Intraarticular adhesion is one of the complications when the patients suffer from operations of knee-joint. Hydroxycamptothecin (HCPT) can selectively inhibit the activity of topoisomerase I and has shown anti-proliferative effect in previous researches. The aim of our experiment was to investigate the effect of topical application of HCPT on preventing intraarticular scar adhesion by activating the PERK signal pathway. As the results showed, HCPT could prevent the intraarticular scar adhesion and the inhibitory effect was in a dose-dependent manner. Furthermore, we assumed that ER stress mediated the suppression effect of the intraarticular scar adhesion. The results of immunohistochemistry showed that the expression of GRP78 and CHOP, which were hallmarks of ER stress. Proteins associated with PERK signal pathway such as p-PERK and p-eIF2α were upregulated after treated by HCPT determined by western blot analysis. Knockdown of PERK decreased the ratio of Bax/Bcl-2, GRP78 and CHOP were increased together with the tendency of HCPT. When human fibroblasts were treated with HCPT in vitro, cell viability was inhibited according to the results of CCK-8 assay and Hoechst staining, and cell apoptosis was induced according to the results of AV/PI and western blot analysis. HCPT-induced apoptosis was correlated with elevation of GRP78 and CHOP, which were hallmarks of ER stress. Proteins associated with PERK signal pathway such as p-PERK and p-eIF2α were upregulated after treated by HCPT determined by western blot analysis. Knockdown of PERK decreased the ratio of Bax/Bcl-2, GRP78 and CHOP expression, suggesting that PERK signal pathway was involved in HCPT-induced fibroblast apoptosis. Our findings indicate that topical application of HCPT can prevent intraarticular scar adhesion. ER stress plays an important role in this effect by inducing fibroblasts apoptosis that may be mediated by PERK signal pathway.
cations, protein folding as well as the maintenance of intracellular calcium homeostasis (Shi et al., 2013; Leitman et al., 2013). These processes are tightly regulated as the ER is sensitive to alterations of homeostasis. The perturbations in Ca^{2+} homeostasis and imbalance between the load and capacity of protein folding could induce the ER stress (Selimovic et al., 2013; Ron and Walter, 2007; Rao et al., 2001). Numerous stimuli, including Ca^{2+} ionophores, inhibitors of glycosylation and hypoxia could trigger ER stress and eventually lead to cell death (Hitomi et al., 2004).

RNA-dependent protein kinase (PKR)-like ER kinase (PERK), inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6) can sense the presence of unfolded/misfolded proteins and transduce signals to the cytoplasm and the nucleus (Moennier et al., 2007). During the accumulation of unfolded/misfolded proteins, the glucose regulated protein 78 (GRP78/BiP) dissociate from PERK, IRE-1a and ATF-6, results in their activation and then triggers the unfolded protein response (UPR) (Mori, 2000; Barateiro et al., 2012). Activated PERK phosphorylates α-subunit of eukaryotic initiation factor 2 (eIF2α) and induces activation transcription factor 4 (ATF4), which subsequently increased C/EBP homologous protein (CHOP/GADD153) expression (Roussel et al., 2013; Jiang and Wek, 2005). CHOP is downstream of PERK signal pathway (Zinszner et al., 1998) and has been proved for the pro-apoptotic effect during ER stress by increasing the expression of B-cell lymphoma 2 (Bcl-2) family proteins, such as Bax and Bcl-2 (Wu et al., 2013a, 2013b; Rodriguez et al., 2011). Once the CHOP is activated, it can regulate the Bax/Bcl-2 ratio and initiated the apoptotic process (Wu et al., 2013a, 2013b). As CHOP expression is upregulated after PERK phosphorylation, we infer that HCPT prevent intracellular adhesion by inducing fibroblasts apoptosis through the activation of PERK signal pathway.

2. Materials and methods

2.1. Fibroblast culture and treatment

The experimental research was approved by the Ethical Committee and Research Committee of Subei Hospital of Jiangsu Province. The primary human fibroblast cell line was obtained from GuangZhou Jenino Biotech Co., Ltd. Fibroblasts were cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) containing 20% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo, USA). Human fibroblasts in exponential growth phase between passages 4 and 7 were used for the experiments. Fibroblast monolayers were cultured in different specifications petri dishes until reaching approximately 50–80% density, then washed with PBS (pH 7.4) and treated with various concentrations of HCPT (Santa Cruz, CA) in different times. The cells were treated with 5-min applications of PBS only as controls. After treatment, the cells were immediately washed three times with PBS and maintained in the growth medium for subsequent experiments.

