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Original Article

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Intestinal Absorption and Transformation of the Increased Dose of Paracetamol in Streptozotocin-Induced Diabetic Rats

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Abstract

Background: Luminal disappearance of the paracetamol and the appearance of its metabolites (paracetamol-β-D-glucuronide (PG), paracetamol sulfate (PS), paracetamol cysteine (PC) and paracetamol mercapturate (PM)) were investigated in control and in experimental diabetic rats of the perfused paracetamol substrate.

Methods: Experimental diabetes was induced by the administration of streptozotocin (STZ) (65 mg/kg, intravenous (IV)). The paracetamol solution was luminally perfused through the small intestine of anesthetized rats, and the parent compound and its metabolites were determined from the perfusion solution with an isocratic reverse phased high performance liquid chromatography (RP-HPLC) method.

Results: The excreted amount of paracetamol metabolites increased after the STZ pretreatment, and the concentration of the glutathione (GSH) in the small intestine tissue homogenate showed a decreasing tendency, although the perfused paracetamol does not accelerate the observed changing tendency. The oxidative stress caused by the STZ contributed to the formation of the oxidized glutathione (GSSG), and its level was elevated by the effect of the paracetamol administration. The paracetamol administration alone did not provoke the detectable appearance of the GSSG.

Conclusions: The elevated paracetamol concentration and the experimental diabetes negatively influenced the absorption of the paracetamol. The protective GSH level showed a decreasing tendency, while the level of the oxidative stress indicator GSSG was higher.

Keywords: Paracetamol; Conjugation reactions; Glutathione

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Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is one of the most important antioxidant low-molecular-weight tripeptide, which can be found in all mammalian tissues (0.5 - 10 mmol/L). The majority of GSH is present in the liver (10 mmol/L) [1-3], and it also plays an important role in the intestinal function [4, 5]. GSH serves many essential functions especially in the detoxification of exogenous compounds by conjugation as a nucleophile molecule to form GSH adducts [2, 6]. Some non-steroidal anti-inflammatory drugs decrease the level of GSH by phase II conjugation. Noteworthy examples include the metabolism of paracetamol and diclofenac [6-8].

The significance of the intestinal tract is attributed to its location because this is the site where drugs can be absorbed, and also the formed metabolites can be excreted into the circulation or back to the intestinal lumen. Therefore, the small intestine is an important site of drug absorption and metabolism, where both phase I and phase II enzymes and transporters are expressed [9, 10].

The tested compound, paracetamol (acetaminophen, 4-hydroxy-acetanilide, N-acetyl-para-aminophenol) is a weak acid with moderate water solubility and a low molecular mass. At a physiological pH, it is unionized, and therefore it is absorbed by passive diffusion theoretically along the entire gastrointestinal tract but mostly in the proximal part of the small intestine [11]. At higher dose, the surface of the membranes for passive diffusion and the available transporters can be saturated, and the pharmacokinetics of the drug can be altered [12].

Paracetamol, a frequently used analgesic and antipyretic, is mostly metabolized in the liver, but the small intestine and the kidneys are involved too. It is transformed into inactive paracetamol-β-D-glucuronide (PG) and paracetamol sulphate (PS) [13, 14]. A minor fraction is mostly oxidized by CYP3A4, CYP2E1, CYP1A2, CYP2A6 and cyclooxygenase into a highly reactive metabolites, including N-acetyl-p-benzoquinone imine (NAPQI) and 3-hydroxy-paracetamol [15-17]. NAPQI is formed through two intermediate products: the N-acetyl-p-benzoquinone imine radical and the 4-aminophenoxyl radical [18, 19]. Then NAPQI conjugates with GSH to form an inactive intermediate, which is subsequently transformed into paracetamol cysteine (PC) and with deacetylation into paracetamol mercapturate (PM) [20]. Also, one of the several important metabolites is 4-aminophenol, which plays an important role in the mechanism of ac-

tion [21, 22]. It can form 4-aminophenol-glucuronide and 4-aminophenol sulfate, and it is able to form a conjugate with GSH to produce a nephrotoxic product [23, 24]. Generally, paracetamol is administered in doses of 500 - 1,000 mg. However, because of its high number of its generic forms, and numerous trade names, the duplication of the single dose might be relatively common. This can lead to elevated, but not toxic drug levels, which may provide alterations in its pharmacokinetics [25].

Diabetes mellitus is an endocrine metabolic disorder that affects a significant proportion of the population of the world and shows a growing tendency [26]. In this experiment, paracetamol metabolism was investigated under pathological condition (diabetes). Metabolites that were observable and excreted by the small intestinal enterocytes were PG, PS, PC, and PM [27].

Pathological conditions such as prolonged hyperglycemia or untreated diabetes can influence the metabolism of xenobiotics [28-30], and it is able to cause an elevation of reactive oxygen species (ROSs) and free radicals, therefore leading to oxidative stress [31, 32]. Oxidative stress plays a major role in the development of both types of diabetes mellitus and the related micro- and macrovascular complications such as retinopathy, nephropathy, neuropathy and endothelial disfunctions [33-35]. These ROSs, free radicals and endogenous compounds are eliminated by the conjugation with GSH. This process can occur spontaneously, be catalyzed by the enzyme GSH transferase [36, 37], or can be reduced with GSH either. Besides the glucose metabolic disorders, other factors that can increase the amount of the formed reactive intermediates of paracetamol and elevated doses can contribute to the formation of the oxidative stress [38].

