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Leptin promotes proliferation and inhibits apoptosis of prostate cancer cells by regulating ERK1/2 signaling pathway

C.-J. XU[1], L.-L. DONG[2], X.-L. KANG[1], Z.-M. LI[3], H.-Y. ZHANG[4]

[1]Department of Urology, Hainan General Hospital, Hainan Affiliated Hospital of Hainan Medical University, Haikou, China
[2]Department of Oncology, Yantai Yuhuangding Hospital, Yantai, China
[3]Department of Health Management, PLA Rocket Force Characteristic Medical Center, Beijing, China
[4]Laboratory Medicine, Linyi Tumor Hospital, Linyi, China

Congjie Xu and Liangliang Dong contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of leptin (Lep) on the proliferation, invasion and apoptosis of prostate cancer cells through the extracellular regulated protein kinase 1/2 (ERK1/2) signaling pathway.

MATERIALS AND METHODS: Prostate cancer DU145 cells in the logarithmic growth phase were randomly divided into Lep (10, 20, 40, 80, 160 and 320 ng/mL) groups and blank control (Con) group. After culture, the cells were treated for 6 h, 12 h and 24 h, respectively. The effects of Lep on the proliferation and invasion of DU145 cells were detected via methyl thiazolyl tetrazolium (MTT) assay and transwell chamber assay, respectively. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out to examine the messenger ribonucleic acid (mRNA) expressions of ERK1/2, b-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) in DU145 cells after Lep treatment for 24 h. Thereafter, immunofluorescence assay was performed to detect the localization of ERK1/2 protein in prostate cancer DU145 cells. In addition, the expressions of phosphorylated (p)-ERK, ERK1/2 and apoptosis-related proteins, Bcl-2, Bax and cleaved cysteinyl aspartate specific proteinase (c-Caspase 3) in prostate cancer DU145 cells after treatment with different concentrations of Lep for 24 h were examined by Western blotting.

RESULTS: MTT assay results showed that the proliferation rate of DU145 cells increased significantly at 6 h, 12 h and 24 h after 5-320 ng/mL of Lep treatment ($p<0.05$). Transwell assay manifested that the number of invasive cells was significantly raised after Lep treatment for 24 h ($p<0.05$). Meanwhile, the invasion ability of cells increased gradually with the elevation of Lep concentration. Subsequent qRT-PCR results demonstrated that after treatment with different concentrations of Lep, the mRNA expressions of ERK1/2 and Bcl-2 rose markedly ($p<0.05$). However, the mRNA expression of Bax was remarkably down-regulated ($p<0.05$) with the increase of Lep concentration in a concentration-dependent manner. According to the detection using a laser scanning confocal microscope, ERK1/2 red fluorescence showed punctiform aggregation, which was gradually raised with the increase of Lep concentration for 24 h. Moreover, Western blotting results denoted that with the increase of Lep concentration, the protein expressions of p-ERK, ERK1/2 and Bcl-2 were notably elevated ($p<0.05$), while those of Bax and c-Caspase 3 were distinctly reduced ($p<0.05$).

CONCLUSIONS: Lep activation induces the proliferation, promotes the invasion and inhibits the apoptosis of prostate cancer cells through the ERK1/2 signaling pathway.

Key Words:
Leptin, Prostate cancer, ERK1/2, Proliferation.

Introduction

Prostate cancer is a common malignant tumor caused by prostatic epithelial lesions. According to different pathological types, it is mainly classified into urothelial cancer, ductal adenocarcinoma, acinar adenocarcinoma, adenosquamous carcinoma and squamous cell carcinoma. Prostate cancer ranks second among male malignant tumors worldwide, showing a year-by-year up-

