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Antagonism of the transient receptor potential ankyrin 1 (TRPA1) attenuates hyperalgesia and urinary bladder overactivity in cyclophosphamide-induced haemorrhagic cystitis

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ABSTRACT

The aim of this study was to investigate the involvement of the transient receptor potential ankyrin 1 (TRPA1) in haemorrhagic cystitis, the main side effect of cyclophosphamide-based chemotherapy. Hannover female rats received intraperitoneal (i.p.) injection of cyclophosphamide (three doses of 100 mg/kg, every other day, in a total of five days). This treatment was followed by the treatment with TRPA1 antagonist HC 030031 (50 mg/kg, p.o.). The threshold for hindpaw withdrawal or abdominal retraction to von Frey Hair and the locomotor activity were measured. The treatment with the TRPA1 antagonist HC 030031 significantly decreased mechanical hyperalgesia induced by cyclophosphamide without interfere with locomotor activity. Urodynamic parameters were performed by cystometry 24 h after a single treatment with cyclophosphamide (200 mg/kg, i.p.) in control and HC 030031 treated rats. Analyses of the urodynamic parameters showed that a single dose of cyclophosphamide was enough to significantly increase the number and amplitude of non-voiding contractions and to decrease the voided volume and voiding efficiency, without significantly altering basal, threshold or maximum pressure. The treatment with HC 030031 either before (100 mg/kg, p.o.) or after (30 mg/kg, i.v.) cyclophosphamide inhibited the non-voiding contractions but failed to counteract the loss in voiding efficiency. Our data demonstrates that nociceptive symptoms and urinary bladder overactivity caused by cyclophosphamide, in part, are dependent upon the activation of TRPA1. In this context, the antagonism of the receptor may be an alternative to minimise the urotoxic symptoms caused by this chemotherapeutic agent.

1. Introduction

Cyclophosphamide is an alkylating agent that has long been used in the treatment of solid tumours, lymphoma, myeloma and chronic lymphocytic leukaemia. More recently, cyclophosphamide has been employed for the treatment of non-neoplastic diseases, including thrombocytopenic purpura, rheumatoid arthritis, systemic lupus erythematosus and as a conditioner before bone marrow transplantation [1–3]. Side effects for the treatment with cyclophosphamide were described by Coggins and co-workers as early as 1960 [4]. Among the side effects, the urological disturbances present the major limiting factor in its use. Effects vary from transient irritating voiding symptoms, such as urinary frequency, dysuria, urgency, suprapubic discomfort and strangury with microhaematuria, to life-threatening haemorrhagic cystitis. Urinary bladder fibrosis, necrosis, contracture and vesicoureteral reflux were also associated with cyclophosphamide therapy [4,5].

The urotoxicity of cyclophosphamide is not directly related to its alkylating activity but rather to the formation of hydroxylated metabolites, particularly acrolein, which is formed from hepatic microsomal enzymatic hydroxylation for further renal excretion [6]. The storage of acrolein within the urinary bladder causes irritation of the urothelium resulting in inflammation that is characterised by urinary bladder hyperreflexia [7]. In addition, acrolein is reported to be the most reactive of the α,β-unsaturated aldehydes, and rapidly binds to and depletes cellular nucleophiles such as glutathione. Acrolein can also react with some proteins residues and nucleophilic sites in DNA. Of note, Bautista and collaborators (2006) demonstrated that acrolein produced a robust increase in calcium influx that was selectively dependent on TRPA1 [8,9].

TRPA1 is a calcium-permeable nonselective ion channel with six transmembrane domains and a unique ankyrin repeat in the amino terminal portion that contains cysteine residues that are sensitive

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to alkylation and activation [9,10]. The cysteine residues are the targets for electrophilic compounds and so the receptor is sensitive to a broad range of naturally occurring compounds including: allyl isothiocyanate; cinnamaldehyde; allicin; the environmental irritant acrolein; the volatile irritant formalin and the end-products of bacterial, inflammatory and oxidative stress hydrogen sulphide, hypochloride, hydrogen peroxide, 4-hydrononenal and 4-oxo-nonenal [11,12]. TRPA1 is highly expressed in unmyelinated and thinly myelinated sensory neurons [10,13,14]. The receptor was identified in neuronal and non-neuronal structures of the lower urinary tract, including unmyelinated nerve fibres innervating the epithelial cells (urothelium), the suburothelial space, muscle layers and blood vessels of the rodent urinary bladder [13,15] and in the human urethra [16].

The role of TRPA1 in pathological processes of the lower urinary tract has been suggested because the receptor is overexpressed in urinary bladder mucosa of patients suffering outlet obstruction of this organ and in the urothelium and neurons innervating the urinary bladder of rats undergoing spinal cord injury [17,18]. TRPA1 mediates the contractile response caused by allyl isothiocyanate and cinnamaldehyde in the rat urinary bladder [19] and the intravesical administration of TRPA1 agonists cause urinary bladder overactivity [15,20].

