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Hyperoside Facilitates Gastric Cancer (GC) Cell Apoptosis and Inhibits Viability via NF-κB/PTEN/JAK2 Pathway

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ABSTRACT This study investigated impacts of hyperoside on viabilities and apoptosis of gastric cancer (GC) cells. CCK-8 and flow cytometry were applied for examining HGC-27 cell viabilities and apoptosis, indicating that hyperoside treatment suppressed cell viabilities but facilitated cell apoptosis. Additionally, RT-qPCR results revealed that mRNA expressions of Bcl-2 in HGC-27 cells were downregulated while Bax and caspase-3 were elevated with hyperoside treatment. Furthermore, ELISA examined changes about protein concentrations of NF-κB/PTEN/JAK signalling pathway in HGC-27 cells after being treated by hyperoside. Results showed that protein concentrations of phosphorylated NF-κB/p65 were reduced with hyperoside treatment while PTEN and JAK2 protein concentrations were promoted.

INTRODUCTION

Gastric cancer is a chronic malignancy of the digestive system with high mortality and morbidity worldwide (Siegel et al. 2018). Due to the difficulties of identification and inconspicuous symptoms for the gastric cancer in the beginning, the clinical result for the gastric cancer patients is very poor. According to the latest report, only one fifth of the gastric cancer patients were successfully diagnosed at early stage all over the world (Pirini et al. 2017). Based on recent studies, genes have been discovered to participate in the progression of gastric cancer. For instance, various genes have been identified that exert their functions in the formation of cancer starting from tumorigenesis to metastasis such as TERT, BCAS1, HSP90AA1, CASP3, and P53 genes that play critical functions in the GC survival (McFarlane et al. 2018), and therapeutic strategies and their outcomes also affected by these genes (Yusefi et al. 2018). In order to find the effective molecular treatment strategies, it is important to have enough research about the molecular mechanism behind the growth and pathogenesis of gastric cancer (Wu et al. 2015).

Traditional Chinese Medicine (TCM) has caught concerns for treating various of disorders including cancers (Teng et al. 2016). Evidence indicated that TCMs have been used to inhibit inflammatory responses and induce apoptosis (Huang et al. 2021; Yang et al. 2017). Hyperoside, a flavonoid derived from quercetin 3-o-β-d-galactopyranoside, has anti-cancer (Wei et al. 2021), anti-oxidant (Xing et al. 2020), and anti-inflammatory properties (Kim et al. 2011). Hyperoside exerted its anti-cancer impacts via increasing human endometrial cancer cells apoptosis (Li et al.

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2012). Hyperoside suppressed the activation of NF-κB signalling pathway via suppressing reactive oxygen species, resulting in breast cancer cell apoptosis (Qiu et al. 2019). Increasing evidence indicated that hyperoside acted as a suppressor in colon cancer (Zhang et al. 2017), pancreatic cancer (Boukes and van de Venter 2016), lung cancer (Li et al. 2018), and prostate cancer (Yang et al. 2015). However, the therapeutic effect of hyperoside on gastric cancer still needs to be elucidated. This research aimed at examining the effects and underlying mechanisms about hyperoside in modulating GC in vitro.

NF-κB belongs to the class of transcription factors, which can form heterodimers and homodimers and suppress or up-regulate the expressions of various genes (Neumann and Naumann 2007). NF-κB has been found to perform pro-tumorigenic effects that can be activated in various types of cancers (Lalle et al. 2021; Pflug and Sitcheran 2020). A previous study exposed that NF-κB activation enhances the proliferation of GC cells (Kang et al. 2008). In addition to that, hyperoside has been discovered to reduce the activation of NF-κB signalling pathway, leading to apoptosis of pancreatic cancer cells (Li et al. 2016). Also, hyperoside inhibited inflammatory responses and increased apoptosis in lung cancer via decreasing NF-κB signalling pathway (Lü 2016). Phosphatase and tension homolog (PTEN) is considered as a tumour suppressor containing phosphatase independent and dependent action (Hopkins et al. 2014). The PTEN expression was inhibited in GC and tumour growth was enhanced by the inactivation of PTEN (Yu et al. 2019). The JAK2 exerts an important function in the differentiation, apoptosis and cell viability (Li 2008). A recent study revealed that the activation of NF-κB/JAK2 significantly down-regulated PTEN expression in pancreatic cancer cells (Zhang et al. 2020). Nevertheless, the potential mechanism of hyperoside and PTEN/JAK2 as well as NF-κB signalling in GC cells still needs to be explored. This current study directed to dig out impacts of hyperoside on modulating apoptosis and cell proliferation in GC in vitro. Moreover, the researchers analysed the impacts of hyperoside on NF-κB, PTEN, and JAK2 signalling pathways.

