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Research Article

1-Hydroxy-3,7,8-Trimethoxyxanthone Suppresses the Malignant Proliferation of Human Bone Marrow Mesenchymal Stem Cells in Colon Cancer Microenvironment

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Abstract

Background and Objective: Human bone marrow mesenchymal stem cells (HMSCs) have been widely used to study tumor gene therapy. However, they may be induced malignant proliferation in some cancers. Therefore, how to decrease the side effects of HMSCs has been becoming a hot topic. In this study, the role of 1-hydroxy-3,7,8-trimethoxyxanthone (HTX) on HMSCs in colon cancer microenvironment *in vitro* was evaluated. **Materials and Methods:** Non-contact co-culture system of colon cancer cells SW480 and HMSCs was established using Transwell cell culture chambers. 20, 10, 5 μg mL^{-1} of HTX was used to treat HMSCs in the colon cancer microenvironment. The proliferation of HMSCs in each group was tested using the MTT assay. The activity of HMSCs in each group was tested using Transwell invasion and migration assays. RT-PCR assay, Western Blot assay and ELISA assay were used to detect the expression of related genes and proteins. **Results:** In colon cancer microenvironment, the proliferation, invasion and migration of HMSCs significantly increased, however, supplemented with HTX isolated from Tibetan medicine *Gentianopsis paludosa* significantly improved the side effects. Furthermore, HTX inhibited the expression of IL-6 and the phosphorylation of STAT3 signalling pathway in HMSCs which was reduced by colon cancer microenvironment. **Conclusion:** All the results indicated that HTX inhibited the malignant proliferation of HMSCs in colon cancer microenvironment by inhibiting IL-6/STAT3 signaling pathway. The HTX may be a novel chemical inhibitor for gene therapy of colon cancer with HMSCs as carrier.

Key words: 1-hydroxy-3,7,8-trimethoxyxanthone (HTX), malignant proliferation, human bone marrow mesenchymal stem cells (HMSCs), colon cancer

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colon cancer is one of the most common digestive system malignant tumor in the world[1] and the second most common cause of cancer-related death in the United States[2]. The standard therapy for colon cancer is surgical treatment and followed by adjuvant radiochemotherapy[3]. However, the therapeutic effect is not entirely satisfactory. Therefore, it is urgently needed to develop a novel treatment strategy for colon cancer. Tumor gene therapy had been reported to treat various cancers such as colon cancer[4], liver cancer[5], lung caner[6], ovarian cancer[7] and glioma[8] both *in vitro* and *in vivo*. Human bone marrow mesenchymal stem cells (HMSCs) had been used as an ideal vector for tumor gene therapy since the favorable characteristics of easy separation and culture *in vitro*[8], induction by exogenous genes and drugs[9-11], migration to tumor microenvironment[12] and low immunogenicity[13]. Despite a series of advances has been obtained in the study of HMSCs as a vector cell for gene therapy, the safety of HMSCs in clinical application is still controversial. Previous studies have shown that the proliferation and migration of HMSCs significantly increased in certain cancer, including lung cancer[14], gastric cancer[15] and gliomas[16]. The results indicated that the tumor microenvironment improved the proliferation and migration of HMSCs and even the malignant transformation. However, there is not literature on the effect of colon cancer microenvironment on HMSCs at present. In this study, HMSCs undergone significant malignant proliferation in colon cancer microenvironment and a natural compound was explored to inhibit the side effects of HMSCs.

1-hydroxy-3,7,8-trimethoxyxanthone (HTX) is a natural xanthone that was obtained from Tibetan medicine *Gentianopsis paludosa* and used to treat colon fibrosis, liver cancer, promyelocytic leukemia[17-19]. The previous studies have demonstrated that *Gentianopsis paludosa* inhibits proliferation and improves the apoptosis of SW480 cells[20,21]. Therefore, HTX may have the effect of inhibiting malignant transformation of HMSCs in colon cancer microenvironment.

In recent years, the interleukin-6 (IL-6) mediated signal transducer and activator of transcription (STAT3) signaling has attracted more attention in anti-cancer research. The sustained activation of IL-6/STAT3 signaling is related to tumor angiogenesis, immunosuppression, tumor invasion and metastasis[22] and the over activation of STAT3 signaling lead to colon cancer[23-25]. Therefore, the study is to investigate whether HTX inhibits the malignant proliferation of HMSCs in colon cancer microenvironment through IL-6/STAT3 signaling.

