# Evaluation of Oxidative Status in Acetaminophen Treated Rat Hepatocytes in Culture

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*Short title:* Evaluation of oxidative status in AAP treated hepatocytes

**Summary**

The present study describes estimation of acetaminophen (AAP) toxicity in cultured rat hepatocytes. We used different concentrations of AAP – 1, 2.5, 5, 10 and 20 mM, to test influence of AAP on cellular viability, functional capacity and oxidative status at given time intervals. WST-1 test showed decrease of dehydrogenase activity in 5, 10 and 20 mM AAP to 75% of control after 1 hour of incubation. At 12 h of treatment, any AAP concentration led to decrease WST-1 signal; no enzyme activity was found since 18 h in 20 mM AAP treated cells according to LDH leakage test at 24 h of incubation. Functional capacity was tested by albumin assay where the decrease was strictly related to AAP dose. Intracellular oxidative status was assessed by analysis of GSH/GSSG levels and time course of ROS production and glutathione reductase (GR) activity. Increased ROS production was found already after 3 h of incubation in 2.5, 5, 10 and 20 mM AAP, respectively. The highest ROS production was measured after 12 h treatment. GR activity was decreased already after 3 h of incubation and remained also decreased in 2.5, 5, 10 and 20 mM AAP treated cells during further time of incubation.

**Key words:** Acetaminophen • Cultured hepatocytes • Hepatotoxicity • Glutathione • ROS

## Introduction

Acetaminophen overdose is the most frequent cause of drug-induced acute liver failure in men (James *et al.* 2003; Lee 2003). It causes typical centrilobular necrosis in the liver, described in detail by Mitchell (Mitchell *et al.* 1973a). Acetaminophen (AAP) is metabolised to major extent by conjugation with glucuronic and sulfuric acid in the liver. The remaining part is metabolized by different isoenzymes of cytochrome P450 to active metabolites (Jaeschke and Bajt 2006), presumably to N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is detoxified by glutathione (GSH) to form glutathionyl conjugate. After the cellular glutathione stores are depleted, NAPQI binds to SH-moieties of proteins within the cell (Mitchell *et al.* 1973b). Consequently, there have been found many mechanisms contributing to the cell death – impairment of mitochondrial respiration, induction of oxidative stress and lipoperoxidation. In addition, the opening of mitochondrial permeability transition pore due to AAP treatment was described recently (Kon *et al.* 2004). The various mechanisms proceeding during the injury are described in detail in numerous current reviews (Jaeschke and Bajt 2006; Jaeschke *et al.* 2003; James *et al.* 2003). Despite substantial progress in our understanding, some mechanisms of the acetaminophen-induced liver injury remain still unknown.

It is generally accepted that AAP-injury is related with oxidative stress. The primary cause of oxidative stress is a decrease of intracellular GSH levels due to the conjugation of GSH with NAPQI and an increase of glutathione disulfide (GSSG) levels (Knight *et al.* 2001; Mitchell *et al.* 1973b). In addition, it was proved that the reactive oxygen species (ROS) are produced as a consequence of decreased levels of GSH after administration of acetaminophen in hepatocytes (Bajt *et al.* 2004).

The acetaminophen toxicity was firstly studied in *in vivo* experiments in mouse and rat models (Jollow *et al.* 1973; Mitchell *et al.* 1973a). Consequently, *in vitro* studies have been used to estimate of AAP toxicity and recently, both types of experiments are used for

characterization of mechanisms involved in AAP toxicity (Bajt *et al.* 2004; Kon *et al.* 2004; Reid *et al.* 2005). Since large variation in acting of different AAP concentrations has been reported, the aim of our study was to describe and compare in detail mechanisms of injury in cultured rat hepatocytes under different AAP concentrations. We focused mainly on investigation of oxidative status in hepatocytes, especially using intracellular ROS probes.

## Methods

### *Chemicals*

Medium Williams´ E (without phenol red), fetal bovine serum, penicillin, streptomycin and glutamine were purchased from PAN BIOTECH GmbH (Germany). Collagenase cruda was obtained from SEVAC (Czech Republic), insulin (Actrapid, Hoechst, Germany), glucagon (Novo Nordisk, Denmark), prednisolon (Solu-Decortin, Merck, USA), Rat Albumin ELISA Quantification Kit (Bethyl Lab. Inc., USA), Cell Proliferation Reagent WST-1 (Roche, Germany) and CM-DCFDA (Molecular Probes, USA). Type I collagen, trypan blue, kit for lactate dehydrogenase (Diagnostic Systems, Germany) and all other chemicals, if not specified in the article, were purchased from Sigma-Aldrich (USA).

