DOI: 10.2478/10004-1254-65-2014-2434

Original article

Cytoprotective effects of silafibrate, a newly-synthesised siliconated derivative of clofibrate, against acetaminophen-induced toxicity in isolated rat hepatocytes

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Received in August 2013
CrossChecked in August 2013
Accepted in January 2014

Acetaminophen (N-acetyl para amino phenol, APAP) is a widely used antipyretic and analgesic drug responsible for various drug-induced liver injuries. This study evaluated APAP-induced toxicity in isolated rat hepatocytes alongside the protective effects of silafibrate and N-acetyl cysteine (NAC). Hepatocytes were isolated from male Sprague-Dawley rats by collagenase enzyme perfusion via the portal vein. This technique is based on liver perfusion with collagenase after removing calcium ions (Ca^{2+}) with a chelator. Cells were treated with different concentrations of APAP, silafibrate, and NAC. Cell death, reactive oxygen species (ROS) formation, lipid peroxidation, and mitochondrial depolarisation were measured as toxicity markers. ROS formation and lipid peroxidation occurred after APAP administration to rat hepatocytes. APAP caused mitochondrial depolarisation in isolated cells**.** Administration of silafibrate (200 μmol L^{-1}) and/or NAC (200 μmol L^{-1}) reduced the ROS formation, lipid peroxidation, and mitochondrial depolarisation caused by APAP**.** Cytotoxicity induced by APAP in rat hepatocytes was mediated by oxidative stress. In addition, APAP seemed to target cellular mitochondria during hepatocyte damage. The protective properties of silafibrate and/or NAC against APAP-induced hepatic injury may have involved the induction of antioxidant enzymes, protection against oxidative stress and inflammatory responses, and alteration in cellular glutathione content.

KEY WORDS: *drug-induced liver injury (DILI); fibrates; mitochondria; oxidative stress; reactive oxygen species (ROS)*

Acetaminophen (N-acetyl para amino phenol, APAP) is a widely used analgesic and antipyretic drug, which if applied excessively can cause severe hepatic damage or even death in experimental animals and individuals (1). At recommended doses, APAP is mainly metabolised by sulphation and glucuronidation (2). A small proportion of APAP is metabolised through cytochrome CYP2E1 and, to a lesser extent, CYP1A2 and CYP3A4, which produce the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI). This reactive intermediate is efficiently detoxified by conjugation with glutathione (3). However, in overdoses, a large amount of APAP is metabolised through the P450 family of cytochromes leading to GSH depletion by NAPQI conjugation followed by the covalent binding of NAPQI to proteins (4). Cellular mitochondria seem to be the target for acetaminophen-induced hepatotoxicity (5).

Although the precise mechanism of APAP hepatotoxicity is not fully understood, some studies

(5, 6) have suggested that NAPQI exerts a cytotoxic effect through its covalent binding to cytosolic or microsomal proteins and membrane components, inducing oxidative stress in cells, inhibition of mitochondrial respiration, and depletion of cellular ATP. Research on acetaminophen-induced hepatotoxicity and finding new approaches to prevent it is a dynamic field of research due to high incidence of hepatotoxicity induced by this drug accidentally or in suicidal attempts (6,7).

Silafibrate (ethyl 2-(4-(trimethylsilyl) phenoxy)-2-methylpropanoate) (Figure 1) is a siliconated derivative of the drug clofibrate (8). Fibrates belong to the peroxisome proliferator-activated receptor α (PPAR-α) drug group, which has been reported to have anti-inflammatory effects (9). Furthermore, PPAR ligands' antioxidant and oxidative stress modulating properties have been shown in previous studies (10,11). It has been reported that the trimethylsilyl group in silafibrate (Figure 1) increases the anti-inflammatory properties of clofibrate and enhances its pharmacological effect on PPAR receptors (8). It has also been shown that PPAR receptors have a crucial role in preventing APAP-induced hepatic damage (12). The role of silafibrate, as a new and more potent analogue of clofibrate (8) against APAP-induced hepatotoxicity in isolated rat hepatocytes was evaluated in this study. The protective role of N-acetylcysteine (NAC) as a gold standard treatment for acetaminophen-induced hepatotoxicity (13), was also studied and compared with silafibrate in this investigation.