To verify the hypothesis, a non-contact co-culture model of colon cancer cell line SW480 and HMSCs was established by the transwell chamber to simulate colon cancer microenvironment and treated with HTX. This study may provide a potential chemical inhibitor for gene therapy of colon cancer with HMSCs as carrier.

MATERIALS AND METHODS

Drugs: *Gentianopsis paludosa* (Lintan County, Gansu province) was identified as *Gentianopsis paludosa* (Munro) Ma. by professor Chen Yuan of Gansu Agricultural University. The HTX was isolated from the ethyl acetate extract of *Gentianopsis paludosa* according to the method of Lu *et al*.[18] and detected by silica gel TLC method and purified by recrystallizing. The compound was dissolved in dimethyl sulfoxide to 1 mg mL^{-1}. In the preliminary experiments, 20 μg mL^{-1} or less of HTX inhibited human colon cancer cells line (SW480) proliferation and promoted HMSCs proliferation. Therefore, 20, 10 and 5 μg mL^{-1} of HTX was used to treat the cells.

Cell culture: The SW480 cells and HMSCs were purchased from cell bank of Chinese Academy of Science in Shanghai and ScienCell Research Laboratories in America, respectively. The SW480 cells were seeded into the dulbecco's Modified Eagle Medium (DMEM) (HyClone, America) containing 1% Penicillin/Streptomycin (HyClone, America) and 10% fetal bovine serum (Clark, America)[26], the HMSCs were seeded into the Mesenchymal Stem Cell Medium (MSCM, America). The cells were cultured at 37°C with 5% CO_2.

Cell co-culture system: The DMEM and MSCM (ratio, 3:7) were mixed and added to the upper and lower chamber of trans well cell culture chambers (0.4 μm pore size) for pre-processing. The fourth-generation HMSCs (1×10^5 cells per well) were seeded into the bottom chamber and cultured for 24 h. When cells adherence occurred, the SW480 cells (5×10^5 cells per well) were seeded into the upper chamber. Then 20, 10 and 5 μg mL^{-1} of HTX were added into the upper chamber as dose groups. Meanwhile, the HMSCs and the co-culture of HMSCs and SW480 cells (co-HMSCs) were acted as the control group and model group, respectively. All groups were cultured for 7 days.

Cell proliferation detection: The inhibitory effect of HTX on co-HMSCs proliferation was determined using MTT assay.

Briefly, HMSCs in each group were firstly cultured in 96 well plate for 1, 2, 3, 4, 5 and 6 days. Freshly prepared 20 μL of MTT (5 mg mL^{-1}, Solarbio, Beijing, China) solutions were added to the cells and incubated for 4 h. About 150 μL of dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) was added into each well to dissolve the formazan crystal[27]. The optical densities (OD) value of the product was measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc, America) and DMSO wells were set as blank control group.

Transwell invasion and migration detection: Transwell invasion and migration assays were carried out in 24 well modified Boyden chambers (8 μm pore size, Corning, America) pre-coated with (invasion) or without (migration) Matrigel (Solarbo, Beijing, China)[27]. HMSCs (1×10^5 cells per well) of each group in serum-free MSCM were seeded onto the trans-membrane in the upper chamber with 10% FBS MSCM in the lower chamber as a chemoattractant. About 24 h later, HMSCs that had migrated through the membrane were fixed with 4% fixative solution (Solarbo, Beijing, China) and stained with 0.1% crystal violet solution (Solarbo, Beijing, China). Then the cells were counted under magnification in 5 randomly selected high power fields with a microscope (Thermo Fisher Scientific, America).

Real time reverse transcription-polymerase chain reaction (RT-PCR): The total RNA of HMSCs in each group was extracted by TRIzol Reagent Kit (Thermo Fisher Scientific, America) and reverse transcribed to cDNA with a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, America). The cDNA was amplified with an amplification reaction kit (Go t*aq*@ qPCR master mix) and an FTC-3000P RT-PCR system (Funglyn Biotech Inc, Canada)[23]. The RNA primers (Takara Bio Inc, Japanese) used were (all 5′-3′): STAT3 forward primer:

