The Cytoprotective Effects of *Allium cepa* Methanolic Extract in Freshly Isolated Hepatocytes

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Abstract

Acetaminophen (acetyl-para-amino phenol; APAP)-induced hepatotoxicity is the most common form of drug-induced liver injury (DILI) worldwide. APAP is also used as a model drug to assess hepatoprotective strategies against DILI. In the current study, the potential cytoprotective effects of *Allium cepa* (Onion) extract (OE) was investigated in APAP-treated hepatocytes. Isolated hepatocytes were prepared with the collagenase perfusion of rat liver. Isolated hepatocytes (10 mL, 106 cells/mL) were incubated in the Krebs Henseleit buffer (pH=7.4) in continuously rotating 50 mL round bottom flasks, under an atmosphere of carbogen (95% O2 and 5% CO2) in a 37 °C water bath. Cytotoxicity, ROS formation, and mitochondrial membrane potential collapse were assessed as oxidative stress markers. APAP administration to rat hepatocytes (500 µM) was accompanied by cytotoxicity, ROS formation, depletion of cellular glutathione (GSH) reservoirs, and mitochondrial depolarization. It was found that OE administration (100 µL) significantly reduced cell death, ROS formation, and its consequences, such as the decrease in cellular GSH and mitochondrial injury induced by APAP. These results indicate that the crude extract of Allium cepa exhibits hepatoprotective action, probably through antioxidative properties and protecting vital cellular organelles such as mitochondria.

Keywords: *Allium cepa*, Cytotoxicity, Hepatocytes, Mitochondrial Membrane Potential, Onion Extract.

1. Introduction

*Allium cepa* (Onion) is a bulbous plant widely cultivated worldwide. Onion is rich in proteins, carbohydrates, sodium, potassium, and phosphorus (1). Antibacterial, antiviral, anti-parasitic, antifungal effects have been attributed to onion (2, 3). The antihypertensive, hypoglycemic, antithrombotic, antihyperlipidemic, anti-inflammatory, and antioxidant activity of onion and its derived chemicals also has been repeatedly investigated (4-6). Organosulfur chemicals are found in the Alliaceae family (e.g., *Allium cepa*) in large quantities (7). It has been shown that organosulfurs had protective effects against xenobiotics-induced cellular damage and oxidative stress in many cases, such as carbon tetrachloride (CCL\(_4\)) (8), cyclophosphamide (9), and aflatoxin B1 (10). N-acetylcysteine (NAC), is also an organosulfur compound that is clinically administered as a stan-
standard treatment against acetaminophen hepatotoxicity.

Acetaminophen (Acetyl-para-aminophenol; APAP) is the most frequent cause of drug-induced liver injury due to unintentional or deliberate overdose (11). APAP is also widely used as a model drug to investigate the mechanisms of drug-induced cytotoxicity and finding therapeutic strategies against drug-induced liver injury (DILI) (12-14). APAP is metabolized through cytochrome p-450 (CYP2E1) to produce the reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which covalently binds to critical intracellular targets and consequently causes cellular injury and death (15). In the current study, APAP-induced toxicity toward isolated hepatocyte was used as an in vitro model to evaluate the potential cytoprotective effects of OE. The results could help the development of therapeutic strategies against xenobiotics-induced liver injury.

2. Material and method
2.1. Chemicals
N-acetyl cysteine (NAC), (4-(2-hydroxyethyl)1-piperazine-ethane sulfonic acid (HEPES), trichloroacetic acid (TCA), ethylene glycol-bis (p aminoethyl ether)-N, N’, N’ tetra acetic acid (EGTA), thiobarbituric acid (TBA), and trypan blue were obtained from Merck (Darmstadt, Germany). Albumin bovine type was purchased from Roche diagnostic corporation (Indianapolis USA). Rhodamine 123, acetaminophen, and collagenase enzyme from clostridium histolyticum were obtained from Sigma Aldrich (St. Louis, USA).

