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DNA damage and apoptosis in blood neutrophils of inflammatory bowel disease patients and in Caco-2 cells *in vitro* exposed to betanin

Uszkodzenia DNA i apoptoza w neutrofilach krwi obwodowej pacjentów z nieswoistym zapaleniem jelit i w komórkach Caco-2 *in vitro* poddanych działaniu betaniny

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Inflammatory bowel diseases (IBD) are chronic, relapsing, inflammatory disorders of the gastrointestinal tract, and continuing colonic inflammation is considered an important risk factor in the development of colorectal cancer. Our previous studies showed that beetroot (*Beta vulgaris* var. *rubra*) products and their major component betanin modulate the reactive oxygen species (ROS) production and DNA damage in 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated human polymorphonuclear neutrophils of healthy volunteers.

The aim of the present study was to evaluate the effects of betanin on the oxidative DNA damage and apoptosis in neutrophils isolated from blood of patients with inflammatory bowel disease – ulcerative colitis (UC) and Crohn's disease (CD). The results were compared with those obtained in colon carcinoma-derived Caco-2 cells.

Betanin treatment at the concentration of 100 μM for 24 h increased DNA damage assessed by comet assay in IBD patients' neutrophils. A similar effect although less pronounced was observed in Caco-2 cells. Treatment of Caco-2 cells with H_2O_2 caused a 4-fold increase of DNA strand breaks in comparison to untreated cells, but pre-treatment with betanin reduced DNA damage in these cells. Betanin also induced procaspase-3 cleavage and caspase-3 activity accompanied by the loss of mitochondrial transmembrane potential, indicating its pro-apoptotic activity.

These results suggest that betanin may support mechanisms that lead to the release of ROS and apoptotic cell death. In this way betanin may exert anti-inflammatory and potentially cancer preventive activity.

Key words: **betanin • ulcerative colitis • Crohn's disease • polymorphonuclear neutrophils • Caco-2 cells • DNA damage • apoptosis**

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Wykaz skrótów: **CD** – Crohn's disease; **CDAI** – Crohn's Disease Activity Index; **IBD** – inflammatory bowel diseases; **MTP** – mitochondrial transmembrane potential; **PMNs** – polymorphonuclear leukocytes; **ROS** – reactive oxygen species; **TPA** – 12-O-tetradecanoylphorbol 13-acetate; **UC** – ulcerative colitis.

Introduction

Inflammatory bowel diseases (IBD) are chronic, relapsing, inflammatory disorders of the gastrointestinal tract. The earliest histological lesion in IBD is a focal infiltration of polymorphonuclear neutrophils (PMNs) which produce reactive oxygen species (ROS) [2].

Thus oxidative stress is considered one of the most important etiological and/or triggering factors for IBD [19,16,13]. ROS are very unstable and because of their high reactivity cause lipid peroxidation and oxidative damage to DNA and proteins [5]. Several studies have shown a decrease in total antioxidant activity and increased ROS level in patients with IBD [16,13,21,6]. On the other hand, it was demonstrated that the antioxidant activity of some herbs can positively change the IBD parameters [9]. For example, extract of *Withania somnifera* (Dunal, Solanaceae) root scored positively on histopathological parameters such as necrosis, edema and neutrophil infiltration in a TNBS-induced IBD rat model [18]. One of the most important consequences of chronically active ulcerative colitis (UC) or Crohn's disease (CD) – the two major forms of IBD – is the development of colon cancer. ROS generated by the inflammatory infiltrate are thought to contribute to the generation of dysplastic lesions [20].

Numerous *in vitro* and animal studies have indicated the potential colon cancer chemopreventive properties of phytochemicals such as curcumin, resveratrol, epigallocatechin gallate, quercetin or genistein, usually exhibiting pleiotropic, including antioxidant effects [22].

