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Chronic high-protein diet induces oxidative stress and alters the salivary gland function in rats

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ABSTRACT

Objective: Chronic high protein intake leads to an increase in reactive oxygen species (ROS) generation. However, there is no data on the impact of high-protein diet on the antioxidant barrier, oxidative stress and secretory function in the salivary glands of healthy individuals.

Design: 16 male Wistar rats were randomly divided into 2 groups (n = 8): normal protein (C) and high-protein diet (HP) for 8 weeks. Salivary antioxidants: peroxidase (Px), catalase (CAT), superoxide dismutase 1 (SOD 1), uric acid (UA), total antioxidant status (TAS), total oxidant status (TOS) and the oxidative stress index (OSI), as well as protein carbonyls (PC), 4-hydroxynonenal protein adduct (4-HNE protein adduct), 8-isoprostanes (8-isoP), 8-hydroxy-2′-deoxyguanosine (8-OHdG) and protein content were determined in the salivary glands and plasma. Salivary unstimulated and stimulated flow rates were examined.

Results: Parotid Px, TAS, UA, TOS, OSI, PC were significantly higher, the total protein content was statistically lower in the HP group as compared to the control. Submandibular UA, TOS, OSI, 8-isoP, 4-HNE-protein adduct, 8-OHdG were statistically elevated, SOD 1 and Px were significantly lower in the HP group as compared to the control rats. The unstimulated salivary flow rate was significantly depressed in the HP group as compared to the controls.

Conclusions: Higher antioxidant capacity in the parotid glands of HP rats *vs.* control rats seems to be a response to a higher ROS formation. In the submandibular glands severe oxidative modification of almost all cellular components was observed. Administration of HP resulted in the weakening of the salivary gland function.

1. Introduction

Evidence shows that protein intake increases in an industrialized population and reaches a level more than twice the WHO recommended intake (WHO, 1985). There is almost no data on the benefits or side effects of high protein intake for healthy adults. It was documented that undesirable metabolic changes occur when the protein intake is 1.6 or more times higher than the recommended values (Metges & Barth, 2000). However, physiological and functional consequences of a chronic high protein intake have not been fully explained.

In contrast to fatty acids and glucose, proteins, when present in excess of the current demand, cannot be stored and they are metabolically processed immediately. Evidence shows that an adaptation to HP is associated with an increase in the activity of enzymes involved in protein digestion and increased amino acid metabolism, including amino acid transport, transamination, oxidative deamination, as well as, the capacities mainly for gluconeogenesis less ureagenesis, to maintain amino acid homeostasis in the body (Jean et al., 2001; Peters & Harper, 1985). It should be underlined that oxidative deamination of amino acids is related to the production of reducing equivalents, that must be reoxidized in the mitochondrial respiratory chain. This may contribute to an increased electron flow along the respiratory

Abbreviations: 4-HNE protein adduct, 4-hydroxynonenal protein adduct; 8-isoP, 8-isoprostanes; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CAT, catalase; OSI, oxidative stress index; PC, protein carbonyls; Px, peroxidase; ROS, reactive oxygen species; SOD 1, superoxide dismutase 1; TAS, total antioxidant status; TOS, total oxidant status; UA, uric acid

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chain and increased ROS formation (Petzke & Proll, 1994). This point of view is supported by data, which shows that chronic high protein intake results in an increased thermogenic response, which e.g. decreases nutritional efficiency of energy utilization, increases oxygen consumption and impairs oxidative phosphorylation capacities (Klein & Hoffmann, 1993; Petzke & Proll,1994; Porrata-Maury, Aust, Noack, & Eschrich, 1987).

An increased production of ROS, in the case of failure of the antioxidant systems to combat extra free radical generation, may lead to oxidative stress (OS) (Pamplona & Barja, 2006). One of its consequences is oxidative modification of polypeptide chains, as well as, single amino acid residues. This results in the formation of, for example, of reactive protein carbonyl derivatives (PC), the generation of peroxides due to a damage in polyunsaturated fatty acids (e.g.) 4-hydoxynonenal protein adduct (4-HNE protein adduct) or 8-isoprostanes (8-isoP) or the oxidative modification of DNA, leading to the formation of 8-hydroxy-D-guanosine (Lushchak, 2014b).

The question of whether higher protein intake induces physiologic OS remains controversial. It has been shown that high protein intake increases plasma oxidative modified protein bound amino acids of adult rats fed 60% casein but does not increase plasma protein carbonyl concentration (Petzke, Proll, Bruckner, & Metges, 1999). Camiletti-Moirón et al. (2015) showed that brain thiobarbituric acid reactive substances and protein carbonyls were higher in the high protein group compared to the normal protein group, which is contrary to the results of Petzke, Elsner, Proll, Thielecke, and Metges (2000).

