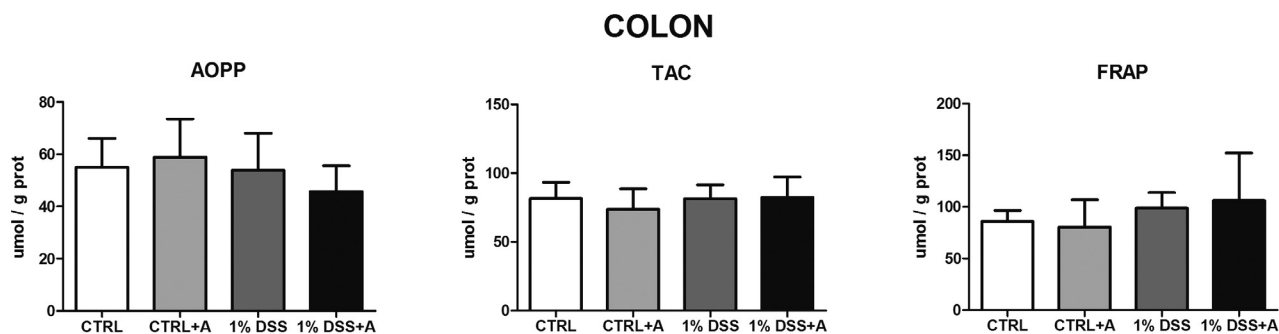
COLON

Fig. 4. Markers of oxidative stress and antioxidant status measured in the colon homogenates of control and 1% DSS groups. Data are presented as mean + SD.
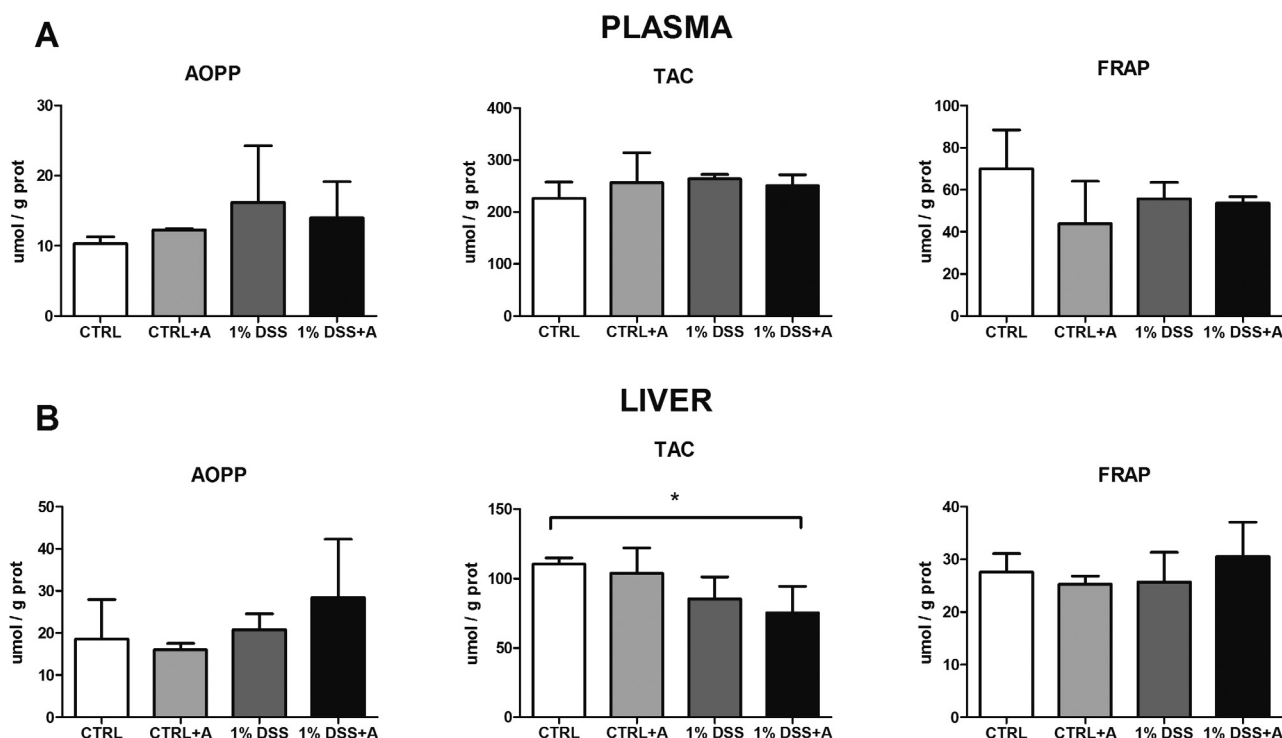
A   **PLASMA**

B   **LIVER**

Fig. 5. Markers of oxidative stress and antioxidant status analysed in the **A**) plasma and **B**) liver of both CTRL and 1% DSS groups

* denotes $P < 0.05$, data are presented as mean + SD.

and DSS+A showed areas with both crypt damage and inflammation (Fig. 3C and 3D). No differences between DSS and DSS+A were found.