Objectives

This study aimed at investigating hyperoside on modulating viabilities and apoptosis in HGC-27 cells and Bcl-2, Bax and caspase-3 mRNA expressions and protein concentrations of NF-κB/p65, PTEN and JAK2.

MATERIAL AND METHODS

Main Regents

The main reagents used for this study included Human Gastric Carcinoma cell line HGC-27 (Sigma Aldrich, USA), RPMI 1640 (VWR, USA), FBS (Gibco, USA), hyperoside (Sigma Aldrich), MTT solution (Abcam, UK), DMSO (Sigma Aldrich), Propidium Iodide (BioVision, USA), Annexin V-FITC (BioVision), ELISA kit (Abcam), RIPA buffer solution (Sigma Aldrich), TRIzol reagent (Thermo Fisher, USA), PrimeScript RT Master Mix Kit (TakaraBio, Sweden), SYBR Premix Ex Taq II Kit (Bioz Inc., USA), NF-κB/p65 ELISA kit (pS536, ab176647, Abcam), NF-κB/p65 ELISA kit (ab176648), PTEN ELISA kit (ab206979), and phosphorylated JAK2 ELISA kit (ab279840).

Cell Culture

Human Gastric Carcinoma cell line HGC-27 bought from Sigma Aldrich (MO, USA) was cultured in RPMI 1640 (VWR, ON, USA) having ten percent FBS at 37^0C with five percent CO_2 environment. The medium was substituted every 48 hours with fresh medium. These cultured cells were preserved with hyperoside (0, 15, 20, 25 μM) for 24 hours.

MTT Assay

HGC-27 cells were raised for 24 hours in a 96-well plate using an incubator comprising five percent CO_2 at 37^0C. Thereafter, hyperoside (0, 15, 20, 25 μM) was applied for treating cells for 24 hours and MTT solution (50 mL; Abcam, UK) was added. When the medium was removed for 4 hours, DMSO solution (150mL; Sigma Aldrich, MO, USA) was added and shaken by using orbital shaker for 30 minutes in darkness. The absorbance at 590 nm was observed by a microplate reader (LabX, ON, Canada). All the steps were repeated in triplicate.

Flow Cytometry

HGC-27 cells were collected in a logarithmic phase and incubated for 24 hours in a 96-well

plate using an incubator with five percent CO_2 at 37^0C follow by treatment with hyperoside (0, 15, 20, 25 μM). Thereafter, cells were rinsed twice using PBS and re-suspended by using binding buffer solution (500 μl). Propidium Iodide and Annexin V-FITC (5 μl respectively; Biovision, CA, USA) were added and cells were raised for 5 minutes in the absence of light and examined by flow cytometry (Biovision, CA, USA) using the CELLQUEST program (BD, Eysins, Switzerland). The absorbance at 530 nm was observed with a microplate reader (LabX, ON, Canada). All the steps were repeated in triplicate.

ELISA Assay

ELISA assay kits (Abcam) were used to evaluate protein concentrations of NF-κB/p65 and tumour suppressor genes, PTEN and JAK2. A 24-well plate was used to incubate cells and cells were lysed by RIPA solution (Sigma Aldrich). These incubated cells were centrifuged at 14,000×*g* for 5 minutes. ELISA kits of NF-κB/p65 (pS536, ab176647, Abcam), NF-κB/p65 (ab176648), PTEN (ab206979) and phosphorylated JAK2 (ab279840) were applied for examining protein concentrations following manufacturer's protocols. All the steps were repeated three times.

