Inhibiting IL-6/STAT3/HIF-1α signaling pathway suppressed the growth of infantile hemangioma

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Research

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Abstract

Purpose

This study aimed to evaluate the expression of Interleukin 6 (IL-6) in IH patients and investigate the role of IL-6/signal transducers and activators of transduction-3 (STAT3)/hypoxia inducible factor-1α (HIF-1α) pathway in the progression of infantile hemangioma (IH).

Methods

Serum samples obtained from IH patients and normal infants were measured for IL-6 expression. Hemangioma-derived stem cells (HemSCs) were transfected with siRNAs targeting IL-6, HIF-1α or STAT3. And then, cell-viability assay and wound healing assay were conducted. After that, the tumor mouse model of HemSCs was established. The *in vivo* anticancer effect of IL-6 inhibitor was investigated.

Results

IH patients had much higher IL-6 levels as comparing to the healthy controls (P=0.005). HemSCs transfected with IL-6 siRNA had significantly lower viability and migration rate than normal HemSCs. And HemSCs transfected with STAT3 siRNA or HIF-1α siRNA had the similar tendency. On tumor bearing mice, IL-6 inhibitor treatment significantly delayed the tumor growth. Compared with control group, Caspase 3 was significantly increased in IL-6 inhibitor group (P<0.05), whereas Ki67 was decreased in IL-6 inhibitor group (P<0.05). In TUNEL assay, IL-6 inhibitor group had much higher apoptosis rate than control (P<0.05).

Conclusion

Our findings indicated that inhibiting IL-6/STAT3/HIF-1α signaling pathway could suppress IH growth.

Introduction

Infantile hemangioma (IH) is a common vascular tumor during infancy caused by endothelial cell (EC) proliferation(1). It often appears on head or face at approximately 2 weeks of age(2). The tumor rapidly proliferates within 6-10 months, and slowly regress over several years(1). Although IH is a benign tumor, it can cause facial deformation, vision obstruction, and can be life-threatening(3). Thus, studying the mechanisms of IH and finding the appropriate treatment strategies are necessary.

It has been reported that many cytokines were elevated in IH patients including Interleukin 6 (IL-6)(2, 4). IL-6 is a pleiotropic cytokine secreting by various cell types such as immune cells and tumor cell, playing an important role in tumor growth(5). IL-6 related pathways were reported to be associated with epithelial-

mesenchymal transition (EMT)(6) and immune reaction in tumor microenvironment(7). Furthermore, the role of IL-6 signaling pathway on angiogenesis has also been previously studied. Zegeye et al. found that IL-6 could regulate the sprouting angiogenic function of ECs(8). Hegde et al. demonstrated that IL-6 induced the expression of vascular endothelial growth factor receptor 2 (VEGFR2) in endothelial cells, leading to the disorder of angiogenesis in breast cancer(9). Signal transducers and activators of transduction-3 (STAT3) is an important downstream molecule of IL-6. After being phosphorylated by IL-6, STAT3 binds to the promoter of the gene encoding vascular endothelial growth factor (VEGF), stimulating the formation of tumor blood vessels(10). Thus, activating IL-6/STAT3 pathway was found to facilitate angiogenesis(11), while inhibiting this pathway was found to reduce angiogenesis and cancer cell progression(12).

The initiation of IH was found to be related to the dysfunction of the balance between pro- and anti-angiogenic signals(13). As mentioned above, IL-6 related signaling pathway plays a vital role in tumor angiogenesis. Thus, IL-6 may also participate in the formation and progression of IH. A study conducted by Fu et al. found that IL-6 was significantly increased in hemangioma cells as comparing to the normal ECs(13). And inhibiting IL-6 could suppress the invasion and proliferation of hemangioma cells(13). However, the definite role of IL-6 in the progression of IH has not been determined.

In this study, we aimed to evaluate the expression of IL-6 in IH patients and investigate the role of IL-6/STAT3/hypoxia inducible factor-1α (HIF-1α) pathway in the progression of IH.

Materials And Methods

Patients

Serum samples were obtained from 15 IH patients. Control serum samples were obtained from sex-/age-matched 15 normal infants. All collected serum samples were immediately stored in liquid nitrogen. Human samples were obtained with written informed consent from the guardian of the children, according to the Declaration of Helsinki. *The study was approved by the Ethics Committee of XX.*

Cell culture

Hemangioma-derived stem cells (HemSCs) were obtained from Procell Life Science&Technology Co,. Ltd (Wuhan, China), and maintained in DMEM medium (Gibco, Grand Island, NY, USA), accompanied by 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution penicillin-streptomycin (Sangon Biotech, Shanghai, China) under the condition of 37 °C and 5% CO_2.

Cell transfection

Three small interfering RNA (siRNA) targeting IL-6 (IL-6 siRNA1, IL-6 siRNA2 and IL-6 siRNA3), three siRNA targeting STAT3 (STAT3 siRNA1, STAT3 siRNA2 and STAT3 siRNA3), three siRNA targeting HIF-1α (HIF-1α siRNA1, HIF-1α siRNA2 and HIF-1α siRNA3), and the non-targeting si-RNA (si-NC) were obtained from

GenePharma (Shanghai, China). HemSCs were seeded in 24-well plates. The aforementioned plasmids were transfected into HemSCs using Lipofectamine 2000 (Invitrogen, Carlabad, CA, USA) according to the manufacturer's instructions.

Cell-viability assay

Cell-viability was assessed by CCK-8 assay. HemSCs were seeded at 5×10^4 cells/mL density and cultured for 24 h. Then, HemSCs were transfected with si-NC, IL-6 siRNA, STAT3 siRNA and HIF-1α siRNA. After being cultured for 24h, 48h and 72h, cells in each well were incubated with CCK8 solution at 37°C for 1 hour. Then, the absorbance of each well was detected by a microplate reader (BioTek, Winooski, VT, USA) at 450 nm.

