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Original Paper

α-Lipoic Acid Supplementation Was Not Effective in Restoring Salivary Flow Rate and Salivary Glands Redox Equilibrium in the Hyperglycaemic Rats

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Key Words
Antioxidants • α-lipoic acid • Hyperglycaemia • Oxidative stress • Salivary glands

Abstract
Background/Aims: The aim of the present study was to investigate whether α-lipoic acid (ALA) could reverse/prevent high fat diet (HFD)-induced salivary gland dysfunction and oxidative damage in the salivary glands of rats, and strengthen their antioxidant defense. ***Methods:*** The enzymatic and non-enzymatic antioxidants as well as their redox status, oxidative damage products and salivary flow rate were investigated in the parotid (PG) and submandibular (SMG) glands of Wistar rats exposed to a high-fat diet and then supplemented with ALA for a period of 4 weeks. The rats in the study were divided into 4 groups of 10 animals each: C (control), HFD, C + ALA, HFD + ALA. ***Results:*** The HFD + ALA group in comparison to the HFD group showed normalization of the activity of antioxidant enzymes to the levels observed in the C group only in the case of the SMG. Additionally, ALA supplementation was more effective in reducing the value of oxidative damage products in the PG compared to the SMG. ALA supplementation in the HFD group was not able to restore the disturbed total antioxidant capacity (TAC) of the salivary glands to the level observed in the C group. In the group of HFD + ALA rats, both unstimulated and stimulated salivation and the protein concentration in the SMG did not differ significantly from the parameters recorded in the group fed with HFD. ***Conclusion:*** ALA supplementation by rats fed the HFD diet prevents/reverses oxidative damage in the PG to a greater extent than in the SMG and is unable to completely restore disturbed TAC to the levels seen in C rats. Moreover, we observed that ALA supplementation did not improve the impaired secretory function of the salivary glands.

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Introduction

Obesity is a chronic condition classified as one of the diseases of civilization which are characterized by abnormal and excessive accumulation of adipose tissue. Data of the World Health Organization (WHO) indicate that the number of obese people nearly tripled between the years 1975 and 2016 [1]. Unfortunately, according to the latest 2019 epidemiological study, the problem continues to worsen. In 2019, 52.7% of the European Union's adult population was overweight. This nearly 53% rate included 17% of people suffering from obesity. It is an alarming fact that overweight/obesity is accompanied by––or perhaps it would be more appropriate to say: results in––numerous associated diseases, including insulin resistance, type 2 diabetes, hypertension and cancer. Excess body weight is also a risk factor for the development of neurodegenerative diseases, including Alzheimer's disease [2]. Our previous studies showed that obesity/overweight leads to impairment of salivary gland function and entails changes in salivary redox parameters [3-5]. Reduced oxidative modification of biomolecules in the saliva was observed six months after bariatric surgery; however, bariatric surgery related to weight loss was not effective in restoring salivary flow rate and oral redox equilibrium [3, 4]. In a physiological sense, saliva is as important for the oral cavity as blood for the entire body: it nourishes, moisturizes and protects the structures present in the mouth. Moreover, it facilitates proper digestion, phonation and speech, thus determining the so-called physical and mental well-being of humans. Its defense systems as well as antioxidant systems not only determine good condition of the oral cavity but also constitute a protective barrier for the gastrointestinal tract, and thus to the entire body [6].

Pharmacological preparations, including dietary supplements, are an option of managing body weight-related complications. Scientific evidence indicates the beneficial effect of numerous dietary supplements with antioxidant properties, such as phenolic compounds (catechins, resveratrol), vitamins and minerals (vitamin C, E, selenium, zinc) [7-10]. Antioxidants are compounds that inactivate the action / prevent the formation of oxygen free radicals (ROS), thus counteracting the development of oxidative stress (OS) [11]. Oxidative stress leads to oxidation of cellular components, dysfunction of tissues and organs and therefore to the occurrence of numerous diseases [12]. It has been evidenced that ROS participate in the development of obesity-related organ complications [10, 13-17]. Studies on the protective effect of antioxidants with respect to obesity complications demonstrated that these compounds may help inhibit adipocyte differentiation, reduce gastrointestinal fat adsorption, reduce lipogenesis and enhance apoptosis processes in adipocytes [7-10]. Our study indicates that N-acetyl-cysteine prevents/reverses the state of hyperglycemia and hyperinsulinemia. Thus, it stabilizes glucose metabolism and prevents the development of insulin resistance [14, 18, 19]. Through an inhibitory mechanism in the intestinal absorption of the ingested chow, it reduced the body weight of rats exposed to a high-fat diet (HFD) [14, 18-20]. NAC supplementation neutralized the effects of OS in the parotid parenchyma and increased stimulated saliva secretion, with only a slight reduction of excessive ROS level in the submandibular gland. The secretory function of the submandibular glands of rats exposed to HFD diet, despite NAC supplementation, did not return to the levels observed in the control group of healthy rats [14, 18, 19].

Another antioxidant important in terms of combating obesity-related complications is α-lipoic acid (ALA). This compound stabilizes glucose metabolism via insulin receptor activation and enhancement of glucose uptake by muscles [21], prevents AGE formation and reduces plasma concentrations of lactic acid and pyruvic acid [22]. ALA itself behaves as an antioxidant, directly scavenging ROS and reactive nitrogen species (RNS) [23]. It boosts the synthesis of the most important endogenous antioxidant: glutathione (GSH) and reduces oxidized forms of other antioxidants such as vitamin C, E and coenzyme Q10 [24], and by chelating some metals it lowers the probability of Fenton's reaction [25]. Endogenous ALA does not affect the action of mitochondrial enzymes; however, substantial reduction of OS improves overall cellular metabolism.

In search of antioxidants that would prevent the adverse effects of ROS-related obesity complications, we decided to investigate whether ALA could reverse/prevent HFD-induced salivary gland dysfunction and oxidative damage in the salivary glands of rats, and strengthen their antioxidant defense.

Materials and Methods

The study was conducted on 40 male Wistar rats with a baseline body weight of 60–80 g. After being delivered to the laboratory, the animals underwent a week-long period of adaptation to the new living conditions. They had access to drinking water and food throughout the experiment, with all rats receiving standard LSM (Agropol, Motycz, Poland) breeding feed composed of: 10.3% fats, 24.2% proteins and 65.5% carbohydrates. The rats were housed under standard rearing conditions (ambient temperature: 20–21°C; 12 hours of daylight / 12 hours of darkness; humidity dependent on external environmental conditions), two rats per cage. After six days of adaptation, the rats were randomly divided into two groups of 20 individuals each.

For the next six weeks, Group 1 was fed a standard LSM (Agropol, Motycz, Poland) feed containing: 10.3% fats, 24.2% proteins and 65.5% carbohydrates, while Group 2 obtained high-fat diet (Research Diet, USA, catalogue number D12492) composed of 59.8% fats, 20.1% proteins and 20.1% carbohydrates [15].

Each breeding cage housed two rats from the same group.

After six weeks, each rat was weighed and had blood drawn from the caudal vein to check blood glucose level (AccuCheck). Each rat in the group 2 demonstrated a glucose level above 250 mg/dL, whereas in the group 1 blood sugar level was up to 90 mg/dL (data not shown).

Next, the rats in each group were divided into two sub-groups of 10 individuals each. Group 1 has been divided into two groups marked with symbols C and C + ALA, group 2 was divided into two groups marked with symbols HFD and HFD+ALA. Two rats from the same group were housed in each breeding cage.

For the next four weeks, Group C was fed standard LSM feed as described above and had a 1% solution of Tween 80 in saline administered intragastrically once a day.

