MEDICAL SCIENCE MONITOR

The Dome Wall of Bladder Acts as a Pacemaker Site in Detrusor Instability in Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to confirm that the interstitial cells of Cajal (ICCs) in the dome wall of the bladder are pacemaker cells, and that the dome wall of the bladder acts as a pacemaker site in the detrusor instability (DI) rat model.

Material/Methods: The model of DI in Wistar rats was established and urodynamic studies measuring the bladder volume and pressure were performed. The detrusor excitability was investigated using the amplitude and frequency of phasic contraction of strips. The localization and quantity of ICCs was identified by immunohistochemistry and c-KIT protein expression in the rat bladder. PCR assay and Western blot were used to assess the expression of HCN2 and Cx43.

Results: The bladder capacity, residual volume, voiding volume, and maximum voiding pressure were significantly increased in the DI group. The contraction frequency and amplitude of the strips from the dome of the bladder in the DI group were higher than the triangle, body, and base parts. Both the concentration of c-KIT positive ICCs cells and expression of the c-KIT protein in the dome wall were higher than in other parts of the bladder. The expression of HCN2 and Cx43 in each part of the DI rat group were obviously higher than each part in the control group. Compared to the body, base, and triangle parts, the expression of HCN2 and Cx43 in the dome wall were obviously higher in the DI group.

Conclusions: The quantity of ICCs was higher in the dome wall and the dome wall of bladder acts as a pacemaker site in the DI rat model.

MeSH Keywords: Urinary Bladder Calculi • Urinary Bladder Diseases • Urinary Diversion

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Background

Detrusor instability (DI) was first coined as "unstable bladder" by Bates in 1971 [1]. After that, DI has been found to be one of the most common bladder micturition dysfunction in the clinical setting [2,3]. The incidence rate is about 42% in patients with lower urinary tract obstruction [4]. DI patients often suffer from urinary frequency, urinary urgency, and urgency incontinence. Recent research has shown that the bladder detrusor is a special kind of excitable muscle, and large conductance voltage- and Ca2+-activated K+(BK) channel activation increases detrusor smooth muscle contractility. The change in excitability of the detrusor may play a significant role in the occurrence of DI [5,6]. IN addition, scientists have also found that this kind of excitability probably originates from the interstitial cells of Cajal (ICCs), which have been found in the bladder detrusor layers [7,8]. As is well-known, ICCs possess self-excitability and act as primary pacemakers stimulating smooth muscle contractions, generating electrical slow waves, and driving peristalsis in the gastrointestinal tract [9,10]. Also, research indicates that ICC-like cells (ICC-LC) are present in the urinary system, such as the renal pelvis, ureteropelvic junction (UPJ), ureter, urinary bladder, and urethra, and act as pacemakers driving ureteropelvic peristalsis [11–13]. Despite significant progress in the study of ICCs function and distribution on bladder dysfunction, their specific function in DI remains elusive [10].

In the bladder, ICCs express many markers including tyrosine kinase receptor KIT, adhesion molecule CD34, and the platelet-derived growth factor receptor alpha (PDGFRα) [14,15]. Thus, the antibodies of c-KIT can be used as the identification marker of ICCs, and to study the functional role of the bladder [16]. The hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) channel currents play an essential role in the pace making activity of the sinoatrial node and cardiac rhythmicity [17]. Under certain abnormal conditions the expression of HCN channel disorder, such as decreases in the sinoatrial node but increases in the atrium, can trigger ectopic tachyarrhythmia [18]. In the case of brain pain and chronic periodontitis, the higher expressions and HCN2 currents aggravate chronic pain through contributing to neuronal excitability and inflammation [19,20]. In the bladder wall, there are two cell types, ICCs and intramural neurons, which coordinate the autonomous activity through connexin43 (Cx43), a gap junction protein in the urinary bladder [21]. Many researchers have reported that higher expression of Cx43 enhances intercellular electrical activity through sensitizing chemical transmission, and increases micturition frequency in an unstable bladder [22].

