Mycophenolate mofetil reduces epidural fibrosis by regulating TGF- β 1/Smad2/3 axis to repress the proliferation, migration and differentiation of fibroblasts

Dongming Zhu

Dalian Medical University

Jie Zhang

Central South University Xiangya School of Medicine

Xiaobo Zhang

Dalian Medical University

Yun Liu

Yangzhou University Medical Academy

Lianqi Yan (✉ yanlianqi@126.com)

Northern Jiangsu People's Hospital

Yu Sun

Northern Jiangsu People's Hospital

Research article

Keywords: Mycophenolate mofetil (MMF), Fibroblast proliferation, Fibroblast migration, Fibroblast differentiation, Epidural fibrosis

Posted Date: May 22nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-27906/v1>

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Abstract

Background

Excessive fibroblasts proliferation is believed as a major reason in the process of epidural fibrosis, which is known as a troublesome complication of lumbar laminectomy clinically. Mycophenolate mofetil (MMF) is a kind of immunosuppressant, previous studies had shown that it has the function of preventing fibrosis, but the role and mechanism are still unclear. In this study, we determined the repressed effect of MMF on fibroblasts proliferation, migration and differentiation in surgical-induced epidural fibrosis and the underlying mechanism.

Methods

In vitro, the impacts of MMF on cell viability were measured by cell counting kit-8 (CCK-8) assay and the fibroblasts proliferation rates were determined by using EdU incorporation, cell cycle assays and western blot. Additionally, the impacts of MMF on fibroblasts migration and differentiation were analyzed by cell wound scratch assay, transwell assay, immunofluorescence analysis and western blot. In vivo, we built epidural fibrosis models in rats and locally applied MMF. Histological and immunohistochemical staining were used to determine the effect of MMF on reducing epidural fibrosis and fibroblasts functions.

Results

CCK-8 assay demonstrated that MMF repressed fibroblasts proliferation in a time- and a dose-dependent manner. EdU incorporation assay and cell cycle analysis proved the inhibition effect of MMF on the proliferation of fibroblasts. Cell wound scratch assay, transwell assay and immunofluorescence proved the inhibition effect of MMF on the migration and differentiation of fibroblasts. Western blotting analysis proved that MMF inhibited the expression of TGF- β 1, p-Smad2 and p-Smad3. The expression of proliferation proteins, migration proteins and differentiation proteins were downregulated. In addition, histological and immunohistochemical stain showed that MMF reduces epidural fibrosis by repressing the proliferation, migration and differentiation of fibroblasts.

Conclusion

MMF could inhibit the proliferation, migration and differentiation of fibroblasts, it reduced surgical-induced epidural fibrosis as well. TGF- β 1/Smad2/3 axis may be the latent mechanism in MMF reduced epidural fibrosis. It might provide a new notion for mitigating surgery-induced epidural fibrosis.

1. Background

One of the major complications after lumbar laminectomy is epidural fibrosis, which can seriously affect the surgical outcome[1]. An important factor contributes to this complication is the massive proliferation of fibroblasts, which form a matrix of scar adhesions outside the epidural, causing many related symptoms such as low back pain[2]. As the number of patients undergoing laminectomy continues to increase, the incidence of epidural fibrosis is increasing year by year. The main way to solve this complication is re-surgical, which not only reduces the patient's quality of life, but also increases the patient's financial burden[3]. This troublesome postoperative complication is an urgent problem for contemporary spine surgeons.

Many researchers have explored various ways to prevent epidural fibrosis, such as the use of antimetabolites, injecting drugs at the surgical site, and locally implanting synthetic materials to fill the postoperative defect area[4–6]. These measures have achieved some curative effects more or less, but there are many side effects and limitations[7].

Related reports in recent years indicate that the main cause of epidural fibrosis after the operation is fibroblasts undue proliferation[8–10]. Many articles have concluded that inhibiting fibroblast proliferation can effectively reduce epidural fibrosis and reduce scar tissues hyperplasia. Some articles show that the TGF- β 1 / Smad2/3 axis is extensively participated in the formation of fibrosis, and repression of the channel can inhibit the proliferation of fibroblasts[11, 12]. In addition, many studies have indicated that inhibition of fibroblasts migration and differentiation can also achieve better anti-fibrotic effects[13, 14].