Wound Healing Assay

After transfection with si-NC, IL-6 siRNA, STAT3 siRNA and HIF-1α siRNA, HemSCs were seeded in 6-well plates at 1×10^7 cells/well and incubated overnight. Wounds with the size of 0.5-1.0 cm were produced by a sterile 200 μl plastic pipette tips. Cells were further cultured with medium for 48 h. Images were acquired by microscope (OLYMPUS, Japan) at 4 × 40 magnification.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from patients' serum samples and cells using TRIZOL reagent (Invitrogen). RNA was reverse-transcribed using the cDNA Synthesis Kit (TaKaRa Bio, USA). qRT-PCR was performed with an ABI 7500 real-time PCR System (Applied Biosystems, USA). The relative gene expression were calculated using the $2^{-\triangle\triangle Ct}$ method. Primers used are as follows: HIF-1α, 5'-AAGTTCACCTGAGCCTAAT-3' (forward primer) and 5'-ATGGGTTCTTTGCTTCTGT-3' (reverse primer); IL-6, 5'-TTCGGTCCAGTTGCCTTCT-3' (forward primer) and 5'- GTGCCTCTTTGCTGCTTTC-3' (reverse primer); STAT3, 5'- TTTTGTCAGCGATGGAGTA-3' (forward primer) and 5'- TTGTTGACGGGTCTGAAGT-3' (reverse primer); GADPH, 5'-AGAAGGCTGGGGCTCATTTG-3' (forward primer) and 5'- AGGGGCCATCCACAGTCTTC-3' (reverse primer).

Western blotting

The proteins extracted from cells were separated by SDS-PAGE. And then the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). After blocked for 1 h, blots were incubated with primary antibodies overnight at 4°C. Antibodies against HIF-1α (1:1000, Cell Signaling Technology, Danvers, MA, USA), IL-6 (1:1000, Cell Signaling Technology), STAT3 (1:1000, Cell Signaling Technology), and GAPDH (1:3000, Cell Signaling Technology) were incubated. Then, membranes were incubated with antirabbit or antimouse IgG–HRP-conjugated secondary antibodies (1:2000, Cell Signaling Technology) for 1 hour at room temperature. ECL Chemiluminescence Detection System (Thermo Fisher Scientific, Rochester, NY, USA) was applied to visualize protein bands.

Tumor growth assay

IL-6 inhibitor, Corylifol A(14) (purity >98%), was purchased from Shanghai Winherb Medical Technology Co., Ltd (Shanghai, China).

Four-week-old male Nu/nu athymic nude mice (Cavens Biotechnology, Changzhou, China) were raised under specific pathogen-free (SPF) conditions. A total of 5×10^5 HemSCs were injected into the left armpit of the nude mice. Tumor size was measured by calipers (length and width) every 3 days. The tumor volumes were calculated with the formula $V = a\times b^2/2$, where a is the larger and b is the perpendicular shorter tumor axis. When the tumor volume reached to 100 mm^3, the mice were divided into two groups: IL-6 inhibitor group (n=20) and control group (n=20). Each mouse was treated with either 200 μL of IL-6 inhibitor (Corylifol-A, 20.8 mg/mL) or the same volume of normal saline (control group) through intratumor injection on day 1. On day 7, 14, 21 and 27, 4 mice in each group were randomly chosen for tumor tissues collection, respectively. *Animal experiments were performed under a project license granted by the Animal Care and Use Committee of XX*.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded sections (5 μM) from tissues at different time point were incubated with rabbit anti-mouse primary antibodies directed against Caspase3 and Ki-67 (Cell Signaling Technology) overnight at 4˚C. Biotinylated goat anti-rabbit anti-immunoglobulin G (Wuhan Boster Biological Technology) was used as the secondary antibody. Proteins were visualized under a light microscope.

TUNEL staining

The cell apoptosis in each group was detected by TUNEL staining. The right amounts of reagent 1 (TdT) and reagent 2 (dUTP) in the TUNEL kit (Beyotime, Shanghai, China) were mixed in a ratio of 1:9 and added to cover the slices. After TUNEL labeling, the cells were counterstained with 4-6-diamidino-2-phenylindole as a fluorescent tracer to detect nuclei. TUNEL positive cell numbers were counted automatically using ImageJ software.

Statistical analysis

Data analyses were performed using the SPSS statistics 22.0 software (IBM Corp., Armonk, NY, USA). The continuous variables were expressed as mean ± SD. The significance of the differences between groups were determined by one-way analysis of variance (ANOVA) followed by LSD test or student's *t* tests. P values of less than 0.05 were considered statistically significant.

Results

The expression of IL-6 was elevated in IH patients

The expression of IL-6 in IH patients and sex-/age- matched normal infants was detected by qRT-PCR. As shown in figure 1, IH patients had much higher IL-6 levels as comparing to the healthy controls (1.17 ± 0.20 vs. 0.96 ± 0.18, P=0.005).

Knockdown of IL-6, HIF-1α or STAT3 inhibited HemSCs viability and migration

HemSCs were transfected with siRNAs targeting IL-6, HIF-1α or STAT3, and the levels of IL-6, HIF-1α or STAT3 were detected by qRT-PCR. As shown in figure 2A, comparing to the control group, the levels of IL-6 were significantly decreased in IL-6 siRNA1, IL-6 siRNA2 and IL-6 siRNA3 groups (P<0.05). IL-6 siRNA1 group had the lowest IL-6 level. The levels of HIF-1α or STAT3 were also decreased in 3 HIF-1α siRNAs groups or 3 STAT3 siRNAs groups, respectively (figure 2B-C). And STAT3 siRNA3 group had the lowest STAT3 level. HIF-1α siRNA2 group had the lowest HIF-1α level. Thus, IL-6 siRNA1, STAT3 siRNA3 and HIF-1α siRNA2 were chosen for the further studies. Western blot analysis further confirmed that HemSCs transfected with IL-6 siRNA, STAT3 siRNA or HIF-1α siRNA had lower IL-6, STAT3 or HIF-1α level

than normal HemSCs, respectively (P<0.05, figure 3).