Saliva produced by the salivary glands plays an important role in oxidant/antioxidant redox homeostasis. Saliva may be therefore considered as a major component of the oral host defenses, which constitute a first line of defense against ROS-induced agents in tobacco smoke, alcohol, drugs, as well as other xenobiotics in the diet (Nagler, Klein, Zarzhevsky, Drigues, & Reznick, 2002). The moisturizing properties of saliva facilitate articulation, swallowing and digestion. Saliva protects the surfaces of the teeth and the mucous membranes of the oral cavity against biological, chemical and mechanical insults (Sonesson, Wickström, Kinnby, Ericson, & Matsson, 2008). Thus, factors which compromise the salivary glands' function, change the amount and composition of the saliva secreted in the oral cavity and have adverse effects on oral health and the quality of life.

Inadequate nutrition is thought to be a key factor in the development of pathological changes of oral homeostasis and it also alters the salivary glands' function (Elverdin et al., 2006; Fathy El-Maghraby, 2012; Huumonen & Larmas, 2005; Johnson, Lopez, & Navia, 1995; Kołodziej et al., 2017; Zalewska et al., 2014). However, still little is known about the impact of a high-protein diet on the secretory function and antioxidant barrier as well as OS markers in the salivary glands. Therefore, it is important to explain whether the chronic intake of a high level of protein contributes to salivary gland dysfunction and results in disturbed redox balance in the salivary glands.

2. Materials and methods

The study was conducted in accordance with the Guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and was approved by the Committee for Ethics Use of Animals in the Medical University in Bialystok, Poland (protocol number 89/2015, 2015/109).

2.1. Animals

16 male Wistar rats, weighing between 67–72 g, were housed individually in steel cages, maintained at 22–24 °C, under standard lighting conditions from 8.00 a.m.–8.00 p.m. The animals remained in constant eye contact with each other. Before the feeding experiment, all rats consumed commercially available rodent chow (Agropol Motycz Poland, 13.5 mg% of fat, 24 mg% of protein, 62.5 mg% of carbohydrates, energy value 0.011 mJ/g). After 5 days of adaptation, the rats were randomly (based on a computer generated sequence of numbers) divided into two dietary groups: control (C) and experimental (HP).

2.2. Diet

During the 8 weeks of the experiment, animals assigned to the control group were fed a pelleted feed (Agropol Motycz Poland, 13.5 mg% of fat, 24 mg% of protein, 62.5 mg% carbohydrates, energy value 0.011 mJ/g). Rats assigned to the experimental group were fed a high protein diet (Research Diets, Inc.; D03012801, containing 14 mg% fat, 44 mg% proteins, 33 mg% carbohydrates, energy value 0.0158 mJ/g).

During the adaptation period and the 8 weeks of the experiment, rats from both groups had unlimited access to drinking water and food.

2.3. Salivary flow measurement

The food intake and body weight were monitored weekly. After 8 weeks, following an overnight fasting, rats were anesthetized with phenobarbital (80 mg/kg body weight, i.p.). Next, the stimulated and unstimulated saliva secretion rates were measured. In order to make measurements of saliva secretion, rats were placed on a couch preheated to 37 °C, placed at an angle of 30°. Non stimulated salivary secretion was measured for 15 min, using a pre-weighted cotton ball inserted underneath the tongue (Knaś, Maciejczyk, Daniszewska et al., 2016; Knaś, Maciejczyk, Sawicka et al., 2016). After 5 min, the rats were peritoneally injected with 5 mg/kg BW pilocarpine nitrate (Sigma Chemical Co, St. Louis, MO, USA). A five minutes after the pilocarpine administration, pre weighted cotton ball was inserted into the oral cavity and the whole stimulated saliva was collected for 5 min (Picco et al., 2012). The volume of saliva collected was measured by subtracting the initial weight from the final weight of the cotton ball. One milligram of whole saliva corresponds to 1.0 μL.

2.4. Blood and salivary glands collection

Finally, whole blood was drawn from the abdominal aorta into chilled heparinized tubes. The blood was centrifuged at 4 ° C, 5 min, 3000*g* (MPW 351, MPW Med. Instruments, Warsaw, Poland) and thus obtained plasma was precooled in liquid nitrogen and stored at −80 °C. Afterwards, the salivary glands from one side were removed on one side, freeze-clamped with aluminum tongs, precooled in liquid nitrogen and stored at −80 °C. The glands on the other side were fixed in 10% formalin and then processed for paraffin embedding.

2.5. Preparation of the blood and salivary glands for biochemical and histological assays

The salivary glands and plasma were slowly thawed (4 °C) the day on which the biochemical determinations were performed. The salivary glands were weighed (laboratory balance KERN PLI 510–3 M) and placed in glass tubes, containing ice cold PBS (1:10). The salivary pieces intended for the determination of carbonyl groups were diluted (1:10) in a 50 mM phosphate buffer. The protease inhibitor (1 tablet/10 mL of the buffer) (Complete Mini Roche, France) and the antioxidant butylhydroxytoluene (10 μL 0.5 M BHT in acetonitrile per 1 mL of the buffer) (Sigma-Aldrich, Germany) were added to all samples (plasma and salivary glands). Next, the glands were homogenized on crushed ice (Omni TH, Omni International, Kennesaw, GA, USA), and sonificated (1800 J/sample, 20 s three times, on ice; ultrasonic cell disrupter, UP 400S, Hielscher, Teltow, Germany). Finally, homogenates were centrifuged for 20 min, 4 °C, 5000 × *g* (MPW Med Instruments, Warsaw, Poland) and supernatants were taken for biochemical analysis.

