The histopathological effects of intracavernosal mitomycin-C injection in a rat Peyronie's disease model

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Abstract

Introduction: We aimed to evaluate whether or not mitomycin-C (MMC) has an antifibrotic effect on transforming growth factor-beta (TGF-β)-induced Peyronie's disease (PD) in a rat model.

Methods: Eighteen 12-week-old male Sprague-Dawley rats were divided into three groups: Group 1=TGF-β1 (n=7); Group 2=TGF-β1+MMC (n=7); and Group 3=Sham group (0.25 ml bovine serum albumin injected) (n=4). All groups were sacrificed on the sixth week of the procedure and their penises were excised. All penis specimens were evaluated semi-quantitatively and quantitatively with histochemical, immunohistochemistry, and image analysis.

Results: Both Group 1 and Group 2 had significantly higher fibrosis scores and lower elastic fibers in both outer surface of tunica albuginea (TA) and subsinusoidal area compared with Group 3. When compared with Group 1, the amount of collagen was significantly decreased in Group 2. Intracavernosal MMC injection (Group 2) ended up with lower elastic fibers when compared with Group 1. According to the quantitative analyses, when compared with Groups 1 and 3, lower dorsal, ventral, and trabecular thickening values were seen in Group 2. These parameters were only statistically significant when compared with Group 1, suggesting the antifibrotic effect of TGF-β1-induced fibrosis. Both Groups 1 and 2 showed lower decorin staining levels in subsinusoidal areas of tunica albuginea (SATA) and subsinusoidal areas of trabecular wall (SATW) when compared with Group 3. The statistically significant difference was only detected between Group 1 and Group 3.

Conclusions: Our study demonstrates the antifibrotic effects of MMC on PD. Further clinical studies are necessary to make inferences regarding its clinical use.

Introduction

Peyronie's disease (PD) is characterized by the formation of fibrous plaques in the tunica albuginea (TA). It causes penile curvature, erectile dysfunction, and pain during erection.[1,2] The general prevalence of PD is between 3.2% and 8.9%.[3]

PD contains two phases: acute and chronic. Pain, plaque formation, and progression in penile curvature are observed in acute inflammatory phase (6–12 months). Following this period, chronic phase starts and it consists of stability of the penile deformity, pain resolution, and symptoms of erectile dysfunction (ED).[4]

Trauma is the main etiological reason of the disease. Repeated microtrauma or macrotraumatic buckling of the rigid phallus cause abnormal wound healing following fibroblast proliferation and collagen deposition.[5] Transforming growth factor-beta (TGF-β), a cytokine, has the key role for PD pathogenesis. Expression of TGF-β was identified in both human and rat plaque formation.[6] It has been shown that successful results are obtained by using anti-TGF-β agent in animal models.[7,8]

Decorin, an antifibrotic agent, regulates collagen fibrils and is mostly expressed by fibroblasts, myocytes, and smooth muscle cells.[9,10] Decorin has been shown to organize the formation of collagen fibrils and inhibit TGF-β.[11] Decorin staining can be used for evaluating the fibrosis level.

Mitomycin-C (MMC), a chemotherapeutic agent, shows antitumoural and antifibrotic activity. Occleston et al reported that MMC reduced fibrosis on Tenon capsule by affecting TGF-β and fibroblast growth factor (FGF). This effect decreases type 1 collagen and fibronectin production.[12] MMC has been used in ophthalmological, orthopedic, and otolaryngological surgery due to its antifibrotic effect.[13-15]

In this study, we aimed to evaluate whether or not MMC has an antifibrotic effect on TGF-β-induced PD in a rat model. To our knowledge, this is the first study evaluating the antifibrotic effect of MMC in PD.

Methods

This study was performed in accordance with the guidelines of the Experimental Animal Care and approved by the Ethical Committee for Animal Studies at our centre. Eighteen

12-week-old male Sprague-Dawley rats (350–470 g) were divided into three groups: Group 1=TGF-β1 (n=7); Group 2=TGF-β1+MMC (n=7); and Group 3=sham group (0.25 ml bovine serum albumin injected) (n=4).