Taking into account the role of TRPA1 in the pathophysiological processes of the lower urinary tract and its sensitivity to the cyclophosphamide-metabolite acrolein, we hypothesised that antagonism of TRPA1 by a selective antagonist, HC 030031, would minimise the urotoxic symptoms caused by cyclophosphamide treatment and in turn, decrease the side effects of this chemotherapeutic drug. Therefore, we evaluated the role of the TRPA1 antagonist HC 030031 in the urodynamic parameters and in the nociceptive responses in rats that were treated with cyclophosphamide.

2. Material and methods

2.1. Animals

Female Hannover rats weighing 150–250 g, age 6–7 weeks, were used. Animals were housed in cages (up to four per cage) in racks model ALERK-dg-50 (Alesco Ind. & Com. Ltda, São Paulo, Brazil) with ventilated control. The room was maintained at a constant temperature of 22 ± 2 °C under a 12 h light/12 h dark cycle at 60–80% humidity with food and water available *ad libitum*. The rats were maintained together as long as they were born to ensure that they were under the same estrous cycle during the analyses. In our hands, cyclophosphamide presented broader toxicity to male than female and this caused a limitation to the experimental model in males. The experimental procedures were approved by the ethics committee of the Federal University of Santa Catarina (PP00607) and followed the National Institutes of Health Animal Care Guidelines (NIH Publications N° 80-23).

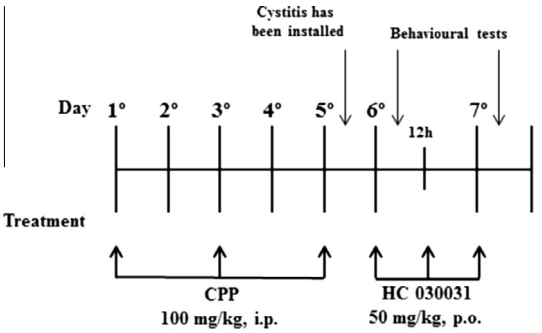

Diagram 1.

2.2. Treatment protocol

For behavioural assessment, rats ($n = 6$ per group) received three doses of 100 mg/kg cyclophosphamide via intraperitoneal (i.p.) on the first, third and fifth days (Diagram 1). This schedule of treatment allows the installation of urinary cystitis before starting any therapeutic treatment and mediates a sub chronic inflammatory situation. This schedule of treatment was followed as described before for female rats [21], with minor modifications. Twenty-four hours after the third injection, on the sixth day, rats received the first treatment with HC 030031 (50 mg/kg) by gavage (p.o.). Treatment with HC 030031 was repeated every twelve hours, with three doses of HC 030031 in total. The mechanical threshold and the locomotor activity were evaluated before the treatments (baseline) and after the first and third doses of HC 030031. The rats were not fed during behavioural tests.

To understand the mechanisms of cyclophosphamide-induced urotoxicity, the urodynamic parameters were taken twenty-four hours after a single dose of cyclophosphamide (200 mg/kg, i.p.) [22]. This acute treatment allows investigating earlier mechanisms of cyclophosphamide-induced urotoxicity. When investigating the effects of the TRPA1 receptor antagonist, reproducible micturition cycles were recorded before (used as baseline values) and during 45 min following administration of HC 030031 (30 mg/kg, 0.1 ml/min for 10 min) or vehicle (8% DMSO + 2% Tween 80 in saline), intravenously (i.v., caudal vein) (Diagram 2A). The administration of HC 030031 24 h after cyclophosphamide intended to evaluate the role of TRPA1 receptor after installation of haemorrhagic cystitis. The dose of the antagonist was selected on the basis of previous studies and this percentage of DMSO has not altered cystometric parameters [17]. To evaluate whether the antagonism of TRPA1 would display some preventive effect against haemorrhagic

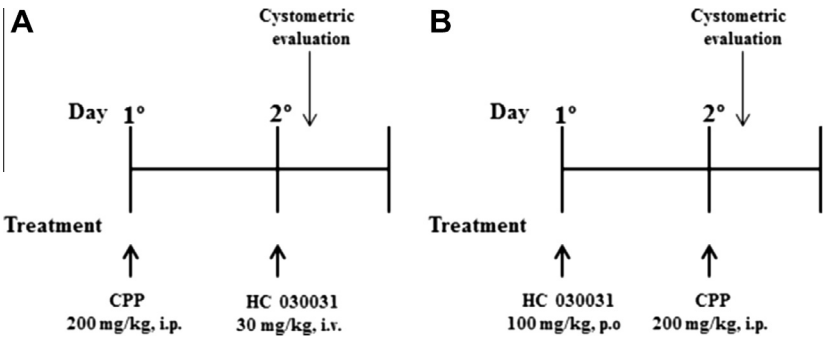

Diagram 2.

cystitis, another scheme of treatment was performed by treating rats with HC 030031 (100 mg/kg, p.o.) one hour before cyclophosphamide (Diagram 2B). The groups ($n = 6$ rats per group) were divided in naïve; cyclophosphamide (200 mg/kg, i.p.); HC 030031 (100 mg/kg, p.o.) one hour before cyclophosphamide (200 mg/kg, i.p.); HC 030031 (30 mg/kg, i.v.) 24 h after cyclophosphamide (200 mg/kg, i.p.).

