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Original article

The protective effect of green tea catechins on ketamine-induced cystitis in a rat model

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ABSTRACT

Objective: To investigate the protective effect of green tea epigallocatechin gallate (EGCG) on long-term ketamine-induced ulcerative cystitis (KIC) using a ketamine addiction rat model.
Materials and methods: Thirty Sprague-Dawley rats were divided into three groups which received saline, ketamine (25 mg/kg/d), or ketamine combined with EGCG (10 μM/kg) for a period of 28 days. In each group, cystometry and a metabolic cage micturition pattern study were performed weekly. Masson's trichrome study was done to evaluate the morphologic changes. Western blot analyses were carried out to examine the expressions of inflammatory protein [transforming growth factor-β (TGF-β)] and fibrosis proteins (fibronectin and type I collagen) in bladder tissues.
Results: Chronic ketamine treatment resulted in bladder hyperactivity with a significant increase in micturition frequency and a decrease in bladder compliance. These alterations in micturition pattern were accompanied by increases in the expressions of inflammatory and fibrosis markers, TGF-β, fibronectin, and type I collagen after long-term ketamine treatment. Masson's trichrome stain showed that ketamine treatment decreased urothelium thickness while increasing the collagen to smooth muscle ratio and exacerbating interstitial fibrosis. By contrast, simultaneous EGCG and ketamine treatment reversed ketamine-induced damage to almost control levels, showing the protective effect of EGCG.
Conclusion: This protective effect of EGCG may come from its antiinflammatory and antifibrotic properties.

1. Introduction

Recently, several studies have demonstrated that ketamine addition affects the urinary bladder, resulting in an increase of urination frequency, urgency, nocturia, hematuria, and painful micturition.[1–3] Traditionally, ketamine has been used for induction of anesthesia. How ketamine induces bladder damage remains unclear; however, formation of an active metabolite of ketamine might result in such urinary tract damage. It is plausible that ketamine and its metabolites accumulate in the urine and induce significant bladder irritation. The higher concentration and the longer time ketamine metabolites remain in the bladder might cause direct toxic effects on the bladder interstitial cells and trigger mucosal and submucosal inflammatory responses.[4]

Several pathological conditions of the urinary bladder are associated with the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with increased oxidative stress.[5–7]

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In cyclophosphamide- and substance P-induced bladder inflammatory changes, both ROS and RNS are increased. Our recent work also showed an increase in oxidative stress in ketamine-induced ulcerative cystitis (KIC).[8]

Green tea is a popular health drink worldwide. Previous studies have identified strong antiinflammatory/antioxidant properties of green tea and its associated polyphenols. Green tea contains several polyphenolic compounds, including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate, and epicatechin. Many studies have suggested that the biological activity of green tea is mediated by its major polyphenolic compound, EGCG.[9] This compound can prevent and/or reduce the deleterious effects of oxygen-derived free radicals associated with various chronic diseases, such as neurodegenerative disorders.[10,11] Many studies have indicated that green tea could offer a potential dietary protection against numerous cancers and have activities in bladder tumor inhibition in different animal models and clinical trials.[12–14]

Our previous study has shown EGCG could ameliorate ovariectomy-induced overactive bladder (OAB) symptoms through decreasing bladder intramural nerve damage and diminishing M2 and M3 muscarinic receptor overexpression.[15,16] To investigate the mechanism of stimulation of fibrosis production in KIC, we determined the amounts of transforming growth factor beta (TGF-β), type I collagen- and fibronectin-specific protein in control, ketamine-treated, and ketamine combined with EGCG-treated bladder tissue samples. The aim of this study was to evaluate whether green tea extract, EGCG, could prevent KIC through lessening oxidative stress and decreased interstitial fibrosis underlying the response to ketamine addition in a rat model.

2. Materials and methods

2.1. Animals and ketamine administration

Experiments were performed on 36 adult female Sprague-Dawley rats (animal center of BioLASCO, Taipei, Taiwan) weighing between 200 g and 250 g. These rats were housed under a 12-hour light/dark cycle with free access to food and water at 21°C. Thirty Sprague-Dawley rats were divided into three groups. Twelve rats received intraperitoneal (IP) injection of saline (0.5 mL, control group), 12 received ketamine daily injection (25 mg/kg/d diluted in 0.5 mL saline), and 12 received a daily injection of ketamine and EGCG, 10 μM/kg IP, over a period of 1 month. EGCG was initially dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L. This study was approved by the Animal Care and Treatment Committee of Kaohsiung Medical University. All experiments were conducted according to the guidelines for laboratory animal care. All efforts were made to minimize animal stress/distress.

