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ORIGINAL ARTICLE

Effect of puerarin on apoptosis of human hepatocellular carcinoma cells under oxidative stress and its mechanisms

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Summary

Purpose: *To investigate the effect of puerarin on the apoptosis of human hepatocellular carcinoma cells induced by hydrogen peroxide and its mechanism.*

Methods: *Experiments were divided into control group, model group, and puerarin group. Normal saline (200 μmol/L) was used in the control group, 200 μmol/L H_2O_2 was used to induce oxidative stress in the model group, and 25 μmol/L, 50 μmol/L, and 100 μmol/L puerarin were used in the puerarin group to treat hepatocellular carcinoma SMMC-7721 cells for 24 h on the basis of 200 μmol/L H_2O_2, respectively. Contents of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) in SMMC-7721 cells were determined by colorimetry. Apoptotic rate of SMMC-7721 cells was determined by flow cytometry.*

Results: *Compared with the control group, MDA content in the H_2O_2 group increased significantly, and SOD activity and GSH content decreased significantly Compared with the control group, SOD activity and GSH content in SMMC-7721 cells of puerarin group decreased significantly ($p<0.05$). Compared with the H_2O_2 group, content of MDA in SMMC-7721 cells of the puerarin group decreased significantly, while SOD activity and GSH content ($p<0.05$) increased significantly. Activity of SOD and content of GSH in SMMC-7721 cells incubated with 50 μmol/L and 100 μmol/L puerarin were significantly higher than that in cells treated with 25μmol/L puerarin ($p<0.05$). Activity of SOD and content of GSH in SMMC-7721 cells incubated with 100 μmol/L puerarin were significantly higher than those in cells treated with 50 μmol/L puerarin ($p<0.05$). Apoptosis rate of SMMC-7721 cells incubated with different concentrations of puerarin was significantly lower than that of the H_2O_2 group ($p<0.05$).*

Conclusion: *Puerarin has protective effect on hepatocellular carcinoma SMMC-7721 cells under oxidative stress. It is suggested that puerarin should be carefully used when the proliferation of hepatocellular carcinoma cells results in the production of large amounts of ROS.*

Key words: *puerarin, hydrogen peroxide, oxidative stress, hepatocellular carcinoma*

Introduction

Liver cancer is one of the most common malignancies in the world, and its incidence rate ranks fifth among all malignancies. High mortality rate and strong invasive ability characterize this disease [1,2]. The treatment of hepatocellular carcinoma is increasingly standardized, but there is still the problem of limited drug efficacy, mainly reflected by the difficulties in the selection of drugs

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and drug resistance [3]. Hepatocellular carcinoma (HCC), as the most common type of liver cancer, accounts for more than 90% of all orthotopic liver cancers [4]. Occurrence and development of HCC is a complex and diverse process, with multiple genes and multi-step interactions involved, in which oxidative stress plays a key role [5].

It has been reported that all HCC animal models have the common feature of elevated reactive oxygen species (ROS) levels in liver cells [6-8]. An increase in ROS levels means that ROS scavenging capacity is reduced or ROS production increased [9]. Due to its oxidative properties, ROS can damage DNA and oxidize lipids, causing cellular damage and further oxidative stress [10]. Studies show that the development of liver cancer and the tolerance of chemotherapy are related to the tolerance of oxidative stress. Liver cancer cells have stronger antioxidant capacity than normal liver cells, making them easier to survive in liver's oxidative environment, which is one of the mechanisms leading to the occurrence of liver cancer. Most liver cancer chemotherapy drugs exert therapeutic effects by inducing increase of ROS in cells to cause the death of liver cancer cells, but the excessively strong antioxidant capacity of liver cancer cells can protect them from death, resulting in insignificant chemotherapy effects [11]. H_2O_2 is an active oxygen source that can enter and damage cells, and is now widely used in in vitro experiments to simulate peroxidative damage of cells [12].

Puerarin is a well-known antioxidant and has been used clinically. Due to the anti-tumor properties of puerarin, it has attracted much attention in recent years [13]. It has been reported that Pueraria lobata is similar to other isoflavones and has an estrogenic effect, and high concentrations of puerarin can inhibit the proliferation of breast cancer cells [14]. Studies have also shown that puerarin can promote the apoptosis of colon cancer cells [15].

