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Liraglutide promotes apoptosis of HepG2 cells by activating JNK signaling pathway

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Abstract. – OBJECTIVE: To study the effect and mechanism of liraglutide on the apoptosis of human hepatocellular carcinoma HepG2 cells.

MATERIALS AND METHODS: HepG2 cell was treated with different concentrations of liraglutide at 0, 1, 10, 100, and 1000 nmol/L. The effect of liraglutide on HepG2 proliferation was detected by Cell Counting Kit-8 (CCK-8) method; the effect of liraglutide on the protein expression of c-Jun NH2-terminal Kinase (JNK) and phosphorylated JNK (p-JNK) was detected by Western blot; the degree of HepG2 apoptosis was observed by flow cytometry, and JNK pathway blocker SP600125 was used to further confirm that liraglutide promoted HepG2 apoptosis by regulating JNK signaling pathway.

RESULTS: The proliferation inhibition rate of HepG2 cells increased with time and the increase in the concentration of liraglutide. The proliferation inhibition rate was the strongest when cultured for 48 h, and the IC50 (half maximal inhibitory concentration) was about 100 nmol/L of liraglutide. 100 nmol/L liraglutide was selected as the intervention condition for subsequent use of SP600165. The apoptosis rate of HepG2 cells increased with the increase of liraglutide's concentration. The apoptosis rate of HepG2 cells at blocker SP600125+100 nmol/L liraglutide was significantly lower than that at 100 nmol/L liraglutide alone ($p<0.05$). There was no significant difference in the expression of JNK protein in HepG2 cells at different concentrations of liraglutide ($p>0.05$). There was no significant difference in the expression of JNK protein in HepG2 cells using JNK pathway blocker SP600125 ($p>0.05$), while using JNK pathway blocker SP600125 significantly up-regulated the expression of p-JNK protein in HepG2 cells than 100 nmol/L of liraglutide alone ($p<0.05$).

CONCLUSIONS: Liraglutide can promote the apoptosis of hepatocellular carcinoma HepG2 cells in a dose-dependent manner, and its mechanism may act by promoting the activation of the JNK signaling pathway.

Key Words
Liraglutide, JNK signaling pathway, HepG2, Apoptosis.

Introduction

The global incidence of liver cancer is the sixth in cancer, whose mortality rate is second among tumor diseases[1,2]. The incidence of liver cancer is related to the living standard, and which is about 83% in developing countries[3]. China is a big country with hepatitis B virus, and the infection rate is very high, about 10%[4]. However, it is well known that hepatitis B, cirrhosis and liver cancer are developed from viral hepatitis[5]. Therefore, it is of great significance to study the pathogenesis of liver cancer and to seek new targets for liver cancer treatment.

Liraglutide is a novel hypoglycemic drug and a Glucagon-like peptide (GLP-1) receptor agonist[(6)]. GLP-1 is an incretin that can continuously activate receptors to promote islet cell proliferation, inhibit apoptosis, delay gastric emptying, and promote insulin secretion[7,8]. A study[9] has found that liraglutide can inhibit the proliferation of pancreatic cancer cells and promote cell apoptosis by down-regulating the expression of GLP-1 and phosphorylation of its downstream signal proteins Extracellular Signal-regulated Kinase1/2 (Erk1/2) and PKB Protein Kinase (Akt).

c-Jun NH2-terminal Kinase (JNK) is a member of the mitogen-activated protein kinases (MAPKs) family and has a dual role in promoting or inhibiting different tumors. Inflammatory factors are involved in the activation of the JNK pathway promoted by liver damage, resulting in phosphorylation of JNK protein[10]. This indicates that the JNK signaling pathway has the complexity of regulating cell proliferation and apoptosis[(11,12)]. However, there is no report on the effect and mechanism of liraglutide on the biological function of hepatocellular carcinoma cells at present.