Cell death, oxidative stress induction (ROS formation), lipid peroxidation, and mitochondrial injury were assessed as toxicity markers induced by acetaminophen (5, 6).

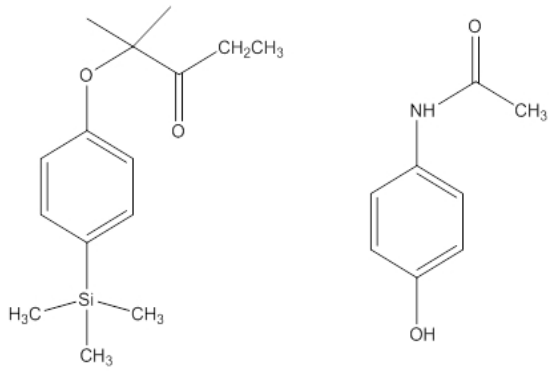

Figure 1 *Chemical structure of silafibrate (left) and acetaminophen (right)*

MATERIAL AND METHODS

Chemicals

Acetaminophen was purchased from Medisca Pharmaceutique Incorporation (Montreal, Canada). N-acetyl cysteine (NAC) and (4-(2-hydroxyethyl) 1-piperazine-ethanessulfonic acid (HEPES) were obtained from Acros (New Jersey, USA). Albumine bovine type was purchased from Roche Diagnostics Corporation (Indianapolis USA). Rhodamine 123 and Collagenase from *Clostridium histolyticum* were obtained from Sigma Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), ethyleneglycol-bis (ρ-aminoethylether)-N,N,N′,N′-tetra acetic acid (EGTA), and Trypan Blue were obtained from Merck (Darmstadt, Germany). A siliconised analogue of clofibrate, silafibrate, was synthesised in the Chemistry and Chemical Engineering Research Center, Tehran, Iran. Thiobarbituric acid (TBA) was obtained from SERVA (Heidenberg, New York). All salts used for preparing buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

Hepatocyte preparation

Male Sprague-Dawley rats (three animals for each test, N=15) weighing 250–300 g were housed in plastic cages at ambient temperature (25±3 °C). Animals had free access to food and water. Collagenase perfusion method was used to isolate rat hepatocytes (14). This technique is based on liver perfusion with collagenase after the removal of calcium ion (Ca^{2+}) with a chelator (EGTA 0.5 mol L^{-1}). The liver was perfused with different buffer solutions through the portal vein. Collagenase-containing buffer solution destructed liver interstitial tissue and caused hepatocytes to be easily isolated. Isolated hepatocytes (10 mL, 10^6 cells mL^{-1}) were incubated in the Krebs-Henseleit buffer (pH 7.4) under an atmosphere of 95 % O_2 and 5 % CO_2, in 50 mL round bottom flasks which were continuously rotating in a water bath at 37 °C. For more precise details on isolated rat hepatocytes preparation, readers are referred to Moldéus et al (15). As CYP2E1, the acetaminophen-metabolising enzyme expression is low in rat liver (16), we used the enzyme induction method by administering β-naphtoflavone (40 mg kg^{-1}, *i.p.*, for three consecutive days) (17) to accelerate acetaminophen-induced toxicity in rat hepatocytes. After this, hepatocytes were isolated and used. All animals received humane care and were used

according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (18), which was approved by the local ethic committee in Tabriz University of Medical Sciences, Tabriz, Iran.

Different concentrations of acetaminophen were added to the cellular media to find the dose response of the drug in rat hepatocytes and the LC_{50} concentration (the concentration which leads to 50 % cell death after 120 min of incubation) for the next experiments.

Cell viability

Trypan blue dye exclusion staining was used to assess the percentage of dead cells (19). Hepatocyte viability was determined at different time intervals to evaluate the effect of acetaminophen on cell viability. LC_{50} dose of the drug was determined and the protective effects of silafibrate and NAC against cell death induced by acetaminophen were tested. Hepatocytes were at least 85 % viable before their use, as recommended in previous studies (20-27).