The purpose of this study was to investigate the effects of puerarin on MDA, SOD, GSH and apoptosis in HCC SMMC-7721 cells under H_2O_2-induced oxidative stress and also to explore the possible mechanisms.

Methods

Ethics approval

The study was approved by the Ethics Committee of The Second Hospital of Shandong University.

Reagents and equipment

Human HCC cell line SMMC-7721 was purchased from the cell bank of the Chinese Academy of Sciences. Bovine serum was purchased from NanJing SenBeiJia Biotechnology Co., Ltd. RPM1640 medium was purchased from Shanghai ZZBio Co., Ltd. Puerarin was purchased from Shanghai Fuxin Pharmaceutical Technology Co., Ltd. SOD, GSH, and MDA kits were purchased from Nanjing Jiancheng Bioengineering Institute. Trypsin-EDTA digestion solution was purchased from Beijing Solarbio Science and Technology Co., Ltd. Annexin V-FITC cell apoptosis assay kit was purchased from KeyGEN BioTECH Co., Ltd. 722 spectrophotometer was purchased from Shanghai Meipuda Instrument Co., Ltd. Epics Altra flow cytometer was purchased from Beckman company (Germany).

Cell culture

Cells were cultured with RPM1640 medium containing 10% fetal bovine serum (FBS) in a constant temperature incubator at 37°C. Cells were digested with 0.25% trypsin for subculture, and cells with good condition were harvested during logarithmic growth phase.

Grouping and treatment

Experiments were divided into the control group, model group and puerarin group. Normal saline (200 μmol/L) was used in the control group, 200 μmol/L H_2O_2 was used to induce oxidative stress for 24 h in the model group, and 25 μmol/L, 50 μmol/L, and 100 μmol/L puerarin were used in the puerarin group to treat cells for 24 h, and on this basis, 200 μmol/L H_2O_2 were added to incubate for another 24 h, respectively. Contents of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) in SMMC-7721 cells were determined by colorimetry. Apoptotic rate of SMMC-7721 cells was determined by flow cytometry.

Colorimetric assay for measurement of intracellular MDA, SOD, and GSH levels

SMMC-7721 cells were inoculated in 6-well plates and allowed to adhere to grow. Different concentrations of puerarin were prepared. Cultured cells were disrupted with ultrasound and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was assayed for colorimetric determination of SOD activity and GSH and MDA contents. The experimental operation was carried out in strict accordance with the manufacturers' instructions.

Flow cytometry detection of cell apoptosis

Cell density was adjusted to 1×10^5 cells/ml and cells were cultured at 37°C with 5% CO_2. After 70% confluence, 0, 25, 50, and 100 μmol/L puerarin were added and cells were incubated for 24 h. Cells were then treated with 200 μmol/L H_2O_2 for 24 h and then were washed twice with PBS at room temperature. Cells were digested with 2.5 g/L trypsin, and the collected cells were resuspended in 0.01 mol/L cold PBS. Cell suspension containing 1×10^5 cells was centrifuged at 1800 rpm for 5 min to discard the supernatant. 195 μL of Annexin V-FITC conjugate was added to resuspend the cells which were then transferred to flow cytometry tube; 5 μL of AnnexinV-FITC and 5 μL of propidium iodide (PI) were added into each tube. After incubation in the dark at room temperature for 15 min, apoptosis was detected within 1 h, and each sample was examined 3 times.

Table 1. Effect of puerarin on SOD, MDA and GSH in SMMC-7721 cells treated by H_2O_2

Groups	*SOD(U/mL)*	*MDA(nmol/mL)*	*GSH(mg/g prot)*
Control group	16.46±0.67	1.31±0.25	30.52±0.54
H_2O_2 group	10.53±0.46*	3.67±0.36*	16.85±0.35*
Puerarin group			
25 μmol/L	12.52±0.58$^{*\#}$	2.65±0.31$^{*\#}$	18.34±0.32$^{*\#}$
50 μmol/L	13.68±0.37$^{*\#\&}$	2.12±0.14$^{*\#}$	20.45±0.42$^{*\#\&}$
100 μmol/L	14.82±0.41$^{*\#\&\square}$	1.68±0.17$^{\#\&\square}$	23.73±0.66$^{*\#\&\square}$
F	58.37	37.82	391.80
p	<0.001	<0.001	<0.001

*compared with control group, $p<0.05$; # compared with H_2O_2 group, $p<0.05$; & compared with 25 μmol/L puerarin group, $p<0.05$; □ compared with 50 μmol/L puerarin group, $p<0.05$.

