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Homoharringtonine prevents surgery-induced epidural fibrosis through endoplasmic reticulum stress signaling pathway

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ABSTRACT

Surgery-induced epidural fibrosis after laminectomy often results in poor clinical outcomes. Fibroblasts proliferation is considered to be one of the major causes of epidural fibrosis formation. Previously, there was no research about the effect of Homoharringtonine(HHT) on inhibiting fibroblast proliferation and reducing epidural fibrosis. Here, we performed in vitro and in vivo experiments to explore the effect of HHT on inhibiting fibroblast proliferation, inducing fibroblast apoptosis and preventing epidural fibrosis formation. In vitro, the effect of HHT on inhibiting fibroblasts was detected by CCK-8 assay. Besides, the effect of HHT on causing fibroblast apoptosis was investigated via Western blots, flow cytometry and TUNEL assay. Results suggested that HHT could inhibit fibroblasts proliferation and induce apoptosis. And the marker proteins of endoplasmic reticulum (ER) stress were also changed positively. In vivo, histological macroscopic assessment, hydroxyproline content analysis and histological staining were used to detect the effect of HHT on reducing epidural fibrosis. The results showed that HHT had positive suppressive effects on epidural fibrosis following laminectomy in rats. TUNEL assay in epidural tissue suggested that HHT could obviously induce fibroblasts apoptosis. Immunohistochemistry staining showed that the expression of two important ER stress markers(78-kDa glucose-regulated protein and C/EBP homologous protein) were also increased. In conclusion, this research showed that HHT could reduce epidural fibrosis formation after laminectomy, and the potential mechanism might through inhibiting fibroblasts proliferation and inducing fibroblasts apoptosis via ER stress signaling pathway. It might provide a novel agent for reducing epidural fibrosis after laminectomy surgery.

1. Introduction

Lumbar laminectomy is one of the most common therapeutic modalities in the treatment for disc herniation. In spite of the improvement of operative technique, the rate of complications after lumbar spine surgery was still unsatisfactory (Fiume et al., 1995; North et al., 1991a, 1991b). Epidural fibrosis was considered to be one of the major causes for the complications, such as radicular nerve pain, persistent lower back pain and so on (Burton, 1991). Thus, preventing epidural fibrosis formation is believed to be the best approach to manage these problems. For many years, two major methods including local application of drugs in the surgery sites and materials implantation outside the exposed dura mater were performed to prevent postoperative epidural fibrosis (Sun et al., 2008; Tao and Fan, 2009). However, the results from these studies were not without complications, and more research was needed before complete clinical application. Therefore, a method with little side effects to prevent epidural fibrosis is urgent needed.

Fibroblast proliferation was considered to be a major cause in the formation of epidural fibrosis (Su et al., 2012). Recently, many researchers have done a lot of experiments to prevent epidural fibrosis via inhibiting fibroblast proliferation (Sun et al., 2016; Cho et al., 2011). Furthermore, some of them found that the reduction of epidural fibrosis was accompanied with the rising apoptotic rate of fibroblast (Sun et al., 2015a, 2015b). And some even believed that the reduction of epidural fibrosis might be realized by promoting fibroblast apoptosis (Shi et al., 2013). However, the exact mechanism was still in the process of exploration.

Homoharringtonine, a cephalotaxine ester extracted from the evergreen tree, have been used to treat hematonosis for 40 years in China (Kantarjian et al., 2015; Lv and Wang, 2014). In 2012, American Food and Drug Administration (FDA) approved HHT could be used in the treatment of chronic myelogenous leukemia(CML). In a previous study, HHT had been proven to be safe and effective in the treatment of fibrosis development following glaucoma filtering surgery of human (Peng et al., 1998), which implicated that it might be also useful in the

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treatment of epidural fibrosis.

As a critical organelle, endoplasmic reticulum (ER) was known to be involved in intracellular calcium regulation, protein folding, synthesis and transport. Once the unfolded or misfolded proteins assembled under a certain degree, unfolded protein response (UPR) would provide a series of reactions to protect the cells from injury. Besides, the unfolded or misfolded proteins in ER might lead to apoptosis once if it was left unsolved. And the apoptosis was known to be mediated via C/EBP homologous protein (CHOP) pathway (Zinszner et al., 1998). Recently, it was reported that ER stress mediated apoptosis was beneficial in the treatment of fibrosis disease (Shi et al., 2013).

Therefore, we investigated whether HHT could induce fibroblast apoptosis via ER stress and whether it could reduce and prevent surgery-induced epidural fibrosis after laminectomy. We hoped that it might provide a new method to treat epidural fibrosis in the future.

2. Materials and methods

2.1. Cell culture and HHT treatment

Fibroblasts were gained from epidural fibrosis scar tissue of rats that received reoperation of laminectomies 4 weeks later. Briefly, the scar tissues around L1 level were separated by lancet and rinsed thrice immediately with phosphate-buffered saline (PBS). Then, we cut the scar tissues into 1 × 1 mm^2 sections with eye scissors (Sun et al., 2016). Then, they were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 15% foetal bovine serum (Clark-Bioscience, USA), 1% penicillin/streptomycin (Gibco, CA, USA) in the stable environment of 5% CO2 at 37 °C. Cells between passages 4–6 were used for all experiments. Fibroblasts were then cultured in various dishes overnight. After reaching 50–60% density, the cells were washed with phosphate buffered saline and treated with HHT in various concentrations(0, 0.5, 1, 2, 4, 8 μg/ml). HHT was purchased from Dalian Meilun Biotechnology Co., Ltd (Liaoning, China).