The C + ALA group was fed a standard LSM feed for a period of four subsequent weeks, and had a solution of ALA (at a dose of 30 mg/kg body weight) administered via a gastric tube at a volume of 2 ml/kg body weight.

The HFD group was fed the HFD (by Research Diet, USA) described above, and received a 1% solution of Tween 80 in saline, administered via a gastric tube once a day, for the next four weeks.

The HFD + ALA group was fed the above-mentioned HFD (Research Diet, USA), and once a day had intragastric administration of ALA solution (at a dose of 30 mg/kg body weight) at a volume of 2 ml/kg body weight, via a gastric tube, for the next four weeks.

The antioxidant solutions were prepared in a 1% solution of Tween 80 in saline. Intragastric administration of the substance is the only route of administration that provides reliable information about the intake of a full dose of the drug by an animal during the experiment. The substance was administered on a daily basis, always at 9 o'clock, and the procedure was carried out by two authorized and experienced researchers (M. M., J. M.).

The dose of ALA was determined by analyzing the available literature and selecting the most commonly applied doses of antioxidants [26, 27]. Considering the dosage of ALA, the rats were weighed every other day.

All rats lived to the end of the experiment in good general condition. Four weeks after the inclusion of antioxidant supplementation, the rats were sedated (phenobarbital, 80 mg x kg^{-1}, intraperitoneally) after overnight starvation. Blood was drawn from their caudal vein to assess glycemia. Unstimulated saliva secretion was measured to assess submandibular gland function, and stimulated saliva (pilocarpine hydrochloride at a dose of 5 mg/kg body weight, intraperitoneally; Sigma, Chemical Co., St Louis, MO, USA) was assayed to assess parotid gland function. A detailed description of the study can be found in our previous works [14, 15, 18, 19, 28-30]. Next, blood was collected from the abdominal artery, which was equivalent to killing the rats. The blood was centrifuged in order to obtain plasma. Plasma was enriched with 10 µL of 0.5 M BHT in acetonitrile per 1 mL of plasma (BHT Sigma-Aldrich, Germany) to prevent spontaneous

oxidation of the sample. The parotid and submandibular glands were removed by routine surgery. The salivary glands were pre-cleaned (to remove fascia, fat and blood) and weighed. The entire material was frozen in liquid nitrogen and then stored at -80°C.

On the day of biochemical determinations, the material was thawed. The salivary glands were reweighed, suspended at a rate of 1:10 in PBS enriched with protease inhibitors (1 tablet/10 mL) and BHT. The material was homogenized with Omni TH (Omni International, Kennesaw, GA, USA) and sonicated (1800 J/20 sec, 3 times, on ice, UP400S, Hielscher, Teltow, Germany). Then the suspension was centrifuged (15 minutes, 4°C, 5000 g, MPW, Med. Instruments, Warsaw, Poland). The obtained supernatant was used for biochemical determinations.

Biochemical determinations

Antioxidants. Salivary peroxidase (Px, E.C. 1.11.1.7) activity was measured according to Mansson-Rahemtulla et al. [31]. This colorimetrical method is based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to thionitrobenzoic acid. Absorbance was measured five times at 30-second intervals (at 412 nm wavelength).

Catalase (CAT, E.C. 1.11.1.6) activity was calculated based on the spectrophotometric method developed by Aebi [32] at 240 nm wavelength. This method is based on evaluating the decomposition rate of hydrogen peroxide (H_2O_2). One unit of CAT activity was defined as the amount of the enzyme able to break down 1 nmol of H_2O_2 in the sample within one minute.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically according to the method by Misra and Fridovich [33], based on oxidation of adrenaline to adrenochrome at 480 nm. One unit of SOD activity was determined as the amount of the enzyme that reduces adrenaline oxidation by 50%.

The ratio of SOD to Px + CAT was estimated according to the formula: SOD/Px + CAT.

Uric acid (UA) concentration was calculated by the colorimetric method with the use of a commercial kit (QuantiChrom™ Uric Acid DIUA-250; BioAssay Systems, Harward, CA, USA) according to the manufacturer's instructions. This method is based on the reaction of 2,4,6-tripyridyl-s-triazine with Fe^{3+} in the presence of UA. The absorbance was measured at 630 nm.

Reduced glutathione (GSH) concentration was measured according to Ellman's colorimetric procedure, based on the reduction of 5,5′-dithiobis-2-nitrobenzoic acid to 2-nitro-5-mercaptobenzoic acid under the influence of GSH at 412 nm wavelength [34].

Total antioxidant/oxidant status. Total antioxidant capacity (TAC) was determined by the colorimetric method as described by Erel [35], using 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation. Changes in the absorbance of $ABTS^{\cdot+}$ solution were measured at 660 nm and calibration curve was prepared for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Total oxidant status (TOS) was calculated colorimetrically according to the Erel method [36]. Briefly, in the presence of oxidants contained in biological materials, Fe^{2+} ions are oxidized to Fe^{3+} ions, and Fe^{3+} ions are detected with the use of xylenol orange (XO).

Oxidative stress index (OSI) was measured based on the formula: TOS/TAC × 100%.

Products of oxidative damage. Protein advanced glycation end products (AGE) were examined spectrofluorimetrically according to the method described by Kalousová et al. [37] by measuring AGE-specific fluorescence at the excitation wavelength 350 nm and emission wavelength 440 nm.

The concentration of advanced oxidation protein products (AOPP) was estimated colorimetrically by determining the oxidative capacity of iodine ions at a wavelength of 340 nm. Samples were diluted in PBS (pH 7.2) at a ratio of 1:5 (v/v) [37].

The concentrations of 4-HNE protein adduct were measured using a commercial enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions (OxiSelectTM HNE Adduct Competitive ELISA Kit, Cell Biolabs Inc. San Diego, CA, USA).

The concentrations of DNA/RNA oxidative damage products were measured by means of commercial ELISA kits (DNA/RNA oxidative damage ELISA Kit, Cayman Chemicals, Ann Arbor, MI, USA, respectively) according to the manufacturer's instructions. The test revealed all three oxidized guanine species: 8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA and 8-hydroxyguanine from either DNA or RNA.

The Protein Assay kit (Thermo Scientific PIERCE BCA Rockford, IL, USA; reading wavelength of 562 nm; bovine serum albumin as a standard) was used to measure protein concentration in salivary gland homogenates.

Plasma insulin concentration in all rats was determined using a commercial ELISA kit (Shibayagi Co., Gunma, Japan).

Tissue sensitivity to insulin was assayed by means of the Homeostasis Model Assessment of Insulin Resistance index: HOMA-IR index (= fasting insulin [U/mL] x fasting glucose [mM]/22.5) [38].

Statistics

GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) was used for statistical data analysis. Homogeneity of variance was checked based on Levene's test, and normality of distribution was checked by the Shapiro-Wilk test. Values were presented as mean ± standard deviation. Three-factor (diet, antioxidant, salivary gland type) and one-factor ANOVA analyses of variance were performed, followed by Tukey Honest Significant Difference (HSD) post-hoc test. Correlation analysis was performed using Pearson's correlation coefficient. The significance level was set at $p \leq 0.05$.

Results

General characteristics of rats

Body weight. The group fed the HFD only was characterized by significantly higher body weight (↑29%, $p < 0.0001$) compared to the control group. ALA supplementation led to a reduction in the body weight of rats in the HFD + ALA group, compared to the HFD group (↓24%, $p < 0.0001$), to the levels observed in the control group. Interestingly, the group of rats fed a standard diet with additional ALA supplementation had a significantly higher body weight (↑18%, $p = 0.001$) compared to the control group, and a significantly lower body weight (↓15%, $p = 0.001$) in comparison with the HFD group (Table 1).