Beetroot (*Beta vulgaris* ssp. *Vulgaris* var. *rubra*, Chenopodiaceae) is a common ingredient of Eastern and Central European diets [14,17]. Beetroot juice is also used as a popular folk remedy for the treatment of liver and kidney diseases as well as for the stimulation of immune and hematopoietic systems. Recently, considerable interest in the anticancer properties of beetroot has emerged and in the use of the products of beetroot or its constituents as dietary supplements in cancer prevention. Among the various mechanisms that may be involved in the chemopreventive properties of beetroot at the cellular level, antioxidant, free radical-scavenging, anti-proliferative, anti-inflammatory, and pro-apoptotic potentials have been considered [14]. Beetroot is one of the vegetables with the highest antioxidant power, due to the presence of pigments known as betalains [17,1]. Of their red components (betacyanins) present, 75-95% is betanin, considered the principal pigment and active phytochemical of beetroot [11]. Thus, it can be assumed that mainly betanin may be responsible for the beneficial effects of beetroot extract or juice.

Our previous studies showed that beetroot products and betanin modulate the ROS production and DNA damage in 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated human PMNs of healthy volunteers [25,26]. Moreover, our studies also showed that betanin may activate the nuclear factor erythroid-2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway. Subsequent induction of the expression of genes controlled by this factor in human liver cell lines suggested their contribution to anticarcinogenic effects of these beetroot components [15].

The aim of the present study was to further explore the possible mechanisms of anti-inflammatory and anti-carcinogenic effects of betanin by evaluating its influence on oxidative DNA damage and apoptosis in neutrophils of ulcerative colitis and Crohn's disease patients and in the human colon cell line Caco-2.

Materials and methods

Chemicals

Betanin (5-O-glucose betanidine) (Figure 1) was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany). Ac (N-acetyl)-DEVD (Asp-Glu-Val-Asp)-AMC (7-amino-4-methylcoumarin) fluorogenic substrate was obtained from BD Biosciences (Heidelberg, Germany). DMSO, diphenylene iodonium (DPI), n-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), low melting point

(LMP) agarose, luminol, RPMI 1640 medium, 12-O-tetradecanoylphorbol 13-acetate (TPA) and trypan blue were provided by Sigma Chemical Co. (St. Louis, MO, USA).

Patients

Patients with ulcerative colitis (UC) (n = 15) and Crohn's disease (CD) (n = 12) (women and men, aged 42 ± 12 years) were recruited. Disease progress was assessed using the Crohn's Disease Activity Index (CDAI) and the Ulcerative Colitis Symptoms Score (UCSS) for CD and UC, respectively. Peripheral blood was collected from patients by standard venipuncture methods in a sterile tube containing preservative-free heparin (10 U/mL blood). The study was approved by Poznan University of Medical Sciences Ethics Committee.

PMNs isolation, culture and betanin treatment

Fresh blood was layered on a density gradient medium (Gradisol G, d = 1.115 g/mL, Aqua-Medica s.c., Poland) and centrifuged at 500 x g for 30 min. Neutrophils were collected and washed twice with PBS. The final suspension of neutrophils contained more than 96% viable cells, as evaluated by trypan blue exclusion.

PMNs (2 x 10^6 cells) were maintained in RPMI 1640 medium (supplemented with 10% FBS, L-glutamine (2 µM), glucose (2 µg/mL), penicillin (100 units/mL), streptomycin (100 µg/mL), and 5 µM HEPES) in 40 mm dishes. Cells were preincubated with PBS alone (controls) or betanin in the concentrations of 100 and 200 µM and 200 nM TPA at 37°C in a humidified 5% CO_2 atmosphere. The concentrations of betanin and TPA were selected based on the results of our previous study [26]. Three culture dishes were used for each condition. After 2 or 24 h of exposure 1% trypsin solution was used to detach adherent cells.

Caco-2 cell culture and betanin treatment

Caco-2 cells (ATCC, HTB-37) were maintained in DMEM with 20% FBS, 1% nonessential amino acids and gentamycin (50 µg/mL) at 37°C and 5% CO_2 in a humidified air. To assess the effect of betanin on the measured parameters 2 x 10^5 cells/mL of culture medium were seeded. The cells were incubated for 24 h with betanin at the concentration range of 20-200 µM. Subsequently to induce DNA damage and apoptosis the cells were exposed to H_2O_2 (100 µM) at 37°C for 30 minutes.