The present investigation was designed to study the effect of hyperglycemia and higher concentration of paracetamol on its metabolism and the level change of GSH.

Materials and Methods

Chemicals and reagents

Paracetamol, sodium citrate dehydrate, citric acid, Tris hydrochloride, streptozotocin (STZ), reduced GSH, DL-dithiothreitol (DTT), triethylamine, formic acid and urethan were purchased from Sigma Aldrich (Budapest, Hungary); potassium chloride (KCl), calcium chloride ($CaCl_2 \cdot 2H_2O$), sodium chloride (NaCl), glucose, sucrose, sodium hydroxide (NaOH), perchloric acid ($HClO_4$) and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) were purchased from Molar Chemicals Kft. (Halasztelek, Hungary); magnesium sulfate ($MgSO_4$); theophylline, acetonitrile and methanol were purchased from VWR International (Radnor, PA, USA); potassium dihydrogen phosphate (KH_2PO_4), oxidized glutathione (GSSG) and mannitol were purchased from Reanal Labor (Budapest, Hungary). Hepes was purchased from Alfa Aesar (Haverhill, Massachusetts, USA). Phosphoric acid was purchased from Thomasker (Budapest, Hungary). The solvents were on high performance liquid chromatography (HPLC) grade. The used standard isotonic perfusion medium had the following decomposition: 96.4 mM NaCl, 7.0 mM KCl, 3 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.9 mM phosphate buffer (pH = 7.4), 29.5 mM Tris buffer (pH = 7.4), 14.0 mM glucose, and 14 mM mannitol. Distilled water was purified using a Purelab Option Q7 Water System (ELGA LabWater, Woodridge, IL, USA) at the Department of Pharmaceutical Chemistry, University of Pecs. A Sanxin MP512 Precision pH meter and a Mettler Toledo LE410 electrode were used to adjust the pH. Blood glucose level was controlled using an AccuChek blood glucose meter (Roche, Basel, Switzerland). Homogenizer (witeg Labortechnik GmbH, Wertheim, Germany) was used to prepare the homogenates.

Animals and experimental procedure

Four treatment groups were established: a control group, an STZ-pretreated group, a paracetamol-perfused group, and a group that was both STZ-pretreated and paracetamol-perfused.

Male Wistar-Hanover rats (weighing 240 - 300 g) were used. The rats were anesthetized with urethane (1.2 g/kg, intraperitoneal (IP) [39]. The abdomen was opened by a midline incision, and approximately 10 cm of the jejunal loop was isolated and cannulated. The lumen of the jejunal loop was flushed with warmed isotonic solution (30 - 40 mL) to remove digesta and food residues, and then blown empty with 4 - 5 mL air. Perfusion through the lumen of the jejunal loop with 500 µM solution of paracetamol was performed at the rate of 12 mL/min in a recirculation mode for 90 min (Fig. 1). The initial perfusion volume was 16.5 mL. During the 90-min experiment, 250 µL samples were collected 14 times (0, 0.5, 1, 3, 5, 10, 15, 22, 29, 36, 45, 60, 75, 90 min). The temperature of perfusion was maintained at 37 °C.

Experimental diabetes was induced by intravenous administration of STZ at a dose of 65 mg/kg (dissolved in 0.1 M citrate buffer, pH 4.0) 1 week before the experiment [40]. Control and STZ-treated rats were treated with a vehicle. Blood glucose levels were measured before the STZ treatment and 1 week prior to the start of the experiments. Animals were considered diabetic from a value of 13.9 mmol/L. Blood sugar levels were measured immediately before starting experiments. The experimental rats were provided with standard rat chow (ToxiCoop, Budapest, Hungary). Food was withdrawn 12 h before glucose levels were measured. Small intestine perfusate and bile samples were obtained from the same rats in four different groups. After the experiments, the organs (liver, small intestine, kidneys) were removed, and the animals were over-anesthetized by urethane. The samples were stored in refrigerator (-70 °C) until the analysis was performed. Then they were measured in 2 weeks. For the measurement of oxidized and reduced GSH, the same experiment was performed with Krebs-Tris buffer perfusion without paracetamol in four different groups (control and STZ treated). Each group had five rats.

Determination of paracetamol and its metabolites from the intestinal perfusate

The protocol to determine paracetamol and its major metabolites in the intestinal perfusate was developed and validated in our previous study [27]. Before the reverse phased high performance

