# *IN VITRO* STUDY ON PARACETAMOL BEHAVIOUR AS POTENTIAL ANTIOXIDANT AND CYTOPROTECTOR

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**Abstract.** The aim of the present study is to evaluate the antioxidant and cytoprotective effects of Paracetamol, using different *in vitro* models of toxicity on sub-cellular (isolated rat liver microsomes) and cellular (isolated rat hepatocytes) levels.

On isolated rat liver microsomes, in conditions of non-enzyme lipid peroxidation, paracetamol revealed good statistically significant time- and concentration-dependent antioxidant activity (decreasing malondialdehyde (MDA) production – marker for lipid peroxidation).

On isolated rat hepatocytes, we determined the main parameters of the hepatocytes' functional and metabolic status: cell viability (measured by trypan blue exclusion), levels of lactate dehydrogenase (LDH), reduced glutathione (GSH) and MDA.

In carbon tetrachloride-induced toxicity, paracetamol showed statistically significant concentration-dependent cytoprotective and antioxidant activities. Paracetamol prevented the loss of cell viability and GSH depletion, decreased LDH leakage and MDA production.

These cytoprotective and antioxidant activities on different toxicity models might be due to a possible metabolic interaction between paracetamol and toxic agents.

**Keywords:** paracetamol, isolated rat microsomes, isolated rat hepatocytes, cytoprotection, antioxidant

## Introduction

In the recent years there is an information of the ability of some phenol derivatives, including paracetamol, to act as inhibitors of the membrane lipid peroxidation and as scavengers of free radicals. Literature data suggest that at low concentrations, paracetamol protects different tissues against free radicals (7, 8, 11). Regarding the literature data, the aim of the present study was to evaluate the antioxidant and protective effect of paracetamol, using different models of toxicity in different levels in rats.

## Materials and methods

*Chemicals*

The chemicals used in the biological experiments were: pentobarbital sodium (Sanofi, France), HEPES (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), $NaHCO_3$ (Merck), $KH_2PO_4$

(Scharlau Chemie SA, Spain), $CaCl_2.2H_2O$ (Merck), $MgSO_4.7H_2O$ (Fluka AG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich), albumin, bovine serum fraction V, minimum 98 % (Sigma Aldrich), EGTA (Sigma Aldrich), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck), lactate dehydrogenase (LDH) kit (Randox, UK), acetaminophen (Sigma Aldrich) and carbon tetrachloride (Valerus, Bulgaria).

*Animals*

Animals were purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization was allowed before the commencement of the study. The health was monitored regularly by a veterinary physician. The vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and made according Ordinance № 15/2006 for humaneness behavior to experimental animals.

*Isolation of liver microsomes*

Liver is perfused with 1.15 % KCl and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH=7,4. The liver homogenate was centrifuged at 9 000 x g for 30 min at 4°C and the resulting post-mitochondrial fraction (S9) was centrifuged again at 105 000 x g for 60 min at 4°C. The microsomal pelletes were re-suspended in 0.1 M potassium phosphate buffer, pH=7.4, containing 20 % Glycerol. Aliquots of liver microsomes were stored at -70°C until use (12). The content of microsomal protein was determined according to the method of Lowry and co-workers, using bovine serum albumin as a standard (6).

*$FeSO_4$/Ascorbinic acid-induced lipid peroxidation in vitro*

As a system, in which metabolic activation may not be required in the production of lipid peroxide, 20 μM $FeSO_4$ and 500 μM Ascorbinic acid were added directly into rat liver microsomes and incubated for 20 min at 37°C (5)

*Lipid peroxidation (LPO) in microsomes*

After incubation of microsomes (1 mg/ml) with the compounds, we added to the microsomes 1 ml 25 % (w/v) trichloroacetic acid (TCA) and 1 ml 0.67 % 2-Thiobarbituric acid (TBA). The mixture is heated at 100°C for 20 min. The absorbance was measured at 535 nm, and the amount of MDA was calculated using a molar extinction coefficient of 1.56 x 105 $M^{-1}cm^{-1}$ (2).