2.6. Biochemical analysis

The plasma glucose and insulin concentrations were measured in a duplicate. The activity of peroxidase (Px), catalase (CAT) and superoxide dismutase 1 (SOD 1), as well as concentrations of 4-HNE protein adduct, 8-isoprostanes (8-isoP), 8-hydroxy-D-guanosine (8-OHdG), protein carbonyls (PC), uric acid (UA), and total protein were measured in the plasma and salivary glands in duplicate samples. Total antioxidant status (TAS) and total oxidant status (TOS) were measured in a triplicate. The final results were the arithmetical mean of the readings. The results were standardized for the milligrams of the total protein. Such a presentation of the results allows for the observation of the differences in the ratio of examined parameters present in the salivary glands or plasma.

The fasting glucose concentration was determined by a glucometer (Accu-check glucometer, Byer, Germany).

2.7. Antioxidants assays

The activity of Px was measured according to the method described by Mansson- Rahemtulla, Baldone, Pruitt, and Rahemtulla (1986). A 10 μL of sample was incubated (25 °C) with 200 μL 50 μM dithiobis-2-nitrobenzoic acid (DTNB) in 0.1 a M potassium-phosphate buffer (pH 5.6), 10 μL 4 mM KSCN (potassium thiocyanate) and 10 μL 100 μM H_2O_2 (hydrogen peroxide) (Sigma Aldrich, Germany). The interaction between peroxidase and DTNB in the presence of β-mercaptoethanol (β-ME) resulted in a color change which was determined spectrophotometrically at 412 nm (6 measurements for two minutes, each every 30 s).

Catalase (CAT) activity was determined colorimetrically by the Aebi (1984) method, which is based on measuring the decrease in absorbance of hydrogen peroxide (H_2O_2). Samples were incubated (25 °C) with 13.2 mM H_2O_2 in a 50 mM phosphate buffer (pH 7.0) (Sigma Aldrich, Germany) and absorbance changes was measured during 3 min at 240 nm using the Infinite M200 PRO Multimode Microplate Reader, Tecan. One unit of CAT activity was defined as the amount of the enzyme-catalyzed decomposition of one millimole of H_2O_2 per min.

Superoxide dismutase 1 (SOD 1, copper and zinc-containing SOD 1) activity was estimated by the Misra and Fridovich (1972) method in the modification of Sykes, McCormack, and O'Brien (1978). 200 μL of tissue homogenates/plasma were incubated (25 °C) with a reaction mixture containing 0.3 mM epinephrine and 0.1 mM EDTA (ethylenediaminetetraacetic acid) in 0.05 M sodium carbonate (pH 10.2) (Sigma Aldrich, Germany). All samples were protected from light. The standard curve was prepared for superoxide dismutase from bovine erythrocytes (Sigma Aldrich, Germany). Changes in absorbance were measured colorimetrically at 480 nm using the Infinite M200 PRO Multimode Microplate Reader, Tecan. Determination of SOD 1 activity is based on the ability of the enzyme to inhibit the autoxidation of epinephrine at pH 10.2. One unit of SOD 1 activity was defined as the amount of enzyme, which inhibits epinephrine oxidation by 50%.

The concentration of UA was assayed with a commercial kit supplied by BioAssays System QuantiChrom™Uric Acid Assay Kit DIUA-250; BioAssay Systems, Harvard, CA, USA. In this assay 2,4,6-tripyridyl-s-triazine in the presence of uric acid, formed a blue complex with iron. The intensity of the color, measured spectrophotometrically at 490 nm, was directly proportional to the uric acid concentration in the supernatants

2.8. Oxidative damage products, TAS, TOS and OSI determination

The concentration of PC was determined by 2,4-dinitrophenylhydrazine (2,4-DNPH) (Reznick & Packer, 1994). A 100 μL sample was incubated with 400 μL 10 mM 2,4-DNPH in 2.5 M HCl (1 h in the dark, 25 °C) (Polskie Odczynniki Chemiczne POCH, Gliwice, Poland). Then, 500 μL of cold 20% trichloroacetic acid (TCA, w/v) (Sigma Aldrich, Germany) was added and samples were centrifuged for 5 min (6000 × *g*, 25 °C). The supernatants were discarded. Next, 400 μL of cold 10% TCA was added and pellets were washed 3 times with 400 μL of ethanol-ethyl acetate (1:1) (v/v). The final precipitates were dissolved in 2 mL of 6 M guanidine hydrochloride solution (containing 20 mM potassium phosphate adjusted to pH 2.3) (Sigma Aldrich, Germany) and were left for 10 min at 37° with general vortex mixing. The principle of the method is based on the reaction of 2,4-DNPH with carbonyl groups in the oxidatively damaged proteins, which results in hydrazone determined spectrophotometrically at 355 nm. PCO content was calculated using an absorption coefficient for 2,4-DNPH (22,000 $M^{-1}cm^{-1}$).