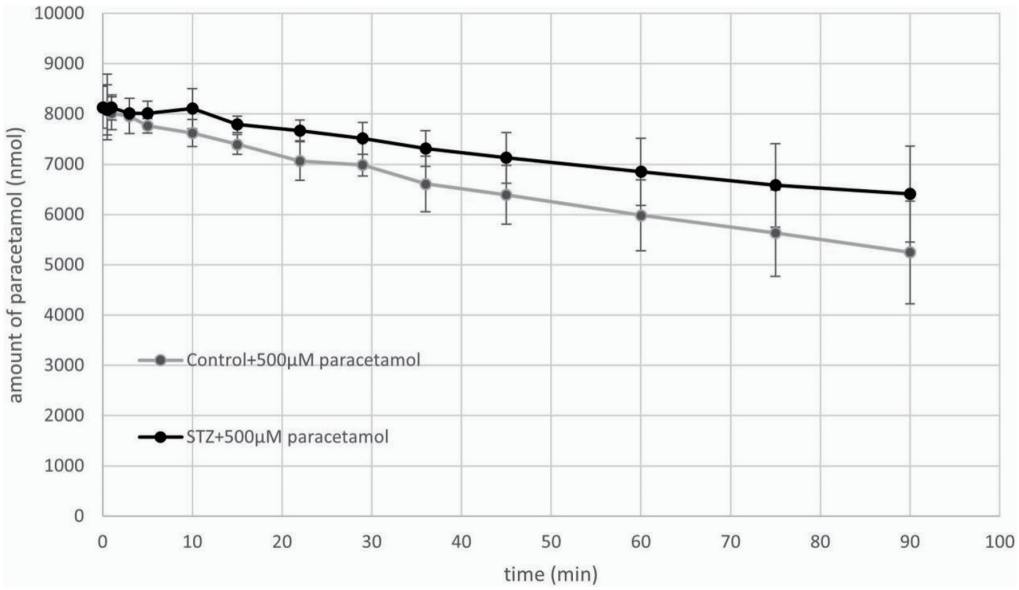

Figure 1. Time course of the disappearance of paracetamol in rat's small intestinal perfusate during perfusion of the jejunal loop with isotonic medium containing 500 µM paracetamol. Each value represents the mean of five independent experiments of five rats. No statistical significance was found within the tested parameters. STZ: streptozotocin.

liquid chromatography (RP-HPLC), UV/Vis analysis was performed. The collected perfusate samples were allowed to reach the ambient temperature and then vortex-mixed for 5 s. Then 75 µL of each sample was mixed with 25 µL 0.00159 M theophylline (dissolved in Krebs-Tris buffer). The samples were vortex-mixed for 5 s, and then 10 µL of 3.88 M $HClO_4$ solution was added, vortexed for 5 s, then centrifuged at 10,000 × g for 10 min to sediment the precipitated proteins. After centrifugation, 50 µL was transferred and mixed with 5 µL of 3.96 M NaOH. After vortexing and centrifugation processes, 20 µL sample was injected.

Analysis of the perfusates was performed using Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), which was equipped with a quaternary HPLC pump (G1311A), degasser (G1322A), an autosampler (G1313A), a thermostated column compartment (G1316A), and a UV-Vis detector (G1314A). Data were recorded and evaluated using Agilent ChemStation software (Rev.B.03.02-SR2). Reversed-phase PerfectSIL 120 ODS C18 (4.6 × 100 mm, 5 µm particle size) (MZ-Analysentechnik GmbH, Mainz, Germany) were used for analytical separation.

The mobile phase consisted of water, acetonitrile, triethylamine in a ratio of 92.95:7.00:0.05 v/v% with the pH of 2.25 (adjusted with formic acid). The flow rate of the eluent was 1 mL/min. The measurements were done at ambient temperature, and the wavelength of the detection was 245 nm.

Determination of the quantities of the reduced GSH and GSSG

Organ samples were placed into homogenization buffer (250 mM sucrose, 1 mM DL-dithiothreitol, 10 mM Hepes-Tris buffer, pH = 8). The ratio of buffer to the tissue was 10:1 (mL/g) (v/m). Organs were disrupted with homogenizer. Into 1 mL homogenate, 100 µL 0.388 M perchloric acid was added, and the mixture was vortex-mixed for 5 s, and then centrifuged at 14,000 × g for 5 min to sediment the precipitated proteins. After centrifugation 800 µL was transferred, and it was mixed with 80 µL 0.396 M sodium-hydroxide. After vortexing and centrifugation process, a 10-µL sample was injected.

Analysis of the homogenates was performed using Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), which was equipped with a quaternary HPLC pump (G1311A), degasser (G1322A), an autosampler (G1313A), a thermostated column compartment (G1316A), and a UV-Vis detector (G1314A). Data were recorded and evaluated using Agilent ChemStation software (Rev.B.03.02-SR2). Reversed-phase Kinetex Hypersil C18 (4.6 × 150 mm, 5 µm particle size) was used for analytical separation. The mobile phase consisted of 0.1% phosphoric acid with pH of 2, adjusted with NaOH and methanol. The ratio of the buffer and the methanol was 9:1. The flow rate of the eluent was 1 mL/min. The volume of the samples was 10 µL, and the measurements were done at 25 °C. Detection was performed at a wavelength of 215 nm.

Calculations and statistical analysis

Data were presented as the means ± standard error (SE) of five independent experiments. Data were analyzed by one-way analysis of variance (ANOVA). The difference among groups was determined by the Student's *t*-test.

