Protective effect of apple polyphenols on hepatocytes injury induced by carbon tetrachloride in vitro

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Apple polyphenols are considered as natural antioxidants. The purpose of this paper was to evaluate the cytoprotective effects of apple polyphenols against rat primary hepatocytes oxidative injury induced by carbon tetrachloride in vitro. Some biochemical assays were carried out to determine the cytoprotective effects of apple polyphenols on the hepatocytes subjected to oxidative injury, including cell viability, the content of malondialdehyde in culture medium, as well as alanine aminotransamine, aspartate aminotransaminase and lactate dehydrogenase leakage into culture medium. Statistical results showed that treatment of the cultured hepatocytes with apple polyphenols at concentrations of 10 mg/l at the same time of oxidative injury of the cells could provide cytoprotective effect to the cells to improve cell viability, significantly reduce alanine aminotransamine, aspartate aminotransaminase and lactate dehydrogenase leakage into culture medium and decrease the formation of malondialdehyde compared to control group, while treatment of the cultured cells after oxidative injury could help injured cells self-repairing. It was shown that apple polyphenols displayed stronger cytoprotective and repairing effect against oxidative injury of the rat primary hepatocytes.

Key words: Apple polyphenols, oxidative injury, carbon tetrachloride, primary hepatocytes.

INTRODUCTION

Liver is the most important organ involved in the biotransformation of exogenous chemicals and the major target for toxic substances. During the detoxification of xenobiotics, reactive oxygen species (ROS) are generated which begin a series of reactions of lipid peroxides within the hepatocytes, and subsequently cause oxidative stress, cell death and liver disease, such as hepatocellular carcinoma, viral and alcoholic hepatitis and so on (Kohen and Nyska, 2002; Vitaglione et al., 2004). Liver diseases are a world wide problem. Therefore, scavenging of ROS or protection against oxidative stress in the liver is very essential process for health. Natural antioxidants, especially those in daily foods, such as polyphenols, attract much more attention of many researchers.

Apple is the fruit of the Malus genus belonging to the rose family Rosaceae. Traditionally, apples have been regarded as a healthy fruit in many historical cultures, as seen from the popular proverb “one apple a day keeps the doctor away.” In these years, association between apple consumption and several health benefits has been established. Apples are rich in phytochemicals, particularly carotenoids, flavonoids, isoflavonoids and phenolic acids (Boyer and Liu, 2004). The major phenol compounds in apples are quercetin glycosides,
Table 1. Experimental groups and project of culture solution.

<table>
<thead>
<tr>
<th>Time</th>
<th>Initial 0 h</th>
<th>Initial 8 h</th>
<th>Initial 12 h</th>
<th>Initial 36 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h before model</td>
<td>Start modeling</td>
<td>End modeling</td>
<td>24 h after modeling</td>
<td></td>
</tr>
<tr>
<td>Control seeding cells</td>
<td>Normal medium</td>
<td>Normal medium</td>
<td>Normal medium</td>
<td></td>
</tr>
<tr>
<td>Model seeding cells</td>
<td>Modeling medium</td>
<td>Modeling medium</td>
<td>Modeling medium</td>
<td></td>
</tr>
<tr>
<td>AP-pre seeding cells in culture medium containing AP</td>
<td>Modeling medium</td>
<td>Normal medium</td>
<td>Normal medium</td>
<td></td>
</tr>
<tr>
<td>AP-m seeding cells</td>
<td>Modeling medium containing AP</td>
<td>Normal medium</td>
<td>Normal medium</td>
<td></td>
</tr>
<tr>
<td>AP-rep seeding cells</td>
<td>Modeling medium containing AP</td>
<td>Normal medium</td>
<td>Normal medium containing AP</td>
<td></td>
</tr>
</tbody>
</table>

In the present study, the cytoprotective effect of apple polyphenols against the oxidative injury induced by CCl₄ was studied on primary rat hepatocytes, isolated from normal SD rat liver tissue. To examine and compare the effect of apple polyphenols in three different treating ways on hepatocytes, cell viability, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and lactate dehydrogenase (LDH) leakage into culture medium, malondialdehyde (MDA) formation were assayed, for these biochemical indices are considered as important indicators of cell injury and lipid peroxidation. The aim of this study is to reveal the cytoprotective way of apple polyphenols in vitro.

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MATERIALS AND METHODS

Chemicals

Apple polyphenols were obtained from Tianjin Jianfeng Natural Product Co., Ltd, (Tianjin, China). The reagents included from fetal bovine serum (FBS), RPMI-1640 medium (Hyclone), 3-[4,5-dimethylthiazol-2-yl]-2,5- dephenyl tetrazolium bromide (MTT, sigma), dimethylsulfoxide (DMSO, sigma). All other chemicals were analytical agents.

Experiments were conducted following approval and in accordance with the guidelines set by the Animal Experimental Ethical Committee, Tianjin University of Science and Technology. Hepatocytes were isolated from normal SD rat liver tissue following the method improved by Zhang et al. (2008) and incubated in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin G and 100 mg/ml streptomycin at 37°C in an incubator of humidified air with 5% CO₂. The cells were seeded onto 96-well plates at 5 × 10⁴ cells/well for cell cytotoxicity and viability assay, or 24-well plates at 5 x 10⁶ cells/well for MDA, AST, ALT and LDH determination.

