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Assessment of human 4-hydroxynonenal, 8-isoprostane concentrations and glutathione reductase activity after synbiotics administration

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ABSTRACT

Purpose: Probiotics and prebiotics have become an object of intense research, to identify methods of mitigating oxidative stress. Over the past few years, the number of *in vitro* and *in vivo* studies, related to antioxidant properties of probiotics/prebiotics has significantly increased. The aim of the present study was to assess whether probiotic in combination with prebiotic influences the level of human 4-hydroxynonenal, 8-isoprostane and glutathione reductase activity.

Material/methods: Experiments were carried out on healthy volunteers (male and female). All oxidative stress markers were measured in blood plasma pre- and post-administration of synbiotic.

Results: The administration of synbiotic resulted in a significant decrease in 4-hydroxynonenal in the female-synbiotic group ($p < 0.05$), 8-isoprostanes in the female-synbiotic group and male-synbiotic group ($p < 0.05$) and non-significant increase in the activity of glutathione reductase ($p > 0.05$) *vs.* control.

Conclusions: The present results show that supplementation of synbiotics contributed to the decrease in oxidative stress parameters in the female patients.

1. Introduction

Reactive oxygen species (ROS) are generated as a result of incomplete one-electron reduction of molecular oxygen and can be divided into two groups. The first one includes compounds called radicals, whereas the other one - compounds which are non-radical forms. Both groups can demonstrate highly reactive properties. The main feature differentiating radical and non-radical forms is their electron structure [1,2].

It is proved that there are three basic sources of free radicals generation [3,4]. The first source includes: metabolic processes in mitochondria and other organelles, redox reactions taking place in the mitochondria, carried out by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase and flavoprotein oxidase. The second source of ROS is the external environment which contains both natural and artificial toxins that either are free radicals themselves or they produce free radicals. This negative environment consists of air pollutants, toxic wastes, pesticides, solar radiation and ionizing radiation, bacteria or viruses. The third source is chain reactions, initiated by other radicals.

ROS are involved in pathological conditions [3,5]. Their targets include lipids, proteins and nucleic acids. Lipid components of biological membranes (side chains of fatty acids) are particularly susceptible to effects of free radicals. They may be damaged in the process of lipid peroxidation - free radical oxidation of unsaturated fatty acids and other lipids. Mainly residues of polyunsaturated fatty acids are involved in this process. They build phospholipids, which results in forming lipid peroxides, which, in turn, contributes to lipid membrane damage. End-products of lipid peroxidation are α, β-unsaturated aldehydes (mainly malondialdehyde and human 4-hydroxynonenal) which are considered second messengers of lipid peroxidation [5,6].

Probiotics are living microorganisms which have beneficial health effects if they are administered in appropriate amounts. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth or/and activity of one/or a limited number of bacteria in the colon [7]. Many authors suggest that oxidative stress (OS) protection is another beneficial property of probiotics/prebiotics. *In vitro* studies, many parameters can be taken into account and for numerous strains can be analyzed. The authors investigate antioxidant properties of a wide range of bacterial species *e.g.* 11 strains of *Lactobacillus*, 7 strains of *Bifidobacterium*, and 6 strains of *Lactococcus* [8]. *Lactobacillus* strains have been found to have higher total antioxidant activity than other examined strains [9]. Animal experiments are highly useful in identifying the antioxidant properties of probiotics. An interesting study of the antioxidative effect of *L. casei* spp. in Wistar rats was performed over a 90-day period [10]. A significantly higher

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CAT activity and lower levels of lipid peroxidation were observed in the liver, after probiotic supplementation. In recent years, the antioxidant properties of probiotics have also been tested in humans. Such studies are necessary, because they provide direct guidance about effectiveness of their actions. Thankfully, it is now possible to evaluate a large number of strains and conduct studies on many subjects. A potential role of probiotics in the improvement of antioxidant status in type 2 diabetic patients was a subject of one study [11]. The results were very promising: probiotic yogurts can significantly increase the total antioxidant status, GPx and SOD level compared with the control group.

The aim of the study was to evaluate the concentration of: 8-isoprostane (isoP), human 4-hydroxynonenal and glutathione reductase (GR) activity after administration of synbiotic in the human plasma of healthy volunteers. There are no studies on HNE and isoprostanes concentrations after *L.casei* + inulin administration in the plasma of healthy volunteers. To develop novel synbiotic products with the potential one (s) for preventing oxidative stress, the search for specific probiotic strains which offer the most effective prevention and mitigation of oxidative stress needs to be continued. Other studies are also needed to reveal the complete antioxidative properties of potential synbiotics.

2. Materials and methods

Thirty-two healthy volunteers (20–35 years old) were recruited for the study. There were 16 males and 16 females. The study was carried out in Poland in years 2014-2015. Subject were asked to fill in a questionnaire regarding their medical condition. Subjects with a history of gastrointestinal disease, food allergy, acute infections, alcoholism, addiction to cigarettes, administration of antimicrobial, anti-inflammatory or nonsteroidal drugs over last three months or administration of vitamins/probiotics were excluded from the study. Healthy subjects who were not on special diets which might affect antioxidant properties of plasma, or subject who were not taking antioxidant vitamins/yogurts were included in the study. Blood samples from the forearm veins were collected pre- and post-administration of synbiotics (after 7-weeks). The study was approved by the Ethical Committee of the Medical University of Lodz (number RNN/801/14/KB). All subjects gave their informed consent. A synbiotic was purchased from ICN Polfa Rzeszow S.A., Poland. The capsule contained 4×10^8 CFU lyophilized *Lactobacillus casei* plus 400 mg of inulin. Subjects were administered one capsule of synbiotic per day for 7 weeks [12].