Both behavioural and urodymic analysis were performed in a double-blinded scheme for the treatment and measurements.

2.3. Mechanical hyperalgesia

The mechanical hyperalgesia induced by the i.p. injection of cyclophosphamide was evaluated through the application of von Frey Hair to the rat hindpaw and abdominal cavity, according to the method previously described [23,24], with some modifications. Rats were individually placed in clear Plexiglas boxes (13.8 cm × 18.0 cm × 68.2 cm) on an elevated wire-mesh platform (23.0 cm × 39.8 cm × 72.7 cm) to allow access to the abdominal and plantar surface of both hindpaws. The animals were acclimatised for at least 1 h prior to the behavioural test. The withdrawal response was quantified by means of the electronic pressure-meter test for rats, which consisted of a hand-held force transducer fitted with a 0.7 mm^2 polypropylene tip (electronic von Frey anesthesiometer, IITC Inc., Life Science Instruments, USA). The stimulus was applied on the plantar surface of the left hindpaw and on the lower abdominal cavity. The intensity of the stimulus (g) was automatically recorded when the paw was withdrawn or when the abdomen was retracted. The stimulus was applied three times alternately and the mean of these values was registered as the mean force supported by the rat. The nociceptive responses were evaluated at different time-points following cystitis induction. All groups were evaluated before cyclophosphamide injection in order to determine the baseline mechanical thresholds.

2.4. Locomotor activity

The locomotor performance was scored, evaluating the number of crossings and rearings by the open-field test. For this purpose, rats were separated in four groups: vehicle, cyclophosphamide, HC 030031, cyclophosphamide plus HC 030031, as described in the treatment protocol, and placed in the centre of a round open-field (50 × 98 × 80 cm), in which the floor was divided into 12 areas. The number of areas crossed and the rearing responses was recorded for 5 min.

2.5. Cystometric parameters

For the urodynamic studies, rats were anaesthetised with urethane (1.1 g/kg, i.p.). A PE-60 polyethylene catheter (Clay Adams, Parsippany, NJ, USA) was inserted via a midline abdominal incision into the bladder through its dome. The intravesical catheter was