Corresponding Author: Haiyan Zhang, BM; e-mail: zhanghaiyan1111@126.com

trend. Epidemiological statisticians estimate that there will be 1.7 million new cases in 2030[1]. In China, with the aging of the population, people's lifestyle is constantly changing, and their living standards are improving year by year. However, it is noteworthy that the incidence rate of prostate cancer also displays a year-by-year upward trend. Currently, prostate cancer ranks sixth in terms of the incidence and death rates among male malignant tumors in China. Prostate cancer is triggered by the interaction of multiple signaling pathways [including the PI3K/PTEN/Akt/mTOR and Raf/MEK/extracellular regulated protein kinase (ERK) signaling pathways] between epithelial cells and mesenchymal cells. These signaling pathways exert crucial roles in the occurrence and development of prostate cancer. Meanwhile, they also provide new therapeutic ideas and effective targets for its treatment and outcome[2-5]. Nevertheless, the function and relevant mechanisms of ERK, a vital target of prostate cancer, in the occurrence, development, metastasis and invasion of prostate cancer remain elusive. Several studies have suggested that the ERK1/2 signaling pathway may be a potential kinase target in prostate cancer. In prostate cancer cell models, E26 Transformation-Specific (ETS) variant transcription factors are able to record MAPK transcription program without ERK kinase[6]. However, clinical proteomics data manifest that ERK1/2 phosphorylation is strong and frequent in prostate cancer, which is in line with the direct activation of the kinase[7].

ERKs, pivotal enzymes in cells, can transmit signals from cell surface to the nucleus. They include two substructures, namely ERK1 and ERK2. ERK1/2 can be activated by cell growth factors, hydrogen peroxide and some external stress conditions (such as particle radiation). Afterwards, this can phosphorylate ERK protein and enter the nucleus to perform function[8]. The occurrence and development of tumor cells have been confirmed closely correlated with the ERK1/2 signaling pathway. Activated ERK signaling pathway can facilitate normal cells to transform into malignant tumors, suppress the apoptosis of tumor cells, and promote the progression of cell cycle[9]. After surgical treatment, the activity of ERK in tumor tissues is 2-18 times higher than that in normal tissues. Meanwhile, it tends to increase continuously, indicating that ERK is closely associated with the invasion and metastasis of diverse tumor cells[10]. When ERK inhibitor PD98059 is applied to SKOV3 cells, the toxicity of cisplatin to SKOV3 cells can be increased. Furthermore, the apoptosis of SKOV3 cells can be promoted after the inhibition of ERK1/2 activity[11].

Leptin (Lep), a protein hormone secreted by adipose tissues, can inhibit adipocyte synthesis. Lep is encoded by OB gene on chromosome 7, consisting of 167 amino acids with a size of 17 kDa. Lep mainly regulates appetite and body weight[12]. Meanwhile, it exerts effects on the pathogenesis of different cancers, including breast cancer, ovarian cancer, hepatocellular carcinoma, colorectal cancer and pancreatic cancer[13-15]. Nevertheless, the relationship between Lep and prostate cancer has not been fully elucidated. Although many factors can influence the occurrence and development of prostate cancer, there are few reports on the effects of Lep on the proliferation, invasion and apoptosis of prostate cancer cells through the ERK pathway. Therefore, the exact role of Lep on the ERK1/2 pathway in prostate cancer cells remains elusive. In this study, we aimed to explore the effects of the ERK signaling pathway on the proliferation, invasion and apoptosis of prostate cancer cells through the intervention of Lep. All our findings might help to provide certain theoretical and experimental bases for the treatment of prostate cancer.

Materials and Methods

Experimental Materials

Prostate cancer DU145 cell line was purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China), glycine, methylene acrylamide and Hoechst33342 fluorescent dyes from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China), human Lep from Sigma-Aldrich (St. Louis, MO, USA), rabbit anti-human ERK1/2 polyclonal antibody from Invitrogen (Carlsbad, CA, USA), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS) and trypsin from Hyclone (South Logan, UT, USA), a polyvinylidene difluoride (PVDF) membrane from Milipore (Billerica, MA, USA), and methyl thiazolyl tetrazolium (MTT) from Life Technologies (Gaithersburg, MD, USA).