### *Animals*

Male albino Wistar rats (Biotest, Czech Republic) were housed at 23±1 °C, 55±10 % relative humidity, air exchange 12-14 times/h, and 12-hour light-dark cycle periods (6:00 h to 18:00 h). The animals had free access to standard laboratory rat chow (DOS 2B, Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set by the Institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

*Hepatocyte isolation, cultivation and treatment*

Hepatocytes were isolated from rats mentioned above with a body mass of 220-250 g by collagenase perfusion (Berry *et al.* 1991). The viability of freshly isolated hepatocytes was more than 90 % as confirmed by trypan blue exclusion. Isolated hepatocytes were suspended in Williams' E medium supplemented with fetal bovine serum (6 %), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (10 mg/ml), insulin (0.08 IU/ml), prednisolon (0.5 μg/ml), glucagon (0.008 μg/ml) and plated in collagen-coated Petri dishes (60 mm), at a density of $2x10^6$ cells/Petri dish. Hepatocytes were allowed to attach in a gassed atmosphere (5 % $CO_2$) at 37 °C for 2 h.

After the establishment of monolayers, the medium was removed and replaced with fresh medium containing dissolved acetaminophen at concentrations of 1, 2.5, 5, 10 or 20 mmol/l. Then the hepatocytes were incubated for appropriate time-period, mostly for 24 h. After incubation, the medium was collected for required biochemical assays and cells were harvested or sonicated.

*Biochemical assays*

Acetaminophen toxicity was determined by lactate dehydrogenase activity (LDH) in the culture medium using a commercial kit from Diagnostic Systems (Germany). The functional capacity of cultured hepatocytes was evaluated by the amount of albumin secreted into the culture medium during incubation period using commercial ELISA kit from Bethyl Lab. Inc., (USA).

After cell lysis in freezer (-80°C, 10 min) and harvesting of hepatocytes, antioxidative status of hepatocytes was determined by the measurement of glutathione content. Reduced and oxidized form of glutathione were analyzed by reverse-phase high-performance liquid chromatography (Shimadzu, Japan) using modified method of Hissin and Hilf (Hissin and

Hilf 1976; Kanďár *et al.* 2007). The separation was performed on reverse-phase column Discovery C18, 15 cm x 4 mm, 5 μm (Supelco, USA) followed by fluorometric detection (excitation wavelength 350 nm, emission wavelength 420 nm). The CSW32 program (DataApex, Czech Republic) was used for collecting and processing of the chromatographic data.

*WST-1 assay*

Cell viability was also evaluated by the WST-1 assay (Roche, Germany). WST-1 assay measures the activity of intramitochondrial and extramitochondrial dehydrogenases. Briefly, tetrazolium salts are cleaved by dehydrogenases of viable cells to produce formazan and the change of absorbance is detected spectrophotometrically. Dehydrogenase activity was assayed in collagen-coated 96 well-plates (density of $3.10^4$ cells/well). At required time, the medium was removed and the WST-1 reagent (diluted 1:10 in PBS) was added. The cells were incubated in a gassed atmosphere (5% $CO_2$) for 60 minutes. The changes in absorbance were measured using a microtiter plate reader TECAN Infinite M200 (Austria) at wavelength 440 nm.

*Glutathione reductase assay*

Glutathione reductase (GR) activity was assayed by the method described by Carlberg (Carlberg and Mannervik 1975). The cells were sonicated by Bandelin Sonopuls sonicator (Germany) and the lysates were centrifuged (4°C, 10 min, 8000 g). Glutathione reductase activity in supernatants was estimated by monitoring of NADPH decrease by 340 nm for 7 minutes on microtiter plate reader spectrophotometrically and was expressed as units of enzyme per mg protein. The protein concentration was assayed according to Bradford (Bradford 1976).

*Detection of reactive oxygen intermediates*

The production of reactive oxygen species (ROS) was evaluated using 5- and 6-chloromethyl-2´,7´-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA; Molecular Probes, USA). CM-$H_2$DCFDA is nonfluorescent until hydrolyzed by esterases and oxidized by ROS in cells. After incubation, the cells were rinsed in phosphate-buffered saline (PBS) and consequently loaded by 1 µM CM-$H_2$DCFDA for 45 minutes. The increase of fluorescence intensity was monitored in thermostated fluorescence plate reader TECAN Infinite M200 (Austria) for 1 hour using an excitation and emission wavelength 485 and 535 nm, respectively. For better comparison of several samples wherein big variation in the density of attached cells may occur, the results have been expressed as a ratio of the change in fluorescence intensity at the end and at the start of measurement.