The concentration of insulin, 8-OHdG and of 4-HNE protein adducts was determined by ELISA using commercially available kits: BioVendor, Brno, Czech Republic, USCN Life Science, Wuhan, China; Cell Biolabs, Inc. San Diego, CA, USA, respectively.

The concentration of 8-isoP was determined using a commercial enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical Company, Ann Arbor, MI, USA). The principle of the method is based on the competition between 8-isoprostanes and 8-isoprostanes conjugate with acetylcholinesterase (Tracer) for binding with an antibody specifically directed against the 8-isoP. The binding capacity of the conjugate with 8-isoP, acetylcholinesterase and the antibody is inversely proportional to the concentration of 8-isoP in the sample.

TAS was determined using a commercial kit supplied by Randox (Crumlin, UK) according to the manufacturer's instructions. In this method, metmyoglobin is converted into ferrylmyoglobin in the presence of iron ions. The result of the reaction between ferrylmyoglobin with the Randox ABTS reagent was a green product, the absorbance of which was measured at 600 nm. The TOS concentration was determined using the commercial kit Per-OX TOS/TOC (Immune Diagnostics, Bensheim, Germany). The reaction of peroxidase with lipid hydroperoxides led to the production of reduced phospholipid products of green color, which changes to yellow upon addition of the stop reagent. Absorbance was measured at 450 nm. The index of oxidative stress was calculated based on the formula = TOS/TAS x 100 (Şen et al., 2014).

The total protein concentration (BC) was measured using the BCA™ Protein Assay Kit BCA™ (Pierce, No.23225, Rockfold, IL, USA), with bovine albumin as a standard.

2.9. Histology

The salivary glands, fixed in 10% formalin, were processed for paraffin embedding. Next, they were cut in five micron sections. Directly before staining, the paraffin was removed from the salivary gland sections by bathing twice in xylene, followed by rehydration in decreasing concentrations of ethanol from pure to 70%. The sections were then placed in water, followed by full immersion in hematohylin for 8 min. The next step was rinsing in water for 15 min before staining with 1% eosine with one drop of acetic acid and a final rinse with distilled water. The sections were observed under light microscope (OPLYMPUS BX 51, OLYMPUS, 40× and 60× magnification) and analyzed by a histologist.

2.10. Statistical analysis

The results were reported as median, minimum and maximum. All analyses were performed using Statistica 12.0 (Statsoft, Cracow, Poland). The control and HP groups were compared using the U Mann-Whitney test. The relationships between the antioxidants, oxidative stress markers, protein concentration, salivary flow rate in the salivary glands and plasma were analyzed using the Spearman Correlation Coefficient. Differences with $p \leq 0.05$ were considered significant.

Table 1
Effect of high-protein diet on body weight, fasting plasma glucose and insulin concentration and food intake.

	C n = 8 M (min–max)	HP n = 8 M (min–max)
Body weight (g)	322 (303–373)	317 (305–357)
Glucose concentration (mg/dL)	99.7 (94.3–100.7)	101.7 (94.2–105.6)
Insulin concentration (μU/mL)	4.4 (4.0–5.2)	4.8 (3.9–5.7)
Food intake (g)	22.85 (15.1–23.65)	16.98 (13.17–18.25)↓*
Energy intake (mJ)	0.248(0.156–0.267)	0.268(0.197–0.281)

Abbreviation: C-control group, HP-high protein group, M- median, min- minimum, max-maximum, * $p < 0.05$, ↓- decrease.

3. Results

3.1. Final body weight, food intake, plasma glucose and insulin concentrations, salivary gland weight, total protein concentrations in the salivary glands and salivary flow

HP rats consumed significantly less chow than to the control group ($p = 0.02$), while energy intake was comparable between these two groups. After 8 weeks of the experiment the body weight of the HP and control rats was similar. The high protein intake did not alter glucose homeostasis, as fasting glucose and insulin levels did not differ significantly among the groups (Table 1).

The effects of the high-protein diet on the salivary gland weight, unstimulated and stimulated saliva secretion and protein content are presented in Table 2. The median weight of the submandibular and parotid glands of the HP group was higher than in the control groups, however, only in the case of the submandibular glands was the observed increase was statistically significant ($p = 0.007$). The median of the stimulated flow rate did not differ between the HP and the control group. However, we noticed a significant (40%) drop in unstimulated salivary flow in the HP group as compared to the control rats ($p = 0.015$). The total protein concentration in the parotid glands was significantly reduced in the HP rats as compared to the control rats (29%, $p = 0.04$), while the protein concentration in the submandibular glands of the HP group and control groups did not differ significantly.

