

Prooxidative and fluorescence properties of paracetamol during interactions with mitochondria

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Abstract. This study was carried out to investigate isolated liver mitochondrial functions after paracetamol administration by monitoring of liver mitochondrial fluorescence properties as well as prooxidative properties of paracetamol. Paracetamol was administered to rat (in subtoxic $500 \text{ mg} \cdot \text{kg}^{-1}$ dose) *in vivo*. The effect of this dose was compared *with* the subtoxic and toxic dose of paracetamol added to mitochondria *in vitro* (1 and 1.5) mg paracetamol/mg mitochondrial protein. Subtoxic dose of paracetamol *in vitro* did not change mitochondrial fluorescence, but it significantly decreased mitochondrial fluorescence *in vivo* in comparison with control mitochondrial group. Toxic dose of paracetamol *in vitro* significantly decreased mitochondrial fluorescence. The enzymatic activity of superoxide dismutase (SOD) significantly decreased after paracetamol administration *in vitro* and *in vivo*. While both activities of glutathione peroxidase (GPx) and glutathione reductase (GR) significantly increased in dependence upon paracetamol doses. Our experiment showed, that paracetamol participates in formation of free radicals and confirms previous studies, in which paracetamol administration caused elevation of antioxidative enzymes activities in dependence on dose, which is considered therapeutically as subtoxic and toxic.

Keywords: Paracetamol, superoxide dismutase, glutathione peroxidase, glutathione reductase, fluorescence, synchronous fluorescence spectra

1. Introduction

Paracetamol (N-acetyl-p-aminophenol, acetaminophen, Fig. 1) is a well-known analgetic–antipyretic drug widely used in medical practice. This drug exhibits mild toxic side effects in therapeutical doses. The liver mitochondria, as the formation centre of free radicals are very vulnerable site of paracetamol's overdose [17].

Symptoms of allergy and abnormalities in blood count are some of paracetamol's unfavourable effects [9]. Paracetamol in high doses increased accumulation of the hepatotoxic intermediate, N-acetyl-p-benzoquinone imine (NAPQI) and formation of peroxides [21]. In addition, hepatotoxic effect was observed in children after taking paracetamol [14]. Repetitive long-term administration of paracetamol ameliorates its hepatotoxicity, likely as a result of adaptation to paracetamol by elevating the activities of enzymes such as glutathione reductase and glucose-6-phospho-dehydrogenase [15].

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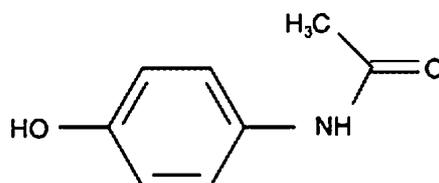


Fig. 1. Molecular structure of paracetamol.

Paracetamol administration induced significant decrease in intracellular reduced glutathione (GSH) concentrations (both cytosolic and mitochondrial) along with an increase in lipid peroxidation in conjunction with mitochondrial dysfunction, depleting of ATP content, mitochondrial swelling injury as documented by Ca^{2+} -induced opening of mitochondrial permeability transition pores and leakage of cytochrome c [13].

Although mitochondria were demonstrated as primary targets in acetaminophen hepatotoxicity, the mechanism for mitochondria-mediated toxicity has not been defined yet. There is a possible hypothesis for mechanisms of acetaminophen hepatotoxicity including two phases, a metabolic phase and an oxidative phase. The metabolic phase involves binding of paracetamol to proteins and depletion of reduced glutathione (GSH). The oxidative phase involves cell death, which is caused by increased formation of reactive oxygen species (ROS) and by loss of mitochondrial membrane potential [20].

Previous studies with liver mitochondria demonstrated that paracetamol effect *in vivo* on microsomal oxidation, respiration and phosphorylation as well as on mitochondrial structure depends on the size and age of experimental animals, paracetamol dose and many other factors [23].