RT-Qpcr

TRIzol reagent (Thermo Fisher, NJ, USA) was used for isolating total RNA from HGC-27 cells following the manufacturer's guide. Then reverse transcription of these RNA was done into cDNA by PrimeScript RT Master Mix Kit (TakaraBio, Goteborg, Sweden). SYBR Premix Ex Taq II Kit (Bioz Inc., CA, USA) was used to amplify the primers for caspase-3, Bax, and Bcl-2 in a Step One Plus real Time PCR system. $2^{-\Delta\Delta Ct}$ was used to examine the results of PCR and the primers used are given in the Table 1.

Table 1: Primers used in RT-qPCR assays

Primers	*Sequence*	
Caspase-3	Forward	3ACTGGACTGTGGCATTGAGA
Caspase-3	Reverse	GTTTCAGCATGGCACAAAGC
Bcl-2	Forward	2GTGGTGGCAGATGTGCTTAG
Bcl-2	Reverse	TTCAGAGCCACAAACAAGGC
Bax	Forward	TCCAAGAAGCCCTAACGTGT
Bax	Reverse	ATGATCGTCTGGCTGCTGTA
â-actin	Forward	ACCCAGAAGACTGTGGATGG
â-actin	Reverse	TCAGCTCAGGGATGACCTTG

Statistical Analysis

The data were evaluated by using SPSS 19.0 (IBM, NY, USA) and were presented as the mean ±SD. Student's t-test as well as one-way or two-way ANOVA were performed for the comparison among two or more groups, respectively. Graphpad Prism 7.0 (Graphpad Software, CA, USA) was used for statistical analysis of the data and the $P < 0.05$ was deliberated as statistical significance. All steps were performed thrice.

RESULTS

Impact of Hyperoside on HGC-27 Cell Viability and Apoptosis

MTT assay examined the HGC-27 cell viability and the findings elucidated that the increase in the concentration of hyperoside decreased the HGC-27 cells viabilities (Fig. 1A, $^{**}P<0.05$). Also, the flow cytometry results revealed that increasing concentrations hyperoside facilitated HGC-27 apoptosis rate (Fig. 1B, $^{**}P<0.05$). Furthermore, caspase-3, Bax, and Bcl-2 mRNA levels were assessed using RT-qPCR and the results revealed that increase in the concentration of hyperoside suppressed the Bcl-2 mRNA level but enhanced caspase-3 and Bax mRNA expressions in HGC-27 cells (Fig.1C, $^{**}P<0.05$). Therefore, hyperoside treatment could suppress HGC-27 cell viabilities and accelerate apoptosis via downregulating Bcl-2 mRNA expressions and promoting Bax and caspase-3 mRNA expressions.

Impact of Hyperoside on Modulating NF-κB Signalling Pathway in HGC-27 Cells

NF-κB pathways are responsible for the regulation of cell apoptosis and phosphorylated NF-κB/p65 is the key factor for the activation of NF-κB pathway. ELISA assay indicating that hyperoside treatment downregulated the protein concentrations of phosphorylated p65 in a dose-dependently, while no substantial change in the total protein of p-65 was detected with the increased concentrations of hyperoside (Fig. 2, $^{**}P<0.05$). Hence, hyperoside treatment restrained HGC-27 cell progression through suppressing the phosphorylation of NF-κB/p65.