3.2. The effect of a high protein diet on plasma UA, TAS, TOS, OSI, 4-HNE-protein adduct, 8-isoP, 8-OHdG and PC

The plasma concentrations of UA (↑75%, $p = 0.02$), TAS (↑74%, $p = 0.03$), TOS (↑145%, $p = 0.02$), OSI (↑86%, $p = 0.03$), 8-isoP (↑55%, $p = 0.031$), 4-HNE-protein adduct (↑43%, $p = 0.028$), and PC (↑83%, $p = 0.01$) were higher in the plasma of the HP rats than in the control rats. The plasma 8-OHdG concentrations were not significantly different among both groups (Table 3).

Table 2
Effect of high-protein diet on salivary glands weight, unstimulated and stimulated saliva secretion and protein content.

	C n = 8 M (min–max)	HP n = 8 M (min–max)
Parotid gland weight (mg)	0.095 (0.088–0.104)	0.101 (0.074–0.12)
Submandibular gland weight (mg)	0.26 (0.23–0.3)	0.302 (0.263–0.356)↑**
UWS (μL/min)	0.42 (0.28–0.66)	0.25 (0.09–0.48)↓*
SWS (μL/min)	96.79 (86.88–151.12)	109.9 (97.1–125.4)
Total protein mg/dL parotid glands	5080 (3186.9–7553)	3627.2 (3016.8–4011.8)↓*
Total protein mg/dL submandibular glands	5023.35 (4179–5251.2)	4910.75 (3375.1–5736.3)

Abbreviation: C- control group, HP- high protein group, UWS- unstimulated saliva secretion, SWS- stimulated saliva secretion, * $p < 0.05$, ** $p < 0.0001$, ↑- increase, ↓- decrease.

Table 3
Plasma UA, TAS, TOS, OSI, 8-OHdG, 8-isoP, 4-HNE protein adduct, PC of the control and HP rats.

plasma	C n = 8, M (min–max)	HP n = 8, M (min–max)
UA (ng/mg of protein)	4.0 (1.0–4.3)	7.0 (6.0–8.0)↑*
TAS (μmol/mg of protein)	0.075 (0.065–0.097)	0.131 (0.105–1.16)↑*
TOS (μmol/mg of protein)	0.795 (0.25–0.745)	1.95 (1.41–3.25)↑*
OSI	903.2 (35.7–1010.09)	1687.8 (1021–2133.3)↑*
8-OHdG (fg/mg of protein)	5.1 (4.125–6.422)	5.4 (4.734–5.945)
8-isoP (fg/mg of protein)	2.0 (1.96–2.71)	3.1 (3.034–4.38)↑*
4-HNE protein adduct (ng/mg of protein)	0.39 (0.322–0.45)	0.56 (0.51–0.59)↑*
PC (nmol/mg of protein)	0.08 (0.06–0.11)	0.147 (0.101–0.194)↑*

Abbreviation: C- control group, HP-high protein group, M-median, min-minimum, max-maximum, UA-uric acid, TAS- total antioxidant status, TOS- total oxidant status, OSI oxidative status index, 8-OHdG- 8- hydroxy-D- guanosine, 8-isoP- 8 isoprostanes, PC-protein carbonyl, * $p < 0.05$, ↑- increase, ↓- decrease.

3.3. Antioxidants and oxidative stress markers in the submandibular glands

We observed that the concentrations of UA (↑81%, $p = 0.0009$), TOS (↑150%, $p = 0.002$), OSI (↑131%, $p = 0.004$), 8-OHdG (↑25%, $p = 0.03$), 8-isoP (↑37.5%, $p = 0.024$) and 4-HNE protein adduct (↑53%, $p = 0.03$) in the submandibular glands of rats fed a high-protein diet were significantly higher compared to the control glands. In contrast, the specific activity of SOD 1 (↓34%, $p = 0.009$) and Px (↓56%, $p = 0.003$) in the submandibular glands of the HP group decreased significantly, compared to the control. Moreover, the specific activity of CAT, TAS and PC concentrations in the submandibular glands of the HP group did not differ from the control group (Table 4).

3.4. Antioxidants and oxidative stress markers in the parotid glands

The effects of the high-protein diet on parotid gland antioxidants and oxidative stress biomarkers are shown in Table 5. We noted that the specific activity of Px, as well as the concentrations of UA, TAS, TOS, OSI and PC in the parotid glands of HP rats showed a significant increase when compared to the control group, by 48% ($p = 0.003$), 116% ($p = 0.0009$), 10% ($p = 0.002$), 94% ($p = 0.002$), 59% ($p = 0.02$) and 106% ($p = 0.004$) respectively. Both the HP and control groups showed similar specific activity of SOD 1 and CAT as well as 8-OHdG, 8-isoP

Table 4
Submandibular glands UA, TAS, TOS, OSI, 8-OHdG, 8-isoP, 4-HNE protein adduct, PC of the control and HP rats.