Surgical procedure

The rats were anesthetized with pentobarbital (35 mg/kg). A small pubic incision was performed to visualize the left proximal section of the TA of the penis in all groups. A total of 0.5 μg/0.25 ml TGF-β1 (T 7039, 0.5 μg in a total volume of 0.25 ml; Sigma Chemical Co., St. Louis, MO, U.S.) was injected into TA by a 26-G needle in Groups 1 and 2. A total of 0.25 ml bovine serum albumin, which was used for TGF-β1 preparation, was injected in sham group rats. Afterwards, Group 2 rats received intracavernosal MMC (a total of 0.3 mg/ml) injection for four consecutive days starting from the second day of the TGF-β1 injection, and sham group rats received the same doses of intracavernosal saline injection.

All groups were sacrificed on the sixth week of the procedure and their penises were excised. All penis specimens were evaluated semi-quantitatively and quantitatively using routine and histochemical staining methods, immunohistochemistry, and image analysis.

Histopathological examination

Following 10% buffered formalin fixation, all penile specimens was divided into right and left side in the longitudinal axis. After automated tissue processing and cutting procedures, all sections were stained with hematoxylin and eosin (H&E), Masson's trichrome (to evaluate amount of collagen bundles in TA and trabecular wall, which are stained with a blue colour, and proportion of smooth muscle cells in same areas, which are stained with a red colour), and Verhoeff-Van Gieson (to evaluate amount of elastic fibers in subsinusoidal areas of TA and trabecular wall, and subcutaneous connective tissue) methods. For all stained tissue sections, morphological parameters consisted of TA thickening, trabecular wall thickening and sinusoidal narrowing, attenuation or thinning in the smooth muscle layer of TA, increasing of trabecular smooth muscle cells, and reduction of elastic fibers in outer layer of TA and sinusoidal wall were semi-quantitatively evaluated by a single pathologist. Thickening of ventral and dorsal TA and sinusoidal narrowing in tissue sections stained with Masson's trichrome were measured under x40 objective using an image analysis program (Image J 10.2).

Semi-quantitative evaluation

To determine amount of the fibrous tissue and elastic fibers, semi-quantitative examination was performed by a single pathologist using the following scoring system: 0=no thickening or attenuation or changes; 1(+)=1.25 times thickening or increasing/thinning or decreasing; 2(+)=1.5 times thickening or increasing/thinning or decreasing; 3(+)=2 times thickening or increasing/thinning or decreasing (Table 1).

Image analysis (quantitative) evaluation

To determine the amount of the fibrous tissue, all images stained with Masson's trichrome (a total of 72 images) that were processed by pathologist were loaded into the Image J 10.2 program and all measurements were performed under x40 objective. All measurements were automatically recorded as pixels and afterward all values were converted to mm using a formula.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 20.0 software (SPSS 20.0 for Windows, Chicago, IL, U.S). Descriptive statistics were noted with mean, standard deviation, minimum, and maximum. Kurtosis, skewness, Kolmogorov-Smirnov, and Shapiro-Wilk tests were used to assess the variables' normalization. Mann-Whitney U test and Student t test were used for between-group comparisons. Comparisons of multiple groups were made using Mann-Whitney-U test and Student t test. A probability of $p<0.05$ was accepted as statistically significant.

Results

Histopathological results

Semi-quantitative evaluation

When compared with Group 3 (sham group), previously described semi-quantitative scores evaluating fibrosis were higher in both Group 1 and Group 2, and this finding was statistically significant (Table 1) (Figs. 1a–c); however, the changes regarding collagen amount were significantly decreased in Group 2 compared with Group 1, suggesting the protective effect of MMC in Group 2 (Fig. 2a). Similarly, decrease of elastic fibers in both the outer surface of TA and the subsinusoidal area was significantly higher in both Group 1 and Group 2 (Table 1) (Figs. 1d–f, 2b). Intracavernosal MMC injection (Group 2) resulted in a decrease of elastic fibers in both TA and sinusoidal wall when compared with Group 1, again suggesting the protective effect of MMC against TGF-β1-induced fibrosis.

