

Basic Science

Toxicity Effects of Methylene Blue on Rat Intervertebral Disc Annulus Fibrosus Cells

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Disclaimer: There was no external funding in the preparation of this manuscript.

Conflict of interest: Liang Zhang and Yang Liu are the corresponding author. Each author certifies that he or she, or a member of his or her immediate family, has no commercial association (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted manuscript.

Manuscript received: 05-31-2018

Revised manuscript received: 08-13-2018

Accepted for publication: 09-17-2018

Free full manuscript:
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Background: There is an increasing local application of methylene blue (MB) in the treatment of discogenic low back pain (LBP) and percutaneous transforaminal endoscopic discectomy (PTED) procedures. MB could generate DNA damage and induce apoptosis in different cell types; however, the effects of MB on intervertebral disc (IVD) annulus fibrosus (AF) cells are not clearly understood.

Objective: The objective of this study was to investigate the effects of different concentrations of MB on rat AF cells in vitro.

Study Design: This study used an experimental design.

Setting: This research was conducted at the Orthopaedic Institute of the Clinical Medical College of Yangzhou University.

Methods: AF cells were isolated and cultured with different concentrations of MB (0, 2, 20, and 200 µg/mL) and assessed to determine the possible cytotoxic effects of MB. The cell proliferation was detected by Cell Counting Kit-8 (CCK-8) assay. The inverted phase-contrast microscopy was used to perform morphological observation of apoptotic cells, and flow cytometry was used to measure the incidence of cell apoptosis. The mRNA and protein expression levels of apoptosis-associated genes (caspase-3, Bcl-2, and Bax) and other related genes (collagen type I, transforming growth factor β1 [TGF-β1], fibroblast growth factor [bFGF], and tissue inhibitor of metalloproteinase-1 [TIMP-1]) were analyzed by quantitative real-time PCR (RT-PCR) and Western blotting.

Results: Our results indicated that MB reduced cell viability in a concentration- and time-dependent manner. MB also induced marked AF cell apoptosis in a concentration-dependent manner observed by inverted phase-contrast microscopy, flow cytometry, and indicated by the increased expression of caspase-3. Both RT-PCR and Western blotting revealed significant up-regulation of Bax and caspase-3 expression levels accompanied by decreased expression of Bcl-2 in a concentration-dependent manner. Moreover, collagen type I, TGF-β1, bFGF, and TIMP-1 mRNA and protein levels were also found to be decreased by MB in a concentration-dependent manner.

Limitations: Limitations of this study were the in vitro study design and lack of in vivo validation of the observed effects of MB on human IVD cells.

Conclusions: Our results indicate that a high concentration of MB can not only inhibit proliferation and paracrine function of AF cells, but can also induce cell apoptosis in a concentration-dependent manner, suggesting that it is necessary to choose low concentrations of MB in practical application and limit the use of MB in the treatment of discogenic LBP to research protocols.

Key words: Methylene blue, annulus fibrosus cell, proliferation, apoptosis, paracrine

Pain Physician 2019; 22:155-164

Low back pain (LBP) is one of the most common and costly musculoskeletal disorders in many countries. The high morbidity of LBP causes severe incapacity that increases medical expenses and affects the workforce, resulting in huge socioeconomic costs (1,2). Internal disc disruption, identified as the most common cause of LBP through provocation discography, has a reported prevalence of up to 42% (3).

Since methylene blue (MB) was first synthesized in 1876, it has been used in many different areas of clinical medicine (4,5). Due to its neurolytic effect, MB injected into the nucleus pulposus (NP) can spread into radial fissures and destroy the nerve endings or nociceptors that have grown into the painful disc. The results of a randomized controlled trial (RCT) published in 2010 (6) showed statistically and clinically meaningful reduction in pain and disability in patients with discogenic LBP. However, the effectiveness of MB in treating discogenic LBP is controversial. A multicenter prospective study published in 2016 (7) concerning intradiscal MB injection demonstrated that only 40% of the patients reported 30% pain relief after 6 months. Furthermore, the success rate of MB used in the treatment of discogenic LBP was only 12.5% to 25%, as reported by Levi et al in 2014 (8) and Gupta et al in 2012 (9). Due to its tissue dye effect, MB is also commonly used in percutaneous transforaminal endoscopic discectomy (PTED) to better stain the intervertebral disc (IVD) in order to reduce neurological complications (10). Intradiscal MB injection treatment commonly consists of 1 mL of MB, 0.5 mL of lidocaine hydrochloride 2%, and 0.5 mL of contrast dye (11), while IVD staining consists of 1 mL of MB and 9 mL of contrast dye (10). However, little is known about the effect of high concentrations of MB on IVD cells, especially in the local application of PTED procedure. Laszlo et al (12) demonstrated that MB could exert neurotoxic effects on the central nervous system and thus raised questions regarding the safety of using this drug at high doses during parathyroid gland surgery. Simone et al (13) further found that MB can generate DNA damage and induce apoptosis of zebrafish cell lines.

