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NITRITE MITIGATES LIPID PEROXIDATION IN RAT GASTRIC MUCOSA

AZOTYN OBNIŻA PEROKSYDACJĘ LIPIDÓW W ŚLUZÓWCE ŻOŁĄDKA SZCZURA

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Sodium nitrite, a nitric oxide (NO) donor in the acid pH, has been found to mitigate lipid peroxidation in rat gastric mucosa, and it elevated both Cu, Zn- and Mn-forms of the superoxide dismutase in this tissue. Putrescine, a simple polyamine of anti-oxidant properties has been shown to normalize lipid peroxidation levels in nitrite-treated animals.

INTRODUCTION

Nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$) have been found to have opposite effect(s) towards lipid peroxidation and/or pro-oxidant shift(s) in animals [17]. To our knowledge, the $^{\cdot}NO$ radical was recognized as a chain-breaking anti-oxidant molecule, which protects cells against the detrimental effects of reactive oxygen species (ROS) [9]. In contrast, the peroxynitrite anion ($ONOO^-$) was shown to have pro-oxidant properties, affecting both lipid and/or nucleic acid components towards pro-oxidant directions [38]. Since lipids peroxides mainly decompose to malondialdehyde (MDA), enhancing DNA adducts and elucidating both mutagenic and carcinogenic properties, more recent studies have been focused to examine a potent role of inorganic nitrite and its red-ox counterparts, including $^{\cdot}NO$ and $ONOO^-$ in gastrointestinal pathologies [18].

Inorganic nitrite (NO_2^-) is known as a food-born contaminant and water pollutant, which unfortunately causes a number of adverse effects in the gastrointestinal tract of humans and animals [15, 43]. Although nitrite-induced toxicity was mainly associated to methemoglobinaemia and nitrosation of amines, leading to gastrointestinal cancer [5], the non-enzymatic formation of $^{\cdot}NO$ from nitrite in the acid gastric pH [18, 30] as well as $^{\cdot}NO$ production from the amino acid L-arginine due to nitric oxide synthase (NOS) [31], and more recently peroxynitrite ($ONOO^-$) formation due to reaction between the $^{\cdot}NO$ radical and superoxide anion ($^{\cdot}NO + O_2^{\cdot -} \rightarrow ONOO^-$) [37] made the situation regarding gastrointestinal nitrite toxicity much more complex that it was initially assigned [1, 16, 28].

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In the present studies, we tried to gain further insights into the toxic effect(s) of sodium nitrite in the pyloric stomach of rats, elucidating both lipid peroxidation and anti-oxidant enzyme activities, especially superoxide dismutase (SOD), an enzyme which catalyzes the dismutation of superoxide radicals to yield hydrogen peroxide ($O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$) in this tissue. Since putrescine, a first-step polyamine from the L-arginine/L-ornithine pathway was previously shown to have anti-oxidant properties in nitrite-dosed animals [13], a more through approach have been done to study putrescine's effects in the gastric mucosa of animals.

MATERIAL AND METHODS

Male Wistar rats (220 ± 20g) were used in the studies. Before the experiment, the animals were acclimatized for one week under standard conditions (room temperature 22°C, humidity 40-70%, and 12/12 hr light/darkens), and they were given a standard chow (Murigran pellets, Motycz, Poland) and water *ad libitum*. The animals were selected into 2 groups of 14 rats in each, and they were treated *per os* with either an aqueous solution of sodium nitrite (10 mg/kg b.w./day) or normal saline (control) for 14 days. On day 7th of the experiment period, the half of randomly selected rats in each groups were also treated *per os* with putrescine (10 mg/kg b.w./day) dissolved in a normal saline, and the polyamine was given at 3-4 hours post-nitrite/saline *per os* for 7 days only (days 7-14). The animals were sacrificed by cervical dislocation at 24 hours after the last nitrite or saline dosages, and thiobarbituric acid-reactive substances (TBARS) as a biomarker of lipid peroxidation, and superoxide dismutase (SOD) isoforms as well as glutathione peroxidase (GPX) and protein-bound (P-SH) and nonprotein-bound (NP-SH) sulfhydryl groups were analyzed in the gastric mucosa of animals.