Statistics

SPSS 19.0 statistical software was used for all analyses (Shanghai Cabit Information Technology Co., Ltd.). Quantitative data was shown as mean±SD. One-way ANOVA was used for the comparisons of the quantitative data among multiple groups, and least significant difference (LSD) test was used for comparison between two groups. $P<0.05$ was considered to be statistically significant.

Results

Effect of puerarin on SOD, MDA and GSH in SMMC-7721 cells treated with H_2O_2

The SOD activity of SMMC-7721 cells in the H_2O_2 group and different concentrations of the puerarin group were significantly lower than those in the control group ($p<0.05$). The activity of SOD in SMMC-7721 cells incubated with different concentrations of puerarin was significantly higher than that in the H_2O_2 group ($p<0.05$). The activity of SOD in SMMC-7721 cells incubated with 50 μmol/L and 100 μmol/L puerarin was significantly higher than that in cells treated with 25 μmol/L puerarin ($p<0.05$). The activity of SOD in SMMC-7721 cells incubated with 100 μmol/L puerarin was significantly higher than that in cells treated with 50 μmol/L puerarin ($p<0.05$).

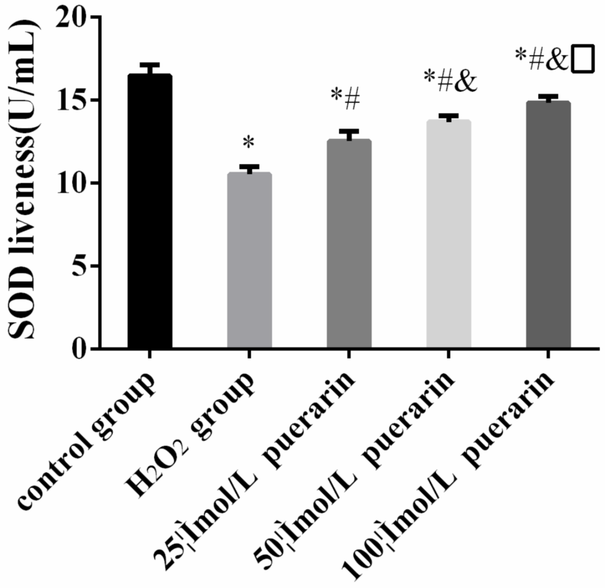

Figure 1. Effect of puerarin on SOD activity in SMMC-7721 cells with H_2O_2 treatment. Results of colorimetry showed that SOD activity of SMMC-7721 cells in the H_2O_2 group and different concentrations of the puerarin group were significantly lower than those in the control group ($p<0.05$). Activity of SOD in SMMC-7721 cells incubated with different concentrations of puerarin was significantly higher than that in the H_2O_2 group ($p<0.05$). Activity of SOD in SMMC-7721 cells incubated with 50 μmol/L and 100 μmol/L puerarin was significantly higher than that in cells treated with 25 μmol/L puerarin ($p<0.05$). Activity of SOD in SMMC-7721 cells incubated with 100 μmol/L puerarin was significantly higher than that in cells treated with 50 μmol/L puerarin ($p<0.05$).
*compared with control group, $p<0.05$; # compared with H_2O_2 group, $p<0.05$; & compared with 25 μmol/L puerarin group, $p<0.05$; □ compared with 50 μmol/L puerarin group, $p<0.05$.