An electronic analytical balance (Mettler Toledo, Columbus, OH, USA), a CO_2 thermostatic cell incubator (Sanyo, Osaka, Japan), a high-speed desktop frozen centrifuge (Hermle, Reichenbach,

Germany), an inverted optical microscope (Leica, Wetzlar, Germany), a clean bench (HDL Apparatus, Beijing, China), a digital gel imaging system (Shanghai Tanon Science & Technology Co., Ltd.), a laser scanning confocal microscope (Leica, Wetzlar, Germany), a Model-680 microplate reader (Bio-Rad, Hercules, CA, USA), a decolorizing shaker (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China), an ultrasonic cell pulverizer (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China), a high temperature/pressure steam sterilizer (Shanghai Boxun Co., Ltd., Shanghai, China), a water bath pot (Shanghai Jinghong Experimental Equipment Co., Ltd., Shanghai, China), protein electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), and protein tans-blot apparatus (Bio-Rad, Hercules, CA, USA).

Methods

Cell Culture

Prostate cancer DU145 cells, adherent cells, were cultured in RPMI-1640 medium containing 10% of newborn fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a cell incubator with 50% CO_2 at 37°C. The cells were digested by 0.25% trypsin for passage once every 2 days.

MTT Assay

After digestion by 0.25% trypsin, prostate cancer DU145 cells in the logarithmic growth phase were collected into a centrifuge tube and washed with RPMI-1640 culture solution. After diluted into 1×10^9/L cell suspension, they were inoculated into 96-well plates (200 μL/well). Subsequently, the cells were cultured in serum-free RPMI-1640 culture medium in an incubator with 50% CO_2 at 37°C for 24 h. Next, the cells were divided into blank control (Con) group and Lep (10, 20, 40, 80, 160 and 320 ng/mL) groups for subsequent experiments, with 5 replicate wells in each group. After culture for 6 h, 12 h and 24 h, respectively, 100 μL of MTT was added to each well for continuous culture for 4 h. Then, the supernatant was discarded, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. Following vibration at room temperature for 10 min, optical density (OD) at the wavelength of 492 nm in each well was measured using a micro-plate reader. Cell proliferation rate = $[(OD_{control} - OD_{blank}) - (OD_{experimental} - OD_{blank})]/(OD_{control} - OD_{blank}) \times 100\%$.

Transwell Chamber Assay

24-well transwell chambers were coated with 50 μL of Matrigel, and the plates were incubated in an incubator with 50% CO_2 at 37°C for 30 min. Next, cells were seeded into plates at a density of 4.5×10^4/well, with a total volume of 250 μL per well. Then, they were randomly divided into four groups, including: Con group, 40 ng/mL Lep group, 80 ng/mL Lep group and 160 ng/mL Lep group. After culture in an incubator with 50% CO_2 at 37°C for 24 h, the cells were fixed with 4% formaldehyde solution for 30 min, stained by crystal violet for 20 min, and rinsed with clear water. The number of invasive cells passing through Boyclen cell carbonate membranes was finally counted under a microscope.

Fluorescence Quantitative Polymerase Chain Reaction (qPCR)

After digestion by trypsin, the cells were collected into a centrifuge tube. Total ribonucleic acids (RNAs) in cells were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The purity and concentration of RNAs were determined by an ultraviolet spectrophotometer. Reverse transcription (RT) was carried out according to the RT kit instructions. Using complementary deoxyribose nucleic acids (cDNAs) as templates, target genes were amplified according to the instructions of qRT-PCR kit. Fluorescence qPCR system was composed of 10 μL of qPCR Master Mix (2×), 1 μL of PCR forward primer, 1 μL of PCR reverse primer, 2 μL of cDNAs and 6 μL of ddH_2O. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences for qRT-PCR were listed in Table I.