2.2. Hepatocytes isolation procedure
Male Sprague–Dawley rats (250-300 g) were prepared from Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. Animals were housed in plastic cages on a wood-chip bedding. The ambient temperature of 23±1 °C. Rats had free access to a normal rodents’ diet (Behparvar®, Tehran, Iran) and tap water. A local ethics committee in Tabriz University of medical sciences, Tabriz, Iran, approved the animal procedures. Collagenase perfusion via portal vein was used as a method to isolate rat hepatocytes (16). This method is based on liver perfusion with collagenase after the removal of calcium ion (Ca2+) with a chelator (EGTA 0.5 mM). The liver was perfused with several buffer solutions throughout the portal vein (17, 18). The isolated hepatocytes (10 mL, 10 6 cells/mL) were incubated in Krebs Henseleit buffer (pH=7.4) under carbogen atmosphere (95% O2 and 5% CO2), in 50 mL round bottom flasks which constantly rotating into at 37 °C in a water bath (18-20). The expression of CYP2E1, the enzyme responsible for acetaminophen-metabolization, is low at in rat liver (21). Therefore, to accelerate acetaminophen-induced toxicity in rat hepatocytes, the CYP2E1 enzyme was induced using β-naphtoflavone (22). Briefly, CYP induced hepatocytes were prepared by pre-treating rats by β naphtoflavone (40 mg/kg, i.p, for three consecutive days) (22), then hepatocytes were isolated and used. The protective role of N-acetylcysteine as a gold-standard treatment for acetaminophen-induced hepatotoxicity (23), was studied and compared with OE supplementation.

2.3. Cell viability
Trypan blue exclusion test was used to evaluate cell death in isolated hepatocytes (24, 25). Hepatocyte viability was determined at scheduled time intervals. Briefly, 100 µL of trypan blue (0.1% w: v) was added to 1 mL of isolated hepatocytes (10 6 cells/mL). The percentage of dead cells (Blue nucleus) was determined using a light microscope (24, 26). Hepatocytes were at least 80% viable before their use.

2.4. Reactive oxygen species (ROS) formation
The fluorescent probe 2, 7 dichlorofluorescein diacetate (DCFH-DA) was used to assess ROS formation in isolated rat hepatocytes (27). Briefly, 10 µL of DCFDH-DA (Final concentration of 10 µM) was added to 1 mL of isolated hepatocytes (106 cells/mL). The percentage of dead cells (Blue nucleus) was determined using a light microscope (24, 26). Hepatocytes were at least 80% viable before their use.

2.5. Lipid peroxidation Measurement
Hepatocyte lipid peroxidation was deter-
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mined by evaluating the amount of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides. Briefly, 1mL aliquots of hepatocyte suspension (10^6 cells/mL) was treated with 250 µL of trichloroacetic acid (70% w/v) and 250 µL thiobarbituric acid (0.8% w/v) (29). The mixture was heated (100 ºC, 20 min). Finally, samples were centrifuged (10000 g, 15 min), and the absorbance of appeared color was determined at λ=532 nm (30, 31).

### 2.6. Mitochondrial membrane potential

Rhodamine 123 was used to estimate the mitochondrial depolarization (32-36). Samples (1 mL) were taken from the cell suspension at arranged time points and centrifuged (1000 rpm, 1 min). The pellet was then resuspended in 2 mL of new incubation containing 1.5 µM rhodamine 123 and gently mixed. Samples were incubated (10 min) at 37 ºC. Then, hepatocytes were separated by centrifugation (3000 g, 1 min, 4 ºC), and the amount of rhodamine 123 appearing in the supernatant was measured fluorometrically at λ=490 nm excitation and λ=520 nm emission wavelengths (37-39).

### 2.7. Onion extract preparation

Raw onion (*Allium cepa* L.) was purchased from a retail food store (Tabriz, Iran) and identified by botanists in the herbarium of Tabriz University. Onion bulbs were peeled, weighed, and ground to obtain the juice. Samples were homogenized in methanol (10 mL methanol: 1 g Onion), and the homogenized mixture was filtered. A rotary evaporator was used to remove the methanol content. Afterward, samples were freeze-dried and stored at -70 ºC until use. On the day of the experiment, 100 mg of OE was dissolved in 10 ml of the incubation buffer, and 100 µL of the prepared solution was added to isolated hepatocytes. The organosulfur content of OE was characterized by a GC-MS method (Figure 1).