Reactive oxygen species (ROS) formation

To determine the extent of ROS generated during acetaminophen metabolism, 2,7-dichlorofluorescein diacetate (DCFH-DA; 1.6 μmol L^{-1}) was added to the hepatocyte incubate. DCFH-DA was first hydrolysed to non-fluorescent DCFH in hepatocytes. DCFH then reacted with ROS to form the highly fluorescent DCFH. 1 mL (approximately 10^6 cells) of hepatocyte suspension was taken and the fluorescence intensity was measured using a Jasco® FP-750 spectrofluorometer (Jasco Corporation, Tokyo, Japan) with excitation and emission wavelengths of 500 nm and 520 nm, respectively (28).

Lipid peroxidation measurement

Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides. After treating 1 mL aliquots of hepatocyte suspension (10^6 cells mL^{-1}) with trichloroacetic acid (70 % w/v) and boiling the supernatant with thiobarbituric acid (0.8 % w/v) for 20 min, the absorbance of the observed colour was determined using an Ultrospec® 2000 UV spectrophotometer at 532 nm (Pharmacia Biotech Cambridge, England) (29).

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed as an indicator of toxicity induced by methimazole or N-methylthiourea. The fluorescent dye, rhodamine 123, was used as a probe to evaluate the mitochondrial membrane potential in rat hepatocytes. Samples (1 mL) were taken from the cell suspension at scheduled time points, and centrifuged at 1000 *g* for 1 min. The cell pellet was then resuspended in 2 mL of fresh incubation medium containing 1.5 μmol L^{-1} rhodamine 123 and gently shaken in a thermostatic water bath at 37 °C for 10 min. Hepatocytes were separated by centrifugation (402 *g* for one min) and the amount of rhodamine 123 appearing in the incubation medium was measured fluorimeterically at 490 nm excitation and 520 nm emission wavelengths using a Jasco® FP-750 spectrofluorometer (30).

Statistical analysis

Results are given as mean±SE for at least three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test. $P<0.05$ was considered as significant difference.

RESULTS

In isolated rat hepatocytes, acetaminophen hardly caused cytotoxicity and very high concentrations of the drug were needed to induce toxicity (Figure 2). Hence, to accelerate the acetaminophen-induced toxicity, we used the enzyme-induced hepatocyte model. The LC_{50} of acetaminophen was found to be 750 μmol L^{-1} (Figure 2). Different compounds (APAP in combination with proposed protective agents such as silafibrate and/or NAC) were added to the incubation medium, aimed to determine their ability to modulate the toxic response of acetaminophen. Silafibrate and/or NAC caused no significant toxicity in hepatocytes as compared to the control cells when administered alone at given concentrations.

Administration of NAC (200 μmol L^{-1}) or silafibrate (200 μmol L^{-1}) effectively reduced cell death (Figure 3), a significant amount of formed ROS (Figure 4), and lipid peroxidation (Figure 5) caused by acetaminophen. Acetaminophen also caused mitochondrial depolarisation in rat hepatocytes (Figure 6) and NAC or silafibrate proved to have a preventive role in this regard (Figure 6).