This article aims to study the drug treatment of liver cancer patients *in vitro*, and use JNK pathway blocker SP600125 to further verify the effect of liraglutide on HepG2 cells, and provide a theoretical reference for the clinical decision of patients taking liraglutide drugs.

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Materials and Methods

Cell Culture

Human hepatocellular carcinoma HepG2 cells (Shanghai Xinyu Biotechnology Co., Ltd., Shanghai, China; Item number: AD0101) were cryopreserved in liquid nitrogen at -180°C. The cell suspension was placed in Roswell Park Memorial Institute-1641 (RPMI-1640) cell culture medium (Beijing Solarbio Technology Co., Ltd., Beijing, China; item number: SH30809.01B), mixed, centrifuged at 1000 rpm for 5 min, and the supernatant was discarded, and the sample was inoculated in RPMI-1640 medium with 10% fetal bovine serum (Beijing Solarbio Technology Co., Ltd., Beijing, China; Item Number: 11011-6125), cultured at 37°C, inoculated at 4×10^5 /mL; when the cell confluence reached 80%, digested by trypsin (Shanghai Shifeng Biological Technology Co., Ltd., Shanghai, China; Item Number: EB04590) and passaged at 1:3, medium changed at 24 h, passaged at 72 h.

Experimental Methods

HepG2 cells cultured for 48 h were added with 0, 1, 10, 100, 1000 nmol/L liraglutide (Shanghai Kindu Biotechnology Co., Ltd., Shanghai, China; Item number: JD-42931) to study the effects of different concentrations of liraglutide on HepG2 cell proliferation, apoptosis, and JNK signaling pathway. Then, the optimal concentration of liraglutide (100 nmol/L) was selected, and HepG2+ 100 nmol/L of liraglutide +1 μM SP600125 (TargetMol China, Shanghai, China; Item number: T3109) was set as observation group; HepG2+100 nmol/L Liraglutide was used as the control group; SP600125 was intervened for 1 h in advance, further confirming that liraglutide promoted HepG2 apoptosis by regulating JNK signaling pathway.

Detection of Cell Counting Kit-8 (CCK-8) on Cell Proliferation Inhibition

The logarithmic growth phase HepG2 was inoculated into 96-well plates at a cell density of 1×10^5 cells per well, and cultured at 37°C until the logarithmic growth phase, and then, it was incubated with RPMI-1640 medium supplemented with 10% fetal bovine serum for 12 h at 37°C. The supernatant was discarded, and according to the above grouping, CCK-8 solution (10 μL/well) was added 24, 48, and 72 hours after administration respectively. Then, after 2 hours of continuous culture in the incubator, the light absorption value was measured at 450 nm by CLARIOstar microplate reader (HongKong Biogene Technology Co., Ltd., HongKong, China), measured 3 times per well to calculate the average value. Then, the cell growth inhibition rate was calculated according to the formula: cell survival rate (%) = (absorbance value of experimental group – absorbance value of control group) / (absorbance value of control group - absorbance value of blank group) *100%.

Detection of the Effect of Different Concentrations of Liraglutide on Apoptosis of HepG2 by Flow Cytometry

The logarithmic growth phase cells were inoculated into culture flasks. The cells were intervened according to the above grouping and drug concentration for 48 hours, and the cells were digested with trypsin and made into single cell suspension, they were rinsed twice using PBS (poly butylene succinate) (Shanghai LMAI Bioengineering Co., Ltd., Shanghai, China; Item No.: LM0221A), and next, they were centrifuged for 10 minutes at a speed of 1000 rpm. After centrifugation, the supernatant was removed and the cells were suspended, adding Annexin V-FITC solution (Xiamen Huijia Biotechnology Co., Ltd., Xiamen, China; item number: MC-251) 10 μL in the dark for 15 min; 300 μL Binding Buffer (Beijing Sobio Biotech Co., Ltd., Beijing, China; item number: R2131) and 5 μL PI (propidium iodide) (Beijing Chreagen Biotechnology Co., Ltd., Beijing, China; item number: 88378) was added to each well, detected at a wavelength of 488 nm by using Attune NxT flow cytometer (Jiangsu Bomeida Life Science Co., Ltd., Suzhou, China) and repeated for 3 times.

