



# The use of Hypochlorous Acid as a Model for Investigating Bladder Overactivity

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## ABSTRACT

Involuntary detrusor contractions play an important role in the development of urge incontinence. Also in an in-vitro situation contractions which develop spontaneously can be seen; a parallel with the in vivo observations is likely. In order to study this muscle overactivity we investigated the possibility to induce this phenomenon with oxidative stress using hypochlorous acid (HOCl).

**Materials and Methods:** Urinary bladder muscle strips from pigs were mounted in a custom made organ bath and incubated for 20 minutes in Krebs solution. Next HOCl (10 $\mu$ M) was added to the organ bath and the onset of overactive contractions was closely followed. Overactivity was defined as a development of more than 5 phasic detrusor contractions per minute without any other provocation in the 30 minutes following addition of HOCl to the organ bath.

**Results:** Of the 50 strips which were used 36 (72%) became overactive after exposure to HOCl during 30 minutes recording. In 76% of the overactive strips overactivity occurred within 5 minutes, in 19% between 5 and 15 minutes, and in 5% it took longer than 15 minutes. The overactivity could be stopped by washing out HOCl for 10 minutes after which still a significant contraction after EFS and ACh stimulation was seen.

**Conclusions:** It can be concluded that an oxidative stressor, like HOCl, is capable of inducing smooth muscle overactivity. This model can be used for the development and testing of new treatment modalities for the overactive detrusor. Furthermore, this study provides evidence for a causal relationship between oxidative stress and detrusor overactivity.

## ARTICLE INFO

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## INTRODUCTION

Overactivity bladder (OAB) has become the focus of intense interest because this term is used to describe the symptom complex of urinary urgency with or without urge incontinence, usually with frequency and nocturia (1). Drug treatment continues to have an important role of women with OAB.

Concerning the basic mechanisms of bladder overactivity, increasing evidence has shown

that the generation of free radicals plays a role in the development of overactivity bladder (2).

Previous in vitro research showed that during low extracellular calcium concentrations a relatively fast rate of force development was observed (3,4). A similar rate of force development could be demonstrated in spontaneously generated contractions. Therefore it was hypothesized that spontaneously generated contractions might be more dependent upon calcium release from intracellular stores. At least these contractions were

evoked by a process with a fast pathway to the contractile units. From our previous study we could conclude that selective inhibition of the  $IP_3$  pathway with the calcium blocking agent Xestospingon C significantly ( $P = 0.036$ ) slowed down the rate of force development while still a significant ( $P = 0.003$ ) contraction amplitude remained through the CICR pathway. We could assume that the  $IP_3$  pathway is the prevailing pathway for the 'fast' spontaneously developing detrusor muscle contractions.

Overactivity is seen when in vitro detrusor muscle strips are cut. To some extent they become more overactive when they are cut smaller but in the majority of the cases they become overactive at all (5). In order to induce overactivity we designed an in vitro model for the development of researchers on the field of new treatment modalities for the overactive detrusor, using oxidative stress.

## MATERIALS AND METHODS

Experiments were performed on pig urinary bladders obtained from the slaughterhouse approximately 30 minutes after slaughter. Strips of 2 x 2cm were cut from the dorsal side of the bladder dome and transported to the laboratory in oxygenated Krebs solution. The mucosa and the submucosal fat layer were removed using a binocular microscope, and strips of 0.3mm diameter and length between 1 and 2mm were excised. To facilitate diffusion the thin layer covering the muscle fiber was opened and for the greater part removed. Each strip was positioned horizontally in our custom made organ bath (IDEE University Maastricht) between two tweezers of which one was attached to the KG4 force transducer connected to the BAM4C amplifier (Scientific instruments, Heidelberg, Germany). The other tweezer was connected to a translation stage which was regulated by a controller (translation stage M111,1DG; Mercury Controller C860, Physic Instrumente). An electrical field was generated between two platinum electrodes connected to the HM8130 Function Generator from Hameg Instruments. Flow in the organ bath (volume 0.23mL) was regulated with separate in and outflow syringe pumps (Vickers Medical,  $IP_4$ ). Temperature was kept at 37°

C using infrared radiation from a halogen lamp (Philips, Eindhoven, Netherlands 12V, 20W, 6°) and controlled using a 200 $\mu$ m diameter thermocouple (Omega ChAl/005).

