Original Article
Inhibition of a calcium channel blockers on human urethral scars in vitro and in vivo

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Abstract: Objectives: The aim of this study was to investigate the outcome and potential mechanism of verapamil on human urethral scars in vitro and in vivo. Methods: To evaluate anti-fibrosis effects of verapamil, three kinds of observation indexes, including electron microscope observation, cell movement ability analysis, and expression of collagen I proteins, were investigated in primary cultured human urethral scar fibroblasts with various doses of verapamil. Moreover, an implanted nude mice urethral scar model was established, upon which 4 mg/kg of verapamil per day was locally injected. Scars were excised and, after 4 weeks, measured by immunohistochemistry, Van Gieson’s staining, and hematoxylin-eosin staining. Results: The migratory ability of urethral scar fibroblasts was obviously suppressed after verapamil treatment. Furthermore, verapamil induced micromorphological changes and reduced collagen I protein expression of urethral scar fibroblasts. In vivo, verapamil downregulated collagen I protein and changed the density of collagen fibers. Conclusion: Verapamil acts against urethra scars, possibly inhibiting excessive biological behaviors of urethral scar fibroblasts. Verapamil also decreases collagen levels in urethral scars.

Keywords: Urethral scar, calcium channel blockers, antifibrosis, verapamil

Introduction
Urethral stricture, caused by hypertrophic scar formation after urethral injuries, is a common and serious complication, a challenging problem for urologists. Urethral scar (US) formation can lead to severe urine flow dynamic change and dysfunction such as dysuria, urinary tract infections, sexual dysfunction, and male infertility, leading to kidney damage [1]. Common treatments for urethral stricture include urethral dilatation and surgical operations, but these have high rates of recurrence. Currently, some drugs such as verapamil (VP), fluorouracil, bleomycin, corticosteroids, and interferon alfa-2b are employed in the treatment and prevention of hypertrophic scars [2]. However, none of them are widely used in the United States.

Recently, based on clinical and experimental results, VP was reported to be an excellent choice as a scar modulator [3-5]. VP is a widely applied calcium channel antagonist and has been shown to inhibit synthesis/secretion of extracellular matrix molecules including collagen, glycosaminoglycans, and fibronectin [3]. Many studies have indicated the anti-inflammatory [6], anti-fibrosis [2], anti-scar [7], anti-cancer [8], and neuroprotective [9] effects of VP pharmacodynamics. Urethral scars, closely linked to inflammatory response and scar formation, have a similar formation mechanism as hyperplastic scars [6]. Therefore, these things indicate the important role of VP as a scar modulator in human urethral scars. In the present study, electron microscope observation, cell movement ability analysis, expression of collagen I (Col) proteins, and density of collagen fibers were used as observation indexes to measure its anti-fibrosis effects in vitro and in vivo.
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Materials and methods

Main reagents

Verapamil was obtained from SH Harvest Inc. (Shanghai, CN). Anti-Collagen I [ab21286] antibodies and anti-Vimentin antibodies [ab92-547] were obtained from Abcam Inc (Cambridge, MA, USA). Anti-GAPDH [D190090] was acquired from BBI Life Sciences (Shanghai, CN). Fetal bovine serum (FBS) was acquired from Tianhang Biotechnology Co., Ltd (Zhejiang, CN). Dulbecco’s Modified Eagle Medium with 4.5 g/L D-glucose (DMEM) and Penicillin-Streptomycin liquid were obtained from Thermo Fisher Scientific Inc (USA). Hank’s Balanced Salt mixture, without magnesium and calcium, (D-Hank’s) was obtained from Solarbio Science & Technology Co., Ltd (Beijing, CN). The 0.25% Trypsin-EDTA (Gibco) was purchased from Life Technologies.

Patient samples

Fresh urethral scar tissues were obtained from trauma urethral stricture patients, without any antifibrosis therapeutic intervention, in the Urology Department, Xiangya Hospital, Central South University. The cohort of 16 urethral stricture patients provided written informed consent. Collection and usage of clinical specimens were approved by the Xiangya Hospital Medical Research Ethics Committee. Experimental methods were approved by the Scientific Research Project 201503-230 (Histopathological Application) of Xiangya Hospital.

