

Association between intestinal and antioxidant barriers in children with cancer

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Objective: Reactive oxygen species (ROS) play a role in cancerogenesis processing and damage tissues. Furthermore, oncological treatment may impair proper function of the gut barrier. The aim of this study was to measure intestinal permeability in children in clinical remission for solid tumours and to search for a possible relationship between free radicals and the intestinal barrier. No such investigation in children has been reported so far. **Research Methods and Procedures:** The prospective study consisted of 19 paediatric patients with cancer after completion of chemotherapy. 32 healthy children from the outpatients clinics were recruited for measurement of intestinal permeability and antioxidant barrier as a control group. Intestinal permeability was assessed by measurement of urinary lactulose and mannitol after oral challenge. Antioxidant enzymes: superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in erythrocytes were assessed. Ischemia modified albumin (IMA) concentration was measured in serum. **Results:** Cancer patients excreted less mannitol and more lactulose versus controls. The ratio of lactulose to mannitol was significantly higher in oncological children vs control (mean 0.188 and 0.0453, respectively, $p=0.0006$). Significantly higher IMA level in the oncological group vs control was noted (mean 123.8 and 87.3 U/ml, respectively, $p=0.0037$). No correlation between intestinal permeability and oxidative stress barrier was found. **Conclusions:** Our data shows that intestinal barrier is damaged in paediatric cancer patients after chemotherapy. IMA is believed to play a protective role in the defence against tissue damage. No correlation was found between these two barriers.

Key words: intestinal permeability, oxidative stress, childhood cancer

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INTRODUCTION

The side-effects of chemotherapy in paediatric oncological patients have been of increasing interest in recent years. More intensive multi-drug chemotherapy may lead to changes in normal tissues, especially those with a high proliferative rate like epithelia of the gastrointestinal tract (Trier, 1968). One of the most uphill post-chemotherapy complications is mucosal barrier injury (MBI), including gastrointestinal mucositis (Vliet *et al.*, 2009; Sonis *et al.*, 2004). It is important to note, however, that this kind of inflammation occurs mainly in the small intestine.

Currently, a five-stage model for MBI is in use. The first two stages are characterised by local inflammation of the intestine, while the third stage is dominated by the loss of enterocytes and submucosal cells. Next is an ulcerative stage during which bacteria and their products are proposed to translocate. The fifth and final stage is the healing one. It leads to a structurally normal mucosal barrier, but it is not known if the healed barrier is fully functional (Sonis *et al.*, 2004; Sonis, 2004). Lesions of the small bowel mucosa are associated with nutritional problems, leading to malnutrition and the necessity to establish a proper diet. They may also cause a reduction of cytostatics doses, implicating a decrease in the overall survival in cancer patients (Eltong *et al.*, 2003). A damaged intestinal barrier may allow passage of toxic substances or antigenic macromolecules or bacteria, which may be the reason of some severe adverse-effect such as typhlitis, enterocolitis, infective colitis, perforation, gastrointestinal haemorrhage, ischemic colitis and obstruction (Cronin *et al.*, 2009). However, few reports describe sub-clinical damage and its role in the absorption of nutrients and drugs such as fluoropyrimidines, alkylating compounds, anthracyclines and a taxane/platinum combination (Melichar *et al.*, 2005; Melichar *et al.*, 2007).

Cytostatics may provoke intestinal damage by a decrease in the functioning of the intestinal barrier according to cell proliferation inhibition, and macrophages and neutrophils infiltrate into the inflammatory invasion of endotoxins (Maeda *et al.*, 2010). In a biophylaxis reaction, macrophages and neutrophils infiltrate into the inflammatory area and produce reactive oxygen species (ROS) (Gao *et al.*, 2001; Miyazono *et al.*, 2004). Free radicals, among other effects, play a role in DNA damage and clonogenic cell death in the epithelial layer and in activation of nuclear factor- κ B, which up-regulates inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and molecules of adhesion. This inflammatory-related transcription factor may be involved in small bowel inflammation (Sonis *et al.*, 2004; Sethi *et al.*, 2008; Mantovani *et al.*, 2008). ROS regulate the paracellular permeability and tight junction opening (Sheth *et al.*, 2003). Defence mechanisms of cells are programmed to protect cells from reactive oxygen species. Superoxide dismutase and glutathione peroxidase belong to the antioxidant repair