Detection of Protein Expression of JNK and Phosphorylated JNK (p-JNK) by Western Blot

The cells in logarithmic growth phase were obtained and washed with 5 mL of cold PBS and repeated 3 times. Appropriate amount of RIPA lysate (G-Clone (Beijing) Biotechnology Co., Ltd., Beijing, China; item number: EX6010-100 ml) containing 100 mM phenyl methane sulfonyl fluoride (PMSF; Shanghai LMAI Bioengineering Co., Ltd., Shanghai, China; Item Number: LM5005) was added and the sample was shaking on ice for 30 minutes to fully lyse the cells, and total protein was extracted. Total protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Shanghai LMAI Bioengineering Co., Ltd., Shanghai, China; Item Number: LM0014A) electrophoresis, polyvinylidene fluoride (PVDF) membrane (Suzhou Renold Biotechnology Co., Ltd., Suzhou, China; Item number.: 25900446) was closed at room temperature for 2 h after wet transfer, and JNK rabbit anti-human monoclonal antibody dilution and p-JNK (9H8) antibody (1:1000) was added, shaking for 30

min, incubated at 4°C overnight, and the membrane was washed with Tris-Buffered Saline Tween (TBST; Beijing Sobio Biotechnology Co., Ltd., Beijing, China; item number: T1085-500) 3 times, and then the goat-anti-rabbit IgG (H+L) secondary antibody dilution (1:1000) was added and incubated for 120 min at room temperature, and exposed in dark cells. β-actin (Shanghai Yodubio Biotechnology Co., Ltd., Shanghai, China; Item number: JC-409) was used as an internal reference. Grayscale scanning was performed by using ChemiScope chemiluminescence imaging system (Shanghai Genesci Medical Technology Co., Ltd., Shanghai, China), and the relative expression of the protein was determined, and the measurement was repeated three times.

Statistical Analysis

The analysis was performed by SPSS 17.0 statistical software (Shanghai Cabit Information Technology Co., Ltd., Shanghai, China). The measurement data were analyzed by the *t*-test. The comparison between groups was analyzed by the variance analysis. The comparison of different time points in the group was performed by repeated measures analysis of variance, and LSD test was used for multiple comparisons. $p<0.05$ indicated the difference was statistically significant.

Results

Effects of different concentrations of liraglutide on the proliferation inhibition rate of HepG2

The proliferation inhibition rate of HepG2 cells increased with time and the increase in the concentration of liraglutide. The inhibition rate of proliferation at 48 h was significantly higher than that at other time points at each concentration ($p<0.05$). IC50 (half maximal inhibitory concentration) of liraglutide was closest to 100 nmol/L after 48 hours of intervention. Therefore, 100 nmol/L liraglutide was subsequently selected as an intervention condition for apoptosis experiments and pathway validation by using SP600165 (Table I).

Effects of different concentrations of liraglutide and blocker SP600125 on apoptosis of HepG2 cells

The apoptosis rate of HepG2 cells increased with the concentration of liraglutide (Figure 1, Tables II, III).

Blocker SP600125+100 nmol/L liraglutide significantly reduced the apoptosis rate of HepG2 cells than 100 nmol/L liraglutide alone, and the difference was statistically significant ($p<0.05$) (Figure 2).

Effect of different concentrations of liraglutide on the relative expression of JNK and p-JNK in HepG2 cells and the effect of blocking agent SP600125 on the expression of JNK and p-JNK

There was no significant difference between the effect of different concentrations of liraglutide on the expression of JNK protein in HepG2 cells ($p>0.05$). There was no significant difference between the effect of JNK pathway blocker SP600125 on JNK protein expression in HepG2 cells and 100 nmol/L liraglutide on JNK protein expression in HepG2 cells ($p>0.05$).