Mycophenolate mofetil (MMF) is a kind of immunosuppressant that is mainly used to prevent rejection and treat refractory rejection in patients with allogeneic kidney transplantation[15]. Recently, many experiments have reported that MMF has a therapeutic effect on a variety of fibrotic diseases[16–18]. This study aims to explore whether MMF is effective in inhibiting epidural fibrosis after laminectomy, and to explore its possible mechanism of action: Whether MMF could inhibit fibroblast proliferation via TGF- β 1 / Smad2/3 axis. To find a more effective and safe treatment to help prevent this surgical complication.

2. Materials And Methods

2.1 Reagent and antibodies

MMF (C23H31NO7), it was bought from Aladdin Biotechnology (Shanghai, China). Its relative molecular mass is 433.49(Fig. 1A), and the purity is 98.00%. TGF- β 1 was bought from Cell Signaling Technology (CST, America). The rabbit monoclonal antibodies against TGF- β 1 (#3711), Smad2 (#5339), p-Smad2 (#18338), Smad3 (#9523), p-Smad3 (#9520), α -SMA (#19245), PCNA (#13110), CyclinD1 (#2978) and GAPDH (#5174) were bought from CST (America). The rabbit monoclonal antibody against vinculin (#ab129002) was purchased from Abcam (British). The mouse monoclonal antibody against tubulin (#ab52866) was purchased from Abcam (British). We bought horseradish peroxidase-conjugated goat anti-mouse (#7056) and goat anti-rabbit (#7074) secondary antibodies from CST (America) as well.

2.2 Fibroblasts collection and MMF treatment

We obtained a primary fibroblast cell line from previous study. Then, fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin, 15% fetal bovine serum, at 37°C in 5% CO₂, and the humidity was saturated. In vitro treatments, dissolved 1.25 mg of MMF in dimethyl sulfoxide (DMSO). Before added MMF to the cell culture, it was diluted to various concentrations. And we insured the concentration of DMSO in the solution was less than one thousandth.

2.3 Cell viability inspection

We assessed the impact of MMF on viability of the fibroblasts via Cell Counting Kit-8 (CCK-8). We determined the cell density as 1×10^4 cells/well, then inoculated the cell suspension in 96-well plates. Each concentration group had been repeated for three times. Incubated the plates for 24 h at 37°C in the incubator, then, treated various concentration groups for 12 h, 24 h, 36 h, and 48 h. After that, we pipetted 10 µl of CCK-8 solution into the wells of these treatment groups in these plates, incubated these plated for another 2 hours. We detected the optical density at 450 nm by microplate absorbance reader (Tecan, Switzerland). Calculated the fibroblasts viability according to the method on the instructions.

2.4 EdU incorporation assay

We used Cell-Light KFluor 488 EdU Kit (KeyGEN BioTECH, Jiangsu, China) to inspect fibroblasts proliferation after the treatments of MMF or agonists. The plates were stored for 24 h at 37°C, then treated cells with various concentration of MMF (0, 0.1, 1, 10 µM) or TGF-β1 (5 ng/ml) for 24 h. After it, we added 10 µM EdU solution to treatment well to hatched the cells about 24 h. Fixed these fibroblasts in 4% paraformaldehyde for 10 min, then we used 0.5% Triton X-100 to permeate them for 15 min. Used Hoechst 33342 to stain cellular nucleus. After completed all of the steps, Zeiss inverted fluorescence microscope were used to observe the positive cells with fluorescent signals. We used Image J to count the number of the positive cells.

2.5 Cell cycle analysis

To explored the principle of the MMF which induced proliferation of the fibroblasts, we used Cell Cycle Testing Kit (KeyGEN BioTECH, Jiangsu, China) to analysis the cell cycle distribution of various concentration groups. We cultured fibroblasts in 10 cm dishes, and treated cells with different concentrations of MMF (0, 0.1, 1, 10 µM) or TGF-β1 (5 ng/ml) in the logarithmic growth phase. Collected cells after the treatment then centrifuged at 1000 g/min for 5 min, throwed away the supernatant. Used the cold phosphate buffered saline to wash the wells once and used 70% ice-cold ethanol to fixed them. In dark room, resuspended the fibroblasts in 0.5 ml propidium iodide (PI) dye solution with 37°C water bath about 30 min. We detected the cells by flow cytometry (MoFlo™ XDP, German). Used ModFit software to analyze cell cycle distribution.

2.6 Cell wound scratch assay

Inoculated the cells in 6 well plates, and grown until 85–90% confluence. Then wounded the cells with white pipette tips and washed three times with phosphate buffered saline. Then hatched the cells with MMF (0, 0.1, 1, 10 μ M) or TGF- β 1(5 ng/ml) in medium supplemented that with 2% serum for 24 h. We took the photo of the scratch area under an inverted microscopy. We measured the wounded area from treatment wells at three randomly selected wound sites for statistical analysis, and we calculated three independent scratch-wound experiments.