Immunofluorescence Assay

Coverslips (10 mm × 10 mm) were first placed in 24-well plates. Then, cells were inoculated into the 24-well plates at 1×10^8/L and 500 μL/well, followed by culture in an incubator with 50% CO_2 at 37°C overnight. When cell density reached 80%, they were randomly assigned into Con group, 40 ng/mL Lep group, 80 ng/mL Lep group and 160 ng/mL Lep group. Next, the cells were continuously cultured for 24 h. After discarding the culture medium, 200 μL of 4% paraformaldehyde was added for 10 min of fixation. Afterwards, the cells were washed with 0.1% Triton-100 phosphate-buffered saline (PBS) for 5 min and sealed with goat serum for 30 min. Then, the cells were incubated with corresponding ERK1/2 antibodies at 4°C overnight.

Table I. Primer sequences for qPCR.

Primer	Sequence
ERK1/2	5'-GAACTCCAAGGGCTATACCAAGT-3' 5'-GGAGGGCAGAGACTGTAGGTAGT-3'
B-cell lymphoma 2 (Bcl-2)	5'-CCGGGAGATCGTGATGAAGT-3' 5'-ATCCCAGCCTCCGTTATCCT-3'
Bcl-2-associated X protein (Bax)	5'-GTGGTTGCCCTCTTCTACTTTG-3' 5'-CACAAAGATGGTCACTGTCTGC-3'
GAPDH	5' AGGTCGGTGTGAACGGATTTG 3' 5' TGTAGACCATGTAGTTGAGGTCA 3'

After washing with PBS, the cells were incubated with corresponding HRP-labeled goat anti-mouse antibody for 30 min, followed by washed again with PBS. The cells were then incubated with 50 μL of DIPA for 5 min, washed by PBS and excited with the wavelength of 632 nm after mounting. Finally, the cells were observed under a laser confocal microscope and photographed.

Western Blotting

After trypsinization, the cells were collected into a centrifuge tube and washed with PBS for 3 times. After centrifugation, the supernatant was discarded. 200 μL of radioimmunoprecipitation assay (RIPA) lysate containing phenylmethylsulfonyl fluoride (PMSF) was added for 30 min of lysis (Beyotime, Shanghai, China). The supernatant was collected as the total protein of cells. Subsequently, protein loading buffer was added, mixed well and boiled for 10 min. Next, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After sealing with 5% skim milk powder solution at room temperature for 1 h, the membranes were incubated with primary antibodies (diluted at 1: 1000) at 4°C overnight. On the next day, the membranes were washed with Tris Buffered Saline and Tween-20 (TBST) for 3 times, with 5 min each time. Next, they were incubated with corresponding diluted secondary antibody containing horseradish peroxidase (1:5000) at 37°C for 1 h. Immuno-reactive bands were exposed by color developing solution. Quantitative analysis was finally carried out using ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was adopted for all statistical analysis. Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Impacts of Lep on Prostate Cancer DU145 Cell Proliferation Detected Via MTT Assay

The impacts of different concentrations of Lep (10, 20, 40, 80, 160 and 320 ng/mL) on prostate cancer DU145 cell proliferation were detected by MTT assay after they were added for 6 h,12 h and 24 h, respectively. The results (Figure 1) revealed that Lep significantly promoted the growth of prostate cancer DU145 cells within a certain range ($p<0.05$) in time- and dose- dependent manners.

Impacts of Lep on Prostate Cancer DU145 Cell Invasion

As shown in Figure 2, after DU145 cells were treated with Lep for 24 h, the number of invasive cells increased obviously ($p<0.05$). Compared

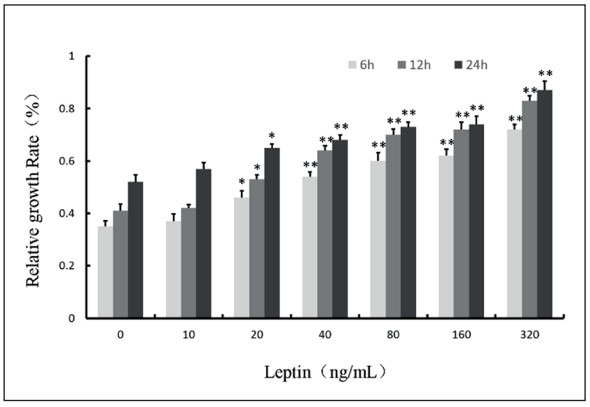

Figure 1. Impacts of different concentrations of Lep on the proliferation of prostate cancer DU145 cells. Note: *$p<0.05$ and **$p<0.01$ *vs.* Con group (0 ng/mL Lep group).