The expression of the p-JNK protein in HepG2 cells decreased gradually with increasing dose concentration, but the expression of p-JNK protein in HepG2 cells was significantly higher than that in 100 nmol/L liraglutide-treated HepG2 cells after using JNK pathway blocker SP600125 ($p<0.001$) (Figures 3, 4 and Tables IV, V).

Table I. Effects of different concentrations of liraglutide on the proliferation inhibition rate of HepG2.

Concentration	Proliferation inhibition rate (%)				F	*p*
	12 h	24 h	36 h	48 h		
0 nmol/L	0	0	0	0		
1 nmol/L	5.63±0.13	11.46±0.78*	19.31±0.86*#	21.25±1.13*#&	237.100	<0.001
10 nmol/L	12.98±0.14	18.78±0.86*	26.52±1.42*#	39.37±1.28*#&	354.000	<0.001
100 nmol/L	20.34±0.83	27.13±0.97*	44.33±1.35*#	50.86±1.24*#&	491.900	<0.001
1000 nmol/L	32.75±1.53	43.34±0.85*	59.34±1.32*#	70.78±1.15*#&	555.700	<0.001
F	523.400	747.500	617.000	895.800		
p	<0.001	<0.001	<0.001	<0.001		

Note: *compared with 12 h, $p<0.05$; # compared with 24 h, $p<0.05$; & compared with 36 h, $p<0.05$.

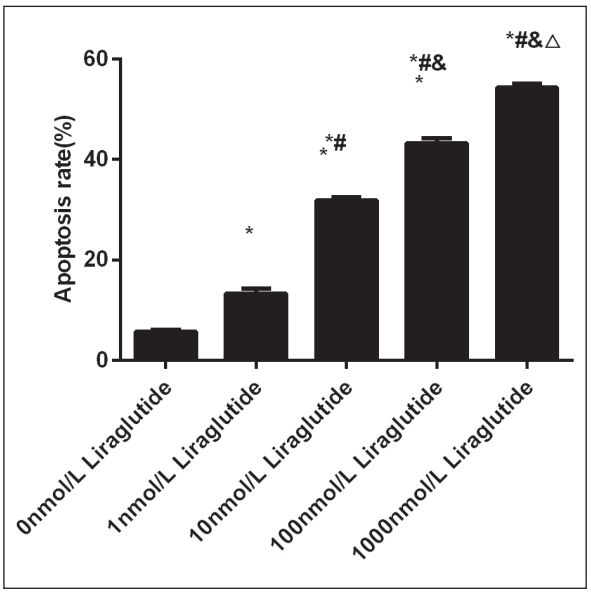

Figure 1. Effect of different concentrations of liraglutide on apoptosis of HepG2 cells. The results of flow cytometry showed that the effects of 0-1000 nmol/L of liraglutide on apoptosis of HepG2 cells were significantly different (p<0.001), and the apoptosis rate of HepG2 cells increased with the concentration of liraglutide. *p<0.05, compared with 0 nmol/L liraglutide; $^{\#}p$<0.05, compared with 1 nmol/L liraglutide; $^{\&}p$<0.05, compared with 10 nmol/L liraglutide; $^{\Delta}p$<0.05, compared with 100 nmol/L liraglutide.

Table II. Effect of different concentrations of liraglutide on apoptosis of HepG2 cells.

Group	Apoptosis rate (%)
0 nmol/L liraglutide	5.71±0.34
1 nmol/L liraglutide	7.28±0.97*
10 nmol/L liraglutide	21.85±0.68*$^{\#}$
100 nmol/L liraglutide	43.21±0.96*$^{\#\&}$
1000 nmol/L liraglutide	44.33±0.77*$^{\#\&\Delta}$
F	1366.000
p	<0.001

Note: *compared with 0 nmol/L liraglutide, p<0.05; $^{\#}$compared with 1 nmol/L liraglutide, p<0.05; $^{\&}$compared with 10 nmol/L liraglutide, p<0.05; $^{\Delta}$compared with 100 nmol/L liraglutide, p<0.05.