Cytotoxicity assay

Betanin cytotoxicity was evaluated by MTT assay (Roche, Germany). In brief, cells were plated into 96-well plates at 1 x 10^4 Caco-2 cells per well. Cells were treated with various concentrations of betanin, ranging from 2 to 500 µM (1-250 µg/mL), incubated for 24 h at 37°C, and then washed twice with PBS. The dissolved formazan crystals were quantified using a microplate reader (TECAN infinite M200) at 570 nm.

Comet assay

The cell suspensions were embedded in the low melting point agarose. After cell lysis, DNA unwinding, electrophoresis and neutralization, the slides were dehydrated in 70% ethanol and dried overnight. DNA was stained with SYBRGold. Microscopic evaluation was performed under 200 x magnifications on an epifluorescence microscope (Zeiss Axiovert 200). Image analysis software (CometScore, Tn Tek Corp., USA) was used for quantification of DNA damage. At least three slides for each experimental condition, with 70 randomly selected cells per slide, were analyzed. The results were expressed as the percentage of DNA in the comet tails.

Mitochondrial transmembrane potential (MTP)

The change in MTP was evaluated using Mitochondrial Staining Kit (Sigma, USA). Cells were incubated with 10 µM JC-1 fluorescent dye (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolo-carbocyanine iodide) for 20 min at 37°C and washed with PBS. Fluorescence emission was measured by a FACScan Flow Cytometer (Becton Dickinson, USA) using a 488 nm band pass filter for JC-1 aggregates (red fluorescence) indicating high or normal MTP, and a 525 nm band pass filter for JC-1 monomers (green fluorescence), which represents disrupted mitochondria.

Fluorometric analysis of caspase-3 activity

Caspase-3 activity was measured using the caspase-3 fluorogenic substrate Ac-DEVD-AMC (Caspase-3 assay kit BD, USA). Briefly, cultured cells were washed with ice-cold PBS and lysed in whole cell lysis buffer. Cell lysates (100 µg of protein) and substrate Ac-DEVD-AMC (20 µM) were mixed with standard reaction buffer. After 1 h of incubation at 37°C, the fluorescence of AMC released from Ac-DEVD-AMC was determined in a Hitachi F-2500 spectrofluorimeter (λ_{ex} = 388 nm, λ_{em} = 450 nm). The activity of caspase-3 was expressed in the AMC fluorescence units.

Western blot analysis of procaspase-3 cleavage

Cell lysates were obtained by extracting PMNs or Caco-2 cells in RIPA buffer. Sixty micrograms of protein was resuspended in sample buffer and separated on 12% Tris-glycine gel using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel proteins were transferred onto nitrocellulose membrane (0.2 µm; Sigma, USA), which was blocked with 5% skimmed milk in Tris-buffered saline/Tween. The immunodetection was performed with 1:1000 diluted polyclonal rabbit antihuman caspase-3 antibodies (Santa Cruz Biotechnology, USA), which recognize both inactive procaspase-3 and its cleavage products. Thereafter, the blots were incubated for 1 h with goat anti-rabbit IgG-HRP conjugated (HRP-horseradish peroxidase) antibody (Santa Cruz Biotechnology, USA). The membranes were reblotted with anti-actin HRP-conjugated antibodies to ensure equal protein loading of the lanes. Bands were revealed using LumiLight Western Blotting kit (Roche, Germany).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA). All results were presented as mean ± SEM and compared between experimental groups. P values were determined using one-way analysis of variance ANOVA. The differences were considered statistically significant at $p<0.05$.

Results

Effect of betanin on Caco-2 cell viability

Figure 1 presents the data on Caco-2 cell viability. Betanin at the concentration range of 2-500 µM did not significantly reduce the cell viability. After 24 h incubation with the highest dose of betanin 90% of the cells were viable. In comparison with healthy PMNs, Caco-2 cells were slightly more sensitive to the betanin cytotoxic effect (95% viability at the concentration of 500 µM) [26]. Based on the results of this assay, in the subsequent experiments concentrations of 20-200 µM of betanin were used.