Submandibular glands	C n = 8, M(min–max)	HP n = 8, M (min–max)
SOD 1 (mU/mg of protein)	0.029 (0.022–0.043)	0.01 (0.001–0.019)↓***
CAT (nmol H_2O_2/min/mg of protein)	0.053 (0.013–0.078)	0.035 (0.023–0.068)
Px (μU/mg of protein)	0.069 (0.054–0.085)	0.044 (0.036–0.052)↓*
UA (ng/mg of protein)	0.011 (0.008–0.017)	0.02 (0.017–0.025)↑***
TAS (μmol/mg of protein)	1.39 (0.98–2.12)	1.45 (0.85–2.01)
TOS (μmol/mg of protein)	0.01 (0.009–0.0175)	0.025 (0.019–0.032)↑**
OSI	0.714 (0.55–1.167)	1.655 (1.005–2.62)↑**
8-OHdG (fg/mg of protein)	0.81 (0.42–1.1)	1.15 (0.81–1.3)↑*
8-isoP (fg/mg of protein)	0.4 (0.37–0.47)	0.55 (0.52–0.62)↑*
4-HNE protein adduct (ng/mg of protein)	28.1 (22.0–33.0)	43.11 (36.0–49.0)↑*
PC (nmol/mg of protein)	0.053 (0.01–0.075)	0.047 (0.023–0.09)

Abbreviation: C- control group, HP-high protein group, M-median, min-minimum, max-maximum, SOD 1- superoxide dismutase 1, CAT- catalase, Px- peroxidase, UA-uric acid, TAS- total antioxidant status, TOS- total oxidant status, OSI oxidative status index, 8-OHdG- 8- hydroxy-D- guanosine, 8-isoP- 8 isoprostanes, PC- protein carbonyl, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.00001$↑- increase, ↓- decrease.

Table 5
Parotid glands UA, TAS, TOS, OSI, 8-OHdG, 8-isoP, 4-HNE protein adduct, PC of the control and HP rats.

Parotid glands	C n = 8, M (min–max)	HP n = 8, M (min–max)
SOD 1 (mU/mg of protein)	0.027 (0.01–0.047)	0.026 (0.015–0.03)
CAT (nmol H_2O_2/min/mg of protein)	0.037 (0.011–0.05)	0.02 (0.012–0.11)
Px (μU/mg of protein)	0.087 (0.07–0.11)	0.129 (0.116–0.16)↑**
UA (ng/mg of protein)	0.018 (0.011–0.03)	0.039 (0.031–0.055)↑***
TAS (μmol/mg of protein)	2.15 (1.57–2.35)	2.38 (2.11–2.91)↑**
TOS (μmol/mg of protein)	0.11 (0.09–0.145)	0.214 (0.198–0.24)↑**
OSI	5.12 (3.84–8.4)	8.19 (7.21–11.32)↑*
8-OHdG (fg/mg of protein)	0.92 (0.53–1.11)	0.82 (0.805–0.91)
8-isoP (fg/mg of protein)	0.53 (0.51–0.54)	0.55 (0.49–0.57)
4-HNE protein adduct (ng/mg of protein)	24.13 (21.55–31.5)	30.1 (28.12–33.87)
PC (nmol/mg of protein)	0.049 (0.036–0.72)	0.101 (0.081–0.95)↑**

Abbreviation: C- control group, HP-high protein group, M-median, min-minimum, max-maximum, SOD 1- superoxide dismutase 1, CAT- catalase, Px- peroxidase, UA-uric acid, TAS- total antioxidant status, TOS- total oxidant status, OSI oxidative status index, 8-OHdG- 8- hydroxy-D- guanosine, 8-isoP- 8 isoprostanes, PC- protein carbonyl, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.00001$↑- increase, ↓- decrease.

and 4-HNE protein adduct concentrations in their parotid glands.

3.5. Histological observation

Typical acini and duct architecture of the parotid and submandibular glands of HP rats were observed, however, in the microscopic view, cytoplasmic vacuolization of alveolar cells in both salivary glands of HP rats was noted. Cytoplasmic vacuolization was not observed in the control glands. The number and size of the vacuoles were comparable between the parotid and submandibular glands of the HP group (Fig. 1).

3.6. Correlations

A positive relationship was observed between plasma UA and parotid ($p = 0.01$, $r = 0.57$) and submandibular ($p = 0.02$, $r = 0.49$) UA concentrations. A negative correlation between UA and protein concentrations in the parotid glands was observed ($p = 0.002$, $r = -0.79$), as well as, a positive correlation between UA and the 4-HNE protein adduct in the submandibular glands ($p = 0.01$, $r = 0.54$) of HP rats.

4. Discussion

The purpose of the present experiment was to analyze the influence of high-protein intake on salivary gland function and its redox balance. The main findings of our study were: 1. High protein intake increased parotid Px, TAS, UA, TOS, OSI, PC and decreased the total protein content as compared to the control group. 2. Submandibular UA, TOS, OSI, 8-isoP, 4-HNE protein adduct and 8-OHdG were statistically elevated but SOD 1 and Px were significantly lower in the HP group as compared to the control rats. 3. Finally, the unstimulated salivary flow rate was significantly reduced in the HP group as compared to the controls.