Lipid peroxide levels were measured by thiobarbituric acid test, as TBARS, according to the method described by *Ohkawa* et al. [34]. Briefly, samples of 10% tissue homogenates (prepared in 1.15% KCl) was added to 8.1% SDS. Thereafter, a solution of 20% glacial acetic acid and 0.8% 2-thiobarbituric acid (v/v) was added to the reaction mixture. To start the reaction, the mixtures were heated for one hour at 95°C, and then were cooled in tap water. The mixtures were then extracted with a spectral pure *n*-butanol and centrifuged (4000 x g) for 10 minutes at 4°C. All butanol extracts were measured spectrophotometrically at 532 nm, using a standard 1,1,3,3-tetraethoxypropane instead of homogenate.

Superoxide dismutase (superoxide: superoxide oxidoreductase, EC, 1.15.1.1, SOD) was assayed as described in details by *Grudziński* and *Frankiewicz-Jóźko* [13]. In the present study, Cu,ZnSOD and MnSOD izozymes were determined using a diagnostic RANSOD kit with xantine/xantine oxidase to generate superoxide anion ($O_2^{\cdot-}$), which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formazon dye (Randox Laboratories Ltd., Antrim, UK). The superoxide dismutase activity was measured spectrophotometrically by the degree of inhibition of this reaction at 505 nm. Briefly, gastric mucosa tissues were homogenized in 50 mmol/dm^3 potassium phosphate buffer, pH 8.6, and the supernatant obtained by centrifugation at 20,000 x g for 30 minutes was used for Cu/ZnSOD assays. In the present studies, MnSOD activity was measured using supernatants from mitochondrial pellets, which were preliminary resuspended in 50 mmol/dm^3 potassium phosphate buffer, pH 8.6, and lysed by digitonin (2.5 mg/ml) before centrifugation at 12,000 x g for 15 minutes [7]. A reaction mixture contained 0.05 mmol/dm^3 xantine, 0.025 mmol/dm^3 INT, 40 mmol/dm^3 CAPS buffer, pH 10.3, 0.94 mmol/dm^3 EDTA, 80 U/dm^3 xantine oxidase and supernatants containing 0.1-0.2 mg protein.

Glutation peroxidase (glutathione: hydrogen peroxide oxidoreductase, E.C. 1.11.1.9, GPX) was assayed by the method of *Paglia* and *Valentine* [36], which measured the rate of reduced glutathione (GSH) oxidation to the oxidized form (GSSG) by hydrogen peroxide (H_2O_2) as catalyzed by GPX ($2GSH + H_2O_2 \rightarrow GSSG + H_2O$). The rate of GSSG formation in the reaction was then measured by

following a decrease in absorbance of the reaction mixture at 340 nm as NADP was converted to NADPH. Briefly, GPX activity was measured using the supernatant obtained by centrifugation of the tissue homogenate(s) (prepared in 50 mmol/dm^3 potassium phosphate buffer, pH 8.6) for 30 minutes at 20,000 g. A reaction mixture contained 50 mmol/dm^3 potassium phosphate buffer, pH 7.0, 5 mmol/dm^3 reduced glutathione (GSH), 1 unit (U) glutathione reductase (GR), 280 mmol/dm^3 NADPH, 70 mmol/dm^3 H_2O_2, 3.75 mmol/dm^3 sodium azide (catalase inhibitor) and supernatant containing 0.1-0.2 mg protein.