Cell culture

Apple polyphenols were dissolved in RPMI-1640 medium containing 10% FBS, CCl₄ were dissolved in DMSO and mixed well with the medium so that the final concentration of DMSO was not ≥ 0.1% (v/v). The cells in test groups were incubated with apple polyphenols at 10 mg/l and oxidative injured with CCl₄ (10 mmol/L, 4 h) according to Table 1. The cells in control group, containing equal volume of DMSO but without any addition of AP, were not oxidative injured. Being incubated for 24 h, all groups were processed immediately for biochemical assays. Test groups were divided into three according to Table 1. The group of hepatocytes which were pretreated with AP for 24 h before oxidative injury named AP-pre group. The hepatocytes of AP-m group suffered oxidative injury while be incubated with AP. The cells in AP-rep group which had been oxidative injured were incubated with AP for 24 h.

Biochemical assays

Cell viability

Cell viability of the hepatocytes was measured by MTT assay. After the cells were treated according to Table 1, twenty microliter of MTT solution (5 g/L) was added to each well of the 96-well plate. After 4 h of incubation, the plate was centrifuged at 1800 g for 5 min at 4°C and the MTT solution was removed from the wells by aspiration. One hundred microliter of DMSO was added to each well to resolve the formazan generated from MTT. The absorbance of each well was recorded on a microplate reader (Thermo, MA, USA) at the wavelength of 490 nm. Cell viability in each test group and model group was expressed as percentage of the control group. Cells in control group were as 100% viable.
Table 2. Cytotoxicity of apple polyphenols on the hepatocytes incubated for 24 h.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Model</th>
<th>AP-pre</th>
<th>AP-m</th>
<th>AP-rep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell viability %</td>
<td>100.0 ± 6.0a</td>
<td>89.2 ± 4.2c</td>
<td>89.7 ± 4.8c</td>
<td>99.3 ± 5.9a</td>
<td>121.9 ± 4.5bd</td>
</tr>
</tbody>
</table>

Note: a: indicates p < 0.05 when compared with model group, there was significant differences; b: indicates p < 0.01 when compared with model group, there was significant differences; c: indicates p < 0.05 when compared with control group, there was significant differences; d: indicates p < 0.01 when compared with control group, there was significant difference.

Table 3. The effects of apple polyphenols on AST, ALT, LDH leakage of the hepatocytes injured by addition of CCl4 in vitro.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Model</th>
<th>AP-pre</th>
<th>AP-m</th>
<th>AP-rep</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH (U/L)</td>
<td>30.19 ± 5.33</td>
<td>36.87 ± 5.48</td>
<td>26.10 ± 1.95a</td>
<td>23.13 ± 2.11b</td>
<td>24.64 ± 0.79a</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>10.29 ± 0.41b</td>
<td>24.94 ± 1.47d</td>
<td>17.62 ± 1.59bd</td>
<td>9.37 ± 0.75b</td>
<td>9.73 ± 0.66b</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>2.86 ± 0.11b</td>
<td>4.13 ± 0.35d</td>
<td>4.11 ± 0.33bd</td>
<td>2.70 ± 0.31b</td>
<td>2.88 ± 0.23bd</td>
</tr>
</tbody>
</table>

Note: a: indicates p < 0.05 when compared with model group, there was significant differences; b: indicates p < 0.01 when compared with model group, there was significant differences; c: indicates p < 0.05 when compared with control group, there was significant differences; d: indicates p < 0.01 when compared with control group, there was significant difference.

AST, ALT and LDH leakage

After cell treatment followed the introduction of Table 1, the culture medium in 24-well plates was centrifuged at 1000 g for 10 min and the culture medium was collected for further tests. AST, ALT and LDH leakages were quantified using commercial enzymatic kits from Nanjing Jiancheng (Nanjing, China), respectively.

MDA assay

The contents of MDA in the cells culture were measured using commercial enzymatic kits from Nanjing Jiancheng (Nanjing, China), respectively.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). SPSS were used to analyze and report the data. The differences between the mean values of multiple groups were analyzed by one-way analysis of ANOVA with Duncan’s Multiple Range Test. ANOVA data with a p < 0.05 were classified as statistically significant.

RESULTS

Cell viability

Comparing to the cells in control group, treatment of the hepatocytes with CCl4 (10 mmol/l, for 4 h) resulted in a significant decrease in cell viability in model group and AP-pre group (p < 0.05). The cell viability in model group and AP-pre group decreased to 89%, indicating that oxidative injury occurred in the hepatocytes induced by CCl4 and being pretreated with apple polyphenols could not prevent from the injury. If the hepatocytes were incubated with apple polyphenols at the same time of oxidative injury happening, oxidative injury of the cell could be alleviated, at 10 mg/l (p < 0.05). Cell viability of hepatocytes in AP-m group was similar to the ones in control group. Additionally, Cell viability in AP-rep groups was enhanced clearly (p < 0.01), even much higher (p < 0.01) than normal hepatocytes in control group (Table 2).

