



Experimental Study of Rhein Inhibiting the Invasion and Migration of the HepG2 Cells by Extracellular Signal-Regulated Kinase Signaling Pathway

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Abstract

Background: The antitumor mechanism of Rhein has attracted much attention in the current study, and it was found that Rhein could inhibit the growth of the human hepatoma cell line (HepG2).

Objectives: This study aimed to investigate the effect of rhein on the proliferation, invasion, and migration of the HepG2 cells through the extracellular signal-regulated kinase (ERK) signaling pathway.

Methods: HepG2 was treated with different concentrations of rhein (Rhein treatment group) and cultured in a culture medium alone (control group). The proliferation activity of the cells was determined by methyl Thiazolyl Tetrazolium colorimetry. Transwell assay detected the invasion and migration of the cells in each group. Cell scratch test was used to detect the migration ability of cells in each group. Excella-phospho ERK (p-ERK) activity was determined by ELISA after treatment with 50 μmol/L rhein at different times. Western blot was employed to detect the ERK protein expression in the HepG2 cells treated with 50 μmol/L of rhein.

Results: The proliferation activity, invasion, and migration ability of the HepG2 cells in the rhein-treatment group were all decreased, compared to the control group ($P < 0.05$), and the p-ERK relative activity of the HepG2 cells treated with rhein was decreased ($P < 0.05$).

Conclusion: Rhein inhibits the invasion and migration of the hepatocellular carcinoma cells possibly by inhibiting the ERK pathway.

Keywords: Cell invasion, Cell migration, ERK pathway, HepG2 cells, Rhein

1. Background

Hepatocellular carcinoma (HCC) is the most common primary liver cancer recently, ranking second among the malignant tumors. About 700,000 people die of HCC every year (1). Hepatitis B virus and hepatitis C virus had a high incidence rate of liver cancer in China. According to China's cancer statistics in 2015, HCC accounted for the second and fifth of the total cancer-related deaths in men and women, respectively (2). In recent years, the treatment measures for patients with liver cancer include surgery, chemotherapy, and radiotherapy (3-5). However, due to the lack of effective interventions for HCC, the metastasis rate and mortality are high, and it is one of the urgent concerns to find more effective treatments in the field of hepatobiliary surgery. At present, the effect of traditional Chinese medicine in the treatment of advanced cancer is significant, and it has been gradually recognized in clinical studies. Rhein is one of the active components of anthraquinones in rhubarb, which has antioxidant, antiviral, anti-inflammatory, anti-tumor, and anti-fibrosis properties, as well as liver protection, kidney protection, and other functions (6,7).

Rhein can regulate cytochrome P450 enzymes, protects liver cells from damage, and prevents the progression of liver fibrosis in rats; moreover, oral

rhein significantly accelerates energy expenditure and reduces cholesterol and liver triglyceride levels in hepatitis B virus-transgenic high-fat diet mice. It also helped anti-fibrosis in carbon tetrachloride/ethanol-induced liver fibrosis.

2. Objectives

This study investigated the effectiveness of rhein in the human hepatoma cell line (HepG2) by cell experiment in vitro; moreover, the mechanism of rhein inhibiting tumor invasion and migration by inhibiting the extracellular signal-regulated kinase (ERK) pathway of the HepG2 cells was also explored, which provided a theoretical basis for the treatment of HCC with rhein.

3. Methods

3.1. Material

Rhein, ERK and excella-phospho ERK (p-ERK) antibodies, as well as methyl thiazolyl tetrazolium (MTT) kits, were purchased from Sigma-Aldrich (the USA). Moreover, AK strain transforming (AKT) and phosphorylated AKT antibodies were obtained from Cell Signaling Technology Inc. (the USA). In addition, β-Actin antibody and Matrigel were obtained from Abcam Inc. (the UK) and BD (the USA), respectively.

RPMI-1640 and trypsin were obtained from Gibco (the USA). CO₂ incubator and ELISA were purchased from Thermo (the USA). The quantitative PCR and Gel Doc XR were obtained from Strategene (the USA) and LICOR (the USA), respectively.

