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Characterization of Acetaminophen Toxicity in Human Kidney HK-2 Cells

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Summary

Acetaminophen (APAP) overdose causes liver injury, but in some cases it is associated also with renal impairment. While several studies exist in relation to acetaminophen nephrotoxicity, no reports have been published describing intracellular changes related to APAP nephrotoxicity *in vitro*. Because proximal tubular cells are considered to constitute a secondary site of drug-induced injury after hepatocytes, our study's aim was to estimate the toxicity in the human HK-2 cell line. We used a range of APAP concentrations (1-10 mM) to examine toxicity in the cells (1-48 h). We evaluated cell viability using the WST-1 and LDH tests. Cells impairment was also determined by monitoring ROS production, glutathione levels. We proved that HK-2 cells are able to metabolize acetaminophen. We observed moderate impairment of cells already after 1 h of treatment based on a finding of increased ROS production and decreased cell viability. After 24 h, the results showed significant cellular impairment at all tested concentrations except for 1 mM APAP, but no glutathione depletion was found. We conclude that HK-2 cells are susceptible to acetaminophen toxicity but, unlike hepatocytes, it might be not linked to glutathione depletion.

Key words

Acetaminophen • Nephrotoxicity *in vitro* • HK-2 cells • Oxidative stress

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Introduction

Acetaminophen (APAP) is the most commonly used antipyretic and analgesic. When taken at therapeutic doses, it is considered to be a safe drug. Overdose can result in both liver and renal injuries, however, as has been observed in both humans and laboratory animals (Bessems and Vermeulen 2001). The incidence of acetaminophen overdose is over 20,000 cases a year in the UK and the USA (Lee 2004). Although many investigators have studied APAP's toxicity mechanism, the entire mechanism of organ injury is still not completely understood.

Acetaminophen is conjugated with glucuronate and sulfate to form more water-soluble compounds that are eliminated from liver and blood mainly *via* bile and urine, respectively. At therapeutic doses, a small proportion of the administered APAP is oxidized by microsomal cytochrome P450 to the toxic electrophile N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is detoxified by conjugation with reduced glutathione (GSH) to form acetaminophen-glutathione conjugate (APAP-SG) (James *et al.* 2003). Consequently, enhanced APAP-SG formation occurs together with GSH depletion, which leads to injury and cell death (Jaeschke and Bajt 2006). Presently, it is not certain whether APAP toxicity is caused by protein arylation, increase of oxidative stress due to GSH depletion, or through some other toxic effects.

Acetaminophen-induced nephrotoxicity is less commonly seen than is hepatotoxicity. In some cases

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after overdose, acute renal failure can occur as a complication of hepatotoxicity (Loh and Ponampalam 2006, Satirapoj *et al.* 2007, Wilkinson *et al.* 1977). Renal insufficiency occurs in approximately 1-2 % of patients (Mazer and Perrone 2008). Several reports describe APAP nephrotoxicity without liver failure (Blakely and McDonald 1995, Campbell and Baylis 1992, Prescott *et al.* 1982). There are reports in the literature of lower renal GSH depletion and reduced covalent binding of APAP to proteins compared to values in the liver after APAP treatment in animals (McMurtry *et al.* 1978, Mudge *et al.* 1978). On the other hand, most *in vitro* studies have been intended to characterize only APAP hepatotoxicity (Bajt *et al.* 2004, Potter *et al.* 1973, Reid *et al.* 2005, Rousar *et al.* 2009).

A human kidney cell line (HK-2) derived from normal adult human proximal tubular cells that were immortalized by human recombinant papilloma virus (HPV 16) was established by Ryan *et al.* (1994). These cells express normal phenotypic characteristics of proximal tubular tissue of human kidney and retain functional characteristics, i.e. membrane transporters, enzymes, etc., consistent with *in vivo* system and primary proximal tubular cells (Arbillaga *et al.* 2007, Racusen *et al.* 1997, Ryan *et al.* 1994, Trifillis *et al.* 1985). A number of investigators have used HK-2 cells to study nephrotoxicity for a variety of compounds (Arbillaga *et al.* 2007, Gunness *et al.* 2010, Jennings *et al.* 2007, Wu *et al.* 2009). Therefore, this cell line has been generally accepted as a suitable *in vitro* model for the study of nephrotoxicity.