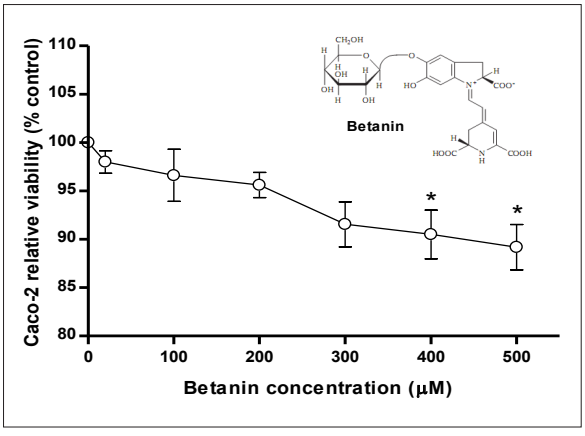

Fig. 1. Effect of betanin on viability of Caco-2 cells. Mean values from three experiments ± SEM are shown. The viability of vehicle-treated cells was considered 100%.

Effect of betanin on DNA damage

DNA damage was evaluated using the alkaline version of the comet assay. In PMNs of IBD patients DNA damage was assessed after treatment with betanin alone or in combination with TPA. Treatment with betanin alone at the concentration of 100 µM increased the DNA content in comet tails, and this effect was more pronounced in PMNs of UC patients in comparison with CD patients. No effect of betanin on DNA damage was observed in PMNs stimulated with TPA (Figure 2A). In Caco-2 cells betanin alone increased DNA damage in a concentration-dependent manner (Figure 2B). Treatment of Caco-2 cells with H_2O_2

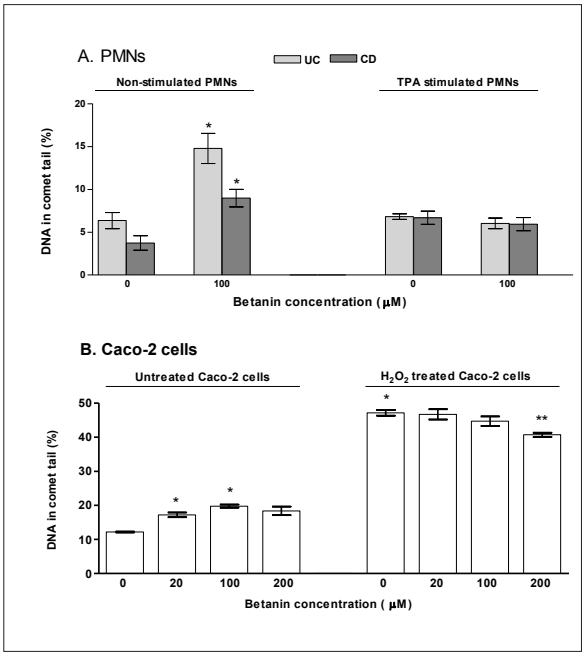

Fig. 2. DNA damage assessed by comet assay A. in DNA of neutrophils from ulcerative colitis patients and Crohn's disease patients or B. in Caco-2 cells. DNA strand breaks were measured with the comet assay, after 24 h incubation with betanin. The data represent mean ± SEM, * $p<0.05$ significantly different from control cells, ** $p<0.05$ significantly different from TPA- or H_2O_2-treated cells

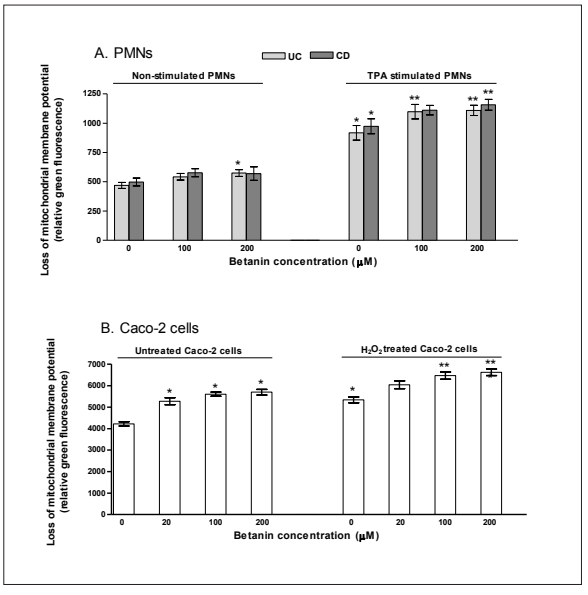

Fig. 3. Loss of mitochondrial membrane potential A. in non-stimulated and stimulated neutrophils and B. Caco-2 cells incubated with betanin as determined by flow cytometry using JC-1 staining. Values shown are the means (± SEM) from UC (n=15) or CD (n=12) neutrophils or Caco-2 cells (n=3), * $p<0.05$ significantly different from control cells, ** $p<0.05$ significantly different from TPA- or H_2O_2-treated cells