The aim of our work was to study liver mitochondrial functions after paracetamol administration *in vivo* 500 $\text{mg} \cdot \text{kg}^{-1}$ and *in vitro* (1 and 1.5) $\text{mg paracetamol} \cdot \text{mg}^{-1}$ mitochondrial protein by determining activity of antioxidative enzymes SOD, GPx and GR and by monitoring liver mitochondria by fluorescence spectroscopy.

Mitochondrial synchronous fluorescence spectrum (SFS) is a method, which in a simplistic form defines the outer mitochondrial membrane. It is considered as a characteristic “fingerprint”, as it is specific for a given mitochondrial suspension after isolation and this method enables to monitor the indirect interaction of mitochondria and paracetamol.

2. Material and methods

Paracetamol, dimethyl sulfoxide (DMSO), dipotassium hydrogen phosphate (K_2HPO_4), ethylenediaminetetraacetic acid (EDTA), magnesium chloride (MgCl_2), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium succinate ($\text{NaOOCCH}_2\text{CH}_2\text{COONa} \cdot \text{H}_2\text{O}$) and Tris HCl were obtained from Sigma, Germany. The respiration medium (pH 7.4) containing EDTA (0.78 mM), MgCl_2 (6 mM), Tris HCl (15 mM), KCl (0.08), K_2HPO_4 (0.3 M) and KH_2PO_4 (0.3 M) was prepared using bidistilled water, 1 mM sodium succinate.

2.1. Mitochondria isolation and determination of proteins

Male Wistar rats (Velaz Praha, Slovakia) weighting 250–300 g, fed with a standard laboratory diet and tap water were used for experiments. Animals (6 in one group) were sacrificed by decapitation, their livers were quickly removed. Mitochondria were isolated according to [12]. The protein content of the

isolated mitochondria was determined by the method of [10]. The experiments were approved by the Ethics Committee of the Faculty of Medicine of Pavol Joseph Šafarik University in Košice.

2.2. Experimental

Mitochondria were divided into control group and experimental group. Paracetamol was added into isolated mitochondria (experimental group) in a dose of (1 and 1.5) $\text{mg} \cdot \text{mg}^{-1}$ mitochondrial proteins, that corresponds to the oral dose (500 and 750) $\text{mg} \cdot \text{kg}^{-1}$ of the drug *in vivo*, respectively [5]. The animals were decapitated 24 h after *in vivo* administration of paracetamol.

The interaction of mitochondria with different doses of paracetamol in the respiration medium in the ratio 1:1000 in experimental groups was measured by synchronous fluorescence spectra [19].

2.3. Fluorescence synchronous spectra measurement

The fluorescence synchronous spectra (SFS) of mitochondrial suspension at the constant difference $\Delta\lambda = 70$ nm between excitation and emission monochromators and their interaction with mitochondria were run on a Perkin–Elmer Model LS 55 Luminiscence spectrometer using 1 cm pathlength quartz cuvette at room temperature. The scan speed of both monochromators was $1200 \text{ nm} \cdot \text{min}^{-1}$. Setting of instrument's excitation slit was 10 nm and emission slit was 15 nm. Data processing was managed by the Winlab (Perkin–Elmer) software package. The protein concentration of the investigated mixture of fresh isolated mitochondria was $20 \mu\text{g} \cdot \text{ml}^{-1}$.

The practical advantages of fluorescence technique and spectra (SFF) application on mitochondria are high sensitivity, reproducibility, minimal quantity of mitochondria (20 μl) and rate of a measurement (5 min).

2.4. Mitochondrial superoxide dismutase

Activity of mitochondrial superoxide dismutase (E. C. 1.15.1.1) in control and experimental groups was measured by [22]. Decrease in absorption at 480 nm was measured at 310.15 K during 10 min. Activity of superoxide dismutase was expressed as $\text{U} \cdot \text{mg}^{-1}$ mitochondrial protein.