*Statistical analysis*

Experiments were repeated at least three times using different hepatocyte preparations. The results are expressed as mean ± SD. After testing of normality, the statistical significance was analyzed using one-way ANOVA test followed by Dunnett´s post hoc test for comparison between control group and the others. (GraphPad Prism 4.03 for Windows, GraphPad Software, USA). $p < 0.05$ was considered as significant.

**Results**

We examined the toxicity of acetaminophen in cultured rat hepatocytes treated with various concentrations of AAP (1, 2.5, 5, 10 and 20 mM). We used LDH assay and WST-1 test to determine the cell viability. LDH activity in medium (Fig. 1A) increased significantly in three highest concentrations of AAP till at 12 h; after 4 h of incubation with AAP, no difference in LDH activity was found (compared to control group). The LDH activity in medium was

increased after 24 h in a concentration dependent manner of AAP concentration in dose dependent manner. However, the medium LDH activity in cells treated with 20 mM AAP was lower in comparison with those exposed to 5 or 10 mM AAP. As results from LDH leakage in cells treated in 20 mM AAP (Fig. 1B), the decrease of LDH activity found in medium in 20 mM AAP is probably caused by total impairment of the enzyme. LDH leakage at 24 h was increased proportionally to AAP dose, although 1 mM AAP seems to have no effect on LDH leakage.

WST-1 test evaluates the overall activity of cellular dehydrogenases. We used WST-1 at following time points of incubation – 1, 3, 6, 12, 18 and 24 hours (Fig. 2). The enzyme activities in 5, 10 and 20 mM AAP treated cells were significantly reduced to less than 80 % of control already during the first hour. At 12 hour-interval, all acetaminophen concentrations caused decrease in activity of dehydrogenases - from 60 % of controls in 1 mM, to 24 % of controls in 20 mM AAP, respectively. Interestingly, no enzyme activity was found in cells treated with 20 mM AAP after 18 h of incubation. The other doses of AAP caused further decline of the enzyme activity in dose dependent manner.

For evaluation of functional capacity of hepatocytes, we used the analysis of albumin synthesis within 24 hours of incubation. Results shown in figure 3 depict the decrease in albumin production in AAP treated cells. Albumin production was reduced proportionally to AAP dose (5, 10, 20 mM AAP – to 52, 37 and 10 % of control values, respectively). The levels of glutathione were assessed only after 24 h of incubation. As expected, the results showed total disappearance of reduced form of glutathione. GSH levels were detectable only in controls, 62.9 ± 3.8 µM and in 1 mM AAP, 41.29 ± 2.8 µM ** (n=6). The levels of oxidized form, glutathione disulfide (GSSG) were found in all cells. However, the decrease of GSSG levels regarding concentration was also observed. The GSSG levels in controls, 1, 2.5, and 5 mM AAP were: 5.9 ± 1.7, 5.54 ± 1.1, 1.5 ± 0.3 ** and 0.48 ± 0.1 µM **, respectively.

In hepatocytes incubated with 10 and 20 mM AAP, the GSSG levels were similar to 5 mM AAP. (* , $p < 0.05$; ** , $p < 0.01$ – compared to control)

For better description of intracellular oxidative state, ROS production and activity of glutathione reductase were assayed. ROS production was evaluated at 3, 12 and 24 h. As shown in Table 1., a significant occurrence of ROS was found in 10 and 20 mM AAP treated cells after 3 h of incubation. At 12 and 24 h time points, significant increase was identified in all groups except 1 mM AAP. The 12 h incubation with AAP caused dose dependent increase of ROS production – 2.5, 5, 10 and 20 mM AAP, increased to 200 %, 410 %, 490 % and 770 %, respectively (compared to control). After 24 h, the increase was 0 %, 87 %, 490 % and 400 %, respectively. Figure 4 represents influence of incubation time and AAP concentration on glutathione reductase activity. As shown, GR activity was already reduced after 3 hours in all of cells in comparison to control. In addition, there is a trend of dose dependent reduction in GR activity levels. After 24 hours, the resulting activities were in control and 1, 2.5, 5, 10 and 20 mM AAP, 56.5 ± 1.8, 52.2 ± 3.4, 49.5 ± 1.0, 44.5 ± 1.7, 34.9 ± 4.5 and 21.8 ± 0.7 U/mg protein, respectively.