In the cell-viability assay, HemSCs transfected with IL-6 siRNA had significantly lower viability than normal HemSCs after being cultured for 24h, 48h and 72h (P<0.05, figure 4). HemSCs transfected with STAT3 siRNA or HIF-1α siRNA had the similar tendency (figure 4).

In the wound healing assay, the migration rate for normal HemSCs was 100% after being cultured for 48h. But the migration rate for HemSCs transfected with IL-6 siRNA (49.07 ± 3.54 vs. 100.00, P<0.001), STAT3 siRNA (44.98 ± 3.91 vs. 100.00, P<0.001) or HIF-1α siRNA (43.15 ± 1.02 vs. 100.00, P<0.001) was significantly decreased as comparing to the normal HemSCs (figure 5).

IL-6 inhibitor inhibited HemSCs growth in vivo

HemSCs xenograft mouse models were created and randomly assigned to receive IL-6 inhibitor or normal saline (control). Our results indicated that IL-6 inhibitor significantly delayed the tumor growth of the mice (figure 6A-B).

Tumor tissues from mice in each group were obtained at day 7, 14, 21 and 27 after treatment. And IHC was used to assess the expression of Ki67 and Caspase 3 in tumors. Compared with control group, Caspase 3 was significantly increased in IL-6 inhibitor group at each time point (P<0.05, figure 7), whereas Ki67 was decreased in IL-6 inhibitor group (P<0.05, figure 8), indicating IL-6 inhibitor could inhibit HemSCs proliferation in *vivo*.

TUNEL assay was used to detect cell apoptosis in each group. As shown in figure 9, IL-6 inhibitor group had much higher apoptosis rate than control at day 7 (0.16 ± 0.01 vs. 0.12 ± 0.01, P=0.008), day 14 (0.21 ± 0.04 vs. 0.10 ± 0.01, P=0.010), day 21 (0.35 ± 0.02 vs. 0.15 ± 0.01, P<0.001) and day 27 (0.43 ± 0.01 vs. 0.17 ± 0.03, P<0.001) after treatment, respectively.

Discussion

In the present study, we found that the expression of IL-6 was elevated in IH patients. Knockdown of IL-6, HIF-1α or STAT3 could significantly inhibit HemSCs viability and migration *in vitro*. Using IL-6 inhibitor could delay tumor growth in IH mouse models *in vivo.* Our results indicated that regulating IL-6/STAT3/HIF-1α signaling pathway might inhibit IH growth.

IL-6 is a inflammatory cytokine that strongly correlated with tumor progresssion(15). In IH patients, the serum levels of IL-6 were found to be increased(2, 16). Elevated IL-6 expression is asscoiated with IH progression. Fu et al. found that elevated IL-6 triggered the malignancy of IH via induction of proliferation and migration of hemangioma cells (13). In the present study, we also found that IH patients had much higher IL-6 levels as comparing to the healthy controls. IL-6 binds to its receptor, leading to the the phosphorylation of STAT3. IL-6/STAT3 is a well-known signaling pathway, which has a profound effect on tumor initiation and tumor growth including the process of regulating tumor immune microenvironment, protecting tumor cells from apoptosis, driving tumor cell proliferation, and promoting tumor angiogenesis(10, 17). HIF-1α is a transcription factor expressed in cells under hypoxia condition(15). The activation of HIF-1α is associated with the growth of multiple tumors such as lung cancer(15), gastric cancer(18) and colorectal cancer(19). The IL-6/STAT3/HIF1α autocrine loop has been found in several tumor types(20). IL-6 induces STAT3 phosphorylation, then, HIF-1α interacts with phosphorylated STAT3 by blocking HIF-1α degradation and accelerating synthesis(15). Disrupting the IL-6/STAT3/HIF1α autocrine loop was reported to reduce tumor growth significantly(21).

In the present study, HemSCs were transfected with siRNAs targeting IL-6, HIF-1α or STAT3 to disrupt the IL-6/STAT3/HIF1α pathway. As expected, HemSCs transfected with IL-6 siRNA had significantly lower viability and migration rate than normal HemSCs. And HemSCs transfected with STAT3 siRNA or HIF-1α siRNA had the similar tendency. In Fu's study, they used anti-IL-6 and STAT3 inhibitors to suppress IL-6/STAT3 pathway in hemangioma cells, and found that inhibiting IL-6/STAT3 pathway could suppress the proliferation and migration of hemangioma cells(13). Consistent with Fu's study, our results indicated that inhibiting IL-6/STAT3/HIF1α pathway, reducing the expression of IL-6, HIF-1α or STAT3, could inhibit HemSCs proliferation and migration.

Our *in vivo* study further confirmed the conclusion that inhibiting IL-6/STAT3/HIF-1α signaling pathway could suppress IH growth. After IL-6 inhibitor treatment, the tumor growth of mice was significantly delayed. Corresponding with the results shown in tumor growth measurements, the expression of Ki67 after IL-6 inhibitor treatment was significant decreased. Ki67 is one of the most extensive cell proliferation marker, which can be expressed at all stages of the cell cycle except in G0 phase(22). IL-6 inhibitor group had lower Ki67 positive cells than control group, indicating that inhibiting IL-6/STAT3/HIF1α pathway could suppress IH cell proliferation.

Apoptosis is considered one of the important mechanisms to prevent the development of cancer. It has been reported that IL-6/STAT3 signaling pathway could up-regulate the anti-apoptotic protein, protecting tumor cells from apoptosis(10, 23). Inhibiting IL-6/STAT3 signaling pathway could promote tumor cell

apoptosis in ovarian cancer(24), glioblastoma(25) and colorectal cancer(26). In the present study, IL-6 inhibitor group had much higher apoptosis rate than control group. Furthermore, Caspase 3 was significantly increased in IL-6 inhibitor group. Caspase-3 is a protein at the end of the caspase cascade which could be activated by both the intrinsic and extrinsic death pathways in apoptosis(27). Our results indicated that inhibiting IL-6/STAT3/HIF1α pathway could promote tumor cell apoptosis of IH. However, the underlying mechanisms how IL-6/STAT3/HIF1α pathway regulates IH cell apoptosis remains unclear, which needs us to explore in the further studies.