2.5. Mitochondrial glutathione peroxidase

Activity of mitochondrial matrix glutathione peroxidase (E. C. 1.11.1.9) was measured by [8] by measuring NADPH oxidation at 360 nm during 5 min. Enzyme activity is expressed as $\text{U} \cdot \text{mg}^{-1}$ mitochondrial protein.

2.6. Mitochondrial glutathione reductase

Activity of mitochondrial glutathione reductase (E. C. 1.6.4.2) in control and experimental groups was measured by [4]. Decrease in absorption at 360 nm due to the reduction of NAD^+ to NADPH was measured at 310.15 K during 5 min. Activity of glutathione reductase was expressed $\text{U} \cdot \text{mg}^{-1}$ mitochondrial protein.

2.7. Statistical analysis

The statistical significance between control and paracetamol treated groups was calculated by non-parametric Mann–Whitney *U*-test by use of STATISTIKA 6.0 software (Base StatSoft, Inc., 2001).

3. Results

3.1. Mitochondrial fluorescence

Paracetamol administration *in vivo* $500 \text{ mg} \cdot \text{kg}^{-1}$ significantly decreased mitochondrial fluorescence functions in comparison with control mitochondrial group and paracetamol administration *in vitro* $500 \text{ mg} \cdot \text{kg}^{-1}$, as we can see in Fig. 2(a). Fluorescence of mitochondria decreased after toxic dose $750 \text{ mg} \cdot \text{kg}^{-1}$ of paracetamol *in vitro* Fig. 2(b).

3.2. Paracetamol antioxidative properties

Mitochondrial antioxidative function is described by the activities of SOD, GPx and GR enzymes after administration of subtoxic and toxic doses of paracetamol. While enzymatic SOD activity after paracetamol administration *in vitro* and *in vivo* significantly decreased, the enzymatic GPx and GR activities significantly increased in dependence upon paracetamol dose 1 mg ($p < 0.01$) and 1.5 mg ($p < 0.001$), respectively in comparison to control mitochondria group (Table 1).

These results showed antioxidative properties of paracetamol and higher toxic effect of subtoxic paracetamol *in vivo* than *in vitro*.

4. Discussion

Mitochondria are high organized integrated multifluorescent system of fluorophores. The spectral characterization and interpretation of mitochondria are difficult and were practically unsolvable in the past. Recently it is possible to use the highly efficient fluorescence spectrophotometer with a computer processing of spectra. This method enables to monitor dynamic changes of mitochondria.

