Bulgarian Chemical Communications, Volume 56, Special Issue D1 (pp.142-147) 2024 DOI: 10.34049/bcc.56.D.S1P54

Measurement of oxidative stress-related markers in gastro-intestinal damages in Bulgarian pediatric patients

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Received: November 3, 2023; Revised: April 11, 2024

Cow's milk allergy (CMA), affects ~2 - 7.5% of the infants, and results in an immunological response to casein and α-/β-lactalbumins such as: skin rashes, respiratory and gastrointestinal disturbances. Inflammatory bowel disease (IBD), affecting adolescents, is sustained by an impaired immune/ inflammatory response against intestinal microorganisms. Reactive oxygen/nitrogen (ROS/RNS) overproduction in the gastrointestinal tract damages the mucosa in CMA and IBD. The aim of the present study was to determine ROS/RNS and oxidative disturbances in the intestinal mucosa influence in CMA and IBD. We investigated the levels of advanced protein (OPC), protein carbonyls (PCC), nitric oxide (•NO) and total antioxidant capacity (TAC) in blood serum in CMA and IBD patients. The results showed a significant increase in PCC ($p < 0.0001$), OPC ($p < 0.0001$), and increased •NO deposition ($p < 0.001$) and TAC ($p < 0.0001$) that likely induce oxidative damage in CMA *versus* IBD patients. In conclusion, the ROS/RNS accumulation, and oxidative damage to the protein skeleton in the colonic mucosa play a key role in the CMA and IBD pathophysiology, especially in the initiation, duration and maintenance of mucosal inflammation.

Keywords: CMA, IBD, OPC, PCC, oxidative damages

INTRODUCTION

Cow's milk protein allergy (CMA) is a form of food allergy in infants and children and is a common cause for gastrointestinal mucosal inflammation (2 - 7.5 % frequency) [1-3]. Epidemiological studies indicate that 5-15% of children exhibit symptoms suggestive of CMA [4]. The most common allergens are β-lactoglobulin, α-lactoalbumin, bovine serum albumin, globulin and casein. Other important allergens are egg whites, ovalbumin, soy protein, peanuts, fish, and (beef, pork, chicken) meat [5].

Clinical presentation includes general and gastro-intestinal symptoms; skin manifestations; respiratory injuries; and the complaints start in the first two hours in case of IgE-mediated allergy and several days/weeks in the case of non-IgE-mediated allergy [3, 5] after CMA ingestion. Gastrointestinal food allergy is usually considered as non-IgE mediated, whereas eosinophilic dominant disorders could be mixed IgE and non-IgE allergies. Infants less than <1 year, are victims of gut-affecting non-IgE-mediated allergies [2, 6]. The antigens cross the intestinal barrier and are recognized by M-cells in the mucosa, which convey the information to antigen presenting cells. Thus, APCs present the antigen to T-helper lymphocytes (Th0), which cause overexpression of the Th2 response, so that cytokines such as interleukins- IL-4 and IL-13, are released and stimulate B-lymphocytes to synthesize specific IgE [7]. In cases of non-IgE-mediated allergy, IL-5 and TNF-α are synthesized which promote recruitment of neutrophils and eosinophils activation and infiltration and determine the appearance of edema, pain and abnormal functioning [6-8]. CMA induces an increase in eosinophils in the intestinal mucosa, with affection on the rectum and duodenum. Focal erythema, erosions, lymphoid nodular hyperplasia and partial villous atrophy were reported [6, 8]. In 62% of the gastrointestinal biopsies inflammation and eosinophilic infiltration were found [3, 6-8]. The allergic inflammation in the intestinal mucosa leads to an increase in some oxidative stress (OS) markers, in particular nitric oxide (•NO) and protein levels [9].

Inflammatory bowel diseases (IBD) are chronic, idiopathic and complex diseases of the gastro-intestinal tract, with two forms: ulcerative colitis (UC) and Crohn's disease (CD) [10, 11]. The IBD incidence and prevalence was increased [12, 13] and CD/ UC pathogenesis involves genetic, immune, and environmental factors leading to disruption of the delicate homeostasis between host immunity and the gastrointestinal microbiome [10, 11, 13, 14]. Mucosal tissue infiltration with activated phagocytic immune cells generating ROS/RNS induces