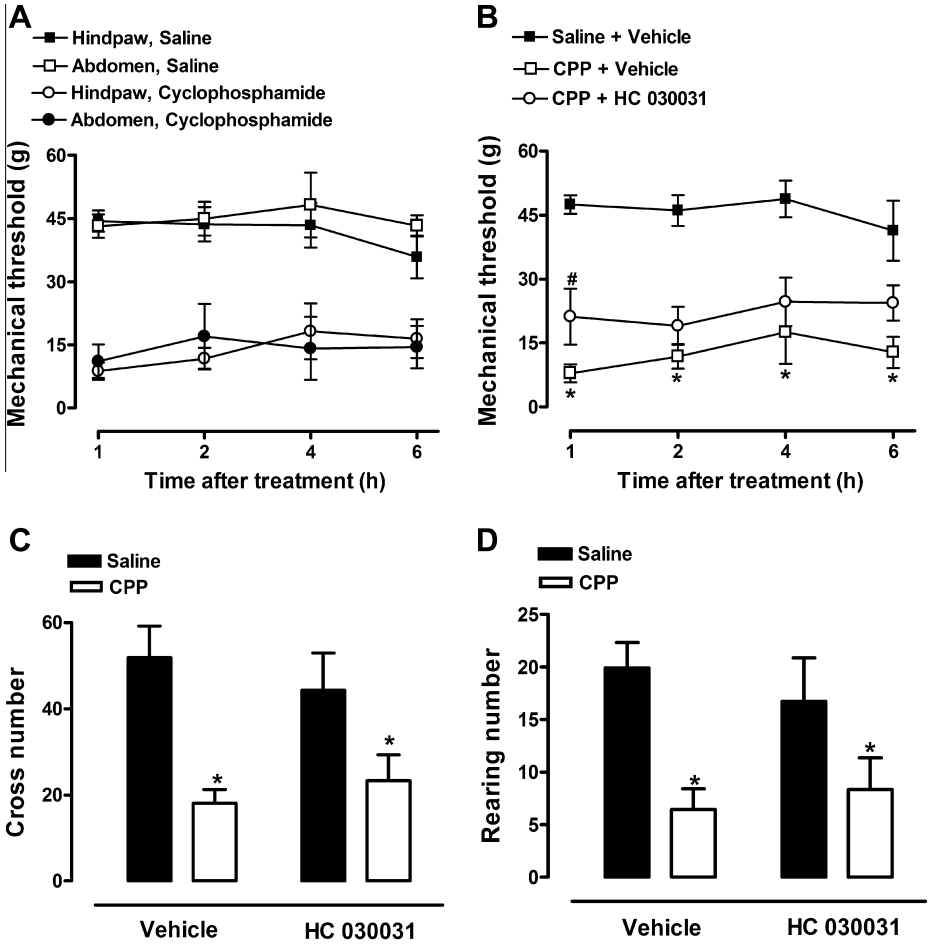

Fig. 1. Mechanical hyperalgesia and locomotor activity in cyclophosphamide (CPP)-induced haemorrhagic cystitis. Mechanical hyperalgesia was assessed by the application of von Frey Hair (VFH) (A) on the abdominal area or (B) on the plantar surface. The rats received three doses of cyclophosphamide (100 mg/kg, i.p.) on the days 1st, 3rd and 5th. Mechanical hyperalgesia was assessed after the third dose of HC 030031 (50 mg/kg, p.o.). (C) The crossing and (D) the rearing numbers in the open-field test were evaluated 6 h after the last treatment with HC 030031. Data are the mean ± S.E.M. of six individuals. The asterisks denote a statistically significant difference $^{*}P < 0.05$ from the saline-treated group and $^{\#}P < 0.05$ from the cyclophosphamide-treated group (two-way ANOVA followed by Newman–Keuls Multiple Comparison test).

connected via a three-way stopcock to a pressure transducer (ADInstruments Pty Ltd., Castle Hill, Australia) and to a micro-infusion pump (Insight Equipamentos Científicos, Ribeirão Preto, SP, Brazil) for recording intravesical pressure and infusing saline into the bladder, respectively. Intravesical pressure was continuously recorded using data acquisition software (PowerLab 8/30, ADInstruments). After catheter implantation, rats were left undisturbed for the next 30 min for bladder stabilisation. After this period, saline at 37 °C was infused at a rate of 0.1 ml/min.

We assessed the micturition pressure (MP, maximum bladder pressure during micturition), basal pressure (BP, the lowest bladder pressure between micturitions), and threshold pressure (TP, bladder pressure immediately before micturition). The number and mean amplitude of non-voiding contractions (NVCs) were also measured. Non-voiding contractions were defined as a rhythmic intravesical pressure increase greater than 5 mmHg from baseline pressure without release of saline from the urethra.

The volume of voided saline from urethral meatus was collected and measured to determine voided volume [25]. In order to measure residual volume, the saline infusion was stopped at the beginning of the voiding contraction, and the residual volume (RV) was measured by harvesting saline through the intravesical catheter and then manually expressing the remaining intravesical contents by exerting pressure on the bladder abdominal wall. The bladder capacity (BC) was calculated as the voided volume plus the residual volume, and the voiding efficiency (VE) was determined as a percentage using the following equation: VE = [(VV/BC) × 100]. The cystometric parameters were calculated from voiding cycles obtained during 45 min.

2.6. Statistical analysis

Results are presented as mean ± S.E.M of six animals. Data were analysed by one or two-way analyses of variance (ANOVA) followed by Newman–Keuls test when appropriate. P values less than 0.05 ($P < 0.05$) were indicative of significance.

3. Results

The systemic treatment with cyclophosphamide caused a persistent hyperalgesia as evidenced by a decrease in withdrawal response threshold to von Frey Hair stimuli [$F(4; 16) = 6.929$; $P < 0.002$]. There was a similar response when the stimulus was applied on the abdominal area or on the hindpaw surface [$F(4; 16) = 1.278$; $P > 0.05$] (Fig. 1A). This suggests that cyclophosphamide