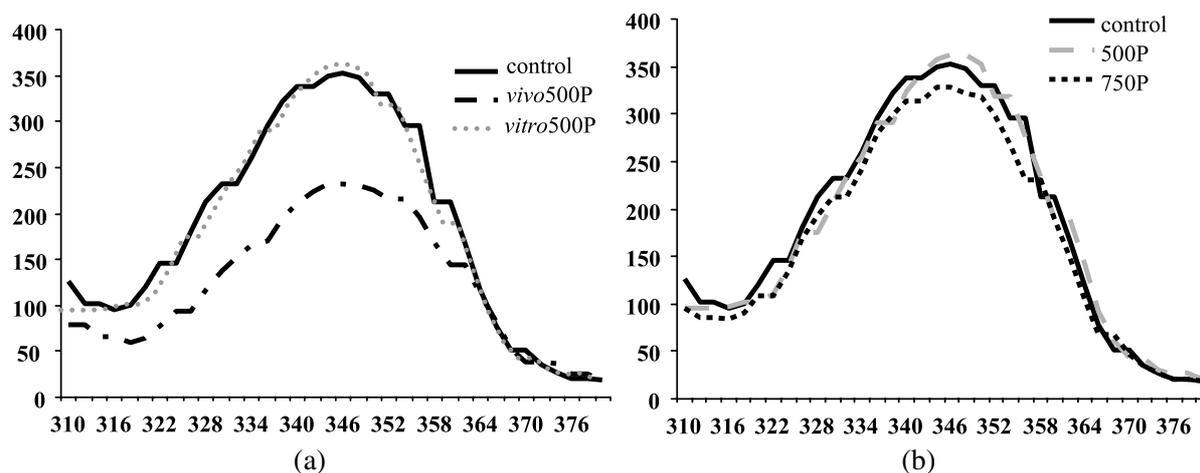


Fig. 2. Effect of paracetamol *in vitro* and *in vivo* was studied on mitochondrial model (a) and (b). Effect of subtoxic ($500 \text{ mg} \cdot \text{kg}^{-1}$ weight) and toxic ($750 \text{ mg} \cdot \text{kg}^{-1}$ weight) doses of paracetamol on outer mitochondrial membrane measured by SFS at the constant $\Delta\lambda = 70 \text{ nm}$ is shown in (b). Label “control” in the graph legend responds to the intact mitochondria, “P” to paracetamol administered into mitochondria. In the graph is a fluorescence on y axis plotted against emission wavelength on x axis.

Table 1
Effect of paracetamol on mitochondrial SOD, GPx and GR enzymatic activities

$\bar{x} \pm s_d$	SOD ($U \cdot mg^{-1}$)	GPx ($U \cdot mg^{-1}$)	GR ($U \cdot mg^{-1}$)
Control	7.919 ± 0.906	0.357 ± 0.025	4.382 ± 0.769
Paracetamol			
1.0 mg <i>in vitro</i> ^a	$4.646 \pm 1.410^{**}$	$0.565 \pm 0.058^{**}$	$6.0533 \pm 1.056^{**}$
1.5 mg <i>in vitro</i> ^a	$3.494 \pm 1.364^{***}$	$0.633 \pm 0.099^{***}$	$6.850 \pm 0.4162^{***}$
500 mg <i>in vivo</i> ^a	$2.548 \pm 0.818^{***}$	$0.451 \pm 0.05^*$	$5.677 \pm 0.6523^*$

Notes: ^aExperimental mitochondria group after subtoxic paracetamol administration ($1.0 \text{ mg} \cdot \text{mg}^{-1}$) mit. proteins and toxic paracetamol dosage ($1.5 \text{ mg} \cdot \text{mg}^{-1}$) mit. proteins, paracetamol dose $1.0 \text{ mg} \cdot \text{mg}^{-1}$ mit. proteins *in vitro* corresponds to paracetamol dosage $500 \text{ mg} \cdot \text{kg}^{-1}$ *in vivo* (Section 2). *Mean significance $p < 0.05$; ** mean significance $p < 0.01$; *** mean significance $p < 0.001$; \bar{x} – average; s_d – standard deviation.

The aim of this work was investigation of interaction between small molecules of paracetamol and mitochondrial surface by SFS as well as the measurement of SOD, GPx and GR enzymatic activities in matrix and intermembrane space of mitochondria. Our results showed, that paracetamol had different effects *in vivo* and *in vitro*. The interaction paracetamol–mitochondria was proved by correlation between the mitochondrial autofluorescence and the activities of GPx in matrix as well as SOD and GR in mitochondrial intermembrane space.

Maximum of outer mitochondrial membrane autofluorescence ($\lambda_{\text{ex}} = 346 \text{ nm}$) was determined by SFS at the constant $\Delta\lambda = 70 \text{ nm}$.

The comparison of control and experimental mitochondria showed changes in the presence of paracetamol by SFS ($\Delta\lambda = 70 \text{ nm}$). Autofluorescence of mitochondria after paracetamol administration *in vivo* $500 \text{ mg} \cdot \text{kg}^{-1}$ was significantly decreased in comparison with the control mitochondria. Paracetamol administration in order $500 < 750 \text{ mg} \cdot \text{kg}^{-1}$ *in vitro* significantly decreased fluorescence.

Paracetamol administration *in vitro* does not form a toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). Paracetamol can react with oxidized glutathione (GSSG) and can form conjugates. These statements are in accordance with observations of increased GR activity and GSSG level. After paracetamol administration in isolated hepatocytes *in vitro* concentration of GSH decreased [11]. Paracetamol significantly decreased mitochondrial respiratory control in our previous experiments *in vitro* [5] along with reduction in ATP level in isolated mouse liver mitochondria as also was observed [3].