2.2. Metabolic cage study and data analysis

The rats were placed in individual R-2100 metabolic cages (Lab Products, Rockville, MD, USA). After this time, a known volume of water was measured and placed in the drinking bottles of the animals. Micturition frequency and urine output were determined using a cup especially fitted to an FT03 force transducer (Grass Technology, Warwick, RI, USA). The volume of liquid consumed, 24-hour micturition frequency, and urine production were recorded for 3 days and an average value was determined.

2.3. Cystometrogram and data analysis

Cystometry was performed before euthanizing the animals. The cystometrograms (CMGs) were carried out according to the method previously described.[17] In brief, in each experiment, rats were anesthetized with Zoletil-50 (1 mg/kg IP injection). Before the beginning of each CMG, the bladder was emptied; a polyethylene-50 catheter from the urethra was inserted into the bladder and was connected via a T tube to a pressure transducer and a microinjection pump (CH-4103, Infors, Bottmingen, Switzerland). Then, the bladder was infused with saline at a steady rate (0.08 mL/min). A voiding contraction was defined as an increase in bladder pressure that resulted in urine loss. CMG was recorded until the bladder pressure was stable and at least five filling/voiding cycles were measured on each rat. CMG parameters recorded for each animal included peak micturition pressure, bladder volume, the frequency of voiding contractions, and the frequency of nonvoiding contractions (without urine leakage during bladder infusion).[18]

2.4. Histological study and collagen/detrusor smooth muscle ratio

After cystometric studies, experimental rats were perfused with a saline solution through the left ventricle. The bladders were then removed, cut open, and further fixed overnight. The tissue samples were embedded in paraffin blocks with the same area in different groups, and serial sections 5-μm thick were obtained. Deparaffinized sections were stained with Masson's trichrome stain (Sigma-Aldaich, St. Louis, MO, USA, Masson's trichrome Stain Kit HT15). The standard Masson's trichrome staining procedure, which stains connective tissue blue and detrusor smooth muscle (DSM) red, was performed. The urothelial thickness was compared between groups according to the layers of urothelial cells, and the connective tissue distribution was compared between groups. Each specimen was captured using a digital camera in five random nonoverlapping frames at 400× magnification. The color setting and the image-associated quantification were determined using image analysis software (Image-Pro Plus, Rockville, MD, USA, Media Cybernetics, MD, US). The blue-stained collagen and the red-counterstained DSM were highlighted in each image. The total areas occupied by collagen and DSM were then determined, and the ratios of collagen to DSM (collagen/DSM) were calculated. The histology slides were reviewed by two different pathologists who were unaware of the experimental groups.

2.5. Protein isolation and Western blot analysis for inflammatory and fibrosis markers

Frozen tissues of bladder were homogenized on ice in the buffer containing the Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL, USA) at 100 mg/mL. Protein concentration in the supernatant was determined using the bicinchoninic acid protein assay (Pierce) against a *bovine serum albumin* protein standard. An equal amount of protein (30 μg) from the bladders was loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride (Immobilon-P, Millipore, Billerica, MA, USA) membranes with Towbin buffer. Immobilon-P membranes were then blocked and incubated with the primary antibody. Monoclonal antibodies to TGF-β (R & D, Minneapolis, MN, USA, 1:1000), fibronectin (BD, Franklin Lakes, New Jersey, USA, 1:1000), type 1 collagen (Abcam, Cambridge, MA, USA, 1:1000), and cyclooxygenase-2 (COX-2) (Cayman, Ann Arbor, Michigan, USA, 1:1000) were used to determine the changes in the expressions. COX-2 was used to determine the changes in inflammatory markers; TGF-β, fibronectin, and type 1 collagen were used to determine the changes in interstitial fibrosis. In each experiment, negative controls without the primary antibody and protein molecular weight markers were carried out to exclude nonspecific bands. Band intensity was normalized with respect to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Upstate Signaling, Charlottesville, VA , USA, 1:5000). After treatment with the primary antibody,