Our experimental animals were fed a diet containing 44 mg% proteins as opposed to the control group which received 24mg% protein in their diet. The addition of protein replaced an almost equivalent amount of carbohydrates (Jean et al., 2001). The choice of the protein concentration in the control and high protein diets was based on the previously published results. It was established that an adequate dietary protein requirement for laboratory rats is 17 mg% for growth, but 13.8-25.7mg% to maintain the redox balance (Petzke et al., 2000). We showed that the HP group displayed a reduced food intake when compared to the control rats, which was probably due to the fact that high protein intake reduces hunger and induces satiety (Leidy et al., 2015). It is also believed that the main reasons for decreased food intake are a low glycemic index for protein and a greater thermic effect of proteins than carbohydrates and fat (Astrup, Raben, & Geiker, 2015) and a higher calorific value of the high-protein diet (0.0158 mJ/g) in comparison to the control diet (0.011 mJ/g) (Antonio, Peacock, Ellerbroek, Fromhoff, & Silver, 2014; Camiletti-Moirón et al., 2015). We also noted that the high-protein diet did not alter glucose homeostasis in HP rats, since we noted unaltered fasting glucose and insulin

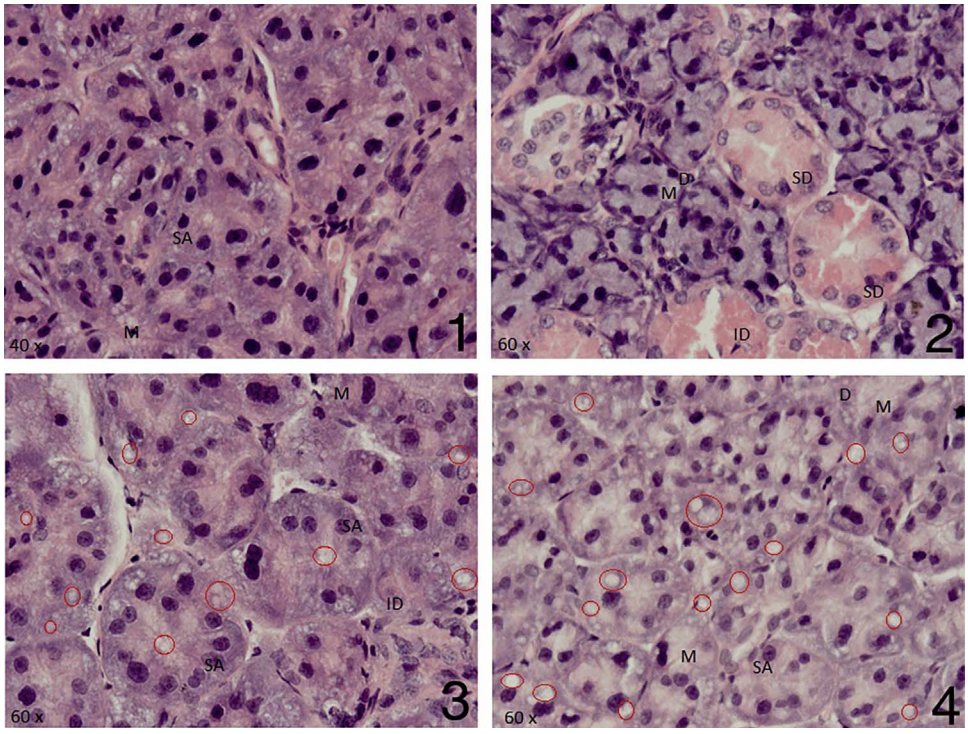

Fig. 1. The impact of high protein intake on histological observation of the salivary glands.
Abbreviation: 1 – parotid gland of the control rats, 2 – submandibular gland of the control rats, 3 – parotid gland of rats fed a high protein diet (HP), 4 – submandibular gland of rats fed a high protein diet (HP), red circles indicate vacuoles, SA- serous acini, M-mucous acini, SD- striated ducts, ID- intercalated ducts, D- demilune.

concentrations as compared to the control rats.

Physiologically, the formation of ROS is compensated by the elimination or prevention of formation, through the antioxidant systems. From the point of view of the redox balance of the salivary glands, the most important antioxidants produced only in the salivary glands is salivary peroxidase (Px) and plasma-born uric acid (UA) (Nagler et al., 2002). Other enzymatic (e.g. SOD, CAT) and non-enzymatic antioxidants are of secondary importance, however they also influence the total antioxidant status (TAS). TAS provides information about the capacity of the salivary glands in the fight against oxygen free radicals. Instead of determining each ROS separately, the notion of the total oxidant status (TOS) was introduced (Erel, 2005; Knaś, Maciejczyk, Daniszewska et al., 2016; Knaś, Maciejczyk, Sawicka et al., 2016; Ogawa, Shimizu, Muroi, Hara, & Sato, 2011). TAS and TOS are inextricably linked to the oxidative stress index (OSI), which is commonly used in assessing the intensity of oxidative stress (Şen et al., 2014).