Glucose. In the group of HFD rats we observed a significantly higher concentration of glucose (↑41%, $p < 0.0001$) compared to the control group. ALA supplementation in the group of HFD rats led to a significant reduction (↓34%, $p < 0.0001$) in glucose concentration compared to the HFD group without ALA supplementation, but this concentration was still significantly higher (↑12%, $p = 0.03$) compared to the controls. ALA supplementation in the control group significantly lowered (↓37%, $p < 0.0001$) glucose level compared to the HFD group without ALA supplementation (Table 1).

Table 1. Rats general characteristics. C- control, HFD- high fat diet group, C+ALA- control supplemented with α-lipoic acid (ALA), HFD+ALA- high fat diet group supplemented with α-lipoic acid (ALA), HOMA-IR- Homeostatic Model Assessment of Insulin resistance, PG- parotid gland, SMG- submandibular gland. Values followed by $^{\bullet,\Delta}$ in columns are significantly different ($p<0.05$) : $^{\bullet}$ vs C, $^{\Delta}$ vs HFD

Parameter	C	HFD	C+ ALA+	HFD+ALA+	ANOVA P
Body weight (g)	279.6 ± 9.246$^{\Delta}$	395.9 ± 34.85$^{\bullet}$	337.6 ± 40.8$^{\bullet,\Delta}$	302 ± 34.85$^{\Delta}$	<0.0001
Glucose (mg/dL)	89.09 ± 5.983$^{\Delta}$	150.6 ± 5.835$^{\bullet}$	94.67 ± 7.009$^{\Delta}$	99.6 ± 12.01$^{\bullet,\Delta}$	<0.0001
Insulin (μU/mL)	74.19 ± 5.724$^{\Delta}$	163 ± 14.64$^{\bullet}$	89.01 ± 18.76$^{\Delta}$	90.82 ± 12.49$^{\bullet,\Delta}$	<0.0001
HOMA-IR	1.635 ± 0.1931$^{\Delta}$	6.062 ± 0.6073$^{\bullet}$	2.072 ± 0.4263$^{\Delta}$	2.243 ± 0.4695$^{\bullet,\Delta}$	<0.0001
PG weight (mg)	98.88 ± 6.67$^{\Delta}$	79.2 ± 8.879$^{\bullet}$	92.35 ± 11.21$^{\Delta}$	110.4 ± 7.244$^{\bullet,\Delta}$	<0.0001
SMG weight (mg)	246.3 ± 18.29	258.5 ± 17.8	273.8 ± 44.58	287.4 ± 33.48$^{\bullet}$	0.0277
PG total protein (μg/mL)	3832 ± 468.7$^{\Delta}$	2441 ± 488.6	3781 ± 421.7$^{\Delta}$	3028 ± 85.1$^{\Delta}$	<0.0001
SMG total protein (μg/mL)	3690 ± 679.6$^{\Delta}$	2630 ± 398.3	3554 ± 516.8$^{\Delta}$	2296 ± 63$^{\bullet}$	0.0006
Stimulated salivary flow (μL/min)	129.9 ± 28.27$^{\Delta}$	74.91 ± 14.77	107.9 ± 21.02$^{\Delta}$	84.8± 0.09$^{\bullet}$	<0.0001
Unstimulated salivary flow (μL/min)	0.56±0.05	0.314±0.02$^{\bullet}$	0.52±0.01	0.35±0.05$^{\bullet}$	<0.002

Insulin. Insulin concentration was significantly higher (↑55%, $p < 0.0001$) in the HFD group compared to the group fed a standard diet. Supplementation with ALA in the HFD group led to a significant reduction (↓44%, $p < 0.0001$) in insulin level compared to the HFD group without ALA supplementation. Moreover, insulin concentration was still significantly higher (↑22%, $p = 0.04$) than in the control group. ALA supplementation in the control group resulted in a significantly lower (↓45%, $p < 0.0001$) insulin concentration compared to the HFD-fed group without ALA supplementation (Table 1).

HOMA-IR. The HFD-fed group demonstrated a significantly higher HOMA-IR value (↑73%, $p < 0.0001$) compared to the control group. ALA supplementation in the HFD group was responsible for a significant decrease (↓63%, $p < 0.0001$) in the HOMA-IR value compared to the group fed only the HFD; however, that value was still significantly higher (↑44%, $p = 0.02$) than in the control group. The addition of ALA to the diet of the control rats significantly lowered the value of HOMA-IR (↓66%, $p < 0.0001$) compared to the HFD group (Table 1).

Weight of salivary glands. The weight of the parotid gland was significantly reduced (↓20%, $p < 0.0001$) in the HFD group compared to the controls. The group receiving ALA supplementation considerably increased the weight of the parotid gland in HFD + ALA rats compared to the HFD group and the control group without this supplementation (↑28%, $p < 0.0001$, ↑11%, $p = 0.02$, respectively). The control group supported with ALA supplementation demonstrated a significantly higher (↑14%, $p = 0.009$) weight of the parotid gland compared to the group fed only the HFD (Table 1).

There was no significant difference between the control group and the HFD group. Interestingly, the weight of the submandibular glands in the HFD + ALA group was significantly higher (↑14%, $p = 0.02$) compared to the control group.

Total protein. The result of applying the HFD was a significant reduction in parotid and submandibular protein levels compared to the control group without supplementation (↓36%, $p = 0.003$; ↓27%, $p = 0.002$, respectively). ALA supplementation raised parotid protein levels in the HFD + ALA group compared to the group receiving the HFD alone (↑23%, $p = 0.02$), to the levels observed in group C. In the submandibular salivary glands, despite the inclusion of ALA supplementation, protein levels in the HFD + ALA group did not differ from those in the HFD group, and remained at a significantly lower level compared to group C (↓37%, $p = 0.001$) (Table 1).