2.1. Measurement of 4-HNE concentration in human plasma

To measure 4-HNE concentration, Human 4-Hydroxynonenal ELISA Kit (Item No. MBS006597), manufactured by MyBioSource (P.O. Box 153308, San Diego, CA 92195-3308, USA), was used. This method is based on 4-HNE antibody - 4-HNE antigen reactions (immunosorbency) and HRP colorimetric detection system to detect 4-HNE antigen targets in samples [13]. Human 4-Hydroxynonenal ELISA Kit (Item No. MBS006597) consisted of: Standards, Sample Diluent, HRP - Conjugate Reagent, Wash Solution (20x), A and B Chromogen Solution and Stop Solution. At first, 50 µl of Standard, sample or Sample Diluent were added to respective wells. Then, 100 µl of HRP-conjugate reagent was added to wells. The cuvette was incubated for 60 min at 37 °C. Next step was to wash the microtiter plate 4 times. Then, 50 µl of Chromogen Solution A and Chromogen Solution B were added to each well. The cuvette was incubated for 15 min at 37 °C. Finally, 50 µl of Stop Solution was added to each well. The color in the wells changed from blue to yellow. Absorbance was read at 450 nm with a plate reader (TECAN Sunrise with software Magellan Standard).

2.2. Measurement of 8-isoprostanes concentration in human plasma

To measure 8-isoprostanes concentration, 8-Isoprostane ELISA Kit (Item No. 516351) manufactured by Cayman Chemical Company, Ann Arbor, MI (BIOKOM, Ul. Wspolna 3, 05-090 Janki, Poland), was used. This method is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate for a limited number of 8-isoprostane-specific rabbit antiserum binding sites [14]. 8-Isoprostane ELISA Kit (Item No. 516351) consisted of: 8-isoprostane Antiserum, 8-isoprostane-AchE Tracer, 8-isoprostane ELISA Standard, ELISA Buffer Concentrate (10x), Wash Buffer Concentrate (400x), Polysorbate 20, Mouse anti-rabbit IgG coated plate, Ellman's reagent, ELISA Tracer Dye and ELISA Antiserum Dye. A suggested plate format (Blk- blank; TA – total activity; NSB – non- specific binding; B0 – maximum binding; S1-8 – standards 1-8) was used. At first, 100 µl of ELISA Buffer was added to NSB wells and 50 µl of this buffer was added to B0 wells. After preparation of 8-isoprostane Standard, 50 µl of ELISA Standard was added to S1-8 and 50 µl of sample was added to wells. Then, 50 µl of AChE Tracer was added to wells except TA and Blk wells. 50 µl of 8-isoprostane ELISA Antiserum was added to each well except TA, NSB and Blk wells. The plate was incubated for 18 h at 4 °C. The next step was to wash the microtiter plate 5 times. Then, 200 µl of Ellman's regent was added to each well and 5 µl of tracer was added only to TA wells. The assay developed in 90-120 min. Absorbance was read at 405 nm.

2.3. Measurement of glutathione reductase (GR) activity in human plasma

To measure GR activity, the Glutathione Reductase Assay Kit (Item number 703202), manufactured by Cayman Chemical Company, Ann Arbor, MI (BIOKOM, Ulica Wspolna 3, 05-090 Janki, Poland), was used. This kit determinates GR activity by measuring the rate of NADPH oxidation. Oxidation of NADPH to $NADP^+$ is related with decrease in absorbance at 340 nm [15]. Glutathione Reductase Assay Kit Item number 703202 consisted of: GR Assay Buffer (10x), GR Sample Buffer (10x), GR glutathione reductase-Control, GR NADPH and GR glutathione disulfide (GSSG). To prepare the background or non-enzymatic wells, 120 µl of final Assay Buffer and 20 µl of GSSG to three wells were added. To prepare positive control wells 100 µl of final Assay Buffer, 20 µl of GSSG and 20 µl of diluted GR to three wells were added. To prepare sample wells, 100 µl of final Assay Buffer, 20 µl of GSSG and 20 µl of sample to three wells were added. To initiate the reaction, 50 µl of NADPH was added to all used wells and the plate was carefully mixed for a few seconds. Absorbance was read every minute at 340 nm with a plate reader to obtain readings at 5 time points.

2.4. Statistical analysis

The data are presented as MEAN ± SEM in each group. Groups were compared using the Student's t-test and Mann-Whitney test. The selection of appropriate tests depended on the distribution of the obtained data. A p value below 0.05 was considered significant.

3. Results

3.1. Evaluation of 4-HNE concentrations

Levels of 4-HNE in the female-control group and male-control group were no significantly higher than those of the control group before experiment. A significant decrease in 4-HNE levels was observed in the female-synbiotic ($p < 0.05$) group but insignificant in male-synbiotic group ($p > 0.05$) in comparison to the their control groups before experiments (Fig. 1).

3.2. Evaluation of 8-isoprostane concentrations

Levels of 8-isoprostanes in the female-control group and male-control group were no significantly higher than those of the control group before experiment ($p > 0.05$). Administration of synbiotic significantly reduced 8-isoprostanes levels in the female-synbiotic group