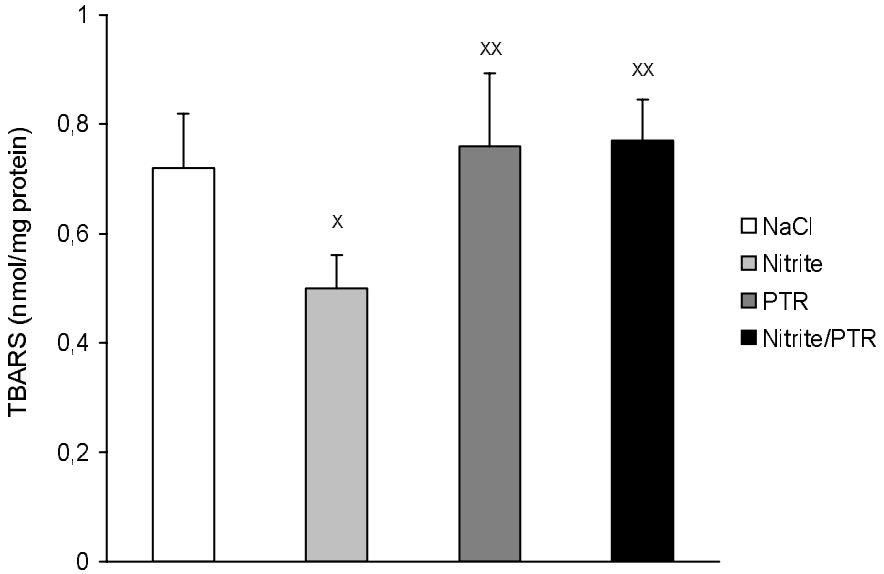
Protein-bound (P-SH) and nonprotein-bound (NP-SH) sulfhydryl group concentrations were examined with the *Ellman's* reagent, as described by *Sedlak* and *Lindsay* [39]. For a total level of thiol groups, tissue homogenates were prepared in 0.02 mol/dm^3 EDTA and they were mixed with 0.2 mol/dm^3 Tris buffer, pH 8.2, containing 0.01 mol/dm^3 5,5'-dithiobis-(2-nitro-benzoic acid) (DTNB) and absolute ethanol. The mixtures were centrifuged at 3000 x g for 15 min (room temperature), and supernatants were assayed at 412 nm. For the NP-SH groups, aliquots of the tissue homogenates (as before) were mixed with 50 % trichloracetic acid (TCA) and centrifuged at 3000 x g for 15 minutes at room temperature. Supernatants were mixed with 0.4 mol/dm^3 Tris buffer, pH 8.9 and 0.01 mol/dm^3 DTNB and measured at 412 nm. The total level of P-SH groups was then calculated by substracting the NP-SH from total SH groups. In the assay, glutathione (GSH), a reduced form, was used as a standard.

Protein content was measured by the method of *Lowry* et al. [26] with bovine serum albumin as a standard.

The results were subjected to statistical analysis by *Student's* t-test for unpaired samples. Differences were considered significant when probability (p) values were less than 0.05.

RESULTS AND DISCUSSION

Studies showed that sodium nitrite ($NaNO_2$), a nitric oxide (NO) donor in the acid pH, mitigated lipid peroxidation in the gastric mucosa of rats (Fig. 1). These results were found in accordance with those reported by *Nicolescu* and associates who also noted that nitrate and nitrite inhibited lipid peroxidation in murine synaptosomes [32]. Since opposite direction towards nitrite-mediated lipid peroxidation have been recognized in the gastric and small intestinal mucosa of animals [13], a direct anti-oxidant effect(s) of nitrite in the gastric tissue was mainly postulated due to nitric oxide (NO) formation in the acid pH of the stomach environment [30]. In accordance, *Kim* and *Kim* [21] showed that nitric oxide (NO) donors, such as L-arginine and sodium nitroprusside, mitigated hydrogen peroxide (H_2O_2)-induced lipid peroxidation in isolated rabbit gastric glands. More recently, *Kwiecień* et al. [24] have also found that nitric oxide donors such as SIN-1 (molsidomine metabolite), S-nitroso-N-acetyl-penicyllamine (SNAP), and nitroglycerin (NG) reduced water immersion restraint stress (WRS)-induced rat gastric injuries, and the agent(s) mitigated malondialdehyde (MDA) and 4-hydroxynonenal, lipid peroxide products in murine gastric tissues. Interestingly, nitric oxide (NO) releasing aspirin (ASA), a novel non-steroidal anti-inflammatory drug candidate, coupling of the ASA and NO moiety (ASA-NO), has been also found to mitigate mucosal lesions and MDA levels in the gastric mucosa of WRS-stressed rats [23]. Since the ASA-NO agent raised the luminal content of NO/nitrite in rats, a reduced number of gastric lesions was associated in NO-linked gastric hyperemia and the attenuation of lipid peroxidation processes [23]. It should be noted that WRS-induced gastric stress also increased gastric mucosal concentration of nitrite/nitrate, a breakdown product(s) of endogenous NO, and it was occurred with a drastic increase in inducible nitric oxide synthase

