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Synergistic anti-glioma effect of Hydroxygenkwanin and Apigenin *in vitro*

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ABSTRACT

Apigenin (AP) and Hydroxygenkwanin (HGK) are two natural flavonoid compounds. Previous studies have already demonstrated the anti-tumor capability of AP. However, it is not clear whether HGK has such property. In the current study, the anti-glioma activities of HGK and its synergistic anti-glioma effects with AP on C6 glioma cells were investigated. In addition, the possible mechanisms were also studied.

MTT assay and morphologic analysis including acridine orange/ethidium bromide (AO/EB) and 4′,6-diamidino-2-phenylindole (DAPI) staining were used in the research, and the results indicated that the treatment with AP or HGK could inhibit C6 glioma cell proliferation respectively. Moreover, when AP was administrated simultaneously, the anti-glioma effect of HGK was dramatically enhanced in a dose-dependent manner, which is obviously better than that of carmustine (BCNU) at the concentration 25 μM for treating of 24 h. Compared with control, mitochondrial membrane potential (MPP) loss and mitochondrion damage were detected by JC-1 fluorescence probes (JC-1) and transmission electron microscopy (TEM) after treatment. Obvious DNA damage and cell cycle S phase arrest were detected by alkaline comet assay and flow cytometric analysis (FCM). Additionally, up regulation of TNF-α level, activations of caspase-3, -8, over expressions of BID and BAK protein and BCL-XL protein down expression were also observed after treatment by the combination of AP and HGK.

The results indicate that HGK may be an effective natural product to treat glioma, and the combination of AP and HGK may be a promising method for glioma chemotherapy.

1. Introduction

Glioma is one of the most commonly seen primary brain tumors in central nervous system (CNS), which is dreadful for its malignant biological behaviors such as invasive growth, high recurrence and mortality rate [1]. Many breakthroughs have been made in the treatment of glioma including surgery, chemotherapy, radiotherapy and their combination. However, the outcome is not satisfactory. The overall 2-year survival rate of patients with glioma is about 27.2% [2]. Although the most commonly applied treatment for glioma is tumor resection, chemotherapy is also essential to prolong the life span, especially for unresectable glioma [3]. However, only a few commonly used anti-tumor chemotherapeutics can be used in brain tumors at present due to the blood-brain barrier (BBB) [4]. Clinically, it is proved effective to combine applications of a variety of chemotherapeutic agents which act in different mechanisms to treat glioma. But severe side effects such as gastro-intestinal reaction, liver toxicity and bone marrow depression can not be avoided.

Therefore, there is an urgent need for new therapeutic agents. Nowadays, chemicals extracted from herbs may be promising since a lot of natural compounds including some flavonoid compounds have been demonstrated to have the functions of anti-tumor [5,6], reversing multi drug resistance (MDR) and the permeability of BBB [7–9].

Lilac Daphne (*Daphne genkwa Sied. Et Zucc.*) has been used for thousands of years in Traditional Chinese Medicine (TCM), which has functions such as anti-inflammation, anti-tumor, relieving cough, and alleviating abscess. The earliest record about its medicinal value can be dated back to the "*Treatise on Febrile Diseases*" (in Chinese "*Shang Han Lun*") between the year 200–205 A.D.

HGK – a member of flavonoid compound, with the molecular weight of 300.26, has been demonstrated as (Fig. 1A) one of the main components of Lilac Daphne by modern pharmacological studies, whose content has also been considered as one of the criteria for the quality of Lilac Daphne [10]. Its extraction and

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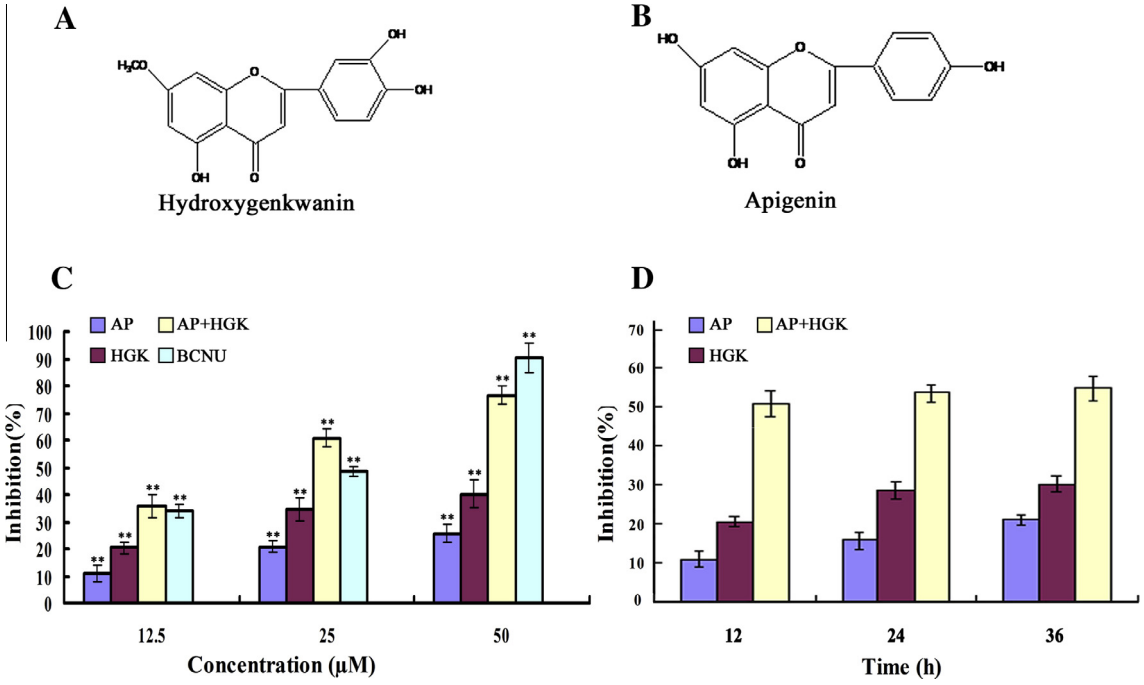