- GCAGCTGACTACACTGGCAGAGA and reverse primer
- ATTGTCCAGCCAGACCCAGAA, Bcl-XL forward primer
- GAGTGACGAGTTTGTGGACTCCT and reverse primer
- CAAGTTCCGATCCCACCAG, CyclinD1 forward primer
- GTGCATCTACACCGACAACTCC and reverse primer
- GTTCCACTTGAGCTTGTTCACC, β-actin forward primer
- GGAGATTACTGCCCTGGCTCCTA and reverse primer
- GACTCATCGTACTCCTGCTTGCTG. RT-PCR conditions: 94°C for 2 min, 95°C for 15 sec, 60°C for 60 sec, 60°C for 15 sec, 40 cycles, 3 replicates were set for each sample. The expression of target genes was normalized to phosphoglycerate kinase 1 (PGK-1) using the $2^{-\Delta\Delta Ct}$ method

Enzyme-linked immunosorbent assay (ELISA): The IL-6 protein levels were measured by human IL-6 ELISA kit (Neobioscience, Shenzhen, China) according to the manufacturer's instructions[23]. Different concentrations of IL-6 standard solution were prepared to make the standard curve. OD values were measured at 560 nm using an enzymatic marker (Bio-Rad Laboratories, Inc, America).

Western blot: Total protein in HMSCs was extracted using a high-efficiency RIPA Tissue/Cell Lysates (Solarbo, Beijing, China). The protein concentration of each group was detected using BCA protein concentration assay kit (Solarbo, Beijing, China). The total protein (10 μg) denatured at 100°C for 10 min, then it was loaded onto 7.5-10% SDS-PAGE gels (Solarbo, Beijing, China) and electrotransferred to polyvinylidene fluoride (PVDF) membranes[28]. After blocking in 5% non-fat milk for 2 h, the membranes were incubated with primary antibodies against P-STAT3 (1:2,000), CyclinD1 (1:5,000), Bcl-XL (1:1,000) or GAPDH (1:5,000) overnight at 4°C and with the secondary antibody (1:5,000) for 2 h. The protein bands were analyzed by the western blot detection system (Azure biosystems, America).

Statistical analysis: The SPSS 18.0 software was used for statistical analysis. All data were presented as Means±Standard Deviation (SD). Statistical significance was determined using analysis of one-way ANOVA. Correlations were analyzed by Pearson's correlation analysis. The $p<0.05$ were considered statistically significant.

RESULTS

Morphological analysis: As shown in Fig. 1, SW480 cells were dispersed and shaped like short spindles (Fig. 1a). HMSCs in the normal control group were dispersed and shaped like long spindles or polygon (Fig. 1b). The proliferative ability of co-HMSCs in the model group was significantly higher than HMSCs in the normal control group (Fig. 1c). After treatment with HTX, the proliferation of HMSCs was inhibited in a dose-dependent manner (Fig. 1d-f).

Effect of HTX on the proliferation of co-HMSCs: The MTT assay was used to measure the effect of different dose of HTX (20, 10, 5 μg mL^{-1}) on co-HMSCs. As shown in Fig. 2, comparing with HMSCs in the normal control group, the proliferation level of HMSCs in the model group was significantly promoted ($p<0.05$). Supplemented with HTX significantly inhibited the proliferation of co-HMSCs in a dose-dependent manner. These results showed that