Image analysis (quantitative) evaluation

Group 2 revealed lower dorsal, ventral, and trabecular thickening values when compared with Groups 1 and 3 (Table 2, Fig. 2c). This difference was statistically significant when

Table 1. The results of semi-quantitative and statistical analyses of morphological changes among groups

Groups*	Semi-quantitative analysis of morphological changes (mean value±SD)											
	Histochemistry**						Immunohistochemistry***					
	Masson's trichrome staining				Elastic staining: Decreasing of elastic fibers		TGF-β1			Decorin		
	Tunica albuginea thickness	Trabecular wall thickness + sinusoidal narrowing	Attenuation or thinning of smooth muscle layer	Increasing of trabecular smooth muscle cells	Outer surface of tunica albuginea	Subsinusoidal area	SATA	SATW	SCA	SATA	SATW	SCA
1	2.71±0.39	2.14±0.37	2.00±0.28	2.00±0.40	1.78±0.26	1.57±0.18	0	0	0	1.14±0.24	1.01±0.06	0
2	1.57±0.34	0.92±0.44	0.71±0.26	1.35±0.37	0.64±0.24	0.5±0.00	0	0	0	1.30±0.27	1.20±0.27	0
3	0	0	0	0	0	0	0	0	0	2.75±0.35	2.75±0.35	2
Statistical analysis of morphological changes (p value)												
1 & 2	0.001	0.001	0.001	0.017	0.001	0.001	-	-	-	0.485	1	1
1 & 3	0.018	0.017	0.015	0.018	0.016	0.017	-	-	-	0.05	0.04	0.001
2 & 3	0.017	0.016	0.019	0.015	0.017	0.018	-	-	-	0.095	0.092	0.001

*Group 1=TGF-β1 (n:7), Group 2=TGF-β1+MMC (n:7), and Group 3=Sham group (n:4). **Scoring for histochemistry: 0=No thickening or attenuation or changes/thinning or decreasing; 1(+)=1.25 times thickening or increasing/thinning or decreasing; 2(+)=1.5 times thickening or increasing/thinning or decreasing; 3(+)=2 times thickening or increasing/thinning or decreasing. ***Scoring for immunohistochemistry: 0=no staining, 1(+)=weak, 2(+)=moderate, 3 (+)=strong. SATA: subsinusoidal areas of tunica albuginea; SATW: subsinusoidal areas of trabecular wall; SCA: subcutaneous areas; SD: standard deviation.

compared with Group 1 (p=0.001), suggesting the antifibrotic effect of TGF-β1-induced fibrosis. Group 2 and the sham group values were similar and the difference was not statistically significant (p>0.05).

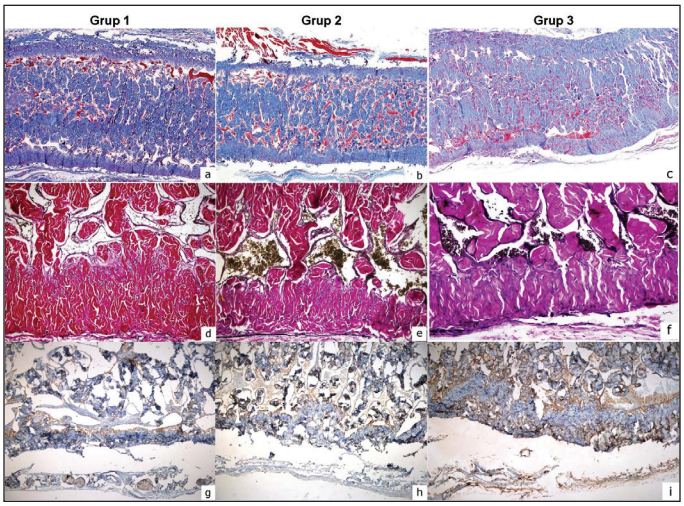

Fig. 1. (a, b, c) The thickening of tunica albuginea and trabecular wall, and sinusoidal narrowing in Group 1 is slightly more than Groups 2 and 3 (Masson's trichrome stain, x40). ***(d, e, f)*** The amount of elastic fibers in outer surface of tunica albuginea and subsinusoidal area in Group 1 is lower than Groups 2 and 3, whereas the amount of elastic fibers in Group 2 is almost similar to Group 3 (Verhoeff-Van Gieson's elastic stain, x200). ***(g, h, i)*** Decorin staining in subsinusoidal areas of tunica albuginea (SATA) and trabecular wall (SATW) in Groups 1 and 2 is lower than Group 3. No decorin staining is observed in subcutaneous connective tissue of Groups 1 and 2, unlike Group 3 (Immunohistochemistry, x100).

Immunohistochemical analysis

The amount of decorin staining in subsinusoidal areas of tunica albuginea (SATA) and subsinusoidal areas of trabecular wall (SATW) are summarized in Table 1. While the decorin levels were lower in Groups 1 and 2 when compared with Group 3, a statistically significant difference was observed between Group 1 and Group 3 (Figs. 1g–i, 2d). No staining for decorin in subcutaneous areas (SCA) was detected in either Group 1 or Group 2, contrary to Group 3 (Table 1). In all groups, no TGF-β1 staining in SATA, SATW, and SCA was observed because of its speed circulation and elimination in the cavernous system of the penis.