In our previous work, we found an interesting phenomenon: all the strips isolated from bladders exhibited spontaneous contraction activity which was little affected by neural factors, and there was an increase of contractive frequency in the unstable detrusor strips [23]. On the basis of these findings, we speculated that the ICCs in the bladder wall were pacemaker cells that could generate spontaneous excitability of the detrusor; and that there might be a possible pacemaker site, where a larger number of ICCs assemble.

The aim of this study was to confirm that the ICCs in the dome wall of the bladder are pacemaker cells using a rat bladder detrusor instability (DI) model.

Material and Methods

Experimental model

Female Sprague-Dawley rats of 2–3 months, weighing from 220–280 g (n=40), were divided into a DI group (n=20) and a normal control group (n=20). In the DI group, the proximal urethra was partial ligated for six weeks to produce the models of partial bladder outlet obstruction [24]. Rats were anesthetized with pentobarbital sodium (38 mg/kg) or urethane (1.1 g/kg) injected intraperitoneally before surgery. All the experimental rats received a standard diet before being killed. This study was approved by the local animal ethics committee.

Functional studies

Rats were killed by cervical dislocation in accordance with experimental protocols. Bladders and urine dynamics detectors (Nidoc 970A, USA) were interlinked by epidural catheter-tee-microperfusion pump (AVI271, USA, 2 mL/minute), to monitor intravesical pressure. After emptying the bladder, cystometry was completed with normal saline infuse (0.2 mL/minute). The bladder capacity, residual volume, voiding volume, and maximum voiding pressure were measured.

Bladders were opened by a ventral incision. Longitudinal detrusor strips (2×7 mm) were removed from the body, base, triangle, and dome parts of bladder, and placed in Kreb bicarbonate solution (NaCl 6.96 g, KCl 0.35 g, CaCl$_2$ 0.278 g, MgSO$_4$ 0.296 g, KH$_2$PO$_4$ 0.164 g, NaHCO$_3$ 1.249 g, Glu 2.07 g, distilled water 1,000 mL, 38 °C), equilibrating for 30 minutes. The detrusor excitability was investigated by the amplitude and frequency of the phasic contraction test of strips. Detrusor strips showing no phasic activity were discarded.

Immunofluorescence studies for ICCs

Bladders were opened by a ventral incision. The detrusor strips (15×15 mm) were removed from the body, base, triangle, and dome parts of the bladder, and placed in liquid nitrogen for immunofluorescence studies. The detrusor sections (20 um) were fixed for 10 minutes in acetone solution and washed in

Table 1. List of primers used in PCR.

Gene	Primer sequence (5'-3')	Length (bp)
β-actin	F: CTGGAGAAGAGCTATGAGCTG R: AATCTCCTTCTGATCCTGTC	246
HCN2	F: GTGTGCGGGCTGACACCTACTGT R: CTGCCTGCTGCACCATCT	255
Connexin43	F: CATTGGGGGGAAGGCGTGAGG R: AGCGCACGTGAGAGATGGGGAAG	188

phosphate buffered saline (PBS, 0.01 mol/L, pH7.4) three to five times. Then we quenched the endogenous peroxidase activity and blocked the nonspecific combination of antibody protein by treating with 0.3% H_2O_2 in 60% methanol solution and 1% BSA in PBS for 30 minutes, respectively. Tissues sections were incubated with primary antibody dilutions made up in 0.2% BSA/0.01 MPBS: goat anti-mouse c-KIT (1: 100, Santa Cruz, Germany), rabbit anti-mouse HCN2 (1: 100, Chemicon, Japan), and rabbit anti-mouse Cx43 (1: 100, Cell Signaling, USA) overnight at 4°C, respectively. After washing three to five times in PBS (0.01 M), the tissue sections were incubated with 1: 100 dilutions of secondary antibodies: Rhodamine (TRITC) AffiniPure donkey anti-goat for c-KIT, FITC-conjugated AffiniPure Mouse Anti-Rabbit for HCN2, and FITC-conjugated AffiniPure Mouse Anti-Rabbit Cx43 (Jackson, USA) at 25°C for 60 minutes. In the negative control, the tissue sections were incubated in 0.01 M PBS with 5% normal goat serum only. Then we added 2 ug/mL DAPI nuclear stains (Bioss, China) for 20 minutes at room temperature. After several washes, tissues were fixed with glycerin, and were examined using a fluorescence microscope (Olympus, Japan); ICCs quantity variance=ICCs (DI group)–ICCs (control group).