2.7 Transwell assay

We prepared transwell chambers to conduct the experiment. The cells were collected in serum-free medium, its final concentration were 1×10^5 cell/ml. Then treated with MMF (0, 0.1, 1, 10 μ M) or TGF- β 1(5 ng/ml) for 24 h at 37°C. 200 μ l cell suspensions with serum-free medium were added to the upper compartment, and 500 μ l medium containing 15% fetal bovine serum was placed in the lower compartment. Following hatched for 18 h, we stained the migrated cells on the lower surface with purple crystal (Beyotime, Jiangsu, China). Then we quantified by manual counting and chosen three randomly fields in each group for analyzing.

2.8 Immunofluorescent staining for tubulin, vinculin and α -SMA

Treated the fibroblasts with different concentrations of MMF, at indoor temperature, we used 4% paraformaldehyde in PBS to fix the cells for 10 min. Then used 0.1% Triton X-100 in PBS to permeabilize the cells at indoor temperature for 15 min. At 4°C, we used anti-vinculin antibody (1:100 dilution) and anti- α -SMA antibody (1:100 dilution) to hatch the cells all night. Then at indoor temperature, we washed the cells and hatched them with anti-rabbit IgG H&L (Alexa Fluor 555) antibody (1:200 dilution) (Abcam, British) for 1 h. And the cells were hatched with anti-tubulin antibody (1:100 dilution) all night at 4°C, then at indoor temperature, hatched them with anti-mouse IgG H&L (Alexa Fluor 555) antibody (1:200 dilution) (Abcam, British) about 1 h.

2.9 Western blotting analysis

After treatment with MMF (0, 0.1, 1, 10 μ M) or TGF- β 1 (5 ng/ml), we lysed the cells with equal volume lysis buffer (CST, American) on ice. Then used ultrasonic wave to treat cell lysate. Centrifugated at 15000r, 4°C for 10 minutes. Measured the protein concentrations by BCA Protein Assay Kit (Thermo, USA). We used 10% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, Jiangsu, China) to separate equal amount protein extracts, then transferred onto polyvinylidene difluoride membranes (Millipore, America). After transferred, at indoor temperature, immersed the polyvinylidene fluoride membrane in 5% skim milk in 1x TBST about 2 h. Then hatched with diluted primary antibodies at 4°C all night according to the instruction books. Washed the membranes 3 times with 1x TBST for 10 minutes and then hatched with secondary antibody (anti-mouse or anti-rabbit IgG, CST, America) for 2 hours at indoor temperature. Membranes were washed for 3 times with 1x TBST as well after incubated. Finally, added ECL reagent (enhanced chemiluminescence detection) on the membranes then used the ChemiDoc XRS + system (Bio Rad, America) to explore the membranes.

2.10 Animals

We bought SD male rats weighing between 300 and 350 g from the experimental animal center of Yangzhou University (Yangzhou, China). Animal Ethics Committee of Yangzhou University ratified this study about animals, and we cared each rat carefully. We divided the rats into four groups (12 rats per group) randomly: control group (saline) and MMF groups (low, medium and high dose groups).

2.11 Rats laminectomy model and topical application of MMF

Established laminectomy models according to the methods in previous study. Briefly, we anesthetized the rats through intraperitoneal injection with 1% sodium pentobarbital (40 mg/kg), then located the lumbar 1 position by pinching the rib pedicle. Revealed fascia and isolated the paravertebral muscles bluntly. Then we used mini-biting forceps to remove the L1 vertebral plate and expose the 5 × 3 mm defect in the dura mater district. After the surgical area got adequate hemostasis, we washed the area with saline. Then, the gauzes thorough soaked with 0.2 mg/ml saline and MMF in different concentrations were placed on the operation area for 15 min. Then we washed the surgical area and sutured the wounds layer by layer. For the sake of preventing notch infection, each rat had intramuscular injection with penicillin (Lukang, Shandong, China) in once a day for three days, and injected 50 mg of penicillin per kilogram of body weight each mouse.