Ethical approval

The study was designed and conducted according to the European Legislation (Directive 2010/63/E.U.) and Hungarian

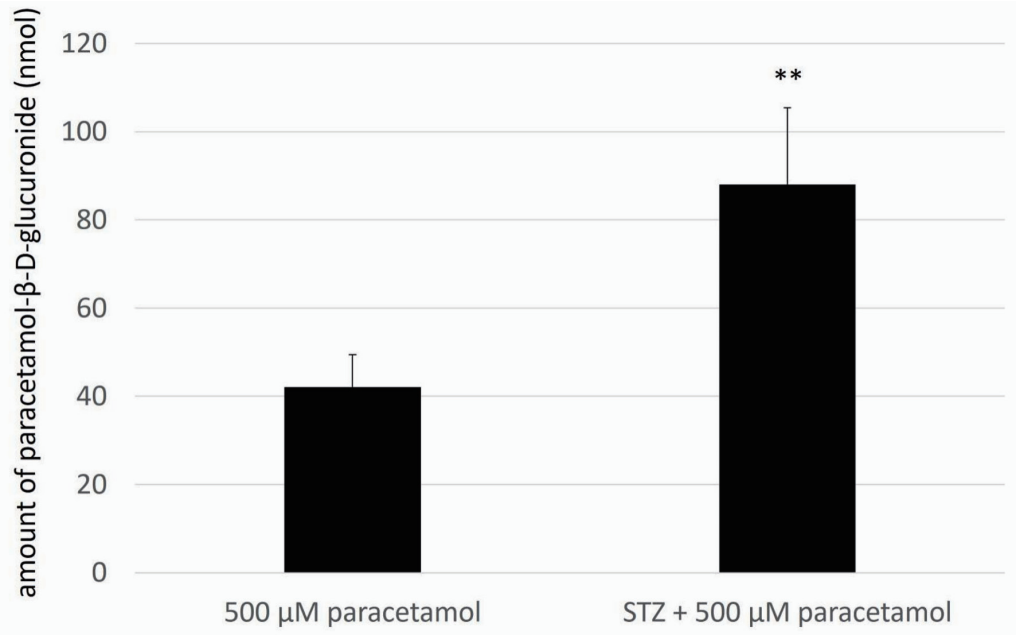

Figure 2. Alteration in the total excreted amount of paracetamol β-D-glucuronide (PG) into the lumen of the small intestine of rat during perfusion of the jejunal loop with isotonic medium containing 500 µM paracetamol. Each value represents the mean of five independent experiments ± standard error. **P < 0.01. STZ: streptozotocin.

Government Regulation (40/2013., II.14.) on the protection of animals that are used for scientific purposes. The project was approved by the Animal Welfare Committee of the University of Pecs and by the Government Office of Baranya County (license No. BAI35/51-61/2016 (license supplement No. BAI35/90-5/2019) and BA02/2000-53/2023).

Results

The onset of hyperglycemia was monitored before the experiments, and their values were 6.56 ± 1.05 mmol/L in the control, and 6.84 ± 1.67 mmol/L at the paracetamol-perfused group. After STZ pretreatment, in the buffer perfused group, the average of the blood sugar levels was 20.16 ± 2.83 mmol/L, while in the paracetamol-perfused group, it was 21.56 ± 2.97 mmol/L.

Figure 1 shows the slow, mainly passive absorption of the paracetamol during the 90-min experiment. The tendency of the rate of the absorption is reduced at the group of the animals treated with STZ. During the 90-min period, the scale of difference is increasing with final values of 5,245.94 ± 1,022.29 nmol of paracetamol at the non-pretreated group, while the STZ pretreatment modify the remaining paracetamol quantity in the perfusion buffer to 6,406.17 ± 954.21 nmol.

Figures 2 and 3 show that the appearance of PG (42.12 ± 7.31 nmol) and PS (6.68 ± 1.07 nmol) metabolites. These derivatives change parallelly toward the increasing direction under the influence of the STZ (PG (88.07 ± 17.33 nmol) and PS (8.45 ± 1.95 nmol)). The alteration at the PG was found to be significant.

Figures 4 and 5 show that the significant elevation in the PC (4.57 ± 0.68 nmol) and PM (0.74 ± 0.69 nmol) levels can be seen after STZ treatment (PC (8.91 ± 2.19 nmol), and PM (4.07 ± 1.27 nmol)) in the perfusion medium, and both of the alterations were found to be significant.

The detection of the GSH and the GSSG was performed by a simple reversed phase HPLC method after the appropriate sample preparation. The chromatogram of the applied HPLC method can be seen in Figure 6.

By the effect of the STZ-induced hyperglycemia and paracetamol perfusion, the control GSH concentration in the small intestinal tissue homogenate (78.84 ± 7.24 µM) is going to change toward a decreasing direction at all treatments (in case of STZ pretreatment (63.10 ± 9.20 µM), at 500 µM paracetamol perfusion (77.21 ± 9.27 µM) and with STZ pretreatment and 500 µM paracetamol perfusion (72.60 ± 14.53 µM) (Fig. 7).

The formation of the GSSG cannot be detected in the small intestinal homogenate with our method of those animals, which were not treated previously with STZ. But the STZ pretreatment contributed to the appearance of the oxidative derivative (0.1539 ± 0.0592 µM), and its level has increased after the perfusion of 500 µM paracetamol (0.6686 ± 0.1183 µM) (Fig. 8).