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Abbreviations: BPH, benign prostatic hyperplasia; BUN, blood urea nitrogen; GSH-Px, glutathione peroxidase; IMA, ischemia modified albumin; LMER, lactulose-mannitol excretion ratio; MBI, mucosal barrier injury; ROS, reactive oxygen species; SOD, superoxide dismutase.

system (Gadjeva *et al.*, 2008). This study is a continuation of our earlier study concerning the antioxidant status in children with neuroblastoma and soft tissue sarcomas (Stachowicz-Stencel *et al.*, 2011). One of the most promising oxidative stress markers in paediatric oncological patients seems to be ischemia-modified albumin. Free radicals may modify the N-terminal region of albumin and thus increase IMA levels (Roy *et al.*, 2006). While numerous studies concerning the role of IMA in ischemic disorders have been published, only one paper evaluating the role of this marker in paediatric oncology was found (Stachowicz-Stencel *et al.*, 2011).

The aim of the present study was to measure intestinal permeability in children in clinical remission after chemotherapy for solid tumours. The lactulose-mannitol test is a simple, reliable, non-invasive and safe tool to estimate early-stage intestinal mucosal damage. Thus it seems useful in clinical practice to establish a diet which could help in recovery the proper function of gastrointestinal tract. Furthermore, we made the necessary attempt to find a relationship between antioxidant barriers parameters and the intestinal barrier.

MATERIALS AND METHODS

Patients. Nineteen paediatric patients with cancer, who were diagnosed and treated in the Department of Paediatrics, Haematology, Oncology and Endocrinology, Medical University of Gdansk, in 2008 and 2009 were enrolled in the study. There were 12 males and 7 females, aged from 2 to 20 years, mean age 10.5 years, median age 9.8 years. All patients had no symptoms of infection (thus excluding any influence of inflammation) at the time of recruiting and before. A complete blood count, C-reactive protein concentration, erythrocyte sedimentation rate, alanine and aspartate transaminases, blood urea nitrogen (BUN), serum creatinine and albumin were performed to check the health status. No patients were receiving vitamin supplementation. The studies were performed at least two years after finishing oncological treatment. This period seems to be sufficient to recover the normal function of damaged organs. All patients received multi-drug chemotherapy. In our study we compared intestinal permeability between healthy control and oncological patients (Table 1).

Control group. Thirty-two healthy children from the outpatient clinics were recruited for measurement of intestinal permeability and antioxidant barrier as a control group (18 girls and 14 boys), aged between 2 and 18 years, mean age 9.97 years, median age 10.92 years. At the time of the study, on the basis of detailed medical history and clinical examination, all children were found to be in complete health with no contemporaneous disease. None of the participants was taking vitamin and/or antioxidant supplements for at least 8 weeks prior to the beginning of the study.

The study was approved by the Ethical Committee of the Medical University of Gdansk.

Laboratory analysis. All assays were performed in the laboratory of the Department of Clinical Nutrition, Medical University of Gdansk, Poland.

Measurement of intestinal permeability. Intestinal permeability was assessed by measurement of urinary lactulose and mannitol after oral challenge. After a 6 hour overnight fast the bladder was emptied completely. Then urinary collection started. Each patient drank a solution containing 10 g/l of mannitol and 75 g/l of lactulose in quantiles 100 ml/m² body surface.

Table 1. Intestinal permeability parameters in the controls and cancer patients

	Control											Cancer patients											P
	X	M	S.D.	min	max	25Q	75Q	n	X	M	S.D.	min	max	25Q	75Q	n							
Age	9.97	10.92	4.26	2.33	18.00	6.00	13.67	32	10.5	9.8	6.2	2.0	20.0	4.0	17.0	18	0.848						
Sex F/M	18/14								7/12								0.293						
Body surface area (m ²)	1.18	1.30	0.39	0.58	1.92	0.81	1.45	32	1.12	1.10	0.42	0.50	1.76	0.70	1.48	19	0.718						
Urine volume (ml)	153.4	110.0	128.8	40.0	738.0	84.0	165.0	30	298.2	250.0	211.9	60.0	900.0	180.0	400.0	19	0.0007						
Volume of oral intake of lactulose/mannitol (ml)	118.1	130.0	39.0	58.0	192.0	81.0	146.0	32	112.3	110.0	41.6	50.0	176.0	70.0	148.0	19	0.675						
Mannitol excretion (%)	10.7	10.6	8.5	1.3	44.5	4.3	13.8	32	5.50	2.46	7.03	0.00	26.97	1.25	7.89	19	0.0014						
Lactulose excretion (%)	0.360	0.285	0.278	0.036	1.270	0.155	0.495	32	1.26	0.52	2.81	0.06	12.74	0.30	0.99	19	0.0088						
LMER	0.0453	0.0349	0.0344	0.0067	0.1501	0.0191	0.0662	32	0.188	0.167	0.158	0.000	0.589	0.081	0.271	19	0.0006						