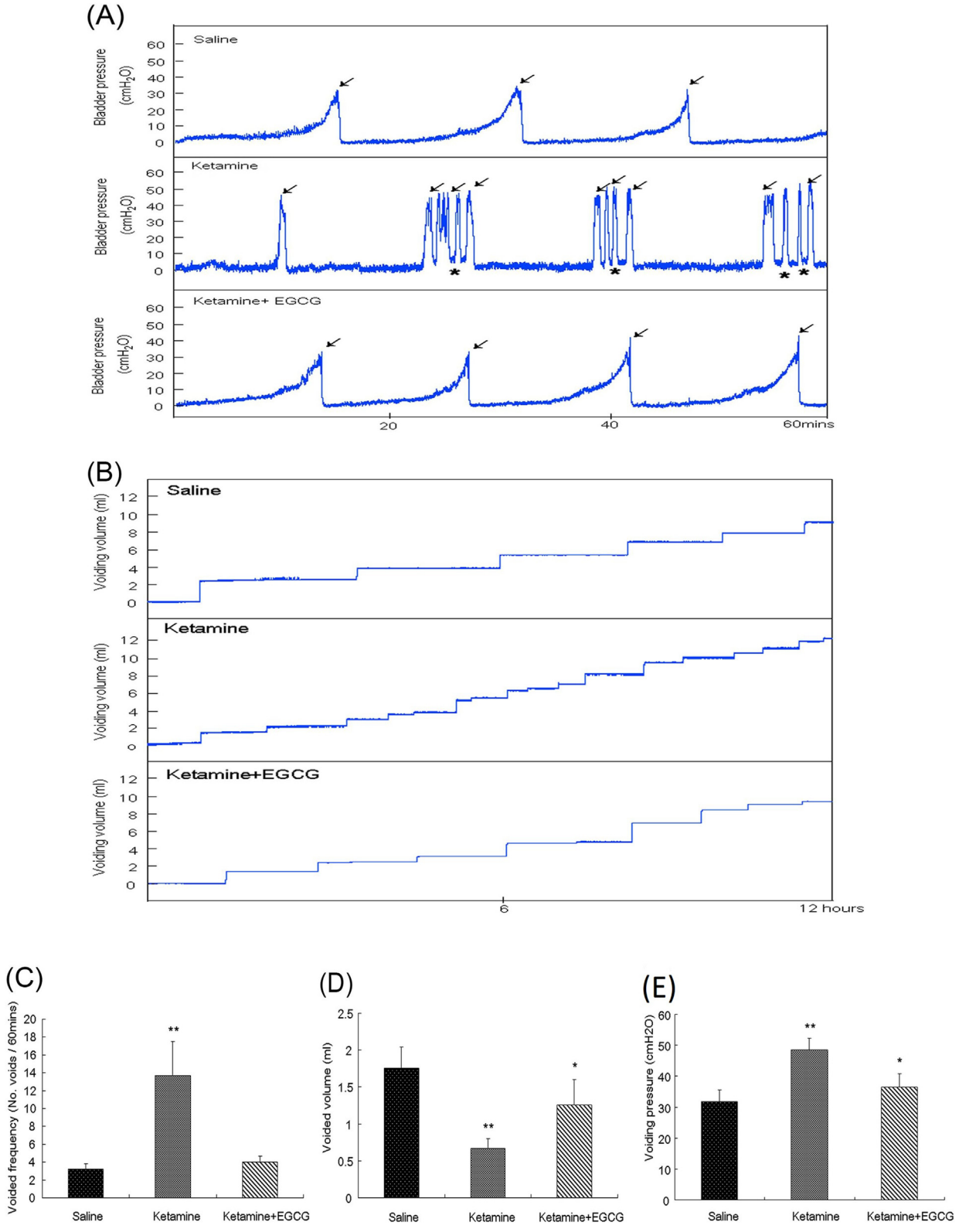

Fig. 1. Effects of ketamine treatment on cystometric parameters and voiding behavior in different groups. (A) Cystometry recordings illustrated micturition pressure and voiding frequency, including voiding contractions (arrows) and non-voiding contractions (stars) on anesthetized rats. (B) Voiding behavior evaluated by metabolic cage voiding diary as voided volume. (C-E) Trace recordings illustrating voided frequency (C), voided volume (D) and maximal voiding pressure (E) were calculated for different groups. Each bar was the mean ± standard deviation (SD) of the mean for $n = 10$. *Indicates significant difference from the control (saline) group ($p < 0.05$). **Indicates significant difference from the control (saline) group ($p < 0.01$).