HepG2 cells were obtained from the Fourth Hospital of Hebei Medical University and Hebei Cancer Hospital, Hebei, China. The cell culture medium (RPMI-1640) contained 5% fetal bovine serum, 0.01% sodium pyruvate, 2 mmol/L glutamine, and 5 μmol/L 2-mercaptoethanol. The cells were cultured in 5% CO₂ (37°C). Rhein stock solution (100 μm) was prepared with dimethyl sulfoxide (DMSO) and diluted accordingly. Before the experiment, the cells were digested with trypsin.

3.2. Proliferation Assay

The density of the HepG2 cells was adjusted to 1×10⁴ and placed on a 96-well plate. The cells in the rhein group were treated with 10, 30, and 50 μmol/L of rhein solution, respectively, while the control group was cultured in a medium alone. A total of 20 μL MTT solution was added into each well four hours before detection, and the culture medium was discarded when it reached the detection time. Following that, 150 μL DMSO was added to each well to dissolve the blue-purple crystal and shook for 10 min under dark conditions. The absorbance value of each well was measured by ELISA, and the average value was taken. The cell activity was expressed by the absorbance percentage with the control group.

3.3. Transwell Experiment

The Matrigel at a -20°C refrigerator was melted at a 4°C refrigerator, mixed with a precooled medium according to the concentration of 1:6, and 40 μL Matrigel was added into each Transwell and kept at a 37°C incubator for 2 h. The logarithmic phase cells of each group were collected, and the cell density was detected. In the next stage, 10% fetal bovine serum was then taken into the lower Transwell and cultured at 37°C for 48 h. The cells on the bottom of the Transwell and Matrigel were wiped off with a wet cotton swab. After being stained by 0.1% crystal violet (10 min), five areas were photographed and counted under the microscope, and the mean value was taken to show the invasion of the cells. For migration assay, Matrigel was not added into Transwell.

3.4. Wound Healing Assay

The wound-healing assay was started after 24 h of the cell culture. The method was as follows: a sterilized 20 μL pipette nozzle was used to make a scratch along the bottom of a 6-well culture plate. The scratch area was gently beaten upon serum-free culture medium, poured out the culture medium, and repeated three times. The scraped cells were washed clean as far as possible, and 10% fetal bovine serum was used for culture. The growth of cells on both sides of the scratch

line was observed at 0 h and 48 h after scratch, and cell migration was observed under a microscope.

3.5. Detection of p-ERK Activity

The HepG2 cells had been fixed with 4% paraformaldehyde (30 min) at room temperature, incubated with 1% hydrogen peroxide and 0.5% Triton X-100/PBS (10 min), and blocked in 10% fetal bovine serum (1 h). Mouse p-ERK (1:200) was incubated at 37°C for 1 h, and mouse IgG (1:2000) was incubated for 1 h at 37°C. After being incubated with tetramethylbenzidine peroxidase substrate and treated with 0.1 mol/L of hydrochloric acid, the absorbance at 450 nm was determined by ELISA, and the relative activity of p-ERK was expressed by the absorbance ratio of the control group.

3.6. Western blot

According to the actual concentration of the protein sample, the actual sample volume of each group of samples was calculated according to the sample loading volume of 30 μg in each well. The samples were mixed with 6×buffer, water bath for 5 min at 95°C, and then immersed in ice for 10 min.

SDS-PAGE electrophoresis (5% concentration gel, 12% separation gel, 90 V, 35 mA, 1 h, after reaching the separation interface, changing voltage 120 V, 30 mA, 1.5 h) was utilized to separate the sample protein, remove the gel, lay the PVDF on the gel, place between the two layers of filter paper, put the gel on the negative electrode, put the PVDF in the positive electrode and into the electrophoresis tank, carry out the imprinting electrophoresis on the ice (constant current 190mA, 90min), and transfer the protein from PAGE to PVDF. Following that, the PVDF was taken out, rinsed with TTBS for 10 min, and put into 30 ml 5% confining liquid at 37°C for 2 h. Subsequently, the confining liquid was discarded without washing, and the primary antibody diluted was added to the TBST and set overnight at 4°C.