2.12 Histological analysis

1 month later, killed the rats with fetal dose of sodium pentobarbital after laminectomy. Then we perfused the rats in cardiovascular with 4% paraformaldehyde. After that, we removed the whole L1 spinal column and immersed in 4% paraformaldehyde. We decalcified the samples in ethylenediamine tetraacetic acid (EDTA) about 1 month and then embedded in paraffin. Continuous 4 μm samples were made of paraffin lumps which selected from these rats randomly. Then we used hematoxylin-eosin (H&E) and Masson's trichrome to stain the samples respectively. We used light microscope to take photos of epidural fibrotic tissues under 40 and 200fold microscope. The study calculated the fibroblasts in three counting fields (100 × 100 mm each) of the surgical areas by using Image J, the magnification was × 200.

2.13 Immunohistochemical staining

We used immunohistochemical staining to explore the impacts of MMF on fibroblasts proliferation, migration and differentiation in epidural fibrosis. PCNA can be a sign of recent cell proliferation, tubulin can be a sign of cell migration, and α-SMA can be a sign of cell differentiation. Therefore, we used immunohistochemical staining to detect the content of PCNA, tubulin and α-SMA in fibrotic tissues. After deparaffinization and alcohol dehydration, we used citrate buffer to acting on the samples to repair

antigen, and then exposed the samples in 3% H₂O₂ to interdict endogenous peroxidase activity. We washed these slices with PBS three times, and hatched them with anti-PCNA, anti-tubulin and anti- α -SMA antibodies all night at 4°C. Followed by incubation with anti-rabbit or mouse IgG at RT for 2 h. Then we used DAB kit to analysis the slices and detect the binding of antibodies. In the end, we counterstained the slices with hematoxylin and used light microscope to observe them. For the study, we used Image J to calculate the proportion of positively cells per five 200 × magnification fields.

2.14 Statistical analysis

We used SPSS 19.0 statistical tool to analyzed the results in our experiments, and used mean $\pm$ standard deviation (S.D.) to indicate all results. The one-way analysis of variance and independent-samples T test was used to compare the data of each group. P value < 0.05 is considered with statistical significance.

3. Results

3.1 MMF suppress fibroblasts proliferation

As shown in Fig. 1B, the results of CCK-8 assay demonstrated that MMF repressed fibroblasts proliferation with the increased concentration and longer effect time. The results of EdU incorporation assay proved the inhibition effect of MMF on the proliferation of fibroblasts (Fig. 1C). To further confirmed how MMF regulated the fibroblasts proliferation, we used flow cytometry to explore the cell cycle. As the results in Fig. 1D, MMF prevented the cell cycle course in the G0/G1 phase. The analysis about Western blotting indicated that the synthesis of proliferation proteins, for example, PCNA and Cyclin D1, decreased as MMF concentration increased (Fig. 1E). The results of the above experiments proved that MMF could inhibit the proliferation of fibroblasts.

3.2 MMF inhibits migration of fibroblasts in vitro

Cell wound scratch assay demonstrated that structure and quantitative damage of the cytoskeleton led to the reduction of migration capacity (Fig. 2A), the percentage of scratch reduction area decreases with increasing MMF concentration. In the transwell assay, we proved the inhibited impact of MMF on the migration of fibroblasts as well. The amount of cells went through the transwell was reduced along with the increase concentration of MMF (Fig. 2B). Immunofluorescence demonstrated that MMF repressed the synthesis of migration proteins and changed structure of the cytoskeleton (Fig. 2C). As the increase of MMF concentration, the direction of the migrating proteins changed from uniform to chaotic and diffused.

Western blot was used to detect the synthesis levels of the migration proteins like vinculin and tubulin (Fig. 2D). MMF repressed the synthesis of vinculin and tubulin obviously. Our results indicated that MMF repressed migration of fibroblasts in vitro.

3.3 MMF inhibits differentiation of fibroblasts in vitro

We explored the impacts of MMF on the fibroblasts differentiation. The outcomes displayed that the quantitative decrease synthesis of differentiation protein like α -SMA was indicated by immunofluorescence (Fig. 3A). Besides, the synthesis quantity of α -SMA was decreased with concentrations of the drug increased (Fig. 3B). These results indicated that MMF inhibits differentiation of fibroblasts in vitro.

3.4 MMF regulates TGF- β 1/Smad2/3 axis expression

To explore whether the MMF regulated TGF- β 1/Smad2/3 axis expression, we treated fibroblasts with MMF of 0, 0.1, 1, 10 μ M, then used Western blot to detect channel correlative protein expression. As the results in Fig. 4A, along with the treatment of various concentration of MMF, the synthesis quantities of TGF- β 1, p-Smad2, p-Smad3 were decreased with concentrations of the drug increased. TGF- β 1, which was known as an activator of TGF- β 1/Smad2/3 axis, partially increased the low under-expression quantities of p-Smad2, p-Smad3 induced by MMF (Fig. 4B). The above results showed that MMF could regulate TGF- β 1/Smad2/3 axis expression.