Considering the cytotoxic effects of MB, high concentrations of MB may affect the proliferation and apoptosis of annulus fibrosus (AF) cells. Therefore, the present study aimed to determine the toxic effect of MB on AF cells *in vitro*, including cell proliferation, apoptosis, and paracrine function.

METHODS

Isolation and Culture of AF Cells

The protocol for the present work was approved by the Ethical Committee of the Clinical Medical College of Yangzhou University. The AF cells were isolated from the AF of coccygeal spines of 4 Sprague-Dawley rats (weight, 300-400 g; age, 4-6 months) based on a previously published protocol (14). Briefly, the AF tissue was harvested from the coccygeal IVD after being killed by an intraperitoneal overdose injection of 10% chloral hydrate and cut into small pieces. The minced AF tissues were subjected to enzymatic digestion with 0.2% collagenase type II (Gibco, New York, NY) at 37°C for 3 hours and 0.25% trypsin (Gibco, New York, NY) at 37°C for 10 minutes. Then washing with phosphate-buffered saline (PBS) twice and centrifuging at 1000 rpm for 5 minutes, the cell pellets were resuspended in a DMEM/F12 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin (Gibco, New York, NY). Finally, cells were plated at a density of 2×10^5 cells/mL into 25 cm²-cell culture flasks and incubated at 37°C with 5% CO₂. The medium was replaced every 3 days, and the growth status was observed under an optical microscope. When adherent cells reached 80% confluence, they were harvested with 0.25% Trypsin-EDTA for 3 minutes at 37°C and subcultured at 1:3. At third passage, cells were harvested for subsequent experiments.

Treatment Group

The AF cells were treated with the following concentrations of MB at 37°C with 5% CO₂ for 72 hours: 2 µg/mL (MB-2), 20 µg/mL (MB-20), and 200 µg/mL (MB-200). The control group was exposed to the same media without MB under the same conditions (MB-0). The effects of MB on the proliferation, apoptosis, and paracrine function were determined by Cell Counting Kit-8 (CCK-8), flow cytometry, activity of caspase-3, real-time quantitative polymerase chain reaction (RT-PCR), and Western blotting.

Morphological Observation

AF cells were plated in 6-well plates at a density of 1×10^5 cells/well with different concentrations of MB for 2, 4, 6, 12, 24, or 72 hours, then observed by inverted phase-contrast microscopy (Olympus, Japan) and photographed with a digital camera (Nikon, Japan).

Cell Proliferation Assay

AF cells were plated at a density of 1,000 cells/well

in 96-well plates with different concentrations of MB at 37°C with 5% CO₂. At 2, 4, 6, 12, 24, and 72 hours after the initial plating, 10 µL CCK-8 reagent were added into each well of the plate. After incubating for 4 hours, the absorbance at 450 nm was detected by a microplate reader (Tecan Infinite, Switzerland) and cell viability was normalized as a percentage of control.

Apoptosis Assay

The Annexin V-fluorescein isothiocyanate/propidium Iodid (Annexin V-FITC/PI) apoptosis detection kit (KeyGen, China) was used to detect apoptotic and dead cells. Because a preliminary test showed significant inhibition of cell viability after 4 hours of incubation with different concentrations of MB, the 4-hour time point was chosen to analyze the effect of MB on cell apoptosis. AF cells plated at a density of 1 × 10⁵ cells/well in 6-well plates were treated with different concentrations of MB at 37°C with 5% CO₂ for 4 hours, then cells were washed with PBS and collected by trypsinization. These cells were suspended in PBS and then incubated with 5 µL of Annexin V-FITC and 5 µL of PI for 15 minutes in the dark. All samples were subjected to flow cytometry (Beckman Coulter, Brea, CA) using CellQuest software (BD Biosciences, San Jose, CA).

Caspase-3 Activity Assay

Caspase-3 activity was detected using a caspase-3 activity kit (Keygen, China). The third passage AF cells were placed in 6-well plates at a density of 1 × 10⁵ cells/well and treated with different concentrations of MB at 37°C with 5% CO₂ for 4 hours. According to the protocol, these cells were collected and disrupted in lysis buffer (containing 0.5 µL dithiothreitol/50 µL). The lysates were then incubated with acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) in 96-well microtiter plates at 37°C for 4 hours. The optical density (OD) value at 405 nm was measured with a microplate spectrophotometer (Tecan Infinite, Switzerland). The relative activity of caspase-3 was expressed as the ratio of enzyme activity of different concentrations of MB groups relative to the control group.