PVDF was washed with TBST for 5 min 3 times; 1 ml of peroxidase-labeled secondary antibody diluted by TBST was added and set at 37°C for 1 h and 20 min. In addition, the PVDF was washed with TBST for 5 min 3 times, and PVDF was washed with TBS for 5min. Odyssey imaging system was used for color rendering and data analysis. Antibodies used in this assay were as followed: p-ERK (1:1000) and ERK (1:1000) that were purchased from Sigma-Aldrich (the USA); p-AKT (1:1000) and AKT (1:1000) (from Cell Signaling Technology Inc, the USA), and β-Actin (1:10000) (Abcam Inc, the UK).

3.7. Statistical Analysis

The obtained data were analyzed in SPSS software (version 17.0), and the data were expressed as mean±SD. The comparison of the two groups was performed by ANOVA. A p-value less than 0.05 was considered statistically significant.

4. Results

4.1. Effect of Rhein on the Proliferation Activity of the HepG2 Cells

With the increase of rhein concentration, the relative activity of the HepG2 cells treated with 10, 30, and 50 $\mu\text{mol/L}$ of rhein decreased, compared to the control group ($P < 0.05$, Figure 1).

4.2. Effect of Rhein on the Invasion Ability of the HepG2 Cells

The number of cells passing through the membrane in the rhein-treated group was significantly less than that in the control group ($P < 0.05$, Figure 2), indicating that rhein significantly inhibited the invasion ability of the HepG2 cells in a concentration-dependent manner.

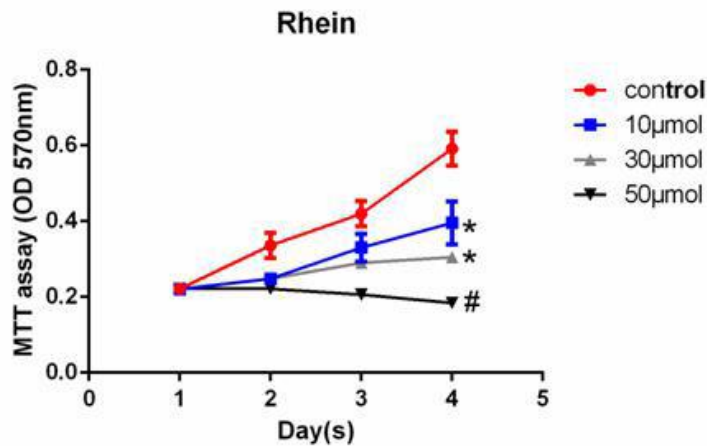


Figure 1. Proliferation activity of the HepG2 cells treated with different concentrations of rhein