Table 1
General characteristics and urodynamic parameters between different experimental groups.

	Saline	Ketamine	Ketamine + EGCG
No. of rats	12	12	7
General characteristics			
Body weight (g)	276.0 ± 17.2	260.0 ± 14.2	268.0 ± 15.8
Bladder weight (mg)	112.0 ± 13.8	128.0 ± 16.0*	116.0 ± 14.6
Urodynamic parameters			
Frequency (no. voids/60 min)	3.2 ± 0.6	13.7 ± 3.8**	4.0 ± 0.7
Voiding pressure (cm H_2O)	31.3 ± 3.8	48.6 ± 3.7**	36.4 ± 4.4*
Voided volume (mL)	1.76 ± 0.28	0.67 ± 0.13**	1.26 ± 0.34*
No. of nonvoiding contractions between micturition (no./60 min)	0	3.7 ± 0.50**	0
Water intake (mL)/24 h	32.6 ± 4.6	31.0 ± 4.3	32.4 ± 4.8
Urine output (mL)/24 h	26.5 ± 3.5	25.2 ± 3.9	30.5 ± 5.2

Data are presented as mean ± standard error.
*$p < 0.05$.
**$p < 0.01$.

membranes were washed with buffer and incubated with the secondary antibody (goat anti-mouse IgG). The expression of the protein band was visualized by adding ECL-Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1–2 minutes. The membrane was sealed in a hybridization bag, scanned, and analyzed using a Kodak, Rochester, NY , USA Image Station 440CF and Kodak ID image analysis software.

3. Results

3.1. The effects of ketamine and EGCG treatment on cystometric parameters

CMG parameters recorded micturition pressure, micturition frequency, voiding contractions, and nonvoiding contractions as shown in Fig. 1. Ketamine-injected rats showed bladder hyperactivity with increases in voiding contractions (arrows) and nonvoiding contractions (stars), micturition frequency, and micturition pressure (Figs. 1A and 1B). There was a significant decrease in micturition interval and increase in micturition pressure (1.6-fold; Figs. 1A and 1C) and micturition frequency (2.3-fold) compared with the saline (control) rats (Figs. 1B and 1D). Fig. 1C demonstrated that maximal micturition pressure (pdt max) was significantly increased by ketamine treatment. Moreover, bladder volume was also significantly decreased in the ketamine group ($p < 0.01$ in comparison with the control; Fig. 1D). However, treatment with EGCG significantly attenuated the frequency of nonvoiding contractions (Figs. 1A and 1D) and maximal micturition pressure (Figs. 1A and 1C), but increased bladder volume (Figs. 1B and 1D) compared with the ketamine group. The voiding pattern and bladder volume were similar to the control and showed good storage and emptying functions.

3.2. Tracing analysis of voiding behavior and bladder storage

Table 1 shows tracing analysis of voiding behavior by metabolic cage. On tracing analysis, ketamine treatment increased micturition frequency and lessened voiding volume compared with the saline rats. These results demonstrated that ketamine treatment impaired bladder storage function. However, EGCG treatment improved bladder storage function with decreased micturition frequency and increased voiding volume compared with the ketamine rats (Table 1).

3.3. The histological features of ketamine-associated bladder damage

The histological features of ketamine-associated bladder damage were analyzed using Masson's trichrome stain. In the control group (Figs. 2A and 2A′), there were three to five layers of urothelium (black star) and only sparse collagen (blue color) distributed between the smooth muscle bundles (Fig. 2A′). In the ketamine group (Figs. 2B and 2B′), the bladder tissues were characterized by ulcerated mucosa with a thinner layer of epithelial cells (black star), erythematous patches, and increased interstitial fibrosis stained in dark blue (black arrow) between the DSM bundles were shown prominently. However, after EGCG administration (Figs. 2C and 2C′), the thickness of urothelium (star) increased to three layers (black star), and interstitial fibrosis decreased (black arrow). In Fig. 3, the ratio of collagen to DSM showed that ketamine injection over 1 month significantly increased the severity of interstitial fibrosis ($p < 0.01$ in comparison with the control), whereas EGCG administration lessened interstitial fibrosis and erythematous patches.