3.5 MMF regulates proliferation, migration and differentiation via TGF- β 1/Smad2/3 axis

Along with the stimulation of TGF- β 1, the low synthesis levels of PCNA (Fig. 4C), tubulin, vinculin (Fig. 4D) and α -SMA (Fig. 4E) were partly enhanced. Meanwhile, the results of EdU assay (Fig. 5A), cell cycle analysis (Fig. 5B), wound scratch assay (Fig. 6A), transwell assay (Fig. 6B) and the immunofluorescent staining (Fig. 6C, Fig. 7) indicated the consistent significance as the analysis of Western blot assay. Above results revealed that negative regulation of TGF- β 1/Smad2/3 axis induced by MMF had a significantly impacts on the proliferation, migration and differentiation of fibroblasts.

3.6 MMF repressed fibroblasts proliferation, migration, differentiation, and reduced epidural fibrosis in rats

The results of H&E-staining showed that applied topically of MMF was able to reduce fibrotic tissues in epidural. Compared with the saline group, the topical application of MMF could significantly arrest the epidural fibrosis, and its action trend was basically consistent with the concentration. The fibrotic tissues in 0.16 mg/ml group were obviously less than that in medium, low concentration groups and saline group (Fig. 8A). We calculated the amount of fibroblasts in fibrotic tissues taken from surgical areas demonstrated that MMF could obviously repressing the fibroblasts proliferation (Fig. 8B, Fig. 8C). Meanwhile, we used Masson's trichrome staining to confirm collagen content in fibrotic tissues from the surgical areas. Optical density analysis proved that the content of collagen in the experimental groups was significantly reduced compared with which in the saline group (Fig. 8D). The PCNA immunohistochemistry staining in fibroblasts also reflected that the proliferation of cells in treatment group was weaker than the proliferation of cells in control group (Fig. 9A). Moreover, the tubulin

immunohistochemistry staining suggested that MMF could inhibit the migration of fibroblasts (Fig. 9B). The α -SMA immunohistochemistry staining proved that MMF could inhibit the differentiation of fibroblasts (Fig. 9C).

4. Discussion

Epidural fibrosis is a deformed healing of the incision site after laminectomy. In this process, fibroblasts accumulate at the incision after a large number of proliferation[19]. As a pathological process, epidural fibrosis can cause a large amount of fibrotic tissue to form in the surgical site after laminectomy, which seriously affects the surgical effect of patients[20]. The mechanism of the proliferation of fibroblasts is relatively complicated, and there are still many related problems that need to be solved. However, it is generally recognized that excessive proliferation of fibroblasts is the main factor causing tissue fibrosis[21–23].

TGF- β 1 has extensive biological functions, including regulating cell growth, differentiation, migration, matrix formation, and damage repair[24–26]. It is the most in-depth and most powerful profibrotic cytokine currently studied[27–29]. TGF- β 1 is a powerful chemokine for monocytes and fibroblasts[30]. As well as, it can increase the proliferation ability of fibroblasts, promote their secretion of extracellular matrix such as collagen and fibronectin[31]. TGF- β 1 can induce fibroblasts phenotype Transformation to myofibroblasts phenotype, characterized by high synthesis of α -SMA, causing matrix contraction and participating in accelerating the extracellular matrix deposition[32]. It also has the function of repressing extracellular matrix degradation and promoting apoptosis of epithelial cells[33]. At the same time, it is an important cell that induces epithelial-mesenchymal transition, making epithelial cells lose their polarity, adhesion molecules such as cadherin, and obtain characteristics of mesenchymal cells, such as the synthesis of fibronectin, α -SMA, and exhibit migration[34]. It can be seen that it has a significantly impacts on the mechanism of epidural fibrosis formation.

The Smad protein is the main signal transduction factor of TGF- β 1, which transfers the signal of TGF- β 1 from the cell to the nucleus regulates target gene transcription[35]. Co-regulates the transcription of target genes with other transcription factors[36]. Many articles indicate that repress the TGF- β 1 channel can inhibit fibroblasts functions[37–39].