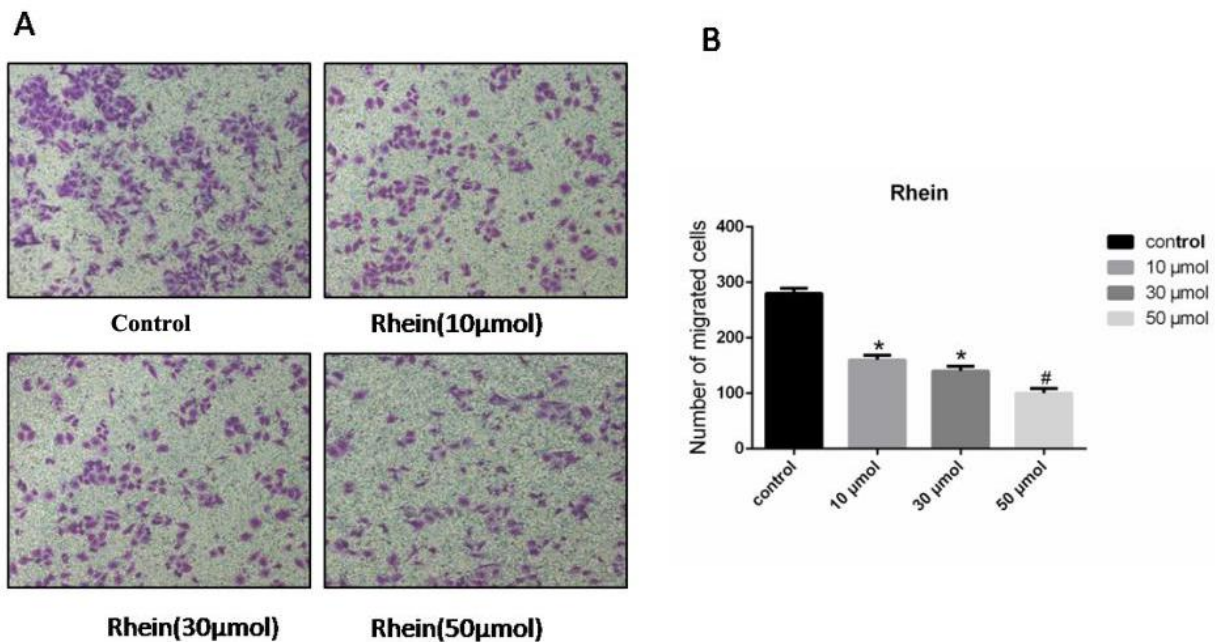


Figure 2. Invasion ability of the HepG2 cells treated with different concentrations of rhein

4.3. Effect of Rhein on the Migration Ability of the HepG2 Cells

The number of cells passing through the membrane in the rhein-treated group was significantly less than that in the control group ($P < 0.05$, Figure 3), indicating that rhein significantly inhibited the migration ability of the HepG2 cells in a concentration-dependent manner.

4.4. Wound Healing Assay

The healing area of the cells treated with rhein at different concentrations (10, 30, 50 $\mu\text{mol/L}$) was significantly lower than that in the control group ($P < 0.05$, Figure 4). In conclusion, 50 $\mu\text{mol/L}$ of rhein can affect the invasion and migration of the HepG2 cells more significantly.

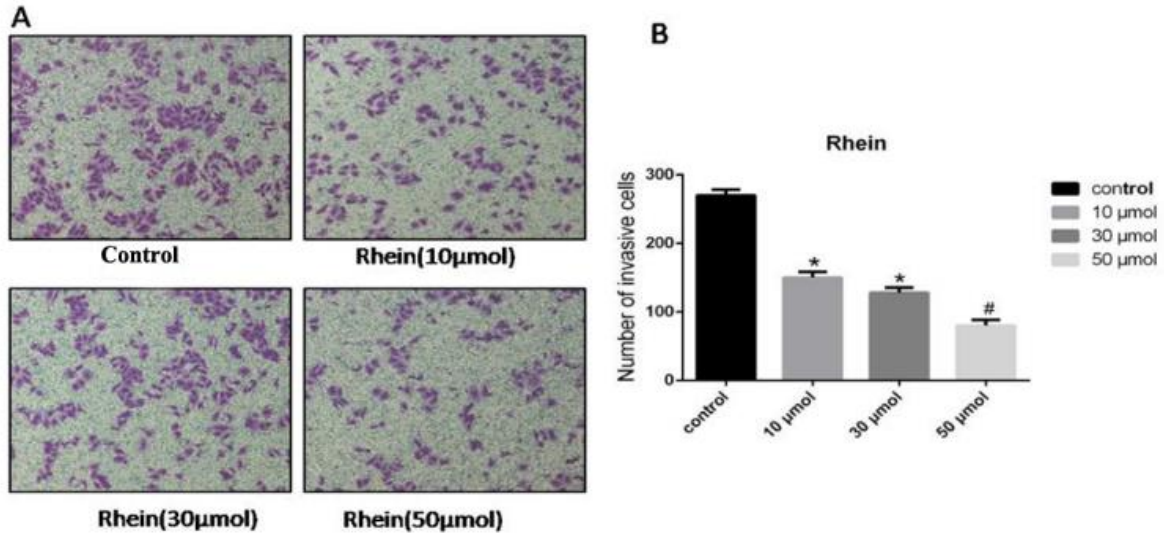


Figure 3. Migration ability of the HepG2 cells treated with different concentrations of rhein