Recently, only little research has been carried out with a focus on APAP nephrotoxicity *in vitro* in both animal and human cells (Wu *et al.* 2009, Zhang *et al.* 2007). Therefore, the aim of our study was to estimate cellular changes in HK-2 cells after APAP treatment. We aimed especially to examine oxidative stress in HK-2 cells after APAP treatment and to characterize the relationship of reactive oxygen species (ROS) production, glutathione depletion and cell viability.

Material and Methods

Chemicals

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (with/without phenol red), insulin, transferrin and sodium selenite were purchased from Sigma-Aldrich (USA). Fetal bovine serum, pyruvate, penicillin, streptomycin, epidermal growth factor and all other chemicals, if not otherwise specified, were purchased from Invitrogen-Gibco (USA).

Cell line

Human kidney (HK-2) cells, a proximal tubular epithelial cell line derived from normal adult human kidney cells immortalized by transduction with human papillomavirus (HPV 16) DNA fragment (Ryan *et al.* 1994), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12 = 1:1) supplemented with 5 % (v/v) fetal bovine serum, 1 mM pyruvate, 50 µg/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml sodium selenite, and 5 ng/ml epidermal growth factor and maintained at 37 °C in a sterile, humidified atmosphere of 5 % CO_2 and 95 % O_2. All the experiments were conducted using the HK-2 cells (14-18 passages).

Cell viability tests

HK-2 cells were seeded into 96-well plates at density of 3 x 10^4 cells/well and incubated for 24 h. After seeding, medium was removed and replaced in the wells with the exposure medium containing acetaminophen dissolved in distilled water at final concentrations of 1, 5 and 10 mM. The cells were then incubated for specific time periods. Cell viability was evaluated by assay of lactate dehydrogenase activity in culture medium using a commercial kit from Roche (Germany) and also using the WST-1 test (Roche, Germany). The WST-1 assay measures the activity of intramitochondrial and extramitochondrial dehydrogenases. At the required time, the WST-1 reagent was added to the cultured cells (1:10 final dilution). The cells were incubated in a gassed atmosphere (5 % CO_2) for 60 min and the absorbance change between 0 h and 1 h was measured spectrophotometrically at wavelength of 440 nm using a Tecan Infinite M200 plate reader (Tecan Austria). Finally, the xCELLigence System (Roche, Germany) was also used for estimating cellular proliferation. The cells were seeded at density of 2 x 10^4 cells/well on 96 E-plates for 24 h. Then, upon reaching the logarithmic growth phase, the cells were incubated with acetaminophen (1, 5 and 10 mM) for 72 h. The growth curves were normalized as a cell index corresponding to the electrical impedance measured in every well. Real-Time Cell Analysis 1.2.1 software was used for data analysis.

Detection of reactive oxygen intermediates

HK-2 cells were seeded into 96-well plates (density 3 x 10^4 cells/well) and incubated for 24 h. After removal of medium, the cells were treated with APAP (1, 5 and 10 mM) and incubated for specific time periods. The production of reactive oxygen species was assessed using 5-,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, Eugene, OR, USA). This probe is nonfluorescent until the acetate groups are hydrolyzed by intracellular esterases and oxidized by ROS in cells. After incubation, the cells were loaded by 1 μM CM-H_2DCFDA for 120 min. The increase of fluorescence intensity was monitored in the Tecan Infinite M200 plate reader for 1 h at excitation and emission wavelengths (485 and 535 nm, respectively). The results were expressed as a ratio of the change in fluorescence intensity at the end and at the start of measurement relative to the fluorescence signal at the start.

Glutathione and acetaminophen-metabolite levels

To measure glutathione concentration, HK-2 cells were seeded into 24-well plates at density 1.5 x 10^5 cells/well for 24 h and treated with acetaminophen. The medium was then removed and ice-cold 5 % metaphosphoric acid was added. The cells were scratched and the lysate was centrifuged (4 °C; 18,000 rpm; 5 min). Reduced glutathione content was assessed using a spectrofluorimetric method (Rousar *et al.* 2012a). The levels of acetaminophen-glutathione and acetaminophen-cysteine conjugates in cell lysates were measured using a LC/MS/MS method (Cesla *et al.* 2013). Briefly, mobile phase consisted of water, acetic acid (0.1 % v/v) and methanol (gradient 10-70 %). Detection of acetaminophen metabolites was carried out at MRM transitions m/z 454.9/271.9 and 271/140 for acetaminophen-glutathione and acetaminophen-cysteine conjugate, respectively. Synthetized and purified conjugates were used as standards (final purity >98 %).