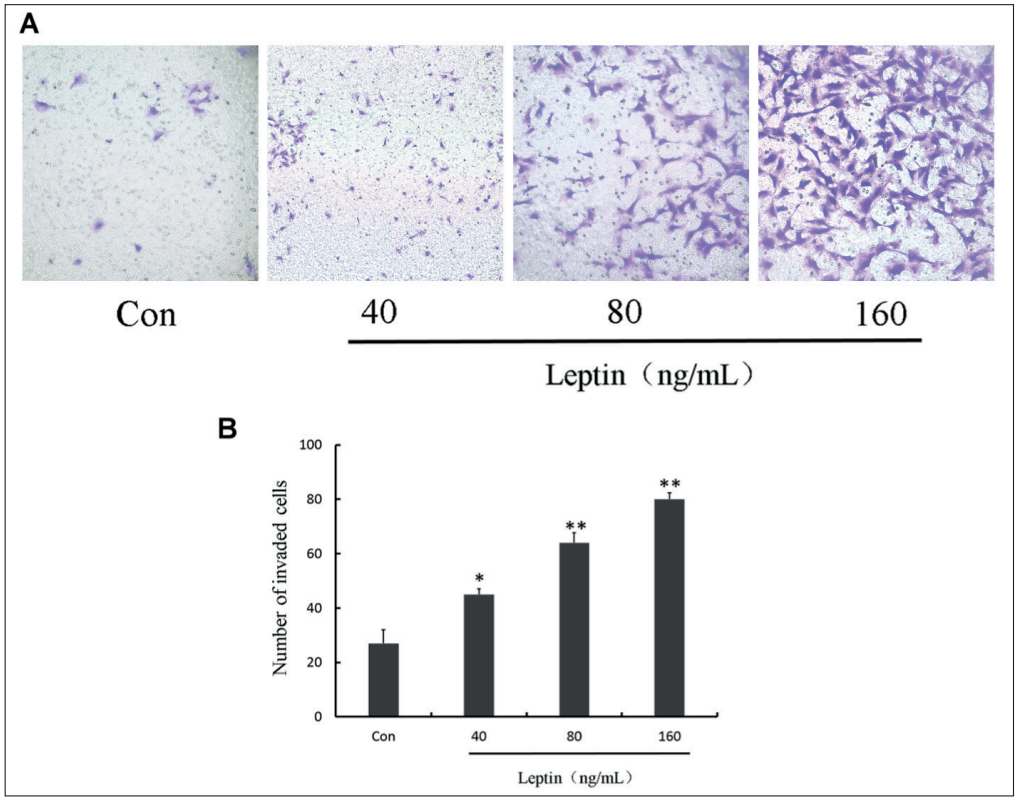

Figure 2. Morphology of prostate cancer DU145 cells in each group. **A,** Invasion pattern of prostate cancer DU145 cells in each group. (magnification: 200×) **B,** Quantitative analysis of invasive cells. Note: *p<0.05 and **p<0.01 *vs.* Con group.

with Con group, the number of invasive cells increased remarkably in 40 ng/mL Lep group (p<0.05) and rose extremely notably in 80 ng/mL and 160 ng/mL Lep groups (p<0.01).

Messenger RNA (mRNA) Expression Changes of ERK1/2, Bcl-2 and Bax In Prostate Cancer DU145 Cells

According to the results (Figure 3), in comparison with Con group, 40 ng/mL Lep group exhibited an obviously reduced mRNA expression of Bax (p<0.05). 80 ng/mL Lep group exhibited extremely notably raised mRNA expressions of ERK1/2 (p<0.01) and Bcl-2 (p<0.05), and significantly markedly decreased mRNA expression of Bax (p<0.01). Meanwhile, 160 ng/mL Lep group displayed extremely considerably elevated mRNA expressions of ERK1/2 and Bcl-2 (p<0.01), and extremely evidently decreased mRNA expression of Bax (p<0.01).