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prooxidants generation [15, 16]. In addition, ROS/ RNS are predominant mediators responsible for the intracellular damages of proteins, lipids, nucleic acids, and being highly reactive, upregulate the gene expression and reduce immune responses adaptation [16-19]. ROS/ RNS and OS are related to inflammatory responses [20] and have been implicated in the IBD exacerbation [14, 21-23]. The increased intracellular ROS/ RNS production, but higher superoxide dismutase (SOD), glutathione peroxidase (GP_X) and catalase (CAT) activities [15] and higher nitric oxide (•NO) [24] were found in UC patients, than in controls. Moreover, Lih-Brody *et al.* [25] observed increased protein carbonyls content, DNA oxidation and iron content, but decreased/ increased activity of copper and Cu-Zn SOD in CD and UC biopsies. The study concluded that an imbalance in the formation of ROS and antioxidant trace elements may be important in the pathogenesis of tissue damage in IBD and may provide a rationale for therapeutic modulation with antioxidants.

On these bases, the aim of the present study was to investigate and compare the oxidative stress biomarkers and the clinical characteristics of a group of children with gastrointestinal problems, e.g. CMA and IBD.

EXPERIMENTAL

The group included 35 CMA patients (15 boys, 20 girls), at age 1 month - 12 years (29 infants – median age range 5.2 months; and 6 toddlers at 2 - 12 years, median age range 7.6 years) and 18 IBD patients (8 males; 10 females) at age 13 - 17 years (median age range 14.1 years). All patients have been treated in Trakia Hospital and UMAT Hospital "Stoyan Kirkovich", Stara Zagora, Bulgaria, between 2022-2023 (Table 1).

Table 1. Demographic and clinical characteristics of CMA and IBD patients; p-values *p < 0.01, **p < 0.001, **p < 0.0001 by Students t-test or Mann–Whitney test.

Demographic and clinical characteristics	CMA (n= 35)	IBD (n= 18)	p
Gender			
-male	15 (39 %)	8 (49 %)	p < 0.0001
-female	20 (61 %)	10 (52 %)	p < 0.0001
BMI (kg/m^2)	≤ + 1 SD	≤+1SD, ~34,9%	-
Age (years) :			-
Infants	~5.2 months	-	
Toddlers	~7.6 years	~14.1 years	-
Disease duration (months/years)	2 months	3 years	p < 0.001
			p = 0.01
Diagnostic delay (months)	1 month	6 months	
			p = 0.083
Disease behavior			
Clinical signs of inflammation	100%	100%	
Colonoscopy	-	(+) positive	p = 0.00
Histological study	-	-	-
Active IBD	-	2 (9%)	-
Remission	1	16 (95%)	-
Fecal calprotectin	-	~1290 μg/g	p <0.0001
			-
Extra-intestinal disease			
-Skin	14 (47 %)	No	
-Arthritis			-
Allergies yes/no			
Esonophilia	21 (64%)	No	
Atopic dermatitis	14 (47 %)	-	-
Bronchial asthma	2 (3.9 %)	-	
Eosinophilic esophagitis	3 (4.3 %)	-	
Familial CMA/ IBD yes/no	16 (49%)	No	
			-
Pharmacotherapy			
5-aminosalicylates (5-ASA)	No	10	
Steroids	No	1	
Azathioprine	No	4	
Biologics	No	3	

The assessment of patient health status was based on medical history, physical examination, laboratory data and imaging studies.

The exclusion criteria were: 1) evidence of intestinal infection; 2) other acute infection.

The inclusion criteria were: 1) CMA group - positive elimination and challenge test for cow milk (infant formula) with/ without blood eosinophilia and increased IgE; 2) IBD group - positive colonoscopy findings and histology and increased fecal calprotectin.

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethic Committee by Trakia Hospital and UMAT "St. Kirkovich" Hospital (code: 10-816/ 12 Oct. 2019). All subjects or legal guardians gave written consent.

Serum samples (1.2 ml) were collected from patients after an all-night rest, at 7.30 a.m., and centrifuged (2000×g; 4 ˚C, 10 min).

Advanced oxidation protein content (OPC): 30 µl serum, 970 µl PBS, 50 µl KI (1.20 M) and100 µl acetic acid were mixed and immediately read at 340 nm. OPC were quantified in µmol/mg proteins by using the standard chloramine-T (Sigma-Aldrich, Germany) [26].

Protein carbonyl content (PCC): Serum PCC was evaluated by an immune-enzyme method using commercially available ELISA kit in nmol/mg (126287/BioVision Inc., CA, USA) [27].