MMF is a derivative of mycophenolic acid (MPA), and it is a new immunosuppressant. Rapid hydrolysis of MMF after oral administration into the active metabolite MPA in the body. MPA inhibits the synthesis of guanine nucleotides by repressing the key rate-limiting enzyme inosine monophosphate dehydrogenase (IMPDH), a new channel for the synthesis of purine nucleotide[40]. Due to its specificity, MMF has recently been used in the treatment of proliferative fibrotic diseases and has achieved good results. Badid et al.[13]found that 72 hours after MMF treatment, MMF can dose-dependently inhibit rat fibroblasts proliferation, reduce infiltration of interstitial myofibroblasts and deposition of type III collagen. Liu et al.[41] found that MMF improves renal function and reduces renal interstitial fibrosis in a rat renal interstitial fibrosis model with 5/6 resection of the kidney. Nina[42] and other studies found that

MMF can inhibit the expression of collagen genes, the contraction of extracellular matrix, and the migration of fibroblasts suggest that it may have an anti-fibrotic effect. Our experimental results also proved the anti-fibrosis effect of MMF. Dubus et al.[43] found that MMF can reduce TGF- β 1-induced human epithelial cell proliferation and extracellular matrix production (such as type I collagen and laminin deposition). It can be seen that MMF can not only inhibit lymphocyte proliferation and recruitment, but also have a direct anti-fibrosis effect, but there is no related research on epidural fibrosis. We speculate that MMF can terminate the signal transduction of TGF- β 1-related channel, thereby inhibiting the proliferation, migration and differentiation of fibroblasts. Our study showed that MMF could also inhibit the differentiation of fibroblasts, which was barely involved in the above studies, and our study on cell differentiation helped deepen the understanding of the mechanism of MMF anti-fibrosis.

Therefore, we speculated that MMF may repress the functions of fibroblasts by regulating the TGF- β 1 channel. In this research, we used Western blot to research fluctuation in related protein synthesis. The results showed that the synthesis quantities of TGF- β 1, p-Smad2, p-Smad3, PCNA and Cyclin D1 decreased after MMF treatment. These results indicate that MMF can regulate TGF- β 1 channel and its channel proteins to repress fibroblasts proliferation.

In vitro experiments, we conducted a number of cell experiments to investigate whether MMF can inhibit the proliferation, migration, and differentiation of fibroblasts. Our results indicate that MMF can play these roles. At the same time, we also found that MMF significantly repressed the TGF- β 1 / Smad2/3 axis. Therefore, we used a potent activator, TGF- β 1, to activate the channel. According to the results of Western blot analysis, we found that TGF- β 1 effectively activated the TGF- β 1 / Smad2/3 axis. The activated channel attenuated impacts of MMF on the proliferation, migration, and differentiation of fibroblasts.

In vivo experiments, we established a laminectomy model in rats. After laminectomy, different concentrations of MMF were applied locally to rat incisions. The selection of the MMF concentrations was based on previous studies in rats. H&E-staining histological analysis of epidural fibrotic tissues demonstrated that fibrosis in the fibrotic tissues from the surgical areas in the MMF treatment group was significantly weaker, and the number of fibroblasts was significantly reduced. We used Masson's trichrome staining to evaluate collagen content in fibrotic tissues. Our results showed that MMF can significantly inhibit collagen content. Further research on histochemical staining demonstrated that the synthesis of PCNA, tubulin and α -SMA in fibrotic tissues in the group treat with MMF was obviously less than in the saline group. These studies indicate that MMF could reduce epidural fibrosis caused by operation.

In conclusion, based on above results, we can preliminary explain the role of MMF on inhibiting epidural fibrosis. In short, when applying MMF to the laminectomy site, the TGF- β 1 / Smad2/3 axis in fibroblasts is repressed, and finally the proliferation, migration and differentiation of fibroblasts are inhibited, thereby reducing the fiber formation.

The mechanism of epidural fibrosis formation is intricacy, there are still some limitations in our research. Firstly, MMF was administered topically. Although all animals survived until the study finished, a few side effects may happen on other regions. Secondly, we only studied the effect of TGF- β 1 / Smad2/3 axis in inducing fibroblasts proliferation, migration, and differentiation, without further exploring MMF may affect fibroblasts proliferation through other signaling pathways. These are the shortcomings of this research, and we will start to conduct more in-depth research in these areas in the future.

5. Conclusion

In summary, we have initially verified that MMF can inhibit fibroblasts proliferation, migration, and differentiation. Then we propose a new concept: MMF may affect these three fibroblasts activities by repressing the transduction of the TGF- β 1 / Smad2/3 axis. To this point, further exploration is needed in the future.