RT-PCR Assay

The expressions of related genes were determined by RT-PCR. The third passage AF cells were placed in 6-well plates at 1 × 10⁵ cells/well with different concentrations of MB. The total RNA of AF cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and underwent reverse transcription to cDNA with reverse

transcriptase (ToYobo, Japan). Quantities of gene expression were calculated with standard samples and normalized with glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) values. The relative gene expression level was calculated by the comparative computed tomography (CT) method. Primers for the amplification of genes encoding collagen type I, basic transforming growth factor β1 (TGF-β1), fibroblast growth factor (bFGF), tissue inhibitor of metalloproteinase-1 (TIMP-1), caspase-3, Bcl-2, Bax, and GAPDH were synthesized (Genechem, China); these are listed in Table 1.

Western Blotting Assay

Total proteins were extracted using a protein extraction kit, and the protein concentrations were quantified using the BCA Protein Assay Kit (Beyotime, China). Each sample (30 µg protein) was loaded onto 12% SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA). The blots were blocked using 5% nonfat dry milk for one hour at room temperature, and then incubated with primary antibodies overnight. The following antibodies from Proteintech (Rosemont, IL) were used: anti-collagen type I (1:2000 dilution), TGF-β1 (1:1000 dilution), bFGF (1:1000 dilution), TIMP-1 (1:1000 dilution), Bcl-2 (1:1000 dilution), Bax (1:1000 dilution), and caspase-3 (1:1000 dilution); and anti-Tubulin (1:8000 dilution; Beyotime, China). After 3 washes with phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST), membranes were incubated with horseradish peroxidase (HRP)-

Table 1. *Primer sequences for target gene.*

Gene	Prime Sequence
β-actin	Forward: 5'-CCCAGCCCGTGTTCCT-3' Reverse: 5'-GTCCAGTTGGTGACGATGG-3'
Collage type I	Forward: 5'-TAGGAGTCGAGGGACCCAAG-3' Reverse: 5'-AGGCTCTCCCTTAGGACCAG-3'
TGF-β1	Forward: 5'-GACTCTCCACCTGCAAGACC-3' Reverse: 5'-AGCCTGTATCCGTCTCTCT-3'
bFGF	Forward: 5'-ACCCTATCCCTTCACAGCCT-3' Reverse: 5'-CCGCTTGGGATCCTTGAAGT-3'
TIMP-1	Forward: 5'-TTTGCATCTCTGGCCTCTGG-3' Reverse: 5'-TAGCCCTTCTCAGAGCCCAT-3'
Caspase-3	Forward: 5'-CCTCAGAGAGACATTCATGG-3' Reverse: 5'-GCAGTAGTCGCCTCTGAAGA-3'
Bcl-2	Forward: 5'-GGTTGTTCCTAGGCTGGGTC-3' Reverse: 5'-AATAGGGGAAGGGCGTGA-3'
Bax	Forward: 5'-CACTACTAGGGTCCCCAGCT-3' Reverse: 5'-GAGGAAGTCCAGTGTCCAGC-3'

conjugated secondary antibodies (Sanying, China) for 2 hours on a shaker at room temperature. Bands were visualized using an enhanced chemiluminescence (ECL) system and analyzed with ImageQuant LAS 400 software (GE Healthcare Life Sciences, Pittsburgh, PA). Tublin was used as the loading control.

Statistical Analysis

Statistical analysis was performed with SPSS Version 22.0 (IBM Corporation, Armonk, NY). All measurements were performed in triplicate, and results were expressed as mean \pm standard deviation. One-way analysis of variance (one-way ANOVA) was used to assess differences among different groups. *P* values < 0.05 were considered statistically significant.

RESULTS

Morphological Observation

As shown in Fig. 1, AF cells cultured without MB grew well, and their shapes were mostly polygonal. However, the cells treated with different concentrations of MB (2, 20, and 200 $\mu\text{g/mL}$) presented shrinkage phenomena and typical degenerative changes, which increased significantly in a concentration-dependent manner (Fig. 1A) and in a time-dependent manner (Fig. 1B).

MB Inhibits AF Cell Growth

To determine the effects of different concentrations of MB on AF cells, CCK-8 assay was used to evaluate cell proliferation. Two to 72 hours after exposure to different concentrations of MB, the viability of AF cells was significantly inhibited in a concentration- and time-dependent manner, except in the MB-2 group at 2 hours (Fig. 2). The inhibition of cell proliferation was most pronounced at 4 hours, and then entered the plateau phase gradually. Therefore, this time point was chosen to study the effects of MB on AF cells in the following experiment.