2.2. Cell viability

Cell viability was measured using a Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan). The fibroblasts were cultured in triplicate in 96-well plates and treated with 0, 0.05 μg/ml, 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 4 μg/ml and 8 μg/ml HCPT. In another group, the fibroblasts were treated with 1 μg/ml HCPT maintained in DMEM for 0, 12, 24, 36, 48 or 72 h. After treated with HCPT, cells were incubated with 10 μl CCK-8 solution for 1 h at 37 °C. Cells that stained positively with CCK-8 were considered viable cells and expressed as a percentage compared with the control cells.

2.3. Western blot analysis

Treated cells were lysed in RIPA buffer (Beyotime, Hangzhou, China) according to the manufacturer’s instructions. The protein concentration was determined by the BCA Protein Assay Kit (Beyotime, Hangzhou, China). Equal amounts (60 μg/lane) of total proteins were subjected to electrophoresis on a 6%, 10% or 12% SDS-polyacrylamide gel and transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in TBST for 2 h at room temperature, and then incubated with the primary antibodies. Anti-78-kDa glucose-regulated protein (GRP78), anti-phospho-eIF2α, anti-eIF2α, anti-CHOP, anti-activated-caspase-3, anti-Bax, and anti-Bcl-2 antibodies were obtained from Abcam (USA). Anti-phospho-PERK was obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, USA). Anti-poly ADP-ribose polymerase (PARP), anti-PERK, anti-β-actin antibody and were purchased from Cell Signaling Technology (USA). The membranes were next washed three times in TBST and incubated with secondary antibodies (Santa Cruz Biotechnology, USA) (diluted 1:5000) for 2 h and then washed with TBST for three times. The immune complexes were visualized via fluorography using an enhanced ECL system (Millipore, USA).

Fig. 1. Histological analysis of fibroblast in intraarticular scar tissue treated with saline, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml of HCPT. (A) Dense scar tissue was found in the operative areas treated with saline, moderate scar tissue was found in 0.1 mg/ml and 0.5 mg/ml group and in the 1 mg/ml group. The amount of scar tissues in 1 mg/ml group was less than that of other groups. (B) The number of the fibroblast was decreased with the concentration of HCPT increased. The results showed that HCPT could inhibit the proliferation of fibroblast in a dose-dependent manner. The sections were stained with hematoxylin–eosin and the magnification is × 200. The values represent the Mean ± S.E.M of three separate experiments. *P < 0.05, **P < 0.01.
2.5. Hoechst staining

The cells were incubated in the dark for 15 min at room temperature before the double-staining according to the manufacturer’s protocol. The mixture and incubated with AV-FITC and PI (BD Biosciences, Singapore) for referred to the web version of this article.

2.6. Generation of stable PERK-silenced fibroblasts

Lentiviral vector, which contained target genes PERK, was purchased from Shanghai Genechem Co. Ltd. (Genechem, China). Fibroblasts were incubated with the lentiviral vector at an MOI of 20 in the presence of 2 mg/ml polybrene (Gibco, USA) overnight. Then the medium was removed and fresh complete medium was added. Following transfection 48 h, the fibroblasts were cultured with 2 mg/ml puromycin (Sigma, USA) for 96 h. Stable transfected cells with reduced PERK for subsequent experiments.

2.7. Animals

24 mature male New Zealand white rabbits weighing 2.0–3.0 kg used in the study were purchased from the experimental animal center of Yangzhou University. All animals received care in compliance with the principles of Laboratory Animal Care of international recommendations and the experimental protocol was approved by the Animal Care and Research Committee of the Yangzhou University, China. The rabbits were randomly and equally divided into four groups: 0.1 mg/ml group; 0.5 mg/ml group; 1.0 mg/ml group or control (saline) group.

Before the experiment, the rabbits were acclimated to the environment for 1 week before the experiment.

2.8. Animal mode

The animal model of intraarticular adhesion was developed according to the procedure in the previous study (Liang et al., 2014). After general anesthesia by intravenous administration of 2% pentobarbital (1.5 ml/kg), the hairs around the location of knee joint were shaved, antiseptics of the exposed skin was performed with iodophor. The femoral condyle of the left femur was exposed through a medial approach. Approximately 10 mm × 10 mm of the cortical bone was removed from both sides of the femoral condyle with a dental burr, and the underlying cancellous bone was exposed. The articular cartilage was left intact. After disinfection and hemostasis of the operative region, HCPT in various concentrations of 0.1, 0.5 and 1 mg/ml or saline were administered to the decorticated areas with cotton pads for 10 min. The surrounding tissues were covered by wet gauze to avoid getting in touch with the agent. After the cotton pads were removed, the decorticated areas were irrigated with saline to get rid of the remaining HCPT immediately. The wounds were sutured. After the operations, an intramuscular injection of Cefazolin sodium was administered and the rabbits to prevent infection after the operation for 3 days, and the surgical knee joints were fixed extra-articular in a flexed position with Kirschner wires for 4 weeks.