3.4. The effects of ketamine and EGCG treatment on fibrosis marker expression

To investigate the fibrosis process after ketamine treatment or with EGCG, the expressions of TGF-β, fibronectin, and type I collagen at protein level were examined using Western blotting. The results showed that TGF-β (11.5-fold; Fig. 3A), fibronectin (5.0-fold; Fig. 3B), and type I collagen (11.2-fold; Fig. 3C) expressions were significantly increased 1 month after ketamine administration. In addition, treatment with EGCG significantly decreased TGF-β (3.8-fold; Fig. 3A), fibronectin (1.9-fold; Fig. 3B), and type I collagen (5.5-fold; Fig. 3C) expressions compared with ketamine rats. These findings showed that administration of EGCG reduced the fibrotic biosynthesis and protected the bladder from ketamine-induced inflammatory damage.

4. Discussion

In the present study, we demonstrated that ketamine injection significantly increased bladder overactivity 28 days later. These results demonstrated that ketamine treatment caused bladder hyperactivity by altering maximal micturition pressure, increased micturition frequency, enhanced bladder nonvoiding contraction, and lessened bladder capacity. By contrast, EGCG administration improved voiding pattern and bladder capacity. Ketamine treatment significantly decreased urothelial thickness, increased suburothelial hemorrhage, and enhanced interstitial fibrosis; however, EGCG treatment reversed these changes to near the control levels.

In the present study, ketamine addition was related to a significantly greater collagen/smooth muscle (SM) ratio, which was compatible with previous studies.[19,20] These histological changes might be associated with decreased bladder compliance and bladder capacity as demonstrated in urodynamic studies. Investigations have associated bladder wall mechanical properties with detrusor muscle elasticity, content of collagen, and collagen distributions.[19] In studying dysfunctional bladders, Landau et al[21] showed dysfunctional bladders were significantly less compliant than normal controls, and these bladders have increased collagen deposition and collagen/SM ratios. These pathological ultrastructural changes were reflected by abnormal low bladder volume and decreased bladder compliance, and these could be indicators for a loss of bladder wall elasticity.