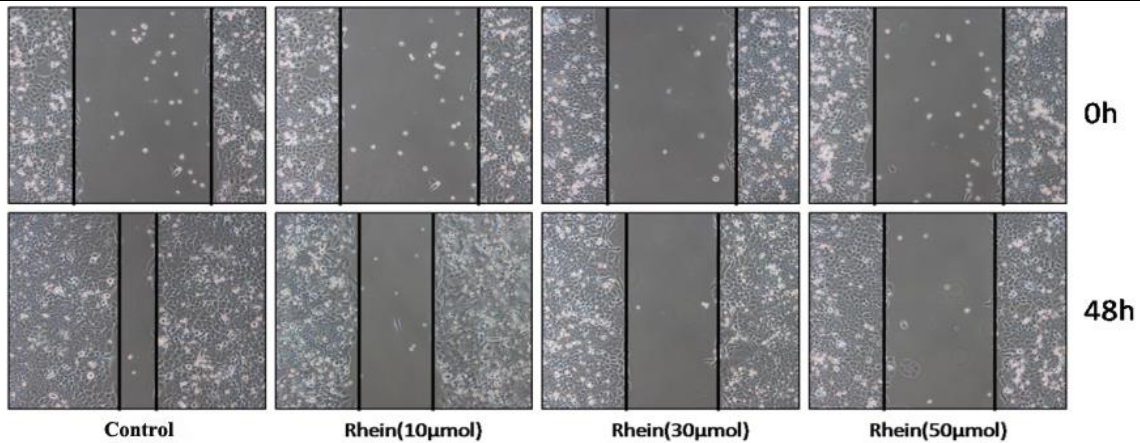


Figure 4. Healing ability of the HepG2 cells treated with different concentrations of rhein

2.5. Changes in the p-ERK Activity of the HepG2 Cells Treated with Rhein

The relative activity of the p-ERK in the HepG2 cells treated with 50µmol/L of rhein for 60, 120, 180, and

240 min decreased gradually with time (P<0.05, Figure 5), compared to that in the control group. Therefore, rhein inhibited the invasion and migration of the HepG2 cells by inhibiting the activity of the ERK pathway.

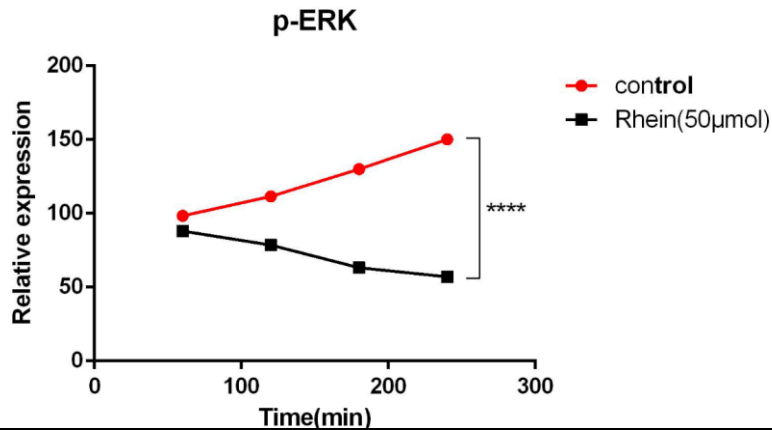


Figure 5. Changes of the p-ERK activity in the HepG2 cells treated with rhein for different time

2.6. Expression of ERK Protein in HepG2 Cells Treated with Rhein

The activity of the p-ERK and p-AKT in the

HepG2 cells treated with rhein was significantly lower than that in the control group ($P < 0.05$, Figure 6).