The strips were incubated in modified Krebs solution: NaCl, 118mM; KCl, 4.7mM; NaHCO<sub>3</sub>, 25mM; KH<sub>2</sub>PO<sub>4</sub>, 1.2mM; CaCl, 1.8mM; MgSO<sub>4</sub>, 1.2mM; glucose 11mM; pH 7.4; aerated with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. All agents were manufactured by Calbiochem. For pharmacological stimulation a 10 $\mu$ M acetylcholine solution (Sigma, St-Louis, MO, USA) was used.

## Measurement protocol

The muscle strips were placed in the organ bath and incubated in Krebs. We determined  $L_0$  the length at which maximum isometric force was developed at 37° C. After that, the strips were stimulated at this length. Before incubation with HOCl the strips were stimulated in a random order twice electrically and once with acetylcholine. EFS was applied (10s, 15V amplitude, 50ms, 100Hz). When a muscle strip developed less than 100 $\mu$ N force it was excluded from further measurements. Between each stimulation, an interval of 10 minutes was introduced. After these initial stimuli 50 strips were incubated with the HOCl solution in order to see whether they became overactive. Control group with 10 muscle strips underwent the same stimulation protocol without treating them with HOCl. Overactivity in this study was defined as a development of more than 5 phasic detrusor contractions per minute without any other provocation in the 30 minutes following addition of HOCl to the solution. After washing out HOCl for twenty minutes a last EF and pharmacological stimulation with acetylcholine was applied.

At the end of the experiment the strips were analysed in order to quantify the isoprostane 8-epi PGF<sub>2</sub> alpha amount.

## Data analysis

All isometric contractions were sampled at a rate of 100 Hz with a PCI-DAS 1000 card from Computer Boards® in a Pentium® 4 computer. Phase plots, which represent the first derivative of force as a function of the force itself, were calculated. Normally these phase plots of mono

exponential isometric smooth muscle contractions can be characterized by a straight line where  $F$  is the measured force,  $F_{iso}$  is the maximum extrapolated isometric force,  $t$  is time, and  $C$  (Eq.1) is the time constant for isometric force development. The time constant is an indicator of the rate limiting process in the excitation-contraction coupling and indicates in which period of time 66% of the saturation level of maximum force development is reached. The smaller the value of  $C$ , the faster the rate of force development.

$$F = F_{iso} [ 1 - e^{-(t/C)} ] \quad \text{Eq.1}$$

The value of EF-stimulation at  $L_0$  was seen as the optimal stimulation and therefore as the baseline value of  $F_{iso}$  and time constant. This is maximum force development without treatment with the HOCl solution. When the strips became overactive,  $F_{iso}$  and  $C$  of the spontaneously developed contractions were calculated. After washing out the HOCl solution the percentage of reduction of force development was compared to the initial force development. These normalized percentages were averaged for all 49 muscle strips. The time constants were not normalized.

### Oxidative Products

EIA of the oxidatively modified product isoprostane 8-epi PGF2alpha was performed. Briefly, HOCl-treated and control bladder tissues were equilibrated for 2 hours in culture medium at 37° C. The medium was exchanged with fresh medium every hour. After the last hour of incubation the levels of isoprostane 8-iso PGF2alpha in supernatant were assayed in triplicate with commercially available EIA kits (Cayman Chemical, Brazil). Microtiter assay plates were scanned with a SpectraMax Plus 384 computer controlled microplate reader. The quantity of isoprostane 8-epi PGF2alpha was standardized as pg/100mg wet weight of tissue per hour.

### Statistical analysis

A paired t-test was used for statistical analysis. Changes in provoked electric and pharmacological stimulation with acetylcholine were

calculated.  $F_{iso}$  and rate of force development of the overactive contractions were also calculated. All calculations were processed in Matlab® 6.1. For statistical analysis SPSS® version 10 was used.