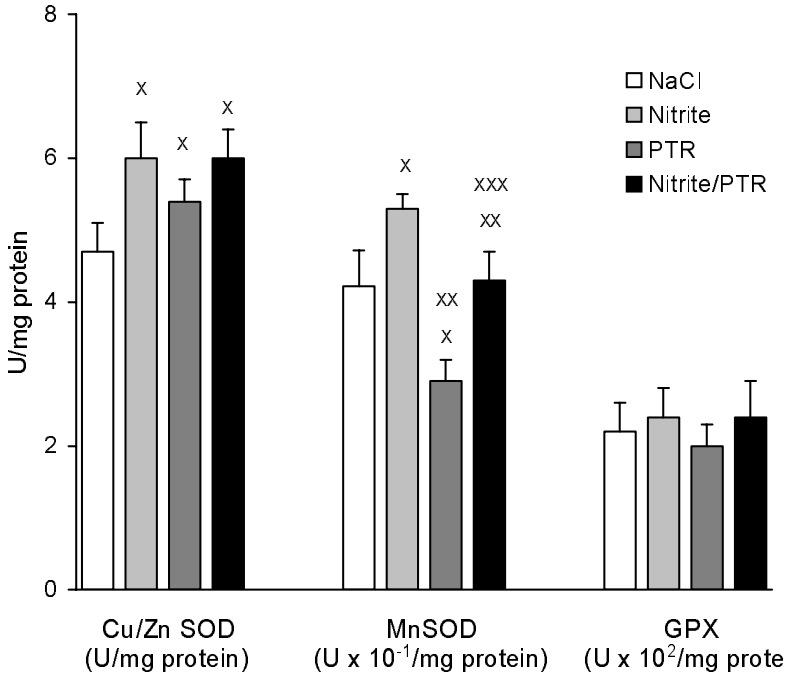


Wistar rats were treated *per os* with normal saline, 0.9 % NaCl (control) or sodium nitrite, $NaNO_2$ (10 mg/kg b.w.) daily for 14 days, and they were dosed with putrescine, PTR (10 mg/kg b.w.) *per os* for 7 days only (days 7-14) (see material and methods for details). Results are mean ± SE, n=7, x vs. saline $P<0.5$, xx vs. nitrite $P<0.05$.

Fig. 1. Lipid peroxidation in the gastric mucosa of rats treated with sodium nitrite and putrescine.

(iNOS) activity in the gastric mucosa of animals [33]. Nitric oxide (NO) from its donors was also found to elevate lipid peroxidation in murine gastric tissue since the local intra-arterial infusion of SNAP and nitroprusside caused extensive gastric mucosal damages and provoked lipid peroxidation in the pyloric stomach of rats [25].