### 2.8. Statistical analysis

Results are given as the mean±SEM for at least three independent experiments. A one-way analysis of variance (ANOVA; followed by Tukey’s post hoc test) was used to compare the obtained data. A P<0.05 was considered a statistically significant difference.

### 3. Results

APAP-induced cytotoxicity was evaluated using the trypan blue exclusion test (Table 1). It was found that APAP (500µM) significantly caused cell death at different time intervals (Table 1). On the other hand, OE administration (100 µL) significantly decreased APA-induced cell death.

![GC-MS spectroscopic analysis of main organosulfur compounds in the onion extract used in the current study.](image1)

**Figure 1.** GC-MS spectroscopic analysis of main organosulfur compounds in the onion extract used in the current study.
Table 1. Preventing Acetaminophen (APAP)-induced cell death by different concentrations of onion extract (OE).

<table>
<thead>
<tr>
<th>Incubation time (min):</th>
<th>Cytotoxicity (% Trypan blue uptake)</th>
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<tbody>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Control (only hepatocytes)</td>
<td>19±1</td>
</tr>
<tr>
<td>+OE 100µL</td>
<td>22±2</td>
</tr>
<tr>
<td>+APAP 500 µM</td>
<td>52±4*</td>
</tr>
<tr>
<td>+OE 100 µL</td>
<td>22±3a</td>
</tr>
<tr>
<td>+NAC 100 µM</td>
<td>19±2a</td>
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</tbody>
</table>

Isolated rat hepatocytes (106 cells/mL) were incubated at 37 °C in continuously rotating round bottom flasks under carbogen (95 % O₂ and 5 % CO₂) atmosphere in Krebs-Henseleit buffer (pH=7.4). NAC: N-acetyl cysteine. The results shown represent the mean± SE for three independent experiments. *Significantly different from the control group (P<0.05). aSignificantly different from the APAP-treated group (P<0.05).

Table 1). NAC (100 µM) treatment also significantly decreased APAP cytotoxicity in isolated rat hepatocytes (Table 1). There was no significant difference between the cytoprotective effects of OE and NAC, as judged by the trypan blue exclusion test (Table 1).

APAP administration (500µM) also caused significant ROS formation in isolated rat hepatocytes. OE treatment alleviated APAP-induced ROS formation (Figure 2). The effects of NAC on APAP-induced ROS formation (Figure 2). On the other hand, there was no significant difference between OE and NAC in preventing ROS formation in APAP-treated hepatocytes (Figure 2).

The TBARS level as an index of lipid peroxidation was significantly higher in APAP-treated rat hepatocytes (Figure 3). It was found that OE and/or NAC treatment significantly mitigated the level of lipid peroxidation in the APAP-treated group (Figure 4). No significant difference between hepatocytes TBARS levels was found between NAC and OE-treated groups (Figure 4).

Figure 2. Acetaminophen (APAP)-induced reactive oxygen species (ROS) formation and the role of onion extract (OE) administration. NAC: N-acetyl cysteine. Data are given as mean±SEM for three independent experiments. *Indicates a significant difference as compared with the control hepatocytes (P<0.05). aIndicates significant difference as compared with the acetaminophen-treated cells (P<0.05).
It was found that the cellular glutathione reservoir (GSH) was decreased after APAP administration (Figure 4). OE significantly prevented cellular GSH depletion induced by APAP (Figure 4). NAC treatment also significantly preserved GSH levels in APAP-treated hepatocytes (Figure 4). There was no significant difference between NAC and OE when their effects on hepatocytes GSH content were compared (Figure 4).

The effect of APAP on cellular mitochondria and the role of OE administration was studied. It was found that APAP caused mitochondrial membrane potential (ΔΨm) collapse (Figure 5). APAP-induced mitochondrial depolarization was significantly alleviated by OE (100 µL) and/or NAC (100 µM) administration (Figure 5). No significant difference was found between the effects of NAC and OE on APAP-induced mitochondrial depolarization in isolated rat hepatocytes (Figure 5).