Discussion

PD is a progressive fibrotic process related with TGF-β1, which can cause sexual dysfunction. Several animal models have been reported for PD in the literature. Injection of cytomodulin, fibrin, and TGF-β1 show fibrotic effect in an animal model of PD, as they induce production of TGF-β1.[5-7,16] In a six-week period after injection, chronic cellular infiltration is accompanied by focal diffuse elastosis, thickening and disorganized collagen fibers, and elastin fragmentation.[7] Fibrous plaque, which appears with profibrotic agent injection, is histologically similar to human plaque formation.[16]

In terms of PD treatment, anthocyanin extract, an anti-inflammatory and antifibrotic agent, was evaluated in a rat model by Sohn et al.[17] When the anthocyanin-treated group was compared to the PD group, TGF-β1 expression

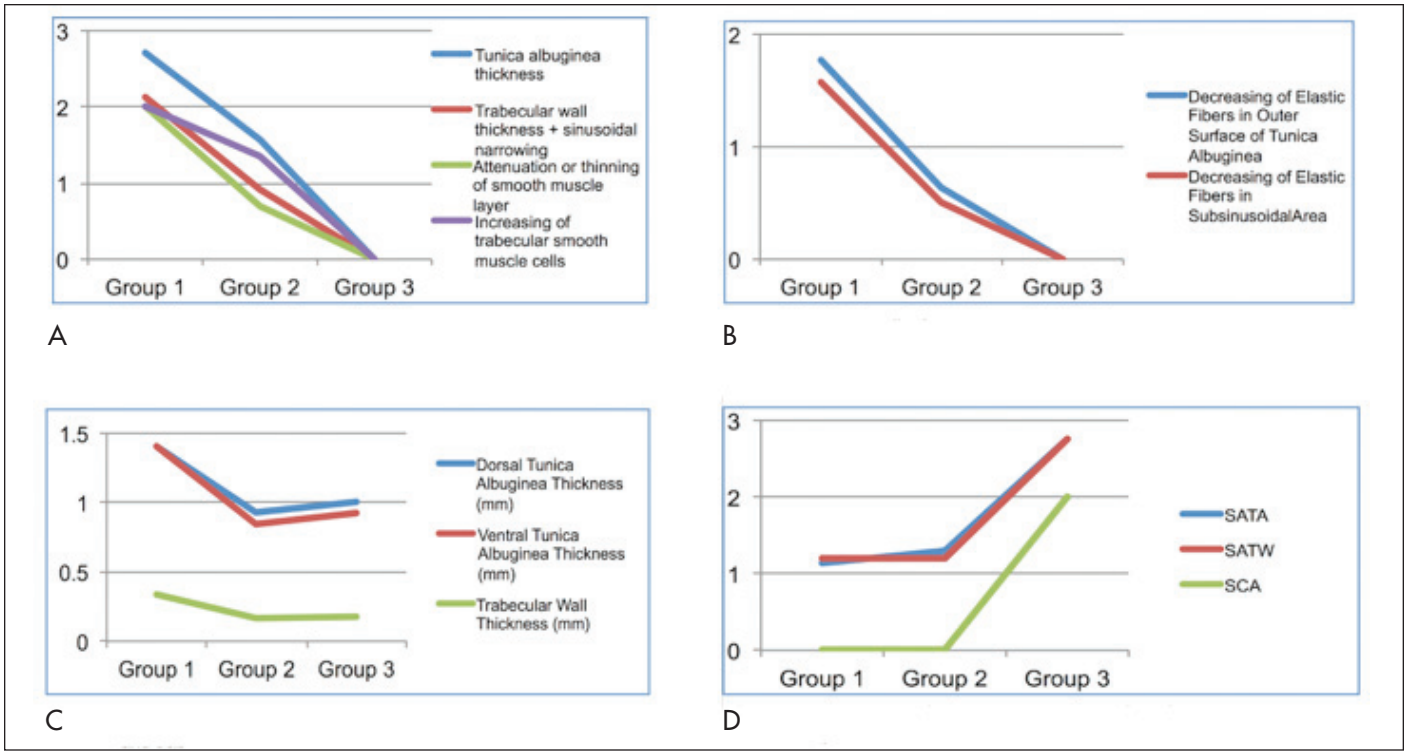

Fig. 2. (A) Histopathological changes in tunica albuginea and trabecular wall among groups. ***(B)*** The distribution and amount of elastic fibers among groups. ***(C)*** The results of quantitative analysis of the thickness of tunica albuginea and trabecular wall among groups. ***(D)*** The proportion of decorin staining in subsinusoidal areas of tunica albuginea (SATA), subsinusoidal areas of trabecular wall (SATW), subcutaneous areas (SCA) among groups.