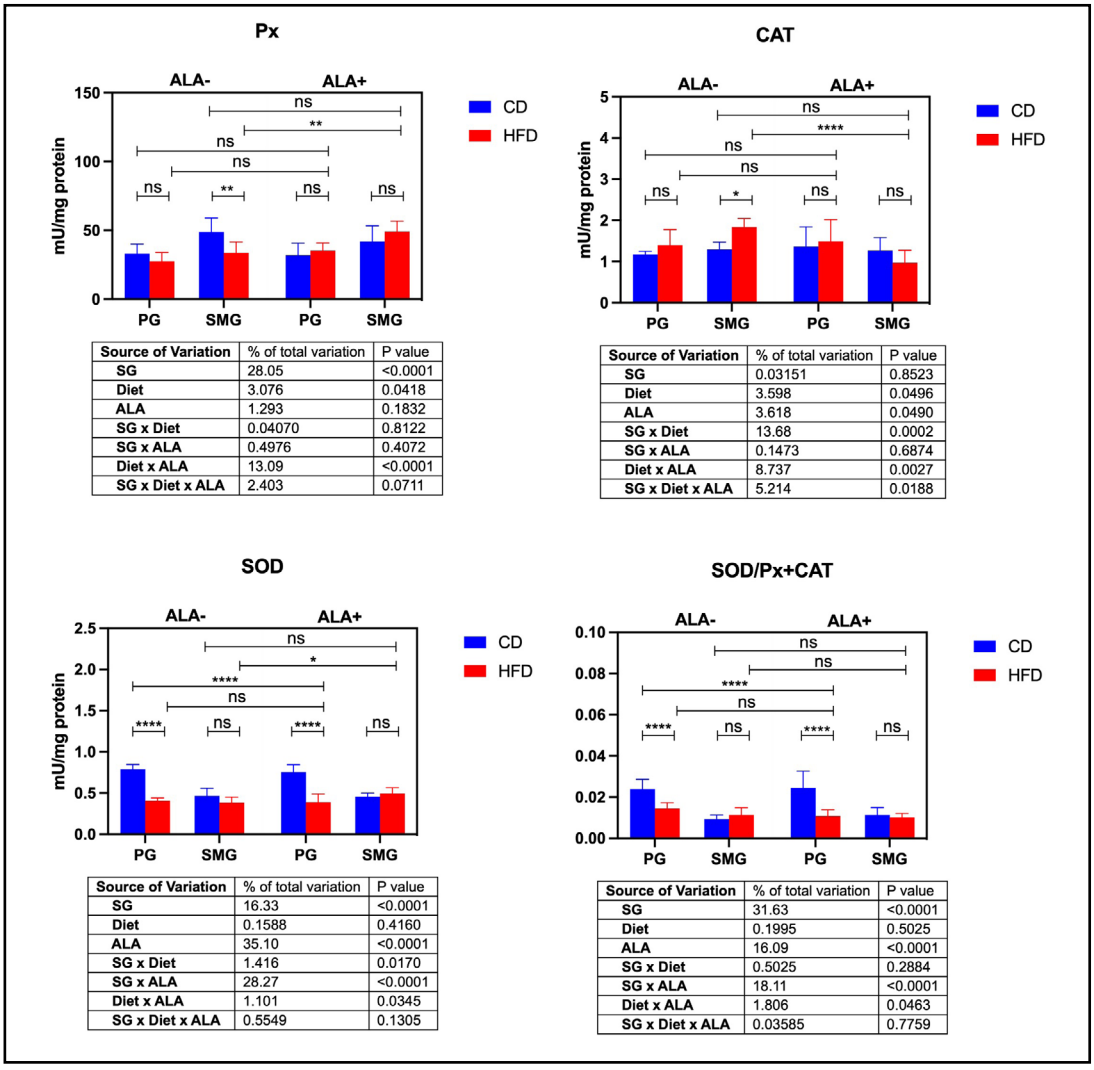
Salivary flow. Stimulated (↓42%, $p = 0.001$; ↓34%, $p = 0.002$, respectively) and unstimulated (↓44% $p = 0.0001$; ↓37.5%, $p = 0.002$, respectively) saliva secretion in both groups fed the HFD, i.e., HFD and HFD + ALA groups remained significantly lower compared to group C. It was not notably different between HFD and HFD+ALA groups (Table 1).

Redox parameters

Parotid glands. There was no significant difference in Px and CAT activity between the HFD group and the group fed a standard diet, and there was no considerable difference in the activity of the above-mentioned antioxidant enzymes between the control + ALA group and the HFD/HFD + ALA groups (Fig. 1).

The activity of SOD in the parotid glands of HFD rats was significantly lower (↓48.5%, $p < 0.0001$) compared to the control group. ALA supplementation failed to increase SOD activity in the HFD + ALA group compared to the HFD group. The group fed a standard diet + ALA showed significantly higher SOD activity compared to the HFD + ALA group (↑48%, $p < 0.0001$, respectively) (Fig. 1). The activity of SOD in the HFD + ALA group was significantly lower compared to the group of rats fed a standard diet (↓51%, $p < 0.0001$).

Similarly, the ratio of SOD to Px + CAT in the HFD group was significantly lower (↓39%, $p < 0.0001$) than in the control group. ALA supplementation had no effect on the ratio of



Source of Variation	% of total variation	P value
SG	28.05	<0.0001
Diet	3.076	0.0418
ALA	1.293	0.1832
SG x Diet	0.04070	0.8122
SG x ALA	0.4976	0.4072
Diet x ALA	13.09	<0.0001
SG x Diet x ALA	2.403	0.0711

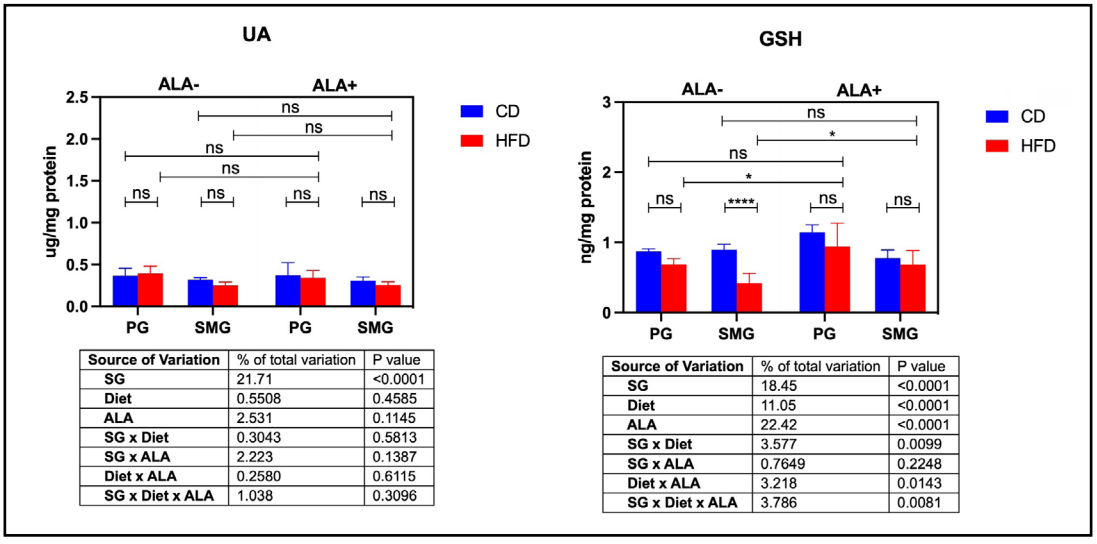
Source of Variation	% of total variation	P value
SG	0.03151	0.8523
Diet	3.598	0.0496
ALA	3.618	0.0490
SG x Diet	13.68	0.0002
SG x ALA	0.1473	0.6874
Diet x ALA	8.737	0.0027
SG x Diet x ALA	5.214	0.0188

Source of Variation	% of total variation	P value
SG	16.33	<0.0001
Diet	0.1588	0.4160
ALA	35.10	<0.0001
SG x Diet	1.416	0.0170
SG x ALA	28.27	<0.0001
Diet x ALA	1.101	0.0345
SG x Diet x ALA	0.5549	0.1305

Source of Variation	% of total variation	P value
SG	31.63	<0.0001
Diet	0.1995	0.5025
ALA	16.09	<0.0001
SG x Diet	0.5025	0.2884
SG x ALA	18.11	<0.0001
Diet x ALA	1.806	0.0463
SG x Diet x ALA	0.03585	0.7759

Fig. 1. Effect of ALA supplementation on enzymatic salivary antioxidant barrier of rats. C, control rats; HFD, high-fat fed rats; -ALA, without ALA supplementation; +ALA, with ALA supplementation; SOD, superoxide dismutase; CAT, catalase; Px, salivary peroxidase; SOD/ Px+CAT, ratio of SOD to Px + CAT; ns, not important significantly; $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$, $^{****}p<0.0001$.

SOD to Px + CAT in the parotid glands of the HFD group compared to the HFD + ALA group. The control group supported by ALA supplementation demonstrated a significantly higher value of ratio SOD to Px + CAT in the parotid glands compared to HFD + ALA groups (↑56%, $p < 0.0001$, respectively) (Fig. 1). The ratio of SOD to Px + CAT in the HFD + ALA group was significantly lower compared to the group of rats fed a standard diet (↓55%, $p < 0.0001$). Interestingly, there were no considerable differences in the concentration of GSH and UA in the parotid glands between the HFD group and the control, but ALA supplementation led to a significant increase in GSH concentration in both groups (↑23%, $p = 0.0152$; ↑27%, $p = 0.0079$, respectively). In the group fed a standard diet + ALA, there was no notable difference in the concentration of UA and GSH in relation to the group of HFD + ALA. Interestingly, the control + ALA group was characterized by significantly higher GSH values (↑40%, $p < 0.0001$) and no significant difference in UA values compared to the HFD group without ALA supplementation (Fig. 2).