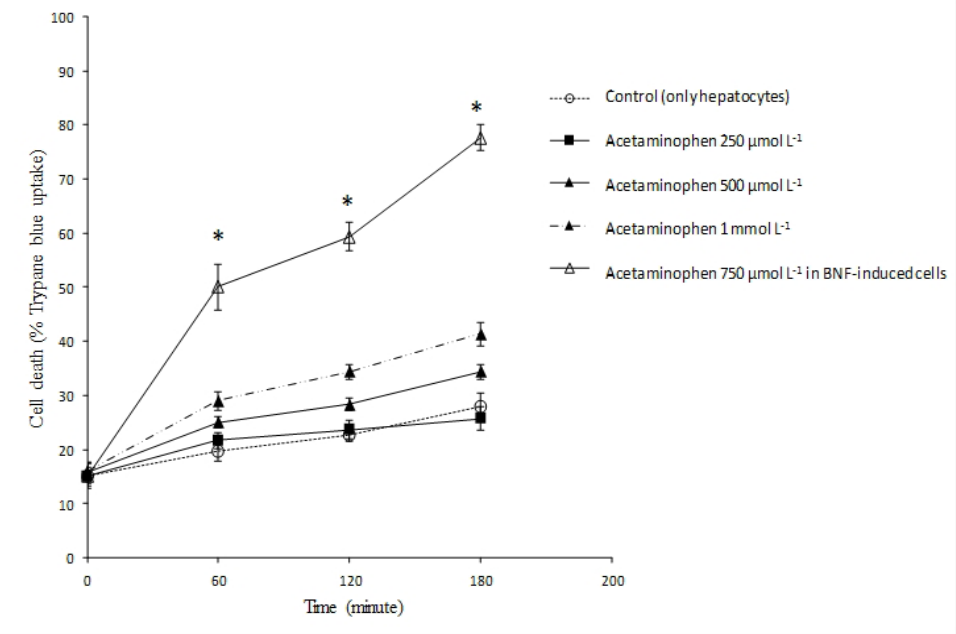

Figure 2 *Dose-response of acetaminophen-induced cytotoxicity in isolated rat hepatocytes.*
BNF: β-naphtoflavone
** Indicates the significantly higher cell death than that of control group* ($p<0.05$).
Hepatocytes cell death was not significant in the control group at different time intervals as assessed by trypan blue exclusion test.

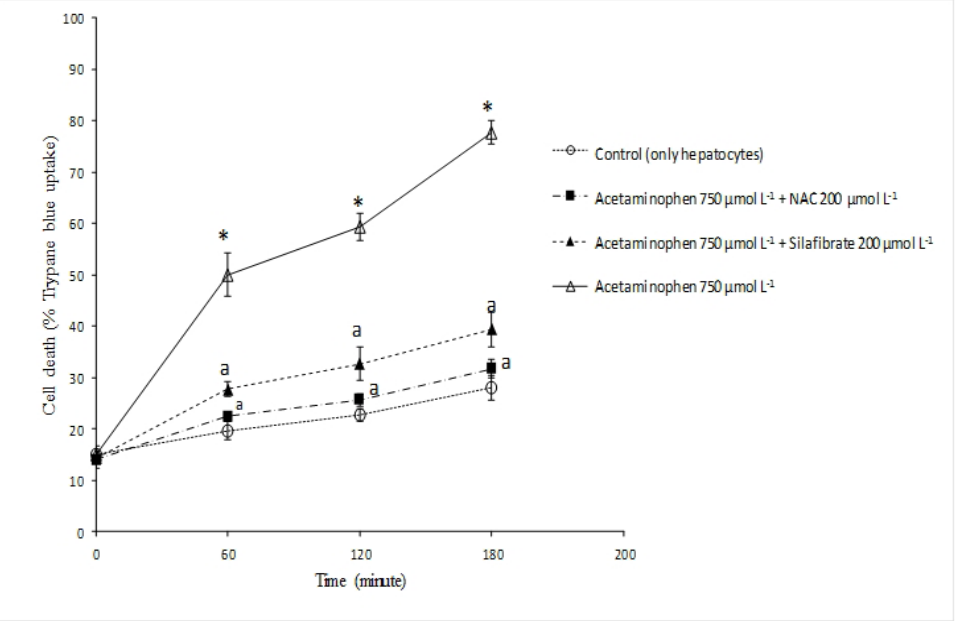

Figure 3 *The protective effect of N-acetyl cysteine (NAC) and silafibrate against acetaminophen-induced cytotoxicity in rat hepatocytes.*
** Indicates significantly higher cytotoxicity as compared to the control group ($p<0.05$).*
[a] Indicates significantly lower cytotoxicity as compared to the acetaminophen-treated group ($p<0.05$)