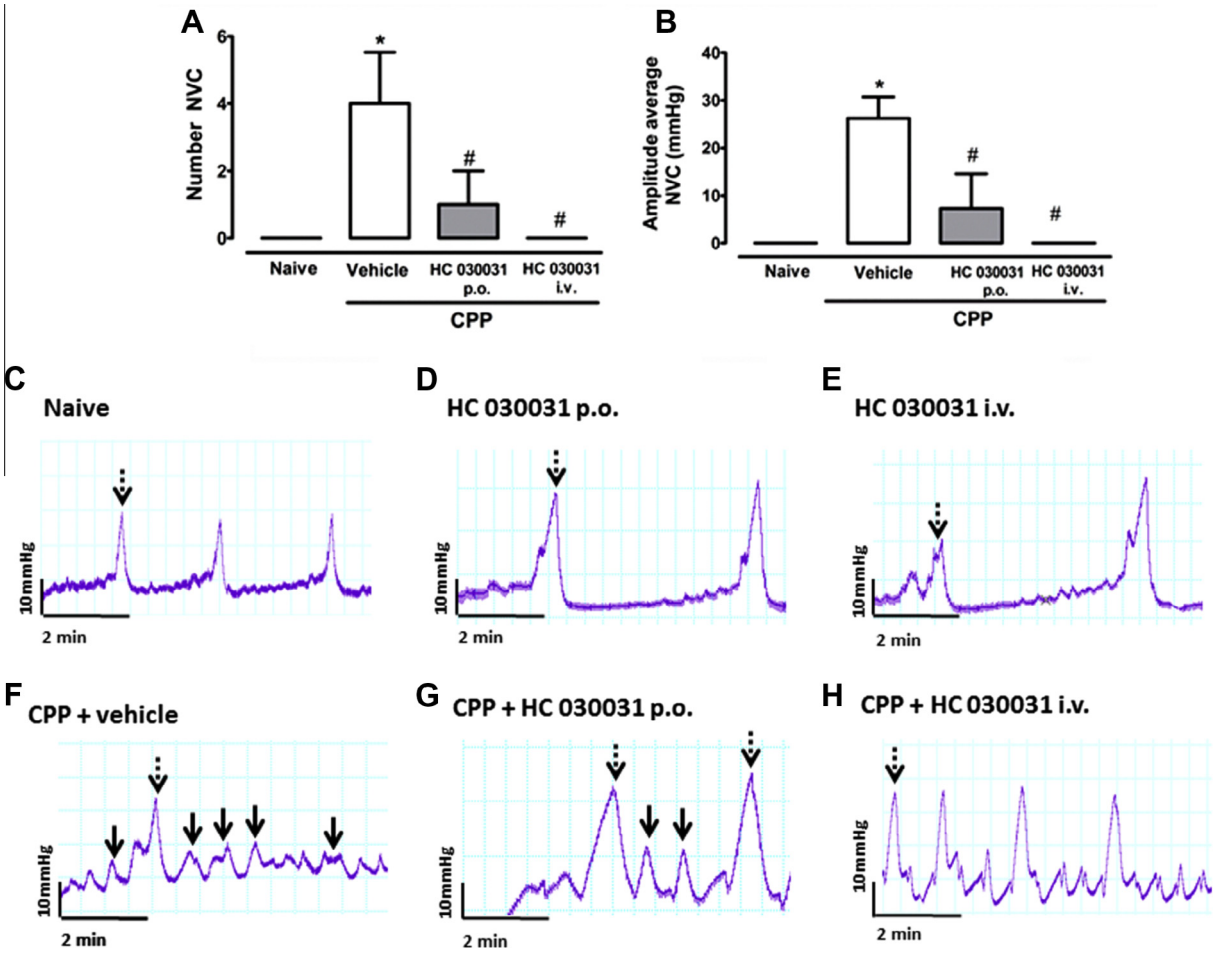

Fig. 2. Urodynamic profile in CPP-induced haemorrhagic cystitis. The number (A) and amplitude (B) of non-voiding contractions (NVCs) were evaluated in rats 24 h after a single injection of cyclophosphamide (200 mg/kg, i.p.). The HC 030031 (100 mg/kg) was administered by oral gavage (p.o.) one hour before cyclophosphamide or by intravenous route (i.v., 30 mg/kg, 0.1 ml/min for 10 min) 24 h after cyclophosphamide. In the i.v. treatment the measurements were performed for 45 min following administration of HC 030031. Each column represents the mean, and the vertical lines indicate the S.E.M. of six animals. $^{*}P < 0.05$ from the saline-treated group and $^{\#}P < 0.05$ compared with the cyclophosphamide-treated group (one-way ANOVA followed by Newman–Keuls Multiple Comparison test). The representative traces of cystometry during bladder-filling phase in (C) naïve; (D) 24 h after HC 030031 p.o.; (E) following HC 030031 i.v.; (F) cyclophosphamide plus vehicle for HC 030031 (8% DMSO plus 2% Tween 80 in saline); (G) cyclophosphamide plus HC 030031 (100 mg/kg, p.o.); (H) cyclophosphamide plus HC 030031 (30 mg/kg, i.v.). The continuous and the dotted arrows over the cystometograms represent the non-voiding and voiding contractions, respectively. Each peak that is a voiding contraction represents a micturition.

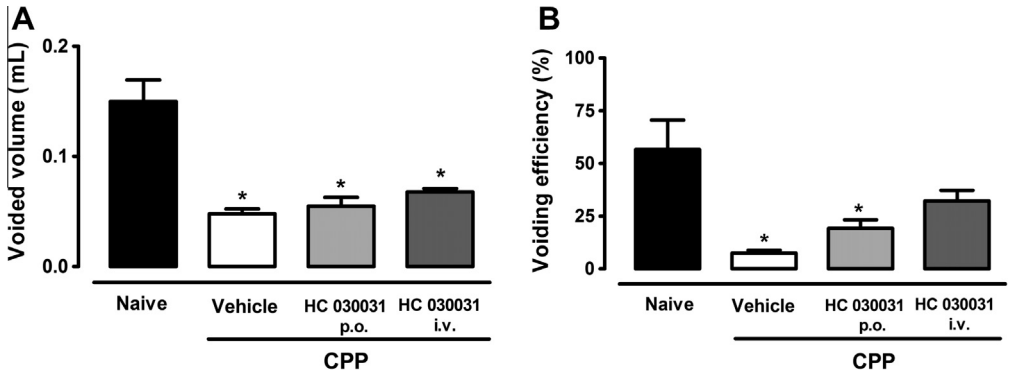

Fig. 3. Urodynamic profile in CPP-induced haemorrhagic cystitis. (A) The voided volume and (B) voiding efficiency in naive or cyclophosphamide-treated (200 mg/kg, i.p.) rats that were under a pre-treatment with vehicle (8% DMSO plus 2% Tween 80) or HC 030031 (100 mg/kg, p.o.) or under a post-treatment with HC 030031 (30 mg/kg i.v.). Each column represents the mean, and the vertical lines indicate the S.E.M. of six animals. $^{*}P < 0.05$ from the saline-treated group (one-way ANOVA followed by Newman–Keuls Multiple Comparison test).