The content of MDA in SMMC-7721 cells of the H_2O_2 group was significantly higher than that in the control group ($p<0.05$). There was no significant difference in MDA content between SMMC-7721 cells treated with 100 mol/L puerarin and the control group ($p>0.05$). Compared with the control group, MDA content in SMMC-7721 cells incubated with 25 μmol/L and 50 μmol/L puerarin was significantly different ($p<0.05$). The content of MDA in SMMC-7721 cells incubated with different concentrations of puerarin was significantly lower than that in the H_2O_2 group ($p<0.05$). There was no significant difference in MDA content in SMMC-7721 cells incubated with 25 μmol/L and 50 μmol/L puerarin ($p>0.05$). The content of MDA in MMC-7721 cells incubated with 100 μmol/L puerarin was significantly lower than cells treated with 25 μmol/L and 50 μmol/L puerarin (p<0.05).

The GSH content in SMMC-7721 cells of the H_2O_2 group and different concentrations of puerarin groups was significantly lower than those in the control group (p<0.05). The content of GSH in SMMC-7721 cells incubated with different concentrations of puerarin was significantly higher than that in the H_2O_2 group (p<0.05). The content of GSH in SMMC-7721 cells incubated with 50 μmol/L and 100 μmol/L puerarin was significantly higher than that in cells incubated with 25 μmol/L puerarin (p<0.05). The content of GSH in SMMC-7721 cells incubated with 100 μmol/L puerarin was significantly higher than that in cells incubated with 50 μmol/L puerarin (p<0.05) (Table 1 and Figures 1-3).

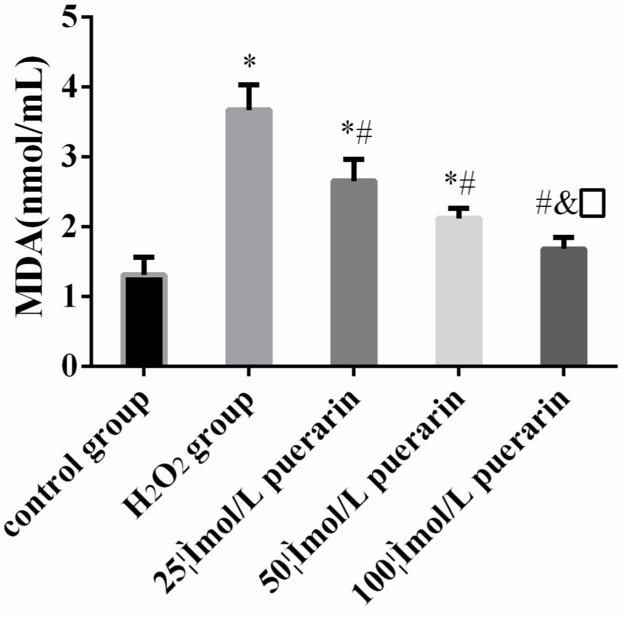

Figure 2. Effect of puerarin on MDA content in SMMC-7721 cells with H_2O_2 treatment. Results of colorimetry showed that the content of MDA in SMMC-7721 cells of H_2O_2 group was significantly higher than that in the control group (p<0.05). There was no significant difference in the MDA content between SMMC-7721 cells treated with 100 mol/L puerarin and the control group (p>0.05). Compared with control group, MDA content in SMMC-7721 cells incubated with 25 μmol/L and 50 μmol/L puerarin was significantly different (p<0.05). The content of MDA in SMMC-7721 cells incubated with different concentrations of puerarin was significantly lower than that in the H_2O_2 group (p<0.05). There was no significant difference in MDA content in SMMC-7721 cells incubated with 25 μmol/L or 50 μmol/L puerarin (p>0.05). The content of MDA in MMC-7721 cells incubated with 100 μmol/L puerarin was significantly lower than in cells treated with 25 μmol/L and 50 μmol/L puerarin (p<0.05).
*compared with control group, p<0.05; # compared with H_2O_2 group, p<0.05; & compared with 25 μmol/L puerarin group, p<0.05; □ compared with 50 μmol/L puerarin group, p<0.05.

Effect of puerarin on apoptosis of SMMC-7721 cells induced by H_2O_2

The apoptosis rate of SMMC-7721 cells in the H_2O_2 group was significantly higher than that in the control group (p<0.05). The apoptosis rate of MMC-7721 cells incubated with different concentrations of puerarin was significantly lower than that of the H_2O_2 group (p<0.05). The apoptosis rate of SMMC-7721 cells incubated with 50 μmol/L and 100 μmol/L puerarin was significantly lower than in cells incubated with 25 μmol/L puerarin (p<0.05). The apoptosis rate of SMMC-7721 cells incubated with 50 μmol/L was not significantly different from that of cells treated with 100 μmol/L puerarin (p>0.05) (Table 2 and Figure 4).