**Discussion**

Acetaminophen is well-known hepatotoxin whose toxic action is highly dependent on dose. In our study, rat hepatocytes in primary culture were used for estimation of AAP toxicity. Rat hepatocytes are much more resistant to toxic action of acetaminophen, compared to mice and hamster (Mitchell *et al.* 1973a; Tee *et al.* 1987). Since recently, mouse hepatocytes have been used in most of studies interested in AAP toxicity, we decided to use rat hepatocytes where higher resistance was expected and proved in comparison to other results using mice. Hepatocytes were treated with different concentrations of AAP for various time periods and the changes in viability and cellular oxidative state were measured. Viability of cells was

estimated by two different methods. The first method is commonly used assay of extracellular LDH activity and LDH leakage. The second assay is WST-1 test evaluating the activity of intra- and extramitochondrial dehydrogenases. Results obtained by both of these tests show decline in cell viability detectable at 12 h of incubation. However, the LDH test did not show any changes of viability at 4 h of incubation. The WST-1 is a more sensitive assay that provided information about decrease of enzyme activity in hepatocytes already at 1 hour of treatment. Moreover, the significant changes of enzyme activity in cells exposed to AAP 1 mM are apparent in WST-1 after 12 hours although LDH tests show no difference even after 24 h. In addition, our WST-1 results documented that there is no dehydrogenase activity detectable in the cells treated with AAP 20 mM after 18 h of treatment. The comparison of both of tests is well consistent with findings published recently where WST-1 test reflected the changes in viability of hepatocytes more precisely and earlier compared to LDH testing (Kikkawa *et al.* 2005).

We have monitored the production of reactive oxygen species for evaluation of oxidative stress. We have used the fluorimetric method based on the change in fluorescence intensity of intracellular probe, DCFDA, after oxidation. As the results show, the increase of fluorescence intensity present in all time points is strictly dose dependent. The enhanced level of fluorescence in all of groups, including control, at 3 h of incubation may be detected due to higher oxidative stress linked to hepatocyte isolation. Therefore, we decided to express these data as a ratio between fluorescence of AAP treated cells and controls, where the effect of enhanced levels of fluorescence also in controls is eliminated. We have shown that AAP 1 mM exerts mild influence on ROS production. The other AAP doses caused enhanced production of ROS. In addition, we have detected repeatedly high ROS levels in AAP 20 mM and 10 mM at 24 h, although cell viability was highly decreased or even completely lost at this time interval. This finding is in good accordance to results in mouse hepatocytes

published recently (Bajt *et al.* 2004). Since a number of various compounds is able to react with DCFDA, the fluorescence signal in dead cells may be caused by reaction of the probe with stable compounds still persisting in dead cells (Halliwell and Whiteman 2004). There are few reports that applied DCFDA to monitor the implication of ROS in AAP toxicity by now (Bajt *et al.* 2004; Reid *et al.* 2005). Thus, it is quite difficult to compare our results with the others; especially, if we used rat hepatocytes unlike other researchers using mouse hepatocytes.

Glutathione reductase is an enzyme playing essential role in antioxidant defense. The GR function is to reduce the oxidized disulfide form of glutathione back to the reduced one. We have found that the activity of glutathione reductase is diminished in cells treated in most of AAP concentrations; GR activity has not been decreased in AAP 1 mM treated cells. These results are in good accordance with findings published recently by Zhu and Lei (Zhu and Lei 2006). The authors studied the influence of AAP concentration on two groups of knockout mice in glutathione metabolism-related enzymes; as a control group, they used wild-type mouse hepatocytes. They found the decrease in glutathione reductase activity, as well as in thioredoxin reductase activity within treatment of cells with acetaminophen, however, the explanation of decrease of enzyme activities has not been described.

Once the GSH stores are exhausted in acetaminophen-induced injury of hepatocytes, NAPQI binds to a number of compounds, especially to SH- moieties of proteins and the NAPQI adducts are detected in cells consequently. Many enzymes have been determined to react with NAPQI by now (Jaeschke and Bajt 2006; James *et al.* 2003), but in most of them no or very weak decrease of enzyme activity has been detected (Pumford *et al.* 1997). NAPQI adducts have been proved also in two enzymes contributing to glutathione metabolism – glutathione peroxidase and glutathione transferase (Cohen *et al.* 1997; Qiu *et al.* 1998). However, glutathione reductase has not been ever determined to react with NAPQI.

Glutathione reductase has been shown to be quite sensitive to oxidative stress. The decrease of enzyme activity was described in reaction with products of lipoperoxidation, especially with 4-hydroxynonenal (Vander Jagt *et al.* 1997). On the other hand, influence of hydrogen peroxide on GR activity has been studied too, but no decrease in GR activity has been found (Vessey and Lee 1993). Therefore, the decrease of glutathione reductase activity could be explained by above described reason.