Source of Variation	% of total variation	P value
SG	21.71	<0.0001
Diet	0.5508	0.4585
ALA	2.531	0.1145
SG x Diet	0.3043	0.5813
SG x ALA	2.223	0.1387
Diet x ALA	0.2580	0.6115
SG x Diet x ALA	1.038	0.3096

Source of Variation	% of total variation	P value
SG	18.45	<0.0001
Diet	11.05	<0.0001
ALA	22.42	<0.0001
SG x Diet	3.577	0.0099
SG x ALA	0.7649	0.2248
Diet x ALA	3.218	0.0143
SG x Diet x ALA	3.786	0.0081

Fig. 2. Effect of ALA supplementation on non-enzymatic salivary antioxidant barrier of rats. C, control rats; HFD, high-fat fed rats; -ALA, without ALA supplementation; +ALA, with ALA supplementation; UA, uric acid; GSH, reduced glutathione; ns, not important significantly; $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$, $^{****}p<0.0001$.

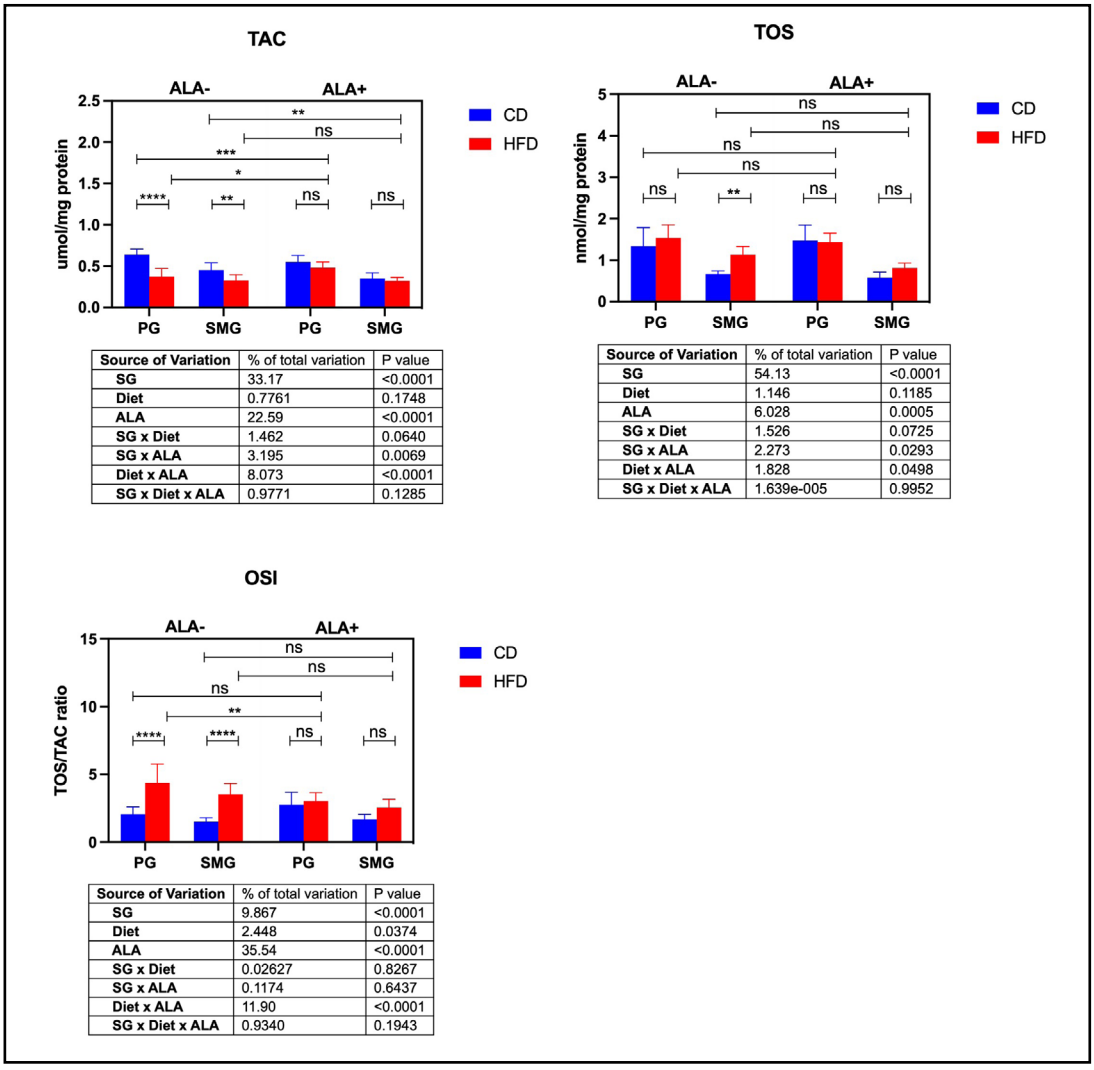
In the parotid glands of HFD rats, TAC values were significantly lower (↓42%, $p < 0.0001$) compared to the group provided with a standard diet. ALA supplementation led to a significant increase (↑22%, $p = 0.0406$) in TAC concentration in the HFD + ALA group compared to the HFD group (Fig. 3). Levels of TAC in the HFD + ALA group were significantly lower compared to the control group fed a standard diet (↓64%, $p = 0.0002$).

There was no significant difference in TOS value in the parotid glands between the HFD group and the controls, and there was no considerable difference between the control given ALA supplementation and the HFD/HFD + ALA group (Fig. 3).

The values of OSI and AGE were significantly higher (↑53%, $p < 0.0001$, ↑19%, $p = 0.0307$, respectively) in the HFD group compared to the control group. ALA supplementation led to a significant reduction of OSI and AGE values (↓31%, $p = 0.0036$; ↓53%, $p < 0.0001$, respectively) in the HFD + ALA group compared to the HFD group. The controls supported with ALA supplementation presented significantly higher levels of AGE compared to the HFD + ALA group (Fig. 3, 4). In terms of AGE, we observed a significant decrease of its content in the HFD + ALA group compared to the control group fed a standard diet (↓42%, $p < 0.0001$).

The values of AOPP, 4-HNE and DNA/RNA oxidative damage products in the parotid glands of HFD rats were significantly higher (↑27%, $p = 0.0093$; ↑51%, $p < 0.0001$; ↑37%, $p = 0.0145$, respectively) compared to the control group. ALA supplementation in the HFD + ALA group resulted in a significant reduction in the concentration of 4-HNE and DNA/RNA oxidative damage products compared to the HFD group (↓29%, $p = 0.0081$; ↓40%, $p < 00001$, respectively) to the levels observed in the parotid glands of the control group. Unfortunately, ALA supplementation was not able to lower AOPP values in the HFD + ALA group compared to the HFD group (Fig. 4).

Submandibular glands. Px activity in the HFD group was significantly lower (↓31%, $p = 0.0022$) and CAT – significantly higher (↑29%, $p = 0.0146$) compared to the control group. ALA supplementation led to a significant increase in Px activity (↑31%, $p = 0.0018$) and a decrease in CAT activity (↓47%, $p < 0.0001$) in the submandibular glands of the HFD + ALA group compared to the HFD group, to the values observed in the control group. There was no significant difference in Px and CAT activity between the group fed a standard diet + ALA and HFD + ALA group (Fig. 1).