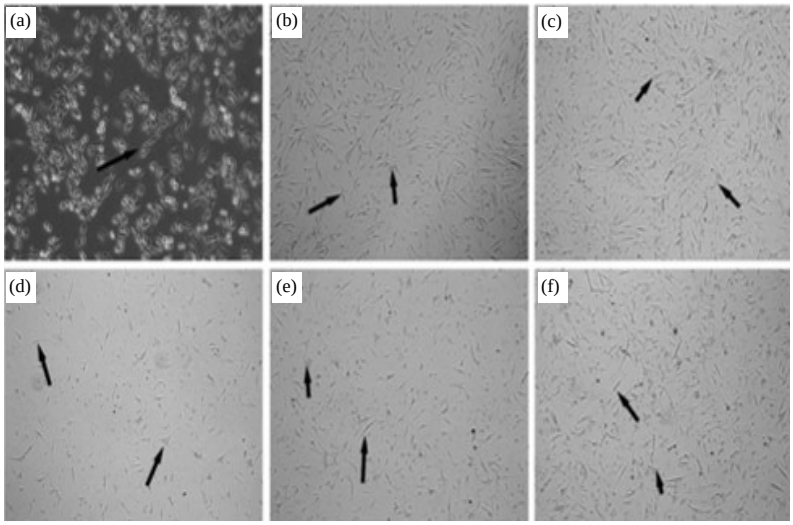

Fig. 1(a-f): Morphological analysis of SW480 cells and HMSCs: (a) SW480, (magnification, ×100, scale bar: 100 μm), (b) HMSCs in normal control group (magnification, ×200, scale bar: 200 μm), (c) Co-HMSCs in model group (magnification, ×200, scale bar: 200 μm) and (d-f) Co-HMSCs treated with HTX (20, 10, 5 μg mL^{-1}, respectively) (magnification, ×200 scale bar: 200 μm)

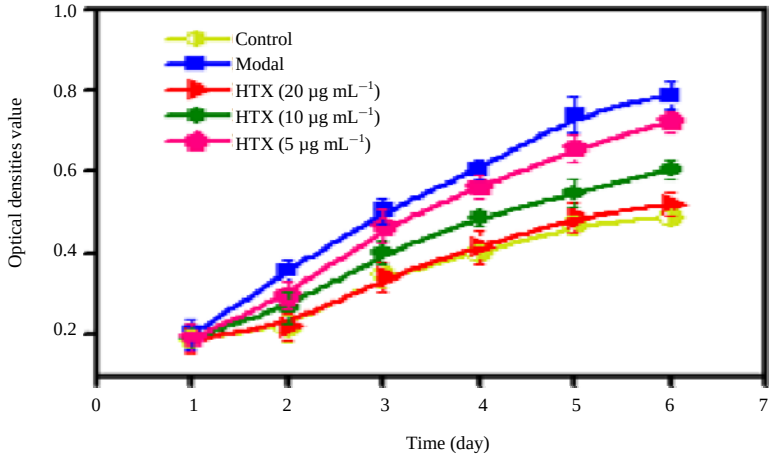

Fig. 2: Cell growth curve of HMSCs in each group

co-HMSCs grew significantly faster than HMSCs and HTX can effectively suppress their development.

Effect of HTX on the invasion and migration of co-HMSCs: Transwell invasion and migration assay were used to determine the effects of HTX on regulating the metastasis of HMSCs cells. In transwell invasion assay, HMSCs in the control group had little cell invasion (Fig. 3a), however, the number of HMSCs cells in the model group significantly improved ($p<0.05$). After treatment with 20, 10 and 5 μg mL^{-1} of HTX, the number of co-HMSCs decreased in a dose-dependent manner ($p<0.05$) (Fig. 3b). In transwell migration assay, compared with the control group, the model group had more number of HMSCs ($p<0.05$) (Fig. 3c). After treatment with different concentration of HTX, the number of HMSCs cells significantly decreased ($p<0.05$) (Fig. 3c-d). The results showed that the invasion and migration of co-HMSCs were significantly promoted and HTX observably suppressed the invasion and migration of co-HMSCs.