MB Promotes AF Cell Apoptosis

To assess whether MB-mediated growth inhibition was related to the induction of apoptosis, AF cells were stained with Annexin V-FITC and PI for flow cytometric analysis. As shown in Fig. 3A, MB increased the percentage of necrosis and early apoptotic cells in a concentration-dependent manner. After cells were treated with MB at concentrations of 2, 20, and 200 $\mu\text{g/mL}$ for 4 hours, the apoptotic rates of AF cells were 16.07%, 74.95%, and 90.09%. Compared with

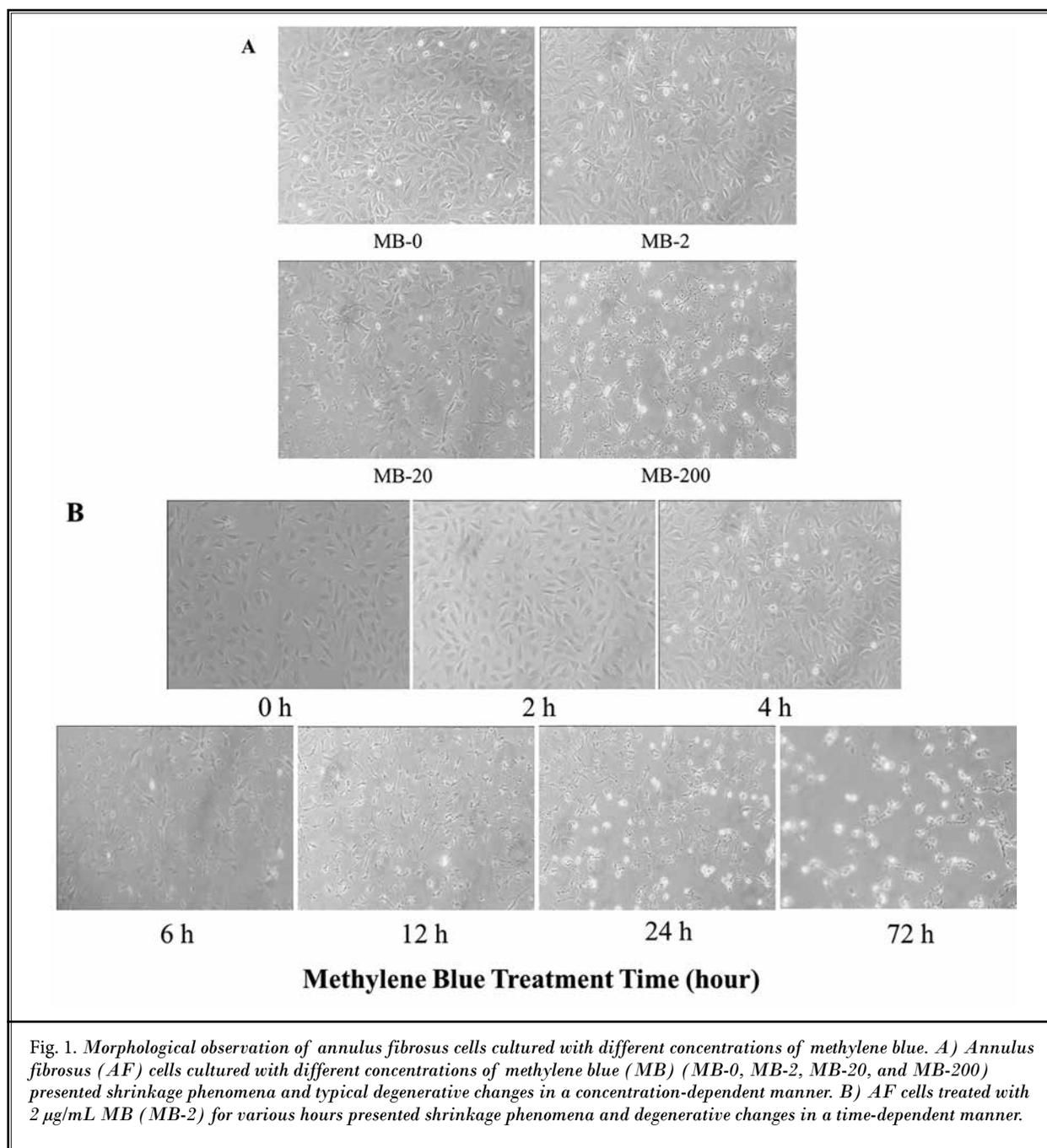
the MB-0 group, the cell apoptotic rates of the MB-2, MB-20, and MB-200 groups significantly increased ($P < 0.05$). The cell apoptotic rates of the MB-20 and MB-200 groups were greater than that of MB-2, and the cell apoptotic rates of the MB-200 groups were also greater than that of the MB-20 group ($P < 0.05$) (Fig. 3B). The data indicated that MB induced apoptosis of AF cells in a concentration-dependent manner.