Source of Variation	% of total variation	P value
SG	33.17	<0.0001
Diet	0.7761	0.1748
ALA	22.59	<0.0001
SG x Diet	1.462	0.0640
SG x ALA	3.195	0.0069
Diet x ALA	8.073	<0.0001
SG x Diet x ALA	0.9771	0.1285

Source of Variation	% of total variation	P value
SG	54.13	<0.0001
Diet	1.146	0.1185
ALA	6.028	0.0005
SG x Diet	1.526	0.0725
SG x ALA	2.273	0.0293
Diet x ALA	1.828	0.0498
SG x Diet x ALA	1.639e-005	0.9952

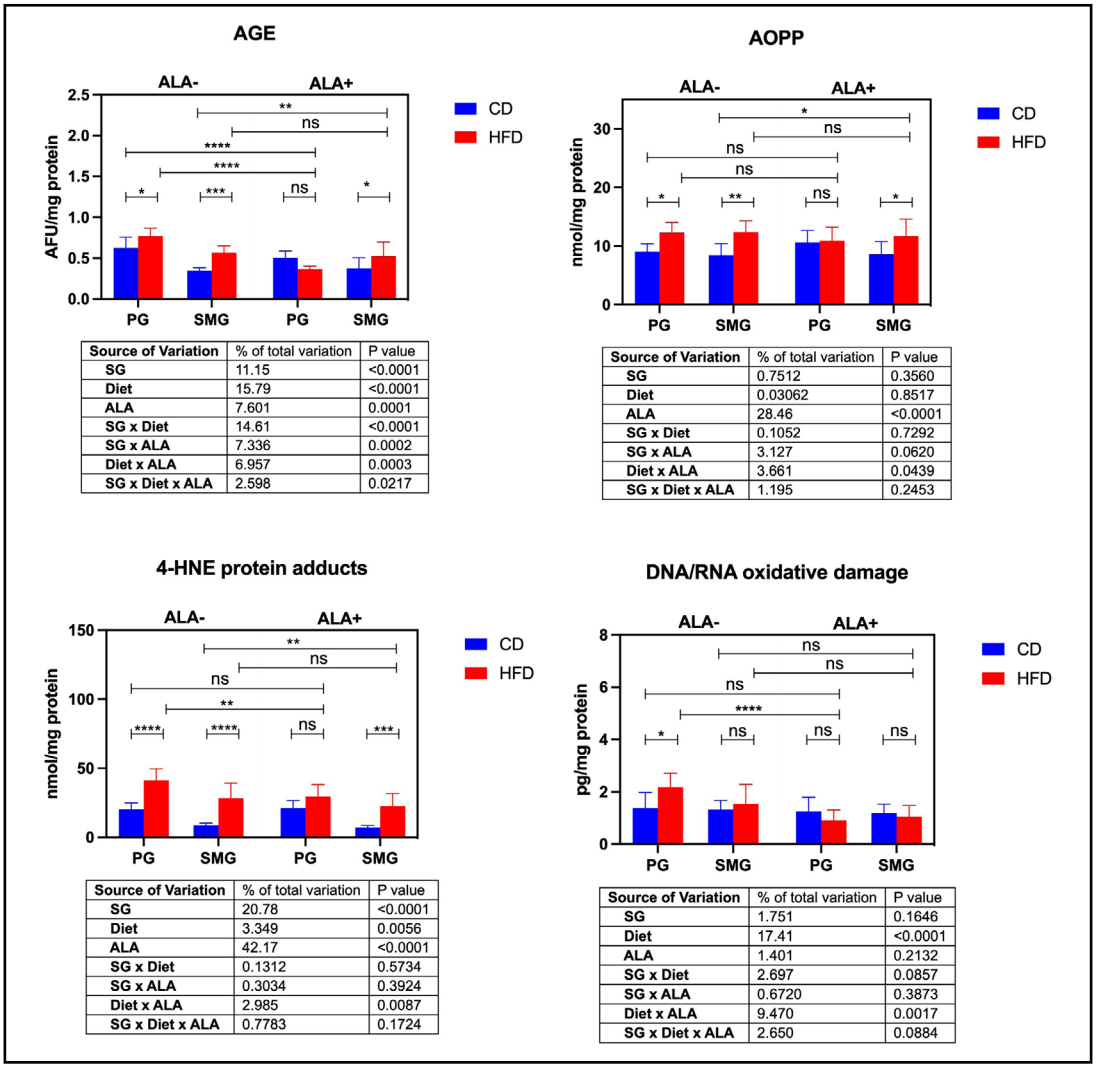
Source of Variation	% of total variation	P value
SG	9.867	<0.0001
Diet	2.448	0.0374
ALA	35.54	<0.0001
SG x Diet	0.02627	0.8267
SG x ALA	0.1174	0.6437
Diet x ALA	11.90	<0.0001
SG x Diet x ALA	0.9340	0.1943

Fig. 3. Effect of ALA supplementation on salivary redox status of rats. C, control rats; HFD, high-fat fed rats; -ALA, without ALA supplementation; +ALA, with ALA supplementation; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative status index; ns, not important significantly; $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$, $^{****}p<0.0001$.

There was no significant difference in SOD activity in the submandibular glands between the HFD group and the control group. However, a considerable increase (↑23%, p = 0.0188) in SOD activity was observed in the group fed the HFD after ALA supplementation compared to the HFD group without ALA supplementation. There was no significant difference in SOD activity between the control + ALA group and the HFD + ALA group (Fig. 1).

Similarly, no significant difference was found in the SOD to Px + CAT ratio and UA concentration, both before and after ALA supplementation, between the HFD and control groups. Moreover, there was no notable difference in SOD to Px + CAT ratio between the control + ALA group and the HFD + ALA group (Fig. 1).

The concentration of GSH in the submandibular glands of HFD rats was significantly lower (↓53%, p < 0.001) compared to the rats fed a standard diet. ALA supplementation led to a significant increase (↑38%, p = 0.012) in GSH concentration in the submandibular glands of HFD + ALA rats compared to the HFD group, to the levels observed in the control group. The concentration of GSH in the control group after ALA supplementation revealed no significant differences in relation to the HFC + ALA group (Fig. 2).



Source of Variation	% of total variation	P value
SG	11.15	<0.0001
Diet	15.79	<0.0001
ALA	7.601	0.0001
SG x Diet	14.61	<0.0001
SG x ALA	7.336	0.0002
Diet x ALA	6.957	0.0003
SG x Diet x ALA	2.598	0.0217

Source of Variation	% of total variation	P value
SG	0.7512	0.3560
Diet	0.03062	0.8517
ALA	28.46	<0.0001
SG x Diet	0.1052	0.7292
SG x ALA	3.127	0.0620
Diet x ALA	3.661	0.0439
SG x Diet x ALA	1.195	0.2453

Source of Variation	% of total variation	P value
SG	20.78	<0.0001
Diet	3.349	0.0056
ALA	42.17	<0.0001
SG x Diet	0.1312	0.5734
SG x ALA	0.3034	0.3924
Diet x ALA	2.985	0.0087
SG x Diet x ALA	0.7783	0.1724

Source of Variation	% of total variation	P value
SG	1.751	0.1646
Diet	17.41	<0.0001
ALA	1.401	0.2132
SG x Diet	2.697	0.0857
SG x ALA	0.6720	0.3873
Diet x ALA	9.470	0.0017
SG x Diet x ALA	2.650	0.0884

Fig. 4. Effect of ALA supplementation on oxidative modification of the salivary glands of rats. C, control rats; HFD, high-fat fed rats; -ALA, without ALA supplementation; +ALA, with ALA supplementation; AGE, advanced glycation end product; AOPP, advanced oxidation protein product; ns, not important significantly; $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$, $^{****}p<0.0001$.

The values of TAC in the submandibular glands of HFD rats were significantly lower (↓27%, p = 0.0092) and of TOS – significantly higher (↑41%, p = 0.0047) compared to the control group, and these values did not significantly increase/decrease (respectively) after ALA supplementation (Fig. 3). Moreover, TAC levels in the submandibular glands of rats from the HFD group supplemented with ALA were significantly lower than in the control group (CD-ALA) (↓26%, p = 0.002).