Statistical analysis

All experiments were repeated three times independently. The results are expressed as mean ± S.D. Statistical significance was analyzed using one-way ANOVA test followed by Tukey's post-hoc test to compare results with controls (OriginPro 9.0.0, USA). The cutoff for statistical significance was set at $p<0.05$ (* $p<0.05$; ** $p<0.01$, *** $p<0.001$).

Results

Cellular viability and function

Toxicity of APAP was examined in the HK-2 cell line treated with various concentrations of APAP (1, 5 and 10 mM). To prove that HK-2 cells can metabolize acetaminophen through oxidation pathway, we measured acetaminophen-glutathione and acetaminophen-cysteine (APAP-Cys) levels in cells after 24 h treatment with acetaminophen. We found dose-related production of both metabolites in cells (Table 1). Neither APAP-SG nor APAP-Cys measurable concentration was found in cell medium. Intracellular APAP-SG and APAP-Cys levels showed that HK-2 cells possess a capacity to metabolize acetaminophen.

Table 1. Levels of acetaminophen metabolites.

	APAP-SG	**APAP-Cys**
Control	–	–
1 mM APAP	3.7 ± 0.5 μM	1.6 ± 0.1 μM
5 mM APAP	6.6 ± 0.1 μM	2.9 ± 0.4 μM
10 mM APAP	7.2 ± 0.1 μM	4.0 ± 0.3 μM

The levels of acetaminophen metabolites (APAP-SG, acetaminophen-glutathione conjugate; APAP-Cys, acetaminophen-cysteine conjugate) in HK-2 cells were measured after treatment with 1, 5 and 10 mM acetaminophen for 24 h. After removal of the culture medium, cells were lysed with ice-cold 5 % metaphosphoric acid. The lysates were centrifuged and the levels of APAP-SG and APAP-Cys were determined using a LC/MS/MS method. The results are expressed as mean ± S.D. (n=3).

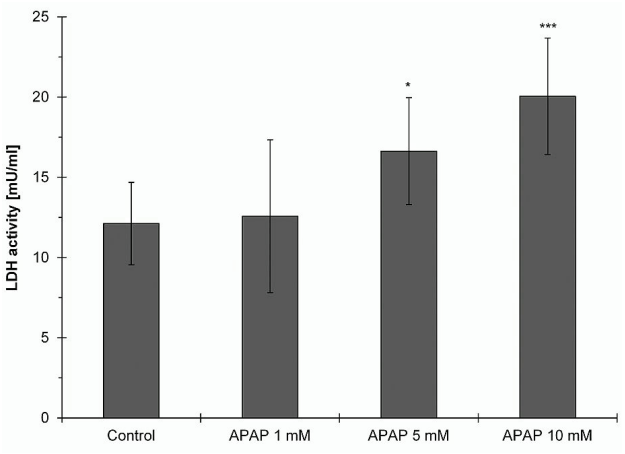

Fig. 1. LDH activity in medium. The viability of HK-2 cells was assayed by measuring LDH activity in medium after treatment with acetaminophen (1, 5 and 10 mM APAP) for 24 h. The results are expressed as mean ± S.D. (n=6). * $p<0.05$; *** $p<0.001$ (compared to control).

Lactate dehydrogenase assay and the WST-1 test were used to estimate cell viability. In addition, the xCELLigence system was used to measure cellular proliferation. The activity of LDH in medium (Fig. 1) was found to be increased significantly after 24 h of incubation with 5 and 10 mM APAP. No differences in LDH activity were found in other time periods (1, 3 and 6 h; data not shown) compared to a control group.

The overall dehydrogenases activity was evaluated using the WST-1 test. We determined the change in the enzyme activity during APAP treatment up to 48 h of incubation (Fig. 2). After 1 h, the dehydrogenases activity was significantly reduced in cells treated with 5 mM and 10 mM APAP (by 20 % and 32 % respectively). Decrease in WST-1 signal was found also at other time intervals. After 24 h, for example, the signals in cells treated with 5 mM and 10 mM APAP were 90±8 % and 65±13 %, respectively, compared with controls. After 48 h, the dehydrogenase activity continued to be reduced in 10 mM APAP to 50±3 % of the control signal. On the other hand, an increase in enzyme activity was detected in 1 mM APAP after 48 h of incubation.