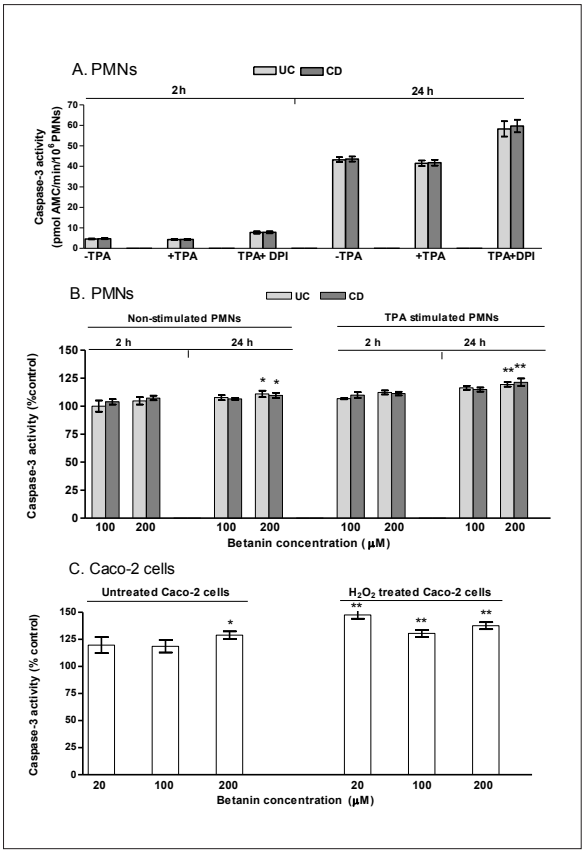

Fig. 4. Caspase-3 activity assayed by measuring fluorescence of AMC, liberated from Ac-DEVD-AMC A. in neutrophils undergoing spontaneous and TPA-stimulated apoptosis; B. after treatment of neutrophils with betanin for 2 and 24 h or C. after treatment of Caco-2 cells with betanin for 24 h. Data represent mean ± SEM, * $p<0.05$ significantly different from control cells, ** $p<0.05$ significantly different from TPA- or H_2O_2-treated cells

significantly increased DNA damage (by ~4 fold) in comparison with untreated cells. Pretreatment of Caco-2 cells with betanin and challenge with H_2O_2 reduced DNA damage (~14%) only at the highest concentration of betanin.

Effect of betanin on mitochondrial transmembrane potential (MTP)

The dissipation of the mitochondrial transmembrane potential is known as an early event in apoptosis. Treatment with betanin did not lead to loss of MTP in neutrophils of IBD patients in comparison with non-stimulated control cells. However, simultaneous treatment with TPA induced the loss of mitochondrial membrane potential in these cells, and no differences between PMNs of UC and CD patients were observed (Figure 3A). Treatment with H_2O_2 alone had a significant effect on the integrity of mitochondrial membrane of Caco-2 cells (i.e. higher green fluorescence) ($p<0.05$), and pretreatment with betanin led to greater loss of its potential in both untreated and H_2O_2 treated cells (Figure 3B).

Caspase-3 activation

The pro-apoptotic protein caspase-3 is known as a major factor in regulation of the cellular apoptotic cascade. The effect of betanin on apoptosis in neutrophils was compared with that of DPI, a flavoprotein inhibitor of NADPH oxidase in neutrophils. Previously we have shown that triggering the neutrophil oxidative burst with TPA blocks caspase activation measured by cleavage of the fluorogenic caspase substrate Ac-DEVD-AMC [26]. NADPH oxidase inhibitor (DPI) reversed the suppression of caspase-3 activity induced by TPA in UC and CD neutrophils at both time points tested (Figure 4A).

Exposure of UC and CD neutrophils to 200 μM of betanin for 24 h significantly enhanced caspase-3 activity in non-stimulated (Figure 4B) as well as in TPA-stimulated neutrophils.