Molecular studies

Bladders were opened by a ventral incision. The detrusor strips (7×7 mm) were removed from the body, base, triangle, and dome parts of bladder, and placed in liquid nitrogen for molecular studies. Total RNA was extracted from detrusor tissues using TRIzol reagent (Invitrogent, UK) according to the manufacturer's instructions. HCN2 and Cx43 expression levels were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the SYBR®Green (TaKaRa, Japan) dye detection method on ABI StepOne PCR instrument using β-actin as an internal control. The primers are listed in Table 1. The expression levels of HCN2 and Cx43 were calculated using Ct ratio (Ct ratio=$Ct_{RNA}/Ct_{\beta-actin}$), with higher Ct ratio value indicating higher expression.

The detrusor strips were solubilized using standard methods, then 20 μg of protein samples were separated by 5% SDS-PAGE. Separated protein bands were transferred to nitrocellulose membranes, and membranes were blocked (5% skim milk, 2% normal goat serum, 10 mM Tris (pH 8), 0.15 M NaCl, and 0.05% Tween-20) for 60 minutes. The primary antibodies against c-KIT, HCN2, Cx43, and GAPDH were diluted according to the instructions, and incubated in blocking solution overnight at 4°C. Then horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1: 1,000 and incubated at room temperature for one hour. The membranes were washed with PBS four times and the immunoreactive bands were visualized using ECL Plus Kit.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Comparisons between control group and DI group were performed using an unpaired Student's *t*-test. Statistical analysis was performed in GraphPad Prism. Differences were considered significant at $p<0.05$.

Results

Two rats died in the DI group six weeks post operation. The success rate of the DI models was 69.2%, and the cytometry graph of the models conforms to the DI definition (Figure 1). There were no differences in weights of rats between the two groups.

Urodynamic change

Compared to the control group, the bladder capacity (mL), residual volume (mL), voiding volume (mL) and maximum voiding pressure (cm H_2O) were significantly increased in the DI group (1.34±0.05 versus 4.07±0.13; 0.42±0.07 versus 2.11±0.22; 0.92±0.09 versus 1.96±0.22; 49.89±2.21 versus 59.34±3.33, respectively) ($p<0.05$). There was no difference in the contraction frequency and amplitude of the strips among the four parts in the control group, but they were lower compared to the DI group (Figure 1). In the DI group, the contraction frequency and amplitude of the strips from the dome of the bladder were higher than the triangle, body, and base parts (Figure 1A, 1B).