Conclusion

In conclusion, our findings revealed that the expression of IL-6 was elevated in IH patients. Inhibiting IL-6/STAT3/HIF-1α pathway could significantly suppress HemSCs viability and migration *in vitro*, and delay tumor growth in IH mouse models *in vivo.* Our results indicated that regulating IL-6/STAT3/HIF-1α signaling pathway could inhibit IH growth.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Children's Hospital of Xinjiang Uygur Autonomous Region.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Competing interests

All of the authors had no any personal, financial, commercial, or academic conflicts of interest separately.

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Authors' contributions

Maimaiti A and Aierken F conceived of the study, and He J and Zhou L participated in its design and Li SX coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

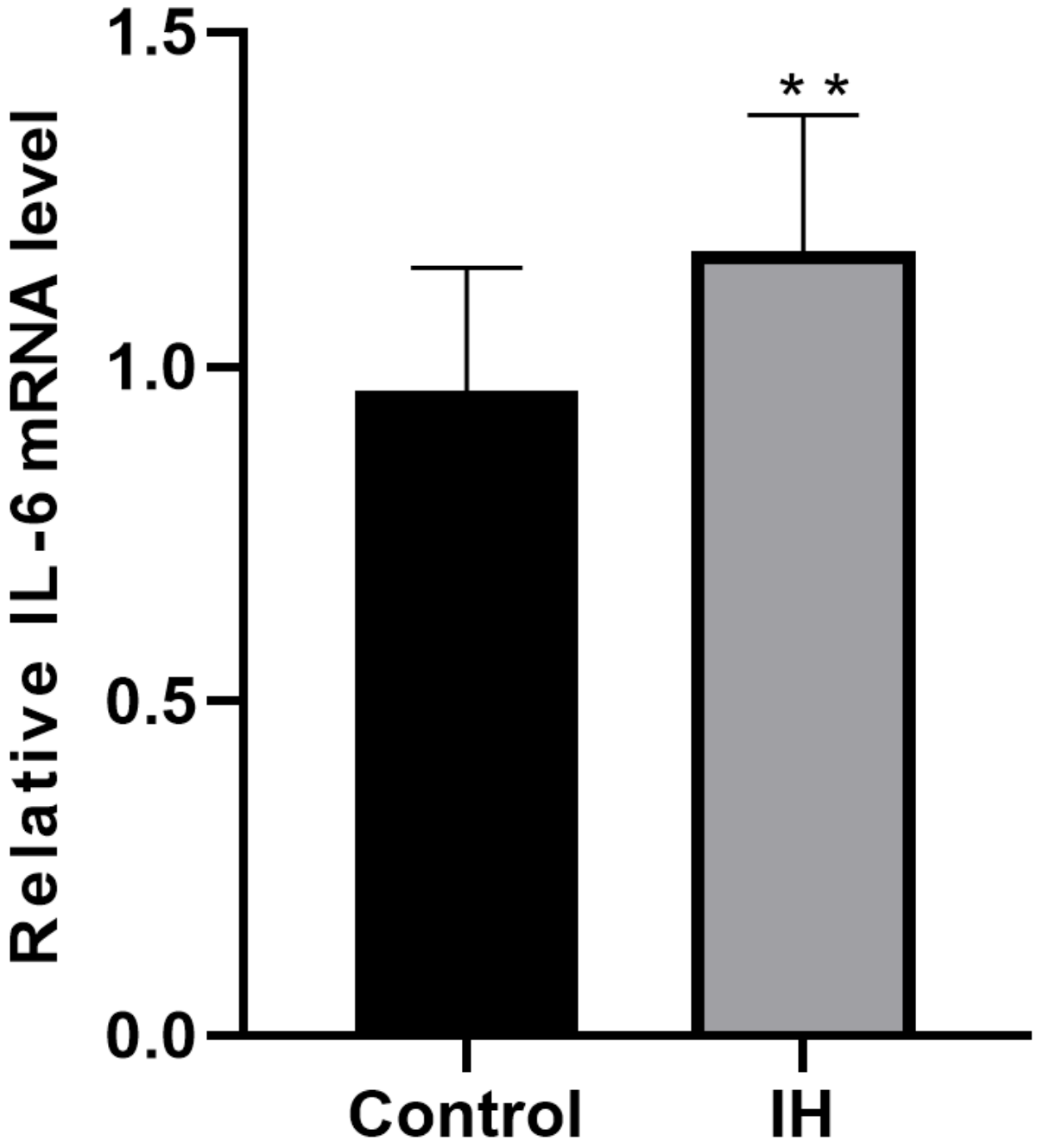

Figure 1

IH patients had much higher IL-6 levels as comparing to the healthy controls. IH, infantile hemangioma. **P<0.05 vs. control group.