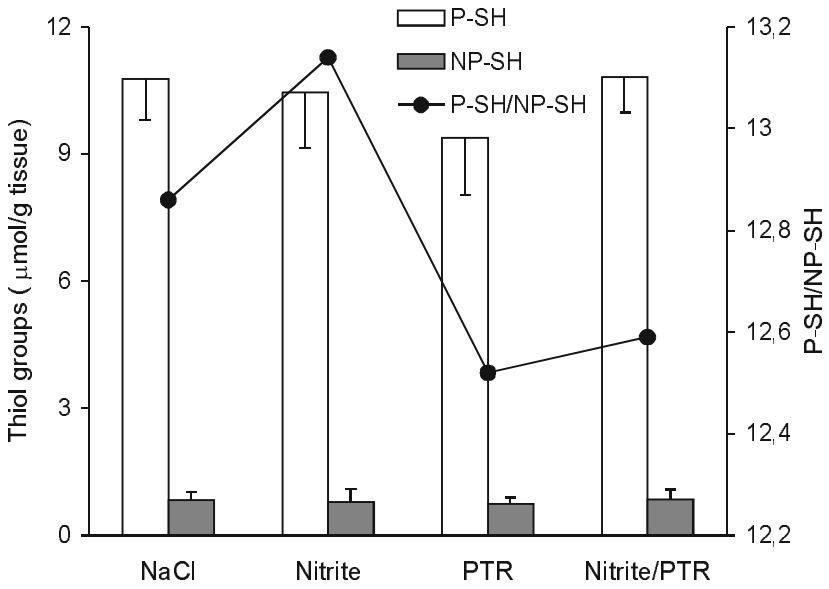
Present results clearly show that supplementation of animals with putrescine, a first-step polyamine from L-arginine/L-ornithine pathway, normalized lipid peroxidation processes in nitrite-dosed rats (Fig. 1). Previous studies from our laboratories also evidenced that putrescine decreased the elevated level of lipid peroxidation in the small intestinal mucosa of nitrite-treated rats, elucidating anti-oxidative effectiveness in the gastrointestinal tract of animals [13]. It is well known that polyamines, among other functions in cells, are recognized to act as free radical scavengers and cellular anti-oxidants [27]. For example, spermidine and spermine was found to mitigate lipid peroxidation in rat brain exposed to sodium nitroprusside, quinolinic acid and iron (Fe^{+2}) [4]. Anti-oxidant activities of polyamines, including putrescine, spermidine and spermine were also reported in human red blood cells exposed *in vitro* to hydrogen peroxide [12]. More recently, oral spermine administration to mouse inhibited NO-mediated intestinal damages and levels of systemic inflammatory mediators in mouse endotoxin model [40]. Spermine-NONOate, a donor of NO composing of the spermine agent and NO moiety, was also found to inhibit macrophage-dependent oxidation of low density lipoproteins (LDL), as induced by the copper ion (Cu^{+2}) and azo-bis-amidinopropane, and the spermine-NONOate diminished photo-oxidized LDL [41]. In other



Results are mean ± SE, n=7, x vs. saline P<0.5, xx vs. nitrite P<0.05, xxx vs. putrescine P<0.05 (see figure 1 for abbreviations and comments).

Fig. 2. Activity of cytosolic (Cu/ZnSOD) and mitochondrial (MnSOD) superoxide dismutase in the gastric mucosa of rats treated with sodium nitrite and putrescine.

studies, spermine without NO-linkage and putrescine prevented lipid peroxidation induced by essential fatty acids in human breast cancer cells [8], and putrescine dosed alone, also attenuated malondialdehyde (MDA) contents in the liver tissue of *tert*-butyl hydroperoxide (TBHP)-treated rats [32]. It should be noted that polyamines also decreased carbon tetrachloride- and paraquat-induced lipid peroxidation in rat liver cells [20], and it also diminished xantine oxidase-mediated lipid peroxidation in polymorphonuclear leucocytes [19]. As evidenced by *Mizui* et al. [29] polyamines mitigated iron-induced lipid peroxidation processes and gastric lesions in rats, and they also decreased malondialdehyde formation and lipid peroxidation in rat liver microsomes [22]. These results and other cited papers indicate that polyamines, including putrescine are anable to play a pivotal anti-oxidant role in cells. As shown in figure 1, putrescine shifted lipid peroxidation in the gastric mucosa of rats to the physiological background, and the polyamine also elevated superoxide dismutase (SOD) activity, a cytosolic form of the enzyme (Cu,ZnSOD) in murine gastric tissue (Fig. 2). In contrast to animals pretreated with nitrite, polyamine also decreased superoxide dismutase (MnSOD), a mitochondrial enzyme in rat stomach mucosa (Fig. 2). In the studies, sodium nitrite ($NaNO_2$) elevated both Cu,ZnSOD and MnSOD activities in the gastric mucosa of animals, and it also increased MnSOD enzymes in putrescine-treated rats (Fig. 2). No effects of nitrite or even putrescine (if dosed alone) were found to affect the



Results are mean ± SE (see figure 1 for abbreviations and comments)

Fig. 3. Protein (P-SH) and non-protein (NP-SH) thiol group levels in the gastric mucosa of rats treated with sodium nitrite and putrescine.