induces a referred hyperalgesia and we chose the hindpaw area to perform the next experiments as it offers a more reliable method for evaluating withdrawal response. Cyclophosphamide induced a consistent and reproducible hyperalgesia from one to six hours; hence we assumed that this scheme of treatment was consistent with the progress of urinary bladder cystitis. Once urinary bladder cystitis had been installed, the treatment with TRPA1 antagonist HC 030031 (50 mg/kg, p.o.) was started. After the third dose of HC 030031 (second day of treatment) there was a mild but significant reduction of hyperalgesia. The anti-hyperalgesic effect was significant only one hour after HC 030031 treatment (Fig. 1B).

Rats treated with cyclophosphamide also presented locomotor alterations, which were likely related to the abdominal discomfort caused by cystitis (Fig. 1C and D). As a result, we detected a significant decrease in the numbers of crossing [$F(1; 24) = 14.314$; $P < 0.05$] and rearing [$F(1; 24) = 17.588$; $P < 0.05$] in rats that received cyclophosphamide. The treatment with three doses of HC 030031 in healthy rats did not alter either crossing [$F(1; 24) = 0.026$; $P > 0.05$] or rearing [$F(1; 24) = 0.078$; $P > 0.05$] numbers, showing that HC 030031 did not cause any sedative effect at this schedule of treatment. In addition, HC 030031 did not significantly attenuate the reduction in locomotor activity caused by cyclophosphamide. No significant interactions were detected for the combined treatment in crossing [$F(1; 24) = 0.779$; $P > 0.05$] and rearing [$F(1; 24) = 0.128$; $P > 0.05$] tests (Fig. 1C and D).

The urotoxic effect of cyclophosphamide was confirmed through cystometric analyses. The histogram at the Fig. 2A and B illustrates the number of the non-voiding contractions (NVCs) and the amplitude of these NVCs contractions. The NVCs were defined as the rhythmic intravesical pressure greater than 5 mmHg from baseline pressure, without the release of saline from the urethra. The NVCs are identified by the continuous arrows over the peaks (Fig. 2F and G). Naive rats did not show any NVCs during the bladder-filling phase and each peak at the trace represents a micturition (Fig. 2C, dotted arrow). Oppositely to the naïve rats, the treatment with cyclophosphamide induced a marked number of NVCs, with high amplitude in these contractions, indicating spontaneous detrusor overactivity (Fig. 2F). Treatment with cyclophosphamide induced polymorphic micturition cycles with improper storage and voiding phase leading to bladder distension. Interesting, the systemic pre-treatment with HC 030031 (100 mg/kg, p.o.) decreased NVCs by 68.75 ± 1.3% (Fig. 2G). Additionally, the post-treatment with HC 030031 (30 mg/kg, i.v.) completely abolished the NVCs induced by cyclophosphamide (Fig. 2H). The administration of HC 030031 either by oral or intravenous route did not alter cystometric parameters by itself (Fig. 2D and E).

Rats treated with cyclophosphamide also presented a decreased voided volume and voiding efficiency, achieving only 32 ± 2.9% and 13 ± 2.6% activity of the control, respectively (Fig. 3A and B). The intravenous administration of HC 030031 partially recovered voided volume by 45 ± 2.3% and to a better degree the voiding efficiency, with 57 ± 8.8% activity of the healthy control (Fig. 3B). The oral treatment with HC 030031 prevented neither voided volume nor voiding efficiency impairment. The cystometric analysis also revealed a slight increase in basal and threshold pressure and a slight decrease in maximum pressure; none of these alterations were significant. Both schedules of HC 030031 treatment presented similar profiles to that with the cyclophosphamide treated group (Fig. 4).

4. Discussion

The urological symptoms caused by cyclophosphamide are well characterised side effects during chemotherapeutic interventions [4,5,26,27]. In this context, acute or chronic treatments with cyclophosphamide have been widely used as models for haemorrhagic cystitis in rodents, either to identify the intracellular pathways involved in haemorrhagic cystitis or to search for new drugs to offset the pathological state. Chronic administration of cyclophosphamide causes abdominal mechanical hyperalgesia and urinary bladder overactivity in mice, as assessed by von Frey Hair application and cystometry [28]. Furthermore, acute and chronic treatment with cyclophosphamide causes bladder dysfunction in female rats [29]. In this study, we have extended these findings and demonstrated that treatment with cyclophosphamide induces mechanical hyperalgesia and overactivity of the urinary bladder in rats.