To further confirm the induction of apoptosis by MB, the caspase-3 activity was detected using a caspase-3 activity kit. After AF cells were treated with MB at concentrations of 2, 20, and 200 $\mu\text{g/mL}$ for 4 hours, the caspase-3 activity was 1.22, 2.21, and 2.78, respectively. Compared with the MB-0 group, the caspase-3 activity of the MB-2, MB-20 and MB-200 groups significantly increased ($P < 0.05$). The caspase-3 activity of the MB-20 and MB-200 groups was greater than that of MB-2, and the caspase-3 activity of the MB-200 groups was also greater than that of MB-20 ($P < 0.05$).

To assess the potential pathway in the apoptotic process, we detected the expression of apoptosis-related genes by RT-PCR and Western blotting. As shown in Fig. 4, AF cells treated with MB showed significant up-regulation of Bax and caspase-3 mRNA levels in a concentration-dependent manner, which was accompanied by decreased expression of Bcl-2. Western blotting also showed that the protein levels of caspase-3 and Bax increased and that of Bcl-2 decreased (Fig. 5). Compared with MB-0, the apoptosis-related gene expressions of the MB-2, MB-20, and MB-200 groups increased significantly in a concentration-dependent manner ($P < 0.05$). The apoptosis-related gene expressions of the MB-20 and MB-200 groups were greater than those of MB-2, and the apoptosis-related gene expressions of the MB-200 groups were also greater than those of MB-20 ($P < 0.05$).

MB Inhibits Paracrine Function of AF Cell

To investigate the effect of MB on the paracrine function of AF cells, we detected the expression of related genes by RT-PCR and Western blotting. As shown in Figs. 4-5, AF cells treated with MB showed significant down-regulation of collagen type I, TGF- β 1, bFGF, and TIMP-1 mRNA and protein levels in a concentration-dependent manner. Compared with the MB-0 group, the paracrine function-related gene expressions of the MB-2, MB-20, and MB-200 groups increased significantly in a concentration-dependent manner ($P < 0.05$). The paracrine function-related gene expressions of the MB-20 and MB-200 groups were greater than those of MB-2, and the paracrine function-related gene expressions



of the MB-200 groups were also greater than those of MB-20 ($P < 0.05$).

DISCUSSION

MB, a partially liposoluble vital dye, has been used since it was first synthesized in 1876 in many different

areas of clinical medicine. MB has now been widely used to stain IVD in the PTED procedure (10) and as a redox agent in the treatment of discogenic LBP in some clinical trials (6-9). However, little is known about the effect of high concentrations of MB on intervertebral disc cells, especially during local application of PTED proce-

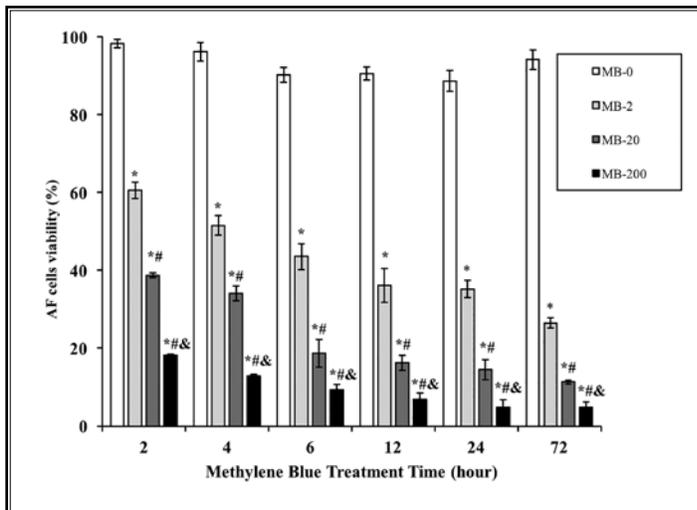


Fig. 2. The viability of annulus fibrosus cells cultured with different concentrations of methylene blue for 72 hours. The viability of annulus fibrosus (AF) cells was significantly inhibited in a concentration- and time-dependent manner. * $P < 0.05$ compared to methylene blue (MB)-0 group, # $P < 0.05$ compared to MB-2 group, & $P < 0.05$ compared to MB-20 group.

ture. The AF cells belong to fibroblasts and can participate in the tissue repair process by proliferating and secreting large amounts of collagen and other extracellular matrix (ECM) (15). Repairing of the tears remaining in the AF and posterior longitudinal ligament after PTED should be accomplished by AF cells and fibroblast cells (16). This is the first study of the toxic effects of MB on AF cells. Our results are similar to those found for other tissues; these studies also showed cell toxic effects after MB treatment (8,12,13,17). The present study demonstrated that MB can not only inhibit cell proliferation and expression of collagen type I, but also promote cell apoptosis in a concentration-dependent manner. Therefore, it is essential to choose low concentrations of MB in practical application. In addition, symptoms should be observed closely after the use of MB to enable earlier detection of complications.