2.9. Histological analysis

All the rabbits were anesthetized and killed for histological analysis after four weeks and the surgical sites were reopened through original incision. The knee joints were excised including all connective tissues and adhesive scars. Then the samples were fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Transverse sections of 5 mm perpendicular to the femoral axis were stained with Hematoxylin–eosin (HE) and Masson’s Trichrome. Intraarticular scar adhesion and intrarticular collagen tissues were evaluated by light microscopy using 200 × magnifications.

2.10. Immunohistochemistry

Immunohistochemistry for GRP78 and CHOP were performed using the avidin-biotin-peroxidase complex method. For antigen retrieval, the sections for GRP78 and CHOP were treated with 0.5% trypsin at 37 °C for 10 min. After blocking with 1% skim milk, tissue sections were incubated with anti-GRP78 and anti-CHOP polyclonal antibody (1:300) (Abcam, USA) overnight at 4 °C and then washed in phosphate-buffered saline solution. Biotinylated anti-rabbit IgG were used as secondary antibody and the sections were visualized as described previously.

2.11. Statistical analysis

The statistical analysis was performed by SPSS 19.0 statistical software. The data were expressed as the mean ± S.E.M. Statistical significance was defined as a P value < 0.05.

3. Results

3.1. The effect of HCPT on intraarticular scar adhesion in histological analysis

The HE images showed that topical application of HCPT could reduce intraarticular scar adhesion. Compared to the control group, topical application of HCPT could markedly decrease scar tissues and the tendency of the effect was coincidence with the concentrations, the scar tissues in 1 mg/ml group were significantly less than those in

Fig. 2. The effect of HCPT on intararticular collagen tissue in rabbits. The collagen tissue is blue in the section with Masson’s trichrome staining under the light microscope × 200. The results showed that HCPT could reduce collagen synthesis in a dose-dependent manner. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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0.1 mg/ml and 0.5 mg/ml and control group. Low concentration groups including 0.1 mg/ml and 0.5 mg/ml have the moderate content of the fibrous adhesion tissue, but 0.5 mg/ml group is still less compared with 0.1 mg/ml group (Fig. 1A). The number of fibroblasts in intraarticular scar tissue measured by image pro plus showed that the fibroblast number was decreased in a dose-dependent manner (Fig. 1B). These results revealed that HCPT could reduce intraarticular scar adhesion in rabbits.

### 3.2. Effect of HCPT on intraarticular collagen density

Masson’s trichrome-stained images showed that HCPT treatment could reduce the collagen density of intraarticular adhesion tissue. In the control group, the collagen density was more than those in HCPT treated groups. The collagen density in 1.0 mg/ml HCPT group were significantly less than those in other HCPT-treated groups. Moreover, the degree of collagen density in 0.5 mg/ml group was moderate, which is also a subtle decreased compared to that in 0.1 mg/ml group (Fig. 2). This result demonstrated that topical application of HCPT could reduce collagen density in intraarticular adhesion tissue.

### 3.3. HCPT suppresses the intraarticular adhesion through ER stress

To further investigate whether ER stress was involved in the role of HCPT in preventing intraarticular scar adhesion, GRP78 and CHOP expressions were detected through immunohistochemical analysis, which were the markers of ER stress. The results of the immunohistochemical analysis showed that the immunestained granules of cytoplasmic GRP78 (Fig. 3A–B) and CHOP (Fig. 3C–D) were significantly increased in the HCPT-treated groups compared to that of control group. Furthermore, the increasing expressions of GRP78 and CHOP were in a dose-dependent manner. These laboratory findings were strongly suggested that ER stress was activated in HCPT-mediated prevention of intraarticular scar adhesion.
3.4. Apoptotic effect of HCPT on human fibroblasts