Caco-2 cells treated with 20-200 μM betanin also showed an increase in caspase-3 activity after 24 h incubation (Figure 4C).

The activation of caspase-3 was further confirmed by Western blot, which showed an increased level of caspase-3 cleavage products (17 kDa) as a result of treatment with betanin (100-200 μM), when compared with control stimulated neutrophils after 24 hours (Figure 5A). There was also an increase in the active p17 fragment of caspase-3 in Caco-2 cells following treatment with betanin for 24 h of both resting and H_2O_2-treated cells (Figure 5B).

Discussion

ROS, including superoxide ($O_2^{\bullet-}$), H_2O_2, and hydroxyl radicals, are produced by the partial reduction of oxygen. Under physiological conditions, formation of ROS is counterbalanced by endogenous antioxidant defense systems. When ROS production exceeds cellular antioxidant capacity, oxidative stress can damage DNA, proteins and lipids [23]. Several studies have shown decreased total antioxidant activity and increased ROS production in patients with IBD [16,13,21,6]. Similarly, decreased antioxidant defense was observed in patients with cancer, including colon cancer, which is the major risk factor related to IBD [20]. Since the principal source of ROS in IBD patients is infiltrating PMNs, an increased level of DNA damage in peripheral blood neutrophils might also be expected. However, the results of this study do not confirm this suggestion. The basic levels of DNA strand breaks in PMNs of IBD patients were even lower than those found in our earlier studies conducted in healthy volunteers [26]. Moreover, in contrast to healthy cells, TPA treatment did not increase the level of DNA damage in IBD patients' neutrophils. TPA initiates the signaling pathway leading to activation of NADPH oxidase, whose primary function is generation of ROS [23]. The lack of difference in the level of DNA oxidative damage in non-stimulated and TPA-stimulated PMNs may indirectly indicate that in