The expression of c-KIT and the quantity change of ICCs cell

The expression of c-KIT and the quantity change of ICCs cells were obtained by Western blot and immunofluorescence studies, respectively. The quantity of ICCs in the dome of the bladder was significantly higher than in the other three parts for both groups. The quantity change of ICCs cells was significantly higher in the dome wall of the DI rat bladder (Figure 2A, 2C).
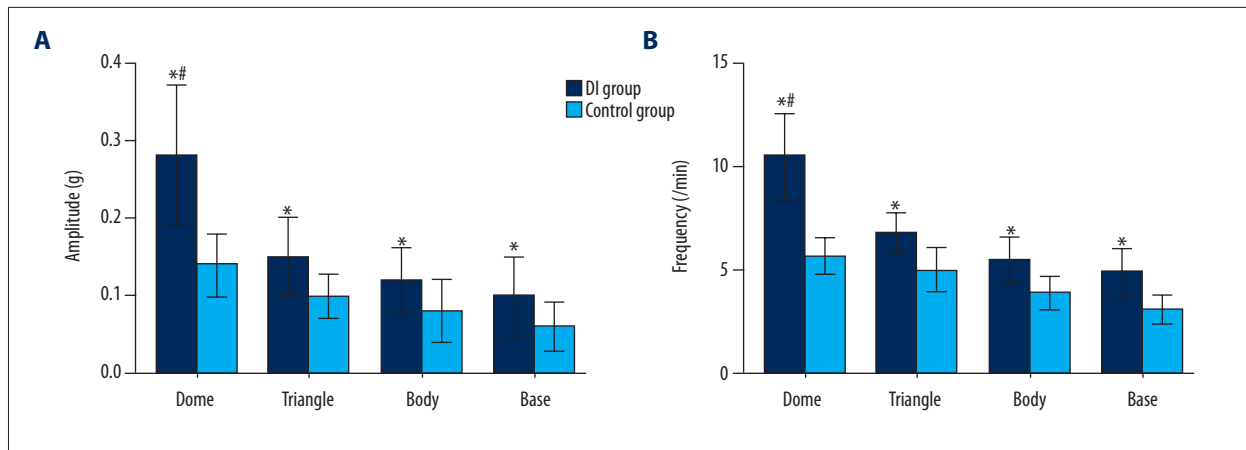
Figure 1. The detrusor excitability of strips in detrusor instability (**A, B**). (**A**) The frequency and (**B**) amplitude of phasic contraction in normal bladder and detrusor instability strips. * $P<0.05$ *vs.* paired normal phasic activity; # $P<0.05$ *vs.* other strips in DI group.
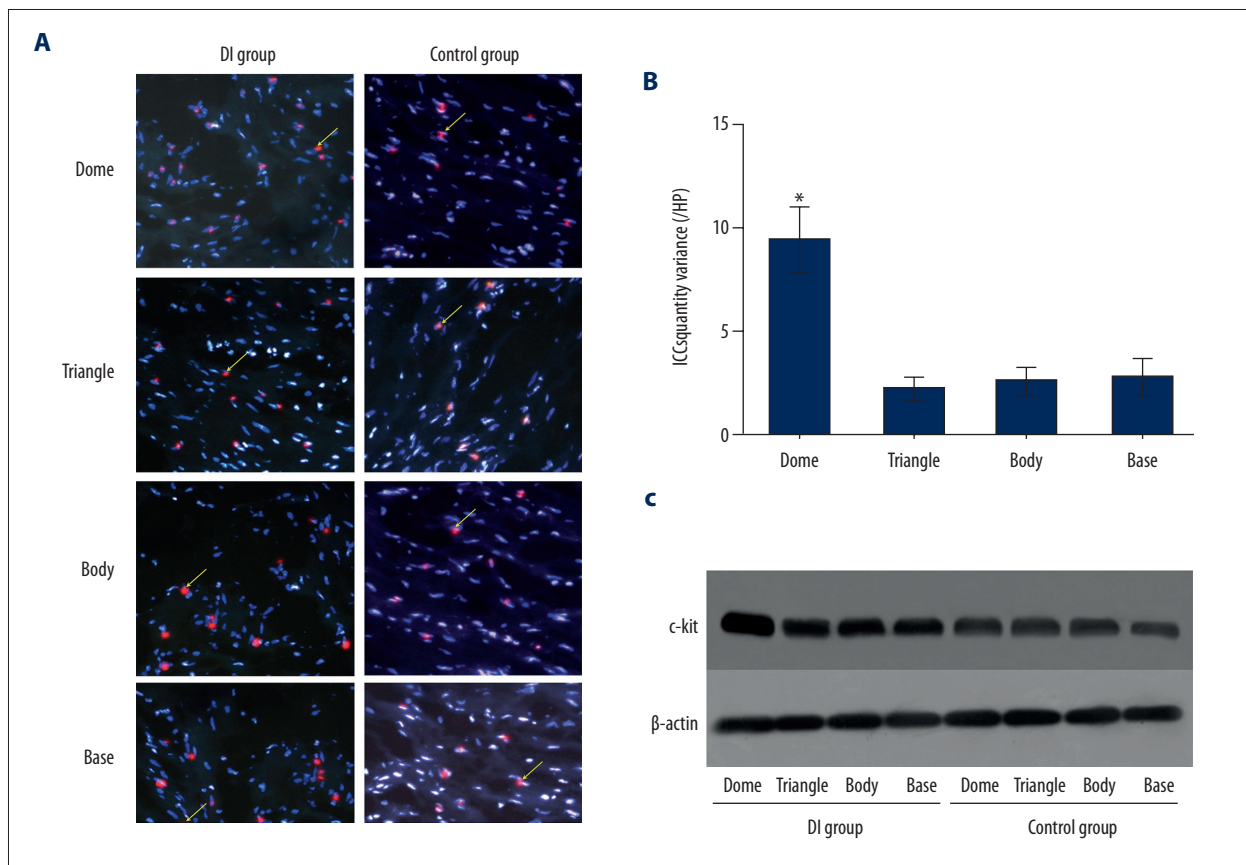