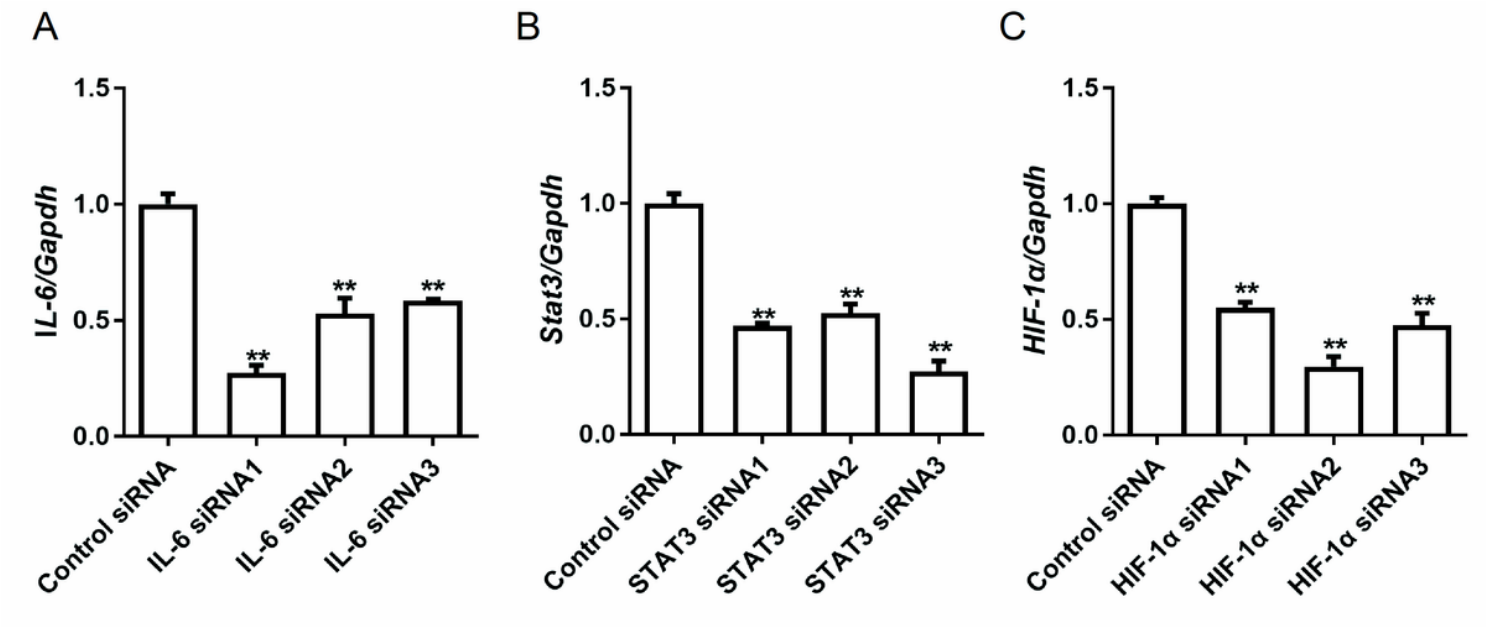

Figure 2

HemSCs were transfected with siRNAs targeting IL-6 (A), STAT3 (B), or HIF-1α (C) and the levels of IL-6, STAT3 or HIF-1α were detected by qRT-PCR. **P<0.05 vs. control group.

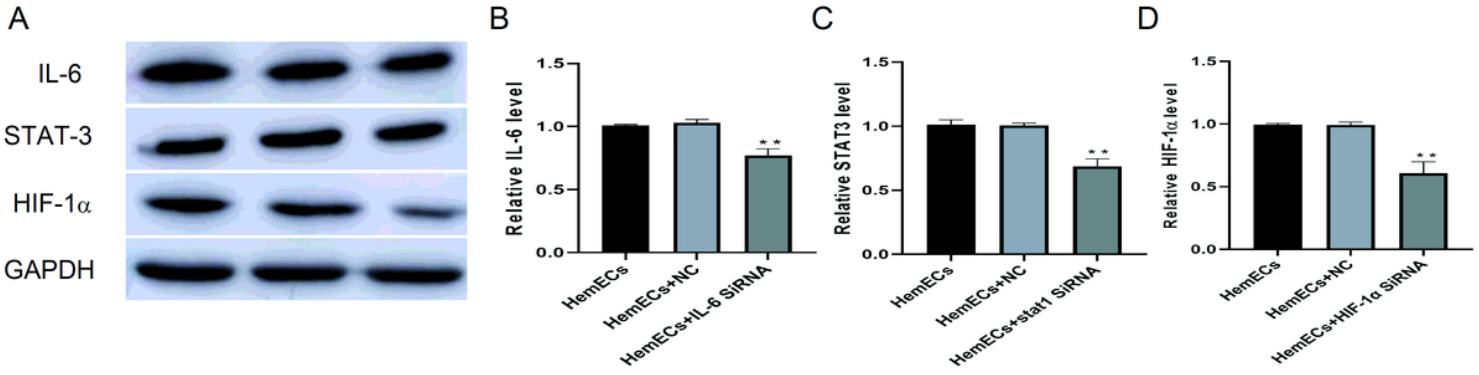

Figure 3

HemSCs were transfected with siRNAs targeting IL-6, STAT3, or HIF-1α and the levels of IL-6, HIF-1α or STAT3 were detected by western blot. (A) the representative images; (B) the relative IL-6 expressions; (C) the relative STAT3 expressions; (D) the relative HIF-1α expressions. **P<0.05 vs. normal HemSCs group (control).