Abbreviations

MMF:Mycophenolate mofetil; IF:immunofluorescence; IHC:immunohistochemical; DMEM:Dulbecco's modified Eagle's medium; DMSO:dimethyl sulfoxide; CCK-8:Cell Counting Kit-8; PI:propidium iodide; SDS-PAGE:sodium dodecyl-sulphate-polyacrylamide gel electrophoresis; EDTA:Ethylenediamine tetraacetic acid; H&E:Hematoxylin and eosin; PBS:Phosphate-buffered saline; S.D.:standard deviation

Declarations

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its supplementary materials.

Ethics approval and consent to participate

The research was conducted in accordance with the guidance of the Animal Ethics Committee of Yangzhou University, China, and pathology laboratory of Yangzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by Jiangsu Provincial Medical Innovation Team (grants#CXTDB2017004).

Authors' contributions

DZ and JZ performed the whole experiments and were responsible for the data and drafting of the article. YS and LY designed the study and contributed to the preparation of the manuscript. XZ, XL, and YL helped in the performance of animal surgeries and the interpretation of data. All authors read and approved the final manuscript.

Acknowledgements

We thank the authors of the included studies for their help.

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Figures

Figure 1

The molecular structure of MMF and its inhibitory effect on cell growth and cell proliferation in fibroblasts. (A) Chemical structure of MMF. (B) After different concentrations of MMF treated fibroblasts for different time, CCK-8 test results show that MMF can inhibit the growth of fibroblasts in a concentration and time-dependent manner. (C) EdU incorporation analysis showed that after 24 h treatment with MMF, the percentage of EDU-positive cells decreased in a concentration-dependent

manner. Five different fields were randomly selected under a fluorescence microscope to calculate the percentage of EDU-positive cells. Each group counted more than 50 fibroblasts for EDU analysis. (D) Cell cycle analysis showed that after 24 hours of different concentrations of MMF treatment, MMF could limit the growth of fibroblasts to G1 phase. (E) Western blot analysis showed that MMF could reduce the expression of PCNA and Cyclin D1 in a concentration-dependent manner. GAPDH was used as a control group. The histogram represents the mean $\pm$ S.D. of three replicates. * Compared with control group, $P < 0.05$.

A

B

C

D

Figure 2

MMF inhibits migration of fibroblasts in vitro. (A) Wound scratch assay showed that MMF could reduce the percentage of scratch reduction area in a concentration-dependent manner. (B) Transwell assay showed that the amount of cells went through the transwell was reduced along with the increase concentration of MMF. (C) The reduced tubulin and vinculin protein content is accompanied by structural changes as shown by immunofluorescence. Tubulin fibers lose their straight directed orientation and vinculin is decreased in the cytoplasm after treatment with MMF. (D) Western blot analysis showed that MMF decreased the expression of tubulin and vinculin in a concentration-dependent manner. GAPDH was used as a control, and the bar chart showed the means $\pm$ SD deviation of three repeated tests. *Compared with the control group, $P < 0.05$.

A

B

Figure 3

MMF inhibits differentiation of fibroblasts in vitro. (A) Immunofluorescence staining of α -SMA showed that after treat with MMF for 24 hours, the fibroblasts expressed a lower amount of α -SMA in the cytoplasm. The data were calculated as means $\pm$ SD deviation of three random visual fields (at least 50 cells per field; *P < 0.05). (B) Western blot analysis showed that MMF decreased the expression of α -SMA in a concentration-dependent manner. GAPDH was used as a control, and the bar chart showed the means $\pm$ SD deviation of three repeated tests. *Compared with the control group, P < 0.05.

Figure 4

MMF inhibits the activation of the TGF- β 1 / Smad2/3 axis, and the inhibition of this pathway may affect the proliferation, migration, and differentiation of fibroblasts. (A) Fibroblasts were treated with different doses of MMF for 24 hours. Western blot results showed that after MMF acted on fibroblasts, the phosphorylation levels of Smad2 and Smad3 decreased in a concentration-dependent manner. (B) After treated with activator TGF- β 1, the expression of the phosphorylation levels of Smad2 and Smad3

increased. (C) After treated with activator TGF-β1, the expression of cell proliferation-related protein increased. (D) After treated with activator TGF-β1, the expression of cell migration-related protein increased. (E) After treated with activator TGF-β1, the expression of cell differentiation-related protein increased. GAPDH was used as control. The histogram shows the gray value of three repeated tests of Western blot results. MMF group compared with MMF mixed TGF-β1 group, *P < 0.05.