*Isolation and incubation of hepatocytes*

An optimized *in situ* liver perfusion using less reagents and shorter time of cell isolation was performed (10). The method resulted in higher amount of live and metabolically active hepatocytes.

Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g). After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) + 0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85) and finally HEPES buffer containing collagenase type IV (50 mg/200 ml) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised, minced into small pieces, and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1 % bovine serum albumin.

Cells were diluted with KRB to make a suspension of about 3 x $10^6$ hepatocytes/ml. Incubations were carried out in flasks containing 3 ml of the cell suspension (i.e. 9 x $10^6$ hepatocytes) and were performed in a 5 % $CO_2$ + 95 % $O_2$ atmosphere.

The rat liver microsomes and hepatocytes were incubated with different concentrations paracetamol.

The cell viability was measured by using Trypan blue (0.05 %) as reagent (10). Initial

viability averaged 89-90 %.

*Lactate dehydrogenase (LDH) release*

LDH release in isolated rat hepatocytes was measured spectrophotometrically using a LDH kit (10).

*Reduced glutathione (GSH) depletion*

At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular GSH, which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm (10).

*Malondialdehyde (MDA) assay*

Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20 % (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67 % (w/v) 2-thiobarbituric acid (TBA) and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of MDA 1.56 x $10^5$ $M^{-1}cm^{-1}$ (10).

***Statistical analysis***

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for 6 experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant. Three parallel samples were used.

**Results and Discussion**

Effect of paracetamol, in rat microsomes, pre-incubated for 10 minutes, modified induced LPO. The results in Table 1 showed that paracetamol, at all studied concentrations: 0.003 mM, 0.03 mM, 0.1 mM, 0.3 mM and 1 mM, statistically significant decreased MDA quantity, compared to the toxic agent ($Fe^{2+}$/AA). The protective effect of paracetamol, against LPO was time- and concentration-dependent. The effect observed was the most prominent at the 5-th minute after the LPO induction, followed by the 10-th and 20-th minutes.

***Table 1.*** *Effect of paracetamol (PC) in 5 increased concentrations, under conditions of non-enzyme-induced LPO, on MDA quantity measured in dynamic.*

| Group | Time (min) | MDA (nmol/mg protein) |
|---|---|---|
| Non-treated microsomes | 5 | 0.09 ± 0.01 |
| | 10 | 0.12 ± 0.01 |
| | 20 | 0.59 ± 0.01 |
| $Fe^{2+}$/AA | 5 | 0.96 ± 0.01 *** |
| | 10 | 1.19 ± 0.01 *** |
| | 20 | 1.51 ± 0.01 *** |
| $Fe^{2+}$/AA + 0.003 mM PC | 5 | 0.81 ± 0.01 + |
| | 10 | 1.04 ± 0.01 + |
| | 20 | 1.4 ± 0.009 + |

| | | |
|---|---|---|
| $Fe^{2+}$/AA + 0.03 mM PC | 5 | $0.42 \pm 0.01^{+}$ |
| | 10 | $0.76 \pm 0.01^{+}$ |
| | 20 | $1.14 \pm 0.01^{+}$ |
| $Fe^{2+}$/AA + 0.1 mM PC | 5 | $0.26 \pm 0.01^{++}$ |
| | 10 | $0.61 \pm 0.01^{++}$ |
| | 20 | $1.01 \pm 0.01^{++}$ |
| $Fe^{2+}$/AA + 0.3 mM PC | 5 | $0.18 \pm 0.01^{+++}$ |
| | 10 | $0.51 \pm 0.01^{++}$ |
| | 20 | $0.77 \pm 0.01^{+++}$ |
| $Fe^{2+}$/AA + 1 mM PC | 5 | $0.09 \pm 0.01^{+++}$ |
| | 10 | $0.13 \pm 0.02^{+++}$ |
| | 20 | $0.62 \pm 0.02^{+++}$ |

*** $P < 0.001$ vs control (non-treated microsomes)
$^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$; $^{+++}$ $P < 0.001$ vs toxic agent ($Fe^{2+}$/AA)