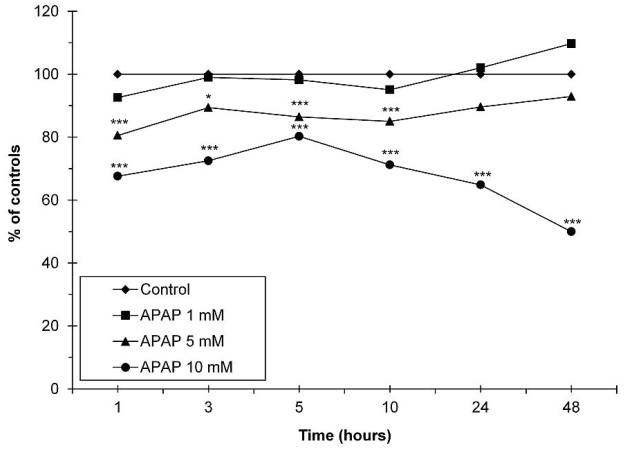

Fig. 2. WST-1 test in HK-2 cells (after 1, 3, 5, 10, 24 and 48 h). The cell viability was evaluated after treatment of HK-2 cells with acetaminophen (1, 5 and 10 mM APAP) for each time period. After addition of WST-1 reagent, the absorbance increase (λ=440 nm) was detected between 0 h and 1 h. The results were expressed as mean ± S.D. (n=6). Although only means are shown in the figure, each S.D. value was lower than 15 % of the mean signal. * $p<0.05$; *** $p<0.001$ (compared to control for the same time period).

The likely increase of cell proliferation after longer incubation was also confirmed in experiments carried out using the xCELLigence System. Here, the cellular response in 1 mM APAP treated cells was significantly enhanced after 24 h and 48 h compared to controls. On the other hand, cell proliferation was continuously inhibited in the cells treated with 10 mM APAP after 24 h incubation (Fig. 3).

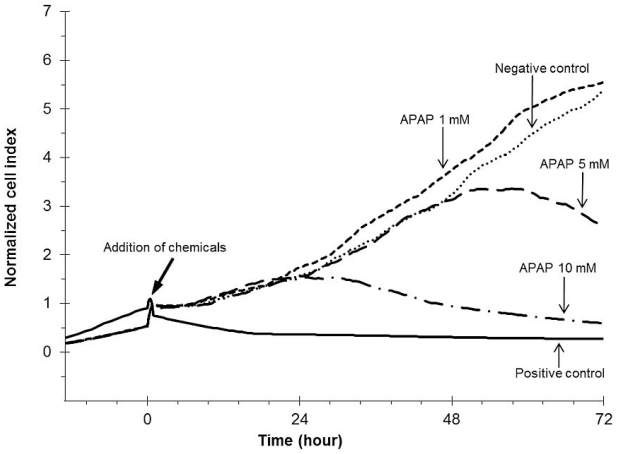

Fig. 3. Real-time analysis of cell proliferation using the recorded xCELLigence System. Each curve illustrates the HK-2 cell proliferation after treatment with acetaminophen (1, 5 and 10 mM APAP). The time of acetaminophen addition is indicated by the arrow. Negative control means HK-2 cells were treated with culture medium; cells incubated with 5 % dimethyl sulfoxide were used as positive control. The changes in cellular proliferation were expressed as a normalized cell index (n=4).

Cellular oxidative status

ROS production and GSH concentration were assayed to characterize the intracellular redox state. ROS production was evaluated in cells treated with various APAP concentrations (1, 5 and 10 mM) and for various time periods (1, 3 and 24 h). After 1 h, as shown in Table 2, ROS production had increased significantly at all tested APAP concentrations. We found the fluorescence to be increased in all cases in relation to the APAP dose. The largest production of ROS was determined in 10 mM APAP after 24 h, for which there was a 83 % increase in fluorescence versus that measured in controls. Glutathione concentrations were measured after 24 h. The signals in 1, 5 and 10 mM APAP treated cells and in controls were 96±7 %, 104±4 %, 91±9 % and 100±4 %, respectively. Therefore, no significant glutathione depletion was observed in cells treated with acetaminophen compared with controls after 24 h. No significant change in GSH concentration was found also after treatment for 6 h (data not shown).