We studied the effect of acetaminophen treatment in cultured rat hepatocytes and several methods were used to detect changes in oxidative status, viability and functional capacity of cells regarding dose and time interval of AAP treatment. The results show apparent relation of dose and toxic acting of AAP. The treatment with 1 mM AAP did not cause almost any significant changes after 24 hours (except of WST-1 test and GR). On the other hand, the highest AAP dose, 20 mM, caused very intensive damage of cells even earlier then after 24 hours. Therefore, an optimal acetaminophen dose which is recommended to be used for subsequent evaluation of AAP acting, eventually study of hepatoprotective substances in AAP toxicity (e.g. S-adenosylmethionin, N-acetylcysteine) (Kučera *et al.* 2006), seems to be 5 mM AAP in cultured rat hepatocytes, especially in 24 hour incubation.

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**Abbreviations:** AAP, acetaminophen; DCFDA, dichlorodihydrofluorescein diacetate; GSSG, glutathione disulfide; GSH – glutathione (reduced form); GR, glutathione reductase; LDH,

lactate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; NAPQI, N-acetyl-p-benzoquinone imine; PBS, phosphate buffered saline; ROS, reactive oxygen species.

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**Table 1.** Time course of ROS production in cultured rat hepatocytes.

| | **3 hours** | **12 hours** | **24 hours** |
|---|---|---|---|
| **control** | 90.2 ± 5.7 % | 24.7 ± 9.6 % | 35.5 ± 9.3 % |
| **AAP 1 mM** | 137.0 ± 54.9 % | 45.1 ± 6.3 % | 22.2 ± 3.6 % |
| *(ratio to control)* | (1.51) | (1.82) | (0.63) |
| **AAP 2.5 mM** | 178.8 ± 39.6 % ** | 74.3 ± 21.2 % | 35.3 ± 16.0 % |
| *(ratio to control)* | (1.98) | (3.00) | (1.00) |
| **AAP 5 mM** | 147.0 ± 32.5 % | 126.2 ± 47.1 % ** | 66.7 ± 12.9 % * |
| *(ratio to control)* | (1.63) | (5.11) | (1.88) |
| **AAP 10 mM** | 189.3 ± 35.1 % ** | 146.1 ± 30.4 % ** | 211.4 ± 9.4 % ** |
| *(ratio to control)* | (2.10) | (5.91) | (5.96) |
| **AAP 20 mM** | 177.4 ± 16.2 % ** | 216.6 ± 19.1 % ** | 178.4 ± 19.7 % ** |
| *(ratio to control)* | (1.97) | (8.76) | (5.03) |

After removal of the cell culture medium, cells were washed two times in PBS and DCFDA solution (1 µM, in PBS) was added. Hepatocytes were loaded for 45 minutes and then the solution was removed and PBS was added again. The increase of fluorescence was monitored for 1 h and the results are expressed as a percent ratio of difference between 0 h and 1 h and the fluorescence intensity at 0 h. In addition, the ratio of signal of AAP treated cells and control was expressed too – noted in brackets. The results of fluorescence increase are expressed as mean ± SD (n = 4). * , $p < 0.05$; ** , $p < 0.01$ (compared to control at appropriate time period).