Localization of ERK1/2 Protein In Prostate Cancer DU145 Cells In Each Group

Subsequent results using the laser confocal microscope revealed that compared with Con

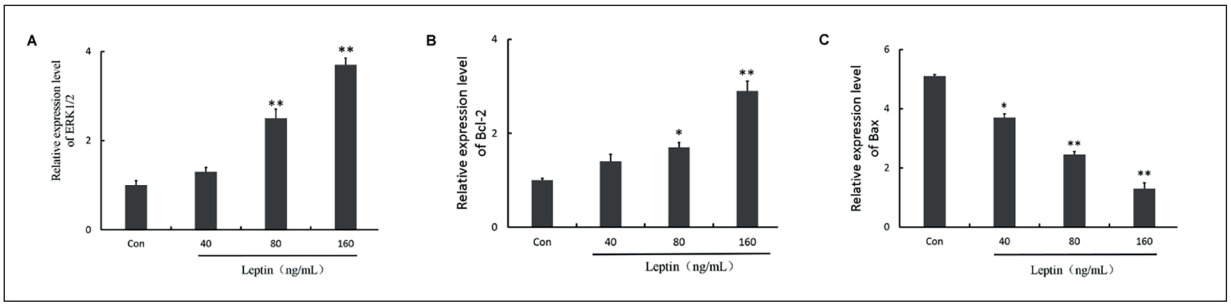

Figure 3. MRNA expression changes of ERK1/2, Bcl-2 and Bax in prostate cancer DU145 cells in each group. **A-C,** Represented tmRNA expression changes of ERK1/2, Bcl-2 and Bax, respectively. Note: *p<0.05 and **p<0.01 *vs.* Con group.

group, the punctiform aggregation of ERK1/2 red fluorescence appeared. It was gradually aggravated after different concentrations of Lep treatment on prostate cancer DU145 cells for 24 h in Lep groups. In 160 ng/mL Lep group, the punctiform aggregation of ERK1/2 was distinctly aggravated (Figure 4).

Effects of Lep on the protein expressions of ERK1/2, Bcl-2, Bax and cysteinyl aspartate specific proteinase (Caspase) 3 in prostate cancer DU145 cells

As displayed in Figure 5, in comparison with Con group, the protein expression of Bcl-2 extremely rose ($p<0.01$), while the protein expressions of Bax and Caspase 3 were markedly reduced ($p<0.05$) in 40 ng/mL Lep group. In 80 ng/mL Lep group, the protein expressions of ERK1/2 and Bcl-2 increased significantly ($p<0.01$). Meanwhile, the protein expression of Bax rose remarkably ($p<0.05$), whereas the protein expression of Caspase 3 extremely prominently decreased ($p<0.01$). In 160 ng/mL Lep group, the protein expressions of ERK1/2 and Bcl-2 were extremely up-regulated ($p<0.01$), whereas those of Bax and Caspase 3 extremely significantly declined ($p<0.01$).

Discussion

Prostate cancer is a common malignant tumor in Europe and the United States, which seriously endangers the life and health of men. Currently, the incidence and mortality rates rank highest among male malignant tumors[16]. In China, with the rise in life expectancy, the serious aging of population, the change of diet structure and the improvement of living standards, the incidence and mortality rates of prostate cancer are increasing year by year. Meanwhile, affected patients show a year-by-year younger trend[17]. The pathogenesis of prostate cancer remains not very clear. Currently, it is mainly treated by surgery, endocrine therapy and radiotherapy. However, the therapeutic effect is far from satisfactory. There-