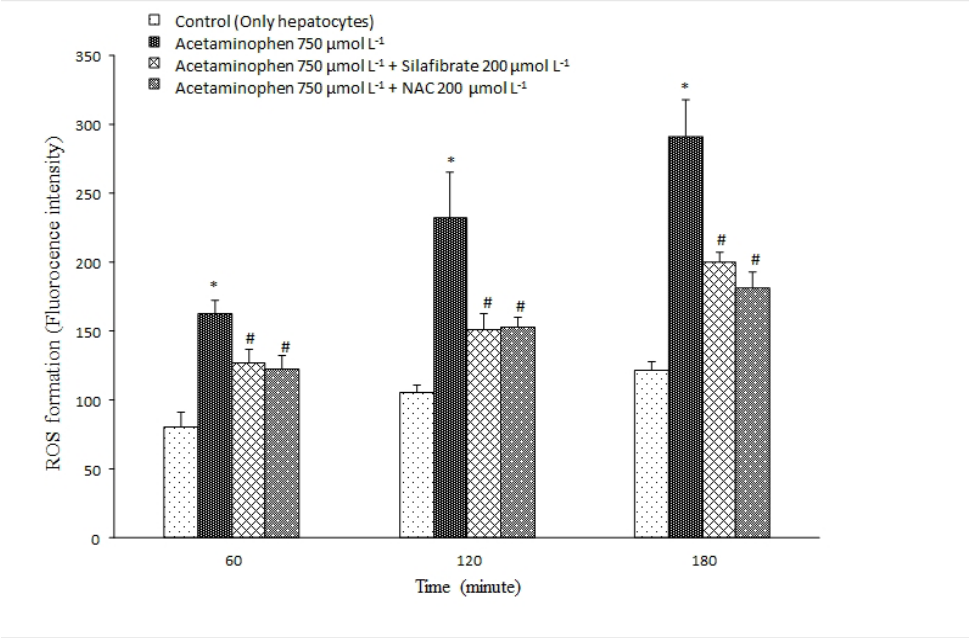

Figure 4 *Acetaminophen-induced ROS formation in isolated rat hepatocytes and the effect of NAC and silafibrate administration.*
** Significantly higher ROS level than control group (*p<*0.05).*
*# Significantly lower ROS level than acetaminophen-treated hepatocytes (*p<*0.05)*

DISCUSSION

Drug-induced liver disease is the most common cause of acute liver failure, and acetaminophen accounts for the bulk of these (6). Incubation of enzyme-induced rat hepatocytes with acetaminophen caused cell death concentration-dependently. The LC_{50} for acetaminophen was 750 µmol L^{-1}. Acetaminophen caused ROS formation, lipid peroxidation, and mitochondrial depolarisation in isolated rat hepatocytes. Administration of NAC and/or silafibrate diminished the toxic effects of acetaminophen in rat hepatocytes.

In previous studies, it has been shown that administration of PPAR ligands such as clofibrate diminished acetaminophen-induced hepatotoxicity in *in vivo* models (31). In addition, it has been found that PPARs null mice (PPARs -/-) were more susceptible to hepatotoxicity induced by acetaminophen (32). Furthermore, the protective effects of PPAR ligands in *in vitro* experiments have been shown (33).

However, the mechanism(s) underlying the hepatoprotection afforded by peroxisome proliferators have yet to be clarified, but the induction of antioxidant enzymes (34), alteration in cellular glutathione content (35), and protection against oxidative stress and inflammatory responses (36-38) are the proposed protective mechanisms. It has been found that PPAR ligands have a role in modulating oxidative stress and its deleterious consequences in different tissues such as liver (39), nervous (40), and vascular systems (41). These mechanisms could be involved in the protective properties of silafibrate against APAP-induced cytotoxicity in freshly-isolated rat hepatocytes. Currently there is no data available on the direct effect of silafibrate on ROS in biological systems. However, the chemical structure of this drug and its direct effects on reactive species might also be attributed to its protective properties in isolated rat hepatocytes. This characteristic might arise from high electron-donating nature of trimethylsilyl (TMS) group in the drug.

NAC is a standard clinical treatment against APAP-induced hepatotoxicity (42). It provides protection mainly by counteracting oxidative stress (43), and scavenging APAP reactive metabolite (44). Moreover, it replenishes hepatic glutathione reservoirs as a crucial defence barrier against xenobiotics (44).

We found that acetaminophen caused ROS formation in isolated rat hepatocytes (Figure 4). Since PPARα ligands showed anti-oxidative stress properties in previous studies (36, 37, 45), one of the mechanisms by which the drug silafibrate may protect rat hepatocytes against acetaminophen might be its effect