After 5 min of incubation, MDA quantity decreased by 16 % ($p < 0.05$) at concentration 0.003 mM; by 56 % ($p < 0.05$) at 0.03 mM; by 73 % ($p < 0.05$) at 0.3 mM; by 81 % ($p < 0.05$) at 0.1 mM and by 91 % ($p < 0.05$) at 1 mM. These results correlate with the results of Dinis et al. (3) on $Fe^{2+}$/ascorbate-induced LPO in sarcoplasmatic reticulum. They found out that at concentration range 0.05 – 1 mM, paracetamol inhibits LPO, as a function of concentration. The lowest concentration was the less effective. Van Dayke et al. (14) showed that paracetamol dramatically inhibit peroxynitrite formation. The authors suggest, that the obtained results, might be due to the ability of some phenol derivatives, including paracetamol, to act as a scavenger of free radicals. Merrill et al. (9) showed that during ischemia and reperfusion in guinea pig heart, paracetamol attenuated the release of hydroxyl radicals and peroxynitrite.

Chou and Greenspan (1) studied the effect of paracetamol on the myeloperoxidase-hydrogen peroxide mediated oxidation of LDL. They found out that the presence of paracetamol (25-250 μM) prevented the modification of LDL and inhibited the production of thiobarbituric acid-reactive substances in leucocytes. Our results also determined antioxidant effect of paracetamol, assessed in rat microsomes, at concetrations including part of the concentrations, used by Chou and Greenspan (1).

The second part of our study investigate the effect of paracetamol (at concentrations 10 μ M and 50 μM), administered together with carbon tetrachloride ($CCl_4$) (at concentration 86 μM) in isolated rat hepatocytes. The principle reasons for using liver cells is their capacity for xenobiotic biotransformation.

The effect of paracetamol and carbon tetrachloride, administered alone and in combination, on the assessed parameters: trypan blue exclusion, LDH leakage into the medium, content of GSH and MDA quantity, in isolated rat hepatocytes, is shown in Table 2.

***Table 2.*** *Effect of paracetamol (PC) (10 μM and 50 μM), administered together with carbon tetrachloride ($CCl_4$) (86 μM) on trypan blue exclusion, LDH leakage into the medium, GSH content and MDA quantity, in isolated rat hepatocytes.*

| Group | Trypan blue exclusion, % | LDH, nmol/min/mill cells | GSH, nmol/mill cells | MDA, nmol/mill cells |
|---|---|---|---|---|
| Control | 87 ± 6.5 | 0.167 ± 0.02 | 40 ± 8.6 | 0.56 ± 0.04 |
| $CCl_4$ | 45 ± 4.6 *** | 0.753 ± 0.08 *** | 19 ± 4.5 *** | 0.831 ± 0.07 ** |
| 10 μM PC | 64 ± 8.2 ** | 0.354 ± 0.06 ** | 29 ± 6.5 ** | 0.537 ± 0.01 |
| 50 μM PC | 53 ± 8.5 *** | 0.530 ± 0.08 *** | 23 ± 4.1 *** | 0.578 ± 0.03 |
| 10 μM PC + $CCl_4$ | 58 ± 8.5 ** ++ | 0.469 ± 0.05 *** +++ | 24 ± 4.4 ** | 0.537 ± 0.02 +++ |
| 10 μM PC + $CCl_4$ | 69 ± 2.0 * +++ | 0.297 ± 0.03 ** +++ | 30 ± 3.5 ** ++ | 0.528 ± 0.08 +++ |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs control (non-treated hepatocytes)
++ $p < 0.01$; +++ $p < 0.001$ vs toxic agent (carbon tetrachloride)

Carbon tetrachloride causes liver damage following the cleavage by cytochrome P450 to form the trichlormethyl free radical, which covalently binds to lipids and other cellular macromolecules. Consequently, hepatic unsaturated fatty acids in microsomal lipids undergo peroxidation, resulting in the production of lipid peroxides (13). In our study, carbon tetrachloride as a potent hepatotoxic agent, showed its toxicity, affecting statistically significant all assessed parameters.

Paracetamol, administered alone, affected the studied parameters, except MDA, in concentration-dependent manner but not in that extent like carbon tetrachloride.