Primary cell culture

Human urethral scar fibroblasts (USFs) were obtained by explant culture. Within 4 hours after the surgical procedure, tissues were washed with D-Hank’s. They were cut to 1 mm² in diameter of 10 cm tissue culture plates containing DMEM, 20% FBS, and 100 U/mL of penicillin/streptomycin. Sheared tissues were then moved to 25 mm² tissue culture bottles, using tweezers, and interval adherent was placed (interval distance is 0.3~0.5 mm). Culture bottles were vertically placed so that tissues adhered to the bottle wall without medium. They were changed to horizontal, for complete medium immersion of tissues two hours later, in a humidified incubator at 37°C containing 5% CO₂. Tissues were fed once per three days with complete medium. Serum concentrations of the complete medium would be adjusted to 10% FBS after the third generation of cells. The fourth to eighth generations of cells were used in the experiments. Morphologies of cells were evaluated, initially, by optical microscopy. Purity of the USFs enriched pellet (> 98%), after more than 3 passages of purified culture, was determined by enumerating five random microscopic views per slide and was expressed as the mean percentage of USFs with a fibrocyte morphology. Purity was further checked by immunofluorescence assay using vimentin (Figure 1).

Transmission electron microscopy

Cell ultrastructure observation by Transmission Electron Microscopy (TEM). TEM (HT7700, Japan) was employed to observe the subcellular structure. Briefly, USFs were seeded on the samples in a 6-well plate at a density of 5 ×

Figure 1. The primary cultured human urethral scar fibroblasts and cell identification. A: Urethral scar fibroblasts climbed out from tissues by tissue culture method. B: The fourth generation of fibroblasts by immunofluorescence test, cytoplasmic vimentin present green fluorescence, cell nucleus appears blue fluorescence by DAPI re-dyeing.
Cells were incubated in 10% FBS supplemented DMEM with VP (100 uM) or solvent for 24 hours after incubating overnight. They were starved for 24 hours in DMEM without FBS at 37°C in 5% CO₂. All samples were fixed with 2.5% glutaraldehyde overnight at 4°C. Acetone with a series of concentration gradients of 50, 70, 90 and 100 v/v% was used sequentially to dehydrate the samples. Subsequently, epoxy resin (EPON-812) embedding, toluene ammonia blue staining, and ultrathin sectioning (LKB-III, Sweden) were conducted in sequence. All samples were stained with uranyl acetate and lead nitrate for observation.

**Scratch assay detecting cell migration**

Urethral scar fibroblasts, in each group, were seeded in 6-well plates at a cell density of 5 × 10⁵ cells/well and incubated overnight at 37°C in 5% CO₂. When the cells reached 80%-90% confluence, a 200 µL sterile pipette tip drew a vertical line from the middle of each hole from top to down. PBS was used to rinse cells, the complete medium with verapamil (50 uM, 100 uM, 150 uM) or solvent was added, and cells were cultured for 24 hours. Width variations, in three crawling slides, were observed under a light microscope after cells were treated for 0 hours, 12 hours, and 24 hours. Average cell migration distances were measured using Image-Pro Plus analysis software.

**Western blot for detection of protein expression**

In performing Western blot, USFs were seeded on the samples in 6-well plates at a density of 5 × 10⁵ cells/well. Cells were incubated in 10% FBS supplemented DMEM with verapamil (100 uM) or solvent for 24 hours, after incubating overnight and starving for 24 hours in DMEM without serum at 37°C in 5% CO₂. After collection from sample surfaces, cells were lysed with a protein extraction regent containing protease inhibitor cocktail (Roche, Germany) and RIPA lysis buffer (Cwbiotech, CN). The obtained protein concentration was measured using BCA protein assay kit (Cwbiotech, CN). Equal amounts of protein from different samples were then separated on SDS-polyacrylamide gel electrophoresis (PAGE) and elec-
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trotransferred to a polyvinylidene difluoride membrane (PVDF, Pall, USA). Membranes were incubated with rabbit anti-collagen I antibody (dilution, 1:1000) and mouse antibody anti-GAPDH (dilution, 1:5000) overnight at 4°C. Finally, the membranes were visualized using horseradish peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-mouse, using ECL plus reagents (Cwbiotech, CN) and Gene Tools imaging system.