Discussion

The determination of the metabolites from the small intestinal perfusate was performed with an isocratic, validated RP-HPLC UV-Vis method, which was developed for the quantification of paracetamol and its major metabolites (PG, PS, PC and PM) [27]. The concentration of the paracetamol was aimed

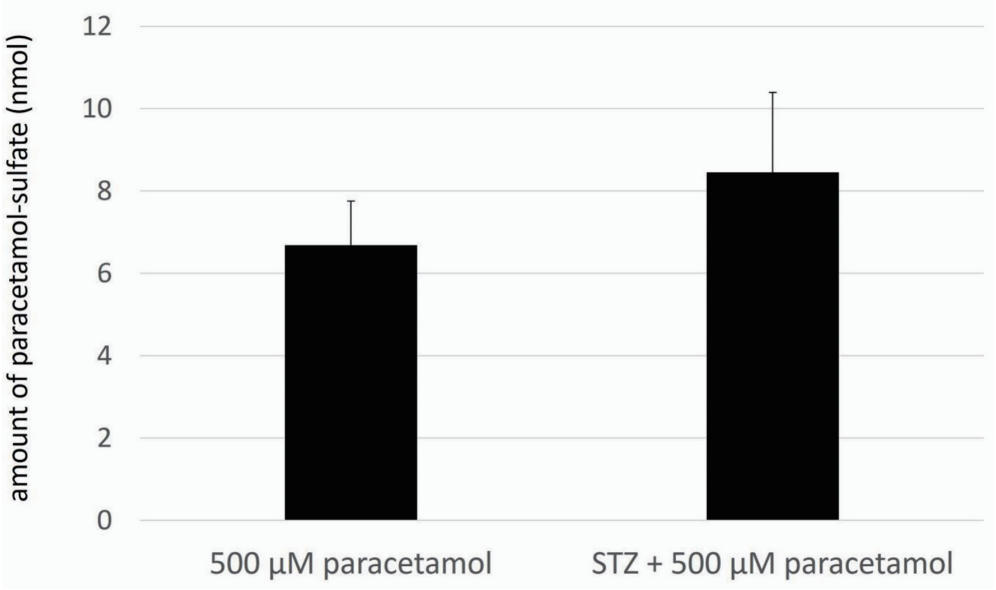

Figure 3. Alteration in the total excreted amount of paracetamol sulfate (PS) into the lumen of the small intestine of rat during perfusion of the jejunal loop with isotonic medium containing 500 µM paracetamol. Each value represents the mean of five independent experiments ± standard error. No statistical significance was found within the tested parameters. STZ: streptozotocin.

to model an elevated dose between the regular and the maximal doses on one occasion. Some of the experimental animals were treated with STZ to provoke a diabetic condition. We found that the experimental diabetes caused changes in intestinal absorption and metabolism, resulting in an increase of all of the metabolic processes [27].

In Figure 1, the predominantly slow and passive absorption of the paracetamol can be observed, and the absorption tendency is reduced in the STZ-pretreated animals. Although the STZ-induced diabetes can increase the permeability of the membrane of the gastrointestinal mucosa, at higher concentrations of the administered paracetamol molecule, the saturation of the transporters, and the slower change of the concentration gradient can be emphasized. The number of the transporters can limit the rate of the absorption, although their function can be important as well. The STZ pretreatment during the modelling of a long-term diabetes increases the membrane permeability, but inhibits the function and the expression of P-gp

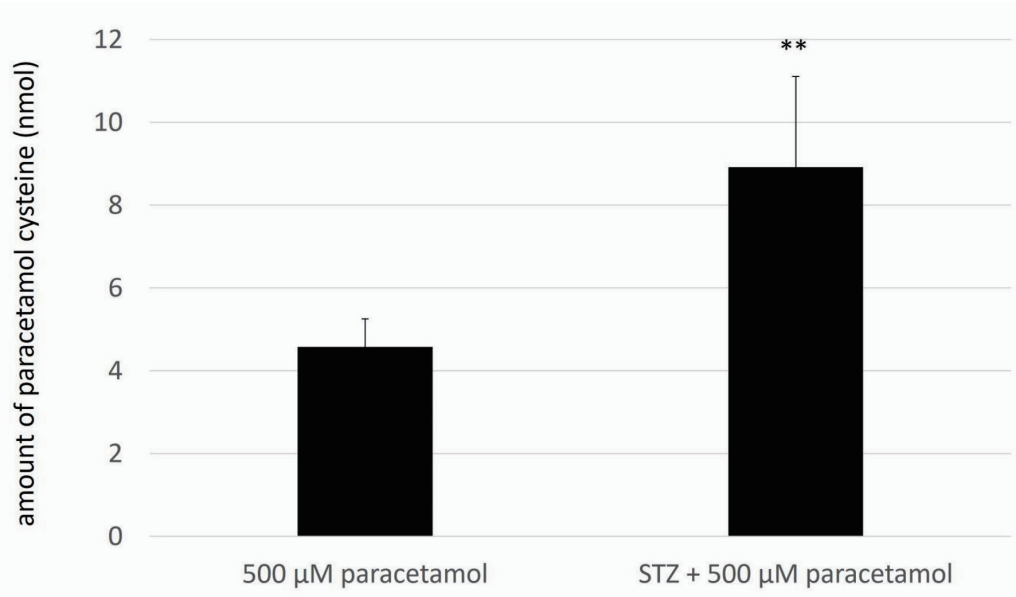

Figure 4. Alteration in the total excreted amount of paracetamol cysteine (PC) into the lumen of the small intestine of rat during perfusion of the jejunal loop with isotonic medium containing 500 µM paracetamol. Each value represents the mean of five independent experiments ± standard error. **P < 0.01. STZ: streptozotocin.