Figure 2. The expression of c-kit and the quantity change of ICCs cell. (**A, C**) The quantity change of ICCs cells was obtained by immnofluorescence methods in DI rat bladder. (**B**) The c-kit expression was obtained by Western blot methods in the dome wall of DI group. * $P<0.05$ *vs.* other parts in DI group.

The expression of c-KIT of each part in the DI group was significantly increased compared to the control group, especially in the dome wall (Figure 2B).

Expression levels of HCN2 and Cx43 mRNAs and protein

HCN2 and Cx43 were both co-expressed with c-KIT in ICCs cells (Figure 3C, 4C), and the expression of HCN2 and Cx43 mRNAs and proteins of each part in the DI group were obviously higher
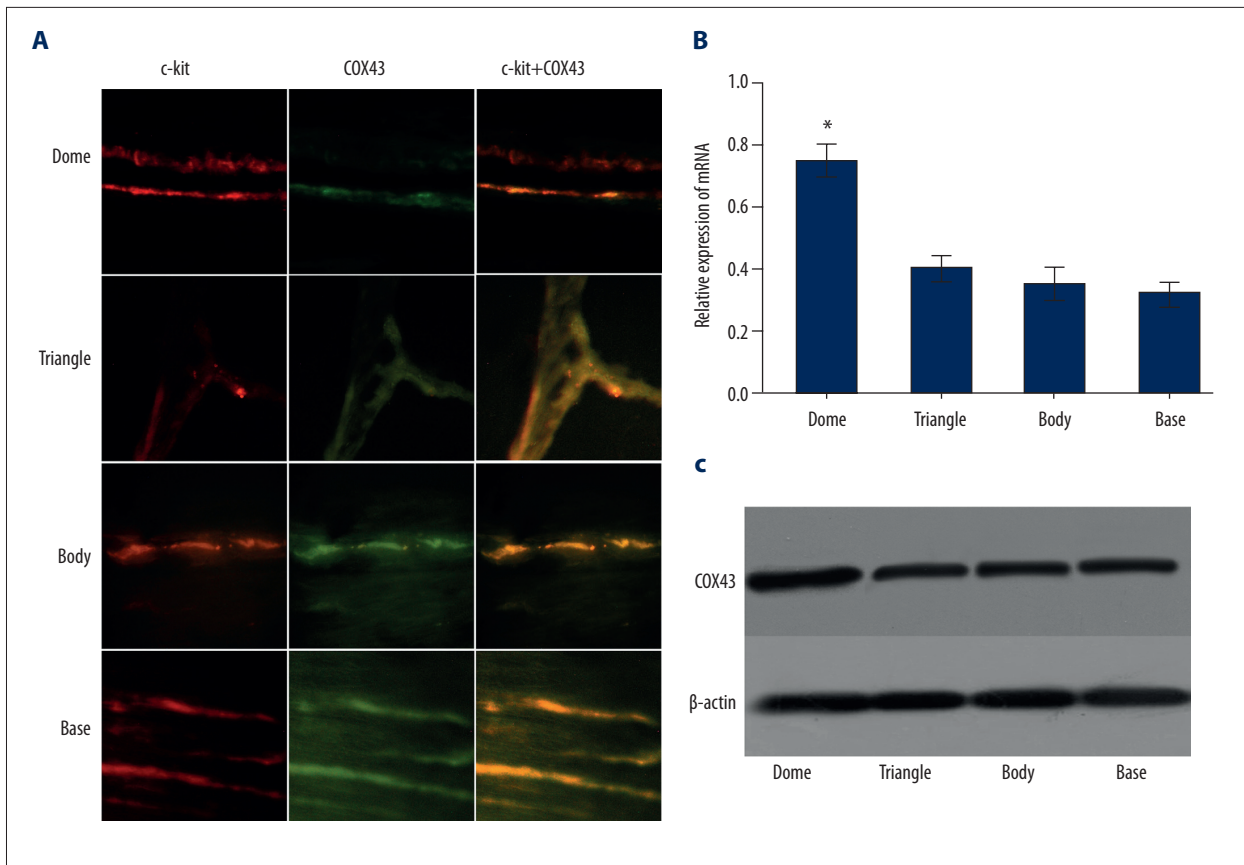
Figure 3. The expression of Connexin43. (**A**) Connexin43 mRNAs and (**B**) protein expression in DI group. (**C**) Co-expression of Connexin-43 and c-kit-positive ICCs in the DI detrusor. * $P<0.05$ *vs.* other parts in DI group.