Effect of paracetamol administration (subtoxic and toxic dose) on rat liver mitochondria was studied mostly in experiments *in vivo*. Paracetamol is a hepatotoxic substance after administration *in vivo* 750 and $800 \text{ mg} \cdot \text{kg}^{-1}$. Paracetamol added to mitochondria at concentration 1 and $1.5 \text{ mg} \cdot \text{mg}^{-1}$ mitochondrial protein in our experiments *in vitro* corresponds to subtoxic $500 \text{ mg} \cdot \text{kg}^{-1}$ and toxic $750 \text{ mg} \cdot \text{kg}^{-1}$ paracetamol administration *in vivo*, had been used in many previous experiments [6,24].

Paracetamol is metabolised primarily in the liver into non-toxic products. Three metabolic pathways are notable: glucuronidation, sulfation (sulfate conjugation), N-hydroxylation and rearrangement, then GSH conjugation [2].

Paracetamol is in organism metabolized into toxic intermediate product NABQI N-acetyl-p-benzoquinone-imid by cytochrome P450 [7]. This metabolite spontaneously reacts with GSH and forms conjugate compounds. Paracetamol overdose leads to the accumulation of NAPQI, which undergoes conjugation with glutathione and makes covalent complexes. Conjugation depletes intracellular glutathione GSH, a natural antioxidant and reduces the level of cytochrome c, decreases mitochondrial respiration, increases production of superoxide radicals, which can cause mitochondrial dysfunction. Accumulation of GSSG in mitochondria *in vitro* causes mitochondrial damage and collapse of membrane potential. This

is in accordance with the results of [1], which observed that paracetamol tablet in dose $500 \text{ mg} \cdot \text{kg}^{-1}$ could be toxic as a consequence of different sensitivity of each adult individual as well as children for which could be toxic also dose $200 \text{ mg} \cdot \text{kg}^{-1}$. Formation of reactive oxygen after paracetamol administration $200 \text{ mg} \cdot \text{kg}^{-1}$ to infants have observed [14]. The result of multiple interactions of administered paracetamol $500 \text{ mg} \cdot \text{kg}^{-1}$ is 20–25% depleting of normal physiological level of glutathione [11]. This in combination with direct cellular injury by NAPQI, leads to cell damage and death [3,9,18].

Paracetamol administration in higher than therapeutic dosage caused activation of antioxidant enzymes. Superoxide dismutase SOD is the first antioxidative enzyme, which protects the cell against reactive superoxide radicals. Hydrogen peroxide increases during this process. The activity of GPx is induced by substrates (hydrogen peroxide or organic peroxide), which are decomposed by GPx to water [18]. Our results showed significant elevation of GPx activity after paracetamol administration, probably as the result of ROS production.

At the same time GPx is induced by reduced glutathione GSH, which is oxidized into GSSG. GPx activity is increased simultaneously with GR activity, which protects cell against accumulation of oxidized glutathione by converting GSSG back to reduced form of glutathione GSH. When intracellular concentration of GSH decreases, GR activity increases. Increase of GPx and GR activity *in vivo* according our results after paracetamol administration is a result of ROS production and covalent complex formation of paracetamol with oxidized GSSG. Increase of GR (61–62%) and glucose-6-P-dehydrogenase activity until (110–130%) after paracetamol administration 980 and $1200 \text{ mg} \cdot \text{kg}^{-1}$ *in vivo* was also observed [16].

Insufficiency of antioxidants accumulates a negative effect of paracetamol [24]. Generally, effect of oxidants can be eliminated by antioxidative therapy. Our experiment showed, that paracetamol participates in formation of free radicals and confirms previous studies, in which paracetamol administration elevated antioxidative enzymes activities in dependence on dose, which is considered therapeutically as subtoxic and toxic.

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