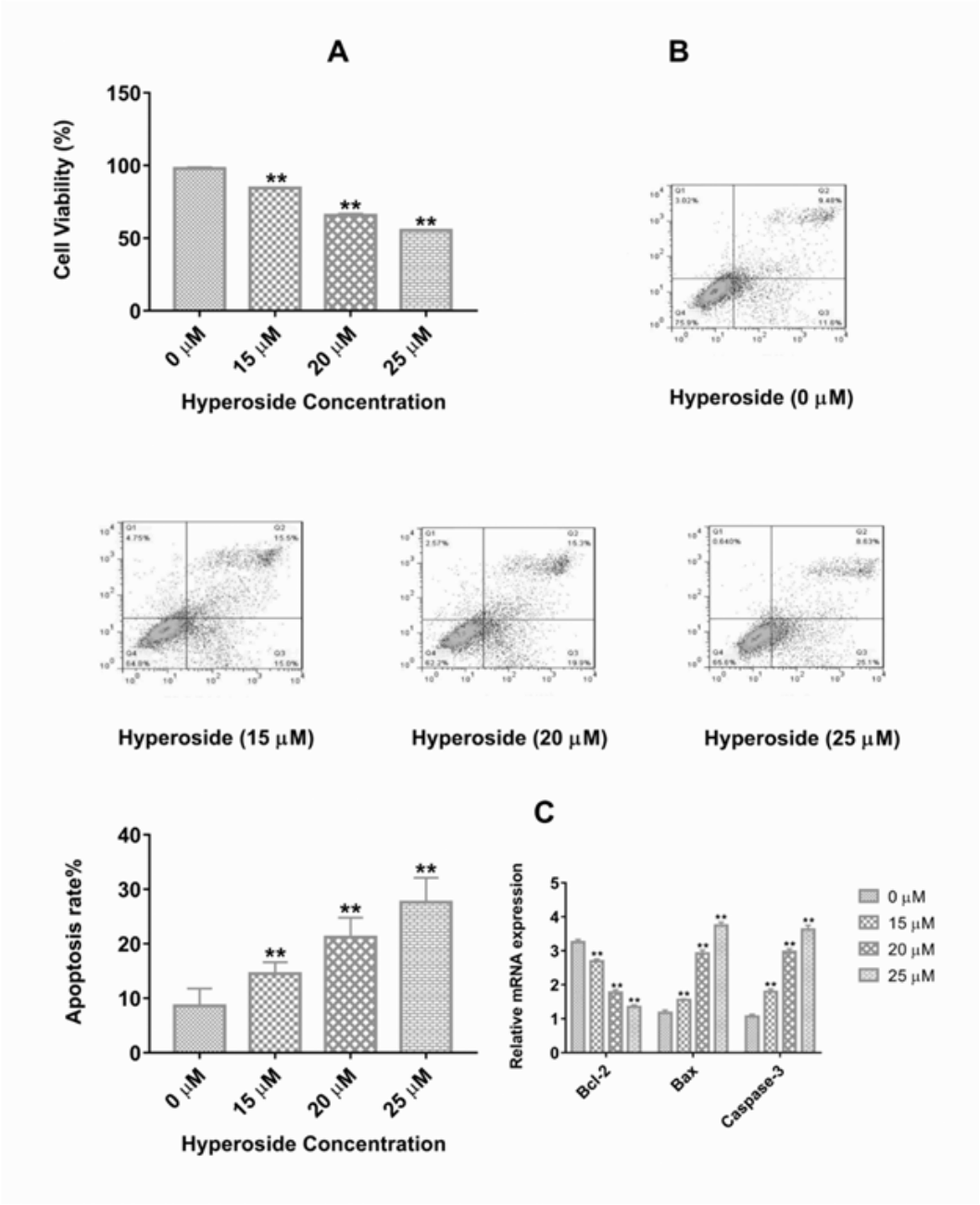

Fig. 1. Impacts of hyperoside on regulating HGC-27 cell viabilities and apoptosis:
A) MTT assay was applied to examined the HGC-27 cell viabilities.
B) Flow cytometry was performed to evaluating HGC-27 cell apoptosis
C) RT-qPCR was used for analyzing Bcl-2, caspase-3 and Bax mRNA expressions