than in the control group. There were no differences in expression of HCN2 and Cx43 mRNAs and proteins among the four parts of bladder wall in the control group. In contrast, expression of HCN2 and Cx43 mRNAs and proteins were significantly higher in the dome than in the other three parts while the difference was not obvious in the triangle, body, and base parts (Figures 3A, 3B; 4A, 4B).

Discussion

With the development of prostatic hyperplasia, many patients suffer from urinary frequency, urinary urgency, and urgency incontinence, which are considered symptoms of detrusor instability (DI) [2–4]. The effect of surgery and drug conservative management for DI is still unsatisfactory. Thus, to discover DI pathogenesis would help patients who are suffering from a DI bladder. Over the past decades, several studies have provided evidence for neurogenic and myogenic contraction of smooth muscles in the bladder wall [25]. DI results from the absence of nerve stimulation. Many researchers have found that denuded detrusor strips exhibit spontaneous contraction under smaller tension load [26]. Moreover, in the unstable bladder,

the frequency and amplitude of unstable detrusor strips are significantly increased compared to the normal bladder. These phenomena indicate that the spontaneous contraction of detrusor strips in DI may mainly be related to myogenesis. Recently, there has been increasing evidence that the interstitial cells of Cajal (ICCs), but not the smooth muscle cells, are the pacemaker cells in the bladder [27]. Just like the heart and gastrointestinal tract, the bladder is considered an automatic pacemaker organ. Our study confirmed that the contraction frequency and amplitude of the detrusor strips from all the locations of DI bladder were significantly higher than the corresponding locations of the normal bladder, and contraction frequency and amplitude of the strips obtained from the dome were higher than those obtained from the triangle, body, and base (Figure 1A, 1B). So we assumed that the dome was the pacemaker site, where larger numbers of ICCs assemble in the DI bladder.