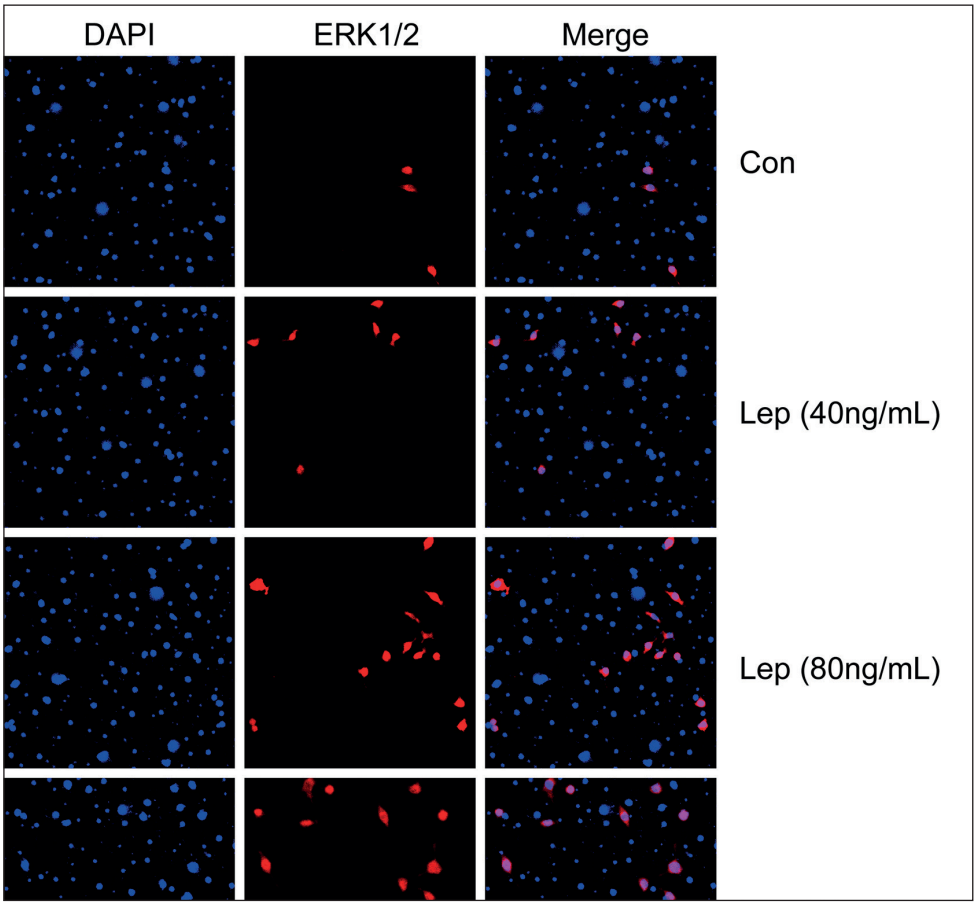

Figure 4. Localization of ERK1/2 protein in prostate cancer DU145 cells in each group, (magnification: 200×).