As for the mechanism during the apoptosis process, the ratio of anti-apoptotic proteins

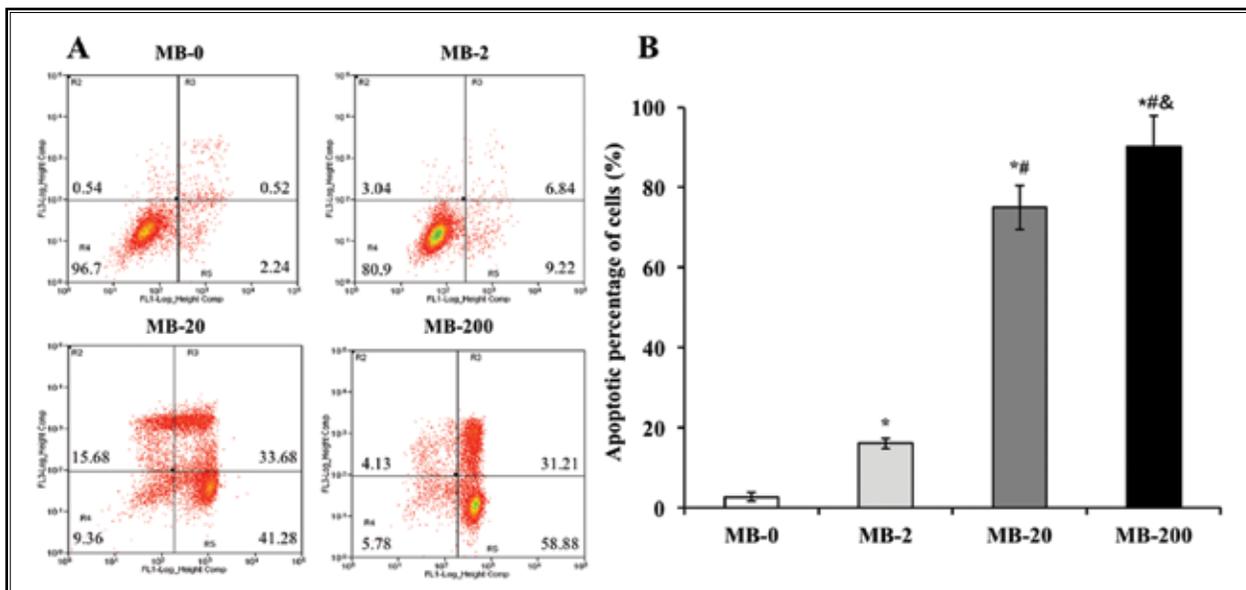


Fig. 3. Determinations of the apoptotic rate of annulus fibrosus cells following 4-hour exposure to methylene blue. A) Representative graphs obtained by flow cytometry analysis after double staining with Annexin V-FITC and PI. The apoptotic rates of annulus fibrosus (AF) cells were 16.07%, 74.95%, and 90.09% after cells were cultured with methylene blue (MB) at concentrations of 2, 20, and 200 $\mu\text{g}/\text{mL}$ for 4 hours. B) Histogram for statistical analysis shows that the apoptosis rate of the AF cells was significantly increased in the MB-2, MB-20, and MB-200 groups when compared to the MB-0 group, and the cell apoptotic rate of the MB-200 group was greater than those of the MB-2 and MB-20 groups. * $P < 0.05$ compared to MB-0 group, # $P < 0.05$ compared to MB-2 group, & $P < 0.05$ compared to MB-20 group.