AST, ALT and LDH leakage

As shown in Table 3, injury of the hepatocytes in model group with CCl4 caused more AST, ALT and LDH leakage into culture medium. Treatment of the cells with apple polyphenols led to the injured cells in test groups with less AST, ALT and LDH leakage, especially in AP-m group and AP-rep group. Pretreatment of the cells with apple polyphenols in AP-pre group reduced LDH leakage from 36.87 - 26.10 and the AST from 24.94 - 17.62 significantly while no effect on ALT. When apple polyphenols were added at the moment of oxidative injury to the cells in AP-m group, LDH, AST or ALT leakage could be reduced significantly (p < 0.01) from 36.87, 24.94 or 4.13 - 23.13, 9.37 or 2.70. The hepatocytes of AP-rep group showed the similar results to the AP-m groups as well as the normal cells in control group.

MDA assay

MDA content is used as a marker of lipid peroxidation. Comparing to the MDA formation in the cells in control group, treatment of the hepatocytes with CCl4 caused more MDA formation in model group (p < 0.01, Table 4), indicating lipid oxidation occurred in the groups during cell injury. MDA content in cells culture medium in model group was 1.43 nmol/ml, much higher than that in control
Cell damage induced by free radicals has been known as the predominant mechanism of hepatotoxicity (Gregus and Klaasen, 1995). The mechanism by which CCl₄ causes cell oxidative injury involves that cytochrome P-450 system transforms CCl₄ into CCl₃ and CCl₃ is transformed into a more reactive CCl₃O₂, CCl₃O₂ causes lipid peroxidation, disturbs Ca²⁺ homeostasis and kills cells finally (Farombi, 2000; Wang et al., 2004). The critical process underlying CCl₄ hepatotoxicity is the combining effect of both lipid peroxidation and the covalent binding of CCl₄ reactive metabolites to lipids and proteins (Masuda and Nakamura, 1990). Protective effects of natural antioxidants had been widely studied. In the study carried by Yau et al. (2002), an aqueous extract of Rubus chingii fruits showed strong protective effect on primary rat hepatocytes. A study about protective effect of tea polyphenols on human hepatocytes was reported recently (Li et al., 2010). It has been shown that CCl₄ induced lipid peroxidation can be obstructed by natural antioxidants (Subramanian et al., 1999; Wang et al., 2000). The identification of inhibitors of peroxidation resulting in cell damage could therefore lead to important new strategies for disease prevention (Subramanian et al., 1999).

The activities of AST, ALT are most commonly used biochemical makers of liver damage (Sturgill and Lambert, 1997). Also, because of the difference in location, AST locates in mitochondria while ALT locates in cytoplasm, the activities of AST and ALT indicates the degree of cells damage (AST) or the amount of cells injured (ALT). LDH is another important index of cell viability. The present study showed, comparing model group to control group, exposure of the hepatocytes to CCl₄ led to cell death, membrane damage, lipid peroxidation and so on, which were confirmed by some biochemical assays as cell viability, ALT, AST and LDH leakage, MDA formation in cells culture. The hepatocytes in AP-pre group had lower cells viability, and the activity of ALT was much higher comparing to the control group, which indicated that the pretreatment could not prevent from the membrane damage. However, the lower activities of LDH and AST meant the damage degrees were reduced. The hepatotoxic effect of CCl₄ has been attributed to its metabolism by Cytochrome P450 to yield toxic trichloromethyl radicals that act as free radical initiators (Kim et al., 2003). Action of this free radicals increase hepatic lipid peroxidation level in CCl₄ toxicity. This finding is in accordance with the known hepatotoxic effect of CCl₄ that causes oxidative damage in the liver (Farber and Gerson, 1984).

The results of AP-m group in cell viability and the activities of intracellular enzyme in culture medium were similar as the control group. The authors could presume that AP terminated the reaction at the beginning of the oxidative injury induced by CCl₄ to protect the cells. The cells damage induced by free radicals was usually a series of reactions. AP solution was added into the cells culture medium of AP-rep after the hepatocytes had been oxidative injured immediately. Almost all the indicators of this group closed to AP-m group and control group, which indicate that AP could inhibit further damage companying with Lipid peroxidation to protect the cells. There was another possibility that AP could help hepatocytes self-repairing after being injured.

**Conclusions**

In summary, the results showed that apple polyphenols could improve the cell viability of oxidative injured hepatocytes, reduce AST, ALT and LDH leakage into culture medium and decrease the formation of MDA significantly, which indicate that they had cytoprotective effect against hepatocytes oxidative injured by CCl₄, and could help injured cells self-repairing. Compare with some plant extract or active ingredients which can protect the hepatocytes by pretreatment for short time (Zhao and Zhang, 2009; Li et al., 2010), the way of AP to protect the cells prefer to be scavenging free radicals, stopping lipid peroxidation and promoting self-repair. However, there is a need for more studies in animal models in vivo for further confirmation, which is already in progress in the laboratory.

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REFERENCES