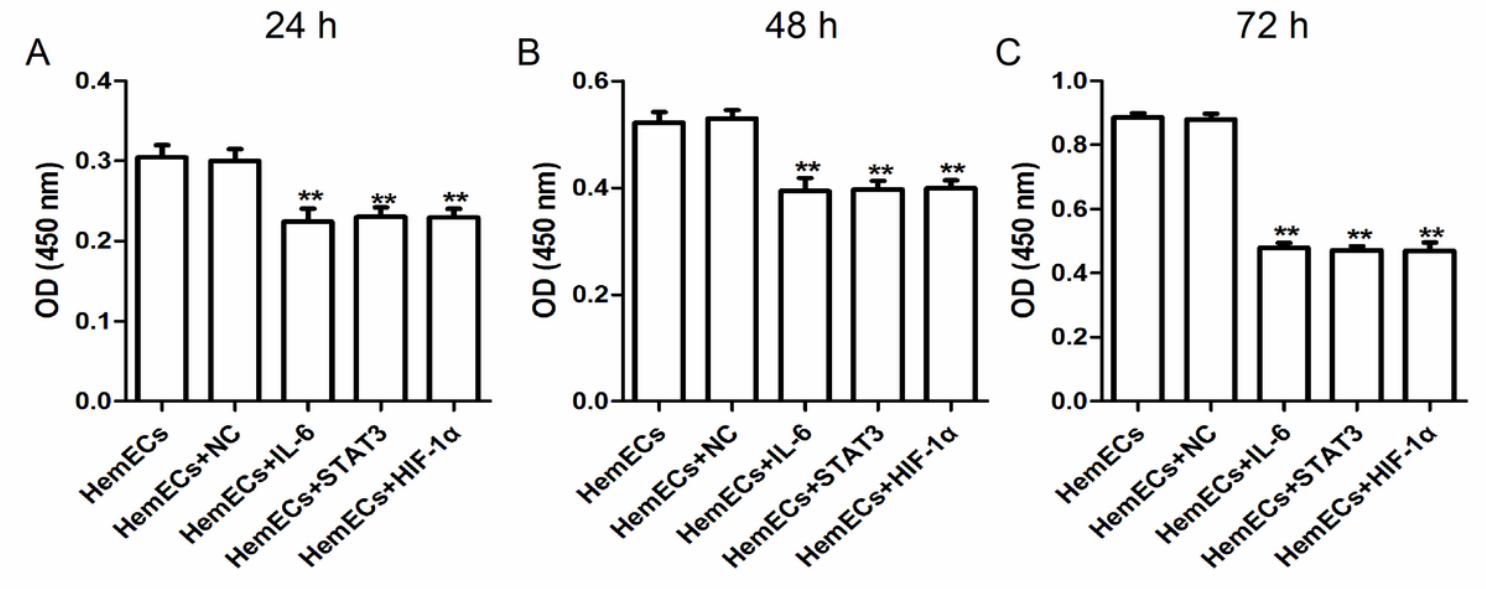

Figure 4

HemSCs were transfected with si-NC, IL-6 siRNA, STAT3 siRNA and HIF-1α siRNA, and the cell viability was measured at 24h (A), 48h (B) and 72h (C) after culture. **P<0.05 vs. normal HemSCs group (control).

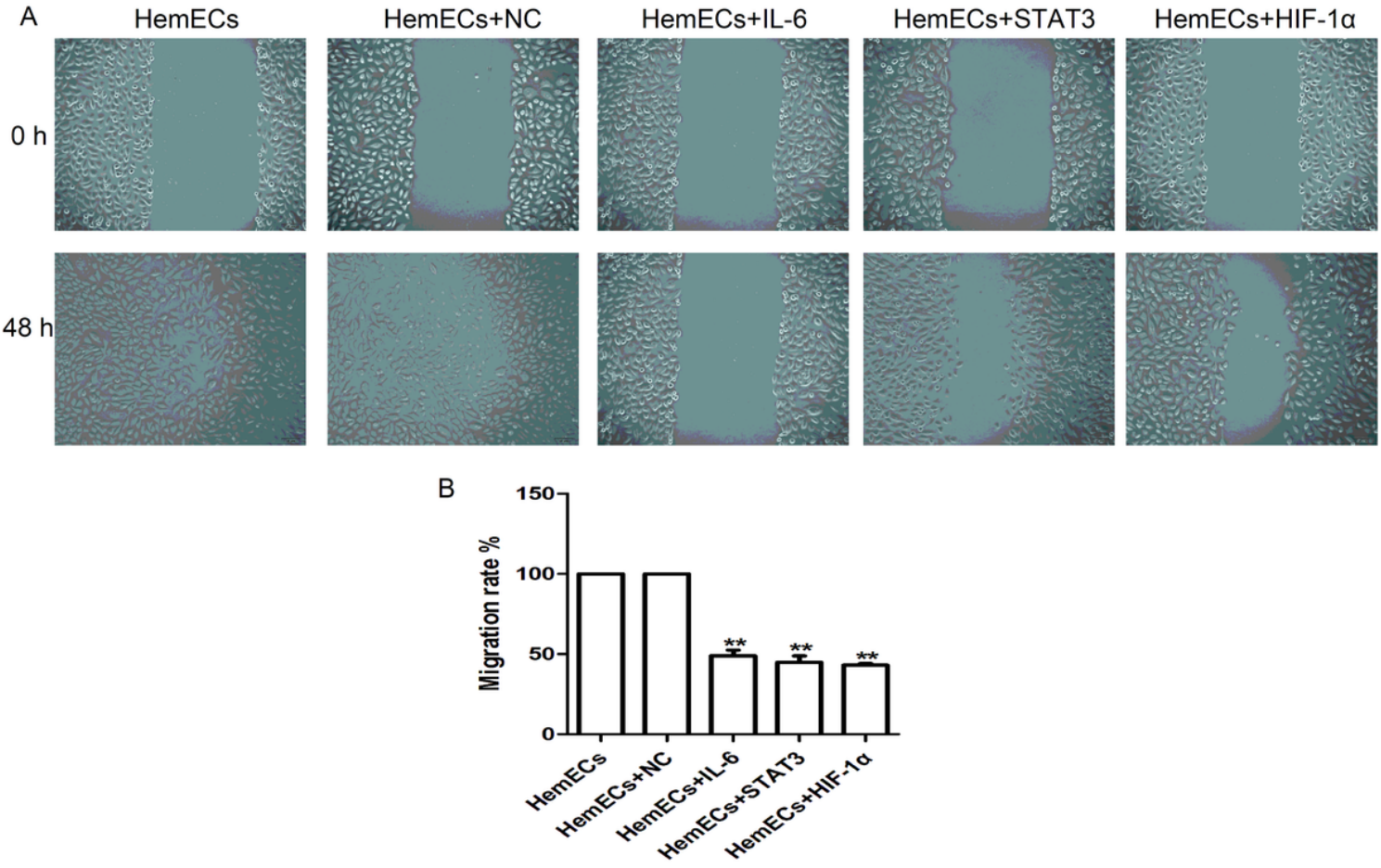

Figure 5

HemSCs were transfected with si-NC, IL-6 siRNA, STAT3 siRNA and HIF-1α siRNA, and the migration rate was measured at 48h after culture. (A) the representative images at 0h and 48h; (B) the comparisons among groups. **P<0.05 vs. normal HemSCs group (control).

Image not available with this version

Figure 6

HemSCs xenograft mouse models were created and randomly assigned to receive IL-6 inhibitor or normal saline (control). (A) the final tumor volume on day 27; (B) tumor growth curve in each group.