was significantly decreased. The amount of the TGF-β1-positive cells in the PD group was higher than control and anthocyanin group.[17] In another study, decorin, antifibrotic agent was administered into the corpus cavernousum in a rat model. Thickening of TA was identified in the PD group and PD+decorin injection group as 87.5% and 37.5%, respectively. It was reported that decorin protected erectile response by inhibiting TGF-β1.[7]

Clostridium collegenase (CC) injection is the only medical treatment option for PD; it is approved by United States Food and Drug Administration. In a study by Gelbard et al, CC treatment showed significant benefit for penile curvature and outcomes of symptom bother scores.[18]

Repeated injection of intralesional verapamil is reported to lead to regression in 78% of patients with minimal side effects in the early stages of PD.[19]

Trost et al evaluated 127 patients in terms of the effects of interferon-α2B. Intralesional therapy with interferon-α2b provided significantly improved curvature without impacting penile vascular parameters.[20]

Besides being a widely used chemotherapeutic agent, MMC is a potent inhibitor of fibroblast via its ability to induce apoptosis.[21] MMC has been used various capacities in urology. It's been reported that MMC reduces the recurrence rate of urethral stricture after internal uretrotomy.[22,23] It has been further reported that MMC decreases hemosid-

Table 2. The results of quantitative and statistical analyses of morphological changes among groups

Groups	Quantitative results of morphological changes (mean value±SD)		
	Dorsal TA thickness (mm)	Ventral TA thickness (mm)	Trabecular wall thickness (mm)
1	1.4±0.17	1.40±0.61	0.3371±0.02
2	0.93±0.16	0.84±0.09	0.1671±0.01
3	1.00±0.18	0.93±0.18	0.1766±0.01
Statistically analysis of quantitative results of morphological changes (p value)			
1 & 2	0.001	0.001	0.001
1 & 3	0.017	0.117	0.017
2 & 3	0.517	0.667	0.383

SD: standard deviation; TA: tunica albuginea.

erin-laden macrophages, mononuclear cell infiltration, and fibrosis after internal ureterotomy compared with control groups in a rat model.[24]

In our study, we evaluated the antifibrotic effects of MMC on PD. Both Group 1 and Group 2 had significantly higher fibrosis scores compared with the sham group (Group 3). When compared with Group 1, the amount of collagen was significantly decreased in Group 2. At the same time, decrease of elastic fibers in both the outer surface of TA and the subsinusoidal area was significantly higher in Groups 1 and 2. Group 2 ended up with lower elastic fibers in both TA and sinusoidal wall when compared with Group 1. These findings support the protective effect of MMC against TGF-β1-induced fibrosis.

According to the quantitative analyses, when compared with Group 1 and Group 3, lower dorsal, ventral, and trabecular thickening values were seen in Group 2. These parameters were only statistically significant when compared with Group 1, suggesting the antifibrotic effect of TGF-β1-induced fibrosis. The difference was not statistically significant when compared to control group.

Both Group 1 and Group 2 showed lower decorin staining levels in SATA and SATW when compared with Group 3. The statistically significant difference was only detected between Group 1 and 3. No staining for decorin in SCA was observed in both Group 1 and Group 2, contrary to Group 3. Although there were no statistically significant differences, lower decorin staining was detected in MMC against TGF-β1-induced fibrosis.

Histopathological (semi-quantitative) and image analysis program (quantitative) demonstrated that the amount of collagen bundles decreased and elastic fibers increased in the MMC group (Group 2) compared to the TGF-β1 group (Group 1). These results were statistically significant.

Conclusion

MMC shows antifibrotic effects on a rat PD model. New animal and further clinical studies are warranted.

Competing interests: The authors report no competing personal or financial interests.

This paper has been peer-reviewed.

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