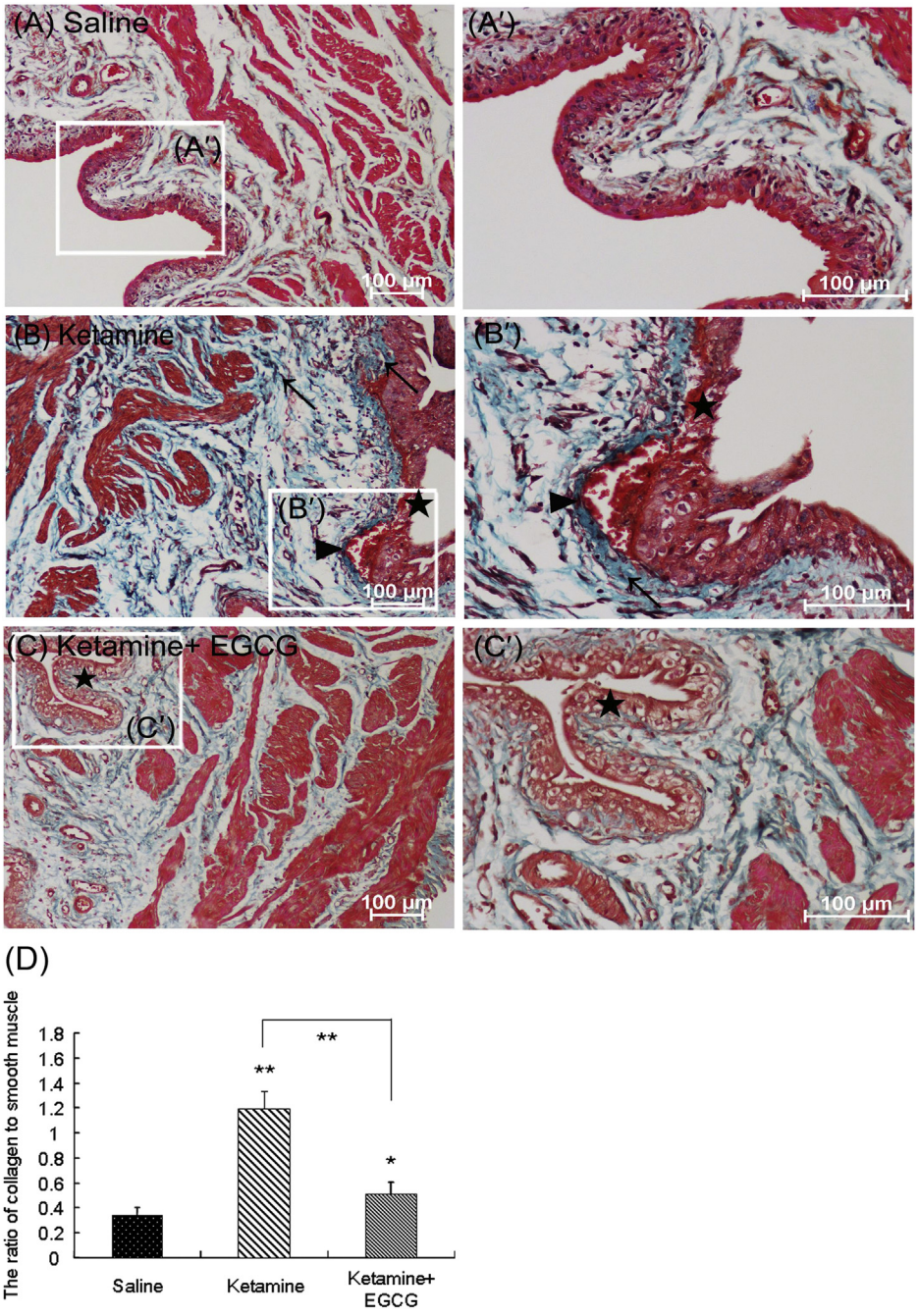

Fig. 2. Representative microphotographs of (A) control group, (B) ketamine group and (C) ketamine-combined EGCG groups as shown by Masson's trichrome staining. (D) The ratio of collagen to smooth muscle. The blue-stained collagen and red-counterstained smooth muscle (SM) were highlighted for each image. The total areas occupied by SM and collagen were calculated. Scale bar represents the unit of 50 μm. Each bar represents the mean ± SD of the mean for $n = 10$. *Indicates significant difference from the control (saline) group, $p < 0.05$; ** Indicates significant difference from the control (saline) group, $p < 0.01$.

Bladders with more SM expression would be expected to have more elastic properties and higher compliance than bladders with a higher proportion of collagen. EGDG treatment reversed collagen/SM ratios, lessened interstitial fibrosis level, and restored bladder storage function.

In ketamine-treated bladders, TGF-β expression was significantly increased when compared with the control. TGF-β is a multifunctional growth factor involved in the cell differentiation, proliferation, and extracellular matrix remodeling after binding with cell surface receptors for TGF-β.[22] In obstructive renal disease, increased TGF-β expression has led to enhanced macrophage infiltration, myofibroblast formation and type I collagen accumulation.[23,24] Our previous study demonstrated that there are increased fibronectin and type I collagen expressions in ovariectomized bladders,[15] showing that these profibrotic and fibrosis markers are important in bladder interstitial fibrosis and are the pathophysiological mechanisms underlying OAB.

EGCG is the major constituent and displays the most potency in the inhibition of cell growth. In the present study, EGCG treatment could ameliorate ketamine-induced TGF-β overexpression. Another study also mentioned that EGCG could ameliorate the overproduction of proinflammatory cytokines and mediators, reduce the activity of nuclear factor-κB (NF-κB), inhibit the expression of inducible nitric oxide synthase, and reduce the inflammatory