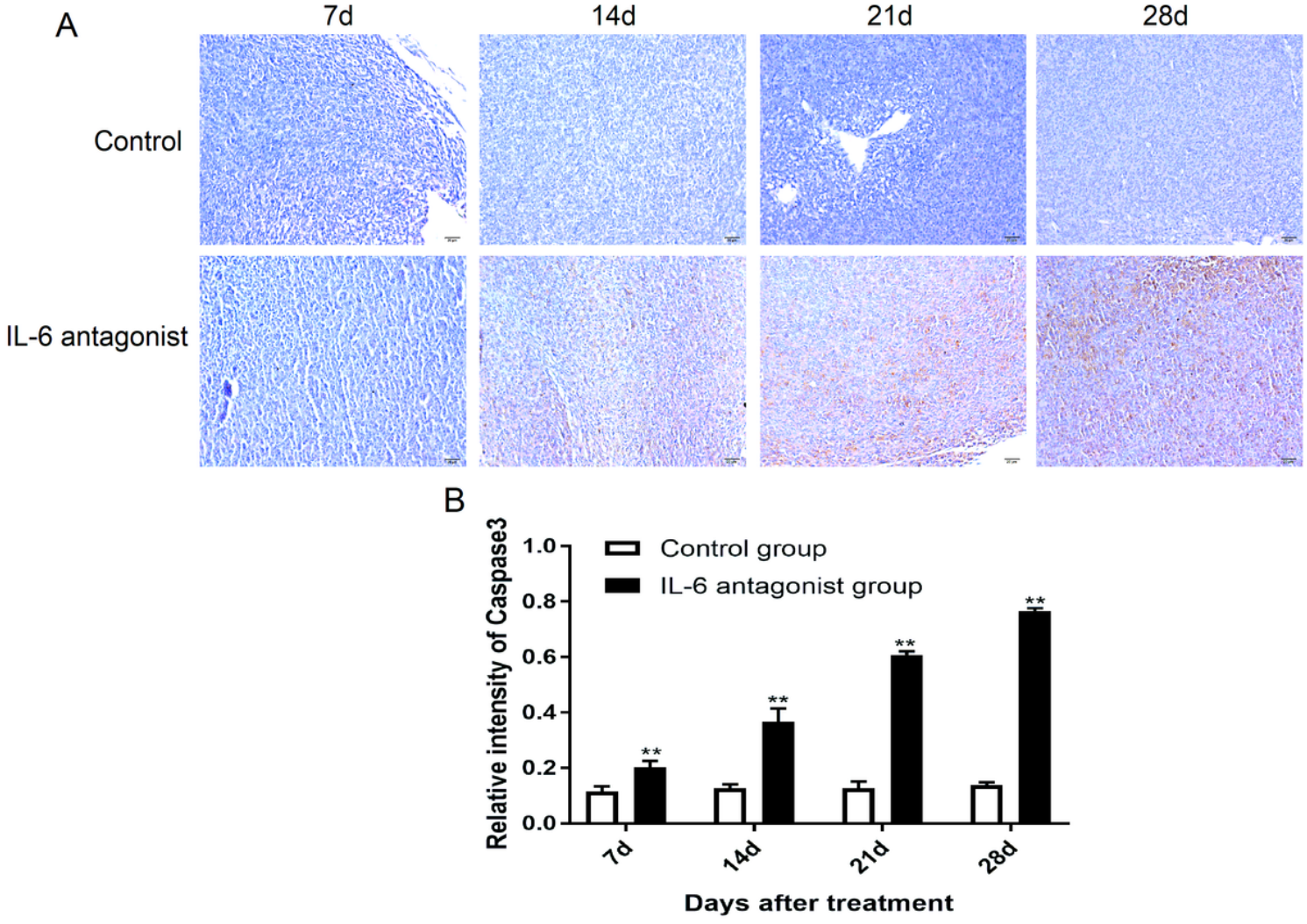

Figure 7

The expression of Caspase 3 in each group. (A) the representative images (×400); (B) the comparisons of Caspase 3 expression between groups at different time point. **P<0.05 vs. control group.