## RESULTS

### Percentage of overactivity after HOCl treatment

After treatment with HOCl, 72% (36 out of 50) of the muscle strips became overactive, 20% (10 out of 50) of the muscle strips did not show overactivity, defined as a development of more than 5 phasic detrusor contractions per minute without any other provocation in the 30 minutes following addition of HOCl. 8% (4 out of 50) of the muscle strips became overactive spontaneously within a few seconds before the HOCl solution was added.

### Time the strips became overactive

Most of the muscle strips (76%) showed overactivity within 5 minutes. In 19% they became overactive between 5-15 minutes and in 5% it took 15-30 minutes. In 5 muscle strips the recording was continued for 60 minutes and after that time, none of them showed overactivity.

In Figure-1 the bars represent the rate of force development ( $C$ ). It shows no significant difference between Electrical Field Stimulation (2.45 seconds) and pharmacological stimulation with acetylcholine (2.55 seconds). Using a paired t-test the provoked overactive contractions have a faster rate of force development (1.20 seconds) ( $P = 0.001$ ) compared to the Electrical Field Stimulation and acetylcholine stimulated contractions.

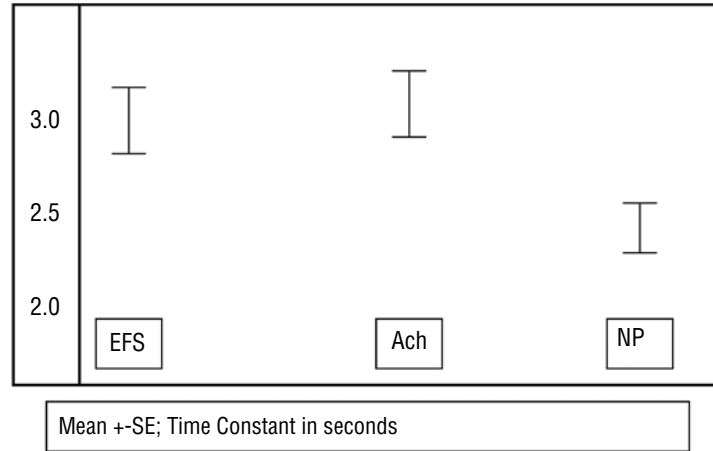
### Marker of Oxidative Stress

Isoprostane 8-epi PGF2alpha levels significantly increased in HOCl-treated bladders compared to control group (Figure-2).

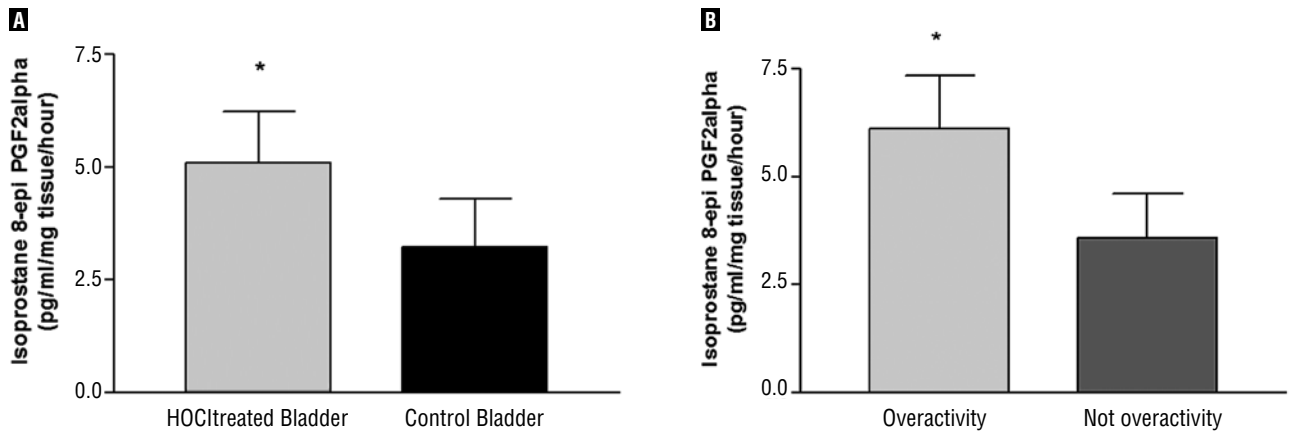
## DISCUSSION

An important research topic in functional urology has become the search for a way to inhibit the spontaneously developed involuntary contractions and leave the voluntary or evoked contractions unchanged. The development of involuntary contractions can be caused by both a dysfunction