After human fibroblast cells were exposed to various concentrations of HCPT (0, 0.05 μg/ml, 0.5 μg/ml, 1 μg/ml, 4 μg/ml and 8 μg/ml) for 24 h, the results showed that cell viability was decreased sharply along with the concentration of HCPT increased by CCK-8 assay (Fig. 4A). Additionally, fibroblasts were then incubated with 1 μg/ml of HCPT for different time durations and the results of CCK-8 assay showed that the cell viability was decreased in a time-dependent manner (Fig. 4B). Furthermore, to ascertain whether the effect of HCPT on cell viability was caused by apoptosis, fibroblasts were treated with 1 μg/ml of HCPT for 24 h and underwent the hoehst33342 staining, the results revealed that HCPT caused chromatin condensation and typical morphological changes of apoptosis (Fig. 4C). Consistent with the
3.5. HCPT induces ER stress

After incubated with different concentrations of HCPT, proteins were collected and tested by western-blot analysis. The results found that the expressions of CHOP and Bax were increased and anti-apoptotic Bcl-2 expression was downregulated. The ration of Bax/Bcl-2 was increased and peaked at 4 μg/ml HCPT for 24 h (Fig. 4D–E). In addition, active-caspase 3 and cleaved PARP, regarded as biochemical markers of apoptosis, were increased after treated with 1 μg/ml of HCPT for various durations (Fig. 4F). Collectively, these data suggested that HCPT treatment could reduce the cell viability via apoptosis.

3.6. PERK signal pathway is activated in HCPT-induced fibroblasts apoptosis

To investigate the relation between HCPT-induced apoptosis and PERK signal pathway, we collected the fibroblasts treated with various concentrations of HCPT. The results demonstrated that phospho-PERK and phospho-eIF2α were upregulated by HCPT treatment with increasing concentrations (Fig. 6A). Regarding time-dependent kinetics, the levels of phospho-PERK, phospho-eIF2α, and CHOP were increased with the time extension after treatment of 1 μg/ml HCPT (Fig. 6B).

Next, we further investigated the role of PERK in HCPT-induced fibroblasts apoptosis. Knockdown of PERK (PERK−) using lentivirus shRNA significantly inhibited expression of the PERK in fibroblasts by western blot analysis (Fig. 6C), increased cell viability by CCK-8 assay (Fig. 6D) and decreased the apoptosis rate by Hoechst 33342 staining (Fig. 6E). Meanwhile, results of western-blot analysis found that the ER stress markers such as GRP78, CHOP and the ratio of downstream Bax/Bcl-2 were both down-regulated in PERK knockdown group (Fig. 6F–G). When PERK-knockdown fibroblasts were treated with 1 μg/ml HCPT for 24 h, the increased expressions such as CHOP, GRP78, Bax/Bcl-2 as well as the fibroblasts apoptosis by HCPT treatment were inhibited by PERK-knockdown. Moreover, the inhibited cell viability by HCPT treatment was increased by PERK-knockdown. These results suggested that PERK signal pathway was activated by HCPT treatment and involved in HCPT-induced fibroblast apoptosis.

4. Discussion

Camptothecin (CPT) and its derivatives are a series of cell cycle-specific alkaloids that selectively inhibit the activity of type topoisomerase I. Increasing reports have shown that CPT is an important enzyme in uncoiling of the super-twisted DNA structure and directly participating in DNA replication, repair, genetic transcription, which can lead to cell differentiation, proliferation and apoptosis. Due to the pro-apoptosis effects, CPT was widely used as anti-tumor agent to treat many diseases in clinical practice (Pu et al., 2009). 10-hydroxycamptothecin (HCPT), one of the Camptothecin (CPT)’s analogues, is a naturally occurring alkaloid anticancer agent extracted from the Chinese plant Camototheca acuminate. HCPT received considerable attention because of its strong anti-tumor activity and low toxicity, which has been used in clinical practice to treat gastric carcinoma, hepatoma, bladder carcinoma, and lung cancer (Yang et al., 2011). Previous researches have proved that HCPT could induce apoptosis of human tenon’s capsule fibroblasts and reduce scar adhesion after laminectomy through apoptotic-promoting effect in fibroblasts.