It is well documented that under increased ROS production, an increase in the activity of antioxidant enzymes or antioxidant concentration is usually connected with their *de novo* synthesis (Lushchak, 2011) or activation of inactive molecules (Semchyshyn, 2009). These effects are directed at enhancing cell resistance to OS. So, it is highly probable that the observed significant increase in TAS and Px activity in parotid glands of HP group can be an adaptive response induced by increased ROS formation in this gland (↑TOS). On the other hand, up-regulation of the parotid antioxidant barrier may be an attempt to compensate for inefficient submandibular glands in terms of antioxidant defense. A significant decrease in the SOD 1 and Px activity in submandibular glands of HP rats may result from the use of the enzymes in the elimination of excessive amounts of ROS or due to the oxidative modification of the polypeptide chain of SOD 1, Px, resulting in a significant reduction in enzyme activity.

Changes in the activity/concentration of the antioxidants do not allow for an assessment of the severity of oxidative stress and its effects. The most reliable marker of oxidative stress assumes an increase in the concentration of oxidative modified molecules (Lushchak, 2014a,b), as well as the oxidative stress index (OSI) (Maciejczyk et al., 2017). The most commonly used markers of oxidative damages include: 4-HNE protein adducts, 8-isoprostanes, protein carbonyl groups and 8-OHdG.

The results of our study revealed a greater degree of diversity and oxidative damage as well as intensity of OS to the submandibular glands compared to the parotid glands of rats exposed to a high-protein diet. This different size and type of oxidative damage present in the parotid and submandibular glands may be due to the fact that the parotid gland presents an aerobic metabolism (Feinstein & Schramm, 1970), while the submandibular one has a lower demand for oxygen (Fernandes & Junqueira, 1953). Finally, evidence showed that parotid glands are capable of a more effective antioxidant response to counterbalance an excess of ROS and effectively prevent oxidative modification of their biomolecules *vs.* submandibular glands (Nagler et al., 2002).

We found that the pattern and intensity of oxidative modifications in salivary glands were not reflected in the plasma oxidative modification. Although we have shown that the concentration of UA in the parenchyma of both salivary glands was positively related to plasma UA levels. We have not found other correlations between the studied parameters in the plasma and salivary glands.

In addition to the antioxidant properties (Patterson, Horsley, & Leake, 2003), UA can act as a pro-oxidant agent, and it may be responsible for the propagation of oxidative stress in aerobic cells (Al-Rawi, 2011). A significant negative relation between UA and protein concentration in the parotid glands as well as a positive association between UA and 4-HNE protein adduct concentration in submandibular glands of HP rats suggest that salivary UA creates a rather pro-oxidative environment than a protective response to increased ROS formation.

Unstimulated whole saliva is mainly composed of submandibular secretion with the substantial participation of sublingual and minor salivary glands (Dawes, 2008). Therefore, high-protein related changes in the secretion of the unstimulated saliva can be related to a dysfunction of the submandibular glands. A similar degree of parenchyma vacuolization observed in both glands, rather rules out an attenuate secretion of non-stimulated saliva by reduction in the active secretory surface of the submandibular glands. It seems that severe oxidative modification of cellular elements of submandibular glands of HP rats carries a remodeling of the extracellular matrix, which can interfere with neurotransmission between neural and residual secretory units and lead to a reduction of secreted saliva.

Somewhat surprising is the fact that despite the higher intensity OS in the submandibular glands of HP rats, the acinar mechanism involved in the synthesis/secretion of protein in these glands seems to be unaffected. The finding of a decreased concentration of protein in the parotid glands could suggest that its protein machinery is more susceptible to oxidant attack generated in the course of a high-protein diet *vs.* the submandibular one. On the other hand decreased protein concentrations in the parotid glands of HP rats may be due to their reduced food intake. It was proven that decreased food intake was a cause of disturbances in exocytosis or protein synthesis only in the parotid, a phenomenon not found in the submandibular gland cells, which was observable as an elevated storage of granules/vehicles with secretory proteins and decreased protein concentrations in those glands (Sreebny & Johnson, 1968).

While analyzing the presented results, one needs to take into account that we only evaluated some markers of oxidative modifications and elements of the antioxidative barrier. The evaluation of other oxidative stress markers may completely or partially change our observations and assumptions. Moreover, the observed result might be due to changes in the concentration of carbohydrates that were replaced by the proteins. Finally, the results should be analyzed with caution, given the small size of the study groups.

5. Conclusions

1. The parotid and submandibular glands showed different responses to high-protein feeding. The greater oxidative modification to the cellular components were observed in the submandibular glands. The antioxidant defense system in the parotid glands was more effective in preventing oxidative damage, induced by a high-protein diet.
2. Administration of a high-protein diet resulted in the weakening of the salivary gland function.

Conflict of interest

Authors declare no conflict of interest.

Ethical approval

The study was conducted in accordance with the Guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and was approved by the Committee for Ethics use of Animals in the Medical University in Bialystok, Poland (protocol number 89/2015, 2015/109).

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