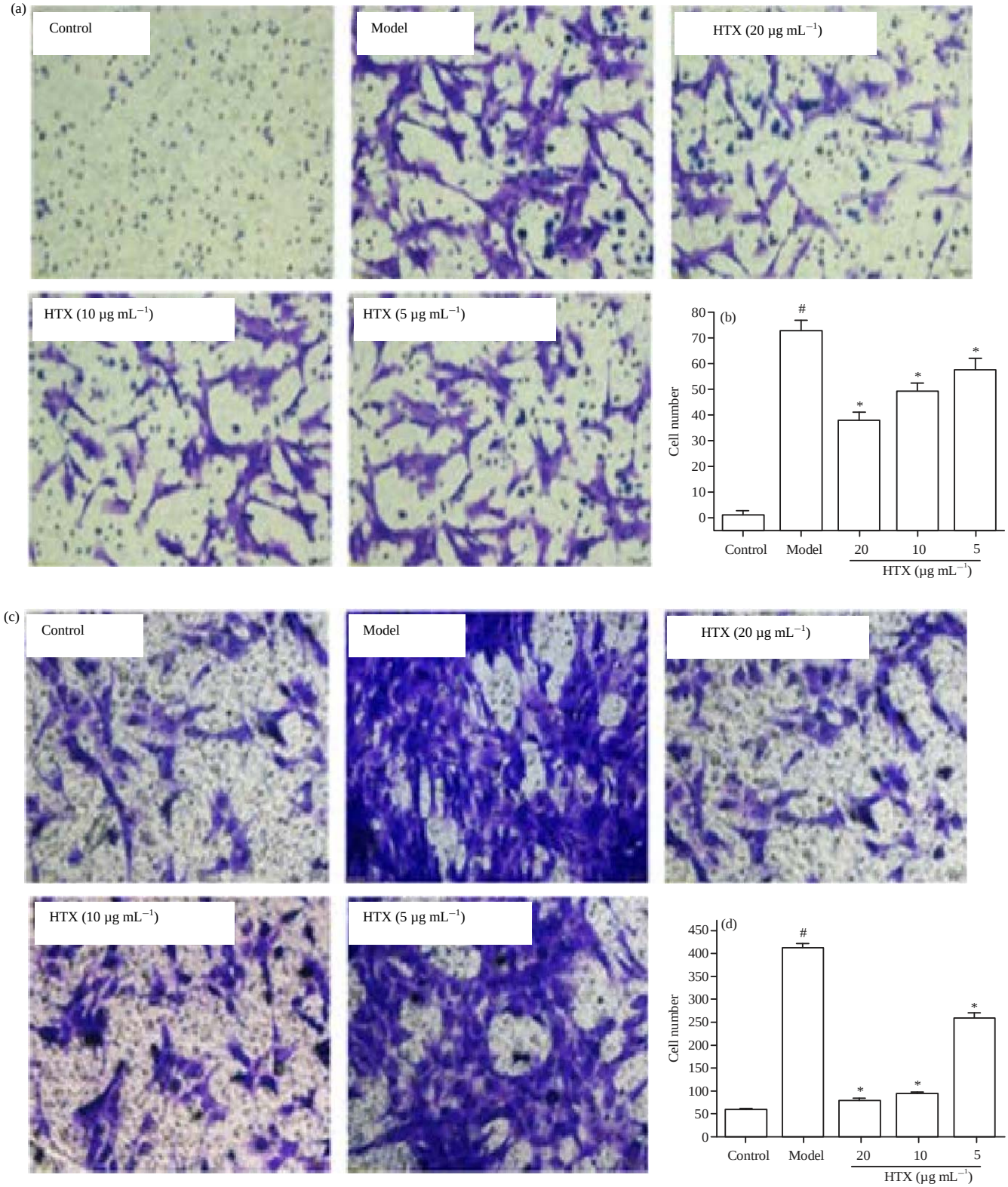

Fig. 3(a-d): Cell invasion and migration assay of HMSCs in each group, (a-b) Cell invasion test and data analysis and (c-d) Cell migration test and data analysis

Compared with the control group, the difference in the model group was statistically significant (#p<0.05), Compared with the model group, the difference in the HTX group was statistically significant (*p<0.05)

Effects of HTX on STAT3, Cyclin D1 and Bcl-XL expression: After treatment with different concentration of HTX for 7 days, RT-PCR analysis was used to detect the mRNA expression of signaling gene (STAT3) and downstream genes (Cyclin D1 and Bcl-XL) in HMSCs cells. The results showed that the mRNA expression of STAT3, Cyclin D1 and Bcl-XL significantly increased in the model group compared with the control group ($p<0.05$). After treatment with HTX, the expression of STAT3, Cyclin D1 and Bcl-XL decreased in a dose-dependent manner ($p<0.05$) (Fig. 4a-c). Next, the IL-6 protein level of HMSCs was measured by ELISA assay. The results showed that supplemented with HTX significantly decreased the IL-6 protein level induced by co-HMSCs ($p<0.05$) (Fig. 5a-b). To further investigate the effects of HTX on the expression of P-STAT3, Cyclin D1 and Bcl-XL, western blot was used to measure the protein expression of P-STAT3, Cyclin D1 and Bcl-XL. The results showed that compared with the normal control group, the protein expression of P-STAT3, Cyclin D1 and Bcl-XL in model group significantly increased.

Supplemented with HTX dramatically decreased the protein expression of P-STAT3, Cyclin D1 and Bcl-XL (Fig. 6a-d).