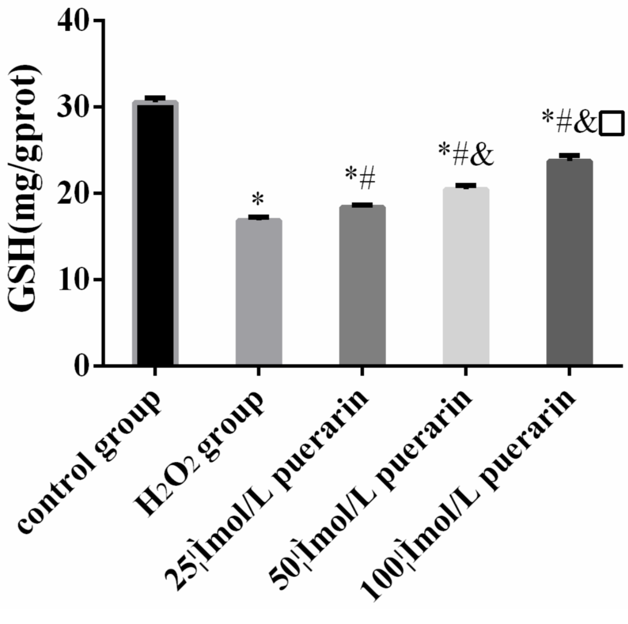

Figure 3. Effect of puerarin on GSH content in SMMC-7721 cells with H_2O_2 treatment. The GSH content in SMMC-7721 cells of the H_2O_2 group and different concentrations of puerarin groups were significantly lower than those in the control group (p<0.05). The content of GSH in SMMC-7721 cells incubated with different concentrations of puerarin was significantly higher than that in the H_2O_2 group (p<0.05). The content of GSH in SMMC-7721 cells incubated with 50 μmol/L and 100 μmol/L puerarin was significantly higher than that in cells incubated with 25 μmol/L puerarin (p<0.05). The content of GSH in SMMC-7721 cells incubated with 100 μmol/L puerarin was significantly higher than that in cells incubated with 50 μmol/L puerarin (p<0.05).
*compared with the control group, p<0.05; # compared with the H_2O_2 group, p<0.05; & compared with 25 μmol/L puerarin group, p<0.05; □ compared with 50 μmol/L puerarin group, p<0.05.

Discussion

Causes of HCC usually include environmental factors, virus contamination, alcohol consumption, and genetic factors such as genetic inheritance [16]. Even though the predisposing factors for HCC are diverse, the data from a large number of animal models of HCC indicate that oxidative stress is generally involved [17]. Oxidative stress may be involved in the genesis of HCC from the following two pathways: First, oxidative stress stimulates proliferation of hepatocytes to a certain extent, and ROS increase accordingly, resulting in a significant increase in the probability of gene mutation and activation of oncogene expression [18]. Second, high-intensity oxidative stress promotes hepatocyte apoptosis and induces compensatory proliferation of other hepatocytes to induce HCC [19]. HCC cells maintain a proliferative state with high-intensity oxidative stress.