Urethral scar mouse model

Four to six-week old BALB/C-nu nude male mice were used for animal studies. All animals were maintained in specific pathogen-free (SPF) conditions. Construction procedures of scar mouse model: Sheared 1 mm² tissues were transplanted, subcutaneously, into nude mice (8 per group) and 4-0 absorption line sutured wounds prevented implanted tissue from slipping out. Scar tissue growth was monitored by caliper measurement, once or twice a week, for at least 4 weeks. During a period of four weeks, four mice died from surgery-induced injuries, due to inflammation or infection. For the in vivo scar treated assay, VP 4 mg/kg·d or saline 100 µl/d negative control were local injected into the model mice (6 per group). The mice were sacrificed after 4 weeks of treatment. At the time of killing, scars were removed and immediately fixed in 4% (w/v) paraformaldehyde, overnight, and tested for immunohistochemistry, Van Gieson’s staining, or hematoxylin-eosin staining.

Immunohistochemistry (IHC)

10% formalin-fixed and paraffin-embedded tissue sections (4 µm) were stained with polyclonal rabbit anti-Collagen I (1:250). Expression scores (staining intensity: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) were multiplied by the proportion of stained cells (0, < 5% stained; 1, 6-25% stained; 2: 26-50%; 3: ≥ 50%). Final IHC scores were then calculated by adding the two above scores. Scores of 0-2 were considered as low expression while scores of 3-6 were defined as high expression. All IHC stained slides were scored by two independent researchers.

Scar staining

Mice were euthanized under deep anesthesia and scars were excised. For histology, excised scars were formalin-fixed and paraffin-embedded. Embedded scars were cut into 4-µm-thick sections and mounted onto poly-l-lysine-coated glass slides. Hematoxylin-eosin and colla Van Gieson’s (CvG) staining were performed, according to standard procedures. Collagen volume fraction (CVF) was quantitatively analyzed with Image J digitalized microscopic images, as previously described.

Statistical analysis

All data were analyzed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Count data are expressed as percentage and were tested using X²-test. Measurement data are expressed as mean ± standard deviation. Comparison between two groups was analyzed using two-sample t-test and comparison among multiple groups was measured using analysis of variance. P < 0.05 indicated a statistically significant difference.

Results

Fibroblast ultrastructural changes

By transmission electron microscopy, it was possible to detect normal ultrastructural features of fibroblasts in untreated samples (Figure 2A1-2A3). It was clearly observed that cell surface microvilli were broomy and primary lysosomes were the major form of intracytoplasmic lysosomes in the untreated group. However, the microvilli presented a recessionary state in verapamil treated samples and secondary lysosomes increased significantly (Figure 2B1, 2B2). In the drug treated group, electron microscopic images further showed that subcellular structures, including autophagosomes and vesicles, increased markedly. Also, there was a reduction and atrophy of intracytoplasmic rough endoplasmic reticulum (Figure 2B2, 2B3) and the cell morphology tended to be spherical, compared with the untreated group (Figure 2B1). Mitochondria did not show an obvious change, however, in the two groups.

Inhibited urethral scar fibroblast migration of VP

Figure 3 shows that VP significantly inhibited the migratory capacity of USFs. The number of migrating cells was significantly higher in VP untreated (Figure 3A2, 3A3) and low-dose (50
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Verapamil (VP) significantly inhibited the migratory capacity of urethral scar fibroblasts (USFs), which were presented as a dose-dependent and time-dependent factor. (A1-A3) show that USFs in the untreated samples. The migration partner distances were difficult to be observed after USFs incubated in 10% FBS supplemented DMEM for 24 hr (A3). The migration distances were significantly wider ($P < 0.01$) with VP treated samples (B2, C2, D2) than the untreated group (A2) after incubated for 12 hr. There were significant differences ($P < 0.01$) in the comparison of migration ability between groups after treated 24 hr (A3, B3, C3, D3). There were no significant differences of the scratch width in (A1, B1, C1, D1) groups ($P < 0.05$).