Total antioxidant capacity (TAC): The 30 µl serum was mixed to ABTS•$^{+}$ solution (3-ethylbenzothiazoline-6-sulfonic acid) immediately read at 660 nm in µmol/mg protein, by using the standard Trolox (Sigma-Aldrich, Germany) [28].

Nitric oxide generation (•NO): The mixture of 30 µl serum, 50 µM carboxy-PTIO.K, 50 mM Tris (pH 7.5) and DMSO at a ratio 9:1, was centrifuged at 4000 rpm, 10 min, 4°C. The spin-adduct between carboxy-PTIO and •NO radicals was measured in a.u. [29].

Statistical analysis: The data were analyzed using Statistica (Version 10.0, StaSoft, Inc., USA), and performed using one-way ANOVA. Student's t-test or Mann-Whitney–U test was used to compare the groups. EPR spectral processing was performed using Win-EPR and Simfonia software after consecutive replicates. The $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

OS is not only a consequence of acute and chronic inflammation, but has an essential role in the development and maintenance of inflammation levels, and therefore, abnormal immune response in CMA and IBD in infants and toddlers [5, 20, 30]. Our results demonstrated significant differences in gender ($p < 0.0001$), in median age range ($p < 0.0001$), in disease duration ($p < 0.01$), in diagnostic delay ($p = 0.083$), and in inflammation level ($p = 0.01$), (Table 1). In our study, only 2 patients of the CMA group presented bronchial asthma (3.8%); tree more patients (4.3 %) had eosinophilic esophagitis; 14 patients (47 %) had presents of atopic dermatitis; 21 patients (64%) had presents of esonophilia. The clinical data demonstrated significant differences in familial CMA presents in 16 (49%) infants, and no familial history for IBD group. Based on the international WHO norms for age/ sex-specific BMI, the significant differences were not measured. Ambulatory fecal calprotectin (~1290 µg/g; FC) and 18 positive (+) colonoscopy were used for IBD confirmation. Patients in active IBD were 2 (9%) and in IBD remission - 16 (95%; $p < 0.0001$) *versus* CMA children.

It is widely accepted that the use of >3 medicaments significantly taints a clinical study. For this reason, we used patients in IBD group, taking only 1 medicament: 10 patients took 5-aminosalicylates (5-ASA) medicaments; 1 patient ttook steroids medicaments; 4 patients took azathioprine medicaments; 3 patients had biologics pharmacotherapy.

In regard to OS, our results show an overall increase in oxidative biomarkers in patients with CMA compared to IBD, highlighting that severe allergic activity directly reflects acute oxidative stress-induced protein damages (Table 2). Among the markers analyzed, OPC (mean 12.73 (4.12 – 16.19) µmol/g *vs*. 9.47 (3.75 – 10.71) µmol/g of oxidize proteins; $p < 0.0001$) and PCC (mean 11.48 (7.53 – 12.49) nmol/mg *vs*. 7.69 (5.42 – 11.061) nmol/mg of oxidize proteins; $p < 0.0001$) demonstrated the greatest statistically significant difference in CMA from IBD patients. In CMA children, probably the acute-phase induced excessive formation of di-tyrosine-containing cross-linked products, after the activation of serum proteins (albumin) with chlorinated compounds [30, 31], compared to IBD children.

Table 2. Mean values of oxidative markers in serum samples from CMA patients and IBD patients. The means ± SD and **p < 0.0001 by Student's t-test or Mann–Whitney test were statistically significant; a.u.: arbitrary units.

Oxidative stress markers	CMA (n =35)	IBD (n = 18)	p
OPC (μmol/mg advanced proteins)	12.73 (range 4.12 – 16.15)	9.477 (range 3.75 – 10.14)	p < 0.0001
PCC (nmol/mg)	11.46 (range 7.12 – 12.49)	7.78 (range 5.13 – 11.06)	p < 0.0001
TAC (μmol/mg protein)	3.97 (range 0.87 – 4.11)	1.59 (range 0.91 – 2.07)	p < 0.0001
•NO deposition (a.u.)	7.39 (range 5.16 – 8.22)	5.09 (range 2.99 – 6.044)	p < 0.0001

In this sense, OS-induced protein damage in CMA allergy is associated with excessive ROS/RNS production that depletes serum antioxidants two-fold. As in the IBD disorders, mucosal ROS-infiltration only overwhelms tissue antioxidant defenses [30, 32]. AOPP and PCC contents activate protein kinase C and nicotinamide adenine, NADPH oxidase, as well as the NF-κB-dependent oxidative and inflammatory pathways [30, 33].