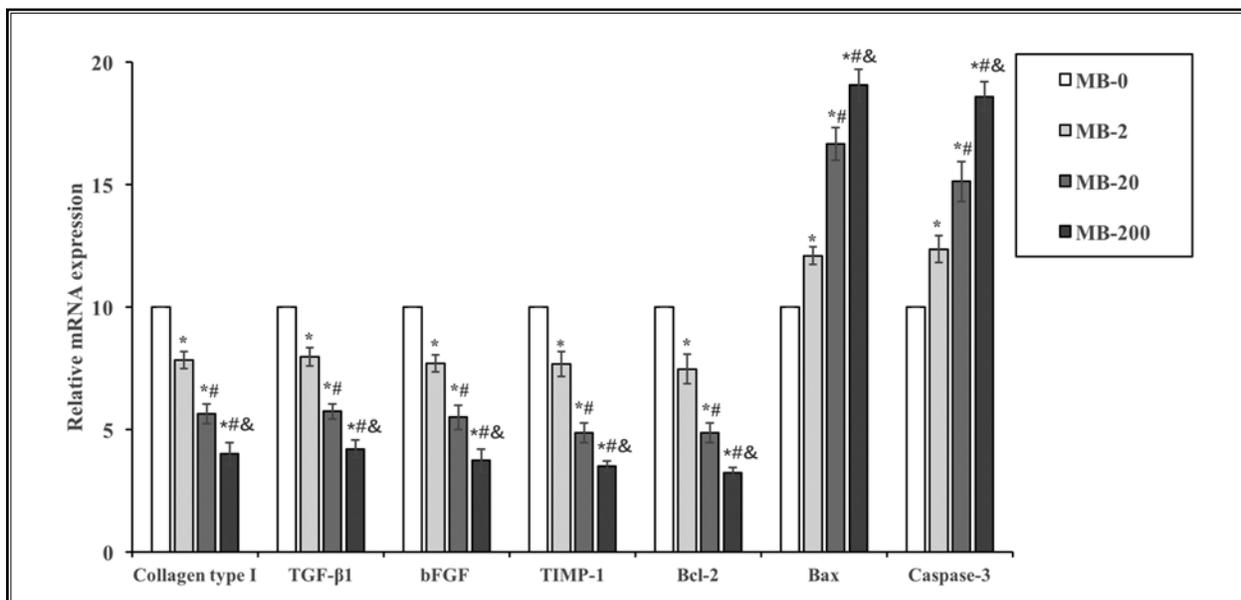


Fig. 4. Relative mRNA expressions of collagen type I, TGF-β1, bFGF, TIMP-1, caspase-3, Bcl-2, and Bax. Annulus fibrosus (AF) cells cultured with methylene blue (MB) showed significant down-regulation of collagen type I, TGF-β1, bFGF, TIMP-1, and Bcl-2 levels, and up-regulation of caspase-3 and Bax in a concentration-dependent manner. Compared to the MB-0 group, the relative gene expressions of the MB-2, MB-20, and MB-200 groups significantly increased in a concentration-dependent manner ($P < 0.05$). The relative gene expressions of the MB-20 and MB-200 groups were greater than those of MB-2, and the relative gene expressions of the MB-200 groups were also greater than those of MB-20 ($P < 0.05$).

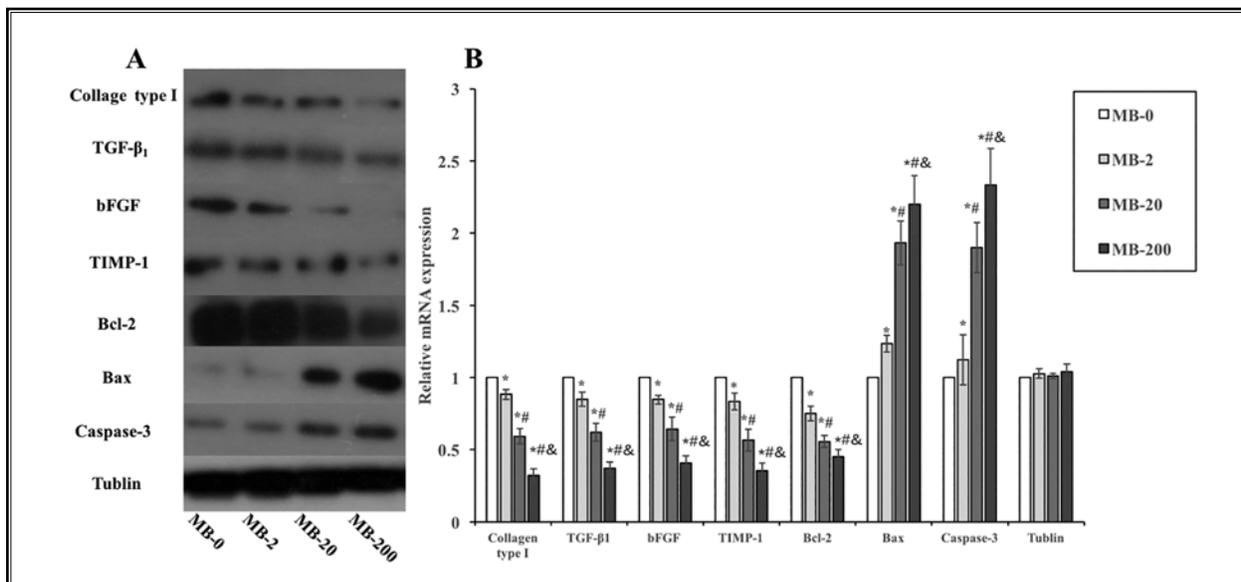


Fig. 5. Relative protein levels of collagen type I, TGF-β1, bFGF, TIMP-1, caspase-3, Bcl-2, and Bax by western blotting. A) Western blotting analysis for collagen type I, TGF-β1, bFGF, TIMP-1, caspase-3, Bcl-2, and Bax in annulus fibrosus (AF) cells cultured with different concentrations of methylene blue (MB). B) Histogram for statistical analysis shows that the relative protein levels of the MB-2, MB-20, and MB-200 groups were significantly increased when compare to the MB-0 group. The relative protein levels of the MB-20 and MB-200 groups were greater than those of MB-2, and the relative protein levels of the MB-200 groups were also greater than those of MB-20 ($P < 0.05$). * $P < 0.05$ compared to MB-0 group, # $P < 0.05$ compared to MB-2 group, & $P < 0.05$ compared to MB-20 group.