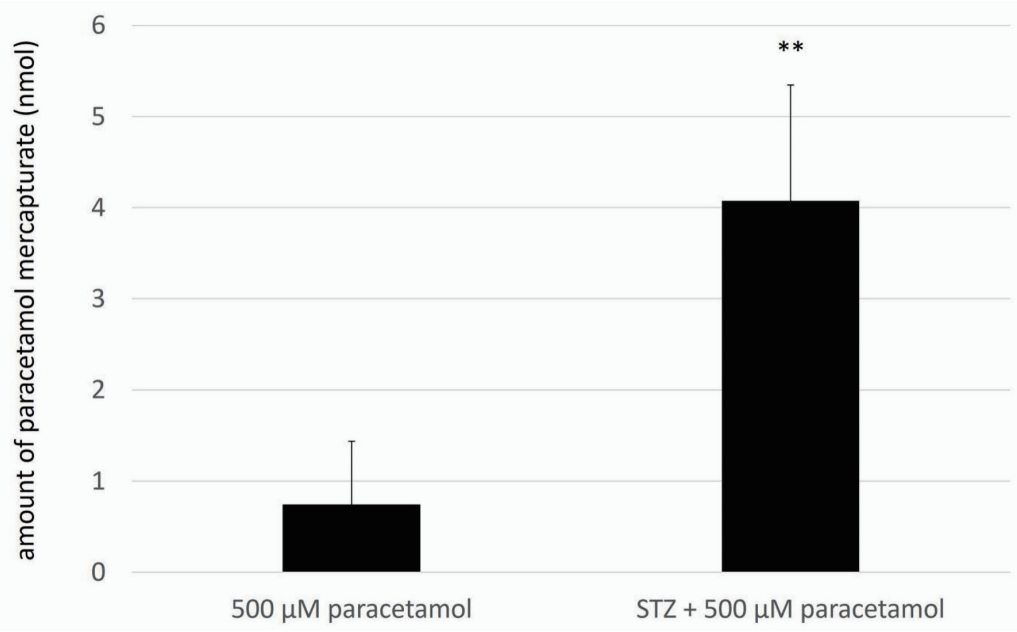

Figure 5. Alteration in the total excreted amount of paracetamol mercapturate (PM) into the lumen of the small intestine of rat during perfusion of the jejunal loop with isotonic medium containing 500 µM paracetamol. Each value represents the mean of five independent experiments ± standard error. **P < 0.01. STZ: streptozotocin.

transporter in the small intestine [41, 42]. In short-term diabetes (9 days), the decrease of the P-gp expression is reported [42-44]. Besides affecting the small intestine, the experimental diabetes reduces the activity of efflux transporters in the liver and the kidney, such as MDR1B (multidrug resistance transporter, P-gp), multidrug resistance-associated protein 2, and breast cancer resistance protein [29].