DISCUSSION

HMSCs as a vector of tumor gene therapy have been carried out in multiple tumors[4-8]. The cells maintain potential original multi-directional differentiation and stably and sustainably express the foreign genes after gene modification. After transfection and culture *in vitro*, HMSCs can survive and migrate to tumor tissue *in vivo*. The low immunogenicity of HMSCs reduce the occurrence of immune rejection after

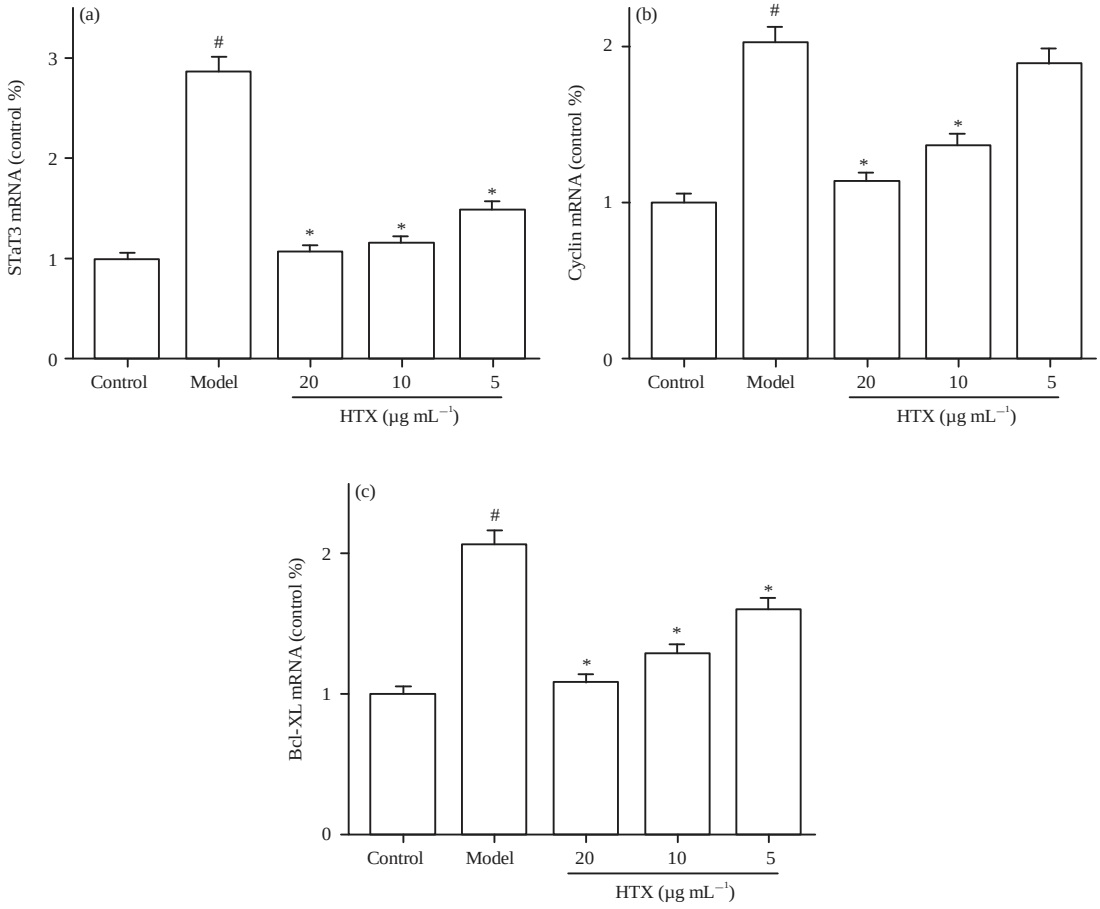

Fig. 4(a-c): mRNA expression analysis of HMSCs in each group by RT-PCR, (a) mRNA expression of STAT3, (b) mRNA expression of Cyclin D1 and (c) mRNA expression of Bcl-XL

Compared with the control group, the difference in the model group was statistically significant (#$p<0.05$), Compared with the model group, the difference in the HTX group was statistically significant (*$p<0.05$)