Up to now, the pathophysiological mechanism of intraarticular scar adhesion is still uncertain and need to be further clarified. It is well known that many growth factors and inflammatory cytokines participate in the formation of scar adhesion after being activated by cytokines such as fibroblast growth factor (FGF) and transforming growth factor-beta (TGF-β). Fibroblasts migrate to the operative areas, proliferate and synthesize collagen fibers, which eventually form intraarticular scar adhesion and result in a series complications after operations (Fukui et al., 2001b; Yan et al., 2010). In our present study, in vivo and in vitro experiments were used to investigate the effect and mechanism of HCPT preventing intraarticular scar adhesion by inducing human fibroblast. Multiple parameters were used in our experiment to evaluate the intraarticular scar adhesion,
such as HE staining, Masson’s trichrome staining and fibroblast counting. The HE staining demonstrated that 1 mg/ml HCPT was better than those in low concentration of HCPT and control group. Fibroblast counting showed a dose-dependent manner in preventing intraarticular scar adhesion. Masson’s trichrome staining, which is parameter of collagen in scar tissue, was coincidence with the results of HE staining. The density of collagen in 1 mg/ml HCPT group was less than those in low concentration of HCPT and control group. Moreover, human fibroblasts were treated by HCPT in vitro experiment and results showed that HCPT could decrease the cell viability and inhibit fibroblasts proliferation in a dose- and time- dependent manner. After being treated with 1 μg/ml HCPT for 24 h, the results from flow cytometry analysis and heochst33342 staining demonstrated that the fibroblast apoptosis were increased. Cleaved-PARP and active-caspase-3 increased by HCPT treatment also indicated that HCPT was involved in fibroblasts apoptosis. These data above suggested that HCPT could promote fibroblasts apoptosis and reduce production of collagen fiber, which may be the effect in preventing intraarticular scar adhesion.

Apoptosis, also called programmed cell death, is an important way of cell death. In mammalian cells, there are two major pathways: receptor-mediated or mitochondrial-mediated apoptosis, which are also known as the extrinsic pathway and intrinsic pathway. Recently, it is reported that ER stress participated in cell apoptosis (Oyadomari and Mori, 2004). ER is a multifunctional organelle that is important to

**Fig. 6.** PERK signal pathway was participated in HCPT-induced apoptosis in fibroblasts. (A-B) The doses-dependent and time-dependent manner effects of HCPT on proteins associated with PERK signal pathway, including p-PERK, PERK, p-eIF2α and eIF2α, were determined by Western blot analysis in cells. β-actin was used as a control. (C) The expression levels of the targeted transcripts were determined by Western blot. The presented data represents the results of two independent experiments. (D) PERK-/- and PERK+/+ fibroblasts were treated with HCPT as described before and then cell viability was measured after 24 h. (E) Cells stained by heochst 33342 were observed under a fluorescence microscope. (F-G) PERK-/- and PERK+/+ fibroblasts were treated with 1 μg/ml HCPT for 24 h, the detected by western blotting with antibodies specific for GRP78, CHOP, Bax, Bcl-2 and β-actin. This experiment was performed in triplicate. The histograms in each panel represent the mean ± S.E.M of three independent experiments. *P < 0.05 versus control group (0 μg/ml).
calcium homeostasis and synthesis of proteins. After ER stress was induced by numerous conditions and stimuli, unfolded protein response (UPR) can prevent the accumulation of misfolded/unfolded proteins in the ER lumen and restore the normal function of the cells. However, chronic or prolonged ER stress will eventually lead to apoptosis by regulating CHOP. PERK is a critical regulator of CHOP expression and can phosphorylate eIF2α and ATF4 after activation, which together with C/EBP-b then binds to the composite site and transactivates the CHOP promoter (Pan et al., 2012).

In our study, the expressions of cytoplasmic GRP78 and CHOP were significantly increased in a dose-dependent manner in the HCPT-treated groups in vivo and in vitro, indicating that ER stress was activated. In vitro experiment, the expressions of CHOP and Bax were upregulated, whereas the Bcl-2 was downregulated when fibroblasts were treated with different concentrations of HCPT. Moreover, the proteins of PERK signaling pathway such as phospho-PERK, phospho-eIF2α were also upregulated after treated by HCPT and showed in a dose-dependent manner and time-dependent manner together with the tendency of CHOP, suggesting PERK signaling pathway was involved in HCPT-induced apoptosis. Furthermore, knockdown of PERK using shRNA result in the increase of cell viability and reduction of apoptosis. Therefore, these results indicated that HCPT induced fibroblasts apoptosis via PERK signal pathways that is involved in apoptotic process.

In conclusion, our current study suggests that topical application of HCPT can prevent the intracartilaginous scar adhesion. ER stress plays an important role in inducing fibroblasts apoptosis that is may be mediated by PERK signal pathway. As a scientific and rigorous experiment, our study provided a new insight in preventing intraarticular scar adhesion by topical application of HCPT, but the safety and toxicity still need more experiment to verify.

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