In the gastrointestinal tract and urethra, the ICCs are identified by c-KIT-immunization and possess self-excitability and act as primary pacemakers stimulating smooth muscle contractions [28,29], and these ICCs cells form a complex network with the neighboring nerves and detrusor muscle through the
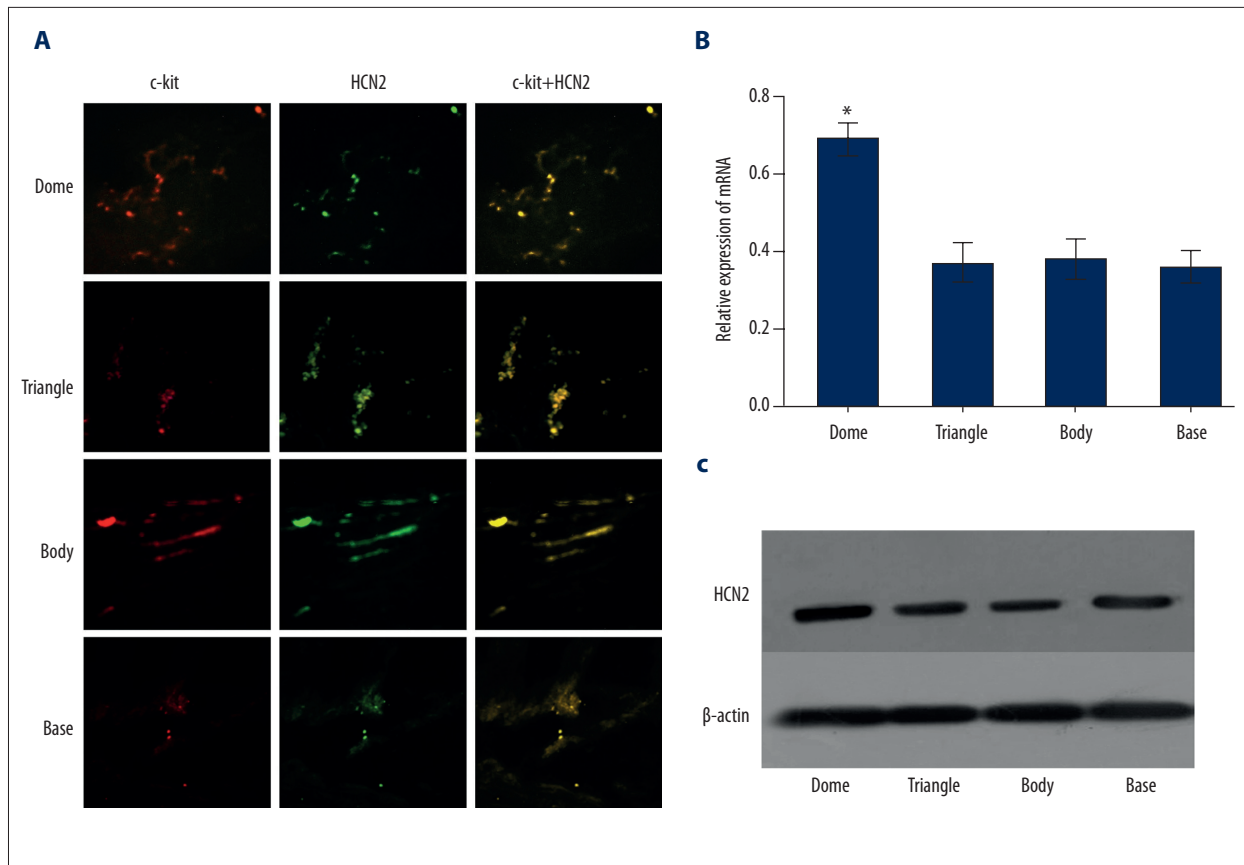
Figure 4. The expression of Connexin43. (**A**) HCN2 mRNAs and (**B**) protein expression in DI group. (**C**) Co-expression of HCN2 and c-kit-positive ICCs in the DI detrusor. * $P<0.05$ *vs.* other parts in DI group.