2.2. Reagent

Homoharringtonine(molecular formula:$C_{29}H_{39}O_9$) was purchased from Dalian Meilun Biotechnology Co., Ltd. The purity of HHT is 98%.

2.3. Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) was applied to test the effect of HHT on cell viability of fibroblast. Briefly, cells at exponential stage were seeded in triplicate into 96-well plates overnight. After applying various concentrations of HHT in each well for 12 h, the cells were further treated with 10 μl CCK-8 solution in each well for another 2 h. In another group, cells were treated with HHT of 2 μg/ml for 0, 12, 24, 36, 48, 60 h as described above. Microplate absorbance reader(Bio-Tek, Elx800, USA) was used to determine the optical density at 450 nm. Cell survival rate was calculated according to the instruction book.

2.4. Flow cytometry analysis of cell apoptosis

Cells were harvested and then seeded into 6-well plates and incubated overnight. Half of the wells were set as control group. After treatment with 2 μg/ml HHT for 12 h in the other wells, cells in all the wells were collected and then wash cold PBS buffer for three times. Cells were then resuspended in 1 ml 1× Binding Buffer, from which 100 μl solution was transferred to a tube. Then, 5 μl FITC Annexin V and 5 μl PI were added to every tube. After incubation by FITC Annexin V and PI for 15–20 min in the condition of protection from light at room temperature and with the addition of 400 μl 1× Binding Buffer, the mixture was detected by flow cytometry.

2.5. Western blot analysis

After various treatments, fibroblasts were collected. Resuspended fibroblasts were lysed by radioimmuno-precipitation assay (RIPA) buffer (Beyotime, Hangzhou, China) on ice. BCA Protein Assay Kit (Beyotime, Hangzhou, China) was performed to detect the concentration of protein. 40 μg of each protein lysate was used for Western blot at a 5–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following soaking in the blocking buffer for 2 h at room temperature, the polyvinylidene difluoride membranes (Millipore, Bedford, MA) were incubated with primary and secondary antibodies successively according to the instructions. The primary antibodies used were anti-cleaved-poly ADP-ribose polymerase (cleaved PARP), anti-Bax, anti-Bcl-2, anti-−78-kDa glucose-regulated protein (GRP78), anti-CHOP, anti-PERK, anti-phospho-PERK (P-PERK), anti-eukaryotic translation initiation factor 2a (eIF2a), anti-phospho-eIF2a (P-eIF2a), anti-activating transcription factor 6 (ATF6), anti-inositol-requiring enzyme 1a (IRE1a), and anti-phospho IRE1a (P-IRE1) and anti-β-actin antibodies (Cell Signaling Technology, Beverly, MA, USA). The anti-mouse or anti-rabbit IgG were also purchased from Cell Signaling Technology.

2.6. TUNEL assay in fibroblasts

Apoptosis of fibroblasts caused by HHT was detected by the TUNEL test system (KeyGEN, Nanjing, China). Fibroblasts were seeded to 6-well plates, which contained a glass slide in each well overnight. Following treatment of 2 μg/ml HHT for 12 h, 4% paraformaldehyde was used to fix the cells at room temperature for 20 min. The TUNEL staining steps were based on the manufacturer's instructions. After brief steps of staining, the apoptosis cells were detected by a fluorescence microscope.

2.7. Animals

SD male rats weighing 250–300 g were purchased from the experimental animal centre of Yangzhou University (Yangzhou, China). The animal study was approved by the Animal Ethics Committee of Yangzhou University, all rats received meticulous care. The rats were split into three groups randomly (12 rats per group): HHT (0.1 mg/ml), HHT (0.05 mg/ml), and control (saline).

2.8. Laminectomy model and local application of HHT

Laminectomy models were built according to a previous study (Sun et al., 2007). Briefly, after anesthesia by 1% pentobarbital sodium (40 mg/kg body weight) via intraperitoneal injection, we exposed the fascia and the paraspinal muscles by a midline skin incision, then we used rongeur forceps to remove the vertebral plate of L1 carefully and the dura mater was exposed. After satisfactory hemostasis around the operative region, cotton pads (4 × 4 mm) with different concentrations of HHT were applied to the epidural defect areas for 5 min. Following removing the cotton pads, the surgery areas were washed by saline to get rid of the remaining drugs immediately. The wounds were sutured in layers finally.

2.9. Macroscopic assessment of epidural fibrosis

4 weeks after laminectomy, six rats were randomly picked from three groups. According to a previous report (Rydell, 1970), the amount of fibrosis was judged based on the following classification: grade 0, no adhesions were appeared around the dura mater; grade 1, thin adhesions were observed at the outside of the dura mater, and the adhesions were easily dissected; grade 2, moderate adhesions were appeared around the dura mater, and it was hard to dissect the adhesions from the dura mater; grade 3, dense fibrous adhesions was tightly adhesive