LMER, lactulose/mannitol ratio; X, mean; M, median; S.D., standard deviation; n, number of patients

All urine passed for the following 5 h was collected in urine container. No food was allowed for the duration of the test, although very small amount of water was available. At the end of the urine collection the bladder was emptied completely. The volume of the urine samples was noted and preserved at -20°C until analysed.

Urinary estimations of lactulose and mannitol were measured by the enzymatic analyses described by Northrop *et al.* (1990) and Lunn *et al.* (1989), respectively.

The lactulose-mannitol excretion ratio (LMER) was calculated by means of the following ratio after correction for basal samples: $\text{LMER} = \frac{\text{percentage of lactulose excreted in 5 h}}{\text{percentage of mannitol excreted in 5 h}}$.

Oxidative stress measurement

Superoxide dismutase (SOD) assay in erythrocytes. SOD activity in erythrocytes was assessed according to the Ransod (Randox, Crumlin, United Kingdom) procedure. The increase of absorbance was followed at 505 nm for 3 min on a UV-VIS spectrophotometer (LKB Pharmacia, Great Britain United Kingdom). Results were expressed in U/g Hb.

Glutathione peroxidase (GSH-Px) assay in erythrocytes. GSH-Px in erythrocytes was measured according to the Ransel (Randox, Crumlin, United Kingdom) procedure. The decrease of absorbance was recorded at 340 nm for 3 minutes. GSH-Px was expressed in U/g Hb.

Ischemia modified albumin (IMA) in serum. The concentration of ischemia-modified serum albumin was determined by Bhagavan *et al.* (2003). The absorbance of assay mixtures was read at 470 nm with a LKB Spectrophotometer. The value of absorbance was given in units [U/ml serum] with the use of ACB Roche calibrator of 221 U/ml as a standard.

Statistical analysis. For each parameter mean (X), median (M), standard deviation (SD), range (min, max), and lower and upper quartile (25Q, 75Q) were calculated. Statistical significance between means for different groups was calculated by one-way analysis of variance (ANOVA), alternatively using the non-parametrical Kruskal-Wallis test, when the variances in groups were not homogeneous (the homogeneity of variance was determined by the Bartlett's test) or the number of cases in groups was too small to use parametrical tests. Statistical significance between frequencies was calculated by the chi-square test with Yate's correction.

Table 2. Oxidative stress barrier parameters in the controls and cancer patients

	Cancer patients										p						
	X	M	SD	min	max	25Q	75Q	n									
SOD (U/gHb)	2144.2	1982.0	770.5	1382.0	6167.0	1714.0	2416.0	32	1859.4	1890.3	544.8	980.0	3320.0	1600.0	1830.0	19	0.147
GSH-Px (U/gHb)	21.3	21.4	5.6	9.0	31.0	18.0	26.0	32	23.8	23.0	7.56	12.3	39.1	20.3	28.1	19	0.319
IMA (U/ml)	87.3	95.8	38.3	4.9	133.3	63.1	119.9	32	123.8	117.2	33.5	53.6	190.1	95.9	150.8	19	0.0037

SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; IMA, ischemia modified albumin

Relation between two parameters was assessed using correlation analysis and Pearson correlation coefficients were calculated. A p value of less than 0.05 was required to reject the null hypothesis. Statistical analysis was performed using EPIINFO Ver. 3.4.3 (08-11-2007) software package.