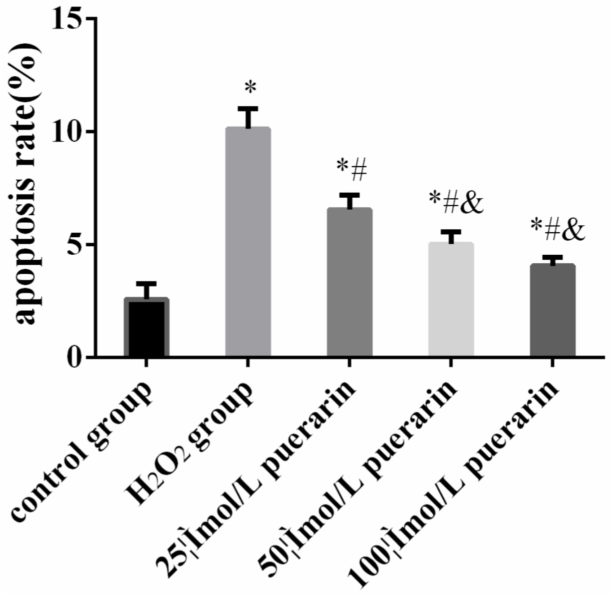

Figure 4. Effect of puerarin on apoptosis of SMMC-7721 cells induced by H_2O_2. Flow cytometry results showed that the apoptosis rate of SMMC-7721 cells in the H_2O_2 group was significantly higher than that in the control group ($p<0.05$). The apoptosis rate of MMC-7721 cells incubated with different concentrations of puerarin was significantly lower than that of the H_2O_2 group ($p<0.05$). Apoptosis rate of SMMC-7721 cells incubated with 50 µmol/L was not significantly different from that of cells treated with 100 µmol/L puerarin ($p>0.05$). The apoptosis rate of SMMC-7721 cells incubated with 50 µmol/L and 100 µmol/L puerarin was significantly lower than in cells incubated with 25 µmol/L puerarin ($p<0.05$).
*compared with control group, $p<0.05$; # compared with H_2O_2 group, $p<0.05$; & compared with 25 µmol/L puerarin group, $p<0.05$.

Table 2. Effect of puerarin on apoptosis of SMMC-7721 cells induced by H_2O_2

Groups	*Apoptosis rate (%)*
Control group	2.57±0.68
H_2O_2 group	10.12±0.89*
Puerarin group	
25 µmol/L	6.54±0.66*#
50 µmol/L	5.02±0.53*#&
100 µmol/L	4.05±0.38*#&
F	58.88
p	<0.001

*compared with control group, $p<0.05$; # compared with H_2O_2 group, $p<0.05$; & compared with 25 µmol/L puerarin group, $p<0.05$.

SOD and GSH all have the ability of scavenging ROS. The production of MDA will increase the damage of membrane lipids and indirectly reflect the level of ROS in vivo [20]. The results of this study showed that SOD activity and GSH content in HCC SMMC-7721 cells were significantly lower and MDA content was significantly higher in the H_2O_2 group than in control group ($p<0.05$). It was demonstrated that the oxidative stress model of liver cancer cells was successfully established. In this study, SOD activity and GSH content were significantly higher, while MDA content was significantly lower in the puerarin group than in the H_2O_2 group after treatment with different concentrations of puerarin ($p<0.05$). This shows that puerarin can increase the SOD activity and GSH content of hepatoma SMMC-7721 cells, reduce MDA content, inhibit oxidative stress, and effectively reduce the damage of H_2O_2 to HCC SMMC-7721 cells. The protective effect of puerarin increases with increasing puerarin concentration. The findings of Li et al. [20] are basically consistent with ours. Puerarin has a protective effect against traumatic brain injury through nrf2 and is a signal pathway transduced through the resistance to oxidative stress [21]. This shows that puerarin has protective effects on oxidative stress-induced SMMC-7721 injury, and can increase the antioxidative capacity of HCC cells. Cell apoptosis experiments showed that H_2O_2 promoted the apoptosis of SMMC-7721 cells. After treatment with different concentrations of puerarin, the apoptosis rate of HCC SMMC-7721 cells was significantly lower than that of the H_2O_2 group ($p<0.05$). The findings of Zhou et al. [22] are similar to ours. Puerarin reduces oxidative stress-induced apoptosis in epithelial cells via MAPK signaling pathways both in vivo and in vitro [22]. This shows that puerarin can effectively reduce the apoptosis rate of SMMC-7721 cells induced by H_2O_2, and the protective effect of puerarin on SMMC-7721 cells increases with increasing concentrations.

Conclusions

The results of the determination of oxidative stress index and the apoptosis rate of HCC cells both showed that puerarin has a protective effect on H_2O_2-induced oxidative stress in human HCC SMMC-7721 cells. It is suggested that puerarin should be used with caution when HCC cells proliferate rapidly and produce large amounts of ROS.

Conflict of interests

The authors declare no conflict of interests.

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