**Figure 3.** Acetaminophen (APAP)-induced lipid peroxidation in isolated rat hepatocytes. OE: Onion extract, NAC: N-acetyl cysteine. Data are represented as mean±SEM for three independent experiments. *Indicates a significant difference as compared with control hepatocytes (P<0.05). aIndicates significant difference as compared with acetylsalicylic-treated cells (P<0.05).

**Figure 4.** Cellular glutathione levels in acetaminophen (APAP)-treated hepatocytes. OE: Onion Extract, NAC: N-acetyl cysteine. Data are represented as Mean±SEM for at least three separate experiments. *Indicates a significant difference as compared with control hepatocytes (P<0.05). aIndicates significant difference as compared with acetaminophen-treated cells (P<0.05).
4. Discussion

Hepatocytes are continuously exposed to a wide range of xenobiotics. Several chemicals with hepatotoxic properties have been identified (40-42). Therefore, finding compounds with hepatoprotective properties has great clinical value. In the current study, it was found that the OE administration tended to suppress APAP-induced cytotoxicity as judged by lower cell death, ROS formation, increased GSH content, and improved mitochondrial function.

Different extracts from alliacea family have been shown to exhibit a wide range of biological activities, including potent antioxidant properties (5, 43). Most of the researches performed on OE is to determining the bioactive constituents of this extract (43). Many organosulfur compounds are derived from OE in different previous investigations (44, 45). These active ingredients exhibit antioxidative and protective properties in different studies (44, 45) (Figure 1). Oxidative stress and its consequences are the most critical cause of xenobiotics-induced hepatotoxicity, including different drugs (46). Oxidative stress affects a vast range of intracellular targets, including lipids, proteins, cellular mitochondria, and DNA (47). In the current study, APAP administration was accompanied by a significant ROS formation and lipid peroxidation. OE decreased the ROS formation and lipid peroxidation induced by acetaminophen (Figures 2 and 3). The role of onion extract in attenuating oxidative stress might be due to the role of organosulfur compounds in chelating metal ions (45) and/or scavenging reactive species (48). Decreased cellular GSH content (Figure 3) endorses the occurrence of oxidative stress in rat hepatocytes. As shown in Figure 4, the OE administration prevented GSH consumption by APAP. Administration of NAC (100 µM) also increased hepatocytes GSH reservoirs (Figure 4). Based on these data, the antioxidant properties of OE could play a pivotal role in its cytoprotective mechanism.

It has been repeatedly mentioned that APAP could induce mitochondrial injury and respiratory chain dysfunction (49). Mitochondria have a significant role in maintaining cellular homeostasis and play an essential role in xenobiotics-induced cell death, especially in oxidative stress conditions (50). On the other hand, cellular mitochondria are significant sources of ROS (51). Hence, xenobiotics-induced mitochondrial impairment could play a significant role in elevated ROS levels and oxidative stress. In this study, it was found that the mitochondrial membrane potential (ΔΨm), as a critical indicator of mitochondrial function, was collapsed in APAP-treated hepatocytes.

Figure 5. Effect of acetaminophen (APAP) on hepatocytes, mitochondrial depolarization, and the role of onion extract (OE) administration. NAC: N-acetyl cysteine. Data are represented as mean±SEM for at least three separate experiments. *Indicates a significant difference as compared with control hepatocytes (P<0.05). a Indicates significant difference as compared with acetaminophen-treated cells (P<0.05).
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cytes (Figure 5). As onion extract administration diminished mitochondrial injury caused by APAP (Figure 5), protecting subcellular organelles could be another mechanism involved in the protective properties of OE. It is noteworthy to mention that the cytoprotective properties of OE and NAC were not significantly different in the current model. These data mention the significant cytoprotective role of OE and its organosulfur compounds.

The data collected in this study indicate significant cytoprotective properties of OE. On the other hand, further studies are required to determine the exact hepatoprotective agent(s) and the hepatoprotective mechanism(s) of OE. Moreover, different in vitro and in vivo experiments could be carried out in the future on the hepatoprotective effects of OE, especially when the occurrence of oxidative stress is suspected.

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Conflict of Interest

None declared.

5. References


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