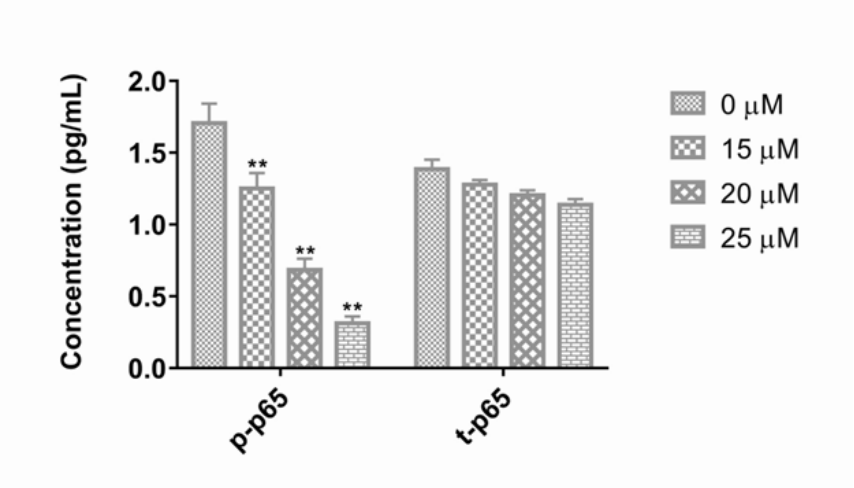

Fig. 2. Impact of hyperoside on modulating NF-κB pathway in HGC-27 cells: Protein concentrations of phosphorylated p65 and total p65 were validated in HGC-27 cells with hyperoside using ELISA assay **P <0.05

Impacts of Hyperoside on PTEN and JAK2 Pathways

Further, the researchers observed the impact of hyperoside on modulating tumour suppressor genes, PTEN and JAK2. ELISA assay was used to determine the protein concentrations of PTEN and p-JAK2. The findings elucidated that the expression of PTEN and phosphorylation level of JAK2 were enhanced with increasing concentrations of hyperoside in HGC-27 cells (Fig. 3A-B, $^{**}P<0.05$). Based on these detections, hyperoside treatment restrained HGC-27 cell progression through promoting expressions of PTEN and JAK phosphorylation.

DISCUSSION

In the current research, the researchers examined the role of hyperoside on HGC-27 cell viabil-

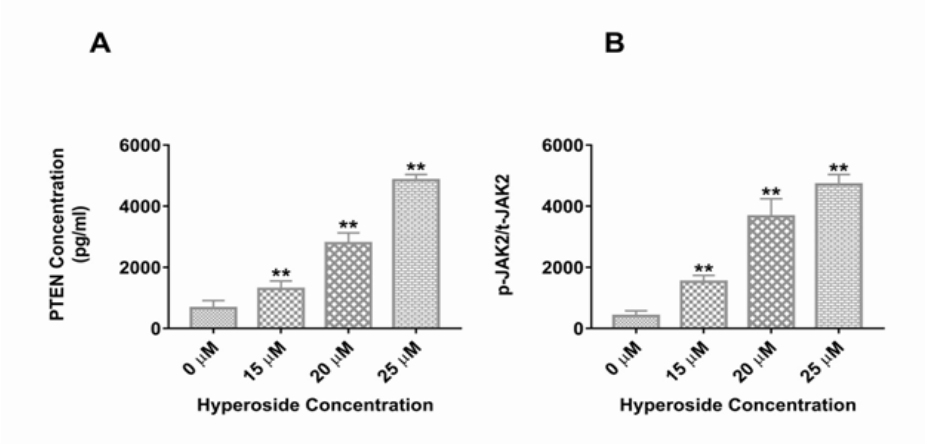

Fig. 3. Impact of hyperoside on PTEN and JAK2 pathways:
A) The protein concentration of PTEN was determined in HGC-27 cells after treatment with different concentrations of hyperoside
B) The ratio of phosphorylated JAK2/ total JAK2 was determined in HGC-27 cells after treated by hyperoside $^{**}P < 0.05$

ities and apoptosis. Besides, the researchers explored the mechanism of hyperoside in HGC-27 cells, revealing that increase in the concentration of hyperoside reduced the HGC-27 cell viabilities and enhanced apoptosis. Besides, hyperoside suppressed the NF-κB pathway and activated the PTEN/JAK2 signalling pathways. The findings revealed that hyperoside suppressed NF-κB pathway and activated PTEN protein expression and the phosphorylation of JAK2 pathway in gastric cancer HGC-27 cells, thereby inducing apoptosis.