The analysis of oxidative stress markers revealed no significant differences between analysed groups in all measured markers in the colon (Fig. 4), plasma and liver samples (Fig. 5A and 5B, respectively). We did not find out deterioration of oxidative stress markers in DSS groups compared to CTRL groups. DSS solution did not affect the markers of oxidative stress and antioxidant status in colon, plasma and liver samples. Thus, anthocyanin-rich food did not influence the markers of oxidative and antioxidant status because there was no effect of DSS on these markers in our experimental model of colitis.

Discussion

In the current experiment, we used DSS solution for the induction of colitis. We observed gradually declining stool consistency during the DSS treatment, but returning back to normal after water supplementation. On the other hand, the body weight as another parameter of the disease was decreased throughout the experiment. Numerous studies dealing with IBD previously reported shortened colon or increased colon weight/length ratio in mice with colitis (Marin et al., 2013; Shigeshiro et al., 2013; Gardlik et al. 2014). The colon length has been used as a parameter of inflammation (Gardlik et al., 2012). In this study, there was no difference between both DSS groups and, thus, anthocyanins did not manifest any effect on improving the colon length after colitis induction. This result is inconsistent with the result

observed by Xiao and colleagues in mice treated with dried, polyphenol-rich cranberries before and during colitis induction. These authors observed significantly longer colon in the group administered with cranberries compared to the colitis group without dried cranberry access (Xiao et al. 2015). In addition, the effect of polyphenols as a group of antioxidants was described, but authors did not include solely the anthocyanin group. Histological damage was not different between DSS and DSS+A group.

Oxidative stress markers could represent useful indicators of IBD activity and, in fact, many studies were focused on investigation of the association of oxidative status and intestinal disorders (Sundaram et al., 2003; Roessner et al., 2008). We analysed oxidative stress in the colon as a target organ and in the liver, which is often affected by various hepatic diseases in patients with IBD (Albuquerque et al., 2011). In our experiment, the induced colitis did not influence the oxidative stress markers in the colon and other analysed tissues in both colitis groups compared to the control groups. This could be why the anthocyanins did not improve the concentration of oxidative stress markers or antioxidant markers. Oxidative stress markers could have fast dynamics and their concentrations could also be influenced by the DSS/water change. On the other hand, one of the symptoms of colitis, loss of weight, was further deteriorating after exchange of 1% DSS with water.

Several previously published studies focused on the protective effects of anthocyanins with regard to various diseases (Grace et al., 2009; Khoo et al., 2013). Although many of these studies explored the effect of polyphenols as a group including anthocyanins, none of them tested only the specific effect of anthocyanins. Orsi et al. (2014) investigated the effect of pre-treatment with tropical fruit *Hymenaea stigonocarpa* with high polyphenol content on the colitis induced by TNBS acid in rats (five days before colitis inducing and two days during TNBS acid treatment). They observed lower colon MDA in the colitis rats after the treatment with 200 mg/kg stem bark extract concentration and 10 % fruit pulp flour from *Hymenaea stigonocarpa* compared to TNBS-induced colitis without treatment. Mandalari et al. (2011) investigated the effect of natural almond skin polyphenols on the dinitrobenzene sulfonic acid-induced colitis in mice. The authors observed that natural almond skin significantly decreased the TBARS amount analysed in the colon tissue of mice with colitis. In addition, another study reported the effect of pomegranate extract treatment on the DSS-induced colitis in rats. Significantly higher concentrations of FRAP were observed in the plasma of the DSS group treated with pomegranate extract compared to the DSS group without pomegranate extract treatment (25 days before colitis induction and five days during the DSS treatment) (Larrosa et al., 2010). In this study, DSS treatment did not affect the oxidative stress markers. Anthocyanins were administered to animals only three days before DSS treatment.

The study was not focused on long-term intake of anthocyanins or their preventive effect.

The main limitation of our study was the food composition. The food contained only wheat or wheat producing anthocyanins. Higher consumption of fibre could decrease water absorption in the intestine based on the water retention ability. The regular fibre intake could cause the softer stool or diarrhoea (Bosaeus, 2004). This fact could be the reason why the stool consistency was also temporarily deteriorated in the CTRL group. In addition, animals in each group were housed together, so the precise amount of anthocyanin-rich food taken by each animal could not be ensured. On the other hand, we assume that food intake by each mouse was equal because we observed similar dynamics of body weight in animals from one group.

In conclusion, anthocyanin-rich food showed no protective effect on the colitis in the DSS-induced model of IBD in mice. In the future, it would be appropriate to analyse the effect of a mixture of anthocyanins with other types of polyphenols or various concentrations of polyphenol compounds in the food.

Acknowledgement

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