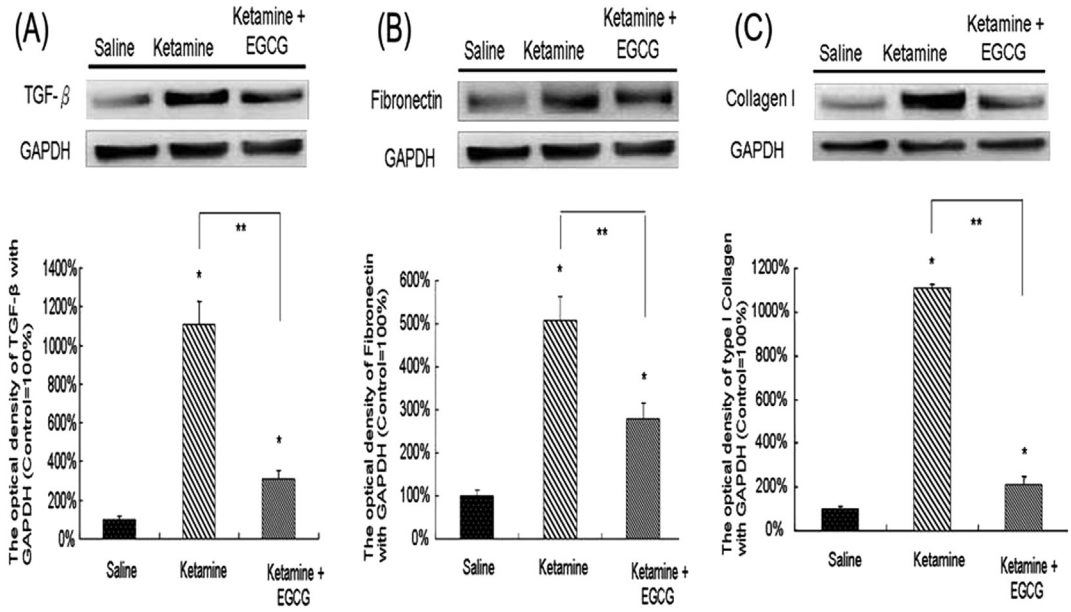

Fig. 3. The effects of ketamine and epigallocatechin gallate (EGCG) treatment on profibrotic and fibrosis marker expressions. Representative Western blots of bladder tissue homogenate probed with (A) an antibody specific to transforming growth factor-β (TGF-β), (B) fibronectin, and (C) type I collagen in each group. Quantifications of the ratios of TGF-β, fibronectin, and type I collagen expressions to actin are shown in the lower of the top figures. Results are normalized as the control = 100%. Each bar is the mean ± standard deviation (SD) of the mean for $n = 8$. * Indicates significant difference from the control (saline) group, $p < 0.05$; ** Indicates significant difference from the control (saline) group, $p < 0.01$. GADPH = glyceraldehyde 3-phosphate dehydrogenase.

process.[25] Therefore, EGCG might ameliorate ketamine-induced bladder damage through multifunctional activities. Firstly, EGCG is a powerful hydrogen-donating antioxidant, a free radical scavenger of ROS and RNS. EGCG could accumulate in the mitochondria where it acted locally as a free radical scavenger. Then, the protective effect of EGCG may involve the regulation of naturally occurring antioxidant protective enzymes. EGCG was found to elevate the activity of two major ROS metabolizing enzymes, superoxide dismutase and catalase,[26] thus protecting tissues from oxidation injuries. Third, EGCG may modulate cell survival/death genes.

In diabetic mice, EGCG significantly increased the immunoreactivity of Ki-67 (cellular marker for proliferation), CD-31 (used primarily to demonstrate the presence of endothelial cells and evaluate the degree of angiogenesis), and α-smooth muscle actin expressions. These results suggest that EGCG could benefit wound healing by accelerating re-epithelialization and angiogenesis. In addition, several reports have shown that EGCG was a powerful hydrogen-donating antioxidant and free radical scavenger of ROS and RNS. EGCG can inhibit iron ascorbate-induced mitochondrial membranes and synaptosomes lipid peroxidation,[27,28] thus protecting brain tissue from free radical damages.

5. Conclusion

This preliminary study showed that EGCG had protective effects on KIC and OAB symptoms. This protective mechanism may arise from reducing profibrotic markers and lessening interstitial fibrosis. However, further studies for investigating the protective effects of EGCG on bladder tissues are required to elucidate the underlying mechanisms and signal pathways.

Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

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