Table III. Effect of blocker SP600125 on apoptosis of HepG2 cells.

Group	Apoptosis rate (%)
Control	43.21±0.96
Observation	28.78±0.94
t	18.600
p	<0.001

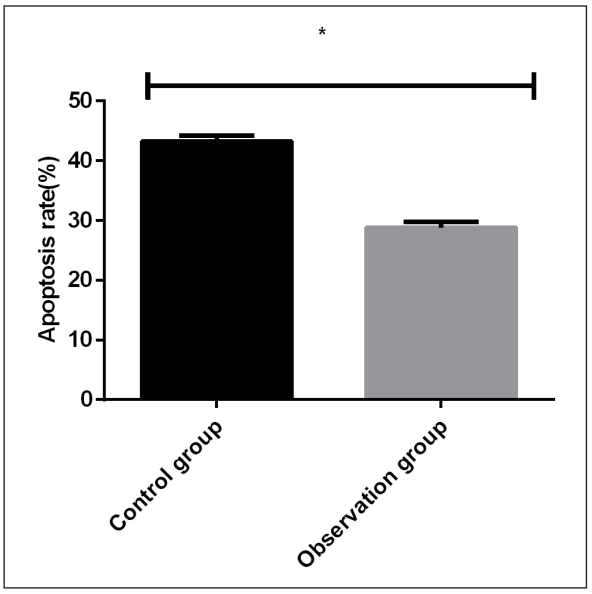

Figure 2. Effect of JNK pathway blocker SP600125 on apoptosis of HepG2 cells. The results of flow cytometry showed that the apoptotic rate of HepG2 cells at SP600125+ 100 nmol/L liraglutide was significantly lower than that at 100 nmol/L liraglutide alone, and the difference was statistically significant (p<0.05). *, p<0.05.

Discussion

Liver cancer is one of the common malignant tumors. The occurrence and development of liver cancer are usually caused by the proliferation of cancer cells and the decrease of apoptosis. However, the signaling pathway of liver cancer plays an important role in the development of tumors[14,15]. It has been found[16,17] that MAPK signaling pathway is not only involved in the development of inflammation and tumor, but also plays a regulatory role in the growth, proliferation, and metastasis of various tumors. It is known that the MAPK family and the PI3K/Akt family signaling pathway are closely related to apoptosis, and JNK is a kinase in the MAPK family that plays an important role in apoptosis[18]. According to the literature[19], JNK can promote the development of hepatocellular carcinoma and has a regulatory effect on the proliferation, apoptosis, invasion, and metastasis of hepatoma cells. Under a variety of cellular stress responses, JNK can be activated by MAP kinase kinase (MKK)4/7 to phosphorylate, and activated JNK binds to the amino terminus of transcription factors, activates the corresponding transcription factors and enhances its transcriptional activity, thereby regulating cell proliferation and apoptosis[20]. At present, the mechanism of liraglutide in improving