The OSI value in the submandibular glands was significantly higher (↑57%, p < 0.0001) in the HFD group compared to the control group (Fig. 3).

AGE concentration in the HFD group was significantly higher (↑39%, p = 0.0001) compared to the control group. Unfortunately, ALA supplementation did not significantly lower this value in the HFD + ALA group compared to the HFD group (Fig. 4). Moreover, AGE values in the submandibular glands of HFD + ALA rats were considerably higher compared to the CD-ALA group (↑38%, p = 0.0002).

The values of AOPP and 4-HNE in the submandibular glands of rats fed the HFD were significantly higher (↑32%, p = 0.0007, ↑69%, p < 0.0001, respectively) compared to the

control group. The addition of ALA to the diet did not effectively decrease the values of AOPP and 4-HNE in HFD + ALA rats compared to HFD rats. The values of 4-HNE and AOPP were significantly higher in the HFD + ALA group compared to the control + ALA group (↑68%, $p = 0.0002$; ↑30%, $p = 0.0002$), as well as compared to the CD-ALA group (↑31%, $p = 0.003$; ↑47%, $p = 0.0001$, respectively). There was no significant difference in 8-OHdG values between the group fed the control diet + ALA and the HFD / HFD + ALA group (Fig. 4).

A three-way ANOVA analysis of variance showed that Px activity was correlated with salivary gland type ($p < 0.0001$), diet ($p = 0.04$) and interaction of diet and ALA ($p < 0.0001$) (Fig. 1).

A three-way ANOVA analysis of variance demonstrated that CAT activity depended on diet ($p = 0.04$), ALA supplementation ($p = 0.04$) and relationships of: diet and salivary gland type ($p = 0.0002$), diet and ALA ($p = 0.003$) as well as salivary gland type, diet and ALA ($p = 0.02$) (Fig. 1).

A three-way ANOVA analysis of variance showed that SOD activity was connected with salivary gland type ($p < 0.0001$), ALA supplementation ($p < 0.0001$) and correlations of: diet and salivary gland type ($p = 0.02$), ALA and salivary gland type ($p < 0.0001$) as well as diet and ALA ($p = 0.03$) (Fig. 1).

By means of a three-way ANOVA analysis of variance we demonstrated that SOD/Px + CAT ratio depended on salivary gland type ($p < 0.0001$), ALA supplementation ($p < 0.0001$), interaction of ALA and salivary gland type ($p < 0.0001$) and co-dependence of diet and ALA ($p = 0.04$) (Fig. 1).

A three-way ANOVA test demonstrated that UA concentration was correlated only with a salivary gland type ($p < 0.0001$); however, GSH concentration depended not only on the type of a salivary gland ($p < 0.0001$) but also on diet ($p < 0.0001$), ALA supplementation ($p < 0.0001$) and the interactions of: salivary gland type ($p = 0.01$), diet and ALA ($p = 0.01$) as well as of salivary gland type, diet and ALA ($p = 0.01$) (Fig. 2).

A three-way ANOVA analysis of variance showed that TAC was dependent on salivary gland type ($p < 0.0001$), ALA supplementation ($p < 0.0001$), the interaction of: salivary gland type and ALA ($p = 0.0002$), diet and ALA ($p < 0.0001$) (Fig. 3).

A three-way ANOVA analysis of variance revealed that TOS level was correlated with salivary gland type ($p < 0.0001$), ALA supplementation ($p = 0.0005$) and the interaction of: salivary gland type and ALA ($p = 0.03$) as well as diet and ALA ($p = 0.04$) (Fig. 3).

A three-way ANOVA test showed that OSI was dependent on salivary gland type ($p < 0.0001$), diet ($p = 0.005$), ALA supplementation ($p < 0.0001$) and the interaction of diet and ALA ($p < 0.0001$) (Fig. 3).

According to the performed three-way ANOVA analysis of variance, AGE concentration depended on salivary gland type ($p < 0.0001$), diet ($p < 0.0001$), ALA supplementation ($p = 0.002$) and the relationship between: diet and salivary gland type ($p < 0.0001$), ALA and salivary gland type ($p = 0.002$) and diet and ALA ($p < 0.0001$) (Fig. 4).

A three-way ANOVA analysis of variance demonstrated that AOPP concentration was correlated with salivary gland type ($p = 0.01$), ALA supplementation ($p < 0.0001$) and the interaction of diet and ALA ($p = 0.0004$) (Fig. 4).

A three-way ANOVA analysis of variance showed that 4-HNE protein adduct concentration depended on salivary gland type ($p < 0.0001$), diet ($p = 0.006$), ALA supplementation ($p < 0.0001$) and the interaction of diet and ALA ($p = 0.009$) (Fig. 4).

A three-way ANOVA test showed that the concentration of DNA/RNA oxidative damage products was related to diet ($p < 0.0001$) and the interaction of diet and ALA ($p = 0.002$) (Fig. 4).

Correlations

A positive correlation was observed between GSH and TAC concentrations ($r = 0.81$, $p = 0.005$) in the parotid glands of HFD + ALA rats. The same group showed a negative correlation between the concentrations of GSH and 4-HNE ($r = 0.67$, $p = 0.037$) and GSH and AGE ($r = 0.79$, $p = 0.007$) and a negative correlation between AGE content in the homogenate of the submandibular salivary glands and unstimulated saliva secretion ($r = -0.94$, $p < 0.0001$).

Discussion

ALA is a naturally occurring dithiol compound synthesized in mitochondria from octanoic acid. The resulting ALA plays an important role in energy production by serving as a cofactor for mitochondrial α-ketoacid dehydrogenase. It has been scientifically evidenced that exogenously supplied ALA cannot be used as a booster of mitochondrial metabolism, but it exhibits a unique set of biochemical interactions with a potential pharmacotherapeutic value against numerous pathophysiological factors. ALA is known as a detoxifying agent and antidiabetic drug, and has been used to ameliorate age-related cardiovascular, cognitive, and neuromuscular deficits as a modulator of various inflammatory signaling pathways [39-41]. As already mentioned in the introduction to this paper, ALA demonstrates antioxidant activity [42]. The main purpose of the presented study was to investigate whether ALA, like N-acetyl-cysteine, can reverse/minimize oxidative stress in the salivary glands of rats exposed to a high-fat diet (HFD). Furthermore, we were interested in whether ALA supplementation can restore secretory function of salivary glands, impaired as a result of a diet and abnormal sugar metabolism.

The HFD fed to rats for six initial weeks constitutes an experimental model that leads to increased body weight, hyperglycemia, hyperinsulinemia and insulin resistance [13, 18, 19, 30, 41], as confirmed by the present study. The results of our experiment demonstrated that 4-week ALA supplementation boosts carbohydrate metabolism to some extent, which is probably due to the fact that ALA is a compound that improves tissue insulin sensitivity and activates insulin signaling pathways [43]. In the HFD + ALA group, the concentrations of glucose and insulin, and HOMA-IR index reached significantly lower levels than in the HFD group; yet, these parameters remained at considerably higher levels compared to the control group. According to research conducted so far on the subject, ALA reduces body fat mass via inhibition of hypothalamic AMP-activated protein kinase (AMPK), decreases daily energy intake [44], reduces lipoprotein lipase activity [45] and increases lipolysis while inhibiting lipogenesis [46]. As expected, we observed a significant decrease in the body weight of HFD rats supported with ALA supplementation compared to the group fed the HFD only, to levels observed in the standard diet group. It should be noted that ALA exhibited virtually opposite effect on control rats versus HFD rats in regard to general characteristics. Perhaps this different effect is related to the fact that ALA shows its protective effect only in tissues subjected to oxidative stress [42]. A similar mechanism was observed after the use of NAC, the protective effect of which was shown only in tissues with glutathione deficiency [14, 15, 18].