In a recent review, Geppetti and collaborators reported that cyclophosphamide induces a neurogenic inflammation in the lower urinary tract, suggesting that its metabolite, acrolein, directly activates TRPA1 receptors from sensory neurons. As a consequence, the activation of TRPA1 may be due to a secondary inflammation involving *c-fos* overexpression, release of inflammatory peptides and activation of α_1-adrenergic receptors [30]. Our findings demonstrate that the effects of cyclophosphamide/acrolein do not involve the synthesis of *de novo* TRPA1 because mRNA expression was similar in naive and cyclophosphamide-treated groups (data not shown). However, these results do not exclude the possibility of post-translational changes in the immature form of the protein.

Acrolein, an important environmental irritant present in tear gas and vehicle exhaust, also targets TRPA1 from pulmonary tissue [8]. *In vivo*, acrolein is formed in conjunction with phosphoramide mustard by hydroxylation of cyclophosphamide through hepatic

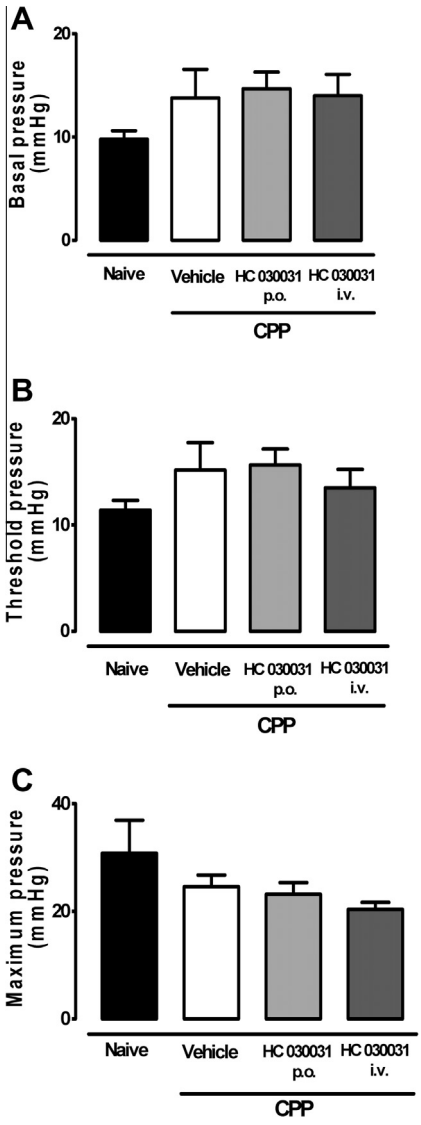

Fig. 4. Urodynamic profile in CPP-induced haemorrhagic cystitis. (A) Basal, (B) threshold, and (C) maximum pressure in naive and cyclophosphamide-treated (200 mg/kg, i.p.) rats. The rats received a single dose of vehicle or HC 030031 (100 mg/kg, p.o.) one hour before cyclophosphamide or HC 030031 (30 mg/kg, i.v.) 24 h after cyclophosphamide. Each column represents the mean, and the vertical lines indicate the S.E.M. of six animals. Statistical were performed by one-way ANOVA followed by Newman–Keuls Multiple Comparison test.

microsomal enzymes [6,31]. Furthermore, acrolein is able to rapidly enter the uroepithelium because of its chemical nature, where it causes its main toxic effect. In agreement with this, the direct intravesical injection of acrolein increased vascular bladder permeability and plasma exudation [32].

The direct activation of TRPA1 by acrolein has been demonstrated *in vitro* [8] and this may be the main mechanism by which acrolein induces urotoxicity. However, taking into account the low concentration of acrolein in urine of patients undergoing cyclophosphamide treatment, it may also cause urotoxicity by other mechanisms [33]. As reported by Korkmaz and collaborators, a disruption in the redox status of the cell, driven by cyclophosphamide/acrolein, could be the main inducer of urotoxicity [5]. This fact is interesting because the cysteine residues contained in the ankyrin sequence of TRPA1 are, in fact, sensors for the impairment of oxidation-reduction status of the cell. Therefore, TRPA1 may be a direct target for acrolein and also an indirect target for the oxidised substances generated by acrolein accumulation. Of note, the disruption of redox status in the cell may affect other proteins and this nonspecific effect might also contribute to acrolein toxicity [34].

In the present study, we show an involvement of TRPA1 in the urotoxic symptoms of cyclophosphamide treatment. The visceral inflammation and pain induced by cyclophosphamide sensitizes a somatic area at the spinal cord which causes referred pain [24,35,36]. In accordance with this, rats treated with cyclophosphamide presented a similar sensitivity when the stimulus (von Frey Hair) was applied to either the abdominal area or to the ventral surface of the hindpaw.