In the small intestine, the excreted quantity of the PG in-

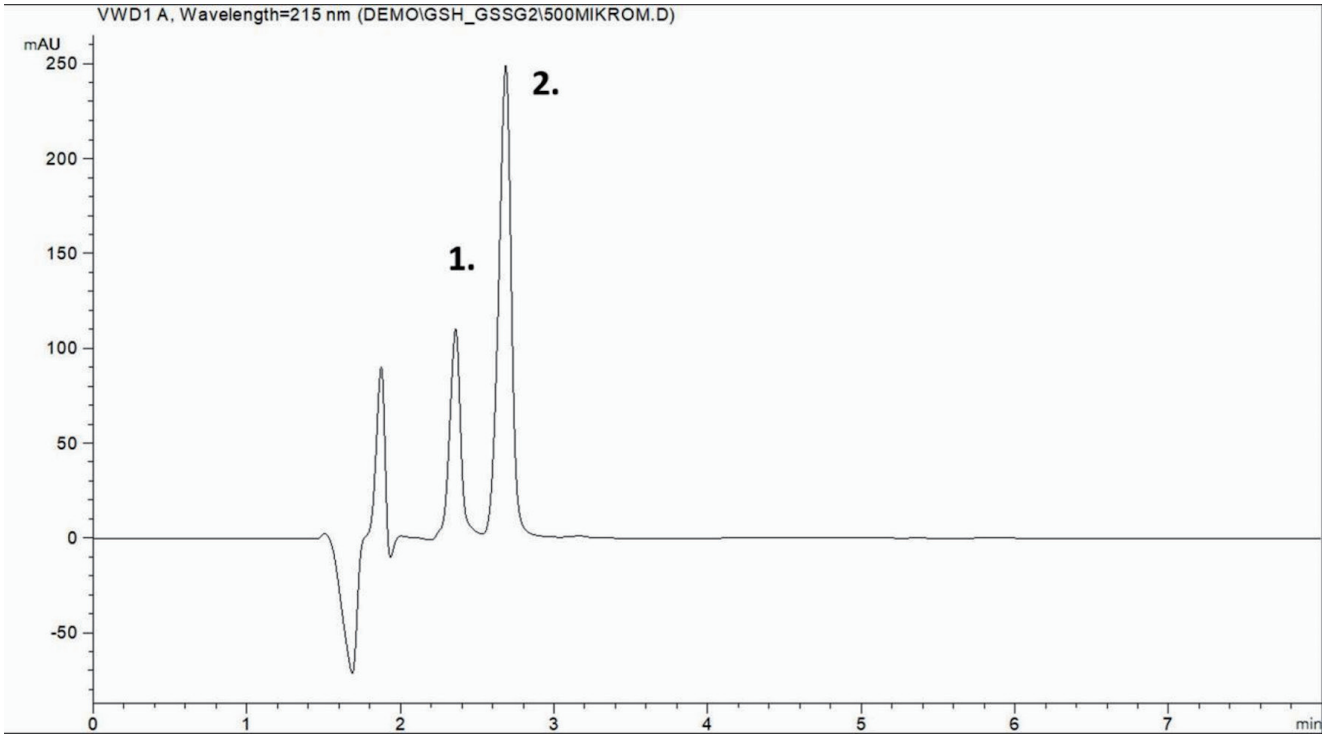

Figure 6. HPLC chromatogram of the glutathione (GSH) and oxidized glutathione (GSSG) standards prepared with the blank perfusate. Peak 1 represents the reduced GSH (tR = 2.454 min); peak 2 represents the GSSG (tR = 2.776 min).

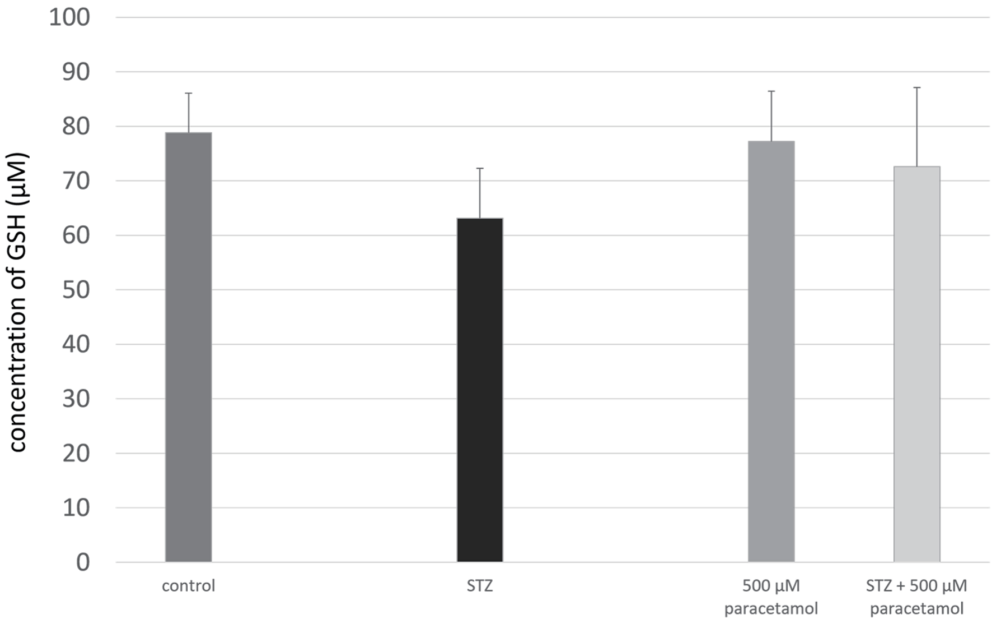

Figure 7. Concentration of reduced glutathione (GSH) (the ratio of buffer to the wet tissue was 10:1 (mL/g)) in the small intestine, liver and kidney in control animals. Each value represents the mean of five independent experiments of five rats ± standard error. STZ: streptozotocin.

creased significantly. Although the PS showed an increasing tendency, the change was not significant after STZ pretreatment. These alterations correspond with the changes that were found at the tests for activity of the UDP-glucuronyltransferase and the sulfotransferase enzymes in the control and STZ-pretreated rats [45], and these results were directly proportional with the applied concentrations [27].

The changes of the PC and PM levels can be explained with the reactive metabolites formed after metabolic transformation by spontaneous and enzyme-catalyzed pathways [36]. The oxidation itself can be facilitated by the CYP3A4 and prostaglandin H-synthase in the small intestine [46]. The numbers