Increased OS damages and protein modification (↑ OPC and ↑ PCC) in serum correlated with reduced antioxidant barrier, significantly increased TAC (mean 9.37 (0.78–4.11) μmol/mg *vs*. 1.58 (0.9–2.03) μmol/g proteins; p < 0.001), in children with CMA, compared to IBD children. The TAC content was almost 8-fold higher in the blood of children with CMA (~5.2 months age), which suggests an un-adaptive antioxidant response of the organism to ROS/ RNS overproduction. Although we did not directly evaluate ROS concentration, our hypothesis can be confirmed by a positive correlation between increased protein content and and TAC levels in CMA children, compared to IBD children.

The nitric oxide (NO/ •NO radical) is the most important vasodilator helping the proper function of blood vessels [34, 35]. Interestingly, we found a sharp increase in serum •NO radical concentration (mean 7.44 (5.14 – 8.25) a.u. *vs*. 5.07 (2.99 – 6.04) a.u.; p < 0.001) in CMA children, compared to IBD children. The main mechanism increasing the •NO radical bioavailability is direct oxidation with superoxide anion radical ($•O_2^-$) leading to the formation of highly reactive peroxynitrite with strong oxidizing properties, which interferes with mitochondrial function and further oxidizes thiol groups of enzymes and signaling proteins [33, 36, 37]. This can be confirmed by a positive correlation between increased TAC *vs*. •NO (r = 0.86, p < 0.005), PCC vs •NO (r = 0.81, p < 0.002), •NO *vs*. OPC (r = 0.81, p < 0.001) levels in CMA children, compared to IBD children. Intestinal mucosal inflammation promotes ROS/RNS through the activation of NOX systems and inducible nitric oxide (iNOS) synthase, which directly damages cytoskeletal proteins and escalates OS-induced intestinal inflammation, probably in both groups, CMA and IBD children [15].

A positive correlation between markers was observed for: PCC vs •NO (r = 0.81, p< 0.002); TAC *vs*. •NO (r = 0.86, p< 0.005); TAC *vs*. PCC (r = 0.81, p< 0.003); PCC *vs*. OPC (r = 0.81, p< 0.0002); •NO *vs*. OPC (r = 0.81, p < 0.001) in CMA children, compared to IBD children.

In fact, OS and ROS/RNS uncontrolled production are a key pathologically-contributing factors responsible for the development of several gastrointestinal tract pathological disorders, such as CMA and IBD. In the context of mucosal (intestinal epithelium) inflammation (CMA and IBD), the activation by inflammatory cytokines leads to the superoxide anion radical ($•O_2^-$) and nitric oxide radical (•NO) production by IEC cells, neutrophils, and macrophages [15]. However, Maor *et al*. and Beltran *et al.* [36, 37] identified that OS rates were higher in active IBD phase compared to patients in remission. In our study, 16 patients (95%) were in IBD remission phase; probably the reduced inflammation levels, as well as the application of pharmaceutical treatment (5-ASA, steroids) dually regulate ROS/ RNS concentrations and reduce OS disorders. We hypothetize that the statistical increase of OS markers in the CMA group (infants and toddlers) is directly affected by the duration of the symptomatology, by the distinct diagnosis at a younger age, but in the CMA allergic condition, to the inflammation and OS maximum [15, 36, 37]. In addition, the high OS observed in CMA patients, witnessed by the increase in OPC, PCC, •NO radical

and TAC [38-39] markers measured, correlated to CMA patients examined before starting drug treatment and implementing a diet.

The limitations of our study are: 1) the relatively small sample size; 2) the relatively low uniformity of the lips; 3) repeated measurements during the course of the disease, necessary to validate the relevance of the use of OS markers in clinical settings.

CONCLUSIONS

Despite limitations, our results provide evidence that circulating OPC, PCC, •NO radical and TAC are significantly elevated in CMA patients, suggesting that these parameters could be evaluated in a prospective, larger study on the CMA and IBD progression, as biomarkers for diagnosis or monitoring of CMA and IBD patients. The search for new non-invasive diagnostic methods is of great importance, especially for children and adolescents with CMA and IBD.

***Acknowledgement**: This research was funded by the scientific project No.17/2023 and Ministry of Education and Science BG-RRP-2.004-0006 "Development of research and innovation at Trakia University in service of health and sustainable well-being".*

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