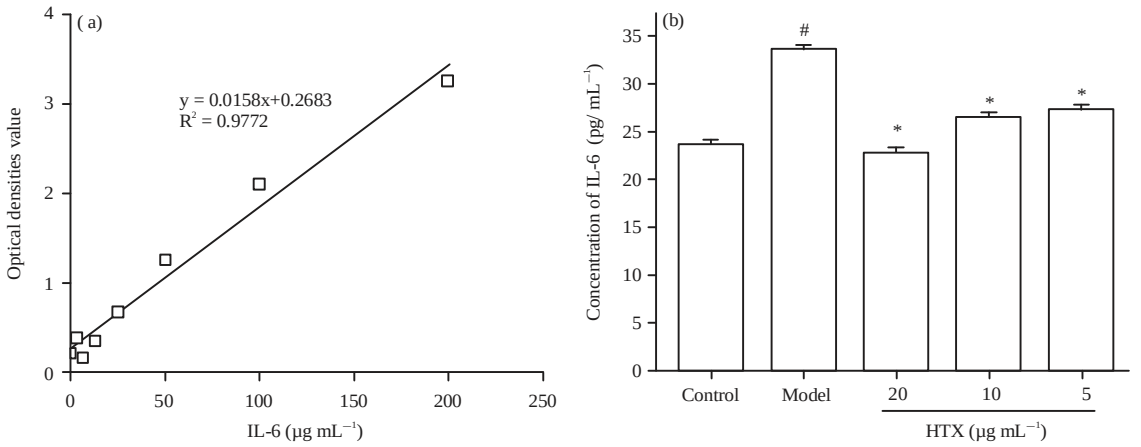

Fig. 5(a-b): IL-6 expression analysis of HMSCs in each group, (a) Standard curve of IL-6 detected by ELISA assay and (b) Detection the expression of IL-6 protein by ELISA assay

Compared with the control group, the difference in the model group was statistically significant (#p<0.05). Compared with the model group, the difference in the HTX group was statistically significant (*p<0.05)