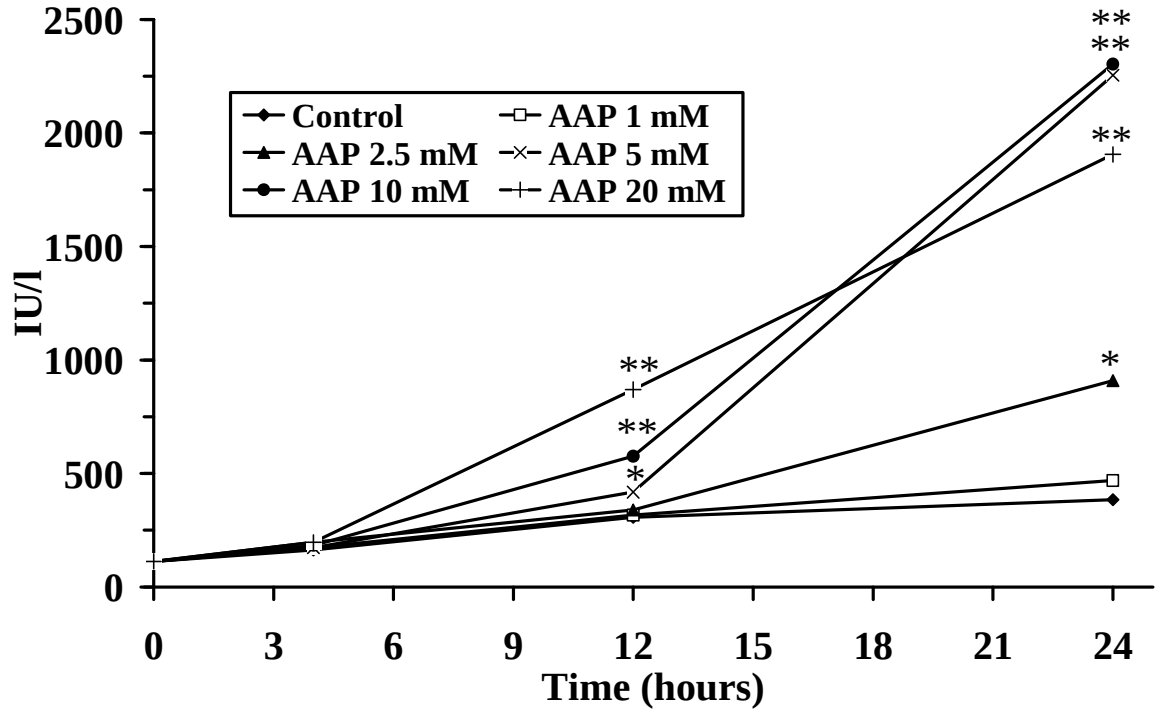


**Fig. 1A.** LDH activity in medium (time course – 4, 12 and 24 h). The viability of cells was assayed by measurement of LDH activity at time points after treatment with different AAP concentration (1, 2.5, 5, 10 and 20 mM). The results were expressed as mean ± SD (n = 3) although only means are showed at the figure. * , $p < 0.05$; ** , $p < 0.01$ (compared to control at appropriate time period).

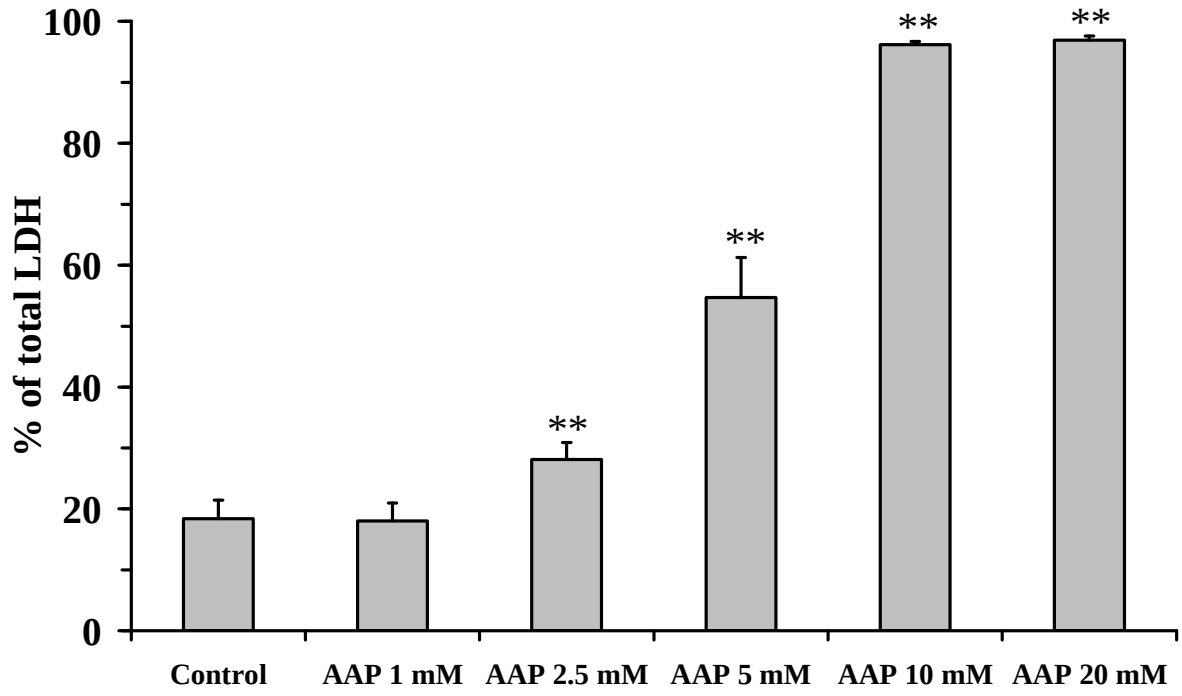


**Fig. 1B.** LDH leakage. The effect of AAP treatment (1, 2.5, 5, 10 and 20 mM) on LDH leakage was assayed in hepatocytes after 24 h. The results are expressed as mean ± SD (n = 4). ** , $p < 0.01$ (compared to control).