Administered together with carbon tetrachloride, paracetamol showed cytoprotective effects. It reduced the effect of carbon tetrachloride on: cell viability by 29 % ($p < 0.01$) at 10 μM and by 53 % ($p < 0.001$) at 50 μM; on LDH leakage – by 38 % ($p < 0.01$) at 10 μM and by 61 % at 50 μM. The GSH content was preserved by 26 % ($p < 0.01$) and by 58 % ($p < 0.001$) at 10 μM and 50 μM paracetamol, respectively. Paracetamol, at both concentrations, reduced the increased by carbon tetrachloride MDA quantity by 36 % ($p < 0.001$).

It is known that metabolic activation of paracetamol is catalyzed by cytochrome P450 and may involve CYP1A2, CYP2E1, CYP3A4, depends on the dose (4, 13). Carbon tetrachloride-induced lipid peroxidation appeared to be dependent on CYP2E1 and CYP1A2 (4, 13). According to these data we supposed a possible metabolic interaction of the two compounds that might partly explain the observed effects.

## Conclusion

The results of our study suggest the protective and antioxidant effect of paracetamol using different models of toxicity *in vitro*, which might be due to possible metabolic interaction.

**References**

1. Chou TM, Greenpan P. Effect of acetaminophen on the myeloperoxidase-hydrogen peroxide-mediated oxidation of LDL. Biochem Biophys Acta 2002; 1581(1-2): 57-63.

2. Deby C, Goutier R. New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. Biochem Pharmacol 1990; 39: 399-405.

3. Dinis T, Madeira V, Almeda L. Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosaliylate) as inhibitors of membrane lipid peroxidation and as phenoxyl radical scavangers. Arch Biochem Biophys 1994; 315: 161-169.1.

4. Hazai E, Vereczkey L, Monostory K. Reduction of toxic metabolite formation of acetaminophen. Biochem Biophys Res Commun 2002, 291(4): 1089-1094.

5. Kim HJ, Chun YJ, Park JD, Kim SI, Roh JK, Jeong TC. Protection of rat liver microsomes against carbon tetrachloride-induced lipid peroxidation by red ginseng saponin through cytochrome P450 inhibition. Planta Med 1997; 63: 415-418.

6. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin Phenol reagent. J Biol Chem 1951; 193: 265-275.

7. Maharaj DS, Saravanan KS, Maharaj H. Acetaminophen and aspirin inhibit superoxide anion generation and lipid peroxidation, and protect against 1-methyl-4-phenyl piridinium-induced dopaminergic neurotoxicity in rats. Neurochem Int 2004; 44(5): 355-360.

8. Merril GF. Acetaminophen and low-flow myocardial ischemia: efficacy and antioxidant mechanisms. Am J Physiol Heart Circ Physiol 2002; 282(4): 1341-1349.

9. Merrill GF, Goldberg E. Antioxidant properties of acetaminophen and cardioprotection. Basic Res Cardiol 2001; 96: 423-430.

10. Mitcheva, M., M. Kondeva, V. Vitcheva, P. Nedialkov, G. Kitanov , Effect of benzophenones from Hypericum annulatum on carbon tetrachloride-induced toxicity in freshly isolated rat hepatocytes. Redox Rep 2006; 11: 3-8.

11. Orhan H, Sahin G. In vitro effects of NSAIDs and paracetamol on oxidative stress-related parameters of human erythrocytes. Exp Toxicl Pathol 2001; 53(2-3): 133-140.

12. Simeonova RL, Vitcheva VB, Kondeva-Burdina MS, Krasteva IN, Nikolov SD, Mitcheva MK. Effect of purified saponin mixture from Astragalus corniculatus on enzyme- and non enzyme-induced lipid peroxidation in liver microsomes from spontaneously hypertensive rats and normotensive rats. Phytomedicine 2010; 17(5): 346-9

13. Timbrell J. In: principles of Biochemical Toxicology, 3-rd Edition, Taylor&-Francis Ltd, 2000: 89.

14. Van Daykek, Sacks M, Quazi N. A new screening method to detect water soluble antioxidants: acetaminophen (Tylenol) and other phenols react as antioxidants and destroy peroxynitrite-based luminol-dependent hemiluminescence. J Biolumin Chemilumin 1998; 13: 339-348.

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