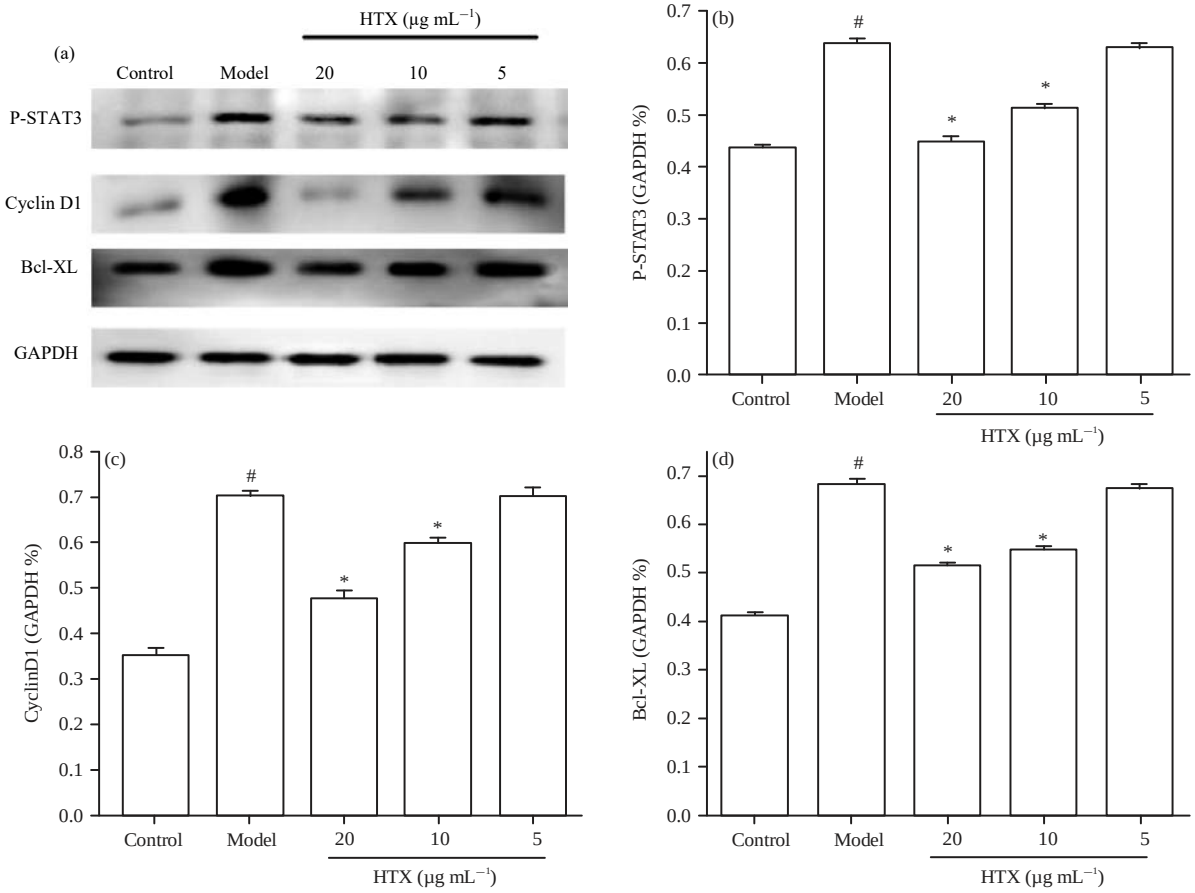

Fig. 6(a-d): Proteins expression analysis of HMSCs in each group, (a) Detection the protein expression of P-STAT3, Cyclin D1 and Bcl-XL in HMSCs before and after HTX treatment by Western Blot, (b) Quantitative analysis of P-STAT3 protein, (c) Quantitative analysis of Cyclin D1 protein and (d) Quantitative analysis of Bcl-XL protein

Compared with the control group, the difference in the model group was statistically significant (#p<0.05), Compared with the model group, the difference in the HTX group was statistically significant (*p<0.05)

transplantation. HMSCs can be transplanted into human body after gene modification, which avoid the immune rejection caused by allogeneic transplantation. Although the advantages of HMSCs as ideal vector cells for gene therapy, the clinical safety of HMSCs has attracted more attention in recent years. Previous studies showed that HMSCs cells would undergo malignant transformation or even carcinogenesis induced by tumor microenvironment[14-16], which is an unfavorable factor for the clinical application of HMSCs in gene therapy.

In this study, the transwell chamber was used to simulate the tumor microenvironment of HMSCs *in vitro*. SW480 cells were added to the upper chambers and HMSCs cells were added to the lower chambers. The cytokines secreted by SW480 cells freely passed through the semipermeable membrane of the transwell chamber to affect the development of HMSCs. Both micromorphological analysis and MTT assay showed that the proliferation of HMSCs cells was promoted in colorectal microenvironment. Cell invasion and migration assays showed that the regulation of metastasis of HMSCs was implicated in colorectal microenvironment. These results indicated that colorectal microenvironment improved the malignant proliferation of HMSCs. Therefore, a natural compound was explored to inhibit the side effects of HMSCs.

The previous studies have demonstrated that *Gentianopsis paludosa* inhibits proliferation and improves the apoptosis of SW480 cells[20,21]. The HTX also has been proved to possess an inhibitory effort on colorectal precancerous lesions: colon fibrosis[18]. In this study, HTX was further confirmed to suppress the malignant proliferation of HMSCs in colon cancer microenvironment in a dose-dependent manner.

Previous studies showed that IL-6/STAT3 signaling regulates the proliferation, invasion and metastasis of many kinds of tumors, including liver cancer, colon cancer, lung cancer, pancreatic cancer[23-25,29]. However, the effect of IL-6/STAT3 signaling on the malignant proliferation of HMSCs in colon cancer microenvironment has not been reported. In this study, the expression of IL-6 in the co-culture system was significantly increased than the normal control. After treatment with HTX, the expression of IL-6/STAT3 signaling was significantly inhibited. As one of the cell cycle regulators, the overexpression of Cyclin D1 is a characteristic of many human primary tumors, which is of great significance to the diagnosis and prognosis of human tumors[30]. Over expression of apoptosis suppressor gene Bcl-XL inhibits apoptosis pathway[31]. The low expression of Bcl-XL protein increase cell apoptosis and inhibit cell growth in colon cancer. In this study, the expression of cyclin D1 and Bcl-XL of HMSCs in colon cancer microenvironment was higher than that the normal control group and HTX inhibited the expression of cyclin D1 and Bcl-XL.

CONCLUSION

This study discovers that HTX can inhibit the malignant proliferation and metastasis of HMSCs in the colon cancer microenvironment by regulating the IL-6/STAT3 signaling pathway.

SIGNIFICANCE STATEMENT

This study will help the researcher to uncover the critical areas of gene therapy inhibitor of colon cancer with HMSCs as carrier that many researchers were not able to explore. Thus a new theory on HTX as an inhibitor of malignant transformation of HMSCs may be arrived at.

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