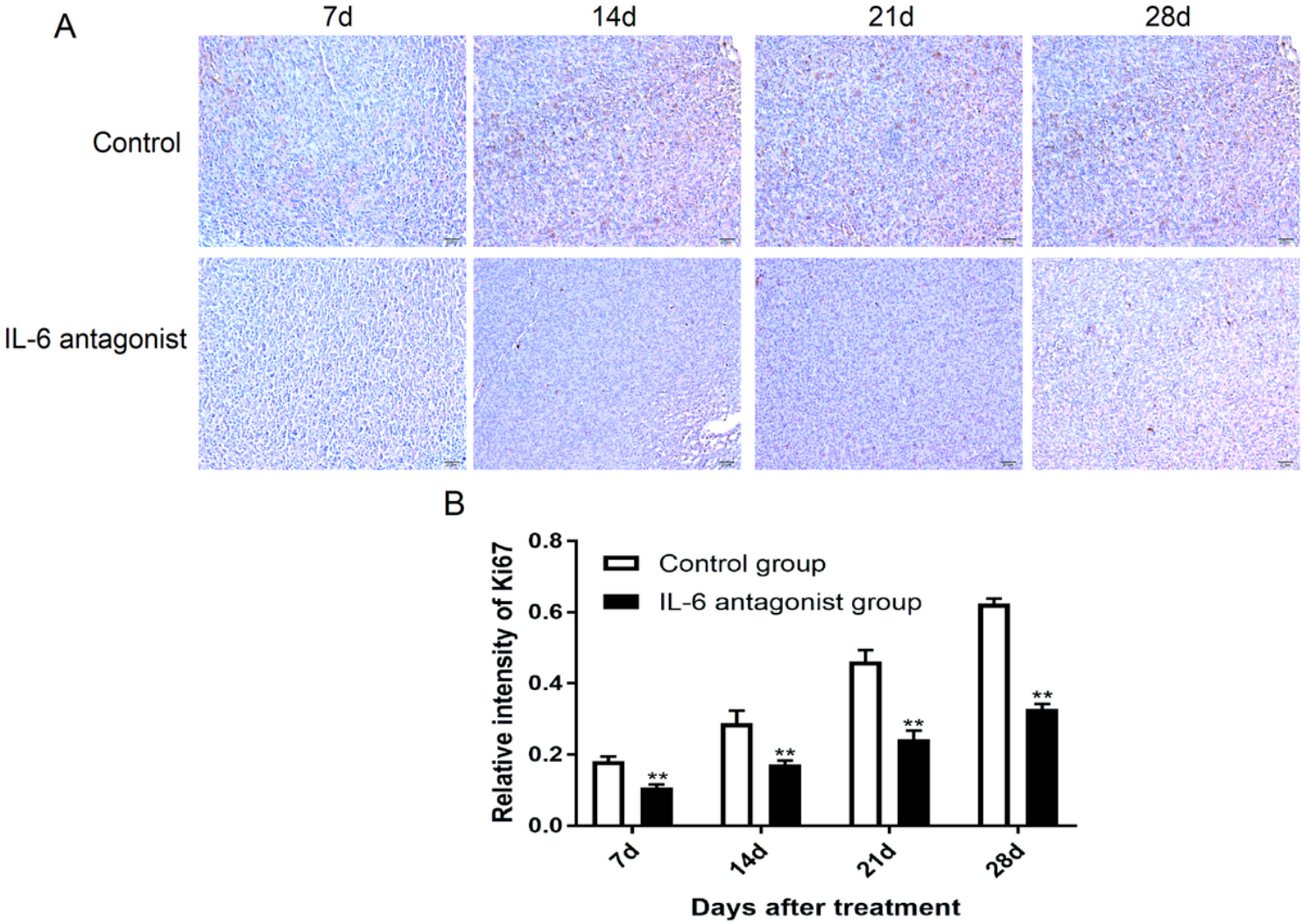

Figure 8

The expression of Ki67 in each group. (A) the representative images (×400); (B) the comparisons of Ki67 expression between groups at different time point. **P<0.05 vs. control group.

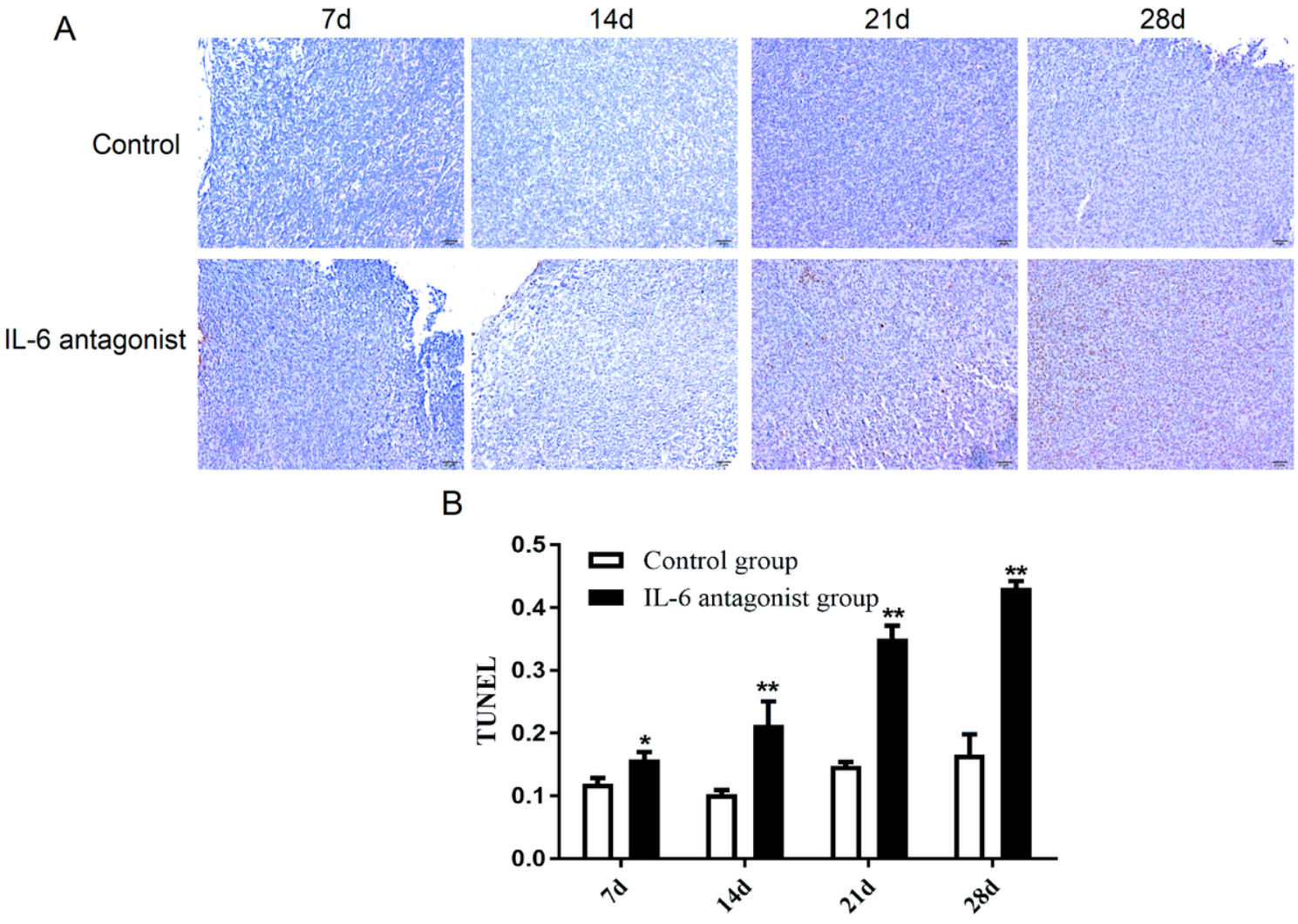

Figure 9

Cell apoptosis in each group were detected by TUNEL assay. (A) the representative images (×400); (B) the comparisons of apoptosis rate between groups at different time point. **P<0.05 vs. control group.