Fig. 1. (A) Structure of HGK. (B) Structure of AP. (C) Concentration dependent anti-proliferation effect of AP, HGK, AP + HGK and BCNU. Cells were treated in different drug concentration for 24 h; inhibition (%) was determined by MTT assay and compared to the control group ($^{*}p < 0.05$ and $^{**}p < 0.01$). (D) Time dependent inhibition (%) of AP, HGK and AP + HGK. Cells were treated with AP (25 μM), HGK (25 μM), AP + HGK (12.5 μM + 12.5 μM) for 12 h, 24 h and 36 h. Inhibition (%) was determined by MTT assay and compared to the control ($^{*}p < 0.05$ and $^{**}p < 0.01$).

purification process has already been clear [11,12]. And previous studies have also indicated that HGK has anti-oxidant ability and can act as tissue factor (TF) inhibitor to prevent thrombosis [13,14]. However, the reports about its anti-tumor function are absent.

AP (Fig. 1B) is another flavonoid compound, which is widespread in vegetables and fruits, with the molecular weight of 270.24. It has attracted many researchers due to its versatile biological effects such as anti-tumor, anti-inflammation, anti-biotic and antioxidation effects [15–18]. A research conducted by Das et al. [15] indicated that AP induce apoptosis of glioblastoma cells line T98G and U87MG (glioma multiform) without any effect on human normal astrocytes. Because of its wide distribution in food, more and more researchers have recognized that AP plays an important role in diet therapy of cancer, and it can even prevent cancer metastasis due to its involvement in adhesion, motility, angiogenesis, and invasion [19,20].

This study is to explore the anti-glioma ability of HGK with and without AP, meanwhile to investigate the potential mechanisms. The results indicated that both AP and HGK had anti-proliferation effects on C6 glioma cells, where HGK showed stronger anti-glioma activity. When the two chemicals were administrated together (AP + HGK), the anti-glioma effect was increased significantly.

HGK inhibits C6 glioma cell proliferation via tumor necrosis factor-alpha (TNF-α) induced activations of caspase-3, -8 which results in apoptosis, other mechanisms including mitochondrial apoptosis pathway, DNA damage and cell cycle arrest are also involved. AP acts as a sensitizer in the synergistic anti-glioma effect of AP + HGK.

Additionally, we compared the anti-glioma capability of HGK + AP with that of carmustine (BCNU), one of the most prescribed chemotherapeutics for brain tumors, and assessed the impact of HGK on normal neurons (PC12 cell line). All the results suggested that HGK might be another effective anti-glioma agent which had no toxicity on normal neurons at low concentration. Drug combination of AP and HGK may also be used for glioma treatment.

2. Materials and methods

2.1. Chemicals and reagents

AP and HGK (purity: over 98%) were obtained from Y-J biological (Shanghai, China). BCNU was obtained from Tianjin King York (Tianjin, China). Cell proliferation kit (MTT) and propidium iodide (PI) were purchased from Roche Diagnostics (Mannheim, Germany). TNF-α radioimmunoassay kit, Caspase-3 and -8 activity assay kits, mitochondrial membrane potential assay kit (JC-1), cell cycle and apoptosis analysis kits, BCA protein assay kit, RIPA lysis buffer and phenylmethanesulfonyl fluoride (PMSF) were all purchased from Beyotime Institute of Biotechnology (Shanghai, China). 4,6-diamidino-2-phenylindole (DAPI), Tris and SDS were provided by Sigma (St. Louis, MO, USA). FBS and L-DMEM culture medium were obtained from Thermo (Beijing, China). Antibodies against Bcl-2, Bcl-XL, BAX, BAK, BID, GAPDH, horseradish peroxidase-conjugated goat anti-rabbit IgG, and horseradish peroxidase-conjugated goat anti-mouse IgG were supplied by Proteintech Group, Inc. (Chicago, USA). Comet assay kits and slides were purchased from Cell Biolabs, Inc (USA).

2.2. Cells and cell culture

C6 glioma cell line and well-differentiated PC12 cell line were purchased from Shanghai Cell Biology Institute of Chinese Academy of Sciences (Shanghai, China). After recovery, cells were cultured in L-DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated in 5% CO_2 atmospheric incubator at 37 °C. Medium was renewed every three days. When cells were proliferated to confluence of 80–90%, the experiments were carried on.