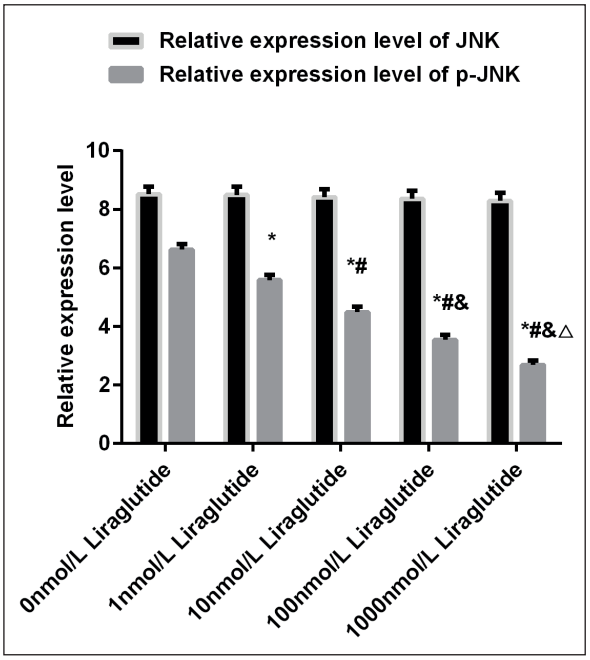

Figure 3. Expression of JNK and p-JNK proteins in HepG2 cells at different concentrations of liraglutide. Western blot showed that there was no significant difference in the expression of JNK protein in HepG2 cells at 0-1000 nmol/L liraglutide (p>0.05). The expression of p-JNK protein in HepG2 cells decreased with the increase of the dose concentration of liraglutide at 0-1000 nmol/L. *p<0.05, compared with 0 nmol/L liraglutide; $^{\#}p$<0.05, compared with 1 nmol/L liraglutide; $^{\&}p$<0.05, compared with 10 nmol/L liraglutide; $^{\Delta}p$<0.05, compared with 100 nmol/L liraglutide.

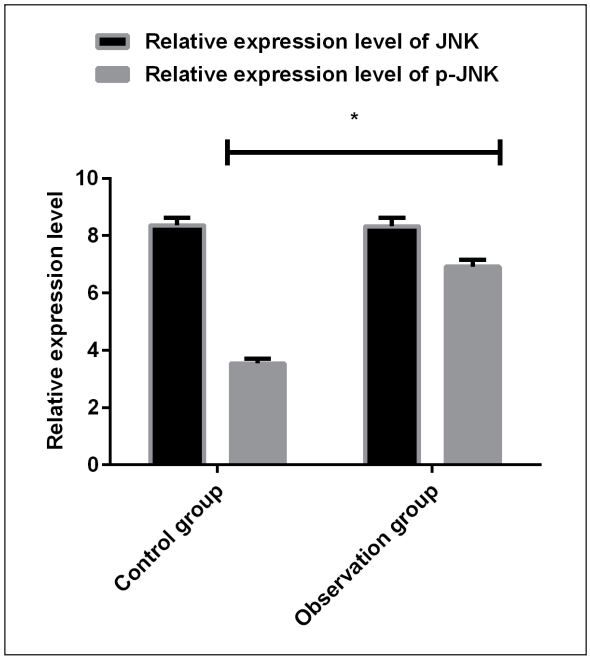

Figure 4. Effect of JNK pathway blocker SP600125 on JNK and p-JNK expression. Western blot analysis showed that the effect of JNK pathway blocker SP600125 on JNK protein expression in HepG2 cells was not significantly different from that of 100 nmol/L liraglutide (p>0.05). The expression of JNK protein in HepG2 cells was significantly higher than that of p-JNK protein by using 100 nmol/L liraglutide and SP600125+100 nmol/L liraglutide (p<0.05). *, p<0.05.

liver cancer has not been clarified, which may be related to inhibition of inflammatory response and reduction of insulin resistance[21]. According to reports in the literature, liraglutide can inhibit the proliferation of MCF-7 human breast cancer cells, and promote their apoptosis by down-regulating the expression of miR-27a[22]. Therefore, this paper studied the effect and mechanism of liraglutide on apoptosis of liver cancer HepG2 cells.

Firstly, different concentrations of liraglutide were used to intervene in HepG2 cells; the growth tendency was observed, then the optimal concentration was selected for subsequent experiments. The results showed that the proliferation inhibition rate of HepG2 cells gradually increased with time and the liraglutide's concentration. The inhibition rate was the strongest at 48 h of culture. Zhang et al[23] selected five concentrations of liraglutide at 0, 1, 10, 100, and 1000 nmol/L to intervene in cardiac microvascular endothelial cells, and 100 nmol/L of liraglutide were selected as intervention conditions for subsequent experiments, which is consistent with our conclusion. SP600125 is a selective JNK antagonist that can reduce the expression of p-JNK, and inhibit the activation of the JNK signaling pathway, which is a common tool for studying the JNK signaling pathway[13]. Therefore, we used the flow cytometry to detect the effect of different concentrations of liraglutide on the apoptosis of HepG2, and JNK we used the pathway blocker SP600125 to test the

Table IV. Relative expression of JNK and p-JNK in HepG2 cells.