A

B

Figure 5

MMF inhibits fibroblasts proliferation by inhibiting TGF- β 1 / Smad2/3 axis. (A) In vitro, we detected the proliferation of fibroblasts by EdU incorporation assay. All nuclei were blue and the nuclei of EdU-positive cells were green. Five different fields were randomly selected under a fluorescence microscope to calculate the percentage of EDU-positive cells. Each group counted more than 50 fibroblasts for EDU analysis. After treatment with the activator TGF- β 1, MMF-induced fibroblasts proliferation inhibition was reversed to a certain extent. MMF group compared with MMF mixed TGF- β 1 group, *P < 0.05. (B) Cell cycle analysis showed that after treatment with the activator TGF- β 1, the proportion of cells in G1 phase was decreased. The proliferation inhibition of fibroblasts induced by MMF was reversed by the activator TGF- β 1. MMF group compared with MMF mixed TGF- β 1 group, *P < 0.05.

A

B

C

Figure 6

MMF inhibits fibroblasts migration by inhibiting TGF-β1 / Smad2/3 axis. (A) Wound scratch assay showed that after treatment with the activator TGF-β1, the percentage of scratch reduction area were higher than the treatment group which only treated with MMF (10μM). The migration inhibition of fibroblasts induced by MMF was reversed by the activator TGF-β1. MMF group compared with MMF mixed TGF-β1 group, *P < 0.05. (B) Transwell assay showed that after treatment with the activator TGF-

$\beta 1$, the amount of cells went through the transwell was increased. MMF group compared with MMF mixed TGF- $\beta 1$ group, $*P < 0.05$. (C) Immunofluorescence staining of tubulin and vinculin showed that after treatment with the activator TGF- $\beta 1$, the amount of these protein which expressed in the cytoplasm of fibroblasts were increased.

Figure 7

MMF inhibits fibroblasts differentiation by inhibiting TGF- $\beta 1$ / Smad2/3 axis. Immunofluorescence staining of α -SMA showed that after treatment with the activator TGF- $\beta 1$, the amount of α -SMA in the cytoplasm of fibroblasts were increased. MMF group compared with MMF mixed TGF- $\beta 1$ group, $P < 0.05$.

A

B

Figure 8

Histological analysis of the laminectomy areas. (A) In the HE images, we found dense or thick fibrosis tissues (*) with extensive or firm adherence (arrow) to dura mater in control group (0 mg/kg). We observed medium fibrosis tissues (*) with medium adherence (arrow) to dura mater in laminectomy defect areas of 0.04 mg/ml MMF-treated group. Then we found sparse fibrosis tissues (*) with few adhesions to dura mater (arrow) in laminectomy defect sites of 0.08 mg/ml MMF-treated group. Looser fibrosis tissues (*) with fewer adhesions to dura mater (arrow) were observed in 0.16 mg/ml MMF-treated group. The laminectomy defect site was marked by "L", and spinal cord was marker by "S". All sections were stained by the mean of HE, and the magnification was $\times 40$. (B) The images of fibroblasts in epidural fibrosis tissues of each group showed that the number of fibroblasts was decreased along with the

increase of MMF concentration. The magnification was $\times 200$. (C) Fibroblasts number was calculated by randomly selecting three counting areas from every section. * Compared with the control group, $P < 0.05$. (D) After the slices were stained with Masson's trichrome, the images showed that collagen content in epidural fibrosis tissues decreased in MMF treatment group. The magnification was $\times 200$. Optical density analysis of Masson staining images showed that MMF could reduce collagen production. * Compared with the control group, $P < 0.05$.

Figure 9

Immunohistochemical staining of PCNA, tubulin and α-SMA in epidural fibrosis tissues. (A) The PCNA immunohistochemistry staining in fibroblasts reflected that MMF treatment reduced the number of PCNA-positive cells in a concentration-dependent manner in vivo. (B) The tubulin immunohistochemistry

staining in fibroblasts reflected that MMF treatment reduced the number of tubulin-positive cells in a concentration-dependent manner in vivo. (C) The α -SMA immunohistochemistry staining in fibroblasts reflected that MMF treatment reduced the number of α -SMA-positive cells in a concentration-dependent manner in vivo. The percentages of positive cells were calculated by Image J. Approximately 200 cells or nuclei were counted in five fields of each slice. * Compared with the control group, $P < 0.05$.