gap junctions [30]. On the contrary, the loss or damage of the ICCs network leads to many gut and gastrointestinal diseases, such as Crohn's disease, Hirschsprung disease and Pancolonic decrease [31]. ICCs were also found in the bladder by Smet et al. [32], and located within the lamina propria and detrusor layers in the urinary bladder [33]. The distribution and the quantitative of ICCs change in the pathological urinary bladder [34]. Similarly, we found ICC-like cells located in the detrusor muscles of the rat bladder, and there are more c-KIT-positive ICCs cells in the DI bladder than in the normal bladder. There were more ICC-like cells in the dome than in the triangle, body, and base wall in the DI bladder (Figure 2). In the literature, by blocking c-KIT receptors of ICCs, urinary bladder capacity, voided volumes, frequency, contraction thresholds, and spontaneous motor activity were reduced [35]. Our study identified a relationship between the excitability of strips and the distribution and the quantity of ICCs in the DI bladder. Our findings provide support for the hypothesis that the dome wall is the pacemaker site in the DI bladder.

Cx43 is a gap junction protein that is found in the rat heart [36]. Gutstein et al. report that knockout of Cx43 could significantly slow the ventricular conduction velocity in cardiac arrhythmic mice, meaning that the gap junction remodeling contributes to the increased propensity for cardiac arrhythmogenesis [37]. In myogenic detrusor hyperreflexia, the intermediate junctions of muscle cells were absent or reduced, and instead had dominant intimate cell appositions with much narrower junctional gaps [38]. Expression of Cx43 and Cx26 were both significantly increased in the ICCs of bladder outlet obstruction rats and contributed to the response of the bladder wall to increased voiding pressure through enhancing intercellular communication [22,30,39]. As expected, PCR, Western blotting, and immunofluorescence studies showed that Cx43 was higher when co-expressed with c-KIT positive ICCs in the DI detrusor (Figure 3), along with a higher increase of contraction frequency and amplitude of the strips.

HCN2 acts as a channel current protein and is a promising marker to use to recognize the pacemaker role of cells [40]. Valiunas et al. [41] showed that the inward current evoked in HCN2-expressing cell, which also express Cx43, was delivered to the cardiac myocyte via gap junctions and generated action potentials such that the cell pair could function as a pacemaker unit. I_f is "funny" current that forms by HCN, and is a key player in driving pacemaker activity [42]. Moreover, ICCs cells

expressing HCN channels are known to participate in spontaneous bladder contractility and voiding through different signaling pathways [43]. Modifications in expression or function of HCN2 currents could alter the rate of spontaneous activity [44]. Mahendra et al. [45] observed that HCN channels were expressed in detrusor of human and rat bladders and that the bladders can be relaxed when blocking the HCN channel. In our study, we observed an increased expression of HCN2 (I_f) mRNA and protein in the DI rat bladder. HCN2 was higher when co-expressed with c-KIT positive ICCs in the DI detrusor (Figure 4). Thus, the dome wall might be a possible pacemaker site, where lager numbers of HNC2-positive ICCs assemble.

Conclusions

In conclusions, based on the previous studies and our studies, we postulate that all ICCs cells of the detrusor are innervated under normal conditions, and the excitatory response generating from the pacemaker site is suppressed by the special characteristics that low frequency, less intracellular gap junction, and lack of electricity coupling. The low inner pressure of the bladder is maintained by the low smooth muscle contraction frequency and amplitude. The inner pressure will significantly increase when the bladder outlet obstructs, as well as when the nerve in the bladder wall is disorder or damaged under the condition of ischemia, hypoxia, and abnormal pH. The urinary bladder wall will remodel along with the quantity, distribution, and ultrastructural feature changes of ICCs, especially in the dome wall. Under these conditions, the ICCs of the dome wall act as pacemaker cells leading to the detrusor instability. However, Tekin A, et al. [46] found that the density of ICCs decreased in the neurogenic bladder of fetal rats with myelomeningocele. This phenomenon was explained as follows. The development and differentiation of ICCs need neural support in the fetuses. And the changing of ICCs quantity is along with the whole life or under different disease and pathological conditions. So more studies should be reviewed and further studies should be conducted to investigate the function of ICCs in abnormal bladders.

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