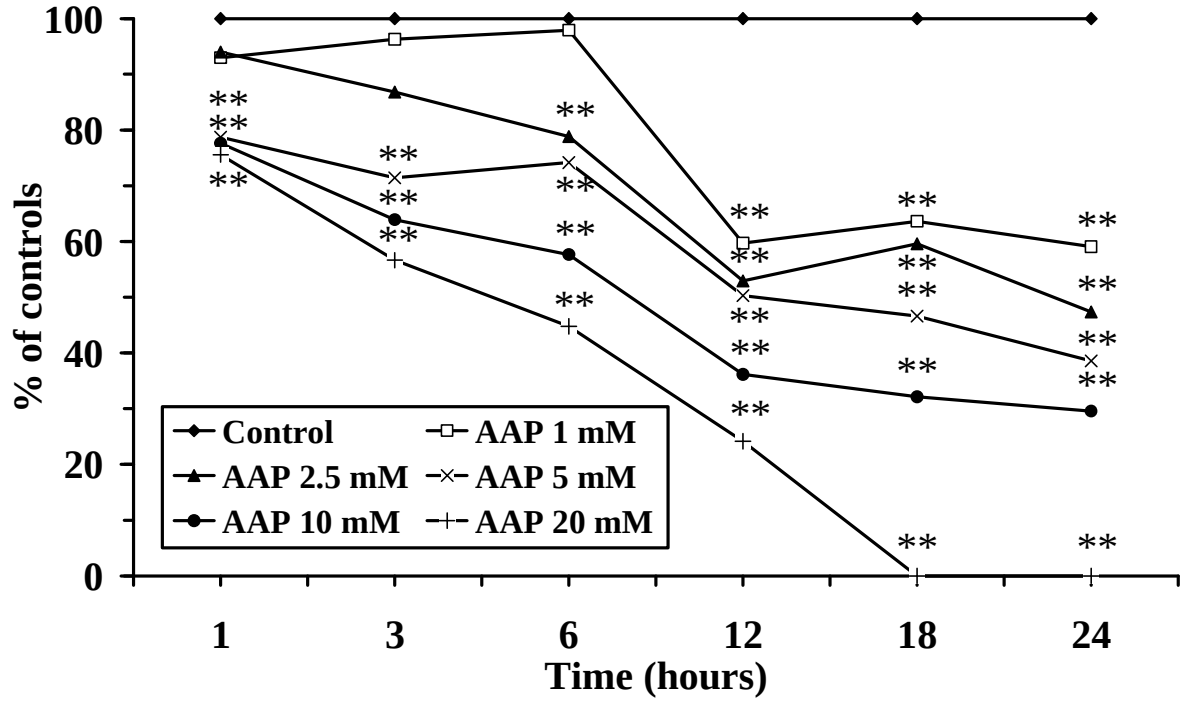


**Fig. 2.** WST-1 test (time course – 1, 3, 6, 12, 18 and 24 h). Cell viability was assayed by detection of cellular dehydrogenase activity. After AAP treatment (1, 2.5, 5, 10 and 20 mM), hepatocytes were incubated with WST-1 cell proliferation reagent and the increase of absorbance ($\lambda$ = 440 nm) was detected during 1 hour. The results were expressed as mean ± SD (n = 8) in percents of control signal, although only means are showed at the figure. ** , $p < 0.01$ (compared to control at appropriate time period).