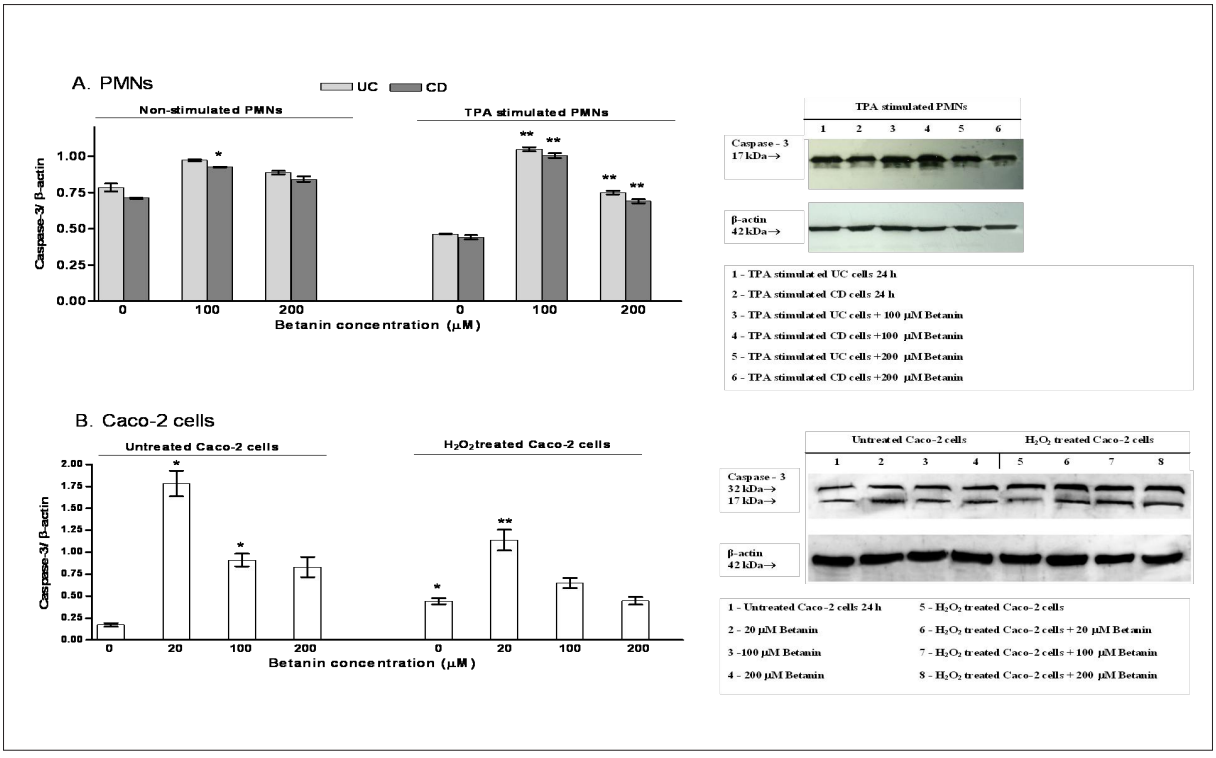

Fig. 5. Western blot analysis of procaspase-3 cleavage A. in PMNs and B. Caco-2 cells. Cells were incubated for 24 h with various concentrations of betanin (20-200 μM) in the presence of stimulants or equivalent volumes of the solvent. The cell lysates were then subjected to SDS-PAGE and western blotting analysis using antibodies against the proform or the cleaved form of caspase-3 (right part). Beta-actin was the internal control. The left part indicates densitometry analysis of the blots. The data represent mean ± SEM, * $p<0.05$ significantly different from control cells, ** $p<0.05$ significantly different from TPA- or H_2O_2-treated cells

IBD patients the TPA signaling pathway is disturbed. Surprisingly, however, increased DNA damage was observed as a result of betanin treatment, particularly in PMNs of UC patients. Moreover, such a tendency was also noted in Caco-2 cells not challenged with H_2O_2. Betanin is one of the most potent naturally occurring antioxidants [11]. It is now recognized that naturally occurring antioxidants can also act as pro-oxidants catalyzing DNA degradation in the presence of metal ions such as copper [12]. Thus it is possible that betanin may support mechanisms that lead to the release of ROS through reduction of Cu(II) to Cu(I), particularly in PMNs of IBD patients. Such a mechanism further supports the results of our earlier studies indicating that chokeberry and apple juices, rich in polyphenols, increased hepatic DNA damage measured in animals treated with the potent carcinogen and oxidative stress inducer N-nitrosodiethylamine (NDEA) [4]. Moreover, the enhancement of DNA damage in PMNs of healthy volunteers was also observed as a result of treatment with phenolic acids or resveratrol *in vitro* (unpublished data).

To clarify the betanin DNA damaging effect, further studies are required with the application of a modified protocol for single cell gel electrophoresis allowing the assessment of oxidation of purine and pyrimidine bases [10] as well as DNA repair kinetics [7].

Treatment of Caco-2 cells with H_2O_2 significantly (~4 fold) increased the DNA strand breaks which are predominant lesions in DNA induced by H_2O_2, although some oxidation of bases also occurs. Strand break rejoining is normally rapid, but treatment with H_2O_2 induces additional breaks and slows down the repair process [8]. Pretreatment with betanin, particularly at the highest dose, tended to accelerate this process and reduced DNA damage in these cells. This effect seems to confirm the phenomena observed in the earlier reports describing genotoxic action of some naturally occurring compounds on human cells, comparable with activity of food mutagens, while in combination with food mutagens these compounds provided anti-genotoxic effects in the comet assay [3].

It was suggested that generating ROS through mobilization of nuclear copper could be an alternative, independent of Fas and mitochondria-mediated programmed cell death, particularly in cancer cells in which copper levels are usually elevated [24].

It cannot be excluded that such a mechanism of cell death is activated also by betanin, although treatment with this compound also induced other forms of apoptosis. Moderate loss of mitochondrial potential as a result of betanin treatment was observed in PMNs of IBD patients and in Caco-2 cells. Betanin also induced procaspase-3 cleavage and caspase-3 activity. The relatively moderate effect

might be related to the doses applied, which were basically non-toxic.

Collectively the results of the present study suggest that betanin may support mechanisms that lead to the release of ROS followed by apoptotic cell death. In this way betanin may exert anti-inflammatory and potentially cancer preventive activity. Further studies are necessary to confirm this suggestion.

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The authors have no potential conflicts of interest to declare.