Figure 3. Verapamil (VP) significantly inhibited the migratory capacity of urethral scar fibroblasts (USFs), which were presented as a dose-dependent and time-dependent factor. (A1-A3) show that USFs in the untreated samples. The migration partner distances were difficult to be observed after USFs incubated in 10% FBS supplemented DMEM for 24 hr (A3). The migration distances were significantly wider ($P < 0.01$) with VP treated samples (B2, C2, D2) than the untreated group (A2) after incubated for 12 hr. There were significant differences ($P < 0.01$) in the comparison of migration ability between groups after treated 24 hr (A3, B3, C3, D3). There were no significant differences of the scratch width in (A1, B1, C1, D1) groups ($P < 0.05$).
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Verapamil downregulated Collagen I protein expression levels, the result was significantly lower than in untreated samples.

After USFs were incubated overnight and starved for 24 hours at 37°C, cells were treated with verapamil (100 uM) for 24 hours. Verapamil downregulated Col I protein expression levels by 68.2%, significantly lower than the Col I expression levels in untreated samples (Figure 4, P < 0.01). Relative expression levels of the protein were normalized with GAPDH.

Decreased protein levels of collagen I and changed density of collagen fibers of VP in vivo.

To further verify the suppressive effects of VP on US in vivo, an implanted mice urethral scar model was employed, as described previously. Immunohistochemistry results showed the same trend as Western blot. Notably, verapamil obviously inhibits expression of Col I compared with saline and the sham group had a larger claybank staining, indicating presence of abundant collagen (P < 0.05, Figure 5A, 5D). Finally, Van Gieson’s and hematoxylin-eosin staining were performed to identify the presence and amount of collagen fibers in the US (P < 0.01, Figure 5B, 5C, 5E). Van Gieson’s staining results showed that collagen fibers became sparser and more disorderly arranged in VP-treated group than those in control group.

Discussion

Verapamil is a calcium channel blocker that blocks the influx of calcium from the extracellular matrix to the cytoplasm by acting specifically on the L-type calcium channels. The pharmacological effects are often used in cardiovascular disease. Lee et al. [7], in 1994, first described the use of verapamil for treatment of hypertrophic scars, verifying the positive effects. Since then, various articles have been concerned about the effects of calcium channel blockers (CCBs) on keloids and fibrosis [3-5]. To the best of our knowledge, CCBs have rarely been reported to treat urethra scars. Therefore, through in vitro and in vivo experiments, we explored verapamil’s effects on biological behaviors of primary cultured human urethral scar fibroblasts and inhibition of collagen deposition.

Calcium plays vital and diverse roles in the complicated process of wound healing [10]. Because calcium-antagonists can effectively reduce intracellular free calcium [11], it has been applied to study of hypertrophic scars and keloids by many clinicians and researchers. CCBs were first clinically used in the collagen matrix on connective tissue remodeling, by Lee [12]. Several researches have shown that intralesional injections of verapamil are successful in the management of keloids and hypertrophic scars [4, 7, 13]. CCBs have not only been used in plastic surgery but also used to treat fibrosis diseases caused by excessive activation of fibroblasts. Relevant literature has reported that verapamil inhibits both the formation of more free radicals and rate of lipid peroxidation, increasing antioxidant effects [14,
Mishima K et al. suggested that N-type Ca\(^{2+}\) channels participate in multiple steps of renal fibrosis and its blockade may, thus, be a useful therapeutical approach for prevention of renal fibrosis [16]. Topcu SO et al. stated that verapamil significantly prevented impairment in renal function and also prevented upregulation of p53, Fas, and PCNA during urinary tract obstruction [17]. Kazama I et al. demonstrated that benidipine slowed the progression of renal fibrosis in rat kidneys with advanced chronic renal failure [18]. A study revealed that verapamil suppresses NE-induced cardiac fibroblast activation by inhibiting Ca\(^{2+}\) channels [19].