Discussion

Acetaminophen-induced liver toxicity has been studied very extensively while the extrahepatic

Table 2. Reactive oxygen species production in cultured HK-2 cells.

	1 h	3 h	24 h
Control	82 ± 4 %	77 ± 5 %	81 ± 4 %
1 mM APAP	108 ± 6 % ***	98 ± 9 % ***	100 ± 7 % ***
(ratio to control)	(1.31)	(1.26)	(1.24)
5 mM APAP	137 ± 6 % ***	124 ± 17 % ***	127 ± 4 % ***
(ratio to control)	(1.67)	(1.61)	(1.57)
10 mM APAP	145 ± 8 % ***	144 ± 8 % ***	148 ± 5 % ***
(ratio to control)	(1.77)	(1.86)	(1.83)

Reactive oxygen species production in cultured HK-2 cells (after 1, 3 and 24 h) when treated with 1, 5 and 10 mM acetaminophen. After removal of the culture medium, CM-H_2DCFDA (1 μM) diluted in culture medium was added to the cells and loaded for 120 min. The increase of fluorescence was monitored for 1 h and the results were expressed as the percentage changes in fluorescence intensity between 0 min and 60 min. The results are expressed as mean ± S.D. (n=6). *** $p<0.001$ (compared to control at corresponding time period).

manifestations of APAP toxicity are not sufficiently described in the literature. Although renal impairment resulting from APAP overdose is not seen as commonly as is liver injury, that impairment appears to cause other complications. Kidneys can also metabolize APAP to an arylating intermediate *via* the microsomal cytochrome P-450 systems, albeit to a somewhat lesser extent than do hepatocytes (McMurtry *et al.* 1978, Mudge *et al.* 1978). The oxidation of acetaminophen to NAPQI has been proven by the detection of acetaminophen-protein adducts in kidneys (Hart *et al.* 1991, 1994). The greatest activities of the cytochrome P-450 systems are found in the renal cortex, and especially in the proximal tubules (Kaloyanides 1991). Hence, APAP renal toxicity is presumably restricted to this part of the kidneys. Other possible mechanisms involved in APAP-induced renal toxicity are the actions of prostaglandin synthetase and N-deacetylase (Fowler *et al.* 1993, 1991, Lock *et al.* 1993).

Only a few studies have been reported that mention renal APAP toxicity *in vitro* (Wu *et al.* 2009, Zhang *et al.* 2007). Most of those studies focused on APAP nephrotoxicity had been carried out under *in vivo* conditions (Hart *et al.* 1996, Jones *et al.* 1979, Mudge *et al.* 1978, Newton *et al.* 1986, Richie *et al.* 1992, Stern *et al.* 2005a, b, Tarloff *et al.* 1996, Zaher *et al.* 1998). Those experiments tested a variety of animals, including rats, mice, rabbits and hamsters (Gregus *et al.* 1988, Hart *et al.* 1994, McMurtry *et al.* 1978, Tarloff *et al.* 1996). Although some species differences appeared, it is generally accepted that APAP nephrotoxicity is manifested as proximal tubular necrosis due to the intensive metabolic activity therein (Kaloyanides 1991) and the occurrence of a number of transporting mechanisms in the membrane of proximal tubular cells (Anders 1980, Lash 1994, 2005). Considering the absence of *in vitro* data in human kidney cells, we endeavored to characterize APAP toxicity in HK-2 cells. This cell line is believed to provide a suitable model for studying nephrotoxicity because the cells express some cytochrome P450 isoforms including 1A and 2C (Arbillaga *et al.* 2007, Naud *et al.* 2011) and renal uptake and efflux drug transporters, i.e., multidrug resistance-associated proteins 2 and 4, organic anion transporters 1 and 3, and monocarboxylate protein (Naud *et al.* 2011, Wang *et al.* 2006). Based on these outcomes, HK-2 cells have been used for nephrotoxicity *in vitro* testing of a variety of compounds, i.e. heavy metals (Bao *et al.* 2013, Fujiki *et al.* 2014), endotoxin (Quoilin *et al.* 2012, Wang *et al.* 2015), commonly used toxic chemicals like cisplatin, polymyxin and cyclosporine A (Genc *et al.* 2014, Keirstead *et al.* 2014, Vizza *et al.* 2013) and also for characterization of oxidative stress after H_2O_2 treatment (Lee *et al.* 2013, Lin *et al.* 2014). These experiments showed that HK-2 cells are substantially rather sensitive to oxidative damage and that is why this cell line has been predominantly accepted as a suitable *in vitro* model for studying nephrotoxicity.