The body's cells have mechanisms to prevent over-production of ROS. One of them is the interaction of three groups of enzymes cooperating in the neutralization of ROS, namely SOD1, CAT and GPx. Non-enzymatic compounds also play an important role. GSH is one of the most important intracellular antioxidants, which limit the impact of OS and protect cellular components against oxidation and UA derived from blood. The latter limits free radical processes through the capture of hydroxyl radicals, lipid peroxides and singlet oxygen, and the formation of stable complexes with iron, copper and manganese ions, which stabilizes the reduced form of vitamin C [11]. The organ's antioxidant failure results in oxidative modification of cellular components. This condition is expressed by increased levels of oxidatively modified cellular components and an increase in OSI (oxidative stress index). ROS have the ability to modify all cellular elements, therefore, for the full range of OS, several types of oxidative modification should be assessed. We choose from the most frequently assessed oxidative modification products: AOPP, AGE for the evaluation of protein oxidative damage, 4-HNE protein adduct for the evaluation of lipid peroxidation and DNA/RNA for the evaluation of oxidative modification of genetic material [28, 29]. Consistent with the previous studies, the effects of introducing a high-fat diet include: the impairment of antioxidant systems observed by us in relation to the control group (in the parotid glands: ↓SOD, ↓SOD/Px + CAT, ↓TAC; in the submandibular glands: ↓Px, ↑CAT, ↑GSH, ↓TAC) [13-15, 18, 19, 30, 41, 47]. In our opinion, the most important parameter is TAC, i.e., the total antioxidant

capacity of a given sample, reflecting the capacity to combat ROS/RNS. Although ↑TOS (number of ROS/RNS in a given sample) was only observed in the submandibular salivary glands, ↑OSI (↑AGE, AOPP, 4-HNE and DNA/RNA) were observed in both salivary glands of HFD rats.

The addition of ALA to the diet of rats did not significantly improve the activity of antioxidant enzymes in the parotid glands (no increase in SOD and SOD/Px + CAT activity, vs. the HFD group), but changed the activity of all antioxidant enzymes of the submandibular salivary glands (↑Px, ↓CAT, ↑SOD) vs. the HFD group. The activity of submandibular gland enzymes was completely normalized to the levels observed in the control group. However, it should be noted that after ALA supplementation, TAC was significantly higher in the parotid glands than in case of the HFD group (it did not change in the submandibular glands), and in both these types of salivary glands it remained at a significantly lower level than in the group of rats receiving a standard diet. The latter observation demonstrates that ALA supplementation accompanying the HFD diet is unable to restore the impaired antioxidant potential of salivary glands to the level observed in control rats. ALA has a great capacity to reduce oxidized form of GSH, thus increasing the pool of reduced GSH [48]. This fact presumably explains the increase in reduced GSH concentration observed in our study in both salivary glands of rats from the HFD +ALA group compared to the HFD group. Increased concentration of GSH is probably responsible for elevated TAC in the parotid glands vs. the HFD group, as confirmed by the positive correlation between GSH and TAC.

A three-way ANOVA showed that the behavior of antioxidant systems is mainly dependent on the type of the salivary gland; ALA supplementation as well as the interaction of different components of the experiment (salivary gland type, supplementation and diet) also play an important role. The parotid gland is the gland in which the predominant type of metabolism is the aerobic one, whereas in the submandibular gland energy is mainly produced in the course of anaerobic glycolysis. Furthermore, the parotid gland is thought to be the major source of antioxidants in the oral cavity and it is believed that this gland is physiologically prepared to prevent oxidative stress [49]. Therefore, additional supplementation with an exogenous antioxidant, despite having no effect on antioxidant enzymes, results in a significant increase of the antioxidant potential primarily in the parotid salivary glands, with no effect on TAC in the submandibular glands of HFD + ALA rats, similar to NAC supplementation [19].

Similarly, ALA effectively decreases lipid peroxidation, DNA/RNA oxidative damage and AGE formation in the parotid glands, and only AOPP in the submandibular glands. These observations may suggest that ALA prevents/reverses oxidative damage in the parotid glands to a greater extent than in the submandibular glands. The negative correlation between GSH concentration with 4-HNE and AGE indicates a leading role of GSH in protecting this gland from oxidation. In addition to the thiol groups of proteins, GSH is the redox buffer with the highest capacity and the so-called rapid-response system which is primarily used to reduce the content of oxidants and xenobiotics [50]. GSH may also directly neutralize ROS, thus replacing inefficient antioxidant enzymes. It is also involved in the detoxification of lipid peroxidation products such as 4-HNE [51]. Another explanation for the decreased concentration of oxidative damage to the parotid gland in the HFD + ALA group of rats is the intensified degradation of the oxidation products produced, as observed in the parotid glands of rats exposed to a protein-rich diet [52].

We further observed that ALA supplementation did not improve the impaired secretory function of salivary glands. In the HFD + ALA group of rats, both unstimulated and stimulated saliva secretion as well as protein concentration in the submandibular glands did not significantly differ from those parameters recorded in the group fed a fat-rich diet, and remained at considerably lower levels than in the control group. It was observed that AGE decreases the activity of nitric oxide synthase (NOS) and reduces nitric oxide (NO) concentration by its consumption in the process of peroxynitrite formation [53]. The negative correlation between AGE levels in the submandibular salivary gland homogenate of HFD + ALA and HFD rats and unstimulated secretion may be helpful in defining the pathomechanism of reduced saliva secretion. Indeed, NO signaling is thought to play a

key role in salivary gland secretion. NOS is synthesized in postganglionic parasympathetic neurons surrounding salivary gland alveolar cells as well as in the cells themselves [54, 55]. NO regulates not only the blood flow through alveolar cells but also saliva secretion in non-adrenergic non-cholinergic transmission upon cholinergic activation [54]. On the other hand, AGE formation on extracellular matrix proteins may disrupt both matrix-matrix and matrix-cells interactions, which has been reported in the literature as one of the reasons for decreased saliva secretion in the course of Sjögren's syndrome [56-59]. Reduction/dysfunction of the active surface area of the submandibular glands caused by persistently increased glycation of its protein elements may be one of the causes of reduced protein secretion by these salivary glands.

Conclusion

1. ALA restores/prevents weight gain, but reduces the hyperglycemia, hyperinsulinemia and HOMA-IR index only when a HFD diet is consumed.
2. ALA supplementation accompanying the HFD diet cannot restore the impaired total antioxidant capacity (TAC) of the salivary glands to the level observed in the salivary glands of control rats fed a standard diet.
3. ALA supplementation accompanying the high-fat diet prevents/restores oxidative damage in the parotid glands to a greater extent than in the submandibular glands.
4. ALA does not improve the impaired secretory function of salivary glands of rats fed the HFD.

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Author Contributions

A.Z., conceptualization, formal analysis, funding acquisition, investigation, methodology, material collection, supervision, validation, writing original draft, final approval of the version to be published. S.Z., data curation, formal analysis, material collection, final approval of the version to be published. P.K.S., formal analysis, material collection, investigation, final approval of the version to be published. J.M., formal analysis, material collection, data curation, final approval of the version to be published. M.M., conceptualization, formal analysis, funding acquisition, investigation, methodology, material collection, supervision, visualization, final approval of the version to be published.

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Statement of Ethics

Animal experiments conform to internationally accepted standards. The experiment was approved by the Local Committee for Experiments on Animals in Olsztyn, Poland (21/2017).

Disclosure Statement

The authors declare that no conflicts of interest exist.

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