RESULTS

We found statistical significant difference for lactulose and mannitol urine excretion between patients and controls ($p=0.0088$ for lactulose and $p=0.0014$ for mannitol). The cancer patients, who received chemotherapy, excreted less mannitol (mean 5.50%) than the controls (mean 10.7%). In contrast, excretion of lactulose in the oncological group was higher (mean 1.26%) than in the control group (mean 0.360%). The ratio of lactulose to mannitol (LMER), which is an estimation of intestinal wall permeability, was significantly higher in children with cancer (mean 0.188) than in the controls (mean 0.0453) ($p=0.0006$).

In the same group of patients we also measured parameters of oxidative stress barrier. These were: SOD, GSH-Px and IMA (Table 2). Our study revealed very interesting results for IMA. The ischemia modified albumin level was significantly higher in the patients with neoplasms (mean 123.8 U/ml) than in the control group (mean 87.3 U/ml) ($p=0.0037$).

In this particular group of patients there was no correlation between intestinal permeability (considered as LMER) and SOD ($r=0.17$, $p=0.510$), LMER *vs* GSH-Px ($r=0.12$, $p=0.629$) and LMER *vs* IMA ($r=0.09$, $p=0.723$).

DISCUSSION

The measurement of the intestinal barrier integrity seems to be a useful tool in the diagnosing and monitoring of the severity of damage of the intestinal mucosa in neoplastic diseases. We have attempted here to establish of useful markers for the antioxidant barrier in paediatric oncological patients. It is known that during cancer both the intestinal and antioxidant barriers are damaged (Van Huyen *et al.*, 1998; Brown & Mayer, 2007). For this reason we made an attempt to find a relationship between parameters of these two barriers.

As we examined oncological paediatric patients, who very often are in a poor physical and psychological condition, it was necessary to find a safe and non-invasive method of measurement of small bowel permeability. The

lactulose-mannitol test meets these criteria and is used in clinical practice (Katz *et al.*, 1989; Zhang *et al.*, 2000). It is assessed by measuring orally administered substances in urine. There are a few hypotheses concerning the passage of substances of different sizes through the mucosa. In healthy people small molecules pass through small and large size pores, while large molecules only via large ones. Another theory describes paracellular route through intercellular junctions (e.g. the lactulose) and transcellular route through cellular membrane (e.g. the mannitol). The single paracellular permeation model, which is another potential explanation, tells about the tightness of intercellular tight junctions between epithelium cells and an increase of sugar migration from crypts to the villus tip (Brown & Mayer, 2007; Vilela *et al.*, 2008). It was observed that in children with diarrhoea there was an increase in the mucosal permeability for lactulose and a decrease for mannitol, which resulted in an increase of LMER (Zhang *et al.*, 2003). Pearson *et al.* (1984) noticed the same situation in paediatric patients with acute lymphoblastic leukemia. Our study is in agreement with those authors. We observed reduced permeation of small molecules such as mannitol and an increase for the larger lactulose. The lactulose to mannitol ratio was significantly higher in cancer patients than in the control group. A study of Pledger *et al.* (1988) on a group of patients with childhood tumours of different histological types revealed decreased permeation of mannitol, but no changes in the intestinal permeability of lactulose. The authors made the suggestion that the reason for the observed difference were various types of cytostatics and treatment schedule (continuous or pulses of chemotherapy). In contrast, Benjamin *et al.* (2008), who examined patients with Crohn's disease, reported no difference in mannitol secretion, but a significant increase in lactulose excretion. For this reason the LMER was also abnormal. All measurements were compared with a control group. Similar findings were observed in studies of Pearson *et al.* (1982) and Katz *et al.* (1989). The authors suggest that the lack of difference in mannitol urine excretion between patients with Crohn's disease and healthy controls is connected with the mode of sugar transmission through the intestinal mucosa. Mannitol uses tight junctions in the villous tips, not the pores in the villous crypts as lactulose does. This could be a reason for such result. It is described that, in patients with Crohn's disease, generally there is no villous atrophy, so mannitol excretion may be unaffected in this situation. In oncological patients there is diffuse villous atrophy, so both lactulose and mannitol urine excretion is abnormal (Van Huyen *et al.*, 1998).