Concentration	JNK relative expression	p-JNK relative expression
0 nmol/L	8.52±0.25	6.62±0.19
1 nmol/L	8.49±0.28	5.58±0.18*
10 nmol/L	8.41±0.29	4.49±0.18*$^{\#}$
100 nmol/L	8.36±0.27	3.55±0.16*$^{\#\&}$
1000 nmol/L	8.29±0.28	2.69±0.15*$^{\#\&\Delta}$
F	0.322	253.000
p	0.890	<0.001

Note: *compared with 0 nmol/L liraglutide, p<0.05; $^{\#}$compared with 1 nmol/L liraglutide, p<0.05; & compared with 10 nmol/L liraglutide, p<0.05; $^{\Delta}$compared with 100 nmol/L liraglutide, p<0.05.

Table V. Effect of blocker SP600125 on the expression of JNK and p-JNK.

Group	JNK relative expression	p-JNK relative expression
Control	8.36±0.27	3.55±0.16
Observation	8.33±0.29	6.92±0.24
t	0.131	20.240
p	0.902	<0.001

effect of liraglutide on HepG2 apoptosis. The results were as follows: the apoptosis rate of HepG2 cells increased with the liraglutide's concentration. Blocker SP600125+100 nmol/L liraglutide significantly reduced the apoptosis rate of HepG2 cells than 100 nmol/L liraglutide alone. This result is basically consistent with the results of Qing et al[24]. They found that JNK signaling pathway blocker SP600125 can significantly reduce the apoptotic rate in the intervention of neuroblastoma cells by trimethyltin chloride.

Our results showed that there was no significant difference in the expression of JNK protein in HepG2 cells at different concentrations of liraglutide ($p>0.05$). There was no significant difference in the effect of JNK pathway blocker SP600125 on the expression of JNK protein in HepG2 cells ($p>0.05$). However, the expression of the p-JNK protein in HepG2 cells treated with JNK pathway blocker SP600125 was significantly higher than that in HepG2 cells treated with 100 nmol/L liraglutide ($p<0.001$). Our results are basically consistent with the researches of Zhang et al[25] and Natalicchio et al[26]. In the mouse experiment, Zhang et al found that liraglutide can down-regulate the JNK pathway and the inflammatory factors TNF-α and NF-KB65, which improves non-alcoholic fatty liver. Natalicchio et al[27] found that exenatide can inhibit JNK activation. Since the two GLP-1 receptor agonists, liraglutide and exenatide, are mainly used clinically, it is suggested that GLP-1 receptor agonists can inhibit the JNK signaling pathway. The use of JNK pathway blocker SP600125 significantly up-regulated the expression of the p-JNK protein in HepG2 cells ($p<0.05$), further verifying our results. However, a study[28] indicated that the proliferation of HepG2 cells was reduced after liraglutide treatment without changing the level of oxidative stress. However, it was found that liraglutide may inhibit the proliferation of HepG2 cells by inducing autophagy and senescence through the increase of TGF-β1, which is different from our conclusion. We speculate that the effect of liraglutide on HepG2 cells may be due to its influence on multiple signaling pathways, which needs to be further explored in the future.

Conclusions

We found that liraglutide enhanced the apoptosis of HepG2 cells in a dose-dependent manner, and its mechanism may act by promoting the activation of the JNK signaling pathway. However, there is no relevant literature to further verify the conclusions of this study. In the follow-up experiments, we will further verify this, and hope that many scholars can carry out relevant experiments.

Conflict of Interest

The authors declare that they have no competing interests.

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