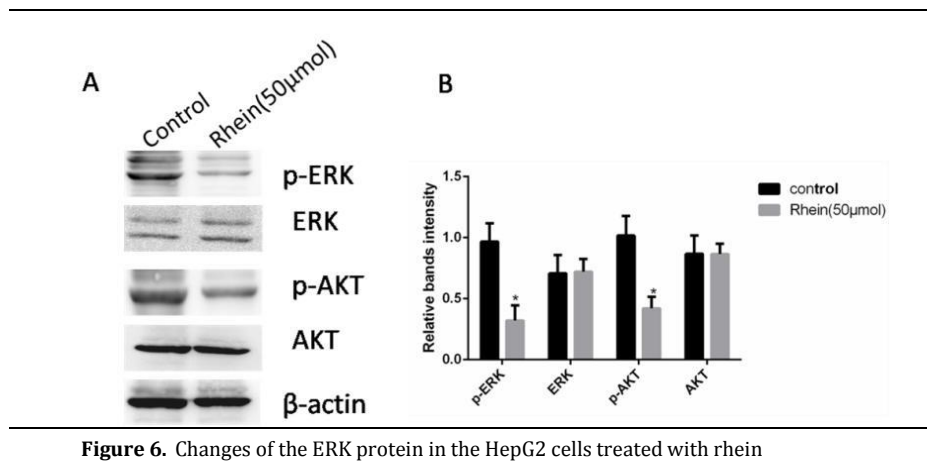


Figure 6. Changes of the ERK protein in the HepG2 cells treated with rhein

5. Discussion

Liver cancer has become a serious threat to human health and life, and the way to treat it has been an urgent issue of modern medicine. Traditional Chinese medicine in our country has been used to treat cancer, which can not only improve the quality of life of patients and prolong the survival period of patients but also plays a role in promoting apoptosis by directly targeting cancer cells, while other traditional Chinese medicine may control tumor growth and metastasis by regulating the immune system. (8-10)

Rhubarb was a popular traditional Chinese medicine for a long time. In previous studies, researchers have pointed out that rhein had anti-inflammatory, antiviral, antioxidant, and other biological activities in vivo and in vitro (11-13) along with anti-tumor activity against breast cancer, lung cancer, colon adenocarcinoma, ovarian cancer, and other tumors (14).

At the same time, the anti-tumor mechanism of rhein has attracted much attention. Recently it has been found that rhein can induce the apoptosis of the human gastric cancer of the SGC-7901 cells (15) and Blacher revealed that rhein could inhibit the process of glioma by inhibiting the activity of the CD38 enzyme (16). Huang et al. investigated the effect of rhein on the breast cancer of the MCF-7 and MDA-MB-231 cells and found that the mechanism of rhein-induced breast cancer cell apoptosis was mainly caused by the promotion of cell protein misfolding and the imbalance of cell redox state. (17)

The endoplasmic reticulum is stress-related, and rhein decreases the mRNA level of MMP-9, which plays an important role in various human cancers related to tumor invasion and metastasis. Moreover, it reduces the levels of MMP-2 and urokinase u-PA and inhibits migration and invasion in human

tongue cancer of the SCC-4 cells (18). Rhein induces the elimination of mitochondrial membrane potential and the cleavage of Bid protein in human cervical cancer cells (19). Furthermore, it reduces the Bcl-2 levels and increases the caspase-3 gene resulting in DNA fragmentation(20).

This study found that rhein inhibited the growth of the HepG2 cells. Moreover, the proliferation of the HepG2 cells was decreased along the concentration gradient after being treated with 10, 30, and 50 μmol/L of rhein. It was also found that different concentrations of rhein had different effects on the HepG2 cells. Rhein at 50 μmol/L had the strongest inhibitory effect on the HepG2 cells. In addition, the activity and protein expression of the p-ERK in the HepG2 cells were significantly decreased after treatment with 50 μmol/L of rhein.

In conclusion, rhein can inhibit the invasion and migration of the HepG2 cells by the ERK signaling pathway. The therapeutic effect and mechanisms of rhein on the HCC will be further studied through cell or animal experiments.

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Footnotes

Authors' contributions: YW designed the study. YW and LL participated in writing and revising the manuscript. YZ and WL contributed to data analysis. WX, QW, and MC helped to perform the cell studies. All authors have read and approved the final manuscript

Ethical Approval: This study was conducted in accordance with the declaration of Helsinki. The study protocol was approved by the Ethics

Committee of Hebei University of Chinese Medicine, Hebei, China.

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Conflicts of Interest: The authors declare that they have no competing interests.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Informed Consent: Not applicable.

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