The consecutive treatment with the TRPA1 antagonist HC 030031 partially decreased mechanical hyperalgesia suggesting that TRPA1 is a putative target to counteract symptoms related to urinary bladder cystitis. The neuronal inputs caused by the agonism of TRPA1 in pelvic nerves is an important mechanism to cause visceral pain and, therefore, the antagonism of TRPA1 has been suggested as a promisor therapy against somatic pain [37,38]. TRPA1 activation is associated with nerve growth fact (NGF) synthesis and phosphorylation of p38 [14], a mechanism that will maintain hyperalgesia even in the absence of the initial stimulus. Of interest, NGF was overexpressed within bladder and in L_6/S_1 dorsal root ganglia in acute and subacute cystitis induced by instillation of acrolein and the systemic treatment with NGF-neutralizing antiserum or intravesical treatment with a trkA antagonist supressed mechanical hyperalgesia [35]. In fact, NGF has been described to contribute to urinary bladder hyperreflexia [39–42] and somatic sensitivity [43–45].

In addition, the treatment with cyclophosphamide induced an overexpression of interleukins: IL-1β, IL-4, IL-18 and interferon γ [46]. This might contribute to a diffuse inflammatory response and activation of intracellular messengers. In fact, the treatment with cyclophosphamide induced urinary bladder hyperreflexia related to the activation of JAK-STAT pathway [21], phosphorylation of Akt [29] and extracellular signal regulated kinase (ERK) [47,48]. As a consequence of this inflammatory signaling, the synthesis of kinins, mast-derived trypsin, fatty acids metabolites, chemokines, nitric oxide and nitrate derivative compounds will further sensitize TRPA1 [49–55].

The urodynamic parameters have confirmed the capability of cyclophosphamide to induce bladder overactivity. Overactive urinary bladder is a syndrome characterized by exacerbated contractions of the urinary bladder during the filling phase, associate with detrusor sphincter dyssynergia and inefficient voiding [56,57]. We found that a single acute treatment with cyclophosphamide significantly increased the number and amplitude of non-voiding contractions, decreased voided volume and voiding efficiency, but only slightly increased basal and threshold pressure. It is noteworthy that voiding efficiency is the ratio between the voided volume and the urinary bladder capacity, whereas the capacity is the total voided volume plus residual volume; therefore, a high number of non-voiding contractions in conjunction with a low voiding efficiency support an overactivity of the organ that is not associated with micturition. This is a feature of the bladder dysfunction during cystitis. Of relevance, these urodynamic parameters are in agreement with previous findings that used acute treatment with cyclophosphamide to induce cystitis [21,58].

The non-voiding contractions are represented by increased spontaneous activity of the detrusor smooth muscle cells during the filling phase, which may cause bladder overactivity. It has been shown that the spontaneous phasic activity of the detrusor tissue is myogenic in origin [59] and that the urothelium plays a significant role in modulating the nature of these contractions [60]. Additionally, the modulation of ion channels in sensory fibers and urothelial cells by mechanical stimuli and inflammatory cytokines can lead to voiding disorders [61]. Recently, TRPA1 receptor expression has

been described in both C fibers innervating urinary bladder and in urothelial cells, where it is suggested to act as mechanosensor and nociceptor in either physiological or pathological states [15,20]. In agreement to these findings, our study demonstrated that the TRPA1 antagonism either before or after urinary bladder cystitis development efficiently decreased the number and the amplitude of non-voiding contractions. This suggests that urothelium actively contributes to bladder contractile response mediated by TRPA1. Previous studies have reported the role of TRPA1 in bladder overactivity; for instance, the TRPA1 agonists allyl isothiocyanate, sodium sulphide or cinnamaldehyde increase the micturition frequency, and decrease voiding volume and intercontration interval [15,20,62]. In addition, both the antagonism by HC 030031 and the down-regulation by anti-sense oligonucleotide of TRPA1 significantly reduced the number and amplitude of non-voiding contractions in bladder overactivity induced by spinal cord injury [17].

It is noteworthy to mention that the dysfunction of the external urethal sphincter triggers the development of detrusor-sphincter dyssynergia. In our study, we observed marked alterations in voiding caused by the external urethral sphincter dysfunction such as reduced voided volume and voiding efficiency and greater urinary bladder capacity. These events were only partially recovered by TRPA1 antagonism. The lack of effectiveness of HC 030031 could be explained by a restriction in TRPA1 function, i.e., the receptor does not seem to exert any descending control of spinal motor neurons that regulate external urethral sphincter. However, TRPA1 has a significant role in to regulate the activity of the urothelium.

5. Conclusion

In summary, our findings demonstrate that treatment with cyclophosphamide induces haemorrhagic cystitis with evident abdominal discomfort. The urinary bladder overactivity and the reduction in the nociceptive threshold were dependent on the activation of TRPA1. As mentioned previously, the cyclophosphamide metabolite, acrolein might be the main agent responsible for TRPA1-dependent urinary bladder dysfunction. Therefore, antagonism of TRPA1 may be a strategy to counteract the urotoxic side effects of some chemotherapeutic drugs.

Conflict of interest

The authors state that there are no conflicts of interests in respect to the work reported in this paper.

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