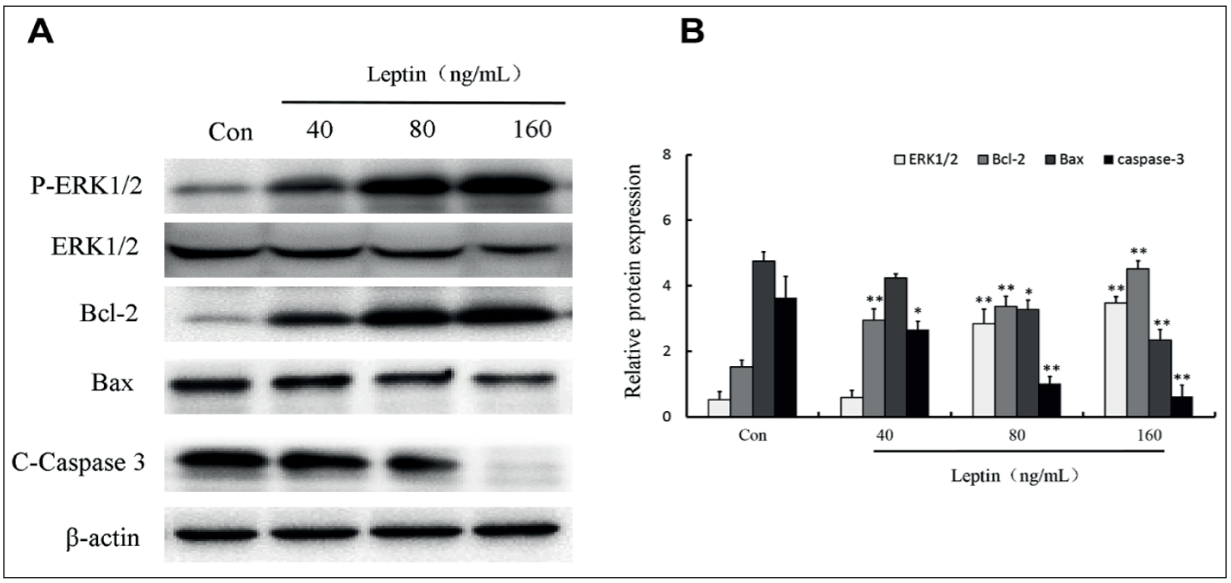

Figure 5. Protein expressions of ERK1/2, Bcl-2, Bax and Caspase 3 in prostate cancer DU145 cells in each group. Note: *p<0.05 and **p<0.01 *vs.* Con group.

fore, searching for novel targets and effective treatment methods for prostate cancer is the top priority in advanced medicine.

In this study, we aimed to explore the influences of the ERK1/2 signaling pathway on the proliferation and apoptosis of prostate cancer DU145 cells through treatment with different concentrations of Lep. When different concentrations of Lep acted on prostate cancer DU145 cells for 6 h, 12 h and 24 h, the proliferation rate of prostate cancer DU145 cells was significantly promoted, showing a concentration- and time-dependent manner. It can be seen that Lep facilitates the proliferation of prostate cancer DU145 cells. Transwell cell assay results manifested that the number of invasive cells increased significantly after Lep treatment on prostate cancer DU145 cells for 24 h. Meanwhile, the invasion ability of cells increased gradually with the elevation of Lep concentration. This indicates that Lep is able to promote the invasion ability of prostate cancer DU145 cells. However, the action mechanism is still unknown. Hence, the mRNA expression of ERK1/2 in prostate cancer DU145 cells was detected *via* qRT-PCR. It was found that Lep could influence the mRNA expressions of ERK1/2, anti-apoptosis proteins, Bcl-2 and Bax, in prostate cancer DU145 cells. After ERK1/2 protein was indirectly labeled *via* immunofluorescence in DU145 cells, it was observed under a laser confocal microscope that ERK1/2 protein showed evident aggregation after Lep treatment. It was aggravated remarkably in 160 ng/mL Lep group, with nuclear localization. The above results suggest that Lep promotes the proliferation of prostate cancer DU145 cells through the ERK1/2 signaling pathway. The above conclusion was finally verified by Western blotting.

Nevertheless, the exact mechanism of Lep in influencing the proliferation and apoptosis of prostate cancer DU145 cells may also be associated with other signaling pathways. Exogenous NO can activate messenger conduction through NO-cGMP pathway to stimulate prostate cancer ALAV-31 cell growth[18]. Meanwhile, it can also probably modulate the expression of cell proliferation-related genes during cell signal conduction, eventually contributing to the growth of prostate cancer cells. However, NO released by high-concentration SNP can up-regulate p21 expression and arrest the proliferation cycle of prostate cancer cells at G1 phase through its protein expression. Ultimately, this suppresses the proliferation and induces the apoptosis of prostate cancer cells[19]. In this study, only the influence of Lep on the ERK signaling pathway was verified, with no clarification of its effects on other signaling pathways. Our findings will lay an experimental foundation for further research on the mechanism of Lep in the proliferation and apoptosis of prostate cancer.

Conclusions

In short, lep activation induces the proliferation, promotes the invasion and inhibits the apoptosis of prostate cancer cells through the ERK1/2 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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