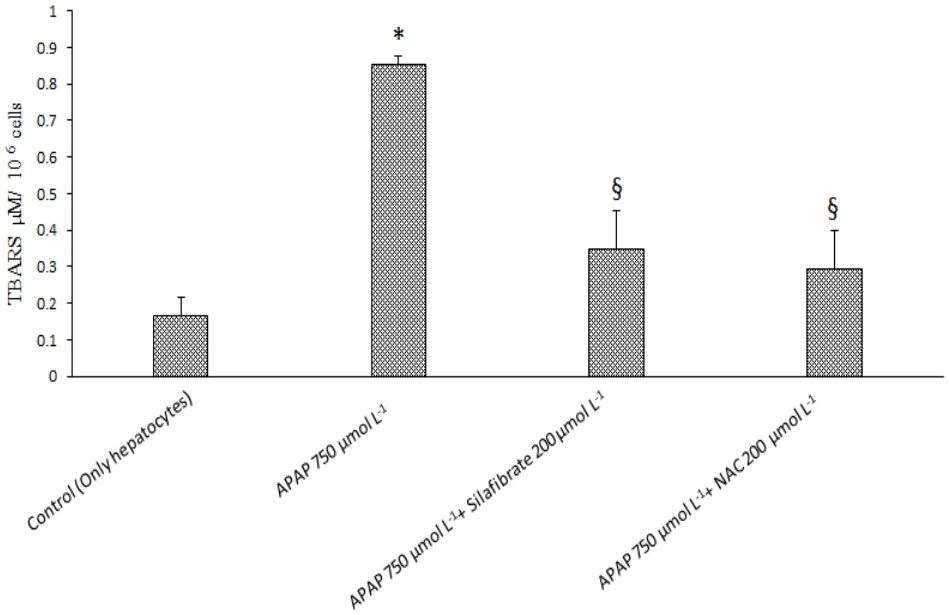

Figure 5 *Lipid peroxidation caused by acetaminophen (APAP) in isolated rat hepatocytes and the protective role of N-acetyl cisteine (NAC) and silafibrate.*
**Significantly higher TBARS level than control group (*p*<0.05).*
*§ Significantly lower TBARS level than acetaminophen-treated group (*p*<0.05)*

on alleviating oxidative stress (Figure 4). The effect of silafibrate on lipid peroxidation induced by acetaminophen could be attributed to its effect on reducing the ROS level, which is a major cause of lipid peroxidation in cells (46).

It has been shown that oxidative stress is one of the major causes of mitochondrial damage (47). A part of silafibrate protection against acetaminophen-induced cytotoxicity may be due to its effect in preventing mitochondrial injury caused by this drug. In the current investigation we found that silafibrate as a new and more potent analogue of clofibrate (8) showed protective effects against APAP-induced cytotoxicity. These results might provide new therapeutic strategies against APAP-induced hepatotoxicity.

CONCLUSION

Silafibrate as a newly synthesised analogue of clofibrate protected rat hepatocytes against acetaminophen-induced toxicity. The protective effects of silafibrate could be attributed to its role in counteracting oxidative stress and/or its consequences such as lipid peroxidation and mitochondrial damage. The induction of antioxidant enzymes (34), alteration in cellular glutathione content (35), and protection against oxidative stress and inflammatory responses (36-38) might be involved in the cytoprotective properties of silafibrate. On the other hand, the duration of study in this in vitro model of isolated rat hepatocytes might not be sufficient to observe some silafibrate protective properties, such as induction of antioxidant enzymes. Future *in vivo* investigations will provide more insights on silafibrate hepatoprotective properties.

Acknowledgements

This study was carried out at Biotechnology and Drug Applied Research Centres (DARC) of Tabriz University of Medical Sciences. The authors thank Biotechnology Research centre and Drug Applied Research Center for providing financial supports and facilities to carry out this investigation. This paper was a part of the Pharm.D. thesis for Sara Nafisi and was supported technically by the students' research committee of Tabriz University of Medical Sciences, Tabriz, Iran. The authors thank students' research committee for their support.

Conflict of interest

The authors declare no conflict of interest.