It is widely known that the intestinal mucosa in normal conditions is an absorbent and a defense organ. In patients with cancer it is often observed that malnutrition leads to cachexia; the cachexia-anorexia syndrome is associated with lack of ability of using aggressive chemotherapy and leads to poor response to therapy (Argilés, 2005). In malnutrition, the small intestine morphology and physiology change (Monteleone *et al.*, 2004). Some studies show an increased risk of systemic infections and post-operative sepsis in malnourished patients (Meguid *et al.*, 1988; Deitch, 1992).

The intestinal mucosal barrier may be disrupted in increased oxidative stress condition, leading to its increased permeability (Musch *et al.*, 2006). During oxidative stress there is an imbalance between generation of ROS and the antioxidant defence mechanisms (Hamer *et al.*, 2009; Stocker & Keaney, 2004), as evidenced by various oxidative stress biomarkers such as GSH-Px, SOD and IMA.

SOD is the first line of defence against production of ROS, which eliminates the superoxide radical (O_2^-). GSH-Px and catalase together detoxify H_2O_2 by converting it into water and molecular oxygen (Michiels *et al.*, 1994). In the present study SOD activity in erythrocytes of paediatric cancer patients was lower than in the control group. The decreased activity of SOD causes accumulation of cellular O_2^- , damages mitochondrial membranes and leads to apoptosis of cancer cells (Huang *et al.*, 2000). The similar observations were made by Gadjeva *et al.* (2008) who described significantly lower erythrocyte SOD activity in patients with melanoma, and Kato *et al.* (2003) in adult patients with leukaemia. By contrast, in a study by Devi *et al.* (2000), on children and adults with different types of leukaemias the superoxide dismutase activity was higher *versus* controls. Badjatia *et al.* (2009) suggested that lower levels of antioxidants in patients with urothelial carcinoma of the bladder were connected with progression of cancer. Other authors thought that this condition may be related to the scavenging of lipid peroxides and their sequestration by tumour cells (Sharma *et al.*, 2007). In patients with gastric cancer SOD and GSH-Px activities were lower. Superoxide is transmitted through membranes and may have destructive influence on sites far from the tumour. In cancer patients lipid peroxidation of erythrocytes is probably due to O_2^- produced by the tumour and low activity of SOD. The low level of GSH-Px may be in relation with increased scavenging of lipid peroxides (Arivazhagan *et al.*, 1997). However, observations made by Dursun *et al.* (2006) concerning adult patients with oesophageal and gastric cancer showed high activity of SOD but low activity of GSH-Px in erythrocytes. The authors explained that the increased activity of SOD leads to an increase in the conversion of O_2^- into H_2O_2 . H_2O_2 may be converted into hypochlorous acid and hydroxyl radical (OH) by myeloperoxidase. OH as a more reactive radical may cause oxidative damage (Dursun *et al.*, 2006).

To the best of our knowledge, there is no published study that describes relation of IMA to intestinal permeability in cancer. Our study revealed significantly increased levels of IMA in cancer patients. Most likely, ischemic tissues modify the albumin N-terminus making it unable to bind cobalt (Bar-Or *et al.*, 2001; Polk *et al.*, 2008). Free radicals may modify the N-terminal region of albumin resulting in increased in IMA levels (Cichota *et al.*, 2008; Roy *et al.*, 2006). We are aware of only one report evaluating IMA in prostatic diseases. The authors observed a slight increase in IMA level in prostatic cancer and a significant increase in benign prostatic hyperplasia (BPH). The cause of the increase IMA may be inflammation connected with BPH that may lead to release of free radicals (Mastella *et al.*, 2009). It is suggested that very low or very high serum albumin levels may influence IMA concentration and lead to unreliable results (Apple *et al.*, 2005; Gaze *et al.*, 2006). Some authors showed a statistically significant negative correlation between serum albumin and IMA in both healthy adult outpatients and in patients with a suspicion of acute coronary syndrome (Lippi *et al.*, 2007; Lee *et al.*, 2007). In our previous paper, we observed the same inverse relationship between IMA levels and serum albumin concentrations in pediatric cancer patients (Stachowicz-Stencel *et al.*, 2011).

In summary, our data shows that the intestinal barrier is damaged in paediatric cancer patients after chemotherapy. As to the oxidative stress barrier parameters, only IMA level was significantly higher in the oncological patients after treatment *vs* controls. Further studies

are needed in order to improve its role as a protective substance against damage of tissue. No correlation was observed between the antioxidant and intestinal barriers.

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