Hyperoside is an active compound obtained from hypericum perforatum which contains various pharmacological properties (Choi et al. 2010). Previous research revealed that hyperoside induced apoptosis in non-small cell lung cancer cells via suppressing Akt/mTOR pathway (Fu et al. 2016). Hyperoside also inhibited the cell viability and enhanced apoptosis in HT-29 cells via activating caspases (Guon and Chung 2016). In the present study, the researchers studied effects of hyperoside on HGC-27 cell viability and the results exposed that hyperoside decreased the HGC-27 cell viability. The activation of caspases is the key point to apoptosis and is regulated by various factors including Bcl-2 family exerting its function in promoting or inhibiting the cell death (Solà-Riera et al. 2020; Warren et al. 2019). FCM apoptosis was used to determine apoptosis rate in HGC-27 cells, indicating that the rate of apoptosis was enhanced with increased hyperoside concentrations in HGC-27 cells. Moreover, caspase-3 and Bax mRNA expressions were enhanced and Bcl-2 mRNA expressions was reduced in HGC-27 cells dose-dependently. These findings confirmed the therapeutic role of hyperoside in the inhibition of GC.

Previous research revealed that inhibition of NF-κB pathway enhanced apoptosis in GC cells through activating caspas-3 and caspase-9 (Shang et al. 2019). Moreover, inactivation of NF-κB pathway suppressed the growth and cell viability and increased apoptosis through upregulating caspase-3 in glioma cells (Geeviman et al. 2018). Another study showed that 25-Hydroxycholestrol (25-HC) enhanced migratory and invasive capacities of gastric cancer cells via activating NF-κB signalling pathway (Wang et al. 2019). Furthermore, inactivation of NF-κB pathway reduced the cell viability in vitro and suppressed the metastasis and tumorigenesis of gastric cancer in vivo (Zheng et al. 2019). Also, hyperoside inhibited the viability and enhanced apoptosis in pancreatic cancer cells via suppressing NF-κB pathway (Li et al. 2016). However, whether hyperoside exerts its function in the modulation of NF-κB pathway in gastric cancer remains unknown. PTEN has been widely known for tumour suppressor ability and was researched in numerous types of cancers including gastric cancer (Yu et al. 2019). Also, PTEN suppresses the progression of tumour in gastric cancer via negatively regulating PI3K signalling pathways (Ma et al. 2017). Another study revealed that the repression of PTEN expression activated the NF-κB pathway in vivo and vitro model of breast cancer (Xie et al. 2018). Moreover, PTEN suppressed the pathogenesis of colon cancer by suppressing NF-κB pathway (Zhang et al. 2015). Recent research indicated that NF-κB pathway activation enhanced the cell viability via JAK2 pathway in vitro model of colon cancer (Fang et al. 2017). In this work, the researchers examined the protein concentration of PTEN and the phosphorylation level of NF-κB JAK2 in HGC-27 cells treated with hyperoside by using ELISA assay. The findings exposed that the protein concentration of PTEN and p-JAK2 were enhanced in hyperoside-treated HGC-27 cells while NF-κB/p65 protein concentrations were decreased in a concentration dependent manner.

Taken together, hyperoside could promote apoptosis and suppress cell viability via activating PTEN/JAK2 pathway and inhibiting NF-κB signalling pathway suggesting a promising role of hyperoside for diagnosing and treating GC.

CONCLUSION

Hyperoside treatment suppressed HGC-27 cell viabilities and facilitated cell apoptosis via inhibiting phosphorylated NF-κB/p65 and upregulating PTEN and JAK2, suggesting that hyperoside might be a promising drug to treat GC.

RECOMMENDATIONS

Hyperoside treatment restrained HGC-27 cell viabilities and promoted apoptosis. Hyperoside also suppressed Bcl-2 mRNA expressions but elevated Bax and caspase-3 mRNA expressions. Moreover, hyperoside reduced protein concen-

trations of phosphorylated NF-κB/p65 but increased PTEN and JAK2 protein concentrations.

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