Since acetaminophen is a lipophilic compound, its ability to diffuse across the membrane supports the validity of use of HK-2 cell line in acetaminophen toxicity study. In addition, our results on production of acetaminophen metabolites after APAP treatment showed that HK-2 cells possess a capacity to metabolize acetaminophen through oxidation pathway. Although 2E1 isoform of cytochrome P450 has not been estimated in

HK-2 cells yet, our determination of APAP-related products might show that HK-2 cells may contain also this P450 isoform related to APAP oxidation. In addition, 3A4 mRNA and protein synthesis was proven in these cells recently (Gunness *et al.* 2010).

HK-2 cells were treated with acetaminophen and the changes in cell viability and oxidative status were measured. We used a range of APAP concentrations (1-10 mM). These APAP concentrations seem to be of limited clinical relevance because acetaminophen plasma levels can occur at 1 mM maximally shortly after overdose (Rumack 2002, Rumack and Matthew 1975). We used them to compare the outcomes with studies on hepatocytes treated with up to 20 mM APAP (Bajt *et al.* 2004, Kheradpezhouh *et al.* 2014, Lewerenz *et al.* 2003, Rousar *et al.* 2009, Toyoda *et al.* 2012). Cell viability was evaluated using the WST-1 test, LDH activity assay, and also cell proliferation using the xCELLigence System. Although the WST-1 test showed significant changes in enzyme activity of HK-2 cells exposed to 5 and 10 mM APAP after 1 h, the other methods showed toxic changes only after 24 h and later. In the case of 1 mM APAP, our WST-1 and xCELLigence test results showed that viability of cells was enhanced after 24 and 48 h. These findings might be caused by induced reparation process in mildly injured cells which could lead to higher level of proliferation. Having compared these outcomes with our results published earlier for cultured rat hepatocytes (Rousar *et al.* 2009), we can conclude that the susceptibility of HK-2 cells to APAP appears to be somewhat similar. On the other hand, such extent of increase in extracellular LDH activity as corresponds to the rupture of cell membrane found in hepatocytes treated with 10 mM of APAP was not found in HK-2 cells (Rousar *et al.* 2009).

We examined the production of ROS using an intracellular fluorescent probe for evaluating oxidative stress. As the results show, there was a significant, stable and dose-related increase of ROS production through all time periods. These observations in HK-2 cells are comparable with findings for hepatocytes *in vitro*, but the ratio of signal relative to controls in hepatocytes is different (Bajt *et al.* 2004, Rousar *et al.* 2009). According to our previous studies on toxicity of APAP-SG, here presented results might be explained also with respect to APAP-SG ability to induce ROS production (Nydlova *et al.* 2014, Rousar *et al.* 2012b). On the other hand, the levels of APAP-SG detected in HK-2 cells seem to be much lower than those inducing ROS production. We also characterized intracellular redox status by measuring GSH levels in HK-2 cells. Mild glutathione depletion occurred only after 24 h of incubation and in cells treated with higher acetaminophen doses. This finding is interesting in comparison with that for hepatocytes, because complete glutathione depletion is a typical result of acetaminophen overdose in liver cells. Our results are in accordance with findings from *in vivo* experiments the authors of which have described that acetaminophen overdose causes poorer and slower glutathione depletion in kidney cortex compared to liver (McMurtry *et al.* 1978, Mudge *et al.* 1978).

In conclusion, we have found that susceptibility of human HK-2 cells to acetaminophen toxicity is lower in comparison with hepatocytes *in vitro*. HK-2 cells possess a capacity to oxidize acetaminophen but more investigation is needed to localize the site and type of cytochrome P450. The toxic effect was presumably apparent as a decrease in mitochondrial dehydrogenase activity, induction of ROS production, but no significant glutathione depletion.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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