Figure 5. Verapamil decreases protein levels of Collagen I, changes the collagen volume fraction (CVF) in vivo. A. Representative images of collagen I proteins of urethral scar cross-section with immunohistochemistry; B. Representative images of histological section of CVF (VG staining); C. Representative images of histological section of urethral cross-section (HE staining); D. Quantitative analyses results of Collagen I protein expression (mean ± SEM, n = 6, P < 0.05); E, F. Quantitative analyses of CVF (mean ± SEM, n = 6, P < 0.01).
Other publications have demonstrated that CCBs could inhibit iron entry into cardiomyocytes and reduce collagen volume in heart tissues [20-22]. Similarly, CCBs are potential therapeutic agents that can be used to address hepatic fibrosis during iron overload [23]. One article illustrated that verapamil has a significantly prophylactic effect against induced liver fibrosis [24]. Azelnidipine inhibits TGF-β1- and Ang II-induced HSC activation in vitro and attenuates CCl(4)- and thioacetamide-induced liver fibrosis in mice [25]. Tetrandrine exerts antifibrotic effects in both hepatic stellate cells-T6 and in rats with dimethylnitrosamine-induced fibrosis [26]. Collectively, data has shown that nifedipine disrupts Ca (2+) oscillations in fibroblasts and prevents impairment of lung function in the bleomycin model of pulmonary fibrosis by altering the profibrotic response to bleomycin [27]. Lately, intralesional injections of verapamil have achieved reasonable effects in Peyronie's disease [28]. Verapamil has also inhibited areca nut extract-induced buccal mucosa fibroblast contraction [29].

Although, CCBs have been researched for numerous fibrosis diseases, they have been rarely used to study urethral scars. The results of this study show that verapamil inhibited USFs proliferation and presented a dose-dependent and time-dependent feature [30]. This article also explored the effects of calcium channel blockers (VP) on US. According to results, the migratory ability of USFs was suppressed after VP treatment. These results are consistent with the results of many previous studies concerning hypertrophic scars and keloids [19, 31]. A previous study showed that USFs responds to verapamil when stimulated with an appropriate inhibitory concentration (100 μM) [30]. Therefore, this concentration was also selected as the optimum dose for the experiments. Fibroblasts were accepted with verapamil at concentrations of 100 μM. Continuous 24 hour treatment was used for electron microscope observation and collagen I protein detection, since cell proliferation was significantly inhibited and did not present widespread cell apoptosis in this condition. Furthermore, this study discovered a number of micromorphological changes of USFs with VP treatment. The sparse microvilli of the cell surface might explain reduced migratory ability. The secondary lysosomes and autophagosomes increased significantly, illustrating that cell proliferation was inhibited after VP treatment. Like the common pathological mechanism of scar formation, urethral scar formation is also due to excessive extracellular matrix accumulation. Similarly, in this study, there was evidence that VP reduced collagen I protein expression of USFs [4, 32]. It was believed that secreted protein expression down-regulated was associated with rough endoplasmic reticulum reducing and atrophy. Similarly, cell migration disability is associated with cell morphological alterations. In order to verify the anti-fibrosis effects of VP in vivo, this study used verapamil to treat the urethral scar mouse model. These findings show that VP reduced protein expression levels of Col I and decreased the density of collagen fibers in tissue sections. At present, there are some studies show that anti-fibrosis effects of CCBs may involve inhibition of transforming growth factor-β1 signal transduction and influence the production of cytokines such as PDGF, VEGF, and IL-6 [6, 33, 34].

In conclusion, this present study demonstrates that VP could effectively modulate biological behaviors of USFs and decrease scar collagen levels. The potential mechanisms of VP antifibrosis are likely attributed to cell ultrastructural alterations, decrease of cell proliferation, inhibition of biological ability, regulation of extracellular matrix metabolism, and availability management of collagen fibers. Further study of the precise molecular mechanisms underlying all of these events would be helpful in better understanding the pharmacodynamics of VP antifibrosis effects.

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Disclosure of conflict of interest

None.

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