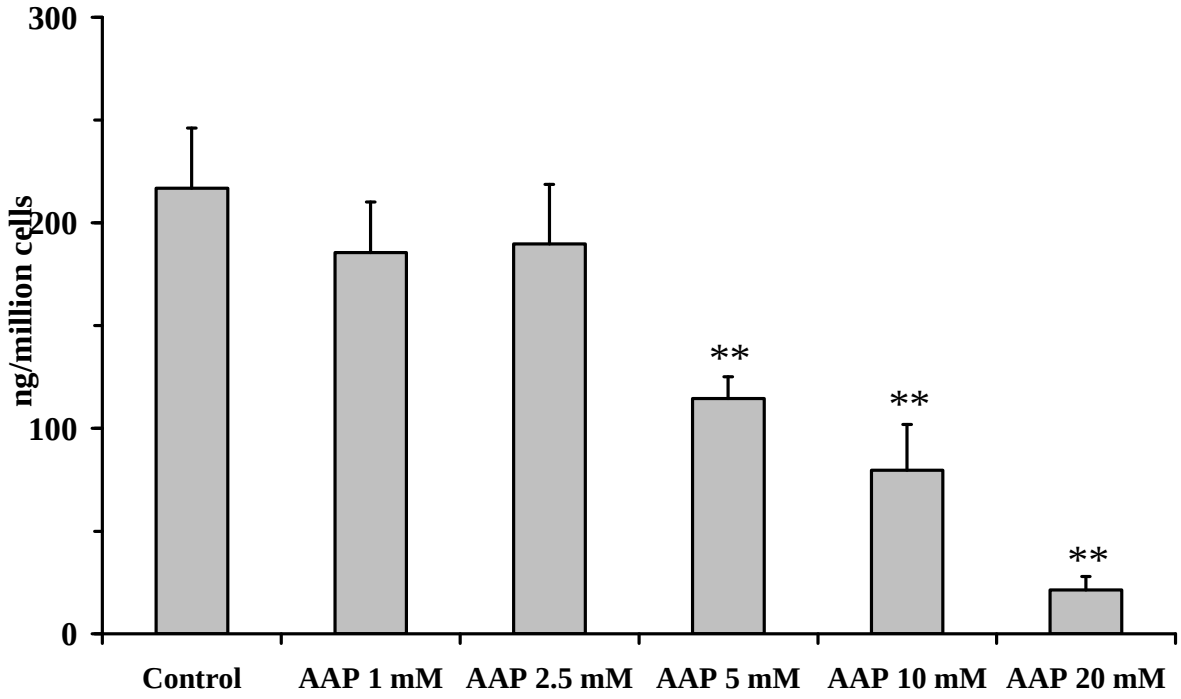


**Fig. 3.** Albumin production. The effect of AAP treatment (1, 2.5, 5, 10 and 20 mM) on hepatocytes albumin production was measured after 24 h. Results are expressed as mean ± SD (n = 4). ** , $p < 0.01$ (compared to control).

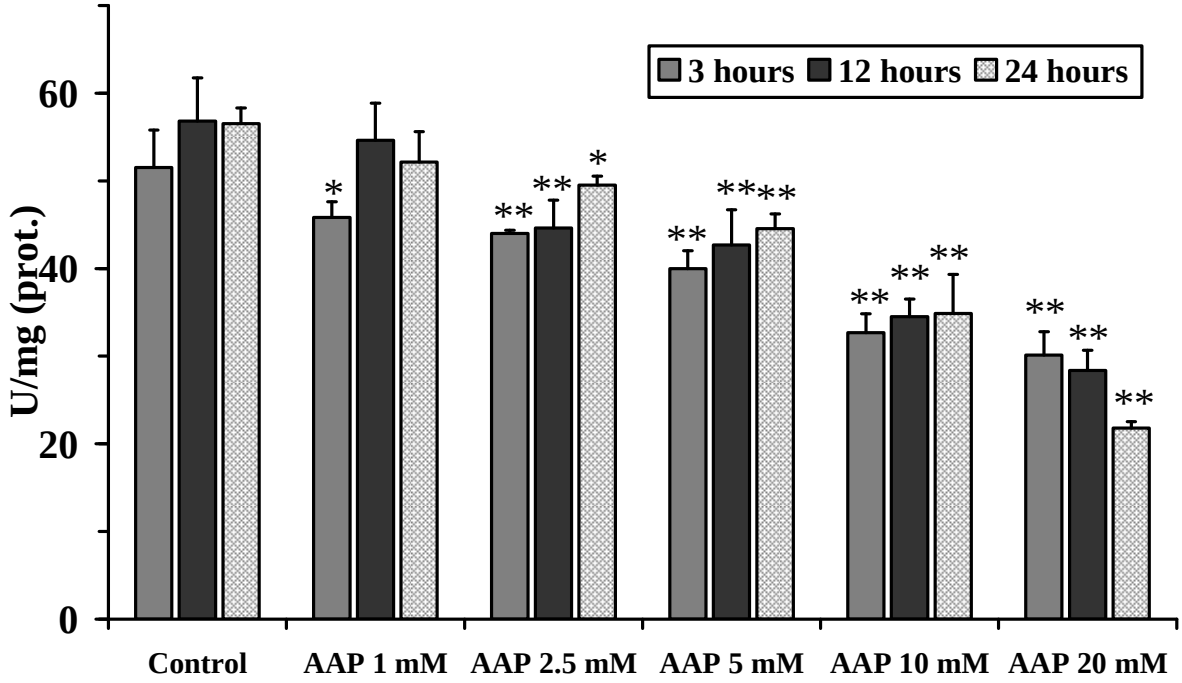


**Fig. 4.** Activity of glutathione reductase (time course – 3, 12 and 24 h). AAP treated cells (1, 2.5, 5, 10 and 20 mM) were sonicated and then GR activity was assayed in cell lysates. The activity of glutathione reductase was measured as decrease of NADPH absorbance at $\lambda = 340$ nm and expressed as Unit of enzyme activity per mg of protein (1 U was defined as nmol of GSSG reduced per minute, using a molar extinction coefficient of $6.22 \times 10^3$ for NADPH). The results are expressed as mean ± SD (n = 4). * , $p < 0.05$; ** , $p < 0.01$ (compared to control at appropriate time period).