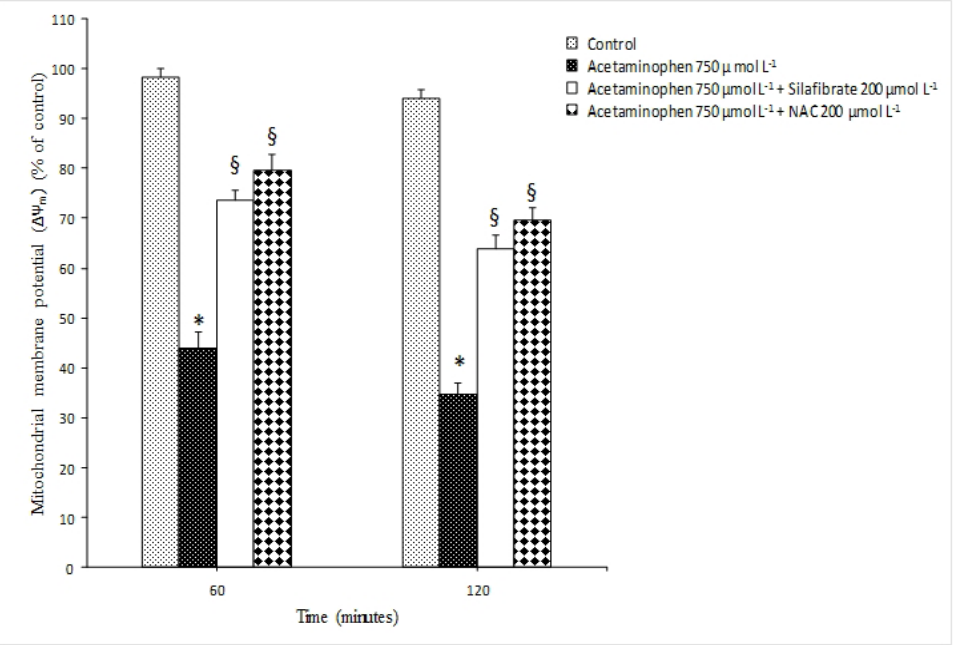

Figure 6 *Acetaminophen-induced mitochondrial depolarisation. NAC and silafibrate administration effectively prevented mitochondrial injury caused by acetaminophen.*
* *Indicates significantly lower membrane potential as compared to the control group (*p*<0.05).*
§ *Indicates a significantly higher membrane potential as compared to the acetaminophen-treated group (*p*<0.05)*

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Sažetak

Citoprotektivni učinci silafibrata, novosintetiziranog silikoniranog derivata klofibrata protiv acetaminofenom izazvane toksičnosti u izoliranim hepatocitima štakora

Acetaminofen (N-acetil-para-aminofenol, APAP) često je korišteni antipiretik i analgetik koji može izazvati oštećenja jetara. Na modelu izoliranih hepatocita štakora istražili smo toksične učinke APAP-a i protektivne učinke silafibrata i N-acetilcisteina (NAC). Hepatociti su izolirani iz mužjaka štakora soja Sprague-Dawley perfuzijom jetara i uvođenjem enzima kolagenaze putem portalne vene. Ta se tehnika zasniva na perfuziji jetara kolagenazom nakon uklanjanja kalcijevih iona (Ca^{2+}) kelatorom. Stanice su tretirane različitim koncentracijama APAP-a, silafibrata i NAC-a. Kao markeri toksičnosti mjereni su smrt stanica, stvaranje reaktivnih kisikovih vrsta (ROS), lipidna peroksidacija i depolarizacija mitohondrija. Primjena APAP-a u štakora izazvala je stvaranje ROS-ova i lipidnu peroksidaciju. APAP je uzrokovao depolarizaciju mitohondrija u izoliranim stanicama. Primjena silafibrata (200 μmol L^{-1}) i/ili NAC-a (200 μmol L^{-1}) smanjila je stvaranje ROS-a, lipidnu peroksidaciju i depolarizaciju mitohondrija uzrokovanu APAP-om. Utvrdili smo da je citotoksičnost APAP-a posredovana oksidativnim stresom. Nadalje, čini se da su mitohondriji ciljni stanični organeli za oštećenja hepatocita izazvanih APAP-om. Moguće je da su protektivna svojstva silafibrata i/ili NAC-a protiv APAP-om induciranog oštećenja jetara uključivala i indukciju antioksidacijskih enzima, zaštitu od oksidativnog stresa i upalnih odgovora te promjenu razine staničnoga glutationa.

KLJUČNE RIJEČI: *fibrati; mitohondriji; oksidativni stres; oštećenje jetara izazvano lijekom; reaktivne kisikove vrste (ROS)*

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