**Figure 1 - Time constants for EFS, ACh and non provoked (NP) overactive contractions.**



**Figure 2 – A) Levels of oxidatively modified product isoprostane 8-epi PGF2alpha after HOCl to be added to organ bath vs control bladder. B) It represents the levels of isoprostane 8-epi PGF2alpha in strips that became overactive vs not overactive strips.**



\* p < 0.05

of the efferent nerve supply of the bladder or by an intrinsic overactivity of the detrusor muscle itself (1). Previous research also indicated that a denervation of the bladder leads to an increase in the development of overactive bladder contractions (5). It appears that an adequate innervation reduces the overactive detrusor contractions. Our own experimental in vitro observations showed the same results: to some extent the muscle strips became more overactive, the smaller they were cut.

This study showed that a small number (4%) of the muscle strips became overactive spontaneously while more than half (53%) the strips became overactive after incubation with HOCl. This validates the results seen in previous studies where the role of several reactive oxidative stressors like hydrogen peroxide and hypochlorous acid led to overactivity of smooth muscle (6-8).

This model makes it possible to study overactivity of smooth muscle in more detail. The

**Table 1 - Three month follow-up of patients with the first episode of spontaneous AUR.**

Patients with successful TWOC	Combination therapy	Monotherapy	P value
During 24 hours after catheter removal	(41/50) 82%	(37/51) 72.5%	0.039
7th day after catheter removal	(35/50) 70%	(32/51) 62.7%	0.3
One month following first episode of AUR	(32/50) 64%	(28/51) 55%	0.07
Three months following first episode of AUR	(26/50) 52%	(24/51) 47%	0.45

contractions caused by HOCl have the same rate and amplitude as the spontaneously developing contractions. Therefore, the induction of overactivity-resembling (overactivity-like) contractions with HOCl makes it possible to study which intracellular pathway is more or less responsible for the development of overactivity in smooth muscle and in particular smooth muscle of the urinary bladder. Changes in amplitude and rate of force development can be observed by using different intracellular pathway inhibiting agents.

During incubation with the oxidative stressor (HOCl 10 $\mu$ M) and after washing out, EFS and acetylcholine were still able to provoke a similar contraction. Therefore it can be assumed that exposure to a 10 $\mu$ M concentration of HOCl causes overactivity but minimal damage.

In our model incubation longer than 15 minutes has no benefit. HOCl is capable of modulating the intracellular calcium pathways or at least cause changes to the contractile units.

Experimental studies that have examined the association between oxidative stress and bladder dysfunction have supported the hypothesis that the generation of free radicals might be linked to bladder dysfunction (9-11). Possibly, the detrusor dysfunction is caused by activation of specific hydrolytic enzymes including calpain and phospholipase A<sub>2</sub> with subsequent damage to intracellular organelles such as the mitochondria and sarcoplasmic reticulum and via generation of reactive oxygen species and subsequent membrane lipid peroxidation (12,13).

A common effect of the activation of these pathways is a change in pattern of gene expression mediated largely through modulation of

the activities of transcription factors. Accordingly, a large number of oxidative stress-responsive transcription factors and genes have been identified (14) and some of these have been implicated in influencing aging processes as well as overactive bladder.

## CONCLUSIONS

In conclusion, it can be concluded that the HOCl can induce in vitro smooth muscle overactivity. Thus, it can be very helpful for the investigation of the different pathways responsible for overactivity of the detrusor, mainly in cases where oxidative stress can associated to the disease.

## CONFLICT OF INTEREST

None declared.

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