to pro-apoptotic proteins is a key factor in determining apoptosis. It is reported that the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax play crucial roles in apoptosis by regulating the permeabilization of the mitochondrial membrane (18,19). Alteration of the equilibrium between Bcl-2 and Bax may lead to formation of a pro-apoptotic signal (20). It has been found that Bcl-2 protects normal IVD cells from apoptosis (21). Our results revealed that the expression of Bcl-2 was markedly declined, and that of Bax concomitantly increased, in AF cells after exposure to different concentrations of MB. Caspases are a well-characterized family of proteins involved in promoting a cell apoptotic mode involving DNA fragmentation and cell shrinkage. Caspase-3 is downstream of the apoptotic cascade and the major protease executing apoptosis (22). The present study demonstrated that MB also increased caspase-3 expression accompanied by the increased apoptosis rate in AF cells. Taken together, we conclude that MB may inhibit the expression of Bcl-2, increase the expression of Bax, and lead to an imbalance in the Bcl-2/Bax ratio, thus activating the downstream caspase-3 to induce apoptosis of AF cells. These observations may indicate that the Bcl-2/Bax/caspase-3 pathway may be impeded in the apoptotic process of AF cells induced by MB.

The ECM of AF cells is rich in collagens, especially collagen type I (23). The physiological balance and integrity of the collagens are essential to maintaining the mechanical function of normal IVD. In the present study, our data suggested that MB could decrease the expression of collagen type I in a concentration-dependent manner. Extensive studies have shown that MB can reduce surgery-induced peritoneal adhesions (24) and epidural fibrosis formation after lumbar laminectomy (17), and it seems that the mechanism is probably related to the inhibition of proliferation and function of fibroblast. It is well known that the healing process of AF tissue is mainly achieved through fibrotic scar proliferation and may last up to one year post operative period (16,25). Therefore, the toxic effect of MB may also impair the repair ability of AF cells when used as a stain in the PTED procedure for IVD degenerative disease. At the same time, the toxic effect of MB can further impair the function of AF cells and aggravate the IVD degeneration process when used as a redox agent for discogenic LBP. These observations may indicate that it is essential to reduce the concentration of MB as much as possible.

The molecular mechanism involved in MB impairing the paracrine function of AF cells has not been

well established. It has been reported that TGF- β 1 and bFGF play an important role in promoting cell proliferation and collagen type I expression of AF cells (26,27). It is known that ECM remodeling is enabled by the regulation of matrix metalloproteinases (MMPs) and "TIMPs" (28). TIMP-1 can inhibit the activity of MMP-1, which mainly breaks down collagen type I and other ECM (29). Aoki et al (30) further confirmed that MMP-1/TIMP-1 complexes were suppressed, and increased degradation of collagen type I was observed when fibroblasts were transduced with small interfering RNAs targeting TIMP-1. In this study, the results showed that the expressions of TGF- β 1, bFGF, and TIMP-1 were all suppressed by MB in a concentration-dependent manner. Considering AF cells belong to a type of fibroblasts, we can conclude that MB may decrease the expression of collagen type I via inhibiting the expression of TGF- β 1 and bFGF, and promote the degradation of collagen type I through inhibiting the expression of TIMP-1.

CONCLUSION

In summary, we found that high concentrations of MB can not only inhibit the proliferation and paracrine function of AF cells, but can also induce apoptosis of AF cells in a concentration-dependent manner. Further, the Bcl-2/Bax/caspase-3 pathway may be involved in the apoptotic process. In addition, our results showed that MB may decrease the expression of collagen type I via inhibiting the expression of TGF- β 1 and bFGF, and promote the degradation of collagen type I through inhibiting the expression of TIMP-1. Overall, based on our results of AF cytotoxicity and in consideration of the previously reported neurotoxicity, we suggest that it is essential to choose low concentrations of MB in practical application and limit the use of MB in the treatment of discogenic LBP to research protocols.

Limitation

This study still had some limitations. First, we only observed the toxic effects of MB on rat AF cells in vitro; the toxic effect of MB on IVD cells should be further studied in vivo. Secondly, we only observed the toxic effects of MB on healthy rat AF cells; the toxic effects on human healthy and degenerative IVD cells should be further studied.

Conflict of Interest

The authors declare no conflicts of interest in regard to the present manuscript.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (81401830) and Young Medical Scholars Major Program of Jiangsu Province (QNRC2016342).

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