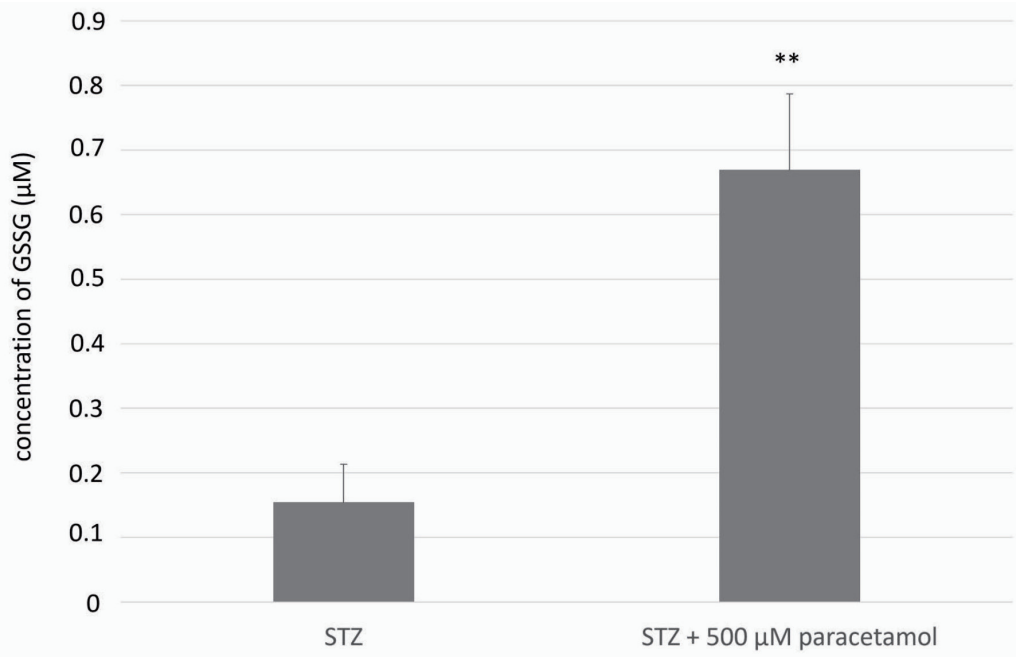

Figure 8. Concentration of oxidized glutathione (GSSG, the ratio of buffer to the tissue was 10:1 (mL/g)) in the small intestine without and with paracetamol perfusion in case of diabetic rats. Each value represents the mean of five independent experiments ± standard error. **P < 0.01. STZ: streptozotocin.

are similar to those obtained with treatments at lower substrate concentrations [27]. This phenomenon also can be explained by the limited capacity of the GSH conjugate degradation process or the interorgan shift of the cysteine and mercapturate derivatives [47]. It can be noted that during the elimination process of paracetamol metabolites, especially paracetamol GSH derivatives, these can enter the bloodstream from the kidney and liver. Moreover, the applied method is available not only for the investigation of the small intestinal absorption but also for detection of the excreted intestinal metabolites. Because the intestinal vascularization is intact, the metabolic roles of the livers and kidneys can also be important [48, 49]. Besides the covalent connection to the reactive metabolites, the formation of the reactive intermediates (phenoxy- or N-acetyl benzosemiquinone radicals) theoretically allows for the direct oxidation of the GSH to GSSG, although this transformation can also occur due to the elevated oxidative stress provoked by the experimental diabetes.

In the small intestine, the most metabolically active layer is composed of the enterocytes of the mucosa, which contains the GSH in the highest concentration [4]. When the GSH content of the small intestine is compared with that in the hepatic and renal tissues, it tends to be the lowest. However, diurnal variations in GSH levels, which can be measured in the liver tissue homogenate, cannot be detected. It partly can be explained by the hepatic resupplementation of the GSH [50, 51].

Due to the STZ-induced hyperglycemia, the GSH concentrations are reduced at all treatments (or show a decreasing tendency). As shown in Figure 7, the reduction of the GSH level can be observed. The obtained results, at least partly, were comparable to those obtained with other methods [27]. Surprisingly, at the recently perfused paracetamol concentration (500 µM), an increase can be observed. This phenomenon can be explained with the observation that the presence of the paracetamol can inhibit the activity of the glutathione-S-transferase enzyme at higher concentrations in the liver homogenate of the mice and in the gills of the eel fish [52, 53]. Moreover, the oxidative stress might induce a protective compensatory increase in the synthesis of the endogenous antioxidant GSH [54]. The formation of the GSSG (Fig. 8) is only observable in the STZ-pretreated animals and is even increased by the effect of the paracetamol.

After the STZ administration, there could be an increased oxidative stress in the metabolizing cells. These results suggest that the quantity of the reactive species formed during the metabolism can transform to O-, or N-centered derivatives or reactive fragment molecules, which can directly interact with GSH [31]. The obtained results can also be the result of the activity change of the enzymes involved in the GSH biosynthesis, e.g., GSH synthetase and γ-glutamylcysteine synthetase [55]. The influence of the GSH resupplementation can be further investigated by experiments on intact tissues [56].

Conclusions

Following the administration of higher (500 µM) dose of paracetamol, all the tested metabolites (PG, PS, PC and PM) appear in elevated quantities after STZ pretreatment. The increase in PC and PM indicates the formation of the oxidative metabolite. With the introduced HPLC method, the GSH and GSSG levels were measured. There are no significant decreases in GSH levels by the effect of the paracetamol and the STZ; but GSSG only appears after STZ pretreatment, in the small intestine. At higher doses of the paracetamol administration, the physico-chemical character of the gastrointestinal mucosa might change, and educed tendency of the absorption can be observed in experimental diabetes. The main metabolic pathways (glucuronidation and sulfation) are intact, although the GSH levels show a downhill tendency both after paracetamol perfusion and STZ pretreatment. After STZ pretreatment the elevation of the GSSG is an indicator of oxidative stress. The exact role of the liver and kidney in the formation of the PC and PM metabolites is to be clarified. In diabetes, the absorption of paracetamol and the protective GSH level can be lowered, and the level of the oxidative stress is higher.

Acknowledgments

None to declare.

Financial Disclosure

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Conflict of Interest

Authors declare no conflict of interest.

Informed Consent

The study is not a human research, no informed consent was obtained.

Author Contributions

A. Almasi: conceptualization, methodology, visualization, and writing. S. Kovacs: formal analysis. P. Meszaros: investigation, data curation, writing, and statistics. All authors have read and agreed to the published version of the manuscript.

Data Availability

Any inquiries regarding supporting data availability of this study should be directed to the corresponding author.

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