activity of glutathione peroxide (GPX) and protein- and nonprotein-bound thiol (SH) group levels in animals (Figs. 2, 3). It is of interested to note that polyamines, including putrescine inhibited nitric oxide synthase (NOS) in lipopolysaccharide (LPS)-activated macrophages, and they down-regulated endogenous nitrite levels, a break-down product of NO from the amino acid, L-arginine [42]. In contrast, modulations by alpha-difluoromethylornithine of the polyamines biosynthesis, through the inhibition of the rate-limiting enzyme, ornithine decarboxylase (ODC) were found to enhance LPS-stimulated nitrite production in J774 cells [3]. Polyamines such as spermine and putrescine, resulting from increased activity of ODC have been also shown to have gastro-protective and mucosal growth-promoting effects in animals [2,6]. Oral spermidine administration was noted to inhibit NO/nitrite-mediated intestinal damages and systemic inflammatory mediators [40]. Based on data from *Banan* et al. [2] and *Otani* et al. [35] it was found that increased gastric levels of polyamines and ODC activity also accompanied with NaCl-induced damages in the gastric mucosa of rats. In accordance, pretreatment of rats with sodium nitrite increased ODC activity in murine gastric tissues [14]. More recently, gastric mucosa tissues from patients with *Helicobacter pylori* infection have been found to increase malondialdehyde (MDA) and SOD activity [11].

Reactive nitrogen species (RONS), including NO and its red-ox forms, such as peroxynitrite ($ONOO^-$), have been postulated to play a key role in both peptic ulcers and gastritis, and lipid peroxidation have been recently shown to promote murine gastrointestinal lesions [10]. Since the normalized level of lipid peroxidiation, resulting from a discrete balance

between pro-oxidation and anti-oxidation states, accompanied with putrescine pretreatment in animals, a modulatory effect(s) of this polyamine was found in sodium nitrite-mediated gastrointestinal toxicities.

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NITRITE MITIGATES LIPID PEROXIDATION IN RAT GASTRIC MUCOSA

Summary

Sodium nitrite ($NaNO_2$), a nitric oxide (NO) donor in the acid pH has been found to decrease lipid peroxidation (TBARS) in the gastric mucosa of rats pretreated in a daily oral dosage of 10 mg $NaNO_2$/kg b.w. for 14 days. It was shown that nitrite also elevated Cu,Zn and Mn isoforms of the superoxide dismutase (SOD) in the gastric tissue, but no effect of nitrite was observed for glutathione peroxidatse (GPX) activity and protein- and nonprotein-bound thiol groups (SH) levels. Pretreatment of nitrite-dosed rats *per os* with putrescine (10 mg/kg b.w./day) for seven days, starting from a day 7th of the experiment, normalized lipid peroxidation in animals. It was found that polyamine elevated Cu,ZnSOD activity in the gastric tissue, but it also decreased MnSOD, respectively. No effect(s) of putrescine was observed for GPX and SH groups in rats treated with or without nitrite.

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AZOTYN OBNIŻA PEROKSYDACJĘ LIPIDÓW W ŚLUZÓWCE ŻOŁĄDKA SZCZURA

Streszczenie

Azotyn sodowy ($NaNO_2$), donor tlenku azotu (NO) w kwaśnym pH obniżał poziom peroksydacji lipidów (TBARS) w śluzówce żołądka szczurów otrzymujących azotyn w dziennej dożołądkowej dawce 10 mg $NaNO_2$/kg m.c. przez okres 14 dni. Wykazano, że azotyn zwiększał aktywność izoenzymów Cu,Zn and Mn dysmutazy ponadtlenkowej (SOD) w błonie śluzowej żołądka, czego nie odnotowano w ocenie aktywności glutationowej peroksydazy (GPX) oraz poziomu grup tiolowych (SH) związanych i niezwiązanych z białkami w tej tkance. Podawanie putrescyny (10 mg/kg m.c./dzień) przez okres 7 dni szczurom otrzymującym azotyn (rozpoczynając od 7-ego dnia doświadczania), normalizowało poziom peroksydacji lipidów u zwierząt. Poliamina zwiększała aktywność Cu,ZnSOD w tkance żołądka, ale również obniżała aktywność MnSOD. Nie odnotowano wpływu putrescyny na zmiany aktywności GPX oraz poziomu grup SH u szczurów otrzymujących i nie otrzymujących azotyn.

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